Peer Review File

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Reviewer A

I must point out several areas of concern:

1. General grammar/spelling errors should be addressed.
2. Quantification of of rosette areas appears subjective and needs to be recalculated by excluding non-retinal areas.
3. If bleaching is incompatible with staining of wholemounted retinas from pigmented strains then this should be explained/described in detail. Which stains are incompatible? Also, if this method is limited to non-pigmented retinas, its usefulness is quite limited.

ARRIVE GUIDELINES 2.0 – Met by authors
RECOMMENDED SET – Followed

> ***To reviewer A's concerns and comments, grammar/spelling errors were addressed and corrected. Quantification of rosette areas were recalculated by excluding non-retinal areas, and then the areas were normalized by unit area. The normalized areas and the ratio of rosette areas to the actual retina area were graphed. The staining method that was incompatible with the bleaching (hydroperoxide) method used is now explained. We address these comments one by one, below.***

COMMENTS

1. Methods state that C57BL/6J wholemounts were bleached. Where is the data?

> ***Reply1: The data of rosettes formed by sodium iodate in C57BL/6J mice were in Supplemental Figure S3 (New, Lines 230-233). Rosette formation was reported in rats, but not enough has been described in mice. Here, we studied majorly rosette formation induced by sodium iodate in the non- pigmented wholemount retinas of BLAB/C mice, but we checked as well the rosette formation in the pigmented whole mount retinas of C57BL/6J mice after hydroperoxide bleaching.***

2. Methods for rosette area calculation (Line 160)

- States that for each calculation, the 1500x1500pixel area was used as total area, and the area of rosettes (aqua) deleted from that total.
- However, the total area should be recalculated by excluding areas that do not include retinal tissue (purple region).
- Example areas that are missed, yet appear to contain rosettes (yellow) – not exhaustive.

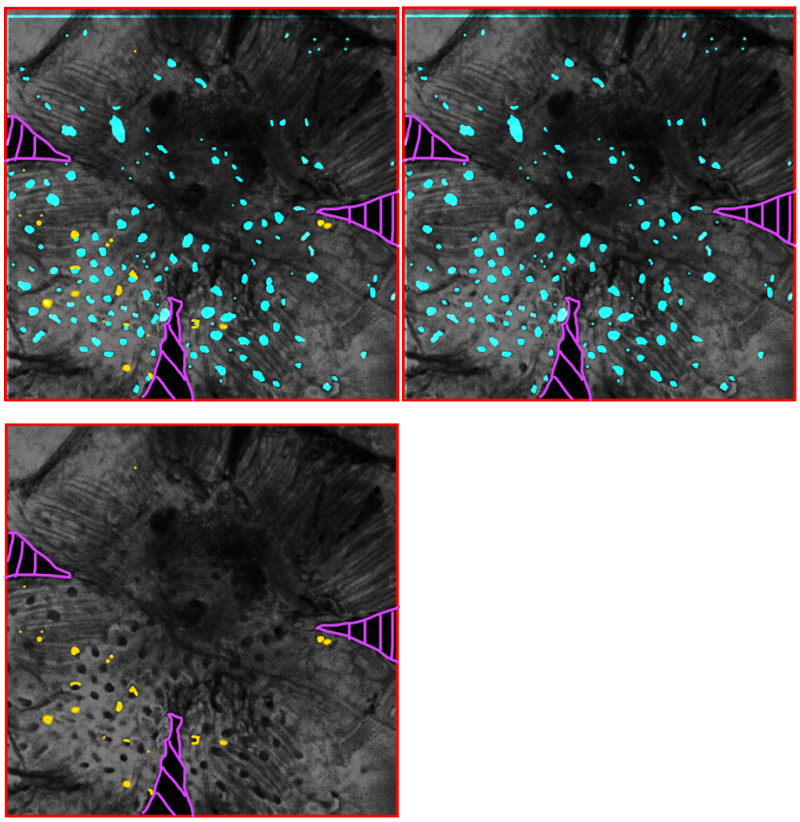

Image above – derived from Figure S3

This method seems to be very inaccurate. Analysis should be performed again with attention to detail in the determination of rosette area and total retinal area.

> ***Reply2: According to the reviewer A's comments, we re-calculated the occupied rosettes areas by excluding areas that do not include the retinal tissues (purple region above). We quantified areas by using ROI particle analysis in ImageJ and added the area ratio of rosettes to retina region in a graph. We revised the manuscript: methods (New, Lines:152-168), Results (New, Lines 237-242; 244-253), and updated Figure 7 and its legend (New, Lines 453-459), and Supplemental Figure S1 (Old, S3), and the legend (New, Lines 465-472).***

3. Discussion (lines 281-284) implies that absence of bleaching pigmented strain retinas for wholemount (eg. C57BL/6J) is incompatible with some staining – but this explanation or evidence for this is not indicated within the references provided (10 & 14).

