Glutamine downregulates TLR-2 and TLR-4 expression and protects intestinal tract in preterm neonatal rats with necrotizing enterocolitis

Wei Zhou a,*, Wei Li b, Xiao-Hui Zheng b, Xiao Rong a, Long-Guang Huang a

a Department of Neonatology, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, China
b Department of Pediatrics, Dongguan Taiping People's Hospital, Dongguan, China

A R T I C L E   I N F O

Article history:
Received 14 April 2013
Received in revised form 31 January 2014
Accepted 17 February 2014

Key words:
Necrotizing enterocolitis
Glutamine
Toll-like receptor 2
Toll-like receptor 4
Cell apoptosis

A B S T R A C T

Background/Purpose: Toll-like receptor (TLR)-4 and TLR-2 play an essential role in the pathogenesis of necrotizing enterocolitis (NEC). In this study, we investigated the protective effect of glutamine (Gln) in an NEC neonatal rat model, and the potential association with TLR-4 and TLR-2 expression in local intestinal tissues.

Methods: Preterm neonatal rats were randomly divided into 3 groups: normal control; NEC model; and NEC plus Gln intervention. NEC was induced by feeding with artificial milk substitutes, plus exposure to hypoxia and cold stress. All preterm rats were sacrificed at 3 days after birth. The intestinal tissues were taken for pathological analysis. Protein and mRNA expression of TLR-2, TLR-4, and caspase-3 was examined by immunohistochemistry and real-time RT-PCR, respectively.

Results: Compared with the normal control, the NEC neonatal rats showed mucosal injury and upregulated mRNA and protein expression of TLR-2, TLR-4, and caspase-3 in ileum and colon. Gln intervention significantly reduced the mucosal injury and suppressed the upregulated expression of TLR-2, TLR-4, and caspase-3 in the ileum and colon of NEC neonatal rats.

Conclusions: Gln protects the intestinal tract of NEC neonatal rats, which may be associated with the reduction of TLR-2 and TLR-4 expression in intestines

© 2014 Elsevier Inc. All rights reserved.

Necrotizing enterocolitis (NEC) is an inflammatory bowel disease of neonates with significant morbidity and mortality in newborn preterm infants, especially in premature with birth weight < 1500 g [1,2]. Although the exact pathogenesis is not clear, preterm birth, hypoxic–ischemic events, formula feeding, and abnormal bacteria colonization are regarded as the risk factors. The combined effect of these factors can cause intestinal immune dysfunction, excessive release of inflammatory mediators, eventually leading to intestinal hemorrhage and necrosis [3,4].

Toll-like receptors (TLRs) are important innate immune recognition receptors. TLR-2 and TLR-4 are involved in intestinal mucosal immune response [5], and are overexpressed in human fetal enterocytes but their expression is downregulated one month after birth and becomes undetectable in healthy adult intestinal epithelium [6]. Both TLR-4 and TLR-2 are overexpressed during NEC in a full term neonatal rat model induced by formula feeding plus hypoxic stress [6]. TLR-4-deficient C3H/HeJ mice are protected from the development of NEC [7]. It was also reported that intestinal epithelial TLR-4 regulates goblet cell development and is required for NEC in mice, suggesting that TLR-4 plays an essential role in the development of NEC [8]. In an NEC preterm rat model, both TLR-2 and phospho-nuclear factor-kappa B (pNF-κB) are increased in ileal epithelium, which is correlated with the severity of mucosal damage. Increased apoptosis was also observed in the ileal epithelium [7]. These results suggest that both TLR-4 and TLR-2 play an important role in the pathogenesis of NEC.

