Caspase-1 Inhibitor Reduces Pyroptosis in Kidney Injury Induced by Brain Death

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Research Article

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Abstract

Brain death (BD) induces an organ-level inflammatory response, and BD donor kidneys show inferior survival and recovery rates. However, the underlying mechanisms have not been fully elucidated. Here, we investigated the role of caspase-1-mediated pyroptosis in BD-induced kidney injury in rats. A BD model was established in Sprague-Dawley rats. The rats were intravenously injected with a caspase-1 inhibitor (Z-YVAD-FMK) 1 h before BD, and sham-operated rats served as controls. After 0, 1, 2, 4, and 6 h of BD, renal function, renal injury, and renal expression of NLRP3, caspase-1, caspase-11, gasdermin D (GSDMD), IL-1 β , and IL-18 were assessed using quantitative reverse transcriptase-polymerase chain reaction, western blotting, and immunohistochemistry. Blood urea nitrogen and serum creatinine levels were measured. Additionally, renal tubular epithelial cells (NRK-52E) were subjected to 3 h of hypoxia followed by 6 h of reoxygenation and incubated with Z-YVAD-FMK before hypoxia and reoxygenation. Caspase-11 was knocked-down using small interfering RNA technology. Cell viability and levels of pyroptosis-associated proteins were assessed thereafter. NLRP3, caspase-1, GSDMD, IL-1 β , and IL-18 expression levels were upregulated in BD rats. Treatment with Z-YVAD-FMK reduced mRNA and protein levels, improved renal function, and alleviated renal injury. Z-YVAD-FMK effectively reduced pyroptosis in BD rats; however, it did not affect caspase-11 expression *in vivo* or *in vitro*. Thus, it could be considered as a therapeutic target for BD-induced kidney injury.

Introduction

Brain death (BD) induces organ injury in donors by stimulating an inflammatory response [1, 2]. Many retrospective analyses and randomized controlled studies have confirmed that kidneys derived from brain-dead organ donors show inferior survival and delayed functional recovery than those derived from living donors [3, 4]. However, the mechanism underlying the effect of BD on donor organ function has not been fully elucidated. Brain-dead donors show inflammatory responses at the organ level, and the degree of response is related to the extent of organ dysfunction after transplantation [5, 6].

The kidney is particularly sensitive to ischemia and hypoxia, and cell death in kidney diseases have been studied primarily in the context of tubular injury. The renal tubule is the key site of BD-associated injury and an important source of inflammatory cytokines. Renal cell death is a core pathophysiological factor in any renal disease. Thus, exploring the mechanisms of cell death and tissue damage can provide major insights for disease treatment.

Pyroptosis is a highly specific type of inflammatory programmed cell death that differs from necrosis and apoptosis [7]. Pyroptosis is activated by caspase-1 (human and mouse), caspase-4 and caspase-5 (human), or caspase-11 (mouse). Ge et al. identified the pathological roles of NLRs and AIM2 inflammasomes in the damaged blood-brain barrier after traumatic brain injury [8]. Caspase-1 inhibitor Ac-YVAD-CMK inhibits pyroptosis in brain microvascular endothelial cells. The role of inflammasome and caspase-1 activation pathway in stroke and traumatic brain injury has been previously confirmed [9, 10].

Yang et al. [11] found pyroptosis of renal tubular epithelial cells to be the key event in mouse kidney ischemia-reperfusion injury. Meanwhile, Wang et al. [12] found renal injury and pyroptosis to be typical events after renal ischemia-reperfusion. However, whether the canonical and non-canonical pyroptosis pathway mediates renal injury following BD remains unclear.

Although pyroptosis has gained attention in the field of inflammation, research on organ injury-related pyroptosis in brain-dead donors remains insufficient. Therefore, we aimed to investigate the role of caspase-1-mediated pyroptosis in BD-induced rat kidney injury, and the effect of a caspase-1 inhibitor (Z-YVAD-FMK) on it to reveal potential target genes for future intervention.

Materials And Methods

Experimental animals

Male Sprague-Dawley rats, weighing 250–300 g, were purchased from the Animal Center of the Medical College of Zhengzhou University. The rats were housed at 18–22°C, with moderate humidity, 12 h light/dark cycle, and a quiet environment. They were allowed access to chow and drink ad libitum. All experiments were approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (No. 2019-KY-019).

