https://doi.org/10.31925/farmacia.2022.5.16 **ORIGINAL ARTICLE**

BEHAVIOURAL AND MOLECULAR STUDY OF THE EFFECTS OF ROSUVASTATIN ON ACQUISITION AND RETENTION OF SPATIAL MEMORY IMPAIRED BY H-89 IN RATS

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Abstract

There is controversy on the effect of statins on cognitive functions such as spatial memory. In the present study, effect of ten-day oral gavage of rosuvastatin (Ros, 20 mg/kg) on spatial learning and spatial memory retention impaired by H-89, was investigated in male rats. This study was comprised of two sets of experiments each including the following 3 groups (n = 8): Control group treated with DMSO; H-89 group received bilateral intra-hippocampal H-89 (10 µM/side, in DMSO) and Ros-H-89 group orally treated with Ros (20 mg/kg) and H-89 (similar to the H-89 group). For spatial learning (acquisition phase) assessment, from day 7 of Ros gavage, rats were trained in the Morris water maze (MWM) for four days (one block of 4 stages each day) and received daily H-89, 30 min after Ros gavage. On day 11, the probe test was performed. Also, to assess spatial memory retention, from day 7 to 10 of Ros gavage, rats were trained in MWM but received H-89 on day 10 only. On day 12, the probe test was performed. Besides, CREB and *p*-CREB protein expression was assessed in hippocampal samples and oxidative stress status was assessed in serum samples. We observed that H-89 led to a clear impairment of the spatial learning and spatial memory recall, increased levels of lipid peroxidation and downregulated CREB and *p*-CREB proteins, compared to the control group. However, Ros prevented H-89-induced deleterious consequences which might be probably in part due to its ameliorative effects on lipid peroxidation index and CREB and *p*-CREB expression.

Rezumat

Există controverse cu privire la efectul statinelor asupra funcțiilor cognitive precum memoria spațială. În studiul de față, efectul gavajului oral de zece zile cu rosuvastatină (Ros, 20 mg/kg) asupra învățării spațiale și asupra păstrării memoriei spațiale afectate de H-89, a fost investigat la șobolani masculi. Acest studiu a fost compus din două seturi de experimente, fiecare incluzând următoarele 3 grupuri (n = 8): grup de control tratat cu DMSO; grupul H-89 a primit H-89 intra-hipocampal bilateral (10 µM/parte, în DMSO) și grupul Ros-H-89 tratat oral cu Ros (20 mg/kg) și H-89 (similar cu grupul H-89). Pentru evaluarea învățării spațiale (faza de obținere), din ziua 7 a gavajului cu Ros, șobolanii au fost antrenați în labirintul de apă Morris (MWM) timp de patru zile (un bloc de 4 etape în fiecare zi) și au primit zilnic H-89, la 30 de minute după gavajul cu Ros. În ziua 11, a fost efectuat testul sondei. De asemenea, pentru a evalua păstrarea memoriei spațiale, din ziua 7 până la 10 de gavaj Ros, șobolanii au fost antrenați în MWM, dar au primit H-89 numai în ziua 10. În ziua 12, a fost efectuat testul sondei. În plus, expresia proteinei CREB și p-CREB a fost evaluată în probe de hipocamp și statusul stresului oxidativ a fost evaluat în probe de ser. Am observat că H-89 a condus la o afectare clară a memoriei spațiale, niveluri crescute de peroxidare a lipidelor și *down-reglarea* proteinelor CREB și p-CREB, comparativ cu grupul de control. Cu toate acestea, Ros a prevenit consecințele dăunătoare induse de H-89, care s-ar putea datora probabil parțial efectelor sale de ameliorare asupra indicelui de peroxidare a lipidelor și a expresiei CREB și p-CREB.

