

Rosmarinic acid mitigates learning and memory disturbances in amyloid β (25–35)-induced model of Alzheimer's disease in rat

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Background and Objective: Alzheimer's disease (AD) is a weakening neurodegenerative disorder typified by increased β -amyloid (A β) deposition and neuronal dysfunction causing to impaired learning and memory. Among proposed risk factors, induced oxidative stress is a main cause for incidence of the disease. The aim of this study was to determine the effects of the rosmarinic acid on learning and memory impairments with emphasis on its antioxidant properties.

Materials and Methods: In the present study, the effect of the intraperitoneally administration of rosmarinic acid (10 and 20 mg/kg) on learning and memory impairments was assessed in intrahippocampal A β (25–35)-injected rats.

Results: The results showed that the intrahippocampal A β (25–35)-injected rats exhibit lower spontaneous alternation score in Y-maze tasks ($p < 0.005$) and impaired retention and recall capability in the passive avoidance test ($p < 0.05$). Rosmarinic acid at both doses significantly improved alternation score in Y-maze task ($p < 0.05-0.001$) and restored retention and recall capability in the passive avoidance test only at a dose of 20 mg/kg ($p < 0.005$). Meanwhile, measurements of oxidative stress markers in hippocampal tissue of A β -injected rats showed significant elevation of malondialdehyde (MDA) and nitrite content ($p < 0.05-p < 0.01$) and rosmarinic acid significantly attenuated the increased MDA and nitrite content ($p < 0.05$) but did not affect SOD activity.

Conclusion: This study indicates that rosmarinic acid pretreatment ameliorates A β -induced impairment of learning and memory in rats via attenuation of oxidative stress burden.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that progressively affects memory and learning, correct assessment, communication with others, and proper behaviors (1). It has been shown that the population older than 65 years is disturbed by AD (2). Marked pathological features of AD include deposition of senile plaques comprising from β -amyloid and the occurrence of intracellu-

lar neurofibrillary tangles in hippocampal and cerebral cortex. In AD, neuronal deterioration in the hippocampus and amygdala affect learning and memory abilities (3,4). The main causes of the AD incidence are dysfunction of cholinergic system, accumulation of free radicals, amyloid deposition, initiation of inflammatory cascade, excitotoxicity and steroid hormones inadequacy (2). Amongst them, amyloid deposition has a key role in development of AD (5). It seems that oxidative

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stress resulting from lipid peroxidation and accumulation of free radicals is necessary for neurotoxicity due to β -amyloid (6). Based on oxidative stress hypothesis, antioxidants could mitigate the AD-induced learning and memory disorders in rats (7) and slow down its clinical progression in humans (8).

In the search for new drugs to treat age-related neurodegenerative diseases such as AD, attention has been focused to some extent on the potential neuroprotective effect of polyphenols (9). A large number of biological activities have been described for rosmarinic acid. Its chief activities are antioxidative, anti-inflammatory, anti-mutagen, anti-bacterial and anti-viral potential. The anti-inflammatory properties of rosmarinic acid are based on the inhibition of lipoxygenases and cyclooxygenases and the its intervention with the complement cascade. Rosmarinic acid is rapidly eliminated from the blood circulation and shows a very low toxicity after intravenous administration. Rosmarinic acid can provide protection against cancer and contributes to the anti-oxidant activity of plants used in the cosmetic industry (10). In this study, we examined the efficacy of acute rosmarinic acid pretreatment on alleviation of β -amyloid-induced deficits in learning and memory with emphasis on its antioxidative role.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (Pasteur's Institute, Tehran), weighing 200–250 g at the start of the experiment were housed three to four per cage in a temperature-controlled colony room under light/dark cycle. Animals were given free access to tap water and standard rat chow. All behavioral experiments were carried out between 10 a.m. and 4 p.m. This study was conducted according to guidelines of Research Council of Iran University of Medical Sciences (Tehran, Iran).

2.2. Chemicals

All chemicals excluding those sources indicated in the text were procured from SigmaAldrich (USA) and Merck (Germany).

