Low-dose Dexamethasone Increases Autophagy in Cerebral Cortical Neurons of Juvenile Rats with Sepsis Associated Encephalopathy

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Abstract—Studies have shown that a certain dose of dexamethasone can improve the survival rate of patients with sepsis, and in sepsis associated encephalopathy (SAE), autophagy plays a regulatory role in brain function. Here, we proved for the first time that small-dose dexamethasone (SdDex) can regulate the autophagy of cerebral cortex neurons in SAE rats and plays a protective role. Cortical neurons were cultured in vitro in a septic microenvironment and a sepsis rat model was established. The small-dose dexamethasone (SdDex) or high-dose dexamethasone (HdDex) was used to intervene in neurons or SAE rats. Through fluorescence microscopy and western blot analysis, the expressions of microtubule-associated protein 1 light chain 3 (LC3), p62/sequestosome1 (p62/SQSTM1), mammalian target of rapamycin (mTOR) signaling pathway related proteins, and apoptosis-related proteins were detected. The results show that compared with those in SAE rats, the cortical pathological changes in SAE rats treated with SdDex were improved, and damaged substances were encapsulated and degraded by autophagosomes in neurons. Additionally, similar to neurons in vitro, cortical autophagy was further activated and the mTOR signaling pathway was inhibited. After HdDex treatment, the mTOR signaling pathway in cortex is inhibited, but further activation of autophagy is not obvious, the cortical pathological changes were further worsened and the ultrastructure of neurons was disturbed. Furthermore, the HdDex group exhibited the most obvious apoptosis. SdDex can regulate autophagy of cortical neurons by inhibiting the mTOR signaling pathway and plays a protective role. Brain damage induced by HdDex may be related to the activation of apoptosis. © 2019 The Author(s). Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Keywords: Dexamethasone, Autophagy, Sepsis, Neurons, mTOR, Apoptosis.

INTRODUCTION

The clinical manifestations of severe sepsis are manifestate of multiple organ dysfunctions induced by the systemic inflammatory response to infection (Balk et al., 2000; Singer et al., 2016). Acute brain dysfunction, characterized by altered mental status, ranging from delirium to coma, seizure or focal neurological signs, and accompanied by abnormal electroencephalography (EEG), often occurred before alterations in other organ function (Adam et al., 2013; Hosokawa et al., 2014; Kaur et al., 2016). These manifestations are common complications of severe sepsis and have been referred to as sepsis-associated encephalopathy (SAE) (Goffton et al., 2012; Dal-Pizzol et al., 2014). Its pathogenesis involves a number of mechanisms, including oxidative stress (Berg et al., 2011; Zhou et al., 2012), cytokine storm (Jacob et al., 2011), mitochondrial dysfunction (Liu et al., 2015a,b,c), cell apoptosis (Liu et al., 2015a,b,c) and cerebral blood flow reduction (Yeh et al., 2015; Gerard et al., 2016).

Despite a large number of clinical and basic studies on the use of corticosteroids in sepsis, there is still controversy on their safety and effectiveness (Annane et al., 2009; Patel et al., 2012; Allen et al., 2014). In a study describing the effect of corticosteroids on survival...
and shock during sepsis, patients have benefitted from corticosteroid therapy (Minneci et al., 2004). In a recent study on its mechanism, using experimental meningococcal sepsis in mice treated with dexamethasone (Dex), a synthetic glucocorticoid, showed that Dex significantly increased blood levels of interleukin-10 (IL-10), reduced levels of tumor necrosis factor alpha (TNF-α) and lowered bacterial blood load (Levy et al., 2015). However, other clinical studies showed that corticosteroid treatment was associated with increased adjusted hospital mortality, and could not improve the prognosis of severe sepsis (Casserly et al., 2012; Atkinson et al., 2014). As a result of not forming a unified understanding, the application of corticosteroids during severe sepsis was strictly limited in the international guidelines for management of severe sepsis and septic shock: 2016 (Rhodes et al., 2017). Thus, further studies should be done to elucidate the reasons for the emergence of these opposing results and provide a basis for the reasonable application of corticosteroids, including Dex and hydrocortisone. We first examined the inflammatory response in sepsis and the effect of different doses of Dex on inflammation.

Autophagy is also known as programmed cell death type II (Gozuacik et al., 2004; Mukhopadhyay et al., 2014; Petersen et al., 2014), characterized by the formation of autophagic vacuoles in the cytoplasm, which plays an important role in the regulation of cell metabolism (Kawaguchi et al., 2016; Lewis et al., 2016), protein secretion (Son et al., 2016; Wang et al., 2016), and cell-mediated immune responses (Baginska et al., 2013; Crouwels et al., 2015). The core machinery of autophagic vacuoles consists of a set of autophagy associated gene (ATG) proteins. Among these, microtubule-associated protein 1 light chain 3 (LC3) and p62/sequesosome1 (p62/SQSTM1) are essential to the formation of autophagic vacuoles in mammalian cells, and is used as a specific marker for autophagy (Fritzen et al., 2016). During this process of formation, the cytoplasmic LC3 (LC3-I) located in the cytoplasm is transformed into membrane-bound LC3 (LC3-II) by the binding of p62/SQSTM1 to phosphatidylic ethanolamine. Then LC3-II is released into the cytoplasm during the fusion of autophagic vacuoles with lysosomes and transforms into LC3-I (Mizushima et al., 2007). Thus, the conversion of LC3-I to LC3-II is usually used to research the level of autophagy. In other words, autophagy is increased if the LC3-II expression or ratio of LC3-II to LC3-I is upregulated (Peng et al., 2016).

