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Title: Restraint Stress Induces and Exacerbates Intestinal Inflammation in IL-10 Deficient Mice

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Dear Editor,

We appreciate the reviewers' comments and the opportunity to improve the manuscript.

The manuscript has been improved according to the suggestions of reviewers:

1) Format has been updated

2) Revision has been made according to the suggestions of the reviewer as the following

3) References and typesetting were corrected

The point-by-point response to each comment suggested by the reviewer is given on separate pages that I have enclosed. For the reviewers' convenience, the alterations were highlighted in yellow.

Reviewer #1 :

This study aim at investigating restraint stress effect on colitis in IL-10 deficient mice, which represent a spontaneous IBD animal model.

Major Comments;

Question 1-1) These IL-10 mice are also treated with piroxicam which is a chemical inducer of rapid colitis. The results obtained are similar to that published using chemically induced Colitis (TNBs or DSS in references 9 and 21. There is no new findings. At least 8 animals should be used for each treatment instead of the very few used since the model gives very variable data as indicated by the authors.

Reply

Thank you very much for your critical comments. Some studies have previously demonstrated that stress aggravates intestinal inflammation. However, we believe that our findings are novel for the following reasons. DSS-induced murine colitis exhibits ulcerative colitis-like features. However, IL-$10^{-/-}$ colitis is a representative model for Crohn's disease. TNBS-induced colitis, a Th1 immune-related model, has the advantages of identifying pro-inflammatory cytokines and inflammatory mediators as well as epithelial repair (Han et al., 2009; Pereira-Fantini et al., 2010). However, TNBS-induced colitis is limited because it is chemically-induced and represents self-limiting colitis (Koboziev et al., 2011). In addition, the epithelial insult induced by rectal administration of TNBS may be affected by the concentration or duration of application. The IL-10 deficient colitis model represents a conjunction of several key factors implied in the pathogenesis of IBD, including genetic predisposition and loss of immune tolerance to colonic commensal bacteria. Therefore, we believe that the IL-$10^{-/-}$ model is suitable for evaluating the effect of restraint stress in intestinal inflammation. We performed *in viv*o studies with or without piroxicam administration to address potential issues regarding the chemically induced colitis model. In our study, we attempted to minimize the number of animals used for ethical reasons. We used four mice in the restraint stress group showing statistical significance in the results.

Question 1-2) Feeding behaviour should be detailed

Reply

Thank you very much for your helpful comment. The mice had ad libitum access to water and standard rodent food. In the piroxicam-induced colitis model, we assessed the amount of piroxicam-containing chow daily. IL-$10^{-/-}$ mice consumed approximately 1.5~2 g of piroxicam-containing chow; similar amounts were consumed in both two groups regardless of stress exposure. We revised our manuscript as follows:

MATERIALS AND METHODS section page 7 line 7-8

Mice had ad libitum access to water and standard rodent food.

MATERIALS AND METHODS section page 8 line 1-3

IL-$10^{-/-}$ mice were consumed approximately 1.5~2 g of piroxicam-containing chow, which was similar in both two groups regardless of stress exposure.

Question 1-3) More inflammatory cytokines should be explored

Reply

Thank you very much for your thoughtful comment. We admit that determination of more inflammatory cytokines provide additional information to the present study. However, we did not perform experiments of various cytokine expressions. In this study, we conclude that restraint stress aggravates intestinal inflammation as proved by the effect of restraint stress using two different animal models. In addition, our results are consistent with those of previous studies that used chemically-induced models. Further studies are needed to examine various cytokine expressions and

the molecular mechanisms underlying stress-induced intestinal inflammation.

Minor Comments,

Q1-4) some english text should be revised for improvements

Thank you very much for your kind comment. The manuscript has been revised by an English-editing company

Reviewer #2:

The manuscript "Restraint Stress Induces and Exacerbates Intestinal Inflammation in IL-10 Deficient Mice" is interesting. The relationship between stress and intestinal disorders has been noted; however, the underlying mechanism between stress and the pathogenesis of intestinal inflammation has not been fully elucidated yet.

Comments:

Q2-1) The number of mice in the second experiment is not precised

Reply

We revised our manuscript as reviewer's suggestion.

MATERIALS AND METHODS section page 7 line 14-15, 21- 22

The first experiment compared the effect of restraint stress on the development of intestinal inflammation in wild-type (stress positive, n=4; stress negative, n=4) and IL-10-/- mice (stress positive, n=4; stress negative, n=10).

The IL-10-/- mice (stress positive, n=4; stress negative, n=6) were exposed to restraint stress for 2

hours per day for 3 consecutive days, and then treated with piroxicam for 4 days at a dose of 200 ppm administered in the rodent chow.

Q2-2) Exposure to restraint stress doesn't exhibited histological intestinal inflammation in distal colon. This result was not discussed.

