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Insulin-like growth factor I reduces the occurrence of necrotizing enterocolitis by reducing inflammatory response and protecting intestinal mucosal barrier in neonatal rats model

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Abstract. – OBJECTIVE: To observe the effect of enteral supplement of insulin-like growth factor I (IGF-1) on dynamic changes of TLR4, NF-κB, IL-6, SIgA and MUC2 in intestinal tissues of neonatal rats, and to investigate the protective effects and possible mechanisms of IGF-1 on necrotizing enterocolitis (NEC).

MATERIALS AND METHODS: Specific pathogen free (SPF) neonatal Sprague Dawley (SD) rats aged 3 days old were randomly divided into 3 groups, namely, normal control group, NEC model group and IGF-1 intervention group. In NEC group, the neonatal NEC rat models were established using artificial feeding, hypoxia and cold stimulation. In IGF-1 intervention group, the models were established by means of artificial feeding plus hypoxia and cold stimulation, and IGF-1 (22 ug/L) at a physiological concentration similar to the breast milk was added to milk replacer for intervention. The rats in the three groups were killed after the blood was collected from the heart at 24, 48 and 72 h, respectively, following the establishment of models; then, 3 cm of the terminal ilea were dissected and used for histopathological examination, RT-PCR and ELISA studies after hematoxylin and eosin (HE) staining.

RESULTS: Symptoms in IGF-1 intervention group were significantly relieved, and the incidence rate of NEC was lowered remarkably. In NEC model group, the peak expression of TLR4 mRNA occurred later than that of NF-κB mRNA and IL-6, and the expressions of TLR4 mRNA, NF-κB mRNA and IL-6 were decreased at 72 h after IGF-1 intervention. In NEC model group, the expression of MUC2 showed a transient decrease, the expression of SIgA was on the decline, but the expressions of MUC2 and SIgA were increased after IGF-1 intervention.

CONCLUSIONS: The enteral administration of IGF-1 at a physiological concentration can ameliorate the clinical symptoms in neonatal NEC rat models and decrease the occurrence rate. The possible mechanism is that IGF-1 down-regulates the TLR4 mRNA expression to inhibit the production of inflammatory mediators, and it up-regulates the expressions of MUC2 and SIgA to protect the mechanical and immuno-barrier functions of the intestinal mucous.

Key Words:
IGF-1, Necrotizing enterocolitis, Inflammatory, Intestinal mucosal barrier.

Introduction

Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency in the neonatal period, which mainly occurs in preterm infants of very low birth weight[1,2]. Its incidence rate among live-born infants is about 1%, and the rate reaches 7% among preterm infants of very low birth weight. With the development of modern Neonatal Intensive Care Unit (NICU) technology and neonatology, the survival rate of preterm infants of very low birth weight is constantly rising, and the incidence rate of NEC shows an increase trend. The mortality rate of the disease is high up to 15-30%[3], and the survivors often have different degrees of sequelae, including intestinal stenosis, short bowel syndrome, recurrent sepsis, growth retardation and neuro-developmental disorders[4,5]. The severe cases are often accompanied by ne-

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crosis of intestinal wall, perforation, peritonitis, systemic inflammatory response syndrome and multiple organ failure, of which the mortality rate is as high as 100%. Although premature birth, artificial feeding, intestinal hypoxia-ischemia and abnormal bacterial colonization are the known high risk factors for NEC, the specific pathogenesis of the disease has not been understood completely, and there are no effective preventive and therapeutic measures[6,7]. The latest studies hold that inflammatory cascades have played an important role in the pathogenesis of neonatal NEC. Some scholars believe that NEC is caused by uncontrolled inflammatory responses, which are induced by characteristic intestinal bacterial colonization in premature infants[8,9]. A large number of inflammatory mediators, receptors and signal transduction pathways are involved in the pathophysiological processes of the disease, but it is still unclear which factors have played the crucial roles in the inflammatory responses and can be used as potential regulating targets for prevention and treatment.

