Protective effects of citicoline in neonatal rats with necrotizing enterocolitis

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Abstract

Background: This study aimed to analyze and evaluate the protective and therapeutic effects of citicoline (CDP-choline) on neonatal rats with necrotizing enterocolitis (NEC).

Methods: Forty 3-day-old Sprague-Dawley rats, of either gender, were randomly divided into four treatment groups of 10 rats each: control, NEC model, NEC+CDP-choline, and CDP-choline. After the experimental period, intestinal tissue was extracted, stained with hematoxylin and eosin (HE), and observed under a light microscope. Additionally, the samples were scored according to the method of Shiou et al. Immunofluorescence was used to detect beclin-1 and LC3A/B to allow a comparison of autophagy and apoptosis in each group. The inflammatory factors TNF-α, IL-1β, and IL-6 were detected by enzyme-linked immunosorbent assay (ELISA).

Results: HE staining revealed that the inflammatory response in the NEC+CDP-choline treatment group significantly improved compared with that in the NEC model group; furthermore, a pathological examination showed a significant difference in Shiou scores (P<0.05). Beclin-1 and LC3A/B levels significantly decreased in intensity and the levels of inflammatory factors TNF-α, IL-1β, and IL-6 significantly decreased (P<0.05).

Conclusions: CDP-choline exerted protective and therapeutic effects on neonatal rats with NEC.

Introduction

Necrotizing enterocolitis (NEC) is a common gastrointestinal emergency for newborns, with a mortality rate of 20–30% [1]. However, there is still no clear and effective clinical therapeutic strategy for it owing to the inexact pathogenesis of the disease [2]. Recent studies have shown that NEC intestinal mucosal injury results from the release of inflammatory mediators mediated by oxygen free radicals. It was also reported that inflammatory responses and inflammatory factor release significantly increased the incidence of nervous impairment in children with NEC [3]. Phosphatidylcholine, the main lipid of the gastrointestinal mucosal barrier, exerts a protective effect on the mucosa as a hydrophobic intestinal surfactant and regulates the inflammatory signaling system. Moreover, studies have demonstrated that defect or damage of the phospholipid layer causes inflammation and increases susceptibility to NEC [4]. Thus, exogenous phosphatidylcholine may exert a therapeutic effect on NEC. This study planned to examine the protective effects against NEC-induced intestinal inflammation, necrosis, and apoptosis following the administration of citocline (cytidine 5'-diphosphocholine, CDP-choline) in neonatal rats with NEC, which provided a basis for investigating the mechanism responsible for the development of NEC and a new search strategy to effectively prevent and treat NEC.

Materials and methods

Experimental animals

Forty 3-day-old neonatal rats were divided into four groups. 1) The control group was conventionally fed after separation from mother rats without any intervention or stimulation [5]. 2) In the NEC model group, rats were fed with intestinal formula milk after separation from the mother rats and the method of hypoxia-hyperoxia exposure and cold stress were used to induce NEC. First, neonatal rats were placed in an environment with 100% CO₂ for 10 min. Then, they were subjected to 4°C for 5 min followed by 97% O₂ for 5 min. This process was performed twice daily for three consecutive days and then saline was intraperitoneally injected. 3) In the NEC+CDP-choline treatment group, CDP-choline was intraperitoneally injected once daily at a dose of 300 mg/kg/day for three consecutive days after NEC was successfully induced. 4) Lastly, in the CDP-choline group, neonatal rats were conventionally fed after separation from the mother rats. CDP-choline was administered by intraperitoneal injection once daily at a dose of 300 mg/kg/day for three consecutive days. At day 7, the rats were sacrificed and gastrointestinal tissue from the end of duodenum to ileocecum was extracted. The institutional and national guide for the care and use of laboratory animals was followed.

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Key words: autophagy and apoptosis, citicoline, inflammatory factors, necrotizing enterocolitis (NEC), Shiou score

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Experimental methods
Pathological examination of intestinal tissue in rats by hematoxylin and eosin (HE) staining

Main reagents: (1) Xylene (Kelong Chemical Reagent Factory, Chengdu); (2) Eagle (Maikun Chemical, Shanghai, batch number: 20120831); (3) Hematoxylin (Sigma, batch number: 041M0014V).

