Neutrophilic Inflammation and Reactive Oxygen Species Production Promotes Necrotizing Enterocolitis induced Lung Injury in Mice

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Research Article

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Abstract

This study was designed to investigate the role and mechanism of neutrophils in lung injury in mice with NEC. Combinational treatment with formula milk, hypoxia, and LPS was performed to establish NEC in 5-day-old mice. The pathological changes in intestinal and lung tissues were examined by HE staining. The terminal ileum was taken out for RNA-seq. Ly6G, NE, MPO staining, and flow cytometry were used for qualitative and quantitative analysis of neutrophils in lung tissues respectively. The mRNA relative expression levels of inflammatory factors in intestinal and lung tissues were detected by RT-qPCR; the ROS release level in neutrophils stimulated by fMLP was detected by an enzyme-labeled instrument. The role of ROS in NEC-induced lung injury was further validated by the NAC, a ROS-specific inhibitor. The expression levels of genes in the Keap1—Nrf2 pathway were determined by RT-qPCR. Results showed that compared with the Ctrl group, the intestinal and lung tissues of the mice in the NEC group were significantly damaged. The mRNA relative expression levels of inflammatory factors and anti-oxidant genes were increased, while the expression levels of anti-ROS genes were downregulated. Mouse intestinal RNA-seq results suggested a strong enrichment in the neutrophil degranulation pathway. The levels of NE, MPO, and ROS released by neutrophils were significantly higher than those in the Ctrl group. And the ROS released by neutrophils is involved in NEC-induced lung injury by regulating the Keap1-Nrf2 pathway as the inhibition of the release of ROS can alleviate the injury of intestinal and lung tissues. Thus our study demonstrates that NAC has therapeutic potential for NEC-induced lung injury.

Introduction

NEC is an extremely serious disease of the neonatal digestive system, which mainly occurs in premature infants[1]. So far, the pathogenesis of NEC has been thought to be multifactorial, including immaturity of the intestinal barrier system, intestinal ischemic injury, and excessive inflammation due to the immaturity of the immune system[2]. It has been reported that approximately 30% of infants with NEC die of sepsis and other intestinal complications, and surviving neonates are often accompanied by multiple organ damage[3]. So far, there are no highly effective drugs for preventing and treating NEC in clinical practice[4].

While the initial determinants of survival in NEC patients reflect the extent of intestine disease, the long-term outcome can be attributed to the extent of disease in other organs, especially the lung[5]. In particular, NEC-induced lung damage is more severe and difficult to treat than BPA, which affects many premature infants; and is a major complication of NEC[6]. The pathogenesis of NEC-induced lung injury is largely unknown, and the dysregulated immune processes were reported to be an important mediator by reversing the Treg/Th17 imbalance to alleviate the NEC-induced lung damage[7, 8]. On the other hand, lung disease and its management in preterm infants may also in turn influence gut oxygenation, microbiota, and other risk factors of NEC[9]. Neutrophils also play important roles in acute and chronic inflammatory diseases[10], but little literature has reported their specific role in NEC lung injury and the mechanism that regulates their recruitment to the lung, so further research is urgently needed to address the role of neutrophils in NEC-induced lung injury.
After chemotactic recruitment into the site of inflammation, neutrophils can use multiple weapons including phagocytosis, the release of ROS, degranulation, NETs, etc to eliminate pathogenic microorganisms[11]. It has been found that inhibiting the NETs formation of neutrophils in NEC mice can moderate their intestinal damage[12]. Neonatal lung disease was thought to be accompanied by the production of ROS, which can damage lung tissues[13]. However, there is no report on the role of the ROS release function of neutrophils in NEC lung injury. Therefore, in this study, the ROS production was quantified from the purified neutrophils from the lung tissues of NEC mice, and the inhibition of the ROS release was performed to rescue and mitigate the lung injury caused by NEC.

Many studies have suggested that regulation of Keap1-Nrf2 signaling pathways can affect the development of lung injury[14]. Specifically, Huang et al.[15] suggest that this pathway regulates endoplasmic reticulum stress, apoptosis, and autophagy to attenuate LPS-induced ALI. Hu et al. [16] demonstrate that the Keap1-Nrf2- ARE signaling pathway can eliminate oxidative stress and it is associated with inflammation to improve traumatic lung injury-related pathology and a Keap1-Nrf2 inhibitor designed by Zhang et al. is expected to be a novel protective agent of ALI[17]. However, whether LPS and NAC play a role in NEC-induced lung injury inflammation responses via the Keap1-Nrf2 pathway has not yet been investigated. We can relieve the lung injury brought by NEC by regulating the key target genes of this pathway.

Material And Methods

Laboratory Animals

Eighty 5-day-old C57BL/6J mice were purchased from the Animal Experiment Center of Chongqing Medical University, male or female, with a weight of 2.0–3.0 g, and were raised in the Animal Experiment Center of Chongqing Medical University.

