Neuroprotective actions of taurine on hypoxic-ischemic brain damage in neonatal rats

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Highlights

1. Taurine, a natural amino acid, exerts neuroprotective effect against cerebral hypoxic-ischemic damage in neonatal rats.
2. Taurine has demonstrated anti-oxidative effect on cerebral hypoxic-ischemic damage in neonatal rats.
3. Taurine regulates the Bcl-2/Bax balance on cerebral hypoxic-ischemic damage in neonatal rats.
4. Taurine inhibits AIF and Cyt c over expression on cerebral hypoxic-ischemic damage in neonatal rats.
5. Taurine may have potential as a little side-effect, safe and effective protective agent for newborn hypoxic ischemic encephalopathy.

Abstract

Taurine is an abundant amino acid in the nervous system, which has been proved to possess antioxidation, osmoregulation and membrane stabilization. Previously it has been
demonstrated that taurine exerts ischemic brain injury protective effect. This study was designed to investigate whether the protective effect of taurine has the possibility to be applied to treat neonatal hypoxic-ischemic brain damage. Seven-day-old Sprague-Dawley rats were treated with left carotid artery ligation followed by exposure to 8% oxygen to generate the experimental group. The cerebral damage area was measured after taurine post-treatment with 2,3,5-triphenyltetrazolium chloride (TTC) staining, Hematoxyline-Eosin (HE) staining and Nissl staining. The activities of superoxide dismutase (SOD), malondialdehyde (MDA), glutathione peroxidase (GSH-Px), total antioxidant capacity (T-AOC), myeloperoxidase (MPO), ATP and Lactic Acid productions were assayed with ipsilateral hemisphere homogenates. Western-blot and immunofluorescence assay were processed to detect the expressions of AIF, Cyt C, Bax, Bcl-2 in brain. We found that taurine significantly reduced brain infarct volume and ameliorated morphological injury obviously reversed the changes of SOD, MDA, GSH-Px, T-AOC, ATP, MPO, and Lactic Acid levels. Compared with hypoxic-ischemic group, it showed marked reduction of AIF, Cyt C and Bax expressions and increase of Bcl-2 after post-treatment. We conclude that taurine possesses efficacious neuroprotective effect after cerebral hypoxic-ischemic damage in neonatal rats.

Key words: Taurine; Neonatal; Hypoxic-ischemic; Neuroprotection; cell death; oxidative stress

1. Introduction

Neonatal hypoxic-ischemic brain damage (HIBD) is a relatively common malignant complication caused by clinical perinatal asphyxia in infants and young children (Chen et al., 2015; Thatipamula et al., 2015), which occurs in 1 to 6 of every 1,000 live term births(Gu et al., 2016; Koonrungsesomboon et al., 2014). Statistics suggests that approximately 40% of the affected infants die in the neonatal period and an additional 30% have lifelong neurological deficits including cerebral palsy, epilepsy and cognitive disabilities (Hristova et al., 2016). The treatment and care for the sequelae of HIBD require extensive resources. What is unfortunate is that current treatment regimens are not optimal, even remained ineffective. Moreover, even with the best care, these children only have little improvement in the overall ability. Accordingly, HIBD is a major public health issue which globally leads to substantial socio-economic burden of the individual, family and healthcare system (Ding et al., 2016). Altogether, efficient pharmacological strategies for the sanitation and therapy of HIBD are restricted by safety and toxicity considerations. Given the magnitude of the problem, it is urgent and unmet to focus on how to supply safe and effective neuroprotective medicine, which would promote prognosis of HIBD infants by promoting after-injury repair.

Although the precise pathogenesis of HIBD is still inconclusive, it is valid that oxidative stress, oxidative metabolism and apoptosis are the significant components of cell death following HIBD (Deanna L.Taylor et al., 1999; Ding et al., 2016). Oxidative stress and oxidative metabolism play a pivotal role in the procedure of pathogenesis after the occurrence of HIBD which ultimately trigger cell death. Additionally, on account of the immature brains possess fairly high polyunsaturated fatty acid concentration, meanwhile low concentration of
antioxidants, they are considered to be particularly prone to tissue damage due to oxidative stress after hypoxia-ischemia injury (Zhang et al., 2014). Pathological accumulation of excessive reactive oxygen species (ROS) and subsequent oxidative stress results in brain necrosis and apoptosis (Chang et al., 2016). Endogenous antioxidants such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), possess the ability of scavenging overproduction of oxidants to prevent deleterious ROS generation (Chen et al., 2015; Zhao et al., 2015).

