Dig Dis Sci
DOI 10.1007/s10620-012-2161-0

ORIGINAL ARTICLE

Moxibustion Inhibits Apoptosis and Tumor Necrosis Factor-Alpha/Tumor Necrosis Factor Receptor 1 in the Colonic Epithelium of Crohn's Disease Model Rats

Chun-Hui Bao · Lu-Yi Wu · Huan-Gan Wu · Yin Shi · Hui-Rong Liu · Rong Zhang · Li-Qing Yu · Jin-Hai Wang

Received: 25 April 2011 / Accepted: 29 March 2012
© Springer Science+Business Media, LLC 2012

Abstract
Background Previous studies have shown that moxibustion on Tianshu (ST25) and Qihai (CV6) is effective for treating Crohn's disease. However, the mechanism of moxibustion has not been clearly elucidated.
Aim The purpose of this study was to investigate the effect of moxibustion on the inhibition of colonic epithelial cell apoptosis and on tumor necrosis factor alpha (TNF-alpha) and tumor necrosis factor receptor TNF receptor-1 (TNFR1) and TNFR2 and to determine the mechanism of its protective effect using Crohn's disease (CD) model rats.
Methods and Results The experimental CD rat models were established by the administration of trinitrobenzene sulfonic acid. In the herbs-partitioned moxibustion (HPM) and mild-warm moxibustion (MWM) groups, moxibustion was administered to Tianshu (ST25) and Qihai (CV6) acupoints once daily for 14 days. In the salicylazosulfapyridine (SASP) group, SASP was administered twice daily for 14 days. A normal control (NC) group and a model control (MC) group were also studied. The levels of TNF-alpha and its mRNA, TNFR1 as well as the rate of colonic epithelial cell apoptosis were significantly decreased in the HPM, MWM and SASP groups compared with the MC group. The HPM and MWM groups had lower mRNA expression and lower protein levels of TNF-alpha compared to the SASP group. The HPM and MWM groups exhibited less apoptosis than the SASP group.
Conclusions Moxibustion may inhibit colonic epithelial cell apoptosis by reducing the high expression of TNF-alpha and TNFR1 to protect the defective colonic epithelial barrier in CD model rats.

Keywords Moxibustion · Colonic epithelial cell apoptosis · Tumor necrosis factor-alpha · Tumor necrosis factor receptor-1 · Crohn's disease · Rat

C.-H. Bao · H.-G. Wu
Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai 200437, China

C.-H. Bao · H.-G. Wu · Y. Shi (✉) · H.-R. Liu · R. Zhang · L.-Q. Yu · J.-H. Wang
Shanghai Institute of Acupuncture-Moxibustion and Meridian, Shanghai University of Traditional Chinese Medicine, Shanghai 200030, China
e-mail: flysy0636@163.com

L.-Y. Wu
Shanghai Qi Gong Institute, Shanghai University of Traditional Chinese Medicine, Shanghai 200030, China

Introduction

Crohn's disease (CD) is a full-layer inflammation of the digestive wall that may affect any part of the gastrointestinal tract from the mouth to the anus. Its exact cause is still unknown. In recent years, studies [1–5] have shown that damage to the intestinal mucosa barrier, mediated by numerous inflammatory cytokines, plays an important role in the occurrence and development of CD. Among these inflammatory cytokines, tumor necrosis factor alpha (TNF-alpha) has been considered a key factor in CD [1–3]. It induces the release of various other inflammatory mediators, such as IL-1 and IFN-γ, and activates the systemic and local intestinal immune systems, causing an immune chain reaction and initiating a series of immune induction events [4, 5]. It also increases the mucosal permeability by changing the shape and function of colonic epithelial cells [6, 7] and induces colonic epithelial apoptosis by inducting of TNF receptor-1 (TNFR1) and/or TNFR2 [8–10], thus

perpetuating intestinal epithelial barrier damage and intestinal inflammation. Therefore, further study is required to clarify the relationships between colonic epithelial cell apoptosis, TNF-alpha and TNFR.

Clinical studies indicate that circulating serum levels of TNF-alpha are increased in CD patients [11]. Neutralizing TNF monoclonal antibodies has been shown to reverse disease activity in CD patients [12, 13], further supporting the pathogenic role of TNF in the colon epithelium. In our previous clinical study, we observed that HPM therapy is effective for CD patients with mild disease activity [14], but its mechanism is unclear. Moxibustion, both herbs-partitioned moxibustion (HPM) and mild-warm moxibustion (MWM), decreased TNF-alpha levels, which were significantly elevated in CD model rats, and alleviated the symptoms of colonitis [15]. We also observed that moxibustion therapy can inhibit the apoptosis of epithelial cells in rats with ulcerative colitis [16]. To date, neither the relationship between moxibustion and apoptosis of the colonic epithelium in CD nor the pathway by which moxibustion regulates TNF-alpha and TNFR levels has been defined.

Therefore, in this paper, we aim to observe the effect of moxibustion on the regulation of colonic epithelial cell apoptosis, TNF-alpha, TNFR1 and TNFR2 in the colonic mucosa and the probable relationship between them.

Methods

Animals

Sprague–Dawley (SD) rats (male, SPF, 140 ± 10 g) were purchased from the Shanghai University of Traditional Chinese Medicine. All rats were provided with humane care in a temperature-controlled room with a 12-h light–dark cycle and free access to food and water in the home cage. All animal experiments in this study were performed under the guidelines approved by the Animal Ethics Committee of the Shanghai University of TCM.

CD Model Establishment

Fifty-five male SD rats were randomly divided into normal control (NC, $n = 12$) and model control (MC, $n = 43$) groups. The models were prepared according to Morris' method [17]. Prior to model establishment, all rats were fed with water only for 24 h, and then anesthetized with 30 mg/kg 2 % sodium pentobarbital via intraperitoneal injection. The model rats were administered TNBS/ethanol (100 mg/kg TNBS + 50 % ethanol 0.25 ml) through the anus by a rubber tube; the solution was retained in the gut cavity at a depth of 6–8 cm. Rats were fixed in a handstand posture for 1 min after the rubber tube was drawn away to prevent solution overflow. This procedure was performed on days 7, 14, 21 and 28. NC rats were administered saline by the same method as stated above for the model rats. When the experimental CD rat models were prepared, one rat was randomly selected from each group to ascertain whether the CD models were successful by hematoxylin and eosin staining of colonic tissues.