Main instruments: (1) Rotary slicer (RM2235, LEICA company); (2) Bleaching machine for pathological tissue (Tec 2500, Hao Silin Instrument Equipment Co., Ltd, Changzhou); (3) Microscope (BX43, OLYMPUS); (4) Waterproof constant-temperature incubator (PYX-DHS500BS-II, Yuejin Medical Equipment Co., Ltd, Shanghai).

Experimental steps: (1) Intestinal tissue was equilibrated to room temperature; (2) preparation of reagent; (3) sample loading and incubation; (4) addition of HRP and the standard product; (5) washing; (6) addition of antibodies and incubation; (7) washing; (8) addition of TM and incubation; (9) termination of the reaction and detection of the absorbance; and (10) result calculation: An average of two measurements of the OD values of the standard product and the sample minus the OD value of zero standard was used, standard curve and formula were established, and the concentrations of each sample were calculated by the detected OD value.

Statistical analysis
SPSS19.0 software was used for statistical analysis. Continuous data were expressed as the mean ± standard deviation (SD), with comparisons between groups conducted by one-way ANOVA with SNK post hoc test. The value of \( P < 0.05 \) was considered to be statistically significant.

Results
Body weight gain
At 7 days of life, all pups in both the control group and the CDP-choline group survived but in these studies 2.3 animals died in the NEC model group and the NEC+CDP-choline treatment group prior to the end-point respectively, and these pups were excluded from all further analyses. Over the 7 days of study, the body weight gain of pups in the control group, NEC model group, NEC+CDP-choline treatment group and CDP-choline group was 5.102 ± 1.517, 0.053 ± 0.085, 0.276 ± 0.171, 4.572 ± 1.927 respectively and the difference was statistically significant (\( F=38.543, P<0.01 \)). The NEC+CDP-choline treatment group and the NEC model group gained significantly less weight from birth, when compared with the NEC model group (\( P < 0.05 \)). However, there was no significant difference in weight gain between the NEC+CDP-choline treatment group and the NEC model group (\( P>0.05 \)).

Pathological examination of intestinal tissue in rats by using HE staining

The HE staining of intestinal tissue in each group was examined under a light microscope. In the control group, the intestinal mucosal epithelium was complete and continuous, cells and glands were arranged regularly, and there was no inflammatory cell infiltration in the mucosal and submucosal layers. In the NEC group, intestinal villus edema was visible and there was a large amount of inflammatory cell infiltration in the mucosal lamina propria and the submucosal layer (mainly neutrophils and eosinophils). Moreover, intestinal capillary congestion and even intestinal epithelial necrosis were visible. In the NEC+CDP-choline group, the intestinal mucosal epithelium was complete and continuous, and cells as well as glands, were regularly arranged. Furthermore, there was neither intestinal villus edema nor intestinal capillary congestion, but a small amount of inflammatory cell infiltration was visible in the lamina propria. There were no obvious inflammatory lesions in the normal+CDP-choline group (Figure 1).

Intestinal pathology was scored according to the new double-blind standard by Shiou et al. [6], which uses the following scoring system: 0 points, intestinal mucosal villi integrity was retained and tissue structure was normal; 1 point, slight edema and separation in the submucosal layer and/or lamina propria; 2 points, moderate separation in the submucosal layer and/or lamina propria and edema in submucosal and/or muscular layers; 3 points, severe separation in the submucosal layer and/or lamina propria, edema in submucosal and/or muscular layers, and shedding of local villi; 4 points, disappearance of intestinal villi combined with intestinal necrosis. The induction of NEC was considered successful when a pathological score of ≥2 points was obtained.

The scores of the CDP-choline group, the NEC+CDP-choline treatment group, the NEC model group and the control group were...
0.5 ± 0.707, 0.7 ± 0.823, 4.3 ± 0.823 and 0.4 ± 0.699 respectively. The scores of the NEC groups were significantly higher than those of the control groups ($P < 0.05$). There were no significant differences between the NEC+CDP-choline treatment, the normal control, and the CDP-choline groups ($F = 0.42, P = 0.661$).

**Immunofluorescence detection of LC3II and beclin-1 and comparison of autophagy levels in rat intestinal tissue**

The expression of fluorescence intensity for beclin-1 and LC3II in the NEC model group was significantly higher than that of the other three groups. However, there were no differences between the NEC+CDP-choline treatment group, the normal control group, and the CDP-choline group (Figures 2 and 3).

