

The Effect of Nerol on Behavioral, Biochemical and Histological Parameters in Male Wistar Alzheimer's Rats

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ABSTRACT

One of the most important reasons for dementia is Alzheimer's disease (AD) characterized by amyloid plaques and neuronal death. Using natural therapies has always been a great concern for AD. Herein, nerol, as a natural monoterpene, was applied to examine its protective and therapeutic effects on a rat model of AD. Fifty six Wistar, male rats were randomly assigned to 7 groups (n = 8 rats/group), including control group with no A β 42 injections, group 2 with A β 42 injection into rats' hippocampus, group 3 with injection of phosphate buffer saline, as A β buffer, into rats' hippocampus, group 4, 5 and 6 that received nerol at 30, 60 and 90 mg kg⁻¹, respectively, after being injected with A β 42, and group 7 that received nerol (60 mg kg⁻¹) in a protective mode before A β injection. Behavioral, biochemical and histological parameters were evaluated in all groups. Alzheimer's-induced group showed impairment in lipid profile, antioxidant enzyme level, long-term memory, enzymes activity along with loss of neuronal cells. Treatment with nerol improved the lipid profile, antioxidant enzyme level, number of amyloid plaques, and memory function. Protection with nerol (60 mg kg⁻¹) also demonstrated similar improvements against AD. Hence, nerol has shown capability of improving the deficiency of neuronal cells, memory as well as antioxidant enzyme level associated with AD.

Keywords: Alzheimer's disease, A β 42, Nerol, Amyloid plaques, Memory

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative condition and the most common cause of dementia. It has been characterized by the presence of aggregates of amyloid β (A β) peptide along with deposits of hyperphosphorylated tau protein inside the brain [1]. It has been proposed that A β accumulation in the brain causes both synaptic loss and dysfunction leading to neuronal death and eventually cognitive dysfunction [2]. A β 42 (containing 42 amino acids) is considered a toxic specie involved in AD pathophysiology and its presence in cerebrospinal fluid could be a reliable predictor of AD progression [3]. In order to study A β aggregation *in vivo*, intra-cerebroventricular administration of A β peptide into rodent brain has been used to stimulate AD. This injection could induce histological and biochemical changes, oxidative damage, and inflammatory responses resulting in memory deficits [3,4].

With this animal model, *in vivo* studies could be performed to test potential new candidates for AD therapy.

Nerol (*cis*-3,7-dimethyl-2,6-octadien-1-ol) is a monoterpene found in many essential oils (Fig. 1) [5]. It is a colorless liquid with a pleasant, sweet rose-like odor which, unlike that of geraniol, is considered to be fresher. Nerol is originally isolated from neroli oil, obtained from the blossoms of the bitter orange tree, *Citrus aurantium*. It is also one of the major constituents of rose oil. Nerol is greatly used in both perfumery, for its particular freshness, and in flavor, for bouqueting citrus flavors [6]. Bitter orange, as the source of nerol, has been found with anti-cancer and antianxiety characteristics while being therapeutically effective in aiding digestion, relieving flatulence, and providing cardiovascular health and stroke treatment [3]. Current studies have also shown that vapors of neroli oil could help reduce anxiety levels [7]. However, the putative neuroprotective effects of nerol is unknown. Therefore, the objective of this study was to assess the effects of nerol, as a natural compound, on amyloid- β plaque accumulation in Alzheimer's disease rat models.

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MATERIALS AND METHODS

Compounds

A β 42 and nerol were obtained from Sigma (St. Louis, MO, USA). A β 42 was dissolved in PBS and placed in an incubator at 37 °C for 1 week before use [8]. Nerol doses (30, 60 and 90 mg kg⁻¹) were prepared in distilled water. Commercial kits used for the evaluation of low-density lipoprotein cholesterol (LDL), high-density lipoprotein (HDL), triglycerides (TG), cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline aminotransferase (ALP) and superoxide dismutase (SOD) were purchased from Zist Shimi Company, Iran.

Animals

The experiments were conducted on fifty six male Wistar rats (200 ± 5 g; Pasteur Institute, Tehran, Iran). The animals were kept in an animal room that was maintained at 20-25 °C with 50-70% relative humidity under a 12-h light-dark cycle. The rats had free access to food (rodent pellets) and water. To induce Alzheimer's disease (AD), animals were anesthetized by ketamine and xylasin injection and placed within the stereotactic device. Using stereotaxy and brain atlas, 2 μ l of A β 42 solution was injected with a hamilton syringe in the ventricle of the animal's brain. Injection was slowly carried out in the CA region on both sides of the hippocampus. After surgery, animals recovered for one hour in a warm box before they were returned to their cages. The animals were given 7 days of recovery. After one week, amyloid plaques were formed in the animal's brain which were visible by the use of histological methods (detailed below). All experiments were performed in accordance with the international guidelines set in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and approved by the Research and Ethics Committee of Science and Research Branch, Islamic Azad University.

