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Research Article

Metforminimproves learning and memory in streptozotocin-induced diabetic rats

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ABSTRACT
Alzheimer's disease (AD) is a neurodegenerative disease characterized by cognitive impairment,and characterized by the accumulation of extracellular amyloid-β (Aβ) plaques, and intracellular hyperphosphorylated Tau protein in the brain. Increasing evidence has indicated that AD is closely associated with impaired insulin signaling in brain. It has been shown that diabetic mice had increased tau phosphorylated proteins and Aβ levels in their brains and treatment with Metformin (Met) attenuates the increase of tau phosphorylated proteins. In present study, we aimed to investigate the therapeutic efficacy of Meton learning and memory, in streptozotocin (STZ) -induced diabetic rats.Animals were divided into 4 groups randomly: :(1) Control group (n = 8), which was the normal rats and received saline intraperitoneally (0.1 ml/100 g), (2) Vehicle group (DM), which was the diabetic rats and received saline as Vehicle of Met intraperitoneally (0.1 ml/100 g), (3) DM+ Met groups, which were diabetic rats and treated with Met (100, and 200mg/kg per d) for 20 days. All rates were trained in the Morris water maze (MWM) and in shuttle-box apparatus respectively. One-way ANOVA followed by Tukey's test for multiple comparisons, was used to analyze data. P values less than 0.05 were considered to be statistically significant. Results : our results show that pre-training injection of Met improves spatial learning and memory in STZ-induced diabetic rats in a dose dependent manner, so that rats of Met groups found platform in less time and with less distance traveled, in comparison with DM group. Met also increased the percentage of time elapsed and the distance swum in the target quadrant in STZ-induced diabetic rats, in probe test. In the Passive avoidance test,Met also dose-dependently increased the step-through latency and total time spent in the light area in STZ-induced diabetic rats. Conclusion: An ip injection of STZ resulted in a significant decline in spatial learning and memory and treatment with Met can enhance learning and memory. Met dose-dependently improved spatial learning and memory and also enhanced retention performance in STZ-induced diabetic rats. The results show that Metas an antidiabetic drug and K-ATP channel blocker through, blocking of K-ATP channels or by sensitizing insulin in the brain improves learning and memory storage in a dose-dependent manner and so is useful for AD treatment.
Keywords:;Streptozotocin; Metformin; Morris Water Maze; diabetic rats

INTRODUCTION
Recent study has indicated that AD is closely associated with impaired insulin signaling in brain. [1]. First, diverse evidence has demonstrated a decreased insulin level in CSF and impaired insulin signaling in patients with AD [2,3]. Second, disruption of cerebral insulin receptors functions by intracerebroventricular (icv) injection of STZ, leads to AD-like changes and progressive cognitive impairment [4]. By contrast, icv injection of insulin improves memory formation [5]. Clinical evidence suggests that diabetic patients treated with insulin may not develop AD [6,7]. Administration of insulin and glucose increases the memory of AD patients to a greater extent than injection of glucose alone [8]. Furthermore, depletion of insulin by injection of STZ, causes obviously increased levels of Aβ plaques and hyperphosphorylated Tau protein and

spatial memory deficits in mice [9,10,11,12], suggesting that insulin deficiency is involved in tau phosphorylation and Aβ generation. It was shown that animals with type 1 and type 2 Diabetes Mellitus (DM) have increased hyperphosphorylated Tau protein and Aβ expression in their brains. (13, 14, 15, 16, 17) Increasing evidence has indicated that brain insulin dysfunction is a risk factor for AD. Both forms of type I and II diabetes are associated with cognitive function impairment (18). Many Studies have indicated that DM and hyperinsulinemia, increases the risk for dementia and AD (19-22). (Brands et al., 2005),AD is characterized by the accumulation of extracellular amyloid-β (Aβ) plaques, and intracellular hyperphosphorylated Tau protein (23, 24). Insulin has been shown to influence both Aβ levels and Tau phosphorylation, through the PI3K pathway or insulin degrading enzyme (IDE). IDE not only degrades insulin but also Aβ (25). Some investigations have shown that insulin signaling is important for neuronal survival (26, 27). Findings that in AD brains the function of multiple players in the insulin signaling are changed, has led to use the term "Type 3 diabetes" for AD (28). Therefore investigating the role of pharmacological agents such as Met that could improve neuronal insulin resistance merit attention in AD therapeutics.Met, is one of the most widely used insulin sensitizer against peripheral insulin resistance.

