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Protective effects of intestinal trefoil factor on neonatal rats with necrotizing enterocolitis

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Abstract: Objective The intestinal trefoil factor (ITF) is closely related with gastrointestinal epithelial cells injury repair. Studying the effects of ITF on necrotizing enterocolitis (NEC) would be helpful to the treatment of NEC. This research investigated the effect of ITF on intestinal histopathological changes, expression of cyclooxygenase-2 (COX-2) and the production of prostaglandin E 2 (PGE_2) and thromboxane B 2 (TXB_2) in neonatal rats with NEC. **Methods** Forty one-day-old Wistar rats were randomly divided into 5 groups: Groups A, B, C, D and E ($n = 8$ each). Group A served as the normal control group. Rats in Groups B, C, D and D were made into NEC models by hypoxia and re-oxygenation for 3 consecutive days. Groups D and E were treated with 0.5 mL ITF (0.5 mg) intraperitoneal injection or 0.2 mL ITF (0.2 mg) subcutaneous injection once respectively after damage, while Groups B and E were injected with normal saline intraperitoneally or subcutaneously respectively. On the 4th day all the subjects were sacrificed and intestinal tissues were obtained to examine the histological changes, COX-2 expression, and PGE_2 and TXB_2 productions. **Results** Intestinal histopathology of rats in Group A was normal, and the pathologic scores were 0. As compared with the corresponding NEC group (Groups B and C), histopathological injuries of NEC were remarkably relieved after ITF treatment (Groups D and E) ($P < 0.01$). The pathologic scores of rats in Groups B and C were 1-4, while those of Groups D and E were 0-2. PGE_2 and TXB_2 contents significantly increased in Groups B and C, while dramatically decreased after ITF treatment (in Groups D and E). No significant differences were observed for the PGE_2 and TXB_2 contents between Groups D, E and A. Immunohistochemistry staining indicated positive expression of COX-2 in Groups B and C, which were significantly higher than Groups A, D and E ($P < 0.05$). Mild positive expression of COX-2 was observed in Groups D and E, which was stronger than Group A. **Conclusions** ITF can decrease the productions of PGE_2 and TXB_2 by suppressing the expression of COX-2, which may be underlying protective mechanisms of ITF on NEC.

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Key words: Intestinal trefoil factor; Enterocolitis, necrotizing; Cyclooxygenase-2; Neonatal rat

肠三叶因子对新生大鼠坏死性小肠结肠炎的保护作用

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[**摘　要**] **目的**　肠三叶因子(ITF)对消化道粘膜上皮细胞的损伤修复具有重要作用。本实验通过研究 ITF 对新生大鼠坏死性小肠结肠炎(NEC)模型肠组织病理学改变,环氧合酶-2(COX-2)表达及前列腺素 E2(PGE_2)、血栓素 B2(TXB_2)生成的影响,以探讨 ITF 对 NEC 是否有治疗作用。**方法**　40 只新生 1 日龄 Wistar 大鼠随机分为 5 组,每组 8 只。A 组为正常对照组。B 和 C 组大鼠为 NEC 模型鼠,分别予以0.5 mL 生理盐水腹腔或0.2 mL 皮下注射。D 和 E 组大鼠亦为 NEC 模型鼠,分别予以 0.5 mL ITF(0.5 mg)腹腔或0.2 mL(0.2 mg)皮下注射。连续 3 天新生大鼠予以缺氧－复氧处理制成 NEC 模型。第 4 天处死所有大鼠,取肠组织检查组织病理学改变,COX-2 表达和 PGE_2 与 TXB_2 的生成。**结果**　A 组的肠组织病理学未见异常,病理评分为 0 分。与相应的 NEC 组(B 和 C 组)比较,ITF 治疗后(D 和 E 组)NEC 导致的组织病理学改变明显减轻($P < 0.01$)。B 和 C 组的病理学评分为 1～4 分,而 D 和 E 组评分为 0～2 分。与 A 组比较,B 和 C 组 PGE_2 与 TXB_2 浓度显著增高,但 ITF 治疗后(D 和 E 组)显著下降,与 A 组无明显差异。免疫组化结果显示 B 和 C 组的 COX-2 表达显著高于 A,D,E 组($P < 0.05$)。D 和 E 组弱表达 COX-2,其强度高于 A 组,但显著低于 B 和 C 组。**结论**　ITF 通过抑制 COX-2 的表达,减少了 PGE_2 和 TXB_2 含量,减轻肠组织炎症反应,这可能是 ITF 治疗 NEC 的机制。　[**中国当代儿科杂志,2005, 7(1): 20-24**]

