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EMERSON SANTOS DA SILVA

PEIXE-ZEBRA COMO UM MODELO DE ESTUDO PARA ENCEFALOPATIA HIPÓXIA PERINATAL: AVALIAÇÃO DE DISTÚRBIOS SOCIAIS, SUSCETIBILIDADE A CRISES EPILÉTICAS E RESPIRAÇÃO MITOCONDRIAL CEREBRAL NA IDADE ADULTA

PORTO ALEGRE

Março, 2019

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Orientador: Profª. Drª. Maria Elisa Calcagnotto
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"Se consegui ver mais longe é porque estava aos ombros de gigantes"

Isaac Newton

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APRESENTAÇÃO

Esta dissertação está dividida em seis partes:

PARTE I: resumo, *abstract*, lista de abreviaturas, introdução e objetivos;

PARTE II: os resultados que fazem parte desta dissertação foram apresentados na forma de artigo científico, **Capítulo I**, o qual será submetido ao periódico *Translational Stroke Research*. O capítulo está subdividido em: Introdução, Materiais e Métodos, Resultados, Discussão, Conclusão e Referências Bibliográficas;

PARTE III: discussão;

PARTE IV: conclusão;

PARTE V: referências bibliográficas referentes à Parte I e Parte III.

PARTE I

RESUMO

A encefalopatia hipóxica perinatal (EHP) é considerada uma das principais causas de morte no período neonatal. As consequências a longo prazo incluem dificuldades sociais e de aprendizado, e aumento do risco de transtornos psiquiátricos e neurológicos, como epilepsia. Dado que a informação sobre a influência da EHP no cérebro adulto é muito limitada, nós investigamos a habilidade de peixes-zebra submetidos à hipóxia poderem ser usados como um novo modelo de EHP. Para atender este objetivo nós investigamos os efeitos sobre a interação social, suscetibilidade a crises epiléticas e consumo de oxigênio (O_2) dos principais componentes do sistema transportador de elétrons (STE) durante a idade adulta de peixes submetidos à hipóxia na fase larval. Para induzir EHP, larvas de peixe-zebra foram colocadas durante 2 horas em um aquário tipo "nursery" com níveis reduzidos de O_2. Depois disso, os animais foram transferidos para um aquário contendo água normóxica para recuperação espontânea da hipóxia por 120 dias. Nós observamos que os animais com EHP exibiram aumento na taxa de mortalidade, diminuíram o tempo de interação com animais co-específicos e aumentaram a suscetibilidade a crises epiléticas induzidas durante a vida adulta. No entanto, o consumo de O_2 mitocondrial do STE foi equivalente aos controles. Embora não tenha sido observado qualquer efeito sobre a respiração mitocondrial cerebral, o peixe-zebra submetido a hipóxia na fase larval apresentou os efeitos clássicos da EHP na idade adulta. Portanto, nossos dados indicam o potencial uso deste novo modelo para futuros estudos relacionados à EHP.

ABSTRACT

Perinatal hypoxic encephalopathy (PHE) is a neurological injury considered a major cause of acute neonatal death. The long-term consequences include social and learning disabilities, and increased risk for psychiatric and neurological disorders such as, epilepsy. Given that the information about the influence of PHE on adult brain is very limited, we investigated the ability of zebrafish subjected to hypoxia to be used as a new model of PHE. To meet this purpose, we evaluated the effects on social interaction, seizures susceptibility and oxygen (O_2) consumption of the main components of electron transporter system (ETS) during adulthood of fish subjected to hypoxia in larval stage. To induce PHE, zebrafish larvae were placed for 2 hours in a nursery with reduced O_2 levels. After that, animals were transferred to an aquarium containing normoxic water for spontaneously recovery from hypoxia for 120 days. We observed that animals with PHE exhibited increase mortality rate, decrease interactive time with conspecific animal and increase susceptibility to seizure like-behavior during adulthood. However, the mitochondrial O_2 consumption of ETS was equivalent to controls. Although no effect on brain mitochondrial respiration was observed, zebrafish-undergoing hypoxia in the larval phase showed the classic effects of PHE in adulthood. Therefore, our data indicate the potential use of this new model for future studies related to PHE.

LISTA DE ABREVIATURAS

EHP: Encefalopatia hipóxica Perinatal

O_2: Oxigênio

TEA: Transtorno do Espectro Autista

TDAH: Transtorno de Déficit de Atenção e Hiperatividade

SNC: Sistema Nervoso Central

ATP: Adenosina Trifosfato

dpf: Dias Pós-Fertilização

hpf: Horas Pós-Fertilização

phd: Pós dia da hipóxia

PTZ: Pentilenotetrazol

STE: Sistema Transportador de Elétrons

INTRODUÇÃO

1. ENCEFALOPATIA HIPÓXICA PERINATAL

A encefalopatia hipóxica perinatal (EHP) é um transtorno cerebral que afeta 4-6 em 1.000 neonatos (SCHENDEL; NELSON; BLAIR, 2012). Ela é caracterizada pela redução ou bloqueio da oferta de oxigênio ao cérebro de recém-nascidos, resultando em prejuízos cognitivos, crises epiléticas e retardo do desenvolvimento (DU PLESSIS; VOLPE, 2002). Qualquer evento que comprometa o fornecimento de sangue ou oxigênio contribui para a ocorrência da EHP, como a falta de cuidados pré-natais, desproporção céfalo-pélvica, parto pélvico, idade gestacional, hemorragia, embolia do líquido amniótico, colapso hemodinâmico (doença cardíaca, tromboembolismo, sepse, overdose, eclâmpsia, hemorragia intracraniana, anafilaxia), eventos placentários (descolamento ou deterioração) e eventos dos cordões umbilicais (envolvimento no pescoço-enforcamento, ruptura ou compressão) (GILLAM-KRAKAUER; CARTER, 2012). Apesar dos esforços consideráveis para encontrar uma farmacoterapia neuroprotetora clinicamente segura e eficaz, a única terapia atualmente aprovada é a hipotermia moderada. Contudo, apesar da introdução da hipotermia terapêutica (TH) como padrão de tratamento para crianças com EHP, 40-50% das crianças afetadas ainda terão uma deficiência neurológica significativa (EDWARDS et al., 2010).

O desenvolvimento de lesões cerebrais depois da EHP é um processo em evolução que se estende para a fase de reperfusão (ANTONUCCI; PORCELLA; PILLONI, 2014). Em estudos com modelos animais neonatos, observou-se 2 fases de falha energética, a primária e a secundária (LOREK et al., 1994). Na fase primária, observou-se reduções no fluxo sanguíneo cerebral (juntamente com a oferta de O_2) e em compostos fosforilados de alta energia (ATP e fosfocreatina), além da acidose tecidual proeminente (MARTINELLO et al., 2017). Esta falha energética primária está associada a uma série complexa de

alterações intracelulares agudas, incluindo perda da homeostase iônica da membrana, osmoregulação defeituosa, alteração na liberação/recaptação de neurotransmissores excitatórios e alterações na homeostase do cálcio intracelular que desencadeia ativação de proteases, lipases e endonucleases (SIESJÖ; BENGTSSON, 1989). A falha energética secundária é caracterizada por declínios nos níveis de fosfocreatina e ATP sem acidose cerebral (LOREK et al., 1994). Neste processo, mecanismos neurotóxicos secundários são ativados. Ocorre acumulação extracelular de neurotransmissores excitatórios, principalmente glutamato. Isto provoca a ativação excessiva de receptores de glutamato neuronal, principalmente o receptor N-metil-D-aspartato (NMDA), o que resulta num influxo intracelular excessivo de cálcio. O acúmulo de cálcio intracelular resulta na ativação de enzimas de degradação celular e apoptose (lipases, fosfolipases, proteases e endonucleases); produção de espécies reativas de oxigênio através da ativação de xantina oxidase, aumento da síntese de prostaglandinas e ativação da enzima óxido nítrico (NO) sintase (LOREK et al., 1994). Como resultado combinado do aumento de cálcio intracelular e quantidades excessivas de radicais livres, ocorre a deterioração da função mitocondrial (ANTONUCCI; PORCELLA; PILLONI, 2014). A disfunção mitocondrial leva a liberação adicional de espécies reativas de oxigênio; liberação de citocromo c que provoca apoptose neuronal através da ativação de uma cascata proteolítica, incluindo caspases e liberação de proteínas capazes de induzir a apoptose através de um mecanismo independente de caspase (JOHNSTON MV, 2001). Assim, esse esgotamento do oxigênio que ocorre na EHP impede a fosforilação oxidativa, o que acarreta falha de energia mitocondrial primária e inicia uma cascata de eventos que levam à disfunção celular e, inevitavelmente, à morte celular (ANTONUCCI; PORCELLA; PILLONI, 2014).

Os efeitos a longo prazo causados pela EHP em indivíduos que atingem a vida adulta é de difícil investigação uma vez que, a maioria dos estudos foca na detecção de alguma disfunção em idades precoces. A associação existente entre dificuldades cognitivas e comportamentais podem ser esperadas pelas lesões cerebrais geradas pelo EHP. O hipocampo e o estriado estão entre as estruturas cerebrais que podem ser afetadas (ALLEN;

BRANDON, 2011; DE HAAN et al., 2006; GADIAN et al., 2000; MAÑERU et al., 2003; MATTIESEN et al., 2009; TOFT, 1999; VAN HANDEL et al., 2007). Essas estruturas têm sido associadas a funções cognitivas específicas, como memória e atenção, e supostamente desempenham um papel na patogênese do Transtorno do Déficit de atenção e hiperatividade (TDAH), Transtorno do Espectro Autista (TEA) e esquizofrenia (BURSTYN et al., 2011; GETAHUN et al., 2013; MIGUEL et al., 2018; MURRAY et al., 2016; VAN HANDEL et al., 2007).