Toll-like receptor is a member of the pattern-recognition receptors of innate immunity, which is widely expressed in various cells of human tissues; it can recognize and conjugate pathogen associated molecular patterns (PAMPs), trigger a series of signal transduction, activate the immune cells to release cytokines and initiate adaptive immune responses, linking innate immunity and acquired immunity[10]. Toll-like receptor 4 (TLR4) is the first human TLR that has been discovered, of which the effect in the pathogenesis of neonatal NEC has been attracting more attention in recent years. Activated TLR4 can activate NF-κB, and then induce the expressions of downstream inflammatory factors, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), thus initiating inflammatory cascades[11,12]. Large quantities of inflammatory mediators can lead to injury of intestinal epithelial cells and destruction of mucosal defense mechanism, which finally develop into NEC; meanwhile, they can induce bacterial translocation and activation of leukocytes, stimulate release of local and systemic cytokines, and trigger systemic inflammatory response syndrome, sepsis and multiple organ failure.

Breast milk contains multiple bioactive substances, which have anti-inflammatory and anti-microbial actions and can slow down the occurrence of NEC[13,14]. Insulin-like growth factor I (IGF-1) is a kind of pleiotropic cytokine in the breast milk; some studies have indicated that IGF-1 has interactions with inflammatory factors, which have unique physiological functions[15,16]. For example, it can promote growth and development of the gastrointestinal tract or healing of intestinal tissue wound; however, whether IGF-1 has effects on NEC still remains unclear at present. In this research, the neonatal NEC rat models were established by means of artificial feeding, hypoxia and cold stimulation, and IGF-1 at physiological concentration was added to milk replacer for intervention, so as to observe the effect of enteral supplement of IGF-1 on dynamic changes of TLR4, NF-κB, IL-6, secretory immunoglobulin A (SIgA) and mucin2 (MUC2) in intestinal tissues of neonatal rats, and to investigate the protective effects and possible mechanisms of IGF-1 on NEC.

Materials and Methods

Laboratory Animals and Model Preparation

SPF Sprague-Dawley (SD) rats aged 3 days old, regardless of gender and with a body mass of 5.12-10.20 g, were provided by the Laboratory Animal Center of Taishan Medical College. The models were prepared via neonatal NEC rat models established by means of artificial feeding plus hypoxia and cold stimulation. This study was approved by the Animal Ethics Committee of Taishan Medical College Animal Center. The neonatal SD rats were separated from their maternal rats at 48 h after birth and then placed in a self-made incubator (the temperature in the incubator was controlled at 28-30°C, and the humidity at 45-65%); the rats were fed with rat milk replacer (preparation of milk replacer: the content and calories of the three major nutrients were 116.3 g/L of fats, 107 g/L of proteins and 27 g/L of sugars, and the heat was 6616 KJ/L) using a self-made gastric tube (silicone cannula of B. Braun indwelling needle was connected to a 1 mL syringe); the milk replacer was given through orotracheal intubation (with an insertion depth of 1.5-2.0 cm) 4 times every day, of which the amount was 0.15 mL at the first time and then 0.1 mL of amount was increased every 24 h. Neonatal SD rats were placed in a hypoxic chamber; after the oxygen analyzer was zeroed, the transducer was connected to the outlet pipe of the hypoxic chamber; then the flow meter of purified nitrogen cylinder was turned on to adjust the nitrogen flow rate to 10 L/min. Timing was started when the oxygen concentration in the hypoxic chamber was zero, and the nitrogen valve was turned off 90 s later. Next, the hypoxic

chamber was opened, and the neonatal rats were taken out and then put into a refrigerator; the rats were given cold stimulation at 4°C for 10 min. After that, they were put into the incubator again. Hypoxia and cold stimulation were performed once at 9 a.m., 3 and 9 pm., respectively, every day for 3 consecutive days.

