World Journal of Gastroenterology Manuscript Revision-Manuscript NO: 73068

Dear Lian-Sheng Ma (Editor-in-chief),

We are grateful to the Reviewers for the helpful comments on the original version of our manuscript. We have taken all these comments into account and submit, herewith, a revised version of our paper. Please, find below our responses to all the points raised by the reviewers. Small corrections and new texts are highlighted using the "Track Changes" function in Microsoft Word, in the revised manuscript. We hope that the revised version of our paper is now suitable for publication in **World Journal of Gastroenterology**, and we are looking forward to hearing from you at your earliest convenience.

Yours sincerely,

Gerly A. C. Brito

Full Professor of Human Embriology and Histology

Federal University of Ceara –School of Medicine - Phone: +55 (85) 33668571

E-mail: gerlybrito@gmail.com

Reviewer #1:

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Major revision

Specific Comments to Authors: It has demonstrated that lack of a functioning P2X7 receptor leads to increased susceptibility to toxoplasmic Ileitis (PLoS One. 2015 Jun 8;10(6):e0129048.), which supports the thesis that P2X7R, a well-documented activator of pro-inflammatory cytokine production, also plays an important role in the regulation of intestinal inflammation. In this paper, authors found that TcdA induced the upregulation of the P2X7 receptor, which promoted enteric neuron loss, S100B synthesis, tissue damage, inflammation and cell death in the ileum of mice, these results are interesting and some innovations. However, there are some concerns as follows:

1. P2X7R has been found to mediate mast cell-dependent intestinal inflammation and inflammation-induced death of enteric neurons via an inflammasome-dependent pathway. P2X7R is also well known as an activator of the inflammasome, a complex of cytosolic proteins that regulates caspase-1 activation and the processing of IL-1β and IL-18 from inactive to active forms. In the present study, caspase-1 activation and IL-18 levels should be examined.

Answer: Thanks for your suggestion. The role of inflammasome in *C. difficile* infection has been demonstrated before [1, 2]. Thus, in our study, we evaluated the IL-1β, one of the final products of this pathway.

In addition, the severity of the ileum damage should be further determined by analysis ileum permeability, not only by histological analysis

Answer: The ileum loop is a model to study the directly interaction between the host and virulence factors released by the pathogen. We did not measure permeability because in this model, the ileum is blocked with double ligature.

2. In the METHODS of abstract, the sentence in Line 57 'To investigate the role of P2X7, BBG (50 mg/kg, i.p.) and A438079 (0.7 μg/mice, i.p.) were injected one hour prior to a TcdA-challenge.' should be revised as 'To investigate the role of P2X7 receptor, Brilliant Blue G (BBG, 50 mg/kg, i.p.), a nonspecific P2X7 receptor antagonist or A438079 (0.7 μg/mice, i.p.), a competitive P2X7 receptor antagonist, were injected one hour prior to a TcdA-challenge.'

Answer: The sentence was revised as suggested.

3. In introduction, P2X7R (Line 129) should be described before the existing problems in this area (Line 125, However, a knowledge gap remains regarding the population of enteric neurons affected by TcdA and the role of the P2X7 receptor in TcdA-induced alterations in enteric neurons and enteric glial cell (EGC)-derived mediators, particularly S100B.) were put forward.

Answer: P2X7R was described previously to the motivation of the study as recommended.

4. In the Methods and Results, levels of IL-1β, IL-6, IL-8 and TNF-alpha should be expressed as pg/mg of tissue, not pg/mL.

Answer: The results were revised.

In the methods, levels of IL-1β, IL-6, IL-8 and TNF-alpha were detected by enzyme-linked immunosorbent assay. However, in the results, levels of IL-1β, IL-6, KC and TNF-alpha were present, the description should be consistent.

Answer: Mouse KC is a human IL-8 analog. We replaced KC for IL-8 in the description.

5. In Statistical analysis, the histological score should be tested by nonparametric test.

Answer: Nonparametric test was used to compare histological scores. We added in statistical analysis and figure legends.

6. In the results, the effects of P2X7 receptor antagonists on nNOS+, Calr+ and ChaT+ neurons should be described in detail.

