IN VIVO HIGH THROUGHPUT SCREENING ASSAY TO TEST POTENTIAL NEUROPROTECTIVE THERAPIES FOR ISCHEMIC STROKE

Romero Bernal, Marina[1], Menéndez Valladares, Paloma[1], González Díaz, Ángela[1] ,
Del Río Mercado, Carmen[1*] and Montaner Villalonga, Joan[1*]

[1]Investigación Neurovascular. Instituto de Biomedicina de Sevilla (IBiS), Campus Hospital Universitario Virgen del Rocío. Avda. Manuel Siurot, s/nº 41013, Sevilla, España

INTRODUCTION

Stroke is a cerebrovascular accident that produces ischemia causing damage to the brain tissue. The main types of stroke are hemorrhagic stroke, which is due to the rupture of a blood vessel in the brain, and ischemic stroke resulting from the blockage of a cerebral artery by a clot. Stroke is the second cause of death worldwide and a leading cause of disability, with ischemic stroke accounting for 85% of all stroke cases.

During ischemic stroke, the sudden deprivation of blood flow reduces the delivery of O_2 and glucose available to the brain cells. The subsequent suppression in ATP production and the failure of different cellular processes causes irreversible injury leading to cell death due to necrosis in the so called ischemic core. The surrounding ischemic penumbra is defined as brain region at risk of progressing to infarction. Cell death that occurs in the ischemic penumbra is heterogeneous. Necrosis occurs minutes after the stroke while apoptotic cell death can take up to 24-48 hours. Therefore, cerebral tissue in the ischemic penumbra is potentially salvageable if reperfused. Paradoxically, the restoration of blood flow and O_2 levels produce an increase of Reactive Oxygen Species (ROS), which can damage proteins, lipids and nucleic acids leading to the activation of cell death mechanisms such as apoptosis.

Up to date, the only approved medical treatment for stroke is reperfusion therapy. This includes the use of thrombolytics (TPA or TKN) and mechanical thrombectomy. However, reperfusion therapies have some limitations such as the increased probability of hemorrhage as well as a very narrow therapeutic window (4.5 h after symptoms onset). Despite many years of research trying to develop neuroprotective therapies results are not encouraging. This may be due to the fact that most studies have focused on a single therapeutic target, while the pathophysiology of ischemic stroke encompasses numerous physiological mechanisms. Therefore, we believe that neuroprotection would be more feasibly achievable through targeting several pathways at the same time by using drug cocktails.

Our objective is to establish a reliable and reproducible high-throughput screening protocol using *D. melanogaster* to study the impact of different drug combinations already identified by *in silico* drug reprofiling as therapeutic approaches for hypoxia-reoxygenation injury. This organism has short life of about 10 days as well as an easy husbandry. In addition, the entire hypoxia signaling cascade is conserved and it has a high degree of homology with approximately 75% of human disease associated genes represented in Drosophila. These features allow *D. Melanogaster* to be used to study complex pathways in biomedical research, including cancer, cellular development, addictions or in our case, stroke.

MATERIALS AND METHODS

Drosophila Melanogaster husbandry: The *D. Melanogaster* wild-type strain Oregon-R was kindly provided by Dr. Luis María Escudero. Flies were raised and maintained in plastic vials containing standard food at 25°C under a 12 h light/12 h dark cycle. 1-3 days old flies were sexed under CO_2 anesthesia and placed in plastic vials containing Nutri-fly instant food. Anoxia (0% O_2) was induced by displacing air oxygen by a gas mix (90% N_2, 5% H y 5% CO_2) using a Coy Vinyl Glove Box chamber. Hypoxia was induced using the Coy O_2 Control InVitro Glove Box for Tissue Culture chamber. Air oxygen was replaced by N_2 continuous infusion into the chamber. We regulated the parameters defining a concentration of 1% of O_2, 25°C and a humidity range of 30-40%. Control groups were kept in normoxia.

Negative geotaxis assay: Climbing analysis was performed 5 h after severe hypoxia. In brief, groups of 20 flies per vial were tapped to the bottom and flies crossing a line at 6 cm height within a time period of 10 second was scored.

Drosophila activity monitoring (DAM) assay: To evaluate activity after hypoxia, flies were transferred into a Drosophila Activity Monitoring system (Model DAM3, Trikinetics, Inc., USA). Activity was monitored by recording the light beam interruptions caused by the fly passing through the light. Flies were kept in the DAM system for 4 h.

