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Potassium Aspartate Attenuates Brain Injury Induced by Controlled Cortical Impact in Rats Through Increasing Adenosine Triphosphate (ATP) Levels, Na^+/K^+-ATPase Activity and Reducing Brain Edema

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Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Potassium aspartate (PA), as an electrolyte supplement, is widely used in clinical practice. In our previous study, we found PA had neuroprotective effects against apoptosis after cerebral ischemia/reperfusion in rats. In this study, we examine whether PA has protective effects on traumatic brain injury (TBI).

Material/Methods: TBI was induced by controlled cortical impact (CCI) in rats. Vehicle treatment (control) or PA treatment was administered intraperitoneally at 30 minutes after CCI. The modified neurological severity score (mNSS) and cortical lesion volume were examined. Brain edema and blood-brain barrier (BBB) integrity were measured, as well as brain ATP contents, lactic acid levels, and Na^+/K^+-ATPase activities.

Results: We found that CCI induced cortical injury in rats. Acute PA treatment at the dose of 62.5 mg/kg and 125 mg/kg significantly improved neurological deficits ($p<0.05$ and $p<0.001$, respectively) and decreased the cortical lesion volume ($p<0.05$ and $p<0.001$, respectively) compared with vehicle-only treatment. PA treatment at the dose of 125 mg/kg attenuated brain edema and ameliorated BBB integrity. In addition, PA treatment significantly reduced the loss of ATP ($p<0.01$), reduced lactic acid levels ($p<0.001$), and increased the activity of Na^+/K^+-ATPase ($p<0.01$).

Conclusions: Our results indicate PA has neuroprotective effects on TBI through increasing ATP levels, Na^+/K^+-ATPase activity, and reducing brain edema. It provides experimental evidence for the clinical application of PA.

MeSH Keywords: Aspartic Acid • Brain Injuries • Myosins

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Background

Traumatic brain injury (TBI) is the leading cause of death and disability in young people [1]. TBI-induced functional deficits can be caused by both primary and secondary insult. Primary insult results from direct biomechanical forces; secondary injury evolves over a period of hours to days and is the result of complicated pathophysiological events that ultimately lead to neuronal cell death [2]. These pathophysiological events include mitochondria functional impairment, imbalance of ion homeostasis, and brain edema. The mitochondria functional impairment causes reduced oxidative phosphorylation and decreased ATP levels [3]. The gradual decrease in ATP disturbs the activity of ion pumps such as Na^+/K^+-ATPase. The decreased Na^+/K^+-ATPase activity after TBI causes dysregulation of cell volume and osmolality and results in cytotoxic brain edema, accounting for astrocytes and neuronal swelling [4]. Effective therapy may require targeting multiple pathophysiological events after TBI.

Potassium aspartate (PA), as an electrolyte supplement, is widely used in clinical practice. Potassium is an important intracellular cation involved in a variety of enzymatic reactions. Aspartic acid, which is a precursor of oxaloacetate *in vivo*, plays an important role in the citric acid cycle, and participates in ATP generation. Aspartic acid has a strong affinity to cells and can increase the K^+ influx [5]. Excessive K^+ efflux and intracellular K+ depletion are key early steps in apoptosis. Our previous study found that PA had neuroprotective effects against apoptosis after cerebral ischemia/reperfusion in rats [6]. In this study, we wanted to know whether PA had neuroprotective effects on TBI through decreasing ATP levels, increasing Na^+/K^+-ATPase activity, and reducing brain edema. There are many models used for TBI research. We selected controlled cortical impact (CCI) as the study TBI model. Compared to other models, CCI has many advantages, such as being more accurate, easier to control, and most importantly, inducing TBI similar to that seen in humans [7].

