Original Article

Behavioral and Histological Analysis of Crocus Sativus Effect in Intracerebroventricular Streptozotocin Model of Alzheimer Disease in Rats

Mohsen Khalili[1], Zahra Kiasalari [1], Batol Rahmati [1], Jamshid Narenjkar [2]

1. Dept of Physiology and Neuroscience Research Center, Shahed University of Medical Science, Tehran, Iran
2. Dept of Pharmacology, Shahed University of Medical Science, Tehran, Iran

ABSTRACT

Background and Objectives: **There is well established the beneficial effects of Crocus sativus extract in learning and memory improvement. In the present study the effect of this plant in memory behavioral impairment and forbrain histological damage induced by STZ-icv model of Alzheimer disease were investigated.**

Materials and Methods: **This study was conducted at Shahed University (Tehran) in 2007. Forty five male rats were divided into three 15 number groups: 1- Control which received CSF bilaterally two times in 1 and 3 days (10 µl in each injection) 2- STZ-icv, streptozotocin (3 mg/kg) dissolved in CSF was injected (icv) to the animals. 3- STZ+CSE, the STZ-icv animals received the plant extract (30 mg/kg; i.p) one other day as treatment ones. All of the animal groups were weighted and subjected to memory behavioral passive avoidance test and brain histological damage analysis.**

Results: **STZ caused selective injury to the fornix and hippocampus and an enlargement as well as loss of ependymal cell in third ventricle. However, STZ-icv treated animals with CSE (30 mg/kg, i.p) one other day starting one day pre-surgery for three weeks show higher correct choice and lower errors in shuttle box test than vehicle-treated STZ-injected rats. But the same CSE treatment rats did not show any antagonizing effects on STZ-icv induced histological impairment.**

Conclusion: **Our findings provide an explanation for effectiveness of CSE in preventing the cognitive deficits caused by STZ-icv in rats, which mediated by enzymes, metabolisms (glucose utilization) and other biochemical pathways, but not via histological injury repair.**

Keywords: Memory, Crocus sativus, Streptozocin, Neurotoxicity Syndrome, Rat

Received: 20 May 2009
Accepted: 26 Augest 2009
Address communications to: Dr Mohsen Khalili, Dept of Physiology and Neuroscience research center, Shahed university of Medical Science, Tehran, Iran
Email: najafabady@yahoo.com

Introduction

Intracerebroventricular (ICV) injection of streptozotocin (STZ) in rats is followed by long-term and progressive deficits in learning, memory, and cognitive performance in rats that is similar to sporadic kind of Alzheimer's disease (SAD), as indicated by behavioral tests including passive avoidance paradigm (1). It was obvious that ICV injection of STZ could reduce regional glucose utilization occurs in the temporal and parietal cortex (2, 3), and histological damages to septal, fornix and third ventricle. Regarding to the close relationship between cortical and hippocampal cholinergic transmission, glucose utilization and cognitive function (4, 5), the reduction in glucose utilization and disruption in spatial memory seen after STZ-icv could be secondary to damage to the fornix. Meanwhile, loss of choline acetyltransferase (ChAT) activity in the hippocampus (6) could produce a decrease in the transport of nerve growth factor (NGF) from the hippocampus to the septum and finally reduction of the weight of septum (7). Similar changes in the activity chAT and NGF occur after lesions to the fornix, a structure that contains axons communicating between the septum and hippocampus (8). Moreover, it was shown transection of the fornix could yield both impairment of learning and glucose utilization in the hippocampus and cingulate cortex (9). There is also experimental evidence that Crocus sativus (saffron) and its components are involved in cognition. It has been shown that administration either of extracts of C. sativus, or of its constituents crocins, reduced ethanol-induced memory impairment in the passive avoidance in mouse (10, 11). Recently, it has been demonstrated that saffron extracts counteracted recognition memory deficits and persist against scopolamine-induced performance impairments in the passive avoidance task in rat (12).

