Original articles

Necrostatin-1 prolongs latency to convulsion in mice exposed to high oxygen partial pressure

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Key words
Animal model; Central nervous system; Inflammation; Necroptosis; Oxidative stress; Oxygen toxicity

Abstract
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Introduction: Exposure to very high oxygen partial pressure may cause central nervous system oxygen toxicity (CNS-OT). The role of necroptosis in the pathogenesis of CNS-OT is still unclear.
Methods: In experiment one, male C57BL/6 mice in the oxygen toxicity (OT) group (n = 5) and necrostatin-1 (Nec-1; a necroptosis inhibitor) (1.5 mg·kg^{-1}, intraperitoneal) group (n = 5) were exposed to pure oxygen at 600 kPa, and the latency to tonic-clonic seizure was recorded. In experiment two, mice were divided into three groups: control group (n = 11), OT group (n = 12) and Nec-1 group (n = 12). Nec-1 was intraperitoneally administered 30 min before oxygen exposure. Mice in the OT group and Nec-1 group were exposed to pure oxygen at 400 kPa for 30 min, and then sacrificed; the brain was harvested for the assessment of inflammation, oxidative stress and necroptosis.
Results: Experiment one. Nec-1 pre-treatment significantly prolonged the latency to seizure (245 [SD 18] seconds in the OT group versus 336 (34) seconds in the Nec-1 group). Experiment two. Nec-1 pre-treatment markedly reduced inflammatory cytokines and inhibited cerebral necroptosis, but failed to significantly suppress cerebral oxidative stress.
Conclusions: These findings indicate necroptosis is involved in the pathogenesis of CNS-OT, and inhibition of necroptosis may prolong seizure latency, but the specific mechanisms should be investigated further.

Introduction

It has been confirmed that exposure to oxygen at a high partial pressure and/or for a long period may cause damage to the central nervous system (CNS) and pulmonary system. Generally, exposure to a pressure of oxygen (PO_2) above 140 kPa may lead to nausea, numbness, dizziness, twitching, hearing, visual disturbances, and even convulsions and unconsciousness, known as CNS oxygen toxicity (CNS-OT).[1] In diving and clinical practice, the PO_2 and duration of oxygen exposure are strictly controlled to avoid oxygen toxicity and thus the actual incidence of oxygen toxicity is relatively low. Currently, the pathogenesis of CNS oxygen toxicity is still poorly understood. Proposed mechanisms include: excess production of reactive oxygen species (ROS);abrupt increase of cerebral blood flow following vascular constriction, imbalance between excitatory and inhibitory neurotransmitters and others.[2]

Programmed cell death is the deliberate suicide of an unwanted cell in a multicellular organism. To date, several types of programmed cell death have been identified, including apoptosis, necroptosis, ferroptosis, autophagy and others.[3] It has been revealed that oxygen toxicity may

induce apoptosis of neuronal cells,[4,5] and inhibition of intrinsic apoptosis is helpful for the prevention of neonatal oxygen induced brain damage.[6] A previous study indicated that necroptosis was involved in the pathogenesis of acute hyperoxia-induced lung injury and anti-oxidative treatment inhibited the necroptosis and thereafter improved the lung injury.[7] However, whether CNS-OT may also induce necroptosis in the brain and whether inhibition of necroptosis protects from CNS-OT *in vivo* have never been investigated. This study aimed to investigate the role of necroptosis in CNS-OT in a mouse model.

Methods

The study protocol was approved by the Institutional Animal Care and Use Committee of the Naval Medical University (NMU 2020-0239), and efforts were made to minimise suffering to the animals used in this study.

