

The Changes of Brain-gut SP and VIP Levels in the Rats with Functional Dyspepsia and the Intervention of Pingwei Capsule

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ABSTRACT

AIM: To explore the levels of substance P (SP) and vasoactive intestinal peptide (VIP) in the brain-gut axis of rats with functional dyspepsia (FD) and the intervention of Pingwei capsule.

METHODS: FD model rats were established by stimulating semi-starved rats via tail damping, provocation, and forced exercise fatigue. Then, the rats were treated with domperidone and traditional Chinese medicine, Pingwei capsule, for 3 weeks. The rates of the rat intestinal propulsion and gastric emptying were observed and the levels of SP and VIP were detected in serum by the enzyme-linked immunosorbent assay (ELISA), and in antrum, duodenum and hypothalamus by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry.

RESULTS: FD rats were less active, nervous, anxious and dysphoria, loose stool was observed, the bodyweight and food intake decreased obviously, and their intestinal propulsion and gastric emptying slowed down markedly. Molecular biological experiments showed that the mRNA and protein expressions of SP and VIP increased significantly in antrum, duodenum, and hypothalamus of FD rats, but the SP level markedly decreased in serum compared with that in the control group. Domperidone and Pingwei capsule could improve the behavioral performances, and intestinal propulsion and gastric emptying, and decrease the expressions of SP and VIP in brain-gut axis of FD rats.

CONCLUSION: There appear abnormal behaviors, gastrointestinal dysmotility and parasecretion of brain-gut SP and VIP in FD rats. Similarity to domperidone, Pingwei capsule has a degree of therapeutic effects in rats with FD.

Key words: Brain-gut axis; Functional dyspepsia; Pingwei capsule; substance P; Vasoactive intestinal peptide

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INTRODUCTION

Functional dyspepsia (FD), is a non-organic disease that mainly presents with epigastric pain, nausea, epigastric fullness, and belching. It is a common clinical functional gastrointestinal disorder. An epidemiological investigation has revealed an increase in FD incidence consequent to more quick rhythms of life and the increasing pressure of work. FD incidence is approximately 20%-25% in the West compared with 8%-23% in Asia^[1-2].

So far, the pathogenesis of FD is not understood completely. Data indicates that visceral hypersensitivity is the main cause of gastrointestinal disorders^[3-4]. Visceral hypersensitivity is a common symptom in patients with irritable bowel syndrome, FD, and functional gastrointestinal disorders, and the patients suffer visceral hypersensitivity in multiple parts of the gastrointestinal tract^[5]. Visceral hypersensitivity refers to increasing reactivity and sensitivity of visceral tissues on stimulation, including visceral allodynia, hyperalgesia, decreasing pressure threshold during colorectal balloon distension, and so forth. It is thought that visceral hypersensitivity and pathogenesis of FD may be related to functional disorder in the brain-gut axis. A disturbance in the brain-gut peptide level in the brain-gut axis may lead to FD. Therefore, it has become a common concern in researches on related peptide indexes of visceral hypersensitivity, such as substance P (SP), vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), and so forth.

The mechanisms of western medicines for the treatment of FD includes acid suppression, protecting gastric mucosa, promoting gastric motility, anti-*Helicobacter pylori*, and so on. However, Chinese medicine has better efficacy and hence has gained attention of worldwide scholars. The clinical and literature research indicate that liver stagnation and spleen deficiency are the main syndromes of FD.

Pingwei capsule is made of *Magnolia officinalis*, bran-fried *Atractylodes*, *Fructus aurantii* fried with bran, tangerine peel, and other traditional Chinese herbs, which trace back to Pingwei San from *Formularies of the Bureau of People's Welfare Pharmacies* published in Song dynasty. The capsule is developed through modern pharmacological research and years of clinical experiments. The modern pharmacological studies show that cang zhu (*Atractylodes*) has antiulcer, anti-inflammatory, and antibacterial effects and modulates gastrointestinal motility^[6]. *M. officinalis* has antioxidative, antitumor, anti-inflammatory, and antibiotic effects^[7]. *Radix Aucklandiae* improves gastrointestinal functions, promotes the contraction of gallbladder, and has anti-inflammatory, antiulcer, and antitumor effects^[8]. *Citrus aurantium* is involved in dual modulation of gastrointestinal smooth muscle^[9]. The Chen Pi decoction remarkably improves gastric emptying in mice as well as accelerates the intestinal propulsion^[10]. Bupleurum improves stomach evacuation, enhances small intestinal movement, and has analgesic and anti-inflammatory effects^[11]. *Gallus gallus domesticus* Brisson promotes secretion of digestive enzymes and gastric motor function, and helps in digestion^[12]. *Fritillaria* bulbs have analgesic, antibacterial, antitumor, and anti-inflammatory effects. Also, they activate blood and dissolve stasis^[13].

Pingwei capsule was permitted in 2012 to be used as a preparation at the Affiliated Hospital of Gansu University of Chinese Medicine. Ni *et al*^[14], through clinical observation of 80 patients with FD, indicated that Pingwei capsule combined with domperidone could improve symptoms of FD. Wang *et al*^[15], through clinical observation, pointed out that Pingwei capsule could improve epigastric pain, nausea, epigastric fullness, and so forth. Under the guidance of the

theories of traditional Chinese medicine and Pharmacy, based on the brain-gut axis research, Pingwei capsule has been tested for its clinical effects through detecting the brain-gut peptide level in FD model rats. The mechanism of FD therapy was also explored to provide an experimental basis for the clinical application of Pingwei capsule.

