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Intestinal trefoil factor increased the Bcl-2 level in a necrotizing enterocolitis neonate rat model

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Background/aim: The aim of this study was to investigate the therapeutic effect of intestinal trefoil factor (ITF) on necrotizing enterocolitis (NEC) by observing the pathological changes and detecting the protein level differences in Caspase-3, Bax, and Bcl-2 in an NEC neonate rat model.

Materials and methods: A Wistar rat model of NEC was established and 30 one-day-old neonate Wistar rats were randomly divided into three groups including a normal control (group A), NEC rats treated with 0.2 ml physiological saline through intraperitoneal (i.p.) injection (group B), and NEC rats treated with 0.2 mg ITF by i.p injection (group C).

Results: Compared with group B, there were statistically significant differences in Caspase-3, Bax, and Bcl-2 levels in groups A and C ($P < 0.05$). Moreover, there was a significant difference in the Bcl-2 level between groups A and B ($P < 0.05$).

Conclusion: ITF alleviated injury of the intestinal tract in neonate rats with NEC and this mechanism was possibly related to a reduction in the expression of Caspase-3 and Bax and the increase in Bcl-2 expression.

Key words: Intestinal trefoil factor, necrosis, caspase-3, Bax, Bcl-2, neonate rat

1. Introduction

Necrotizing enterocolitis (NEC) is one of the most severe diseases threatening newborn lives (1), with an incidence rate ranging from 0.72% to 2.10%. Moreover, there is a high incidence rate of NEC in low birth weight infants (2). Furthermore, only 7%–15% of all NEC cases occur in term or late preterm infants. The age of onset of NEC is inversely related to the postmenstrual age of the mother at the time of birth (3). Additionally, the mortality rate of NEC is 16%–20%, and there is a higher mortality rate in infants with serious NEC, very low birth weight infants, and NEC infants who need surgical therapy (4,5). Presently, NEC is caused by bowel necrosis due to many factors such as premature birth, infection, hypoxia, enteral feeding, and intestine ischemia. The main therapy for NEC is surgery to remove the necrotic bowels. However, the surgery can cause short bowel syndrome and inadequate absorption of nutrients. Thus, better therapeutic measures are needed (6,7). Studies have indicated that the scores for neonatal acute physiology-perinatal extension-II and metabolic derangement acuity were insufficient measures for deciding on surgical treatment for the patients affected by NEC (8). A study from Hong Kong found that the intestinal trefoil factor (ITF) 3 level was higher in NEC patients than in non-NEC patients, suggesting that NEC affects ITF production (9). ITF is a member of the trefoil family and may protect the mucosa from insult, stabilize the mucus layer, and affect healing of the epithelium (10). The current study explores the effect of ITF on the expression of Caspase-3, Bax, and Bcl-2 in order to better understand the relationship between these three proteins and NEC.

2. Materials and methods

2.1. NEC model

Thirty one-day-old Wistar rat pups (5–10 g) were provided by the Hubei Provincial Center for Disease Control and Prevention, Wuhan, China. An AE2200 electronic balance was obtained from Mettler Toledo Company, Shanghai, China. Infant formula (Dumex, Dumex Infant Food Co., Ltd. Shanghai, China) was fed to the neonate rats (200 kcal/kg per day) every 3 h, with an initial volume of 0.1

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mL per feeding that was increased as the weight of the rats increased (4). The room temperature was maintained at 15%–25 °C and rats were exposed to natural light. Hypoxia was induced by exposing each rat pup to 100% nitrogen (maintaining 99.96 ± 0.2% for 60 s measured by an RSS-5100 portable digital oxygen recorder (Shanghai REX Instrument factory, Shanghai, China)), followed by exposure to cold (4 °C) for 10 min two times daily. The newborn rats were returned to the female rats to feed following treatment for 3 days. All newborn rats were killed by decollation on day 4. The intestinal tract tissues from the duodenal inferior to the upper rectum were isolated. Indicators of NEC were noted including colored intestine, bleeding, tympanites, or narrowing of the ileum. Segments of 1–2 cm from the near distal duodenum, small bowel, and Terminal caecum were fixed with 10% formol saline and embedded with paraffin. Then, the expression of caspase-3 was detected by tectological examination and spectrophotometry. Bax and Bcl-2 protein levels were measured using the immunohistochemical technique. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Wuhan University.

2.2. Animals and groups

Thirty rat pups were randomly divided into three groups and each group had 10 rats. In group A, the rats were left untreated as a control. Group B rats were injected with 0.2 mL of physiological saline solution intraperitoneally, and group C NEC rats were injected with 0.2 mL of ITF.

