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Effects of STZ-Induced Long-Term Hyperglycemia on the Lumbar Dorsal Gray Column of Albino Rats- A Histomorphometric Study

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Original Research Article

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Abstract: One of the common clinical observations regarding long-standing diabetes is peripheral neuropathic pain, probably due to its destructive effects on the pain modulating neurons of the dorsal gray column of the spinal cord. Accordingly, the current study was aimed to analyze the effect of experimental hyperglycemia on pain modulating neurons in the lamina I-III of lumbar region of spinal cord of albino rats. Thirty-six albino rats with average weight ~250 g were grouped equally into six. Diabetes was induced with a single dose of streptozotocin (STZ, 60 mg/kg, i.p.). At the end of each experimental period, rats were euthanized by deep ether anesthesia, blood was collected and animals were perfused with Karnovsky fixative. Spinal cord was dissected and processed for histopathological and morphometric parameters and blood for biochemical analysis. Biochemical analysis of all diabetic groups revealed increased serum creatinine and reduced serum total protein. Histopathology and histomorphometry of dorsal gray column and ependymal cells and surrounding structures revealed that with the progressively increasing duration of hyperglycemia was associated with decreased number of pain modulating neurons in the lamina I-III as well as ependymal cell and in addition deposition of collagen fibers in the tunica adventitia of spinal arteries and around the small spinal vessels. The associations of long-standing hyperglycemia with reduction of dorsal gray column inter neurons, demyelination of nerve fibres and excessive deposition of collagen fibers in the tunica adventitia of blood vessels appear to be important contributing factors likely to be responsible for the diabetes-induced peripheral neuropathic pain.

Keywords: Collagen; Diabetes; Dorsal gray column; Neuropathic pain; Streptozotocin

INTRODUCTION

Diabetic myelopathy is a complication of diabetes mellitus characterized by gradually progressive degeneration of spinal cord [1] and manifested as hyperreflexia and non dermatomal extremity numbness [2]. Many animal studies have confirmed that increased oxidative stress and altered balance between pro- and antioxidants, probably plays a major role in the development of diabetic complications including diabetic neuropathy through common final common pathway inciting cellular injury [3,4]. Increased neuronal glucose level and oxidative stress may have association with many glucose-related pathogenetic pathways [5,6] and related neurotoxicity may induce variety of functional and structural abnormalities in both central and peripheral nervous systems [7]. Generally sensory neurons are more vulnerable to conditions of oxidative stress like those in diabetes and their association may provide explanation for prominent sensory loss and pain in diabetic patients. Excessive polyol flux, micro angiopathy, and oxidative stress involving alteration of neuron phenotype, mitochondrial dysfunction, ion channel alterations, and abnormal growth factor signaling, molecular changes in sensory neurons have been identified in diabetic models patients [8]. Another related study revealed that the long-term diabetics lead to the poly neuropathy that is coupled with limb numbness, allodynia, and insensitivity to injury, which leads to foot ulceration and may necessitate amputation [9].

Rexed's laminae (Lamina I to X), is an architectural classification based on the cytological differentiations of the neurons in different regions of the gray matter of the spinal cord. The dorsal gray column contains 90% interneuron's and 10% tract cells [10]. Laminae I and II are the superficial part of the dorsal horn. Lamina 1 also called Lamina marginalis and Layer of Waldeyer [10]. Modulation of nociceptive and pruritoceptive transmission occur in the dorsal part of gray column [11,12] and is transmitted to CNS from PNS [10]. Laminae I-III of the dorsal horn contains a large number of inhibitory interneuron's that modulate sensory information via GABA and or Glycine before this is transmitted to the brain and to other regions of the spinal cord and these neurons are considered to have

anti-nociceptive function [13,14]. In addition some researchers also found excitatory (glutamatergic) neurons which overlaps with other neurons [11] consisting of two morphological classes vertical and radial cells [15]. High threshold nociceptive afferent fibres terminate mainly in its superficial layers (laminae I and II) and low-threshold mechanosensitive afferent fibres mainly innervate the deep dorsal horn (laminae III–V) [10]. Chronic pain such due to the increased sensitivity to noxious stimuli (hyperalgesia) and the painful perception of input from non-nociceptive fibres (allodynia) are at least partially due to dysfunctions of dorsal horn inter neurons [16].