Glutamine (Gln) is the most abundant free amino acid in human body. The Gln level in the blood decreased significantly when stress such as in trauma, major surgery and infection occurred, and Gln supplementation appropriately had protective effect to maintain intestinal mucosal integrity [9,10]. Gln is the main energy source to intestinal tissue. It can protect the intestine through reducing intestinal permeability, enhancing gut immunity, preventing mucosa cells from apoptosis and reducing intestinal flora displacements. Sukhotnik et al. [11] reported that oral glutamine prevented gut mucosal from endotoxemia-induced injury and improved mucosal recovery in rats. Li et al. [12] reported that Gln decreased intestinal damage through reducing levels of inflammatory mediators such as tumor necrosis factor-α (TNF-α) and reducing the intestinal inflammatory response induced by lipopolysaccharide (LPS). In a rat neonatal NEC model induced by feeding with a special rodent formula, enteral glutamine supplementation attenuated the local intestinal damage in rats with NEC [13]. Clinical studies have demonstrated that Gln reduces intestinal permeability in very-low-birth-weight infants and is beneficial to NEC/septicemia in premature neonates [14–16]. Recently, we reported that Gln significantly reduced the expression of...
TLR-4 and caspase-3, and provided protective effects on the intestines in a NEC preterm rat model induced by formula feeding plus cold and hypoxia stimulation [17]. However, it remains unknown whether Gln has any effect on TLR-2 expression in this model.

In this study, we employed an NEC neonatal rat model to investigate if TLR-2 expression could also be influenced by Gln intervention. We found that Gln reduced the expression of both TLR-2 and TLR-4, and significantly inhibited cell apoptosis and alleviated ileum injury in the rat NEC model. These results suggest that Gln can protect intestinal tract of preterm rats, which may be through reducing the expression of TLR-2 and TLR-4 and inhibiting apoptosis of intestinal mucosa.

1. Materials and Methods

1.1. Animal grouping

Ten specific-pathogen-free (SPF) pregnant Sprague–Dawley (SD) rats were purchased from the Animal Center of Guangzhou Traditional Chinese Medicine University (Guangzhou, China). The neonatal rats were obtained by caesarean section at day 21 after pregnancy and were placed in home-made incubators. Premature rats were randomly divided into NEC model group, Gln intervention group and control group (n = 20 each group).

1.2. Animal model preparation and tissue harvest

The newborn rats in model group and Gln intervention group were hand-fed 2 h after birth with formula milk (PreNAN, Nestlé, Beijing, China) [18]. Gln (Sigma, Saint Louis, MO, USA) was added to the formula milk in the Gln intervention group at the dose of 0.3 g/kg. The newborn rats were hand-fed via a home-made gastrostomy tube with 0.15 ml formula milk every 4 h and 0.20 ml formula after 24 h. After the first feeding, the premature rats were put into the hypoxia tank and were exposed to nitrogen with flow at 10 L/min. After the oxygen concentration inside the hypoxia tank shown in TED-60 T oxygen analyzer (Teledyne, City of Industry, CA) reached zero, the premature rats were kept for 90 s before turning off the nitrogen valve. The premature rats were immediately taken out and placed in the refrigerator freezer with simultaneous exposure to cold (4 °C) for 10 min. After that, the preterm rats were put into incubators. Preterm rats were stressed with anoxia and cold stimulation once in the morning and evening respectively for three days. The preterm rats in the model group and the Gln intervention group, together with the premature rats in the control group, were sacrificed at the same time. The entire small intestine was harvested for analysis.

1.3. Pathological changes in intestinal tissue

Jejunum, ileum and colon with 1 cm length were fixed in 10% formalin, and then embedded in paraffin. Coronal plane was stained with hematoxylin and eosin HE staining and the pathological changes were observed under light microscope.

1.4. Histology and injury assessment

The ileum injury was pathologically scored according to the following criteria [15]: 0 point: intact villi in intestinal mucus membrane; 1 point: slight edema of villi, and epithelial shedding only at the top of the villi; 2 points: necrosis in middle part of villi; 3: loss of villi but crypt can still be identified; 4 points: mucosal epithelium complete absence or transmural necrosis. The extent of intestinal injury was determined according to the highest score of injury observed in slices. NEC was defined when histological score was equal or greater than 2 points.

