Iranian Biomedical Journal 13 (2): 65-72 (April 2009)

Morphological Identification of Cell Death in Dorsal Root Ganglion Neurons Following Peripheral Nerve Injury and Repair in Adult Rat

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Received 7 April 2008; revised a September 2008; accepted 1 September 2008

ABSTRACT

Background: Axotomy causes sensory neuronal loss. Reconnection of proximal and distal nerve ends by surgical repair improves neuronal survival. It is important to know the morphology of primary sensory neurons after the surgical repair of their peripheral processes. **Methods:** Animals (male Wistar rats) were exposed to models of sciatic nerve transection, direct epineurial suture repair of sciatic nerve, autograft repair of sciatic nerve, and sham operated. After 1 and 12 weeks of the surgery, the number of L5 dorsal root ganglion (DRG) and ultrastructure of L4-L5 DRG neurons was evaluated by fluorescence and electron microscopy, respectively. **Results:** Nerve transection caused sensory neuronal loss and direct epineurial suture but no autograft repair method decreased it. Evaluation of morphology of the neurons showed classic features of apoptosis as well as destructive changes of cytoplasmic organelles such as mitochondria, rough endoplasmic reticulum and Golgi apparatus in primary sensory neurons. These nuclear and cytoplasmic changes in primary sensory neurons were observed after the surgical nerve repair too. **Conclusion:** The present study implies that the following peripheral nerve transection apoptosis as well as cytoplasmic cell death contributes to neuronal cell death and reconnection of proximal and distal nerve ends dose not prevent these processes. *Iran. Biomed. J. 13 (2): 65-72, 2009*

Keywords: Dorsal root ganglion (DRG), Cell death, Surgery, Morphology

INTRODUCTION

Peripheral nerve transection induces the primary sensory neuronal death [1- 3]. There are many various reports of primary sensory neuron death after peripheral nerve lesion in adults [4, 5]. Between 7% and 50% of primary sensory neurons die after peripheral nerve injury [1, 6]. A variety of stimuli may initiate neuronal death, although loss of target-derived neurotrophic support appears to be significant [7, 8]. Axotomy and target deprivation frequently have been reported to induce standard apoptosis [9] or in some cases, autophagic neuronal death involving strong endocytosis [10] and cytoplasmic cell death [11].

The dorsal root ganglion (DRG) provides a relatively isolated system for study and sufficient similarities exist between the mechanisms underlying axotomy-induced neuronal death within the peripheral nervous system (PNS) and central nervous system (CNS) [12]. Any treatment which protects primary sensory neurons may also have therapeutic implications for treatment of traumatic CNS. Since injury to peripheral sensory or mixed nerves is the commonest form of nervous system trauma [13], neuronal death remains a significant clinical issue. Although various factors are implicated in the poor sensory outcome [14], the single most important factor is probably the death of primary sensory neurons [6, 15], since quality of sensation relates to the number of innervating neurons and the size of their sensory fields [8].

Exogenous neurotrophic factor administration is partially neuroprotective after experimental PNS and

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CNS lesions, and neurotrophic factors have been a leading therapeutic target in the prevention of neuronal death [3]. One way to improve neuronal survival might be to re-establish neurotrophic support from the periphery as soon as possible by early re-connection of proximal and distal nerve ends by primary repair or by nerve grafting [15]. It is important to know the fate of primary sensory neurons after the surgical repair of their peripheral processes to test the hypothesis that the extent of functional recovery is dependent on the anatomical nature of the injury. There are some reports about the number of DRG neurons after surgical nerve repair [15, 16] but no comparative studies have been undertaken to assess ultrastructure and morphology of these neurons following surgical repair.

The aims of this study were therefore to confirm whether cells in the adult lumbar DRG die by apoptosis after nerve transection and if the surgical repair can prevent this process. To achieve hese aims, we have identified sensory neurons at the fluorescence microscopic level using immuno-histochemistry techniques followed by subsequent Hoechst 33342 nuclear staining. Confirmation of the presence of apoptotic neurons in the axotomy and repaired groups was achieved morphologically by electron microscopy.

