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Original Article

Streptozotocin-induced diabetes mellitus affects the NMDA receptors: Role of caffeine administration in enhancing learning, memory and locomotor deficits

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ABSTRACT

Objective: To investigate the deterioration of the brain functions by diabetes mellitus (DM) and the beneficial effect of caffeine.

Materials and Methods: First, the component of N-methyl-D-aspartate receptors (NMDA) of the field excitatory postsynaptic potential (fEPSP) were recorded in streptozotocin (STZ)-induced DM and compared with control animals. Later, 40 mice were divided randomly into five groups (8 mice in each): (1) Normal control (Cont), (2) diabetic group (DM), (3) animals pretreated with i.p. caffeine before the induction of DM (Pre Caf), (4) acute caffeine-treated group (Ac Caf), and (5) chronic caffeine group (Ch Caf). Learning and memory were assessed in Morris-Water maze, and motor coordination was tested by rotarod.

Results: A significant reduction in the NMDA-component of the fEPSPs responses was recorded in the hippocampus of the diabetic animals. All the DM-groups demonstrated defects in learning and memory tasks; only the Ac Caf group could reverse the deteriorating effect of DM. This group showed a significantly lower latency values to reach their target (submerged platform) in the water maze in comparison to the DM, Pre Caf, and Ch Caf groups. Their performance was not significantly different from the control animals. Rotarod testing showed significant role of acute, but not chronic, caffeine administration in enhancing the motor skills.

Conclusion: STZ -induced DM resulted into defects in memory tasks which are associated with a reduction in the hippocampal NMDA-receptor component of the fEPSP. Acute, but not chronic administration of caffeine could reverse the deteriorating effect of DM on learning and memory.

Keywords: DM, caffeine, hippocampus NMDA, learning and memory, mice

Introduction

Impairment in the brain cognitive functions is well documented in patients with diabetes mellitus (DM).[1,2] The hippocampal synaptic plasticity, in the form of long-term potentiation (LTP) and depression (LTD), was implicated in the mechanism of learning and memory.[3,4] The induction and expression of hippocampal synaptic plasticity require activation of the specific N-methyl-D-aspartate (NMDA) receptors.[3-5] Activation of these receptors causes Ca^{+2} entrance into the cells and the triggering of a cascade of reactions leading into synaptic strengthening.[6,7] Defects in NMDA receptors cause deterioration in synaptic plasticity induction as well as deterioration in the learning and memory functions.[8] It was shown that the induction of LTP and the performance in the water maze learning test were both defected in mice with NMDA receptor 1 gene deletion.[9] In a previous report, we demonstrated a defect in LTP induction and enhanced LTD expression in the CA1, CA3 and dentate gyrus of the hippocampus of diabetic rats.[10,11] Defects in synaptic plasticity were associated with a significant deterioration in learning and memory tasks.[10,12] In addition, DM affects the release and synthesis of neurotransmitters involved in learning and memory consolidation.[13] It is well documented that diabetes mellitus impairs the neuronal excitability, Ca^{2+} homeostasis of the neurons and the activity of protein kinase in various tissues.[14,15] The present study was constructed to test the ability of caffeine to modify DM-induced deficits. Caffeine was shown to enhance synaptic plasticity and learning and memory deficits in a wide range of animal models of diseases ranging from Alzheimer's disease[16] to chronic stress.[17] In addition, it has neuroprotective effects in diabetic rats against synaptic defects, axonal degeneration, and scar formation as a result of astrogliosis.[18]

In this study, we demonstrated by *in vitro* extracellular recording from hippocampal slices taken from streptozotocin (STZ)-induced DM animals a significant reduction in the NMDA-expressed responses of the field excitatory postsynaptic potentials (fEPSP) compared to control animals responses.

In another set of experiments, we showed that administration of caffeine to diabetic mice could enhance the animal's performance in memory and learning tasks which were tested in the water maze test. The beneficial effect of caffeine was also demonstrated on the motor coordination examined by the Rotarod setup.