The mammalian target of rapamycin (mTOR), a member of the phosphatidylinositol-3-kinase family, integrates a variety of signaling pathways to regulate cellular growth (Avet-Rochex et al., 2014; Yi et al., 2015), proliferation (Moore et al., 2016; Wang et al., 2016a,b), inflammatory response (Dănoi et al., 2015; Fazolini et al., 2015; Vangan et al., 2016), and autophagy (Itakura et al., 2013). mTOR activation can contribute to tissue damage through inflammatory response (Liu et al., 2014a,b; Hsu et al., 2015). It can also lead to transient activation of the downstream effectors, p70 KDa ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), which regulates the initiation of protein translation and inhibits autophagy. Moreover, mTOR inhibition can downregulate the expression levels of downstream effectors and increases autophagy. Therefore, there is a direct link between autophagy and the mTOR signaling pathway (Gordon et al., 2015). mTOR has been recognized as an important negative regulator of autophagy. Several studies show that mTOR reduced by rapamycin treatment enhances autophagic degradation of aggregate proteins, and can effectively treat neurodegenerative diseases, such as Parkinson’s (Jiang et al., 2013), Alzheimer’s (Liu et al., 2015a,b,c), and Huntington’s (Williams et al., 2008). It has been acknowledged that the systemic inflammatory response manages the progression of these diseases. Thus, the crosstalk between inflammatory response and autophagy has attracted much attention in recent years. However, the relationship between autophagy and brain injury during sepsis is poorly understood.

In this study, we established the SAE juvenile rat model and primary cultured neurons, observed autophagic vacuoles in cortical neurons by transmission electron microscopy, measured the expression levels of LC3, p62/SQSTM1, mTOR, p70S6K and 4E-BP1 through immunofluorescence (IF), performed western blot and image analysis, and investigated the small-dose Dex (SdDex) and high-dose Dex (HdDex) effects on autophagy of cortical neurons, to provide a basis for the reasonable application of Dex in the treatment of SAE.

**EXPERIMENTAL PROCEDURES**

**Cell culture and treatments**

Primary cortical neurons were prepared from the cortices of 1-d-old Wistar rats, as described previously in reference (Zhang et al., 2018). For biochemical analyses, the cells were plated at a density of 6 × 10⁵ cells/dish on 6-well poly-L-lysine precoated dishes. For immunocytochemistry, the cells were plated at a density of 1.5 × 10⁴ cells/well on 24 well plates. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂ and half of the media was changed every 3 – 4 d.

To analyze the effects of different doses of dexamethasone on cells, cells were treated with equal volumes of dexamethasone (10 µM, 50 µM and 100 µM, Tianjin Jin Yao Pharmaceutical Co., Ltd., China) and phosphate-buffered saline (PBS; pH 7.3) for 1–5 days. Subsequently, 100 µl of the culture medium containing 10 µl Cell Count Kit-8 (CCK-8, ab228554, Abcam, Cambridge, UK) was used to replace the medium, and the cells were incubated for 1 h. According to the manufacturer’s protocol, the cell growth curve was determined using the CCK-8. In addition, cell viability was assessed using Trypan Blue exclusion assay (ab233465, Abcam); cells harvested after treatment were stained with 0.4% Trypan Blue; total number of cells and number of dead cells were counted by a hemocytometer; percentage of viable cells is calculated as follows: Viable cells (%) = (Total number of cells - Number of dead cells)/Total number of cells × 100% (Pang et al., 2017).

Hence, for simulating microenvironment in vivo, we added 5% peripheral blood serum of rats with SAE in
the culture medium. Primary cortical neurons were cultured in dulbecco minimum essential medium (DMEM) containing 5% peripheral blood serum of rats with SAE, 5% fetal bovine serum (Gibco, 10099–141), 10 U/ml penicillin, 10 U/ml streptomycin and 0.5 mM glutamine at 37 °C in a humidified atmosphere with 5% CO₂. At 10 DIV, cultured neurons were treated with Dex (10 μM for SdDex, 100 μM for HdDex (Liu et al., 2014a, b)) for 24 hours. Besides, we also intervened with autophagy inhibitor 3-methyladenine (3-MA, 189490, Merck, Darmstadt, Germany) 5 mM (Li et al., 2009) in addition to SdDex to study the effect of autophagy inhibition on the mTOR signaling pathway.

Animals and treatments

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Health, China); the protocol was approved by the Animal Care Committee of Sichuan University Medical Center in China. A total of 156 clean, healthy, thirty-day-old male Wistar rats (weight ranged from 100 to 120 g) were purchased from Sichuan Jianyang Dashuo Animal Science and Technology Co., Ltd (Shu ICP 09003144). All rats were housed with free access to food and water in a 22–25 °C, 55–58% relative humidity environment on a 12 h light/dark cycle. Rats were maintained with isoflurane in 100% O₂. Normal fluid balance was maintained by subcutaneous injection of saline. Drying of the cornea was prevented with eye ointment. Subsequently, the animal was placed in a stereotactic apparatus (model 963, Ultra Precise Small Animal Stereotaxic, David Kopf Instruments, Tujunga, CA, USA). Body temperature, respiratory rate, heart rate, in- and expired CO₂ and SpO₂ were monitored continuously for assessment of anaesthetic depth and anaesthetic drug administration was adjusted appropriately. Following an about 3 cm skin incision along mid-sagittal plane of skull, the peristium was removed periostium from the neurocranium after local anesthesia with lidocaine solution (3 mg/kg). Three small wired stainless steel screws were implanted epidurally, including cortical electrode (S1: 2.5 mm caudal to bregma, 2.5 mm right from midline), and left and right frontal electrode (10 mm rostral to bregma, 1 mm lateral from midline). All electrodes were wired to an 8 pin receptacle (Mecap Preci-Dip 917–93–108–41-005, Preci-Dip Dural SA, Delémont, Switzerland) and fixed to the skull with antibiotic bone cement (Simplex™P bone cement with tobramycin, Stryker Nederland B. V.). The skin was stitched in a single layer around the receptacle. Moreover, two SEP-evoking stimuli electrodes were positioned on the left lateral part of the tail base, and spaced at 3 mm from each other. EEG instrument recorded delta waves (0.5–3 Hz), theta waves (4–8 Hz), alpha waves (8–13 Hz) and beta waves (13–30 Hz). SEPs were elicited by multiple square-wave pulses of the 2 ms duration, stimulus intensity of 5–15 mA, wave width of 10 ms and frequency of 3 Hz, generated with a Grass stimulator (Model S-88, Grass Medical Instruments, Quincy, Mass, USA). And then P1 and N1 waves were recorded.

