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Inhibition of Panx1 reduces neuroinflammatory response and alleviates brain damage in septic mice

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Abstract. Objective: This study mainly investigates whether Pannexin 1 protein (i.e. Panx1) plays a role in the progression of brain injury in sepsis mice and its potential mechanisms. Methods: A total of 45 clean grade adult male C57BL/6 healthy mice were selected and divided into three groups using a random number table method, namely the sham group, the cecal ligation perforation (CLP) group, and the CBX pretreatment plus cecal ligation perforation (CBX+CLP) group, with 15 mice in each group. Excluding the Sham group, the remaining two groups of study mice were used to prepare sepsis brain injury models using the CLP method. The CBX+CLP group was injected into the lateral ventricles of both sides of the mouse half an hour before the modeling process, with a total amount of 1 μL CBX, which was injected into the bilateral lateral ventricles of the other two groups of mice with the same dose of physiological saline solution. 24 hours after the completion of modeling, three groups of mice were evaluated for neurological behavior. Subsequently, all mice were euthanized. After the cardiac perfusion phase, brain tissue of the mice was taken to observe the pathological damage of the hippocampal tissue. The effective content of IL-1β and TNF-α in the mouse's hippocampal tissue was measured, and the expression of Panx1 protein, Caspase-1, NLRP3 protein, as well as NLRP3, Panx1, Caspase-1, TNF-α, IL-1β were measured the mRNA of corresponds to its expression status. Results: Compared with Sham group mice, the neurobehavioral score of CLP group mice significantly decreased, and the pathological damage degree of hippocampal neurons in mice was significant. The expression levels of inflammatory factors IL-1β and TNF-α were increased, and the corresponding expression levels of Panx1, NLRP3, Caspase-1 proteins and mRNA were increased. The mRNA expression levels of inflammatory factors IL-1β and TNF were also increased (P<0 05), compared to the CLP group mice, the CBX+CLP group mice showed an increase in neurobehavioral scores, a reduction in pathological damage to hippocampal tissue, a decrease in the expression of inflammatory factors IL-1β and TNF-α, a decrease in the expression levels of NLRP3, Panx1, Caspase-1 proteins, and corresponding mRNA, and a corresponding decrease in the mRNA expression of the two inflammatory factors (P<0 05). Conclusion: Inhibiting Panx1 can reduce the degree of neural inflammatory response, thereby improving brain damage in sepsis mice, which may be related to the limited activation of NLRP3 inflammasomes.

Key words: Panx1, Sepsis, Brain injury, Inflammatory response, Hippocampal tissue.

Pannexin, also known as Panx, is one of the newly discovered gap junction proteins. One of its subtypes, Panx1, has been confirmed to be involved and play a certain role in the inflammation and immune processes of the body [1]. Sepsis is a key factor leading to mortality in ICUs, with a total of around 200000 deaths caused by sepsis annually worldwide. The effectiveness of simple anti infection interventions is significant. Early excessive inflammatory response and late immunosuppressive state are the pathological and physiological characteristics of sepsis, and immunotherapy for sepsis has become a current research hotspot [2]. A certain amount of researches [3-5] has shown that reducing the activation of the NLRP3/Caspase-1 pathway can significantly improve sepsis induced neuroinflammation and alleviate brain damage in sepsis. The Pannexin 1 (Panx1) channel has been shown to act upstream of NLRP3 inflammasomes, exacerbating liver and lung inflammation in septic mice by mediating ATP release to the extracellular domain, and

exacerbating liver and lung damage in septic mice [6]. There is currently no literature on the role of this channel in sepsis induced brain injury and its relationship with NLRP3 inflammasomes. This study mainly investigates whether Panx1 plays a role in the progression of brain injury in sepsis mice and its potential mechanisms. The following report.

1. Materials and Methods

1.1. Animal sources

A total of 45 C57BL/6 mice were provided by the animal experimental center of a certain hospital. All male, SPF grade, aged 6 to 8 weeks, with a weight range of 18 to 20 g. Adaptive feeding lasts for one week, with free intake of water and 12 hours of light and dark conditions. The ambient temperature is set between 20 and 25 ℃. During the experiment, humanitarian care was given to mice. All mouse animals met the national guidelines for the protection and use of experimental animals, and were approved by the experimental animal ethics committee of the hospital.

