

Establishment of an acute ventral closed spinal cord injury model

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Abstract

Objective. Acute closing spinal cord injury (SCI) is getting more common in clinic while no ideal animal model has been established. Here, we firstly established a new model by ventral violence and examined its neural functional and pathological changes. **Methods.** A self-designed impinger which coup momentum could be regulated by weight \times height (CM). 92 adult male rats were divided into 4 groups: group A (CM: 350 g \times 28 cm), group B (CM: 280 g \times 28 cm), group C (CM: 210 g \times 28 cm) and group D (CM: 0 g \times 28 cm). Animal model was made by free falling method at anterior border of T11 centrum under inferior sober state. Complications and mortality were observed. Basso, Beattie and Bresnahan (BBB) score was recorded postoperatively. Morphology and pathological changes of SCI were examined. **Results.** Coup momentum could be regulated precisely. There were significant differences in BBB scores not only between experimental group and control group but also among 3 experimental groups. In group A and group B, contusion, hematoma, subarachnoid hemorrhage appeared at 1–6 hours after injury. Edema was obvious and inflammatory cells were infiltrated at 6–48 hours. Cicatricial contracture and porosis formed at week 3, while group C only showed sporadic punctate hemorrhage. Glial fibrillary acidic protein (GFAP) expression in group B changed dynamically compared with group D. Multiple complications presented in experimental groups. The mortality among groups had significant differences. **Conclusion.** A novel acute SCI animal model was set up by shape-suitable impinger in simple procedure. At meantime, neural function deficiency, pathological changes and mortality were consistent with severity controlled by coup momentum. [Life Science Journal. 2009; 6(1): 18–26] (ISSN: 1097–8135).

Keywords: animal model; spinal cord injury; ventral; closed

1 Introduction

The incidence of spinal cord injury (SCI) has been increasing partially due to high speed transportation and ultimate athletic sports currently. Although some of the mildly injured recovered well, the majority of SCI finally lead to devastating motor, sensory, and autonomic dysfunctions. The suffering not only involved the survivors but also extended to the whole families, friends and society as well. They have to endure emotional, physical, and financial burdens in providing for necessary surgeries,

care, and rehabilitation.

Except the mechanical damage caused by primary SCI instantly, there is a secondary complicated injury cascade afterwards which leads to progressive neural cell death and impair endogenous recovery processes^[1]. Possible cures were investigated to alleviate the traumatic SCI including a series of strategies such as the interventions to attenuate or overcome the secondary injury cascade, enhancing the endogenous repair mechanisms, improving axons regeneration, replacing lost cells, and measures for rehabilitation^[2,3]. Thus, it is particularly important to set up an ideal animal model. Till now, various SCI model have already been reported by crosscut, oppression, contusion, photochemical injury and ischemia-reperfusion in-

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jury etc^[4-8]. These models represented some characterization of SCI, whereas all of them had some limitations in application such as specialized equipments needed, high expenses, not repetitive well, or inconsistency with actual situation. In clinic, acute closing SCI by ventral violence is very common. But no suitable animal model has been found. Here, we firstly established a new model using self-designed shape-suitable impinger by ventral violence in simple procedure and examined its neural functional and pathological changes. This model could replicate the processes of different severity of SCI and contributed to further pathological mechanism studies during injury.

2 Materials and Methods

2.1 Shape-suitable impinger

Shape-suitable impinger was consisted of orbit, adjustable weight and drive rod. This device was shown in Figure 1.

2.2 Experimental animals

Ninety-two healthy SD adult rats, weighting 220 g – 250 g, provided by Experimental Animal Center of Zhengzhou University. The protocol of this experiment followed the regulations of ethics.

2.3 Experimental procedure

2.3.1 Modeling. 92 rats were divided into four groups according to force momentum calculated by weight and height (CM). Experimental groups: group A (350 g × 28 cm), group B (CM: 280 g × 28 cm), and group C (CM: 210 g × 28 cm); Control group: group D (sham, CM: 0 g × 28 cm). They were fasted for 12 hours and water intake was stopped 3 hours prior to surgery. Midazolam anesthesia was administrated by intraperitoneal injection (0.5 mg/kg). The rats were fixed in dorsal position and the four limbs were stretched under the inferior sober state. The caudal centrum of T10 was suspended in mid-air and then the internal organs were pushed away by the drive rod. The tips stick to the T11 centrum closely and attack the piston end by vertical falling of the force momentum. Rats demonstrated spasmodic twitching of the tail and both lower extremities after injury and lost partial or complete motor function of hindlimbs. Those rats that regained consciousness without dying in 48 hours were considered to be the successful model. Group D underwent sham surgery without attacking the drive rod. There were each 26 rats in groups A and B, 18 rats in groups C and D. After surgery each rat was placed in a single cage and food-intake was restricted to 1/10 of the body weight.