ANIMALS AND GROUPS

Male C57BL/6 mice weighing 20 (SD 2) g were purchased from Shanghai SLAC Experimental Animal Centre and housed at 24 (1)°C, humidity of 54 (2)% and a 12/12 h light/dark cycle. All the animals were given *ad libitum* access to food and water. In experiment one, mice were divided into two groups: CNS-OT group (n = 5) and necrostatin-1 (Nec-1; an inhibitor of necroptosis) group (n = 5). In the Nec-1 group, mice were intraperitoneally injected with Nec-1 (Selleck, TX, USA; S8641) at 1.5 mg·kg^{-1} 30 min before oxygen exposure (see below), and the latency to convulsion was recorded and analysed. Nec-1 was dissolved in 1% dimethylsulfoxide in sterile saline, and Nec-1 solution was prepared immediately before administration. In experiment two, mice were randomly divided into 3 groups: control group (n = 11), CNS-OT group (n = 12), and Nec-1 group (n = 12). In the control group, mice were exposed to normobaric air in a chamber. In the CNS-OT and Nec-1 groups mice were exposed to hyperbaric oxygen (HBO). In the Nec-1 group, mice were intraperitoneally injected with Nec-1 at 1.5 mg·kg^{-1} 30 min before oxygen exposure; mice in the CNS-OT group were intraperitoneally injected with 1% dimethylsulfoxide in sterile saline of the same volume 30 min before oxygen exposure.

HBO EXPOSURE

Animals were placed singly in an animal compression chamber (RDC150-300-6, Naval Medical University, Shanghai, China). In experiment one, the chamber was flushed with pure oxygen (> 99%) for 5 min and then pressurised to 600 kPa at a rate of 100 kPa·min^{-1}. The latency to convulsion was recorded, and then animals were de-pressurised at a rate of 50 kPa·min^{-1}. The pressure of HBO was 600 kPa because the latency to convulsion was longer when the pressure was at a low level. In experiment two, animals were exposed to pure oxygen at 400 kPa for 30 min (avoiding onset of a convulsion), and then de-pressurised at a rate of 50 kPa·min^{-1}. Animals were subsequently anaesthetised with intraperitoneal 1% pentobarbital sodium (50 mg·kg^{-1}) and then sacrificed for sample collection. During HBO exposure the chamber was continuously ventilated with 100% oxygen at 0.5 L·min^{-1}. The whole brain was harvested for further examination. In the control group, seven mice were sacrificed for biochemical examinations and four mice for immunohistochemistry. In the CNS-OT group and Nec-1 group, seven mice were sacrificed for biochemical examinations and five mice for immunohistochemistry.

LATENCY TO CONVULSION

When the chamber pressure reached 600 kPa, the latency was recorded. The latency to convulsion was defined as the time from the arrival of chamber pressure at 600 kPa to the onset of tetanic contraction of the whole body and persistent spasm.[8]

BIOCHEMICAL EXAMINATION

In experiment two, brain tissues were homogenized in phosphate buffer solution (PBS), followed by centrifugation at 3,000 rpm for 15 min. The supernatant was harvested for the detection of protein concentration with BCA assay (Beyotime, Jiangsu, China). Then, the malonaldehyde (MDA) content, superoxide dismutase (SOD) activity and reduced glutathione (GSH) content were detected with commercially available kits (Beyotime, Jiangsu, China) according to manufacturer's instructions.[7] Similarly, the contents of interleukin-1β (IL-1β), interleukin-10 (IL-10) and tumour necrosis factor-α (TNF-α) were detected with enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions.[7]

The receptor interaction protein 1 (RIP1), receptor interaction protein 3 (RIP3) and phosphorylated mixed lineage kinase domain-like protein (p-MLKL) are three crucial proteins involved in necroptosis. During necroptosis, RIP1 kinase activates RIP3, which then gains the ability to phosphorylate and activate MLKL.[9] The expression of RIP1, RIP3 and p-MLKL was detected in the brain tissues. In brief, the brain was collected and lysed in lysis buffer (20 mmol·L^{-1} Tris-HCl, pH 7.5, 150 mmol·L^{-1} NaCl, 1% Triton X-100; 1 mmol·L^{-1} ethylenediaminetetraacetic acid, 1 mmol·L^{-1} ethyleneglycol-tetraacetic acid, 2.5 mmol·L^{-1} pyrophosphate, 1 mmol·L^{-1} β-glycerophosphate) containing protease inhibitor mixture (Roche Applied Science, USA) on ice, followed by centrifugation at 3,000 rpm at 4°C for 15 min. After determination of protein concentration, equal amounts of protein were separated with 10% SDS-PAGE gels and polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). After blocking with 5% milk, membranes were incubated with primary antibodies to RIP1 (1:1000), RIP3 (1:1000), p-MLKL (1:500), and β-actin (1:2000) (Abcam; Danvers, MA, USA) at 4°C overnight. Blots were washed with tris-buffered saline with Tween 20 thrice (6 min for each). After washing, blots were incubated with secondary

antibodies (1:2000) for 2 h at room temperature. Finally, bands were visualised with an Electro-Chemi-Luminescence (ECL) Substrate Kit (Amersham, Rahn AG, Zurich, Switzerland) and quantified with Bio-Rad Quantity One software (Bio-Rad, Hercules CA, USA).