Animal groups and treatment

The BD rat model was established based on previous studies in our laboratory [13] and available literature [14, 15, 16]. Rats were randomly categorized into four experimental groups: BD group (n = 30), controls (group N, n = 6), caspase-1-specific inhibitor-pretreated BD group (BD + Z-YVAD-FMK group, n = 30), and Z-YVAD-FMK-pretreated controls (N + Z-YVAD-FMK group, n = 6). Rats were fasted for 6 h before the experiment but allowed free access to water. BD was induced by inflating a subdural balloon catheter to induce slow and intermittent intracranial compression. Blood samples from the abdominal aorta and kidney tissues were collected at 0, 1, 2, 4, and 6 h after BD. Group N comprised sham-operated rats serving as controls. All operations were performed in the same manner as that in the BD group, but BD was not established. Z-YVAD-FMK (Abmole Bioscience Inc., Shanghai, China) was dissolved in 14 µL of dimethyl sulfoxide (DMSO) and intravenously administered at 300 ng/kg 1 h before BD. Rats were euthanized under general anaesthesia and were sacrificed at different time points as indicated. The abdominal cavity was opened, kidney was removed, and macroscopic ischemia, necrosis, and other lesions were visually observed. Lower portion of the right kidney was collected and fixed in 4% paraformaldehyde solution, followed by paraffin embedding, sectioning, and hematoxylin and eosin staining. Paller score was used to evaluate pathological changes in the kidney [17, 18]. Expression of caspase-1 and caspase-11 in renal tissues was determined by immunohistochemistry (IHC).

Cell culture and treatment

NRK-52E cells (rat ductal epithelial cells; Procell Life Science & Technology Co., Ltd., Wuhan, China) were categorized into four groups: normal control, hypoxia/reoxygenation (H/R), Z-YVAD-FMK, and DMSO

control. In the normal control group, a serum-free medium(Dulbecco's modified Eagle's medium, Solarbio, Beijing, China) was added and cells were cultured for 8 h. In the H/R group, the culture dishes were incubated in 5% CO₂ and 1% O₂ at 37°C for 3 h (BD-induced hypoxia stimulation). Then, the cells were reoxygenated for 2, 4, 6, 8, and 12 h. In the Z-YVAD-FMK group, different concentrations of Z-YVAD-FMK (5, 10, 25, and 50 μM; DMSO volume = 80 μL) were added to each culture dish, and their effects on hypoxia-stimulated NRK-52E cells were detected. Finally, in the DMSO control group, DMSO (80 μL) was added to each dish, and the cells were incubated in 5% CO₂ and 1% O₂ at 37°C for 3 h. Each experiment was repeated thrice.

Next, NRK-52E cells were transfected with small interfering RNA (siRNA) negative control (NC) in one group and with caspase-11 siRNA in another. Then, the cells in both groups were subjected to hypoxic conditions for 3 h, and reoxygenation for 6 h. Finally, we harvested the cells, extracted RNA, reverse transcribed RNA into cDNA, and extracted cell proteins as previously described[19]. Transfection effects of siRNA were determined by RT-qPCR and western blotting.

Cell viability check by cell counting kit-8 (CCK-8)

NRK-52E cell suspension was seeded in 96-well plates at 100 μL/well, and precultured at 37°C and 5% CO₂. After treating the cells as described above, CCK-8 reagent (10 μL) was added to each well and incubated for 1 h. Absorbance was recorded at 450 nm. Each experiment was repeated thrice.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The mRNA levels of NLRP3, caspase-1, caspase-11, IL-1β, and IL-18 were measured by RT-qPCR. Total RNA was extracted from tissues using the TRIzol (Thermo Fisher Scientific, Shanghai, China), and reverse transcription was performed as described previously [20]. Primers were designed based on the gene sequences acquired from PubMed. The primers were synthesized by Invitrogen (Shanghai) Trading Co., Ltd., China, and are shown in Table S1. The primers were diluted appropriately, PCR amplification was performed, and RT-qPCR results were analyzed using a relative quantitative method as described previously [20].

