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Research Article

THE EFFECTS OF DEPRENYLONSYNAPTIC ZONE AFTER SPINAL CORD INJURY IN RATS

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ABSTRACT
In order to distinguish spinal cord injury (SCI) caused by compression model, we demonstrated ultrastructural and morphometric changes in synaptic lesion after SCI. 72 female Sprague Dawley rats weighining 250-300g, were randomly divided into four groups (N =18). The sham groups were only subjected to laminectomy. All administered 2.5 mg/kg Deprenyl (CIPLA/ India, dissolved in 1cc saline) or equal volume of saline. At the time of operating for sham or SCI surgery groups daily injection were done and continued till they were sacricfied: group A: SCI + Deprenyl, group B: SCI + vehicle, group C: Sham + Deprenyl, and group D: Sham + vehicle. In every group, after 1, 2, 4weeks 6 animals were sacrificed. SCI caused motoneuron decreased in Anterior horn of spinal cord with hole and hemorrhage. Deprenyl decreased this process ($P \leq 0.05$). The most pattern of synaptophysin in group A which received Deprenyl, was cytoplasmic ($P \leq 0.05$). Synaptic and neuronal mitochondria changes were analyzed in a blinded manner for qualitative ultrastructural changes. By SCI, pathological changes including irregularity of the synaptic membrane and synaptic cleft with displacement of synaptic vesicles and irregularity of mitochondria were seen beside synaptophysin reduction. Deprenyl decreased pathological changes and maintained motoneurons after SCI.

Keywords: *Deprenyl, Spinal Cord Injury, Synapse, Rat*

INTRODUCTION
Spinal Cord Injury (SCI) causes a majority of disability and costly human condition with worldwide incidence of 10-40 cases per million. Following SCI tissue damage accurred, neurobiological disability depend on mechanical pressure such as vertebrae dislocation, compression or vertebrae traction. Induce of spinal cord injury (SCI), neurons have been reported to undergo cell death (Nielson *et al.,* 2011) where apoptosis was confirmed by using ultrastructural study (Wong *et al.,* 2012) and TUNEL (Loo, 2011). The molecular changes were characterized by an increase in pro-apoptotic gene expression such as Bax (Nickells *et al.,* 2008) or activation of caspase 3 (De-Bilbao *et al.,* 2000). These studies were used to define the type and structure of the dead cell. Other investigators tried other approaches to characterize the histo-functional feature of apoptotic cells, especially in synaptic zone. Findings were reported about the expression of synaptophysin, as a membrane protein in synaptic vesicle filled with neurotransmitters. When an action potentials depolarizes the presynaptic plasma membrane, Ca^{2+}-channels open, and Ca^{2+} flows into the nerve terminal to trigger the exocytosis of synaptic vesicles, releasing their neurotransmitters into the synaptic cleft. Pathological changes this process lead to neurodegenerative dieases (Shojo *et al.,* 2006). The efficacy of some drugs have been studied in various models of SCI. Results show monoamine oxidases type-B inhibitor (MAOB-Is) Selegiline and (-) Deprenyl, used in Parkinson dieases have a lot of pharmacological activities beside its MAOB inhibitory potency, can protect neurons from neural degeneration, mitochondrial impairerment, oxidative stress, enhances the synthesis of neurotrophic factors and anti-apoptotic Bcl-2 protein family (Tatton *et al.,* 1996; Maruyama *et al.,* 2013). In present study the effects of Deprenyl on motoneuron survival, pattern of synaptophysin expression and ultrastructural of synapse were evaluated. Morphometric parameters were used to evaluate the trend of changes quantitatively and patterns of synaptophysin were demonstrated by immunohistochemistry.

