ejpmr, 2017,4(12), 499-507

SJIF Impact Factor 4.161

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

A HISTOPATHOLOGIC AND HISTOMORPHOMETRIC STUDY ON THE HIPPOCAMPUS OF STZ-INDUCED DIABETIC ALBINO RATS

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Article Received on 21/10/2017 **Article Revised on 12/11/2017** **Article Accepted on 02/12/2017**

ABSTRACT

Aim of the study: The chronic hyperglycemia has been associated with dysfunction of various part of brain resulting into cognitive defects, alteration of behavior, memory and emotion which is possibly due to its harmful effect of on the neurons of the hippocampus. Therefore, the current study was aimed to look for the alteration on the histological and histomorphometric parameters of discrete parts of hippocampus. **Material and methods:** After clearance from the Institutional Animal Ethical Committee, 36 adult rats were divided into six groups of six rats each having control and diabetic groups. Diabetes was induced with streptozotocin (60 mg/kg; i.p.). Rats having blood glucose >250 mg/ dl were considered as diabetic. At the end of each experiment rats were euthanized by deep ether anesthesia and blood samples were collected for biochemical analysis. Tissues were fixed in Karnovsky fixative and processed for paraffin sectioning. Routine and special stained sections were studied under the light microscope and relevant images were recorded for histological and histomorphometric analysis. **Results:** It was observed that prolonged hyperglycemia was associated with increased serum creatinine and reduced serum total protein. There was obvious reduction of neuronal density in granular layer of dentate gyrus and CA3 pyramidal neuron in conjunction with remarkable increase in the collagen of tunica adventitia of hippocampal blood vessels and choroid plexus. **Conclusion:** These findings suggest that hyperglycemia-induced neuronal loss and perivascular excessive deposition of collagen may constitute important contributing factor in the development of diabetes-related memory alteration, depression and cognitive impairments.

KEYWORDS: CA3, Collagen, Dentate gyrus, Diabetes, Hippocampus, STZ-induced.

INTRODUCTION

Diabetes mellitus is an endocrine disorder well known for its serious impact on central nervous system.[1] Prolonged hyperglycemias have been associated with increased vulnerability to stress[2] and cognitive dysfunction.[3] Oxidative stress at cellular as well as systemic level followed by initiation of cellular injury and high levels of polyunsaturated lipids in the brain, direct lipid peroxidation frequently occur causing lipid membrane disruption and consequent slow progressive structural neurodegenerative changes.[4] Many deleterious events contribute to oxidative damage to neurons in diabetes in the form of glucose neurotoxicity[5] and mitochondrial dysfunction which is followed by neuronal apoptosis.[6] Hippocampus responsible for storing and retrieving short and long term memories[7,8], declarative and spatial learning and memory[9] are a very vulnerable and sensitive region of the brain to extreme hyperglycemia and hypoglycemia.[10] Long-term diabetes have been linked to abnormalities in the hippocampal neuronal cells as well as associated fibers[11], altered hippocampal synaptic plasticity[12], hippocampal atrophy[13], cognitive deficits[14], prolonged depression[12], impairments in spatial learning, memory and problem solving.[15]

Many researchers[16,17] have tried to demonstrate morphological and histopathological changes in the neuronal alterations and apoptosis without using any special stain. Therefore, the present study is aimed at demonstrating these changes and possibly others like neuronal laminar alterations, arrangement of collagen fibers around hippocampal laminar vessels and neuronal structure by using special stain for collagen (Picro-syrus red- PSR) and neuron (Cresyl violet- CV) in conjunction with histopathological, histomorphological and biochemical parameters after 2 week (2W), 1 month (1M), 2 month (2M), 4 month (4M) and 6 month (6M) periods of experimental induction of diabetes in adult albino rats.

MATERIALS AND METHODS

The current experimental study was performed at the Department of Anatomy, J.N Medical College, faculty of Medicine, Aligarh Muslim University. UP. India. Animal guidelines on the care, experimental usage and approval

from the Institutional Animal Ethics Committee (No: 9025/2014) were obtained before study.

