METHOD

An Efficient and Reliable Assay for Investigating the Effects of Hypoxia/Anoxia on *Drosophila*

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Abstract Stroke is a leading cause of death worldwide. Up to one thousand potential drugs or interventions have been developed to treat stroke, out of which ~160 have gone on to clinical trials. However, none of them has been successful. New insights into the molecular and cellular mechanisms of ischemia-induced injury are needed for discovering new therapeutic targets. Recently, *Drosophila* has been used to uncover new hypoxia-related genes. In this study, we describe an efficient and reliable assay with a sophisticated apparatus for studying the effects of oxygen deprivation on flies. Using this assay, wild-type flies were exposed to an anoxic environment for varying lengths of time, then the cumulative death rate and mobility recovery were systematically analyzed. We found that anoxia for over one hour caused lethality. The cumulative death rate on day 5 after anoxia was linearly and positively correlated

with the duration of anoxia, and reached 50% when the duration was 2.5 h–3 h. We also found that the mobility recovery in normoxia was slow, as the climbing ability remained largely unchanged 4 h–6 h after 2.5-h of anoxia. We suggest that 2.5 h–3 h of anoxia and 4 h–6 h of recovery before mobility analysis are appropriate for future use of the anoxia assay.

Keywords Hypoxia · Anoxia · *Drosophila* · Mortality · Locomotor activity

Introduction

Anoxia and hypoxia (complete and partial lack of oxygen) may have detrimental consequences. The brain is much more sensitive to anoxia/hypoxia than other organs due to its high energy demand. When cerebral blood flow decreases, as in stroke, cell and tissue death results [1]. Stroke ranks second among the causes of death above the age of 60 and is a leading neurological cause of death and disability worldwide [2]. It is estimated that, every 3 min–4 min, someone dies of stroke, or suffers from serious disability or other neurological disorder due to stroke, resulting in a huge economic burden worldwide. Thus, the prevention and treatment of stroke is not only a medical problem but also a social issue [3].

Anoxia/hypoxia can be caused by ischemia, the reduction of blood supply to tissues resulting in a lack of oxygen and nutrients. Ischemia can cause severe tissue injury by activating cellular death. However, reperfusion (restoration of the blood supply) can induce even more damage in what is known as ischemic/reperfusion injury. Repeated reperfusion after long durations of hypoxia can reduce mitochondrial activity and harm respiratory control systems,

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causing increased oxidative stress and damage or inflammation [4–7].

A number of hypotheses about ischemia-induced damage have been proposed in the past decades and the dominant hypothesis is that the energy deficit leads to a massive release of glutamate, disruption of ionic balance, and oxidative stress [1]. More than 1000 potential drugs or interventions have been developed to target the processes or molecules involved in this hypothesis, some of which have gone on to ~160 clinic trials, but none has been successful in clinical translation [8]. One way to change this situation is to uncover unidentified molecules or signaling pathways to regulate an organism's susceptibility or tolerance to anoxia/hypoxia through genetic or chemical screening.

Drosophila melanogaster (fruit fly) has a short lifespan and can reproduce large numbers of progeny with low consumption of resources. The *Drosophila* genome contains ~14,000 genes and many are well-conserved in mammals. Approximately 75% of known human disease genes have a recognizable match in the genetic code of fruit flies. Moreover, the *Drosophila* research community has accumulated a variety of mutants, transgenic strains, and tools/techniques for manipulating genes. Therefore, *Drosophila* is frequently used to model human diseases, especially neurological diseases [9, 10], and they can be further used for genetic and chemical screening to identify new molecules mediating/regulating anoxia/hypoxia-induced injury and potential drugs.

Compared with mammals, *Drosophila* possesses an extraordinary ability to tolerate oxygen deprivation for extended periods of time. Flies subjected to an extended period of anoxia can recover and become active again when placed in room-temperature air [6]. Nevertheless, anoxia/reoxygenation (A/R) stress produces injury in flies. A portion of flies die in the days after exposure to anoxia for several hours [11, 12]. This, along with the above advantages, makes *Drosophila* useful for studying the molecular mechanisms of susceptibility and tolerance to anoxia/hypoxia *in vivo* [13–15], and identifying potential targets and drugs for treating oxygen deprivation-induced injury *via* genetic and chemical screening. To assist in these studies, we developed an apparatus for completely depriving flies of oxygen, and very reliable assays for quantifying the mortality and recovery of motor ability in flies subjected to anoxia.