2.3. Experimental procedure

Rats ($n = 42$) were randomly allocated to the following groups: (1) sham (SH; $n = 6$); (2)

Rosmarinic acid treated-sham (SH-ROS₂₀; $n = 6$); (3) A β injection (A-beta; $n = 10$); and (4,5) rosmarinic acid treated-A β injection (A-beta-ROS10 and -ROS20; $n=10$ each). For stereotaxic surgery, rats were anesthetized with a combination of ketamine (100 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.) and then placed in a Stoelting stereotaxic apparatus (incisor bar -3.3 mm, ear bars positioned symmetrically). Animals in the A β group were bilaterally injected in the dorsal hippocampus at coordinates of -3.5 mm posterior to bregma, 2 mm lateral to sagittal suture, and 2.8 mm below dura, according to the stereotaxic atlas (Paxinos & Watson, 1986) with a solution containing 10 mg aggregated A β (25-35) (5 mg/ μ l) or 0.9% normal saline (sham-operated) of the same volume. To produce neurotoxicity, saline-diluted A β (25-35) was incubated at 37 °C for 7 days to allow fibril formation. The amount of A β (0.5 nM/ μ l dissolved in 0.9% normal saline; pH = 8.0) was chosen based on our earlier experiment and the solution was prepared according to a previously described protocol (11). Rosmarinic acid was dissolved in 10% Cremophor and administered intraperitoneally at doses of 10 and 20 mg/kg body weight one hour before surgery. Post-operatively, the rats were given special care until spontaneous feeding was restored. Behavioral tests were conducted two weeks after the surgery and were evaluated blind to the treatments by the observer.

2.4. Y-maze task

Spatial recognition memory was assessed by recording spontaneous alternation behavior in a single-session Y-maze on the 14th day post-surgery, as described elsewhere (12). The maze was made of black Plexiglas. Each arm was 40 cm long, 30 cm high and 15 cm wide. The arms converged in an equilateral triangular central area that was 15 cm at its longest axis. The procedure was as follows: each rat, naive to the maze, was placed at the end of one arm and was allowed to move freely through the maze during an 8-min session. The series of arm entries were recorded visually. Entry was considered to be complete when the base of the animal's tail was entirely within the arm. Alternation was defined as successive entries into the three arms on overlapping triplet sets. The maximum number of possible spontaneous alternations was determined as the total number of arms entered - 2, and the percentage was calculated as the ratio of actual to possible alternations $\times 100$.

2.5. Single-trial passive avoidance test

The apparatus (40 cm long; 20 cm wide; 30 cm high) consisted of an illuminated chamber connected to a dark chamber by a guillotine door. Electric shocks were delivered to the grid floor by an isolated stimulator. On the first and second days of testing, each rat was placed in the apparatus for 15 min to habituate. On the third day, an acquisition trial was performed. Rats were placed individually in the illuminated chamber. After a habituation period (5 min), the guillotine door was lifted, and, after the rat had entered the dark chamber, the door was lowered and an inescapable scrambled single electric shock (1 mA, 1 s) was delivered. In this trial, the initial latency (IL) of entrance into the dark chamber was recorded, and all rats had ILs greater than 60 s and were included in the study. Twenty-four hours later, each rat was placed in the illuminated chamber for retention trial. The interval between placement in the illuminated chamber and entry into the dark chamber was measured as step-through latency (STL, up to a maximum of 300 s). This test was conducted on 17-20 days after surgery.

2.6. Determination of hippocampal MDA concentration

The rats were anesthetized with ketamine (100 mg/kg) and decapitated. Hippocampal blocks were isolated and blotted dry and then weighed and prepared as a 5% tissue homogenate in ice-cold 0.9% saline solution. After centrifugation ($1000 \times g$, 4 °C, 10 min), the supernatant was aliquoted and stored at -80 °C until assayed. The concentration of malondialdehyde (MDA), used as a marker of lipid peroxidation, was calculated by measuring thiobarbituric acid reactive substances (TBARS) in the supernatant as described previously (13). Briefly, trichloroacetic acid and TBARS reagent were added to aliquots of the supernatant, which were subsequently mixed and incubated at 100 °C for 80 min. After cooling on ice, the samples were centrifuged at $1000 \times g$ for 10 min, and the absorbance of the supernatant was read at 532 nm. The results of TBARS measurements were expressed as MDA equivalents, using tetraethoxypropane as standard.

