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The protective effect of metformin in scopolamine-induced learning and memory impairment in rats

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ABSTRACT

Background: Alzheimer's disease (AD) is the most common neurodegenerative disease in the world. One of the most commonly prescribed oral antidiabetic drug, metformin, has been shown to have beneficial effects on restoring impaired cognitive function. In the present study, we investigated the effects of metformin on spatial memory in terms of alleviating scopolamine-induced learning and memory impairments in rats by using the Morris water maze (MWM) test and the modified elevated plus-maze (mEPM) test. Furthermore, we investigated the possible mechanisms of action of metformin in preventing cognitive dysfunction.

Methods: Male Wistar rats received metformin (50, 100, or 200 mg/kg/day) *via* gavage feeding for three weeks. Scopolamine was administered intraperitoneally before the probe step of the MWM test or the acquisition session of the mEPM test.

Results: The learning and memory impairment induced by scopolamine was reversed by metformin. In addition, metformin improved the level of phosphorylated AMP-activated protein kinase and cAMP responsive element binding protein. However, metformin pretreatment had no impact on inhibiting the scopolamine-induced changes in acetylcholine levels. Furthermore, metformin exerted its antioxidant effect by significantly reversing scopolamine-induced changes in malondialdehyde, total antioxidant status, and superoxide dismutase levels in the hippocampus.

Conclusion: Our results indicate that one of the most commonly used antidiabetic drug, metformin, has the potential to prevent the development of dementia and be a novel therapeutic drug for the amelioration of cognitive dysfunction in AD.

Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the world. Dementia develops with the progressive loss of memory, language, problem-solving, and other cognitive skills that affect the ability of a person to perform daily activities [1]. Nearly 50 million people suffer from dementia worldwide, with a projected increase to 152 million by 2050 [2]. The majority of cognitive symptoms in AD are attributed to cholinergic dysfunction in the basal forebrain [3]. Treatment to slow or stop the destruction of neurons is currently not available. The current treatment involves mostly acetylcholinesterase inhibitors to prolong the availability of acetylcholine at cholinergic synapses. Unfortunately, drugs are only symptomatically effective; furthermore, adverse effects and limited efficacy restrict their therapeutic success [4].

Metformin is one of the most commonly prescribed oral antidiabetic medications for type II diabetes. Metformin decreases insulin resistance and ameliorates hyperglycemia without causing low blood sugar levels [5]. In addition to improving glucose metabolism, it has antioxidant [6,7] and neuroprotective [8] effects. Furthermore, metformin was shown to promote neurogenesis and enhance spatial memory [9].

Metformin activates AMP-activated protein kinase (AMPK), which modulates long term potentiation and memory formation [10]. Moreover, the brain cholinergic system and brain-derived

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neurotrophic factor (BDNF) activate hippocampal neurogenesis and improve cognitive function [11].

The scopolamine-induced amnesia model is the most commonly used pharmacological model related to AD. Scopolamine causes memory impairment by antagonism of the muscarinic acetylcholine receptors [12].

Therefore, in this study, we studied the effects of metformin on spatial memory in terms of alleviating scopolamine-induced learning and memory impairment in rats. Furthermore, to elucidate the mechanism of action of metformin on cognitive dysfunction, we evaluated the levels of acetylcholine (ACh) to assess its effect on the cholinergic system. The levels of total antioxidant status (TAS), superoxide dismutase (SOD), and malondialdehyde (MDA) were measured to evaluate their antioxidant effects. Additionally, we assessed the effects of metformin on the levels of molecules such as *p*AMPK, BDNF, and CREB in the hippocampus of rats involved in cognition.

Material and methods

Subjects

Five-month-old male Wistar rats were used in this study. They were maintained on a standard 12 h light/dark cycle at the environmental temperature (22 ± 2 °C) and with free access to tap water and chow pellets. Behavioral experiments were performed between 08:00 and 13:00 in a semi-soundproof and semi-dark laboratory. The study was approved by the Kocaeli University Animal Research Ethics Committee (30 November 2014, Number: 11/5-2014).

Drugs and experimental design

A total of 80 rats were used in the study. They were divided into four main groups (n = 20 per group) and eight subgroups (n = 10 per group) and were given either physiological serum (2 ml/kg/day) or metformin HCl (Metfull, Vitalis, Istanbul, Turkey; 50, 100, or 200 mg/kg) *via* gavage feeding for three weeks (Table 1). Scopolamine HCl (Sigma, St Louise, USA; 1 mg/kg) was administered intraperitoneally (*ip*) 30 min before the behavioral experiments to one of the subgroups of each main group (Fig. 1).

