Impact of Experimental Hyperglycemia on the Lumbosacral Dorsal Root Ganglia of Albino Rats

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ABSTRACT

Background: One of the typical manifestations of prolonged hyperglycemia is peripheral neuropathy perhaps due to destructive effects on the primary sensory neurons in the dorsal root ganglia (DrG). Therefore, the current study was designed to analyze the impact of experimental hyperglycemia on DrG of laboratory animals. Material and Methods: 30 adult albino rats were divided into five groups, having six rats per group: control, two week, two month, four month and six month. Diabetes was induced by a single dose of streptozotocin administered through intraperitoneal route (60 mg/kg). Animal body weight and blood sugar were measured at biweekly interval. At the end of the experimental duration, animals were euthanized by deep ether anesthesia and blood samples were collected by direct puncture of heart into sterilized plastic vials for biochemical analyses. Animals were perfused with Karnovsky fixative. After 48 hours tissue samples were collected and processed for light microscopical studies. Results: Biochemical analyses and histopathological features revealed that progressively increasing duration of hyperglycemia was associated with reduced serum total protein and increased serum creatinine; decrease proportion of small and medium-sized neurons, increasing frequency of dark and dead neurons. It was also associated with increase in the amount and thickness of collagen in the capsule of ganglia, perineuronal capsule and endoneurium. Conclusion: Long-standing hyperglycemia with increased neuronal death and deposition of collagen fibers in sensory ganglia may be among the important contributing factors in the development of diabetic peripheral neuropathy.

KEYWORDS: Collagen, Diabetes, Dorsal root ganglia, Neuropathy.

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 of various organs, especially the eyes, kidneys, nerves, heart and blood vessels [2,3,4]. Prolonged hyperglycemia leads to glucose neurotoxicity [5] and this is likely to cause a variety of functional and structural disorders in both central and peripheral nervous systems [6].

Even though the sensory neurons are highly sensitive to oxidative stress in diabetes, the DrG lacks blood–brain and blood-nerve barriers [7,8,9]. Oxidative stress and mitochondrial dysfunction have been implicated in the neuronal cell death and neuronal apoptosis [10,11]. The DrG is a highly vulnerable site in diabetic neuropathy [12], which results in the progression of micro-environmental changes in the ganglia which may even transform neuron phenotype, ion channel alterations, mitochondrial dysfunctions and abnormal growth factor signaling [13,14]. Various researchers like Lennertz et al.[15], Kennedy et al.[16] have shown that the long-term hyperglycemia leads to the abnormalities in the micro-architecture of sensory ganglion cell and nerve fibers. Peripheral neuropathy mainly affects lower limb due to the involvement of longest nerve fibers [17].

There are some related studies available on the morphological and histopathological changes in the trigeminal ganglion of adult rat [18] and lumbosacral DrG of neonatal albino rat [19]. Therefore, the present study aims at the assessment of alteration in the morphometric and microscopic parameters of sensory neurons, glial cells, nerve fibres and supporting tissue with respect to the progressive duration of hyperglycemic state.
MATERIALS AND METHODS

**Animal Preparation:** After approval from Institutional Animal Ethics Committee (No. 9025/2014) the albino rats of either sex weighing around 250g each 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:** A total number of 30 animals were divided into following five groups (one healthy control and four experimental groups of: two week, two month, four month and six month) having six rats in each group:

**Induction of Diabetes:** After 12 hours fasting, diabetes was induced by single dose of Streptozotocin (60 mg/kg, IP). Blood sugar level was monitored with Glucometer before beginning of the experiment and after 48h of streptozotocin injection. Animals with blood sugar level at 250 mg/dl and above were considered as diabetic. Weight and blood glucose levels of all animals in each group were monitored biweekly [20].

**Tissue preparation:** At end of the each experimental period animals were euthanized with over dose of ether general anesthesia and tissues were fixed in Karnovsky fixative by perfusion method.

