The Comparation of Two Instruments for Rat Cervical 2 Hemisection Model

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Abstract

The lateral C2 hemisection (HS) rat is the most studied reclinical model in the study of respiratory function after high cervical spinal cord injury. There are two main surgical methods in several studies—microscissors or microscalpel. This study is to evaluate the experimental results between those two methods. In this study, we performed rat lateral C2HS by microscissors (group A) or microscalpel (group B). We record cut frequency during hemisection as well as recovery of diaphragm electrophysiology by electromyogram (EMG) on the 14th day post injury. On the 14th day post injury, we record survival rate and evaluated the injury extent by hematoxylin-eosin (HE) stain. As a result, we found that group A had milder C2 injury extent than group B, higher survival rate on the 14th day post injury, and higher percent of peak root mean square (RMS) EMG post injury to that before injury. However, group A had larger cut frequency during hemisection. Weigh the advantages and disadvantages, microscissors seem had superiority over microscalpel.

1 Introduction

High cervical spinal cord injury (SCI) results in neuromotor deficits and what most challenge neuroscientists is respiratory pathway collapse which lead to diaphragm paralysis. Fortunately, respiratory pathway had plasticity and the part recovery of EMG diaphragm occurred a few weeks after high cervical SCI. Therefore, a mushrooming number of neuroscientists try to understand the cellular mechanisms attribute to phenomenon. In this field, the emergent animal model is adult rat lateral C2HS [1–10].

The hemidiaphragm is innerved by ipsilateral phrenic nuclei located in C3~6 and phrenic nuclei are innerved by bilateral rostral ventral respiratory group (rVRG) in medullary. Lateral C2HS interrupts descending bulbospinal respiratory pathways and results in temporary ipsilateral hemidiaphragm paralysis. The “crossed-phrenic phenomenon” (CPP) is defined as that the partial recovery of ipsilateral phrenic nuclei or hemidiaphragm activity in response to respiratory stressors [11, 12] such as phrenicotomy [7, 13], hypercapnia and hypoxia. Crossed phrenic activity is broadly defined as any recovery of phrenic nuclei or hemidaphragm activity ipsilateral to injury, which occurs spontaneously (sCPP) or in response to respiratory stressors (CPP) [1]. The CPP in adult rat can be explained that the loss of ipsilateral rVRG input to the phrenic nuclei is compensated by the input from contralateral rVRG fibers crossed over the spinal cord midline below C2 or ipsilateral phrenic dendrites crossed over spinal cord midline to receive contralateral rVRG fibers. sCPP is a time-dependent recovery of ipsilateral phrenic nuclei or hemidiaphragm without any intervene, which need several weeks [14–17] or months [18, 19]. And it has been attributed to the formation of new synapse projecting to phrenic nuclei [20–23] because the formation of new synapse requires 3 to 4 weeks [24–27]. In this study, we observed the sCPP during two weeks after C2HS.

There are two kinds of surgery methods in C2HS models. Some investigators used microscissors to perform C2 hemisection, such as Kenneth H. Minor [28],Wayne W.Liou [29], Gregory J Basuraused [30], Yonglu Huang [31], and D.D. Fuller [32]. Some investigators preferred microscalpel to perform C2HS, such as Tatiana Bezdudnaya [33], Brendan J. Dougherty [34], Warren J. Alilain [35], Francis J. Golder [36], Kun-Ze Lee [37, 38], Carlos B. Mantilla [39], Ricardo Siu [40], and Heather M. Gransee [41]. According the different structure characteristics of microscissors and microscalpel, there may be heterogeneity in some results among several studies.

In order to determinate those heterogeneity, we performed rat lateral C2HS by those two methods and recorded cut frequency during hemisection and the percent of peak RMS EMG on the 14th day post injury to that before injury. On 14thday post injury, we recorded survival rate and evaluated the C2 injury extent by HE stain.

2. Material And Methods

2.1 Animals

Twenty 12 weeks old female Sprague Dawley rats with initial body weight 280~320g were used and were randomly assigned to group A and group B in this study. Anesthesia was performed with isoflurane inhalation (O_2 velocity of flow 500-700 ml/min, induced concentration 3-4%, maintain concentration 2-2.5%).

