Metabolic Brain Disease (2019) 34:1431–1446
https://doi.org/10.1007/s11011-019-00448-7

ORIGINAL ARTICLE

Time-dependent impairments in learning and memory in Streptozotocin-induced hyperglycemic rats

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Received: 4 September 2018 / Accepted: 10 June 2019 / Published online: 8 July 2019

Abstract

The sedentary lifestyle is responsible for the high prevalence of diabetes which also impairs cognition including learning and memory. Various studies have highlighted the learning and memory impairments in rodent models but data regarding the timeline of their development and their correlation to biochemical parameters are scarce. So, the present study was designed to investigate the type of memory which is more susceptible to hyperglycemia and its correlation with biochemical parameters such as inflammatory cytokines, cAMP response element binding (CREB) and protein kinase B (Akt) activation. Hyperglycemia was induced using streptozotocin (STZ, 45 mg/kg i.p.) and confirmed by measuring fasting blood glucose levels after 1 week of STZ injection. Learning and memory deficits were evaluated using the Novel Object Recognition Test (NORT) and Morris water maze (MWM), and correlated with biochemical parameters (TNF-α, IL-1β, and dopamine) at 3, 6 and 9 weeks. STZ-injected rats after 3 weeks of injection demonstrated moderate hyperglycemia (blood glucose = 7.99 ± 0.62 mM) with intact learning and reference memory; however, their working memory was impaired in MWM. Severe hyperglycemia (blood glucose = 11.51 ± 0.69 mM) accompanied by impaired short, long, and working memory was evident after 6 weeks whereas learning was intact. After 9 weeks of STZ injection, hyperglycemia was more pronounced (13.69 ± 1.43 mM) and accompanied by a learning deficit in addition to short, long, and working memory impairments. The extent of hyperglycemia either in terms of duration or severity resulted in enhanced inflammation, down-regulation of the level of dopamine, protein expression of AKT and CREB, which possibly affected learning and memory negatively.

Keywords Hyperglycemia · Learning and memory · Novel object recognition test · Morris water maze · Dopamine · AKT · CREB

Ayaz Ahmed and Guirong Zeng shared the first author

Ayaz Ahmed and Guirong Zeng authors contributed equally to the experimentation and preparation of the manuscript.

Research highlights • Learning and memory impairment is time and hyperglycemia severity dependent.
• Working memory impaired firstly among other cognitive declines.
• Early and enhanced inflammation might be responsible for cognitive decline.
• AKT and CREB expressions is reduced with hyperglycemia severity.

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Introduction

Diabetes mellitus is the most complicated metabolic syndrome of global concern. It has ranked 4th among non-communicable diseases with rapidly emerging global prevalence (WHO 2016). Hyperglycemia, the hallmark of diabetes, is attained by impaired insulin function (insulin resistance and hypoinsulinemia). Among many end-organ complications of diabetes, it also affects the central nervous system-associated cognition, especially learning and memory (Nooyens et al. 2010; Long and Dagogo-Jack 2011; Rask-Madsen and King 2013; Feinkohl et al. 2015). Diabetes increases the risk of dementia by 60% (Chatterjee et al. 2016). Hyperglycemic individuals showed impaired working memory in task-oriented work (Mayeda et al. 2015; Redondo et al. 2016). The actual mechanisms behind diabetes-induced effects on learning and memory processes are still inconclusive. However, compromised learning and memory functions have been reported in various hyperglycemic animal models (Benedict et al. 2012; Koekkoek et al. 2015).

Animal models of diabetes, where hyperglycemia was induced by chemical or genetic methods, manifested learning and memory impairments. Among them, streptozotocin (STZ)-induced hyperglycemic model was more reliable, popular, cost-effective and has no direct effect on the brain (Schnedl et al. 1994; Deeds et al. 2011). Streptozotocin injection (40–195 mg/kg) in rats induced significant hyperglycemia (>11.1 mM) with memory and learning deficits accompanied by imbalanced neurotransmitters (corticosterone and dopamine) (Biessels et al. 1998; Stranahan et al. 2008; Alvarez et al. 2009). The behavioral studies indicate that after 8–10 weeks of hyperglycemic conditions, the spatial learning and memory impairments deteriorated noticeably with the passage of time (Beauquis et al. 2010; Matsunaga et al. 2016).

It has been reported that the learning and memory impairments under hyperglycemic conditions are mainly due to alteration in the hippocampus region of the brain via impaired insulin receptor pathway, neurotransmitter dysfunction, and elevated inflammation mediators (Chen et al. 2017; Noor and Zahid 2017; Wang et al. 2017). cAMP response element binding (CREB) is a major constituent of a variety of neurons and it is a part of brain-derived neurotrophic factor (BDNF) genes (Yossifoff et al. 2008; Gao et al. 2011; Landeira et al. 2016). It needs to be activated (i.e. phosphorylation) by different kinases including protein kinase B (Akt) at ser 133 to exert its function (Rosa and Fahnestock 2015). Both CREB activation and BDNF expression are required for neuronal plasticity as well as long-term potentiation (LTP). The activation of both CREB and Akt are suppressed in the presence of inflammatory cytokines (TNFα and IL-1β), the hallmark of the diabetic brain (Dong et al. 2018; Culley et al. 2014; Murray et al. 2012). Majority of the studies showed learning and memory deficit among hyperglycemic rodent models after 7–10 weeks of hyperglycemia but none showed at what stage of hyperglycemia the behavioral changes started to appear, and at what stage hyperglycemia modulates metabolic changes (i.e. inflammation, dopamine level, CREB, and Akt Expression) (Stranahan et al. 2008, 2010). The information regarding the duration of hyperglycemia affecting learning and memory (short term: capacity for holding information for a short period of time without manipulating it; Long term: type of information which stayed indefinitely; Working memory: a type of short term memory which can be manipulated) is scarce. Therefore, the present study was designed to explore the possible effects of duration (3, 6 and 9 weeks) and the severity of hyperglycemia on learning and memory among STZ-induced hyperglycemic rats. The impact of hyperglycemia on learning, short, long and working memory impairments were evaluated using the Novel Object Recognition Test (NORT) and Morris Water Maze (MWM). Further, the possible association of behavioral alterations with the corresponding levels of the impaired neurotransmitter (dopamine), inflammatory modulators (TNF-α; IL-1β) followed by protein expression of protein kinase B (Akt) and cAMP recognition element binding (CREB) protein at each respective time point was also investigated.

Material & method

Animals

Male Sprague-Dawley (SD) rats ($n = 80$, weighing 180–220 g) were purchased from Hunan SJA Laboratories Animal Company Ltd. (Changsha, China). The animals were acclimatized for 5 days under controlled conditions (24 ± 1 °C, 40–50% humidity) with 12 h light and dark cycles with food (standard chow diet) and water ad libitum. All the experimental protocols were prepared according to the National Institute of Health (USA) guidelines and approved by the Ethical Committee of Hunan Drug Safety Evaluation (Study # 2016016).

Induction of hyperglycemia and experimental plan

Hyperglycemia was induced by single intraperitoneal (i.p.) administration of streptozotocin (STZ, 45 mg/kg; Sigma, USA), which could cause the destruction of pancreatic islet β-cells. The dose was selected on the basis of previous experiments in our laboratory, considering the degree of glycemia and mortality rate. STZ was freshly prepared in sodium citrate

(0.1 M) buffer with pH 4.5, and administered to 16 h fasted rats. After 7 days, fasting blood glucose (FBG) from tail vein was measured using GA-6 blood glucometer (SANNUO, China). Rats with ≥7.0 mM FBG were considered hyperglycemic, and were used for the study. During the course of study, rats were fed with standard chow diet.

For behavioral experiments, rats were divided into control and hyperglycemic groups ($n = 10$; respective group for each time point). The behavior parameters that indicate memory impairment were evaluated at 3, 6 and 9 weeks using NORT and MWM tests in both groups. Rats were placed in their respective behavioral rooms 24 h prior to experimental procedures. Detailed experimental design is elaborated in Fig. 1.

Behavioral assessment

Locomotion activities

The locomotion profile of the rat was analyzed using a computer-aided rectangular open field arena (Shanghai Xinruan Information Technology Co., China). The dimensions of the rectangular field was 100 × 100 × 40 cm, enclosed in a metallic chamber with illumination source of 120 lx. Individual rat (control or diabetic) was placed in the center of the arena and after 2 min of acclimatization, locomotion behavior such as speed and time of movement were recorded for a period of 10 min and analyzed using super-maze software (Shanghai Xin-ruan Information Technology Co., China).

