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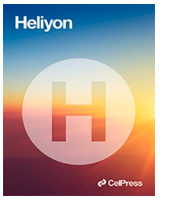

Jian-Pi-Yin decoction attenuates lactose-induced chronic diarrhea in rats by regulating GLP-1 and reducing NHE3 ubiquitination and phosphorylation

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A R T I C L E I N F O

Keywords:
Glucagon-like peptide-1
$NA^{+}H^{+}$ exchanger 3
NHE3 ubiquitin
Diarrhea

A B S T R A C T

Objectives: Jian-Pi-Yin decoction (JPY), a prescription derived from the traditional Chinese medicine Shen-Ling-Bai-Zhu-San, has shown good clinical efficacy in the treatment of diarrhea caused by lactose intolerance. However, the mechanism of action of JPY in the treatment of diarrhea is not fully understood.
Design: In this study, a rat diarrhea model was induced by high lactose feeding combined with standing on a small platform to investigate the ameliorating effect of JPY on hyper lactose-induced diarrhea in rats and its possible mechanism.
Methods: The rat model of hyper lactose diarrhea was given high, medium, and low doses of JPY and the positive control drug Smida by gavage for 1 week. At the same time, $NA^{+}H^{+}$ exchanger 3 (NHE3) inhibitor Tenapanor was administered orally for 3 weeks. Body weight, food intake, water intake, grip strength, and severity of diarrhea symptoms were measured in rats throughout the study. The serum, colon, and jejunum tissues of the model and drug-treated rats were collected for histopathological examination and analysis of relevant indicators.
Results: JPY significantly alleviated the symptoms of fatigue, diet reduction and diarrhea in the model group. Glucagon-like peptide-1 (GLP-1) and cyclic adenosine monophosphate (cAMP) expression were also down-regulated after JPY treatment. JPY can significantly promote NHE3 in intestinal tissues of rats with diarrhea, and the mechanism is related to the decrease of GLP-1, inhibition of cAMP/PKA pathway activation, an increase of ubiquitin-specific protease 7 (USP7) and USP10 expression, and decrease of NHE3 ubiquitination and phosphorylation.
Conclusion: JPY can reduce the expression of GLP-1, reduce the ubiquitination and phosphorylation of NHE3, regulate the expression of NHE3, at least partly improve ion transport in the

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intestinal epithelium, and improve the imbalance of electrolyte absorption, thus significantly reducing the diarrhea symptoms of rats with high lactose combined with small platform standing. *Innovation:* In this study, we explored the mechanism of intestinal GLP-1 activation of cAMP/PKA signaling pathway from multiple dimensions, and increased its expression by reducing phosphorylation and ubiquitination of NHE3, thereby treating chronic diarrhea associated with lactose intolerance.

1. Introduction

Chronic diarrhea is a common and confusing disease that often continues to recur chronically. It affects up to 5% of the population at a given time, and its incidence is increasing worldwide [1]. Among them, with the increase in dairy intake, lactose malabsorption or intolerance and diarrhea caused by high lactose have gradually received more and more attention. This is due to diarrhea when excessive lactose intake or reduced lactase expression exceeds the capacity of the small intestine to absorb lactose [2]. Diarrhea caused by lactose intolerance is known to adversely affect the quality of life of patients and impose a significant financial burden on patients and the healthcare system as a whole. And the clinical effect is poor and easy to repeat, patient problems can't be solved, and there exists an unmet need for lactose-induced chronic diarrhea management.

Many patients hence seek help from Traditional Chinese Medicine (TCM) as an alternative treatment method. TCM has been used to treat various gastrointestinal (GI) conditions for hundreds of years in clinical practice [3]. The efficacy of TCM for lactose-induced chronic diarrhea is not solely anecdotal. Several well-designed randomized controlled clinical trials have demonstrated the effectiveness of TCM for chronic diarrhea [4].

Shen-ling-bai-zhu-san (SLBZS) is a classic prescription for the treatment of diarrhea. It can be used to treat clinical chronic diarrhea, ulcerative colitis, functional dyspepsia, and other diseases, and has achieved remarkable results. At the same time, modern pharmacological studies have confirmed that it can protect the intestinal barrier, improve the immune capacity of the body, improve intestinal microecology, enhance gastrointestinal motility, and promote nutrition absorption [5]. Jian-Pi-Yin decoction (JPY, meaning Tonifying Spleen Drink) was modified from SLBZS according to the classical theory of spleen deficiency and the pathogenesis of diarrhea under the guidance of clinical practice. It mainly includes *Codonopsis pilosula*, *Atractylodes lancea*, *Coix lacryma-jobi*, *Zingiber officinale*, *Dolomiaea souliei*, *Portulaca oleracea*, *Paeonia lactiflora* and *Glycyrrhiza uralensis*. The previous studies of our group demonstrated that JPY could significantly ameliorate the clinical symptoms and possessed potent strengthening of the spleen and stopping diarrhea effects in lactose-induced diarrhea, indicating that JPY is a promising therapy for lactose-induced chronic diarrhea (unpubl. data).

The pathogenesis of lactose intolerance is not fully understood, but there is an imbalance in the absorption and secretion of intestinal water and electrolytes, which is accomplished by transporters on epithelial cell membranes [6,7]. Previous studies showed that intestinal epithelial membrane transporters, particularly NA^{+}-H^{+} exchanger 3 (NHE3), were abnormally expressed in patients with diarrhea, NHE3 activity is regulated by ubiquitination and phosphorylation of NHE3 [7]. Glucagon-like peptide-1 (GLP-1) is a polypeptide hormone with a wide range of pharmacological effects and is secreted by intestinal endocrine L cells. The main effect of GLP-1 is to lower blood sugar by regulating insulin secretion, inhibiting gastric emptying, and reducing appetite and food intake. It has been confirmed that elevated GLP-1 levels in vivo act on GLP-1 receptor (GLP-1R), inhibit renal NHE3, and cause diarrhea, but the role of intestinal NHE3 remains unclear [8].

Therefore, the study aims to examine the effects of JPY on GLP-1 secretion, also the effects of GLP-1 on lactose-induced chronic diarrhea. To further corroborate the role of GLP-1 in the inhibition of intestinal NHE3 and its possible related mechanisms, a lactose-induced chronic diarrhea model were performed.

2. Materials and methods

2.1. Composition and preparation of JPY

The preparation of JPY accords with the Chinese Pharmacopeia 2015 (Batch numbers: 180720, Shanghai Hutchison Pharmaceuticals, China). According to the clinical dosage of Chinese patients (73.5 g/day), and the body surface area conversion method (coefficient 0.0018), we calculated the mouse dosage = human dose (mg/kg/day) *70 kg *0.0018/0. 2 kg = 0.6615 g/kg/day. Taking this dose as a theoretical reference, we select different concentrations. Based on volume of intragastric administration of rats was 1 ml/100 g body weight, low, medium, and high concentrations of JPY were 0.386 g/ml, 0.772 g/ml, 1.544 g/ml, respectively. All Chinese herbal medicines were purchased from Xiyuan Hospital, China Academy of Chinese Medical Sciences, and prepared by the Preparation Department of Xiyuan Hospital.

2.2. Component analysis of JPY with UPLC

A Waters UPLC HSS T3 (1.8 μm, 2.1 mm × 100 mm) was used to separate the aqueous extract of JPY. The mobile phase was 0.1% aqueous formic acid (A) and acetonitrile (B). The MS analysis was carried out with Waters XEVO TQ-X's tandem quadrupole mass spectrometry system in both positive and negative ion modes. The contents of the Lobetyolin, Paeoniflorin, Glycyrrhizic_acid, 6-

Table 1
The standard curves and contents of the five chemical standards of JPY.

Standard	Standard curve	JPY (ng/mL)
Lobetyolin	$Y = 463.7x - 612.78$ ($R^2 = 0.9930$)	5942.95
Paeoniflorin	$Y = 147.12x - 17.3393$ ($R^2 = 0.9978$)	335296.99
Glycyrrhizic_acid	$Y = 59.6843x - 662.942$ ($R^2 = 0.9912$)	65990.76
6 - Gingerol	$Y = 7.25x - 42.6195$ ($R^2 = 0.9973$)	26678.89
Dehydrocostus_lactone	$Y = -0.08^2 + 247.495x + 504.201$ ($R^2 = 0.9983$)	1888.01

Gingerol and Dehydrocostus_lacton were calculated based on pre-constructed standard curves, and the results were listed in Table 1.

2.3. Medicinal materials and reagents

Montmorillonite powder (Smida, F17193) is provided by Bofu Yisen (Tianjin, China) Pharmaceutical Co., LTD., (F17193). According to the above calculation method, the dose of Smida for rats was 0.0945 g/ml. NHE3 inhibitor Tenapanor (GC19352) was purchased from GlpBio (Mo, Ca, USA). Amylase (C016-2-1), D-xylose (A035-1-1), lactic acid (A019-2-1), cAMP (H164), and GLP-1 (H294-1) kits were purchased from Nanjing Jiancheng Company (Nanjing, China). Anti-Sodium/Hydrogen Exchanger 3/NHE-3 antibody (ab95299), Anti-HAUSP/USP7 antibody [EPR4253] (ab108931), Anti-HAUSP/USP10 antibody [EPR4261] (ab108931), Anti-GLP-1R antibody [EPR21819] (ab218532) and Anti-PKA R2/PKR2 antibody [Y116] (ab32514) were provided by Abcom (Massachusetts, US). HA-Tag (C29F4) Rabbit mAb (#3724), Phospho-(Ser/Thr) PKA Substrate Antibody (#9621) were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.4. Animal and experimental design

Laboratory procedures followed the guidelines and practices of the Animal Care Ethics Committee of Xiyuan Hospital (Permission code: 2019XLC004-2). This procedure was following the regulations of the Beijing Laboratory Animal Management Office. Healthy male Wistar rats (weight: 200 g $\pm$ 20 g) were housed under an artificial 12-h light-dark cycle (lights turned on at 8:00 a.m.), with five rats per cage, standard diet, and free water.