In addition to its antidiabetic potential, Met has been proved to be a therapeutically effective drug candidate in various CNS disorders like AD and Parkinson's disease (29, 30). It is found to be neuroprotective by inhibiting apoptosis in neuronal cortical cells (31).

It has been shown that Met promotes neurogenesis and enhances the spatial memory formation (32). It was also observed that long-term treatment with Met increases health span and lifetime (33). Previous studies suggest that Met prevents the oxidative stress-related cellular death (34, 35). It has been reported that prolonged hyperinsulinemic conditions in differentiated N2A cells led to development of pathological indices of AD. Treatment with Met prevented appearance of pathological indices of AD (36).In this regard Li et al (2012) study show that treatment with Met attenuates the increase of tau phosphorylated proteins in diabetic mice (obese, leptin-resistant mice) (17). The primary objective of the present study was to evaluate the therapeutic efficacy of Metformin on learning and memory in STZ - induced diabetic rats

MATERIALS AND METHODS

80 Adult male Wistar rats (Razi Institute, Karaj, Iran), weighing 200–300 g were used for Morris Water Maze apparatus and Passive avoidance test respectively. Animals were kept in an animal house with a 12/12-h light–dark cycle and controlled temperature (22 ± 2 °C). Animals had free access to food and tap water except during the limited periods of experiments. Teen animals were used in each group; each animal was used once only and killed immediately after the experiment. Behavioral experiments were done during the light phase of the light/dark cycle (light on 07:00). The diabetic model was induced by intra peritoneal injection of a single dose of 65 mg/kg STZ which was freshly dissolved in citrate buffer (pH 4. 4, 0. 1 M) for three successive days to induce hyperglycaemia in rats. The control animals were injected with citrate buffer. Seven days after STZ injection, fasting blood glucose levels were determined. Animals were considered diabetic if plasma glucose levels exceeded 7.8 mmol/L (37). Animals were divided into 4 experimental groups:(1) Control group (n = 10), which was the normal rats and received saline intraperitoneally (physiological saline 0.1 ml/100 g), (2) Vehicle group (DM) ,which was the diabetic rats and received saline as vehicle of Met intra peritoneally(0.1 ml/100 g), (3) DM+ Met groups, which were diabetic rats and treated with Met (100, and 200 mg/kg per d) for 20 days. All drugs were prepared immediately prior to use and given intra peritoneally (i.p.) in a volume of 0.1 ml per 100 g body weight of rats (17). Learning performance of the rats was evaluated in the MWM and shuttle -box starting 24 h after the last

(24th day), Met or saline injection. All experiments were executed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication No. 80-23, revised 1996) and were approved by the Research and Ethics Committee of Qazvin University of Medical Sciences. Met was a gift fromMahbanchemi co (Tehran, Iran).and STZ were purchased from SIGMA-ALDRICH Company, STZ, was dissolved in 0.9% saline

Assessment of spatial learning and memory using the Morris water maze

After Met treatment for 20 days, the morris water maze tests were conducted to assess the learning and memory performance. The escape latency (s) and path length (cm) were analyzed in each trial and averaged over four trials for each rat. The frequency the rat reached the former placement of the platform as well as the time spent in the former platform quadrant were detected within 60 s (37, 38).To assess spatial learning and memory of animals, MWM tests were performed according to (37, 38). Briefly, in MWM animal learns to escape to a hidden platform by swimming in circular water tank. This tank consisted of a large circular black colored pool (140 cm in diameter and 60 cm high) that was filled with 25±1 °C water to a depth of 25 cm. The MWM protocol was a stringent protocol of four trials per day for six consecutive days. During each trial, each rat was placed into the water at one of the four cardinal points of the compass (N, E, S, and W), which varied from trial to trial in a quasi-random order. The rat had to swim until it climbed onto the escape platform. Animals that failed to find the platform within the allocated time were gently guided to the platform. At the end of each trial, animals were allowed tostay on the platform for 20 s. The escape latency (platform search time) for each trial was recorded. Afterthe last trial, the animal was towel dried and returned to the home cage. The platform was removed during the spatial probe test, which was performed 2 days after the last acquisition trial. The rats were allowed to swim for 60 s, and we recorded the latency to reach the platform location, the time spent swimming within a zone [i.e., a 20-cm radius that was centered either on the original training location (target zone) or on an equivalent location in the opposite quadrant (opposite zone)], and the proximity (the average distance in centimeters of rats from the center of the platform location across the 60-s test). The velocity of each rat was also calculated. The analysis of the latency to reach the platform location, and time spent within a specified radius (zone) are consistently more sensitive measures of the MWM probe test performance in terms of detecting group differences