MATERIALS AND METHODS

Animals and surgical procedures. Male Wistar rats (n = 36, weighing between 200-300 g) of the experimental and control groups were anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitonealy and their left sciatic nerves were exposed in midthigh. In axotomy group (n = 9), a 9- to 10-mm portion of nerve was excised. Spontaneous regeneration was prevented by ligating both nerve stumps (5-0 nylon). In direct epineurial suture group (n = 9), the sciatic nerve was divided and then repaired immediately using 10-0 nylon sutures. In autograft group (n = 9), a 9- to 10-mm portion of the nerve was excised and after 180° rotation, sutured to proximal and distal segment. For sham groups (n = 9), left sciatic nerves were exposed in midthigh but were not cut.

Neuronal counting. After 12 weeks, three animals from each group were anaesthetized ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitonealy and pre-fixation was achieved by transcardiac perfusion with 150 ml normal saline then by 200 ml 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. The left and right L5 DRG were removed and post-fixed in 4% PFA then 30% sucrose, both at 4ºC for 24 h. The ganglia were blocked in tissue freezing medium and stored at -80ºC. Each entire ganglion was cut into serial 15-µm cryosections and 1 from 4 sections mounted onto gelatine-coated glass slides and dried overnight. Neuron counts were performed using Hoechst 33342 (Sigma, B2261, USA). Hochest 33342 is a non-specific nuclear stain which allows excellent determination of nuclear morphology. For staining, at first, the slides were washed 3 times for 5 min each in 0.1 M PBS at room temperature. Then, the slides were placed in Hoechst 33342 (1 µg ml^{-1} in PBS) at room temperature for 15 min. At the end, the slides were washed 3 times for 5 min each in 0.1 M PBS at room temperature, mounted under glass coverslips, and viewed by fluorescence microscope (Olympus, AX70, Japan). By a camera (DP11 camera) mounted on top of the microscope, images from the sections were prepared at magnifications of ×100, ×400, and ×1000 and normal neuronal nuclei was counted (Fig. 1A). Neuron loss was calculated by subtracting the number of neurons in ipsilateral ganglion from that in their contralateral controls. Loss was then expressed as a percentage of the neuron count in the control ganglia. All data were expressed as mean ± SEM. The statistical test of one-way analysis of variance (ANOVA) was used for comparison among all groups and unpaired student's *t*-test for each of the two groups. $P<0.05$ was adopted for rejection of null hypothesis.

Immuno-histochemistry. Some sections were stained for phosphor-c-Jun as a marker of sensory neuron axon damage [17] by immuno-histochemistry method. In this method [18], fixed sections containing L5 DRG were incubated with anti phosphor-c-Jun Ser^{63} (1:1000, Sigma, USA) in 0.01M PBS at 4°C overnight, pH 7.4, containing 0.3% Triton X-100. After 3 washes with PBS, the sections were incubated with CY3-conjugated secondary antibody (1:200, Sigma, USA) at room temperature for 3 h. Then, the sections were incubated with Hoechst 33342 (1 µg ml^{-1} in PBS) at room temperature for 15 min to visualize nuclei. At the end, the slides were washed 3 times for 5 min each in 0.1 M PBS at room temperature, mounted under glass coverslips, and viewed by fluorescence microscope (Olympus, AX70, Japan).