Novel object recognition test (NORT)

NORT was used to evaluate the memory dysfunction among the hyperglycemic and the control rats as described previously, with minor modifications (Revsin et al. 2009). Rats were placed in an open field arena. The overall test comprised of habituation, familiarization, and trial phase. In the habituation phase rats were allowed to explore the arena for 10 min. After 24 h, rats were familiarized for 5 min with two identical objects. It was followed by an exploration phase which was considered positive if the rats reached the object with its head oriented towards it, sniffing or contacting the object at ≤2 cm with its nose. After every test session, olfactory clues and objects in the arena were cleaned with distilled water and ethanol (50%). After 1 h of the familiarization phase, the test was conducted by replacing one of the familiar objects with the new object. The percentage of preference to two similar objects (FOT) or new object (NOT) in familiarization and trial phase was calculated to assess memory dysfunction.

Morris water maze

Biochemical paramater analysis

Spatial learning and memory of the diabetic rats were evaluated using a maze described previously, with slight modifications (Morris 1984; Xu et al. 2016a). The test comprised of: a) Acquisition phase, b) Probe trial phase and c) Working

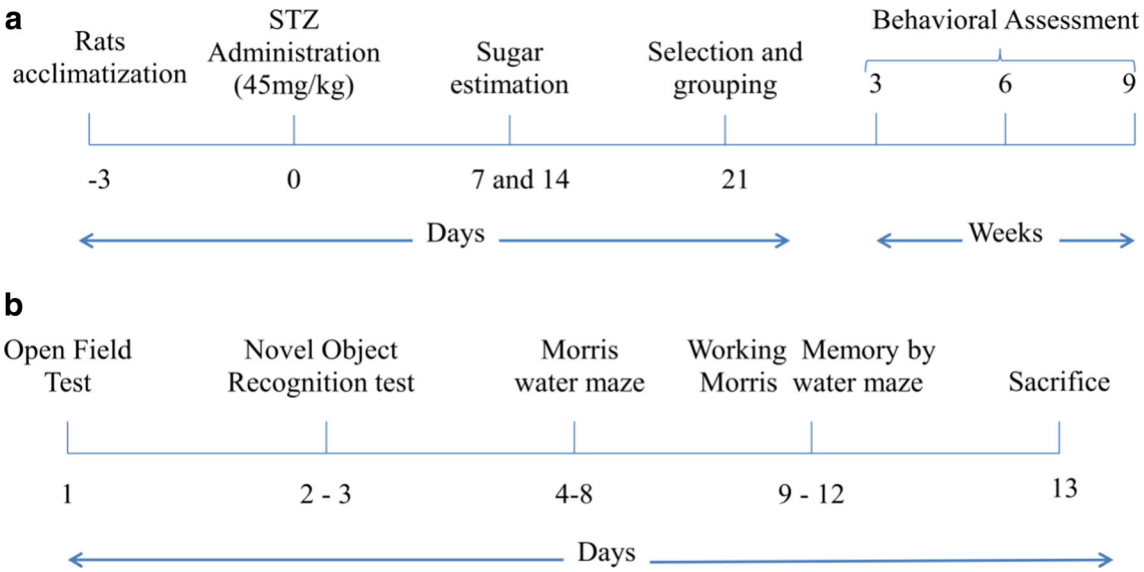

Fig. 1 Study Design for Hyperglycemia Induction and Behavioral Assessment. **a** Acclimatization of rats to the lab environment for 3 days. Hyperglycemia was induced by 45 mg/kg streptozotocin (STZ) administration (Day 0). Hyperglycemic condition was evaluated by estimating fasting blood sugar at 7 and 14 days after STZ administration. At day 21 rats were grouped for behavioral assessment at 3, 6 and 9 weeks. **b** Behavioral assessment of diabetic and control group was evaluated by open field test (Day 1), Novel object Recognition test (Day 2–3), Reference memory (Day 4–8), Working Memory (Day 9–12). Animals were killed (Day 13) and blood and tissue samples were collected

memory trail. The maze consisted of a circular steel tank (diameter = 180 cm, and height = 40 cm), filled with water (3/4th of the tank), and a cylindrical platform (diameter = 9 cm, height = 25 cm) submerged 1.5 cm below the water surface. The water was made opaque using non-toxic white paint and temperature was maintained between 23 and 26 °C. The pool was further divided into four quadrants i.e. North-East, South-East, South-West, and North-West.

During the acquisition phase, the platform position was fixed in the center for north-east quadrant providing the only escape route for the animal. In each trial, rats were released from three different release points. The rats were given 60 s to explore the pool and to reach the platform. However, upon failure, it was gently guided towards it and permitted to sit there for 20 s. Each animal underwent three trials per day for four consecutive days. Behavioral patterns of the rats such as time to reach the platform, swimming speed, and distance as a measure of spatial memory were recorded by the camera fixed over the center of the pool.

After the last probe trial training session, the platform was removed and the rat was released from the opposite quadrant where the platform was located in the acquisition phase. They were allowed to swim freely for a period of 60 s and time spent in the target quadrant and the number of target-crossing were recorded by a camera-linked software.

The spatial working memory of the rats was evaluated as described earlier (Xu et al. 2016b). The training was initiated by releasing rats from fixed entry point while changing locations of the probe on each trial. Working memory trial was conducted for three successive days and each rat was tested 3 times per day.

Body weight and lipid profile and insulin levels

Body weight of the rats was measured before and after 3, 6 and 9 weeks of STZ administration and compared with controls. Serum collected from 3, 6 and 9 weeks of STZ-treated diabetic and control rats was used to determine the lipid profile including low-density lipoprotein (LDL), high-density lipoprotein (HDL), total glycerides (TG) and total cholesterol using a MODULAR P800 automated biochemist analyzer (Roche, Basel, Switzerland). However, insulin levels were monitored using rat insulin ELISA kit (Solarbio Science & Technology Co., China) following manufacturer's protocol. The absorbance determinations at 450 nm of the plate were made by using ELISA plate reader (Tecan, USA).

Levels of tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) in hippocampus

Hippocampus isolated from the brain of the control and the hyperglycemic rats were homogenized in RIPA lysis buffer (Solarbio Science & Technology Co., China) containing protease and phosphatase inhibitor (Thermo Scientific, USA) cocktail. To remove debris, homogenates were centrifuged (12,000 rpm) for 10 min at 4 °C. The protein concentration of each homogenate was noted using bicinchoninic acid protein assay (Solarbio Science & Technology Co., China). Levels of tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) in the hippocampus were determined using rat TNF α and IL-1β ELISA kits (Solarbio Science & Technology Co., China) according to manufacturer instructions. The absorbance determination at 450 nm of the plate was read by using ELISA plate reader (Tecan, USA).

Dopamine concentration

Dopamine concentrations in the cerebrospinal fluid (CSF) of the control and hyperglycemic rats at 3, 6 and 9 weeks of STZ treatment were also estimated. Dopamine concentration was analyzed using reverse phase high-performance liquid chromatography (HPLC) consisted of C18 reverse phase column (Water 150 mm × 4.6 mm, 5 μm), an electrochemical detector (ESA, 5600A, Bedford, MA, USA), and a liquid chromatography work station. Cerebrospinal fluid was centrifuged at high speed to remove debris and passed through a 0.2 μm filter and processed for HPLC-ED analysis. The analysis was conducted by using parameters as described earlier by our group (Dang et al. 2009).

Western blot analysis

Homogenate of hippocampus for ELISA were further utilized to estimate the levels of protein expression of CREB/pCREB, AKT/pAKT. Proteins in samples were separated by using SDS -10% PAGE and were transferred to PVDF membrane, and protein expression was determined by using antibodies for cAMP response element binding (CREB / pCREB), protein kinase B (AKT / pAKT) and actin (1:1000; Abcam, USA) respectively, as described earlier by Xu et al. (Xu et al. 2016a, b).

Statistical analysis

All the data presented as mean ± standard error of the mean (SEM). Statistical significance between the groups at different weeks for MWM was analyzed using one way ANOVA followed by LSD post-hoc analysis. Whereas, significance between the hyperglycemic and the control groups at each time interval were determined by Student's t test. The outcome of cognitive test at each respective time (i.e. 3, 6 and 9 weeks) compared with metabolic markers in a linear regression model. Model was created by keeping cognitive test (i.e. MWM and NORT) constant, whereas, glucose, insulin, IL1-β, TNF-α, and

dopamine used as independent variables. All the statistical analysis was conducted using SPSS version 20 (IBM, USA). A p value <0.05 was considered the significant level.