**Histopathology and Histomorphometry**

Fixed lumbosacral dorsal root ganglion samples were used for light microscopic studies. Five µm thick paraffin sections were stained with Cresyle Violet (CV) and PicroSirius with Luxol Fast Blue (PSR with LFB). In histomorphometry, CV-stained sections were used for measuring the neuronal diameter. Sections were visualized under x400 trinocular microscope (Olympus, BX40, Japan) and representative photomicrographs from different samples were recorded with digital camera (Sony 18.2 MP, Japan) in order to achieve images of over 1000 neurons for each group. Measurements were made by using software Motic image version 2.0 [20]. Only those neurons having a clear nucleus with nucleolus were used for the histomorphometry. Based on diameter, neurons were divided into small (< 20 µm), medium (20-30 µm) and large-sized (>30 µm) and data achieved from this was used to calculate the proportion of different size of neuron in different groups.

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

**Biochemical Estimation and Analysis:** Blood sugar was estimated biweekly with Glucometer. Blood for this purpose was obtained from lateral tail vein. At the end of each study period, blood samples were obtained through direct puncture of heart and collected into sterilized plastic vials. Blood samples were allowed to clot, centrifuged at 2500 rpm for 30 minutes and the serum was separated and stored in separate sterilized plastic vials then assayed for serum total protein content and serum creatinine level.

**Statistical Analysis**

The differences in the proportion of neurons, serum creatinine, and serum total proteins 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<0.05 was considered as statistically significant.

**RESULTS**

**General observation:** During the experimental period after induction of diabetes entire diabetic groups showed the classical clinical symptoms of diabetes such as polydipsia, polyphagia, and polyuria.

**Body weight:** The mean body weights of all diabetic groups were reduced as compared to control group during experimental period as reported earlier [20]. The changes observed between age-matched control and 2W diabetic group was statistically not significant but they were significant (P<0.05) among other groups (Table 1).

**Blood glucose level:** After 48h of induction of diabetes rise of blood sugar level was observed above 500 mg/dl and the level was maintained throughout experimental period. After two weeks a significant difference (P<0.05) among all experimental groups was noticed with respect to their age-matched controls. (Table: 1).

**Microscopic observations**

**Histopathology**

In the current study in age-matched control and 2W experimental group the DrG revealed typical features of sensory ganglion containing groups of neuronal somata interspersed with nerve fascicles. Neurons were oval, or round with centrally placed large euchromatic nucleus and prominent nucleolus. Each neuron was surrounded with 4-6 satellite glial cells. The special stain for collagen revealed only minimal amount of collagen in periganglionic connective tissue, perineuronal capsule and nerve fascicles. But in 2M, 4M and 6M diabetic groups, instead of normal micro arrangement; visible changes in myelination were observed. In addition to this feature, these groups also showed plentiful, thickened collagen fibers in the periganglion capsule, perineuronal capsule and along the nerve bundles. Intra ganglionic blood capillaries were quite commonly seen close to the neuronal cell body in all groups. More perineuronal spaces were observed only in 4M and 6M diabetic group as compared with other groups (Figure: 1).

Light and dark stained neurons and neurons with eccentric nuclei were either associated with or without chromatolysis and were noticed in all groups in varying numbers. In diabetic groups, the occurrence of such neurons were more and clearly observed with increasing duration of hyperglycemic state. Fewer neurons were seen to have peripheral rim of chromatin granules. Disorganized arrangements of satellite cells were clearly observed in 6M diabetic group as compared to control group. In all groups Schwann cells and fibrocytes were seen parallel to the nerve fibers (Figure 2).
Figure 1: Photomicrograph from rat lumbosacral DrG showing myelinated nerve fibers (↑↑), blood capillaries (↑), and perineuronal space (*).

Note the prominence of collagen fibers (Red colour) in 4M and 6M diabetic group. PSR with LFB stained sections. Initial magnification x400

Figure 2: Photomicrograph from rat lumbosacral DrG showing eccentric nuclei (→), dark neuron (*), fibrocytes (>), peripheral rim of Nissl substance (»). The inset shows neuron with eccentric nucleus and peripheral rim of Nissl substance. Cresyl violet stain, initial magnification x 400.

Histomorphometry

In diabetic groups the proportion of small and medium-sized neurons among 1000 neurons was significantly (P<0.05) decreased and the proportions of large sized neurons were significantly (P<0.05) increased as compared to age-matched control groups (Table: 2).

