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Potencial efeito neuroprotetor do pré-condicionamento em peixe-zebra (*Danio rerio*) adulto submetido ao modelo de hipóxia

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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Bacharel(a) em Biomedicina.

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SUMÁRIO

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RESUMO

Um dos fenômenos mais comuns em patologias cerebrais é a falta de oxigenação encefálica. Essa situação é uma das principais desencadeadoras de danos mitocondriais que pode ter como resultado a morte neuronal. Dentre os danos encefálicos conhecidos, o acidente vascular encefálico (AVE) é a principal causa de morte. Apesar da relevância deste tipo de doença para à saúde pública, existe uma necessidade crítica para o desenvolvimento de terapias seguras e eficazes. No presente momento, o agente trombolítico Ativador do Plasminogênio tissular (t-PA), constitui a única terapia aprovada pela FDA (Food and Drug Administration) para AVE isquêmico agudo. No entanto, esse fármaco possui vários inconvenientes, incluindo um potencial risco hemorrágico. Dessa forma, destaca-se a necessidade de novas abordagens terapêuticas. Uma estratégia pode ser o pré-condicionamento (PC) que se refere a sobreviver a estímulos nocivos criando um estado mais preparado contra um seguinte estímulo letal. Muitos ambientes de água doce são caracterizados por flutuações espaciais, diárias ou sazonais de disponibilidade de oxigênio. Por isso, várias espécies de peixes, como o peixe-zebra têm evoluído fisiologicamente e anatomicamente para lidar com longos períodos de hipóxia. Portanto, as estratégias adaptativas desses animais poderiam ser estudadas para o desenvolvimento de terapias neuroprotetoras mais efetivas e estudos de mecanismos de tolerância na hipóxia-isquemia. Portanto, neste trabalho propomos a investigação do PC hipóxico como uma estratégia terapêutica que poderia levar o encéfalo de peixes-zebra a tolerar um quadro de hipóxia severa letal, devido a ação sobre a respiração mitocondrial cerebral. Para indução do PC, peixes zebra adultos foram colocados em um recipiente de vidro com níveis reduzidos de O_2 pela adição de gás nitrogênio. Baixo nível de O_2 foi mantido no grupo PC severo (1,8 mgO_2/L) por 10 minutos e 2h no grupo com PC moderado (3,5-4,0 mgO_2/L). Posteriormente os animais foram separados em diferentes grupos (6, 12, 24, 48, e 72h) para recuperação em normóxia (~8,0 mg/L). Após estes períodos os animais foram anestesiados e eutanasiados para remoção dos encéfalos e processamento das amostras. Observamos que animais expostos ao PC severo com 24h de recuperação apresentaram aumento da resistência à hipóxia, diminuição no consumo de O_2, aumento na atividade do complexo I da cadeira respiratória e mantiveram a produção normal de ATP. Nossos resultados demonstraram que o peixe-zebra pré-condicionado melhora sua eficiência mitocondrial, pois consume menos oxigênio e produz a mesma quantidade de ATP, sugerindo um efeito benéfico dessa estratégia a nível mitocondrial. Assim, essa investigação demonstra o efeito benéfico do pré-condicionamento hipóxico usando o modelo de peixe-zebra.

1. INTRODUÇÂO

1.1. Modelo de hipóxia

Hipóxia é caracterizada pela redução ou bloqueio da oferta de oxigênio a um determinado tecido ou órgão (do grego *hipo* = falta, escassez e *oxis* = oxigênio). Esse fenômeno pode desencadear infarto tecidual, principalmente no encéfalo devido a seu alto consumo de oxigênio (O_2) (Magistretti et al., 1999). A hipóxia encefálica é uma das principais desencadeadoras de acidentes vasculares encefálicos (AVE). Devido à alta exigência metabólica, as células neurais são extremamente sensíveis à privação de oxigênio, podendo começar a morrer dentro de poucos minutos nessa condição. Infelizmente, a intervenção direta é limitada e, até o momento, os suportes terapêuticos apresentam resultados insatisfatórios na hipóxia (Marler, 2007; Cheng et al., 2004). A persistência da hipóxia por um período longo de tempo gera um prognóstico ruim para o paciente, que vê sua qualidade de vida ser obliterada. A intervenção terapêutica, por mais limitada que seja, previne algumas complicações como coma, convulsões, e até mesmo a morte cerebral (Lu-Emerson e Khot, 2010).

Já é bem conhecido que em episódios de privação de oxigênio, como aqueles que ocorrem na isquemia cerebral, as células neurais submetidas aos baixos níveis de O_2 e de energia exibem uma falha no metabolismo que acarreta em um desequilíbrio energético (Obrenovitch et al., 1995). Isto conduz à excitotoxicidade via liberação excessiva de glutamato neuronal e ativação anormal de receptores excitatórios, o que resulta no influxo exacerbado de cálcio intracelular (Ca^{2+}) (Choi et al., 1990) e, por consequência, em apoptose e necrose celular (Besancon et al., 2008). Esse conjunto de eventos geram regiões cerebrais infartadas (Murphy et al., 2008), seguido pela perda de funções motoras e sensoriais (Hossmann et al., 2006). Atualmente, os métodos terapêuticos não trazem informações claras sobre o possível aumento ou diminuição da excitabilidade devida ao glutamato. O dano encefálico causado pela hipóxia-isquemia pode ser devastador, mas, ainda assim, muitos indivíduos sobrevivem a um evento inicial, quando alguns mecanismos celulares são acionados pela subsequente reoxigenação/reperfusão da região lesada.

Dentre os danos encefálicos conhecidos, o AVE é a principal causa de morte e incapacidade intelectual dos indivíduos (Saver et al., 2009). De acordo com dados do Ministério da Saúde de 2011, apenas no Sistema Único de Saúde (SUS), o percentual de mortes por infarto oscila entre 10% e 15%, e o AVE atinge 16 milhões de pessoas a cada ano no mundo, sendo que grande parte dos sobreviventes permanecem com algum tipo de sequela. Apesar da relevância deste tipo de doença para a saúde pública, existe uma necessidade crítica para o desenvolvimento de terapias seguras e eficazes, bem como para a investigação de novas moléculas neuroprotetoras a fim de melhorar o prognóstico clínico de pacientes com AVE (Marler, 2007; Sarver et al., 2009). Estudos epidemiológicos têm revelado que pelo menos 44% das ocorrências de injúria encefálica traumática (IET) resultam em hipóxia, a qual tem sido associada com consequências neurológicas adversas (Jeremitsky et al., 2003; McHugh et al., 2007). Segundo a Organização Mundial da Saúde (OMS), o AVE foi a segunda maior causa de morte no mundo em 2011 (WHO, 2011). Entretanto, além do número expressivo de mortes, milhões de pessoas, após ser acometida pela hipóxia-isquemia encefálica, apresentam incapacidade crônica, por debilitar determinadas condições motoras e sensoriais uma vez que determinados neurônios submetidos à privação de oxigênio apresentam danos estruturais (Obrenovitch et al., 1995; Frederickson, et al., 2004). A isquemia cerebral é acompanhada por um aumento rápido de mediadores inflamatórios que causam danos irreversíveis aos neurônios no núcleo isquêmico. Clinicamente, a reperfusão pode ocorrer espontaneamente ou através da utilização de agentes farmacológicos. No presente momento, o agente trombolítico Ativador do Plasminogênio tissular (Altaplase), constitui a única terapia aprovada pela FDA (Food and Drug Administration) para AVE isquêmico agudo. No entanto, esse fármaco possui vários inconvenientes, incluindo um potencial risco hemorrágico, janela terapêutica e eficácia limitadas. Dados obtidos do “Get With The Guidelines-Stroke database” mostra que 4-7% de todos os pacientes com AVE isquêmico agudo são tratados com tPA (Bulkley et al., 2000), uma observação que destaca a necessidade de novas abordagens terapêuticas com maior eficácia.

