Original Article

Delayed Synaptic Changes in Axotomized Spinal Motoneurons of Newborn Rats Associated with Progressive Neuronal Loss: Immunohistochemical, Ultrastructural, and Quantitative Study

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ABSTRACT

Background and Objective: **Sciatic nerve transection is characterized by a rapid wave of motoneuron death associated with progressive synaptic lesions. The purpose of this study was to evaluate the long term synaptic changes.**

Materials and Methods: **This basic study was carried out on paraffin- or resin-embedded tissue blocks for evaluation of synaptophysin and choline acetyl transaferase (CHAT) immunoreactivities and the ultrastructural changes in the synapses of spinal motoneurons following sciatic nerve axotomy in the newborn rats.**

Results: **The results showed that there was a progressive decrease in the percentage of survived motoneurons and high percentage of chromatolytic motoneurons. There was also a high percentage of degenerated motoneurons with dotted pattern synaptophysin immunoreactivity, low percentage of intact motoneurons with complete synaptophysin labeling, and high percentage of motoneurons with low CHAT labeling. The ultrastructural study showed that there were many motoneurons with synaptic pathological changes including irregularity of the synaptic membrane and displacement of synaptic vesicles.**

Conclusion: **The findings of this study indicate that there is a delayed synaptic lesion in axotomized motoneurons of newborn rats.**

Key words: **Motoneuron, Axotomy, Sciatic nerve**

Introduction

Axotomized spinal neurons have been reported to undergo cell death (1-2). In additions, synaptic changes may occur in some other neurons where apoptosis has been confirmed using ultrastructural study (3) and TUNEL (4). The molecular changes are characterized by an increase in pro-apoptotic gene expression such as Bax gene (5) and caspase (6). Comet assay has also been used to evaluate DNA changes in apoptotic neurons (7). These studies have been used to define the type and structure of

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the dead cell. On the other hand, it is very important to distinguish synaptic changes regarding earliness or lateness. Other investigators have tried other approaches to characterize the histo-functional features of apoptotic cells. Axotomy has been reported to decrease the expression of choline acetyl transferase (CHAT) (8). In this respect, Friedman et al revealed that spinal motoneurons lose CHAT immunoreactivity following axotomy (9). Similar findings have been reported about the expression of synaptophysin which is associated with degenerative changes in the synaptic ultrastructure at the early stages of sciatic nerve transection (10) using a time course study designed to investigate the rapid phase (within 72 hrs) of synaptic changes occurring in axotomized motoneurons in neonates.

Therefore, in this study, the late changes (3 weeks post-axotomy) in synapses of axotomized motoneurons of newborn rats and the pattern of the changes in the expression of both CHAT and synaptophysin in the axotomized spinal motoneurons were evaluated using immunohistochemical methods. Morphometric parameters were used to evaluate the trend of changes quantitatively. Ultrastructure investigation of the axotomized motoneurons was also done to characterize synaptic lesions.

