

## Post-Operative Time Effects after Sciatic Nerve Crush on the Number of Alpha Motoneurons, Using a Stereological Counting Method (Disector)

Morteza Behnam-Rasouli<sup>\*1</sup>, Mohammad Reza Nikravesh<sup>2</sup>, Naser Mahdavi-Shahri<sup>1</sup> and Maryam Tehranipour<sup>1</sup>

<sup>1</sup>Dept. of Biology, School of Sciences, Ferdowsi University of Mashhad; <sup>2</sup>Dept. of Anatomy, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

### ABSTRACT

There are extensive evidences that show axonal processes of the nervous system (peripheral and/or central) may be degenerated after nerve injuries. Wallerian degeneration and chromatolysis are the most conspicuous phenomena that occur in response to injuries. In this research, the effects of post-operative time following sciatic nerve crush on the number of spinal motoneurons were investigated. Twelve adult male Wistar rats, whose left sciatic nerves were highly compressed for 30 s, assigned to experimental groups 1 and 2 (n = 6). After 3 and 8 weeks post-operative (in groups 1 and 2 respectively) the lumbar segments of spinal cord were sampled, processed, sectioned serially and stained with toluidine blue (pH 4.65). By using stereological quantitative technique (physical disector), the number of alpha motoneurons in the right and the left ventral horns of spinal cord were counted and compared with each other. Statistical analyses showed a remarkable reduction in the number of alpha motoneurons in the left side (experimental or operated) when compared with the right side (control or unoperated) both in 3 and 8 weeks post-operative groups. This reduction may be due to the blockade of retrograde axonal transport. *Iran. Biomed. J. 4: 45-49, 2000*

*Keywords:* Peripheral nerves, Neuronal degeneration, Alpha motoneurons, Stereology, Chromatolysis

### INTRODUCTION

A few days after axotomy, changes occur in the cell bodies of the most types of neurons. This phenomenon is referred to as chromatolysis [1]. If the neuron successfully regenerates its axon and restores the connections with other cells in the nervous system, the cell body usually returns to its former appearance [2]. Failure to contact a new target cell leads to atrophy and death [3]. The studies on neural development have identified several neurotrophic factors that are released by the targets of neurons and retrogradely transport to neuronal cell body [2, 4]. These factors are necessary and important for neuronal survival and growth [4-6].

To demonstrate the importance of axonal retrograde transport of neurotrophins, it has been shown that exogenous NGF enhances survival of axotomized neurons in adults and crushed neurons in neonatal rats [7]. Recently, it has been demonstrated

that retrograde transport of neurotrophins in intact axons can be abolished by application of drugs such as colchicine [8-10] and neural lesions such as compression [11, 12]. In rodents after peripheral axotomy, some motoneurons survive and undergo typical reactive changes classic for chromatolysis, while other motoneurons undergo changes that lead to cell death. It was also reported that particularly immature rat motoneurons are sensitive to axotomy, with a greater than 80% loss of neurons occurring within 1 week after nerve injuries in newborn rats [13].

The purpose of the present research is to study the post-operative time effects after sciatic nerve compression on the rate of neuronal death. To evaluate this, the numbers of alpha motoneurons in spinal cord segments, L4 to L6 were estimated.

\*Corresponding Author. Tel. (98-21) ext. 52; Fax (98-51) 838032; E-mail: Behnam@sciencel.um.ac.ir

## MATERIALS AND METHODS

**Animal model and experimental design.** Twelve inbred young male Wistar rats weighing 250-300 g (Serum Production Institute, Hesarak, Karaj) were housed in pairs in plastic cages in the animal house and given rat chow and water *ad libitum*. Animals were coded and their ears were marked at the time of surgery and randomly assigned to 1 and 2 experimental groups (n = 6). All assessments were made in a blinded fashion and decoded at the end of study.

