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Original Research Article

Effect of Experimental Hyperglycemia on the Trigeminal Ganglia of Albino Rats

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ABSTRACT

One of the most common presentations of long standing hyperglycemia is peripheral neuropathy which is possibly due to its deleterious effect on the primary sensory neurons located in the sensory ganglia. Therefore, the present study was aimed at to explore the effect of hyperglycemia on trigeminal ganglia. 24 animals were divided into four groups having six rats in each group; control, two week, two month and four month. Diabetes was induced with single dose of streptozotocin administered through intraperitoneal route (60 mg/kg). Body weight and blood sugar was monitored at biweekly interval. At the end of each experimental period animals were euthanized by deep ether anesthesia and blood samples were collected into sterilized vials by direct puncture of heart for biochemical analysis. Tissue were fixed in Karnovsky fixative and processed for light microscopical studies. Biochemical analysis and histopathological features revealed that increasing duration of hyperglycemia was associated with increased serum creatinine and reduced serum total protein; decrease proportion of small and medium sized neurons, increasing frequency of dark and dead neurons and thickening of capsular and endoneurial collagen and vascularity. It is therefore concluded that association of the long standing hyperglycemia with increased neuronal death and deposition of collagen fibres in sensory ganglia is most likely to be responsible for diabetic peripheral neuropathy.

Key Words: Collagen, Diabetes, Neuronal death, Sensory ganglia

INTRODUCTION

Diabetes mellitus is a common metabolic disorder. [1] Hyperglycemia is believed to be associated with increased systemic and cellular oxidative stress, which initiates cellular injury leading to diabetic complications. [2,3] Increased neuronal glucose levels leads to glucose neurotoxicity [4] and this is likely to causes a variety of functional and structural disorders in both central and peripheral nervous systems. [5] Since sensory ganglia are neither associated with blood–brain barrier nor blood-nerve barrier and have higher metabolic requirements than nerve trunks, therefore are more susceptible to conditions of oxidative stress like those in diabetes. [4,6] Oxidative stress has been shown to cause mitochondrial dysfunction which is followed by neuronal apoptosis. [7] In other studies [7,8] it has been suggested that the primary target of diabetes is sensory ganglia neurons which results in to the development of structural changes in the ganglia even altered neuron phenotype, mitochondrial dysfunction, ion channel alterations, and abnormal growth factor signaling. [9,10] Another study [11] revealed that the long-term diabetics lead to the abnormalities in the ganglion cell and nerve fibres.

The trigeminal ganglia (TrG) is a main relay station of sensory information from the orofacial complex in the human as well as in most mammals and it maintains a

constant control upon the transmitted information from the PNS to the CNS. [12] Other researchers [13, 14] have tried to demonstrate changes in neuron, collagen and myelin loss without using any special stain. Therefore, the present study is aimed at demonstrating these and possibly other changes in the arrangement of collagen fibers, nerve fibres and neuronal structure by using special staining for collagen staining (PSR) and myelin (LFB) with CV staining in conjunction with histopathological, histomorphological and biochemical parameters in experimentally induced diabetic rats after 2W, 2M and 4M periods.

MATERIALS AND METHODS

***Animal Preparation*:** After approval from Institutional Animal Ethics Committee (No: 9025/2014) the albino rats of either sex weighing 200g to 250g, for experiment were obtained from central animal house, AMU, Aligarh. Prior to commencement of the experiments, animals were acclimatized to the new environmental condition for a period of one week. They were kept in a well ventilated room and were supplied standard pellet diet.

***Experimental Design*:** Animals were divided into following four groups having six rats in each group: (1) Non-diabetic: Control (2) Experimental diabetics: Two week, (3) Two month (4) and Four month.

***Induction of Diabetes*:** After 12 hours fasting, experimental group were administered Streptozotocin (60 mg/kg, aquous sol., I.P., only once). Blood sugar level was monitored with Glucometer (Dr Morepen gluco one) before and after 2nd day streptozotocin injection. Animals with blood sugar level 250 mg/dl and above were considered as diabetic and were included in this study. Weight and blood glucose levels of all animals in each group were monitored biweekly.

***Tissue preparation*:** After designated experimental period rats were euthanized with over dose of ether and animals were perfusion-fixed with Karnovsky's fixative.

Histopathology and Histomorphometry

Fixed tissue samples were processed for paraffin embedding. Five µm thick sections were stained with Hematoxyline & Eosin (H & E), Cresyle Violet (CV) and PicroSirus with Luxol Fast Blue (PSR with LFB). Only H & E and CV stained sections were used for measuring neuronal diameter.

