Peer Review File

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First External Peer Review

Reviewer A

Comment 1:

Introduction: there is no mentioning of previous thrombosis models in zebrafish: see ref 5: e.g. FeCl3, phenylhydrazine, laser-induced (e.g. Jagadeeswaran et al. Methods Mol Med. 2006;129:187–195, O'Connor et al Blood. 2009;113:4754-62 and others), but also no mentioning of the already published use of bengal rose to induce thrombosis in ZF larvae (Lee et al J Biophotonics. 2017;10:494-502) and to induce stroke in adult zebrafish (Yu, Li. Acta Neurochir Suppl. 2016;121:115-9). Please refer to these and indicate not only why using zebrafish has advantages over other animal models, but also why bengal rose would be a better model in zebrafish, and why larvae are used and not adult fish (as nowhere the possibility of genetic modification is mentioned).

Reply 1: We acknowledged the reviewer's precious comment. So we mentioned the previous zebrafish thrombosis studies and referred to the other studies (see Page 5, line 90-91). We mentioned the description about the advantages at the cost effectiveness than other higher vertebrates (see Page 5, 88-90). we added the advantage of rose bengal usage and the real time angiography in the larval stage (see Page 5, line 92-Page 6. line 99)

Changes in the text: In particular, intravascular thrombosis animal models using larvae and adult zebrafish were introduced in many studies (5,6,8,9). Mechanical stimulation introduced intravascular or extravascular manners to make a thrombosis (8-11). Photochemically induced thrombosis using Rose Bengal in zebrafish was more feasible to make intravascular thrombosis than another mechanically induced thrombosis. Specially zebrafish larvae present a more translucent body than adult zebrafish (12). Also, genetic modifications of zebrafish maximize in vivo angiographic features. Compared with the AB strain wild-type zebrafish, Tg (flk:gfp) transgenic zebrafish are genetically transformed to express a green fluorescent protein (GFP) within the endothelial cells (12). This transgenic fish allows continuous *in vivo* observation of the vertebrate embryonic vasculature throughout embryogenesis.

Comment 2:

l.93: "This model has enabled the development of new drugs for human diseases.": references needed.

Reply 2: We acknowleged reviewer's comment. However, we thought this sentence is not

appropriate to connect two paragraphes. So we deleted this sentence.

Changes in the text:

Delete “These models has enabled the development of new drugs for human diseases.”

Comment 3:

M&M: overall much more detail on how the experiments have been done is needed, the basic rule is that the description should allow somebody else to repeat the experiments.

Reply 3: The authors acknowledged the reviewer’s comment. We added more detail on how the experiment method.

Changes in the text: We added replies over comment on M&M.

Comment 4:

- using tricaine-3-aminobenzoic 122 acid (0.4 g in 100 mL ethyl ester, adjusted to pH 7 with Tris buffer).: not clear how this was used.

Reply 4: The 7 dpf zebrafish larvae were anesthetized with a solution containing a mixture of the tricaine-3-aminobenzoic acid 0.4 g and 100 mL ethyl ester, adjusted to pH 7 with Tris buffer.(See page 7, 123-124)

Changes in the text: We anesthetized the seven dpf zebrafish larvae (total n=8) using a solution containing a mixture of tricaine-3-aminobenzoic acid (0.4 g) in 100 mL ethyl ester, adjusted to pH 7 with Tris buffer.

Comment 5:

- l127: measured area=399585.09 ㎛2: are all these numbers relevant?

Reply 5: Yes, this number is the average skull area focused by the confocal microscope. (See Page7, 128-129)

Changes in the text: and an area (measured area=399585.09μm^2) in the skull of the larvae was focused ~

Comment 6:

- to induce intracerebral thrombosis: what blood vessel? artery/vein?

Reply 6: Rose bengal and rhodamine mixture were injected into the cardinal vein of the larvae. However we could not focus the exposure of laser on the artery or vein separately.(See Page 7, line 130) In next research, we will utilize the selective confocal laser micoroscope.

