Introduction

Diabetes mellitus is a group of common metabolic disorders that is characterized by hyperglycemia and insufficiency in production or action of insulin. Hyperglycemia is believed to be associated with increased free radicals and cellular oxidative stress, which initiates cellular injury leading to diabetic complications including retinopathy, neuropathy, nephropathy and coronary artery diseases. Hyperglycemia followed by high neuronal glucose levels leads to glucose neurotoxicity, and prolonged high intracellular glucose in neurons leads to a variety of functional and structural disorders in various region of nervous system. Due to high cytosolic oxidative stress, both sympathetic and parasympathetic neurons may be more susceptible to pathological changes of oxidative stress like those in diabetes. Oxidative stress followed by mitochondrial dysfunction initiates neuronal apoptosis and is a major mediator of neurodegeneration in diabetes. Autonomic dysfunction affects normal functioning of the cardiovascular, respiratory, gastrointestinal, genitourinary, thermoregulatory, and neuroendocrine systems.

Autonomic ganglia serve as the final output of discrete CNS structures that control the function of the periphery. The pterygopalatine ganglion (PtG) is the largest parasympathetic ganglion located in the pterygopalatine fossa and is functionally attached to the facial nerve. Sensory fibers from the maxillary branch of trigeminal nerve and sympathetic fibers pass through ganglia, but do not synapse in them. The coeliac ganglia (ClG) are a...
well-defined prevertebral sympathetic ganglia located on the sides of the coeliac trunk having a varying number of neurons with extensive dendritic fields.\textsuperscript{[10]} CIG receive convergent synaptic input from spinal preganglionic neurons and peripheral intestinofugal neurons projecting from the gut.\textsuperscript{[11]} Related studies\textsuperscript{[12,13]} found changes in neurons and alteration of myelinated and unmyelinated nerve fibers and collagen without using any special stain. Therefore, the present study aimed at demonstrating these and possibly other changes in the arrangement of collagen fibers and neuronal structure by using special stainings for collagen, picrosirius red (PSR) and cresyl violet (CV) stainings together with histopathological, histomorphological and biochemical parameters, in experimentally induced diabetic rats after two weeks to six months periods.

**Materials and Methods**

After approval from Institutional Animal Ethics Committee (No: 9025/2014), 36 albino rats of either sex weighing approximately 250 g were obtained from central animal house, Aligarh Muslim University, Aligarh were used for the present study. Prior to commencement of the experiments, all animals were placed to the new environmental condition for a period of one week. Animals were kept in a well-ventilated room and were supplied standard pellet diet and water ad libitum and maintained on a 12/12 h light/dark cycle.

Animals were divided into following six groups having six rats in each group: Group 1: non-diabetic healthy control, age-matched; diabetic experimental groups: Group 2: two week, Group 3: one month; Group 4: two months; Group 5: four months; Group 6: six months.

The experimental diabetic model was induced using streptozotocin (STZ) (60 mg/kg, aqueous sol., I.P., only once; SRL-Sisco Research Laboratories, Mumbai, India) after 12 hours of fasting. Blood sugar level was monitored with a glucometer from the blood obtained from the lateral tail vein before and on the 2nd day 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.\textsuperscript{[14]}

After the designated period, all experimental and age-matched control rats were euthanized with overdose of ether general anesthesia, and the rats were perfusion-fixed with Karnovsky’s fixative.