All the surgical procedures were performed with aseptic techniques. For axotomy, the animals were anesthetized under interaperitoneal injection of 0.2 ml of a mixture (1:2) of 10% ketamin (Bayer, Germany) and 2% xylazine (Boxtel, Holland) and the left sciatic nerve was exposed through a gluteal muscle splitting incision. At this location, the nerve trunk was crushed for 30 seconds period between prongs of #5 clamp forceps. On the right side, a control operation was performed which exposed the sciatic nerve but did not disturb it. The muscle and skin were then closed with 14 mm stainless steel sutures. At the selected post-operative times (group 1, 3 weeks and group 2, 8 weeks), rats were anesthetized and intracardially perfused with cold (20°C) 10% formaldehyde. Immediately following perfusion, a block of the spinal cord segments L4 to L6 (approximately 8 mm length) was removed while sciatic nerve roots of both sides were still attached to it. The spinal blocks were placed in the same fixative for post sampling fixation overnight and then processed and embedded in paraffin. The blocks were sectioned serially at 7 micrometer. A uniform random sampling scheme was employed so that about 10 sections from each block were sampled [14]. Sections were stained with toluidine blue with special buffer of acetic acid 1N (1 ml), sodium acetate 1N (1 ml) and distilled water (98 ml) pH 4.65. After permanent mounting, the number of motoneurons in the left and the right sides of ventrolateral regions of ventral horn of the spinal cord (L4 to L6) were determined, using stereological counting technique; physical disector.

The 'disector' principle [15] was used to determine the numbers of motoneurons in each section. From each section and its adjacent neighbor four photos were taken, two from each section; one from right side and the other one from the left side of ventrolateral region of the spinal ventral horn with a final magnification of 100 (Fig. 1). A two-

dimensional unbiased counting frame [16] was overlaid in a uniform, random manner onto regions of any two photos taken from left sides of both sections. Those cells were nuclei selected by the frame on the reference plane but not appearing on the adjacent "look-up" frame section were deemed to have their 'tops' in the volume described by the product of the area of the counting frame and the distance between sections [15]. A similar operation was done on photos of the right sides. These nuclei were counted (Q) to provide the numerical density of cells ( $N_v$ ) in the ventral horns of spinal cord according to the following equation:

$$N_v = \frac{\sum Q}{\sum(\text{disector}) \times h \times a_{(\text{frame})}}$$

Where Q is the sum of counted neurons, (disector) is the number of disectors, h is the depth of the disector equal to the section thickness (7 micron) and  $a_{(\text{frame})}$  is the scaled area of the disector frame. An example of morphometric calculation is as follow: In this research the area of the frame was  $25 \times 25 = 625 \text{ mm}^2$ , and the linear magnification was  $4 \times 10 \times 2.5 = 100$ . To calculate the  $N_v$  of motoneurons in the right side of spinal cord (Table 1, rat number 1) we should do as follows. A total of 22 neurons were counted in 16 disectors and the depth of the disector (h) was  $7 \times 10^{-3} \text{ mm}$ , and the real area corresponding to each disector frame, at a linear magnification of  $\times 100$ , was:

$$\frac{625}{(100)_2} = 6.25 \times 10^{-2} \text{ mm}^2$$

and the numerical density ( $N_v$ ) of motoneurons of spinal cord was calculated according to the above equation:

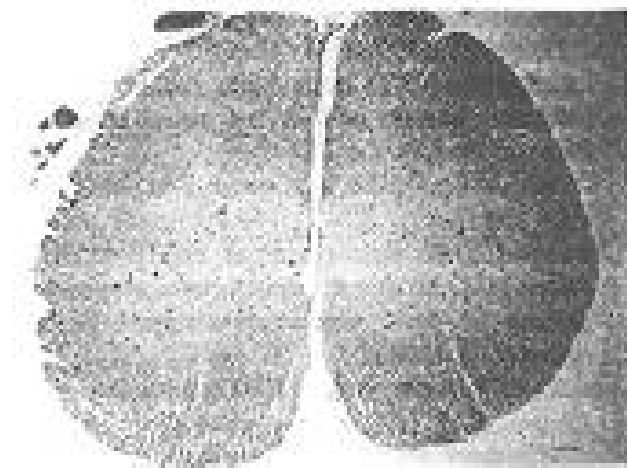
$$N_v = \frac{22}{16 \times 7 \times 10^{-3} \times 6.25 \times 10^{-2}} = 3142/\text{mm}^3$$

The ratio of numerical density of neurons in operated/unoperated sides of spinal cord was then used as an index of neuronal death. All quantitative data were analysed by using ANOVA and *t-test*.