1.2. Main reagents and related instruments

The Panx1 channel inhibitor Carben oxolone (purity of 99.29%, which is glycyrrhetinic acid) was purchased from MCE Corporation in the United States, the ELISA kit for mouse tumor necrosis factor (TNF-α) was purchased from Shanghai Enzymes Biotechnology Co., Ltd, the ELISA kit for mouse interleukin (IL-1β) was purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd, the product number LS-C138631 of Panx1 rabbit derived antibody (i.e. A5167) was purchased from Hangzhou Lianke Meixun Biomedical Technology Co., Ltd, NLRP3 rabbit derived antibody (68102-1-l g) and goat anti rabbit secondary antibody (SA00001-2) were all purchased from Wuhan Feien Biotechnology Co., Ltd.

The electrophoresis instrument (model: DYCZ-20A) was purchased from Beijing Haifuda Technology Co., Ltd, the optical microscope (model: SX-5A) was purchased from Shanghai Optical Instrument Co., Ltd, the gel imaging system (model: Vilber) was purchased in France.

1.3. Specific modeling methods

A total of 45 mice were divided into three groups using random number table method, namely sham surgery (i.e. Sham) group, cecal ligation perforation (i.e. CLP) group, and CBX pretreatment plus cecal ligation perforation (i.e. CBX+CLP) group. Excluding the Sham group, the remaining two groups of study mice were used to prepare sepsis brain injury models using the CLP method.

Half an hour before the surgery, the mice were administered bilateral lateral ventricular injection: 1% pentobarbital sodium was intraperitoneally injected (a total of 50 mg/kg) to anesthetize the mice, which were fixed on a brain stereotaxic device. After routine disinfection, the scalp was cut open to locate the specific injection location of the bilateral lateral ventricles (1 mm behind the anterior fontanel, 1.5 mm deep on both sides of the road, with a depth of 2 at 5mm), a 1 mL syringe tip was used to drill holes, and a micro syringe was inserted into the lateral ventricle of the mouse. After the cerebrospinal fluid was withdrawn and flowed out, CBX was administered to the lateral ventricle, with a total amount of 1 dose μ L (standard 0.2 μ L/min, 2 μ g/ μ L) After the injection is completed, stay for 1 minute, and then exit the needle tip. Mice in the Sham and CLP groups were injected into their bilateral lateral ventricles using the same method 1 μ L physiological saline. Half an hour later, a sepsis model was established using CLP technique. After laparotomy, the Sham group of mice only flipped out the cecum without any treatment, and closed the abdominal cavity after suturing. The other two groups of mice were subjected to CLP modeling.

CLP procedure steps: 5% sevoflurane and 0.8 L/min of oxygen were used to induce anesthesia in mice, 5% sevoflurane plus 0.8 L/min of oxygen for maintenance, remove hair from the center of the mouse's abdomen, after routine disinfection, a 1 cm incision was made to the right of the midline. The cecum of the mouse was free and ligated with a 4-0 suture at the distal half of the cecum. Then,

sterile needle 21 was used to puncture the distal cecum of the mouse twice. After squeezing out a certain amount of feces, the cecum was reintroduced into the mouse's abdominal cavity, suture the peritoneum and skin with specifications 6-0 in sequence, disinfect again, apply a small amount of lidocaine cream to the incision site of the mice, and complete the surgery. The total amount of physiological saline injected subcutaneously into the three groups of mice is 1 mL.

After the successful modeling of mice, infrared radiation was used to keep them warm, and then they were placed in the original breeding environment. After 24 hours, samples were taken and subsequent experimental steps were carried out.

1.4. Observation indicators

1.4.1. Scoring status of neurobehavior

The evaluation of neural behavior in mice after 24 hours of modeling was based on literature review. Evaluate three groups separately

Corneal reflex, auricular reflex, tail flexion, avoidance reflex, and corrective reflex in mice. A score of "0" indicates no reflection, "1" indicates weakened reflection, and "2" indicates normal reflection. The lower the evaluation score, the less optimistic the degree of brain nerve damage in mice.

1.4.2. Pathological changes in mouse hippocampal tissue

Three mice were randomly selected from the three groups 24 hours after successful modeling. After anesthesia, perfusion was performed through incisions in the left ventricle and right auricle of the mice. Then, complete brain tissue was gently peeled off and fixed in a 4% paraformaldehyde solution. Routine paraffin embedding, slicing, dewaxing, hydration, and HE staining were performed. Subsequently, pathological changes in the hippocampus of the mouse brain tissue were observed under a microscope.