There are fewer studies available on the morphological and histopathological changes of dorsal horn of spinal cord [17] therefore, the present study aims at the assessment of alteration in the morphometric and microscopic parameters of inter-neurons, glial cells, nerve fibres, supporting tissue and vasculature in the dorsal horn especially lamina I-III of lumbosacral segments of the spinal cord with respect to the progressive duration of STZ-induced hyperglycemic state of 2W, 1M, 2M, 4M, and 6M periods.

MATERIAL AND METHODS

Non-diabetic rats of either sex (Total- 36 rats) weighing approximately 250g were obtained from central animal house, AMU, Aligarh after approval from Institutional Animal Ethics Committee (No: 9025/2014). Prior to commencement of the experiments, all animals were placed in the clean well ventilated, properly maintained new environmental condition and monitored daily with regard to body weight, for a period of one week. They were supplied with standard pellet diet and water ad libitum and maintained on a 12/12 h light/dark cycle. After one week of acclimatization, animals were divided into following six groups having six rats per group: (1) Non-diabetic healthy Control, age-matched (2) Diabetic Experimental groups: Two week, (3) One month (4) Two month (5) Four month and (6) Six month. After 12 hour fasting, experimental diabetes was induced by single dose of streptozotocin (STZ) (60 mg/kg, aqueous sol., i.p.). Blood sugar level was monitored with Glucometer (Dr Morepen GlucoOne BG03 Blood Glucose Meter) from the blood obtained from lateral tail vein before beginning and after 48 hour of 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. After assigned periods from the onset of diabetics, age-matched control and diabetic rats were euthanized with over dose of ether general anesthesia and whole body was perfusion-fixed with Karnovsky fixative. After two days spinal cord from lumbo-sacral segment were carefully dissected out and processed for paraffin embedding. Five µm thick paraffin sections stained with Hematoxyline & Eosin (H & E), Luxol fast blue (LFB), Cresyle Violet (CV) and PicroSirus (PSR) stained sections were studied under trinocular microscope (Olympus, BX40, and Japan) and relevant images were recorded under x 400 magnification with digital camera (Sony 18.2 MP, Japan). Measurements were made by using software Motic image version 2.0 were used for histopathology and histomorphometry. At the end of assigned experimental periods 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, stored and subsequently assayed for serum total protein content and serum creatinine level by using Avantor Benesphera^{TM} clinical chemistry Analyzer C61. The data related to the density and number of neurons in the lamina I -III, serum total proteins and serum creatinine level were statistically analyzed and the significance calculated using one way 'ANOVA' followed by Tukeys test. All numerical values were expressed as Mean ± SD and the value of $P<0.005$ was considered as statistically significant.

RESULTS

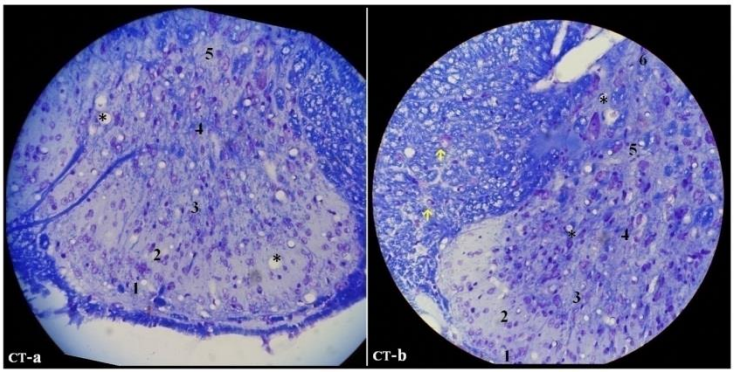
Clinical Observation

General clinical manifestations of diabetes such as polyphagia, polydipsia, and polyuria were observed in all diabetic groups after STZ-induction of diabetes. The mean values of body weight in all diabetic groups were reduced at all experimental stages as compared to control groups and after 2 days of STZ administration. The blood sugar level in all diabetic groups showed the hyperglycemic state (> 500 mg/dl) throughout experimental periods. Data exhibited in our previous studies [18].

