Neurochem Res (2014) 39:353–360
DOI 10.1007/s11064-013-1232-8

ORIGINAL PAPER

Effects of *Zizyphus jujube* Extract on Memory and Learning Impairment Induced by Bilateral Electric Lesions of the Nucleus Basalis of Meynert in Rat

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Received: 17 October 2013 / Revised: 12 December 2013 / Accepted: 14 December 2013 / Published online: 31 December 2013

Abstract Alzheimer's disease (AD) is a common neurodegenerative condition that affects the elderly population. Its primary symptom is memory loss. The memory dysfunction in AD has been associated with cortical cholinergic deficiency and loss of cholinergic neurons of the nucleus basalis of Meynert (NBM). *Zizyphus jujube* (ZJ) activates choline acetyltransferase and may have beneficial effects in AD patients. This study investigates the effect of ZJ extract in intact rats and in rat model of AD. 49 male Wistar rats were divided into seven equal groups (1—control, without surgery, received water), 2—AD (bilateral NBM lesion, received water), 3 and 4—AD + ZJ (NBM bilateral lesion, received ZJ extract 500 and 1,000 mg/kg b.w. per day for 15 days), 5—sham (surgery: electrode introduced into NBM without lesion, received water), 6 and 7—without surgery and lesion, received ZJ extract—the same as groups 3 and 4). The learning and memory performance were assessed using passive avoidance paradigm, and the memory cognition for spatial learning and memory was evaluated by Morris water maze. In shuttle box test ZJ extract (500 and 1,000 mg) significantly increased step-through latency in AD + ZJ groups compared with AD group. In Morris water maze test (in probe day), both AD + ZJ groups receiving extract (500 and 1,000 mg) demonstrated significant preference for the quadrant in which the platform was located on the preceding day as compared with AD group. Our results suggested that ZJ has repairing effects on memory and behavioral disorders produced by NBM lesion in rats and may have beneficial effects in treatment of AD patients.

Keywords *Zizyphus jujube* · NBM lesioned · Memory impairment · Passive avoidance test · Spatial learning and memory

Abbreviations

AD	Alzheimer's disease
MDA	Malondialdehyde
NBM	Nucleus basalis of Meynert
FRAP	Ferric reducing/antioxidant power
ZJ	*Zizyphus jujube*

Introduction

Alzheimer's disease (AD) is the most common cause of dementia in old people, [1]. AD is a progressive chronic multifactorial neurodegenerative disorder which is mainly characterized by memory impairment [2].

Pathogenesis of AD is highly complex. However, extensive in vitro and in vivo experiments have shown that the decreased activity of cholinergic neurons in structures involved in cognitive processes such as neocortex, amygdala and nucleus basalis magnocellularis is responsible for AD [2].

Pathological hallmarks of AD lead to loss of forebrain cholinergic neurons and a decrease in acetylcholine levels can result in devastating cognitive impairment [3].

Experimental studies on AD animal models have shown that, both anticholinergic drugs and lesions of the nucleus basalis of Meynert (NBM) disrupt learning or memory in a number of paradigms such as passive avoidance and Morris water maze tests [4, 5].

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Inflammatory mediators [6] and defects in cholinergic transmission [7] have been demonstrated to be involved in AD. The clinical symptoms of AD are mainly memory impairment and loss of spatial memory [8]. Acetylcholinesterase (AChE) is the key enzyme in brain to breakdown acetylcholine. Inhibition of AChE is considered one of the treatment strategies against several neurological disorders such as AD [9, 10].

One strategy for ameliorating symptoms of AD is the restitution acetylcholine concentration in the synaptic cleft to improve cholinergic neurotransmission. Choline acetyltransferase activators increase the synthesis of acetylcholine to boost the endogenous levels of acetylcholine in the improvement of cognitive function in mild to moderate AD [11]. The free-radical hypothesis suggests that increased production of lipid peroxide and reactive oxygen species, which are produced with free radicals in membrane lipids, cause deterioration of a wide variety of cellular enzymes, subsequently exacerbating neurodegenerative process in AD [12]. Because free radicals are highly reactive, they can cause damage to important biomolecules, such as proteins, lipids, DNA and RNA, leading to cell death. Increased oxidative damage is observed during normal brain aging, and is more prominent in AD [13].

