Author's response to reviews

Title: Effects of carvedilol treatment on cardiac cAMP response element binding protein expression and phosphorylation in acute coxsackievirus B3-induced myocarditis

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Dear Editor:

Thank you for your kind letter of September 26, 2013. We have revised the entire manuscript entitled “Comparison of effects of Ivabradine versus carvedilol in murine model with the coxsackievirus B3-induced viral myocarditis” (MS: 141902641105962) from start to finish in accordance with the comments of the academic editor and reviewers. We acknowledge your help and the reviewer’s comments and suggestions very much, which are very valuable in improving the quality of our manuscript.

Here below is our description on revision according to the reviewers’ comments.

Reviewer 1 (Andreas Henke)

Minor essential revisions:

1. The reviewer’s comment: Next time, please provide a manuscript with numbered lines. This will help a lot to direct comments.

   The authors’ Answer: Corrected accordingly.

2. The reviewer’s comment: M+M section, “Mouse model of viral myocarditis”: Please provide the origin of the CVB3 strain used in the experiments. And please give a better description about the experimental setup in view of the number of mice in each experiments

   and how often you repeated the experiments.

   The authors’ Answer: Corrected accordingly. CVB3 strain was obtained from
ATCC VR30, and originated from JL Melnick (Melnick JL, Ledinko N. Immunological reactions of the Coxsackie viruses. I. The neutralization test; technic and application. J. Exp. Med. 92: 463-482, 1950.) There were 30 mice in each group. Eight surviving mice from each group were killed on day 7 or 14. The Western blot analysis and RT-PCR and ELISA experiments were repeated three times for each samples, respectively, and the plasma noradrenaline was measured repeatedly six times for each samples, and the other experiments for one time.

3. The reviewer’s comment: M+M section, “Detection of apoptosis”: Please rewrite this section because it does not fit with the results presented in Figure 5. You write “nuclei with brown staining indicated…..”, but the pictures show a fluorescent staining. In addition, please use DAPI co-staining to really demonstrate the nuclei.

The authors’ Answer: According to the comment of the reviewer, we rewrite this section (Detection of apoptosis) in detail and recorrect the Figure 5.

4. The reviewer’s comment: Result section, “ELISA analysis of cytokine levels in the heart”: How do you know that the cytokines were really in heart tissues and not in the blood, which was present in your samples as well? Do you have data to show cytokine concentrations in the blood itself?

The authors’ Answer: The myocardial tissue was collected and the cytokine levels in the myocardial tissue were determined by ELISA analysis in the present study. Many previous studies have shown that the concentrations of circulating proinflammatory cytokines, such as TNF-α and IL-6 are elevated in patients with myocarditis and in a murine model of viral myocarditis, and the myocardial expression of TNF-α and IL-6 was increased as well. (Matsumori A, Yamada T, Suzuki H, et al. Increased circulating cytokines in patients with myocarditis and cardiomyopathy. Br Heart J 1994; 72: 561–566; Shioi T, Matsumori A, Sasayama S. Persistent expression of cytokines in the chronic stage of viral myocarditis in mice. Circulation 1996; 94: 2930–2937; Seko Y, Takahashi N, Yagita H, et al. Expression of cytokine mRNAs in murine hearts with acute myocarditis caused by coxsackievirus b3. J Pathol. 1997;183(1):105-8). The severity in the course of the disease appears to correlate with the degree of myocardial production of proinflammatory cytokines (Shioi T, Matsumori A, Sasayama S. Persistent expression of cytokines in the chronic stage of viral myocarditis in mice. Circulation 1996; 94: 2930–2937; Iwasaki A, Matsumori A, Yamada T, et al: Pimobendan inhibits the production of proinflammatory cytokines and gene expression of inducible nitric oxide synthase in a murine model of viral myocarditis. J Am Coll Cardiol 1999,33:1400-7.). Because the amount of blood sample in mice was limited and the blood samples were used for measuring the plasm noradrenaline in the study, we have not blood enough to determine the circulating cytokines. Although we do not have data to show cytokine concentrations in the blood, myocardial production of proinflammatory cytokines were correlated with the severity of myocarditis.
5. The reviewer’s comment: In the description of your Figures please use another word for “myocarditis”, because you find myocarditis in your carvedioli-treated mice as well.

