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Effects of Ginkgo Biloba Extract and Troxerutin on the Hippocampus of Adult Albino Rats after Induction of Diabetes Mellitus

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Authors' contributions

This work was carried out in collaboration among all authors. Author RI designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AM and MA managed the analyses of the study. Author FE managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

This study aimed to assess the effects of Ginkgo Biloba Extract and Troxerutin on the hippocampus of induced diabetes mellitus in adult albino rats using histological methods.50 adult male albino rats were divided into three groups; Group I (Control); Group II (diabetic): subdivided into Subgroup IIa (T1DM)), Subgroup IIb (T1DM+GBE), Subgroup IIc (T1DM+ troxerutin); Group III: subdivided into Subgroup IIIa (GBE) and Subgroup IIIb (troxerutin). The brain was removed and the cerebral hemisphere was coronally cut at the hippocampal level and used for light microscopic study (H&E staining and PCNA immunostaining). There was a statistically insignificant improvement in animal weights in subgroup IIb and subgroup IIc. Subgroup IIb showed a statistically significant reduction of blood glucose levels while the subgroup IIc showed insignificant reduction of blood glucose levels. Diabetes disturbed the light microscopic structure of the hippocampus. In subgroup IIb and subgroup IIc the hippocampus retained an apparently normal appearance and the stratum pyramidale exhibited the pyramidal cells with rounded vesicular nuclei and acidophilic cytoplasm. Diabetic hippocampal sections revealed negative PCNA immunoreactivity in all layers of DG. In subgroup IIb and subgroup IIc, hippocampal sections showed positive immunoreactivity.

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Keywords: Histological study; PCNA; morphometric study; ginkgo biloba extract; troxerutin.

1. INTRODUCTION

Diabetes mellitus is a major health problem worldwide, with an incidence expected to increase by 5.4% in 2025 [1]. It alters metabolism of lipids, carbohydrates and proteins that leads to oxidative stress and cell death in the brain, causing a state of dysfunctions in cognition and behavior [2].Increasing events demonstrates an association between diabetes and hippocampal neuron damagesuggesting that the diabetic condition aggrevates neuronal damage and cognitive failure [3].

The hippocampal formation (formed of the hippocampus proper, the dentate gyrus and the subiculum) is a key brain area for many forms of learning and memory. It is sensitive to changes in glucose homeostasis. Analysis of behavioral performance and hippocampal synaptic plasticity in experimental models of diabetes revealed inconsistent findings [4].

Currently, there is worldwide interest in finding new and safe antioxidants from natural resources. The use of synthetic antioxidants has decreased because of their suspected activity as carcinogenic promoters. Ginkgo biloba is mixture of active compounds extracted from G. biloba leaves. It is prepared as a dry powder and contains two groups of major substances, flavonoid (24%) and terpenoid (6%) fractions. It has been proposed that it has beneficial neuroprotective effects, probably due to its antioxidant action [5].

Troxerutin is a semisynthetic flavonoid, which alleviates the oxidative damage caused by D-galactose in the liver and kidney, as well as cognitive impairment [6,7,8]. A recent study suggested that troxerutin counteracts domoic acid-induced memory deficits in mice by inhibiting the inflammatory response and oxidative stress [9]. However, studies assessing troxerutin's effects on cognitive impairment in the context of diabetes mellitus are scare.

This experimental study was designed to throw a light on the effects of Ginkgo Biloba Extract and Troxerutin on the hippocampus of induced diabetes mellitus in adult albino rats using histological and immunohistochemical methods.

2. MATERIALS AND METHODS

2.1 Experimental Animals

In this study, 50 adult male albino rats about 200 grams weight aging 3-6 months were used and maintained under specific clean conditions in the animal house of Faculty of Medicine, Tanta University. The rats were housed in plastic cages with free access to water and food ad libitum with a constant 12 hours light/ 12 hours dark cycle and at a temperature maintained at 20-30°C. All animal procedures has been carried out in accordance with The Code of Ethics of EU Directive 2010/63/EU for animal experiments and approved by the Institutional Ethics Committee of Tanta University. The animals were divided into into three groups as follow:

Group I (Control): This group consisted of 10 rats and was subdivided into two equalsubgroups: Subgroup Ia (negative control): The rats of this subgroup were kept without any medication for 8 weeks.Subgroup Ib (vehicle control): The rats of this subgroup received 1ml citrate buffer as single intraperitoneal injection at the beginning of the study. Then after 4 weeks, they were given 1ml saline daily by intraperitoneal injection for 4 weeks.

Group II (diabetic group): This group included thirty rats. The animals received single intraperitoneal injection of Streptozotocin at a dose of 60 mg/kg after fasting overnight [10]. After 3 days of diabetes induction, fasting blood glucose level was measured using a portable glucose meter (smart test, ST-9C model). Rats with blood glucose levels exceeding 300 mg/dlfor 2 consecutive days were considered to be diabetic, and assessed in this study [11]. After 4 weeks from diabetic induction, diabetic rats were divided into three equal subgroups; Subgroup IIa(type1 diabetes mellitus (T1DM)):The rats received 1 ml saline daily by intraperitoneal injection for 4weeks. Subgroup IIb (T1DM+GBE):The rats recieved ginkgo biloba extract (GBE) at a dose of 100 mg /kg daily dissolved in saline as mentioned before by intraperitoneal injection for 4weeks [12]. Subgroup IIc(T1DM+ troxerutin):The rats recieved troxerutin at a dose of 60 mg /kg daily dissolved in saline as mentioned before by intraperitoneal injection for 4weeks [13].

Group III: This group included ten rats and was equally divided into two subgroups ;Subgroup IIIa (GBE): The rats recieved ginkgo biloba extract at a dose of 100 mg /kg daily by intraperitoneal injection [12]. Subgroup IIIb (Trox):The rats received troxerutin at a dose of 60 mg /kg daily by intraperitoneal injection [13].

Drinking, eating, urine volume and fur colour were observed. Body weights and fasting blood glucose (FBG) levels of rats were recorded weekly.

2.2 Drugs

Streptozotocin: Streptozotocin was obtained from Cornell lab-chemistry company, Cairo, Egypt. It was freshly dissolved in citrate buffer (PH = 4.5) and given by single intraperitoneal injection at a dose of 60 mg/kg [10].

Ginkgo biloba extract: Ginkgo biloba extract was obtained as capsules (260 mg/capsule) from EMA pharm, Cairo, Egypt. Each hard gelatin capsule contains: Ginkgo Biloba Leaf Powder Extract 260 Mg. Standardized as: Ginkgo Flavones Glycosides NLT 24%. Total Ginkgolides (Lactones) NLT 6 %. The content of the capsule was dissolved in saline and the formed solution concentration was 20 mg/ml. Each rat was given the formed solution at a dose of 100 mg /kg daily by intraperitoneal injection [12].