Tissue homogenization and protein quantification: A group of 10-20 flies per condition was snap-frozen in liquid nitrogen and 30 µl of Tris lysis buffer (pH 7.4) per fly was added. In a set of experiment, flies' head and bodies were collected separately. Samples were sonicated for 15 min in ice-cold water and subsequently centrifuged at 13.000 xg for 30 minutes at 4˚C. The supernatant was used to measure the biochemical parameters. The BCA Protein Quantification Kit was used to quantify protein concentration in each sample.

Determination of Caspase Activation: The APO-ONE Homogeneous Caspase 3/7 Assay Kit (Promega) was used to assess caspase activation after hypoxia.

Determination of ROS: To determine the level of ROS after hypoxia, samples were incubated with 2´7´-dichlorodihydrofluorescein (DCFH-DA) for 30 minutes and then fluorescence intensity was measured.

Carbonylated protein quantification: To measure carbonylated proteins after hypoxia, the Protein Carbonyl Content Assay Kit (Sigma-Aldrich) was used.

Statistical analysis: Data were analyzed using Graphpad Prism version 9. All the data are expressed as the mean ± SD. T-student and One-way Anova tests were performed to determine the statistical significance. The level of significance was set at p<0,05 and asterisks indicate significant between group differences.

RESULTS

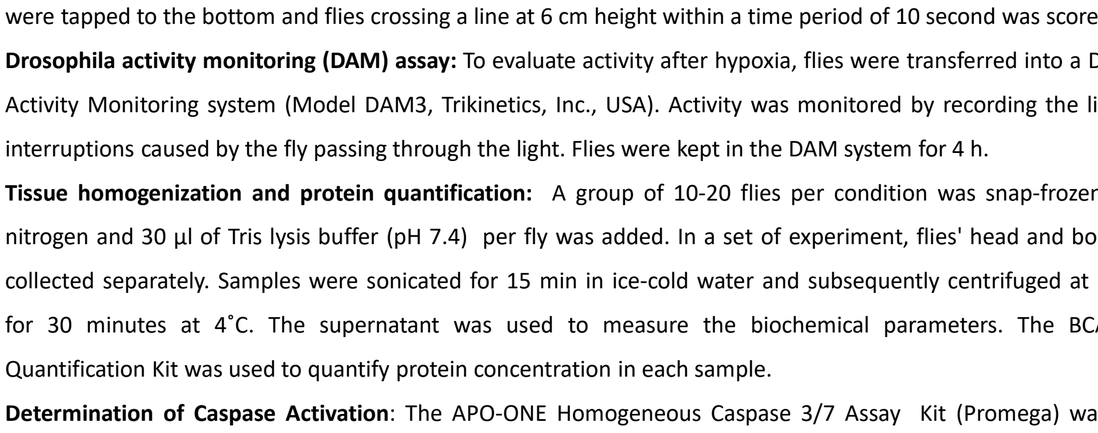

Figure 1. Drosophila Melanogaster as model system. A) Life cycle *of D. melanogaster*. Time-length is variable depending on environmental temperature. At 25˚C life cycle lasts around 10 days and is divided into four different stages: embryo, larva, pupa and adult . B) Objective of the project. We aim to stablish a screening platform using *D. melanogaster* to study neuroprotective therapies for ischemia-reperfusion damage. Mortality rates, locomotor deficit and biochemical changes will be characterized.