1.2. Modelo de Pré – Condicionamento (PC).

Neuroproteção *in vivo* pode ser adquirida por ação de fármacos (compostos neuroprotetores desenvolvidos para minimizar os danos devastadores causados pelo acidente vascular cerebral), porém, essas substâncias têm falhado em ensaios clínicos. A este respeito, torna-se importante o estudo de tolerância contra o AVE, onde se destaca o pré-condicionamento.

O pré-condicionamento (PC) refere-se a sobreviver a estímulos subletais que criam um estado mais preparado para suportar possíveis insultos letais. O termo pré-condicionamento isquêmico foi introduzido na literatura por Murry et al., (1986) e tem como significado a indução de um período de hipóxia, seguido por um período de reperfusão antes de um período mais longo de hipóxia. Eles descreveram um efeito benéfico nos episódios de oclusão coronária, seguidos de períodos de reperfusão, obtendo diminuição no tamanho da área de infarto causado pela isquemia. O papel do pré-condicionamento no aumento da tolerância à hipóxia tem sido descrito em vários órgãos como coração, cérebro, medula espinhal, músculo esquelético, retina, rins, intestino e fígado. Entretanto, estudos sobre o mecanismo protetor do pré-condicionamento não está ainda claramente estabelecido (Bulkley et al., 200). Mesmo assim, a capacidade de resistir, responder e lidar com estresse contínuo é uma propriedade fundamental de todos os seres vivos (Kitagawa, et al., 1990).

A primeira evidência de PC cerebral foi fornecida em 1960 (Dahl et al., 1964; Wells et al., 1963). Os modelos de PC são caracterizados por métodos de reduzir o tamanho do infarto e melhorar o resultado funcional em modelos de acidente vascular cerebral em animais. O primeiro estudo utilizando um modelo de hipóxia/isquemia global mostrou que a uma maior proteção dos neurônios do hipocampo de ratos submetidos a um período de 5 minutos de isquemia quando eles foram expostos a 2 min de PC (Kitagawa et al., 1990). Contudo o PC pode ser causado através de outros estímulos, como o PC caracterizado por um insulto causado por crises epilépticas de severidade moderada que protege os neurônios contra *status epilepticus* e

também contra a isquemia, um fenômeno conhecido como tolerância cruzada (Plamondon et al., 1999). Além disso, em modelos *in vitro* há o pré-emprego de OGD (oxygen-glucose deprivation) para imitar a PC isquêmico *in vivo* (Xu et al., 2002; Grabb e Choi, 1999), que é diferente do PC hipóxico. O efeito sobre o tecido encefálico após a hipóxia é determinado pelo grau e duração da hipóxia. Mesmo sem estar totalmente pré-condicionadas, as células neurais respondem naturalmente à hipóxia pela mobilização de uma série de defesas e respostas para atenuar a lesão e morte celular (Koerner et al., 2006). Em síntese, o PC estimula o sinal de "perigo", induzindo uma proteção endógena até então latente.

Há duas distintas janelas de proteção contra a isquemia proporcionada pelo PC: uma denominada proteção precoce e a outra proteção tardia. A proteção precoce é estabelecida entre 4-6 horas pós estímulo e tem sido observada em relativamente menos estudos do que aqueles expostos tardiamente no cérebro. Entretanto, foi demonstrada que na "segunda janela" ou PC tardio, os efeitos de proteção ocorrem 24 horas após o estímulo e perdurando por até 72 horas. (Yellon et al., 1995).

1.3. Modelo de peixe-zebra e avaliação mitocondrial.

Muitos ambientes de água doce são caracterizados por flutuações espaciais, diárias ou sazonais de disponibilidade de oxigênio. Por isso, várias espécies de peixes têm evoluído fisiologicamente e anatomicamente para lidar com longos períodos de hipóxia (van den Thillart G & van Waarde A (1985); Walsh et al., 2007). Essas estratégias incluem economia de oxigênio (O_2) por redução da taxa metabólica, maior absorção de O_2 por ventilação, respiração superficial aquática, expansão da superfície branquial e do aumento da afinidade da hemoglobina por O_2 (Van der Meer DL et al., 2005; Sollid J & Nilsson GE (2006)). Peixes rotineiramente experimentam hipóxia e anóxia no seu ambiente natural. Para nossa estratégia de estudo, organismos hipóxia-tolerantes, isto é, que apresentam uma resistência inata a níveis hipóxicos de O_2, permitiria a obtenção de novos conhecimentos de mecanismos de tolerância na hipóxia encefálica.

Interessantemente, o peixe-zebra é um desses casos de sucesso adaptativo a hipóxia, sendo isto promovido pela necessidade de sobrevivência às oscilações de O_2 ambientais em seu habitat natural. O peixe-zebra apresenta adaptações que o fazem manter ativo o metabolismo aeróbico mesmo estando com baixos níveis de O_2 (Barrionuevo et al., 1999). Além disso, quando submetido à hipóxia severa, o cérebro desses animais tem apresentado um rápido aumento de moléculas relacionadas à resistência a hipóxia, como a neuroglobina (Burmester et al., 2007) e o fator de choque térmico (HSF1) (Tucker et al., 2011).

O PC em si, pode atuar como um estímulo e induzir mudanças profundas na atividade cardíaca e resistência periférica, e até mesmo estimular a eritropoiese, pois se o PC induzir uma redistribuição do sangue e/ou estimular a produção de células vermelhas (eritrócitos) do sangue no peixe-zebra. Essas alterações poderão servir de base para um possível mecanismo de adaptação. (Thorsten et al., 2003).

A adoção do peixe-zebra como modelo de investigação sobre resistência à hipóxia é suportada ainda mais pelas suas vantagens intrínsecas. Isto se deve a constatação de que esses peixes compartilham muitos dos genes do genoma humano (Barbazuk et al., 2000), tais como os Fatores Indutores de Hipóxia (HIFs) (Rojas et al., 2007). Também a partir de estudos neuroquímicos tem-se observado que o peixe-zebra apresenta uma conservação de áreas cerebrais e sistemas de neurotransmissão semelhantes a mamíferos (Vargas et al., 2012; Rico et al., 2011). Fora isso, a indução de hipóxia em peixe-zebra é atingida através de uma câmara, que permite investigar, de forma simples e não invasiva, os efeitos cerebrais *in vivo* causados por níveis hipóxicos de O_2 nestes animais. Portanto, aproveitando-se destes benefícios, o nosso grupo tem iniciado o desenvolvimento de estudos acerca deste modelo. Como consequência, nós já conseguimos demonstrar que o peixe-zebra consegue, em até 48h, reverter completamente os danos comportamentais e mitocondriais gerados pela hipóxia severa (Braga et al., 2013).