Material and Methods

Drosophila Stocks and Culture

Drosophila melanogaster isogenic wild-type (WT) w^{1118} flies (Bloomington stock number 5905) were maintained in

standard cornmeal-agar-molasses-yeast medium at relative humidity of 50%–70% on a 12 h/12 h light/dark cycle at 25 °C. The recipe for making 1 L medium was: ddH₂O 0.65 L, baker's yeast 16 g, corn flour 80 g, agar 6.5 g, brown sugar 137.5 g, beer yeast 7.5 g, methylparaben 2 g, alcohol 20 mL and propionic acid 6.25 mL. Male and female flies were distinguished under a dissecting microscope. At the time of the study, adult flies were at the age of 4–6 days after pupation.

Anoxia Apparatus

As shown in Fig. 1, The anoxia apparatus consisted of an anoxic chamber (a barrel-like, transparent, airtight container, 12 cm in diameter and 14 cm high), a water tank, and a gas-flow-rate indicator and pipes. In the anoxic chamber was a two-layer circular platform, of which the lower layer was wire netting, and the upper layer was a plastic plate with holes. A 1.5 mL Eppendorf (EP) tube containing 10–20 flies was placed in each hole. The lids of the EP tubes were removed, covered by gauze, and placed upside down on the platform. A plastic funnel was installed in the center of the platform and the rostral end of the funnel was connected to a small serpentine pipe, through which pure nitrogen (N₂) flowed to the central bottom of the anoxic chamber so that oxygen (O₂ is heavier than N₂) flowing through the EP tubes and in the anoxic chamber was expelled through a hole at the center of the lid, through a larger serpentine pipe tightly attached to the hole. The N₂-carrying serpentine pipe was placed in a larger pipe and both pipes led to a water tank, which allows gas from the anoxia chamber to leave, but blocked the entry of air into the chamber. The N₂-carrying serpentine pipe left out the water tank and was connected to the gas-flow-rate indicator and further connected to a N₂ cylinder containing compressed pure N₂ gas. Silica gel and Parafilm were used to prevent gas leakage at connections. The gas tightness at each connection was tested under water.

Anoxia Assay

Flies at 5 days of age were collected quickly under CO₂ (1–2 min) and allowed to recover in vials with standard medium for 18–24 h before anoxia exposure, to minimize any confounding effect of CO₂ anesthesia. Each vial contained an equal number of female and male flies (total 10 or 20). After recovery, all flies in one vial were carefully transferred into a 1.5 mL EP tube with a funnel, and the tube was quickly covered with gauze and rubber bands to avoid escape. Then, the fly-containing EP tubes were transferred into the platform in the anoxic chamber and pure N₂ was introduced at a constant flow rate of 5L/min into the chamber. The time was recorded. Flies in each vial

Fig. 1 Design and set-up of the anoxia apparatus. **A** Schematic representation (upper) and image (lower) of the anoxia apparatus. Green arrows, N_2 flow direction; red arrows and dots, extra gas discharge from the sealed anoxic chamber.

B (i) Flies in 1.5 mL EP tubes with the lid removed and replaced with gauze; (ii) EP tubes upside down in holes of the 2-layer platform.

were subjected only once to a period of anoxia. All the assays were carried out in the same room with the same level of light and noise at 25 °C and 50%–70% humidity.

Mortality Analysis

After exposure to anoxia for 1.0, 1.5, 2, 2.5, 3, 4, or 4.5 h, the flies in each EP tube were returned to their culture vial with standard fly food. The dead flies in each vial were counted daily in the following 5 days and the cumulative death rate for each vial was calculated. To avoid flies being trapped in culture medium, the culture vial was placed horizontally on the first day after anoxia.

