November 8, 2019

RE: Manuscript NO. 51410
Title: Irisin attenuates intestinal injury, oxidative and ER stress in mice with L-arginine-induced acute pancreatitis

Dear Dr. Jin-Zhou Tang:

Thank you very much for your email dated November 5, 2019 in which you informed us that our manuscript has been reviewed and invited us to revise and resubmit the manuscript for further consideration. We also thank you for including the critiques from the referee that were very useful for improving our manuscript. The comments, our point-by-point responses to them, and changes made in the manuscript (which are highlighted by red font) are listed in separate pages.

We sincerely hope that the extensive changes made in the revised manuscript meet with your approval as well as the approval of the referee and, therefore, our manuscript is now acceptable for publication in *World Journal of Gastroenterology.* If there are any further questions, please do not hesitate to contact us.

Sincerely yours,

Rongqian Wu, MD, PhD
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Responses to Reviewers' Comments

Reviewer #1:

The manuscript no 51410 is focused on the possible effects of irisin administration on intestinal injury in experimental model of acute pancreatitis. The MS is appropriately written and well-organized. The data is presented clearly. However, there are some critical comments related to the description of methodology. Also, I would like to recommend to revise the manuscript (at least the discussion) following comments pointed-out below. However, there are few major and minor points below:

Thank the reviewer for the positive comments. We will address the concerns below.

- please, provide the appropriate reference (in „Materials and Methods" section) to the experimental model implemented in this study

We apologize for missing this important information. Acute pancreatitis was induced in mice by 2 hourly intraperitoneal injections of 4.0 g/kg L-arginine (0.2 ml) as described by Dawra et al. (PMID: 17170029) and used by us recently (PMID: 31250660).

- please, provide the information about the total number of mice included in the study and within each experimental group

A total of 24 mice (n=6/group) were used in this study. This information has been provided in the revised manuscript.

- I would recommend to include details regarding „scoring criteria" to „Materials and Methods" section (histological evaluation)

The detailed description of the scoring criteria for histological evaluation was provided in the revised manuscript.

- please, provide the information about the ALL chemicals and drugs used in the study

The detailed information about the chemicals and drugs used in the study was provided in the revised manuscript.

- what was the post-hoc test used for statistical analysis?

The Student-Newman-Keuls test was used for the post-hoc analysis.

- what was the dilution of each antibody used in the study?

This information about the dilution of each antibody was provided in the revised manuscript.

- Few studies were published recently showing that the beneficial or harmful effect of exercise (possibly followed by the moderate or intensive increase in irisin release) within gastrointestinal tract also associated with ROS generation depends on its intensity (e.g. doi: 10.3390/nu11051127; doi: 10.1371/journal.pbio.2006159; doi: 10.26402/jpp.2018.1.13.; doi: 10.1093/ecco-jcc/jjy026). Therefore, it would be interesting to consider by Authors to test the effect of even higher dose of irisin in the experimental model included in this manuscript or at least to discuss the possible limitation of the study.

We thank the reviewer for suggesting these inflammatory bowel disease studies. The following information has been incorporated in the revised manuscript.

Physical exercise can favorably influence outcomes of patients with inflammatory bowel disease (PMID: 27255494, PMID: 31455308, PMID: 31427183, PMID: 31266461). Voluntary exercise increased irisin levels and attenuated experimental colitis in high fat diet-fed mice (PMID: 28425943). However, forced treadmill exercise has been shown to exaggerate the severity of experimental colitis in mice fed a high fat diet (PMID: 31117199). This discrepancy may be related to differences in irisin release after voluntary and forced excise. Unfortunately, irisin levels were not measured in the forced exercise study. In our current study, we only assessed the effect of two different doses of irisin on intestinal injury in AP. It is possible higher doses of irisin may offer even better protection in this model. In this regard, the optimal dose of irisin in AP should be determined in the future.

Reviewer: 2

The manuscript evaluated effect of irisin on intestinal mucosa of mice. The aim was to evaluate oxidative and endoplasmic reticulum stress. However, since oxidative status of an organism is significantly affected by numerous factors, and authors did not provide much information about it, there is serious doubt about the validity of the study and results. My major comments are as follows.

Thank the reviewer for reviewing our manuscript. We will address the concerns below.

Authors wrote: »ARRIVE guidelines statement: All of our experiments follow ARRIVE guidelines. « – From that statement it is clear that authors do not know what ARRIVE guidelines are. ARRIVE guidelines are instructions which information should be stated in the manuscript when experiment is performed on animals. Authors did not provide necessary information about animal experiment and therefore it is not possible to evaluate the quality and validity of the results.

We apologize for missing the necessary information regarding animal experiment. More details on animal experiment were provided in the revised manuscript as described below.

Authors wrote: »All experimental procedures were consistent with international guidelines for the care and use of laboratory animals…«. Which international guidelines? There are many guidelines but international?

We apologize for missing this important information. All experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources) and were approved by the Animal Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University.

Authors wrote: »all animals were housed for one week under standard conditions…« What are standard conditions?!!!! Standard conditions do not exist therefore ARRIVE guidelines were published to help authors what information they should provide when animals are used. I strongly suggest that authors read and follow ARRIVE guidelines and provide the necessary data in their manuscript.