IMMUNOPRECIPITATION OF RIP1/RIP3

The protein concentration was determined in the brain as mentioned above. Immunoprecipitation was used for determining the interaction of proteins and performed as manufacturer's instructions with the Pierce Co-Immunoprecipitation (Co-IP) Kit (Pierce Biotechnology; Rockford, IL, USA). One ml of lysates was mixed with 2 mg of rabbit anti-RIP1 antibody (Abcam, USA) followed by incubation at 4°C overnight. Subsequently, the lysates were mixed with 40 ml of re-suspended Protein A + G Agarose, followed by incubation at 4°C for 3 h with constant shaking. After washing five times in lysis buffer, proteins were boiled with 1× loading buffer for 10 min. The specimens were evaluated by Western blotting.

IMMUNOHISTOCHEMISTRY

Mice were anaesthetised and transcardially perfused with 4% paraformaldehyde and then with normal saline. The brain tissues were harvested and immediately fixed in 4% paraformaldehyde at 4°C. After treatment with 30% sucrose in 0.1 M PBS at 4°C overnight, the brain tissues were embedded in paraffin. 4-µm sections were obtained, deparaffinized and rehydrated in gradient alcohol. After antigen retrieval in citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0), brain sections were blocked in 2.5% normal serum and then treated with anti-RIP1/anti-RIP3 antibody at 4°C, overnight. After rinsing in PBS, sections were treated with secondary antibody, and counterstaining was done with hematoxylin. In negative controls, sections were incubated with PBS instead of primary antibody. Five randomly selected fields at a magnification of 400× (Nikon TE300; Nikon, Japan) were captured, and the immunopositive cells were counted by an investigator who was blinded to the experiment.

STATISTICAL ANALYSIS

Statistical analysis was performed with SPSS version 21.0 (Statistical Product and Service Solutions Inc., Chicago, IL, USA). All data are expressed as mean and standard deviation (SD). Student's *t*-test was used to examine the difference in the latency between the two groups after using the Shapiro-Wilk test for normality. Comparisons were performed with one-way ANOVA among groups, followed by Tukey's post hoc test. A value of $P < 0.05$ was considered to indicate statistical significance.

Results

LATENCY TO CONVULSION

The latency to convulsion was 245 (SD 18) s in the control group. In the Nec-1 group, the latency to convulsion was prolonged to 336 (34) s. The first generalised, tonic-clonic

Figure 1

Oxidative stress and inflammation-related cytokines in the brain of different groups. A. IL-1β concentration; B. TNF-α concentration; C. IL-10 concentration; D. MDA concentration; E. 8-OHdG concentration; F. GSH concentration; G. SOD activity. ** $P < 0.01$ versus control group; # $P < 0.05$, ## $P < 0.01$ versus CNS-OT group. CNS-OT – central nervous system oxygen toxicity; GSH – glutathione; IL-1β – interleukin-1β; IL-10 – interleukin-10; MDA – malonaldehyde; Nec-1 – Necrostatin-1; TNF-α – tumor necrosis factor-α; SOD – superoxide dismutase; 8-OHdG – 8-hydroxydeoxyguanosine