Materials and Methods

Animals

Male BALB/C mice of 1 month age (15-20 g body weight) were housed on sawdust and maintained on a 12 h light: 12 h dark cycle with free access to food and water. DM was induced by a single i.p. injection of STZ (65 mg/kg) (S-0130, Sigma, U.K.) dissolved in citrate buffer (pH4.5). The control group received saline (0.9% NaCl) i.p injection. All experimental protocols were done following the National Institutes of Health guiding principles in animal experimentation which are implicated by the guidelines of the Arabian Gulf University for laboratory animals research.

In the first experiment, the changes in the functional properties of hippocampal cells induced by STZ -induced DM were investigated. The NMDA component of the fEPSP was recorded by extracellular electrodes from hippocampal slices after inhibiting the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. The responses obtained from control animals slices (n = 8) were compared with those obtained from the diabetic animals (n = 8).

In the second experiment, 40 animals were randomly divided into five groups: (1) Normal control (Cont; n = 8), (2) diabetic group (STZ) (DM; n = 8), (3) pretreated with caffeine group (PreCaf; n = 8). The animals were injected with caffeine (40 mg/kg) 20 min before the STZ injection, (4) acute caffeine-treated group: Animals were given caffeine (0.5 g/L in drinking water) after 12 weeks of DM and for 3 days (Ac Caf; n = 8), and (5) chronic caffeine treatment group: Caffeine was provided in the drinking water after the induction of diabetes till the testing of the animals after 12 weeks (Chr Caf; n = 8).

Hippocampal electrophysiology

12 weeks after the induction of DM, the animals were sacrificed, and hippocampal slices were made as described elsewhere.[19] Briefly, decapitation of the animals was done following short inhalation anesthesia with isoflurane (Baxter Health Corporation/USA). The brains were removed from the skull and maintained into ice-cold artificial cerebrospinal fluid (ACSF). Hippocampal transverse slices of 450 µm thickness were made and kept for 1 h in an oxygenated (95% O_2, 5% CO_2) ACSF of the following compositions in mM: 124.0 NaCl, 3.3 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 10.0 glucose, 2.00 $NaHCO_3$, and 2.5 $CaCl_2$. The same ACSF was used as a perfusion medium during recording. Bipolar stainless steel electrodes of 0.1 mm in diameter were used to stimulate the afferent fibers of the stratum radiatum of the CA1 region, while low resistance glass microelectrodes (tip diameter; 3–5 µm; 0.5 MΩ, filled with the same ACSF) were used to record the fEPSP responses from the dendritic layer. The slices in the recording chamber (submerged type) were continuously superfused with the oxygenated ACSF in a rate of about 1–2 ml/min. Slices which gave synaptic responses of more than 1 mV amplitudes were included in the experiment. The stimulation current intensity was between 60 and 120 µA. This intensity evoked half maximum amplitude responses. The stimulation frequency was 0.33 Hz. Data were digitized at a sampling rate of 10 kHz by an A-to-D converter (Cambridge Electronic Design Ltd., UK) and transferred to an IBM-compatible computer. The average fEPSP slopes were calculated every 5 min using Spike2 analysis program (Cambridge Electronic Design, Ltd., UK). The slices were first perfused with the normal ACSF which contained Bicuculline (free base; 10 µmol/L) to block GABAA receptors and responses were recorded for 15 min. Thereafter, the slices were perfused with Mg-free ACSF that contained 10 µmol/L 6,7-dinitroquinoxaline-2,3-dione (DNQX to block AMPA receptor-mediated responses) to isolate NMDA receptor-mediated responses. The responses were then recorded for another 15 min. In some experiments, the NMDA receptor antagonist APV (100 µmol/L) was then added to the perfusing medium, and the EPSPs were recorded for another 15 min. The evoked responses were expressed as percentage changes from the baseline responses (set as 100%).

Behavioral study

Morris water maze test (MWM)

Water maze measures spatial learning and memory.[20] The apparatus is a circular swimming pool with a diameter of 140 cm and a height of 50 cm and which is filled with water (26°C–28°C) to a depth of 30 cm. The maze was housed in a darkened room and illuminated by red light. It was divided into four equal quadrants by two imaginary diagonal lines.

