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RESEARCH PROTOCOL

Necrostatin-1 as a Neurogenic Agent to Ameliorate the Neurogenesis and Motor Outcomes in Neonatal Hypoxic-Ischemic Encephalopathy Through RIP1 Inhibition: A Research Protocol

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Abstract

Introduction: Hypoxic-ischemic encephalopathy (HIE) is a severe neonatal brain injury that disrupts neurogenesis and motor function through RIP1-mediated necroptosis. While Necrostatin-1 (Nec-1) demonstrates neuroprotective potential in adult ischemia models, its effects in neonatal HIE remain poorly understood due to developmental differences in blood-brain barrier permeability and RIP1 signaling. This research protocol investigates Nec-1's capacity to ameliorate HIE-induced neurogenic and motor deficits by inhibiting RIP1-mediated cell death pathways.

Methods: Postnatal day 7 mice will be randomized into three groups: Nec-1-treated HIE, vehicle-treated HIE, and untreated controls (n = 14/group, equivalent sex distributions). HIE will be induced via right carotid artery ligation followed by hypoxia (FiO_2 = 0.08 for 1.5 h). Nec-1 (0.04 mg/kg) will be administered intracerebroventricularly pre- and post-hypoxia. Tissue will be collected at 1 hour, 6 hours, P10, and P21 for immunohistochemical analysis of RIP1-mediated necroptosis via the Ser-166 autophosphorylation site, neurogenesis markers (DCX and Nestin), and apoptosis (caspase-3). MRI will assess brain volume at P21, while motor function will be evaluated via RotaRod and grip strength tests.

Results: We hypothesize that Nec-1 will reduce RIP1 expression and necroptosis in HIE mice, evidenced by decreased Ser-166 activation and p-RIP1 cells. Increased DCX^+ and $Nestin^+$ cells in the hippocampal and cerebellar regions alongside preserved brain volumes in MRI are anticipated. Nec-1-treated mice are expected to show improved motor coordination and strength compared to vehicle- and untreated mice. Sex-specific responses are predicted to emerge, with male mice exhibiting attenuated benefits.

Discussion: This research protocol addresses critical gaps in understanding Nec-1's neurogenic effects in developing brains. Successful outcomes could position RIP1 inhibition as a complementary strategy to the current standard, hypothermia. Limitations include translational challenges of murine models and intracerebroventricular injection in neonates, effects of stress responses throughout handling, and short-term endpoints.

Conclusion: This study evaluates Nec-1's potential to mitigate HIE-induced neurodevelopmental impairments through RIP1 inhibition, with anticipated outcomes including reduced necroptosis, preserved neurogenesis, and improved motor function. By characterizing these mechanisms in neonatal mice, this study aims to inform adjunctive approaches to hypothermia and strengthen the rationale for translating RIP1-targeted interventions into clinical strategies for infant neuroprotection.

Keywords: hypoxic-ischemic encephalopathy; neurogenesis; motor function; necroptosis; receptor-interacting protein 1; Necrostatin-1; neonatal brain injury

Introduction

Hypoxic-ischemic encephalopathy (HIE) is a severe neonatal brain injury caused by perinatal asphyxia, disrupting oxygen delivery to developing cerebral tissue [1]. Affecting approximately 1.5 per 1000 live births, risk factors include low gestation periods, late-trimester bleeding, and maternal infection, with severity depending on hypoxia duration and cerebral growth [2-4]. Clinically, HIE is graded from Stage 1 (mild symptoms, high recovery potential) to Stages 2-3 (severe, greater risk of long-term consequences) [5]. Acute symptoms like seizures, lethargy, and respiratory distress can progress to chronic neurological disabilities if left untreated [6]. Notably, male infants often face greater adverse outcomes, including higher mortality and developmental deficits, due to reduced neuroprotective sex hormones and X-chromosome immune gene-conferred advantages in females [7, 8].

Long-term consequences range from cerebral palsy (CP) to epilepsy, with motor deficits frequently linked to necrosis [9, 10]. Necrosis is a form of premature cell death that disproportionately affects the Purkinje cells in the cerebellum, a brain region essential for learning and

memory [11]. Untreated HIE can lead to significant cerebellar and cerebral volume loss, where the degree of atrophy correlates with symptom severity [12].

