November 12, 2013

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 5206-review.doc).

Title: Sodium alginate ameliorates indomethacin-induced gastrointestinal mucosal injury via inhibiting translocation in rats

Author: Atsuki Yamamoto, Tomokazu Itoh, Reishi Nasu, Ryuichi Nishida

Name of Journal: *World Journal of Gastroenterology*

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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

Reviewer 1

This study is of some significance to Sodium alginate ameliorates indomethacin-induced gastrointestinal mucosal injury via inhibiting translocation in rats. Request according to the instructions for authors of WJG to polish the article. Old references are too much, suggest to join the latest references in 2013.

Answer: According to the reviewer's comment, we revised reference No. 1, 33.

Reviewer 2

Major concerns

1. The authors concluded that the protective effect of AL-Na on small intestine is via inhibition of bacterial translocation several times especially in abstract and last paragraph of Discussion. However, the only evidence of the authors given are the measurement of enterobacterial count. The conclusion is not convincing which may be caused by mucin protection.

Answer: Our studies showed that treatment of AL-Na enhanced mucin production in intestine. It is well-known that mucin works as a barrier against bacterial infusion (PLos One, 2012; 7, 6, e40087). So, we examined the enterobacterial counts. In the results, increasing of bacterial infusions in NSAIDs-treated rats were ameliorated by oral administration of AL-NA. From these, it is suggested that AL-Na protect NSAIDs-induced bacterial translocation via mucin production.

2. The authors looked the stomach and intestinal injuries caused by Indomethacin, but the observations only focused on small intestine. Actually, the injury mechanisms cased by NSAIDs in stomach and small intestine are different. The conclusion the authors got from the current study may not fit in the stomach. I would like have 2 suggestions: one is that this paper only talk about small bowel without mentioning stomach, the other way is do all the studies the authors did on the small bowel to compare if there are the same or not.

Answer: According to the reviewer's comment, we revised the DISCUSSION as follows.

3. The authors only showed graphic and histology of injuries, I would like to show the gross picture too to see the mucosal injuries.

Answer: Mucosal injury induced by NSAIDs was typical. So, we focused the injury in physiological data.

4. The authors use normal (Nor) indicate untreated animals and control (Cont) indicate animals treated with Indomethacin. This is very confusing. In conventional biomedical studies, "Control" indicates untreated or sham groups. It is better the authors change "control " for untreated rats .

Answer: According to the reviewer's comment, we revised the manuscript.

5. The authors are from Pharmaceutical lab, they should understand this kind of study should have enough control groups. For example, Solution used to dissolve indomethacin and Al-Na alone all should be included in the study as controls. If the authors have them already, please mention in the method part.

Answer: According to the reviewer's comment, we revised the MATERIAL AND METHODS as follows.
Page 4, line 7 to 8
Indomethacin-treated control animals were administrated distilled water at the same time.

6. Many spelling and grammar errors, please ask English native speaker who has biomedical ground to read this manuscript before submission.

Answer: The manuscript has been carefully reviewed by American Experts.

7. Method-induction of small intestine injury, are those animal fasted or not?

Answer: In small intestinal studies, the animals were not fasted. According to the reviewer's comment, we revised the MATERIAL AND METHODS.

8. All the studies in small bowel focused in ileum, have the authors compared the differences between duodenum, jejunum or ileum? Clinically, it is also seen injuries in duodenum caused by NSAIDs in patients.

Answer: In animal experience, injury induced by NSAIDs was mainly caused in ileum compared to jejunum. Therefore, we tested ileum tissue in our studies.

9. Figs 2C is a completely normal small bowel histology. I did not see any abnormality in it. The fig 1 showed only oxyntic gland mucosa without showing antral mucosa, any injury in antral mucosa? The Pas stains in Fig 6 also has similar problem, normal mucosa is abnormal and abnormal mucosa was explained normal. I wonder if the authors showed the pictures to somebody who knows pathology of small bowel or stomach?

Answer: As we mentioned in figure legends, Fig 2C showed normal tissue. According to the reviewer's comment, we revised the Results as follows.
As well as Fig 2, we revised the Results in Fig 1 and 6.