Neuronal size: Only those neurons having clearly defined nucleus with nucleolus and cytoplasm were used for diameter measurements and neuronal counting. Based on diameter, neurons were divided into small (< 20 µm), medium (20-30 µm) and large sized (>30 µm). Histological features under x 400 magnification of trinocular microscope (Olympus, BX40, and Japan) were recorded by digital camera (Sony 18.2 MP, Japan) and measurements were made by using software Motic image version 2.0.

***Dark and Light neurons*:** The neurons were identified as dark or light on the basis of their morphology and staining characteristics of cell body and nucleus with visible nucleolus within the nucleus.

***Biochemical Estimation and Analysis*:** Blood glucose levels were measured from lateral tail vein blood biweekly by using Glucometer. At the end of each study period blood samples were collected into sterilized vials through direct puncture of heart. Samples were allowed to clot, centrifuged at 2500 rpm for 30 min, the serum was separated and stored in vials and subsequently assayed for serum total protein content and serum creatinine level.

Statistical Analysis

The data related to number of neurons, 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

RESULTS

General features: In all diabetic groups the typical clinic manifestations of the diabetes

such as polyphagia, polydipsia and polyuria were observed after induction of diabetes.

***Body weight*:** The mean values of body weight in diabetic groups were reduced at all experimental stages as compared to control groups (Table: 1).

Table 1: Initial and final weight (g) of different groups (Mean ± SD)

Body weight (g)	0 Day	2 Week	2 Month	4 Month
Control	249.67 ± 06.15	251.83 ± 05.71	271.33 ± 03.72	339.17 ± 17.74
Diabetic	248.17 ± 05.60	240.17 ± 05.60	201.83 ± 06.59	169.05 ± 09.65

Note: Showing the mean values of body weight were reduced in all experimental stages as compared to control groups.

Blood glucose level: Mean values of blood sugar level in all diabetic group showed hyperglycemic state (> 500 mg/dl) throughout experimental periods (Table: 2).

Table 2: Showing blood sugar (mg/dl) level during the period of study. (Mean ± SD)

Blood Glucose level (mg/dl)	0 Day	2 Week	2 Month	4 Month
Control	120.67 ± 05.85	116.83 ± 08.23	124.12 ± 06.57	128.67 ± 05.85
Diabetic	117.83 ± 04.63	542.66 ± 67.05	530.17 ± 63.51	527.83 ± 62.64

Note: Showing mean values of blood sugar level in all diabetic groups showing hyperglycemic state (> 500 mg/dl) throughout experimental period

Microscopic observations

Histopathology

In all groups the neurons were round to oval in shape. In control and 2-Week diabetic groups these neurons were compactly packed in clusters and separated by bundles of myelinated nerve fibres and few collagen fibres around the neurons and along the nerve fibers. Almost similar features were also observed in 2 month diabetic group however they showed slight difference in the collagen fibres thickening. Whereas in 4 month diabetic group neurons were in the form of uniformly distributed clusters separated by few myelinated nerve fibres and numerous thickened collagen fibers were seen around the neurons and between clusters of neurons and capsule of the ganglia. Perineuronal spaces were found in diabetic groups. Intra ganglionic blood capillaries were often seen close to the neurons in all groups (Figure: 1).

Although light and dark stained neurons were showed in all groups, as compared to control group the occurrence of dark neurons were more often observed with increasing duration of of hyperglycemic state in diabetic groups. Neurons having eccentric nuclei were either associated with or without chromatolysis. Such neurons were more commonly seen in TrG of 4M diabetic group than other diabetic groups. Small population of neurons showed peripheral rim of chromatin granules. Fibrocytes nuclei were seen parallel to the myelinated nerve fibers (Figure 2).

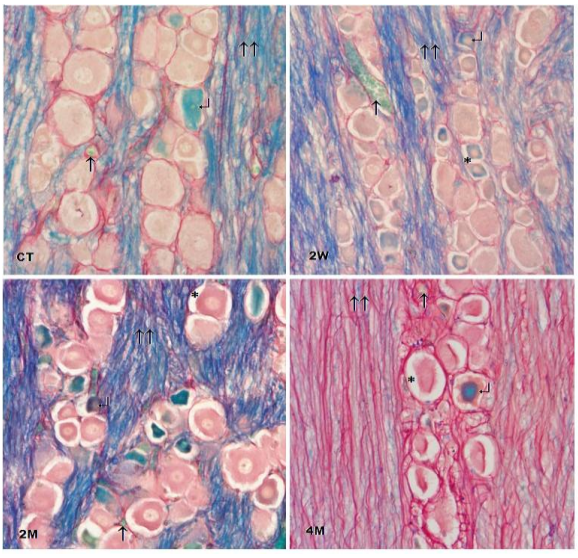

Figure 1: PSR with LFB stained sections, Arrows (↑) pointing the blood capillaries, (↑↑) pointing the myelinated nerve fibres, (↵) pointing dark neuron, (∗) pointing perineuronal space. Collagen fibers (Red Colour) along the nerve fibers and around the neurons, in 4M diabetic group more thickened and numerous collagen fibers were seen around neurons and between clusters of neurons and capsule of ganglia. Initial magnification X400.

