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Effect of Experimentally-induced Diabetes on the Cerebellum of Albino Rats: A Histological and Histomorphometric Study

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ABSTRACT

Diabetes mellitus is a common serious metabolic disorder with well-known serious secondary complications. Long term hyperglycemia induced- neurotoxicity leads to many adverse effects on various parts of both central and peripheral nervous system. The mechanisms responsible for the diabetes-related neuro-chemical alteration and structural abnormalities in the brain are not fully understood as yet. The aim of this current experimental study was to carry out a histological and histomorphological analysis of the diabetes-related changes in the cerebellar cortex of STZ-induced diabetic rats. 36 Albino rats weighing approximately 250 g were included in the study and divided in to control and diabetic groups and each group having 6 rats. Diabetic group received single dose of streptozotocin (60 mg/kg/bw, i.p.) and control animals received normal saline. The induction of diabetes was confirmed by measuring the blood glucose levels from the lateral tail blood and blood sugar level above 250 mg/dL were considered diabetic. After experimental period all groups' rats were sacrificed and coronal sections were taken from the cerebellum and stained with Cresyl violet, LFB, and PSR. The number of Purkinje and thickness of different layers of the cerebellum was evaluated for histomorphometry. Light microscopic studies and biochemical estimation revealed that there is reduction of number as well as diameter of Purkinje cells and reduced thickness of molecular and granular layer of cerebellar cortex. There is progressive increase in the amount of collagen fibers around tunica adventitia of cerebellar cortical as well as medullary vessels and choroid plexus of fourth ventricle. Alteration of biochemical changes in the form of increased serum creatinine level and decreased serum total protein was also noticed with increasing duration of hyperglycemia. It is therefore concluded that long-standing hyperglycemia leads to reduction of number Purkinje cells and thickness of cerebellar cortical layers; increased collagen around cerebellar vessels and choroid plexus in conjunction with the biochemical changes appear to promote the cerebellar functional alterations observed in chronic diabetics.

Keywords: Cerebellum, Collagen, Diabetes, Neuropathy, Purkinje cells, STZ-induced

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INTRODUCTION

Diabetes mellitus is a common metabolic disorder characterized by hyperglycemia[1], altered metabolism of lipids, carbohydrates, and proteins[2]. Diabetes mellitus is also related to the vascular disorder, retinopathy, peripheral neuropathy, and dysfunctions of the central nervous system[3]. Hyperglycemia is considered to be correlated with elevated cellular oxidative stress and hyperlipidemia, which trigger cellular injury leading to high risk of diabetic complications[4]. Chronic high neuronal glucose levels have been shown to lead to glucose neurotoxicity[5] and which promotes to a variety of functional and structural disorders of the nervous system[6]. Hyperglycemia produces functional and structural blood-brain barrier changes[7] and also associated diabetic encephalopathy leading to end-organ damage in the central nervous system[8]. Oxidative stress has been shown to cause mitochondrial dysfunction which is followed by neuronal apoptosis[9]. In other studies, it has been suggested that the hyperglycemia in chronic diabetes initiates neuro pathological changes, such as swelling, gliosis, cell loss, myelin damage, and thickened capillary basement membranes in the cerebrum and cerebellum[10]. Another study revealed that the long-term diabetics lead to the abnormalities in the cerebellar micro vessels and decreased density of cerebellar gray matter[11] and loss of number and size of the Purkinje cells and the granular cells and reduction of thickness of the Purkinje and internal granular layer of the cerebellar cortex[12].