Por conta do impacto na saúde ao longo da vida dos indivíduos acometidos por EHP, vários biomarcadores moleculares de danos cerebrais causados pela EHP foram descritos e testados. Devido à capacidade limitada de atravessar o Barreira Hematoencefálica (BHE), muitos desses biomarcadores moleculares – como proteínas, podem ser detectados no sangue periférico e outras são detectáveis apenas no líquido cefalorraquidiano (LCR) (Hongyan et al. 2015). Em alguns casos, como por exemplo, a enolase específica de neurônios (EEN) e a proteína ácida fibrilar glial (PAFG) tem seus níveis séricos elevados no soro e no LCR em EHP leve e grave (Celtik et al. 2004) e ainda, durante os danos encefálicos, tem sido demonstrado que os níveis séricos de PAFG correspondem ao dano morfológico avaliado por ressonância magnética após hipóxia, sugerindo que PAFG pode ser um biomarcador molecular confiável de EHP (Lv et al. 2015, Ennen et al. 2011). No entanto, as sequelas neurológicas neonatais (por exemplo, crises epilépticas, hipotonia) são o método comum usado para diagnosticar a EHP e continuam sendo a análise que nos permite determinar o prognóstico do recém-nascido.

2. CRISES EPILÉTICAS

A epilepsia é uma desordem neurológica que acomete o SNC (Sistema Nervoso Central), caracterizada por crises epiléticas decorrentes da atividade anormal, excessiva e hipersincrônica nos neurônios do cérebro (ENGEL, 2006). As lesões neurológicas mais graves ocorrem com uma prevalência de aproximadamente 3/1000 em recém-nascidos

vivos, sendo esse prognóstico associado à EHP (PRESSLER et al., 2015). A EHP é a causa mais comum de crises epiléticas na fase perinatal e está também relacionada a um risco aumentado de desenvolver epilepsia na fase adulta (ALLEN; BRANDON, 2011).

As alterações no SNC resultantes da EHP muito provavelmente sejam causadas por mudança na energia celular. Esta mudança pode resultar numa diminuição dos níveis de trifosfato de adenosina (ATP), um desequilíbrio de neurotransmissores inibitórios e excitatórios que incluem a liberação sináptica excessiva de glutamato e a diminuição da recaptação desse mesmo neurotransmissor, produzindo níveis aumentados na fenda sináptica (GILLAM-KRAKAUER; CARTER, 2012). Em estudos de microdiálise *in vivo* no cérebro de ratos imaturos, demonstrou-se que a EHP resulta em níveis elevados de glutamato (BENVENISTE et al., 1984; SILVERSTEIN; NAIK; SIMPSON, 1991). Além disso, a relevância clínica para os estudos experimentais é sugerida devido a demonstração de concentrações elevadas de glutamato no Liquido Cefalorraquidiano (LCR) de crianças após hipóxia (HAMBERGER et al., 1992). Por fim, já se sabe também que o cérebro neonatal é mais suscetível a crises do que qualquer outro período na vida, por causa de uma predominância de neurotransmissores excitatórios em contrapartida a presença de sistemas inibitórios imaturos (GILLAM-KRAKAUER; CARTER, 2012).

3. MODELO DE PEIXE-ZEBRA E INTERAÇÃO SOCIAL

Modelos animais experimentais de distúrbios cerebrais são ferramentas úteis para descoberta dos mecanismos patogênicos de desordens cerebrais (SILVERMAN et al., 2013). Abordagens experimentais utilizando peixe-zebra (*Danio rerio*) estão rapidamente ganhando popularidade na pesquisa em neurociência (STEWART et al., 2014). Isto se deve a evidências experimentais recentes demonstrando que o peixe-zebra possui alta homologia genética e fisiológica com os mamíferos, além de apresentar respostas sociais e cognitivas complexas que são semelhantes aos observados em roedores e humanos (STEWART et al., 2014).

O peixe-zebra é um animal altamente social e passa a maior parte do tempo em cardume, ficando muito próximo de seu co-específico e exibindo uma gama de comportamentos em cardumes como: interação social (DREOSTI et al., 2015; GREEN et al., 2012; KRAUSE et al., 2000; MILLER; GERLAI, 2012; PHAM et al., 2012), agressão direcionada co-específica (JONES; NORTON, 2015), acasalamento (ENGESZER et al., 2008) entre outros (ARGANDA; PEREZ-ESCUDERO; DE POLAVIEJA, 2012). O comportamento social em peixe-zebra reflete uma interação complexa entre números de animais que se movem juntos de maneira coordenada em um determinado ambiente. Sendo assim, a formação de um cardume coeso corresponde a uma unidade funcional em situações aversivas com a finalidade de proteção. O comportamento de grupo em peixe-zebra vem sendo abordado em estudos envolvendo aspectos ontológicos e efeitos de estresse ambiental, como a hipóxia. Dessa forma, o peixe-zebra pode contribuir para a compreensão da patobiologia da interação entre os indivíduos (KALUEFF; STEWART; GERLAI, 2014).

O estresse hipóxico provoca grandes mudanças metabólicas em todos os organismos que necessitam de oxigênio. Entre os métodos mais conhecidos que têm sido empregados para estudar os efeitos da hipóxia durante o desenvolvimento, está a incubação do organismo numa câmara hipóxica. Neste aparato, o peixe-zebra adulto permanece em água contendo menos de 2mg de O_2/L, caracterizando um procedimento técnico não invasivo, que produz uma baixa perda de animais durante os testes de hipóxia (BRAGA et al., 2013; YU; LI, 2011). Até o momento, esses estudos demonstraram que a câmara de hipóxia causa prejuízos no comportamento exploratório de peixe-zebra adulto associado a uma diminuição na atividade mitocondrial do cérebro (BRAGA et al., 2013; YU; LI, 2011). Se considerarmos o modelo animal apropriado, deve-se ter em mente que existem vários fatores que poderiam influenciar os resultados. Um dos mais significativos é temperatura. A hipotermia é hoje aceita como importante procedimento de tratamento e diferentes temperaturas aos animais durante todo o experimento causam resultados diferentes (Vannucci e Vannucci 2005). No modelo da câmara hipóxica, isso não ocorre porque a temperatura é controlada desde o

princípio de cada experimento. Outra limitação dos modelos da EHP e que deve ser levada em conta é a diferença no sexo. Animais têm respostas sexuais específicas frente a hipóxia e nem todas as moléculas ou mecanismos celulares de dano cerebral são muito provavelmente compartilhada entre sexos (Hagberg et al. 2004, Nijboer et al. 2007b). Assim, nosso modelo torna-se vantajoso pelo fato de um maior número de animais exposto ser possível após a hipóxia separar os animais por sexo e fazer qualquer tipo de análise. Portanto, o modelo de câmara hipóxica em peixe-zebra consiste em ensaios rápidos e práticos, que são adequados para pesquisas sobre mecanismos de hipóxia adulta e no desenvolvimento de novas terapias em doenças relacionadas à hipóxia. Dado todas estas características, o peixe-zebra, associado a uma abordagem de privação de O_2 bem estabelecida, pode se tornar uma ferramenta útil para o estudo da EHP ao longo do desenvolvimento.

Justificativa:

Devido às poucas informações sobre as sequelas que a hipóxia perinatal pode causar na fase adulta esse projeto propõe investigar a habilidade de peixes-zebra submetidos à hipóxia poderem ser usados como um novo modelo de EHP.

Hipótese:

Nossa hipótese de trabalho é que peixes-zebra submetidos à hipóxia na fase larval apresentem, na idade adulta, alterações previamente descritas para modelos de EHP como, déficit social e aumento da suscetibilidade a crises epiléticas. Além disso, nós hipotetizamos que estes efeitos poderiam estar associados com um prejuízo no metabolismo mitocondrial.

OBJETIVO

1. OBJETIVO GERAL

Validar a utilização de modelo de encefalopatia em peixe-zebra adulto através da avaliação da interação social, a susceptibilidade a crises epiléticas e o consumo de oxigênio mitocondrial cerebral.

2. OBJETIVOS ESPECÍFICOS

- Avaliar parâmetros comportamentais através da interação social na fase adulta.

- Estudar a suscetibilidade a crises epiléticas através do estudo de latência e intensidade de crises induzidas por pentilenotetrazol (PTZ) na fase adulta.

- Investigar o consumo de oxigênio dos principais componentes do sistema transportador de elétrons de mitocôndrias cerebrais de peixe-zebra adulto exposto ou não ao pentilenotetrazol (PTZ) na fase adulta.

PARTE II

CAPÍTULO I

Artigo em preparação para submissão

Título: *Hypoxia in zebrafish larvae alters social behavior and seizure susceptibility during adulthood*

Autores: da Silva, Emerson Santos; Braga, Marcos Martins; Loss, Cássio Morais; Lazzarotto, Gabriela; Klamt, Fábio; Calcagnotto, Maria Elisa.

