Research Paper Medical Science

Anatomical Characterization of Wistar Rat Brain Tissue After Diabetic Induction by Transmission Electron Microscope and Relative Behavioral Changes

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ABSTRACT

the aim of this study was to study the physiological and histological effects in rats after induction of diabetes by streptozotocin (STZ). Mmethod: the study carried out on 8 male rats (Wister species), weight 353±90 g, about 3 months old, after incubation period of diabetes inducting by STZ (1 week), kept in a laboratory (in a 12/12 h dark-light cycle, 23.4ºC), feed on normal rat chow and tap water, then the experiments were performed. The tests performed were behavioral tests (Hole board, Pole tests) and histological test. Results: the behavioral tests showed that: The total locomotors activity was decreased as significant decreases in the head-dipping episode number ($p<0.05$) and significant increase was observed in the latency to the first grooming episode ($p<0.001$) as well the t-turn for the diabetic rats was increased significantly ($P = 0.004$) relative to control group with about 45%, while their pole time decreased significantly ($P = 0.0005$) relative to control group with about 38 (time pole/s). The histology showed that: there is an increase in meningeal thickness and a higher density of circulating cells in the meninges ($p<0.05$), mitochondria showed alteration in form of swollen and/or vacuolation, alteration in apoptotic process, microglia-like cells in the neuropil and Numerous activated brain macrophages were observed and myelin disturbances in the neuropil.

KEYWORDS Diabetes, Rats, Behavior, Brain, histology

INTRODUCTION

Diabetes mellitus can be defined as a group of at least three metabolic diseases whose hallmark is hyperglycemia resulting from the loss of the ability of the body to produce insulin (insulin-dependent diabetes mellitus or type 1 diabetes), or the loss of the ability of the body to control glucose metabolism in either subjects displaying hyperinsulinemia (non-insulin-dependent diabetes mellitus or type 2 diabetes), or in pregnant women (gestational diabetes or type 3 diabetes) [1]. Diabetes mellitus results in high increases in healthcare costs, loss of labor productivity and decreased rates of economic growth [1]. According to WHO 347 million people worldwide were suffering from diabetes, in 2012, with about 471 billion USD healthcare expenditure, and diabetes-related death toll is expected to double by horizon 2030 [2, 3]. Alarmingly in a diabetes mellitus epidemiological study performed between 2005 and 2007 in pediatric patients of five different medical centers and hospitals in Khartoum state, showed a marked increase in the prevalence of diabetes mellitus as in 2005 were 83,702 against 97,399 in 2007, which is expected to be in the top 10 of diseases leading to hospitalization in Sudan [4, 5]. Also Ali Omer et al, [6] stated that: the diabetes has been as endemic disease in central Sudan (Khartoum & Jazeera) representing 55% and in the west of Sudan representing 38%. In view of diabetes impact in tissue changes and other physiological disorders, Ali Omer et al, [6] stated that: the impact of diabetes duration was a reduction in size significantly as $R^2 = 0.61$ and 0.55 for the left and right kidney respectively. And the kidneys size were so enlarged as 92.4 ± 11.7 and 121 ± 17.1 for the right and left kidney respectively in early stage of diabetes; while in late case of Diabetes, the kidney is more echogenic, atrophied size with loss of corticomedullary differentiation. The experimental evidence also indicates that hyperglycemic conditions are associated often with peripheral and central nervous system alterations [7, 8]. However, while peripheral nervous system alterations in diabetes mellitus are well documented, surprisingly, very few are known about central nervous system alterations and the brain in particular. This is quite surprising considering that, as discussed further, gait disturbances and cognitive impairment, thought to be due to neuronal death in various areas of the central nervous system, are common symptoms and complications of type 1 diabetes mellitus.

The aim of the current study is to highlight the (i) Behavioral activity using pole and hole board tests and (ii) histological changes in brain by light microscopy (iii) Ultra-structural changes by transmission Electron Microscope TEM.

