# Association between injury severity and amyloid β protein levels in serum and cerebrospinal fluid in rats with traumatic spinal cord injury

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Abstract. The aim of the present study was to determine whether amyloid- $\beta$  (A $\beta$ ) protein could be detected in the serum and cerebrospinal fluid (CSF) of rats with traumatic spinal cord injury (SCI) and whether Aß protein levels in serum and CSF correlated with the injury severity. A total of 140 adult female Sprague-Dawley rats were randomly divided into four groups: Sham, mild injury, moderate injury and severe injury. Serum and CSF samples were collected at 12 h, 1, 3, 7, 14, 21 and 28 days post-injury. ELISA analysis for serum and CSF Aß protein was performed. Locomotor function of all animals was assessed using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale. Following SCI, the protein levels of Aβ in serum and CSF samples from SCI groups significantly increased from 12 h (P<0.05) and peaked 3 days after injury. A significant increase of A $\beta$  levels in serum and CSF in the severe SCI group was also observed at 28 days after injury (P<0.05). At 28 days after injury the protein levels of A $\beta$  in serum and CSF were significantly correlated with the severity of injury (serum, R<sup>2</sup>=-0.806, P<0.01; CSF, R<sup>2</sup>=-0.694, P<0.01). A significant correlation between Aß protein level in serum and CSF and neurological deficits (BBB score) was also observed (P<0.01). The protein level of Aβ in serum and CSF

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was severity and time-dependent during the acute phase in rats with traumatic SCI. Monitoring the level of  $A\beta$  protein in serum may improve the evaluation of SCI severity and the neuron functional status following SCI.

### Introduction

Spinal cord injury (SCI) is characterized by sensorimotor deficits below the injury and suffers from a lack of effective treatment. Previous studies have aimed to evaluate the severity and the potential for recovery in patients with traumatic SCI; however, reliable methods for this and prediction of the outcome following traumatic SCI, particularly in the acute stages remain to be elucidated (1,2). The current evaluation methods of injury severity of traumatic SCI is predominantly limited to neurological evaluation and imaging studies, which is often imprecise due to the unstable condition of patients, with occurrences such as spinal shock and the artifacts of metal implants following spinal operations (3-6). This contributes to the difficulty of developing novel treatments for patients with SCI.

Recently, increasing experimental evidence has demonstrated that SCI should be considered a neurodegenerative disease (7,8). A previous study determined that there was degenerative evidence for structural changes in the brain during the early stage of SCI using high-field structural magnetic resonance imaging (MRI) (9). Previous studies revealed that neuron degeneration may be part of SCI pathology. In degenerative diseases, such as multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and Alzheimer's disease, amyloid- $\beta$  (A $\beta$ ) deposition occurs as a marker for neuron degeneration (10-12). Changes in A $\beta$  concentration in serum and cerebrospinal fluid (CSF) were considered to be an indication of the severity of injury to the blood-brain barrier and neuron degeneration in the central nervous system (CNS) (13,14). A recent study reported an increase in  $A\beta$ protein levels in a lesion site of spinal cord in SCI model of rats (15). However, to the best to our knowledge the present study is the first to determine whether  $A\beta$  protein levels in serum and CSF are associated with injury severity in SCI. The present study quantified Aß protein levels in serum and CSF in rats with traumatic SCI. The primary aims were to determine whether A $\beta$  protein was detectable in serum and CSF samples of traumatic SCI and whether A $\beta$  protein level reflected the severity of the injury.

# Materials and methods

*Experimental animals and grouping.* All experiments were performed in accordance with the guidelines established by the Animal Ethics Committee of Chinese PLA Beijing Army General Hospital (Beijing, China). A total of 140 adult female Sprague-Dawley rats (age, 8-9 months; weight, 220-260 g) were purchased from Beijing Haidian Thriving Experimental Animal Center (Beijing, China). All rats were housed in a climate-controlled closed facility with 12 h light/dark cycles at  $24\pm2^{\circ}$ C and free access to food and water for 1 week prior to the experimental procedures. All rats were randomly and equally divided into four groups (n=35 per group).