Groups and Treatment

When the experimental CD rat models were confirmed to be successfully established, they were subdivided into an MC group, HPM group, MWM group and salicylazosulfapyridine (SASP) group. In the HPM group, moxa cones (0.5 cm in diameter and 0.6 cm high) made of refined mugwort floss were placed on the herbal cake (medicinal formula dispensing [radix] Aconiti praeparata, [cortex] Cinnamomi, etc.) at Tianshu (ST25) and Qihai (CV6) and ignited (Fig. 1a). The intestinal functions were regulated by ST25 and CV6. Two moxa cones were used for each treatment, which occurred once daily for 14 days. In the MWM group, moxa sticks were ignited 1–2 cm above ST25 and CV6 for 10 min once daily for 14 days (Fig. 1b), with the temperature of the local area kept at 43 ± 2 °C. The rats in the SASP group were fed with SASP, which was prepared at a proportion of 1:0.018 [18], twice a day for 14 days. The rats in the MC and NC groups did not receive any treatments.

Histological Observation

After treatments, all rats were sacrificed simultaneously. Approximately 6 cm of the colon lesion was reserved at 6–8 cm distance from the anus. A 1-cm length of the dissected colon was removed, washed with iced saline, fixed in 10 % formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, dehydrated in 95, 90 and 70 % ethanol, cleared in xylene, mounted in Permount or Histoclad and observed under a light microscope.

Colonic Epithelial Cell Isolation and Purification in Normal Rats

Colonic epithelial cells were isolated and purified immediately according to the method of Evans et al. [19]. Colon samples of approximately 2–3 cm were washed three times with pre-chilled antibiotic lotions to adequately remove the colon contents, then sectioned into 1 mm^3 tissue pieces and placed into sterile plates. After washing with an antibiotic lotion, the samples were rinsed five times, and then new antibiotic lotion was added. They were then left at room temperature for 30 min. When the antibiotic lotion was

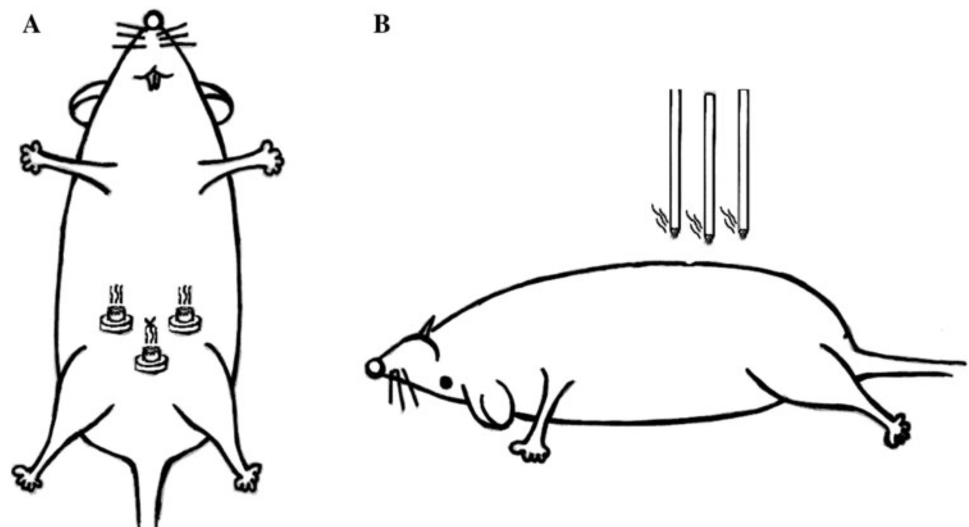

Fig. 1 Herbs-partitioned moxibustion (**a**) and mild-warm moxibustion (**b**) at ST25 and CV6 acupoints

removed, 15 ml of fresh digestive enzymes were added for digestion, and the sample was shaken for 30 min at 37 °C. Afterwards, the samples were blown forcefully for 5 min with a straw, placed into 25 ml sterile centrifuge tubes, and left at room temperature for 60 s. The supernatants were then transferred carefully into another centrifuge tube. The above steps were repeated two to three times. Ten milliliters of DMEM culture solution was added to the supernatant fluid and centrifuged slowly at 500 revs/min ($R = 12$ cm). The supernatant fluid and the precipitate from the MC, HPM, MWM and SASP groups were collected for mixed culture and apoptosis detection, respectively. In the NC group, we discarded the supernatant fluid and divided the precipitate into two parts: one was used for apoptosis detection, and the other was used for cell identification and cultivation. To the latter part, 20 ml of DMEM was added. After blowing, it was centrifuged again at 500 rpm/min for 2–3 min. These steps were repeated five to six times. Then, an appropriate amount of DMEM was added to the precipitate for resuspension. The turbid liquid was then transferred into a culture flask and incubated at 37 °C in a 5 % CO_2 incubator overnight. The next day, colonic epithelial cell identification was performed, and apoptosis was examined by an immunohistochemical SP assay and flow cytometry, respectively.

Immunohistochemical Assay

The cells cultured in vitro from the colon samples of normal rats were identified using an immunohistochemical SP assay in order to confirm that these cells were colonic epithelial cells. The cells were digested by digestive enzymes and blown adequately to create a single cell suspension. The single cell suspension was dripped onto a cover slip in a 24-well culture plate and cultured at 37 °C in a 5 % CO_2 culture case for 24–48 h. Immunohistochemical staining was performed at 90 % confluence. The cover slips harboring cells were rinsed with 0.01 M PBS (pH 7.4) for 3 s, then 3.4 % paraformaldehyde was used to fix them for 30 min, followed by another rinse with PBS for 3 min. One percent H_2O_2 was used to eliminate endogenous peroxidase activity; after 20 min, the cover slips were washed with PBS 3 min × 3 times. For antigen retrieval, the slides were treated twice for 10 min (placed into 0.01 M pH 6.0 citric acid buffer, microwaved at a power level of 3 at 98 °C for 10 min × 2) and then allowed to cool to room temperature. After washing with PBS three times for 3 min, slides were sealed with 1 % normal serum at room temperature for 20 min. Without washing, primary antibody anti-rat keratin 19 (Sigma) was diluted at 1:200, added to the slides and incubated at 37 °C for 1–2 h. After washing with PBS three times for 3 min, secondary antibody was added directly (with no dilution) and incubated at 37 °C for 10–15 min, followed by washing with PBS three times for 3 min. Streptavidin horseradish peroxidase conjugate (S–A/HRP) was added and slides were incubated at 37 °C for 10–15 min. After washing with PBS three times for 3 min, 0.04 % DAB and 0.03 % H_2O_2 were used for color development. After 8 min, slides were rinsed with water and then sealed with neutral gum and stored at 2–8 °C in a light-proof area for further observation.

