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Effect of Human Breast Milk on the Expression of Proinflammatory Cytokines in Caco-2 Cells after Hypoxia/Re-Oxygenation

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ABSTRACT

Background: Neonatal necrotizing enterocolitis is a common and often fatal gastrointestinal disease, especially in premature infants. To study potential mechanisms underlying the protective effect of breast milk on neonatal necrotizing enterocolitis, we induced intestinal inflammation in a Caco-2 cell model of neonatal necrotizing enterocolitis by hypoxia/re-oxygenation to investigate whether breast milk supernatant fluid inhibited the expression of proinflammatory cytokines interleukin-1β, interleukin-6, and tumor necrosis factor-α. **Methods:** Caco-2 cells were divided into normal (control) and neonatal necrotizing enterocolitis groups. Neonatal necrotizing enterocolitis was mimicked by exposing Caco-2 cells to hypoxia/re-oxygenation. Cells were independently maintained in minimal essential medium alone, minimal essential medium containing 5% breast milk supernatant, or 5% boiled breast milk supernatant. Production of interleukin-1β, interleukin-6, and tumor necrosis factor-α was investigated in cell culture supernatants by ELISA, reverse transcription polymerase chain reaction, and immunofluorescence. **Results:** Hypoxia/re-oxygenation significantly increased the expression of interleukin-1β, interleukin-6, and tumor necrosis factor-α. In the normal group, breast milk supernatant and boiled breast milk supernatant markedly downregulated the expression of interleukin-1β, interleukin-6, and tumor necrosis factor-α when compared with the minimal essential medium group, with the reduction in interleukin-1β expression being more pronounced in the breast milk group. In Caco-2 cells undergoing hypoxia/re-oxygenation, both breast milk supernatant and boiled breast milk supernatant significantly reduced the expression of interleukin-1β, interleukin-6, and tumor necrosis factor-α, where the decrease in interleukin-1β expression was greater in the breast milk group. **Conclusions:** Breast milk supernatant fluid inhibited the expression of proinflammatory cytokines interleukin-1β, interleukin-6, and tumor necrosis factor-α in Caco-2 cells, especially after hypoxia/re-oxygenation. This may be one of the mechanisms underlying the protective effect of breast milk on neonatal necrotizing enterocolitis. (REV INVES CLIN. 2016;68:105-11)

Key words: Necrotizing enterocolitis. Human milk. Interleukin-1. Interleukin-6. Tumor necrosis factor-α.

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INTRODUCTION

Necrotizing enterocolitis (NEC) is a severe gastrointestinal disease of neonates, especially premature infants, with a poor prognosis and a mortality of 10-50% in China[1,2]. Apoptosis is the main type of epithelial cell death and a necessary stage in the progression of NEC[3,4]. Cytokines including tumor necrosis factor-α (TNF-α), lipopolysaccharide-binding protein, and platelet-activating factor play important roles in the pathogenesis of NEC[5,6]. A number of studies have demonstrated that multiple cytokines and the signaling pathway mediated by them play important roles in systemic inflammatory responses in children with NEC. Most of these studies focused on proinflammatory cytokines, including interleukin (IL)-1, IL-6, TNF-α, and interferon (IFN)-γ, while few studies were about protective cytokines such as tumor growth factor (TGF)-β and IL-10. Ischemia/reperfusion injury of the intestine, a common cause of intestinal mucosal barrier function injury, has been proved to activate endothelial cells to cause an imbalance of the local cytokine network, with overexpression of some cytokines and adhesion molecules, resulting in tissue injury mediated by white blood cells[7].

Nuclear transcription factor-κB (NF-κB) is one of the hubs of proinflammatory cytokines gene expression[8], including IL-1, IL-6, TNF-α, and INF-γ. One study[9] showed that activated NF-κB could enhance gene expressions of TNF-α and IL-6, increase TNF-α and IL-6 release, and thus upregulate the inflammatory response. In addition, TNF-α and IL-6 could activate NF-κB in a feed-back way and further amplify primary inflammatory signals, causing a cascade of inflammatory responses. A moderate intervention of NF-κB could have positive effects on the clinical therapy of ischemic injury of the bowel mucosa and provide a new direction for research on the prevention of hypoxic/ischemic bowel mucosa injuries.

Although great progress has been made in elucidating the potential mechanisms underlying the pathogenesis of NEC, its molecular biology has not been fully understood and thus effective treatments have not been developed[8,9].