2.7. Measurement of hippocampal SOD activity

The supernatant of hippocampal homogenate

was obtained as described above. Superoxide dismutase (SOD) activity was measured as previously reported (13).

Briefly, supernatant was incubated with xanthine and xanthine oxidase in potassium phosphate buffer (pH 7.8, 37 °C) for 40 min, and then nitroblue tetrazolium (NBT) was added. Thereafter, blue formazan was monitored spectrophotometrically at 550 nm. The amount of protein that inhibited NBT reduction to 50% maximum was defined as 1 nitrite unit (NU) of SOD activity.

2.8. Assay of hippocampal nitrite concentration

Supernatant nitrite (NO₂⁻) content was assayed by the Griess method. The compound NO has a short half-life and is rapidly converted to the stable end products nitrate (NO₃⁻) and NO₂⁻. In the assay used here, NO₃⁻ is converted to NO₂⁻ by cadmium, and this is followed by color development with Griess reagent (sulfanilamide and N-naphthyl ethylenediamine) in acidic medium. The absorbance was determined using a spectrophotometer at 540 nm.

2.9. Protein assay

The protein content of the supernatant was measured by the Bradford method, using bovine serum albumin as the standard (14).

2.10. Statistical analysis

All results were expressed as mean \pm S.E.M. The non-parametric Kruskal-Wallis test was used to analyze the behavioral tests, and if a difference was found to be significant, pair-wise comparison was done using the Mann-Whitney U-test. Parametric one-way ANOVA was used to assess the results of biochemical tests. In all calculations, a difference at $p < 0.05$ was regarded as significant.

3. Results

3.1. Spatial recognition memory in Y-maze

Fig. 1 shows the results for the performance of rats in Y-maze task, in which short-term spatial recognition memory performance as alternation behavior can be examined. In this respect, the alternation score of the A β injected rats was found to be significantly lower ($11.92 \pm 2.03\%$)

as compared to the sham-operated group ($81.5 \pm 13.8\%$) at the end of the study ($p < 0.005$). Meanwhile, rosmarinic acid-treated A β injected rats at a dose of 10 and 20 mg/kg showed a higher alternation score ($27.9 \pm 4.71\%$ and $43.3 \pm 4.24\%$, respectively) as compared to A β group ($p < 0.05$ - $p < 0.001$). To assess compounding effect of locomotor activity on memory processes in experimental groups, total number of arms entered was considered as an index of locomotor activity. In this regard, there was no statistically significant difference between the A β injected rats (19.4 ± 1.96) as compared to the sham-operated group (14.7 ± 1.1). Meanwhile, the total number of arms entered showed no change at both doses of rosmarinic acid treated-A β -injected rats as compared to untreated A β -injected group.

3.2. Passive avoidance test

Fig. 2 shows the performance of rats in passive avoidance paradigm as indicated by STL. Regarding initial latency, there was no significant difference among the groups. In addition, A β -injected rats developed a significant impairment in retention and recall in passive avoidance test ($p < 0.05$), as it is evident by a lower STL. Rosmarinic acid treatment at a dose

of 20 mg/kg did produce an improvement in this respect ($p < 0.005$).