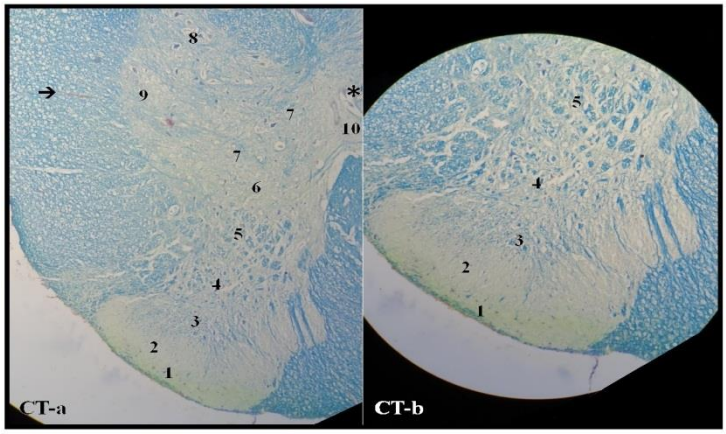
Microscopic observations

General observation of Cyto and myeloarchitectonic characteristics of spinal gray column: In all groups CV, LFB and PSR stained tissue revealed a clear outline of Cyto- and myeloarchitectonic characteristics of spinal gray column. However the lamina I in all groups were observed as a thin cell layer located at the edge of the dorsal horn having loosely dispersed cells consisting of small sized neurons, and myelinated as well as non myelinated nerve fibers. Lamina II or substantia gelatinosa of Rolando had densely packed small sized neurons and non-myelinated nerve fibers. Lamina III appeared curved having small and medium sized neurons, less closely packed than lamina II and thin bundles of myelinated fibers cross lamina III in all groups but in lamina IV had features very similar to lamina III but also contained additional large sized neurons. Lamina V had fewer neurons than lamina I to IV but the thickness and number of the bundles of myelinated nerve fibers were high. Lamina VI located at the base of the dorsal cell column and had

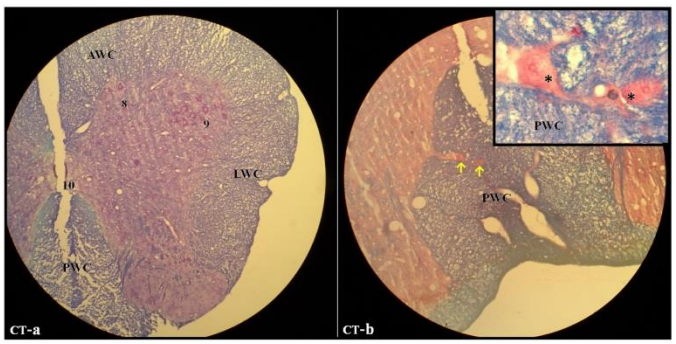
many small and medium-sized neurons at its medial area and multipolar neurons seen at its lateral part. Lamina VII connects ventral and dorsal horn and central part. Lamina VIII located on the medial part of the ventral horn and contains different sized neurons. Lamina IX on the lateral part of the ventral horn contain multi polar motor neurons while lamina X or substantia grisea centralis connects two half of gray column forming anterior and posterior gray commissure. Myelinated nerve fibers and small sized neurons were observed this region (Figure 1, 2 and 3).



