

# Mechanisms of acupuncture and moxibustion in regulation of epithelial cell apoptosis in rat ulcerative colitis

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## Abstract

**AIM:** To investigate the effect of acupuncture and moxibustion on epithelial cell apoptosis and expression of Bcl-2, Bax, fas and FasL proteins in rat ulcerative colitis.

**METHODS:** A rat model of ulcerative colitis was established by immunological methods and local stimulation. All rats were randomly divided into model control group (MC), electro-acupuncture group (EA), herbs-partition moxibustion group (HPM). Normal rats were used as normal control group (NC). Epithelial cell apoptosis and expression of Bcl-2, Bax, fas and FasL proteins were detected by TUNEL and immunohistochemical method respectively.

**RESULTS:** The number of epithelial cell apoptosis in MC was significantly higher than that in NC, and was markedly decreased after the treatment with herbs-partition moxibustion or electro-acupuncture. The expression of Bcl-2, Bax, fas and FasL in colonic epithelial cells in MC was higher than that in NC, and was markedly down-regulated by herbs-partition moxibustion or electro-acupuncture treatment.

**CONCLUSION:** The pathogenesis of ulcerative colitis in rats involves abnormality of apoptosis. Acupuncture and moxibustion can regulate the expression of Bcl-2, Bax, fas and FasL proteins and inhibit the apoptosis of epithelial cells of ulcerative colitis in rats by Bcl-2/Bax, fas/FasL pathways.

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## INTRODUCTION

Ulcerative colitis (UC) is a non-specific inflammatory intestinal disease. The pathogenesis of ulcerative colitis involves abnormality of apoptosis which is affected by a variety of

factors<sup>[1-3]</sup>. At present, increasing evidence suggests that acceleration of apoptosis of epithelial cells and inhibition of apoptosis of inflammatory cells (such as neutrophil) are closely associated with colonic tissue injury and immunological abnormality in ulcerative colitis.

Apoptosis is determined by the relative expression of serial genes involved in the regulation of apoptosis. Fas/FasL is one of the important pathways of epithelial cell apoptosis in UC. In tissues of UC, the number of FasL positive cells is significantly increased, resulting in apoptosis. FasL expression increases in the focal region of active UC, which directly promotes apoptosis of Fas expressing colonic epithelium. The apoptosis promoting gene bax also plays an important role in apoptosis. The ratio of bax/bcl-2 determines whether apoptosis occurs or not. Excessive expression of bax promotes apoptosis.

In the present study, a rat model of UC was established by immunological method and local stimulation. After the treatment with electro-acupuncture and herbs-partition moxibustion, the number of colonic epithelial cell apoptosis and the expression of Bcl-2/Bax and Fas/FasL proteins were detected by TUNEL and immunohistochemistry respectively for elucidating the mechanism of acupuncture and moxibustion underlying colonic epithelial cell apoptosis in rat UC.

## MATERIALS AND METHODS

### Experimental animals and materials

Two hundred male SD rats (weighting 200±20 g) were provided by Experimental Animal Center of Shanghai University of TCM. TUNEL kits was purchased from Boehringer Mannheim (Germany). Bax, Bcl-2 and FasL kits were from Dako (Denmark). Fas was from Santa-cruz (USA).

### Methods

**Animal model and therapeutic methods** Establishment of animal model: According to Experimental Methodology of Pharmacology<sup>[4]</sup>, UC rat model was established by immunological method and local stimulation. Colonic mucosa was prepared from human fresh surgical colonic specimens, homogenized by adding appropriate amount of normal saline and centrifuged for 30 min at 3 000 r/min. The supernatant was removed for the measurement of protein concentration and then mixed with Freund adjuvant. The antigen fluid was first injected into the plantar pedis of the model group rats, then into the plantar pedis, dorsum, inguen and abdominal cavity (no Freund adjuvant in the last injection) on the tenth, seventeenth, twenty-fourth and thirty-first day respectively. When a certain titer of serum anti-colonic antibody was reached, 3 mL 3% formalin and 2 mL antigen fluid (no Freund adjuvant) were administered by enema successively. The rats in NC were administrated with normal saline as the same procedure of MC.

