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Original Article:

Neuroprotective Effects of Salvia Hydrangea Extract through Dietary Uptake in Amyloid Beta-injected Rats

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Abstract

Introduction: Alzheimer's disease (AD) has been identified as a progressive memory and cognitive impairment. Some *Salvia* species are suggested by certain studies for the management of mild to moderate AD. We aimed to evaluate the anti-inflammatory, antioxidant, and anti-apoptotic effects of *S. hydrangea* on amyloid beta-injected rats.

Materials and Methods: Rats were pretreated with *S. hydrangea* for 10 days before amyloid beta (Aβ) injection. Western blotting techniques were used to evaluate protein level of γ-glutamyl cysteine synthetase (γ-GCS), Tumor Necrosis Factor α (TNF-α) and Interleukin-6 (IL-6) in two brain regions: hippocampus and frontal cortex.

Results: Current data show that *S. hydrangea* extract increased γ-GCS protein levels in amyloid beta injected rats, and pretreatment with *S. hydrangea* increased it further. Besides, *S. hydrangea* decreased protein levels of TNF-α and IL-6 in amyloid beta injected rats.

Conclusion: Based on the decreased levels of IL-6, TNF-α, and the increased levels of γ-GCS, it is suggested that the use of *S. hydrangea* could be protective in neurodegenerative diseases.

Keywords: Alzheimer' s disease, Interleukin-6, *S. hydrangea*, Tumor necrosis factor α, γ-glutamyl cysteine synthetase

1. Introduction

Alzheimer's disease (AD) is an age-related category of human psychiatric disorders characterized by cognitive impairment and progressive memory loss, along with the deposition of extracellular amyloid beta (Aβ) plaques [1]. According to Ashabi et al., Aβ has been implicated in the degeneration of neurites through programmed cell death (PCD) [2]. Additionally, the accumulation of intraneuronal neurofibrillary tangles may contribute to the loss of learning and memory [3].

In previous research, the role of oxidative stress in development of AD pathogenesis has been discussed, particularly through lipid peroxidation of cell membrane [4]. Mitochondria, as previously demonstrated, produce reactive oxygen species (ROS), and within this organelle, various enzymes act to defend cells against ROS and decrease their levels

[5, 6]. γ-glutamyl cysteine synthetase (γ-GCS) is an enzyme responsible for producing Glutathione (GSH), abundant in brain tissue; however, its levels decrease in certain brain-related neuropathies such as those observed in AD patients [7].

Akiyama et al., reported that brain-related inflammatory activations increase with elevated factors, including Tumor Necrosis Factor-α (TNF-α). TNF-α, a primary cytokine in inflammatory responses, has been found to be elevated in both the brain and blood of individuals with AD [8]. Interleukin-6 (IL-6) acts as a pro-factor in inflammatory responses throughout the body, and similar to TNF-α, it is elevated in AD patients [9]. Previous studies have indicated that S. hydrangea possesses a high antioxidant capacity [10]. In current study, we evaluated the anti-inflammatory and antioxidant functions of *S. hydrangea* in an AD-animal model, specifically in the hippocampus and frontal cortex of male Wistar rats.

2. Materials and Methods

Reagents

We acquired antibodies against IL-6, TNF-α, γ-GCS and β-actin from Cell Signaling Technology (Beverly, USA). The Polyvinylidene fluoride (PVDF) membrane was purchased from Millipore (Billerica, MA, USA), while the Electrochemiluminescence (ECL) kit was sourced from Amersham Bioscience (Piscataway, USA). Other reagents used in the study were procured from Sigma Aldrich (St. Louis, USA).

Plant material

S. hydrangea was collected from Karaj, Iran, and verified by Dr. A. Sonboli (Department of Biology, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran). A voucher specimen number (1042) was deposited at herbarium of Medicinal Plants and Drug Research Institute, Shahid Beheshti University, Tehran, Iran [10].

Animals

Male albino rats of Wistar strain, weighing approximately 230-260 g, were obtained from the Pasteur Institute (Tehran, Iran). The Rats were kept under standard laboratory conditions, adhereing to a 12h light-dark cycle, and provided with unrestricted access to water and food. All experiment procedures were conducted in accordance with the guidelines set by the Animal Care Committee of Shahid Beheshti University of Medical Sciences.