Material and Methods

CCI model in rats

The current study was approved by the Animal Ethics Committee of Beijing Neurosurgical Institute. All animal experimental procedures were performed in accordance with the ARRIVE Guidelines [8]. Male Spraque-Dawley rats, weighing 300–330 g (3 months old, specific pathogen free (Beijing Vital River Laboratory Animals Technology Co., Ltd., Beijing, China), were kept in a 12/12 hour light/dark cycle with free access to food and water *ad libitum*. Experimental CCI was induced as described previously [9]. The rats were anesthetized with chloral hydrate (400 mg/kg, intraperitoneal) and placed in a stereotaxic frame (#68508, RWD Life Science Co., Shenzhen, China). Following a midline incision, a craniotomy of about 2.5 mm radius was performed. The skull disk was removed without disturbing the dura. CCI was performed using the PinPoint™ PCI3000 Precision Cortical Impactor (Hatteras Instruments, Cary, NC 27518, USA) with a velocity of 1.5 m/s, reaching a depth of 2.0 mm, and remaining in the brain for 85 ms. Body temperature was maintained at 37°C using a warming pad. After injury, the bone flap was immediately replaced and the scalp was sutured closed. Sham-operated rats received identical surgical procedures except for the CCI.

Experimental groups and treatment

In the first part of the experiments, the rats were divided randomly into six groups: (1) sham, no CCI; (2) vehicle, normal saline, 1 mL/kg; (3) PA (bulk drug, 5 g/vial, Batch No. 100602, Liaoning Union Pharmaceutical Co., Ltd., Shenyang, China), 10 mg/kg; (4) PA, 25 mg/kg; (5) PA, 62.5 mg/kg; (6) PA, 125 mg/kg. These rats were used for evaluation of neurological function, cortical lesion volume, brain edema, and blood-brain barrier (BBB) integrity. In the second part of the experiments, rats were randomly assigned to three groups treated with normal saline or PA: (1) sham, no CCI; (2) vehicle, normal saline, 1 mL/kg; (3) PA, 125 mg/kg. These rats were used for the measurement of brain ATP content, lactic acid levels, and Na^+/K^+-ATPase activity. For all experiments, drugs were administrated intraperitoneally 30 minutes after CCI, at a volume of 1 mL/kg.

Neurological function

Neurological function was evaluated by modified neurological severity score (mNSS, Table 1), an 18-point scale that was based on the presence of certain reflexes and the ability to perform motor and behavioral tasks such as beam walking, beam balance, and spontaneous locomotion [10]. The scores were evaluated before CCI and at 24 hours and 72 hours after CCI by an examiner blinded to the experimental groups.

Cortical lesion volume

The cortical lesion volume was determined by hematoxylin and eosin (H&E) staining. At 72 hours after CCI, the rats were anesthetized by chloral hydrate and perfused transcardially with normal saline, followed by 4% paraformaldehyde. Starting at +1.2 mm and ending at –4.8 mm anterior from the bregma [11], coronal brain sections (5 μm) at an interval of 500 μm were obtained and a total of 10 sections of each brain were processed for H&E staining. Images of all sections were photographed with the Leica Upright Microscope System (DM4000 B LED, Leica, Wetzlar, Germany) and analyzed by National

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Table 1. Modified neurological severity score points.

Motor tests	6
Raising rat by tail	3
Flexion of forelimb	1
Flexion of hindlimb	1
Head moved >10° to vertical axis within 30 s	1
Placing rat on floor (normal=0; maximum=3)	3
Normal walk	0
Inability to walk straight	1
Circling toward paretic side	2
Falls down to paretic side	3
Sensory tests	2
Placing test (visual and tactile test)	1
Proprioceptive test (deep sensation, pushing paw against table edge to stimulate limb muscles)	1
Beam balance tests (normal=0; maximum=6)	6
Balances with steady posture	0
Grasps side of beam	1
Hugs beam and 1 limb falls down from beam	2
Hugs beam and 2 limbs fall down from beam, or spins on beam (>60 s)	3
Attempts to balance on beam but falls off (>40 s)	4
Attempts to balance on beam but falls off (>20 s)	5
Falls off; no attempt to balance or hang on to beam (<20 s)	6
Reflex absence and abnormal movements	4
Pinna reflex (head shake when auditory meatus is touched)	1
Corneal reflex (eye blink when cornea is lightly touched with cotton)	1
Startle reflex (motor response to a brief noise from snapping a clipboard paper)	1
Seizures, myoclonus, myodystony	1
Maximum points	18

One point is awarded for inability to perform the tasks or for lack of a tested reflex: 13–18, severe injury; 7–12, moderate injury; 1–6, mild injury.

