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Original Article

Electroacupuncture protects the intestinal mucosal barrier in diarrhea-predominant Irritable Bowel Syndrome rats by regulating the MCs/Tryptase/PAR-2/MLCK pathway

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Abstract: Objective: The pathogenesis of diarrhea-predominant irritable bowel syndrome (IBS-D) is related to damage to the intestinal mucosal barrier function. Based on the Mast cell (MC)/Tryptase/Protease-activated receptor-2 (PAR-2)/Myosin light chain kinase (MLCK) pathway, this study explored the effect of electroacupuncture (EA) on IBS-D rats and its possible mechanism of protecting the intestinal mucosal barrier. Methods: The IBS-D rat model was established by mother-offspring separation, acetic acid enema, and chronic restraint stress. The efficacy of EA on IBS-D rats was evaluated by observing the rate of loose stool (LSP) and the minimum volume threshold of abdominal withdrawal reflex (AWR) in rats. Mast cells and the ultrastructure of intestinal mucosa were observed by H&E staining, toluidine blue staining, and transmission electron microscopy. The expression levels of Tryptase, PAR-2, MLCK, zonula occludens-1 (ZO-1), and Occludin in rats were detected by ELISA, qRT-PCR, and western blot. Results: After 7 days of intervention, compared to the IBS-D group, the loose stool rates of rats in IBS-D + EA group and IBS-D + ketotifen group were decreased ($P < 0.01$), the minimum volume thresholds of AWR were improved ($P < 0.01$), the inflammation of colon tissue decreased, the number of MCs were decreased ($P < 0.01$), the expression of Tryptase, PAR-2, and MLCK were lowered ($P < 0.01$, $P < 0.05$), and the expression of ZO-1 and Occludin were enhanced ($P < 0.01$, $P < 0.05$). Compared to the EA group, there was no significant difference in each index between the ketotifen groups ($P > 0.05$). Conclusion: EA has a good therapeutic effect on IBS-D rats. Regulating the MCs/Tryptase/PAR-2/MLCK pathway may be a mechanism to protect the intestinal mucosal barrier.

Keywords: IBS-D rat, electroacupuncture, MCs/Tryptase/PAR-2/MLCK, intestinal mucosal barrier function

Introduction

Irritable bowel syndrome (IBS) is a highly prevalent functional gastrointestinal disorder [1]. It is characterized by recurrent abdominal pain and changes to fecal traits or frequency [2], and has no structural or biochemical disease markers [3]. The most common IBS subtype is diarrhea-predominant IBS (IBS-D) [4]. The disease affects 4%-10% of the world's population [2]. Although IBS-D is not life-threatening, it has the characteristics of recurrence and remission in most people, which seriously affect the quality of life of patients and impose a heavy economic burden on individuals and society [5].

At present, treatment regimens for IBS-D include prokinetic drugs, antispasmodic drugs, antidiarrheal drugs, intestinal microecological preparations, anti-anxiety and depression drugs, and selective antibiotics [6]. However, these treatment regimens are only aimed at alleviating individual symptoms, and they are relatively ineffective in helping overall health [7]. IBS-D is prone to recurrent attacks, and there is evidence that the long-term use of these drugs often causes side effects such as dry mouth, blurred vision, dizziness, and vomiting [8]. The current treatment cannot meet the needs of IBS-D patients, so complementary and alternative medicine (CAM) provides pa-

tients with options [9]. Acupuncture, as a complementary and alternative therapy [10], has been practiced in China for thousands of years [11], and is becoming more popular and accepted among the public and health care professionals in the west. IBS is among 63 kinds of common diseases for which acupuncture was recommended by the World Health Organization (WHO) in 1996 [12]. Acupuncture treatment is effective and has been widely used in the treatment of gastrointestinal diseases such as IBS-D, inflammatory bowel disease, and functional dyspepsia. In addition, acupuncture treatment can prevent long-term drug side effects [13-16].

The possible mechanism of electroacupuncture (EA) in the treatment of IBS-D has been reported [17], but the mechanism of EA affecting mast cells (MCs) to regulate the intestinal mucosal barrier function in IBS-D is not clear, and further research is needed. It has been reported that EA has a dual regulatory MC activation. EA stimulation of acupoints can activate MCs in the acupoint area and increase their degranulation [18]. Other observations show that acupuncture can inhibit the activation of MCs, reduce their degranulation, inhibit the expression of inflammatory factors, and play an anti-inflammatory and immune role in the treatment of intestinal disease [19]. MCs, as classical immune cells, are involved in the pathogenesis of IBS-D intestinal mucosal barrier destruction [20]. MCs can activate Tryptase, thereby activating protease-activated receptor-2 (PAR-2) [21]. The activation of PAR-2 plays an important role in the pathological mechanism of intestinal barrier damage in IBS-D It directly affects the contraction of the cytoskeleton and changes intestinal mucosal barrier function by triggering the phosphorylation of myosin light chain (MLC) and the subsequent changes in tight junction proteins [22].

In this study, a rat model of IBS-D was established, and the activation of MCs was used as the starting point. The MCs/Tryptase/PAR-2/Myosin light chain kinase (MLCK) signaling pathway was used to explore the possible mechanism of EA intervention on the protection of intestinal mucosal barrier function, and to provide a scientific basis for electroacupuncture treatment of IBS-D.