MATERIALS AND METHODS

All experimental protocols of this study were approved by the animal care committee of Shahed University in accordance with the policies established in the guide to the care and use of experimental animals prepared by animal care and all efforts were made to minimize the number of animals and their suffering. 72 female Sprague Dawley rats (Razi Institute, Karaj/ Iran) weighing 250-300g (10-12 weeks old) were housed in an air-conditioned colony room on a 12/12 light/dark cycle (22-23°C and 30-40% humidity) three to four per cage with standard pelleted diet and tap water add libitum for at least 10 days before the beginning of experiment. Experimental group: After anesthetizing the animals (ketamine 100mg/kg/xylazine 5mg/kg, ip) and exposure of the spinal cord through laminectomy, an extradural compression was applied at T6 level for 1minute by bilateral position oriented by Kerr Loughed aneurysm clip applicator incision. Cephazoline and 5-8 ml Lactate serum was administered intraperitoneally to replace the bleeding and the animals were maintained at an ambient temperature of 25-27°C. The applied compression on the spinal cord resulted in a moderately severe SCI with initial complete paraplegia followed by a delayed partial recovery of hindlimbs (Joshi and Fehling, 2002) Manual compression of bladder was performed three times daily and all efforts were done to prevent bladder infection and any injury due to the sensory disorders. The 72 animals were randomly divided into four groups (N=18). The sham groups were only subjected to laminectomy without any compression applied on the cord. All administered daily 2.5mg/kg Deprenyl (CIPLA/ India, dissolved in 1cc saline) or equal volume of daily saline as the vehicle at the time of operating for the sham or SCI surgery, it continued once a day till they were sacrificed: group A: SCI + Deprenyl, group B: SCI + vehicle, group C: Sham + Deprenyl, and group D: Sham + vehicle. In every group after 1, 2, 4weeks of surgery animals (N=6) were sacrificed and the T6 segment of the spinal cord was transferred in fixative according to their protocol as: (N=3) for motoneuron counting and immunohistochemical technique, (N=3) for ultrastructural studies. For tissue preparing, the spinal segments T6 were removed by laminectomy and the tissues were proccessed for paraffin embedding, eight micrometr serial sections (T6) were taken from the spinal segment and stained with Cresyl violet. Spinal motoneurons counting was done according to (Li *et al.*, 1998), and five micrometer sections (T6) of the animals, labeled with mouse anti-synaptophysin antibody (Chemicon International). The sections were treated with 3% hydrogen peroxide in order to inhibit endogenous peroxidase then treated with diaminobenzidine 0.05% in tris buffer (pH 7.4). Synaptophysin immunolabeled motoneurons in the ventral horn were classified according to the pattern of immunoreactivity of anti-synaptophysin antibody (Tiraihi *et al.*, 2004) which is briefly: 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 anti-synaptophysin 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. 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 T-test and analysis of the variance was used to compare the difference among and within the groups. For ultrastructural study, the spinal cord tissues 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 (30 μm) were cut, stained with uranyl acetate and lead citrate and examined under Ziess EM 900. 2 independent investigators were instructed to examine the synaptic area and neuronal mitochondria. Investigators using an objective grading system for abnormalities in synaptic membrane regularity and synaptic vesicle or synaptic cleft, mitochondrial distribution or shape, matrix density, cristae shape, and appearance of any abnormal structures compared with matched sham controls; each finding was indicated as mild, moderate, or severe depending on its frequency.

Statistical analysis: All statistical analyses were performed using SPSS software (version 10.0 SPSS Inc). Data were showed the mean & SD. Intergroup and among group differences were analysed by t-test and one-way ANOVA. These parametric statistical tests were used normally distribution.

RESULTS AND DISCUSSION

With the use of Nissl staining we have found that chromatolytic motoneurons. The percentage of the survived motoneurons decreased in SCI groups, compare to Sham groups. These reduction are as: Group A to C: for first week 30.8%, second week 42.08%, fourth week 27.74%. Group B to D: for first week 74.10%, second week 76.76%, fourth week 84.12% ($P \leq 0.05$). Comparison between group A and group B which were as SCI groups reveals motoneurons decreased in group B and this is elevated by weeks (Table 1). Percentage of reduction are as: group B to A: first week 62%, second week 59.8% and fourth week 78%. It means chromatolytic motoneurons was significantly higher in group B compared to group A ($P \leq 0.05$). Results show significant differences in both of group A and group B in survived motoneurons for different weeks ($P \leq 0.05$) and it is not seen in group C and D (Table 1).

Table 1: Mean and SD of Motoneurons (T6 segment) and percentage of cell count changes according to weeks in each group

Mean and SD of Motoneurons based on sampling in groups

Weeks & percentage of changes / Group	1	2*	4**
A	**628.33±20.21**	**526.67±20.82(-16.1%)**	**666.67±5.77(+20.9%)**
B	**234.33±21.36**	**211.67±15.28(-9.6%)**	**146.67±12.58(-30.7%)**
C	**908.33±7.64**	**910.00±5.00(+0.18%)**	**922.67±7.51(+3.3%)**
D	**903.33±17.56**	**913.33±20.82(+1.09%)**	**926.67±5.77(+1.4%)**

**comparing first week to second week*
***comparing second week to fourth week*
(- show decreased +show increased)

Synaptophysin immunolabeling results are demonstrated in figures 1 representing different pattern of labeling (intact, partial, cytoplasmic and negative).