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Necrotizing enterocolitis (NEC) is a severe emergency as well as the first cause of death in extremely premature infants. Prematurity, enteral feeding, bacterial colonisation, and intestinal hypoxia-ischemia are considered to contribute to the enteral necrosis[1]. In spite of extensive epidemiological, clinical, and basic research, there is no effective preventative treatment for this disease [2].

Intestinal trefoil factor (ITF), a member of trefoil peptide family, can greatly reduce the damage mediated by many injury factors. It can transform mucin solutions into a gel-like state, significantly increase mucin's viscosity and elasticity, thus enhancing the defense barrier of gastrointestinal tract mucosa[3]. ITF can also promote cell proliferation, migration and anti-apoptosis capacity and so it plays a major role in the self-defense and healing of gastrointestinal tract. There may be some other mechanisms underlying the protective effect of ITF. The reasons for a predilection for prematurity are unclear, but deficiency of ITF may contribute to the susceptibility of premature neonates[4].

Ischemia leads to intestinal hypoperfusion, which may cause intestinal mucosa injury. Thereafter topical microorganisms may invade the mucosal barrier and induce cascade amplification reaction of inflammation, and cause NEC. A series of inflammatory factors upregulate during NEC. COX-2 is an inducible cycloxygenase, which can be quickly induced and activated by variety of inflammatory factors. COX-2 expression in intestinal epithelium of neonates with NEC is significantly higher than other non-inflammation cases[5]. It is believed that the COX/prostaglandin pathway plays a critical role in the pathogenesis of NEC. Increased COX-2 can induce the production of prostaglandin, causes the degradation of cell membrane phospholipids and produce arachidonic acid. With the catalysis of COX-2, prostaglandin E 2 (PGE_2) and thromboxane B 2(TXB_2) are generated from arachidonic acid, which contributes to the pathogenesis of colonitis.

Whether ITF can inhibit inflammation, and decrease the expression of COX-2 and its downstream production during NEC remains unknown. The aim of the present study was to test the effects and mechanisms of peritoneal and subcutaneous administration of ITF on the development of NEC in neonatal rats. In this research, experimental NEC was induced by hypoxia in neonatal rats[6], and the effects of ITF on the development of NEC, expressions of COX-2 and its downstream productions, PGE_2 and TXB_2 were evaluated.

Materials and methods

Experimental animals, reagents and experiment instruments

Neonatal Wistar rats (1 day old, weighing 5-10 g) were provided by the People's Hospital Animal Center of Wuhan University. The neonatal rats were kept together with and fed by mother rats. The room temperature was kept between 12 - 25°C with natural illumination. Recombinate human ITF (hITF) was provided by the State Key Laboratory of Protein Engineering and Plant Genetic Engineering, College of Life Sciences, Peking University. TXB_2 was provided by the Beijing East-Asia Biology Institute. PGE_2 was purchased from Amersham International (USA). Experimental instruments included normal pressure hypoxia chamber (20 cm × 30 cm × 40 cm, with one air inlet and one outlet), RSS-5100 portable digital oxygenmeter (Shanghai Rex Instrument Factory), and SN-682 radioimmunity γ counter (Shanghai Heifu Photoelectric Instrument Factory).