Apoptosis is a form of cellular suicide which is essential for development and tissue homeostasis of all metazoan organisms (Guo et al., 2002). Mitochondrion as a key organelle is the main source of cellular ATP which may also regulate cell death (David J. Granville et al., 2001). It is demonstrated that mitochondria are involved in cell death based on experimental stroke (Sun et al., 2011). A mechanism pertaining to the death of immature neurons is the accumulation of AIF and Cyt c, which is safely sequestered within the mitochondrial intermembrane space in non-apoptotic cells (Guo et al., 2002). On the other hand, anti-apoptotic protein Bcl-2, also known as a mitochondrial membrane protein, blocks the apoptotic death of many cell types (Deanna L. Taylor et al., 1999; Gu et al., 2016), it plays a crucial role in regulation of mitochondria-mediated cell death. Therefore, anti-apoptotic therapies via inhibiting AIF and Cyt c expression and modulating the actions of Bcl-2 family proteins have been proposed to be useful in ameliorating neonatal HIBD.

Taurine, a β-amino acid, presents high concentration in the mammalian tissues, which possesses a number of cytoprotective properties through its actions as neurotransmitter, neuromodulator, osmoregulator, anti-oxidant, membrane stabilizer, anti-inflammation and neuroprotection (El Idrissi, 2008; Georgia B and Park., 2004; Haas and Hosli, 1973; Hussy et al., 1997; Huxtable, 1989; Huxtable, 1992). In recent years, taurine has been demonstrated to function neuroprotective activity in various kinds of in vitro and in vivo brain injury models. It has been reported that taurine reduces caspase-8 and caspase-9 expression in ischemia injury (Taranukhin et al., 2008) and intracellular calcium elevation, as well as depresses calpain activation, thereby attenuating glutamate-induced apoptotic neuronal death and enhancing Bcl-2:Bax ratio. Furthermore, in vivo taurine has been verified to protect brain against experimental stroke in a dose dependent manner with marked protection (Sun et al., 2011; Sun et al., 2012a; Sun et al., 2012b; Wang et al., 2007).

However, no information is available on possible effects of taurine in neonatal brain injury induced by hypoxia-ischemia. It was speculated that taurine might exert a protective effect on neonatal HIBD under this background. To test the hypothesis, the present experiment was designed to investigate the potential neuroprotective effects of post-insult administration of taurine on neonatal HIBD using the neonatal hypoxic-ischemic rat model as well as to further identify its underlying mechanisms.

2. Materials and Methods

2.1. Experiment animals
Female Sprague-Dawley rats with 7-day-old neonates were supplied by the Experimental Animal Center of Ningxia Medical University (Certificate number was SYXK Ningxia 2015-0001). The animals were housed in the temperature-controlled environment (22-24°C) under 12h light and dark cycles and animals had access to food and water ad libitum. The experimental designs and all procedures were in accordance both with the National Guidelines for Care and Use of Laboratory Animals, together with the Animal Care Guidelines issued by the Animal Experimental Committee of by Ningxia Medical University. All surgeries were performed under diethyl ether anesthesia. All efforts were made to minimize suffering, thereby reducing the number of animals used.

2.2. Construction of HIBD Model

The HIBD model was established according to literatures. The method to create hypoxic-ischemic brain damage in the 7-day-old rat is based on the Levine preparation in the adult rat (Vannucci and Vannucci, 2005) except for rat in sham group, the concrete processes were operated as hereunder mentioned. The newborn rats of both sexes were narcotized by ether inhalation, a small incision was made in the left side of the neck, the thyroid, vein, and nerve tissues were stripped to expose the left common carotid artery, cut between double ligatures with 6-0silk surgical suture, and then sutured. Each surgery was completed within 5 min. The neonates were sent back to their cages with their mothers for 1.5 h. After recovery, pups were placed in a lower oxygen tank to maintain constant temperature (37°C) under continuous hypoxia with 8% oxygen/balance nitrogen gas in the container for 2 h. After hypoxic exposure, all surviving pups were returned to their cages with their mothers until they were sacrificed. Animals in sham group were randomly selected from the same litters of hypoxic-ischemic rats, and then treated with anesthesia followed by exposing their left carotid artery without undergoing hypoxic-ischemic.

2.3. Drug administration

For evaluating the effects of taurine on neurological deficits, brain swelling, neutrophil infiltration and infarct volume, the rats were randomly assigned to five groups treated with taurine (Sigma, St. Louis, MO, U.S.A.) dissolved in normal saline (0.9%NaCl) before using. Drugs were administrated intraperitoneally (i.p.) in volume of 0.1ml/10g body weight and administered 15 min prior to testing. Pups of mixed sex from different litters were randomly divided into the following groups (n=48, for each group):

- Sham (sham surgery) with NS group;
- HI (cerebral hypoxic-ischemic) with NS group;
- HI (cerebral hypoxic-ischemic) with taurine (30 mg/kg) group;
- HI (cerebral hypoxic-ischemic) with taurine (60 mg/kg) group;
- HI (cerebral hypoxic-ischemic) with taurine (120 mg/kg) group;

Taurine was given by intraperitoneal injection every 12 h for two consecutive days after HI. Sham and HI groups were treated with saline under the same conditions.