Materials and methods

Animals

Five SPF Sprague-Dawley pregnant rats (350 ± 20 g of body weight), gestational age (18 ± 1) d, were purchased from the Experimental Animal Center of Anhui Medical University (Anhui, China). The rats were raised in the experimental animal center of Anhui University of Traditional Chinese Medicine, with (23 ± 2)°C, 50% ± 10% humidity, and a 12 h/12 h light/dark cycle. Pregnant rats were fed routinely and a total of 32 pups were born and randomly divided into four groups: control group, IBS-D group (without treatment), IBS-D + EA group, IBS-D + ketotifen group. This experiment has been approved by the Animal Ethics Committee of Anhui University of Traditional Chinese Medicine (AHUCM-rats-2020011). The care of animals during the experiment is in line with the provisions of the "Guiding Opinions on Treating Experimental Animals" issued by the Ministry of Science and Technology of the People's Republic of China.

Induction of IBS-D model

The IBS-D model was established by mother-offspring separation, and acetic acid enema, combined with chronic restraint stress stimulation [23, 24]. The neonatal rats that needed to be modeled were separated from their lactating mothers at 2-15 days after birth. They were taken out at 9.00 a.m. every day and raised separately from their lactating mothers for 3 hours, and then put back. Weaning was performed at 24 days, the neonatal rats were fed separately to 30 days, and then the male and female cages were separated. The neonatal rats were normally raised to 35 days until acetic acid stimulation. A single-lumen central venous catheter with a diameter of 1 mm lubricated with paraffin oil was inserted into the rectum for 4 cm through the anus, and 1 mL of 4% acetic acid solution was slowly injected. Then the tail was raised for 60 s, and then 1 mL of PBS buffer was injected to rinse the colon. After acetic acid stimulation, they were rested for 3 days. The rats were subjected to chronic restraint on the 38-44 days after birth. The upper limbs and chest of rats were wrapped and bound with paper tape 3 hours a day. The rat's head could only swing, unable to groom

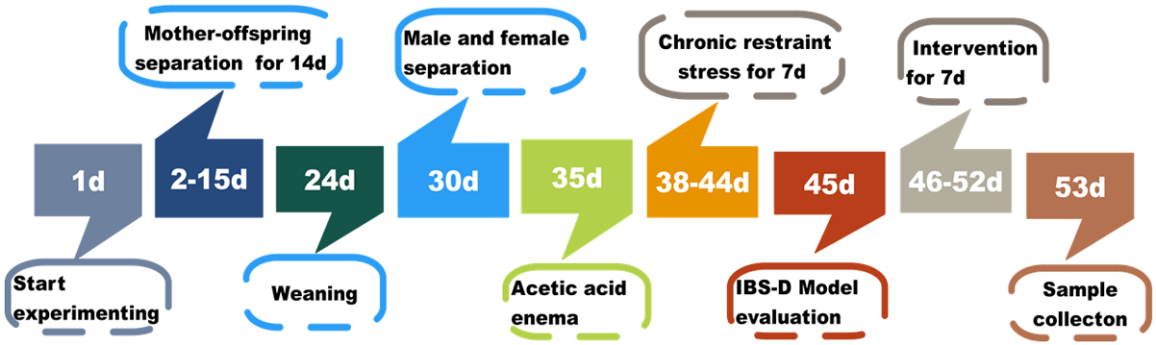

Figure 1. Schematic diagram of the experimental timeline.

his face and hair with the forelimbs (**Figure 1**). The criteria for successful modeling: compared with the control group, the loose stool rate of the IBS-D group increased and the minimum capacity threshold of abdominal withdrawal reflex (AWR) decreased ($P < 0.05$).

Electroacupuncture intervention

Rats in the EA group were treated with electroacupuncture, 20 min/time/d, for 7 days. The limbs of the rats were fixed on the rat board, and the skin of the acupoints was disinfected with 75% alcohol, and then treated with EA. The acupoints were Tianshu (ST 25, located in the abdomen, about 5 mm in the middle of the navel) and Shangjuxu (ST 37, located on the outside of the hind limb, about 10 mm below the fibular head) [25]. A disposable sterile acupuncture needle (Suzhou Medical Products Factory Co., Ltd., China, Φ0.25 × 25 mm) was inserted directly into the needle about 2-3 mm, and connected to the electroacupuncture instrument (SDZ-II, Suzhou, China), to the dilatational wave, frequency 2 Hz/100 Hz, current intensity 0.3 mA. The rats in the other groups received the same fixation without EA.

Drug intervention

Ketotifen was used as a positive control for the effect of EA intervention on IBS-D and the effect of EA intervention on MCs. Ketotifen fumarate tablets (Shanghai Hengshan Pharmaceutical Co., Ltd., China) were finely ground and dissolved in double distilled water to make a suspension. The suspension was shaken before each use. According to the "Table of Equivalent Dose conversion coefficients of different animals" of Pharmacological Experimental Methods [26], the dosage of rats was 0.25 mg/kg/d by gavage at 10 ml/kg, 2 times/d for 7 d continuously.

Loose stool rate (LSR)

Before modeling (35 days), after modeling (45 days), after intervention (53 days) observation. Each group of rats was fed in a single cage with a grid at the bottom of the cage. The tray under the grid was padded with filter paper. After 6 hours, the total numbers of feces and the number of loose stools produced by the rats were counted (the distinction between dry and loose stools was marked by the presence or absence of stains on the filter paper). The loose stool rate (%) = (the number of loose stools/the total number of feces) × 100% [27].

