Study on regulating AQP1, AQP3, AQP4, 5-HT, NOS1 in slow transit constipation rats by Liqi Tongbian mixture

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Conflict of interest
None declared

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Abstract

Background. Liqi Tongbian is a traditional Chinese medicine (TCM) preparation that contains herbs that may treat slow transit constipation (STC). *Atractylodes macrocephala*, *Astragalus membranaceus*, *Fructus aurantii*, radish seed, uncooked *Polygonum multiflorum*, and *Agastache rugosa* were included in the formula for their unique qualities. The control of water transfer in the colon is greatly influenced by aquaporin 3 (AQP3).

Objectives. Based on this, the Liqi Tongbian mixture was used to detect the concentrations of aquaporins (AQPs), 5-HT and nitrix oxide synthase 1 (NOS1) in STC rats, and explore its effect, in order to provide a theoretical basis for the remedy of STC with TCM.

Materials and methods. Zhejiang University of Traditional Chinese Medicine provided 32 three-week-old Sprague Dawley rats of SPF-grade. The pairs licensed under SYXK (Zhejiang) 2021–0012 were kept at 20–25°C and humidity of 50–65%. The compound diphenoxylate caused constipation in the control, model, Liqi laxative (LQTB), and mosapride groups. The Liqi laxative rats were administered a mixture of traditional Chinese herbs after modeling, while mosapride was given to the other group. The levels of 5-HT, NOS1 and AQPs were tested in the feces and intestinal tissues.

Results. Comparing the condition of rat feces, it was found that the model group had significantly lower overall bulk, score and particles within 24 h compared to the control group. In comparison to mosapride, LQTB performed better. The model group had higher levels of 5-HT and NOS1 in intestinal tissue, while the LQTB and mosapride groups had decreased levels of these AQPs. LQTB had lower levels of AQP1, AQP3 and AQP4 than mosapride, while the model group had higher levels of these AQPs.

Conclusions. Liqi Tongbian mixture works better than mosapride in improving constipation symptoms in rats with STC, and its mechanism is related to regulating the level of intestinal AQPs and neurotransmitters.

Key words: 5-hydroxytryptamine, nitric oxide synthase, Liqi Tongbian mixture, slow transit constipation, aquaporins

Cite as
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Background

Slow transit constipation (STC) is a common type of constipation caused by the slow transmission of intestinal contents, mainly manifested as poor defecation, reduced frequency and dry stool.[1] In recent years, with the change of people's lifestyle and dietary structure, the incidence rate of STC has increased year by year, so the research on STC has become important in recent years.[2] At present, the pathogenesis of STC is not fully understood. Some researchers have demonstrated that STC is closely related to intestinal aquaporins (AQPs) and 5-hydroxytryptamine (5-HT) immunoreactivity[3,4] Aquaporin is an important protein that exists widely in mammals to regulate the balance of intracellular and extracellular water. Among them, AQP1, AQP3 and AQP4 are mostly found in gastrointestinal tract and digestive system. Mammals' digestive systems contain aquaporins, including AQP1, AQP3 and AQP4, which regulate water balance within and outside cells. The intricate relationship between AQPs and the gastrointestinal tract suggests a potential link to STC, a condition characterized by sluggish intestinal content transport. While the specific pathways are unknown, studies are being conducted to investigate the role of AQPs in STC. The process of intestinal water transport and play a regulatory role in constipation to some extent.[5]

5-hydroxytryptamine is also widely distributed in the intestines and is a common secretory messenger and neurotransmitter. The 5-HT immunoreactivity is usually enhanced in the intestines of patients with constipation.[6] In addition to 5-HT, nitric oxide synthase 1 (NOS1) is also an important regulatory factor in intestinal diseases.[7,8] Sailer showed that the concentration of NOS1 in intestinal tissue of patients with constipation is notably higher compared to the normal concentration.[9] Therefore, AQPs, 5-HT and NOS1 can be used as important indices to assess STC. At present, the drugs for the STC treatment mainly include gastrointestinal motility regulators and laxatives, but long-term use has great side effects, which will not only cause water and electrolyte disorders, but also lead to gastrointestinal damage.[10] The syndrome differentiation of traditional Chinese medicine (TCM) in the remedy of STC has the advantages of few side effects and remarkable effectiveness.

