

Abdominal Massage Regulates the Irritable Bowel Syndrome Sensitivity through Mast Cells mediated Trypase -PAR2-PKC ϵ pathway

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Research Article

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Abstract

Background: Irritable bowel syndrome (IBS) is a clinical disease mainly manifested by abdominal pain and discomfort, which troubles the majority of human aged 20-50. Abdomen massage is of great medical significance for the health of the body, including promoting intestinal peristalsis, relieving constipation, helping to lose weight. However its function and mechanism in IBS was seldom reported.

Methods Firstly, we established IBS and abdomen massage rat model to detect the distribution of mast cells. Then, we used transmission electron microscopy (TEM), immunohistochemistry or immunofluorescence staining and laser confocal focus to visualize the micromorphology of intestinal mucosa, the expression of TRPV1 and the release of trypase. RT-qPCR and Western Blot were used for the mechanism study.

Results We found that compared with the control group, the MC in IBS group was significantly increased and the increased MC was partially decreased by abdominal massage with or without ketotifen treated. We also found that TRPV1 was up-regulated in IBS group. Abdominal massage with or without ketotifen treated could attenuate the up-regulation of TRPV1 in IBS and massage group. Mechanically, results of IHC and Western Blot suggested abdominal massage reduce the sensitivity of IBS by regulating trypase - PAR2-PKC ϵ pathway.

Conclusion: Overall, our results suggested that abdominal massage play a protective role in IBS through inhibiting MC and trypase -PAR2-PKC ϵ pathway. Ketotifen could promote the effect of abdominal massage on IBS treatment, which would be a potential therapeutic strategy for IBS.

Introduction

Irritable bowel syndrome (IBS) is a group of intestinal dysfunction disorders characterized by persistent or intermittent onset of abdominal pain, abdominal distension, bowel habits, and/or bowel changes as clinical manifestations, but lacking gastrointestinal tract structure and biochemical abnormalities. The prevalence rate of IBS in western countries is 10%-20%[1]. Though the etiology and pathogenesis of IBS are not very clear, the role of mast cell mediated mucosal immune changes in IBS has received extensive attention[2]. The function of Chinese medicine massage on IBS and mast cells is still obscure.

Massage, an important part of traditional Chinese medicine, was widely used to treat diseases, eliminate fatigue and enhance physical fitness[3]. As an effective treatment for disease prevention and health care, massage is a kind of relaxation tendon, activation of collateral ligament, promoting blood circulation, regulating qi and relieving pain[4]. Chinese medicine massage manipulation on the body surface and the local meridians can also affect the viscera including the abdomen.

PAR2, a G protein-coupled receptor composed of 397 amino acids, is widely distributed in gastrointestinal nerves and involved in pathophysiological reactions such as gastrointestinal motional changes, intestinal endocrine and visceral pain[5]. It was reported that PAR2 could be activated by trypsin-like enzymes

secreted by mast cells. PKC can phosphorylate various substrates which are reported to mediate cell division, proliferation, apoptosis and cytoskeleton proteins[6]. PKC located in peripheral sensory nerves, especially PKC ϵ , plays an important role in inflammation induced hyperalgesia through phosphorylation of TRPV1[7]. The function of Chinese medicine massage on TRPV1 and whether Chinese medicine massage could regulate PAR2/PKC pathway in IBS is not clear and needed further exploration.

In this study, we first reported that Chinese medicine massage could inhibit IBS sensitivity by down-regulating MC cells and TRPV1 expression in the gut. The possible mechanism was that abdominal massage reduced the sensitivity of IBS by regulating TRPV1 mediated trypase -PAR2-PKC ϵ pathway.