The Induction of Colonic Epithelial Cell Apoptosis in Normal Rats by the Colonic Mucosa Supernatant in Different CD Model Groups

After 1 week, when the cells of normal rats reached >80 % confluence in the culture flask, we sieved the colonic mucosa supernatants of MC, HPM, MWM and SASP

groups through a 200 mesh screen, and 5 ml of each supernatant was combined with the colonic epithelial cells of normal rats cultured overnight. After mild agitation, we resuspended the cells in the appropriate amounts of DMEM and then incubated them at 37 °C in a 5 % CO_2 culture case for 48 h. Cells were observed microscopically, and observations were recorded daily. The culture liquid was changed once every 2 days. After 1 week, the culture liquid was examined by flow cytometry to detect colonic epithelial cell apoptosis of normal rats induced by the colonic mucosa supernatant from different CD model groups.

Flow Cytometry Assay

Five hundred grams of suspension cells were washed with ice-cold PBS and incubated at 4 °C for 5 min. The supernatant was removed, and the cells were filtered again with ice-cold binding buffer to a concentration of 5×10^5–5×10^6/ml. Five microliters of Annexin V-FITC (100 U/ml, Sigma) and 2.5 μl of propidium iodide (50 μg/ml, Sigma) were added into a 100 μl suspension, gently mixed, and then incubated on ice in darkness for 10 min. Four hundred microliters of ice-cold 1 × binding buffer was added into the suspension. After gently mixing for 30 min, the suspension was analyzed by flow cytometry to determine the cell cycle distribution and proportion of apoptotic cells using a FACSort instrument (Becton–Dickinson, Boston, MA, USA), and the data were analyzed with Cell Quest software (version 3.2.1f1, Becton–Dickinson).

TUNEL Assay

Frozen 8-μm sections of rat colonic samples were prepared, fixed in acetone at 4 °C, and then processed for TUNEL staining (TdT-FragEL DNA fragmentation detection kit, MERCK). The number of TUNEL-positive cells was counted in five visual fields (400×), and the TUNEL positive ratio was calculated.

Enzyme Linked Immunosorbent Assay

The frozen rat colonic samples were homogenized in a homogenization buffer (10 mmol/l phosphate buffer, 250 mmol/l sucrose, 1 mmol/l EDTA, 0.1 mmol/l PMSF, and 0.1 % tergitol, pH 7.5). Homogenates were centrifuged at 43,000×*g* for 10 min at 4 °C, and the supernatant was isolated. Protein concentrations were determined by the Pierce bicinchoninic acid assay. The levels of TNF-alpha were detected by an enzyme linked immunosorbent assay (ELISA) using a TNF ELISA kit (DRG, USA) following the manufacturer's instructions. First, all agents were removed from the kits and allowed to reach room temperature (20–25 °C) for 30 min. A standard preparation of 100 μl and a 100-μl sample were infused in corresponding pores of an enzyme scale plate, with 10 μl bioepiderm HRP tag liquid added, and gently mixed for 30 s and incubated for 30 min at 36 ± 2 °C. Then, we discarded all the liquid in the enzyme scale plate, washed the enzyme scale plate with eluent (350 μl for each pore), and removed the water with drying papers. Washing and drying were repeated five times. After the addition of 100 μl of biotin anti-rat TNF-alpha and incubation for 30 min at 36 ± 2 °C, all of the liquid in the enzyme scale plate was removed. The enzyme scale plate was washed with eluent (350 μl for each pore), and water was removed by drying papers five times. Fifty microliters of A and B coloration liquid were dripped into each pore, gently mixed for 10 s and incubated for 30 min at 36 ± 2 °C away from light. Fifty microliters of stop buffer was added and gently mixed for 30 s. Each pore's OD value was then observed with a 450-nm enzyme scale instrument within 15 min. We drew a standard curve with the OD value represented on the *Y*-axis, and the standard preparation concentration on the *X*-axis. The concentration of samples could be obtained using this standard curve according to their OD values.

Real-Time PCR

Total cellular RNA was isolated from the rat colonic epithelial cell samples using Trizol reagent (Invitrogen). Three micrograms of total RNA was used as a template for reverse transcription using a superscript reverse transcriptase kit (Invitrogen). The cDNA samples were then applied for PCR with the following primer pairs: for *β*-actin, sense 5′-TTGCTGACAGGATGCAGAAGGAGA-3′ and antisense 5′-ACTCCTGCTTGCTGATCCACATCT-3′; and for TNF-alpha, sense 5′-CTGGCCAATGGCATGGATCT CAAA-3′ and antisense 5′-ATGAAATGGCAAATCGGC TGACGG-3′. Real time PCR was performed with a QuantiTect SYBRgreen PCR kit (Qiagen) using an ABI7500 real-time PCR system and 7500 system SDS software (Applied Biosystems). mRNA expression data were calculated with the $2^{-\Delta\Delta C_t}$ method normalized to the expression of *β*-actin.

Western Blotting

Sixty micrograms of protein extracted from the isolated rat colonic epithelial cell samples was separated by SDS–PAGE and transferred to a PVDF membrane. Blots were blocked for 2 h in 5 % milk powder in phosphate-buffered saline and overnight in 5 % bovine serum albumin in phosphate-buffered saline (at 4 °C) before incubation with primary antibodies for 90 min at room temperature. Primary rabbit monoclonal immunoglobulin (Ig) G antibodies

were directed against TNFR1 (1:200) (Santa–Cruz), TNFR2 (1:200) (Santa–Cruz) and β-actin (1:300) (Abcam). The films were visualized using an enhanced chemiluminescence system (Pierce Company, Minneapolis, MN, USA).

Statistical Analysis

Results were expressed as the mean ± SD. Statistical analyses were performed using SPSS 13.0 (SPSS Inc., Wacker Drive, Chicago, IL, USA). Differences in the means were compared by one-way ANOVA. Differences were considered statistically significant if $P < 0.05$.