PrtGs were carefully dissected out from the pterygopalatine fossa along with the maxillary branch of the trigeminal ganglion and the CIG from either sides of the celiac trunk were secured en bloc. Both tissues were processed for paraffin embedding. 5 μm thick sections were stained with Hematoxyline and Eosin (H&E), cresyl CV and PSR. Only H&E and CV stained sections were used for measuring the neuronal diameter. Random photomicrographs were recorded under ×400 magnification of trinocular microscope (Olympus, BX40, Tokyo, Japan) by digital camera (Sony 18.2 MP, Tokyo, Japan) and measurements were made by using software Motic Images Plus version 2.0 (Motic, Kowloon, Hong Kong). Sufficient numbers of random images from both sides of PrtG and CIG were taken in order to get 1000 neurons having a clear nucleus with one or more nucleoli to be used for the histomorphometry. Based on their diameter, neurons were divided into small (<20 μm), medium (20–30 μm) and large sized (>30 μm). Proportions of different size of neurons in randomly selected 1000 neurons were calculated in each group. The neurons were also identified as dark or light on the basis of their morphology and staining characteristics of cell body and nucleus with visible nucleolus.

Blood glucose levels were measured from the lateral tail vein blood at biweekly intervals with a glucometer. 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 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).

The number of neurons, serum total protein and serum creatinine levels were statistically analyzed using one-way ANOVA followed by Tukey’s test. All numerical values were expressed as Mean±SD and p<005 was considered as statistically significant.

**Results**

Consistent with the known effects of induced hyperglycemia, the STZ-treated rats displayed classical clinical symptoms of diabetes such as polydipsia, polyuria and polyphagia. The mean body weights of all diabetic groups were reduced compared to the control group during experimental period as reported earlier.\textsuperscript{[14]} The changes observed between 2W diabetic and age-matched control group were statistically not significant (>0.05), but they were significantly (p<0.05) reduced at all stages of the induced diabetes compared to age-matched controls. Rise of blood sugar level was observed above 500 mg/dl after 48 hours of induction in diabetic groups which remained so throughout experimental period.
After 2W, a significant difference (p<0.01) was observed when comparison was made between 2W, 1M, 2M, 4M, and 6M diabetic rats to age-matched controls and corresponding diabetic group rats, respectively (Table 1).

### Microscopic observations

The PtG was located in the pterygopalatine fossa closely related with maxillary division of trigeminal nerve. Neurons in PtG were closely packed with homogenous distribution. Most neurons appeared round to oval in shape and almost all neurons were in the range of small to medium size with cell body diameter of 15 to 30 Âµm. Each neuron was surrounded by a thin capsule of small satellite cells with delicate connective tissue and unmyelinated nerve fibers between them. Fibrocytes and occasional intraganglionic blood capillaries were also noticed. Each ganglion had its own protective connective tissue capsule. Neuronal cell bodies were characterized by oval large euchromatic nuclei. In control, 2W and 1M diabetic groups, Nissl substance was coarser and sparse in the perikaryal area. However in 2M, 4M and 6M diabetic groups, most of the parasympathetic neurons had peripheral condensed rim of Nissl substance. Most of the literature suggest that nuclei of a sensory ganglion neurons are located centrally and those in the autonomic ganglia are located eccentrically. However, in the current study, we observed that the nucleus appeared to be located centrally in most of the PtG neurons. Similarly, the common textbook description is that the autonomic ganglion neurons are multipolar, but in the present study quite many of the neurons in both PtG and CIG had appearance very much similar to typical sensory ganglion neurons. Interestingly, all nuclei had a single and large densely stained eccentrically placed nucleolus. (Figures 1 and 2).

Collagen fibers were observed in the perineuronal capsule. The unmyelinated nerve fibers and connective tissue fibers were also seen between the neurons. The control and 2W diabetic groups were associated with fewer collagen fibers around the neurons and along the nerve fibers, but in other groups progressively thickened collagen fibers in the periganglionic capsule, perineuronal capsule and also along the nerve bundles were noticed. Perineuronal spaces were commonly noticed in 1M, 2M, 4M, and 6M diabetic groups (Figure 3).