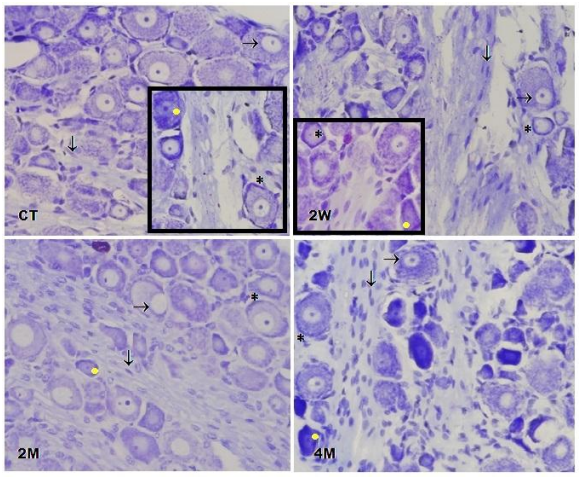

Figure 2: Cresyle Violet stained sections, Arrows (→) pointing eccentric nucleus, (✱) pointing peripheral rim, (↓) fibrocytes, and (Yellow Dot) pointing dark neurons. And Control and 2 W (Yellow Dot) pointing dark neuron and. (∗) pointing peripheral rim (In set) at initial magnification X400.

Dead neurons were rarely observed in control group but these neurons were more in diabetic groups. Disorganized arrangement of satellite cells was clearly observed in 4M diabetic group as compared to all other groups (Figure 3).

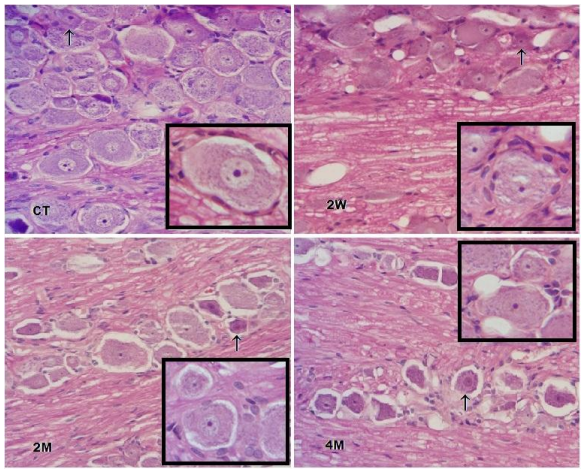

Figure 3: H & E stained sections, Arrows (↑) pointing dead neuron at initial magnification x400.Inset showing organized satellite cells in control group and disorganized cells in experimental groups at initial magnification x1000.

Histomorphology

Among 1000 neurons the proportion of small and medium sized neurons were significantly (P<0.05) reduced in 4M diabetic group compared to all other group. As compared to control group, in 2W and 2M diabetic groups the proportion of these neurons were less but not at significant level. Proportion of large sized neurons were increased in all diabetic groups but at nonsignificant level compared to control group (Figure 4).

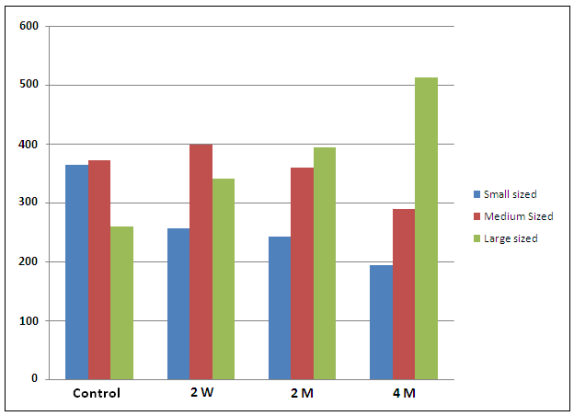

Figure 4: Showing the proportion of small and medium sized neurons was significantly (P<0.05) reduced in 4M diabetic group compared to all other groups.

Biochemical analysis

Serum creatinine level were significantly (P<0.01) increased in 2M and 4M diabetic group compaired to 2W and control groups. In all diabetic groups the serum total protien levels significantly (P<0.01) decreased compaired to control group (Table 3).

