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The Effects of Streptozotocin On Spinal Cord Motor Neurons Count in Rat: A Stereological Study

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ABSTRACT

In this study we investigated effects of Streptozotocin (STZ) administered at different doses on the total number of neurons in segment Thoracic 11 (T11) of the spinal cord of adult rats by using stereological methods. Twenty-eight adult male Wistar albino rats were used. The rats were divided into four groups; control group, saline group (0.5 ml of 0.9% NaCl i.p.), 45mg/kg of STZ (i.p.) group and 65 mg/kg of STZ (i.p) exposed group. At the seventh day, the rats were perfused with 10% neutral buffered formaldehyde. T11, which is one of the transverse spinal cord segments, was isolated and processed for routine histologic examination. Semithin sections (0.5µm thick) were obtained using a ultramicrotome according to systematic random sampling strategies. The sections were stained with Toluidine Blue with Borax. The motor neurons were counted unbiased stereological disector/Cavalieri combination method. The groups were compared statistically. In terms of motor neuron number, no significant difference among the groups was found ($p>0.05$). Our results indicated that exposure to different doses of Streptozotocin has no effect on the T11 segment of the spinal cord total motor neuron number of the adult rats.

Key Words: Rat, spinal cord, stereology, streptozotocin

Introduction

Diabetes mellitus (DM) is a collection and series of metabolic illnesses in which there are high glucose levels present in the blood for long period (1). Uncontrolled state of hyperglycemia due to defects in insulin secretion and action leads a variety of complications including peripheral vascular diseases, nephropathy, neuropathy, retinopathy, morbidity, and/or mortality (2). The prevalence of diabetes Mellitus is rising all over the world. Given that diabetes affects nearly 246 million human in the world, it is estimated that 20–30 million human in the world are affected by symptomatic diabetic neuropathy. Diabetes mellitus is the most well-known reason for neuropathy worldwide (3). The mechanisms that underlie the development and maintenance of diabetic neuropathy are not well understood. The T11 spinal segment control the muscles, glands in the urinary system and uterus and some part of large and small intestinal and sense of this organs, with other segments by producing complex nerve roots that are coming and going from the spinal cord segment (4). The purpose of this study is to investigate the effects of STZ at different doses on the motor neuron number in the T11 segment of the spinal cord using stereological methods. The 45mg/kg is the lower dose, and 65mg /kg is the higher dose for the human body (5,6).

Material and Methods

Application of Experiment : The experiments were performed according to protocols approved by our university Animal Studies Local Ethics Committee (2015, No: 12). Twenty-eight adult male Wistar albino rats (152-256 g.) were used. Animals and kept in 12 hour dark - light period in a constant temperature (21 °C) room. They were fed with rat standard uniform pellet and tap water ad libitum. Diabetes mellitus was induced by intraperitoneal injection of Streptozotocin (Streptozotocin U-9889, Santa Cruz Biotechnology Inc, Dallas, TX, USA). STZ powder was transferred into microfuge tubes and maintained at -20°C. The streptozotocin was kept in unbroken cold chain and freshly dissolved in citrate buffer (0.1 M; pH 4.5) before administration. Rats were randomly divided into 4 groups (n=7): control (I), the saline group (II), with 0.5 ml of 0.9% NaCl, the 45 group (III), was administered with 45mg/kg of STZ and 65 group (IV), was administered 65mg/kg of STZ. All these three groups were injected intraperitoneally while control group was not injected. Weight of all experimental groups were recorded prior to and after the experiment (Table 1). After stopping feeding for

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Table 1. Body weight changes of animals during experiment

Groups (n:7)	Prior to the experiment (g)	Before Sacrification (g)	Difference (g)	Changes in body weight (%)
Control	164.3	172.3	+8.3	4.8%
Saline	208	211	+3	1.4%
G45	229.7	203.6	-26.1	-11.3%
G65	222	181.4	-40.1	-18%