Minimum volume threshold for an AWR score of 3

Evaluation was performed before and after modeling and after intervention. Fasting was enforced for 12-24 hours before assessment, but not water deprivation. After the rats were anesthetized with isoflurane, a single-lumen central venous catheter lubricated with paraffin oil was slowly inserted through the anus to a depth of 6-8 cm from the anal opening. The catheter was fixed to the tail of the rats, and then the rats were placed in a transparent glass box that restricted their turning. After they were awake and calm, 0.9% sodium chloride solution at 26-28°C was slowly injected into the balloon for colorectal balloon distention (CRD). The CRD was maintained by water injection and repeated three times to achieve accurate measurement. The CRD was calculated as the amount of water injected when the AWR score was 3 [28].

Table 1. Primer sequences

Name	Upstream primer sequence (5'→3')	Downstream primer sequence (5'→3')
β-actin	CCCATCTATGAGGGTTACGC	TTTAATGTCACGCACGATTTC
Tryptase	AATAAGGCTGACCCCAACAA	GTCAGCAGGTGGTCATGATA
PAR-2	CATTGGCTTTTTCTACGGCA	ACCAGAAAAATCAGGAGCCA
MLCK	GATAGACACAGAGAACTTGGC	CTTCTCACTTTGGTGGTAACG
ZO-1	CAAGGAGGTAGAGCGAGGCA	CTGGGAACTTTGTTTGAACTGG
Occludin	TGGGAGCCTTGACATCTTGT	GGTGCATAATGATTGGGTTTG

Sample collection

After the intervention (53 days), the rats were anesthetized by intraperitoneal injection of 2% pentobarbital sodium solution (30 mg/kg), and then sacrificed by blood sampling. After opening the abdominal cavity, we collected the abdominal aorta blood (about 5 ml) in a negative pressure anticoagulant tube, 3000 r/min, and centrifuged it 20 min to take the upper serum, for -80°C preservation. Colon tissue (2-3 cm) was collected at 5-8 cm from the anus and washed with 0.9% sodium chloride solution. It was divided into three parts. One was placed in 4% paraformaldehyde for HE staining, toluidine blue staining and immunofluorescence. One was placed in 2.5% glutaraldehyde for transmission electron microscopy, and the other was placed in a freezing tube and placed in liquid nitrogen at -196°C for ELISA, qRT-PCR, and WB detection.

Histological assessment and mast cell counts

Colon tissue samples were taken from 4% paraformaldehyde, dehydrated in gradient ethanol, transparentized in xylene, and sectioned (5 μm) after paraffin embedding. H&E staining and toluidine blue staining were used to observe the morphology of colon tissue under an optical microscope (CX41, Olympus, Japan).

Transmission electron microscope (TEM)

The tissue was cut into 1 mm^3 cubes quickly, fixed in 2.5% glutaraldehyde for 24 hours, then placed in PBS buffer for 6 hours, and fixed in 1% osmic acid for 2 hours. After gradient alcohol dehydration, epoxy resin infiltration, embedding, heating and curing, 70 nm ultrathin sections were obtained by an ultra-thin sectioning machine (UC-7, Leica, Germany). After electron staining (lead staining), mast cells degranulation and intestinal mucosal ultrastructure were observed by electronic transmission electron microscopy (JEM1400, Japan Electronics Co., Japan).

Enzyme-linked immunosorbent assay (ELISA)

The levels of Tryptase, PAR-2, MLCK, Zonula occludens-1 (ZO-1) and Occludin in serum and colon tissues were detected by ELISA kit (Wuhan ColorfulGene Biological Technology Co., Ltd., China). In the above steps, the colon tissue in liquid nitrogen at -196°C was taken, and the temperature of the specimen was maintained at 2-8°C after melting. A certain amount of PBS (PH7.4) was added. The specimen was homogenized and centrifuged for 20 min, 3000 r/min, and the supernatant was collected. At the same time, the supernatant of the above steps stored in the refrigerator at -80°C was taken. The two supernatants were diluted in turn, added with 50 μL of enzyme-labeled reagent in each well, incubated at 37°C for 60 min, washed and patted dry. To each well were added 50 μL of chromogenic agents A and B, and then incubated at 37°C in the dark for 10 min. Each well received 50 μL of stop solution to terminate the reaction. Within 15 min, the microplate reader read the absorbance of each well at 450 nm to calculate the sample concentration.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA extraction was performed using Trizol reagent (Life Technogies, USA); total RNA was reverse transcribed into cDNA. The primer sequence is shown in **Table 1**. Then the fluorescence quantitative PCR amplification reaction was carried out. The PCR reaction conditions were as follows: the first step (pre-denaturation): 95°C 1 min; the second step: 95°C 20 s, 60°C 1 min, for 40 cycles. The relative expression of mRNA was calculated by $2^{-\Delta\Delta Ct}$ method with β-actin as internal reference.

Western blotting (WB)

100 mg of colon tissue was lysed with lysis buffer (Beyotime, China). The protein preparation was separated by SDS-PAGE gel electrophoresis and transferred to PVDF membrane (Millipore, USA). The membrane was blocked with 5% skim milk powder. After washing, primary antibodies Tryptase (1:1000, Bioss, China), PAR-2 (1:1000, Bioss, China), ZO-1 (1:1500, Bioss, China), Occludin (1:2000, Bioss, China), MLCK (1:4000, abcam, UK) were added. After that, HRP-labeled secondary antibody (1:20000, Zsbio, China) was dropped. After 1.2 hours of incubation, PBST was added and washed three times. Finally, the ECL luminescence kit (Thermo, USA) was used to detect the protein. Image J software (National Institute of Health, USA) was used for analysis, and β-actin was used as an internal reference. The ratio of the gray value of the target protein to the β-actin was taken to indicate its relative expression.

