Zebrafish thrombosis models according to the location of thrombus formation

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Background: Ischemic stroke becomes a major cause of death and disability. It can develop due to intravascular or cardiac thromboemboli. Animal models that reflect diverse stroke mechanisms remain under development. Using photochemical thrombosis, we developed a feasible zebrafish model according to the thrombus location (intracerebral *vs.* intracardiac). We validated the model using real-time imaging and thrombolytic agent.

Methods: We used transgenic zebrafish larvae (flk:gfp), which express specific fluorescence in endothelial cells. We injected Rose Bengal, a photosensitizer as a mixture of photosensitizer, and a fluorescent agent into the cardinal vein of the larvae. We then evaluated real-time thrombosis *in vivo* by inducing thrombosis through exposure to a confocal laser (560 nm) and staining the blood flow (RITC-dextran). We validated intracerebral and intracardiac thrombotic models with checking the activity of tissue plasminogen activator (tPA).

Results: The photochemical agent induced the formation of intracerebral thrombi in transgenic zebrafish. Real-time imaging techniques confirmed the formation of the thrombi. The damage and apoptosis of the vessel's endothelial cells were seen in the *in vivo* model. An intracardiac thrombosis model was developed by the same method using photothrombosis, and the model was validated through thrombolysis by tPA.

Conclusions: We developed and validated two zebrafish thrombosis models that are readily available, cost-effective, and intuitive for assessing the efficacy of thrombolytic agents. These models can be used for a broad spectrum of future studies, such as screening and efficacy assessment of new antithrombotic agents.

Keywords: Zebrafish; stroke; thrombosis; photosensitization; embolism

Submitted Mar 09, 2022. Accepted for publication Apr 14, 2023. Published online Jun 19, 2023.
doi: 10.21037/atm-22-1265
View this article at: https://dx.doi.org/10.21037/atm-22-1265

Introduction

Stroke is associated with high morbidity and mortality, and the global incidence of stroke is increasing. Although the quantity and quality of prevention and treatment strategies for stroke have improved, the need to develop new drugs remains critical. Stroke includes ischemic and hemorrhagic stroke. In ischemic stroke, the diverse mechanisms are attributed to the various causes of the disease.

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Ischemic stroke develops mainly due to thrombosis from atherosclerosis or arteriosclerosis and thromboembolism from cardiac disease.

The development of animal models for stroke assessment is an indispensable component of research and development for new drugs. Suitable animal models for stroke research can provide insights into stroke mechanisms and allow researchers to designate and localize the ischemic lesion, predict the final ischemic lesion, and potentially perform *in vivo* functional studies. Moreover, animal models should be easily and economically reproduced. Until now, many animal models have been developed to investigate stroke pathogenesis in which focal or global ischemia was induced by a mechanical or chemical stimulus, with little consideration of stroke mechanisms (1-6). Mainly higher vertebrates have been used for vascular thrombosis animal models and used to discover screen and preclinical testing of new thrombolytic and antithrombotic agents (3,7).

The zebrafish (*Danio rerio*) has emerged as a popular and cost-effective model because its larvae have translucent body and relatively high genetic and functional similarity with humans (5,6,8). In particular, intravascular thrombosis animal models using larvae and adult zebrafish were introduced in many studies (5,6,8,9). Chemical or irradiation damage introduced intravascular or extravascular manners to make a thrombosis (8-11). 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). 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.

This study developed intracerebral and intracardiac thrombosis models using photochemically induced thrombosis in transgenic zebrafish. The intracranial thrombosis model was validated with a real-time imaging study. To demonstrate the prospective application of our intracardiac photochemical thrombosis model, we evaluated the thrombolytic capability of tissue plasminogen activator (tPA) in zebrafish. We present this article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-1265/rc).

Highlight box

Key findings

- We developed a photochemical thrombosis zebrafish model according to the thrombus location and validated the model using real-time imaging.

What is known and what is new?

- Intravascular thrombosis models using zebrafish were introduced in many studies.
- We presented the site specific (intravascular and intracardiac) thrombosis models using photosensitizer in transgenic zebrafish, and validated the model using real-time imaging and thrombolytic agent.

What is the implication, and what should change now?

- These models are feasible, cost-effective, and intuitive for assessing the thrombolytic agent and can be used for a broad spectrum of future studies.