A key pathological feature of HIE is disrupted neurogenesis, when new neurons are generated from neural progenitor cells (NPCs) for brain repair and plasticity, particularly in the subgranular zone (SGZ) and subventricular zone (SVZ) [13]. This impairment is associated with cerebellar hypoplasia and elevated risk for neurodevelopmental disabilities, with structural brain abnormalities associated with severe motor impairments [14, 15]. Disrupted neurogenesis is largely driven by necroptosis, a form of regulated necrosis that exacerbates NPC loss and HIE-related cognitive deficits [16].

Tumour necrosis factor-alpha (TNF-α), an inflammatory cytokine, initiates a signalling cascade by activating receptor-interacting protein 1 (RIP1), a kinase that triggers either apoptosis or necroptosis [17]. Apoptosis, a form of programmed cell death, is the genetically determined elimination of cells through cellular shrinking, chromosome condensation, nuclear fragmentation, membrane blebbing, and non-inflammatory clearance [18]. In contrast, necroptosis features cellular swelling, cytoplasmic granulation, and membrane rupture, releasing intracellular contents and provoking inflammation [19]. Apoptosis involves RIP1 complexing with Fas-associated death domain (FADD) and caspase-8, while necroptosis requires RIP1-RIP3 necrosome formation. Caspase-3 cleavage marks apoptosis, whereas RIP1 Ser-166 autophosphorylation denotes necroptosis and directly promotes inflammation and tissue damage [20]. Both pathways can converge to disrupt plasma membranes, propagate inflammatory signaling, and ultimately cause cell death, resulting in impaired neurogenesis [21, 22].

Targeting necroptosis via RIP1 inhibition has shown promise in stroke and neurodegenerative diseases [23]. Ischemic brain injury models demonstrate significantly reduced infarct volume and neurodegeneration, while HIE models reveal neuroprotection against oxidative damage and neuroinflammation following RIP1 inhibition [24, 25].

The current standard treatment for HIE is mild hypothermia (HT), which demonstrates reductions in neuronal injury and improves outcomes [26]. However, its efficacy is limited in neonates due to infant fragility and instability [27]. Alternative experimental treatments, including antioxidants, antiepileptics, and stem cell transplantation, have shown pre-clinical promise, but face translational challenges regarding efficacy and safety concerns [1]. These emphasize the need for novel therapeutic interventions against neonatal HIE.

Necrostatin-1 (Nec-1) is a small-molecule inhibitor that stabilizes the inactive conformation of RIP1 by targeting its allosteric regulatory adaptive pocket, preventing necrosome formation and autophosphorylation [28]. Although effective in adult models, its applications in neonatal neurogenesis and motor outcomes are complicated by developmental variation in RIP1 and caspase-8 expression. Furthermore, the immature blood-brain barrier's (BBB) heightened susceptibility to toxins entering circulation influences Nec-1's bioavailability, hindering its therapeutic potential and highlighting a critical gap in knowledge [29].

This study aims to investigate Nec-1's efficacy in mitigating neurogenesis and motor consequences for in vivo neonatal HIE mice models via RIP1 inhibition. We hypothesize that Nec-1 will attenuate RIP1-mediated necroptosis, thereby promoting neuronal survival and brain repair.

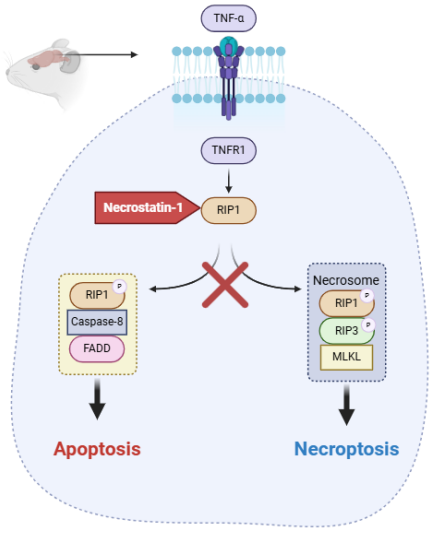

Figure 1: TNF-α signalling pathway: TNFR1 activation directs cellular fate toward apoptosis (via Caspase-8) or necroptosis (via RIP1/RIP3/MLKL). Nec-1 exerts its therapeutic effect by specifically inhibiting RIP1 to block necroptotic signalling. (Created with BioRender.com)