Methods

Animal models

Forty male Sprague Dawley (SD) rats with body weight of 180–220 g were bought from Sipford Biotechnology Co., LTD and raised in SPF environment of light cycle 12h / 12h (light on time) and relative humidity 60%. All the rats were free for drinking water and food for 3 days and then randomly divided into 4 groups based on body weight: normal group, IBS group, abdominal massage group and abdominal massage + ketotifen treatment group. IBS rat model was constructed as described before[8]. The fasted rats were anesthetized and enema was administered with acetic acid (4%, 1 mL/ rat). The colon was then irrigated with 1 mL phosphate buffered saline (PBS) to dilute acetic acid. Normal group was given 1 mL PBS. Abdominal massage was performed in Guan Yuan, Zhongwan acupuncture points about once a day for 5 minutes in 14 days. Animal experiments were conducted in accordance with Guidelines for the Care and Use of Laboratory Animals developed by the National Institutes of Health. All the animal experiments obtained the approval of the ethics committee.

Histological Assessment and Mast Cell Counts

Tissue samples were fixed in 4% paraformaldehyde, dehydrated, transparent, waxed, and embedded conventionally. Continuous sections were made with a thickness of 5 μ m and a distance of 30 μ m. Toluidine blue staining was performed after routine dewaxing, and pictures were taken under high power microscope. The number of MC was counted with ImageJ software.

RT-qPCR

Tissue RNA was extracted with Trizol and then synthesized reversely into cDNA. Realtime PCR was performed with SYBR Premix Ex Taq kit (TaKaRa). Primers used in this study were listed below: GAPDH-F: CGAGATCCCTCCAAAATCAA; GAPDH-R: TTCACACCCATGACGAACAT; TRPV1-F: CGCGGCGTGGGGAAAGACAT; TRPV1-R: CCTTCGGCTGCGGCGTGAT.

Western Blot

An equal amount of protein samples were added into polyacrylamide gel for electrophoresis, followed by semi-dry membrane transfer. Milk was used to block nonspecific antigens before the incubation of anti-TRPV1 antibody (1:500), anti-PAR2 antibody (1:400), anti-PKC ϵ antibody (1:1000), anti-PAR2 antibody (1:500), anti-PIP2 antibody (1:400) (abcam, USA) followed by HRP secondary antibody, and color imaging was performed after inoculation with DAB chromogenic kit (abcam, USA). Grey value was analyzed with ImageJ software and normalized to GAPDH or β -actin.

Immunohistochemistry staining

Paraffin embedded tissues were sectioned continuously, and the tissue sections were dehydrated with gradient alcohol solution and sealed with 3% H₂O₂. Then the primary antibody of tryptase (Abcam, USA) was incubated before the HRP-second antibody incubation. Diaminobenzidine solution was added onto the tissue sections, and the distribution of tryptase was observed under a microscope.

Immunofluorescence staining and confocal laser scanning

The rats were anesthetized, the colon was exposed, and 10 ~ 15 points were taken from the colon-bladder level to the oral cavity about 6cm away. Fluorescent dye 1,1'-bis (octan-1-yl)-3,3',3'-tetramethylindole carbonyl anthocyanin perchlorate (Dil, Invitrogen™, Dil) was injected into each point (Thermo Fisher Scientific Inc.) . In order to prevent dye leakage and contamination of adjacent organs, the injection needle was kept for 1min, and each point was cleaned with 0.9%NaCl solution after injection. One week after Dil injection, the small intestine tissues were quickly separated, fixed in 4% paraformaldehyde solution for 6-8h, and precipitated in 30% sucrose solution at 4°C. The sections were sealed with 10% rabbit serum for 1h, sheep anti-rat TRPV1 polyclonal antibody (1:100 dilution) was added at 4°C overnight, FITC-labeled rabbit anti-sheep IgG/rabbit anti-Rat IgG (H+L) Secondary Antibody, Texas Red (1:50 dilution) was added at 37°C for 30min, Hoechst was stained with nucleation, and the slices were sealed with glycerin. Laser confocal detection was used to calculate the percentage of TRPV1 positive cells in total Dil labeled cells by randomly taking 3 sections from each paraffin block and 3 fields from each section.