Table 2. Blood glucose levels changes in groups

Group	Prior to the experiment	Before Sacrification
Control 1	83 mg/dI	99 mg/dI
Control 2	86 mg/dI	70 mg/dI
Control 3	89 mg/dI	71 mg/dI
Control 4	75 mg/dI	80 mg/dI
Control 5	101 mg/dI	83 mg/dI
Control 6	82 mg/dI	77 mg/dI
Control 7	90 mg/dI	82 mg/dI
	Fasting blood glucose level before injection of Saline	Fasting blood glucose level after injection of Saline
Saline 1	79 mg/dI	84 mg/dI
Saline 2	83 mg/dI	77 mg/dI
Saline 3	91 mg/dI	81 mg/dI
Saline 4	87 mg/dI	93 mg/dI
Saline 5	82 mg/dI	90 mg/dI
Saline 6	84 mg/dI	79 mg/dI
Saline 7	76 mg/dI	80 mg/dI
	Fasting blood glucose level before injection of STZ	Fasting blood glucose level after injection of STZ
G45 1	87 mg/dI	315 mg/dI
G45 2	79 mg/dI	366 mg/dI
G45 3	94 mg/dI	379 mg/dI
G45 4	78 mg/dI	301 mg/dI
G45 5	96 mg/dI	405 mg/dI
G45 6	80 mg/dI	600 mg/dI
G45 7	87 mg/dI	474 mg/dI
G65 1	88 mg/dI	386 mg/dI
G65 2	76 mg/dI	410 mg/dI
G65 3	82 mg/dI	365 mg/dI
G65 4	83 mg/dI	454 mg/dI
G65 5	90 mg/dI	402 mg/dI
G65 6	89 mg/dI	382 mg/dI
G65 7	97 mg/dI	450 mg/dI

12 hour, blood was obtained from tail of all rats by pinprick for examination of the level of glucose in blood by using blood glucometer an injection of STZ after day. In the sixth day of work after 12 hours of cessation of feding, blood was obtained again from the tail of all rats. In the seventh day, all animals were fixed with 10 % neutral buffered formalin under ketamine (50mg/kg) anesthesia. The T11 spinal cord

Table 3. Descriptive statistics and comparison results of the groups

Groups (n:7)	CE	CV	Mean	Min.	Max.	P
Control	0.04	0.1	3489.4	2145.0	4212.0	0.2
Saline	0.04	0.1	3183.4	2174.4	3985.8	
G45	0.05	0.1	2882.3	2340.0	3940.2	
G65	0.05	0.2	2682.9	2095.2	4546.8	

($p<0.05$)

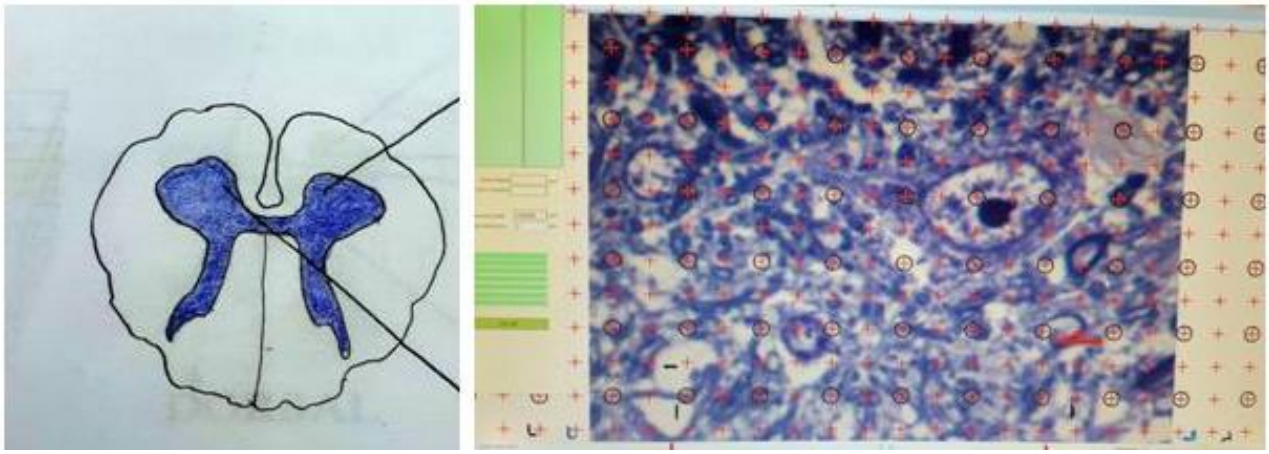

Fig.1. T11 segment of spinal cord sectioning and motor neuron counting protocol. Anterior horns were photographed. Toluidine Blue with Borax,Magnification ×200.

segment was removed by the thoracic vertebrectomy and remained in a 2.5% of glutaraldehyde for 24-48 hours and then post-fixed with 2 % osmium tetroxide (OsO4) at 4 C for 1:45 hours, dehydrated through a graded alcohol series, and embedded in Epon resin for sample processing. For light microscopy 0.5 µm sections (semithin section) were obtained using ultramicrotome according to systematic random sampling strategies. The sections were stained Toluidine Blue with Borax (7). Investigations were performed under light microscopic magnification x200 (Zeiss Axioskop 4 Carl Zeiss Göttingen, Germany).

