

Original Article

Zebrafish as an alternative model for hypoxic-ischemic brain damage

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Abstract: Acute cerebral ischemia is one of the leading causes of mortality and chronic disability. Animal models provide an essential tool for understanding the complex cellular and molecular pathophysiology of hypoxic-ischemia and for testing novel neuroprotective drugs in the pre-clinical setting. In this study we tested zebrafish as a novel model for hypoxic-ischemic brain damage. We built an air-proof chamber where water inside had a low oxygen concentration (0.6-0.8 mg/L) proximate to complete hypoxia. Each zebrafish was placed individually in the hypoxia chamber and was subjected to hypoxia treatment until it became motionless, lying on its side on the bottom of the chamber (time to hypoxia = 679.52 ± 90 seconds, mean \pm SD, n =23), followed by transferring into a recovery beaker. Overall, 60.87% of subjects did not recover from hypoxia while 39% survived. The size and distribution of brain injury were determined by triphenyltetrazolium chloride (TTC) staining. Bilateral, moderate to complete TTC decoloration or demarcation of the infarct after 10 minutes of hypoxic treatment was clearly visible in the optic tectum of the optic lobe. The size of the infarct expanded to the deep structure of the optic lobe with longer hypoxic treatments. The zebrafish that survived hypoxia experienced initial twitching followed by unbalanced erratic movements until they regained coordinated, balanced swimming ability. These data indicate that zebrafish are susceptible to hypoxic attack and suggest that the model we present in this study can be used as an alternative model to evaluate hypoxia-induced brain damage.

Keywords: Ischemia, zebrafish, animal model, stroke, brain injury, triphenyltetrazolium

Introduction

Hypoxic-ischemic brain damage is often seen in patients who suffered a stroke or cardiac arrest. The brain is about 2% of the total body mass, yet 15-20% of blood flow travels from the heart to the brain and the brain accounts for 20% of total oxygen consumption. Due to its high metabolic demand, brain cells are extremely sensitive to oxygen deprivation and can begin to die within five minutes after oxygen supply has been cut off. When hypoxia lasts for longer periods of time without therapeutic intervention, it can cause coma, seizures, and even brain death [1]. Unfortunately, direct intervention is limited and mostly supportive at this time [2, 3]. Among brain damage, ischemic stroke is a leading cause of death and long-term disability in the United States [4]. The only FDA approved drug for the treatment of acute thrombotic stroke is tissue plasminogen activator (tPA). Currently, according to National Institutes of

Health (NIH), only 1-3% of stroke patients in the US receive this therapy. There is a critical need to develop safe and effective therapies or neuroprotective agents to improve the clinical prognosis of stroke patients [3, 4]. While animal models have helped us better understand the pathophysiology of ischemic brain damage, the use of animal models has very limited translational success in acute ischemic stroke therapies [5-9]. There is a need to develop alternative animal models for understanding the complex cellular and molecular pathophysiology of hypoxic-ischemic brain damage and, particularly, for the purpose of drug screening of neuroprotective agents in the pre-clinical setting.

The zebrafish (*Danio rerio*) is a freshwater fish usually found in shallow water or shallow streams. It is an important and widely used vertebrate model organism in biological and biomedical research. The zebrafish has most of the same organs found in mammals and is more

closely related to humans than other simple organisms. Many features of zebrafish development have been characterized, including early embryonic patterning, early development of the nervous system, and aspects of cell fate and lineage determination [10]. In the past decade, the zebrafish has been exploited by researchers interested in disease processes and, accordingly, there is a growing literature on the use of zebrafish to model human diseases [11-14], including neurological disorders and in stress research [15-17]. The use of zebrafish has begun to show a good correlation between zebrafish and rodent data and has demonstrated similarity to mammalian models in toxicity testing. Therefore, zebrafish assays are a potential alternative to rodent assays [18, 19].

