

Neuroprotective effect of ceftriaxone in a rat model of traumatic brain injury

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Abstract Traumatic brain injury (TBI) is a leading cause of mortality and disability in children and young adults worldwide. Neurologic impairment is caused by both immediate brain tissue disruption and post-injury cellular and molecular events that worsen the primary neurologic insult. The β -lactam antibiotic ceftriaxone (CTX) has been reported to induce neuroprotection in animal models of diverse neurologic diseases via up-regulation of GLT-1. However, no studies have addressed the neuroprotective role of CTX in the setting of TBI, and whether the mechanism is involved in the modulation of neuronal autophagy remains totally unclear. The present study was designed to determine the hypothesis that administration of CTX could significantly enhance functional recovery in a rat model of TBI and whether CTX treatment could up-regulate GLT-1 expression and suppress post-TBI neuronal autophagy. The results demonstrated that daily treatment with CTX attenuated TBI-induced brain edema and cognitive function deficits in rats. GLT-1 is down-regulated

following TBI and this phenomenon can be reversed by treatment of CTX. In addition, we also found that CTX significantly reduced autophagy marker protein, LC3 II, in hippocampus compared to the TBI group. These results suggest that CTX might provide a new therapeutic strategy for TBI and this protection might be associated with up-regulation of GLT-1 and suppression of neuronal autophagy.

Keywords TBI · Glutamate excitotoxicity · GLT-1 · Ceftriaxone · Autophagy · Functional recovery

Introduction

Traumatic brain injury (TBI) is a leading cause of mortality in the young aged population and is one of the major reasons for hospital admissions in modern life [1]. Pathological cerebral edema is one of the leading causes of death shortly following TBI with very few therapeutic options. Furthermore, mechanical disruption of neurons triggers a cascade of events leading to neuronal cell death, impaired motor and cognitive functions following TBI [2]. During the last decade, multiple studies have demonstrated that glutamate is the major excitatory neurotransmitter in the brain, with up to 40 % of all synapses being glutamatergic [3]. Accumulation of excess extracellular glutamate and subsequent overstimulation of glutamatergic receptors increase the production of reactive and excitotoxic oxygen/nitrogen species, which induce oxidative stress leading to neuronal death [4]. It has been demonstrated that glutamate transporter subtype 1 (GLT-1; EAAT2) is one of the major glutamate transporters expressed predominantly in astroglia cells responsible for 90 % of total glutamate uptake, and it is

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essential for maintaining low extracellular glutamate, preventing glutamate neurotoxicity [5].

After the pioneer work of Rothstein in relation to GLT-1 mediated neuroprotection by ceftriaxone (CTX) in an animal model of amyotrophic lateral sclerosis disease [6], several other groups instantly studied and tried to investigate the possible action mechanism of CTX in several models, including Huntington disease, stroke, Parkinson's disease, adult motor neuron disease, and spinal muscular atrophy opioid dependence [7–11]. All these studies strongly suggested that the beneficial neuronal effects of CTX were specifically due to up-regulation of GLT-1. However, no experiments have been done in the setting of TBI.

Autophagy is an evolutionarily conserved pathway that leads to degradation of proteins and entire organelles in cells undergoing stress [12]. Although autophagy sometimes harnesses the apoptotic machinery for cell suicide, it is also clear that autophagic cell death can be executed independently of an intact apoptotic machinery [13]. Previous data demonstrated that TBI activated autophagy and increased microtubule-associated protein 1 light chain 3 (LC3) immunostaining mainly in neurons [14]. Afterwards, a recent study suggested that excessive autophagy is a contributing factor of neuronal death in cerebral ischemia and hypoxia [15], but whether CTX treatment is involved in the autophagic neuronal death following TBI remains totally unclear.

In this study, we examined the effect of CTX on post-TBI brain edema and spatial cognitive function in rat model. GLT-1 expression and neuronal autophagy were also assessed. The results would provide an evidence on CTX-mediated neuroprotection in a rat model of TBI.