Keywords: rosuvastatin, protein kinase AII inhibitor, Morris water maze, spatial memory, protein markers

Introduction

Alzheimer's disease (AD) as a progressive neurodegenerative disease commonly seen in the elderly people, presents progressive cognitive and physical decline [4, 28, 36]. AD affects approximately 44 million people worldwide of which, 5.5 million are in the USA making AD the sixth-leading cause of deaths in the country [18]. AD is characterized by cerebral atrophy especially within the hippocampus and temporal and parietal lobes and accompanied by senile plaques, neurofibrillary tangles, and neuronal cell death [36]. Clinically, AD leads to loss of short-term memory and cognitive function [36]. AD molecular pathophysiology involves amyloid β (Aβ) peptide and tau hyperphosphorylation [21]. A considerable body of evidence confirmed that haemostasis of lipids metabolism has a strong effects on AD [4, 28, 37].

Statins have been widely used for management of neurodegenerative conditions like AD and Parkinson's disease [34]. The underlying mechanism of neuroprotective effects of statins is lowering cholesterol levels, decreasing Aβ production, antioxidant effects, decreasing serum apolipoprotein E levels, stimulating anti-inflammatory responses, modifying cognition-related receptors, and upregulating endothelial nitric oxide synthase [14, 28]. Furthermore, statins could downregulate mevalonate, targeting key cell signalling pathways that control proliferation including protein kinase A (PKA), cytokine production, and reactive oxygen species (ROS) generation [4, 28]. Nevertheless, some studies showed that statins have the ability to cause reversible cognitive impairment in some patient [19, 25, 36], but a meta-analysis of prospective cohort study showed that statins uses are associated with reduced risk of dementia [27]. In a study that employed neuroimaging for analysis of AD biomarkers in 1160 individuals, it was found that long-term (over 5 years) therapy with statins, was not associated with differences in AD biomarkers [22].

As a member of statins family, rosuvastatin (Ros) produced neuroprotective effects against cognitive impairment and attenuated neuroinflammation induced by high levels of salt and cholesterol, *via* repression of NF-ƙB pathways [12], improved learning and memory in diazepam-induced amnesia [7], and ameliorated age-associated memory impairment [23] and disorientation and amnesia caused by traumatic brain injury [32].

Our past studies confirmed that H-89, as a protein kinase A inhibitor, impairs memory in learning and retention phases and decreases CREB and *p*-CREB proteins [29, 31]. To the best of our knowledge, it is for the first time that Ros effects on memory deficit induced by H-89 with respect to cAMP/PKA/CREB/p-CREB signalling pathway and oxidative stress status, are reported.

Materials and Methods

Animals

In this study, 48 male Wistar rats (aged 8 - 10 weeks; 180 - 220 g body weight, obtained from Animal Centre of Zabol University of Medical Sciences, Zabol, Iran) were housed in stainless-steel cage, and kept under standard conditions (at 25°C with 12/12 h light/dark cycles) with free access to food and water, *ad libitum*. All animal experiments were approved by the Animal Research Ethics Committee of Zabol University of Medical Sciences and all procedures were carried out according to the guideline of National Institute of Health Guide for the Care and Use of the Laboratory Animals.

Materials

Rosuvastatin (Ros) was purchased from Actoverco (Slovenia). Dimethyl sulfoxide (DMSO) and H-89 were purchased form Sigma-Aldrich, St. Louis, MO, USA. Antibodies against cAMP response-element binding protein (CREB), *p*-CREB (serine 133), β-actin and secondary rabbit antibody were obtained from Cell Signaling (Beverly, Massachusetts, USA). Coomassie (Bradford) Protein Assay Kit was purchased from Thermo scientific (Rockford, Illinois, USA). Ketamine (Alfasan, Holland) and xylazine (Alfasan, Holland) were used for surgical anaesthesia.

Animals grouping and treatment

In this work, the rats were randomly divided into 2 sets of the following 3 groups (n = 8): (1) Control group – rats treated with DMSO; (2) H-89 group – rats administered with bilateral intra-hippocampal H-89 (10 µM/side, dissolved in DMSO); and (3) Ros-H-89 group – rats treated with H-89 and Ros (20 mg/kg, oral gavage for 10 days).

