Round 1

Dear Editor,

We truly appreciate you assigning such qualified reviewers to our manuscript. His/Her efforts and insights were a tremendous help to us during this revision. We would like to express our gratitude to you, the editorial team, and the reviewer whose valuable comments on this paper have significantly improved its quality. We have addressed all the comments in the new version of the paper and provided a point-by-point response to the reviewer's comments. These changes will not influence the content and framework of the paper. The reviewer comments are laid out below in italicized font and specific concerns have been numbered. Our responses are given in normal font and highlighted the revised/added contents with yellow color in the revised manuscript.

We hope that the changes we have made resolve all your concerns about the article. Once again, our thanks for your corrections and comments, and hope that you find the paper acceptable this time.

Reviewer 1:

No comments

Reviewer 2

Comment 1: *The study was superficial without a logical connection between in vivo and in vitro studies. Db/db mice are type 2 diabetes that should be hyperglycemia with hyperlipidemia, however, the in vitro study the authors used 30 mM glucose without any palmitate as most other in vitro studies used.*

Answer 1: Thank you for pointing out this deficiency. In our study, DCM was established in high glucose (HG)-treated cardiomyocytes and db/db mice to form in vitro and in vivo models. We consulted some literatures, the same models of db/db mice and cardiomyocytes treated with HG without palmitate have been used, such as: ① Sirtuin 3 Alleviates Diabetic Cardiomyopathy by Regulating TIGAR and Cardiomyocyte Metabolism. J

Am Heart Assoc 2021, 10: e018913. ② Metrnl ameliorates diabetic cardiomyopathy via inactivation of cGAS/STING signaling dependent on LKB1/AMPK/ULK1-mediated autophagy. J Adv Res 2023, 51: 161-179, etc. But we especially appreciate your suggestion, hyperglycemia and hyperlipidemia are more consistent with the characteristics of type 2 diabetes, only HG without any palmitate in vitro experiment do not exactly match the animal's. Cardiomyocytes should be further cultured with HG and palmitate. We have described these as a limitation in our discussion, please see page20.

Comment 2: *The authors stated "our study shows that beyond glycemic control, empagliflozin improved…..". However, the authors might not see their results in the Table 1 where EMP significantly reduced the FBG and HbA1c. How to eliminate the glycemic control role at the in vivo level? The authors might try to use in vitro to explain whether hyperglycemic control is responsible for the cardiac dysfunction protection, but the model does not match, therefore these studies are two separately two unlinked studies, which can not explain each other now.*

Answer 2: Thank you for your reminder. we established DCM models of db/db mice and treated mice with empagliflozin for 8 weeks, and found empagliflozin observably improved cardiac function together with a reduction of the FBG and HbA1c in diabetic mice in Table 1. In order to exclude the effects of metabolic improvement on hearts in vivo, a vitro experiment in HG conditions was performed. We especially appreciate your suggestion, hyperglycemia and hyperlipidemia are more consistent with the characteristics of type 2 diabetes, only HG without any palmitate in vitro experiment do not exactly match the animal's. Cardiomyocytes should be further cultured with HG and palmitate. We have described these as a limitation in our discussion, please see page20. The modifications and the newly added contents have been highlighted in yellow color in the revised manuscript.

Comment 3: *Results (page 13): The authors tried to state the cardiac apoptotic cell*

death is the key patho-mechanism responsible for cardiac dysfunction, and their improvement by EMP. However, the cardiac cell death was superficial, not quantitative evidence to indicate the mitochondrial apoptotic cell death exited in their mouse model. There was not solid evidence for the apoptotic cell death so far.

Answer 3: Thank you for your nice suggestion. We have added a histogram of the apoptosis rate assayed by TUNEL (Figure 2B). The apoptotic index was calculated as the percentage of TUNEL-positive cells, ten representative fields were evaluated for each group and the average value was calculated. It would be even more meaningful if apoptosis-related genes and proteins were detected in this in vivo study. However, apoptosis-related indicators, such as Bax and bcl-2 mRNA expression and caspase-3 protein levels, were measured in our in vitro study. A few studies have demonstrated that increased cardiac apoptosis has been considered a major risk factor for the development of DCM in both T1DM and T2DM models and T2DM patients, as supported by the evidence that biopsied heart tissues of patients with DCM show 85-fold more cardiomyocyte apoptosis than control nondiabetic hearts, such as references : ① Apoptosis in patients with dilated cardiomyopathy and diabetes: a feature of diabetic cardiomyopathy? Horm Metab Res 2007; 39:672e6. ② Diabetes-related cardiomyopathy: the sweet story of glucose overload from epidemiology to cellular pathways. Diabetes Metab 2019; 45:238e47.