Experimental Design

The rats were allowed to acclimate for seven days prior to their use in the studies. The rats were randomly assigned to seven groups (n = 8 rats/group) as follows:

- Group 1: Control; no A β 42 injection

- Group 2: Beta Amyloid; A β 42 was injected into rats' hippocampus
- Group 3: PBS; phosphate buffer saline, as A β buffer, was injected into rats' hippocampus instead of A β for four weeks
- Group 4: Exp 1; A β was injected into the rats' hippocampus and they received nerol 30 mg kg⁻¹ intraperitoneally for four weeks
- Group 5: Exp 2; A β was injected into the rats' hippocampus and they received nerol 60 mg kg⁻¹ intraperitoneally for four weeks
- Group 6: Exp 3; A β was injected into the rats' hippocampus and they received nerol 90 mg kg⁻¹ intraperitoneally for four weeks
- Group 7: Protective; the rats first received nerol 60 mg kg⁻¹ intraperitoneally for two weeks and then A β was injected into their hippocampus

Behavioral Study

A shuttle-cage consisting of two compartments of equal size (26 × 26 cm), including the starting and shock compartments, separated by a sliding door (8 × 8 cm) was used. The starting compartment was light whereas the shock compartment was dark. Each experiment started with a pre-training trial: the rat was first placed in the starting compartment for 5 s, after which the sliding door was raised and the rat was allowed to stay in the dark compartment for 10 s. The rat was then put back in its cage and stayed there for 30 min after which it was again put into the shuttle cage. At this time, after entering the dark compartment, a footshock (50 Hz, 1 mA and 5 s) was delivered to the rat. The rat was then put back into cage and stayed there for 120 s. When put back in the shuttle cage, if a latency (in the order of 120 s) was observed before entering the dark compartment, successful acquisition of passive avoidance learning was recorded. A similar procedure was used 24 h after training sessions to make a retention test for evaluating long-term memory. Higher or lower latencies are taken as indicative of increase or decrease in memory retention [9].

Biochemical Evaluation

Animals were anesthetized by inhalation of mild diethyl ether and their blood was taken. The blood samples were

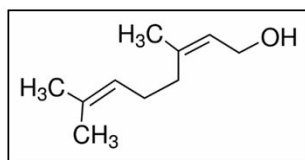


Fig. 1. Chemical structure of nerol.

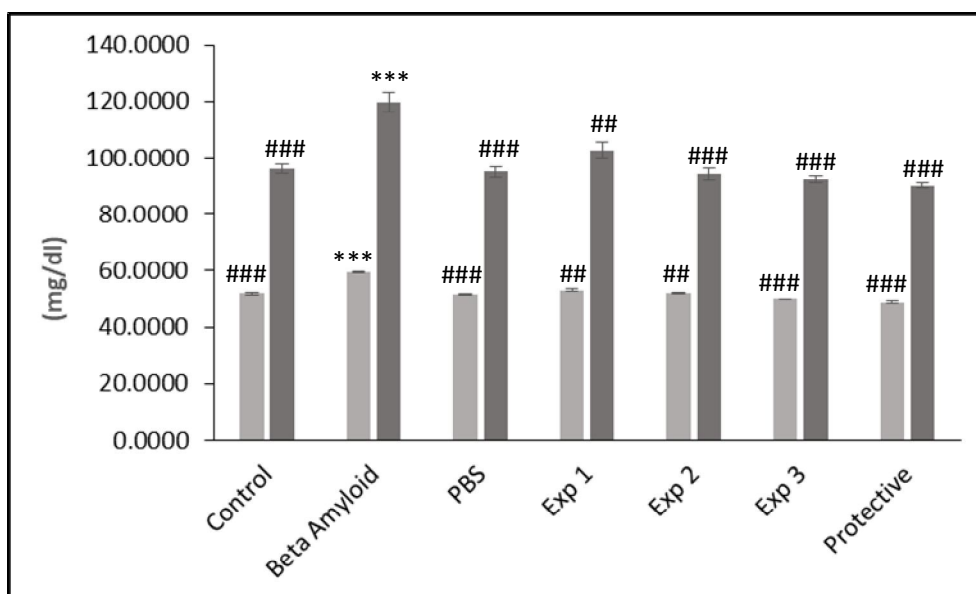


Fig. 2. Overall serum levels of cholesterol and TG in different groups. Cholesterol: light gray; TG: dark gray. ***: $p < 0.001$ compared with control group. #: $p < 0.01$ and ###: $p < 0.001$ compared with the Alzheimer's disease group receiving β -amyloid. Please refer to the Methods section for groups' definition.

allowed to clot for 30 min at room temperature and centrifuged at $1000 \times g$ at $37^\circ C$ for 10 min to separate the serum. Levels of HDL, LDL, TG, cholesterol, ALT, AST, ALP and SOD were determined.