Methods

1. Materials and Reagents

The following materials and reagents were used to conduct the neonatal HIE experiments, including induction of injury, drug administration, tissue processing, and immunohistochemistry. All reagents were sourced from commercial suppliers:

- C57BL/6J neonatal mice (Charles River Laboratories, Strain Code 000664)
- Isoflurane (USP grade)
- Sodium pentobarbital (Sigma-Aldrich, Cat# P3761)
- 6-0 silk suture (Ethicon, Cat# EH7796H)
- Necrostatin-1 (Selleck Chemicals, Cat# S8037)
- Methyl-beta-cyclodextrin (Sigma-Aldrich, Cat# C0926)
- 30% sucrose solution (Thermo Fisher Scientific, Cat# BP220-1)
- Phosphate-buffered saline (Thermo Fisher Scientific, Cat# 10010023)
- 4% paraformaldehyde (Thermo Fisher Scientific, Cat# AA433689M)
- ProLong Gold Antifade Mountant with DAPI (Invitrogen, Cat# P36931)
- Anti-RIP1 Ser166 (Cell Signaling Technology, Cat# 65746)
- Anti-DCX (Abcam, Cat# ab18723)
- Anti-Nestin (Thermo Fisher Scientific, Cat# MA1-110)
- Anti-cleaved Caspase-3 (Cell Signaling Technology, Cat# 9664)
- Goat anti-rabbit IgG HRP-conjugated (Santa Cruz Biotechnology, Cat# sc-2004)
- Alexa Fluor-conjugated secondary antibodies (Invitrogen, various)

2. Equipment

All experiments were performed using specialized laboratory equipment designed for surgical procedures, tissue preparation, imaging, and behavioural testing in neonatal mice. The following instruments and software were employed:

- Tecniplast GM500 ventilated cages (Tecniplast, Cat# 1284L)
- Stereotaxic apparatus
- Leica CM1950 Cryostat (Leica Biosystems)
- Microscope slides (Fisher Scientific, Cat# 12-550-15)
- Nikon Eclipse Ti-U fluorescence microscope (Nikon Instruments, Cat# T3-84000)
- ImageJ software (NIH)
- ITK-SNAP software (version 3.8.0)
- Dragonfly image segmentation software (Object Research Systems)
- Bruker BioSpec 7T MRI scanner (Bruker Biospin, Cat# B-Biospec-70/30)
- 3D bSSFP MRI imaging sequence (Bruker)
- RotaRod apparatus (Ugo Basile, Cat# 47600)
- Grip strength meter (Ugo Basile, Cat# 47200)

3. Animal Model and Experimental Groups

Postnatal day 7 (P7) wild-type Charles River C57/Bl6 mice of either sex will be randomly assigned to: (1) Nec-1-treated HIE, (2) vehicle-treated HIE (methyl-β-cyclodextrin [Sigma-Aldrich, Cat# C4555]), and (3) untreated baseline controls (n = 22/group) [30]. Randomization will be done via a random number generator for group allocation and researchers will be blinded until the end of analysis. Each group will be further divided into cohorts designated for histological analysis or behavioral testing. Four mice will be housed per Tecniplast GM500 rack with outward airflow under standard conditions (22 ± 1°C, 12-hour light/dark cycle), with *ad libitum* access to food and water. Sex-balanced groups will enable evaluation of differential responses, given males' established higher HIE susceptibility, while controlling for hormonal variability [7].

4. Induction of HIE

The modified Vannucci model, a well-established method for stimulating neonatal HIE, will be used [31]. Mice will be anesthetized using isoflurane (4% induction, 2-3% maintenance, FiO_2 = 0.30/N_2O = 0.70), followed by a midline cervical incision and permanent ligation of the right common carotid artery using a 6-0 silk suture [32]. After 1-hour recovery, mice will be placed in a hypoxia chamber (FiO_2 = 0.08) for 1.5 hours at 37°C to mimic the hypoxic environment seen in neonatal brain injury, inducing systemic hypoxia [30]. Untreated controls will undergo anesthesia and sham surgery (carotid artery exposure without ligation) without hypoxia exposure [23]. HIE induction will occur in staggered cohorts with start times offset by 10-minutes to standardise tissue collection. Tracking sheets will log exact hypoxia start and end times to minimize variability.