Reply3: One of the reference papers (Fig 6, 2016, TVST) indicates that the bleaching method hindered CD11b antibody staining and endogenous GFP signal. However, we realized that the second paper (2021, bio-Protocol) did not include the information that phalloidin staining, a commonly used technique, is hindered by the bleaching. We remove both references in that part of the discussion, and now clearly state that the bleaching step

hinders the phalloidin staining. Further, we discussed that it is required to validate the compatibility of antibody staining such as ZO-1 or cadherin with a bleaching method for the application of this method for pigmented mice (New, Lines 277-280).

4. Figure 4: Figure legend does not state strain of mouse.

   Reply4: The strain of mouse, BALB/C, was added to the Figure 4 legend (New, Lines 433)

5. Figure 5: Asterisks defined text (line 226 & 231), but not figure legend itself.

   Reply5: We defined "Asterisks-choroidal vasculatures" in the Figure 5 legend (New, Lines 444-445): Choroidal vasculatures (asterisks) in normal and SI-damaged retinas.

6. Figure 7: Figure legend states 'Figure 5'

   Reply6: It has been corrected. The legend of the Figure 7 states the updated "Figure 7".

7. Retinal wholemount analysis indicates location as a measure from the centre towards the periphery, but does not indicate superior/inferior nasal etc.

   Reply7: During the eyeball preparation, we did not indicate eyeball location information. Analyzation of the rosettes formation, based on the retina location (i.e., the superior/inferior/nasal), is beyond the current study. In the future, the relationship between the formed rosettes and retina location could be clarified.

Reviewer B

In their study, the authors describe features of RPE-whole mounts of animals injected with sodium iodate. The study has several shortcomings, including the design, the discussion and the language.

We appreciate the concerns and comments made by reviewer B. the manuscript was improved by addressing the comments. We describe the changes one by one.

Major concern
The number of animals per group is arbitrary and, concerning many of them, much too small with n = 1 or n =3. In addition, no statistics are provided. This is not sufficient for a scientific publication

Reply1: This report is based on an observation study. We hypothesized that there are rosettes in BALB/C mice, and we clearly exhibited the formed rosettes and the possible application of rosettes quantification in sclerochoroid/RPE/retina whole mounts for the translational study. In the observation study to answer the question hypothesis, we used a total of 64 eyeballs of rats and mice: 10 eyeballs of rats, 10 eyeballs of pigmented mice and 44 eyeballs of non-pigmented mice. We finally added a total number of animals used in method (New, Line 103).

Abstract
I have a hard time understanding the abstract and I am not sure what exactly the new findings in this project are.
Please introduce abbreviations at first mention.
What is meant by "mingled/mixed" nuclei?

Reply2: To clarify the purpose of this study, we revised the Background of the Abstract, but the abbreviation was not used in Abstract according to the journal rule. "Mixed nuclei" mean that nuclei lamination in normal retina is disorganized in Sodium iodate-induced retina damage, and the nuclei from photoreceptor, RPE and choroid layers are observed in one-Z position of confocal images.:
(Old, Lines 30-36): The recently developed sclerochoroid/retina pigment epithelium/retina whole mount method allows us to observe the integral horizontal view of subretinal layer which is not easily addressed in general whole mounts of either retina or retina pigment epithelium side because the subretinal layer should be separate. In this study, subretinal and neighboring layers in retina degeneration induced by sodium iodate were exploited by non-separate sclerochoroid/retina pigment epithelium/retina whole mounts to observe rosettes and to develop the morphological analytic assessment.)
→ (New, Lines 30-37) Sodium iodate is a chemical widely applied to induce retina degeneration in animal models. Sodium iodate treatment caused formation of rosettes/folds in the outer nuclear layer of the rat retina, but it was previously unclear whether sodium iodate also forms rosettes in mice. In addition, sodium iodate induced retina degeneration was never addressed in non-separate sclerochoroid/retina pigment epithelium/retina whole mount. Here we displayed features of retina degeneration including rosette formation in mice and developed a morphological analytic assessment using sclerochoroid/retina pigment epithelium/retina whole mounts.