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TITLE PAGE

RESEARCH PAPER

TITLE

Hypoxia in zebrafish larvae alters social behavior and seizure susceptibility during adulthood

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RESEARCH PAPER

ABSTRACT

Perinatal hypoxic encephalopathy (PHE) is a neurological injury considered a major cause of acute neonatal death. The long-term consequences include social and learning disabilities, and increased risk for psychiatric and neurological disorders such as, epilepsy. Given that the information about the influence of PHE on adult brain is very limited, we investigated the ability of zebrafish subjected to hypoxia to be used as a new model of PHE. To meet this purpose, we evaluated the effects on social interaction, seizures susceptibility and oxygen (O_2) consumption of the main components of electron transporter system (ETS) during adulthood of fish subjected to hypoxia in larval stage. To induce PHE, zebrafish larvae were placed for 2 hours in a nursery with reduced O_2 levels. After that, animals were transferred to an aquarium containing normoxic water for spontaneously recovery from hypoxia for 120 days. We observed that animals with PHE exhibited increase mortality rate, decrease interactive time with conspecific animal and increase susceptibility to seizure like-behavior during adulthood. However, the mitochondrial O_2 consumption of ETS was equivalent to controls. Although no effect on brain mitochondrial respiration was observed, zebrafish-undergoing hypoxia in the larval phase showed the classic effects of PHE in adulthood. Therefore, our data indicate the potential use of this new model for future studies related to PHE.

KEYWORDS

perinatal hypoxia encephalopathy; zebrafish; *Danio rerio*; behavior; social preference test; seizure like-behavior; susceptibility

1. INTRODUCTION

Perinatal hypoxic encephalopathy (PHE) is a neurological injury characterized by partial or complete hypoxia associated with reduction/interruption of the blood flow [1,2]. PHE affects about 4–6 of 1000 live births in developed countries, the incidence is even higher in developing countries [3–6] and is considered a major cause of acute neonatal death [7–9]. In the newborn survivors, the long-term consequences PHE include developmental disabilities, learning deficits, reduced socialization and increased risk for later psychiatric and neurological disorders, such as autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHS), schizophrenia and epilepsy [10–21]. During the perinatal period, PHE is the most common cause of seizures, increasing the risk for development of epileptic seizures in adulthood [22–25]. However, the effects of PHE on increased seizure susceptibility in adulthood are still poorly understood [26–28].

Experimental approaches using zebrafish (*Danio rerio*) are rapidly gaining popularity in neuroscience research (STEWART et al., 2014). This is due to recent experimental evidence demonstrating that zebrafish has high genetic and physiological homology with mammals, as well as presenting complex social and cognitive responses that are similar to those observed in rodents and humans (Stewart et al., 2014). The zebrafish are highly social animals and spend most of their time in school, exhibiting a range of behaviors in schools, such as social interaction (DREOSTI et al., 2015; GREEN et al., 2012; KRAUSE et al., 2000; MILLER; GERLAI, 2012; PHAM et al., 2012). In addition, zebrafish have showed similar behaviors to other models when they are exposed to known compound containing neurochemical action (e.g., kainic acid and penthylene tetrazole (PTZ)) (ALFAROS et al., 2011; MUSSULINI et al., 2018). Finally, this fish species have been used to evaluate the effects caused by hypoxia through a non-invasive technical procedure, which produces a low loss of animals during hypoxia tests (BRAGA et al., 2013; YU; LI, 2011). To date, these studies have demonstrated

that the hypoxia causes impairment in the exploratory behavior of adult zebrafish associated with a decrease in mitochondrial brain activity (BRAGA et al., 2013). Given all these characteristics, zebrafish, associated with a well-established O_2 deprivation approach, can become a useful tool for the study of PHE throughout development.

Since we understand that the development of a more practical PHE model could support a better understanding of the long-term effects of this dysfunction, we aimed to investigate whether zebrafish subjected to hypoxia present the known effects induced by PHE in adulthood. For this purpose, we exposed zebrafish larvae to hypoxia and observed the effects throughout development (e.g., growth and survival). In adulthood, these animals were evaluated in relation to ability to social interaction and susceptibility to seizures induced by PTZ. Moreover, given the role played in hypoxia and seizures, the O_2 consumption of the main components of electron transporter system (ETS) was measured in the brain of the animals, before and after to PTZ exposure.

2. MATERIAL AND METHODS

2.1. ANIMALS

According to handling and animals reproduction described in Baggio et al. 2017 fertilized eggs of zebrafish were collected 2 hours post fertilization (hpf). Eggs not coagulated and translucent based on fertile characteristics descript by Zefin Atlas [43] and approximately 200 fertilized eggs by cohorts (three cohorts were used in the whole study; 600 eggs in total) were selected and divided randomly (manually, without use of software) in 2 equal rearing tanks. Eggs were incubated on Biological Oxygen Demand (B.O.D) at 28 °C until 7 days post fertilization (dpf) and were fed twice a day with paramecium from 2 hpf until 30 dpf. At 7 dpf, the fish were moved into 2.8L rearing tanks (20 fish per tank) in a high-density rack system equipped to re-circulating system with mechanical and biological filtration at 28 °C, pH 7.4 and conductivity 500µS (system water). The zebrafish were housed in holding room illuminated with ceiling-mounted fluorescent lamps asset for 14/10 light/dark photoperiod

(light on at 08:00 am). The animals were fed four times a day with a commercial flake fish food (ALCON BASIC, Alcon Brazil) and nauplii of brine shrimp (Artemia Saline), and were maintained according to the National Institute of Healthy Guide and Use of Laboratory animals (2011). The ethics Committee approved all procedures with animal subjects for use of animals – CEUA (33284) from the Universidade Federal do Rio Grande do Sul. Zebrafish remained on these holding tanks until the end of the experiments (four months of age – 120 dpf).

2.2. HYPOXIA MODEL

At 72 hpf, zebrafish larvae were randomly assigned to one of the following groups: PHE group (exposed to aquatic hypoxic condition; ~1.8mgO_2/L); Normoxia group (exposed to aquatic regular [O_2]; 8mgO_2/L). We choose exposing animals to hypoxia at this developmental stage because neural tube is fully developed at this time, corresponding to the third trimester of human gestational period [44]. To induce the hypoxia, a glass chamber isolated from the atmospheric environment was filled with water. To obtain very low O_2 concentration (~1.8mgO_2/L) nitrogen gas (N_2) was added into the water as previously described [38,42]. The O_2 dissolved in water was maintained at a low level (~1.8mgO_2/L) in order to obtain hypoxic condition. Next, the water from the sealed hypoxic chamber was transferred through a hose to the sealed cover nursery (containing 100 larvae; PHE group). The chamber and the nursery container remained sealed to obtain a closed hypoxic microenvironment. Based on Kimmel [44] the 72 hpf larvae were maintained for 2 hour in this condition. After that, animals were transferred to an aquarium containing water on normoxic condition (8.0 mgO_2/L) and allowed to spontaneously recovery from hypoxia. The other 100 larvae were used as control animals (Normoxia group) and were handled exactly the same way, except that they were maintaining in normoxic conditions during the whole experiment. The day animals were submitted to hypoxia was considered as post-hypoxia day zero (phd0).

2.3. DEVELOPMENTAL MONITORING

Six hundred (4 months-old) wild-type zebrafish (*Danio rerio*) from both sexes were used. All fish were obtained by local breeding through pair-wise crossings and kept at 14h/10h dark/light cycle. Embryos were maintained at embryo medium (NaCl 5.03mM, KCl 0.17mM, $CaCl_2$ 0.33mM, $MgSO_4$ 0.33mM, pH 7.5±0.5, 28±1°C) and housed in Biochemical Oxygen Demand (B.O.D.) incubator (28±1°C) at a density of 1 animal/2.5mL. At 5 days post fertilization (dpf), larval zebrafish were fed with *Paramecium* infusoria *ad libitum*, which was gradually replaced by *Artemia* nauplii at 13 dpf. Young fish (21 dpf) were transferred to a recirculating housing system and kept at following conditions: 28±1° C, pH and of 7.5±0.5, conductivity of 500 µS/cm and density of 3 animals/L. Adult animals were fed four times a day with *Artemia* nauplii (09:00 a.m. and 05:00 p.m.) and commercial flake fish food (11:00 a.m. and 03:00 p.m.) at 4-5% of the total biomass. All animals were experimentally naive, healthy and free of any signs of disease.

2.4. SOCIAL PREFERENCE

When animals reached phd120 they were subjected to social preference test. Before being exposed to the apparatus, animals were allowed to acclimate with the experimental room during 1 hour. The acclimation consisted in placing the home tank (containing fishing net inside the aquarium) in the experimental room. This procedure was adopted to minimize agitation and stress provoked by fishing during testing. All behavioral procedures were conducted between 10:00 am and 4:00 pm with ~200 lux of luminosity.

Social preference test was conducted in a glass tank containing fresh system water, which was virtually divided in three zones: (i) the conspecific zone; (ii) the center zone;
(iii) the opposite to conspecific zone (Fig. 2). Another tank containing three matched zebrafish was placed adjacent to the conspecific side while an empty tank was placed next to the opposite side. Each experimental fish was gently netted from their home tank, individually putted in the center zone of the tank and allowed to explore it for 6 min [45] (n = 10 by cohorts per group; total 30 animals per group). After each test, the water of the

experimental tank was replaced with the fresh system water. Before and during the experiments all efforts were made to minimize external environmental influence (noise, vibration, movement in front of the tank). ANY-maze video-tracking software (Stoelting CO, USA) was used to automatically record and collect behavioral data. The following parameters were collected: a) number of entries in each zone: an entry was registered when the entire body of the fish crossed the virtual line which defined the zones; b) time spent in each zone; c) moving time. Animals that did not enter one of the zones at least three times were removed from the analysis. In addition, animals that did not move at least 80% of the time were also excluded (6 animals of the n total per group) from the analysis. As a measure of sociability, normal fishes are expected to spend more time in the conspecific zone [45].