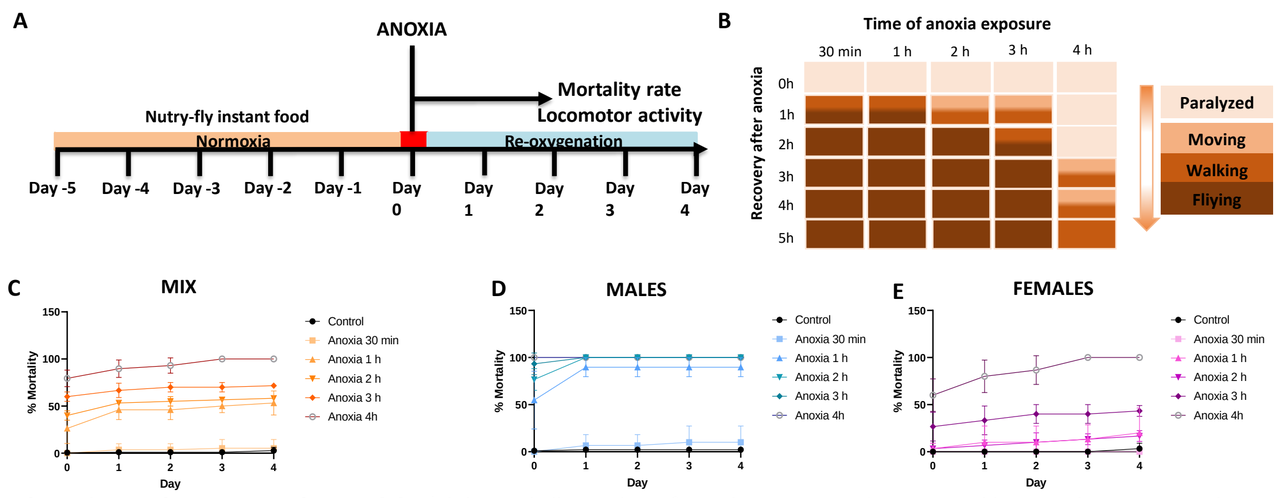

Figure 2. Anoxia-reoxygenation model. A) Schematic ilustration of anoxia-reoxygenation protocol in *D. melanogaster*. 1-3 day old flies were sexed under CO_2 anesthesia and maintained with Nutri-fly instant food for 5 days. On day 0 flies were subjected to different time-lengths of anoxia and mortality was assessed daily for 5 days. B) Recovery after anoxia. The degree and speed of recovery after anoxia is dependent on exposure length. C) Percentage of mortality was evaluated daily. D and E) Percentage of mortality rate in male and female flies, respectively. Sexual dimorphism was observed in the response to anoxia.

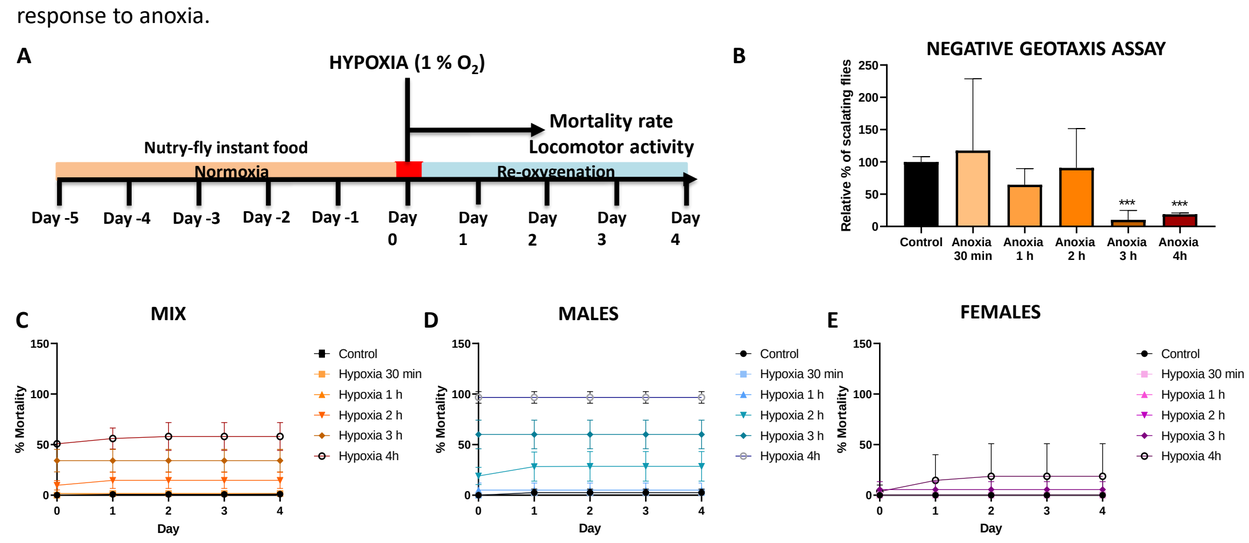

Figure 3. Hypoxia-reoxygenation model. A) Schematic ilustration of hypoxia-reoxygenation protocol in *D. melanogaster*. 1-3 day old flies were sexed under CO_2 anesthesia and maintained with Nutri-fly instant food for 5 days. On day 0 flies were subjected to different time-lengths of severe hypoxia (1 % O_2) and mortality was assessed daily for 5 days. B) Negative geotaxis assay. Four hours after hypoxia, flies were tapped to the bottom of the tube and the number of flies able to climb 6 cm in 10 sec were registered. Periods of hypoxia longer than 2 h resulted in a significant reduction of climbing ability. C) Percentage of mortality was evaluated daily. D and E) Percentage of mortality in male and female flies, respectively. Sexual dimorphism was observed in the response to anoxia.

