Peer Review File

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Reviewer A:
This work reported a strategy to ameliorate intimal hyperplasia in the ballon injury rat model via inhibition of ERK or Akt.

In general, the authors provide convincing data to prove their hypothesis, and the findings highlight the potential of inhibition of ERK or Akt in the treatment of ballon injury-induced intimal hyperplasia.

I have the following comments:
1. Usually, pharmacological manipulation induces compensation effects. Why did the authors not try to inhibit the ERK and Akt using other ways?
Reply:
Thanks for your question. Inhibitors of ERK and Akt are wildly used in *in-vitro* and *in-vivo* studies for efficient and effective inhibitions of ERK and Akt pathways, and the two inhibitors used in this study showed no significant compensation or side effects. Therefore, due to its higher efficiency and potential for therapeutic application, we used pharmacological manipulation with these inhibitors instead of other methods.

2. It seems that both ERK and Akt inhibition could bring similar effects. What about the combination of treatment? I am not asking you to re-conduct all the experiments. I would like to hear some comments on this point or see some discussion.
Reply:
Thanks for your question. Actually, we checked of the effect of a combining treatment of the two inhibitors and found that it showed no significant difference with the result of the single treatment of ERK-inhibitor. We considered that it is probably because that ERK-inhibitor is much effective to achieve a high-level inhibition of ERK/Akt pathway, so that dual-treatment could not lead to a significant change.

3. Pay attention to the writing. I give you an example. There are many square boxes (should be < or >) in the figure legends.
Reply:
Thanks for the comment. The relevant content is checked.

4. How to sacrifice animals? Please provide the details.
Reply:

Thanks for your question and suggestion. The rats were sacrificed by the cervical dislocation method and details have been added into the methods section of the text (in Page 4).

Reviewer B:

I read this paper with interests. Authors investigated the role of conexins in the development of intimal hyperplasia (IH) using a series in vivo analysis with ballon injury (BI) rat models. They found the expression levels of two major conexins (Cx37 & Cx43) and phosphorylation levels of ERK and Akt changed significantly in BI rats. Subsequently they revealed that the inhibition of ERK/Akt could significantly attenuate the IH phenotypes of BI rats. This study is interesting and would be beneficial for a better understanding of the functions of conexins in IH. I only have some minor concerns.

1: How about other conexins? Why did you choose only the four conexins for your test?

Reply:

Thanks for your question. It is because that the four connexins have been reported to be most highly-expressed and have showed major effects on formation/regulation of gap junction between VSMCs and endothelial cells than other connexins. Due to the limited funding and resources, we only studied the connexins in the current study.

2: As ERK/Akt-mediated pathway entails many cellular biological process and have many downstream targets, how could you exclude the possibility that the attenuation of IH by inhibition of ERK or Akt work though other downstream pathway but not conexins?

Reply:

This is a good question. We agree with you that inhibition of ERK/Akt may cause some side effects and probably affects other upstream or downstream targets. But as our data indicated two major connexins that reportedly regulated by ERK/Akt pathway showed significant changes consistent with change of ERK/Akt phosphorylation levels, we therefore consider that IH were attenuated by inhibition of ERK/Akt which directly or indirectly repaired connexins network.

3: Many typos exist in your text. Please do a careful proofreading.

Reply:

Thank you for the comment and we have thoroughly checked the manuscript.

Reviewer C:

This manuscript examines the effects of balloon injury restenosis as a model to elucidate the relevant mechanism associated with connexins in the development of intimal hyperplasia.
In general, the article is well written, and the study is relevant, although not entirely novel. Some considerations should be taken into account:
Main observation:
1- The manuscript lacks important prior information in both introduction and discussion. For example: the abstract states "Connexins (Cxs) participate in atherosclerosis associated intimal hyperplasia (IH), while the function involved in the balloon injury (BI) induced-IH and restenosis is unknown". This statement is in fact inaccurate, as there are a lot of previous papers studying this item in several experimental models, for example:
-Kozlov, K. L., Bolotov, I. I., Lin'kova, N. S., & Drobintseva, A. O. (2017). Expression of Signal Molecules in Culture of Human Endothelial Cells in Atherosclerosis and Restenosis. Bulletin of Experimental Biology and Medicine, 163(4), 566–569. doi:10.1007/s10517-017-3850-7
-Chadjichristos CE, Matter CM, Roth I, Sutter E, Pelli G, Luscher TF, Chanson M, Kwak BR. Reduced connexin43 expression limits neointima formation after balloon distension injury in hypercholesterolemic mice. Circulation 2006;113:2835–2843
-Yeh HI, Lupu F, Dupont E, Severs NJ. Upregulation of connexin43 gap junctions between smooth muscle cells after balloon catheter injury in the rat carotid artery. Arterioscler Thromb Vasc Biol. 1997; 17: 3174–3184
I suggest these reports should be quoted in the paper's introduction or discussion. In addition, there is no reference to previous work showing the relationship between P-AKT and ERK with connexin expression. As this was also already studied, references should be provided (e.g. Connexin37 reduces smooth muscle cell proliferation and intimal hyperplasia in a mouse model of carotid artery ligation Florent Allagnat, Celine Dubuis, Martine Lambelet , Loic Le Gal, Florian Alonso , Jean-Marc Corpataux , Sebastien Deglise, and Jacques-Antoine Haefliger. Cardiovascular Research (2017) 113, 805–816 doi:10.1093/cvr/cvx079.)
Reply:
Sorry for the missing literature review. The function of Connexins in several other experimental models is referred in the discussion part (page 9) of the revised manuscript. And the previous report indicating the link between P-AKT and ERK with connexin expression is also referred in the discussion part (page 9).

Minor observations:
2- A reference of the balloon injury method should be included.
Reply:
Thanks for your constructive advice. The reference has been inserted in the method part.

3- How were the inhibitor doses chosen? The authors should include a reference for this issue or discuss it.
Reply:
Thanks for the advice. The reference has been inserted in the method part (page 5).

4- The SDS-lysis buffer formulation and the homogenization method used should be specified.
Reply:
Thanks for the comment. The relevant information has been added in the method part (page 5).

5- Symbols in figure legends appear as □ instead of <.
Reply:
Thanks for your careful reading. This is caused by the different type of the fonts. The symbols in the figure legends have been corrected.

6- In figure 1C, the authors should state what exactly mRNA levels of Cx30 and Cx37 of BI groups were normalized to.
Reply:
Thanks for the comment. The connexins' mRNA levels were first normalized to respective β-actin mRNA levels and the values were further normalized to the value of 6 hours (been set to be 1) for comparison. Details have been added to the legend of figure 1.

7- The figure order in the manuscript is not right: Figure 2 appears as Figure 3.
Reply:
Thanks for the comment. And we have thoroughly checked the order of the figure as suggested.

8- A reference should be added in the first paragraph of page 8 of the manuscript.
Reply:
Thanks for the advice and the references are added in the first sentence of page 8.

9- In Figure 3A, the authors state that total AKT was regulated in balloon-injured rats, but such increase is not observed in the western image. Please, add a representative image.
Reply:
Thanks for your careful reading. We apologize that the sentence was miswritten and the total Akt showed no significant difference between BI and control rats. The sentence has been revised (in Page 8).

10- In Figures 3D and 3E, the ordinate axes label should not include “au “because it is a ratio and hence has no units.

Reply:

Thank you for your careful scrutiny and suggestion. The axes labels hasve been revised accordingly.

11- In figure 4A, an image of NI artery should be included.

Reply:

Thanks for your constructive advice. An image of NI artery has been added into figure 4A.

