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Research Article

METFORMIN IMPROVES LEARNING AND MEMORY IN STREPTOZOTOCIN-INDUCED RAT MODEL OF SPORADIC ALZHEIMER'S DISEASE

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ABSTRACT
Alzheimer's disease (AD) is closely associated with impaired insulin signaling in brain. Therefore, investigating the role of pharmacological agents similar to Metformin that could improve neuronal insulin resistance merit attention in AD therapeutics. In present study, we aimed to investigate the therapeutic efficacy of Metformin on learning and memory, in streptozotocin (STZ) Rat Model of AD. Animals were divided into 7 groups randomly: control, and groups treated with STZ and STZ plus Saline or Metformin. For induction of AD, STZ (3 mg/kg, 10 µl/injection site) were administered bilaterally into lateral ventricles. Metformin (50,100,200mg/kg, i.p) or saline (0.2ml) were injected daily, one week after operation for 10 days before training. 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. The results show that an i.c.v. injection of 3mg/kg STZ significantly increased escape latency, distance and number of crossed quadrants in comparison with control group ($P<0.01$). Treatment with Metformin dose-dependently protected learning and memory against impairment induced by STZ. So that in the MWM test, rats of Metformin groups found platform in less time and with less distance traveled, in comparison with STZ group. Metformin also increased the percentage of time elapsed and the distance swum in the target quadrant, in probe test. In the Passive avoidance test, Metformin also dose-dependently increased the step-through latency and total time spent in thelightarea in STZ Rat Model of AD. ***Conclusion:*** An i.c.v. injection of STZ resulted in a significant decline in spatial learning and memory and treatment with Metformin can enhance learning and memory. Metformin dose-dependently improved spatial learning and memory and also enhanced retention performance in STZ Rat Model of AD. The results show that Metformin through reducinginsulin resistanceof neurons improves learning and memory storage in a dose-dependent manner and so is usefulin treatment of AD.

Keywords: *Alzheimer's Disease, Streptozotocin, Metformin, Morris Water Maze, Probe Test*

INTRODUCTION
Both forms of type I and II diabetes are associated with cognitive function impairment (Leibson *et al.*, 1997). Many Studies have indicated that Diabetes Mellitus (DM) and hyperinsulinemia, increases the risk for dementia and Alzheimer's disease (AD) (Brands *et al.*, 2005; Ott *et al.*, 1999; Strachan *et al.*, 1997; Luchsinger *et al.*, 2004). AD is characterized by the accumulation of extracellular amyloid-β (Aβ) plaques, and intracellular hyperphosphorylated Tau protein (Hardy, 2001; Hardy and Selkoe, 2002).
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 (McDermott and Gibson, 1997). Therefore, if the insulin level increases in the brain, it would inhibit enzyme activity of IDE, which could increase the level of Aβ and AD progression (Qiu and Folstein, 2006; Shiiki *et al.*, 2004). It was shown that animals with DM have increased hyperphosphorylated Tau protein and Aβ expression in their brains (Li *et al.*, 2007; Jolivalt *et al.*, 2008; Kim *et al.*, 2009; Planel *et al.*, 2007b). Some investigations have shown that insulin signaling is important for neuronal survival (Ryu *et al.*, 1999; de la Monte and Wands, 2002).

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 (Steen *et al.,* 2005). Therefore, investigating the role of pharmacological agents that could improve neuronal insulin resistance merit attention in AD therapeutics. Metformin, is one of the most widely used insulin sensitizer against peripheral insulin resistance. In addition to its antidiabetic potential, Metformin has been proved to be a therapeutically effective drug candidate in various CNS disorders like AD and Parkinson's disease (LI *et al.,* 2010; Ashabi *et al.,* 2014). It is found to be neuroprotective by inhibiting apoptosis in neuronal cortical cells (El-Mir *et al.,* 2008).
It has been shown that Metformin promotes neurogenesis and enhances the spatial memory formation (Wang *et al.,* 2012). It was also observed that long-term treatment with Metformin increases health span and lifetime (Martin-Montalvo *et al.,* 2013). Previous studies suggest that Metformin prevents the oxidative stress-related cellular death in non-neuronal cell lines (Labuzek *et al.,* 2010a; Labuzek *et al.,* 2010b). It has been reported that prolonged hyperinsulinemic conditions in differentiated N2A cells led to development of pathological indices of AD. Treatment with Metformin prevented appearance of pathological indices of AD (Amit *et al.,* 2011). Journal of Li *et al.,* (2012) study show that obese, leptin-resistant mice (diabetic mice) had increased tau phosphorylated proteins and Aβ levels in their brains and treatment with Metforminattenuates the increase of tau phosphorylated proteins. The primary objective of the present study was to evaluate the therapeutic efficacy of Metformin on learning and memory in STZ Rat Model of AD.

