**Basic Study**

# Effect mechanism investigation of herb-partitioned moxibustion on relieving colon inflammation in Crohn disease rats based on neutrophil extracellular traps

## 基于中性粒细胞胞外诱捕网研究隔药灸减轻克罗恩病大鼠结肠炎症的效应机制

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**Abstract**

**Objective**: To explore the mechanism of herb-partitioned moxibustion in relieving rat intestinal inflammation by focusing on the neutrophil extracellular traps (NETs) in Crohn disease (CD) development.

**Methods**: Rats were randomly divided into a normal group, a model group, a herb-partitioned moxibustion group, and a mesalazine group. The CD rat model was prepared with 2,4,6-trinitrobenzene sulfonic acid except for rats in the normal group. Rats in the normal group and model group did not receive any treatment but had the same fixation as the other groups. Rats in the herb-partitioned moxibustion group received herb-partitioned moxibustion at Qihai (CV6) and bilateral Tianshu (ST25). Rats in the mesalazine group received intragastric administration of mesalazine enteric-coated tablets. The general situation of rats in each group was recorded, and the histopathological changes in the colon were observed and scored by hematoxylin-eosin staining. The serum concentrations of NETs DNA (NETs-DNA), neutrophil elastase (NE)-DNA, and myeloperoxidase (MPO)-DNA were detected by ABC enzyme-linked immunosorbent assay, and the citrullinated histone 3 (citH3), MPO, and NE protein and mRNA expression levels in rat colon tissue were observed by immunofluorescence and real-time quantitative polymerase chain reaction.

**Results**: Compared with the normal group, the mucosal ulcer reached the muscularis, the epithelium was incomplete, the goblet cells decreased obviously with significant inflammatory cell infiltration in the colon; the colonic mucosa damage index (CMDI) score increased significantly ($P<0.01$); the serum NETs-DNA, NE-DNA, and MPO-DNA concentrations increased ($P<0.05$); the NE, citH3, and MPO protein and mRNA expression in the colonic tissues increased significantly in the model group ($P<0.01$ or $P<0.05$). Compared with the model group, the mucosal epithelium in the herb-partitioned moxibustion group and the mesalazine group was repaired and the goblet cells increased with a few infiltrating inflammatory cells in the colon; the CMDI score decreased ($P<0.01$); the serum NETs-DNA, NE-DNA, and MPO-DNA concentrations decreased ($P<0.05$); the NE, citH3, and MPO protein and mRNA expression in the colonic tissues was down-regulated ($P<0.01$ or $P<0.05$).

**Conclusion**: Herb-partitioned moxibustion reduced the serum NETs complex and inhibited the protein and mRNA expression of NETs complex in the colon tissue, which may be one mechanism of herb-partitioned moxibustion in relieving colon mucosal inflammation in CD.

**Keywords**: Herb-partitioned Moxibustion; Medicinal Cake-partitioned Moxibustion; Inflammatory Bowel Diseases; Crohn Disease; Neutrophil Extracellular Traps

【摘要】目的: 从中性粒细胞胞外诱捕网(NETs)参与克罗恩病(CD)进展的角度, 阐释隔药灸减轻CD大鼠肠道炎症的效应机制。方法: 将大鼠随机分为正常组、模型组、隔药灸组和美沙拉嗪组。除正常组外, 其余大鼠采用2,4,6-三硝基苯磺酸制备CD模型。正常组和模型组不治疗仅做与其他组相同的固定。隔药灸组予以隔药灸气海和双侧天枢穴。美沙拉嗪组接受美沙拉嗪肠溶片灌胃治疗。比较各组大鼠一般情况, 苏木精-伊红染色观察结肠组织病理学变化并评

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分。采用ABC 酶联免疫吸附测定法检测各组大鼠血清中性粒细胞胞外诱捕网DNA(NETs-DNA)、中性粒细胞弹性蛋白酶DNA(NE-DNA)、过氧化物酶DNA(MPO-DNA)浓度, 采用免疫荧光和实时定量聚合酶链反应技术观察各组大鼠结肠组织瓜氨酸化组蛋白3(citH3)、MPO和NE蛋白和mRNA的表达。**结果:** 与正常组比较, 模型组结肠黏膜溃疡深达肌层, 上皮不完整, 杯状细胞明显减少, 可见大量炎性细胞浸润, 结肠黏膜损伤指数(CMDI)评分显著增加(P<0.01), 模型组血清NETs-DNA、NE-DNA、MPO-DNA浓度均增加(P<0.05), 模型组结肠组织NE、citH3和MPO蛋白和mRNA表达均上调(P<0.01或P<0.05); 与模型组相比, 隔药灸组和美沙拉嗪组结肠黏膜上皮修复, 杯状细胞明显增多, 可见少量炎性细胞浸润, CMDI评分明显降低(P<0.01), 隔药灸组和美沙拉嗪组血清NETs-DNA、NE-DNA、MPO-DNA浓度均下降(P<0.05), 隔药灸组和美沙拉嗪组NE、citH3和MPO蛋白和mRNA表达下调(P<0.01或P<0.05)。**结论:** 隔药灸能降低血清中性粒细胞NETs复合物的浓度, 抑制结肠组织中的NETs复合物的蛋白和mRNA表达, 可能是隔药灸缓解CD结肠黏膜炎症的机制之一。

