Author’s response to reviews

Title: Up-regulating autophagy by targeting the mTOR-4EBP1 pathway: a possible mechanism for improving cardiac function in mice with experimental dilated cardiomyopathy

Authors:

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Author’s response to reviews:

Dear Editor:

Thank you for your thoughtful review of our manuscript BCAR-D-19-00947R1 and for the opportunity to resubmit it. As recommended, we have used colored font to indicate changes in the revised manuscript. We hope it now meets your approval.

Our response to the comments is listed below:

Reviewer 1

The manuscript by Jin et al, focuses on the role of autophagy in DCM. As per the data presented, the authors have demonstrated that autophagy, potentially though mTOR-4EBP1 pathway, improves the cardiac function in mice. The manuscript is well written and sufficient number of animals have been used to support the hypothesis. Appropriate statistics have also been applied for analyzing the experimental data. I just have few minor comments:

Q1: When did you start the treatment with Rapamycin/3-MA, after day 0 of treatment with cardiac myocin or day 7? Please clarify in the methods.

Reply: Thank you. We have clarified this issue in our revised manuscript.
Eight weeks after immunization, rapamycin was administered intraperitoneally at a dose of 2 mg/kg/d for 2 weeks. The mice in 3-MA group received 3-MA at a dose of 15 mg/kg/d as previously described.

Q2. The word "respectively" on line 10 doesn't fit in the sentence.
Reply: The word has been deleted as recommended.

Q3. In the "Animal model of DCM and experimental design" section authors have mentioned that the animals were randomly divided in 4 groups (n=8), in results section however under "General characteristics" they have mentioned that total 24 animals were divided into three groups. Again, in results figure, there are four groups. Please be consistent with the data throughout the manuscript.

Reply: Thank you for your thoughtful review of our manuscript. We selected 8 normal mice as the control group, furthermore, 24 DCM mice were divided into three groups (n = 8 mice per group). Therefore, our experiment included four groups: control group (normal + PBS), DCM group (DCM + PBS), rapamycin group (DCM + rapamycin) and 3-MA group (DCM +3-MA). We revised the manuscript and clarified the issue according to the comments.

Q4. What do the colored lines in Figure 4 indicate? Please explain in figure legends.
Reply: Thank you. The red lines and blue lines indicated LVEDD and LVESD respectively. We have made modifications to improve clarity in figure legends.

Reviewer 2

In the present manuscript, Jin et al report on the role of autophagy regulation in experimental dilated cardiomyopathy. The authors conclude that activation of autophagy contributes to improved cardiac function in mice with dilated cardiomyopathy via mTOR-4EBP1 pathway. These findings are very important, in that they further reinforce the concept that autophagy may play a role in cardiac pathology and may serve as a promising therapeutic target. However, the manuscript has not been presented in high standards; I have enumerated my concerns in the below comments.
Reply: Thank you. The comments above are worthy of careful consideration. Although there are several limitations in the present study, we try our best to revise the manuscript. As recommended, we have used colored font to indicate changes in the revised manuscript.

Introduction

Q1. Background literature is abysmal. In the Introduction, the authors fail to make a convincing case for why the present study was conducted.

Reply: Thank you for your thoughtful review of our manuscript. As recommended, we revised the section and update some literature to clarify why the present study was conducted.

Q2. The background literature review on dilated cardiomyopathy (DCM) is very limited. I suggest an entire paragraph should be devoted to this. The definition should be revised for improvement. The phrase "...ventricular cavity expansion and decline in contraction function..." should be revised as "...left ventricular dilation/dilatation and decline in contractile function..."

Additionally, the authors state that DCM is associated with congestive heart failure. However, it would be noted that CHF is not the only or the most important sequelae of DCM. Life-threatening arrhythmias leading sudden cardiac death (the most common cause of death in this population) and supraventricular arrhythmias are very common, too.

Reply: Thank you for your thoughtful review of our manuscript. Dilated cardiomyopathy is one of the most common cardiomyopathy worldwide characterized by left ventricular dilation and decline in contraction function, which is the third leading cause of congestive heart failure. As recommended, we provided an entire paragraph to review on dilated cardiomyopathy and clarified the issue in our revised manuscript.

Q3. The link between autophagy and cardiovascular diseases needs to be well highlighted. The authors should provide a paragraph wherein they review this intriguing link and how their present study fits into the picture.

Reply: Thank you. The link between autophagy and cardiovascular diseases needs to be well highlighted. As recommended, we provided a paragraph to review it and revised the manuscript.

