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CARACTERIZAÇÃO BIOQUÍMICA DE UM PROTOCOLO DE DISSOCIAÇÃO CELULAR CEREBRAL EM PEIXE-ZEBRA ADULTO PARA RESPIROMETRIA DE ALTA RESOLUCÃO

Porto Alegre
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Orientador: Prof. Dr. Diogo Losch de Oliveira

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SUMÁRIO

APRESENTAÇÃO

Esta dissertação está dividida em três 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 um artigo científico apresentado no Capítulo I, o qual será submetido ao periódico Mitochondrion. Esse capítulo está subdivididos em: Introdução, Materiais e Métodos, Resultados, Discussão, Conclusão e Referências Bibliográficas;

PARTE III: discussão final e conclusão;

PARTE I

RESUMO

A mitocôndria é a principal organela responsável pela biossíntese de ATP nas células eucariontes por meio da fosforilação oxidativa. Esse processo é essencial para a manutenção da vida e alterações nessa via podem estar relacionadas a múltiplas disfunções. Atualmente, é possível medir o consumo celular de O_2 e inferir o estado da fosforilação oxidativa por meio da técnica de respirometria de alta resolução. Apesar dos avanços na área, refinamentos nos protocolos, principalmente em ensaios que determinam a função mitocondrial utilizando células intactas, são necessários. Neste contexto, nosso objetivo foi caracterizar um método de dissociação tecidual de cérebro de peixes-zebra adulto, visando obter um dissociado com o maior número de células viáveis para uso na respirometria de alta resolução, bem como aplicá-lo frente a uma patologia, como a epilepsia. Foram utilizados 272 peixes-zebra adultos (5-6 meses de idade) na proporção de 50:50 machos:fêmeas (CEUA 33922). Os animais foram mortos e seus cérebros retirados. As amostras foram submetidas a quatro diferentes dissociações teciduais: dissociação mecânica com *pasteur* de vidro; dissociação mecânica com pistilo para eppendorf; dissociação enzimática com collagenase IV; e dissociação enzimática com tripsina. A viabilidade celular foi avaliada pela incorporação de azul de Tripan e pela atividade extracelular da enzima lactato desidrogenase (LDH). A respirometria de alta resolução foi realizada no equipamento *Oroboros Oxygraph-2k* através da medida do consumo de oxigênio por meio da adição de inibidores da cadeia transportadora de elétrons e titulação com desacoplador do potencial de membrana mitocondrial. A viabilidade celular medida pelo do método de azul de Tripan não apresentou diferença significativa para as dissociações mecânicas com *pasteur* de vidro e pistilo, porém o grupo homogeneizado com *pasteur* de vidro apresentou menor variabilidade nos dados. Através da avaliação da viabilidade celular dos diferentes protocolos de dissociação tecidual por método enzimático, as concentrações ótimas escolhidas para dissociação com collagenase IV e tripsina foram respectivamente, 0,1mg/mL e 0,01%; ambas com 10 minutos de incubação em banho-maria a 37º C. A avaliação da viabilidade celular através da atividade da enzima LDH não apresentou diferenças significativas entre os grupos testados. Com base nesses dados, utilizamos as dissociações mecânica com *pasteur* de vidro, collagenase IV ou tripsina para avaliar diversos parâmetros da função mitocondrial em células intactas. As dissociações enzimáticas apresentaram redução significativa na respiração de rotina (ROUTINE), na capacidade de fosforilação oxidativa (P/O2) e na respiração extramitocondrial (ROX) quando comparadas a dissociação com *pasteur* de vidro. Desta forma, escolhemos o método de dissociação mecânica como padrão para testar os parâmetros de respiração celular frente a um modelo de crises epilépticas prolongadas induzidas por ácido caínico. Um aumento significativo na capacidade respiratória de reserva (SRC) foi observado nos animais submetidos a crises epilépticas prolongadas quando comparados ao grupo controle. Como conclusão, de acordo com os resultados obtidos nesse trabalho, sugerimos que a avaliação da função mitocondrial em peixe-zebra adulto seja feita através do protocolo de dissociação mecânica com *pasteur* de vidro.

ABSTRACT

Mitochondria are the main source of ATP biosynthesis in eukaryotic cells through oxidative phosphorylation. This process is essential for life maintenance and any changes in this pathway may be related to multiple dysfunctions and neurological diseases. It is now possible to measure cellular consumption of O_2 and, consequently, evaluate oxidative phosphorylation capacity by High Resolution Respirometry technique. Despite the advances, in order to generate reliable and reproducible results, optimization of the metodology used to obtain samples for high resolution respirometry are required, especially in assays that determine mitochondrial function using intact cell. The main goal of the present work was to characterize a method to dissociate adult zebrafish brain tissue into viable single cells to high resolution respirometry measurements, as well as apply these optimized protocol under pathological states, such as epilepsy (CEUA 33922). Subjects were 272 animals (~50:50 male: female ratio) adult zebrafish (*Danio rerio)* (5-6 months-old). Animals were dissected and brain samples were submitted to four different tissue dissociations: mechanical dissociation with glass pasteur pipette: mechanical dissociation with microtube pestle; enzymatic dissociation with collagenase IV; and enzymatic dissociation with trypsin-EDTA. Cell viability was assessed by trypan blue exclusion test and extracellular activity of lactate dehydrogenase (LDH). High resolution respirometry was performed at Oroboros Oxygraph-2k equipment by measuring oxygen consumption after the addition of electron transport chain inhibitors and titration with mitochondrial uncoupler. The results of cellular viability by trypan blue assay did not show significant difference between the two mechanical dissociations compared, but the group homogenized with glass pasteur pipette present less variability. The optimal concentrations selected for dissociation with collagenase IV and trypsin were, respectively, 0.1 mg / mL and 0.01%, both incubated during 10 minutes at 37ºC. Cellular viability by LDH activity did not present significant differences. Based on these data, we evaluate mitochondrial function in intact cells originating from mechanical dissociation with glass pasteur pipette and enzymatic dissociation with collagenase IV or trypsin. Enzymatic dissociations showed a significant reduction in some parameters measured: routine respiration (ROUTINE), oxidative phosphorylation capacity (P/O_2) and extramitochondrial respiration (ROX). Then, mechanical dissociation method was applied to measure respiratory capacity under prolonged epileptic seizures, and a significant increase in respiratory reserve capacity (SRC) was seen in epileptic group when compared to the control group. Concluding, both the mechanical dissociation with glass pasteur pippete and enzymatic dissociation with collagenase IV can be used to evaluate mitochondrial function.