The abdomen of the rat was gently massaged to assist in defecation. In addition, they were injected with 3 ml of 10% glucose via vena dorsalis penis 3 times a week.

2.3.2 Motor function evaluation. 5 rats in each group were randomly picked up for neurological evaluation. Motor functional test was performed at day 1, day 7, day 10, day 14, day 21, day 28 postoperatively with double blind method. Based on three independent tests, neurologic function was recorded according to the Basso, Beattie and Bresnahan (BBB) score and calculate the mean.

2.3.3 Morphology and pathology. Two rats in each group at hour 1, hour 6, hour 48, day 7, day 28, underwent heart perfusion fixation with 4% of paraformaldehyde, fixed overnight and prepared for histology (20 μm thick sections), observed by HE staining (Bi Yun-tian Biotec, China) under immunofluorescence microscope (OLYMPUS, Japan). Specimens were divided into three segments: 0.20 cm surrounding the main injury region (center injury segment, CIS), 0.20 – 0.50 cm from injury (proximal injury segment, PIS) and 0.50 – 0.80 cm from injury (distal injury segment, DIS).

2.3.4 Immunohistochemistry. Glial fibrillary acidic protein (GFAP) expression was determined 1 hour, 6 hours, 2 days, 7 days and 28 days after injury by immunohistochemical SP (streptavidin-peroxidase) method in group B and D. All of the procedures followed the manufacturer's instructions. Sections were observed under the microscope and 4 fields were selected under low power (× 100) and 10 photographs were taken under high power (× 400). By using Image-pro plus 6.0 software, we measured and analyzed the mean optical density (MOD) and positive area (%) of per field respectively, and applied relative content ($RC = MOD \times \text{positive area}$) to represent positive component.

2.3.5 Complication and death. All of the rats were under observation for complications and mortality from day 1 to day 28.

2.4 Statistical analysis

SPSS13.0 software was used for statistical analysis. All data were presented as mean ± SD. Chi-square test was used for analyzing significant difference of rate among groups. Statistical analysis of BBB scores was performed using repeated measures analysis of variance (ANOVA) and a pairwise multiple post hoc comparison using the Bonferroni *t*-test. One-way ANOVA was used to compare means in one time point among groups, *t*-test was used for significant difference analysis of two means between groups. $P < 0.05$ was considered significant.

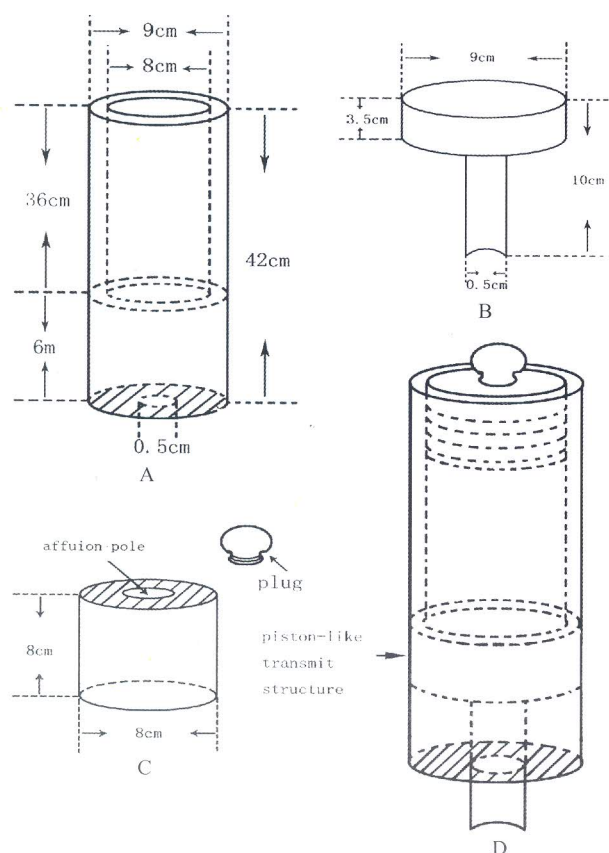


Figure 1. The self-designed impinger and the fabricated device. A: Orbit. Orbit was a circular plastic cannula with smooth inner wall. The tip of the orbit was fit for adjustable weight, and the bottom was constraint the piston end and formed the piston-like transmission structure. B: Adjustable weight. Adjustable weight was made up of capacity bottle and bottle plug. Quality of adjustable weight could be adjusted randomly, so as to guarantee momentum regulation precisely. C: Drive rod. Drive rod had piston end and shape-suitable rod. The front-end of shape-suitable tip was long and narrow and its arc shape similar to the ventral side of spinal for reducing the aortic injury, and ensuring the well-distributed forces on both sides. D: Fabricated device. Those above guarantee the unity of momentum and the direction of each model. Along orbit, adjustable weight hit the drive rod to make the SCI model.