Fig–1: Photomicrographs showing the part of dorsal gray column of control groups. Note: Cytoarchitecture laminae (1-6) and some myelinated nerve bundles. A very uncommon location of neuron within the lateral white column (CT-b) (↑) and blood vessels (*), CV and LFB stains; initial magnification X 100.



Fig–2: Photomicrographs showing myelo-architecture of spinal cord of Control rats. Note: Different lamina (1-10), medullary blood vessels (→) and central canal (*). LFB and PSR stain; initial magnification X 40 (CT-1) and X100 (CT-2)

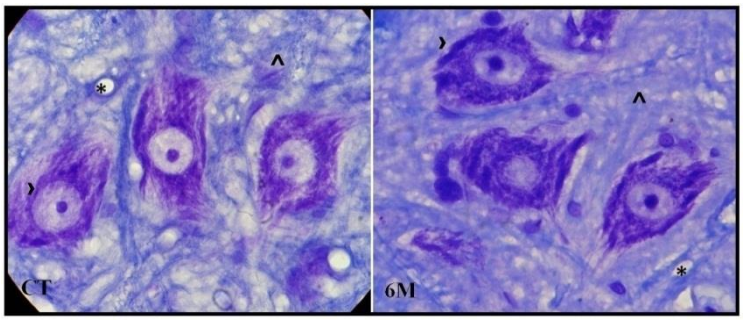


Fig–3: Photomicrographs showing the part of gray column showing lamina 8-10 (CT-a) and unusual location of neuron in posterior white column (CT-b). LFB, PSR and CV stains; initial magnification X 100. Inset: Showing neurons of the posterior white column (*) as in CT-b. X 1000.

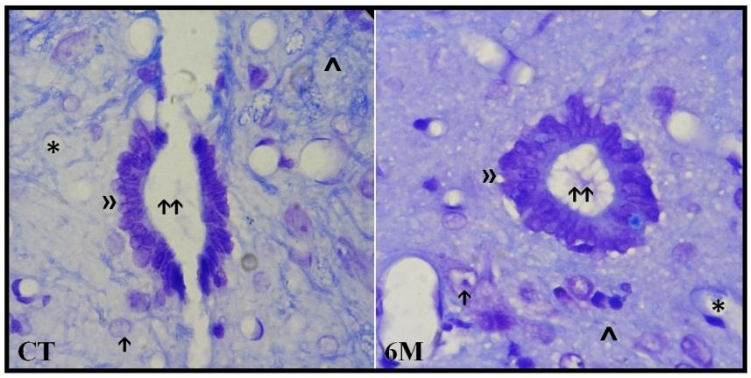
Histopathology

In all groups, generally varying sized neuron was observed in the spinal cord gray matter separated by bundles of myelinated nerve fibers of different thickness. The ventral grey column, in both control and diabetic groups neurones were multipolar and of different sized separated by bundles of myelinated nerve fibres in all groups. Though comparable features were also observed in prolonged diabetic groups, in 6M diabetic group they showed slight alteration in the motor neurons in the form of slight shrinkage and poorly stained myelinated fiber in majority of cases (Figure: 7). Blood capillaries were often seen close to the different sized motor neurons in all groups (Figure: 4). Dorsal horn of control group show groups of varying sized sensory neurons and other inter neurons densely packed with in bundles of myelinated and non-myelinated nerve fibers.