MATERIALS AND METHODS

56 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. Eight 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). Animals were divided into 7 experimental groups: control, Saline, STZ, STZ+ vehicle (saline) and STZ+ Metformin groups. Rats in the vehicle and Metformin groups received saline (0.2 ml) or Metformin (50, 100, and 200 mg/kg, i.p.) for 10 days one week after i.c.v, injection of STZ (Li *et al.,* 2012; Del Prete *et al.,* 1999; Heishi *et al.,* 2008). 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. For induction of AD, STZ (3 mg/kg, i.c.v, 10 µl each) was administered bilaterally into lateral ventricles. In the saline group saline (10 µl) was administered bilaterally into lateral ventricles. Learning performance of the rats was evaluated in the MWM and shuttle -box starting 24 h after the last (18^{th} day) Metformin or vehicle 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. Metformin and STZ were purchased from SIGMA-ALDRICH Company and anesthetic drugs (ketamine and xylazine) are products of Alfasan Company, Holland.

Induction of Experimental Dementia of AD by i.c.v. Administration of STZ in Rats

For induction of AD, Prior to surgery, rats were anesthetized with a combination of ketamine (100 mg/kg, ip) and xylazine (5 mg/kg, ip). The animal's head was fixed in the stereotaxic frame (stolting, USA). The scalp was cleaned with iodine solution, and two holes were drilled in the skull bilaterally over the lateral ventricles. According to Paxinos and Watson's atlas (Paxinos and Watson, 1997), the following coordinates were used for icv injection of STZ: 0.8 mm posterior to the bregma, 1.5 mm lateral to the sagittal suture, and 3.6 mm ventral from the surface of the brain with the tooth bar set at 0 mm. STZ was dissolved in normal saline shortly before use. STZ (3 mg/kg, i.c.v 10 µl each) were administered bilaterally (Sharma and Gupta, 2001; Sodhi and Singh, 2013; Tiwari *et al.,* 2009; Gutierres *et al.,* 2014). The same surgical procedures were also used in the saline group, (10 µl/injection site). To determine that STZ was administered exactly into the cerebral ventricles, some rat (30%) were injected with 5 µl of diluted potent blue dye and their brains were examined macroscopically after sectioning. After surgery,

rats were housed individually, and three weeks later Morris water maze test and passive avoidance learning were performed to assess learning and memory.

Assessment of Spatial Learning and Memory Using the Morris Water Maze

To assess spatial learning and memory of animals, MWM tests were performed according to (Morris *et al.*, 1982; Pourmotabbed *et al.*, 2011; Omrani *et al.*, 2007; Gilbert *et al.*, 2000). 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 of 150 cm diameter, 60 cm height, filled to a depth of 40 cm with water at 25±1 °C. A black colored round platform of 10 cm diameter was placed 1 cm below the surface of water in a constant position in the middle of the target quadrant (Q2) in the pool; the starting point was in the Q1 quadrant in all the trials. The rats could climb on the platform to escape from the necessity of swimming. Only distal visual cues were available. The task was divided into two sessions: place learning and probe test. The rats were given a maximum time of 60 s (cut-off time) to find the hidden platform and were allowed to stay on it for 20 s. Once the animal found it, it was allowed to remain there for 20 s. If it did not find the platform after 60s, it was guided gently onto the platform and allowed to remain there for 20s. The animals were given a daily session of 4 trials per day for 6 consecutive days. The numbers of quadrants crossed by the animals and path length (swimming paths) and latency time to reach the platform were recorded in each trial by water maze software. Twenty-four hours after the last acquisition session, a 'probe trial' was used to assess the spatial memory. During this trial, the platform was removed from the maze and the rat was allowed to search the pool for 60 s. The mean time spent by the animal in the target quadrant (Q2) searching for the hidden platform was measured and noted as an index of memory.