1.5. Expression of TLR-2, TLR-4 and caspase-3 protein

The protein expression of TLR-2, TLR-4 and caspase-3 was detected with immunohistochemistry. Tissue sections were deparaffinized in xylene, rehydrated in a graded series of ethanol, and then rinsed in PBS three times. After incubation with 3% H2O2 for 10 min, the sections were rinsed in distilled water three times. Next, the sections were placed in a 0.01 M sodium citrate (pH 6.0) solution and boiled for at least 5 min in a microwave oven. The sections were cooled to room temperature and blocked by incubation in 5% normal goat serum (Vector Labs, Burlingame, CA) for 20 min. The tissue sections were then incubated sequentially with the rabbit anti-rat caspase-3 monoclonal antibody (1:25 dilution) (Abcam, Cambridge, MA, USA), rabbit anti-mouse TLR-2 polyclonal antibody (1:200) (Cell Signaling, Danvers, MA, USA), rat anti-mouse TLR-4 monoclonal antibody 1:100 (Abcam, Cambridge, MA, USA) for 1 h at 37 °C. After the sections were rinsed in PBS three times, a biotinylated goat-anti-rabbit antibody (1:500; Vector Labs) was added, and the sections were incubated for 30 min at 37 °C. The unbound antibody was then removed by rinsing in PBS three times. Immuno detection was performed using DAB reagent (Dako, Glostrup, Denmark) for 2 min and hematoxylin for 2–3 min. After differentiation in acid alcohol solution, the sections were washed in water for 15 min, and then mounted using neutral resin for dehydrated and transparent treatment. PBS was used as negative control instead of primary antibody. The positive results of caspase-3, TLR-2 and TLR-4 were shown by appearing brown–yellow in cytoplasm or membrane. Five views were randomly selected in each slide under optical microscope at 400× magnification, and analyzed using Image-proPlus 6.0 Color Image Analysis System (Rockville, MD). The Average absorbance of positive expression in each view was the expression values of caspase-3, TLR-2 and TLR-4 in rat intestines.

1.6. mRNA expression of TLR-2 and TLR-4

The mRNA expression of TLR-2 and TLR-4 was measured by real-time fluorescence quantitative reverse transcription polymerase chain reaction (qRT-PCR). (1) Total RNA was extracted respectively from 50–100 mg jejunum, ileum and colon using extraction and purification kit (TaKaRa, dalian, China) according to the instruction book. The mRNA integrity was determined using agarose gel electrophoresis and micro nucleic acid protein quantitative instrument. (2) PCR primers for TLR-2, TLR-4 and β-actin were designed using primer 6.0 software and synthesized by the TaKaRa Company (Dalian, China). TLR-2 upstream primers: 5'-AGCAGATTCTTATGGTGGAAG-3', TLR-2 downstream primers: 5'-ATGATCATTGCGCCGAAC-3'; the length of PCR product is 112 bp. TLR-4 upstream primers: 5'-CCGCTCTGGGATCATCTTCA-3', TLR-4 downstream primers: 5'-CCCACCCTGAGTAAGCTTTG-3'; the length of PCR product is 107 bp. β-actin upstream primers: 5'-GGAGACTTACGTGCGCTGTCTC-3', β-actin downstream primers: 5'-ACTGACTTGACTCTGCTGCTG-3'. The length of PCR product is 150 bp. (3) CDNA was synthesized according to the instructions provided by the kit (Company, City, State, Country). (4) Fluorescence quantitative reverse transcription PCR was performed using SYBR Premix Ex TaqTM kit (TaKaRa, Dalian, China), and the volume of qRT-PCR reaction was 25 μL. The reactions were incubated in a 96-well plate at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 63 °C for 20 s and 72 °C for 45 s. (5) Primer dimer amplification was analyzed with PCR reaction melting curve, and PCR products were identified with 2% agarose gel electrophoresis. (6) To determine amplification efficiency, the cDNA was diluted according to the gradients 1:5, 1:25, 1:125, 1:625, and 1:3125. The slope of standard curve and amplification efficiency was obtained after the 6 samples of TLR-2, TLR-4 and β-actin were
analyzed with qRT-PCR. (7) β-actin was used as the endogenous reference gene, and the relative gene expression level was calculated with $2^{-\Delta \Delta Ct}$ method.

