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Etanercept Improves Cognitive Performance and Increases eNOS and BDNF Expression During Experimental Vascular Dementia in Streptozotocin-induced Diabetes

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Abstract: Diabetes mellitus (DM) is related to an increase in the incidence of vascular dementia. Inflammation is an important cause of endothelial dysfunction and cognitive deficits. The anti -tumor necrosis factor (TNF)-α fusion protein etanercept has been reported to exhibit memory-enhancing effects and endothelial protection. We tested the effect of etanercept on the cognitive endpoints and compared it with the cognitive dysfunction in streptozotocin (STZ)-induced DM rats by using the Morris water maze test (MWMT) and passive avoidance test (PAT). Systolic blood pressure (SBP), thoracic endothelial function, endothelial nitric oxide synthase (eNOS) expression, and hippocampal brain-derived neurotrophic factor (BDNF) expression were assessed. Thirty days after the induction of DM, rats exhibited severe learning and memory deficits associated with endothelial dysfunction and decreased expression of eNOS and BDNF. Chronic treatment with etanercept (0.8 mg/kg, s.c., every week for 30 days) improved cognitive performance, endothelial function, and expression of eNOS and BDNF in DM rats. Furthermore, the memory-improving effects of etanercept were independent of hyperglycemia. These data suggest that etanercept treatment prevents changes in endothelial function, eNOS expression, and hippocampal expression of BDNF and, consequently, vascular dementia in DM rats.

Keywords: Diabetes mellitus, learning, memory, water maze, passive avoidance, rat.

INTRODUCTION

It is well known that diabetes mellitus (DM) is accompanied by moderate impairments in cognitive functions. Furthermore, DM patients were shown to be at a high risk for affective disorders as well as dementia and Alzheimer' s disease (AD) [1-4]. Learning and memory deficits are also observed in streptozotocin (STZ)-induced DM rats, and these effects have been associated (at least in part) with the activation of inflammatory mechanisms [5-10]. There is strong evidence that vascular risk factors have a role in the development of AD or vascular dementia. DM is such a vascular risk factor [11]. Moreover, DM-induced endothelial dysfunction has been associated with vascular dementia (which is the second leading cause of dementia) [12]. In recent years, inflammation has been accepted as a prominent feature of diabetic vascular disease [13]. Like depression, based on various findings in DM models and animal models, the "cytokine hypothesis" may be formulated: diabetic vascular dementia may be result from an increased production of pro-inflammatory cytokines [14]. Indeed, proinflammatory cytokine levels have been found to be significantly increased in brain as well as blood of animal models of DM [15]. There is now evidence that pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, which has peripheral origins, can result in cytokine synthesis in the central nervous system (CNS) [16]. Thus, as reported by Kubera *et al.* [16] peripheral inflammation may induce neuroinflammation. The vascular endothelium has a prominent role in the maintenance of vascular homeostasis. Furthermore, it is involved in vasomotor function, vascular tone as well as the regulation of inflammation, platelet aggregation, and thrombosis [17]. Many of the functions exerted by the intact endothelium are actively mediated by Nitric oxide (NO) [18] and we previously showed that DM causes endothelial dysfunction [19-21]. Our previous studies also demonstrated that reduced NO bioavailability is the primary mechanism behind the endothelial dysfunction in STZ- induced DM [19, 21]. It is well known that DM is strongly correlated with poor vascular endothelial function. TNF-α is a proinflammotory cytokine that is involved in the pathogenesis of vascular dysfunction [22]. In endothelial cell cultures, TNF- α decreases the expression of endothelial nitric oxide synthase (eNOS), which is the primary enzyme involved in NO production in the vasculature [23]. Although there is a growing body of evidence to suggest that inflammation is a key feature in DM and cardiovascular disease

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[24] or in DM and dementia [25], reports focusing on the influence of the anti-TNF-α agents against DM- induced endothelial and cognitive dysfunctions are lacking. Therefore, the present study was designed to investigate the effects of long-term (30 days) etanercept treatment in DM rats on (i) spatial memory, (ii) emotional memory, (iii) blood pressure (BP), and (iv) vascular reactivity. We believed that these experiments may offer insights into how DM-induced endothelial dysfunction may contribute to cognitive impairment.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (Kocaeli University, Experimental Medical Research and Application Center, Kocaeli, Turkey) weighing 250- 300 g were used in the experiment. Rats were kept in an animal colony, (~5 to 6 per cage) for 2 weeks prior to beginning the experiments. Tap water and food pellets were provided ad libitum. All behavioral tests were conducted between 9:00 A.M. and 12:00 P.M. under standard laboratory conditions (22 ± 2°C room temperature; 12-h light/dark cycle with lights on at 7:00 A.M.).

All animals were naive to the experimental tests. Different rats were used in each experiment. The experiments reported in this study were conducted in accordance with the Regulation of Animal Research Ethics Committee in Turkey (July 6, 2006, Number 26220). Ethical approval was granted by the Kocaeli University Animal Research Ethics Committee (Project number: HADYEK 7/3, Kocaeli, Turkey).

Rats were divided into four equal groups of seven rats: two DM groups and two control groups. The grouping was as follows: control, control + etanercept, DM, and DM + etanercept.

Induction of DM and Treatment Schedule

DM was induced in rats by using our previously reported method with a minor modification [26]. In brief, STZ was dissolved in 0.1 M sodium citrate buffer (pH 4.4) and administered at 50 mg/kg via the intraperitoneal route. STZ-treated rats received 5% glucose solution instead of water for the next 24 h in order to reduce the death risk due to hypoglycemic shock. In order to measure glucose levels, blood samples were collected from the tail vein 48 h after STZ injection. Animals with fasting blood glucose levels >200 mg /dL were considered to have DM and used for further study. After confirmation of the induction of DM, separate groups of rats (n = 7) were administered etanercept (0.8 mg/kg, s.c) or vehicle (1 mL/kg, s.c.) every week for the next 30 days. Etanercept dose was selected on the basis of our previous reports [27, 28].

At the end of the treatment schedule, rats were tested in the locomotor activity test, footshock sensitivity test, Morris Water Maze Test (MWMT) or Passive Avoidance Test (PAT). Similar treatments were applied to the control (non-DM) rats. The learning and memory performances of these rats were evaluated during days 31– 35.

At the end of the treatment schedule, separate groups of rats (n = 7, each group) were subjected to systolic blood pressure (SBP) measurement. After SBP measurement, rats were decapitated and thoracic aortae removed for isolated organ-bath studies (which were evaluated during days 31–35).