Troxerutin: Troxerutin was obtained as dry powder from Minapharm, Cairo, Egypt. The powder was dissolved in saline and the formed solution concentration was 12 mg/ml. Each rat was gives the formed solution at a dose of 60 mg /kg daily by intraperitoneal injection [13].

2.3 Sample Collection

At the end of the experiment, all rats were anaesthetized using thiopental sodiumat a dose of 30 mg/kg IV [14]. Then, the rat was decapitated immediately rostral to the first cervical vertebra. Using the scalpel, an incision was made in the middle of the scalp. Then, the skull plates were cut with scissors and separated from the brain. The brain was removed safely and the two cerebral hemispheres were separated from cerebellum and cut at the longitudinal fissure. the cerebral hemisphere was coronally cut at the hippocampal level and fixed in 10% neutral buffered formalin for light microscopic study. Finally sacrificed rats were safely collected in a special package according to safety and health precaution measures to be incinerated later.

2.4 Light Microscopic Study

In order to preserve the tissue, it is fixed in 10% neutral buffered formalin for 24 hours then dehydrated, cleared in xylene and embedded in paraffin. Embedding allows storage of the specimen in a block of wax. Then sectioning is done on a microtome which cuts sections by 5 microns, passed on a water bath then placed on microscope slides. The slides are then dried in an oven or on a hot plate to remove moisture and help the tissue adhere to the slide to be ready for staining.

2.4.1 Hematoxylin and eosin stain [15]

H&E stain were used to study the general histological structure of rat's hippocampus in all groups**.** The first step before staining is removal of wax from the slide by xylene. Sections need rehydration after dewaxing. This is done by putting the slides in descending series of alcohol from 90%, 70% to 50%. Then the slides were immersed in distilled water. The sections were stained in hematoxylin for 15 minutes and washed in tape water for 10 minutes. Then, they were stained in eosin for 1 minute. When a stain is complete the section is covered with a coverglass that makes the preparation permanent. The slides allowed to be dried for few minutes.

2.4.2 Immunohistochemistry (Proliferating cell nuclear antigen - PCNA)

Anti PCNA rabbit polyclonal antibody is used to detect proliferating cells [Primary antibody: PCNA Ab-1, dilution 1: 500 (Clone PC10), Lab Vision Corporation laboratories, CA 94539, USA, catalogue number MS-106-P]. Paraffin sections were deparaffinized in xylene for 1-2 minutes, rehydrated in descending grades of ethanol then brought to distilled water for 5 minutes. Sections were incubated in hydrogen peroxide for 30 minutes then rinsed in phosphate buffer saline (PBS) for 3 times (2 minutes each). Antigen retrieval was done by immersing the slides in 10mm citrate buffer, pH 6.0, for 10-20 minutes at 100°C in a microwave then cooling at room temperature for 20 minutes. To reduce non-specific staining blocking of tissue was done with protein blocking reagent for 30 minutes. Each section was incubated for 60 minutes with 2

drops (= 100 µl) of the primary antibody (PCNA). Slides were rinsed well in PBS (3 times, 2 min. each), incubated for 20 minutes with 2 drops of biotinylated secondary antibody for each section then rinsed well with PBS. Each section was incubated with 2 drops enzyme conjugate"Streptavidin-Horseradish peroxidase" for 10 minutes at room temperature then washed in PBS. Substrate-chromogen(DAB) mixture (2 drops) was applied to each section and incubated at room temperature for 5-10 min. then rinsed well with distilled water. Slides were counterstained with hematoxylin, dehydrated and mounted. Immunoreactive cells showed brown deposits. All steps were performed in a humidity chamber to prevent drying of the tissues [16].

Brain tissue sections were examined using Olympus BX 50 Automated microscope. The images were digitized using a Olympus digital video camera (model NO.E-330 DC 7.4v).

2.4.3 Morphometric study

The image analysis was done by using the software (Image J 1.48) (National Instate of Health,Bethesda, Maryland, USA).Five different non-overlapping randomly selected fields from each slide were quantified for the mean number of PCNA immunopositive cells were measured in DG of all immunostained hippocampal sections (at x 400 magnification).

2.5 Statistical Analysis

Data were tabulated and statistically analyzed to evaluate the difference between the groups as regards the various parameters using the IBM SPSS (version 21) statistical package. All data were expressed as the means ± standard errors of the means (SEM). Comparisons between groups were assessed using one-way ANOVA, followed by Post Hoc LSD multiple comparison tests. Results were considered statistically significant when P was < 0.05.

3. RESULTS

3.1 Effects on Body Weight

There was no statistically significant difference between the mean body weight of the control subgroups, therefore they were pooled in one group(control) and the mean of the body weight was 202.20±5.87 g . The T1DM subgroup IIa was characterized by reduction of body weights (126.70± 8.58 g vs. 202.20±5.87 g, *P*< 0,001) compared with the control group. There was a statistically insignificant improvement in animal weights in subgroup IIb (T1DM+GBE) and subgroup IIc (T1DM+ troxerutin) compared with the T1DM subgroup IIa and their body weight means were 133.60 ± 5.32 g and 132.1 ± 11.0 g respectively vs. 126.70 ± 8.58 g. There was no statistically significant difference between the mean body weight of the control group and group III (Table 1) (Graph 1).

3.2 Effects on Blood Glucose Level

There was no statistically significant difference between the mean blood glucose level of the control subgroups, therefore they were pooled in one group (control) and the mean of blood glucose level was 123.90±8.72 mg/dl. The T1DM subgroup IIa was characterized by a statistically significant increasing of blood glucose levels (527.0±43.9 mg/dl vs. 123.90±8.72 mg/dl, *P*< 0.001). The subgroup IIb (T1DM+GBE) showed a statistically significant reduction of blood glucose levels (482.7± 40.0 vs. 527.0±43.9 mg/dl, P = 0.009) compared with the T1DM subgroup IIa. However, The subgroup IIc (T1DM+ troxerut in) showed insignificant reduction of blood glucose levels (520.2 ± 39.7 vs. 527.0 ± 43.9 mg/dl)compared with the T1DM subgroup IIa. There was no statistically significant difference between the mean blood glucose level of the control group and groupIII (Table 1) (Graph 1).