Methods

Reagents

We used the following drugs and chemicals in this study: Rose Bengal, rhodamine B-isothiocyanate (RITC)-dextran (MW =70,000 Da),1-phenyl 2-thiourea (PTU), tricaine, O-dianisidine, and human tPA from Sigma-Aldrich (St. Louis, MO, USA). The singlet oxygen from Rose Bengal in the vessels injure the endothelium of blood under light exposure. It triggers the coagulation pathway to make thrombosis in the irradiated area (13).

Zebrafish husbandry

We used transgenic zebrafish larvae Tg (flk:gfp) and Tg (mpeg I: GAL4, UAS:eGFP) in this study. We housed adult zebrafish in an aquaculture facility controlled with standard 14-h-light-10-h-dark photoperiod at 28.5 ℃ and mated to obtain zebrafish larvae. We equipped the container with a multistage filter, fluorescent UV ling, and sterilizing filter (Zebrafish AutoSystem, Genomic Design, Daejeon, Korea). Zebrafish embryos were collected seven days post-fertilization (dpf).

Intracerebral thrombosis and quantitative analysis of thrombus (each n=4)

We anesthetized the seven dpf zebrafish larvae (total n=8)

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using a solution containing a mixture of Tris buffer and tricaine-3-aminobenzoic acid (0.4 g) in 100 mL ethyl ester, adjusted to pH 7. The mixture of Rose Bengal (800 μg/mL), and a fluorescent agent (RITC-dextran; 10% in dH_2O, 2 nL) to stain the blood flow were injected three times into the cardinal vein of the larvae with a microinjector (World Precision Instruments, Friedberg, Germany). The larvae were mounted with 1.5% low melting agarose on the glass-bottom chamber, and an area (measured area =3.99585 μm^2) in the skull of the larvae was focused and exposed to a 560 nm confocal laser (Nikon Eclipse Ti, Tokyo, Japan) to induce thrombosis in the intracerebral vessels by photochemical reaction for 10 minutes. Real-time imaging of thrombosis and quantitative analysis of the vascular endothelial cell apoptosis was performed. We validated our zebrafish photochemical thrombosis model with testing the thrombolytic activity of tPA. We removed the larva from the microscope and injected tPA. After injection, we put the larva back in the microscope to observe the thrombolysis. tPA was injected into larvae approximately 120 s after photochemical thrombosis. The blood clots began disappeared when the larva was injected with tPA (intravascular concentration 225 $\mu g \cdot mL^{-1}$).

Intracardiac thrombosis and quantitative analysis of thrombus

Zebrafish larvae (7dpf) (n=15) were anesthetized and classified into three groups. Rhodamine and dimethyl sulfoxide (DMSO) were injected into the control group and the Rose Bengal group (rhodamine, DMSO and Rose Bengal were injected). The heart of the mounted zebrafish larvae was exposed to a confocal laser for 15 min in the Rose Bengal + tPA group (rhodamine and tPA were injected after photoactivation). 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 larvae were observed after incubation at 28 ℃ for 24 hours. Five zebrafish larvae in each group were stained with 1.0 mg/mL O-dianisidine dye solution for 15 min and washed with DMSO three times as described by Chen *et al.* (14).

After O-dianisidine staining, zebrafish were mounted with 1.5% low melting agarose, and images were acquired in identical conditions with a microscope. To quantitatively evaluate the RBCs in the zebrafish heart, stained RBC intensity was analyzed by Image J 1.52a software (https://imagej.nih.gov/ij/).

Statistical analysis

All statistical analyses were performed using SPSS 20.0 for Windows (IBM Corporation, Armonk, NY, USA). Data are expressed as mean ± standard deviation (SD) or median (interquartile range) as the appropriate and statistical analysis was performed using the χ^2 test or Mann-Whitney *U* test. P<0.05 was considered to indicate statistical significance.

Ethical statement

The experimental animal procedures and the animals' care were approved by the Institutional Animal Care and Use Committee at the Korea University College of Medicine (IACUC Number: KOREA-2019-0041) in compliance with the Korean guidelines for the standard operations of the IACUC.