Experimental animal and animal care

36 albino rats of either sex weighing approximately 250 g (4-5 month old) obtained from central animal house, Aligarh Muslim University, Aligarh were used for the this study. Prior to beginning of the experiments, all animals were maintained to the new environmental condition (climate controlled room) for a period of one week. Animals were kept in a well-ventilated room and were supplied standard dry food pellet diet and water ad libitum and maintained on a 12/12 h light/dark cycle.

Drug, Dosage and route

Streptozotocin is a naturally occurring chemical particularly toxic to the beta cells of the pancreas in mammals used for treat metastatic cancer of the pancreatic islet cells. In the current study STZ was used to produce an animal model for hyperglycemia. Streptozotocin (STZ) (SRL-Sisco Research Laboratories, Mumbai, India) dissolved in sterile water to give 60 mg/kg dose intra-peritoneally injection to adult albino rats.

Animal experimental design

After one weeks of acclimation to the diet and the environment, all albino rats were divided into following six groups having six rats in each group: (**I**) Non-diabetic healthy Control, age-matched (**II**) Diabetic Experimental groups: 2W, 1M, 2M, 4M and 6M.

Induction of Diabetes, estimation of blood sugar

After 12 hour fasting, experimental diabetic model was induced by single dose of streptozotocin (STZ) (60 mg/kg, aqueous sol., I. P) while age-matched control group received equal amount of Sterile water via same route. Blood sugar level was sampled from lateral tail vein and monitored with Glucometer (Dr Morepen Gluco One BG03 Blood Glucose Meter) beginning of experiment and after 2nd day streptozotocin injection. Animals with blood sugar level 250 mg/dl and above were considered as diabetic. Both body weight and blood glucose levels of all animals in each group were monitored biweekly.

Tissue Preparation

After assigned periods entire experimental and age-matched control animals were euthanized with over dose of ether general anesthesia and were rapidly perfusion-fixed with Karnovsky fixative.

Histopathology and Histomorphometry

After proper fixation whole brains were carefully dissected out from the cranial cavity. Tissue blocks consisting of 3 mm thick coronal section containing hippocampus 4-5 mm behind optic chiasma were processed for paraffin embedding. Some researches took hippocampus from in-situ block extending from bregma 3.12 mm to 4.68 mm of the primary motor cortex[18] but with this method tissue damage remains inevitable. For light microscopic evaluation, 5 µm thick sections were stained with Cresyle Violet (CV) and Picro Sirus (PSR). Only CV stained sections were used for measuring neuronal density. Random photomicrographs were recorded under x 100, 200, 400 and 1000 magnification under trinocular microscope (Olympus, BX40 and Japan) by digital camera (Sony 18.2 MP, Japan) and measurements were made by using software Motic image version 2.0. Number of granular cells in the granular layer of dentate gyrus and CA3 pyramidal neuron was evaluated in 10000 µm^2 area from the neurons having well defined nucleus visible nucleolus. Histopathological and histomorphometrical parameters for neuron were recorded under100x objective.

Biochemical Estimation and Analysis

Blood glucose levels of all groups were measure from lateral tail vein blood at biweekly interval with the help of Glucometer. After the designated period, blood samples were obtained from direct puncture of heart and collected into sterilized plastic vials. Blood samples were allowed to clot, centrifuged at 2500 rpm for 30 minutes, the serum was separated and stored in sterile plastic vials and afterward assayed for serum total protein content and serum creatinine level by using Avantor BenespheraTM clinical chemistry Analyzer C61 (Avantor Performance Materials, Inc., Center Valley, PA, USA).

Statistical Analysis

The data related to density of granular cells in the dentate gyrus, CA3 pyramidal neuron, serum total proteins and serum creatinine level were statistically analyzed and the significance calculated using one way 'ANOVA' followed by Tukey's test. All numerical values were expressed as Mean ± SD and the value of $P<0.05$ was considered as statistically significant.

RESULT

General and behavioral observations

During the experimental period, STZ-injected hyperglycemic rats showed classical clinical manifestations of diabetes such as polydipsia, polyuria and polyphagia (Data not shown). Prolonged diabetic rats became less active when released from the cage as compared with age-matched control group. Total general activity was reduced markedly in prolonged diabetic group rats.