How can the zebrafish model help the research of hypoxic-ischemic brain damage and ultimately contribute to the advancement of therapeutic drug development? The zebrafish offers several advantages for use as a model system. The zebrafish can easily take dissolved chemicals with water into its body through its gills merely by breathing. Zebrafish circulation is a single circuit: heart-gills-body-heart. Chemicals are typically added to the aquatic environment in which they live, allowing for easy experimental manipulation and observation. Since chemicals are absorbed directly into the circulation system without the need for nociceptive and time-consuming injection or invasive operation, there is no stress or tissue damage associated with rodent stroke models. Another advantage is that the zebrafish has a well studied gene pool and fully-sequenced genome. Since there is availability of well-characterized mutants, it is practical and convenient to use gene knock-outs and knock-ins for further study if needed. The zebrafish has increasingly been used as a whole organism model in which to perform functional high-throughput drug screens, and numerous chemical screens have been performed in zebrafish [20-24]. Compared to other animal models of hypoxia/ischemia, the zebrafish model is also cost effective.

To make a global ischemic model, the zebrafish was placed in an air-proof water chamber (or hypoxia chamber). In this setting, the extent of hypoxia can be regulated by the level of oxygen deprivation to achieve partial or complete hypoxia (anoxia) and by the length of time in the hypoxia chamber (transient or permanent). Therefore, in addition to the advantages of the

zebrafish model mentioned above, this model potentially allows us to study brain damage caused in a variety of hypoxic conditions. In the present study we explored zebrafish as a novel model for hypoxic-ischemic brain damage, particularly the damage caused by global ischemia. The zebrafish was placed in the hypoxia chamber with an extremely low level of dissolved oxygen (less than 0.8 mg/L), which is similar to the conditions in complete global ischemic stroke. We observed abnormal behaviors during and after hypoxic treatments. After that the zebrafish brains were removed for TTC staining to get a histological view of any brain damage. Results showed a correlation between the damage and the extent of hypoxic treatment. Our results suggest that the zebrafish is a promising animal model for studying hypoxic ischemia and related brain damage *in vivo*.

Materials and methods

Animals

All work in this study was conducted in accordance with the Ohio University Institutional Animal Care and Use Committee (IACUC) guidelines. Zebrafish (*Danio rerio*) were procured from a local aquarium supplier and were kept in aquaria under a 12-h light/12-h dark photoperiod with half deionized water plus half dechlorinated water (made with fresh tap water with sodium thiosulfate, a dechlorinating agent, 10-15mg/L) at room temperature [25]. The fish were fed daily on commercially available dry food. The size of the zebrafish (mixed sex) used in the present study ranged from 30 mm to 49 mm total length (mean: 41.56 mm; median: 42.5 mm). Thus, all individuals were well into adulthood.

Hypoxia chamber and hypoxic treatment

Hypoxia chamber was made by 1000 ml clear glass bottle with 800 ml water inside. The chamber had two ports, one connected to a nitrogen (N₂) tank and other one connected from the air space inside to the open air during nitrogen perfusion (**Figure 1A**). Once sealed, this was a closed air tight system. Hypoxia in the chamber was created by flushing pure nitrogen into the chamber and then hermetically sealed immediately after stopping nitrogen perfusion. The dissolved oxygen (DO) reached a nadir of 0.6-0.8 mg/L after less than 6 minutes and remained constant during the test course (**Figure**