LISTA DE ABREVIATURAS

ATP: Trifosfato de Adenosina

CNS: Sistema Nervoso Central

CI: Complexo I ou NADH-ubiquinona-oxidorredutase

CII: Complexo II ou Succinato-ubiquinona-oxidorredutase

CIII: Complexo III ou Complexo Citocromo bc1

CIV: Complexo IV ou Citocromo c oxidase

Complexo V: F1-Fo-ATP sintase

ETS: Sistema de Transferência de Elétrons

GABA: Ácido gama-aminobutírico

KA: Ácido Caínico

LDH: Lactato Desidrogenase

NADH: Dinucleótido de nicotinamida e adenina reduzida

OXPHOS: Fosforilação Oxidativa

ROX: Consumo de Oxigênio Residual

SE: *Status Epilepticus*

SRC: Capacidade Respiratório de Reserva

INTRODUÇÃO

1. PEIXE-ZEBRA COMO MODELO ANIMAL

O peixe-zebra (*Danio rerio*), também chamado de *zebrafish* ou paulistinha, é um pequeno teleósteo (3-4 cm na fase adulta) de água doce da família *Cyprinidae*. O peixe-zebra vive, em média, três anos e meio quando criado em laboratório, e são considerados animais adultos após 3-4 meses de desenvolvimento, período necessário para atingirem a maturidade sexual (GERHARD et al., 2002; REED; JENNINGS, 2011).

O uso do peixe-zebra como modelo animal em pesquisas biomédicas vem se tornando cada vez mais popular devido as diversas vantagens. Dentre elas, pode-se citar a alta homologia fisiológica e genética com mamíferos e a facilidade de manipulação genética, as quais vem auxiliando, através da geração de peixes-zebra mutantes, a compreensão sobre os mecanismos cruciais envolvidos no desenvolvimento embrionário, bem como alterações comportamentais e encefálicas na fase larval e adulta (STEWART et al., 2014). Além disso, esta espécie apresenta um desenvolvimento externo com os ovos e larvas translúcidas, o que permitem ao pesquisador o controle do ambiente de desenvolvimento logo após a fertilização e o monitoramento do desenvolvimento de tecidos e órgãos (Ablain and Zon, 2013).

No campo das neurociências, o peixe-zebra também tem proporcionado diversas contribuições: tanto nas fases de larva quanto adulto são amplamente utilizados para estudar desordens que envolvam o sistema nervoso central (SNC) (SHAMS et al., 2018). Nesse contexto, as características de diversas desordens neuropsiquiátricas, neoplasias cerebrais e doenças como Parkinson, Huntington, Alzheimer e epilepsia vem sendo estudadas. Parâmetros bioquímicos, comportamentais e genéticos, bem como biomarcadores neuronais já foram descritos para patologias do SNC no peixe-zebra (STEWART et al., 2014; XI; NOBLE; EKKER, 2011). Apesar do grande progresso científico, as desordens do SNC precisam ser melhor compreendidas e, nesse contexto, melhores tratamentos precisam ser desenvolvidos. Na ascensão de espécies mais simples (ex: *D. rerio, Drosophila melanogaster, Caenorhabditis. elegans*) como modelos animais promissores para compreensão das desordens do SNC, o peixe-zebra ganhou espaço rapidamente devido a alta complexidade do sistema nervoso quando comparado as demais espécies de invertebrados.

Dentro das desordens do SNC, o peixe-zebra tem grande potencial para modelar as epilepsias e crises epilépticas. Diversos modelos genéticos estão disponíveis devido a aplicação da metodologia de injeção de oligonucleotídeos morfolinos, manipulando genes que resultam em fenótipos epilépticos (MUSSULINI et al., 2016). Somado a isso, os modelos não-genéticos também são amplamente utilizados, como por exemplo, o de crises convulsivas induzidas por agentes químicos como o pentilenotetrazol (PTZ), pilocarpina, ácido domóico e ácido caínico (KA) (STEWART et al., 2012). Alterações neuroquímicas, como a expressão do gene c-Fos, o padrão eletroencefalográfico e alterações comportamentais clássicas das crises epilépticas vistas em ratos ou roedores também são reproduzidas em peixe-zebra (BARABAN, 2015). Apesar de certas limitações, a validade do modelo já está consolidada na literatura para estudo de desordens do SNC (BARABAN, 2015).

2. MITOCÔNDRIAS

A principal função da mitocôndria é converter os substratos energéticos em energia na forma de trifosfato de adenosina (ATP), através do processo de fosforilação oxidativa. Funções mitocondriais adicionais incluem a geração e detoxificação de espécies reativas de oxigênio (ROS), envolvimento no processo de morte celular programada (apoptose), regulação da homeostase de cálcio na célula, síntese e catabolismo de metabólitos (HERST et al., 2017).

