Author’s response to reviews

Title: Rapamycin Prevents Retinal Neovascularization by Downregulation of Cyclin D1 in a Mouse Model of Oxygen-Induced Retinopathy

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Author’s response to reviews:

Dear Editors:

Thank you for your letter and for the reviewer’s comments concerning our manuscript entitled “Rapamycin Prevents Retinal Neovascularization by Downregulation of Cyclin D1 in an Oxygen-Induced Retinopathy Model” (BOPH-D-19-00251). The comments are all valuable and very helpful for revising and improving our paper, as well as providing important guidance significant to our research. We have studied the comments carefully and have made several corrections which we hope will meet with the reviewer’s approval. Revised portions are marked in red in the paper. The main corrections in the paper and the responses to the reviewer’s comments are as follows:

Belal Azab, Ph.D (Reviewer 1):

Concern 1. English language revision is needed, some sentences are not clear and vague, especially the methods section.

Response: We are very sorry for our non-clear and vague writing in the paper. We have this manuscript been reviewed by someone who is fluent in English, and made several corrections to the manuscript.

Concern 2. Figure 1 (B and C), the legend should specify, where the neovascularization is, like how the nonperfused area is point on by the arrows. Figure 2 should specify what the arrows
stand for. In overall the figure legends should be more descriptive and points out all elements of the figure.

Response: We thank the reviewer for the suggestion. As shown in REVISED Fig 1, we have used red arrows to show where neovascularization is. Also, we add more description of the figures in the Figure Legend.

Original:

Revised:

Original: Fig 1. Vascular patterns in retinal flat mount after FITC–dextran perfusion (40×). (A) Control group (without treatment) retina shows perfused well vessels and no neovascularization. (B) OIR group retina presents nonperfused area and neovascularization. (C) RAPA ameliorates the change in OIR retina. White arrow, non-perfused areas.

Fig 2. Light micrograph of retinal cross-sections from P17 mice. H&E 40×. (A) Control group (without treatment) retina. (B) OIR group retina. (C) RAPA group retina. (D) Graphs showing the average number of neovascular nuclei per retinal cross-section. * p < 0.001, significantly different from the CON group; # p < 0.001, significantly different from the OIR group.

Revised: Fig 1. Vascular patterns in retinal flat mount after FITC–dextran perfusion (40×). (A) Control group (without treatment) retina shows perfused well vessels and no neovascularization. (B) OIR group retina presents nonperfused area and neovascularization. (C) RAPA ameliorates the change in OIR retina. White arrow shows non-perfused areas. Red arrow shows neovascularization on retina.

Fig 2. Light micrograph of retinal cross-sections from P17 mice. H&E 40×. (A) Control group (without treatment) retina. (B) OIR group retina. (C) RAPA group retina. (D) Graphs showing the average number of neovascular nuclei per retinal cross-section. * p < 0.001, significantly different from the CON group; # p < 0.001, significantly different from the OIR group. Black arrow shows vascular cell nuclei broke through ILM. (page 6-7, line 171-185) (Revised)

Concern 3. I think the methods should be explained in a way that helps the reader understands why each experiment is used in this study.

Reponses: We thank the reviewer for the excellent suggestion and have rewritten parts of Materials and Methods according to your suggestion.
Four animals from each of the three groups were anesthetized intraperitoneally with pentobarbital sodium (50mg/Kg). Mice were perfused with fluorescein isothiocyanate (FITC)-dextran (Sigma, St. Louis, MO, USA) through left ventricle. Then eyes were enucleated (euthanized intraperitoneally with pentobarbital sodium 800mg/Kg) and fixed in 4% paraformaldehyde at 4°C for 12 hours. Retinas were isolated, flat-mounted on glycerol/gelatin-coated glass slides, and viewed by fluorescent microscope (Zeiss, Oberkochen, Germany), and photographed. Non-perfused areas of retina were quantified by Image-Pro plus 6.0 analysis software for statistical analysis.

Retinal flat mounts were used to show the nonperfusion area and neovascularization on the retina. Four animals from each of the three groups were anesthetized intraperitoneally with pentobarbital sodium (50mg/Kg). Mice were perfused with fluorescein isothiocyanate (FITC)-dextran (Sigma, St. Louis, MO, USA) through left ventricle. Then the eyes were enucleated after euthanasian (intraperitoneally with pentobarbital sodium 800mg/Kg) and fixed in 4% paraformaldehyde at 4°C for 12 hours. Retinas were isolated, flat-mounted on glycerol/gelatin-coated glass slides, and viewed by fluorescent microscope (Zeiss, Oberkochen, Germany), and photographed. Non-perfused areas of the retinas were quantified by Image-Pro plus 6.0 analysis software for statistical analysis.

The degree of RNV was quantified by counting the number of vascular cell nuclei that had broken through the internal limiting membrane (ILM) into the vitreous.

Two eyes of two mice from each group were marked for orientation, enucleated and placed in 4% paraformaldehyde at 4°C for 24 h, after which they were embedded in paraffin. Serial 5 μm sections (each separated by at least 30 μm) through the cornea and parallel to the optic nerve were prepared, stained with hematoxylin and eosin (H&E), and viewed by light microscopy (OLYMPUS Optical Co., Ltd., Japan), for the assessment of the retinal vasculature. The degree of RNV was quantified by counting the number of vascular cell nuclei that had broken through the internal limiting membrane (ILM) into the vitreous.

Concern 4. Rapamycine is known to be somehow safe to the eyes, but it could be a good point to add any side effects this drug has on other organs. It is suggested that it has a potential use as a treatment of retinal neovascularization-related diseases, but it could cause a problem elsewhere, so the potential harm should also be included somewhere in the manuscript, or even the it should be stated that there is no side effects if there is none.
Response: Thank you for your suggestion. It is really true as the reviewer suggested that we need to add if there were any side effects of RAPA on other organs. We had have seen any side effects during our experiments. We have rewritten parts of RESULT according to your suggestion.

Original: RNV was examined in retinal flat mounts using FITC-dextran at P17.

Revised: No side effects were observed in mice given RAPA. RNV was examined in retinal flat mounts using FITC-dextran at P17. (page 7, line 206-223) (Revised)

ELENA Pacella, Ph.D. (Reviewer 2):

Concern 1. I congratulate the authors. The study design is appropriate and correct, has achieved significant results, has a current relevance in clinical practice.

Response: We appreciate the reviewer’s favorable comments.

Concern 2. The study shows that RAPA downregulates the expression of cyclin D1, inhibiting the retinal vascular system could represent a therapeutic potential in the treatment of RNV-related diseases with a different mechanism, compared to the other molecules currently used "anti-VEGF or glucocorticoids" in these diseases, recommend

- Discussion

Add the following reference to line 53 pag. 6


and therefore to be able to preserve the vision of the patient suffering from RNV diseases.

It would be interesting if the authors had indicated, in this work, also the modalities of a possible clinical application in patients suffering from RNV.

Reponses: We thank the reviewer for the suggestion. We have added reference and rewritten parts of the discussion according to your suggestion. (page 6-7, line 171-185) (Revised)
Original: These results demonstrate that RAPA could inhibit retinal vascular endothelial cell proliferation and RNV, with the underlying mechanism of arresting the cell cycle in its transition from the G1- to S-phase via inhibition of cyclin D1 activity.

Revised: These results demonstrate that RAPA could inhibit retinal vascular endothelial cell proliferation and RNV, with the underlying mechanism of arresting the cell cycle in its transition from the G1- to S-phase via inhibition of cyclin D1 activity, which is different from current clinically used anti-VEGF drugs or glucocorticoids [17, 18].