**Treatment:** After the ulcerative colitis rat model was built, the animals were randomly divided into model control group (MC 8), electro-acupuncture group (EA 8), herbs-partition moxibustion group (HPM 8) and normal control group (NC 6). HPM: Moxa cones made of refined mugwort floss were placed on the medicinal formula (medicinal formula dispensing: *Radix Aconiti praeparata*, *cortex Cinnamomi*, et al) for Qihai (RN

6) and Tianshu (ST 25, bilateral) and ignited. Two moxa cones were used for each treatment once a day and 14 times as a course. EA: Tianshu (bilateral) and Qihai were acupunctured and then stimulated by intermittent pulse with 2HZ frequency, 4 mA intensity for 20 minutes once a day and 14 times as a course.

After treatment, four group rats were killed simultaneously. The distal 6 cm long colons were dissected and reserved in formaldehyde solution.

**TUNEL analysis** Formalin fixed specimens were embedded in paraffin using standard procedures. Deparaffinised and rehydrated sections were immersed in 3mL/L  $H_2O_2$  for 30 min at room temperature and digested with proteinase K for 20 min at 37 °C. The sections were immersed in 1g/L Triton-100 and then incubated with TUNEL mixture for 1 hour at room temperature, with streptavidin-HRP(1:400) for 30 min at 37 °C. The sections were stained with 0.4g/L DAB and treated with 3mL/L  $H_2O_2$  for 10 min and with hematoxylin for 1 min. The results were observed with light microscopy. Positive reaction was shown by brown color. The apoptotic cells were counted as the mean of cells in 3 visual fields of one section. The data were analysed by *q* test, using statistical package SPSS.

**Immunohistochemistry** Formalin fixed specimens were embedded in paraffin using standard procedures. Sections attached on carry sheet glass were autoclaved at 58 °C for 24 h. Deparaffinised and rehydrated sections were immersed in 10mL/L  $H_2O_2$  for 20 min and washed three times, each time for 3 min with PBS. Sections were preincubated with 10mL/L normal goat anti rabbit serum for 20 min at room temperature and then incubated with the first antibodies diluted for 18 h at 4 °C and Envision reagent for 30 min at 37 °C. Sections were stained by 0.4g/L DAB with 0.3mL/L  $H_2O_2$  for 8 min and hematoxylin for 30 s. The results were observed under light microscope.

Positive specimens were used as positive controls. The result of PBS instead of the first antibody was used as negative control. The positive reactions showed brown particles. The positive cells expressing Bcl-2, Bax, fas and FasL were counted as the mean of cells in 3 visual fields of one section. The data were analysed by *q* test, using statistical package SPSS.

## RESULTS

The effect of acupuncture and moxibustion on epithelial cell apoptosis in UC rats is shown in Table 1 and Figure 1(A-D).

**Table 1** Results of epithelial cell apoptosis in different groups

Group	<i>n</i>	Number of apoptotic cells (mean±SD)
NC	6	20.61±1.99
MC	8	66.21±8.51 <sup>b</sup>
HPM	8	33.58±3.59 <sup>bd</sup>
EA	8	34.29±2.70 <sup>bd</sup>

<sup>b</sup>*P*<0.01 vs NC; <sup>d</sup>*P*<0.01 vs MC.

Table 1 shows that the number of epithelial cell apoptosis in MC was significantly increased compared with that of NC (*P*<0.01). The number of epithelial cell apoptosis in EA and HPM was remarkably decreased compared with that of MC (*P*<0.01), but was not as low as that of NC (*P*<0.01).