Zebrafish as a hypoxic-ischemic model

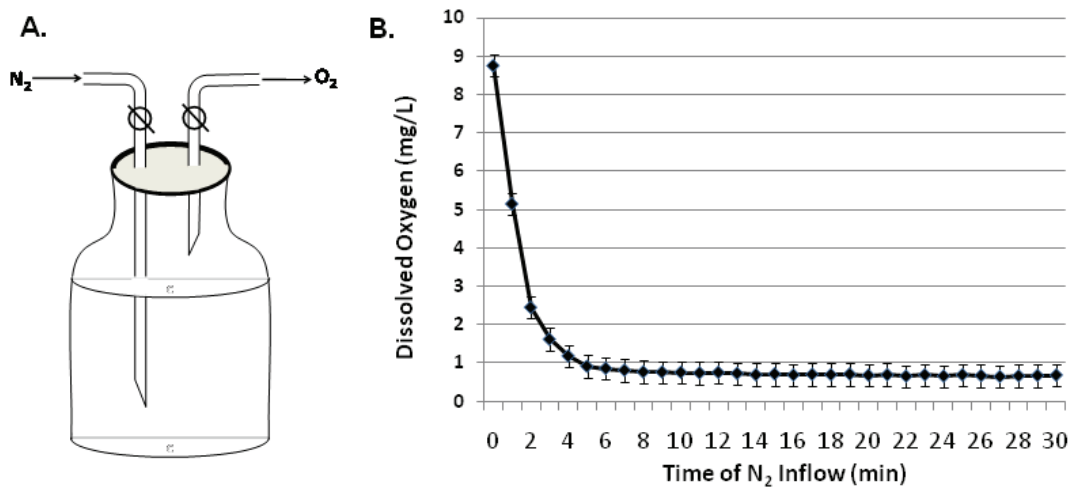


Figure 1. Hypoxia Chamber. **A.** Schematic diagram of the custom-designed hypoxia chamber. **B.** The average of dissolved oxygen (DO) in the water of the hypoxia chamber decreased as the time of nitrogen perfusion increased. It started decreasing at the beginning of nitrogen perfusion and reached the minimum (0.6-0.8 mg/L) in 6 minutes. DO stayed stable during the rest of measurement ($n=3$).

1B). The level of oxygen deprivation in this system is near complete depletion and is considerably below 2 mg/L dissolved oxygen, the hypoxia condition defined by the Committee on Environment and Natural Resources [26]. When the chamber was hypoxia-ready, a zebrafish was transferred into the chamber. The chamber was continuously perfused with a steady stream of nitrogen bubbling for one more minute in the presence of the zebrafish to ensure it was approximate to an anoxia environment. The endpoint of the time in the hypoxia chamber was determined as when the zebrafish lay on one side at the bottom of the chamber for 1 minute, motionless (except for occasionally opercular movement). Taken together, this system allowed for a reliable and fast gas exchange and essentially provided a no-fluctuating hypoxic environment. Immediately after hypoxic treatment, the zebrafish was transferred to the recovery beaker for recovery and behavioral observation. A camera (Nikon D300s) was positioned in front of the hypoxia chamber or recovery beaker. All experiments including hypoxic treatment and recovery were recorded onto the video-camera and later replayed for analysis.

TTC stain

Ischemic brain damages were evaluated by TTC (2,3,5-triphenyltetrazolium chloride, Sigma-Aldrich, St Louis, MO, USA) staining as de-

scribed [27, 28]. TTC solution (2% by weight in PBS solution) was made right before use at room temperature and covered with foil due to the light sensitive property of TTC. Zebrafish brain dissection occurred 6-7 minutes after anesthetizing (MS-222, 200 mg/L) when zebrafish had no opercular movement and no activity responding to stimulation. The optic lobe region of the brain was removed and cut into 1 mm slices using a tissue chopper (McIlwain Tissue Chopper). Brain slices were placed in a petri-dish with TTC solution for 40 minutes at room temperature. The petri-dish was covered with foil during this process. The slices were then transferred into 10% PBS buffered formalin for fixation. High resolution digital images of brain sections were photographed within 2 days under a dissection microscope that was connected to a digital camera (Motican 2500) and supported by Motic Images Plus 2.0 software. Exposure time and gain value were adjusted until clear images were taken and stayed unchanged during the whole TTC image-capturing process. Infarct size was measured by Motic Images Plus 2.0 software and shown as percentage of the total area as described [28].