3.3 Light Microscopic Analysis

Examination of the hippocampal sections of the control subgroups and group III showed the same histological features. The hippocampus of the control rat showed four hippocampal areas, (CA1, CA2, CA3, CA4) and the dentate gyrus(DG), with its upper limb and lower limb. Hippocampal layers was formed from : strata alveus, oriens, pyramidale, radiatum and lacunosum molecular(Fig. 1).

The stratum pyramidale of CA1 in the control group contained small pyramidal cells with rounded vesicular nuclei, prominent nucleoli and apical dendrites that extended into the stratum radiatum. A few small blood vessels were observed (Fig. 2a.) The CA1 region in the T1DM (subgroup IIa) rats displayed small shrunken pyramidal cells with an acidophilic cytoplasm and pyknotic or fragmented nuclei. The apical processes of the cells were nearly lost (Fig. 2b). In subgroup IIb (T1DM+GBE), CA1 retained an apparently normal appearance and the stratum

pyramidale exhibited the small pyramidal cells with rounded -vesicular nuclei and wellformed apical dendrites (Fig. 2c). In subgroup IIc (T1DM+trox), the CA1 stratum pyramidale exhibited a large number of small pyramidal cells with rounded vesicular nucleiand acidophilic cytoplasm. The apical process of the cells can be identified (Fig. 2d).

In the control group, CA3 hippocampal pyramidal cells were well arranged in 4-5 compact layers, with rounded vesicular nuclei and prominent nucleoli (Fig.3a). The hippocampal sections of T1DM subgroup IIa exhibited reduction in thickness and distortion of the stratum pyramidale. Shrunken pyramidal cells with deeply stained nuclei (karyopycknosis), red neurons with pericellar halo and neuropil vacuolations were detected (Fig. 3b). These abnormalities were relatively reduced and retained its apparently normal appearance in sections of subgroup IIb (T1DM+GBE) and subgroup IIc (T1DM+trox) in which the stratum pyramidale (SP) exhibiting multiple layers of closely packed large pyramidal cells with rounded vesicular nuclei and acidophilic cytoplasm. Also, the apical processes of the cells can be identified in the subgroup IIb (T1DM+GBE) (Fig. 3c and d).

The CA4 area contained closely packed pyramidal cells arranged in 2-3 layers with rounded vesicular nuclei and prominent nucleoli in the control group (Fig. 4a). In sections of the T1DM subgroup IIa, the stratum pyramidale showeddistorted small shrunken pyramidal cells with deeply stained nuclei, red neuron with pericellar halo and multiple glial cells (Fig. 4b). In subgroup IIb (T1DM+GBE) and subgroup IIc (T1DM+trox) hippocampal sections, CA4 showed many normal pyramidal cells with large, rounded, vesicular nuclei. A few degenerated and shrunken cells were observed (Fig. 4c and d).

Table 1. The mean values (±SD)of the body weight and blood glucose level in different studied groups

Group	Body weight (mg) (mean± SD)	Blood glucose level (mg/dL) (mean± SD)	ANOVA	
			F	P value
Group I (Control)	202.20 ± 5.87	123.90 ± 8.72	F1=199.042	P1<0.001*
Subgroup IIa (T1DM)	126.70 ± 8.58	527.0 ± 43.9	F2=290.937	P2<0.001*
Subgroup IIb(T1DM+GBE)	133.60 ± 5.32	482.7 ± 40.0		
Subgroup IIc (T1DM+troxerutin)	132.1 ± 11.0	520.2 ± 39.7		
Subgroup IIIa(GBE)	201.1 ± 7.58	119.8 ± 7.26		
Subgroup IIIb(Troxerutin)	195.8 ± 4.25	122.4 ± 9.24		

Post Hoc LSD for body weight

I&IIa	I&IIb	I&IIc	IIa&IIb	IIa&IIc	IIb&IIc
<0.001*	<0.001*	<0.001*	0.63	0.14	0.67

Post Hoc LSD for blood glucose

I&IIa	I&IIb	I&IIc	IIa&IIb	IIa&IIc	IIb&IIc
<0.001*	<0.001*	<0.001*	0.009*	0.675	0.025*

P1and F1 for body weight (BW); P2 and F2 for body glucose (BG); (*) P≤ 0.05=Significant

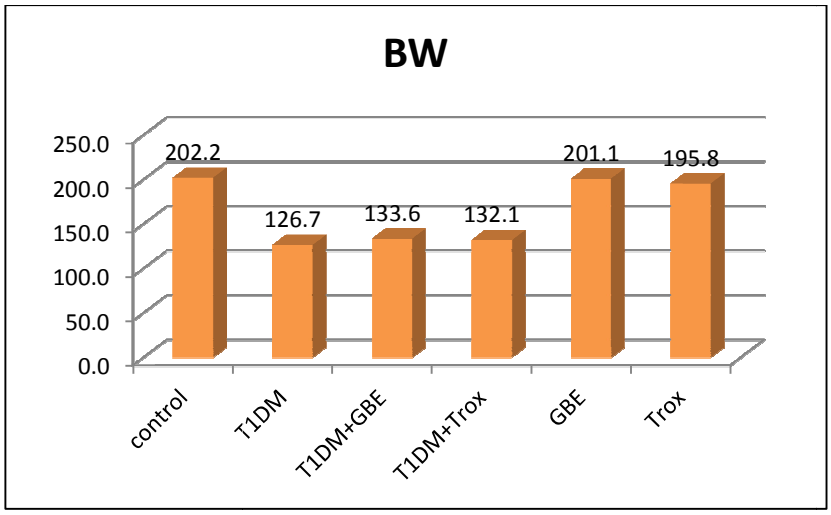
Table 2.The mean values (±SD)of the numbers of PCNA immunopositive cells in dentate gyrus in different studied group

Groups	PCNA immunopositive cells (mean± SD)	*F*	*P* value
Group I (Control)	32.5 ± 6.186	F=49.592	P <0.001*
Subgroup IIa (T1DM)	9.6 ± 4.835		
Subgroup IIb (T1DM+GBE)	23.3 ± 2.869		
Subgroup IIc (T1DM+ troxerutin)	18.9 ± 1.791		
Subgroup IIIa (GBE)	29.9 ± 5.85		
Subgroup IIIb (Troxerutin)	31.5 ± 7.25		