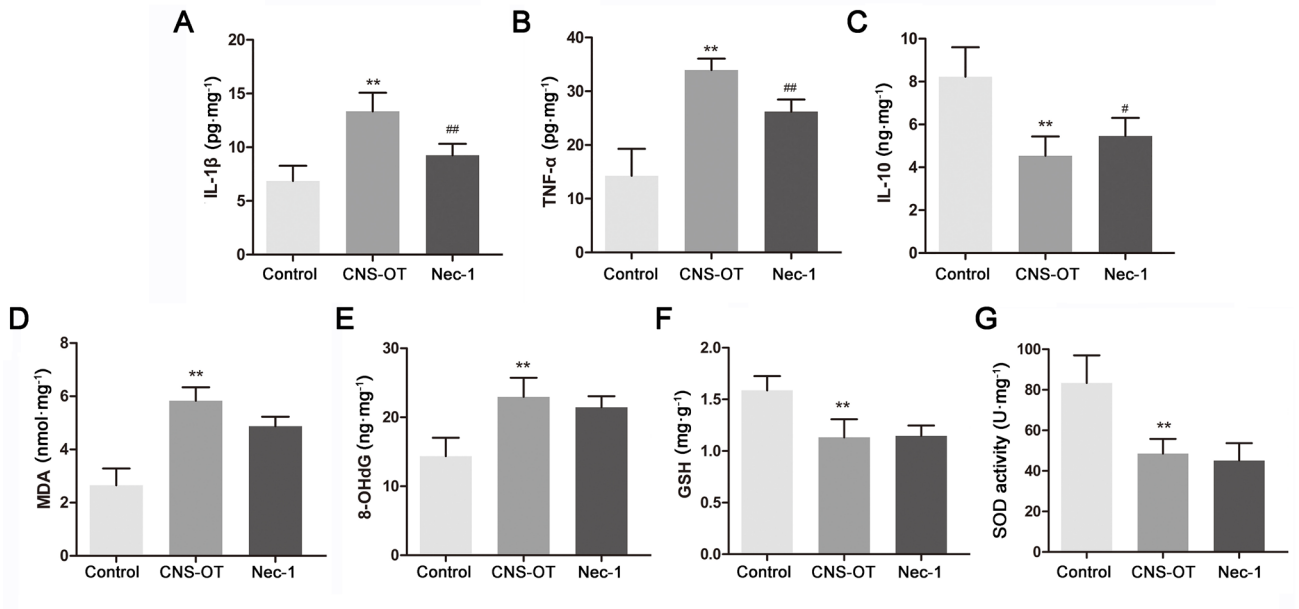


Figure 2

Detection of RIP1, RIP3 and MLKL protein expression in the brain (Western blotting). * $P < 0.05$ versus Control group; # $P < 0.05$ versus CNS-OT group. CNS-OT – central nervous system oxygen toxicity; Nec-1 – Necrostatin-1; RIP1 – receptor interaction protein 1; RIP3 – receptor interaction protein 3; p-MLKL– phosphorylated mixed lineage kinase domain-like protein