All rats were randomly divided into 7 groups with 10 rats in each group: control group, model group, Smida group, Tenapanor group, JPY high-dose (JH), medium-dose (JM), low-dose (JL) groups. Rats in model group, Smida group, JH, JM, and JL groups were fed with 50% high lactose diet for 14 consecutive days. 50% of the high lactose feed was purchased from Beijing Keaoxili Feed Co., LTD. (SCXK (Beijing) 2019-0012). At the same time, the rats were placed on a small platform in a special rectangular water tank from 22:00 to 8:00 the next day, and the platform was filled with water for 14 consecutive days. The Tenapanor group received the Tenapanor dose (0.03 mg/ml) for 21 consecutive days according to the instructions. The control and Tenapanor group received routine feeding, normal water, and diet. After successful modeling, the rats in the Smida group, JH, JM, and JL groups were given gavage for 7 consecutive days according to the above concentrations. The control group was gavaged with distilled water for 7 consecutive days.

2.5. Monitoring treatment effects of the general condition of rats

Throughout the process, general symptoms, weight, grasping force, food intake, and water intake were recorded daily, the method was as previously described [9].

2.6. Biochemical analysis

Serum from the corresponding treatment of rats was collected according to the instructions provided by the biochemical kit manufacturer, and its content was measured using amylase, D-xylose, and lactate kits as needed.

2.7. Analysis of serum and tissue cAMP and GLP-1 levels

cAMP and GLP-1 levels in serum, jejunum, and colon were analyzed using the rat cAMP and GLP-1 Enzyme-linked immunosorbent assay (ELISA) Kit. All procedures were performed according to the manufacturer's instructions.

2.8. Histopathologic examination

The jejunum and colon tissues of rats in each group were immediately fixed overnight with 4% paraformaldehyde and embedded with conventional paraffin wax. Intestinal specimens were evaluated and histopathological changes were photographed with a digital camera.

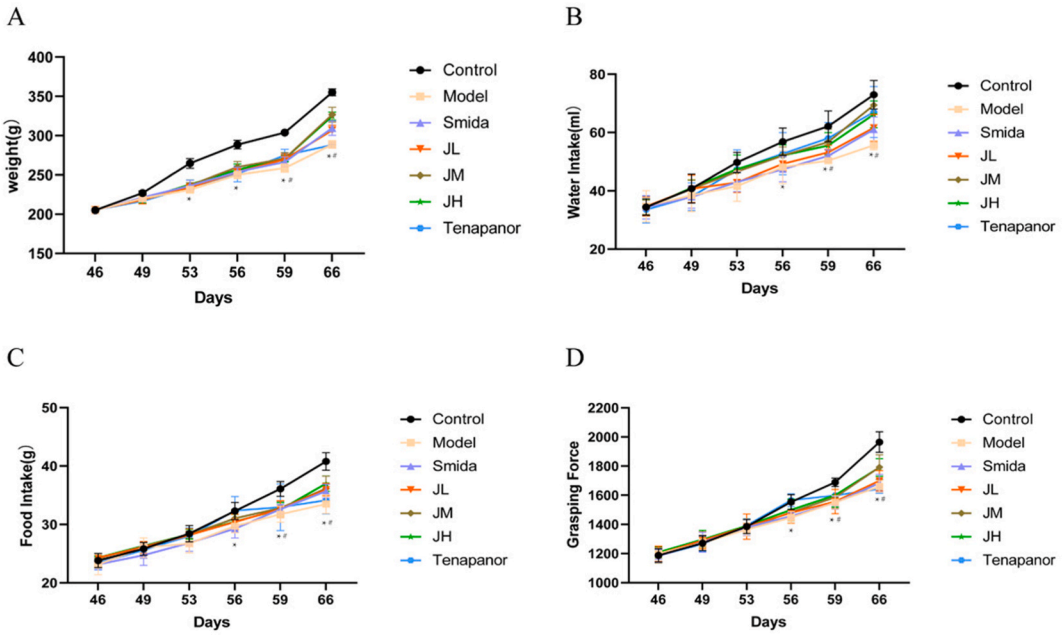

Fig. 1. Effects of JPY on body weight, food intake, water intake and grip strength of rats. (A) Effects of JPY on body weight. (B) Effects of JPY on food intake. (C) Effect of JPY on water intake. (D) Effect of JPY on grip strength. Data were presented as mean ± SEM (n = 6). $^{*}P < 0.05$ compared with the control group and $^{\#}P < 0.05$ compared with the model group.

2.9. Immunohistochemistry (IHC)

IHC was performed as previously described [9]. Antibodies mentioned above were used to detect protein expression in the jejunum and colon tissue. Finally, protein expression was quantified at 200× magnification using a computer-supported imaging system connected to an optical microscope (DM1000, Leica, Germany), and 3 fields of view of each sample were randomly selected to quantify the relative intensity of protein staining.

2.10. Real-time-polymerase chain reaction (RT-PCR) test

After treatment, jejunum and colon tissues were homogenized, and RNA extraction was performed using TIANGEN (Beijing, China) according to the instructions. We performed RT-PCR with reagents and protocols from SG Green qPCR mix kit (2×) (Sinogene Biotech Co., Ltd., China) and the BioRad CFX Connect Real-Time System (Bio-Rad, Hercules, CA). The murine primers used for RT-PCR were as follows:

GAPDH, 5′-TGACATCAAGAAGGTGGTGAAGC and 3’ -GGAAGAATGGGAGTTGCTGTTG; NHE3, 5′-CAAGGTCACCAGTATCGTCCC and 3′-GCATGAAGTATCCAGCATCCAAC; USP7, 5′-CCACCAAGAATTACTCAGAACCC and 3′-AAGGACCGACTCACTCAGTCT; NHE3 ubiquitination, 5′-GACATGGAGCATGGATGGGAA and 3′-GTTCGGCCTAAATTGTCCACT; PKA, 5′-GAGCAGGAGAGCGTGAAAGA and 3′-TCCTTGTGCTTCACGAGCAT; GLP-1R, 5′-ATAGCTGAGGAACTTGGGCG and 3′-TATTCGTCCGCATGCAAAGC.

2.11. Western blot (WB) analysis

We used WB analysis to detect the expression levels of NHE3, USP7, USP10, GLP-1R, phosphorylated NHE3, ubiquitinated NHE3, and PKA proteins in the jejunum and colon of rats. WB was performed as previously described [9]. Finally, analysis was performed using ImageJ software (National Institutes of Health).

2.12. Statistical analysis

SPSS 22.0 software was used for data analysis. All continuous data are expressed as mean ± standard deviation (SD). Before analysis, normality, and variance homogeneity tests were performed for each set of data to be compared. For data conforming to a normal distribution, one-way ANOVA or one-way repeated measure ANOVA was used for comparison between groups. The LSD model was used to compare paired tests between multiple groups. Data that does not conform to a normal distribution. A nonparametric test was performed by Kruskal-Wallis test, and intergroup trends were observed by the rank mean method. Bilateral 95% confidence interval, $P < 0.05$ indicated that the difference was statistically significant; $P > 0.05$ showed no statistical significance.

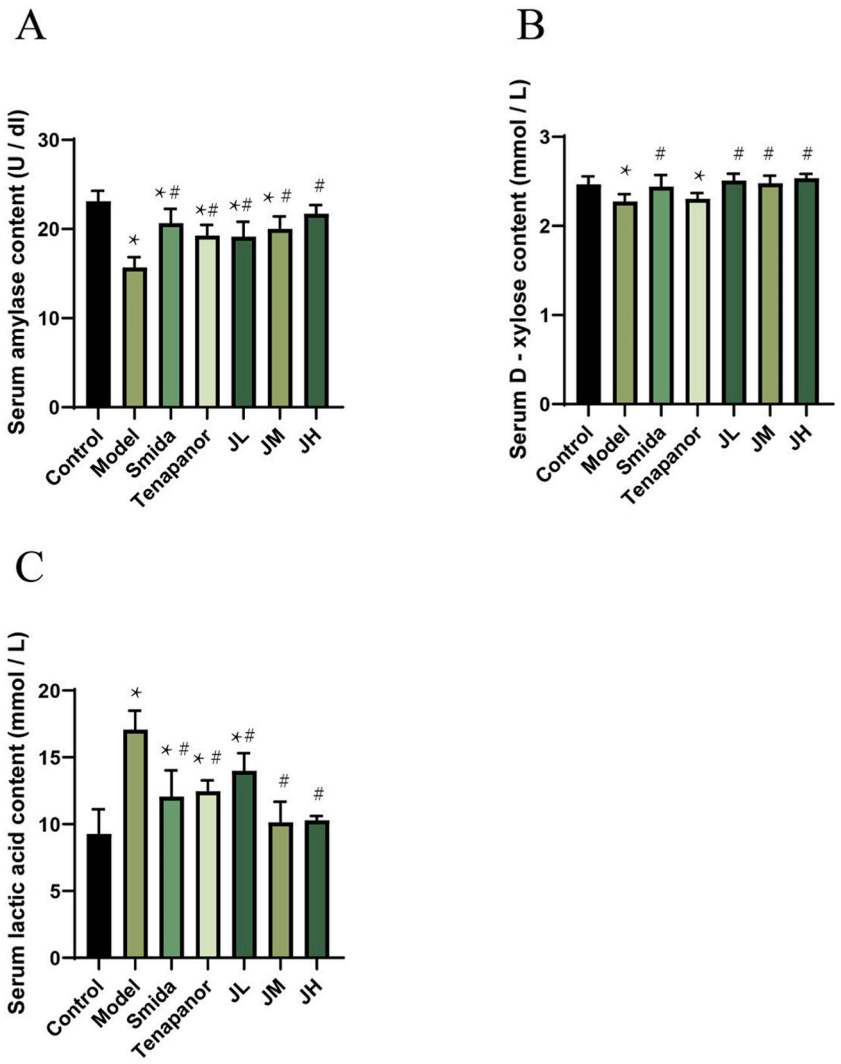

Fig. 2. Effect of JPY on amylase, D-xylose, and lactic acid in rats. (A) Effect of JPY on amylase. (B) Effect of JPY on D-xylose. (C) Effect of JPY on lactate. Data were presented as mean ± SEM (n = 6). $^{*}P < 0.05$ compared with the control group and $^{\#}P < 0.05$ compared with the model group.