Materials and Methods

In this study, 45 Sprague-Dawley newborn rats (as an animal model) (3) were procured from Razi institute (Karaj, Iran). The rats were divided into three groups (15 in each group). The surgery was done at day 3 postnatal (P3). The animals were anesthetized by hypothermia and the sciatic nerves at the left sides were axotomized while the right sides were used as controls. Then, the newborns were warmed and returned to their mother. Housing of the animals was carried out according to gGuidelines for animal experimentation of the ethical committee of the School of Medicine, Shahed University. The animals of the first group were used for motoneuron counting, while the second and third groups were used for immunohistochemical and ultrastructural studies respectively. The animals were sacrificed at day 21 post-surgery. The spinal segments (L4-L6) were removed by laminectomy and the tissues were processed for paraffin embedding, sectioning (at a thickness of 8 μm) and staining with Cresyl violet. Counting of spinal motoneurons was done according to Li et al (11) and the percentage of survived motoneurons was calculated according to Tarihi and Rezaie (5). Also, the percentage of chromatolytic motoneuron (PCM) was estimated. Five micrometer sections were taken from the spinal segments (L4-L6) of the animals in the second group, labeled with mouse anti-synaptophysin antibody (Chemicon international) as primary antibody and followed by labeling with rabbit anti-mouse peroxidase conjugated antibody as secondary antibody (Serotec). The sections were treated with 10% hydrogen peroxide in order to inhibit endogenous peroxidase, then, treated with diaminobenzidine tetrahydrochloride in Tris buffer (pH 7.6) containing fresh hydrogen peroxide. Also, CHAT immunolabeling was carried out with mouse anti-CHAT antibody (Chemicon International), followed by the same procedure used in the previous labeling. Synaptophysin immunolabeled motoneurons in the ventral horn at the intact and axotomized sides were classified according to the pattern of immunoreactivity of antisynaptophysin antibody reported by Tarihi and Rezaie (10). In brief, an intact pattern, where the subplasmallemal labeling completely envelops the motoneurons or covers equal or more than two thirds of the subplasmallema; a partial pattern, where discontinuous antisynaptophysin labeling is present and the labeling with synaptophysin covers less than two-third of the subplasmallemal region; a cytoplasmic pattern, classified either dotted or homogeneous cytoplasmic subtypes; and a negative pattern, where no labeling is detected in the motoneurons. CHAT immunolabeled motoneurons in the ventral horn at the intact and axotomized sides were classified into high, intermediate, and low intensities.

All the parameters were tested for normality using nonparametric S-K test and the results showed that all the parameters were not significantly different from normal ($p > 0.05$). The means were compared using Tukey's test, while the analysis of the variance was used to compare the difference among

the groups. For ultrastructural study, the tissues from L4-L6 spinal segments were sampled after perfusion with Karnovsky's fixative, immersed in 2.5% glutaraldehyde in phosphate buffer (0.1M, pH 7.4) and post-fixed in 1% osmium tetroxide in phosphate buffer. Thin sections were cut, stained with uranyl acetate and lead citrate and examined under Ziess EM 900.

The percentage of pattern reduction (PPR) was calculated as follows:

$$PPR = \frac{PNPAS - PNPUS}{PNPUS} \times 100$$

Where, PNPAS: is percentage of neuronal pattern at axotomized side and PNPUS is percentage of neuronal pattern at un-axotomized side.

Results

The results of this investigation showed that the percentage of the survived motoneurons decrease at the axotomized side, while the percentage of chromatolytic motoneurons (60%) was significantly higher at the axotomized than that of the intact sides. Synaptophysin immunolabeling results are demonstrated in Figures 1 and 2, representing different types of labeling (intact: i, partial: p, dotted: d, homogenous: h and negative: n patterns) and it is decreased. CHAT immunolabeling is shown in Figure 3 at high, intermediate, and low intensities. In detail, the statistical differences

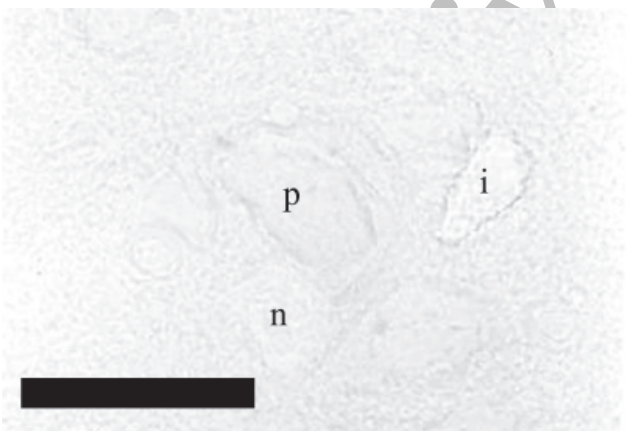

Figure 1: Photomicrograph shows anti-synaptophysin immunoperoxidase labeling of spinal cord motoneurons from the axotomized side of a rat with intact pattern (i) where well preserved synaptophysin immunoreactivity present around the soma, partial pattern (p) where the immunoreactivity less than two third of the soma and negative pattern (n) with immunoreactivity present in the motoneuron (scale bar = 25 µm).