Main Reagents

Similac milk powder was purchased from Abbott Laboratories Company in the United States; milk replacer powder was purchased from Pet Ag Company; LPS (L2880) was purchased from Sigma-Aldrich Company in the United States; 4% PFA was purchased from Beijing Sevier Company; RNA extraction kits were purchased from Nanjing Vazyme Company; DEPC-treated water (NR0001) was purchased from Regen; reverse transcription and RT-qPCR reagents were purchased from MCE Company; primers were synthesized by Shanghai Sangon Biotech Co., Ltd.; rabbit anti-mouse Ly6G antibody (87048S) was purchased from CST Company; rabbit anti-mouse CD45 (ab10558), CD11b (ab8878), and Ly6G (ab25377) antibodies were purchased from Abcam Company; Antigen Retrieval Solution was purchased from Boster Biological Company; Triton X-100 was purchased from Shanghai Tengyue Biotechnology Company; DAB Color development kit was purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.; Universal Histochemical Kit was purchased from Zhongshan SP-9000 Company; 1×D-Hanks (H1040), 10×D-Hanks (H1046) were purchased from Solarbio Company; Collagenase I (C0130), Collagenase IV (C5138), Bovine serum albumin (BSA), and DNase I (DN23) were all purchased from
Sigma-Aldrich Company in the United States; FBS from Mexico (10437) was purchased from GIBCO Company; 70 µm cell sieve (258368) and 5 ml Pasteur tube were purchased from NEST Company; Percoll (17089109) was purchased from Shanghai GE Company; Wright-Gimsa staining solution (BA4017) was purchased from Besso Biological Company; NAC (A9165) was purchased from Sigma-Aldrich Company of the United States.

**Experimental Methods**

**Animal Grouping and Establishment of NEC Model**

SPF level 5-day-old C57BL/6J mice were divided into 4 groups by random number table method: Ctrl group, n = 20; NEC group, n = 20; NEC + PBS group, n = 20; NEC + NAC group, n = 20.

The mice in the Ctrl group were caged with the mother mice and were breastfed without any treatment. The mice in the NEC group were established in the NEC model according to the method of CHEN et al.[18]; and were raised in a constant temperature incubator [temperature (36 ± 1) °C, humidity 45%-60%], formula milk (Similac milk powder 3.33 g + Pet Ag dog milk replacer powder) 2g to 10 ml ddH₂O dissolved and prepared, 0.03–0.05 ml/g) gavage feeding, 5 times a day; hypoxia treatment in a hypoxia box (5% O₂ + 95% N₂) for 10 min, 3 times a day; LPS gavage (5mg/kg), once a day; continuous modeling for 4 days. The modeling method of the NEC + NAC group was the same as that of the NEC group. In addition, 20µl of NAC (25 mg/kg) in total was directly instilled through the nasal cavity, 4 times a day, 5µl each time, for 4 days. The modeling method of the NEC + PBS group was the same as that of the NEC group, and the same dose of PBS as NAC was administered intranasally every day[13].

**Observation of the General Condition**

The mental state, activity, abdominal distension, diarrhea, etc. of the newborn mice were observed, and the daily changes in body weight and death were recorded.

**Organizing Material**

Twelve hours after the end of NEC modeling, all mice were sacrificed by cervical dislocation at the same time, and the entire gut tissues including from the stomach to the rectum were obtained to observe the general shape of the intestine tissues; the intestine tissues of the ileum, jejunum, and duodenum were taken out to be fixed in 4% PFA; the thoracic cavities were quickly opened and the lungs were taken out and divided into two parts, one half was immersed in Hanks1 for neutrophil extraction, and the other half was fixed in 4% PFA for subsequent section staining.

**HE Staining and Tissue Injury Scores of Intestinal and Lung Tissues**

The intestinal and lung tissues of the Ctrl group, NEC group, NEC + PBS group, and NEC + NAC group were fixed with 4% PFA, dehydrated, embedded in paraffin, cut into 4 µm thick slices, and baked in an oven at 62°C for 2 h. After being dewaxed with xylene, HE staining was performed, and the sections were
mounted with neutral resin. The pathological changes in intestinal and lung tissues were observed under an optical microscope. According to the 4-point standard of intestinal injury scoring system by Jilling et al. [19] (Table 1), a double-blind method was used to score, and 3 visual fields were selected for each specimen, and the average value was taken as the score value of each specimen. In addition, according to the acute lung injury scoring standard [20] (Table 2), the same method was used, and the average value was taken as the score value of each specimen.

**RNA-seq of Intestine Tissues**

3 mice were respectively selected from the mice in the Ctrl group and the NEC group, and the most severely damaged intestine tissue parts (usually the terminal ileum) were taken, and the mesentery, peritoneum, and blood on the intestinal surface were removed, and the intestinal cavities were washed with 1×PBS. After cleaning, they were immediately snap-frozen with liquid nitrogen and placed in enzyme-free tubes, stored at -80°C, and then contacted Beijing Nuohezhiyuan Company to send RNA-seq, and bioinformatics technology was used for analysis after the results were obtained.

**Determination of Wet/Dry Mass Ratio and Lung Index in Lung Tissues**

4 mice were respectively randomly selected from the mice in the Ctrl group and the NEC group, and the whole lung tissues were taken out and dried briefly on absorbent paper, transferred to pre-weighed aluminum foil, and the wet weight of the lungs was measured. After being dried in a 58°C incubator for 48 hours, the dried lung mass was remeasured. The degree of pulmonary edema was assessed by calculating the lung wet/dry mass ratio and lung index.