3.3. Markers of oxidative stress

A β -injected rats exhibited significantly elevated levels of MDA (23.5 ± 2.1 nmol/mg protein; $p < 0.01$) and nitrite (21.2 ± 1.6 nmol/mg protein; $p < 0.05$) in hippocampal tissue as compared to the sham-operated group (MDA, 13.4 ± 1.2 nmol/mg protein; nitrite, 12.7 ± 1.4 nmol/mg protein) (Figures 3–5). But there was no significant difference regarding SOD activity between sham and A β -injected rats (6.4 ± 1.1 and 9.1 ± 1.1 unit/mg protein, respectively). Pretreatment of A β -injected rats with rosmarinic acid at a dose of 20 mg/kg significantly attenuated the increased MDA content (16 ± 1.8 ; $p < 0.05$) and nitrite level (14.5 ± 1.7 ; $p < 0.05$) relative to sham-operated rats. However, the levels of SOD did not significantly change by rosmarinic acid treatment.

4. Discussion

Alzheimer's disease is one of the most prominent type of dementia that its major pathological hallmark is the production of senile plaques in the brain particularly in the cortex and

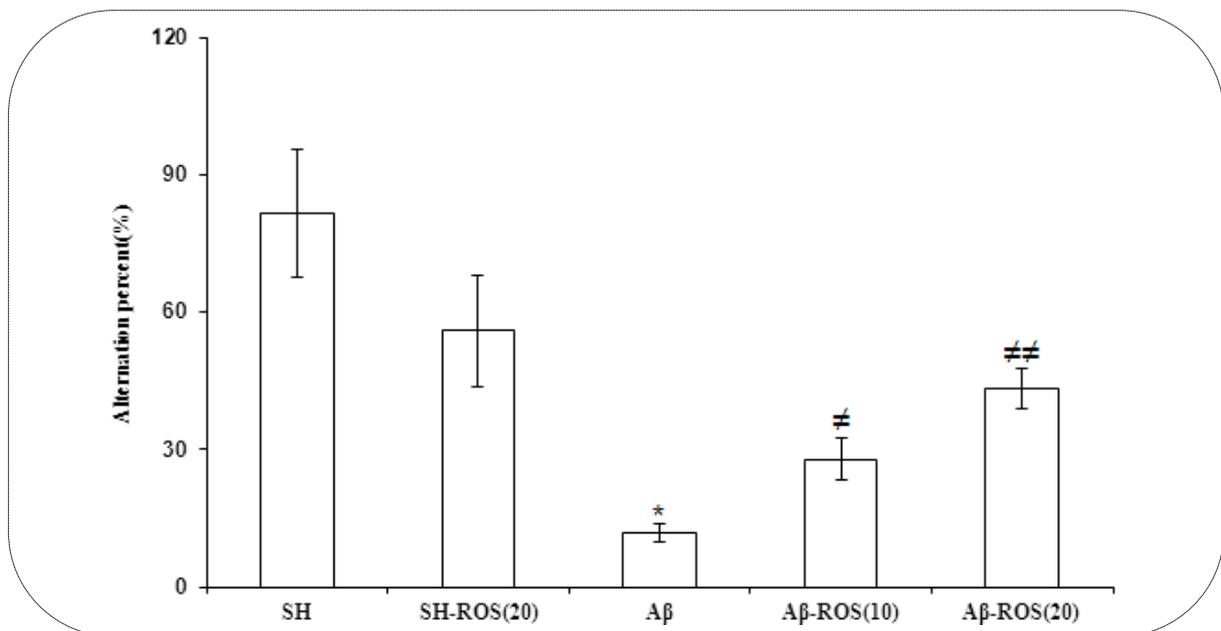


Figure 1. Alternation behavior displayed in the Y-maze by rats. Animals were pretreated with rosmarinic acid at doses of 10 and 20 mg/kg before bilateral hippocampal injection of A β (25-35); 10 μ g of aggregated A β ; 5 μ g/ μ l). Values are means \pm SEM.

* $P < 0.05$ and ** $P < 0.01$ indicate significant differences as compared to PTZ-kindled group.