(Old, Lines 47-48) displayed mingled/mixed nuclei from choroid to photoreceptors at single horizontal images in the sclerochoroid/retina pigment epithelium/retina whole mounts
→ (New, Lines 47-49) displayed mixed nuclei from choroid to photoreceptors, due to layer disorganization, as shown by single horizontal images in the sclerochoroid/retina pigment epithelium/retina whole mounts.

Introduction
The claim that dry AMD has a similar pathology to RP is simply wrong.
"kind of genetic problem" is not an appropriate term to describe RP.

What is meant by “examined majorly”?
I do not understand how the whole mounts relate to the pigmentation.

Reply3: We removed the sentence that Dry form of AMD, geography atrophy (GA), has similar pathological features to RP (Old, Lines 68-69). Additionally, we added the differences between RP and AMD in manuscript (New Lines 65-67: While RP results in photoreceptor degeneration and peripheral vision loss, caused by genetic mutations, aging is the major contributor to macular RPE degeneration in AMD pathogenesis.)
We removed “majorly” from “further majorly examined” because “further” seems to be descriptive enough (Old, Line 83, New Line 84), and the current study limitation and future clarification on the whole mount method application in pigmented mice is discussed (New, Lines 277- 280): it will be advantageous to validate the compatibility of other types of antibody staining, such as ZO-1 and cadherins, to outline the RPE cells when a bleaching step in sclerochoroid/RPE/retina whole mount preparation of pigmented mice is necessary.

Methods
What is meant by “according to policies” (line 104)?
As mentioned above, the number of samples is not sufficient.
There is no statistics mentioned in the method section.
Microscopy, has there been any normalization done to the pictures, any randomization in taking the pictures, any protocol on how many picture were taken per slide?
Line 167, 168, were the eyeballs excluded because of poor handling included or excluded in the n number of animals provided before?

Reply4: We reply questions or describe the updates one by one:

1. (Old, Line 94) according to policies → (New, Line 92) according to institutional policies.

2. Please see the answer of replay 1: (Old, Lines 99-100) The eye numbers examined are as follows → (New, Line 103) A total 64 eyes were examined. The details were as follows

3. Basically, the report is based on the observation, although we quantified the rosette areas induced by sodium iodate. We described that the assessment and quantification was graphed by Prism 6, but we did not use statistics for the difference and comparison between control/normal and SI groups because the difference is distinct, and does not require the statistics.

4. We added the detailed description on image capture:

(Old, Line 145) Image process and measurement → (New, Line 151) Image processing and area quantification of rosettes

(New, Line 154-160) First the optic nerve head was center-XY positioned, and the area was focused and center-Z positioned. Second, while defining the tile regions to cover all areas of the whole mounts, several local areas were focused to verify the z range, and two local areas were chosen to define the first and last of Z position. The acquisition of 8 x 8 tiles and 3 z-slices, under a 10X objective lens, covered the areas of interest in the retina whole mounts, including the ONL depth. The images were stich-processed and projected into a single image for quantification of rosettes.

5. We allotted 4 eyeballs for Week 1 and Week 3 of SI each, but 1 eyeball of Week3 was not included for whole retina scanning, because the large area was damaged by scissors: (Old, Lines 151-153) The eyeballs scarred by scissors due to handing errors were excluded for the measurement → (New, Lines 169-172) Rosette areas were quantified from 6 eyeballs of normal, 4 eyeballs of Week 1 and 3 eyeballs of Week 3. One eyeball allotted for the whole mount of Week 3 (Table 1) was excluded in the quantitation because part of eyeball was scarred by poor scissor handling.

Results

The first paragraph of the result section belongs either in the introduction or in the discussion. Generally, I have a hard time understanding the new findings here.

The authors mention a statistical differences (or the lack hereof, line 256 following) but do not explain how this was calculated.

Reply5: According to the reviewer B's comments, we updated the manuscript by removing the first paragraph from the Results, but some part of the first paragraph of the result was moved into the second paragraph of the Introduction part. For the Second question, we answered at the replay 4.3. The other changes are:

(Old, Lines 71-72) SI-induced retina damage, since it was introduced in 1941, has been applied widely for both basic and translational studies. However, the pathological features

→ (New, Lines 72-75) SI-induced retina damage has been applied widely for both basic and translational studies. SI is known to directly damage RPE and indirectly manage adjacent photoreceptors. Systemic single injection of SI causes complete destruction of RPE and photoreceptor segments and induces macrophage and microglia infiltration in the subretinal space

Figures

The alterations that the authors describe in the text are hard to see in the pictures.

Figure 1, I recommend to show the desired alterations in higher magnification.

Figure 2, why are there 2 rows of "B", what is the difference between them? Also please indicate which color stains what molecules. Please indicate the number of pictures taken in total. Please indicate from where exactly in the eye the pictures were taken.