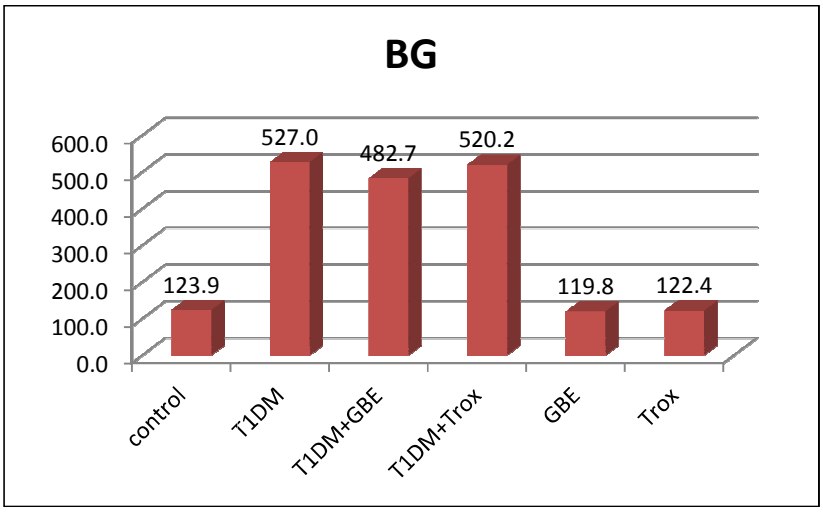
Post Hoc LSD for PCNA immunopositive cells

I&IIa	I&IIb	I&IIc	IIa&IIb	IIa&IIc	IIb&IIc
<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	0.027*

(*) P≤ 0.05 =Significant; SD = standard deviation



(A)



(B)

Graph 1. The means of a) body weight b) blood glucose level in different studied groups

The DG consisted of stratum moleculare, stratum granulosum and stratum pleomorph. The stratum granulosum contained numerous densely packed granular cells with rounded vesicular nuclei and some small dark immature cells with oval nuclei extending among the granular cells from the subgranular zone in the control group (Fig. 5a). In sections of the T1DM subgroup IIathe upper and lower limbs of the DG exhibited a large number of degenerated cells with pyknotic nuclei and a few mature granular cells (Fig. 5b). Neuropil Vacuolation was detected in other sections (Fig. 5b*). In sections of subgroup IIb (T1DM+GBE) and subgroup IIc (T1DM+trox), the stratum granulosum exhibited a large number of mature granular cells and a few number of the degenerated cells. An apparently little neuropil vacuolation was demonstrated comparing with T1DM subgroup IIa (Fig. 5c and d).

Hippocampal sections of Control group revealed positive brown nuclear immunoreactivity mainly in subgranular layer of DG. Few immunoreactive nuclei were detected in molecular and polymorphic layers (Fig. 6a). The sections of T1DM subgroup IIa revealed negative immunoreactivity in all layers of DG (Fig. 6b). In subgroup IIb (T1DM+GBE) and subgroup IIc

(T1DM+trox) hippocampal sections, positive immunoreactivity was detected in the DG (Fig. 6c and d). Hippocampal sections of T1DM subgroup IIa revealed a significant decrease in the mean numbers of PCNA immunopositive cells in comparison with the control rats. In subgroup IIb (T1DM+GBE) and subgroup IIc (T1DM+trox), there was a significant increase in the mean number of PCNA immunopositive cells compared to T1DM subgroup IIa with better result in subgroup IIb (T1DM+GBE). There was insignificant difference concerning the mean number of PCNA immunopositive cells between the control group, subgroup IIIb (GBE) and subgroup IIIc (trox) (Table 2).

4. DISCUSSION

Cognitive dysfunction is present in 30% to 40% of elderly DM patients. The severity of cognitive impairment has a direct relationship with poor glycemic control in diabetic patients [17]. Diabetes affects the content of several exocytotic proteins in hippocampus and induced cognitive impairment and memory loss in diabetic humans and animal models [18].

Uncontrolled hyperglycemia in diabetes can disturb neurochemical profiles and cerebral blood flow with structural abnormalities which may involve direct neuronal damage in the brain. The hippocampus is considered as a special target for abnormalities associated with diabetes [19,20].

Accordingly, the present study was designed to assess the hippocampal damage resulting of experimental diabetes. Also, the effects of Ginkgo biloba extract (GBE) and troxerutin on the hippocampus in T1DM rats induced by STZ were assessed in order to find a better neuroprotective drug for hippocampus against diabetic damage.

Streptozotocin (STZ) is frequently used to induce diabetes in experimental animals through its toxic effects on pancreatic -cells and as a potential inducer of oxidative stress. It has been reported that diabetes induced by STZ is the best characterized system of xenobiotic-induced diabetes and the commonly used model for the screening of antihyperglycemic activities [21,22].

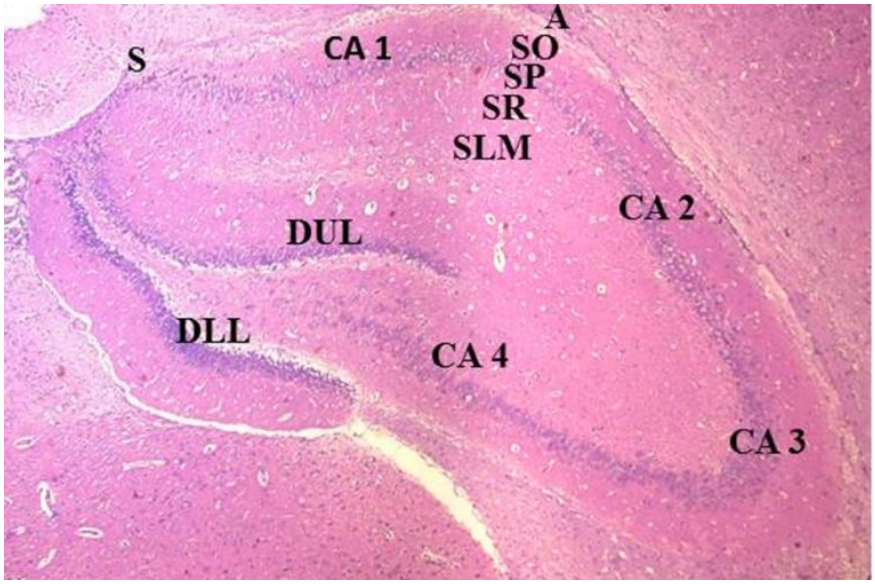


Fig. 1. A photomicrograph of a section in the hippocampus of an adult control albino rat showing different areas of hippocampal formation ; subiculum (S), hippocampus proper and dentate gyrus . The hippocampus proper is divided into four cornuammonis areas (CA1, CA2, CA3 and CA4). Hippocampal layers appear as alveus (A), stratum oriens (SO), stratum pyramidale (SP), stratum radiatum (SR) and stratum lacunosum molecular (SLM). Dentate gyrus is seen surrounding CA4 by its upper limb (DUL) and lower limb (DLL)