Locomotor activity test

Locomotor activity was evaluated to exclude the adverse effects of locomotor activity altering compounds. A locomotor activity cage (May Commat, Ankara, Turkey) was used for this test.

Morris water maze (MWM) test

The circular pool of MWM was divided into four equal quadrants. A platform (10 cm diameter) was placed 1 cm below the surface of the water in one of the quadrants for the first four days. The surface of the water was covered by small white beads. At each day of acquisition session (three trials per session), one of the rats was positioned in one of three randomly selected locations in the pool. A trial was initiated by placing the rat into the pool. The trial was terminated when the rat found and climbed onto the platform, and the mean escape time was determined. The maximum test duration was 60 s. If the rat did not climb onto the platform within 60 s, it was gently placed on the platform and the delay was recorded as 60 s. Between two trials, the rat was held on the platform for 20 s. After the test was repeated at all three starting points, the rats were gently dried and placed in their cages. The 'probe trial' was performed on the fifth day, for assessing the rat's recall of the location of the hidden platform for 60 s. The platform was taken out of the pool during this trial.

Modified elevated plus maze (mEPM) test

The test was performed as previously described by Hlinak and Krejci [13]. The mEPM test was carried out by using a cross-shaped wooden platform consisting of two open (50 × 10 cm) and two closed (50 × 10 cm) arms elevated 50 cm above the ground. On the first day, each rat was placed at the distal end of the open arm, and the time needed to transfer to the closed arm (transfer latency, TL) was recorded. The maximum test duration was 90 s. After entering the closed arm, the rat was released in the maze for 10 s. The test was repeated on the second day and the transfer latency was assessed.

Brain tissue sampling

All rats were sacrificed under ketamine–xylazine anesthesia 1 h after the MWM test. The whole brains were removed and the hippocampi were rapidly separated, frozen in liquid nitrogen, and stored at −80 °C.

Sample preparation

Weighed tissue samples were quickly treated with 0.9% NaCl, then homogenized for 1 min (2,000 rpm/min, 1:10 w/v) with the aid of a stirrer (Stuart SHM 1, UK) in PBS (pH 7.4) solution in an ice bath on the day of the experiment. The homogenate and supernatant were analyzed for protein analysis according to the Lowry method (1951) [14] (Shimadzu UV-1800, Japan).

Measurements of lipid peroxidation

The homogenate MDA levels were detected *via* the single heating method based on the thiobarbituric acid (TBA) reactivity as described in Yoshioka et al. [15]. Briefly, 2.5 ml trichloroacetic acid solution (TCAA) (20%) and 1 ml thiobarbituric acid (0.67%) were mixed with 0.5 ml tissue homogenate and kept at 95 °C in water bath for 30 min. The reaction mixture was cooled with tap water, vortexed, and added to n-butanol (4 ml). It was then centrifuged at 3000 rpm for 10 min. The absorbance of n-butanol at 535 nm was measured after removing the organic layer. The MDA

Table 1
The main groups, subgroups and the treatments.

Groups	Subgroups	n	Treatment for three weeks (*po*)	Treatment before the tests (*ip*)
Group I	Control group	10	1 ml/kg/day Saline	1 ml/kg Saline
	SCO group	10	1 ml/kg/day Saline	1 mg/kg Scopolamine
Group II	Met 50 group	10	50 mg/kg/day Metformin HCl	1 ml/kg Saline
	Met 50 + SCO group	10	50 mg/kg/day Metformin HCl	1 mg/kg Scopolamine
Group III	Met 100 group	10	100 mg/kg/day Metformin HCl	1 ml/kg Saline
	Met 100 + SCO group	10	100 mg/kg/day Metformin HCl	1 mg/kg Scopolamine
Group IV	Met 200 group	10	200 mg/kg/day Metformin HCl	1 ml/kg Saline
	Metn 200 + SCO group	10	200 mg/kg/day Metformin HCl	1 mg/kg Scopolamine

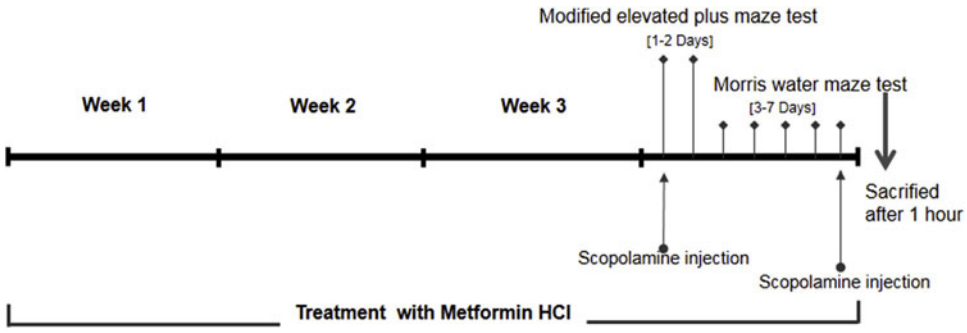

Fig. 1. The experimental design of the study.

