

The Effect of Aqueous *Crocus sativus* L. Extract on Intracerebroventricular Streptozotocin-induced Cognitive Deficits in Rat: a Behavioral Analysis

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Abstract

Intracerebroventricular (ICV) injection of streptozotocin (STZ) causes cognitive impairment in rats. The beneficial effect of *Crocus sativus* L. extract (CSE) was evaluated on ICV STZ-induced learning, memory, and cognitive impairment in male rats. For this purpose, rats were injected with ICV STZ bilaterally, on days 1 and 3 (3 mg/kg). The STZ-injected rats received CSE (60 mg/kg; i.p.) every other day, starting one day before surgery, for three weeks. The learning and memory performance was assessed using passive avoidance paradigm, and for spatial cognition evaluation, Y-maze task was used. It was found that CSE-treated STZ-injected rats show higher correct choices and lower errors in Y maze than vehicle-treated STZ-injected rats. In addition, CSE administration significantly attenuated learning and memory impairment in treated STZ-injected group in passive avoidance test. Therefore, these results demonstrate the effectiveness of CSE in preventing the cognitive deficits caused by ICV STZ in rats and its potential in the treatment of neurodegenerative diseases such as Alzheimer's disease (AD).

Keywords: *Crocus sativus* L; Streptozotocin; Learning; Memory; Spatial cognition; Rat.

Introduction

Intracerebroventricular injection of 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 AD (SAD), as indicated by behavioral tests including passive avoidance paradigm (1). SAD has been known as a chronic debilitating neurodegenerative disorder characterized by progressive cognitive impairment, memory loss, and behavioral disturbances (2) and is considered

as the most common cause of dementia in elderly patients (3). Interventions that could delay SAD onset would have a major public health impact (2). Free radical generation has been associated with cognitive impairment in ICV STZ model of SAD in rats (1, 4) and ICV STZ has also been known to impair cholinergic neurotransmission (5).

Crocus sativus L., commonly known as saffron, is a plant cultivated in various parts of the world such as Iran, China, Spain, Italy and Greece. Chemical analysis of its stigmas has shown the presence of water-soluble carotenoids, small amounts of monoterpene aldehyde and its glucoside (safranal and picrocrocin) and

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flavonoids (quercetin and kaempferol) (6). The pistils of *C. sativus* are used in traditional medicine as an antispasmodic, eupeptic, nerve sedative and emmenagogue (7). Its crude extract and purified chemicals have been demonstrated to prevent tumour formation (8-10), atherosclerosis (11) or hepatic damage (12).

There is also experimental evidence that 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 (13, 14). Recently, it has demonstrated that saffron extracts counteracted recognition memory deficits and persist against scopolamine-induced performance impairments in the passive avoidance task in rat (15).

The aim of the present study was to further investigate the role of *C. sativus* in learning and memory processes. For this purpose, we evaluated the effect of *C. sativus* in antagonizing extinction of recognition memory in ICV STZ-induced model of SAD in the rats using passive avoidance and Y-maze tasks.

Experimental

Materials

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 obtained from local market and then scientifically identified by the department of botany of Shahid Beheshti University (SBU).

Methods

Animals

Male adult Wistar rats (310-350 g) (Pasteur's Institute, Tehran), at the start of the experiment were housed three to four per cage in a temperature-controlled colony room under 12-h light/dark cycle. Animals were given free access to water and kept at 80–85% of their free feeding body weight throughout the experiment. All behavioral experiments were carried out between 11 a.m. and 4 p.m. This

study was carried out in accordance with the policies set forth in the Guide for the Care and Use of Laboratory Animals (NIH) and those of the Research Council of Shahed University of Medical Sciences (Tehran, Iran).

Preparation of crude extract

Ten grams of cleaned *C. sativus* stamen (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 filter paper No. 1) three times. The filtrate was dried in an organ bath (50°C) until 5-6 grams of the concentrated filtrate was remained in the container.