Materials and methods

Animals and TBI model

All experimental procedures were approved by Hebei Medical University Committee for the use and care of animals in research and were in accordance with the guidelines of the Chinese Council on Animal Protection. A total of 150 male Sprague–Dawley rats (aged 10–12 weeks old and weighing 300–330 g, Tangshan, China) were used in the present study. All the rats were free access to water and food, and housed with a standard 12-h light/dark cycle. The rat model of TBI was induced using a modified weight-drop device, as previously described by Marmarou [16]. Briefly, rats were anesthetized with sodium pentobarbital (Nembutal 60 mg/kg) before the surgery. We made a midline incision to expose the skull between bregma and lambda suture lines, and a steel disk (10 mm in diameter and 3 mm thickness) was adhered to the skull using dental acrylic. Afterwards, rats were placed on a foam mattress

underneath a weight-drop device in which a 450 g weight falls freely through a vertical tube from 1.5 m onto the steel disk. Sham-operated animals underwent the same surgical procedure without the weight-drop impact. All the rats were housed in individual cages following surgery and placed on heat pads (37 °C) for 24 h to maintain normal body temperature during the recovery period.

Groups and drug administration

Rats were randomly divided into three groups: sham-operated group (sham, $n = 30$); TBI group (TBI, $n = 60$); and TBI treated with CTX group (CTX, $n = 60$). 200 mg/kg CTX (Shandong Qilu Pharmaceutical, Jinan, China) was given daily intraperitoneal injection in CTX group for 5 days beginning immediately after TBI. Both sham and TBI groups received equal volumes of 0.9 % saline daily injection.

Evaluation of brain edema

Brain edema was evaluated by measuring brain water content with the wet-dry weight method as described previously [17]. Animals were sacrificed by decapitation under deep anesthesia at 1, 3 and 5 days following TBI or sham surgery. Brains were harvested and weighed immediately to get wet weight. Following drying in a desiccating oven for 24 h at 100 °C, dry tissues were weighed again. The percentage of water content in the tissues was calculated according to the formula: % brain water = [(wet weight – dry weight)/wet weight] × 100.

Assessment of spatial learning ability

The spatial learning ability was assessed in a Morris water maze as described previously [18]. The Morris water maze consists on a black circular pool (180 cm diameter, 45 cm high) filled with water (30 cm depth) at 26 °C and virtually divided into four equivalent quadrants: north (N), west (W), south (S) and east (E). A 2-cm submerged escape platform (diameter 12 cm, height 28 cm, made opaque with paint) was placed in the middle of one of the quadrants equidistant from the sidewall and the center of the pool. Rats were trained to find the platform before TBI or sham surgery. For each trial, the rat was randomly placed into a quadrant start points (N, S, E or W) facing the wall of the pool and allowed a maximum of 60 s to escape to a platform, rats which failed to escape within 90 s were placed on the platform for a maximum of 20 s and returned to the cage for a new trial (inter-trial interval 20 s). Maze performance was recorded by a video camera suspended above the maze and interfaced with a video tracking system (HVS Imaging, Hampton, UK). The average escape latency

of a total of five trials was calculated. This test was conducted at 1, 3 and 5 days following TBI or sham surgery.

Western blot analysis

Western blotting was conducted as described previously [19]. Briefly, rats were anesthetized and underwent intracardiac perfusion with 0.1 mol/L phosphate-buffered saline (PBS; pH 7.4). The hippocampus of brain was rapidly isolated, total proteins were extracted and protein concentration was determined by the BCA reagent (Solarbio, Beijing, China) method. Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Separated proteins on the gel were transferred onto PVDF membranes (Roche Diagnostics, Mannheim, Germany). Blots were blocked with 5 % fat-free dry milk for 1 h at room temperature. Following blocking, the membrane was incubated with indicated primary antibodies overnight at 4 °C, including rabbit anti-GLT-1 polyclonal antibodies (Santa Cruz Biotechnology; Santa Cruz, CA, USA, diluted 1:500), rabbit anti-LC3 polyclonal antibody (Santa Cruz Biotechnology; Santa Cruz, CA, USA, diluted 1:500), mouse anti- β -actin monoclonal antibody (Santa Cruz Biotechnology; Santa Cruz, CA, USA, diluted 1:500). And then incubated with horseradish peroxidase conjugated anti-rabbit IgG and anti-mouse IgG (Cell Signaling Technology, Inc., Danvers, MA, USA, diluted 1:5000) for 2 h at room temperature. Following incubation with a properly titrated second antibody, the immunoblot on the membrane was visible after development with an enhanced chemiluminescence detection system and densitometric signals were quantified using an imaging program. Immunoreactive bands of all proteins expression were normalized to intensity of corresponding bands for β -actin. The Western blot was conducted at 1, 3 and 5 days following TBI, and results were analyzed with National Institutes of Health Image 1.41 software (Bethesda, MD, USA).