Immunofluorescence staining

The colon tissue was dehydrated, waxed, embedded, and sliced in turn, and then dewaxed by xylene and gradient ethanol. Antigen repair was performed with citrate buffer and microwave heat induction, and goat serum blocking solution was added (Ebiogo, China). Incubation was done in a 37°C incubator. The primary antibody Mast cell Tryptase (1:200, Bioss, China), MLCK (1:300, Bioss, China), ZO-1 (1:300, Bioss, China), Occludin (1:300, Bioss, China), or PAR2 (1:200, Abcam, UK) was added. This was rinsed in PBS-T, and we added immunofluorescence secondary antibody (goat anti-rabbit 1:400) (Ebiogo, China), incubated in the 37°C incubator, rinsed with PBS-T three times, covered with anti-fluorescence quenching sealer (including DAPI), and scanned the fluorescence slices with a digital slice scanner (Pannoramic MIDI, Hungary 3DHISTECH). An optical fluorescence microscope was used to observe and take pictures, and a medical image analysis system (JD801, China) was used for image analysis.

Statistical analysis

SPSS 26.0 and GraphPad Prism 8.0 were used for statistical analysis. The data were expressed as mean ± standard deviation. The data of each group were in accordance with a normal distribution and homogeneity of variance. LSR and AWR minimum capacity were compared among multiple groups by repeated measures analysis of variance. Other data were analyzed by one-way analysis of variance, and the LSD method was used for pairwise comparison between groups. $P < 0.05$ was considered significant.

Results

EA improved the LSR and AWR minimum capacity in IBS-D rats

Before modeling, there was no significant difference in the LSR and AWR minimum capacity between the groups ($P > 0.05$). After mold making, compared to the control group, the AWR minimum capacity in the IBS-D, IBS-D + EA, and IBS-D + ketotifen groups were markedly decreased ($P < 0.01$), and the LSR was significantly increased ($P < 0.01$). After intervention, compared to the IBS-D group, the AWR minimum capacity of the IBS-D + EA group and the IBS-D + ketotifen group were observably elevated, and the loose stool rate was significantly reduced ($P < 0.01$) (**Figure 2A** and **2B**).

EA improves low-grade inflammation of the intestinal mucosa

H&E staining results revealed that the colon mucosal structure was intact in Control group, epithelial cells and glands were orderly and showed no inflammatory cell infiltration. Compared to the control group, rats in IBS-D group had disturbed glands, blurred mucosa and submucosal structures, visible inflammatory cell infiltration, and mild mucosal edema. Compared to the IBS-D group, rats of the IBS-D + EA group and IBS-D + ketotifen group were relatively complete, and the inflammatory cell infiltration was reduced and not abnormal (**Figure 3**).

EA reduced the number of MCs in IBS-D rats and inhibited the degranulation of MCs

After toluidine blue staining, the mast cells showed microscopic purple particles, diverse morphology, oval, round, irregular, etc. The degranulation of MCs in the colon tissue of each group was observed by comparison between

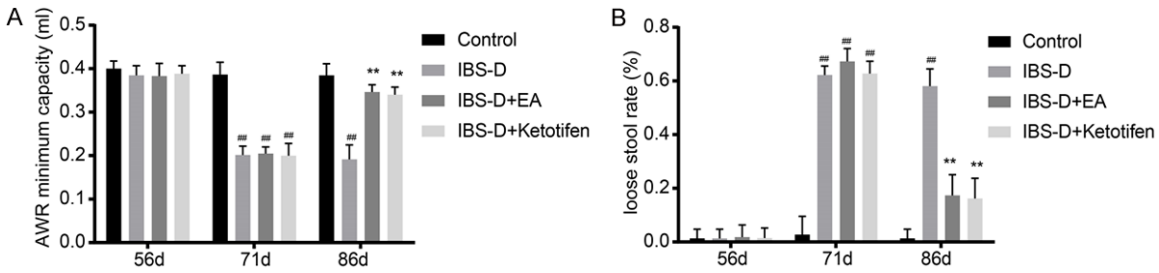

Figure 2. Electroacupuncture (EA) improved the symptoms of diarrhea-predominant irritable bowel syndrome (IBS-D) rats. A. Impact of EA on the abdominal withdrawal reflex (AWR) minimum capacity of IBS-D rats. B. Effects of EA on the loose stool rate of IBS-D rats. $^{\#\#}P < 0.01$ compared to the control group at the same time point, $^{**}P < 0.01$ compared to the IBS-D group at the same time point.

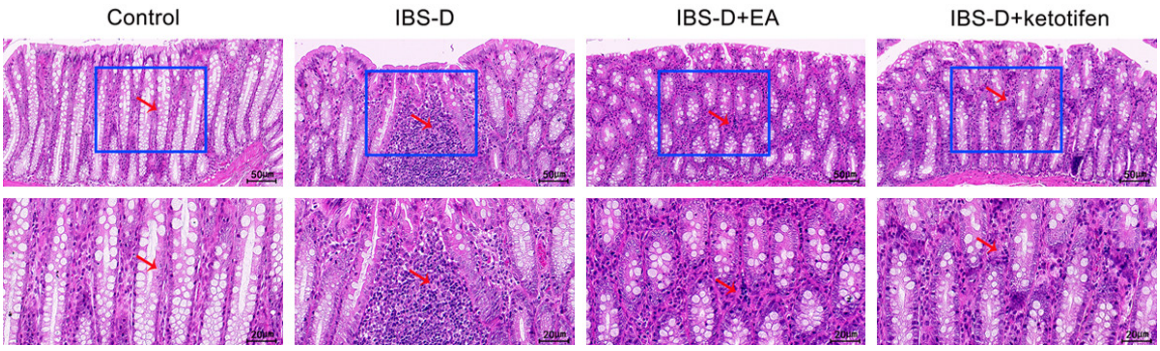

Figure 3. EA improved intestinal mucosal microinflammation (H&E staining) (magnification: 200 ×, 400 ×, respectively) (inflammatory cells: red arrows).