Locomotor Activity and Footshock Sensitivity Test

The procedure for the footshock sensitivity test was designed as described by Schmatz *et al.* [29]. Shock reactivity was assessed in the same apparatus used for inhibitory avoidance. Briefly, the flinch and jump threshold was determined. Control, DM- and etanercept-treated rats were placed on a grid and were habituated for a 3-min period before initiating a series of shocks (1 s) delivered at 10-s intervals. Shock intensities ranged were 0.1–0.5 mA in 0.1- mA increments. Adjustments in shock intensity were made based on the observed response of each animal. Specifically, the shock intensity was increased by one unit if there was no response and it was lowered by one unit if there was a response. A "flinch" response was defined as the withdrawal of one paw from the grid floor, and a "jump" response was defined as the withdrawal of three or four paws. Three measurements of each threshold were made, and the mean of each score was calculated for each animal.

PAT

A one-trial, light-dark passive avoidance test (Ugo Basile model 7551, Italy) was conducted for the assessment of emotional memory based on contextual fear conditioning. [30]. In this test, the animals are trained to avoid a specific location that was associated with an aversive event. The reduction of latency to avoid was used as a learning index. The apparatus was composed of two compartments (22 cm x 21 cm x 22 cm). An illuminated white box was connected to a dark box that was had an electrifiable grid floor. An inescapable electrical shock could thus be delivered via a shock generator. The two boxes were separated by a flat- box partition. There was also an automatically operated sliding door at floor level. A training trial was carried out as described by Monleon and co-workers [31].

In the preacquisition trial (Day 1), rats were placed individually into the light compartment and they were allowed to explore the boxes. In this trial, the door between the two compartments was opened after 30 s and the animal was able to translocate freely into the dark compartment. The acquisition (training) trial was performed fifteen minutes after the preacquisition trial. In the training trial, rats were placed in the light compartment of the passive avoidance apparatus. Following the 30 s-long familiarization with the apparatus, the door between the compartments was opened. Upon the complete entrance of the animal to the dark compartment, the sliding door between the chambers was closed automatically and an electric foot-shock (0.5 mA) of 3-s duration was delivered. The time it took the rat to enter the dark compartment was recorded as the training latency. If the animal did not enter the dark compartment within 300 s, that animal was excluded from the experiment. Following the shock delivery, the animals were put back in their home cages. Both compartments of the box were cleaned thoroughly between each training session for removing any confounding olfactory cues.

The retention test trial was conducted, twenty-four hours after the acquisition trial. In the retention test, the rat was placed in the light compartment and their latency to enter the dark compartment (four paws in) was recorded and used in evaluating the recall of the shock stimulus. No foot shock was delivered during the retention test. If the animal did not enter the dark compartment within 300 s, a latency of 300 s was recorded for that animal.

MWMT

The Morris water maze performance was assessed in a water tank (150 cm in diameter) as previously described [32]. The rats underwent three trials over five daily sessions. The hidden platform (submerged 1.5 cm below the surface of water) was placed in the center of the southwest quadrant during the first four days of testing. Small black pieces of plastic covered the water surface, which made the platform invisible to the rats. The platform position remained unchanged over 4 days, and the latency to find the platform was assessed to quantify spatial learning. A trial was initiated by placing a rat into the pool at one of the three starting positions, facing the wall of the tank. Each starting position (north, east, and west) was used once in a series of three randomly ordered trials. Each trial was terminated as soon as the rat had climbed onto the escape platform or at the end of 60 s. A rat was taken from the platform after 20 s, after which the next trial was started. If a rat could not locate the platform within 60 s, they were placed on the platform by the experimenter and were allowed to stay on it for 20 s. After completing the 3rd trial, rats were dried with a towel, kept warm for an hour, and returned to their home cage.

A 'probe trial' was conducted on Day 5 to evaluate the rat's spatial retention of the location of the hidden platform twenty-four hours after the last acquisition session. The platform was not present during the probe test and the rat was allowed to search the pool for the platform for 60 s before being removed. Animals that have learned the task were expected to spend more time searching in the quadrant that previously contained the hidden platform than in the other three quadrants.

SBP Measurement

At the end of the 30 day period of DM, SBP was recorded using the tail cuff method. Indirect SBP and heart rate (HR) were recorded weekly by using tail-cuff plethysmography (MAY-COM BPHR 200; Commat Iletisim, Ankara, Turkey). To apply this procedure, conscious rats were placed in a restraining holder and their tails protruded from this holder. Local warming of the tail with an infrared light bulb was used for vasodilatation. A cuff and pulse sensor were placed around the tail, and the cuff was inflated until the pulse disappeared. When the cuff was deflated, the point of reappearance of the pulse was used as the value of SBP. The average value of BP and HR in each rat was obtained from three sequential inflation–deflation cycles of the cuff.

Organ-bath Studies

After SBP measurement, rats were killed, and the thoracic aorta from the aortic arch to the diaphragm excised. Vessels were placed in Kreb's solution (see below for com position). They were dissected carefully and rings were prepared. The rings were transferred to 20-mL organ baths containing Kreb's solution that was maintained at 37 °C by using a thermoregulated water circuit and aerated continuously with 95% O2 and 5% CO2. The pH of the solution was 7.4. Tissues were allowed to equilibrate for 60 min before experimentation.

During this period, the bath fluid was changed every 15 min. Resting tension was set at 1 g by repeated adjustments and remained unchanged throughout the experiment. Each ring was connected to a force-displacement transducer (MAY-COM FDT 10A; Commat Iletisim) for the measurement of isometric force, which was displaced continuously and recorded online on a personal computer by using a four-channel transducer data-acquisition system (MP30B-CE; Biopac Systems, Santa Barbara, CA, USA), using software (BSL Pro 3.7; Biopac Systems) that could analyze the data.

Agonist-induced contractions:

In order to test the viability of the preparation, the tissues were exposed to 80 mM KCl for 5 min. Tissues were then washed, and the contractile responses to phenylephrine (10–9–10–4 M) obtained cumulatively.

Agonist-induced relaxations:

Each aortic ring was contracted by treatment with 3× 10–6–1 × 10–5 M phenylephrine. These concentrations resulted in 85–87% of the maximal response to phenylephrine. After the phenylephrine-induced contraction had reached a plateau, the concentration response relationships for carbachol (10–8 10–5 M), sodium nitroprusside (SNP; 10–8–10–4 M), or papaverine (10–5–10– 4 M) were obtained by addition of one of these agents to the bath in a cumulative fashion. The agonist concentration in the bath was incremented approximately threefold at each step after the response to the previous dose had reached a plateau. Between successive concentration response curves, tissues were rinsed with fresh buffer and they wer allowed to recover for 30 min. During this period, tension returned to basal levels.