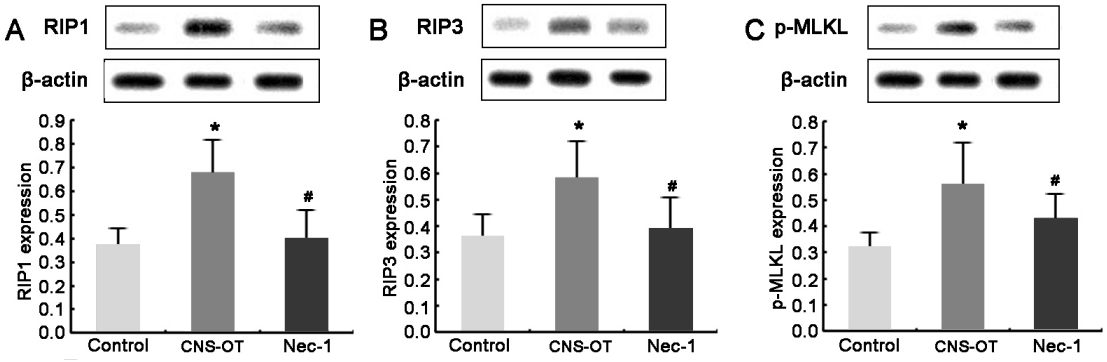
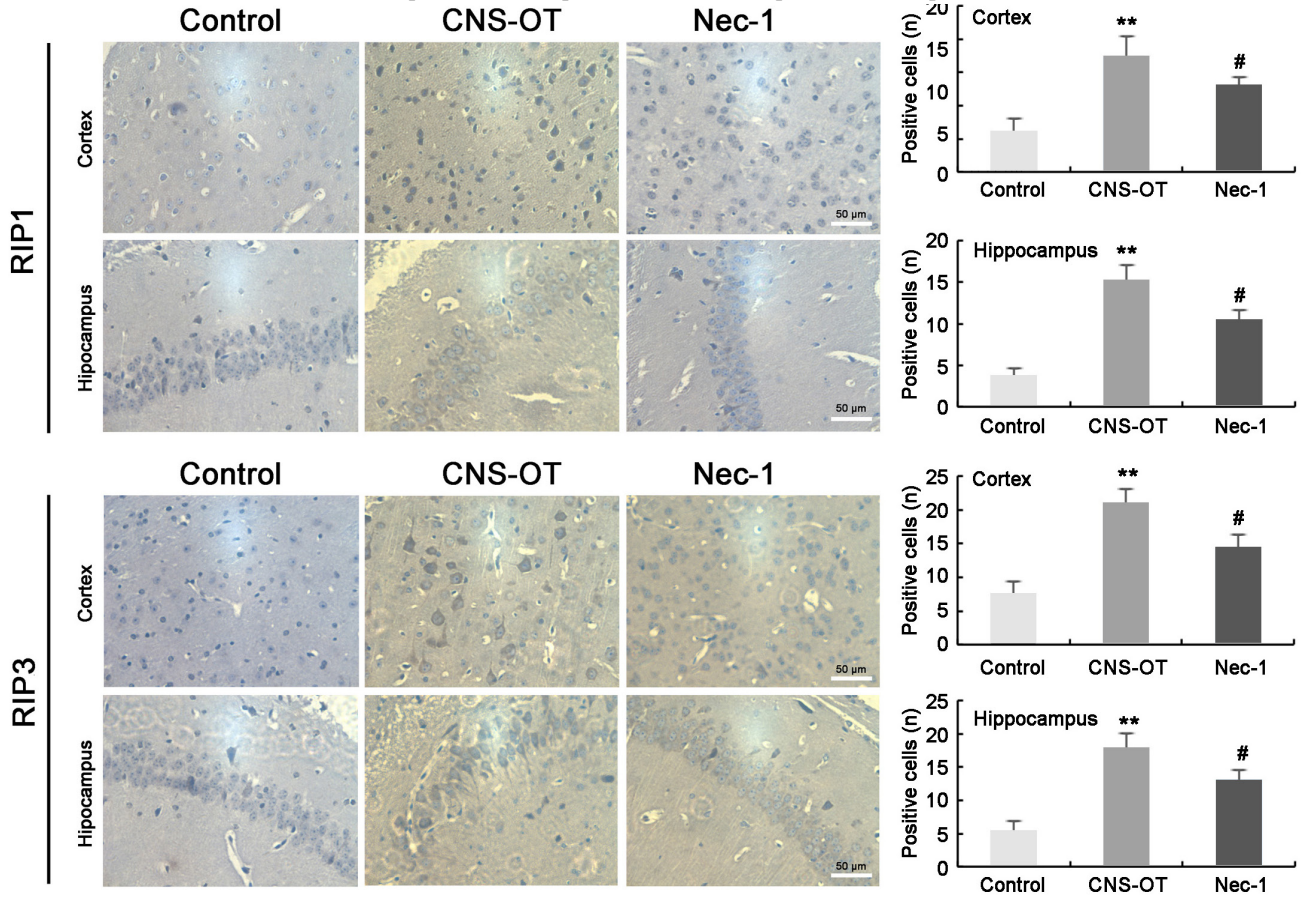


Figure 3

Immunohistochemistry for RIP1 and RIP3 in the cortex and hippocampus (× 400). Positive cells had brown granules. ** $P < 0.01$ versus Control group; # $P < 0.05$ versus CNS-OT group. CNS-OT – central nervous system oxygen toxicity; Nec-1 – Necrostatin-1; RIP1 – receptor interaction protein 1; RIP3 – receptor interaction protein 3



(grand mal) seizure was found at about 4 min after reaching 600 kPa, and thereafter these animals remained calm with significantly reduced activity in the chamber.