Transmission electron microscopy (TEM)

Briefly, the tissue mass was fixed with 2.5% glutaraldehyde, phosphate buffer preparation for 2 hours or longer and then rinsed with 0.1M phosphoric acid rinse solution before fixed 1% osmium solution. The mass was then dehydrated infiltrated and embedded. After section, the slice was stained with citrate before electron microscope observation.

Statistical Analyses

Data were presented in means \pm Standard Deviation. GraphPad Prism 8 software was used for statistical analyses and photograph. Significance of P value was identified with two-tailed Student's t-test.

Results

1. The role of MC in the treatment of visceral hypersensitivity in IBS by abdominal massage.

IBS rat model was successfully constructed. Results of HE staining showed that the morphology of the intestinal tissue in normal rat and abdominal massage is completely normal with long and complete villi, orderly arrangement of intestinal epithelial cells. Compared with the control group, intestinal mucosal layer of rats in IBS group had different degrees of damage. Villi were loose with inflammatory cell infiltration. When IBS rat were treated with MC blocker ketotifen, the protect effect of abdominal massage was abolished reflected by the swollen mucous membrane and acinar cells (Fig. 1A). As shown in the picture of toluidine blue staining, compared with normal control group, the MC in IBS group was significantly increased and the increased MC was partially decreased by abdominal massage. When IBS rats were treated with abdominal massage and ketotifen, the counts of MC was the least among the four groups (Fig. 1B).

2. Abdominal massage protected the microstructural of colonic mucosa.

It is well known that mitochondria are not only involved in cellular energy metabolism, but also mediate cellular inflammatory response. Subsequently, transmission electron microscopy (TEM) was used to detect the effects of IBS and massage on intestinal mucosal mitochondria. As shown in Fig. 2A, compared with the control group, the number of mitochondria in IBS group was significantly decreased, the morphology of mitochondria was also abnormally swollen, and the internal mitochondrial crest was shard and some even broken. On the contrary, abdominal massage could promote the count of mitochondria and help to maintain the integrity of mitochondrial morphology and thicken the mitochondrial crest. Furthermore, this effect of massage was enhanced when MC were blocked with ketotifen.

3. Effect of abdominal massage on TRPV1 expression.

Transient receptor potential cation channel subfamily V member 1 (TRPV1) was reported to be a noxious receptor of signaling transducer to affect mitochondrial function. Inhibition of TRPV1 played an important role in improving gastrointestinal function and reducing visceral hypersensitivity in rats[9, 10].

We then detected the expression of TRPV1 with RT-qPCR and found that TRPV1 was up-regulated in IBS group. Abdominal massage could attenuate the up-regulation of TRPV1 in IBS and massage group. Cooperated with abdominal massage, MC block could further inhibit the expression of TRPV1 (Fig. 3A). The protein expression of TRPV1 was then detected with immunofluorescence. As shown in Fig. 3B, TRPV1 located in the plasma membrane of the cells was high expressed in IBS group and could be inhibited by abdominal massage with or without MC blocker. The above results indicated that abdominal massage could decrease the sensitization of TRPV1 by inhibiting its mRNA and protein expression in IBS.

4. Abdominal massage reduced the sensitivity of IBS by regulating Trypase -PAR2-PKC ϵ pathway.

It was reported that changes in MC microenvironment of mouse peritoneum could result in alteration of MC particle protease, membrane signaling and membrane receptor phenotype [11]. We then detected the distribution of MC tryptase granules with immunohistochemistry. As shown in Fig. 4A, the distribution of tryptase granules in mast cell was the highest in IBS group compared that in the normal group. Compared with the IBS group, the distribution of tryptase particles decreased gradually in abdominal massage or blocker groups. Recent studies indicated that over-activation of PAR2-PKC pathway could mediate TRPV1 phosphorylation, decrease of TRPV1 open threshold, and abnormal visceral pain perception, eventually leading to visceral hypersensitivity in IBS[2]. We then detected the protein expression of PAR2 and PKC with Western Blot and found that PAR2-PKC was up-regulated in IBS group and decreased in abdominal massage group. Combined with abdominal massage, MC blocker could significantly inhibit the expression of PAR2-PKC (Fig. 4B). The downstream signaling PIP2-PLC was then detected with Western Blot too. As we can see in Fig. 4C, PIP2-PLC was up-regulated in IBS group and decreased in abdominal massage group. Combined with abdominal massage, MC blocker could significantly inhibit the expression of PIP2-PLC. These results suggested that abdominal massage may reduce the sensitivity of IBS by regulating Trypase-PAR2-PKC ϵ pathway.