Results

Clinical Evaluation

Compared with the weights in the NC group, those in the MC group were markedly reduced. Compared with the model rats, the weights of rats in the SASP group, MWM group and HPM group were increased significantly; however, there were no significant differences among the SASP, MWM and HPM groups. In addition, the stool of the rats in the MC group presented as pasty, and some even exhibited bloody stool. The rats in the three treatment groups were obviously improved compared with the MC group. Stools were well formed in some rats in the SASP group, and the mucous and bloody stool disappeared. Additionally, stools were well formed in the majority of the rats in the MWM and HPM groups (Table 1).

Histological Analysis

Light microscope observation of NC rats showed the complete structure of the colonic mucosa and the clear structure of the epithelium, and the glands of the colonic epithelium were regularly arranged with no apparent inflammatory cell infiltration (Fig. 2a). In the MC group, the epithelial lesion of the mucus membrane, inflammatory cell infiltration (such as eosinophils, neutrophils and lymphocyte), obvious sub-mucosa congestion, hemorrhage and hydroncus, damaged or absent glands, the proliferation of fibrocytes, the formation of granulation tissue and fissure ulceration were shown (Fig. 2b). The three treatment groups improved to different degrees. In the SASP group, epithelial lesions of part of the mucus membrane, small amounts of inflammatory cell infiltration, sub-mucosa congestion and hydroncus, as well as proliferation of small amounts of fibrocytes were observed, although these symptoms were not as serious as those in the MC group (Fig. 2c). The MWM group demonstrated mild epithelial lesions of the mucus membrane, mild congestion, and proliferation of small amounts of fibrocytes (Fig. 2d). The HPM group rats showed a basic complete structure of the mucosal epithelium, small amounts of inflammatory cell infiltration, mild congestion and hydroncus (Fig. 2e). The colonic inflammation of the MWM and HPM groups was relieved compared with that in the MC group.

Table 1 Body weight ($n = 10$) (mean ± SD)

Group	Body weight (g)
NC	377.20 ± 15.47
MC	281.80 ± 24.30[a]
SASP	324.40 ± 26.05[b]
MWM	348.50 ± 24.51[c]
HPM	341.50 ± 35.82[c]

SD standard deviation, *NC* normal group, *MC* model group, *SASP* salicylazosulphapyridine group, *MWM* mild-warm moxibustion group, *HPM* herbs-partitioned moxibustion group

[a] $P < 0.01$ versus NC group

[b] $P < 0.05$ versus MC group

[c] $P < 0.01$ versus MC group

Identification of the Colonic Epithelial Cells

The expression of keratin 19 was detected by an immunohistochemistry SP assay to identify the cells cultured in vitro from the colon tissues of normal rats. Most cells showed features that are typical of colonic epithelial cells: round, oval or polygonal and positive for keratin 19 (Fig. 3).

Colonic Epithelial Cell Apoptosis In Vivo

Compared with the NC group, the rates of colonic epithelial cell apoptosis in the MC, SASP, MWM and HPM groups were significantly increased. Compared with the MC group, the numbers of apoptotic cells in the MWM, HPM and SASP groups were significantly decreased (Table 2).

Colonic Epithelial Cell Apoptosis in Normal Rats Induced by Colonic Mucosa Supernatants In Vitro

Compared with the MC group, the rates of colonic epithelial cell apoptosis in the SASP, MWM and HPM groups were significantly decreased; the MWM and HPM groups had lower numbers of apoptotic cells than the SASP group (Table 3).

Colonic Epithelial Cell Apoptosis by TUNEL

Compared with the NC group, the rates of colonic epithelial cell apoptosis in the MC and SASP groups were