3 Results

3.1 Motor function

Rats in group D walked normally. Rats in group C returned to normal 7 days after injury. Scores of group A and B showed increasing tendency after SCI. They appeared completely flaccid paralysis of hind limbs immediately after injury and relatively partial recovery in mild activity during day 5 – 7. More obvious recovery presented in day 14, but could not support its whole body in 4 weeks. Scores in Table 1 showed an increasing tendency in ex-

perimental groups. There were significant differences not only between experimental groups and control group but also among experimental groups from day 2 to day 28 ($P < 0.05$). Group A and B didn't show any differences in motor function from day 1 to day 10. However, there was a significant differences since day 14 and last to day 28 (Table 1).

3.2 Pathological changes

3.2.1 Gross appearance. Fracture of centrum was found in few samples. Contusion, hematoma and subarachnoid hemorrhage were observed within 6 hours after injury. Most injured lesions became swollen at hour 6 and subsided 7 days. After 4 weeks, injured region became anolysis and thin. The changes described above were not found in group C (Figure 2).

3.2.2 Histology changes. Sections stained with HE showed the following histopathological changes: 1 – 6 hours after injury, haematoma, contusion, subarachnoid hemorrhage in group A and B (Figure 3 A – C), only dispersed punctate hemorrhage showed in group C (Figure 3 D), no abnormality in group D. Tissue space was still normal, interstitial edema was not obvious (Figure 3 E). 6 – 48 hours later, haematoma and tissue space expanded, and large amount of inflammatory cells infiltrated (Figure 3 F). Enlarged tissue space was not obvious at day 7. Cicatricial constriction and porosis formed after 3 – 4 weeks (Figure 3 G & H). Posttraumatic haematoma and contusion located mainly in coup position, while extensive small lesions were found in cephal-caudal end of grey matter along longaxis of spinal cord in group A and B (Figure 3 I). However, different distributions appeared in group A and B. Group A had lesions both in PIS and DIS, but only PIS had small contend foci while hemorrhagic spots in DIS (Figure 3).

3.3 Expression of GFAP

The expression of GFAP at hour 1, hour 6, day 2, day 7, and day 28 in group B and D was determined by immunohistochemistry. GFAP expression was shown in Figure 4 A & B. As showed in Table 2 and Figures 4 and 5: in group B, there was minimal GFAP expression in gray matter and decreased expression in white matter 1 hour after injury (Figure 4 C & D). 6 hours later, increased GFAP expression at pia mater spinal was near injured region and grey matter near central tunnel (Figure 4 E – H). Expression of GFAP continued to increase especially in DIS 48 hours after injury and located mainly in PIS at 7 days ($P < 0.05$, Table 2), and expression of GFAP developed from local region to hol-region around injured area at day 7 (Figure 4 I). Both white and gray matter had higher ex-

Table 1. Comparison of Basso, Beattie and Bresnahan score

Group	Day 1	Day 7	Day 10	Day 14	Day 21	Day 28
A	0.93 ± 1.44	3.53 ± 1.99	4.66 ± 2.38	7.33 ± 2.47	9.87 ± 2.47	11.27 ± 2.69
B	2 ± 3.53 [#]	3.4 ± 2.13 [#]	4.20 ± 2.62 [#]	10.4 ± 3.18 [*]	13.87 ± 2.90 [*]	13.27 ± 2.89 [*]
C	4.40 ± 4.24	17.93 ± 2.81	18.53 ± 1.60	19.87 ± 1.13	19.60 ± 1.30	19.87 ± 1.19
D	19.87 ± 1.36	20 ± 1.07	19.87 ± 1.13	19.93 ± 1.10	20.47 ± 0.64	20.60 ± 0.74

vs. group A: [#]: $P > 0.05$, ^{*}: $P < 0.05$. $n = 15$. Scores in experimental group showed increasing tendency after SCI. They appeared completely flaccid paralysis of hind limbs after injury, partial recovery during 5 – 7 days and obvious recovery in day 14, but could not support its whole body in 4 weeks. There were significant differences not only between experimental group and control group but also among experimental groups from day 2 to day 28.