To assess whether any motivational factors interfered with the rat's ability to escape, 24h after probe test, a visible platform test was designed in which escape could be guided by proximal rather than distal spatial cues visible platform test (cue learning). During this trial, the platform was elevated above the water surface and extra maze cues were removed from the walls and the rats were allowed to swim freely for 60 s. The distance to platform (swim length), the escape latency and the number of quadrants crossed by the animals were measured. This test was aimed as measuring the visuo-motor abilities and the motivation of the animals.

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 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) (Pourmotabbed *et al.*, 2011; Jafari-Sabet, 2006).

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 and 2 display place learning of different experimental groups in the MWM. As expected, the average escape latency (The latency time to find the hidden platform), escape distance (the path length to find the platform) and number of crossed quadrants 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 and a fewer number of crossed quadrants (Figure 1, 2). An i.c.v. injection of 3mg/kg STZ, however, resulted in a significant decline in spatial learning, with longer latency and distance and more number of crossed quadrants in searching for the underwater platform. These results indicate that STZ could significantly impair spatial learning and memory in rats. So, the different between control and STZ groups on the all of training days was significant ($p < 0.05$).

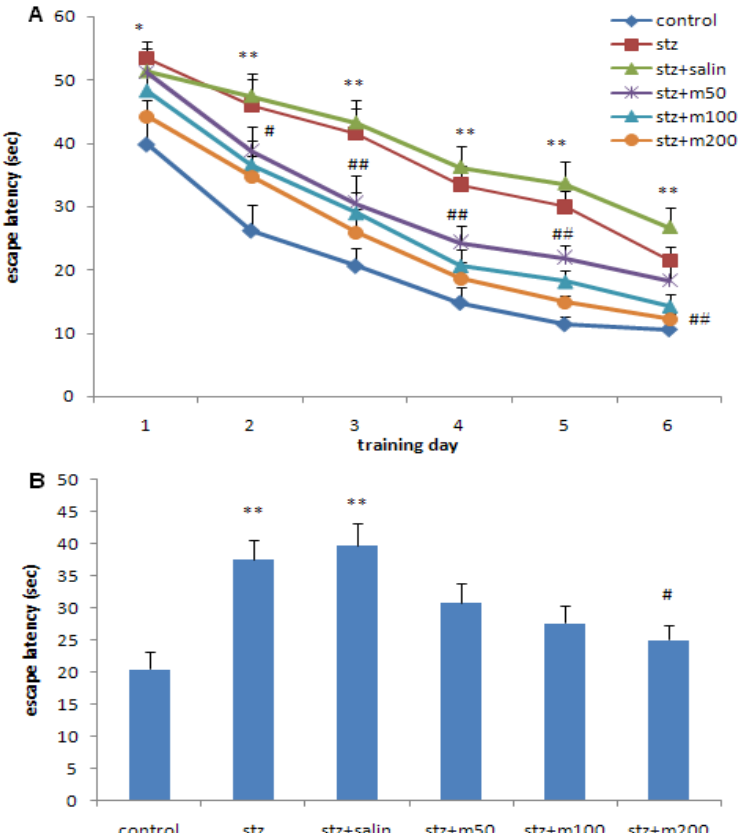

Figure 1: Place Learning

Upper Panel Shows the Escape Latency (The Latency Time to Find the Hidden Platform) of the Experimental Groups During Successive Training Days (four Sessions per day). Lower Panel Shows the Escape Latency in all of the Training Days.