Statistical analysis

Data are expressed as mean \pm standard error and analyzed using SPSS 16.0 (SPSS, Chicago, IL). Statistical analysis was performed using ANOVA and followed by the Student–Newman–Keuls post hoc tests. Statistical significance was inferred at $P < 0.05$.

Results

CTX treatment attenuates brain edema

Traumatic brain injury induced a significant increase in brain edema at 1, 3 and 5 days compared to the sham group

(Fig. 1), and CTX treatment significantly reduced brain edema following TBI ($P < 0.05$, vs. TBI).

CTX treatment improves the learning and memory ability

Since CTX could attenuate brain edema, we next examined whether CTX treatment could improve spatial learning function assessed by Morris water maze at 1, 3 and 5 days following TBI or sham operation. As shown in Fig. 2, TBI caused a significant spatial learning deficit at 1, 3 and 5 days compared to the sham group, and administration of CTX significantly reduced the escape latency at 3 and 5 days compared to the TBI group.

CTX treatment increases GLT-1 expression in hippocampus

GLT-1 protein expression in hippocampus was analyzed by Western blot analysis at 1, 3 and 5 days after surgery. As demonstrated in Fig. 3, there was a significant down-regulation of GLT-1 expression in the TBI group compared to the sham group. And treatment with CTX caused remarkably elevation of GLT-1 at 3 and 5 days compared to the TBI group, the GLT-1 protein level was peaked at 5 days.

CTX treatment suppresses neuronal autophagy in hippocampus

As recent study demonstrated that autophagy marker protein LC3 II expression was remarkably elevated following TBI [20], we examined whether CTX treatment could

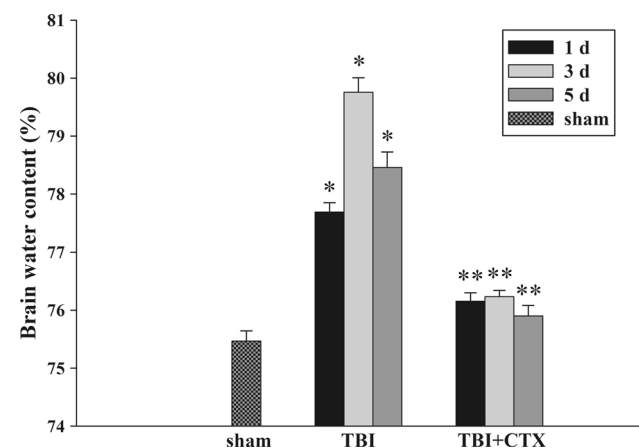


Fig. 1 The effect of CTX on brain edema. Brain water content was measured at 1, 3 and 5 days following TBI. Bars represent mean \pm standard error ($n = 5$, per group). Brain water content increased markedly at 1, 3 and 5 days following TBI (* $P < 0.01$ vs. sham group). Treatment of CTX significantly decreased brain edema (** $P < 0.05$ vs. TBI group), as reflected by a decrease in brain water content

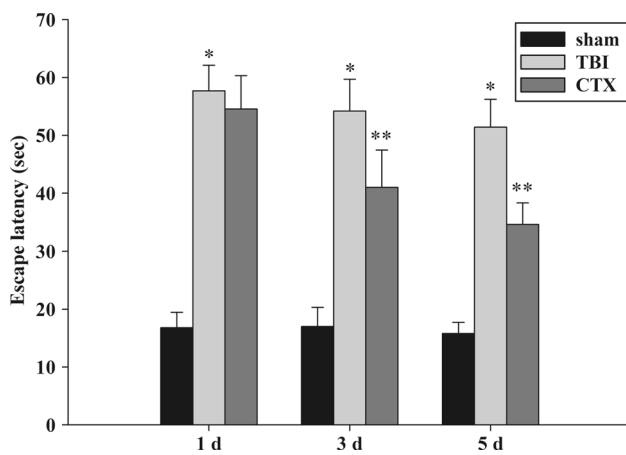


Fig. 2 The effect of CTX on the escape latency performance. Morris water maze was performed at 1, 3 and 5 days following TBI or sham surgery. Bars represent mean \pm standard error ($n = 5$). The escape latency increased remarkably at 1, 3 and 5 days following TBI ($*P < 0.01$ vs. sham group). Treatment with CTX significantly reduced the time to find the platform at 3 and 5 days after injury ($**P < 0.05$ vs. TBI group)

reduce the expression of LC3 II at 1, 3 and 5 days following TBI determined by Western blot analysis. As shown in Fig. 4, at 1 and 3 days following TBI, administration of CTX significantly attenuated LC3 II protein expression in rat hippocampus compared to the TBI group.