Evaluation of Blood Glucose Levels and Body Weight

At the end of the experiment, all rats were weighed and plasma glucose levels were determined. Blood glucose levels were measured with a portable glucometer (Optimum Xceed, diabetes monitoring system, Abbott, UK). In brief, blood was withdrawn from the tail vein. A drop of blood placed on the glucometer strip was loaded in the glucometer for determination of blood glucose level.

Drugs and Solutions

STZ was purchased from Sigma–Aldrich (Saint Louis, MO, USA) and dissolved in citrate buffer (pH 4.4). Etanercept was purchased from Wyeth (Münster, Germany) and it was dissolved in physiologic (0.9%) saline. The ionic composition of Krebs solution (mM) was as follows: NaCl 118, KCl 4.71, MgCl2 1.05, NaH2PO4 1.33, NaHCO3 25, CaCl2 2.7, and glucose 5.6. In the high K+ solution, NaCl was exchanged for equimolar amounts of KCl. Fresh solutions were prepared fresh on the day of testing. Phenylephrine hydrochloride, carbachol chloride, SNP and

Table 1. Body weights, blood glucose and systolic BP of rats in control, DM and etanercept-treated groups.

	Body Weight (g)	Blood Glucose (mg/dl)	SBP (mmHg)
Control (n=7)	362.3±10.9	115.7±6.1	102.2 ± 1.9
Control+Etanercept (n=7)	375.1±6.5	112.9±5.8	104.8 ± 2.1
DM (n=7)	278 ± 9.6*	424.4±15.6*	105.1 ± 2.4
DM+Etanercept (n=7)	296.3 ± 5.6*	437.1±17.4*	103.1 ± 1.6

Values are arithmetic means ±S.E.M., n= number of animals used. * p< 0.05, statistically different from control rats.

papaverine hydrochloride were used (all obtained from Sigma Aldrich). Drugs were prepared fresh every day in distilled water and kept on ice during experiments.

Immunohystochemical Analyses for eNOS and Brain-Derived Neurotrophic Factor (BDNF)

After blood pressure measurements rats were sacrified by decapitation and thoracic aorta tissues were collected from rats and they fixed with 10% neutral buffered formalin. Routine histological tissue procedure was performed and the tissues were embedded in paraffin. Paraffin- embedded sections of 3 μm thickness were taken to poly-L-Lysine slides. Sections were deparaffinized with xylene, hydrated with descending series of ethanol, and they were washed with phosphate-buffered saline (PBS). The tissue sections were then placed in 1 mM citrate buffer (pH:6.0) and antigen retrieval was performed in a microwave oven and incubated with 3% H2O2 in methanol to reduce endogenous peroxidase activity. Without washing PBS, sections were incubated overnight with polyclonal primary antibody against eNOS (sc654, Santa Cruz, California USA). Sections were incubated with secondary biotinylated antibody, streptavidin-peroxidase, and diaminobenzidine (DAB) solution. The slides were mounted after sections were counterstained with hematoxylin.

After behavioral tests rats were decapitated and the brain structures were removed and dissected into hippocampus for the immunohistochemical studies. Paraffin sections were prepared from the rat brains fixed with 10% neutral buffered formalin. Sections were deparaffinized in xylene, rehydrated through a graded alcohol series, and washed with PBS. Next, an antigen retrieval procedure was performed by treating the samples in 10 mM citrate buffer (pH 6.0) in a microwave oven at 600 W for 5 min two times. The samples were allowed to cool for 20 min at room temperature and incubated in 3% H2O2 for 15 min. Sections were incubated in a blocking serum (Histostain-Plus Kit, Broad Spectrum, Invitrogen, Carlsbad, CA, USA) for 10 min at room temperature to block nonspecific binding. The primary rabbit polyclonal anti-BDNF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied overnight at a 1:100 dilution at room temperature. Negative control samples were prepared by replacing the primary antibody with the antibody diluent solution (Ab-diluent reagent solution, Invitrogen, Carlsbad, CA, USA) at the same concentration. After several washes, the slides were incubated with a biotinylated secondary antibody (Histostain-Plus Kit, Broad Spectrum, Invitrogen, Carlsbad, CA, USA) for 20 min at room temperature, and DAB (DAB Substrate Kit, Invitrogen, Carlsbad, CA, USA) was applied for visualization. Sections were briefly counterstained with Mayer's hematoxylin (Invitrogen, Carlsbad, CA, USA) and mounted with ClearMount (Invitrogen, Carlsbad, CA, USA) on glass slides. Both aorta and brain slides were examined under a light microscope (Olympus BX 50, Tokyo, Japan). The photomicrographs were taken with a Leica DM 100 system (Leica DFC 290HD, Wetzlar, Hessen, Germany). All samples were treated following the same protocol.

One independent observer who was blind to the current study graded the staining intensity based on a semiquantitative scale ranging from no expression (0) to very weak (1+), moderate (2+), strong (3+) and very strong (4+) expression. The percentage of positive cells was defined as 0, < 5%; 1, 6–15%; 2, 16–50%; 3, 51-80%; and 4, > 80% positive cell.

Statistical Analyses

Data are the mean ± Standard error of the mean (SEM). Acquisition (1–4 day) latency scores in the MWMT were measured by two-way analyses of variance (ANOVA). The following were measured by one-way ANOVA: scores of the time spent in the escape-platform quadrant in the MWMT; first-day and retention latencies in PAT scores; total locomotor activity scores; footshock sensitivity scores. Further statistical analyses for individual groups were carried out using the Bonferroni test.

In isolated organ-bath experiments, contractile force is expressed as milligrams of developed tension. The relaxant effects of agonists are expressed as the percentage of the precontraction to phenylephrine. Concentration–response curves were fitted by nonlinear regression with the simplex algorithm and maximum responses (Em) and pD2 (–log EC50) calculated using the software of the transducer data-acquisition system. Briefly, the cumulative concentration–response curve data were fitted as described previously to a four- parameter logistic equation: $E = Em/1 + (EC50/[D]n)$, where E denotes the observed effect in grams of tension, [D] denotes the concentration of agonist, Em denotes the calculated maximal effect, EC50 denotes the [D] at 0. 5 Em, and n is the slope factor parameter. The significance was conducted with one-way ANOVA followed by a post hoc Tukey–Kramer test. The immunoreactivity scores were compared by the Kruskal–Wallis test following Dunn's multiple comparison test; $p < 0.05$ was considered significant.