Institutes of Health ImageJ 1.48v software. The volume of the ipsilateral and contralateral hemisphere was calculated using the following formula:

$$\sum\left(A_n + A_{n+1}\right) \times d / 2 \ ,$$

where A is the corresponding hemispheric area and d is the distance between sections. The total lesion volume was calculated by numerical integration of a difference between contralateral hemispheric volume and ipsilateral hemispheric volume.

Brain edema

Brain edema was determined by water content measurement. We measured water content in the ipsilateral hemisphere by the wet-dry weight method, as described previously [12]. Briefly, rats were killed 24 hours after CCI under 10% chloride hydrate anesthesia. The ipsilateral hemisphere was dissected and the surface of the sample was gently blotted with tissue paper to remove adsorbent cerebrospinal fluid. The sample was weighed as wet weight, then dried in a 120°C incubator for 24 hours. Dried tissue was weighed as dry weight after cooling. Tissue water content (%) was calculated as follows: (wet weight – dry weight)/wet weight ×100%.

Blood-brain barrier (BBB) integrity

BBB integrity was determined by assessing extravasation of Evans blue (EB) dye, as previously described [13]. Briefly, 2% EB was injected (4 mg/kg, intravenous) at 24 hours after CCI and allowed to circulation for 2 hours. To remove the intravascular dye, rats were perfused transcardially with saline until clear perfusion fluid was found. Then the rats were decapitated and their brains were removed. The ipsilateral hemisphere

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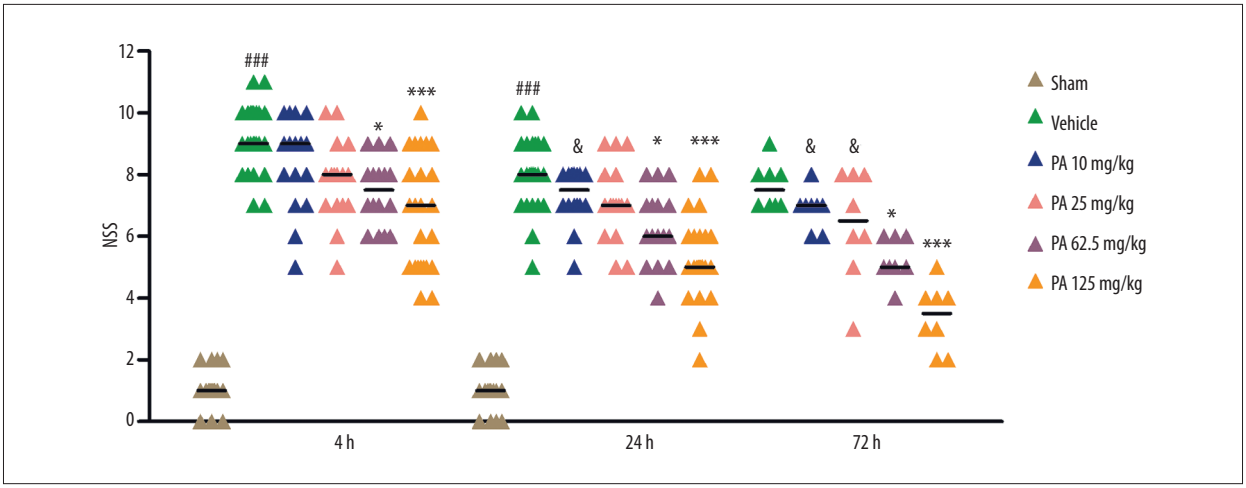

Figure 1. Effects of PA on NSS after CCI. Vehicle or PA was administrated intraperitoneally 30 minutes after CCI, and NSS was performed at 4 hours, 24 hours, and 72 hours after CCI. Data are presented as scatterplots, with bar as the median. n=8. * $p<0.05$ *vs.* vehicle-treated group, *** $p<0.001$ *vs.* vehicle-treated group, & $p<0.05$ *vs.* PA 125 mg/kg group, ### $p<0.001$ *vs.* sham-operated group.

was weighed and 5 mL of formamide (Sigma) was added, then the tissue was incubated at room temperature for 72 hours. The supernatant was centrifuged at 4°C for 15 minutes at 12,000 g and transferred to 96-well black plates. The concentration of EB was determined at 620 nm excitation/683 nm emission with a fluorescence multi-well plate reader (Infinite M200, TECAN, Salzburg, Austria). The tissue was dried in an oven at 120°C for 24 hours and weighed again. The tissue EB content was quantified from a linear standard curve derived from known amounts of the dye and was normalized to tissue weight (μg/g).