5. Nec-1 Administration

Nec-1 (Selleck Chemicals, Cat# S8037) will be dissolved in dimethyl sulfoxide (DMSO [Sigma-Aldrich, Cat# D8418]) to prepare a 20 mM stock solution, and diluted in sterile saline containing 10% (w/v) methyl-β-cyclodextrin immediately before administration. This preparation improves Nec-1 solubility and bioavailability while minimizing DMSO toxicity at higher concentrations. Methyl-β-cyclodextrin was selected as the vehicle due to its established use in enhancing Nec-1 solubility and delivery in rodent studies without exerting neuroprotective effects [24]. Nec-1 (0.04 mg/kg) will be administered via intracerebroventricular (ICV) injection, given its short half-life (90 minutes), using a stereotaxic apparatus to accurately target the right lateral ventricle per the Paxinos and Franklin

mouse brain atlas [24, 33]. A neonatal stereotaxic mouse adaptor will be used to increase precision and reduce variability. ICV injection was chosen as alternative methods pose increased risk of organ puncturing due to the small size and limited blood volume of neonatal mice, resulting in imprecise targeting [34, 35]. The stereotaxic injection will be induced via a 0.5 mm diameter hole drilled 2.2 mm lateral of the bregma and 3 mm below the skull surface [36]. To verify the spatial distribution of the stereotactic injections and accuracy of ICV injections, a subset of 3 mice/group will receive co-injection of 0.05% Evans Blue dye (Sigma-Aldrich, Cat# E2129). Brains will be harvested 30 minutes post-injection, sectioned, and inspected under a dissecting microscope to assess the diffusion patterns of injections. The first dose for each staggered cohort will be administered 30 minutes before hypoxia, and a second dose will be delivered 90 minutes post-hypoxia to sustain RIP1 inhibition. Vehicle controls will receive equivalent volumes of methyl-β-cyclodextrin.

6. Tissue Collection and Processing

Mice will be sacrificed at four post-HIE induction time points to capture distinct phases of injury and recovery: 1 hour (acute necroptosis; n = 3/group), 6 hours (early neuroinflammation; n = 3/group), postnatal day 10 (P10; subacute neurodegeneration/neurogenesis; n = 2/group), and postnatal day 21 (P21; later neurogenesis effects following MRI; n = 6/group) [37]. At each time, mice will be anesthetized with 1.5% sodium pentobarbital (0.06 mL/10 g; Sigma-Aldrich, Cat# P010) in the lower left abdomen and transcardially perfused with phosphate-buffered saline (pH 7.4), followed by 4% paraformaldehyde for tissue fixation [38-40]. Brains will be extracted, post-fixed for 24 hours, cryoprotected in 30% sucrose (Thermo Fisher Scientific, Cat# BP220-1) at 4°C until they sink before freezing, then embedded in Optimal Cutting Temperature compound [41]. Coronal sections (30 µm) will be cut on a cryostat (Leica CM1950) and stored at -20°C until staining [42]. The cerebellum and hippocampus will be isolated for volumetry and histology analyses, given their vulnerability to HIE and relevance to the primary outcomes of motor functions and neurogenesis, respectively. [43, 44]

7. Immunohistochemistry (IHC) and Staining

IHC will be performed to evaluate RIP1 expression, neurogenesis markers, and cell death markers [45]. Free-floating sections will be blocked in 5% normal goat serum (Thermo Fisher Scientific, Cat# 50062Z) for 1 hour and incubated overnight at 4°C with the following primary antibodies [37]:

- Ser-166 (1:500, Cell Signaling Technology, Cat# 65746) to detect necroptosis activation via phosphorylated RIP1.
- Anti-RIPK1 antibody (1:500, Cell Signaling Technology, Cat# 3493) to quantify total RIP1 protein expression.
- Doublecortin (DCX) (1:1000, Abcam, Cat# ab18723) and Nestin (1:500, Thermo Fisher Scientific, Cat# MA1-110) to assess neurogenesis in the SGZ and SVZ.
- Cleaved caspase-3 (1:500, Cell Signaling Technology, Cat# 9664) to evaluate potential effects of apoptosis.