2.3. Spectrophotometry

Dissected intestinal tract tissues were washed in cold normal saline solution and then weighed after drying with filter paper. Then, 5 mg of tissue was added to 100 μL of lysate and the tissues were homogenized on ice, transferred into 1.5 mL Eppendorf tubes, and kept on ice for an additional 5 min. Next, tubes were centrifuged at 16,000 rpm for 15 min at 4 °C. Then, the supernatant was collected, transferred into cold tubes, and stored at –20 °C. The protein quantification and detection of caspase-3 activity were performed using caspase-3 activity test kits (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturers' instructions. The peptide nucleic acid (pNA) level was calculated using standard curves to obtain the caspase-3 activity.

2.4. Immunohistochemistry

The SP immunohistochemical technique was used to detect the Bax and Bcl-2 protein levels in the intestinal tissues. SP staining kits were purchased from Beijing ZSGB-Bio Company, Beijing, China. All samples were fixed with 10% formalin and embedded with paraffin, cut to a thickness of 4 μm, and mounted on glass slides. Paraffin-embedded intestinal sections were deparaffinized, immersed in sodium citrate buffer, and heated at 96–100 °C for 15 min prior to treatment with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Antigen retrieval was performed using a microwave. The specimens were washed twice with distilled water and then with PBS for 3 min each and incubated with 10% normal serum for 10 min at room temperature. Then, samples were incubated overnight with rat antihuman monoclonal Bcl-2 antibody (Beijing ZSGB-Bio Company, Beijing, China) and rat antihuman monoclonal Bax antibody (Beijing ZSGB-Bio Company, Beijing, China) at a dilution of 1:200. The sections were washed three times with PBS for 3 min each and incubated with biotin-labeled anti-rat IgG for 10 min at room temperature. After three washes with PBS for 3 min each, SP was added at 37 °C for 10 min, and the staining was visualized with 3,3'-diaminobenzidine tetrahydrochloride chromogen (Beijing ZSGB-Bio Company, Beijing, China). A nuclei counterstain was performed using hematoxylin.

2.5. Pathological examination

Experimental rat pups were killed on day 4 and 1–2 cm-long specimens were taken from the ileocecal segments of the intestinal tissues for histological study. After 10% formalin fixation and paraffin embedding, the processed and sectioned (4–6 μm) tissues were stained with hematoxylin and eosin. Five sections were obtained from each rat and 5 fields of each section were microscopically observed (×200). The criteria for each histological grade (0–4 score) were as follows: normal (0), no damage and intact chorioepithelilum; mild (1), slight edema of chorioepithelilum; moderate (2), slight middle-end villus necrosis; severe (3), severe middle-end villus necrosis and/or severe edema in the submucosal and muscular layers and regional villus sloughing; necrosis (4).

2.6. Statistical analysis

Statistical analyses were performed using the statistical software package SPSS13.0. The pathological scores were measured by Ridit analysis. Bax and Bcl-2 protein levels were analyzed using the optical intensity values. The image analysis software was from HPIAS2000 (Qianpin Image Engineering Company, Tongji, China). Data are expressed as the means ± standard deviation. The data were analyzed for significant differences with one-way ANOVA. A q-test was used to compare groups. $P < 0.05$ was considered significant.

3. Results

3.1. Expression of Caspase-3, Bax, and Bcl-2

In normal rat pups, there was less expression of caspase-3 in the intestinal tissues when compared with NEC rats. The Caspase-3 contents in group B were the highest, while the Caspase-3 contents in groups A and C were lower. There were significant differences in caspase-3 expression

between groups A and B ($q = 15.88$, $P < 0.05$) and between groups B and C ($q = 14.98$, $P < 0.05$). However, there was no significant difference between groups A and C ($q = 0.9$, $P > 0.05$).

The expression of Bcl-2 protein was negative in the normal mucous membrane. Positive brown-yellow particles indicated that Bax and Bcl-2 were located in the plasma. Bax and Bcl-2 protein levels were analyzed using the optical intensity values. The Bax protein expression was highest in group B, and there was a significant difference between groups A and B ($q = 2.95$, $P < 0.05$) and between groups B and C ($q = 2.79$, $P < 0.05$), but there was no significant difference between groups A and C ($q = 0.17$, $P > 0.05$). Moreover, the protein expression of Bcl-2 was highest in group C, and there was a significant difference between groups A and B ($q = 3.51$, $P < 0.05$) and between groups B and C ($q = 5.30$, $P < 0.05$). There was also a significant difference between groups A and C ($q = 8.81$, $P < 0.05$, Table).

3.2. Comparison of pathological changes

The pathological scores ranged from 0 to 2 in the tissue from group C, which exhibited small congested blood vessels and few necrotic cells of the villi. The pathological scores ranged from 1 to 4 in the tissue from group B, which exhibited superficial epithelium lesions and full mucous membrane necrosis. Moreover, intestinal tract lesion were slighter in groups C and A than in group B, and there was no significant difference between groups A and C.

