Original Article
Curcumin alleviates neuronal apoptosis and cerebral mitochondrial dysfunction in septic mice

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Abstract: The pathogenesis of septic brain injury is quite complicated. Apoptosis and mitochondrial dysfunction is reported to be critical in sepsis. Curcumin (Cur) is an antioxidant and act to scavenge free radicals in the mitochondria. This study, therefore, aims to investigate the effect of Cur on brain injury induced by sepsis in a mouse model of cecal ligation and puncture (CLP). Mice are randomly assigned into sham group, CLP group and CLP+Cur group. Survival rate, brain water content, blood-brain barrier (BBB) integrity, neuronal apoptosis and mitochondrial function were assessed. The expression of Bcl-2 and Bax was detected by western blot. Our results suggest that Cur improved survival rate, attenuates brain edema, enhanced BBB integrity, decreased apoptosis and attenuated mitochondrial dysfunction in septic mice. In summary, Cur is a potential drug for the treatment of sepsis.

Keywords: Curcumin, sepsis, apoptosis, mitochondrial dysfunction

Introduction

Sepsis is a severe disease that is associated with high mortality [1]. Severe sepsis is associated with a systemic inflammatory response syndrome (SIRS) that is characterized by overproduction in reactive oxygen species (ROS) and increased levels of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), macro-phage migration inhibitory factor (MIF), and high mobility group box 1 (HMGB1). These cytokines individually, or in combination, contribute to the recruitment of leukocyte and subsequent organ damage in sepsis [2, 3]. Sepsis can cause various complications, such as cardiac dysfunction, kidney injury, liver disorder and brain injury. In those complications, the brain dysfunction, known as septic encephalopathy in septic patients, is reported to be earlier and more frequently than those in other systems [4]. Sepsis triggers cell death, inflammation, and oxidative stress in the brain and the clinical manifestation ranges from mild delirium to deep coma [4]. Up to now, the therapy for sepsis-induced brain injury is lacking.

Curcumin (Cur), the active component of the traditional Chinese drug *Curcuma longa*, has a wide spectrum of biological functions, including cardioprotective, anti-inflammatory, antioxidant properties [5, 6]. Cur has been tested as a potential therapeutic agent in a number of pathological conditions, including cardiovascular disease and other disorders [7]. In addition, the protective role of Cur has been suggested in several studies [8]. However, the role of Cur in sepsis-induced brain injury has not been investigated. Therefore, the present study aims to explore whether Cur protects the brain from sepsis-induced injury.

Materials and methods

Animals

All experiments were performed on healthy adult male C57BL/6J mice that weighed between 22 g and 25 g. The mice were obtained from the animal center of Shandong University. Mice were fed under a pathogen-free condition at about 22°C on a 12 h light-dark cycle with free access to food and water. This study was

performed according to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (National Institutes of Health Publication No. 85-23, revised 1996) and was approved by the Ethics Committee of Shandong University.

Reagents

Curcumin (Cur), evans blue (EB), EX527, dimethyl sulfoxide (DMSO), and 4',6-diamino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kits were purchased from Roche (Mannheim, Germany). Antibodies against cytochrome c, Bcl-2, Bax, and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). The rabbit anti-goat, goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from the Zhongshan Company (Beijing, China).

Cecal ligation and puncture (CLP) model

Fasting was performed for 8 h for all mice but water was allowed ad libitum before the experiments. The CLP model was established as previously reported with some modifications [9]. In brief, after anesthetized with intraperitoneal injection of 50 mg/kg pentobarbital sodium, mice were put on aseptic operating table. In sterile operation environment, a 1 cm abdominal midline incision was made to expose the cecum, which was ligated below the ileocecal valve and punctured once with a 20-gauge needle. A small amount of stool was squeezed through the puncture site. The bowel was then sent back to the abdomen and the incision was sutured with a sterile 6-0 silk. The mice in Sham-operated group underwent the similar operation without cecal ligation and puncture. Animals among all groups received basic normal saline resuscitation (50 ml/kg) injected subcutaneously.

Experimental protocol

Mice were randomly assigned into three groups: Sham group (n=60); CLP group (n=60); CLP+Cur group (n=60). Cur was dissolved in 1% DMSO (in normal saline). And Cur was administrated intraperitoneally at a dose of 100 mg/kg each time, at 3, 12, 24 hours after surgery. The mice in the Sham group were given equal amount of vehicle at 3, 12, 24 hours after surgery.

Evaluation of survival rate

The mice in each group had free access to food and water. And mice were fed under a pathogen-free condition. The survival rate was evaluated within 7 days after sham or CLP operation.