Brain ATP, lactic acid levels and Na^+/K^+-ATPase activity

At 24 hours after CCI, the rats were anesthetized and decapitated. The ipsilateral hemisphere was dissected and homogenized. Homogenate buffer (0.01 mol/L Tris, 0.0001 mol/L EDTA, 0.01 mol/L sucrose, 0.9% NaCl, pH 7.4) was added to the samples to obtain a 10% tissue homogenization. The homogenate was centrifuged at 3,500 rpm for 10 minutes at 4°C, and the supernatant was collected to measure ATP and lactic acid levels, as well as Na^+/K^+-ATPase activity, determined by a corresponding assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Statistical analysis

All data were presented as mean ± standard error of the mean (SEM), with n indicating the number of rats studied. Differences between groups were analyzed by one-way analysis of variance with a post hoc Dunnett's Multiple Comparison Test. $p<0.05$ was considered significant.

Results

PA reduced neurological severity score after CCI

The modified neurological severity score (mNSS) was used for neurological function assessment after CCI in rats. Compared to a sham group, the vehicle-treated and PA-treated rats showed a significantly increased neurological severity score at 24 hours and 72 hours after CCI (Figure 1). Compared to the vehicle-treated rats, the PA-treated (62.5 mg/kg and 125 mg/kg) rats showed significantly improved scores ($p<0.05$ and $p<0.001$, respectively, Figure 1), while PA-treated (10 mg/kg and 25 mg/kg) rats showed improved tendency, but no significant difference was found at 24 hours and 72 hours after CCI. Compared to the 10 mg/kg and 25 mg/kg PA-treated groups, 125 mg/kg PA treatment showed reduced neurological severity scores at 24 hours and 72 hours after CCI.

PA attenuated CCI-induced cortical lesion volume

The rats were sacrificed at 72 hours after CCI for histological measurement. CCI induced significant cortical lesions. In vehicle-treated rats, the infarct volume was 30.86±2.15 mm^3 (Figure 2A, 2F). PA treatment of 62.5 mg/kg and 125 mg/kg significantly decreased the lesion volume compared with vehicle treatment ($p<0.05$ and $p<0.001$, respectively, Figure 2D–2F). PA treatment of 10 mg/kg and 25 mg/kg showed a tendency toward reduced lesion volume, but no significant difference was found compared with vehicle treatment (Figure 2B, 2C, 2F).