Neurobehavioral test-score

Neural behavior was scored as described in literature (Li et al., 2016), including auricle reflex, corneal reflex, is reflection, tail flick reflex and escape reflex, 0 for no reflection, 1 point for reflection weakened (less than 10 s lack of reflection), 2 points for normal reflection, the highest score was 10 points.

Physiological examination

Anesthesia was completed with 10 % chloral hydrate by intraperitoneal injection. An indwelling tube was placed
in the femoral artery to provide a venous channel for connecting the iWorx biological signal recorder (iWorx Systems, Inc., USA), which was used to monitor the blood pressure and heart rate continually.

Enzyme linked immunosorbent assay (ELISA)

Cell lysate and tissue supernatant were prepared according to the instructions. Rat TNF alpha ELISA Kit (ab100785, Abcam) and Rat IL-1 beta ELISA Kit (ab100768, Abcam) were used to detect tumor necrosis factor (TNF)-α and interleukin (IL)-1β. Absorbance values were analyzed at different wavelengths using microplate readers (Thermo Fisher Scientific, MA, USA).

Histological examination

The brains of rats were harvested and fixed in paraformaldehyde at 4 °C for 24–48 h, and they were cut in a coronal plane from the optic chiasma through to the back (1 cm long) with the segment of the brain tissue paraffin-embedded. Then, brain samples were cut (6 μm thick) and routinely stained with hematoxylin and eosin. Finally, the Leica inverted optical microscope was used to capture images (Leica, USA).

Cerebral edema measurement

The degree of cerebral edema is obtained indirectly by using the wet weight-dry weight technique, to measure the dry and wet weights of brain tissues respectively (Li et al., 2017). After the rats in each group were sacrificed, fresh brain tissue was immediately removed, the cortex at the top of the frontal lobe was isolated, weighed (wet weight), dried in an oven at 100 °C for 24 hours, and then weighed (dry weight). The brain water content (BWC) = (dry weight - wet weight) / wet weight × 100%.

Electron microscopy

The tissue samples for electron microscopy were obtained 2 mm posterior to the optic chiasma in a coronal plane (1 mm long) and were fixed with 2 % paraformaldehyde and 2.5 % glutaraldehyde in phosphate buffer at 4 °C for 24–36 h. They were then washed with the same buffer and postfixed with 3 % glutaraldehyde and 1 % phosphate-buffered osmium tetroxide at 4 °C. Then the samples were dehydrated in acetone, infiltrated with Epon resin (Electron Microscopy Sciences, RT 14120) for 2d, embedded in the same resin, and polymerized at 60 °C for 48 h and embedded in Epon812. Afterwards, sections were cut at 0.12 μm thickness and stained with 0.2 % lead citrate and 1 % uranyl acetate, which were subsequently observed under an H-600IV transmission electron microscope (Hitachi, Japan).

Fluorescence microscopy

The rats were anesthetized with 10% chloral hydrate (1 ml/300 mg, i.p.) and sacrificed by transcardiac perfusion with ice-cold 5 mM sodium PBS and 4% paraformaldehyde. The brains were removed and postfixed in 4 % paraformaldehyde overnight at 4 °C. Brain samples were embedded in 4 % agarose and were cut (40 μm thick) with an oscillate slicer. The slides were washed in PBS and blocked in PBS containing 2% fetal calf serum and 0.2 % Triton X-100 at 4 °C for 1 h.

Primary antibodies, rabbit anti-LC3 polyclonal antibody (1:200, Novus, USA) or mouse anti-NeuN monoclonal antibody (1:500, Abcam) in PBS containing 1 % fetal calf serum and 0.1 % Triton X-100 were added and incubated overnight at 4 °C. The slides were then washed in PBS (10 min each) and incubated with a mixture of DyLight 488-conjugated donkey anti-rabbit IgG (1:800, Jackson ImmunoResearch, USA) and Cy3-conjugated donkey anti-mouse IgG (1:800, Jackson ImmunoResearch, USA) in PBS containing 1 % fetal calf serum and 0.1 % Triton X-100 for 1 h. After washing with PBS, 4,6-diamidino-2-phenylindole (DAPI, 1:5000, Beyotime, China) was used to stain nuclear DNA. After staining, all of the slides were mounted onto glass slides and cover-slipped with antifade mounting medium (Beyotime, China). Using a confocal laser scanning microscope (Olympus, Japan), the slides were observed with the appropriate laser beams and filter settings for green-emitting Dylight 488 (excitation peak 493 nm, emission peak 518 nm) or red-emitting Cy3 (excitation peak 550 nm, emission peak 570 nm). The digital images were captured with FV10-ASW-4.2 software (Olympus, Japan).