Discussion

In this study, we found that CTX has a neuroprotective potential in a rat TBI model. Post-TBI CTX treatment (200 mg/kg/day, up to 5 days) could reduce posttraumatic brain edema and improve learning and memory ability. These results are similar to earlier studies reported that CTX treatment could afford neuroprotection against a variety of neurologic disorders, especially acute brain injury [10, 11]. GLT-1 is the most abundant glutamate transporter in brain, and plays a principal role in keeping the glutamate concentration low by removing the glutamate released at the synapse. Furthermore, a recent study demonstrated that focal overexpression of the glutamate transporter, GLT-1, significantly reduced ischemia-induced glutamate overflow, decreased cell death and improved behavioral recovery in a rat model of stroke [21]. In the present study, we have confirmed that GLT-1 down-regulation is induced following TBI and this phenomenon can be reversed by administration of the CTX. This may be one of the mechanisms of which CTX exerts its neuroprotective activity on TBI.

Furthermore, it is worth noting we also found that following injury, administration of CTX could suppress neuronal autophagy in hippocampus of rat brain. Interestingly,

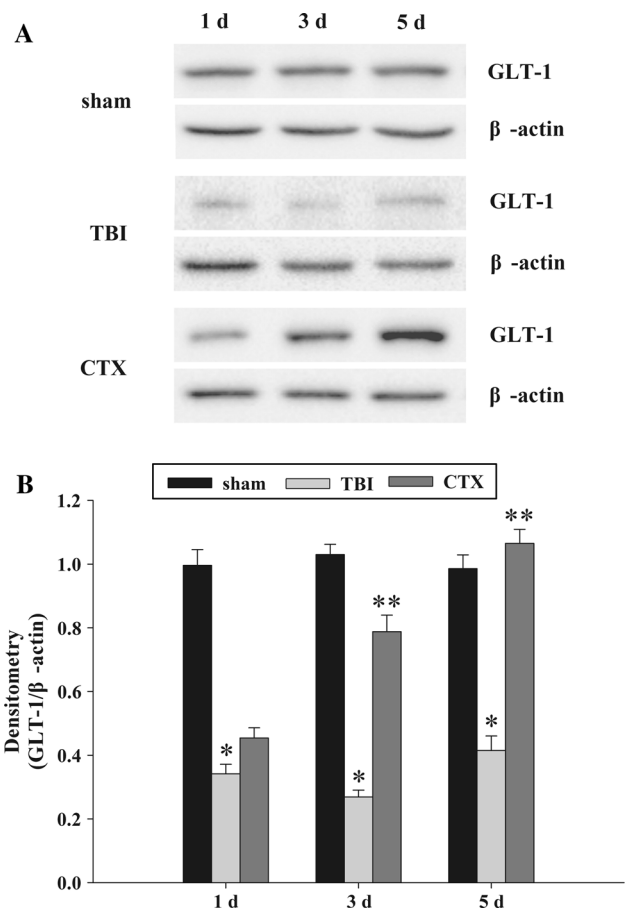


Fig. 3 The effect of CTX on GLT-1 expression. **a** Western blot shows levels of GLT-1 and β -actin in the hippocampus of rats at 1, 3 and 5 days following TBI or sham surgery. **b** The quantitative results of GLT-1 are expressed as the ratio of densitometries of GLT-1 to β -actin bands. Data were expressed as mean \pm standard error ($n = 5$ per group). Results demonstrated a significant down-regulation of GLT-1 expression in the TBI group ($*P < 0.01$ vs. sham group). Treatment with CTX caused significant elevation of GLT-1 expression at 3 and 5 days after injury ($**P < 0.01$ vs. TBI group)

the role of neuronal autophagy after acute brain injury remains uncertain and controversial [14, 20]. Erlich [20] demonstrated that rapamycin, which induces neuronal autophagy via inhibition of mammalian target of rapamycin, improved neurological outcome following TBI. However, Lai et al. [14] have shown that the antioxidant γ -glutamylcysteinyl ethyl ester not only reduced neuronal autophagy but also improved neurological outcome. In the present study, our results are in line with the finding of Lai, and it is therefore conceivable to hypothesize that the neuroprotection of CTX on TBI might be associated with attenuation of neuronal autophagy, which is a contributing factor of neuronal death [22]. Nevertheless, the neuroprotective mechanism of CTX-induced attenuation of neuronal autophagy in hippocampus remains unresolved. Recent study indicated that autophagic stress was induced in the rat hippocampus following kainic acid administration [23].