concentration was calculated by the MDA-TBA complex absorbance coefficient ($\varepsilon = 1.56 \times 105/M/cm$) and expressed as μmol/mg tissue protein in the homogenate or μmol/L in serum. The other half of the homogenate was centrifuged at 5000 g for 5 min. The supernatant was separated and then other analyses were performed.

Assessment of total antioxidant status (TAS)

TAS was detected with a commercially available kit (Total Antioxidant Status Assay kit, Rel Assay Diagnostics, Turkey) by using an automated measurement method. The results are described as millimoles of Trolox equivalent per mg tissue protein in the supernatant.

The enzyme-linked immunosorbent assay (ELISA) tests

Supernatant and Serum SOD (Rat Superoxide dismutase [Cu-Zn] ELISA kit, Fine Test, ER0332, China), Ach (Rat Acetylcholine ELISA kit, Fine Test, ER1466, China), *p*AMPK (Rat Phosphorylated Adenosine Monophosphate-Activated Protein Kinase ELISA kit, Elabscience, E-EL-R0738, China), BDNF (Rat Brain Derived Neurotrophic Factor ELISA kit, Fine Test, ER0008, China) and CREB (Rat Cyclic AMP Response Element Binding Protein ELISA kit, Fine Test, ER0865, China) levels were measured using commercially available kits on an ELISA-reader (Thermo Multiskan FC, USA).

Statistical analysis

Prism 6.0 software (GraphPad Software, Inc., San Diego, CA, USA) was used in data analysis and results were expressed as the mean ± SEM. The acquisition test of MWM was evaluated with two-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* tests. The other results were analyzed with one-way ANOVA followed by Bonferroni *post-hoc* tests. The results were considered to be statistically significant for *p*-values less than 0.05.

Results

The effect of metformin on the total locomotor activity test

Statistical analysis of the data demonstrated that metformin (50, 100, or 200 mg/kg) or scopolamine (1 mg/kg), or their combination, did not significantly modify the number of movements [$F(7,72) = 1.814$; $p > 0.05$] (Fig. 2).

The effect of metformin on the MWM test

The latency to find the hidden platform on the four test trials of animals decreased from day 1 to day 4 (two-way ANOVA, the effect of day, $F(3,76) = 56.87$, $p < 0.05$). A significant decrease in latency on each subsequent day of acquisition testing was shown by Bonferroni's *post-hoc* comparison test for all groups ($p < 0.05$) (Fig. 3A). Metformin and control groups were similar in the acquisition trial.

During the probe trial of the MWM test, there was a significant difference in latencies between the scopolamine group and the control group regarding the time spent in the quadrant with the escape platform (one-way ANOVA, $F(7,74) = 6.341$, $p < 0.05$, Fig. 3B). *Post-hoc* comparisons also showed that scopolamine reduced the time spent in the escape platform's quadrant ($p < 0.0001$). Metformin (100 and 200 mg/kg) + scopolamine co-administration reversed the scopolamine-induced decrease in latency compared to scopolamine alone ($p < 0.05$ for Met-100+SCO group and $p < 0.0001$ for Met-200+SCO group).

The effect of metformin in the mEPM test

Fig. 3 shows the first-day and the second-day latency of the rats. The retention latency was significantly decreased with metformin (50 mg/kg) compared with that in the control group ($p < 0.05$). There was no significant effect of metformin or its combination with scopolamine on the first-day latency in the mEPM test [$F(7,71) = 3.740$, $p < 0.05$; Fig. 4A]. Scopolamine significantly

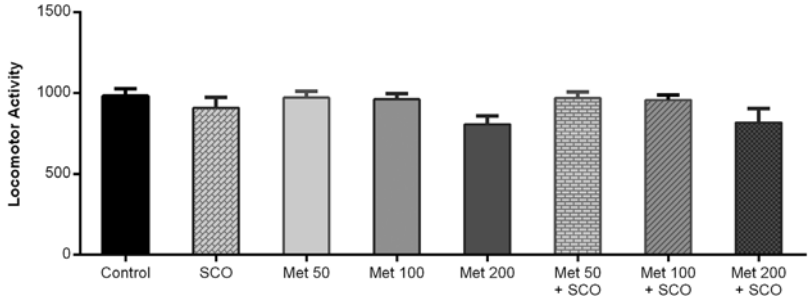

Fig. 2. Effect of metformin (50, 100 and 200 mg/kg) on total locomotor activity.