【关键词】隔药灸疗法; 药饼灸疗法; 炎症性肠病; 克罗恩病; 中性粒细胞胞外诱捕网

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Crohn disease (CD) is a chronic nonspecific inflammatory bowel disease (IBD) with recurrent attacks and unclear pathogenesis. CD is believed to be caused by the interaction of many pathogenic factors (such as genetic susceptibility, environmental factors, intestinal flora disorder, etc.), leading to innate immunity and acquired immunity imbalance[1-2]. In addition to abdominal pain, diarrhea, hematochezia, and other clinical manifestations, CD often affects the whole body with long-term emaciation, anemia, and other symptoms. Long-term recurrent attacks seriously reduce the quality of life and affect the normal work and study of patients. Epidemiological studies show that the incidence of CD has had a global upward trend in the past decades, which has increased the social and economic burden[3]. The acceleration of urbanization increases the incidence of incurable CD in China[4]. Aminosalicylic acid preparations, antibiotics, immunosuppressants, biological agents, and other ways are used in Western medicine to control CD symptoms. However, the adverse reactions and high prices limit their clinical application. In recent years, the curative effect of acupuncture and moxibustion on CD has received more and more attention[5]. Our previous randomized controlled clinical trial confirmed that acupuncture effectively treated mild-to-moderate CD[6-7]. Related animal experiments also discussed the possible mechanisms of acupuncture in treating CD from multiple viewpoints and levels, such as inhibiting the inflammatory factor expression, repairing the colon mucosal barrier, regulating intestinal flora homeostasis, and regulating the autophagy immune-related genes[8-11].

Neutrophils are quickly recruited to the infected or damaged tissue sites to curb inflammation. Neutrophils continue to be activated and over-recruited in the long-term course of CD due to the long-term existence of inflammation. Neutrophils play an important role in intestinal inflammation of CD. They can destroy the epithelial barrier of colon mucosa and recruit monocytes and more neutrophils to the intestinal tract by releasing proteases and promoting inflammatory cytokines and mediators, such as interleukin (IL)-8 and tumor necrosis factor (TNF)-α[12]. Colonic neutrophil infiltration is related to the disease activity of CD[13]. The formation of neutrophil extracellular traps (NETs) increases in the intestinal mucosa, feces, and blood of IBD patients, and is positively correlated with disease activity[14]. The NETs-related protein expression in the colon tissue of CD patients is significantly higher than that of normal subjects. The expression of NETs decreases after anti-TNF-α treatments[15]. Targeting NETs may form a new therapy and promote the general healing of intestinal mucosa. Acupuncture is effective in treating CD, and its mechanism may be related to regulating the biological function of NETs. Therefore, we focused on NETs to explore the protective mechanism of acupuncture on CD colon mucosa and provided a more scientific experimental and theoretical basis for acupuncture treatment of CD in this study.

## 1 Material and Methods

### 1.1 Laboratory animal

Forty male specific-pathogen-free Sprague-Dawley rats, about 4 weeks old and weighing (100±20) g, were provided by the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine [SYXK (Hu) 2020-0009]. Rats were adaptively fed in the environment of 12 h/12 h alternating circadian rhythm, room temperature of (20±2) ℃, and indoor humidity of 50%-70% for 1 week and maintained under the same condition. The animal experiments were approved by the Ethics Committee of Shanghai University of Traditional Chinese Medicine. The ethical approval number was PZSHUTCM210305005. All animal experiments were conducted under the guidance of the Ethics Committee of Shanghai University of Traditional Chinese Medicine.

### 1.2 Main reagents and instruments

1.2.1 Main reagents

Hematoxylin, eosin, neutral gum, xylene, ethanol (Sinopharm Chemical Reagent Co., Ltd., China); pentobarbital sodium (Cat. No. P-010, Merck, Germany); NETs-DNA complex enzyme-linked immunosorbent

assay (ELISA) kit (Cat. No. FD02028, Shanghai Xitang Biotechnology Co., Ltd., China); rabbit anti-citrullinated histone 3 (citH3) antibody (1:1 000, Cat. No. ab5103, Abcam, UK); rabbit anti-myeloperoxidase (MPO) antibody (1:1 000, Cat. No. ab208670, Abcam, UK); rabbit anti-neutrophil elastase (NE) antibody (1:3 000, Cat. No. ab177487, Abcam, UK); goat anti-rabbit second antibody (1:100, Cat. No. 111-543-003, Jackson, USA); Trizol (Cat. No. 15596018, Invitrogen, USA); RT reagent kit (Cat. No. RR047A, TAKARA, Japan); TB Green quantitative polymerase chain reaction (qPCR) kit (Cat. No. RR420A, TAKARA, Japan).

1.2.2 Main instruments

ATP700 (ST) tissue dehydrator (Hestion, China); EG 1160 tissue embedding machine (Leica, Germany); RM 2235 paraffin slicer (Leica, Germany); HI 1220 tissue dryer (Leica, Germany); BX53 optical microscope (OLYMPUS, Japan); FrescoTM 17 4℃ centrifuge, -80 ℃ refrigerator, and microplate reader (Thermo Fisher Scientific Inc., USA); Ascent software for Multiskan; Light Cvcler real-time quantitative polymerase chain reaction (RT-qPCR) apparatus (Roche, Switzerland).

**1.3 Model preparation**

The CD rat model was prepared with 2,4,6-trinitrobenzene sulfonic acid (TNBS)[16]. The rats were anesthetized with 1% pentobarbital sodium [40-50 mg/(kg·bw)]. Mixed 5% (W/V) TNBS and 50% ethanol at a volume ratio of 2: 1 to form an enema. The enema was injected at a dose of 3 mL/(kg·bw) 6-8 cm away from the anus, once a week for 4 weeks.

**1.4 Grouping and intervention methods**

Forty rats were randomly divided into an un-modeled group (n=10) and a modeling group (n=30) with SPSS version 26.0 software. Four rats died during model preparation. Ten un-modeled rats and 26 modeled rats remained. Two rats in each group were randomly selected to determine the result of model preparation. After successful model establishment, 24 rats in the modeling group were randomly divided into a model group, an herb-partitioned moxibustion group, and a mesalazine group, with 8 rats in each group. The remaining 8 un-modeled rats were included in the normal group.

Normal group: Rats in the normal group did not receive any treatment but had the same fixation as the other groups.

Model group: The model group did not receive any treatment but had the same fixation as the other groups.