Zizyphus jujube (ZJ), among 50 tested plants had the highest activator effect on choline acetyltransferase in vitro [14]. ZJ is widely distributed in Iran. It has gained wide attention in native herbal medicine for treatment of broad range of disorders.

The present study evaluated the effects of hydroalcoholic extract of ZJ on learning and memory functions in AD rat model with memory deficits and intact rats through performance in the passive avoidance and Morris water maze tests.

Materials and Methods

Plant Material and Preparation of the Extract

For preparation of hydroalcoholic extract, jujube fruit was milled with core and finely powdered. The powder of jujube fruit was extracted by distilled water and methanol (1:1 v/v) as solvent. This extract was filtered and concentrated under reduced pressure on a rotary evaporator and then lyophilized. The extract was dissolved in water and used when needed [15].

Determination of Radical Scavenging Activity of *Zizyphus jujube* Extract

Radical scavenging activity of ZJ extract was determined as described by Moon and Terao [16] with minor modification, based on ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) stable radicals. Briefly, various concentrations of the extract (20–700 μg/ml) were mixed with DPPH solution in ethanol (100 μl). The reaction mixture was shaken at room temperature in a dark room. After 15 min at room temperature, the absorbance was recorded at 517 nm using a UV–Vis spectrophotometer (Biochrom Ltd, England) [16]. Butylated hydroxytoluene (BHT) was used as a positive control.

Inhibition of free radical by DPPH (%) was calculated as follows:

$$I(\%) = 100 \times \left(A_{control} - A_{sample}\right)/A_{control}$$

Analysis of Total Phenolic Compounds in the Extract

The content of total phenols was measured based on Folin–Ciocalteau method and gallic acid, [17]. The standard curve was plotted using 12.5, 25, 50, 62.5, 100, and 125 mg/L solutions of gallic acid in methanol and water (60:40, v/v). The amount of phenols was obtained based on mg/g in gallic acid equivalent.

Total Flavonoid and Flavonol Determination

The amount of total flavonoids and flavonols in the jujube fruit extract was determined calorimetrically as described by Asgari et al. [18]. The experiments were repeated three times. Total flavonoids and flavonols were expressed in terms of rutin equivalent (mg/g), which is a common reference compound.

Animals

Male Wistar rats, weighing 150–250 g, were obtained from Pasteur institution (Tehran, Iran). Rats were housed in groups of four in cage at 25 °C with controlled 12 h light–dark cycle. Food and water were freely available.

All experiments were executed in accordance with the Guide for the Care and Use at Laboratory Animals and were approved by Research and Ethics Committee at Shahrekord University of Medical Sciences.

Animals were randomly divided into the seven groups with seven rats in each group: 1—Control group without surgery, received distilled water, 2—AD group (bilateral NBM lesion, received water), 3 and 4—AD + ZJ groups (rats with NBM lesion received ZJ extract 500 and 1,000 mg/kg b.w, respectively i.p for 15 days), 5—Sham group (electrode was lowered into the NBM without passing any current, received water), 6 and 7—intact groups without any surgery received ZJ extract 500 and 1,000 mg/kg, respectively i.p for 15 days. Doses were applied according to previously published data [15].

Stereotaxic Surgery

For stereotaxic surgery, rats were anesthetized by intraperitoneal injection of ketamine hydrochloride 110 mg/kg and xylazine, 4 mg/kg then they were placed in stereotaxic apparatus. The surgery area was shaved to prevent infection by hairs. A stereotaxic stage with an embedded heating pad was used to keep the animals warm during surgery. The animal head was firmly positioned within the stereotaxic frame by inserting ear bars into the external ears. Following these steps, lubricating eye ointment was applied to prevent drying.