The aim of the present study regarding to marked counteraction effect of saffron in learning and memory deficits in different animal models, was to investigate the role of C. sativus. in antagonizing histological brain impairment in ICV STZ–induced model of SAD in the male rats.

Materials and Methods

Streptozotocin (STZ) was purchased from Sigma Chemicals Co (St. Louis, MO, USA). Ketamone (10%) and xylazine (2%) were purchased from Alfasan Co (Holland). Chemical constitutes ACSF to make ready from Merck Co (Germany). Saffron were provided from local market and then scientifically identified by the department of botany of Shaheed Beheshti University (SBU).

The present experimental study was performed at Shahed University (Tehran) in 2007 on male Wistar rats (Pasteur's Institute, Tehran) weighing 250–290 g (aged 3.5–4 months). The rats were housed two per cage for 1 week in the animal house at an ambient temperature of 21 ± 1°C and a 12-h diurnal light cycle, before surgery. Protocol of this study was approved by research council of Shahed University that was in accordance with the policies set forth in the Guide for the Care and Use of Laboratory Animals (NIH).

Experimental procedure

Rats (n = 45) were randomly divided into three 15 number groups: 1. Control group (CSF) 2. STZ-injected group (STZ) which received ICV injection of STZ 3. CSE-treated STZ group (STZ + CSE), which also received CSE (30 mg/Kg; i.p.) one other day. STZ and CSE-treated STZ groups were given a bilateral ICV injection of STZ (Sigma, St. Louis, USA) (3 mg/kg). STZ was freshly dissolved in cold artificial CSF (120 mM NaCl; 3 mM KCl; 1.15 mM CaCl2; 0.8 mM MgCl2; 27 mM NaHCO3; and 0.33 mM NaH2PO4 adjusted to pH 7.2) (Merck Chemical, Germany) and at a volume of 10 µl on each side. The injection of STZ was repeated on day 3. For stereotaxic surgery, rats were anesthetized with a combination of ketamin (100 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.), placed in a stereotaxic apparatus (Stoelting, USA). The scalp was cleaned with iodine solution, incised on the midline and a burr hole was drilled through the skull (A, -0.8 mm from bregma; L, 1.4 mm; 3.4 mm below the dura) according to the stereotaxic atlas (13).

Preparation of crude extract

Ten grams of cleaned C. sativus stigma (saffron) was crushed and mixed with 50 ml distilled water. The mixed complex was boiled for 20 min. Then, the aqueous extract was filtered (Whatman No. 1) three times. The filtrate was dried in an organ bath at 50 °C. The yield of the extract was 59% (W/W).

Single trial passive avoidance test

The apparatus (BPT Co., Tehran) consisted of an illuminated chamber connected to 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 on the apparatus and left for 5 min to habituate to the

apparatus. On the third day, an acquisition trial was performed. Rats were individually placed in the illuminated chamber. After a habituation period (2 min), the guillotine door was opened and after the rat entering the dark chamber, the door was closed and an inescapable scrambled electric shock (1 mA, 1 s once) was delivered. In this trial, the initial latency (IL) of entrance into the dark chamber was recorded and rats with ILs greater than 60 s were excluded form the study. Twenty-four hours later, each rat was placed in the illuminated chamber for retention trial. The interval between the placement in the illuminated chamber and the entry into the dark chamber was measured as step-through latency (STL up to a maximum of 150 s). This test was conducted after 3 weeks post-surgery and each rat was tested only once.

Histological study

At the end of behavioral experiments, the rats were deeply anesthetized with high dose of ketamine (150 mg/kg) and perfused through the ascending aorta with 50-100 mL of 0.9% saline followed by 300 mL of fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) followed by 100 mL of 0.1 M PB containing 10% sucrose. Following perfusion, the brains were removed from the skull, the block of brain (-2.56 to -4.16 from bregma) were prepared, and after final step of preparation (30% sucrose for 2 days), sections (nearly 28-32 number) were cut in coronal plane at thickness of 50 µm on a freezing microtome (Leica) and collected in PB (0.1 M). Every second section was Nissl-stained with 0.1% cresyl violet (Sigma).