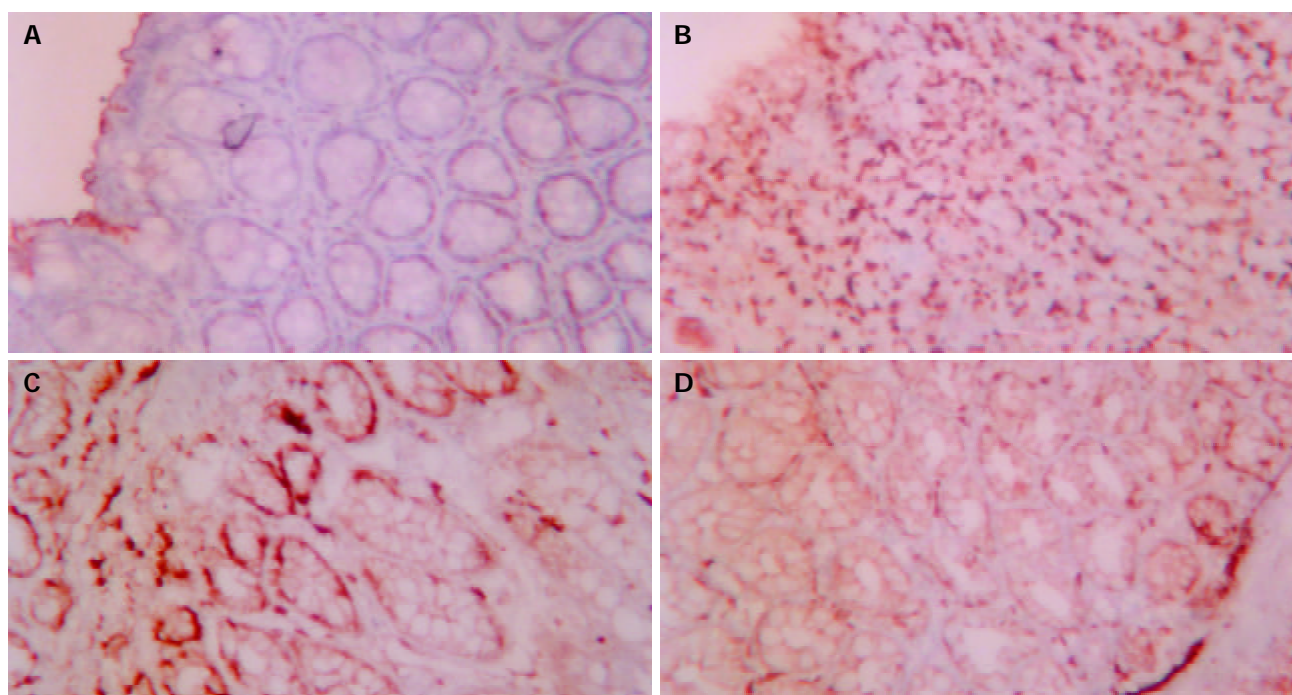
*The effect of acupuncture and moxibustion on Bax expression in colonic epithelia of UC rats is shown in Table 2 and Figure 2(A-D)*

**Table 2** Bax expression in colonic epithelia of different groups (mean±SD)

Group	<i>n</i>	Area of expression	Intensity of expression
NC	6	35 905.06±2 987.97	0.1683±0.0105
MC	8	52 451.13±3 174.10 <sup>b</sup>	0.2558±0.0142 <sup>b</sup>
HPM	8	39 561.50±1 382.94 <sup>d</sup>	0.1900±0.0047 <sup>d</sup>
EA	8	38 477.79±3 309.19 <sup>d</sup>	0.1796±0.0117 <sup>d</sup>

<sup>b</sup>*P*<0.01 vs NC; <sup>d</sup>*P*<0.01 vs MC.

Table 2 shows that the area and intensity of Bax expression in the colonic epithelia of MC were significantly increased compared with that of NC (*P*<0.01). The area and intensity



**Figure 1** A: Epithelial cell apoptosis in NC ×200, B: Epithelial cell apoptosis in MC ×200, C: Epithelial cell apoptosis in EA ×200, D: Epithelial cell apoptosis in HPM ×200.



of Bax expression in the colonic epithelia of PHM and EA were markedly decreased compared with that of MC, but showed no significant difference when compared with that of NC.