2.4. Measurement of Infarct Volume
Pups (n=6, for each group) were anesthetized and euthanized 48 h after HI treatment. Rat brains were removed and sectioned coronally with a self made blade at 2-mm intervals and incubated for 30 min at 37°C in a 2% solution of 2,3,5-Triphenyltetrazolium chloride (TTC) (Sigma, St. Louis, MO, U.S.A.) followed by overnight immersion in 4% formaldehyde solution. The TTC stained sections were recorded with a digital camera to measure the infarct volumes. Areas which were not stained red with TTC were considered injured, calculated by the image-analysis software (Image-Pro plus, USA). Since brain edema might significantly affect the accuracy of infarct estimation, the exact infarct volume was calculated (Lin et al., 1993; Swanson et al., 1990). Brain swelling was determined by subtracting the total volume of the nonischemic hemisphere from that of the ischemic hemisphere (Lin et al., 1993).

2.5. Histopathologic Analysis

In summary, neonatal rats (n=6, for each group) were sacrificed at forty-eight hours after HI, and transcardially perfused with physiological saline from the heart to the systemic circulation until the liver appeared white, followed by 4% paraformaldehyde solution of 4°C. The brains were removed and immersed in the 4% formaldehyde solution overnight at 4°C overnight. Afterwards, the brains were processed through graded alcohols and xylene. By using a brain matrix, the forebrain was cut coronally into two equally spaced slices and embedded in paraffin blocks after detaching the hindbrain. The brain sections located at 1.5 mm behind the bregma in the coronal plane were cut into 5 mm sections with vibratome (Leica, Solms, Germany) and mounted on the glass slides. The sections were deparaffinized in xylene and rehydrated in 100% to 70% gradient ethanol. Finally, they were stained with hematoxylin-eosin (HE staining) or Nissl staining. The slices were washed with double distilled water, dehydrated in ethanol, cleaned with xylene, and examined with light microscopy. The pictures were taken with a digital camera. The sections corresponding to the plate 35 contained the brain atlas were selected for analysis. The images were captured using the computer assisted image analyzer system consisting of a microscope (Olympus BX-51, Tokyo, Japan) magnification at ×400 and ×200. The necrotic cells in the cerebral cortex and the hippocampus CA3 were evaluated by the five-point score that was described previously: 0=normal; 1=damaged neurons were <25%; 2=damaged neurons were 25–50%; 3=damaged neurons were 50–75%; and 4=damaged neurons were >75% (Sun et al., 2011).

2.6. Determination of oxidative stress indicators

The levels of SOD, GSH-Px, T-AOC and MDA in brain were estimated 48 h after HI. The neonatal rats (n=6, for each group) were executed, the ischemic hemispheres were quickly removed and washed in chilled saline, then stored at-80°C. The biochemical analyses were performed within 48h.
10 min. Afterwards, the supernatant was collected to employ for this test. The protein concentration of tissues, the SOD and GHS-Px activities, as well as the T-AOC capability were measured with the microplate as described (1510; Thermo Fisher Scientific, Waltham, MA, USA) according to the instructions of the assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). One unit of the enzyme activity was defined as the quantity of SOD required for inhibiting the cytochrome c reduction rate by 50%. The absorbance change at 550nm was monitored. SOD activity is expressed as units/mg protein. The GSH-PX activity was defined as the GSH-PX in 1mg protein that led to the decrease of 1 mmol/l glutathione (GSH) in the reactive system as one unit. Therefore, the expression of GSH-PX activity was evaluated as units/mg protein. In recent studies, T-AOC has been verified to be a reflection of the enzymatic and nonenzymatic antioxidants, both of which are the criteria for measuring the overall cellular endogenous antioxidative capability. The chromogenic reagent 2,20-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) was implemented (ABTS) with maximal absorbance at 414 nm to estimate all these antioxidants. The T-AOC was expressed as units/mg protein. According to manufacturer’s instruction, BCA method was utilized for protein analysis.

2.6.2. Measurement of lipid peroxidation

Malondialdehyde (MDA) was estimated according to Ohkawa’s method. 200mL of brain homogenate supernatant was added to 50mL of 8.1% sodium dodecyl sulfate, vortexed then incubated for 10 minutes at room temperature. A volume of 375mL of thiobarbituric acid (6%) was added and placed in an boiling water bath for 60 minutes, and then the samples were allowed to cool at room temperature. A mixture of 1.25mL of butanol:pyridine (1.5:1) was added, vortexed and centrifuged at 1000 rpm for 5 minutes. The optical density of the colored layer was measured at 532 nm on a spectrophotometer against reference blank, the rate of MDA formation was expressed as nanomoles of the MDA formed per hour per milligram of protein.

2.7. Determining the activity of MPO

The MPO activity in the homogenate was determined by MPO activity assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). One unit (U) of MPO activity was defined as the amount that degrades 1mol hydrogen peroxide at 37°C normalized to the wet tissue weight (U/g wet tissue). The results were expressed as the percentage of the levels in sham-operated rats.