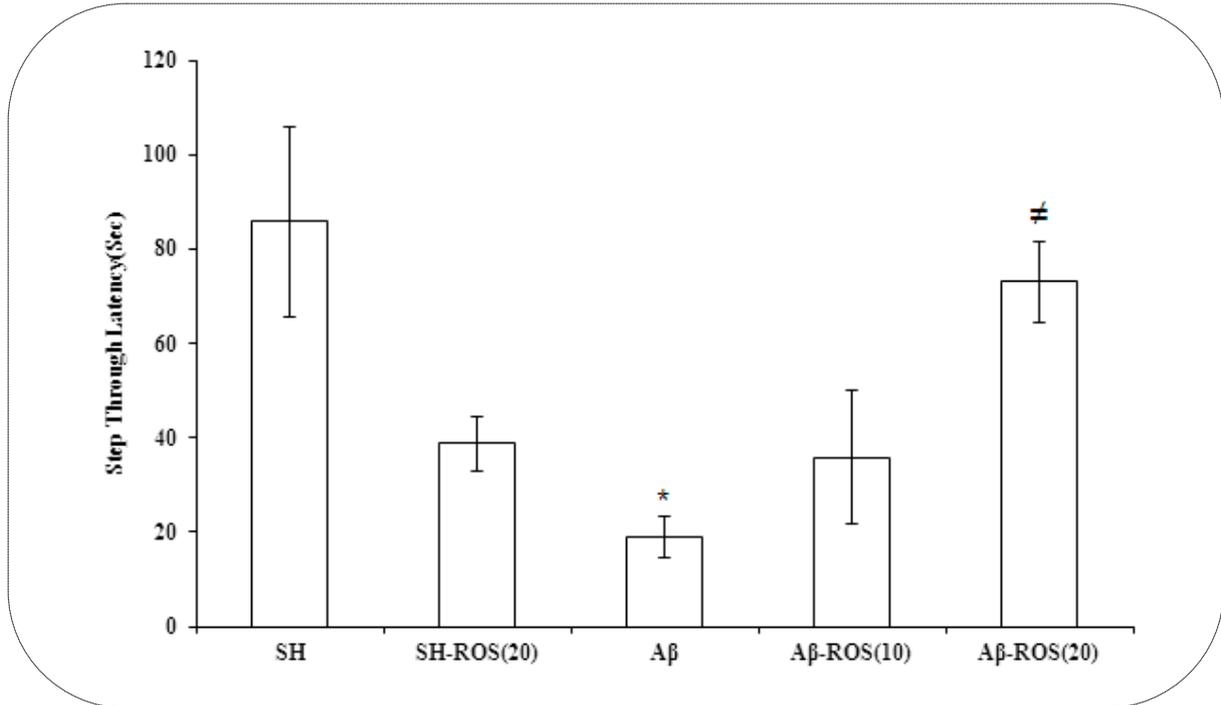


Figure 2. Step-through latency (STL) recorded in a single-trial passive avoidance test for rats. Animals were pretreated with rosmarinic acid at doses of 10 and 20 mg/kg before bilateral hippocampal injection of A β (25-35); 10 μ g of aggregated A β ; 5 μ g/ μ l). Values are means \pm SEM.
 * p<0.05 (vs. sham), # p<0.005 (vs. A-beta).