SCI model induction. Rats were anesthetized via intraperitoneal injection of 10% chloral hydrate (3.0 mg/kg body weight; Shanghai Golden Harvest Biotechnology Co., Ltd., Shanghai, China). The graded contusion model of SCI was performed using the New York University-Multicenter Animal Spinal Cord Injury Study (NYU-MASCIS) impactor as previously described (16). Briefly, a laminectomy was performed at T8 level to expose the spinal cord, and a contusion lesion was produced with the NYU-MASCIS impactor by dropping a 10 g rod onto the exposed dorsal surface of the spinal cord from the following specific heights: i) Mild group (SCI-M), 12.5 mm; ii) moderate group (SCI-Mo), 25 mm; and iii) severe group (SCI-S), 50 mm. Rats in the sham group received laminectomy only. Following the operation, rats were placed back to their cages with heating pads and closely observed until they woke up. As prophylactic for infections, 200,000 U/animal/day penicillin was administered subcutaneously for 3 consecutive days following the operation. Saline was injected subcutaneously immediately following development of the lesion and then daily for 7 days. Food and water were provided ad libitum. Postoperative care included manual expression of bladders twice a day until the rats were sacrificed or bladder function recovered.

*Behavioral testing.* Locomotor functions of all animals were assessed using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale (17). BBB is a 21-point scale used to assess and analyze the hindlimb movements of rats in open-field, at 12 h and 1, 3, 7, 14, 21 and 28 days after the operation. Two investigators, who were blinded to experimental design, assessed the motor function in all the animals.

*ELISA and western blotting.* Rats were sacrificed by intraperitoneal injection of lethal dose of 10% chloral hydrate (30 mg/kg body weight), at 12 h, 1, 3, 7, 14, 21 and 28 days post injury (n=4) after surgery, for the sham group, laminectomy only. Blood samples (5 ml) were taken from the tail vein of the injured rats at 12 h, 1, 3, 7, 14, 21 and 28 days. CSF samples (100  $\mu$ l) were collected by lumbar puncture at the same time point. The blood samples were anticoagulated using ethylenediamine tetraacetic acid and centrifuged at 1,368 x g for 10 min at room temperature to obtain plasma. Plasma and CSF

samples were stored at -80°C prior to analysis of the protein levels of A<sup>β</sup> by ELISA. Proteins for ELISA and western blotting were sequentially extracted as previously described (18), from a total 5 mm spinal cord tissue (2.5 mm rostral and 2.5 mm caudal to the injury site) using diethylamine (DEA) and radioimmunoprecipitation assay buffer. A commercially available kit (rat A $\beta_{1-42}$  ELISA kit) from Xinfan Biotechnology Corporation (Shanghai, China) was used to detect endogenous soluble rat  $A\beta_{1-42}$  as per manufacturer's protocol from the DEA extraction. Anti-A $\beta_{1-42}$  antibody (cat. no. ab10148) for western blotting was purchased from Abcam (Cambridge, UK). Western blot analysis was performed as previously described (19). Total protein (20  $\mu$ g for each) was separated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were first blocked with 5% skimmed milk and then incubated with rabbit anti-A $\beta$  (ab10148; 1:500) or anti- $\beta$ -actin (ab129348; 1:1,000; Abcam) overnight at 4°C. After 5 washes in 1X PBST, membranes were incubated with appropriate horseradish peroxidase conjugated affinity purified secondary antibody (AP187P; 1:1,000; EMD Millipore, Billerica, MA, USA) for 1 h at 37°C, developed using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for detection of proteins. Pixel intensity for each sample and its corresponding  $\beta$ -actin was determined on three different blots using ImageJ software (imagej.nih.gov/ij/). To quantify the results, samples were normalized to  $\beta$ -actin and a bar chart was produced using GraphPad Prism version 5 (GraphPad Software, Inc., San Diego, CA, USA).

Statistical analysis. Data are expressed as the mean ± standard deviation and were analyzed using SPSS version 19.0 (IBM SPSS, Armonk, NY, USA). One-way analysis of variance, followed by the Student-Newman-Keuls test were used to determine the differences between groups. The association between variables was determined by the correlation coefficient method according to the Spearman or Pearson methods. P<0.05 was considered to indicate a statistically significant difference.

