CXCL1/CXCR2 is Involved in White Matter Injury in Neonatal Rats via the Gut-Brain Axis

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Research Article

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Abstract

Background: This study aimed to investigate whether CXCL1/CXCR2 mediates intestinal injury or white matter injury by delivering inflammatory mediators through the gut-brain regulation axis.

Methods: Immature rats were administered 3% dextran sulfate sodium via intragastric administration at different time points to construct necrotizing enterocolitis (NEC) models. Meanwhile, hypoxia and ischemia were induced in 3–7-day-old rats to construct hypoxic-ischemic brain injury (HIBI) and NEC+HIBI models. Hematoxylin-eosin staining was used to observe pathological changes in neonatal rat intestinal and brain tissues. Western blotting detected CXCL1 and CXCR2 expression in NEC, HIBI, and NEC+HIBI rat intestinal and brain tissues.

Results: Compared with normal rats, pathological damage to periventricular white matter was observed in the NEC group. Furthermore, neonatal rats in the HIBI and NEC+HIBI groups showed inflammation and necrosis in the intestinal tissues. Western blotting results suggested that CXCL1 and CXCR2 expression levels were upregulated to varying degrees in the intestinal and brain tissues of NEC, HIBI, and NEC+HIBI neonatal rats compared to that in the normal group. Compared with the HIBI group, the expression of CXCL1 and CXCR2 continued to increase in NEC+HIBI rats at different time points.

Conclusions: CXCL1/CXCR2 may be involved in white matter injury in neonatal rats by delivering intestinal inflammatory mediators through the gut-brain axis.

1. Background

Statistically, approximately 15 million premature babies are born worldwide each year. With an 11% probability of occurrence, there is an inevitable risk of brain damage in these preterm births, despite breakthroughs in modern perinatal medicine [1]. Perinatal brain injury refers to cerebral ischemia and/or hemorrhagic damage in premature infants at the critical stage of development due to hypoxic-ischemic encephalopathy, inflammation, infection, and other pathological factors, which can also lead to long-term sequelae of the nervous system or even death in severe cases [2]. In an epidemiological survey of 3378 premature infants, 798 (23.56%) were diagnosed with brain injury [3]. White matter injury is a unique form of brain injury in preterm infants, and studies have found that preterm infants with periventricular white matter malacia are at an increased risk of cerebral palsy [4, 5]. Therefore, brain injury in premature infants has become a serious public health problem, leading to motor impairment, cognitive impairment, and audio-visual impairment. Necrotizing enterocolitis (NEC) can cause acute intestinal necrosis in premature infants and can potentially cause severe neurological impairment and increase the risk of brain damage [6-8]. Likewise, intestinal inflammation during traumatic brain injury can lead to sustained systemic immune responses and changes in autonomic nerve balance through the gut-brain axis [9]. Feng et al. found that chronic colitis may promote T-cell migration from the intestinal tract to the meninges and influence the M1/M2 macrophage imbalance, exacerbating ischemic brain injury in

mice [10]. Based on these findings, we hypothesized that neonatal brain injury may be associated with NEC. However, the underlying mechanism of the gut-brain regulation axis has not been clarified.

The chemokine family and their receptors control the migration and residence of immune cells and are closely related to inflammatory responses [11]. Moreover, hypoxic-ischemic and inflammatory responses are key factors affecting premature brain injury [12]. As a member of the chemokine family, C-X-C motif chemokine ligand 1 (CXCL1) binds to its specific receptor, C-X-C motif chemokine receptor 2 (CXCR2), resulting in chemotaxis of T-cells, monocytes, and neutrophils in the brain [13]. CXCL1 has also been shown to play a critical role in producing reactive oxygen species, which consequently regulate further inflammation [14]. Furthermore, the CXCL1/CXCR2 pathway may be involved in inflammation-induced hypoxic-ischemic brain injury (HIBI) in neonatal rats and pro-inflammatory microglial activation [15]. Yellowhair et al. illustrated that increased CXCL1/CXCR2 signaling in the brain could stimulate immune cell activation and neutrophil recruitment [16]. CXCL1 was also involved in pathways of reactive oxygen species generation during neutrophil recruitment in the circulating blood of patients with NEC [17]. Downregulated activation of CXCR2-related pathways can improve intestinal injury and inflammatory responses in newborn NEC mice [18]. Based on the above studies, we speculated that CXCL1/CXCR2 is involved in inflammatory responses in NEC and brain injury. However, whether CXCL1/CXCR2 affects neonatal white matter injury by transmitting intestinal inflammation through the gut-brain regulatory axis has not been yet reported.