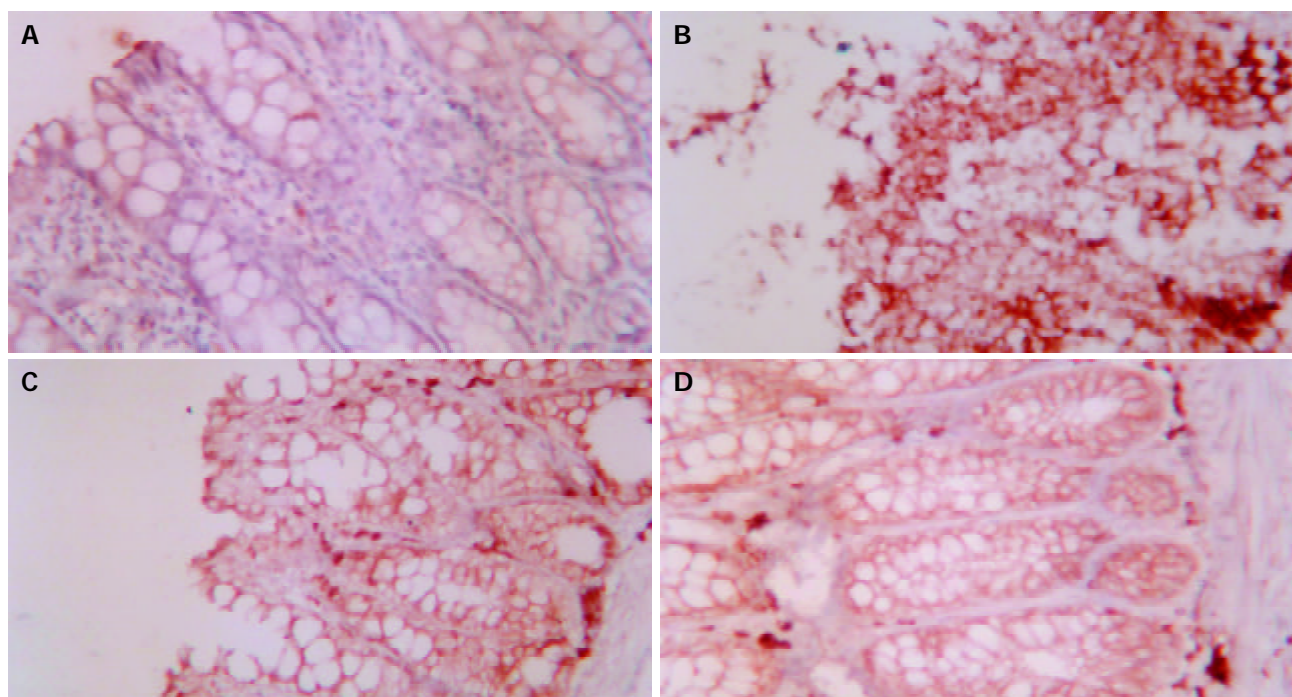
***The effect of acupuncture and moxibustion on Bcl-2 expression in the colonic epithelium of UC rats is shown in Table 3 and Figure 3(A-D)***

Table 3 shows that the area and intensity of Bcl-2 expression in the colonic epithelia of MC were significantly increased compared with that of NC ( $P<0.01$ ). The area and intensity of Bcl-2 expression in the colonic epithelia of PHM and EA were

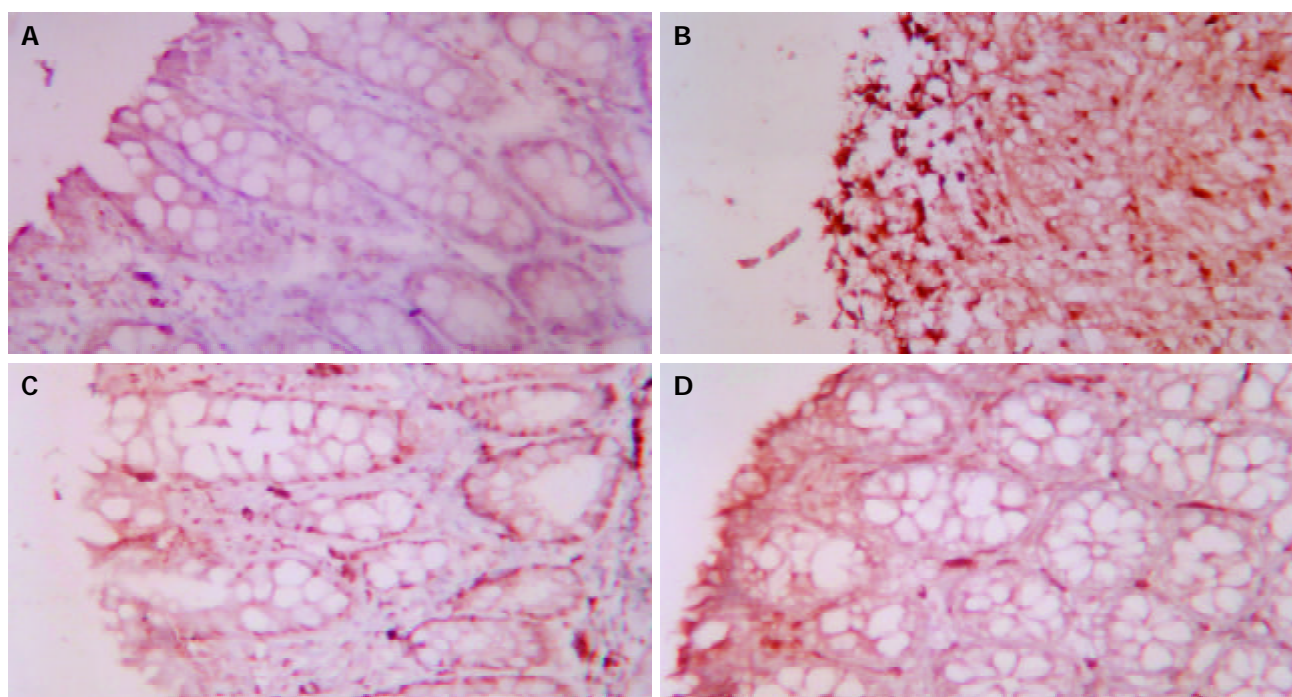
markedly decreased compared with that of MC, but which were not as low as that of NC.