In cresyl violet-stained sections of the celiac ganglion, both control and diabetic groups showed the same basic cellular architecture, consisting of a moderately dense arrangement of sympathetic neurons separated by bundles of unmyelinated nerve fibers and surrounded by well-

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>2W</th>
<th>1M</th>
<th>2M</th>
<th>4M</th>
<th>6M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum total protein (g/dl)</td>
<td>Control</td>
<td>5.97±0.04*</td>
<td>5.95±0.05*</td>
<td>5.99±0.03*</td>
<td>6.01±0.01*</td>
<td>5.98±0.05*</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>5.23±0.01</td>
<td>5.12±0.03</td>
<td>5.00±0.07</td>
<td>4.05±0.03</td>
<td>3.96±0.04</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>Control</td>
<td>0.43±0.02</td>
<td>0.42±0.06*</td>
<td>0.45±0.05*</td>
<td>0.44±0.07*</td>
<td>0.43±0.04*</td>
</tr>
<tr>
<td></td>
<td>Diabetic</td>
<td>0.45±0.07</td>
<td>0.63±0.08</td>
<td>0.78±0.03</td>
<td>0.93±0.09</td>
<td>1.06±0.05</td>
</tr>
</tbody>
</table>

*p<0.05

**Figure 1.** (a-d) Photomicrographs from rat autonomic ganglia showing neuron having peripheral ring (††††), eccentric nucleus (↑↑↑↑), eccentric nucleolus (»»), dark neuron (→→) and fibrocytes (**) (Cresyl violet stain, ×400). Inset showing moderate yellow fluorescence in perikarya occupied by fine Nissl granules (←←) and no fluorescence in the peripheral rim (AA) (PSR stain, ×1000). [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]
defined collagen fibers around the neurons and along the nerve fibers and ganglionic capsule. Most of the neuronal cell bodies inside the CIG had eccentrically placed, large, euchromatic nuclei having deeply stained nucleoli (Figures 1–3) which were also located eccentrically. Fine Nissl granules were distributed uniformly throughout the cytoplasm. Lipofuscin pigments were not obviously seen in both control and experimental groups. All ganglion neurons in the control group were covered by thin sheath of fewer sparsely located satellite glial cells. In prolonged diabetic groups, the satellite cells were less organized as compared to age-matched control groups (Figures 1 and 2). Though collagen fibers were present in variable amounts in the perineuronal capsules, nerve fascicles and periganglionic capsules. The overall amount of collagen and thickness of their fibers revealed direct correlation with the increasing duration of hyperglycemic state as compared to the age-matched controls. Perineuronal spaces were found in the 6M diabetic group. Intraganglionic blood capillaries were more often seen close to the neurons in diabetic groups (Figure 4). The 2W diabetic group revealed two medium sized neurons housed within the same perineuronal sheath as suggested by the absence of satellite cells and fibrous capsule between them (Figure 4).
Histomorphometry

In the PtG, the proportion of small sized neurons among 1000 neurons was significantly (p<0.05) decreased in 2M, 4M and 6M groups and the proportions of medium sized neurons were significantly (p<0.05) increased in 2M, 4M and 6M diabetic groups as compared to the age-matched control groups (Figure 5).

In 1000 neurons of the ClG of all diabetic groups, the proportion of small sized neurons were significantly (p<0.05) decreased. However, in the 2W diabetic group such decrement remained statistically non-significant as compared to the age-matched control group. In ClG of 1M, 2M, 4M and 6M diabetic groups, the proportion of medium and large sized neurons were significantly (p<0.05) increased as compared to the age-matched control group. In the 2W diabetic group, the proportions of the total number of medium and large sized neurons were less compared to the age-matched control group, but not at a statistically significant level (Figure 6).

Biochemical analysis

In all diabetic groups, the serum total protein levels were significantly (p<0.05) decreased compared to age-matched
control groups, while serum creatinine levels were significantly (p<0.05) increased in all diabetic groups except 2W, as compared to age-matched control groups. In 2W diabetic group, the serum creatinine levels were less compared to the age-matched control group, but not at significant level (p>0.05) (Table 1).

Figure 5. The proportion of small sized neurons was significantly (p<0.05) reduced in all diabetic groups, while that of medium sized neurons significantly (p<0.05) increased in all diabetic groups except 2W and 1M compared to age-matched control groups.

