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Chronic Cyanidin-3-glucoside Administration Improves Short-term Spatial Recognition Memory but not Passive Avoidance Learning and Memory in Streptozotocin-diabetic Rats

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This research study was conducted to evaluate the efficacy of chronic cyanidin-3-glucoside (C3G) on alleviation of learning and memory deficits in diabetic rats as a result of the observed antidiabetic and antioxidant activity of C3G. Male Wistar rats were divided into control, diabetic, C3G-treated-control and -diabetic groups. The C3G was administered i.p. at a dose of 10 mg/kg on alternate days for eight weeks. For evaluation of learning and memory, initial latency (IL) and step-through latency (STL) were determined at the end of study using passive avoidance test. Meanwhile, spatial recognition memory was assessed as alternation in the Y-maze task. Oxidative stress markers in brain tissue were also measured. It was found that the alternation score of the diabetic rats was lower than that of control ($p < 0.01$) and C3G-treated diabetic rats showed a higher alternation score as compared to diabetic group ($p < 0.05$). Diabetic rats also developed a significant impairment in retention and recall in passive avoidance test ($p < 0.01$) and C3G treatment of diabetic rats did not produce any significant improvement. Meanwhile, increased level of malondialdehyde (MDA) in diabetic rats was significantly reduced following C3G treatment ($p < 0.05$). Taken together, chronic C3G could improve short-term spatial recognition memory disturbance in the Y-maze test but not retention and recall capability in passive avoidance test in STZ-diabetic rats. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: cyanidin-3-glucoside; learning and memory; spatial recognition memory; diabetic rat.

INTRODUCTION

Diabetes mellitus (DM) has been associated with neurological complications in both the peripheral and the central nervous system (Manschot *et al.*, 2008). The nerve damage observed in streptozotocin (STZ)-diabetic rats parallels in many ways the nerve degeneration seen in human diabetic neuropathy (Jin *et al.*, 2009). In addition, different kinds of neuropathies are one of the major complications contributing to morbidity in patients with diabetes mellitus. Pathological studies have also suggested that diabetes is one of the risk factors for senile dementia of Alzheimer's type (Carlsson, 2010). In recent years, evidence has been accumulating at an increasing pace on the effects of diabetes on the brain itself (Roriz-Filho *et al.*, 2009; Wrighten *et al.*, 2009). Functionally, passive avoidance learning and memory deficits develop in STZ-diabetic rats (Patil *et al.*, 2006; Kucukatay *et al.*, 2007). Impairment of spatial learning in a hippocampus-dependent complex maze has also been reported in such animals (Stranahan *et al.*, 2008a). Changes in hippocampal synaptic plasticity have been reported in diabetes (Iwai *et al.*, 2009). These findings suggest that untreated diabetes results in deficits in those brain regions that are involved in learning and memory processes.

On the other hand, recent interests are focusing on the use of non-vitamin antioxidants such as flavonoids in reducing the devastating complications of diabetes. Plant-based pharmaceuticals including flavonoids have been employed in the management of various human diseases (Laight *et al.*, 2000). They are an essential part of human diet and are present in plant extracts that have been used for centuries in oriental medicine. Antioxidant properties, reactive oxygen species (ROS) scavenging, and cell function modulation of flavonoids could account for the large part of their pharmacological activity (Laight *et al.*, 2000; Xiao *et al.*, 2011). Since diabetes mellitus is considered as a free-radical-mediated disease, there has been renewed interest in the use of flavonoids in the treatment of some diabetes complications (Xiao *et al.*, 2011). Anthocyanins and their aglycone derivatives anthocyanidins are important groups of flavonoids. Of these groups, cyanidin and its derivatives have been reported to exhibit an antidiabetic activity (Sasaki *et al.*, 2007), benefits for the prevention of obesity and diabetes (Tsuda *et al.*, 2003), lipid peroxidation (LPO) inhibition (Mulabagal *et al.*, 2009) and antioxidant activity (Mertens-Talcott *et al.*, 2008), protecting and rescuing the neuronal cells from toxicity induced by amyloid-beta (A beta) peptide (Tarozzi *et al.*, 2008, 2010), antiinflammatory properties (Wang *et al.*, 2008), antiapoptotic activity through inhibition of peroxyl radical-induced oxidative damage (Elisia and