Therefore, this study first induced NEC in neonatal rats. We observed pathological damage in periventricular white matter and increased CXCL1 and CXCR2 expression in intestinal and brain tissues. Subsequently, 3–7-day-old neonatal rats were selected to simulate the highest risk period of preterm brain injury (23–26 weeks of gestation) [4, 19]. We constructed HIBI and NEC+HIBI rat models to observe behavioral and pathological changes and CXCL1 and CXCR2 expression changes in intestinal and brain tissues. Our study proposes potential therapeutic targets for neonatal white matter injury and provides a theoretical basis for further understanding the mechanism of the gut-brain regulation axis in premature brain injury.

2. Materials And Methods

2.1 Mice

Parturient Sprague Dawley (SD) rats were provided by the Experimental Animal Center of North Sichuan Medical College (License No: SYSK [Sichuan] 2018-076). The animals were bred professionally under specific pathogen-free conditions until natural delivery. A total of 43 newborn one-day-old SD rats were used to construct the NEC model. We selected 45 3–7-day-old SD rats weighing 5–7 g each for HIBI and HIBI+NEC model construction.

2.2 Experimental NEC model

Forty-three newborn SD rats were randomly divided into 21 experimental cases and 22 controls. NEC was induced with 3% dextran sulfate sodium salt (DSS) dissolved in normal saline. Each NEC rat was administered 0.1 mL intragastric gavage at an interval of 3 h, four times a day for three days. Rats in the control group were administered the same dose of normal saline at the same time. Based on the histologic injury score of NEC defined by Ginzel et al. (Table S1), neonatal rats with intestinal histopathological scores ≥ 2 were considered NEC-positive [20].

2.3 HIBI and NEC+HIBI model construction and experimental grouping

We randomly assigned 45 rats to the normal, NEC, and NEC+HIBI groups (15 rats in each group). Neonatal rats were anesthetized with isoflurane, and the right common carotid artery and permanent ligation of the proximal and distal ends were separated using silk thread to construct the HIBI model. The rats were placed in an oxygen-deficient chamber (containing 8% oxygen and 92% nitrogen) 30 min after ligation for 3 h for hypoxia treatment. The NEC+HIBI group received 3% DSS, via intragastric administration, at 3 h, 12 h, 24 h, and 72 h, followed by HIBI model construction. Neonatal rats in the HIBI group received normal saline via intragastric administration at the same time after birth, followed by hypoxia treatment. Rats in the control group were given normal saline via intragastric administration after birth, and the right common carotid artery was separated without ligation or hypoxia treatment. After successfully constructing these models, the neonatal rats were killed by decapitation, and whole brain and intestine tissues were frozen in liquid nitrogen for 10 min and stored at -80°C until use.

2.4 Hematoxylin and eosin (H&E) staining

The intestinal and brain tissues of NEC, HIBI, and NEC+HIBI rats 72 h after intragastric administration were histopathologically observed using H&E staining. The preserved tissue samples were prepared as paraffin sections after fixation, dehydration, embedding, and sectioning. Dewaxed paraffin sections received the hematoxylin solution and were incubated for 5 min at room temperature. After washing and differentiation for 30 s, paraffin sections were treated with eosin for 2 min, followed by xylene decolorization and neutral gum sealing. Finally, a light microscope (400×) was used to observe the pathological features of the ileocecal and periventricular tissues.