Animal Grouping and Treatment

60 SPF neonatal SD rats aged 3 days old were randomly divided into three groups (n=20) in accordance with the research project after being weighed. In normal control group, the neonatal rats stayed with their maternal rats in the same cage after birth, and they were fed with breast milk without any intervention. In NEC model group, the neonatal rats were separated from their maternal rats at 48 h after birth and then placed in an incubator; the rats were artificially fed with rat milk replacer, and hypoxia and cold stimulation were given at regular intervals, so as to establish the in neonatal NEC rat models. In IGF-1 intervention group, the models were established by means of artificial feeding plus hypoxia and cold stimulation, and IGF-1 (22 mg/L) at a physiological concentration similar to the breast milk was added to the milk replacer for intervention.

Hematoxylin and Eosin (HE) Staining

The intestinal tissues were fixed in 10% neutral buffered formalin, conventionally embedded in paraffin, and then sliced to 3 μm in thickness. The tissues were stained with HE after being dewaxed in two cylinders of xylene and dehydrated in a gradient of ethanol; finally, the tissues were mounted in neutral balsam and then observed under a microscope.

Enzyme-linked immunosorbent assay (ELISA)

According to conventional processes, the total proteins in the intestinal tissues of the rats were extracted in accordance with the instructions of the ELISA kit, so that the IL-6 content in the intestinal tissues was measured.

RNA Extraction and Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The total RNA in the intestinal tissues of the rats was extracted according to the steps in the instructions of TRIzol Reagent which was used for extraction; PCR amplification was conducted after reverse transcription; at the end of the reactions, the results were automatically analyzed and calculated using a computer on the basis of standard curve.

Statistical Analysis

Statistical Product and Service Solutions (SPSS Inc., Chicago, NY, USA) 17.0 software was used for analyses, and measurement data were expressed as mean ± standard deviation (x±s). The one-way analysis of variance was used for comparisons among multiple groups, and Least Significant Difference (LSD) test was additionally performed for pair wise comparisons when the difference in the analysis of variance was statistically significant. $p<0.05$ suggested that the difference was statistically significant.

Results

General Conditions and Body Mass Changes

In normal control group, the rats ate milk every day, and their defecation was normal, without abdominal distension, milk vomiting and gastric retention; the rats grew and developed well, the body mass was increased stably, and the activity and response to stimuli were good. In NEC model group, following symptoms occurred sequentially among the rats on the second day after model establishment: feeding difficulty, milk vomiting, abdominal distension, diarrhea, reduced activity, drowsiness, slow response, reduced body mass, as well as discharge of greenish-yellow mucus and watery stools. In severe cases, frequent vomiting, cyanosis, respiratory distress and paleness occurred. In IGF-1 intervention group, the above-mentioned symptoms occurred later and milder, and the body mass was increased slowly.

Gross Morphology of Intestinal Tissues and Pathologic Changes Under Microscope

As for gross morphology, it was shown in normal control group that the ilea of the rats were light yellow, and the jejuna were ivory white; both were resilient without pneumatosis. In NEC model group, different degrees of dilatation and luminal pneumatosis could be seen in the intestines, and beaded intestines appeared in severe cases. In IGF-1 intervention group, the symptoms of luminal dilatation and pneumatosis were relieved compared with those in NEC model group. Microscopic histopathology indicated that in normal control group, the intestinal villi and epitheliums were complete, of which the tissue structures were normal, without infiltration of inflammatory cells. In NEC model group, severe necrosis of in-