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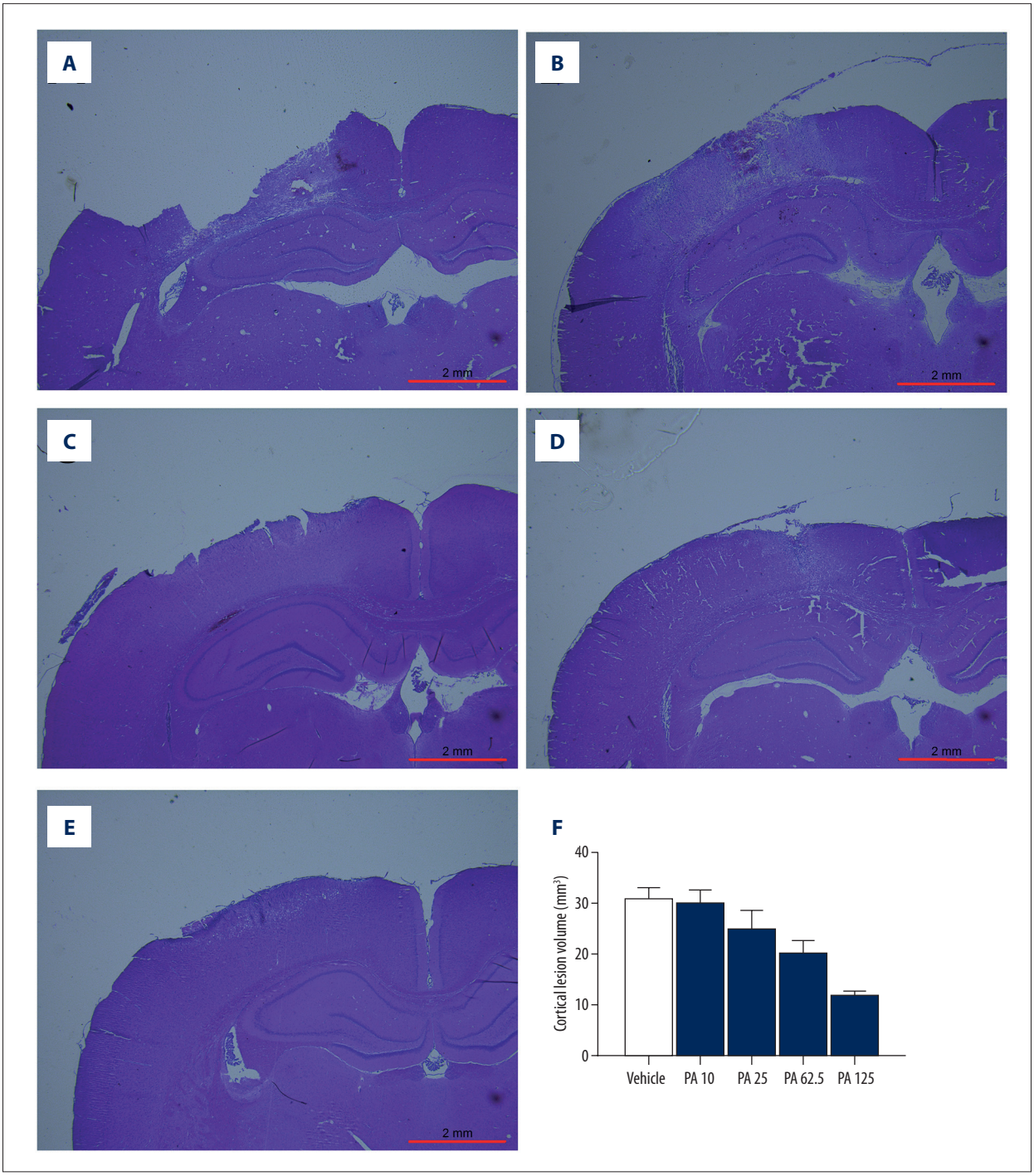

Figure 2. Effect of PA on cortical lesion volume as evaluated by H&E staining 72 hours after CCI. Representative photographs of 5-μm-thick coronal slices (at –3.2 mm from bregma) with H&E staining were showed in **A–E**. (**A**) Vehicle group; (**B**) PA 10 mg/kg group; (**C**) PA 25 mg/kg group; (**D**) PA 62.5 mg/kg group; (**E**) PA 125 mg/kg group. There is a considerable lesion in the injured hemisphere of the vehicle-treated rats, and the lesion in PA-treated rats is much smaller. Scale bar, 2.0 mm. (**F**) Measurement of cortical lesion volume by HE staining in each group (n=8). CCI induced the significant loss of hemispheric tissue, which was reduced in PA-treated groups. Data are expressed as mean ±SEM. * $p<0.05$ *vs.* vehicle-treated group, *** $p<0.001$ *vs.* vehicle-treated group.

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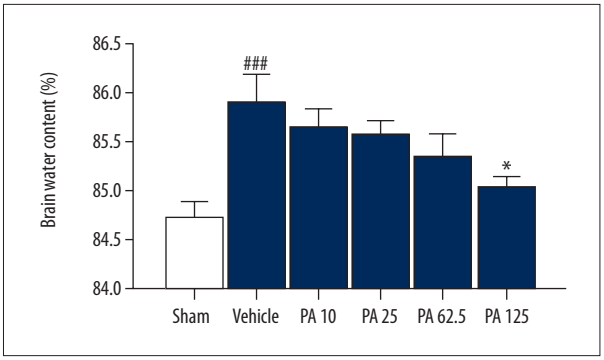

Figure 3. Effects of PA on brain water contents after 24 hours CCI. Vehicle or PA was administrated intraperitoneally 30 minutes after CCI. The results of water contents are given as percentages and data are presented as mean ±SEM. n=8. * $p<0.05$ *vs.* vehicle-treated group, ### $p<0.001$ *vs.* sham-operated group.