All experimental protocols were approved the ethics committee of Tianjin Medical University. All methods were carried out in accordance with relevant guidelines and regulations. All methods are reported in accordance with ARRIVE guidelines

2.2 Electrophysiology

The electrodes were implanted three days before C2HS to avoid the effect of laparotomy to respiratory. In order to verify completeness of C2HS, silence of ipsilateral hemidiaphragm EMG activity was confirmed at anesthesia condition at the time of surgery and on the third days post C2HS. Briefly, rats were placed on a heating pad to maintain a constant body temperature (37℃) and laparotomy was performed to expose the diaphragm and custom-made bipolar electrodes (AS631; Cooner Wire, Chatsworth, CA) were implanted into the both slides mid-costal hemidiaphragm in such a manner that an uninsulated 3 mm segment was embedded within the diaphragm, as previously described [41–45]. The electrode wires gone subcutaneously and gone out in the dorsum of the animal and were used for chronic EMG recordings for up to two weeks. Signals were amplified (2000×) and band pass-filtered (20Hz-1kHz) by amplifiers (IPS 100C-1, BIOPAC Systems) and raw EMG signals were recorded by a Powerlab data acquisition device connected to a computer and analyzed using LabChart 8 Pro software (AD Instruments, Dunedin, New Zealand). The root mean square (RMS) of EMG was integrated (50 ms decay). Motor unit recruitment is reflected by peak RMS EMG. The higer peak value, the more motor unit recruited.

2.3 Lateral C2HS

For each animal, C2 spinal cord exposure was performed following the method of Emilie Keomani [46]. Briefly, perform a posterior cervical midline incision with scissors caudally 30 mm from ear level. Cut the acromiotrapezius muscle rostro-caudally and dissociate the rhomboid muscle to access the spinalis muscles. Then, the C2 vertebral plate with a prominent apophysis was exposed after retracting the spinalis muscle from C1 to C3 vertebra. Remove carefully the both slides C2 vertebral plate with a corneal scissors (Majestic, UK). Use tooth forceps hook up the dura at C2 and cut it with 11#surgical blade.

The right C2 lateral section were performed with microscissors (group A) or microblade (group B) just caudal to the C2 dorsal roots which means cut close to superior margin of C2 vertebral body. There was need to note in this step. In group A, one blade of microscissors initially inserted into cervical cord along anteroposterior at lateral 1/4 C2 cord transverse diameter, and the other blade inserted into the space outside edge. Then cut. If ipsilateral EMG still existed, move insert point to medial line a little and repeat the above actions until ipsilateral EMG disappeared.

In group B, the blade of microscalpel also inserted into cervical cord along anteroposterior at lateral 1/4 C2 cord transverse diameter and made an incision to edge. If ipsilateral EMG still existed, move insert point to medial line a little and repeat the above actions until ipsilateral EMG disappeared.

2.4 Histological evaluation the extent of C2 injury

Two weeks after HS, the survival rats were sacrificed. Rats were transcardially perfused with 200 ml cold 0.9% saline and 200 ml 4% paraformaldehyde. The sample of C1-C3 segment were harvested and followed those procedures: 1) post-fixation in paraformaldehyde overnight, 2) cryoprotection in 50% ethanol 120 min,70% ethanol 180 min,85% ethanol 180 min,95% ethanol 120 min, 90 min, 100% ethanol 30 min, 60 min, 60 min. 3) xylene 20 min,30 min,4) soak in liquid histowax 60℃ 120 min, 90 min,30 min. The slices were cut with Leica RM2245 (German) in the coronal plane at 6 μm thickness and dyed with HE. Slides were then observed with biomicroscope (OLYMMPUS,Japan). Each picture was scan by () and analyzed with Image J software (NIH).

2.5 Data Analysis

Data were expressed as means ± SD (standard deviation). The Statistical Product and Service Solutions (SPSS) 25.0 software (SPSS, USA) was applied for statistical analyses. The cut frequency and the recovery of peak RMS EMG between the two groups were compared by t test. $p < 0.05$ was indicated as statistically significant.