RESULTS

Effects of DM and Systemic Administration of Etanercept on Animal Weight and Blood Glucose Levels

Plasma levels of glucose in DM and etanercept + DM groups were significantly higher than those in control and etanercept + control groups ($p < 0.01$) (Table **1**). Body weight (g) for the control group was 362.3±10.9, whereas it

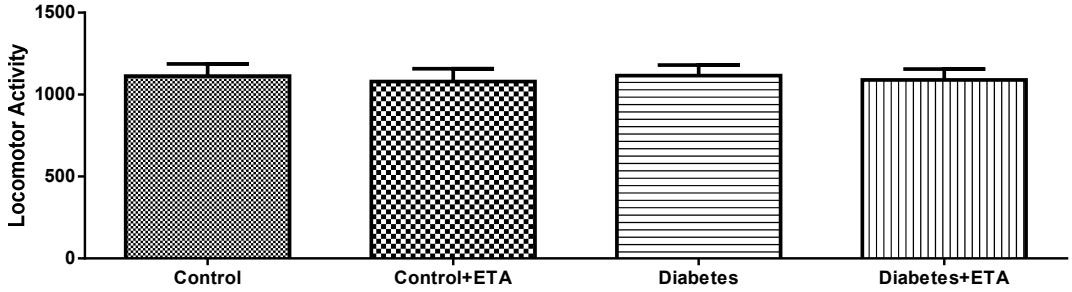

Fig. (1). Total locomotor activity in the activity cage separately for the non-DM control, control + etanercept 0.8 mg/kg, Diabetes, and Diabetes + etanercept 0.8 mg/kg. Each value represents the mean ± SEM. There were no significant differences between the groups. There were seven animals in each group.

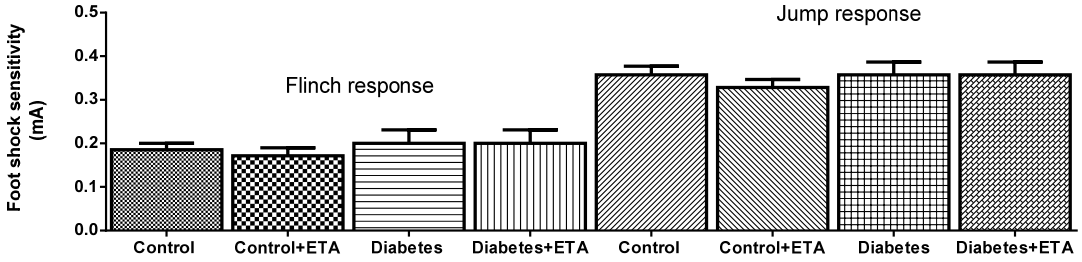

Fig. (2). Footshock sensitivity scores separately for non-DM control, control + etanercept 0.8 mg/kg, Diabetes, and Diabetes + etanercept 0.8 mg/kg groups. Each value represents the mean ± SEM. There were no significant differences between the groups. There were seven animals in each group.

was 375.1±6.5 for the control + etanercept group, 278 ± 9.6 for the DM group, and 296.3 ± 5.6 for the DM + 0.8 mg/kg etanercept group. The average body weight after 30 days of DM was significantly lower than that in control animals [$F(3,27) = 19.23$, one-way ANOVA, Bonferroni test, $p < 0.0001$] but was not significantly different from that of etanercept-treated DM rats ($p > 0.05$). Etanercept did not show any significant effect per se on the serum glucose level and body weight of rats (Table **1**).

Effects of DM and Systemic Administration of Etanercept on Locomotor Activity and Footshock Sensitivity

DM and etanercept treatment did not affect total locomotor activity ($F(3,27) = 0.06311$, $p = 0.9652$, one-way ANOVA, Bonferroni test; Fig. **1**). Moreover, DM did not alter footshock sensitivity, as demonstrated by the similar flinch ($F(3,24) = 0.3056$, $p = 0.8211$, one-way ANOVA, Bonferroni test;) and jump ($F(3,24) = 0.3243$, $p = 0.8077$, one-way ANOVA, Bonferroni test) thresholds exhibited by the animals (Fig. **2**). This finding suggested that neither DM nor etanercept treatment caused gross motor disabilities upon testing.

Effects of Systemic Administration of Etanercept on DM-induced Memory Impairment in the PAT in Rats

There were no significant differences between any groups ($F(3,27) = 0.7183$, $p = 0.5508$, one-way ANOVA, Bonferroni test; Fig. **3a**), during the training session of the light-dark type passive avoidance task, However, there was a significant difference between groups in the retention test ($F(3,27) = 26,91$, $p < 0.0001$, one-way ANOVA Bonferroni test; Fig. **3b**). DM rats had a significantly shorter latency compared with control rats during the retention test conducted 24 h after the training ($p < 0.001$; one-way ANOVA, Bonferroni test; Fig. **3b**). The reduced retention latency suggested impaired retention in the passive avoidance task. The effect of DM was reversed by administration of 0.8 mg/kg of etanercept ($p > 0.05$, *vs.* control; one-way ANOVA, Bonferroni test; Fig. **3b**), though 0.8 mg/kg etanercept had no effect on the memory of control rats ($p > 0.05$ *vs* control; one -way ANOVA, Bonferroni test; Fig. **3b**).

Effects of Systemic Administration of Etanercept on DM-induced Memory Impairment in the MWMT in Rats

We found that inducing DM in rats for 4 weeks resulted in performance deficits in the tasks in the MWMT. The day had an extremely significant effect in the dataset (two-way ANOVA, Bonferroni test, effect of day, $F(3, 96) = 9.31$, $p < 0.0001$; Fig. **4a**). In addition, an extremely significant effect of treatment was demonstrated (two-way ANOVA, Bonferroni test, effect of treatment, $F(3, 96) = 36.26$, $p < 0.0001$). Further analyses revealed that the day × treatment interaction was not significant (two-way ANOVA, day × treatment, $F(9,96) = 0.36$, $p = 0.9522$). Post hoc comparison showed that DM caused a significant disruption in learning and memory as indicated by the increase in the escape latency compared with control animals (two-way ANOVA, Bonferroni test, effect of treatment, $p < 0.01$, $p < 0.001$, $p < 0.01$, $p < 0.001$, respectively, Fig. **4a**). The Bonferroni test suggested that etanercept (0.8 mg/kg/day, s.c.), administered during DM prevented DM-induced impairment of escape latency in the task of the MWMT (two-way ANOVA, effect