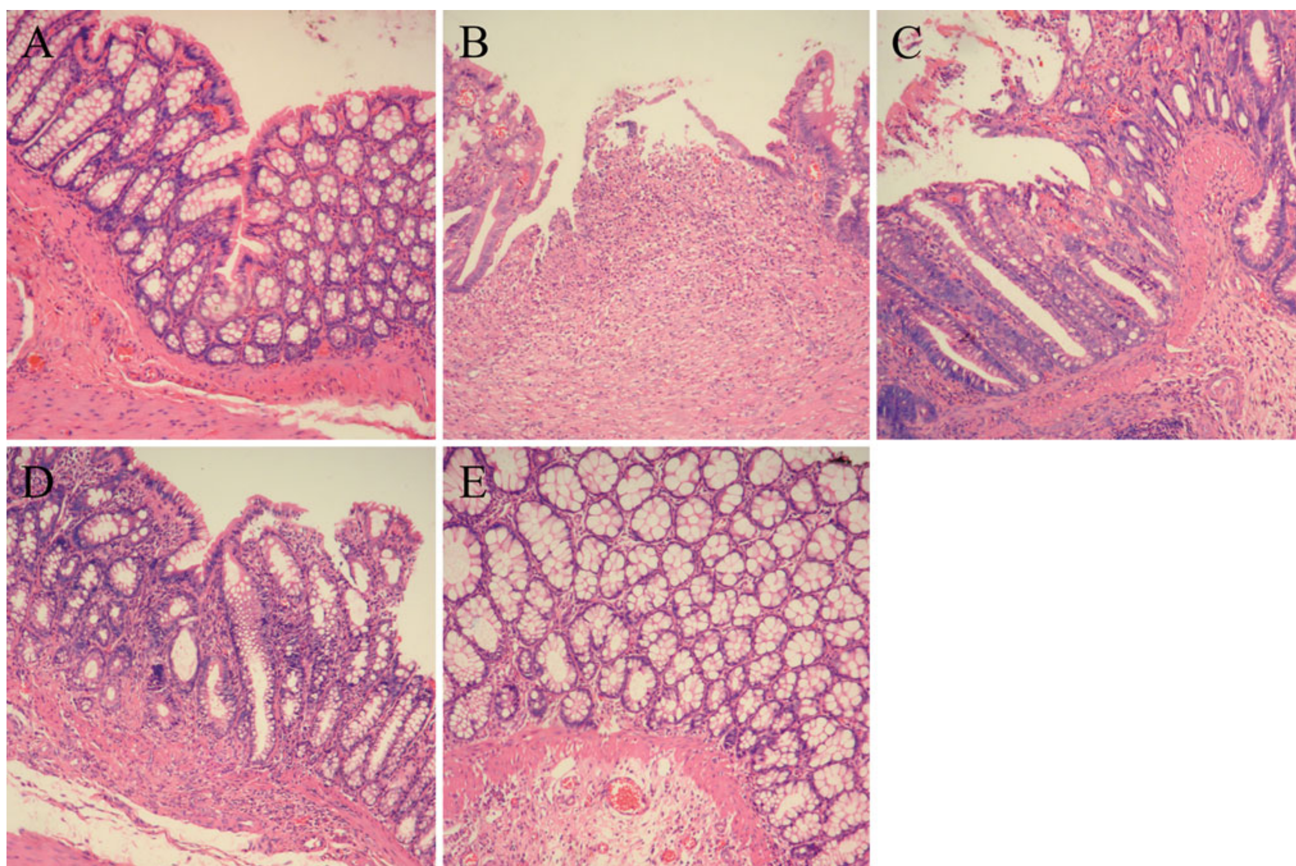

Fig. 2 Histological findings of rat colonic epithelium in different groups. **a** Normal control. **b** Model control. **c** Salicylazosulphapyridine group. **d** Mild-warm moxibustion group. **e** Herbs-partitioned moxibustion group. Original magnifications: ×200

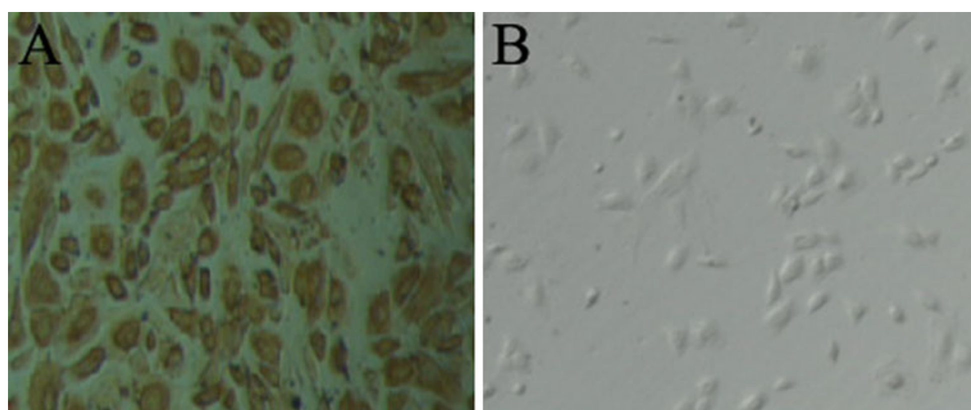

Fig. 3 Identification of the colonic epithelial cells. **a** The expression of keratin 19 was detected by an immunohistochemical assay. **b** Negative control. Original magnifications: ×400

significantly increased (Table 4). Compared with the MC group, the rates of colonic epithelial cell apoptosis in the MWM, HPM and SASP groups were significantly decreased. Additionally, there was a greater number of apoptotic cells in the colonic epithelium in the SASP group than in the MWM or HPM groups (Fig. 4).

Expression of TNF-Alpha

The levels of TNF-alpha in the colonic mucosa in the MC and SASP groups were significantly higher than in the NC group. The MWM and HPM groups had less TNF-alpha than the MC group. The SASP group had less TNF-alpha

Table 2 Rate of colonic epithelial cell apoptosis in vivo by FCM ($n = 10$) (mean ± SD)

Group	AR (%)
NC	20.88 ± 6.72
MC	82.78 ± 2.65[a]
SASP	58.46 ± 13.98[a,b]
MWM	53.01 ± 15.17[a,b]
HPM	54.33 ± 13.32[a,b]

AR apoptosis rate, *SD* standard deviation, *FCM* flow cytometry, *NC* normal group, *MC* model group, *SASP* salicylazosulphapyridine group, *MWM* mild-warm moxibustion group, *HPM* herbs-partitioned moxibustion group

[a] $P < 0.01$ versus NC group

[b] $P < 0.01$ versus MC group

Table 3 Rate of colonic epithelial cell apoptosis in normal rats induced by colonic mucosa supernatants in vitro by FCM ($n = 10$) (mean ± SD)

Group	AR (%)
MC	52.66 ± 7.72
SASP	39.15 ± 5.78[a]
MWM	30.59 ± 9.25[a,b]
HPM	29.61 ± 9.13[a,b]

AR apoptosis rate, *SD* standard deviation, *FCM* flow cytometry, *MC* model group, *SASP* salicylazosulphapyridine group, *MWM* mild-warm moxibustion group, *HPM* herbs-partitioned moxibustion group

[a] $P < 0.01$ versus NC group

[b] $P < 0.05$ versus MC group

Table 4 Rate of colonic epithelial cells apoptosis by TUNEL ($n = 10$) (mean ± SD)

Group	Rate (%)
NC	0.018 ± 0.008
MC	0.080 ± 0.021[a]
SASP	0.041 ± 0.016[a,b]
MWM	0.026 ± 0.010[b,c]
HPM	0.024 ± 0.010[b,c]

SD standard deviation, *NC* normal control, *MC* model control, *SASP* salicylazosulphapyridine group, *MWM* mild-warm moxibustion group, *HPM* herbs-partitioned moxibustion group

[a] $P < 0.01$ versus NC group

[b] $P < 0.01$ versus MC group

[c] $P < 0.05$ versus SASP group

than the MC group but more than the MWM and HPM groups (Table 5).

Expression of TNF-Alpha mRNA

The levels of TNF-alpha mRNA in the colonic mucosa in the MC, SASP, MWM and HPM groups were significantly higher than in the NC group. The SASP, MWM and HPM groups had less TNF-alpha mRNA than the MC group. Compared with the SASP group, the level of TNF-alpha mRNA was significantly increased in the MWM and HPM groups (Fig. 5).

Expressions of TNFR1 and TNFR2

Compared with the NC group, the expression of TNFR2 was significantly increased in the MC, SASP, MWM and HPM groups (Table 6). The expression of TNFR1 was significantly increased in the MC group, with no significant differences among the SASP, MWM, HPM and NC groups. Compared with the MC group, the expressions of TNFR1 in the SASP, MWM and HPM groups were significantly increased. However, there was no significant difference in TNFR2 expression. There were no significant differences among the SASP, MWM and HPM groups in TNFR1 or TNFR2 expression (Fig. 6).