Answer: The aim of this study was to characterize the population of myenteric neurons affected by TcdA and to investigate the role of the P2X7 receptor in ileal damage, inflammation, enteric glial and neuronal changes. Thus, we evaluated the effect of P2X7 receptor antagonist in prevent the loss of non-specific neuron using a pan-neuronal marker, HuC/D.

7. In discussion, the present study demonstrated that P2X7 receptor blockage decreases inflammation, apoptosis and enteric neuron loss during Clostridioides difficile Toxin A-induced ileitis in mice. However, it has demonstrated that lack of a functioning P2X7 receptor leads to increased susceptibility to toxoplasmic Ileitis. The difference should be discussed.

Answer: We added the difference between our study and this previous investigation in discussion.

Reviewer #2:

Scientific Quality: Grade C (Good)

Language Quality: Grade B (Minor language polishing)

Conclusion: Major revision

Specific Comments to Authors:

1【line 154 : Swiss mice (n=5 per group)】【line 591: or PBS alone (Control) (n=4).】【line 593: in 5-6 microscope fields per sample (n=4 subjects per group).】【line 601: challenged mice (n=4 subjects per group).】【line 612: in the ileum myenteric plexus from control and TcdA-challenged mice (n=4).】【line 659: challenged (TcdA, TcdA+A438079) mice (n=4 subjects per】 Why had some experimental groups 5 nude mice? And the other groups had 4 nude mice? The number of animals is different. Can they be compared?

Answer: To better reproduce our data we performed different experiments. For some experiments such as immunostaining analysis we had 4 nude mice in each group. According to the statistical analysis they could be compared.

2【line 591-593 : Quantification of the percentage (mean ± s.e.m.) of the P2X7-immunopositive area in ileum from control and TcdA-challenged mice in 5-6 microscope fields per sample (n=4 subjects per group).】There are only 4 nude mice. Is there sampling error in the test data? Does the test data have normal distribution? Can it be used as mean ± standard deviation to describe?

Answer: The percentage of immunopositive area has been largely used to examine the expression of a tissue protein [3-5] using normal distribution. The analysis of multiple fields in 4 mice is enough to have a confinable result as confirmed by qPCR.

Reviewer #3:

Scientific Quality: Grade D (Fair)

Language Quality: Grade A (Priority publishing)

Conclusion: Major revision

Specific Comments to Authors: The study aimed to characterize a specific population of TcdA-affected myenteric neurons and investigate the role of P2X7 in TcdA-induced ileum inflammation, cell death, and enteric nervous system (ENS) changes in mice. And the authors conclude that TcdA induces the upregulation of the P2X7 receptor, which promotes enteric neuron loss, S100B synthesis, tissue damage, inflammation and cell death in the ileum of mice. These findings contribute to future directions in understanding the mechanism involved in intestinal dysfunction reported in patients with pos-CDI. It was an interesting and a good-writing paper. However, I have some comments.

1. The necessity and hypothesis of this study should be mentioned in the Introduction.

Answer: We added the hypothesis of our study in the introduction.

2. The grouping was not clear.

Answer: We added one paragraph in methodology with the description of each group.

3. The magnifications and the conditions of observers in every histological analysis should be described appropriately.

Answer: We added the magnifications and the conditions of observers in every histological analysis.

4. Primers of β-actin should be presented in real-time PCR section.

Answer: We used GAPDH. We added the primers.

5. The Discussion should be carried out around the data obtained from the study. I noticed that some mechanisms were not clearly elaborated. The authors should reorganize some contents and place their emphases on the mechanisms.

Answer: The discussion was partially changed. Each paragraph emphasizes the data from the study, comparing to the literature and the significance for understanding of mechanism of disease.

6. The limitations and perspective of this present study should be involved in the Discussion. The clinical significances of this study should be mentioned in the Discussion.

Answer: We added in discussion the limitations and perspective, as well as the clinical significance of our study.

7. The images in Figures 1, 2, 3 and 5 were not clear, and the identifying arrow heads were not given explanations in Figure legends.

Answer: We improved the quality of the figures 1, 2, 3 and 5. We also added the identifications for the arrow heads in figure legends.

8. The documents of "73068-Biostatistics Review Certificate" and "73068-Institutional Review Board Approval Form or Document" were not applicable.

Answer: Thank you for your observation.