Quanto ao metabolismo mitocondrial, em comparação com mamíferos, animais hipóxia-tolerantes apresentam uma rápida recuperação da respiração celular após reoxigenação

cerebral, seguida de um insulto hipóxico (Gallig et al., 2014). De fato, algumas adaptações mitocondriais, fazem diminuir o consumo de ATP durante a hipóxia severa (Faccenda et al., 2012), um fenômeno que também é observado em mamíferos intolerantes a O_2 quando pré-condicionados em hipóxia moderada (Faccenda et al., 2012). Portanto, a união de todos estes dados demonstram que as estratégias adaptativas desses animais poderiam ser estudadas para o desenvolvimento mais efetivo de terapias neuroprotetoras da hipóxia-isquemia.

1.1 JUSTIFICATIVA

Devido à falta de substâncias para o tratamento de doenças do SNC envolvendo hipóxia, propõe-se a investigação do PC hipóxico como uma estratégia que poderia levar o encéfalo de peixes-zebra a tolerar hipóxia severa letal devido a ação sobre a respiração mitocondrial cerebral.

1.2 OBJETIVOS

1.2.1 Objetivo geral

Avaliar o desenvolvimento de mecanismos mitocondriais de tolerância cerebral em peixe-zebra adulto após submissão ao PC.

1.2.2 Objetivos específicos

Investigar o efeito promovido pelo PC na atividade mitocondrial cerebral.

Identificar componentes responsáveis pela tolerância à hipóxia em encéfalo de peixe-zebra.

2 ARTIGO CIENTÍFICO

Trabalho experimental na forma de artigo científico

Este trabalho foi escrito em formato de artigo científico, seguindo as normas para submissão de artigo da revista Neuroscience, as quais estão contidas no anexo 1.

Potential neuroprotective effect of preconditioning in adult zebrafish exposed to hypoxia

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ABSTRACT

Stroke is a common brain disorder affecting approximately 800 million people worldwide. Several researchers are searching for new therapies which have satisfactory results on brain damage caused by hypoxia. The preconditioning (PC) under low O_2 levels has been studied as therapy for hypoxia treatment. However, the neurochemical effects are still unknown. The proposal of this work is to investigate the hypoxic preconditioning in zebrafish in order to evaluate the cerebral strategies of tolerance to hypoxia. To induce the PC, adult zebrafish were placed in a glass chamber to reduce the O_2 levels by addition of nitrogen gas. The O_2 was kept at a low level for 10 minutes in the severe PC group (O_2:1.8 mg/L) and for 2 h in the moderate PC group (O_2:~3.5-4.0 mg/L). After PC, the animals were separated into different groups for recovery under normoxia (~8.0 mg/L) at 6, 12, 24, 48, and 72h. Next, the animals were anesthetized and euthanized for brain dissection. We observed that the animals exposed to severe PC with 24 hours of recovery increase their resistance to severe hypoxia, decrease the oxygen consumption, increase the activity in complex I and maintain ATP production equivalent to the control. In addition, the mitochondria in the SNC of these animals have preference for malate, pyruvate and glutamate over succinate as energy substrates. Our results demonstrate that the preconditioning induces a decreased in oxygen consumption and a preference for malate, pyruvate and glutamate by the mitochondria. That seems to be important to maintain the production of ATP in physiological levels, revealing a mitochondrial efficiency. Thus, this investigation demonstrates the beneficial effect of hypoxic preconditioning by using zebrafish model.

Key words: Zebrafish, Hypoxic preconditioning, Mitochondria, Oxygen consumption.

1. Introduction.

Stroke is the main cause of disability (Mozaffarian et al., 2015) and second leading cause of death worldwide (Feigin et al., 2014). Each year ≈795,000 people will experience a new or recurrent stroke (Mozaffarian et al., 2015). Patients who survive this event often have to significant functional limitations that greatly impair their day-by-day life. Currently, clinical treatments are extremely limited. There are two approved treatments for stroke by the Food and Drug Administration (FDA). They are intravenous injection of tissue plasminogen activator (Alteplase) (Hacke, W, 2008) and mechanical thrombectomy (surgical removal of thrombus) (Jovin et al., 2015). These methods have numerous disadvantages, since they are performed 5-8 hours after the onset of symptoms and the side effects are significant (Hacke, W, 2008; Jovin et al., 2015). Besides, many patients do not fit this time window, which leads to inevitable brain lesions. Furthermore, many neuroprotective drugs in animal models have failed in clinical tests (Xu et al., 2013). Therefore, the development of new therapies on stroke is emergent in order to obtain a better survive perspective for patients with stroke.

Preconditioning is a natural adaptive process that can be induced by a variety of (stimulus) that increases the tolerance to aversive conditions, such oxygen (O_2) deprivation during stroke (Veighey et al., 2012). For example, the brain preconditioning may be induced by submitting individuals in alternating periods of hypoxia and reperfusion to release endogenous protector mechanisms (Dirnagl. et al., 2003). The resultant neuroprotection is associated with cellular recruitment of defensive molecules (Dirnagl. et al., 2003). Thus, the brain preconditioning by hypoxia could be beneficial to individuals with propensity to diseases evolving hypoxia, such as stroke.

Under aerobic conditions, the mitochondria is the primary site of ATP production. Without O_2, the final electron acceptor is not available and the electron transport chain will come to a halt (Griffiths 2012). As consequence, protons will accumulate in the mitochondrial matrix generating the fall in ATP production. Over recent years there has been a growing appreciation that this organelle is more than just an isolated energy producer. The oxygen consumption is, therefore, an indicator of cellular function and is the particularly relevant in analysis of mitochondrial dysfunction (Gnaiger et al.,2012). The mitochondrial dysfunction has also been implicate in numerous diseases states, including neurological and cardiovascular disorders ((Gnaiger et al.,2012). As result these association, the mitochondria has been targeted in various therapeutic strategies because the hypoxia per se can inhibits mitochondrial oxidative

phosphorylation in the brain (Caspersen et al., 2008). Thus, in the development of any therapy that acts on the central nervous system, the mitochondrial function should be assessed.

Aerobic aquatic organisms need an adequate concentration of the dissolved oxygen to ensure the functionality of electron transport chain in mitochondria (Caspersen et al., 2008). Fish are commonly challenged to hypoxic and anoxic conditions in their natural habitat and the zebrafish (*Danio rerio*) is among the species with ability to survive to O_2 fluctuations in aquatic environment. To maintain the function of the organism in a low-oxygen environment, zebrafish can induce adaptive changes in mitochondrial and cellular responses. Thus, zebrafish may have developed molecular strategies that can be induced by natural submission to the hypoxic preconditioning.