Figure 6. The proportion of small sized neurons in CIG was significantly (p<0.05) reduced in all diabetic groups except 2W group, while the total number of the medium and large sized neurons significantly (p<0.05) increased in all diabetic groups except 2W compared to age-matched control groups.
**Discussion**

Diabetes mellitus is a group of metabolic disorders of carbohydrate, fat and protein metabolism characterized by low grade inflammatory metabolic disorder\[16,17\] in which a person has high blood sugar, either characterized by insufficient amounts of insulin or because cells do not respond to the insulin that is produced, which leads to hyperglycemia.\[19\] Reduced anabolic insulin hormone in DM, promotes protein catabolism and releasing amino acids for gluconeogenesis\[19\] which leads to increased muscle wasting due to loss of tissue proteins and reduction of body weight.\[20\] In the current study, all diabetic groups maintained the hyperglycemic state throughout the experimental period and showed progressive reduction in body weight in the STZ-induced rats, reflecting an increase in protein catabolism and loss of tissue proteins. This result is in agreement with previous related studies.\[21–23\]

In this study, structure of both sympathetic and parasympathetic autonomic ganglia and orientation of nerve fibers in both control and experimental groups were, in general, found to be similar to those reported earlier.\[10,12–20\] Each neuron in CIG is anatomically and functionally independent. In the present study, a couple of neurons appeared to share a common sheath formed by satellite glial cells and the interneuronal sheath connective tissue was not visible. Significance of such intimate association between certain neurons remains unclear. On one hand, absence of the interneuronal sheath element makes its appearance very akin to a binucleate neuron; similar findings were observed in another study on the trigeminal ganglion.\[25\]

The present study demonstrates a concomitant increase in the thickness of collagen fibers located in the capsule of the ganglia, perineuronal capsule and interfascicular region of PtG and CIG. Previous studies showed progression of fibrosis in a diabetic heart by PKC-β, p38 mitogen activated protein kinase expression in redox reaction,\[29\] and AGE and RAGE interaction increased expression of TGF-β and contributed to the development of submesothelial fibrosis and neoangiogenesis.\[29\] In the current study, in PSR and CV stained sections, control and 2W diabetic groups of PtG, and control, 2W, and 1M diabetic groups of CIG revealed thin collagen fibers around the neurons and along the nerve bundles; however, in 1M and 2M diabetic groups of PtG and in the 2M diabetic group of CIG, the collagen fibers were of moderate thickness. However, in 4M and 6M diabetic groups, both PtG and CIG revealed a remarkable thickening of collagen in the capsule of the CIG ganglia, perineuronal capsule, interfascicular region, and endoneurium. Comparison of these with previous observations reported earlier\[14,16\] indicate that hyperglycemia seems to accelerate fibrosis in terms of the amount and thickness of collagen fibers. In earlier studies on sensory ganglia, the perineuronal spaces in some of the neurons were suggested to be due to either shrinkage or apoptosis of neurons with the progression of hyperglycemia.\[14,30\] In the present study, similar observations were found in 1M, 2M, 4M and 6M diabetic groups of PtG and 6M diabetic group of CIG.

The dark neurons are also considered as apoptotic type neurons or a type of cell degeneration with hyperelectronic density properties and hyperbasophilia.\[31–33\] Many researchers consider neuronal cell death mainly due to apoptotic changes.\[34\] In the present study, it was observed that, on the progression of the duration of hyperglycemic state in both PtG and CIG, the number of dark neurons increased in agreement with earlier studies showing that hyperglycemia and increased free radical generation in diabetes accelerated the formation of dark neurons.\[31\] The number of neurons with a distinct peripheral rim of Nissl substance also seems to increase with the duration of hyperglycemia which may be secondary to chromatolytic changes.\[35,36\]

