Author’s response to reviews

Title: Zhenqing Recipe attenuates non-alcoholic fatty liver disease by regulating the SIK1/CRTC2 signaling in experimental diabetic rats

Authors:
Daofei Song (871993733@qq.com)
Lei Yin (m201776027@hust.edu.cn)
Chang Wang (448329145@qq.com)
XiuYing Wen (345144591@qq.com)

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Author’s response to reviews:

Dear editor:

We are grateful to you for giving us the opportunity to revise our manuscript. We also thank the reviewers for their constructive comments, which are all valuable and very helpful for revising and improving our manuscript. Accordingly, we have carefully revised the manuscript. The detailed point-by-point answers to the reviewers’ comments are listed below this letter. All changes made to the text are highlighted in red in the revised manuscript so that they can be easily identified.

We hope that, with these modifications and improvements based on the reviewer’s comments, the quality of our manuscript meets the publication standard of the journal. Once again, thank you very much for your attention to our manuscript. Looking forward to hearing from you soon.

With kindest regards,
XiuYing Wen

Point-by-point responses to the reviewers’ comments:
Editor:
Thank you for your helpful comments. We have read all these comments carefully and have made correction in the revised manuscript. Our point-by-point responses are listed below.

1. Please include the voucher numbers in the Methods section.
Response: We have added the voucher numbers in the Methods section of the revised manuscript (Page 5-6, Lines 116-118).

2. Please consider the list of authors as it currently stands with reference to our guidelines regarding qualification for authorship.
Response: We have further supplemented the contributions of YL and WC in this article (Page 24-25, Lines 535-537).

Reviewer 1:

Thank you very much for your insightful comments on our manuscript. These comments are all valuable and very helpful for revising and improving our manuscript. Please find below a detailed responses to your comments.

1. Lines 57-59: "Therefore, attenuating the complication of T2DM is equally important to decreasing blood glucose levels in long-term therapies." This statement is not logical as the incidence of a pathological change does not define the importance of therapy, but rather the severity of the changes.
Response: Thank you for your critical comments and we totally agree with your suggestion which might be of great help to improve the quality of our manuscript. Accordingly, we have added something about the serious harm of this disease in the corresponding sections of the revised version (Page 3, Lines 58-59).

2. Line 98: ZOR or ZQR?
Response: We are sorry for the mistake, which has been corrected in the revised version.

3. Lines 105-113: This information is insufficient and does not allow other scientists to repeat this study. The amount of each herb must be included and the compounds present in the extract should be identified. At least, a spectroscopic fingerprint of the extract used in this study must be included, rather than a reference to publication 26 from 2012.
Response: Thank you very much for your insightful suggestion. According to your suggestion, more details about the amount of each herb have been supplemented in the appropriate position (Page 5, Lines 109-110) of the revised manuscript. We totally agree with the point of reviewer that a spectroscopic fingerprint of the ZQR extract used in this study should be included. Because the herbal drugs of ZQR have been verified using fingerprinting techniques in our previous study [1] and the composition and dosage of ZQR used in this study have been the same as those in our previous study[1], a spectroscopic fingerprint of the ZQR extract has not been included in this study. However, we are afraid that we are not able to accomplish additional experiments due to the tight time schedule and limited research fund. We will take into account this work again in the future.


4. Line 140: You cannot approximate a range; please give the range as numbers, or, preferably, give the value as the mean +/- SEM or SD.
Response: Thank you for your professional suggestion. Accordingly, we have given the value as the mean +/- SD (Page 7, Lines 145).

5. Lines 149-151: The high fat diet (HFD) must be completely described so that the reader can assess the role of this diet.
Response: Thank you for your careful review and valuable suggestions. As suggested, more details about the high fat diet have been supplemented in the appropriate position (Page 7, Lines 155-157) of the revised manuscript.
6. Lines 150-151: STZ was injected after 4 weeks, but line 261 notes that "Injection of STZ (week 12)". Which time is correct? Was STZ given as a single dose, or multiple doses?
Response: Thank you for your careful review. We are sorry for unclear descriptions about STZ injection in rats. STZ was given as a single dose. "Injection of STZ (week 12)" means "12 weeks after STZ injection in rats ". Accordingly, we corrected the mistake in the revised version (Page 12, Lines 268).

7. Results: Please present figures 1 and 2 as a Table so that readers can compare changes in a single interventional group across multiple parameters.
Response: Thank you for your professional and constructive suggestion. Accordingly, we have presented figures 1 and 2, respectively, as a Table in our revised manuscript (Page 12, Lines 258-263; Page 13, Lines 283-289).

8. You have referred to the HFD+STZ treatment giving a model of type 2 diabetes (for example, line 383) yet body weight fell. Type 2 diabetics are usually overweight or obese. In contrast, type 1 diabetics, such as with STZ alone, show a decreased body weight as in your study. Please justify the description of your rat model as type 2 diabetes, rather than type 1 diabetes, using the physiological and biochemical data from your project. If the model really is one of type 1 diabetes, then your conclusions that ZQR may be a treatment for type 2 diabetes is an invalid extrapolation of the data.
Response: Thank you for your careful review and valuable suggestions. In the present study, we need to establish a model of diabetic rat, which has the characteristics of human type 2 diabetes mellitus and fatty liver disease. The rats fed with high-fat diet (HFD) alone develop obesity, hyperinsulinemia, and insulin resistance and not frank hyperglycemia or diabetes [1-2], while we need to develop a suitable type 2 diabetic rat model that would closely mimic the natural history of the disease events (from insulin resistance to beta cell dysfunction) as well as metabolic features of human type 2 diabetes and fatty liver. The materialization of the disease pattern can be achieved by combining the feeding of HFD which produces insulin resistance and low dose of STZ treatment that causes the initial beta cell dysfunction and subsequently the frank hyperglycemia in normal rats, which has been confirmed by previous studies [3-6]. The HFD/STZ-induced diabetic rats showed hyperglycaemia (blood glucose level fluctuation from 20.09 to 30.61 mmol/L), thus leading to classic diabetic symptoms such as weight loss, polyuria and polydipsia, which was consistent with our previous studies [3-4].