1.7. Statistical analysis

The statistical analysis was performed with SPSS13.0 software (Armonk, NY) and GraphPad Prism (La Jolla, CA). The data in the normal distribution dose were shown as mean ± standard deviation. The pathological scoring of ileum was checked with Kruskal–Wallis H. The expression difference of caspase-3, TLR-2 and TLR-4 was analyzed with analysis of variance. The correlation between the mRNA expression of TLR-2, TLR-4, caspase-3 and the scoring of pathological injury was analyzed with Pearson correlation test and Spearman’s rank correlation test. The data have statistical significance when P is lower than 0.05.

2. Results

2.1. General status

The activity and weight of premature rats in NEC model group decreased, and premature rats were slow in reaction, having abdominal distension and diarrhea with yellow green grume and bloody stool. The status of premature rats in Gln intervention group was similar to that in model group but fewer rats had bloody stool. The premature rats in control group had shown good activity and increasing weight, took food and defeated normally, and had no abdominal distension or diarrhea. The case fatality rate of premature rats in NEC model group, Gln intervention group and control group was 20%, 10% and 0%, respectively.

2.2. Pathological changes in intestinal tract tissue

The intestinal tract of premature rats in control group had good elasticity; the jejunum and ileocolon presented milky white and faint yellow respectively. The intestinal tract of premature rats in model group was enlarged, became hyperemic to different extent, and presented purple and black. Compared with premature rats in model group, the intestinal tract of premature rats in Gln intervention group expanded less and was less hyperemic.

Under light microscopy, the premature rats in control group had intact structure of ileum and colon, and intact villus, with a little damage on the top. The rats in control group showed normal arrayed enteraden, no vein expansion in lamina propria and submucosa, and no inflammatory infiltrates. The ileum and colon of premature rats in model group had villus falling off, putrescence, enteraden deficiency and even enterobrosis. Compared with model group, the ileum and colon of premature rats in Gln intervention group had less edema in muscularis mucosa and muscular layer, and had less villi falling off and putrescence (Fig. 1). The pathological injury score of ileum tissue in model group, Gln intervention group and control group was $3.10 \pm 0.99$, $2.40 \pm 0.69$ and $0.30 \pm 0.48$ respectively, with statistically significant difference ($P < 0.01$).

2.3. Localization and protein expression of caspase-3, TLR-2 and TLR-4

In the NEC model and Gln intervention groups, caspase-3 was expressed in villus and recess to different extent, while in the control group, it was not or less expressed in intestinal tract villus top (Fig. 2A–C). TLR-2 and TLR-4 in control group were not or less expressed in cell membrane and cytoplasm of villus, but TLR-2 and TLR-4 in the NEC model and Gln intervention group were expressed in cell membrane and cytoplasm of villi to different extent (Fig. 2D–F and G–I). The expression of caspase-3 in the NEC model and Gln intervention groups was higher than that in control group, and the expression of caspase-3 in the NEC model group was the highest in the three groups. The expression of TLR-2 and TLR-4 in jejunum in three groups had no statistical significance ($P > 0.05$). The expression of TLR-2 and TLR-4 in ileum and colon tissue in the NEC model and Gln intervention groups was higher than that in control group, and the expression of TLR-2 and TLR-4 in the NEC model group was the highest among the three groups. The expression of caspase-3, TLR-2 and TLR-4 in ileum and colon tissue was markedly higher than that in jejunum ($P < 0.05$). (Fig. 3).

2.4. mRNA expression of TLR-2 and TLR-4

Compared with the control group, the mRNA expression of TLR-2 and TLR-4 in ileum and colon tissue in the NEC model group and Gln intervention group markedly increased ($P < 0.01$), and the mRNA expression of TLR-2 and TLR-4 in ileum and colon tissue in Gln intervention group was lower than that in the NEC model group ($P < 0.05$) (Fig. 4). The mRNA expression of TLR-2 and TLR-4 in jejunum had no statistical significance among the three groups ($P > 0.05$). The mRNA expression of TLR-2 and TLR-4 in ileum and colon was higher than that in jejunum ($P < 0.01$).

