Author’s response to reviews

Title: High shear stress suppresses proliferation and migration but promotes apoptosis of endothelial cells co-cultured with vascular smooth muscle cells via down-regulating MAPK pathway

Authors:

Qiang Ji (ji.qiang@zs-hospital.sh.cn)
YuLin Wang (wang.yulin@zs-hospital.sh.cn)
LiMin Xia (xlm_1117@sohu.com)
Ye Yang (yang.ye@zs-hospital.sh.cn)
Chun Wang (zscardiacs2016@163.com)
YunQing Mei (drmeiyq2004@tongji.edu.cn)

Version: 1 Date: 06 Aug 2019

Author’s response to reviews:

Response point by point to reviewers’ comments:

To Reviewer #1:

1. Abstract/Background: Authors should not refer to their former research is abstract. rather they should be focused on the background of their research, what is known and how their current study adds to the current knowledge.
R: We have made a change as suggested in the revised manuscript.

2. Methods: In general, the experimental setting is reasonable and I have no major objections to the methods performed.
R: Thank you very much for your approval.

3. Results: Put briefly, under higher level of shear stress condition, cell proliferation and migration of ECs were suppressed, while cell apoptosis was promoted. These results are interesting and may have some implications to the clinical arena.
R: Thank you very much for your approval.

4. Conclusion: Conclusion is grounded on the results and there is a logical sequence. However, given the fact this is translational research I would recommend authors to stress that out clearly so these results may serve just as an impetus for further studies.
R: We have made changes as suggested in the revised manuscript. (See also “5. Conclusion” in the revised manuscript).

This study demonstrated that higher shear stress may suppress proliferation and apoptosis of ECs in a co-culture system with VSMCs but promote cell migration via down-regulating ERK1/2 and p38.
MAPK pathways. This study provides experimental evidences for the clinical application of double-layer vein grafting, although further studies are needed.

To Reviewer #2:

1. English Language: There are minor English language and typographical corrections throughout the paper that need to be addressed.
   R: We have tried our best to correct the errors. Thank you for your advice.

2. Abstract: The abstract is sufficiently clear about the methods, but like the body of the text needs to expand on the size of the project and the number of samples taken etc.
   R: We have made changes as suggested in the revised manuscript.

3. Introduction: The introduction is sufficient to explain why the need for this study exists.
   R: Thank you very much for your approval.

4. Methods: The methods describe the laboratory methods in variable detail. Crucially, the scale of the project is never mentioned and it is unclear how many samples were taken and how many cultures were processed.
   R: We have made changes as suggested in the revised manuscript.

5. The primary hypothesis could be stated more clearly.
   R: In the present study, ECs and VSMCs were co-cultured and synchronized under shear stress using Parallel-Plate Flow Chamber system, and then were incubated with U0126 (ERK1/2 inhibitor) or PD98059 (p38 inhibitor). Cellular processes including proliferation, apoptosis and migration of ECs co-cultured with VSMCs were detected, respectively; and protein expressions of ERK1/2 and p38 MAPK were determined, respectively. This study aimed to evaluate the impacts of shear stress and MAPK pathways on proliferation, apoptosis and migration of ECs co-cultured with VSMCs, to test the hypothesis that high shear stress suppresses proliferation and migration but promotes apoptosis of ECs co-cultured with VSMCs via down-regulating MAPK pathway. (See also the last paragraph of “Introduction”)

6. P8 L58 - unclear which cells were placed in which density.
   R: We have made a change as suggested in the revised manuscript. Briefly, for MTT assay, ECs were seeded in a 96-well plate at a density of 3000 cells/well. (See also the front part of “Cell proliferation” in “Materials and Methods”)

7. P9 L6 - change in tense half way through
   R: We have made a change as suggested in the revised manuscript. The medium was then removed and the cells were incubated for 15min with 100ml of acidic isopropanol (0.08 N HCl) to dissolve the formazan crystals. The absorbance of the MTT formazan was determined at 490 nm in an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Rad, USA). (See also the middle part of “Cell proliferation” in “Materials and Methods”)

8. P9 L28 - unclear what this first sentence means
   R: We have deleted the sentence in the revised manuscript.
9. P9 L37 - disposed?
R: We have made a change as suggested in the revised manuscript.