3. Results

3.1. Effects of JPY on general symptoms, weight, food intake, water intake, and the grasping force of the lactose-treated rats

Before modeling, rats in each group were in normal mental state, agility, rapid reaction, bright hair, normal stool, and yellowish brown. They did not differ significantly in body weight (Fig. 1A), food intake (Fig. 1B), water intake (Fig. 1C), and grip strength (Fig. 1D). After modeling, the model rats were drowsy, unresponsive, yellow hair, loose stool, high water content, watery stool in severe cases, diarrhea rate was 100%, and all indicators were significantly lower than those of the control group ($P < 0.001$). After treatment, all indexes did not return to normal, but the mental state of rats in each group was improved, the reaction was gradually rapid, the hair gradually recovered, the fecal water content decreased, and the body weight, food intake, and grip strength were significantly increased compared with the model group ($P < 0.05$), and the diarrhea rate was reduced to 0%, indicating that the drug improved the symptoms of the model rats. The efficacy of the JM and JH groups was higher than that of the Smida and JL groups. There were no significant differences between the Tenapanor group and the model group, indicating that NHE3 plays an important role in diarrhea. Inhibition of NHE3 can lead to a series of symptoms, such as increased fecal water content, which is worthy of further exploration.

3.2. Effects of JPY on amylase, D-xylose, and lactic acid of the lactose-treated rats

The contents of serum amylase, D-xylose, and lactic acid are the commonly used indexes of spleen deficiency syndrome in TCM,

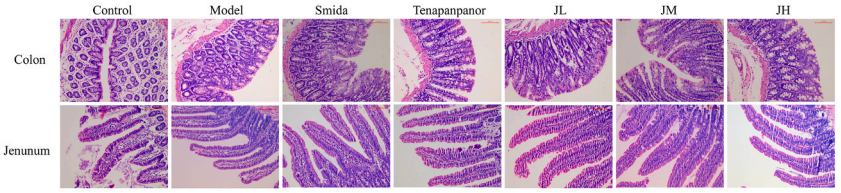

Fig. 3. Effect of JPY on histopathology of colon and jejunum in rats. Representative photos of the histopathological features of the colon and jejunum with H & E staining. Magnification at 200×.

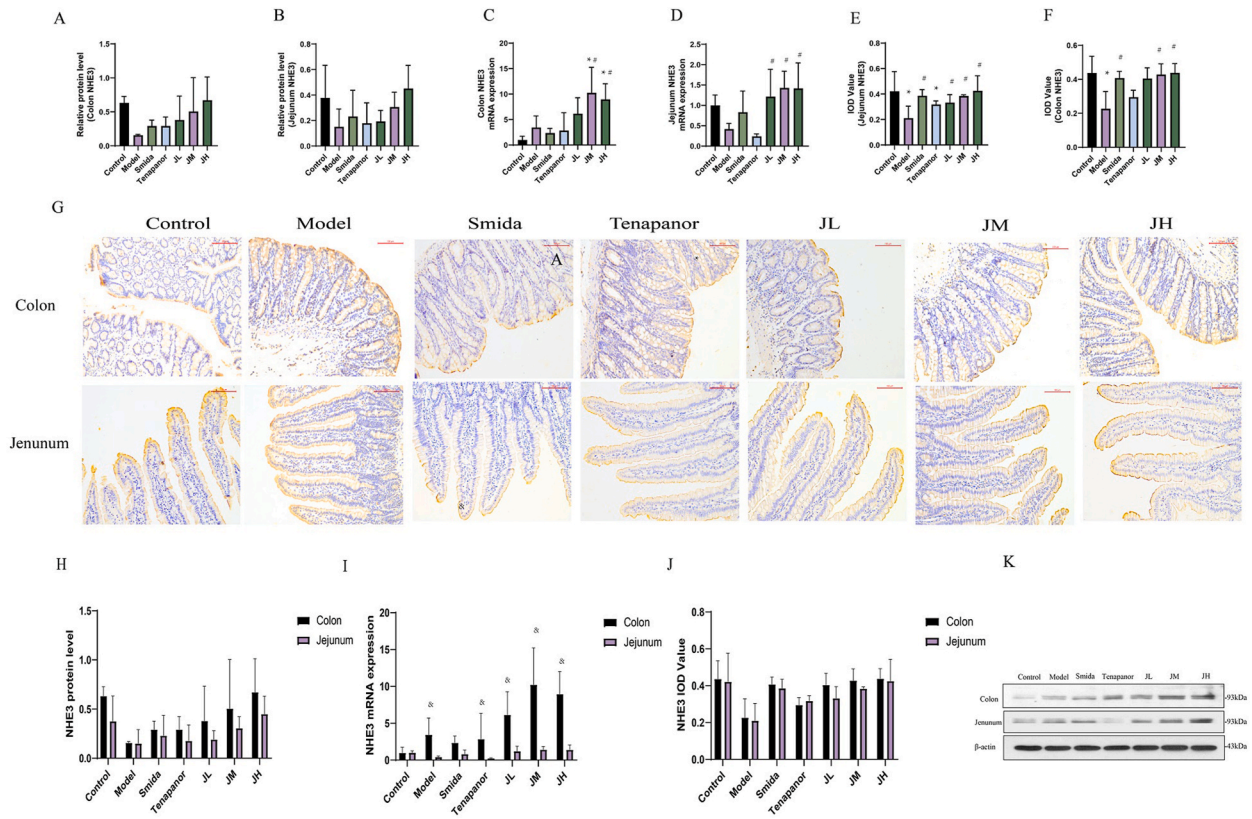

Fig. 4. Effect of JPY on the expression of NHE3 in the colon and jejunum of rats. (A-B) Analysis of the WB bands of NHE3 of colon and jejunum. (C-D) Analysis of mRNA expressions of NHE3 of colon and jejunum. (E-F) Analysis of IHC staining of NHE3 of colon and jejunum. (G) Representative photos of NHE3 of the colon and jejunum with IHC staining. (H-I) Comparison of the WB bands, mRNA expressions, and IHC staining of colon and jejunum NHE3. (K) Representative WB bands of colon and jejunum NHE3. (S4KC) Supplementary material of uncropped versions of colon NHE3. (S4KJ) Supplementary material of uncropped versions of jejunum NHE3. Data were presented as mean ± SEM (n = 3). $^{*}P < 0.05$ compared with the control group and $^{\#}P < 0.05$ compared with the model group. Magnification at 200×.

which can reflect spleen deficiency in rats to a certain extent. The serum amylase (Fig. 2A) and D-xylose (Fig. 2B) contents of the model group were significantly lower than those of the control group ($P < 0.001$), indicating that the digestion and absorption function of the model group was reduced. The lactate content (Fig. 2C) was significantly higher than that of the other groups ($P < 0.001$), which meant that the fatigue degree of the rats increased. After drug treatment, the serum levels of amylase and D-xylose in Smida, JL, JH, and JM groups increased to varying degrees, and the serum lactic acid content decreased, indicating that drug treatment could not only improve diarrhea symptoms in rats but also improve digestion and absorption. Among them, the treatment effect of JH could reach the same level as the control group. The contents of serum amylase and lactic acid in the Tenapanor group were higher than those in the model group, and the content of D-xylose in the Tenapanor group was not significantly different from that in the model group, but significantly different from that in the normal group ($P < 0.05$), indicating that NHE3 inhibitor inhibited the digestion and absorption function of rats.

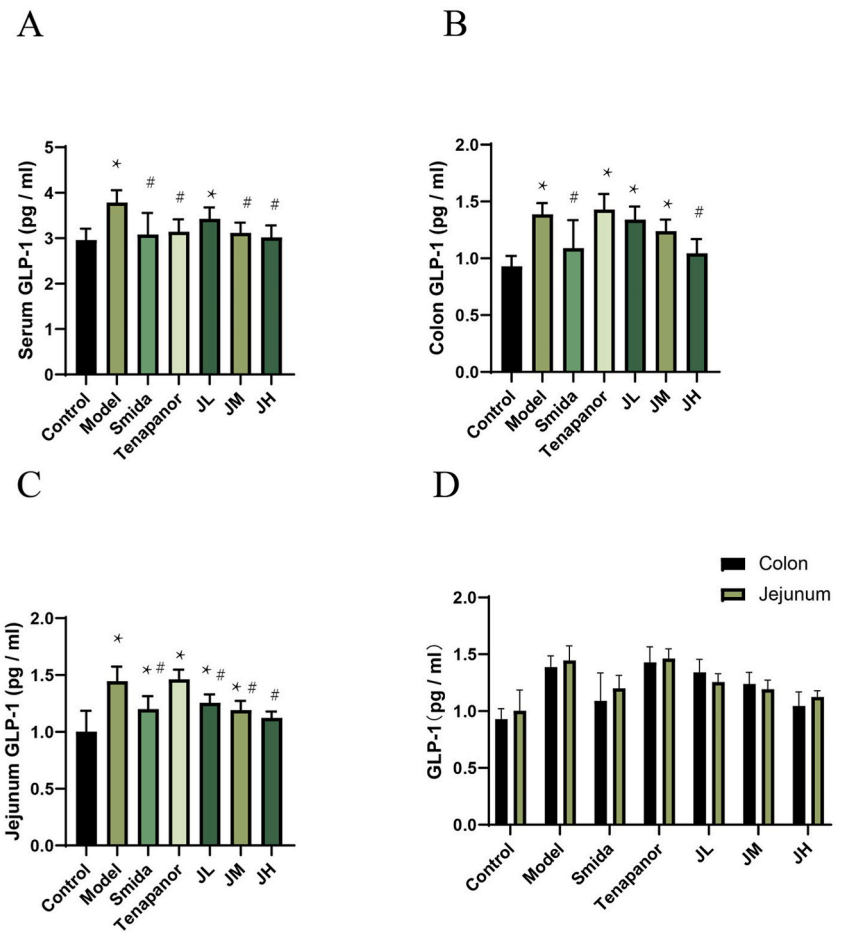

Fig. 5. Effect of JPY on GLP-1 level in rats. (A) Effect of JPY on GLP-1 of rat serum. (B) Effect of JPY on GLP-1 levels in rat colon. (C) Effect of JPY on GLP-1 level in rat jejunum. (D) Comparison of GLP-1 levels in rat colon and jejunum. Data were presented as mean ± SEM (n = 6). $^{*}P < 0.05$ compared with the control group and $^{\#}P < 0.05$ compared with the model group.