Stereological Analysis: The parallel and serial sections at equal distances of 0.5 µm thickness from the constructed blocks were taken at 1/100 (f2) (Cross-sectional sampling share) (Fig.1). The unbiased stereological dissector/Cavalieri combination method, was used in the stereological examination of neurons number (8-10). The unbiased counting frame is 120x110µm. For motor neuron number magnification is 200x Obj. The step size of nerve profiles was done as 1/16 systematic random. The total number of motor neurons in the ventral horn in a spinal segment T11 (Fig. 2) was estimated using the formula:

$$N = \bar{Q}^{-} \times \sum_{p} \times k \times \frac{(a/p)}{a_{(frame)}}$$

where **N** = Total neuron number, $\bar{Q}^{-}$= averaged objects, Σ_p = Total number of volumetric dots, **k** = section thickness, **a/p** (frame) = represents the area of each point on the point counting grid **a**=frame area.

It was reported that it must be a specific value for coefficient error at stereological studies for calculating coefficient error (11). Section sampling number must be determined for calculating Coefficient of error (CE). CE value must be 0.05 or under this value. Additionally, the coefficient of variation value (CV) for every group of animals should be based on 0.10. It was determined that Shtereom 1.5 package program was adequate for this method (12). Stereological estimation values based on our result are given in (Table 2).

Statistical Analysis: The total number of motor neuron groups were compared using the Kruskal-Wallis test. Results were expressed as mean, standard deviation, minimum and maximum value. A p value of less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSS software (Statistical Package for the Social Sciences, version 13.0, SPSS Inc., Chicago, IL, USA)

Results

In the light microscopic general histological appearance examination, no changes were observed in all groups in section of the spinal segment T11 (Fig. 2). In rats, no behavioral abnormalities or daily feeding and drinking rate were observed compared to

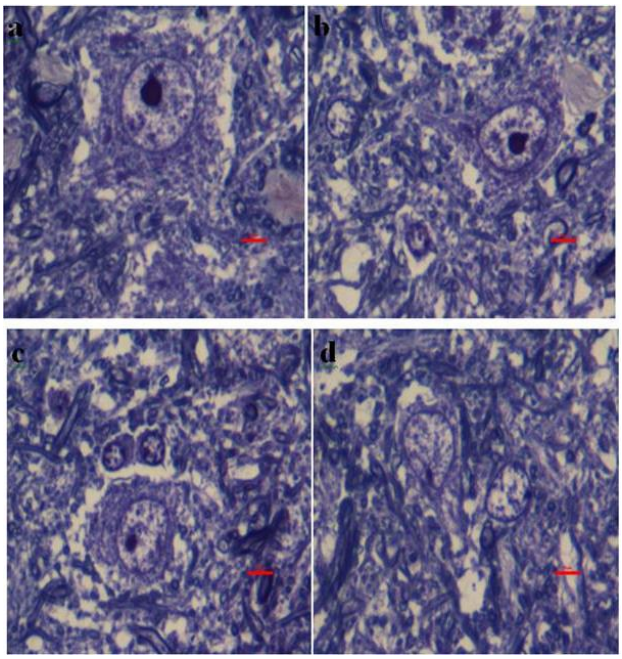

Fig. 2. Transverse section of T11 spinal cord of control group (a), saline group (b), 45 mg STZ group (c), 65 mg STZ group (d) (Toluidine Blue with Borax,Scale bars, 10 μm)

control and saline group. On the other hand in the STZ groups rats suffered from excessive water drinking and Polyuria, drowsiness and decreased interest in food. Weight change among control and saline groups was minimum. There was considerable loss of weights in STZ group rats (Table 1). The blood glucose rate was between normal range (75-125 mg/dl) in the control and saline groups. Both in the 45 and 65 groups after injection by STZ, the rate of glucose in the blood increased, more than 200 mg/dl, indicating that these groups have diabetes (Table 2). The groups were compared for total mean numbers motor neuron (Fig. 3). The results showed no significant differences between groups (Table 3).