Results

Each Zebrafish was placed individually into the hypoxia chamber and remained there until it was lying motionless (except for occasionally

Zebrafish as a hypoxic-ischemic model

Table 1. Effect of hypoxic treatment on Zebrafish

| Zebrafish | Number | % Dead | Hypoxia Time (mean \pm SD, Sec) |
|------------------------|--------|--------|-----------------------------------|
| Total | 23 | | 679.52 \pm 90 |
| Dead after Hypoxia | 14 | 60 | 695.64 \pm 111 |
| Survived after Hypoxia | 9 | 40 | 647.78 \pm 56 |

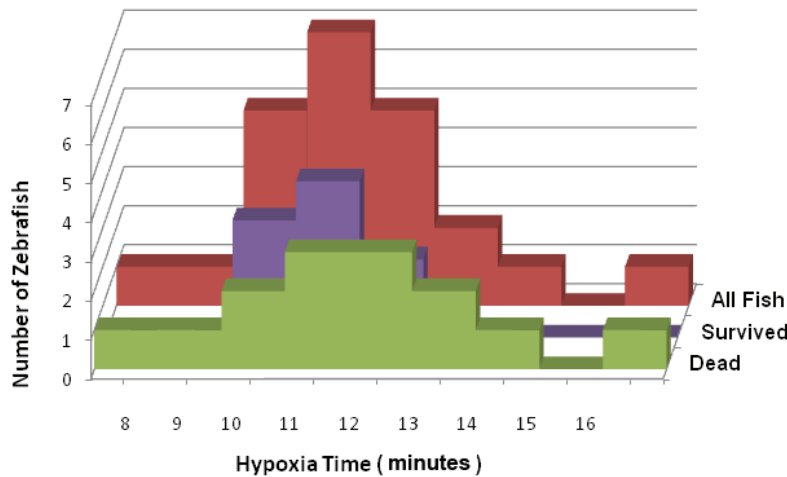


Figure 2. The analysis of zebrafish hypoxia time distribution. Three dimensional diagram shows the distributions of hypoxia or tolerant time of individual zebrafish in the hypoxia chamber (All fish = 23, Survived=9, Dead =14).

opercular movement) on its side at the bottom of the chamber for 1 minute. Once this point was reached, the subject was transferred to a recovery beaker. This measurement was not accumulative, thus, any motionless status (or staying still) that was less than 1 minute was ignored. The period of time that the zebrafish was under hypoxic treatment was defined as hypoxia time. The mean hypoxia time or the average time to the endpoint for tested zebrafish in hypoxia chamber was 679.52 \pm 90 seconds (mean \pm SD, n =23; **Table 1**). The shortest hypoxia time was 540 seconds (9 minutes), which was from a subject that didn't survive. The longest time was 940 seconds (\approx 16 minutes), also from a subject that didn't survive. The distribution analysis of individual zebrafish revealed that hypoxia times for the majority of zebrafish (>70%) were between 10-12 minutes (**Figure 2**).

Immediately after hypoxic treatment, the zebrafish was transferred into the recovery beaker for recovery and behavioral observation. We gener-

ally observed the zebrafish recovery for two hours. Depending on recovery from hypoxic treatment, tested zebrafish were sorted subsequently into dead or survived groups. **Table 1** summarizes the results of zebrafish under hypoxic treatment. Although there was no difference in average hypoxia times between these two groups, 14 (60.87%) did not recover from hypoxia with an average hypoxia time of 695.64 \pm 111 seconds (mean \pm SD) while 9 survived with a hypoxia time of 647.78 \pm 56 seconds (mean \pm SD).

