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# Anxiolytic-like Effects of *Lupinus angustifolious* Protein Hydrolysates in Alzheimer Model Mice †

**Guillermo Santos-Sánchez [1,2,±], Eduardo Ponce-España [1,2,±], Ivan Cruz-Chamorro [1,2], Juan Carlos López-García [3], Ana Isabel Álvarez-López [1,2], Justo Pedroche [4], María Carmen Millán-Linares [5], Francisco Millán [4], Patricia Judith Lardone [1,2], Ignacio Bejarano [1,2], Juan Miguel Guerrero [1,2,6], Antonio Carrillo-Vico [1,2,*]**

[1] Instituto de Biomedicina de Sevilla, IBiS (Universidad de Sevilla, HUVR, Junta de Andalucía, CSIC), Seville 41013, Spain; gsantos-ibis@us.es; eponce-ibis@us.es; icruz-ibis@us.es; aialvarez-ibis@us.es;plardone@us.es; ibejarano@us.es; guerrero@us.es; vico@us.es.
[2] Departamento de Bioquímica Médica y Biología Molecular e Inmunología, Universidad de Sevilla, Seville 41009, Spain.
[3] Departamento de Psicología Experimental, Universidad de Sevilla, Seville 41009, Spain; jclopez@us.es.
[4] Department of Food & Health, Instituto de la grasa, CSIC, Ctra, Utrera Km 1, Seville 41013, Spain; jjavier@cica.es; frmillan@cica.es.
[5] Cell Biology Unit, Instituto de la grasa, CSIC, Ctra, Utrera Km 1, Seville 41013, Spain; mcmillan@ig.csic.es.
[6] Departamento de Bioquímica Clínica, Unidad de Gestión de Laboratorios, Hospital Universitario Virgen del Rocío, Seville 41013, Spain.
* Correspondence: vico@us.es; Tel: +34955923106; Fax: +34954907048 (A.C.V.)
± To be considered as equal first author.

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**Abstract:** Alzheimer's disease (AD), which is characterized by a devastating and progressive loss of memory, is the principal neurodegenerative disease in the elderly population worldwide. As a consequence, AD patients present neuropsychiatric symptoms such as anxiety, causing sleeping difficulty, irritability, agitation, or aggressiveness. Previous studies have demonstrated that a high-fat diet, in addition to exacerbating AD, aggravates anxiety. We have demonstrated that *Lupinus angustifolius* protein hydrolysates (LPHs) have anti-inflammatory and antioxidant effects, key risk factors for AD and anxiety. Thus, this study aimed to evaluate the potential effects of LPHs on spatial memory and anxiety of a preclinical model of AD. ApoE$^{-/-}$ mice fed with a western diet were intragastrically treated with LPHs (or vehicle) for 14 weeks. Spatial memory and anxiety were then assessed through Morris Water- and Elevated Plus- Maze, respectively. The results did not show significant differences in spatial memory between groups. However, a significant increase ($p < 0.05$) in time in open arms, center time, number of crossings, and a reduction of anxiety behavior were observed in LPHs-treated mice. This is the first study showing that a LPHs treatment causes anxiolytic effects, pointing to LPHs as a potential component of future nutritional therapies.

**Keywords:** Alzheimer´s; Anxiety; Lupine Protein Hydrolysates; ApoE$^{-/-}$, Functional Food Ingredient.

## 1. Introduction

Alzheimer´s disease (AD) is a neurodegenerative disorder characterized by a quick progressive cognitive dysfunction, loss of memory, and behavior impairment. AD is the world's biggest cause of

dementia, affecting more than 40 million people [1]. Besides principal symptoms, AD produces a variety of neuropsychiatric effects such as anxiety, which generates sleep disturbances, irritability, agitation, or aggressiveness [2]. Although the pathophysiological mechanisms of AD have not yet been fully described, the diet westernization described as one of the main causes to the increase in risk factors of AD development such as cerebrovascular diseases, diabetes, hypertension, obesity, and dyslipidemia [3,4]. In this context, previous studies have demonstrated that a high-fat diet, in addition to exacerbating AD, aggravates anxiety [5]. The relationship between diet and AD has generated a search for nutritional treatments that ameliorate the symptoms and consequences of AD. Hence, the beneficial effects of vegetable peptides from soy [6] on improving memory activity and anxiety have been demonstrated.