Since the options to treat stroke are restrict with no effective drugs available, we propose to investigate the effect of hypoxic preconditioning in adult zebrafish in order to evaluate the mitochondrial activity as a mechanism of tolerance to hypoxia in the brain.

2. Materials and Methods

2.1. Ethics statement

All procedures with animals have been approved by the Ethics Committee for Use of Animals- CEUA from Universidade Federal do Rio Grande do Sul (protocol number 29723).

2.2. Animals

Adult male and female zebrafish (6-8 months-old) of heterogeneous wild-type stock (standard short-fin phenotype) were obtained from a local commercial supplier (Delphis, RS, Brazil). Fish were housed in 40-L aquariums (70-100 fish per aquarium) for at least 2 weeks prior to the experiments in order to acclimate to the animal facility. They were maintained on a constant 14/10-h light/dark cycle (lights on at 8:00 am). Water from all tanks was aerated, maintained with mechanical and chemical filtration at 26±2°C and water pH at 7.0-8.0. Animals were fed twice a day with commercial flake fish food (alcon, BASICH, Alcon®, Brazil) and twice a day with arthemia. The animals were maintained according to the National Institute of Health Guide for Care and Use of Laboratory Animals (2011).

2.3. Hypoxia model

To induce the hypoxia model, the animals were put in a glass chamber which is isolated from the atmospheric environment to reduce levels of oxygen by the addition of nitrogen gas as described previously (Yu e Li, 2011). This chamber was divided in two equal parts with a porous membrane which allowed the passage of dissolved nitrogen to both sides. The dissolved oxygen in water was maintained at a low level (between 1.2-1.7 mg/L) in order to obtain oxygen deprivation observed in stroke.

2.4 Hypoxic Preconditoning model

The hypoxic preconditioning (PC) consisted in nitrogen gas perfusion within the hypoxic chamber in order to obtain water in moderate oxygen levels (4.0 mgO_2/L) and severe oxygen levels (1.8 mgO_2/L). Fish were placed in hypoxic chamber after the desired oxygen levels have been achieved. Based on previous standardization in our laboratory, animals were maintained for 10 minutes in severe preconditioning and hours in moderate preconditioning, which was preconditioning period tolerated by animals. After preconditioning, the animals were placed in aquarium containing water in normoxic condition. This period was considered the "recovery period", which animals were separated in 5 groups: 6, 12, 24, 48 and 72 hours after the PC. After the recovery period, some animals were used in neurochemical analyzes, while remaining animals were exposed to severe hypoxia in order to investigate the latency to reach the 3rd stage of hypoxia. This stages are: 1st stage, swimming at the top; 2nd stage, loss of posture; 3rd stage, maintenance of opercular beats with brief movements; and 4th stage, death). Finally, control groups were maintained in the same conditions in hypoxic chamber except that under normoxic conditions (standard oxygenation in water containing 8 mgO_2/L).

2.5 Resistance to hypoxia test

After exposure to preconditioning and recovery periods, some zebrafish were exposed to severe hypoxia. During hypoxia trial was evaluated the resistance to hypoxia through the latency to reach the 3rd hypoxia stage as previously reported (Braga et al., 2013) In the chamber was placed a porous membrane that divided it into two parts. On one side was put the control animals and on the other the preconditioned animals. All hypoxia stages were evaluated by one trained observer.

2.6. Measurement of mitochondrial viability in brain tissue

Brain damage by hypoxia was assessed by the method TTC (2,3,5-triphenyltetrazolium chloride) as previously described (Bederson et al., 1986). After exposure to preconditioning period, the animals were anesthetized by immersion in tricaine solution 160 µg/mL, euthanized by decapitation and the brains were removed. Then, the brain was incubated in tubes containing TTC (2%) in PBS solution for 40 minutes at 37°C and, then, fixed in 10% formalin. The tubes were covered with aluminum foil because the TTC has photosensitive properties. Throughout the duration of the incubation tubes were checked for tissue have not adhered to the wall. The brains were dried at 40°C for 2 hours and the tissues were weighed. Next, the brains were transferred to 96 well plates and incubated with 200 ul of dimethylsulfoxide (DMSO). The plate was protected from light and placed in constant stirring for 4 hours for extraction of formazan produced from TTC reaction. The absorbance of the supernatant was read at 490 nm in plate reader. The results were expressed as absorbance per brain weight (g) and were normalized as a percentage to control.

2.7. Mitochondrial function - High resolution respirometry

2.7.1 Oxygen (O_2) measurement

Respiration measurements were performed in 2 ml of 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 at pH 7.2). The O_2 consumption rate were measured polarographically using high-resolution respirometry (Oroboros Oxygraph-O2K). Total brains were incubated respiration buffer – HBSS and the oxygen consumption flow was monitored with energetics substrate to mitochondrial respiration with described by (Muller et al., 2013).

2.7.2 TCA cycle measurement

TCA cycle were performed in 2ml of the MIR05 buffer containing (0.5 mM EGTA, 3 mM $MgCl_2.6H_2O$, 20 mM Taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM Sucrose, 1g/L BSA - Essentially Fatty Acid Free). The O_2 consumption rate were measured polarographically using high-resolution respirometry (Oroboros Oxygraph-O2K). Total brains were homogenized and incubated in MIR05 buffer (free energetic substrates) and the oxygen consumption flow was

monitored with energetic substrates – malate, pyruvate, glutamate and succinate to mitochondrial respiration as described by Muller et al. (2013).

2.8. Statistics

Resistance and mitochondrial viability was expressed by mean±S.E.M and analyzed by the One-way ANOVA followed by the t-test as post hoc.

Analysis in high-resolution respirometry (Oroboros Oxygraph-O2K) data was analyzed or t-test. In all analyses, the significance level was taken as $p \leq 0.05$.

3.0. Results

3.1 Resistance to hypoxia test

In the resistance to hypoxia test, we observed that control animals without severe PC took about ~ 10 minutes to reach the 3rd stage during hypoxic condition (Fig. 1A). These behaviors have been described previously (Braga et al., 2012) and we have considered the 3rd stage, because it is the highest stage prior to death (4th stage). Thus, the increase in time to reach the stages 3rd was regarded as tolerance.

We observed that 6 hours after severe PC, the animals took about 10 minutes to reach the third stage. However, there was an increase in latency for the same stage in the 12 and 24 h groups. The 24 h group after PC had the highest latency to reach the 3rd stage (28 minutes). The latency remained increased in the 48 h group after the PC (18 minutes of hypoxia to reach the 3rd stage). The 72 h group was similar to control, suggesting higher sensibility to severe hypoxia similar to control group (Fig. 1).

When animals were exposed to severe hypoxia after treatment in moderate PC (4.0 mgO_2/L), the control group also took about 10 minutes to reach the stage 3rd (Fig. 1B). The time to reach stage 3rd increased 12, 24 and 72 hours after PC (Fig. 2).