2.8. Measurement of brain ATP and lactic acid levels

The ATP and LA levels were determined by ATP Assay Kit and Lactic acid Assay Kits (Nanjing Jiancheng Bioengineering Institute, China) according to the instructions.

2.9. Immunofluorescence analysis

For observing the expression of AIF, Bcl-2, Bax and Cyt C, we took three consecutive
coronal sections (5 mm) from the ischemic core region using vibratome (Leica, Solms, Germany). Immunofluorescences for ischemic brain tissue were performed on paraffin sections which were prepared using the same method of hematoxylin-eosin (HE) staining. Paraffin-embedded coronal brain sections were subjected to deparaffinization, rehydration followed by microwave oven antigen retrieval (microwave method). The brain sections were incubated with anti-AIF, anti-Bcl-2, anti-Bax and anti-Cyt C as the primary antibodies (AIF, 1:200; Bcl-2, 1:500; Cyt C, 1:100; Abcam, U.K. Bax, 1:200 Proteintech Group, USA) at 4°C overnight. The sections were washed with PBS and incubated for 1 h at 37°C with TRITC-labeled Goat Anti-Rabbit IgG in the next day (1:200; Proteintech Group, USA). The cover slips were stained by 40,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. All samples were prepared in a single batch. Negative controls received an identical treatment except for the primary antibody and showed no positive signal. For immunostaining analysis, the images were captured with Olympus FV1000 confocal microscope to examine the stained brain sections, then utilized a digital camera to photograph all the immunofluorescence images of AIF, Bcl-2, Bax and Cyt C, respectively. The fluorescence images of AIF, Bcl-2, Bax and Cyt C of rat brain sections were randomly photographed (×400 and×200) by a single investigator who was blind to sample.

2.10. Western blot analysis

The neonatal rats (n=6, for each group) were decapitated and ischemic brains were rapidly collected. The prepared brain tissues were homogenized in 1:10 (w/v) ice-cold protein extraction buffer in glass homogenizers. Soluble proteins were collected and centrifuged at 12,000 g for 10 min at 4°C, and then the supernatant was used to detect the level of AIF, Bcl-2, Bax, Cyt C and the total protein. The protein concentration of the samples was determined by BCA Protein Assay reagent kit. Equal amount of protein lysate (50μg) in each group was separated by 8% and 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins on the gel were subsequently transferred onto a nitrocellulose membrane (260mA, 2 h). The membranes were blocked with PBST containing 5% skim milk for 2 h at room temperature and then incubated with primary rabbit monoclonal antibody respectively overnight at 4°C(AIF, 1:100; Bcl-2, 1:200; Cyt-C,1:200; Abcam, U.K Bax, 1:500 Proteintech Group, USA). The membranes were then washed and incubated with secondary antibody (anti-rabbit IgG, 1:3000; Proteintech Group, USA) for 1.5 h at room temperature. The anti-actin antibody (1:1000; Proteintech Group, USA) served as control. The protein bands were visualized with enhanced chemiluminescence reagents (ECL), and the signal densitometry was quantified by an observer blinded to the groups of the animals using western blotting detection system (Quantity One, Bio-Rad Laboratories, USA).

2.11. Statistical analysis

All analyses were performed using SPSS 17.0 Statistical Software (Chicago, IL). The data were presented as mean ± SEM. The statistical differences between more than two groups were assessed with one-way ANOVA followed by post hoc test. The two-group data were analyzed by unpaired t test. A value of P<0.05 was considered statistically significant.
3. Results

3.1. Taurine provides neuroprotection after hypoxia-ischemia

3.1.1. TTC staining
Representative coronal brain sections from vehicle and taurine-treated rats stained with 1% TTC are illustrated in Figure 1A, normal brain tissues appeared uniform red while the infarction region showed white. There was no remarkable cerebral infarct area in the sham-operated group rats, and post-treatment with taurine (30, 60 and 120 mg/kg) group significantly reduced the percentage of infarction to 21.43±1.84% (P<0.05), 17.10±2.88% (P<0.01), 10.93±1.65% (P<0.01) when versus the HI group 24.09±1.29% (Fig.1.B).

3.1.2. Effects of taurine on necrotic cell death
HE and Nissl staining were performed to investigate the morphology of necrotic cells, and the results were illustrated in Fig.2B. In the sham-operated group, morphology of neurons in cortex and hippocampus CA3 region remained intact, the nucleus and cytoplasm were centered with clear staining with the neurons remained well-arranged. In the vehicle-treated group (Fig.2.B.b), neuron arrangement was disordered with loosened and vacuolar neural fiber. A large number of neurons appeared shrunken, swollen and karyopyknosis, even died with disappearance of Nissl’s body in the cytoplasm and the lack of cellular structure. While, the extent of degeneration and necrosis of nerve cells in taurine-treated group was significantly lower, in contrast to the higher number of intact neurons significantly than those in the vehicle-treated group.