***The effect of acupuncture and moxibustion on Fas expression in the colonic epithelium of UC rats is shown in Table 4 and Figure 4(A-D)***

Table 4 shows that the area and intensity of Fas expression in the colonic epithelia of MC were significantly increased compared with that of NC ( $P<0.01$ ). The area and intensity of Fas expression in the colonic epithelia of PHM and EA were markedly decreased compared with that of MC ( $P<0.01$ ), but still had a significant difference compared with that of NC ( $P<0.05$ ).



**Figure 2** A: Bax expression in colonic epithelia of NC  $\times 200$ , B: Bax expression in colonic epithelia of MC  $\times 200$ , C: Bax expression in colonic epithelia of EA  $\times 200$ , D: Bax expression in colonic epithelia of HPM  $\times 200$ .



**Figure 3** A: Bcl-2 expression in colonic epithelia of NC  $\times 200$ , B: Bcl-2 expression in colonic epithelia of MC  $\times 200$ , C: Bcl-2 expression in colonic epithelia of EA  $\times 200$ , D: Bcl-2 expression in colonic epithelia of HPM  $\times 200$ .

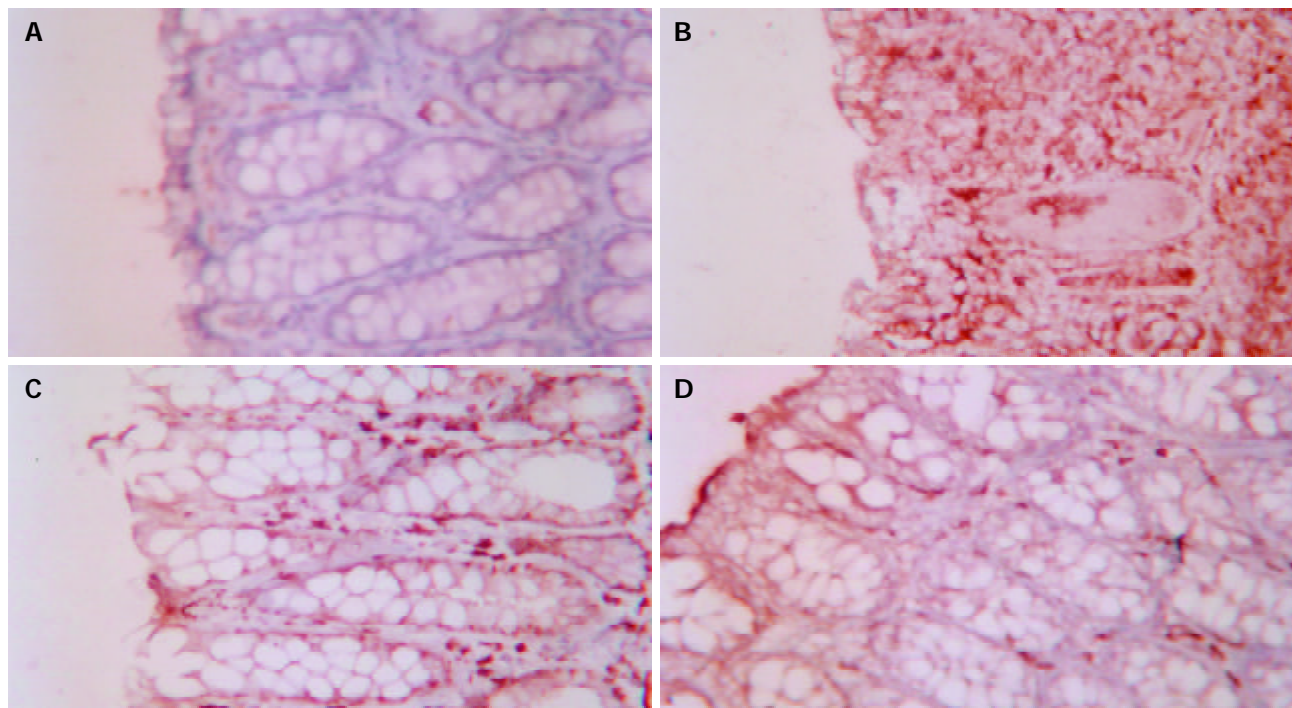
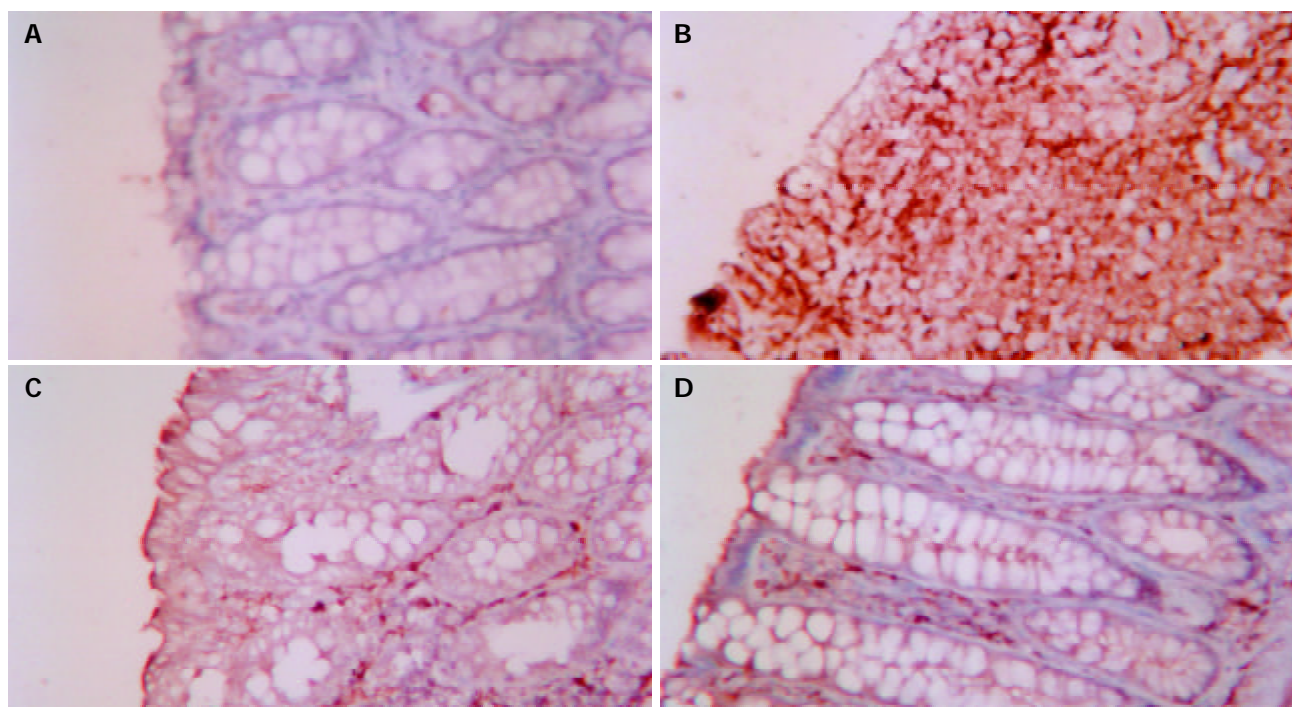


**Table 3** Bcl-2 expression in colonic epithelia of different groups (mean±SD)

Group	n	Area of expression (μm <sup>2</sup> )	Intensity of expression
NC	6	30 863.61±2 273.44	0.1539±0.0114
MC	8	44 757.67±28.1.53 <sup>b</sup>	0.2242±0.0196 <sup>b</sup>
HPM	8	40 061.63±4 937.84 <sup>be</sup>	0.1979±0.0177 <sup>bd</sup>
EA	8	39 219.04±3 449.84 <sup>db</sup>	0.1875±0.0133 <sup>bd</sup>