3.2 . Measurement of mitochondrial viability in brain tissue

After recovery from preconditioning, the mitochondrial viability was assessed prior to severe hypoxia exposure to evaluate the tolerance mechanisms. All groups showed changes in mitochondrial viability. In animals exposed to severe PC (1.8 mgO_2/L), the mitochondrial viability decreased in the 6 h group, but as noted in Fig. 2A, the decrease was not significant. However, it increased 40% in 24 h group after severe PC. In the 72 h group mitochondrial viability decreased 39% (Fig. 2A).

The mitochondrial viability in animals exposed to moderate preconditioning showed different results compared to severe PC. The mitochondrial viability increased in 24 and 48 hours after preconditioning (Fig. 2B). The 24 h group presented the highest mitochondrial activity (34% higher than the control).

3.3 Mitochondrial function - High resolution respirometry.

3.3.1 Oxygen (O_2) measurement.

The tolerance mechanisms in zebrafish exposed to preconditioning was also investigated by the analysis of mitochondrial function prior to severe hypoxia exposure. As the group with 24 hours of recovery period from preconditioning stimulus was the only one with the higher resistance to severe hypoxia and higher mitochondrial activity, this group was chosen for analysis.

In the first analysis the routine respiration (the basal oxygen consumption of brain cells) was performed. Thus, the preconditioned animals consume less oxygen than the controls (Fig. 3A). There was no statistical difference in the oxygen consumption for ATP production (Δ ATP)(Fig. 3B). Although the preconditioned animals present a lower basal respiration; the capacity to produce ATP was not changed.

There was no difference in the maximum efficiency of electron transport in the transport chain (Fig. 3C), suggesting that preconditioned animals consumed less oxygen, while the maximum respiration remained unaltered. Thus, the results showed the adequate functioning of complex IV in these animals. The preconditioned animals had higher reserve capacity of O_2 when compared to controls (Fig. 3D).

There was an increase in the complex I activity (Fig. 4A), whereas no change was observed in complex III (Fig. 4B). The antagonism of the complex I and III may be associated with reactive oxygen species, since there was no mitochondrial oxygen consumption. Thus, we observed a decrease of ROS production in preconditioned animals (Fig. 4C).

3.3.2 TCA cycle measurement

To perform the analysis of the TCA cycle, we used a buffer without energetic substrates. Physiological concentrations of malate, pyruvate, glutamate and succinate were added in to check mitochondrial function.

We observed that preconditioned animals consumed more endogenous substrates that favor the production of NADH to the complex I. The substrates were malate, pyruvate and glutamate (Fig 5 A, B, C). , There was no statistical difference for succinate as energetic substrate (Fig. 5 D).

4.0 Discussion

Considering that zebrafish is able to tolerate hypoxia during your life cycle and it has been used to study several brain disorders (Kalueff et al., 2014), we performed a study to investigate the mechanisms of brain hypoxia tolerance using the hypoxic PC in zebrafish.

Initially we measured the resistance of the fish to oxygen deprivation. We observed that after 24h from the severe (1.8 mg O_2/L) or moderate (4.0 mg O_2/L) PC animal had increased time to reach the 3rd stage of hypoxia (Braga et al,, 2013). These results showed that the PC induced an increase in tolerance to severe hypoxia.

Oxygen deprivation can induce to tissue infarction (Sims et al,, 2002). The measurement of brain area with infarct is an important determinant of stroke consequences. Thus, the staining technique by TTC is used to evaluate viability or vitality of animal cells throughout brain tissue (Perry et al., 2002, Sumii and Lo 2002). Because TTC accepts electrons directly from the electron transport chain (Byth et al., 2001), its reduction is directly linked to the mitochondrial respiratory chain (Comas et al., 2000). It is supported that TTC accepts electrons directly from the low potential cofactors of the NADH dehydrogenase (complex I) (Rich et al., 2001). Several studies showed that TTC assay is mainly associated with complex II and IV activities. However, it has been shown that TTC is reduced by all mitochondrial dehydrogenases, especially the complex I Rich et al., 2001). In addition, mitochondrial damage was detected by the TTC staining using rotenone, a complex I inhibitor (Fukumoto et al., 2012). Accordingly, our group showed that there was an increase in mitochondrial dehydrogenases activities after 24 hours after the severe and moderate PC, which could be associated to elevated activity of complex I.

Over the last 20 years scientists has been intensively studied the function/dysfunction evolving mitochondria and the present work contributes on the role of this organelle in the preconditioning (Pérez-Pinzón, 2004; Pérez-Pinzón et al., 2005; Christophe and Nicolas, 2006; Murphy and Steenbergen, 2007; Halestrap et al., 2007). Based on our data, the animals stimulated by severe PC consumed less oxygen in basal respiration than control animals. Therefore, after 24 hours after the severe PC the central nervous system of these animals showed decreased oxygen consumption (Fig. 3A). Since ATP synthase is associated to the site of O_2 consumption in the mitochondria (complex IV), the blockage of this proton carrier channel by

oligomycin allowed to study ATP production linked to oxygen consumption. The difference on mitochondrial respiration with or without oligomicyn administration corresponded to the quantification of O_2 consumption linked ATP production. Thus, our data showed no change on ATP production in the preconditioned animals though their mitochondria (complex IV) consumed less oxygen (Fig 3B).

Ischemic damage in the brain is caused by reduction of oxygen and glucose supply, which mainly affects mitochondrial ATP production (Dirnagl et. al., 2009). In order to verify the maximum respiration by mitochondria of preconditioned animals, we evaluated the maximum passage of the protons by electron transport chain by using FCCP, an uncoupler agent able to open pores in the mitochondrial membrane. We did not observe any difference in maximum respiration. Therefore, the lowest oxygen consumption performed by mitochondrion from preconditioned fish was not supported by a dysfunction on electron transport chain.

The difference between the maximum respiration and basal respiration correspond to spare capacity. We observed that preconditioned animals had a higher spare mitochondrial activity. Particularly, when preconditioning is followed by reperfusion, the increase in oxygen supply results in a burst of ROS production (Dirnagl et. al., 2009). Thus, preconditioned animals could generate less ROS due to higher mitochondrial efficiency presumed by a normal ATP production under less oxygen consumption, such as reported in mitochondria from primary cerebellar granule neuronal cultures (Jekabson and Nicholls et al., 2004). Our results showed a decrease on ROS levels in these fish, corresponding to the higher efficiency on mitochondrial respiration caused by preconditioning. Studies on superoxide anion (O_2•) generation have shown that mitochondrial complex I and III are the major ROS formation cell sites. There are two oxygen reduction sites in complex I, flavin mononucleotide (FMN) and iron-sulfur cluster, which may be exposed to oxygen when complex I is dissociated from the complex III (Lenaz et. al., 2010). Thus, it is postulate that preconditioning could have stimulated an association between complex I and complex III (like supercomplex I-III_2), avoiding the interaction of oxygen with the prosthetic groups of complex I and, then, decreasing ROS production.