Midline skin incision along scalp was made using sterile scalpel and it was cleaned using sterile cotton tipped applicator.

All the rats were implanted with a twisted bipolar stainless-steel electrode (Plastic Products MS 301/1, 0.25 mm in diameter; Bilaney, Düsseldorf, Germany) in one hemisphere under conventional stereotaxic procedures. The electrode was aimed to the NBM with the incisor bar set at −2.7 mm below the interaural line and according to the following coordinates from the stereotaxic atlas of AP: −1.30 mm from bregma, L: ± 2.8 mm both respect to bregma, and DV: −8.00 mm from cranium surface [19]. The NBM lesions were made by electrolysis using a current intensity of 2 mA for 15 s. The electrode was withdrawn after induction of lesion at each side. The sham operated groups underwent similar surgical procedures without delivery of current. The incision was cleaned and sutured, subsequently the rats were returned to their home cages and allowed 10 days for recovery before behavioral testing [20].

Water Maze Test

A circular water pool was used for a water maze test. A black escape platform was placed into water and submerged 1 cm below the water in one quadrant water maze surface. Animals were placed into water at one of four positions selected randomly, and the time from start to escape onto the platform (acquisition latency) was measured. Animal were given four trials daily for 4 consecutive days, with an inter trial interval of 10 min. On the fifth day, rats were individually subjected to a probe trial session by removing the platform and were allowed to swim for 60 s to search for platform [21].

Passive Avoidance Test

Passive avoidance test was carried out using a shuttle box apparatus. The apparatus consisted of a lighted and dark compartment with a grid floor. This test was performed for each rat during the 4 days. Initial latency (t1) was recorded on third day and step-through latency (t2) for animals was recorded on forth day [22].

Ferric Reducing/Antioxidant Power (FRAP) Assay

Blood samples were collected from intact groups of rats and the antioxidant power assay was performed. The antioxidant power of plasma was determined by measuring its ability to reduce Fe^{3+}–Fe^{2+} with ferric reducing antioxidant power (FRAP) test according to the procedure described by Benzie and Strain [23]. $FeSO_4$ (100–1,000 μM concentration range) was used as a standard in FRAP assay. The results are expressed in μM.

Measurement of Malondialdehyde (MDA)

The Measurement of Malondialdehyde (MDA) level of the plasma was determined as described by Karatas et al. [24] with minor modification. Chromatographic determinations were performed on a high-performance liquid chromatograph equipped with an 1,100 series pump and a UV absorbance detector. An HP 3,395 integrator was employed to record retention times, chromatograms, and evaluate peak heights. A technopak 10u C18 reversed-phase column (emission553 and excitation 515) was used. MDA standards were prepared from 1, 1,3,3-tetraethoxypropane.

The optimized assay was carried out as follows: 50 μl plasma or the standard was treated with 50 μl (0.05 %) BHT (in absolute ethanol), followed by the addition of 400 μl H_3PO_4 (0.44 M) and 100 μl TBA (Butylated Hydroxytoune) (42 mM), vortexed and then incubated for 60 min at 100 C° the reaction was stopped by cooling at 4 C°, then 250 μl of n-butanol was added for extraction of MDA-TBA complex. The solution was vortexed and then centrifuged for 5 min at 14,000 rpm to separate two phases. The supernatant (20 μl) was injected into the HPLC system.

Statistical Analysis

All the results were expressed as mean ± SEM and statistical analyses were performed using SPSS 11.0 statistical software. $p < 0.05$ was considered statistically significant.

Results

Standardization of *Zizyphus jujube* Extract

To standardize the plant extract, total phenolic, flavonoid and flavonol compounds in ZJ extract were measured. Total amount of phenolic compounds in ZJ extract was 48.8 mg/g galic acid equivalent per 1 g dried extract. Total amount of flavonoid and flavonol compounds were 9.1 and 8 mg/g, respectively per 1 g of dry matter.