9. Lines 254-255: "The HFD/STZ-induced diabetic rats showed classic diabetic symptoms of polyuria, polydipsia and weight loss." This statement cannot be validated as food intake, water intake and urine volumes have not been reported.
Response: Thank you for your critical comments and we greatly agree with your suggestions which might be of great help to improve the quality of our manuscript. Although the cages used in our animal experiments could not accurately measure the urine volume, food intake and water intake of rats, we clearly observed that the water intake and food intake of diabetic rats were significantly higher than those of normal rats and the bedding in the cage of diabetic rats was obviously wet and had strong urine smell. In future animal experiments, we will try our best to accurately measure the water intake, food intake and urine volume of diabetic animals.

10. Line 256: "blood glucose level fluctuation from 20.09 to 30.61 mmol/L" - does this refer to individual rats, or to groups? Some groups, especially controls, had fasting blood glucose concentrations markedly lower than 20.09 mmol/L, so please clarify this comment.
Response: We greatly appreciate you for carefully reviewing the manuscript. We are sorry for the misunderstanding due to unclear descriptions in our manuscript. It describes the lowest and highest blood glucose levels of individual rats in the diabetic group. In order to avoid ambiguity, the bracketed content has been deleted in our revised manuscript.

11. Lines 257-260: What is the evidence that these rats died of hyperglycemia?
Response: Thank you for raising this important issue. In this study, those rats that died usually had higher fasting blood glucose levels than other rats and were significantly emaciated, and the bedding in the cage of those dead rats was obviously wet and had strong urine smell. Therefore, we speculated that the dead diabetic rats died of hyperglycemia.

12. Lines 300+: Please use Image J analysis to determine the percentage of area as lipid droplets and also islet and island size. These values can then be included in the new Table.
Response: Thank you very much for your insightful suggestion. According to your suggestion, We have used Image J software to calculate the area of lipid droplets and islet as well as island size in the picture and added the results in Fig 1 of the revised version. Fig 1 was also attached below for the Reviewer’s convenience.

13. Were plasma concentrations of inflammatory markers measured in this study? This would seem to be highly relevant to this study.
Response: Thank you for raising this important issue. In this study, we have not examined plasma concentrations of inflammatory markers. We are afraid that we are not able to accomplish additional experiments due to the tight time schedule and limited research fund. We will take into account this work in the future.

14. Which unique understandings were added to the project by the inclusion of the metformin-treated group? This intervention could have been used to show the importance of AMP kinase up-regulation, but this was not determined.
Response: Thank you for your professional and constructive comment. Metformin, an AMPK agonist and a widely used drug for the treatment of diabetes, also improves hepatic steatosis in both high glucose-stimulated primary hepatocytes and the liver of diabetic animals [1-4]. Furthermore, our previous report showed that metformin promoted SIK1 expression and activation in high glucose-treated HepG2 cells [5]. Therefore, metformin was used as a positive control drug in this study.


15. An additional figure describing possible mechanisms of the ZQR would be appreciated.
Response: Thank you for your professional suggestion, which we agree with. Accordingly, we have drawn an additional figure describing possible mechanisms of the ZQR, which has been represented in Figure 5. Figure 5 was also attached below for the Reviewer’s convenience.

Figure 5. Proposed mechanisms of the hypoglycemic and hypolipidemic effect of ZQR. Schematic representation of the role of ZQR in amelioration of T2DM with NAFLD. ZQR can contribute to preventing hyperglycemia and hepatic lipid accumulation through upregulating the expression of SIK1 and then suppressing gluconeogenesis and lipid storage with a decrease in the expressions of CRTC2, PEPCK and G6Pase in liver, thus reducing fasting blood glucose, serum TC, serum TG and hepatic steatosis. In pancreas, ZQR might exert its hypoglycaemic activities via the stimulation of insulin secretion from pancreatic β cells. ZQR, Zhenqing Recipe; SIK1, salt-induced kinase 1; CRTC2, CREB-regulated transcription co-activator 2; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase; TC, total cholesterol; TG, triglycerides.

Reviewer 2:
Thank you for the time and effort you have spent reviewing our paper, which have guided us to revise and reorganized this manuscript.

Reviewer 3:
Thank you for your thoughtful and helpful comments. We are pleased to note that you have pointed out some problems to help us improve the quality of our work. Motivated by your comments, we have tried
to fix all the problems you mentioned. Our itemized response to your questions, comments and suggestions is as follows:

1. English language needs editing.
Response: Thank you for your critical comments and we totally agree with your suggestion which might be of great help to improve the quality of our manuscript. Accordingly, we have sought American Journal Experts (AJE) for help with English usage, which may be verified on the AJE website using the verification code AB66-D27F-CA1D-044C-2239.

2. Please clarify your idea.
Response: We really appreciate the reviewer for pointing out this confusion. To make it clear, we have sought professional company to help us polish our article and have drawn an additional figure to given schematic representation of the role of ZQR in amelioration of T2DM with NAFLD.

3. The discussion needs to be in the point as well as connection of all parts to become suitable for publication in this journal.
Response: Thank you for your professional suggestion. Accordingly, the text in the manuscript has been revised.