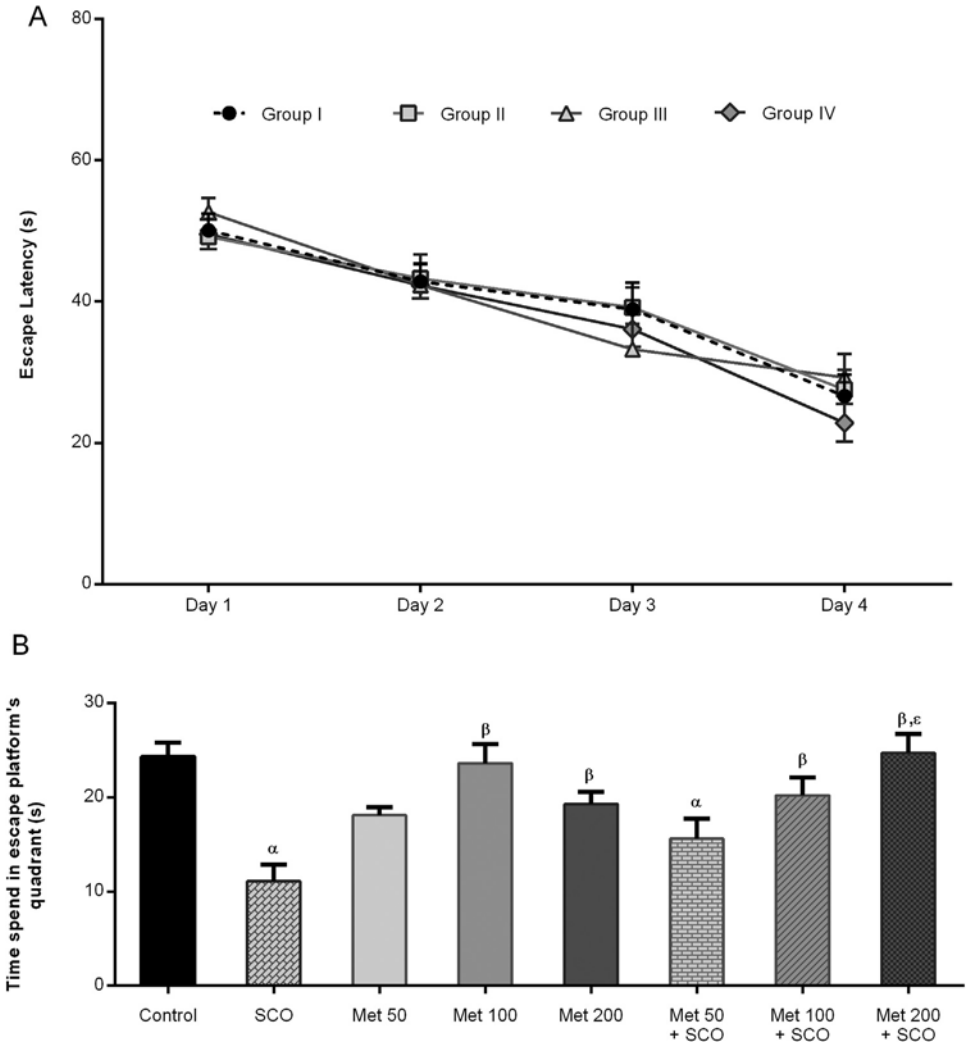

Fig. 3. (A) Effect of metformin (50, 100 and 200 mg/kg) on the acquisition test of MWM. There is no scopolamine administration. Each value represents the mean ± SEM (n = 10). (B) Effect of metformin (50, 100 and 200 mg/kg), scopolamine (1 mg/kg) and the combination of drugs on the probe test of MWM. Each value represents the mean ± SEM (n = 10). $^{\alpha}$ $p <$ 0.05 compared with the control group; $^{\beta}$ $p <$ 0.05, compared with the scopolamine group; $^{\varepsilon}$ $p <$ 0.05 compared with the Met 50 + SCO group.

prolonged the second-day retention latency compared with that in the control group ($p < 0.05$), while metformin pretreatment significantly reversed this effect compared to scopolamine alone ($p < 0.001$ for the Met-50+SCO group, $p < 0.05$ for Met-100+SCO group and $p < 0.05$ for Met-200+SCO group). The retention latency was significantly decreased with metformin, compared to that with scopolamine alone ($p <$ 0.05) [F (7,72) = 6.171, $p < 0.05$; Fig. 4B].