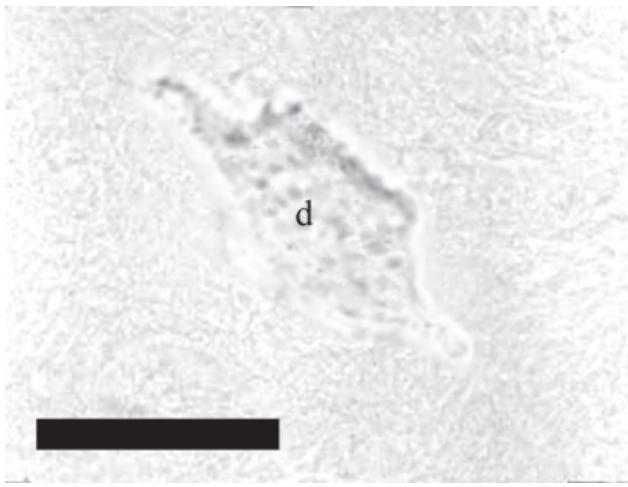

Figure 2:Photomicrograph shows anti-synaptophysin immunoperoxidase labeling of spinal cord motoneurons from the axotomized side of a rat with cytoplasmic pattern (d) (scale bar =25 µm).

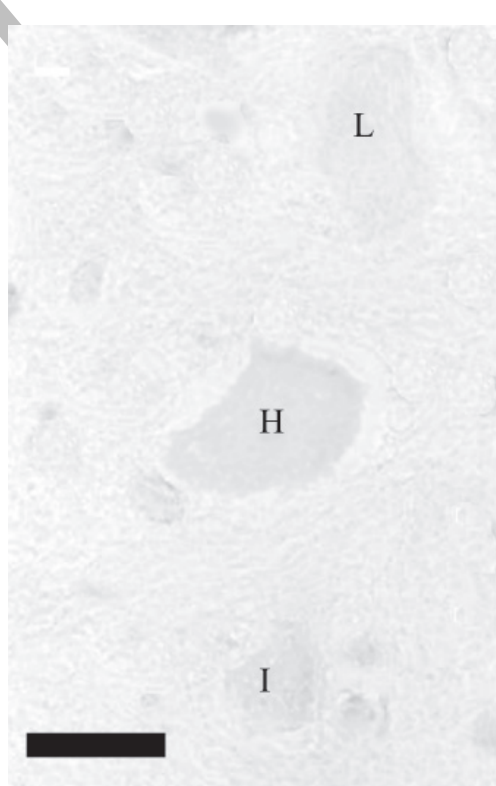

Figure 3:Photomicrograph shows anti-choline acetyl transferase immunoperoxidase labeling of spinal cord motoneurons from the axotomized side of a rat with low (L), intermediate (I), and high (H) intensity labeled motoneurons (scale bar =25 µm).

between the axotomized and un-axotomized sides were significant in all groups except between the intact pattern of synaptophysin labeled motoneurons versus cytoplasmic and negative patterns at the axotomized side; the cytoplasmic pattern at the axotomized side versus the negative pattern at the axotomized and cytoplasmic pattern at the un-axotomized sides, the negative pattern at the axotomized side versus cytoplasmic pattern at the un-axotomized, and the intact and partial pattern at the axotomized side.

