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Zengye decoction regulated the expression of aquaporin in colon tissue of rats with constipation through miR-10a-5p targeting Drd2/AC/cAMP axis

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Summary. Slow transit constipation (STC) is a colonic motor disorder characterized by a marked delay in the movement of substances through the colon. Traditional Chinese medicine (TCM) is a treasure trove of natural compounds, which is effective in treating constipation with relatively minor side effects. Zengye decoction (ZYD), a classic herbal formula in TCM, is used for moistening the intestines and relieving constipation. However, the molecular mechanism of ZYD in the treatment of constipation remains unclear. The present study aimed to investigate the effect and molecular mechanism of ZYD on STC. A loperamide-induced rat model and IEC-18 cells were used *in vitro* and *in vivo*. We found that ZYD increased the intestinal transit rate and fecal water content of STC rats in a dose-dependent manner. Furthermore, ZYD dose-dependently decreased the expression of Aquaporin (AQP)3 and increased AQP9 expression in the colon tissue of STC rats. Mechanistically, ZYD reduced the level of miR-10a-5p, increased the expression of dopamine receptor D2 (Drd2), and inhibited the activity of adenylate cyclase (AC)/cyclic adenosine monophosphate (cAMP) signaling in the colon tissue of STC rats. However, miR-10a-5p overexpression reversed the improvement effect of ZYD on constipation. Moreover, miR-10a-5p targeted Drd2 and enhanced AC/cAMP signaling activation in IEC-18 cells. Also, miR-10a-5p regulated AQP3 and AQP9 expression in IEC-18 cells. Thus, ZYD alleviated constipation and aquaporin expression in STC rats, and its mechanism may be mediated by downregulating miR-10a-5p levels and inhibiting the Drd2/AC/cAMP axis. ZYD may be an efficient therapeutic strategy for constipation.

Key words: Slow transit constipation, Zengye decoction, Aquaporins, miR-10a-5p, Drd2/AC/cAMP axis

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Introduction

Chronic constipation is a common gastrointestinal complaint characterized by decreased bowel movements or defecation straining. The incidence of chronic constipation in the world is about 14% (Camilleri et al., 2017). Chronic constipation is considered to impair the quality of life. Slow transit constipation (STC) is defined as slow-moving intestinal contents due to reduced colon transmission due to various reasons (Bharucha and Phillips, 2021). According to statistics, STC accounts for about 15-30% of constipation (Knowles and Martin, 2000). The symptoms of STC are stubborn and common to senior citizens and women of childbearing age (Black and Ford, 2018). At present, mental status, dietary factors, abnormal gastrointestinal dynamics, and intestinal infections are thought to be some of the causes of constipation (Vilanova-Sanchez et al., 2018). However, the pathology of STC is still not clear to (Vilanova-Sanchez et al., 2018). Laxatives are often used in the management of constipation. However, long-term laxative treatment frequently causes adverse effects, such as melanosis coli (Nusko et al., 2000; Cao et al., 2018), and various kinds of colectomy can cause not only a large wound but it would also be difficult to avoid postoperative complications (Błachut et al., 2004). Thus, a search for alternative treatment is required.

Traditional Chinese medicine (TCM) has a long

history and rich experience in the treatment of constipation and has certain advantages (Rao et al., 2021; Wang et al., 2022). According to TCM theory, constipation is located in the large intestine and is related to the spleen, stomach, liver, lungs, kidneys, and other internal organs (Lin et al., 2009). TCM believes that improper diet, emotional changes, and stagnation of Qi movement are the main causes of constipation (Xu et al., 2022). The mechanism of constipation includes Qi-Blood-Yin-Yang deficiency, Qi stagnation, hot accumulation, and cold coagulation (Xu et al., 2022).

Zengye decoction (ZYD) is composed of Xuan Shen (*Scrophulariae Radix*), Di Huang (*Radix Rehmannia*), and Mai Dong (*Ophiopogonis Radix*). As a traditional ancient prescription, ZYD was reported to effectively alleviate acute kidney injury through modulation of the gut microbiome and serum amino acid metabolism (Dai et al., 2022). Furthermore, ZYD reduced blood glucose levels in diabetic rats by promoting glucose uptake, and the mechanism may be related to the regulation of Akt signaling pathway activity (Wang et al., 2020). It is noteworthy that ZYD is widely used in the treatment of constipation and other chronic diseases in China. In aged constipated rats, ZYT altered metabolically active gut microbiota (Liu et al., 2019). However, the molecular mechanism of ZYD in treating constipation is not clear. Thus, this study explored the molecular mechanism by which ZYD improved STC.

MicroRNAs (miRNAs) are a class of highly conserved non-coding single-stranded RNA molecules, with a length of about 18-25 nucleotides, with regulatory functions in eukaryotes; they can regulate the expression of target gene mRNAs by interacting with them (Mohr and Mott, 2015; Lu and Rothenberg, 2018). There is increasing evidence that miRNAs are closely associated with the occurrence and development of constipation (Singh et al., 2021; Yao et al., 2022). Our results suggested that ZYD could improve the symptoms of STC in rats by regulating the expression of aquaporins (AQPs). ZYD inhibited the expression of miRNA-10a-5p, and miRNA-10a-5p overexpression reversed the improvement effect of ZYD on constipation by targeting the dopamine receptor D2 (Drd2)/adenylate cyclase (AC)/cyclic adenosine monophosphate (cAMP) signaling pathway.

Materials and methods

Preparation of ZYD

The formula of ZYD is Xuan Shen (*Scrophulariae Radix*) 30 g, Di Huang (*Radix Rehmannia*) 24 g, and Mai Dong 24 g (*Ophiopogonis Radix*), which were provided by the School of Integrated Traditional Chinese and Western Medicine Clinical Medicine, North Sichuan Medical College (Nanchong, China). The formula was placed in a casserole and mixed with 2000 mL of distilled water. It is then covered and soaked for 1h, followed by decoction in a heating furnace for 1h. After the initial filtration, the solution undergoes another round of boiling with six times the amount of water for 1h. Subsequently, a second filtration is performed to combine the filtrates from both rounds and concentrate them to achieve a concentration of 0.51 g of raw drug per 1 mL of solution.

High-performance liquid chromatography (HPLC)

The chemical composition of ZYD was analyzed using HPLC. The analysis was conducted with a UHPLC Agilent 1290II system, employing a BEH C18 (2.1×100 mm) 1.7 μm, and a column temperature of 35°C. The mobile phase consisted of 0.02% formic acid (A) and acetonitrile (B), with a flow rate of 0.2 mL/min under gradient elution conditions as follows: 0.00 min, 2% B; 2.00 min, 2% B; 4.00 min, 7% B; 9.00 min, 16% B; 12.00 min, 22% B; 17.00 min, 34% B; 23.00 min, 80% B; and 23.01 min, 2% B. The detection wavelength was set to Diode-Array Detection (DAD) at λ=295 mm.

Animal grouping and model construction

Sixty healthy SD rats (SPF, 220-240 g, male and female) were purchased from Chengdu Dashuo Experimental Animal Co., Ltd. China (SCXY(Chuan) 2020-034). The rats were fed for 3 days and then kept under individually ventilated cage (IVC) conditions with a 12-hour circadian cycle. All rats received standard rat chow and water. Animal studies followed the ARRIVE 2.0 guidelines. Animal experiments conducted were approved by the Ethics Committee of North Sichuan Medical College ([2023]014).