Each mouse was given six acquisition trials in the 1st day (training day) of the experiment to learn the position of a hidden "escape" platform, submerged 2 cm below the water surface, at a fixed location inside the pool. On each trial, the mice were released successively from four predetermined positions on the perimeter of the pool. Animals were given a maximum of 2 min to find the platform and were allowed to remain on the platform for 20 s. Mice that failed to locate the platform were put onto it by the experimenter and allowed to stay there for 20 s. After 48 h, three test trials were done to determine the memory of each animal to locate the hidden platform. The position and movement of the animals in the

pool were captured and analyzed every 0.2 s, the ANY-maze video tracking system (Stoelting Co., Wood Dale, IL, USA). Outcome measures were latency time and distance swum to reach the platform. Performance in each trial was averaged to yield one data point per mouse per test. Speed of swimming (which is a measure of motor function) was measured as a control between the groups. In the following day, a probe test was performed in which the platform was removed, and each animal was allowed to swim for 120 s. The percentage of time spent by the animals in each quadrant was determined.

Rotarod test

Motor coordination and balance were assessed using an accelerating rotarod. Mice were placed on a cylinder that rotates at a pre-assigned speed of 5 m/min. Each mouse was tested for three trials. Latency to fall from the rotating rod was recorded with a maximum trial length of 300 s. All groups of mice were tested within the same experiment to allow comparison of baseline motor performance. All testing was done on the same day.

Statistical analysis

All data are presented as a mean ± standard error of means. Significant differences between the groups in electrophysiological experiments, water maze learning, and rotarod were calculated by analysis of variance (ANOVA) test. Differences in weight gain, blood glucose levels were measured using *t*-test.

Results

After 12 weeks of DM induction, the body weight and blood glucose levels were compared in the control animals and the other four groups of DM. All the diabetic groups showed significant hyperglycemia, and decreased body weight (*t*-test, $P < 0.0005$, t critical two-tail= 2.365) when compared to the control animals [Table 1]. There were no significant differences in the body weight between the different DM groups (*t*-test, $P > 0.05$, t critical two-tail = 2.36). The blood glucose level in the pre-Caf group (22.5 ± 0.8 mmol/l) was significantly lower than in the DM (25.5 ± 0.8, *t*-test, $P = 0.047$, t critical two-tail =2.36; Table 1).

In the first experiment, fEPSP recording in STZ-induced DM animals showed significantly reduced NMDA component when compared to the control animals. The hippocampal slices were stimulated first in normal ACSF perfusion medium which contained Bicuculline (GABAA receptor blocker, 10 umol/l) for 15 min to get rid of the GABA components of the fEPSPs. The perfusion medium was then shifted to a Mg^{+2}-free DNQX medium in addition to Bicuculline. DNQX is an AMPA and kainate receptor antagonist, which means the responses evoked by afferents stimulation would be consisted only of NMDA receptors since all the other receptors are blocked [Figure 1a]. The analysis of data demonstrated significantly reduced NMDA components of the fEPSPs in the diabetic animals when compared to the control (ANOVA; $P < 0.001$, F = 27.21, F critical =4.747, Figure 1a and b). No group differences were recorded in the few experiments where APV (NMDA antagonist) was added to the perfusing medium (data not shown).

In the second set of the experiment; learning and memory were tested by Morris-water maze in the different five groups.

In the control mice, the latency and distance swum to reach the platform decreased gradually during the 6 training sessions in the 1st day of the experiment. The memory of the platform location was retained in this group 48 h later as shown in the test trials 7,8, and 9 [Figure 2a]. The control mice could reach the hidden platform in their last test trial after a latency of 63.12 ± 7.97 s, which was significantly lower than the latency made by the DM (116.67 ± 3.33), pre Caf (101.83 ± 7.08 s), and Ch Caf (94.13 ± 7.87 s) groups (ANOVA test; $P < 0.001$, F = 9.479,

