

The response of Toll-like receptors to glutamine in neonatal rat intestine

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Abstract

Background: There are several barrier systems in the newborn gastrointestinal tract and one of the most important among these are the Toll-like receptors (TLRs). The aim of this study was to examine the effect of glutamine on Toll-like receptors 2 (TLR-2), TLR-4, and TLR-9 levels in the newborn gut.

Material and methods: Rats were randomly assigned to three experimental groups. Group I (control at day 0) (n = 9), group II (control at day 10) (n = 9): oral distilled water; group III (study group) (n = 9): oral glutamine (200 mg/kg/day). After 10 days, the ileojejunal segment was removed for biochemical analyses of TLR-2, -4 and -9.

Results: There was a significant difference between groups for the levels of TLRs. The level of TLR-9 was higher in group I than group II and in group III than group II. There was no statistically significant difference between group I and group III in terms of TLR-9 levels. There were no statistically significant differences between groups for the levels of TLR-2 and TLR-4.

Conclusions: Glutamine administration, by increasing the level of TLR-9, may prevent the increase in the level of TLR-4 in newborn rat intestine. Thus, it may play a protective role in the intestine and reduce the susceptibility to necrotizing enterocolitis (NEC), which is associated with the intensity of TLR-4 expression.

Key words: Toll-like receptor, glutamine, intestine, newborn rat.

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Introduction

In the newborn gastrointestinal tract, there are up to 1×10^9 bacterial cells/gr of colonic content at the end of the first week of life [1]. Balanced intestinal microflora have a direct impact on maintaining gut homeostasis, such as by processing nutritional factors and by inducing immune responses [2]. Microbiota also provides a continuous source of antigens and toxins capable of activating the host immune system [3, 4]. A dysregulated immune response to bacterial derivate molecules can result in excessive mucosal inflammation [5]. The recognition of microbial antigens by the newborn host may be accomplished through two branches of the immune system: the innate immune system, which consists of cells and their receptors; and the adaptive

immune system, which requires prior exposure to antigenic stimuli and the release of antibodies by lymphocytes [6].

Recent work has shed light on the important and exciting role of innate immune receptors of the intestinal epithelium in the development of necrotizing enterocolitis. These receptors, called Toll-like receptors (TLRs), detect unique molecular sequences on bacteria and other potential pathogens [7, 8]. Ten individual TLRs have been identified in humans, termed TLR-1–TLR-10 [9]. Experimental animal data clearly demonstrate that the TLR pathway has a critical role in limiting mucosal injury from enteral toxins by aiding reparative tissue regeneration and promoting clearance of pathogenic bacteria [10–12]. In the newborn infant, mucosal TLR signaling regulates intestinal responses to post-

natal colonization, aids development of tolerance to commensal bacteria, and interacts with components of breast milk to maintain the integrity of the intestinal barrier [13-15].

In this study, we examined the effect of glutamine, an important precursor for nucleotide synthesis and glutathione for enterocytes, on the expression of TLR-2, TLR-4, and TLR-9 in a rat model of breast-fed infant gut.

Material and methods

Animal model

Ethical approval was obtained from the experimental animal ethics committee of the Gulhane Military Faculty of Medicine (Ankara, Turkey). Three time-mated Sprague-Dawley pregnant rats obtained from the Gulhane Military Medical Faculty Experimental Research Unit were delivered spontaneously. Three groups were allocated from 27 pups as follows: group I (control at day 0, $n = 9$); group II (control at day 10, $n = 9$); group III (glutamine, $n = 9$). The rat pups were housed with their mothers in the same cage and maintained at room temperature with a natural day and night cycle with *ad libitum* access to standard rat chow tap water. Baby rats were randomly selected and marked on post-natal day 1 and divided equally into 3 groups. Group I was sacrificed immediately after birth. Starting from day 1 until day 10, group II received breast milk and distilled water; group III received breast milk and 200 mg/kg/day glutamine. Glutamine supplement was available in capsule form (GNC, Pittsburgh, USA), which was dissolved in distilled water and fed to the rat pups through a 24-gauge angiocath with the help of an injector.

Tissue preparation

Baby rat pups were sacrificed by cervical dislocation. The ileojejunal part of the intestine was removed and washed with 0.9% NaCl to remove residual blood, put into tubes, frozen in liquid nitrogen, and stored at -70°C for biochemical analysis immediately after birth from group I and after a 10-day period from group II and III.