**IHC Staining of Lung Tissues**

Using the method of random number table, paraffin sections of lung tissues of 4 mice were respectively selected from each of the 4 groups of mice for IHC staining, and 3 sections were selected from each mouse. The sections were routinely deparaffinized and rehydrated; rinsed with tap water for 3 min; incubated with Boster antigen retrieval solution at 37°C for 30 min; incubated with endogenous catalase blocking agent Triton at RT for 20 min; washed with PBS for 3 times, 3 min each time; covered the surface with 3% H₂O₂, incubated at RT for 10 min; washed with PBS for 3 times, 3 min each time; incubated with goat serum at 37°C for 30 min; washed with PBS for 3 times, 3 min each time; incubated with primary antibodies (Ly6G, NE, and MPO antibodies, 1:100 dilution), overnight at 4°C; washed with PBS for 3 times, 3 min each time; added with dropwise biotinylated goat anti-mouse/rabbit IgG, incubated at RT for 20 min; washed with PBS for 3 times, 3 min each time; added with horseradish enzyme-labeled streptavidin, incubated at RT for 20 min; washed with PBS for 3 time, 3 min each time; added with freshly prepared DAB chromogenic solution dropwise, incubated at RT for 40 s, added in ddH₂O to terminate the reaction, adjusting the time according to the observation results under the microscope; rinsed with tap water for 2 min; counterstained with hematoxylin, transparented, and sealed with neutral gum after gradient dehydration and drying. Image analysis was performed using Image J software.
**Inflammatory Cytokines and ROS-related Genes in the Intestinal and Lung Tissues Detected by RT-qPCR**

4 mice were respectively selected from 4 groups of animals for RT-qPCR analysis by the random number table method. Took the intestinal and lung tissues frozen in enzyme-free tubes at -80 °C, then extracted the total RNA with an RNA extraction kit, next detected the RNA concentration with a spectrophotometer, reversely transcribed 1µg RNA into cDNA, and used cDNA as a template to amplify IL-6, IL-1β, TNF-α, Keap1, Nrf2, H0-1, SOD1, NQO1, ELANE, and MPO; used GAPDH as the internal reference. The reaction condition was set as; 95 °C for 3 min, 95 °C for 30 s, and 55 °C for 20 s, a total of 40 cycles. The primer sequences are as followed:

**Quantitative Ratio of Neutrophils in Lung Tissues Detected by Flow Cytometry**

3 mice were selected from the Ctrl and NEC groups for flow cytometry analysis. Two groups of fresh lung tissues were digested with digestive solution (the composition was 4% FBS + 1.5 mg/ml collagenase I + 0.5 mg/ml DNase, and the solvent was 1640 medium[21]), and the digestion process was heated in the water bath at 37°C continuously. The time should not exceed 1h. The digested tissue fluid was filtered through a 70 µm cell sieve, and the supernatant clear fluid was stained with CD45, CD11b, and Ly6G flow antibodies for flow cytometry detection. The results were analyzed using FlowJo software.

**Isolation and Purification of Neutrophils from Lung Tissues[21]**

15 mice in the Ctrl group and 8 mice in the NEC group were randomly selected for the extraction of neutrophils from lung tissues. Three concentrations of 81%, 60%, and 45% cell separation solutions were prepared with Percoll,10×D-Hanks, and 1×D-Hanks, and the solutions were mixed and stirred thoroughly. Prepared digestion solution, red blood cell lysate, and Hanks1 (1×D-Hanks + 0.5% BSA + 1% HEPES) in advance. Two groups of fresh lung tissues were digested with a digestive solution, and the digestion process was heated in the water bath at 37°C for 45 min. The digested tissue fluid was filtered through a 70µm cell sieve, and then Hanks1 was added to terminate the digestion, and the cells were centrifuged at 750×g for 5 min twice. After being centrifugated, erythrocyte lysis buffer was added, and Hanks1 was added to stop lysing after 3 min. The cells were centrifuged at 750×g for 5 min twice. Gently suspended the cells in 3.5 ml of 45% cell separation solution. Filled a new 15 ml centrifuge tube with 3 ml of 81% cell separation solution and covered the top of the 81% with 60% cell separation solution. Added 45% cell separation solution containing cells to the top of the centrifuge tube and centrifuged for 30 min at 20°C, 1500×g, at the minimum speed of the centrifuge. Collected these cells by pipetting between 81% and 60% of the layers with a clean pipette tip. Hanks1 was added to 14 ml, centrifuged at 1250×g for 5 min, then added to 10 ml, 750×g, and centrifuged again for 5 min. Resuspended in Hanks1, kept cells on ice, and aspirated 100µl for cell counting. The rest of the cells were used for the next experiment.
Detection of ROS Released by Neutrophils in Lung Tissues by an Enzyme-labeled Instrument

Coated a 96-well white plate with Fn, 100ul per well, incubated at 37°C for at least 5 hours; and suspended the neutrophils extracted from the fresh lung tissues of the Ctrl group and the NEC group in Assay Buffer (prepared by Hanks + 0.25% BSA,1 million cells/ 100uL). Seeded 50ul of cells (coated strips) per well, and added 50ul of reaction buffer (added Luminol and Isoluminol) to each well. The bands were placed at 37°C and incubated for 8 min. Put these bands into the microplate reader to read the background value, quickly added the stimulant fMLP to each well, read with the microplate reader 150 times, and performed statistical analysis after the values were determined.

Statistical Analysis

Statistical analysis and graphing software used GraphPad Prism 9.0. For normal distribution test categorical quantitative data, the mean ± standard deviation was used to represent the quantitative data conforming to a normal distribution and homogeneous variance, and the independent samples t-test was used to compare the differences between the two groups; the comparison between multiple groups was performed by one-way ANOVA. Quantitative data that did not conform to a normal distribution or had unequal variances were compared between groups using the Mann-Whitney test. Survival analysis was performed using the Kaplan-Meier test. All the experiments were repeated at least in triplicate, with at least 3 pups per group for experimental NEC assessment. P 0.05 indicates that the difference is statistically significant.

