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Caspase-3 Mediates In Part Hippocampal Apoptosis in Sepsis

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Abstract The brain is one of the first organs affected during sepsis development resulting in apoptosis for a short-term and cognitive impairment for a long-term. Despite its importance, the mechanisms of brain dysfunction during sepsis are not fully elucidated. Thus, we here, in an animal model of sepsis, evaluated apoptosis in the dentate gyrus cell layer of the hippocampus to document the involvement of caspase-3 in the pathogenesis of neuronal apoptosis. Wistar rats sham-operated or submitted to the cecal ligation and perforation (CLP) procedure were killed at 12, 24, 48 h, and 10 days after surgery for the determination of caspase-3 and apoptosis rate. In a separate cohort of animals, a caspase-3-specific inhibitor was administered and animals were killed at 12 h after sepsis. An increase in the number of apoptotic cells 12, 24, and 48 h by histopathological evaluations and an increase of caspase-3 apoptotic cells 12 and 24 h after sepsis induction were observed. The caspase-3 inhibitor decreases the number of apoptotic cells by histopathological evaluations but not by immunohistochemistry evaluations. Caspase-3 is involved in part in apoptosis in the dentate gyrus cell layer of the hippocampus in septic rats submitted by CLP.

Keywords Sepsis · Hippocampus · Caspase-3 · Apoptosis · Brain · Rats

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Introduction

Sepsis remains an important health-associated problem with high rates of morbidity and mortality. Sepsis occurs by the failure to limit the spreading of the infection leading to systemic inflammation and multiple organ failure [1]. The brain is one of the first organs affected during sepsis development, resulting in acute and long-term brain dysfunction in both animal models and humans. However, the mechanisms associated with cognitive impairment are not well understood. Indeed, it is known that at early times, hippocampus alterations are related with cognitive impairment observed in animal models such as cerebral blood flow and blood–brain barrier alterations [2–4], cerebral edema [4, 5], inflammation [1], oxidative stress [6], mitochondrial failure [7, 8], microcirculatory alterations [2–4], and apoptosis [9–11].

Caspase-3 is a central player of programmed cell death because it is responsible in inactivating proteins that protect living cells from apoptosis [12]. In some diseases such as ischemia [13], bacterial meningitis [14], and traumatic brain injury [15], apoptotic cells are colocalized with activated

caspase-3 in the hippocampus. However, there is no clear description of the relation between caspase-3 activity and apoptosis in the hippocampus during acute and chronic phases of sepsis-associated brain dysfunction. Thus, in this study, we evaluate caspase-3-positive apoptotic cells in the dentate gyrus layer of the hippocampus in order to document the involvement of caspase-3 in the pathogenesis of neuronal apoptosis during sepsis.

Material and Methods

Animals

Adult male Wistar rats (220 to 300 g) were used. They were housed five to a cage with food and water available ad libitum and were maintained on a 12-h light/dark cycle (lights on at 7 a.m.). All procedures were approved by the Animal Care and Experimentation Committee at UNESC, Brazil (21/2010).

Cecal Ligation and Perforation

Rats were subjected to cecal ligation and perforation (CLP) as previously described [16]. Briefly, a midline laparotomy was performed to allow exposure of the cecum with the adjoining intestine. The cecum was tightly ligated with a 3.0-silk suture at its base, below the ileocecal valve, and perforated seven times with a 14-gauge needle. The cecum was then gently squeezed to extrude a small amount of feces from the perforation site and then returned into the peritoneal cavity, followed by the laparotomy closure with 4.0-silk sutures. The sham-operated group rats were submitted to all surgical procedures, but the cecum was neither ligated nor perforated. After the surgery, all the groups received 50 mL/kg saline (subcutaneously) immediately and 12 h after CLP.