Histological Study

At the end of experiment, the rats were decapitated under anesthesia and their brain were removed for histological assessments. The brains were fixed in 10% formalin and consequently embedded with paraffin. Serial sections of $6 \mu m$ -thickness were then prepared and stained with hematoxylin and eosin. The slides were examined by light microscopy. Furthermore, Thioflavin-S method was

applied for staining of amyloid plaques [10], in hippocampus cells, for which photomicrographs were prepared and amyloid plaques were counted using ImageJ 1.8.0_112 software.

Statistical Analysis

Data were presented as mean \pm S.D. and analyzed by SPSS version 22 software. Statistical analyses were performed using one-way ANOVA. Tukey multiple comparison tests were used to analyze the significance of the differences between the groups, when appropriate. P values less than 0.05, 0.01 and 0.001 were considered significant.

RESULTS AND DISCUSSION

Effects of Nerol Treatment and Protection on Biochemical Factors

A few factors of lipid profile were first evaluated. As shown in Fig. 2, the level of cholesterol was significantly high in the disease-induced group, injected with β -amyloid, compared to the control group ($p < 0.001$). However, when the disease group were treated with 60 and 90 mg kg⁻¹ of nerol or receiving 60 mg kg⁻¹ of nerol in a protective mode, the amount of cholesterol was significantly lowered ($p < 0.001$). The serum level of TG was also considerably higher in the AD-induced group compared to the control group ($p < 0.001$). On the other hand, as compared to the disease group, TG level was notably improved in the groups treated with 60 and 90 mg kg⁻¹ of nerol as well the protective group receiving 60 mg kg⁻¹ of nerol before AD induction ($p < 0.001$) (Fig. 2).

Regarding the serum level of LDL, it was high in the disease-induced group compared to the control group ($p < 0.01$) while its level was notably lowered in the groups treated with 60 and 90 mg kg⁻¹ of nerol as well as the group receiving 60 mg kg⁻¹ nerol in the protective mode compared to the AD-induced group ($p < 0.01$) (Fig. 3). With respect to HDL, only the group treated with 90 mg kg⁻¹ nerol showed the most increase in the HDL level compared to the disease-induced group ($p < 0.01$), in which the HDL level was notably lower than the control group ($p < 0.01$). The group receiving nerol in a protective mode also demonstrated an increase in HDL level ($p < 0.01$) (Fig. 3).

The activity of several enzymes, including SOD, AST, ALT, ALP were also assessed before and after treatment with nerol. All groups receiving therapeutic or protective amount of nerol showed significant increase in SOD activity compared to the disease-induced group, in which SOD activity was notably lower than the control group ($p < 0.01$) (Fig. 4). As shown in Fig. 5A, the activity of AST was significantly high in the disease-induced group compared to the control group ($p < 0.01$), whereas treatment with all three doses of nerol caused significant reduction in AST activity ($p < 0.05$). Interestingly, the group receiving protective dose of nerol (60 mg kg⁻¹) demonstrated the lowest activity of AST. Meanwhile, the activity of ALT was significantly high in the disease-induced group compared

with the control group ($p < 0.001$), whereas treatment and protection with all three doses of nerol caused significant reduction in ALT activity ($p < 0.001$) (Fig. 5B). Also, the activity of ALP was significantly high in the disease-induced group compared with the control group ($p < 0.001$), whereas treatment with the highest dose of nerol (90 mg kg⁻¹) reduced its activity significantly ($p < 0.001$) (Fig. 5C). Interestingly, the group receiving protective dose of nerol (60 mg kg⁻¹) demonstrated the lowest activity of ALP.

Amyloid Plaque Formation

Amyloid plaques were investigated by Thioflavin S staining, which results into a fluorescence in amyloid plaques that could be distinguished as bright spots (Fig. 6). Upon intra-hippocampal injection of A β 42, a high number of plaques had formed in the brain tissue of disease-induced group compared with the control group ($p < 0.001$) (Figs. 6C and H). Nerol consumption at all doses in both treatment and protective modes caused a significant reduction in the plaque numbers compared to the AD-induced group ($p < 0.001$) (Fig. 6).

Histological Studies

In the histological study, no statistical studies were performed and studies were only microscopic observations on the effect of nerol treatment on the number of pyramidal neurons of the CA1 region of the hippocampus. By looking at the resulting images in Fig. 7, it seemed that the Alzheimer's group faced with reduction of neuronal number compared to the control group. Improvement in the number of neurons was clear in the groups treated with 60 and 90 mg kg⁻¹ of nerol as well as the group receiving protective dose of nerol (60 mg kg⁻¹).

