The Effect of Timing of Decompression on Neurologic Recovery and Histopathologic Findings After Spinal Cord Compression in a Rat Model

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Abstract- Prior animal models have shown that rats sustaining 3-second immediate spinal cord compression had significantly better functional recovery and smaller lesion volumes than rats subjected to compression times of 1 hour, 6 hours, 3 weeks, and 10 weeks after spinal cord injury. We compare locomotor rating scales and spinal cord histopathology after 3 seconds and 10 minute compression times. . Ten rats were assigned into two early (3-second) and late (10-minute) compressive surgery groups. Compressive injury was produced using an aneurysmal clip method. Rats were followed-up for 11 weeks, and behavioral assessment was done by inclined plane test and tail-flick reflex. At the end of the study, the rats were sacrificed, and spinal cord specimens were studied in light and EM. Basso, Beattie and Bresnahan (BBB) locomotor rating scales were significantly better in the early compression group after the 4th week of evaluation (P<0.05) and persisted throughout the remainder of the study. Histopathology demonstrated decreased normal tissue, more severe gliosis and cystic formation in the late group compared to the early group (P<0.05). In EM study, injuries in the late group including injury to the myelin and axon were more severe than the early compression group, and there was more cytoplasmic edema in the late compression group. Spinal cord injury secondary to 3-second compression improves functional motor recovery, spares more functional tissue, and is associated with less intracellular edema, less myelin and axon damage and more myelin regeneration in rats compared to those with 10 minutes of compression. Inclined plane test and tail-flick reflex had no significant difference.

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Introduction

Spinal cord injuries (SCI) are among the most disabling conditions not only to the individual but also to patient's families.(1,2). Despite immense spinal cord injury research, the prognosis remains poor in terms of neurologic recovery after an acute SCI (3-5). Neurological status after SCI primarily depends on the extent of injury to neural tissues (6). Following the primary mechanical injury to the spinal cord, a complex cascade of secondary injuries such as tissue edema, ischemia and inflammatory processes (7-9) result in further neural damage, apoptosis (6) and reducing endogenous recovery (1). Spinal cord decompression and adequate vascular perfusion preservation to the injured spinal cord tissue are the two important strategies believed to be effectual on neurological outcome (10). Although the importance of restoring spinal stability is well established (11,12), the timing of surgical intervention is still debated (3-5,11-14). Time

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frames of surgical intervention differ in human and animal models. Early decompressive surgery in human SCI is usually referred to as surgical intervention within the first 24 hours after injury versus early decompression in animals is referred to decompression at seconds our minutes after injury (15). In animal studies, it has been shown that neurologic recovery depends on the timing of decompression and significantly more favorable results have been seen after an early decompressive surgical intervention (3,11,13,16).

In our previous study (3,17), SCI compression was performed on rats using an aneurysmal clip compression technique. Our results showed that early SCI decompression resulted in less tissue damage compared to late decompression in terms of measurement of the injured spinal cord surface on axial spinal cord sections stained with hematoxylin and eosin (H&E) and better recovery of normal behavior as defined by Basso, Beattie and Bresnahan locomotor rating scales (BBB) (18). In this study, we investigate two additional questions. First, 10-minute SCI compression was investigated as an intermediate time point between 3-second and 1-hour decompression in terms of both sensorimotor recovery and SCI lesion volume. Second, electron microscopic evaluation was utilized to explore axonal injury, myelination, and post-injury integrity of the nucleus, mitochondria and endothelium.

Materials and Methods

The study was conducted at Research Centre for Neural Repair, University of Tehran, during September to December 2010.

Rats

A total of 12 adult female rats (*Ratus norvegicus wistar*) weighing between 200 and 300 grams were obtained from the animal facility at Tehran University of Medical Sciences (TUMS). The rats were assigned into three groups. Five rats were put into a group receiving early decompression after (3 seconds) compressive injury of the spinal cord. In another group, five rats were decompressed after 10 minutes (late compression) and one rat underwent sham treatment. On the injury day, one rat in the late compression group suffered from severe hind limb bleeding and was sacrificed; hence another rat was used to ensure equal sized groups.