Animal grouping and drug treatment are described in Figure 1A. The SD rats were randomly divided into a control group of 20 rats and a model group of 80 rats, with no difference in weight or sex between the groups. The rats in the model group were treated with 10 mg/kg/d of loperamide by intragastric administration to establish the STC model. The rats in the control group were treated with the same dose of normal saline. The number of fecal pellets, fecal weight, and the weight of the rats were recorded every 5 days. After 7 days of modeling, rats were considered to have been successfully modeling when they showed signs of low spirits, dull fur, and dry, hard feces. There were ten groups and each group had six rats (n=6 per group). ZYD-low/medium/high rats were respectively administered 0.5 mL/1 mL/2 mL ZYD solution (0.51 g/mL) and 1.5 mL/1 mL/0 mL ultrapure water (Equivalent to 5, 10, and 20 times the daily dose for adults, respectively) by gavage for 8-14 days. Rats in the ZYD + miR-10a-5p agomir group were treated with 2 mL ZYD (0.51 g/mL) by gavage, and 100 μL (5 nmoL) miR-10a-5p agomir was injected through the tail vein every 48h for 4 consecutive injections (8, 10, 12, and 14 days). For control, rats in the ZYD + NC agomir group

were transfected with negative control (NC)-agomir.

Measurement of fecal water content

Fresh feces were collected from each rat within 6 h of day 14 and weighed in wet weight. Then, the feces were dried at 60°C for 24h and weighed again. The fecal water content was calculated as follows: Water content = (wet weight - dry weight)/wet weight.

Intestinal propulsive ratio

The rats fasted for 24h before the experiment. During the experiment, each rat was administered 2 mL activated carbon suspension by gavage, and the rats were sacrificed by cervical dislocation after 30 min. The abdomens were opened and the small intestine from the pylorus to the caecum was carefully removed. Propulsion rate of carbon powder = propelling length of carbon powder /whole length of the small intestine.

Small RNA transcriptome sequencing

Total RNA was extracted from the colonic tissues of rats in the control and STC groups. After RNA purification, RNA libraries were constructed using the TruSeq Small RNA Sample Pre Kit (Illumina, CA, USA). The libraries were enriched by PCR amplification and purified by gel electrophoresis. After that, the library quality was confirmed by the Agilent High Sensitivity DNA Kit (Agilent Technologies, CA, USA). The optimal loading amount was quantitatively selected by the Quant-IT PicoGreen dsDNA Assay Kit (Invitrogen, CA, USA). Sequencing was performed using the Illumina NovaSeq 6000 sequencing platform. Annotation and quantification of the miRNA were performed by miRDeep2.

Cell culture and treatment

Rat intestinal epithelial IEC-18 cells were purchased from the American Type Culture Collection (Manassas, VA, USA; CRL-1589). The cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) at 37°C with 5% CO_2 in a humidified incubator. Lipofectamine 3000 transfection reagent was used for the transient transfection of NC mimic, miR-10a-5p mimic into IEC-18 cells (1.0×10^5 cells/well). Meanwhile, cells were incubated with NC inhibitor or miR-10a-5p inhibitor

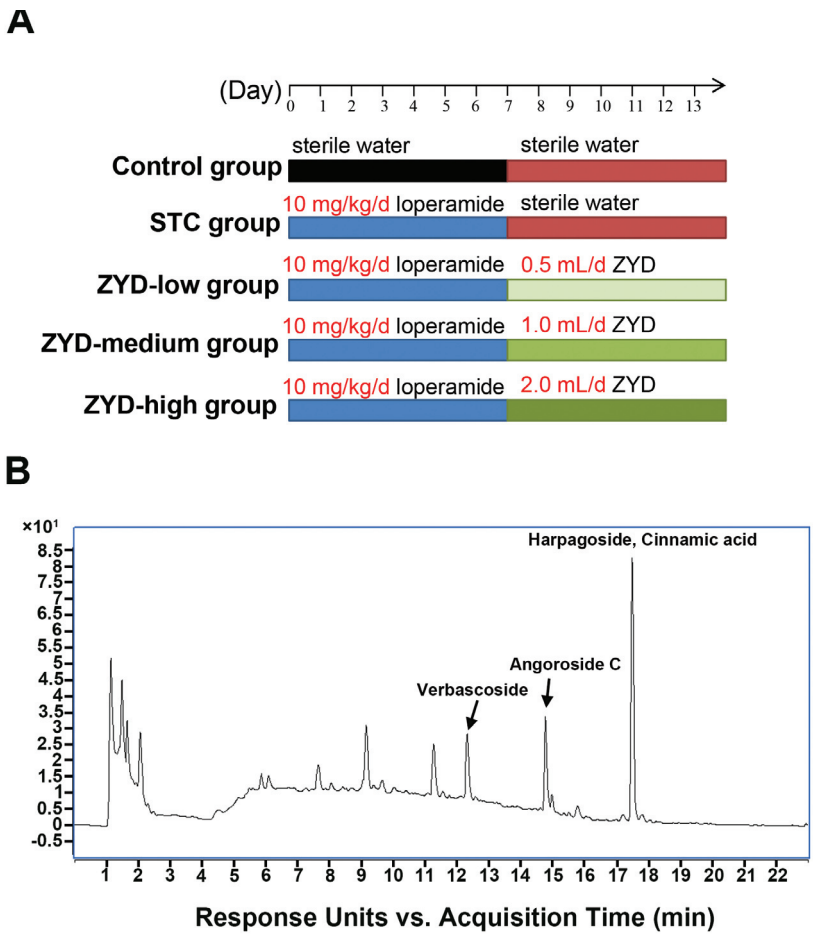

Fig. 1. Schematics of experiment workflow and active ingredient analysis of ZYD. **A.** Schematic flowchart of experiments. **B.** The active ingredient composition of the ZYD using High-performance liquid chromatography (HPLC).

according to the manufacturer's instructions.

Luciferase reporter assay

Analysis of miR-10a-5p target genes was performed with the online bioinformatics database Starbase. The wt-Drd2 target junction fragment or mut-Drd2 target binding site fragment was ligated to psiCHECK-2 plasmid. 293T cells were co-transfected with wild-type or mutated luciferase reporter vector, NC or miR-10a-5p mimic using transfection reagent, and the dual-luciferase activity was measured 24h after transfection.

Enzyme-linked immunosorbent assay (ELISA)

According to the manufacturer's instructions, the content of AC and cAMP in rat colon tissue was detected by ELISA with an automatic microplate reader (Thermo Scientific, Waltham, MA, USA) at 450 nm absorbance. The optical density (OD) value was used as the ordinate, and the content of the substance was used as the abscissa. Curve Expert 1.3 software was used to draw the standard curve and the content of analytes was calculated.

Extraction of RNA and Real-time fluorescence quantitative polymerase chain reaction (RT-qPCR)

RNA was extracted from colon tissue or IEC-18 cells using the TRIzol® reagent (Thermo Fisher, Massachusetts, USA). Two μL of 1 g/L total RNA was used for reverse transcription. RT-qPCR was performed using 5 μL of reverse transcription product as a template, with specific primers and PCR reaction system added. The ΔΔCt method was used to analyze the Ct value, β-actin was used as an internal reference gene to calculate the Ct value, and $2^{-\Delta\Delta Ct}$ was used to represent gene expression. RT-qPCR primer sequences were as follows: AQP3, forward primer (5′-3′) ATT GTC TCC CCA CTC CTG, reverse primer (3′-5′) TCA CAT TCT CTT CCT CGG. AQP9, forward primer (5′-3′) CTT CCA CCA TCC TTC CAC, reverse primer (3′-5′) TGA GCA ATA GAG CCA CAT C. β-actin, forward primer (5′-3′) CTC CAT CGT CCA CCG CAA ATG CTT CT, reverse primer (3′-5′) GCT CCA ACC GAC TGC TGT CAC CTT C. U6, forward primer (5′-3′) CTC GCT TCG GCA GCA CA, reverse primer (3′-5′) AAC GCT TCA CGA ATT TGC GT. miR-10a-5p, forward primer (5′-3′) CGC TAC CCT GTA GAT CCG AA, reverse primer (3′-5′) GTG CAG GGT CCG AGG T.