3.2. Effect of taurine on altered redox status induced by hypoxic ischemic
To demonstrate the effect of taurine on the oxidative stress induced by HI, MDA contents and SOD activities were measured 48 h after HI injury. As shown in Fig.3, the level of MDA significantly increased in the ischemic hemisphere of HI group compared with the sham group (P<0.01), however, after the administration of taurine (120 mg/kg), MDA content significantly attenuated compared with HI group (Fig.3A, P<0.01). The result indicated that SOD, GSH-Px, and T-AOC enzyme activities significantly decreased in the HI group compared with the sham group (all P<0.01), however, the taurine (30, 60, 120 mg/kg) treatment group remarkably restored their activities in a dose-dependent manner, especially at the dose of 120 mg/kg (Fig.3B-D).

3.3. Effects on brain ATP and lactic acid levels
The ATP and lactic acid levels of vehicle-treated rats were significantly lower than those of sham-operated rats (all P< 0.01, Fig. 4), while taurine (120 mg/kg) treatment showed significantly increased ATP and lactic acid levels compared with vehicle treatment (P < 0.01).

3.4. Effect of taurine on MPO activity induced by hypoxic ischemic
The effects of taurine on the MPO activity induced by HI were investigated by the level of MPO which was measured after 48 h of HI injury. When compared with the sham-operated group, the MPO concentration significantly increased (Fig. 5, \( P < 0.01 \)) in the vehicle-treated group, however, MPO activity significantly attenuated compared with HI group in the post-treatment with taurine (120 mg/kg) \( (P < 0.01) \).

3.5. Taurine affects the expression of apoptosis-related protein assessed by immunofluorescence and western blot

Based on the above results, it indicates that taurine may have neuroprotective effects against neonatal HI brain injury. We further determined the apoptosis-related proteins which might be involved in the effect. We examined the expression of AIF, Bcl-2, Bax and Cyt-C in the hippocampus CA3 and cortex regions with immunofluorescence staining, and the expression of AIF, Cyt C, Bax and Bcl-2 with western blot analysis. In conformity with other studies (Sun et al., 2011; Wang et al., 2014), we found that AIF, Cyt C and Bax protein expressions were significantly greater compared with the sham-operated group 48 h after HI (Fig. 6C, \( P < 0.01 \); Fig. 7C, \( P < 0.01 \); Fig. 8C, \( P < 0.01 \)), while the expression of Bcl-2 protein was lower (Fig. 9B, \( P < 0.01 \)) as well as a lower ratio of Bcl-2/Bax protein (Fig. 9D, \( P < 0.01 \)) in the HI group compared with the sham group. In contrast, post-treatment with taurine (120 mg/kg) significantly reduced the AIF, Cyt C and Bax levels (Fig. 6C, \( P < 0.01 \); Fig. 7C, \( P < 0.01 \); Fig. 8C, \( P < 0.01 \)), and remarkably increased Bcl-2 expression (Fig. 9C, \( P < 0.01 \)), while the ratio of Bcl-2/Bax (Fig. 9D, \( P < 0.01 \)) approximately returned to the control level in comparison to the vehicle-treated group. Compliance with the western blot results, immunofluorescence analysis exhibited that the expression of AIF, Cyt C and Bax in the vehicle-treated group was greater in ipsilateral hippocampus CA3 and cortex region, however, in the taurine-treated (120 mg/kg) group, the expression of AIF, Cyt C and Bax was markedly reduced (Figs. 6A, 7A, 8A). As opposed to the expression of Bcl-2, which was decreased in hippocampus CA3 and cortex regions in the HI group, the expression of Bcl-2 was restored by taurine (120 mg/kg) post-treatment (Fig. 9A).

4. Discussion

Some researchers have reported protective functions of taurine against experimental stroke through preserving the mitochondrial functions and blocking the mitochondria-mediated cell death pathway in the penumbra and core. In view of these findings of neuroprotective effect of taurine on adult rats, we investigated the hypothesis of the potential neuroprotective effect of taurine on the brain injury due to HIBD. Using TTC staining in the present study, we proved that post-treatment of taurine could decrease brain infarct volume 48 h after HI in neonatal rats. Meanwhile, taurine post-treatment could enhances the level of ATP, down-regulate the lactic acid level, MPO activity and MDA content, up-regulate the activities of SOD, GSH-PX and T-AOC. It was suggested that post-treatment of taurine could exert a neuroprotective effect on the brain subjected to HIBD. Moreover, according to the observation of the dose-dependent effect, we found the dose of 120 mg/kg, i.p. taurine could produce the most protective effect on the brain of rats after HIBD. Under this dosage,
approaches of HE staining, Nissl staining, immunofluorescence and Western-blot analysis revealed that taurine could alleviate histopathology changes, mitigate necrosis cell death of the ischemic brain tissue, and inhibit expression of AIF, Cyt c and Bax at the mean time increase Bcl-2 protein expression and the ratio of Bcl-2/Bax. These results provide convincing evidences which verify that taurine may be used clinically as a neuroprotective drug for treating neonatal suffering from hypoxic-ischemic brain damage.