Compression method

Methods of surgery and anesthesia and care of the rats have been mentioned in our previous study (3). Briefly, rats were anaesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Fascia and paravertebral muscles were gently dissected until the lamina and transverse processes of T9 were exposed. Laminectomy was carried out, and spinal cords of rats were exposed and compressed extradurally by an aneurysm clip [Yasargil aneurysm clips FE716K] at the T9 level. The spinal cord decompression was performed at 3 seconds in the early group and 10 minutes in the late group by removing the aneurysm clip. The sham rat was anesthetized and underwent laminectomy without spinal cord compression. After surgery, intraperitoneal normal saline (10 cc) was injected twice daily for a week, and prophylactic antibiotics (Gentamicin sulfate 1 mg/kg and cephazolin 75 mg/kg) were administered twice daily. Manual bladder emptying was performed three times a day in the first week after injury and thereafter twice daily.

Neurological assessment

The neurological results after SCI were assessed on the 1st, 4th and 7th day after injury; further assessments were done weekly and continued for 11 weeks. The BBB (18), TFR (19,20) and inclined plane test (21) were used in the neurological evaluation of rats. In BBB scoring system, motor function is rated by strength and positioning of the hind limbs. The score ranges from 0 for a completely paralyzed animal without any movement to 21 for a normal, healthy, walking animal. TFR is a flexor withdrawal reflex that functions even in decerebrated rats with intact spinal cord function. It was performed by pinching the rats tail and the movement was assessed. The inclined plane test performed by placing the rat on an adjustable inclined plane to provide a slope of varying grade and the maximum angle at which the rat can maintain its position without falling could be assessed (21). Functional outcome of the rats was measured by this test once during the study period.

In every assessment session, two examiners evaluated each rat individually. Each rat was weighed and assessed separately for 4 minutes in an open area; the behavioral recovery is presented as the mean BBB score for the hind limbs and the presence or absence of the TFR was recorded. Whenever two BBB scores differed between examiners, the average score was recorded.

Pathology

After 11 weeks, rats were anaesthetized by an

intraperitoneal injection of ketamine (100 mg/kg) and Xylazine (10 mg/kg) and were sacrificed by intracardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.35). Spinal cords were dissected out and sections containing at least 1.5 centimeters rostral and caudal from the injury site were resected. The sample cords were fixed in glutaraldehyde 2.5% solution.

From each group, tissue samples were obtained in axial sections at the site of the injured spinal cord for electron microscopy and two additional (caudal and rostral to the injury site) sections were used for the light microscopy. An 1×1 mm specimen, containing both gray and white matter was trimmed from the injury site of the same specimens for electron microscopy preparation. The tissue pieces were stored in the 2.5% glutaraldehyde fixation solution (*p*H=7.4) for 24 hours. Then, the samples were washed in phosphate buffer saline and post-fixed in 1% osmium tetroxide (*p*H=7.4). Ultrathin sections of samples, 70-100 nm thickness, were stained with uranyl acetate and lead citrate.

For light microscopy assessment, spinal cord specimens were fixed overnight in 4% paraformaldehyde, dehydrated in graded ethanol solutions, immersed in xylene and embedded in paraffin. Serial 5 µm cross sections, separated by 50 µm, were cut from areas caudal and rostral to the lesion's center using a vibratory microtome. H&E staining was performed for all sections. ImageJ (an open source java based program developed by NIH) (22) was used to measure spared tissue, using pathological features like cystic cavitation and gliosis as well as the total spinal cord area with area selection and analysis feature of Image J (23). The percentage of spared tissue of spinal cord was calculated by dividing the area of normal appearing tissue over the entire tissue in the most injured cross section of the spinal cord (24).

Statistical analysis

Statistical analysis was done by GraphPad Prism 5.0. A two-way ANOVA test was used for the analysis of BBB scores and Mann-Whitney U test for analyzing histopathology findings.