The cultured neurons were washed once in PBS and fixed with 4% paraformaldehyde for 20 min. After rinsing with PBS, the cells were permeabilized with 0.2% Triton X-100 in PBS with 2% fetal calf serum albumin for 1 h at 4 °C. Other manipulations as mentioned above are completed.

Western blot analysis

Brain tissue or cortical neurons were collected in lysis buffer (2% SDS, 1% Triton X-100, 50 mM Tris-HCl and 150 mM NaCl, pH 7.5), and a protease inhibitor cocktail (Roche, USA). Cells were harvested and lysed immediately in lysis buffer (2% SDS, 1% Triton X-100 [Sigma, 9002–93-1], 50 mM Tris-HCl and 150 mM NaCl, pH 7.5). Protein lysates were separated by SDS-PAGE electrophoresis, transferred to nitrocellulose membrane and analyzed by immunoblotting. Antibodies were diluted in a blocking solution containing TBS-T (150 mM NaCl, 8 mM K2HPO4, 1.99 mM KH2HPO4, 0.1% Tween). The membranes were incubated with the following primary antibodies overnight at 4 °C: anti-LC3 (NB100-2220) rabbit polyclonal from Novus Biologicals; anti-p62/SQSTM1 (ab56416) mouse monoclonal from Abcam; anti-β-actin (4967L) rabbit polyclonal, anti-mTOR (2972 s) rabbit polyclonal, anti-p70S6K (5707 s) rabbit monoclonal, anti-4E-BP1(2855S) rabbit monoclonal, anti-caspase-3 (9662) rabbit polyclonal, anti-Bax (2772S) rabbit polyclonal, and anti-bcl2 (3498S) rabbit monoclonal from Cell Signalling Technology. After washes in TBS-T for 2 h; polyclonal anti-rabbit or anti-mouse secondary antibody (1:3000, ZSGB-BIO, China) in TBS-T for 1 h. Proteins were...
Statistical analysis

The values are presented as mean ± SEM of at least 5 experiments. The student-newman Keuls test and one-way ANOVA statistical analyses were performed with the Statistical Package for Social Sciences (SPSS, Version 19.0, Chicago, IL, USA). Values of P < 0.05 were considered statistically significant.

RESULTS

SdDex has no cytotoxic effect on cortical neurons

To study the cytotoxic effects of Dex, equal volumes of Dex (10 μM, 50 μM, and 100 μM) and phosphate buffered saline (PBS) were used to intervene cortical neurons in vitro. After cells were cultured for 1–5 days, Cell Counting Kit-8 (CCK-8) assays and Trypan Blue staining were used to observe the effects of Dex on the growth and viability of cortical neurons respectively. When compared with the PBS group, it is noted that 10 μM Dex had no effect on cell activity and viability. Fifty micromolar Dex reduced growth and viability of cortical neurons gradually until the fifth day. One hundred micromolar Dex impacted activity and viability of cortical neurons with increasing deterioration between the third and fifth day. (P < 0.05, Fig. 1A and B). Therefore, in a later study, we selected SdDex (10 μM) to intervene in the SAE model to observe if it could play a protective role in the related mechanism. Simultaneously, the causes of harmful effects of HdDex (100 μM) on cell growth curve and cell viability were also analyzed.

Juvenile rats with SAE show abnormal behavior, and characteristics of EEG and SEP

At 12 hours after cecal ligation and puncture (CLP), Wistar rats appeared crouched, with piloerection and shivering. To confirm whether CLP rats were suffering from SAE, we measured the neurobehavioral test-scores, EEG and somatosensory evoked-potential (SEP). CLP rats would be diagnosed with SAE if they appeared to have lower neurobehavioral test-scores, abnormal EEG and SEP. Table 1 shows that the neurobehavioral test-scores and mean arterial pressure (MAP) were significantly lower in the SAE group than in the sham operated group. Conversely, the heart rate (HR) was significantly higher in the SAE group than in the sham-operated group.

The amount of alpha wave in EEG was significantly lower, the amount of delta wave was significantly higher,
and the amplitude of the P1 wave in SEP were also significantly lower. The latencies of P1 and N1 waves were significantly longer in the SAE group than in the sham-operated group (Table 1). Thus, the EEG of rats with SAE is characterized as the decrease of alpha wave frequency and the increase of delta wave frequency. SEP shows the decrease of P1 amplitude, delay of P1 and N1 latency.

**SdDex improves neurobehavioral test-scores, vital signs, EEG and SEP in juvenile rats with SAE**

To further investigate the effects of Dex at different doses on SAE rats, neurobehavioral test-scores, vital signs, EEG and SEP were measured 3 days after injection.

**SdDex and HdDex reduces inflammation in cerebral cortical cells from rats with SAE**

*In vitro* and *in vivo*, inflammatory cytokines in rat cortical neurons were detected, as shown in Fig. 2. The levels of TNF-α and IL-1β in SAE group were significantly higher than those in the sham operation group. The levels of TNF-α (Fig. 2A and C) and IL-1β (Fig. 2B and D) decreased in different degrees after SdDex and HdDex treatment. However, there was no significant difference between the SdDex and HdDex groups in detecting the level of IL-1β in cortical neurons (Fig. 2B) and TNF-α in the cortical supernatant (Fig. 2C).
SdDex improves cortical pathological changes in juvenile rats with SAE

After the pathological sections were stained, the cortex at the top of the frontal lobe was selected for observation. No significant changes were observed in the sham group under optical microscope (Fig. 3A). In the SAE group, parenchyma was loose, the number of neurons was reduced, the cell body was atrophic with more space around (white arrows) and the neutrophils infiltrated neurons (black arrows) in the early stage of SAE (Fig. 3B). In the SdDex group, parenchyma was slightly loose and the neurons were plump compared with the SAE group (Fig. 3C). In Fig. 3D, although no obvious inflammatory cell infiltration was observed in the HdDex group, the number of cells was significantly reduced, parenchyma was looser, the capillaries collapsed, and the neuron cell body was atrophic (white arrows).