Experimental Design

Four groups were utilized in this study: (1) Group one (PBS group) received oral gavage of water daily for 10 days before the intrahippocampal injection of 3 µl vehicle phosphate-buffered saline (PBS); (2) Group 2 animals were given oral gavage of water daily for 10 days before the injection of Aβ (10 ng/µl) in both CA1 region; (3) Group 3 animals received oral gavage of *S.hydrangea* for 10 days (50mg/kg) [11] before the injection of 3 µl PBS in both CA1 region; and (4) Group 4 animals were given oral gavage of *hydrangea* for 10 days (50mg/kg) before the injection of Aβ (10 ng/µl), in both CA1 region.

Surgery and microinjection

The rats were deprived of water and food for 9 hours, followed by administration of 10 ml of *S. hydrangea* extract for 10 days. After this period, anesthesia was induced through an intraperitoneal injection of Xylezine (4 mg/kg) and also Ketamine Hydrochloride (50 mg/kg). The rats were then secured in a stereotaxic apparatus. In the next step, following the atlas of Paxinos and Watson, a skin incision was made overlying the skull to reveal the coordinates for CA1 of the hippocampus. Subsequently, bilateral injections of 3µl/side phosphate buffer saline (PBS) (vehicle) and/or Aβ were performed using the following coordinates: vertical: 7.2mm (from dura); anterocaudal: -3.2mm; lateral: ±1.8 (both with respect to bregma) [12]. Seven days after Aβ injection, rats were decapitated, and their brains were removed for further experimentation.

Amyloid beta 1-42 preparation

The Aβ 1-42 was dissolved in PBS (0.1 M) at a concentration of 200 ng/µl, and the solution underwent incubation at 37˚C for 5 days. Subsequently, this solution was diluted with PBS to achieve a a concentration of 10 ng/µl, reaching the final Aβ concentration on the surgery day.

Determining the total components of S. hydrangea

For HPLC quantification of compounds of the extract, the liquid chromatographic apparatus (Agilent LC, G1311A, USA) was deployed, consisting of a controller coupled to a G1315D photodiode array detector and an in-line degasser pump. An automatic injector interfaced with chromatography manager software (Agilent Chemstation) was utilized. Separation occurred on a reverse-phase-C18 analytical column (Knauer, Berlin, Germany) with dimensions of 300 × 4 mm i.d, 5µm particle size, conducted at room temperature (27˚C) and a flow rate of 1ml/min. Detection spanned the range of 200-600 nm. Elution employed a ternary non-linear gradient of the solvent mixture CH3OH/H2O/CH3COOH (10:88:2, V/V/V) as solvent A, CH3OH/H2O/CH3COOH (90:8:2,

v/v/v) as solvent B, and CH3OH as solvent C. Solvent B composition increased from 15% to 30% over 15 min, reached 40% in 3 min (maintained for 12 min), and then surged to 100% in 5 min. Following this, solvent C composition increased to 15% in 2 min, elevated to 30% in 11 min, and returned to initial conditions in 2 min. Retention times were compared to authentic standards under UV spectra for element indication. Identical analysis conditions were maintained using our in-house PDA library. Notably, standards and sample solutions were replicated three times for robustness.

Western blot analysis

The animals were anesthetized using CO2, decapitated, and their hippocampus and frontal cortex were promptly replaced in liquid Nitrogen for molecular experiments. The isolated tissues were homogenized in a protein extraction buffer containing a protease inhibitor cocktail, followed by centrifugation at 4,000×g at 4 ˚C, as per Niimura's method [13]. Protein concentration was determined using Bradford's method [14]. Subsequently, 60 µg of total protein was subjected to sodium dodecyl sulfate-polyacrylamide (SDS)-PAGE and transferred to a polyvinylidene difluride membrane. (Millipore, Billerica, MA, USA). The membranes were blocked and then incubated with primary antibodies against Interleukin-6 (IL-6; 1/500), γ-GCS, TNF-α (1/500), followed by a horseradish peroxidase-conjugated secondary antibody. Chemiluminescence method was used to visualize immunoreactive proteins. We scanned the bands by densitometric quantification using ImageJ software.

Data analysis

The experiments were replicated a minimum of three times. Mean± SEM (standard error of mean) was calculated and processed using Graph Pad Prism® 5.0 to present the data. Post hoc analysis employing Turkey's and one way analysis of variance (ANOVA) were used to evaluate Western blot and Enzymatic data. A significance level of $P < 0.05$ was considered statistically significant.