2.5. Correlation of pathological injury in intestinal tract with the mRNA expression of TLR-2, TLR-4 and caspase-3 in ileum

In order to obtain an insight into the role of TLR-2 and TLR-4 in the pathogenesis of NEC in this model, we investigated the relationship between TLR-2/TLR-4 expression and the pathological injury in intestinal tract. The correlation analysis revealed that the mRNA expression of TLR-2 and TLR-4 had a positive correlation with the expression of caspase3, and the values of r were 0.71 and 0.90 ($P < 0.01$), respectively. The mRNA expression of TLR-2 and TLR-4 had a positive correlation with scoring of pathological injury in intestinal tract, and the values of r were 0.69 and 0.77 ($P < 0.01$), respectively. The expression of caspase3 had a positive correlation with scoring of pathological injury in intestinal tract, and the value of r was 0.81.

Fig. 1. Pathological changes in the ileum (HE × 200). (A): The structure of preterm intestinal mucosa in control group was normal; (B): ileum muscle edema, necrosis of villi, and some missing epithelial structures were observed in the premature rat in model group; (C): ileum tissue injury of preterm rat in Gln intervention group was less serious, and submucosal edema and necrosis of central villi were also less observed.
3. Discussion

In this study, we found that Gln administration reduces the expression of both TLR-2 and TLR-4, as well as caspase-3, and significantly alleviates the ileum injury in rat NEC model. These results corroborate the protective effect of Gln against intestinal injury in preterm NEC rats, which may be through reducing the expression of TLR-2 and TLR-4.

TLR-4 can recognize LPS in cell wall of Gram negative bacteria, activate NF-κB transcription factor and promote the synthesis of inflammation factors, such as TNF-α, interleukin-1 and interferon-γ [19,20]. TLR-2 not only recognizes peptidoglycan and lipoteichoic acid of Gram positive bacteria but also co-expresses with CD14, enhancing the recognition of LPS in cell wall of Gram negative bacteria [21]. In addition, TLR-2 can cooperate with TLR-4 to promote the synthesis of anti-inflammatory factor [22]. Both TLR-2 and TLR-4 are upregulated in the course of NEC [23], and the expression of TLR-2 and TLR-4 precedes the pathological injury of intestinal tract [6], suggesting that both TLR-2 and TLR-4 play an essential role in NEC. As reported by Le Mandat Schultz et al. [23], increased expression of TLR-2 and TLR-4 is associated with increased apoptosis in the intestinal tissues of NEC rat mode. The increased apoptosis in mucous membrane epithelial cells can enhance hemorrhage and necrosis in intestinal tract [24,25]. Sodhi et al. [26] reported that TLR-4 can inhibit enterocyte proliferation and aggravate hemorrhage and necrosis in intestinal tract by breaking down β-catenin pathway. Another mechanism underlying TLR-4-mediated apoptosis may be through the p53-up-regulated modulator of apoptosis (PUMA) [27]. In the present study, the expression of TLR-2 and TLR-4 in intestinal tract was markedly increased in the intestinal tissues in the NEC rat model, and the TLR-2 and TLR-4 expression levels correlate with the expression of caspase-3 and the pathological injury in intestinal tissues, suggesting that TLR-2 and TLR-4 may play an important role in the apoptosis and pathological injury in intestinal tissues in this NEC premature rat model.

The NEC model in this study was built in premature newborn rats by feeding with formula plus cold and hypoxia stimulation. The content of TLR-2 and TLR-4 in ileum and colon increased in this study, which is consistent with the formula–hypoxia–reoxygenation NEC rat model [23]. The increased expression of TLR-2 and TLR-4 in this model may be due to: (1) Formula feeding. As reported by Liu et al. [6], feeding with formula milk can increase the content of TLR-2 and TLR-4 in intestinal tissues of neonatal rats [6,28]; (2) Hypoxia. It has been reported that hypoxia can activate the differentiation of TLRs and increase the content of TLR-2 and TLR-4, causing a large number of cell apoptosis [29]. In addition, hypoxia can induce intestinal tract circulation disorder so that the colonizing bacteria increased. The colonizing bacteria having no pathogenicity in mature intestinal tract may be pathogenic bacteria in immature intestinal tract, activating TLRs pathway [30,31].