The cerebellum, a part of the central nervous system, containing more than 50% of all neurons in the brain[13], is responsible for the control and accurate execution of almost all motor activities, sensorimotor synchronization and may reflex activities[14, 15, 16]. The previous report observes that cerebellar lesions can be from asymptomatic to blatant pathology and damage may cause disturbance of motor coordination and precision qualities that are important in producing a smooth and sequential movement[17]. Some researchers[10, 18, 19] have tried to demonstrate changes in neuron, thickness of white matter of cerebellum[20], collagen and myelin loss by biochemical and structural analysis in diabetes without using any special stain. Therefore, this experimental study was designed to demonstrate these and possibly other changes on the cerebellum by using special staining for collagen staining (PSR) and myelin (LFB) in conjunction with histopathological, histomorphological and biochemical parameters in experimentally induced diabetic albino rats after 2W, 1M, 2M, 4M and 6M periods.

MATERIALS AND METHOD

Study approval, Experimental animals, Animal Care, and Housing preparation:

This experimental study was performed at the JN Medical College, Department of Anatomy, Faculty of Medicine, Aligarh Muslim University, Aligarh, India. Untreated albino rats of either sex (young, weight ~ 250g, number- 36 rats, 5-month-old) obtained from the central animal house, AMU, Aligarh were used for the current study. Guidelines on the care and use of laboratory animals and approval of the ethics committee were obtained before the study. All animals were housed in the new well maintained environmental condition for a period of one week in clean polypropylene cages and maintained under standard laboratory environmental conditions (12/12 h light/dark cycle) with free access to the standard pellet diet and water ad libitum. The Institutional Animal Ethical Committee of Aligarh Muslim University, Aligarh approved the protocol of the present study (Ref. No.9025/2014).

Animal groups and treatment:

Animals were divided into following six groups having six rats in each group: (1) Non-diabetic healthy Control, age-matched (did not receive any active compound); (2) Diabetic Experimental groups: Two week, (3) One month (4) Two month (5) Four-month and (6) Six month. After one week of acclimation to the diet and the environment, all rats were placed without food but free access to water. After 12- hour fasting, experimental diabetic model rats received the single dose of streptozotocin (STZ) (Sigma-Aldrich Canada, Oakville, Ontario, Canada) (60 mg/kg, aqueous sol., I.P). Control groups receiving an equivalent volume normal saline injection intra peritoneally (IP).

Blood Sugar Measurements:

Blood sugar level was monitored with Glucometer (Dr. Morepen Gluco One BG03 Blood Glucose Meter) in the blood obtained from lateral tail vein before the beginning of the experiment and after 2nd- day streptozotocin injection for checking induction of diabetes. Animals with fasting 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.

Light microscopic preparation

After assigned periods all experimental and their corresponding controls were sedated and euthanized with an overdose of ether general anesthesia and thereafter for the light microscopic preparation of the cerebellum, the rats were rapidly perfusion-fixed with Karnovsky fixative for the histological procedure.

Histopathology and Histomorphometry:

After proper fixation, the cerebellum was exposed and dissected out from posterior cranial fossa and then subjected to the standard histological procedures of dehydration, clearing and paraffin

embedding (58-60^{o}C melting point). The sagittal section, (5µm thick) were stained with Hematoxyline Eosin (H & E) for general histological features, Cresyl Violet (CV) and Luxol fast blue for histopathology and histomorphometry; while PicroSirus Red (PSR) was used for collagen. Random photomicrographs were recorded under oil immersion (x1000 magnification) with trinocular light cum fluorescent microscope (Olympus, BX40, Japan) with the digital camera (Sony 18.2 MP, Japan). Purkinje cells having well-defined nucleus were used for the histomorphometry. Data achieved from these were used to calculate the mean number and mean diameter of Purkinje cells were determined in different groups.

The morphometric analysis of cerebellum includes the thickness of cerebellar cortex (under x40) and for this purpose 25 random visual fields from each groups having only simple folia were include in the light of high degree of laminar thickness variation in complex folia; linear density Purkinje cell (the number of Purkinje cells/mm line of the whole folia (under x10), diameter of the Purkinje cells (under x40) and for this purpose adequate number of random images were taken in order to get 500 Purkinje cells having clear nuclei.