2.5 Western blot analysis

We used western blotting to detect CXCL1 and CXCR2 expression at the protein level in neonatal rats' intestinal and brain tissues under different treatment conditions. Total protein was extracted using cell lysis buffer (P0013, Beyotime) and quantified using a BCA Protein Assay Kit (P0009, Beyotime). We used a polyacrylamide gel electrophoresis (PAGE) preparation kit (PG112, EpiZyme) to perform sodium dodecyl sulfate (SDS)-PAGE, before transferring the proteins to an Immobilon-PSQ PVDF membrane (ISEQ00010, Sigma-Aldrich). The membranes were incubated with anti-CXCL1 (1:1000, AF5403, Abcam), anti-CXCR2 (1:1000, DF7095, Abcam), and anti-β-actin (1:100000, AC026, ABclonal) antibodies.β-actin was used as an internal reference. After incubation with the secondary antibody (1:5000, goat anti-rabbit IgG(H+L), ab6721, Abcam), the membranes were treated using an ECL

luminescence kit (KF001, Affinity) and visualized using a Universal Hood II gel imager (Bio-Rad). A detailed protocol for western blotting analysis can also be found in Kurien et al. [21].

2.6 Statistical analysis

Statistical analysis was performed using SPSS 23.0, and all data are presented as the mean ± standard deviation in this study. Comparisons between multiple groups were performed using a one-way ANOVA. The least significant difference test was used for homogeneity of variance, and Tamhane's T2 test was used for heterogeneity of variance. Statistical significance was set at $P < 0.05$.

3. Results

3.1 Pathological manifestations of intestinal and brain tissues in NEC neonatal rats

Compared with the control group, intestinal tissues of newborn NEC rats showed intestinal swelling, apparent intestinal gas deposition, and intestinal wall thinning 72 h after intragastric administration (Figure 1A). H&E staining suggested that the intestinal tissue structure of the control group was intact, but the villi of the NEC group were damaged, accompanied by a certain number of inflammatory cell infiltrations (Figure 1B). The intestinal tissues in the experimental group were pathologically scored ≥ 2, further indicating NEC positivity. Furthermore, compared to brain tissue of the control group, the NEC group had unclear tissue hierarchy, loose periventricular white matter, and reduced glial cells 72 h after intragastric administration (Figure 1C). This also suggested that NEC may cause several pathological changes in brain tissues.

3.2 CXCL1 and CXCR2 expression is upregulated in the intestines and brains of NEC rats

Using western blotting, we further detected CXCL1 and CXCR2 expression differences in the brain and intestinal tissues of control and NEC rats 3, 24, and 72 h after intragastric administration. The results suggested that in the intestinal tissues (Figure 2A), the expression of CXCL1 was significantly increased in NEC rats 3 and 72 h after intragastric administration compared to the control group ($P < 0.05$). There was no significant difference in CXCR2 expression between the two groups at different gavage times. Furthermore, in brain tissues (Figure 2B), CXCL1 was significantly upregulated in NEC rats 72 h after intragastric administration compared to the control group ($P < 0.001$). CXCR2 expression was also notably upregulated in the NEC group 3, 24, and 72 h after intragastric administration, compared to the control group ($P < 0.01$). These results indicated that NEC may promote CXCL1 and CXCR2 expression in intestinal and brain tissues.

3.3 Behavioral and pathological features of HIBI and NEC+HIBI rats

In terms of behavior and state, neonatal rats in the HIBI group showed cyanosis, limb twitching, abdominal distension, diarrhea, reduced activity, growth retardation, and a mortality rate of approximately 20% (Figure 3A). Furthermore, neonatal rats in the NEC+HIBI group had a mortality rate of 30% and showed behavioral changes, such as pale skin, bloody stools, and growth restrictions (Figure 3A).

Compared with the control group 72 h after intragastric administration, the intestinal tissues of the HIBI group showed moderate hyperemia and edema in the mucosa and submucosa, while the intestinal tissues of the NEC+HIBI group showed severe necrosis, villi abscission, and gland arrangement disorder (Figure 3B). We also found that the white matter fibers around the right ventricle were loose and disordered in the HIBI group. Brain tissues in the NEC+HIBI group showed loose and irregularly arranged fibrous structures, reduced nerve cells, and formed mesh-like softening foci (Figure 3C).