It has been reported that the incidence of NEC in formula-fed babies is higher than that in infants exclusively fed breast milk. The basic mechanism for this protective role of human milk, however, has not been extensively investigated. In the present study, in an *in vitro* model of NEC, Caco-2 cells underwent hypoxia/re-oxygenation and were then treated with breast milk[10]. Expression of proinflammatory factors was detected in these cells to explore the potential mechanism underlying the protective effect of breast milk on NEC.

MATERIALS AND METHODS

Breast milk samples

Colostrum was collected from 12 mothers at 2-4 days after normal deliveries at the Department of Obstetrics in the Combined Traditional Chinese and Western Medicine Hospital in Jiangsu Province, from March to June 2013. Approximately 15-20 ml of colostrum was collected from each mother, stored at 4 °C, and centrifuged within 24 hours at 12,000 rpm/min for 10 minutes to remove fat and cell fragments. Milk supernatants were randomly pooled and assigned to one of two groups of six samples each. One group was stored (un-boiled) at 4 °C for further use, and the other was boiled for five minutes to inactivate the proteins or peptides (boiled). The boiled supernatant was centrifuged for 10 minutes at 12,000 rpm/min and the inactivated milk supernatant was collected. Both un-boiled and boiled milk supernatants were stored at −20 °C for further use.

Culture of Caco-2 cells

Caco-2 cells (Shanghai Cell Bank of the Chinese Academy of Sciences) were cultured in a 75 cm^2 dish and maintained at 37 °C in a humidified environment with 5% CO_2 in a medium containing 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, and 1% penicillin-streptomycin; cells were untreated (control) or treated with boiled or un-boiled milk supernatant. Passaging was performed at a ratio of 1:3 once every five days, reaching cell fusion seven days later. When cell confluence reached approximately 80%, 6-7 days later, cells were harvested and processed for further analysis.

Hypoxia/re-oxygenation of Caco-2 cells

Six days after passaging, 90% of medium was removed to minimize the gas-diffusion distance, leaving

Table 1. Concentration of proinflammatory cytokines in the supernatant of the control and hypoxia/re-oxygenation Caco-2 cell groups

Group		IL-1β	IL-6	TNF-α
Normal	MEM	146.87 ± 7.95	126.37 ± 1.38	938.46 ± 180.80
	BM	65.45 ± 3.83	99.27 ± 6.07	694.29 ± 49.58
	BMb	76.38 ± 2.59	111.48 ± 10.22	704.51 ± 74.31
Hypoxia/re-oxygenation	MEM	277.38 ± 11.56	363.87 ± 43.42	3,918.01 ± 761.54
	BM	117.30 ± 23.16	197.21 ± 6.59	1,751.92 ± 6.95
	BMb	167.43 ± 28.53	230.46 ± 24.47	1,896.24 ± 86.55

IL: interleukin; TNF-α: tumor-necrosis factor α; MEM: minimal essential medium; BM: 5% breast milk supernatant; BMb: 5% boiled breast milk supernatant.

the remaining medium to keep cells alive. Hypoxia/re-oxygenation was induced as follows: cells were placed in a closed container connected to a negative-pressure generator and flushed with high purity nitrogen, followed by incubation for another 90 minutes (hypoxia). Then the medium was added to reach the original volume and cells were grown for another 30 minutes (re-oxygenation)[11].

Cell groups

Caco-2 cells were seeded into a six-well plate at a density of 10^5 cells per well. Cells were divided into two groups: normal group and hypoxia/re-oxygenation group; cells were further subdivided into three subgroups: (i) minimal essential medium (MEM) group; (ii) 5% breast milk supernatant fluid (BM); (iii) 5% boiled breast milk supernatant fluid (BMb). The contents of proinflammatory factors (IL-1β, IL-6, TNF-α) secreted by Caco-2 cells were determined by ELISA in culture supernatants in the six subgroups after a six-hour culture.

Detection of nuclear factor-κB p65 messenger RNA expression by reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was performed following the manufacturer's instructions (Reverse Transcription System, MBI Fermentas Co., USA), and repeated three times. The PCR primers were as follows: (i) GAPDH primers: F:5'-ACCACAGTCCATGCCATCAC-3', R:5'-TCCACCACCCTGTTGCTGTA-3'; (ii) NF-κB p56 primers: F:5'-AGGCTCCTGTGCGTGTCTCC-3', R:5'-GGGTGGGCTTGGGGGCAGGT-3'.

