Necrostatin-1 decreases necroptosis and inflammatory markers after intraventricular hemorrhage in mice

Abstract
Necrostatin-1, an inhibitor of necroptosis, can effectively inhibit necrotic apoptosis in neurological diseases, which results in the inhibition of inflammation, endoplasmic reticulum stress, and reactive oxygen species production and substantial improvement of neurological function. However, the effects of necrostatin-1 on intraventricular hemorrhage (IVH) remain unknown. In this study, we established a mouse model of IVH by injecting autologous blood into the lateral ventricle of the brain. We also injected necrostatin-1 into the lateral ventricle one hour prior to IVH induction. We found that necrostatin-1 effectively reduced the expression levels of the necroptosis markers receptor-interacting protein kinase (RIP)1, RIP3, mixed lineage kinase domain-like protein (MLKL), phosphorylated (p)-RIP3, and p-MLKL and the levels of interleukin-1β , interleukin-6, and tumor necrosis factor-α in the surrounding areas of the lateral ventricle. However, necrostatin-1 did not reduce ependymal ciliary injury or brain water content. These findings suggest that necrostatin-1 can prevent local inflammation and microglial activation induced by IVH but does not greatly improve prognosis.

Key Words: ependymal cilia; hydrocephalus; inflammation; intraventricular hemorrhage; microglia; MLKL; necroptosis; necrostatin-1; RIP1; RIP3

Introduction
Intraventricular hemorrhage (IVH) is a common neurological disorder with a high mortality rate (Hanley et al., 2017; Bosche et al., 2020). In adults, IVH is often secondary to physical brain trauma, hypertensive intracerebral hemorrhage (ICH), or subarachnoid hemorrhage (Jabbari et al., 2016; Moullaili et al., 2017; Kowalski et al., 2021). In preterm infants, IVH is usually caused by germinal matrix hemorrhage adjacent to the lateral ventricle (Segado-Arenas et al., 2018). When the combination occurs, it is known as germinal matrix hemorrhage-IVH, which occurs in approximately three per 1000 live births and has a mortality rate of 20–30% in the USA (Klebe et al., 2020). Researchers have found that certain treatments, such as celecoxib and minocycline, can improve the prognosis of IVH in animals, and these studies may lead to promising treatments for IVH patients (Ko et al., 2018; Ballabh and de Vries, 2021). At present, there are no in-depth studies on the pathogenesis and development of IVH and secondary injuries. Therefore, it is critical to clarify the pathological mechanism of brain injury after IVH and design effective intervention measures to improve the clinical prognosis of IVH patients.

Necroptosis is a type of programmed cell death that was discovered recently. Necroptosis is a caspase-independent mode of cell death that is mediated by death receptors and results in morphological changes that are characteristic of necrotic cells (Yuan et al., 2019a). The formation of a necrotic death complex (necosome), which includes receptor-interacting protein kinase 1 (RIP1), receptor-interacting protein kinase 3 (RIP3), and mixed lineage kinase domain-like protein (MLKL), plays a critical role in necroptosis, and deregulation of necroptosis is associated with pathological conditions in various systems, including inflammatory diseases (Wallach et al., 2016). Evidence has emerged that suggests necroptosis is involved in various neurological diseases (Caccamo et al., 2017; Morrice et al., 2017; Naito et al., 2020). Necrostatin-1 (Nec-1), a small molecule compound, inhibits the activity of RIP1 and is a specific inhibitor of necroptosis (Degterev et al., 2005). RIP3 and MLKL are key proteins involved in the process of necroptosis. A large number of animal experiments have shown that Nec-1 can effectively inhibit necroptosis in neurological diseases, thereby inhibiting inflammation, endoplasmic reticulum stress, production of reactive oxygen species and significantly improving neurological function (Li et al., 2019; Cao and Mu, 2021). However, it remains unknown whether necroptosis occurs during IVH and whether Nec-1 may be neuroprotective under this condition. In this study, we investigated whether necroptosis occurs in the periventricular tissue after IVH using a mouse model. In addition, we evaluated the potential protective effects of the necroptosis inhibitor Nec-1 during IVH.