3 Results

3.1 Cut frequency

In both groups, EMG disappeared immediately after C2HS (Figure1). It means both microscissors and microscalpel can be used to cut spinal cord. However, there was a difference in cut frequency between two groups (Figure2). Group A had litter cut frequency than group B. Less cut frequency meat smoother incisal edge and narrower lesion, which benefits axon regeneration through scar.

3.2 The extent of C2 lesion

We chose one representative HE stain picture from each group. We can see C2 lesion in group B was larger than that in group A (figure 3). Obviously, it was nearly impossible that cut at one initial site on C2 cord too many times.

3.3 Peak RMS EMG

On the 14th days post injury, a variety of EMG recovery happened (figure 4). Group A had larger percent of peak RMS EMG post injury to that before injury than group B (figure 5). That means the more motor unit recruited in group A.

3.4 Survival rate

We calculated the 14-day post injury survival rate in group A and group B. The survival rate in group A was 80 % and in group B was 60%. All death happened in 1-day post injury. There was no pneumothorax during electrode implant or hemorrhage during laminectomy in both groups. We assumed the respiratory inhibition caused by C2HS can attribute for all the death.

4. Discussion

The present study, we made rat lateral C2HS model separately with microscissor and microscalpel. the first time cut frequency, 14 days survival rate, the extent of C2 injury, and percent of peak RMS EMG post injury to that before injury on performed with two different surgical procedures during anesthesia. As a result, we found that microscissor, comparing microscalpel, caused milder C2 injury extent, higher survival rate on the 14th day post injury, and higher percent of peak RMS EMG post injury to that before injury. However, microscissor need larger cut frequency during hemisection.

Structure characteristics of Spinal canal

We had two reasons to performed lateral C2 hemisection just above superior margin of C2 vertebral body. First, we dissected two rats and found C2 nerve root arise just above superior margin of C2 vertebral body and performing lateral C2 hemisection close to superior margin of C2 vertebral body can avoid C3 hemisection erroneously. Second, it is difficult for the microscissor to cut the cervical cord edge at vertebral level because of the curved sidewall of spinal canal.

Structure characteristics of hemisection tool

As for microscissor, the length of blade of microscissor seem to go into dilemma. Both long blade and short blade are disadvantage to cut the cervical cord edge. Too long blade was disadvantage for fine

manipulation and too short blade cannot cut C2 spinal cord once. In this study, we use microscissors with 14mm long blade and we found its blade length were appropriate.

Tough dura mater was easy to out of shape before pierced and this deformation caused C2 spinal cord crush injury which was devastating. The lateral dura mater in this study cannot be cut due to pedicle of vertebral arch. As for microscalpel, cutting cervical cord from middle to lateral avoid piercing lateral dura mater. As for microscissor, one blade must pierce lateral dura mater before cut spinal cord. Therefore, microscalpel seem better.

The extent of C2 injury

Fuller DD reported that the sparing of ventromedial (VM) tissue caused different ventilation and phrenic nerve activity ipsilateral to C2 lesion from complete C2HS and confirmed that descending respiratory projections from the brainstem were present in VM tissue by anterograde neuroanatomical tracing [47]. Lipski further proposed that rVRG projects ipsilateral axons in the lateral funiculus and contralateral axons in the ventral funiculus [48]. This idea was indirectly confirmed by Vinit and he reported that the transection of median (include VM) did not abolish the ipsilateral hemidiaphragm activity but the lateral one did [6]. Similar to Vinit, Kenneth H. Minor reported that lateral C2 area transection with the ventral funiculus sparing leads to a functional silencing of the ipsilateral hemidiaphragm. However, if lateral and lateral ventral funiculus transection were made initially, the recovery was failure [28]. Therefore, we assumed that the lateral area of the ventral funiculus is indispensable for sCPP response and it is sufficient to induces complete ipsilateral hemidiaphragm paralysis by lateral C2 area transection. Similiar to other studies [49], we performed C2 lateral HS and ipsilateral hemidiaphragm activity disappeared immediately. It's important to note that it was difficult to cut C2 spinal cord lateral edge with microcissors due to the shield effect of adduction structure of pedicle of vertebral arch. Therefore, one blade of microcissors have to insert spinal cord closed to post middle line so as to the other blade can cut C2 spinal cord lateral edge and this action caused larger but unnecessary lesion area.