3.3. Effect of JPY on intestinal histopathological changes in lactose-treated rats

As shown in Fig. 3, the mucosal structure of the colon and jejunum of rats in each group was normal, and no histopathological changes were observed. Compared with the control group, there was no significant difference between the model group and the Tenapanor group, with obvious congestion and edema in the jejunum and colon, and no obvious neutrophil infiltration and interstitial edema. Mild hyperemia and edema were observed in the JL group, whereas no obvious abnormalities were observed in the colon and jejunum tissue of the Smida, JM, and JH groups. Compared with the model group, the congestive edema injury of rats was alleviated to varying degrees after drug treatment, especially in the JH group, which returned to normal levels.

3.4. Effect of JPY on intestinal NHE3 expression in lactose-treated rats

NHE3 is a transmembrane transporter of intestinal sodium ions, which can promote the absorption of intestinal sodium ions and form a concentration gradient. Decreased NHE3 expression can lead to an imbalance of intestinal ion transport, leading to diarrhea. The expression of NHE3 mRNA and protein in intestinal tissue showed the same trend. In the model group and the Tenapanor group, the expression of mRNA and protein of NHE3 in the colon (Fig. 4A, C, E, K, S4KC) and jejunum (Fig. 4B, D, F, K, S4KJ) were significantly decreased ($P < 0.05$) and were increased after drug treatment ($P < 0.05$). IHC images confirmed that intestinal NHE3 was mainly distributed on the membrane surface (Fig. 4G), which was consistent with previous studies. We analyzed the results and found that NHE3 mRNA expression was improved to a greater extent in the colon than in the jejunum (Fig. 4H, I, J). The therapeutic effect of the colon is better than that of the jejunum. Among them, the JH group had the best effect, which could restore the mRNA and protein expression of NHE3 to normal levels in rats with diarrhea.

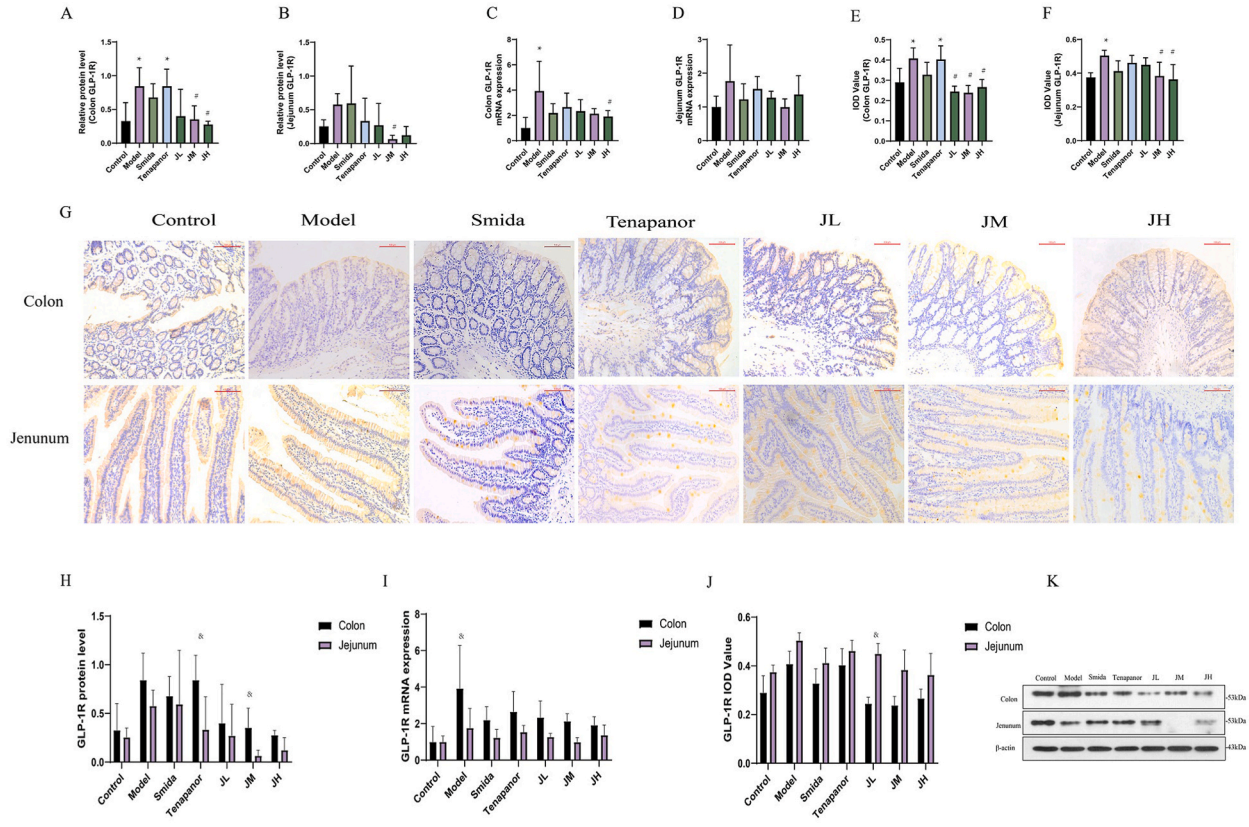

Fig. 6. Effect of JPY on the expression of GLP-1R in the colon and jejunum of rats. (A-B) Analysis of the WB bands of GLP-1R of colon and jejunum. (C-D) Analysis of mRNA expressions of GLP-1R of colon and jejunum. (E-F) Analysis of IHC staining of GLP-1R of colon and jejunum. (G) Representative photos of GLP-1R of the colon and jejunum with IHC staining. (H-I) Comparison of the WB bands, mRNA expressions and IHC staining of colon and jejunum GLP-1R. (K) Representative WB bands of colon and jejunum GLP-1R. (S6KC) Supplementary material of uncropped versions of colon GLP-1R. (S6KJ) Supplementary material of uncropped versions of jejunum GLP-1R. Data were presented as mean ± SEM (n = 3). $^{*}P < 0.05$ compared with the control group and $^{\#}P < 0.05$ compared with the model group. Magnification at 200×.

3.5. Effect of JPY on GLP-1 level and GLP-1R in the lactose-treated rats

GLP-1 and GLP-1R agonists are commonly used to treat type 2 diabetes. The main mechanism of action is to inhibit Na^{+} absorption by inhibiting NHE3 in renal tubules, a common side effect of which is diarrhea [10,11]. GLP–1. We hypothesized that it is also involved in the inhibition of intestinal NHE3 and therefore examined the content of GLP-1 in intestinal tissues and serum of different groups of rats. The content of GLP-1 in serum (Fig. 5A), colon (Fig. 5B), and jejunum (Fig. 5C) in the model group was significantly increased ($P < 0.05$). The tenapanor group did not change significantly indicating that the NHE3 inhibitor may not affect the metabolism of serum GLP-1. After drug treatment, GLP-1 levels in the Smida group, JM group, and JH group decreased significantly ($P < 0.05$). There was no significant difference in GLP-1 content in tissues of the Tenapanor group compared with the model group, suggesting that Tenapanor may inhibit intestinal GLP-1 metabolism. Next, we analyzed the GLP-1 content in the colon and jejunum, and the difference was not statistically significant (Fig. 5D). GLP-1 content in tissue and serum correlated with NHE3 expression. Therefore, we propose that GLP-1 can inhibit intestinal NHE3 expression, and JPY can play a role in regulating NHE3 expression by regulating GLP-1.

GLP-1 activates signal transduction primarily by binding GLP – 1 receptor (GLP -1R), so we examined GLP-1R mRNA and protein expression. The content of GLP-1R protein in the colon (Fig. 6A, E, K, S6KC) and the jejunum (Fig. 6B, F, K, S6KJ) of the model group and Tenapanor group was significantly increased ($P < 0.05$) and decreased to varying degrees after the drug treatment, among which the JM group and JH group were the most significant ($P < 0.05$). The mRNA expression of GLP-1R was significantly increased in the colon (Fig. 6C) and jejunum (Fig. 6D) compare with the model group ($P < 0.05$), and the decrease returned to normal levels after drug treatment. Using IHC (Fig. 6G), we found that GLP-1R was mainly distributed on the cell membrane, and there was a more pronounced change in protein expression level. Meanwhile, GLP-1R mRNA and protein expression was higher in the colon than in the jejunum (Fig. 6H, I, J). The results showed that inhibition of NHE3 function resulted in increased GLP-1R expression and that JPY may reduce signaling by reducing GLP-1R expression.