Herb-partitioned moxibustion group: The rats were covered with breathable gloves and protected from light. After the stress state was over, the rats were fixed on a treatment frame with the abdomen upward using straps. Qihai (CV6) and bilateral Tianshu (ST25) were selected for herb-partitioned moxibustion[17]. The powder of processed Fu Zi (*Radix Aconiti Lateralis Praeparata*), *Rou Gui* (*Cortex Cinnamomi*), and *Huang Lian* (*Rhizoma Coptidis*), etc. were mixed with yellow wine into thick pastes, and the herbal cakes of 0.8 cm in diameter and 0.4 cm in thickness were made by a mold. Moxa cones made of refined moxa wools (about 90 mg in mass) were used with 2 Zhuang at each point once a day for 7 consecutive days[18].

Mesalazine group: Rats in the mesalazine group received intragastric administration of mesalazine enteric-coated tablets (State Food and Drug Administration Approval No. HJ20202002, Losan Pharma GmbH, Germany) daily according to the ratio of 56:1 between the human adult (70 kg in body mass, 4 g/d) and the rat (200 g in body mass), once a day, 1 mL each time for 7 d[19].

**1.5 Sample collection and processing**

Rats were anesthetized with 2% pentobarbital sodium [40-50 mg/(kg·bw)], and the abdominal cavity was opened. About 3 mL of arterial blood was collected from the abdominal aorta using a 5 mL BD disposable vacuum non-anticoagulant blood collection vessel, kept at room temperature for 30 min, and centrifuged at 4 ℃ and 3 000 r/min for 10 min. Collected the supernatant (i.e. serum) and stored it with aliquots in a -80 ℃ refrigerator for later use. The colon segment 2-10 cm away from the anus was cut along the mesenteric margin and washed with physiological saline. The distal 1 cm colon tissue was fixed with 4% paraformaldehyde. The rest of the colon tissue was cut, mixed evenly in EP tubes, frozen for 1 h, and then stored in the refrigerator at -80 ℃ for later use.

**1.6 Testing items**

1.6.1 Morphological observation of colonic epithelium

The colonic tissues fixed for 24 h were dehydrated, embedded in paraffin wax and made into sections of 4 µm thick. The colonic sections were dewaxed to water, then treated in turn with dimethylbenzene Ⅰ and dimethylbenzene Ⅱ for 15 min, 100%, 90%, 80%, and 70% ethanol for 5 min, double-distilled water rinsing for 5 min twice, hematoxylin staining solution for 2 min, running water rinsing for 10 min, 1% hydrochloric acid alcohol differentiation for 5 s, running water rinsing for 5 min, eosin staining solution for 2 min, dehydration with 70%, 80%, 90%, and 100% ethanol for 8 s, dimethylbenzene Ⅰ and dimethylbenzene Ⅱ transparency for 15 min, and neutral resin mounting. After hematoxylin-eosin (HE) staining, colonic epithelium, glands, and inflammatory cell infiltration were observed under the light microscope[20].

1.6.2 Detection of NETs-DNA in peripheral blood

The NETs-DNA concentration in each sample was calculated using the double antibody sandwich ABC ELISA with standard curves. The standard substance or samples to be tested were added into the designated wells of a 96-well plate and then kept at 37 ℃ for 40 min. Washed the plate fully with the washing buffer 4-6 times and absorbed the residual washing buffer

with filter papers. Added 50 μL of distilled water and 50 μL of primary antibody working solution into each well (except the blank wells). The plate was allowed to stand at 37 ℃ for 20 min after being fully mixed. After the plate was washed, 100 μL of enzyme-labeled antibody working solution was added into each well. The plate was kept at 37 ℃ for 10 min. After the plate was washed, 100 μL of substrate working solution was added to each well to react in the dark at 37 ℃ for 15 min. Added 100 μL of termination solution into each well and mixed it well. The absorbance value was measured at 450 nm with the microplate reader within 30 min.

### 1.6.3 NETs complex protein and mRNA expression in colon tissue

The NETs complex protein expression in the colon tissue was detected by immunofluorescence. Repaired the antigen after the slices were dewaxed to water, washed with 1×PBS 3 times, blocked with 5% BSA blocking buffer at room temperature for 40 min, incubated overnight with rabbit primary antibody at 4 ℃, incubated with goat secondary antibody at 37 ℃ for 60 min after washing with 1×PBS 3 times again, incubated with DAPI solution at room temperature for 10 min, and then observed under the fluorescence microscope.

The NETs mRNA expression was observed by RT-qPCR. Total RNA was extracted from cryopreserved colon tissue by Trizol method, and the concentration and purity were measured. It was reverse transcribed into cDNA using the kit after quantification. RNA was amplified by Roche RT-qPCR system after preparing the reaction solution under the reaction conditions of being pre-denatured at 93 ℃ for 2 min, annealed at 93 ℃ for 1 min, annealed at 58-60 ℃ for 1 min and 72 ℃ for 1 min according to primers. A total of 40 cycles were carried out and finally extended at 72 ℃ for 7 min. The data were analyzed and calculated using the $2^{-\triangle\triangle Ct}$ method after the reaction was completed. The primer information of each target gene and internal reference gene is shown in Table 1.

### 1.7 Statistical methods

The SPSS version 22.0 software was used for statistical analysis. All measurement data were in normal distribution and expressed as mean ± standard deviation ($\bar{x} \pm s$). One-way analysis of variance was used to compare the measurement data with homogeneous variance. With α=0.05 as the test size, $P<0.05$ indicated the statistically significant difference.

**Table 1　Primer sequences (5'-3')**

| Primer name | | Primer sequence (5'-3') | Amplification length/bp |
|---|---|---|---|
| NE | Upstream primer | ACGATCGTAGAGGGACGGAA | 83 |
| | Reverse primer | GCATATGGGTTCCCAGGCTT | |
| citH3 | Upstream primer | TTCAAGACCGACCTGCGTTT | 234 |
| | Downstream primer | CTTGAAACCGTGTGTGGCTC | |
| MPO | Upstream primer | TTCCTACCAGGGTTGGAGTG | 70 |
| | Downstream primer | CAGGGAAGATGTAGCAACGC | |
| GAPDH | Upstream primer | TTCAACGGCACAGTCAAGG | 114 |
| | Downstream primer | CTCAGCACCAGCATCACC | |

Note: NE=Neutrophil elastase; citH3=Citrullinated histone 3; MPO=Myeloperoxidase.