2. Methodology:

2.1 *Materials and equipments*

Experimental animals

- Twenty healthy young adult male Wistar rats (weight 353±90 g, about 3 months old) were kept in a laboratory (in a 12/12 h dark-light cycle, 23.4ºC), and the experiments were performed after an acclimation time of 1 week. Animals had access to food (normal rat chow) and tap water. All procedures were approved by the institutional ethical committee.
- Board test (The hole board test was developed by Boissier and Simon 1965[9]. The arena comprised of a square Plexiglas box measuring 80 cm × 80 cm with equally spaced 16 holes in the floor 6 cm in diameter)
- The vertical pole (150 cm long, 3 cm in diameter)
- Digital camera (Digital camera (HD 720p video calling and HD video recording 1280 x 720 screen resolution)
- Transmission Electron Microscope (TEM): JEOL transmission electron microscope (JEM1010) with accelerating voltage of 80 kV. Digital images were taken using a Gatan Orius SC 1000 CCD camera and Digital Micrograph 3.11.0 software (Gatan, Pleasanton, CA).
- Light Microscope: (Leica Digital Microimaging Device 108)
- Paraffin and dyes (Haematoxylin and eosin)
- Specimen tubes
- Rotary Microtome: Leica RM2125
- Ultra Microtome: Leica ultra cut UCT-GA-D/E-1/00, consisting of the mechanical sectioning device, type 706201 powered by control unit, type 65620; 90-240VAC. 1.25A slow blown, 120W.

2.1 Method

Two experimental groups were devised: a group of rats made diabetic by single injection of streptozotocin (60 mg/kg body weight, i.p.) dissolved in citrate buffer (pH = 4.5) (n=12 rats), and a control group (n=8 rats) injected with an equivalent volume of the vehicle solution. Diabetes mellitus was confirmed (> 200mg/dl) by checking rat fasting blood sugar level with a glucometer (One Touch II Glucometer, Life Scan, USA) in the third day following injection. Animals were monitored for 8 months (long-term diabetes model). Then, they were submitted to two behavioral tests and histological studies.

2.1.2 Behavioral tests:

2.1.2.1 Method of Hole board test

Rats were individually subjected to the test. During the test, each rat was placed at the center of the platform and the activity in the arena was video recorded for a 5 min period. The video recordings were scored and the following exploratory behavior parameters were measured: the latency, number and total duration of episodes of head dipping through the holes ("nose-poking", which represent a possible way to escape from the aversive environment and therefore reflect the escape response of the animal, which is a normal cognitive ability [10, 11], the total distance travelled on the arena (ambulation score) and the latency to the first grooming were recorded. The apparatus was cleaned with 95% alcohol after every trial.

2.1.2.2 Method of Pole test

The vertical pole test has been used to assess basal ganglia-related movement disorders in rodents [12, 13]. The rats are placed head-up on a cloth-tape covered vertical pole (150 cm long, 3 cm in diameter); when placed on the pole, animals orient themselves downward and descend the length of the pole. Animals with deficits in motor coordination and balance will fall off the pole [14]. Animal time to orient downward (t-turn, reflect of cognitive abilities [15, 16] and the total time the animals stays on the pole (t-total, reflect of motor coordination ability [14,16] are determined from video recordings for a maximum of 180 seconds.

2.1.3 Preparation for histological studies:

After behavioral observations, animals were sequentially perfuse with phosphate buffer saline (pH 7.4) and Karnovsky's Fixative (5% Glutaraldehyde, 4% Formaldehyde in 0.08M buffer) under deep anesthesia with isoflurane (5%). Brains were dissected out, post-fixed for 2-h, and cut in two halves in the sagittal plan (following cerebral hemispheres). Brain left halves were processed for H&E histopathological studies, and ultrastructural studies.

2.1.3.1 H&E staining

Right side halves of brains were embedded in paraffin and cut (thickness 5µm) in series of 6 sections in the sagittal plan. A series of sectioned paraffin-embedded cerebellar tissues was processed for H&E staining, while other series were processed for ultrastructural study. Stained sections were analyzed for detecting and characterizing the histopathological changes, particularly in the Purkinje cell layer and in the neighboring molecular and granular layers, but also in the large neurons of the dentate nucleus of the cerebellum. Histopathological changes were also assessed in the CA1, CA2 and CA3 layers of the hippocampus, with focus on changes affecting pyramidal cells. Meningeal thickness was determined in sections of the central cerebellum area (about Bregma -12.0 mm) using Sturrock's morphometric method [17, 18]. These analyses were performed using a light microscope under 20x and 40x objectives.

2.1.3.2 Method of TEM study:

Blocks made from brain left halves (hippocampal CA1 area) were processed for ultrastructural studies, on the basis of H&E observations using standard methods for brain tissue processing and observations [19, 20, 21]. Briefly, semi-thin sections were made from the blocks. After toluidine blue observation, brain ultrathin sections were cut with a diamond knife (70–80 nm thickness), mounted on 200 mesh copper grids, and stained with 4% aqueous uranyl acetate for 15 min followed by Reynolds' lead citrate for 8 min. The sections were observed using TEM. Chromatin and mitochondrial changes were assessed in cells with marked changes revealed by toluidine blue observation and in the neuropil, where changes in myelin sheath were assessed.