Quantitative analyses

Calculation of ventricular volume: the 3rd ventricle was assumed to be a cylinder oriented horizontally at the base of the brain, and along the anterior–posterior axis of the brain. The cross-sectional area of the 3rd ventricle was thus taken as the base of the cylinder. However, since the cross sectional area of the 3rd ventricle varies along its anterior–posterior extent, it was measured in coronal sections, at three levels posterior to bregma: 1.8 mm, 2.56 mm and 3.30 mm. The cross-sectional area at each level was multiplied by 0.75 mm, the anterior–posterior distance which represents the height of the cylinder at each level.

The size of the fornix adjacent to the site of STZ injection was quantified as the cross-sectional area at the junction between the fornix and anterior hippocampus at three levels posterior to bregma: 0.92, 1.4 and 2.3 mm. The Image tools software were used for measurement of fornix size and 3rd ventricle volume.

Statistical analyses

All results were expressed as mean ± S.E.M. For the passive avoidance test, nonparametric Kruskal-Wallis test was used, which if significant, was followed by Mann-Whitney U-test for pair-wise comparisons. Anatomical data (fornix and ventricular size) were also analyzed by ANOVA test.

Results

Effect of STZ and CSE on memory retention deficit in passive avoidance test

The initial latency was 29.9, 41.5 and 29.9 s in Control, STZ-icv and STZ+CSE groups respectively. The mean initial latency was statistically different between STZ and control groups ($P < 0.05$). Step through latency in STZ-icv (17.98) and STZ+CSE (31.9) groups reduced markedly in comparison to control (50.9) animals. On the other hand, the STZ + CSE group exhibited significant reversal of STL ($P < 0.05$) as compared to STZ-icv group, indicating improved acquisition or retention of memory (Fig. 1).

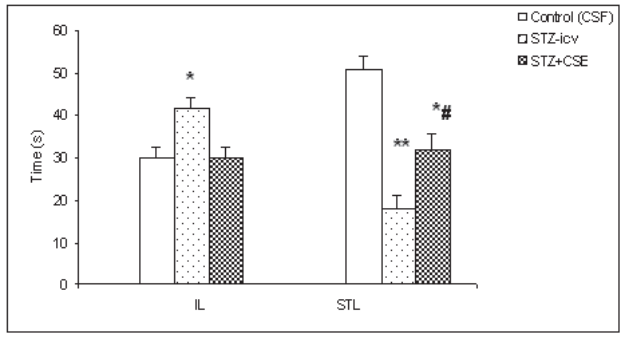

Fig. 1. The effect of CSE treatment (30 mg/kg) on passive avoidance performance after ICV injection of STZ in rats as indicated by initial and step-through latencies after 3 weeks. Values are expressed as means ± S.E.M. * $P < 0.05$, ** $P < 0.01$ (in comparison with control group); # $P < 0.05$ (STZ + CSE vs. STZ-icv) (non-parametric Kruskal-Wallis and Mann-Whitney U-tests

Histopathology

Effects of STZ-icv and CSE on the size of fornix ICV-injedtion of STZ reduced the size of the fornix and anterior hippocampus significantly (Fig. 2). Not

that the CS extract could not antagonized the mentioned STZ-histopathological effects (quantification show in Fig. 3). However a shrinkage of fornix (arrows in B and C) resulting from the enlargement of left ventricular (LV) space could shown. Also, there was shown a marked fornix cellular fragmentation in STZ and STZ+CSE groups (arrowheads in E and F) in comparison to not degeneration CSF control cells (D). Analysis of fornix size on three distance from bregma (mm) in all groups (Fig. 3), show a significant difference in STZ and STZ+CSE groups in comparing to control (CSF) ones.