Detection of nuclear factor-κB p65 messenger RNA expression by immunofluorescence

Cells in the logarithmic phase were inoculated in a 24-well plate, with a density of 70-80% per well. Cells were fixed with 4% iced paraformaldehyde for 20 minutes, washed with phosphate buffered saline (PBS) for 15 minutes, and incubated with 0.1% Triton X-100 at room temperature for 10 minutes. The first antibody (NF-κB p65 antibody, CST Ltd., Chicago, IL, USA) was incubated overnight at 4 °C, and the second antibody, labeled with Texas red, was incubated for one hour. After washing with PBS, cells were sealed with 3% bovine serum albumin for one hour. The slides were dyed with the fluorescent stain DAPI and sealed; cells were observed under fluorescence microscope and photographed.

Statistical analysis

Statistical analysis was performed using SPSS version 17.0. Quantitative data were expressed as means ± standard deviation ($\bar{X}$ ± S); one-way analysis of variance was performed for comparisons between groups. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Concentration of proinflammatory cytokines in Caco-2 cells

When compared with the normal group, the concentration of IL-1β, IL-6, and TNF-α markedly increased in the hypoxia/re-oxygenation group (t = 16.112,

Figure 1. Interleukin-1β concentration in Caco-2 cells. Milk supernatant inhibited the expression of proinflammatory cytokine IL-1β in Caco-2 cells, especially the un-boiled supernatant, which induced a more potent inhibition of IL-1β compared to boiled supernatant. *Compared with the normal group, the concentration of IL-1β markedly increased in hypoxia/re-oxygenation group (t = 16.112, p= 0.000). #In Caco-2 cells undergoing hypoxia/re-oxygenation, the concentration of IL-1β markedly decreased in the BM group (p = 0.000) and BMb group (p = 0.001) compared with the control (MEM) group. ##The decrease in IL-1β in the BM group was significantly greater than in the BMb group (p = 0.033).
IL: interleukin; MEM: minimal essential medium; BM: 5% breast milk supernatant; BMb: 5% boiled breast milk supernatant.

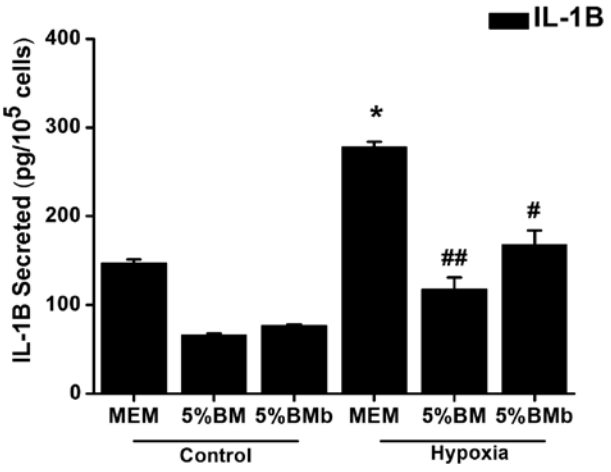


Figure 2. Interleukin-6 content of Caco-2 cells. Milk supernatant inhibited the expression of proinflammatory cytokine IL-6 in Caco-2 cells. *Compared with the normal group, the concentration of IL-6 significantly increased in the hypoxia/re-oxygenation group (t = 9.468, p = 0.001). #In Caco-2 cells undergoing hypoxia/re-oxygenation, the concentration of IL-6 significantly decreased in the BM (p = 0.000) and BMb (p = 0.001) groups compared with the control (MEM) group.
IL: interleukin; MEM: minimal essential medium; BM: 5% breast milk supernatant; BMb: 5% boiled breast milk supernatant.