***p*‹0.05; ****p*‹0.001; Relative to Healthy Group, One-Way ANOVA Followed by the Tukey Post hoc Test**

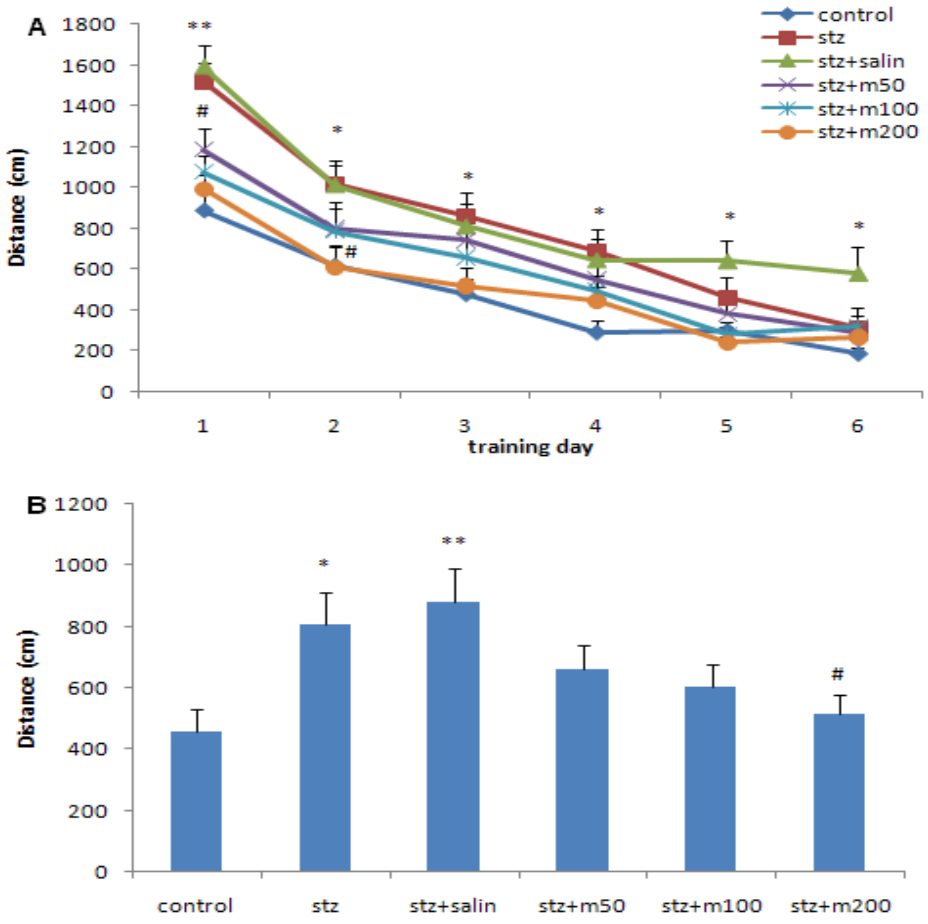

Figure 2: Place Learning
Upper Panel Shows Distance (The Path Length to Find the Platform) of the Experimental Groups During Successive Training Days. Lower Panel Shows the Distance in all of the Training Days *p<0.05; **p<0.001; Relative to Healthy Group, One-Way ANOVA Followed by the Tukey Post hoc Test

On the other hand, Metformin treatment (50,100, 200mg/kg) of rats attenuated STZ-induced impairment in learning processes in a dose dependent manner. In the other word, STZ increased escape latency, distance and number of crossed quadrants in the all of training days in comparison with control group whereas Metformin dose-dependently decreased these parameters in the STZ plus Metformine groups. So that rats of STZ plus Metformine groups found platform in less time and with less distance traveled, in comparison with STZ/STZ+ Saline groups. There was no significant difference between control and STZ plus Metformine groups, whereas the difference between control and STZ/STZ+ Saline groups in the most of training days were significant (figure 1, 2).

The difference between STZ and STZ+ Saline were not significant whereas The difference between these two groups with STZ+ Metformine (200mg/kg) group were significant ($p < 0.05$). Also, our results show that swimming speed of all groups of rats increased in the consecutive training days. But there was no significant difference between experimental groups indicating both STZ and Metformin treatment had no effect on the motor activity of rats.

Probe Test

In the probe test, healthy rats spent most time and swum most in the target quadrant indicating memory consolidation were took place well in this group. However, in theSTZ and STZ+ saline groups, time spent

and swimming distance in the target quadrantwere significantly less than those in control group. On the other hand, in the STZ + Metformin treatment (50,100, 200mg/kg) groups, time spent and swimming distance in the target quadrant were close to those in healthy rats. The percentage of time spent and distance swimming in the target quadrant in the STZ plus Metformine (50,100, 200mg/kg) groups rats similar to control group rats were significantly higher than those in the other STZ-induced AD groups (figure 3).

Metformin dose-dependently increased time spent and swimming distance in the target quadrant in probe test. Indicating Metformin treatment attenuated STZ- induced impairment in memory consolidation. However, Metformin treatment had no effect on the swimming speed.