2.5. SEIZURE PHENOTYPES

To evaluate hypoxia effects on seizure phenotypes, another group of animals (n = 12 by cohort per group; total n = 36 per group) was subjected to PTZ- induced seizures. PTZ was readily dissolved in water to reach a concentration of 7.5mM. The apparatus consisted of a tank (20 cm width x 613 cm height x 67 cm length) filled with 1.5L of PTZ solution. A webcam (Logitech® HD Pro Webcam C920) was placed 30 cm from the tank to capture animal's behavior. In order to avoid animals' reflex in the walls and floor of the tank and also to ensure a uniform background for the video analysis, the floor plus three of the tank walls were coated with white background cover. All necessary precautions were taken to ensure representative behavioral results and to avoid handling stress by gently transferred between home tanks, beakers, and experimental apparatus. All fish were tested in the same room between 10:00 am and 4:00 pm. Animals were individually placed in the tanks and their behavior was video recorded during 20 min for posterior seizure behavioral assessment [46]. Fish behaviors were analyzed across time through the following score system: score 0, swimming in the bottom area (standard swimming of the fishes); 1, Increased swimming activity (propulsive movements); 2, Erratic movements (Fast forward swim with large bend angles and sharp changes in direction or velocity); 3, Circular swimming in the top (Repetitive swimming in a

circular-rotation direction); 4, Clonic-seizure like-behavior (rapid movements of the body without direction or order hyperactivity circling, spasms, tremor, head shake movements and jittery locomotion); 5, Loss of body posture; 6, death. The videos were analyzed for two investigators who were blinded to the groups. Main attention was given to score 3, 4, and 5, where the following parameters were evaluated: a) latency to reach each score; b) number of events for each score; c) mean events duration for each score: calculated as the total time spent in a specific score divided by total number of events in that score.

2.6. MITOCHODRIAL FUNCTION

A third group of animals (adult zebrafishes; phd120) were used for neurochemical analyzes of mitochondrial function. Animals from both PHE and Normoxia groups were euthanized by decapitation and their brains were removed, weighed, and polled so that each sample unit (n) was composed by two brains (n = 12 by cohort per group; total n = 36 per group). Brains were mechanically dissociated in 2mL mitochondrial respiration buffer (Hanks Balanced salt solution; HBSS) containing 137mM NaCl, 0.63mM Na_2HPO_4, 4.17mM $NaHCO_3$, 5.36mM KCl, 0.44mM KH_2PO_4, 1.26mM $CaCl_2$, 0.41mM $MgSO_4$, 0.49mM $MgCl_2$ and 1.11mM glucose, pH 7.2. Next, each sample unit was transferred to high-resolution oxygraph (Oxygraph-2k Oroboros Instruments, Innsbruck, Austria) and O_2 consumption was measured at 28°C. The O_2 flux (in pmol O_2.s-1.mL-1.), which is directly proportional to O_2 consumption, was recorded continuously using DatLab software 6 (Oroboros Instruments). After stabilization of the steady state of O_2 consumption, the routine O_2 consumption (basal respiration), phosphorylation coupled to ATP synthesis, maximum capacity of the mitochondrial electron transport system, Complex I and Complex III activities, and residual oxygen respiration (ROX) were measured in each sample unit by the addition of the Oligomycim (4 mg/mL), FCCP (0.1mM), Rotenone (1mM), Antimycin A (5mM), respectively. Results are expressed as consumption of pmol $O_2.s^{-1}.mL^{-1}$. A part of animals (adult zebrafishes; phd120) was used for neurochemical analyses of mitochondrial function in

animals that were affected by seizures. These animals were subjected to exactly the same procedures described above, except that they were exposed to PTZ-induced seizures protocol before euthanasia.

2.7. STATISTICS

Data presenting Gaussian Distribution (tested by Kolmogorov-Smirnov normality test) are expressed as mean ± SD and were analyzed by appropriated parametric test (see below). Data that did not assume Gaussian Distribution are expressed as median and the interquartile ranges and were analyzed by appropriated non-parametric test (see below). Data of survival were analyzed by Mantel–Cox test. For the Social Preference task, we tested the hypothesis that animals did not present preference for one of the zones (conspecific zone). For this, the social discrimination index was calculated as follows: TC/(TC+TO) where TC means the time spent in conspecific zone and TO means time spent in opposite zone. For each group, using One-sample t test, the social discrimination index was tested against 50% of chance of not preferring any of the zones. Moreover, Fisher's exact test was used to analyze the frequency of preference for each zone. Regarding epileptic seizure behavioral assessment, latency for each seizure score and number of seizures for each score were analyzed by Unpaired Student t test, while mean seizure duration for each score was analyzed by Mann Whitney test. For the mitochondrial function assessment, data were analyzed by Paired t test. An $\alpha = 0.05$ was set for all analysis. The data collection was made without knowledge of conditions.

3. RESULTS

3.1. EFFECT OF HYPOXIA ON ANIMAL SURVIVAL AND BODY SIZE

The survival rate was evaluated from phd0 to phd120. Animals exposed to hypoxia present greater probability of dying than animals maintained in Normoxia (Chi square= 16.54; $P < 0.0001$, Mantel-Cox). Graphical representation indicates that the first two weeks post hypoxia was a critical period and about 44% of the animals died. The mortality rate of PHE animals was higher when compared to Normoxia group (about 19%) (Fig. 1).

In relation to body size, animals from PHE group were larger than Normoxia group at phd60 ($P = 0.0067$), phd90 ($P < 0.0001$) and phd120 ($P = 0.0012$) but not at phd30 ($P =0.3605$) (Supp. Fig. 1).

3.2. EFFECT OF PHE ON SOCIABILITY OF ZEBRAFISH DURING ADULTHOOD

We analyzed whether PHE would alter adult zebrafish social behavior. Animals from Normoxia group spent more time in the conspecific zone ($P < 0.0001$, One-sample t) while animals from PHE group did not ($P = 0.4633$, One-sample t) (Fig. 2b). We also observed that the frequency of animals that spent more than 50% of the testing time in the conspecific zone was higher in Normoxia group (92%) than in PHE group (67%) ($P< 0.0432$) (Fig. 2c). The behavioral pattern of each experimental group is shown in Figure 2a.

3.3. EFFECT OF HYPOXIA ON PTZ-INDUCED SEIZURE SUSCEPTIBILITY IN ADULT ZEBRAFISH

The seizure behavior during PTZ exposure was similar to those previously described by (Mussulini et. al. 2013). Animals from both groups presented increased swimming activity and erratic movements followed by circular swimming at the apparatus surface (score 3) which culminate in clonic seizure-like behavior (score 4) and loss of posture (score 5). Only three animals from Normoxia group (less than 9%) died during the PTZ exposure. We did not observe influence of hypoxia on latency to score 3 ($P = 0.3278$), score 4 ($P = 0.1398$) or score 5 ($P = 0.6427$) (Fig 4a). However, hypoxia increased the number of clonic seizures-like behavior (score 4) ($P < 0.0001$) and the mean duration of score 4 ($P = 0.0414$) (Fig. 4b and c). That also indicates that the animals from PHE group spent more time presenting clonic seizures-like behavior than animals from the Normoxia group. A representation of seizure behavior across time is depicted in Figure 3.

3.4. EFFECT OF HYPOXIA ON MITOCHONDRIAL FUNCTION OF ADULT ZEBRAFISH

Once hypoxia increased animal's susceptibility to PTZ-induced seizures, we tested the hypothesis that hypoxia caused long-term mitochondrial dysfunction, which would be related to the increased predisposition to seizures. We did not observe any influence of hypoxia on mitochondrial O_2 consumption regardless of whether the animals were exposed to PTZ (Supp. Fig. 2) or not (Fig. 5 a-g). However, we observed a decrease on extra mitochondrial respiration (ROX) ($P = 0.0237$) in animals from PHE group that were not exposed to PTZ (Fig. 5h). ROX alteration was not observed between groups in animals that were exposed to PTZ (Supp. Fig. 2).

4. DISCUSSION

We investigated the on social interaction, seizures susceptibility and function of the main components of electron transporter system (ETS) in adulthood of zebrafish subjected hypoxia in larval stage. Our main findings are that hypoxia increased the mortality rate, lead to social deficits and increased the susceptibility to seizure like-behavior in adulthood. Interestingly, these results are similar to those observed in PHE models [47,48], indicating that zebrafish subjected to hypoxia could be a useful model to support a better understanding of the effects caused by PHE.

In our study, we showed that animals subjected to hypoxia had increased mortality rate (approximately 20%) in the first month of development. Interestingly, human neonates affected bysevere perinatal hypoxic-ischemic encephalopathy had 40-81.5% of mortality during the first weeks of life [49], reducing significantly the number of deaths after the sixth month of life [49]. In the present study, we observed that the highest mortality occurred in the first two weeks of life and after the first month, no deaths were detected. Thus, these data indicated that the mortality on our study using zebrafish is similar to other clinical and classical PHE models.

Early brain damage caused by PHE can significantly impair the cognition and social behavior in adulthood [50]. Based on social test, our results showed lower permanency of PHE animals in the conspecific zone, which is interpreted as less preference to interact with conspecific animal. We cannot rule out the possible visual impairment of the animals, since hypoxia can be associated with blindness and retinopathy [51]. However, we believe that this factor did not take part on social interaction in the PHE animals, once the frequency in conspecific zone was approximately 70%. Hyperactivity and impulsive behavior have been found in studies with PHE models [10,15,52]. These findings suggest that PHE-affected individuals could present a higher propensity to behavioral impairments, such as elevated rates of hyperactivity, attention deficit hyperactivity disorder (ADHD) and autism spectrum disorder

(ASD) [18–21]. In fact, previous study had demonstrated that 4.2% from PHE-affected children were diagnosed with ASD and about 6-fold increase in ASD risk when compared to children who did not have encephalopathy [53]. There is also a strong correlation between schizophrenia and hypoxia at birth [54–57]. Similar to what has being reported to PHE, our study using zebrafish showed the ability of hypoxia in larval stage to cause long-term social abnormalities.

