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Ellagic acid protects against diabetes-associated behavioral deficits in rats: Possible involved mechanisms

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ABSTRACT

Aims: Diabetes mellitus (DM), a chronic metabolic disease, is associated with behavioral deficits. It has been suggested that ellagic acid (EA), a natural polyphenol compound, has potent anti-diabetic, anti-inflammatory, and neuroprotective properties. The present study was aimed to explore the potential protective effects of EA against diabetes-associated behavioral deficits and verified possible involved mechanisms.

Main methods: Fifty adult male Wistar rats were randomly divided into five groups: i.e., CON: normal rats treated with vehicle (5 ml/kg/day; P.O.), EA: normal rats treated with EA (50 mg/kg/day; P.O.), STZ: diabetic rats treated with vehicle (5 ml/kg/day; P.O.), STZ + INS: diabetic rats treated with insulin (6 IU/rat/day; S.C.), STZ + EA: diabetic rats treated with EA (50 mg/kg/day; P.O.). All the groups were under treatment for eight consecutive weeks. During the seventh and eighth weeks, behavioral functions of the rats were assessed by commonly used behavioral tests. Subsequently, pro- and anti-inflammatory cytokines, neurotrophic factors, and also histological changes were evaluated in both cerebral cortex and hippocampus of the rats.

Key findings: Chronic EA treatment attenuated anxiety/depression-like behaviors, improved exploratory/locomotor activities, and ameliorated cognitive deficits in diabetic rats. These results were accompanied by decreased blood glucose levels, modulation of inflammation status, improved neurotrophic support, and amelioration of neuronal loss in diabetic rats. In some aspects, treatment with EA was even more effective than insulin therapy.

Significance: The current work's data confirms that EA could potentially serve as a novel, promising, and accessible protective agent against diabetes-associated behavioral deficits, owing to its anti-hyperglycemic, anti-inflammatory, and neurotrophic properties.

1. Introduction

Diabetes mellitus (DM) is defined as a group of metabolic diseases characterized by chronic hyperglycemia, and is caused by defects in insulin secretion, insulin action, or both. According to the report of the International Diabetes Federation (IDF), there are > 350 million diabetic people worldwide and this number is expected to rise to 592 million by 2035 [1]. Chronic hyperglycemia in DM can lead to neurodegeneration, brain aging, brain atrophy, and increased risk of a wide range of behavioral and psychiatric disorders such as anxiety, depression, stress, low intelligence quotient (IQ) and, locomotor and cognitive impairments [2–5]. The complete spectrum of mechanisms, through which diabetes could mediate these damages are not clearly identified. Nevertheless, it seems that neuroinflammation, oxidative stress impairments, mitochondrial malfunction, neurotransmitters' changes, neuronal apoptosis, altered neurogenesis, loss of neurotrophic support, and dysfunction of cell signaling pathways are involved in the pathophysiology of brain damage and behavioral deficits associated with diabetes [4–7].

An imbalance of pro- and anti-inflammatory cytokines plays a critical role in the progression of brain tissue injuries and development of neurodegenerative complications [8–10]. It has been suggested that

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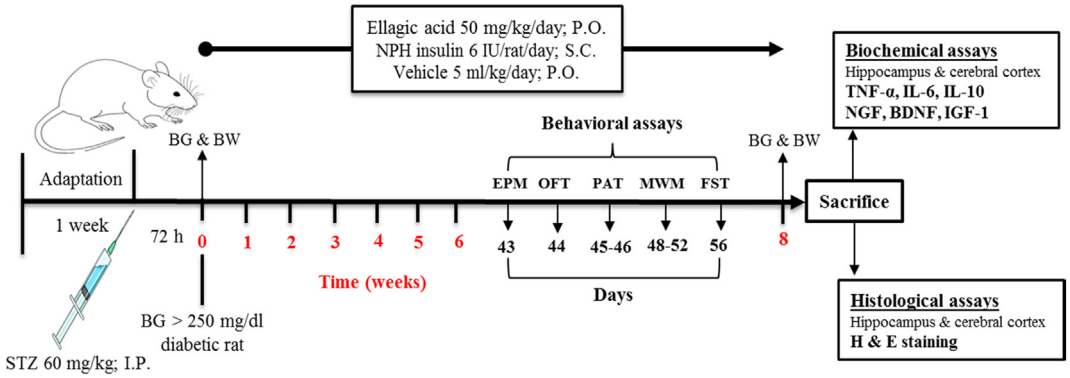


Fig. 1. Schematic diagram of experimental design. STZ: Streptozotocin; I.P.: Intraperitoneal; BW: Body Weight; BG: Blood Glucose; P.O.: Per Oral; S.C.: Subcutaneous; EPM: Elevated Plus Maze; OFT: Open Field Test; PAT: Passive Avoidance Test; MWM: Morris Water Maze; FST: Forced Swimming Test; TNF-α: Tumor Necrosis Factor α; IL-6: Interleukin 6; IL-10: Interleukin 10; NGF: Nerve Growth Factor; BDNF: Brain-Derived Neurotrophic Factor; IGF-1: Insulin Growth Factor 1; H & E: Hematoxylin and Eosin.

hyperglycemia is associated with diabetes' consequences including increased proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) [7], as well as decreased anti-inflammatory cytokines such as interleukin 10 (IL-10) [11]. These deleterious alterations may trigger neuronal degeneration, and subsequently cause behavioral deficits [11,12].

Neurotrophic factors have an important role in growth, neuronal survival, and maintaining physiological brain functions. It is well known that growth, cell survival, synaptic connectivity, plasticity, and cell differentiation are affected by neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and insulin growth factor 1 (IGF-1) [13,14]. Reduction of neurotrophins in DM may injure the central nervous system (CNS) and lead to progression of behavioral deficits [6,15–17].

Ellagic acid (EA) (2,3,7,8-tetrahydroxybenzopyrano[5,4,3-*cde*]benzopyran-5-10-dione), a natural polyphenolic compound, can be found in various natural products such as raspberries, strawberries, grapes, pomegranates, walnuts, and many other plant-based foods [18]. EA has attracted a lot of interest in recent years due to its wide array of biological properties, including antioxidant [19], anti-inflammatory [20,21], anti-diabetic [22,23], neuroprotective [24,25], antidepressant [26], antianxiety [27], antinociceptive [28], and cognitive-improving effects [29,30]. The scientific reports in the literature suggest that the beneficial effects of EA on CNS are comparable to the effects of reference substances used in medical practice [31]. However, the influence of EA on behavioral functions in experimental models of diabetes has not yet been elucidated.

Considering all of the above-mentioned points, this study was designed in order to examine the efficacy of chronic oral administration of EA on behavioral functions, inflammation status, neurotrophins' action, and histological changes in both cerebral cortex and hippocampus of streptozotocin (STZ)-induced diabetic rats.