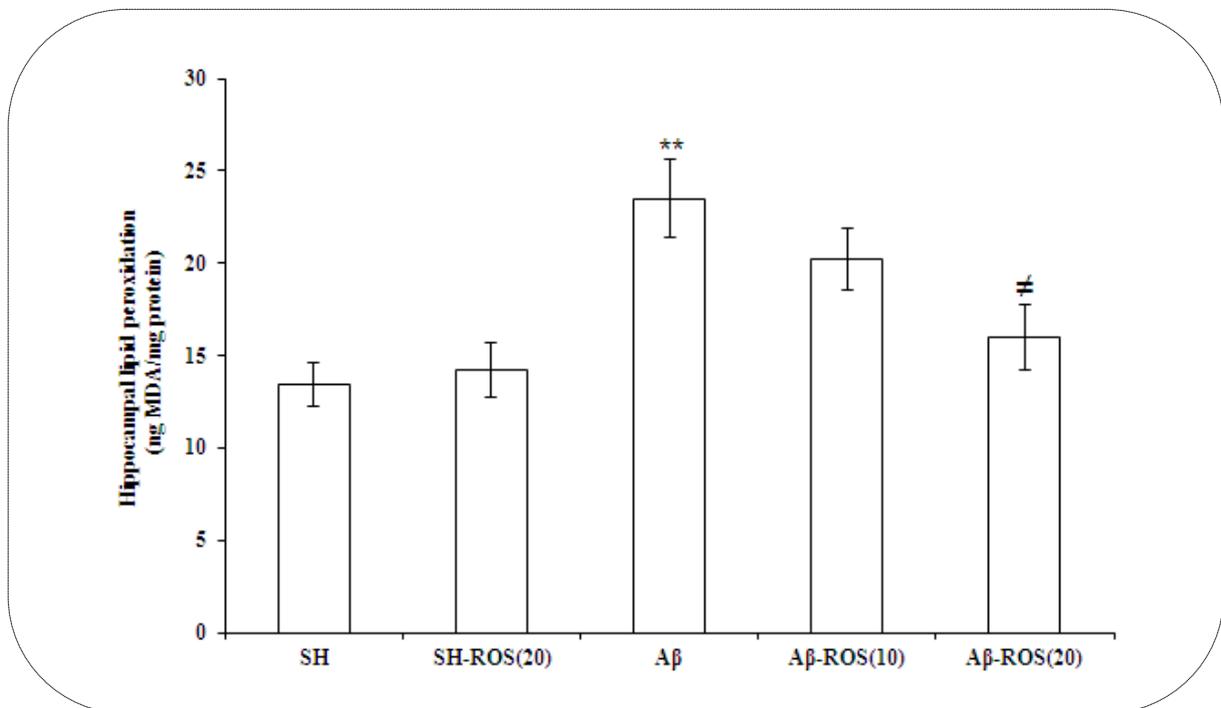


Figure 3. Malondialdehyde (MDA) content in hippocampal homogenate from different groups. Animals were pretreated with rosmarinic acid at doses of 10 and 20 mg/kg before bilateral hippocampal injection of A β (25-35); 10 μ g of aggregated A β ; 5 μ g/ μ l).
 * p<0.01 (vs. sham); # p<0.05 (vs. A-beta). (means \pm SEM)