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Kitts, 2008) and neuroprotective effect (Yao and Vieira, 2007). In addition, due to their antioxidant properties anthocyanins, like cyanidins, could represent a promising class of compounds useful in the treatment of pathologies where free radical production plays a key role (Acquaviva *et al.*, 2003). In addition, berry fruits contain high amounts of anthocyanins, which play a major role as free radical scavengers, which could inhibit H_2O_2-induced lipid peroxidation in the rat brain homogenates (Noda *et al.*, 2002). Therefore, this study was undertaken to evaluate the efficacy of chronic cyanidin-3-glucoside (C3G) on alleviation of learning and memory deficit in streptozotocin-diabetic rats using passive avoidance and Y-maze tests.

MATERIALS AND METHODS

Animals. Male albino Wistar rats (Pasteur's institute, Tehran, Iran) weighing 265 ± 13 g (11–13 weeks old) were housed in an air-conditioned colony room on a light/dark cycle (21–23 °C and a humidity of 30–40%) and supplied with standard pelleted diet and tap water *ad libitum*. Procedures involving animals and their care were conducted in conformity with the US National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

Experimental procedure. The rats ($n = 40$) were randomly allocated and similarly grouped into four groups: normal vehicle-treated control, C3G-treated control, vehicle-treated diabetic and C3G-treated diabetic. The rats were rendered diabetic by a single intraperitoneal injection of 60 mg/kg streptozotocin (STZ) (Pharmacia and Upjohn, USA) freshly dissolved in cold normal saline. Control animals received an injection of an equivalent volume of normal saline and vehicle. Diabetes was confirmed by the presence of hyperglycaemia, polyphagia, polydipsia, polyuria and weight loss. One week after STZ injection, non-fasting blood samples were collected under light ether anaesthesia from the retro-orbital capillary plexus and serum glucose concentrations were measured using the glucose oxidation method (Zistshimi, Tehran). Only those animals with a non-fasting serum glucose level higher than 250 mg/dL were selected as diabetic for the following experiments. The day on which hyperglycaemia had been confirmed was designated as day 0. Cyanidin-3-glucoside (Polyphenols Laboratories, Norway) was administered i.p. at a dos of 10 mg/kg body weight on alternate days one week after STZ injection for a period of 8 weeks. The dose of C3G was chosen according to our pilot study and a study by Matsui *et al.* (2002). Cyanidin-3-glucoside was dissolved in Cremophor (Sigma, USA) with further dilution in normal saline before use. Changes in body weight, food consumption and water intake were observed regularly (but not quantitatively measured) during the experimental period. Behavioural tests including passive avoidance and Y-maze were performed blind to treatments by two observers at the end of the study as described below.

Y-maze task. Short-term spatial recognition memory performance was assessed by recording spontaneous alternation behaviour in a single-session Y-maze as described before (Nitta *et al.*, 2002). The maze was made of black-painted Plexiglas. Each arm was 40 cm long, 30 cm high and 15 cm wide. The arm converged in an equilateral triangular central area that was 15 cm at its longest axis. The procedure was basically as follows, and is the same as that described previously: each rat, naive 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 series of arm entries was recorded visually. Arm entry was considered to be completed when the base of the animal's tail had been completely placed in the arm. Alternation was defined as successive entries into the three arms on overlapping triplet sets. The number of maximum spontaneous alternation was then the total number of arms entered minus 2 and the percentage is calculated as the ratio of actual to possible alternations (defined as the total number of arm entries minus two).

Single-trial passive avoidance test. This test was conducted 2–3 days after the Y-maze task and was as according to a previous study (Baluchnejadmojarad and Roghani, 2006). The apparatus (BPT Co., Tehran) consisted of an illuminated chamber connected to a 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 (5 min), the guillotine door was opened and upon the rat entering the dark chamber, the door was closed and an inescapable scrambled electric shock (1 mA, 1 s once) was delivered. 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 a retention trial. The interval between the placement in the illuminated chamber and entry into the dark chamber was measured as step-through latency (STL up to a maximum of 600 s as cut-off).