2. Material and methods

2.1. Chemicals and drugs

The chemicals and drugs used in this experiment were the following: EA, streptozotocin (STZ), dimethyl sulfoxide (DMSO), sodium citrate, citric acid (Sigma-Aldrich; St. Louis, MO, USA); Protease inhibitor cocktail, phosphate-buffered saline (PBS) (Roche, Basel, Switzerland); Interleukin-6 (IL-6), interleukin-10 (IL-10), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), insulin growth factor 1 (IGF-1) (ZellBio, Germany); Tumor necrosis factor alpha (TNF-α) (Diaclone, France); Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA); Protamine-Zinc insulin (NPH) (EXIR Pharmaceutical Co, Borujerd, Iran); Hematoxylin and eosin (H & E) (Merck, Germany); Ketamine, and xylazine (Alfasan Chemical Co, Woerden-Netherlands). EA was freshly prepared by dissolving it in 10% DMSO and normal saline solution. STZ was dissolved in citrate buffer solution (0.1 M, pH 4.5), and in order to avoid the degradation of STZ, all the procedures were performed in darkness and on ice. Insulin was freshly diluted with normal saline solution. All the other chemicals utilized in this experiment were of the analytical grade.

2.2. Animals and experimental design

Fifty adult male Wistar rats (200 ± 20 g) were obtained from the animal house of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. The animals were retained under standard laboratory conditions of humidity, temperature (22 ± 2 °C), and 12 h light/dark cycle (light from 7:00 to 19:00), with ad libitum access to food and tap water. All the protocols were approved by the Animal Ethics Committee Guidelines for the use of experimental animals (Ethic code: IR.AJUMS.REC.1395.629), which are based on the "NIH Guide for the Care and Use of Laboratory Animals". The best possible efforts were made in order to minimize the suffering of the animals and the number of rats required to produce reliable scientific data.

The experimental design is illustrated in Fig. 1. After one week of acclimatization, the experimental animals were randomly divided into five groups (n = 10 per group) as follows:

1. CON group: Normal control rats, received 10% DMSO vehicle (5 ml/kg/day; P.O., for eight consecutive weeks).
2. EA group: Normal rats, received EA (50 mg/kg/day; P.O., for eight consecutive weeks).
3. STZ group: Diabetic control rats, received 10% DMSO vehicle (5 ml/kg/day; P.O., for eight consecutive weeks).
4. STZ + INS group: Diabetic rats, received NPH insulin (6 IU/rat/day; S.C., 2 IU in the morning and 4 IU in the afternoon, for eight consecutive weeks) as previously described [32,33].
5. STZ + EA group: Diabetic rats, received EA (50 mg/kg/day; P.O., for eight consecutive weeks).

The dose of EA used in this study was selected based on previously conducted studies on its neuroprotective effects against oxidative damage in a rat model of diabetes [34], its anti-inflammatory action, as well as its improvement of motor and cognitive disorders in different models of brain injury in rats [24,29,30,35,36]. Additionally, a pilot study was conducted to determine the best hypoglycemic ability of EA in the laboratory. In terms of safety, oral administration of EA up to 3 g/kg/day for 90 days in male rats showed the no-observed-adverse-effect level (NOAEL) [37]. Therefore, the dosage used in the present study was considerably lower than the NOAEL. All behavioral tests were

performed during the seventh and eighth weeks (from 43rd to 56th day of the study). After behavioral tests, the rats were analyzed biochemically and histologically.

2.3. Diabetes induction

Experimental diabetes was induced by a single intraperitoneal injection of STZ (60 mg/kg in citrate buffer) [4,38]. Three days after STZ injection, blood glucose levels were estimated by a digital glucometer (Accu-Chek Active®, Roche, Germany) by a blood sample which was obtained from the tail prick. The day on which hyperglycemia was confirmed was considered as the onset of study. Rats with blood glucose levels of above 250 mg/dl were considered as diabetic animals. Non-diabetic rats were injected with the same volume of citrate buffer. Body weights and blood glucose levels of all groups were measured at the onset and end of the experiment [4,6].

2.4. Behavioral tests

2.4.1. Open field test (OFT)

According to the method described by Pardon et al. [39], OFT was run in order to investigate locomotor activity and exploratory behavior. The apparatus was placed in an isolated noise-free room under mild lighting conditions. It was basically consisted of a black metal box (50 × 50 × 50 cm) subdivided into 9 equal squares by white lines. The 'center' field is defined as the central square. In order to perform the test, an animal was placed in the center of the arena and was filmed non-stop with a video camera device (A4TECH® PK-710G, Taiwan) for 5 min. The total distance traveled (cm) and the time spent in the central square of the arena were recorded, and video recordings were analyzed by two independent observers blinded to the group setups. At the end of each test, the box was cleaned with 70% ethanol to eliminate olfactory cues.

2.4.2. Elevated plus maze (EPM)

EPM was conducted in order to assess the locomotor activity and anxiety-like behaviors in accordance with the method described by Pellow and File [40]. Briefly, the apparatus consisted of two closed arms (50 × 10 × 40 cm) and two open arms (50 × 10 cm) intersecting at a central square (10 × 10 cm). At the start of each 5-min trial, each animal was placed at the center of the maze, facing an open arm and it was permitted to freely explore the device in a soundproof room.

The behavioral parameters scored during the testing sessions were: number of entries into closed and open arms, and total time spent in closed and open arms. Subsequently, number of total entries (open + closed arms) and percentage of time spent in the open arms were calculated respectively as locomotor activity and anxiety-like behaviors. The maze was wiped with 70% ethanol between trials to eliminate olfactory cues.

2.4.3. Passive avoidance test (PAT)

Details of the experimental apparatus and general procedure for assessing passive avoidance learning and memory functions have been described previously [41,42]. In brief, the apparatus (Borj Sanat Co, Tehran-Iran) was composed of two equal light and dark chambers with a grid floor made of stainless steel rods (3 mm in diameter, spaced 10 mm apart), which are separated by a mobile guillotine door (8 × 8 cm). The floor of the dark chamber was connected to a shock generator (0.3 mA, 75 V, 50 Hz). On the habituation day, each animal was placed in the lit chamber of PAT with the doors open and it was allowed to explore freely for 10 min.

An acquisition phase was performed 24 h later. In this phase, each rat was again placed in the lit chamber facing away from the guillotine door and 30 s later, the door was opened. When the rat entered into the dark chamber, the door was closed and an electrical foot shock was immediately delivered through the grid floor for 3 s. Entrance latency to the dark chamber from the time the door was lifted, was recorded as initial latency (IL).

On the retention phase that occurred 24 h after the PAT acquisition phase, each rat was placed into the lit chamber and 30 s later, the guillotine door was raised. Consequently, the step-through latency (STL) was regarded as the measure of retention performance and cut-off time was set at 300 s while no shock was delivered in this day. Short latencies indicated poorer cognition.

2.4.4. Morris water maze (MWM)

Rats' spatial learning and memory were tested using MWM [43]. The MWM apparatus (150 cm diameter, 80 cm height) was divided into four equal quadrants filled with water (23 ± 1 °C) to a depth of 40 cm.

A hidden circular platform (10 cm diameter) was submerged 1 cm below the water surface in the center of designated target quadrant. All animals were given four consecutive training trials per day for four days, with 10 min inter-trial intervals.

Each trial lasted until either the animal found the hidden platform, or up to a maximum of 60 s had been elapsed. If a rat failed to find the hidden platform within the allotted time, the animal was guided to the platform by a technician and was allowed to remain there for 30 s. The traveled distance and the escape latency to reach the hidden platform were obtained using a video-tracking software (Maze Router V3.1, Techniq Azma Co, Tabriz-Iran). Learning process was estimated as the traveled distance and the escape latency to reach the hidden platform [44]. On the fifth day, the animals were subjected to a probe trial to assess their consolidated memory, while the platform was removed and the rats were allowed to swim freely for 60 s. The time spent in the target quadrant was calculated as a measure of spatial memory.