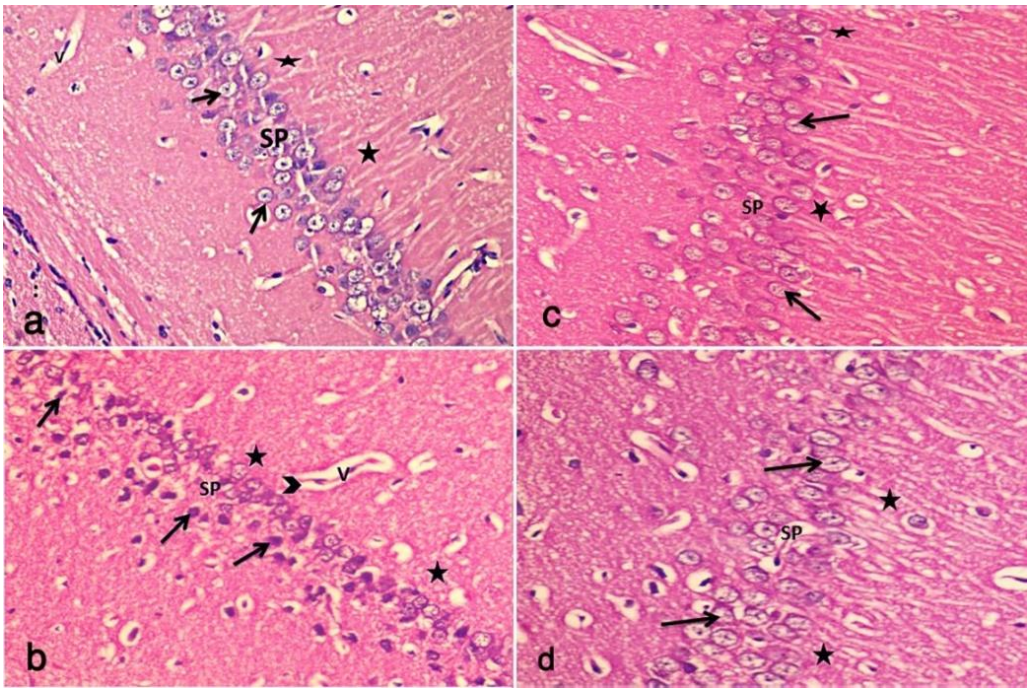


Fig. 2. A photomicrograph of the hippocampus of an adult albino rat showing CA1 area. a) The control group: The stratum pyramidale (SP) contains 3-4 layers of pyramidal cells having rounded vesicular nuclei, prominent nucleoli (arrows) and apical dendrites (stars) extending into the stratum radiatum . A few small blood vessels are observed (v). b) The T1DM subgroup IIashowingdecrease in the stratum pyramidale (SP) thickness into 1-2 layers that exhibits a large number of small shrunken pyramidal cells with pyknotic nuclei and acidophilic cytoplasm(arrows). The apical processes of the cells were nearly lost (star). Dilated blood vessels (V) with wide perivascular spaces (arrow head) are observed.c) The subgroup IIb (T1DM+GBE) showing normal appearance of the stratum pyramidale (SP) which exhibits a large number of small pyramidal cells with rounded vesicular nuclei and acidophilic cytoplasm (arrows). The apical processes of the cells (stars) is well formed. d) thesubgroup IIc (T1DM+trox) showing an apparently normal appearance. The stratum pyramidale (SP) exhibits a large number of small closely packed pyramidal cells with rounded vesicular nuclei and acidophilic cytoplasm (arrows). The apical process of the cells can be identified (stars)

In the present study, STZ caused a significant weight loss of the recieved rats and a significant increase in fasting blood glucose. Swanston-Flatt et al. [23] reported that weight loss of rats was due to increased muscle wasting and due to loss of tissue proteins. Kavalali et al. [24] found that STZ-induced diabetis was caused by destruction of β-cells of the islets of Langerhan.

Treatment of T1DM animals with GBE in this study, resulted in insignificant improvement of animal weight. Cheng et al. [25] reported that treatment with GB at different concentrations suppressed the decrease in the body weight due to hyperglycemic condition which indicates the prevention of muscle tissue damage.

The treatment of T1DM animals with GBE in this study, resulted in significant reduction of blood glucose level. Accordingly, [26,27] reported that blood glucose reduction is time and dose dependent and blood glucose values returned to near normal values after administration of GBE at a dose of 300 mg / kg for 30 days. Zhou et al. [28] reported that ingestion of 120 mg of GBE as a single dose for 3 months for individuals leads to an increase in pancreatic β cell function .It was proposed that GBE improved insulin sensitivity mainly by enhancing insulin receptor substrate 2 transcription and preventing insulin resistance. Also, [25] added that reduction of blood glucose may be either due to the increased level of plasma insulin in diabetic rats which may influence the stimulation of pancreatic insulin

secretion from βcells in islets of Langerhans, or due to the enhanced transport of blood glucose to peripheral tissue. So, GBE is a promising antidiabetic drug.

In the present study, treatment of T1DM animals with troxerutin resulted in no significant improvement of both animal weight and blood glucose level. This is in accordance with the studies of [29] on the effect of troxerutin in STZ induced diabetic retinopathy and [30] on the beneficial effect of troxerutin on diabetes-induced vascular damages. On the other hand, [31] found that troxerutin significantly reduced the level of blood glucose in type II diabetic rats induced by high cholesterol diet. Similarly, hypoglycemic effect of troxerutin was reported by [32] in mice fed with high fat-fructose diet and by [33] in sucrose-induced type II diabetic rats. This contrary may be due to the type of diabetic models used in different experiments.

Troxerutin may have no effect on pancreatic beta cells and insulin secretion and subsequent changes in blood glucose levels in STZ-induced type I diabetic models as beta cells were destructed by STZ, which makes the cells less active leading to poor sensitivity of insulin for glucose uptake by tissues and results in chronic hyperglycemia [34]. In type II diabetes, beta cells are intact and therefore troxerutin may facilitate insulin secretion and/or its signaling leading to uptake of glucose from the blood that represents the most important process to regulate glucose homeostasis [33].