EMG during anesthesia and eupnea

The full extent of spontaneous ipsilateral hemidiaphragm recovery is significantly attenuated by anesthesia, such as ketamine/xylazine, isoflurane, and urethane. Some people reported that there were litter peak RMS EMG in anesthesia rats compared with the awaken rats [50 51]. In this study, rats were anesthetized with 10% chloral hydrate (0.3 ml/100g). Therefore, it should be worthy of noting that anesthesia or awaken condition when use this rat model to evaluate EMG.

Some studies have showed robust correlation between transdiaphragmatic pressure (Pdi) and peak RMS EMG[52, 53]. Therefore, there was significance in peak RMS EMG consistency among studies which used different methods. Our result showed that there was no significant difference in peak RMS EMG during anesthesia and eupnea condition between scissors group and knife group at any time point post HS. Therefore, if there was significant difference in percent of peak RMS EMG post HS normalized to that

before HS among studies which used different methods, the significant difference cannot be attribute to microscissors and microscalpel.

Electrode implantation site

In some study, they detected the sternal, costal and crural regions of the hemidiaphragm in order to avoid this result what some activity may have been missed due to only one area of the hemidiaphragm detected [54]. That result, however, rare happened. For example, some animals showed an absence of EMG activity in the crural hemidiaphragm also had no EMG activity in the other two regions of the hemidiaphragm in a study of 44 animals [55]. Addition, sternal hemidiaphragm was too small and attribute limitedly to hemidiaphragm movement. Therefore, it was acceptable to electrode implant at costal region alone like this study. More implantation sites mean larger probability of pneumothorax which could be avoid.

The Schedule of HS

In this study, we performed C2HS on the 3rd days after electrode implantation in order to avoid diaphragm disfunction induced possibly by laparotomy. Following upper abdominal surgery, diaphragm disfunction [56–59] such as abnormal respiratory frequency, tidal volume (Vt) and transdiaphragmatic pressure (Pdi) appeared because of diaphragmatic reflex inhibition instead of structural impairment [60] and persisted for 1-2 day [61] after upper abdominal surgery until pain relief [57]. In P. A. Easton study [62], for example, rats without any peridiaphragmatic contact also had diaphragm reflex inhibition only due to abdomen incision. Therefore, it is better to performed C2HS on the 3rd days after electrode implantation.

Technical difficulties of electrode implantation

The details of electrode implantation were unclear in many literatures. In this study, electrode puncture needle was custom-made with 25G syringe needle. The puncture needle through full layer diaphragm was a challenge due to the high incidence of pneumothorax when the diaphragm was perforated. Therefore, we try half layer diaphragm with electrode puncture needle. And the electrode implantation site located in the diaphragm crural region and on the border line between diaphragm and chest wall. In the early stage of this study, the formation of scar tissue about 21 day after surgery at the site of muscle insertion decreased the signal output and this phenomenon is similar to the study of Philippa M. Warren [55]. His solution was that the electrode implant was repeated in a rat every recording. However, multiple abdominal surgeries must inevitably affect diaphragmatic reflex. Not to mention, EMG comparation under different electrode implantation site condition had no significance. Fortunately, scar tissue within 14 days was not enough to affect EMG and that was the reason why EMG measurement ended up on the 14th day after C2HS.

Gender-related differences in survival rate

As M Farooque reported, female mice on the 14th day after thoracic 10 compression SCI have less severity initial injury and higher Basso, Beattie and Bresnahan scores than male mice. The mechanism(s) of neuroprotection effects of estrogen on pathophysiological processes such as blood flow, leukocyte migration inhibition, antioxidant properties, and inhibition of apoptosis might attribute this differences [63]. Similarly, in our pre-study, all the three months old male rats dead eight hours after and all the three months old female rats survived. We had speculated this difference might attribute to weight because female rats only weight 3/5 male rats at same yearth level. Therefore, in our pre-study, we repeat C2HS using 300g male rats and 300g female rats. However, this result did not change. Therefore, weight cannot contribute to that difference and estrogen might, although not yet elucidated, contribute to that difference like M Farooque's study. Therefore, in this study, we only use female rats so as to avoid death.

