

General comments Yin and Wang et al. described TXYF relieves IBS in rats by mechanisms involving regulation of enteric nervous system. The article is informative and well-presented. The reviewer has some comments.

Here are some problems which need to be modified.

1. The title The author only observed the changes in the expression of 5-HT and SP. They are only a very small part of Enteric Nervous System, so the title was not so appropriate.

**Answer: We changed the title accordingly**

2. The abstract The abstract describe the objective, the model, key results, and the conclusion. In the ‘background’ part, the authors describe the objective, not the background.

The methods only include the model and the positive controls, there are no detective methods and the indexes description in the method part.

Key results are not so clear in the abstract. There is no description about the background in the abstract. So, the author should rewrite the abstract and modified it’ s structure.

**Answer: We changed the abstract accordingly**

3. The material and methods

About the rats: In the 'IBS model' part, all the rats were 8-day old, and after the model establishment, they are 21-day old. In the 'Animal groups and administration' part, the rats were 12-week old. Are they the same rats? Are the drugs were given after the rats model establishment and after they grew up to 12-week old, or the model was established and meanwhile treated?

**Answer: Please see the new Fig. 1 which is a flow chart of the study design and experiment procedure.**

About the methods: Usually,  $\beta$ -actin was used as the reference for the protein expression in the western blotting methods, and GAPDH was used as the reference for the mRNA expression in RT-PCR. But in this research, the authors use GAPDH for western blotting and  $\beta$ -actin for RT-PCR. Are there any spelling mistake? Or did the authors do like this?

**Answer:  $\beta$ -actin is a commonly used and excellent internal reference for PCR and Western blotting. Common uses of protein internal reference include GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and cytoskeletal protein  $\beta$ -actin.**

4. The statistical methods The authors use one-way ANOVA to compare the differences among the groups and post hoc were compared with SNK test. This method is used for data which are in accordance with normal distribution and homogeneity of variance. But the authors didn't describe whether the data are of normal distribution and homogeneity of variance.

**We thank the reviewer for their comments on statistical analysis. We have now re-calculated the results according to their valuable suggestions. First, we checked the distribution and homogeneity of variance of all original data from each group and each treatment. All data used in the study are in accordance with a normal distribution and homogeneity of variance. Second, we chose to use one-way ANOVA with post hoc SNK (student Newman Keuls-q) method with Prism 6.0 software (GraphPad Software, San Diego, CA, USA), as we know that the SNK method in Prism 6.0 was designed with a Bonferroni correction.**

5. The Discussion In this part, the authors review some researches before, and compared them with the present research. At the same time, the authors described the information about SP in the IBS research in detail, but the information about 5-HT is less.

The authors should supplement information about 5-HT.

**Answer: We have now edited the Introduction according to the reviewers' suggestion and added additional information about 5-HT cited with references**

And they are two independent neurotransmitter, what' s the relationship of them?

**Answer: In the current study, we made a preliminary investigation of the relationship between IBS and 5-HT or SP, respectively. We did not specifically investigate the relationship between 5-HT and SP.**

Why did the authors investigate these two indexes?

**Answer:**

**5-HT**

**In the formation of visceral high sensitivity, peripheral nerve biology plays an important role. The changes of the gastrointestinal neurotransmitter expression such as intestinal mucosa serotonin (5 - hydroxytryptamine, 5 - HT) and somatostatin (SS), vasoactive intestinal peptide (VIP) in IBS patients lead to intestinal immune cells releasing allergic medium [i], which acts on the nerve terminals to cause sensitization, leading to enhanced visceral sensitivity to pain.**

Some study shows 5 - HT as ghrelin plays an important role in the process and development of IBS visceral high sensitivity. Release, secretion, distribution and metabolism of 5HT in the digestive tract are complex processes of neurotransmitter regulation, which are not only influenced by the expansion, ischemia and stress factors, but also regulated by its receptor. A variety of 5 - HT receptors were distributed on the mucous membrane of the digestive tract. The receptor regulation of intestinal EC not only releases the 5 - HT, but also has the effect on regulating gastrointestinal function [ii].

[i] Irritable bowel syndrome and nerve - endocrine - immune network relations [J]. 中国中西医结合消化杂志, 2013, 21 (6): 106-109.

[ii] The effect of ShuGanJianPi prescription on the serotonin-related colonic mucosa epithelial secretion in diarrhea type IBS rats. [J]. 中华中医药杂志, 2012, 27 (12): 3092

## SP

Previous study revealed [i] that substance P (Substance P, SP) content on intestinal mucosa in IBS patient was increased. SP in different parts of mast cells (MC) was found in the cytoplasm, and confirmed that SP receptors exist in MC surface [ii], [iii]. Intestinal mucosal MC is activated to release the medium whose effect on the enteric nervous system and on adjacent endocrine cells of nerve fibers to release the neuropeptide, such as SP. The SP combined with surface receptors of the MC further induces MCs degranulation, resulting in pathophysiological pain, stimulating and accelerating gastrointestinal peristalsis. Therefore, SP showed the roles of neurotransmitters and modulation factor.

[i] Palsson OS, Mo rteau O, Bozymski EM, et al. Elevated vaso active intestinal peptide concentrations in patients with irritable bowel syndrome [J]. Dig Dis Sci, 2004, 49 (7-8) : 1236-1243.

[ii] Marchand JE, Sant GR, Kream RM. The Increased expression of substance P receptor - encoding mRNA in bladder biopsies from patients with interstitial cystitis. Br J Urol 1998;81:224-228

[iii] Pothoulakis C, Castagliuolo I, Leeman SE, Wang, CC, Li H, Hoffman, BJ, Mezey e. Substance P receptor expression in intestinal epithelium in clostridium difficile toxin A enteritis in rats. Am J Physiol 1998;275: G68 - G75

This is a very interesting study that deserves to be published.