The size and distribution of brain injury were determined by TTC staining. The brains of

healthy zebrafish were bilaterally stained deep red (**Figure 3A and B**). Following hypoxic treatment TTC staining reliably delineated the infarct (pale or unstained areas versus deep red colored brain tissue). Bilateral, moderate to complete TTC decoloration or demarcation of the infarct after 10 minutes of hypoxic treatment was clearly visible in the tectum of optic lobes (**Figure 3C and D**). Bilateral TTC decoloration expanded to the deep structure of the optic lobe with 20 to 35 minutes of hypoxia (**Figure 3E-H**). We measured the infarct size as percentage of the total area. In 10 minutes of hypoxia, the percentage was around 11.6 \pm 5.97 % (Mean \pm SD, n = 3), and this number was increased to 24.79 \pm 3.82 % (n = 3) with 20 minutes of hypoxia and up to 60 \pm 27.26 % (n = 5) after 30-35 minutes of hypoxia.

Of tested zebrafish, 9 (39.13%) survived hypoxic treatment and recovered in the recovery beaker, although the mean hypoxia time (647.78 \pm 56 seconds, mean \pm SD, n = 9; **Table 1**) was not

Zebrafish as a hypoxic-ischemic model

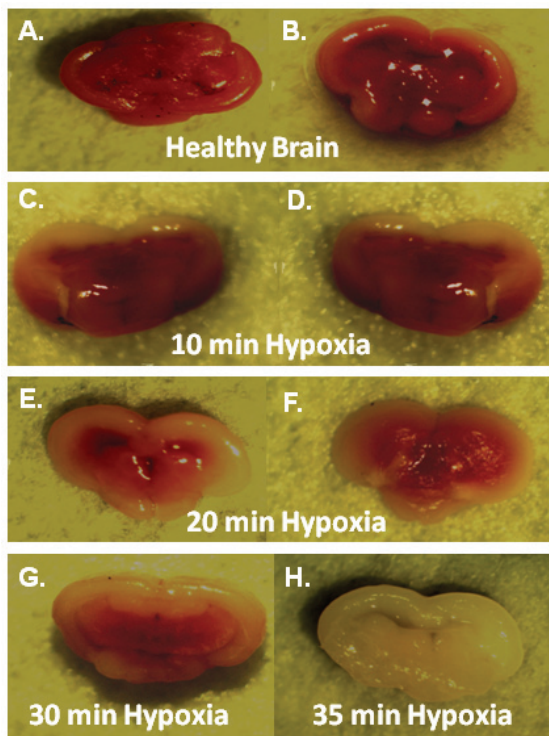


Figure 3. Brain injury of zebrafish detected by TTC staining. **A.** TTC stained healthy brain sections. **B-H.** TTC stained brain sections following various hypoxic treatments (10, 20, 30, 35 min) in the hypoxia chamber.

significantly different from the dead zebrafish group. We recorded the movements and behaviors of the tested zebrafish once it was transferred to the recovery chamber. The zebrafish that survived hypoxic treatment showed rapid opercular movement in a few minutes but didn't move until about 10 minutes after being placed in the recovery beaker. Through recovery following hypoxic treatment, all surviving zebrafish experienced initial twitching followed by unbalanced or erratic movements before balanced movement was regained. The times when surviving fish started each movement are listed in **Figure 4**. We did not observe any erratic movement from control zebrafish that underwent the transfer from one environment to another without the hypoxic treatment. Unbalanced movement was defined as swimming associated with erratic behaviors such as circling, rotating, and upright swimming. Circling and rotating were both observed on the same zebrafish, but did not necessarily have to happen at the same time (**Figure 5**). Sometimes, zebrafish circled