Clinical data

The body weight changes in prolonged hyperglycemic group after 2W of STZ-induced diabetic rats. The control rats showed increasing body weight as advancement of experimental periods, at the same time the STZ group had decreasing body weight. The mean values of body weight in diabetic groups were reduced at all experimental stages and significant ($P= <0.005$) in prolonged hyperglycemic group except 2W diabetic group as compared to age-matched control groups. After 2 days of STZ administration blood sugar level showed

hyperglycemic state (> 500 mg/dl) in all diabetic groups throughout experimental periods as compared to age-matched control groups as presented earlier.[19]

Microscopic Observation

Histopathology

In Cresyl violet-stained sections the dentate gyrus revealed three laminae namely molecular layer, granular layer and polymorphic layer (Figure 1). In general, the location and laminar arrangement of right and left hippocampus were almost alike in all groups. The CA3 region of hippocampus proper was located in the outer part of the hilum of dentate gyrus (Figure 1). CA3 region of hippocampus was clearly observed in all groups. Small sized pyramidal cells were observed in all groups of CA3 of hippocampus proper (Figure 1 and 2). The prolonged hyperglycemic group showed evidences of neuronal damage in the form of apoptotic or dark neuron and perineuronal space around the damaged neurons were more pronounced in CA3 region and granular layer (Figure 2). Blood capillaries were often seen in all laminae of hippocampus in all groups and some capillaries were located in a very close relation to neurons (Figure 3). In all groups, collagen fibers were observable in variable amounts in the adventitia of intra laminar vessels of hippocampus and choroid plexus of ventricles. However in control group, intra laminar vessels and choroid plexus had fewer collagen fibres and in 6M diabetic group the collagen fibres in the adventitia revealed additional thickening. Generally the amount of collagen and thickness of their fibers showed direct relationship with the duration of hyperglycemic state as compared to the age-matched controls (Figure 4 and 5).

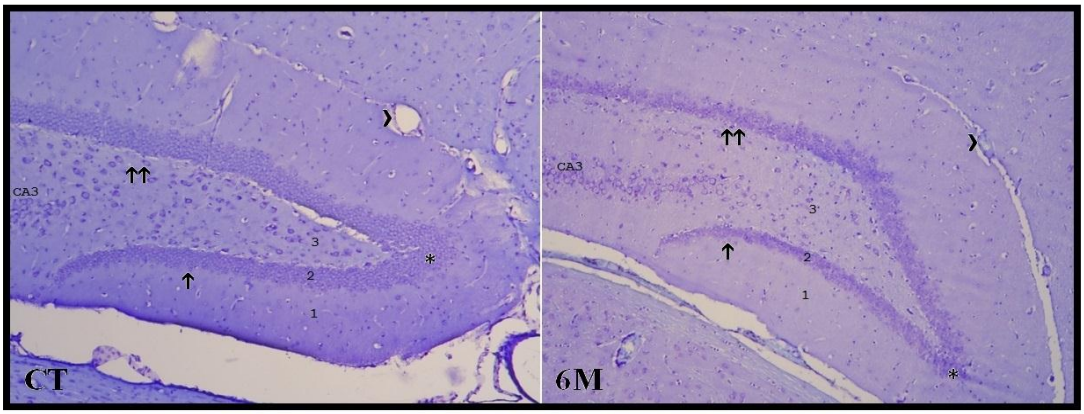

Figure 1: Photomicrographs showing the part of hippocampus of control and 6M diabetic groups. Note: almost uniformly arranged laminae of dentate gyrus showing molecular layer (1), granular layer (2), polymorphic layer (3), superior pyramidal blade of granular layer (↑↑), inferior pyramidal blade of granular layer (↑), crest of granular layer (*) and blood vessels (>). CV stains; initial magnification X 200.