Frequentemente, linhagens ou organismos geneticamente modificados desenvolvidos para estudar diversas patologias, apresentam disfunções mitocondriais, principalmente aquelas relacionadas ao comprometimento do metabolismo energético. Dentre as desordens associadas a desregulações bioenergéticas estão as doenças cardiovasculares, doenças psiquiátricas (principalmente o autismo) e, especialmente, doenças que comprometem o sistema nervoso central, como Parkinson, Huntington e epilepsia (BRAND; NICHOLLS, 2011). Em relação as epilepsias, é comum pacientes epilépticos apresentarem disfunções mitocondriais associadas a alterações no sistema de transporte de elétrons (TENNEY et al., 2014). Além disso, durante as crises epilépticas, relata-se um aumento no consumo de ATP e um aumento nos níveis de glicose, que poderiam estar relacionados a uma redução bioenergética mitocondrial (LEE et al., 2012).

Em torno de 1500 proteínas são necessárias para que a mitocôndria desempenhe sua função, e essas proteínas são codificadas, em sua maioria, por genes do DNA nuclear.

No entanto, o DNA mitocondrial (mtDNA) também é responsável por codificar uma parcela das proteínas mitocondriais (RUSTIN; MUNNICH, 2003). Dentro das proteínas codificadas pelo mtDNA, encontra-se aquelas relacionadas a cadeia transportadora de elétrons. O mtDNA do peixe-zebra, em comparação com os mamíferos, possui semelhanças tanto genéticas quanto funcionais (BROUGHTON; MILAM; ROE, 2001).

O centro do metabolismo energético mitocondrial é a fosforilação oxidativa (OXPHOS). A OXPHOS requer tanto o transporte de elétrons para o oxigênio, quanto a fosforilação do difosfato de adenosina (ADP) para produção do trifosfato de adenosina (ATP). Com base na hipótese quimiosmótica, à medida que os elétrons passam pelas proteínas da cadeia transportadora de elétrons – complexos I, II, III, IV, Coenzima Q e citocromo C, estes são entregues ao oxigênio, o qual vai sendo consumido e convertido em água. Ao mesmo tempo, um gradiente de prótons é formado na membrana mitocondrial interna, o qual impulsiona, por fim, a produção do ATP no complexo V (também chamado de ATP sintase). Por isso, podemos dizer que parte do consumo de oxigênio mitocondrial está acoplado à síntese de ATP.

3. RESPIROMETRIA DE ALTA RESOLUÇÃO

Entre as diversas técnicas para avaliar função e disfunção mitocondrial, encontra-se a respirometria de alta resolução. Através de equipamentos contendo um eletrodo de Clark (como o Oxygraph-2K Oroboros®, que realiza medições do consumo de O_2 em uma determinada amostra em tempo real) é possível inferir e avaliar diversos parâmetros relacionados a fisiologia mitocondrial.

Através de diferentes preparações de amostras (ex: células intactas, células permeabilizadas, mitocôndrias isoladas, etc) é possível calcularmos diversos parâmetros fundamentais da respiração celular (BRAND; NICHOLLS, 2011). A metodologia que emprega células intactas apresenta diversas vantagens e desvantagens, quando comparada a outros métodos de obtenção da amostra. Entre as principais vantagens, destacam-se: a preservação do ambiente celular; a rapidez da preparação da amostra, o que exclui o viés dos artefatos criados pelos longos processos de extração mitocondrial; uma menor quantidade de tecido ou células em relação a quantidade necessária para utilização de mitocôndrias isoladas. Como desvantagens, destaca-se a difícil interpretação dos resultados obtidos, a dificuldade de extrair células viáveis de tecidos animais e o viés da

normalização dos resultados, no qual a média obtida muda de acordo com a normalização escolhida (número de células, massa tecidual, quantidade de proteína, etc).

Dentre as principais variáveis avaliadas, de acordo com o livro *Mitochondrial Pathways and Respiratory Control: An introduction to OXPHOS Analysis*, temos: a respiração de rotina (R), a capacidade da cadeia transportadora de elétrons (ETS), o consumo de oxigênio proveniente do vazamento de prótons (H^+ Leak), a capacidade da fosforilação oxidativa (P/O_2), a capacidade respiratória de reserva (SRC) o consumo de oxigênio residual (ROX). Essas medidas podem ser feitas através do protocolo chamado controle de acoplamento, visto na figura 1.

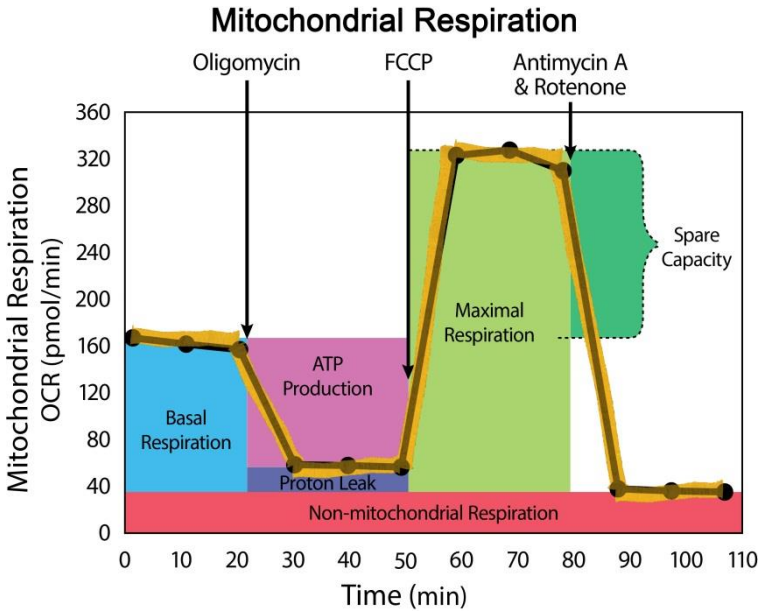

Figura 1. Parâmetros fundamentas da função mitocondrial. Através da adição de inibidores da cadeia transportadora de elétrons (Oligomicina, Rotenona e Antimicina A) e do desacoplador FCCP, é possível medir seis parâmtros: respiração de rotina (azul), capacidade da fosforilação oxidativa (rosa), H^+ Leak (azul), capacidade máxima da cadeia transportadora de elétrons (verde claro), a capacidade respiratória de reserva (verde escuro), e o consumo de oxigênio residual (vermelho). Fonte: *Mitochondrial reserve capacity is calculated by subtracting basal respiration from maximal respiratory capacity. doi:10.1371/journal.pone.0085436.g001*

OBJETIVO

1. OBJETIVO GERAL

Nosso objetivo foi otimizar um protocolo de dissociação tecidual de cérebro de peixe-zebra adulto, visando obter um dissociado de células intactas, para caracterização de processos da respiração celular através da técnica de respirometria de alta resolução.