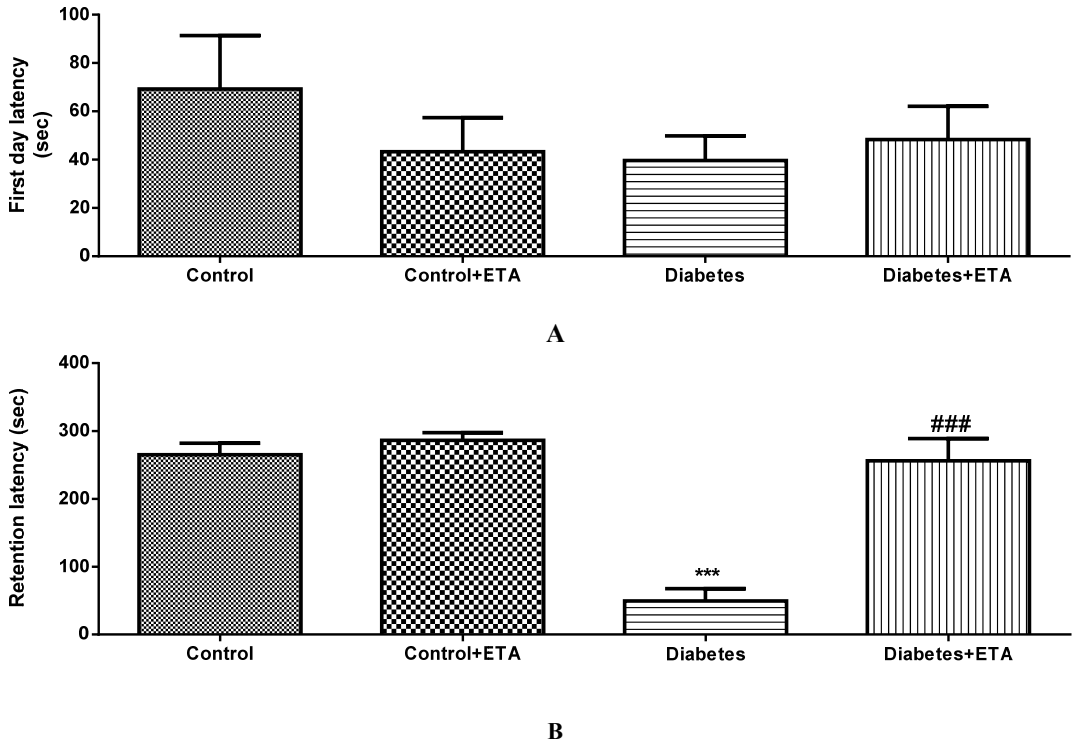

Fig. (3). a) Acquisition and b)Retention latencies in Passive Avoidance Test separetely non- DM control, control + etanercept0.8 mg/kg, Diabetes, and Diabetes + etanercept 0.8 mg/kg groups. Each value represents the mean ± SEM. There were no significant differences between the groups in the acquisition test. On the other hand, DM rats exhibited a significantly lower latency compared to control rats (*** $p < 0.001$), and DM + etanercept group showed higher latency compared to DM rats (### $p < 0.001$) in the retention test (One way ANOVA, Bonferroni corrected. There were seven animals in each group.

of treatment, $p < 0.05$, $p < 0.01$, $p < 0.05$, $p < 0.01$, respectively). In addition, etanercept treatment had a significant effect on acquisition latency in the MWMT in the first 1–4 days compared with saline-treated control rats ($p > 0.05$).

There was a significant difference between DM and control groups in terms of the time spent in the correct quadrant during the probe trial of the MWMT (one-way ANOVA, Bonferroni test, $F(3,27) = 9.619$, $p = 0.0021$; Fig. **4b**). However, 0.8 mg/kg etanercept increased the time spent in the escape-platform quadrant, suggesting that etanercept administration reversed the reduction in the time spent in the escape-platform quadrant by DM rats in a dose-dependent manner. Post hoc comparisons also showed that 0.8 mg/kg etanercept had no effect on the time spent in the correct quadrant in the MWMT compared with controls ($p > 0.05$, Bonferroni's test).

Effects of DM and Systemic Administration of Etanercept on Expression of BDNF Protein

In the CA1 region of the hippocampus, levels of BDNF protein were decreased significantly by DM ($p < 0.05$, Kruskal–Wallis test after Dunn's multiple comparison test; Fig. **5**), whereas treatment with 0.8 mg/kg etanercept significantly increased levels of BDNF protein in the CA1 of the hippocampus in DM rats ($p < 0.05$, Kruskal–Wallis test after Dunn's multiple comparison test; Fig. **5**). In DM rats receiving 0.8 mg/kg etanercept, levels of BDNF protein were similar to those in the control group. In the CA3 region of the hippocampus, levels of BDNF protein were significantly lower in the DM group compared to the control group ($p < 0.05$, Kruskal–Wallis test after Dunn's multiple comparison test; Fig. **5**). In the 0.8 mg/kg etanercept group, levels of BDNF protein were significantly increased compared with those in the DM group ($p < 0.05$, Kruskal–Wallis test after Dunn's multiple comparison test; Fig. **5**; Table **2**).

Effects of DM and Systemic Administration of Etanercept on SBP and Vascular Reactivity

No significant change was observed in the SBP of animals (control = 102.2 ± 1.9 mmHg; control + etanercept = 104.8 ± 2.1; DM = 105.1 ± 2.4 mmHg; DM + etanercept = 103.1 ± 1.6 mmHg; $n = 7$ for each group; $p > 0.05$) (Table **1**). The contractile response to 80 mM KCl did not change in all groups. There were no significant difference between the maximum responses of rings of the etanercept-treated DM, DM, non-DM control, or etanercept-treated control rats ($p > 0.05$) (Table **3**). The ability of carbachol or SNP to relax arteries precontracted with a submaximal concentration of phenylephrine was determined. The precontractile tone was similar in all groups (Table **3**). In thoracic aortic rings precontracted with phenylephrine at submaximal concentrations (3×10-6– 1×10–5 M), carbachol (10–8–10–5 M) evoked concentration-dependent relaxations. The endothelium-dependent relaxation to carbachol was decreased significantly in the DM group compared with that in the control, etanercept-treated control, and etanercept treated- DM