Surgery

Before initiation of the experiments, rats were anesthetized using ketamine (100 mg/kg, intraperitoneal (i.p.)) and xylazine (25 mg/kg, i.p.) and as described previously, guide cannulas were inserted bilaterally into the dorsal hippocampus (CA1 region) of the anesthetized rats using a stereotaxic instrument [29, 31].

Behavioural training and probe test

The behavioural study was comprised of two sets of experiments. (i) In Experiment set (*i.e.* spatial learning experiment), 24 animals were assigned to three groups of eight animals. For evaluation of spatial learning (acquisition), animals orally received Ros 20 mg/kg/d for 10 days. From day 7 to 10, four consecutive daily administrations of bilateral intra-hippocampal H-89 (10 µM/side) were

done 30 min after oral gavage of Ros. Animals were trained by the Morris water maze (MWM) from day 7 to day 10; each training session included one block of four trials. Spatial learning was assessed by measuring escape latency, travelled distance, and swimming speed using the EthoVision tracking system (Noldus Information Technology, Wageningen, The Netherlands), as described in previous studies [5, 29]. Twenty-four hours after the last training trial, on day 11, the probe test (to find the hidden platform) was done and the time spent in the target quadrant for 60 s was recorded. (ii) In Experiment set 2 (*i.e.* spatial memory retention), in order to assess the effects of treatments on spatial memory retention, 24 rats were randomly divided in 3 groups of 8. From day 7 to 10 of Ros administration, male rats were trained in MWM (similar to Experiment set 1), but received H-89 only on day ten, 30 min after oral gavage of Ros. Forty-eight-hour post final training session (*i.e.* on day 12), the probe test for evaluation of spatial memory retention was performed.

Biological samples collection

Following the probe test (on day 11 for set 1 and on day 12 for set 2), animals were sacrificed by induction of deep anaesthesia, and serum and hippocampus samples were collected.

Western blot analysis

As previously described, 200 mg of hippocampi obtained from animals, was homogenized, sonicated, and centrifuged and supernatant was separated for assessment of CREB and *p*-CREB protein expression [11]. For this purpose, about 10 - 20 µL of supernatant was subjected to acrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane, for protein detection using primary and secondary antibodies of CREB, *p*-CREB and beta-actin. After blot detection by Syngene Chemidoc, samples were analysed by GeneTools software. Eventually, the expression level of each protein was normalized against beta-actin [8, 30].

Assessment of Oxidative stress

Blood samples taken from rats hearts and collected in heparinized tube, were homogenized in cold 1.15% potassium chloride to yield a 10% homogenate [8]. Then, 0.5 mL of the homogenate was mixed with 3 mL thiobarbituric acid 0.8% (W/V), boiled for 45 min at 95°C and centrifuged at 12,000 g for 10 min. Next, samples were cooled at the room temperature and the supernatant absorbance was measured at 532 nm using a spectrophotometer (Jenway 6305; Bibby Scientific Ltd.) [1].

Statistical analysis

GraphPad Prism version 5.0 was used for data analysis. One-way analysis of variance followed by Newman-Keuls *post hoc* test was used to compare the behavioural and molecular data among the groups. The differences were considered statistically significant at $p < 0.05$.

Results and Discussion

Effects of Ros on H-89-induced spatial learning (acquisition) and spatial memory retention impairment in MWM

The results showed that Ros ameliorated spatial learning deficit induced by 4-day bilateral intra-hippocampal H-89 (from day 7 to 10). Our results showed that H-89 significantly increased both travel distance (Figure 1B) and escape latency (Figure 1A) compared to DMSO-treated control animals ($p < 0.001$). Ros (20 mg/kg given orally for 10 days) decreased escape latency significantly (Figure 1A, $p < 0.05$) but did not affect travelled distance ($p > 0.05$) compared to the H-89 group. Ros-H-89 treated rats showed increased time (to near that of the control group) spent in the target quadrant compared to the H-89 group (Figure 1C, $p < 0.05$). In the spatial memory retention experiment (*i.e.* experiment set 2), differences between the H-89 group and the control group and between Ros-H-89 and H-89 group were significant (Figures 1D and 1E, for both cases $p < 0.01$). Also, Ros-H-89 group, compared to the H-89 group, spent more time in the target quadrant (Figure 1F, $p < 0.05$) reaching the level of the control group.