Table 1 DPPH radical scavenging activities for various concentrations of *Zizyphus jujube* extract and BHT as positive control

Sample	Concentration (μg/ml)	DPPH radical scavenging activity Inhibition (%) IC_{50} (μg/ml)
Zizyphus jujube extract	20	3.7
	50	6.2
	100	11.8
	250	28.4
	500	**53.5 (IC_{50})**
	700	70.5
BHT	20	8.8
	50	14.4
	100	**30.7 (IC_{50})**
	250	68.2
	500	79.3
	700	95.3

IC_{50} values are highlighted in bold

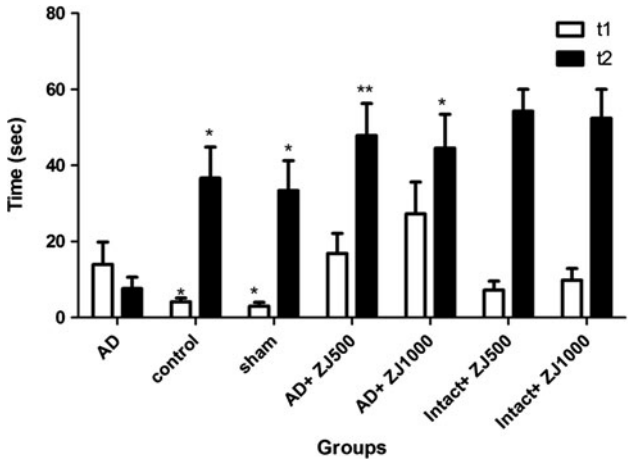

Fig. 1 The initial latency and step-through latency in the passive-avoidance response. *t1* initial latency, *t2* step-through latency. The data are expressed as mean ± SD; n = 7 in each group. *$p < 0.05$; **$p < 0.01$ AD versus control, sham, AD + ZJ 500, and AD + ZJ 1,000 groups (n = 7). *ZJ Zizyphus jujube*, *AD* Alzheimer's disease

Radical Scavenging Activity *Zizyphus jujube* Extract

IC_{50} values for radical scavenging activity of ZJ extract are shown in Table 1.

Effect of *Zizyphus jujube* Extract on Initial and Step-Through Latency in the Passive-Avoidance Response in Rat Model of AD

The mean initial latency increased statistically in AD group when compared with control group ($p = 0.035$). Step-through latency reduced markedly in AD group compared with control and sham groups ($p = 0.038$ and 0.030, respectively). Step-through latency increased significantly in AD + ZJ 500 and AD + ZJ 1,000 when compared with AD group ($p = 0.007$ and $p = 0.015$, respectively) (Fig. 1).

Zizyphus jujube extract (1,000 and 500 mg/kg) had no remarkable effect ($p > 0.05$) in the test session on intact rats when compared with control group.

Effect of *Zizyphus jujube* Extract on Morris Water Maze Swimming Test

Figures 2 and 3 show the effect of ZJ extract on Morris water maze swimming test. AD rats spent significantly less time in the correct quadrant (zone1) compared with control group in the probe trail ($p = 0.022$).

In the probe test (on fifth day of test), the control, sham, AD + ZJ 500 mg/kg and AD + ZJ 1,000 mg/kg groups demonstrated a significant preference for the quadrant in which the platform was located on the preceding day (zone1) when compared with AD group ($p = 0.022$, $p = 0.002$, $p = 0.043$ and $p = 0.001$ respectively). There were no significant differences between the control and sham groups in the probe test. Platform was eliminated on the probe day (Figs. 2, 3).