Reply

Thank you very much for your critical comment. In the present study, restraint stress significantly aggravated the severity of colitis in the proximal colon. As known, IL-$10^{-/-}$ mice exhibits minor histological change in the distal colon, compared to that in the proximal colon. Although we could not confirm statistical significance, the trend toward aggravating severity of inflammation was shown for the distal colon.

We revised our manuscript as follows:

DISCUSSION section page 15 line 1-4.

Second, restraint stress significantly aggravated the severity of colitis in the proximal colon. As known, IL-$10^{-/-}$ mice exhibits minor histological change in the distal colon, compared to that in the proximal colon. Although we could not confirm statistical significance, the trend toward aggravating severity of inflammation was shown for the distal colon.

Q2-3) - * $P<0.05$ compared with stress negative controls: the stress negative controls are wild-type and/ or IL-10-/- mice. You must precise the control group.

Reply

Thank you very much for your kind comment. The stress negative controls are IL-10-/- mice. We revised our manuscript as stated above.

Reviewer #3:

Q3-1) To the authors, Importantly, the authors are not very clear about the number of animals used in the Methods or Results section (also in the Figure legends…). They are only stating that they tried to minimize the number of animals exposed to restraint stress. This could generate some questions about the statistics and data interpretation.

Reply

Thank you very much for your helpful comment. We revised our manuscript as reviewer's suggestion.

MATERIALS AND METHODS section page 7 line 14-15, 21- 22

The first experiment compared the effect of restraint stress on the development of intestinal inflammation in wild-type (stress positive, n=4; stress negative, n=4) and IL-10-/- mice (stress positive, n=4; stress negative, n=10).

The IL-10-/- mice (stress positive, n=4; stress negative, n=6) were exposed to restraint stress for 2 hours per day for 3 consecutive days, and then treated with piroxicam for 4 days at a dose of 200 ppm administered in the rodent chow.

Q3-2) Why the mice were killed 5 days after the restraint stress? Why not a day after (as in most of the behavioural tasks in the literature) or why not immediately after the stressor exposure?

Reply

Thank you very much for your thoughtful comment. In the present study, we investigated the effect of restraint stress on intestinal inflammation in a chronic colitis model. Therefore, we evaluated the severity of colitis using a clinical and histopathological index. For five days after the last exposure of restraint stress, we assessed daily body weight change, hematochezia, and stool consistency. We did not observe any differences in the clinical index between the groups with or without restraint stress, as described in the manuscript. However, histological grading showed that restraint stress significantly aggravated the overall score for colitis.

Q3-3) I think it should be interesting if the authors will mention in the Discussion section also the relevance of the oxidative stress in this matter, since there is a lot of research regarding oxidative stress status in IBD and also there are very well know connections between inflammation markers determined here and oxidative stress.

Reply

Thank you very much for your helpful comment. We added a paragraph regarding the suggestions in the discussion section as follows:

DISCUSSION section page 14, line 1-9

Although we have demonstrated that restraint stress aggravates intestinal inflammation, the molecular mechanism remains obscure. It is suggested that oxidative stress is one of the etiological factor of IBD. In several studies, patients with IBD demonstrated excessive reactive oxygen molecules in various specimens including colon tissues. More importantly, an experimental colitis study in rats showed that immobilization stress increased susceptibility to oxidative damage. Based on these results, oxidative stress seems to be an important factor in the molecular pathogenesis of stress-induced colitis. Further studies are needed to elucidate the molecular pathogenesis between stress and intestinal inflammation.

Q3-4) There is a recent paper by Mozaffari et al. in 2011 which used the restrained stress to generate an animal model of irritable bowel syndrome…How do you comment on that, especially since you very well noted in the paper that psychological stress have been noted to have increased rates of irritable bowel syndrome ? I think the discussions on stress and irritable bowel syndrome vs. stress and inflammatory bowel disease (IBD) should be treated with care by the authors…

Reply

Thank you very much for your thoughtful comment. We added a paragraph regarding the suggestions in the discussion section as below.

DISCUSSION section page 14, line 10-19

Irritable bowel syndrome (IBS) and IBD are distinct diseases; however, they have some important overlapping features such as genetic factor, impaired gut barrier function, and immune activation. Interestingly, stress can activate both IBS and IBD symptoms. However, it remains unclear whether stress overlaps in the pathogenesis of both diseases. Previously, *Mozaffari* et al. used restraint stress to create an animal model of IBS. In this study, five-day restraint stress induced rapid small bowel and colonic transit. Our data showed that restraint stress aggravates the severity of histopathology in IL-10-/- mice. Therefore, we believe that restraint stress animal models may be useful in investigating the common pathogenesis of IBS and IBD.

Thank you again for reviewing our manuscript in the *World Journal of Gastroenterology.*

Sincerely,

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