6 EDITORIAL OFFICE'S COMMENTS

Authors must revise the manuscript according to the Editorial Office's comments and suggestions, which are listed below:

(1) Science editor:

The manuscript studied the role of P2X7 in TCDA induced ileal inflammation, cell death and changes of intestinal nervous system (ENS) in mice. This is an interesting and good paper. However, I have some comments. 1. This study is not particularly innovative and not very in-depth. 2. The limitations of this study should be included in the discussion. 3. The picture quality is not good. Please check carefully and give high-quality pictures.
Language Quality: Grade B (Minor language polishing)
Scientific Quality: Grade C (Good)

Answer: We included the limitations of our study in the discussion section. We also improved the quality of the pictures.

(2) Company editor-in-chief:

I have reviewed the Peer-Review Report, the full text of the manuscript, and the relevant ethics documents, all of which have met the basic publishing requirements

of the World Journal of Gastroenterology, and the manuscript is conditionally accepted. I have sent the manuscript to the author(s) for its revision according to the Peer-Review Report, Editorial Office's comments and the Criteria for Manuscript Revision by Authors. Before final acceptance, uniform presentation should be used for figures showing the same or similar contents; for example, "Figure 1Pathological changes of atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...". Please provide decomposable Figures (in which all components are movable and editable), organize them into a single PowerPoint file. Please authors are required to provide standard three-line tables, that is, only the top line, bottom line, and column line are displayed, while other table lines are hidden. The contents of each cell in the table should conform to the editing specifications, and the lines of each row or column of the table should be aligned. Do not use carriage returns or spaces to replace lines or vertical lines and do not segment cell content. In order to respect and protect the author's intellectual property rights and prevent others from misappropriating figures without the author's authorization or abusing figures without indicating the source, we will indicate the author's copyright for figures originally generated by the author, and if the author has used a figure published elsewhere or that is copyrighted, the author needs to be authorized by the previous publisher or the copyright holder and/or indicate the reference source and copyrights. Please check and confirm whether the figures are original (i.e. generated de novo by the author(s) for this paper). If the picture is 'original', the author needs to add the following copyright information to the bottom right-hand side of the picture in PowerPoint (PPT): Copyright ©The Author(s) 2022.

Answer: We provided in **Image file** the pictures in PowerPoint (PPT) and added Copyright ©The Author(s) 2022 to the bottom right-hand side of the images.

References

Costa, D.V.S., Moura-Neto, V., Bolick, D.T., Guerrant, R.L., Fawad, J.A., Shin, J.H., Medeiros, P., Ledwaba, S.E., Kolling, G.L., Martins, C.S., et al. (2021). S100B Inhibition Attenuates Intestinal Damage and Diarrhea Severity During Clostridioides difficile Infection by Modulating Inflammatory Response. Front Cell Infect Microbiol *11*, 739874. 10.3389/fcimb.2021.739874.

Cowardin, C.A., Kuehne, S.A., Buonomo, E.L., Marie, C.S., Minton, N.P., and Petri, W.A., Jr. (2015). Inflammasome activation contributes to interleukin-23 production in response to Clostridium difficile. mBio *6*. 10.1128/mBio.02386-14.

Martins, C.S., Costa, D.V.S., Lima, B.B., Leitao, R.F.C., Freire, G.E., Silva, G.F.M., Pacifico, D.M., Abreu, J.G., and Brito, G.A.C. (2020). Clostridioides difficile Toxin A-Induced Wnt/beta-Catenin Pathway Inhibition Is Mediated by Rac1 Glucosylation. Front Microbiol *11*, 1998. 10.3389/fmicb.2020.01998.

Ng, J., Hirota, S.A., Gross, O., Li, Y., Ulke-Lemee, A., Potentier, M.S., Schenck, L.P., Vilaysane, A., Seamone, M.E., Feng, H., et al. (2010). Clostridium difficile toxin-induced inflammation and

208 intestinal injury are mediated by the inflammasome. Gastroenterology *139*, 542-552, 552 e541-543.
209 10.1053/j.gastro.2010.04.005.
210
211 Souza, R.F., Evangelinellis, M.M., Mendes, C.E., Righetti, M., Lourenco, M.C.S., and Castelucci, P.
212 (2020). P2X7 receptor antagonist recovers ileum myenteric neurons after experimental ulcerative
213 colitis. World J Gastrointest Pathophysiol *11*, 84-103. 10.4291/wjgp.v11.i4.84.
214

215