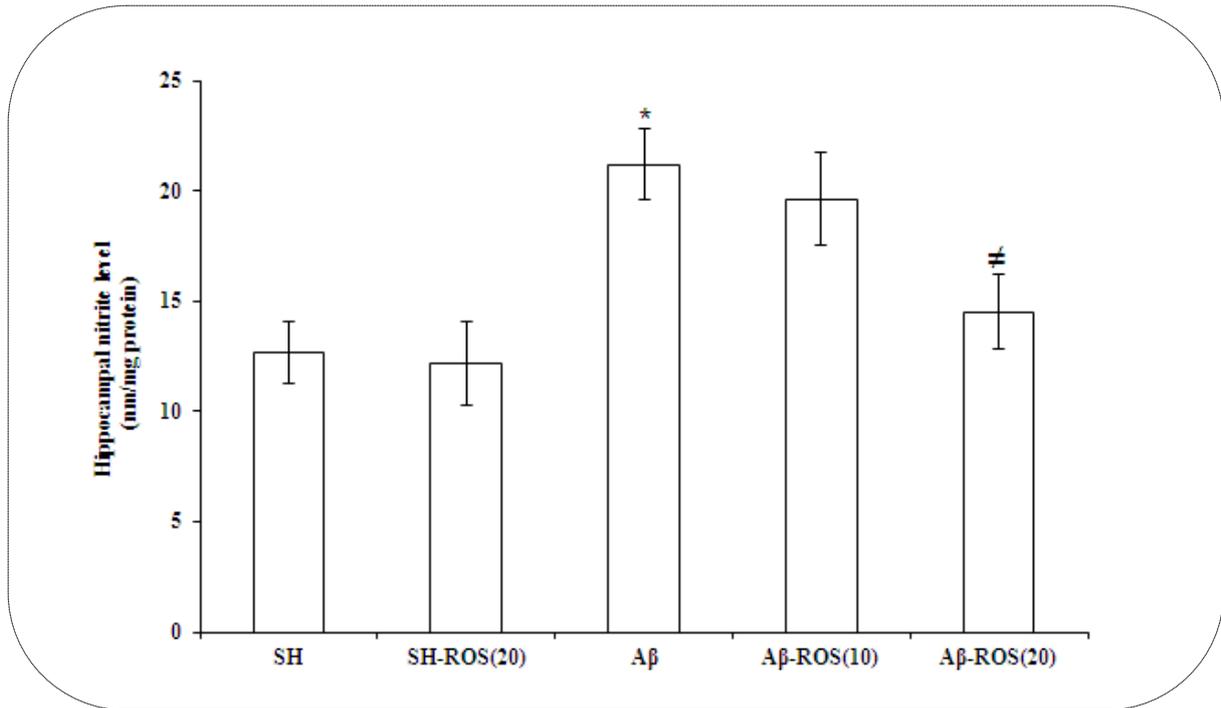


Figure 4. Nitrite content in hippocampal homogenate from different groups. Animals were pretreated with rosmarinic acid at doses of 10 and 20 mg/kg before bilateral hippocampal injection of A β (25-35); 10 μ g of aggregated A β ; 5 μ g/ μ l).

* $p < 0.05$ (vs. sham); # $p < 0.05$ (vs. A-beta). (means \pm SEM).

