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• *BASIC RESEARCH* •

Expression of intestinal trefoil factor, proliferating cell nuclear antigen and histological changes in intestine of rats after intrauterine asphyxia

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Abstract

AIM: To study the expressions of intestinal trefoil factor (ITF) and proliferating cell nuclear antigen (PCNA) and histologic changes in intestine, to investigate the relationship between ITF and intestinal damage and repair after intrauterine hypoxia so as to understand the mechanism of intestinal injury and to find a new way to prevent and treat gastrointestinal diseases.

METHODS: Wistar rats, pregnant for 21 d, were used to establish animal models of intrauterine asphyxia by clamping one side of vessels supplying blood to uterus for 20 min, another side was regarded as sham operation group. Intestinal tissues were taken away at 0, 24, 48 and 72 h after birth and stored in different styles. ITF mRNA was detected by RT-PCR. PCNA expression was measured by immunohistochemistry. Intestinal tissues were studied histologically by HE staining in order to observe the areas and degree of injury and to value the intestinal mucosa injury index (IMDI).

RESULTS: ITF mRNA appeared in full-term rats and increased with age. After ischemia, ITF mRNA was decreased to the minimum (0.59±0.032) 24 h after birth, then began to increase higher after 72 h than it was in the control group (P<0.01). PCNA positive staining located in goblet cell nuclei. The PCNA level had a remarkable decline (53.29±1.97) 48 h after ischemia. Structure changes were obvious in 48-h group, IMDI (3.40±0.16) was significantly increased. Correlation analyses showed that IMDI had a negative correlation with ITF mRNA and PCNA (r = -0.543, P<0.05; r = -0.794, P<0.01, respectively).

CONCLUSION: Intrauterine ischemia can result in an early decrease of ITF mRNA expression. ITF and PCNA may play an important role in the damage and repair of intestinal mucosa.

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Key words: ITF; PCNA

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INTRODUCTION

Intestinal trefoil factor (ITF) is a member of the trefoil peptide family[1,2], which is important in maintenance and repair of the intestinal mucosal barrier[3] and was first discovered and named by Suemori *et al*[4], in 1991. Researchers have demonstrated that ITF can protect cells of intestinal mucosa from damage mediated by many kinds of injury factors[5,6]. It can-not only stimulate cell migration and proliferation, promote epithelial cell repair[7,8], but also interact with mucus, stabilize mucus gel by perhaps interacting with intestinal mucin and increasing the viscosity[9]. So it is important in the self-protection mechanism of intestine.

Proliferating cell nuclear antigen (PCNA) is a kind of intranuclear protein, which is an assistant protein of DNase[10]. It has no specificity of species, genus and tissue, exists in the cells, expressing in phages G1 and S, so it has been widely used to mark cells of S phage. In this point PCNA is a perfect marker to evaluate cell proliferation[10].

There are almost 70% neonates with birth asphyxia complicating varied degree damages of organs such as heart, brain, kidney and gastrointestine[11]. Among which the rate of gastrointestinal injury is 33% and even higher than that of brain damage.

Whether the damage of intestine has a relationship with ITF and PCNA remains unknown[12]. In this study, the model of intrauterine asphyxia of rats and the methods of RT-PCR and immunohistochemistry were used to explore the expressions of ITF and PCNA, to observe the histologic changes of intestine so as to understand the mechanism of intestinal injury after asphyxia and to find a new way to prevent and treat gastrointestinal diseases.

MATERIALS AND METHODS

Animal model

Sixty-three Wistar rats (53 females and 10 males, weighing 270±30 and 300±20 g respectively) were provided by the Animal Center of China Medical University No. 2 hospital. According to the methods of Mamoru *et al*, and Terry *et al*[13,14], models of intrauterine acute ischemia were established by clamping one side of vessels supplying blood to uterus of 21-d pregnant Wistar rats for 20 min and the other side was regarded as sham operation group. When the prescribed time was reached, uterus horn was opened rapidly and pups were taken out. A total of 144 surviving baby rats were enrolled in this study, which were fed by other step-mother rats for 0, 24, 48 and 72 h, respectively, then 18 baby rats in each group were killed and 100-200 mg intestinal tissue (taken from 3 to 4 baby rats) was stored at -80 ℃ as samples for ITF mRNA, five samples at each time point. In each intestinal tissue, 0.5-cm intestinal tissue was fixed in 40 g/L formaldehyde for HE staining and PCNA immunohistochemistry study.