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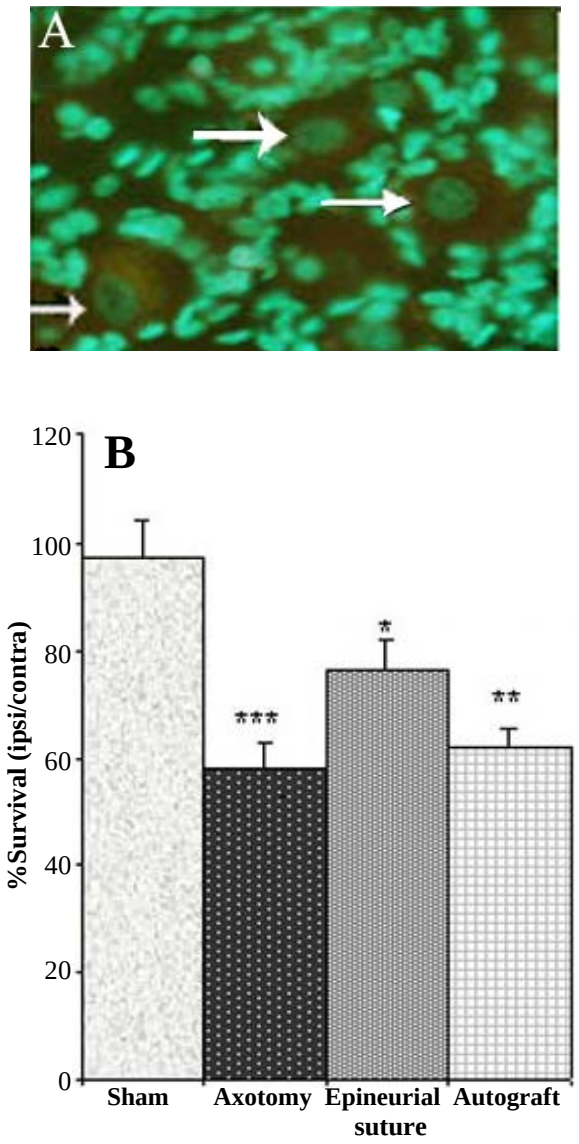

Fig. 1. Hoechst staining and percentage of surviving neurons after the surgery in four groups. **(A)** Hoechst staining of L5 DRG neurons. Arrows show normal nucleuses of DRG neurons (1000×); **(B)** quantitation of surviving neurons from sham, axotomy, epineurial suture, and autograft groups 12 weeks after the surgery. The data are presented as % survival (ipsi/contra) and is the mean ± S.E. $^{*}P<0.04$, $^{**}P<0.001$, $^{***}P = 000$.

Electron microscopy . Three rats from each group were killed 1 and 12 week(s) after operation. They were deeply anaesthetized with ketamine and xylazine intraperitonealy and transcardially perfused with 3.5% PFA and 2.5% glutaraldehyde in 0.1 M phosphate buffer. The left and right L4-L5 DRG were removed, post-fixed in 2.5% glutaraldehyde for a further 2 h and then 1% osmium tetroxide for 1.5 h and dehydrated through a series of acetone solutions and finally embedded in Araldite 502. Semithin and ultrathin sections were cut, semithin sections (500 nm) were mounted on glass slides, stained with toluidine blue and observed with an Olympus Provis light microscope. The ultrathin sections (50-70nm) were mounted on grids, contrasted with uranyl acetate and lead citrate and observed by transmission electron microscope (Zeiss 900 transmission electron microscope, Germany).

RESULTS

The levels of c-Jun immuno-staining in sham DRG were comparable in unlesioned normal rat. The expression of c-Jun was induced in DRG neurons following injury with positive neurons spread randomly throughout the DRG. Axotomy of the sciatic nerve and the interruption of its contact with the target tissue resulted in the upregulation of c-Jun expression in DRG neurons ipsilateral to the lesion. This increased expression was observed for 12 weeks after nerve transection. In animals whose sciatic nerve repaired, there was an increase in the number of neurons exhibiting nuclear c-Jun staining.

In axotomized animals, a loss of 42% of L5 neurons occurred during the12 weeks after nerve transection. At 12 weeks, neuron loss was not affected by sham treatment, while in epineurial suture repair and autograft repair groups, it was 23.7% and 34.1%, respectively (Fig. 1B).