3.4 CXCL1 and CXCR2 expression is upregulated in HIBI neonatal rats via the gut-brain axis

Western blotting results suggested that CXCL1 expression levels were significantly increased 12 h after hypoxic-ischemic induction ($P < 0.05$), and CXCR2 expression was significantly upregulated at 72 h ($P < 0.05$) in HIBI rats' intestinal tissues compared to the control group (Figure 4A). Similarly, CXCL1 and CXCR2 expression in the NEC+HIBI group was significantly elevated 12 h after hypoxic-ischemic induction compared to the control group ($P < 0.05$). Compared with the HIBI group, CXCL1 expression in the NEC+HIBI group increased significantly at 3, 24, and 72 h ($P < 0.01$), while CXCR2 expression was notably upregulated only at 24 h ($P < 0.001$). Furthermore, in the brain tissues (Figure 4B), CXCL1 levels in HIBI neonatal rats increased significantly 12 h after hypoxic-ischemic induction, and CXCR2 levels increased only after 12 h ($P < 0.01$). CXCL1 and CXCR2 expression levels were also significantly higher in NEC+HIBI rats than in normal rats at all time points ($P < 0.05$). Compared with the HIBI group, CXCL1 expression in the NEC+HIBI group was significantly upregulated at all time points ($P < 0.05$), while that of CXCR2 was significantly increased 3, 12 and 24 h after hypoxic-ischemic induction ($P < 0.05$). These results showed that CXCL1 and CXCR2 expression was upregulated to varying degrees in the intestinal and brain tissues of HIBI and NEC+HIBI neonatal rats, and that they may be involved in white matter injury via the gut-brain axis.

4. Discussion

NEC is a common complication in preterm infants. About 10% of preterm infants develop NEC, of which only 50% survive, and of those who survive, nearly half suffer from long-term neurological sequelae [22]. Biouss et al. experimentally showed that NEC severity in newborn rats is related to brain injury severity [23]. Martin et al. found that 24-month-old preterm infants with a history of NEC had an increased risk of neurodevelopmental dysfunction and microcephaly [24]. The present study also confirmed the prevalence of periventricular white matter injury and decreased glial cells in neonates with NEC. As a potential regulatory mechanism of premature brain injury, the relationship between intestinal and nervous system diseases has been widely discussed in recent years, and the concept of the gut-brain axis has been derived.

The gut-brain axis is a communication system consisting of interactive and bidirectional neural, hormonal, and immune signals between the gut and brain, of which gut microbes are major contributors [25]. Ma et al. found changes in colon morphology and increased intestinal permeability in mice with traumatic brain injury [26]. Metagenomic sequencing has revealed that brain injury can affect

the composition of the intestinal flora [27]. Studies have also shown that patients with central nervous system disorders, such as Parkinson's disease, may experience gastrointestinal dysfunction before diagnosis [28]. These studies suggest that brain injury may affect intestinal injury through reverse signal transmission via the gut-brain axis. Our findings confirmed the above viewpoints and found that the intestinal tissues of neonatal rats were also damaged after HIBI. Likewise, intestinal and brain injuries were more severe in NEC+HIBI rats, suggesting that there may be a mechanism of gut-brain axis regulation in neonatal rats with hypoxic-ischemic brain injury. However, the molecular mechanisms that mediate the gut-brain axis have not been clearly studied.

The gut is an important immune organ in the human body; gut epithelial cells, immune cells, and microorganisms maintain intestinal immune homeostasis through cytokine interaction [27]. When the intestinal immune system is imbalanced, pro-inflammatory cytokines may participate in brain inflammation through the gut-brain axis, thereby leading to brain damage [29]. CXCL1 is a pro-inflammatory chemokine that mediates immune cell migration after binding to CXCR2 and participates in biological effects, such as inflammation and immune responses. Previous reports have found that NEC disrupts intestinal immune homeostasis and increases CXCL1 expression in pathological intestinal tissue [30]. Meanwhile, CXCL1 induces peripheral neutrophil migration to the central nervous system by binding to CXCR2 [31]. Our study found that CXCL1 and CXCR2 expression was upregulated to varying degrees in the brain and intestinal tissues of NEC, HIBI, and NEC+HIBI neonatal rats, suggesting that CXCL1/CXCR2 may affect brain injury by delivering intestinal inflammatory mediators through the gut-brain axis. An intestinal flora-based study can explain our findings and indicate that changes in intestinal flora can induce NEC, allowing intestinal pro-inflammatory cytokines to penetrate the intestinal and blood-brain barriers, leading to systemic inflammatory responses and inflammatory brain injury [29]. Activated intestinal immune cells can also migrate to the site of brain injury and secrete pro-inflammatory cytokines to participate in the inflammatory response and aggravate brain injury [32]. Combined with our results, we conclude that intestinal immunity, inflammatory responses, and brain injury are interrelated, and that CXCL1/CXCR2 may play an important role in inflammatory transmission.