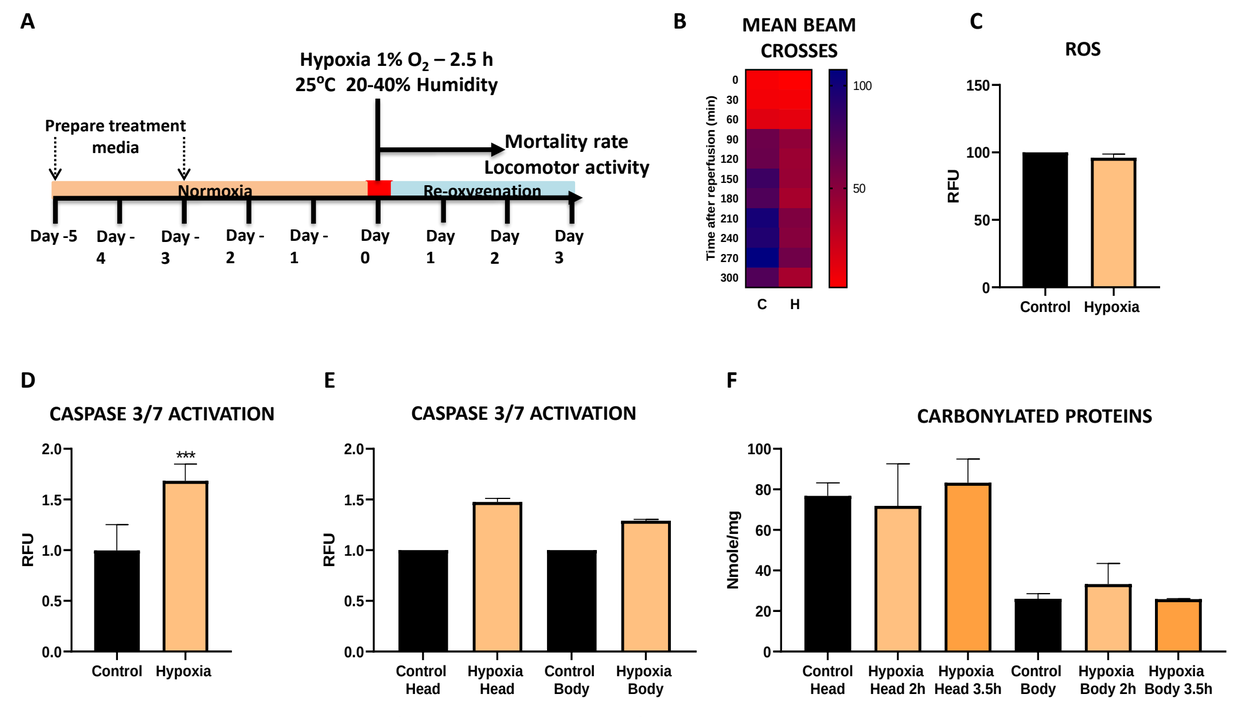

Figure 4. Protocol characterization. A)Schematic ilustration of hypoxia-reoxygenation protocol in *D. melanogaster*. 1-3 day old male flies were sexed under CO_2 anesthesia and maintained with Nutri-fly instant food for 5 days. On day 0 flies were subjected to 2.5 h of severe hypoxia (1% O2) under controlled temperature (25°C) and humidity (20-40 %). Immediately after hypoxia, locomotor activity was measured for 4 h using the DAM activity monitor and mortality was recorded for 3 days. B) Heatmap showing locomotor activity. Reduced number of movements was observed in flies subjected to hypoxia compared to the control group. C) ROS measurement 2 h after of the exposure to hypoxia. No significant changes were observed. D) Measurement of caspase 3/7 activation 2 hours of the exposure to hypoxia. Hypoxia significantly induced caspases activation. E) Caspase activation measurement in flies' heads and bodies separately two hours after hypoxia exposure. An increase in caspases was observed in both tissues compared to control. F) Quantification of carbonylated proteins in flies' heads and bodies separately two hours after hypoxia exposure. No significant changes were observed.

CONCLUSIONS

In conclusion, we established a reliable and reproducible operating protocol to study the impact of hypoxia-reoxygenation damage in *D. melanogaster* which will allow the screening of drug cocktails against ischemic stroke. In addition, our protocol and the availability of mutants and transgenic strains may facilitate the study of stroke treatments and potentially, other neurodegenerative diseases.

REFERENCES

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