Brain water content

Mice were anesthetized and sacrificed and the brains were removed at 48 h after operation. Brain water content was measured using the standard wet-dry method [10]. Brain samples were immediately weighed on an electronic balance to obtain the wet weight. Then brain samples were dried in an oven at 100°C for 24 h to obtain the dry weight. Brain water content was calculated as follows: (wet weight-dry weight)/ wet weight ×100%.

Blood brain barrier (BBB) permeability

Evans blue (EB) dye was used to evaluate the BBB permeability as the method previously described with some modifications [11]. At 48 h after operation, mice were injected with EB (2% in saline) in a dose of 3 ml/kg through tail vein and allowed to circulate for 2 h. Under deep anesthesia, they were transcardially perfused with saline and sacrificed. The brain in each group was removed and weighted, homogenized in formamide (1 ml) and incubated at 37°C for 48 h. After centrifugation, the optical density of the supernatant was measured at OD 625 nm by a microplate reader (Multiskan Spectrum, Thermo Scientific, USA). According to a linear standard curve, the amount of EB (mg/g wet weight) was quantified and expressed as relative amount.

TUNEL staining

Neuronal apoptosis was analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Briefly, 50 μL TUNEL reaction mixtures were added on each sample, and the slides were incubated in humidified atmosphere for 60 min at 37°C in the dark and then rinsed with PBS (pH 7.4). To detect the nuclei, the slides were incubated with DAPI for 5 min at room temperature in the dark, rinsed with PBS, and observed with a fluorescence microscopy. The TUNEL-positive cells showed green fluorescence and nucleus were stained with blue fluorescence. Apoptotic index was
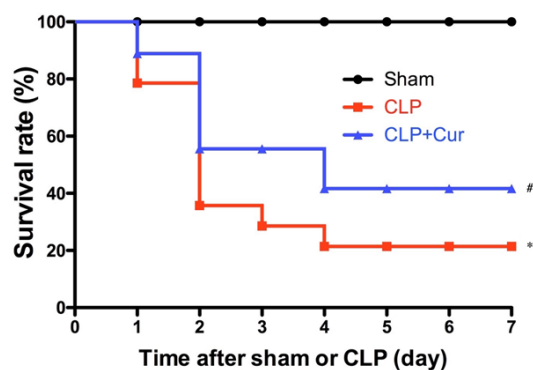
Figure 1. Effect of curcumin on the 7-day survival rate after CLP surgery. Values are expressed as survival percentage, n=20 in each group. $^{*}P<0.05$ versus Sham group, $^{#}P<0.05$ versus CLP group.

determined as the ratio of the number of TUNEL-positive neurons to the total number of neurons.

Mitochondria and cytosolic fraction isolation

The brain tissues were homogenized in an ice-cold isolation buffer (0.25 mM sucrose, 1 mM K-EDTA, 10 mM Tris-HCl, pH 7.4) with a Teflon pestle. The homogenate was immediately centrifuged at 2000 g for 3 min, the supernatant was centrifuged again at 2000 g for 3 min, and the second supernatant was decanted and centrifuged at 12000 g for 8 min. The supernatant was discarded, and the pellet was resuspended in isolation buffer without KEDTA. Then, the suspension was centrifuged at 12000 g for 10 min. The resulting brown mitochondrial pellet was resuspended in the same buffer. The supernatant represented the cytosolic fraction. The procedures were carried out at 4°C. Cytosolic and mitochondrial fractions were stored at -20°C until use.

Mitochondrial membrane potential (MMP) measurement

According to the methods reported previously [12], MMP was determined using JC-1. Mitochondria samples (0.5 mg/mL, 1 mL) were incubated with 19 mL JC-1 staining buffer according to the manufacturer's instructions (Sigma-Aldrich). At the end of the experiments, valinomycin was added as a negative control. Fluorescent intensity was determined at 37°C in a fluorescence spectrophotometer. The ratio of aggregates (red in the Web version, 590 nm)

to monomer (green in the Web version, 525 nm) was calculated as an indicator of MMP.

Mitochondrial reactive oxygen species (ROS) production detection

Mitochondrial ROS production was measured using dichlorodihydrofluorescein diacetate (H_2-DCFDA) according to the instructions. Mitochondrial protein was incubated in a total volume of 200 µL respiration buffer at 37°C for 15 min in the presence of 10 µM H_2DCFDA. The relative amount of mitochondrial H_2O_2 and free radical production was measured using a plate reader with 490-nm excitation and 526-nm emission filters.

