行政院國家科學委員會專題研究計畫 成果報告

急性脊髓損傷之基因治療實驗研究 探討利用腺病毒載體攜帶 對受傷神經組織恢復之療效

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Abstract

Background and purpose: Animal models are extremely important for studying pathophysiology of spinal cord injury (SCI) and developing the experimental therapeutic strategies. There have been a wide variety of animal and injury techniques of SCI; weight drop and clip compression in rat are two most common models. A study comparing the reproducibility and consistence, also the nature of injury and the damage of the complex tracts of these two SCI animal model is imperative in minimizing the methodological flows. Furthermore, there has been also no previous study has assessed the best neuromonitoring technique in SCI model. The purposes of the current study were to evaluate the electrophysiologic changes after different SCI models, and to compare these findings with behavioral and histological features.

Materials and Methods: Experimental SCI was induced in sixteen rats by New York University impaction injury (NYU model, 8 rats) and clip compression injury (Rivlin and Tator model, 8 rats). Various spinal somatosensory evoked potentials (SSEP) and compound muscle action potential (CMAP) were monitored immediately before and after compression, and at 3-day intervals for fifteen days. Behavioral observation by Basso, Beattie, Bresnahan scale (BBB scale) in every 3-day interval and the days need for a bladder emptying, and the final histological examinations were performed.

Results: At measurement immediate after the injury, the amplitude of either SSEP or CMAP abolished completely, and the BBB scale also drop to 0 from the initial few days after injury in both groups. There was no change of amplitude of SSEP in both groups in the 2-week follow-up. However, the amplitude of CMAP showed a
consistent trend toward increase (25 % of pre-injury level in average), and the BBB scale recovered to 14.5 points of the clip compression group; meanwhile, the CMAP and BBB scale showed no trend of any recovery at the end of follow-up. These electrophysiological and behavioral changes were also correlated well with histological change in the extent and degree of damage. Discussion: The NYU device provides a more reproducible and consistent trauma without spontaneous recovery in 14 days after injury than clip model by behavioral, electrophysiological, and histological observations. The ability of a single electrophysiological test in defining the degree of experimental SCI is limited, and should not be the primary criteria for the SCI. Besides, we suggested that the sensitivity of electrophysiological study is higher if multi-tests added.
Introduction

The NYU impactor and clip compression techniques are two most commonly techniques in creating acute SCI animal, because it allows easy manipulation and producing injury mimicking clinical spinal cord injury. However, when the spinal cord is improperly damaged, it can recover, which can mislead the effect of therapy. Reproducible and consistent damage of spinal cord is gold standard in creating such acute SCI model. Therefore, a comparative study in evaluation of these two common used SCI models is needed to avoid the bias brought by the initial injury.

Several conventional models of electrophysiologic monitoring during spinal surgery are effective in the diagnosis and assessment of neurologic compromise resulting from spinal cord injury; however, these models used in acute SCI model is variable in report and with consistent acceptance of their ability to detect injury. Our recent work suggests that detailed multi-model electrodiagnostic system is warranted to establish an optimal monitoring method and elucidate the pathophysiology of neural damage. Here we produced the acute SCI as two published model commonly used in rats. We investigated SSEP, CMAP, and conductive SCEP; as well as functional and histologic changes after injury caused by NYU impactor and clip of the spinal cord in the T12/13 in rats. We describe how these changes correlated with the injured spinal cord taken after surgery to document the injury location, to provide a comprehensive basis for determining the sensitivity and efficacy of different electrophysiologic monitoring technique, and to validate the consistence of the spinal cord injury induced by these two techniques.
Materials and Methods

24 wistar male rats weighing 250-300 grams were used in this study. Each rat was housed individually in our animal care center with a 12/12-hr light/dark cycle and free access to food and water. The experimental protocol was reviewed and approved by the appropriate authorities.