Biochemical Estimation and Analysis:

Blood glucose levels were measured from lateral tail vein blood at the biweekly interval with the help of Glucometer (Dr. Morepen GlucoOne BG03 Blood Glucose Meter). At the end of each study period, blood samples were obtained from direct puncture of heart and collected into sterilized plastic vials. Samples were allowed to clot, centrifuged at 2500 rpm for 30 minutes, the serum was separated and stored in sterile plastic vials and subsequently assayed for serum total protein content and serum creatinine level by using Avantor Benesphera^{TM} clinical chemistry Analyzer C61.

Statistical Analysis:

The data related to counting of Purkinje cells, 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 $P<0.005$ was considered as statistically significant.

RESULTS AND DISCUSSION

General Observations, Body weight, and Blood sugar:

In the current study, all diabetic groups exhibit the classical clinical symptoms of diabetes such as polydipsia, polyphagia, and polyuria. The mean body weights of all diabetic groups were reduced compared to the age-matched control group during the experimental period. Blood sugar

level significantly (P<0.01) increases in all diabetic group (>500 mg/dl) and maintained throughout the experimental periods as compared to the control group.

Microscopic observations

Histopathology

The cerebellar cortex of control and diabetic groups is seen to consist distinct three different cortical layers: it is made up of the outermost molecular layer, on cell thick Purkinje layer, and an inner granular layer. The central medullary region consists of white matter. The molecular layer contains unmyelinated fibers and few small scattered stellate cell neurons located superficially, glial cells, and few scattered basket cells in the deeper parts near Purkinje cell bodies. The Purkinje layer was observed to be sandwiched between the molecular and the granular layers. Large flask-shaped Purkinje cells have clear nuclei, prominent nucleoli, and a thin basophilic cytoplasm. The Purkinje cells are also surrounded by few astrocytes. The granular layer is composed of abundant tightly packed small neurons called the granule cells, which have large, rounded prominent, deeply stained nuclei and thin cytoplasm (Figure 1).