Hypoxic-ischemic brain damage (HIBD) resulting from perinatal cerebral hypoxia-ischemia is the leading cause of neonatal death and nervous system development disorders. The pathogenesis of perinatal hypoxic-ischemic brain damage is complex, which involves acidosis, excitotoxicity, oxidative metabolism, free radical mediated toxicity and loss of cell ion homeostasis, meanwhile, it has been validated that impaired blood–brain barrier permeability and growth factor deficiency present in the immature brain (Oja and Kontro, 1983; Pasantes Morales et al., 1982; Wade et al., 1988). Previous literatures have pointed out the weak point of “too little and too late” of the current neuroprotective agents which is disappointing in clinical trials of stroke treatment. (Jonas et al., 2001). Therefore, however, there is no safe and effective neuroprotective drug available for neonatal HIBD, which aims to salvage hypoxic-ischemic tissue, limit infarct size or minimize hypoxic-ischemic injury.

Taurine as a neurotransmitter, neuromodulator, antioxidant and membrane stabilizer is used for experimental therapy against neuronal damages, in the present study of which is consistent many observations reported before. Schurr et al (Schurr et al., 1987) has indicated that pretreatment of taurine (0.5, 1 or 2 mmol/L) can resume the synaptic function of rat hippocampal slice following a standardized hypoxic insult. Whereas other studies have exhibited that intracerebroventricular administration of taurine significantly increased the survival time of mice exposed to hypoxia and protected mice from learning impairment induced by hypoxia(Malcangio et al., 1989). As to in vivo rat brain ischemic models, several studies (Sun et al., 2011; Sun et al., 2012a; Sun et al., 2012b; Wang et al., 2007) have documented neuroprotective effect of taurine. Moreover, taurine has been used with varying degrees of success in the clinical therapy of epilepsy and other seizure disorders (Birdsall, 1998). These data provide convincing evidence which prove that taurine can cross blood–brain barrier and reach the ischemic area to exert its cytoprotection and conditions with a broad range of doses when it is administered intravenously after hypoxia-ischemia.

The current studies exhibit that these animals are chosen since the brain tissue of a 7-day-old rat is histologically similar to that of a 32–34 week gestation human fetus or infants (Vannucci and Vannucci, 2005). The infarct volume plays a vital role in evaluating the validity of cerebrovascular drugs for ischemic brain diseases. Upon our preliminary experiments, we observed the brain infarct volume at 24, 48 and 72 h after the HI insult, and we observed that point-in-time of 48 h indicated moderate tissue damage (Zhao et al., 2015). TTC staining of the brain tissue 48 h after HI in neonatal rats showed that the infarct volume was dramatically reduced in the dose-dependent manner in taurine-treated animals, especially at the dose of 120 mg/kg. In addition, the degree of ischemic damage was observed through HE staining, which is commonly employed to identify the histopathology changes and to link the development of
HIBD, however, the size of the neuronal necrosis regions induced by cerebral ischemia was significantly decreased by taurine post-treatment. Nissl staining, which can estimate the necrotic and apoptotic cell death, is another important observation method. The data showed that the neurons in cortex and hippocampus displayed prominent morphological injuries after HI. Nevertheless these morphological damages were mitigated in the taurine post-treatment group. Therefore, the early histological results suggest that taurine has a protective effect following HIBD.

In order to investigate the possible mechanisms underlying neuroprotective effect of taurine, we evaluated its effects on oxidative metabolism, oxidative stress status and cell death. It has been known that decline in energy status defined as a tissue depletion of phosphocreatine, ATP, or both compounds is due to neonatal hypoxia–ischemia (Azzopardi et al., 1989; Hamilton et al., 1986; Hope et al., 1984). Rapid decrease of ATP has shown after HIBD, which leads to the secondary injuries (Li et al., 2012). Accordingly, hypoxia-ischemia leads to inadequate supplies of glucose and oxygen, thus reduces ATP generation(Cox et al., 1988; Ljunggren et al., 1974). In the present studies, taurine treatment attenuates the loss of ATP 48h after HIBD. LA is used as an energy substrate in developing brain. Seventy percent of the cerebral metabolic requirements are met by LA in the immediate postnatal period and ketone bodies are important during the suckling period(Xu et al., 2015). Nevertheless, under the condition of hypoxia, glycolysis formed pyruvate. The metabolism of pyruvate further proceeded via lactic acid fermentation, accompanied by the elevated LA level in ischemic tissues. Taurine treatment indeed attenuated the ischemic injury, meanwhile, a large number of cells remained in low-oxygen conditions, which led to the increase of LA contents (Gu et al., 2013). According to the above results, it has been verified that taurine can alleviate HIBD in neonatal rats at least partly due to oxidative metabolism dysfunction.