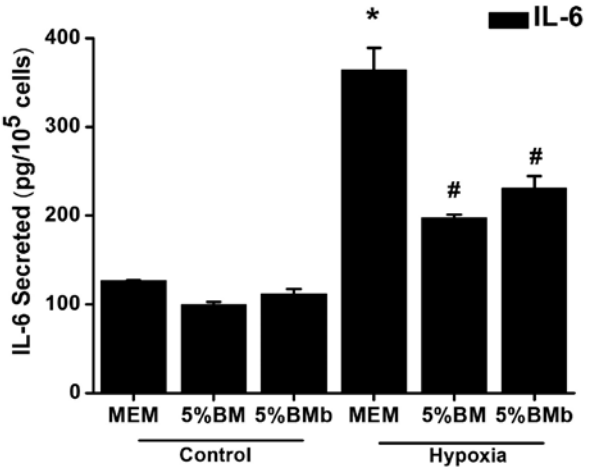
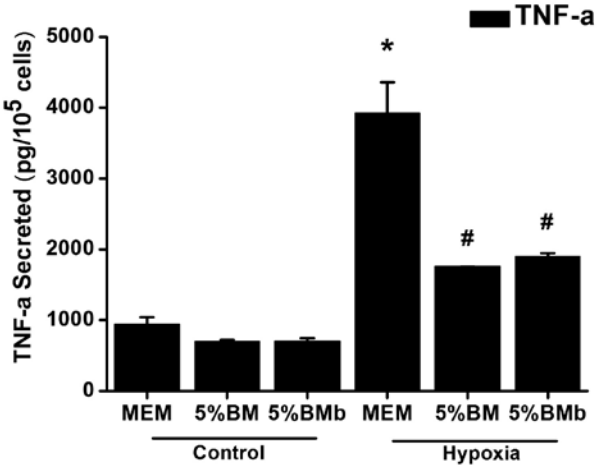


Figure 3. Tumor necrosis factor-α content of Caco-2 cells. Milk supernatant inhibited the expression of proinflammatory cytokine TNF-α in Caco-2 cells. *Compared with the normal group, the concentration of TNF-α significantly increased in the hypoxia/re-oxygenation group (t = 6.593, p = 0.003). #In Caco-2 cells undergoing hypoxia/re-oxygenation, the concentration of TNF-α significantly decreased in the BM (p = 0.001) and BMb (p = 0.001) groups compared with the control group.
TNF: tumor necrosis factor; BM: 5% breast milk supernatant; BMb: 5% boiled breast milk supernatant.



p = 0.000; t = 9.468, p = 0.001; t = 6.593, p = 0.003, respectively) (Table 1, Fig. 1-3). This suggests that the expression of proinflammatory cytokines increases significantly after hypoxia/re-oxygenation.

In the normal group, one-way analysis of variance showed that IL-1β, IL-6, and TNF-α concentrations reduced significantly in the BM and BMb groups when compared with the MEM group ($p < 0.05$). Further paired comparisons revealed that the IL-1β decrease in the BM group was greater than in the BMb group ($p < 0.05$), but the reductions in IL-6 and TNF-α were similar in both groups. This suggests that milk supernatant may inhibit the expression of proinflammatory cytokines (IL-1β, IL-6, TNF-α) in Caco-2 cells, and especially the un-boiled supernatant has a more potent ability to inhibit IL-1β as compared to boiled supernatant.

Table 2. Relative nuclear factor-κB p65 messenger RNA expression in Caco-2 cells ($\bar{X} \pm S$, n = 3)

Group		Relative NF-κB p65 mRNA expression
Control group	MEM	1.00 ± 0.10
	BM	0.48 ± 0.05[#]
	BMb	0.45 ± 0.08[#,§]
Hypoxia/re-oxygenation group	MEM	2.12 ± 0.22[*]
	BM	0.63 ± 0.07[†,‡]
	BMb	0.97 ± 0.25[†]

*p < 0.01 when compared with MEM group in control group; †p < 0.01 when compared with MEM group in hypoxia/re-oxygenation group; ‡p < 0.05 when compared with BMb group in hypoxia/re-oxygenation group; #p < 0.05 when compared with MEM group in control group; §p > 0.05 when compared with BMb group in control group.
NF-κB p65: nuclear transcription factor κB p65 protein; mRNA: messenger RNA; MEM: minimal essential medium; BM: 5% breast milk supernatant; BMb: 5% boiled breast milk supernatant.

In Caco-2 cells undergoing hypoxia/re-oxygenation, one-way analysis of variance revealed that the concentration of IL-1β, IL-6, and TNF-α markedly decreased in the BM and BMb groups when compared with the MEM group (p < 0.05). Further paired comparisons indicated that the IL-1β reduction in the BM group was greater than in the BMb group (p < 0.05), but the reductions in IL-6 and TNF-α were similar in the BM and BMb groups. This indicates that milk supernatant may inhibit the expression of proinflammatory cytokines in Caco-2 cells after hypoxia/re-oxygenation, and especially un-boiled supernatant had a more potent ability to inhibit IL-1β as compared to boiled supernatant.