**Comparison of the contents of inflammatory factors**

The levels of the inflammatory factors TNF-α, IL-1β, and IL-6 showed a significant difference between the four groups ($P < 0.05$). The content of TNF-α, IL-1β, and IL-6 in the NEC model group was significantly higher than the other groups ($P < 0.05$), but there were no significant differences between the NEC+CDP-choline treatment group, the normal control group, and the CDP-choline group ($P > 0.05$) (Tables 1 and Figure 4).

**Discussion**

In this study, we used the comprehensive stress method of enteral formula feeding, hypoxia or hyperoxia exposure, and cold stimulation to establish a neonatal rat model of NEC. We found that CDP-choline intervention significantly improved the Shiou score of intestinal tissue; reduced levels of autophagy, apoptosis, and inflammatory factors (INF-α, IL-1β, and IL-6); and exerted intestinal protective effects.

NEC is the most common neonatal gastrointestinal emergency and has a high mortality rate. Studies have shown that NEC intestinal mucosal injury is a result of the release of inflammatory mediators, such as oxygen free radical-mediated platelet activating factor (PFA), leukotriene, and tumor necrosis factor-α (TNF-α). The release of inflammatory mediators is the last common pathway or key link for the occurrence of NEC (4). We also found that the content of inflammatory factors (TNF-α, IL-1β, and IL-6) in the NEC model...
The mucous layer in the gastrointestinal tract is a key factor in the protection of the gastrointestinal tract; phosphatidylcholine (lecithin) is the main lipid of the gastrointestinal mucosal barrier, which, as a hydrophobic intestinal surfactant, has protective effect on mucosa and can regulate the inflammatory signaling system. The occurrence of NEC was related to the defect or damage of this system; exogenous lecithin administration has shown a good effect on ulcerative colitis [4,5]. CDP-choline, an activated form of choline that plays an important role in lecithin synthesis, reduces the production of free fatty acids, increases triglyceride levels, and is commonly used as a metabolic activator of brain cells in clinical practice. Moreover, it has important significance, especially for delayed neurotoxicity, prevention of the inflammatory markers and oxidative stress to effectively protect against NEC-induced ileal degeneration.

In summary, CDP-choline can inhibit the intestinal inflammatory cascade by reducing the expression of inflammatory mediators such as TNF-α, IL1-β, and IL-6. Moreover, it can inhibit apoptosis and apoptosis of the intestinal mucosal epithelial cells, which exerts a protective effect against the intestinal injury of neonatal rats with NEC.

Cytokine | The CDP-choline group (n=10) | The NEC+CDP-choline treatment group (n=10) | The NEC model group (n=10) | The control group (n=10) | F/P value
--- | --- | --- | --- | --- | ---
The content of INF-α | 10.041 ± 4.721 | 8.265 ± 5.585 | 42.722 ± 18.46* | 8.731 ± 5.699 | 26.679/<0.001
The content of IL-1β | 43.012 ± 30.951 | 43.534 ± 40.862 | 312.250 ± 204.261* | 54.485 ± 34.405 | 15.475/<0.001
The content of IL-6 | 0.528 ± 0.215 | 0.664 ± 0.335 | 2.291 ± 0.898* | 0.867 ± 0.672 | 18.726/<0.001

Note: *P = 0.05, compared with the other group.

A = INF-α, B = IL-1β, C = IL-6. Each bar represents mean ± SD, n=10 rats per group. *P<0.05 versus the other groups.

## Table 1. ELISA detection of INF-α, IL-1β and IL-6 in intestinal tissue.

Figure 4. ELISA detection of INF-α, IL-1β and IL-6 in intestinal tissue of rats.

(A). The content of INF-α. (B). The content of IL-1β. (C). The content of IL-6. Each bar represents mean ± SD, n=10 rats per group. *P<0.05 versus the other groups.
Authors’ contributions

Shuxia Liang, Jie Xu and Mingxing Ding conceived and coordinated the study, designed, performed and analyzed the experiments, wrote the paper. Xiaobing Li, Panjian Lai, Jinzhi Mei and Lingjian Zhang carried out the data collection, data analysis. Yuanshu Fang and Fangming Guo performed and analyzed the experiments. Yunguang Bao and Kaixuan Wang revised the paper. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The institutional and national guide for the care and use of laboratory animals was followed.

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