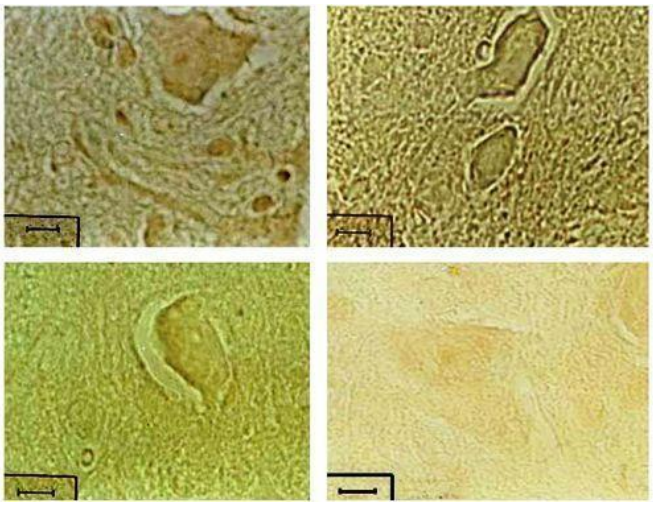

Figure 1: A photomicrograph shows anti-synaptophysin immunoperoxidase labeling of spinal cord motoneurons after SCI in rat with intact pattern (up right) where well preserved synaptophysin immunoreactivity present around the soma, partial pattern (up left) where the immunoreactivity less than two third of the soma and negative pattern (down right) with no immunoreactivity present in the motoneuron and cytoplasmic pattern (down left) where immunoreactivity are as homogeneous cytoplasmic (scale bar =25 µm)

The percentage of the patterns of synaptophysin-labeled motoneurons indicate in group (A, B, C and D) in weeks (1, 2 and 4) have no significant differences. For intact pattern comparison between two groups A

and B (as SCI), C and D (as Sham) have no significant differences (Table2). Comparison between group A to C, and group B to D have significant differences (P≤0.05) in groups C and D are significantly higher than that of two other groups A and B (P≤0.05).

Percentage of reduction synaptophysin intact pattern in group A compare to group C is 99.8% and B to D is 99.95%. Partial pattern of synaptophysin-labeled motoneurons comparison between groups A (as SCI), C and D (as sham) have no significant differences (table2), but comparison between group A and B (as SCI) have significant differences (P≤0.05) with 61.86% reduction in group B.

The cytoplasmic pattern of synaptophysin-labeled motoneurons comparing between groups B (as SCI), C and D (as sham) have no significant differences. As though comparison between group A (as SCI) with B (as SCI), C and D groups (as sham) has significant differences in weeks, as it is increased (46.5%).

Negative pattern are only seen in group A and B, with significant differences between these two groups. It is increased in group B (139%).

On the whole partial and cytoplasmic patterns are significantly higher at the group A compared to the group B (p≤0.05). However, negative pattern are significantly higher in group B (Table 2). Following SCI there was a decreased percentage of survived motoneurons paralleled by an increased percentage of neurons with partial and cytoplasmic synaptophysin pattern (Table 2).

Table 2: Mean and SD of percentage of synaptophysin pattern in motoneurons (T6 segment) in groups by weeks