groups. Compared to the control group, the number of mast cells in the colon tissue of the IBS-D group was increased ($P < 0.01$). Compared to the IBS-D group, the number of mast cells in the colon tissue of the IBS-D + EA group and the IBS-D + ketotifen group was significantly reduced ($P < 0.01$) (**Figure 4A**). The degranulation of mast cells was observed by TEM. In the control group, the MCs membranes were intact, the cytoplasm contained a large number of particles, the particles were evenly distributed and the density was high, and no obvious degranulation was observed. In the IBS-D group, the MCs capsule was incomplete, and the low-density particles in the cytoplasm were increased. After partial degranulation, a gap appeared at the edge of the cell, and the membrane between the particles was fused to form a channel after degranulation. In the IBS-D + EA and IBS-D + ketotifen groups, the degranulation of MCs decreased, the capsules were intact, the particle density in the cytoplasm was increased, and the particle distribution was more uniform (**Figure 4B**).

EA reduced the expression of Tryptase, PAR-2, and MLCK in IBS-D rats

Compared to the control group, Tryptase, PAR-2, and MLCK were significantly increased in IBS-D group ($P < 0.01$). After EA treatment or ketotifen treatment, the levels were diminished compared to those in the control group ($P < 0.01$) (**Figure 5A**). In order to further explore the effect of EA on Tryptase, PAR-2, and MLCK, we performed qRT-PCR, WB, and immunofluorescence. The results were consistent with ELISA results, where Tryptase, PAR-2, and MLCK were raised in IBS-D group ($P < 0.01$), and Tryptase, PAR-2, and MLCK were lowered in colon tissues after EA or ketotifen treatment ($P < 0.01$, $P < 0.05$). It was observed that the results of EA or ketotifen on Tryptase, PAR-2, and MLCK were similar (**Figure 5B-D**).

EA increases the expression of tight junction proteins in IBS-D rats

It was found by TEM that the microvilli on the surface of the intestinal mucosa in the control

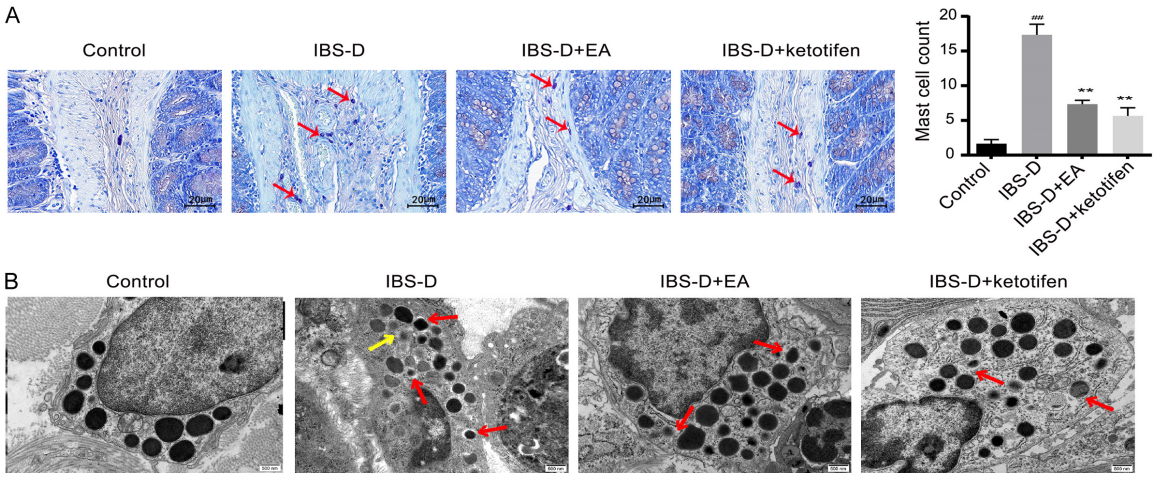

Figure 4. EA reduced the number of mast cells (MCs) and inhibited MCs degranulation in IBS-D rats. A. Toluidine blue staining used to detect mast cells counts (magnification: 400 ×) (mast cells: red arrows). B. Mast cell degranulation observed by Transmission Electron Microscope (TEM) (magnification: 25000 ×) (edge gap: red arrows, membrane fusion: yellow arrows). $^{\#\#}P < 0.01$ compared to the control group, $^{**}P < 0.01$ compared to the IBS-D group.

group were rich, dense, regular, and clear in structure. In the IBS-D group, the microvilli were sparse, arranged in disorder, the structure was blurred, and some defects were found. In the IBS-D + EA group and IBS-D + ketotifen group, the microvilli were relatively uniform, but the arrangement was still neat, and the loss and shedding were reduced (**Figure 6A**). In order to further explore the effect of EA on tight junction proteins, we used ELISA to detect tight junction proteins. The results showed that compared to the control group, there was a significant reduction in ZO-1 and Occludin in serum and colon tissue of IBS-D group ($P < 0.01$). The levels of ZO-1 and Occludin in serum and colon tissue were significantly increased after treatment in the EA or ketotifen group ($P < 0.01$) (**Figure 6B**). We observed similar results by qRT-PCR and WB analysis (**Figure 6C**, **6D**). The results of immunofluorescence were consistent with those of qRT-PCR and WB. Compared to the control group, the fluorescence expression of ZO-1 and Occludin in the colon tissue of the IBS-D group were diminished ($P < 0.01$). Compared to the IBS-D group, the fluorescence expression of ZO-1 and Occludin in the colon tissue of the EA group and the ketoprofen group were substantially improved ($P < 0.01$) (**Figure 6E**).