Determination of brain malondialdehyde (MDA) concentration. The rats were anaesthetized with ketamine (100 mg/kg), decapitated, brains were removed, blotted dry, weighed, then made into 10% tissue homogenate in ice-cold 0.9% saline solution, centrifuged (1000 × **g**, 4 °C, 10 min), and the obtained supernatant was placed in an aliquot and stored at −80 °C until assayed. The MDA concentration (thiobarbituric acid reactive substances, TBARS) in the supernatant was measured as described previously (Roghani and Baluchnejadmojarad, 2009). Briefly, trichloroacetic acid and TBARS reagent were added to the supernatant, then mixed and incubated at 100 °C for 80 min. After cooling on ice, samples were centrifuged at 1000 × **g** for 20 min and the absorbance of the supernatant was read at 532 nm. The TBARS results were expressed as MDA equivalents using tetraethoxypropane as standard.

Measurement of brain superoxide dismutase activity. The supernatant of brain homogenate was obtained as described earlier. Superoxide dismutase (SOD) activity measurement was in accordance with previous work (Roghani and Baluchnejadmojarad, 2009). Briefly, supernatant was incubated with xanthine and xanthine oxidase in potassium phosphate buffer (pH 7.8, 37 °C) for 40 min and NBT was added. Blue formazan was then

monitored spectrophotometrically at 550 nm. The amount of protein that inhibited 3-nitrotetrazolium blue chloride (NBT) reduction to 50% maximum was defined as 1 nitrite unit (NU) of SOD activity.

Assay of brain nitrite concentration. Supernatant nitrite content was assayed by the Griess method. Because NO is a compound with a short half-life and is rapidly converted to the stable end-products of nitrate ($NO3^-$) and nitrite ($NO2^-$), the principle of the assay is the conversion of nitrate into nitrite by cadmium and followed by colour development with Griess reagent (sulphanilamide and N-naphthyl ethylenediamine) in acidic medium. The total nitrite was measured by Griess reaction. The absorbance was determined at 540 nm with a spectrophotometer.

Protein assay. The protein content of the supernatant was measured with the Bradford method using bovine serum albumin (Sigma Chemical, St Louis, MO) as the standard (Bradford, 1976).

Chemicals. All chemicals excluding those sources indicated in the text were procured from Sigma (USA) and Merck (Germany).

Statistical analysis. All data are expressed as mean ± SEM. For behavioural and biochemical tests, a parametric one-way ANOVA test was applied. Body weight and serum glucose levels at different weeks were analysed using repeated measure one-way ANOVA. In all calculations, a difference at $p < 0.05$ was regarded as significant.

RESULTS

General considerations

One week after STZ injection, three rats (two rats from vehicle-treated diabetic and one rat from C3G-treated diabetic groups were excluded from the study due to their serum glucose level being lower than 250 mg/dL). After 8 weeks, the weight of the vehicle-treated diabetic rats was found to be significantly decreased as compared with control rats ($p < 0.05$) and C3G treatment caused a less significant decrease in diabetic rats as compared with diabetics ($p < 0.05$). In addition, diabetic rats had an elevated serum glucose level over those of control rats ($p < 0.001$) and treatment of diabetic rats with C3G for 4 and 8 weeks caused a significant decrease in the serum glucose ($p < 0.005$) relative to vehicle-treated diabetics. Meanwhile, C3G treatment of control rats did not produce any significant change regarding serum glucose level (Fig. 1).

Alternation behaviour in the Y-maze

Figure 2 shows the results for the performance of rats in the Y-maze task, in which short-term spatial recognition memory performance as alternation behaviour can be examined. In this respect, the alternation score of the diabetic rats was lower than that of the control ones at the end of the study ($p < 0.01$). Meanwhile, C3G-treated diabetic rats showed a higher alternation score as compared with the untreated diabetic group ($p < 0.05$) at the end of the study. Meanwhile, C3G treatment of control rats did not produce any significant change regarding this variable. To avoid compounding the effect of locomotor activity on memory processes in experimental groups, especially diabetics, the total number of arms entered was considered as an index of locomotor activity. In this respect, although total number of entrances was slightly lower in diabetic animals, especially in untreated diabetic rats, this difference was not statistically significant when compared with controls (Fig. 2).

Passive avoidance test

Figure 3 shows the performance of treated-control and diabetic rats in the passive avoidance test as indicated by initial and step-through latencies. Regarding initial latency, there was no significant difference among the groups. In addition, diabetic-treated rats developed a significant impairment in retention and recall in the passive avoidance test ($p < 0.01$), as was evident by a lower STL and C3G treatment did not significantly increase this variable. Furthermore, retention and recall of C3G-treated control rats was not significantly increased as compared with untreated control.