1.4.3. Determination of effective levels of IL-1β and TNF-α in mouse hippocampal tissue

Take 30 mg of mouse hippocampal tissue in an environment of -80 ℃, add RIPA lysate and mix well. Centrifuge at a speed of 12000 r/min for 15 minutes, separate the supernatant of the upper layer, and follow the instructions of the ELISA kit to detect the effective levels of IL-1β and TNF-α in mouse hippocampal tissue.

1.4.4. Determination of Panx1 expression in mice

The brain tissue is fixed to the slice, which is consistent with the method used in 1.4.2 is the same. Antigen repair is performed on tissue slices, endogenous peroxidase is added, serum is sealed, and Panx1 primary antibody is added overnight. After PBS rinsing for 5 minutes, secondary antibody is added, and the reaction is carried out at 37 ℃ for one hour. PBS rinsing is performed for 2 minutes each time, with a total of 3 rinses. DAB develops color at room temperature, and PBS rinsing is performed for 2 minutes each time, with a total of 3 rinses followed by re staining and dehydration until it appears transparent, sealing operation, taking photos using an optical microscope to observe the actual expression of Panx1 in the hippocampus tissue of mice.

1.4.5. Detection of effective expression of NLRP3, Panx1, and Caspase-1 in mice

Take approximately 30 mg of hippocampal tissue from each group of mice, add pre cooled protein extraction solution, and extract total protein. Adjust the concentration of each sample to be consistent using the BCA kit method. Mix it with the sample buffer at a ratio of 1:4 and boil for 10 minutes. Then, after SDS-PAGE gel electrophoresis, the protein was electrotransferred to the NC membrane. The NC membrane was placed in 5% skimmed milk and sealed at room temperature for 1 hour. After cutting the membrane, the 1:1000 diluted Panx1, Caspase-1, GAPDH and 1:2000 diluted NLRP3 antibodies were incubated in a shaking table at 4 ℃ overnight. On the second day, the 1:10000 diluted HRP secondary antibody was incubated at room temperature for 1 hour. The strips were exposed in

the gel imaging system. The strip gray values were analyzed with ImageJ software, and Panx1, NLRP3 The relative expression level of Caspase-1 protein.

1.4.6. Detection of effective mRNA expression of NLRP3, Panx1, Caspase-1, TNF-α, and IL-1β in mice

Under an environment of -80 ℃, 30 mg of mouse hippocampal tissue was taken, and total RNA was extracted from the hippocampal tissue of different three groups of mice using centrifugation column method. Then, cDNA was obtained by reverse transcription, and finally, PCR kit 20 μ L reaction system was used, with a program of pre denaturation at 95 ℃ for 3 seconds, 95 ℃ for 15 seconds, and 60 ℃ for 30 seconds, repeated for 40 cycles to obtain the cycle curve and cycle threshold (Ct). The effective mRNA expression of NLRP3, Panx1, Caspase-1, TNF-α, and IL-1β was calculated, and the relative expression levels of different genes were analyzed using Graph Pad.

1.5. Statistical Methods

The data processing was carried out using Graph Pad Prism 8.4.3 software, and the normal distribution of econometric data was expressed in the form of mean ± standard deviation ($\bar{x} \pm s$). The comparison between groups was performed using one-way ANOVA, and P<0.05 showed statistically significant differences between groups.

2. Experimental results

2.1. Final scoring results of neurobehavior

The Sham group mice had the strongest reflexes, the reflex responses of CLP group mice were weak or even disappeared, the mice in the CBX+CLP group showed enhanced reflex responses, compared to the Sham group, the neurobehavioral score of CLP group mice decreased (P<0.05), compared to CLP, the neurobehavioral score of CBX+CLP group mice increased (P<0 05), as shown in Table 1.

Table 1. Final scoring results of neurological behavior in three groups of mice (points), ($\bar{x} \pm s$)

group	Neurobehavioral score (points)
Sham group	8.65±0.60
CLP group	4.29±0.59
CBX+CLP group	6.31±1.14

2.2. Final results of HE staining of hippocampal tissue in three groups of mice

The hippocampal neurons of Sham group mice showed neat arrangement, normal morphology, and intact nuclei observed, the hippocampal neurons of CLP group mice showed disordered and relatively loose arrangement, with deep staining and pyknotic changes observed in the nucleus, and a decrease in the number of hippocampal neurons, the hippocampal neurons of CBX+CLP group mice showed neat and compact arrangement, significantly improved degree of damage, and significantly increased number of hippocampal neurons. As shown in Figures 1 to 3.