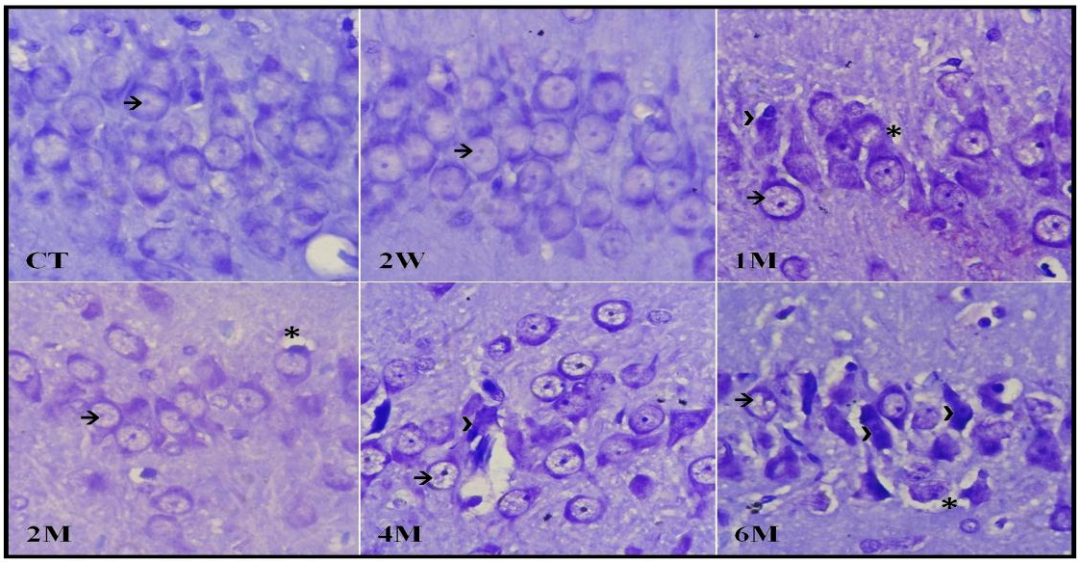

Figure 2: Photomicrographs showing the CA3 pyramidal neuron of all diabetic groups. Note almost uniformly arranged CA3 pyramidal neuron having clear nucleus and visible nucleolus (→) in all groups, Dark pyramidal neuron (>) and perineuronal space (*) are seen as unstained empty spaces of varying size and shapes around the neurons of prolonged hyperglycemic groups. CV stains; initial magnification X 1000.

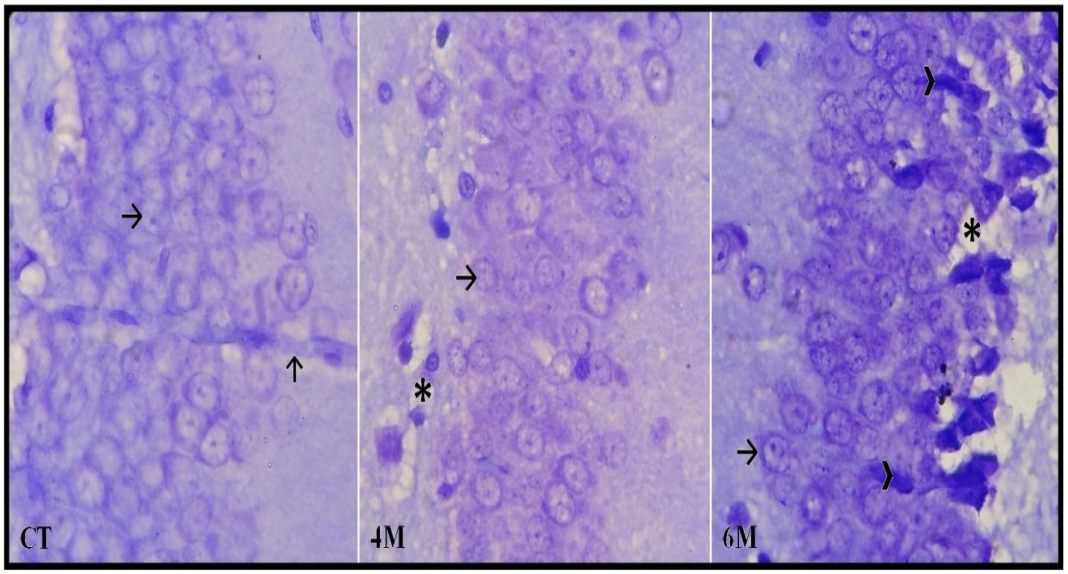

Figure 3: Photomicrographs showing the granular cells in the dentate gyrus of control, 4M and 6M diabetic groups. Note: almost regularly arranged granular cells having vesicular nucleus and dark nucleolus (→), Dark granular cells (>) and perineuronal space (*) prominently seen in 6M group. CV stains; initial magnification X 1000.