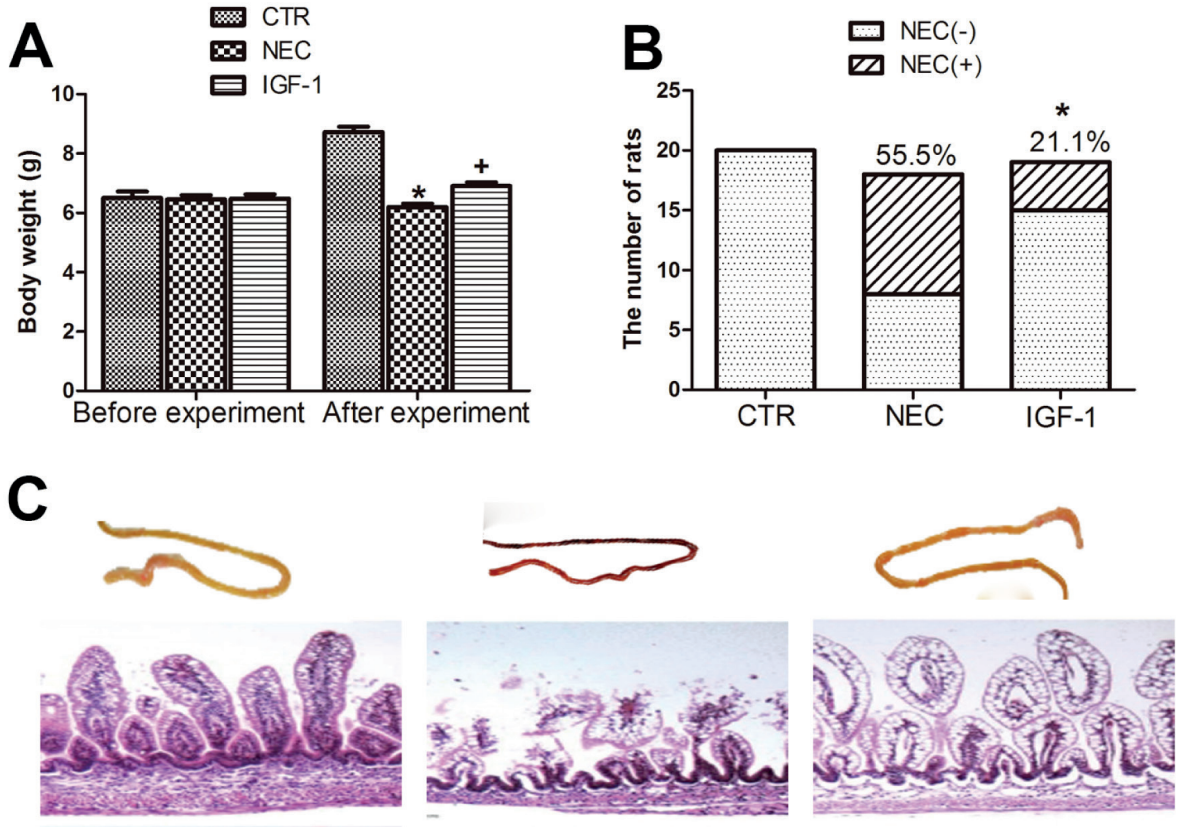

Figure 1. The protective effect of IGF-1 on necrotizing enterocolitis in a neonatal rat model. ***(A)*** The analysis of body weight of the rats before and after experiment. ***(B)*** The analysis of necrotizing enterocolitis incidence in different group. ***(C)*** Gross morphology of intestinal tissues and pathologic changes under microscope. $^{*}p<0.05$ *vs.* CTR group.

testinal tissues occurred: the intestinal villi were not well-arranged and with hydropic degeneration; a part of villi were mortified, desquamated and disappeared; many villus fragments were visible; the glands were arranged irregularly; the submucosa was severely separated from the lamina propria, edema occurred in the submucosa and muscle layer, the muscularis was thinned and a large number of inflammatory cells infiltrated. In IGF-1 intervention group, the hyperemia, edema and desquamation of intestinal villi were milder compared with those in NEC model group, and the edema in the submucosa and muscle layer was also milder. The NEC incidence rate in each group of rats was determined in accordance with the histopathological scores. The results showed that the occurrence rate was 0% in normal control group, 60.1% in NEC model group, and 20.1% in IGF-1 intervention group. The occurrence rate in IGF-1 intervention group was lower than that in NEC model group.

Dynamic Expressions of TLR4 mRNA in Ileum Tissues in Each Group

In normal control group, the TLR4 mRNA was constantly lowly expressed at each time point; in NEC model group, the TLR4 mRNA was highly expressed at 24 h, and the expression reached the peak at 48 h and was slightly decreased at 72 h; in IGF-1 intervention group, the expression of TLR4 mRNA was also elevated at 24 h, but it was lowered rapidly later, which approached the level in normal control group at 72 h. The expression levels at each time point in NEC model group were significantly increased compared with those in normal control group; the expression level at 24 h in IGF-1 intervention group was higher than that in normal control group, and there were no statistical differences at 48 h and 72 h. The expression levels at 48 h and 72 h in IGF-1 intervention group were remarkably decreased compared with those in NEC model group.

