Curcumin improves necrotising microscopic colitis and cell pyroptosis by activating SIRT1/NRF2 and inhibiting the TLR4 signalling pathway in newborn rats

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Abstract
This study aimed to explore comprehensively the biological function of curcumin, and its underlying mechanism, in protecting from necrotising microscopic colitis in newborn rats. A total of 20 normal healthy rats were selected, and a necrotising enterocolitis (NEC) model was established. After hypoxia and hypothermia stimulation, these rats were treated with different doses of curcumin (control group, NEC model group, NEC + 20 mg/kg curcumin and NEC + 50 mg/kg curcumin). Inflammation was identified using hematoxylin and eosin staining, and inflammatory factors were detected via ELISA. The mRNA and protein levels of SIRT1, NRF2, TLR4, NLRP3 and caspase-1 were determined by quantitative RT-PCR and Western blotting, respectively. Curcumin improved the inflammatory condition of NEC and inhibited the expression of inflammatory factors in NEC newborn rat intestinal tissue. Furthermore, the SIRT1/NRF2 pathway was inhibited in the intestinal tissue of NEC newborn rats, whereas curcumin treatment induced the activation of the SIRT1/NRF2 pathway and inhibited TLR4 expression in these animals. In addition, curcumin could also inhibit the expression of inflammatory factors and alleviate the LPS/ATP-induced focal death pathway in intestinal epithelial cells through the SIRT1 pathway. Curcumin can improve necrotising microscopic colitis and cell pyroptosis by attenuating NEC-induced inhibition of SIRT1/NRF2 and inhibiting the TLR4 signalling pathway in newborn rats.

Keywords
Curcumin, necrotising microscopic colitis, cell pyroptosis, SIRT1/NRF2, TLR4

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Introduction
Neonatal necrotising enterocolitis (NEC) is an acquired disease which is caused by damage to the intestinal mucosa, leading to diffuse or local necrosis of the small intestine and colon.¹,² This mainly occurs in premature or sick neonates, with abdominal distension and blood in the stool as the main symptoms.³,⁴ At present, the death rate of NEC in China is approximately 10–50%.⁵ With the improvements in diagnosis, treatment and nursing technology, the death rate of NEC is decreasing. However, 90–95% of cases occur in premature infants with a gestational age of < 36 wk, and most cases of NEC occur within 2 wk after birth.⁶ The younger the gestational age and the lighter the birth mass, the later the incidence of NEC.

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The occurrence of NEC could be caused by one or a combination of preterm delivery, hypoxia, ischaemia, infection, exotoxin, excessive feeding, rapid increase in milk formula, erythrocytosis and exchange transfusion.\(^7,8\)

Curcumin is a diketone component extracted from the rhizomes of some plants in the family Zingiberaceae and Araceae.\(^9\) Medical research has revealed that curcumin has anti-tumour, anti-inflammatory, gall-bladder and antioxidant functions, as well as lowering blood fat. Inflammation is a complex process, which is triggered by cell infection and/or tissue damage, and it creates a chain reaction that leads to the rapid development of certain chronic diseases.\(^10\) Therefore, anti-inflammatory drugs are needed to prevent this. Although various anti-inflammatory drugs, such as cimicoxib, aspirin, ibuprofen and butazone, can be used to treat inflammatory diseases, most of these have side effects. Curcumin has anti-inflammatory activity comparable to steroidal and non-steroidal drugs, but its role and mechanism in necrotising microscopic colitis have not been fully reported.\(^11\)

Pyroptosis, which is also known as cell inflammatory necrosis, is a form of inflammatory programmed cell death, which manifests through the constant expansion of cells until the membrane ruptures, leading to the release of cell contents and the activation of a strong immune response. Pyroptosis is an important natural immune response, and plays an important role in fighting infection.\(^12\) Pyroptosis could be characterised by up-regulated caspase-1 expression and the release of a large number of pro-inflammatory cytokines. Studies have shown that pyroptosis plays an important role in the occurrence and development of infectious diseases, nervous system-related diseases and atherosclerotic diseases.\(^13\) In-depth research on pyroptosis would be helpful to understand its correlations with NEC.