5 Conclusion

The rat model of lateral C2 hemisection was an emergent tool to study CPP. However, there were mainly two experiment methods in several studies—microscissors or microscalpel. We compared those two methods and found microscissors csused milder C2 lesion than microscalpel, higher 14 days survival rate, and higher percent of peak RMS EMG 14 days post C2SH to that before injury. However, microscissors had larger cut frequency during hemisection. Weigh the advantages and disadvantages, microscissors seems had superiority over microscissors.

Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

Contribution

Kai Wang and Liang Zhang contributed to the study conception and design. LinLin Shen performed experiment and wrote the manuscript. Chen Song made statistics and figures.

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Figures

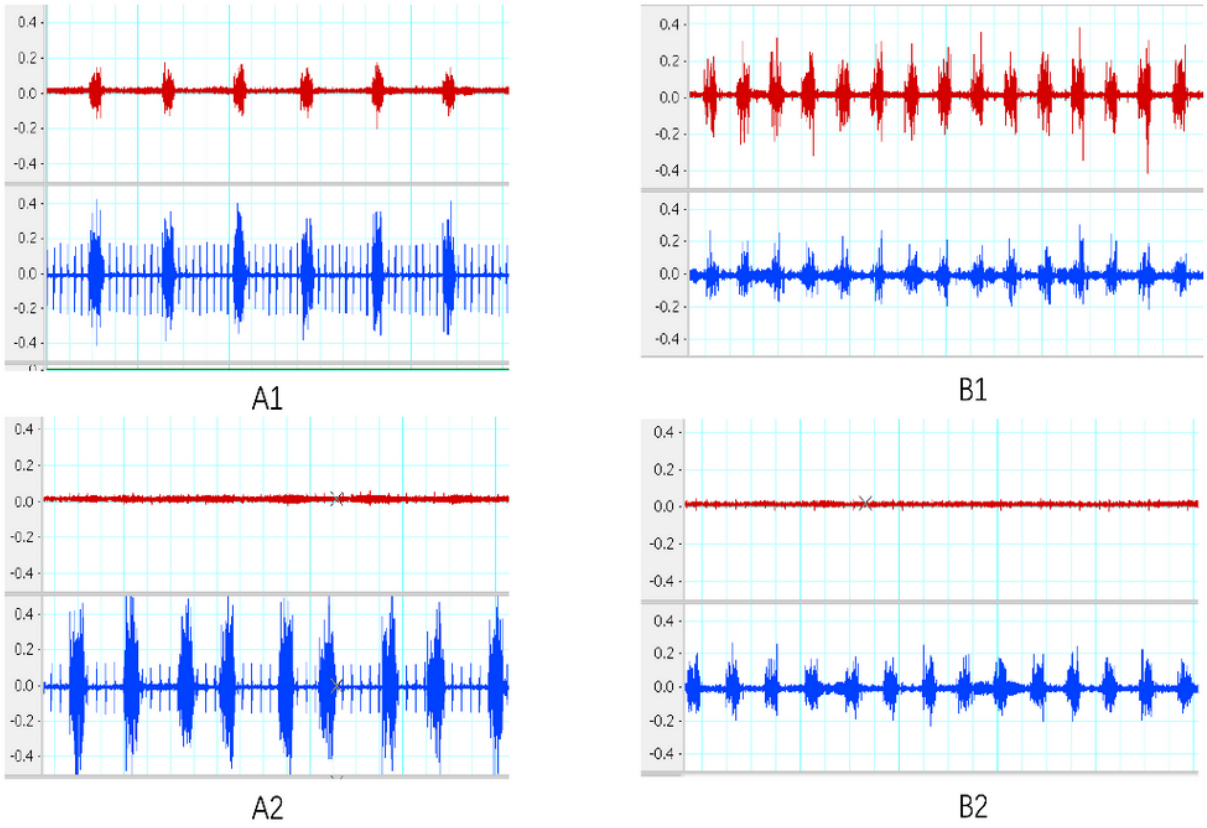

Figure 1

EMG recorded a few miniutes before and after C2HS. The EMG of a rat from group A before (A1) and after (A2) C2HS. The EMG of a rat from group B before (B1) and after (B2) C2HS.