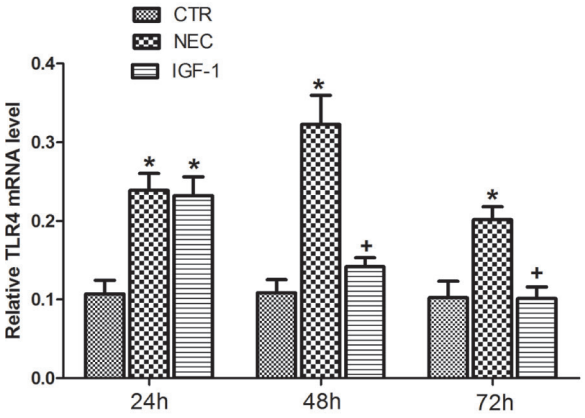

Figure 2. The dynamic change of TLR4mRNA in different groups at different time. $^{*}p<0.05$ *vs.* CTR group, $+p<0.05$ *vs.* NEC group.

Dynamic Expressions of NF-κB mRNA in Ileum Tissues in Each Group

In normal control group, the NF-κB mRNA was constantly lowly expressed at each time point; in NEC model group, the NF-κB mRNA was highly expressed at 48 h and reached the peak at 72 h; in IGF-1 intervention group, the expression of NF-κB mRNA was also elevated at 48 h, but it approached the level in normal control group at 72 h. The expression levels of NF-κB mRNA at 48 h and 72 h in NEC model group were higher than those in normal control group; the expression level at 48 h in IGF-1 intervention group was higher than that in normal control group, and there were no statistical differences at 24 h and 72 h. The expression level at 72 h in IGF-1 intervention group was significantly decreased compared with that in NEC model group.

Dynamic Expressions of IL-6 in Ileum Tissues in Each Group

In normal control group, the expression of IL-6 was steady at each time point; in NEC model group, the expression of IL-6 showed an increase tendency and reached the peak at 72 h; in IGF-1 intervention group, the expression of IL-6 was also increased at 48 h, but it approached the level in normal control group at 72 h. Compared with those in normal control group, the expression levels of IL-6 at 48 h and 72 h in NEC model group were significantly elevated; the expression level at 48 h in IGF-1 intervention group was higher than that in normal control group, and there were no statistical differences at 24 h and 72 h. The expression level at 72 h in IGF-1 intervention group was obviously lowered compared with that in NEC model group.

Dynamic Expressions of MUC2 in Ileum Tissues in Each Group

The expression of MUC2 was steady on average in normal control group; in NEC model group, the low expression of MUC2 occurred at 24 h, but it rapidly returned to the level approximate to that in normal control group at 48 h and remained steady at 72 h. In IGF-1 intervention group, the expression level of MUC2 was not decreased at 24 h after model establishment, but it quickly exceeded the level in normal control group at 48 h and reached the peak at 72 h. The expression level at 24 h in NEC model group was lower than that in normal control group, but except that, there were no statistical differences at 48 h and 72 h. The expression level at 72 h in IGF-1 intervention group was increased compared with that in normal control group and NEC model group.

Dynamic Expressions of SIgA in Ileum Tissues in Each Group

The expression of SIgA was steady on average in normal control group; in NEC model group, the expression of SIgA was on the decline, of which the levels at each time point were lower than those in normal control group. In IGF-1 intervention group, SIgA was also lowly expressed at 24 h, which rose gradually at 48 h and approached the level in normal control group at 72 h. By comparing with normal control group, the expression level of SIgA was decreased at 24 h and 48 h in IGF-1 intervention group, but there was no statistical difference at 72 h. In IGF-1 intervention group, the expression level of SIgA was remarkably elevated at 72 h compared with that in NEC model group.