Then the data related to training—learning of animals was analyzed. AD group showed significant increase in latency before finding the escape platform across the training days when compared with control group. In control, sham, AD + ZJ 500 and AD + ZJ 1,000 rats the latency before reaching the platform decreased gradually during 4 days of training. Performance of sham rats was not significantly different from control rats. The decrease in the escape latency improved significantly on day 3 in control, sham, AD + ZJ 500 and AD + ZJ 1,000 when compared with AD group ($p = 0.044$, $p = 0.021$, $p = 0.006$ and $p = 0.008$ respectively). The latency before reaching platform on day 4 significantly decreased in control, sham, AD + ZJ 500 and AD + ZJ 1,000 when compared with control group ($p = 0.001$, $p = 0.000$, $p = 0.005$, $p = 0.001$, respectively) Fig. 4a.

Administration of ZJ extract with doses of 500 and 1,000 mg/kg/day in intact rats caused a slightly reduction (not significant, $p > 0.05$) of the latency before finding the escape platform compared with the control group (Fig. 4b).

Effect of *Zizyphus jujube* Extract on the Plasma Antioxidant Level

Figure 5 shows, the plasma level antioxidant power (FRAP assay) in all experimental groups. In the intact group, ZJ

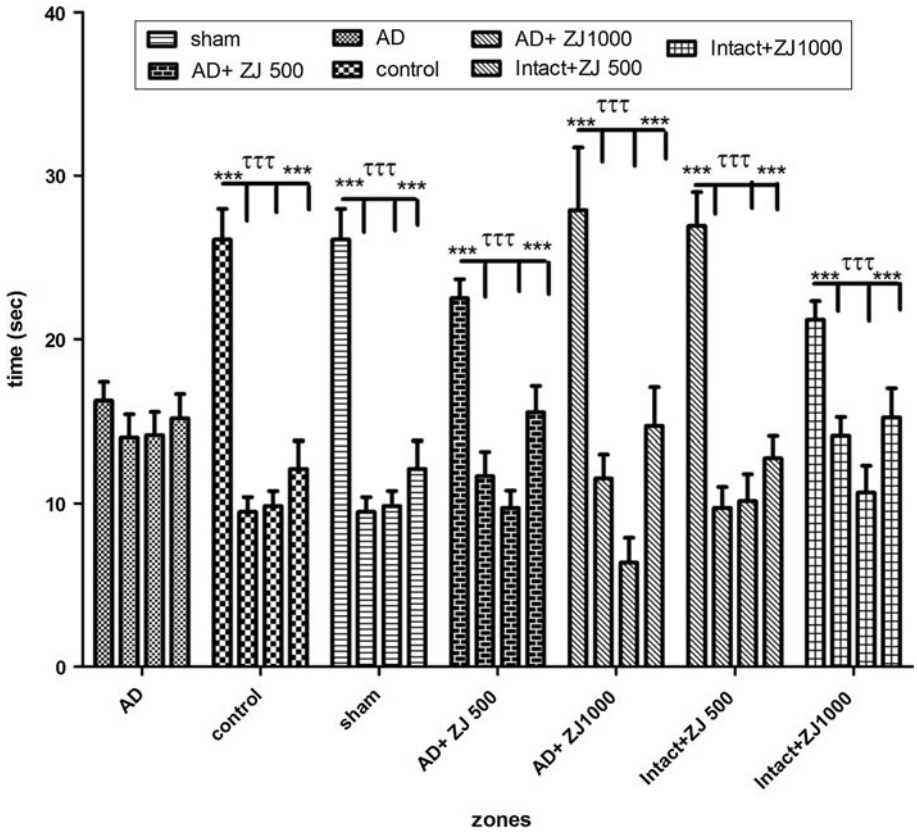

Fig. 2 Spent time in each quadrant (from *left* to *right*: zone1, zone2, zone3, zone4 in each group) during the probe trial in experimental groups. The data are expressed as mean ± SD; n = 7 in each group. ***, $^{\tau\tau\tau}p < 0.001$ zone 1 versus zone 2, 3, and 4 (n = 7). *ZJ Zizyphus jujube*, *AD* Alzheimer's disease