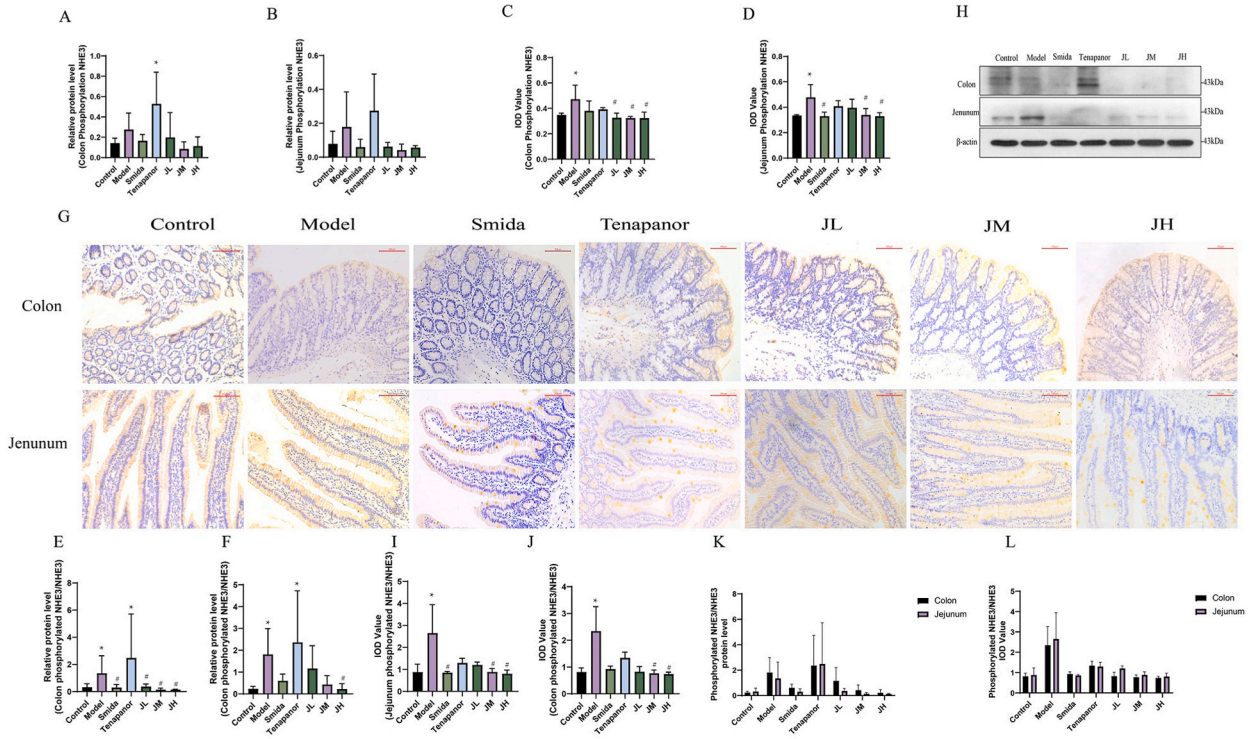

Fig. 7. Effect of JPY on the expression of phosphorylated NHE3 in the colon and jejunum of rats. (A-B) Analysis of the WB bands of phosphorylated NHE3 of colon and jejunum. (C-D) Analysis of IHC staining of phosphorylated NHE3 of colon and jejunum. (G) Representative photos of phosphorylated NHE3 of the colon and jejunum with IHC staining. (H) Representative WB bands of colon and jejunum phosphorylated NHE3. (E-F) Analysis of the WB bands of phosphorylated NHE3/NHE3 of the colon and jejunum. (I-J) Analysis of the IHC staining of phosphorylated NHE3/NHE3 of the colon and jejunum. (K, L) Comparison of the WB bands and IHC staining of colon and jejunum phosphorylated NHE3/NHE3. (S7HC) Supplementary material of uncropped versions of colon phosphorylated NHE3/NHE3. (S7HJ) Supplementary material of uncropped versions of jejunum phosphorylated NHE3/NHE3. Data were presented as mean ± SEM (n = 3). $^{*}P < 0.05$ compared with the control group and $^{\#}P < 0.05$ compared with the model group. Magnification at 200×.

3.6. *Effect of JPY on ubiquitination and phosphorylation expression of intestinal NHE3 in lactose-treated rats*

The activity of NHE3 in the apical membrane of epithelial cells is regulated by several mechanisms, including transcriptional regulation, protein phosphorylation, ubiquitination, protein-protein interactions, and transport. Studies have shown that phosphorylation and ubiquitination of NHE3 are important factors regulating the active expression of NHE3 [12]. Phosphorylated and ubiquitinated NHE3 was increased in the colon (Fig. 7A, C, E, I, H, S7HC, Fig. 8A, E, L, N, K, S8KC) and jejunum (Fig. 7B, D, F, J, H, S7HJ, Fig. 8B, F, M, O, K, S8KJ) of the model rats compared with the normal group and decreased to varying degrees after drug treatment. IHC study showed that phosphorylated and ubiquitinated NHE3 was distributed in the cytoplasm and cell membrane (Figs. 7G and 8G). The mRNA and protein expression levels of ubiquitinated NHE3 in the colon (Fig. 8C) and jejunum (Fig. 8D) of rats in each group showed the same trend. It was increased in the model group and decreased after drug treatment, but there was no significant difference. This indicates that drug treatment may only alter protein expression and have little effect on its mRNA expression. There were no significant differences in NHE3 phosphorylation and ubiquitination levels in the jejunum and colon (Figs. 7K, L, 8H, I, J). The levels of phosphorylated and ubiquitinated NHE3 in the jejunum and colon of the Tenapanor group were not significantly different from those of the model group, showing that inhibition of NHE3 function leads to phosphorylated and ubiquitinated NHE3 increase. The experimental results show that the JPY drug treatment can reduce NHE3 phosphorylation and ubiquitination level.

3.7. *Effect of JPY on intestinal USP7 and USP10 expression in lactose-treated rats*

Ubiquitin-specific protease 7 (USP7) and USP10 have been identified as two DUBs that interact and regulate NHE3 ubiquitination. The results showed that USP7 and USP10 were mainly distributed in the nucleus and cytoplasm (Figs. 9G and 10G). Compared with the control group, the expression of USP7 and USP10 in the colon (Fig. 9A, E, K, S9KC, Fig. 10A, E, K, S10KC) and jejunum (Fig. 9B and F, S9KJ, Fig. 10B and F, S10KJ) of rats in the model and Tenapanor groups was significantly decreased ($P < 0.05$). After drug treatment, the Smida and JH groups increased significantly ($P < 0.05$) and returned to the same level as the control group. The changing trend of USP7 and USP10 mRNA in the colon (Figs. 9C and 10C) and jejunum (Figs. 9D and 10D) of rats in each group was the same as the protein expression level, but there was no significant difference. Inhibition of NHE3 function by Tenapanor reduces the expression of