Markers of oxidative stress

Regarding brain lipid peroxidation and oxidative stress markers (Fig. 4), STZ-induced diabetes resulted in significant elevation of MDA ($p < 0.01$) and nitrite content ($p < 0.05$) and significant reduction of SOD activity ($p < 0.05$) and treatment of diabetic rats with C3G significantly attenuated the increased MDA content ($p < 0.05$). However, the level of SOD was not significantly higher and the level of nitrite was not significantly lower in C3G-treated diabetics as compared with the diabetic group.

DISCUSSION

The main findings of this study were twofold. First, long-term STZ-diabetes deteriorated animal performance in passive avoidance and Y-maze tasks. Second, 8-week administration of C3G only improved short-term spatial recognition memory performance in the Y-maze test and its administration could not prevent retention and recall abnormality in diabetic rats as evaluated by the passive avoidance test.

Although the multifactorial pathogenesis of cognitive and memory impairments in diabetes is not completely understood, several factors such as metabolic impairments, vascular complications and enhanced release of free radicals have been implicated (Stewart and Liolitsa, 1999; Biessels *et al.*, 2007; Kucukatay *et al.*, 2007). First, prolonged hyperglycaemia is a primary cause of most complications of diabetes. Indeed, chronic hyperglycaemia is thought to lead to cognitive impairments in diabetes (Tuzcu and Baydas, 2006; Biessels *et al.*, 2007). Hyperglycaemia in type I diabetic patients has also been associated with learning impairments (Brands *et al.*, 2005). Therefore, the restoration of some cognitive

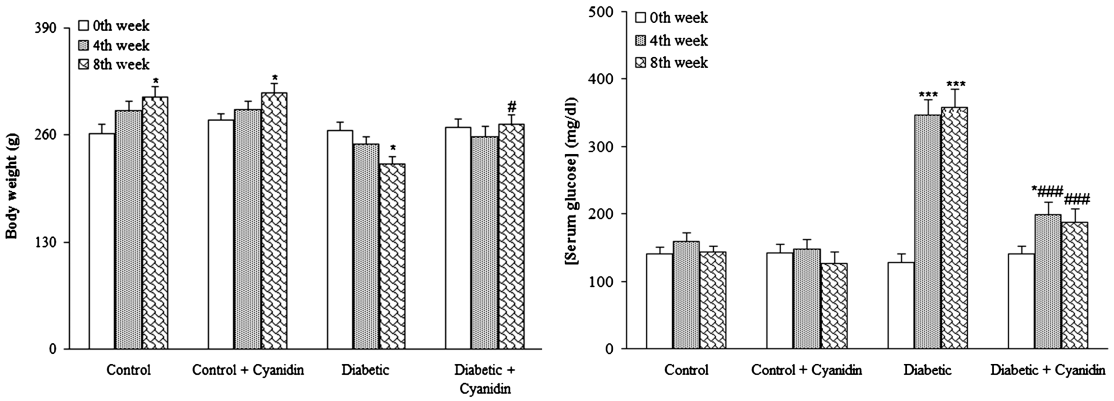

Figure 1. Body weight and serum glucose concentration in different weeks (means ± SEM). $*p < 0.05$, $***p < 0.001$ (as compared with week 0 in the same group). $\#p < 0.05$, $\#\#\#p < 0.005$ (as compared with diabetics in the same week).

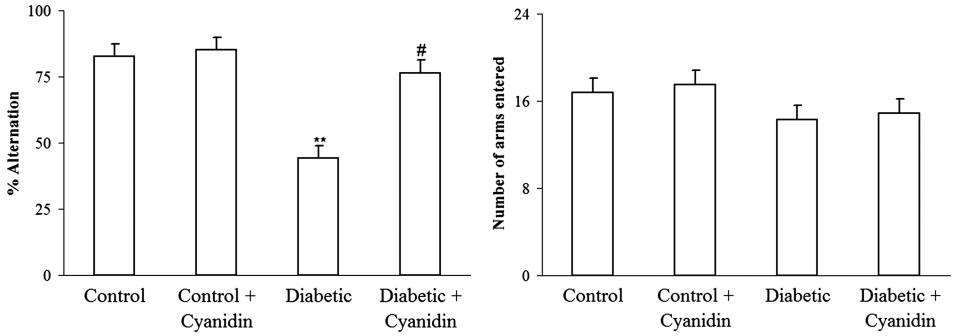

Figure 2. Alternation behaviour of treated-control and -diabetic rats and total number of arms entered in Y-maze task in different groups as an index of locomotor activity in Y-maze task. $**p < 0.01$ (as compared with control). $\#p < 0.05$ (as compared with diabetic).