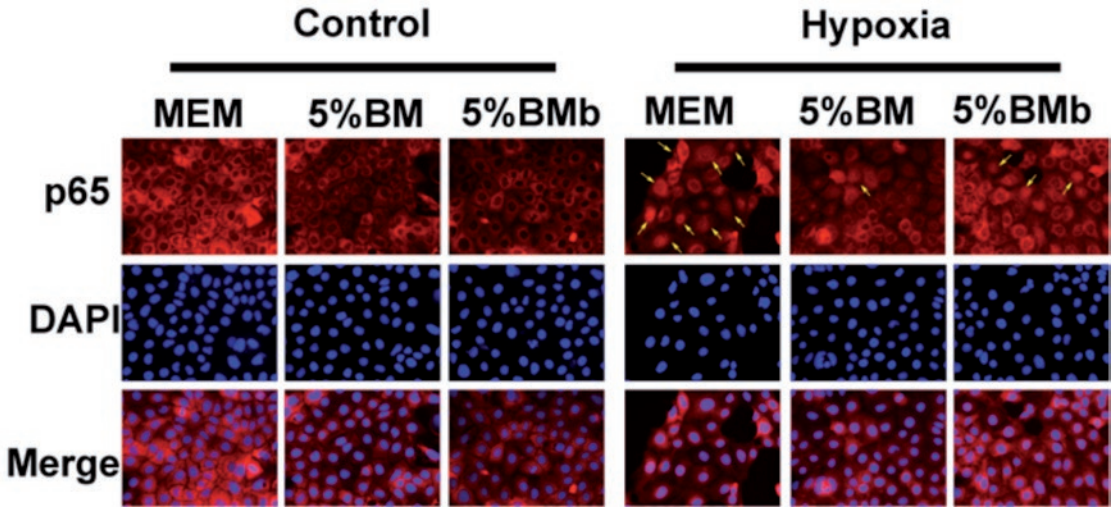
Reverse transcription polymerase chain reaction for detecting nuclear factor-κB p65 messenger RNA expression in Caco-2 cells

Results of RT-PCR (Table 2) show that the relative expression of NF-κB p65 mRNA in the MEM subgroup of hypoxia/re-oxygenation increased significantly when compared with the MEM subgroup of the control group (p < 0.01), indicating that NF-κB p65 mRNA was highly expressed after hypoxia/re-oxygenation, which proved a successful establishment of the model. When the BM and BMb subgroups in the hypoxia/re-oxygenation group were compared with those in the MEM group, the expression of NF-κB p65 mRNA significantly decreased (p < 0.01); and when the BM and BMb sub-groups in the control group were compared with the MEM group, the expression of NF-κB p65 mRNA also decreased significantly (p < 0.01), indicating that BM and BMb could downregulate NF-κB p65 mRNA expression in both control and hypoxia/re-oxygenation groups, and that both BM and BMb could downregulate NF-κB p65 mRNA expression in Caco-2 cells before and after hypoxia/re-oxygenation. Relative NF-κB p65 mRNA expression in the BM subgroup in the hypoxia/re-oxygenation group was lower than that in the BMb group (p < 0.05), while there was no significant difference in relative NF-κB p65 mRNA expression between BM and BMb subgroups in the control group (p > 0.05), suggesting that the downregulation effect of BM on NF-κB p65 mRNA expression was more significant than that of BMb after hypoxia/re-oxygenation of Caco-2 cells.

Immunofluorescence to detect nuclear factor-κB p65 messenger RNA expression in Caco-2 cells

Results of immunofluorescence (Fig. 4) showed that there was almost no red fluorescence in Caco-2 cell nuclei in the control group, while there was red fluorescence in all Caco-2 cell nuclei in the hypoxia/re-oxygenation group, which was most significant in the MEM group and least in the BM group. This indicated that NF-κB p65 in the plasma of Caco-2 cells after hypoxia/re-oxygenation was activated and entered and expressed in cell nuclei; BM could inhibit the entrance and expression of NF-κB p65 in nuclei, which was more significant than the BMb. These were consistent with the results of RT-PCR.