Biochemical analysis

The total serum protein was significantly (P<0.05) decreased in all diabetic groups as compared to control group. Serum creatinine levels were significantly (P<0.05) increased in all diabetic groups as compared to age-matched control groups. (Table: 3).
Table 1: Initial and final body weight (gm) and blood glucose levels (mg/dl) during the period of study (Mean ± SD) of diabetic and control groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Control</th>
<th>Diabetic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>2-Week</td>
<td>251.83 ± 05.71</td>
<td>240.17 ± 05.60</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>2-Month</td>
<td>271.33 ± 03.72</td>
<td>201.83 ± 06.59</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>4-Month</td>
<td>339.17 ± 17.74</td>
<td>169.05 ± 09.65</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>6-Month</td>
<td>348.35 ± 18.70</td>
<td>162.12 ± 08.55</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Blood Sugar (mg/dl)</td>
<td>2-Week</td>
<td>116.83 ± 08.23</td>
<td>542.66 ± 67.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>2-Month</td>
<td>124.12 ± 06.57</td>
<td>530.17 ± 63.51</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>4-Month</td>
<td>128.67 ± 05.85</td>
<td>527.83 ± 62.64</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>6-Month</td>
<td>125.12 ± 06.45</td>
<td>510.32 ± 46.85</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Note: In diabetic groups as compared to the control the body weight significantly (P<0.05) decrease and blood sugar level remained significantly increased throughout the experimental period.

Table 2: Showing the proportion of neurons (in 1000 neurons)

<table>
<thead>
<tr>
<th>Proportion of neurons (in 1000 neurons)</th>
<th>Group</th>
<th>Control</th>
<th>Diabetic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small and medium sized</td>
<td>2-Week</td>
<td>462.35 ± 32.23</td>
<td>377.69 ± 18.45</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>2-Month</td>
<td>469.26 ± 18.43</td>
<td>302.04 ± 21.15</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>4-Month</td>
<td>455.19 ± 27.74</td>
<td>250.28 ± 26.35</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>6-Month</td>
<td>465.22 ± 29.18</td>
<td>213.74 ± 21.50</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Large sized neurons</td>
<td>2-Week</td>
<td>537.65 ± 25.43</td>
<td>622.31 ± 16.18</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>2-Month</td>
<td>548.26 ± 32.43</td>
<td>697.96 ± 20.31</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>4-Month</td>
<td>539.19 ± 20.55</td>
<td>749.72 ± 22.51</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>6-Month</td>
<td>521.75 ± 25.42</td>
<td>786.26 ± 20.18</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Note: The proportion of small and medium-sized neurons significantly (P<0.05) reduced while that of large sized neurons significantly (P<0.05) increases in all diabetic group compared to control group.

Table 3: Biochemical Parameters during the period of study (Mean ± SD) of diabetic and control groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Control</th>
<th>Diabetic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum total protein (g/dl)</td>
<td>2-Week</td>
<td>5.97 ± 0.04</td>
<td>5.23 ± 0.01</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>2-Month</td>
<td>5.99 ± 0.03</td>
<td>5.00 ± 0.07</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>4-Month</td>
<td>6.01 ± 0.01</td>
<td>4.05 ± 0.03</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>6-Month</td>
<td>5.98 ± 0.05</td>
<td>3.95 ± 0.04</td>
<td>&lt;0.05</td>
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</table>
DISCUSSION

Diabetes mellitus is considered to be a complex low-grade inflammatory metabolic disorder [21] either characterized by insufficient amounts of insulin, or in which tissues fail to respond appropriately to insulin, which leads to hyperglycemia [22]. In diabetics, the reduction of body weight is primarily due to lack or reduced anabolic insulin hormone [23] which leads to increased muscle wasting as a result of loss of tissue proteins [24]. In this study, all diabetic groups maintained the hyperglycemic state throughout experimental period and showed the marked reduction of body weight. This result is in agreement with previous related studies [25,26,27].

In the present study, structure and orientation of nerve fibers in both control and experimental groups were found to be similar to those reported earlier [28]. However, the myelin sheaths were not obviously seen around the nerve fibers in 4M and 6M diabetic groups which may be due to subtle defect in the myelin of nerve fibers.