Q4. Last paragraph on study hypothesis and aims should be revised for improvement. Currently, it lacks focus.
Reply: Thank you. The comments above are worthy of careful consideration. As recommended, we have clarified this issue in our revised manuscript. Therefore, we pinpointed that up-regulating autophagy by targeting the mTOR-4EBP1 pathway as the possible molecular mechanisms in cardio-protection in DCM mice.

Methods

Q1. Under the model of DCM and experimental design section, the creation of DCM model has not been well described. Currently, it is unclear how the development of DCM was confirmed. This should be revised.

Reply: Thank you. The experimental model was established in BALB/c mice by immunization with porcine cardiac myosin to induce DCM. In the present study, we confirmed myosin-induced DCM model by histomorphological study and echocardiographic assessments. As recommended, we have clarified this issue in our revised manuscript.

Q2. The authors also claim that four groups were developed: control group, DCM group, rapamycin group, and 3-MA group, respectively. However, it is unclear whether rapamycin and 3-MA groups also had DCM. The authors should elaborate more these groups.

Reply: Thank you for your thoughtful review of our manuscript. We selected 8 normal mice as the control group, furthermore, 24 DCM mice were divided into three groups (n = 8). Therefore, our experiment included four groups: control group (normal + PBS), DCM group (DCM + PBS), rapamycin group (DCM + rapamycin) and 3-MA group (DCM + 3-MA). We clarified the issues in the revised manuscript according to the comments.

Q3. The anaesthesia has not been well described. The authors should elaborate on the type and dosage of anaesthetics used.

Reply: Thank you for your thoughtful review of our manuscript. We supplemented the information in the revised manuscript.

After intraperitoneal injection of sodium pentobarbital (75 mg/kg body weight), all mice were sacrificed by cervical dislocation while anesthetized.
Q4. For Histopathology, the authors state that they used five random fields of view for quantifying fibrosis. I wonder why only five views were, especially given that these may not be truly representative of the entire digital slide section. The authors should also provide the name and address of the supplier of "IMS Cell Image Analysis System" software used for fibrosis quantitation.

Reply: Thank you. In the present study, five random fields of view per mouse (n=8) were evaluated for CVF analysis across the left ventricular section. We clarified the issue in the revised manuscript according to the comments. Furthermore, the CVF was determined by quantitative morphometry of specimens with IMS Cell Image Analysis System (Shen Teng, Shanghai, China). We provided the name and address of the supplier of the software according to the comments.

Q5. Under the western blotting experiments, the authors should comment on how tissues were stored. The dilutions of all antibodies used should be provided, too. The National Institute of Health should be acknowledged for the use of Image J.

Reply: Thank you for your thoughtful review of our manuscript. We supplemented the information in the revised manuscript according to the comments.

Q6. The statistical software package used for the statistical analysis has not been described.

Reply: Thank you. GraphPad Prism version 6.0 software was used for data analysis in our present study. We revised the manuscript and clarified the statistical software according to the comments.

Results

Q1. The general characteristics of the groups should summarised in a table and cited in-text. This has not been done. The authors comment that no significant difference was noted in body weight; however, the animal weights have not been reported. The authors should improve these.

Reply: The comments above are worthy of careful consideration. We provided the general characteristics of the groups in Table 1 and cited in-text as recommended in the revised version.
Table 1 The general characteristics of the four experimental groups

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>DCM group</th>
<th>Rapamycin group</th>
<th>3-MA group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of death</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>19.87±2.40</td>
<td>19.72±2.22</td>
<td>19.69±2.16</td>
<td>19.59±2.27</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>0.22±0.02</td>
<td>0.21±0.01</td>
<td>0.22±0.02</td>
<td>0.23±0.03</td>
</tr>
<tr>
<td>HW/ BW (mg/g)</td>
<td>11.09±1.25</td>
<td>10.94±1.08</td>
<td>11.15±1.21</td>
<td>11.53±1.33</td>
</tr>
</tbody>
</table>

HW/ BW: Heart weight/ Body weight (mg/g); Each group, n = 8.

Q2. The morphological images are very pixelated and should be replaced with images of higher quality.

Reply: Thank you for your thoughtful review of our manuscript. We revised the morphological images and confirmed that the figures with at least 300 dpi resolution.

Citations

The use of excessive review articles is worrying. It is expected that the authors should consult the original research papers and draw their own conclusions, rather than basing this on the opinions of review authors.

