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# Dose-dependent S-allyl cysteine ameliorates multiple sclerosis disease-related pathology by reducing oxidative stress and biomerkers of dysbiosis in experimental autoimmune encephalomyelitis

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ABSTRACT

Garlic is a component of the Mediterranean diet. S-allyl cysteine (SAC), the most common organosulphur present in garlic, possesses neuroprotective properties. This investigation was performed to evaluate the dose-dependent protective action of SAC on oxidative damage, inflammation and gut microbiota alterations biomarkers. Experimental autoimmune encephalomyelitis (EAE) as a model of multiple sclerosis (MS) was induced by the myelin oligodendrocyte glycoprotein (MOG), whose effects were quantified by examining the changes in: clinical score, lipid peroxidation products, carbonylated proteins, glutathione system, tumor necrosis factor alpha (TNFα), and lipopolysaccharide membrane bacteria (LPS). Our results reveal that MOG induces paralysis, oxidative damage and increases in LPS binding protein (LBP) and LPS levels. In this work, two doses of SAC were compared with two dose of N-acetyl cysteine (NAC). SAC was more effective than NAC and it prevented the harmful effects induced by MOG more effectively at the dose of 50 mg/kg than that of 18 mg/kg. Surprisingly, NAC increases LBP levels while SAC had not such negative effect. In conclusion the data show the ability of SAC to modify EAE evolution.

## 1. Introduction

The Mediterranean diet is considered to be a good, healthy dietary model. It consists of fresh, local and seasonal food, especially fruit and vegetables and fish, as well as olive oil, a moderate consumption of red wine and garlic and others (Borghi and Cicero, 2017; Caruana et al., 2016; Pallauf et al., 2013). Furthermore, dietetic habits are related to the characteristics and quality of gut microbial flora. Dietary components influence the microbiota and its life stages, and all these are linked to neurodegenerative disorders (Del Chierico et al., 2014; Leulier et al., 2017).

Different studies have shown that garlic (*Allium savitum* L.) exerts cardioprotection, as well as neuroprotective activity. S-allyl-cysteine (SAC) is the main organosulphur compound of garlic which possesses these characteristics. The protective capacity of garlic and its compounds, especially SAC, have showed protective effects in different processes (Colin-Gonzalez et al., 2015, 2014; Ray et al., 2011).

Oxidative damage plays a relevant role in the development and evolution of neurodegenerative diseases such as demyelinating disorders, including multiple sclerosis (MS) (Haider, 2015; Lassmann, 2013, 2014; Ljubisavljevic, 2016; Mahad et al., 2015). Additionally, the importance of oxidative damage in the development of MS is evidenced by the latest treatments whose effects derive from their antioxidant potential, regulating the nuclear factor (erythroid-derived 2)-like 2 (Nrf2). This nuclear factor modifies the expression of genes encoding antioxidant proteins, vitagenes, as well as proinflammatory molecules (Bahamonde et al., 2014; Tasset et al., 2012a, 2012b).

A widely accepted and used model for the study of the

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physiopathology of MS as well as for the design of new therapeutic strategies is the experimental autoimmune encephalomyelitis (EAE) induced by the myelin oligodendrocyte glycoprotein (MOG). Data from our group and others have shown that this model has similar characteristics to MS i.e. an intense inflammatory response, oxidative and cell damage, neuronal loss and demyelination (Escribano et al., 2017).

Finally, many studies show a close relationship between the microbiota and central nervous system (CNS) disorders, such as MS. Gut microbes are essential for the correct development and maturation of the immune system and the CNS. Dysbiosis could be the basis of CNS illnesses (Escribano and Túnez, 2014). These alterations may be modified by feeding (Escribano and Túnez, 2014; Esposito et al., 2017; O'Toole and Jeffery, 2015; Weiss and Hennet, 2017). Escribano et al. (2017) showed the existence of MOG-induced changes on the levels of bacterial lipopolysaccharide (LPS) and the LPS binding protein (LBP). In addition, the treatments applied reversed these changes towards normality.

The main aim in this work was to evaluate dose-dependent neuroprotective effects of SAC, by determining: i) the changes in clinical score; ii) level of lipopolysaccharide bacteria (LPS) and LPS-binding protein (LBP) as indicators of changes in intestinal flora; iii) the tumor necrosis factor alpha (TNF-α) concentration as, an inflammation marker; and iv) the oxidative stress biomarkers.

## 2. Materials and methods

### 2.1. Chemical reagents and administered products

Heat-inactivated *Mycobacterium tuberculosis* (H37Ra) was acquired from DIFCO (Detroit, MI, USA), and mielyn oligodendrocyte glycoprotein (MOG, fragment 35–55), complete Freund's adjuvant and other reagents were purchased from Sigma-Aldrich (Madrid, Spain).

### 2.2. Animals

All the rats were cared for in accordance with European Communities Council (86/609/ECC) and RD 53/2013 approved by the Presidency Minister of Spain (BOE 8 February 2013) and by the Bioethics Committee at the University of Cordoba, Spain. All animals were obtained from the Animal Experimentation Center (University of Cordoba, Spain). Fifty-five 8-week old male Dark Agouti rats weighing 190–220 g were used. Each group was individually housed in plastic cages. They were kept under controlled temperature conditions (20–23 °C) and illumination (12 h light/12 h dark cycle, lights on at 08:00 h), and supplied with food (Purina®, Spain) and water *ad libitum*.