To evaluate the functionality of complex III we use of Antimycin A, a complex III inhibitor. As result, no difference was found in the complex III activity. Furthermore, we evaluated the complex I, which is a NADH dehydrogenase. The complex I was inhibited by rotenone, since it is a blocker of the active site leading to an inhibition around 100% of this complex. After rotenone administration there was an increase in complex I activity. Therefore, the increase on complex I activity could be caused by a compensatory mechanism induced by a deficiency on complex II activity. There is evidence that formation of supercomplexes I-III_2 does

increase the stability and activity of complex I more than complex III (Bultema et. al., 2009; Wittig et. al.; 2006). Moreover, studies have reported that complex III deficiency lead to complex I dysfunction (Petrosillo et al., 2009). As we observed only an increase in complex I activity, our data supports a probable supercomplex I-III_2 formation caused by preconditioning, providing an adaptive mechanisms to increase mitochondrial efficiency and prevent generation of ROS (Panov et al., 2007).

In TCA cycle analysis, we observed that brain of preconditioned animals consumed predominantly malate, pyruvate, and glutamate, the energetic substrates associated to production of NADH. Interestingly, these data corroborated with the higher activity of complex I observed in these animals, suggesting that oxidation of those energetic substrates would be readily favored in relation to other substrates. In fact, the lower preference by succinate was observed in preconditioned fish, since this substrate generates FADH as coenzyme, which it has affinity for the complex II.

In summary, here we demonstrated for the first time the beneficial effect of hypoxic preconditioning in zebrafish model it that is. Our data showed that, both severe and moderate PC, increased the resistance to hypoxia associated to an elevation in the mitochondrial viability in the brain 24h after the preconditioning stimulus. Further investigation on fish exposed to severe PC indicated that hypoxia resistance is linked to normal ATP production with less oxygen consumption by brain mitochondrion. Even preliminary, these results support the potential use of hypoxic preconditioning as therapeutic treatment in dysfunctions evolving oxygen deprivation. If future studies confirm the benefits of hypoxic preconditioning, similar strategy could be applied translationally (e.g., in hyperbaric chambers, physical exercises) in people prone to diseases related to hypoxia.

5. Conflict of interest

None of the authors have any conflict of interest to disclose.

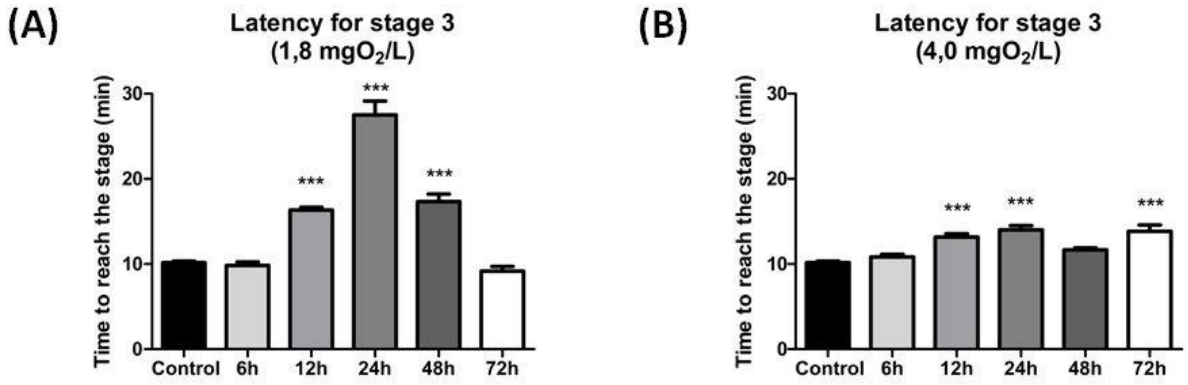

Fig. 1: Resistance to hypoxia test showing time to reach 3rd stage in each animal over time. (n=6). (A) Each animal was a 1.8 mgO2 / L PC prior to the severe hypoxia. (B) Each animal was a PC 4.0 mgO2 / L prior to the severe hypoxia. p <0.05 (ANOVA One way, post hoc Tukey test).

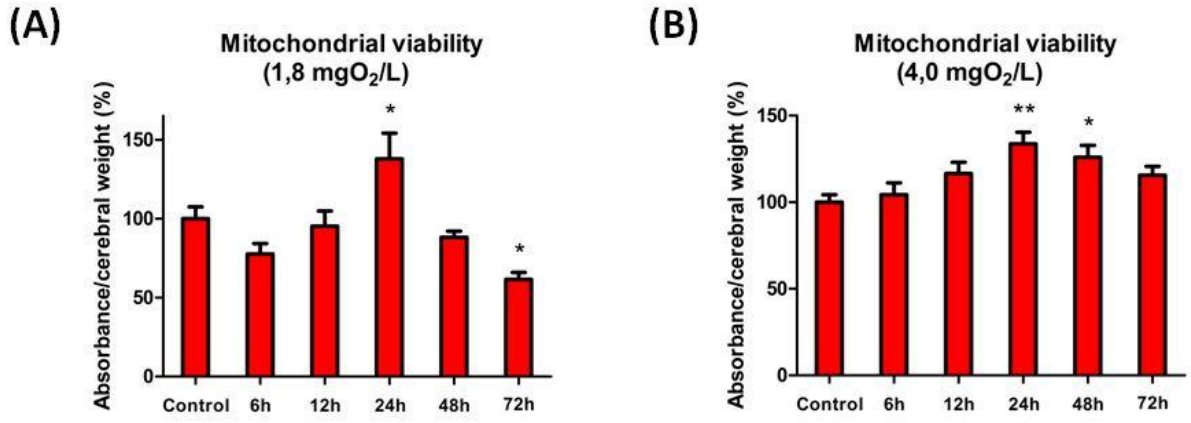

Fig. 2: Preconditioning Effect of enzymatic parameters related to the activity of mitochondrial dehydrogenases. (A) Each animal was a PC 1.8 mgO_2 / L. (n = 6). $p < 0.05$ (ANOVA One way, post hoc Tukey test. (B) Each animal underwent a PC 4.0 mgO_2 / L. (n = 6). $p < 0.05$ (ANOVA One way, post hoc Tukey test).