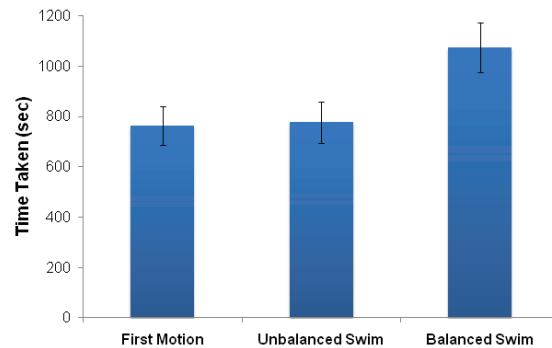


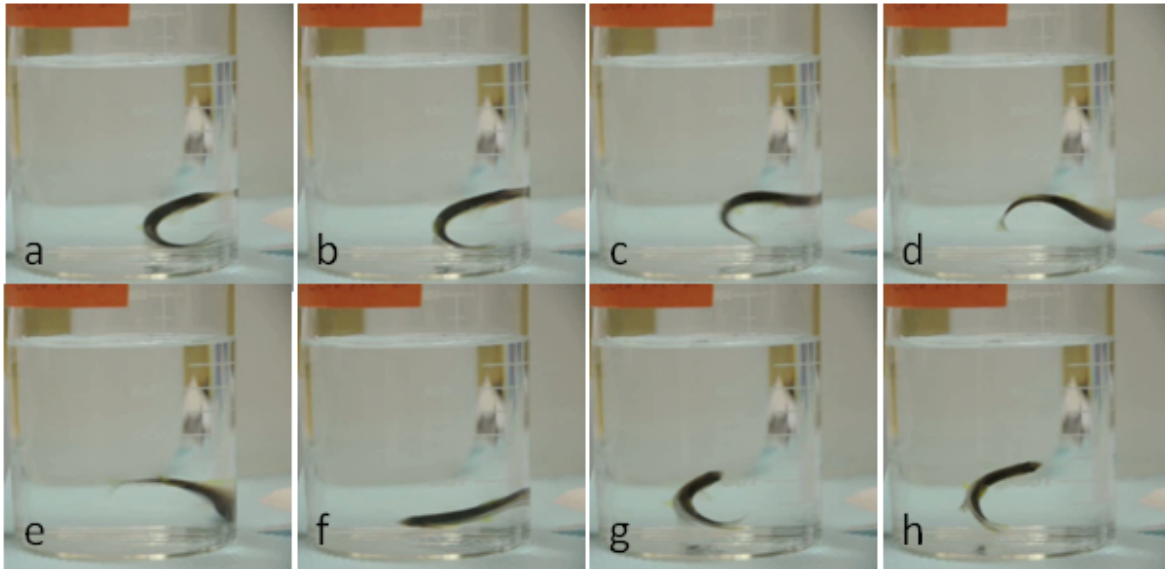
Figure 4. Swimming capability of zebrafish during recovery. Bars were times when zebrafish regained each pattern of swimming (first motion, unbalanced swim, and balanced swim) after hypoxic treatment (Mean \pm SD, n = 9).

first, proceed by a couple of seconds of rotating, and then they were circling again. The surviving fish began to show balanced movement after about 15 minutes of recovery, which was when the zebrafish regained normal and coordinated swimming, indicating sufficient recovery in their swimming ability.

Discussion

In this study we demonstrated that hypoxic treatment effectively induced zebrafish brain damage. Under near complete hypoxia conditions (DO = 0.6-0.8 mg/L), zebrafish quickly lost motion and reduced opercular movement with the average hypoxia time of 679.52 seconds or about 11 minutes. The distribution analysis of individual zebrafish revealed that hypoxia times for the majority of zebrafish (>70%) were between 10-12 minutes. Essentially, 60.87% of tested zebrafish did not recover or died from hypoxia. These data indicate that zebrafish are sensitive to hypoxic attack. Furthermore, the fact that TTC-defined brain damage was observed and became worse with increasing length of hypoxic treatment indicates the clear correlation between hypoxia time and brain damage and further suggests that the hypoxia time or hypoxic treatment reliably predicts subsequent severe hypoxic brain damage in zebrafish. About 40% of tested zebrafish survived hypoxic treatment and recovered by regaining coordinated swimming. Through recovery following hypoxic treatment, all surviving zebrafish experienced erratic movements, including circling and