Changes in the text: to induce thrombosis in intracerebral vessels

Comment 7:

- l131: “The thrombosis model was validated using tPA injection”: details? How much, how administered, at what time after induction of thrombosis…

Reply 7: To validate the thrombosis model, we tested the thrombolytic activity of tPA on our zebrafish photochemical thrombosis model. The larva was removed from the microscope and injected with tPA. After injection, the larva was placed back in the microscope again to observe the thrombolysis. tPA was injected into larvae approximately 120s after photochemical thrombosis. The blood cells ceased to adhere to the clots when the larva was injected with tPA(intravascular concentration 225μg $mℓ^{-1}$) (See Page 7, line 132-137)

Changes in the text: To validate the thrombosis model, we tested the thrombolytic activity of tPA on our zebrafish photochemical thrombosis model. The larva was removed from the microscope and injected with tPA. After injection, the larva was placed back in the microscope again to observe the thrombolysis. tPA was injected into larvae approximately 120s after photochemical thrombosis. The blood cells ceased to adhere to the clots when the larva was injected with tPA(intravascular concentration 225μg $mℓ^{-1}$)

Comment 8:

- l137: “rhodamine and t-PA were injected after photoactivation” and “stained with O-dianisidine”: how? how much?

Reply 8:

To format the intracardiac thrombosis model and validate the thrombolysis of tPA, the larvae were injected with rhodamine and t-PA in the same manner and doses in intracerebral thrombosis formation and validation. The O-dianishine staining method was to quantify the heart red cells (RBCs) within zebrafish. Larvae placed in 0.1% DMSO were considered to be the vehicle control. The morphology of zebrafish larvae was evaluated after incubation at 28°C for 24h. In brief, 5 zebrafish larvae of each group were stained with 1.0mg/mL O-dianisidine dye liquor for 15min and washed with DMSO three times (Chen et al., 2018). (See Page 8, lines 146-149)

Changes in the text: For quantitative analysis of thrombosis in the zebrafish heart, laser-exposed zebrafish larvae were stained with O-dianisidine to quantify the heart red blood cells (RBCs). Larvae placed in 0.1% DMSO were the vehicle control. The morphology of zebrafish larvae was evaluated after incubation at 28°C for 24h. In brief, 5 zebrafish larvae of each group were stained with 1.0mg/mL O-dianisidine dye liquor for 15min and washed with DMSO three times as described by Chen et al. 2018. (14)

Comment 9:

Results:

- l340: Fig1: “Blood flow in intracerebral arteries disappeared”: what about veins?,

“and endothelial linings' fluorescence intensity slightly decreased”: please indicate on the figure where this can be seen

Reply 9:

We replaced the word “arteries” with “vessels”. We added the circle in figure 1 to indicate the endothelial lining’s decreased intensity. (See page 18, line 352, Fig1 B, E)

Changes in the text: Blood flow in intracerebral vessels disappeared, and endothelial linings' fluorescence intensity slightly decreased (D-F).

Comment 10:

can an indication be given on the figure on how large the laser-exposed area was. At what time after the 10 min laser are the pictures D-F taken? Did the larvae survive this? And if not, would this apparently massive cerebral thrombosis be a good stroke model?

Reply 10: We describe the whole area covered by the confocal laser and the average area (measured area=399585.09 ㎛2). We took a picture of the larvae 24 hrs later after 600 seconds of laser exposures. In preliminary experiments followed by Lee et al J Biophotonics. 2017;10:494-502, 750s exposure could induce massive cerebral thrombosis and death. After 600s exposure, all fishes were alive the next day.

Changes in the text: no change.

Comment 11:

- l343 Fig.2: unclear what this shows: real time imaging: where is the time scale? What are the red figures, how are the grey/white figures generated? What are the three dimensions? Representative of how many repeats? Statistics?

