Peer Review File

Article Information: http://dx.doi.org/10.21037/atm-20-3426

Reviewer A

The title"Adaptation of endothelial cells to shear stress under atheroprone conditions by modulating internalization of vinculin and VE-cadherin" and the group aimed to We quantified the response of endothelial monolayers in a parallel-plate flow chamber to laminar shear stress (LSS) and oxidized Low- Density Lipoprotein (oxLDL) in terms of cell viability, barrier Integrity, VE-cadherin availability, focal adhesion remodeling, and monocyte- endothelial interactions. The design had four groups. 1- static, 2- static+oxLDL, 3- Low shear+oxLDL and High shear+oxLDL.

Few important points to raise:
1- the paper focused on studying the effect of oxLDL under different shear conditions rather than investigating the effect of low shear on barrier integrity. it seems two very important controls if you can call them is are missing which are high shear stress alone and low shear stress alone.

Reply 1: The purpose of this article is to observe the changes of endothelial cells in early atherosclerosis, and to simulate the conditions of hypercholesterolemia and atherosclerosis in vivo. In this kind of pathophysiological process, the combined effects of shear stress and oxLDL can not be separated. So the conditions of high shear stress alone, and low shear stress alone were not included in the present study. However, we now understand the importance of these two groups (high shear stress alone and low shear stress alone) as controls, and any design of future experiment should take this into consideration. Thank you .

2- line 246 "Exposure of VECs to LSS of 25 dynes/cm2 lead to a marked shift in cell shape from typical cobblestone to elongated, spindle-like morphology, tend to parallel to the flow direction, and a 26.68% increase in retention rate compared to the Static oxLDL group ($P<0.05$) (Figure 1A, C)". this does not relate to the title about athroprone as this simply compare high shear? not clear about oxLDL to static group.

Reply 2: In our study, endothelial cells were affected by both shear stress and oxLDL. For this sentence of Line 246, we aim to describe the difference between VECs subjected to shear stress or cultured statically when they both stimulated by oxLDL. But we did not explain it accurately. This sentence has been revised. In order to describe the group more accurately, when referring to the shear+oxLDL group, we revised it as "simultaneously subjected to LSS and oxLDL", or the endothelial monolayer under oxLDL plus LSS.

Thank you for catching our defective description and helping us improve.

Changes in the text: marked green

1. Line 226: Although stimulated by oxLDL at the same concentration and duration, the VECs exposed to 25 dynes/cm^2 LSS had a marked shift in cell shape: they became elongated, spindle-like, and tended to be parallel to the flow direction, with a 26.68% increase in retention rate compared to the static controls (P<0.05) (Figure 1A, C).
2. Line 239: Endothelial cells simultaneously subjected to shear stress and oxLDL exhibited a dramatic decline in LDH release.
3. Line 251: In contrast, monolayers simultaneously subjected to 25 dynes/cm^2 LSS and oxLDL displayed a 24.97% increase in TEER compared with the static oxLDL- stimulated endothelial cells.
4. Line 271: Immunofluorescence staining of VE-cadherin in the endothelial monolayer under oxLDL plus 25 dynes/cm^2 LSS showed an increase in membrane localization of VE-cadherin.

3- there is no consistency in the text regarding which groups to compare as some time the group compare static to high shear and some time low shear to high shear etc...

Reply 3: As you pointed out, the design of our study had four groups. 1- static, 2- static+oxLDL, 3- Low shear+oxLDL and 4-High shear+oxLDL. Statistically, we conducted a pairwise comparison of each of the four groups. Due to space limitations, in the result section, we only discuss comparisons with statistically significant differences. Sometimes, the differences between the Low shear+oxLDL and static+oxLDL can not reach statistically significance. Sometimes, the differences between Low shear+oxLDL and High shear+oxLDL has a trend but it is not statistically significant. We truly describe the results of the experiment, although sometimes it is not so perfect.

4- 276 "Shear Stress Alters Endothelial Cell-cell Interactions" this is not purely shear its the combination of shear and oxLDL so title needs adjustment to clarify and this is similar in all subheading.

Reply 4: Yes, this is the combination of shear and oxLDL. The title, “shear stress under atheroprone conditions” has already taken it into account, however, the subheadings and several sentences need to be modified. Thank you for your advice.

Changes in the text:

1. Line 219: ***Shear stress affects endothelial cell viability and ameliorates endothelial barrier damage evoked by oxidized low-density lipoprotein***
2. Line 257：***Shear stress alters endothelial cell-cell interactions and reduces vascular endothelial cadherin internalization evoked by oxidized low-density lipoprotein***
3. Line 281： ***Shear stress affects endothelial cell-substrate interactions and attenuates focal adhesion remodeling evoked by oxidized low-density lipoprotein***
4. Line 337；***Shear stress decreases monocyte adhesion and transendothelial migration evoked by oxidized low-density lipoprotein***
5. Line 278：These results suggest the role of physiologically higher levels of LSS in protecting cell-cell interactions and the availability of VE-cadherin on the endothelial cell surface under atheroprone conditions.
6. Line 278：Of the endothelial cells exposed to 25 dynes/cm^2 LSS plus oxLDL, only 38.52 % were shed, displaying an evident resistance to trypsin
7. Line 299: Peripheral plaque-like staining was difficult to find in the static oxLDL-treated group (Figure 3A).
8. Line 312: whereas the simultaneous application of 25 dynes/cm^2 LSS and oxLDL resulted in fewer but larger FAs.
9. Line 329: The OxLDL challenge produced diffuse and prominent integrin β1 and FAK in static cells, and the simultaneous application of 25 dynes/cm^2 LSS and oxLDL down-regulated the expression of integrin β1 and FAK.
10. Line 341: Therefore, we investigated thecombined impact of shear stress and oxLDL on the resistance of the endothelial barrier to monocytes in a flow chamber.
11. Line 351-357: The simultaneous exposure of endothelial cells to 5 dyne / cm^2 LSS and oxLDL could alleviate this effect. The simultaneous exposure to 25 dyne / cm^2 LSS and oxLDL could further alleviate this effect by reducing the adhesion and migration of THP-1 cells (62.83% and 57.28%, respectively, $P< 0.05$ versus static+oxLDL group) (Figure 5 A, B, D, E). The expression levels of VCAM-1 and MCP-1 were also reduced in endothelial cells receiving 25 dyne / cm^2 LSS plus oxLDL ($P< 0.05$ versus static+oxLDL group) (Figure 5 C, F-H).