## RESULTS

The results of the morphometric analyses of numerical density of alpha motoneurons in the ventral horns of spinal cord are shown in Table 1.

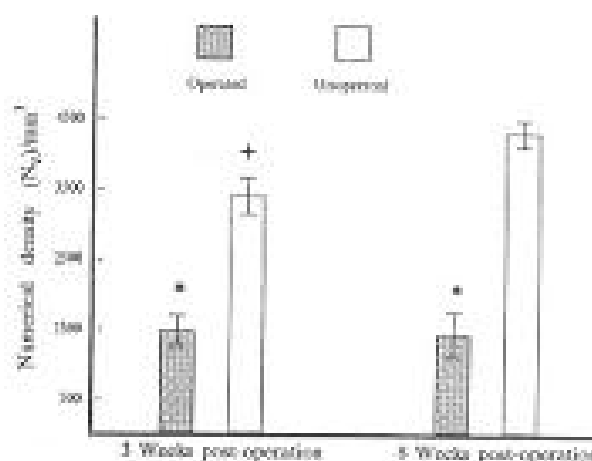
After sciatic nerve crush, the spinal ventral horn motoneuron counts show a decline in number. Reduction of cell number appeared complete at 3 weeks post-operative with no additional loss noted at 8 weeks (1489 and 1438 respectively). Although in operated sides of spinal cord of both groups the numerical density is decreased and remained constant but in unoperated sides the numerical density of alpha motoneurons is higher in group 2 (4353 vs 3441). Statistical analyses show that the reduction in the alpha motoneurons in treated sides of spinal cord of both groups, when compared with unoperated sides is significant ( $p < 0.0001$ ) (Table 1, Fig. 2). Comparison of numerical density of motoneurons among operated sides in both groups showed no significant differences but when unoperated sides were compared, there was a significant difference between neuronal density ( $p < 0.01$ ).



**Fig. 1.** Photomicrographs of toluidine blue stained transverse section from L4-L6 spinal cord following unilateral crushing of sciatic nerve, 3 weeks post-operation. Note the decrease in the numbers of motoneurons in the operated (left) side.

## DISCUSSION

It has been well known that the survival and functional maintenance of neurons are clearly dependent upon retrogradely transport of neurotrophins [17, 18]. Peripheral nerve transection or crush, blockades axonal transport and therefore



**Fig. 2.** The comparison of numerical density of motoneurons of spinal ventral horn in operated and unoperated sides, in 3 and 8 weeks post-operation time.

might produce chromatolysis and cell death. In this research, we investigated the post-operative time effects following sciatic nerve crush on the numerical density of alpha motoneurons. The present results, are based on using method which provides absolute estimates of cell number rather than ratios [15, 19, 20]. This method has been rapidly gaining acceptance, because not only it is the most efficient quantification method, but also unbiased and simple to employ.

Statistical analyses of data indicate that, in comparison with unoperated side of spinal cord, the numerical density of alpha motoneurons in the operated side significantly decreases. However, there are no remarkable differences between group 1 and 2, which indicates that post-operative time has no effect on neuronal loss. It is possible that, the site of compression was very close to the spinal cord (gluteal region) and therefore after a few days following axotomy alpha motoneurons undergo degeneration. It has been shown that particularly immature rat motoneurons are sensitive to axotomy, with a greater than 80% loss of neurons occurring within 1 week after nerve injuries in newborn rats [10]. The magnitude of motoneuronal death observed in this research is consistent with that of previously reported [13]. Because sciatic nerve is a mixed nerve, it seems very likely that after compression efferent and also afferent pathways are damaged [21-23]. Therefore, it is possible that,

**Table 1.** Comparison of numerical density of motoneurons of spinal ventral horn in operated (left) and unoperated (right) sides, in 3 and 8 weeks post-operative time.