Comment 4: *There was control group of EMP.*

Answer 4: Thank you for your valuable comment. It is true that we should set a positive drug control, which can make the results more accurate. We have described this point as a limitation in our Discussion. Next step, positive drug control will be established in vivo, the effects of empagliflozin on DCM will be further clarified, please see page 20. The modifications and the newly added contents have been highlighted in yellow color in the revised manuscript.

Comment 5: *In page 7: Animal models with n= 7 – 11, why all results showed n=3.*

Answer 5: We think this is a nice comment. In fact, the results of Western only showed n=3, we obtained consistent and statistically significant trends from the three samples in western blot. While, other results such as assay of fasting blood samples and echocardiography indicators showed n=7-11.

Comment 6: *Page 13: There was no description what kind of cardiac function change with these variables, diastolic and systolic function changes?*

Answer 6: Thanks for your nice comment. We have added the diastolic and systolic functions on page 13 in our manuscript. The modifications have been highlighted in yellow color in the revised manuscript.

Finally, we sincerely thank you for your valuable feedback so that we can use the feedback to improve the quality of our manuscript. Thank you again for your valuable time and effort in this study.

Reviewer 3:

Comment 1: *It is very important to change and modify the title. the title is not appropriate.*

Answer 1: Thank you for your valuable suggestion. According to your suggestion, we have changed the title of our manuscript: " Empagliflozin ameliorated diabetic cardiomyopathy via activating AMPK/PGC-1α and inhibiting the RhoA/ROCK pathway".

Comment 2: *Are the objectives and the rationale of the study clearly stated?.*

Answer 2: Thank you for your honest advice. We have modified the objectives and the rationale in the Abstract and Introduction of the manuscript. We highlighted the added contents with yellow color in the manuscript. Please see Page 2: "Diabetic cardiomyopathy (DCM) increases the risk of hospitalization for heart failure (HF) and mortality in patients with diabetes mellitus. However, no specific therapy to delay the progression of DCM has been identified. Mitochondrial dysfunction, oxidative stress,

inflammation and calcium handling imbalance play a crucial role in the pathological processes of DCM, ultimately leading to cardiomyocyte apoptosis and cardiac dysfunctions. Empagliflozin, a novel type of glucose-lowering agents, has been confirmed to reduce the risk of hospitalization for HF in diabetic patients. Nevertheless, the molecular mechanisms by which these agents provide cardioprotection remain unclear". We tried to explore the effects of empagliflozin on the development of DCM. The objective is to investigate whether empagliflozin can improve mitochondrial injury and cardiac dysfunction, prevented HG-induced oxidative stress and cardiomyocyte apoptosis, along with the underlying molecular mechanism.

Comment 3: *In the abstract, the research gap was not clearly stated. In addition, the authors need to rewrite the study objectives to be more academic writing.*

Answer 3: Thank you for your suggestion. We have emphasized the background and significance of our study in the Abstract. "Diabetic cardiomyopathy (DCM) increases the risk of hospitalization for heart failure (HF) and mortality in patients with diabetes mellitus. However, no specific therapy to delay the progression of DCM has been identified.. Empagliflozin, a novel type of glucose-lowering agents, has been confirmed to reduce the risk of hospitalization for HF in diabetic patients. Nevertheless, the molecular mechanisms by which these agents provide cardioprotection remain unclear. The purpose is to investigate the effects of empagliflozin on high glucose (HG)-induced oxidative stress and cardiomyocyte apoptosis and the underlying molecular mechanism". We have rewritten the study objectives to be more academic writing. We highlighted the added contents with yellow color in the manuscript (page 2).

Comment 4: *In the introduction, include the study's significance and novelty. What makes the study different from the rest and what does it add to the current knowledge?*

Answer 4: Thank you for your nice comment. "Clinical trials have

demonstrated that SGLT2 inhibitors substantially reduced the risk of hospitalization for HF in patients with DM [8-10]. In addition, the cardiac benefits of empagliflozin have been demonstrated in non-diabetic patients with HF and reduced ejection fraction (HFrEF). However, the mechanism by which these observed benefits are mediated remains unclear. Further experiments are required to validate the molecular mechanisms underlying the benefits of SGLT2 inhibitors on the hearts. Cardiomyocyte apoptosis is believed to be the initial factor contributing to HF in DCM. However, there is a scarcity of studies on SGLT2 inhibitors and cardiomyocyte apoptosis in DCM". We highlighted the added contents with yellow color in the Introduction of the manuscript, please see Page 5-6.