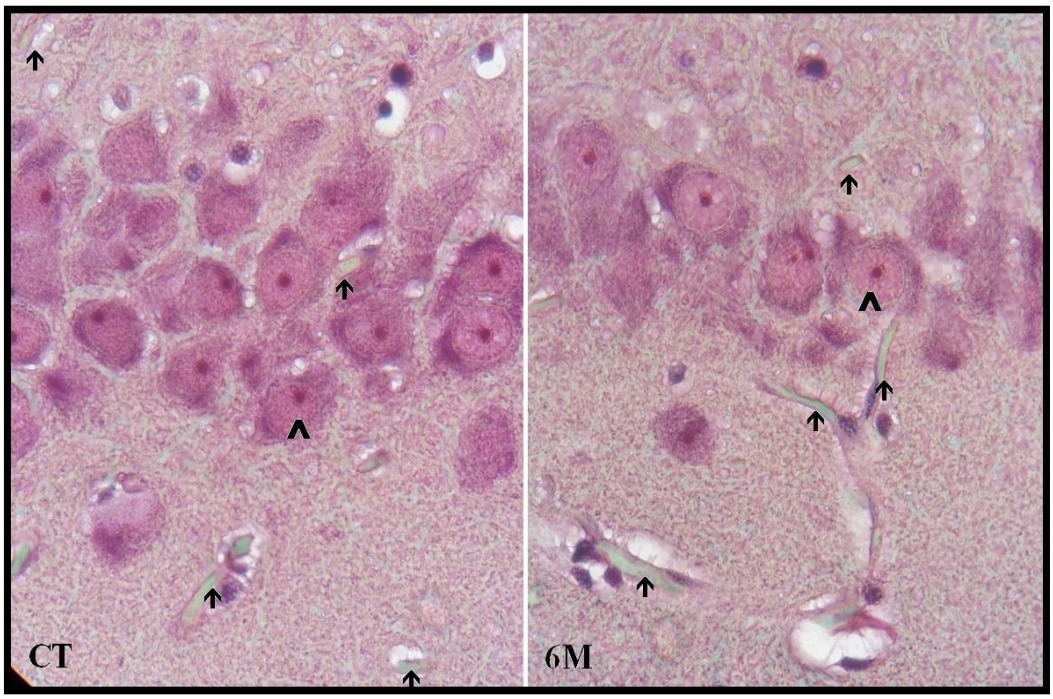

Figure 4: Photomicrograph showing the CA3 pyramidal neuron of 6M diabetic and corresponding control groups. Note almost uniformly arranged CA3 pyramidal neuron having uniformly stained pinkish nucleus and centrally placed dark prominent nucleolus (∧) and blood capillaries (↑) are distributed in and around part of CA3 region. PSR and CV stains; initial magnification X 1000.