In this study, we demonstrated that Gln intervention confers significant protection against intestinal injury and apoptosis in this NEC premature rat model. As an abundant amino acid in human, Gln provides a major source of energy for intestinal tract. Gln not only maintains the integrity of intestinal tract, reducing the infectious morbidity and mortality in seriously ill patients, but also protects intestinal tract by reducing cell apoptosis in intestinal mucous membrane [32]. Kessel et al. [33] demonstrated that Gln can decrease the expression of TLR-4 in rats with endotoxemia, with reduced epithelial cell apoptosis in intestinal mucous membrane and alleviated intestinal tract injury. Larson et al. [34] reported that Gln reduces the expression of caspase-3 and the degradation of DNA, leading to reduced apoptosis in rat intestinal epithelial (RIE-1) cell lines.

![Protein expression of caspase3, TLR-2, and TLR-4 in ileum (SP × 400). The caspase-3 expression was not or less expressed in control group (A) but significantly increased in model group (B). In model group, the expression of caspase-3 was located in hair and even the whole crypt. Compared with model group, the expression of caspase-3 in the Gln intervention group decreased, and caspase-3 was mostly expressed in the top and middle of villi, and less expressed in the crypt (C). TLR-2 was expressed only in cell membrane of intact villi in control group (D). The expression of TLR-2 in model group significantly increased, and TRL-2 was mostly expressed in the cell membrane and cytoplasm of seriously damaged villi (E). The expression of TLR-2 in the Gln intervention group was lower than that in model group (F). TLR-4 was only expressed in the cell membrane of intact villi in control group (G). The expression of TLR-4 significantly increased in model group, and TLR-4 was mostly expressed in the cell membrane and cytoplasm of seriously damaged villi (H). The expression of TLR-4 in the Gln intervention group was lower than that in model group (I).](image-url)
Fig. 3. Protein expression of caspase-3, TLR-2 and TLR-4 in intestinal tissue of preterm rats. Five views in each slide under optical microscope at 400× magnification were analyzed using Image-proPlus 6.0 Color Image Analysis System (Rockville, MD). The Average absorbance of positive expression in each view represents the expression of caspase-3 (A), TLR-2 (B) and TLR-4 (C) in rat intestines. NS, *P > 0.05, **P < 0.05, ***P < 0.01.

Fig. 4. mRNA expression of TLR-4 and TLR-2 in different intestinal segments. (A): TLR-4 expression in jejunum, ileum, and colon from control rats, NEC model, and Gln intervention group. (B): TLR-2 expression in jejunum, ileum, and colon from control rats, NEC model, and Gln intervention group. **P < 0.05, ***P < 0.01.
Our results showed that Gln administration down-regulated TLR-2, TLR-4 and caspase-3 in intestinal tract, and alleviated intestinal tract injury. These effects may be mediated by heat shock protein 70 (HSP70). HSP70 is involved in basic mechanisms for cellular protection, and higher levels of serum HSP70 correlate with improved survival in patients following severe trauma [35]. Recently, it was reported that HSP70 has potent efficacy against NEC [36]. Gln administration can increase HSP70 expression in a mouse model of sepsis-induced kidney injury [37], and Gln’s protection against sepsis and lung injury is dependent on HSP70 expression [38]. It was also reported that HSP70 downregulates TLR-4 and suppresses the activation of TLR-4 signaling pathway [39]. Therefore, it is reasonable to speculate that Gln may attenuate NEC through the upregulation of HSP70, thereby suppressing TLR-4 expression and signaling activation, leading to the protection against NEC.

Another possible mechanism by which Gln protects premature rats from NEC may be through the modulation of autophagy in enterocytes. It was recently reported that TLR-4 can induce autophagy in enterocytes, which plays a critical role in the pathogenesis of NEC [40]. Gln can activate mammalian TORC1 (mTORC1) by enhancing glutaminolysis and alpha-ketoglutarate production, which blocks autophagy [41]. It was also reported that HSP70 attenuates heat-stimulated cell autophagy, although in cardiomyocytes [42]. Because TLR-4-induced autophagy in enterocytes plays a critical role in the pathogenesis of NEC [40], it appears that the protective effect of Gln on NEC could be through either direct activation of mTORC1, or upregulation of HSP70, both of which lead to the reduction of TLR-4-induced autophagy, thus alleviating NEC.