Western blot

Rat colon tissue was added to 1 mL 1% PMSF protein lysate and homogenized on ice for 30 min. For cell experiments, cells were harvested and lysed with 2 μL RIPA lysate containing 1% PMSF. The supernatant was extracted by centrifugation for 5 min at 4°C and 12000 r/min. Protein concentration was assessed by the bicinchoninic acid (BCA) method. Protein samples were standardized and electrophoresed on 10% SDS–PAGE gel, then transferred to a polyvinylidene fluoride transfer membrane (Immun-Blot PVDF membrane, Westang, Shanghai, China) at 300 mA for 1h. The PVDF membrane was washed with 0.1% TBST three times for 15 min each, at room temperature on a shaker. Then, the PVDF membrane was blocked using 5% skim milk powder. The membrane was incubated with primary antibody (anti-Drd2, anti-Protein Kinase A (PKA), anti-p-PKA, anti-cAMP Response Element-Binding protein (CREB), and anti-p- CREB; 1:1000) at 4°C overnight, followed by the addition of secondary antibody at 37°C for 1h. Enhanced chemiluminescence (ECL) reagent (Affinity Biosciences, Cincinnati, Ohio, USA) was used to observe the bands. The relative protein expression was analyzed, and β-actin was used as an internal control.

Immunofluorescence (IF) staining

We focused on the proximal colon for examining the expression levels of AQP3 and the distal colon for AQP9. Paraffin sections of colon tissue were dewaxed and hydrated. The sections were incubated in QuickBlock™ Blocking Buffer (Beyotime Biotechnology, Jiangsu, China, P0260) for 30 min at room temperature. Then, the sections were incubated with the anti-AQP3 (GB12533, Servicebio, Wuhan, China, 1:300), or anti-AQP9 (bs-2060R, Boaosen, Beijing, China, 1:350) at 4°C overnight and washed three times with phosphate-buffered saline (PBS). FITC-conjugated goat anti-rabbit antibody (GB22303, Servicebio, Wuhan, China, 1:100) and CY3-labeled goat anti-mouse antibody (GB21301, Servicebio, Wuhan, China, 1:100) were used as the secondary antibody. DAPI (Abcam, ab104139; 1/2000) was added dropwise onto the sections for 5 min. The staining was observed under a fluorescence microscope BX53 (Olympus, Tokyo, Japan) at 100× magnifications.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 (IBM Corp., Armonk, NY, USA). The data are expressed as the mean ± standard deviation. Differences among multiple groups were compared by one-way analysis of variance (ANOVA) with Dunnett's *post-hoc* test, and differences between two groups were compared by the Dunnett-t test. $p<0.05$ was considered to indicate statistical significance, and <0.01 was considered highly significant.

Results

ZYD promoted defecation and intestinal mobility in loperamide-induced STC rats

We first confirmed the active ingredient composition

of the ZYD using HPLC. In the chromatographic profile of ZYD, Verbascoside, Angoroside C, Harpagoside, and Cinnamic acid were tested (Fig. 1B). To explore the influence of ZYD on STC, we established a rat STC model and applied different dosages of ZYD. As shown in Fig. 2A, the intestinal propulsive rate in the STC group was significantly lower than in the control group. However, the intestinal propulsive rate in ZYD-low, ZYD-medium, and ZYD-high groups was increased, in comparison with the STC group (Fig. 2A,B). Meanwhile, the fecal water content of the STC group was significantly decreased compared with the control group (Fig. 2C). At day 14, fecal water content was recovered following ZYD treatment in the ZYD-low, ZYD-medium, and ZYD-high groups (Fig. 2C). These results indicated that the administration of ZYD promoted defecation and enhanced colonic mobility in STC rats.

ZYD regulated the expression of AQP3 and AQP9 in the colon of STC rats

It is known that STC could be caused by changes in the expression of aquaporin, which is responsible for the transport of water molecules. Thus, we determined the effect of ZYD on the expression of AQP3 and AQP9 in the colon of STC rats. As shown in Fig. 2D,E, the mRNA levels of AQP3 were elevated in the proximal colon of rats with STC, while AQP9 mRNA levels were decreased in the distal colon of STC rats compared with the control group. ZYD reduced mRNA levels of AQP3 and induced mRNA levels of AQP9 in a concentration-dependent manner (Fig. 2D,E). In addition, IF staining showed that treatment of ZYD-low, ZYD-medium, and ZYD-high decreased the expression of proximal colon AQP3 and increased the expression of distal colon AQP9 compared with the model group (Fig. 2F-I).

ZYD decreased miR-10a-5p, increased Drd2, and inhibited AC/cAMP signaling pathway activation in loperamide-induced STC rats

Then, we explored the mechanisms of ZYD in relieving constipation. Small RNA sequencing results showed the top 15 differentially expressed miRNAs between the control and STC group, including miR-10a-5p. As shown in Figure 3B, the expression levels of miR-10a-5p were increased in the colon of STC rats compared with the control group, which was dose-dependently decreased by ZYD. Moreover, loperamide induction resulted in decreased Drd2 levels in the colon of rats (Fig. 3C,D). The expression level of Drd2 was dose-dependently elevated in the ZYD group compared with the STC group (Fig. 3C,D). Furthermore, ELISA proved that loperamide induction led to AC activation and subsequent cAMP stimulation in the colon of STC rats compared with the control group (Fig. 3E,F). However, the treatment of ZYD-low, ZYD-medium, and ZYD-high negatively regulated AC expression, thereby blocking cAMP induction (Fig. 3E,F). Western blot results indicated that the increased expression of PKA, p-PKA, CREB, and p-CREB in the colon of STC rats was decreased by ZYD administration in a dose-dependent manner (Fig. 3G-K).

MiR-10a-5p overexpression reversed the improvement effect of ZYD on constipation

The effects of ZYD and miR-10a-5p agomir on constipation and aquaporin expression were determined. As shown in Figure 4A,B, treatment with ZYD effectively enhanced the intestinal propulsive rate and fecal water content of STC rats. Co-treatment with ZYD and miR-10a-5p agomir further reversed the promotion of intestinal propulsive rate and fecal water content in response to ZYD (Fig. 4A,B). Then, the effects of ZYD and miR-10a-5p agomir intervention on aquaporin expression in the colon of STC rats were examined. IF staining results demonstrated that miR-10a-5p overexpression effectively counteracted the ZYD-induced reduction of AQP3 expression and elevation of AQP9 expression in the colon of STC rats (Fig. 4C-F).

MiR-10a-5p overexpression blocked the inhibitory effect of ZYD on the Drd2/AC/cAMP axis

To investigate the effect of ZYD and miR-10a-5p agomir on the Drd2/AC/cAMP signaling pathway, ELISA and western blot analysis were performed. The results showed that treatment with ZYD dramatically suppressed AC and cAMP expression, while this effect was notably abrogated by miR-10a-5p overexpression (Fig. 5A,B). In addition, treatment of ZYD notably enhanced Drd2 expression in the colon of STC rats (Fig. 4C,D). Consistently, the above effect was also reversed following STC rat transfection with miR-10a-5p agomir (Fig. 5C,D). Subsequently, the data revealed that after ZYD treatment of STC rats, the protein expression levels of PKA, p-PKA, CREB, and p-CREB were decreased (Fig. 5C,E-H). However, the levels of these proteins were notably restored following miR-10a-5p overexpression (Fig. 5C,E-H).