After washing, sections will be incubated with goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Cat# sc-2004), a conjugated secondary antibody, for 1 hour at room temperature for fluorescent dying. Sections will be counterstained with hematoxylin to visualize cell nuclei [23].

Images will be acquired on a Nikon Eclipse T300 fluorescence microscope with optimized excitation and emission filters to quantify staining [37]. For each marker, five images per hippocampus and cerebellum will be taken (20x magnification). Regions of interest (ROIs) will be selected based on anatomical landmarks defined in the Paxinos and Franklin mouse brain atlas for sample consistency [33]. ImageJ (NIH, Bethesda, Maryland, USA) will be used to count the number of positively stained cells and quantify the intensity of RIP1 expression, detected via anti-RIP1, and phosphorylated RIP1, detected using Ser-166, by mean fluorescence intensity within ROIs [23]. Background signal will be excluded by setting a threshold based on average background fluorescence in adjacent unstained areas. Data will be normalized as ROI area and expressed as cells/mm^2. All values will be compared to age-matched untreated controls to assess relative changes in expression.

8. Magnetic Resonance Imaging (MRI)

To assess brain/lesion volume and structural changes, subsets (n = 6/group) will undergo MRI at P21 [23]. Mice will be anesthetized with 1.5% isoflurane via an oxygen mixture through a nose cone and imaged using a 7T MRI scanner (Bruker BioSpec). T2-weighted images will be acquired to evaluate brain volume, focusing on the cerebellum and hippocampus [45]. Anatomical images will be acquired using a 3D balanced Steady-State free precession sequence, and volumetric analysis will be performed using ITK-SNAP software [46]. MRI analysis of ROIs will occur through manual tracing using Dragonfly segmentation tools [23]. Volume measurements will be taken for the hemispheres, hippocampus, and cerebellum based on the MRI atlas of the brain [23].

9. Motor Function Analysis

To evaluate Nec-1's impact on motor function, a dedicated cohort of mice (n = 8/group) will undergo behavioural testing starting week 6 [30]. Mice will be

acclimated to the testing environment for 10 minutes/day over 3 consecutive days to minimize stress. The following tests will be conducted for 3 days and 3 trials/day, with the average score used for analysis:

RotaRod [47]: Mice will be placed on an accelerating rotarod apparatus (Ugo Basile), increasing from 4 to 40 rpm over 5 minutes. Latency to fall will be recorded to evaluate short-term and long-term motor coordination via 10 trials, with 200 s between each. A single test will last from the time the mouse is placed on the rod until it falls or 5 minutes have elapsed. If a mouse can make a complete revolution, the latency to the first complete revolution is recorded. Latency values below 100 s will indicate significant motor impairment, while values above 200 s will suggest preserved or enhanced motor coordination.

Grip Strength [30]: Forelimb and hindlimb grip strength will be measured using a grip strength meter (Ugo Basile). Mice will be placed on a 45 cm metal wire over a foam pad for 60 s with tension induced by gravity. Peak pull force (tension) achieved by the limbs before losing grip will be recorded to assess neuromuscular function. Values below 80 g will indicate significant neuromuscular weakness, while values above 120 g will suggest preserved or enhanced strength.

Following completion of behavioural testing, mice will be humanely euthanized with 1.5% sodium pentobarbital (0.06 mL/10 g).

10. Statistical Analysis

Quantitative IHC data will be analyzed using GraphPad Prism to evaluate RIP1 expression, neurogenesis markers (DCX and Nestin), and cell death markers (cleaved caspase-3) [23]. One-way ANOVA with Tukey's post hoc correction will be conducted for pairwise comparisons between groups. For behavioural data, repeated-measures ANOVA will be used for multiple testing sessions over time. To account for hormonal and genetic factors causing specific responses, data will be sex-stratified and analyzed separately within each group. All data will be presented as the mean ± standard error of the mean (SEM), and $p<0.05$ will be considered statistically significant [23].