Passive Avoidance Test

Alzheimer's-induced group showed loss of long-term memory by entering the dark chamber on an average time of 35 s. However, all experimental groups being treated with different doses of nerol showed significantly longer step-through latency compared to the Alzheimer's group ($p < 0.001$) in the test day (Fig. 8). The group that received nerol (60 mg kg⁻¹) in a protective mode also exhibited improved long memory by showing longer step-through

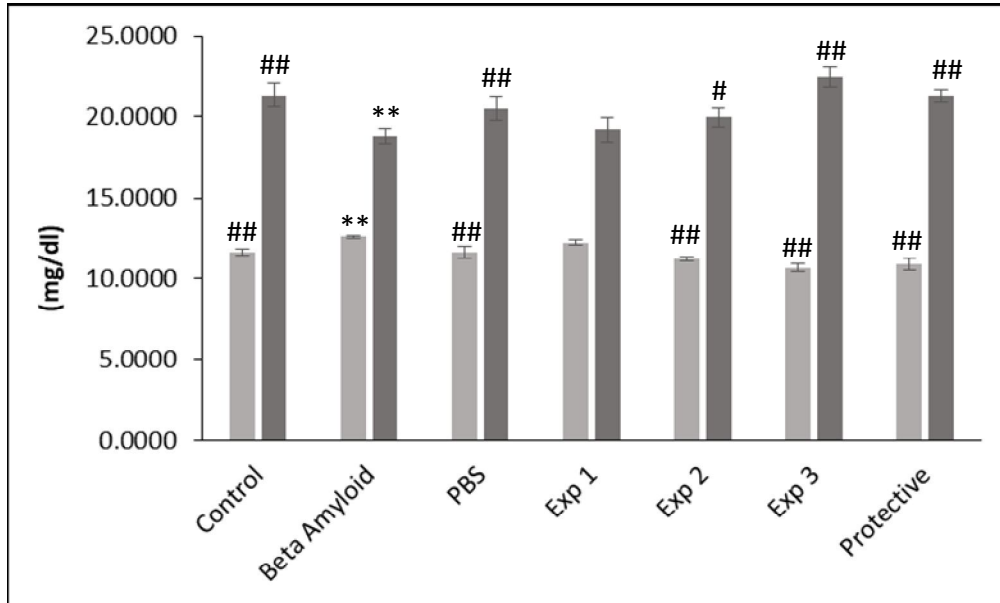


Fig. 3. Overall serum levels of LDL and HDL in different groups. LDL: light gray; HDL: dark gray. * $p < 0.05$ compared with the control group. #: $p < 0.05$ and ##: $p < 0.01$ compared with the Alzheimer's disease group receiving β -amyloid. Please refer to the Methods section for groups' definition.

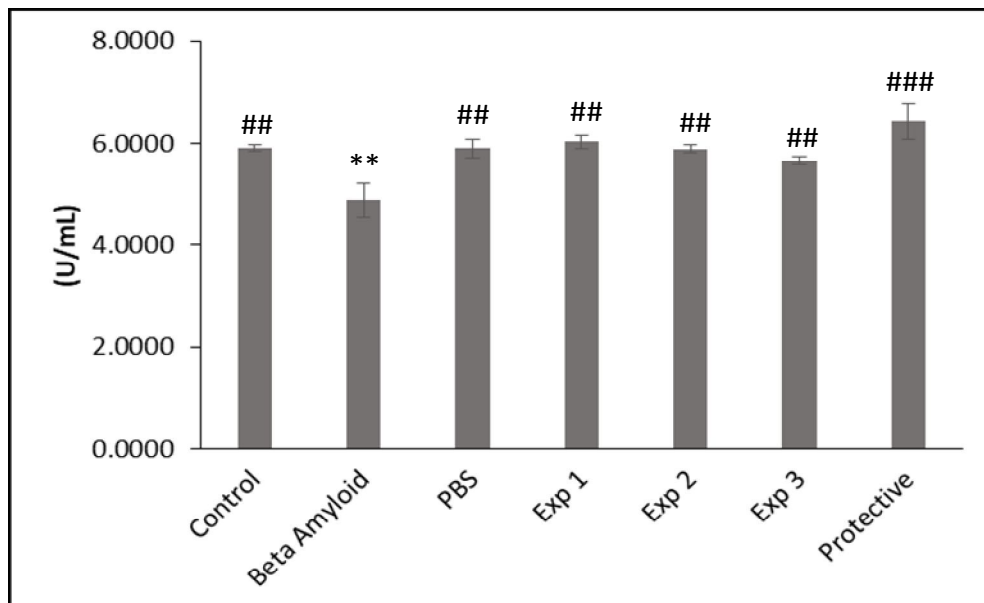


Fig. 4. Overall serum activity of SOD in different groups. ** $p < 0.01$ compared with the control group. ##: $p < 0.01$ and ###: $p < 0.001$ compared with the Alzheimer's disease group receiving β -amyloid. Please refer to the Methods section for groups' definition.