Prior to the experiments, all animals were housed in Perspex cages, 5 mice per cage, at the animal facility of Xi'an Jiaotong University Health Science Center for one week under standard conditions (25 ± 2 °C, 12 h/12 h light/dark, 50 % humidity) to acclimate to the surroundings. The mice were housed. The mice were fed on a standard Purina mouse chow diet and allowed water (tap) *ad libitum*.

Important data about experimental design that should be stated in the manuscript are: - Authors stated that animal fasted for 12 hours before experiment – WHY?

Since we were investigating intestinal injury, fasting the mice for 12 hours before experiment is necessary for obtaining reproducible results.

were animals in metabolic cages i.e. without bedding, enrichment etc.

No.

- How many animals were in one cage (singly housed or in groups – how many animals per cage?), bedding material, diet (type and manufacturer), water (tap, autoclaved, sterilized, acidified…)

Prior to the experiments, all animals were housed in Perspex cages, 5 mice per cage, at the animal facility of Xi'an Jiaotong University Health Science Center for one week under standard conditions (25 ± 2 °C, 12 h/12 h light/dark, 50 % humidity) to acclimate to the surroundings. The mice were housed. The mice were fed on a standard Purina mouse chow diet and allowed water (tap) *ad libitum*.

- Microbiological state – health monitoring report – microbiological state can significantly affect results Housing conditions (temperature, humidity etc), light/dark period etc. All above mentioned factors (which are also stated in ARRIVE guidelines) are very important factors that significantly affect oxidative enzymes and consequently validity of the results.

Prior to the experiments, all animals were housed in Perspex cages, 5 mice per cage, at the animal facility of Xi'an Jiaotong University Health Science Center for one week under standard conditions (25 ± 2 °C, 12 h/12 h light/dark, 50 % humidity) to acclimate to the surroundings. The mice were housed. The mice were fed on a standard Purina mouse chow diet and allowed water (tap) *ad libitum*.

In addition, the protocol is very purly explained: - It is not clear why animals received »2 hourly intraperitoneal injections of L-arginine (4.0 g/kg L-arginine, A5006, Sigma-Aldrich, Shanghai, China).«

We apologize for missing this important information. Acute pancreatitis was induced in mice by 2 hourly intraperitoneal injections of 4.0 g/kg L-arginine (0.2 ml) as described by Dawra et al. (PMID: 17170029) and used by us recently. The mice received a total of **TWO** intraperitoneal injections of L-arginine, not 36 injections in 72 hours. Please note that intraperitoneal injection of L-arginine in mice is a commonly used model of acute pancreatitis (PMID: 25688985, PMID: 24365745). Three new references (ref. No. 11, 17, 18) have been provided in the revised manuscript.

Authors did not provide the volume injected and the reason why such protocol was used.

The volume injected was provided in the revised manuscript.

- It is not clear why only one application of irisin was used, and why the dose was used. Again, authors did not provide the injected volume.

The volume injected was provided in the revised manuscript. The doses of irisin used in this study were chosen on the basis of our previous experience in acute pancreatitis (PMID: 31250660) and ischemia reperfusion injury (PMID: 30388684).

- It is not clear why authors killed mice 69 hours after irisin treatment and why not 7 or 14 day or 30 day after (time that is recommended to get valid results).

According to the literature (PMID: 25688985), the peak of organ injury in this model happens at 72h after the first injection of L-arginine (i.e., 69 hours after irisin treatment in our current study). Please note that L-arginine injection in mice is a commonly used model of acute pancreatitis.

Histologic evaluation section: authors wrote: »Three sections were randomly selected for each group; two fields were randomly photographed for each section...«. 6 mice per group were used, suffered and then only 3 sections for each group were used? This is not ethical!!! Authors should macroscopically and histologically evaluate all animals used in experiment according to good laboratory standard procedures.

We thank the reviewer for the suggestion. Accordingly, all 6 sections from each group were evaluated. The data have been incorporated in the revised Figure 2.

Statistics section. Authors wrote: »One-way ANOVA was used to analyze the differences between groups ». Since the authors have 4 groups, MANOVA with post hock test should be used and because of small samples mean +/- SEM (and not standard deviation) should be used.

All measurement data are expressed as the means ± standard error of the mean (SEM). Since we were not assessing multiple dependent variables simultaneously in the current study, the differences between groups were compared by one-way ANOVA and Student-Newman-Keuls test.

There is serious doubt regarding the protocol and ethical justification of animal use. - Mice received intraperitoneal injection every 2 hours in 72 hours, which is 36 intraperitoneal injections in 3 days. Why they did not use minipumps instead 36 intraperitoneal injections in 3 days!!! This is very painful and stressful for the animals (mice need time to rest-day and time to activity-night – so if they were disturbed every two hours at night the mice did not have dark period, which affects the melatonin production and oxidative stress etc) and therefore the results are questionable, especially because the authors investigated oxidative stress – such protocol is not appropriate and significantly affects not only animal welfare but most importantly the results.

We apologize for causing this confusion. Acute pancreatitis was induced in mice by 2 hourly intraperitoneal injections of 4.0 g/kg L-arginine (0.2 ml) as described by Dawra et al. (PMID: 17170029) and used by us recently. The mice received a total of **TWO** intraperitoneal injections of L-arginine, not 36 injections in 72 hours. Please note that intraperitoneal injection of L-arginine in

mice is a commonly used model of acute pancreatitis (PMID: 25688985, PMID: 24365745). Three new references (ref. No. 11, 17, 18) have been provided in the revised manuscript.