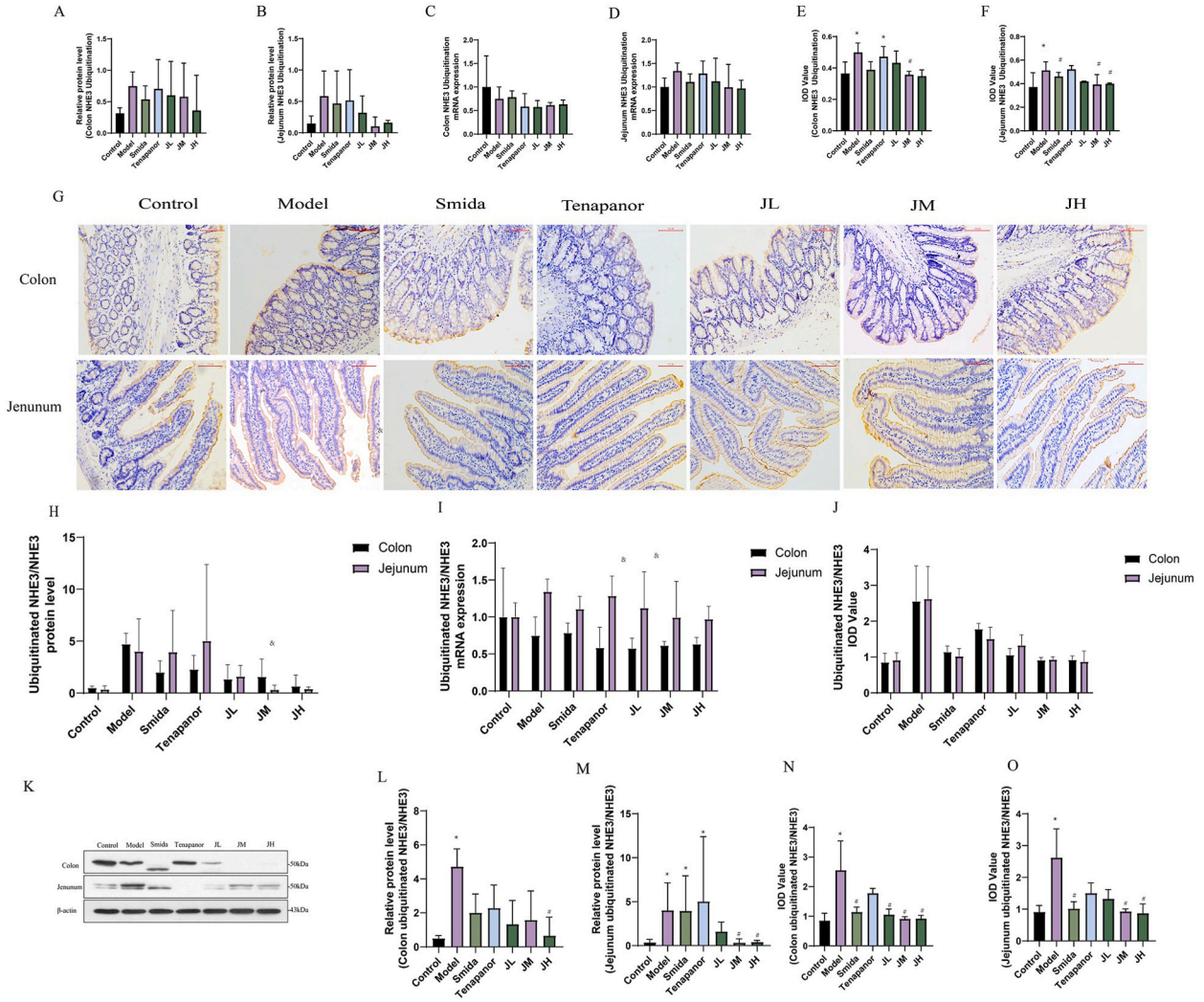

Fig. 8. Effect of JPY on the expression of ubiquitinated NHE3 in the colon and jejunum of rats. (A-B) Analysis of the WB bands of ubiquitinated NHE3 of colon and jejunum. (C-D) Analysis of mRNA expressions of ubiquitinated NHE3 of colon and jejunum. (E-F) Analysis of IHC staining of ubiquitinated NHE3 of colon and jejunum. (G) Representative photos of ubiquitinated NHE3 of the colon and jejunum with IHC staining. (H-I) Comparison of the WB bands, mRNA expressions and IHC staining of colon and jejunum ubiquitinated NHE3/NHE3. (K) Representative WB bands of colon and jejunum ubiquitinated NHE3. (L-M) Analysis of the WB bands of ubiquitinated NHE3/NHE3 of the colon and jejunum. (N-O) Analysis of the IHC staining of ubiquitinated NHE3/NHE3 of the colon and jejunum. (S8KC) Supplementary material of uncropped versions of colon ubiquitinated NHE3/NHE3. (S8KJ) Supplementary material of uncropped versions of jejunum ubiquitinated NHE3/NHE3. Data were presented as mean ± SEM (n = 3). $^{*}P < 0.05$ compared with the control group and $^{\#}P < 0.05$ compared with the model group. Magnification at 200×.

USP7 and USP10 in intestinal tissues. JPY treatment can improve the protein expression of USP7 and USP10, but their mRNA expression is less regulated.

3.8. *Effect of JPY on cAMP/PKA signaling pathway in lactose-treated rats*

cAMP is the second messenger produced by GLP-1 binding to GLP-1R, and cAMP regulates NHE3 phosphorylation and ubiquitination by activating cAMP-dependent protein kinase (PKA) [13]. Therefore, we first examined cAMP changes in serum and intestinal tissues. Compared with the normal group, the serum cAMP (Fig. 11A) level in the model group increased significantly and decreased significantly after drug treatment. There was no significant difference in serum cAMP content between the Tenapanor and normal groups. The content of cAMP in the colon (Fig. 11B) and jejunum (Fig. 11C) of the model group was significantly higher than that of the normal group, but there was no significant difference from that of the Tenapanor group. After drug treatment, the levels were significantly decreased in all groups and returned to normal in the JH group. There was no difference in cAMP content between the colon and jejunum (Fig. 11D). The above results indicate that inhibition of NHE3 increases cAMP content in intestinal tissues, but may have no effect on serum cAMP metabolism. Treatment with JPY could restore cAMP level to normal level, and the JH group had the

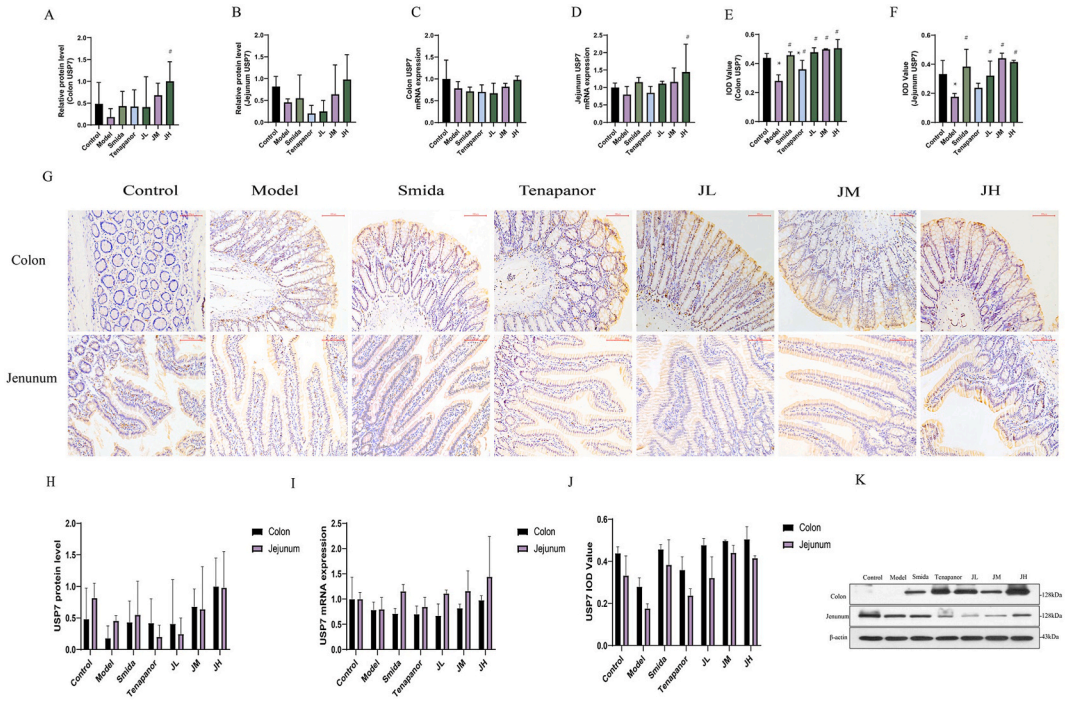

Fig. 9. Effect of JPY on the expression of USP7 in the colon and jejunum of rats. (A-B) Analysis of the WB bands of USP7 of colon and jejunum. (C-D) Analysis of mRNA expressions of USP7 of colon and jejunum. (E-F) Analysis of IHC staining of USP7 of colon and jejunum. (G) Representative photos of USP7 of the colon and jejunum with IHC staining. (H-I) Comparison of the WB bands, mRNA expressions and IHC staining of colon and jejunum USP7. (K) Representative WB bands of colon and jejunum USP7. (S9KC) Supplementary material of uncropped versions of USP7. (S9KJ) Supplementary material of uncropped versions of jejunum USP7. Data were presented as mean ± SEM (n = 3). $^{*}P < 0.05$ compared with the control group and $^{\#}P < 0.05$ compared with the model group. Magnification at 200×.

most significant effect.

PKA is mainly expressed in the cytoplasm and cell membrane (Fig. 12G). The protein expression of PKA in the colon (Fig. 12A, E, K, S12KC) and jejunum (Fig. 12B, F, K, S12KJ) of the model and Tenapanor groups increased compared with the control group ($P < 0.05$) and decreased after drug treatment ($P < 0.05$). PCR results showed that there was no significant difference in the expression level of PKA mRNA in the colon (Fig. 12C) and jejunum (Fig. 12D) of rats in each group. There was also no significant difference in PKA expression between the colon and jejunum of rats in the same group (Fig. 12H, I, J). In conclusion, inhibition of NHE3 by Tenapanor and diarrhea can lead to elevated intestinal PKA levels. JPY treatment can regulate the expression of NHE3 by reducing the expression of PKA protein, and the effect of JH is obvious.

4. Discussion

TCM has always been considered as a common method for the treatment of diarrhea, and its safety and effectiveness in relieving diarrhea have been documented in the literature. UPLC analysis showed that JPY was rich in Paeoniflorin and Glycyrrhizic_acid. It has been confirmed that chronic treatment with Paeoniflorin suppressed the activations of JNK and p38 MAPK, but enhanced ERK activation [14] and Glycyrrhizic_acid can inhibit activation of PERK-eIF2α-CHOP and MAPK pathway induced by UV-B [15]. The above findings provide sufficient evidence for us to study the rationality and effectiveness of JPY. On the basis of the anti-diarrhea effect of JPY, the possible molecular mechanism of JPY was explored through a rat diarrhea model induced by high lactose.

High lactose-induced diarrhea occurs when the intake of lactose exceeds the capacity of the small intestine to absorb lactose, and the metabolites and unhydrolyzed lactose lead to increased osmotic pressure in the intestinal cavity, which in turn leads to an imbalance in the intestinal absorption and secretion of water and electrolytes, resulting in diarrhea [16]. At the same time, traditional Chinese medicine believes that the occurrence of diarrhea is closely related to spleen deficiency. Symptoms of spleen deficiency include fatigue, decreased levels of digestion and absorption. As a result, we combine a small platform standing in the diarrhea model caused by high lactose. On the one hand, rats were surrounded by a humid environment, moisture in the body, trapped spleen; On the other hand, to avoid falling into the water, the rats were subjected to great stress both mentally and physically, mimicking the increasing mental stress and physical consumption of modern humans, leading to spleen deficiency. After modeling, the rats in each group showed different degrees of diarrhea, yellow hair, poor physical strength, and poor mental state, which were consistent with the symptoms of lactose-induced diarrhea patients. The contents of serum amylase, d-xylose, and lactic acid are the commonly used indexes of spleen deficiency syndrome in TCM, which can reflect spleen deficiency in rats to a certain extent. Amylase in serum breaks