A. Zebrafish circling behavior



B. Zebrafish rotating behavior

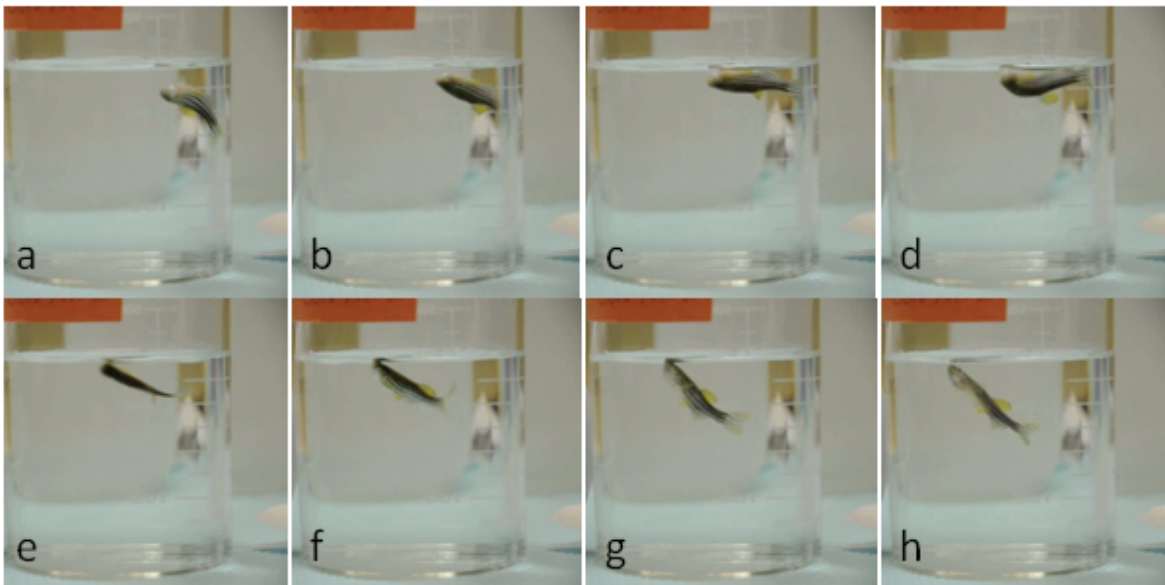


Figure 5. Behavioral observations of zebrafish recovery following hypoxic treatment. **A.** Sequential images of zebrafish circling movement from 1 second of video. **B.** Sequential images of zebrafish rotating movement from 1 second video. Images were taken by converting video into images sequences at 10 frames per second using software A4 image video converter.

rotating, before sufficiently recovering their swimming ability. These results suggest that the model we present in this study can be used as an alternative model to evaluate hypoxia induced brain damage.

Using fish in oxygen deficiency research is not

entirely new. Many studies have addressed the environmental impact of ecological hypoxia or anoxia killing fish globally [29]. However, no study has reported the use of fish or zebrafish as a model for hypoxic-ischemic brain damage, even though the zebrafish are one of the most widely used animal model systems in biological

Zebrafish as a hypoxic-ischemic model

research, ranging from embryonic to behavioral studies. Because the zebrafish is freshwater fish that were originally found in shallow streams and rice paddies (eastern India and Burma), it is not surprising that zebrafish are not tolerant of hypoxic treatment. As shown in this study, they were only able to sustain near complete hypoxia for a considerably short duration of time with a mean duration of about 11 minutes, supporting that zebrafish can be developed as an alternative ischemic model.

In the present study we chose the endpoint of hypoxic treatment as such: the zebrafish was motionless with occasional opercular movements for one minute. To determine an endpoint can be a considerable task since insufficient hypoxia or time in the hypoxia chamber allowed zebrafish to survive without brain damage, and on the other hand, over treatment ensured zebrafish death. The endpoint set in this study appears reliable. We had about 60% of zebrafish died and 40% survived following hypoxic treatments, implying that this is a responsive animal model system for use in developing therapeutic interventions for hypoxic-ischemic brain damage.