MATERIALS AND METHODS

Animals and instruments

The study was performed on 60 healthy male Wistar rats, weighing between 180 and 220 g, provided by the Experimental Animal Center of Lanzhou University [Qualified No. 2001A032, Certification SCXK(Gan)2013-0002], and in accordance with the guidelines from the International Association for the Study of Pain.

Enzyme-labeling instrument (Thermo, America); Confining and standing temperature cultivator (GNP-9080, Shanghai Jinhong Experimental Instrument Co.Ltd); E60 frozen microtome (Thermo, America), BX-50 optical microscope (Olympus, Japan); high-speed freezing centrifuge (Eppendorf, Germany); ultra-cold storage freezer (Thermo America); Instrument for Polymerase Chain Reaction (Thermo, America); Gel Doc EQ Imager (Bio-Rad, United States).

Drugs and reagents

Pingwei capsule, 0.5g/capsule (made from *M. officinalis*, bran-fried *Atractylodes*, and other traditional Chinese herbs), was used in this study. It was a preparation of the Affiliated Hospital of Gansu University of Chinese medicine (Approval code: Z120022224). Domperidone: 10mg/pill, produced by Xi'an-JANSEN Pharmaceutical Co. Ltd (Approval code: H10910003). Kits for SP and VIP ELISA in rats (R&D Systems, Minneapolis, United States). Anti-SP antibody, anti-VIP antibody and anti-actin antibody (Bioss, Beijing, China). Trizol reagent (Invitrogen, Carlsbad, United States). A reverse-transcription kit (Promega, Madison, United States). The forward primer, sequence of SP was 5'-GTTTGCAGAGGAAATCGGTGCCAA-3', and the reverse primer sequence of SP was 5'-GCATCCCCTTTGCCATTAATCCA-3'. The forward primer sequence of VIP was 5'-TGCCTTAGCGGAGAATGACA-3', and the reverse primer sequence of VIP was 5'-CCTCACTGCTCCTCTTCCCA-3'. The forward primer sequence of β -actin was 5'-TCCTGTGGCATCCATGAAACT-3', and the reverse primer sequence of β -actin was 5'-GAAGCACTTGCGGTGCACGAT-3'. (These primers were synthesized by Shanghai Shengong Bioengineering Co. Ltd.). 2% agarose gel (Invitrogen, Carlsbad, United States).

Animal groups and drug administration

A total of 60 Wistar male rats were randomly grouped according to weight; 10 of them were sampled as the healthy group without modeling. The rest were randomly divided into five groups after modeling: FD model group, domperidone group, and low-, medium-, and high-dose Pingwei capsule groups (10 rats in each group). The doses administered through a gastric tube were determined using the body surface area conversion ratio between rats and humans, which amounted to 0.08, 0.16 and 0.24 g/mL Pingwei capsule suspension for the low-, medium-, and high-dose Pingwei capsule groups, respectively; 0.5 mg/mL motilium suspension for the domperidone group, and physiological saline for the healthy and FD model groups. Each dose was 1 mL/100 g by gavage twice a day.

Animals model

The rat model of liver depression and spleen deficiency FD syndrome was established using composite factors of overstrain and intemperance of taking food. The tail of rats were clipped for stimulation, according to the method reported by GuoHaijun^[10]. After 1 week of adaptive breeding, the rats in the healthy group received no stimulation, ate, drank, and moved freely. The rats in the other groups started modeling. The distal ends of the tails of rats were clipped at one third of the length with a long oval sponge clamp for 30 mins so that they were irritated and fought with each other. They were then put on rotating rods to run for 10 min at a speed of 50 rpm. This modeling was repeated every 3 h, four times a day for 10 days, fed with alternate day, and hair color, chilly extremities, hypodynamia, and irritability were observed. After that, bodyweight, food intake, water intake, and stool of rats were recorded. The gastric emptying and intestinal propelling were used to evaluate gastrointestinal motility.

Measurement of intestinal propulsion and gastric emptying

After 10 days of modeling, five rats were randomly separated from the model and healthy groups. Then, gastric evacuation and intestinal propelling were performed to confirm whether modeling was successful. The rats were fasting for 24 h before experiment. The rats were given 5% graphite powder with milk and glucose in water (weighted and recorded as A1,) by oral gavage and absorbed 30 min later, then sacrificed. The stomach and attached small intestine of rats, immediately exposed by laparotomy, were carefully removed and placed on a wooden board to observe the leading edge of the graphite powder in the intestine after ligation of esophagogastric, gastroduodenal, and ileocaecal junctions. Length of the small intestine from pylorus to ileocecal and the length of graphite powder promoting were measured. The formula for calculating intestinal propulsion was as follows: intestinal propulsion rate = length of graphite powder/whole length of small intestine. The stomach was cutted out, weighted (recorded as A2) and immersed into 0.9% saline solution to clean the rest of graphite powder out. After blotted dry with absorbent paper to remove any surface moisture, the stomach was weighed again (recorded as A3). The formula for calculating gastric emptying rate was as follows: gastric emptying rate = (A2-A3) / A1.