MiR-10a-5p targeted Drd2 and regulated AC/cAMP signaling pathway activation in IEC-18 cells

Next, the potential binding sequence between miR-10a-5p and the target gene *Drd2* was predicted by TargetScan (Fig. 6A). The result of the dual-luciferase experiment suggested that luciferase activity of the wild-type of 3' UTR *Drd2* was significantly reduced, which was restored by the mutation sequence of 3' UTR Drd2 (Fig. 6B). IEC-18 cells were transfected with miR-10a-5p mimic, miR-10a-5p inhibitor, or their negative controls; transfection did not affect cell viability (Fig. 6C). RT-qPCR results showed that miR-10a-5p

expression was increased by miR-10a-5p mimic and was decreased by a miR-10a-5p inhibitor (Fig. 6D). As shown in Fig. 6E, miR-10a-5p mimic inhibited Drd2 expression and miR-10a-5p inhibitor promoted Drd2 expression. ELISA analyses demonstrated that the contents of AC and cAMP in IEC-18 cells were induced by a miR-10a-5p mimic and reduced by a miR-10a-5p inhibitor (Fig. 6F,G). Meanwhile, miR-10a-5p mimic enhanced the expression of PKA, p-PKA, CREB, and p-CREB in IEC-18 cells, and miR-10a-5p showed the

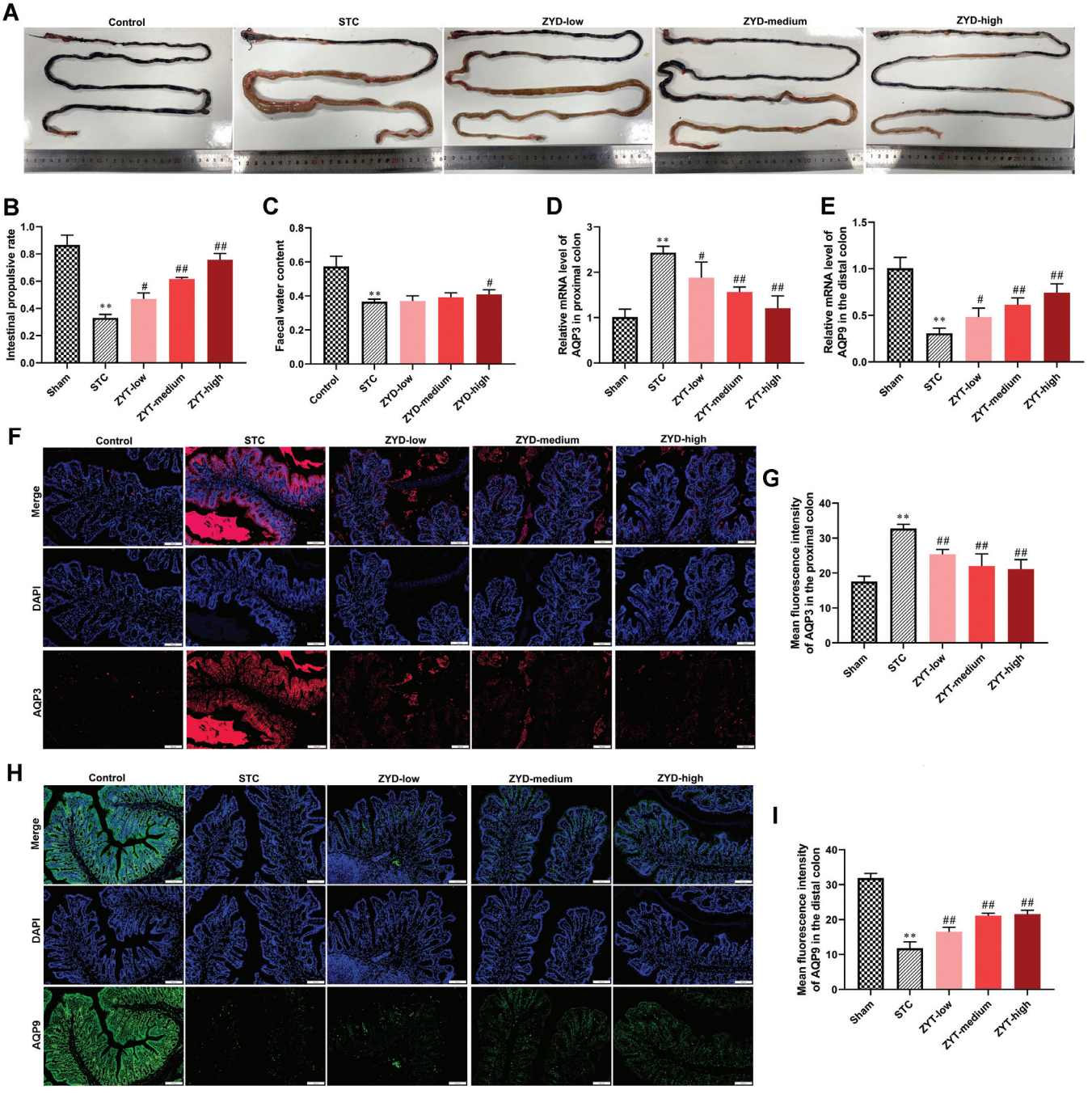

Fig. 2. ZYD promoted defecation and intestinal mobility and inhibited aquaporin expression in loperamide-induced STC rats. **A, B.** Intestinal transport was analyzed by the intestinal propulsive movement of carbon ink. **C.** The fecal water content of STC rats treated with ZYD. **D, E.** RT-qPCR analysis of AQP3 and AQP9 expression in the colon of rats. **F, G.** Immunofluorescence (IF) microscopy analysis of AQP3 expression in the colon of rats. **H, I.** AQP9 expression detection by IF. $^{**}p<0.01$ vs. control, $^{\#}p<0.05$ vs. STC, $^{\#\#}p<0.01$ vs. STC. × 100.