Qi is a core concept in TCM: not only the basic substance that makes up the human body, but also the driving force that maintains the vital activities of the human body. Liqi Tongbian mixture contains *Atractylodes macrocephala*, *Astragalus membranaceus*, *Fructus aurantii*, radish seed, raw *Polygonum multiflorum*, and *Agastache rugosa*. Among them, *Atractylodes macrocephala* has the effect of invigorating spleen, replenishing qi and promoting diuresis. *Astragalus* invigorates spleen and qi. *Fructus Aurantii* breaks qi, eliminating accumulation and removing ruffians. Radish seeds have been found to promote qi and aid in digestion. Raw *Polygonum multiflorum* has the effect of moistening intestines and relieving constipation, and *Agastache rugosa* removes dampness. The combination of various medicines plays the role of regulating qi and invigorating the spleen and reaching the 6 viscera.

Objectives

Liqi Tongbian mixture was used to detect the concentrations of AQPs, 5-HT and NOS1 in STC rats, and to explore its effect to provide a theoretical basis for the treatment of STC with TCM.

Materials and methods

Animals

Thirty-two 3-week-old Sprague Dawley rats of SPF-grade were purchased from the Animal Experiment Center of Zhejiang University of Traditional Chinese Medicine (Hangzhou, China). License for the use of laboratory animals was SYXK (Zhejiang) 2021-0012. The rats were kept in cages with 2 rats in each cage, in the animal center laboratory of our hospital, at a temperature of 20–25°C and a relative humidity of 50–65%. The experiment was approved by the Animal Ethics Committee of Zhejiang University of Traditional Chinese Medicine (approval No. I ACUC-20220905-05).

Grouping and modeling

A total of 32 rats were fed adaptively for 1 week and randomly divided into control group (n = 8), model group 2 mL of normal saline was used in the model group (n = 8), Liqi laxative group (LQTB, n = 8), and mosapride group (n = 8). Slow transit constipation rat models were prepared by intragastric administration of compound diphenoxylate except the control group. The suspension of compound diphenoxylate was prepared with 10 mg/kg of compound diphenoxylate and 2 mL of normal saline. The rats were fed with water normally, rested for 1 day after 6 days of gavage, and continued to gavage for 21 days. After 24 h of gastric perfusion, the fecal particles and wet weight of each subgroup of rats were counted. If the number of stool particles and wet weight of stool of rats decreased, the divergence was statistically notable, indicating that the model was successful. If the number of fecal particles and fecal wet weight of rats decreased, the difference was statistically significant, indicating that the model was successful. Therefore, we did not further verify the success of the model through hematoxylin and eosin (H&E) staining or immunohistochemistry.

Drug intervention

After modeling, the control subgroup and model subgroup were given normal saline by gavage. Rats in the LQTB subgroup were given 30 g of *Astragalus root*, 12 g of *Atractylodes macrocephala*, 12 g of radish seed, 10 g of stir-fried *Fructus aurantii*, betel nut, and *Agastache rugosa*. A total of 6 g of raw *Polygonum multiflorum* was soaked in pure water, decocted twice and concentrated to 1 g/mL after filtering. Processed *Polygonum multiflorum* was stored in 4°C and heated to 37°C. With reference to the equivalent dose converted from the body surface area of humans and animals, 18.75 g/(kg) of the Liqi Tongbian mixture was administered by gavage. The rats in the mosapride subgroup were treated with 1.35 mg/(kg/d)[3] mosapride (Chengdu Kanghong Pharmaceutical Group Co., Ltd, Chengdu, China). Liqi Tongbian mixture and *Mosapride aqueous* solution was administered by gavage for 21 days according to the equivalent dose converted from human and animal body surface areas.

Record of fecal granules and wet weight of rats

The fecal granules and wet weight of rats in each subgroup were recorded at the time of modeling and within 24 h after intragastric treatment for 21 days, and the feces of rats were scored. Scoring standard of rat feces was as follows: When the rats' defecation presents scattered dry ball like stool, which was difficult to be discharged – 1 point. When the stool was segmented and sausage-shaped – 2 points. When the feces were sausage-like, with cracks on the surface – 3 points. When the stool was sausage-like or snake-like, smooth and soft – 4 points. When the stool was soft, appeared lump, and the edge was clear – 5 points. When the stool was soft, with rough edges or pasty stool – 6 points. When the stool was watery and had no solid component – 7 points.