Serum and tissue preparation

All rats were killed after treatment with drugs for 3 weeks. Then, overnight fasting blood was collected, kept at room temperature for 1-2 h, and centrifuged at 3500-4000 rpm for 10 min at 4°C. Thus, the supernatant or serum was obtained and stored separately in a centrifuge tube in a freezer at -80°C. The hypothalamus, antral, and duodenal tissues were collected and the parts of the tissues were stored in a freezer at -80°C for mRNA detection, another parts of the tissues were fixed with 4% paraformaldehyde for immunohistochemistry experiment.

General behavior observation

Each group was observed for change in hair color, chilly extremities, hypodynamia, irritability, mobility, spirit, and stool.

Determination of diet and bodyweight

Food intake and bodyweight were recorded daily. All rearing cages and feed boxes were checked for leakage to prevent accidents. Diet was given at the same time daily. The amount remaining was subtracted from the total diet to determine food intake.

Detection of SP and VIP in plasma

The levels of SP and VIP protein in serum were quantitated by ELISA (R&D Systems, Minneapolis, United States) according to the manufacturer's protocol. Optical densities of the ELISA samples were determined within 30 min on a microplate reader set to 450 nm.

RT-PCR detection of SP and VIP mRNA

Total RNA was isolated from the hypothalamus and gastrointestinal tissues with Trizol reagent and reverse transcription reaction was performed with a reverse transcription kit according to a manufacturer's protocol. PCR reactions, and then products were separated on 2% agarose gel and color imaging was performed.

Immunohistochemistry

The antrum, duodenum and hypothalamus were fixed in neutral 4% paraformaldehyde in 0.1 M sodium phosphate buffer, embedded in paraffin blocks, and cut into 4- μ m-thick sections on slides. Deparaffinized sections were incubated in 0.01 M citrate buffer (Solarbio) in a microwave oven for heat-induced epitope retrieval. Endogenous peroxidase activity was blocked with 3% H₂O₂ (Zsbio, Beijing, China), and nonspecific binding was blocked by incubation with 5% normal goat serum (Zsbio) in phosphate-buffered saline for 5 min. The samples were then incubated with anti-SP antibody and anti-VIP antibody (Bioss, Beijing, China) overnight at 4°C. After washing, the slides were incubated with secondary biotinylated antibody (Zsbio) for 20 min at room temperature. Visualization was achieved using a DAB detection kit (Solarbio).

Statistical Analysis

SPSS 19.0 software was used for the statistical analyses. All experimental data are expressed as mean \pm SD. One-factor analysis of variance (ANOVA) was used for multi-group comparisons. For inter-group comparisons, the least significant difference (LSD) method was used to assess the homogeneity of variance. Statistical significance was accepted for *p*-values < 0.05.

RESULTS

Observations of rat general condition and behavioral performances

Amounts of diet and drinking water were stable for rats of the healthy group. The normal rat hair was bright and clean, soft and smooth. The control rats were highly sensitive and active, with heightened senses. The stool was well formed and moderately desiccated in control group. The rats in the model group were less active, nervous, and anxious with chilly extremities on day 2. After 5 days of modeling, the rats were less excited and active. They moved stiffly, were slow in response, and withered. Such symptoms were more significant with increasing days of modeling. Apathy to stimulation, occasional dysphoria, unstable mood, and loose stool was observed as symptoms of liver depression and spleen deficiency compared with the normal group. After treatment with domperidone and Pingwei capsule, the above symptoms relieved obviously.

Rates of intestinal propulsion and gastric emptying

The rate of intestinal propulsion was decreased (*p* < 0.01) and gastric emptying slowed down significantly (*p* < 0.001) in the model group compared with the control (Figure 1). Pingwei capsule, same as domperidone could improve gastric emptying markedly (Figure 1).

Effect of Pingwei capsule on the diet and bodyweight of rats

Food intake and bodyweight decreased significantly in the model

group compared with that in the normal group ($p < 0.001$). Bodyweight increased significantly in the domperidone group ($p < 0.01$), and in medium- and low-dose Pingwei capsule groups (all $p < 0.05$) compared with that in the model group. Food intake increased significantly in the domperidone group ($p < 0.05$) and in medium- and low-dose Pingwei capsule groups (all $p < 0.01$) compared with that in the model group (Table 1).

Effects of Pingwei capsule on the levels of serum SP and VIP in FD rats

The plasma SP level decreased significantly in the model group compared with that in the control group ($p < 0.01$). However, the plasma VIP level increased significantly in the model group compared with those in the control group ($p < 0.001$). Compared with the model group, the plasma SP level in the domperidone group and high-dose Pingwei capsule group increased significantly (all $p < 0.01$). The plasma VIP levels in the domperidone group, and in low-, medium- and high- Pingwei capsule groups decreased significantly (all $p < 0.001$) (Figure 2).

Expression of SP mRNA in the antrum, duodenum, and hypothalamus

Compared with the control group, the SP mRNA level in the model group increased significantly in the antrum, duodenum and hypothalamus (all $p < 0.001$). Compared with the model group, the SP mRNA levels decreased significantly in the antrum and duodenal tissue (all $p < 0.001$) after treatment with domperidone. The SP mRNA levels in the low- and medium-dose Pingwei groups significantly reduced in the antrum (all $p < 0.001$). The SP mRNA levels in the low- and high-dose Pingwei groups decreased significantly in duodenum (all $p < 0.001$) (Figure 3).