Passive avoidance performance (shuttle box):

To assess memory retention of animals, Passive avoidance test were performed. In this task, the animal learns that a specific place should be avoided since it is associated with an aversive event. Decrease in step through latency (STL) indicates an impairment in memory in the PA task. The passive avoidance apparatus consisted of two light (Plexiglas) and dark (Black) compartments of the same size (20×20×30 cm3) separated by a door. The floor of the dark compartment (i.e. conditioning chamber) was made of stainless-steel bars (0.5 cm diameter) separated by a distance of 1 cm. Intermittent electric shocks (50 Hz, 3 s), 1 mA intensity, were delivered to the floor of the dark compartment by an isolated stimulator.Inhibitory-avoidance training. The rats were allowed to become familiar with the laboratory environment 1 h before each of the training. Each animal was placed in the light compartment for 20 s, after which the door was opened and the time the animal waited before crossing to the dark (shock) compartment was recorded as the latency. The animal was removed from the experiment when it waited for more than 180 s to cross to the other side. Once the animal completely crossed to the dark compartment, the door was closed and a 1 mA foot shock was delivered for 3 s. The rat was then removed from the apparatus and 2 min later, the procedure was repeated. Training was terminated when the rat remained in the light compartment for 120 consecutive seconds. All the animals were trained

with a maximum of two trials. Retention test. 24h after training, each animal was placed in the light compartment for 20 s, the door was opened, and the latency for entering into the shock compartment was measured as STL. During these sessions, no foot shock was applied and the test session ended when the animal entered the shock compartment or remained in the light compartment for 600 s (criterion for retention) (39).

STATISTICAL ANALYSES:

Data are expressed as mean ± SEM (standard error of mean). In order to compare the latency time, the number of quadrants that the animals crossed and path length to reach the platform (distance) and values for the probe trial in MWM and values for the shuttle box separately were assessed by one-way ANOVA followed by Tukey's test to detect statistical differences between the groups. P values less than 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Place learning

Figures 1 A, and B display place learning of different experimental groups in the MWM. As expected, the average escape distance (the path length to find the platform) and escape latency (The latency time to find the hidden platform), in searching for the hidden platform decreased with the increase in training days. In the control group there was shorter average escape latency and a shorter escape distance.

An i.p injection of 65 mg/kg STZ for three days to induce hyperglycaemia in rats, however, resulted in a significant decline in spatial learning, with longer latency and distance in searching for the underwater platform. These results indicate that diabetes induced by STZ could significantly impair spatial learning and memory in rats. So the different between control and DM groups on the all of training days was significant ($p < 0.05$).

On the other hand, Mettreatment (100, and 200mg/kg for 20 days) of rats attenuated DM - induced impairment in learning processes in a dose dependent manner.

In the other word DM –induced by STZ increased escape latency, and distance in searching for the hidden platform in the all of training days in comparison with control group whereas Met dose-dependently decreased these parameters in the DM+ Met groups.So that rats of DM+ Met groups found platform in less time and with less distance traveled, in comparison with DMgroup.There were no significant difference between control and DM+ Met groups, whereas the difference between control and DM group in the most of training days were significant. The difference between DM+ Met groups with DMgroup were significant ($p < 0.05$).Also, our results show that swimming speed of all groups of rats increased in the consecutivetraining days. But there was no significant difference between experimental groups indicatingboth STZ and Mettreatment had no effect on the motor activity of rats.