Mitochondrial complex I activity measurement

Mitochondrial complex I was measured according to previous reports [13]. This method involves catalytic oxidation of NADH to NAD^{+} with the subsequent reduction of cytochrome c. The reaction was initiated by the addition of requisite amount of solubilized mitochondrial sample and followed absorbance change at 550 nm for 2 min.

Western blot

The brain cortex samples were collected and protein extractions were obtained 48 h after operation. The protein was quantified by using a BCA Protein Assay kit. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto Immobilon NC membranes (Millipore, Boston, MA, USA). The membranes were blocked with 5% non-fat milk in TBST at room temperature and then incubated with antibodies against Bcl-2, Bax, and β-actin (1:1000) overnight at 4°C, followed by three times wash with TBST, 10 min for each time. The membranes were then incubated with the appropriate secondary antibodies (1:5000) at room temperature for 1.5 h and washed with TBST. The protein bands were detected using a Bio-Rad imaging system and quantified using the Quantity One software package.

Statistical analysis

Data are presented as the mean ± S.E.M.. SPSS 13.0 was used to analyze data in this study. Fisher exact test probability method was
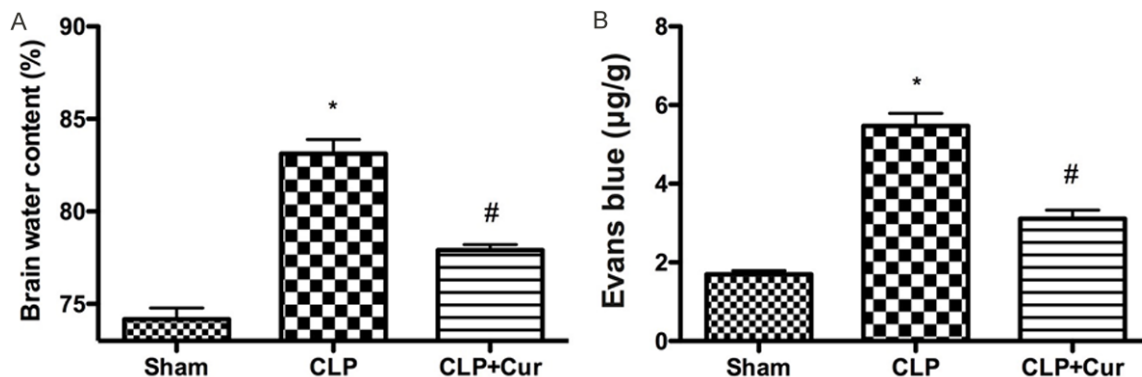
Figure 2. Effect of curcumin on brain water content and BBB integrity. A. Effect of melatonin on brain water content. B. Effect of melatonin on blood-brain barrier (BBB) integrity. The results are expressed as the mean ± S.E.M., n=6 in each group. *P<0.05 versus Sham group, #P<0.05 versus CLP group.
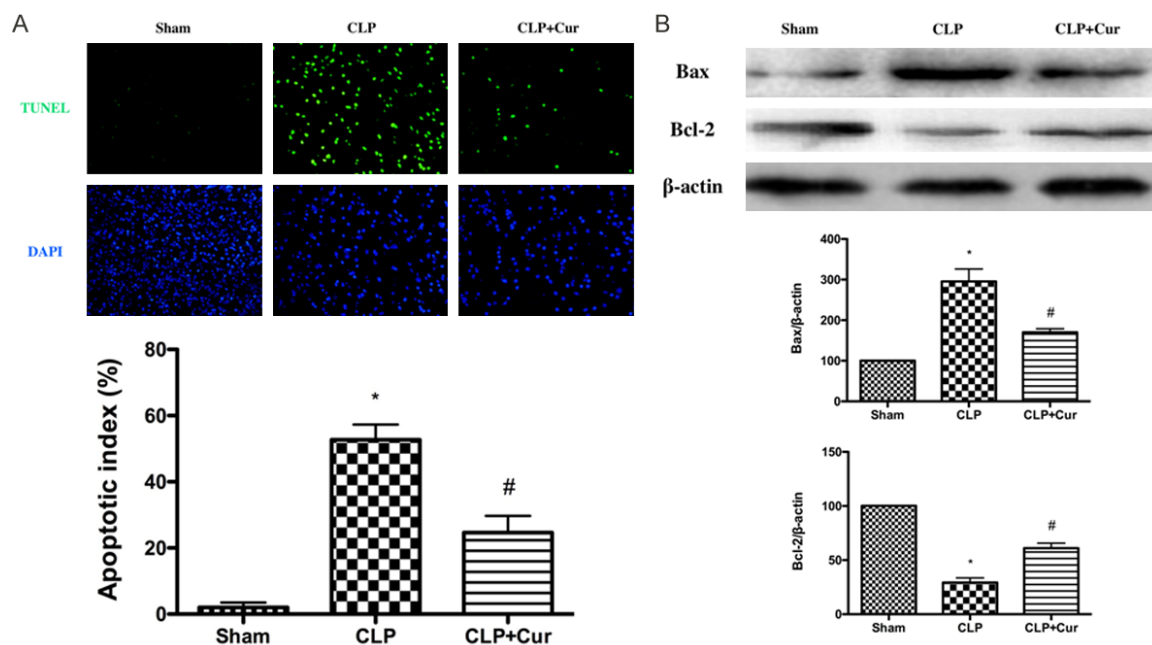