2. OBJETIVOS ESPECÍFICOS

- Avaliar a viabilidade celular resultante dos protocolos de dissociação mecânicas e enzimáticas através da metodologia de azul de tripan

- Avaliar a viabilidade celular resultante dos protocolos de dissociação mecânicas e enzimáticas através da medida da atividade extracelular da enzima lactato desidrogenase (LDH);

- Descrever o perfil respiratório do tecido cerebral do peixe-zebra adulto resultante dos protocolos que apresentarem maior viabilidade celular através da técnica de respirometria de alta resolução;

- Analisar o perfil respiratório do peixe-zebra frente a crises epilépticas prolongadas induzidas pelo ácido caínico a partir da metodologia/otimização da dissociação tecidual mais apropriada através da técnica de respirometria de alta resolução.

PARTE II

CAPITULO I

Artigo em prepração para submissão

Título: An optimized method for adult zebrafish brain-tissue dissociation to access mitochondrial function.

Autores: Luana Moro, Giovana Rech, Amanda Linazzi, Thainá Garbino, Diogo Losch de Oliveira.

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Justificativa: Atualmente, através da técnica de respirometria de alta resolução, é possível determinar diversos parâmetros relacionados a função mitocondrial em estados fisiopatológicos. A utilidade do peixe-zebra como ferramenta de estudo é inegável e, até o presente momento, não há protocolos de respirometria de alta resolução disponíveis na literatura para esse modelo animal.

Objetivo Geral: Otimizar um protocolo de dissociação tecidual de cérebro de peixe-zebra adulto, gerando um dissociado de células intactas para posterior caracterização de parâmetros da respiração celular através da técnica de respirometria de alta resolução.

An optimized method for adult zebrafish brain-tissue dissociation to access mitochondrial function.

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1. Introduction

Mechanisms implicated in neurodegenerative disorders are a challenging topic in neurobiology. Progress in modeling human degenerative disorders in zebrafish has been reported in literature (MARTÍN-JIMÉNEZ; CAMPANELLA; RUSSELL, 2015). Well established advantages include the basic structure of zebrafish central nervous system (CNS): all the major domains are similar to mammalian brain and same neurotransmitters such as Gamma aminobutyric acid (GABA), glutamate, dopamine, noradrenaline, serotonin, histamine and acetylcholine are found. Moreover, there are similar expression patterns of genes involved in degenerative disorders, such as Alzheimer, Parkinson, Huntington, and some types of epilepsy (PANULA et al., 2010). Particularly, human epilepsies have been successfully modeled in zebrafish through acute or chronic seizure induction protocols (MUSSULINI et al., 2016). Electroencephalography (EEG) responses, neurochemical, genetic and behavioral aspects of these pathology are recapitulated in the zebrafish model (GRONE; BARABAN, 2015).

There are strong evidences correlating the role of mitochondria in the pathophysiology of several nervous system disorders (Lax et al., 2017, Johri and Beal, 2012). Mitochondrial dysfunctions related to neurodegenerative diseases include impairment of calcium influx, dissipation of potential mitochondrial membrane, accumulation of mutant proteins or mtDNA deficiencies, and defects of mitochondrial oxidative phosphorylation (OXPHOS) (GOLPICH et al., 2017). OXPHOS defects are associated with both genetic and acquired epilepsy (KOVAC et al., 2017). Decrease in ATP levels leading to energy depletion in neurons and accumulation of intracellular calcium and ROS are common features related in the disorder (HERST et al., 2017). Even though the relation between mitochondrial dysfunction, energy metabolism and epilepsy remains to be elucidated, these organelles are clearly involved in the disease manifestation. Therefore, the interest in evaluate mitochondrial performance, content and structure in nervous tissue have been increased worldwide.

Physiological performance of mitochondria can be monitored by high-resolution respirometry technique. Based on measurement of O_2 consumption, appropriated protocols were designed using different cell preparations (e.g. isolated mitochondria, intact cells, permeabilized cell, tissue homogenates and permeabilized tissue) and all of them provide relevant information about OXPHOS (MAKRECKA-KUKA;

KRUMSCHNABEL; GNAIGER, 2015). Studies have shown increased physiological relevance of working with intact cells: maintenance of cellular environment, such as cytosolic factors and cellular interaction, minimization of tissue and time needed for measurements, as well as no artefacts due to mitochondrial isolation interfere in the experimental procedures (BRAND; NICHOLLS, 2011). Although zebrafish is a promising model to study neurological disorders, information about mitochondrial physiology and bioenergetics under both healthy and pathological conditions are missing.

Despite the progress in this field, we still need increased understanding of mechanisms in neurodegenerative disease, as well as better treatments. Therefore, the major aim of the present study is to establish a method which would provide detailed analysis of mitochondrial function in zebrafish CNS. So, we first isolate viable single cell from adult zebrafish nervous system to characterize respiration rates of tissue dissociation preparations. The second step was to analyze respiration rates in a pathological condition, particularly epilepsy.

2. Methods

2.1 Chemicals

Collagenase type IV (125 CDU/mg) from *Clostridium histolyticum* and Trypsin-EDTA (280 U/mg) were purchased from Sigma-Aldrich (Cat.- C5138-25MG) and Gibco (Cat- 15400-054 100ML), respectively. Kainic acid was purchased from Cayman Chemical Company (CAS N° 58002-62-3).