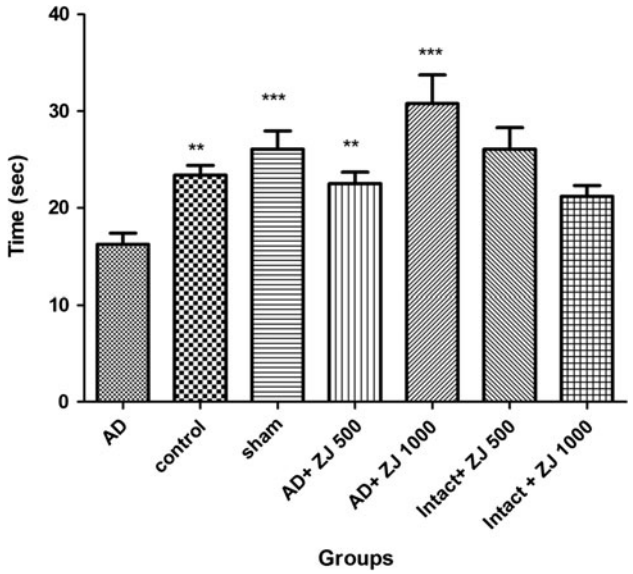

Fig. 3 Spent time in zone1 during the probe trial in experimental groups. The data are expressed as mean ± SD; n = 7 in each group. $^{**}p < 0.05$; $^{***}p < 0.01$ AD versus control, sham, AD + ZJ 500, and AD + ZJ 1,000 (n = 7). *ZJ Zizyphus jujube*, *AD* Alzheimer's disease

extract (500 and 1,000 mg/kg/day) increased plasma levels of antioxidant significantly as compared with the control group ($p = 0.001$ and $p = 0.046$, respectively).

No significant change ($p > 0.05$) was observed in plasma level antioxidant power between control group and sham group (Fig. 5).

Effect of *Zizyphus jujube* Extract on the Plasma Level of Malondialdehyde

Figure 6 shows, the plasma level of malondialdehyde in each experimental animal group. In the intact group, administration of ZJ extract resulted in a significant ($p < 0.001$) reduction in plasma MDA levels in the doses of 500 and 1,000 mg/kg/day ($p = 0.034$, $p = 0.037$, respectively) when compared with control group. No significant difference ($p > 0.05$) was observed between sham and control groups.

Discussion

AD is one of the most common neurodegenerative diseases, which proceeds from mild and moderate to severe stages and gradually destroys the brain. Brain aging is known to be related to decrease in acetylcholine level, neuronal loss, increase in inflammation, and oxidative stress [25].

The drugs accepted for the AD therapy usually act by counteracting the acetylcholine deficit, and are employed to enhance the acetylcholine level in the brain [26].