Detection of 5-HT and NOS1 concentrations in intestinal tissues of rats

The intestinal ileum tissue of rats was taken. The tissue was washed with pre-cooled phosphate-buffered saline (PBS) and cut into homogenate. The supernatant was centrifuged from homogenate and underwent enzyme-linked immunosorbent assay (ELISA) detection.

Detection of AQPs in intestinal tissues of rats

Rat intestinal tissues were taken and total proteins were extracted by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was transferred to polyvinylidene fluoride (PVDF) membrane using semi-dry method. The PVDF membrane was placed in 5% skimmed milk powder at room temperature and sealed for 2 h, added the primary antibody and secondary antibody for binding, incubated for 2 h. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference protein. The color-developing solution was used for color development, and then absorbance analysis was performed.

Statistical analyses

The data from each group were statistically analyzed using GraphPad Prism v. 8.0.2 (GraphPad Software, San Diego, USA) and IBM SPSS software v. 25 (IBM Corp., Armonk, USA). The measurement data were reported as the median (1st quartile (Q1) and 3rd quartile (Q3)). The normality of the distribution was tested using the Kolmogorov–Smirnov test. Since all the distributions were normal, the Brown–Forsythe test was used to establish the equality of variances, and then significant differences between multiple groups were analyzed using the Kruskal–Wallis test. Dunn's post hoc test was used for multiple comparisons. If $p < 0.05$, the data divergence was statistically notable. All tests in this study were bilateral.

Results

Comparison of stool condition of rats

There was no mortality among the rats tested. In 24 h, the model group had significantly lower total fecal bulk, score and particles than the control group. The LQTB subgroup outperformed the mosapride subgroup regarding these measures (Fig. 1A–C, Table 1).

Comparison of 5-HT and NOS1 contents in rat intestinal tissue

Compared with the control group, the concentrations of 5-HT and NOS1 in the intestinal tissues of rats in the model group were notably increased. Compared with the model group, the concentrations of 5-HT and NOS1 of rats in the LQTB group and the mosapride group were notably reduced. The concentrations of 5-HT and NOS1 were lower in LQTB group compared to the mosapride group (Fig. 2A,B, Table 1).

Comparison of AQPs content in rat intestinal tissue

Compared with the control group, the concentrations of AQP1, AQP3 and AQP4 in the intestinal tissues of rats in the model group were notably increased; the concentrations of AQP1, AQP3 and AQP4 in the intestinal tissues of rats in the LQTB group and the mosapride group notably decreased compared to the model subgroup. The concentrations of AQP1, AQP3 and AQP4 were lower in the LQTB group compared to the mosapride group (Fig. 3, Table 1).