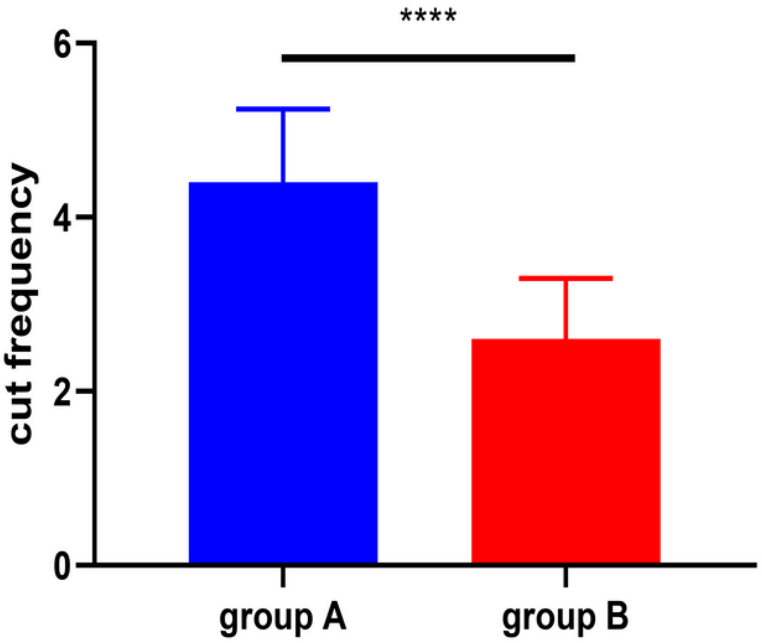

Figure 2

The cut frequency during hemisection in group A was larger than that in group B. **** p<0.0001