<sup>b</sup>*P*<0.01 vs NC; <sup>d</sup>*P*<0.01 vs MC; <sup>e</sup>*P*<0.05 vs MC.**Table 4** Fas expression in colonic epithelia of different groups (mean±SD)

Group	n	Area of expression (μm <sup>2</sup> )	Intensity of expression
NC	6	33 764.67±4 422.37	0.1722±0.0153
MC	8	50 262.08±4 780.34 <sup>b</sup>	0.2500±0.0212 <sup>b</sup>
HPM	8	37 992.29±3 239.23 <sup>de</sup>	0.1825±0.0200 <sup>d</sup>
EA	8	38 913.21±4 669.80 <sup>de</sup>	0.1850±0.0138 <sup>d</sup>

<sup>b</sup>*P*<0.01 vs NC; <sup>d</sup>*P*<0.01 vs MC; <sup>e</sup>*P*<0.05 vs NC.**Figure 4** A: Fas expression in colonic epithelia of NC ×200, B: Fas expression in colonic epithelia of MC ×200, C: Fas expression in colonic epithelia of EA ×200, D: Fas expression in colonic epithelia of HPM ×200.**Figure 5** A: FasL expression in colonic epithelia of NC ×200, B: FasL expression in colonic epithelia of MC ×200, 5: FasL expression in colonic epithelia of EA ×200, D: FasL expression in colonic epithelia of HPM ×200.

**The effect of acupuncture and moxibustion on FasL expression in the colonic epithelium of UC rats is shown in Table 5 and Figure 5(A-D)**

**Table 5** FasL expression in colonic epithelia of different groups (mean±SD)

Group	n	Area of expression (μm <sup>2</sup> )	Intensity of expression
NC	6	33 063.56±3 347.24	0.1561±0.0080
MC	8	44 566.58±4 637.23 <sup>b</sup>	0.2600±0.0105 <sup>b</sup>
HPM	8	38 825.58±2 495.51 <sup>bd</sup>	0.1838±0.0156 <sup>bd</sup>
EA	8	38 553.29±3 489.38 <sup>bd</sup>	0.1850±0.0108 <sup>bd</sup>

<sup>b</sup>P<0.01 vs NC; <sup>d</sup>P<0.01 vs MC.

Table 5 shows that the area and intensity of FasL expression in the colonic epithelia of MC were significantly increased compared with that of NC ( $P<0.01$ ). The area and intensity of FasL expression in the colonic epithelia of PHM and EA were markedly decreased compared with that of MC ( $P<0.01$ ), which had a significant difference compared with that of NC ( $P<0.01$ ).

## DISCUSSION

UC is a non-specific inflammatory intestinal disease. The incidence of UC in our country has an increasing trend yearly. The pathogenesis of ulcerative colitis in rats involved in the abnormality of apoptosis<sup>[5-8]</sup>. Increasing evidence showed that acceleration of epithelial cell apoptosis and inhibition of inflammatory cell apoptosis were closely associated with colonic tissue injury and immunological abnormality in ulcerative colitis<sup>[9-11]</sup>.

Studies showed that cell proliferation, differentiation and apoptosis of epithelial cells in intestines mucosa were a dynamic equilibrium process, and neonate epithelial cells migration along crypt villi from pit cells became mature villous epithelial cells. In physiological condition, apoptosis only occurred on superficial epithelial cells of intestine. In pathologic status, this sequence was destructive. For example, at the area of active inflammation, the apoptotic rate of neonate epithelial cells is accelerated and pit cells were superproliferative<sup>[12-14]</sup>. This alteration would lead to destruction of epithelial barrier of colon and imbalance of intestinal function of absorption and excretion.

Apoptosis is a procedure of death adjusted by a flock of apoptotic genes, the cell apoptosis was determined by the relative gene expression level of a series of apoptosis genes<sup>[15-17]</sup>. The bcl-2 gene kindred is an important apoptosis adjusting gene, the position of Bcl-2 protein is at mitochondrial membrane, endoplasmic reticulum and nuclear membrane. As it could prolong the life of cells, it has been generally accepted as an antiapoptosis gene<sup>[18-23]</sup>. Bax is a new member of bcl-2 gene kindred, it could form a dimer with bcl-2 to inhibit its function<sup>[24-27]</sup>. The relative expression ratio of bcl-2 and bax determines whether apoptosis happens in cells or not. When expression of bax gained advantage, apoptosis would occur and when the expression of bcl-2 gained advantage, the cells would continue to exist<sup>[28-32]</sup>. Many studies have shown that abnormal apoptosis in ulcerative colitis could be affected by many agents<sup>[33-36]</sup>.