Materials and Methods
Animals
Estrogen has a neuroprotective effect on intracerebral hemorrhage-related diseases (Ding et al., 2014), which may have influenced the results of this study. Therefore, a total of 165 male C57BL/6 mice (weighing 23–27 g, 7–9 weeks of age, special pathogen-free level) were purchased from the

https://doi.org/10.4103/1673-5374.339488

Date of submission: June 29, 2021
Date of decision: October 20, 2021
Date of acceptance: January 20, 2022
Date of web publication: April 29, 2022

Graphical Abstract
Mechanism of necroptosis after intraventricular hemorrhage

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Laboratory Animal Center of Sichuan University (license No. SCXK (Chuan) 2018-026). All mice were housed in a room with 25°C and humidity control, under a 12-hour light/dark cycle and free to access food and water. All protocols and experiments were conducted strictly in accordance with the approved procedures by the Sichuan University Animal Protection and Use Committee on April 15, 2020. All experiments were designed and reported in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Perec de Sert et al., 2020).

Intracerebroventricular injection

The animals were randomly divided into three groups: sham (n = 51), IVH (n = 36), Nec-1 (n = 36) group. The sham group was injected with normal saline (0.3% hydrogen peroxide for 15 minutes). The sections were washed three times for 15 minutes each and transferred to 1% osmium tetroxide solution for 2 hours. Tissue blocks were washed three times for 15 minutes each and transferred to a 1% osmium tetroxide solution for 2 hours. Tissue blocks were washed three times for 15 minutes each. The samples were placed on the metal stub with a carbon sticker, and the gold foil was sprayed for 30 minutes. Finally, tissue samples were observed, and images were captured with a scanning electron microscope (SuB100; Hitachi, Tokyo, Japan).

Magnetic resonance imaging

For detecting secondary hydrocephalus on day 3 after modeling, mice were anesthetized by inhalation of 2% isoflurane (RWD) and subjected to magnetic resonance imaging (MRI). A 7.0-Tesla MR scanner (Bruker; Karlsruhe, Germany) with a T2 fast spin echo sequence (matrix, 256 × 256; echo time, 2.5 ms; repetition time, 100 ms) was used for image acquisition of mouse heads. A total of 18 coronal slices were obtained for each scan with a view field of 35 × 35 mm and slice thickness of 1.0 mm.

Scanning electron microscopy

On day 3 after modeling, mice were anesthetized with pentobarbital sodium and their intact brains were removed and placed on pre-weighed and numbered tin foil paper. Wet tissues were weighed on an electronic balance (Sartorius; Göttingen, Germany). After drying the tissue for 72 hours at 105°C, the dry weights of the tissues were measured. The formula for calculating brain water content was as follows: (weight − dry weight)/weight × 100%.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated from periventricular tissue 3 days after IVH induction. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed after reverse transcription. The primers used were as follows: Rpp3 forward, 5′-GCC GAG ACC AGC GAC CAA GAG-3′; reverse, 5′-AGC CAG GCG CGA CCT TTC TTC-3′; Rpp4 forward, 5′-CAC ATT TGA AGG CTT TCC TTA G-3′; reverse, 5′-CAG AAA TCC TAC CGT CCA C-3′; Gapdh forward, 5′-CCT CGG CTC GAA AAA ATG-3′; reverse, 5′-TGA GGT CAA TGA ACG GGT CAT-3′. The fold changes were calculated using the ΔΔCT method (Tabatabaeian and Hojati, 2013).

Statistical analysis

GraphPad Prism software (version 6.01; GraphPad; San Diego, CA, USA, www. graphpad.com) was used for statistical analysis. All data are presented as the mean ± standard error of mean (SEM). Statistical differences among groups were analyzed using unpaired Student’s t-tests, and statistical significance was set at P < 0.02.

Results

The IVH model was successfully established and necroptosis occurred in the periventricular tissues after IVH induction.