Results

Hyperglycemia induction and its progression

In STZ-administered rats, the parameters such as body weight, blood glucose, and serum insulin in hyperglycemic condition were monitored and compared with that of control rats (Table 1). In STZ-treated hyperglycemic group there was a gradual increase in fasting blood glucose (FBG) (i.e. FBG >7 mM was evident after 1–3 weeks of STZ induction which drastically elevated to 11.51 and 13.69 mM after 6 and 9 weeks respectively). Overall 75% of the rats demonstrated hyperglycemic condition with 10% mortality during the experiment. Hyperglycemic rats also showed a significant reduction in body weight by 29%, 38% and 41% after STZ treatment at 3, 6 and 9 weeks, respectively. In these animals, serum insulin levels were reduced by 26.4%, 24.7% and 50.9% as compared to the respective controls (Table 1). Polydipsia, polyuria, and hyperphagia were also observed in hyperglycemic rats (data not shown). Hyperglycemic rats also showed dyslipidemia as evident by a significant increase in the LDL-C, total cholesterol and triglycerides levels after 6 to 9 weeks of STZ administration (Table 1).

Hyperglycemia and behavioral changes

Locomotion activity

The open field was used to evaluate the effect of hyperglycemia on the movement and speed of rats. After STZ treatment 3, 6 and 9 weeks, the hyperglycemic rats displayed a significant reduction in the movement and speed as compared to their respective controls ($p < 0.05$, $p < 0.01$) (Fig. 2a, b). The movement of the hyperglycemic rats in 6 weeks group, the speed in the 6, 9 weeks groups was significantly decreased compared to the 3 weeks group ($p < 0.05$, $p < 0.01$). The movement of the hyperglycemic rats in 9 weeks was decreased compared to the 6 weeks group ($p < 0.05$).

Novel object recognition test (NORT)

In NORT, the control rats demonstrated a better preference to explore the novel object as compared to the hyperglycemic rats (Fig. 3a, b). In the trail phase, although at 3 weeks no significant difference was observed between the hyperglycemic and the control rats, at 6 and 9 weeks the novel object preference in the hyperglycemic rats was reduced significantly by 35% and 10% respectively as compared to their respective controls (55% and 65%) suggesting loss of object recognition memory in hyperglycemic animals ($p < 0.05$) (Fig. 3b). In the familiarization phase, both the control and the hyperglycemic group showed similar preference toward the similar objects (Fig. 3a).

Morris water maze test (MWM)

In the acquisition phase negligible difference was observed between the control and the 3, 6 weeks of STZ treatment rats (Fig. 4a). However, after 9 weeks of STZ treatment, the hyperglycemic rats displayed significant higher escape latency during all four consecutive days of training as compared to the control counterpart ($p < 0.05$) (Fig. 4a). In STZ treatment 3 weeks hyperglycemic rats the memory was intact as the time spent in the target quadrant and the number of platform crossing were similar to the control (Fig. 4a, b). However, at STZ treatment 6 and 9 weeks groups, significant memory impairment in hyperglycemic rats were observed as they spent less

Table 1 Biochemical and weight comparison among diabetic and control rats

Parameters	3 week		6 week		9 week	
	Control	Diabetic	Control	Diabetic	Control	Diabetic
Fasting Blood Glucose (mM)	3.45 ± 0.05	7.99 ± 0.62	3.44 ± 0.11	11.51 ± 0.69	3.92 ± 0.12	13.69 ± 1.43
Weight (grams)	453.7 ± 8.08	346.0 ± 22.68**	509.6 ± 12.23	337.2 ± 8.29***	541.89 ± 6.57	293.78 ± 13.77***
Insulin (IU/L)	10.93 ± 1.2	8.04 ± 0.31*	9.87 ± 0.36	7.43 ± 0.3**	10.65 ± 1.23	5.21 ± 0.67 ***
LDL-C (mmol/L)	0.344 ± 0.03	0.173 ± 0.01***	0.236 ± 0.04	0.378 ± 0.02**	0.377 ± 0.02	0.628 ± 0.08**
HDL-C (mmol/L)	0.869 ± 0.05	0.484 ± 0.05***	0.705 ± 0.12	0.981 ± 0.06*	0.741 ± 0.03	1.194 ± 0.3***
TG (mmol/L)	0.509 ± 0.05	0.358 ± 0.08	1.583 ± 0.43	0.749 ± 0.13*	1.733 ± 0.22	3.648 ± 0.98*
TC (mmol/L)	1.47 ± 0.08	0.84 ± 0.08***	1.263 ± 0.2	1.688 ± 0.1*	1.556 ± 0.06	2.7 ± 0.31**

Weight and Biochemical Parameters of diabetic and non diabetic rats at 3, 6 and 9 weeks respectively. Data was represented as mean ± S.E.M. Statistical significance of the data was represented as $p > 0.05$*, 0.01* and 0.001***. **LDL-C** = Low density Lipoprotein; **HDL-C** = High density Lipoprotein; **TG** = Total glyceride; **TC** = Total cholesterol

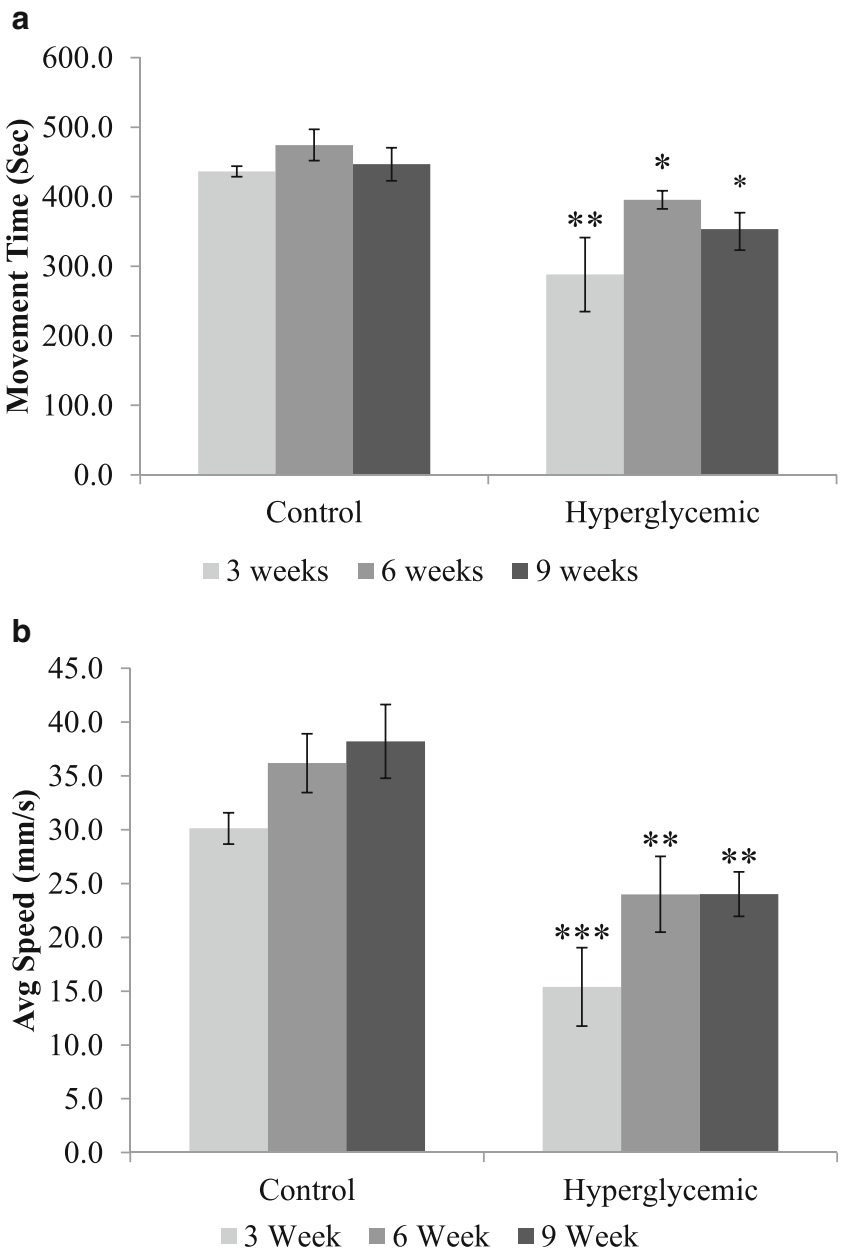

Fig. 2 Locomotory activities among control and hyperglycemic rats in open field arena at 3, 6 and 9 week. Each bar shows Mean ± S.E.M values. **a** Movement time (**b**) Speed of rats. One way ANOVA was used to evaluate the statistical significance (* = $p < 0.05$, ** = $p < 0.01$ and *** $p < 0.001$) of data

time in the target quadrant and the frequency to cross-platform was also reduced ($p < 0.05$; Fig. 4a, c). Moreover, STZ treatment 3, 6 and 9 weeks hyperglycemic rats displayed non-significant changes in the swimming speed and distance travel (Fig. 4d, e). At STZ treatment 9 weeks group, two out of ten rats developed cataract (observed as white eyes) and excluded from the study.