Animal preparation and recording of evoked potential

The animals were anesthetized with sodium thiopentone (50mg/kg, i.p.; Pentothal, Abbott, Australia). The depth of anesthesia was determined by noting the withdrawal reflex upon tail pinch. The animals were premedicated with Gentamycin (8 mg/kg, i.m.; Yung Shin Taiwan), and an aseptic procedure was performed. Core temperature was monitored with probes in the rectum and connected to a multichannel thermometer (model 3087, Yokogawa Hokushin Electric, Tokyo, Japan). The rat was then placed in prone position with its head fixed firmly in a stereotatic frame. A 5-cm longitudinal incision was made in the back, from the T-L junction to the upper sacral spine. Dorsal incisions in both thighs were used to expose a 1.5-cm segment of both sciatic nerves. Stimulation of the bilateral sciatic nerves was via hook-needle electrodes (Nihon Koden, Tokyo, Japan) placed at the sciatic nerves just proximal to the bifurcation of the peroneal branch. Rectangular impulses of 0.2 msec duration were presented at a rate of 7 Hz, and stimulus intensity was set at supramaximal level. SSEP was recorded from a needle electrode inserted in the thoracolumbar junctional space, a reference electrode placed in subcutaneous tissue just proximal to the recording electrode, and a ground electrode placed in the pelvic girdle ipsilateral to the side stimulated. Potentials were averaged 20 to 50 times at a band-pass filter setting of 50 to 5000 Hz with a 20-msec time base.
Experimental protocol of acute spinal cord injury

After the dorsal midline incision of the lower back, the paravertebral muscles were dissected to the lateral aspect of the facets and transverse processes to expose the posterior elements of upper thoracic spine to lower lumbar spine under a 1.5× loupe. The rats were divided into three groups. In each Group-1 rat (sham, n = 8), the spinal cord at the T12-T13 was exposed by laminectomies without any neural damage, and then the wound was closed as in experimental groups. In each Group-2 rat (weight drop NYU, n = 8), injuries was produced using force of 50 gram centimeter impacting on the spinal cord at T12-13 after laminectomies. This was achieved with a 10 gms impactor dropped through a height of 5cm to strike the exposed dura. The wound was closed in anatomical layers. And in each Group-3 rat (clip compression, n = 8), compression of the spinal cord at T12-13 was produced by placing a modified aneurysm clip around the cord extradurally after laminectomies. After application, the clip was rapidly removed from the applicator producing immediate acute cord compression. The clip was compressed for 3 minutes and then rapidly released. The level was verified by preoperative radiography.

Stimulation and Recording Techniques of Evoked Potentials.

Three different kinds of electrophysiological surveillance systems, D-SSEP, conductive-SCEP, and CMAP were set up. DSSEP was recorded from a needle electrode (Nihon Koden, Tokyo, Japan) inserted in the T8/9 interspinous ligament after a proper marking and radiological confirmation, and a reference electrode was placed in subcutaneous tissue just proximal to the recording electrode. D-SSEP was elicited by a pair of subcutaneous needle electrodes with an interelectrode distance of 2 mm in the lateral aspect of the left forefoot, which corresponds to the L5 dermatomal field. The
conductive-SCEP elicited by direct spinal cord stimulation was recorded by the same electrodes inserted into the thoracolumbar interspinous ligament. The stimulation was prepared by making a 2-cm longitudinal incision the back of neck, from the base of the skull to the upper cervical spine, and stimulation of the cervical spinal cord was via two needle electrodes placed 3 mm apart and inserted into the C2/3 interspinous ligament with the cathode being proximal. CMAP was recorded from the monopolar myographic needle electrodes placed in each belly of the bilateral intrinsic plantar muscles by the stimulation of the spinal cord at T12-13 using needle electrodes in the interspinous ligament. The acquisition parameters were similar to those for SSEP, but the presentation rate of stimulation decreased to 1/sec, and the low and high linear range of the filter was between 1 Hz and 2000 Hz. A ground electrode was placed subcutaneously between the stimulus and the recording site. At least three sequential single-sweep runs (i.e., without averaging) with similar waveforms were recorded to check and verify the consistency of the responses. Rectangular impulses of 0.2 msec duration were presented at a rate of 5 Hz, and stimulus intensity was set at supramaxial level, i.e., 4 to 5 times greater than required to produce a visible twitch of the paw. A ground electrode was placed in the pelvic girdle for the recording of SSEP and CMAP, and in the shoulder girdle for the recording of conductive SCEP. The potentials were averaged 20 to 50 times at a band-pass filter setting of 10 to 5000 Hz with a 20-msec time base.