Results

Intracerebral thrombosis model

Formation of intracerebral thrombosis

We induced intracerebral thrombosis in transgenic zebrafish larvae [Tg (flk:gfp)]. In the transgenic zebrafish larvae, *in vivo* angiography visualized the injury and collapse of the vessels compared with the control group (*Figure 1*). We found that the blood flow in small-sized cerebral vessels disappeared after microinjection of the photosensitizer mixture and exposure to the confocal laser.

Real-time imaging of intravascular thrombosis

The formation of intracerebral thrombosis after rhodamine injection was recorded through *in vivo* angiography. We quantitatively analyzed the amount of blood flow per cross-sectional area of the total vasculature pre-and post-exposure with the confocal laser. The dye intensity reflecting the blood flow per cross-sectional area in the Rose Bengal group markedly decreased around the thrombi compared with the control group (*Figure 2*).

Analysis of vascular endothelial cell damage

We found that the number of vascular endothelial cells decreased in the intracerebral vessel of transgenic zebrafish larvae [Tg (flk:gfp)] 2 days after photothrombosis. The

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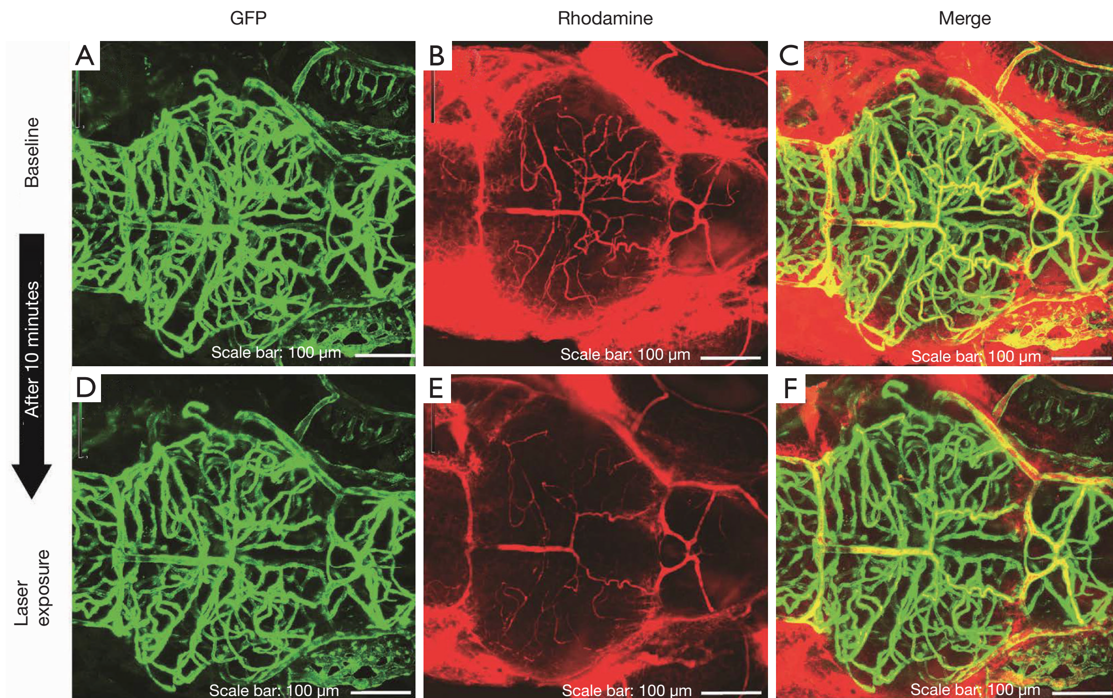

Figure 1 *In vivo* images at pre-and post-photoactivation. (A) *In vivo* cerebral angiography of vascular endothelial cells in transgenic zebrafish larvae [Tg (flk:gfp)] (GFP). (B) Evaluation of blood flow using the 560 nm laser fluorescence microscope (rhodamine). (C) Evaluation of blood flow in the endothelial lining by merging GFP and rhodamine. Blood flow in intracerebral vessels disappeared, and endothelial linings' fluorescence intensity slightly decreased (D-F). GFP, green fluorescent protein.

control group (n=2) showed intact fluorescence of the vascular endothelial cells (*Figure 3A*). Photochemical dye-injected larvae showed decreased fluorescence intensity of damaged vascular endothelial cell layers (*Figure 3B*). The intensity of fluorescence of endothelial cells in the Rose Bengal group was significantly lower than that in the control group ($P<0.01$) ((*Figure 3C*).