The ability of rats to learn new messages by changing platform orientation was assessed by working memory in the MWM. Working memory was impaired at early stages i.e. at 3 weeks in hyperglycemic rats that was reflected by an increase in escape latency time as compared to the control **(**Fig. 5**)**. At 6 weeks hyperglycemic rats the escape latency time on day 1 and 2 were significantly longer than that of control ($p < 0.001$ and $p < 0.05$). On the other hand 9-week hyperglycemic rats showed significantly increase in escape latency on day 2 and 3 compared to its control ($p < 0.05$).

Hyperglycemia and biochemical markers

Inflammatory markers

In hyperglycemic rats, brain levels of inflammatory markers TNF-α and IL-1β were also altered. In the hippocampus of STZ treatment 3, 6 and 9 weeks hyperglycemic rats, the level of TNF-α and IL-1β were significantly higher compared to their respective control (3 weeks, $p < 0.05$; 6 and 9 weeks, $p <$

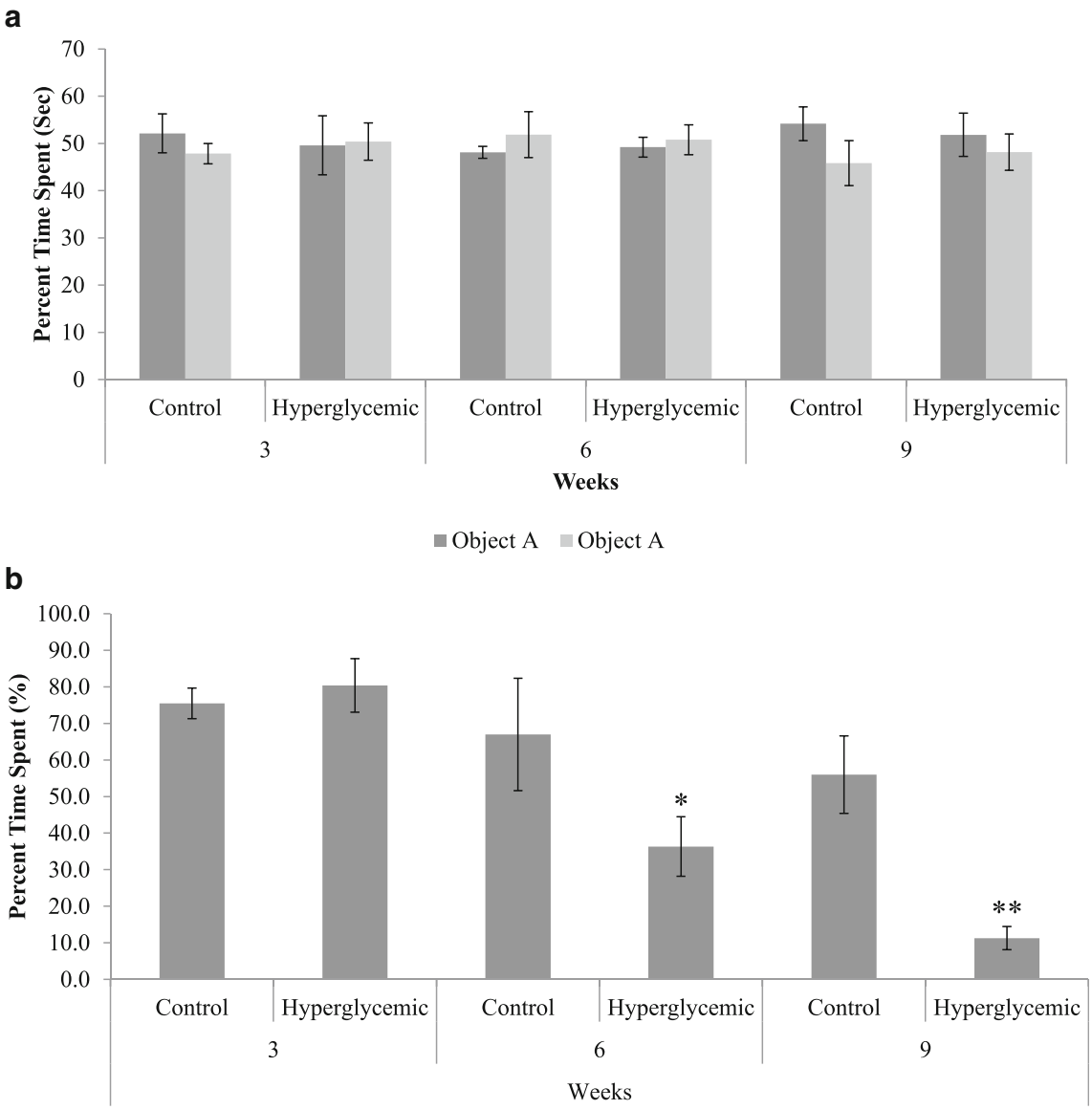

Fig. 3 Effect of hyperglycemia on short term memory deficiency in novel object recognition test at 3, 6 and 9 week of hyperglycemia. **a** Percent time exploration on the familiarization phase (**b**) Percent time spent toward new object. Each bar show Mean ± S.E.M. Student *t* test was used to evaluate statistically significant ($p < 0.05$*, $p < 0.01$**) of data

0.001). The levels at the 6 and 9 weeks were significantly raised as compared to 3 weeks in hyperglycemic rats ($p < 0.01$) (Fig. 6a, b). However, the memory decline in the MWM and NORT compared with different factors (glucose, insulin, dopamine, TNF-α, and IL-1β) positively correlated with only inflammatory markers with *p* value less than 0.05 at 6 and 9 weeks (Table 2).

Dopamine level

Although the dopamine levels decreased among 3 and 6 weeks hyperglycemic rats, the reduction was not statistically significant compared to non-hyperglycemic controls. However, at 9 weeks, dopamine levels were significantly ($p < 0.01$) reduced in the hyperglycemic group as compared to its respective control (Fig. 7).

Expression of p-AKT/AKT and p-CREB/CREB

Western blot analysis showed the altered temporal expression of activated CREB and AKT. The ratio of p-AKT/AKT and p-CREB/CREB reflected the activation state. At 3 weeks hyperglycemic brains the activation of AKT and CREB did not differ significantly which further confirmed the intact learning and memory in rats (short and long term) (Fig. 8a, b and c). On the other hand, the levels of p-AKT and p-CREB expression differ significantly among the hyperglycemic brains and the control brains at 6 and 9 weeks which positively correlated