3.1 Results & Discussion:

Figure 1 shows the effect of long-term streptozotocin-induced diabetes mellitus on Rat performance in a hole board arena. The total locomotors activity was decreased ($p<0.05$) (Figure 1B), while only a slight and non-statistically significant change was observed in the latency to the first head-dipping (Figure 1C). However, significant decreases were observed in the head-dipping episode number ($p<0.05$) (Figure 1D) and duration ($p<0.01$) (Figure 1E). In addition, a significant increase was observed in the latency to the first grooming episode ($p<0.001$) (Figure 1F). Such result ascertaining the facts that: diabetes could influence locomotors activity in rats significantly; however such effects seemed quite difficult to be induced in diabetic human. The decrement of locomotors activities in rats could be ascribed to myelin sheath of axon alteration and cerebral cortex as mention by Juan et al, [22].

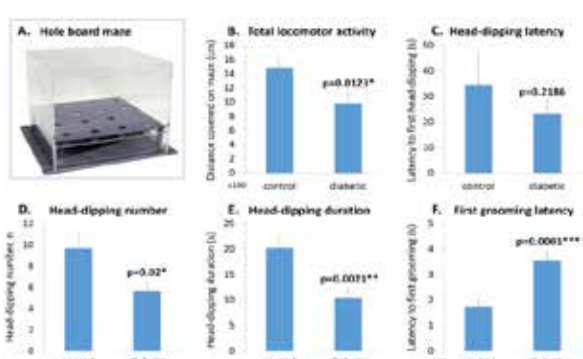


Figure 1: shows the effect of long-term streptozotocin-induced diabetes mellitus on Rat performance in a hole board arena. * P<0.05, ** P<0.01, *** P<0.001, U-test. Data are

mean±SEM.

Figure 2: shows the effect of induced diabetes mellitus on rats cognitive based on vertical pole performance test. The results showed that: the t-turn for the diabetic rats was increased significantly (P = 0.004) relative to control group with about 45%, while their pole time decreased significantly (P = 0.0005) relative to control group with about 38 (time pole/s); such results could be due to induced effects by streptozotocin as diabetes in the cerebral cortex and hippocampus as well. In comparison to these facts, Juan et al, [23], found that: the hippocampus was affected and significantly atrophied following diabetes progress and these changes were accompanied by a progressive enlargement of the ventricles in db/db mice as they aged. Cortical and hippocampal thicknesses were also reduced, which may account for the overall reduction in cortical and hippocampal sizes.

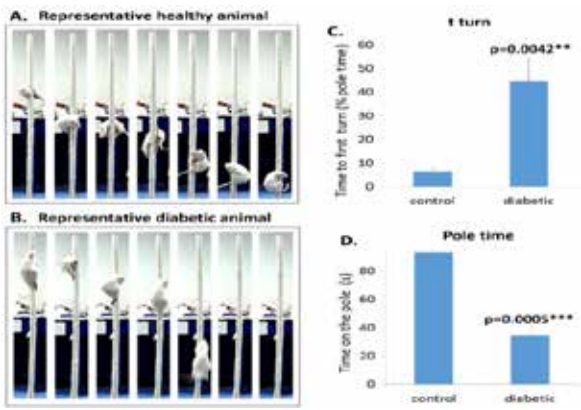


Figure 2: shows the effect of induced diabetes mellitus on rats cognitive based on vertical pole performance test. ** p<0.01, *** p<0.001, U-test. Data are mean ± SEM.

Figure 3 shows a light microscope image for Rat brain for (A) Non diabetic and (B) diabetic with relative meningeal thickening. It reveals that there is an increase in meningeal thickness and a higher density of circulating cells in the meninges were observed in diabetic animals compared with control group (Figure 1A-B). The determination of meningeal thickness confirmed such observation as this parameter was significantly increased in the diabetic group (p<0.05) as shown in relative Figure (E). Also there is appearance of hemorrhage as multiple foci spread in brain tissue as shown in Figures (C & D). Such results in agreement with studies done by Juan et al, [22] in which they proved that: structural and ultrastructural brain changes of streptozotocin rats were accompanied by depression in diabetic animals.

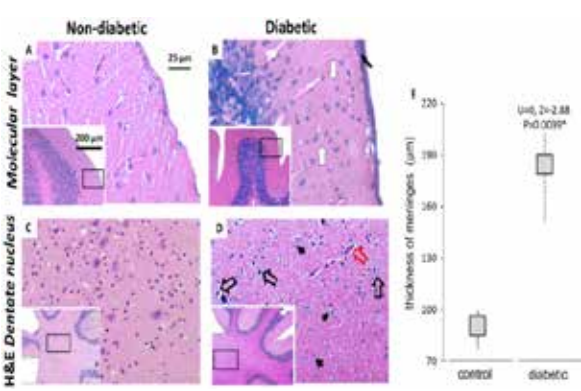


Figure 3 shows a light microscope image for Rat brain for (A) Non diabetic and (B) diabetic with relative meningeal thickening at U test, P=0.004.