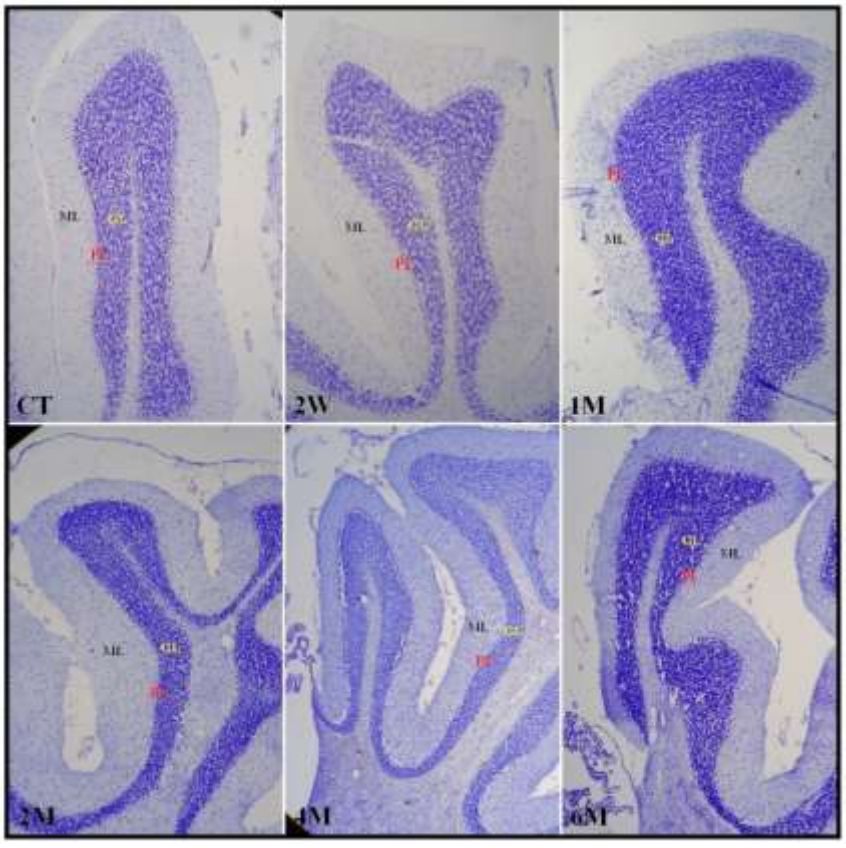

Figure 1: Photomicrograph showing control and diabetic groups. ML: molecular layer, PL: Purkinje cell layer and GL: granule cell layer, CV stains; initial magnification X 100.

In PTAH stained sections of cerebellar cortex of STZ-treated albino rats, Control, 2W, and 1M diabetic groups showed typical tri-laminar structure. However, in the diabetic groups the Purkinje cells were affected sporadically between the normal ones, while the molecular and the granular layers were almost similar to the control. The diabetic groups from 2 Month onwards revealed progressive increment in the qualitative and quantitative parameters in the form of frequent focal reduction in the thickness of molecular layer and its adhesion with the adjacent folia; loss of unique linear arrangement of Purkinje cells, distortion of cell shape, reduction of number, cell death and perineuronal space (Figure 2) were seen in Purkinje cell layer and in addition decrement of thickness and cell count of granular layer was also noticed. Cortical blood vessels were quite commonly observed in the molecular layer and granular layer of all groups. In PSR and LFB stain revealed the connective tissue surrounded the medullary vessels and the choroid plexus in the fourth ventricle which had fewer collagen fibers in control group. However, in the prolonged hyperglycemic groups the collagen fibers revealed added the thickening in the tunica adventitia of cerebellar vessels and the choroid plexus of fourth ventricle (Figure 3). Interestingly in the current study, sample from 2M diabetic group exhibited thin lamina of the white matter of superior medullary velum and the 4M diabetic group showed abnormal fusion of part of adjacent folia (Figure 4).

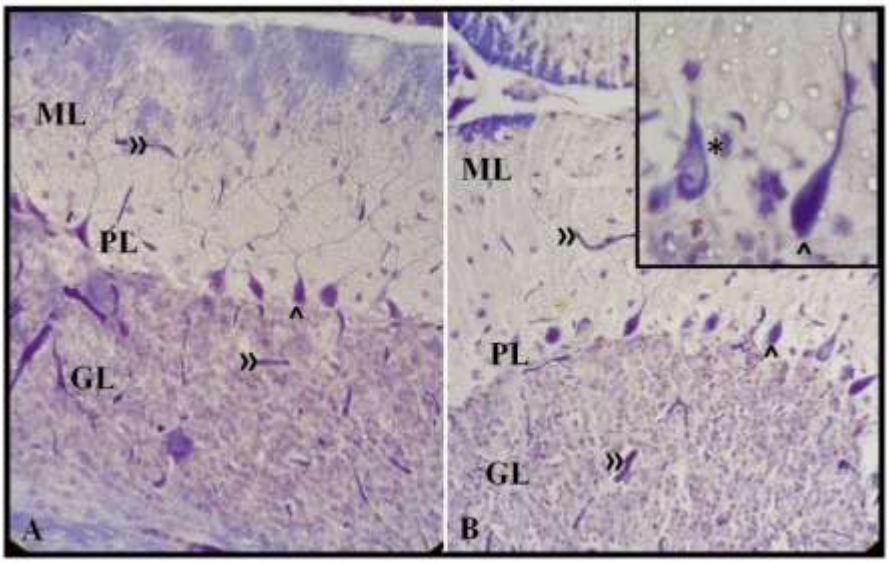

Figure 2: Photomicrograph from 4M (A) and 6M (B) diabetic group cerebellum showing ML: molecular layer, PL: Purkinje cell layer and GL: granule cell layer, Blood capillaries (>>), Perineuronal space (*), degenerated Purkinje cells (∧). Note: Many of the Purkinje cells appeared distorted and shrunken, losing their pyriform shape. PTAH stain, initial

magnification x 400. The inset shows Purkinje cells with the clear nucleus having perineuronal space around the cell and degenerated Purkinje cells. PTAH stain, initial magnification x 1000.