Expression of VIP mRNA in the antrum, duodenum, and hypothalamus

The VIP mRNA levels in the model group increased significantly in the antrum ($p < 0.001$), duodenum ($p < 0.001$), and hypothalamus ($p < 0.05$) compared with that in the control group. Compared with the model group, the VIP mRNA levels decreased significantly in the antrum, duodenum, and hypothalamus (all $p < 0.001$) after treatment with domperidone. The VIP mRNA levels in the medium-dose and high-dose Pingwei groups decreased significantly in the duodenal tissues (all $p < 0.001$). The VIP mRNA levels in the low-dose ($p < 0.001$) and high-dose Pingwei group ($p < 0.05$) decreased significantly in the hypothalamus (Figure 4).

Effect of PWC on the expression of SP and VIP

Compared with the normal control, the model group had increased SP and VIP expression in antrum, duodenum and hypothalamus, however, after treatment with domperidone and Pingwei capsule, the expression of SP (Figure 5A) and VIP (Figure 5B) decreased.

DISCUSSION

Gastric hypersensitivity was reported in 34%-87% patients with FD^[16-18]. The brain-gut peptides, including SP, VIP, CGRP, and 5-hydroxytryptamine, were found to be related to gastric hypersensitivity. SP, VIP, and CGRP are widely distributed in the central nervous system and gastrointestinal tract. Previous studies showed a significant increase in the SP content of gastric mucosa in patients with FD compared with the control group. Visceral hypersensitivity can be alleviated by decreasing plasma SP levels,

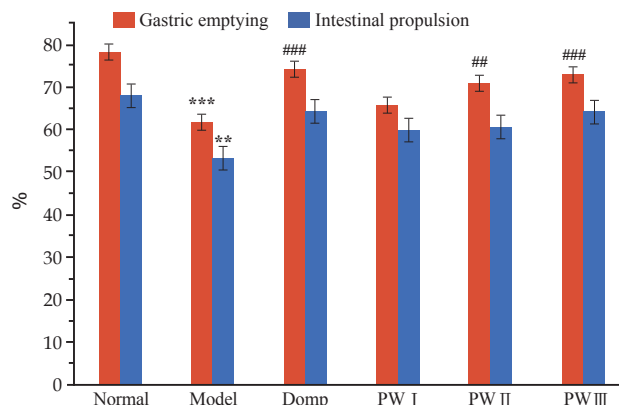


Figure 1 Effects of Pingwei capsule treatment on rates of intestinal propulsion and gastric emptying in FD rats. Domp: $10 \text{ mg} \times \text{kg}^{-1} \times \text{d}^{-1}$ domperidone; PW I: $1.6 \text{ g} \times \text{kg}^{-1} \times \text{d}^{-1}$ Pingwei capsule; PW II: $3.2 \text{ g} \times \text{kg}^{-1} \times \text{d}^{-1}$ Pingwei capsule; PW III: $4.8 \text{ g} \times \text{kg}^{-1} \times \text{d}^{-1}$ Pingwei capsule. Statistical significance to normal group (** $p < 0.01$, *** $p < 0.001$); (## $p < 0.01$, ### $p < 0.001$) to model group.

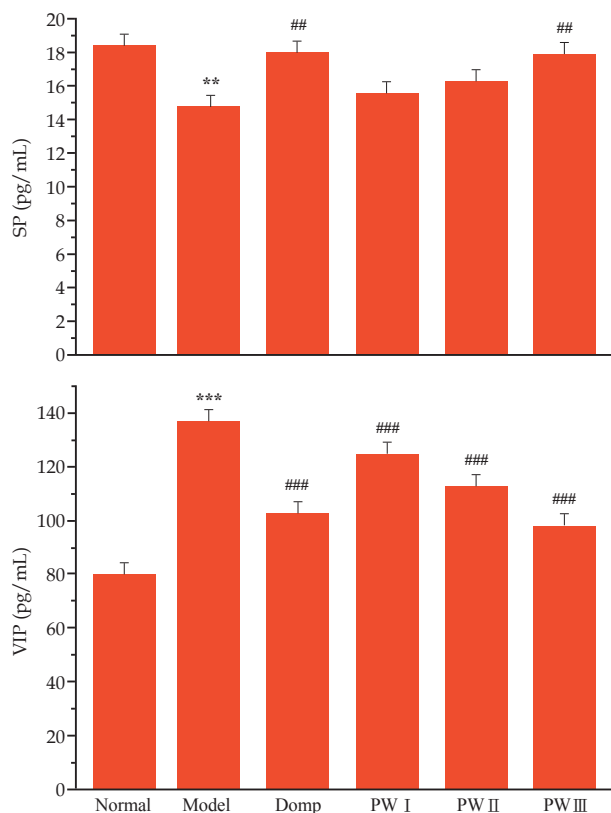


Figure 2 Effects of Pingwei capsule on SP and VIP in serum. Statistical significance to normal group (** $p < 0.01$, *** $p < 0.001$); (## $p < 0.01$, ### $p < 0.001$) to model group. $n = 4-9$.

thus enhancing gastrointestinal pain threshold^[19]. Researchers found that motilin (MTL) and cholecystokinin (CCK) levels in both fasting or meal FD group were higher than those in the control group. The plasma VIP level was also higher than that in the control group, but the effect of VIP level on FD is yet to be explored completely^[20].

Based on the symptoms such as postprandial fullness, early satiety, anorexia, and belching, FD is termed as “Epigastralgia,” “Epigastric Fullness,” and “noisy stomach” in traditional Chinese medicine. It is believed that the location of FD is stomach, also closely related to the function of spleen and liver. Spleen deficiency, liver qi invasion, and disorders of liver and middle energizer (stomach fails to propel

Table 1 Effects of Pingwei capsule on bodyweight and food intake in FD rats.