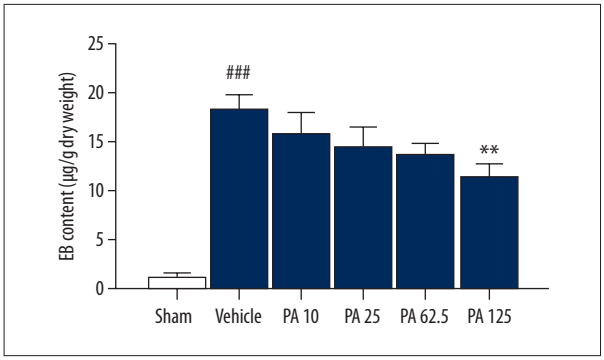

Figure 4. Effects of PA on BBB integrity after 24 hour CCI. Vehicle or PA was administrated intraperitoneally 30 minutes after CCI. Integrity of the BBB is presented by contents of Evans blue (EB) dye, and data are given as mean ±SEM. n=8. ** $p<0.01$ *vs.* vehicle-treated group, ### $p<0.001$ *vs.* sham-operated group.

Table 2. The effect of PA on ATP, LA levels and Na^+/K^+-ATPase activity after 24 h CCI.

	n	Sham	Vehicle	PA
ATP (μmol/g prot)	8	33.25±1.92	15.01±2.30#	25.87±2.59*
LA (mmol/g prot)	8	0.47±0.01	0.57±0.01#	0.46±0.02**
Na^+/K^+-ATPase (U/mg prot)	8	9.76±0.59	6.24±0.33#	8.25±0.37*

# P<0.001 *vs.* sham-operated group; * P<0.01 *vs.* vehicle-treated group; ** P<0.001 *vs.* vehicle-treated group.

PA reduced CCI-induced brain edema

Brain edema was determined by measuring brain water content at 24 hours after CCI. CCI significantly increased brain water content. Compared to the vehicle-treatment group, PA treatment of 10 mg/kg, 25 mg/kg, and 62.5 mg/kg showed a tendency toward reduced water content, but no significant difference was found. PA treatment of 125 mg/kg markedly reduced water content compared with the vehicle-treatment group ($p<0.05$, Figure 3). However, there was no significant difference among the groups treated with different doses of PA.

PA ameliorated BBB integrity in CCI rats

BBB integrity was determined by assessing EB extravasation at 24 hours after CCI. CCI significantly increased brain EB content. Compared to the vehicle-treatment group, PA treatment of 10 mg/kg, 25 mg/kg, and 62.5 mg/kg showed a tendency toward reduced brain EB content, but no significant difference was found. PA treatment of 125 mg/kg markedly reduced brain EB content compared with the vehicle-treatment group ($p<0.05$, Figure 4). However, there was no significant difference among the groups treated with different doses of PA.

PA increased brain ATP, decreased lactic acid levels and increased Na^+/K^+-ATPase activity after CCI

Compared to sham-operation rats, vehicle-treatment rats showed low ATP levels, increased lactic acid levels, and decreased Na^+/K^+-ATPase. PA treatment (62.5 mg/kg) significantly increased ATP levels, decreased lactic acid levels, and increased the Na^+/K^+-ATPase activity compared with vehicle treatment (Table 2).

Discussion

In the current study, we found that acute, post-injury administration of potassium aspartate (PA) markedly improved neurological sensorimotor function at 24 hours and 72 hours, and significantly attenuated cortical lesion volume at 72 hours after TBI-induced CCI in rats. This is the first report regarding the ability of PA to improve neurobehavioral function and attenuate cortical lesion volume in an experimental model of TBI. The current results suggest that PA has a neuroprotective effect on TBI. The study further showed that PA has neuroprotective effects on TBI through increasing ATP levels, Na^+/K^+-ATPase activity, and reducing brain edema.