Oxidative stress was hypothesized as the first response to the neonatal brain injury. The neonatal brain, with its high rate of oxygen consumption, low concentrations of antioxidants, and presence of redox-active iron, is thought to be particularly prone to tissue damage caused by oxidative stress(Xu et al., 2015). Several studies have looked at the possibility of reducing morbidity by ameliorating the effects of oxidative stress in the newborn (Saugstad, 1996). During and after the period of hypoxia-ischemia, a breakdown in cellular defense systems contributes to oxidative stress with a net increase in ROS, subsequent lipid, protein, and DNA damages, with which induce key cellular functions in activation and ultimately cell death (Barry Halliwell, 1992; Stoian et al., 1999). ROS is widely recognized that it plays a major role in the pathology of neuronal ischemia, additionally can be considered as both initiators and perpetrators of neurotoxicity(Brassai et al., 2015). Normally, oxidative stress results from the imbalance between the free radicals production and the cell ability to combat them(Stefan W et al., 2007). In addition, endogenous antioxidant enzymes such as SOD, glutathione peroxidase and catalase play an important role in the maintenance of low concentration oxidants and redox homeostasis in the pathogenesis of neonatal HIBD(Chan, 1996; Chan, 2001). Nevertheless, lipid peroxidation such as MDA is well established after HI, and it causes alterations in cell membrane fluidity including the increased permeability of membranes and the decrease in membrane ATPase activity, thus leading to cell injury(Chen et
Therefore, it is reasonable that taurine may suppress the fore-mentioned MDA level and the decrease of SOD, GSH-PX, T-AOC activity due to hypoxia-ischemia, which suggests that taurine could mitigate HIBD in neonatal rats.

Oxidative stress is considered to be a major mediator of apoptosis in several cellular systems including neurons. Traditionally, two major apoptotic pathways are characterized as follows: cell death receptor-mediated extrinsic pathway and mitochondrial mediated intrinsic pathway. In the extrinsic pathway, the activation of death receptors leads to the cleavage of caspase-8 and then activation of caspase-3/-7 (Dickens et al., 2012; Hassan et al., 2014). The key event of intrinsic pathway is the permeabilization of the mitochondrial outer membrane, which results in releasing of Cyt C and AIF, together with the activation of caspase-9 and caspase-3/-7 (Tait and Green, 2010), subsequently including Bcl-2 family (Zhou et al., 2015). Bcl-2 is a mitochondrial membrane protein that suppresses cell death (Hockenbery et al., 1990). Consistent with this role, cytochrome-c release can be initiated by the pro-apoptotic protein Bax (Skulachev, 1998) and blocked by Bcl-2 (Yang et al., 1997). In view of this fact, Bcl-2 family play a crucial roles in the regulation of mitochondria-mediated cell death during neonatal stroke (Gross et al., 1999; Lipton, 1999; Nakka et al., 2008). This study demonstrates that taurine at the dose used in our study apparently inhibits hypoxia-ischemia brain injury through modulating the actions of Bcl-2 family proteins, nevertheless, concurrently suppress the expression of AIF and Cyt C, depress apoptotic and necrotic cell death, finally block the expansion of ischemic lesion. The above results strongly support the notion that taurine has an anti-cell death activity which is in line with previously reported results (Sun et al., 2011).

Our study has several potential weaknesses. Particularly, we investigated the mechanism of taurine which performed as an individual therapy for HIBD. In view of the fact that HIBD is a complicated disease which possesses multiplex procedure, more thorough approaches should be established to pinpoint its mechanism. Hopefully, we would utilize taurine together with other medicines to achieve combination therapy, thereby to treat HIBD safely and defiantly. Taken together, in the most recent animal stroke studies, it has been proved that numerous neuroprotective agents are affective. Meanwhile, none of them achieved their primary outcome when utilized in clinical trials (Philip et al., 2009). Taurine has been used for the clinical therapy of epilepsy and other seizure disorders, which should be of good safety for newborns due to its naturalness. In this study, taurine exhibits protective effect against cell death after HIBD in neonatal rats. It might provide the experimental evidence for the clinical application of taurine.

Further studies are required to elucidate the detailed pharmacological mechanisms of taurine and combination therapy against HIBD to assess the long-term cognitive of infant, as well as the level of learning-memory disorganized for neonatal stroke.

5. Conclusions

Our data demonstrate that the post-treatment of HIBD in neonatal rats with taurine exerts significant neuroprotective effect. Taurine treatment exhibits remarkably reduced infarct
volume and suppressed cell death. Histopathology injury is ameliorated, antioxidant enzyme activities are increased and lipid peroxide is alleviated. In addition, the levels of ATP, lactic acid and MPO are decreased. The inhibition of AIF and Cyt C expression, together with the modulation of the actions of Bcl-2 family proteins are involved in the neuroprotective effect of taurine. These actions might block the expansion of ischemic lesion after HIBD. Therefore, the present study implies a potential taurine therapy for HIBD. For the sake of future clinical treatment, long-term safety and efficacy of HIBD therapy remain to be evaluated.