Discussion

Crohn's disease (CD) is a chronic, recurrent, refractory disease. Despite currently available treatments, many patients experience frequent relapses and significant complications [20]. For thousands of years, moxibustion therapies have been used to treat several different types of disorders in China, notably gastroenterological diseases, such as Crohn's [21], ulcerative colitis [22], and irritable bowel syndrome [23]. However, it is still difficult to establish the biological basis of moxibustion therapy.

In the present study, a histological analysis showed that the colonic inflammatory cells in the lamina propria and submucosa were reduced in the MWM, HPM and SASP groups to different degrees. The colonic epithelium in the MWM and HPM groups was more complete than that in the SASP group. Moxibustion was shown to relieve the intestinal inflammation observed in CD model rats.

As an important immune and proinflammatory factor, TNF-alpha plays indispensible roles in the CD inflammatory reaction, initiation and lasting immune response, especially in the inflammatory reaction of intestinal mucosa [24–26]. A great number of CD colonic epithelial cells quickly succumbed to apoptosis; this disruption of the balance between epithelial proliferation and apoptosis destroys the structure of the intestinal mucosa. It has been shown that apoptosis in defective epithelial cells was increased in patients with CD [27, 28]. Our data from TUNEL and FCM both demonstrated that colonic epithelial cell apoptosis was significantly down-regulated in HPM and MWM groups compared with the MC group in vivo and in vitro. Additionally, the colonic epithelial cell

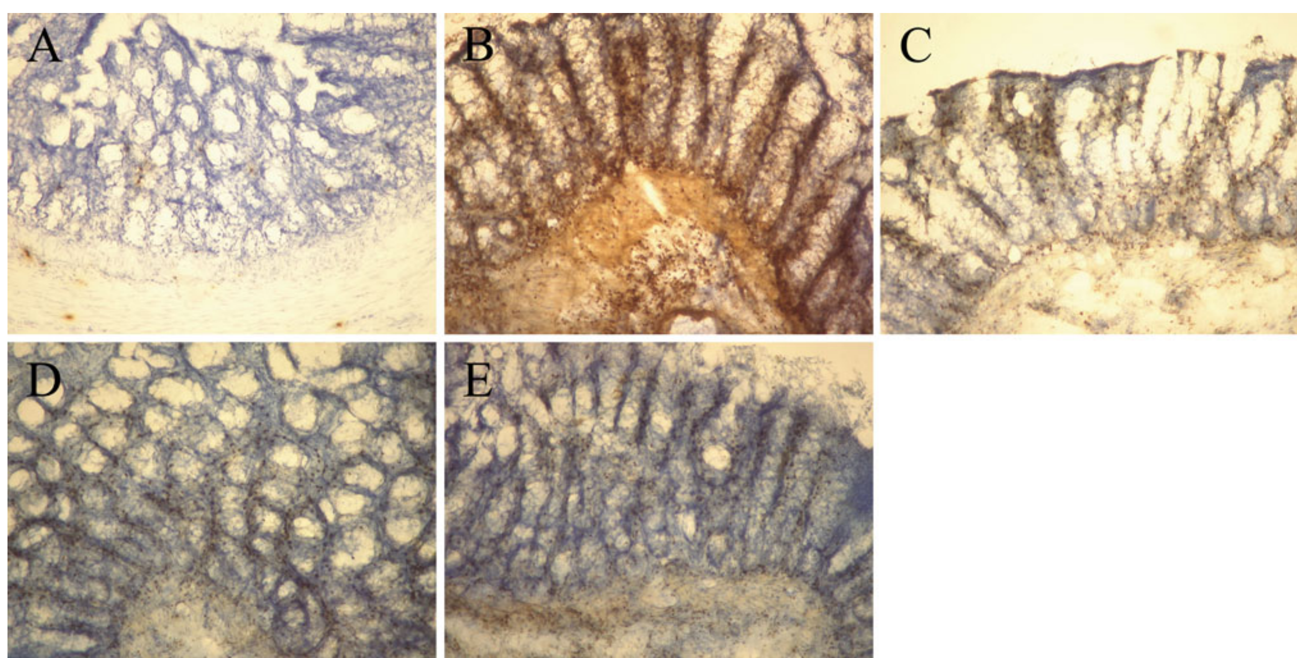

Fig. 4 Apoptosis of colonic epithelial cells. **a** Normal control. **b** Model control. **c** Salicylazosulphapyridine group. **d** Mild-warm moxibustion group. **e** Herbs-partitioned moxibustion group. Original magnifications: ×100

Table 5 TNF-alpha protein level in colonic epithelium ($n = 10$) (mean ± SD)

Group	TNF-alpha (pg/ml)
NC	11.06 ± 1.17
MC	16.03 ± 2.03[a]
SASP	13.65 ± 1.31[a,b]
MWM	12.23 ± 1.39[b,c]
HPM	12.32 ± 1.18[b,c]

TNF tumor necrosis factor, *SD* standard deviation, *NC* normal group, *MC* model group, *SASP* salicylazosulphapyridine group, *MWM* mild-warm moxibustion group, *HPM* herbs-partitioned moxibustion group

[a] $P < 0.01$ versus NC group

[b] $P < 0.01$ versus MC group

[c] $P < 0.05$ versus SASP group

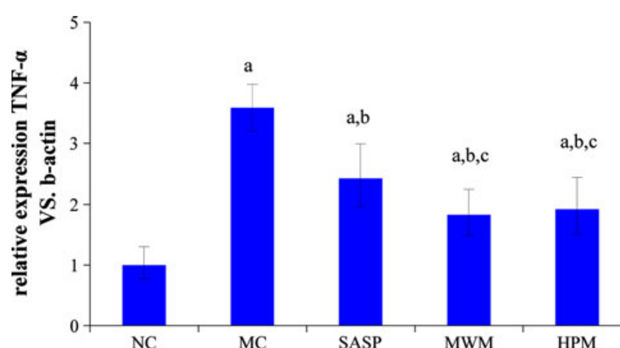

Fig. 5 Expressions of tumor necrosis factor (TNF)-alpha mRNA in rat colonic epithelium. *NC* normal group, *MC* model group, *SASP* salicylazosulphapyridine group, *MWM* mild-warm moxibustion group, *HPM* herbs-partitioned moxibustion group. [a]$P < 0.01$ versus NC group. [b]$P < 0.01$ versus MC group. [c]$P < 0.01$ versus SASP group

apoptosis in these two groups was lower than in the SASP group using TUNEL and FCM in vitro, but there was no significant difference between them using FCM in vivo. The levels of TNF-alpha and its mRNA expression were lower in the MWM and HPM groups than in the MC group or in the SASP group. The TNFR1 levels were also lower in the MWM and HPM groups than in the MC group but not in the response to TNFR2 levels. Therefore, we presume that moxibustion may inhibit colonic epithelial cell apoptosis via the reduction of the high expression of TNF-alpha and TNFR1, but not TNFR2, in CD model rats.