10. P9 L59 - what was the chemoattractant?
R: We have made a change as suggested in the revised manuscript. This assay involved a two-compartment system where cells were induced to migrate from an upper compartment through a porous membrane into a lower compartment following the gradient of a chemokine. (See also the middle part of “Cell migration” in “Materials and Methods”)

R: We have made a change as suggested in the revised manuscript. Total cellular protein was extracted in radio immunoprecipitation assay (RIPA) buffer (containing 1% Triton X-100, 1% deoxycholate, and 0.1% SDS). (See also the front part of “Protein expression” in “Materials and Methods”)

12. P11 L1 - was normality tested for? Was t-test, mean and SD appropriate?
R: We have made a change as suggested in the revised manuscript. Continuous variables were expressed as mean ± standard deviation and were compared between different groups using one-way analysis of variance and Dunnett post hoc test. (See also the front part of “Statistical analysis” in “Materials and Methods”)

13. Results: P11 L22 - no need to repeat what was done. The results section should be a more concise description of what was noted from the experiments and the interpretation left to the discussion.
R: We have made a change as suggested in the revised manuscript. The sentence of “Primary ECs and VSMCs from porcine great saphenous vein were isolated and cultured” has been deleted.

14. P11 L28 - what was the percentage of ECs measured against?
R: We have made a change as suggested in the revised manuscript. The result of immunofluorescence showed the positive cells of vWF were found in over 95% of the ECs of the second generation of culture, and fluorescence staining could be seen in the cytoplasm (as shown in Figure 2A), indicating that ECs were collected successfully. (see also the first paragraph of “Isolation and identification of ECs and VSMCs” in “Results”)

15. Figure 2 - there are three images in part A and three in part B and it is not clear what this figure is demonstrating. Each of the three images should be appropriately described and, if necessary, arrows used to indicate key findings.
R: We have made changes as suggested in the revised manuscript.

16. Figure 3 - actual values including p values would be useful, which are also absent from the prose.
R: We have made changes as suggested in the revised manuscript.

17. Figure 4 - both in the legend and in the body of the text, these images are not adequately explained. A brief explanation of what the graph shows and how to interpret this information would be of benefit to the generic cardiac surgical reader.
R: We have made changes as suggested in the revised manuscript.

18. Figure 5 - what is the unit of measure here? What are the values and the exact p-values?
R: We have made changes as suggested in the revised manuscript.

19. Figure 6 - another ambiguous figure with lots of bars causing a loss in the readability of this image.
The legend and prose again are not clear in what is being demonstrated.
R: We have made changes as suggested in the revised manuscript.

20. Discussion: P13 L33 - the extrapolation of the effects of the extra-vascular support is unwarranted here. If the findings are to be tied back to a previous clinical study, it should be more tentatively.
R: We have made a change as suggested in the revised manuscript. The sentence of “In this study, we have identified another mechanism by which extra vascular stent may increase wall shear stress of vein graft and then protect from pathogenesis of neointimal hyperplasia of vein graft” has been deleted.

21. P13 L49 - the authors seem to be in contradiction with the perceived wisdom of lower stress being beneficial. This paragraph requires elaboration and referencing if it is to be accepted as given.
R: We have made changes as suggested in the revised manuscript. In this study, results of both MTT and BrdU assays showed that higher shear stress compared with lower shear stress may suppress ECs proliferation, FACS analysis showed that higher shear stress compared with lower shear stress may promote ECs apoptosis, and Transwell assay showed that higher shear stress compared with lower shear stress may suppress ECs migration, all of which suggesting that higher shear stress may suppress proliferation and migration of ECs co-cultured VSMCs but promote cell apoptosis. This study implied that higher shear stress instead of lower shear stress was beneficial for prevention and treatment of neointimal hyperplasia of vein graft. Souilhol and colleagues have reported that low shear stress promoted vascular dysfunction and atherosclerosis; conversely, high shear stress was protective. This evidence was in line with the results of this study. Additionally, Liu and colleagues found that gradually increasing shear stress could improve EC retention on vascular grafts. This finding was consistent with that in this study. (See also the last part of the first paragraph of “Discussion”)

22. P14 L58 - this paragraph is unclear, appearing to conflate the findings of the authors' previous study with the findings of this one. Again, the association between the two should not be overstated.
R: We have made changes as suggested in the revised manuscript. It is believed that hemodynamics environmental change is an initiation factor of neointimal hyperplasia of vein graft, and neointimal hyperplasia of vein graft is responsible for vein graft failure. Our previous study demonstrated that double-layer vein grafting compared with conventional single-layer vein grafting was effective in restraining early excessive distension of vein graft and ameliorating early neointimal hyperplasia via enhancing intravenous surface shear stress in animal experiments. The current study found higher shear stress compared with lower shear stress may suppress proliferation and migration of ECs in a co-culture system with VSMCs but promote cell apoptosis, and higher shear stress suppressing ECs proliferation and migration but promoting apoptosis may be via inactivation of ERK1/2 and p38 MAPK pathways. This study implies that increase of wall shear stress and inactivation of MAPK pathway may be promising strategies for preventing and treating vein graft failure. Additionally, this study may contribute to increasing the understanding of the mechanisms of vein graft failure. More importantly, this study provides experimental evidences for the clinical application of double-layer vein grafting, although further studies are needed. (See also the last paragraph of “Discussion”)

To Reviewer #3:

1. First of all, the language is not acceptable and must be corrected by somebody with better language abilities.
R: We have tried our best to correct the errors. Thank you for your advice.
2. More importantly the methodology must be explained much better. How is the double vein constructed? How is the shear stress measured? How is it explained that shear stress is higher in the double layer graft? Instinctively I would think that it was lower, when we had a supporting external layer. I think it is necessary to have drawings of how this double saphenous vein is constructed and how the shear stress is measured. It is not adequate to refer to previous articles as our readers cannot be expected to look this up.