## 2 Results

### 2.1 Comparison of rat general conditions among groups

Rats in the normal group moved and ate as usual with normal stools and smooth furs. Rats in the model group were in poor condition, showing thin and small body shapes, reduced diet and movements, dim and filthy furs, and loose stools; some rats had bloody stools and abdominal bloating. Compared with the model group, the overall state of rats in the herb-partitioned moxibustion group and the mesalazine group was significantly improved, showing enlarged body sizes, less lustrous furs, flat abdomens, more formed feces without bloody stools, with occasional loose stools and normal activities.

### 2.2 Comparison of rat colonic histopathological changes among groups

HE staining of rat colon tissue showed that the colon tissue structure was complete and crypts were arranged neatly with a large number of goblet cells and no inflammatory cell infiltration in the normal group. The ulcer reached the muscularis and the colonic epithelium was incomplete, the local mucosa was missing, the crypt morphology was abnormal, and the goblet cells decreased obviously with many infiltrating inflammatory cells in the model group. The colonic mucosal ulcer was healed, and the crypts were arranged neatly with more goblet cells and little inflammatory cell infiltration in the herb-partitioned moxibustion group and the mesalazine group (Figure 1).

<div align="center">Normal group       Model group</div>

<div align="center">Herb-partitioned moxibustion group     Mesalazine group</div>
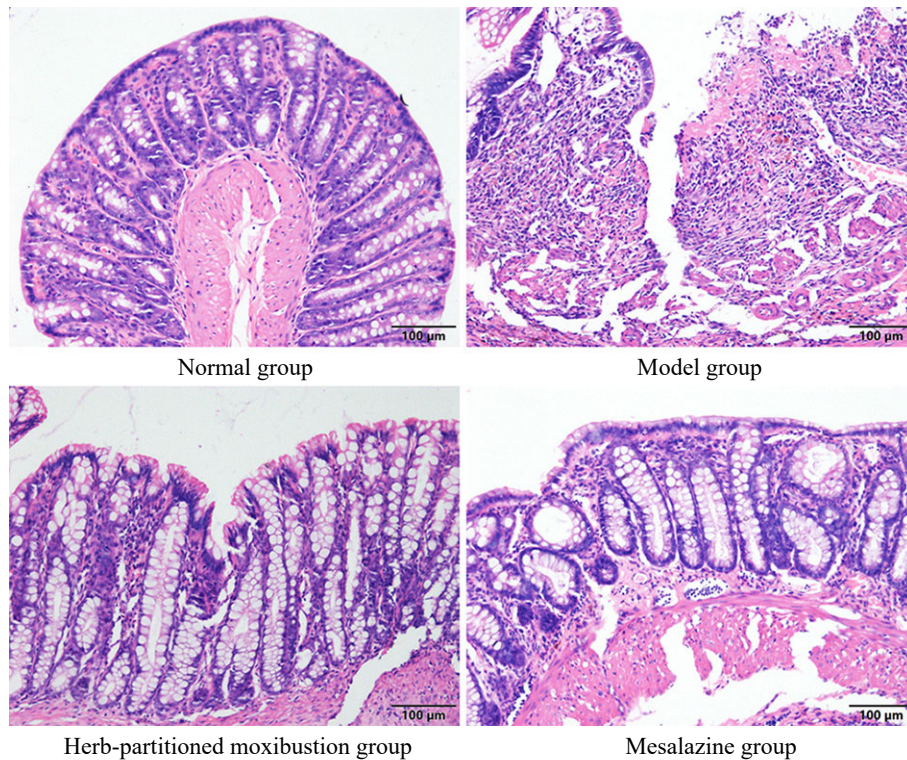
**Figure 1   stopathological observation of rat colons in each group (hematoxylin-eosin staining, ×200)**

Compared with the normal group, the colonic mucosa damage index (CMDI) score was significantly higher in the model group ($P<0.01$); it was significantly lower in the herb-partitioned moxibustion group and the mesalazine group versus the model group ($P<0.01$). See Figure 2.
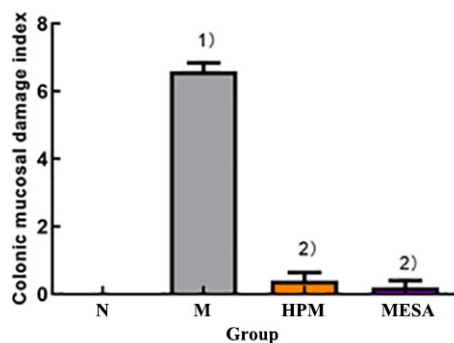


Note: N=Normal group; M=Model group; HPM=Herb-partitioned moxibustion group; MESA=Mesalazine group; compared with the normal group, 1) $P<0.01$; compared with the model group, 2) $P<0.01$.

**Figure 2   Comparison of colonic mucosa damage index score among groups ($n$=8)**

**2.3 Comparison of NETs-DNA, MPO-DNA, and NE-DNA protein concentration among groups**

The serum protein quantitative results of NETs and their complexes showed that the protein concentration of NETs-DNA in the model group was significantly higher than that in the normal group ($P<0.01$), while the protein concentration of NETs-DNA in the herb-partitioned moxibustion group and the mesalazine group was significantly lower than that in the model group ($P<0.01$). The serum protein concentrations of MPO-DNA and NE-DNA, the main complexes of NETs, increased significantly in the model group compared with the normal group ($P<0.05$). The serum concentrations of MPO-DNA ($P<0.05$) and NE-DNA ($P<0.01$) in the herb-partitioned moxibustion and the mesalazine groups were decreased significantly versus the model group (Figure 3).