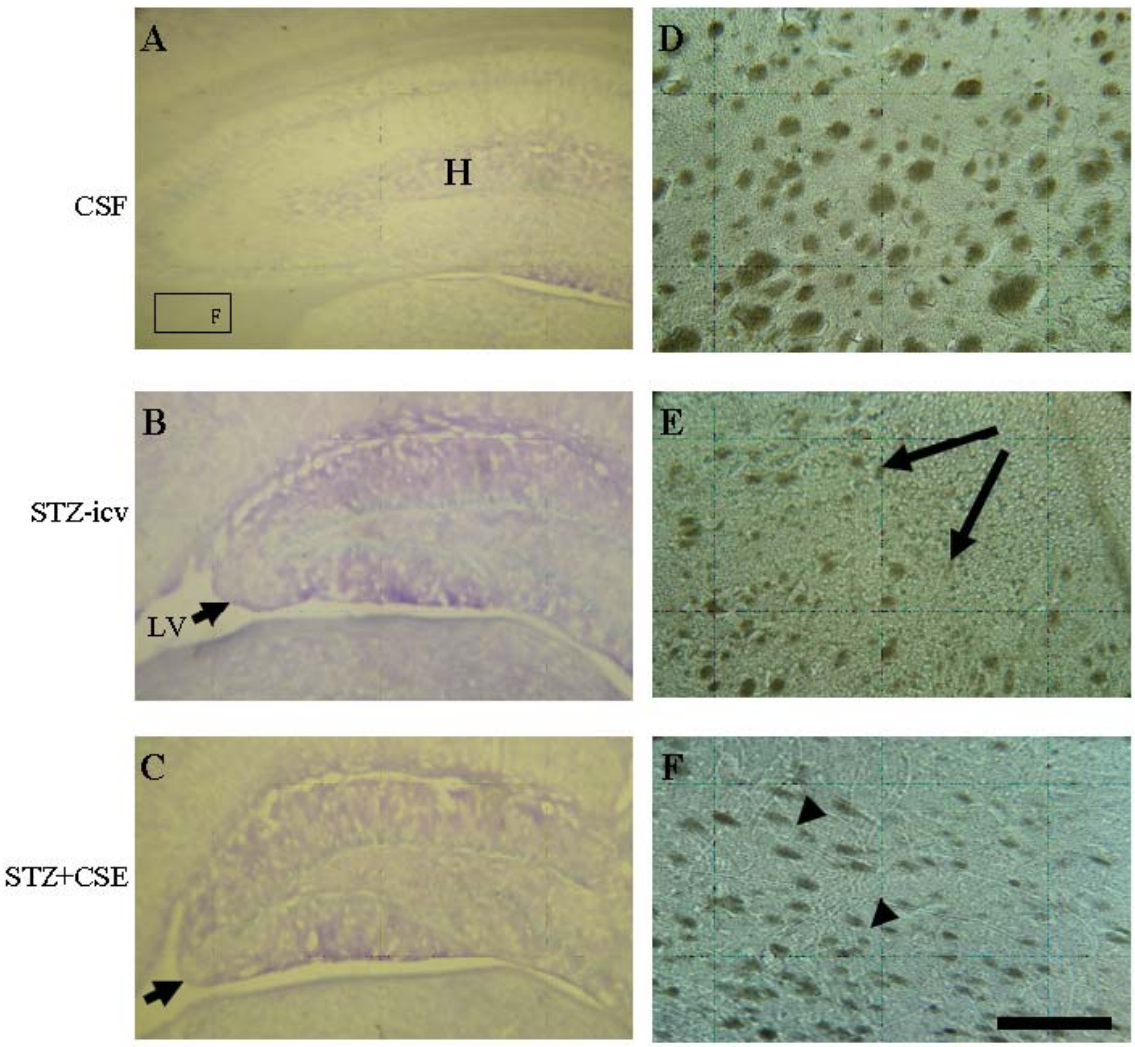

Fig. 2. Cresyl violet staining of hippocampus (H) and fornix (F) in control (A) STZ (B) and (STZ+CSE) groups in coronal plane. As shown CS extract (C) could not reverse the shrinkage of fornix and enlargement of LV. Fornix cellular fragmentation (arrowheads in E and F) is obvious in STZ (E) and STZ+CSE (F) groups. Calibration BAR = 400 μm in A–C and 100 μm in D-F.