	Normal	Model	Domp	PW I	PW II	PW III
Body weight (g)	293.78 ± 28.91	225.22 ± 11.72***	262.36 ± 19.54##	256.66 ± 16.86#	254.08 ± 16.34#	251.68 ± 8.71
Food intake (g)	35.62 ± 1.916	25.70 ± 2.684***	30.11 ± 2.602#	30.53 ± 2.731##	30.27 ± 3.367##	29.00 ± 1.415

Domp: 10 mg × kg⁻¹ × d⁻¹ domperidone; PW I : 1.6g × kg⁻¹ × d⁻¹ Pingwei capsule; PW II : 3.2g × kg⁻¹ × d⁻¹ Pingwei capsule; PW III : 4.8g × kg⁻¹ × d⁻¹ Pingwei capsule. Statistical significance to normal group (***)*p* < 0.001); (#*p* < 0.05, ## *p* < 0.01) to model group.

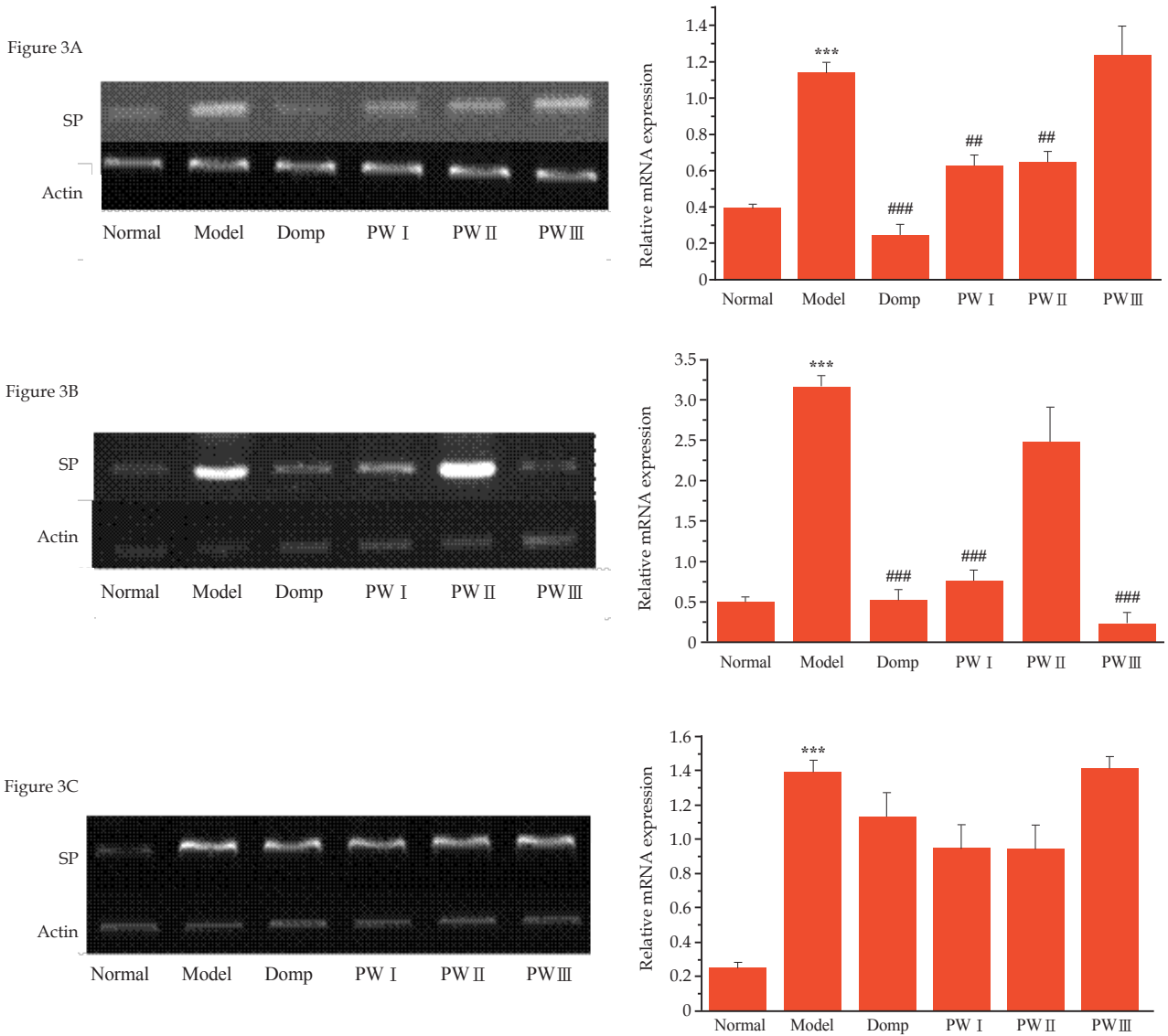


Figure 3 Effects of Pingwei capsule on SP mRNA in FD rats. Expression of VIP mRNA in homogenates of antrum (A), duodenum (B), hypothalamus (C). Statistical significance to normal group (***)*p* < 0.001); (#*p* < 0.01, ###*p* < 0.001) to model group.