Seizures are common neurological manifestation of PHE [58], mainly in childhood, and they can occur in adulthood as well [59]. In humans, encephalopathy can be associated with status epileptics and chronic recurrent seizures can be predictors of neurological disability and epilepsy [60–62]. Our study provided an evaluation on PTZ- induced seizures in adult zebrafish that suffered hypoxia at early life. That allowed us to observe that PHE animals presented increased duration and frequency of seizures, indicating a low threshold to seizure like-behavior. Recently, continuous video-EEG monitoring shown that animal models (rodents) exhibited higher frequency to spontaneous recurrent seizures at 6 months after perinatal hypoxia [23,25,63], indicating that PHE can play a role on development of epilepsy later in life. Moreover, a recent study reported that survival individuals from PHE had a five-fold increased risk to develop epilepsy when compared with those healthy individual [64]. Although the need for further studies, these data could be helpful to reinforce the idea that adult individuals with history of PHE should be carefully evaluate regarding seizure precipitating factors or the use of drugs class, individual medications associated with seizures [65].

To date, there is no published information regarding brain mitochondrial function in adulthood of individuals affected by PHE. Here, we hypothesized that our results of low seizure threshold could be related to the altered brain mitochondrial function in adulthood caused by perinatal hypoxia. As mitochondrial injury is a short-term effect of PHE [66–69], our data showed no changes on O_2 consumption of electron transport system components of brain mitochondria of adult animals that had hypoxia.

Therefore, the adult zebrafish behavior alterations describe here cannot be related to modifications of mitochondrial respiration at basal state. It has been shown that genetic factors are able to modulate mitochondrial function (e.g., heat shock factors) and they play an important role in stress situations, such as seizures [70,71]. Thus, it could possible that PHE animals could sustain a lower seizure threshold due to the increased inability to maintain the mitochondrial respiration during stress. To test this hypothesis, we exposed animals from both groups, Normoxia and PHE, to PTZ prior to mitochondrial O_2 consumption evaluation and the results remained unaltered. This finding suggested that there is no relation between the hypoxia-induced reduction in seizure threshold and mitochondrial O_2 consumption in neural cells.

In additional analysis, we observed a decrease in the Residual Oxygen Consumption (ROX) in PHE animals. Although mitochondria are responsible for the vast majority of cellular O_2 consumption, a number of biological processes (e.g., protein folding, lipid and collagen synthesis, and DNA and histone demethylation) involve reactions that utilize oxygen directly [72]. In contrast, studies report that ROX can represents oxidase activities in the cells, indicating the possibility for Reactive Oxygen Species (ROS) formation [72,73]. Thus, there is no plausible explanation for a concrete interpretation for this finding.

5. CONCLUSIONS

Taken altogether, our data suggested that hypoxia at early developmental stages in zebrafish induced similar effects to those observed in humans with PHE such as problem in the social interaction, high mortality and propensity to seizures. Moreover, these effects were

not related to dysfunction on mitochondrial O_2 consumption. Therefore, future investigations will be required to fully understand the cellular and neurochemical mechanisms involved on susceptibility to seizures induced by PHE. Nevertheless, it is remarkable that our data support the potential use of this new model in studies related to PHE.

6. ABBREVIATIONS

ASD; Autism Spectrum Disorder; ADHD; attention deficit hyperactivity disorder; dpf, days post fertilization; PHE, Perinatal Hypoxia Encephalopathy; PTZ, pentylenetetrazol; hpf, hours post fertilization; phd; post hypoxia day.

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8. DISCLOSURES

The authors have no conflict of interest.

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10. FIGURE AND LEGENDS

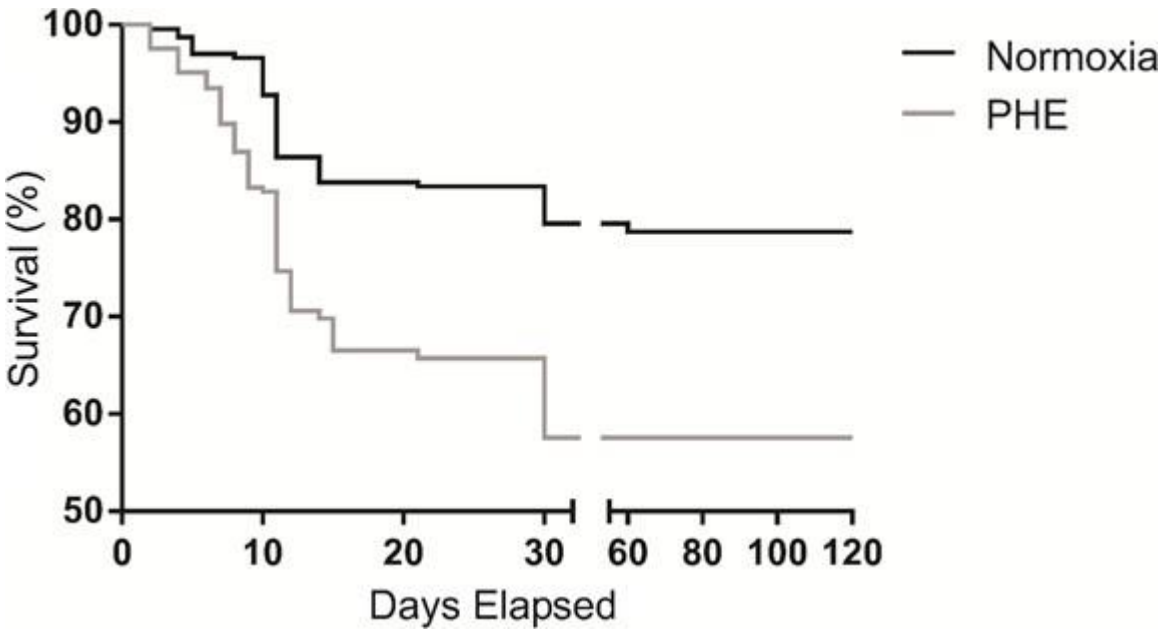


Figure 1. Survival Rate. Survival rate until PHD 120 (~4 months). Animals EHP showed a higher mortality in the PHD30 when compared to control. The black represents the control group and the grey the PHE group. (n = 100/group), Chi square= 16.54; P < 0.0001, Mantel-Cox).

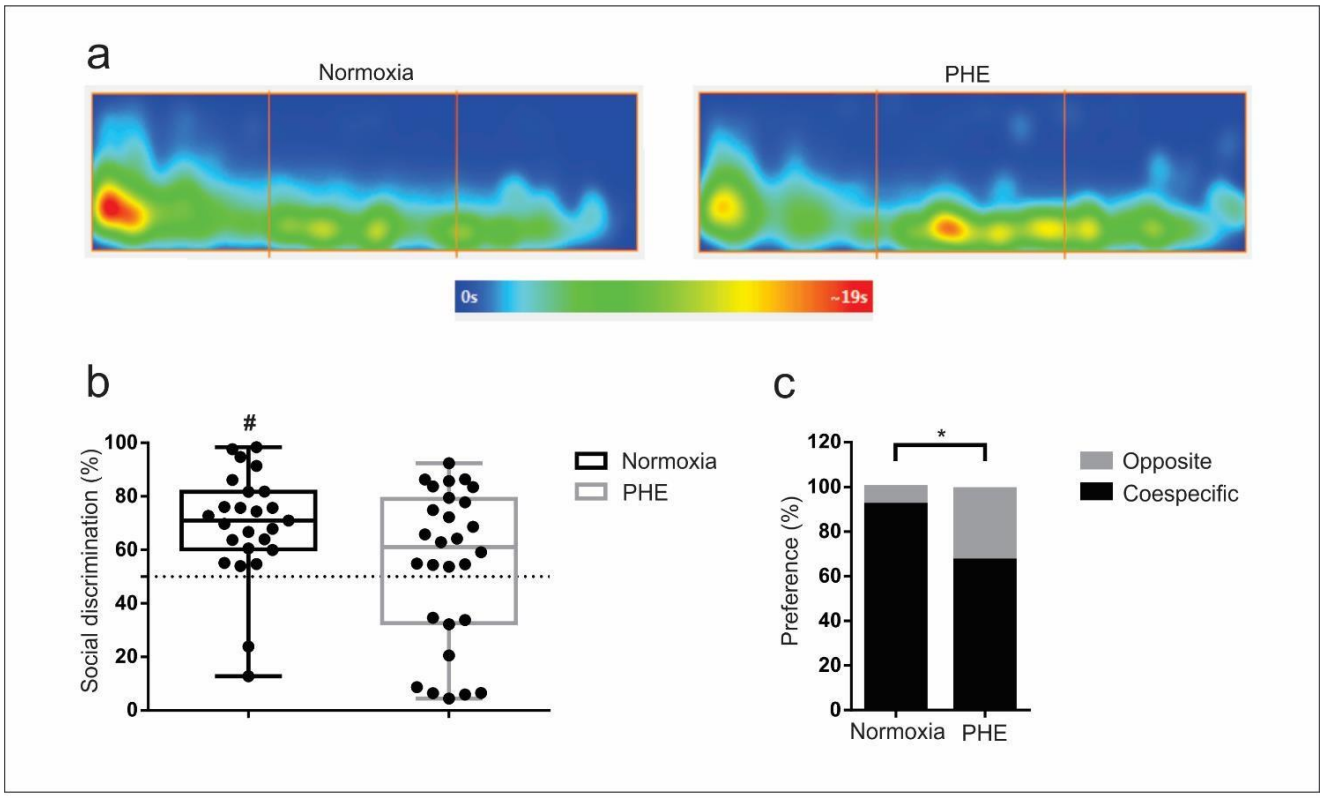


Figure 2. Social Preference Test. A. Track Plot showing the heat map mean of the group´s. Reddish tones represent a highest time in the zone and blueish tones represent the opposite. B. Social discrimination index. The PHE animals show lower preference by spent less time in the conspecific zone; however, its do not characterizes an aversion by conspecifics. C. Conspecific preference. Percentage time in opposite and conspecific side in the apparatus is represented by gray and black, respectively. ($n = 30$/group), ($p < 0.05$).