The authors’ Answer: We interpreted the meaning of “Control”, “Myocarditis” and “Carvedilol” in the Figures. (Control, normal mice treated with normal saline solution; Myocarditis, infected mice treated with normal saline solution; Carvedilol, infected mice treated with carvedilol). We can not find another word for “myocarditis” which is not associated with “carvedilol”-treated mice with myocarditis.

6. The reviewer’s comment: Figure 6: Instead of demonstrating PCR results it would be much more appropriate to show viral titers. Please add these results.

The authors’ Answer: Corrected accordingly. According to the comment of the reviewer, we investigated the viral titers and added the results in the Figure 6.

7. The reviewer’s comment: Figure 8: Next time, please provide data based on quantitative RT-PCR.

The authors’ Answer: Corrected accordingly. According to the comment of the reviewer, we investigated the cytokines mRNA by quantitative RT-PCR and added the results in the Figure 10.

Reviewer 2 (Rudin Pistulli)

Major concerns:

The reviewer’s comment: 1. The authors have chosen TUNEL staining for their analyses of apoptosis. The quantification of the apoptotic cells is described in the methods section as the percentage of total cells counted in randomly selected fields by microscope.

a. How did the authors count “total cells” in the selected fields?

The authors’ Answer: We manually count the all myocardial cells in the randomly selected filed.

b. How were these “fields” defined in size?

The authors’ Answer: Positive staining cells were manually counted in 10 randomly selected fields of each slide by microscope (200× magnification field). Cell death was expressed as the average percentage of total cells counted.

c. The microscopic photographs in figure 5 do not show “brown” DAB+staining, as described in methods (!). They resemble rather fluorescence microscopy images
of surely something else. The authors must therefore re-describe their characterization of apoptosis by a clearer quantification method and appropriate example photographs. As TUNEL staining cannot differentiate between cellular apoptosis and tissue necrosis, I would also suggest a second more specific staining method for apoptotic cells.

The authors' Answer: According to the comment of the reviewer, we rewrite this section (Detection of apoptosis) in detail. In addition, we show the DAB staining images for apoptotic cells.

Minor concerns:

2. The reviewer's comment: The reported heart rate of the control balb/c mice is too low (around 450b/min, instead of the normally reported 600b/min). Can the authors please mention this and the possible reasons such as stress-induced bradycardia or the limitations due to the tail-cuff method?


3. The reviewer's comment: The authors characterized the severity of myocarditis merely through HE and fibrosis staining. The lack of any echocardiographic data should be at least mentioned as a study limitation.

The authors' Answer: We agree with the reviewer that echocardiography should be investigated in the study, which might further improve the strength of the study. However, our and other previous studies (Li YC, Ge LS, Yang PL, et al. Carvedilol treatment ameliorates acute coxsackievirus B3-induced myocarditis associated with oxidative stress reduction. Eur J Pharmacol. 2010;640:112-116. Pauschinger M, Rutschow S, Chandrasekharan K, et al. Carvedilol improves left ventricular function in murine coxsackievirus-induced acute myocarditis association with reduced myocardial interleukin-1beta and MMP-8 expression and a modulated immune response. Eur J Heart Fail. 2005; 7: 444-452. Yuan
ZY, Shioji K, Kihara Y, et al. Cardioprotective effects of carvedilol on acute autoimmune myocarditis: anti-inflammatory effects associated with antioxidant property. Am J Physiol-Heart Circ Physiol. 2004; 286: 83–90.) examined the effects of carvedilol on cardiac function. Thus, we believe that the results from other studies are available. Indeed, the lack of echocardiographic data was a limitation of the present study. We agree with the reviewer that we should recognize and emphasize the limitation. Corrected accordingly. See the paragraph “Study limitations”.

4. The reviewer’s comment: Recent publications on the cellular mechanisms in murine myocarditis are missing.

The authors’ Answer: According to the comment of the reviewer, we have mentioned the cellular mechanisms in the recent publications in murine myocarditis in the discussion section of the manuscript. Corrected accordingly.

Many grammatical or typographical errors have been revised.

All the pages indicated above are in the revised manuscript.

Thank you and all the reviewers for the kind advice.

Sincerely yours,

Li yue-chun

2013.10.23