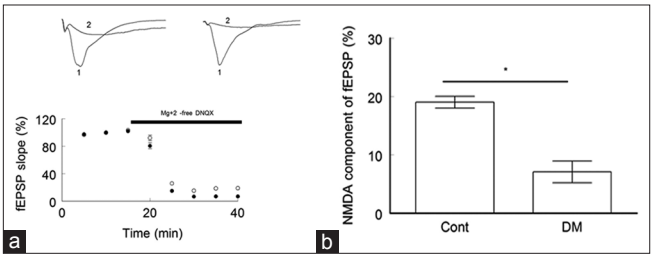

Figure 1: N-methyl-D-aspartate (NMDA) receptor-mediated field excitatory postsynaptic potentials (fEPSPs) in the CA1 field of the hippocampus of STZ-induced diabetic animals. (a) NMDA receptor-mediated EPSPs were isolated by removing Mg^{2+} ions from the medium and adding 10 µmol/L DNQX (to block α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [AMPA] receptors). The absolute value of the baseline fEPSPs was similar in both groups during 15 min of recording. 10 min after switching to the Mg^{2+}-free/10 µmol/L DNQX medium the fEPSP in slices from STZ-rats (●; $n = 7$) was reduced to 7.09 ± 1.86%, whereas in slices from control animals (○; $n = 7$) EPSPs were reduced to 19.02% ± 1.01%. Inset above: Typical averaged five examples of mixed AMPA receptor and NMDA receptor-mediated EPSP (recorded in normal ACSF containing 10 µmol/L Bicuculline; line 1) and the isolated NMDA receptor-mediated EPSP (recorded in in Mg^{2+}-free/10 µmol/L DNQX medium; line 2) from a control (left) and STZ-animals (right), (b) the relative NMDA component of the fEPSP in STZ-induced diabetic animals was significantly smaller (*$P < 0.05$) than what is recorded in the control group

Table 1: Body weight and blood glucose level at the end of the experiment

Variable	Cont	DM	Pre Caf	Ac Caf	Chr Caf
Body weight (g)	28±0.6	22.88±0.5	23.38±0.46	22.8±0.3	22.13±0.64
Blood glucose (mmol/l)	5.2±0.14	25.5±0.8	22.5±0.8	24.8±0.7	23.8±0.9

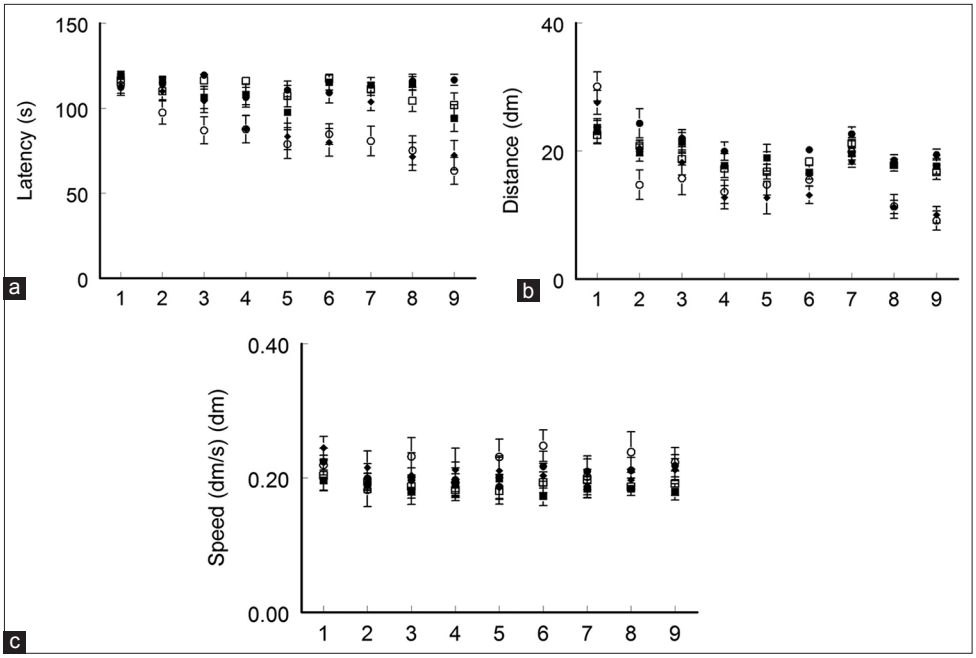

Figure 2: Streptozotocin (STZ)-induced diabetes mellitus (DM) in mice (●, *n* = 8) results in impaired performance in the hidden platform version of the Morris water maze. Caffeine administration effects on the performance of the animals are shown. Escape latency (a), distance swum to reach the hidden platform (b), and the speed of swimming in the pool (c) during 6 training trials in the 1st day and three testing trial after 48 h are presented. Escape latency was increased ($P < 0.05$) in STZ-induced DM group compared with control (Cont;○, *n* = 8) mice. ANOVA test revealed an enhanced performance in acutely administered caffeine group (Ac Caf; ♦, *n* = 8) when compared to the DM, chronically administered caffeine (Ch Caf; ■, *n* = 8) and the Pre Caf (Pre Caf; □, *n* = 8) groups. No significant difference between the Ac Caf and the control groups was recorded. Similar results were recorded concerning the distance swum to reach the hidden platform (b). The speed of swimming was not different between the groups indicating that the difference in latency and distance was not due to swimming speed differences