Grouping of experimental animals

Forty neonatal rats were randomly divided into 5 groups (A, B, C, D and E, $n = 8$ rats each. Groups B, C, D and E were made into NEC models as follows: CO_2 was added to the hypoxia chamber till CO_2 concentration reached 100% (maintained at 99.7% ± 0.2%). Then neonatal rats were then put into the hypoxia chamber. After 5 minutes, O_2 was added into the chamber quickly until O_2 concentration reached 100% (maintained at 99.0% ± 0.8%) for another 5 minutes. This process was carried out for a successive 3 days. After damage, Groups B and C were injected with 0.5 mL normal saline (NS) intraperitonealy or 0.2 mL NS subcutaneously once respectively, while Groups D and E were injected with 0.5 mg ITF (0.5 mL) or 0.2 mg ITF (0.2 mL) respectively [7,8]. Rats in Group A were not subjected to either hypoxia or re-oxygenation and any injection. All subjects were sacrificed on the 4th day and intestines from the lower duodenum to the upper colon were

removed. One to two cm of ileocecal intestine was fixed using 10% formaldehyde, then paraffin imbedded for hematoxylin-eosin staining and immunohistochemistry analysis. The rest of the tissues were kept in -70°C for further use.

Histopathology analysis of intestine

Four slices (4 μm) of intestinal tissue of each rats and 5 views (×100) of each slice were analyzed. The histopathological changes were scored as 0 (normal villi), 1 (epithelial cell sloughing), 2 (midvillous necrosis), 3 (complete villous necrosis), or 4 (transmural necrosis) in double-blind way[9]. The mean score of fields was calculated for statistical analysis.

Measurement of PGE_2

The procedures were carried out according to the manufacturers' instructions. Samples were added into dehydrated alcohol-NS (1∶4) at a concentration of 20 mg/mL. After homogenate, 2.0 mL supernatant was collected, and the pH was adjusted to 3.5. Five mL acetic ether was mixed with the supernatant. After 2 minutes, the supernatant was centrifuged at 3 500 rpm for 15 minutes. The extraction fluids were frozen at -20℃.

Measurement of TXB_2

Measurement was carried out according to the manufacturers' instructions. Samples were added into dehydrated alcohol-NS (1∶9) at a concentration of 20 mg/mL. After homogenate, samples were stored at -20℃. During measurement, samples were centrifuged at 3 500 rpm at 4°C for 15 minutes. Two mL supernatant was collected.

Immunohistochemistry analysis of intestine

Samples were dewaxed, dehydrated, and heat antigen repaired. One mL goat anti antibody (1∶50) was added and COX-2 incubated at 4° C overnight. Biotinylated rabbit anti goat IgG working solution was added and incubated at 37°C for 15 minutes. Incubation with HRP labeled streptavidin (S-A/HRP) working solution (37°C for 15 minutes) was carried out. Color was developed with DAB and the sections were counterstained with hematoxylin. Immunohistochemistry results were analyzed using HPIAS 2000 Imaging Software (Tongji Qiangping Imaging Company). Positive staining area was measured in 4 slices (5 regions of each slice) of each rat. Positive rate was calculated by comparing with total area; mean values were used for statistical analysis.

Statistical analysis

Statistical analysis was carried out using SPSS 11.5. Data were expressed $\bar{x} \pm s$. One-way ANOVA was performed to analyze differences among various groups, then the q test was used to analyze differences between two groups. Pathologic scores were evaluated using Ridit assay.

Results

Manifestations of rats during hypoxia-reoxygenation

During the experiment, no abnormalities were observed in Group A. Remarkable abdominal distension, mucous loose stools even bloody purulent stools were observed in Groups B and C. Rats in Groups D and E had less severe abdominal distension and a minority presented with mucous loose stools.

Histopathological changes of intestine

The intestinal histopathology of rats in Group A was normal, and the pathologic scores were 0. Compared with the corresponding NEC group, histopathological injuries of NEC were greatly relieved after ITF treatment ($P < 0.01$). The pathologic scores of rats in Groups B and C were 1-4, while those of Groups D and E were 0-2 (Figure 1).

Contents of PGE_2 and TXB-2 in various groups

The PGE_2 and TXB_2 contents in NEC intestinal tissue homogenate significantly increased in Groups B and C, but dramatically decreased after ITF treatment (in Groups D and E). No significant differences were observed between Groups D, E and A (Table 1).