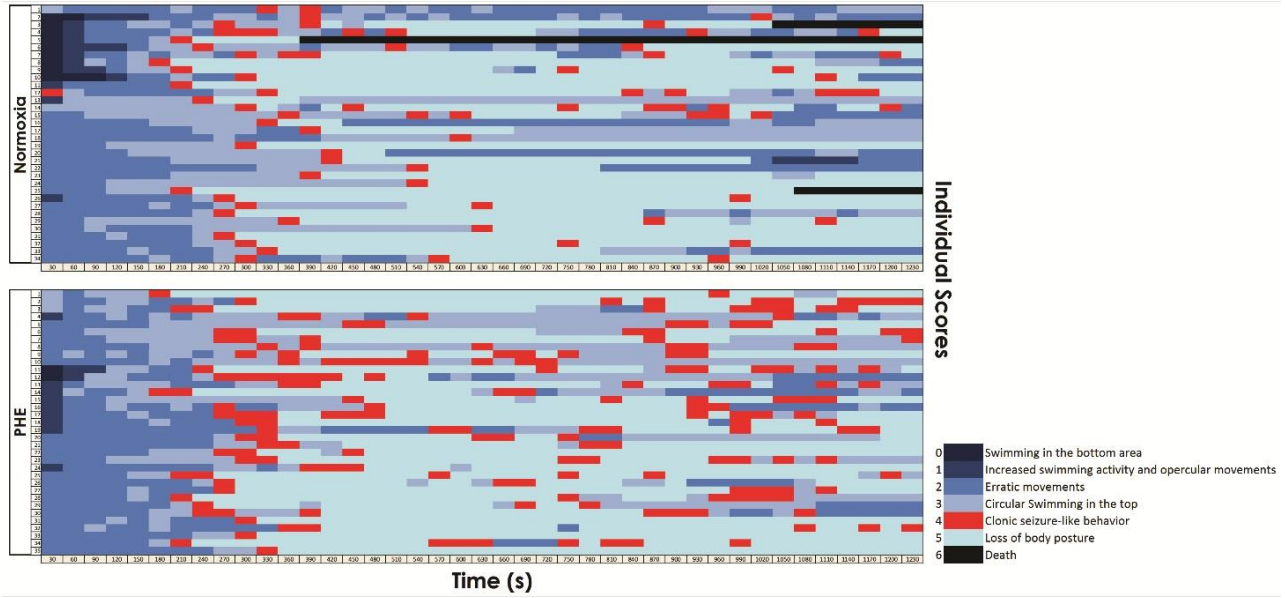


Figure 3. Ethogram of the main behavior scores. Individual scores are represented every 30 sec across 20 min exposure to PTZ. The red color represent the score 4 (seizure).

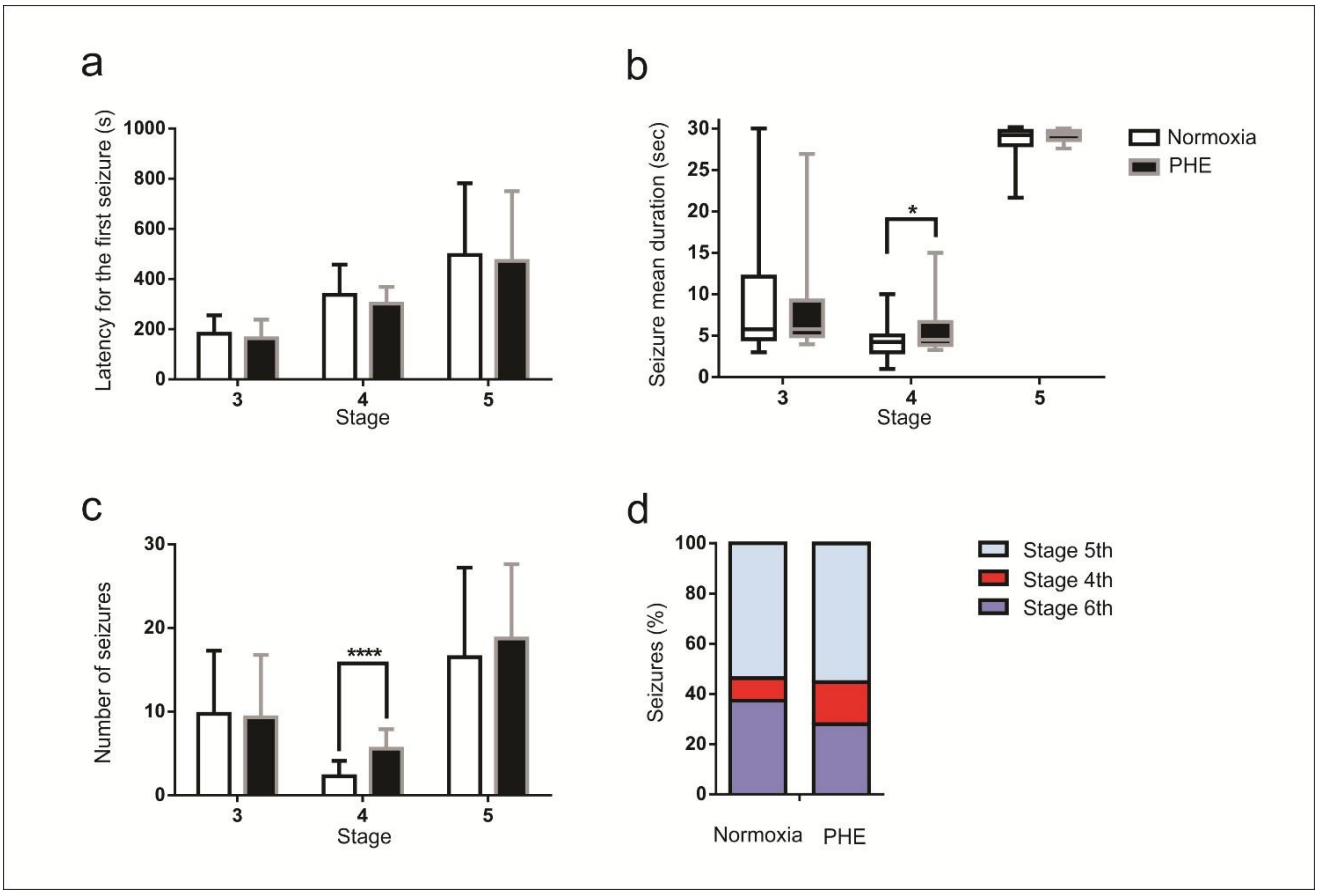


Figure 4. Analysis of Seizure. A. Latency for Seizure. Represent the time spent to manifest the first seizure. B. Seizure duration. C. Number of the seizures. Represent the number of seizures in 20 min test. D. Total Percent of seizure. (n = 36/group), (P <0.0001).