2.4.5. Forced swimming test (FST)

FST experimental procedure was performed in order to estimate depression-like behavior in accordance with the method that has been described previously [45,46]. In this test, a rat was dropped into a plexiglass cylinder (20 cm in diameter, 50 cm in height) containing water to a height of 30 cm at a temperature of 23–25 °C. The durations of swimming (the animal making horizontal movements in the water), climbing (vertical movements along the wall of the beaker), and immobility (floating in the water without struggling) were recorded for 5 min. The animals were subjected to a 15 min adaptation session under the same conditions 24 h before the actual experiment. In order to have the same water level, fresh water was refilled between each trial.

2.5. Biochemical assay

At the end of the treatment period, six rats were randomly selected from each group and anesthetized deeply with a ketamine-xylazine mixture (100 mg/kg–10 mg/kg, respectively). Following decapitation, the animal brains were rapidly taken out, and cerebral cortex and hippocampus tissues were quickly dissected on ice, rinsed with saline, and frozen at −80 °C until they were used. The frozen tissues were thawed and homogenized in a cold PBS with a pH of 7.4 (100 mg tissue per 1 ml of the buffer), which contained protease inhibitor cocktail. The samples were centrifuged at 10,000 ×*g* for 20 min at 4 °C, and subsequently, clear supernatants were collected carefully, divided into aliquots, and stored immediately at −80 °C until analysis. Total protein concentrations of the supernatants were determined using a Bio-Rad protein assay kit according to the manufacturer's protocols.

The values of cytokines/neurotrophic factors including TNF-α, IL-6, IL-10, BDNF, NGF, and IGF-1 in both cerebral cortex and hippocampus were assessed and quantified using the enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. These concentrations were expressed as picograms per milligram of total protein (pg/mg protein).

2.6. Histological assay

2.6.1. Hematoxylin and eosin (H & E) staining

To determine histological changes in both cerebral cortex and hippocampus, four animals were randomly chosen from each group and perfused with 5% formalin through the left ventricle under deep anesthesia. Rats were decapitated, their brains were removed and fixed with 10% paraformaldehyde solution for 72 h and embedded in paraffin. In the next step, tissue sections (5 μm thick) were stained by H & E. Six slides per animal were selected, then intact neurons (normal cells) and dark neurons (dead cells) from cerebral cortex and hippocampal regions (CA1: cornu ammonis 1, CA3: cornu ammonis 3, and DG: dentate gyrus) were evaluated under a light microscope (Olympus PX 50 F3 model, Japan).

2.7. Statistical analysis

All data analyses were performed using Statistical Package of Social Sciences (SPSS) software program, version 16 (SPSS; Chicago, IL, USA). Traveled distance and escape latency to reach the hidden platform of MWM in training days were analyzed by two-way analysis of variance (ANOVA), and differences between individual groups were determined using one-way ANOVA and Tukey's post-hoc test. Other data was analyzed by one-way ANOVA. The data is presented as mean ± standard error of mean (SEM), and a P value of < 0.05 was considered statistically significant.

3. Results

3.1. Effects of EA on body weight and blood glucose levels in diabetic rats

At the onset of the study, the non-diabetic animals and the animals that were to receive STZ, had similar body weights ($F_{4,\ 45} = 0.699$; $P > 0.05$), while body weights were severely decreased in the STZ group compared to the CON group at the end of the study ($F_{4,\ 45} = 25.621$; $P < 0.001$, Table 1). This effect was significantly improved in the STZ group after insulin treatment ($P < 0.001$). However, EA treatment did not affect the body weight of the diabetic rats, displaying that EA consumption did not prevent weight loss in diabetic rats.

Three days after STZ or citrate buffer injection, blood glucose levels of the rats, which had been treated by STZ, were significantly higher than those treated by citrate buffer ($F_{4,\ 45} = 62.852$; $P < 0.001$). At the end of the treatment course, diabetes induction increased blood glucose levels ($F_{4,\ 45} = 803.738$; $P < 0.001$), while they were significantly reduced in the groups treated with insulin or EA ($P < 0.001$), suggesting that insulin or EA treatment provided protection against severe hyperglycemia in diabetic rats (Table 1).

3.2. Behavioral results

3.2.1. Effects of EA on OFT in diabetic rats

OFT was carried out to determine any possible effects of diabetes induction and treatment by EA on locomotor activity and exploratory behavior. As shown in Fig. 2A and B, the total distance traveled ($F_{4,\ 45} = 7.532$; $P < 0.001$) and the time spent in the central square of the arena ($F_{4,\ 45} = 4.796$; $P < 0.01$) were significantly decreased in the STZ group as compared with the CON group, indicating obvious locomotor deficits as well as decreased exploratory behavior in the diabetic rats. These effects were improved in the STZ group after EA treatment ($P < 0.05$). In addition, insulin therapy markedly increased the total distance traveled in the diabetic animals ($P < 0.001$), although the time spent in the central square of the arena was non-significantly higher in the STZ + INS group compared to the STZ group.

3.2.2. Effects of EA on EPM in diabetic rats

The anxiety-like behaviors and locomotor activity were evaluated by EPM. Statistical analyses revealed that the percentage of time spent in open arms ($F_{4,\ 45} = 10.108$; $P < 0.001$), the number of closed arms entries ($F_{4,\ 45} = 11.477$; $P < 0.01$), and number of total arm entries ($F_{4,\ 45} = 10.482$; $P < 0.001$) were lower in the STZ group versus the CON group (Fig. 2C, D and E). This data indicated that STZ decreased locomotor activity and increased anxiety-like behaviors in diabetic rats, where administration of EA significantly attenuated anxiety-like behaviors ($P < 0.05$) and improved locomotor activity ($P < 0.001$) in diabetic rats. Likewise, insulin therapy markedly improved locomotor activity in diabetic animals ($P < 0.01$), but it was not capable of ameliorating anxiety-like behaviors.

3.2.3. Effects of EA on PAT in diabetic rats

The results of the animals' performance in the acquisition phase of PAL showed that the IL was similar in all the experimental groups ($F_{4,\ 45} = 1.146$; $P > 0.05$). Though in the retention phase, the STL in the STZ group was markedly less than that of the CON group ($F_{4,\ 45} = 3.924$; $P < 0.01$). The STL was significantly improved after administration of EA in STZ-induced diabetic rats ($P < 0.05$), with insulin having no significant improving effect (Fig. 2F).

3.2.4. Effects of EA on MWM in diabetic rats

To determine the association of DM-induced hyperglycemia with the spatial learning and memory performance, animals were trained in the MWM paradigm. In this task, learning was associated with reductions in the traveled distance and escape latency to reach the hidden platform during four consecutive days of training trials.

Two-way repeated ANOVA analysis of the traveled distance and escape latency to find the hidden platform showed the overall significant effects of the treatment [($F_{4,\ 45} = 7.853$; $P < 0.001$, for traveled distance) and ($F_{4,\ 45} = 17.869$; $P < 0.001$, for escape latency)], and day [($F_{3,\ 135} = 26.646$; $P < 0.001$, for traveled distance) and ($F_{3,\ 135} = 41.123$; $P < 0.001$, for escape latency)], and day × treatment interaction ($F_{12,\ 135} = 2.230$; $P = 0.021$, for escape latency) in all the

Table 1
Effects of EA (50 mg/kg/day; P.O., for 8 weeks) on body weight and blood glucose levels of STZ-induced diabetic rats.