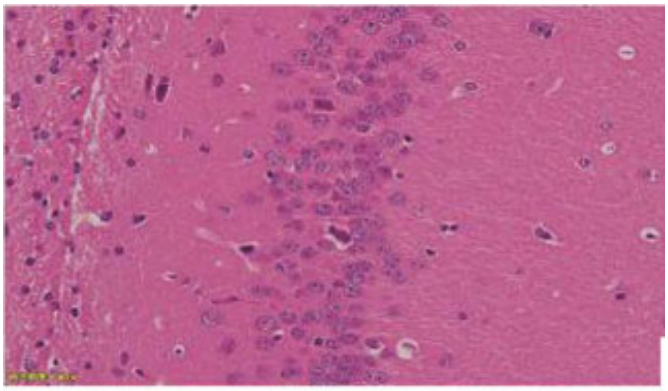

Figure 1. Display results of hippocampal tissue lesions in Sham group mice (HE) × 400)

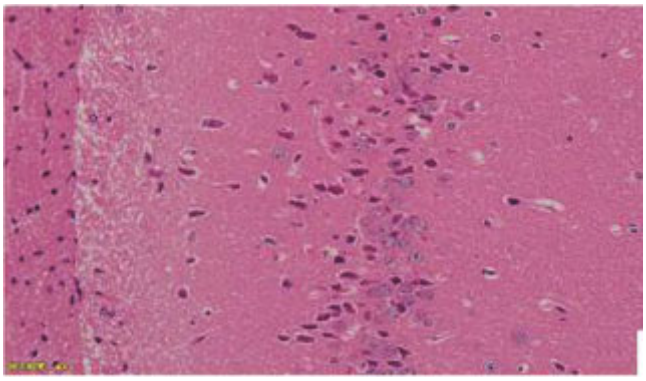

Figure 2. Display results of hippocampal tissue lesions in CLP group mice (HE) × 400)

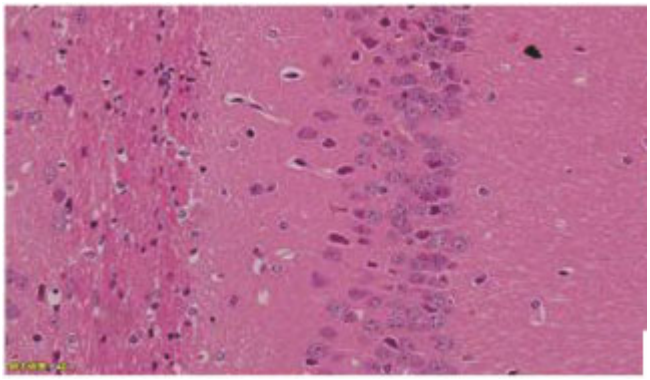

Figure 3. Display results of hippocampal tissue lesions in CBX+CLP group mice (HE) × 400)

2.3. Effective levels of IL-1β and TNF-α in the hippocampus of three groups of mice

Compared to the Sham group mice, the levels of IL-1β and TNF-αin the CLP group mice were significantly increased (P<0.05). compared to the CLP group, the levels of IL-1β and TNF-αin the CBX+CLP group mice were significantly reduced (P<0.05). See Table 2.

Table 2. Effective levels of inflammatory factors in the hippocampus of three groups of mice ($\bar{x} \pm s$)

group	IL-1β(pg/ml)	TNF-α(pg/ml)
Sham group	8.01±0.23	25.11±1.02
CLP group	15.02±0.19	60.10±2.03
CBX+CLP group	8.00±0.21	37.40±3.50

2.4. Detection sesults of Panx1 expression level in hippocampus of three groups of mice

Immunohistochemistry and Western Blot were used to detect the effective expression of Panx in the three groups of mice. Immunohistochemical references were used to analyze the final results of staining using the Histochem istry score (also known as histochemical score).

Under light microscopy, all three groups of mice showed positive staining for Panx1 in their hippocampal neurons (visible in brown). Compared with the Sham group, the expression level of Panx1 in the CLP group mice was significantly increased (P<0.05), compared with the CLP group, the expression level of Panx1 in the CBX+CLP group mice significantly decreased (P<0.05), the final results of Western Blot showed that compared to the Sham group, the expression level of Panx1 protein in CLP group mice was significantly increased (P<0.05), compared with the CLP group, the effective expression level of Panx1 protein in the CBX+CLP group mice was significantly reduced (P<0.05), as shown in Table 3.