Western blot analysis

Cell and tissue proteins were extracted as previously described [13], and bicinchoninic acid method was used to determine the protein concentration. Nitrocellulose membranes were incubated with either primary or secondary antibodies (anti-NLRP3, anti-IL-18, Proteintech Group, Inc., Chicago, IL,USA; anti-GSDMD, Abbexa Ltd, Cambridge, United Kingdom; anti-caspase-1, anti-IL-1β, anti-cleaved caspase-1, anti-cleaved IL-1β, Affinity Biosciences, Cincinnati, OH, USA; anti-caspase-11 p20, Santa Cruz Biotechnology, Inc. Dallas, Texas, USA), and the membrane was scanned using an Odyssey CLx imaging system(LI-COR Biosciences, Lincoln, NE,USA) as previously described [13].

Biochemical determination

Blood samples extracted from the abdominal aorta were centrifuged at 10,000 *g* for 20 min at 4°C. Frozen serum in the upper layer was collected, and serum creatinine (Cr) and urea nitrogen levels were measured using a Commercial Kit (Jiancheng Biotech, Nanjing, China) following the manufacturer's instructions.

Statistical analysis

SPSS 19.0 (SPSS Inc., Chicago, USA) was used for statistical analysis. Student's *t*-test was used to calculate the difference between the data obtained from two groups. One-way analysis of variance was used to calculate the difference across the data of multiple groups. Results are expressed as mean ± standard deviation. Results with *P* values < 0.05 were considered as statistically significant.

Results

Pyroptosis occurring in BD rats promoted inflammation and induced kidney injury

Paller scores and renal function results suggested that kidney injury increased with time after BD. Paller scores in the BD group were higher than those in the control group at 1, 2, 4, and 6 h ($p < 0.05$; Fig. 1B). Proportion of necrotic renal tubular cells increased 6 h after BD (Fig. 1A), and the proportion of cells with positive caspase-1 (Figs. 2) and caspase-11 staining (Figures S1) increased in BD rats. Cr and urea nitrogen levels were also higher in the BD group (Figs. 1C-D). In the BD + Z-YVAD-FMK group, Paller scores were reduced at 6 h (Fig. 1B). Renal function was better at 4 h and 6 h after BD (Figs. 1C-D), and the proportion of caspase-1-positive staining decreased (Fig. 2C), thereby suggesting that pyroptosis was involved in renal injury in BD rats. Z-YVAD-FMK alleviated renal injury and improved renal function after BD by inhibiting pyroptosis.

Effects of Z-YVAD-FMK on mRNA and protein expression of pyroptosis-related molecules in brain-dead rats

RT-qPCR and western blotting results showed that the expression of NLRP3, caspase-1, caspase-11, GSDMD, IL-1 β , and IL-18 in the BD (6 h) group were significantly higher than those in the control group (Fig. 3). However, Z-YVAD-FMK treatment reduced the mRNA and protein levels of caspase-1, GSDMD, IL-1 β , and IL-18 in the BD + Z-YVAD-FMK (6 h) group, although caspase-11 expression remained unchanged (Fig. 3). mRNA expression of NLRP3 in the BD + Z-YVAD-FMK (6 h) group was significantly lower than that in the BD (6 h) group (Fig. 3); however, the protein expression of NLRP3 remained unchanged (Figs. 3).

Effects of caspase-1 or caspase-11 inhibition on cell activity after H/R

NRK-52E cells with different concentrations of Z-YVAD-FMK were treated for 6 h and 12 h in normal environment. There was no significant difference in the cell activity (as measured by CCK-8) between the two groups, indicating that Z-YVAD-FMK had no toxic effect on NRK-52E cells at a concentration of 50 μ M. Cell activity decreased significantly after H/R and was stable at 6 h compared with that in the normal control group (Figure S2D). The cell activity recovered to some extent with prolonged reoxygenation time, and reoxygenation for 6 h was chosen as the optimal treatment in this experiment.

Next, we analyzed the activity of NRK-52E cells treated with different concentrations of Z-YVAD-FMK in the H/R environment (Figure S2E). Cell viability in the 25 μ M and 50 μ M Z-YVAD-FMK-treated groups was significantly higher than that in the DMSO-treated group. However, treatment with 25 μ M and 50 μ M showed no significant differences. Therefore, 25 μ M Z-YVAD-FMK was used for subsequent experiments.

Transfection effects of siRNA were determined by RT-qPCR and western blotting (Figures S2 A-C). Caspase-11 expression was downregulated in NRK-52E cells transfected with siRNA-1, siRNA-2, and siRNA-3, and its expression was lower in siRNA-2-transfected group than in the siRNA-1- and siRNA-3-transfected groups. Therefore, siRNA-2 was selected for subsequent experiments.