Analysis of Evoked Potentials and Correlation with Neurologic Function Observation and Histologic Studies

The electrophysiological data were collected, stored, and analyzed on an electrodiagnostic device (Neuropack Z; Nihon Koden, Tokyo, Japan). Baseline potential
was determined seven times: before operation; one hour; three, five, seven, ten, and fourteen day post-operation. The amplitude of the major peaks in these recordings was expressed as a percentage of the pre-operation values. The latency of the response was measured from the onset of electrical shock artifact to the initial positive peak.

The neurologic deficit in each of the animals was assessed by Basso, Beattie, Bresnahan scale (BBB scale) before every electrophysiological examinations. All animals were tested between 2:00 and 5:00 p.m., and all observations were conducted by a blinded observer. Animals operated upon had their bladder expressed manually twice daily during the first week and thereafter once daily. The days they needed bladder massage was also recorded.

After the final electrophysiological and functional observation by BBB scale, the animals were deeply anesthetized using intraperitoneal sodium thiopentone, after which an intracardiac perfusion with warm lactated Ringer’s saline solution and glutaraldehyde was performed. After perfusion and careful posterial dissection of the thoracic vertebrae, gross examination of the relation of the damaged spinal cord was done. The injured spinal cord of all rats were removed and fixed in 2% glutaraldehyde in 0.1% PBS (pH 7.4). Whole lengths of the spinal cord were then prepared and examined in 1.0-µm-thick cross-sections and stained with hematoxylin and eosin, and Massion stain.
Results

Electrophysiological findings

Normal Evoked Potentials (D-SSEP, Conductive SCEP and CMAP)

Figure 1 shows representative recordings of the typical waveforms of three different potentials. D-SSEP and conductive SCEP show a typical initial small positive wave and a major negative wave. The amplitude of D-SSEP and conductive SCEP varied between 60.5 and 225.5 µV (average, 75.2 ± 44.2) and between 32.5 and 219.2 µV (average, 80.9 ± 41.7); the onset latency ranged from 2.83 to 4.98 ms (average, 3.56 ± 0.66) and 3.22 to 5.64 ms (average, 3.88 ± 0.53) respectively. Stimulation by the interspinous electrode yielded a reproducible CMAP response from the intrinsic foot muscles. Because the monopolar needle placed on the belly of the foot muscles is very stable, and the electrode can also be firmly attached to the feet, similar waveforms could be obtained even though the hind limbs moved in response to each stimulation. There were no significant differences in amplitude and the latency between the left and right sides. The CMAP response occurred bilaterally in response to each stimulation. The amplitude is very large and stable, and the latencies were consistent in spite of variations in morphology and polarity between the two hind feet.

Immediate and Long-Term Effect of spinal Cord Lesioning on Evoked Potentials, and the Difference between Different Injury Models (NYU and Clip Compression)

In the sham group, there were no significant changes in amplitude, latency of all three kinds of electrophysiologic tests when comparing every time-point during and three months after the operation with the preoperative baseline (Tables 2-4). In the two experimental groups, all the parameters showed significant changes after the operation,
and the degree of change in amplitude of all kinds of evoked potentials varied between these two groups. Figures 1-3 show typical changes in D-SSEP, conductive SCEP, and CMAP after the acute injury. In general, the major-spike wave of SSEP and NAP tended to be sensitive to this acute injury: amplitude decreased and latency increased.

In the NYU group (Group II), the injury caused an almost total disappearance of the waveform in all three kinds of evoked potentials. These obvious intraoperative changes in D-SSEP and CMAP showed no recovery in every time-point for two weeks during the follow-up. However, the amplitude of conductive SCEP showed a restoration of to 36.7%, a significant recovery in the post-operative 14 days. (Fig 1 and 2; Tables 1 and 2).