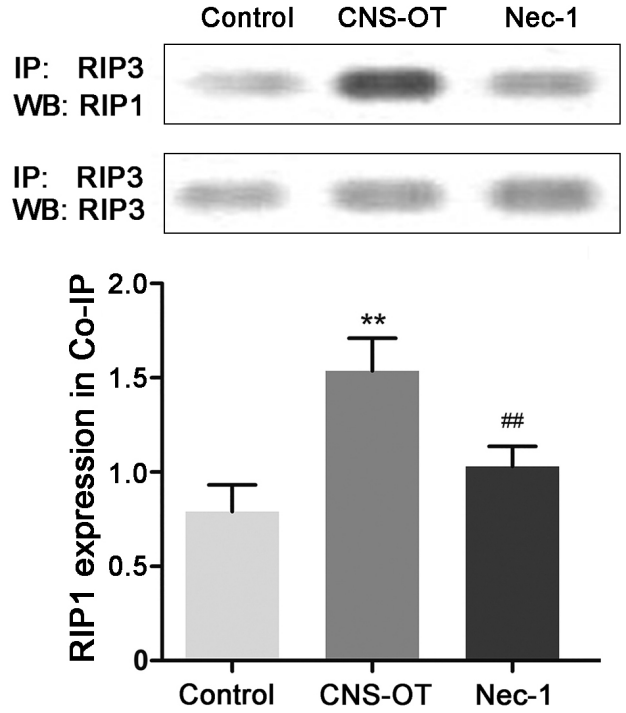
INFLAMMATION

HBO exposure increased cerebral contents of IL-1β and TNF-α ($P < 0.01$), but reduced the IL-10 content. Moreover, Nec-1 pre-treatment markedly reduced the contents of IL-1β and TNF-α ($P < 0.01$) and increased IL-10 ($P < 0.05$) as compared to the CNS-OT group (Figure 1 A, B, C).

OXIDATIVE STRESS

HBO exposure significantly increased cerebral MDA content, but markedly reduced the SOD activity and GSH

Figure 4
Co-Immunoprecipitation of RIP1 and RIP3 in the brain. ** $P < 0.01$ versus Control group; ## $P < 0.01$ versus CNS-OT group. CNS-OT – central nervous system oxygen toxicity; Nec-1 – Necrostatin-1; RIP1 – receptor interaction protein 1; RIP3 – receptor interaction protein 3; Co-IP – co-immunoprecipitation



content, but Nec-1 pre-treatment failed to significantly alter the MDA content, SOD activity and GSH content (Figure 1 D, E, F, G).

RIP1, RIP3 AND p-MLKL EXPRESSION

HBO exposure significantly increased the expression of RIP1, RIP3 and p-MLKL ($P < 0.05$ versus control group), and this increase was markedly inhibited by the pre-treatment with Nec-1 ($P < 0.05$ versus CNS-OT group) (Figure 2).

Numbers of RIP1-positive cells and RIP3-positive cells increased significantly in the hippocampus and cortex ($P < 0.05$ versus control group). However, in the presence of Nec-1 pre-treatment, the numbers of RIP1 positive cells and RIP3 positive cells reduced markedly in both hippocampus and cortex ($P < 0.05$ versus CNS-OT group) (Figure 3).

INTERACTION BETWEEN RIP1 AND RIP3

An interaction between RIP1 and RIP3 was significantly reinforced after HBO exposure, but pre-treatment with Nec-1 significantly inhibited the RIP1-RIP3 complex formation after HBO exposure ($P < 0.05$) (Figure 4).

Discussion

These results showed that high PO_2 exposure significantly increased oxidative stress and inflammation in the brain, which was accompanied by the elevation of cerebral necroptosis. However, inhibition of necroptosis before HBO exposure was able to prolong the latency to convulsion of mice, which was not related to the oxidative stress.

Although the pathogenesis of CNS-OT is still poorly understood, some mechanisms have been proposed: excess production of ROS, abrupt increase of cerebral blood flow following vascular constriction, imbalance between excitatory and inhibitory neurotransmitters and others.[2] Thus, some strategies targeting these potential mechanisms are also developed for the prevention of CNS-OT.