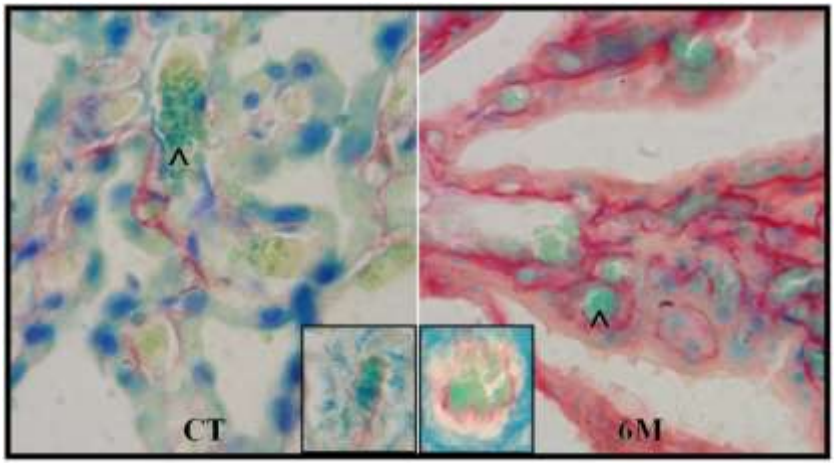

Figure 3: Photomicrograph showing the choroid plexus from the control and 6M diabetic groups. RBC (^) and collagen in the choroid plexus (stained red). Note thin collagen fibers in control and added the thickening collagen in 6M group. The inset shows medullary vessels of the corresponding group surrounded by red-stained collagen fibers. PSR stain, initial magnification x 1000.

Figure 4: Photomicrograph showing thin lamina of the white matter of superior medullary velum (long red ←) in the 2M diabetic group (PSR and LFB stain) and abnormal fusion of

part of adjacent folia (short yellow →) in the 4M diabetic group (PSR stain). Initial magnification x 100.

Histomorphometry:

The diameter of Purkinje cells (µm):

From 500 Purkinje cells having the clear nucleus, the results showed that the mean diameter was significantly (P<0.005) reduced in 4M and 6M diabetic groups compared to the age-matched control group. But in 2W, 1M, and 2M diabetic groups mean diameter of Purkinje cells were though less but not statistically significant le (Table: 1).

The number of Purkinje cells/mm length: Linear density analysis of Purkinje cells of only those having clear nucleus and prominent nucleolus (i.e. sectioned through middle part) showed that the decrease in the mean total number was statistically significant (P=<0.005) in all groups except 2W diabetic group.

The thickness of molecular and granular layers:

There was significant (P <0.005) decrease in the mean thickness of the molecular layer and granular layer of cerebellar cortex in the prolonged hyperglycemic groups of 2M, 4M, and 6M as compared to the age-matched control group. The 2W and 1M diabetic groups although showed reduction in the mean values which was not statistically significant (P >0.005) (Table: 1).

Table 1: **Show cerebellar laminar thickness, Purkinje cell number and their diameter in different groups (mean±SD).**

