Author's response to reviews

Title: MicroRNA Profiling In Ischemia-Reperfusion Injury Of The Gracilis Muscle In Rats

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Author's response to reviews: see over
Dear reviewer Dmitry Grigoryev:

Thank you for your time, effort and professional comments in regard to our manuscript entitled “MicroRNA Profiling In Ischemia-Reperfusion Injury Of The Gracilis Muscle In Rats” to BMC musculoskeletal disorders. I have revised the document according to your suggestions and highlighted those areas in green color.

They are listed in the following:
1. There seemed to be no problem for another reviewer to approach the Table 1, we had cautiously confirmed the attachment of this file in the revision form.
2. In this study, four one-color miRNA arrays for miRNA expression and four two-color whole rat genome arrays for mRNA expression were used (originally we had indicated 2 replicate experiments for miRNA array in Method/Expression of miRNAs and 1 specimen at each of the abovementioned time points for whole genome array in Method/Whole Genome Microarray Analyses). Upon request, we had indicated and highlighted this point more clearly in the revised text (Method/Expression of miRNAs/lines 3-4 and Method/Whole Genome Microarray Analyses/lines 2-3).

In addition, we can understand the concern of Yours in the number of whole genome array used in each experimental group of this study. We have to address that because the accuracy of the array is impossible 100%, there was the existence of some false positive and false negative targets from the array experiment. While false positive predictions can be eliminated by experimental validation studies, the number of false negative predictions remains unknown. With increase of the $n$ (number) of the array experiments, the identified targets would be more accurate, but at the sacrifice of missing some targets (because of the unknown false negative rate). Therefore, in our original design, we used the inclusion of downregulated genes at all 4 time points of the given miRNA to make a more reasonable approach for the miRNA:mRNA pairing. In addition, further real time PCR may be helpful to eliminate the false positive targets, although this step so far is halted by lack of knock-out rodent and expensive antisense oligonucleotide (like LNA-antimiR).
3. Upon this very good query, we had written and added one paragraph to the
Discussion regarding the data in Figure 2 (*Discussion/3*rd *paragraph*).

4. So far, none of these four identified genes (*Nqo1*, *Pdpn*, *CXCL3*, and *Rad23b*) could be correlated to the illness of ischemia-reperfusion of the muscle in reviewing the literature. Although *Nqo1* was suggested to play a role in ischemia-reperfusion injury in renal tissue and neuronal cells, there existed opposing opinions regarding the role of cytoprotection in these two conditions. We had re-written and highlighted in the revised text to address this point (*Discussion/page 17/lines 9-13, 19-22*).

5. Under your kind suggestion, we had revised the Y axis of the Figure 3 into a negative numbers to make the illustration more clear.

6. The conclusion was re-written with mentioning the data and the meaning.

7. The page numbering had been added.

We hope the revised article and the explanation could answer well your comment and query. If required, we are very delighted to make further change or revision.

Thank you very much

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Dear reviewer Natarajan Aravindan:

Thank you for your time, effort and professional comments in regard to our manuscript entitled “MicroRNA Profiling In Ischemia-Reperfusion Injury Of The Gracilis Muscle In Rats” to *BMC musculoskeletal disorders*. I have revised the document according to the suggestions of reviewer and highlighted those areas in green color. They are listed in the following:

1. The reason we choose the 4 h ischemia but rather than 1 or 2 h ischemia is because 1 or 2 h ischemia of the muscle will not induce remarked tissue injury with clinical significance. In clinical, all the plastic or orthopedic surgeons quite frequently use the pneumatic tourniquet in the operative field to acquire a bloodless operative field (I am a plastic surgeon too) and understand that in clinical the maximal allowable time of the pneumatic tourniquet time is 2 h (please also see Solonen KA, Hjelt L. Morphological changes in striated muscle during ischaemia. Acta Orthop Scand 1968;39:13-19. and I. R. Fletcher and T. E. Healy. The arterial tourniquet. *Ann R Coll Surg Engl.* 1983 November; 65(6): 409–417.) Some surgeons even proposed that ischemia 3 h should be regarded as the upper limit of safety (Kleenerman L. Tourniquet time--how long? *Hand.* 1980 Oct;12(3):231-4.). The reason is that, generally, within 2 h, there is no remarked sequela to the muscle or the limb will happen; more than 2 h, the muscle might get injury or some irreversible change (like fibrosis). This is why most of the articles studying the skeletal muscle ischemia-reperfusion injury would choose the 4 h as the ischemic time. For example, if you search the PubMed with the key word “gracilis muscle ischemia reperfusion injury” with limitation of within 10 years and English-written article, there are 21 articles in the list. Among the 21 articles, 15 articles study only 4 hours or a longer ischemic time (13 articles 4 hr, 1 article 5 hr, 1 article 6 hr, or listed below).

1. *Ischemia-reperfusion-induced apoptotic endothelial cells isolated from rat skeletal muscle.*
   
   Wang WZ, Fang XH, Stephenson LL, Khiabani KT, Zamboni WA.
   