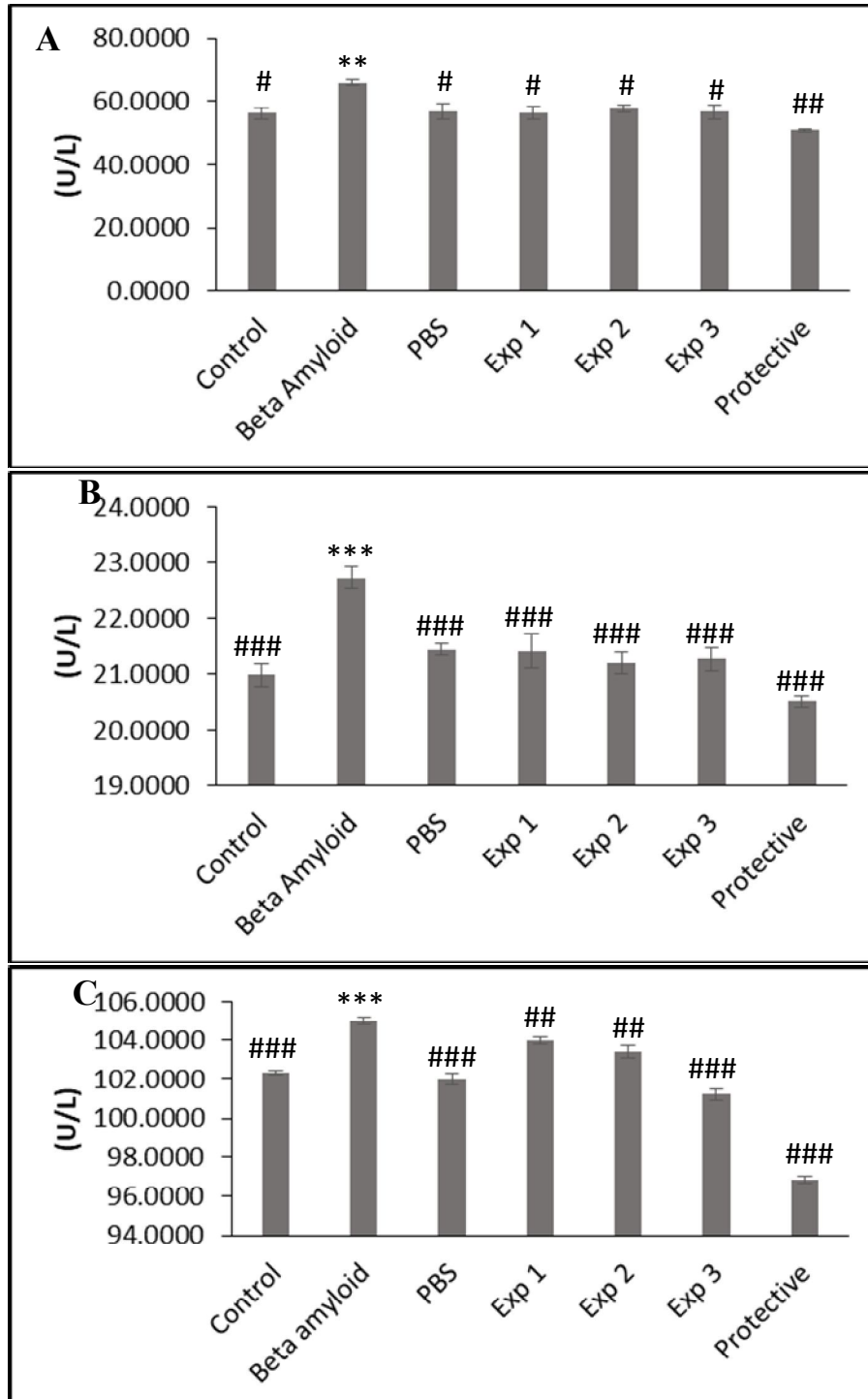


Fig. 5. Overall serum levels of AST, ALT, and ALP in different groups. (A) AST; (B) ALT; (C) ALP. ** $p < 0.01$ and ***: $p < 0.001$ compared with the control group. #: $p < 0.05$, ##: $p < 0.01$, and ###: $p < 0.001$ compared with the Alzheimer's disease group receiving β -amyloid. Please refer to the Methods section for groups' definition.