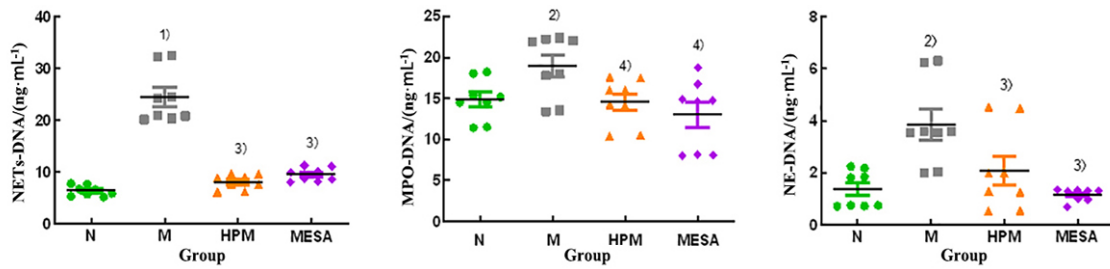**2.4 Comparison of NETs complex protein expression in colon tissue among groups**

2.4.1 Comparison of NE protein expression in colon tissue among groups

The NE protein expression was detected by immunofluorescence staining. Compared with the normal group, the NE (green) expression was more significant in inflammatory site neutrophils of colon tissue in the model group; compared with the model group, the NE positive expression of colon tissue was less significant in the herb-partitioned moxibustion group and the mesalazine group (Figure 4).

2.4.2 Comparison of citH3 protein expression in colon tissue among groups

Immunofluorescence results showed that the positive expression of citH3 protein (green) was less in the normal group, more in the model group, and less in the herb-partitioned moxibustion group and the mesalazine group (Figure 5).

Note: N=Normal group; M=Model group; HPM=Herb-partitioned moxibustion group; MESA=Mesalazine group; compared with the normal group, 1) $P<0.01$, 2) $P<0.05$; compared with the model group, 3) $P<0.01$, 4) $P<0.05$.