Discussion

Massage is important for fitness and healing of healthy individuals or patients. Abdomen massage which can affect the function of the gastrointestinal tract by promoting digestion and absorption strengthen the physical fitness and improve immunity of individuals. Studies showed that abdominal massage could significantly improve the symptoms of constipation[12], insomnia[13], faecal incontinence[14]. It was also reported that abdomen massage can serve as alternative medicine treatment of IBS. But the mechanism of abdomen massage in IBS treatment was still obscure.

In our study, we used IBS rat model to explore the mechanism of abdomen massage in IBS treatment. We found that the MC in intestinal mucosa of IBS rat was higher than that in normal rats, which could be

inhibited by abdomen massage. MC blocker combined with abdomen massage has a synergistic effect on MC inhibition. Abdomen massage and MC blocker could significantly attenuated the perception of broken mitochondria and help maintain the integrity of mitochondria. This result was in consistence with the study from Tianjin University of Traditional Chinese Medicine. In that study, regular abdominal massage decreased mitochondrial levels to improve the ultrastructure of intestinal glial cells[15].

We then reported that TRPV1 was up-regulated in IBS rats, which could be inhibited by abdomen massage. MC blocker combined with abdomen massage has a synergistic effect on TRPV1 inhibition. TRPV1 can transfer the signaling of noxious heat and capsaicin [16], which could be sensitized by mast cells mediators such as histamine[9]. Published on the journal of *Neurogastroenterology*[9], data indicated that endogenous anti-inflammatory lipid mediators could modulate TRPV1 activation for IBS treatment. So in our study, we used ketotifen, a systemic antihistamine for the treatment of allergic rhinitis, allergic bronchitis by antagonizing histamine H1 receptor and inhibiting the release of anaphylaxis medium, to treat IBS rat and found that abdomen massage could increase the sensitivity of ketotifen. This result demonstrated the great prospect of the abdomen massage in the management of IBS.

Different from the study of Tianjin University of Traditional Chinese Medicine, we found that Trypase-PAR2-PKC ϵ pathway could regulate the sensitivity of IBS treated with abdominal massage. It was reported that over-activation of PAR2-PKC ϵ pathway phosphorylated TRPV1 protein, resulting in decreased TRPV1 opening threshold and abnormal visceral pain perception, ultimately leading to the occurrence of visceral hypersensitivity[7, 17]. In our study, we highlighted that ketotifen combined with abdominal massage stabilized the mast cells and then inhibited the PAR2-PKC ϵ -TRPV1 pathway in IBS treatment.

The limitation of this study is that when the experiment operator carried on abdomen massage with rats, the behavior itself may cause psychological stress in rats, which may lead to the weakening of the therapeutic effect of abdominal massage. This part of the factors may lead to the bias of experimental results. There are still many clinical trials to be done on abdominal massage and ketotifen used for the treatment of IBS in humans.

In conclusion, we found abdominal massage could stabilize the release of mast cell degranulation, further inhibit the PAR2-PKC ϵ -TRPV1 signaling pathway, and increase the sensitivity of ketoplifen drug therapy, which has unexpected effects in the treatment of IBS.

Declarations

Acknowledgments

Not applicable.

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Availability of data and materials

All the data generated and/or analyzed during this study are included in this published article

Statement of data

The data is available upon reasonable request

Competing interests

The authors declare that they have no competing interests.