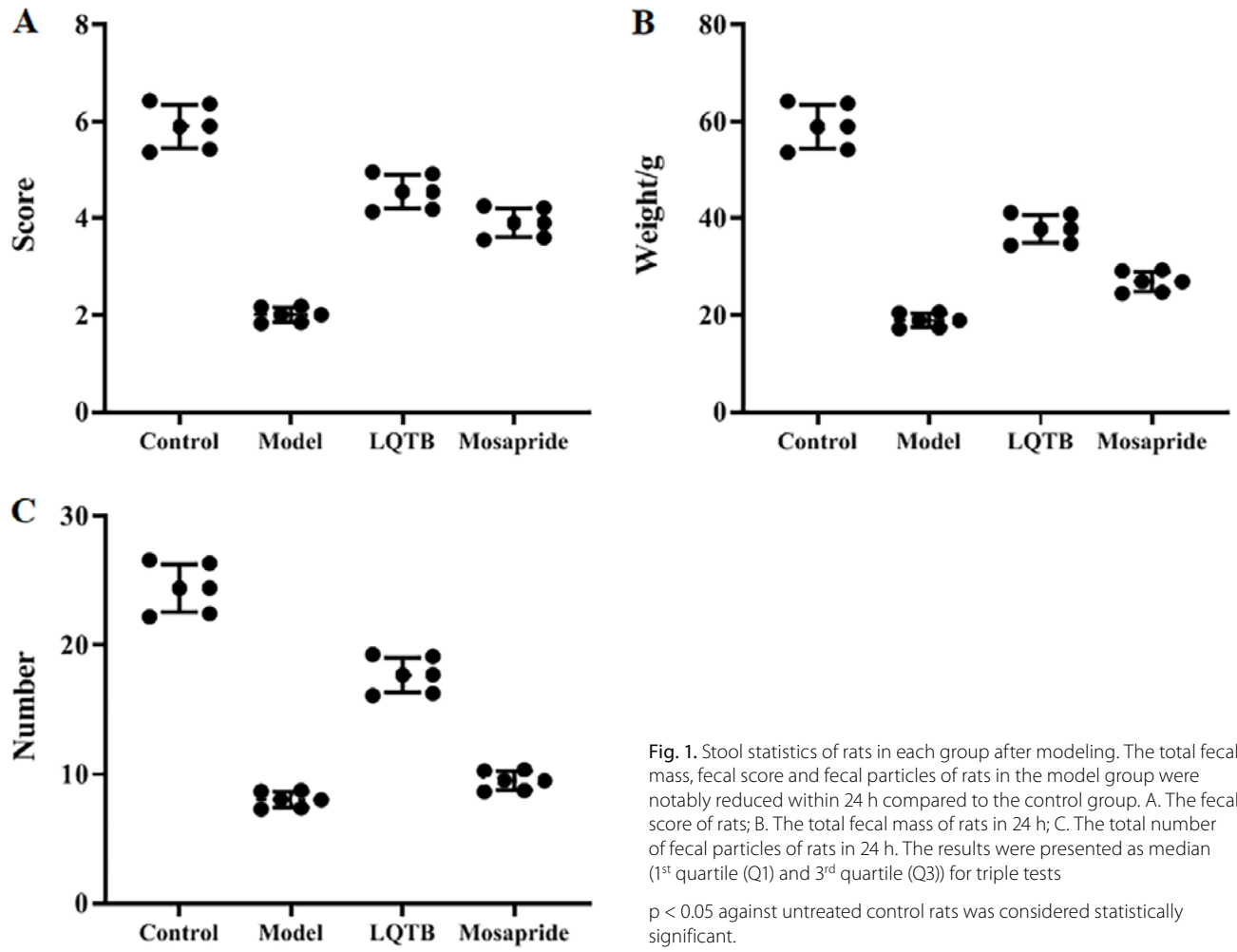

Fig. 1. Stool statistics of rats in each group after modeling. The total fecal mass, fecal score and fecal particles of rats in the model group were notably reduced within 24 h compared to the control group. A. The fecal score of rats; B. The total fecal mass of rats in 24 h; C. The total number of fecal particles of rats in 24 h. The results were presented as median (1st quartile (Q1) and 3rd quartile (Q3)) for triple tests

$p < 0.05$ against untreated control rats was considered statistically significant.