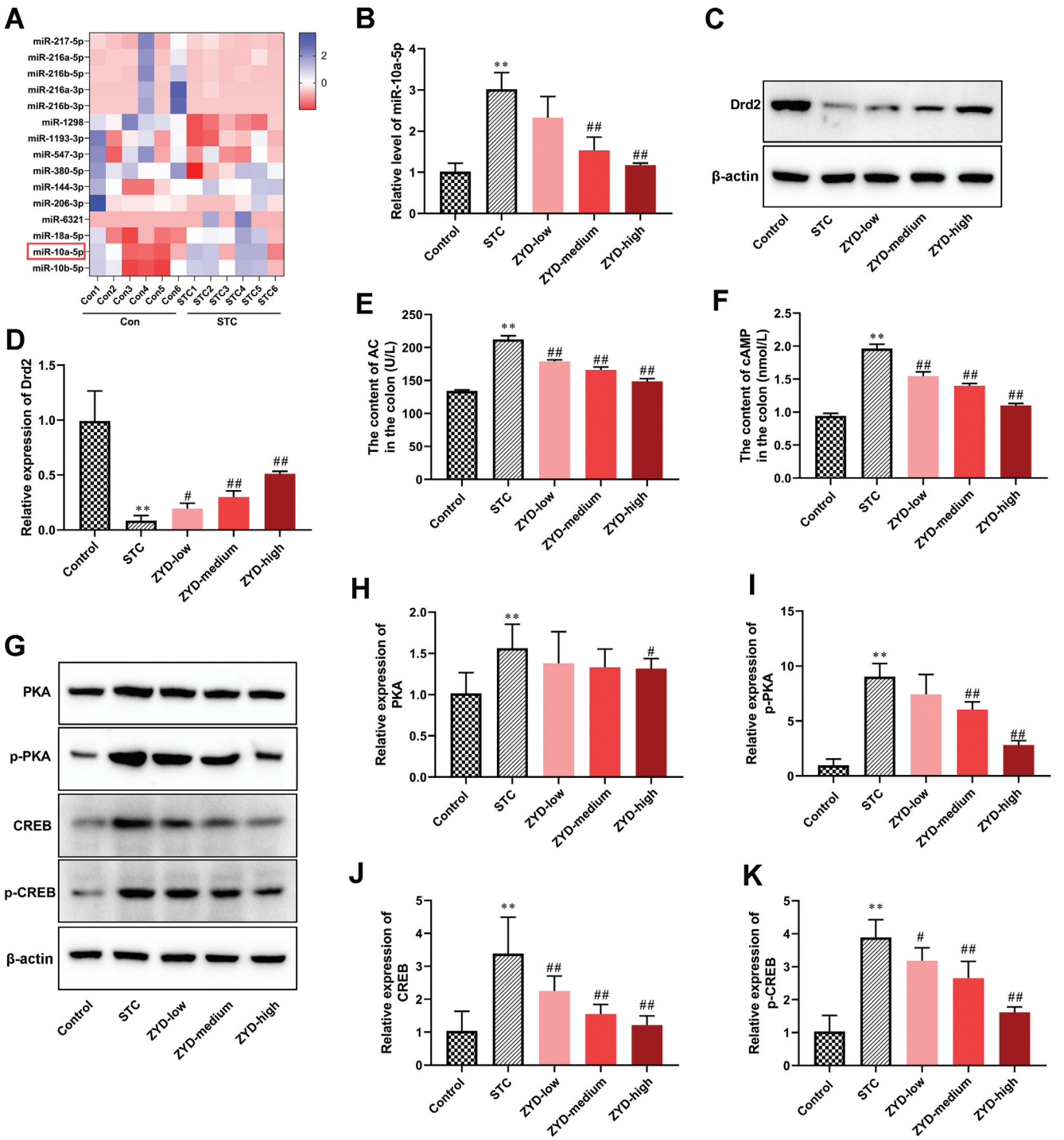

Fig. 3. ZYD decreased miR-10a-5p, increased Drd2, and inhibited AC/cAMP signaling pathway activation in loperamide-induced STC rats. **A.** The cluster analysis of differentially expressed miRNAs between the control and STC groups was illustrated with a heatmap. **B.** The level of miR-10a-5p was quantified by RT-qPCR. **C, D.** Drd2 protein expression in the colon was detected by western blot with β-actin as an internal reference. **E, F.** ELISA for AC and cAMP production. **G-K.** Analysis of PKA, p-PKA, CREB, and p-CREB protein expression by western blot with β-actin as an internal reference. $^{**}p<0.01$ vs. control, $^{\#}p<0.05$ vs. STC, $^{\#\#}p<0.01$ vs. STC.

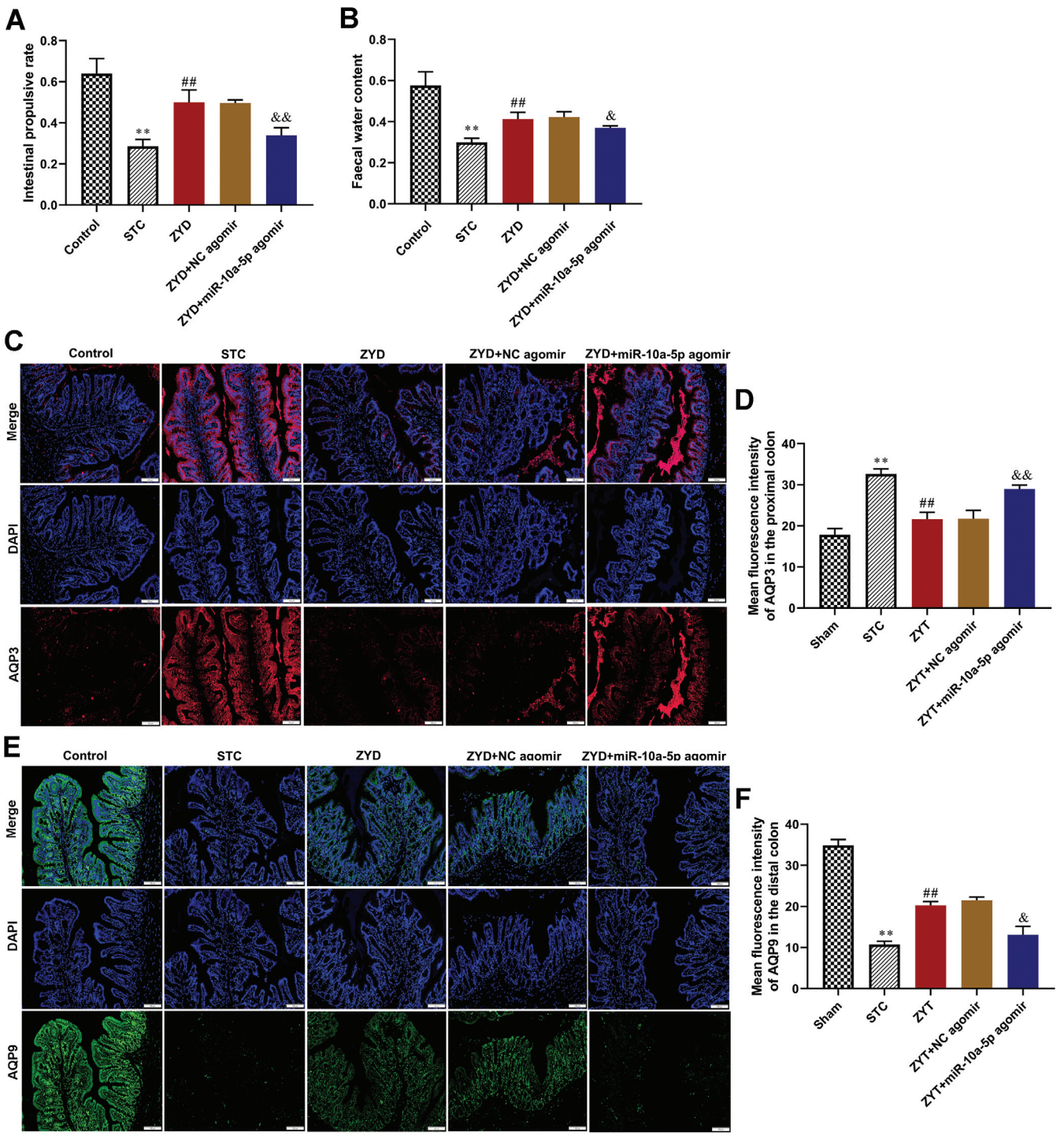

Fig. 4. MiR-10a-5p overexpression reversed the improvement effect of ZYD on constipation. **A.** Intestinal transport was analyzed by the intestinal propulsive movement of carbon ink. **B.** The fecal water content of STC rats treated with ZYD and/or miR-10a-5p agomir. **C, D.** Immunofluorescence (IF) microscopy analysis of AQP3 expression in the colon of rats. **E, F.** AQP9 expression detection by IF. $^{*}p<0.05$ vs. control, $^{\#\#}p<0.01$ vs. STC, $^{\&}p<0.05$ vs. ZYD+NC agomir, $^{\&\&}p<0.01$ vs. ZYD+NC agomir. × 100.

opposite results (Fig. 6H-L).