Ros effects on oxidative stress induced by H-89

Oxidative stress in both sets of experiments (spatial learning; Figure 2A and spatial memory retention; Figure 2B) was significantly (***$p < 0.001$ and *$p < 0.05$, respectively) increased by H-89 compared to the control (DMSO-only treated) animals. In Ros-H-89 group, compared to H-89 group, oxidative stress was significantly decreased only in spatial memory retention experiment (Figure 2B, $p < 0.05$).

The effect of Ros on the expression of CREB and p-CREB proteins

Intra-hippocampal infusion of H-89 decreased the amount of CREB and *p*-CREB proteins in the spatial memory retention experiment (Figures 3C and 3D, respectively) compared to the control group. However, Ros significantly increased the expression of these protein markers (Figures 3C and 3D, #$P < 0.05$) compared to H-89-treated animals in spatial memory retention test. In spatial learning experiment, H-89 did not significantly affect he expression of these proteins as compared to the control group but Ros significantly increased the expression of the proteins compared to DMSO-treated animals (Figures 3A and 3B).

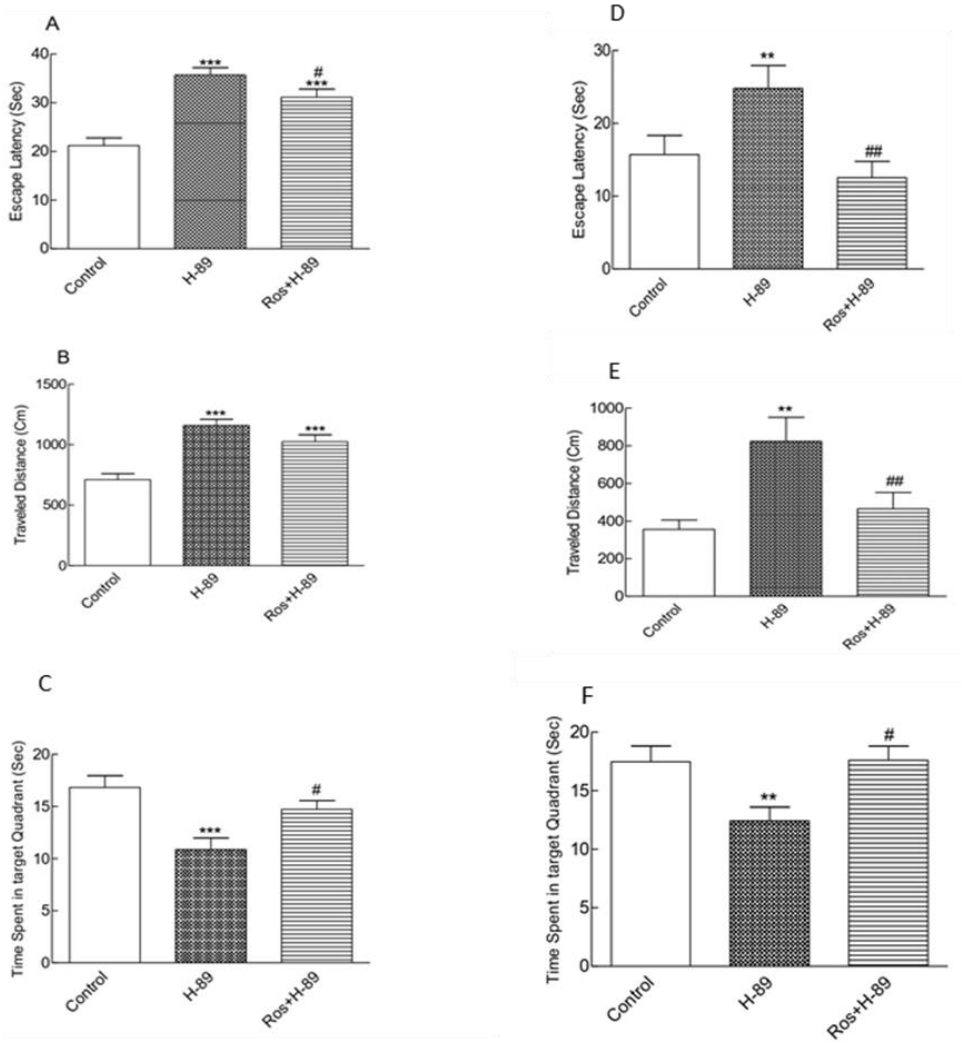

Figure 1.

Effects of Ros on H-89 -induced spatial learning (A, B and C) and spatial memory retention (D, E and F) impairment in MWM in rats. Average of escape latency (A and D), travelled distance (B and E) and time spent in target quadrant (C and F) is shown in these six graphs

**p < 0.01 and ***p < 0.001 indicate significant differences from the control (DMSO-only treated) animals

#p < 0.05 and ##p < 0.01 represent significant differences compared to the H-89 group

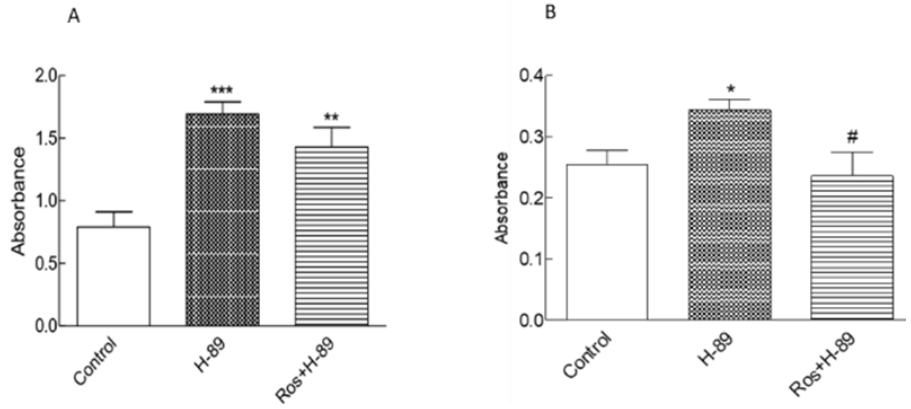

Figure 2.

The effect of Ros on oxidative stress induced by H-89 in the plasma of rats (A: spatial learning experiment and B: spatial memory retention experiment)

*p < 0.05, **p < 0.01 and ***p < 0.001 reflect significant differences from the control (DMSO-only treated) animals