Experimental Procedure

Sham and CLP animals were killed 12, 24, 48 h, and 10 days after surgery and perfused by the left cardiac ventricle with 4 % paraformaldehyde (PFA), the brain was dissected, post-fixed for 2 days at 4 °C, and cryoprotected for another 2 days in an 18 % sucrose solution. Then brains were frozen in 2-methylbutane (−50 °C), and cryosections (45 μm for apoptosis analysis and 10 μm for immunohistochemistry analysis) obtained using a Cryostat (Jung CM1800, Leica, Glattbrugg, Switzerland) were transferred onto polylysine-coated glass slides to evaluate apoptosis. Brain slices were collected in phosphate-buffered saline (PBS), transferred onto chrome–alum–gelatin-coated glass slides, briefly dried at room temperature, and rinsed in PBS to determine caspase-3 immunohistochemistry.

Quantification of Apoptosis

Glass slides for apoptosis were then put in xylol, hydrated, stained with Nissl solution, dehydrated, and mounted with Entellan (Merck, Darmstadt, Germany). The quantification of apoptosis in the dentate gyrus of the hippocampus was quantified by bright-field microscopy. A series of typical morphological changes occur in the course of the apoptotic process. Here, the identification of apoptotic cells was based on the recognition of round or oval apoptotic bodies in Nissl-stained sections. Because most cells have multiple apoptotic bodies, a cell was defined as apoptotic if it had two or more round, regularly shaped, dark chromatin clumps. Cells presenting these features were counted in three visual fields at×400 magnification in each of the four blades of the dentate gyrus for quantitative assessment of apoptosis as described previously. The counts were expressed in absolute numbers, and the mean value per brain was calculated and used for statistical evaluation. Histopathological evaluations were performed by a blinded investigator.

Immunohistochemistry

Cryosections on glass slides were washed in PBS and were then incubated separately for 1 h at room temperature (37 °C) with CM1, a rabbit polyclonal antibody caspase-3 (Idun Pharmaceuticals, La Jolla, CA) at a dilution of 1:400. Sections for negative controls were incubated with BSA-TBS. After washing three times for 10 min in PBS, the samples were incubated for 45 min with a goat anti-rabbit secondary antibody (dark room) (Sternberger Monoclonals, Lutherville, MD) at a dilution of 1:100. The samples were again washed three times for 10 min in PBS in a dark room. Afterwards, they were stained with DAPI (1:2,000) for 1 min (dark room) and then the slices were washed three times for 10 min in PBS in a dark room and mounted with Mowiol (EMS, Fort Washington, PA) and were covered with a coverslip.

Intervention

The animals were randomly divided to receive an intracisternal injection of 10 μL sterile PBS or 200 μg caspase-3-specific inhibitor Ac-DEVD-CHO (Bachem, Bubendorf, Switzerland) in 10 μL sterile PBS immediately and 6 h after sepsis. Animals were killed 12 h after surgery and perfused with ice-cold 4 % PFA.

Statistical Analysis

The data were presented as mean±SD and analyzed using one-way ANOVA followed by post hoc Tukey test. Values of $p<0.05$ were considered to be significant.