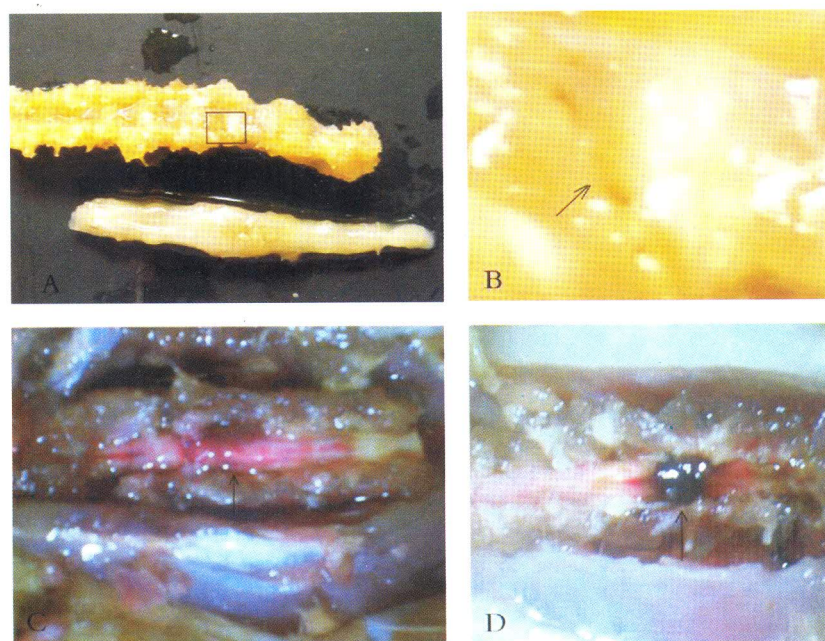


Figure 2. Gross appearance. A: Vertebral fracture and spinal cord injury shown at the same level. B: Magnified field as shown in Figure 2 A, vertebral fracture line could be seen clearly (arrow). C & D: Subarachnoid hemorrhage, contusion and hematoma could be found clearly 6 hours after injury (arrow).

pression of GFAP 28 days after injury (Figure 4 J).

3.4 Complications and mortality

30 rats were performed pathological examination and 40 rats' complications and mortality were observed after operation. During day 2 to day 28, 8 rats died in group A and B (2 were excluded because of human factor), and no rat died in C and D. Three rats died from day 2 – 7, one died of bladder rupture, the other died of intestinal obstruction and hematuria, and another by pulmonary infection. Three rats died from day 8 to day 14 after injury because of intestinal obstruction. Two rats died from day 15 to day 28 as the result of vesical rupture due to inappropriate massaging of the bladder. Complication and

death were shown in Table 3 and Table 4. There was obvious difference of mortality among groups in (reject 2 because of human factor) ($P = 0.018$).

4 Discussion

At present, the incidence of SCI has been increasing while pathologic mechanism was complicated. Common causes were traffic accidents, sports or height crashed^[9,10]. No matter what the violence was by buckling, axial or shear force, and leads to compression fractures, burst fractures, chance fracture, most patients resulted in various kinds SCI under sober state, part of them belong to acute