Validation of intracerebral photothrombosis model through tPA

The thrombolytic activity of tPA was visually confirmed by *in vivo* angiography in zebrafish larvae and measured by a confocal microscope. *Figure 4A* shows the dorsal longitudinal vein of zebrafish larvae taken by a confocal laser microscope before and after exposure to the confocal laser. The tPA group showed a reduction of the thrombus (*Figure 4B*). The area of the thrombus in the control group (n=4) was 568.5 (100%), and that in the tPA group was 283 (49.78%±7.090%, mean ± standard deviation) (*Figure 4C*).

Intracardiac thrombosis model

We induced intracardiac thrombus formation by the same photothrombosis method and simultaneously validated the model. We established three groups: control group (n=5), Rose Bengal group (n=5), and Rose Bengal + tPA group (n=5). After exposure to a confocal laser for 15 min, comparing with the control (*Figure 5A*), the RBC intensity in the heart markedly decreased in the Rose Bengal group (24.33%±2.899%) (*Figure 5B*). The RBC intensity in the heart in Rose Bengal + tPA groups (63.53%±14.07%) did not decreased (*Figure 5C*). The quantitative image analysis of the heart RBC intensity validated the intracardiac thrombosis formation and thrombolytic activity of tPA (*Figure 5D*).

Discussion

This study developed vascular thrombosis models according to the location of the thrombosis and validated the

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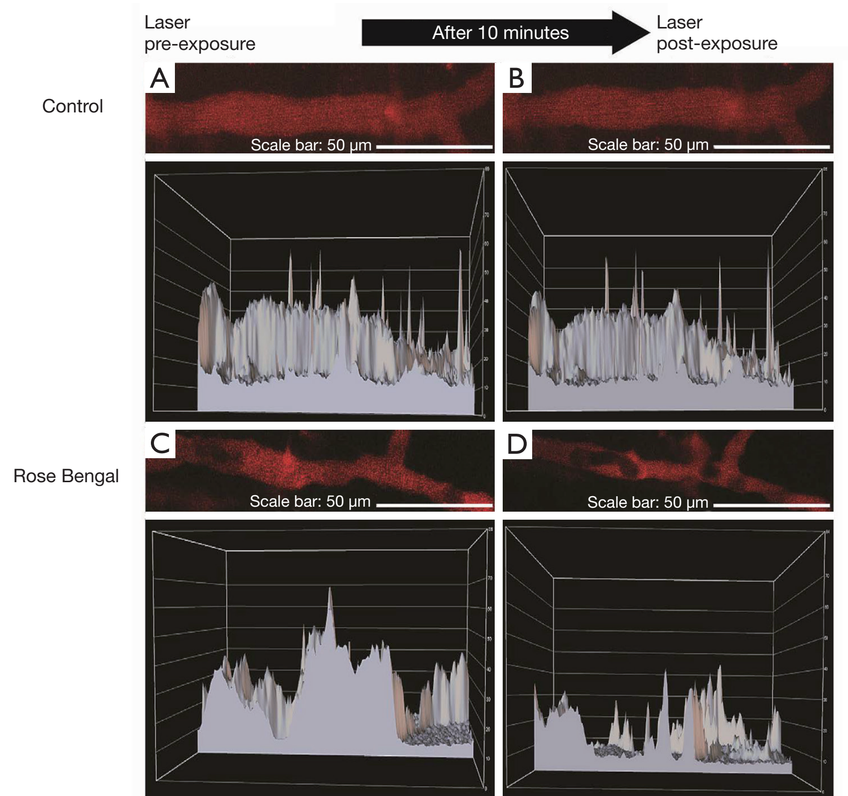

Figure 2 Real-time imaging of thrombosis in the vasculature (*in vivo* angiographies and blood flow chart per cross-sectional area of total vasculatures). Compared with the pre-photoactivation state (A,C), no thrombus or change of blood flow was observed at the post-activation state in the control group after photocoagulation (B), while thrombus formation and decreased blood flow were observed after photocoagulation in the Rose Bengal group (D).