MiR-10a-5p regulated AQP3 and AQP9 expression in IEC-18 cells

Finally, we explored the effect of miR-10a-5p on AQP3 and AQP9 expression in IEC-18 cells. As shown in Figure 7A,B, the expression of AQP3 was enhanced by miR-10a-5p overexpression and decreased by miR-10a-5p silencing. However, the AQP9 in IEC-18 cells showed the opposite results (Fig. 7C,D).

Discussion

AQPs play important roles in the water transport system in the human body, which provides channels for rapid water transport across membranes (Gonen and Walz, 2006). The 13 isoforms of AQP (AQP0-AQP12) are widely expressed in epithelial and endothelial cells, which are closely related to fluid absorption and secretion (Borgnia et al., 1999). In recent years, various studies confirmed that AQPs played a key role in gastrointestinal fluid metabolism (Krane and Goldstein, 2007). In our study, we specifically focused on the expression levels of AQP3 in the proximal colon and AQP9 in the distal colon of STC rats. This choice was made on the basis that different regions of the colon show different functional features, which may affect aquaporin expression, and the known roles of these aquaporins in each region. Previous studies have demonstrated that the proximal colon exhibits a higher overall count of dendritic cells and a more advanced immune profile in comparison with the distal colon (Bernardo et al., 2015). This regional disparity in immune cell composition may potentially impact the expression and functionality of aquaporins, particularly AQP3, which plays a crucial role in water transport and gut homeostasis (Kon et al., 2018). Additionally, the involvement of AQP9 in the distal colon is particularly intriguing due to its association with neutrophil recruitment and inflammatory responses (Thon et al., 2024). Our study demonstrated a significant up-

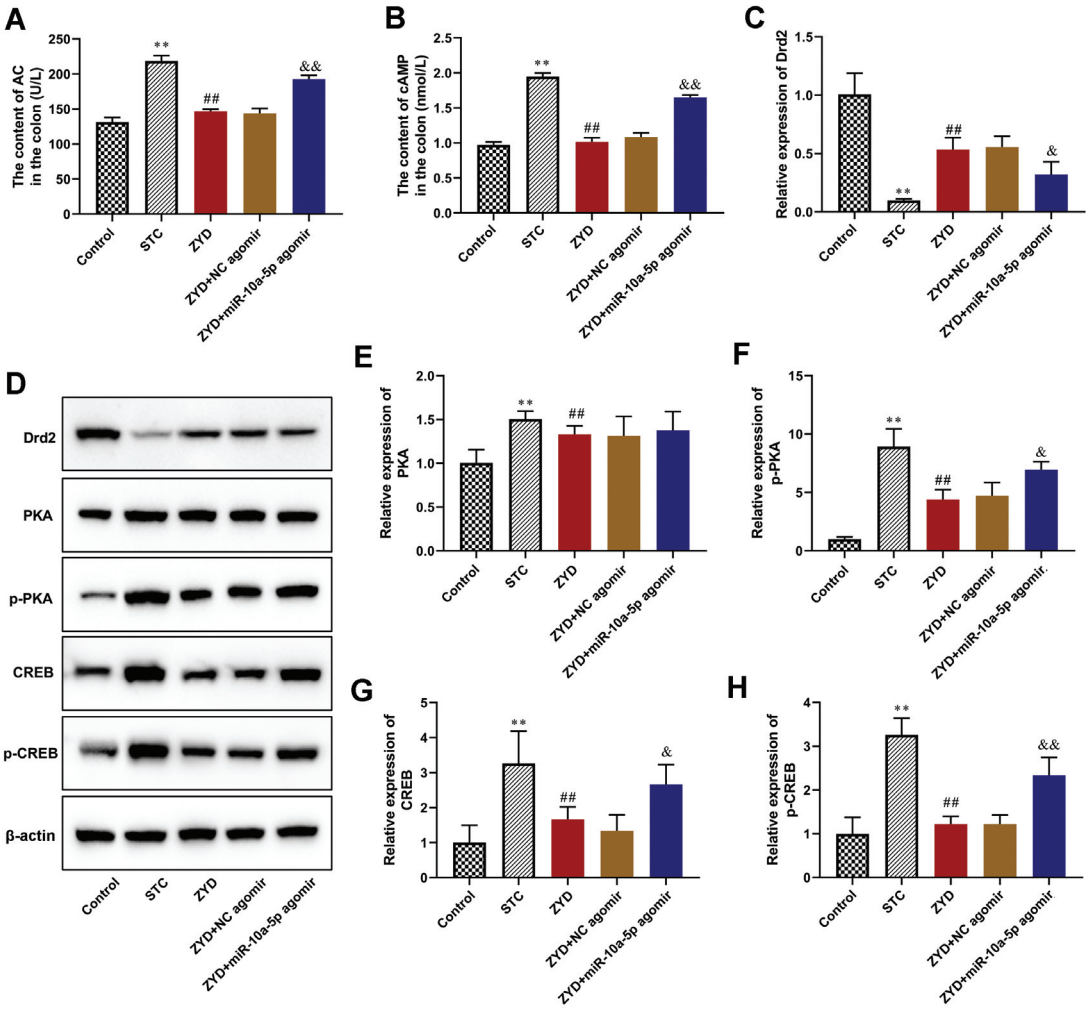

Fig. 5. MiR-10a-5p overexpression blocked the inhibitory effect of ZYD on the Drd2/AC/cAMP axis. **A, E.** AC and cAMP production were detected by ELISA. **H-L.** Analysis of PKA, p-PKA, CREB, and p-CREB protein expression by western blot with β-actin as an internal reference. $^{*}p<0.05$ vs. control, $^{\#\#}p<0.01$ vs. STC, $^{\&}p<0.05$ vs. ZYD+NC agomir, $^{\&\&}p<0.01$ vs. ZYD+NC agomir.