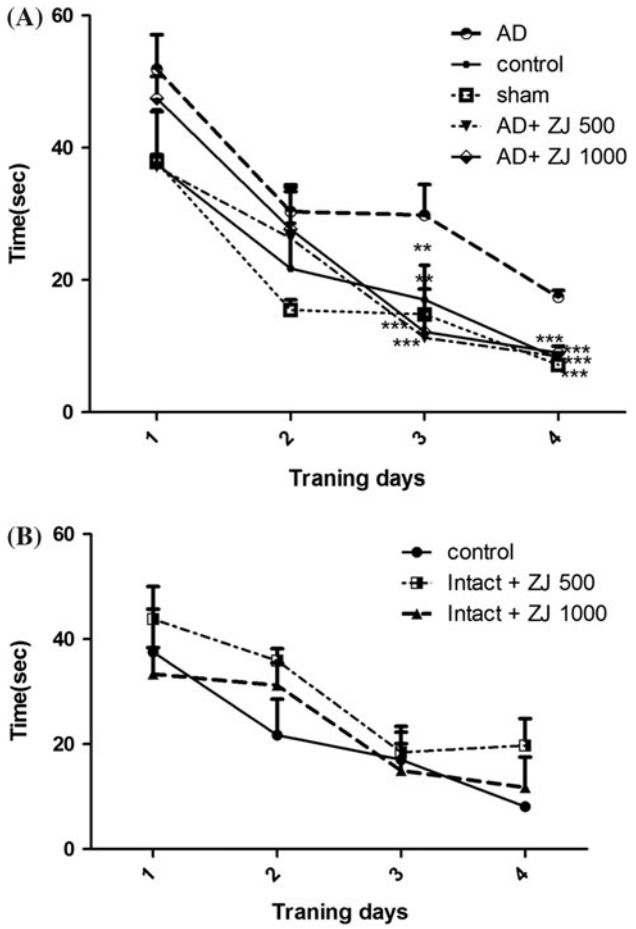

Fig. 4 **a**, **b** Spatial learning in a hidden platform model in experimental groups during four training days. The data are expressed as mean ± SD; n = 7 in each group. $^{**}p < 0.05$; $^{***}p < 0.001$ AD versus sham, control, AD + ZJ 500, and AD + ZJ 1,000 groups (n = 7). *ZJ Zizyphus jujube*, *AD* Alzheimer's disease

Most basal forebrain magnocellular neurons, including those in NBM, are cholinergic [20]. The basal forebrain has been implicated in a number of important behavioral functions such as learning and memory, attention, arousal, sleep, sensorimotor integration and locomotor activity [20].

Studies have shown that the severity of dementia in AD depends on the loss of neurons in the basal nucleus with large cells (NBM) and significant decrease in the amount of acetylcholine transferase enzyme in brain cortex and amygdalea results in impaired learning and memory [27].

Studies have also demonstrated that the ZJ extract has a high activator effect on choline acetyltransferase in vitro [14].

Our results have shown that the NBM lesions by electric current in rats have induced significant learning and memory disturbance in passive avoidance paradigm and spatial cognitive deficit in Morris water maze, whereas treatment of rats with ZJ extract for 15 days could significantly attenuate these abnormalities.

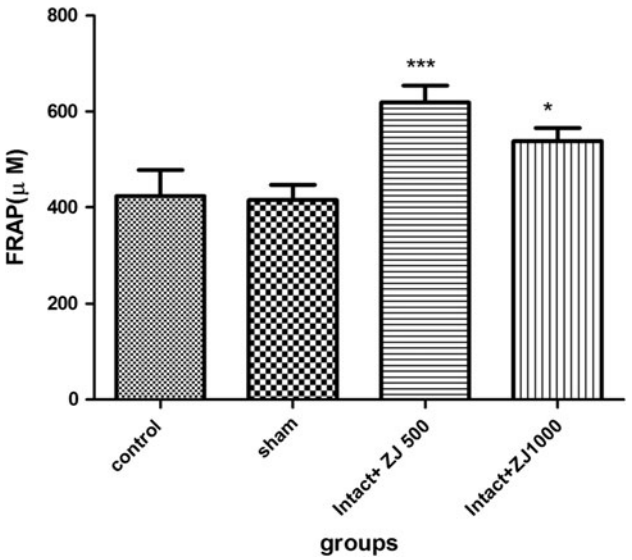

Fig. 5 Plasma antioxidant capacity (FRAP) in experimental groups. The data are expressed as mean ± SD; n = 7 in each group. $^{*}p < 0.05$; $^{***}p < 0.001$ control versus Intact + ZJ 500 and Intact + ZJ 1,000 (n = 7). *ZJ Zizyphus jujube*