There are a few limitations associated with our study. First, we could not analyze CXCL1 and CXCR2 expression in intestinal and brain tissues at the transcriptional level—doing so could have improved our study overall. Additionally, the current study lacks an understanding of the immune response mechanisms involved in the CXCL1/CXCR2 pathway. In a follow-up study, we will conduct a blocking experiment on CXCR2, detect peripheral and central immune cell enrichment, and identify glial cell activation in rats to further explore CXCL1 regulatory mechanisms in the gut-brain axis.

5. Conclusions

Through the construction of animal models, white matter injury was found in NEC rats, while intestinal inflammation was observed in HIBI rats, potentiating a bidirectional regulation mechanism of the gut-brain axis in intestinal and brain injuries. Furthermore, upregulation of CXCL1 and CXCR2 expression was

detected in the intestinal and brain tissues of NEC, HIBI, and NEC+HIBI neonates, further indicating that CXCL1/CXCR2 may transmit inflammatory mediators through the gut-brain axis to influence brain injury.

Declarations

Ethics approval and consent to participate

The animal use protocol listed below has been reviewed and approved by the Medical Ethics Committee of the Affiliated Hospital of North Shichuan Medical College (Approval No. AEWC-2020ER038-3). All methods were carried out in accordance with relevant guidelines and regulations. The study was carried out in compliance with the ARRIVE guidelines. Consent to participate as Not Applicable.

Availability of data and materials

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

Consent for publication

Not applicable.

Availability of data and materials:

The data used to support the findings of this study are available from the corresponding author upon request.

Competing Interests

The authors declare that they have no competing interests.

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Authors' contributions

Jing Zhao and Can Yang designed the research and drafted the manuscript. Zhiyuan Feng and Ling He acquired data and analyzed data. Linlin Yin, Hong Deng and Lu Dai acquired data and performed statistical analysis. All authors read and approved the final manuscript.

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Figures

Figure 1

Pathological intestine and brain changes in NEC neonatal rats 72 h after intragastric administration.

(A) Pathological intestinal tissue features in the control group and NEC neonatal rats. **(B)** H&E staining results suggest that NEC neonatal rats displayed pathological intestinal tissue changes, with pathological scores greater than 2, accompanied by inflammatory cell infiltration. **(C)** Pathological changes in the periventricular white matter of NEC neonatal rats observed using H&E staining.

Figure 2

CXCL1 and CXCR2 expression is upregulated in the intestine (A) and brain (B) tissues of NEC rats compared with the control group 3, 24, and 72 h after intragastric administration.

*P < 0.5, **P < 0.1, and ***P < 0.001 compared with the normal group.

Figure 3

Pathological intestine and brain tissue changes in HIBI and NEC+HIBI rats.

(A) Behavioral manifestations of HIBI and NEC+HIBI in neonatal rats compared to the normal group. **(B-C)**: Pathological changes in the intestinal (B) and brain (C) tissues observed under a 400× microscope 72 h after intragastric administration.

Figure 4

CXCL1 and CXCR2 expression changes in intestinal (A) and brain (B) tissues of HIBI and NEC+HIBI neonatal rats.

*P < 0.5, **P < 0.1, and ***P < 0.001 compared with the normal group. $^{\#}P$ < 0.5, $^{\#\#}P$ < 0.1, and $^{\#\#\#}P$ < 0.001 compared with the HIBI group.

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