However, minor English language edits and edits for content are required to make the paper more easy to follow. Specific suggestions are:

1 – The abbreviations 5-HT and SP should be spelled out at their first occurrence in the paper, i.e. the abstract.

**The full names of 5-HT, 5-hydroxytryptamine and SP, substance P have now been added to the abstract as suggested by the reviewer.**

2 – The conclusion “and that these hormones/neurotransmitters are involved in the pathogenesis of IBS” is not warranted (since the authors only examine a model of IBS); this part of the last sentence in the abstract should be taken out.

**At the request of the reviewer, we have now removed the sentence that states that 5-HT and SP are involved in the pathogenesis of IBS. We now wrote:**

**TXRF can modulate the activity of the enteric nervous system and alter 5-HT and SP activities, which may contribute to the symptoms of IBS.**

3 – In the description of the IBS model, it is unclear to me why maternal separation is an essential part of the model. The way I understand the papers the authors use visceral hypersensitivity as a model for IBS. What does maternal separation have to do with IBS, either in rats or in humans?

**Answer: Regarding neonatal stress and gastrointestinal disorders, we refer to the following references.**

1) Coutinho SV<sup>1</sup>, Plotsky PM, Sablad M, Miller JC, Zhou H, Bayati AI, McRoberts JA, Mayer EA. Neonatal maternal separation alters stress-induced responses to viscerosomatic nociceptive stimuli in rat. *Am J Physiol Gastrointest Liver Physiol.* 2002 Feb;282(2):G307-16.

2) Mayer EA, Naliboff BD, Chang L, Coutinho SV. V. Stress and irritable bowel syndrome. *Am J Physiol Gastrointest Liver Physiol.* 2001 Apr;280(4):G519-24.

3) Mayer EA1, Collins SM. Evolving pathophysiologic models of functional gastrointestinal disorders. *Gastroenterology*. 2002 Jun;122(7):2032-48.

4 – I could not understand the rationale for including a “positive” drug. This should be explained better. Please clarify the schedule on which the drugs were given. Daily?

**Answer: The studied drug (TXYF) is a Chinese herbal compound, the positive control is a western drug, pinaverium bromide. Pinaverium bromide has exactly the same therapeutic effect on IBS. With this kind of comparison, we can understand the effect of Chinese herbs on IBS.**

- Please see Fig. 1 (flow chart of study design). It gives the protocol of the experimental method in a simpler form as requested by the reviewer. Briefly, IBS modeling from day 8 to 21; then, no treatment for all rats until they are 12 weeks-old. Daily administration of positive drug or TXYF for IBS+ positive group and IBS + TXYF group, respectively, continued for 2 weeks.

5 – It would be very helpful to include a flowchart of the experimental design. I had trouble following what the different sub-groups of rats were, and it is not always clear how many rats were used in each experiment.

**A ‘Flow chart’ has now been added (Fig. 1).**

1) This is an important issue. Irritable bowel syndrome is a common disease that is associated to negative events. The manuscript is very well written and the English seems correct.

**We are pleased that the reviewer thinks that the manuscript is very well written with correct English.**

However, some cautions that need to be reviewed.

I suggest avoid short name in title and abstract;

**We accept the reviewer's suggestion and have now removed all short names (abbreviations) from the title of the manuscript, which is now entitled:**

***“Tong Xie Yao Fang Relieves Irritable Bowel Syndrome in Rats by Mechanisms Involving Regulations of 5-hydroxytryptamine and substance P”***

in the last paragraph of introduction: delete the sentence: “A rat model of IBS was developed and produced visceral stimulants that were used as hypersensitivity positive controls” .

**Answer: In accordance with the reviewer's suggestion, we have deleted the following sentence from the Introduction:**

**“A rat model of IBS was developed and stimulants that produced visceral hypersensitivity were used as positive controls.”**

This topic must be detailed in section “methods” . The sentence “the mechanism of action on rat colon txyf motility and the regulation of the brain-gut axis were also analyzed” refers to purpose, I believe. I think you may leave it lighter.

**Answer: we changed the manuscript accordingly**

Still in the last paragraph, please delete the last sentence: “it is concluded that txyf is a useful therapy to alleviate the symptoms of IBS” .

**Answer: In agreement with the reviewer's wishes, we have deleted the following sentence: "It is concluded that TXYF is a useful therapy to alleviate the symptoms of IBS."**

In section methods: what is the total number of animals? What is the specimen? After the end of the procedures, what the destination of the animals?

**Answer: Please see the Flow Chart. At the end of the procedures, all rats were sacrificed and colon and hypothalamus tissues were collected.**

In the second paragraph of section "methods" there is a symbol like a square preceding the word "actin" what needs to be reedited here and along the text.

**Answer: The square symbol was due to a missing font and should have been a  $\beta$ . It has now been corrected throughout the manuscript to read  $\beta$ -actin.**

In conclusion, I suggest delete "and that these hormones/neurotransmitters are involved in the disease pathogenesis of ibs."

**Answer: At the request of the reviewer, we have now removed the sentence that states: ...and that these hormones/neurotransmitters are involved in the pathogenesis of IBS. We now wrote: 'Increased motility of colon smooth muscle in IBS model rats was significantly reduced by TXYF. These stimulatory actions were significantly regulated towards normal control levels after the application of TXYF. The results showed that TXYF can modulate the activity of the enteric nervous system by altering 5-HT and SP activity and that these hormones/neurotransmitters may contribute to the symptoms of IBS.'**