Light microscopic observations. In control animals, semithin sections of the DRG neurons showed a regular shape with large round nuclei and prominent nucleoli. In the axotomy and repair groups, chromatolysis reaction was seen evidently. At 1 week after axotomy and repair, no obvious destructive changes were observed in most of the DRG neurons. In a few neurons, the cytoplasm had slightly darker staining of toluidine blue. At 12 weeks after axotomy and surgical repair, some of the neurons in the DRG neurons displayed dramatic morphological changes. In some of the neurons, many vacuoles were found in the cytoplasm (Fig. 2B and C). For darkly stained neurons, the chromatin aggregated into many irregular dense clumps and the boundary of cytoplasm became obscure (Fig. 2A). These changes decreased in direct epineurial suture repair but no in autograft group.

Electron microscopic observations. In control animals, DRG neurons had round nuclei with evenly distributed chromatin and clear nucleoli (Fig. 3A). No discontinuities were found in cytoplasmic membrane and nucleolemma. In the cytoplasm,

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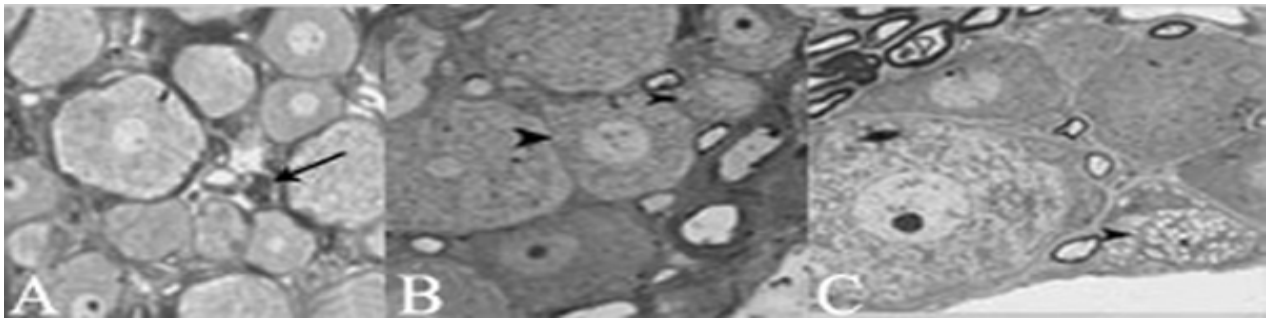

Fig. 2. Semithin sections of L5 DRG 12 weeks after direct epineurial suture repair. Photomicrograph of **(A)** reveal apoptotic neuron (arrow) and **(B)** and **(C)** cytoplasmic vacuolation (arrow head) (1000×).

well-developed rough endoplasmic reticulum (RER) arranged in small parallel stacks; the polyribosomes formed characteristic rosettes; Golgi apparatus contained scattered vesicles and parallel stacks of empty cisterns. Mitochondria had electron dense matrix and narrow intracristal spaces (Fig. 4C and D).

At first week (Fig. 3B and C) and 12 weeks (Fig. 3D-F) after surgery in the axotomy and repair groups, noticeable changes occurred in the nucleus and cytoplasm of some neurons. In a small portion of neurons, the chromatin aggregated into several clumps and the invaginations of nucleolemma became evident indicating the shrinkage of the nuclei. No swollen organelles were found in these neurons. Ultrastructural examination of the ganglia in axotomy and repair groups confirmed that neurons exhibited classical features of apoptosis. Some of the apoptotic neurons were shrunken with nuclei that exhibited masses of condensed and margination of chromatin (Fig. 3B-F) and unravelled nuclear membranes. In some cases, the nucleus was severely shrivelled resulting in pseudopodia formation (Fig. 3F). The cytoplasm condensed and subsequently the cell shrinked in size, while the plasma membrane remained intact. The mitochondria, RER and Golgi apparatus in apoptotic neurons generally appeared normal.