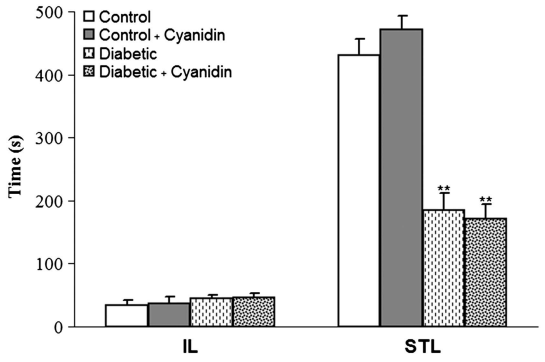

Figure 3. Initial (IL) and step-through (STL) latencies of treated-control and -diabetic rats in single-trial passive avoidance test. $**p < 0.01$ (vs. control).

functions observed in diabetic animals in this study may be due partly to the ability of C3G to attenuate hyperglycaemia. Second, brain vascular abnormalities may also contribute to cognitive impairment in diabetes (Stewart and Liolitsa, 1999; Biessels *et al.*, 2007; van Deutekom *et al.*, 2008). It has been reported that C3G enhances vascular endothelial nitric oxide synthase activity and improves vascular function (Xu *et al.*, 2004) and this may underlie the improvement in spatial recognition memory in the present study. Third, oxidative damage is associated with cognitive dysfunction (Fukui *et al.*, 2002; Kucukatay *et al.*, 2007). Therefore, treatment with antioxidants could be a therapeutic approach in various types of neurodegenerative diseases (Baluchnejadmojarad *et al.*, 2009). Oxidative stress contributes to increased neuronal damage and death through protein oxidation, DNA damage and peroxidation of membrane lipids (Hawkins and Davies, 2001). It has been observed that use of antioxidants and neuroprotective agents may decrease the risk of memory deficits (Rasoolijazi *et al.*, 2007). As oxidative stress is thought to play a crucial role in development of memory impairment in diabetes (Kucukatay *et al.*, 2007), the antioxidant properties of C3G, as was observed in this study may provide an underlying mechanism for some nootropic effect of prolonged antioxidant therapy in diabetic rats. In our study, chronic diabetes was accompanied by increased levels of MDA and nitrite and decreased activity of SOD that was consistent with previous reports (Baluchnejadmojarad and Roghani, 2011) and C3G was capable to some extent of attenuating such abnormal changes within the brain. Fourth, type I diabetes causes apoptosis-induced neuronal loss in the hippocampus and to a lesser extent in the frontal cortex of rats, which is associated with cognitive impairment in diabetes (Li *et al.*, 2002). Since anthocyanins can inhibit apoptosis (Elisia and Kitts, 2008), this may have attenuated memory abnormality in the Y-maze test in diabetic rats in this study. Part of the effects of C3G in this study (i.e. antioxidant activity) required its passage through the blood brain barrier (BBB) to achieve effective concentration in the brain. The capability of oral C3G for crossing BBB has been established previously (Marczylo *et al.*, 2009). In this respect, it has been shown that some flavonoids including C3G are capable of crossing cell models of BBB in a time-dependent manner and this process is independent of alcohol as an organic solvent (Faria *et al.*, 2010). Even some reports indicate that polyphenolic compounds such as anthocyanins are able to cross the BBB, localized in