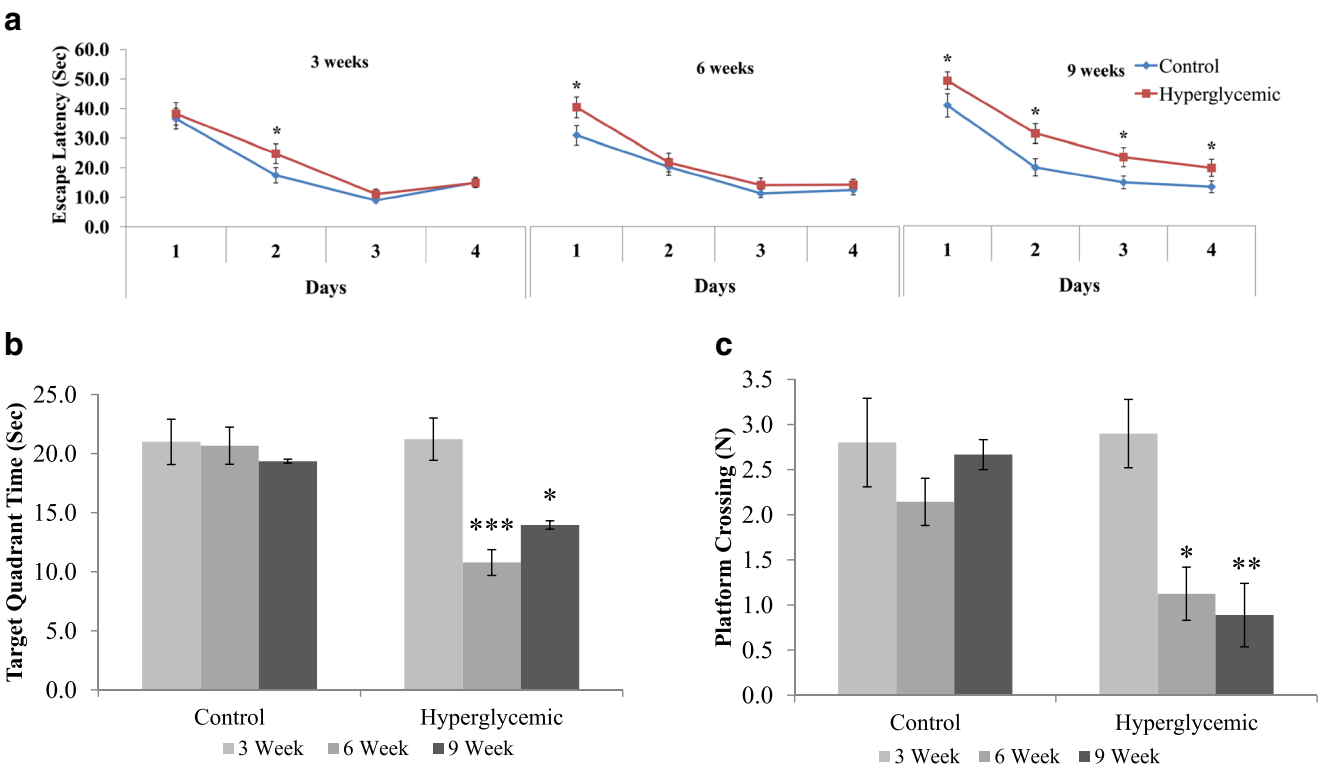

Fig. 4 Effect of hyperglycemia duration (3, 6, and 9 weeks) on reference memory impairment among rats in Morris water maze. **a** Escape Latency were measured for four consecutive days of training. **b** Time spent in the target quadrant (**c**) platform crossing were recorded during the probe trial (**d**) swimming distance (**e**) Average speed (**f**) Pattern of control and hyperglycemic rats movement. Data are presented as mean ± SEM (n = 8–10 rats in each group). One way ANOVA was used to determine the statistical significance of the data $p < 0.05$*, <0.01**, <0.001*** among controls and hyperglycemic rats

with the memory impairments among hyperglycemic rats at these time points ($p < 0.05$) (Fig. 8a, b and c).

Discussion

In diabetes, the hyperglycemic condition has been reported to adversely affect various organs including brain in terms of memory and learning. However, the time of their emergence, type of memory impaired, and its correlation with biochemical markers are still ambiguous. Therefore, in the present study, duration of the hyperglycemic condition and its impact on short, long or working memory and learning were addressed by monitoring their learning and memory behavior and correlates with alterations in biochemical parameters such as inflammatory markers, dopamine and AKT, and CREB expression at various time points after induction of diabetes.

STZ induced progressive hyperglycemia i.e. 7 mM glucose at 3 weeks increasing to 13 mM at 9 weeks with less mortality and high success rate (Data not shown). Hyperglycemia-induced by using this dose was also reported in different studies and our data is in good agreement with the results of the previous studies i.e. increasing hyperglycemia, weight reduction, and impaired biochemical profiles (Table 1) (Gajdosik et al. 1999; Sadek et al. 2017). A time-dependent increase in the glucose level was evident in STZ-treated rats suggesting the direct relation of duration and severity of the disease. After validation of hyperglycemia, rats were subjected to behavioral studies to identify impairment in learning and memory at 3, 6 and 9 weeks. With the increase in hyperglycemia i.e. moderate to hyperglycemia working memory was impaired initially followed by reference memory and learning. These memory deficits further correlate with the reduced dopamine level, enhanced inflammation and altered expression of activated AKT and CREB in the brain with the passage of time.

The learning and memory impairments in hyperglycemic rats were evaluated by considering short- and long-term memories. Behavioral assessment using an open field, NORT and MWM were selected by considering the fact that long term memory requires consolidation of short term memory i.e. acquisition and encoding in the hippocampal region of the brain.

Different studies have highlighted the impact of hyperglycemia on learning and memory impairments in rodents. However, data regarding temporal and sequential emergence of memory impairment (short, long or working memory) is still lacking. Novel object test to evaluate short-term memory was selected on the basis of rodent inborn preference to novelty which evaluated non-spatial memory independent of the

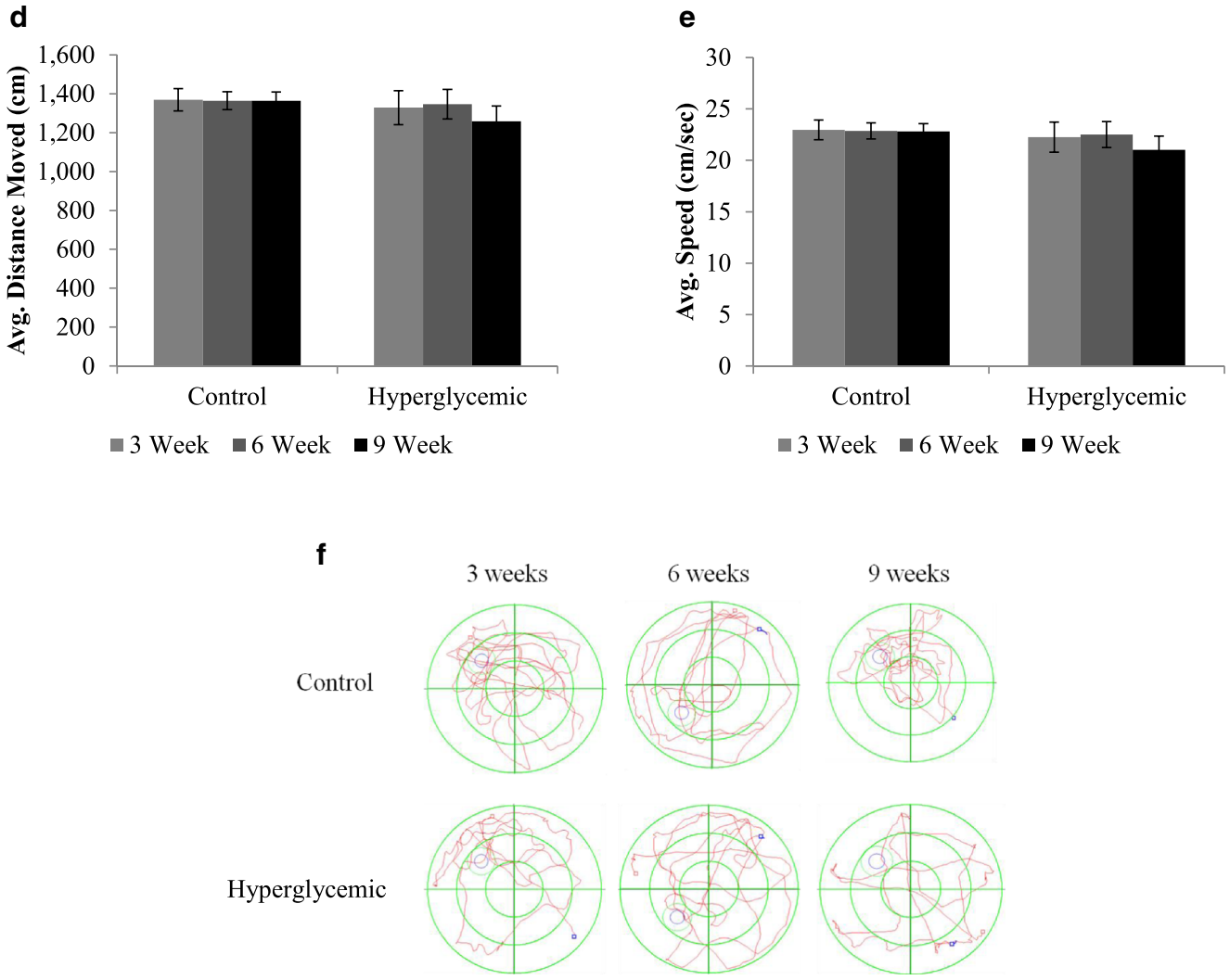

Fig. 4 (continued)

hippocampus (Revsin et al. 2009; Langston et al. 2010; Johnson et al. 2016). Memory will be considered intact when the rats showed preference to explore novel objects and retain the memory of familiarized object(s) (Ennaceur 2010). At each time point i.e. 3, 6 and 9 weeks the control rats showed intact memory as they spent more time to explore novel objects with respect to familiar one. On the contrary, the hyperglycemic rats demonstrated intact short-term memory after