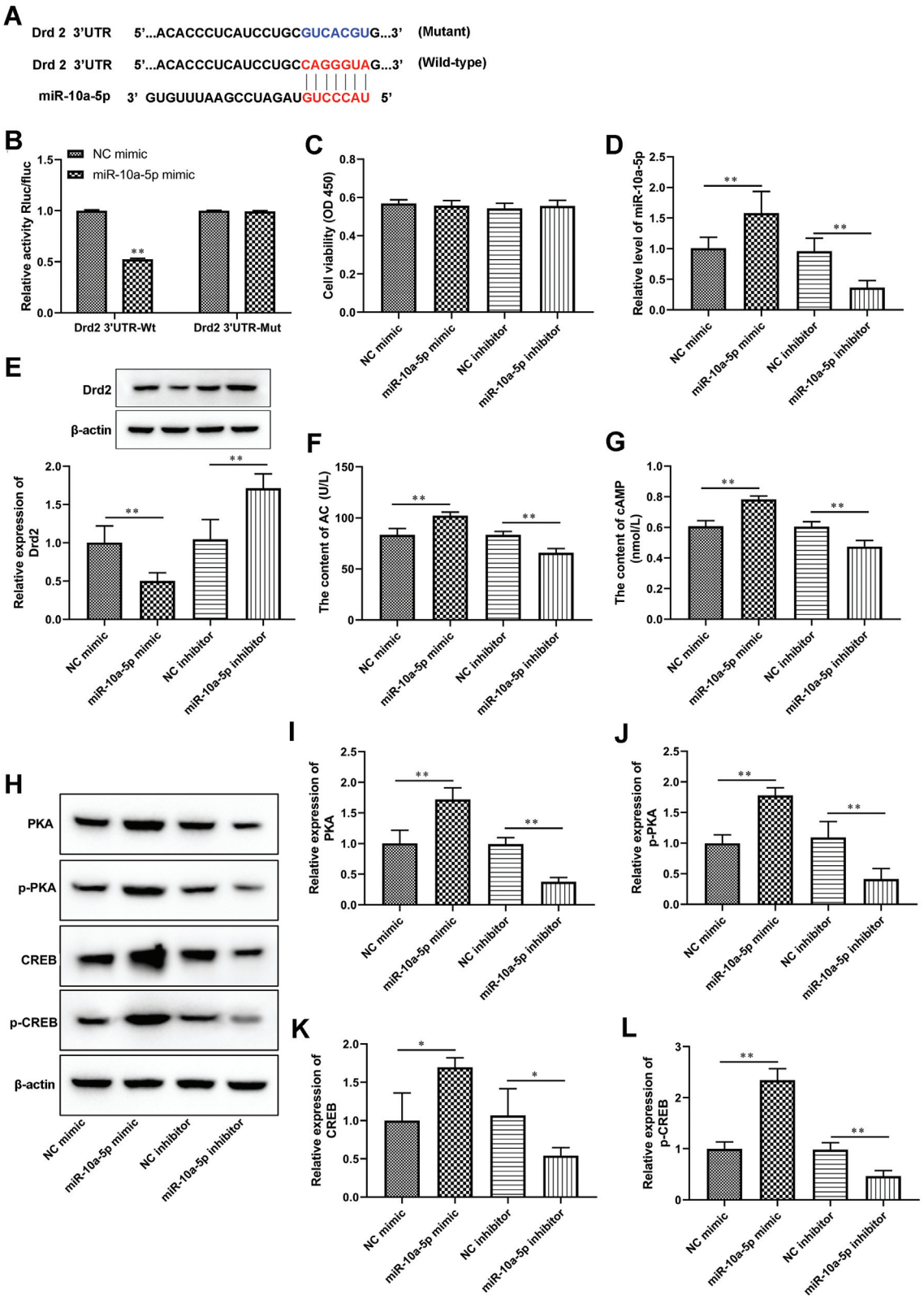

Fig. 6. MiR-10a-5p targeted Drd2 and regulated AC/cAMP signaling pathway activation in IEC-18 cells. **A.** TargetScan was used to predict miR-10a-5p targets. **B.** Luciferase activity of UGT1A 3'-UTR luciferase reporter vectors co-transfected with miR-10a-5p mimic or NC mimic in 293T cells. **C.** Cell viability was measured by CCK-8. **D.** The level of miR-10a-5p was quantified by RT-qPCR. **E.** Drd2 protein expression in the colon was detected by western blot with β-actin as an internal reference. **F, G.** ELISA for AC and cAMP production. **H-L.** Analysis of PKA, p-PKA, CREB, and p-CREB protein expression by western blot with β-actin as an internal reference. *p<0.05 vs. NC mimic or NC inhibitor, **p<0.01 vs. NC mimic or NC inhibitor.

regulation of AQP3 expression in the proximal colon of STC rats compared with the control group, which was significantly attenuated by ZYD treatment. Conversely, AQP9 exhibited contrasting results in the distal colon.

AQP3, a predominant isoform found in the gastrointestinal tract, has been recognized for its ability to selectively transport water, glycerol, and hydrogen peroxide (H_2O_2) (Yde et al., 2016). AQP3 has garnered significant attention for its crucial roles in maintaining water transport, cell volume regulation, intestinal permeability, fluid secretion, and absorption homeostasis (Zhu et al., 2016; Ikarashi et al., 2019). Growing evidence suggests that AQP3 in the colon plays a significant role in fecal characteristics and may contribute to the development of constipation by facilitating water absorption from the luminal to the vascular side (Kon et al., 2015). For instance, research by Kon et al. found that AQP3 mRNA expression in the colon was elevated in constipated rats induced by morphine, likely due to increased serotonin secretion (Kon et al., 2015). Indeed, the expression of AQP3 in the ascending colon mucosa of patients with functional constipation was higher than that of healthy volunteers, while the expression of AQP9 in the descending colon was lower than that of healthy volunteers (Yuan et al., 2008). Another study found that AQP3 was highly expressed in the colon of patients with morphine-induced constipation, indicating that AQP3 played an important role in regulating the water content of intestinal feces (Ikarashi et al., 2016). Moreover, it was reported that the treatment with the atractylodes macrocephala Koidz (AMK) and paeonia lactiflora Pall (PLP) reduced the expression of AQP1, AQP3, and AQP8 in the colon of constipated rats (Meng et al., 2022). Luteolin decreased the upregulation of AQP3, AQP4, and AQP8 in the colon of functional constipation