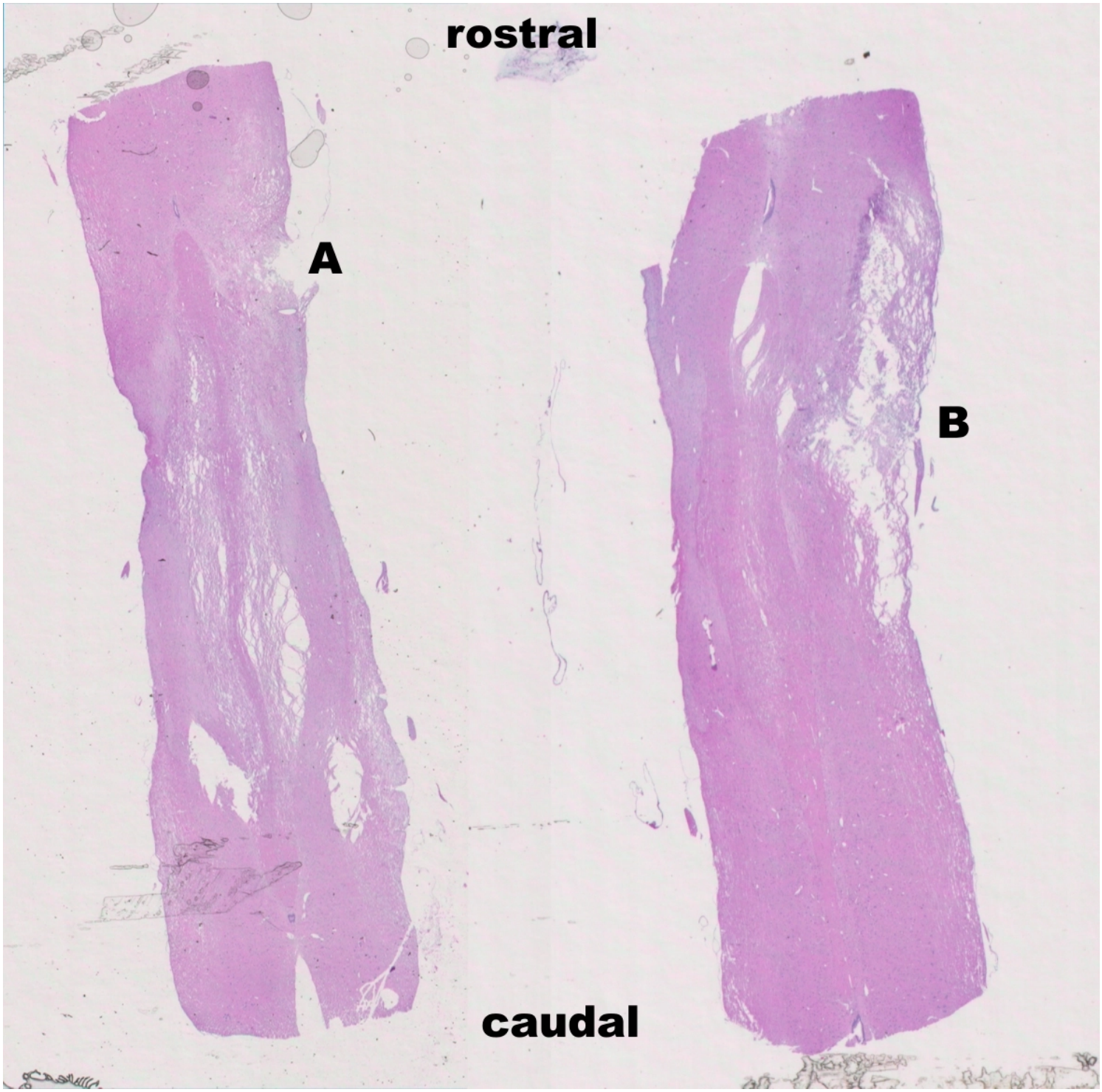

Figure 3

C1~C3 spinal cord coronal plane. Two representative HE stain pictures from Group A (left) and group B (right) separately. “A” and “B” indicate the cut location. The port closed to medial line was spared in both groups. And the lesion in group B in was larger than that in group B in rostral-caudal level and lateral level.