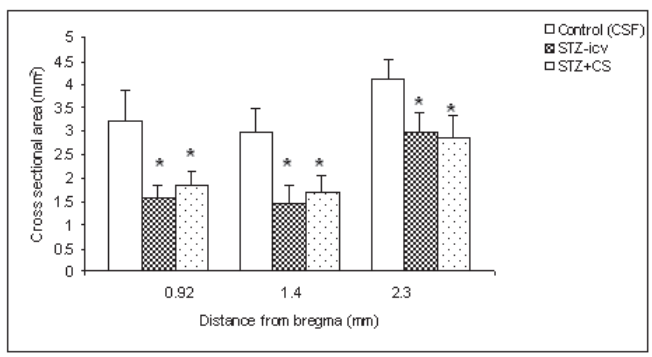

Fig. 3. Effects of STZ-icv and CS extract on the size of fornix. The abscissa depicts the distance from bregma at which measurement of the fornix cross-sectional area was measured. * Significantly different from CSF (Contro), $P < 0.05$.

Effects of STZ and CSE on volume of third ventricle
Injection of STZ-icv caused significant enlargement of the 3rd ventricle (Fig.4 B, small arrows) in comparing to controls (A). However treatment of the animals with CSE extract could not reverse STZ-icv patohistolocal effects (Fig.4C). Also, a halo around 3rd ventricle resulted from periventricular damge will produce due to STZ-icv application (Fig. 4 big arrows). However, CS extract could not prevent from this degeneration (Fig.5C). Quantification analysis show that if there is an anatomical increase in the volume of the 3rd ventricle with increasing distance posterior from bregma, the effect of STZ and STZ+CSE were superimposed on the normal pattern of change in 3rd ventricle volume as the distance from the bregma increased (Fig .5).