   *Plast Reconstr Surg.* 2009 Feb;123(2 Suppl):131S-8S.

   
   Khiabani KT, Bellister SA, Skaggs SS, Stephenson LL, Nataraj C, Wang WZ, Zamboni WA.
3. Ischemia/reperfusion-induced necrosis and apoptosis in the cells isolated from rat skeletal muscle.
   Wang WZ, Fang XH, Stephenson LL, Khiabani KT, Zamboni WA.

   Khiabani KT, Stephenson LL, Gabriel A, Nataraj C, Wang WZ, Zamboni WA.

5. The expression of proinflammatory cytokines in the rat muscle flap with ischemia-reperfusion injury.
   Zhang F, Hu EC, Gerzenshtein J, Lei MP, Lineaweaver WC.

6. Regulation of inducible nitric oxide synthase in ischemic preconditioning of muscle flap in a rat model.
   Zhang F, Oswald T, Holt J, Gerzenshtein J, Lei MP, Lineaweaver WC.

7. Lack of chlorpromazine effect on skeletal muscle metabolism after ischemia and a short reperfusion period.
   Piccinato CE, Salles Roselino JE, Massuda CA, Cherri J.

8. Effect of L-arginine on leukocyte adhesion in ischemia-reperfusion injury.
   Gabriel A, Porrino ML, Stephenson LL, Zamboni WA.
9. Noninvasive remote ischemic preconditioning for global protection of skeletal muscle against infarction.

10. Local hypothermia during early reperfusion protects skeletal muscle from ischemia-reperfusion injury.
   Mowlavi A, Neumeister MW, Wilhelmi BJ, Song YH, Suchy H, Russell RC.

11. Age-related differences of neutrophil activation in a skeletal muscle ischemia-reperfusion model.

12. Effects of adenosine pretreatment on detection of free radicals in ischemic and reperfused canine gracilis muscle flaps by use of spin-trapping electron paramagnetic resonance spectroscopy.
    Brisson BA, Miller CW, Chen G, McCutcheon LJ, Janzen EG.

    Mowlavi A, Ghavami A, Song YH, Neumeister M.
Olivas TP, Saylor TF, Wong HP, Stephenson LL, Zamboni WA.

15. Glycine preserves function and decreases necrosis in skeletal muscle undergoing ischemia and reperfusion injury.
Ascher E, Hanson JN, Cheng W, Hingorani A, Scheinman M.

Therefore, study of a shorter time ischemia like 1h or 2 h also may induce some different miRNAs expression, but if these miRNAs are not to be induced in a longer hour ischemia, the investigation of this target may be devoid of clinical importance or attention. However, we had demonstrated in this study that the 4 h ischemia-induced miRNAs (miR-21, 200c, and 205) were actually induced in an earlier time like 1h or 2 h, implying the epigenetic regulation would be mediated earlier before the remarked pathophysiologic change could be observed in clinical. We had indicated this point in the Discussion of the revised manuscript (Discussion/3rd paragraph).

2. We have to admit that the four predicted genes may be a false positive; So far, this is an inevitable flaw with this computational and experimental approach with array. Because the accuracy of the array is impossible 100%, there would be existence of some false positive and false negative targets from the array experiment. While false positive predictions can be eliminated by experimental validation studies, the number of false negative predictions remains unknown. If we can use loss-of-gene approach, the results will be more solid. however, so far, there are only very few miRNA-knock out mice in the market (miR-150, miR-155, miR-206, miR-17 to 92 cluster…etc) and the antisense oligonucleotide like LNA-antimiR is too expensive to afford in vivo experiment (10 mg = 7000 US dollars, generally in vivo effective dosage for rat = 25mg/kg, that is, for a 300-350 g rat, you have to inject the drug at
the expense of around 5000 US dollars, albeit the unexpectable knock down effect).

3. In this study, the use of the isopentane (or 3:1 propane-isopentane) before the specimen being cooled by liquid nitrogen to -175°C is an old (http://jcb.rupress.org/content/4/5/593.abstract) but common technique for best morphological and histochemical preservation for the tissue sample, in particular the muscle tissue, which is fragile and easy to lose its morphological maintenance. Although we did not perform morphological investigation in this study, this is the standard procedure of our laboratory to preserve any harvested muscle specimen.

4. In this study, the rats were randomly assigned to the sham-operated control group and the I/R group, we had indicated that in the original text (Methods/Animal surgery and tissue preparation/lines 2-3).

5. We used the inclusion of downregulated genes at all 4 time points of the given miRNA from the whole genome array experiment to make a more reasonable approach for the miRNA:mRNA pairing. Because of the screening characteristic of array, no statistics of the mRNA expression were demonstrated. We believe that, in the future, with an available and affordable in vivo loss-of-function study, a quantitative real time PCR experiment is more suitable to measure the expression statistically.

We hope the revised article and the explanation could answer well your comment and query. If required, we are very delighted to make further change or revision.

Thank you very much

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