Reply 11: The red figures were indicated as *in vivo* angiography and the grey/white figures were generated by the dye intensity reflecting the blood flow/cross-sectional area of a vasculature. We added the sentence “(In vivo angiographies and blood flow chart per cross-sectional area of total vasculatures)” (see page 18, lines 355-356). We indicated the time flow on a large black arrow (After 10 minutes) and the “Control” and “Rose Bengal” groups (See Fig.2) Three dimension figures are quantitatively summations of blood flow per cross-sectional area of the whole length of vasculatures. The each group included 4 fish. We were able to check the decreased blood flow by comparing the area, not statistics.

Changes in the text: Figure 2. Real-time imaging of thrombosis in the vasculature (In vivo angiographies and blood flow chart per cross-sectional area of total vasculatures).

Figure 2

Comment 12:

- l.349: Fig.3 please indicate also here that this is two days after photoactivation

Reply 12: We added the indication in figure 3 (C) " 2 days after photoactivation". And we added the statistics to chart (C). (see page 18 lines 361-365)

Changes in the text: Figure 3. Analysis of vascular endothelial cell apoptosis at pre-and post-photoactivation (after 2 days). (A) In vivo angiography of the control group, (B) In vivo angiography of photosensitizer injection group (c) fluorescence intensity: Control: 27.83±0.56 (mean, standard deviation) photocoagulation (Rose Bengal + rhodamine): 17.28±2.08 (measured by Image J1.52a software). (*, $p<0.01$).

Comment 13:

- l.355: Fig.4: give more details: state that this is in the dorsal longitudinal vein, how much time after photoactivation.

- l.186: SD? Statistics?

Reply 13: We added the indication for the "Rose Bengal" group and the " Rose Bengal+tPA" group and the time flow on the black arrow. We indicated the "Dorsal longitudinal vein"(see Figure 4). We added the sentence " Compared to Rose Bengal (n=4) group, Rose Bengal +tPA (n=4) group shows a decreased thrombus area (49.78±7.090 %, mean, standard deviation)." (See page 10 195 and page 18, lines 369-370)

Changes in the text: Figure 4. legend (C) Comparison of thrombus area using Image J1.52a (Mann Whitney test, $P<0.05$). Rose Bengal +tPA (n=4) group shows a decreased thrombus area (49.78±7.090 %, mean, standard deviation). and Figure 4

Comment 14:

- l.360: Fig.5: do I understand correctly that upon photoactivation, less red blood cells are present in the intracardiac area? Is the blood supply to the heart blocked? Did the larvae survive?

Reply 14: Yes. Correctly understood. The stained RBC in the larvae. All larvae survived. These depend on laser the exposure time. We added the sentence the statistics as well.(see page 10 line 200-203)

Changes in the text: After exposure to a confocal laser for 15 min, the RBC intensity in the heart markedly decreased in the Rose Bengal group (24.33±2.899%) (Figure 5B) compared with the control (Figure 5A) and Rose Bengal + tPA groups (63.53±14.07%) (Figure 5C).

Comment 15:

Discussion:

l.245: "the clotting time of zebrafish plasma is shorter than that of humans (22). The clotting time of zebrafish plasma is also much shorter than that of human plasma (23)." twice the same statement?

Reply 15: We deleted the duplicated sentence. (See Page 12-13, lines 253-255)

Changes in the text: The density of thrombocytes (24) is lesser, and the clotting time of zebrafish plasma is shorter than that of humans (25).

Reviewer B

Comment 1:

Cerebrovascular events are associated with significant co-morbidity with resultant mortality in certain cases. The authors in the study have highlighted the role of tPA using real time imaging in Zebrafish models. It would be interesting to evaluate this in human studies with obvious ethical clearance. The authors should be commended on their efforts in undertaking this vital research.

Reply 1: Yes, we do

Comment 2:

The dysfunction of the endothelial glycocalyx is the main proponent to formation of vascular thrombosis.

Reply 2: Yes we acknowledge the reviewer's comment.