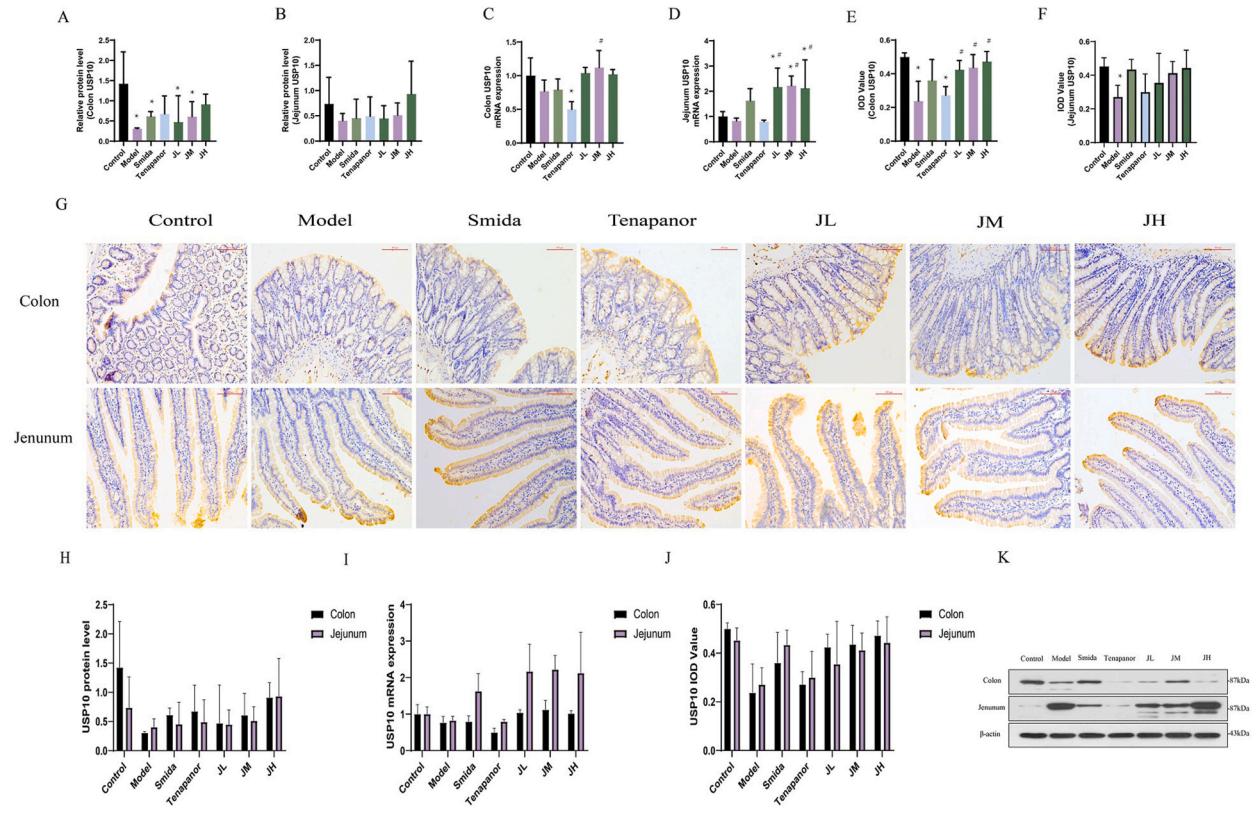

Fig. 10. Effect of JPY on the expression of USP10 in the colon and jejunum of rats. (A-B) Analysis of the WB bands of USP10 of colon and jejunum. (C-D) Analysis of mRNA expressions of USP10 of colon and jejunum. (E-F) Analysis of IHC staining of USP10 of colon and jejunum. (G) Representative photos of USP10 of the colon and jejunum with IHC staining. (H-I) Comparison of the WB bands, mRNA expressions and IHC staining of colon and jejunum USP10. (K) Representative WB bands of colon and jejunum USP10. (S10KC) Supplementary material of uncropped versions of USP10. (S10KJ) Supplementary material of uncropped versions of jejunum USP10. Data were presented as mean ± SEM (n = 3). $^{*}P < 0.05$ compared with the control group and $^{\#}P < 0.05$ compared with the model group. Magnification at 200×.

down polysaccharides and shows the body's digestive function [17]. d-xylose is a small molecule of pentose that does not exist in the blood of normal people or rats [18]. After being taken orally, it is absorbed into the bloodstream of the small intestine. The digestive and absorption function of functional diarrhea rats with spleen deficiency was impaired, so the serum amylase content should be reduced. Serum lactic acid is mainly produced after exercise and can indirectly reflect the fatigue state of rats [19]. Smida is a commonly used drug for the treatment of chronic diarrhea [20]. It contains dioctahedral montmorillonite powder, which has a layered structure and plasticity, and uneven charge distribution. As a result, Smida can reduce the interference and damage of intestinal cells and maintain the normal secretion and absorption function of intestinal cells. Therefore, in this experiment, we chose Smida and JPY for comparison. Tenapanor is an inhibitor of NHE3, and excessive ingestion of tenapanor selectively inhibits the intestinal absorption of sodium and reduces the intestinal absorption of water and electrolytes, causing diarrhea [21]. The mechanism of action is the same as that of diarrhea caused by high lactose. It can be used specifically to observe the improved effect of JPY on NHE3 inhibition. After drug treatment, diarrhea in model rats was significantly relieved, serum amylase and D-xylose contents increased, and lactic acid content decreased, indicating that JPY drug treatment could not only improve diarrhea symptoms, but also improve mental, dietary physical strength and relieve fatigue. Diarrhea caused by lactose intolerance is not associated with organic changes. The mucosal structure of the jejunum and colon was basically normal, with only congestive edema. After drug treatment, the congestive edema injury of rats was alleviated to varying degrees, and the JH group had the best efficacy.

Intestinal water and electrolyte absorption and secretion are mainly related to ion channels and transporters on intestinal epithelial cells, in which NHE3 plays a key role [22]. NHE3 is highly expressed in the apical membrane of the proximal tubules of the small intestine and colon, colon, and kidney, and is mainly responsible for neutral electrical uptake of Na^{+}. NHE3 usually interacts with Cl^{-}/HCO^{3-}, NaCl exchanger downregulated in adenomas (Dra; SLC26A3) [23] or possibly anion transporter 1 (PAT1; SLC26A6) [24] were conjugated to function. Previous studies have shown that $NHE3^{-/-}$ mice have defects in intestinal and renal ion absorption, resulting in acid-base imbalance and deranged sodium levels [25]. In the present study, NHE3 expression was found to be significantly reduced in the jejunum and colon of rats with hyperlactose-induced diarrhea, confirming that NHE3 is closely associated with diarrhea. Meanwhile, rats gavaged with the Tenapanor showed the same symptoms as the model rats, which further verified the correlation. JPY treatment significantly increased NHE3 mRNA and protein expression, indicating that JPY restores intestinal absorption of

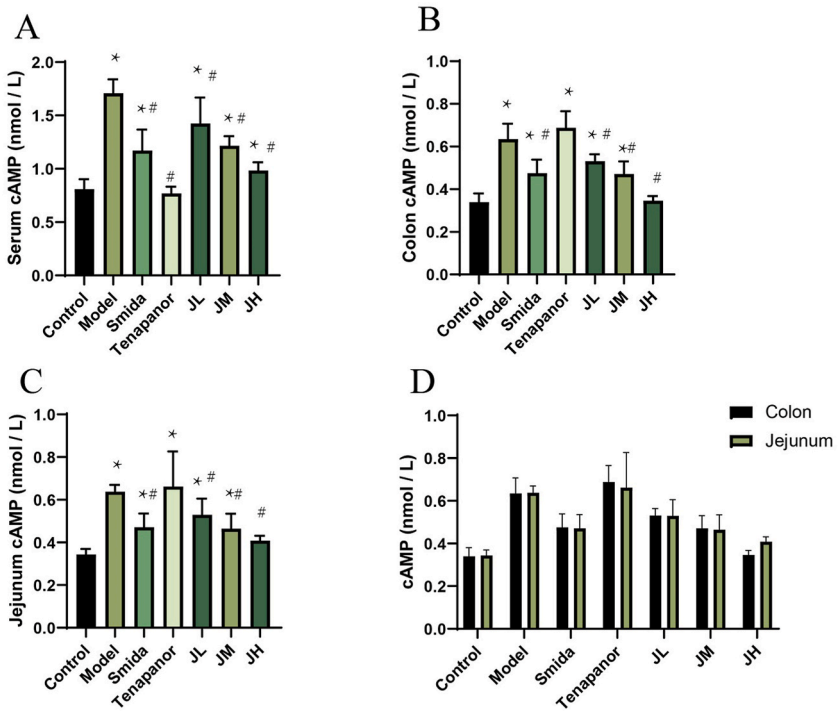

Fig. 11. Effect of JPY on cAMP level in rats. (A) Effect of JPY on cAMP of rat serum. (B) Effect of JPY on cAMP of rat colon. (C) Effect of JPY on cAMP of rat jejunum. (D) Comparison of cAMP levels in rat colon and jejunum. Data were presented as mean ± SEM (n = 6). $^{*}P < 0.05$ compared with the control group and $^{\#}P < 0.05$ compared with the model group.

electrolytes and relieves diarrhea by increasing NHE3 expression.

GLP-1 is an insulin hormone, and there is increasing evidence that it has a wide range of physiological effects on our health. GLP-1 was found to inhibit the function of NHE3 in the renal proximal tubule, while diarrhea is a common side effect of GLP-1R agonist drugs. However, there are few studies on the relationship between GLP-1 and intestinal NHE3 in diarrheal rats [26]. In the present study, we confirmed an increase in GLP-1 in serum and intestinal tissues of rats with diarrhea, together with an increase in GLP-1 in tissues of rats in the NHE3 inhibitor group, suggesting that increased GLP-1 content is associated with functional inhibition of intestinal NHE3. Previous investigators have found that increased GLP-1 content is associated with multiple factors and that increased intracellular $Ca^{2+}$and cAMP can mediate GLP-1 secretion by enteroendocrine L cells through various receptors and second messenger pathways [27]. Our study also confirmed an increased serum cAMP level in rats with diarrhea, which may be related to the increase in GLP-1. However, the specific mechanism still needs further investigation. Meanwhile, GLP-1 can bind to its selective G protein-coupled receptor GLP-1R and form cAMP through Gs signaling to regulate downstream protein function, indicating a synergistic interaction between cAMP and GLP-1. We also found that the expression of GLP-1R was also significantly increased in the intestinal tissue of rats in the model group, indicating that all three are associated with NHE3 functional inhibition. After drug treatment, the expression of GLP-1, cAMP, and GLP-1R decreased, and JPY may reduce its inhibitory effect on downstream proteins by reducing the expression of these three proteins.