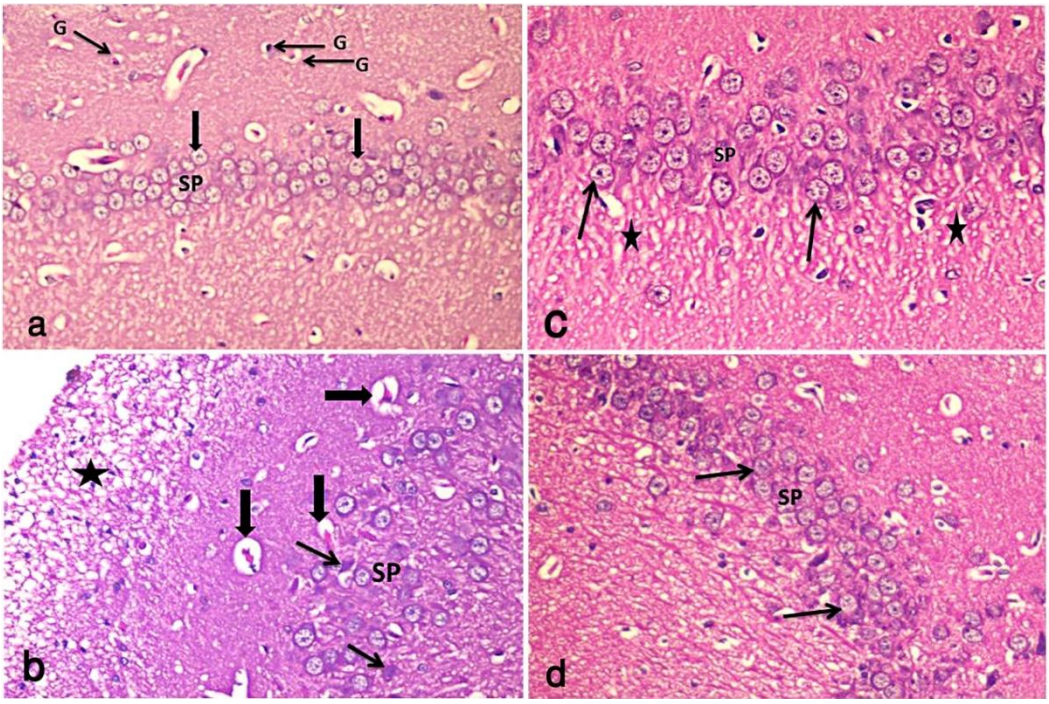


Fig. 3. A photomicrograph of the hippocampus of an adult albino rat showing CA3 area.a) The control group: The stratum pyramidale (SP) contains 4-5 compact layers of pyramidal cells. These cells have rounded vesicular nuclei and prominent nucleoli (thick arrows). Many glial cells (G) appear among the neuronal processes. b) The T1DM subgroup IIashowing reduction and distortion of stratum pyramidale (SP). Small shrunken pyramidal cells with deeply stained nuclei (thin arrows) and acidophilic cytoplasm are observed. Also, there are red neurons (thick arrow) with pericellar halo and marked neuropil vacuolations (star).The subgroup IIb (T1DM+GBE) showing normal appearance of the stratum pyramidale (SP) which exhibits multiple layers of closely packed large pyramidal cells with rounded vesicular nuclei and acidophilic cytoplasm (arrows). The apical processes (stars) of the cells can be identified. d) thesubgroup IIc (T1DM+trox) showing an apparently normal appearance of stratum pyramidale (SP) with a large number of closely packed large pyramidal cells with rounded vesicular nuclei and acidophilic cytoplasm (arrows)

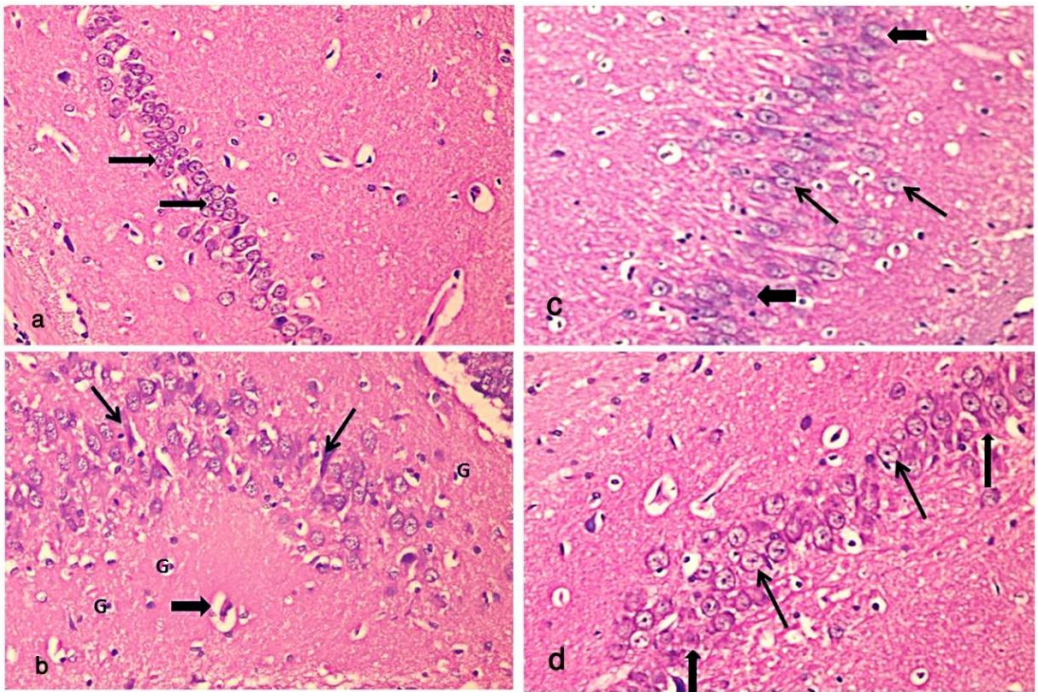


Fig. 4. A photomicrograph of the hippocampus of an adult albino rat showing CA4 area.a) The control group:The stratum pyramidale (SP) is composed of closely packed 2-3 rows of pyramidal cells (arrows) with rounded vesicular nuclei and prominent nucleoli. b) The T1DM subgroup IIashowing distorted small shrunken pyramidal cells with deeply stained nuclei and acidophilic cytoplasm (thin arrows). Also, there are multiple glial cells (G) and a red neuron (thick arrow) with pericellar halo. c) The subgroup IIb (T1DM+GBE)showingmany normal pyramidal cells with rounded vesicular nuclei (thin arrows) and a few degenerated cells cells with deeply stained pyknotic nuclei (thick arrows). d) thesubgroup IIc (T1DM+trox) showing large number of normal pyramidal cells with rounded vesicular nuclei and acidophilic cytoplasm (thin arrows). A few degenerated cells with deeply stained pyknotic nuclei can be seen (thick arrows)

In this research, examination of hippocampus sections of the diabetic rats revealed marked effects of diabetes in the form of cell death in several areas with disruption of normal layer organization. The hippocampus of the T1DM subgroup displayed small shrunken neuronal cells with an acidophilic cytoplasm and pyknotic or fragmented nuclei with reduction in thickness and distortion of the layers. Neuropil vacuolation was also detected.