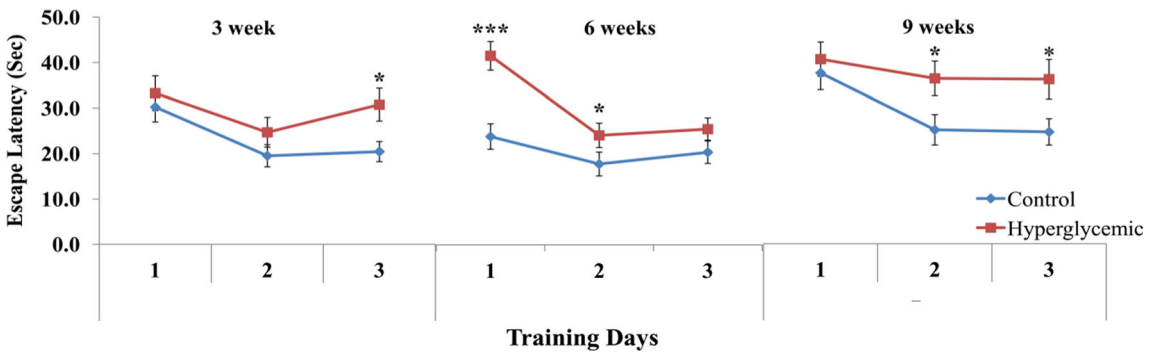

Fig. 5 Effect of STZ induced hyperglycemia duration (3, 6 and 9 weeks) on working memory impairment among rats in Morris water maze. Escape Latency were measured for three consecutive days of training with changing platform positions. Data are presented as mean ± SEM (n = 8-10rats in each group). $p < 0.05$*, <0.01**, <0.001*** as compared to their respective controls (One way ANOVA)

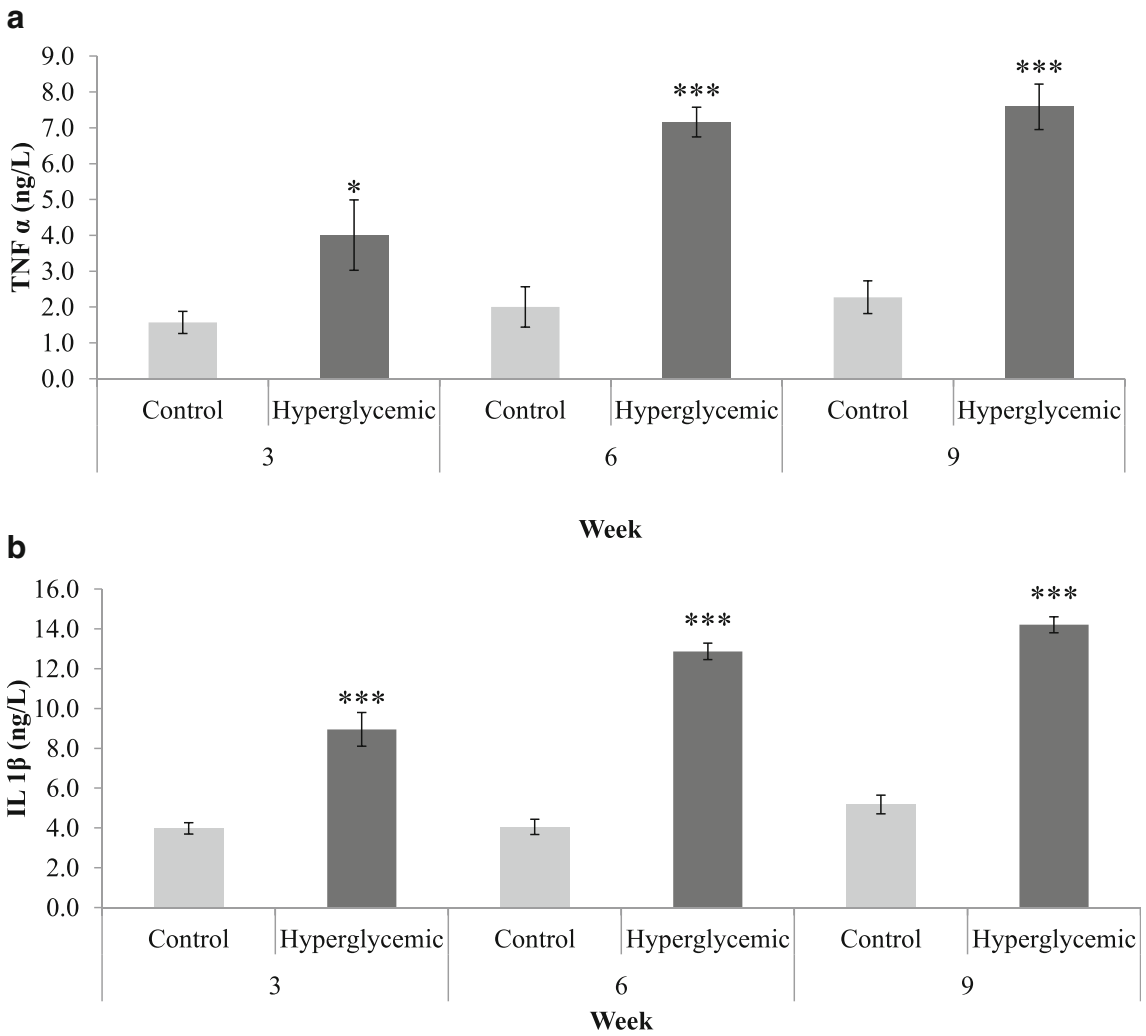

Fig. 6 Effects of hyperglycemia duration (3, 6 and 9 weeks) on inflammatory markers (TNF-α and IL 1β) in the hippocampus. **a** TNFα and (**b**) IL1β. Values are represented as mean ± S.E.M. t-test was used to determine significance ($p < 0.05$* and < 0.001***) between control and hyperglycemic rats

3 weeks of STZ induction, which was impaired with the passage of time (6 and 9 weeks) and correlated with increasing hyperglycemia. Moreover, in the case of familiarization phase the hyperglycemic rats showed approximately similar preference to both the similar objects. Our results displayed reduced preference to the novel object in the hyperglycemic rats after STZ-treatment 6 and 9 weeks followed reduced locomotor activities in the open field test. The influence of reduced locomotor activities was prevented by evaluating the percentage of preference of the novel object. Our results were in agreement with the previous study which showed reduced locomotor activities among hyperglycemic rats with a reduced preference towards novel objects (Revsin et al. 2009). Thus the memory impairment manifested STZ-treatment 6 and 9 weeks are positively correlated with the subsequent increase in hyperglycemia which might affect the NMDA expression, its function in central vagal circuitry contributed to persistently elevated glutamate release in the dorsal motor nucleus of the vagus nerve (DMV) and subsequent modulation of visceral function associated with systemic glucose dysregulation, a hallmark of diabetes (Warburton et al. 2013; Bach et al. 2015).