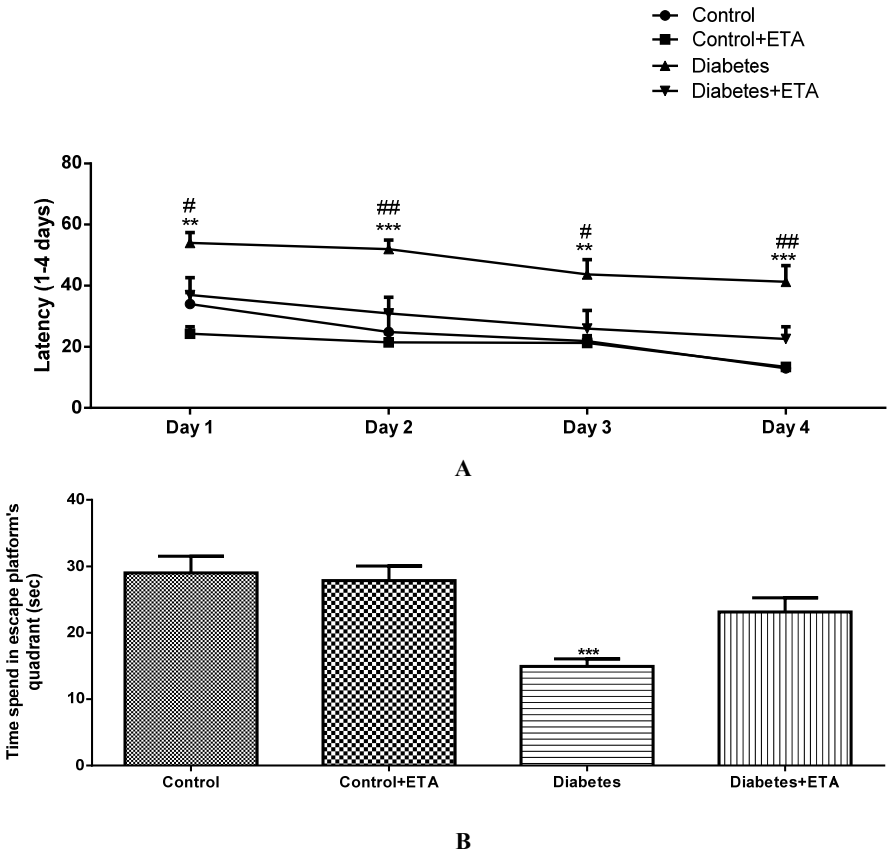

Fig. (4). Morris Water Maze a) Acquisition and b) Probe test performance separately the non-DM control, control + etanercept 0.8 mg/kg, Diabetes, and Diabetes + etanercept 0.8 mg/kg groups. DM resulted in a significant disruption in learning as indicated by an increase in the escape latency compared with the control animals in the acquisition test (two-way ANOVA, Bonferroni test, effect of treatment, **p < 0. 01, ***p < 0.001, **p < 0.01, ***p < 0.001, respectively). In addition, etanercept treatment had a significant effect on acquisition latency in the MWMT in the first 1–4 days compared to DM rats (#p < 0.05, ## p < 0.01, #p < 0.05, ## p < 0.01, respectively). There was a significant difference between DM and control groups in the time spent in the correct quadrant during the probe trial of the MWMT (one-way ANOVA, Bonferroni test, F(3,27) = 9.619, p = 0.0021). Etanercept prolonged the time spent in the correct quadrant.There were seven animals in each group.

Table 2. Semi-quantitative distribution of BDNF-immunoreactive neurons of CA1 and CA3 region of hippocampus and eNOS-immunoreactivity of aorta in rats.

		CA1			CA3			Aorta	
Animals	**Control**	**DM**	**DM+ETA**	**Control**	**DM**	**DM+ETA**	**Control**	**DM**	**DM+ETA**
1	2+	1+	2+	3+	0	2+	3+	1+	3+
2	2+	1+	3+	2+	1+	2+	1+	0	2+
3	3+	2+	2+	2+	2+	2+	3+	2+	2+
4	3+	1+	2+	3+	1+	3+	2+	1+	2+
5	2+	0	1+	3+	0	1+	3+	1+	1+
6	3+	2+	2	2+	1+	3+	2+	1+	2+
7	2+	0	1+	1+	1+	1+	2+	0	2+

The staining intensity was classified as no expression (0), very (1+), moderate (2+), strong (3+), to very strong (4+) expression. The immunopositivity was decreased in DM group compared to the control group (p<0.01, Kruskal-Wallis test). In the etanercept-treated group, both BDNF and eNOS immunoreactivity was similar to that of the control group.

Table 3. E_{max} (% of 10^{-6} M Phenylephrine) values for carbachol, sodium nitroprusside, and papaverine, E_{max} values (g) for 80 mM KCl and pD_2 values (-log EC_{50}) for carbachol, and sodium nitroprusside in rings of thoracic aorta obtained from the all groups of rats.

	Control (n=7)	DM (n=7)	DM+Etanercept (n=7)
Carbachol			
E_{max} (mg)	53.4±2.6	24.8±3.0*	47.7±3.4
pD_2 (-logEC_{50})	6.82±0.06	6.59±0.04	7.09±0.03
SNP			
E_{max} (mg)	100±0	100±0	100±0
pD_2 (-logEC_{50})	6.47±0.02	6.35±0.03	6.40±0.04
KCl			
E_{max} (g)	0.73±0.03	0.79±0.06	0.69±0.07
Papaverine			
E_{max} (mg)	100±0	100±0	100±0

Each ring was obtained from different rats. Values are arithmetic means±S.E.M. n= the number of thoracic aortic rings used. * $p<0.05$, statistically different from all other groups.