Climbing Assay

After anoxia, flies were allowed to recover in normoxia in their culture vials for 5 h, or other periods as indicated, then subjected to the automated rapid iterative negative geotaxis (aRING) assay as previously described [16]. Briefly, 10 flies in the same group were transferred into a transparent plastic cylinder with an inner diameter of 2.0 cm and height of 20.0 cm. This testing vial was vertically mounted on an aRING apparatus, which automatically tapped all flies to the bottom of the vial. Then, the flies were allowed to climb up the walls of the testing vials and this was recorded on a video. The video was

Fig. 2 Effect of anoxic period length on A/R-induced mortality. **A** Plots of the cumulative death rates of flies exposed to different periods of anoxia against the time after anoxia exposure ($n = 5$ for each data point). **B** Plot and straight line fit of the cumulative death rate on day 5 after anoxia against the duration of anoxia (inset, best fit

equation and R^2 value). The data are mean $\pm$ SEM, $n = 5$ groups and 20 flies/group for each data point; $P < 0.05$, one-way ANOVA, Tukey's multiple comparison t -test. Flies were assessed 5 days after eclosion.

transferred to a computer and a snapshot at 5 s after the initiation of climbing was taken and analyzed using Rfly detection, a home-made software, and the climbing height of each fly at 5 s was measured.

Immunostaining and Imaging

Briefly, brains from flies exposed to 0 or 2.5 h of anoxia were dissected out in cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde diluted in PBS for 45 min. After three washes in PBS-0.25% Triton-5% bovine serum albumin, brains were incubated with primary antibody (cleaved caspase-3, Cell Signaling Technology, 9664, Danvers, MA) for 10–12 h. After incubation with primary antibody, preparations were then washed three times with PBS. Samples ($n \geq 5$ /group) at 5 days after eclosion were then incubated for 2 h with fluorescein isothiocyanate FITC-conjugated secondary antibody and imaging was carried out using confocal microscopy (Zeiss LSM 710 METANLO). An $n \geq 5$ was done for each group at 5 days after eclosion.

Data Analysis and Statistics

All data sets are expressed as mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA and $P < 0.05$ was considered statistically significant. GraphPad Prism was used for statistical analysis. All experiments were run at least in triplicate.

Results

The design of the anoxia apparatus is shown in Fig. 1. Using this system, the A/R-induced injury in young w^{1118} flies was examined by investigating the effects of the

duration of anoxia on mortality and recovery of mobility. In the anoxic chamber with N_2 flowing at 5 L/min, all flies dropped to the gauze within 3 min, and remained motionless thereafter. In room-temperature air, all flies recovered and became active again. The length of the recovery time depended on the duration of anoxia; the longer the anoxic period, the longer the recovery time (data not shown). However, when the anoxic period was ≥ 6 h, most of the flies did not recover. In contrast, when the anoxic period was < 1 h, all flies recovered and did not die in the following 5 days (data not shown).

This study quantitatively investigated the effects of 7 anoxic periods on the mortality of flies: 1, 1.5, 2, 2.5, 3, 4, and 4.5 h. WT flies at 5 days after eclosion were divided into 7 groups, and each was subjected to anoxia for only one of the 7 periods. For each group, 100 WT flies were equally divided into 5 subgroups, each with 10 male and 10 female flies. Within the 5 days after exposure to anoxia for ≥ 1.5 h, a portion of the flies died on each day so that the cumulative death rate increased with time in the first 3 days, and reached a plateau on day 4 (Fig. 2A). Moreover, the longer the anoxic period, the greater the cumulative death rate. Plotting the cumulative death rates on day 5 against the anoxic periods and fitting a straight line equation to the data points showed that the cumulative death rate was linearly and positively correlated with the length of the anoxic period. The correlation coefficient R^2 was 0.9893, with high significance ($P < 0.0001$, Fig. 2B). Notably, when the anoxic period was between 2.5 and 3 h, the cumulative death rate at day 5 was $\sim 50\%$.

Using the aRING assay, the effects of reoxygenation time on the recovery of mobility were also investigated. Fifty male and 50 female flies at 5 days of age were equally divided into 10 groups and subjected to 2.5 h of anoxia, then returned to vials containing standard culture medium for reoxygenation. The period of anoxia exposure was set

Fig. 3 Mobility of flies after anoxia and reoxygenation using the climbing assay. **A** After exposure to anoxia for 2.5 h and reoxygenation, the motor ability of the flies was assessed by analyzing the climbing height (cm) of each fly. **B** To further extend the study, after exposure to anoxia for different times and 5 h after the onset of

reoxygenation, the motor ability was assessed as climbing height (cm) ($n = 20$ flies/group; mean $\pm$ SEM; $*P < 0.05$ using one-way ANOVA and Tukey's multiple comparison t -test). Flies were assessed 5 days after eclosion.