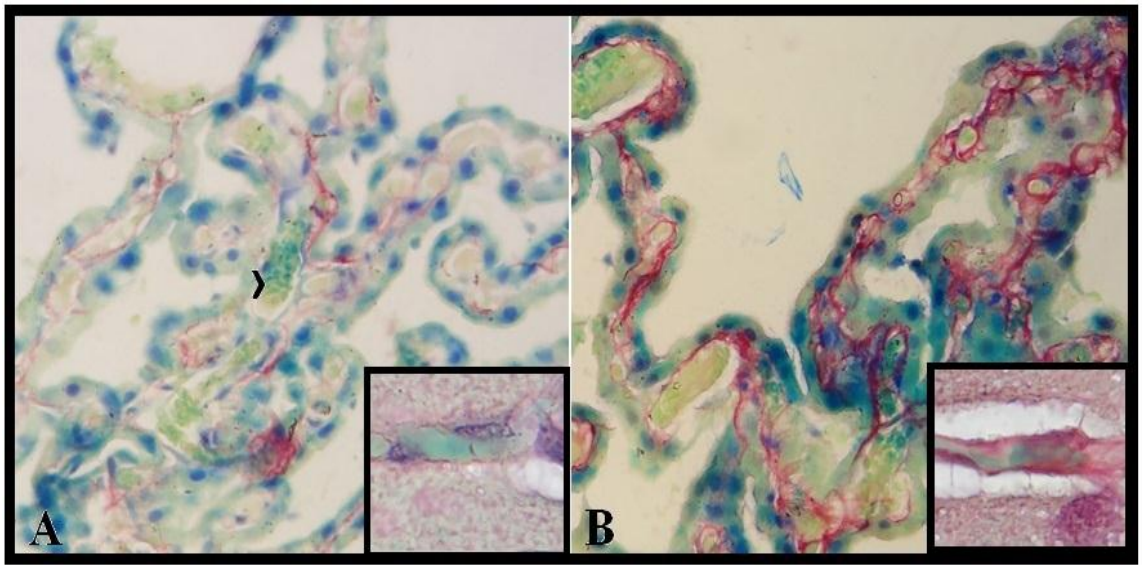

Figure 5: Photomicrograph showing the choroid plexus from the control (A) and 6M (B) diabetic groups. Note: RBC (>) and choroid plexus of the control group and 6M diabetic group is associated with red-stained thin collagen fibers and while 6M group shows added the thickening of connective tissue of tunica adventitia collagen connective tissue fibers. The inset shows hippocampus laminar capillaries of the corresponding groups the amount of collagen associated with them. PSR and CV stain. initial magnification x 1000.

Histomorphometry

The granular cell number in the given area (10000µm^2) of different parts of dentate gyrus

The granular cells in 10000 µm^2 area of the crest, superior and inferior pyramidal blade of the dentate gyrus revealed decrement of mean number of the granular cells in all regions of dentate gyrus of diabetic group as compared with age- matched controls. However, the decrement is significant (P<0.05) only in 2M, 4M and 6M diabetic groups. In addition, it was observed that the granular cell number in the given area of the superior pyramidal blade remained always more than the number in the inferior pyramidal blade of the same sample, there by suggesting more dense packing of neurons in the superior blade than the inferior one (Figure 3 and Table 1).

Table 1: Show the granular cell number in the given area (10000µm^2) of different parts of dentate gyrus of diabetic and age-matched control groups (mean±SD).

Groups	Crest	Superior pyramidal blade	Inferior pyramidal blade
2W-Control	41.99 ± 01.90	42.28 ± 00.89	41.88 ± 01.69
2W-Diabetic	41.56 ± 01.54	41.33 ± 01.21	41.33 ± 00.82
1M-Control	40.71 ± 02.43	42.13 ± 00.77	41.24 ± 01.06
1M-Diabetic	39.65 ± 02.58	39.33 ± 02.07	40.83 ± 01.72
2M-Control	41.28 ± 02.28	41.97 ± 01.35	40.83 ± 00.75
2M-Diabetic	36.96 ± 02.06	34.52 ± 01.06	35.67 ± 01.51
4M-Control	40.18 ± 02.24	41.43 ± 00.88	40.33 ± 01.37
4M-Diabetic	29.71 ± 02.57	27.44 ± 00.73	33.02 ± 01.50
6M-Control	39.77 ± 02.15	41.67 ± 01.04	40.11 ± 01.33
6M-Diabetic	21.30 ± 01.49	22.99 ± 00.86	32.20 ± 02.75

The density of CA3 pyramidal neurons (Area 10000µm^2)

The number of CA3 pyramidal neurons per 10000 µm^2 area of hyperglycemic significantly (P= < 0.005) reduced as compared with age-matched control groups except in 2W and 1M diabetic groups. (Figure 2 and 6).