Figure 4 shows ultrastructural changes for induced diabetic Rat's brain tissue parenchyma imaged by TEM. It shows that: (4-A) Necrotic neuron-like cell (black arrows) between two activated brain macrophages 'M'. (4-B) Higher magnification of micrograph (4-A) showing that astrocytes (white asterisks) close to the brain macrophages are karyolitic (in necrosis). (4-C) shows myelin disturbances in the neuropil as white arrows. (4-D) shows alteration in apoptotic process, microglia-like cells in the neuropil 'AM'. Space bar = 2 µm. Numerous activated brain macrophages were observed, as well as necrotic neuron-like cells, karyolitic astrocytes and mitochondria showed alteration in form of swollen and/or vacuolation (4-E). These findings were not found in the section of control group Figure (5), which showed normal microglia cell with continues intact nuclear membrane, undisturbed cytoplamic organelles and absence of activated macrophages, Mylinated sheaths show normal appearance, Astrocyte shows normal morphology and along the neuropil no glycogen or glycogen like granules noticed (5-A – 5-D).

In contrast with previous studies, Yang et al, [2] have found that: the induced diabetic with STZ in rats showed mitochondrial dysfunction and loss of cerebellar Purkinje neurons and, subsequently, associated with motor coordination deficits.

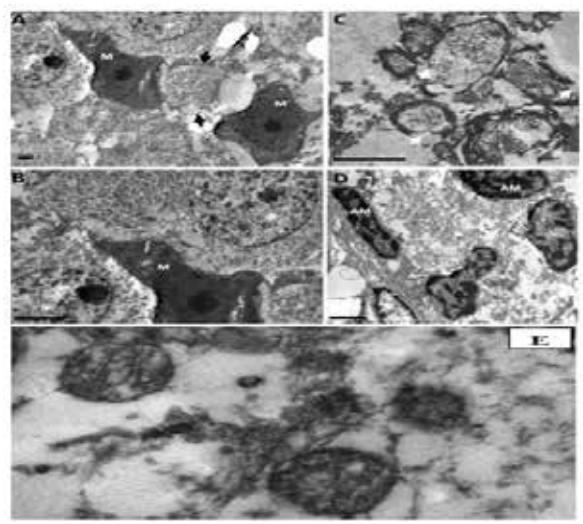
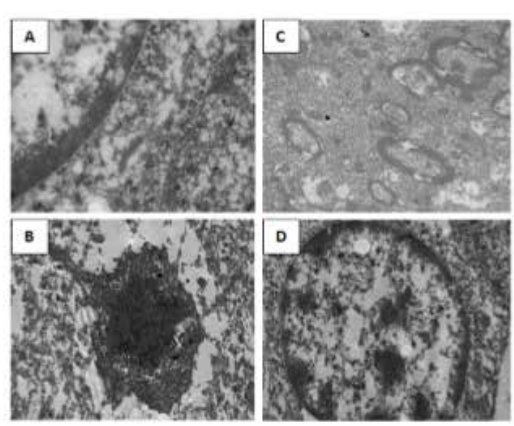


Figure 4 shows ultrastructural changes for induced diabetic Rat's brain tissue parenchyma imaged by TEM: (4-A) shows Necrotic neuron-like cell (black arrows), (4-B) shows higher magnification of micrograph (4-A), (4-C) shows myelin disturbances in the neuropil as white arrows and (4-D) shows alteration in apoptotic process, microglia-like cells in the neuropil 'AM' where Space bar = 2 µm. (4-E) shows the swollen and vacuolation.



5 shows ultrastructural for control group of parenchymal Rat's brain tissue image by TEM shows normal nucleus ,nuclear membrane and endoplasmic reticulum(A) also unchanged

mylinated figure(C) and normal neurons and neuropil (B) and(D).

Conclusion:

For the aim of this study which deal with studying of induced diabetes in rats behavior and histological effects by using STZ, the scored fact is that: STZ could induce diabetes which in turn affects the physiological and histological changes in forms of (*myelin disturbances in the neuropil, alteration in apoptotic process, microglia-like cells in the neuropil, Numerous activated brain macrophages, and mitochondria swollen and/or vacuolation*),which reflected in animal behavior as decrease locomotors activity, and cognitive behavior which is quite difficult to be express in human.

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