Results

Behavioral assessment

Analysis of rats undergoing compression at the two time points of 3 seconds and 10 minutes after SCI showed that recovery in behavioral scales (BBB) was significantly better in the early compression group (P<0.05) after the 4th week (difference: -6.100, Confidence Interval (CI) 95%: -9.468 to -2.732) (Figure 1). The sham rat was fully recovered after the 5th week of follow up (BBB score of 21 thereafter). In all rats, BBB score improved during the study. The inclined plane test was used once during the study and showed that rats in early compression could gain stability on a table with an inclination of 32.7±6.8 degrees and rats in the late group established stability with an average table inclination of 35±5.7 degrees, a non-significant difference with the early group. Tail flick reflex (TFR) was positive in all rats the day after SCI and remained positive throughout all 11 weeks of the study.

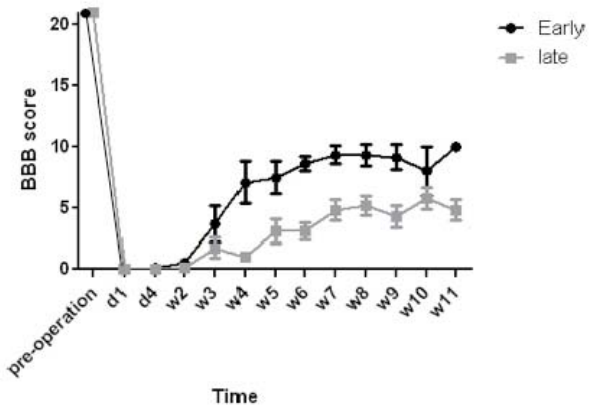

Figure 1. BBB score of two groups.
BBB scores of two groups are shown. First day post injury BBB scores of all rats were 0, which indicates a complete injury. A significant difference between mean BBB scores of two groups is seen from 4th to 11th weeks post injury (P<0.05).

Histopathologic findings

Histopathologic evaluation revealed less tissue loss and more preserved tissue in the 3-second group compared to the 10-minute group (Figure 2). On light microscopy evaluations, the absolute cross sectional area was decreased in both early and late intervention groups compared with the rat which underwent sham intervention. Light microscopy showed no sharp border between gray and white matter in either the early or late compression groups. Our study demonstrated that gliosis and cyst formation was more severe in the late SCI compression group than in the early group (P<0.05). In the late compression group, large central cavitation was seen with partially spared peripheral tissue and intact ependymal lining; both ventral and dorsal horns were distorted (Figure 2). In the early compression group,

tissue loss occurred in smaller areas, mostly gray matter (Figure 2). Tissue sparing averaged 76% in axial spinal cord sections in the early compression group, which exceeded tissue sparing in the late compression group (13% (P<0.05)). Clip compression caused injury to the dorsal columns of the spinal cord characterized by cavitation, gliosis, and atrophy (Figure 2).