5- 334 "Under static conditions, compared with untreated cells,
oxLDL treatment lead to a 60% reduction in vinculin membrane fractions, a 2.09- fold increase in cytosol content, and a 2.23- fold increase in total cell lysate (P< 0.05)" not clear why we are comparing static to treated to untreated in this paper.

Reply 5: It seems like that our statement is not very clear and accurate. In this sentence, we aimed to compare VECs treated with or without oxLDL, both of which are statically cultivated. Now this sentence has been revised.

Changes in the text:

Line 319: Under static conditions, compared with non-oxLDL-treated cells, oxLDL-treated VECs displayed a 60% reduction in vinculin membrane fractions, a 2.09-fold increase in cytosol content, and a 2.23- fold increase in total cell lysate ($P< 0.05$) (Figure 3D, E).

6- figure2 and 3 staining seems visually that both low and high shear groups have the barrier disrupted compared to static and static+oxLDL and both visually seems to have less staining compared to static + oxLDL which needs clarification

Reply 6: Yes, you observe very carefully and correctly. Both the low and high shear group suffered from the same oxLDL treatment in the perfusion medium, so both groups had different levels of barrier disruption. For static culture, there were two conditions: with or without oxLDL. The most damaged is the static+oxLDL group. High shear improves oxLDL damage more effectively than low shear. In figure 2 and 3, the abnormal expression (staining) of VE-cadherin and vinculin induced by oxLDL is most severe in the static+oxLDL group.

the data presented is the result of hard work and was done well however the manuscript did not give the data the correct interpretation deserved.

the manuscript need to be focused on answering the question proposed or the question asked in my opinion need to be changed to something like role of oxLDL under different flow conditions.

Reply 7: Thank you for catching the inaccurate statement in this article. We have answered your suggestions and criticisms point by point and indicated the revisions we have made. And the article had been revised by the professional language editing service (AME). We have made every effort to to improve the language.

Thank you for your advice and consideration.

Reviewer B

The submitted manuscript titled "Adaptation of endothelial cells to shear stress under atheroprone conditions by 2 modulating internalization of vinculin and VE-cadherin" by TingtingZhong et al. is a scientifically sound research article that aims to investigate the the phenotype and barrier function of endothelial cells in response to shear stress under pro-atherogenic conditions. The results reported and further developed in a very well-written discussion provide some insight into the quantitative assessment of the response of endothelial monolayers in a parallel-plate flow chamber to laminar shear stress (LSS) and oxidized Low-Density Lipoprotein (oxLDL) in terms of cell viability, barrier Integrity, VE-cadherin availability, focal adhesion remodeling, and monocyte- endothelial interactions and are of considerate scientific interest for this specific research field, while also falling within the scope of the journal. The paper is well-written and documented, and contains an interesting discussion. We would like to advise the authors to use the following articles in their article: Cellular Mechanisms of Human Atherogenesis: Focus on Chronification of Inflammation and Mitochondrial Mutations. Alexander M. Markin, Igor A. Sobenin et al. Frontiers in Pharmacology, 11, 5 2020. https://doi.org/10.3389/fphar.2020.00642; Poznyak, Anastasia V et al. “.” Cells vol. 9,3 584. 1 Mar. 2020, doi:10.3390/cells9030584. After discussing these articles, we see no obstacles to publication.

Reply: Thank you for your kind advice. We read these two articles carefully and found that they are so interesting and greatly enrich our knowledge about atherosclerosis. We would like to put its information in the Introduction section for reference 1 and 5. It is really helpful for us to improve the manuscript. We appreciate it. We made corresponding changes to the introduction section.

Changes in the text:

LINE 59-72: Vascular smooth muscle a-actin-positive resident cells and macrophages are now considered to be the two most significant sources of foam cells. The majority of lipids during foam cell formation come from modified low-density lipoprotein (LDL), not native LDL. LDL undergoes multiple post-translational modifications (such as oxidation and glycosylation) and acquires atherogenic properties (1, 2). The endothelium serves as a semi-selective barrier between the bloodstream and surrounding tissues, and controls the exchange of proteins, solutes, and liquid, as well as the migration of cells. If the permselective barrier function of the endothelium is weakened, molecules, such as modified LDL, and monocytes will enter the subendothelial space via circulation to initiated chronic inflammation and immune disorders. According to current consensus, the primary event in atherosclerotic lesion development is local endothelial activation and increased permeability, which may be associated with hemodynamic forces occurring at the sites prone to atherosclerosis (3-5).