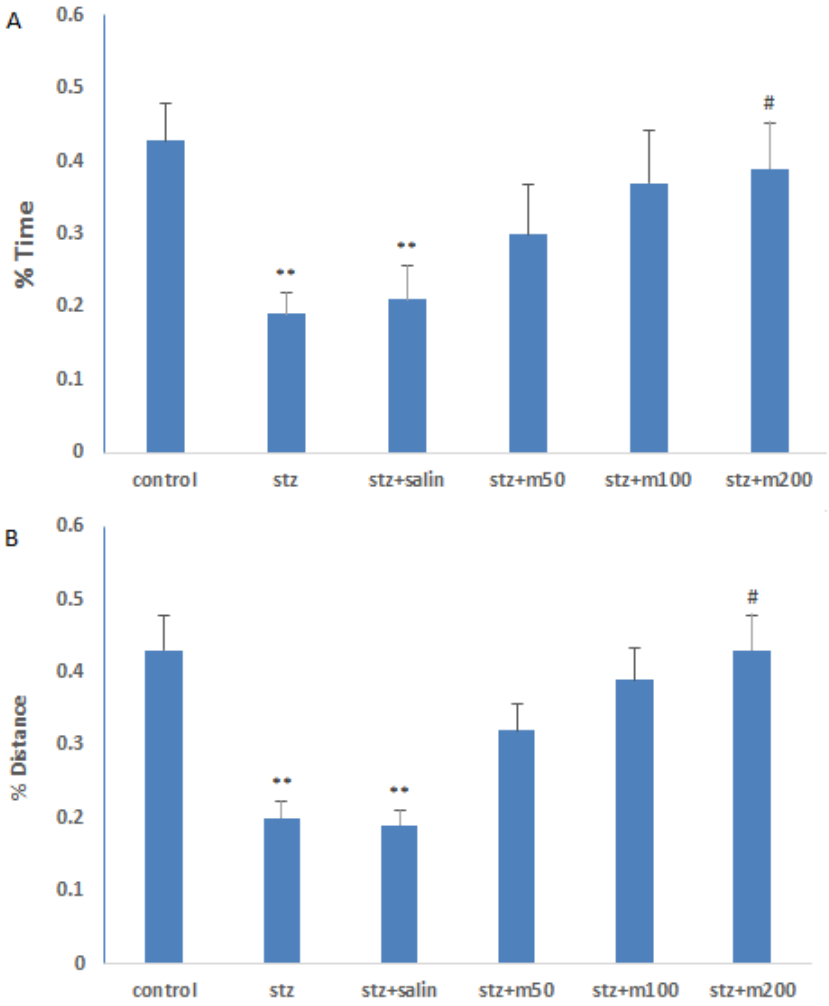

Figure 3: Probe Test

Upper Panel Shows the Percentage of Time Spent in the Target Quadrant; Lower Panel Shows the Percentage of Distance Swimming in the Target Quadrant During Only One Day in Different Experimental Groups

***p*‹0.05; Relative to Healthy Group, One-Way ANOVA Followed by the Tukey Post hoc Test**

Passive Avoidance

Figures 4 and 5 shows the effects of Metformin treatment on memory retention of passive avoidance learning. The data showed that the STL and total time spent in light chamber of STZ/STZ+ saline groups rats were significantly reduced compared to control group rats. The STL and total time spent in light

chamber in the STZ plus Metformine (50,100, 200mg/kg) groups rats similar to control group were significantly higher than those in the other STZ-induced AD groups. So, Metformine could improve memory retentiin in STZ Rat Model of AD.