3. Results

S. *hydrangea* elevated the Level of γ-GCS in Hippocampus and Frontal Cortex in Amyloid Beta-injected Rats

In this study, we utilized the Western blot method to assess the γ-GCS protein levels. Our findings indicated a 1.25- and 1.37-fold increase in the γ-GCS protein level in the frontal cortex and hippocampus, respectively, in Aβ-injected rats. Furthermore, in *S. hydrangea*-fed rats, a significant increase of 2.12-fold in frontal cortex was observed compared to control rats, while no significant change was noted in the hippocampus. It is noteworthy that pretreatment with *S. hydrangea* led to a substantial increase in γ-GCS levels by 2.5- and 1.6-fold in the frontal cortex and hippocampus, respectively, compared to control rats (Figure 1).

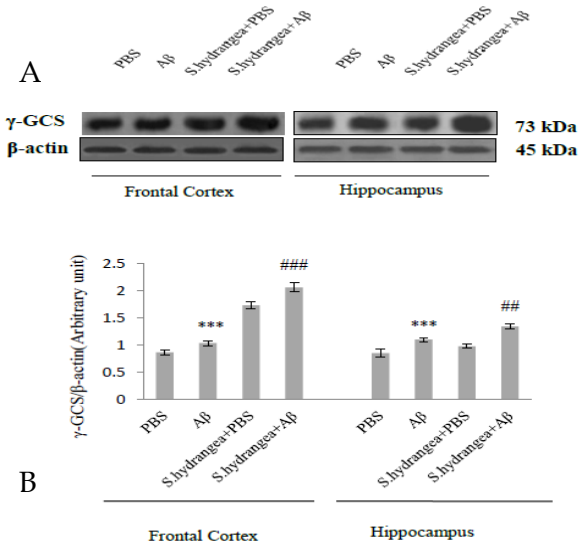

Figure 1. Western blots analysis to measure the efects of S. hydrangea on γ-GCS in the hippocampus and prefrontal cortex of Aβ-injected rats. Sixty µg proteins were loaded on SDS-PAGE ,
western blotted, Block by nonfat milk, probed with γ-GCS primary antibody and reprobed with anti-β-actin antibody. (One of the six western blot were shown; n=6). B The densities of γ-GCS bands were measured by Image.J program and their ratio was calculated .

TNF-α and IL-6 Level Decreased in Consequence of Pretreatment with S. hydrangea in Amyloid Beta-injected rats

In the current study, we investigated the levels of TNF-α and IL-6 using Western blot analysis. As depicted in Figure2, the level of TNF-α increased significantly by 2.75- and 2.15- fold in frontal cortex and hippocampus, respectively, in Aβ-injected rats compared to the control group. Also, the level of IL-6 increased by 1.63-fold in Aβ-injected rats in these mentioned areas compared to the control rats. Additionally, pretreatment with *S. hydrangea* decreased the level of IL-6 and TNF-α notably in the frontal cortex and hippocampus compared to Aβ-injected rats .

*Significantly different from PBS group. # Significantly different from Aβ-injected rats, ***p<0.001, ##p<0.01 and ###p<0.001.