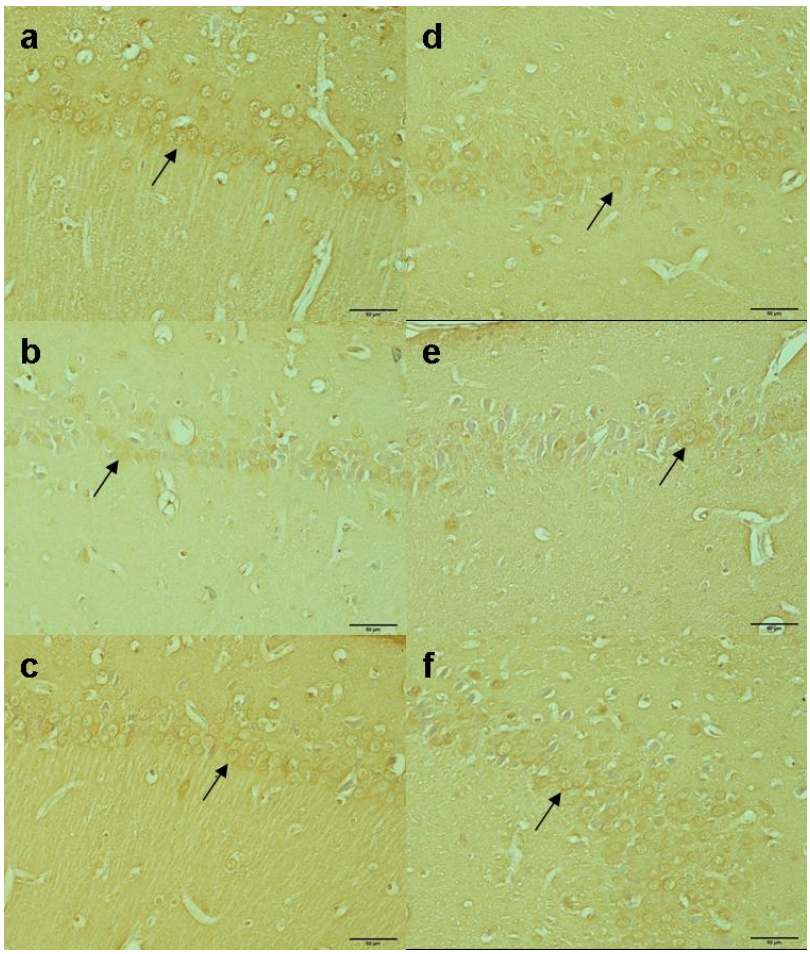

Fig. (5). Representative image illustrating BDNF expression (arrows) in hippocampal formation. Control (**a**), Diabetes (**b**) and Diabetes+etanercept (**c**) group in CA1 region, and control (**d**), Diabetes (**e**) and Diabetes+etanercept (**f**) group in CA3 region. BDNF expression was decreased in Diabetes in the both CA1 and CA3 regions of the hippocampus, whereas In Diabetes+ etanercept rats BDNF expression was similar to those in the control group in the both regions. Scale bars: 50 μm.

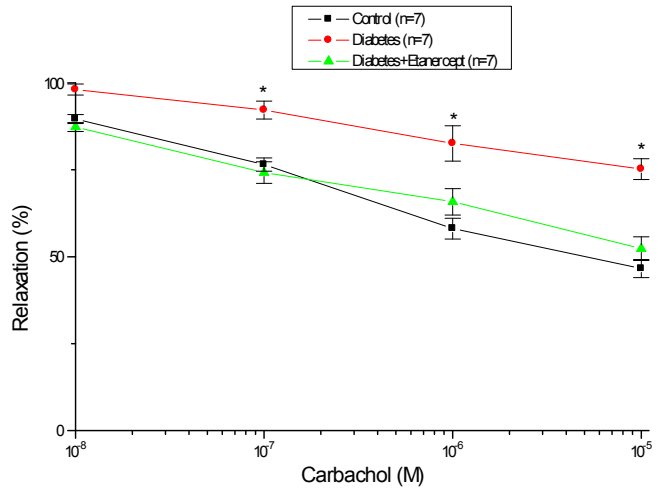

Fig. (6). Carbachol concentration–response curves in isolated thoracic aortic rings precontracted with phenylephrine (3 × 10–6–1 × 10–5 M). The concentration– response curve for carbachol was shifted to the right with significantly lower values of Em and pD2 in the DM group than in controls (*p < 0.05).
Impairment of relaxation in the etanercept treatment group was returned to that seen in the controls. Each point is expressed as a percentage of the contraction induced by phenylephrine and is represented as the mean ± SEM. The values in parentheses indicate the number of preparations used; *p < 0.05, different from the response of tissue rings from non-DM rats.

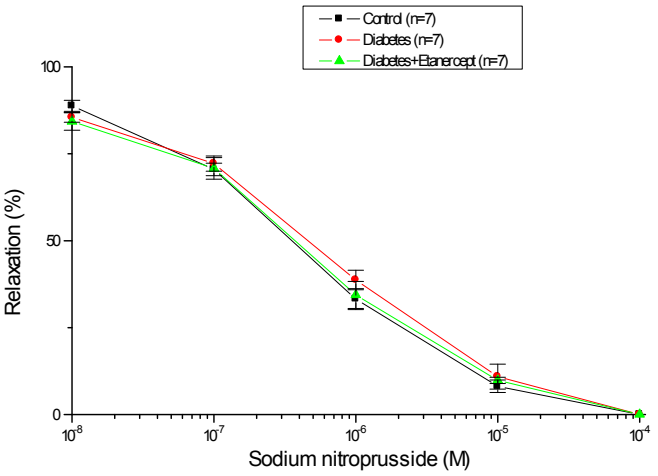

Fig. (7). Sodium nitroprusside concentration–response curves in isolated thoracic aortic rings precontracted with phenylephrine (3 × 10–6–1 × 10–5 M). The relaxation response to SNP was similar among vascular tissues from all groups. Each point is expressed as a percentage of the contraction induced by phenylephrine and is expressed as the mean ± SEM. The values in parentheses indicate the number of preparations used.

groups (p > 0.05, Fig. **6**). The concentration–response curve for carbachol was shifted to the right with significantly lower values of Em and pD2 in the DM group than in controls (p < 0.05, Table **3**, Fig. **6**). Impairment of relaxation in the etanercept treatment group was returned to that seen in controls. There was no significant difference in values of pD2 and Em between control and etanercept treatment groups (p > 0.05, Fig. **6**, Table **3**).

In aortic rings precontracted with phenylephrine, the NO donor SNP (10–8–10– 4 M) caused concentration-dependent

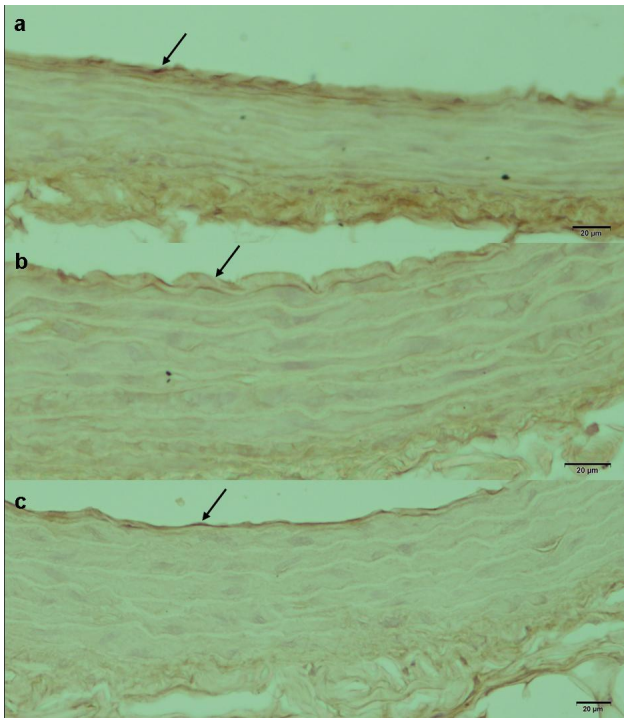

Fig. (8). Representative light microscopy of control, DM and etanercept-treated DM groups in the endothelium of the aorta. Decreased eNOS (arrows) (b) immunoreactivity in the aorta in DM rats compared to controls (a) and increased eNOS (c) immunoreactivity in endothelial tissue in the etanercept-treated DM group compared to DM groups (b). Scale bars: 20 µm.

relaxation (Fig. **7**), but there were no significant differences in values of Em and pD2 among the groups (Table **3**). In addition, the relaxation response to papaverine was similar among vascular tissues from all groups (Table **3**).