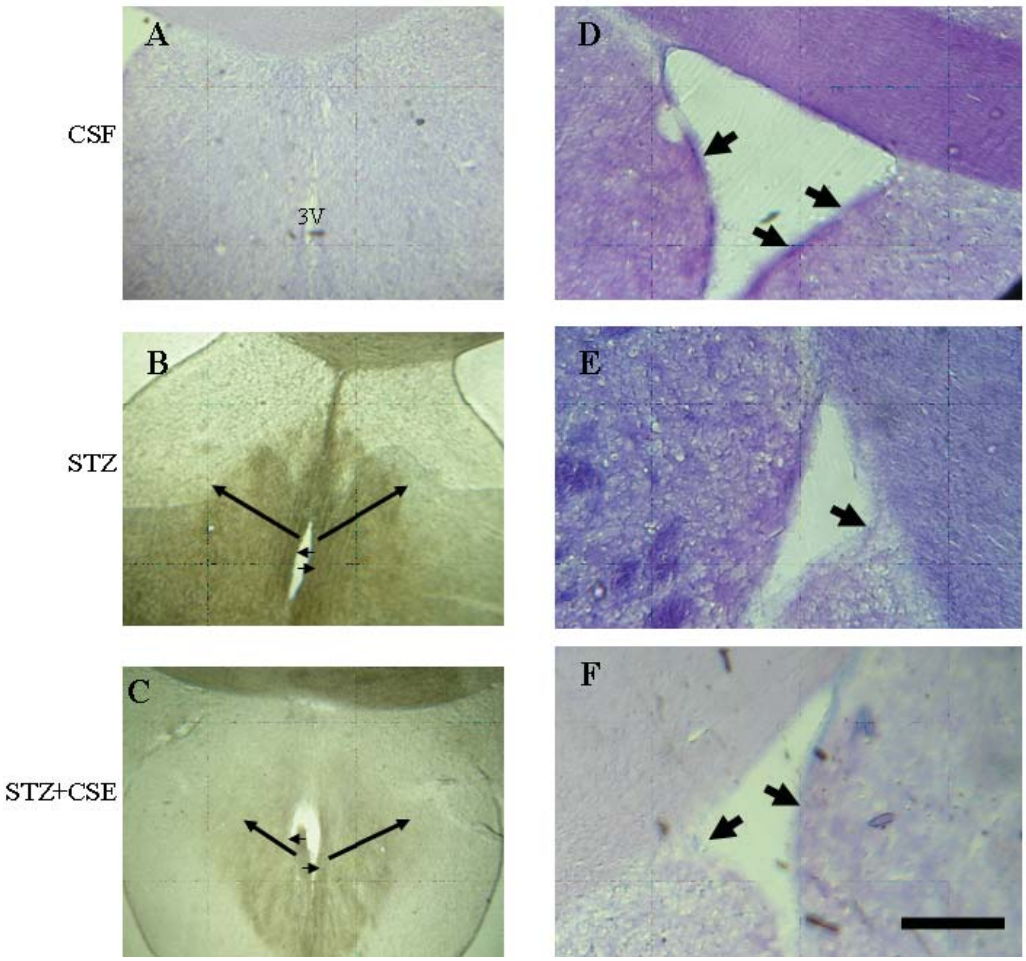

Fig. 4. Coronal plane sections cresyl violet staining show the marked enlargement of 3rd ventricle (B and C small arrows) and periventricular halo damage (B and C big arrows) in STZ-icv and STZ+CSE were shown. The normal periventricular wall ependymal cell layer (Fig. 4 D arrows) in CSF group have significantly damaged in STZ-icv and STZ+CSE animals (arrows in E and F). Calibration BAR = 400 µm in A–C and 100 µm in D-F

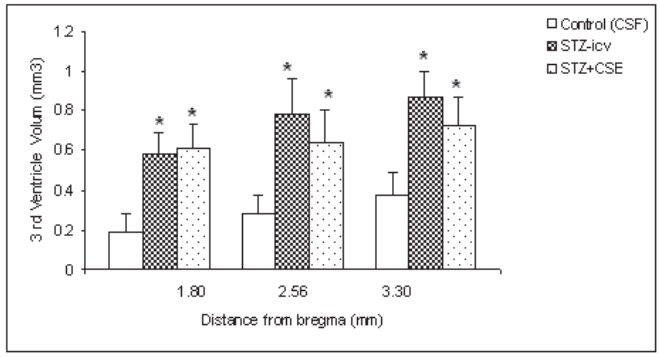

Fig. 5. Effects of STZ and CSE on volume of third ventricle. The abscissa depicts the distance from bregma (mm) at which the ventricular volume was measured. * Significantly different from CSF (Control), $P < 0.05$

Effects of STZ and STZ+CSE on periventricular wall ependymal cell layer

In CSF-injected rats, an ependymal cell layer lined the periventricular wall (Fig. 5 D). In STZ-injected animals, this layer was severely damaged or missing (Fig. 5 E). Not that the CSE (Fig. 5 F) could not alleviate this effect.

Discussion

The results of the present study demonstrated that treatment of STZ-icv rats with CSE (30 mg/kg) for 3 weeks could significantly ameliorate cognition deficits, but not fornix, hippocampal and other histopatological abnormalities. In the present study, STZ at a dose of 3 mg/kg was used. This dose has been shown not to cause any change in the peripheral blood glucose level, although this dose induces a significant cognitive impairment in all of the animals (14). The results from the passive avoidance test in concomitant with other reports (15) showed that the STZ-injected rats reveal significantly reduced retention latencies (STLs), suggesting an impairment in learning and memory processes. Also, our histological data in conformity with the Shoham report (16) show that injection of STZ-icv inflicted severe damage to the fornix and myelinated fornical axons, thereby disrupting septohippocampal activity. Disrupt transport of NGF via fornical axonal fibres from septum to hippocampus could explain the selective reduction of chAT activity in hippocampus (6). There was reported that the main memory deficits mechanisms for STZ-icv injection is related to induction of oxidative stress (17) to which myelin is particularly vulnerable (3). In addition, evidence of lipid peroxidation in whole brain homogenates was provided by the finding of an increase in malondialdehyde and a decrease in glutathione 3 weeks after icv injection of STZ (18). Therefore, that neuroprotective (19), antioxidant (20, 21) and free radical scavengering action of CSE (22) could describe the antagonized extinction recognition memory action of the CSE.