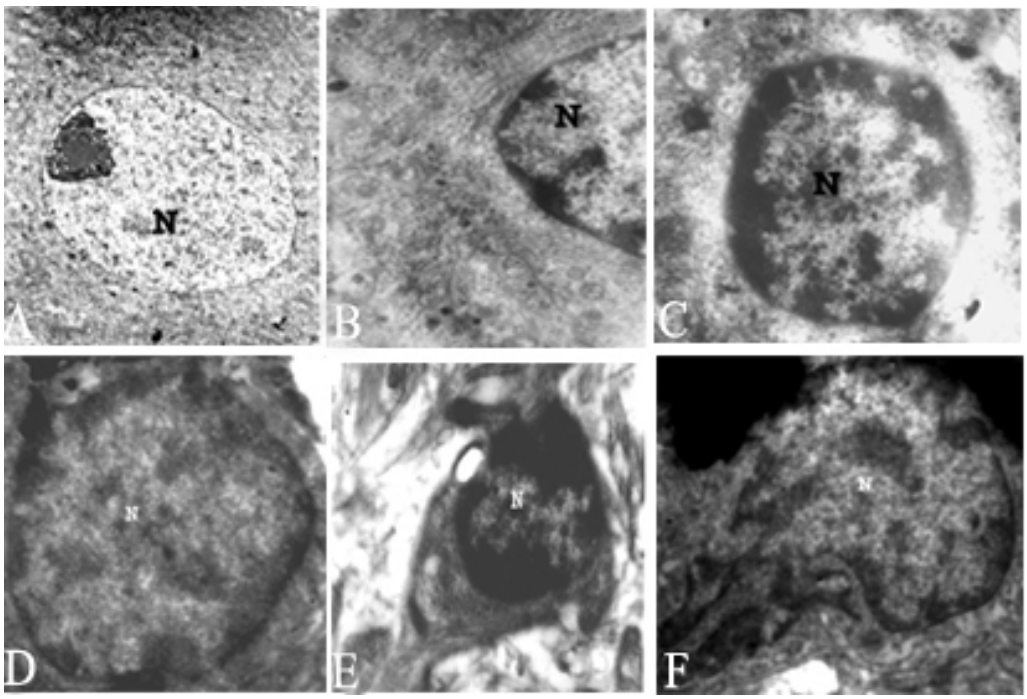

Fig. 3. Electron micrographs of L5 dorsal root ganglion neurons nuclei in axotomy and repair groups. **(A)** normal neuron; **(B)** and **(C)** 1 week and **(D)**, **(E)**, and **(F)** 12 weeks after the surgery. **(B-F)** show apoptotic changes including pyknosis, chromatin condensation and margination. N, nucleus; magnifications were (A), 3000×; (B) and (C), 12000×; (D), 4646×; (E), 6000× and (F) 12000×.

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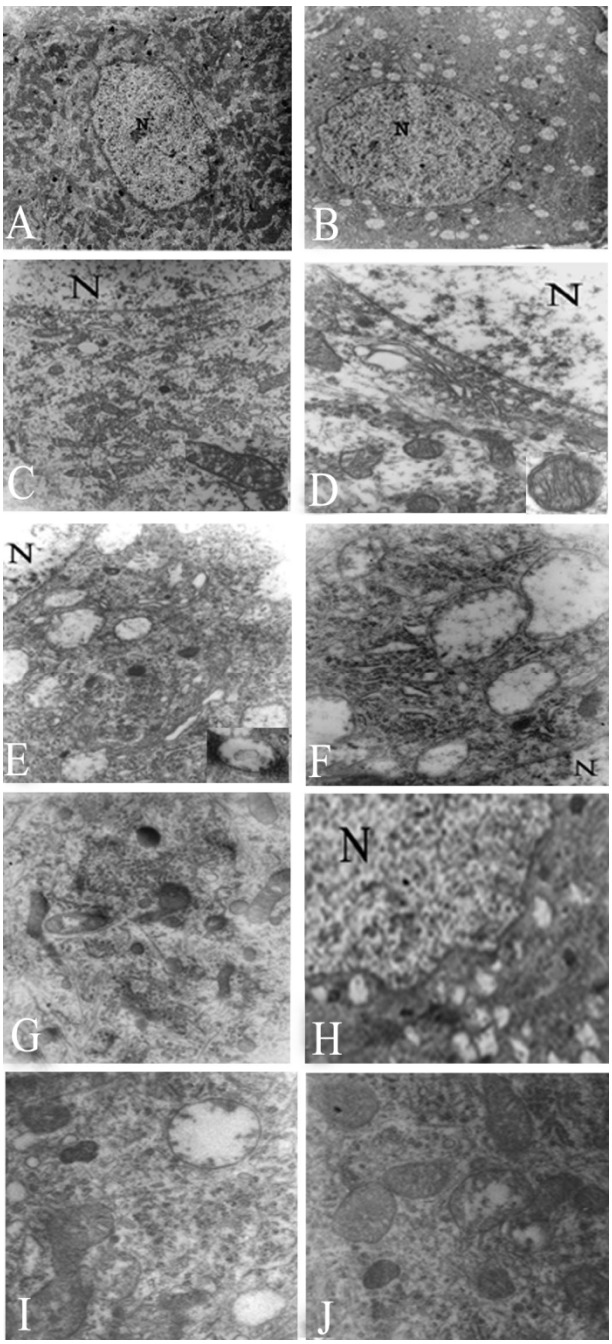