Comment 5: *In the introduction, the authors should have explained the purpose of this study and the existing gaps in this field and explained why this study was conducted.*

Answer 5: Thank you for pointing out this deficiency. We have explained the purpose and rationale of this study and the existing gaps in this field in the Introduction. Cardiomyocyte apoptosis is believed to be the initial factor contributing to HF in DCM. "However, there is a scarcity of studies on SGLT2 inhibitors and cardiomyocyte apoptosis in DCM. The aim of this study was to elucidate the molecular mechanisms underlying the protective effects of empagliflozin on cardiomyocytes". We highlighted the added contents with yellow color in the Introduction of the manuscript, please see Page 6-7.

Comment 6: *Are the methods clear and replicable? Do all the results presented to match the methods described?*

Answer 6: Thanks for your nice comment. Our laboratory technology is mature. At the cellular level, our study has a consistent trend with high reliability and good repeatability (n=3). We can provide the original graph with three consistent trends. We have provided detailed experimental

methods in the manuscript, which are clear and reproducible. All the results presented to match the methods described.

Comment 7: *If relevant are the results novel? Does the study provide an advance in the field? Is the data plausible?*

Answer 7: Thank you for your questions. "To date, there has been few studies on the effects of empagliflozin on HG-induced cardiomyocyte apoptosis and the underlying mechanism. Cardiomyocyte apoptosis is believed to be the initial factor contributing to HF in DCM. This study offers a novel molecular foundation for the anti-HF effects of empagliflozin.". We highlighted the added contents with yellow color in the Introduction of the manuscript, please see Page 20. Our data is credible since it has been validated at least three times and the trend is consistent.

Comment 8: *References are relevant, correct, and not recent. The number of references should be increased. please add some references. since this is a scientific review, all the sentences need to be supported with references. This study is very beautiful. I liked the sequence and enjoyed reading. Please add more references on similar studies*

Answer 8: Thank you for your valuable suggestions. According to your nice suggestions, we have read and cited some excellent papers to support our findings. Our references have been increased to 50. We highlighted the added contents with yellow color in the revised manuscript.

Comment 9: *There are a lot of grammatical errors. This must be taken care of and addressed. .*

Answer 9: Thank you for your careful checks. We have sent our revised manuscript to a professional English language editing company, and spelling and grammar issues have been revised. We will pay more attention to grammar in the future. We provide a language certificate along with the

manuscript.

Comment 10: *What are the limitations of the study? A description of limitations is missing at the end of the discussion section. • If your manuscript is related to mine, you can cite it (ORCID: https://orcid.org/0000-0002-5107-5550)*

Answer 10: According to your nice suggestions, we have added some limitations in the Discussion of our manuscript. We highlighted the added contents with yellow color in the revised manuscript. We have read your article about oxidative stress and learned a lot from your article. We have cited this excellent article as reference 4.

Once again thanks for your courtesy in examining our manuscript. Your comments have enriched our paper and helped to improve the quality of our paper.

E-mail address

With best regards,
Sincerely yours,
Hong Zhou

20-Oct-2023

Round 2

Reviewer 1: The authors were not well addressed the second reviewer's comments, instead of debating with the reviewer by providing a few published works to address these works used same models. In fact, different studies have different working focuses so that under certain conditions, their usage of the similar models might be acceptable. These facts do not mean that the authors can use what the previous works used to support the authors used a correct one. Therefore, these kinds of debates are not well addressing the second reviewer's concern. However, the authors have added the

limitation of this study to explain their usage of this not well fit model, which is an acceptable way. Therefore, this work can be accepted as long as the authors can change their title "Empagliflozin ameliorated diabetic cardiomyopathy via activating AMPK/PGC-1 α and inhibiting the RhoA/ROCK pathway" to "Empagliflozin ameliorated diabetic cardiomyopathy probably via activating AMPK/PGC-1α and inhibiting the RhoA/ROCK pathway" since their conclusion is too strong and was not support by the experimental evidence, but if it read like " ..... probably via ....." is acceptable.

Answer : we have changed our title "Empagliflozin ameliorated diabetic cardiomyopathy via activating AMPK/PGC-1 α and inhibiting the RhoA/ROCK pathway" to "Empagliflozin ameliorated diabetic cardiomyopathy probably via activating AMPK/PGC-1α and inhibiting the RhoA/ROCK pathway".

Thank you again for your valuable comments and suggestions. Your comments have enriched our paper and helped to improve the quality of our paper.

E-mail address

With best regards,

Sincerely yours,

Hong Zhou

28-Oct-2023