2.2 Ethical note

All experimental procedures were performed according to the Brazilian Law for Laboratory Animal Care and Use (Law 11794/2008) and were previously approved by the Institutional Committee for Animal Care and Use from Universidade Federal do Rio Grande do Sul (protocol number #33922). All experimental designs were developed in order to minimize the number of fish and their discomfort or suffering.

2.3 Animals and tissue preparation

Two hundred and seventy two adult (5-6 months-old) zebrafish (*Danio rerio*) of short-fin wild type strain were used (~50:50; male:female ratio). Fish were obtained from

local breeding through pair-wise crossing. Adult zebrafish were maintained in 3.5 L aquaria in an automatic recirculating water system (Zebtec,Tecniplast, Italy) at a 14h/10h light:dark photoperiod (lights on at 8 a.m.). Water was maintained at the following parameters: conductivity 500±100 uS/cm, pH 7,0±0,5, temperature of 28±1° C. Adult zebrafish were housed in groups of 5 fish/L (REED; JENNINGS, 2011) and they were fed four times a day, twice with commercial flake fish food (Alcon BASIC®, Alcon, Brasil) and twice with live brine shrimp (*Artemia s.p.*).

2.4 Brain tissue dissociation

Adult zebrafish were anesthetized with tricaine 160 µg/ml and then euthanized by decapitation. After brain removal, freshly isolated brains were cleaned once with Hank's Balanced Salt Solution (HBSS) for removing red blood cells: 137 mM NaCl; 0.63 mM Na_2HPO_4; 3.0 mM $NaHCO_3$; 5.36 mM KCl; 0.44 mM KH_2PO_4; 1.26 mM $CaCl_2$; 0.90 mM $MgSO_4$; 5.55 mM glucose; and 20 mM HEPES, pH 7.4, temperature 28±1 °C. A pool of two brains were suspended in 1 mL HBSS or HBSS+enzymes for tissue digestion **(Fig. S1).** The following dissociation methods were conducted:

I) mechanical dissociation with glass Pasteur (*n*= 4): gently dissociate the brain tissue by slowly pipetting up and down 4-5 times with the glass Pasteur pipette slight flambéed (thicker tip). After, pipette up and down 4-5 times with another glass pasteur pipette highly flambéed (thinner tip);

II) mechanical dissociation with microtube pestle (*n*= 4): brain tissue was gently dissociated by slowly pipetting up and down 8-10 times with microtube pestle;

III) enzymatic dissociation with Collagenase type IV from *Clostridium histolyticum*: tissue was incubated with different enzyme concentrations (0, 0.1, 0.5, 1 mg/mL) (*n*= 4 for each group), during 30 min at 37° C. After determination of the optimal concentration, samples were incubated for 10, 30 and 60 min (*n*= 4 for each group) at 37°C. Tissue was gently dissociated mechanically with glass Pasteur pipette in the middle and at the end of each incubation time (2-3 times each).

IV) enzymatic dissociation with Trypsin-EDTA: tissue was incubated with different enzyme concentrations (0, 0.01, 0.05, 0.25%) (*n*= 4 for each group), during 30' at 37°C. After optimal concentration was chosen, samples were incubated for 10', 30' and 60' (*n*=

4 for each group) at 37°C. Tissue was gently mechanically dissociated with glass Pasteur pipette in the middle and at the end of each incubation time (2-3 times each).

2.5 Cellular viability evaluation

In order to define a protocol for adult zebrafish brain dissociation to single cells, cellular integrity was measured by two methods: trypan-blue exclusion and lactate dehydrogenase activity.

For trypan-blue exclusion, after tissue dissociation cell suspension were centrifuged at 400g for 5 min at 4º C. The supernatant was discarded and the pellets containing cells were re-suspended in 1 mL of phosphate buffered saline (PBS) 0.3M. A sample of the cell mixture was stained with trypan-blue and evaluated as previously described (STROBER, 2015).

A LDH UV commercial kit (Bioclin, Ref. K014) was used for lactate dehydrogenase (LDH) activity measurement. Brain tissue was dissociated in 1 mL HBSS and centrifuged. Extracellular activity of LDH was measured from an aliquot of 5 *u*L from the supernatant. Then, pellet was re-suspended and the cell suspension was lysated with insulin syringe 29G in order to measure total intracellular LDH activity. Both samples were mixed with 250 *u*L of working reagent and incubated at 37 ºC. Decomposition of Nicotinamide Adenine Dinucleotide (NADH) was measured at 340 nm.

2.6 High-resolution respirometry

The Oxygraph 2k (O2k, OROBOROS Instruments, Innbruck, Austria) was used for measuring cellular respiration. Respiration of intact cells from mechanical dissociation with glass Pasteur pipette, collagenase IV and trypsin-EDTA was measured in Oxygraph-2k (O2k, OROBOROS Instruments, Innsbruck, Austria) at 28°C (MAKRECKA-KUKA; KRUMSCHNABEL; GNAIGER, 2015) by applying a coupling control protocol (*n*=4 for each group, pool of two brains/sample). Briefly, after stabilization of respiration (Routine respiration), Oligomycin (ATPase inhibitor, final concentration in the chamber 4ug/mL) was added to obtain coupling efficiency respiration (p/O_2) and Leak (H^+ Leak). Then, FCCP-titrated (a protonophore and uncoupler of oxidative phosphorylation, final concentration in the chamber 0.05 µM) was added to obtain electron transfer system (ETS) capacity and spare respiratory capacity

(SRC). Rotenone (a complex I inhibitor, final concentration in the chamber 0.5 µM) and Antimycin-A (a complex III inhibitor, final concentration in the chamber 2.5 µM) were added after FCCP to determine residual oxygen consumption (ROX). Oxygen concentration and flux were simultaneous recorded and analyzed by Oroboros DatLab software and oxygen flux was expressed on the basis of tissue wet weight. For each measurement, two brains were used (~7-13 mg wet weight), and both were weighed by difference into an Eppendorf containing 100 *u*L of HBSS solution.