Groups	Treatments	Body weight (g)		Plasma glucose (mg/dl)	
		Onset of study	End of study	Onset of study	End of study
CON	Vehicle (5 ml/kg; P.O.)	185.9 ± 2.24[a]	309.3 ± 9.69[a]	105.2 ± 4.97[b]	111.2 ± 4.12[c]
EA	EA (50 mg/kg; P.O.)	186.6 ± 3.74[a]	295.1 ± 5.87[a]	106.3 ± 3.13[b]	108.2 ± 3.03[c]
STZ	Vehicle (5 ml/kg; P.O.)	181.0 ± 2.92[a]	214.7 ± 10.71[b]	459.7 ± 24.37[a]	569.3 ± 9.19[a]
STZ + INS	Insulin (6 IU/rat; S.C.)	189.2 ± 5.30[a]	329.6 ± 14.03[a]	456.0 ± 30.29[a]	136.7 ± 7.70[bc]
STZ + EA	EA (50 mg/kg; P.O.)	188.0 ± 3.83[a]	231.6 ± 7.20[b]	411.9 ± 34.47[a]	147.1 ± 8.75[b]

Data is shown as mean ± SEM of 10 animals per group. Values within the same column that do not share a common superscript letter differ significantly at $P < 0.05$ (one-way ANOVA, Tukey's post-hoc test).

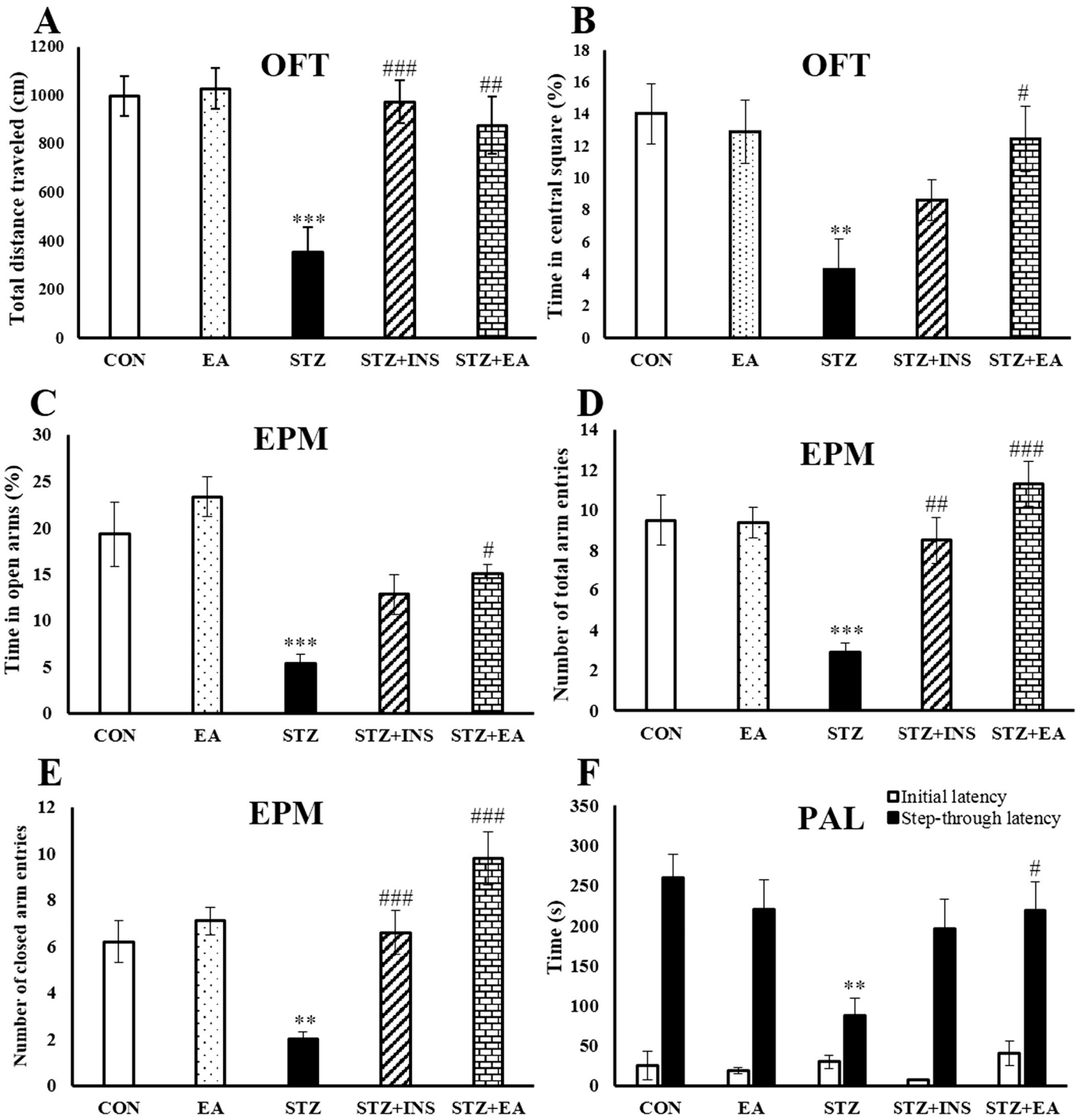


Fig. 2. Effects of EA (50 mg/kg/day; P.O., for 8 weeks) on total traveled distance (A), percentage of time spent in central square (B) of OFT, percentage of time spent in open arms (C), number of total arm entries (D), number of closed arm entries (E) of EPM, time of initial and step-through latencies (F) of PAL in STZ-induced diabetic rats. Each bar represents mean ± SEM of 10 animals per group; *P < 0.05, **P < 0.01, ***P < 0.001 vs. the CON group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. the STZ group (one-way ANOVA, Tukey's post-hoc test).

training trial days. However, there was no significant effect of day × treatment interaction ($F_{12,\ 135} = 1.437$; $P > 0.05$) for the distance traveled during the consecutive training trials.

The following Tukey's post-hoc test showed that STZ group displayed significantly more traveled distance as well as escape latency than the CON group on four consecutive days ($P < 0.001$, and $P < 0.05$, respectively), indicating noticeable learning deficits in the diabetic animals. However, prolonged treatment of diabetic rats with EA or insulin markedly improved the traveled distance and the escape latency impairments induced by STZ ($P < 0.05$ for each comparison, Fig. 3A, B).

In the probe trial, the hidden platform was removed, and each rat was allowed to swim for 60 s, which evaluated the spatial memory of the animals. The percentage of time spent in the target quadrant was used to evaluate memory consolidation.