Results

Mice in the NEC Group were in a Poor Mental State and had a Constant Loss of Weight

All the modeling strategies are shown in Fig. 1A. Compared with mice in the Ctrl group, the mice in the NEC group were in a poor mental state and poor in activity, accompanied by varying degrees of abdominal distention, diarrhea, and occasionally bloody stools occurred. The weight of the mice in the Ctrl group was increased gradually, while the weight of the mice in the NEC group was decreased day by day (Fig. 1B). And the NEC group mice showed a high lethality (about 55%, Fig. 1C).

NEC Group Mice Exhibited Inflammation and Tissue Damage Both in the Intestinal and Lung Tissues

The intestines of Ctrl group mice were colored pale yellow, with good elasticity, without expansion, swelling, or gas accumulation. On the contrary, the intestine tissues of the NEC group mice showed poor elasticity, obvious gas accumulation, expansion in the intestinal tubes, and part of bead-like changes (Fig. 2A). Intestine HE staining of NEC group mice showed that the ileocecal region was the most severely damaged part of the intestinal tract, and the jejunum and duodenum were also damaged to varying
degrees. The intestine tissue structure of the mice in the NEC group was disorganized, and the villi were completely shed, accompanied by a large number of inflammatory cell infiltration (Fig. 2B-C).

We further examined the lung morphology and phenotypes both in the Ctrl and NEC groups mice. The lungs of NEC mice were colored bright red with obvious edema and congestion (Fig. 2D). HE staining of lung tissues showed that the structure of the lung tissues in the Ctrl group was intact, with no thickening of the bronchial intima and no inflammatory cell infiltration. However, the alveolar septum and bronchial intima of the mice in the NEC group were thickened, accompanied by a large number of inflammatory cell infiltration, and pulmonary bullae were occasionally observed (Fig. 2E). The results of lung injury quantification confirmed lung tissue damage in the NEC mice (Fig. 2F). Consistently, the lung wet/dry mass ratio and lung index quantification data further proved lung damage in the NEC group mice (Fig. 2G). In addition, RT-qPCR results showed that compared with the Ctrl group, the mRNA relative expression levels of IL-6, IL-1β, and TNF-α in the intestinal and lung tissues of the NEC group were significantly increased (Fig. 2I-M), suggesting a robust inflammatory response in the intestinal and lung tissues of NEC group mice.

Neutrophilic Inflammation might be Involved in NEC Disease

RNA-seq was performed on the intestines of mice in the Ctrl and NEC groups, and the results indicated that there was an obvious enrichment of the neutrophil signaling pathway (Fig. 3A), suggesting that neutrophils play a more important role in the disease process of NEC. The expression of signaling molecules related to neutrophil recruitment and activation, such as DPEP1, CCL20, IL-17b, and S100A9, were highly up-regulated, and DPEP1 was the most significantly up-regulated. RT-qPCR results further confirmed the upregulation of DPEP1 in the intestinal and lung tissues of NEC group mice (Fig. 3B-C).

The Pulmonary Neutrophil Recruitment was Increased in the NEC Group Mice

The results of Ly6G IHC staining of lung tissue sections showed that compared with the Ctrl group, the neutrophils in the lung tissues of the NEC group mice were significantly increased with an enrichment in the alveolar interstitium (Fig. 4A). The results of NE and MPO IHC staining showed that compared with the Ctrl group, the NE and MPO released by neutrophils in the lung tissues of NEC group mice were significantly increased (Fig. 4B-C). The flow cytometry also reconfirmed the increased infiltration of neutrophils in the lung tissues of NEC group mice (Fig. 4D-E). Next, we examined whether the infiltrated neutrophils contributed to lung injury by producing inflammatory cytokines. Indeed, RT-qPCR results showed that compared with the Ctrl group, the mRNA relative expression levels of IL-6, IL-1β, and TNF-α in the purified neutrophils from NEC group mice lung tissues are obviously increased, as well as the NE and MPO secreted by the neutrophils in the lungs (Fig. 4F-J).
The Release of ROS from Neutrophils in the Lung Tissues of Mice in the NEC Group was Significantly Increased

The purity of neutrophils extracted from lung tissues by flow cytometry is 87.8% (Fig. 5A), which is feasible for detecting the function of neutrophils. Under the stimulation of fMLP, compared with the Ctrl group, the intracellular and extracellular ROS released by neutrophils in the lung tissues of the NEC group mice were apparently increased (Fig. 5B-C).

NAC could Attenuate Intestinal and Lung Tissue Damage and Reduce Inflammation in Mice of the NEC Group

Intestinal HE staining showed that the intestine tissue structure of the mice in the NEC + PBS group was disappeared, the villi were completely shed, and a large number of inflammatory cells were infiltrated. However, in the NEC + NAC group, the intestine tissue structure was almost intact, the intestinal epithelial cells were continuous, the villi were intact, and there was no detectable inflammatory cell infiltration with lower injury scores (Fig. 6A-B), suggesting that the intestinal injury in the NEC + NAC group mice was evidently alleviated. In addition, HE staining of lung tissues showed that the alveolar septum and bronchial intima of the mice in the NEC + PBS group were thickened and pulmonary bullae were occasionally observed. In contrast to the NEC + PBS group, the lung tissue structure of the NEC + NAC group was intact, and there was no thickening of the bronchial intima and no inflammatory cell infiltration, and also lower lung injury scores (Fig. 6C), indicating that the lung injury of the NEC mice was visibly relieved. Ly6G IHC staining of lung tissues showed that the number of neutrophils in the lung tissues of the NEC + NAC group was distinctly lower than that of the NEC + PBS group (Fig. 6D). RT-qPCR results indicated that the NE and MPO released by neutrophils were fewer in the NEC + NAC group than those in the NEC + PBS group. RT-qPCR results also showed that compared with the NEC + PBS group, the mRNA relative expression levels of IL-6, IL-1β, and TNF-α in the intestinal and lung tissues of the NEC + NAC group were significantly decreased (Fig. 6G-L), indicating the ROS functionally contributed to the lung injury in the NEC group mice.