SdDex reduced cortical cerebral edema in juvenile rats with SAE

The cortical parenchyma was observed to be loose under the optical microscope (Fig. 3B), so it was considered that cerebral edema may occur in SAE. Cerebral edema is a common clinical manifestation of acute brain injury, so we detected brain water content (BWC) in different groups. As shown in Fig. 4, compared with the sham operation rats, the BWC of SAE rats was significantly increased, and the treatment with SdDex showed a certain degree of improvement, but HdDex had no effect on cerebral edema.

SdDex increases autophagy of cerebral cortical neurons from rats with SAE

Transmission electron microscopy is an important method for observing autophagic vacuoles in cells. Thus, we used it to observe autophagic vacuoles in cortical neurons. In autophagy, the first vesicles with a double-membrane structure are generated. These wrap the damaged organelles or protein fragments, and then combine with lysosomes to form autophagosomes that degrade intracellular materials.

Fig. 5A showed the cortical neurons from sham-operated rats that displayed a nearly normal structure and proper organelle distribution with low or absent autophagy. No alteration of tissue integrity was observed. Fig. 5B showed the initiation of autophagy, the autophagic vacuolization in cortical neurons of SAE rats. A number of irregularities, including autophagic vacuoles with loss of discernable organellar fragments in the cytoplasm, prominent matrix granules and crystalline-like inclusions, were seen sporadically in high-power electron microscopic images. Fig. 5C showed further activation of autophagy, a large autophagosome containing mitochondria and other organelles and herniation of the outer membranes of the endoplasmic reticulum into adjacent lysosomal structures. However, there were a few prominent matrix granules and crystalline-like inclusions in the cortical...
neurons of SdDex-treated SAE rats. Fig. 5D showed the structure of cortical neurons in HdDex-treated SAE rats in which organelles and nuclear membranes are destroyed.

Fig. 5. SdDex induced autophagy in cortical neurons of rats with SAE. (A) The cortical neurons of sham-operated rats were displayed very low or absent autophagy. (B) A small number of autophagy vacuoles (arrows) appeared in cortical neurons of SAE rats. (C) Cortical neurons of SdDex-treated SAE rats showed autophagy vacuoles containing damaged materials (arrows). (D) The cell structures, including organelles and nuclear membrane, were destroyed in cortical neurons from HdDex-treated SAE rats. Scale bar = 4 μm. The number of autophagy vacuoles in each group was described by a histogram. All experiments were repeated 5 times and error bars depict mean ± SEM; ** P < 0.01.

Fig. 6. SdDex increased conversion of LC3-I to LC3-II in cortical neurons of SAE rats. (A) SdDex increased LC3 expression in cerebral cortex neurons of SAE rats. Rats were treated as described above. Brain sections were processed for immunofluorescence of LC3 (green), NeuN (red) and the nuclear marker DAPI (blue). In cerebral cortex, the number of LC3 positive neurons was relatively increased in SdDex-treated SAE rats. Under the same visual field, LC3 fluorescence signals of each group were quantitatively analyzed and described by histogram. Scale bar = 40 μm. (B) The above results were further verified by the primary culture of neurons in vitro. Scale bar = 10 μm. (C) Western blotting analyzed the levels of LC3-I and LC3-II of primary culture of neurons. All experiments were repeated 5 times and error bars depict mean ± SEM; * P < 0.05; ** P < 0.01.
The number of autophagy vesicles was observed in the field of 10,000×, and it was found that the SAE group was more than the Sham group, the SdDex group was the most, and the HdDex group was even less than the SAE group (Fig. 5).

**SdDex increases conversion of LC3-I to LC3-II in cortical neurons of rats with SAE**

There are many types of cells in the cerebral cortex, including neurons and glial cells. LC3 is essential to the formation of autophagic vacuoles in mammalian cells and is used as a specific marker for autophagy (Fritzen et al., 2016). To identify LC3 expression in cortical neurons, we labeled the neurons in the cerebral cortex with an anti-neuronal nuclei antibody (NeuN), frequently used as a neuron-specific marker of vertebrates (Lavezzi et al., 2013; Gusel’nikova et al., 2015). Immunofluorescence analysis showed that the number of LC3 positive neurons increased in SdDex-treated SAE rats. In contrast, these cells were markedly lower in HdDex-treated SAE rats (Fig. 6A). Similar results were also obtained in vitro experiments of primary cultured neurons. Moreover, the amount of LC3 positive particles in neurons was higher in co culture of SdDex and serum of rats with SAE than in co culture of HdDex and serum of rats with SAE (Fig. 6B).

To further confirm that the conversion of LC3-I to LC3-II was occurring in the neurons, we examined the LC3 expression of primary cultured neurons through western blot analysis. Our results showed that the ratio of LC3-II/LC3-I of neurons was increased in the cortical neurons cultured in SdDex and serum of rats with SAE, when compared to the control or only the serum of rats with SAE (Fig. 6C). Interestingly, this ratio was also elevated in co culture of HdDex and serum of rats with SAE, though its ratio was lower than that in co culture of SdDex and serum of rats with SAE (Fig. 6C). Taken together, these data suggest that SdDex could increase conversion of LC3-I to LC3-II in cortical neurons of juvenile rats with SAE.