Reply: The comments above are worthy of careful consideration. We replaced some review articles with original research papers in the reference section in our revised manuscript.


Minor comments

Q1. There should be a continuous line numbering throughout this manuscript to aid with the review.

Reply: Thank you. We provided the continuous line numbering throughout this manuscript as recommended in the revised version.

Q2. The address of the electron microscope should be provided. There should be a comma before respectively.

Reply: As recommended, we supplemented this information in the method section.

Q3. I suggest the authors describe the Echocardiographic assessments before histopathology, electron microscopy, and western blots experiments.

Reply: Thank you. The comments above are worthy of careful consideration. We describe the echocardiographic assessments before histopathology, electron microscopy, and western blot experiments in method section in the revised manuscript.

Q4. The scar bars on the figure materials in Figure 2A are not very clear. They can be improved by changing the font color to white. There should be some arrows indicating which substructure is autophagosome.
Reply: Thank you. The scar bars in Figure 2A are original marks by transmission electron microscopy. Therefore, it is difficult to change the font color to white. As recommended, we supplemented the arrows indicating autophagosomes in revised Figure 2A.

Q5. The blot bands should have corresponding molecular weights of the antibodies.
Reply: As recommended, we supplemented the corresponding molecular weights in the Figure 3A.

Q6. In the 1st paragraph of the Discussion, the phrase "cardio-protection effects" should be corrected as "cardio-protective effects".
Reply: The phrase has been revised as recommended.

Reviewer 3
The study by Jin et al investigates the role of autophagy regulation with the inhibitors and activators of mTOR/4EBP1 pathway in the development and progress of dilated cardiomyopathy in mouse model. Authors present interesting and potentially translatable findings that stimulation of autophagy with rapamycin has therapeutic value in a mouse model, both on the levels of attenuated morphological changes in myocardium and improved hemodynamics. While the study itself is adequately planned and executed, and the results seem reliable, there are several shortcomings that need to be addressed.

Reply: Thank you for your thoughtful review of our manuscript. As recommended, we have used colored font to indicate changes in the revised manuscript.

Q1. It is not clear from the Discussion, whether the induction of autophagy in myocardium of mice immunized with porcine cardiac myosin is the element of DCM pathogenesis or a part of the reparative processes induced to limit DCM progression.

Reply: Thank you for your thoughtful review of our manuscript. Our previous study indicated that autophagic activity was up-regulated in a rat model of early-stage dilated cardiomyopathy, which was a part of the reparative processes during DCM progression. In our present study, the rapamycin-induced autophagy activation successfully reversed myocardial fibrosis and improved cardiac function in DCM mice. In contrast, down-regulating autophagy inhibited the formation of autophagosomes in the 3-MA group, which induced severe myocardial fibrosis and decreased cardiac function. We clarified the issue in our revised manuscript.

Q2. DCM in humans is a disease of multifaceted etiology. To what extent does a mouse model reflect the pathogenesis of human disease?

Reply: Thank you. The comments above are worthy of careful consideration. Dilated cardiomyopathy is a disease of multifaceted etiology in humans. Myocarditis is regarded as a precursor of dilated cardiomyopathy, and 21% of patients with acute myocarditis ultimately developed into dilated cardiomyopathy by long-term follow-up studies. Autoimmune response of cardiac tissue plays an important role in the pathophysiological process of DCM. Experimental autoimmune myocarditis in mice is a typical animal model which mimics dilated cardiomyopathy in order to allow assessment of the therapeutic effects on this disease. We clarified the issue in the discussion section in our revised manuscript.

Q3. The universal nature of autophagy in clinical DCM and a positive correlation of autophagy indices with better outcomes was shown previously by Saito and coauthors (Autophagy, 2016; 12:579-587). Authors missed this crucial reference.

Reply: Thank you. As recommended, we supplemented the crucial reference in our revised manuscript.


Q4. Methods section lacks critical information about the starting time and duration of 3-MA and rapamycin administration, and their timing relative to the myosin/adjuvant injections. The total dose of administered myosin for DCM induction is also missing.

Reply: Thank you. As recommended, we have supplemented the information in the revised manuscript.
Q5. Fig. 2 - please indicate with arrows the double membrane autophagosomes referred in the text.

Reply: Thank you for your thoughtful review of our manuscript. As recommended, we supplemented the arrows indicating the double membrane autophagosomes in revised Figure 2A.

Sincerely,

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