We have previously demonstrated that *Lupinus angustifolius* protein hydrolysates (LPHs) reduce inflammation and oxidative stress [7], key risk factors for AD and anxiety. Thus, this study was aimed to evaluate the potential effects of LPHs on spatial memory and anxiety of a preclinical model of AD, the ApoE$^{-/-}$ mice.

## 2. Materials and Methods

### *2.1. LPHs Preparation*

LPHs were synthesized as described previously [7].

### *2.2. Animals and Experimental Design*

The experimental design is shown in Figure 1. Twelve male ApoE$^{-/-}$ (B6.129P2-ApoEtm1Unc/J) mice were housed under specific pathogen-free conditions in a room with controlled temperature (22 ± 2 °C), humidity (<55%), and 12-h light-dark cycle. Animals had free access to water and food. Animals were initially classified in 2 groups: mice that were fed with a standard diet (SD, $n$ = 4) or Western diet (WD, $n$ = 8) from the Special Diets Production Section of the University of Granada (Spain). 6 week-old mice from the WD group were randomly divided into two groups and treated intragastrically with LPHs (100 mg/kg) or vehicle for 14 weeks, respectively. Individual body weight was measured and recorded weekly. Behavioral tests were performed at the Laboratory of Animal Behavior & Neuroscience (Facultad de Psicología, Seville). Before performing the behavioral tests, animals were conditioned to these installations for a week. After the experiments, mice were euthanized with an intraperitoneal injection of sodium thiopental (50 mg/Kg, B. Braun Medical SA, Barcelona, Spain). The experimental procedures were approved by the Virgen Macarena-Virgen del Rocío University Hospital ethical committee (reference number 21/06/2016/105) and conducted under Spanish legislation and the EU Directive 2010/63/EU for animal experiments.

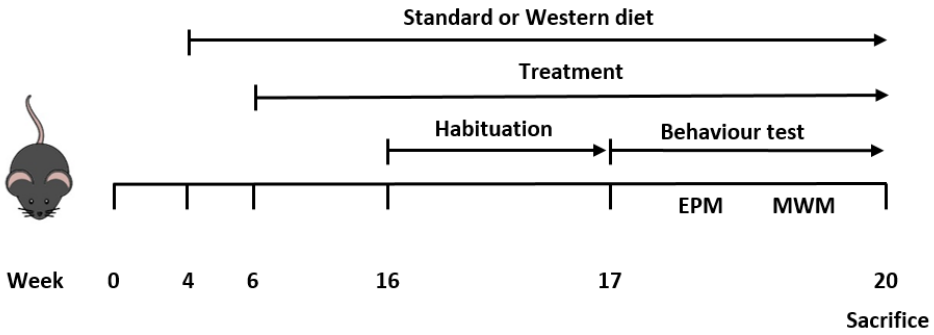


**Figure 1.** Experimental design and timeline. Schematic diagram of the experimental design of the study showing mice groups, dietary, and intervention. EPM, Elevated Plus Maze; MWM, Morris Water Maze.

### *2.3. Behavioral Test*

#### 2.3.1. Elevated Plus Maze

Anxiety-like behavior was evaluated using the Elevated Plus Maze (EPM) test at 17 weeks of age, after twelve weeks of consumption of SD, WD, or WD+LPHs. The test was performed as previously described [8]. All the sessions were recorded using a camera located over the maze. Other anxiety-related behaviors such as head dips and rears were considered. Afterwards, the recording was processed using the plugin Animal Tracker for ImageJ software and the time spent in the arms and center of the maze were measured.

#### 2.3.2. Morris Water Maze

Morris Water Maze (MWM) was performed at 19 weeks to study the processes of spatial memory and learning. Experimental procedures were performed as described by Janseen et al [9]. All sessions were recorded with a video tracking system overlooking the pool from above. The test started at 11:30 am during the light phase of light-dark cycle and nobody stayed in the room during the test. Latency time, distance traveled and the time spent in each quadrant were analyzed using the plugin Animal Tracker for ImageJ software.