Table 3. Test results of Panx1 expression level in the hippocampus of three groups of mice ($\bar{x} \pm s$)

group	Panx1
Sham group	0.50±0.03
CLP group	1.27±0.11
CBX+CLP group	0.51±0.02

Note: The detection results of Western blot method

2.5. Effective expression of NLRP3 and Caspase-1 proteins in the hippocampus of three groups of mice

Compared to the Sham group, the expression levels of NLRP3 and Caspase-1 proteins in CLP group mice were increased (P<0 05), compared to the CLP group mice, the expression levels of NLRP3 and Caspase-1 proteins in the CBX+CLP group mice decreased (P<0.05). see Table 4.

Table 4. Effective expression of NLRP3 and Caspase-1 proteins in the hippocampus of three groups of mice ($\bar{x} \pm s$)

group	NLRP3	Caspase-1 protein
Sham group	0.50±0.02	0.57±0.02
CLP group	1.01±0.03	1.25±0.05
CBX+CLP group	0.63±0.03	0.61±0.03

Note: The detection results of Western blot method

2.6. mRNA expression results of relevant indicators in the hippocampus of three groups of mice

The final results of the detection showed that compared to the Sham group, the mRNA expression levels of NLRP3, Panx1, and Caspase-1 in the CLP group were upregulated (P<0.05); Compared to the CLP group, the mRNA expression levels of NLRP3, Panx1, and Caspase-1 in the CBX+CLP group mice were downregulated (P<0 05), see Table 5.

Table 5. mRNA expression results of relevant indicators in the hippocampus of three groups of mice

group	NLRP3	Panx1	Caspase-1	IL - 1β	TNF -α
Sham group	1.00±0.19	0.99±0.04	1.00±0.19	0.89±0.18	0.80±0.20
CLP group	2.99±0.30	1.70±0.48	2.42±0.50	2.83±0.50	3.54±0.21
CBX+CLP group	1.40±0.18	0.63±0.13	1.44±0.05	1.36±0.19	1.80±0.14

Note: The detection results of Western blot method

3. Discussion

Previous studies [7-8] have pointed out that uncontrolled systemic inflammatory response is a characteristic of early sepsis. The levels of inflammatory factors in the plasma of sepsis patients significantly increase, with an increase in monocyte chemotactic protein-1 levels indicating the migration of a large number of immune cells from the bone marrow, and an increase in IL-1β levels indicating the activation of inflammatory bodies [9]. These processes are all related to the activation of the PanxI channel. It can be speculated that blocking Panx1 in the early stages of sepsis can reduce the body's inflammatory response and tissue damage [10]. In the middle stage of sepsis, the innate immune level decreases and the acquired immune level increases. Panx1 is involved in the activation and proliferation of T cells, as well as in the formation of immune synapses [11]. At this time, Panx1 plays an important role in acquired immunity. Immunosuppression or immune paralysis is a characteristic of late sepsis, with extensive apoptosis of T cells being the main cause. It can be speculated that Panx1 is in an inactive state in late sepsis [12-13]. However, further research is needed to determine the role of Panx1 in the progression of sepsis and its underlying mechanisms.

A brain injury model of sepsis mice was prepared with reference to the literature. After 24 hours of modeling, the mice showed signs of mental decline, decreased activity, decreased depth of water intake, trembling, hair erection, purulent secretions visible in the corners of the eyes, and purulent feces discharged from the anus. The neurological performance score was significantly reduced. Pathological results showed disordered and loose arrangement of neurons in the hippocampus, with hyperchromatic and pyknotic changes in the nucleus, the significant decrease in the number of hippocampal neurons reflects the success of the sepsis brain injury model prepared. Moreover, the results of this study ultimately showed that compared to the Sham group mice, the CLP group mice showed an increase in Panx1 expression level and mRNA expression, as well as a significant increase in the effective content of inflammatory factors IL- 1β and TNF -α in the hippocampus tissue, and an increase in mRNA expression; Compared to the CLP group mice, the CBX+CLP group mice showed a decrease in Panx1 expression level and mRNA expression, as well as a significant decrease in the effective content of inflammatory factors IL- 1β and TNF -α, and a decrease in mRNA expression, it reflects that both Panx1 and inflammatory factors are involved and play a certain role in sepsis induced brain injury. Inhibiting Panx1 can alleviate brain injury in sepsis mice by reducing neuroinflammation.

In summary, Panx1 participates in and exacerbates brain injury in sepsis mice. Inhibiting Panx1 can reduce the neuroinflammatory response to brain injury in sepsis mice, and has a protective effect on sepsis brain injury. As an upstream signaling pathway of NLRP3 inflammasomes, Panx1's protective effect is likely related to the reduced activation of NLRP3 inflammasomes. The specific mechanism still needs further research in cell experiments.

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