One-way ANOVA revealed that the STZ group spent the least amount of time in the target quadrant in comparison to the other groups ($F_{4,\ 45} = 8.045$; $P < 0.01$, Fig. 3C). However, it was completely

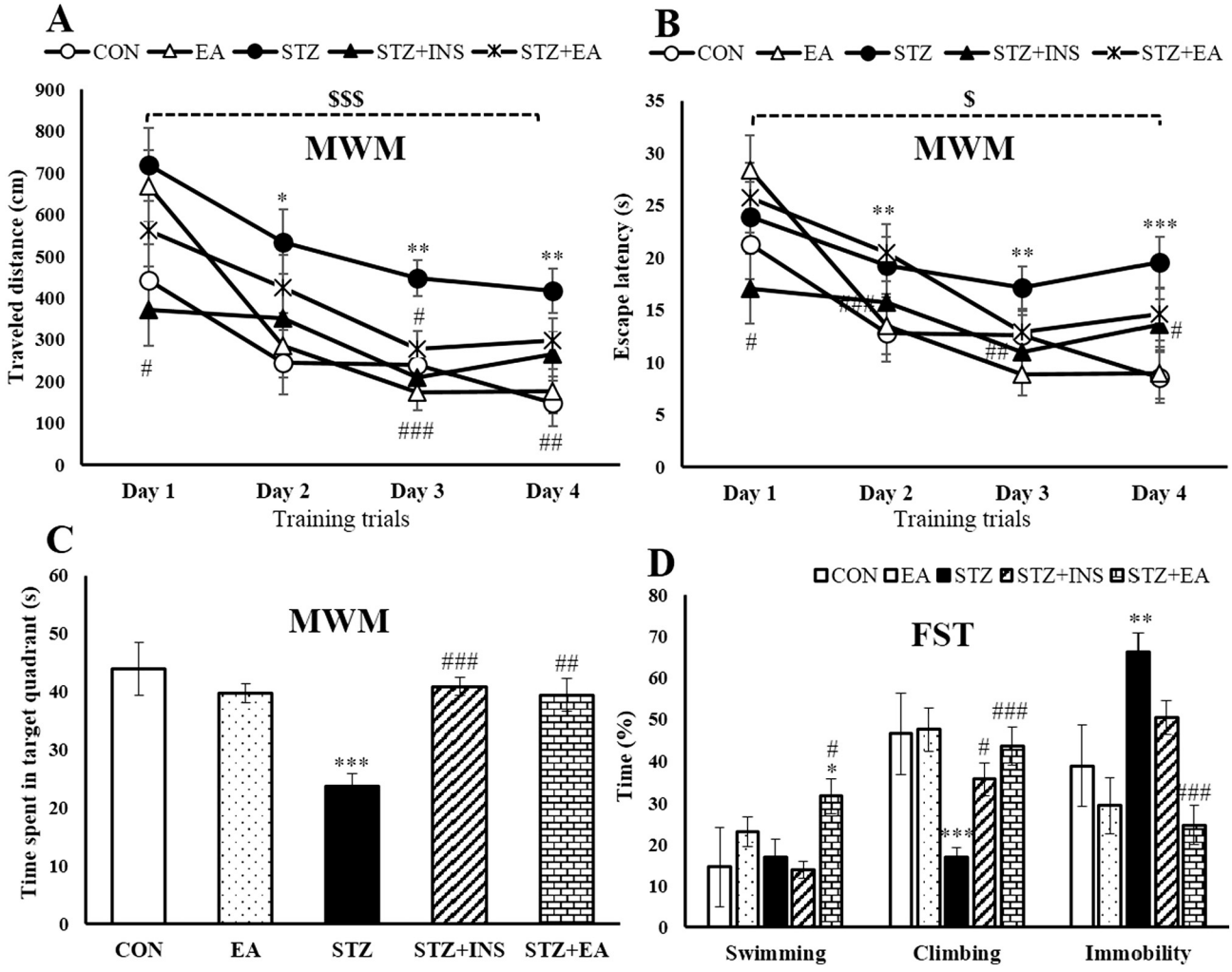


Fig. 3. Effects of EA (50 mg/kg/day; P.O., for 8 weeks) on traveled distance (A), escape latency (B), time spent in target quadrant (C) of MWM, and percentages of swimming, climbing, & immobility (D) behaviors of FST in STZ-induced diabetic rats. Each bar represents mean ± SEM of 10 animals per group. $P < 0.05, $$ $P < 0.001 (repeated measures two-way ANOVA); *P < 0.05, **P < 0.01, ***P < 0.001 vs. the CON group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. the STZ group (one-way ANOVA, Tukey's post-hoc test).

reversed for the diabetic rats after chronic administration of EA or insulin (P < 0.01 for each comparison).

Based on these results, the diabetic rats were not able to remember the location of the hidden platform, while STZ-induced memory impairments were ameliorated in the EA or insulin-treated rats.

3.2.5. Effects of EA on FST in diabetic rats

The depression-like behavior was assessed in FST. As shown in Fig. 3D, diabetic rats spent the least amount of time on climbing ($F_{4, 45} = 9.682$; P < 0.05) and the greatest amount of time on being immobile ($F_{4, 45} = 10.966$; P < 0. 01) as compared to the other groups, indicating that STZ produced depression-like behaviors in the diabetic animals. In the same experimental conditions, administration of EA markedly attenuated depression-like behaviors in diabetic rats (P < 0.001). Insulin therapy significantly increased the time spent on climbing in diabetic animals (P < 0.05), though it was not capable of improving the duration of immobility. On the other hand, swimming time was greater in the STZ + EA group compared to the CON, STZ, and STZ + INS groups ($F_{4, 45} = 4.352$; P < 0.05, P < 0.05, and P < 0.01, respectively), but interestingly, there was no significant difference between the STZ + EA group and the EA group.

3.3. Biochemical results

3.3.1. Effects of EA on TNF-α, IL-6, and IL-10 levels on the cerebral cortex and hippocampus of diabetic rats

As an impact of diabetes, elevated TNF-α and IL-6 levels were found in both cerebral cortex and hippocampus of the STZ group compared to the CON group [TNF-α: ($F_{4, 25} = 17.718$; P < 0.001 for cerebral cortex) and ($F_{4, 25} = 31.296$; P < 0.001 for hippocampus)] and [IL-6: ($F_{4, 25} = 24.484$; P < 0.001 for cerebral cortex) and ($F_{4, 25} = 28.415$; P < 0.001 for hippocampus)].

Treatment with EA or insulin markedly suppressed the elevation of TNF-α levels in both cerebral cortex (P < 0.01 for each comparison) and hippocampus (P < 0.001 for each comparison) of diabetic animals. Furthermore, EA or insulin significantly decreased IL-6 levels in the examined brain areas of the diabetic rats (P < 0.01 for each comparison).

The level of anti-inflammatory cytokine IL-10 was significantly declined by STZ in the cerebral cortex ($F_{4, 25} = 11.329$; P < 0.001) and hippocampus ($F_{4, 25} = 43.382$; P < 0.001). Notably, EA increased the levels of IL-10, in both the EA and the STZ + EA groups, which is indicative of the general anti-inflammatory effects of EA in these brain areas. The results of the statistical analyses are presented in Fig. 4A, B, and D.

