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Original Article

Stereological Study on the Neurons of Superior Cervical Sympathetic Ganglion in Diabetic Rats

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Abstract

Background: Most research on autonomic dysfunction of diabetes mellitus is conducted on ganglions innervating gastrointestinal (GI) tract and there are limited works focusing on cervical sympathetic ganglia. The effects of diabetes mellitus (DM) on the neurons of superior cervical sympathetic ganglion (SCSG) are investigated by stereological methods.

Material and Methods: Female rats (n=72) randomly divided into DM (blood glucose =400-600 mg/dl) and control (n=36) groups. Rats were sacrificed at 4-, 8- and 12-weeks of induction of DM (65 mg/kg streptozotocin, ip). The same procedure followed chronologically in control group. SCSG of both groups were removed, fixed, and embedded in cylindrical blocks. Isotropic uniform random sections obtained and stained. The mean particle volume (according to the method of volume-weighted mean particle estimation) of the perikarya and nuclei of ganglion cells (neurons) were estimated using the point-sample intercepts method.

Results: There was no significant difference between the mean perikaryal and nuclear volumes of DM and control rats after 4-, 8- and 12-weeks. There was, however, a significant increase in the mean volume of perikarya and nuclei of the neuronal cells of DM rats at 8- and 12-weeks diabetes as compared with those of 4-weeks.

Conclusion: The mean volume of SCSG and their nuclei were not significantly reduced after 4-, 8-and 12-weeks in DM rats and these cells continued their normal growth.
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Keywords ● Diabetes mellitus ● Stereology ● Autonomic ganglia

Introduction

Impaired function of the autonomic nervous system is a serious and common complication of diabetes mellitus.[1,2] The sympathetic and parasympathetic nervous systems are involved with manifestations of cardiovascular, respiratory, gastrointestinal, skeletal, eye abnormality, psudomotor, and vasomotor symptoms.[1] It has been reported that sympathetic nerve fibers regulate the thickening and the number of pericyte basement membrane in the rat retina, and sympathetic denervation may play a role in diabetes retinopathy.[3] Because the prime target of autonomic neuropathy is GI tract,[1,2] most researches on diabetes mellitus (DM) have focused on changes

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of sympathetic ganglia that innervate the gasterointestinal (GI) tract whereas, cervical sympathetic ganglion received less attention.[1] Cervical sympathetic ganglia innervate various sites, such as cerebral vessels, thyroid glands, retina, pupil, etc.[1,3] Furthermore, some studies have revealed that cerebrovascular and cardiovascular strokes are the most frequent cause of mortality in diabetic patients.[2] The probable involvement of superior cervical sympathetic ganglion (SCSG) in DM might explain the increasing disturbances observed in cardiovascular, cereberovascular, and retinopathy in DM. Therefore the goal of this research was study of quantitative parameters of SCSG in DM.

Our previous study showed that DM decreases the volume of the somatic sensory of dorsal root ganglia.[4] Herein, the effects of DM on cervical sympathetic ganglion are examined by studying the structural changes that may occur in SCSG neurons (e.g. mean volume of perikarya and nuclei) using a stereological method at different periods of DM in the rat.

Materials and methods

Animals were treated in accordance with the protocols established in the NIH/NRC Guide for Care and Use of Laboratory Animals, reviewed and authorized by the Ethical Committee of the University. 72 female Sprague-Dawley rats weighing 220-280g (University animal house, Shiraz, Iran) were housed in standard cages in a temperature (22-24 ºC), humidity (40-60%), and light/ dark period of 07.00-19.00 controlled environment. Rats had free access to water and food pellets (Pars Dam, Iran). They were randomly divided into two normal (control; n=36) and DM (n=36) groups. Each group was further subdivided randomly into three equal subgroups (n=12). The first, second and third subgroups of control and DM rats sacrificed at 4, 8 and 12 weeks respectively.