Fig. 4. Electron micrographs of L5 DRG after left sciatic nerve surgery. **(A)** and **(B)**, two neurons after sciatic nerve transection: **(A)** shows mitochondrial accumulation in the cytoplasm and **(B)** shows cytoplasm vacuolation; **(C)** and **(D)**, two neurons in sham group as well as two normal mitochondria (insets); **(E)** and **(F)**, two neurons after 12 weeks nerve transection. Mitochondria (panel E and F plus inset) and RER (Panel F) dilation and vacuolation is showed. These cytoplasmic changes have seen after direct epineurial suture repair **(G)** and **(H)** and autograft repair **(I)** and **(J)**. N, nucleus; RER, rough endoplasmic reticulum. Magnification: (A) and (B) 3000×, (C) 20000×, insect 30000× (D) 7000×, insect 30000× (E) 30000×, insect 30000×, (F) 20000×, (G) 12000×, (H) 12000×, (I) and (J) 24000×.

In some neurons of axotomy and repair groups, at 1 week mitochondrial accumulation in the cytoplasm was observed (Fig. 4A) but at 12 week, the mitochondria and/or endoplasmic reticulum and/or Golgi complex were dilated and vacuolated that left empty spaces (Fig. 4B, E-J). In these neurons, nuclei had normal features (Fig. 4B, E, F and H); also. We did not observe necrotic changes such as cellular and nuclear swelling, loss of integrity of the plasma membrane, and dissolution of the nuclear envelope.

DISCUSSION

The present study showed that after 12 weeks from the surgical nerve repair, sensory neuronal loss decreased. Sciatic nerve transection caused apoptosis as well as other types of cell death in adult L5 DRG sensory neurons and the surgical nerve repair did not prevent these processes.

Nerve injury results in dramatic changes in the transcriptional program of the injured neuron, some of which are mediated by the induction of immediate- early transcription factor of c-Jun [19]. The expression of c-Jun has been shown to increase in DRG cells following transection of the sciatic nerve, and is maintained if the damaged nerve is ligated, but returns to basal levels if the peripheral nerve is allowed to regenerate [20]. In our study, increasing c-Jun transcription factor was demonstrated in neurons axotomized and repaired and is consistent with the observations of cell death in these groups.

During the developmental cell death period, peripheral neurons are absolutely dependent on trophic factors produced by their targets, and those developing neurons that fail to compete successfully for sufficient target support die by apoptosis. In adulthood, peripheral neurons no longer depend on target-derived growth factors for survival [21], and axotomy results only in delayed and restricted neuronal loss [4, 22]. In adults, in spite of central axotomy [23], sensory neurons are more sensitive to peripheral axotomy than motor neurons, probably because they depend more on neurotrophic molecules released by peripheral target organs [15]. Our study showed that direct reconnection of the proximal nerve stump with its distal stump will reduce the sensory neuronal loss from 42% to 23.7%. In the present study, the importance of distal stump and peripheral neurotrophic support was clearly demonstrated.