(the quantitative data is presented in Table 1), where the percentage of the intact pattern of synaptophysin-labeled motoneurons at the un-axotomized side was significantly higher than that of the axotomized side, while the negative and partial patterns were significantly higher at the axotomized side than those at the un-axotomized side. However, the differences in the cytoplasmic pattern were not significant. The data of CHAT in detail shows that the statistical differences between: the axotomized and un-axotomized sides were significant in all groups except between the low intensity at the un-axotomized side and high intensity of choline acetyl transferase at the axotomized side, low and intermediate intensities at the un-axotomized side; and the high intensities at the axotomized and low and intermediate intensities at the un-axotomized sides (Table 2).

Table 1. The means and standard error of the means of the percentage of all types of synaptophysin-labeled motoneurons at the un-axotomized and the axotomized sides (intact; i, partial; p, cytoplasmic; c, and negative; n).

	U-side	A-side	PPR
i	42.3 ± 4	10.8 ± 14	-74
p	38.8 ± 3.9	73.3 ± 26	89
C	19.2 ± 5.6	10 ± 13	-48
N	0 ± 0	2.5 ± 7.9	in

PPR: percentage of pattern reduction
In: inconclusive result

The statistical differences between the axotomized and un-axotomized sides were significant in all groups except between: the intact pattern of synaptophysin labeled motoneurons versus cytoplasmic and negative patterns at the axotomized side, the cytoplasmic pattern at the axotomized side versus the negative pattern at the axotomized and cytoplasmic pattern at the un-axotomized sides, the negative pattern at the axotomized side versus cytoplasmic pattern at the un-axotomized, and the intact and partial pattern at the axotomized side.

Table 2. The means and standard error of the means of the percentages of CHAT positive motoneurons with high (H), intermediate (I), and low (L) intensities at the un-axotomized and the axotomized sides.

	U-side	A-side	PPR
H	68.6± 2	18.5 ± 2.5	-73
I	12.2± 1.6	29.7 ± 2.6	143
L	20.1 ± 1.5	50.2 ± 1.7	150

PPR: percentage of pattern reduction

The statistical differences between the axotomized and un-axotomized sides were significant in all groups except between the low intensity at the un-axotomized side and high intensity of choline acetyl transferase at the axotomized side;, low and intermediate intensities at the un-axotomized side, and the high intensities at the axotomized and low and intermediate intensities at the un-axotomized sides.

The high intensity pattern was significantly higher at the un-axotomized side than that of the axotomized one, while the intermediate and low intensities were significantly higher at the axotomized than the un-axotomized sides. The data of PPR in synaptophysin showed that there was an increase in the percentage of partial pattern, while both the percentage of intact and cytoplasmic patterns declined, while CHAT showed progressive decline in the intensity of CHAT, where the highest percentage was in the low intensity group of neurons. The trend in CHAT intensity was parallel with the progressive decline in the percentage of

survived motoneurons.

Figure 4 shows that that there are displaced synaptic vesicles in the synapses at the axotomized side, while Figure 5 shows irregularities of synaptic membranes in an area with collapsed synaptic cleft. It also shows presynaptic and postsynaptic membranes detachment. There was low electron density in the synaptic active zone that its region was ensheathed by astrocytic processes (Figure 6).

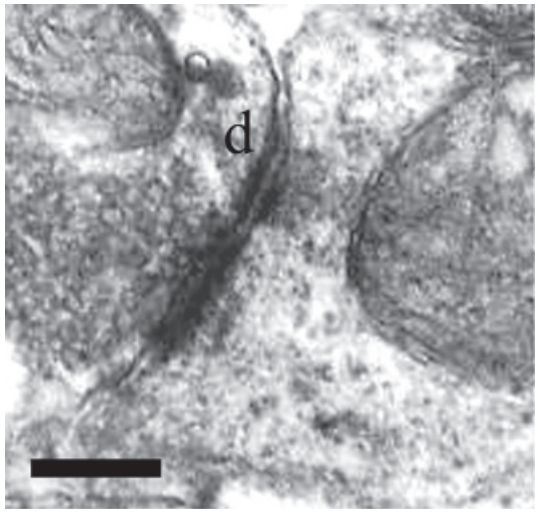

Figure 4:An electron micrograph of synapse in a motoneuron from the spinal cord of the axotomized side which shows displacement of the synaptic vesicles (d) (scale bar = 0.22 µm).