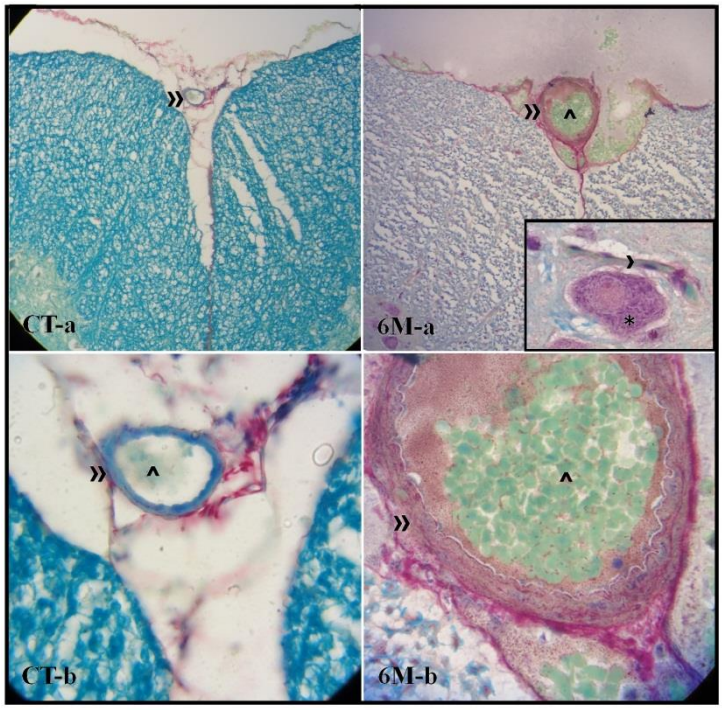
Ependymal cells were seen as simple cuboidal or low columnar type of lining cells having variable number of cilia and microvilli of all STZ- induced diabetic and age-matched control groups. In control group central canal was clearly surrounded by ependymal cells and disorganized arrangements as well as loss of ependymal cells around the central canal were clearly observed in 6M diabetic group as compared to control group (Figure: 5). In all groups blood vessels were quite commonly observed in the spinal cord and capillaries were located very close to neuronal stoma. The PSR and LFB stains revealed the connective tissue in the tunica adventitia of the anterior spinal vessels and capillaries of spinal cord which had fewer collagen fibers in control groups. However, in the prolonged hyperglycemic group of 6M the collagen fibers revealed added the thickening in the tunica adventitia of anterior spinal vessels and capillaries of spinal cord. Commonly the amount of collagen thickness showed a direct correlation with the duration of diabetes as compared to the age-matched controls (Figure 6).



Fig−4: Photomicrographs showing the part of ventral gray column of control and 6M diabetic groups. Note: almost uniformly arranged myelinated nerve fibers (∧), multi polar motor neurons (>), and blood capillary (*). CV stains; initial magnification X 1000.



Fig−5: Photomicrographs showing central canal lined by ependymal cells of control and 6M diabetic groups (>>), central canal (↑↑), myelinated nerve fiber (∧), neuron (↑), and blood vessels (*). Note: the ependymal cells in 6M appear to be more numerous having denser apical specialization and also associated with focal disruption in their continuity possibly due to cell death. CV stains; initial magnification X 1000.

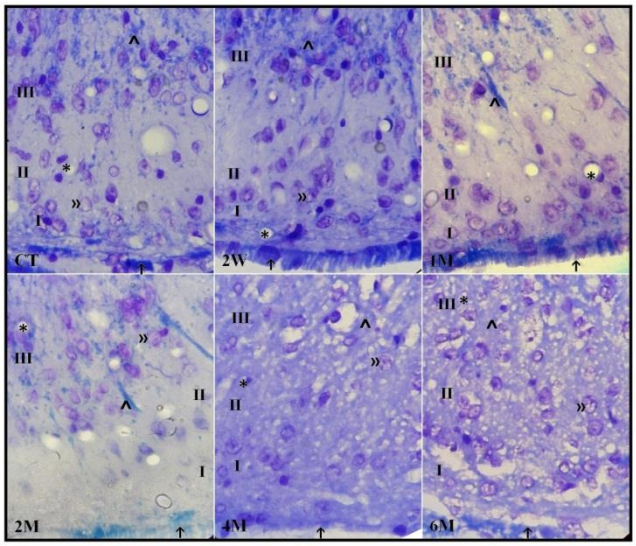


Fig−6: Photomicrographs showing red stained collagen (>>) in the adventitial of anterior spinal arteries of control and 6M diabetic groups. Note heaping up of collagen in 6M as compared to control, myelinated nerve fiber (↑), RBC (∧). LFB and PSR stains; initial magnification upper pannel X200 and lower panel X 1000. The inset shows (6M-a) motor neuron (*) with blood capillaries (>) in its close vicinity, x 1000.