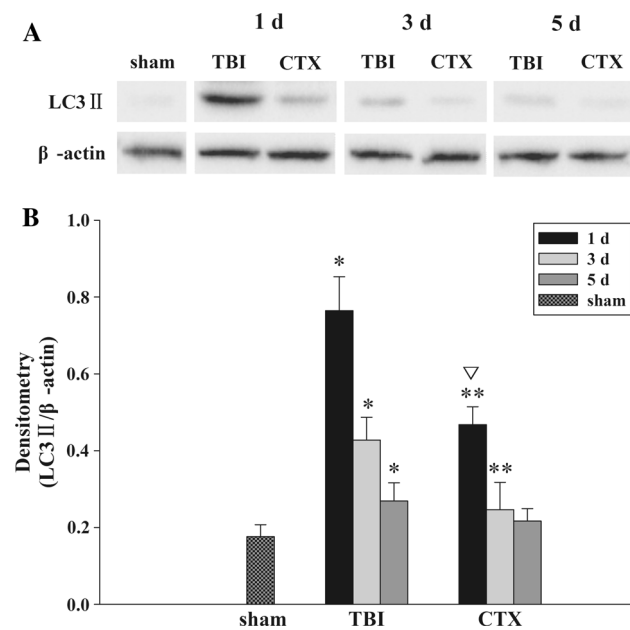


Fig. 4 The effect of CTX on LC3 expression. **a** Western blot shows levels of LC3 and β -actin in hippocampus at 1, 3 and 5 days following TBI or sham surgery. **b** Densitometry of LC3 II band related to β -actin band. Data were expressed as mean \pm standard error ($n = 5$ per group). Results demonstrated that LC3 II protein expression was significantly elevated at 1, 3 and 5 days following TBI ($*P < 0.05$ vs. sham group). Administration of CTX significantly decreased the level of LC3 II protein expression at 1 and 3 days ($**P < 0.05$ vs. TBI group), but the level of LC3 II protein was still higher compared with untreated rats at 1 day ($\nabla P < 0.05$ vs. sham group)

And in our study, we hypothesize that the suppression of neuronal autophagy might be a responder of anti-excitotoxicity via GLT-1 up-regulation. On the other hand, one way in which CTX can affect neuronal and astrocytic functions, irrespective of the potential for increasing GLT-1 expression, relates to some anti-inflammatory action. Recently, Wei et al. [24] have shown that single dose of CTX could reduce the multiplex inflammatory cytokine levels (interleukin-1 β , interferon- γ , and tumor necrosis factor- α) following TBI. We also hypothesize that the autophagic pathway might be influenced by CTX via down-regulating of inflammatory cytokine as neuronal autophagy was suppressed via CTX treatment in hippocampus of rat brain. In fact, although the mechanism of neuroprotection by CTX remains unknown, on the basis of our data we could admit that autophagy is recruited as a physiologic response to protect neurons from insults derived by TBI, thus acting with a synergic mechanism together with CTX. Therefore, this topic requires further investigations.

In addition, CTX is the most widely used antibiotic has no substantial toxic central nervous system actions at normal antibacterial dose. Since the drug has been safely

used for a long time, the pharmacokinetics of CTX and its pattern of adverse reactions are universally known. Furthermore, CTX may easily cross the blood–brain barrier. It is strongly suggested that CTX is an attractive potential drug candidate for TBI. However, the usual adult daily dose of CTX is 1–2 g given once daily in adults, and 50–100 mg/kg once daily in pediatric patients. Thus, the dose used in this study (200 mg/kg per day) is relatively high as compared with clinical practice. Nevertheless, it has been demonstrated that the concentration for 50 % of maximal effect required to increase GLT-1 expression by CTX is 3.5 $\mu\text{mol/L}$ (6), which is comparable to the known central nervous system levels attained during therapy for meningitis (0.3–6 $\mu\text{mol/L}$) [25]. Therefore, normal CTX doses are likely to be sufficient to up-regulate GLT-1 and attenuate neuronal autophagy following TBI in humans.

In summary, the results of the current study demonstrated that post-injury administration of CTX could enhance cognitive functional recovery and attenuate brain edema. Afterwards, CTX treatment caused remarkable elevation of GLT-1 and suppresses neuronal autophagy induced following TBI in rats. Furthermore, the study also suggests that CTX, an attractive potential drug, might provide a novel clinical efficacy on TBI.

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