### *2.4. Statistical Analysis*

All results were presented as mean ± SEM using GraphPad Prism 7 software (San Diego, CA, USA). Statistical differences were evaluated using the non-parametric Mann-Whitney test and $p < 0.05$ was considered statistically different. The analysis was performed using SPSS® software v24 (IBM Corporation, Armonk, NY, USA)

## 3. Results and Discussion

### *3.1. LPHs Treatment does not Change Body Weight*

As shown in Table 1, no differences were observed in the baseline body weight (BBW), final body weight (FBW), and body weight gain (BWG) between groups.

**Table 1.** Body weight parameters.

| Parameter | SD | Ctrl | LPHs |
|---|---|---|---|
| **BBW (g)** | 20.35 ± 0.41 | 20.98 ± 0.36 | 20.88 ± 0.49 |
| **FBW (g)** | 26.20 ± 0.87 | 26.50 ± 0.54 | 27.15 ± 0.69 |
| **BWG (g)** | 5.85 ± 1.18 | 5.53 ± 0.65 | 6.28 ± 1.09 |

Baseline body weight (BBW), final body weight (FBW), and body weight gain (BWG) in ApoE-/- mice. Values are shown as the mean and standard error of the mean of each group ($n = 4$). Ctrl, control group. LPHs, lupine protein hydrolysates group. SD, standard group.

With these results we can conclude that the effects observed in this study were not related to BWG of mice, that remained unchanged between the three experimental groups.

### *3.2. LPHs Exert an Anxiolytic-like Effect*

Representative images of the tracks of mice in the maze appear in the Figures 2A–C. WD mice spent significantly less time in opened arms and more in closed arms compared to SD group. This effect was reversed after LPHs supplementation (Figure 2D–E). Moreover, the time spent in the center was significantly lower in WD compared to SD and WD+LPHs (Figure 2F). Furthermore, other anxiety-related behaviors were evaluated. The number of head dips was significantly lower after WD intake compared to SD, but not in WD+LPHs group (Figure 2G). There were no differences in the rears between groups (Figure 2H).