2.7 Kainic acid-induce *status epilepticus*

Kainic acid was diluted in phosphate buffered saline (PBS). Adult zebrafish (*n*= 8 for each group, pool of two brains/sample) were anesthetized with tricaine (concentration…) and then injected with kainic acid (KA 6 mg/kg group) or PBS (control group) intraperitoneally. Volume injection was 10 µL/g of fish weight. After injection, fish were immediately transferred to individual 1.5 L tanks containing reverse osmosis water reconstituted with marine synthetic salt (Instant Ocean®). Animals were monitored for 60 min and seizure behavior was quantified as previously described (MUSSULINI et al., 2018). Only animals that reached scores IV-VI in a period of 30 min were included. For quantifying in order to explore the effects of *status epilepticus* on mitochondrial respiration, coupling control protocol was performed with control and KA-treated group as described above.

2.8 Statistical analyses

Data from cellular viability were represented as mean±S.D. Viability of mechanical dissociations were analyzed by Unpaired T-test. Viability of enzymatic dissociations were analyzed by one-way ANOVA followed by Tukey *post-hoc* test. All data of O_2 consumption were presented as mean±S.D. and analyzed by Unpaired T test.

3. Results

3.1 Cellular viability by Trypan-blue dye exclusion

There was no difference between mechanical dissociations (**Fig. 1A**; unpaired t test, p=0.9157), however tissue dissociated with glass flambéed Pasteur pipette showed less data variation than microtube pestle (coefficient of variation: 18.63 and 53.06%, respectively).

We also evaluated if enzymatic tissue digestion before mechanical dissociation could improve cellular viability. Cellular viability was decreased only when tissue was digested during 30 min with collagenase IV at concentration of 1 mg/mL (one-way ANOVA followed by Tukey *post hoc* test, p=0.0021) (**Fig. 1B**). Since there was no difference in the number of viable cells among the other concentrations, we evaluated the dissociative ability of the collagenase IV (0.1 mg/mL) during 10, 30 and 60 min of incubation. Digestion with collagenase IV at 10 min generated a cell suspension with higher viability than the other groups (one-way ANOVA followed by Tukey *post hoc* test, p<0.0001) (**Fig. 1C**).

Tissue digestion during 30 min with trypsin-EDTA at 0.01 and 0.05% generated 51,1 and 47,5% of viable cells, respectively ((**Fig. 1D**). At concentration of 0.25%, trypsin killed all cells (one-way ANOVA followed by Tukey *post hoc* test, p<0.0001). Thus, we evaluated the cellular viability of trypsin-EDTA at the lowest concentration (0.01%) during 10, 30 and 60 min incubation. Only at 60 min of incubation, cellular viability decreased significantly (ANOVA, p<0.0001) (**Fig. 1E**).

3.2 Cellular viability by lactate dehydrogenase activity

We also analyzed viability of the cell suspensions by the lactate dehydrogenase activity assay. There was no difference in cell viability between the two mechanical dissociations (unpaired t test; p=0.9969; **Fig. 2A**). Similar to those observed with trypan blue exclusion, there was also no difference in cellular viability in samples treated with both enzymes: collagenase IV (one-way ANOVA followed by Tukey *post hoc* test; p=0.7530; **Fig. 2B**) and trypsin-EDTA (one-way ANOVA followed by Tukey *post hoc* test; p=0.1403; **Fig. 2C**). Therefore, for the further assays we chose collagenase IV (0.1 mg/mL during 10 min of incubation) and trypsin-EDTA (0.01% during 10 min of incubation).

3.3 Respiratory profile of intact cell from zebrafish brain dissociated by different methods

We found significant differences in respiratory indices between mechanical and enzymes dissociation methods for adult zebrafish brain (**Fig. 3**). When mechanical glass Pasteur, which we choose as optimal mechanical method for tissue dissociation, was compared to dissociation with Collagenase IV and Trypsin-EDTA reduced the Routine respiration (unpaired t test; p=0.012 and p=0.026, respectively; **Fig 3A**), p/O_2 (unpaired t test; p=0.002 and p=0.045, respectively; **Fig 3B),** and ROX (unpaired t test; p=0.001; and p=0.043, respectively; **Fig 3F**) when compared to mechanical dissociation with glass Pasteur pipette. Other mitochondrial respiratory indices did not differ between Collagenase IV or Trypsin-EDTA and glass Pasteur pipette dissociation: H^+ Leak (unpaired t test; p=0.098 and p=0.074, respectively; **Fig 3C**), ETS (unpaired t test; p=0.089 and p=0.339, respectively; **Fig 3D**), and SRC (unpaired t test; p=0.147 and p=0.641, respectively; **Fig 3E**).

The Coefficient of Variation (CV) was calculated for all respiratory variables in each group: mechanical glass dissociation, collagenase IV, and trypsin-EDTA. For all parameter measured, CVs originated from cell suspension dissociated with mechanical glass pasteur and collagenase IV were similar **(Table 1).** In contrast, CVs calculated from trypsin-EDTA group were predominantly higher than mechanical dissociation group **(Table 1)**.

3.4 Respiration profile of zebrafish brain after KA-induced *status epilepticus*

In order to evaluate our characterization of zebrafish brain mitochondrial respiration, we measured mitochondrial respiration from animals submitted to KA-induced *status epilepticus* (SE). We used mechanical glass Pasteur pipette as the dissociation method. Animals submitted to KA-induced SE showed a tenuous increased in the reserve capacity (SRC) when compared to control group (unpaired t test, p=0.026) (**Fig 4E**). We did not find differences in any other analyzed variables, such as Routine respiration (unpaired t test, p=0.441) (**Fig 4A**), P/O_2 (unpaired t test, p=0.413) (**Fig 4B**), H^+ Leak (unpaired t test, p=0.507) (**Fig 4C**), ETS (unpaired t test, p=0.660) (**Fig 4D**), and ROX (unpaired t test, p=0.745) (**Fig 4F**).