Experimental procedure

Rats (n=60) were randomly divided into the following groups: 1. Control group, 2. Sham-operated group (SH) that received bilateral ICV injection of artificial CSF (ACSF) (10 µL on each side) as the solvent of STZ, 3. CSE-treated control group (CSE), 4. STZ-injected group (STZ) which received ICV injection of STZ, and 5. CSE-treated STZ group (STZ + CSE), which also received CSE (60 mg/Kg, i.p.) every 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 and at a volume of 10 µL on each side. The injection was repeated on day 3. In the sham group, only 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) was ICV injected. 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 (16).

Behavioral tests

Spatial Y-maze memory

The experimental apparatus for Y-maze consisted of a black-painted maze made of

Plexiglas. Each arm of the Y-maze was 40 cm long, 30 cm high and 15 cm wide (17, 18) and positioned at an equal angle (labeled A, B and C) and converged in an equilateral triangular central area with 15 cm at its longest axis. Each rat, naïve to the maze, was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The sequence of each arm entry recorded manually (i.e., ACBABCACBCACAC, etc.). A spontaneous alternation behavior, which is regarded as a measure of spatial memory, was defined as the entry into all three arms on consecutive choices in overlapping triplet sets (i.e., ACB, ABA, CAC, BCA, CAC) (18). The percentage of spontaneous alternation behavior was calculated as the ratio of actual to possible alternations. Percent Alternation = [Actual Alternation (i.e., ACB, BCA=6)/Maximum Alternation (i.e., ACBABCACBCACAC= 15-2=13)] \times 100= (6/13) \times 100=46.15%. Maximum Alternation was calculated as total number of arms entered minus 2 (18). Each test was done once on each animal.

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 from 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.

Psychomotor coordination (PMC) index

This test was done by Rota-rod Treadmill with shock facility apparatus (Harvard model 865) after Y-maze task. First, animals were trained by their placing on the rolling bar where they had to walk on it. Then, for 5 times, they were placed in the case with these characteristics: initial speed=4 rpm, final speed=30 rpm, initial to final speed time=4 min, shock intensity=1.1 mA, shock duration=0.2-0.8 sec, experimental length time=5 min, interval between experiments=2 min. The mean stay time on the rod per trial was considered as PMC index (18). Also, the mean number of fallings for each animal was recorded. Each animal was experimented in this test only once.

Statistical analysis

All results were expressed as mean \pm standard error of mean (SEM). For the passive avoidance test, nonparametric Kruskal-Wallis test was used, which was followed by Mann-Whitney U-test for pair-wise comparisons, if significant. Data for the Y-maze task were evaluated by Wilcoxon's rank sum test. In all calculations, a difference at $P < 0.05$ was regarded as significant.

Results

Effect of CSE on memory retention deficit in passive avoidance test

The initial latency was 29.9, 27.1, 25.4, 41.5 and 29.9 s in Control, SH, CSE, STZ, 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 (17.98) and STZ + CSE (31.9) groups reduced markedly in comparison to control animals (50.9). On the other hand, the STZ + CSE group exhibited significant reversal of STL ($P < 0.05$) as compared to vehicle-treated STZ group, indicating improved acquisition or retention of memory (Figure 1).

Effect of CSE on spatial cognition deficit in Y-maze task

As shown in Figure 2, the mean scores of alternation behavior for control, SH, CSE, STZ and STZ + CSE were 69.9%, 89.9%, 59.9%, 33.2% and 51.6 %, respectively. There was a

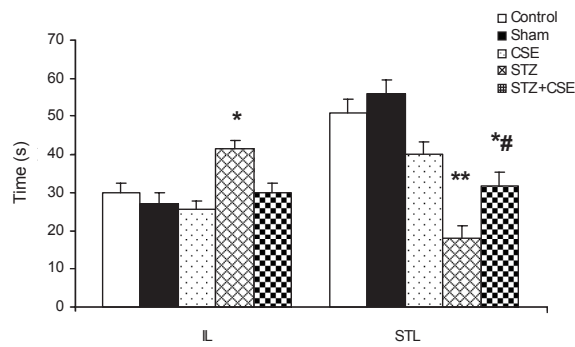


Figure 1. The effect of CSE treatment (60 mg/kg) on passive avoidance performance after ICV injection of STZ in rats as indicated by initial and step-through latencies after 3 weeks. Values are expressed as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$ (in comparison with control group); # $P < 0.05$ (STZ + CSE vs. STZ) (non-parametric Kruskal-Wallis and Mann-Whitney U-tests).