F critical =2.439) [Figure 2a]. The Ac Caf group performance on the other hand (latency 72.37 ± 8.62 s) was significantly better than the other diabetic groups with lower latency to reach the platform. No significant difference was measured between the Cont group and the Ac Caf group (ANOVA $P = 0.434$, F = 0.621, F critical =3.998). Measurements of the distance swum to reach the platform demonstrated the same results seen with the latency measurements. Control and Ac Caf groups of animals could reach the platform after swimming shorter distances (9.1 ± 1.5 and 10 ± 1.3 dm, respectively) than the other groups (DM; 19.4 ± 0.9, pre-Caf; 16.7 ± 1.1, and Chr Caf; 17.6 ± 1.2 dm) [Figure 2b]. Figure 2c shows the velocity of swimming in the swimming pool during the water-maze testing. No significant differences were measured between the groups (ANOVA; $P = 0.388$, F = 1.041, F critical =2.435) which indicates that the shorter latency and distance swum by the Cont and Ac Caf to reach the platform was due to better learning and memory in these animals rather than higher speed of swimming.

At the end of this experiment, the platform was removed, and the animals were allowed to swim for 120 s. The percentage of time spent by the animals in each quadrant of the pool was measured. The more time spent by the animals in the platform quadrant indicates significant memory. Both Cont and Ac Caf groups exhibit a selective search strategy, i.e., swum significantly above chance of 25% in the platform quadrant (32.9 ± 3.9% and 32.9 ± 5.6%, respectively) when compared to the non-significant time spent by the other groups in the platform quadrant (DM = 21.2 ± 2.9%, Pre Caf = 19.5 ± 5.2%, and Chr Caf 21.1 ± 5.2%) [Figure 3].

Motor coordination and learning were tested by the rotarod. The time the animal could stay on a rotating rod (at a speed of 5 m/s) was calculated. The control mice stayed on the rotating rod before falling (33.15 ± 3.4 s) significantly longer time than the other diabetic groups (DM = 12.1 ± 1.02 s, Pre Caf = 12.2 ± 0.8 s, Ac Caf = 18.6 ± 2.2 s, and Chr Caf= 12.3 ± 2.01s; ANOVA $P < 0.001$, F = 7.237, F critical = 2.438). Analysis of the result showed also a significant difference between the Ac Caf group and the other diabetic groups (ANOVA $P < 0.05$, F = 4.11, F critical = 2.75) [Figure 4].

Discussion

This paper searches in the basic mechanisms of the deteriorating effects of induced DM on the higher brain functions and the role of caffeine as a psychostimulant on these defects. All the injected animals with STZ showed significant hyperglycemia. However, the Pre Caf group showed relatively lower glucose levels than the other diabetic groups. This result confirms other reports indicating that the severity of hyperglycemia induced by STZ injection was modified by pre-caffeine treatment of animals.[18,21,22,23] This may be caused by the stimulating

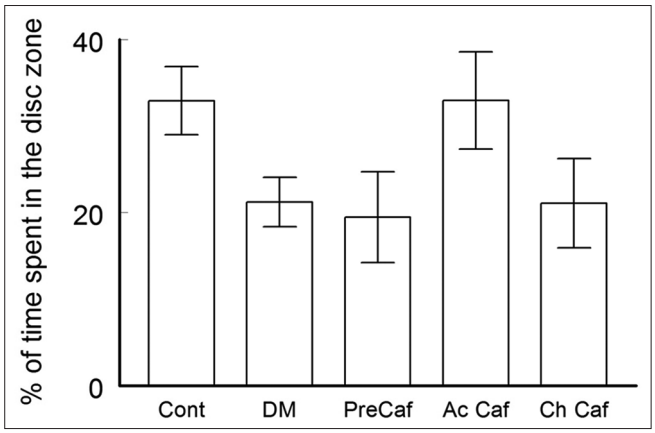

Figure 3: The results of the probe test performed at the end of water-maze testing. The platform was removed from the swimming pool, and each animal was allowed to swim for 120 s. The percentage of time spent by the animals in each quadrant was determined. Significant learning (above the chance percentage of 25% in the platform quadrant) was recording in the Cont and Ac Caf groups (ANOVA, $P < 0.05$)