Gln also decreased specificity protein 3 (Sp3), a highly conserved zinc-finger transcription factor [43], in intestinal epithelial IEC-6 cells [44]. This effect, as well as Gln-mediated inhibition of apoptosis, was both abolished by siRNA silencing of Sp3 gene [44], suggesting that Sp3 plays a critical role in the inhibitory effect of Gln on apoptosis. This could be another mechanism underlying protective effect of Gln on NEC.

Regarding the potential signal transduction pathways that are responsible for the protective effects of Gln in intestinal cells, Larson et al. [34] reported that extracellular signal-related kinase (ERK) signaling pathway plays a critical role in Gln-mediated inhibition of apoptosis in intestinal cells. Recently, it was reported that epidermal growth factor receptor expression and signaling are essential in Gln's cytoprotective mechanism in heat-stressed intestinal epithelial-6 cells [45]. Besides, Gln can activate Akt and JNK signaling pathways [46], which may potentially influence TLR-4 and TLR-2 expression.

As demonstrated by this study and others [6,23], the expression of both TLR-2 and TLR-4 is upregulated in the intestines of NEC rats. The role of TLR-2 in NEC has not been well characterized. It was reported that TLR-2 signaling induced by Lactobacillus lactis provides protective signaling in models of inflammatory colitis [47], and an increased injury score was found in an intestinal ischemia model of C57BL/6 TLR-2-deficient mice [48], suggesting that TLR-2 may provide protective signaling in NEC. The mechanism underlying the upregulation of TLR-2 expression in NEC may be through the upregulation of TLR-2, as TLR-2 signaling can induce TLR-2 expression, which may be via NrfB [49,50]. Also, the TLR-4 ligand LPS can stimulate the expression of TLR-2 expression [51]. In this study, we have observed that Gln downregulates both TLR-2 and TLR-4. It is possible that the downregulation of TLR-2 may be secondary to the downregulation of TLR-4 by Gln, which needs further investigation. It is also noteworthy that Gln only partially downregulates the expression of TLR-2, TLR-4 and caspase-3, as well as the histological grade of injury in NEC rat model, suggesting that other mechanisms may also exist that underlie the beneficial effects of Gln on NEC.

In summary, Gln administration confers protection against intestinal injury in NEC premature rat model, and inhibits the upregulated intestinal TLR-2, TLR-4 and caspase-3 expression. These results help to obtain mechanistic insights into the antiapoptotic effects of Gln in the intestine, and provide basis for clinical management of this disease with Gln.

Funding
This study was supported by grants from the Guangdong Natural Science Fund Committee (No. 8151012000100002).

Ethical approval
The study was approved by the hospital's Ethics Committee.

Competing interest
No benefits in any form have been received or will be received from any commercial party related directly or indirectly to the subject of this article.

Contribution
Zhou W proposed the study and wrote the first draft. All authors contributed to the design and interpretation of the study and to further drafts. Zhou W is the guarantor.

References
Kessel A, Toubi E, Pavlotzky E, et al. Treatment with glutamine is associated with

Beutler B. Inferences, questions and possibilities in Toll-like receptor signaling.

Auestad N, Korsak RA, Bergstrom JD, et al. Milk-substitutes comparable to rat's


Hirata N, Yanagawa Y, Ebihara T, et al. Selective synergy in anti-inflammatory
cytokine production upon cooperated signaling with TLR4 and TLR2 in murine


and PNF-kappaB in a neonatal rat model of necrotizing enterocolitis. PLoS ONE
2007;2:e1102.

recognition complex in the human healthy and inflamed premature and adult gut.
Inflamm Bowel Dis 2010;16:68–75.

necrosis in an experimental rat model of neonatal necrotizing enterocolitis.