Learning and memory consolidation (long term memory) plays a crucial role in performing daily life functions and personal health care. It was reported that MWM was positively correlated with long-term memory of rodents (Jing et al. 2008; Johnson et al. 2016). The rodent's ability to navigate, learn or memorize cues is the basis of this test which makes it a popular behavioral paradigm to evaluate long term reference and working memory. Similar to NORT, the 3 weeks hyperglycemic rats showed intact memory as they spent almost similar time to learn the cues to reach the platform and remember the location as reflected by time spent in the target quadrant and platform crossing. With the passage of time and increase in hyperglycemia, rats showed memory impairment which was evident in STZ treatment 6 weeks rats and further learning deficit in STZ treatment 9 weeks rats, while, the

Table 2 Multiple regression analysis to compare cognitive tests (MWM and NORT) at 3, 6 and 9 weeks after adjusting for glucose, Insulin, IL1-β, TNF-α, and dopamine

Parameter	3 Weeks				6 Weeks				9 Weeks			
	MWM ($R^2 = 0.85$)		NORT ($R^2 = 0.91$)		MWM ($R^2 = 0.92$)		NORT ($R^2 = 0.90$)		MWM ($R^2 = 0.95$)		NORT ($R^2 = 0.93$)	
	β (95% CI)	*p* value	β (95% CI)	*p* value	β (95% CI)	*p* value	β (95% CI)	*p* value	β (95% CI)	*p* value	β (95% CI)	*p* value
Glucose	1.58 (−0.70; 3.85)	0.13	−1.54 (−45.91; 42.82)	0.93	−0.33 (−0.83; 0.17)	0.14	−3.48 (−13.90; 6.95)	0.41	0.08 (−1.02; 1.17)	0.86	−2.06 (−23.13; 19.01)	0.80
Insulin	0.94 (−0.77; 2.66)	0.2	−2.32 (−35.78; 31.13)	0.86	0.63 (−0.66; 1.91)	0.25	−5.42 (−32.30; 21.46)	0.61	−0.08 (−0.94; 0.78)	0.80	1.09 (−15.45; 17.62)	0.86
IL1-β	−0.43 (−2.26; 1.41)	0.55	1.97 (−33.87; 37.82)	0.89	0.32 (−0.34; 0.97)	0.25	−6.67 (−20.30; 6.96)	0.25	−0.38 (−3.13; 2.37)	0.01	−2.70 (−55.59; 50.19)	0.02
TNF-α	−0.71 (−2.44; 1.01)	0.31	−0.44 (−34.08; 33.20)	0.97	−0.07 (−1.05; 0.92)	0.04	7.93 (−12.61; 28.48)	0.03	0.29 (−1.33; 1.91)	0.001	5.38 (−25.74; 36.51)	0.002
Dopamine	−0.21 (−0.75; 0.32	0.33	−2.85 (−13.37; 7.66)	0.49	0.11 (−0.04; 0.26)	0.11	0.11 (−2.99; 3.21)	0.93	−0.26 (−0.85; 0.33)	0.29	3.11 (−14.44; 8.23)	0.49

Data is represented as mean differences (β), 95% confidence intervals (CI), and values from linear regression with MWM and NORT as dependant variable markers of memory decline with Glucose, Insulin, IL1-β, TNF-α and dopamine as independent variables

IL1-β Interleukin 1 Beta; *TNF-α* Tumor Necrosis Factor alpha; *MWM* Morris water maze; *NORT* Novel Object Recognition Test

control rats' learning and memory were intact at corresponding time points. The other two parameters such as swimming speed and distance covered by the hyperglycemic and the control rats displayed non-significant changes among 3, 6 and 9-week groups. Long term potentiation (LTP) was reported to be impaired after 8 weeks of hyperglycemia, which clearly correlates with the finding of this study i.e. after STZ treatment (70 mg/kg) 6 and 9 weeks memory retention was impaired in the hyperglycemic rats (Stranahan et al. 2008; Stranahan et al. 2010). The intact memory in the STZ treatment 3 weeks hyperglycemic rats is probably due to different factors including short duration of diabetes, insufficient hyperglycemia, and absence of inflammation, which were evident in the STZ treatment 6 and 9 weeks rats. A retrospective study on diabetic patients also displayed timely increments of blood glucose over time and improper glycemic control responsible for late-life cognitive complications (Ravona-Springer et al. 2014). It is well known that NMDA receptor and CREB activation is an integral part of long term memory and was not significantly reduced after 4 weeks of post hyperglycemic as several weeks or elevated hyperglycemia for changes to materialize at receptor level (Gardoni et al. 2002; Tian et al. 2016). This might be the most plausible reason for the intact memory and learning observed in the STZ treatment 3 weeks hyperglycemic rats. Whereas, studies showed learning and memory impairment in STZ treatment 4–5 weeks of diabetic rats but differs from this study in term of strain of rats, STZ dose used (60-65 mg/kg) and severe glycemia i.e. >11.1 mM as compared to 7.9 mM in this study (Alvarez et al. 2009; Ayoub 2009; Jayanarayanan et al. 2013; Tian et al. 2016). The memory impairments after 6 and 9 weeks might be due to the impaired consolidation process due to altered protein expression in the hippocampus which was reported to be deteriorated with the duration of diabetes, hyperglycemic state and age of animal (Kamal et al. 2000, 2012). Impaired long-term potentiation was reported after 8 weeks of STZ (70 mg/kg hhyperglycemia and consistently maintained after 12 weeks of diabetes (Stranahan et al. 2008, 2010). Neurogenesis was also reported to be altered after STZ treatment (60-70 mg/kg) 6–12 weeks of hyperglycemia (Alvarez et al. 2009; Wongchitrat et al. 2016; Choi et al. 2017).

The working memory trial evaluated in the MWM was used to further confirm the impairments in working memory where rats learned new clues by changing the platform position in each trial. Working memory was also considered as short-term memory as it maintains small parts of information through active rehearsal (Baddeley and Hitch 1974). In the escape latency, hyperglycemic rats showed impaired working memory and needed more time to learn new cues as compared to the control rats. After 3 weeks of STZ administration hyperglycemic rats also required more time to reach the platform which was inconsistent in each trial. At day 3, the hyperglycemic rats required significantly more time to reach the

platform as compared to day 1 and day 2. It displayed contradictory results with NORT which showed intact memory after 3 weeks. However, the working memory was impaired further after 6 and 9 weeks of STZ administration, which was similar to the effect observed in the NORT.

Thus the behavioral experiments clearly indicated that working memory was more susceptible to even slight hyperglycemic conditions as compared to reference memory. Thereby, with the passage of time and elevated hyperglycemia (11.1 mM), all three parameters such as learning, short- and long-term memory were affected.

Hyperglycemia has been shown to be associated with increased inflammatory mediators in hippocampus, possibly affecting learning and memory (Tsao et al. 2013; Wang et al. 2017). Our results also showed a significant increase in pro-inflammatory cytokines IL-1β and TNF-α in hyperglycemic rats. Under hyperglycemic condition accumulation of reactive oxygen species due to increased oxidative damage or malfunctioning of mitochondria also contributed to inflammatory conditions (Kuhad and Chopra 2008). The impaired working memory after moderate hyperglycemia i.e. 3 weeks is most likely due to a significant increase in pro-inflammatory cytokines as inflammation has been reported to delineate reference and working memory in various situations (Culley et al. 2014). Another study reported impaired working memory deficit among animal model with moderate inflammation whereas, their performance was normal in Y or T-maze tests (Murray et al. 2012). Therefore, working memory is highly sensitive to inflammation as it requires complex processing of information i.e. integrating new information with the previous one in a short passage of time (Sparkman et al. 2006; Chen et al. 2008).

Hyperglycemia and inflammation were also reported to affect levels of neurotransmitters in the brain. Interleukin-1 (IL-1) administration intracerebroventricularly (ICV) induced memory impairments with disturbed neurotransmitter levels such as dopamine (Lacosta et al. 1998; Song et al. 2008). Dopamine is among one of the important neurotransmitter responsible to modulate various cognitive domains including learning, reference or working memory (Small 2017; Edelmann and Lessmann 2018; Gutierrez et al. 2018). The dopamine receptors (D1 and D2) also play a crucial role in the activation of AKT and CREB (Dudman et al. 2003; Chen et al. 2012). Learning in the different behavioral task such as spatial navigation in MWM requires intact dopaminergic system and to promote further consolidation of memory by activating AKT and its downstream CREB protein (Gasbarri et al. 1996; Edelmann and Lessmann 2018). Our findings demonstrated reduced dopamine levels soon after the induction of hyperglycemia. This attenuation is positively correlated with the reduced activation of AKT and CREB (i.e. the decreased p-AKT/AKT and p-CREB/CREB). The activation of AKT and its downstream pathway involving CREB is critical for memory consolidation and retrieval. Learning and memory deficit among hyperglycemic rats after 6 and 9 weeks positively correlated with reduced dopaminergic system. This further affected associated downstream pathways in the hyperglycemic brains (Chu et al. 1986; Ezzeldin et al. 2014). Inflammatory markers especially TNF-α via JNK pathway has been reported to promote insulin resistance and hence further halt the downstream pathway i.e. activation of AKT and CREB (Verdile et al. 2015). Therefore, results suggest for the first time that a series of temporal changes in the overall expression of neurotransmitter (dopamine), inflammatory markers (IL-1β and TNF-α) at early stages of hyperglycemia may probably lead to alter protein expression of activated AKT and CREB influencing the learning and memory in the hyperglycemic brain. Other investigators determined the behavioral deficiencies among STZ (45-65 mg/kg) treated after 8 to 10 weeks of diabetic rats., where the