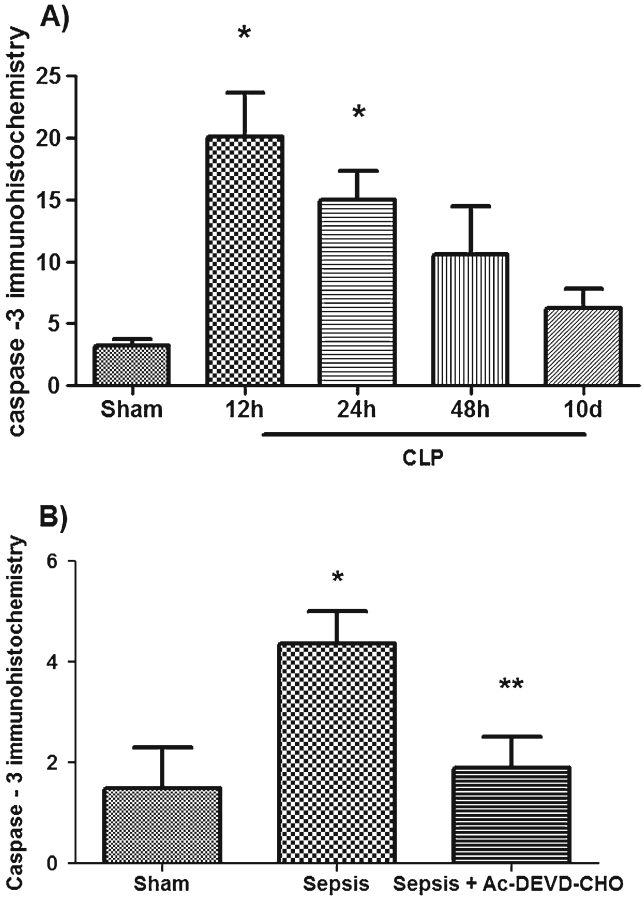

Fig. 1 Number of caspase-3-positive apoptotic cells in the hippocampal dentate gyrus. **a** Impact of sepsis on caspase-3 immunohistochemistry in the dentate gyrus of the hippocampus. Sham had significantly lower apoptosis scores than CLP 12 and 24 h after induction. **b** Effect of the caspase-3-specific inhibitor Ac-DEVD-CHO on caspase-3-positive apoptotic cells in the dentate gyrus of hippocampus 12 h after sepsis induction. Treatment with the inhibitor significantly reduced capsase-3 immunohistochemistry comparing sepsis and saline-treated animals. *Bars* represent mean and standard deviation. *$p<0.05$ vs. sham; **$p<0.05$ vs. CLP; $n=10$

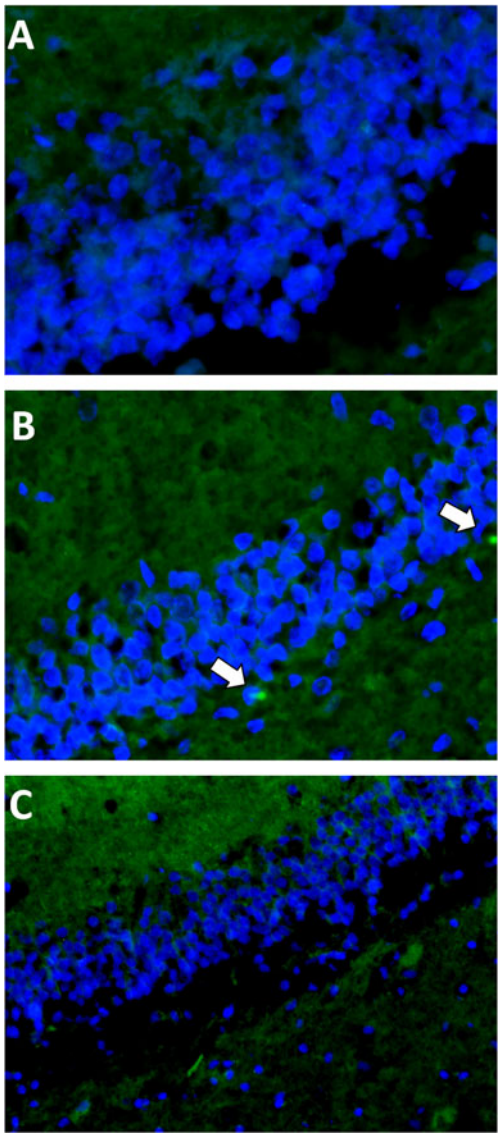

Fig. 2 Documentation of caspase-3-positive apoptotic cells in experimental sepsis in the dentate gyrus of hippocampus: **a** sham, **b** CLP, and **c** CLP plus caspase-3-specific inhibitor Ac-DEVD-CHO

