Author's response to reviews

Title: The Effects of apoptosis vulnerability markers on the myocardium in depression after myocardial infarction

Authors:

Yiming Wang (yimingw66@yahoo.com)
Xingde Liu (lxd@gmc.edu.cn)
Dongfeng Zhang (53588@163.com)
Shuzheng Liu (1057386401@qq.com)
Michael Berk (mikebe@barwonhealth.org.au)

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Author's response to reviews: see over
Dear Sabina

Many thanks for your great help, following are point by point responses to reviewers' comments.

**Answer questions:**

Firstly reviewer: Guy ROUSSEAU

1. Did some rats in the depression group present data in the behavioural tests that are similar to the MI group. In other words, did all rats in the depression perform similarly in the behavioural tests?

   **Answer:**

   Yes, all rats performed same behavioural test in the depression group or MI group or others. The data is similar between the depression group and post-MI depression group.

2. If I understand correctly, the measurements of caspase-3 or Bax-Bcl-2 were performed on tissues that were previously used in the determination of infarct size (TTC staining). Do you know if TTC influences the results?

   **Answer:**

   Rats in every group were randomly divided into two subgroups, in one of subgroups TTC staining was performed, and in the other, caspase-3 or Bax-Bcl-2 in the different tissues was measured.

3. More importantly, how can you claim that apoptosis is important whereas the only apoptotic marker presenting a difference is the Bax/Bcl2 ratio. Is it possible that Bax/Bcl-2 did not only reflect apoptosis but other mechanisms?

   **Answer:**

   Some published articles have showed that the Bax/Bcl-2 ratio is a measure of a cell’s vulnerability to apoptosis; Up-regulation of the Bax/Bcl-2 ratio suggests greater apoptotic activity [16-17].
Michael Berk  cont’d…

There are both kinds of apoptotic pathways in mammalian cells, the mitochondrial-mediated pathway of apoptosis is regulated by the bcl-2 family of antiapoptotic (bcl-2, bcl-x, mcl-1) and proapoptotic proteins (bax, bad, and bak), and bcl-2 inhibits apoptosis by interacting and forming inactivating heterodimers with bax/bak. It has been suggested that the bax/bcl-2 ratio may be more important than either promoter alone in determining apoptosis. The Bax/Bcl-2 ratio is a measure of a cell’s vulnerability to apoptosis, therefore, in our study, the use of a more sensitive, Bax/Bcl-2 ratio was chosen to reflect apoptosis.

Revised manuscript
Evan Blue staining is used to determine the area at risk and not to measure infarct size.

Answer:
Pg.2, Methods, line 7-8, revised and highlighted in red.

Background section:
1. It is difficult to follow this section. The link between depression, MI and apoptosis is difficult to understand.

Answer:
There is an increased incidence of major depressive disorder (MDD) (15-30%) in individuals after myocardial infarction (MI), but the pathophysiological processes mediating this association are unclear. Depression is linked to a 2.0-2.5 fold increased risk in new cardiovascular events and increased cardiac mortality. Our previous study demonstrated an increase in pro-apoptotic pathways in the myocardium and hippocampus in MDD, which was reversed by venlafaxine. This study aimed to attempt to confirm whether apoptosis vulnerability markers were present in the myocardium of rats after MI. We consequently hypothesised that active pro-apoptotic pathways in the myocardium may be involved in the nexus between cardiovascular disorders and depression.

2. The description of caspase-3 in this section is very weak compared to Bax/Bcl2.
- Humans are not used in ref 23.

Background: We have revised and added the contents of caspase-3 in the pg 5, line 6-9, highlighted in red.
ref 23 is Wann [23], Pg 5.

Methods:
1. A student's t test could not be performed in this kind of experiments. You should perform an ANOVA followed by post-hoc tests such as Bonferonni.

Answer:
We have revised this as suggested in the Statistical analysis, pg12, line 1-4, highlighted in red.
2. How did you divide the rats between MI and post-MI depression groups. What are the criteria.