NAC might Reduce NEC-induced Lung Injury by the Keap1-Nrf2 Pathway

To further explore whether the anti-oxidant Keap1-Nrf2 pathway was involved in the NEC-induced lung injury in vivo, we also measured the levels of the Keap1-Nrf2 related genes in the NEC mice model by detecting RT-qPCR. We found that LPS increased the Keap1 gene level in the lung tissues, whereas NAC significantly reduced the gene level of Keap1 (Fig. 7B, G). And the total Nrf2 gene level was decreased after NAC treatment (Fig. 7C, H). Then, the mRNA relative expression levels of Keap1-Nrf2 pathway downstream genes were evaluated. We found that the mRNA relative expression levels of HO-1, SOD1, and NQO1 were decreased with LPS treatment compared with the Ctrl group (Fig. 7D-F). But NAC led the mRNA relative expression levels of HO-1, SOD1, and NQO1 to be increased in the NEC + NAC treatment group (Fig. 7I-K). The results further showed that ROS was involved in NEC-induced lung injury.
Discussion

Characteristics of NEC Mouse Model and Successful Establishment of Lung Injury Model

NEC occurs in preterm infants and is the leading cause of death from gastrointestinal diseases in preterm infants[22]. As the survival rate of premature and low-birth-weight infants gradually improves, the morbidity and mortality of NEC are still on the rise[23]. The onset of NEC is rapid, with abdominal distension and blood in the stool as the main symptoms, and its characteristic pathological manifestations are intestinal mucosal ischemia and necrosis. The lesions most often occur in the distal ileum and proximal colon. Due to the immaturity of the immune system in premature infants, the inflammatory response of the NEC intestines often progresses to systemic inflammation[24].

The intestine is the primarily involved organ of NEC, but its inflammatory cascade can cause damage to multiple extraintestinal organs, and lung injury is one of them[25]. Children with NEC are often at increased risk of developing pulmonary complications, such as neonatal respiratory distress syndrome, prolonged mechanical ventilation, and BPD[9, 26]. In this study, 5-day-old C57BL/6J mice are used for modeling. The pathogenesis of NEC is traditionally believed to be associated with multiple factors including premature birth, inflammatory response, hypoxia-ischemia, and improper feeding[27]. Therefore, in this study, the classical NEC modeling methods of formula feeding, LPS gavage, and hypoxia were used for modeling to better simulate the multifactorial pathogenesis of NEC and explore the effect of NEC on the lung tissues of distant organs.

The results showed that the survival rate of the mice in the NEC group was reduced with intestinal swelling, gas accumulation, disordered intestinal structure, and thinned muscle layer. We further found the lungs of the mice in the NEC group were accompanied by obvious edema and congestion with thickened bronchial intima. Those observations indicated that the NEC mouse lung injury model was successfully established.

Inflammation and Massive Neutrophil Infiltration in the Lungs of NEC Mice

Neutrophils are the body's first line of defense against foreign germs[27]. However, their over-recruitment can lead to abnormal organ function and serious lesions: (1) the lack or dysfunction of neutrophils can aggravate lung tissue damage; (2) over-recruitment or non-regression of neutrophils may cause additional damage through the release of toxic products and ROS[28].

Our intestine tissue RNA-seq data suggested that neutrophil degranulation was involved in the NEC disease. It has been reported in the literature that the number of neutrophils in the intestine of 5-day-old C57BL/6J mice after NEC modeling is only 100 thousand[29], however, more than 1 million neutrophils are required to assay for neutrophil-related functions in vitro. This technical difficulty brings insurmountable challenges to analyzing functions and phenotypes with purified intestinal neutrophils.
Once inflammation occurs, neutrophils will travel among various organs in the body with the blood. As a storage pool of neutrophils, the lungs are necessary to study the immune process[30].

The physical properties of neutrophils and the pulmonary capillary bed play an important role in the accumulation of neutrophils in the lungs. Neutrophils must have good deformability to pass through the pulmonary capillary bed, resulting in a slower passage of neutrophils through the pulmonary capillaries. And under the action of direct or indirect inflammatory factors, the retention time of neutrophils will be significantly prolonged, thus forming a neutrophil retention pool in the lungs[31]. The members of the Johns Hopkins Children's Center research team have been devoted to working on the mechanism of NEC disease for many years. They claim that NEC may be a lymphocyte-mediated disease and that gut-lung signaling through TLR4 in the lung epithelium is required to induce NEC-induced lung injury by altering lymphocyte populations in the neonatal lung, and reversing Treg/Th17 imbalance attenuates NEC-induced lung damage[7–9]. The structure of NETs arises from the release of neutrophil chromatin binding to specific granule proteins, such as NE and MPO of neutrophils, thereby trapping microorganisms and promoting microbial killing[32]. However, neutrophils also play an important role in the acute and chronic inflammatory and immune processes[33], but few kinds of literature have reported their specific role in NEC lung injury and the mechanism that regulates their recruitment to the lungs.