4. Discussion

Protocols for high-resolution respirometry provides relevant information about mitochondrial function and dysfunction. Limited mitochondrial respiratory capacity is observed in different tissues and disorders (OST et al., 2018). There are different approaches to assess respiratory capacity of mitochondria, and in this work, we described and characterized a new protocol for intact cells from zebrafish brain. Therefore, our goal was to describe, under physiological conditions, the mitochondrial respiration from adult zebrafish brain by using different mechanical and enzymatic methods for tissue dissociation. After mitochondrial respiration was characterized, we explored mitochondrial respiratory capacity under a pathological condition, such epileptic seizures, to apply and validate the experimental protocol chosen.

Several methods are used to dissociate brain tissue from different organisms into viable single cells, including mechanical dissociation (e.g. meshing, trituration with a pipette, tip), enzymatic digestion, or combination of both (VOLOVITZ et al., 2016). We first evaluate cell viability by trypan-blue dye exclusion test. Different dissociation methods yield distinct percentage of viable single cells **(Figure 1 and 2)**. Although the two mechanical dissociation methods used did not differ statistically **(Fig. 1A)**, tissue dissociated with glass Pasteur pipette produce a cell mixture (cells and their surrounding solution) with less undissociated cell clumps than microtube pestle, and also less variation in the percentage of viable cells (±40% viability). Mechanical dissociation needs to be delicate enough to generate a live single cell suspension to avoid leaving behind too many clumps of tissue (BREWER; TORRICELLI, 2007).

Mechanical dissociation with glass Pasteur pipette and enzymatic dissociation with collagenase IV at low concentration (0.1 and 0.5 mg/mL) did not show statistical differences **(Fig. 1B)**; however, the produced cell mixture with different dissociation quality, in which enzymatic dissociation present less undissociated cell clumps and cellular debris **(Fig. S2)**. We also found the highest percentage of viability in tissue dissociated with trypsin-EDTA at lower concentrations similar (0.01 and 0.05%) **(Fig 1D)**. According to the cell viability we found, we selected the lowest concentrations of collagenase IV (0.1 mg/mL) and trypsin-EDTA (0.01%) to test different incubation times. We observed critical decrease in number of viable cells in tissue exposed to a longer time digestion, especially 1h **(Fig 1C and 1E).**

We also evaluate cellular viability by lactate dehydrogenase activity in order to compare mechanical dissociation groups and also the lowest concentration of each enzyme – collagenase IV and trypsin-EDTA, at different incubation times tested previously. We did not find any significant difference in cellular viability **(Fig. 2A, 2B, 2C).** The differences found in viability measured by trypan-blue methodology did not reproduce with lactate dehydrogenase activity, and these difference could be related to the size of the pore needed to each of them transpose the membrane of the cell **(Fig. 1 and 2)**.

Comparing the two best viability we found with collagenase IV and trypsin-EDTA at the lowest concentration at the shorter time, and considering the fact that the literature describes some negative aspects of using trypsin-EDTA in nervous tissue (MARIC; BARKER, 2004), we chosen two protocols to measure respirometry variables: enzymatic dissociation with collagenase IV at the lowest concentration (0.1 mg/mL) at shorter time (10 min).

In order to describe respiratory profile of zebrafish CNS, after viability assays, we applied the coupling control protocol for intact cells originated from mechanical dissociation and enzymatic dissociation with collagenase IV or trypsin-EDTA. The differences we found in Routine, P/O_2, and ROX **(Fig. 3A, 3B and 3F)** in the current study could be explained by methodological limitations. First, water temperature of 28.5 °C is widely cited as the optimum temperature for zebrafish in which they grow satisfactory, otherwise, maintenance in suboptimal temperatures will have a metabolic cost that may affect development and welfare (REED; JENNINGS, 2011). As the optimal temperature for both enzymes collagenase IV and trypsin-EDTA is 37°C, it is possible that temperature variation could lead to metabolism changes.

Second, it is know that the meaning of results changes with normalization of the oxygen flux presents (BRAND; NICHOLLS, 2011). In our work, we express oxygen flux corrected by tissue wet weight (mg) in which the tissue mass is determined before addition of samples to the Oxygraph chamber. In contrast to mechanical dissociation with glass Pasteur, both protocol in which brain tissue is digested with enzymes (collagenase IV or trypsin-EDTA), we have an additional centrifugation step to remove enzyme medium. This step probably caused a decrease in total tissue weight, which did not occur in mechanical dissociation protocol since tissue was rapidly dissociated and added direct to the Oxygraph chamber. We did not perform a protocol in which mechanical

dissociation go through centrifugation, or heating (37C) as control of the experiment, once our goal was just characterizing respiratory parameters from zebrafish CNS front of different tissue dissociation methodologies. We also tried an alternative normalization method by quantify protein from tissue recovered from the Oxygraph chamber (GNAIGER, 2014). Measurements of protein content was limited for mechanical dissociation method due to the few tissue clumps remained in cell mixture, it was difficult the complete recovery of the tissue from the chamber.