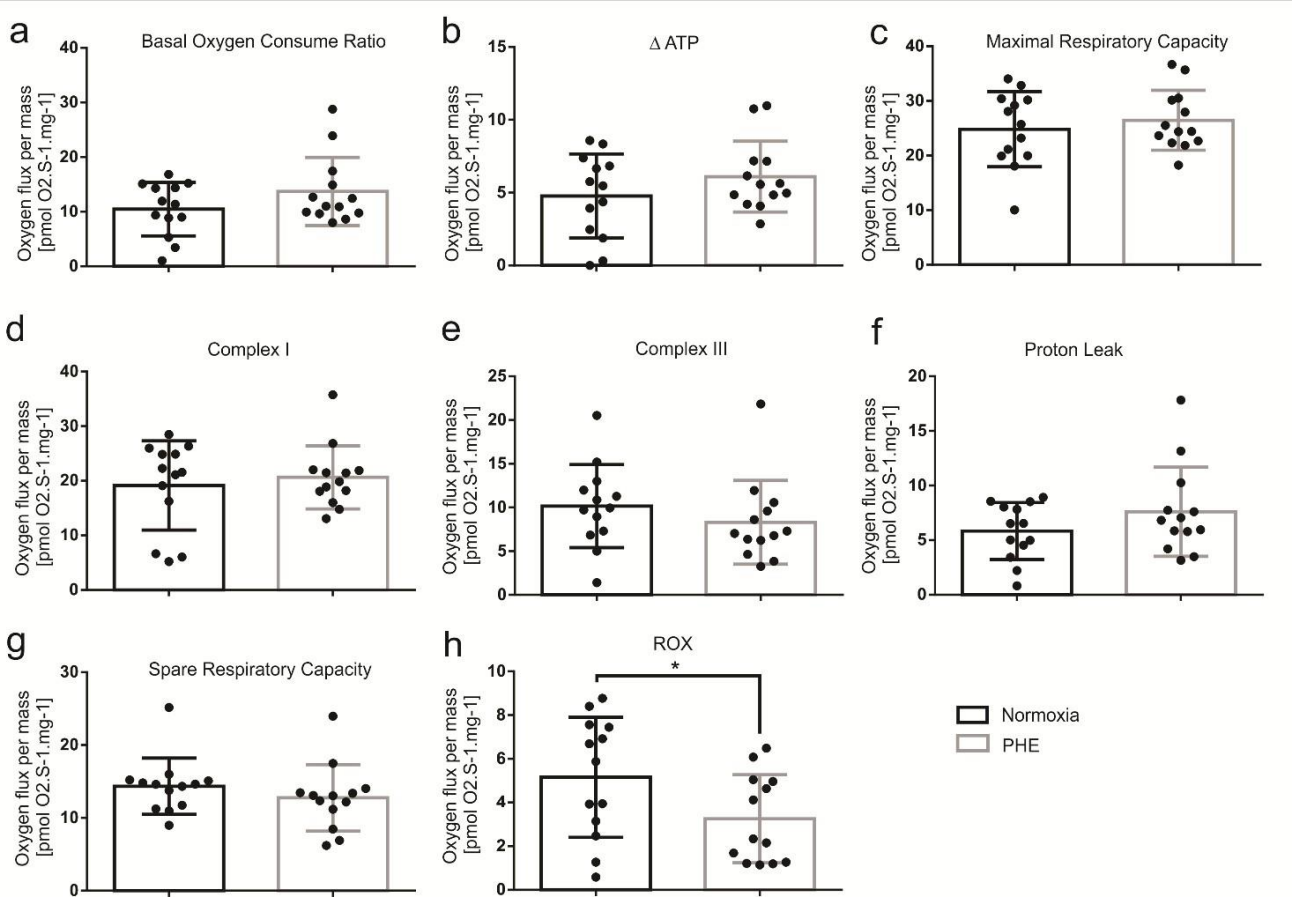
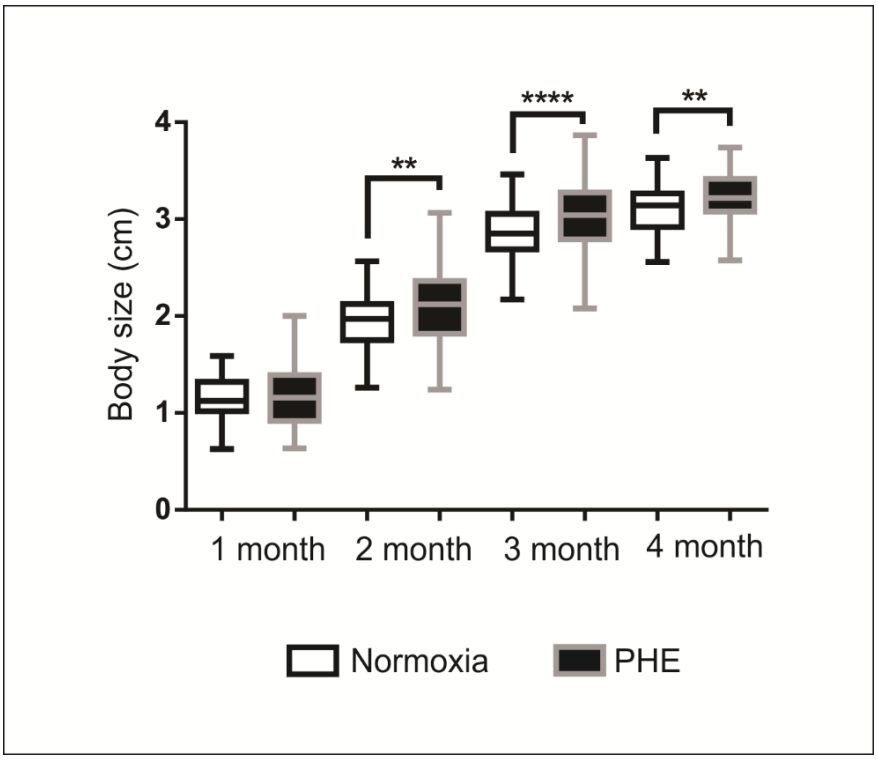
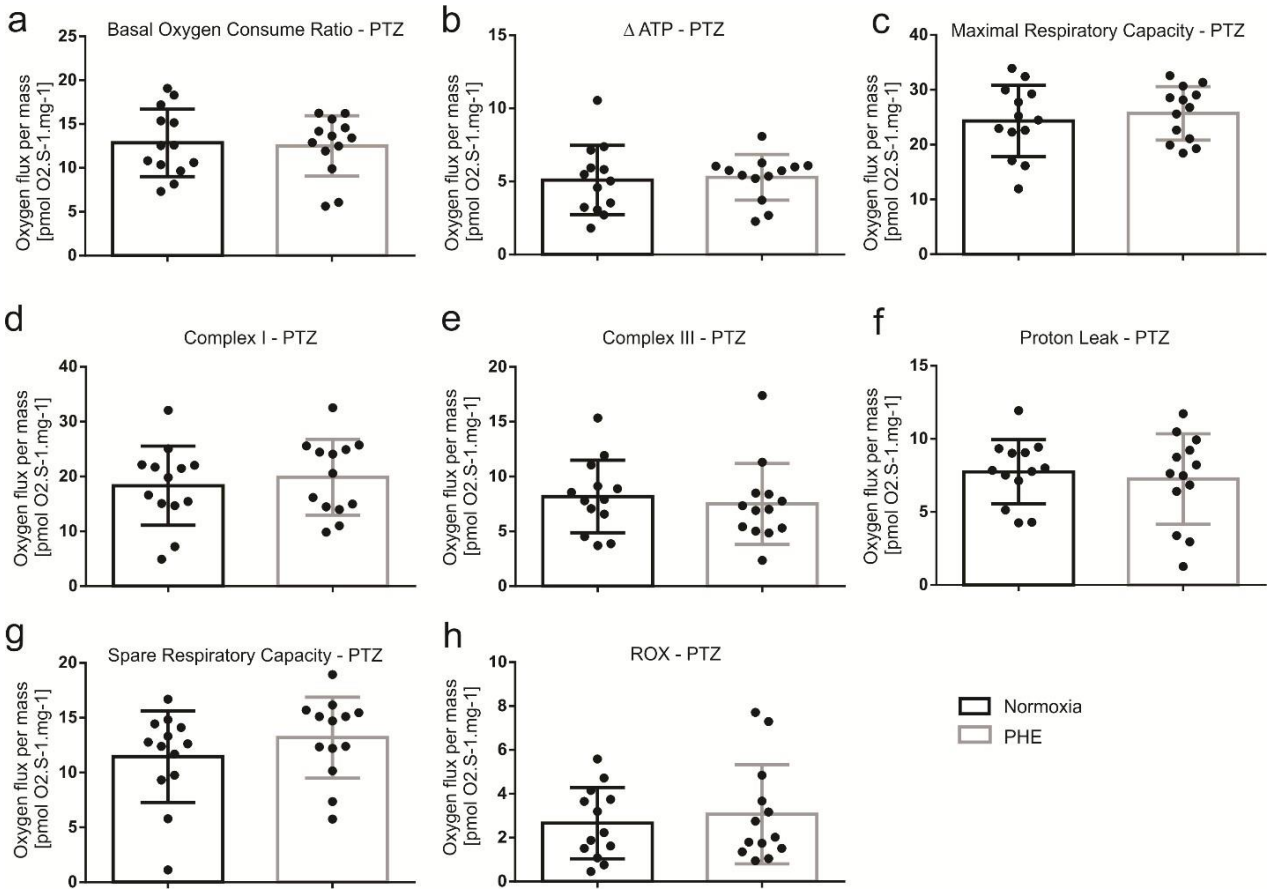


Figure 5. Mitochondrial Respiratory analysis. The analysis consisted by an evaluation of the oxygen level consumed in different components of the electron transport system. A. Basal Oxygen Consumption Ratio. B. Δ to ATP production. C. Maximal Respiratory Capacity. D. Complex I Activity. E. Complex III Activity. F. Proton Leak. G. Spare Respiratory Capacity. H. Residual Oxygen Consumption (ROX). (n = 36/group), ($p < 0.05$).



Supplementary Figure 1. Body size growth. Demonstrate the increase of the body size during four months after hypoxia. ($p < 0.005$).



Supplementary Figure 2. Mitochondrial Respiratory analysis. The analysis consisted by an evaluation of the oxygen level consumed in different components of the electron transport system. A. Basal Oxygen Consume Ratio. B. Δ to ATP production. C. Maximal Respiratory Capacity. D. Complex I Activity. E. Complex III Activity. F. Proton Leak. G. Spare Respiratory Capacity. H. Residual Oxygen Consumption (ROX). These animals were exposed to PTZ (7.5mM) in prior to mitochondrial function analysis. (n = 36/group), ($p < 0.05$).

PARTE III

DISCUSSÃO

Crescentes evidências em estudos clínicos e pré-clínicos elucidam que o estresse intrauterino pode ter um impacto leve ou severo na estrutura e função cerebral durante o desenvolvimento fetal, levando a uma maior vulnerabilidade a disfunções tardias. Por conta disso, foram desenvolvidas intervenções terapêuticas para minimizar a lesão cerebral em neonatos que sofrem de encefalopatia hipóxico-isquêmica. Contudo, resultados benéficos são observados somente em aproximadamente 30% dos pacientes, os demais 70% podem desenvolver sequelas neurológicas leves, moderadas ou graves na vida adulta (ALLEN; BRANDON, 2011). Devido a esta questão, vários modelos animais têm sido utilizados para estudar lesões cerebrais perinatais, incluindo primatas não humanos, ovelhas, coelhos e roedores, e todos eles contribuem para elucidar mecanismos e novos tratamentos da EHP (JACOBSON MISBE et al., 2011; KOEHLER et al., 2018; ROOHEY; RAJU; MOUSTOGIANNIS, 1997). Aqui, devido a facilidade de manipulação embrionária e indução de estresse hipóxico, nós selecionamos o peixe-zebra para estudar o efeito da hipóxia em estágio larval sobre o comportamento social, a suscetibilidade a crises epiléticas e a relação destes efeitos com respiração mitocondrial cerebral em indivíduos adultos. Embora nós não observamos nenhuma alteração sobre a respiração mitocondrial cerebral, a hipóxia aumentou a taxa de mortalidade, causou déficits sociais e aumentou a suscetibilidade a crises epiléticas na idade adulta.