Fig. 4 Active caspase-3 staining in the brain of *Drosophila* exposed to anoxia. **A** and **B** Representative images of the brain of a fly with no exposure (**A**) and after exposure to anoxia for 2.5 h (**B**). Increased

staining (arrow) for caspase-3 is seen in the brain after exposure to anoxia ($n \geq 5$ /group).

at 2.5 h as this caused a $\sim 50\%$ cumulative death rate (Fig. 2). At each of 3, 4, 5, 6, and 7 h of reoxygenation, two of the 10 groups were assessed by the climbing assay and the average climbing height of 20 flies was calculated. The longer the reoxygenation, the better the recovery, and the climbing height remained stable between 4 and 6 h of reoxygenation (Fig. 3A).

Further, the effects of different durations of anoxia (0, 1.5, 2, 2.5, and 3 h) on climbing after 5 h of reoxygenation were evaluated on 50 male and 50 female flies (5 days old, equally divided into 10 groups, and 2 of the 10 groups were subjected to either 0, 1.5, 2, 2.5, or 3 h anoxia followed by 5 h reoxygenation). The longer the anoxic period, the worse the climbing ability (Fig. 3B), showing a negative dose-dependence of anoxic exposure and motor function (5.19 cm at 1.5 h, 3.35 cm at 2 h, 1.54 cm at 2.5 h, and 0.85 cm at 3 h compared to 8.24 cm with no anoxia).

To obtain direct evidence of cell death in flies after anoxia, brains were collected and stained for active caspase-3 (Fig. 4). Flies exposed to 2.5 h of anoxia were immunostained for caspase-3 and visualized using confocal

microscopy. Compared with controls, flies exposed to anoxia showed an increased active caspase-3 staining.

Discussion

Hypoxic injury involves a complicated and dynamic process that is not fully understood. Further studies are needed to understand the pathways involved in hypoxic injury and identify new molecular targets involved in hypoxia, anoxia, and A/R-induced injury. *D. melanogaster* is a powerful model used in screening for new genes involved in different phenotypes [17]. Our assays and apparatus provide an efficient and reliable way to screen for genes involved in hypoxia-induced injury or for pharmacological compounds that can reduce hypoxia-induced injury in flies.

Several other laboratories have aimed to investigate hypoxia- or A/R-induced injury [18–22]. In their studies, a hypoxic environment was generated by placing flies in a sealed plastic tube or chamber, expelling the O_2 by

injecting pure N₂, and controlling the timing of the N₂ flush by monitoring the O₂ level (<1%). The effect of hypoxia on flies was examined by monitoring the survival rate thereafter or the mortality at a certain time point, such as 48 h after hypoxia, or monitoring the recovery of mobility by recording the time for the first fly and all flies to start moving, or by traditional climbing or flight assays [13, 18, 23]. At the beginning of the present study, we also used these reported methods to examine hypoxia/anoxia- or A/R-induced injury and found large variations among different researchers and experiments. Therefore, we developed a more efficient and reliable assay. Using this assay, we performed a systematic analysis of the effect of different anoxic periods on mortality and the recovery of mobility. In *w¹¹¹⁸* flies at 5 days after eclosion, anoxia for >1 h was lethal, and the mortality rate 5 days after anoxia was linearly and positively correlated with the duration of anoxia, reaching 40%–60% when the duration was 2.5 h–3 h. Thus, anoxia for 2.5 h–3.0 h might be an appropriate time for quantifying the positive or negative effects of genetic manipulation or chemical application on mortality rate. In addition, the recovery of mobility was slow after 2.5 h of anoxia, as the climbing height remained largely unchanged 4 h–6 h later. Thus, 2.5 h–3 h of anoxia and 4 h–6 h of recovery before mobility analysis are recommended for future anoxia assays.

In summary, we showed that this assay is superior to other traditional hypoxia/anoxia screening apparatus due to its ease of use, suitability for large-scale studies, and ability to produce repeatable results. The anoxia apparatus can be used quickly, efficiently and at a low cost. This apparatus will aid in providing new insights into understanding the mechanisms involved in injury to tissues after exposure to a compromised O₂ supply, such as ischemic stroke.

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