However, ameliorating effect of potent antioxidant chemicals (like vitamin E) and plants (like turmeric) on STZ-icv cognition deficits (23, 24) consolidate the hypothesis antioxidant action of CSE on memory impairment. Hence it has been suggested that elevation of the acetylcholine (ACh) level might be helpful in attempts to improve the symptoms of cognitive deficits in SAD, reports on antagonizing action of scopolamine-induced memory deficits by CSE, potentiate the direct or indirect hypothesis of cholinergic mimicking action of the CSE (the main neurotransmitter involved in learning and memory). Finally our histological data show the impotency of CSE for antagonizing of anatomical abnormality due to STZ-icv application. Regarding to in vivo and in vitro neuronal injury protection effect of carotenoids extracted from saffron (25), perhaps some minor cellular protection were occurred by the extract which could not show with our used methodological histology experiments.

Conclusion

By comparison of the behavioral and histological results, it was reveal that improvement of the learning and memory in STZ+CSE treatment animals could mediated via metabolism or enzyme mechanisms, but anatomical structural repair were not probably involved.

Acknowledgements

We wish to thank Fariba Ansari from Department of Physiology, School of Medicine, Shahed University, for the preparation of the aqueous *Crocus sativus* extract. The authors declare that they have no conflicts of interest.

References

1. Veerendra Kumar MH, Gupta YK. Effect of Centella asiatica on cognition and oxidative stress in an intracerebroventricular streptozotocin model of Alzheimer's disease in rats. Clin Exp Pharmacol Physiol 2003;30(5-6):336-42.

2. Arnaiz E, Jelic V, Almkvist O, Wahlund LO, Winblad B, Valind S, *et al.* Impaired cerebral glucose metabolism and cognitive functioning predict deterioration in mild cognitive impairment. Neuroreport 2001;12(4):851-5.

3. Smith KJ, Kapoor R, Felts PA. Demyelination: the role of reactive oxygen and nitrogen species. Brain Pathol 1999;9(1):69-92.

4. Hoyer S, Nitsch R, Oesterreich K. Predominant abnormality in cerebral glucose utilization in late-onset dementia of the Alzheimer type: a cross-sectional comparison against advanced late-onset and incipient early-onset cases. J Neural Transm Park Dis Dement Sect 1991;3(1):1-14.

5. Wree A, Kaever C, Birgel B, Schleicher A, Horvath E,

Zilles K. Local cerebral glucose utilization in the brain of old, learning impaired rats. Histochemistry 1991;95(6):591-603.

6. Blokland A, Jolles J. Spatial learning deficit and reduced hippocampal ChAT activity in rats after an ICV injection of streptozotocin. Pharmacol Biochem Behav 1993;44(2):491-4.

7. Terwel D, Prickaerts J, Meng F, Jolles J. Brain enzyme activities after intracerebroventricular injection of streptozotocin in rats receiving acetyl-L-carnitine. Eur J Pharmacol 1995 Dec 4;287(1):65-71.

8. Hagg T, Fass-Holmes B, Vahlsing HL, Manthorpe M, Conner JM, Varon S. Nerve growth factor (NGF) reverses axotomy-induced decreases in choline acetyltransferase, NGF receptor and size of medial septum cholinergic neurons. Brain Res 1989 Dec 25;505(1):29-38.

9. Galani R, Obis S, Coutureau E, Jarrard L, Cassel JC. A comparison of the effects of fimbria-fornix, hippocampal, or entorhinal cortex lesions on spatial reference and working memory in rats: short versus long postsurgical recovery period. Neurobiol Learn Mem 2002 ;77(1):1-16.