4. Discussion

NEC is caused by several factors such as prematurity, enteral feeding, and infection (11). The trefoil factor family consists of small polypeptides secreted by mucins in the gastrointestinal tract. Suemori et al. (12) found ITF in rat jejunums in 1991. Under physiological conditions, ITF is mainly expressed in the goblet cells of the intestines and colon, and is also found in the ducts of the pancreas, uterus, hypothalamus, and pituitary gland (13). The cloverleaf pattern structure made by the TFF3s exhibits antiprotease hydrolysis, acid digestion, and heat resistance. The present study indicated that ITF was cryoprotective and reduced the impairment of the intestinal mucosa mediated by many kinds of injury factors. Interestingly, ITF not only promoted cell proliferation and migration, but also special physical function; for example, it helped to stabilize the mucus layer by binding to mucous glycoprotein (14,15). Additionally, experiments have shown that ITF plays an important role in protecting the integrity of the intestinal mucosa and promoting its reconstruction after injury (16–19). Mice lacking ITF did not heal well after mucosa injury. In this study, the histopathological results showed that ITF alleviated the intestinal lesions in NEC rats.

Caspases or cysteine-aspartic proteases are essential for helping to regulate apoptosis in cells. Thus, caspases are also called executioner proteases. Caspase 3 is the key protease that mediates cell apoptosis (20). Moreover, caspase 3 is the most critical protease in the caspase cascade reaction. As an executioner caspase, the caspase-3 zymogen has virtually no activity until it is cleaved by an initiator caspase after apoptotic signaling events have occurred. Our results showed that the expression of caspase-3 was significantly increased in the NEC rat group compared with the control group, but was decreased in the NEC group after treatment with ITF.

Bcl-2 is derived from B-cell lymphoma 2 and is classified as an oncogene. When the expression of Bcl-2 protein is increased, apoptosis is inhibited, increasing the cell survival time (21). Bax was the first proapoptotic member of the Bcl-2 protein family to be identified. Bax can form Bax-Bax homodimers that act as proapoptotic regulators, but Bcl-2 may affect the formation of these homodimers. An abnormal increase in Bcl-2 results in the increased separation of Bax-Bax homodimers and the formation of more stable Bax-Bcl-2 heterodimers, with Bcl-2 suppressing cell apoptosis. Therefore, the ratio of Bcl-2 and Bax is an important factor determining the sensitivity of the apoptosis signal stimulated by cells. The overexpression of Bcl-2 protein could inhibit cell apoptosis, but an increase in Bax homodimers promotes cell apoptosis. Chan et al. (22) found that antisense ITF significantly enhanced cell apoptosis induced by adriamycin, and Taupin et al. (23) also suggested that endogenous ITF protected the colonic

Table. Expressions of Caspase-3 (μmol), Bax and Bcl-2 in rat intestinal tissues.

Group	N	Caspase-3 (μmol)	Bax	Bcl-2
A	10	6.72 ± 1.45	2.19 ± 0.61	1.64 ± 0.38
B	10	22.60 ± 2.93	5.15 ± 0.14	5.14 ± 0.54
C	10	7.62 ± 2.16	2.36 ± 0.40	10.45 ± 1.79
F value		40.23	152.3	162.78
P value		<0.05	<0.05	<0.05

cell line from apoptosis induced by serum starvation and ceramide-C2. This effect was dependent on the activity of PI3-kinase. This study demonstrated that ITF may prevent p53-dependent and p53-independent apoptosis. In addition, some studies implied that ITF inhibited cell apoptosis through activation of the Akt/PKB/NF-κB (p65) pathway (17,24). Our results indicated that Bax and Bcl-2 were expressed more in the NEC group than in the control group, and the expression of Bax was decreased in the ITF treatment group. Conversely, the expression of Bcl-2 was increased in the ITF treatment group compared with the NEC group. Both Bax and Bcl-2 proteins are downstream regulators of cell apoptosis following the activation of the Akt or P53 pathway. Thus, our results are consistent with previous findings.

It has been indicated that cytC is released from the mitochondrial inner membrane in the early stages of apoptosis in order to launch the mitochondrial apoptosis pathway (25). The release of cytC results in the release of the mitochondrial transition pores and the induction of the proapoptosis gene Bax. However, the antiapoptotic gene Bcl-2 can block the release of cytC and the activation of caspase (26). This study showed that the expression of Caspase-3 and Bax were increased in the NEC groups with and without ITF treatment, and their expression decreased in intestinal tissues, while the expression of Bcl-2 significantly increased. The protective effect of ITF on intestinal cells should be further studied.

In summary, this study demonstrated that the expressions of Caspase-3 and Bax were decreased in intestinal tissues after treatment of NEC rats with ITF, but the expression of the Bcl-2 gene was increased. The findings indicate that these three genes are involved in the occurrence and development of NEC. The results from this study provide critical information needed for the future development of a target-gene therapy for NEC.

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