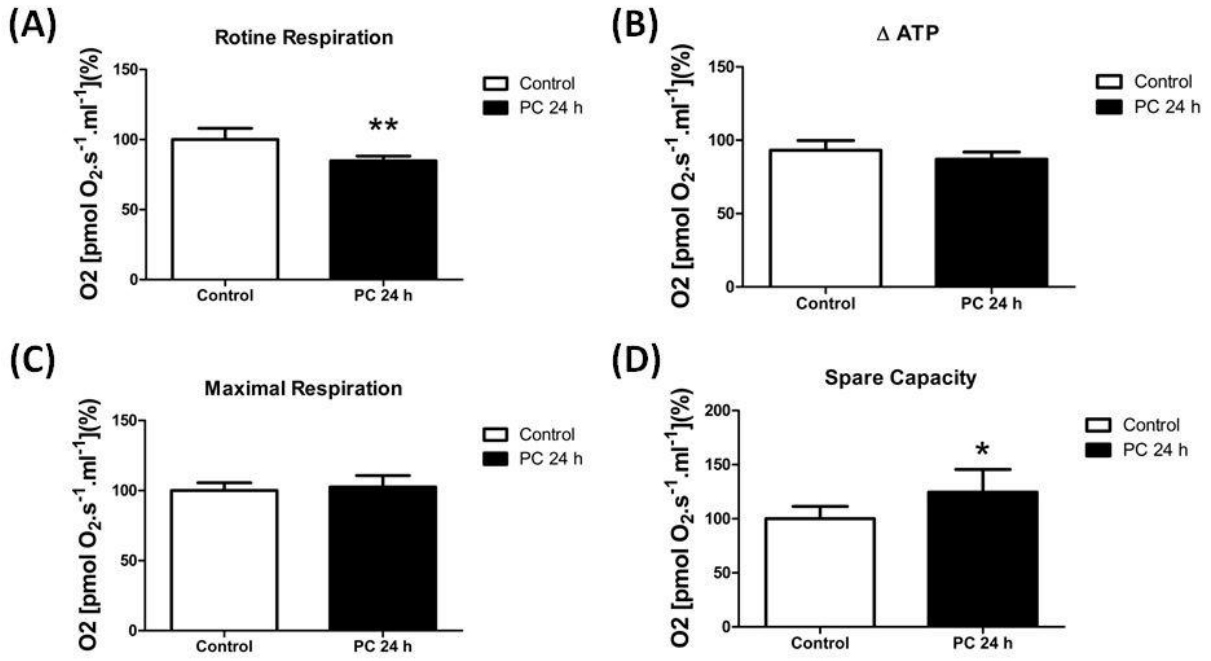

Fig. 3: Analysis in high-resolution respirometry (Oroboros Oxygraph-O2K). (A) Rotine respiration (B) Oxygen consume to ATP production (Δ ATP). (C) Maximal Respiration. (D) Spare capacity. (n= 6 per group). $p < 0.05$, t-test.

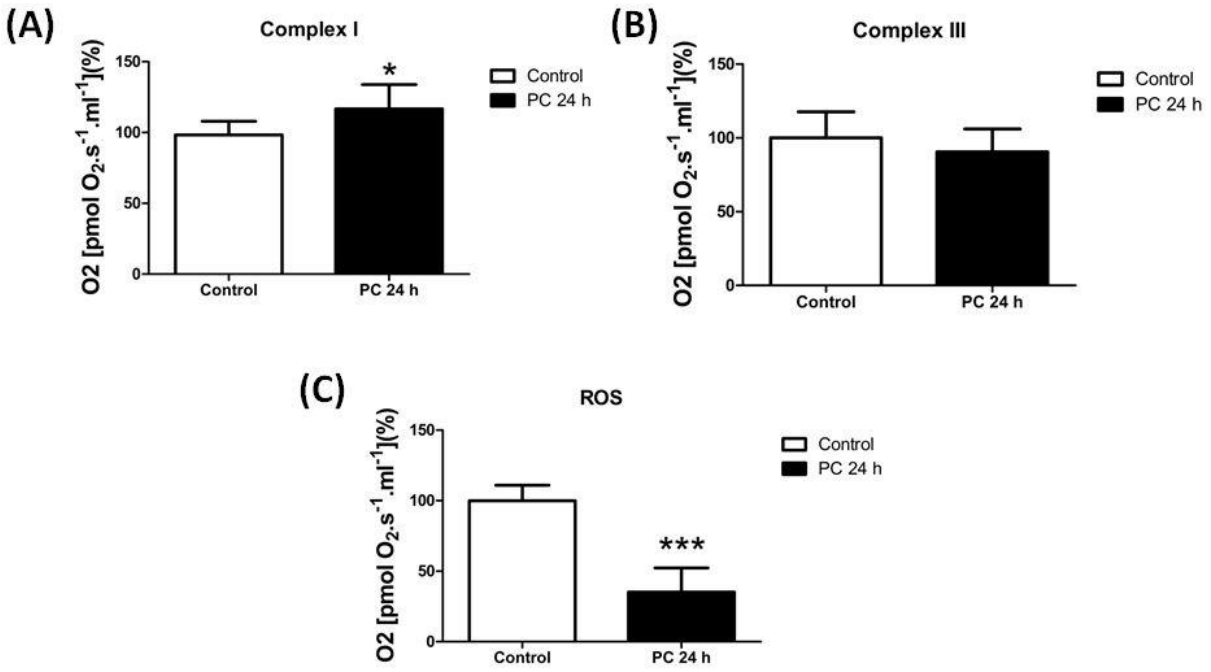

Fig. 4: Analysis in high-resolution respirometry (Oroboros Oxygraph-O2K). (A) Complex I. (B) Complex III. (C) Production of the oxygen-reactive species (ROS). (n= 6). $p < 0.05$, t-test.

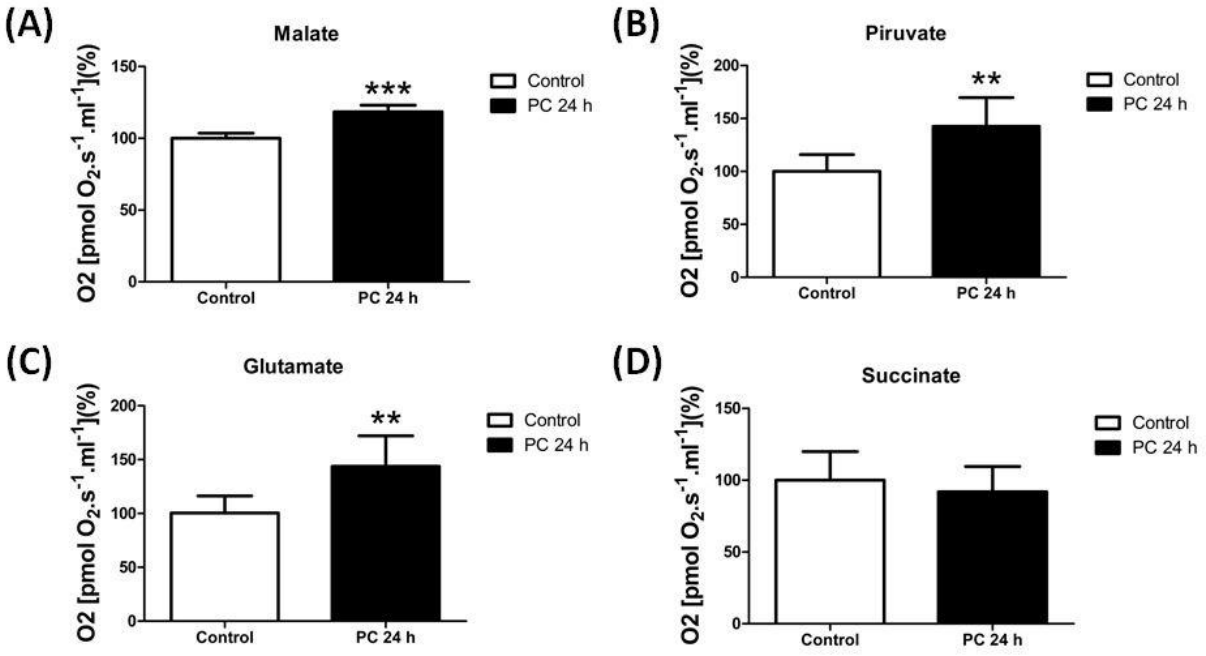

Fig 5: Analysis in high-resolution respirometry (Oroboros Oxygraph-O2K). Preference for energetic substrates. (A) Malate. (B) Piruvate. (C) Glutamate. (D) Succinate. (n= 6). $p < 0.05$, t-test.