Discussion

Irritable bowel disease with diarrhea (IBS-D) is a common disease that seriously affects the quality of life of patients, and its treatment is still challenging [29]. As an effective treatment of traditional Chinese medicine, acupuncture can play a benign regulatory role in IBS-D [30]. In this study, the LSR of rats decreased and the AWR minimum capacity increased after EA, which is consistent with the clinical improvement of abdominal pain and diarrhea in IBS-D patients [31], indicating that EA can regulate gastrointestinal homeostasis and relieve intestinal symptoms.

Evidence shows that MCs play a very important role in the pathogenesis of IBS-D [32]. MCs can activate downstream signaling pathways by secreting cytokines or mediators such as tryptase and histamine, leading to visceral hypersensitivity, or triggering the release of inflammatory mediators, leading to changes in epithelial permeability, intestinal epithelial barrier defects, thereby aggravating abdominal pain and diarrhea symptoms [33]. Increased mast cells were observed in both IBS-D patients and animal models [34, 35]. In view of the important role of MCs in IBS-D, this study found that the intestinal mucosa of IBS-D showed low-grade inflammation. After comparison between groups, it was found that the number of degranulated MCs in the IBS-D group increased. After intervention in the IBS-D + EA group or IBS-D + ketotifen group, the number of degranulated MCs decreased significantly, the MCs particles became more uniform, and the degranulation phenomenon decreased, indicating that EA

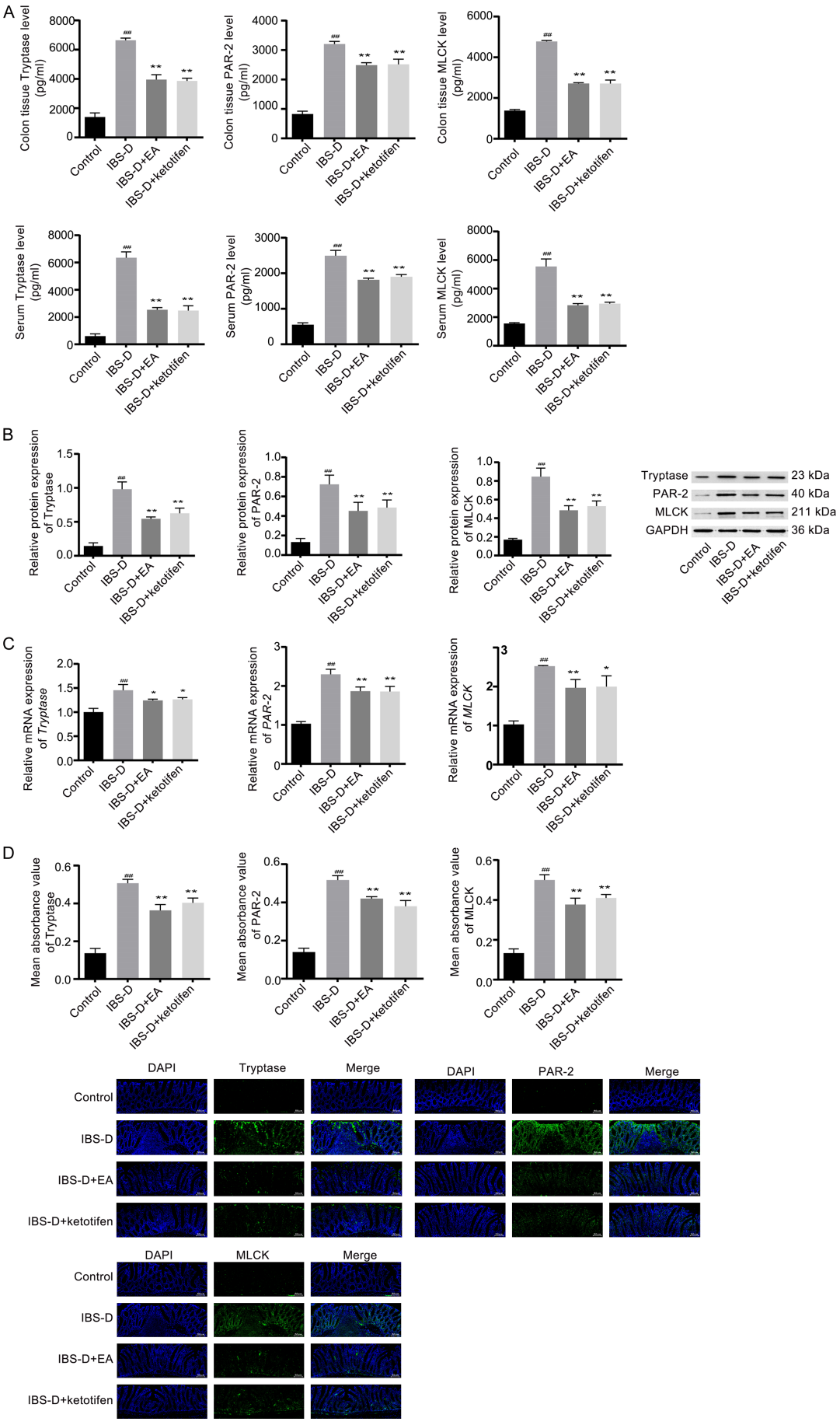
A
Colon tissue Tryptase level (pg/ml)
Colon tissue PAR-2 level (pg/ml)
Colon tissue MLCK level (pg/ml)
Serum Tryptase level (pg/ml)
Serum PAR-2 level (pg/ml)
Serum MLCK level (pg/ml)
B
Relative protein expression of Tryptase
Relative protein expression of PAR-2
Relative protein expression of MLCK
Tryptase 23 kDa
PAR-2 40 kDa
MLCK 211 kDa
GAPDH 36 kDa
C
Relative mRNA expression of Tryptase
Relative mRNA expression of PAR-2
Relative mRNA expression of MLCK
D
Mean absorbance value of Tryptase
Mean absorbance value of PAR-2
Mean absorbance value of MLCK
Control
IBS-D
IBS-D+EA
IBS-D+ketotifen
DAPI
Tryptase
Merge
PAR-2
MLCK