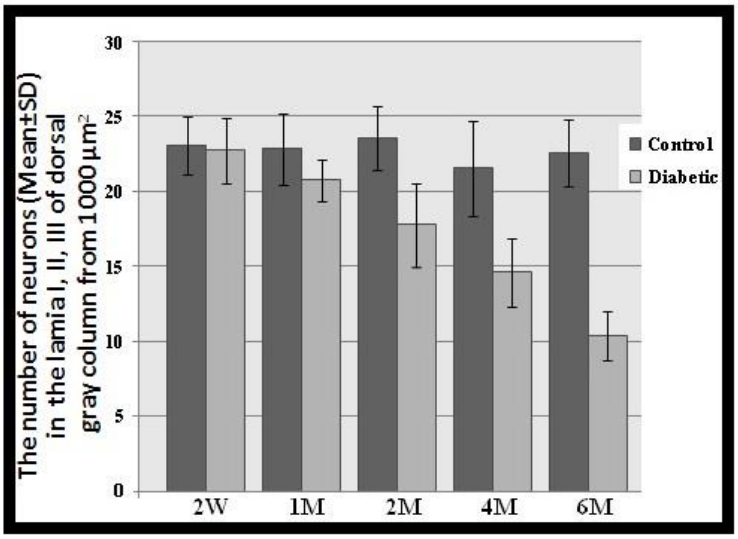
Histopathological Observation

Total neuronal number in given area (10000µm^2)

The total neuronal number in 10000 µm^2 areas of the dorsal gray column (lamina I-III) of the rat spinal cord revealed decrement of mean number of the neurons in the lamina of I-III of diabetic group as compared with age- matched controls. However, the decrement is significant (P<0.005) only in 1M, 2M, 4M and 6M diabetic groups (Figure 7, 8 and Table 1).



Fig−7: Photomicrographs showing the lamina I-III of dorsal gray column of all diabetic groups. Note lamina I-III, myelinated nerve fiber (∧) inter neurons (>>), blood capillaries (*) and dorsolateral tract (↑). LFB and CV stains; initial magnification X 1000



Fig−8: Showing that the number of total neurons in the lamina I-III was decreases with advancement of duration of hyperglycemia as compared with age-matched control groups

Table−1: Showing changes in the number of ependymal cells lining the central canal and neurons of dorsal gray column Lamina I-III (mean±SD).

Groups	2W	1M	2M	4M	6M
Control	23.11±1.96	22.86±2.35	23.58±2.15	21.54±3.19	22.60±2.19
Diabetic	22.75±2.15	20.76±1.37	17.82±2.78	14.61±2.27	10.42±1.59

Biochemical analysis

In the current study, the serum creatinine level were significantly (P<0.05) increases in all diabetic groups as compared to age-matched control groups except 2W diabetic group. The serum total protein levels significantly (P<0.05) decreases in all diabetic groups as compared to corresponding control group as presented earlier [19].

DISCUSSION

Hyperglycemia occurs due to reduced secretion of anabolic insulin hormone from the beta-cells of pancreas or decreased tissue response to insulin [20]. This is followed by complex low-grade inflammation [21] and reduction of body weight due to increased muscle wasting as a result of increased protein catabolism in muscles and release of amino acids for gluconeogenesis [22,23]. In the current study, STZ-induced diabetic groups exhibit the hyperglycemic state as well as marked reduction of mean body weight throughout experimental period as compared with age-matched control groups. Pervious related studies also observed hyperglycemia and reduction of body weight during the experimental periods [3,24] and this was found to be in agreement with present study.