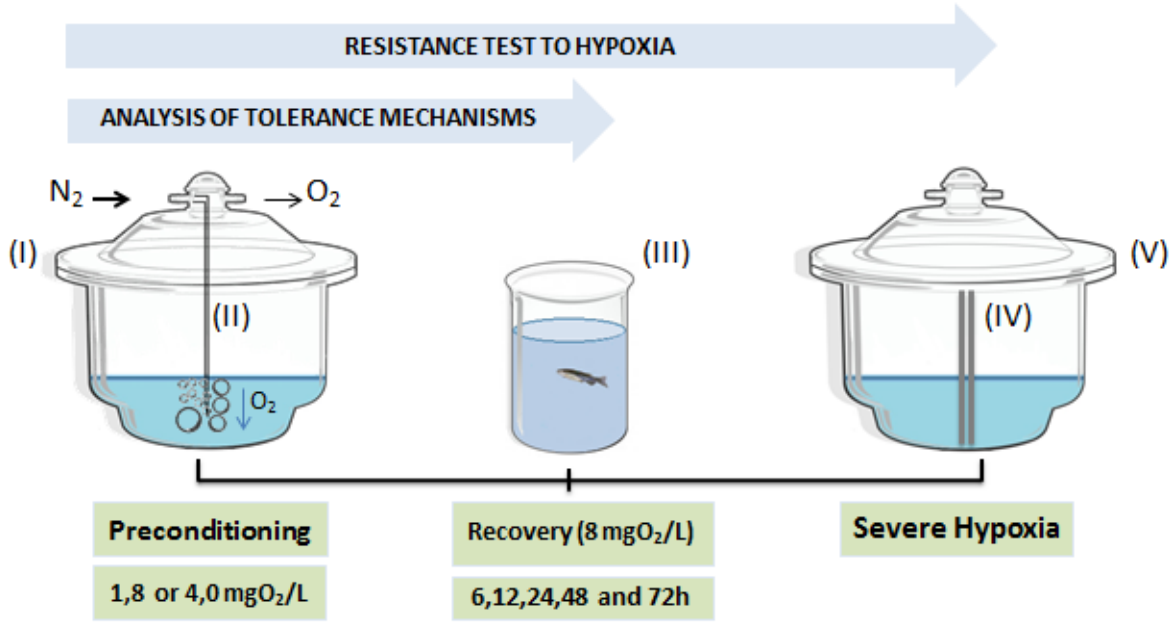

Fig 6: Complementary Figure. Representation of the experimental time line. (I) Chamber of hypoxic preconditioning. See the nitrogen bubbling inside the chamber. (II) Establishment of oxygen concentration inside the chamber (preconditioning of 1.8 or 4.0 mgO2 / L). (III) Aquarium for recovery of animals with water under normoxic conditions (8 mgO2 / L). The recovery time was 6, 12, 24, 48 and 72 hours in each group. (IV) Division of the hypoxia chamber in the half. Thus, preconditioned animals and controls can be placed in the same hypoxic condition and evaluate resistance. (V) Hypoxic chamber to available tolerance mechanism.

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Divide your article into sections according to the headings listed below. Main sections (Introduction, Experimental Procedures, Results, etc.) and sub-section headings should appear on their own separate line. Use the section and sub-section names for internal cross-referencing: do not just refer to "the text.

Research papers should be organized in the following four main sections: Introduction, Experimental Procedures, Results, Discussion

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Introduction

This should provide the scientific rationale for the research that is reported. No results should be reported but it should finish with a succinct description of the main finding and conclusion. The heading "Introduction" should be used.

Experimental procedures

Procedures used in the research should be described in sufficient detail to permit the replication of the work by others. Previously published procedures should be referenced and briefly summarized. The source of all materials, including animals and human tissue, must be provided. The location of each supplier should be detailed on first use in the text. The author(s) also agree(s) to make freely available to colleagues in academic research any clones of cells, nucleic acids, antibodies, etc. that were used in the research reported and that are not available from commercial suppliers. Authors must clearly describe all manipulations made to digital data that were collected as images, and images which have been scanned and printed for publication.

Results

This section presents findings without discussion of their significance. Subsections should be used in order to present results in an organized fashion.

Discussion

This section presents the authors' interpretations of their findings and an assessment of their significance in relation to previous work. Avoid repetition of material presented in the Results section. The Results and Discussion sections may *not* be combined.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of the Discussion section.

Glossary

Please supply, as a separate list, the definitions of field-specific terms used in your article.

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Material appearing in Appendices should augment the main manuscript narrative, by providing details otherwise not readily amenable to include in the main narrative. Examples of material that could appear in an Appendix include, but are not limited to: mathematical derivations; results of genetic screens; and lengthy reports of neuroimaging results. If there is more than one appendix, they should

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A concise and factual abstract is required. The abstract should state briefly in a single paragraph (in <250 words) the purpose of the research and the principal results obtained. The abstract should conclude with a final statement summarizing the major conclusions in such a way that the implications of the work to the field would be clear to a general neuroscience reader. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

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term, e.g. acetylcholinesterase (AChE). The abbreviations should then be used consistently thereafter and appear at least twice in the text. A comprehensive list of the abbreviations used should be put on a separate page that follows the title page.

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Título: POTENCIAL EFEITO NEUROPROTETOR DO PRÉ-CONDICIONAMENTO EM PEIXE-ZEBRA ADULTO SUBMETIDO AO MODELO DE HIPÓXIA-ISQUEMIA

Vigência: 01/09/2015 à 31/08/2017

Pesquisadores:

Equipe UFRGS:

MARIA ELISA CALCAGNOTTO - coordenador desde 01/09/2015
DIOGO LOSCH DE OLIVEIRA - pesquisador desde 01/09/2015
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Marcos Martins Braga - pesquisador desde 01/09/2015
Denis Broock Rosemberg - pesquisador desde 01/09/2015
Eduardo Pacheco Rico - pesquisador desde 01/09/2015

Comissão De Ética No Uso De Animais aprovou o mesmo em seus aspectos éticos e metodológicos, para a utilização de 702 peixes zebra, Danio rerio, adultos, machos e fêmeas, provenientes do fornecedor Delphis, RS – IBAMA nº 4298655, 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.

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