Results

Histological and Molecular Markers of Necroptosis and Apoptosis

IHC analysis of Ser-166 staining is expected to reveal significantly reduced RIP1 expression in Nec-1-treated mice compared to other groups, indicating the inhibition of RIP1-mediated necroptosis. Comparing the right and left hemispheres for RIP1 immunofluorescence intensity, the immunofluorescence ratio of RIP1 is anticipated to be significantly reduced in the hippocampal dentate gyrus of the right hemisphere, based on prior models showing regional specificity in necroptosis reduction. [48]

Caspase-3 immunofluorescence is expected to show a lower number of caspase-3-positive apoptotic cells in Nec-1-treated mice, particularly in the hippocampus and cerebellum, suggesting indirect inhibition of apoptosis [49]. Male mice are predicted to exhibit less reduction than females, coinciding with reported sex differences in caspase-3 pathway activation [50].

Neurogenesis Markers and Brain Volume Preservation (MRI)

Evaluation of neurogenesis markers is expected to reveal significant increases in DCX-positive and Nestin-positive cells within the SGZ and SVZ of Nec-1-treated mice. Effects sustained through P10 and P21 would indicate the long-term preservation of NPC populations. MRI analysis at P21 is anticipated to demonstrate protection of cerebellar and hippocampal brain tissue in Nec-1-treated mice [23]. Volumetric measurements using ITK-SNAP software will solidify the significant decrease in hippocampal and right hemisphere tissue loss in Nec-1-treated mice. Sex-specific analyses are expected to demonstrate male mice exhibiting greater tissue loss [51].

Motor Function Assessments (RotaRod and Grip Strength)

Nec-1-treated mice are anticipated to outperform vehicle- and untreated mice in behavioural assessments. RotaRod testing is predicted to show a longer mean latency to fall, indicating preserved cerebellar and hippocampal volumes and enhanced neurogenesis [47]. Vehicle- and untreated mice are likely to exhibit weakened learning and worse performance. The grip strength test is expected to reveal significantly higher peak tension in the limbs of Nec-1-treated mice, indicating improved neuromuscular function [52]. As with cellular and structural outcomes, male mice are expected to experience more severe motor function deficits than females [53].

Discussion

This research protocol provides a comprehensive investigation of Nec-1's potential as a neuroprotective agent in mitigating the neurogenic and motor consequences of neonatal HIE. By targeting necroptosis through RIP1 autophosphorylation inhibition, we aim to address neuronal loss, enhance neurogenesis, and improve motor function, targeting limitations of current HIE treatments. Findings would contribute to growing evidence that necroptosis plays a prominent role in neonatal brain injury and that its inhibition may offer a viable therapeutic strategy.

Interpretations

Ischemia-induced RIP1 phosphorylation via Ser-166 specifically marks necroptotic cells, and the presence of p-RIP1 cells would support the role of RIP1 as a biomarker for necroptosis in imminent cell death [54]. Beneficial effects observed in preserved brain volumes on MRI at P21 in ROIs would indicate Nec-1's role in reducing neuroinflammation and oxidative stress, improving structural outcomes. In motor function assessments, significant improvements in Nec-1-treated mice in motor

coordination and neuromuscular function, compared to deficits in skill acquisition and learning in other groups—likely due to motor-related brain injury—would support RIP1 inhibition for functional recovery. These anticipated results would align with studies linking motor deficits in HIE to cerebellar and hippocampal damage [10].

This study aims to build on prior evidence that RIP1 inhibition offers neuroprotection. For instance, Qu et al. found reduced brain volume loss one week after HIE with RIP1 inhibition [55]. Confortim et al. reported minor motor aberrations in HIE mice models, such as inhibited control and coordination [10]. However, Marlicz et al. found that HIE mice were able to catch up in motor learning performance with control, suggesting potential for neural repair [47]. Notably, Chevin et al. did not find sex-specific differences in any assessments, contrasting sex-specific improvements expected in this study that align with previous findings regarding male infants' greater vulnerability to severe HIE outcomes [23, 7].