In conclusion, we selected lung tissues as the object to study neutrophil function. We found that the mRNA relative expression levels of inflammatory factors such as IL-6, IL-1β, and TNF-α were significantly increased in both intestinal and lung tissues in NEC group mice, as well as the neutrophils extracted from the lungs. The IHC staining results of Ly6G, NE, and MPO in lung tissue sections showed that the neutrophils in the lung tissues of the mice in the NEC group were significantly increased, and the NE and MPO released by the neutrophils were significantly increased, as was shown by RT-qPCR as well. Flow cytometry also showed that the proportion of neutrophils in the lung tissues of the NEC group mice was significantly increased. Therefore, we can speculate that neutrophils ought to have a vital effect on NEC-induced lung injury, and studying their related functional phenotypes may have guiding significance for preventing and treating NEC distant organs in the future.

**Significantly Increased ROS Release from Neutrophils in the Lung Tissues of NEC Mice**

Neonatal lung disease is thought to develop with the production of ROS, which causes severe cellular damage, leading to lung cell death and alveolar damage. In addition, lung injury during other inflammatory processes often involves the accumulation of ROS[13]. After the recruitment and chemotaxis migration of neutrophils in the inflammatory area, neutrophils can remove pathogenic microorganisms by releasing ROS, phagocytosis, degranulation, NETs, and other mechanisms. Therefore, we want to explore the changes in ROS released by neutrophils in the lung tissues of NEC mice to provide a theoretical basis for subsequent lung injury rescue experiments. Under the stimulation of fMLP, the intracellular and extracellular ROS released by neutrophils in the lung tissues of mice in the NEC group
were significantly increased. Next, we can use ROS-specific inhibitors to explore what happens to lung injury and intestine injury after inhibiting the ROS release.

**NAC can Alleviate Intestine and Lung Tissue Damage in NEC Group Mice by the Keap1-Nrf2 Pathway**

NAC, or N-acetyl-L-cysteine, is a redox scavenger that reduces ROS release[13]. Clinically, NAC is a mucolytic agent, with a strong phlegm-dissolving effect. The sulfhydryl group contained in the molecule can break the disulfide bond in the glycoprotein polypeptide chain in the sputum, thereby reducing the viscosity of the sputum and making the sputum liquefied and easy to cough up.

The standard of care for NEC-induced lung injury includes supporting the infants' needs for gas exchange (for example, delivery of oxygen to tissues and removal of carbon dioxide), including the use of advanced ventilation strategies, selective use of medications including corticosteroids, steroids, monoxide nitrogen, vitamin A, and minimize ventilator-induced lung damage. These basic principles of treatment are similar to those of BPD, but NEC causes more severe lung damage than other forms of neonatal lung disease and requires longer treatment. Our study confirmed that NAC could alleviate inflammation in the intestinal and lung tissues by inhibiting the release of ROS, thereby protecting the lung tissues from being damaged. Therefore, NAC could be used not only in patients with primary lung inflammatory diseases; but also in pulmonary complications caused by NEC.

As previously reported, the classical Keap1-Nrf2 pathway is pivotal for regulating oxidative stress, and in oxidative stress conditions, the activated Nrf2 transfers from the cytoplasm to the nucleus to activate the downstream anti-oxidant effectors through binding to ARE[14]. Through LPS promotion and NAC suppression, we found that the related genes on the pathway of Keap1-Nrf2 had changed accordingly. Our results suggested that NAC attenuated NEC-induced lung injury inflammation responses via the Keap1-Nrf2 pathway.

**Limitations and Prospects**

We found NAC could rescue NEC-induced lung injury via the Keap1-Nrf2 pathway. Besides, we can knock out or knock down the genes involved in the pathway or use related inhibitors to rescue NEC-induced lung injury. As a broad-spectrum inhibitor of ROS release, NAC not only inhibits ROS release from neutrophils. In the future, we need to verify whether neutrophil-derived ROS plays a leading role in mice with neutrophil-specific knockout of Nox2, to further provide new clinical ideas for diagnosing and treating NEC patients with lung injury.

Moreover, the results of intestine RNA-seq indicated that the expression of signaling molecules related to neutrophil recruitment and activation was up-regulated, among which the DPEP1 was the most significant. Therefore, we speculated that neutrophils played a key role in NEC lung injury, and DPEP1 might be its key regulator. In 2019, the journal "Cell" reports a novel neutrophil adhesion receptor, DPEP1, which exists in lung endothelial cells and recruits neutrophils in the lung, and is involved in sepsis-induced lung injury. After inhibition of DPEP1, LPS-induced aggregation is greatly reduced and pulmonary
lesions are alleviated induced by sepsis, suggesting that targeted inhibition of DPEP1 may have clinical potential to alleviate and treat sepsis-induced pulmonary lesions[34].

Considering the limited time of the experiment, we have not conducted thorough research on it, and we can verify the upstream and downstream regulatory genes of the pathway where the gene is located and its corresponding inhibitors.

In addition, intestine RNA-seq can only reflect genes and pathways related to intestine tissues, and cannot directly and intuitively connect intestinal and lung tissues. Subsequent RNA-seq of lung tissues can be improved, and the results of the two parts are taken at an intersection to find common pathways and key genes.