Researchers have shown that TNF-alpha's induction of colonic epithelial apoptosis through the TNF receptor (TNFR) can specifically contribute to intestinal epithelium barrier defects and chronic intestinal inflammation [29, 30]. TNF-alpha induces colonic epithelial apoptosis by binding TNFR on the colonic epithelial cell membrane [31]. Fries et al. [32] confirmed that the proapoptotic effect is mediated through receptor 1 for TNF-alpha and that enterocyte apoptosis was completely prevented by anti-TNF treatments and in TNFR-1−/− mice. Moreover, the same results were achieved using TNFR-1 knockout mice, confirming that an apoptotic detachment of enterocytes was mediated by this receptor [9]. Interestingly, knockout mice for TNFR-1 showed more serious mucosal damage and a higher mortality in TNBS-induced colitis [33, 34], whereas

Table 6 TNFR1 and TNFR2 protein levels in colonic epithelium ($n = 10$) (mean ± SD)

Group	TNFR1	TNFR2
NC	0.19 ± 0.01	0.29 ± 0.02
MC	0.80 ± 0.11[a]	0.85 ± 0.16[a]
SASP	0.24 ± 0.04[b]	0.65 ± 0.06[a]
MWM	0.23 ± 0.02[b]	0.63 ± 0.08[a]
HPM	0.25 ± 0.03[b]	0.67 ± 0.12[a]

TNF tumor necrosis factor, *SD* standard deviation, *NC* normal control, *MC* model control, *SASP* salicylazosulphapyridine group, *MWM* mild-warm moxibustion group, *HPM* herbs-partitioned moxibustion group

[a] $P < 0.01$ versus NC group

[b] $P < 0.01$ versus MC group

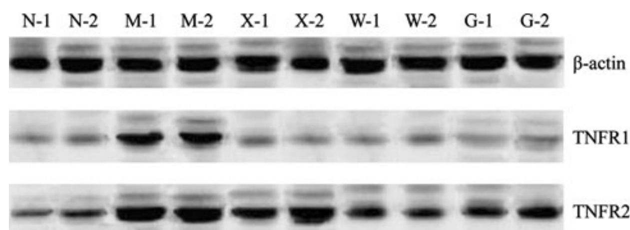

Fig. 6 Expressions of TNFR1 and TNFR2 in rat colonic epithelium. *TNF* tumor necrosis factor, *N* normal group, *M* model group, *X* salicylazosulphapyridine group, *W* mild-warm moxibustion group, *G* herbs-partitioned moxibustion group

those that lacked TNFR-2 developed a less severe disease despite higher serum TNF-alpha levels. Blocking TNF-alpha by a combination of TNF-alpha- and TNFR1-binding peptides effectively improves the symptoms of TNBS-induced colitis and alleviates colonic pathological damage in rats [35].

Further studies revealed that TNF-alpha signals through two distinct cell surface receptors: TNFR1 (P55, CD120α) and TNFR2 (P75, CD120β). Each receptor promotes different cellular responses in a cell context-dependent manner [36, 37]. TNFR1 signaling involves the sequential formation of two complexes, known as complex I and complex II (or DISC) [38, 39]. Immediately after TNF-alpha stimulation, TRADD (TNFR1-associated death domain protein) is recruited to TNFR1. At the same time, TRAF2, RIP1 and cIAP-1/2 translocate to the receptor [38–40]. This immediate TNFR1 signaling complex has been named complex I. Approximately 30–60 min later, while TNFR1 and FADD compete for the same binding site as TRADD and TRADD, TRAF2, RIP1 and cIAP-1/2 dissociate from the receptor. Thus, complex II, which contains TRADD, TRAF2, RIP1 and, importantly, FADD and caspase 8 [38–40], is assembled, and this complex promotes the activation of the caspase cascade resulting in apoptosis.

In conclusion, our results suggest that moxibustion (MWM and HPM) administration of Tianshu (ST25) and Qihai (CV6) acupoints may inhibit colonic epithelial cell apoptosis via the reduction of the high expression of TNF-alpha and TNFR1 to protect the defective colonic epithelial barrier in CD model rats. These findings suggest the possibility of moxibustion therapy as a useful method for the treatment of CD.

Acknowledgments We thank Qing-wen Sun professor and Guo-qing Ji PhD at Fu Dan University for their technical assistance. This work was supported by the National Natural Science Foundation of China, No. 30772831; National Basic Research Program of China (973 program), No. 2009CB522900; and the Shanghai Leading Academic Discipline Project, No. S30304.

Conflict of interest None.

References

1. van Dullemen HM, van Deventer SJ, Hommes DW, et al. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). *Gastroenterology*. 1995; 109:129–135.
2. Ferguson LR, Huebner C, Petermann I, et al. Single nucleotide polymorphism in the tumor necrosis factor-alpha gene affects inflammatory bowel diseases risk. *World J Gastroenterol*. 2008; 14:4652–4661.
3. Kindler V, Sappino AP, Grau GE, et al. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. *Cell*. 1989;56:731–740.
4. Stewart RJ, Marsden PA. Biologic control of the tumor necrosis factor and interleukin-1 signaling cascade. *Am J Kid Dis*. 1995; 25:954–966.
5. Breese EJ, Michie CA, Nicholls SW, et al. Tumor necrosis factor alpha-producing cell in the intestinal mucosa of children with inflammatory bowel disease. *Gastroenterology*. 1994;106:1455–1466.
6. Ma TY, Boivin MA, Ye D, et al. Mechanism of TNF-{alpha} modulation of Caco-2 intestinal epithelial tight junction barrier: role of myosin light-chain kinase protein expression. *Am J Physiol Gastrointest Liver Physiol*. 2005;288:G422–G430.
7. Hilsden RJ, Meddings JB, Sutherland LR. Intestinal permeability changes in response to acetylsalicylic acid in relatives of patients with Crohn's disease. *Gastroenterology*. 1996;110:1395–1403.
8. Gitter AH, Bendfeldt K, Schulzke JD, et al. Leaks in the epithelial barrier caused by spontaneous and TNF-alpha-induced single-cell apoptosis. *FASEB J*. 2000;14:1749–1753.
9. Piguet PF, Vesin C, Guo J, et al. TNF-induced enterocyte apoptosis in mice is mediated by the TNF receptor 1 and does not require p53. *Eur J Immunol*. 1998;28:3499–3505.
10. Zeissig S, Bojarski C, Buergel N, Mankertz J, et al. Downregulation of epithelial apoptosis and barrier repair in active Crohn's disease by tumour necrosis factor alpha antibody treatment. *Gut*. 2004;53:1295–1302.
11. Wang J, Fu YX. Tumor necrosis factor family members and inflammatory bowel disease. *Immunol Rev*. 2005;204:144–155.
12. Yoshizawa S, Hibi T. New therapy in inflammatory bowel disease (infliximab). *Nippon Rinsho*. 2008;66:1279–1285.
13. Peyrin-Biroulet L, Deltenre P, de Suray N, Branche J, et al. Efficacy and safety of tumor necrosis factor antagonists in