Answer:
The model of post-MI depression was determined using open field and sucrose preference tests. Using this model of depression after MI, we showed significantly decreased scores of both horizontal and vertical movement and sucrose consumption compared to sham group, and a similar pattern in the depression group.

Results:
The size of area at risk (expressed as % of left ventricle) is not clearly described. Similarly, I believe that infarct size is expressed as % of area at risk. This is not clear.

Answer:
We have revised in pg 12, the Infarct size paragraph, Results, line 2-3, highlighted in red.

Discussion:
1. The pertinence to discuss about extrinsic and intrinsic pathways is questionable. Caspase-3 is an effector enzyme of apoptosis and can be activated by both pathways, whereas bax/Bcl-2 is involved mainly in the intrinsic pathway.

Answer:
Both kinds of apoptotic pathways were observed simultaneously in the same experiment assessing activation and non-activation of caspase-3. This may cause cleavage of substrates and cell death. The mitochondrial-mediated pathway of apoptosis is regulated by the bcl-2 family of antiapoptotic (bcl-2, bcl-xl, mcl-1) and proapoptotic proteins (bax, bad, and bak), and bcl-2 inhibits apoptosis by interacting and forming inactivating heterodimers with bax/bak. It has been suggested that the bax/bcl-2 ratio may be more important than either promoter alone in determining apoptosis. The Bax/Bcl-2 ratio is a measure of a cell’s vulnerability to apoptosis, therefore, in our study, the use of a more sensitive, Bax/Bcl-2 ratio was used to reflect apoptosis. We have revised this in the Discussion: page 14-15, highlighted in red.

2. In page 15, the authors indicate that caspase-3 may not be activated during post-MI time-course [21] in this reference the authors studied the hippocampus and not the myocardium. However the argument to explain the low activity of caspase-3 is very weak. Is it possible that the caspase-3 activity is low because the apoptotic process in the myocardium is finished after 2 weeks post-MI?

Answer:
Yes, it is possible that the caspase-3 activity is low because the apoptotic process in the myocardium continues until 2 weeks post-MI. We have revised this in the Discussion: pg 16. Second paragraph, line 6-7, highlighted in red.
2. The section on limitations is not convincing.

Answer: We have revised in the limitations: pg 17, line 12-20, highlighted in red.

Second reviewer: Lucia Carboni

1. Experimental design

About the experimental design, from the section entitled “subjects” of the methods it appears that the group labelled as MI-depressed was selected within the MI group because of the response in the open field and sucrose preference tests. The animals were labelled as MI-depression by splitting the MI group based on results obtained in the tests aimed at evaluating depressive-like behaviours. Therefore, the results in these tests cannot be displayed as results of the experimental groups, since they were adopted to define the groups themselves. If the belonging to a group was defined based on results in sucrose intake and open field tests, performing statistical analyses after the selection is meaningless. Thus, the results section is reduced to the comparison of bax/bcl-2 and caspase levels in the four groups. Moreover, the selection criteria to include an animal in the MI-depressed group should be clearly defined beforehand and described in the methods section.

Answer:
We have revised in the methods section as suggested above: pg 8, last paragraph, line 11-15, pg 9, 1st paragraph, line 3-10, highlighted in red.

2. The title is misleading since no stress plus MI infarction group was examined.

Answer:
We have revised the title: pg 1, highlighted in red.

3. The sham, MI, and MI-depressed groups experienced surgery, whereas the chronic unpredictable stress-depressed group did not. I wonder if this difference may introduce bias. Reasons for this choice should be given in the methods.

Answer:
We think that the design of the experiment accounts for this. The use of a chronic unpredictable stress-depressed (depression) group on which surgery is performed as an alone “control” group would assist in interpretation of these results. This is a goal for future studies, and we have also added this in the “limitations”: page 17, line 13-15, highlighted in red.
2. Choice and description of the methods

The method for measuring the expression levels of Bax and Bcl-2 is not sufficiently detailed. The indicated provider apparently does not show its products or protocols in a web site. The American provider does not list the indicated kit among its products. Additional information should be provided to ensure reproducibility of the data. In particular, the method for quantitative measure of immunostaining should be reported in detail.