This study showed that persistent inflammation resulted in the abnormal increase of epithelial cell apoptosis in UC rats. Meanwhile, the area and intensity of Bcl-2 and Bax expression in colonic epithelia of MC were significantly increased compared with that of NC, suggesting that epithelial cell apoptosis is abnormally active. The upregulation of Bcl-2 and Bax expression in colonic epithelia increased the number of

apoptotic cells, which might be one of the important mechanisms of colonic pathological changes in UC. After the treatment with electro-acupuncture and herbs-partition moxibustion, the ulceration of colonic tissues in both groups was markedly improved and the tissue structure was well restored. The number of apoptotic cells in colonic tissues in EA and HPM was significantly decreased compared with that of NC. The area and intensity of Bcl-2 and Bax expression in colonic epithelia of PHM and EA were markedly decreased compared with that of MC. Especially, Bax expression was much downregulated. The above results showed that electro-acupuncture and herbs-partition moxibustion could inhibit colonic epithelial cell apoptosis of UC rats by decreasing Bcl-2 and Bax expression. The extent of Bcl-2 and Bax expression in colonic epithelia of UC rats downregulated by acupuncture and herbs-partition moxibustion was different, thus the relative ratio of Bcl-2 and Bax expression in colonic epithelia was changed due to the inhibition of the abnormal increase of epithelial cell apoptosis in UC. The above results showed that down-regulation of the inflammatory reaction of colonic epithelia in UC rats, inhibition of the injurious effect of a variety of proinflammatory cytokines on colonic tissues, decrease of Bcl-2 and Bax expression of epithelial cells and regulation of the relative ratio of Bcl-2 and Bax expression could change the active state of colonic epithelial cell apoptosis due to its decrease. This is one of the important mechanisms of acupuncture and moxibustion in regulating apoptosis and treating UC.

Many studies<sup>[37-39]</sup> have shown Fas/FasL is an important pathway of epithelial cell apoptosis in UC. Fas is also named Apo-1 or CD95, belongs to tumour necrosis factor receptor (TNFR) kindred, it has comprehensive expressions in various histocytes and can bind anti-Fas antibody or FasL to change the constitution of cell surface. So it can transmit signals in cells to switch on apoptosis mechanism, leading to apoptosis of cells that express Fas. The function of Fas/FasL is to maintain immune stability of body and balance of body's apoptosis<sup>[40-44]</sup>. Normally colonic epithelium could express Fas, and a small quantity of cells could express FasL in the site where the number of apoptosis cells was markedly increased. When UC occurred, because of stimulation by inflammation, the increase of FasL expression would cause apoptosis<sup>[45,46]</sup>. The high expression of FasL in active UC could cause apoptosis of cells expressing Fas<sup>[47-49]</sup>, this would accelerate migration and activity of neutrophils and lymphocytes, causing progressive mucosal lesion of UC<sup>[50-52]</sup>. Therefore, the study of epithelial cell apoptosis may develop a new effective therapeutic approach to UC.

The study showed that the area and intensity of Fas and FasL expression in colonic epithelia of MC were significantly increased compared with that of NC as apoptosis increased, suggesting that the high expression of Fas and FasL plays an important role in epithelial cell apoptosis in UC. After the treatment with electro-acupuncture and herbs-partition moxibustion, Fas and FasL expressions in colonic epithelia of both groups were markedly downregulated compared with MC, and the number of apoptosis cells was also decreased. The above results showed that regulating the abnormal expression of Fas and FasL in colonic tissues of UC rats and decreasing its epithelial cell apoptosis might be one of the important mechanisms of acupuncture and moxibustion in treating UC. Our previous study showed that acupuncture and moxibustion could markedly inhibit the expression of proinflammatory cytokines such as IL-1β and IL-6<sup>[53-56]</sup>. It is suggested that acupuncture and moxibustion can regulate Fas and FasL expression in colonic tissues of UC rats, and may be associated with the inhibition of the activation of macrophages in colonic epithelia and decrease of the expression of proinflammatory cytokines such as IL-1β and IL-6. Further activation of

macrophages in the blocked initial cascade reaction of inflammation and immunity in colonic epithelia can be effectively controlled. Stimulation of inflammatory cytokines on colonic tissues is relieved and stability of immunological function in UC rats is restored.

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