We isolated and purified neutrophils from lung tissues and only tested their function of released ROS, and testing the in vitro function of neutrophils did not directly reflect the in vivo status of neutrophils. To make the results of the experiment more convincing, the next step is needed to supply the detection of neutrophil function in vivo. In addition, neutrophils also play an immune role through functions such as chemotaxis, adhesion, phagocytosis, and spreading. However, the role of other functions of neutrophils in NEC lung injury has not been reported. Therefore, to make up for this part shortly, we plan to completely detect the chemotaxis, adhesion, phagocytosis, and other functions of neutrophils in the lungs of NEC mice; and inhibit the related functions according to the results to rescue and alleviate the lung injury caused by NEC.

In our current study, the lung injury caused by NEC is acute inflammation, and its long-term survival rate and prognosis have not been explored. As a result, further experiments are needed to clarify its related mechanism.

**Conclusion**

This study proves that neutrophils play an important role in NEC-induced lung injury. The ROS released by neutrophils is involved in NEC-induced lung injury by regulating the Keap1-Nrf2 pathway as the inhibition of the release of ROS can alleviate the injury of intestinal and lung tissues. Our findings may potentially provide strategies to improve the prognosis and treatment of infants with NEC by targeting neutrophil inflammation and ROS production.

**Abbreviations**

NEC, necrotizing enterocolitis  
LPS, lipopolysaccharide  
RNA-seq, RNA transcriptome sequencing  
Ly6G, lymphocyte antigen 6 complex, locus g  
NE, neutrophil elastase  
MPO, myeloperoxidase
Declarations

AUTHOR CONTRIBUTION

All the authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Wei Zhao, Yujuan Su, Yujuan Yu, Mou Zou, and Ying Xu. The first draft of the manuscript was written by Wei Zhao and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

FUNDING

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AVAILABILITY OF DATA AND MATERIALS

The datasets generated and analyzed during the present study are available from the corresponding author upon reasonable request.

This study has been approved by the Ethics Review Committee of the Animal Experiment Center, Children's Hospital Affiliated with Chongqing Medical University (February 2020). All mice were raised and conformed to the Institutional Animal Care and Use Committee of China Agricultural University (AW72602202-1-7).

Consent to Participate and Consent for Publication Not applicable.
The authors declare no competing interests.

References


### Tables

**TABLE 1. INTESTINE INJURY SCORING SYSTEM**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>grade 0</td>
<td>no injury (normal)</td>
</tr>
<tr>
<td>grade 1</td>
<td>sloughing of villous tip cells</td>
</tr>
<tr>
<td>grade 2</td>
<td>mid-villous damage</td>
</tr>
<tr>
<td>grade 3</td>
<td>denudation of epithelium with loss of villi, crypts intact</td>
</tr>
<tr>
<td>grade 4</td>
<td>transmural necrosis</td>
</tr>
</tbody>
</table>

**TABLE 2. LUNG INJURY SCORING SYSTEM**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Score per field</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Neutrophils in the alveolar space</td>
<td>none</td>
</tr>
<tr>
<td>B. Neutrophils in the interstitial space</td>
<td>none</td>
</tr>
<tr>
<td>C. Hyaline membranes</td>
<td>none</td>
</tr>
<tr>
<td>D. Proteinaceous debris filling the airspaces</td>
<td>none</td>
</tr>
<tr>
<td>E. Alveolar septal thickening</td>
<td>&lt;2x</td>
</tr>
</tbody>
</table>

\[
\text{Score} = \frac{[(20 \times A) + (14 \times B) + (7 \times C) + (7 \times D) + (2 \times E)]}{(\text{number of fields} \times 100)}
\]
### TABLE 3. PRIMERS SEQUENCES

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Forward Sequence (5’→3’)</th>
<th>Reverse Sequence (5’→3’)</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>Mouse</td>
<td>CCAATTTCATGTCTTCTCT</td>
<td>ACCACAGTGAGGAATGTCGA</td>
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<tr>
<td>IL-1β</td>
<td>Mouse</td>
<td>CAGGTTCATCTCCAGAGGAG</td>
<td>CTTCTTTGGATTTTGGATGC</td>
<td>175</td>
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<tr>
<td>TNF-α</td>
<td>Mouse</td>
<td>ATGCTGAGCTCTTTTCTCAT</td>
<td>GCTTGTCACTCGAATTTGAGA</td>
<td>179</td>
</tr>
<tr>
<td>ELANE</td>
<td>Mouse</td>
<td>CAGAGGGGCTGGAGGGCTATT</td>
<td>GAAGATCCGCTGACAGAGA</td>
<td>174</td>
</tr>
<tr>
<td>MPO</td>
<td>Mouse</td>
<td>GACAGAGTCCAGAGATGAAGGTAC</td>
<td>TTGATGCTTTCTCTCGCTCC</td>
<td>189</td>
</tr>
<tr>
<td>Keap1</td>
<td>Mouse</td>
<td>TGCCCTGTTGGTCAAAGTG</td>
<td>GCTTGGGTTACCGTCCTGC</td>
<td>104</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Mouse</td>
<td>CAGCATAGAGCAGAATGGGAG</td>
<td>GAACAGGCTGATATCAGGAGCAG</td>
<td>166</td>
</tr>
<tr>
<td>HO-1</td>
<td>Mouse</td>
<td>GAACAGGCGTATGATCAGGCCAG</td>
<td>GTGTCTCTGTGCAGCTCACCC</td>
<td>152</td>
</tr>
<tr>
<td>SOD1</td>
<td>Mouse</td>
<td>AACCAGGGGCTGGAGGGCTATT</td>
<td>CCACATGCTCTTTAGGATGAGG</td>
<td>139</td>
</tr>
<tr>
<td>NQO1</td>
<td>Mouse</td>
<td>AAGATGGGAGGTACTGGAATC</td>
<td>AGGCCGCTCTTTCTATATGCTA</td>
<td>144</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Mouse</td>
<td>TGAAGCGAGCATCTTGAGGG</td>
<td>CGAAGGTGGAAGAATTGGAGG</td>
<td>102</td>
</tr>
</tbody>
</table>