CCK-8 results revealed that NRK-52E cell viability was significantly decreased in the H/R environment and significantly increased upon Z-YVAD-FMK treatment. However, caspase-11 knockdown with siRNA did not exhibit a protective effect on cell viability after H/R by inhibiting the non-classical pathway of pyroptosis (Fig. 4C).

Protein and mRNA expression in NRK-52E cells after inhibition of caspase-1 or caspase-11 in H/R environment

Real-time qPCR and western blotting results showed that the expression of NLRP3, caspase-1, caspase-11, IL-1 β , IL-18, and GSDMD in NRK-52E cells was upregulated under H/R conditions (Fig. 4). Expression of IL-1 β , IL-18, caspase-1, and GSDMD was lower in the Z-YVAD-FMK group than in the H/R group ($p < 0.05$); however, there was no significant change in NLRP3 or caspase-11 expression (Fig. 4). Further, mRNA and protein expression of GSDMD and caspase-11 were significantly lower in the siRNA group than in the H/R group ($p < 0.05$); however, there was no significant change in NLRP3, caspase-1, IL-1 β , and IL-18 in the siRNA group (Fig. 4).

Discussion

Changes in blood circulation in brain-dead organ donors can lead to severe ischemia-reperfusion injury, resulting in acute tubular necrosis and delayed organ function after kidney transplantation [21, 22]. During BD, an inflammatory storm occurs, which causes drastic inflammatory changes in the donor organ before transplantation [23, 24].

Studies support the role of apoptosis in acute kidney injury [25, 26]. Proximal tubular epithelial cells are susceptible to apoptosis, and damage to this region results in organ failure. However, preventing

apoptosis alone cannot significantly improve renal function after transplantation; therefore, we aimed to explore the mechanism of pyroptosis in brain-dead donors. Here, our results suggested that pyroptosis was induced in kidney tissues after BD, and Z-YVAD-FMK treatment effectively improved the renal function and reduced renal injury thereafter.

We examined the expression of classical and non-classical pathway-related molecules in a brain-dead rat model. Caspase-1/11 belong to the proinflammatory caspase subfamily and play key roles in immune response-related signaling. Mice with caspase-1/11 gene knockout are more tolerant to *Escherichia coli*-induced septic shock than those lacking caspase-1 and IL-1 β , suggesting that caspase-1/11 associated pathways act together in mice along with septic shock. Initially, caspase-1 and caspase-11 were thought to be associated with independent pathways; however, later, they were discovered as part of a complex regulatory network with mutual correlation and interaction [27, 28]. Here, we found that both caspase-1 and caspase-11 were increased in BD rats and associated with BD-induced kidney injury.

Cao et al. [29] confirmed that NLRP3 inflammasome activation mediated blood-brain barrier dysfunction in cerebral ischemia, and inhibition of the same reduced blood-brain barrier injury after ischemia [29]. In the BD model, expression of NLRP3 in the kidney was significantly increased, suggesting that it was one of the main receptors associated with inflammasome formation and initiation of the canonical pyroptotic pathway. NLRP3 promotes the activation of caspase-1, induces release of IL-1 β and IL-18, and leads to renal injury, confirming that certain stimulating factors induced by BD activate NLRP3 in rats and promote occurrence of canonical pyroptosis thereafter.

GSDMD, a substrate of both caspase-1 and caspase-11/4/5, is primarily expressed in immune cells and shows unique structural characteristics of a perforating protein [30]. Here, a significant increase in GSDMD was detected in the kidneys of BD rats, demonstrating that GSDMD cleavage was necessary and sufficient for inflammatory caspase activation-induced pyroptosis. Both mRNA and protein expression of GSDMD in the BD + Z-YVAD-FMK group were significantly decreased, indicating that caspase-1 expression was inhibited by Z-YVAD-FMK, and the expression of GSDMD correspondingly decreased.

Pyroptosis is involved in the cryopreservation and auto-transplantation of mouse ovarian tissues, and its inhibition can improve ovarian graft function [31]. In our study, Z-YVAD-FMK effectively protected renal function in BD rats. In the H/R model, we verified that caspase-1, caspase-11, and GSDMD were significantly upregulated; whereas, addition of Z-YVAD-FMK abrogated this effect. NRK-52E cell viability decreased significantly in the H/R environment and Z-YVAD-FMK treatment increased the cell viability significantly.