Probe test

In the probe test, control group rats spent most time and swum most in the target quadrantindicating memory consolidation were took place well in this group (Figures 1 C, and D). However, in theDM group, time spent and swimming distance in the target quadrantwere significantly less than those in control group. On the other hand, in the DM+ Met (100, and 200mg/kg) groups, time spent and swimming distance in the target quadrant were close to those in control group rats.

The percentage of time spent and distance swimming in the target quadrant in the DM+ Met (100,and 200mg/kg)groups rats similar to control group rats were significantly higher than those in the DM groups. Metdose-dependently increased time spent and swimming distance in the target quadrant in probe test.IndicatingMet treatment attenuated DM- induced impairment in memory consolidation.

However, Met treatment had no effect on the swimming speed.

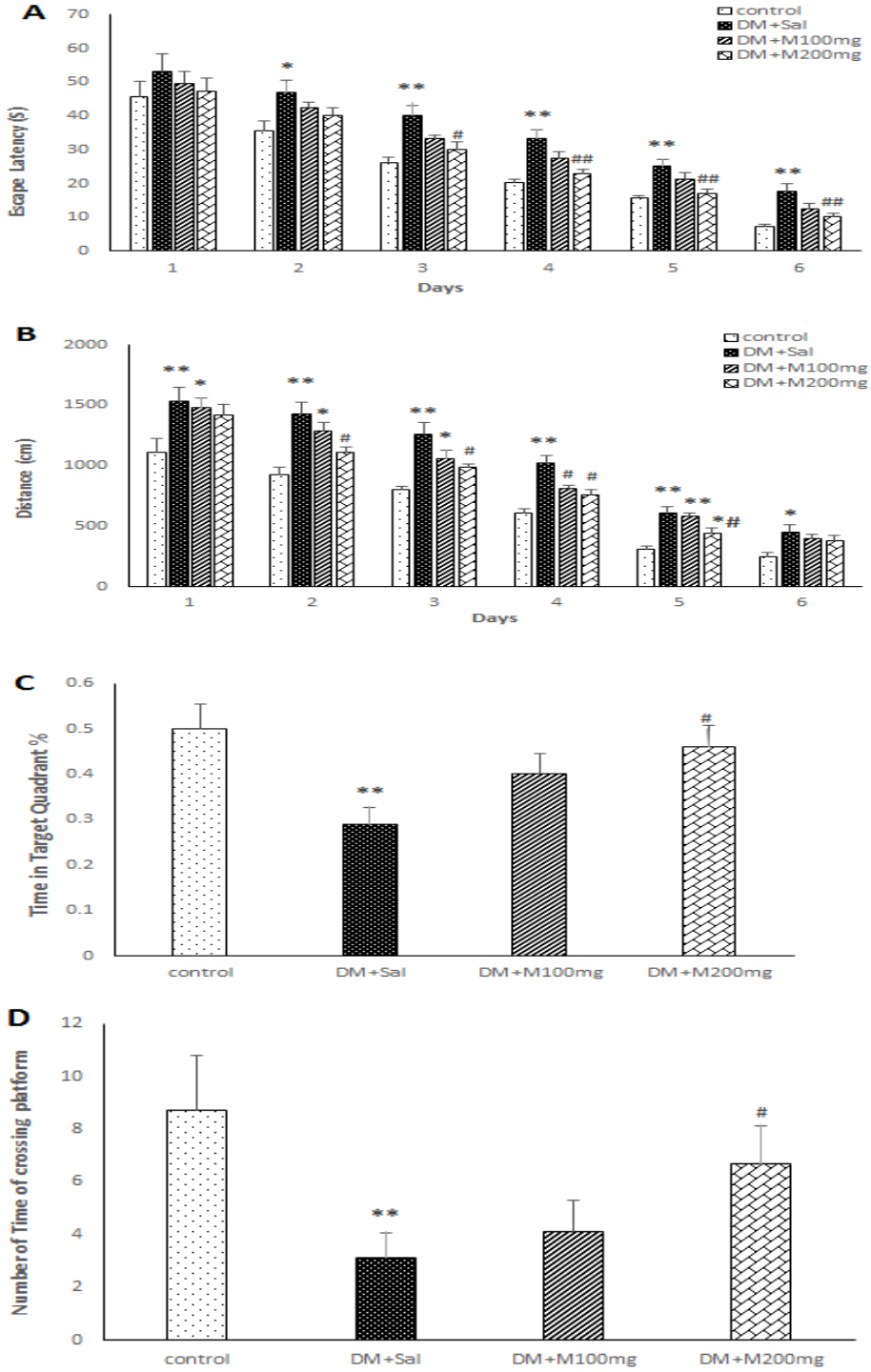

Fig.1. Place learning. Panel A shows the escape latency (The latency time to find the hidden platform). Panel B shows distance (the path length to find the platform) (four sessions per day) of the experimental groups during successive training days

Panel C shows the percentage of time spent in the target quadrant (Probe test). Panel D shows the number of times of crossing platform during only one day in different experimental groups (Probe test). . *$p < 0.05$, **$p < 0.01$; relative to control group, #$p < 0.05$, ##$p < 0.01$; relative to DM+Saline group, One-way ANOVA followed by the Tukey post hoc test.

Passive avoidance

Figures 2 shows the effects of Met treatment on memory retention of passive avoidance learning. The data showed that the STL and total time spent in light chamber of DM group rats were significantly reduced compared to control group rats. The STL and total time spent in light chamber in the STZ plus Met (100, and 200mg/kg) groups rats similar to control group rats were significantly higher than DM group rats. So Met could improve memory retention in STZ induced diabetic rat.

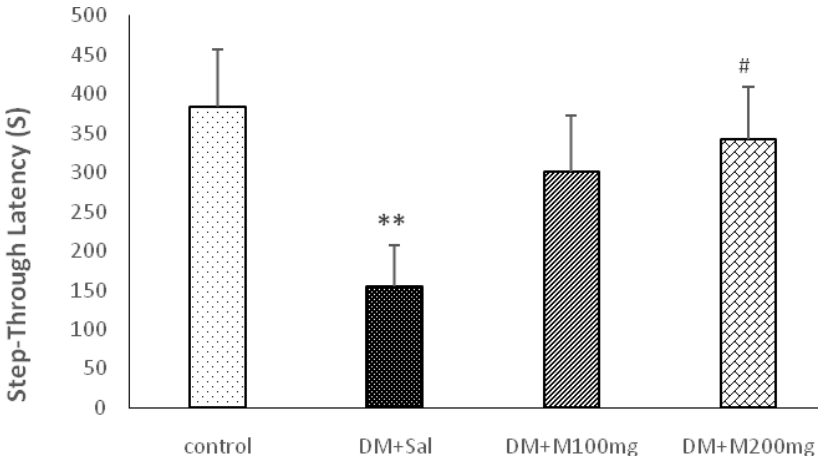

Fig. 2. Comparison of STL during the Passive Avoidance test. Each value represents the mean ± SEM of the latency before entering the dark compartment. **p<0.001 relative to the control group, #p < 0.05, relative to the DM +Saline group.