Some investigators have shown progression of fibrosis in a diabetic heart by PKC-β and p38 mitogen activated protein kinase expression in redox reaction [29] and also due to AGE and RAGE interaction and increased expression of TGF-β which contributes to the development of submesothelial fibrosis and Neangiogenesis [30]. In the current observation, the control, 2W and 2M diabetic groups special stain revealed thin collagen fibers around the neurons and along the nerve bundles. In 4M and 6M diabetic groups, there was a remarkable thickening of collagen in the capsule of the ganglia, perineuronal capsule, interfascicular area, and also in the endoneurium. These results indicate that the hyperglycemia seems to promote fibrosis in terms of amount of collagen as well as the thickness of collagen fibres which is in agreement with previous observations [19,20].

In DrG, the perineuronal spaces in some of the neurons have been suggested to be due to either shrinkage or apoptosis of neurons with the progression of hyperglycemia [19]. Somewhat similar observations have been made in our previous study Faizal et al.[20] as well as in the current study.

Many researchers consider dark neurons as apoptotic type of neuron [31] or a type of cell degeneration with hyper electron density properties and hyper basophilia [32,33]. In the current study it was noticed that on the progression of hyperglycemic state in diabetic groups the number of dark neurons increases and this is in agreement with other studies suggesting that in diabetes the hyperglycemia and increased free radical generation accelerates the dark neuron formation [31].

Sensory neurons commonly display centrally placed nucleus and the eccentricity of nucleus is of rare occurrence [34,35]. In the current study also the neurons having eccentrically placed nucleus is only seen occasionally while their frequency seems to increase with the progressive duration of hyperglycemic state. Such neurons do not always show typical chromatolytic changes. Similar observations made earlier on the occurrence of neurons having eccentric nucleus suggest that they may be partly due to aging process [34,35,36]. Occurrence of neurons having a distinct peripheral rim of Nissl substance also seems to increase with duration of hyperglycemia which may either suggest a specific subset of neuron or it may be secondary to mild grade of chromatolytic changes [37,38].

Small and medium-sized neurons are considered to be nociceptors which are mainly concerned with pain and temperature [39] and in trigeminal ganglion the small-sized neurons modulate pain sensation in migraine [40]. In our study it was noticed that the proportion of small and medium-sized neurons in lumbarosacral dorsal root ganglia were significantly (P< 0.05) decreased in all diabetic groups. Therefore, these findings possibly indicate that in diabetes the gradual loss of neuropathic pain sensation is probably due to altered function and or loss of small and medium-sized neurons.

Large sized neurons are concerned with mechanoreception and have the myelinated nerve fibres [18]. Some study suggest that size of spinal ganglion neurons increase with ages and thus many immature small and medium sized neuron transform to large size neuron and thus increase in the proportion/number of large size neuron is an expression of function of age [41]. Since diabetes is also known to enhance the ageing process, it is quite likely that both diabetes and ageing may influence the proportion of large size neuron in a synergistic manner as observed in the present study.

Our results also showed that the serum creatinine level increased and the serum total protein levels were reduced in all diabetic groups compared with control group depending on the duration of hyperglycemia suggestive of diabetic nephropathy a finding very similar to the other related studies [42,43].

CONCLUSION

Based on biochemical, histomorphological and histological findings, it is concluded that prolonged hyperglycemic state leads to alteration of biochemical changes suggestive of diabetic nephropathy; alteration in the proportion of different sized neurons possibly due to oxidative stress and

<table>
<thead>
<tr>
<th>Serum creatinine levels (mg/dl)</th>
<th>2-Week</th>
<th>2-Month</th>
<th>4-Month</th>
<th>6-Month</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.43 ± 0.02</td>
<td>0.45 ± 0.07</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.45 ± 0.05</td>
<td>0.78 ± 0.03</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.44 ± 0.07</td>
<td>0.93 ± 0.09</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.43 ± 0.04</td>
<td>1.06 ± 0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

Note: In all diabetic groups the serum total protein levels significantly (P<0.05) decreased compared to age matched control group. Serum creatinine levels were significantly (P<0.05) increased in 2M, 4M and 6M diabetic groups.
ageing process and progressively increasing amount of collagen and thicker collagen fibers in and around the sensory ganglia appear to be an important co-factor in the development of peripheral neuropathy in chronic diabetes.

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Conflict of Interest: None

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