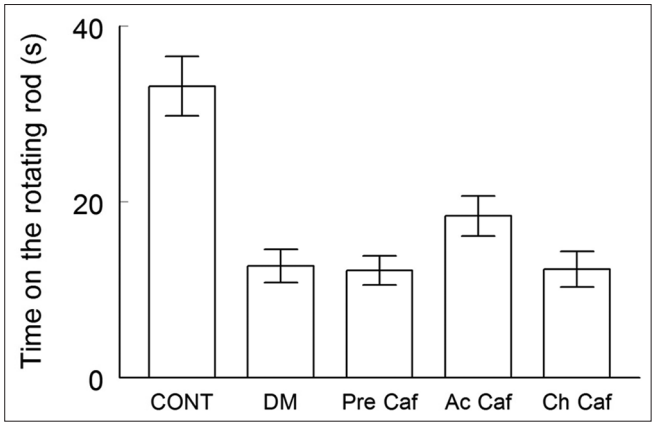

Figure 4: Muscle power and coordination were tested by the performance of the animals in the rotarod test. The diabetic group (diabetes mellitus) and all the diabetic groups treated with caffeine showed significantly lower time staying on the rotating rod (at a speed of 5 m/min) when compared with the control animals. However, the Ac Caf group showed slightly better motor coordination than the other group

effect of caffeine on the beta islet cells of the pancreas.[24] Although it was reported that caffeine affects glucose uptake in the skeletal muscles[25] as well as the brain[26] we did not record any significant difference in the random blood glucose levels after acute or chronic caffeine administration. Subtle differences may be detected with more sensitive tests such as fasting blood glucose levels, HbA 1c, or glucose tolerance test. However, concerning the interrelation between DM and caffeine consumption, some contradictory results were reported.[27,28] On the other hand, the diabetic animals in all the groups failed to show a similar increase in body weight that was seen in the control animals.

The electrophysiological data obtained from recording of fEPSPs in the hippocampus of diabetic animals demonstrated a reduction in the NMDA component of the evoked responses. Similar results were seen by intracellular recording as well.[29] However, it was reported that a remnant of ATP-mediated P2X receptors can be recorded from hippocampal slices after blocking the other receptors.[30,31] In this experiment, the difference between the two groups in the evoked fEPSPs was eliminated by the addition of APV, suggesting that this difference was due to NMDA components of the responses. Activation of NMDA receptors increases the Ca^{+2} entrance into the cells and the subsequent activation of cascades of reactions and increase in the activity of kinases resulting into augmentation of the AMPA receptors components of the fEPSPs. In the previous study, we showed that the expression of specific type of NMDA receptors subtype, namely, the NR2A was significantly reduced in STZ-induced DM in rats.[5,29] As already confirmed by several previous reports, activation of NMDA receptors is crucially important in the induction and expression of hippocampal synaptic plasticity in the form of LTP and LTD.[32,33] Both of these forms of synaptic plasticity are considered as the memory and learning mechanisms at cellular levels. It is widely accepted that impaired synaptic plasticity or decreased activation of NMDA receptors would consequently resulted in spatial memory and learning defects. In previous studies, we showed that STZ-induced DM in rats resulted in defects in LTP and LTD induction and this was associated with deterioration in the learning and memory functions.[19]