Biochemical analysis

Homogenate preparation

The frozen tissues were homogenized in phosphate buffer solution (pH: 7.4) by means of a homogenizer (Heidolph DiAx 900; Heidolph Elektro GmbH, Kelheim, Germany) on ice. Homogenates were centrifuged at 14,000 rpm at 4°C for 10 min. The supernatants were used for the entire assays. The protein content of tissue homogenates was measured by the method of Lowry [16].

Measurements of rat Toll-like receptor 2 (TLR-2), 4 (TLR-4) and 9 (TLR-9)

We used enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Cusabio

Biotech Co., Ltd., Hubei Province, P.R. China) to determine the tissue TLR-2, TLR-4, and TLR-9 concentrations. In brief, the microtiter plate was pre-coated with an antibody specific to TLR-2, TLR-4, or TLR-9. Standards or samples were added to the appropriate microtiter plate wells with biotin-conjugated antibodies specific for TLR-2, TLR-4, and TLR-9. Subsequently, avidin conjugated to horseradish peroxidase was added to each microplate well and incubated. TMB (3,3',5,5'-tetramethylbenzidine) substrate solution was then added to each well. Only those wells containing TLR-2, -4, and -9, biotin-conjugated antibody and enzyme-conjugated avidin exhibited a change in color. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color change measured spectrophotometrically at a wavelength of 450 ± 2 nm. The concentrations of TLR-2, -4, and -9 in the samples were then determined by comparing the O.D. of the samples to the standard curves. The intra-assay and inter-assay coefficients of variation for all assays were $< 8\%$ and $< 10\%$, respectively. We measured all samples in duplicate. Tissue TLR-2, TLR-4 and TLR-9 concentrations were expressed as pg/mg protein, ng/mg protein and ng/mg protein, respectively.

Statistical analysis

SPSS for Windows version 15.0 (SPSS, Chicago, IL) was used for statistical analyses. $P < 0.05$ was accepted as indicating a statistically significant difference. A Kruskal-Wallis test was used for testing differences and a Mann-Whitney *U*-test was used for comparing groups with each other and with the control group to determine the source of the difference among study groups.

Results

Figure 1 summarizes TLR-2, TLR-4, and TLR-9 levels in homogenates of intestinal tissue taken from all of the groups. There was a significant difference between groups for TLR-9 level ($p < 0.05$). The level of TLR-9 were significantly higher in group I than group II ($p = 0.002$). There was also a significant difference between group II and group III ($p < 0.001$). The levels of TLR-9 were higher in group III than group II. There was no statistically significant difference between group I and group III in terms of TLR-9 level ($p > 0.05$). There was also no significant difference between groups for TLR-2 and TLR-4 levels ($p > 0.05$) (Table 1).

Discussion

In this study, we evaluated the alteration and the effect of oral glutamine on TLR-2, TLR-4, and TLR-9 levels in newborn rat intestine in the first ten days of postnatal life. There was no significant difference between study and control groups for TLR-2 and TLR-4 levels, whereas TLR-9

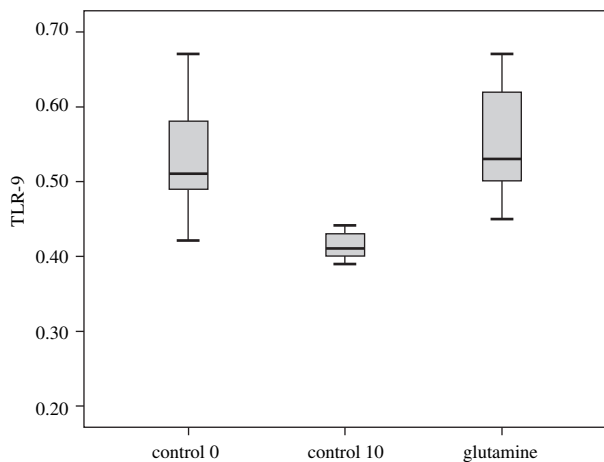


Fig. 1. Toll-like receptor 9 level according to groups

level in group II was significantly lower than group I and III. The level of TLR-9 did not decrease with time and remained high during the study period in group III.