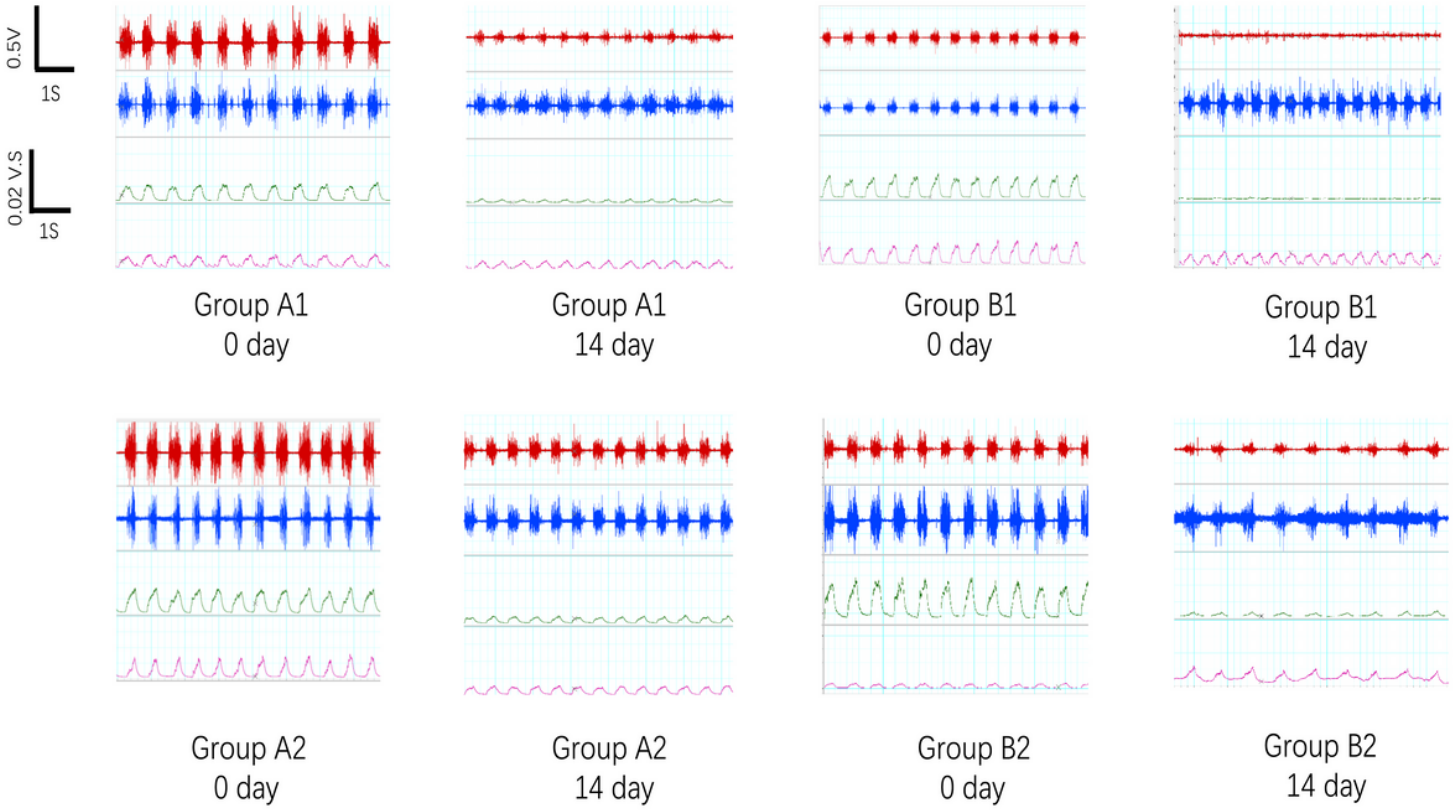

Figure 4

The representative EMG that recorded on the 3 days before C2HS as well as the 14th day post C2HS of two rats in each group. A1 and A2 were two rats from group A. B1 and B2 were two rats from group B. In each EMG, upper 2 panel were raw EMG, red: right hemidiaphragm. blue:left hemidiaphragm. Lower 2 panel were RMS EMG. green: right hemidiaphragm. purple: left hemidiaphragm.

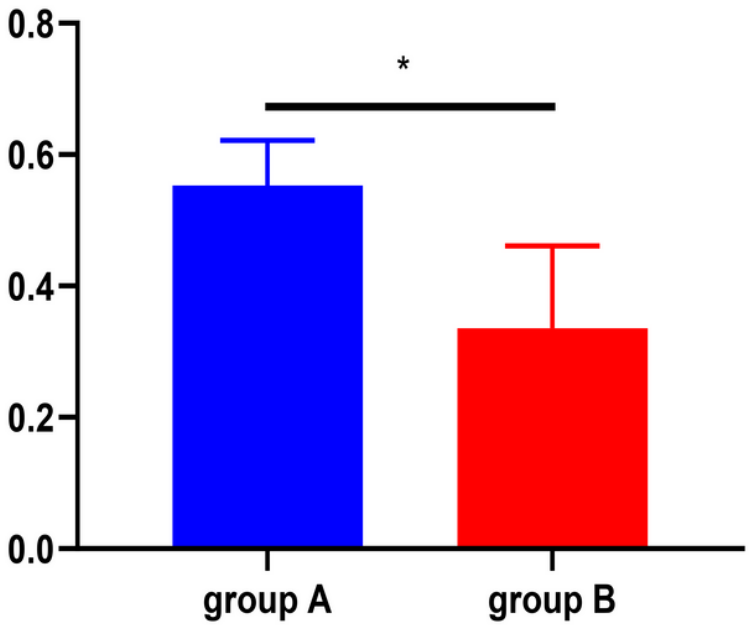

Figure 5

The group A had higher percent of peak RMS EMG any time point post injury to that before injury. And that means larger motor unit recruitment was happened in group A. * $p<0.05$