RT-PCR for detection of ITF mRNA

Total RNA was isolated from intestine samples using TRIzol reagent (Promega Co., USA). Two microliters of total RNA were used as a template to synthesize cDNA. The resulting cDNA was used as a template for subsequent PCR (TaKaRa Co., Ltd). A 236-bp fragment of ITF was amplified from single-stranded DNA by PCR using two oligonucleotide primers to ITF sequence: sense primer, 5'TTT GAC TCC AGC ATC CCA 3', and antisense primer, 5'CGC AAT TAG AAC AGC CTT G 3' (synthesized by AuGCT Biotechnology Co., Beijing). Meanwhile, amplification of β-actin was performed on the same RNA samples to assess RNA integrity. Reaction mixture for PCR contained cDNA template 4 μL, dd H_2O 11.8 μL, 5× buffer 5 μL, dNTPS 2 μL, TaqE 0.2 μL, primers A and B each 1 μL. Forty-five cycles of PCR were conducted at 94 ℃ for 4 min, at 60.6 ℃ for 1 min, at 72 ℃ for 90 s, at 72 ℃ for 7 min. Agarose gel electrophoresis was used to detect the amplified ITF products. The density of bands was assessed and the amount of ITF mRNA was determined according to the ratio to β-actin[15].

Immunohistochemistry for PCNA

The samples were fixed in 40 g/L formaldehyde and embedded in paraffin. Five-micrometer thick serial sections of paraffin blocks were dewaxed and rehydrated. PCNA monoclonal antibody and SABC correlated reagents were purchased from Zhongshan Biotechnology Co., Beijing. Detection was carried out according to instructions. Five views were randomly selected in each tissue section, measured under a 400× microscope and analyzed with Meta Morph software to assess the average gray density.

Determination of intestinal mucosa damage index (IMDI)

Samples were fixed in 40 g/L formaldehyde and embedded in paraffin, then cut into 5-μm sections and stained with HE. Three sections of each tissue, five sights of each section were selected randomly to observe under a microscope, the areas and degree of injury and IMDI were evaluated by an expert pathologist using double blind. The standard was suggested by Chiu *et al*[17], and Okur *et al*[18].

Grade 0: Normal mucosal villi.

Grade 1: Development of subepithelial Gruenhagen's space, usually at the apex of the villus, often with capillary congestion.

Grade 2: Extension of the subepithelial space with moderate lifting of epithelial layer from the lamina propria.

Grade 3: Massive epithelial lifting down the sides of villi. A few tips might be denuded.

Grade 4: Denuded villi with lamina propria and dilated capillaries exposed. Increased cellularity of lamina propria might be noted.

Grade 5: Digestion and disintegration of lamina propria, hemorrhage and ulceration.

Statistical analysis

Data were expressed as mean±SD. Results were analyzed with *t* test and LSD test (*q* test). Spearman's method was used for correlation analysis by SPSS 10.0 software.

RESULTS

Changes of ITF mRNA expression in intestine after intrauterine asphyxia

RT-PCR showed that expression of ITF mRNA appeared in full-term rats and increased with age. After ischemia, ITF mRNA expression decreased to the minimum (0.59±0.032) 24 h after birth, and then began to increase. It was even higher 72 h after birth than it was in the control group ($P<0.01$) (Figure 1 and Table 1).

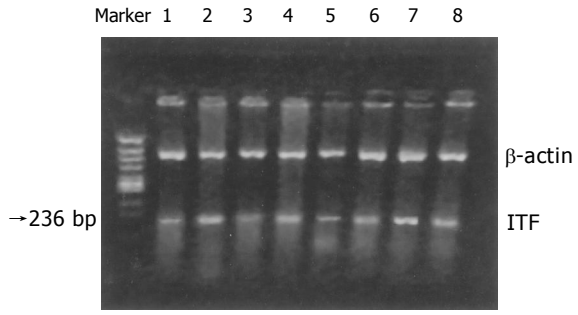

Figure 1 ITF mRNA expression in intestine after intrauterine asphyxia detected by RT-PCR. Lane 1: after intrauterine asphyxia 0-h group; lane 2: control group; lane 3: after intrauterine asphyxia 24-h group; lane 4: control group; lane 5: after intrauterine asphyxia 48-h group; lane 6: control group; lane 7: after intrauterine asphyxia 72-h group; lane 8: control group.