Em nosso estudo, mostramos que os animais submetidos a hipóxia apresentaram um aumento na taxa de mortalidade (aproximadamente 20%) no primeiro mês de desenvolvimento. Sendo a mortalidade mais acentuada nos primeiros 15 dias. Curiosamente, neonatos humanos afetados por encefalopatia hipóxico-isquêmica perinatal grave tiveram 40-81,5% de mortalidade durante as primeiras semanas de vida (TAGIN et al., 2012), reduzindo significativamente o número de mortes após o sexto mês

de vida. No presente estudo, observamos que a maior mortalidade ocorreu nas duas primeiras semanas de vida e após o primeiro mês, não foram detectadas mortes. Assim, estes dados indicam que a mortalidade no nosso estudo utilizando peixe-zebra é semelhante a outros estudos clínicos e a outros modelos animais de EHP.

No presente trabalho nós observamos que houve uma mortalidade significativa também no grupo controle das quais foram resultantes de outras causas que não a hipóxia. Durante o desenvolvimento fetal humano várias condições podem modificar o ambiente intrauterino, deixando vulnerável o cérebro em desenvolvimento (FAGIOLINI; JENSEN; CHAMPAGNE, 2009). No caso do desenvolvimento de larvas de peixes geralmente podem ocorrer fortes mudanças em seu hábitat como hipóxia e mudanças de temperatura, que podem exercer influência significativa em todos os processos biológicos desses animais (DIMITRIADI et al., 2018; JIN et al., 2017; KOPP et al., 2014; LAHIRI et al., 2005; LONG et al., 2015; MARQUES et al., 2008; SCOTT; JOHNSTON, 2012; TON; STAMATIOU; LIEW, 2003). Uma vez que a mortalidade não só do grupo controle como do grupo HIPÓXIA foi maior nos períodos de mudanças da água do aquário-moradia (dias 5, 10, 15, 20 e 30) é plausível concluir que, no presente trabalho, mudanças similares no ambiente dos peixes (mudanças de temperatura e parâmetros osmolares) vieram a somar nos efeitos da hipóxia sobre a mortalidade dos animais.

Lesões cerebrais causadas por EHP podem afetar o desenvolvimento individual, refletindo no comprometimento das habilidades cognitivas e do comportamento social em idades mais avançadas (DUBOVICKÝ, 2010). Neste estudo, avaliamos a habilidade social do peixe-zebra adulto submetido ao modelo de hipóxia em 72 hpf, quando se dá início ao estágio de desenvolvimento do seu SNC (KIMMEL et al., 1995). Nossos resultados mostraram uma menor preferência dos animais HIPÓXIA em interagir com os seus co-específico, pelo fato de permanecer menos tempo na zona co-específica. (DRISCOLL et al., 2016), usando o teste de interação social, demonstraram que os ratos

afetados por EHP aumentam o tempo gasto correndo sozinho e diminuíram o tempo gasto correndo em pares, refletindo uma redução no comportamento de interação. Assim, nossos dados vão de encontro com dados pré-clínicos (MARQUES et al., 2008; MOZAFFARIAN et al., 2015) e clínicos mostrando que anormalidades sociais tem reflexos há longo prazo e não são incomuns após a EHP.

Em humanos, a EHP pode estar associada ao status epilépticos e crises recorrentes crônicas podem ser predecessoras de incapacidade neurológica e epilepsia (CLANCY; LEGIDO, [s.d.]; NELSON; BROMAN, 1977; SHINNAR et al., 1985). Aqui, tentamos fornecer uma compreensão maior das crises epiléticas induzidas por Pentilenotetrazol em peixe-zebra adultos submetidos à hipóxia. Isso nos permitiu observar que animais com hipóxia tiveram um aumento da duração e frequência das crises, indicando um menor limiar para crises epiléticas. Recentemente, a monitorização contínua por vídeo-EEG mostrou que os modelos animais (roedores) exibiram maior frequência de crises recorrentes espontâneas aos 6 meses após a hipóxia perinatal (KANG; KADAM, 2015; LIU; YU; LÜ, 2016; WANG et al., 2015), indicando que a EHP pode desempenhar um papel no desenvolvimento da epilepsia em um período mais tarde da vida. Além disso, um estudo recente relatou que os indivíduos sobreviventes de EHP tinham um risco cinco vezes maior de desenvolver epilepsia quando comparados àqueles saudáveis (DARIPA et al., 2013; SCHENDEL; NELSON; BLAIR, 2012b). Apesar da necessidade de mais estudos, esses dados podem ser úteis para reforçar a ideia de que indivíduos adultos com histórico de EHP devem ser cuidadosamente avaliados em relação a fatores desencadeantes de crises epiléticas ou ao uso de medicamentos individuais associados a crises epiléticas (DE RIU et al., 1995).

Até o momento, não há informações publicadas sobre a função mitocondrial cerebral na idade adulta de indivíduos afetados por EHP. Nossa hipótese foi que o baixo limiar para crises epiléticas poderia estaria relacionado à alteração da função mitocondrial cerebral. Embora o dano mitocondrial seja um efeito de curto prazo do EHP (FINESCHI et al., 2017; HUANG; CASTILLO, 2008; NUÑEZ et al., 2018), nossos dados não

mostraram mudanças no consumo de O_2 dos componentes do sistema transportador de elétrons de mitocôndrias cerebrais de animais adultos que sofreram hipóxia na fase larval. Portanto, as alterações descritas previamente para os animais que sofreram hipóxia não podem estar relacionadas à respiração mitocondrial observada em seu estado anterior às crises epiléticas. É de conhecimento na literatura que fatores genéticos são capazes de modular a função mitocondrial (por exemplo, fatores de choque térmico) (DAS; ENGELMAN; KIMURA, 1993; KOIKE; HATANO; USHIMARU, 2018) e desempenhar um papel importante em situações de estresse, como crises epiléticas (DAS; ENGELMAN; KIMURA, 1993; HASHIMOTO-TORII et al., 2014; KOIKE; HATANO; USHIMARU, 2018; MAGALHÃES et al., 2005). Assim, seria plausível hipotetizar que os animais do grupo HIPÓXIA poderiam manter um menor limiar para crises epiléticas devido à maior incapacidade de manter a respiração mitocondrial durante as crises. Para testar esta hipótese, expusemos animais de ambos os grupos, Normoxia e PHE, a PTZ antes da avaliação do consumo de O_2 mitocondrial e os resultados permaneceram inalterados. Este achado sugere que não há relação entre a redução induzida no limiar para as crises epiléticas por EHP e o consumo de O_2 mitocondrial em células neurais.

Em análise adicional, observamos uma diminuição no consumo de oxigênio residual (ROX) em animais submetidos à hipóxia. Embora as mitocôndrias sejam responsáveis pela grande maioria do consumo de O_2 celular, vários processos biológicos (por exemplo, dobramento de proteínas, síntese de lipídios e colágeno e desmetilação de DNA e histona) envolvem reações que utilizam oxigênio diretamente (BANH et al., 2016; PERRONE et al., 2016). Em contraste, estudos relatam que a ROX pode representar atividades de oxidase nas células, indicando a possibilidade de formação de Espécies Reativas de Oxigênio (BANH et al., 2016; PERRONE et al., 2016). Assim, não há explicação plausível para uma interpretação concreta para esse resultado.

PARTE IV

CONCLUSÃO

Em conjunto, nossos dados sugerem que a hipóxia nos primeiros estágios de desenvolvimento do peixe-zebra induziu efeitos semelhantes aos observados em humanos que tiveram EHP. Além disso, esses efeitos não foram relacionados ao consumo de O_2 mitocondrial dos principais componentes do STE. Além disso, nossos dados demonstram que o modelo de hipóxia empregado em peixe-zebra pode ser útil no estudo de efeitos a longo prazo na EHP. Portanto, futuras investigações serão necessárias para entender completamente os mecanismos celulares e neuroquímicos envolvidos na suscetibilidade a crises epiléticas induzidas por EHP. Contudo, é notável que nossos dados suportam o potencial uso deste novo modelo em estudos relacionados a EHP.

PARTE V

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PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 33284
Título: AVALIAÇÃO DE PARÂMETROS COMPORTAMENTAIS E NEUROQUÍMICOS EM PEIXE-ZEBRA ADULTO SUBMETIDO AO MODELO DE ENCEFALOPATIA HIPÓXICA PERINATAL

Vigência: 01/10/2017 à 30/09/2019

Pesquisadores:

Equipe UFRGS:

MARIA ELISA CALCAGNOTTO - coordenador desde 01/10/2017
FABIO KLAMT - pesquisador desde 01/10/2017
DIOGO LOSCH DE OLIVEIRA - pesquisador desde 01/10/2017
SUELEN BAGGIO - Aluno de Mestrado desde 01/10/2017
EMERSON SANTOS DA SILVA - Aluno de Mestrado desde 01/10/2017

Equipe Externa:

Marcos Martins Braga - pesquisador desde 01/10/2017

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 10/07/2017 - SALA 331 DO ANEXO I DO PRÉDIO DA REITORIA - CAMPUS CENTRO , em seus aspectos éticos e metodológicos, para a utilização de 328 embriões de peixes-zebra (Danio rerio) wild-type, a partir da sexta geração, machos e fêmeas, provenientes do Biotério do Departamento de Bioquímica/UFRGS; de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Sexta-Feira, 28 de Julho de 2017

MARCELO MELLER ALIEVI
Coordenador da comissão de ética

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