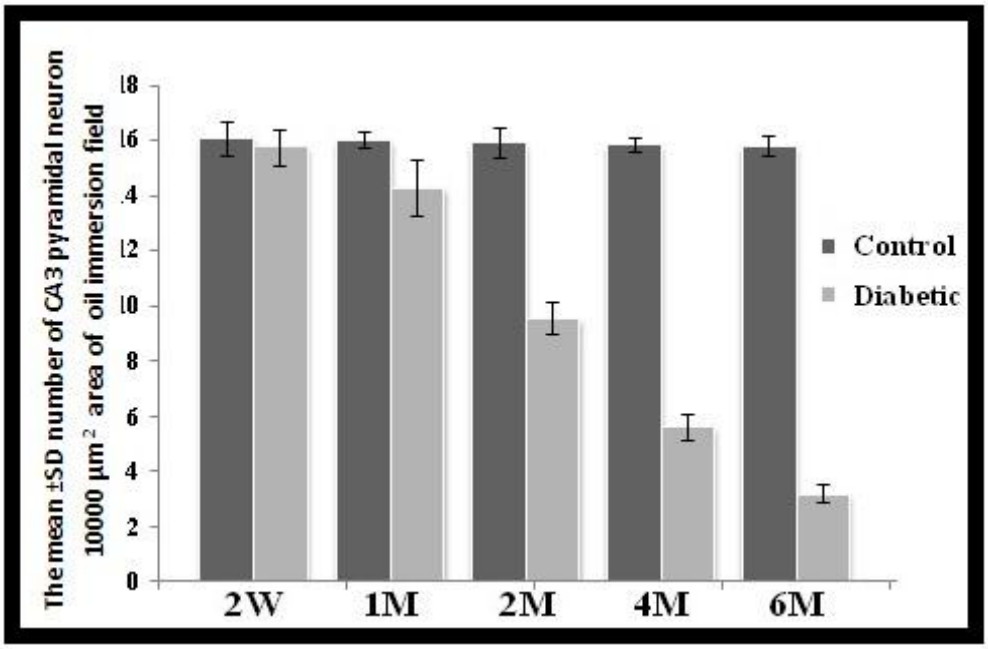

Figure 6: Shows that the number of CA3 pyramidal neurons decreases with advancement of duration of hyperglycemia as compared with age-matched control groups.

Biochemical analysis

Serum creatinine level were increased significantly ($P<0.05$) in all diabetic groups as compared to age-matched control groups except 2W diabetic group. while serum total protein levels significantly ($P<0.05$) decreases in all diabetic groups as compared to corresponding control group as presented earlier.[19]

DISCUSSION

In the current study the STZ-induced diabetic rats had significantly increased blood glucose levels than the corresponding group of control and it remained high throughout the experimental period similar to one shown in previous reports.[2] Generally, hyperglycemia occurs as a result of insufficient amounts of insulin, or in which tissues fail to respond appropriately to insulin.[20] Persistent chronic hyperglycemia is the main source of increased generation of free radicals through auto-oxidation of glucose that increases the flux of glucose through the polyol pathway.[21] Excessive production of free radicals beyond the scavenging capacities of endogenous antioxidant capacity leads to macro-and micro vascular dysfunction.[22] And these changes are also associated with dysfunctions of neuron like alteration of hippocampal neuron density of the central nervous system.[23] In addition decreased secretion of anabolic insulin hormone from the beta cells of pancreas produces hyperglycemia, increases protein catabolism and releasing amino acids for gluconeogenesis.[24] In prolonged hyperglycemic state loss of tissue protein leads to fall of body weight.[25] In the current study, the mean body weight at the end of the experimental period in all diabetic groups was reduced and weight losses remained throughout the experimental period. Previous studies have also reported that body weights were reduced in diabetic group[26,27] and this was found to be in agreement with present study.

The structure and orientation of hippocampus architecture were reported to be similar in the control and diabetic groups[28,29] which are in agreement with the findings of the present study. In the current study, some granular cells in the dentate gyrus and CA3 pyramidal neuron have shown perineuronal spaces in 4M and 6M diabetic groups, indicating the extent of damage due to prolonged hyperglycemia to the neurons in the hippocampus. Somewhat similar observations have been made in previous studies on sensory ganglia[30,31] and autonomic ganglia[32] and those were suggested to be due to either shrinkage or apoptosis of neurons with the progression of hyperglycemia. Oxidative stress and associated free radical generation accelerates the dark neuron formation and it is considered as apoptotic type of neuron[33] or a type of cell degeneration with hyper electron density properties and hyper basophilia.[34,35] In the current study also it was noticed that, the number of dark neurons increased on the progression of the duration of hyperglycemic state in both granular cells in dentate gyrus and CA3 pyramidal neuron.