#p < 0.05 shows significant differences from H-89 group

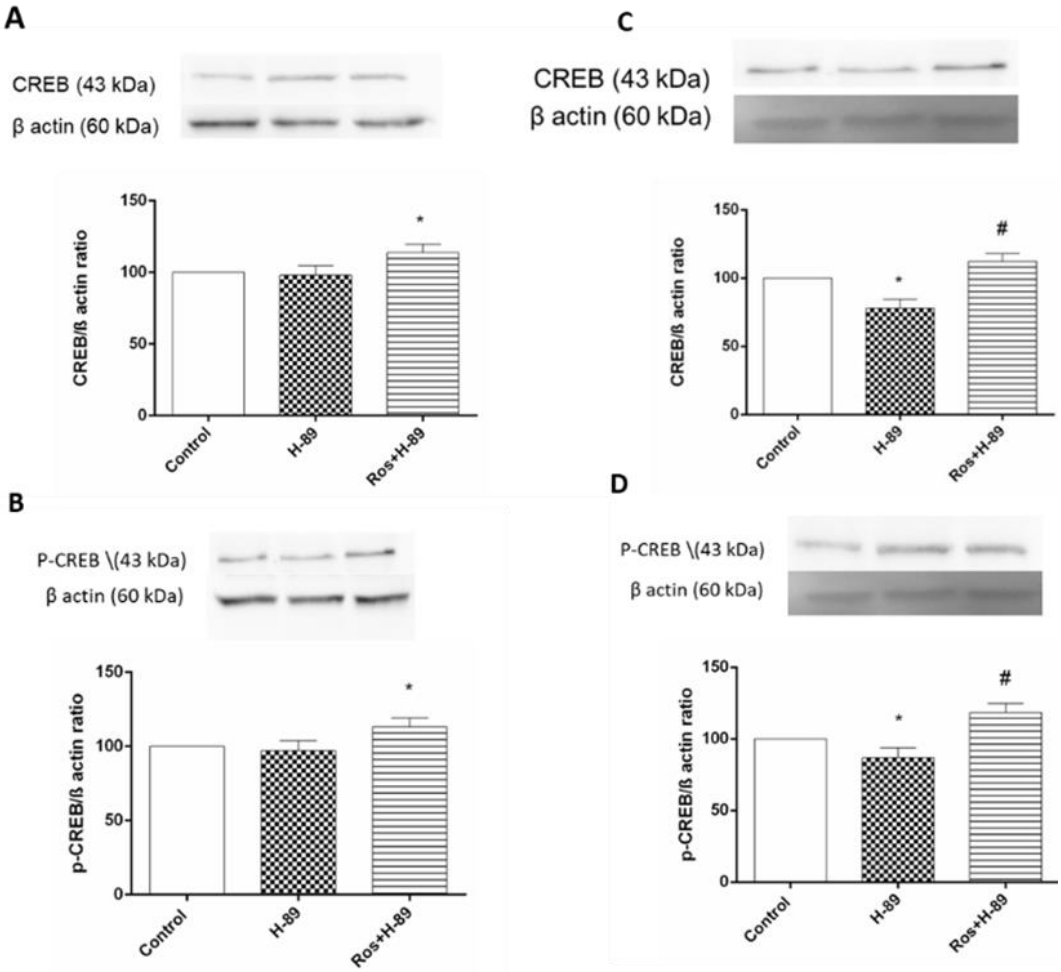

Figure 3.

Effects of DMSO, H-89 and H-89/ROS on the CREB and *p*-CREB protein expression in spatial learning (A and B) and spatial memory retention (C and D) in rat' hippocampus samples using western blot

*p < 0.05 significantly different from the control (DMSO-treated) animals

#p < 0.05 significantly different from the H-89 group

Statins are antihyperlipidaemic drugs with neuroprotective effects [12, 16]. Assessment of the effect of different members of this family on dementia and AD led to controversial results [6, 19, 22]. Nevertheless, Ros effect has not been studied in H-89-induced learning and memory deficit model.

Our results confirmed that Ros prevented H-89-provoked spatial learning and memory retention impairments in MWM and increased H-89-induced decrement of CREB and *p*-CREB protein in the hippocampus. Furthermore, Ros had favourable effects on H-89-induced oxidative stress.

H-89 is an inhibitor of PKA which is used to clarify the role of PKA signal transduction in the brain [29]. PKA inhibitors were shown to negatively affect spatial learning and spatial memory retention. It was accordingly shown that PKA has an important role in the hippocampus-dependent memories and it was suggested that cAMP/PKA/CREB signalling activation potentially enhances memory [26]. CREB and *p*-CREB proteins are involved in the synaptogenesis and neurogenesis in the brain [28]. H-89 as a PKA inhibitor could inhibit cAMP/PKA/CREB/*p*-CREB signalling that stimulates synaptogenesis and neurogenesis in the brain has and has preventive effects on neurodegenerative diseases like AD [28].