Hyperglycemia was induced by a single ip injection of streptozotocin (65 mg/kg; Zanosar, Upjohn, USA) as stated elsewere.[5,6] Blood samples were taken from the tail vein. To do this the animal's tail placed in warm water for a few minutes and cleaned with alcohol solution and then the end piece of the tail was cut and blood was collected using a capillary tube. Random blood glucose (RBG) evaluated just before DM induction and also 2 days and 2 weeks after induction of diabetes. Two weeks after induction of diabetes rats with RBG between 400-600 mg/dl were selected and considered for the study.[6,7]

Preparation of the tissue

Diabetic and control rats were sacrificed 4, 8 and 12-weeks after induction. They were anesthetized with ether and perfused transcardially with approximately 500 ml of a solution of neutral buffered formaldehyde delivered in 10 min time at a pressure of 120 mmHg, using a bottle equipped with a pomp and barometer. The left SCSG were dissected under a stereomicroscope and were immersed, for one week, in the same fixative solution.[1]

Isotropic uniform random (IUR) sections are necessary for the evaluation of volume-weighted mean volume of principal ganglion cells of SCSG (i.e. selecting cells according to their volumes and estimation of their mean volume). Orientator method was used, as described by Gundersen and Mattfeldt and their colleagues,[8,9] to generate IUR sections. Briefly, each ganglion was embedded into a plastic syringe-its tip was cut off-filled with warm paraffin. After cooling, the paraffin cylinder was pulled out from the syringe and then in the center of a circle with 36 equidistant divisions along the perimeter. A random number between 0-36 was selected using a random table and the cylinder was cut in the selected direction. The first cut was performed in tissue free space of the cylinder. Then the cut edge of the cylinder was placed parallel to 0-0 direction of a second circle with the sine-weighted non-equidistant 97 divisions along its perimeter. A new random number between 0-97 was chosen and the specimen was cut in the new direction. The second cut was also performed in tissue free space of the cylinder. This new cut surface was the isotropic face of the cylinder. Complete serial sections (5 µm thickness) were then cut from the isotropic face of the embedded tissue (SCSG).

Stereological study

After staining with Heidenhain's azan, ten sections were selected in a systematic random manner and cell volume was measured using a projection microscope (Reichert, Austria). The fields of vision were projected at magnification of x1200. On the average five systematic sampling fields were selected by movement of the microscopic stage in X- and Y-directions in equal intervals. Using point-sampled intercept method, as stated by Gundersen et al,[8] a grid system with lines and associated points, was superimposed on the projected images to estimate volume-weighted mean volume of the cells and their nuclei. Volume-weighted mean volume is a method of volume estimating that the particles are selected according to their volume. When a random point on a random section hit a cell or its

nucleus, the intercept (l_0) through the point was measured and the mean volume (Vv) estimated using following formula:

$$Vv = \frac{\pi}{3} \cdot \overline{l_0^3}.$$

Statistical analysis

Data are presented as Mean±SD. Two comparisons were made; first between DM and control groups and second comparison of three subgroups of DM and control groups with each other to disclose changes that were related to the duration of DM. Statistical comparison of data of control and of DM rats was performed using Mann-Whitney U-test, one-way ANOVA and Duncan multiple range test and P<0.05 was considered as significantly different.

Results

The mean volume of the perikarya and nuclei of the ganglionic cells revealed no significant difference between diabetic and control groups during 4, 8 and 12-weeks period of the study (Table 1). The comparison of three subgroups of DM and control groups with each other to disclose changes that were related to the duration of DM indicated that the mean perikaryal volume of ganglion cells of DM animals showed a ~43% and ~32% increase at 8 and 12-weeks of DM as compared to those of 4-weeks (p<0.003). The corresponding increases in the control groups were ~44% and ~23%, at 8 and 12-weeks, respectively as compared with those of 4-weeks (Table 1; p<0.03). The mean nuclear volume in diabetic rats increased by ~39% and ~30%, at 8 and 12-weeks as compared with those of 4-weeks respectively, whereas, in control group, these increases were ~51% and ~39%, at 8 and 12-weeks with respect to that of 4-weeks (Table 1, p<0.007).