10. Sugiura M, Shoyama Y, Saito H, Nishiyama N. Crocin improves the ethanol-induced impairment of learning behaviors of mice in passive avoidance tasks. Proc Jpn Acad 1995;(71):319-24.

11. Zhang Y, Shoyama Y, Sugiura M, Saito H. Effects of Crocus sativus L. on the ethanol-induced impairment of passive avoidance performances in mice. Biol Pharm Bull 1994;17(2):217-21.

12. Pitsikas N, Sakellaridis N. Crocus sativus L. extracts antagonize memory impairments in different behavioural tasks in the rat. Behav Brain Res 2006 Oct 2;173(1):112-5.

13. Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. 2 ed. San Diego: Academic Press; 1986.

14. Sharma M, Gupta YK. Chronic treatment with trans resveratrol prevents intracerebroventricular streptozotocin induced cognitive impairment and oxidative stress in rats. Life Sci 2002 Dec 11;71(21):2489-98.

15. Lannert H, Hoyer S. Intracerebroventricular administration of streptozotocin causes long-term diminutions in learning and memory abilities and in cerebral energy metabolism in adult rats. Behav Neurosci 1998;112(5):1199-208.

16. Shoham S, Bejar C, Kovalev E, Weinstock M. Intracerebroventricular injection of streptozotocin causes neurotoxicity to myelin that contributes to spatial memory deficits in rats. Exp Neurol 2003;184(2):1043-52.

17. Feillet-Coudray C, Rock E, Coudray C, Grzelkowska K, zais-Braesco V, Dardevet D, *et al.* Lipid peroxidation and antioxidant status in experimental diabetes. Clin Chim Acta 1999;15;284(1):31-43.

18. Sharma M, Gupta YK. Effect of chronic treatment of melatonin on learning, memory and oxidative deficiencies induced by intracerebroventricular streptozotocin in rats. Pharmacol Biochem Behav 2001;70(2-3):325-31.

19. Fukui K, Omoi NO, Hayasaka T, Shinnkai T, Suzuki S, Abe K, *et al.* Cognitive impairment of rats caused by oxidative stress and aging, and its prevention by vitamin E. Ann N Y Acad Sci 2002;959:275-84.

20. Saleem S, Ahmad M, Ahmad AS, Yousuf S, Ansari MA, Khan MB, *et al.* Effect of Saffron (Crocus sativus) on neurobehavioral and neurochemical changes in cerebral ischemia in rats. J Med Food 2006;9(2):246-53.

21. Papandreou MA, Kanakis CD, Polissiou MG, Efthimiopoulos S, Cordopatis P, Margarity M, *et al.* Inhibitory activity on amyloid-beta aggregation and antioxidant properties of Crocus sativus stigmas extract and its crocin constituents. J Agric Food Chem 2006;54(23):8762-8.

22. Assimopoulou AN, Sinakos Z, Papageorgiou VP. Radical scavenging activity of Crocus sativus L. extract and its bioactive constituents. Phytother Res 2005;19(11):997-1000.

23. Tiwari V, Kuhad A, Bishnoi M, Chopra K. Chronic treatment with tocotrienol, an isoform of vitamin E, prevents intracerebroventricular streptozotocin-induced cognitive impairment and oxidative-nitrosative stress in rats. Pharmacol Biochem Behav 2009;93(2):183-9.

24. Ishrat T, Hoda MN, Khan MB, Yousuf S, Ahmad M, Khan MM, *et al.* Amelioration of cognitive deficits and neurodegeneration by curcumin in rat model of sporadic dementia of Alzheimer's type (SDAT). Eur Neuropsychopharmacol 2009;19(9):636-47.

25. Ochiai T, Shimeno H, Mishima K, Iwasaki K, Fujiwara M, Tanaka H, *et al.* Protective effects of carotenoids from saffron on neuronal injury in vitro and in vivo. Biochim Biophys Acta 2007;1770(4):578-84.