The effect of metformin on the levels of ACh in the hippocampus

ACh levels in the hippocampus were significantly decreased in the scopolamine group ($p < 0.05$), and in combination groups as compared with the control group [F (7,40) = 6,906; $p < 0.05$; Fig. 5].

The effect of metformin on the levels of pAMPK, CREB and BDNF in the hippocampus

Levels of *p*AMPK in the hippocampus were significantly decreased in the scopolamine group ($p < 0.05$). Metformin pretreatment significantly increased *p*AMPK levels compared with scopolamine alone. İnterestingly, metformin treatment alone at 100 mg/kg also markedly increased *p*AMPK levels compared with the control group [F (7,40) = 28.43; $p < 0.05$]. Levels of CREB in the hippocampus were significantly decreased in the scopolamine group, compared with the control group. Metformin significantly increased CREB levels both alone and with scopolamine compared with scopolamine alone [F (7,40) = 15.50; $p < 0.05$]. Levels of BDNF in the hippocampus were decreased in the scopolamine group compared with the control group [F (7,40) = 16.56, $p < 0.05$]. Metformin pretreatment (100–200 mg/kg) significantly reversed this effect but was still significantly lower than in the control (Fig. 6).

The effect of metformin on the levels of MDA, TAS, and SOD in the hippocampus

MDA levels were significantly increased in the hippocampus of scopolamine group compared with the control group [F (7,40) = 133.8, $p < 0.05$]. Metformin alone significantly decreased the MDA levels in the hippocampus compared with scopolamine alone. However, pretreatment with metformin significantly attenuated MDA elevation, which did not return to the control group values. Scopolamine significantly depleted the levels of antioxidant in the hippocampus including TAS and SOD, compared with the control group [F (7, 40) = 10.62, $p < 0.05$ and F (7,40) = 21.42, $p < 0.05$ respectively] (Fig. 7). These changes were reversed by metformin pretreatment. Interestingly, SOD levels in

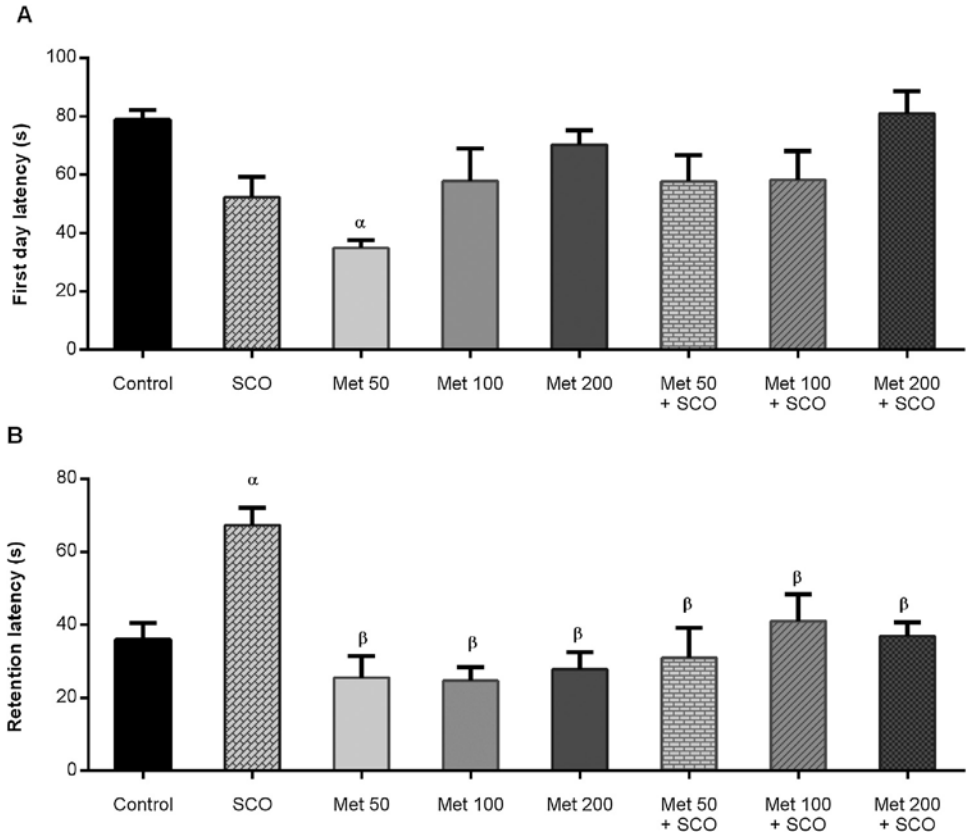

Fig. 4. Effects of metformin (50, 100 and 200 mg/kg), scopolamine (1 mg/kg) and the combination of drugs on (A) transfer latency 1 (TL-1) and (B) transfer latency 2 (TL-2) (n = 10) in the mEPM test. Each value represents the mean ± SEM (n = 10). $^{\alpha}$ $p < 0.05$ compared with the control group; $^{\beta}$ $p < 0.05$ compared with the scopolamine group.