Figure 3. Effect of curcumin on neuronal apoptosis. A. TUNEL staining. B. Western blot analysis of Bcl-2 and Bax expression. The results are expressed as the mean ± S.E.M., n=6 in each group. *P<0.05 versus Sham group, #P<0.05 versus CLP group.

used to analyze the survival rates. The comparison of multiple groups was tested by one-way analysis of variance. The LSD t-test was used to make the intergroup comparison. Values of P<0.05 was considered to be statistically significant.

Results

Curcumin improved survival rate after sepsis

As shown in **Figure 1**, the 7-day survival rate of the sham group was 100%. Seven days after

CLP surgery led to a significant decrease in survival rate (21.4%) (versus sham group, P<0.05). With the administration of Cur, the survival rate was elevated to 41.7% in CLP+Cur group (versus CLP group, P<0.05).

Curcumin attenuated brain edema and preserved BBB integrity after sepsis

The mice in CLP group exhibited an increase in brain water content and a decrease in blood-brain barrier integrity. Cur administration attenuated brain edema and enhanced BBB integrity
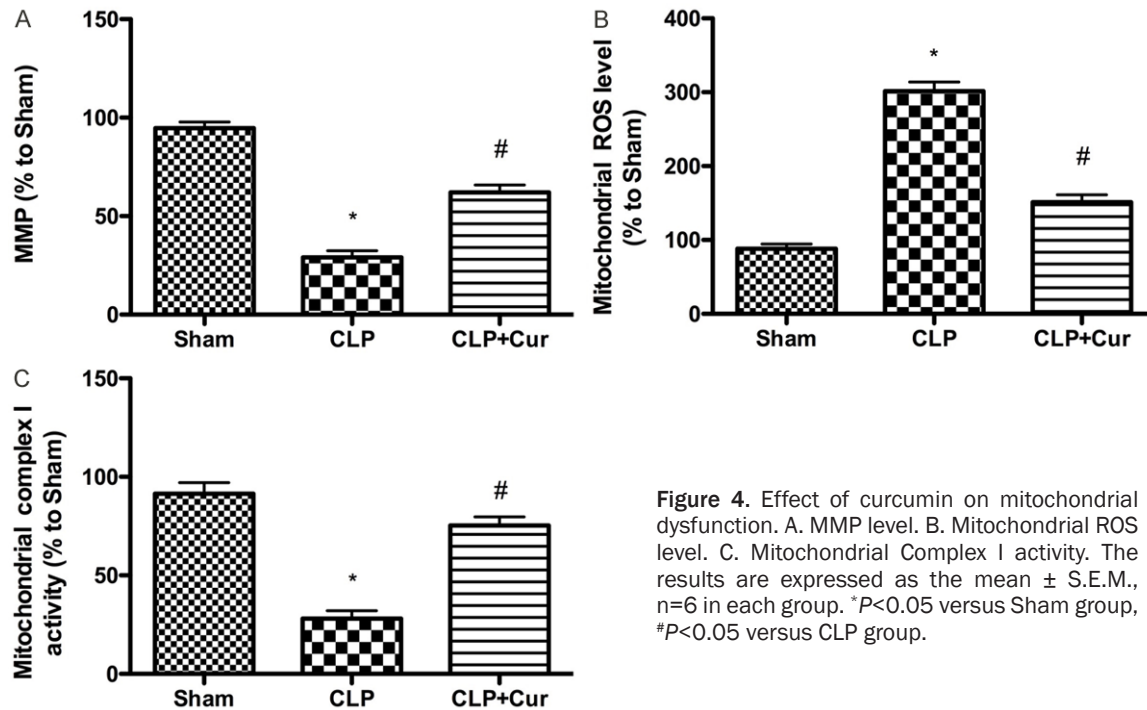
Figure 4. Effect of curcumin on mitochondrial dysfunction. A. MMP level. B. Mitochondrial ROS level. C. Mitochondrial Complex I activity. The results are expressed as the mean ± S.E.M., n=6 in each group. *$P<0.05$ versus Sham group, #$P<0.05$ versus CLP group.

in CLP+Cur group (versus CLP group, $P<0.05$) (**Figure 2**).