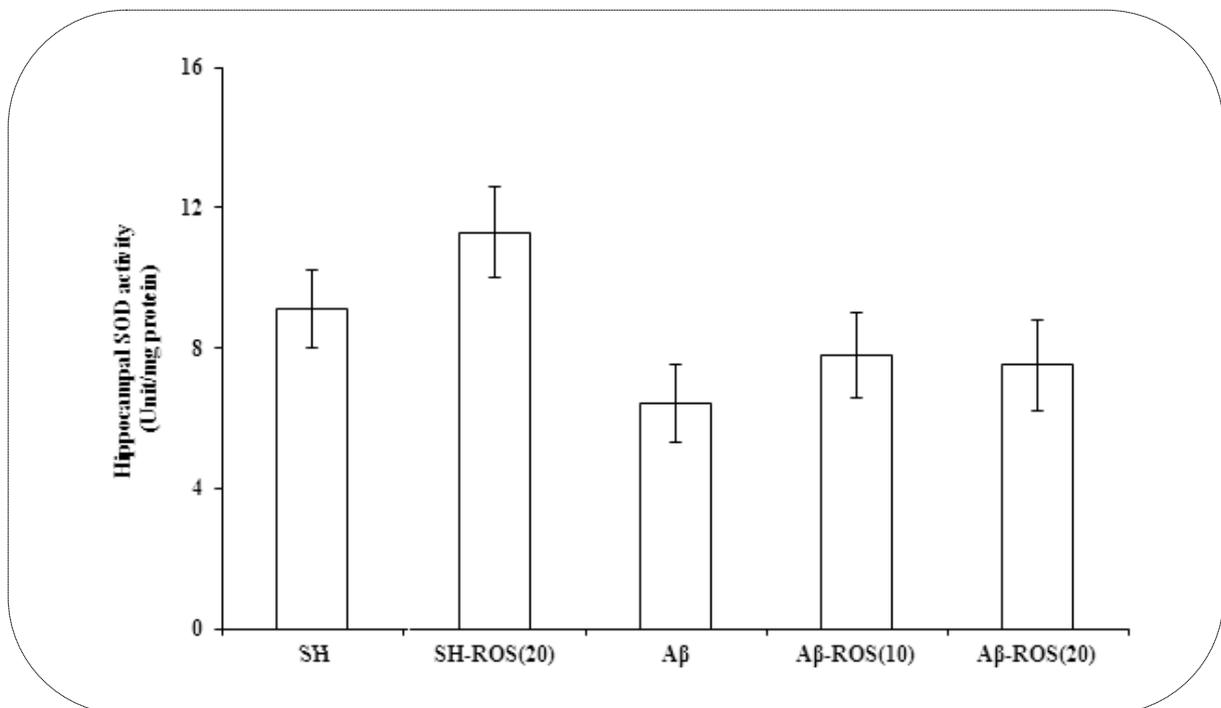


Figure 5. Superoxide dismutase (SOD) activity in hippocampal homogenate from different groups. Animals were pretreated with rosmarinic acid at doses of 10 and 20 mg/kg before bilateral hippocampal injection of A β (25-35); 10 μ g of aggregated A β ; 5 μ g/ μ l). (means \pm SEM)

hippocampus (15). The main component of senile plaques is amyloid β protein ($A\beta$), which derives from the proteolysis of amyloid precursor protein (APP) and consists of 39-43 amino acids (16). Experiments *in vivo* or *in vitro* have shown that while the full-length of $A\beta$ molecule is neurotoxic (17) but $A\beta_{25-35}$, a short synthetic fragment of $A\beta$ has the same neurotoxicity as that natural full-length of $A\beta$ molecule (18).

On the basis of "the amyloid cascade hypothesis", infusion of $A\beta$ into the cerebral ventricles resulted in neuronal dysfunction, neurodegeneration and impaired learning and memory (19, 20). In addition, $A\beta$ infusion decreases choline acetyltransferase activity in the cerebral cortex and hippocampus (21) and activates glial cells, seen as increased immunoreactivity for glial fibrillary acidic protein (22). Furthermore, following infusion of $A\beta$ into the hippocampus and cerebral cortex, marked reduction in nicotine- and/or KCl-induced release of acetylcholine as well as reduced dopamine release is observed in the striatum (23). These data suggest that infusion of $A\beta$ into the brain can affect different neuronal pathways. Regarding the neurodegenerative effect of $A\beta$, some investigators (7, 24) have found morphological signs of cell damage after $A\beta$ injection in the brain. In the current study, we observed impaired learning and memory in rats after bilateral injections of soluble $A\beta_{25-35}$ into the dorsal hippocampus, which agrees with the results of previous investigations (22).

Furthermore, pretreatment of rosmarinic acid improved the development of memory loss in the rats, and this enhancement may be related to the beneficial effect of rosmarinic acid on some of neuronal pathways.

Oxidative stress plays an important role in the development and progression of AD. $A\beta$ interacts directly with the mitochondria and induces production of free radicals, mitochondrial dysfunction, and cell death (25); for these reasons, antioxidants such as α -tocopherol have a protective effect against learning and memory deficits induced by $A\beta$ (7). Superoxide dismutase, glutathione peroxidase and catalase are the main enzymes involved in cellular protection against damage caused by oxygen-derived free radicals (26). In the present study, rosmarinic acid reversed the $A\beta$ -induced increase in MDA and nitrite production but it could not improve SOD activity, suggesting that the beneficial effect of

rosmarinic acid does not occur mainly via its antioxidant capacity. We can not however, exclude the possibility that longer pretreatment with rosmarinic acid might inhibit the induction of oxidative stress.

As mentioned earlier, $A\beta$ can lead to cholinergic dysfunction and cognitive impairment (27). Choline acetyltransferase and acetylcholinesterase are important for maintaining a stable level of acetylcholine in the brain. Flavonoids like rosmarinic acid can moderately inhibit acetylcholinesterase and in this way delay degradation of the neurotransmitter (28). Further research is needed to determine possible effects of rosmarinic acid on the cholinergic pathway in the rat model of AD used in our study. Rosmarinic acid can also inhibit the activity of tyrosine kinase (29), which is expressed extensively in hippocampus and is involved in the induction of long-term potentiation (LTP) that is the basis of learning and memory (30).

In conclusion, our results suggest that rosmarinic acid pretreatment by attenuation of oxidative stress prevents $A\beta$ (25-35)-induced impairment of short-term spatial recognition memory in a Y-maze and learning and memory in the passive avoidance test.

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