Figure 4. Nuclear factor-κB p65 messenger RNA expression in Caco-2 cells by immunofluorescence.
NF-κB p65 dye gives a red fluorescence; the DAPI dye of cell nuclei gives a blue fluorescence. Activation of NF-κB may be identified by its entrance to the nucleus. There was almost no red fluorescence (NF-κB p65) in cell nuclei in the control group, while red fluorescence was seen in all Caco-2 cell nuclei in the hypoxia/re-oxygenation group. This was most intense in the control (MEM) group and least in the BM group.
NF-κB: nuclear factor-κB; MEM: minimal essential medium; BM: 5% breast milk supernatant.



DISCUSSION

Neonatal NEC is a common but severe digestive tract disease in which a number of unanswered questions regarding its diagnosis and treatment still remain. To date, the etiology and pathogenesis of NEC are still poorly understood. Studies have proposed that a variety of factors, such as premature birth, hypoxia, hypothermia, improper feeding, intestinal ischemia, and infections, synergistically cause NEC. On the other hand, breast-feeding is a protective factor. For neonates, breast-feeding is recommended for adequate growth and development and the prevention of diseases. It has been reported that the incidence of NEC in formula-fed neonates is higher than among those receiving breast milk. The osmotic pressure of breast milk is 286 mmol/l and it contains a large amount of epidermal growth factors, which are beneficial in the prevention of NEC[1,12]. In the present study, Caco-2 cells, human colon cancer cells with characteristics similar to human intestinal epithelial cells, were cultured *in vitro* to investigate at the cellular and molecular levels the potential mechanisms underlying the protective effect of breast milk on NEC. In this study, we tested colostrum collected after 2-4 days of delivery, which contains significantly more adequate cytokines than mature breast milk, making it more suitable for our research purposes.

Caco-2 cells are derived from human colon adenocarcinoma and have homology with human intestinal cells. Caco-2 cells have spontaneous epithelial differentiation and may form tight junctions between cells. In addition, the morphology, expression of functional enzymes, and osmolality of Caco-2 cells are similar to those of intestinal cells. Thus, these cells can be used to investigate drug transport, absorption, and metabolism of intestinal epithelial cells *in vitro* and have been widely used in studies. Cytokines refer to small molecular polypeptides secreted by immune cells, mainly mediate and regulate immunity, inflammation, and hematopoiesis, and are important for the regulation of infection, immune response, inflammation and trauma. Proinflammatory cytokines include IL-1, IL-6, TNF-α, and IFN-γ, among others. Intestinal ischemia/reperfusion is a common cause of mucosal barrier dysfunction of the intestine. It has been confirmed that ischemia/reperfusion may activate endothelial cells and cause an imbalance of the cytokine network and overexpression of cytokines and adhesion molecules, resulting in leukocyte-mediated injury[7]. In the

present study, results showed that hypoxia/re-oxygenation significantly increased the expression of proinflammatory cytokines (IL-1β, IL-6, TNF-α), which was consistent with previous findings[11], and further confirms that the imbalance of the cytokine network is related with the pathogenesis of NEC. In the cytokine network, the corresponding anti-inflammatory cytokines to proinflammatory cytokines, such as TGF-β and IL-10, also play important roles in the pathogenesis of NEC. In this study, both un-boiled and inactivated milk supernatant could downregulate levels of proinflammatory cytokines IL-1β, IL-6, and TNF-α in cells with hypoxia/re-oxygenation injuries, suggesting that there may be other active substances involved. In future studies we will aim to clarify the regulation mechanisms from both pro- and anti-inflammatory cytokines.

In previous studies, 1 and 5% breast milk supernatants could exert inhibitory effects on Caco-2 cells, with the effects of 5% supernatant being more pronounced, but no significant difference was observed ($p > 0.05$). In the present study, 5% breast milk supernatant was used. In the normal group (not subjected to hypoxia/re-oxygenation), BM and BMb could downregulate the expression of IL-1β, IL-6, and TNF-α. Moreover, IL-1β reduction was greater in the BM than in the BMb group. In the hypoxia/re-oxygenation group, BM and BMb were found to markedly reduce the expression of IL-1β, IL-6, and TNF-α, and the reduction of IL-1β was greater in the BM than in the BMb group ($p < 0.05$). These results show that boiling the supernatants decreases, but does not eliminate, their protective function, implying that not only heat denaturalizing-prone elements are participating in this phenomenon. It is important to consider that BM also contains other substances besides cytokines, for example, soluble human milk oligosaccharides that strongly attenuate inflammatory processes in the intestinal mucosa. These findings suggest that breast milk is able to downregulate the expressions of proinflammatory cytokines (IL-1β, IL-6, TNF-α) in Caco-2 cells, especially after hypoxia/re-oxygenation. This may be one of the mechanisms underlying the protective effect of breast milk on NEC. Breast milk has many components with biological activities, and boiling may affect the biological activities of heat-sensitive proteins and polypeptides. When compared with boiled milk supernatant, reduction of IL-1β was greater after treatment with un-boiled milk supernatant. In addition, the concentration of breast milk supernatant may be another contributing factor, and the influence of milk supernatant concentration on NEC will be further investigated in our future studies[13].