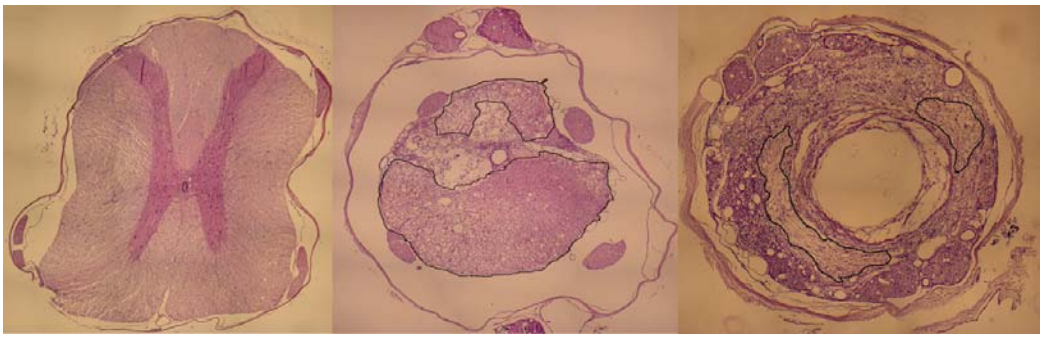

Figure 2. Light microscopic findings of rat decompression at T9.
A normal cross-sectional histology of an operated rat after anesthesia and laminectomy with clip-compression injury is shown (Left). Early decompression histopathology (Middle) – 3-second clip-compression transverse section of rat which shows 76% saved cross-section of spinal cord on both anterior (large) and posterior (small) sides in white and gray matter. Necrotic cells with gliosis and inflammatory cells can be seen even after 11-week of injury. Late 10-minute (Right) clip-compression histopathology at ninth thoracic vertebra (T-9) transverse section of rat which shows 13% saved cross-section of spinal cord on both lateral sides in white and gray matter. There are extensive areas of necrotic cells with gliosis and inflammatory cells after 11weeks of injury. Large syringomyelia has been demonstrated in the center of spinal cord. Original magnification ×200.

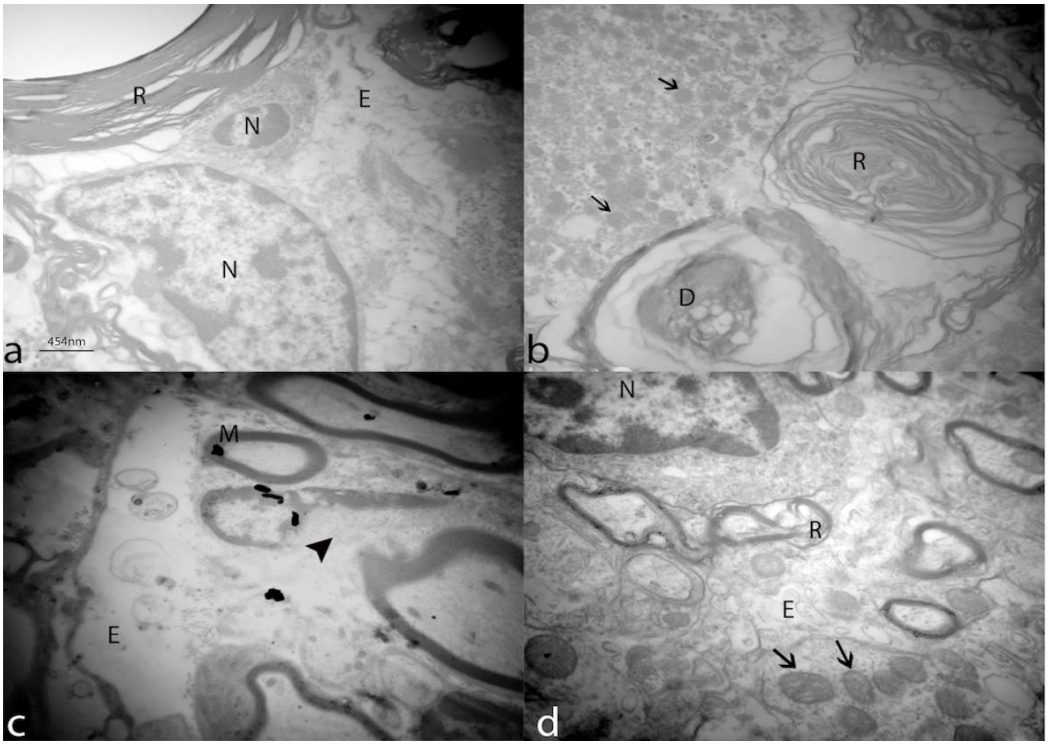

Figure 3. Electron microscopic findings of Early and Late rat decompression
Mitochondria (arrows), Nucleus (N), Intact myelin (M), Regenerated myelin (R), Degenerated myelin (D), Edema (E) and Collagen fibers (arrow head).
Figure 3a and 3b shows several layers of remyelination, degenerated myelin and intercellular and intracellular edema, intact nucleus along with engorged mitochondria in the early 3-second decompression group. Figure 3c and 3d shows layers of remyelination, collagen fibers, engorged mitochondria, intact nucleus and intracellular and intercellular edema in the late 10-minute decompression group. Original magnification ×11000.