Previous studies on pyroptosis [32] have mainly focused on the role of caspase-1 in the canonical pathway; here, we focused on whether caspase-11-mediated pyroptosis could be involved in BD-related organ injury. The level of caspase-11 in brain-dead kidney tissues was significantly increased, as determined by IHC and mRNA and protein expression. However, caspase-11-mediated atypical pyroptotic pathway was not affected by the caspase-1 inhibitor. Caspase-11 was knocked down by an siRNA, and the results revealed that H/R activated both canonical and non-canonical pyroptosis. Z-YVAD-FMK

inhibited the expression of IL-1 β and IL-18, thereby indicating the increased protective effect of Z-YVAD-FMK on cell viability after H/R. However, caspase-11 knockdown did not exhibit a protective effect on cell viability after H/R. Therefore, we concluded that canonical pyroptosis was the major pathway that affected H/R injury in NRK-52E cells.

This study has a few limitations. First, the maintenance time(6 h) in BD rats was limited. Second, we did not explore the role of pyroptosis in kidney injury beyond 6 h. Thus, further studies are required to understand the potential mechanisms of action.

In summary, our study shows that pyroptosis may occur in brain-dead donors, promote inflammation, and induce kidney injury, and could be considered as a therapeutic target for BD-induced kidney injury.

Abbreviations

BD, brain death; DMSO, dimethyl sulfoxide; GSDMD, gasdermin D; IHC, immunohistochemistry; H/R, hypoxia/reoxygenation; NRK52E, renal tubular epithelial cells

Declarations

Acknowledgments

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Author contributions

W.L., D.Y., J.S., P.W., S.C., W.G., and S.Z. conceived and planned the experiments. W.L., D.Y., J.S., J.Z., Z.W., B.H. and X.S. carried out the experiments. J.Z., Z.W., B.H., X.S. and S.C. contributed to sample preparation. W.L., D.Y., J.S., W.G. and S.Z. processed the experimental data, performed the analysis, drafted the manuscript and designed the figures. W.L., D.Y., J.S., P.W., S.C., W.G., and S.Z. contributed to the interpretation of the results. W.L. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data availability statement

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Conflicts of interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics approval

All experiments were approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (No. 2019-KY-019) and the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments.

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Figures

Figure 1

Hematoxylin and eosin staining , Paller scores and renal function results (A) Hematoxylin and eosin staining of the brain-death (BD) and BD + Z-YVAD-FMK groups. (B) Paller scores of the BD and BD + Z-YVAD-FMK groups. (C) Serum creatinine levels in each group at different time points. (D) Urea levels in each group at different time points. * $p < 0.05$, ** $p < 0.01$, $n = 6$.

Figure 2

Immunohistochemical (IHC) analysis of caspase-1 in brain-death (BD) rats (200×). (A) BD group. Magnification: 200×. (B) BD + Z-YVAD-FMK group (200×). (C) IHC score of caspase-1 in different groups. * $p < 0.05$, ** $p < 0.01$, $n = 6$; N = sham-operated controls.

Figure 3

Protein and mRNA expression of pyroptosis-related molecules in brain-dead rats. (A) mRNA expression of pyroptosis-related molecules in brain-dead rats. (B) Western blotting results of the BD and BD + Z-YVAD-FMK groups at 6 h. (C) BD-induced pyroptosis-related protein expression in rat kidneys. * $p < 0.05$; ** $p < 0.01$, $n = 6$; N = sham-operated controls.

Figure 4

Effect of Z-YVAD-FMK and caspase-11 inhibition by siRNA on cell activity, protein and mRNA expression in NRK-52E cells after hypoxia/reoxygenation(H/R). (A) mRNA levels of pyroptosis-related molecules in NRK-52E cells after H/R. (B, D) Expression of proteins in NRK-52E cells after caspase-1 or caspase-11 inhibition. (C) Effect of caspase-1 inhibition by Z-YVAD-FMK and caspase-11 inhibition by siRNA on cell

activity, as detected by CCK-8. DMSO, dimethyl sulfoxide; NC, negative control; N = normal control; *p < 0.05, **p < 0.01, n = 3.

Supplementary Files

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