**SdDex increases conversion of LC3-I to LC3-II in cortical neurons of rats with SAE**

To investigate the expression of LC3 in rat cerebral cortical cells, we first analyzed the LC3 expression of cerebral cortical cells using immunofluorescence. Our results showed higher amounts of LC3 positive cells in the cerebral cortex of rats with SAE or SdDex-treated SAE rats than in sham-operated rats or HdDex-treated SAE rats (Fig. 7A). This was also demonstrated by image analysis (Fig. 7B). Western blot analysis of cell lysates showed that SdDex treatment increased the ratio of LC3-II/LC3-I in cortical cells as compared to sham-operated, or SAE rats (Fig. 7C). Interestingly, HdDex treatment also elevated the ratio of LC3-II/LC3-I, though its ratio was lower than that of SdDex treatment (Fig. 7C).

![Fig. 7](image-url)
SdDex reduces the expression of p62/SQSTM1 in cortical neurons of rats with SAE

In mammals, p62/SQSTM1 seems to be a selective substrate for autophagy that binds LC3 and ubiquitin, so when autophagy occurs, it acts as an adaptor protein that connects ubiquitin to the autophagosome (Mizushima et al., 2007). Therefore, we used western blot analysis to detect the expression of p62/SQSTM1 in cortical neurons of rats, and we obtained roughly the same results as we did with the cerebral cortical cells of rats. Compared with sham operation, the expression of p62/SQSTM1 was decreased in SAE rats (Fig. 8A). Compared with SAE rats, p62/SQSTM1 continued to decrease in SdDex treatment and HdDex treatment, but interestingly, the expression of HdDex treatment is a little bit higher than that of the SdDex treatment (Fig. 8A).

SdDex reduces the expression of p62/SQSTM1 in cerebral cortical neurons of rats with SAE

Similarly, to better understand the process of autophagy, western blot analysis was used to detect p62/SQSTM1 expression in cerebral cortical cells of different groups. The results showed that SdDex treatment reduced the expression of p62/SQSTM1 compared with the sham treatment group or SAE rats (Fig. 8B). At the same time, HdDex treatment also reduced the expression of p62/SQSTM1, but was higher than that of the SdDex treatment (Fig. 8B).

SdDex inhibits mTOR signaling pathway in cortical neurons from rats with SAE

Other studies have demonstrated a direct link between autophagy and the mTOR signaling pathway (Neufeld, 2012; Itakura et al., 2013; Liu et al., 2014a,b; Hsu et al., 2015). The mTOR activation inhibits autophagy. Conversely, mTOR inhibition increases autophagy. The mTOR signaling pathway has been recognized as an important negative regulator of autophagy. However, it is unknown whether SdDex-induced autophagy of neurons is regulated by the mTOR.

To confirm SdDex-induced autophagy in cortical neurons through the mTOR signaling pathway, we detected the expressions of mTOR and downstream effectors, p70S6K and 4E-BP1, in primary culture neurons and in cerebral cortex cells. Western blot showed that the expression of mTOR, p70S6K, or 4E-BP1 in culture neurons of SdDex-treated SAE rats was downregulated as compared to sham-operated rats or SAE rats (Fig. 9A); but these expressions were higher than those of HdDex-treated SAE rats. Similar results were observed in cerebral cortex cells (Fig. 9B).

These results show SdDex induces autophagy of cortical neurons from rats with SAE by inhibiting the mTOR signaling pathway. In previous studies, we have found that the autophagy process in the hippocampus of rats with sepsis might be blocked by the activation of the NF-κB signaling pathway (Su et al., 2015).

After autophagy is inhibited, mTOR signaling pathway is activated again in cortical neurons from rats with SAE

Our study found that SdDex can activate autophagy more effectively than HdDex (Figs. 5–8), and inhibit the mTOR signaling pathway more (Fig. 9). Therefore, to study the correlation between autophagy and the mTOR signaling pathway after SdDex intervention, we further explored using an autophagy inhibitor 3-MA in addition to SdDex.

Similar results were obtained in cultured primary cortical neurons (Fig. 10A) and cortical samples (Fig. 10B). Western blot analysis showed that, compared with the SdDex group, the expression of mTOR and its downstream p70S6K and 4E-BP1 were significantly upregulated in the 3-MA group. This indicates that inhibition of autophagy after the addition of 3-MA can reactivate the mTOR signaling pathway.

HdDex activates the caspase-3 signaling pathway in cortical neurons from rats with SAE

Interestingly, we did not find that autophagy was increased, although, HdDex treatment increased conversion of LC3-I to LC3-II in neurons (Fig. 6C and Fig. 7C), reduced the expression of p62/SQSTM1 (Fig. 8) and inhibited the mTOR signaling pathway (Fig. 9). Moreover, HdDex treatment resulted in damage of the cortex (Fig. 3D) and neuronal structure (Fig. 5D). There was a contradiction between these phenomena and the traditional

Fig. 8. SdDex reduced the expression of p62/SQSTM1 in SAE rats. (A) Western blotting analysis of the expression levels of p62/SQSTM1 in cultured cortical neurons of rats with SAE. (B) Western blotting analysis of the expression levels of p62/SQSTM1 in cerebral cortex cells of rats with SAE. The ratios of p62/SQSTM1 to β-actin are described by histograms. All experiments were repeated 5 times and error bars depict mean ± SEM; * P < 0.05; ** P < 0.01.
understanding of the mTOR signaling pathway. Therefore, we would like to know if HdDex induced neuronal apoptosis.