Figure 5. EA reduced the expressions of Tryptase, Protease-activated receptor-2 (PAR-2) and Myosin light chain kinase (MLCK) in IBS-D rats. A. Serum and colon Tryptase, PAR-2, and MLCK levels measured by ELISA. B. Relative protein levels of Tryptase, PAR-2, and MLCK determined by WB. C. Relative mRNA levels of Tryptase, PAR-2, and MLCK determined by qRT-PCR. D. Immunofluorescence micrographs (magnification: 200 ×) and its mean absorbance value (The nuclear expression was blue, the positive expression was green, and Merged was co-expressed). $^{\#\#}P < 0.01$ compared to the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the IBS-D group.

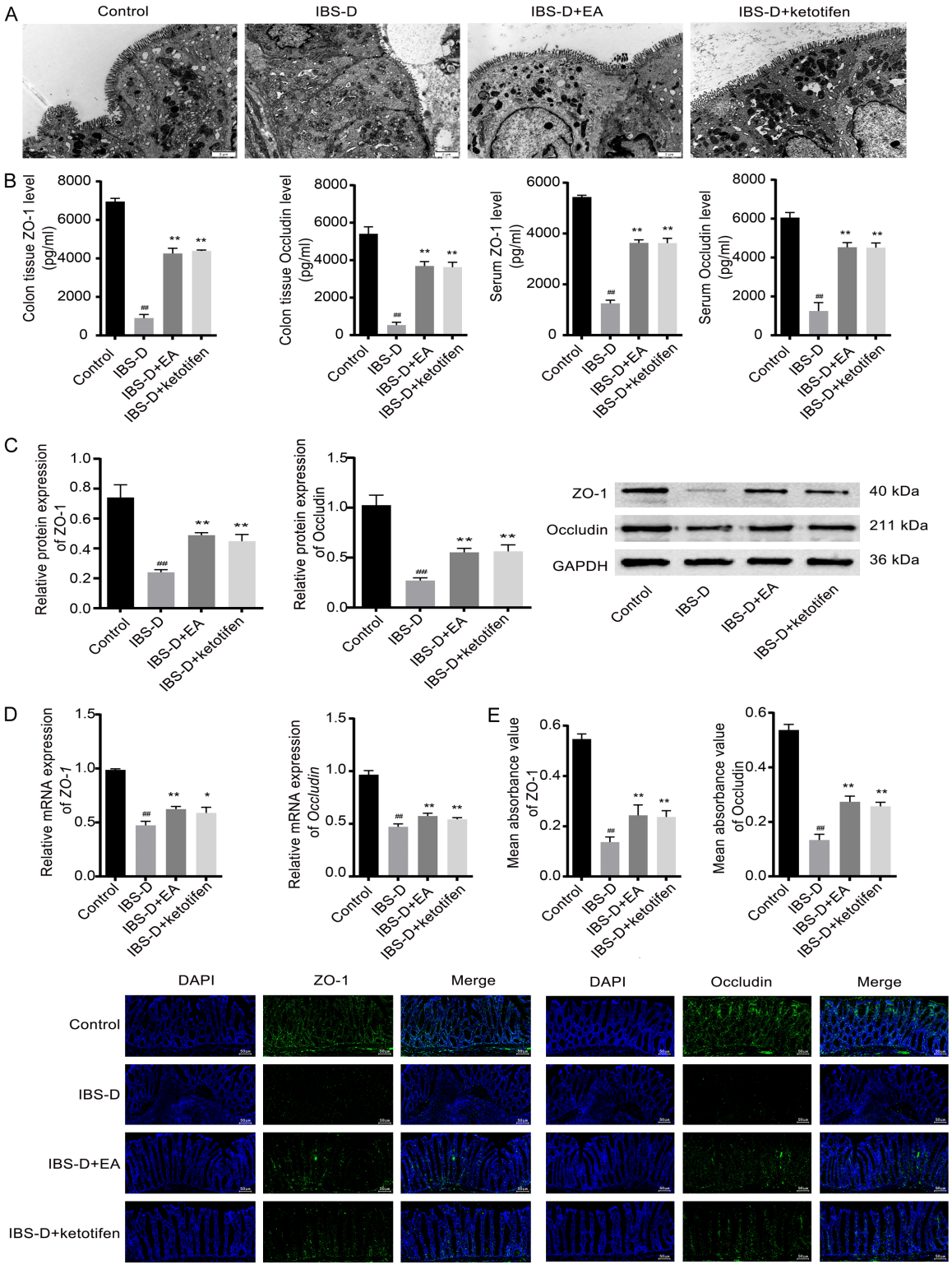

Figure 6. EA increased the expression of tight junction proteins (TJs) in IBS-D rats. A. Ultrastructural changes of the colon observed by TEM (magnification: 10000 ×). B. Serum and colon zonula occludens-1 (ZO-1) and Occludin levels measured by ELISA. C. Relative protein levels of ZO-1 and Occludin determined by WB. D. Relative mRNA levels of ZO-1 and Occludin determined by qRT-PCR. E. Immunofluorescence micrographs (magnification: 200 ×) and its mean absorbance value. $^{\#\#}P < 0.01$ compared to the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the IBS-D group.

could downregulate the number of MCs and inhibit the MCs of rat intestinal mucosa to a certain extent, consistent with other observations [36].