Page 7, line 21
Histological comparisons of treated and untreated tissues indicated that indomethacin caused an inflammatory reaction that was characterized by epithelial losses, ulcers; inflammatory infiltration into the lamina propria, submucosa, and serosa; and shortening of crypts (Fig. 2D) compared with indomethacin-untreated groups (Fig. 2C).
Page 7, line 9
Histological comparisons of treated and untreated tissues indicated that indomethacin caused exfoliation of gastric epithelial cells and disrupted the mucosal layer of the stomach (Fig. 1D) compared with

normal (Fig. 1C).
Page 9, line 21
Indomethacin treated animals had depleted goblet cell numbers compared with untreated animals (Fig. 6C) compared with indomethacin-untreated groups (Fig. 2B).

And then, mucosal injuries induced by NSAIDs were typical. So, we focused the physiological data in Fig.1.

10. Same as concerns #9, the authors called indomethacin reduced length of small intestine. Are you sure you are talking about "length" of small intestine, not villous height?

Answer: We tested intestinal length in the experiments. Treatment of indomethacin caused reduction intestinal length. And, Administration of AL-Na inhibited the decreasing of length.

11. Page 9, regarding body weight, anemia, etc. I assume the authors mean that in the small intestine injury group. Please make clear. Are they that fast to have these changes in acute use of indomethacin?

Answer: Double treatment of indomethacin caused body weight loss and decreasing of hemoglobin levels next day. In the stomach ulcer experiments in 4 h after single treatment of indomethacin, there are no changes in blood cells. From these, it is suggested that repeat times and period are important.

12. As to the mucin depletion stained by PAS, I would suggest do one more IHC stain by using MUC2 antibody. It would be more convincing that PAS stain alone and mucin measurement.

Answer: We think immunohistochemistry is very useful to reveal mucus involvement. But, it is too expensive.

Minor concerns

1. PCNA IHC stain in the method is not complete.

Answer: According to the reviewer's comment, we revised the MATERIAL AND METHODS as follows.

Page 6, line 9
Sections were incubated overnight at 4℃ with monoclonal mouse anti-proliferating cell nuclear antigen (PCNA; Dako, Denmark). After washing with PBS, slides were incubated for 30 min with biotinylated horse anti-mouse serum (Vector, Burlingame, USA) followed by avidinconjugated horseradish peroxidase (Vector, Burlingame, USA). The enzyme activity was detected using DAB (3,3'-diaminobenzidine).

2. Many abbreviations are not given full name for their first time use, especially in the abstract.

Answer: According to the reviewer's comment, we revised the Abstract.

3. Title is not appropriate.

Answer: As answer #1, we suggested that AL-Na protect NSAIDs-induced bacterial translocation via mucin production. So, we think our title is appropriate.

4. Titles and subtitles should be bold.

Answer: According to the reviewer's comment, we revised the manuscript.

Reviewer 3

Authors used rats indomethacin-induced gastrointestinal mucosal injury model to show the effects of drugs including sodium alginate. This manuscript has interesting data, which is new findings. However, I have some comments and question to the authors.
major comments:

1)The title of this manuscript "Sodium alginate ameliorates indomethacin-induced gastrointestinal mucosal injury via inhibiting translocation in rats." may be inappropriate. Because authors have not a direct evidence of translocation(may be bacterial translocation?). Moreover, neither ref#7 nor 36 did not show the evidence of BT.

Answer: we suggested that AL-Na protect NSAIDs-induced bacterial translocation via mucin production. So, we think our title is appropriate. According to the reviewer's comment, we revised the references.

2)Authors showed both the stomach and small intestine data. I do not understand completely why authors included the stomach data.

Answer: Clinically, it is important that NSAIDs caused both stomach and small intestine. Our studies showed that treatment of AL-Na protects indomethacin –induced gastric and small intestinal injury. Therefore, we revealed that AL-Na is very useful medicine on NSAIDs-induced side effects.

3)In Introduction, authors commented PPI but not rebamipide. However, throughout this paper, authors compared the effects of sodium alginate and rebamipide. Why did not authors introduce rebamipide in Introduction?