It is generally accepted that the PtG contains both vasomotor and secretomotor neurons\[37\] and that they are independent of each other.\[38\] In the present study, were observed both small and medium sized neurons in control and experimental groups. In another study on the otic ganglion of the cat, small and large sized neurons were described as vasomotor and secretomotor, mainly with blood vessel vasomotor activity and glandular secretion of the salivary gland.\[39\] In our study, there is evidence for a minor change in neuronal proportion in the parasympathetic ganglia of diabetic rats. The proportion of small sized neurons in PtG significantly (p<0.05) decreased in all diabetic groups. Therefore, these findings indicate that in diabetes the isolated asymptomatic progressive involvement of lacrimal secretion in progression of hyperglycemia. The impaired tear secretion in prolonged hyperglycemic state which is commonly believed to be due to age-dependent decline in glandular function and excessive fibrosis\[40,41\] may also be partly attributed to the loss of small sized neurons of PtG observed in the present study.

CIG is the part of sympathetic ganglia located near the celiac trunk, a complex relay and integrative center for projections from the parasympathetic, sensory, spinal cord-derived motor, and retrograde intestinofugal sources.\[42\] Most neurons are noradrenergic and supply the stomach, spleen, pancreas, small intestine and mesen-
teric blood vessels. In the current study, we identified small, medium and large-sized neurons. Such neuronal population has earlier been functionally divided into pilomotor, vasomotor and secretomotor neurons. In our study, there is evidence for a minor change in neuronal proportion in the CIG. It was noticed that the proportion of small-sized neurons in CIG were significantly (p<0.05) decreased, however medium and large-sized neuron were significantly (p<0.05) increased in 1M, 2M, 4M and 6M diabetic groups. Therefore, these findings indicate that diabetes leads to progressive dysregulation of the sympathetic control of motility in the stomach and small intestine, and of fluid exchange in the small intestine with progression of hyperglycemia probably due to altered function and or loss different neurons along with age-dependent decline in glandular function and excessive fibrosis.

In the present study, dead neurons were easily differentiated due to their dark staining characteristics as compared to normal neurons in the control group. In 4M and 6M diabetic groups, the neurons with dark nuclei appeared shrunken, nuclear membranes became less distinct and nucleoplasm no more remained open-faced as similar to described earlier. However, with the progression of hyperglycemic state in diabetic groups, the number of dead neurons increased in agreement with an earlier study, suggesting diabetes enhances neuronal death.

Lipofuscin pigment (senile pigment) is described to be one of the typical features of the sympathetic ganglia and can be shown described by H&E stain. In our present study, lipofuscin pigment could neither be located in the control nor in any experimental groups after using with different stains. Certain literature suggest that lipofuscin pigment shows auto-fluorescent property with blue light. Diabetes is said to accelerate accumulation of fluorescent granules in sensory and sympathetic neurons which could not be confirmed in the present study. However, the perikarya of CIG neurons showed a low-grade yellow fluorescence in the region occupied by fine Nissl substance (Figure 1).

In this study, it has been shown that, as shown earlier, abnormally high levels of serum creatinine are consistent with impaired kidney function and the serum creatinine level increased in all diabetic groups parallel to the severity of hyperglycemia, but the serum total protein levels were reduced and altered in serum total protein relating the hyperglycemia to a low grade inflammatory process. Similar findings have been shown in the other related studies.

Conclusion

Based on histopathological, histomorphological and biochemical findings, it is concluded that prolonged hyperglycemic state leads to increased serum creatinine level, reduced serum total protein and proportion of small sized neurons, increase in dark and dead neurons and thickening of collagen fibers in autonomic ganglia. Therefore, it appears that one of the important contributory factors in development of autonomic neuropathy in chronic diabetes might be the hyperglycemia-induced neuronal cytotoxicity and altered perineuronal microenvironment in terms of increased fibrosis within the autonomic ganglia.

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References

Effect of streptozotocin-induced diabetes on the autonomic ganglia of albino rats


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