Comment 3:

The choice over the use of the Zebrafish compared to other mammals is justified from an ethical perspective and reflects responsible research with integrity

Reply 3: Yes we agree

Comment 4:

Could the authors kindly evaluate how their research provides additional information to already performed procedures e.g., catheter-directed thrombolysis using t PA?

Reply 4: We acknowledge the reviewer's comment. Unfortunately, the introduction part is already too long. We could not evaluate the additional information of other procedures.However, we added the previous study to evaluate the tPA in other animals.

Changes in the text: none.

Comment 5:

The study is comprehensive with the analysis of the results from each of the treatment group.

Reply 5: Yes, we acknowledged the reviewer's comment.

Comment 6:

With the increased use of early goal directed therapy, early management of intracardiac

thrombosis could potentially prevent more proximal propagation of the thrombus.

Reply 6: We acknowledged the reviewer's comment.

Comment 7:

The authors have also highlighted the main limitations to their study, although comparative research has been undertaken these results may not be extrapolated in human subjects.

Reply 7: We acknowledged the reviewer’s comment.

Second External Peer Review

Comment 1: For Reply 1

"Mechanical stimulation introduced intravascular or extravascular manners to make a thrombosis (8-11)."

Comment 1a: In the studies mentioned, no mechanical stimulation has been used: all are either chemical or irradiation damages

Reply 1a: Authors admitted reviewer's comment. We exchanged the word "mechanical" with "chemical or irradiation damage".

Changes in the text: Chemical or irradiation damage introduced intravascular or extravascular manners to make a thrombosis (8-11)

"Photochemically induced thrombosis using Rose Bengal in zebrafish was more feasible to make intravascular thrombosis than another mechanically induced thrombosis."

Comment 1b: again these are not mechanically induced thrombosis models

Reply 1b: We admitted reviewer's comments. We changed the word "mechanically" with "chemical or irradiation damage".

Changes in the text: Photochemically induced thrombosis using photosensitizer and focal irradiation are more feasible to make site specific intravascular thrombosis than other chemical or irradiation damage induced thrombosis (8).

Comment 1c: why is Rose Bengal administration and irradiation more feasible then e.g.laser-induced injury?

Reply 1c: Through photochemical excitation, a thrombus was induced to form at a selected section of the vessels of larval zebrafish, which had been injected with photosensitizers. Sosuch photochemical thrombosis can be controlled to occlude the target vessel.

Comment 2: For Reply 4

"We anesthetized the seven dpf zebrafish larvae (total n=8) using a solution containing a mixture of tricaine-3-aminobenzoic acid (0.4 g) in 100 mL ethyl ester, adjusted to pH 7 with Tris buffer."

Were the larvae put in this ethyl ether solution?

Reply 2: Yes. They were.

Comment 3: measured area=399585.09 ㎛ 2: are all these numbers relevant? I meant is the accuracy up to 8 digits OK? Or something like 400.103 µm2 better?

Reply 3: Authors acknowledge your consideration**.** We change the numbers into 3.99585 x 10^5 μm^2

Comment 4: For Reply 7

"To validate the thrombosis model, we tested the thrombolytic activity of tPA on our zebrafish photochemical thrombosis model. The larva was removed from the microscope and injected with tPA."

Comment 4a: how, how much?

Reply 4a: We injected tPA solution as same manner as the injection of the mixture of photosensitizer into the cardinal vein of the larvae using a microinjector. The intravascular concentration of tPA was 225μg ml^{-1}**.**

"The blood cells ceased to adhere to the clots when the larva was injected with tPA (intravascular concentration 225μg ml-1)"

Comment 4b: Is this thrombolysis? As described, this seems to be an arrest of thrombus formation, which could be due to different processes. Did the thrombi that were formed actually resolve? Or thus did thrombolysis happen?

Reply 4b: When the intravascular tPA concentration of increased up to 225 μg ml -1, the thromboses began disappearing. We changed the sentence.

Changes in the text: The blood clots began disappeared when the larva was injected with tPA(intravascular concentration 225μg ml^{-1}).