Groups	Diameter of Purkinje cells (µm)	Purkinje cell number (Per 1 mm)	Molecular layer thickness (µm)	Granular layer thickness (µm)
2WC	09.14 ± 01.35	21.14 ± 01.45	169.22 ± 17.48	267.37 ± 40.74
2WD	09.06 ± 01.43	20.84 ± 00.59	167.28 ± 14.85	266.20 ± 49.87
1MC	09.22 ± 01.54	20.49 ± 01.90	170.10 ± 17.26	266.28 ± 43.86
1MD	08.95 ± 01.67	16.26 ± 02.68	162.88 ± 11.06	256.20 ± 31.66
2MC	09.24 ± 01.42	20.27 ± 01.38	169.22 ± 17.48	265.27 ± 35.47
2MD	08.85 ± 01.28	13.03 ± 02.24	158.65 ± 18.39	225.84 ± 45.41
4MC	09.46 ± 00.89	20.51 ± 01.16	169.22 ± 17.48	264.80 ± 35.95
4MD	08.61 ± 01.06	09.61 ± 01.43	146.32 ± 11.00	215.48 ± 37.74
6MC	09.43 ± 00.86	20.87 ± 01.29	171.09 ± 17.15	261.99 ± 28.85
6MD	08.50 ± 01.31	06.36 ± 01.81	135.85 ± 07.64	207.10 ± 43.12

Biochemical analysis

All diabetic groups have shown significantly (P<0.005) decreased serum total protein levels as compared to age-matched control group and serum creatinine levels were significantly (P<0.005)

increased in all diabetic groups as compared to age-matched control group except 2W diabetic group. Data presented in our previous study[21].

DISCUSSION

Diabetes mellitus is a complex metabolic disorder commonly characterized by defective metabolism of carbohydrate, fat and protein due to insufficient amount or the action of insulin. Prolonged hyperglycemia is harmful for both peripheral and central nervous systems[22]. Reduction in body weight in the prolonged hyperglycemic state is principal as a result of increased muscle wasting and due to loss of tissue proteins during the release of amino acids for gluconeogenesis[23]. In the current study weight reduction in all prolonged diabetic groups, which reflected the increase in protein catabolism and the loss of tissue proteins in STZ-induced diabetic rats which agrees with previous related studies[24, 25].

The cerebellum is also called little brain, is a part of hindbrain which is responsible for the coordination of movements and preservation of balance. It is extensively used in studies on the motor system since cerebellar dysfunctions are generally related to motor disorders because the neurons in the cerebellum are placed among sensory and motor pathways of the body[26]. Differences in volume, morphology and histology of the cerebellum appear to relate to the performance of other types of tasks[27]. In the current study the structure and laminar orientation of cerebellum of the control and all diabetic groups similar and in agreement with the light microscopic findings of the previous studies[28, 29]. However, the prolonged diabetic groups showed some morphometric alterations in terms of reduced Purkinje cell diameter, the reduced thickness of molecular as well as granular layer and reduced number of Purkinje cells/mm length of the folia. These observations are in agreement with studies described in STZ-induced diabetes in the rat[30] which may be due to irreversible damage of cerebellar cortex due to hyperglycemia induced neuro-toxicity. Interestingly, in the current study, 2M diabetic group the superior medullary velum and in 4M diabetic group fusion of part of adjacent cerebellar folia were also noticed. Importance of such folial adhesion remains unclear.

The Purkinje cells are the largest and morphologically prominent neuronal group in the cerebellum and send inhibitory impulses to deep cerebellar nuclei mediated through GABA[31]. Around 15.4 millions of Purkinje cell neurons are said to be found in the human cerebellum[32]. However α-synuclein has been linked to synaptic plasticity, neurotransmitter release, neuronal differentiation, and regulation of neuronal viability[33]. In one study it has been shown that during hyperglycemia, Purkinje cells were vulnerable to damage by oxidative stress and chronic

inflammation followed by formation of glycated α-synuclein which accumulates in Purkinje cells in the cerebellum of the diabetic rat model[34]. While other study suggests that it is not only the glycation of α-synuclein but also the program cell death or block of neurogenesis in CNS[18, 35] as well as tissue acidosis[36] which could result in the reduction of Purkinje cells in hyperglycemia.