Curcumin alleviated neuronal apoptosis induced by sepsis

The neuronal apoptosis increased dramatically as evidenced by the increased TUNEL positive neurons, the decreased Bcl-2 expression and the increased Bax expression in CLP group. Cur administration attenuated neuronal apoptosis markedly in CLP+Cur group (versus CLP group, $P<0.05$) (**Figure 3**).

Curcumin attenuated mitochondrial dysfunction after sepsis

Sepsis induced a reduction in MMP level and mitochondrial complex I activity, and an increase in ROS production. Cur treatment significantly increased MMP level and mitochondrial complex I activity, and decreased ROS production (versus CLP group, $P<0.05$) (**Figure 4**).

Discussion

In the present study, our results suggest that Cur protects the brain from injury induced by sepsis. Cur attenuates brain edema and enhanced BBB integrity in septic mice. Cur alle-viates mitochondrial dysfunction induced by sepsis.

First, Cur administration after CLP significantly increases the 7-day survival rate. Then, we found that Cur attenuates brain edema, neuronal apoptosis and BBB disruption. Additionally, we found that Cur alleviates mitochondrial dysfunction as evidenced by deceased ROS production and elevated MMP level and mitochondrial complex I activity.

Sepsis-induced brain injury is a severe compliment of sepsis, and its clinical manifestations include confusion, delirium and even coma. What is worse, sepsis can lead to cognitive decline, progressive immunosuppression, and even metabolic and hydroelectrolyte imbalance [14]. Patients with sepsis-induced brain injury are inclined to have a higher mortality. Sepsis-induced brain injury is associated with the overproduction of proinflammatory cytokines, oxidative stress, and mitochondrial dysfunction, resulting in cell death and organ damage [15]. Additionally, the injury to endothelial cells and astrocytes lead to BBB disruption, triggering the leakage of immune cells and inflammatory mediators and the brain injury is aggravated greatly [16]. Brain edema induced by sepsis is related to the reduction of tight junction protein

occludin, which is inversely correlated with the degree of cerebral edema [17]. Our results suggest that Cur attenuates brain edema and enhanced BBB integrity after sepsis. Moreover, Cur mitigates neuronal apoptosis induced by sepsis as supported by the decreased number of TUNEL-positive neurons, decreased expression of Bax, and increased expression of Bcl-2.

Mitochondrial dysfunction is a key pathological process involved in sepsis [18]. It has been suggested that mitochondrial dysfunction is augmented in sepsis-induced acute kidney injury and polydatin attenuates mitochondrial dysfunction induced by sepsis [19]. Sepsis results in ATP deletion, leading to mitochondrial dysfunction and brain injury. In addition, it has been suggested that sepsis-induced brain mitochondrial dysfunction is associated with altered mitochondrial Src and PTP1B levels [20]. In this study, we investigated mitochondrial function in the brain. The results clearly indicate that the mitochondrial MMP was well preserved due to Cur treatment. This effect was accompanied by significantly elevated mitochondrial Complex I activity and reduced level of mitochondrial ROS, indicating that the mitochondrial oxidative damage induced by sepsis was remarkably attenuated. Mitochondrial oxidative damage has been implicated in a variety of pathological situations, including sepsis [21]. The occurrence of mitochondrial oxidative damage contributes to an increased loss of efficiency in mitochondrial function, which, in turn, further aggravates the mitochondrial oxidative damage and ultimately aggravates sepsis-induced brain damage.

Cur has been widely used as a potential antioxidant and anti-inflammatory agent in clinical and experimental studies [22, 23]. It is suggested that Cur attenuates chronic ethanol-induced liver injury by inhibition of oxidative stress via mitogen-activated protein kinase/nuclear factor E2-related factor 2 pathway in mice [24]. Additionally, Cur protects against oxyhemoglobin-induced injury in rat cortical neurons via attenuating oxidative stress [25]. In the present study, the results suggest that Cur alleviates mitochondrial ROS overproduction induced by sepsis, indicating that Cur attenuates oxidative stress in sepsis.

In summary, we documented that Cur could improve survival of mice with sepsis and ame-liorated brain injury, which may be related to the reductions in neuronal apoptosis, and mitochondrial dysfunction.

Disclosure of conflict of interest

None.

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