Table 3: Showing serum total protein and serum creatinine level. (Mean ± SD)

Analysis	Control	2 Weeks	2 Months	4 Months
Serum total protein (g/dl)	5.97 ± 0.04	5.23 ± 0.01	5.00 ± 0.07	4.05 ± 0.03
Serum Creatinine (mg/dl)	0.43 ± 0.02	0.45 ± 0.07	0.78 ± 0.03	0.93 ± 0.09

Note: Serum creatinine level were significantly (P<0.01) increased in 2M and 4 M diabetic group compaired to 2W and control groups. In all diabetic groups the serum total protien levels significantly (P<0.01) decreased compaired to control group.

DISCUSSION

Diabetes mellitus is considered to be a complex low grade inflammatory metabolic disorder [15] either characterized by insufficient amounts of insulin, or in which tissues fail to respond appropriately

to insulin, which leads to hyperglycemia. [16] In Diabetes the reduction in body weight is primarily due to increased muscle wasting and due to loss of tissue proteins. [17] Previous studies [18, 19] have reported that body weight were reduced in diabetic group and this was found to be in agreement with present study that all diabetic groups maintained the hyperglycemic state throughout experimental periods and there was a progressive weight reduction in all diabetic groups.

The structure and orientation of nerve fibers were reported to be similar in the control and diabetic groups [20] which are agreement with the findings of the present study. However, the 4M diabetic group showed some structural changes in terms of poor staining myelin indicating demyelination in majority of nerve fibres.

In one study the connective tissue capsule was found to be thicker in diabetic group than that of the control group. [13] In the present study the control and 2W diabetic groups showed groups of neurons separated by bundle of myelinated nerve fibre and thin collagen fibres around the neurons and along the nerve bundles. 2M diabetic group showed features very similar to that of control and only slight difference in the thickening collagen fibres. In 4M diabetic group the special stain revealed remarkable presence of collagen in capsule of the ganglia, perineuronal and interfasciclular region and even in the endoneurium.

Regularly arranged clusters of neurons separated by few myelinated nerve fibres and numerous thickened collagen fibers around the neurons and between clusters of neurons were also noticed.

One study in dorsal root ganglia (DrG) [13] has shown perineuronal spaces in some of the neurons are due to either shrinking or apoptotic on progression of hyperglycemic state was also comparable to some of the finding in the present study.

Dark neurons were included in apoptotic type of neuron [21] and type of cell degeneration with hyper basophilic and hyper electron density properties. [22, 23] The present study revealed that on progression of hyperglycemic state in diabetic groups the numbers of dark neurons were increased, this is in agreement with other studies that in diabetes the hyperglycemia and increased free radical generation accelerate the dark neuron formation. [21]

In this study, more eccentric nuclei with and without chromatolysis were notice with advancement of hyperglycemic state in diabetic groups but in control group these type of nuclei were only occasionally seen. In other related studies [24, 25] reported that in DrG eccentric nuclei are rarely seen and it may be due to aging process. Migration of the nucleus to an eccentric position in the neurons and a loss of Nissl substance in the neuronal perikarya are due to chromatolytic changes [26] and these changes were more obvious in diabetic groups. The present study only few neurons have shown features having a prominent peripheral rim of Nissl substance which in accordance with the observation made earlier for both TrG and DrG. [24, 25, 27]

Small and medium sized neurons are nociceptors which are mainly concerned with pain and temperature [28] and in trigeminal ganglion the small sized neurons modulate pain sensation in migraine. [29] In our study it was noticed that the proportion of small and medium sized neurons were significantly ($P< 0.05$) decreased in all diabetic groups, these findings indicate that in diabetes the gradual loss of neuropathic pain sensation is possibly due to altered function or loss of small and medium sized neurons.

Dead neurons were standout due their dark staining characteristics as compared to typical normal neurons. The nucleus in such neuron also turns dark and become shrunken, nuclear membrane becomes less distinct and nucleoplasm no more remains open-faced very similar to one described [30] about dead neurons as being red stained acidophilic neurons with shrunken, triangular shaped dense purple nuclei. In this study only occasional dead

neurons were noticed in control group. However with progression of hyperglycemic state in diabetic groups, number of such neurons increased; this finding is correlates with another study [30] that the diabetes enhances the neuronal death.

It has been shown that abnormally high levels of serum creatinine are consistent with the impaired kidney function. [31] Our result showed that the serum creatinine level was increased in all diabetic groups parallel to the severity of hyperglycemia but the serum total protein levels were reduced. Similar observations have been shown in the other related studies. [32, 33]

CONCLUSION

Based on histopathological, histomorphological and biochemical findings it is concluded that the prolonged hyperglycemic state leads to increased serum creatinine level, reduced serum total protein; decrease in the proportion of small and medium sized neurons and increase in dark and dead neurons and thickening of collagen fibres. Therefore, it appears that peripheral neuropathy in chronic diabetes might be due to hyperglycemia-induced neuronal cytotoxicity, demyelination and fibrosis.

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***Conflict Of Interest**:* None

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