Results

Figure 1 shows the number of caspase-3-positive apoptotic cells in the hippocampal dentate gyrus. We observed in septic animals an increase in the number of caspase-3-positive cells 12 h ($F_{(4,\ 48)}=9.63$, $p=0.0001$) and 24 h ($F_{(4,\ 48)}=9.63$, $p=0.003$) after surgery when compared with the sham group. There were no differences between the groups 48 h ($F_{(4,\ 48)}=9.63$, $p=0.222$) and 10 days ($F_{(4,\ 48)}=9.63$, $p=0.851$) (Fig. 1a). When rats were treated with caspase-3-specific inhibitor Ac-DEVD-CHO, a significant decrease of caspase-3-positive apoptotic cells ($F_{(2,\ 23)}=4.92$, $p=0.041$) was observed 12 h after surgery when compared with the sham group (Fig. 1b). As seen in Fig. 2, positive staining for activated caspase-3 was seen exclusively in CLP animals.

Figure 3 shows the number of apoptotic cells based on the recognition of round or oval apoptotic bodies in Nissl-stained sections. An increase in the number of apoptotic cells 12 h ($F_{(4,\ 47)}=12.74$, $p=0.0001$), 24 h ($F_{(4,\ 47)}=12.74$, $p=0.0001$), and 48 h ($F_{(4,\ 47)}=12.74$, p=0.038) after sepsis was observed when compared with the sham group. Ten days after surgery, there was no difference in the number of apoptotic cells ($F_{(4,\ 47)}=12.74$, $p=0.830$) when compared with the sham group (Fig. 3a). There was a trend for a decrease in the number of apoptotic cells with caspase-3 inhibitor treatment CLP + Ac-DEVD-CHO when compared with the CLP group ($F_{(2,\ 23)}=11.72$, $p=0.094$) by histopathological evaluation (Fig. 3b).

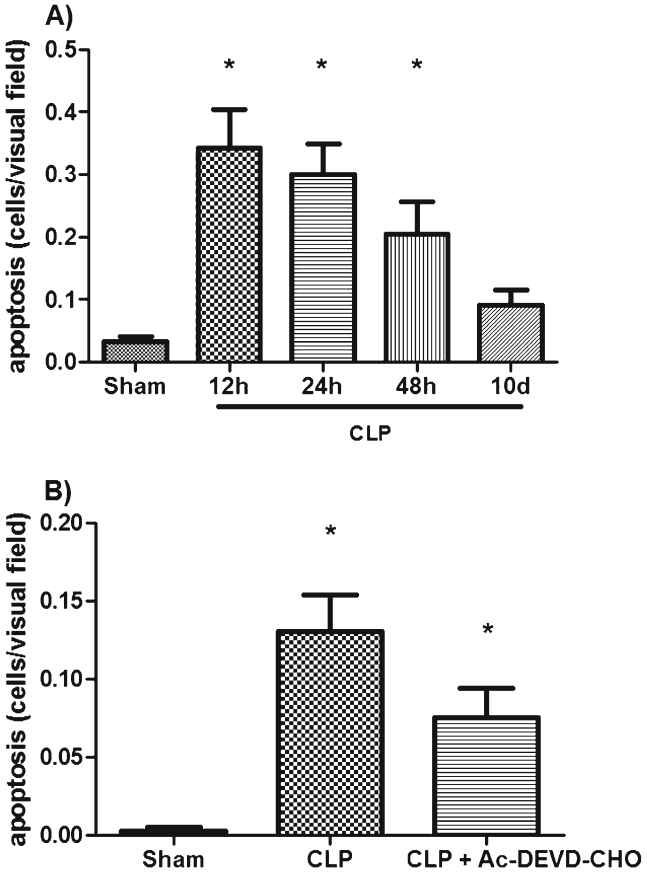

Fig. 3 Number of apoptotic cells by histopathological evaluations. The impact of sepsis on apoptotic injury in the dentate gyrus of the hippocampus was observed in Fig. 1a. CLP animals had significantly higher apoptosis scores 12, 24, and 48 h after sepsis induction when compared with sham animals. The effect of a caspase-3-specific inhibitor Ac-DEVD-CHO on apoptotic injury in the dentate gyrus of hippocampus at 12 h after induction is showed in **b**. Treatment with the inhibitor significantly did not reduce hippocampal apoptosis due to sepsis compared to animals treated with saline. *Bars* represent mean and standard deviation. *$p<0.05$ vs. sham; $n=10$