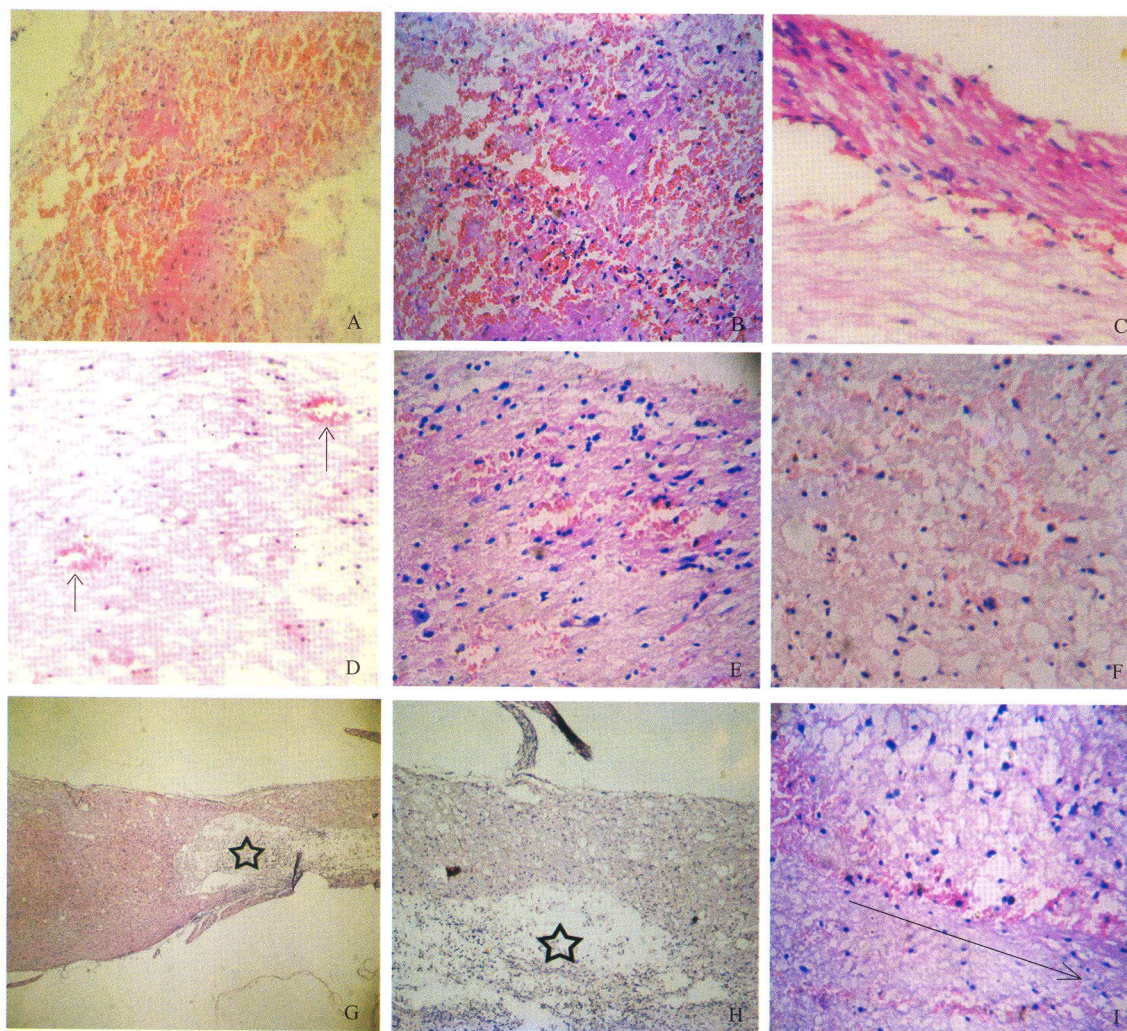


Figure 3. Microscopic observation (HE staining). A – C: 1 – 6 hours after injury. Hematoma, contusion, subarachnoid hemorrhage were shown in group A & B ($\times 100$, $\times 200$, $\times 400$). D: 6 hours after injury. Small hemorrhagic lesion in group C (arrow, $\times 100$). E: 1 – 6 hours after injury. Histological structure of spinal cord was densification: edema was not obvious in tissue space in group A – C ($\times 400$). F: 6 – 48 hours after injury. Tissue space near contusion and hematoma was expanded. Edema was obvious, with mononuclear inflammatory cell infiltrated abundantly in group A & B ($\times 200$). G & H: 4 weeks after injury. Scar tissue and cavitation formed at the injury site in group A & B (asterisk, $\times 40$, $\times 100$). I: 6 hours after injury, multiple contusion could be seen in the gray matter of cephal and caudal end of force spot along the axis of ordinates in group A & B (arrow, $\times 200$).

Table 2. Expression of GFAP in control group and group B

Group		Hour 1	Hour 6	Hour 48	Day 7	Day 28
B	P-W	34.87 ± 5.56^a	37.06 ± 5.44^a	$43.33 \pm 4.40^{b,c,d,e}$	$64.84 \pm 10.38^{a,d,e}$	$95.40 \pm 20.62^{a,d,e}$
	P-G	$2.84 \pm 0.78^{b,c}$	$4.49 \pm 1.28^{b,c}$	$12.05 \pm 1.50^{c,d}$	$24.33 \pm 5.71^{b,c}$	$51.48 \pm 5.84^{b,c}$
	D-W	35.69 ± 5.75^a	36.07 ± 5.93^a	54.78 ± 6.85^a	59.74 ± 3.99^a	68.81 ± 8.42^a
	D-G	$6.71 \pm 2.04^{b,c}$	$4.04 \pm 0.97^{b,c}$	$17.93 \pm 2.86^{b,c}$	$19.05 \pm 1.44^{b,c}$	$22.62 \pm 3.55^{b,c}$
D	W	44.37 ± 5.26	43.23 ± 5.88	44.59 ± 5.18	42.12 ± 3.89	47.87 ± 3.00
	G	12.02 ± 3.12	13.49 ± 4.41	12.95 ± 2.19	11.41 ± 1.92	12.43 ± 2.36

vs. D-W: ^a: $P < 0.05$, ^b: $P > 0.05$; vs. group D-G: ^c: $P < 0.05$; vs. B-DW: ^d: $P < 0.05$. $n = 10$. P: proximal injury segment; D: distal injury segment; W: white matter; G: gray matter; B: group B; D: group D. In group B, GFAP expression was minimal in gray matter and decreased in white matter 1 hour postoperatively. 6 hours later, it increased at white matter. Expression of GFAP increased especially in distal segment of injured area 48 hours postoperatively and located mainly in the proximal injured segment at 7 days. Expression of GFAP developed from local region to hol-region around injured area at day 7. Both white and gray matter had higher expression of GFAP at day 28 after injury.