Our results showed that Ros alleviates H-89 - induced spatial learning and memory deficits in rats. Previous reports showed that statins increased neurogenesis in the dentate gyrus, restored cognitive deficit, and reduced delayed neuronal death in the hippocampal CA3 region [3, 15, 16]. In this context, Duyne Lu *et al.* showed thatatorvastatin promoted the restoration of spatial memory function in a traumatic brain injury and stroke model [15]. Consistently, Chauhan *et al.* confirmed that simvastatin and pravastatin improved spatial learning in a mice model of

traumatic brain injury [3]. The data reported by these two studies were in accordance with the results of the present work.

H-89 represses PKA signalling pathway, suppresses synaptogenesis and neurogenesis and reduces CREB and p-CREB in the hippocampus. In our study, Ros could prevent H-89 effect and increase CREB and *p*-CREB in rats' hippocampus and improve spatial memory. Based on the literature, statins pose neuroprotective effects against deleterious effects of stroke and oxygen and glucose deprivation animal models, through induction of CREB and its active form, *p*-CREB [9, 13]. Guirao, *et al*. showed that ortho-hydroxy atorvastatin in an oxygen/glucose-deprived neuronal model, boosts the intrinsic pro-survival factor *p*-CREB in large-GABAergic cortical neurons [9].

Simvastatin as a statin drug, alleviated ischemic brain damage in immature rats via Akt and cAMP response element binding protein (CREB) activation [2]. CREB was shown to modulate learning and memory function [35]. Besides, statins improved spatial memory and learning *via* decrement of neuroinflammation, Aβ production and oxidative stress by modulation of cAMP/PKA/CREB/p-CREB pathway.

In another study done in a C57/BL/6 mice model of stroke, activation of CREB protein reversed spatial memory impairment after focal cerebral ischemia [17]. Our study confirmed that H-89 decreases the amount of CREB and *p*-CREB protein levels whereas Ros significantly upregulated these proteins and as shown previously, *p*-CREB increment could reverse spatial memory impairment. Beside this mechanism of action, statins as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, were shown to induce neurotrophin in brain cells. These cholesterol-lowering agents mainly modulate mevalonate pathway. In this context, statins could improve memory via binding to PPARα and upregulating neurotrophin expression in the brain and their neurotrophic function is done via PPARα-CREB pathway [24]. Consistent with our study on Ros effect, simvastatin improved memory in an animal model of AD *via* increasing brain-derived neurotrophic factor (BDNF) and a PPARα-mediated mechanism [24].

Statins are known to exhibit antioxidant effects in the cerebrovascular system. Spatial memory improvement by using antioxidants was also described before [10, 20, 33, 38]. For instance, geniposidic acid isolated from *Eucommia ulmoides* Oliv. Bark with anti-aging, anti-oxidant, anti-inflammatory and neurotrophic effects on neurons, could improve spatial memory deficit *in vivo* [38]. Thus, possibly anti-oxidant properties of Ros, observed in the present work, also helped in improvement of spatial memory damaged by H-89.

As another naturally occurring compound, crocin, which is a carotenoid with antioxidant and anti-inflammatory functions, showed protective effects againt spatial memory deficit in rats possibly via suppression of oxidative stress [20].

Ethanol deleterious effects on brain function include impairment of cognitive performance and other neurological consequences. Interestingly, it was reported that oxidative stress is one of the main mechanisms underlying spatial memory impairment. The above-noted evidence confirm that suppression of oxidative state (as induced by Ros in this work) may attribute to a protective effect on spatial memory [33].

Conclusions

Together, our study showed that Ros could be potentially considered a preventive measure/treatment against AD as it could protect rats against deleterious effects of H-89 on spatial learning and spatial memory retention. These promising findings necessitate further evaluations in preclinical and eventually clinical settings.

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Conflict of interest

The authors declare no conflict of interest.

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