Apical membrane NHE3 activity in epithelial cells is regulated by a variety of mechanisms, and acute regulation focuses on NHE3 cycling between the plasma membrane and intracellular compartments, which refers to the reciprocal flow of NHE3 from the surface of the epithelial cell membrane and intracellular vesicles [28]. The phosphorylation and ubiquitination of NHE3 play an important role in the complex regulatory mechanism of endocytosis and are important factors regulating the active expression of NHE3 [29]. Ubiquitination of NHE3 refers to the interaction of NHE3 with the E3 ubiquitin (Ub) ligase NEDD4-2 on the surface membrane, and Ub binding can be reversed by deubiquitinases (DUBS), of which USP7 and USP10 are two DUBS that interact and regulate NHE3 ubiquitination [30]. GLP-1 binds to GLP-1R to generate cAMP, which activates PKA by binding to the PKA regulatory subunit, RIS or RIS. On the one hand, the catalytic subunit (C) of PKA is released from the PKA tetramer and phosphorylates NHE3 at Serine 552, reducing plasma membrane NHE3 expression [31]. On the other hand, it can reduce the expression of USP7 or USP10, increase the ubiquitination level of NHE3, enhance the endocytic transport of NHE3, and reduce the surface membrane abundance of NHE3, both of which can lead to a significant reduction in the total amount of NHE3 protein [32]. In our study, we found that ubiquitination and phosphorylation of NHE3 were significantly increased in model rats, while USP7 and USP10 expressions were decreased. Therefore, JPY can treat diarrhea caused by high lactose by reducing the phosphorylation and ubiquitination of NHE3.

Above we mentioned the cAMP/PKA signaling pathway, whose activation begins with ligand-dependent activation of G protein-coupled receptors (GPCR), followed by Gs activation, adenylate cyclase (AC) activation, and cAMP production [33]. cAMP then

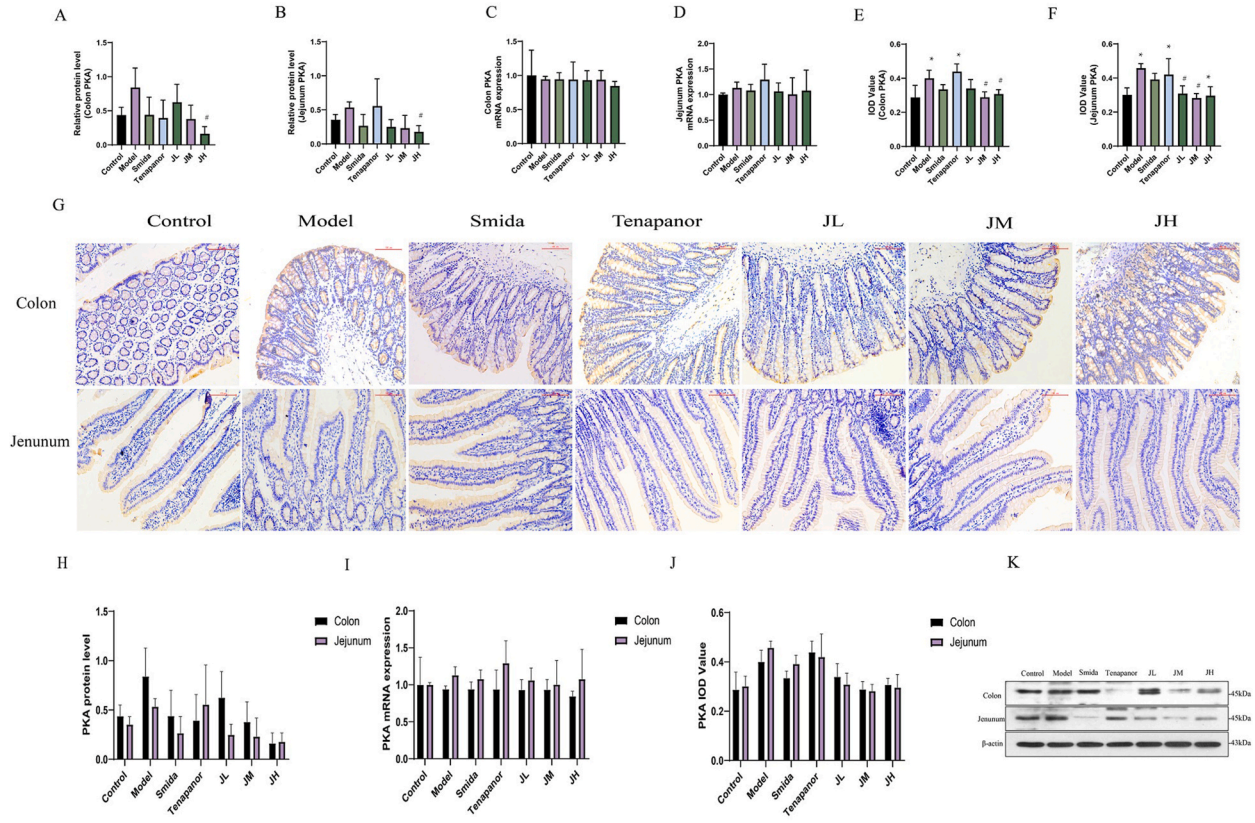

Fig. 12. Effect of JPY on the expression of PKA in the colon and jejunum of rats. (A-B) Analysis of the WB bands of PKA of colon and jejunum. (C-D) Analysis of mRNA expressions of PKA of colon and jejunum. (E-F) Analysis of IHC staining of PKA of colon and jejunum. (G) Representative photos of PKA of the colon and jejunum with IHC staining. (H-I) Comparison of the WB bands, mRNA expressions and IHC staining of colon and jejunum PKA. (K) Representative WB bands of colon and jejunum PKA. (S12KC) Supplementary material of uncropped versions of PKA. (S12KJ) Supplementary material of uncropped versions of jejunum PKA. Data were presented as mean ± SEM (n = 3). $^{*}P < 0.05$ compared with the control group and $^{\#}P < 0.05$ compared with the model group. Magnification at 200×.

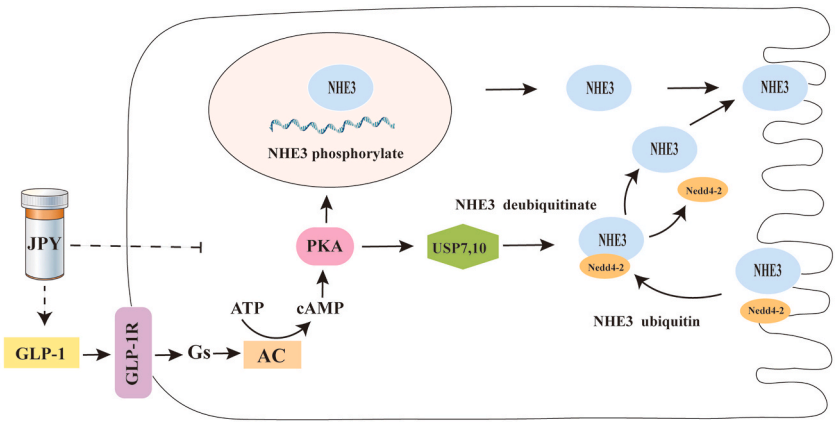

Fig. 13. This schematic summarizes the molecular mechanism of JPY against diarrhea in a rat model of hyper lactose-induced diarrhea. Its mechanism of action includes reducing the expression of GLP-1 and its related receptors, inhibiting the activation of the cAMP/PKA pathway, reducing the ubiquitination and phosphorylation of NHE3, and increasing NHE3 expression to restore intestinal absorption function.

activates PKA by binding to the PKA regulatory subunit. Therefore, it is worth investigating whether the increase in cAMP content is accompanied by an increase in PKA expression. This experiment found increased expression of PKA in the model group, confirming that increased GLP-1 activates cAMP/PKA signaling. Meanwhile, previous researchers have found that both the small intestine and

colon can absorb water and electrolytes [34]. To explore the differences between them, jejunum and distal colon tissues were taken to detect associated proteins and mRNAs. The results showed that the contents of each protein in the two parts were slightly different, but the difference was not significant. The improvement effect of the drug on the jejunum and colon was the same.

Our study also has certain limitations. Firstly, the mechanism by which cAMP decreases GLP-1 expression remains unclear and warrants further investigation. Second, whether GLP-1 activates other pathways after acting on GLP-1R needs to be analyzed based on the changes in downstream proteins following inhibition of the cAMP/PKA pathway.

5. Conclusions

In conclusion, JPY ameliorated hyperlactose-induced diarrhea in rats and restored the balance between intestinal water and electrolyte absorption and secretion. The anti-diarrheal effect was achieved, at least in part, by reducing GLP-1 content, inhibiting cAMP/PKA pathway activation, reducing ubiquitination and phosphorylation of NHE3, and restoring NHE3 function in intestinal epithelial cells (Fig. 13).

Author contribution statement

JinXin Ma: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ting Chen; Hong Xue; MIN ZHANG; Zhongyu Li; Xuan Li; Yitian Wang: Performed the experiments; Analyzed and interpreted the data.

Nan Kang: Analyzed and interpreted the data.

Fengyun Wang; Xudong Tang: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e17444.

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