Figure 3, it is not clear where the different regions (1-3) are located. From the figure, they all seem to be at the same place in the mount.

Figure 4, please indicate which channels are seen in which picture. Also, please indicate what is seen in the black and white picture.

Figure 5, I do not understand what I am seeing here. Please give correct specifications for a-d.

Figure 6 is depicted as Figure 4 (?).

Figure 7 is depicted as Figure 5 (?). It is not clear what is to be seen in the pictures. Please magnify.

Reply6: We updated the figures, figure legends and manuscript according to reviewer B's suggestions:

Figure 1: The magnified images were added.
Figure 2: The differences between 2 rows of Figure 2B were explained in Figure2' legend. The below one is overlaid DIC images.
Secondary antibody was also indicated in the figure legend with primary antibody in Figure 2' legend.
Figure 3: R1 is more centre-located and R3 is peripheral. We described the location of R1-3 in Figure 3' legend: R1 is near to optic nerve head and R3 is outside the muscle, and R2 is between R1 and R3.
Figure4: In Figure4, the channels were added in each image, and the black and white image of three channels was removed because it was not helpful to readers, and redundant with channel images.
Figure 5: Specification of a-d was explained in Figure5 legend.
Figure 6: Label is correctd.
Figure 7: Magnified images were inserted.
The Figures and the Figure legends were all correctly checked, and the legends were updated.

Discussion
The discussion is hard to follow. The authors are vaguely talking about aspects like "looks less damaged" etc, which is not a scientific discussion.
Also Line 278 following, what is meant with "SI makes retina of mice thinner quicky, disappearing even formed rosettes…", also lines 289/290, I simply cannot understand the authors' English.
What exactly is the meaning of the rosettes? What consequences do they have?
What is new about these findings that have been done in a well-known model?

Reply7: In the Discussion, we now explain what our founding is, and we removed vague language like "looks less damaged". For the part of Discussion about FAF in BLAB/C, we also clearly reformed. Rosettes/folds here means ONL undulations, thus we tried to keep both words-"rosettes/folds" at least once in each section and added the definition in the discussion part.

(New, Line 259) SI-formed ONL undulations referred to as rosettes/folds

(New, Lines 259-263) the SI-formed ONL undulations referred to as rosettes/folds were not clearly described and reported in mice. Here, we used non-separate sclerochoroid/RPE/retina whole mounts to maintain retina layer integrity, and observed RPE degeneration, RPE inward movement, retinal layer dislocation, photoreceptor degeneration and ONL rosettes from a new vantage point.

(Old, Lines 245-255) The comparison of SI-induced retina damages between C57BL/6J and BLAB/C mice displayed all similar pathological aspects of retinal degeneration between them, but BLAB/C looks less damaged than C57BL/6J, and BLAB/C displayed distinct fundus autofluorescence (FAF) compared to C57BL/6J, which led us to hypothesize that SI induces

rosettes formation in BLAB/C mice, and the rosettes display FAF, but Here we found that SI induced ONL folds/rosettes in both C57BL/6J and BLAB/C mice as well as SD rats. The its detailed examination is beyond the purpose of this study. However, we suspect that the reason of distinct FAF in SI-damaged retina of BLAB/C mice might be due to the different severity of degeneration: SI makes retina of C57BL/6J mice thinner quickly, disappearing even formed rosettes, while less severe retina degeneration in BLAB/C might maintain rosettes longer time than C57BL/6J.

-> (New lines 264-270)

we initially hypothesized that SI would induce rosette formation in BLAB/C mice, we thought it might not in C57BL/6J, based on published literature, because BLAB/C mice display distinct fundus AF (FAF) compared to C57BL/6J, and FAF can be caused by rosettes. A detailed investigation on the reason why FAF was distinct in BLAB/C is beyond the purpose of this study, but we suspect that the reason of distinct FAF in SI-damaged retina of BLAB/C mice might be due to a difference in the severity of degeneration and/or AF. SI makes the retina of C57BL/6J mice thin quickly, resulting in the disappearance of formed rosettes, while less severe retina degeneration in BLAB/C might maintain rosettes for a longer period of time, and there might be different microglia and macrophages recruited, causing AF.

One sentence was added at (Old, Line 255; New, Line 273-274): **here might be different microglia and macrophages recruited, causing AF**

Language

The language is sometimes not understandable for the reader. A native speaker is strongly suggested to review the paper and make it understandable for potential readers.

Reply8: A native speaker has helped us to edit our English. Thank you