Epidural fibrosis and chronic inflammation were seen at the level of the compression in both groups. This was accompanied by discrete areas of Wallerian degeneration and demyelination in specimens undergoing immediate decompression. Severe central necrosis at the level of compression and as much as 2 cm cephalad to the level of compression was seen when decompression was performed on a delayed basis. There was marked axonal degeneration in the long tracts of the spinal cord which was most prominent in the dorsal columns. Caudal to the level of the compression, there was a loss of axonal architecture with increased numbers of Schwann cells, fibrosis, and axonotmesis was noticeable. In transverse sections of clip-compressed spinal cord, dorsal cord necrosis and cavitation were less severe in the 3-second group compared to the delayed group. The qualitative comparison of the injured groups of rats did not reveal any meaningful difference regarding the following histopathologic findings: Inflammation, congestion, hemorrhage, fibrosis, and demyelination.

In EM, although every effort was made to select the junction of gray and white matter, severe distortion of histopathology prevented the accurate identification of the gray-white matter junction. EM shows that myelin layers were significantly more regenerated in the early compression group (Figures 3a and 3b). In EM sections, (Figure 3c and 3d), injuries to myelin (degeneration and vesiculation) axon, mitochondrial engorgement, organelle accumulation and collagen formation were more severe in the late compression group. Moreover, there was significantly more extra- and intracellular edema in late compression samples.

Discussion

This study shows that early decompression of injured spinal cord lesion results in better functional recovery and preservation of more anatomic histopathologic features. EM findings revealed that ultrastructural damages were more intense in the late decompression group.

Although clinical studies show better outcomes in early decompression of SCI in animal SCI models (25), timing of surgical decompression in SCI is still controversial (3-5,11-14,25). However, there are ongoing prospective studies to determine the role and timing of surgical decompression in traumatic SCIs (2, 26). Early surgical decompression has been shown to result in a better neurological outcome in animal models of SCI (3,11,13,27,28). Improved neurologic recovery in early decompression is due to removal of direct mechanical cord compression and reversal or minimization of secondary mechanisms of injury including ischemia and apoptosis (6-8,29). Studies have worked on medical or surgical interventions to minimize the secondary pathways of injury (6,27,30-34). However, basic to any intervention to decrease the secondary degeneration of spinal cord injured tissue is the mechanical decompression of the neural elements, in which early decompression helps preserve more spinal cord tissue, decreases lesion volume and enhances behavioral recovery (13,25). In our previous experiment (3) with sixty-three rats, the BBB scores were significantly better in the early (3-second) decompression group compared with the late (1-hour) decompression group as early as the 4^{th} day after injury, a difference which persisted for the next six weeks. Several weeks after SCI, the BBB scores of rats in the 3-second group of our previous study were worse than the present study. This difference can be explained by a longer duration of compression in our previous study, less force applied by the aneurysmal clips in this study, and/or better post-injury care provided to the SCI rats with the benefit of more experience. The observation of significant difference in BBB scores(but not the incline plane test and tail-flick reflex) suggests that BBB scoring systems may be a more sensitive test in differentiating small amounts of difference in motor function among spinal cord injured rats.

There is an association between BBB and spared spinal tissue in histopathologic examination (35). Poon et al. have shown that the force of clip compression injury in the rat thoracic cord has been correlated with both functional and histologic outcome measures (35). Our previous and present studies have demonstrated that the 3-second duration of clip compression injury in the rat thoracic cord has been correlated with both better functional and histologic outcome measures.

Previous investigations have utilized EM microscopy in the study of SCI in rats using a 5-minute compressive SCI mechanism. At sacrifices, 24 hours after SCI, myelin damage, vesicular degeneration, and intracellular and axonal edema were demonstrated along with mitochondrial damage and inflammatory cells infiltration (29). In studies by Liu et al. (36), and Zhu *et al.* (37), spinal cord sections after acute SCI demonstrated apoptotic and necrotic changes. Cytoplasmic shrinkage, plasma membrane budding, coarse chromatin condensation, and breakdown of the nucleus into discrete, membrane-bounded bodies characterize apoptotic cells while necrotic changes are

characterized by cell, nuclear, and mitochondrial swelling and cellular membrane breakdown (36,37). In our study, fibrosis and vesiculation in H&E stain were correlated with massive collagen fibers, edema and organelle accumulation in EM sections.

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