Rat	3 weeks post-injury					8 weeks post-injury				
	$\Sigma_{disec}$	left side		right side		$\Sigma_{disec}$	left side		right side	
		$\Sigma Q$	$N_v$	$\Sigma Q$	$N_v$		$\Sigma Q$	$N_v$	$\Sigma Q$	$N_v$
1	16	08	1143	22	3142	18	24	3048	34	4317
2	16	11	1571	29	4142	18	10	1270	31	3936
3	10	04	0914	12	2742	18	16	2032	36	4571
4	14	11	1796	23	3755	14	13	2122	29	4735
5	08	07	2000	12	3428	16	10	1429	33	4714
6						16	12	1714	27	3857
Mean $\pm$ SD		1489 $\pm$ 455 <sup>a</sup>		3441 $\pm$ 530 <sup>b</sup>		1438 $\pm$ 630 <sup>a</sup>		4353 $\pm$ 383		

<sup>a</sup> $p < 0.0001$ , Student's *t*-test compared with contralateral, <sup>b</sup> $p < 0.01$ , Student's *t*-test compared with the same side of other group. *disec.*= number of disectors, *Q* = number of counted motoneurons,  $N_v$ =numerical density (/mm<sup>3</sup>).

in addition to motoneurons, some interneurons which receive inputs from afferent pathways undergo degeneration. Since motoneurons of unoperated side also receive inputs, at least partly, via crossed interneurons, the transneuronal degeneration might affect cell bodies of motoneurons of unoperated side. By passing time, the remaining interneurons try to re-innervate further motoneurons. If this is the case, the appearance of motoneuronal cell bodies of the unoperated side becomes normal and therefore falls into counting. This explanation may be the cause of elevation of numerical density in unoperated side of group 2 (4353 vs 3441). The measurement we have used here allows an unbiased assessment of numerical density, and the result suggests that, although degeneration processes are completed as early as week 3 after compression, a partial recovery in numerical density of motoneurons in unoperated side may be possible.

#### ACKNOWLEDGMENT

This work was supported by the grant #3-2/5272 from Office of Research Affairs of Ferdowsi Mashhad University. The authors wish to thank Dr. A. R. Fazel, Mashhad University of Medical Sciences, for kindly reviewing the manuscript and M. Khaksar and M. Sabzehbin for their technical assistance and animal care.

#### REFERENCES

1. Matthews, M.R. and Nelson, V.H. (1975) Detachment of structurally intact nerve endings from chromatolytic neurons of rat superior cervical ganglion during the depression of synaptic transmission induced by post-ganglionic axotomy. *J. Physiol. (Lond.)* 245: 91-135.
2. Seniuk, N.A. (1992) Neurotrophic factors: role in peripheral neuron survival and axonal repair. *J. Reconstr. Microsurg.* 8: 399-404.
3. Crouch, M.F., Heydon, K., Garnaut, S.M., Milburn, P.J. and Hendry, L.A. (1994) Retrograde axonal transport of the alpha-subunit of the GTP-binding protein GZ in mouse sciatic nerve: a potential pathway for signal transduction in neurons. *Eur. J. Neurosci.* 6: 626-631.
4. Rich, K.M., Luszczynski, J.R., Osborne, P.A. and Johnson, E.M. (1987) Nerve growth factor protects adult sensory neurons from cell death and atrophy caused by nerve injury. *J. Neurocytol.* 16: 261-268.
5. Maisonpierre, P.C., Belloscio, L., Feriedman, B., Alderson, R.F., Wiegand, S.J., Furth, M.E., Lindsay, R.M. and Yancopoulos, G.D. (1990) NT-3, BDNF and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. *Neuron* 5: 501-509.
6. Wong, V., Arriaga, R., Ip, N.Y. and Lindsay, R.M. (1993) Theneurotrophins BDNF, NT-3, and NT-4/5, but not NGF, up-regulate the cholinergic phenotype of developing motor neurons. *Eur. J. Neurosci.* 5: 466-474.
7. Eriksson, N.P., Lindsay, R.M. and Aldskogius, H. (1994) BDNF and NT-3 rescue sensory but not motoneurons following axotomy in the neonate. *Neuroreport* 5: 1445-1448.