We established a mouse IVH model by injecting 50 μL of autologous blood into the lateral ventricle of mice. The brain tissue in the IVH group was stained by hematoxylin-eosin (Figure 1A). Brain tissue sections demonstrated that blood was present in the lateral ventricles at all levels, and the blood was observed in the gap between the hippocampus and corpus callous (Figure 1B). T2-weighted MRI images revealed the existence of the lateral ventricles from the lateral ventricle on day 3 after IVH induction (Figure 1C). Furthermore, we chose days 1, 3, 5, and 7 after IVH modeling as the time points to observe changes in expression of the necroptosis markers RIP3 and MLKL. Western blots showed that the expression of RIP3 and MLKL in the periventricular tissues was highest on day 3 after IVH modeling compared with expression in the sham group (P < 0.01; Figure 2A–C). In addition, immunohistochemistry demonstrated that positive staining for p-RIP3 and p-MLKL within the periventricular tissues increased by day 3 after IVH induction compared with day 1 after IVH induction (Figure 2D). These results indicated that necroptosis occurred in the area of the lateral ventricle after IVH induction and was predominant on day 3 after IVH modeling.
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Spatial expression of p-RIP3 and p-MLKL in periventricular tissues 3 days after IVH induction
To determine the cellular localization of activated RIP3 and MLKL. The box represents the tissue-extraction site for Western blot assay. (A, B) Representative images of immunostaining for p-RIP3 (D) and p-MLKL (E) in the sham and IVH groups on day 3 post-IVH induction. No significant positive results were observed for p-RIP3 or p-MLKL in the sham group, while p-RIP3 and p-MLKL were expressed in the IVH group. Scale bars: 50 μm. (F, G) Statistical analysis of p-RIP3 (F) and p-MLKL (G) expression in tissue from the sham and IVH groups on day 3. Data are expressed as the mean ± SEM (n = 6). *P < 0.05, **P < 0.01, vs. sham group (unpaired Student’s t-test).

Nec-1 decreased microglial activation and expression of inflammatory factors in the tissue surrounding the lateral ventricle 3 days after IVH
Changes in microglia levels were observed with immunofluorescent staining. The numbers of Iba1-positive cells were increased in the area of the lateral ventricles after IVH induction compared with that in the sham group. Moreover, fewer Iba1-positive cells were noted around the lateral ventricle in the IVH + Nec-1 group than that in the IVH group (P < 0.01; Figure 6A and B). In addition, we selected IL-1β, IL-6, and TNF-α as markers to assess the effect of Nec-1 on inflammatory factors after IVH. Immunofluorescent staining of IL-6 was performed to determine whether Nec-1 demonstrated an anti-neuroinflammatory effect after IVH. While the relative fluorescent intensity of IL-6 was increased after IVH induction, Nec-1 treatment partially reversed this effect (P < 0.01, vs. IVH group; Figure 6C and D). Western blots showed that the expression of the three inflammatory factors was increased in the IVH group compared with that in the sham group (P < 0.01), and Nec-1 treatment attenuated the expression of these inflammatory markers (P < 0.05, vs. IVH group; Figure 6E–H).

Nec-1 did not improve hydrocephalus after IVH induction
We found that there was obvious hydrocephalus 3 days after IVH modeling by using T2-weighted MRI. However, Nec-1 treatment did not reduce the degree of hydrocephalus (Figure 7A). In addition, we found that Nec-1 treatment did not significantly reduce water content in the brains when compared with that in the IVH only group (Figure 7B). Injury to the ependymal cilia is closely associated with the development of hydrocephalus. We observed the ependymal cilia using scanning electron microscopy. In the IVH group, the T2 images of the ventricular system demonstrated a low signal, indicating blood in the ventricle. H&E: Hematoxylin-eosin staining; IVH: intraventricular hemorrhage; MRI: magnetic resonance imaging; Nec-1: necrostatin-1; SEM: scanning electron microscope.
Necroptosis is widespread in various neurological diseases. However, it is currently unclear whether necroptosis occurs after IVH. The present study revealed three major findings: (1) necroptosis occurred in the mouse model of IVH, and the expression level of necroptosis markers reached a peak on day 3 after modeling; (2) the key markers of necroptosis, p-RIP3 and p-MLKL, were mainly expressed in neurons and Nec-1 administration reduced the expression of p-RIP3, p-RIP3, p-MLKL, and MLKL, as well as necrotic cell death after IVH; and (3) although Nec-1 treatment reduced microglial activation and the expression of inflammatory factors, this inhibitor had a limited effect on secondary hydrocephalus caused by IVH.