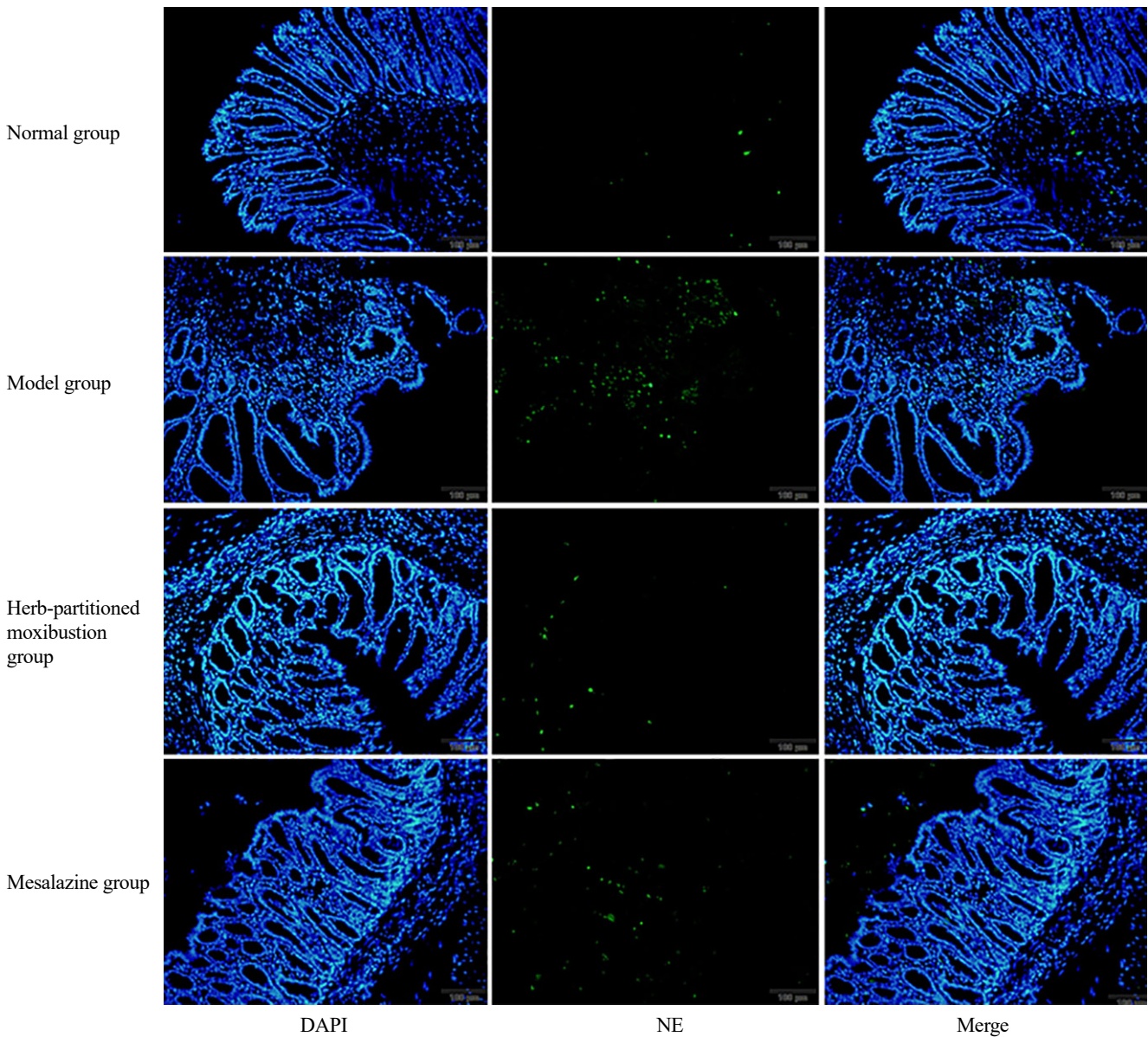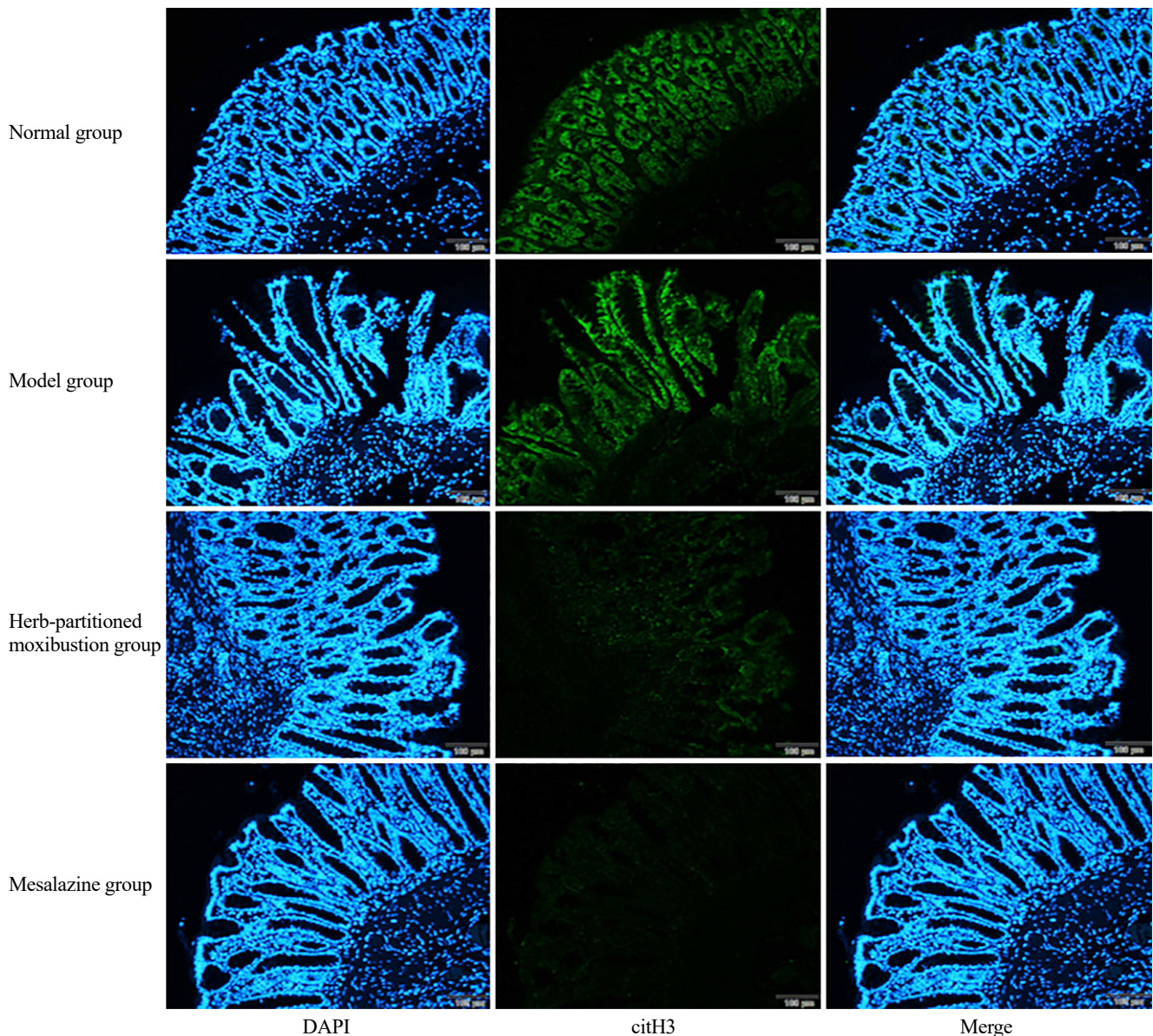**Figure 3　Comparison of the rat serum NETs complex DNAs among groups ($n$=8)**



|  | DAPI | NE | Merge |

**Figure 4　Protein expression of neutrophil elastase in rat colon tissue of each group (immunofluorescence, ×200)**

|  | DAPI | citH3 | Merge |

**Figure 5   Protein expression of citrullinated histone 3 in rat colon tissue of each group （immunofluorescence, ×200)**