Discussion

NEC is the most common gastrointestinal emergency in the neonatal period, which mainly occurs in preterm infants of very low birth weight. Premature birth, artificial feeding, intestinal hypoxia-ischemia and abnormal bacterial colonization are the known high risk factors for NEC. In this research, SPF neonatal rats aged 3 days old were taken as the research objects. The establishment of animal model was integrated with multiple risk factors, including premature birth, artificial feeding, hypoxia and cold stimulation

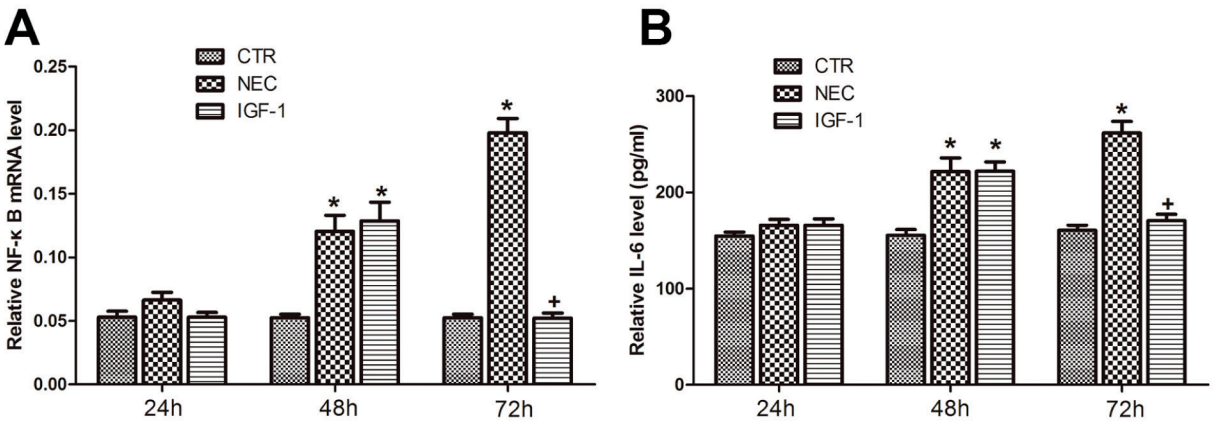

Figure 3. The dynamic changes of NF-κB mRNA and IL-6 protein in different groups at different time. ***(A)*** The analysis of NF-κB mRNA. ***(B)*** The analysis of IL-6 by ELISA. $^{*}p<0.05$ *vs*. CTR group, $+p<0.05$ *vs.* NEC group.

as well as bacterial colonization, etiologically conforming to the pathogenesis of NEC. During the experiment, it was found that in NEC model group, evident symptoms occurred successively in rats on the second day after the model establishment. As for gross morphology of intestinal tissues, different degrees of intestinal dilatation and luminal pneumatosis were observed, and beaded intestines appeared in severe cases. It was discovered under a light microscope that there was severe necrosis of intestinal tissues and massive infiltration of inflammatory cells, which were close to the clinical and pathological manifestations of NEC. It indicated that the neonatal NEC rat models had been successfully established.

There are great differences in clinical manifestations of NEC: the mild symptoms can rapidly develop into necrosis of intestinal wall, perforation, peritonitis, systemic inflammatory response syndrome, multiple organ failure, etc. within a few hours. Therefore, prevention is particularly important. Currently, some scholars[17,18] have put forward several preventive measures, such as regulation of intestinal microecology via probiotics, intestinal supplement of arginine[19] and glutamine[20] and application of glucocorticoid[21]. However, these therapies are not mature yet, and the applications are still controversial. Therefore, searching for safe and effective preventive methods has become a research hotspot. The breast milk can effectively prevent the occurrence of NEC, and IGF-1 is a growth factor with a high concentration in the breast milk. Some studies have indicated that IGF-1 has interactions with inflammatory factors, which can have unique physiological functions; for example, it can promote growth and