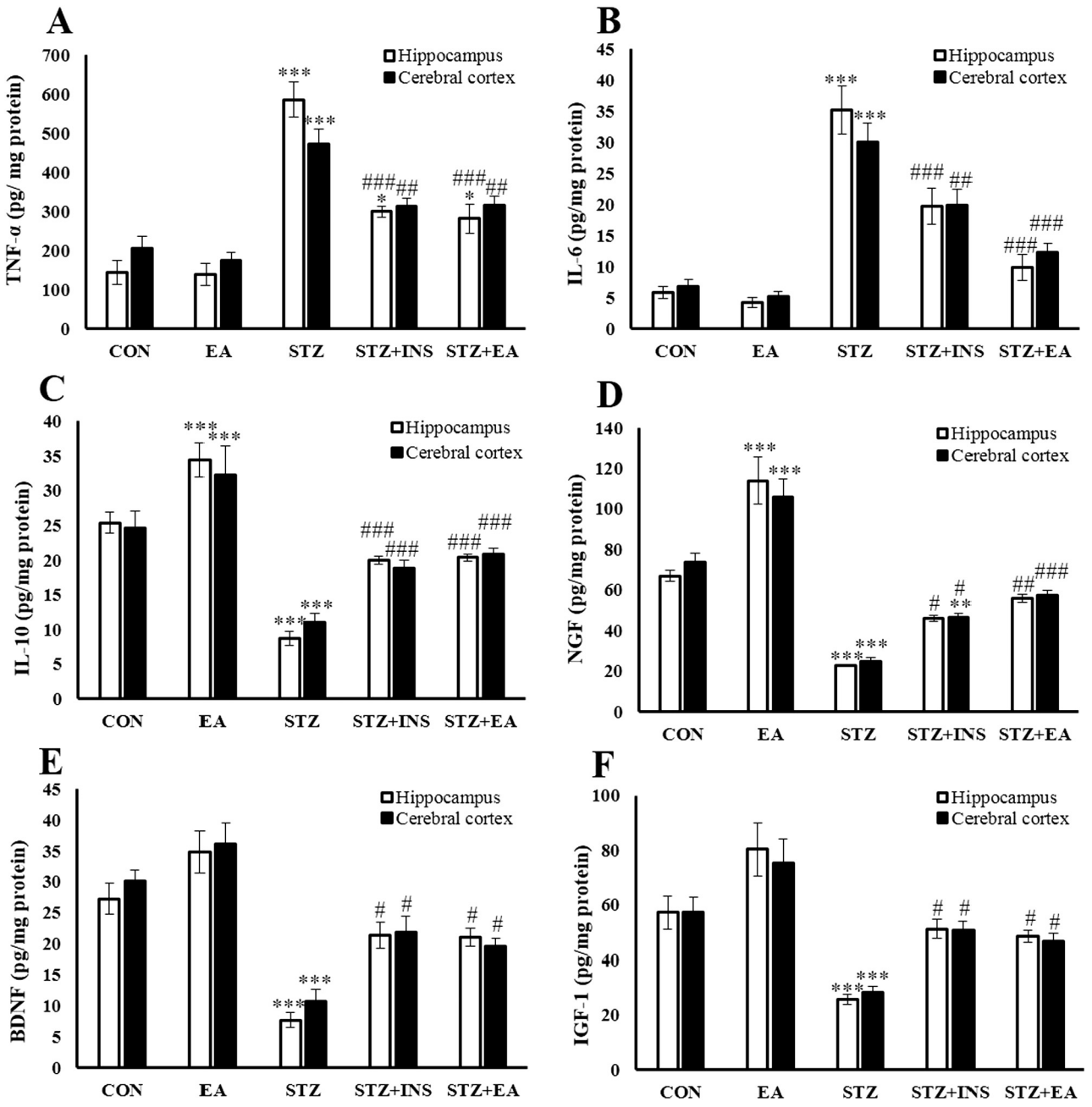


Fig. 4. Effects of EA (50 mg/kg/day; P.O., for 8 weeks) on TNF-α (A), IL-6 (B), IL-10 (C), BDNF (D), NGF (E) and IGF-1 (F) levels in both hippocampus and cerebral cortex of STZ-induced diabetic rats. Each bar represents mean ± SEM of 6 animals per group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the CON group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. the STZ group (one-way ANOVA, Tukey's post-hoc test).

3.3.2. Effects of EA on NGF, BDNF, and IGF-1 levels on the cerebral cortex and hippocampus of diabetic rats

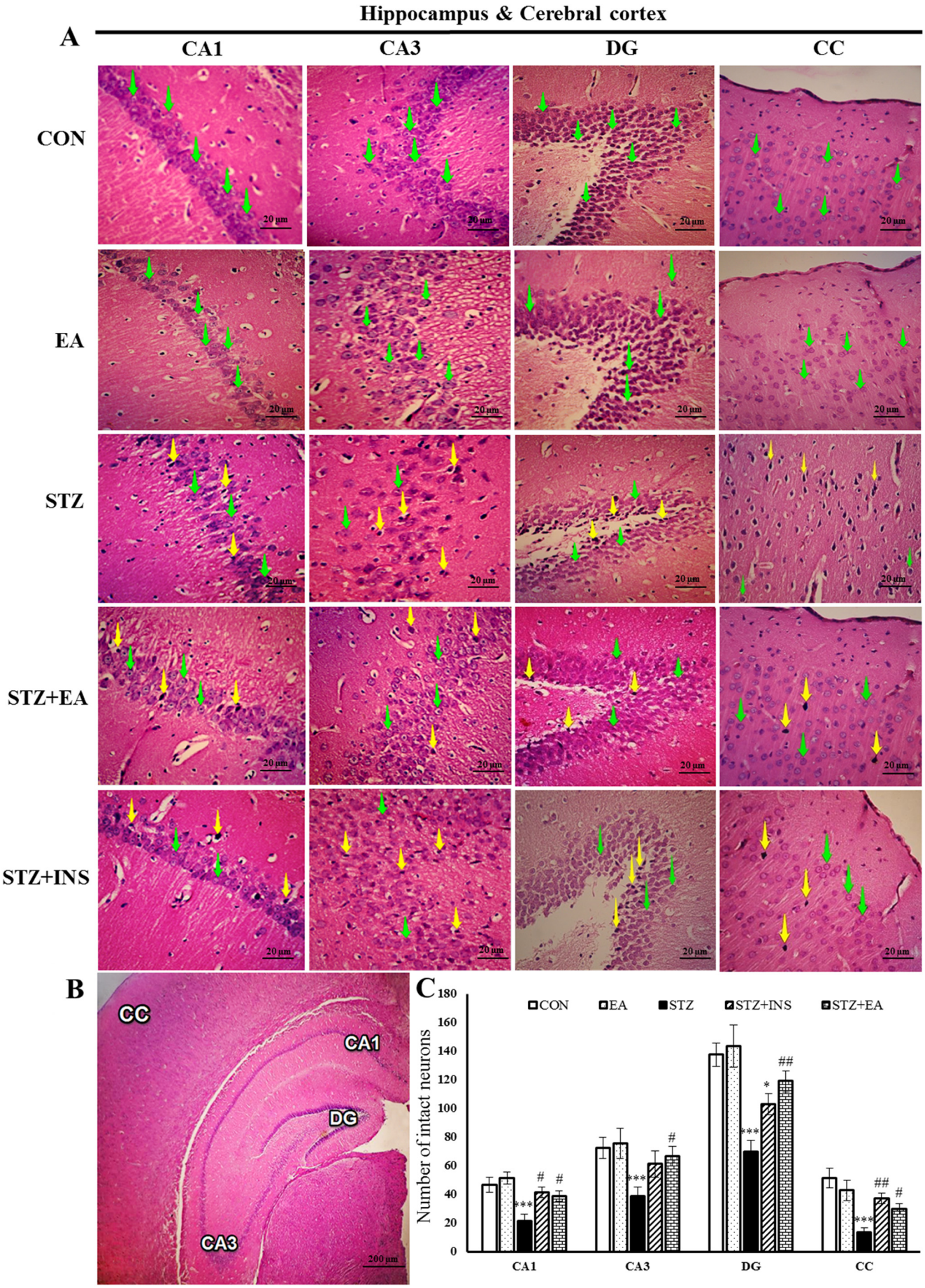
As depicted in Fig. 4D, E, and F, there were significant decreases in the levels of NGF, BDNF, and IGF-1 in both cerebral cortex and hippocampus of the diabetic rats compared to the CON group [NGF: ($F_{4, 25}$ = 41.333; P < 0.001 for cerebral cortex) and ($F_{4, 25}$ = 37.437; P < 0.001 for hippocampus)], [BDNF: ($F_{4, 25}$ = 17.601; P < 0.001 for cerebral cortex) and ($F_{4, 25}$ = 19.197; P < 0.001 for hippocampus)], and [IGF-1: ($F_{4, 25}$ = 11.090; P < 0.001 for cerebral cortex) and ($F_{4, 25}$ = 12.694; P < 0.001 for hippocampus)].