There is evidence that oxygen toxicity may induce the apoptosis of neuronal cells[4,5] and inhibition of intrinsic apoptosis is helpful for the prevention of neonatal oxygen induced brain damage.[6] Necroptosis is another type of programmed cell death. Necroptosis was initially recognised as a caspase-independent cell death mechanism induced by TNF in the presence of a pan-caspase inhibitor. Thereafter, numerous studies have revealed that necroptosis can also be activated by some other factors (such as ROS, calcium, Toll-like receptor agonists and interferon). During necroptosis, RIP3 is activated by RIP1 kinase and then gains the ability to phosphorylate and activate MLKL.[9] In our previous study, necroptosis was found to be involved in the pathogenesis of acute hyperoxia-induced lung injury and anti-oxidative treatment was able to inhibit the necroptosis and thereafter improve the hyperoxia induced lung injury.[7] The present study investigated whether necroptosis was involved in the pathogenesis of CNS-OT.

Experiment one showed that pre-treatment with Nec-1 (a RIP1 inhibitor) 30 min before HBO exposure significantly prolonged the latency to convulsion. This indicates that necroptosis is involved in the pathogenesis of CNS-OT. Thereafter, in experiment two, the key molecules (RIP1, RIP3, p-MLKL) in the necroptosis-related pathway and the interaction between RIP1 and RIP3 were examined. HBO exposure at 400 kPa for 30 min markedly increased the expression of RIP1, RIP3 and p-MLKL as well as the interaction between RIP1 and RIP3. There is evidence that necroptosis is closely related to inflammation and may act as a trigger of inflammation,[10] oxidative stress can induce necroptosis[11] and inhibition of oxidative stress suppresses necroptosis.[12] In a pulmonary oxygen toxicity model, edaravone, a known free radical scavenger, was able to inhibit necroptosis in the lung.[7] Thus, we speculate that excessive production of ROS induced by HBO exposure is, at least partially, responsible for the activation of necroptosis in the brain. In the present study, inflammatory cytokines and oxidative stress-related molecules were also detected in the

brain. Our results showed HBO exposure at 400 kPa for 30 min could increase both inflammation and oxidative stress. Nec-1 pre-treatment significantly reduced the expression of RIP1, RIP3 and p-MLKL and inhibited the production of inflammatory cytokines in the brain, but it failed to markedly inhibit the oxidative stress in the brain following HBO exposure. This may be explained by oxidative stress being an 'upstream' event in necroptosis.

There is evidence that HBO-induced seizures may cause a transient impairment of cognitive function in mice.[13] The hippocampus is involved in learning and memory. In the present study, RIP1 and RIP3 expression was measured in the hippocampus by immunohistochemistry. There was increased expression of both RIP1 and RIP3 in the hippocampus after HBO exposure at 400 kPa for 30 min. Whether necroptosis in the hippocampus is involved in the transient impairment of cognitive function is still unclear.

There were limitations in the present study. Nec-1 was administered at only one dose, and a dose-response effect on CNS-OT was not delineated. Although Nec-1 is protective in some types of brain injury,[14] the blood-brain-barrier (BBB) permeability increases following brain injury and Nec-1 may enter the brain under this condition. In our model, there was no evidence of increased BBB permeability. Of note, Nec-1 was also found to exert neuroprotective effects on prediabetic rats,[15] which might reflect that Nec-1 can enter the brain or is BBB-permeable. More studies are needed to confirm this issue. In addition, the toxicity of Nec-1 and the safety of Nec-1 used before HBO exposure should be further evaluated in more basic and clinical studies.

Conclusions

This study indicates that necroptosis is activated in the brain of mice following exposure to a high PO_2 with risk of CNS-OT, and that inhibition of necroptosis with Nec-1 delays the onset of convulsions following oxygen exposure. These findings suggest necroptosis plays a role in the pathogenesis of CNS-OT, and provide a potential strategy for the prevention of CNS-OT. More studies are needed to confirm these findings and investigate the possibility of Nec-1 being used in the prevention of CNS-OT.

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Conflicts of interest and funding

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