In the present study, the significant decrease in the number of Purkinje cells in all diabetic groups except 2W as compared with age-matched control groups and this was, however, more in those having long term hyperglycemia. In addition this result was also correlated with the decreased thickness of the granular and molecular layers. Some researchers observe decrease in the Purkinje cell density with advancement of diabetes[19, 37] similar to neurodegenerative diseases like Alzheimer's disease[38]. Hyperglycemia-associated reduction in neuronal population has been show earlier as well[39, 40].

Since the molecular layer is the superficial layer of the cerebellum having few perikaryon and many unmyelinated nerve fibers[41] and small cells likely include migrating granule cells. Here dendrites of the Purkinje cells, which make synapses to the other cells molecular layer including the basket and the stellate cells and their processes, receive input from the mossy or climbing fibers from various area of the central nervous system[42]. Any pathological alteration of neuron and processes in the molecular layer may reflect in the form of structural alterations. The current study revealed that a significant reduction of the molecular layer thickness in the prolonged hyperglycemic state is also eventually reflected in the overall decrease in cerebellar cortical thickness. The exact significance of the decrease in molecular layer thickness is not clear, but some researchers observed age-related reduction in the number of neurons in molecular and granular layers of cerebellum[43, 44] and age-related reduction and degeneration of Purkinje cells along with its dendrites[45] may be involved in the reduction of molecular layer thickness and this might result in cerebellar dysfunction.

The granular layer contains the majority of cerebellar neurons[46] which are responsible for sequential and coordinative movements[47]. In the present study, light microscopic results of the prolonged hyperglycemic groups of 2M, 4M, and 6M showed reduction in thickness, reduction of neuronal density and myelinated axons reflected in the form of reduced thickness of cortical layer as compared with age-matched control group. These findings were in agreement with those of some authors, who suggest that the reduction in the thickness of the granular layer may be either due to decreased number of granular cells[48], modifications of the blood vessels and area occupied by them[41] or else age-related neurodegeneration[44]. And these pathological alterations

may contribute in the represent etiologic factors for cerebellar atrophy and associated clinical changes.

Special stained sections from the control groups revealed thin collagen fibers both around cortical, medullary vessels and choroid plexus of the fourth ventricle but in the 6M diabetic group the collagen fibers were of remarkable thickness both around cortical and medullary vessels and also the choroid plexus suggestive of progressive fibrosis. Some researchers observed that AGE and RAGE synergy increases expression of TGF-β which promoted the development of fibrosis and neoangiogenesis[49]. Fibrosis is characterized by extracellular matrix accumulation and often by a change in the quality of the extracellular matrix as well as angiogenesis[50]. In contrast to these observations with current result suggests that the hyperglycemia seems to accelerate fibrosis in terms of the amount and thickness of collagen fibers around medullary vessels and choroid plexus in the fourth ventricle. Our previous studies also found sub-mesothelial fibrosis around autonomic ganglia sensory ganglia[40, 51].

Some researchers observed perineuronal spaces in some of the neurons of sensory ganglia[52], as well as autonomic ganglia[40] which were either due to shrinkage or apoptosis of neurons due to hyperglycemia-related neurotoxicity. Similar observations were found in prolonged diabetic groups of cerebellar Purkinje cell neurons.

In the current study, our results showed that the serum creatinine level increased in all prolonged diabetic groups as compared with age-matched control groups is parallel to the severity of hyperglycemia suggestive of derangement of renal function. The serum total protein levels were reduced in all diabetic groups compared with control group depending on the duration of hyperglycemia relating the hyperglycemia to a low-grade inflammatory process[53] and finding very similar to the other related studies[54].

CONCLUSION:

From the present histomorphometric and biochemical data it is concluded that long-standing hyperglycemia leads to reduction of number Purkinje cells and thickness of cerebellar cortical layers; increased collagen around cerebellar vessels and choroid plexus in conjunction with the derangement in biochemical parameters appear to promote the cerebellar functional alterations observed in chronic diabetics.

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