Combined with relevant research and analysis, findings susggest that the effect of EA on intestinal MCs may be achieved through somatic sensory nerve-autonomic nerve reflex [37]. This is in view of the fact that the superficial nerve innervating the "Tianshu" and "Shangjuxu" points and the intestinal splanchnic nerve are the same ganglion segment (T11-12 and L1-2) [38]. When EA is performed, the peripheral sensory nerve fibers in the dorsal root ganglion (DRG) or trigeminal ganglion are activated, and then the sensory information is transmitted to the spinal cord and brain, thereby activating the peripheral autonomic nerve to achieve an effect on intestinal function and MC degranulation [39].

Tryptase is an important mediator released during mast cell degranulation. It is contained almost exclusively in mast cell granules [40] and has been used as a marker of MC activation. It can activate intestinal PAR-2 [21]. PAR-2 is highly expressed in the gastrointestinal tract [22]. PAR-2 activation is the cause of visceral hypersensitivity, excessive inflammatory response, and intestinal barrier dysfunction in IBS-D patients [41, 42]. The activation of PAR-2 on the intestinal mucosa will directly affect the contraction of the cytoskeleton and increase the expression of MLCK. MLCK is a typical tight junction regulator that phosphorylates the myosin II regulatory light chain (MLC) in the surrounding actomyosin ring [43], thereby changing the structure and distribution of tight junction proteins (TJs) (ZO-1 and Occludin), downregulating tight junction proteins, remodeling and loosening the cytoskeleton, expanding the distance between cells, loosening the connections in TJs and forming gaps, and destroying the structure of the intestinal mucosal barrier, leading to diarrhea and other symptoms [44]. The results of this study suggest that the expression of Tryptase, PAR-2, and MLCK in serum and colon tissue of IBS-D rats was increased, and the expression of Occludin and ZO-1 decreased. After EA or ketotifen intervention, Tryptase, PAR-2, and MLCK in serum and colon tissue of rats decreased, and the expression of Occludin and ZO-1 increased. Combined with the above literature [40-43], it is speculated that EA intervention affects the intestinal mucosal barrier by affecting the expression of MC/Tryptase/PAR-2/MLCK signaling pathway.

The intestine can be exposed to potentially harmful substances. The intestinal mucosal barrier is an important structure to ensure the balance between the inside and outside of the intestine. Impaired integrity of the intestinal mucosal barrier can lead to abnormal activation of immune cells and chronic inflammation. Therefore, maintaining and protecting the intestinal mucosal barrier can effectively prevent IBS-D [3, 45]. Tight junction proteins are a key factor in intestinal barrier function, and their expression level is closely related to intestinal mucosal barrier function [46]. Related studies have shown that promoting ZO-1 and improving tight junction proteins can improve intestinal mucosal function [47]. The results of this study showed that the colonic mucosal structure of rats in the IBS-D group was blurred under the microscope, the villi were sparse, and the expression of Occludin and ZO-1 decreased, which is consistent with the relevant reports [48]. At the same time, we observed that after EA or ketotifen intervention, the structure of intestinal mucosa was improved, and the expression of tight junction proteins Occludin and ZO-1 was increased, suggesting that EA could improve the integrity of intestinal mucosal barrier.

Relevant studies have shown that ketotifen, as a new choice for the treatment of IBS-D, can significantly alleviate the gastrointestinal symptoms of IBS-D patients [49, 50]. Ketotifen is a mast cell stabilizer, which stabilizes the mast cell membrane, prevents degranulation, reduces the release of histamine, Tryptase and other media, and then reduces the subsequent cas-

cade reaction [51]. In this study, ketotifen was used as a positive control of EA intervention MCs to explore the mechanism of EA intervention on the intestinal mucosal barrier protection of IBS-D through MCs. This study found that the changes in each index after EA intervention were similar to those in the ketotifen group, indicating that EA can inhibit mast cell degranulation, and reduce the release of trypsin and other media. This improves the intestinal mucosal barrier and alleviates abdominal pain and diarrhea symptoms in IBS-D rats.

There are some limitations to this study. The "MCs/Tryptase/PAR-2/MLCK" pathway was deduced based on the effect of MC activation combined with its effect on downstream molecular substances, which needs to be further verified. At the same time, the treatment of IBS-D by EA is a comprehensive effect, and its mechanism is multi-factorial with multiple targets [52]. This article studied only one pathway, and its downstream signaling pathway and complex signal transduction mechanism need to be further studied. Further research will supplement the current findings.

In summary, this study found that electroacupuncture had a good therapeutic effect on IBS-D rats, and its mechanism of protecting intestinal mucosal barrier may be related to the regulation of MC/Tryptase/PAR-2/MLCK pathway.

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Disclosure of conflict of interest

None.

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