Our data show a significant decrease in TTC staining after zebrafish underwent hypoxic treatment. In normal tissue, dehydrogenase reduces TTC to formazan, which stains red [30], which has been widely used to demonstrate irreversibly damaged ischemic cerebral tissue in rats and other rodents. To the best of our knowledge it was the first time that TTC staining has been used in zebrafish. The border between stained and unstained tissues was well demarcated and could be identified easily by visual inspection. Decreasing TTC staining or infarct size resulted from increasing time of hypoxic treatment was consistent with previous observations in other animal models.

The fish gill is the primary regulatory interface between internal and external milieu. Because of the single circuit: heart-gills-body-heart, fish are regarded to be extremely susceptible bio-indicators of environmental changes [31, 32]. Among ischemic stroke models, the induction of global ischemia is comprised by the presence of the circle of Willis that gives collateral blood supply to the forebrain. The Mongolian gerbils are widely used as a model for global ischemia due to a species-specific incomplete circle of

Willis. However, the gerbils might present various problems when used in models for global cerebral ischemia, as some animals do not have a totally incomplete circle of Willis [33, 34]. In this study our custom made hypoxia chamber allowed for the quick creation of an extremely low oxygen environment which was maintained at a steady non-fluctuating hypoxic state throughout the testing period. Reducing the dissolved oxygen gradually depleted oxygenated blood in the zebrafish circulation, which is critical to brain function. When dissolved oxygen reached below 0.8mg/L, effectively, the zebrafish became a model for complete global cerebral ischemia that affects the entire brain [33, 34].

Besides its simplicity and susceptibility in making ischemic brain damage, other advantages of this model are that it is not invasive and doesn't mechanically injure vasculature and tissues. Contemporary models for the study of the pathophysiology and therapies for cerebral ischemia can be separated into focal and global ischemia models. One disadvantage with them is that injuring vasculature or brain structures is unavoidable in some circumstances, which may contribute to the failure in making a reliable model, increase animal mortality, and create difficulty in evaluating data [34, 35]. Another unexpected effect of tissue injuries resulting from making an animal model is that tissue injury may trigger the local or global stress responses that may pre-condition the targeted regions to hypoxic/ischemic insults. The zebrafish model presented in this study has therefore the advantage of being noninvasive and technically easier and faster than other stroke models.

There is ongoing debate of the effectiveness of current stroke models [5-9]. Experimental models of stroke are available in a variety of species, including primates, pigs, sheep, dogs, cats, Mongolian gerbils, rabbits, rats and mice. Whether zebrafish can be used to be a model for ischemic brain damage remains to be seen since there is no literature available about hypoxic-ischemic brain damage in zebrafish. Our results suggest that the zebrafish is a reliable and reproducible model appropriate for use in the study of hypoxic-ischemic brain damage. Hypoxic brain damage in zebrafish evaluated by TTC staining closely resembles hypoxic-ischemic brain damage documented in other animal

models or species. The zebrafish allows studies of ischemic, especially global ischemic, damages under well-controlled, noninvasive, reproducible conditions. Another unique advantage of this zebrafish system is that hypoxia can be easily reversible and drugs can be directly added into the bath to accelerate brain restoration after hypoxic treatment, supporting the versatility of zebrafish as an ischemic model. Future studies will test some neuroprotective agents to demonstrate that the zebrafish is a useful model for the screening of anti-stroke treatments. Additional obvious advantages of developing a translational system in zebrafish include the ability to create genetically mosaic animals, efficient *in vivo* tests for genetic interactions, conserved cellular architecture of the central nervous system, and tractable behavior [36, 37]. Zebrafish are the only vertebrates who have a completed genome synthesized. Forward and reverse genetic screenings have been successfully applied in zebrafish. Recent breakthrough evidence has shown the feasibility and successful use of zebrafish for high-throughput behavioral-based drug screening [16, 38]. These will help us develop and refine the argument that the zebrafish is a useful animal model for studying brain damages resulting from hypoxic-ischemic stroke.

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