# Results

*Behavioral scoring.* Significant differences in the behavioral score using the BBB locomotor rating scale between the SCI groups and the sham group were identified (P<0.05). Higher SCI injury groups had significantly lower behavioral scores (P<0.05; Fig. 1). Over the period of four weeks, gradual recovery was observed in all the SCI groups. Significant motor functional improvement was detected in the mild SCI group when compared with moderate and severe SCI group 12 at 7, 10, 14 and 28 days after SCI (P<0.05).

 $A\beta$  protein levels in serum and CSF of SCI rats. Serum and CSF  $A\beta$  protein levels observed were similar (Figs. 2 and 3). In the sham group, the serum and CSF  $A\beta$  levels were low for all time points. The  $A\beta$  levels in SCI-S group at 12 h after injury were significantly increased compared with the sham group (P<0.05; Fig. 2). At 1 and 3 days after injury, the serum  $A\beta$  protein levels were increased significantly in all SCI groups (P<0.05; Fig. 2). It is of note that the serum  $A\beta$  levels in the SCI-M group were significantly reduced compared with the SCI-Mo and SCI-S



Figure 1. BBB score following SCI (n=4). \*P<0.05, \*\*P<0.01 vs. sham/SCI group. BBB score, Basso, Beattie and Bresnahan score; SCI, spinal cord injury; SCI-M, SCI-mild group; SCI-Mo, SCI-moderate group; SCI-S, SCI-severe group.



Figure 2. A $\beta$  protein levels in serum following SCI (n=4). \*P<0.05 vs. sham/or SCI groups, \*\*P<0.01 vs. SCI-M group. A $\beta$ , amyloid- $\beta$ ; SCI, spinal cord injury; SCI-M, SCI-mild group; SCI-Mo, SCI-moderate group; SCI-S, SCI-severe group.

groups at 3 days (P<0.05; Fig. 2). Subsequently, the serum A $\beta$  levels were gradually reduced in all SCI groups; however, A $\beta$  expression level was significantly higher in the SCI-S group compared with the SCI-M group at 28 days after injury (P<0.05; Fig. 2). The CSF A $\beta$  level in the SCI-S group was significantly higher compared with SCI-M group (P<0.01) and SCI-Mo group (P<0.05) at 1 and 3 days after injury (Fig. 3). At 28 days after injury, A $\beta$  levels in the SCI-S group were significantly higher compared with the SCI-S group were significantly higher compared with the SCI-S group were significantly higher injury, A $\beta$  levels in the SCI-S group were significantly higher compared with the SCI-M and SCI-Mo groups at 28 days after injury (P<0.05; Fig. 3).

 $A\beta$  protein levels in injured spinal cord tissue. To determine whether the increase of  $A\beta$  in injured spinal cord tissue may be associated with the protein expression levels observed in serum and CSF, rats in moderate group (n=4) were sacrificed at 12 h, 1, 3, 7, 14, 21 and 28 days and an ELISA was performed to detect the  $A\beta$  changes in tissues following injury. Over the course of the experiment, changes of  $A\beta$  protein levels in injured spinal cord tissue were similar to those occurred in CSF. The  $A\beta$  protein levels in spinal cord tissues were significantly higher in the SCI-M group at 1 and 3 days after injury compared with the sham group (P<0.05; Fig. 4). Rats in all



Figure 3. Aβ protein levels in cerebrospinal fluid following SCI (n=4). \*P<0.05 vs. sham/or SCI groups. \*\*P<0.01 vs. SCI-M group. Aβ, amyloid-β; SCI, spinal cord injury; SCI-M, SCI-mild group; SCI-Mo, SCI-moderate group; SCI-S, SCI-severe group.



Figure 4. A $\beta$  protein content in injured spinal cord tissue of spinal cord injury-moderate group (n=4). \*P<0.05 vs. sham group. A $\beta$ , amyloid- $\beta$ .

the SCI groups and the sham group (n=4) were then sacrificed 3 days after injury and western blots were performed to detect changes in injured spinal cord tissue. The A $\beta$  protein level in the SCI-S group was significantly higher compared with the SCI-M group at 3 days after injury (P<0.05; Fig. 5).