Answer: According to the reviewer's comment, we revised the INTRODUCTION as follows

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Page 2, line 14 to 16
It was reported that rebamipide, one of mucosal protective agent, suppressed NSAIDs-induced intestinal injury [15, 16]. But, the development of more therapeutic agent is demanded.

4)Do authors have any references regarding this indomethacin-induced mucosal injury model? In addition, why did authors chose the timing of drug administration (ie.30min and 6h)?

Answer: We use the Takeuchi's methods. So, we added the reference No.27.

5)Results
(Fig1) Mucosal layer is clearly shown in Fig1F. But I do not see the layer in Fig1G. In addition, the layer in Fig1F seems to be thicker than that in Fig1C (control). What is authors interpretation?

Answer: Mucosal injury induced by NSAIDs was typical. So, we focused the injury in physiological data. There may be a slightly difference between Fig.1C and F. But, the pictures were representative examples of those groups.

(Fig2) I do not see the correlation between Fig2A and Fig 2C,2F,2G. The mucosal damage in Fig2E seems to be worst among the groups.

Answer: The picture using in Figure 2 are representative examples. Fig 2A showed the injury areas which calculated using Image J software. Therefore, there may be slightly different.

(Fig4A) Authors evaluated the length of intestine. I would measure the height of intestinal wall. Why did authors evaluate the length to analyze the effects of drugs on indomethacin-induced atrophy?

Answer: It was reported that intestinal length was useful as intestinal injury by NSAIDs. (Imaoka H et al., Am J

Physiol Gastointest Liver Physiol 299: G311-G319, 2010. Tanahashi S et al., European J Phramcol. 714: 125-131, 2013.) Therefore, we measured intestinal length in this study.

Authors described that rebamipide had no effect on PCNA staining and AL-Na had strong effects on PCNA staining. But I do not see the difference of PCNA staining between Fig4D and 4F. On the other hand, PCNA staining of Fig4E is impressive.

Answer: According to the reviewer's comment, we revised the RESULTS as follow.

Page 8, line 22 to 24
Oral administration of AL-Na at 250 or 500 mg/kg significantly preserved the erythrocyte numbers, hemoglobin levels, and hematocrit. In contrast, rebamipide at 100 mg/kg had no significant effect on these parameters.

(Fig6) A PAS staining of Fig6A seems to be not so strong, which result is dissociated from that of Fig6A. The different way of sectioning might affect this dissociation.

Answer: Figure 6A showed MUC2 protein levels of intestinal tissues. It is well known that MUC2 is the major mucin produced by goblet cells of intestinal mucosa (J Biomed Biotechnol. 2010; 2010: 305879.). PAS-stained methods are useful for observation of polysaccharide. In the intestine, polysaccharide which stained by PAS are mainly goblet cells. Therefore, we showed figure 2.

minor comments:
The "hematocrit" is wrong in spelling in Fig3C.

Answer: According to the reviewer's comment, we revised Figure 3.

Reviewer 4
1) Authors indicate the phenomenon of physical protective mechanism of AL-Na to the mucosal surface. Is there some molecular biological mechanism of AL-Na to the mucosa?

Answer: Our studies revealed that AL-Na treatment enhanced mucin production and protect NSAIDs-induced injury. Therefore, we think there is some molecular mechanism for mucin production. But, it is unclear yet.

2) In Figure legends of Fig.1, (B) is not explained this MPO activity graph. Moreover, the explanation of (G) in Fig.1 dose not find in Figure legends. Author should correct the Figure legends of Fig.1.

Answer: According to the reviewer's comment, we revised Figure legend.

3) In Results of Fig.1, Fig.1C is seen like severe gastric injury. Fig.1G is seen like normal mucosa. Author should check the order of the Figures.

Answer: In Fig.1C, the animal was not treated indomethacin. This stomach seems to be approximately normal. In addition, we used a tissue of stomach of rebamipide treated animal.

4) In Fig.4C, PCNA positive crypt is not clear. Author should indicate these positive by arrows.

Answer: According to the reviewer's comment, we revised Figure 4.

3 References and typesetting were corrected

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

Sincerely yours,

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