Prolonged hyperglycemia as well as reduced intra cellular anti oxidants is believed to be related with the systemic and cellular oxidative stress followed by high generation of free radicals beyond the scavenging abilities of endogenous antioxidant defenses resulting in macro-and microvascular dysfunction [25] which initiates cellular injury leading to many diabetic complications [26]. Long standing hyperglycemia leads to glucose neurotoxicity [27] and non-enzymatic protein glycation and enhanced the flux of glucose through the polyol pathway [28] which leads to alterations of high levels of polyunsaturated lipids in the brain, direct lipid peroxidation causing lipid membrane disruption and consequent slow progressive functional and structural neurodegenerative changes [7,29].

The structure and orientation in terms of cyto- and myelo-architecture of gray column of the spinal cord were reported to be similar in the control and diabetic groups [30] which are in agreement with the findings of the present study. Moreover in all groups different laminar arrangement by different sized myelinated nerve bundles and aggregation of different sized neurons can easily be observed. However, in the prolonged diabetic groups especially the 6M diabetic group showed some structural alterations in terms of poor staining myelin possibly indicating partial demyelination in majority of nerve fibres. One related study observed alterations of myelin in different regions of the nervous system due to decreased myelin-associated glycoprotein, auto antibodies to myelin basic protein, myelin damage induced by nitric oxide, impairment of oligodendrocyte function, and inhibition of glial cell proliferation [31]. Another study explains that insulin necessary for the expression of myelin glycoprotein P0 and also necessary for the maintenance of myelin structure [32]. Generally tract neurons and other interneurons were located within gray column. In

the current study in the control groups, some tract neurons were also located inside the white matter mainly lateral and posterior white column. Significance of such displaced neurons is not yet clear.

Ependymal cells are non-neuronal and modified glial cells characterized by simple ciliated columnar cells which line the central canal of the spinal cord. These cells are believed to be responsible for the exchange of nutrients from CSF to extracellular fluid in the neuropil the spinal cord [33, 34], in addition they also provide mechanical, chemical as well as metabolic support [35]. However it acts as spinal cord-CSF crossing point. They express the glucose transporters GLUT1 and GLUT2, the $Na^+/K^+/2Cl^-$ co-transporter, and the monocarboxylate transporter 1, all of which can move water against an osmotic gradient [36]. Brain glycogen is metabolized by enzymes, glycogen phosphorylase and glycogen synthase [37]. The glycogen phosphorylase brain isoform occurs mainly in astrocytes, but interestingly it can also be found in several other cell types, such as the choroid plexus cells and ependymal cells [38]. In hyperglycemia their biological activity and quantity is also reduced [39]. In the current study, the central canal in all groups is covered by a various sized ependymal cells. However, in prolonged hyperglycemic group structural alteration of ependymal cells could be noticed. This result was in agreement with those of other investigators who reported structural and functional disturbances of ependyma in prolonged hyperglycemia [39].

Basement membrane thickening is the most prominent and characteristic feature of diabetic microangiopathy [40]. Insoluble frame work of collagen, elastin, glycoproteins, proteoglycans – hyaluronans and integrins in the extra cellular matrix of vascular adventitia which is responsible for the mechanical supports as well as complex interaction between cells or between cells and extra cellular matrix of vessels [41]. The basic components of the collagen is an amino acids which is wound together to form triple-helices of elongated fibrils [42]. In fibrosis, collagen fibrils is produced by fusion of short and thin fibrils with tapered ends and extracellular matrix alterations by the activation of pro-sclerotic cytokines and protease or anti-protease systems initiate alterations of the extra cellular matrix and angiogenesis [43]. Types I, II, and III collagen are the fibrillar – interstitial collagens which responsible for diabetic induced fibrosis [41]. In our previous study [44] the collagen in the tunica adventitia of anterior cerebral artery was found to be thicker in diabetic group than that of the control group. Fibrosis in relation to dorsal root ganglion [45] has also been described. In the present study, the control group showed thin collagen fibres around the tunica adventitia of anterior spinal artery while the prolonged diabetic group of 6M showed remarkable presence thick collagen fibres. Fibrosis in aforementioned locations may be explained by the fact that PKC-β and p38 mitogen activated protein kinase expression in redox reaction was found in diabetic heart which is responsible for the progression of fibrosis [46]. Moreover AGE and RAGE interaction and increased expression of TGF-β which accelerate to the development of fibrosis and neoangiogenesis [47].