downward or middle energizer cannot regulate the body's vital energy) all lead to FD. According to the principles of traditional Chinese medicine, FD is categorized into five syndromes: spleen deficiency syndrome, lack of coordination between the liver and the stomach, dampness-heat of spleen and stomach, deficiency cold of spleen and stomach syndrome, syndrome of intermingled heat and cold^[21]. Investigations show that the prevalence of two syndromes are 45.9% (spleen deficiency syndrome) and 43.28% (lack of coordination between the liver and the stomach)^[22]. Therefore, liver depression and spleen deficiency are the main syndromes of FD. *Atractylodes*, a component of Pingwei capsule, activates and invigorates spleen as well as eliminates dampness. *M. officinalis*

eliminates dampness as well as removes food stagnation; it is the important medicine to alleviate distention. *F. aurantii* alleviates distention by promoting the flow of qi. A therapy lacking the treatment of liver is ineffective. Therefore, Pingwei capsule was a combination of Bupleurum, tangerine peel, and *R. Aucklandiae* to smooth the liver. *Fritillaria* bulbs reduce phlegm and resolve masses to inhibit acid secretion. *Rhizoma coptidis* clears away heat and toxic materials. *Rhizoma sparganii* and *Radices zedoariae* relieve blood as well as promote qi. Common *Bletilla pseudobulb* generates blood and new flesh. Cuttlebone inhibits acid secretion to relieve pain. *Rhizoma corydalis* and *Radix paeoniae rubra* promote blood circulation and remove blood stasis. Pingwei capsule aims at soothing liver and

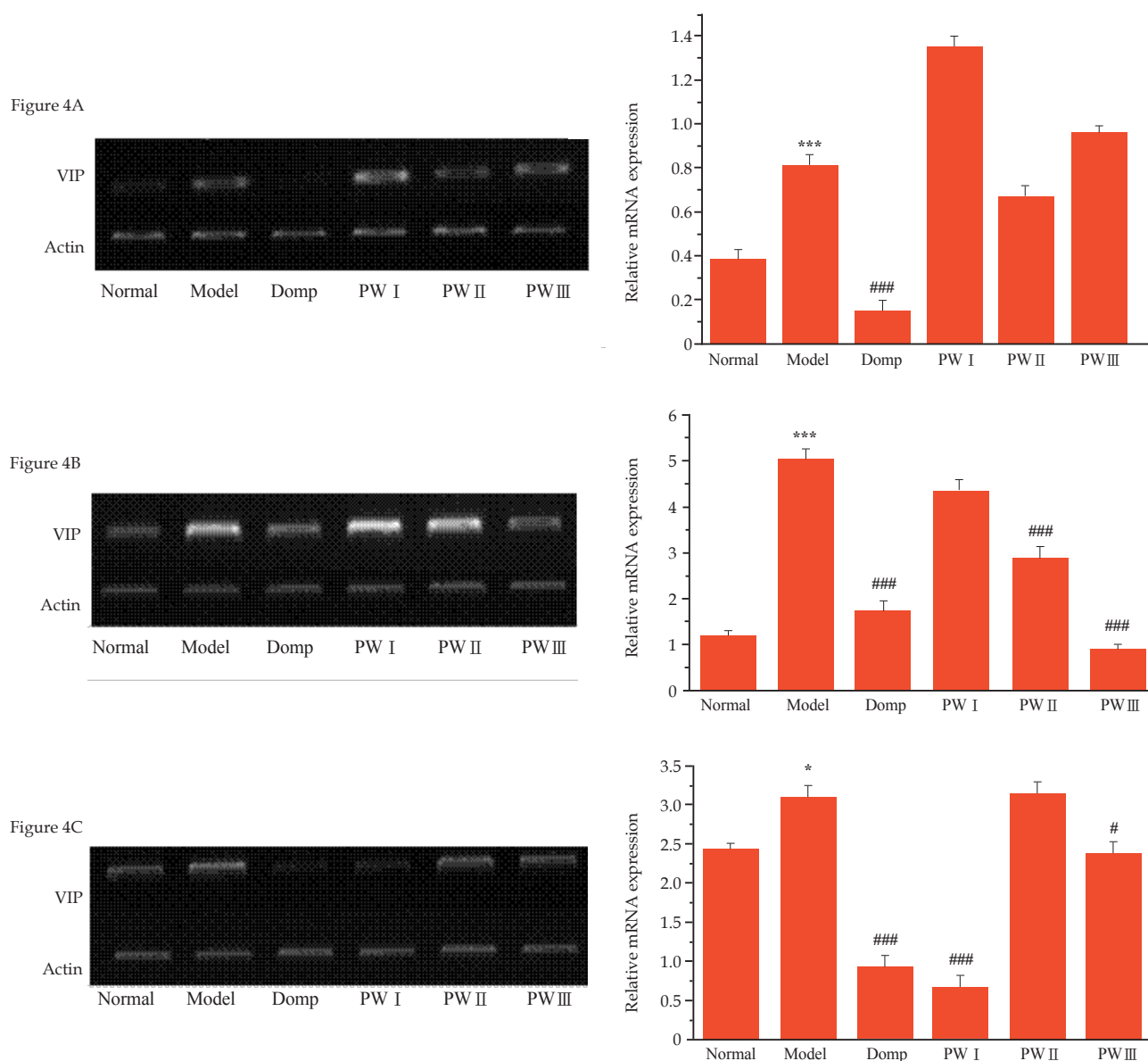


Figure 4 Effects of PingWei capsule on VIP mRNA in FD rats. Expression of VIP mRNA in homogenates of antrum (A), duodenum (B), hypothalamus (C). Statistical significance to normal group (* $p < 0.05$, *** $p < 0.001$); (# $p < 0.05$, ### $p < 0.001$) to model group.