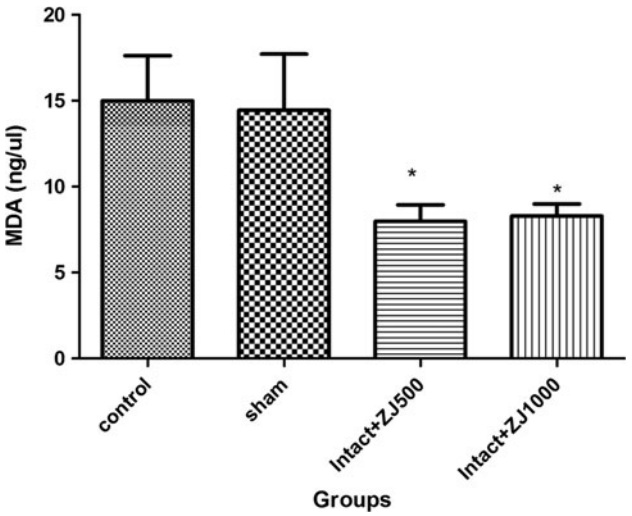

Fig. 6 Plasma malondialdehyde level (MDA) in experimental groups. MDA level decreased in both intact treated groups with extract ($^{*}p < 0.05$ in comparison with control group). The data are expressed as mean ± SD; n = 7 in each group. *ZJ Zizyphus jujube*

In our study ZJ extract significantly increased the step-through latency in NBM-lesioned group receiving extract in shuttle box test. The passive avoidance test is generally used to evaluate the treatments on three stages of memory, such as learning acquisition, memory retention, and the retrieval process [28].

Preacquisition electrical stimulation of the NBM improved the acquisition of the two-way active avoidance task and stimulated in the NBM reached the criterion level

during the first training session, while the control rats achieved it on the last training session [29].

Since many processes, such as motivation or sensorimotor function in addition to learning and memory, affect behavior all these factors could account for the facilitation in acquisition of active avoidance [30–32].

The electrical lesion of NBM did not affect shock sensitivity, therefore enhancement of acquisition after NBM stimulation does not seem to be a consequence of non-specific effects, such as sensorimotor function, and could be associated with attention processes. Microdialysis studies have shown a link between indices of cholinergic function and attention performance [29].

Neuropharmacological treatments increasing AChE efflux improve retention performance in behavioral paradigms or place recognition tasks [33].

Our results suggest that effect of ZJ extract on enhancing learning and memory in AD rat model may be related to mediation of the cholinergic neurotransmitter system.

The escape latency significantly reduced in AD + ZJ rats when compared with NBM-lesion rats. In the probe test, ZJ extract treatment of rats with NBM-lesion significantly increased time searching for hidden platform while these doses had no effect on intact rats. Our study indicates that improved spatial learning and memory in rat AD model may be related to mediation of cholinergic neurotransmitter system.

NBM lesion impairs performance of acquisition in Morris water maze and soybean can prevent impairment induced by NBM lesion [34]. There is some amount of isoflavone in soybean plant that is responsible for beneficial effects on learning and memory in rats. Lesion of NBM had no significant effect on swim speed in water maze test [34].

The brain and nervous system are considered to be more susceptible to oxidative damage than other tissues due to the high content of their polyunsaturated lipid-rich neural parenchyma, high oxygen utilization accounting for one-fifth of the total systemic consumption and low activity of antioxidant enzymes. Brain aging shows increased oxidative damage during normal brain aging, as well as in AD [35].

Pretreatment with hydroalcoholic extract of ZJ (250, 500 and 1,000 mg/kg) significantly increased the brain glutathione levels and significantly decreased brain MDA levels in rat [15].

In order to elucidate the mechanism of anti-amnesic activity of ZJ extract, lipid peroxidation and serum antioxidant levels were measured following the shuttle box and Morris water maze test. Determination of serum MDA levels is still the most commonly applied assay for lipid peroxidation in biomedical experiments, since MDA is one of the major aldehydes formed after breakdown of lipid hydroperoxides. Therefore, it is considered a good biomarker of the involvement of free radical damage in pathologies associated with oxidative stress [36].

Our results showed that pretreatment with ZJ extract significantly decreased serum MDA levels in experimental groups. Our study indicated that improved spatial learning and memory in rat model of AD may be related to decrease in oxidative stress in brain.

Conclusion

In conclusion, the present study clearly demonstrates that ZJ extract treatment can significantly prevent the cognitive impairments following NBM lesion and thus suggesting the therapeutic potential of this extract in aging and age-related neurodegenerative disorders. Further work is required to extend these observations.

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