It has generally been reported that chronic hyperglycemia enhances neuronal alterations. The present study described the alterations in the neuronal number in the laminaI-III of rat dorsal gray column of the spinal cord resulting from prolonged STZ- induced hyperglycemia. There was a statistically significant loss of neurons in the laminaI-III of 2M, 4M and 6M diabetic groups as compared with age-matched control groups. Some experimental studies reported that hyperglycemia causes decrease in the number of neurons in gray matter [48]. Hyperglycemia accelerates polyol pathway and aerobic glycolysis followed by accumulation of excess amount of lactic acid in the brain, leads to brain acidosis [49, 50] and dehydration, both involved in reduction of blood flow and ischemia [51]. Ischemia-related edema involves stimulation of Na-K-Cl co-transporter system facilitating edema formation and swelling of endothelial cells [52]. Moreover production of oxidative stress and excessive formation of free radical followed by consumption of NADPH [53] and reduction in levels of protective endogenous antioxidants [54,55] also involved. Related biochemical alterations induces mitochondrial fragmentation, AGE as well as RAGE increased secondary neuronal death by oxidizing proteins, damaging DNA, and inducing the lipid peroxidation of cellular membranes [29, 56]. Programmed cell deaths in diabetes mellitus also affect neurons because of disturbed trophic support possibly due to decreased insulin or insulin-like growth factor signaling, or an increase in cytokines such as TNFa [57, 58]. These evidences and current results which point out that prolonged hyperglycemia accelerate alterations of the dorsal gray column neuron of the spinal cord. In addition, gradual loss of neuropathic pain sensation is possibly due to altered function or loss of pain modulating neurons in the lamina I-III of the dorsal gray column of spinal cord. Some investigators reported hyperglycemia induces alterations of gray matter and white matter [59]. However, experimental studies suggested that hyperglycemia and associated oxidative stress have subsequent negative effects on neurons. In this context, the pattern of white matter abnormalities may be related to the progression of the age because hyperglycemia is known to induce acceleration of brain ageing [60]. One research finding also suggests that blood-brain barrier glucose transport activity is lower in white matter than the gray matter [61].

Creatinine has been found to be a fairly reliable biomarker of kidney function. Elevated creatinine level due to pure clearance of creatinine signifies impaired kidney function and also associated with greater protein catabolism [62]. In general creatinine is an amino acid derived from muscle metabolism and it is cleared by tubular secretion [63]. Insulin resistance followed by high muscle metabolism results elevated serum creatinine due to diabetic nephropathy [62,64]. The current study revealed that high serum creatinine level in all STZ-induced diabetic groups is corresponding to the duration of hyperglycemia. The current and previous results demonstrated a positive correlation between duration of hyperglycaemia and the development of diabetic related nephropathy. Total serum protein decreases in all STZ-induced diabetic groups compared with control. Similar results have been shown in other related studies [65,66]. One study suggests reduction of total serum protein due to low-grade inflammatory process in prolonged hyperglycemia [67].

CONCLUSION

Based on the histopathological, histomorphological and biochemical findings it is concluded that hyperglycemia-induced neuronal cytotoxicity, reduction of neurons of lamina I-III and alteration of micro-vascular environment appear to be the significant contributing factors in the development of neuropathic pain and dorsal gray column dysfunction in chronic diabetes.

Conflict of interest

The authors declare that they have no conflict of interest

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