The majority of current adult IVH animal models use small rodents, such as rats or mice. In previous reports, the IVH model in C57BL/6 mice was established using 25 μL autologous blood for the lateral ventricular injections (Zhu et al., 2014a; Chen et al., 2019b). However, in this study, we found that when 23–27 g C57BL/6 mice were used for IVH modeling, injecting 50 μL autologous blood into the lateral ventricle established a relatively stable IVH model without high mortality. We speculated that increasing the injection volume of autologous blood would induce greater pathological damage after IVH modeling, thereby producing more frequent complications after IVH, such as hydrocephalus, and aiding the study of brain damage caused by IVH. In this study, we confirmed the successful establishment of the IVH model using pathological tissue sections and T2-weighted MRI. Based on this model, we investigated whether necroptosis occurred after IVH induction and whether the necroptosis inhibitor Nec-1 provided neuroprotection.
Necroptosis is a newly discovered type of programmed cell death that is initiated by the tumor necrosis factor receptor and the Toll-like receptor families, regulated by RIP3 and MLKL, and manifests the characteristics of necrosis (Weinlich et al., 2017). Emerging studies have indicated that anti-necroptosis treatment promotes recovery from several central nervous system or related disorders, including subarachnoid hemorrhage, traumatic brain injury, and atherosclerosis (Karunakaran et al., 2016; Yuan et al., 2019b; Zhu et al., 2021). It has been reported that necroptosis is an important cell death method in ICH, and inhibition of necroptosis may be a potential strategy to improve the prognosis of ICH patients (Shen et al., 2017). At present, the detailed mechanism of cell death after IVH is unclear. In this study, we hypothesized that necroptosis would occur in the periventricular tissues of mice after IVH and inhibition of necroptosis may have a neuroprotective effect. A previous study demonstrated that the expression of RIP1 and RIP3 in the perihematoma tissues was highest on day 3 after ICH (Su et al., 2015). The current view is that the execution of necroptosis is dependent on RIP3 and MLKL (Yang et al., 2018). Therefore, RIP3, p-RIP3, p-MLKL, and MLKL were the key necroptosis markers evaluated in this study. Similar to the results from the ICH model, the expression of RIP3 and MLKL increased and peaked on day 3 after ICH modeling. Immunohistochemistry confirmed that 3 days after ICH modeling, p-RIP3, p-MLKL, and MLKL were increased in the periventricular tissues. After confirming the elevated expression of p-RIP3 and p-MLKL, it was important to explore the location of p-RIP3 and p-MLKL after ICH. Of note, previous studies have shown that necroptosis markers, such as RIP3, were mainly expressed in the neurons of mice with subarachnoid hemorrhage and ischemic stroke; however, there were no studies on the location of necroptosis markers after ICH (Chen et al., 2018; Hu et al., 2020). Our results showed that p-RIP3 and p-MLKL were mainly co-localized in neurons and rarely observed in astrocytes and microglia after IVH.

To further confirm whether the inhibition of necroptosis after IVH had a neuroprotective effect, Nec-1 was used. Nec-1 is known as a specific inhibitor of RIP1 and can inhibit necroptosis (Degterev et al., 2008). At present, Nec-1 treatment has been shown to have neuroprotective effects after ischemic stroke and traumatic brain injury (Zhu et al., 2014b, 2021). Interestingly, recent studies indicated that Nec-1 treatment prevents an increase in hematoma volume after ICH (Chang et al., 2014). Whether Nec-1 exerted a neuroprotective effect by inhibiting necroptosis after IVH was unclear. Consistent with a previous study (You et al., 2008), our current study showed that Nec-1 decreased PI-positive cells after IVH. However, we found that Nec-1 treatment did not significantly alleviate periventricular and ependymal cilia injury.

Recent studies have revealed that either RIP3 deficiency or RIP1 inhibition may have therapeutic potential for tissue damage caused by inflammation and necroptosis (Patel et al., 2020; Zhang et al., 2020). Prior studies have shown that RIP3/MLKL-dependent necroptosis induced inflammation in a rat model of fluid percussion brain injury, while Nec-1 and GSK872 treatment inhibited the expression of pro-inflammatory cytokines (Liu et al., 2016). Therefore, RIP3, p-RIP3, p-MLKL, and MLKL were increased in the periventricular tissues. After confirming the elevated expression of p-RIP3 and p-MLKL, it was important to explore the location of p-RIP3 and p-MLKL after ICH. Of note, previous studies have shown that necroptosis markers, such as RIP3, were mainly expressed in the neurons of mice with subarachnoid hemorrhage and ischemic stroke; however, there were no studies on the location of necroptosis markers after ICH (Chen et al., 2018; Hu et al., 2020). Our results showed that p-RIP3 and p-MLKL were mainly co-localized in neurons and rarely observed in astrocytes and microglia after IVH.