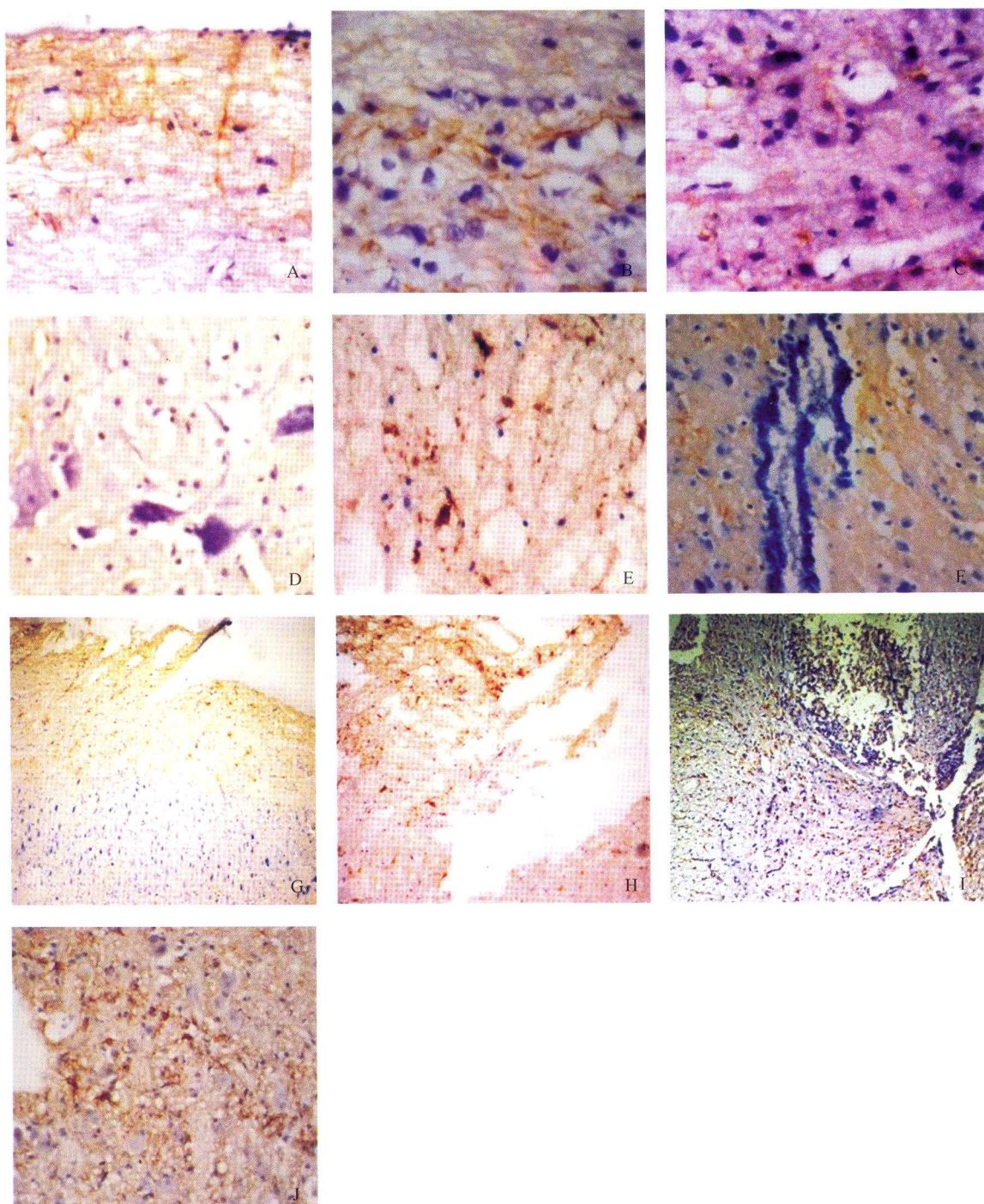


Figure 4. Expression of GFAP (SP staining). A & B: Expression of GFAP in normal spinal cord. Positive area closed to the junction of spinal pia mater and grey matter. There was a low expression in gray matter in group D ($\times 100$, $\times 200$). C & D: 1 hour after injury in group B. Decreased GFAP expression in white matter and minimal expression in gray matter ($\times 400$, $\times 400$). E – H: 6 hours later after injury in group B, GFAP expression appeared and enhanced in white matter near injured region, pia mater spinalis, grey matter near central tunnel ($\times 100$, $\times 400$, $\times 100$, $\times 400$). I: Axial view. Positive cells of GFAP located mainly in near injured segment at 7 days in group B (Axial view, $\times 100$). J: Both white and gray matter had higher expression of GFAP 28 days after injury in group B ($\times 200$).