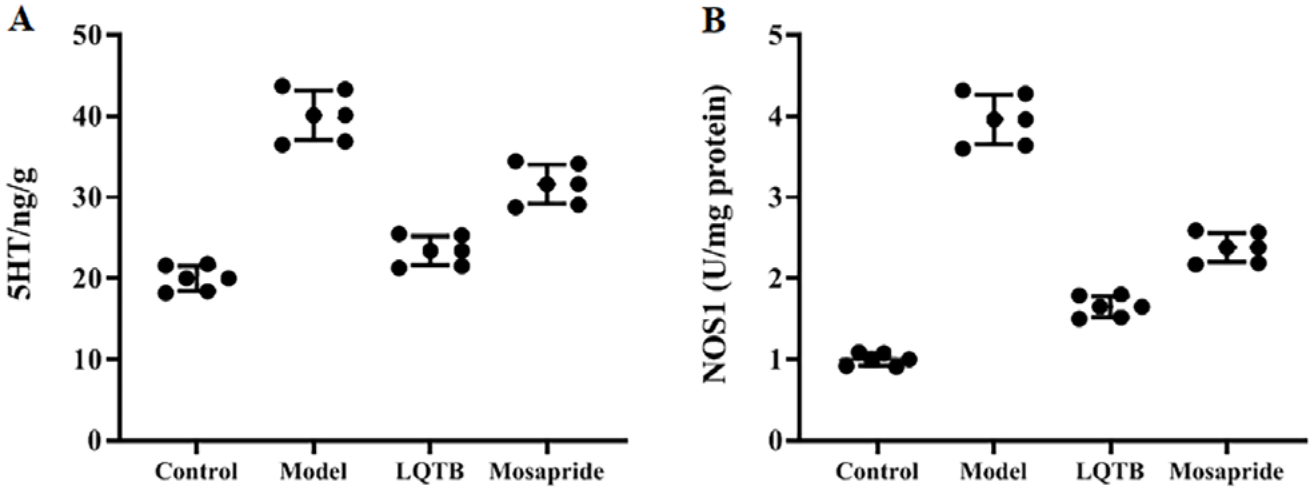

Fig. 2. Detection results of 5-HT and nitrix oxide synthase 1 (NOS1) concentrations in rat intestinal tissues. The concentrations of (A) 5-HT and (B) NOS1 in intestinal tissues of rats were compared with the control group. The results were presented as median (1st quartile (Q1) and 3rd quartile (Q3)) for triple tests

$p < 0.05$ against untreated control rats was considered statistically significant.

Discussion

The lesion of constipation is located in the large intestine, which is also closely related to the dysfunction of the viscera. From the perspective of TCM, the remedy should start with the spleen, liver, kidney, and lung.[11] In this study, Liqi Tongbian mixture was used to treat STC in rats.

Rat is a common animal model for studying STC.[7] In this experiment, the STC rat model was prepared by intragastric administration of compound diphenoxylate, and the feces