The pathogenesis of NEC is complex. Studies have shown that it is associated with the abnormal activation of toll-like receptor and nuclear transcription factor signal transduction[14,15]. New studies have been conducted to investigate the molecular mechanisms regulating the expression of nuclear transcription factors and toll-like receptor in intestinal epithelial cells after breast milk intake.

REFERENCES

1. Hans Van G, Chen C, Zhang R. Hot points at necrotic enterocolitis in newborns. Chin J Evi Bas Pedia. 2011;06:321-3.
2. Dilli D, Eras Z, Ulu HÖ, Dilmen U, Şakrucu ED. Does necrotizing enterocolitis affect growth and neurodevelopmental outcome in very low birth weight infants? Pediatr Surg Int. 2012; 28:471-6.
3. Karatepe HO, Kilincaslan H, Berber M, et al. The effect of vascular endothelial growth factor overexpression in experimental necrotizing enterocolitis. Pediatr Surg Int. 2014;30:327-32.
4. Yurttutan S, Ozdemir R, Canpolat FE, et al. Beneficial effects of Etanercept on experimental necrotizing enterocolitis. Pediatr Surg Int. 2014;30:71-7.
5. McElroy SJ, Prince LS, Weitkamp J-H, Reese J, Slaughter JC, Polk DB. Tumor necrosis factor receptor 1-dependent depletion of mucus in immature small intestine: a potential role in neonatal necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol. 2011;301:G656-66.
6. Richter JM, Schanbacher BL, Huang H, Xue J, Bauer JA, Giannone PJ. Lipopolysaccharide binding protein enables intestinal epithelial restitution despite lipopolysaccharide exposure. J Pediatr Gastroenterol Nutr. 2012;54:639.
7. Geng Y, Li J, Wang F, et al. Epidermal growth factor promotes proliferation and improves restoration after intestinal ischemia-reperfusion injury in rats. Inflammation. 2013;36:670-9.
8. Gane B, Bhat BV, Adhisivam B, et al. Risk factors and outcome in neonatal necrotising enterocolitis. Indian J Pediatr. 2014; 81:425-8.
9. Deshpande G, Rao S, Patole S, Bulsara M. Updated meta-analysis of probiotics for preventing necrotizing enterocolitis in preterm neonates. Pediatrics. 2010;125:921-30.
10. Kasivajjula H, Maheshwari A. Pathophysiology and current management of necrotizing enterocolitis. Indian J Pediatr. 2014; 81:489-97.
11. Sun BW, Zhao XC, Wang GJ, Li N, Li JS. Changes in biological function of dipeptide transporter in Caco-2 cells treated with normal and hypoxia/reoxygenation and regulatory effects of growth hormone. Med J Chin Peo Lib Army. 2003;28: 1025-7.
12. Gregory KE, Walker WA. Immunologic factors in human milk and disease prevention in the preterm infant. Curr Pediatr Rep. 2013; 1:222-8.
13. Herrmann K, Carroll K. An exclusively human milk diet reduces necrotizing enterocolitis. Breastfeed Med. 2014;9:184-90.
14. Winterberg T, Vieten G, Feldmann L, et al. Neonatal murine macrophages show enhanced chemotactic capacity upon toll-like receptor stimulation. Pediatr Surg Int. 2014;30:159-64.
15. Liu YY, Fatheree NY, Mangalat N, Rhoads JM. *Lactobacillus reuteri* strains reduce incidence and severity of experimental necrotizing enterocolitis via modulation of TLR4 and NF-κB signaling in the intestine. Am J Physiol Gastrointest Liver Physiol. 2012;302:G608-17.