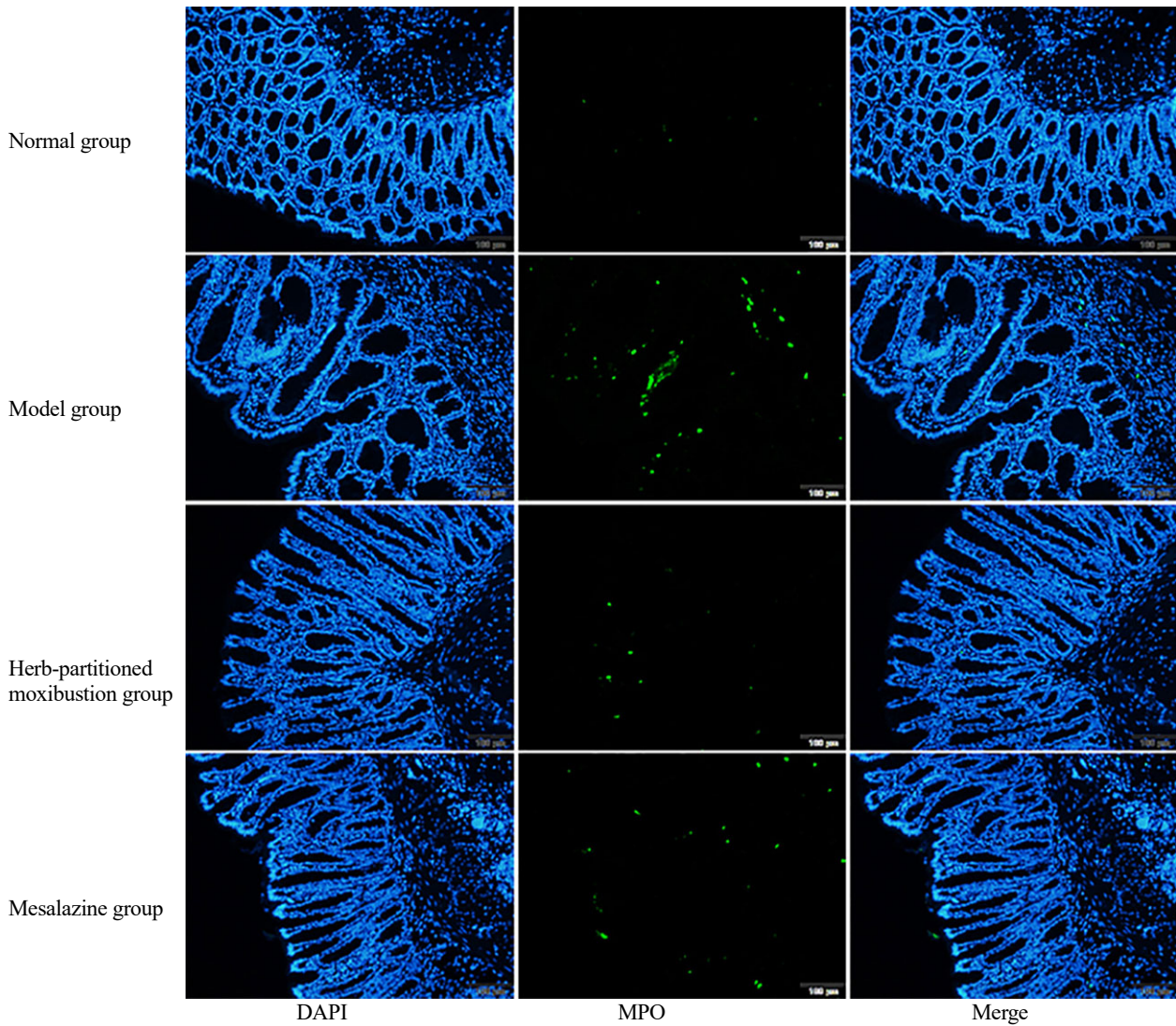2.4.3 Comparison of MPO protein expression in colon tissue among groups

Immunofluorescence staining showed that the positive MPO expression of the rat colon tissue (green) was less in the normal group, more in the model group, and less in the herb-partitioned moxibustion group and the mesalazine group (Figure 6).

ImageJ was used for the fluorescence quantitative analysis of proteins (Table 2). Compared with the normal group, the NE, citH3, and MPO protein expression levels in the colon tissue of the model group were significantly higher than those of the normal group ($P<0.01$); compared with the model group, the NE, citH3, and MPO protein expression in the herb-partitioned moxibustion group and the mesalazine group decreased significantly ($P<0.01$).

## 2.5 Comparison of NETs complex mRNAs in colon tissue among groups

RT-qPCR results showed that the mRNA expression levels of NE and citH3 in the model group were significantly higher than those in the normal group ($P<0.05$). Compared with the model group, the mRNA expression of NE and citH3 in the herb-partitioned moxibustion group and the mesalazine group decreased significantly ($P<0.01$ or $P<0.05$). Compared with the normal group, the MPO mRNA expression in the model group increased significantly ($P<0.01$), while that in the herb-partitioned moxibustion group and the mesalazine group decreased compared with the model group ($P<0.01$). See Figure 7.

**Figure 6 Protein expression of myeloperoxidase in rat colon tissue of each group（immunofluorescence, ×200)**

| DAPI | MPO | Merge |

**Table 2 Comparison of AOD results of NE, citH3, and MPO in rat colon tissue among groups**

| Group | $n$ | NE | citH3 | MPO |
|---|---|---|---|---|
| Normal | 8 | 130.90±18.42 | 120.11±10.70 | 123.74±10.04 |
| Model | 8 | 169.54±14.74[1] | 146.10±11.03[2] | 144.92±4.04[2] |
| Herb-partitioned moxibustion | 8 | 135.41±17.06[3] | 119.93±8.54[4] | 122.78±4.72[4] |
| Mesalazine | 8 | 130.03±12.04[4] | 120.47±10.34[4] | 117.34±3.98[4] |

Note: AOD=Average optical density; NE=Neutrophil elastase; citH3=Citrullinated histone 3; MPO=Myeloperoxidase; compared with the normal group, 1) $P<0.01$, 2) $P<0.05$; compared with the model group, 3) $P<0.01$, 4) $P<0.05$.
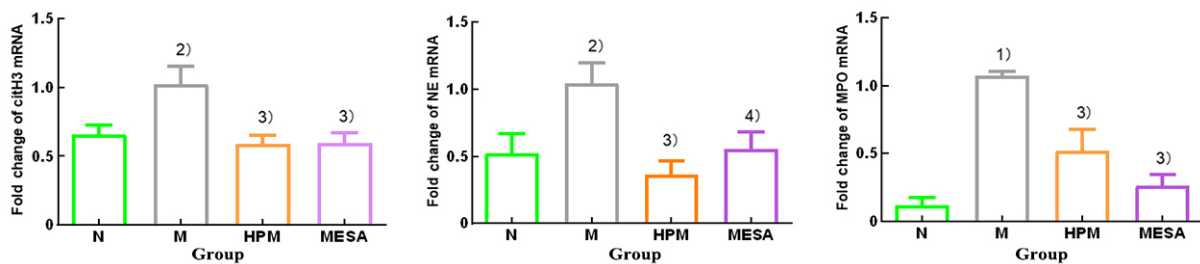


Note: N=Normal group; M=Model group; HPM=Herb-partitioned moxibustion group; MESA=Mesalazine group; compared with the normal group, 1) $P<0.01$, 2) $P<0.05$; compared with the model group, 3) $P<0.01$, 4) $P<0.05$.