Table 1 Contents of PGE_2 and TXB_2 and COX-2 expression in various groups ($n = 8$, $\bar{x} \pm s$)

Group	PGE_2 (pg/mg · tissue)	TXB_2 (pg/mg · tissue)	COX_2 (%)
A	17 ± 3	138 ± 29	30.2 ± 1.1
B	37 ± 3[a]	321 ± 37[a]	64.4 ± 1.4[a]
C	36 ± 3[a]	314 ± 37[a]	64.1 ± 1.5[a]
D	20 ± 3[c]	140 ± 30[c]	38.9 ± 1.1[b,c]
E	19 ± 3[d]	139 ± 28[d]	39.5 ± 1.1[b,d]
F	74.57	73.45	1313.75
P	<0.001	<0.001	<0.001

Compared with Group A a $P < 0.01$, b $P < 0.05$; c Compared with Group B $P < 0.01$; d Compared with Group C $P < 0.01$

Immunohistochemistry analysis of COX-2

Immunohistochemistry staining indicated positive expression of COX-2 in intestinal tissue inflammation cells, smooth muscle cells and some intestinal epithelium of rats in Groups B and C, which were significantly

higher than Groups A, D and E ($P<0.05$). Mild positive expression of COX-2 was observed in Groups D and E, which was stronger than in Group A, but was much less than Groups B and C (Table 1 and Figure 1).

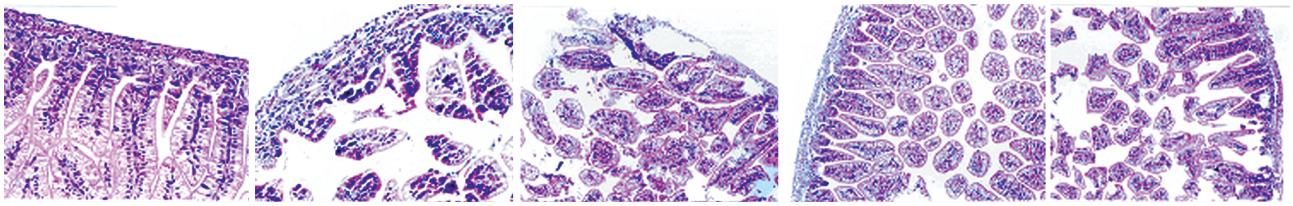

Figure 1 Histopathological changes of intestines in various groups (Hematoxylin-eosin staining, ×200). Group A, the villi were normal, and the pathological score was 0. Group B, the cores of villi were separated, and there were significant submucasal edema and epithelial sloughing. The pathological score was 3. Group C, there were denudation of epithelium with loss of villi and full thickness necrosis, significant epithelial sloughing in the intestinal lumen. The pathological score was 4. Group D, there were villi core seperation and minimal seperation of the mucosa from the basement membrane. The histopathological change was less than that of Group B, and the pathological score was 2. Group E, the villi core seperation and seperation of the mucosa from the basement membrane were slighter than that of the Group C. The pathological score was 2.

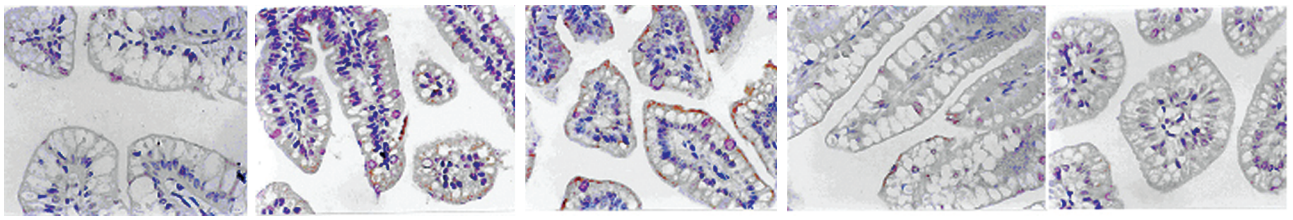

Figure 2 Expressions of COX-2 in intestines of various groups (SP staining, ×400). Group A, there was little COX-2 expressed in the intestinal tissue of normal neonatal rats. Group B, much COX-2 was expressed in the crypt and villi of the intestinal epithelium and in the cytoplasm of inflammatory cells in mucosa and muscularis layers. Group C, the expressions of COX-2 were almost as much as those of Group B. Group D, the expressions of COX-2 were much less compared with those of Group B. Group E, the expressions of COX-2 were much less compared with those of Group C.