Effects of DM and Systemic Administration of Etanercept on Expression of eNOS Protein

In the control group, eNOS immunoreactivity was detected in the cytoplasm of the endothelial cells of the aorta (Fig. **8A**). Immunopositivity decreased in the DM group compared with that in controls (p < 0.01, Kruskal–Wallis test after Dunn's multiple comparison test; Fig. **8B**). In the etanercept-treated DM group, eNOS immunoreactivity was similar to that of the control group (Fig. **8C**; Table **2**).

DISCUSSION

Our study demonstrated that etanercept treatment can reverse the impaired cognition induced by DM in a rat model. In addition to the maintenance of cognitive function, reversal of endothelial disruption was also seen in rats treated with etanercept. DM was induced by a single intraperitoneal injection of STZ. STZ-induced DM is a widely used model of type-I DM, which is characterized by hyperglycemia and weight loss. This model has been used extensively to assess cognition deficits in rats [33].

The present study elicited five major findings. First, data from the present study and those from previous studies suggest that DM decreases eNOS expression, thereby decreasing

NO production [34, 35]. It has been reported that increased TNF-α levels are associated with significant impairment of endothelium-dependent relaxations in DM [22]. Evidence suggest that TNF-α impairs endothelium-dependent and NO-mediated vasodilation in various vascular beds [36, 37].

In accordance with those studies, STZ in our investigation induced significant endothelial dysfunction as reflected by the impairment of endothelium–dependent (but not endothelium-independent) relaxation and reduction in eNOS expression. STZ treatment has been documented to enhance the production of proinflammatory cytokines, eventually leading to inflammation in the periphery and CNS [15]. Authors have reported that STZ-induced increases in the levels of TNF-α and IL-1β in plasma denote inflammation, and are probably major contributing factors in STZ-induced endothelial dysfunction [15]. In accordance with previous studies, we found that neutralization of TNF-α by chronic treatment with etanercept improved endothelial function in diabetic arteries and increased eNOS expression. In addition, we found that TNF-α inhibition does not significantly affect carbachol-induced responses or vascular eNOS expression in non-DM control animals. These data suggest a link between DM, TNF-α and endothelial dysfunction. This hypothesis is consistent with previous studies demonstrating that anti-TNF-α therapies may improve inflammation-related endothelial dysfunction in various pathophysiological conditions, including hypertension, aging and rheumatoid arthritis [38-40]. In addition, our recent studies also showed that, in rats exposed to unpredictable mild stress, etanercept and infliximab also improved endothelium-dependent vascular relaxations and increased the expression of eNOS [41, 28]. Studies have also shown that anti-TNF-α treatment with infliximab improves endothelial dysfunction in humans with vascular inflammation [42] and etanercept treatment enhances endothelium-dependent relaxation and eNOS expression in estrogen-deficient rats [43]. These findings suggest that anti TNF-α therapy may exert vasculoprotective effects in subjects with DM.

Second, DM is associated with moderate cognitive function impairments, and patients carry a high risk of dementia [44, 4, 1]. The present and previous studies have demonstrated that STZ-induced DM rats perform poorly on the MWMT and PAT, indicating impairment in their abilities and memory capabilities [45, 46, 29]. It is well known that DM patients have neuropathy in the CNS that can disrupt cognitive processes and neuroplasticity [47-50]. In the current study, DM was induced in rats for 30 days and then treated with etanercept. In the MWMT, untreated DM rats had significantly-longer-than-normal escape latencies to find the escape platform, and exhibited a deficit in spatial learning. In the etanercept- treated DM group, rats had shorter escape latencies than those in the untreated DM group. Moreover, the untreated DM rats performed poorly in subsequent testing compared with control rats in the probe trials 24-h later, indicating impaired memory retrieval. Consistent with a previous study [25], etanercept prevented these impairments and restored spatial learning and memory in DM rats. A similar effect was observed in the PAT. Etanercept prevented DM-induced cognitive abnormalities in retention trials 24-h later, indicating improvement in emotional learning and memory. Therefore, the present study confirms our hypothesis that etanercept can ameliorate dysfunction of spatial and emotional learning memory induced by DM.

Third, there was a significant decrease in expression of BDNF in DM rats. BDNF is known to have a role in memory formation, and it can be used as a memory marker [51-53]. Furthermore, hippocampus-dependent learning and memory deficits (which could be correlated to a reduction in neurogenesis) have been observed in mouse models of AD [54]. It is well known that STZ-induced DM affects the proliferation and neurogenesis of hippocampal cells as well as regulation of BDNF immunoreactivity in rats [55, 56]. Not only DM but also vascular dementia disorders are associated with gene expression. For example, pretreatment of repetitive transcranial magnetic stimulation improved BDNF protein expression levels in the vascular dementia rats, thereby improving cognitive functions [57]. Xu and co-workers reported that sigma-1 receptor agonists have anti-inflammatory and antiamnestic properties and increased expression of BDNF in vascular dementia [58]. In agreement with these observations, we found that BDNF expression was decreased by DM in the hippocampus and that this decrease was blocked by etanercept treatment. These findings suggest that the anti-neuroinflammatory and/or neuroprotective properties of etanercept may help protect against the cognitive deficits induced by DM.

Fourth, the lack of difference between the DM and non-DM rats in terms of the locomotor counts indicated that the impaired performance of DM rats was associated with cognitive dysfunction rather than alterations in the motor function. In addition, footshock sensitivity in all groups was similar, suggesting that their motor performance was not affected by persistent hyperglycemia and/or etanercept treatment.

Fifth, hyperglycemia has been reported to contribute to most diabetic complications (including cognitive deficits). In addition, anti-hyperglycemics and insulin sensitizers prevent the cognition and better glycemic control has been found beneficial in diabetes-induced cognitive dysfunction [59, 60]. However, we observed that etanercept treatment did not affect blood glucose levels, but did improve memory dysfunction in STZ-treated rats. Hence, the effects of etanercept on cognitive dysfunction in DM rats could be independent from their glucose-lowering potential, but further experiments are needed to fully elucidate this relation.

Taken together, we propose that DM -induced dysregulation of TNF-α expression contributes to endothelial dysfunction, impairment of neurotrophin expression and cognitive deficits. Thus, the vasculoprotective and neuroprotective effects of anti-TNF-α treatments may be beneficial in DM patients.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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STATEMENT OF HUMAN AND ANIMAL RIGHTS

This article does not contain any studies with human or animal subjects performed by the any of the authors.

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