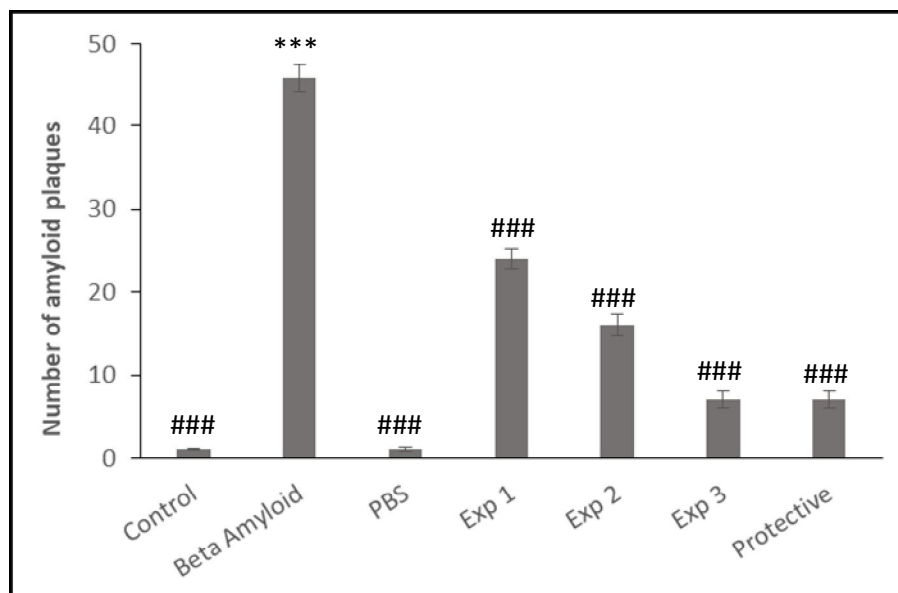


Fig. 6. Number of amyloid plaques in the hippocampus CA1 region. Please refer to the Methods section for groups' definition.

latency compared to the Alzheimer's group ($p < 0.001$).

DISCUSSION

Although the exact pathogenesis of AD is still under study, several observations have proved that A β plays an important role in the pathophysiology of AD and the neurodegeneration process that occurs in the disease course [2]. A β toxicity occurs after fibril formation and its oxidative toxicity on neuronal cells is subsequently a principal event in AD [11]. Many studies have worked on the inhibitory effects of small molecules on A β fibril formation [12]. Many reports have also demonstrated that the inhibitory effects of natural products on A β fibril formation are related to their antioxidative properties [13]. Nerol is the main monoterpene found in the essential oil of bitter orange. This compound has shown several biological properties, including anti-cancer and antioxidant activities [14]. Based on the facts that nerol is an aromatic compound with antioxidant properties, it was thus hypothesized to have anti-amyloid property.

Alzheimer's disease has shown to affect the vital brain cells, and consequently memory, thinking, and behavior

[15] and many studies have been performed on A β aggregation [2] and its derivatives [12,16]. Specifically, injections of A β 35 and A β 40 have shown to cause memory defects [17]. In agreement with the present study, Rasoolijazil *et al.* reported that A β 41 injection in rat's hippocampus can lead to neuronal degeneration [18]. It has been documented that the process of aging is associated with memory impairment [2]. In the present study, injection of A β 42 into the rat's hippocampus also resulted in neuronal degradation and reduction of antioxidant strength leading to the formation of amyloid plaques. Applying nerol to AD-induced rats exhibited a significant reduction in the number of amyloid plaques, in both treated and protective modes, leading to significant improvement of learning and memory impairment, proven by the behavioral test.

In the present study, the level of SOD was decreased in AD-induced rats. SOD is considered to be one of the most active enzymes whose activity is sufficient for inactivation of superoxide anions produced during oxidative stress in cells [19]. Abramov *et al.* reported that beta amyloid enters the nerve cells and produces oxygen free radicals which ultimately leads to oxidation of proteins and lipid peroxidation [20]. In aging brain, the memory impairment is