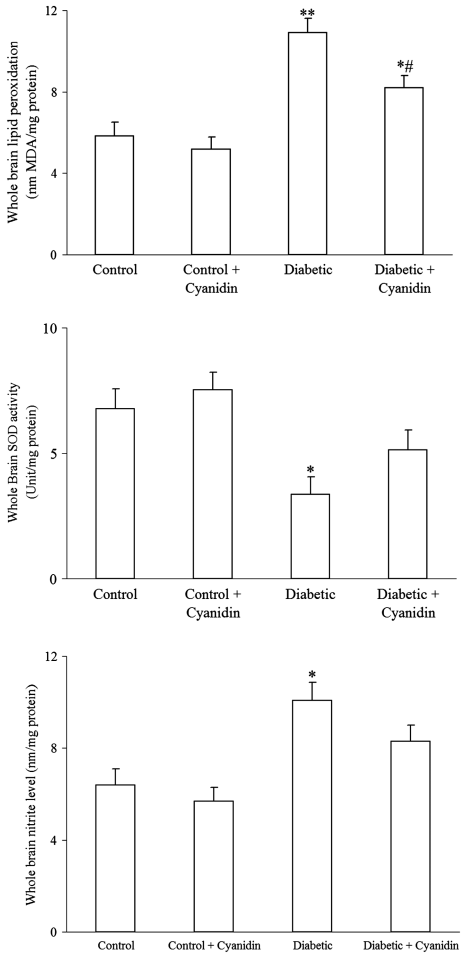

Figure 4. MDA concentration, superoxide dismutase (SOD) activity, and nitrite content in whole brain homogenate from different groups. $*p < 0.05$, $**p < 0.01$ (in comparison with control). $\#p < 0.05$ (in comparison with diabetic).

various brain regions important for learning and memory, and may even deliver their antioxidant and signal modifying capabilities centrally (Andres-Lacueva *et al*., 2005). Accumulating evidence also suggests that flavonoids can cross the BBB, accumulate in the brain at nanomolar concentrations (Youdim *et al*., 2004; El Mohsen *et al*., 2006) and exert neuromodulatory effects through selective actions on different components of a number of protein kinase and lipid kinase signalling cascades (Schroeter *et al*., 2002). It appears that beneficial effects of flavonoids such as C3G may also occur via alternative mechanisms that are dependent on their ability to modulate critical intracellular signalling cascades. In this regard, STZ-diabetic rats exhibit increased phospholipase A2 activity (Rhee *et al*., 2005) and this change in the hippocampus may lead to some cognitive deficits and inhibition of such enzymes could diminish neurotoxicity and memory impairment (Sanchez-Mejia *et al*., 2008). Anthocyanidins like cyanidin can inhibit such enzymes (Dreiseitel *et al*., 2009) and in this way we have had an improvement in performance of diabetic rats in the Y-maze task. In addition, it has also been reported that diabetes via the hypothalamic–pituitary–adrenal axis and its related steroid corticosterone can also cause cognitive dysfunction and derangements in hippocampus-dependent memory, perforant path synaptic plasticity and adult neurogenesis. It is very probable that cognitive impairment in diabetes as a stressful condition may result from glucocorticoid-mediated deficits in neurogenesis and synaptic plasticity (Stranahan *et al*., 2008b). In this respect, cyanidin can exert an antiageing effect in H_2O_2-treated WI-38 cells through decreasing mRNA and protein expressions of nuclear factor-kappaB, cyclooxygenase-2 and inducible nitric oxide synthase (Choi *et al*., 2010), and this may have also occurred in the present study.

In our study, long-term administration of C3G at a dose of 10 mg/kg, contrary to our expectation, did not improve retention and recall disturbance of diabetic animals in the passive avoidance test, which is in agreement with one previous report on an inhibitory avoidance task (Ramirez *et al*., 2005). These researchers found that one-month oral administration of an anthocyanin-rich lyophilized berry could significantly enhance short-term memory, but not long-term memory in an inhibitory avoidance task (Ramirez *et al*., 2005). Previous reports found that one-trial inhibitory avoidance in rats involves the activation of two separate memory types, short-term (i.e. conducting retention test 90 min after training) and long-term (i.e. conducting retention test 24 h and 7 days after training) (Barros *et al*., 2002; Ramirez *et al*., 2005). In addition, testing for short-term memory has been found not to affect long-term memory retention, indicating that different and separate mechanisms may be involved in their expression (Izquierdo *et al*., 1998). In our study, a retention and recall test was conducted 24 h after the training session, being considered a long-term memory process and possibly for this reason, chronic C3G treatment did not improve learning and memory in the passive avoidance test.

In conclusion, chronic C3G could improve short-term spatial recognition memory disturbance in the Y-maze test but not retention and recall capability in the passive avoidance test in STZ-diabetic rats. Further studies are warranted to investigate the detailed involved mechanisms.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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