Table1: Volume-weighted perikaryal (Vp) and nuclear volume (Vn) of the neurons of SCSG in control and diabetic (DM) groups (n=12) at 4-, 8- and 12-weeks after induction of diabetes. Data are presented as mean±SD

Time	Group	Vp (μm³)	Vn (μm³)
4 wks	Control	2296±631	251±81
	DM	2084±535	242±29
8 wks	Control	3317±1159♦	379±135*
	DM	2981±430♦♦	337±70**
12 wks	Control	2827±737♦	348±53*
	DM	2765±791♦♦	316±108**

♦ P<0.03, control group (8 or 12 wks after induction) *vs.* control group (4 wks after induction)
♦♦ p<0.003, DM group (8 or 12wks after induction) *vs.* DM group (4 wks after induction)
* P<0.01 control group (8 or 12 wks after induction) *vs.* control group (4 wk after induction)
** P<0.007 DM group (8 or 12 wks after induction) *vs.* DM group (4 wks after induction)

Discussion

The unbiased stereological methods used in the present study demonstrated that the volume of perikarya and nuclei of SCSG cells in diabetic rats were not significantly different from those of control group. Our previous experiment showed a reduction in the pericaryal nuclear volume of dorsal root ganglion cells 6 weeks after initiation of diabetes in the rat.[4] These findings show that SCSG cells have individual properties that may support them against diabetes.

One of the most important factors in maintenance of cell body size is neural growth factor. This factor controls neurofilament gene expression, which is believed to be important in maintaining cell body size and axon diameter.[7,10] It has been revealed that nerve growth factor can promote the survival, maintenance and regeneration of neurons subject to the noxious effects of diabetes.[7,11] Additionally, it has been demonstrated that transport of neural growth factor in axons of the SCSG of diabetic rats is normal.[7] The relative success of diabetic patients in maintaining normal morphology and function of their nerves ultimately may depend on normal expression of this factor.[11] Thus, it may be one cause for normal remaining of the SCSG cells in diabetes mellitus.

It has been revealed that some biochemical substances are related to diabetic neuropathy. Vasoactive intestinal polypeptide, substance P and gastrin releasing peptide/bombesine are some example.[6] But the SCSG contains less vasoactive intestinal polypeptide and also axons containing substance P and gastrin releasing peptide/bombesine than prevertebral autonomic ganglions.[6]

There are few dystrophic axon terminals in the SCSG of DM animals in comparison with celiac and superior mesenteric ganglia.[12] There are many studies that show the characteristic of SCSG cells in vivo or in vitro are different from the other autonomic ganglia.[12,13] One of the important characteristic of SCSG cells is their histological structure that may protect them from the distracting influences of DM. Studies of SCSG have revealed that neuronal and satellite cells were closely apposed, leaving a small extracellular space as compared to the sensory ganglia and satellite cells in SCSG comprise an effective barrier in general.[14,15]

In addition to histological characters some other factors also support this ganglion. It has been revealed that all fibroblast growth factor-2 isoforms are found in the nuclei of satellite cells surrounding postganglionic perikarya of the SCSG.[16] This factor participates in neuron-

glial interactions of sympathetic ganglia, and may involved in sympathetic nervous survival or nerve regeneration after nerve lesion.[15]

One of the other factors that should be considered in the effects of DM on ganlionic cells is the time of initiation of diabetes. It has been demonstrated that, a few dystrophic axons were found in close proximity to the neurons of SCSG, 8 months after diabetes, but ganglion cells appeared normal.[1] In addition, it has been shown that the absolute frequency of axonopathy increased in diabetic animals with longer time of diseases compared with short duration.[5] Thus SCSG may also express morphometric changes in more chronic duration of diabetes.

The strain of animal under study may change the results of DM. This can be concluded from the study that has been showed Sprague-Dawley animals demonstrated considerably greater severity of axonopathy as well as earlier development of axonal changes than that found in Wistar-Futh-Lewis or Wistar-Lewis animals. Thus, it is possible that the choice of strain may underlie some of apparent controversies in this area of research.[5]

Our study, therefore, demonstrated that superior cervical sympathetic ganglion cells seem to have a normal growth in diabetes mellitus rats the same as of normal animals.

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