Ethical approval

This study was approved by the Research Ethics Committee at the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine

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Figures

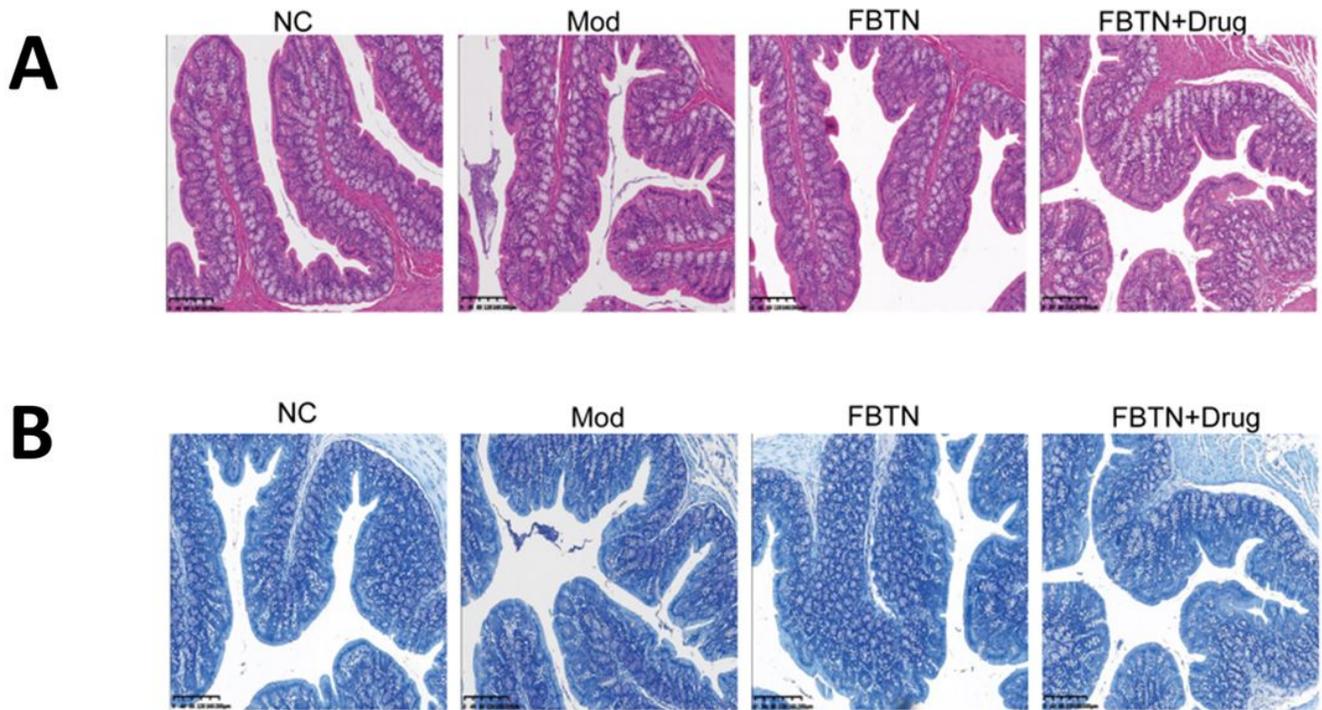


Figure 1

Abdominal massage increased the number of MC in the treatment of visceral hypersensitivity.

A. Morphological changes of small intestinal mucosa were detected by HE in normal, IBS, abdominal massage and ketotifen treatment groups (scale bar 50 μ m); **B.** Mast cells were detected by toluidine blue staining in normal, IBS, abdominal massage and ketotifen treatment groups (scale bar 50 μ m).