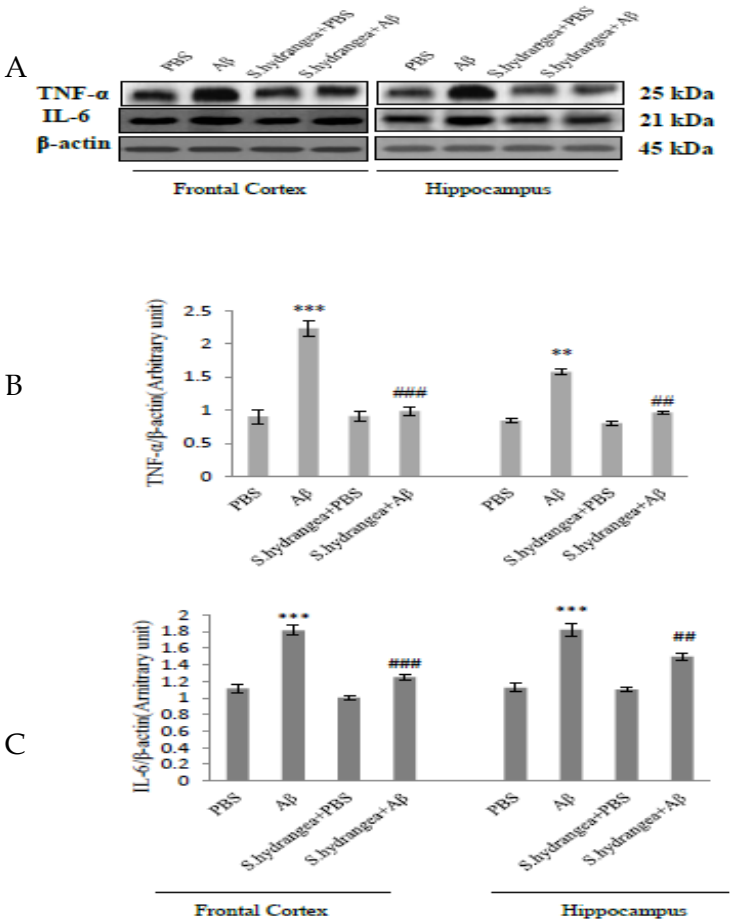

Figure 2. Western blots analysis to measure the efects of S. hydrangea on IL-6 and TNF-α in the hippocampus and prefrontal cortex of Aβ-injected rats. Sixty µg proteins were loaded on SDS-PAGE ,western blotted, Block by nonfat milk, probed with IL-6 and TNF-α primary antibodies and reprobed with anti-β-actin antibody. (One of the six western blot were shown; n=6). B The densities of IL-6 and TNF-α bands were measured by Image.J program and their ratio was calculated .
*Significantly different from PBS group. # Significantly different from Aβ-injected rats, ***p<0.001, **p<0.01, ##p<0.01 and ###p<0.001.

4. Discussion

GSH, acting as an antioxidant in the brain, reacts with ROS [14]. Glutathione deficiency, contributing to oxidative stress, plays a crucial role in certain neuropathies like AD [15, 16]. Furthermore, polyphenolic compounds from the widely spread genus *Salvia* have been shown to reduce oxidative stress [11]. These compounds exhibit high antioxidant activity and can extend and protect neurons [17]. Our study results demonstrate an elevation of γ-GCS protein levels in the hippocampus and frontal cortex. Notably, *S. hydrangea* prevented the decrease in GSH levels in Aβ-injected rats by upregulating the levels of γ-GCS in these areas. The ability of *S. hydrangea* to exhibit this effect serves as evidence for its effectiveness in mitigating the adverse effects of Aβ injections.

Inflammation constitutes a significant physiological immune response to various factors such as trauma and diseases. Akyama et al. reported that insoluble Aβ peptide deposits could serve stimuli for inflammation. Furthermore, the accumulation of these plagues over many years is a significant factor in exacerbating the AD pathogenic process [18]. Decourt et al. showed that TNF-α signaling exacerbates Aβ pathogenesis, and inhibition of TNF-α might decrease cognitive impairment in AD patients [19]. Additionally, Cong Zheng et al. have suggested the robust potential of anti-TNF-α therapies against AD [20]. The data indicated higher TNF-α protein level in the two mentioned brain regions in Aβ-injected rats. Here, we demonstrated that *S. hydrangea* extract decreased

TNF-α protein levels under oxidative stress conditions. Thus, the ability of *S. hydrangea* to modulate TNF-α signaling serves as another indication of the protective effect of *S. hydrangea* extract.

Pro-inflammatory cytokines like IL-6 increase in the patients with AD and interestingly, in the early stage of AD, plasma IL-6 was determined as a potential biomarker [21]. M. Hull et al. have demonstrated that activation of inflammatory responses such as IL-6 may lead to neuritic degeneration. Therefore, suppressing the elevation of IL-6 can stands as therapeutic way against AD [22]. Our results here indicated that total extract of *S. hydrangea* exhibited marked anti-inflammatory activity.

5. Conclusion

In summary, the current research highlights the potential of *S. hydrangea* methanol extract to mitigate the detrimental effects of the accumulation of extracellular Aβ plagues. Collectively, the demonstrated neuroprotective effects, coupled with antioxidant activity, suggest the potential therapeutic use of this this plant fin treating AD.

Ethical Considerations

Compliance with ethical guidelines

All experiments were conducted according to the ethical guidelines of Tehran Medical Sciences, Islamic Azad university and Shahid Beheshti University of Medical Sciences.

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Author's contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declare no conflict of interest.

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