Table 3. Mortality comparison among experimental groups

Group	Dead	Survival	Mortality (%)
A	5	11	31.25
B	1	15	6.25
C	0	16	0

Mortality from day 2 to day 28 in 3 groups. By comparing the mortality among 3 groups, it showed a significant difference ($P = 0.018$. Two were excluded because of human factors).

Table 4. Occurrence of complications and fatality 28 days after injury

Complication	Suffered (n, %)	Dead (n, %)
Blood urine	3 (7.50)	0 (0.00)
Urine retention	40 (100)	1 (16.67)
Urinary system infection	4 (10.00)	0 (0.00)
Pulmonary infection	4 (10.00)	1 (16.67)
Edma	9 (22.50)	0 (0.00)
Pressure sore	0 (0.00)	0 (0.00)
Selfmutilation	0 (0.00)	0 (0.00)
Mutual mutilation	0 (0.00)	0 (0.00)
Intestinal obstruction	15 (37.50)	4 (66.67)

40 rats were observed complications and mortality after operation. During day 2 – day 28, 8 rats dead in group A and B, 0 in C and D. 3 rats died during day 2 – 7.

ventral closing SCI. Some investigators have successively set up opening SCI models by ventral force^[11–13]. However, acute ventral closing SCI was not reported presently. Here, we made the acute ventral closing SCI model without opening the vertebral canal and adopted an instantaneous violent manner under the basal anesthesia.

In this study, Midazolam was adopted as anaesthetic characterized by less irritation, rapid revival, less influence on breath and cardiovascular system^[14,15], contributed to inferior sober state rapidly, lighten psycho-stress and stress reaction, made the animal's passive position close to the real situation of clinic when subjected to external force. This made the procedure conformed not only to ethic but also to clinic.

Ventral violence may incur abdominal organs injury, which made it difficult to establish. We found an anatomic space between diaphragm and liver which doesn't have important organs except of aorta from the view of anatomical analysis. At horizontal plane of this gap, pushing skin to spinal column and avoid squeezing organs surrounded, the violence can transferred to spinal cord through the shape-suitable impinger (Figure 1). In order to establish an acute ventral closing SCI model with good repeatability, well regulated and simple procedure,

we made our unique machinery.

We carried out neural functional analysis of each group shown in Table 1. There were significant differences not only between experimental group and control group but also among experimental groups ($P < 0.05$). It demonstrated that this method could made SCI successfully and regulated degree of SCI resulted in corresponding neural function deficiency. Furthermore, the primary injury of hematoma, contusion, subarachnoid hemorrhage and secondary changes such as inflammatory cell infiltration, edema, cicatricial contracture and porosis were observed in this model. The model reflected pathological changes of SCI completely consistent with other reports on SCI^[16–20]. In addition, the distinctive distribution of lesions in group A and B, and the punctate hemorrhage found in group C. It showed that this animal model could not merely imitate the pathologic change of the existing animal model, but also had distinctive pathological changes, such as slight spinal damage (concussion of the spinal cord)^[21]. In Table 3, analysis of mortality among groups had been carried out, the mortality has significant difference, proving that the quality of coup momentum could cause different mortality. All of the changes including neural function, pathologic change and mortality had consistency to the coup momentum in our experiment, which revealed that severity of SCI could be controlled by adjustable coup momentum.