**Figure 7 Effect of herb-partitioned moxibustion on NETs complex mRNA expression in CD rat colon tissue ($n$=8)**

## 3 Discussion

CD belongs to the "diarrhea" and "abdominal pain" categories in traditional Chinese medicine. Moxibustion has been used in the treatment of diarrhea since ancient times. Tianshu (ST25) is the key point for treating intestinal diseases and the Front-Mu point of the large intestine. Tianshu (ST25) plays a key role in intestinal diseases such as abdominal pain and diarrhea, and is the first-choice point for treating CD[21]. The Conception Vessel runs in the middle of the abdomen and is closely related to the stomach, large intestine, small intestine, and other Zang-Fu organs. Qihai (CV6) is the place where innate Yuan-primordial Qi meets and the sea to generate Qi. It is an important point for health care with the function of tonification and good effects on abdominal pain and diarrhea. The combination of Tianshu (ST25) and Qihai (CV6) is effective in improving intestinal symptoms of CD confirmed by clinical trials and animal experiments[6,22-25].

BRINKMANN V, et al[26] found that the extracellular fibers released granular proteins and chromatin to degrade toxicity and kill bacteria by combining with gram-negative bacteria and gram-positive bacteria after the neutrophils were activated. This special structure, which uses chromatin as the basic skeleton and attaches to granular proteins and other components, is named NETs. The co-expression of granular proteins (such as MPO, NE, and citH3) is a marker of NETs' release[27]. NETs have attracted more and more researchers' attention in recent years due to their important role in the pathological process of many diseases, such as cancer[28], systemic lupus erythematosus[29], psoriasis[30], and IBD[15]. With more research, the double-edged sword characteristics of NETs have been gradually revealed, such as killing pathogenic microorganisms[26], promoting tumor metastasis[31], participating in thrombosis, and promoting atherosclerotic plaque formation[32]. NETs may become a new target for CD treatment to promote the complete healing of intestinal mucosa due to the new understanding of NETs in the inflammatory process of many diseases[33].

NETs play a defensive role when colonic mucosa is exposed to high levels of pathogenic microorganisms (such as bacteria, viruses, fungi, etc.). However, excessive release of NETs can also lead to aggravation or persistence of intestinal inflammation[34]. Many studies have confirmed that abnormally high expression of NETs can be detected in feces, blood samples, and colon tissue of CD patients. With the support of gas chromatography-mass spectrometry proteomics research, LEHMANN T, et al[34] found that NETs, key proteins MPO and NE in the feces of IBD patients were significantly more than those of healthy subjects.

MASUDA S, et al[27] found that the MPO-DNA complex expression level in the peripheral blood of CD patients increased, and the NE and citH3 expression in the damaged colon mucosa increased significantly. The concentration of MPO in NETs complex is positively correlated with the histopathological severity of the colon in CD patients. The three-dimensional image formed by NETs markers in the intestinal biopsy tissue of CD patients was captured by the specific fluorescence staining technique[35]. CD is a chronic inflammation with a long-time existence of intestinal mucosal barrier immune dysfunction. Repeated inflammation changes the structure and function of the colon. The inflammatory state of the intestinal tract is obviously different between CD active and remission stages with different roles of neutrophils.

In this study, we identified that herb-partitioned moxibustion at Tianshu (ST25) and Qihai (CV6) significantly improved loose stools, hematochezia, and other symptoms, reduced CMDI score, alleviated colon mucosal inflammation, and promoted the repair of damaged mucosa of TNBS-induced CD model rats. The above results are consistent with our previous identifications[11,22-25]. Moxibustion at Tianshu (ST25) and Qihai (CV6) warms the spleen and stomach and has a good anti-inflammatory effect on CD. Based on the effect of moxibustion on alleviating colitis in CD rats, we studied the involvement of NETs in intestinal inflammation. It was found that the serum concentrations of NETs-DNA, MPO-DNA, and NE-DNA proteins in CD rats were significantly higher than those in the normal rats, and the protein fluorescence of NETs complex, including the NE, MPO, and citH3, in colon tissue was significantly enhanced. Clinical trials revealed that the MPO-DNA complex expression in the peripheral blood and the NE and citH3 expression in inflammatory site colon mucosa of CD patients were significantly increased[27]. The findings of the peripheral blood MPO-DNA and the fluorescence of NE and citH3 protein in the colon tissue of CD rats in this study are consistent with the results of clinical trials. Here, we also showed that herb-partitioned moxibustion inhibited colonic inflammation in CD rats, which may be related to herb-partitioned moxibustion decreasing the serum DNA and protein of NETs complex and the mRNA and protein expression of NETs complex (NE, MPO, and citH3) in the colon tissue.

In conclusion, we found that herb-partitioned moxibustion improved colonic symptoms, relieved colon mucosal inflammation, and promoted the repair of damaged colon mucosa in CD rats. By focusing on the abnormal release of NETs, we confirmed that herb-partitioned moxibustion reduced the serum concentration of NETs complex and inhibited the protein and mRNA expression of NETs complex in the colon tissue of CD rats, which may be one mechanism

of herb-partitioned moxibustion interfering with CD, but we still lack a more comprehensive and in-depth understanding of this mechanism. The action pathway of herb-partitioned moxibustion in inhibiting NETs complex needs further studies. Furthermore, the role of neutrophils in the pathogenesis of CD is still controversial. Inhibition of neutrophils is beneficial to alleviating intestinal inflammation in CD, while excessive inhibition will bring negative effects. Moreover, MPO is closely related to oxidative stress in the formation of NETs. In the future, we can introduce MPO knockout mice and MPO inhibitors to further explore the effect mechanism of herb-partitioned moxibustion on relieving intestinal inflammation in CD based on the signal pathway related to oxidative stress of neutrophils.

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