Following eight weeks of treatment with EA or insulin, tissue levels of the above-mentioned neurotrophic factors were significantly increased in the cerebral cortex and hippocampus of STZ-induced diabetic rats (P < 0.05). On the other hand, EA increased the levels of NGF in the EA group (P < 0.001), indicating a general neurotrophic action in these brain areas.

3.4. Histological results

3.4.1. Effects of EA on histological changes in the cerebral cortex and hippocampus of diabetic rats

Based on the results shown in Fig. 5A, B, and C, histological changes in the cerebral cortex and hippocampal CA1, CA3, and DG areas were assessed by H & E staining. A significant decline was found in the number of intact neurons of the cerebral cortex ($F_{4, 15}$ = 5.006; P < 0.001) and hippocampal CA1 ($F_{4, 15}$ = 4.502; P < 0.001), CA3 ($F_{4, 15}$ = 3.112; P < 0.001), and DG ($F_{4, 15}$ = 4.768; P < 0.001) areas of the STZ group compared to the CON group, indicating neuronal loss



(caption on next page)

Fig. 5. Effects of EA (50 mg/kg/day; P.O., for 8 weeks) on histological changes in the cerebral cortex (CC) and hippocampal CA1, CA3, and DG regions (H & E stain, 400 × magnification, scale bar 20 μm) of STZ-induced diabetic rats. "A" illustrates the revealing photographs of intact neurons (clear cells with distinct round nuclei, identified by green arrows) and dark neurons (shrinkage of cells with pyknotic nuclei, identified by yellow arrows), "B" represents photomicrograph of cerebral cortex and hippocampal regions of the rat (H & E stain, 40 × magnification, scale bar 200 μm), and "C" includes the quantitative data of the number of intact neurons. Each bar represents mean ± SEM of 4 animals per group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the CON group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. the STZ group (one-way ANOVA, Tukey's post-hoc test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in diabetic animals. Treatment with EA markedly protected the cerebral cortex and hippocampal neurons of the diabetic rats ($P < 0.05$ for each comparison). Furthermore, insulin treatment of diabetic animals significantly increased the number of intact neurons in the cerebral cortex ($P < 0.01$) and hippocampal CA1 ($P < 0.05$) with no significant improvements in the CA3 and DG when compared to the STZ group.

4. Discussion

In this study, STZ-induced diabetes resulted in a wide spectrum of behavioral disorders including increased anxiety-like behaviors accompanied by decreased exploratory and locomotor activities in EPM and OFT, impaired learning and memory performances in PAL and MWM, and enhanced depression-like behaviors in FST. These disturbances were associated with reduced levels of neurotrophic factors, increased inflammation status, and also neuronal loss in both cerebral cortex and hippocampus of diabetic rats. In contrast, treatment with EA for eight consecutive weeks ameliorated all the above-mentioned changes in STZ-induced diabetic rats.

The hippocampus and cerebral cortex are vital centers in the mammalian brain, and play a recognized role in cognitive and behavioral functions. It is believed that these brain regions have a high susceptibility to diabetes-induced hyperglycemia. Hence, these brain areas are considered as specific target tissues for the changes related to diabetes within the CNS in animal models of diabetes [6,7,15,47,48]. Previous studies have demonstrated that neurobehavioral dysfunctions are induced during the seventh and eighth weeks after STZ-diabetes induction [2,4,6,49]. Therefore, in the present study, the experimental period was also considered to be eight weeks long.

It is a well-established fact that induction of diabetes by STZ, is associated with a progressive deterioration of behavioral functions [50]. Poor performance in behavioral functions including enhanced anxiety-like behaviors along with reduced exploratory and locomotor activities in EPM and OFT, decreased cognitive capacity in MWM and PAL, and increased depression-like behaviors in FST, had been shown previously in STZ-induced diabetic rats [4,38,49,51], and it is corroborated by the current work's results. Interestingly, EA improved all the behavioral deficits in the diabetic animals. Amelioration of motor and cognitive functions following the administration of EA have been reported in different models of brain injury [19,24,25,30,35,36,41,52,53]. However, to the authors' knowledge, this is the first and only study that investigates the influences of EA on behavioral functions in experimental models of diabetes.

In this study, STZ significantly increased blood glucose levels and severe loss of body weight in diabetic rats. This diabetogenic effect of STZ is probably due to its deleterious effect on pancreatic beta-cells. Chronic EA treatment improved hyperglycemia of STZ-diabetic rats, which is supported by previous studies [21–23,54,55]. It has been well-documented that chronic hyperglycemia is associated with critical complications such as cognitive and behavioral deficits in both animal models of diabetes and diabetic patients [51,56,57]. Good glycemic control in diabetes may help in preventing or slowing the rate of development of behavioral disorders related to diabetes [58,59]. Some beneficial effects of EA on diabetes-related behavioral dysfunctions may be due to its ability to ameliorate hyperglycemia. The hypoglycemic activity of EA may be attributed to its capability to stimulate insulin secretion, decrease glucose intolerance [22], modulate GLUT4 expression [60], increase insulin sensitivity [54], reduce oxidative stress generated by STZ in beta-cells [23], and enhance pancreatic beta-cells viability via antioxidant activity [55].

Neuroinflammation is a pathophysiological hallmark of chronic DM, which is involved in proliferation and activation of microglia and subsequent astrocyte cells [61]. In the present study, overproduction of proinflammatory cytokines such as TNF-α and IL-6, and decrease of anti-inflammatory cytokine IL-10 were observed in the brains of STZ-diabetic rats, which is in conformity with earlier experimental reports [7,11]. It has been shown that TNF-α and IL-6 are involved in modulation of neuronal microenvironment, behavioral functions, synaptic plasticity, and induced neuronal loss of cortical neurons [62–64]. Moreover, among the anti-inflammatory cytokines, IL-10 plays an important role in modulation of the inflammatory response [11,65]. It has been suggested that even a low level of neuroinflammation can lead to a damage in the synaptic function that might result in cognitive and behavioral deficits [66]. Elevated inflammation in the cerebral cortex and hippocampus of diabetic rats may induce neuronal cell death and initiate behavioral deficits. In accordance with this hypothesis, it has been shown that neuroinflammation in STZ-diabetic animals is correlated with induced neuronal loss and behavioral deficits [7,47,48]. Chronic oral administration of EA significantly modulated the tissue levels of TNF-α, IL-6, and IL-10 in the hippocampus and cerebral cortex of the diabetic rats. Similar to these results, it has been reported that EA can inhibit the excessive production of TNF-α and IL-6 in cardiac tissue [20] and serum [21,67] of experimental diabetic models, and also increase IL-10 levels in acute lung injury in mice in both in vitro and in vivo conditions [65]. Thus, EA may attenuate behavioral deficits through modulating the inflammation status in the brain tissue of the diabetic rats. Collectively, these results suggest that improvement of diabetes-mediated behavioral disturbances in the EA-treated rats may be partly due to its protective effects against diabetes-induced neuroinflammation.