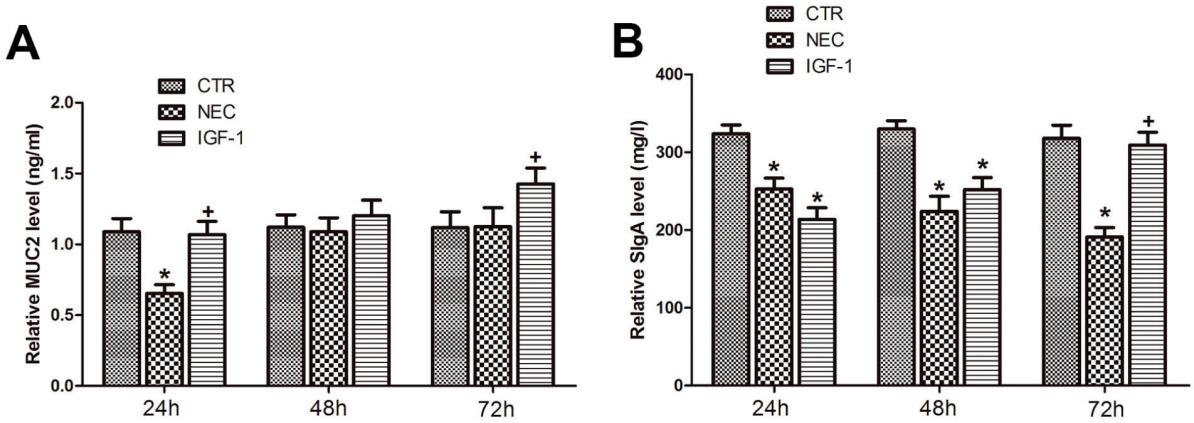

Figure 4. The dynamic changes of MUC2 and SIgA protein in different groups at different time. ***(A)*** The analysis of MUC2 protein. ***(B)*** The analysis of SIgA protein. $^{*}p<0.05$ *vs*. CTR group, $+p<0.05$ *vs.* NEC group.

development of the gastrointestinal tract or healing of intestinal tissue wound. In this work, IGF-1 was administered through the intestines. With a concentration approximate to the physiological concentration of breast milk, the medicine could significantly improve the symptoms of NEC, alleviate the pathologic damage to the intestinal tissues and reduce the occurrence rate. The results suggested that the addition of IGF-1 at a physiological concentration to formula for preterm infants can prevent or slow down the occurrence of NEC, which may provide a new thought for prevention and treatment of the disease.

NEC is a result of the joint action of multiple factors, and inflammatory cascade is the final common pathway of its pathogenesis. As a result, it is one of the directions for treatment of NEC to look for and regulate the key targets of inflammatory responses. In recent years, more importance has been attached to the effect of TLR4 in the pathogenesis of neonatal NEC. TLR4 is lowly expressed in normal intestinal epithelial cells; however, it is highly expressed in NEC children and animal models, and the high expression of TLR4 occurs even before injury of NEC tissues[22,23]. The effect of TLR4 in the pathogenesis of NEC is mainly associated with the inflammatory injury and repair disorders; the activated TLR4 can activate NF-κB and then induce the up-regulated expressions of downstream inflammatory cytokines, such as TNF-α, IL-6 andIL-8. Large quantities of inflammatory mediators can lead to injury of intestinal epithelial cells and destruction of mucosal defense mechanism, which finally develop into NEC. In this research, the dynamic changes of TLR4 mRNA, NF-κB mRNA, IL-6 and intestinal injury were observed. The results showed that TLR4 mRNA had been obviously highly expressed at 24 h, which reached the peak at 48 h and fell back at 72 h; but the expression level was still high. The expressions of NF-κB and IL-6 at 24 h were not significantly different from those in normal control group; the high expression occurred at 48 h, and the peak expression occurred at 72 h, which lagged behind that of TLR4. It suggests that on responding to the stimulation of artificial feeding, hypoxia, cold stimulation and other high risk factors, the body first initiates the innate immune responses and up-regulates the TLR4 expression; next, the activated TLR4 initiates the adaptive immune responses, activates NF-κB and further induce the transcription, translation and massive release of IL-6 gene, the inflammatory cytokine. Furthermore, IL-6 can promote the neutrophils to recruit and release a large number of active oxygen radicals in the lesion areas by virtue of chemotaxis, and it can play a cytotoxic effect in coordination with other inflammatory cells. The vicious cycle formed by positive feedback stimulation of the inflammatory mediators can lead to excessive inflammatory responses, injury and necrosis of intestinal tissues and destruction of mucosal barrier, thus developing into NEC. IGF-1 at a physiological concentration was given to the model animals through the intestines for intervention; the results showed that although the expression of TLR4 in IGF-1 intervention group was elevated at 24 h, it was lowered rapidly later and approached the level in normal control group at 72 h. By comparing with NEC model group, the expressions at 48 h and 72 h in IGF-1 intervention group were remarkably decreased. NF-κB mRNA and IL-6 were also highly expressed at 48 h, but the expressions were lower than those in NEC model group at 72 h. It suggests that IGF-1 may rapidly initiate the negative regulation mechanism to inhibit the TLR4/NF-κB pathway while TLR4 is mediating the inflammatory responses to eliminate pathogenic factors, resulting in moderate expressions of inflammatory factors and appropriate inflammatory responses in the body. Therefore, the immune balance can be restored as soon as possible, and injury and necrosis of intestinal tissues caused by excessive inflammatory responses can be avoided, thus alleviating intestinal injury and lowering the occurrence rate.