In the clip compression group (Group III), the acute compressive injury on the spinal cord also caused a completely disappearance of wave in D-SSEP and CMAP, and a significant reduction of amplitude in conductive SCEP (42.9% and 69.0%) immediately and two hours after the injury. However, there were significant further reductions of amplitude (19.0%, 14.3%, 9.5%, 4.8% of the post-operative 1, 3, 5, 7 day respectively) in conductive SCEP duration the first experimental week period (Table 2). At the same time, the waves of D-SSEP and CMAP reappeared but with significantly reduced amplitude at the seven days after the operation (11.6% and 17.5% respectively). In the second week (the postoperative recording at 10 and 14 days), there was further significant increase in the amplitude of D-SSEP and conductive SCEP, but not the CMAP. In addition, a cross-group ANOVA on the degree of deterioration of amplitude of all three kinds of evoked potentials showed significant differences between the two experimental groups and the sham group.
Behavioral Assessment and Functional Verification of Acute Spinal Cord injury by Basso, Beattie, Bresnahan scale (BBB scale)

The majority of the animals in the experimental groups showed clear behavioral alterations characteristic of both hindlimbs. The average BBB values in all groups during the 2-week experiment are shown in Table 3. Comparing BBB in the same group at different time points revealed no significant differences in the sham group. There were, however, statistically significant differences at the 10 and 14-day postoperative observations between the experimental groups. There was also a tendency (not always significant) toward an increase of BBB in both experimental groups, particularly the group III (clip compression) showed a significant recovery in the second week after injury as the electrophysiological observations.

Histologic observation

Histologic examination of the cross-sections showed that in the specimens from Groups II and III, the acute injury had caused damages of different severities in serial sections. The injured spinal cords showed the gross appearance of significant hemorrhage in all animals. Microscopic examination of the lesions showed the morphological features of hemorrhage, necrosis with cavity, vacuolation in white matter with swollen axons, and loss of neurons (Fig. 3).
Discussion

The establishment of reliable experimental model of SCI and valid evaluating system

Over the past century, many animal models of acute spinal cord injury have been used to investigate the pathophysiology and injury mechanism; and provide the injured animal to study and find the effective method to manage spinal cord injury in man. Despite there has been many reported models since the first paper describing a weight drop on dog’s spinal cord by Allen in 1911, using the animal models of spinal cord injury has been limited by a major factor- creating a consistent, reproducible lesion. Clip compression method and modified weight drop model by NYU impactor are two most common reported models in the current series of such studies. Inconsistence of the injury induced by these two methods remains the major and troublesome problem, and there has been no comparative study in evaluating the diversity of these models.

Until now, evaluation of the SCI animal model has been based on the functional observation, histological and biomolecular analysis and less common, electrophysiological examinations. Although these methods are routine, traditional, and considered to be reliable evaluation methods have the disadvantages of inherent, unavoidable bias in functional behavioral evaluation; lack of continuous, dynamic, and longitudinal observations on a same subject in histological and biomolecular analysis. Electrophysiological test seem to meet to resolve those problem, but the discrepancy of the accuracy or misdiagnosis are also reported in several clinical and experimental studies. One major bias of the diversity of diagnostic results is different electrophysiological examinations such as EMG, MNCV, CMAP, NAP-Sciatic Nerve Action Potential (SNAP)/mixed-nerve NAP (MNAP), F and H wave, and SSEP is used to evaluate the
neurological integrity. Knowledge of electrophysiological change after such lesions from well-controlled animal studies may help our understanding and use of electrodiagnostic tests, but no comparative study of using multi-model electrodiagnostic test in evaluating the conventional acute SCI models have been reported.