DISCUSSION

Any impairment in the metabolism of insulin in the brain may put bad effects on neuronal survival and memory, for example it has been shown that hyperglycemia impairs cognitive performanceand DM increases the risk of developing AD (19-22). Insulin resistanceand DMcan impair spatial learning and memory (19, 21, and 44). In parallel, up to 80% AD patients have DM (42) Consistent with these phenomena, our study showed that escape latency, and distance in searching for the hidden platform in the DM group, were significantly more than those in control group and conversely in Probe test thetime spent and swimming distance in the target quadrant in this group, were significantly less than those in control group.The results from the passive avoidance test of our study also show that STL and total time spent in light chamber in DMgroup, were significantly less than those in control group, which suggesting impairment in learning and memory in the DM group animals. Consistent with our results, a previous study showed that diabetic mice had impaired spatial memory assessed by MWM (43). The main finding of this study is that Met treatment is capable to attenuateDM -induced impairment in learning and memory consolidation. Since there was no significant difference in the swimming speed between experimental groups including DM and DM+Metgroups, this effect of Met treatment was not due to improve in motor activity of rats. Therefore, Met treatment probably attenuated DM-induced neuronal damage in brain.Our results show that Met treatment improves spatial learning and memory inSTZ induced diabetic rat. in a dose dependent manner, so that rats of Met groups found hidden platform in less time and with less distance traveled, in comparison with DMgroup. Mettreatment also dose-dependently increased the percentage of time elapsed and the distance swum in the target quadrant, in probe test. Although our findings suggest that DM disrupt spatial cognition, it is possible that the observed deficits in performance could have been a result of general behavioral or sensorimotor impairment, rather than a result of spatial learning and memory deficits. To investigate these possibilities, a visible