Percentage of Synaptophysin pattern

Group & Week for sampling / Pattern		Intact	Partial	Cytoplasmic	Negative
A	**1**	**0.09±0.01***	**0.24±0.03****	**0.37±0.07*****	**0.30±0.10******
	2	**0.08±0.04***	**0.25±0.04****	**0.48±0.07*****	**0.29±0.07******
	4	**0.07±0.03***	**0.22±0.04****	**0.44±0.03*****	**0.27±0.05******
B	**1**	**0.03±0.00**	**0.10±0.02****	**0.16±0.04*****	**0.64±0.10******
	2	**0.01±0.02**	**0.09±0.03****	**0.28±0.02*****	**0.73±0.18******
	4	**0.02±0.02**	**0.09±0.04****	**0.25±0.11*****	**0.64±0.17******
C	**1**	**0.50±0.05***	**0.31±0.05**	**0.19±0.01**	**0.00±0. 0**
	2	**0.47±0.02***	**0.29±0.03**	**0.23±0.04**	**0.00 ±0.0**
	4	**0.52±0.03***	**0.24±0.04**	**0.24±0.05**	**0.00±0.0**
D	**1**	**0.41±0.03**	**0.32±0.09**	**0.20±0.09**	**0.00±0. 0**
	2	**0.44±0.04**	**0.35±0.02**	**0.21±0.06**	**0.00±0.0**
	4	**0.40±0.08**	**0.26±0.07**	**0.29±0.02**	**0.00±0.0**

**Comparison between group A to C for intact pattern(P<0.05)*
***Comparison between group A to B for partial pattern(P<0.05)*
**** Comparison between group A to C for cytoplasmic pattern(P<0.05)*
***** Comparison between group A to B for negative pattern(P<0.05)*

Ultrastructural studies showed, SCI induces, displaced and dispread synaptic vesicles in presynaptic region at synapses with mithocondriavesiculated. Irregularities of synaptic membrane and swollen mitochondria, which are moderately to severely swollen by the weeks, and increased mitochondria matrix density with collapsed synaptic cleft (Figure 2), Increasing numbers of round structures of the same size as intact mitochondria with poorly defined cristae which might be associated with presynaptic and postsynaptic membranes detachment (Figure 3), and low electron density in the synaptic active zone, which might be ensheathed by astrocytic processes (Figure 4). As the whole comparing animals belong to 1, 2 and 4 weeks in group B changes are most visible after 4 weeks. These changes are less visible in group A according by week. The synaptic region and mitochondria in group C and D (as sham) were intact.

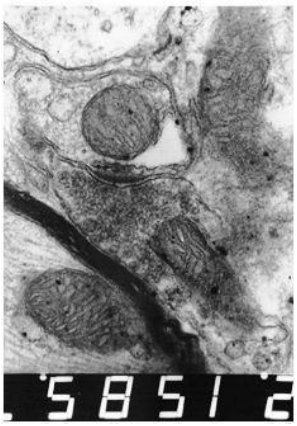

Fig2-Electron micrograph of a synaptic region from spinal cord after SCI. Vesiculated mitochondria with increased mitochondria matrix density. Irregularity in synaptic cleft with collapsed synaptic cleft. 3000X

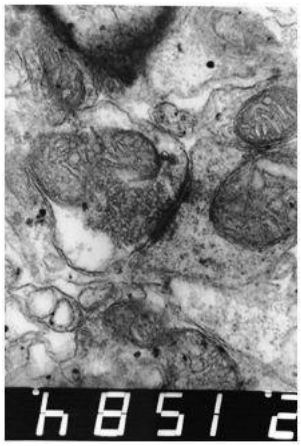

Figure 3: Electron micrograph of a synaptic region from spinal cord after SCI. which shows synapse with irregularity in pre and post synaptic membrane. Mitochondria vesiculated and some round structures of the same size as intact mitochondria with poorly defined cristae. 3000X.

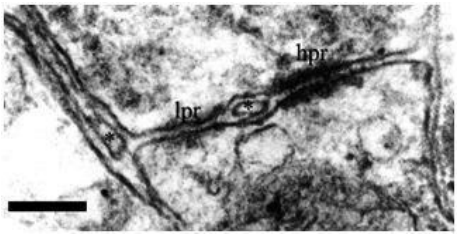

Figure 4: An electron micrograph of synapse in a motoneuron from the spinal cord after SCI, which shows a perforated synapse with low electron density presynaptic zone (lpr: type 1 lesion) of the synapse while other region of the synapse shows high electron density (hpr: type 2 lesion). The astrocytic process ensheathment can be seen in type 1 lesion (*) (scale bar = 0.22 µm) or 5000X