Crohn's disease: meta-analysis of placebo-controlled trials. *Clin Gastroenterol Hepatol*. 2008;6:644–653.
14. Shi Y, Wu HG. The clinical study on herbs-partitioned moxibustion treatment of Crohn's disease. *Jiang Xi J TCM*. 2003;38:16–17.
15. Shi Y, Wu HG, Qin XD, et al. Moxibustion effect on TNF-α and TGF-β1 contents in intestinal mucosa with Crohn's disease rats. *Chin J Basic Med Tradit Chin Med*. 2009;15:864–867.
16. Wu HG, Gong X, Yao LQ, et al. Mechanisms of acupuncture and moxibustion in regulation of epithelial cell apoptosis in rat ulcerative colitis. *World J Gastroenterol*. 2004;10:682–688.
17. Morris GP, Beck PL, Herridge MS, et al. Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology*. 1989;96:795–803.
18. Xu SY, Bian RL, Chen X. *Experimental Methodology of Pharmacology*. Beijing: People's Health Publishing House; 1982: 1184.
19. Evans GS, Flint N, Somers AS, et al. The development of a method for the preparation of rat intestinal epithelial cell primary cultures. *J Cell Sci*. 1992;101:219–231.
20. Clark M, Colombel JF, Feagan BC, et al. American Gastroenterological Association consensus development conference on the use of biologics in the treatment of inflammatory bowel disease, June 21–23, 2006. *Gastroenterology*. 2007;133:312–339.
21. Joos S, Brinkhaus B, Maluche C, et al. Acupuncture and moxibustion in the treatment of active Crohn's disease: a randomized controlled study. *Digestion*. 2004;69:131–139.
22. Zhou EH, Liu HR, Wu HG, et al. Down-regulation of protein and mRNA expression of IL-8 and ICAM-1 in colon tissue of ulcerative colitis patients by partition-herb moxibustion. *Dig Dis Sci*. 2009;54:2198–2206.
23. Anastasi JK, McMahon DJ, Kim GH. Symptom management for irritable bowel syndrome: a pilot randomized controlled trial of acupuncture/moxibustion. *Gastroenterol Nurs*. 2009;32:243–255.
24. Braegger CP, Nicholls S, Murch SH, et al. Tumor necrosis factor alpha in stool as a marker of intestinal inflammation. *Lancet*. 1992;339:89–91.
25. Tracey KJ, Cerami A. Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. *Ann Rev Med*. 1994;45:491–503.
26. Neurath MF, Fuss I, Pasparakis M, et al. Predominant pathogenic role of tumor necrosis factor in experimental colitis in mice. *Eur J Immunol*. 1997;27:1743–1750.
27. Schulzke JD, Bojarski C, Zeissig S, et al. Disrupted barrier function through epithelial cell apoptosis. *Ann NY Acad Sci*. 2006;1072:288–299.
28. von Boyen GB, Steinkamp M, Geerling I, et al. Proinflammatory cytokines induce neurotrophic factor expression in enteric glia: a key to the regulation of epithelial apoptosis in Crohn's disease. *Inflamm Bowel Dis*. 2006;12:346–354.
29. Kontoyiannis D, Pasparakis M, Pizarro TT, et al. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity*. 1999;10:387–398.
30. Hering NA, Sohulzke JD. Therapeutic options to modulate barrier defects in inflammatory bowel disease. *Dig Dis*. 2009;27:450–454.
31. Shen L, Su L, Turner JR. Mechanisms and functional implications of intestinal barrier defects. *Dig Dis*. 2009;27:443–449.
32. Fries W, Muja C, Crisafulli C, et al. Infliximab and etanercept are equally effective in reducing enterocyte APOPTOSIS in experimental colitis. *Int J Med Sci*. 2008;5:169–180.
33. Nakai M, Sudo K, Yamada Y, et al. The role of the tumor necrosis factor receptor in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice. *Dig Dis Sci*. 2005;50:1669–1676.
34. Ebach DR, Newberry R, Stenson WF. Differential role of tumor necrosis factor receptors in TNBS colitis. *Inflamm Bowel Dis*. 2005;11:533–540.
35. Yin B, Hu X, Wang J, et al. Blocking TNF-α by combination of TNF-α- and TNFR-binding cyclic peptide ameliorates the severity of TNBS-induced colitis in rats. *Eur J Pharmacol*. 2011;656:119–124.
36. Abu-Amer Y, Erdmann J, Alexopoulou L, et al. Tumor necrosis factor receptors types 1 and 2 differentially regulate osteoclastogenesis. *J Biol Chem*. 2000;275:27307–27310.
37. Ferrero E, Zocchi MR, Magni E, et al. Roles of tumor necrosis factor p55 and p75 receptors in TNF-alpha-induced vascular permeability. *Am J Physiol Cell Physiol*. 2001;281:C1173–C1179.
38. Micheau O, Tschopp J. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell*. 2003;114: 181–190.
39. Schneider-Brachert W, Tchikov V, Neumeyer J, et al. Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles. *Immunity*. 2004;21: 415–428.
40. Jin Z, El-Deiry WS. Distinct signaling pathways in TRAIL-versus tumor necrosis factor-induced apoptosis. *Mol Cell Biol*. 2006;26:8136–8148.