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The type of cell death is characterized by morphological changes observed at the ultrastructural level [24]. The occurrence of adult sensory neuron death following axotomy [1, 25] and nerve repair [15] has been reported, but the mechanisms by which it may occur have remained controversial. Groves *et al.* [26] showed that at least part of the neuronal loss in DRG that is seen after axotomy is due to apoptosis. In our study, some of the DRG neurons showed classical apoptotical changes including cell shrinkage, an increase in electron density and chromatin condensation towards the periphery, pyknosis and the maintenance of cytoplasmic organelles until late in degeneration. Pseudopodia formation in the nucleus (Fig. 3F) is the beginning of apoptotic body formation from cells exhibiting this morphology [27]. But, dilation and vacuolation of mitochondria, RER and Golgi apparatus in some of the neurons show probably as well as apoptosis other type of cell death has occurred. Cytoplasmic features were not usually associated with classical apoptosis. Pilar and Landmesser [28] observed that the naturally occurring death of ciliary ganglion cells in chick embryos initially involved dilation of RER and/or mitochondria with relatively little changed in the nucleus. Dilation endoplasmic reticulum, Golgi apparatus, and mitochondria as well as a late increase in the granularity of chromatin in some of the neurons in the present study are features of cytoplasmic cell death [29].

In neurons, cytoplasmic cell death has always been reported in embryos and neonatal animals [11, 24, 28, 30], but in the standard description of this kind of cell death, there is dilation of perinuclear space too [29]. We did not observe this phenomenon in our study; furthermore, we did not observe loss of integrity of the plasma membrane and consequently necrosis. As in non-neuronal cells undergoing apoptosis, the morphological integrity of mitochondria is preserved in apoptotic neurons until late in the progression of neurodegeneration [31, 32] because active mitochondria require for apoptosis [33]. It is possible that the cells may be dying by several different mechanisms or that the cell death is a combination of classic apoptosis and cytoplasmic cell death (Clarke's type non-lysosomal disintegration) [29]. Following peripheral nerve injury, there is a disruption in the supply of retrogradely transported neurotrophic factors, leading to neuronal cell death [3]. Deprivation of retrograde support during the period of naturally occurring cell death can provoke apoptosis [26] or cytoplasmic cell death [11].

Neuronal survival is the first challenge after axotomy and its success depends on a number of intrinsic and external factors [34]. Our study showed destructive changes in neuronal cell bodies decreased in direct epineurial suture repair but no in autograft repair group. The best results for the return of function in the rat peripheral nerve axotomy models, compared with bridge operations such as autograft, will occur when the nerve ends suture together [35] and surgical repair of the proximal nerve stump with its distal stump will reduce the sensory neuronal loss to 50% [16] or lesser than 50% (present study). Some results have indicated that sensory neurons retain their ability to regenerate for at least 8 weeks after nerve injury [36] but early [16] and delayed [36] reconstruction of injured with peripheral nerve grafting fail to rescue neurons in DRG.

In conclusion, the present results showed that after peripheral nerve transection and the surgical nerve repair, both apoptosis and probably cytoplasmic cell death can occur, and the surgical nerve repair did not prevent nuclear and cytoplasmic destructive changes. Our data showed that type of sensory neuronal cell death did not relate to reconnection of the proximal nerve stump with its distal stump.

ACKNOWLEDGMENTS

This research study was financially supported by a grant from Iran University of Medical Sciences (Tehran, Iran). The authors acknowledge the cooperation of the Cellular and Molecular Research Centre of Iran University of Medical Sciences (IUMS) and the Anatomical Sciences Research Centre of Kashan University of Medical Sciences (KAUMS, Kashan, Iran).

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