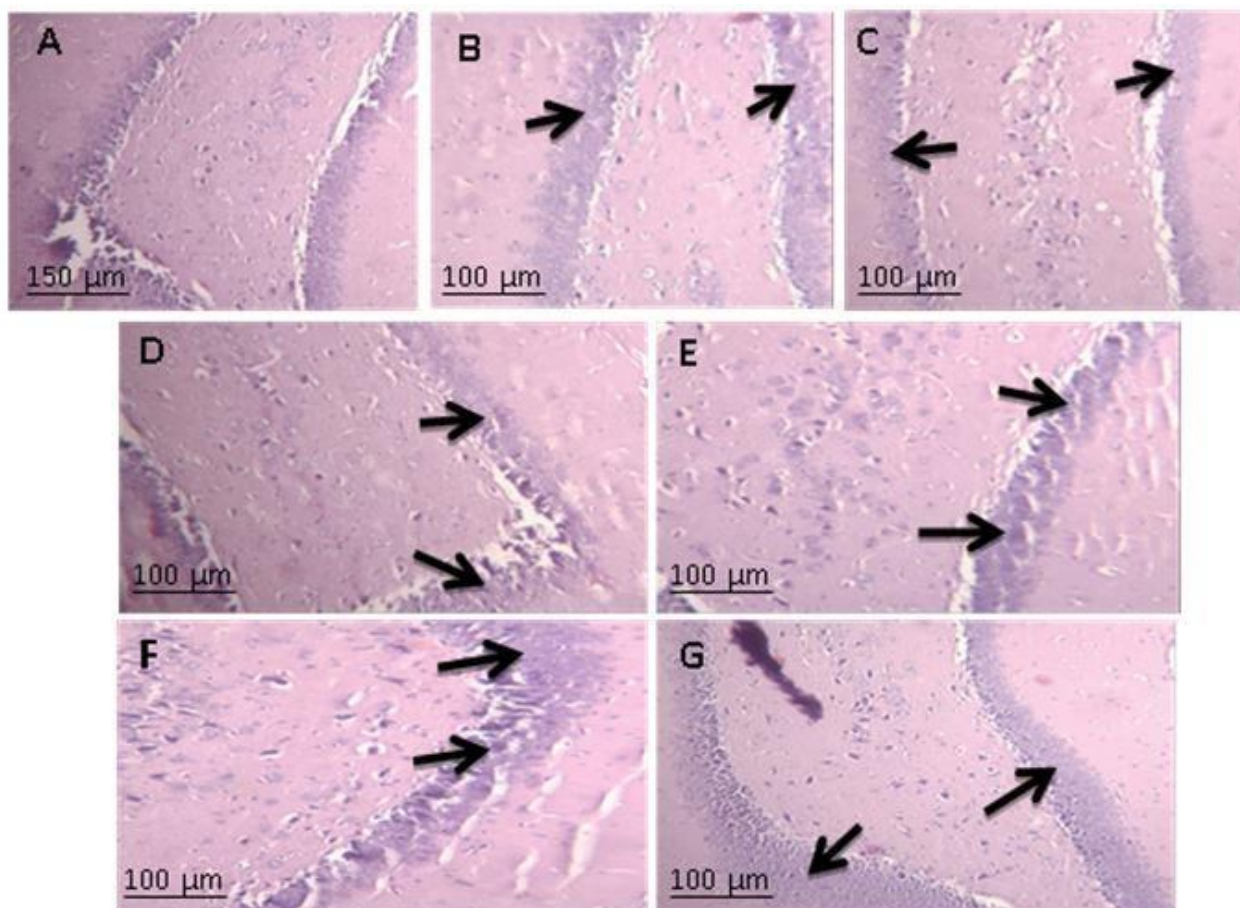


Fig. 7. H & E staining of pyramidal cells in the sagittal sections of hippocampus CA1 region. A: Control group, B: PBS group, C: Beta Amyloid, D: group treated with 30 mg kg⁻¹ nerol, E: group treated with 60 mg kg⁻¹ nerol, F: group treated with 90 mg kg⁻¹ nerol, G: group receiving protective dose of nerol (60 mg kg⁻¹). Black arrows represent the pyramidal cells in the tissue.

also thought to be due to an increased oxidative stress [21] causing the development and progression of Alzheimer's disease [22]. Both aged humans and rodents have demonstrated cognitive impairment being correlated to the accumulation of oxidative damage to lipids, proteins, and nucleic acids [23]. With respect to this fact, it has been shown that injection of intra-hippocampal A β leads to reduced antioxidant activity [24]. Herein, nerol demonstrated the ability of increasing SOD level and therefore could offer protection against A β -induced oxidative stress in the hippocampus.

Linalool, a monoterpene with similar structure to nerol, has also shown to reverse cognitive deficits [25,26] and has

altered the level of the antioxidant (SOD) activity in mice injected with A β [25]. Accordingly, myrtenol, a phenolic monoterpene, has shown to increase the levels of SOD significantly [27]. Previous studies have shown that phenolic monoterpenes, such as thymol could increase the antioxidant potential [28]. Terpenes such as carvacrol, γ -terpinene, *p*-cymene, and thymol have also shown antioxidant effect *in vitro* [29]. The antioxidant of carvacrol has been further confirmed by another study [30]. Moreover, antioxidant activity of monoterpenoids α - and β -pinene, and 1,8-cineole have been shown in the work of Perry and colleagues [30].