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Microglia are resident immune cells in brain tissue that play a key role in neuroinflammation in many neurological diseases. The continued activation of microglia tends to trigger neuroinflammation, which exacerbates brain damage (Salter and Stevens, 2017). Therefore, in this study, we expected that necroptosis would have a pro-inflammatory effect after IVH, and Nec-1 treatment would effectively reduce inflammatory cytokine expression and microglial activation. Compared with that in the IVH only group, treatment with Nec-1 reduced the levels of pro-inflammatory cytokines and inhibited the activation of microglia. These results were consistent with our hypothesis. However, whether inhibition of pro-inflammatory cytokines through Nec-
1 treatment can reduce secondary hydrocephalus after IVH requires further investigation.

IVH can cause immediate obstructive hydrocephalus and delayed communicating hydrocephalus (Bu et al., 2016). Approximately 40% of adult patients with spontaneous ICH present with IVH, and the probability that this proportion of patients will suffer from hydrocephalus reaches 51–89% (Stein et al., 2010; Mustanjoa et al., 2015). Current known mechanisms of hydrocephalus after IVH include alterations in the cerebrospinal fluid drainage pathway, ependyma, blood-brain barrier, aquaporin expression, inflammation, and degradation products of blood, such as iron (Mayfrank et al., 2000; Li et al., 2009; Strahle et al., 2012; Okubo et al., 2013). The IVH model established by injecting 200 μL of autologous blood into the lateral ventricle of rats can induce hydrocephalus 1 day after modeling, and hydrocephalus can be maintained up to 14 days (Wan et al., 2020). In this study, we found secondary hydrocephalus on day 3 after IVH modeling in mice. Inflammation is a key factor in hydrocephalus, and our previous studies have shown that Nec-1 treatment can reduce the expression of inflammatory cytokines. In addition, the ependyma is exposed to the blood itself or increased pressure, and the surface of the ependyma is damaged (Sarnat, 1995; Simard et al., 2011). Defective ependymal cilia are thought to be an important mechanism for hydrocephalus, which can cause changes in the flow of cerebrospinal fluid (Ibañez-Tallon et al., 2004; Del Bigio, 2010). The results of scanning electron microscopy showed that the ependymal cilia were kinked, disorganized, and progressively lost after IVH, and Nec-1 treatment did not significantly reduce this damage. Although we found that Nec-1 alleviated necroptosis and inflammation, T2-weighted MRI showed that Nec-1 treatment appeared to have little effect on the degree of hydrocephalus after IVH.

There were several limitations in this study. First, we observed changes in necroptosis markers from 1 to 7 days after IVH induction and did not explore whether there were changes in necroptosis markers in the longer term. Furthermore, treatment with different doses of Nec-1 may have alleviated long-term IVH-induced hydrocephalus. Second, Nec-1 has limitations, such as its moderate curative effect, and this inhibitor has off-target activity against inflammatory cytokines but not the degree of brain water content and hydrocephalus 3 days after stereotaxic injection of iron. The results of our study indicate that inhibition of necroptosis alone effectively alleviated the level of pro-inflammatory cytokines but not the degree of brain water content and hydrocephalus 3 days after IVH induction.

In conclusion, necroptosis occurred in periventricular tissues after IVH, and Nec-1 effectively inhibited the RIP3/MLKL-mediated necroptosis pathway, which alleviated necrotic cell death, neuroinflammation, and microglial activation in our model. However, Nec-1 treatment had little effect on secondary hydrocephalus 3 days after modeling. Our study is the first to demonstrate a role for necroptosis after IVH induction and may provide new insight into novel therapeutic strategies to manage IVH.

Author contributions: Study design: CL and YC; experiment implementation and manuscript drafting: HWX and LZ; manuscript revising and modifying: KHZ and APT. All authors read and approved the final manuscript.

Conflicts of interest: The authors declare that there are no conflicts of interest associated with this manuscript.

Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Open peer reviewers: Faisal Alami, King Saud bin Abdulaziz University for Health Sciences, Saudi Arabia.

Additional file: Open peer review report 1.

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