These results were in accordance with [35] who reported that the free radicals generated due to oxidative stress may develop several adverse effects commonly seen in diabetes such as neuropathy, nephropathy, retinopathy, and vascular disorders.

Several authors [36,37] reported that diabetes mellitus is associated with increased oxidative stress in central nervous system in particular hippocampus. Rise in free radical activity is suggested to play an important role in lipid peroxidation and protein oxidation of cellular structures resulting in cell injury and implicated in the pathogenesis of vascular disease which are the mainly cause of morbidity and mortality in diabetes [38].

In this study, hippocampal sections of subgroup IIb (T1DM+GBE) retained an apparently normal appearance and the stratum pyramidale exhibited many normal pyramidal cells with rounded -vesicular nuclei and wellformed apical dendrites.The stratum granulosum in DG exhibited a large number of mature granular cells and a few number of the degenerated cells. An apparently little neuropil vacuolation was demonstrated comparing with T1DM subgroup IIa.

GBE serves as a neuroprotective agent, an antioxidant, a free-radical scavenger, a membrane stabilizer, and an inhibitor of the platelet-activating factor [39,40,41,42]. Also, It was reported that the control of hyperglycemia leads to improvement in oxidative stress profile and enhancing antioxidant defense mechanisms in pancreatic islets. GBE is a complex mixture of ingredients with a unique broad spectrum of pharmacological activities so it probably acts through several different mechanisms by increasing levels of free radical scavenging enzymes and/or enhancing antioxidant ability [43].

Nathan [44] reported that GBE improves cognitive function through the interaction with the antioxidant and cholinergic systems. Robertson et al. [45] demonstrated that antioxidants have been shown to brake the worsening of diabetes by improving - cells function in animal models and suggested that enhancing antioxidant defense mechanisms in pancreatic islets may be a valuable pharmacologic approach to managing diabetes. Major antioxidant enzymes are the first line of the antioxidant defense system against Reactive oxygen species (ROS) generated in vivo during oxidative stress and act cooperatively at different sites in the metabolic pathway of free radicals [46]. Reduced activities of antioxidant enzymes in the liver and pancreas have been observed in diabetic rats. Administration of GBE for 30 days increased their activity [25].

Chronic administration of GBE reduced the progressive memory decline and neural circuits loss that are associated with aging and improved working memory and executive processing in both healthy individuals and rats [47,48]. GBE increased the oxidation resisting capacity of the hippocampus so preventing the hippocampus neurons from lipid peroxidation damage [49].

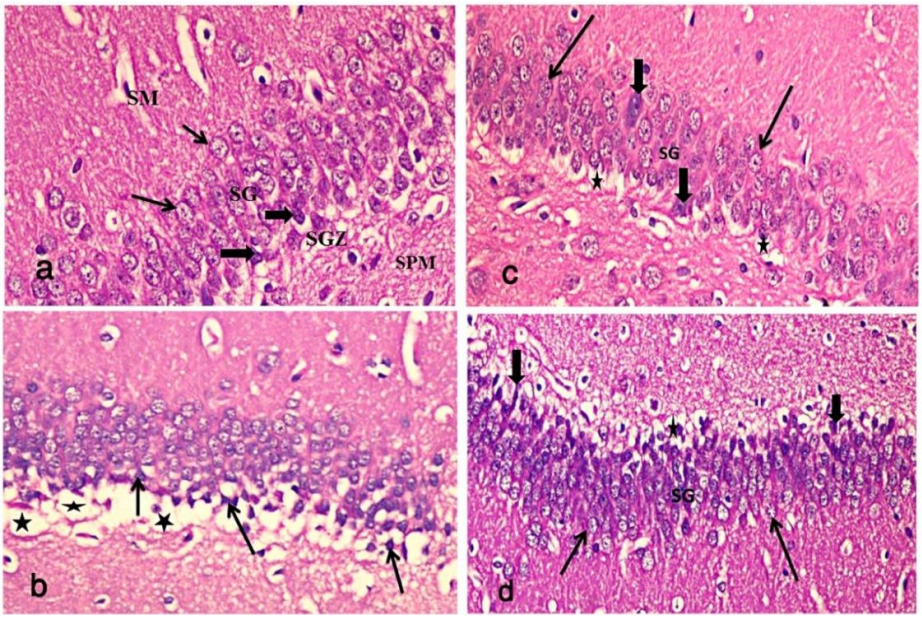


Fig. 5. A photomicrograph of the hippocampus of an adult albino rat showing dentate gyrus.a) The control groupshowing the stratum moleculare (SM), Straumpleomorph (SPM) and stratum granuolosum (SG) that contains numerous densely packed granular cells (thin arrows) with rounded vesicular nuclei. Immature cells (thick arrows) appear as small dark cells with oval nuclei extending among granular cells from the subgranular zone (SGZ). b) The T1DM subgroup IIashowing large number of granular cells with pyknotic nuclei (arrows). Marked vacuolations of neuropil are observed (stars). c) The subgroup IIb (T1DM+GBE)showingthe stratum granulosum (SG) exhibits a large number of mature granular cells with rounded vesicular nuclei (thin arrows). A few degenerated cells with deeply stained pyknotic nuclei (thick arrows) are seen. Apparently little vacuolations (stars) of neuropil are observed. d) the subgroup IIc (T1DM+trox) showing the stratum granulosum (SG) that exhibits numerous densely packed mature granular cells (thin arrows) and few degenerated cells with deeply stained pyknotic nuclei (thick arrows). Apparently little vacuolations (stars)of neuropil are observed in the subgranular zone