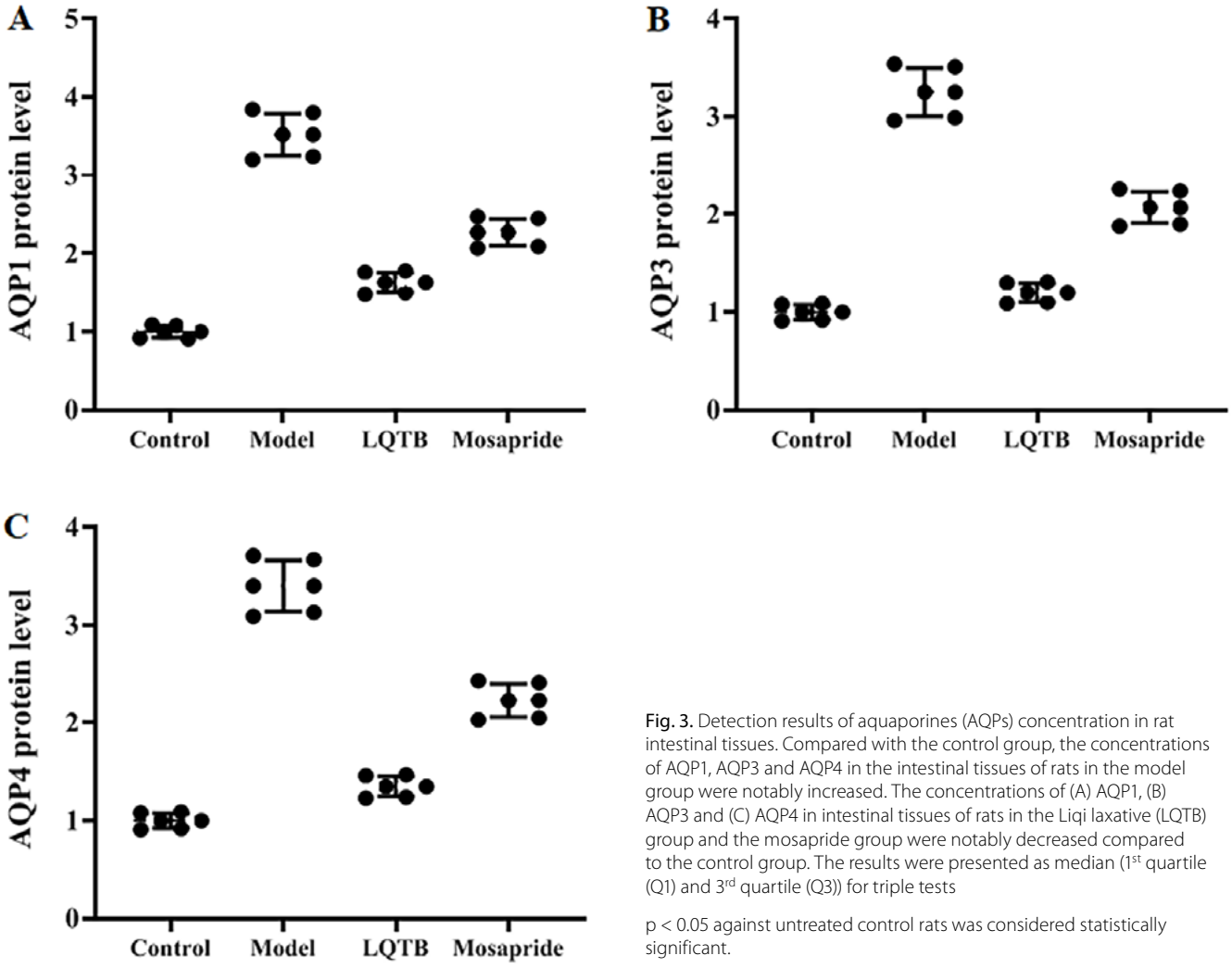

Fig. 3. Detection results of aquaporines (AQPs) concentration in rat intestinal tissues. Compared with the control group, the concentrations of AQP1, AQP3 and AQP4 in the intestinal tissues of rats in the model group were notably increased. The concentrations of (A) AQP1, (B) AQP3 and (C) AQP4 in intestinal tissues of rats in the Liqi laxative (LQTB) group and the mosapride group were notably decreased compared to the control group. The results were presented as median (1st quartile (Q1) and 3rd quartile (Q3)) for triple tests

$p < 0.05$ against untreated control rats was considered statistically significant.

Table 1. Comparison of all studied groups

Variables	Control (n = 6)	Model (n = 6)	LQTB (n = 6)	Mosapride (n = 6)	p-value*
Total fecal mass	58.96 (53.63–64.23)	18.96 (17.24– 20.66)	37.84 (34.42–41.22)	26.95 (24.52–39.36)	p < 0.001
Fecal score	5.90 (5.37–6.43)	2.01 (1.83–2.19)	4.55 (4.14–4.96)	3.91 (3.56–4.26)	p < 0.001
Fecal particles	24.42 (22.21–26.61)	8.03 (7.31–8.75)	8.69 (7.91–9.47)	9.51 (8.65–10.37)	p < 0.001
5-HT	20.01 (18.20–21.80)	40.12 (36.49–43.71)	23.41 (21.29–25.51)	31.63 (28.77–34.47)	p < 0.001
NOS1	1 (0.91–1.09)	3.96 (3.60–4.32)	1.65 (1.50–1.80)	2.38 (2.17–2.59)	p < 0.001
AQP1	1 (0.91–1.09)	3.52 (3.20–3.84)	1.63 (1.48–1.78)	2.27 (2.07–2.47)	p < 0.001
AQP3	1 (0.91–1.09)	3.25 (2.96–3.54)	1.20 (1.09–1.31)	2.07 (1.88–2.28)	p < 0.001
AQP4	1 (0.91–1.09)	3.4 (3.09–3.71)	1.35 (1.23–1.47)	2.22 (2.03–2.43)	p < 0.001

Data were present as median (Q1 and Q3); *p-value was generated from Kruskal–Wallis test. There was a significant difference among all groups in Dunn's test; LQTB – Liqi laxative; NOS1 – nitrix oxide synthase 1; AQP – aquaporine.

of the rats were observed after treatment with Liqi Tongbian mixture to assess the constipation of the rats. It was found that the total fecal mass of rats in the model group was notably reduced, which was related to the absorption of water by the intestine of rats. The boosted absorption of water by the intestinal tissue of rats after the model establishment led to the decrease of the fecal weight of rats, which resulted in constipation in rats. At the same time, the number of particles in the rat defecation decreased notably, indicating that the smooth muscle contraction of the rat fecal intestine was weakened and constipation occurred. Chen et al.[12] have exhibited that the key factor causing STC is insufficient intestinal motility. 5-hydroxytryptamine receptor agonists can promote the release

of acetylcholine to stimulate the gastrointestinal tract and promote intestinal motility. Mosapride is a 5-HT receptor agonist that is widely used in clinical practice.[13] Therefore, we selected the positive control of mosapride in this study.