Mucin is a major component of the intestinal mucus layer, which covers the surface of the intestinal epithelial cells, constituting the first line of defense in the host. Among the 20 kinds of mucin that have been already known so far, MUC2 is the first that has been confirmed, and it is a secretory mucin, which has the highest content in human intestinal cavity. It covers the surface of the intestinal cavity and forms a gelatinous mucus layer, so as to preserve the complete barrier functions[24]. Studies over the past few years have found that MUC2 is involved in the pathogenesis of NEC25,26. In this research, the changes in the expression level of MUC2 in ileum tissues were observed dynamically; the results revealed that the expression of MUC2 was steady on average in normal control group, while in NEC model group, the level of MUC2 protein was significantly lowered at 24 h after the model establishment (i.e., 4 days after birth). The results were basically con-

sistent with those of previous experiments; however, the expression rapidly returned to the level approximate to that in normal control group at 48 h and remained steady at 72 h. In IGF-1 intervention group, the expression level of MUC2 was not decreased at 24 h after the model establishment, but it quickly exceeded the level in normal control group at 48 h and reached the peak at 72 h. It indicates that IGF-1 can accelerate the synthesis and secretion of MUC2 in neonatal rats, thus protecting the mechanical barrier functions of the intestinal mucous.

SIgA is a main immunoglobulin on the surface of the intestinal mucous, which plays an important role in defense of gastrointestinal mucous27,28. In this research, it was discovered that the expressions of SIgA were steady at each time point in normal control group; low expression occurred at 24 h in NEC model group and then the expression was on the decline, which reached the lowest level at 72 h. In IGF-1 intervention group, the expression level of SIgA was decreased at 24 h and 48 h, but it rapidly returned to the level close to that in normal control group at 72 h. It is assumed that in NEC model group, the serious inflammatory responses have destroyed the signal transduction pathways for synthesis and secretion of SIgA and the protein transport system in the intestinal cells of the rats. However, IGF-1 may protect the SIgA synthesis system and maintain the balance of intestinal microecology by relieving the inflammatory responses; therefore, it can induce normal synthesis and secretion of SIgA, protect the immuno-barrier function of the intestines and slow down the malignant progression of disease.

Conclusions

The enteral administration of IGF-1 at a physiological concentration can ameliorate the clinical symptoms in neonatal NEC rat models and decrease the occurrence rate. The possible mechanism is that IGF-1 down-regulates the TLR4 mRNA expression to inhibit the production of inflammatory mediators, and it up-regulates the expressions of MUC2 and SIgA to protect the mechanical and immuno-barrier functions of the intestinal mucous.

Conflict of interest

The authors declare no conflicts of interest.

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