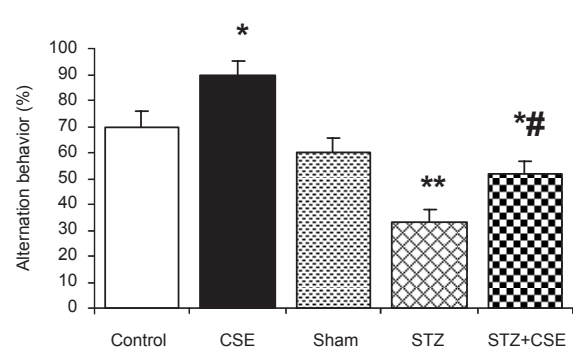


Figure 2. Percentage of Alternation behavior in Y-maze task on the third week after treatment (mean \pm SEM). *, $P < 0.001$ as compared to control group; #, $P < 0.001$ as compared to STZ group.

significant increase in alternation behavior in CSE group compared to control ($P < 0.05$). Also, a marked reduction in alternation score was found in STZ and STZ + CSE groups compared to control animals. However in STZ + CSE group the extract could significantly modify the STZ memory impairment effect ($P < 0.05$).

Psychomotor coordination (PMC) index

Figure 3 shows the results of psychomotor coordination test by Rota-rod treadmill apparatus. The mean PMC indices of experiments for control, SH, CSE, STZ and STZ + CSE were obtained 138 ± 8.78 , 163 ± 11.21 , 144 ± 9.25 , 145 ± 9.56 and 159 ± 7.82 , respectively. Also, the number of animal falling from Rota-rod rolling bar in STZ group (93 ± 9.81) was significantly greater than control ones (68 ± 6.75).

Discussion

The results of the present study shows that ICV STZ injection in rats induces a significant learning and memory disturbance in passive avoidance paradigm and a spatial cognitive deficit in Y-maze task, and treatment of rats with CSE (60 mg/kg) for 3 weeks could significantly attenuate these abnormalities.

It is a well-established fact that ICV injection of STZ is characterized by a progressive deterioration of learning, memory as well as cerebral glucose and energy metabolism, and

this may provide an appropriate and relevant experimental model of SAD (4, 19). 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 (4). The possibility of the effect of increased CSF pressure due to ICV injection was rejected in this study as no behavioral changes reflecting significant increase in intracranial pressure e.g. bulging of eyes were observed. Also, in the sham-operated rats, no apparent signs of raised intracranial pressure were observed. The results from the passive avoidance test showed that the STZ-injected rats reveal significantly reduced retention latencies (STLs), suggesting an impairment in learning and memory processes. In conformity with this, the results from Y-maze task for the first time showed that ICV STZ animals also exhibit a higher score of errors and lower correct choices, indicating an abnormality in spatial cognitive processes. On the basis of the obtained results, it is suggested that impairment in passive avoidance behavior may reflect poorer acquisition and/or retention of memory after ICV STZ injection. The results from the Y-maze task may also indicate a spatial cognition deficit in ICV STZ rats.