Sodhi CP, Shi XH, Richardson WM, et al. Toll-like receptor-4 inhibits enterocyte
proliferation via impaired beta-catenin signaling in necrotizing enterocolitis.

LeBouder E, Rey-Nores JE, Raby AC, et al. Modulation of neonatal microbial
recognition: TLR-mediated innate immune responses are specifically and

Mkaddem SB, Bens M, Vandewalle A, et al. Differential activation of Toll-like

Nanthakumar NN, Fusunyan RD, Sanderson I, et al. Inflammation in the developing
human intestine: a possible pathophysiological contribution to necrotizing

Claud EC, Lu L, Anton PM, et al. Developmentally regulated IkappaBalpha
expression in intestinal epithelium and susceptibility to flagellin-induced inflammation. Proc

Conejero R, Bonet A, Grau T, et al. Effect of a glutamine enriched enteral diet on
intestinal permeability and infectious morbidity at 28 days in critically ill patients
with systemic inflammatory response syndrome: a randomized, single blind,

Kessel A, Toubl E, Pavlovtzky E, et al. Treatment with glutamine is associated with
down-regulation of Toll-like receptor-4 and myeloid differentiation factor 88
expression and decrease in intestinal mucosal injury caused by lipopolysaccharide

Larson SD, Li J, Chung DH, et al. Molecular mechanisms contributing to glutamine-
mediated intestinal cell survival. Am J Physiol Gastrointest Liver Physiol 2007;293:
G1262–71.

Pittet JF, Lee H, Morabito D, et al. Serum levels of Hsp 72 measured early after

a neonatal rat model of early necrotizing enterocolitis. Neonatol 2013;103:
1–6.

inhibition of high mobility group box protein-1 expression during sepsis. Br J Nutr
2010;103:890–8.

Singleton KD, Wischmeyer PE. Glutamine’s protection against sepsis and lung
injury is dependent on heat shock protein 70 expression. Am J Physiol Regul Integr

regulates TLR4 signaling in the newborn intestinal epithelium. J Immunol
2012;188:4543–57.

in the regulation of enterocyte migration and the pathogenesis of necrotizing

Durao RV, Oppliger W, Robizaille AM, et al. Glutaminolysis activates Rag-mTORC1

Hsu SF, Chao CM, Huang WT, et al. Attenuating heat-induced cellular autophagy,
apoptosis and damage in HK2 cardiomyocytes by pre-inducing HSP70 with heat

Philipsen S, Suske G, A tale of three fingers: the family of mammalian Sp/XKLF

Ban K, Kozar RA. Glutamine protects against apoptosis via downregulation of Sp3
in intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 2010;295:
G1344–53.

Niederlechner S, Baird C, Petrie B. Epidermal growth factor receptor expression
and signaling are essential in glutamine’s cytoprotective mechanism in heat-
stressed intestinal epithelial-6 cells. Am J Physiol Gastrointest Liver Physiol

Rhoads M. Glutamine signaling in intestinal cells. JPEN J Parenter Enteral Nutr

Aprahamian CJ, Lorenz RG, Harmon CM, et al. Toll-like receptor 2 is protective of
ischemia-reperfusion-mediated small-bowel injury in a murine model. Pediatr

Foligné B, Nutten S, Steeld L, et al. Recommendations for improved use of the
murine TNBS-induced colitis model in evaluating anti-inflammatory properties of
lactic acid bacteria: technical and microbiological aspects. Dig Dis Sci 2006;
51:390–400.

Fan J, Frey RS, Malik AB. TLR4 signaling induces TLR2 expression in endothelial

Musikacharoen T, Matsuguchi T, Kikuchi T, et al. NF-kappa B and STATs play
important roles in the regulation of mouse Toll-like receptor 2 gene expression.

expression in macrophage response to peptidoglycan and high concentration of
lipopolysaccharide is involved in NF-kappa B activation. Infect Immun 2001;
69:2788–96.

expression in macrophage response to peptidoglycan and high concentration of
lipopolysaccharide is involved in NF-kappa B activation. Infect Immun 2001;
69:2788–96.