platform task was performed. We found that DM did not significantly affect the swim length to escape to the visible platform, a finding that is inconsistent with theidea that disruption of escape to the submerged platform is due to general impairments. Furthermore, in our experiments DMwas sufficient to impair spatial performance, a deficit that is perhaps notreadily attributed to simple sensorimotor impairment. We, therefore, conclude that the memory deficits in the MWM are not due to generalized behavioral impairments. Our results also show that Met dose-dependently increased the STL and total time spent in thelightarea in comparison with DM group.In present study we provide evidences that Met, as an insulin sensitizer against peripheral insulin resistance, could improve cognitive function impairment in STZ induced diabetic rat. In accordance with our results, other investigators have also reported that long-term hyperinsulinemic conditions in Neuro-2a cells led to development of insulin resistance and phosphorylation of tau. This increase of tau phosphorylation is decreased by Metformin (36). The hyperphosphorylated Tau protein and C-jun N-terminal kinase (JNK), a tau kinase, that may be involved in phosphorylation of tau, in the diabetic mouse hippocampus is increased and Met treatment attenuate this increase of the hyperphosphorylated Tau protein and activated JNK(40,44). Also it has been reported that there is a significant increase in the tau phosphorylation two week after i.c.v. administration of STZ in rats (14, 16). This increase can be attenuated by administration of Met (40) and insulin (15). Moreover it has been shown that there is an increased in the expression of Aβ in the brains 30 days after STZ-induced type 1 DM (15, 16, and 45). A recent study shows that insulin reduces Aβ production in neuronal cultures and Met enhances this reduction (46). Similarly, it has been reported that long-term hyperinsulinemic conditions in Neuro-2a cells have increased Aβ production. This increase is attenuated by Met (36). It has shown that Met injection to diabetic mice that have increased plasma insulin levels reduced brain contents of Aβ1-42 compared with the control diabetic mice (40). These results suggest that Metformin in the presence of normal or high insulin levels can reduce Aβ production.Consistent with these phenomena our study showed that an i.p. injection of STZ to rats for induction of DM can significantly decline in learning and memory and Met injection for 20 days to diabetic rats can enhance learning and memory and improve cognitive function impairment in a dose dependent manner. Our results, along with the evidence from previous in vivo and in vitro studies (36, 40, 46), indicate that Met in the presence of normal or high levels of glucose in addition to decrease tau phosphorylation and Aβ generation it can improve cognitive function impairment in different animal Model of DM. Consistent with our results, it has been shown that Met can protect the brain against the oxidative imbalance promoted by type 2diabetes (47).Also it has been shown that Met can act as a neuroprotectant against apoptotic cell death in primary cortical neurons (31). Moreover it has been shown that Met can improve neuronal viability in an in vitro model of ischemia (oxygen-glucose deprivation model) through reduced the elevated activites of the antioxidant enzymes: glutathione peroxidase, superoxide dismutase, and catalase in cerebrum (48, 29, and 51).These results suggest that Met can act as a neuroprotectant against neurodegenerative diseases like Alzheimer, Parkinson and huntington'sdisease. In connection with this hypothesis Positive effects of Mettreatment were shown in a transgenic mouse model of huntington's disease by Ma et al, 2007(49). In a similar line Hwang et al (2010) have shown that Mettreatmentcan normalizes type 2 diabetes-induced decrease in cell proliferation and neuroblast differentiation in the rat hippocampal dentate gyrus (50).

In general, it seems that MetTreatmentprobably through attenuates tau phosphorylation and Aβ generation and increases antioxidant protection, can improve cognitive function (30). These actions may contribute to the beneficial effects of Met onAD treatment and cognitive function improvement in STZ induced diabetic rat.

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