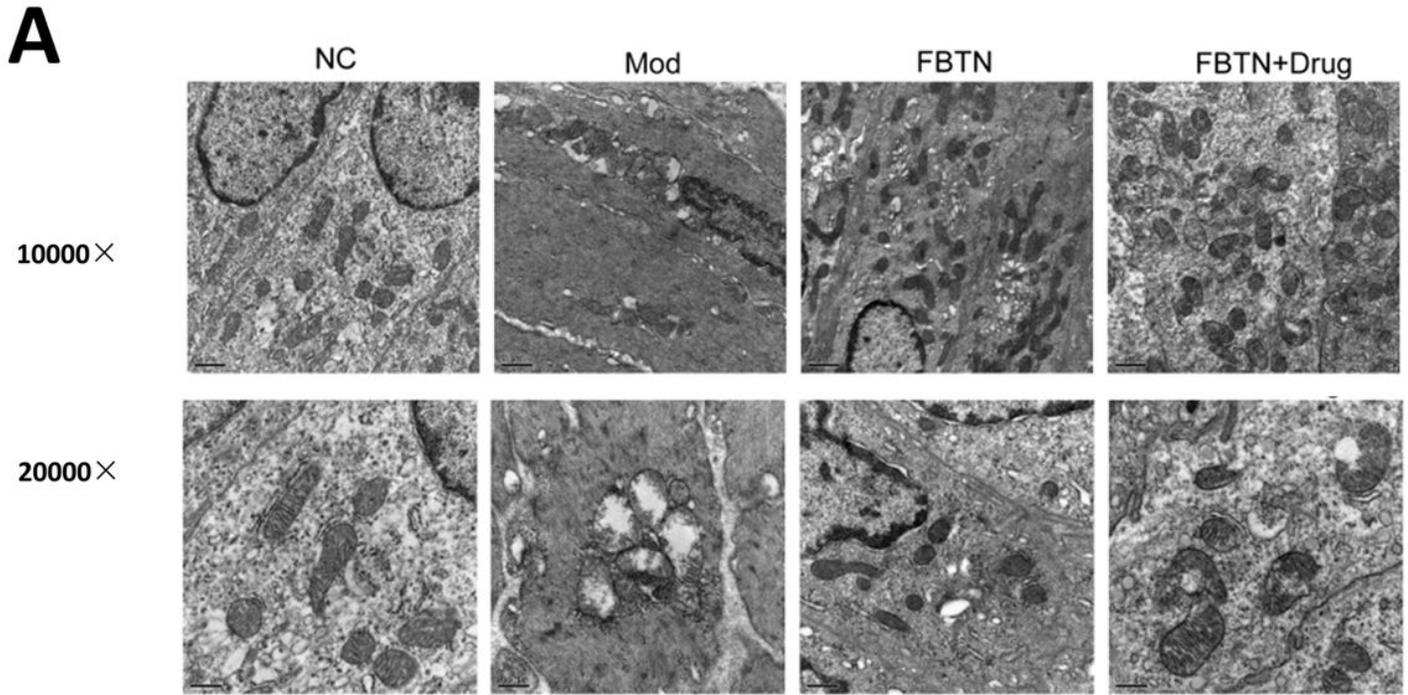


Figure 2

Abdominal massage protected the microstructural of colonic mucosa.

A. Structural evaluation of colonic mucosa by transmission electron microscopy (TEM, upper panel: 10000×, scale bar 0.50μm, down panel: 20000×, scale bar 0.25μm).