Neurotrophic factors have an essential role in neuronal survival and maintaining the physiological functions of the brain. The current results showed that neurotrophic factors such as NGF, BDNF, and IGF-1 were significantly decreased in the cerebral cortex and hippocampus of diabetic rats. Similarly, Ola et al. [17] indicated that NGF, BDNF, and IGF-1 levels were decreased in the brain of STZ-induced diabetic rats. These results reinforce the idea that loss of neurotrophic support may lead to neuronal damage and death in the cerebral cortex and hippocampus and trigger behavioral deficits in diabetic rats. In agreement with this assumption, previous studies have demonstrated that lack of neurotrophic support in the cerebral cortex and hippocampus of diabetic rats is associated with neuronal loss and increased risk of progression of behavioral impairments [6,15,16,68]. Prolonged EA treatment can significantly increase tissue levels of NGF, BDNF, and IGF-1 in the brains of diabetic rats. In this regard, it has been suggested that polyphenols could play a neuroprotective role through modulation and/or potentiation of neurotrophic factors [69]. Therefore, it seems that the improving effect of EA against diabetes-induced behavioral deficits was mediated, at least partially, by the potentiation of neurotrophic factors.

Hyperglycemia-induced neuronal loss, may contribute to behavioral disorders in DM [4,6]. In the current study, histological evaluation of H & E staining showed that diabetes resulted in neuronal loss in both cerebral cortex and hippocampus of the rats. Consistent with the findings of this work, previous studies have shown that diabetes-related

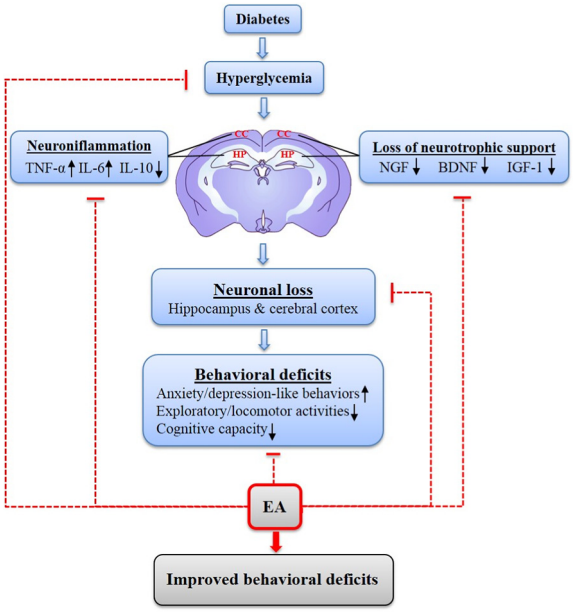


Fig. 6. Schematic representation of the "protective effects of EA against diabetes-associated behavioral deficits in rats: Possible involved mechanisms". CC: Cerebral Cortex; HP: Hippocampus; TNF-α: Tumor Necrosis Factor α; IL-6: Interleukin 6; IL-10: Interleukin 10; NGF: Nerve Growth Factor; BDNF: Brain-Derived Neurotrophic Factor; IGF-1: Insulin Growth Factor 1; EA: Ellagic Acid.

neuronal loss in rats is induced during the seventh or eighth week after diabetes induction [4,6,70]. Interestingly, chronic EA treatment was able to prevent neuronal loss in the cerebral cortex and hippocampus of diabetic rats. It was shown that treatment with EA (50 mg/kg body weight, P.O., for 21 days; initiated one week after chemical induction of diabetes) could improve histopathological changes via decreases in neuronal hydropic degeneration, blood vessels damage, and hemorrhagic focuses of cerebral cortex tissue in STZ-induced diabetic rats [34]. In addition, oral administration of EA at the dose of 50 mg/kg was also able to prevent sporadic Alzheimer's disease (SAD)-induced hippocampal neuronal loss in a rat model [30]. It seems that amelioration of neuronal loss in the present study is due to improvements in hyperglycemia, decrease of neuroinflammation, and increase of the neurotrophic factors following the EA treatment of the diabetic animals.

After EA consumption, it was gradually metabolized via microbiota of the gut, and it produced different types of urolithins (urolithin A and urolithin B), which have better absorption rates compared to the EA. Clinical and animal studies have indicated that plasma concentration of the EA could reach to its peak in about 0.5–1 h after administration of a single oral dose with maximum concentration (Cmax) values of 200 ng/ml, and 93.6 ng/ml in human volunteers and rats, respectively [71,72]. The percentage of plasma protein binding of EA is over 50% and its serum elimination half-life is 8.4 ± 1.8 h [71]. Detection of EA in the brain of rats has been reported following administration of a single oral dose (50 mg/kg), which suggests that EA was in fact able to cross the blood-brain barrier [72]. Therefore, EA could have a possible neuroprotective role for consumers of vegetarian cuisine and specific fruits [73]. In the present study, EA exhibited neuroprotective effects by amelioration of neuronal loss in the cerebral cortex and hippocampus of diabetic animals.

The current study revealed that almost most of the beneficial effects of EA on behavioral deficits were the same as the insulin therapy, which is the standard anti-diabetic drug. In line with previous reports [4,32,70], the findings of the current study showed that insulin therapy not only controlled hyperglycemia, but also improved most of the behavioral dysfunctions and neuronal loss in STZ-induced diabetic rats, which may be due to its anti-inflammatory and neurotrophic properties. Due to the fact that insulin has anti-oxidative [4,32], neuroproliferative [74], and neuroplastic [75] properties, a part of its beneficial effects may be related to these properties.

5. Conclusion

Overall, the results of the present study (Fig. 6) suggest that diabetes-induced hyperglycemia promotes neuroinflammation and loss of neurotrophic support in the hippocampus and cerebral cortex, which in turn, leads to neuronal loss, resulting in behavioral deficits in diabetic animals. In contrast, EA treatment improved behavioral deficits and prevented neuronal loss, at least to the same degree as insulin therapy, which may be caused due to decrease in blood glucose level, modulation of inflammation status (decreased TNF-α and IL-6 and increased IL-10), and increase in tissue levels of the neurotrophic factors (NGF, BDNF, IGF-1) in diabetic rats. It seems that potent anti-hyperglycemic, anti-inflammatory, and neurotrophic properties of EA are possible mechanisms for its ameliorating effects against diabetes-associated behavioral deficits in rats. The present study suggests that EA could be a promising therapeutic agent for treatment and/or prevention of the behavioral deficits associated with diabetes. However, further investigations are required to fully clarify the anti-inflammatory effects of EA and its efficacy on neurotrophic signaling cascade in the diabetes condition.

Conflict of interest

The authors declare that there is no conflict of interest.

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