Silent information regulator 2-related protein 1 (SIRT1) is a multifunctional transcription regulatory factor involved in the regulation of various signalling pathways, which has been reported to play protective roles in inflammatory damage.\(^14\) Meanwhile, SIRT1 can regulate the nuclear factor erythroid 2-related factor 2 (NRF2) pathway, and the activation of the NRF2 signalling pathway can inhibit NLRP3-mediated pyroptosis.\(^15,16\) The expression of TLR4 is also found to be up-regulated under inflammatory conditions, which could further facilitate the transcription and expression of inflammatory cytokines and activate NLRP3 inflammasome and promote pyroptosis.\(^17\) Several studies have suggested that the protective effect of curcumin on necrotising microscopic colitis is correlated to the inhibition of cell pyroptosis.\(^18,19\)

In the present study, various experiments were performed to explore comprehensively the biological functions of curcumin, and its underlying mechanism, in protecting from necrotising microscopic colitis in newborn rats. A total of 20 normal healthy rats were selected, and a NEC model was established via hypoxia and hypothermia stimulation. The biological functions of curcumin at different doses in NEC rats and its underlying mechanism were explored. These results have important significance in improving the NEC disease pathogenesis, providing novel intervention approaches for the treatment of NEC.

**Methods**

**Animals**

The rat NEC model was established by hypoxia (treated at 5% O\(_2\) for 10 min) and hypothermia (treated at 8°C for 10 min) stimulation, while the control group was maintained at 18–25°C in a normal atmospheric environment. Rats displaying symptoms of abdominal distension, rectal bleeding and pneumonia intestinalis which were diagnosed by microscopic examination were regarded as successful NEC models.\(^20\) All rats were treated with different doses of curcumin and divided into four groups: control group, NEC group, NEC rats treated with 20 mg/kg curcumin group (NEC+curcumin (L)) and NEC rats treated with 50 mg/kg curcumin group (NEC+curcumin (H)). Curcumin was dissolved in 1% gum acacia solution and orally administrated using a stomach tube at different doses of 20 and 50 mg/kg by mass. For the control group and NEC model group, rats were orally administrated with 1% gum acacia solution of the same volume.

Hematoxylin and eosin (H&E) staining was used to observe the inflammatory condition of intestinal tissues and the histological score in each group. The expression of SIRT1, NRF2, NLRP3, cleaved caspase-1 and the TLR4 signalling pathway was detected by PCR and Western blot. The expression of the inflammatory markers IL-1β, IL-6, IL-18 and TNF-α in the intestinal tissues (homogenate) of rats in each group was detected by ELISA.

**Cells**

Human intestinal epithelial cell lines (SW480 cells), curcumin and SIRT1 inhibitor sirtinol were used to treat these cells.\(^21,22\) These were divided into the following groups: control, LPS+ATP induction, LPS/ATP+curcumin (H) and LPS/ATP+curcumin (H)+sirtinol. The expression of SIRT1, NRF2, NLRP3, cleaved caspase-1 and the TLR4 signalling pathway were detected by PCR and Western blot, while the
expression of IL-1β, IL-6, IL-18 and TNF-α were detected by ELISA.

**H&E staining**

In the present study, 3-mm punch biopsies (small intestine) were taken from the scar tissue before and immediately after the treatment. The skin pieces were fixed in 10% formalin solution and embedded in paraffin for standard histological examination and immunohistochemical investigation, and were stored in glutaraldehyde for transmission electron microscopic analysis. Consecutive serial sections 5 μm thick were cut and stained with H&E. The dermal thickness was determined in the H&E-stained sections, which was viewed under an Axioplan 2 imaging analyser (Biochemical Department, Shanghai Jiao Tong University School of Medicine). In order to determine the collagen content and organisation of the lesion skin, the deparaffinised sections were processed for Masson’s trichrome stain, which was viewed under an Axioplan 2 imaging analyser (the images were analysed by digital capture).

**ELISA**

The expression of the inflammatory markers IL-1β, IL-6, IL-18 and TNF-α in the intestinal tissues (homogenate) of rats in each group was detected using an ELISA kit (Cyman system), according to the instruction manual.

**Western blot**

The samples were prepared by lysis with a proteinase inhibitor. The protein was quantified using a BCA kit (Pierce Biotechnology, Rockford, IL). Each sample was separated by 15% SDS-PAGE. Then, the membranes were incubated for 12 h at 4°C with Abs, and incubated with goat-anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Afterwards, the signals were detected using electrochemiluminescence reagents.

**RT-PCR**

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer’s instructions. The concentration of RNA was measured using a NanoDrop-1000 (Thermo Fisher Scientific, Waltham, MA), and RNA integrity was determined by 1.5% agarose gel electrophoresis. Furthermore, the cDNA synthesis was performed according to the manufacturer’s recommendation. Briefly, RNA was reverse transcribed using a ReverAid First Strand cDNA kit (Thermo Fisher Scientific). Under experimental conditions, β-actin was used as an internal control for normalising the expression of mRNA. The cDNA products were diluted at 20×, and the 10 μl PCR mixture contained 1 μl diluted reverse-transcribed product, 5 μl SYBR-Green Master Mix, 2 μl RNase-free water, 1 μl forward primer and 1 μl reverse primer. Then, the reaction was incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 65°C for 60 s. Afterwards, the relative mRNA expression was calculated using the 2^ΔΔCt method.