Lipid profile, including levels of TG, LDL and

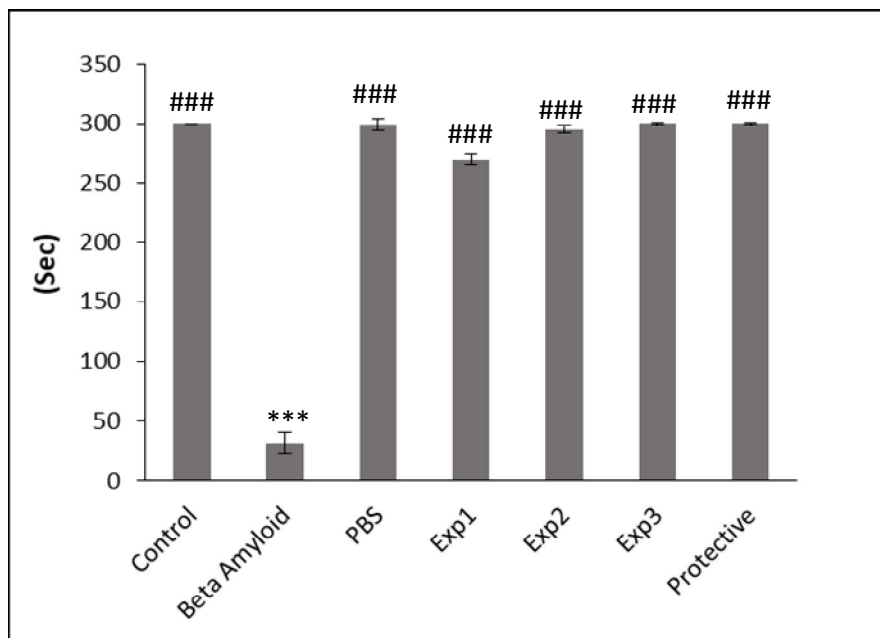


Fig. 8. The mean latency to enter the dark chamber on the test day. ***: $p < 0.001$ compared with control group. ###: $p < 0.001$ compared with the Alzheimer's disease group receiving β -amyloid. Please refer to the methods section for groups' definition.

cholesterol were impaired in AD rats. Presečki *et al.* have reported that TG could be involved in the progress of AD. They have further demonstrated alterations in serum lipid levels, including TG, LDL, HDL and cholesterol in patients with AD and have shown an association between serum cholesterol and LDL-C and severity of the AD. They have concluded that dementia itself modifies lipid levels, due to the changes in diet or metabolism [31]. Shepardson *et al.* also reported high cholesterol levels with the development of AD neuropathology. Regarding the possible mechanism that could connect high cholesterol levels with the development of AD neuropathology, they have suggested that cholesterol may increase the activity of the β - or γ -secretase enzymes that generate $A\beta$ from APP [32]. Another study has suggested that cholesterol levels could influence $A\beta$ aggregation state in the following course: under low-cholesterol conditions, increased interactions of $A\beta$ with the cell membrane may allow greater internalization and degradation of $A\beta$; on the contrary, high levels of cholesterol could cause $A\beta$ dissociation from the cell surface, leading to $A\beta$ aggregation in the extracellular

space [33]. Reed *et al.* have also demonstrated that higher levels of LDL and lower levels of HDL were both associated with brain amyloid. They have suggested that one possible mechanism could be the fact that there is minimal or no exchange of HDL and LDL particles, respectively, across the blood-brain barrier [34]. In accordance with our study, it was found that the use of thymol oil containing thymol and carvacrol demonstrated significant effects on serum levels of TG and cholesterol over a period of 29 days [35].

Nerol could also improve the levels of AST, ALT and ALP enzymes whose levels become affected in disease conditions [36]. AST and ALT are the two aminotransferases of great clinical significance as the serum elevations of these two aminotransaminases may occur with a variety of disorders; thus their assessment could be considered as a disease biomarker [37,38]. Their mechanisms of action have not been shown; however, Riemenschneider and colleagues have suggested that increased aspartate aminotransferase (AST) activity in the cerebrospinal fluid could be used as a marker for

Alzheimer's disease [39]. It was demonstrated that geraniol, a monoterpene with a similar structure to nerol, could effectively improve the levels of AST, ALT and ALP [40].

A research has reported that a few compounds with similar structures, including ginsenosides from *P. ginseng*, ginkgolides and bilobalide from *G. biloba*, and cannabinoids from *C. sativa* have demonstrated beneficial effects against AD by *in vitro* cell-based assays and *in vivo* animal studies [41]. Accordingly, the two terpenes ginkgolides and bilobalide are believed to contain anti-oxidation property as well as protection against amyloidogenesis and A β aggregation [42]. The same result have been reported for the terpene lactones Ginkgolides A, B, C, J and M and bilobalide with the capability to reduce aggregation [43]. Furthermore α - and β -pinene, 1,8-cineole and the monoterpenoid geraniol have relevant properties in treating Alzheimer's disease [30]. Monoterpenes represent a large group of naturally occurring organic compounds whose derivatives exhibit various activities, including analgesic, anti-inflammatory, anticonvulsant, antidepressant, anti-Alzheimer, anti-Parkinsonian, antiviral and antibacterial effects [44]. Therefore, they can play an important role in the creation of new biologically active compounds including drugs.

CONCLUSIONS

It has been demonstrated that the intra-hippocampal injection of A β 42 induced significant learning deficits and nerol administration, in a dose-dependent mode, significantly improved long-term memory. More importantly, nerol could offer protection at a lower dose compared to being used as treatment. Antioxidant and anti-plaque activities may be the mechanisms contributing toward nerol beneficial effects. In this case, valuable therapeutic and protective potential for nerol have been shown in counteracting memory and neuronal cell loss associated with AD, and the compound seems interesting enough to be further investigated.

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University. Authors declare no conflict of study.

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