During cell apoptosis, caspase-3 is known as the "executor" protease because it can degrade multiple substrates in the cytoplasm and nucleus to result in cell death (Xie et al., 2016). Consequently, we tested the expression of cleaved caspase-3 in cerebral cortex cells by western blot. Our results showed that cleaved caspase-3 expression of cerebral cortex cells and cultured neurons was increased markedly in HdDex treatment than in SdDex treatment (Fig. 11A).

B-cell lymphoma-2 (bcl-2) and bcl2-associated X protein (Bax) are upstream effectors of the caspase-3 signaling pathway. Previous studies suggest that a slight change in the dynamic balance of Bax/bcl-2 proteins may result in inhibition or promotion of cell death (Ryu et al., 2015). Therefore, we also tested their expression in cerebral cortex cells through western blot. Our results indicated that the bcl-2 expression of cultured neurons was lower in HdDex-treated SAE rats than in SdDex-treated SAE rats; however, the Bax expression was higher. The Bax/bcl-2 ratio was also elevated (Fig. 11B). Similar results were observed in cerebral cortical cells (Fig. 11B). Taken together, these data suggest that HdDex treatment induces apoptosis of cortical neurons from rats with SAE by the caspase-3 signaling pathway.

**DISCUSSION**

In this study, our results indicate that autophagy is activated by the systemic inflammatory response in cortical neurons of rats with SAE and both SdDex and HdDex are effective in controlling inflammation. Our previous studies have suggested that the main mechanism could be related to the inhibition of the NF-kB signaling pathway (Su et al., 2015). Moreover, SdDex can further induce autophagy by the inhibition of the mTOR signaling pathway. HdDex, however, induces apoptosis by the caspase-3 signaling pathway. These results could provide a basis for the reasonable application of Dex in the treatment of SAE.

After modeling with CLP, we measured neurobehavioral test scores, vital signs, EEG and SEP to analyze whether the SAE rat model was successfully established. Based on the studies completed in relevant literature (Kafa et al., 2010), it can be considered that the SAE rat model in this study was successfully established. Thereafter, on the basis of the established SAE rat model, SdDex and HdDex were used...
for intervention respectively to complete subsequent experiments.

We first found that cortical cells showed severe inflammation in SAE when compared with normal conditions. We found that intervention with dexamethasone at different doses in SAE rats could effectively inhibit cortical inflammation (Fig. 2). However, although corticosteroids can effectively reduce inflammation in patients with sepsis (Levy et al., 2015), some studies have found that using corticosteroids does not improve the prognosis of patients with severe sepsis (Casserly et al., 2012; Atkinson et al., 2014). Therefore, this study will ascertain if Dex can reduce inflammation while treating SAE, and whether it may cause other changes in the brain leading to poor prognosis. Furthermore, we aim to elucidate the therapeutic effects of different doses of Dex.

Autophagy is a new type of programmed cell death, also known as the type II programmed cell death, which plays a key role in the growth, development, and pathogenesis of some diseases (Gozuacik et al., 2004; Baginska et al., 2013; Mukhopadhyay et al., 2014; Petersen et al., 2014; Crauwels et al., 2015; Kawaguchi et al., 2016; Lewis et al., 2016; Son et al., 2016; Wang et al., 2016a,b). Autophagy also allows unneeded proteins to be degraded, and the amino acids recycled for the synthesis of proteins that are essential for survival. The harmful components, including certain toxins, pathogens, protein aggregates, and damaged organelles, are also encapsulated and degraded by autophagy to maintain cellular homeostasis in response to a series of extracellular stimuli. Thus, a certain degree of autophagy can protect the body from harmful stimuli. Conversely, excessive autophagy can also destroy cell homeostasis. In an animal experiment, autophagy of pancreatic tissue cells could prevent exocrine dysfunction of pancreas in septic rats (Chen et al., 2015). In clinical research, it has also been proved indirectly that autophagy had a protective effect on patients with severe sepsis (Kimura et al., 2014). Interestingly, T lymphocyte autophagy was downregulated in sepsis (Lin et al., 2014). Therefore, the sepsis-induced autophagy may relate to the type of cells.

Our previous studies indicate that autophagy is activated by the systemic inflammatory response in the septic hippocampus and provides a neuroprotective function (Su et al., 2015). However, the research of autophagy...
Fig. 11. HdDex activated the caspase-3 signaling pathway in cortical neurons from rats with SAE. (A) Western blotting analysis of the expression levels of cleaved caspase-3 in cultured neurons (left) and cerebral cortex cells (right). (B) Western blotting analysis of the expression levels of Bax and bcl-2 in cultured neurons (left) and cerebral cortex cells (right). The ratios of caspase-3, cleaved caspase-3, Bax or bcl-2 to β-actin are described by histograms. All experiments were repeated 5 times and error bars depict mean ± SEM; ** P < 0.01.
on cortical neurons of SAE is not reported. This study mainly analyzes the autophagy process of cortical neurons. When autophagy is activated, LC3-I in the cytoplasm is transformed into LC3-II and aggregates on the autophagosome membrane; p62/SQSTM1 is the adapter protein linking ubiquitin LC3 and the autophagosome, which is degraded during autophagy (Suzuki et al., 2005; Tsuganezawa et al., 2013). Therefore, the more autophagy is activated, the more LC3-I transforms to LC3-II, and the less free p62/SQSTM1. In this study, we found that the number of LC3 positive cortical neurons and the ratio of LC3-II to LC3-I increased, while the expression level of p62/SQSTM1 decreased in rats with SAE (Figs. 6-8). These results show that the autophagy of cortex neurons is activated.