**Statistical analysis**

The results were analysed using IBM SPSS Statistics for Windows v19.0 (IBM Corp., Armonk, NY), and presented as the mean ± SD of three independent experiments. A P value of < 0.05 was considered statistically significant.

**Results**

**Curcumin could improve the inflammatory condition of NEC newborn rat intestinal tissues**

H&E staining results presented in Figure 1a reveal that curcumin could improve the histological status compared to the NEC group. ELISA was performed to determine the level of IL-1β, IL-6, IL-18 and TNF-α in the intestinal tissues of rats. As shown in Figure 1b, the expressions of these inflammatory factors were significantly up-regulated in NEC group compared to the control group. After treatment with curcumin, the levels of IL-1β, IL-6, IL-18 and TNF-α were dramatically down-regulated. Based on these results, it was found that curcumin could improve the inflammatory condition of NEC newborn rat intestinal tissues, and inhibit the expression of inflammatory factors in NEC newborn rat intestinal tissues.

**Curcumin induced activation of the SIRT1/NRF2 pathway in NEC intestinal tissues and inhibited TLR4 expression**

The expression of SIRT1, NRF2 and TLR4 genes was detected using quantitative RT-PCR and Western blotting. Levels of SIRT1 and NRF2 were lower in the NEC group than they were in the control group, while expression of TLR4 was significantly up-regulated in NEC rats (Figure 2). However, the levels of SIRT1 and NRF2 were increased and the level of TLR4 was decreased in the NEC+curcumin (L) and NEC+curcumin (H) groups compared to the NEC group. These data indicated that curcumin reversed the inhibition of the SIRT1/NRF2 pathway in the intestinal tissues of NEC newborn rats and decreased the TLR4 expression at the same time (Figure 2).
Figure 1. Inflammatory condition in intestinal tissues of control, NEC, NEC+curcumin (L) and NEC+curcumin (H) groups. (a) Histological status observed by H&E staining. (b) Expression of inflammatory factors, including IL-1β, IL-6, IL-18 and TNF-α, detected by ELISA. **P < 0.01 vs. control group; ##P < 0.01 vs. NEC group. NEC: necrotising enterocolitis; H&E: hematoxylin and eosin.

Figure 2. (a) Protein level and (b) mRNA level of SIRT1, NRF2 and TLR4 in control, NEC, NEC+curcumin (L) and NEC+curcumin (H) groups, detected by Western blotting and quantitative RT-PCR, respectively. **P < 0.01 vs. control group; ##P < 0.01 vs. NEC group.
Curcumin inhibited the pyroptosis of intestinal cells by activating the SIRT1 pathway

Quantitative RT-PCR and Western blotting were used to detect the expression of pyroptosis-related genes. Consistently, treatment with curcumin up-regulated the expression of SIRT1 and NRF2 and down-regulated that of TLR4, NLRP3 and cleaved caspase-1 compared to the NEC group (Figure 3). However, the inhibition of SIRT1 using sirtinol resulted in the loss of the protective function of curcumin. This suggests that curcumin could inhibit the pyroptosis of intestinal tissues in NEC rats by activating the SIRT1 pathway.

Figure 3. (a) Protein level and (b) mRNA level of SIRT1, NRF2, TLR4, NLRP3 and caspase-1 in control, NEC, NEC+curcumin (H) and NEC+curcumin (H)+sirtinol groups, detected by Western blotting and quantitative RT-PCR, respectively. **P < 0.01 vs. control group; ###P < 0.01 vs. NEC group.
Curcumin inhibits inflammatory factors induced by LPS/ATP

LPS/ATP was then used to induce inflammatory status in SW480 cells. The levels of pro-inflammatory factors, including IL-1β, IL-6, IL-18 and TNF-α, were remarkably reduced in the LPS/ATP+curcumin group compared to the LPS/ATP group. The levels of these pro-inflammatory cytokines increased again after inhibiting SIRT1 using sirtinol (Figure 4). All the results showed that curcumin could inhibit inflammatory factors induced by LPS/ATP.

Curcumin inhibits LPS/ATP-induced focal death through SIRT1

Consistent with the expression in intestinal tissues of NEC rats, the expression of SIRT1 and NRF2 was significantly up-regulated, while NLRP3, cleaved caspase-1 and TLR4 was dramatically down-regulated in SW480 cells after treatment of curcumin compared to the LPS/ATP group (Figure 5). However, use of the SIRT1 inhibitor sirtinol could reverse the protective effects of curcumin, indicating that curcumin could inhibit LPS/ATP-induced focal death through the SIRT1/NRF2 pathway.