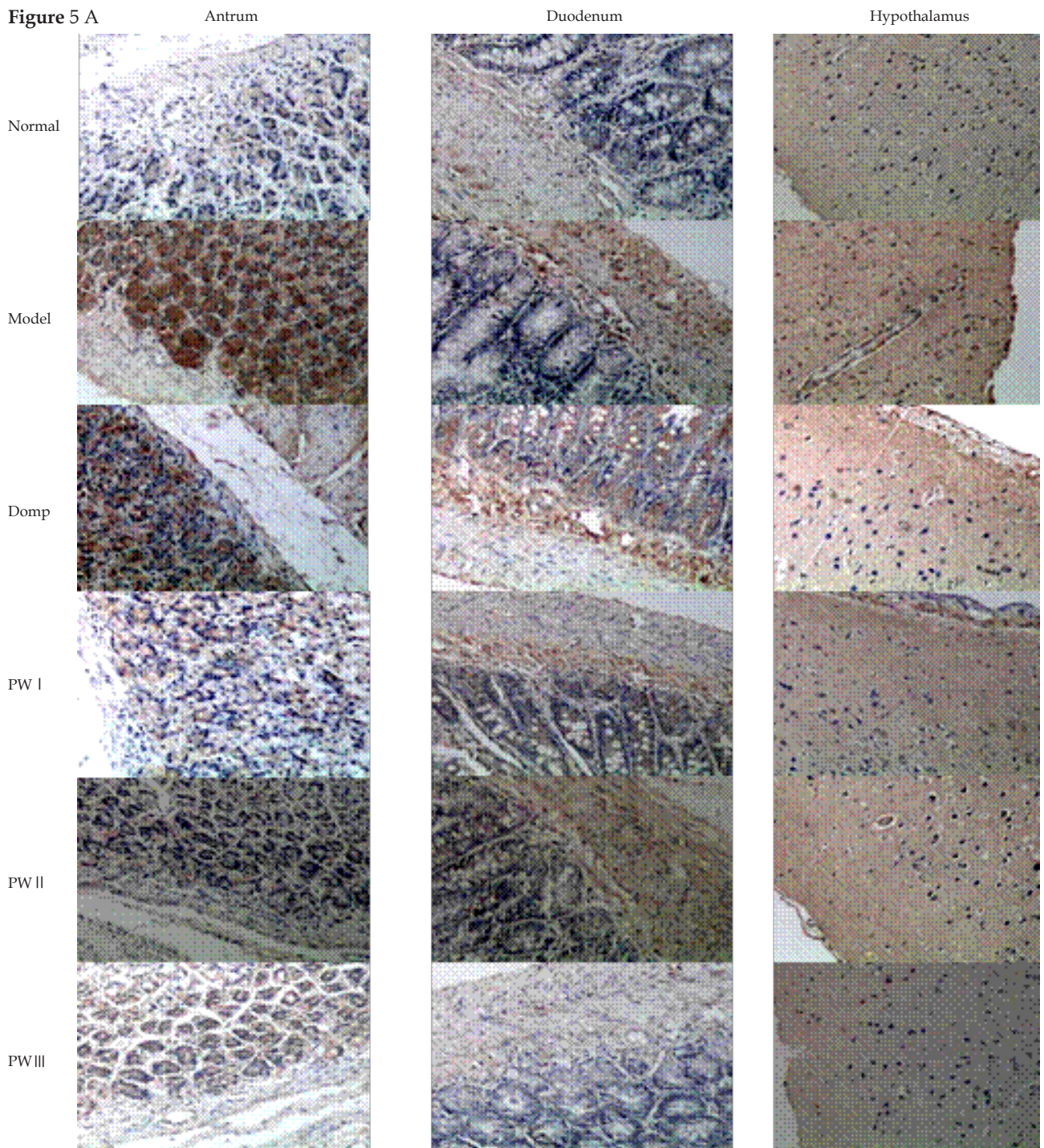
strengthening spleen, as well as clearing heat and removing dampness to inhibit acid secretion to relieve pain, thus reinforces spleen and liver. Therefore, Pingwei capsule has a significant curative effect in the clinical therapy of FD.

In the present study, the FD rats model of liver depression and spleen deficiency was established by clipping the tails of rats for stimulation and using composite factors, and general condition, weight, diet, and the changes of related peptide levels of visceral hypersensitivity such as SP and VIP were recorded. The aim of the study was to explore the molecular mechanism and treatment targets of Pingwei capsule. VIP, which is a neural peptide secreted in the central nervous system (CNS) and enteric nervous system (ENS), participates in secretory activity, motility regulation, and peristaltic reflex suppression in the circular smooth muscle layer^[23-24]. SP is one of the important brain-gut peptides in mammals, which is composed of 11 amino acids. Mainly distributed in the CNS, spinal cord dorsal root, and ENS, SP promotes gastrointestinal peristalsis, protects the gastrointestinal epithelium to repair the impaired

gastrointestinal mucosa, and causes gastric and intestinal mechanical hypersensitivity^[25].