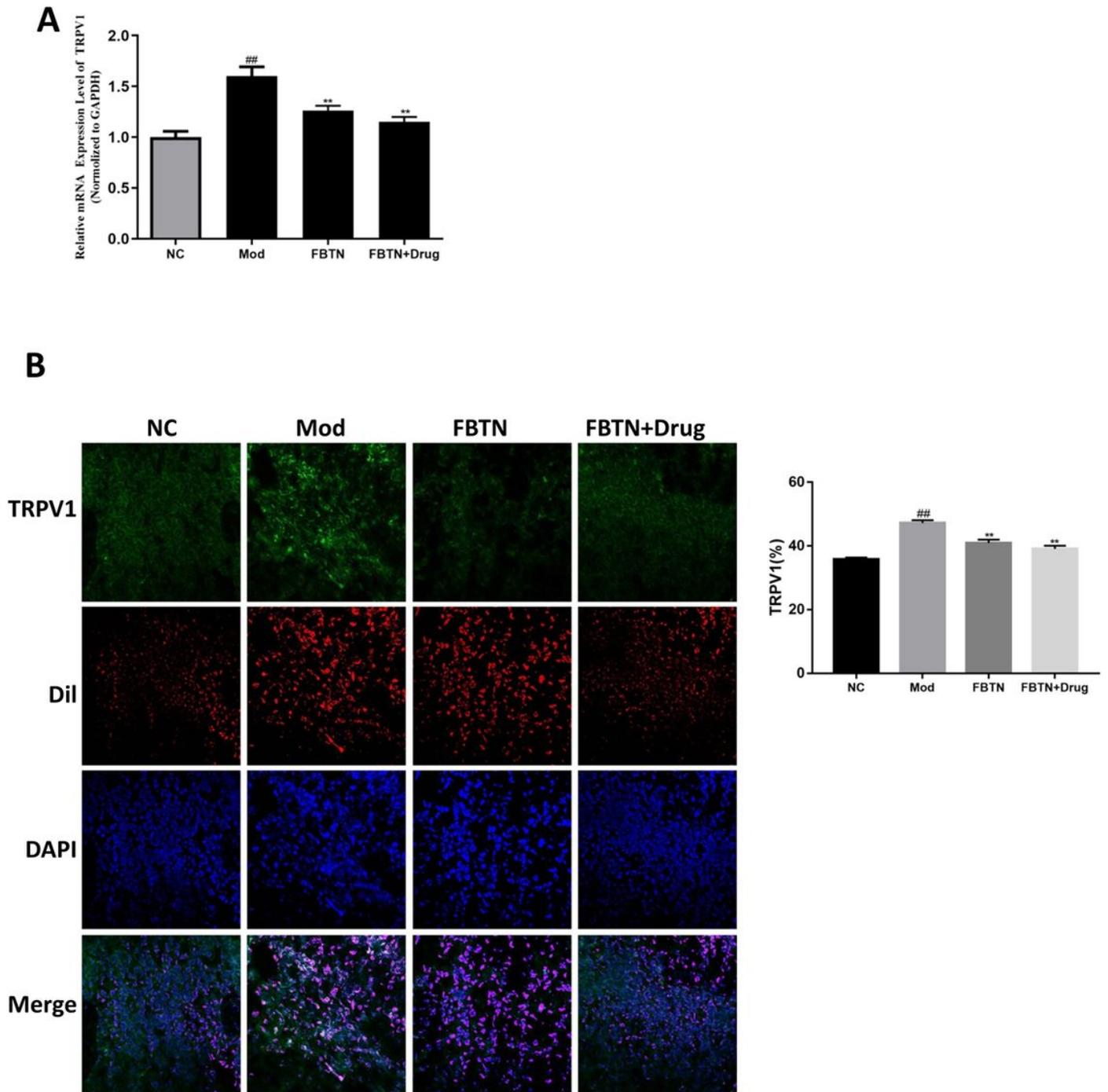


Figure 3

Effect of abdominal massage on TRPV1 mediated IBS sensitization

A Relative mRNA expression of TRPV1 in small intestinal mucosa tissue from the normal, IBS, abdominal massage and ketotifen treatment groups; **B**. Represent immunofluorescence chemical staining images of TRPV1 expression in small intestinal mucosa tissue from the normal, IBS, abdominal massage and ketotifen treatment groups (left panel) and percentage of TRPV1 positive staining (right panel). ## $P < 0.01$, compared with control group; ** $P < 0.01$, compared with IBS group.