Answer:
Details of the quantitative measurement of Bcl-2, Bax and caspase-3 protein using immunohistochemical staining have been added in the Methods section, pg 10, last paragraph, and pg 11.1st paragraph, highlighted in red.

3. The method for measuring caspase activity was not described, unless if expression level was meant. If this is the case, the concerns expressed for bax and bcl-2 hold for caspase as well. In addition, if levels instead of activity were investigated, in the results the comparisons should be described as “difference between caspase expression levels”, not as “difference between caspase activity”, as it is in the current version.

Answer:
The method for measuring caspase level was similar to that used for Bax, Bcl-2. We have also revised our results using“levels” instead of activity. pg 13, line 4-5, highlighted in red.

4. Statistical analysis

Answer:
We have revised the statistical analysis section in pg12, line 1-4, highlighted in red.

Minor essential revisions

1. In the abstract, information should be provided about the animal model used to reproduce major depression symptoms. Thus, chronic mild stress should be mentioned in the abstract as well.

Answer:
Abstract Methods 2th line added “Chronic mild unpredictable stress and separation were used in the depression group”, highlighted in red.

2. Abstract, 6th line in first paragraph: “makers” should be “markers”.

Answer:
Michael Berk  cont’d…

We have used “markers” instead of “makers” in Abstract, 6th line in first paragraph. highlighted in red.

3. Chronic mild stress: how was the isolation carried out if all rats were singly housed as stated in the section “subjects”?
   Answer:
   Yes, all rats were housed singly.

4. There are several language mistakes in figure legends.
   Answer:
   We have revised figure3 highlighted in red.

5. There are format inconsistencies in the References, e.g. in 7, 21, 24, 26, 30, 31, 35, 37 and 43: journal title is not abbreviated; 27: abbreviation is wrong.
   Answer:
   We have revised the format inconsistencies of all References in page 20-25. highlighted in red.

Third Reviewer: Ove Wiborg
Reviewer’s report:
Major Compulsory Revision
design.
1. The design is unclear. Does Post-MI depression mean: MI followed by CMS (MI+ CMS)?
   The reverse order CMS + MI would also have been interesting.
   Answer:
   Yes, recently, we are performing CMS+MI.

2. Generally depression should be replaced by CMS or anhedonic-like. Sucrose pref. is a variable parameter and should rather be measured weekly than just at endpoint. Was suc. pref. measured after MI and thus before CMS? Did only 7 out of 20 rats become anhedonic? Were the rest resilient to stress, like reported by others? In case so they should have been included.
   Answer:
   We have replaced depression by CMS or anhedonic-like in the manuscript, pg 6, line 2, marked in red. It would also have been helpful for the sucrose test to have done weekly in addition to baseline and endpoint readings, we will plan further experiments to clarify this. This has been added to the limitations page 17, line 18-20. The sucrose consumption test was begun 23 hours after water and food deprivation while experiment was begun, and on day 15 after surgery and the day after the end of the procedure of CMS. We have mentioned this in the methods pg 9, line 3-5.
In our experiment, only 7 out of 20 myocardial infarction rats developed an anhedonic-like state compared to sham and depression groups. We have added this to the limitations page 17, line 15-18, highlighted in red.

3. In general heart performance should have been analyzed more detailed.
Authors mentioned in the methods that they have made ECG recordings - it would have been nice to see be nice a fig with representative traces before and after MI. It is important because there is not many groups which are doing this.
Answer:
Yes, we did it before, and we have added the figure of the EEG in figure1.

4. The global problem of the manuscript that it lacks "n" for experiments, graphs, and statistics in the text.
Answer:
Yes, and we have added "n" to Abstract, and table 3-4.