Our experiment demonstrated that FD rats were less active, nervous, anxious and dysphoria, loose stool was observed, the bodyweight and food intake decreased obviously, and their intestinal propulsion and gastric emptying slowed down markedly. The expressions of SP and VIP increased significantly in antrum, duodenum, and hypothalamus of FD rats. Domperidone and Pingwei capsule could improve the behavioral performances and intestinal propulsion and gastric emptying, and decrease the expressions of SP and VIP in brain-gut axis of FD rats. But, in contrast, the SP level of FD rats markedly decreased in serum compared with that in the control group, which needs more experiments to be elucidated.

In summary, similar to domperidone, Pingwei capsule can modulate the secretion of brain-gut axis SP and VIP which probably contribute to the improvement of behavioral performances and gastrointestinal motility, and exert a curative effect on FD liver depression and spleen deficiency syndrome.



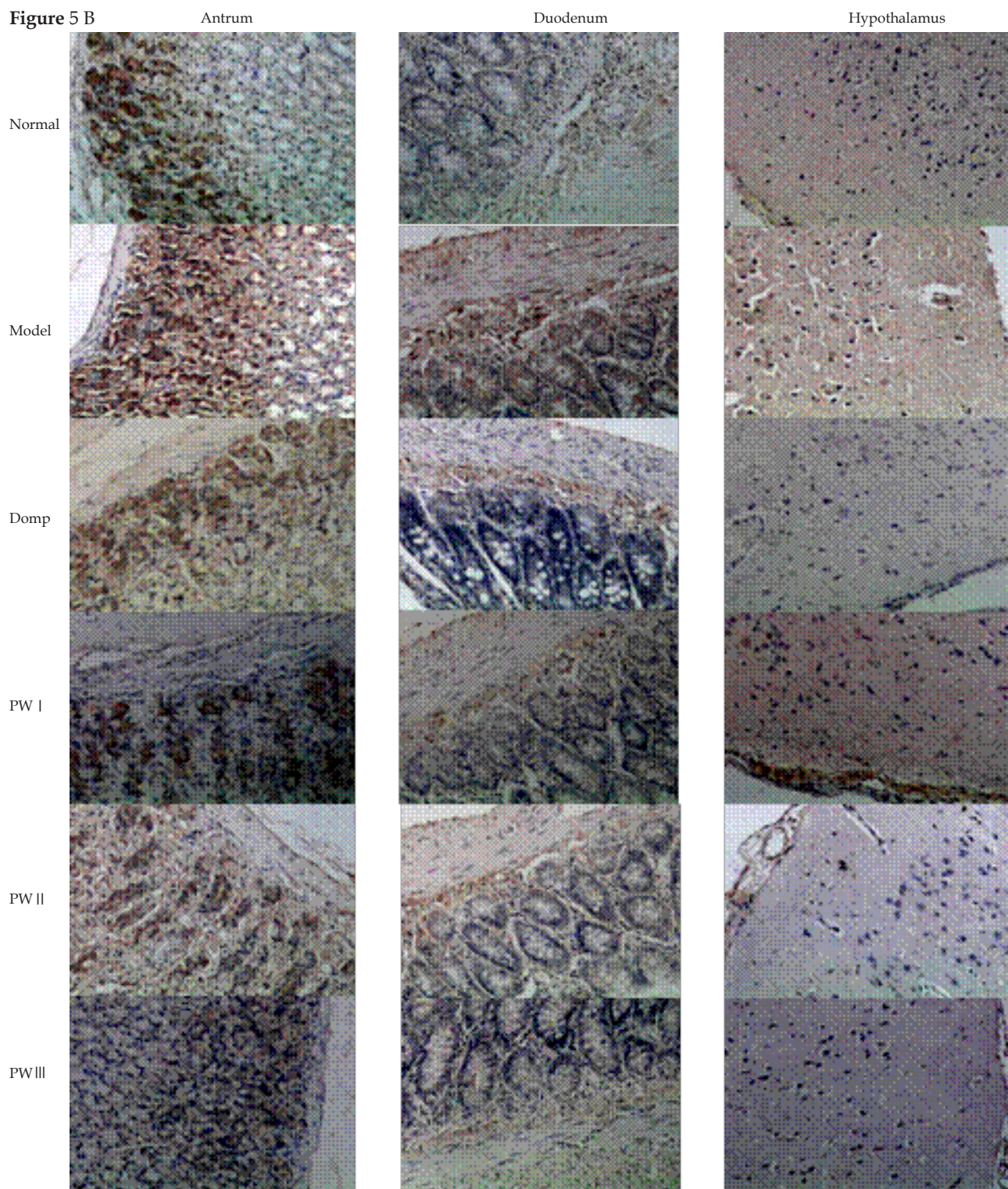


Figure 5 Effects of PWC on SP and VIP protein expression in FD rats. Immunohistochemistry for the expression of SP (A), and VIP protein (B) in the antrum, duodenum, and hypothalamus of rats (magnified $\times 100$). Domp: received $10 \text{ mg kg}^{-1} \text{ d}^{-1}$ domperidone for 21 days; PWC I: received $1.6 \text{ g kg}^{-1} \text{ d}^{-1}$ Pingwei capsule for 21 days; PWC II: received $3.2 \text{ g kg}^{-1} \text{ d}^{-1}$ Pingwei capsule for 21 days; PWC III: received $4.8 \text{ g kg}^{-1} \text{ d}^{-1}$ Pingwei capsule for 21 days. Normal control and model groups (all $n = 6$, respectively): received the same volume of 0.9% sodium daily for 21 days. SP: substance P; VIP: vasoactive intestinal peptide.

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