Conflict of Interest statement

The authors declare that they have no conflicts of interest.

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References


Wang, G.H., et al., 2007. Neuroprotective effect of taurine against focal cerebral ischemia in rats possibly mediated by activation of both GABAA and glycine receptors. Neuropharmacology. 52, 1199-209.


Figure legends:

Fig.1. Dose-responses of Taurine to hypoxic-ischemic brain injury in neonatal rats. (A) TTC staining of representative coronal sections at 48 h after hypoxic-ischemic in vehicle- and Taurine -treated rats. The scale is shown on the left side of each TTC stained brain with 1mm being the shortest interval. (B) Measurement of infarct volume by TTC staining in each group. Data are expressed as mean ± SEM (n=6). ## P<0.01, versus sham group, * P<0.05, ** P<0.01 versus HI group.

Fig.2. Effect of taurine post-treatment on histological alterations and cell death in the ischemic cerebral cortex (×400) and hippocampus CA3 (×200) at 48 h after hypoxic-ischemic. (A) The bar graph reflects the cell death score. (B) HE staining and Nissl staining in the ischemic cerebral cortex (a-c) Ischemic cerebral hippocampus (d-f). Data are expressed as mean ± SEM (n=6). ## P<0.01, versus sham group, * P<0.05, ** P<0.01 versus HI group.

Fig. 3. Taurine attenuates oxidative stress after hypoxic-ischemic in neonatal rats. (A) Effect of taurine on the content of MDA at 48 h after hypoxic-ischemic. (B-D) Effect of taurine on the activities of SOD, GSH-Px, T-AOC at 48 h after hypoxic-ischemic. Data are expressed as mean ± SEM (n=6). ## P<0.01, versus sham group, * P<0.05, ** P<0.01 versus HI group.

Fig.4. Taurine attenuates oxidative metabolism after hypoxic-ischemic in neonatal rats. (A) Effect of taurine on ATP at 48 h after hypoxic-ischemic. (B) Effect of taurine on lactic acid at 48 h after hypoxic-ischemic. Data are expressed as mean ± SEM (n=6). ## P<0.01, versus sham group, * P<0.05, ** P<0.01 versus HI group.

Fig.5. Effects of taurine on the activities of MPO after hypoxic-ischemic in neonatal rats. Data are expressed as mean ± SEM (n=6). ## P<0.01, versus sham group, * P<0.05, ** P<0.01 versus HI group.

Fig.6. Effects of taurine on the expression of AIF. (A) A representative photomicrographs of AIF immunofluorescence staining in the ischemic cortex (×400) and hippocampus CA3 (×200). (B) Representative western blot band of AIF activation in the ischemic brain at 48 h after hypoxic-ischemic. (C) Effect of taurine on the AIF activation in HI neonatal rats at 48 h after hypoxic-ischemic. Data are expressed as mean ± SEM (n=6). ## P<0.01, versus sham group, * P<0.05, ** P<0.01 versus HI group.

Fig.7. Effects of taurine on the expression of Cyt C. (A) A representative photomicrographs of Cyt C immunofluorescence staining in the ischemic cortex (×400) and hippocampus CA3 (×200). (B) Representative western blot band of Cyt C activation in the ischemic brain at 48 h after hypoxic-ischemic. (C) Effect of taurine on the Cyt C activation in HI neonatal rats at 48 h after hypoxic-ischemic. Data are expressed as mean ± SEM (n=6). ## P<0.01, versus sham group, * P<0.05, ** P<0.01 versus HI group.

Fig.8. Effects of taurine on the expression of Bax. (A) A representative photomicrographs of
Bcl-2 immunofluorescence staining in the ischemic cortex (×400) and hippocampus CA3 (×200). (B) Representative western blot band of Bax activation in the ischemic brain at 48 h after hypoxic-ischemic. (C) Effect of taurine on the Bax activation in HI neonatal rats at 48 h after hypoxic-ischemic. Data are expressed as mean ± SEM (n=6). ## P<0.01, versus sham group, * P<0.05, ** P<0.01 versus HI group.

Fig.9. Effects of taurine on the expression of Bcl-2. (A) A representative photomicrographs of Bcl-2 immunofluorescence staining in the ischemic cortex (×400) and hippocampus CA3 (×200). (B) Representative western blot band of Bcl-2 activation in the ischemic brain at 48 h after hypoxic-ischemic. (C) Effect of taurine on the Bcl-2 activation in HI neonatal rats at 48 h after hypoxic-ischemic. (D) Effect of taurine on the ratio of Bcl-2/Bax in HI neonatal rats at 48 h after hypoxic-ischemic. Data are expressed as mean ± SEM (n=6). ## P<0.01, versus sham group, * P<0.05, ** P<0.01 versus HI group.
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