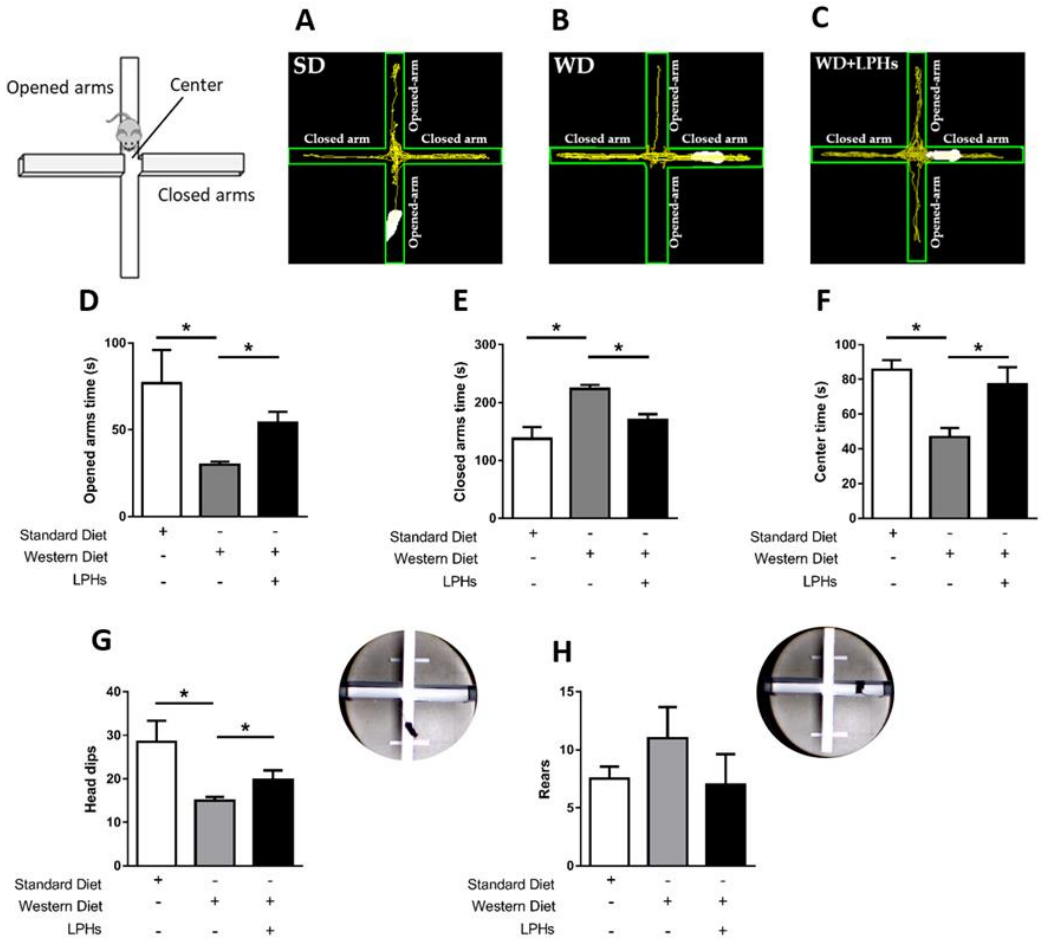


**Figure 2.** Effects of LPHs on WD-induced anxiety behaviors. Representative images of the tracks of mice in the maze (A–C). Time spent in opened arms (D), closed arms (E), and center (F), head dips (G), and rears (H) for the experimental groups. Values are shown as the mean and standard error of the mean of each group ($n = 4$).

It is well known that WD accelerates the cognitive deficits in ApoE$^{-/-}$ mice [5]. In the present study, we observed that WD increases anxiety levels significantly. These results are in accordance to previous studies in humans [10] and mice [11]. LPHs, therefore, shown to be minimizing the anxiolytic effects caused by the intake of the WD. It has already been shown that bioactive peptides from soy [6] may exert an anti-anxiety activity, but to the best of our knowledge, it is the first time that a Lupine hydrolysate shows anxiolytic-like properties.

*3.3. LPHs do not Alter Spatial Memory.*

The latency time, which reflects the ability to learn the platform location, is shown in Figure 3.B. During the training phase, we did not observe any significant differences between groups. This result revealed that all groups are equally capable of learning the task. The probe phase shows that there are no differences in the time spent in the platform zone (Figure 3C) among groups. Interestingly, a decreasing trend in the total distance traveled after WD consumption was observed ($p < 0{,}058$, compared to SD) which was reverted after LPH treatment ($p < 0{,}0571$ compared to WD) (Figure 3D).

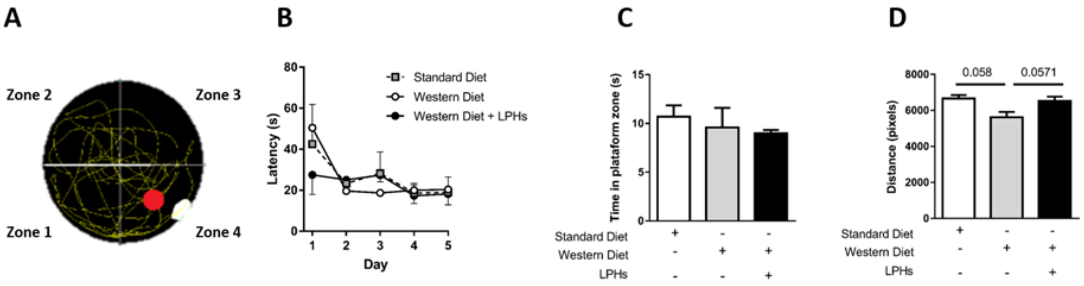


**Figure 3.** Effects of LPHs on WD-induced cognitive deficits. Representative image of the MWM (A); learning effects (B), time in platform zone (C), and distance traveled of each experimental group. Values are shown as the mean and standard error of the mean of each group ($n$ = 4).

Our results reveal no impairment of memory or spatial learning after WD eating. This fact could be associated with the age of mice and time of WD consumption [9]. Unlike other peptides [12], LPHs did not improve either the spatial memory or learning after a 14 weeks treatment.

## 4. Conclusion

This is the first study showing that a LPHs treatment causes anxiolytic effects, pointing to LPHs as a potential component of future nutritional therapies in AD patients with anxiety, a serious side effect of this disease.

**Conflicts of Interest**: The authors declare no conflict of interest.

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