R: The details of double-layer autologous saphenous vein grafting in a porcine model and detection of wall shear stress were shown in the supplementary.

According to the formula (as shown in the supplementary), wall shear stress in middle section of the vein graft was inversely proportional to the inner diameter of the vein graft. Comparing with single-layer vein grafting, double-layer vein grafting restrains early excessive distension of vein graft and reduces the inner diameter of the vein graft, consequently, increases wall shear stress. (See also our published reports: Heart Vessels 2011; 26: 190–5, and J Mech Med Biol 2011; 11: 1059–70)

3. The same applies to the "Parallel chamber flow chamber" which is described, but will be something without meaning for surgeons, I believe. This method must also be described in detail.

R: We have made changes as suggested in the revised manuscript. (See also “2.3 Co-culture of ECs and VSMCs and the flow chamber system” in “Materials and Methods” and Figure 1 in the revised manuscript)

4. The chemicals used to modify the response of the endothelium are not described properly for the journal's readers to understand. This fact makes the whole article more or less unreadable for the regular surgeon. I think that the article must be totally rewritten with the journal's readers in mind or alternatively submitted to a different journal.

R: The members of the MAPK family, including extracellular signal-regulated kinase (ERK1/2) and p38 MAPK, have been proposed as important signaling components mediating extra-cellular stimulation, such as physical stress, oxidative stress and mechanical stress. MAPK pathways play vital roles in regulating a large variety of cellular processes including proliferation, apoptosis and migration. This study aimed to test the hypothesis that high shear stress suppresses proliferation and migration but promotes apoptosis of ECs co-cultured with VSMCs via down-regulating MAPK pathway through evaluating the impacts of shear stress and MAPK pathways on proliferation, apoptosis and migration of ECs co-cultured with VSMCs. To evaluate the impacts of MAPK pathways on ECs processes, U0126 (ERK1/2 inhibitor) and PD98059 (p38 inhibitor) were used.

In addition, the manuscript has been totally rewritten.

To Reviewer #4:
Reviewer #4: This is a well written article (very minor orthographic and language revision), with good background literature search. The right methods and statistical tools were used. The discussion is fair and the conclusion justifiable. Intimal hyperplasia is an established culprit in vein graft failure post coronary artery bypass graft surgery (CABG). The long saphenous vein is still a favourite in such surgery and a lot of research is underway to support and extend the survival of this conduit through minimizing hyperplasia (among other known adverse mechanisms) and even finding suitable synthetic alternatives. Allowing for it retrospective nature, this study confirms previous findings, sheds much light on intimal hyperplasia of vein grafts in CABG surgery. Paving the way to further research, it is set to change clinical practice and improve long term outcomes in vein graft patency. Well done to the authors. (1) Reprinted Article "Pathophysiology of Vein Graft Failure: A Review" Davies, M.G. et al. European Journal of Vascular and Endovascular Surgery, Volume 42, S19 - S29. (2) Vascular Grafting
R: Thank you very much for your approval on our work. And have made changes as suggested in the revised manuscript.

To Associate Editor:

1. The study should be explained in more ‘lay’ terms so that the readers will understand better what is being done. In particular, there should be more detail relating to what is ‘single-layer’ or ‘double-layer’ vein grafting! It is not apparent to this reviewer from the text! Provide more relevance of the study to the clinical arena.

R: The details of double-layer autologous saphenous vein grafting in a porcine model were shown in the supplementary.

2. Is a t-test appropriate for this study? A t-test is used when a control group is compared to a test group; this study has a control group and a number of test groups! ANOVA then post-hoc analysis for multiple comparisons?

R: We have made changes as suggested in the revised manuscript. Continuous variables were expressed as mean ± standard deviation and were compared between different groups using one-way analysis of variance and Dunnett post hoc test. (See also the front part of “Statistical analysis” in “Materials and Methods”)

3. Discussion should be more focused on discussing the results in the context of other similar studies.

R: We have made changes as suggested in the revised manuscript. See also the first and the second paragraphs of “Discussion”.

4. Fig 2 should include arrows to indicate what is trying to be shown.

R: We have made changes as suggested in the revised manuscript.

5. On Fig 3, why does OD450nm indicate proliferation?

R: We have made changes as suggested in the revised manuscript.

6. Fig 4 should include details of what the Panels A-G represent and label Figure.

R: We have made changes as suggested in the revised manuscript.