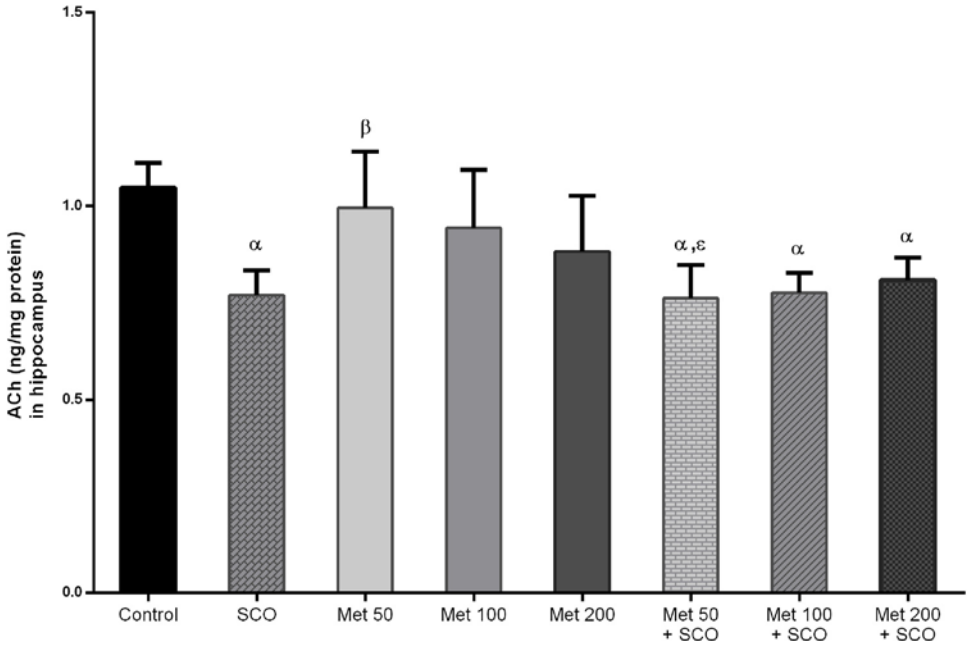

Fig. 5. The effect of metformin on scopolamine-induced changes in Ach levels in the hippocampus. Data are expressed as the mean ± SEM (n = 6). $^{\alpha}$ $p < 0.05$ compared with the control group; $^{\beta}$ $p < 0.05$, compared with the SCO group; $^{\varepsilon}$ $p < 0.05$ compared with the Met 50 group.