Immunohistochemical results of PCNA after intrauterine asphyxia

Goblet cell nuclei were positively stained in intestinal mucosa of full term rats. The PCNA level had a remarkable decline (53.29±1.97) 48 h after ischemia, then began to increase, but was still lower than that in the control group 72 h after birth. There was a significant difference between ischemic and control groups ($P<0.01$) (Figures 2A and B; Table 1).

Table 1 **Changes of expression of ITF mRNA, PCNA and IMDI after intrauterine asphyxia (*n* = 18, mean±SD)**

Age (h)	ITF mRNA		PCNA		IMDI	
	Experimental	Control	Experimental	Control	Experimental	Control
0	0.86±0.043[a,d]	0.97±0.016	56.75±1.18[a,d]	65.24±2.67	0.67±0.16[b,d]	0
24	0.59±0.032[a,d]	0.98±0.011	55.22±2.14[b,d]	66.17±2.10	2.47±0.17[b,d]	0
48	0.83±0.022[a,d]	0.99±0.025	53.29±1.97[b,d]	72.17±3.19	3.40±0.16[b,d]	0
72	1.19±0.023[a,d]	1.07±0.021	61.80±2.72[b,d]	74.48±1.33	1.60±0.21[b,d]	0

[a]$P<0.05$ *vs* control group; [b]$P<0.01$ *vs* control group; [d]$P<0.01$ *vs* other experimental groups.

Change of histology and IMDI

Histologic examinations of normal newborn rats showed that their intestinal tissues were almost mature. After ischemia, lamina propria hyperemia might be noted 24 h after birth, extension of the subepithelial space with moderate lifting of epithelial layer from the lamina propria could be observed. Structural changes were obvious in 48-h group, denuded villi with lamina propria and dilated capillaries were exposed, cellularity of lamina propria was increased, quantity of villi was declined, IMDI (3.40±0.16) was significantly increased. Seventy-two hours after birth, although the quantity of villi was still less than that in the control group, changes recovered remarkably. The intestinal mucosa of control group had almost no damage (IMDI was 0). Correlation analyses showed that IMDI had a negative correlation with ITF mRNA and PCNA (r = -0.543, $P<0.05$; r = -0.794, $P<0.01$, respectively) (Figures 3A and B; Table 1).

DISCUSSION

Neonatal asphyxia is a common disease during perinatal, which happens in uterus and during labor with a high morbidity and mortality in newborns. Previous studies[18,19] showed that the rate of gastrointestinal injury was 33% and even higher than that of brain damage. However, research has been hardly done on the mechanism of intestinal injury.

Studies have demonstrated that there were changes in levels of blood gastrin and motilin in patients with asphyxia[20,21] and they might suffer from more attacks of gastroesophageal acid reflux than the normal controls[22]. There were also changes of free radicals in intestine after hyperoxia-induction[23]. But it is of great value to discuss the maturity and perfection of intestinal mucosal barrier, whether the barrier is damaged and what happens in the proliferation and repair ability after damage.

A previous study showed that among the growth factors, ITF was most closely associated with intestine and was the initiators of mucosal healing[24]. ITF is a new kind of growth factors secreted by goblet cells[25] into the lumen of the intestinal tract[26] with a characteristic structure of trefoil configuration[1], so it not only has the promoting effect on cell proliferation as a common growth factor, but also could combine with mucin glycoproteins to stabilize the mucus

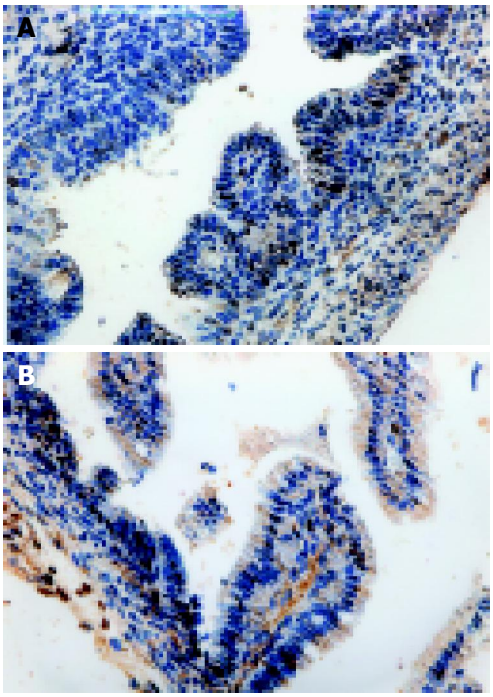

Figure 2 Immunohistochemical results of PCNA (400×). **A**: Positive staining of intestinal mucosal goblet cell nuclei; **B**: Decline of PCNA positive staining 48 h after intrauterine asphyxia.