### 2.3. Treatments and experimental model

The dosage and administration protocols of administration were: i) N-acetyl cysteine (NAC) administered at 50 or 18 mg/kg BW, oral intake daily for 21 days, five days per weeks (CINFA S.A., Pamplona, Spain); and ii) SAC 50 or 18 mg/kg BW/ oral intake daily for 21 days, five days per weeks; the compound was a gift from Canvax Biotech S.L. (Cordoba, Spain) purchased from TCI Europe N.V., Belgium (Reference number: A1468; purity > 98% (T)) (Escribano et al., 2017).

The EAE model was induced by MOG (in phosphate-buffered saline emulsified 1:1 in complete Freund's adjuvant, containing 400 μg heat-inactivated *Mycobacterium tuberculosis*), which was injected in one go at the dorsal base of the tail at a dose of 150 μg and at final volume of 100 μl (Escribano et al., 2017; Perez-Nievas et al., 2010). Treatments were administered for a period of 21 days (3 weeks), beginning at 14 days after MOG administration, five days per week. The length of the study was established on the basis of previous studies from our group (Escribano et al., 2017). At the end of the assays, all the rats were anesthetized and euthanatized, and blood, brain and spinal cord specimens were collected separately. The samples were stored frozen (−80 °C) until their biochemical parameters were measured.

55 rat were distributed as following: i) healthy animals (Control, n = 5 animals per group); ii) control animals treated with complete Freund's adjuvant (Vehicle; n = 5 animals per group); iii) rats injected with MOG (EAE, n = 5 animals per group); iv) EAE + 18 mg of NAC (EAE + 18 NAC, n = 10 animals per group); v) EAE + 50 mg of NAC (EAE + 50 NAC, n = 10 animals per group); vi) EAE + 18 mg of SAC (EAE + 18 SAC, n = 10 animals per group); and vii) EAE + 50 mg of SAC (EAE + 50 SAC, n = 10 animals per group).

### 2.4. Clinical score

Motor evaluation was gauged according to the scale used in previous studies (Escribano et al., 2017; Perez-Nievas et al., 2010), in which: 0 = no sign, 1 = tail paralysis, 2 = hind limb paresis, 3 = hind limb paralysis, 4 = hind limb paralysis plus front limb paresis, 5 = moribund or dead.

### 2.5. Biochemical parameters

At the end of the study, the rats were decapitated under controlled temperature conditions and anesthetized with intraperitoneal injection of 75 mg/kg ketamine (Imalgene 100 mg/mL; Merial Laboratorios, Barcelona, Spain). The brains and spinal cords were extracted and weighed, and homogenates made with a mechanical homogenizer (Tempest Virtis). The buffer used for homogenization was Tris (20 mM) at pH 7.4. Plasma was collected immediately and stored at − 85 °C.

In addition, the quantification of biochemical parameters was measured using a UV-1603 spectrophotometer (Shimadzu, Japan).

#### 2.5.1. Oxidative stress

Lipid peroxidation products as malondialdehyde and 4-hydroxyalkenals (LPO: MDA + 4-HDA; by Bioxytech S.A. kits, Oxis International, Portland, OR, USA; LPO-586) were quantified as oxidative damage indicators. The redox status of the glutathione systems was evaluated by total glutathione (by Bioxytech S.A. kits, Oxis International, Portland, OR, USA; GSH-420) and reduced glutathione (GSH; by Bioxytech S.A. kits, Oxis International, Portland, OR, USA; GSH-400). Oxidized glutathione (GSSG) levels were calculated by subtracting GSSG from total glutathione, and the GSH/GSSG ratio was calculated.

#### 2.5.2. LBP

LBP is up-regulated as part of the acute phase response and is used as a marker for the LPS load (components of the outer membrane of Gram-negative bacteria capable of inducing strong immune responses). Additionally, LBP levels may indirectly reflect changes occurring in the gut microbiota. The LBP was assessed using the LBP soluble (mouse) ELISA Kit (Enzo®, USA), a solid phase sandwich ELISA, which uses a detecting antibody (POD-labeled monoclonal antibody to mouse LBP) and TMB (3,3′, 5,5;-tetramethylbenzidine) as a chromogen.

#### 2.5.3. LPS

LPS has been found to mediate neuroinflammatory processes in the CNS as it is capable of activating microglial cells. LPS was assessed by using the Pierce® LAL Chromogenic endotoxin quantification kit provided by Thermo Scientific (USA). LPS catalyzes the activation of a proenzyme in the modified Limulus Amebocyte Lysate (LAL), which then catalyzes the splitting of p-Nitroamiline (pNA). The activation rate was proportional to the sample endotoxin concentration. The released pNA was photometrically measured at 405–410 nm. Data are expressed as endotoxin units/mg protein. For logistic reasons, LPS was studied in blood and spinal cord.

#### 2.5.4. Total protein and hemoglobin estimation

Protein levels were measured with the Bradford method, using a