Caffeine is considered as a central nervous system stimulant[34] which can enhance the higher cognitive functions of the brain[35,36] this effect is induced by several mechanisms including adenosine and GABA receptors inhibition,[37,38] phosphodiesterase inhibition,[39] catecholamine release, nitric oxide production, inflammation, NMDA receptors and astrocytic function[40] and sensitization of calcium-induced calcium release[41] depending on the dose of caffeine. Caffeine also modulates the brain-derived neurotrophic factor system.[42-45] On the other hand, adenosine receptors are also responsible for the control of hippocampal NMDA receptors and plasticity[46] as well as inflammatory processes.[47,48] Our results showed that all the caffeine administered diabetic animals showed significantly lower latency to reach the hidden platform in the Morris-water maze. However, only the Ac Caf group demonstrated significant search strategy (above than 25% of the time) that means above chance level and indicates learning and memory. The deficits in learning and memory test recorded in diabetic animals could be restored by acute caffeine administration. They reached the platform with a comparable latency (72.4 ± 8.6 s compared to 63.1 ± 7.98 s, ANOVA $P > 0.05$) and they spent statistically similar time in the platform quadrant of the swimming pool in the probe trial at the end of the experiment (32.9 ± 5.6% compared to the control 32.9 ± 3.9%). This Caffeine effects can probably be induced by many of the mentioned mechanisms which could affect the induction and expression of LTP and LTD in the hippocampus. It is worth noted that the dose of caffeine in this study (0.5 g/l) targets mainly the adenosine receptors[49] rather

than GABA receptor or the calcium-induced calcium release mechanism. It would be interesting to study the hippocampal synaptic plasticity in diabetic or healthy animals administered with caffeine. One of the limitations of this study is the lack of such experiments. However, it was shown that caffeine in a non-toxic dose could regulate the hippocampal synaptic plasticity in rodents.[50] Another interesting point would be the study of the effect of caffeine administration on the behavior of control non-diabetic animals. Our preliminary data in this respect showed enhanced memory functions with moderate caffeine dose in normal control animals while high doses have a deteriorating effect (unpublished data). Planning for such experiments will provide more depth to the mechanism of caffeine on brain higher function. Our results showed no significant improvement by chronic administration of caffeine on learning and memory. The reason of this is not very clear, but we think that chronic caffeine in addition to its stimulating effects on the brain, it will interfere with the sleep cycle by inhibition of adenosine.[51-53] Disturbed sleep could be a major cause in the defects in memory construction. Research showed that caffeine can affect both short-term (working) memory and long-term memory. Although chronic caffeine use was beneficial in many conditions and situations;[16,17] for review see,[40] other reports demonstrated opposite effects.[54,55] In fact, data showed negative as well as positive effects of caffeine administration on different aspects of memory.[54] Although Duarte *et al*.[23] had shown in a previous report a protective role of caffeine on the performance of animal's model of type II DM in Y-maze. Our results are in some aspects not consistent with that observation. This could be due to the difference in experimental protocols, as we tested the hippocampus function by the water maze, not the Y-maze in type I, not type II DM.

In addition to its action on cognitive and emotional states of the animals, caffeine is also known to act on pure motor skills.[25,56] By studying the possible effect of caffeine on motor coordination on humans, Terry *et al*.[57] reported a significant difference in time reaction but insensitivity in time perception and time production. In addition, repeated administration of caffeine was reported to cause neurobehavioral sensitization in rodents as indicated by an increased locomotor activity.[58] It was also noted that caffeine enhances motor coordination in mice as indicated by the longer latency of stability on the rotating rod before falling.[58] Our results showed all these positive effects of caffeine in acutely administered doses. However, chronic caffeine administration similar to the effects observed in memory and learning did not demonstrate a significant effect on motor coordination enhancement. This could be caused by the effect of chronic caffeine administration in decreasing the cerebral blood flow[59] or modulation of response or expression of adenosine receptors.[60] In a recent report, Bădescu *et al*. showed in an open field test that caffeine administration in STZ-induced diabetic rats could enhance the mobility time and total distance moved by the animals.[61] However, motor power and motor coordination cannot be measured in such experimental protocol. In this study, we used the rotarod test to document these parameters. Further research is needed in this respect.

We concluded that STZ-induced DM in rodent causes a decrease in NMDA-component of the fEPSP recorded in the hippocampus extracellularly. This effect could be the cause of deterioration in the learning and memory tests performance. Caffeine administration in mice could affect the performance of these animals in water maze learning. The effect is significantly enhanced in animals with acute administration of caffeine, while less pronounced in animals which administered caffeine for 4 weeks.

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