Discussion

We here demonstrated that, after sepsis, apoptosis occurs in the dentate gyrus of the hippocampus, and this can be prevented by the administration of a caspase-3 inhibitor. The importance of apoptosis in the pathogenesis of sepsis is well established, and increased apoptotic cells are seen in different organs both in animal models and patients [17–22]. Sharshar and others had reported that neuronal apoptosis was more evident in autonomic nuclei in septic patients [23]. On the other hand, Semmler and coworkers demonstrated a time-dependent increase in the number of apoptotic cells in the brains of endotoxemic rats and suggested that the hippocampus is the most vulnerable brain region [10]. An increase in neuron apoptosis was also reported in the CA1 region of the hippocampus, choroid plexus, and Purkinje cells of the cerebellum using a CLP model [9, 11]. These findings are in accordance with the data observed in the frontal cortex in a model of experimental sepsis in pigs [24]. Matsuoka and collaborates showed that LPS administration induces delayed neuronal apoptosis in the hippocampus, and this possibly involves excessive nitric oxide production [25].

One of the characteristics of dentate gyrus is to have a high amount of caspase-3 mRNA. This characteristic may reflect in the predisposition of this structure for caspase-mediated apoptosis [26]. This, and the fact that caspase activation occurs in the context of inflammation, might explain the specific vulnerability of the dentate gyrus in sepsis. Recently, an increase for caspase-3-positive cells in the median preoptic nucleus, subventricular zone, dentate gyrus, and CA1 and CA3 regions of the hippocampus in an animal model of sepsis induced by CLP was demonstrated [11]. However, Messaris and collaborates showed that there were no differences in the caspase-8-positive cells early after sepsis, but they demonstrated an increase in Bax immunoreactivity [9]. Thus, caspases can be a therapeutic target for sepsis treatment, in the same way that they are in neurodegenerative diseases. In this study, the use of a caspase-3 inhibitor Ac-DEVD-CHO reduced caspase-3-positive apoptotic cells in the dentate gyrus of the hippocampus by more than 50 %.

Hippocampal formation plays an important role in spatial learning and memory in rats. Many studies had reported that the hippocampus may be more vulnerable to inflammatory and/or circulatory changes during the sepsis (for review, see [27]) and this area is a primary site of adult neurogenesis [28]. It had been previously reported that survivors from sepsis have impaired spatial learning and memory [29] and some cognitive skills do not improve completely in septic patients after 1 or 2 years of follow-up [30]. Indeed, it is tempting to suggest that the increase in apoptotic cell death in dentate gyrus of hippocampus may underlie, at least, some of these impairments [31].

Some limitations must be observed when analyzing our results. First, we evaluated the effects of the caspase-3 inhibitor only at 12 h after sepsis; then, some significant effect of caspase-3 inhibition on later time points is not addressed here. Second, there is a discrepancy between the effect of caspase-3 inhibitor on caspase-3-positive apoptotic cells and on apoptosis accessed by recognition of round or oval apoptotic bodies in Nissl-stained sections. In fact, we believed that the significant effect upon caspase-3-positive cells and the trend towards a protective effect in Nissl-stained sections supports a role for caspase-3 in hippocampus apoptosis, but this must be interpreted with caution. In conclusion, our data suggest that caspase-3 is upregulated after sepsis in the hippocampus and seems, at least in part, responsible to hippocampus cell apoptosis in CLP animals.

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