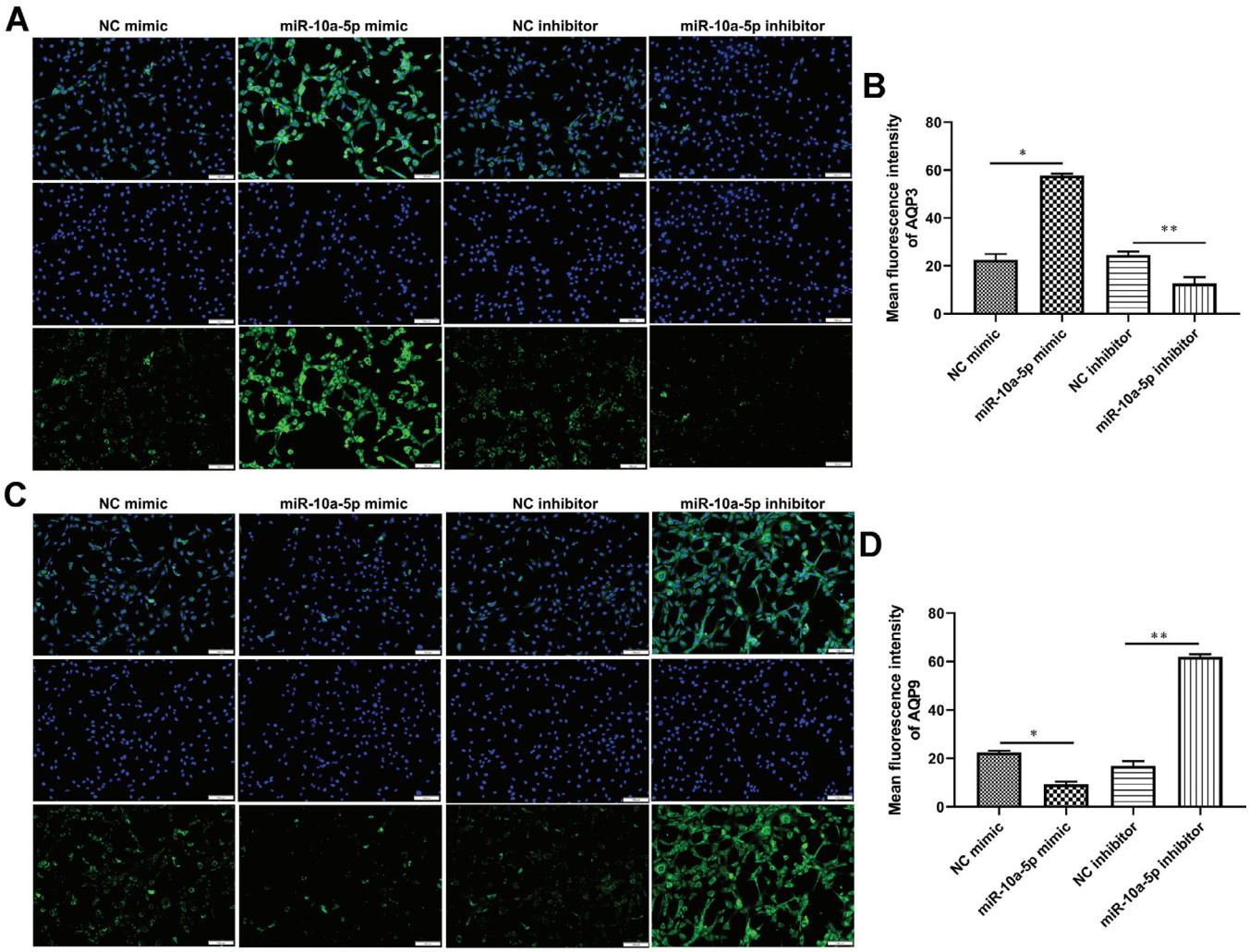

Fig. 7. MiR-10a-5p regulated AQP3 and AQP9 expression in IEC-18 cells. **A, B.** Immunofluorescence (IF) microscopy analysis of AQP3 expression in IEC-18 cells. **C, D.** AQP9 expression in IEC-18 cells was detected by IF. *p<0.05 vs. NC mimic, **p<0.01 vs. NC inhibitor. × 100.

in mice (Wang et al., 2023). Therefore, the majority of these findings provide evidence to suggest that elevated levels of AQP3 may be a significant feature of functional constipation. Conversely, the extracts of Ripe Prunus mume (Siebold) Siebold & Zucc. induced laxative effects by upregulating the expression of AQP3 in rats with loperamide-induced constipation (Na et al., 2022). Naringenin relieved constipation by enhancing the expression levels of AQP3 in STC mice (Yin et al., 2018). Another study demonstrated that sennosides exhibited pronounced laxative effects by inhibiting the expression of most aquaporins (AQP4, 5, 6, 7, and 8) in the colon while promoting AQP3 expression in rats with diarrhea (Cao et al., 2018). On the other hand, AQP9 plays an important role in intestinal health by regulating water balance, glycerol transport, nutrient absorption, and immune regulation. AQP9 was reported to present specifically in the basolateral membrane of the cup cell subpopulation, which has the function of promoting mucus synthesis and/or secretion, protecting the colon mucosa, and lubricating the intestine (Okada et al., 2003). Upregulation of AQP9 was observed in colon biopsies from patients with functional constipation (Lin et al., 2023). A report proved that complex fruit drinks significantly enhanced intestinal motility and significantly upregulated AQP9 mRNA and protein expression levels in constipated mice (Shi et al., 2023). Moreover, compared with the constipation model group, the Lactobacillus plantarum CQPC02 (Yi et al., 2019) and mulberry (Hu et al., 2019) -treated groups showed decreased AQP9 expression. The observed variations in results may be attributed to factors such as treatment dosage, delivery method, administration duration, experimental design, and sampling position, as indicated in the aforementioned studies.

RNA sequencing analysis found that there were at least 17 differentially expressed miRNAs, such as miR-2116-3p, miR-3622a-5p, miR-424-5p, miR-1273-3p, miR-20b, miR-128, and miR-486-3p in the colon of STC patients (Zhao et al., 2018; Yu et al., 2020). Consistently, miR-128 was found to be involved in the pathogenesis of constipation by regulating the p38α/M-CSF inflammatory axis (Hong et al., 2021). MiRNA-222 regulated interstitial cells of Cajal (ICC) apoptosis and excessive autophagy in STC by targeting c-kit (Zheng et al., 2021). To the best of our knowledge, our studies showed for the first time that miR-10a-5p expression was increased in the colon of STC rats. In addition, miR-10a-5p reversed the improvement effect of ZYD on constipation by targeting Drd2 and promoting the AC/cAMP signaling pathway.

Drd2, located on human chromosome 11q23.1, contains 9 exons, several introns, and short tandem repeats (STR). As a dopamine receptor subtype, Drd2 belongs to the G protein-coupled receptors, it inhibits the activity of AC (Jijón-Lorenzo et al., 2018). AC functions as the initial component of the AC/cAMP signaling pathway, serving as a pivotal point for signal transduction (Barodia et al., 2022). Its enzymatic activity is tightly regulated by diverse stimuli; upon cellular stimulation, AC catalyzes the conversion of adenosine triphosphate (ATP) to cAMP, thereby initiating a cascade of intracellular signaling events. cAMP acts as a secondary messenger generated through the action of AC and subsequently activates PKA (Sun et al., 2022). PKA, being a crucial kinase within the cell, phosphorylates multiple substrate proteins including CREB. CREB serves as a transcription factor that becomes activated upon phosphorylation by PKA and consequently stimulates gene transcription. By binding to specific DNA sequences known as cAMP response elements (CREs), CREB exerts regulatory control over target gene expression, thereby influencing various cellular physiological functions (Arora et al., 2021). A previous study demonstrated that acupuncture at Dachangshu (BL25) and Tianshu (ST25) regulated functional constipation in children (FCC) through 13 core targets, including Drd2. Moreover, the expression of Drd2 in the colon of the Parkinson's rat model was reduced. Furthermore, in 6-OHDA-lesioned rats, the combination of Drd2 antagonist and sumanirole remarkably reduced peristaltic activity (Levandis et al., 2015). Our results showed that the expression of Drd2 was reduced in the colon of STC rats, which was enhanced by ZYD treatment. Meanwhile, miR-10a-5p overexpression decreased the expression of Drd2 by targeting the 3′-UTR of Drd2 mRNA. These results suggested that Drd2 played a key role in promoting intestinal peristalsis in constipated mice. On the other hand, it was reported that the cAMP/PKA signaling pathway could regulate AQP3 expression (Hua et al., 2015). β-patchoulene inhibited AQP3 expression via the cAMP/PKA signaling pathway and alleviated diarrhea in intestinal mucositis-induced rats (Wu et al., 2021). In this study, we found that ZYD treatment significantly suppressed AC/cAMP signaling pathway activity, which was blocked by miR-10a-5p overexpression, suggesting that ZYD could improve STC by inhibiting the AC/cAMP signaling pathway. However, whether ZYD could regulate AQP3 and AQP9 expression through the AC/cAMP signaling pathway in the present study needs to be further explored.

Conclusion

In conclusion, this study revealed that ZYD could alleviate the symptoms of constipation and reduce the expression of the AQP protein in the colon of STC rats. Mechanistically, ZYD reduced the expression of miR-10a-5p, increased the expression of Drd2, and inhibited the subsequent AC/cAMP signaling pathway activity. The overexpression of miR-10a-5p hinders the improvement of ZYD on constipation, which may be achieved by regulating the AQP expression level through the Drd2/AC/cAMP axis. This finding could provide a guide for novel treatment options for constipation.

Authors' contributions. Simin Chen and Xuegui Tang conceived and designed the experiments. Simin Chen, Qian Li, Lina Guan, Jun Pang, and Qingfeng Wu performed the experiments. Simin Chen, Qian Li, Lina Guan, Qiuxiao Wang, and Zhibin Zhang analyzed the data. Xuegui Tang contributed to the reagents and materials. Simin Chen wrote the manuscript.

Competing interests. The authors declare that they have no competing interests.

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