5. It is important to see / to measure the cardiac performance in different groups before killing the animals. It can be done directly by the same anesthesia before they take the heart out. All mental state changes could be just due to fall in cardiac output, poor brain (and the rest of the body) perfusion etc.
Answer:
Great suggestion, we will do this in further studies, thanks.

6. Results, pg. 11, Infarct size. I cannot understand how 65% MI in total became 43% and 46% in each group separately.
Answer:
The myocardial infarct risk area of the left ventricular area was 65±2% (mean±SEM) using Evan Blue staining, furthermore the myocardial infarct size in the myocardial infarct risk area was detected by TTC dye, the size of the myocardial infarction was similar in both groups: (MI group 43.2±1.9%; post-MI depression 45.6±2.6%). We have revised in Results, pg.11, Infarct size paragraph, highlighted in red.

7. Results, pg. 11, Bax:Bcl2 ratio. It is not clear from the text whether the authors are talking about mRNA or protein - they have to specify this and address each result separately, even if they are similar.
Answer:
We have added each result separately to Table 3-4 in the results of Bax, Bcl-2mRNA and protein expression.

8. This is very intriguing with Bax:Bcl2 ratio, especially with respect to the most significant
Michael Berk  cont’d…

increase in this ratio in MI+CMS group. Is it consequence of something general in this model of depression? To test this the authors could measure Bax:Bcl2 ratio in different groups, or in another region which have not been exposed to hypoxia. Would there be a difference between the depression and the control here?

Answer:
Yes, it could be the consequence of this model of depression, we will measure Bax:Bcl-2 ratio in different groups, or in another region in further experiments.

9. Authors have not found any difference in Caspase-3. But this is not the only caspase which can be involved in apoptosis. What about others? They comment this possibility in the discussion but they could do a bit more about it. F.ex. they could look in the literature whether other caspases expresses in the heart, or even to do their own test .If they could find other caspase they need to see whether it is affected by MI and depression.

Answer:
In the literature of Lancel et al.(Circulation 2005,242:599-2604), Endotoxininduced caspase -8, and caspase -9-like activities except caspase-3, and reduced contractilereserve and sarcomere disarray at the level of single left ventricular cardiomyocytes. And Condorelli et al.(Proc Natl Acad Sci 2001, 98:9977-9982) viewed that in caspase3 transgenic mice showed increased infarct size and a pronounced susceptibility to die, because its cardiomyocyte-specific overexpression increases infarct size, and implicated that Caspase-3 determined myocardial infarct size after ischemia-reperfusion injury.

At the present, we didn’t find the relationship of between other caspases and MI with depression in heart, it is worthy of further study.

Minor revised
1. They need identify the type of stress in the title. This is especially important because in the "cardiovascular world" stress has, first of all, absolutely another (mechanic) meaning.

Answer:
We have revised the title. highlighted in red.

2. They need to identify the pharmacological origin of venlafaxine (that this is SNRI)

Answer:
We have revised the pharmacological origin of venlafaxine in the background, pg 5, last paragraph, line 4-6, highlighted in red.

3. All abbreviation in the abstract (TTC) need to be explained.
Michael Berk  cont’d…

Answer:
We have revised this in the abstract methods paragraph, line 8.

4. I think that the conclusion of the Abstract is too generalized and has to describe the specific findings in this study.
   Answer:
   We have revised the conclusion of abstract.

5. What is the percentage of rats that survive after MI induction procedure?
   Answer: > 90%

6. Pg.10, Statistical analysis, line 2. "to examine the difference between ."
   Answer:
   We have revised this in the Statistical analysis.pg 12

7. Results, pg. 11, Infarct size, 1st line. Too many labels of %.
   Answer:
   We have revised the Results, pg. 12, Infarct size, highlighted in red

8. Pg. 6 line 7-8: The model of dep. replace by The readout on depression status. Pg. 8 line5: rat model replace by readout
   Answer:
   We have revised this in the methods pg 7, last paragraph, and pg 6, line 4,5, highlighted in red.

Yours sincerely

Michael Berk
Professor of Psychiatry, Deakin University