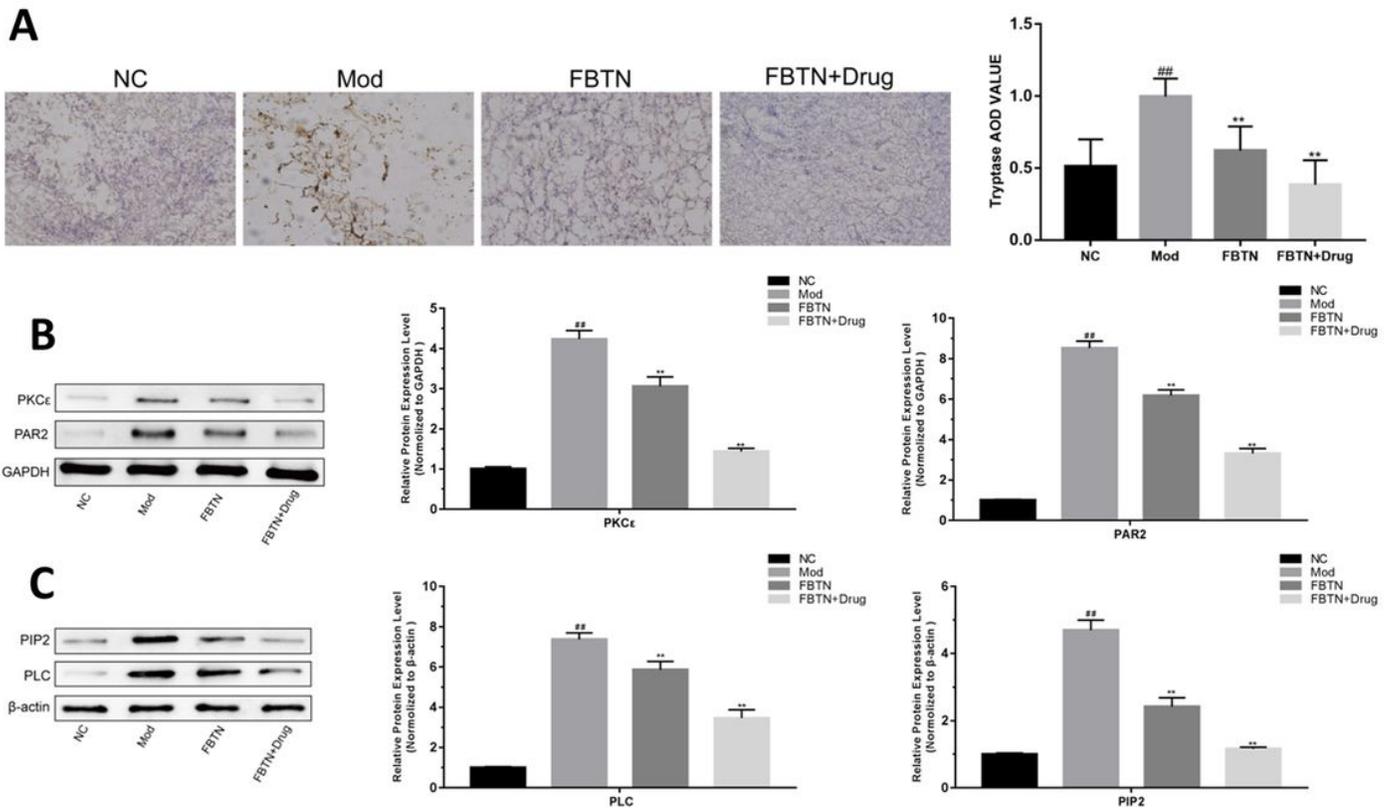


Figure 4

Abdominal massage reduced the sensitivity of IBS by regulating trypase-PAR2-PKCε pathway.

A. Represent photograph of trypase detected with IHC (left panel) and trypase AOD value analyzed with imageJ software(right panel); **B.** Detection of PAR2-PKC protein expression in small intestinal mucosa tissue from the normal, IBS, abdominal massage and ketotifen treatment groups with Western Blot (left panel: represent images of PAR2 and PKC blot; middle panel, relative protein expression of PAR2 in four groups; right panel, relative protein expression of PAR2 in four groups); **C.** Detection of PIP2-PLC protein expression in small intestinal mucosa tissue from the normal, IBS, abdominal massage and ketotifen treatment groups with Western Blot (right panel: represent images of PAR2 and PKC blot; middle panel, relative protein expression of PAR2 in four groups; left panel, relative protein expression of PAR2 in four groups). ## $P < 0.01$, compared with control group; ** $P < 0.01$, compared with IBS group.