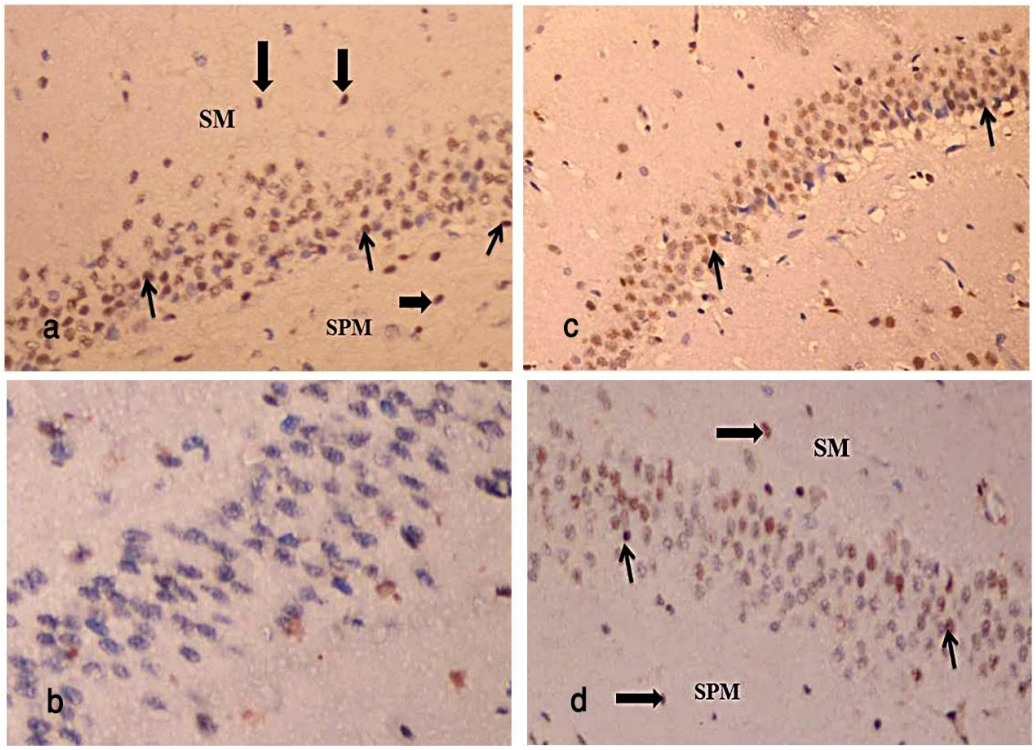


Fig. 6. A photomicrograph of the hippocampus of an adult albino rat showing dentate gyrus.a) The control groupshowing many granule cells with nuclear immunoreactivity. The immunoreactivity occupies mainly sub granular zone cells (thin arrows). Few immunoreactive nuclei are detected in the stratum moleculare (SM) and the straumpleomorph (SPM) (thick arrows). b) The T1DM subgroup IIashowing negative nuclear immunoreactivity in all layers of DG. c) The subgroup IIb (T1DM+GBE)showing many granule cells with nuclear immunoreactivity. The immunoreactivity occupies mainly SGZ cells (arrows). d) thesubgroup IIc (T1DM+trox) showing many granule cells with nuclear immunoreactivity. The immunoreactivity occupies mainly SGZ cells (thin arrows). Few immunoreactive nuclei are detected in the stratum moleculare (SM) and the straumpleomorph (SPM) (thick arrows)

The hippocampal sections of subgroup IIc (T1DM+trox) in the present study retained its apparently normal appearance and the abnormalities were relatively reduced. The stratum pyramidale (SP) exhibiting multiple layers of closely packed pyramidal cells with rounded vesicular nuclei and acidophilic cytoplasm. The stratum granulosum in DG exhibited numerous densely packed mature granular cells and few degenerated cells.

Troxerutin has a variety of biological activities including anti-oxidative, anti-inflammatory and anti-thrombolytic properties. Previous experiments confirmed tissue protective effect of troxerutin in kidney, liver and brain injuries [13,50,51,52,53,31,9,54,55].

Several authors [56,57] indicate that troxerutin alters oxidative stress parameters and improves the antioxidant ability of rats. Troxerutin alleviates memory deficits and cognitive impairment and some studies were done to determine its mechanism. Malondialdehyde (MDA) is an endogenous genotoxic product of enzymatic and oxygen radical-induced lipid peroxidation in DNA isolated from healthy human samples; it increased in diabetes. Superoxide dismutase (SOD) and Glutathione S-transferase (GSH) are important for the antioxidant defense, with activity or level decreased in diabetes [58,59].

Besides its antioxidant effect in improving cognitive impairment, troxerutin may also be involved in platelet aggregation, blood circulation improvement, vascular endothelium protection, and thrombosis prevention. Further studies are needed to test this possibility. Overall, troxerutin alleviates oxidative stress and promotes learning

potential in STZ-induced diabetic rats and should be further assessed for the potential use of troxerutin in the clinic [60].

Because of its close relation to the cell cycle, PCNA is used as a physiological or pathological marker protein of proliferating cells [61]. Proliferating cell nuclear antigen (PCNA) is a 36-kDa protein known as the DNA polymerase δauxiliary factor and it is required for DNA replication and repair PCNA expression shows periodic fluctuation in accordance with the cell cycle. PCNA is synthesized during the late G1-early S phase of the cell cycle, immediately preceding the onset of DNA synthesis, is most abundant during the S phase, and declines during the G2/M phase [61]. In this study,sections of T1DM subgroup IIa revealed a significant decrease in the mean numbers of PCNA immunopositive cells. In subgroup IIb (T1DM+GBE) and subgroup IIc (T1DM+trox), there was a significant increase in the mean number of PCNA immunopositive cells with better result in subgroup IIb (T1DM+GBE).

Proliferation of progenitor cells is observed during the adult period [62,63]. Bayer et al. [64] indicated that most neurons cease proliferating during the early postnatal period. However, some granule cells in the olfactory bulb, dentate gyrus and cerebellum exceptionally continue to proliferate at the juvenile and adult periods especially in the subventricular zone (SVZ) and the subgranular zone of the dentate gyrus. Adult neurogenesis refers to the process by which stem cells located in the subgranular zone of the dentate gyrus undergo sequential stages of proliferation, migration and neuronal differentiation before incorporated into the existing adult hippocampal network [65,66,67].

Recently, the neurogenesis, the proliferation of new neurons in the adult brain, is strongly reduced in STZ-treated mice [19]. So, the understanding and treating of human brain disorders that implicate hippocampal dysfunction like diabetes mellitus may require consideration of the status and potential involvement of ongoing neurogenesis in the dentate gyrus of affected patients.

5. CONCLUSIONS

- The results of the study may be helpful in understanding the complications of diabetes on the hippocampal neurons and in the prevention of this neuronal damage with GBE and troxerutin.
- GBE possesses antihyperglycemic and antioxidant activities in STZ-induced diabetes. It enhances the memory by its protection of all hippocampal areas against diabetic damage.
- Troxerutin alleviates the oxidative stress and relieves the altered hippocampal histology in diabetic rats with its major effect on CA3.
- Diabetes affects the adult neurogenesis in DG. On the other hand, GBE and troxerutin improves adult neurogenesis but further work is necessary to elucidate in detail the mechanism of action of the GBE and troxerutin at the molecular levels.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The Code of Ethics of EU Directive 2010/63/EU for animal experiments and approved by the Institutional Ethics Committee of Tanta University.

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- ✓ Declarations of interest: none.
- ✓ All authors have participated in the research
- ✓ All authors read and approved the final manuscript

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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