DISCUSSION

SCI models such as: weight-drop, contusion and compression have been well established and used for SCI in animal model (Krishna *et al.,* 2013; Poon *et al.,* 2007). As shown in this study, the morphological, histological and ultra structure pathological alterations in SCI compared to that of sham, reproduced and validated previous observations in the compression and other SCI models. Our findings indicated SCI causes a series of fluctuant changes, morphometric analysis of the data showed a progressive decrease in

number of survived motoneurons after SCI. The results are consistent with the results of Rongand *et al.*, who reported SCI causes motoneuron death and it reaches maximum second or even third day after truma (Rong *et al.*, 2012) between the second and third weeks, maximum improvement occurs (Rahimi-Movaghar *et al.*, 2013). A cascade of cellular and inflammatory process lead, primary to progressive secondary damage (Karalija *et al.*, 2012). Apoptosis significantly triggered in secondary damage after SCI, because of mitochondrial changes (Green *et al.*, 1998). Our results indicated SCI cause motoneuron death. After first week survived motoneurons are about 20%, after 4 weeks, about 10% (comparing group B to D). Deprenyl reduce this reduction as in table 1 is shown. It seems Deprenyl can maintaining motoneuron in SCI group about 60%. Comparison between second and fourth weeks, which were treated by Deprenyl showed slightly up regulation, about 10%, it means after 2 weeks post injury 10% survived motoneuron increased. Since motoneuron has been increased neurorescue effect rather than neuroprotective effect of deprenyl has been suggested. Some other findings supported neurorescue effect beside neuroprotective after injury (Al-Nauaimi *et al.*, 2012; Knoll, 1987), these finding demonstrated secondary changes followed primary, due to injury. Functional role and neuropathological changes cause tissue damage through both direct and indirect mechanisms (Ansari *et al.*, 1993). Mechanical injuries cause necrosis in two step, primary and secondary. Neurons directly affected by the force of impact (primary injury phase), then a secondary injury phase is characterised by oxidative and nitrosative stress (Fitzgerald *et al.*, 2010), free radical formation (Vaziri *et al.*, 2004), peroxynitrite and lipid peroxidation, membrane lipids and protein changes, cell homeostasis (Bains *et al.*, 2012). Immunohistochemical study in this study confirmed, Synaptophysin immunoreactive cells decreased in SCI groups. It indicate SCI affect on synaptophysin as a protein in synaptic vesicle, beside loss of cell. The percentage pattern of intact (99.95%) and partial pattern (60.0%) decreased in injured neurons induce of SCI, in spite of negative pattern which is most seen. Although in intact groups most percentage pattern are intact (50%), partial (25%) and cytoplasmic (25%). Deprenyl treated group partial pattern increased as the same as control and the most pattern of synaptophysin induce of deprenyl is cytoplasmic (43%). It means deprenyl mediate neuron from death and these neuron demonstrated synaptophysin as cytoplasmic (43%) and partial (23%). Synaptophysin data showed that due to Deprenyl percentage of negative pattern declined. Chromatolytic motoneurons with the partial pattern demonstrate loss of presynaptic terminals or cell death (Cruz-Sanchez *et al.*, 1996). The negative pattern has been reported in chromatolytic motoneurons (Taraihi *et al.*, 2004). The findings of this investigation are consistent with a previous, which revealed that Deprenyl increased survival motoneuron by inhibiting death receptor expression (Heshmati *et al.*, 2013). Decline in synaptophysin is an important indicator of synaptic stripping or synaptic contact loss (Alvarez *et al.*, 2000). Surviving is focused on synapse and neurotransmitter transferring (Yi *et al.*, 2006). Changes in synaptophysin are consistent with the progressive decline in the percentage of survived motoneurons, a finding reported also by other groups (Yan *et al.*, 1993; Jacobsson *et al.*, 1998; Goettl *et al.*, 2003). However, following traumatic injury, plasticity change is considered as the most important event following neuronal injury (Palkovits, 1995; Miller *et al.*, 1999), reported an increase in synaptophysin immunoreactivity as a result of enlargement of synaptic boutons and accumulation of synaptophysin, While Havton and Kellerth (2001) suggested that the presence of synaptophysin in the cytoplasm maybe due to degenerative changes, however in this study, the statistical difference in the cytoplasmic and partial pattern between the SCI and Sham groups was insignificant. The intact pattern at the SCI groups were significantly lower (approximately one third) than that at the Sham group, and the negative pattern at the SCI groups were significantly higher than those at the Sham groups, these findings revealed that there is progressive degeneration of motoneurons in long standing SCI. On the other hand, the intact pattern of synaptophysin immunoreactivity at SCI was not significantly different from that of the Sham group, parallel with other findings (Tiraihi *et al.*, 2004; Havton, 2001), which may be 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. As mentioned in our results ultra structural changes in synaptic region has occurred. These changes are as displaced, dispread synaptic vesicles in