GFAP is an acidic protein with molecular weight of 50 – 52 kDa. It is a signal skeleton protein of astrocyte and expressed specifically and affluently in astrocyte. When ischemia occurred after injury, astrocyte was induced and synthesized GFAP, so it is a biomarker of astrocyte. In our model, expression of GFAP descended sharply just 1 hour after injury, indicating the visible injury of astrocytic at that time. About 6 hours after injury, glial cell proliferation first appeared in white matter near injured region and dorso-spinal cord under spinal pia mater, appeared near central canal^[22]. What leads to the high expression of GFAP? Nestin was a mark of neural precursor cell in early stage. The fact is that GFAP and Nestin have the same expression domain. It confirmed that the endogenous neural stem cell (ENSC) in spinal cord of adult rat were activated and differentiated into astrocyte consequently under the injured microenvironment, but not neuron. Cheng^[23] reported strong expression of Nestin at central canal vicinity and dorso-spinal cord in rat. Nan^[24] found Nestin expression in adult rat central canal near injured region 24 hours after injury, moreover the cells extend to central spinal cord. Gao^[25] detected positive cells expressing Nestin at adult rat central canal in normal spinal cord, these cells proliferated, budded and migrated and this peaked 8

days after injury. Cao^[26] reported migration of neural stem cell into injured area at week 8 after transplantation. 35% of the survived transplanted cell expressed GFAP, 20% expressed Nestin, none of them expressed NeuN (a sign of mature neurons). It suggested that transplantation of differentiated mature neural precursor cell may contribute to correct differentiation of transplanted cell.

The ENSC distribute in spinal cord indeed, but, the microenvironment changes after injury^[27-31]. ENSC is responsible for neural recovery and could differentiate to astrocyte, these astrocytes have neuroprotective effect and shape into scar tissue by the end, interfere with functional recovery of nervous system.

Seven days after injury, positive expression of GFAP developed from local region to hol-region around injured area, with the RC value firstly decreased and later increased (compare to normal spinal cord, Figure 5), which imply that under the niche or microenvironment after injury, the astrocyte which proliferated from stem cells had migrated to injured region persistently. 28 days after injury, the percentage of astrocyte near injured region was more than that in normal spinal cord. Astrocytes gathered in injured area mostly, contacting each other and formed a grid-like structure, surrounding the injured region and formed the cavity. Around cavity, the islet-like relic neurons surrounded by astrocytes.

In the present experiment, fracture was only found in a small number of samples without obvious displacement. The distinctive distribution of lesions and the punctate hemorrhage found in group C. It was suggested that compressive fracture was by no means essential to SCI, mechanical property of the integral construction consist of spinal cord and its surroundings need to be considered either. Spinal cord, which located in closed vertebra tubes and in viscoplastic state with little hardness^[31,32]. Under the instant violent condition, temple and permanent shifting of bone at the violence point, compression and resilience of vertebra tubes and pressure wave thereafter formed. Damage on the spinal medulla and vascellum, also broke the cell membrane and small vessel wall in wide region, thus formed hematoma, subarachnoid hemorrhage and characteristic contused focus and dispersed punctate hemorrhage of this model. With the theory of hydrostatics and mechanical energy together, the above pathological changes could be explained more completely.

Many complications occurred after injury. Mutual mutilation could be avoided by feeding each rat in a separate cage, decreased intestinal obstruction through diet, intravenous nutrition and abdominal massage, thereby reduce mortality. Intestinal obstruction was the leading cause of the death (Table 4, 66.67%). After injury, transmission-

dysfunction and feces accumulated in the intestine led to mechanical obstruction, and further developed to strangulating intestinal obstruction, resulted in death.

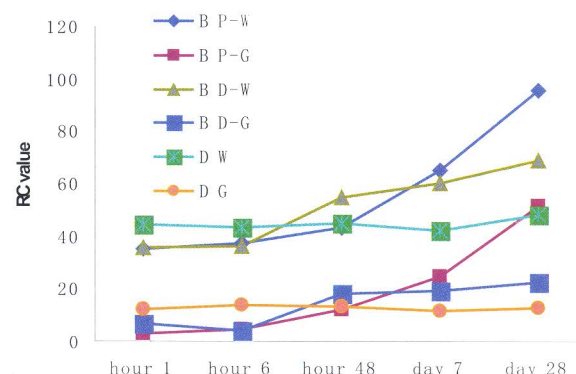


Figure 5. The RC Value of GFAP in normal spinal cord and group B. RC value were to represent the expression of GFAP. It showed gradual improvement in RC value from hour 1 to day 28. The GFAP expression significantly declined at hour 1 to hour 6. Then increased after 6 hours and located mainly in distal injury segment 48 hours later. Eventually, GFAP mainly located to proximal injury segment at day 7 – day 28. P: proximal injury segment. D: distal injury segment. W: white matter. G: gray matter. B: group B. D: group D.

5 Conclusion

Acute ventral closing SCI model could be set up successfully by shape-suitable impinger in simple procedure. Changes of neural function and pathology, different mortality have consistency to the coup momentum. Damage of different degree could be made by adjustable C.M. This model could be regulated and well controlled. Model has distinctive pathological changes. Under the condition of this model, astrocytes were gone through acute damage and proliferated in hyperplasia stage.

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