The onset of STC is closely related to the relaxation of intestinal smooth muscle. There are 3 types of NOS – NOS1, NOS2 and NOS3 – which are distributed in the intestinal nervous system of vascular endothelial cells (VECs) as the "gut brain". It is mainly composed of intestinal neurons and intestinal glial cells. Among them, NOS1 is the main intestinal inhibitory neurotransmitter. Increasing the content of NOS1 in the intestine inhibits the contraction of intestinal smooth muscle and reduces the spontaneous rhythmic contraction of colonic circular muscle.[14] Usually, the increase of NOS in the intestinal tissue of patients with STC leads to the substantial increase of nitric oxide (NO) in colon tissue, which weakens intestinal motility.[9] In addition to NO and NOS, 5-HT is abnormally expressed in intestinal tissues of STC patients. 5-hydroxytryptamine, also known as serotonin, is an indole derivative, which can also enhance the contraction of blood vessels and smooth muscles. Kuang et al.[15] have demonstrated that the enhancement of 5-HT immunoreactivity is one of the main factors leading to gastrointestinal motility disorder. In this study, the concentrations of 5-HT and NOS1 in intestinal tissues of rats were measured. It was found that the concentrations of 5-HT and NOS1 in the intestinal tissue of rats in the model group, the LQTB group and the mosapride group were notably higher than those in the control group, indicating that the rats had constipation symptoms after the use of mosapride. Slow transit constipation (STC) is associated with relaxation of intestinal smooth muscle, NOS (NOS1, NOS2 and NOS3) levels, and abnormal 5-hydroxytryptamine (5-HT) production in intestinal tissue. The concentrations of 5-HT and NOS1 of rats in the LQTB and the mosapride groups were notably reduced, indicating that the constipation symptoms of STC rats were notably improved after the treatment with the Liqi Tongbian mixture. Its mechanism may be related to the reduction of 5-HT and NOS1, which regulates the intestinal smooth muscle of rats.

At present, more than a dozen kinds of AQPs have been found, among which constipation-related AQPs mainly include AQP1, AQP3 and AQP4. Aquaporin 1 is a common AQPs widely expressed in the digestive system, which allows water molecules to pass freely but is not permeable to other ions and small molecules.[16] Aquaporin 3 is mainly distributed on the surface of colonic mucosal epithelial cells. It has been confirmed that the increase of AQP3 leads to spasm of intestinal smooth muscle, thus enhancing intestinal water absorption, reducing intestinal peristalsis frequency and causing constipation.[17] Aquaporin 4 is also one of the common AQPs that cause STC. This kind of AQPs is present in the absorption epithelial cells, mainly participates in the intestinal absorption and has a certain regulatory effect on constipation.[18] The concentrations of AQP1, AQP3 and AQP4 were lower in the LQTB group. This shows that the use of Liqi Tongbian mixture can effectively regulate AQPs in the intestine of rats, thereby improving the constipation symptoms of rats, and the improvement effect is better than mosapride. Mosapride is a gastrointestinal motility drug, which mainly improves constipation by stimulating gastrointestinal peristalsis. Modern pharmacological research[18] shows that *Atractylodes macrocephala* can not only promote intestinal peristalsis, but also increase intestinal water content.

Limitations

It is important to note that this study has several limitations. First, the results are from an animal model, so extrapolation to humans may not be obvious. The study also focused on molecular markers and did not investigate alternative causes or variables of STC. More studies, including clinical trials, are needed to prove that the Liqi Tongbian combination works in humans and to understand its effects on STC. The study did not examine long-term effects or adverse outcomes, so generalizing the results to chronic conditions or prolonged use is risky.

Conclusions

Liqi Tongbian mixture can improve the constipation symptoms of STC rats by regulating the expression of AQPs and by improving the relaxation of intestinal smooth muscle of rats by regulating the concentrations of 5-HT and NOS1. Toxic and side effects of TCM on STC have not been discussed in this study. They will be explored in subsequent experiments.

Supplementary files

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10460804. The package includes the following files:

Supplementary Table 1. The independent-samples median test results of total fecal volume in rats.

Supplementary Table 2. The independent-samples median test results of fecal score in rats.

Supplementary Table 3. The independent-samples median test results of fecal particles in rats.

Supplementary Table 4. The independent-samples median test results of 5HT in rats.

Supplementary Table 5. The independent-samples median test results of NOS1 in rats.

Supplementary Table 6. The independent-samples median test results of AQP1 in rats.

Supplementary Table 7. The independent-samples median test results of AQP3 in rats.

Supplementary Table 8. The independent-samples median test results of AQP4 in rats.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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