Although a large number of clinical and basic studies on the use of corticosteroids in sepsis, there is still a controversy on their safety and effectiveness of their use (Annane et al., 2009; Patel et al., 2012; Allen et al., 2014). Some studies have shown that patients with severe sepsis have benefited from corticosteroid therapy (Minnci et al., 2004; Levy et al., 2015); other studies, however, have shown the opposite results (Casserly et al., 2012; Atkinson et al., 2014). Recent studies were done to elucidate the reasons for the above emergence and thought that it was caused by different doses of corticosteroids (Tsao et al., 2004; Liu et al., 2014a,b). A study has indicated that the small doses of hydrocortisone, namely physiological doses, could improve the survival rate of patients with severe sepsis (Minnci et al., 2004). Therefore, we suspected that the effects of different doses of Dex on autophagy of cortex neurons of SAE rats made a difference.

Our previous experiments have found that autophagy in neurons of sepsis rats may be related to the inhibition of the NF-κB signaling pathway (Su et al., 2015). In recent years, a large number of studies have found that NF-κB is closely related to mTOR (Bai et al., 2018; Kim et al., 2018; Toosi et al., 2018), and previous studies indicate that the mTOR signaling pathway has been recognized as an important negative regulator of autophagy (Itakura et al., 2013; Liu et al., 2014a,b; Gordon et al., 2015; Hsu et al., 2015). The inhibition of mTOR can downregulate the expression of downstream effectors and increase autophagy. To further investigate the mechanism of SdDex-induced autophagy, we detected the expressions of mTOR and downstream effectors, namely p70S6K and 4E-BP1. Our results showed that SdDex decreased the expressions of mTOR, p70S6K, and 4E-BP1 in cortex neurons of rats with SAE (Fig. 9), and after the further use of 3-MA on the basis of SdDex, autophagy was inhibited and the mTOR signaling pathway was activated again (Fig. 10). Therefore, we consider that SdDex could induce autophagy of cortex neurons from juvenile rats with SAE by the inhibition of the mTOR signaling pathway. However, the activation of autophagy could not be related to the mTOR signaling pathway because the mTOR expression in cortex neurons of SAE rats was upregulated in this study (Fig. 9).

Interestingly, we found that HdDex did not activate autophagy more effectively than SdDex, including lower conversion of LC3-I to LC3-II (Fig. 6C and 7C), and higher expression of p62/SQSTM1 (Fig. 8). However, HdDex does inhibit mTOR signaling more (Fig. 9). HdDex treatment of SAE rats resulted in severe cortical damage (Fig. 3D), no improvement in cerebral edema (Fig. 4), and disordered neuron structure (Fig. 5D); neurobehavioral test-score, vital signs, EEG, and SEP did not significantly improve (Table 2). There was a contradiction between these phenomena and the traditional understanding of the mTOR signaling pathway. Since extensive use of Dex can induce excessive apoptosis of cells (Feng et al., 2017), we speculate whether the cortical neuron injury induced by HdDex in this study is also related to apoptosis.

Studies suggest that autophagy and apoptosis may be triggered by common upstream signals, sometimes leading to a combination of autophagy and apoptosis. This means that apoptosis and autophagy mechanisms share common pathways that connect or polarize cellular responses (Mauri et al., 2007). Therefore, this study hypothesized that HdDex induced autophagy of cortical neurons also caused apoptosis, resulting in pathological damage of SAE rat brain tissue. During cell apoptosis, caspase-3 is known as the “executor” protease because it can degrade multiple substrates in the cytoplasm and nucleus that results in cell death (Ali et al., 2004). Consequently, we detected the expressions of cleaved caspase-3 and upstream effectors, namely Bax and Bcl-2 through western blotting analysis. Our results indicated that the Bcl-2 expression of cerebral cortex neurons was lower in HdDex-treated SAE rats than in SdDex-treated SAE rats; however, the Bax expression was higher. The Bax/Bcl-2 ratio was also elevated (Fig. 11). These data suggest that HdDex could induce apoptosis of neurons from juvenile rats with SAE by the caspase-3 signaling pathway.

We believe that the protective effect of SdDex on SAE in rats may be related to the inhibition of the mTOR signaling pathway, while the damage of HdDex on the central nervous system may be related to the activation of caspase-3. This study also has some limitations. For instance, 30-day juvenile rats represent only one age group, and it is not clear whether the same studies will yield the same results in infant or adult rats.

In summary, the mechanisms of brain injury in SAE are very complex. The roles of different signaling pathways in the pathogenesis of SAE are not exactly the same. The roles of the same signal pathway in different stages of the disease are also not exactly the same. The protective effects of SdDex treatment on SAE may be related to the inhibition of mTOR signaling, and the harm of HdDex to the central nervous system may be related to the activation of the caspase-3 signaling pathway. In the systemic inflammatory response, the effects of different doses of Dex on different systems needs to be further evaluated through experiments and clinical trials.
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AVAILABILITY OF DATA AND MATERIALS
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

AUTHORS’ CONTRIBUTIONS
RXZ conducted all the experiments and conducted the statistical analysis. XMS and YYL draft the manuscript, YQ and DZM participated in the design of the study. XMS and OH participated in the physiological examination. XHL designed the project and finalized the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
See methods section ‘Animals and treatments’.

DECLARATION OF COMPETING INTEREST
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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