Discussion

This study indicated that in the intestinal tissues of neonatal rats with NEC, the COX-2 expression increased, and considerable PGE_2 and TXB_2 were pathologically synthesized. However, after peritoneal and subcutaneous injection of ITF, the COX-2 expression was reduced. The contents of PGE_2 and TXB_2 were also significantly reduced.

ITF is a newly discovered growth factor. During acute mucosa injury, ITF expression is up-regulated in the early stage of repair, and this promotes the repair of mucosa and maintains the integrity of mucosa. Our previous research has found that compared to normal premature rats, a series of inflammatory cytokines such as TNF-α and IL-8 are significantly increased in the intestinal tissues of NEC rats, and dramatically decreased after ITF treatment[10,11]. These findings indicate that ITF can inhibit inflammatory factors release, which may underlie the therapeutic effect of ITF in NEC. This study indicated that ITF administration in different methods could exert a mucosa protective function. It is speculated that through the inhibition of COX-2 expression, ITF can inhibit the production of PGE_2 and TXB_2, and therefore exerts a protective function on mucosa. But the detailed mechanism of inhibition effects of ITF on COX-2 expression needs further study.

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·病例报告·

以骨髓坏死为特征的幼儿急性淋巴细胞白血病 1 例

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男,2 岁。因发热 10 d,面色苍黄、骨骼疼痛 2 d 入院。患儿 10 d 前无明显诱因出现不规则发热,体温达 38℃~39℃,伴乏力、食欲差。2 d 前体温升到 39.5℃,并出现面色苍黄、多处骨骼疼痛,血常规示:血红蛋白 86 g/L,白细胞 11.2×10^9/L,血小板 64×10^9/L,予抗感染治疗 2 d 无效入院。查体:体温 39℃,急性热病容,中度贫血貌,面色苍黄,结膜、甲床苍白,足背部、肩部可见少量针尖大小出血点,颌下、颈部可触及 2 粒黄豆大小淋巴结,质软,活动度可,无压痛,其余浅表淋巴结未触及。肝肋下 2.5 cm,脾肋下 2 cm,质中、边缘钝。胸骨、四肢骨骼及指骨、趾骨有明显压痛。实验室检查:血红蛋白 60 g/L,红细胞 2.06×10^{12}/L,血小板 50×10^9/L,白细胞 67.0×10^9/L,分类原始和幼稚淋巴细胞占 0.82;红细胞沉降率 125 mm/h。骨髓象:骨髓液外观呈暗红色稀薄液体;有核细胞增生活跃,粒∶红 = 2∶1;淋巴细胞系比例明显增高,原始及幼稚淋巴细胞占0.885,部分细胞胞浆边缘模糊,外形不完整;细胞之间及片膜背景上可见大量粉红色嗜酸性物质;成熟红细胞边缘模糊不清;全片未见巨核细胞,血小板散在偶见。POX 染色(-)。诊断为急性淋巴细胞白血病(ALL)并骨髓坏死,确诊后转院治疗。

讨论

骨髓坏死主要是指造血细胞和骨髓基质发生面积不等的坏死,主要临床表现为发热、骨痛等。白血病引起骨髓坏死的机制,是由于髓内白血病细胞的过度增生,压迫血窦致血窦扭曲、破裂,髓内血供减少,造成骨髓组织变性和坏死。对于疑有骨髓坏死的病例在骨髓穿刺时应特别注意抽出液的外观。其外观可呈棕红色碘酒样,果酱样或暗红色稀薄液体。涂片染色后镜下可见有核细胞轮廓不清,胞膜及胞核结构模糊,成熟红细胞呈溶解状,细胞之间常有均匀分布的粉红色嗜酸性物质,可能系有核细胞胞质溶解后所释放的蛋白质成分。可大片或局灶性坏死。

ALL 合并骨髓坏死的患儿,由于造血细胞和骨髓基质同时发生坏死,且大多白细胞计数明显增高($>50\times10^9$/L),根据全国小儿血液病学组(1998)提出的标准,多属于高危型 ALL,疗效和预后多较差。

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