8. Zagrebelsky, M., Buffo, A., Skerra, A., Schwab, M.E., Strata, P. and Rossi, F. (1998) Retrograde regulation of growth-associated gene expression in adult rat purkinje cells by myelin-associated neurite growth inhibitory proteins. *J. Neurosci.* 18: 7912-7929.
9. Beske, O. and Gorenstein, C. (1995) Fate of lysosomes transported to the dendrites by a colchicine-induced mechanism. *Brain Res.* 669: 125-129.
10. Crouch, M.F., Hevdon, K., Garnaut, S.M., Milburn, P.J. and Hendry, I.A. (1994) Retrograde axonal transport of the alpha-subunit of the GTP-binding protein GZ in mouse sciatic nerve: a potential pathway for signal transduction in neurons. *Eur. J. Neurosci.* 6: 626-631.
11. Sendtner, M., Stockli, K.A. and Thoenen, H. (1992) Synthesis and localization of ciliary neurotrophic factor in the sciatic nerve of the adult rat after lesion and during regeneration. *J. Cell Biol.* 118: 139-148.
12. Anders, J., Borke, R.C., Woolery, S.K. and Van-demerwe, W.P. (1993) Low power laser irradiation alters the rate of regeneration of the rat facial nerve. *Lasers Surg. Med.* 13: 72-82.
13. Tong, J.X. and Rich, K.M (1997) Diphenyl-piperazines enhance regeneration after facial nerve injury. *J. Neurocyto.* 26: 339-347.
14. Gunderson, H.J.G. and Jensen, E.B. (1987) The efficiency of systematic sampling in stereology and its predication. *J. Microsc.* 147: 229-263.
15. Sterio, D.C. (1984) The unbiased estimation of number and sizes of arbitrary particles using the disector. *J. Microsc.* 134: 127-136.
16. Ulenkate, H.J., Vernagen, M.A., Gispén, W.H. and Jennekens, F.G. (1993) Theneurotrophic analogue ACTH (4-9) reduces the perineuronal microglia reaction after rat facial nerve crush. *Glia* 9: 219-226.
17. Liuzzi, F.J. and Tedeschi, B. (1991) Peripheral nerve regeneration. *Neurosurg. Clin. N. Am.* 2: 31-42.
18. Primi, M.P. and Clarke, P. G. (1997) Early retrograde effects of blocking axoplasmic transport in the axons of developing neurons. *Brain Res.* 99: 259-262.
19. Gunderson, H.J.G. (1986) Stereology of arbitrary particles. *J. Microsc.* 143: 3-45.
20. Cruz-Orive, L.M. (1987) Particle number can be estimated using a disector of unknown thickness. *J. Microsc.* 145: 121-142.
21. Bondok, A.A., Sansone, F.M. (1984) Retrograde and transganglionic degeneration of sensory neurons after a peripheral nerve lesion at birth. *Exp. Neurol.* 86(2): 322-330.
22. Arvidsson, J., Ygge, J., Grant, G. (1986) Cell loss in lumbar dorsal root ganglia and transganglionic degeneration after sciatic nerve transection in the rat. *Brain Res.* 373(1-2):15-21.
23. Himes, B.T., Tessle, A. (1989) Death of some dorsal root ganglion neurons and plasticity of others following sciatic nerve section in adult and neonatal rats. *J. Comp. Neurol.* 284(2):215-230.