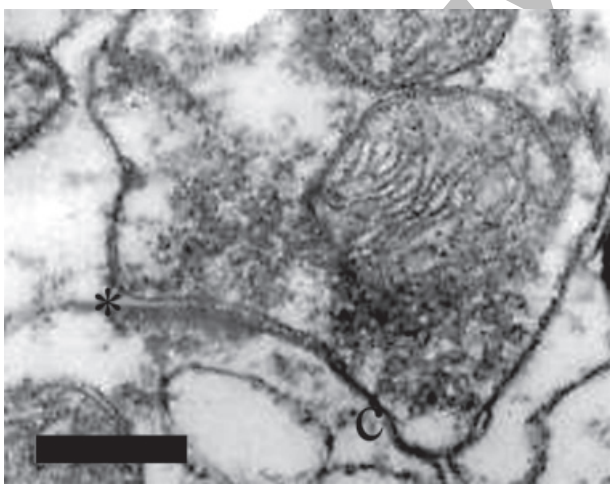

Figure 5:An electron micrograph of synapse in a motoneuron from the spinal cord of the axotomized side which shows collapsed (c) synaptic cleft and an early stage of detachment of presynaptic and postsynaptic membranes (*) (scale bar = 0.22 µm).

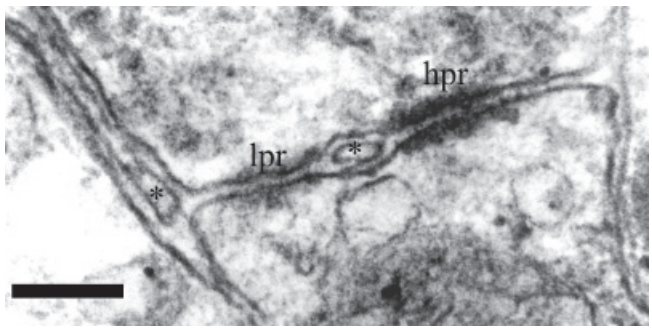

Figure 6:An electron micrograph of synapse in a motoneuron from the spinal cord of the axotomized side, which shows a perforated synapse with low electron density presynaptic zone (lpr: type 1 lesion) of the synapse while other region of the synapse shows high electron density (hpr: type 2 lesion). The astrocytic process ensheathment can be seen in type 1 lesion (*) (scale bar = 0.22 µm).

Discussion and Conclusion

The rate of neuronal loss is too important in histofunctional studies, while the remaining neuron and their function have been noticed by researchers. Progressive decrease in the percentage of survived motoneurons (PSM) at early stages of spinal motoneurons axotomy has been reported (5), where the highest reduction was at the end of the time course (PSM: 66.7%). In this study a further reduction was noticed (43.4 %), which reveals that the trend of neuronal loss continued but at a slower rate. This means that most of the motoneurons in the animals sacrificed three weeks post-axotomy showed more degenerative changes than cell death when compared with those evaluated at early stages (5). Therefore, the ability to conduct nerve stimuli and neuron function is based on neurotransmitter factor. To evaluate this, we used CHAT and Synaptophysin. The changes in the synapse may represent synaptic changes in a degenerating pool of motoneurons. Moreover, the data demonstrated that more than 50% of the axotomized spinal motoneurons had low level of CHAT expression three weeks after axotomy, while the percentage of motoneurons with intense CHAT immunoreactivity was less than 20%. This is consistent with reports from other investigators who reported a decline in the