Clinical Relevance

RIP1 inhibitors are currently being investigated in human clinical trials for other neurodegenerative diseases [56, 24]. Preclinical studies show that combining Nec-1 with HT has promising results in enhancing motor recovery in HIE patients, suggesting that Nec-1 could enhance the efficacy of existing treatments [23]. Our findings could potentially support Nec-1's role in improving coordination and strength and inform targeted therapies for HIE and other disorders involving RIP1-mediated necroptosis. Anticipated improvements observed with Nec-1 appear comparable with the protection provided by HT, supporting Nec-1's potential as a standalone or adjunct therapy.

Limitations

This study's transferability is limited by the use of murine models, whose brain development and responses differ from humans. Findings may not fully replicate the complexity of human HIE, given discrepancies in BBB permeability and neuroimmune responses that may affect Nec-1 pharmacokinetics, as well as the lack of feasibility of ICV in human neonates [57]. Furthermore, small sample sizes (n = 22/group, n = 6 for MRI) limit statistical power in detecting subtle effects. Potential stress to mice during handling may also influence behavioural outcomes. Despite planned efforts to acclimatize mice to testing environments, stress responses are known to cause anxiety, fear, and cognitive impairments, leading to confoundation. Additionally, the short-term focus (up to P21 for neurogenesis and week 6 for motor outcomes) restricts insight into Nec-1's long-term impact and off-target effects on neurogenesis and motor function, including adult-onset cognitive deficits or late-emerging neurodevelopmental disorders. Anticipated challenges include variability in injury severity, individual recovery trajectories, and the various unanalyzed brain regions associated with HIE.

Conclusion

Despite current treatments, many HIE infants continue to experience severe neurological disabilities, underscoring the need for novel therapeutic strategies. This study aims to position Nec-1 as a promising neuroprotective agent in HIE by inhibiting RIP1-mediated necroptosis, thereby preserving neurogenesis and improving motor function. Findings are expected to advance understanding of necroptosis in neonatal brain injury, highlight challenges such as the immature BBB's variable permeability and sex-specific neuroprotection disparities, and support the rationale for translating RIP1-targeted interventions to the clinical setting. Moreover, potential implications may extend beyond HIE to other neurodegenerative conditions involving RIP1-dependent cell death.

List of Abbreviations

BBB: blood-brain barrier
FADD: Fas-associated death domain
HIE: hypoxic-ischemic encephalopathy
HT: hypothermia
ICV: intracerebroventricular
IHC: immunohistochemistry
MRI: magnetic resonance imaging
Nec-1: necrostatin-1
NPCs: neural progenitor cells
RIP1: receptor-interacting protein 1
ROIs: regions of interest
SGZ: subgranular zone
SVZ: subventricular zone
TNF-α: tumor necrosis factor-alpha

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Ethics Approval and/or Participant Consent

Our manuscript did not require ethics approval because it is a research proposal. The proposed research protocol would require the approval of the Research Ethics Board and the Institutional Animal Care and Use Committee (IACUC).

Authors' Contributions

CJ: Substantial contribution to the introduction, interpretation and analysis of hypothetical data, critically drafted and revised the manuscript, and approved the final published version.
RD: Substantial contribution to the methodology, supplementation of figures, interpretation and analysis of hypothetical data, critically drafted and revised the manuscript, and approved the final published version.

Acknowledgements

We extend our deepest gratitude to our mentor, Milica Pavlovic, for her guidance and support throughout this research journey. Her expertise was instrumental as we

refined our ideas, navigated challenges, and explored the complexities of neonatal brain injury and neuroprotection. Milica's patience and encouragement allowed us to grow as scientific writers, and this work would not have been possible without her insightful feedback and commitment to our development.

Funding
This study was not funded.

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Article Information
Managing Editor: Jeremy Y. Ng
Peer Reviewers: Leo Li, Arthur Tung
Article Dates: Received Apr 07 25; Accepted Aug 25 25; Published Nov 05 25

Citation
Please cite this article as follows:
Deng R, Ji C. Necrostatin-1 as a neurogenic agent to ameliorate the neurogenesis and motor outcomes in neonatal hypoxic-ischemic encephalopathy through RIP1 inhibition: A research protocol. URNCST Journal. 2025 Nov 05: 9(10).
https://urncst.com/index.php/urncst/article/view/893
DOI Link: https://doi.org/10.26685/urncst.893