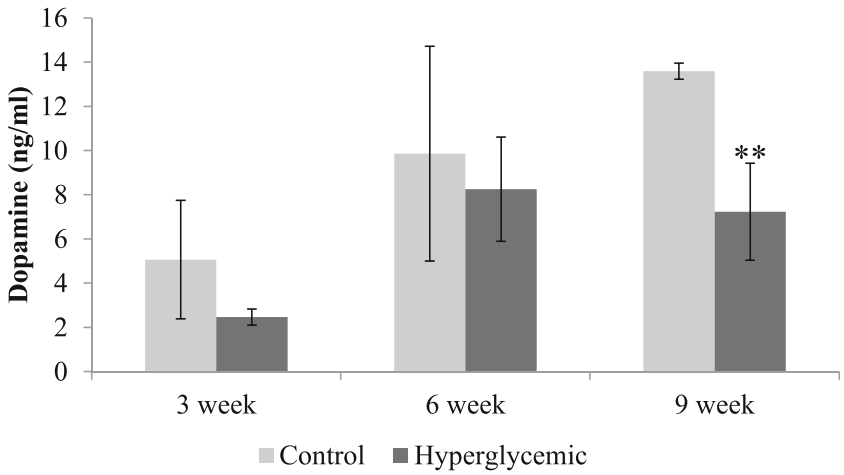

Fig. 7 Effects of hyperglycemia duration (3, 6, and 9 weeks) on dopamine level the cerebral spinal fluid in rats. Values are represented as mean ± S.E.M. t-test was used to evaluate statistical significance of data ($p < 0.01$**) among control and hyperglycemic rats

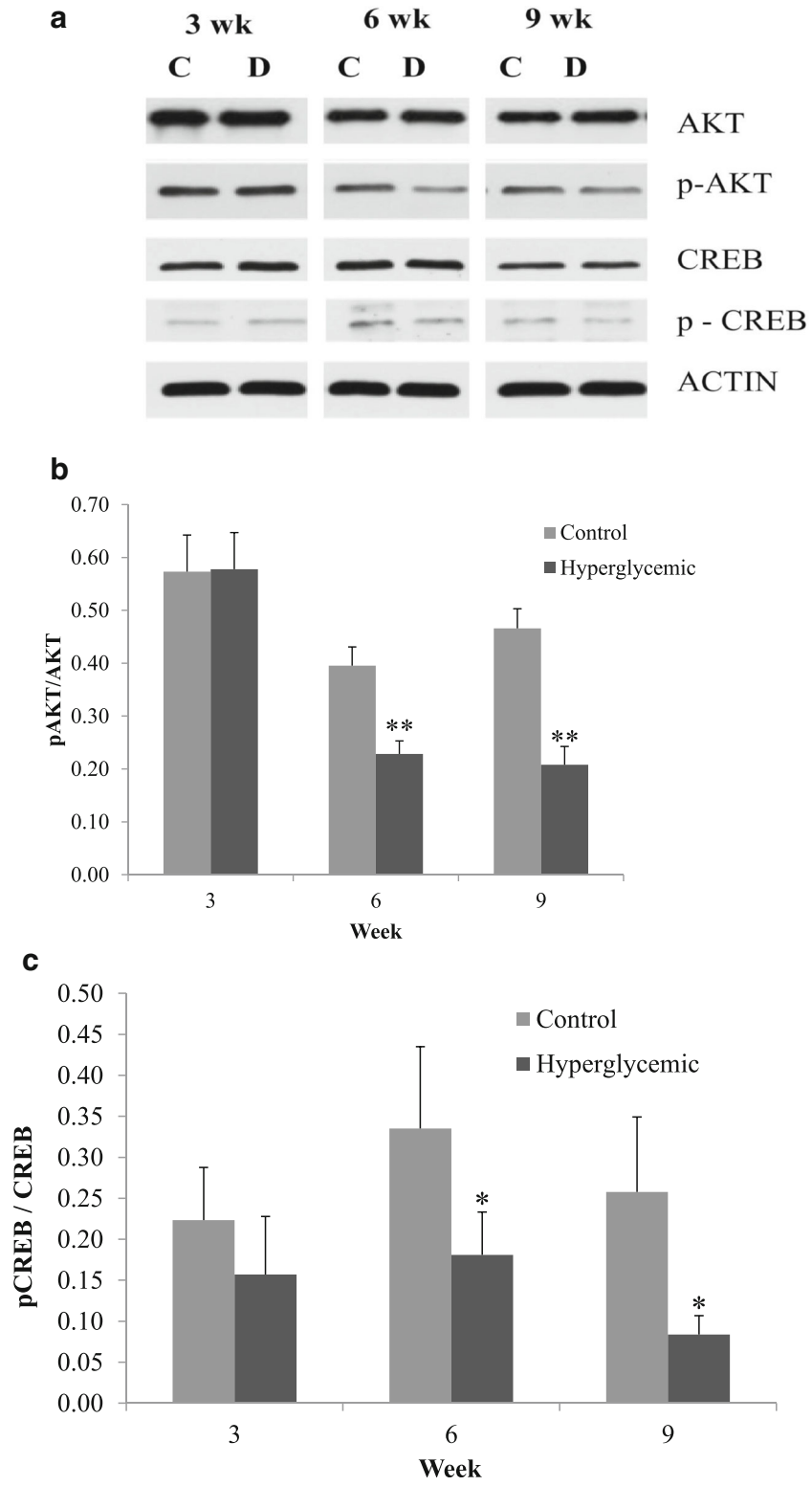

Fig. 8 Temporal expression of pCREB / CREB (cAMP Responsive Element Binding) and pAKT / AKT (Protein Kinase B) in the hippocampus of hyperglycemic brain. **a** Western Blot for pAKT / AKT, pCREB / CREB and actin at 3, 6 and 9 weeks (C = control; D = Diabetic). **b** Ration of pAKT / AKT. **c** Ration of pCREB / CREB. Values represented as mean ± SD. $p < 0.01$**; $p < 0.05$* as compared to their respective controls

expression of related genes, neurogenesis and impaired levels of neurotransmitters were addressed (Stranahan et al. 2008; Stranahan et al. 2010; Choi et al. 2017). However, temporal (3, 6 and 9 weeks of STZ-post-treatment) changes leading to behavioral loss, elevated pro-inflammatory mediators, reduced dopamine levels and its relevant signaling pathway (AKT and CREB) in hyperglycemic conditions have not been addressed.

In conclusion, STZ-induced hyperglycemia in rats adversely affects learning and memory processes in the brain in a progressive manner. This is the first study to highlight the type of memory impaired during the course of hyperglycemia and its correlation with inflammatory markers, dopamine, and activation of CREB and AKT. Working memory was found to be most vulnerable to hyperglycemia rats followed by impaired spatial memory and learning. Enhanced inflammation and reduced dopamine at each time point (3, 6 and 9 post STZ induction) possibly contributed to the reduced activation of AKT (p-AKT) that most likely influenced the activation of CREB (p-CREB) affecting cognition in the hyperglycemic rats.

Acknowledgements This work was supported by CAMS Initiatives for Innovation Medicine (2016-12 M-2-006), the National Key Research and Development Program of China (2016YFE0131800), the Joint Funds of the Southwest Medical University & Luzhou Municipal Government of China 2015LZCYD-S04 (7/15), Hunan Province Science and Technology Plan Projects of China (2015DK3010, the High - end Talents Recruitment Program (Chen Chen group) of Luzhou Municipal People's Government. We would like to thank Dr. Radhakrishnan Rajan, College of Medicine, MBR University, Dubai, UAE, for his kind help to revise the manuscript. We would also like to thank the editor and reviewers of this manuscript whose critical reviews help us to make the draft better.

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