The intestine at birth is thought to be sterile. Bacteria come from the outer environment and intestinal microbiome forms during the neonatal period [17]. Tolerance of the intestinal mucosal immune system may build up through commensal intestinal bacterial stimulation of TLRs [18]. Toll-like receptors are transmembrane components of the innate immune system’s pattern recognition receptor family [8]. Intestinal TLRs preserve homeostasis by balancing the proinflammatory and anti-inflammatory immune responses to both commensal and pathogenic microbiota through recognition of bacterial products. The pathogenesis of NEC may arise from the dysregulation of this immune response [19].

The pathophysiology of NEC in premature infants is multifactorial and complex and comprises a history of a complicated early neonatal course, poor intrauterine environment and perinatal transition. The key factors that have been implicated in NEC include intestinal hypoperfusion, abnormal bacterial colonization, a dysregulated immune response, and feeding [20-23].

It has been reported that TLR-4 expression is increased in the intestinal mucosa of mice, rats, and humans with NEC and that this TLR-4 expression in enterocytes may lead to an increase in death of the cells that line the intestine through the process of apoptosis [24-26]. These findings show that TLR-4 expression may lead to the development of intense and harmful effects in promoting intestinal injury and decreasing the ability of mucosal repair in the newborn small intestine.

As we know from previous studies, oral glutamine has many positive effects on the newborn intestine and is implicated in maintaining the functional integrity of the gut [27-29]. Furthermore, it serves as fuel for enterocytes and other proliferating cells, is involved in nucleotide and glutathione synthesis, provides nitrogen for the synthesis of amino sugars, is necessary for the maintenance of tight junctions, and stimulates crypt proliferation in the human ileum [28, 30-32]. Low plasma glutamine concentrations have been reported to be associated with a higher incidence of NEC [33].

Recent evidence has shown that TLR-9, a homologue of TLR-4 and a receptor of bacterial DNA, may prevent exaggerated TLR-4 signaling [7, 34]. Toll-like receptor 9 is the receptor for bacterial DNA rich in CpG groups in enterocytes and in case of activation may lead to a decrease in TLR-4 signaling that is manifested by reduced cytokine production and decreased apoptosis. In fact, these findings may explain the protective effects of probiotics on NEC [34]. That is to say that these probiotics rich in bacterial DNA are expected to reduce TLR-4 signaling and the severity of NEC via activation of TLR-9 [35]. A recently published study has shown that glutamine supplementation had a beneficial effect on intestinal integrity and reduced the incidence of NEC and septicemia [27]. In another experimental study, it was shown that oral glutamine, by reducing TLR-4 expression, may be protective against NEC [36]. In the present study, a significant increase in TLR-9 levels was noticed in the glutamine supplemented group as compared to group II. We propose that oral glutamine supplementation may lead to a reduction in TLR-4 level by increasing TLR-9 level, which may be protective for the preterm gut.

The other aim of this study was to determine whether glutamine has any effect on the TLR-2, and TLR-4 levels,

Table 1. Comparison of the levels of Toll-like receptors between groups

Groups	Group I	Group II	Group III	p
TLR-2	17.28	16.70	16.52	NS
(pg/mg protein)	16.04; 28.98	11.62; 19.39	15.73; 21.26	
TLR-4	0.15	0.15	0.15	NS
(ng/mg protein)	0.00; 0.90	0.00; 0.88	0.00; 0.89	
TLR-9	0.51	0.41	0.53	0.002* < 0.05
(ng/mg protein)	0.42; 0.67	0.27; 0.50	0.45; 0.67	NS** < 0.001***

All data are expressed as median, minimum and maximum. P < 0.05 are considered as significant. NS – not significant. *comparison of group *1-2; **1-3; ***2-3

the receptors for bacterial ligands associated with gram-positive and gram-negative bacteria, respectively. Toll-like receptor 2 recognizes numerous microbial components of gram-positive bacteria such as peptidoglycan and lipoproteins, despite the fact that TLR-4 is required for lipopolysaccharide responsiveness, which is the outer component of the membrane of gram negative bacteria. Although it has been shown that oral glutamine supplementation has beneficial effects on the newborn intestine, there was no change in TLR-2, and TLR-4 levels during the study period [37-39].

In conclusion, glutamine in the neonatal intestine may play an important and protective role in the pathogenesis of NEC by increasing TLR-9 level in immature preterm intestine via reduction of TLR-4 level. Further studies aimed at evaluating the effects of glutamine on TLRs in a NEC model may shed more light on this issue and contribute to a better understanding of the pathogenesis of NEC.

The authors declare no conflict of interest.

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