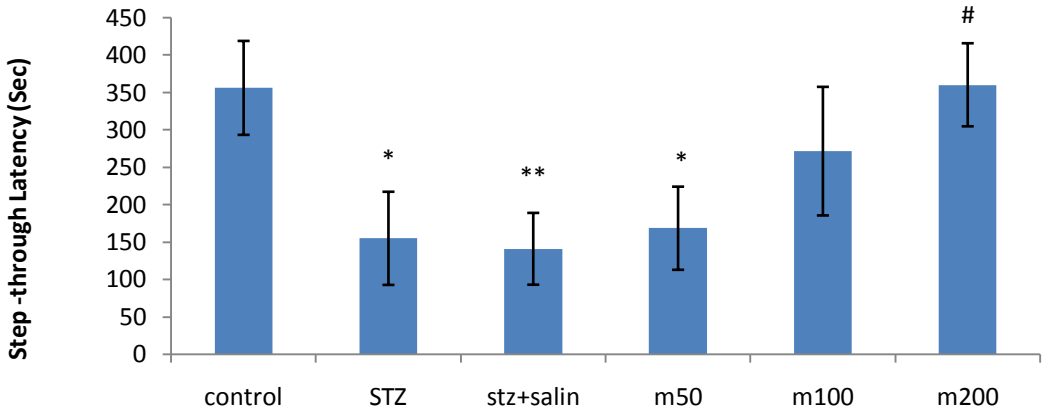

Figure 4: Passive Avoidance Learning
Panel Shows the Step through Latency in Experimental Groups
***p < 0.05, **p < 0.01; Relative to Healthy Group, One-Way ANOVA followed by the Tukey Post hoc Test**

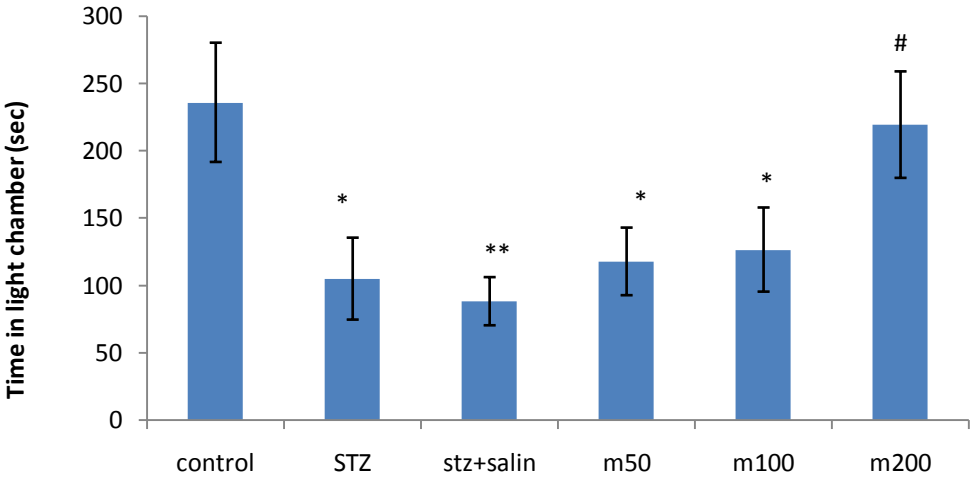

Figure 5: Passive Avoidance Learning
Panel Shows Total Time Spent in Light Chamber in Experimental Groups
***p < 0.05, **p < 0.01; Relative to Healthy Group, One-Way ANOVA Followed by the Tukey Post hoc Test**

Discussion

Insulin, in addition to its vital role in peripheral glucose homeostasis, has been found to have profound effects on the central nervous system, where it can regulates many important processes such asneural proliferation, apoptosis, neuronal survival, synaptic plasticity, learning and memory (Plum *et al.*, 2005; Zhao and Alkon, 2001; Van der Heide *et al.*, 2006).

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 (Brands *et al.,* 2005; Ott *et al.,* 1999; Strachan *et al.,* 1997; Luchsinger *et al.,* 2004). Insulin resistance and DM can impair spatial learning and memory (Brands *et al.,* 2005; Strachan *et al.,* 1997; Stranahan *et al.,* 2008). In parallel, up to 80% AD patients have DM (Janson *et al.,* 2004). It has been reported that induction of brain insulin resistance by intracerebral injection of STZ in animals is enough to produce a number of essential aspects of experimental sporadic AD (Lannert and Hoyer, 1998; Lester-Coll *et al.,* 2006).

Consistent with these phenomena, our study showed that escape latency, distance and number of crossed quadrants in searching for the hidden platform in the STZ 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 STZ group, were significantly less than those in control group, which suggesting impairment in learning and memory in the STZ group animals. Consistent with our results, a previous study showed that diabetic mice had impaired spatial memory assessed by MWM (Li *et al.,* 2002).

The main finding of this study is that Metformin treatment is capable to attenuate STZ-induced impairment in learning and memory consolidation. Since there was no significant difference in the swimming speed between experimental groups including STZ and STZ + Metformin groups, this effect of Metformin treatment was not due to improve in motor activity of rats. Therefore, Metformin treatment probably attenuated STZ-induced neuronal damage in brain.