expression of CHAT in axotomized motoneurons (12). The decline in CHAT intensity was consistent with the progressive decline in the percentage of survived motoneurons. This may reveal that the axotomized motoneurons have more tendencies to reverse the target deprivation to maintain neuronal structural integrity than function which is marked by neurotransmitter synthesis (13). The level of CHAT has been reported to decline 21 day after axotomy in the adult animal (14-15), while Chiu et al (16) reported no decline in CHAT level in axotomized neurons. The possible explanation for this controversy is the age of the animals used in the study, where the axotomy of sciatic nerve in newborns resulted in progressive increase in cell death as compared with adults (1). Synaptophysin data showed that there has been an increase in the percentage of partial pattern, while both the percentage of intact and cytoplasmic patterns declined. The clustering of many neurons with partial pattern may indicate that some neurons tend to return to the intact pattern pool as the regenerative process continue, while other neurons tend to progress toward degeneration (17). Cruz-Sanchez et al (18) have reported that chromatolytic motoneurons are associated with a large number of dot-like around cell body, consistent with the partial pattern, which maybe due to loss of presynaptic terminals or cell death. The negative pattern has been reported to occur in chromatolytic motoneurons (18) that suggest that many motoneurons may undergo degenerative changes. The findings of this investigation are consistent with a previous communication which revealed that motoneuron losses as well as synaptic lesions in axotomized motoneurons in newborn rats (10). Alvarez et al. (19) reported that the decline in synaptophysin is an important indicator of synaptic stripping or synaptic contact loss (20). However, following traumatic injury, plastic change is considered as the most important event following neuronal injury (21). Synaptophysin immunoreactivity is considered as an important marker for plasticity in the central nervous system (22). Lassmann et al (23) reported an increase in synaptophysin immunoreactivity as a result of enlargement of synaptic boutons and accumulation of synaptophysin, which is consistent with this investigation where some neurons showed intense homogenous immunoreactivity (cytoplasmic pattern). While Havton and Kellerth (24) suggested that the presence of synaptophysin in the cytoplasm maybe due to degenerative changes, however, in this study, the statistical difference in the cytoplasmic pattern between the axotomized and un-axotomized sides was non-significant. The intact pattern at the axotomized side was significantly lower (approximately one fourth) than that at the un-axotomized side, and the negative and partial patterns at the axotomized side were significantly higher than those at the un-axotomized side. These findings revealed that there is progressive degeneration of motoneurons in long standing sciatic nerve axotomy. On the other hand, the cytoplasmic pattern of synaptophysin immunoreactivity at the un-axotomized side was not significantly different from that of the axotomized one, which contrasted with the previous finding (10, 24), which maybe due to combined degenerative and regenerative processes occurring at the same time in adult animals, where some population of these motoneurons underwent regenerative changes with a cytoplasmic pattern while other motoneurons with a similar pattern underwent degeneration. The data of CHAT were consistent with that of the synaptophysin intact, partial and negative patterns. Meanwhile, CHAT immunoreactive motoneurons, which show high percentage of motoneurons with low CHAT immunoractivity, corresponded with the trend in synaptophysin decline, which may indicate the high degenerative changes at the axotomized side (25). The results of the ultrastuctural study confirmed the previous finding of Tarihi and Rezaie (10), where displacement of synaptic vesicles, detachment of synaptic membrane, irregularity of synaptic membrane, collapse of synaptic cleft, and astrocytic ensheathment of the degenerated synapse were noticed. Another interesting finding was that some astrocytic processes were covering the portion of synapse with more advanced lesion, while the portion of the synapse with lower lesion showed no astrocytic covering. Moreover, type 1 lesion showed reduction in electron density at the active zone, while both types of the synaptic lesions

were presented with displaced synaptic vesicles. This may indicate that displacement of synaptic vesicle precedes the loss of electron density at the active zone, which may precede the astrocytic ensheathment.

To conclude, axotomized sciatic nerve causes progressive decrease in spinal motoneuron with ultrastructural changes in synapse which is similar to known delayed synaptic changes.

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