In this study, treatment of ICV STZ rats with CSE (60 mg/kg), starting 1 day before surgery, for three weeks caused a significant improvement in learning, memory, and spatial cognitive skills. The beneficial effect of CSE in

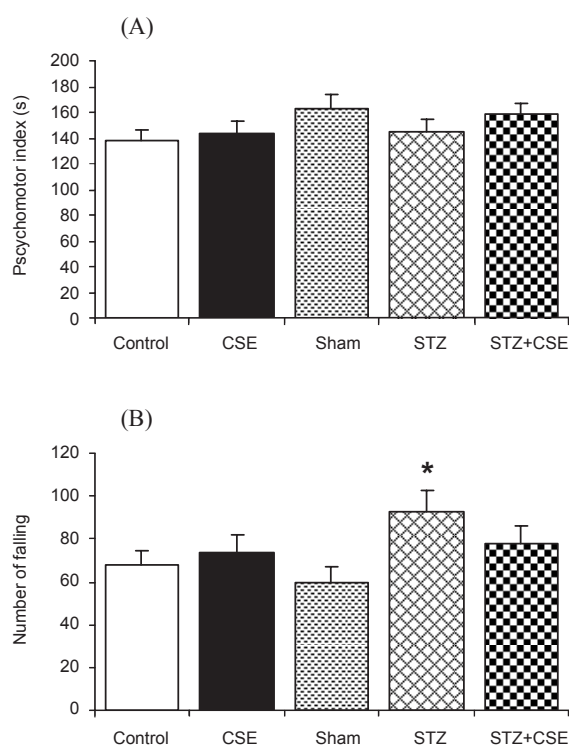


Figure 3. A. Psychomotor coordination by Rota-rod treadmill apparatus (mean±SEM). B. Number of fallings from Rota-rod rolling bar (mean±SEM). *, $P < 0.001$ as compared to control.

this study could be attributed to the following potential mechanisms: firstly, it has been verified that brain damage due to oxidative stress induces the impairment of learning and memory abilities and the development of disturbance in spatial cognitive functions as evaluated by water maze and RAM tasks (20), therefore the neuroprotective (20, 21), antioxidant (21, 22) and free radical scavenging (23) effects of CSE could account for the antagonized extinction recognition memory action of the CSE. Secondly, AD is characterized by alterations at the level of various neurotransmitters and related markers and receptors. Out of these, the most severely affected by far is the cholinergic system (24). The cholinergic system is responsible for the storage and retrieval of items in memory and its degradation correlates well with the severity of cognitive and 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 AD (25). Reports on antagonizing action of CSE on scopolamine-induced memory deficits support the direct or indirect hypothesis of its cholinergic mimicking action. Thirdly, there is strong evidence for the fact that inflammatory processes are associated with the pathophysiology of AD and that treatment with NSAIDs and natural phenolic compounds could reduce the risk for AD (26, 27). Furthermore, it has recently been demonstrated that ICV STZ in rats could also lead to increased expression of beta amyloid in the rat brain which itself may enhance inflammatory processes within the central nervous system (28), and amyloid beta protein (A β)-induced free radical-mediated neurotoxicity is known as a leading hypothesis for a cause of AD. It has been reported that A β increases free radical production and lipid peroxidation in PC12 nerve cells resulting in apoptosis and cell death. However, pretreatment with CSE prevents the generation of the A β -induced reactive oxygen species (29). Fourthly, there is scant evidence for other behavioral effects of CSE (main component crocin). The mechanism(s) of action underlying crocins' role on memory is still under investigation. Among the potential mechanisms, the promotion of hippocampal long-term potentiation (LTP), a form of activity-dependent synaptic plasticity that may underlie learning and memory (30). Finally, our additional experiments show there was no significant changes in total number of entering the animals into the arms and psychomotor index between ICV STZ and CSE operated ICV STZ rats. These data indicate that absolute cognitive learning improvement is related to central cognitive mechanism(s) not the motor coordination paradigms.

In conclusion, the present study clearly demonstrated that CSE treatment could significantly prevent the cognitive impairments following ICV STZ and this suggests the therapeutic potential of this extract in aging and age-related neurodegenerative disorders where cognitive impairment are involved. However, for other behavioral effects of CSE and underlying mechanism(s) of action, further preclinical investigation and the use of genuine models of memory deficits (e.g. old rats) are mandatory for assessing CSE potential on cognition.

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