Our results show that Metformin treatment improves spatial learning and memory in STZ Rat Model of AD in a dose dependent manner, so that rats of Metformin groups found hidden platform in less time and with less distance traveled, in comparison with STZ group. Metformin treatment 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 STZ 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 STZ did not significantly affect the swim length to escape to the visible platform, a finding that is inconsistent with the idea that disruption of escape to the submerged platform is due to general impairments. Furthermore, in our experiments i.c.v. administration of STZ in rats was sufficient to impair spatial performance, a deficit that is perhaps not readily 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 Metformin dose-dependently increased the STL and total time spent in the light area in comparison with STZ group. In present study we provide evidences that Metformin, as an insulin sensitizer against peripheral insulin resistance, could improve cognitive function impairment in STZ Rat Model of AD.

It has been shown that there is a significant increase in the hyper phosphorylated Tau protein in the brains of animals with DM (Li *et al.,* 2007; Kim *et al.,* 2009; Planel *et al.,* 2007b; Li *et al.,* 2012; Clodfelder-Miller *et al.,* 2006). 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 (Kim *et al.,* 2009; Planel *et al.,* 2007b). This increase can be attenuated by administration of Metformin (Li *et al.,* 2012) and insulin (Jolivalt *et al.,* 2008). A recent study has shown that activation of 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 Metformin treatment attenuate this increase of total tau, phospho-tau and activated JNK (Li *et al.,* 2012; Yoon *et al.,* 2010). Also Gupta *et al.,* (2011) observed that long-term hyper insulinemic conditions inNeuro-2a cells led to development of insulin resistance and phosphorylation of tau. This increase of tau phosphorylation is decreased by Metformin.

Extracellular accumulation of Aβ plaques in the brain is another pathological hallmark of AD (Li *et al.*, 2007; Selkoe, 2001). It has been reported that the Aβ1-42 levels in the hippocampus of the diabetic mice were significantly higher than that in the normal mice (Li *et al.*, 2012). 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 (Jolivalt *et al.*, 2008; Planel *et al.*, 2007b; Shuli *et al.*, 2001). A recent study shows that insulin reduces Aβ production in neuronal cultures and Metformin enhances this reduction (Chen *et al.*, 2009). Similarly, it has been reported that long-term hyper insulinemic conditions in Neuro-2a cells have increased Aβ production. This increase is attenuated by Metformin (Gupta *et al.*, 2011). It has shown that Metformin injection to diabetic mice that have increased plasma insulin levels reduced brain contents of Aβ1-42 compared with the control diabetic mice (Li *et al.*, 2012). 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.c.v. injection of STZ to rats can significantly decline in learning and memory and Metformin injection for 10 days to STZ Rat Model of AD that has normal plasma insulin and glucose levels 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 (Gupta *et al.*, 2011; Li *et al.*, 2012; Chen *et al.*, 2009), indicate that Metformin in the presence of normal or high levels of insulin in addition to decrease tau phosphorylation and Aβ generation it can improve cognitive function impairment in different animal Model of AD and therefore, is useful in treatment of AD. Consistent with our results, it has been shown that Metformin can protect the brain against the oxidative imbalance promoted by type 2 diabetes (Correia *et al.*, 2008). Also it has been shown that Metformin can act as a neuroprotectant against apoptotic cell death in primary cortical neurons (El-Mir *et al.*, 2008). Moreover it has been shown that Metformin 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 (Mielke *et al.*, 2006; LI *et al.*, 2010; Abd-Elsameea *et al.*, 2014). These results suggest that Metformin can act as a neuroprotectant against neurodegenerative diseases like Alzheimer, Parkinson and huntington's disease. In connection with this hypothesis Positive effects of Metformin treatment were shown in a transgenic mouse model of huntington's disease by Ma *et al.*, (2007). In a similar line Hwang *et al.*, (2010) shown that Metformin treatment can normalizes type 2 diabetes-induced decrease in cell proliferation and neuro blast differentiation in the rat hippocampal dentate gyrus.

In general, it seems that Metformin Treatment through attenuates tau phosphorylation and Aβ generation and increases antioxidant protection, can improve cognitive function (Ashabi *et al.*, 2014). These actions may contribute to the beneficial effects of Metformin on AD treatment and cognitive function improvement in STZ Rat Model of AD.

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