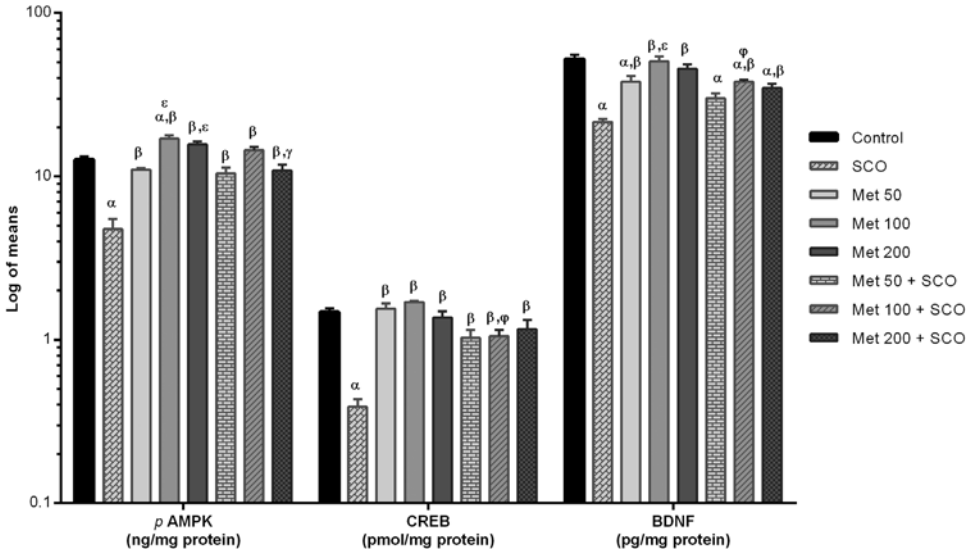

Fig. 6. The effect of metformin on the levels of *p*AMPK, CREB, and BDNF in the hippocampus. Data are expressed as the mean ± SEM (n = 6). $^{\alpha}$ $p < 0.05$ compared with the control group; $^{\beta}$ $p < 0.05$ compared with the SCO group; $^{\varepsilon}$ $p < 0.05$ compared with the Met 50 group; $^{\Phi}$ $p < 0.05$ compared with the Met 100 group, $^{\gamma}$ $p < 0.05$ compared with the Met 200 group in their own group.

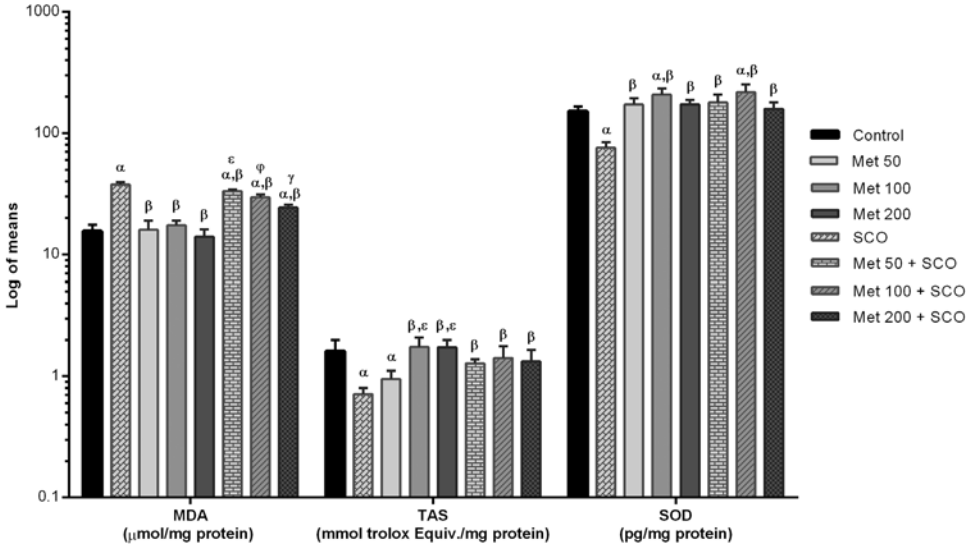

Fig. 7. The effect of metformin on the levels of MDA, TAS, and SOD in the hippocampus. Data are expressed as the mean ± SEM (n = 6). $^{\alpha}$ $p < 0.05$ compared with the control group; $^{\beta}$ $p < 0.05$ compared with the SCO group; $^{\varepsilon}$ $p < 0.05$ compared with the Met 50 group; $^{\Phi}$ $p < 0.05$ compared with the Met 100 group, $^{\gamma}$ $p < 0.05$ compared with the Met 200 group in their own group.

response to 100 mg/kg metformin ($p < 0.05$) and with scopolamine ($p < 0.05$) were significantly enhanced compared with those in the control group.

Discussion

The findings presented here demonstrate that metformin, a commonly used antidiabetic medication, has the potential to prevent the development of dementia associated with AD. Our findings point to four important conclusions. First, we confirmed that metformin has a protective effect on scopolamine-induced spatial learning and memory impairment in rats by using behavioral tests such as MWM and mEPM. Second, our behavioral studies demonstrate that the protective effect of metformin on cognitive function is not due to its effect on acetylcholine in the hippocampus. Third, we report for the first time that metformin significantly improved CREB and *p*AMPK levels reduced by scopolamine. It is well known that CREB is important for memory formation [16]. Fourth, we show metformin exerts antioxidant effects by significantly reversing the effect of scopolamine on MDA, TAS, and SOD levels in the hippocampus.

Age-related dementia and memory deficits observed in AD are correlated with the loss of cholinergic neurotransmission in the basal forebrain [3]. In addition, pharmacological blockage of cholinergic neurons by scopolamine causes impairment of learning and memory in experimental animals [12]. In our study, as expected, scopolamine significantly impaired learning and memory in MWM and mEPM tests. Although metformin treatment alone did not affect cognitive function, metformin pretreatment reversed the impairment of spatial learning and memory induced

by scopolamine. Our data are consistent with a previous report demonstrating that metformin and control groups were similar in their acquisition and probe tests in MWM [9].