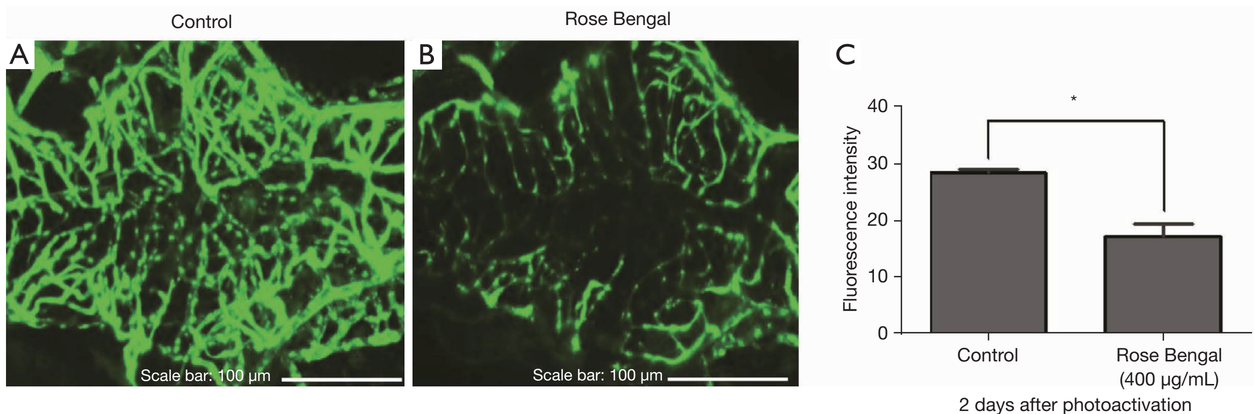

Figure 3 Analysis of vascular endothelial cell apoptosis at pre-and post-photoactivation (after 2 days). (A) *In vivo* angiography of the control group, (B) *in vivo* angiography of photosensitizer injection group, (C) fluorescence intensity: control: 27.83±0.56 (mean ± standard deviation) photocoagulation (Rose Bengal + rhodamine): 17.28±2.08 (measured by Image J 1.52a software). *, P<0.01.

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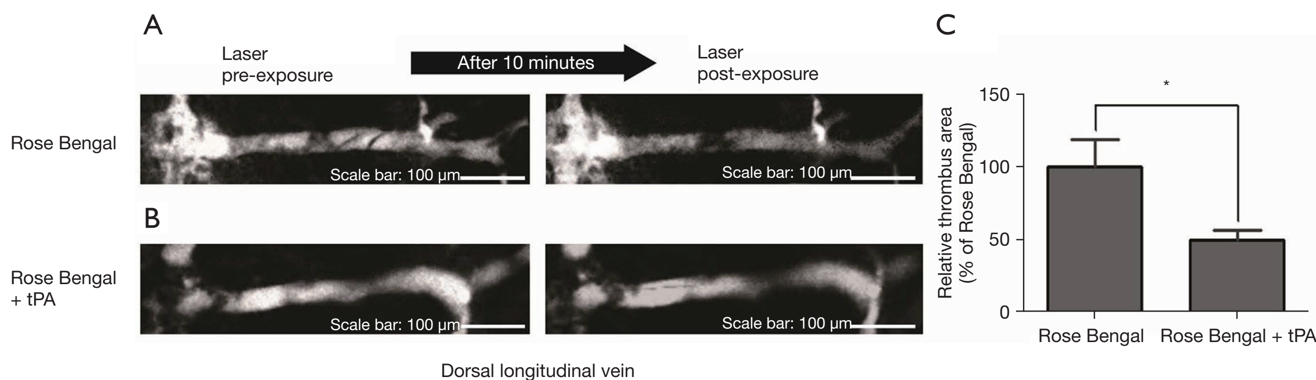

Figure 4 Real-time imaging of the thrombolytic activity of tPA. (A) Thrombus formation after photocoagulation, (B) thrombolytic activity of tPA, (C) comparison of thrombus area using Image J 1.52a (Mann Whitney test, P<0.05). Rose Bengal + tPA (n=4) group shows a decreased thrombus area (49.78%±7.090%, mean ± standard deviation). *, P<0.05. tPA, tissue plasminogen activator.