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In the first part of the experiment, we investigated the dose-response of PA treatment on neurobehavioral function, cortical lesion volume, brain edema, and BBB integrity. Our preliminary experiments found that the median lethal dose (LD_{50}) of PA was 1,250 mg/kg via intraperitoneal injection. The doses of 10 mg/kg, 25 mg/kg, 62.5 mg/kg, and 125 mg/kg are 1/125, 1/50, 1/20 and 1/10 of LD_{50} PA, respectively. Thus, the doses are within a safe range. Our results showed that 62.5 mg/kg and 125 mg/kg PA improved neurological deficits and decreased the cortical lesion volume, while 125 mg/kg PA, but not 62.5 mg/kg PA, attenuated brain edema and ameliorated BBB integrity. Therefore, we speculated that the neuroprotective effect of 62.5 mg/kg of PA may not result in amelioration of BBB integrity and brain edema. In addition, the neuroprotective dose-response of PA on TBI was similar to our previous study in a cerebral ischemia model [6].

In the second part of the experiment, we investigated the effects of PA on brain ATP content, lactic acid levels, and Na^+/K^+-ATPase activity after CCI. Our results revealed that 62.5 mg/kg PA treatment 30 minutes after CCI increased brain ATP, decreased lactic acid levels, and increased Na^+/K^+-ATPase activity. Previous reports showed an "ischemia-like" pattern after TBI that was related to energy depletion and Ca^{2+} homeostasis. In our study, CCI decreased ATP content and increased lactic acid levels, which was consistent with previous reports [14]. Inhibition of PA affect on reduced ATP and increased lactic acid levels suggested PA may act on an "ischemia-like" pattern induced by TBI, but the details of the mechanism need further investigation. The depletion of ATP will cause failure of energy-dependent membrane ion pumps, such as Na^+/K^+-ATPase. Na^+/K^+-ATPase is a ubiquitous plasma membrane protein which plays a key role in the maintenance of intracellular electrolyte homeostasis [15]. It is known that TBI impairs Na+/K+-ATPase activity and that the amelioration of Na+/K+-ATPase activity is beneficial to the treatment of TBI [16]. Our results showed CCI decreased Na+/K+-ATPase activity and PA ameliorated the reduced Na+/K+-ATPase activity. Therefore, the ameliorative effect of PA on Na+/K+-ATPase activity is one of the neuroprotective mechanisms of PA treatment.

The two parts of the experiments showed that PA had neuroprotective effects on TBI through decreasing ATP levels, increasing Na^+/K^+-ATPase activity, and reducing brain edema. The protective effects of PA may be related to potassium and aspartate. Previous studies demonstrated that TBI causes loss of K^+ conductance, which results in the failure of K^+ homeostasis, which in turn promotes abnormal neuronal function [16,17]. Filippidis et al. found a significant injury-induced decrease in [Na^+] and increase in [K^+]e [18]. K^+ efflux causes abrupt neuronal depolarization. Aspartic acid has a strong affinity with cells and can increase the K+ influx. Therefore, PA may reduce TBI through regulation of K^+ homeostasis. In addition, aspartate takes part in the citric acid cycle and the urea cycle. The carbon skeleton of oxaloacetate from the citric acid cycle (in the mitochondrion) is carried to the glyoxysome in the form of aspartate [19]. Aspartate is converted to oxaloacetate, which condenses with acetyl-CoA derived from fatty acid breakdown. Aspartate formed in mitochondria by transamination between oxaloacetate and glutamate can be transported to the cytosol, where it serves as nitrogen donor in the urea cycle reaction catalyzed by argininosuccinate synthetase. So increased ATP generation may be related to the neuroprotective effects of PA.

Klatzo described two major types of brain edema in the 1960s: the cytotoxic or vasogenic edema related to intracellular or extracellular water accumulation due to cellular injury, or BBB breakdown [20]. Our results demonstrated that PA can decrease BBB permeability and improve brain edema after CCI in rats. Previous studies showed that rises in intracellular and extracellular concentrations of lactate can eventually lead to acidosis. The acidosis accompanied cell membrane damage and increased permeability, altered or inhibited cellular function, and caused partial breakdown of the BBB and cerebral edema [21]. PA affects brain function possibly through reducing the lactic acid levels and protecting the BBB against disruption.

Conclusions

PA has been used for clinical therapy as an electrolyte supplement and should have a good safety profile. In this study, we found PA had a neuroprotective effect on TBI through decreasing ATP levels, increasing Na^+/K^+-ATPase activity, and reducing brain edema. This study provides experimental evidence for the clinical application of PA.

Conflicts of interest disclosure

None.

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