Correlation analysis between the serum and CSF A $\beta$  levels and BBB score. The serum and CSF A $\beta$  levels peaked at 3 days after injury and a significant difference between the SCI-S group and the SCI-M group was identified (P<0.01; Figs. 2 and 3). The BBB score improved gradually with time and reached the peak at 28 days after injury with a significant difference between the SCI-S group and SCI-M group (P<0.01; Fig. 1). Therefore, a correlation analysis was performed between A $\beta$  levels in serum or CSF and recovery hind limb function (BBB score). The serum and CSF A $\beta$  levels at 3 days after injury were plotted against the BBB score at 28 days after injury for each sample respectively (for the SCI-S, SCI-Mo and SCI-M groups). A significant negative correlation was identified between the two parameters in the A $\beta$  levels in serum (P<0.01; Fig. 6A) and CSF (P<0.01; Fig. 6B).

# Discussion

The present study determined that there was a significant negative correlation between  $A\beta$  protein levels in the CSF and



Figure 5. Aβ protein levels in injured spinal cord tissue at 3 days after injury (n=4). \*P<0.05 vs. sham group. \*P<0.05 vs. SCI-M group. Aβ, amyloid-β; SCI, spinal cord injury; SCI-M, SCI-mild group; SCI-Mo, SCI-moderate group; SCI-S, SCI-severe group.



Figure 6. Correlation analysis of serum and CSF A $\beta$  protein levels with BBB score. Rats from all SCI groups from the BBB measurements at 28 days after injury, were used for the correlation. (A) Correlation of serum A $\beta$  levels at 3 days after injury with BBB score at 28 days after injury (n=12). Pearson R<sup>2</sup>=-0.806; P<0.01. (B) Correlation of CSF A $\beta$  levels at 3 days after injury with BBB score at 28 days after injury (n=12). Pearson R<sup>2</sup>=-0.694; P<0.01. BBB score, Basso, Beattie and Bresnahan score; A $\beta$ , amyloid- $\beta$ ; CSF, cerebrospinal fluid; SCI, spinal cord injury; SCI-M, SCI-mild group; SCI-Mo, SCI-moderate group; SCI-S, SCI-severe group.

serum and the BBB score of rats with traumatic spinal cord injury.

Spinal cord injury results in mortality and morbidity and currently there are a limited number of effective treatments. The association between inflammation and secondary damage led to the use of a high dose of methylprednisolone therapy within 8 h of injury, and the common surgical procedures made by physicians are stabilization and decompression of spinal cord (2). Successful management of SCI requires an appropriate diagnostic standard following injury, particularly in the first 3 days, which is the acute phase. Neurological examinations provide a general indication of spinal cord neurological function; however, they may be unreliable, particularly during the acute phase following SCI due to spinal shock in patients (2). Neurophysiological examinations, such as determination of somatosensory evoked potentials and motor evoked potentials, are rarely performed in the acute phase due to their low sensitivity at this time (1). MRI has been established as the gold standard for anatomical SCI diagnosis; however, it may not always be able to distinguish neuronal tissue necrosis from apoptosis and edema (5,6).

Serum biomarker examination may provide a quantitative approach for the assessment of real-time tissue injuries and may be useful for making clinical diagnoses. CSF is in direct contact with the CNS and the spinal cord, which may reflect the biochemical state of the central and periphery nervous system under different physiological and pathological conditions (4). Therefore, CSF may be regarded as a potential source for identifying biomarkers for neurological diseases and trauma. A previous study demonstrated that quantifying the blood or CSF level of neuron- or astroglia-specific proteins may be a more sensitive way to assess the severity of traumatic brain injury (TBI) (7). It has been proposed that CSF, which surrounds and protects the brain and spinal cord from traumatic injury, may be a potential target for the diagnosis of a variety of conditions, including TBI and SCI, in addition to Alzheimer's disease, amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS) (10-12). Due to the complexity of biofluids; however, considerable developments are required in the analytical techniques used in order to achieve comprehensive coverage of the proteins present in CSF samples. An ideal biomarker should be exclusively intracellular and present in high concentrations.