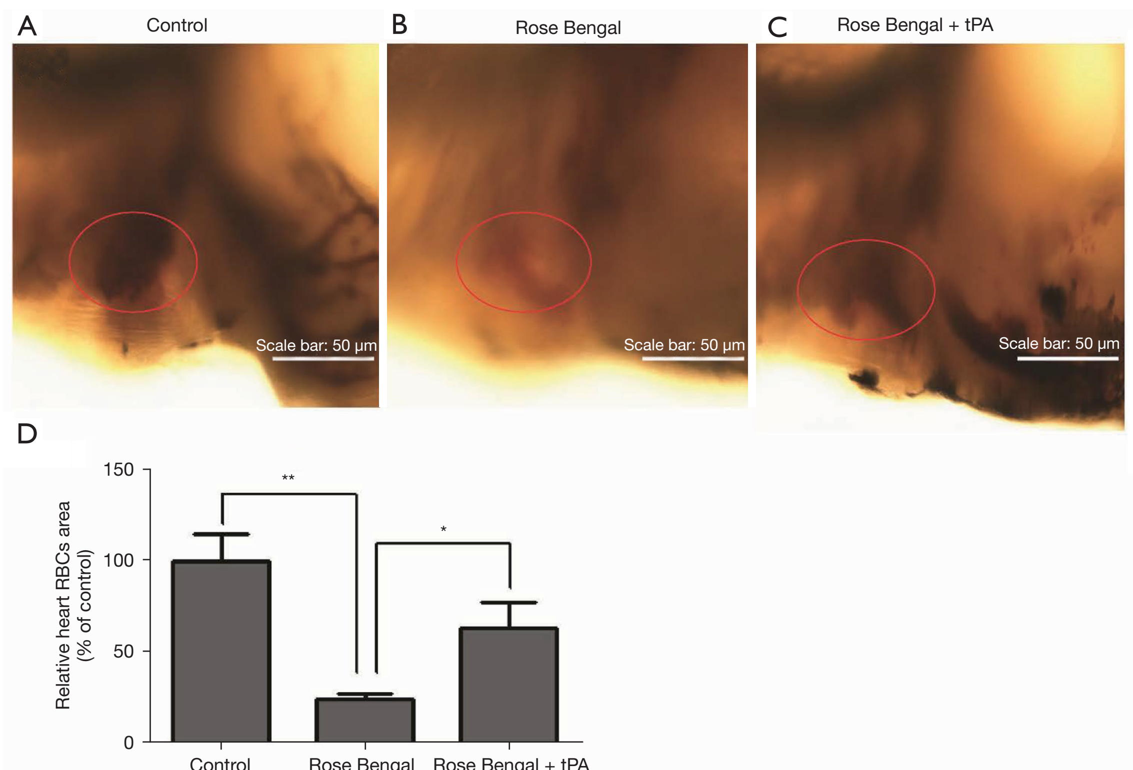

Figure 5 Quantitative assay of intracardiac thrombosis. (A) Control group, (B) Rose Bengal group, (C) Rose Bengal + tPA group, (D) comparison of intracardiac thromboses, red circles indicate thrombus in the zebrafish heart shown by O-dianisidine staining of the red blood cells. *, P<0.05; **, P<0.01. tPA, tissue plasminogen activator.

models as an assessment tool for thrombosis. We achieved intracerebral and intracardiac thrombosis formation on 7dpf transgenic zebrafish larvae by injecting photosensitizing agents and exposing the larvae to a confocal laser (560 nm). We validated thrombosis formation using real-time imaging software and quantitatively assessed the thrombolysis.

Zebrafish demonstrate advantages as a model system for assessing drug efficacy, toxicity, and safety (11,15). Zebrafish

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larvae allow for both ease of *in vitro* fertilization as well as transparency (10). Additionally, they are similar to humans in many aspects (16). In particular, the hematological system in zebrafish, including the platelets and coagulation factors, show similarity with their counterparts in humans (17). Therefore, zebrafish is a useful model for research on thrombus formation and exploring the antithrombotic effect of various drugs. In this study, we used transgenic zebrafish expressing fluorescent vascular endothelial cells. Transgenic zebrafish Tg (flk:gfp) are used for *in vivo* angiography in the animal cerebrovascular model (18). We confirmed vascular endothelial cell damage as the main mechanism in photochemically induced thrombosis through *in vivo* angiography in the larvae. A weaker intensity of fluorescence indicates a smaller number of vascular endothelial cells. The number of cells can decrease due to apoptosis or necrosis. Other research studies using rodent models showed that apoptosis and necrosis might cause cell damage from ROS produced by Rose Bengal and photostimulation (19,20). As zebrafish in larval stage have transparent body, this feature enables the unique possibility of inducing thrombogenesis in specific cerebral vessels and provoking ischemic stroke.