The role of electrodiagnostic test in detecting spinal cord injury

Real-time (immediate) verification of neural injury is warranted in acute SCI for two reasons. First, intraoperative neurophysiological examination can monitor and confirm a consistent acute spinal cord injury by various mechanisms, and, second, a continuous neuromonitoring in longitudinal fashion provide valuable information in the determination of the effect of therapy. Standard CSEP has been used for spinal cord monitoring; however, CSEP is severely suppressed by general anesthesia and various physiologic factors. Furthermore, the acute SCI system is used in T12/13 spinal segments supplying the lower extremities, where the potential elicited is small and needs many averagings. These reasons make CSEP an impractical tool for monitoring acute SCI-related spinal cord injury. SSEP recorded from the interspinous space of the lumbosacral or cervical enlargement after lower- or upper-extremity stimulation has been extensively studied in experiments and used clinically in neuromonitoring in invasive spinal-cord procedures. A major concern about SSEP is that the response is conducted primarily along dorsal nerve roots and afferent tracts in the spinal cord. Consequently, SSEP is limited in detecting motor pathway injuries. One study reported a case in which SEP remained normal, but nerve root injury was detected by abnormal neurotonic discharges identified during intraoperative electromyographic monitoring. It has, therefore, become necessary to investigate the various MEP techniques and compare their
sensitivity with that of conventional SSEP. Although many studies have demonstrated
that several techniques could be used to detect motor pathway dysfunction, some debate
exists in the literature about the proper techniques for stimulating nerves and acquiring
potentials. Electrical stimulation on the motor cortex or on the spinal cord is still the most
commonly reported method of eliciting MEP. It is, however, more often suggested that
the stimulation be delivered to the spinal cord rather than the cranium for prediction of
the motor function of lumbosacral nerve roots. There are two reasons for this: (1) to avoid
the effect of anesthetics on the motor cortex, and (2) less stimulation strength is needed
for spinal cord- than transcranial stimulation. Consequently, we used electrical
stimulation in the mid-thoracic and lower lumbar segments. Our results showed that a
consistent motor response from both lower limbs could be recorded and that it reflected
the motor pathway from the thoracolumbar spinal cord to its innervated muscles. Because
the major spike of conductive SCEP is believed to originate mainly from the lateral
column and partly from the dorsal and anterior columns. We believe conductive SCEP is
a direct measurement to monitor the spinal cord function and can reflect the conductivity
of major neural tracts of spinal cord. In this study of simultaneous using these three
technique- DSSEP, CMAP, and conductive SCEP in evaluation different SCI model, we
found close relation of electrophysiological changes with functional and histological
observations. The present data concur with that: both NYU and clip injury models cause
less damage on the ventral spinal cord indicated by partial and earlier recovery of
conductive SCEP than CMAP, and nearly no return of SSEP with preservation of viable
neural tissue in ventral side than dorsal side in histological analysis. We believe that
accurate neurophysiological evaluation of acute SCI can only achieved by such
multi-model-designed system.

*Monitoring technique and different injury model (NYU and clip compression) rationale in this experiment and its applicative relevance*

An acute clip-compression experiment on a rat spinal cord model monitored by electrophysiological tests, behavioral observation by BBB scale, and histological analysis indicated its less consistence, and possible spontaneous recovery in producing acute SCI model than the NYU technique. Although NYU is not actual traumatic spinal cord injury, this study again verify NYU can produce nearly complete damage of spinal cord in very consistence and should be recommended for further utilization in investigating the acute cord injury before a more standardized and specific experimental design of acute damage to the spinal cord is found. However, clip compression seems to a useful tool in studying the sequential pathological change after acute injury. This experiment demonstrated a close relation of electrophysiological and behavior examinations as simultaneous deterioration in the first post-injured week and recovery in the secondary week; and proved by the pathological findings.

*Conclusion*

These data demonstrate that multi-model electrodiagnosis is an excellent measurement for monitoring pathophysiologic and functional conditions in the acute SCI model. In both groups with NYU impactor or clip compression on the spinal cord showed a significant changes in amplitude compared with the pre-injured level and uninjured control group. These changes in amplitude were consistent with the significant compromise of neurologic function. Complemented with other published techniques, the electrodiagnostic setup for monitoring spinal cord injury can provide total survey of all
neural functions. In addition, NYU impactor technique is advantageous because it is highly reproducible and consistent damage on establishing acute SCI animal.
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**Figure 4.** A: Cross-section of the nerve root with dorsal root ganglion at the injury site reveals the penetrating wire track. (Hematoxylin-eosin stain; original magnification: ×100). B-D: Low and high-power photomicrographs of the same nerve root distal to the injury site reveals focal loss of axon (arrow) and fragmentation of myelin sheaths (arrow head). (Hematoxylin-eosin stain; original magnification: B ×100, C ×200, and D ×1000)