The dentate gyrus is a simple cortical region that is an integral portion of the larger functional part of hippocampus. Dente gyrus receives impulses from the entorhinal cortex via perforant path and ultimately conveys to the CA3 area of the hippocampus.[36] The hippocampus is necessary for normal cognitive function, especially for processing recognition memory and transferring short-term memory items into long-term storage.[37] It has been shown that the process of neurogenesis, which includes cell proliferation, survival, migration and differentiation, continues in the

hippocampal formation well into adulthood in a variety of species.[38] Eventually those cells differentiate into granule neurons.[39] In has been suggested that reduction of neurons in hippocampus can be causes of learning disability and memory in humans and animals.[40,41] One study observed lower neuronal density of dentate gyrus in animals with type 1 and 2 diabetes mellitus.[42] In the current study, 2M, 4M and 6M diabetic groups also showed decrement of density of granular neurons in dentate gyrus and CA3 pyramidal neurons as compared to age-matched control groups. Similar findings were observed in the other related studies[23, 28] suggested that chronic diabetes has a neuro-toxic effect on the hippocampus[28] which is followed by programmed cell death via apoptotic pathway or else it inhibits neurogenesis and migration from the sub-granular zone.[41, 43] Neuronal complications of diabetes mellitus could be due to interference through excessive free radicals generation[44] followed by activation of polyol pathway which results into consumption of NADPH as well as depletion of glutathione anti oxidant, which lower the threshold for intracellular oxidative injury.[45] Reagan and McEwen observed reduced nitric oxide synthase, mRNA and protein concentrations in hippocampal neurons to be the main factors associated with neuronal alterations due to hyperglycemia.[46] These observations and present study results confirm that prolonged hyperglycemia and associated biochemical changes alter the hippocampus neuronal populations of STZ-induced diabetic rats.

One study observed that extra cellular matrix of vascular adventitia contains insoluble frame work of collagen, elastin, glycoproteins, proteoglycans – hyaluronans and integrins which provide both mechanical supports as well as complex interaction between cells or between cells and extra cellular matrix of vessels.[47] In the development of fibrosis which involves predominant extracellular matrix accumulation by the activation of pro-sclerotic cytokines and protease or anti-protease systems which leads to change in the quality of extra cellular matrix and angiogenesis.[48] Types I, II and III collagen are the fibrillar – interstitial collagens responsible for diabetic remodeling fibrosis at neuronal unit.[47] During fibrosis, collagen fibrils are considered to be produced by fusion of short and thin fibrils with tapered ends. One related study observed the progression of fibrosis in the heart by PKC-β and p38 mitogen-activated protein kinase expression in redox reaction[49] and also due to AGE and RAGE interaction and connective tissue of tunica adventitia increased expression of TGF-β which contributes to the development of and neoangiogenesis.[50] In the present study prolonged hyperglycemic groups, PSR and CV Stain revealed that there was thickening of collagen around the adventitia of hippocampal intra laminar vessels and also in the chroid plaexus of ventricles. However this observation indicate that the hyperglycemia seems to promote fibrosis in terms of the amount of collagen as well as the vascular pathology which is in agreement with previous observations regarding link between hyperglycemia and fibrosis.[30, 51]

Creatinine is a byproduct of amino acid metabolism. Basically it is derived from the non-enzymatic conversion of creatinine to phosphor-creatinine in skeletal muscle and in the liver through methylation of guanidine aminoacetic acid to form creatinine, while it is poorly reabsorbed from the renal tubules and eliminated in moderately large amount by tubular secretion due to its molecular weight, is released freely into the plasma and is not reabsorbed or metabolized by the kidney.[52] Defective kidney function in diabetic nephropathy exhibits an irregular high level of serum creatinine. An increased urea concentration is also related with greater protein catabolism.[53] The current study also revealed that elevated serum creatinine in all diabetic groups is also somewhat parallel to the duration of hyperglycemia. It is identified that the proteins contribute to form glucose in severe cases of diabetes mellitus by catabolism of protein via gluconeogenesis. The serum total protein content is also known to be an indicator for the body protein level and severity of hyperglycemia. In the present study the reduction of the total serum protein were observed in the diabetic rats which may be associated with the hyperglycemia due to a low grade inflammatory process.[54] Similar finding has been shown in other related studies.[55,56]

CONCLUSION

Based on the histopathological, histomorphological and biochemical findings it is concluded that hyperglycemia-induced neuronal cytotoxicity, reduction of neuronal number and altered micro-vascular environment seem to be the important contributing factors in the development of hippocampal dysfunction in chronic diabetes.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

The authors would like to gratefully acknowledge all kinds of support and co-operation received from Department of Anatomy and Neuroanatomy laboratory, JN Medical College, Aligarh Muslim University, Aligarh.

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