A $\beta$  has long been used as potentially useful diagnostic biomarker for brain injury. The A $\beta$  peptide is produced by proteolytic cleavage of amyloid precursor protein (APP) by two different enzymes,  $\beta$ - and  $\gamma$ -secretase (20,21). A $\beta$  is derived from the transmembrane APP by proteolytic processing during normal cell metabolism and secreted into the CSF, so the CSF A $\beta$  can serve as the foundation for an A $\beta$  biomarker. The 42-amino acid form of A $\beta$  (A $\beta_{1-42}$ ) is of special interest, as it may have the greatest propensity to deposit into insoluble plaques, which is the pathological characteristic of Alzheimer's disease. Additionally, it may also aggregate into oligomeric A $\beta$  species, reported to be particularly neurotoxic (22-24). Increased level of APP,  $\beta$ - and  $\gamma$ -secretase, and A $\beta$  have been observed with the onset of numerous other neurological disorders, including ALS, MS and microglia activation and associated neuroinflammation (10,11,25,26). When the axon is severely injured, APP and  $A\beta$  are released into the extracellular space via the damaged membrane. Brody et al (13) reported an increase of APP and AB levels in the CSF and peripheral blood following TBI and the increases were positively associated with the severity of injury; however, they were negatively correlated with the functional outcome (13). The purpose of the current study was to determine whether  $A\beta$ protein expression level may be used as a diagnostic marker for SCI in an animal model.

The rat model of SCI in the present study was modified from the methods described by Wrathall et al (27). The severity of SCI was positively associated with the height from which the weight was dropped. The current study did not identify any significant differences between the AB protein levels in the sham group and the SCI-M and SCI-Mo groups 12 h after injury. However, a significant increase in serum and CSF Aß protein levels was observed in the SCI-S group at 12 h. Aβ protein levels peaked at 3 days after injury in all SCI groups, therefore it may accurately reflect the severity during the acute phase of SCI. A previous study suggested that the accumulation of  $\beta$ -APP and increase of A $\beta$  peptide in injured spinal cord tissue were accompanied by axonal dysfunction within 1 day of SCI (28), with a peak at 3 days after injury (29), which is similar to the findings of the present study demonstrated by ELISA and western blotting. The serum and CSF A $\beta$  levels changed similarly with the A $\beta$  level in injured spinal cord tissue, which determined that the  $A\beta$  levels in biofluids may predict the alterations that occur in injured tissue. High levels of A $\beta$  in the CSF may reflect the reduced clearance of APP and the increased deposition of  $A\beta$  in injured spinal cord tissue. At 12 h after injury, although A $\beta$  protein level in the SCI-S group was higher compared with in the sham group, the graded SCI groups did not differ significantly until 1 day after injury. These results are consistent with previous studies on SCI (15,28,29). SCI-S group was significantly different when compared with the SCI-M and SCI-Mo groups at 28 days after injury in the serum and CSF, which may indicate a long-lasting axonal transport destruction and deposition of AB protein as a result of cleavage of APP in the lesion site. By contrast to Pajoohesh-Ganji *et al* (15) where the A $\beta$  expression in injured spinal cord tissue returned to baseline levels at 7 days after injury, the present study revealed the long-lasting increase of  $A\beta$  levels in serum and CSF in the SCI-S group may be due to severe destruction of spinal cord and blood-brain barrier, whereas Aß levels in SCI-M and SCI-Mo groups were consistent with the findings of Pajoohesh-Ganji et al (15).

In conclusion, the present study indicated that serum and CSF A $\beta$  protein level alterations are time-dependent. The peak in the concentration of the biomarker may reflect the mechanical disruption of the spinal cord and blood-brain barrier. The concentrations of the biomarker were negatively correlated with the BBB score. The current study suggested that  $A\beta$  protein may be used as a specific biomarker for SCI diagnosis. This biomarker may also be used for screening of degenerative changes following SCI.

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