presynaptic region, irregularities of pre and post synaptic membrane, collapsed synaptic cleft with mithocondriavesiculated and swollen, increased mitochondria matrix density with poorly defined cristae and low electron density in the synaptic active zone, which might be ensheathed by astrocytic processes. All of these changes demonstrated variability in transferring data or neurotransmitter from cell to cell according to SCI in several weeks. The changes of neurons and axons membrane excitability has been associated to neuronal degeneration in animal models (Beck, 2011; Moldovan, 2009). Extracellular matrix act as important role. Synaptic structure beside homeostasis of the extracellular matrix are critical point of docking in cell signaling, as it is showed is the most important factor for cell survival (Gerst, 1995; Benarroch, 2013). Imbalancing calcium ions cause neuronal death (Panayiotidis *et al.*, 2006). In contrast, regulation of sodium and potassium channels as well as AMPA receptors exert an anti-apoptotic effect (Orlov *et al.*, 2001; Jemmerson *et al.*, 2005). Beside these investigation, mitochondria play an important role in intrinsic apoptotic pathway (Iverson *et al.*, 2004) by cell homeostasis maintaining (Green *et al.*, 1998), by regulating signaling pathways (Bienertova-Vasku *et al.*, 2013)and cell signaling (Putcha *et al.*, 2000). So we evaluated mitochondria structure, results showed SCI causes mitochondria changes. As other results indicated, apoptosis-related alterations in mitochondrial functions (Brooks *et al.*, 2007). Following a death signal, the pro-apoptotic members target and integrate mitochondrial outer membrane (Roy *et al.*, 2013) it is may be form selective channels for cytochrome *c* release and potentially other factors from the intermembrane space such as AIF (Lorenzo *et al.*, 1999). Some channels have been proposed lead to mitochondrial swelling without rupture of outer mitochondrial membrane (Vander-Heiden *et al.*, 1997). Changes mitochondrial compartments (intermembrane space, crista shape and intracristae space) are involved in the death pathways (Zick *et al.*, 2009). One other theory reported Bcl-2 and Bcl-XL inhibit mitochondrial fragmentation and proposed to control the swelling of mitochondria by proposing channel entitled the permeability transition pore (PTP) (Zamzami *et al.*, 1998). Alternatively, BCL-2 family members may represent or regulate smaller ion-selective channels. Calcium inhibits the $BCL\text{-}X_L$ channel activity in vitro (Keinan *et al.*, 2013) Science (-) - Deprenyl (selegiline) has been indicated, protect neurons through mitochondrial apoptotic cascade and induction of prosurvival antiapoptotic Bcl-2 and neurotrophic factors (Naoi *et al.*, 2009) well-known Deprenyl has complex mechanism of action which is not related to its MAOB inhibitory, potency as neroprotection by decreasing the formation of reactive oxygen radical (Magyar *et al.*, 2004). It is supposed in this study it act as a neurotrophic factor and has neuroprotective effect on maintaining synaptic zone as the same as intact, pre and post synaptic membrane, synaptic cleft, mitochondria shape and structure. This is consistent with the findings which reported deprenyl, rasagiline and (-) deprenyl, monoamine oxidase type B inhibitor (MAOB-Is), are the most promising candidate neuroprotective drug. Deprenyl possesses a wide range of pharmacological activities with a complex mechanism of action. In this study a further destruction in mitochondria was reported, which reveals that the trend of neuronal loss continued but at a slower rate. This means that most of the motoneurons sacrificed 4 weeks post-SCI showed mostly degenerative changes rather than cell death when compared with those evaluated at early stages as has been reported by Chen erlier (Chen *et al.*, 2013). Another interesting finding was that some astrocytic processes were covering the portion of synapses. Moreover, reduction in electron density at the active zone, of the synaptic lesions were presented with displaced synaptic vesicles. This may indicate that displacement of synaptic vesicle preceded the loss of electron density at the active zone, which may precede the astrocytic ensheathment.

Conclusion

SCI causes motoneuron death and deprenyl reduce this process. In synaptic vesicle deprenyl has most 0affected on cytoplasmic pattern of Synaptophysin and has no effect on intact pattern. Deprenyl maintaining synapse structure and mitochondria.

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