Acetylcholine is the main neurotransmitter of cholinergic neurons, and acetylcholine deficiency in the AD has been known for years. In studies of scopolamine-induced amnesic animals, an increase in acetylcholinesterase (AChE) activity was observed in the brain tissue after scopolamine administration [17,18]. An *in vitro* study found that metformin inhibits 50% of AChE activity in hemolyzed human erythrocytes [19]. We found that scopolamine significantly decreased the level of ACh in the hippocampus. Pretreatment with metformin did not significantly inhibit the scopolamine-induced changes in the levels of ACh. Nevertheless, metformin pretreatment ameliorated scopolamine-induced learning and memory impairment in the MWM and mEPM tests. In line with our data, Mostafa et al. [20] showed that although metformin treatment did not inhibit the scopolamine-induced increase in AChE activity, it ameliorated scopolamine-induced impairment in MWM test in rats. Although we have not studied acetylcholinesterase activity directly in this study, our results have a number of similarities with these findings. Thus, our data indicate that the protective effect of metformin is not due to its effect on acetylcholine levels in the hippocampus.

Another factor that may be related to the mechanism of action of metformin in memory improvement is AMPK. Several studies have shown that AMPK acts as a long-term potentiation modulator and is necessary for memory formation [10]. Wang et al. suggested that metformin could increase neurogenesis via AMPK [9]. Additionally, it has been shown that AMPK significantly attenuated the decrease in the acetylcholinergic neurons and improved the scopolamine-induced memory impairment in the MWM test [17]. Consistent with previous reports, our study showed that metformin administration significantly enhanced *p*AMPK and improved scopolamine-induced behavioral deficits during the MWM and mEPM tests. Thus, these findings suggest that metformin can improve scopolamine-induced cognitive dysfunction through AMPK enhancement.

AMPK is known to be important for CREB activation through phosphorylation. Activation of CREB creates neuronal plasticity and memory formation by regulating hippocampal neurogenesis [16]. Phosphorylation of CREB decreases in the brain of AD patients [21]. CREB is a downstream target for AMPK and mediates some of the effects of AMPK on gene expression [22]. However, Huang et al. showed that overexpression of AMPK reduced the level of CREB. Therefore, they suggest a dual role of AMPK in the regulation of CREB in the hippocampus, such as decreasing total CREB expression and increasing CREB activity *via* phosphorylation [23]. Here, we report for the first time that metformin treatment significantly improved scopolamine-induced reduction in CREB levels, similar to those of *p*AMPK levels. One of the limitations of our study is that we were not able to determine the levels of phosphorylated CREB.

BDNF, the most recognized neurotrophin of the brain, is also important for learning and memory [24]. BDNF levels are found to decrease in the brain of AD patients [25]. Consistent with previous studies, our study showed a dramatic decrease in BDNF levels in the hippocampus in response to scopolamine administration [11,26]. However, metformin pretreatment did not reverse the effect on BDNF levels. Allard et al. also reported that metformin treatment decreased transcription of BDNF in the brain of mice fed with the high-fat diet [27]. In this study, we found that metformin prevented impairment of spatial memory in the MWM test, despite the reduction in BDNF.

Since oxidative stress contributes to the pathogenesis of neurodegenerative disorders, we also investigated whether metformin had protective effects on oxidative stress in the hippocampus [28]. The increase in oxidative stress levels in the brain can impair hippocampal synaptic plasticity, and create memory deficits [29]. Scopolamine-induced oxidative stress reduced antioxidants and increased MDA levels in the hippocampus [26]. The MDA level is a practical and reliable biomarker of lipid peroxidation, reflecting the damage caused by reactive oxygen species [30]. We found that scopolamine increased the MDA levels and decreased the TAS and SOD levels in the hippocampus. These changes of MDA, TAS, and SOD were significantly attenuated by metformin pretreatment. Our experiment confirms previous data on the beneficial effects of metformin on oxidative stress parameters in the hippocampus [8,9,20].

In conclusion, we found that metformin treatment significantly ameliorated the scopolamine-induced memory deficit. We report that scopolamine treatment decreased the levels of *p*AMPK and CREB, and administration of metformin effectively restored the *p*AMPK and CREB levels in the hippocampus. Metformin treatment significantly increased the levels of TAS and SOD and increased anti-oxidant activity by decreasing MDA levels. In the current study, both oxidative stress and impairments in cognitive function induced by scopolamine were prevented by metformin treatment. Based on literature and our findings, we propose that metformin can be used to prevent the development of dementia and can be a novel therapeutic drug for the amelioration of cognitive dysfunction related to AD.

Conflicts of interest

The authors declare that they have no conflict of interest.

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