Discussion

NEC, one of the most common acute abdominal diseases in premature infants with a low birth mass, could be caused by damage to the intestinal mucosa that is induced by ischaemia and hypoxia, leading to diffuse or local necrosis of the small intestine and colon. Nakame et al. found that inflammatory factors that included TNF-α and IL-6 were up-regulated in NEC rats. Our findings also demonstrated that the level of IL-1β, IL-6, IL-18 and TNF-α were all increased in NEC rats. Curcumin, a chemical component extracted from the rhizomes of some plants in the family Zingiberaeae and Araceae, lowers blood fat content, and has anti-tumour, anti-inflammation, gallbladder and antioxidant properties. Mohammadi et al. found that curcumin could attenuate hepatic oxidative stress, as well as reduce the IL-6 level in polycystic ovary syndrome rats. Curcumin could also attenuate high Glic-induced inflammatory injury in rat thoracic aorta endothelial cells. In this study, various experiments were performed to explore comprehensively the biological functions of curcumin, and its underlying mechanism, in protecting from necrotising microscopic colitis in newborn rats. Consistently, our results indicated that after treatment of curcumin in NEC rats, the levels of IL-1β, IL-6, IL-18 and TNF-α were all down-regulated.

The SIRT1/NRF2 pathway has been reported to participate in attenuating the inflammatory status, while TLR4 could mediate an inflammatory response. Miao et al. reported that curcumin pre-treatment has the ability to alleviate inflammation and mitochondrial dysfunction in experimental stroke by activating the SIRT1 pathway. Ni et al. demonstrated that up-regulation of TLR4 and inflammatory cytokines in the injured rat spinal cord were attenuated by curcumin. In the present study, curcumin could also increase the level of SIRT1 and NRF2 and decrease the level of TLR4 in NEC newborn rats. In addition, SITR1 inhibitor sirtinol partially restored the expression level of TLR4 in curcumin-treated cells, suggesting a negative correlation between SIRT1/NRF2 pathway activation and TLR4 expression.

Pyroptosis is an important natural immune response, and plays an important role in fighting infection. A previous study suggested that the protective

![Figure 4](image-url)  
Figure 4. Expression of inflammatory factors, including IL-1β, IL-6, IL-18 and TNF-α, in control, LPS/ATP, LPS/ATP+curcumin (H) and LPS/ATP+curcumin (H)+sirtinol groups, detected by ELISA. **P < 0.01 vs. control group; ***P < 0.01 vs. LPS/ATP group.
effect of curcumin on necrotising microscopic colitis was correlated to the inhibition of cell pyroptosis.\textsuperscript{31} NLRP3/ASC inflammasome and caspase-1 were found to be upregulated in pyroptotic cells.\textsuperscript{32} Consistently, we found that the expression of NLRP3 and caspase-1 was significantly increased in NEC rats, while curcumin treatment could down-regulate these proteins and attenuate cell pyroptosis. The use of SIRT1 inhibitor could result in the loss of protective ability of curcumin, suggesting that curcumin might participate in improving

Figure 5. (a) Protein level and (B) mRNA level of SIRT1, NRF2, TLR4, NLRP3 and caspase-1 in control, LPS/ATP, LPS/ATP+curcumin (H) and LPS/ATP+curcumin (H)+sirtinol group detected by Western blotting and quantitative RT-PCR, respectively. **\(p < 0.01\) vs. control group; ***\(p < 0.01\) vs. LPS/ATP group.
necrotising microscopic colitis and cell pyroptosis through the SIRT1/NRF2 pathway.

In summary, curcumin can improve the inflammatory condition of NEC, and inhibit the expression of inflammatory factors in NEC newborn rat intestinal tissues. Furthermore, curcumin can reverse the inhibition of the SIRT1/NRF2 pathway in the intestinal tissues of NEC newborn rats, and inhibit TLR4 expression. In addition, curcumin can inhibit the expression of inflammatory factors in intestinal epithelial cells induced by LPS/ATP, and attenuate the LPS/ATP-induced focal death pathway in intestinal epithelial cells through the SIRT1 pathway. These results have important significance in improving the NEC disease pathogenesis, and providing a novel intervention approach for the treatment of NEC. Moreover, further investigation on other potential pathways is needed to understand the molecular mechanisms of curcumin comprehensively in improving necrotising microscopic colitis and cell pyroptosis.

Declaration of conflicting interests
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