### Figures

**Figure 1.**

A)

\[
\text{5d p.p.} \quad \text{hypoxia 5% for 10min} \quad \text{formula milk/LPS} \quad \text{Ctrl} \quad \text{NEC} \quad \text{NEC+PBS} \quad \text{NEC+NAC}
\]

\[
\text{20μl PBS} \quad \text{q 8h for 4 days} \quad \text{20μl NAC (25mg/kg)}
\]

B)

![Graph A](image)

C)

![Graph B](image)
Experimental design and changes in body weight or survival rate of mice in each group. A. Mice within the NEC group were subjected to formula milk/LPS gavage feeding followed by hypoxia at 5% for 10min three times daily for four days. The induction was started on day five postpartum (p.p.). The NEC+PBS group and the NEC+NAC group were given corresponding intervention measures based on the NEC group. For details, please refer to the Experimental methods section. All animals were euthanized on day 9 p.p. at the latest. B. Body mass change (Data are expressed as mean ± standard deviation), *, P 0.05. C. Survival rate.

**Figure 2**

NEC mice appeared inflammation and tissue damage both in the intestinal and lung tissues. A. Gross morphology of intestine tissues. B. Pathological changes of intestine tissues observed by HE staining (100×). C. Statistical analysis of intestinal injury score; ****, P 0.0001. D. Gross morphology of lung tissues E. Observation of pathological changes in lung tissues by HE staining (200×). F. Statistical analysis of lung injury score; ****, P 0.0001. G. Lung wet/dry mass ratio, lung index. H-M: mRNA relative expression of inflammatory factors in the intestinal and lung tissues. *, P 0.05 (n=4, data are expressed as mean ± standard deviation).
Figure 3

Neutrophilic inflammation ought to be related to NEC disease. A. RNA-seq result of mouse intestine tissues (n=3); B, C. mRNA relative expression of DPEP1 in the intestinal and lung tissues (n=4, data are expressed as mean ± standard deviation).

Figure 4

The pulmonary neutrophil recruitment was increased in the NEC mice. A. Ly6G IHC staining (400×). B. NE IHC staining (200×). C. MPO IHC staining (200×). D, E. the proportion of neutrophils in lung tissues detected by flow cytometry and the statistical analysis: *, P 0.05. F, G: RT-qPCR detection of mRNA relative expression of ELANE and MPO: *, P 0.05 (n=4, data are presented as mean ± standard deviation).
H-J: RT-qPCR detection of mRNA relative expression of inflammatory factors in neutrophils. H, J:***, P 0.001; I:****, P 0.0001 (n=3, data are presented as mean ± standard deviation).

Figure 5

The release of ROS from neutrophils in the lung tissues of mice in the NEC group was obviously increased. A. The purity of neutrophils extracted from lung tissues by flow cytometry is 87.8%. B. Luminol detects the release of intracellular and extracellular ROS in neutrophils; C. Isoluminol detects the extracellular ROS release in neutrophils: *, P 0.05 (n=3, data are expressed as mean ± standard deviation).

Figure 6
NAC could relieve intestinal and lung tissue damage and reduce inflammation in mice of the NEC group. A. Chemical structure of NAC (C_{5}H_{9}NO_{3}S)[35]. B. HE staining of intestine tissues and statistical analysis of intestine tissue injury score (200×); C. HE staining of lung tissues and statistical analysis of acute lung injury score (200×). D. Ly6G IHC staining of lung tissues (200×). E, F. RT-PCR detection of the mRNA relative expressions of NE and MPO in the lung tissues of mice in each group. G-I. RT-PCR detection of the mRNA relative expressions of inflammatory factors IL-6, IL-1β, and TNF-α in the intestine tissues of mice in each group. J-L. RT-PCR detection of the mRNA relative expressions of inflammatory factors IL-6, IL-1β, and TNF-α in the lung tissues of mice in each group: *, P < 0.05 (n=4, data are expressed as mean ± standard deviation).

**Figure 7**

NAC might reduce NEC-induced lung injury by the Keap1-Nrf2 pathway. A. After NEC occurs in the intestine, a large number of neutrophils recruit to the lung and cause tissue injury through phagocytosis, ROS release, adhesion, degranulation, NETs, and other mechanisms. The entry of Keap1 and Nrf2 from the cytoplasm into the nucleus requires the dissociation and release of a large number of ROS, and Nrf2 entering the nucleus binds to the ARE on DNA. The released ROS can lead to apoptosis and degranulation, while GSH, HO-1, SOD1, and NQO1 can inhibit the ROS release. B-K. mRNA relative expression of the Keap1-Nrf2 related factors in the lung tissues: *, P < 0.05 (n=4, data are expressed as mean ± standard deviation).