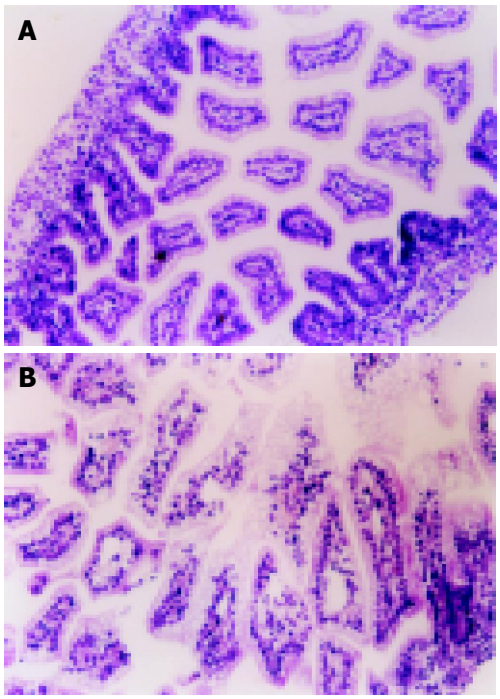

Figure 3 Intestinal tissue HE staining (400×). **A**: Mature intestinal tissue in normal newborn rats; **B**: Obvious structural changes, denuded villi with lamina propria and exposed dilated capillaries increased cellularity of lamina, declined quantity of villi 48 h after intrauterine asphyxia.

gel[3] and prevent the damage caused by proteolytic enzymes and mechanical pressure[27]. In this way, ITF could be looked as a protection factor of intestine[2].

ITF mRNA expression was detected at transcriptional level at different time points after birth in rats with asphyxia in our study. It was found that at birth, the ITF had a certain expression and with time, the expression increased. After asphyxia, ITF mRNA expression decreased. The synthesis ability of ITF of goblet cells decreased and reached the lowest point 24 h after birth, and then increased. It increased more than that in control group 72 h after birth. It was considered as a reflect reaction to injury repair. At this time, the intestinal mucosa began to proliferate fast along with the recovery of intestine function.

The other factor causing damage of the integrity of intestinal mucosal barrier can inhibit intestinal epithelial cell proliferation. Intestinal epithelial cells have the characteristics of short proliferating cycle and strong growth ability, so the intestine could self-repair well. PCNA has no specificity of species, genus, tissue and presense in actively proliferating cells. PCNA is a perfect cell proliferating index in normally proliferating cells and in certain tumor tissues and has already been used as a routing method to test proliferating cells at present[28,29]. It was also demonstrated that the change of PCNA expression increased DNA duplication and cell proliferation[30-32]. The expression of PCNA also increased in intestine of dogs after ischemia and reperfusion[33]. Immunohistochemical technology revealed that the PCNA level had a remarkable decline 48 h after asphyxia, recovered partly after 72 h, but was still lower than that in the control group, suggesting that asphyxia can decrease the proliferating ability of epithelial cells.

At the same time, histologic examination of intestine showed that intestinal mucosa was injured widely and IMDI increased significantly, and then recovered. Correlation analysis showed that IMDI had a negative correlation to ITF mRNA and PCNA. In this way, a low proliferating ability would lead to a low repair ability and perhaps the decline of intestinal mucosa to secrete ITF is associated with dysfunction of mucosal-barrier and the disability of mucosa repair. Whether other factors are involved should be further studied. Feng *et al*[33], studied the relationship between ITF and intestinal damage and repair in rats suffering from severe burns, and found the similar results.

The distinct three-loop secondary structure of ITF could contribute to the remarkable resistance to acid and proteolytic digestion, enabling them to function in the harsh environment of the gastrointestinal tract while maintaining biologic activity[34,35]. It could not affect pH and gastroenteric motility[36], but could stabilize mucus gels so as to protect intestinal mucosa against all kinds of damage factors. Further study should be done to explore whether enteral administration of ITF can prevent and treat intestinal injury caused by asphyxia[37-41].

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