The laser-induced vascular injury model enables intravascular thrombosis in animal cardiovascular disease models. However, in other animal models such as rats and rabbits, establishing an intravascular thrombosis model requires the sacrifice of many mammals. Conventional rat models of vascular thrombosis generally involve complicated procedures that may show irreducibility or risk of inducing extensive thrombosis and inflammation (2). Models of intracardiac thrombus are challenging to establish and have rarely been reported (21). Compared with these models, our transgenic zebrafish models using photochemical thrombosis are highly reproducible and easy to establish. These features may provide real-time visualization of the formation of intracerebral thrombosis and intracardiac RBC aggregation according to the location of the thrombosis. In contrast, higher degree primate models generally require complicated surgery and a substantial investment of resources to assess the effect of new drugs and hence, might suffer from unsatisfactory reproducibility. The photochemical thrombosis were employed in rodents by Nishimura and coworkers (22). However, skull surgery was required to access the cerebral vessels of these animals and confirm the induction of the thrombus.

We present the possibility of establishing the cardiac origin of thromboembolism as the cause of stroke. This method allows selective coagulation. To make intracardiac thromboses, we quantitatively analyzed heart RBCs stained with O-dianisidine. Moreover, tPA thrombolysis was induced. O-dianisidine staining has been used to identify RBCs in the intracardiac thrombosis model (23). We checked the thrombolytic capability of tPA in our model *in vivo*. These results show that our method can be a possible tool for screening and preclinical testing new thrombolytic and antithrombotic agents.

Despite the attractive features of zebrafish models, our study has three limitations. First, zebrafish are considerably different from humans in physical aspects. The lesser density of thrombocytes (24) and the shorter clotting time of zebrafish plasma than that of humans were the different aspects (25). Second, these thrombosis models do not reflect the diverse and complex aspects of human stroke development that are attributed to risk factors, genetic factors, and environmental factors. However, these are inherent problems that can be encountered in animal models in general. Third, although the small laser power employed in our method does not induce extensive photochemical damage, a local thermal effect associated with prolonged exposure may have existed. Therefore, an endothelial injury might aggravate, and the initiation of the thrombi might be partially affected by the high temperature. However, photo stimulated thrombogenesis in transgenic zebrafish was monitored in real-time and confirmed through *in vivo* angiography and injury of the endothelial cells. These models could be a reproducible standard for a thrombosis animal model.

Conclusions

In this study, we developed and validated two zebrafish thrombosis models that could be suitable for *in vivo* screening and efficacy assessment of antithrombotic agents. These zebrafish thrombosis models are precise and readily available with a short testing time and may increase the research and development of antithrombotic drugs.

Acknowledgments

We acknowledge Mi Young Kim, the librarian in Korea university medical library.
Funding: This work was supported by a 2016 Korea University Ansan Hospital R&D Support Project through the support of the Vice President for Medical Affairs of Korea University Special Research Funds (No.

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K1613821), and by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (Information & Communications Technology) (No. NRF-2020R1C1C1009294).

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-1265/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-1265/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-1265/coif). All authors report that this work was supported by a 2016 Korea University Ansan Hospital R&D Support Project through the support of the Vice President for Medical Affairs of Korea University Special Research Funds (No. K1613821), and by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (Information & Communications Technology) (No. NRF-2020R1C1C1009294). JMJ has received: lecture honoraria from Pfizer, Sanofi-Aventis, Otsuka, Dong-A, Hanmi Pharmaceutical Co., Ltd., and Boryung Pharmaceutical Co., Ltd.; a study grant from Il-Dong and Cheiljedang Pharmaceutical Co., Ltd.; and consulting fees from Daewoong Pharmaceutical Co., Ltd. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The experimental animal procedures and the animals' care were approved by the Institutional Animal Care and Use Committee at the Korea University College of Medicine (IACUC Number: KOREA-2019-0041) in compliance with the Korean guidelines for the standard operations of the IACUC.

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Cite this article as: Hwang J, Koun S, Ha Y, Jung JM. Zebrafish thrombosis models according to the location of thrombus formation. Ann Transl Med 2023;11(9):309. doi: 10.21037/atm-22-1265

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