Discussion

Diabetes mellitus causes many abnormalities in the human body organs or it has caused many disorders in different organs, including nervous system disease named as diabetic neuropathy (13). Diabetic neuropathy is a common complication of diabetes mellitus. The definitive pathogenesis of diabetic neuropathy is unknown. However, a number of assumed pathophysiologic mechanisms exist. Hyperglycemia is reported to result in increased advanced glycation end product, oxidative stress, increased polyol activity, nerve hypoxia or ischemia, γ-linolenic acid and growth factor deficiency (14). The neurodegeneration that occurs in diabetes mellitus is unique and cannot be simply related with ischemia,

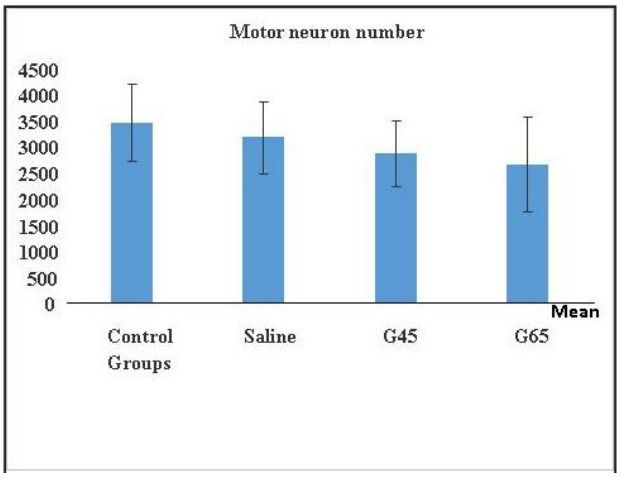

Fig. 3. Graph show motor neuron number in the groups

axotomy or other common mechanisms (15). Peripheral neurons probably undergo a process of terminal retraction where terminals are lost but perikarya or cell bodies are protected (16). There is not sufficient information exists for motor neuron populations in model of diabetes.

Effect of STZ-induced diabetes on motor neuron axon morphometry of T11 has not studied yet. Therefore the stereological method of the dissector/Cavalieri combination was applied for estimation of the total mean number of motor neuron in our study. The result showed that the numbers of motor neuron was not changed statistically. Our results are consistent with literature information. In accordance with Souayah et al. (17) study their findings challenges the belief that motor neuron are resistant to diabetic damage.

Ramji et al. (16) established diabetes in mice via administering different doses of STZ (45, 65 and 85 mg/kg/day) and evaluated motor neuron morphometry. Their results showed that motor neuron structure is conserved. One year after this study Zochodne et al. (18) reported that motor neurons are not among primary targets of diabetes mellitus.

Generally, studies report that the motor neurons diabetes models were changed in ultrastructure and in physiology but not in motor neuron number. This is contrasting to sensory neuron loss which is often seen in long-term diabetes studies (16). Zochodne et al. (18) suggest that motor neurons could be conserved from the loses witnessed in sensory neurons since motor neuron cell bodies are located within spinal cord, limiting their exposure to circulating molecules. Sensory neuron cell bodies are more delicate since they are not protected by the blood-brain barrier. While motor neuronal loss is not witnessed even in long period of time diabetic models, increased

expression of molecules for example, heat shock protein-27 (HSP-27), receptors for advanced glycation end-production (RAGE) and poly (ADP-ribose) polymerase (PARP), which are frequently found in degenerative neurons, occurs in motor neurons (16). Zochodne et al. (18) as in the case of sensory neurons, reported that motor neurons exhibit up-regulated expression of HSP-27, PARP, and RAGE. Thus this points out that although motor neuronal loss has not occurred even at later time points, the motor neurons are yet in a stressed state and are probably more sensitive to neuronal loss.

Ramji et al. (16) proposed two hypothesis for the reason of protection of motor neurons against diabetes mellitus. First of them is the fact that motor neurons are not as sensitive to structural alterations (such as structural protein synthesis) on the contrary to sensory neurons. Secondly terminal retraction which has a role in diabetic neurodegeneration and neuropathy has not proven for motor neurons.

In conclusion, the different doses (low dose: 45 mg, high dose: 65 mg) of STZ has no effect on the segment T11 of spinal cord of total number motor neuron of the adult rats.

Conflict of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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