In order to choose the best dissociation method for high resolution respirometry, we must consider all positive aspects and limitations around the protocols. Mechanical dissociation requires high degree of human manual skills in the preparation of cell suspension, results may not represent biological differences, and reflect operator variability (LARSEN et al., 2014)(ZIAK et al., 2015). Therefore, to obtain reliability and reproducibility of results, operator should be careful and well trained to avoid bias from dissociation technique itself. Although coefficients of variability from tissue dissociated with collagenase IV were satisfactory, the respiratory indices were lower than mechanical dissociation, probably because of the limitations of the methodology discussed before. Therefore, in order to evaluate mitochondrial dysfunctions by these methodology, we probably need more tissue than we used in this work (more than two zebrafish brains). Also, tissue dissociated with trypsin demonstrated the highest coefficients of variability **(Table 1)**. Therefore, we strongly recommend not using this protocol for bioenergetics measures.

We opted for mechanical dissociation to evaluate the effect of prolonged seizure induced by kainic acid in respiratory parameters. We applied the same coupling control protocol for intact cells and we found increased spare respiratory capacity in animals which were injected with KA **(Fig. 4E)**. Spare respiratory capacity is related to the bioenergetics of the cells and the ability of respond to an increase in energy demand, especially neurons, which can experience a variable ATP demand (YADAVA; NICHOLLS, 2007). Increased spare respiratory capacity in fish after prolonged seizure could be explained by the fact that prolonged epileptic seizure requires energy. The other parameters analyzed did not show differences **(Fig. 4A, B, C, D and F)**, as these results are in accordance with previous OXPHOS analysis in rat brain with SE induced by other classical chemical models, such as lithium/pilocarpine (IMRAN; HILLERT; KLEIN, 2015).

5. Conclusion

In the present study, we characterized different methods for dissociate zebrafish brain tissue into single viable cells for using in the high-resolution respirometry. Moreover, we used the best tissue dissociation method to describe the respiratory states of zebrafish brain by using coupling control protocol. Altogether, we concluded that both mechanical dissociation with glass Pasteur pipette and collagenase IV are both useful tools to study mitochondrial function and dysfunction in zebrafish brain tissue.

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Table 1. Coefficient of variation (CV) from each variable measured at high resolution respirometry.

	Mechanical Glass Pasteur pipette (n=4)	Collagenase IV (0.1 mg/mL, 10 min) (n=4)	Mechanical Glass Pasteur pipette (n=4)	Trypsin-EDTA (0.01%, 10 min) (n=4)
Routine	25.27%	23.00%	21.09%	46.70%
P/O_2	23.83%	25.93%	40.82%	31.83%
H^+ Leak	30.01%	24.12%	27.64%	16.39%
ETS	20.91%	20.53%	12.74%	44.33%
SRC	7.56%	37.13%	13.31%	44.33%
ROX	31.75%	48.53%	42.39%	88.03%

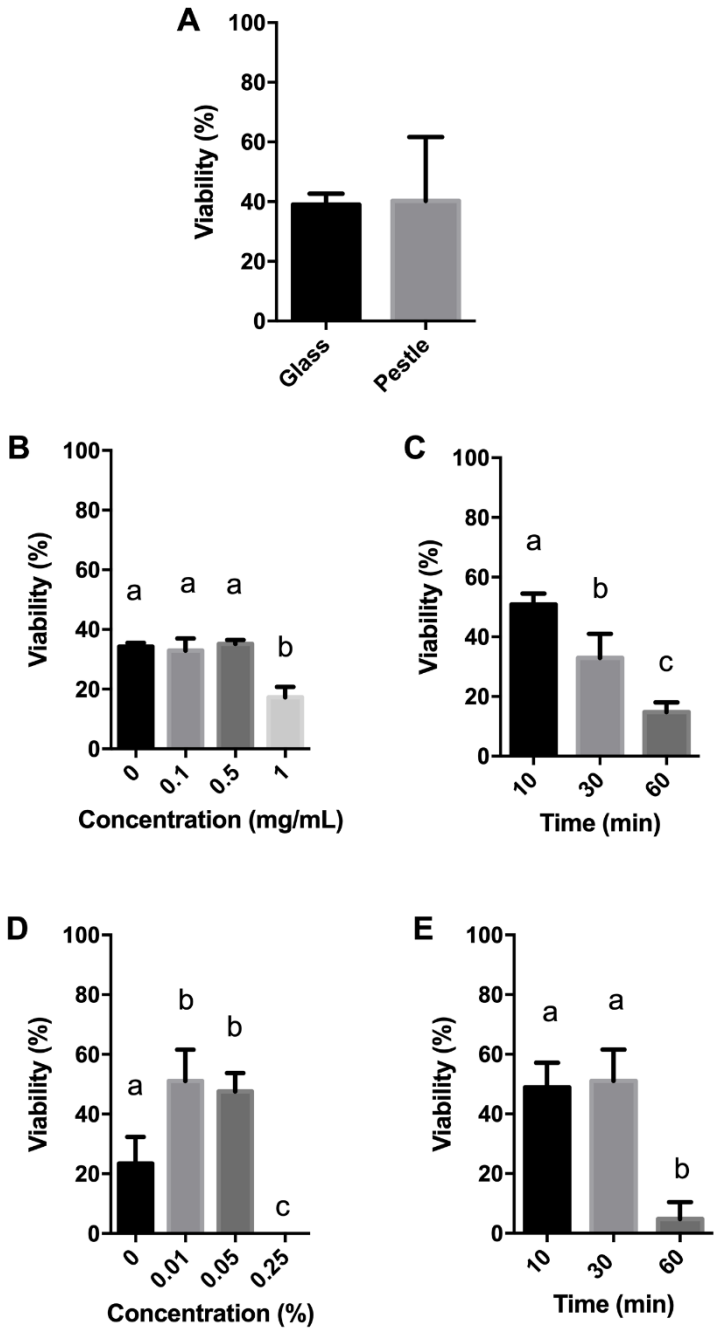

Figure 1. Cellular viability of zebrafish brain tissue dissociated with different methods using trypan-blue dye exclusion. (A) brain mechanically dissociated with glass pasteur and microtube pestle. **(B)** Optimal enzyme concentration and **(C)** optimal dissociation time of brain tissue using collagenase IV. **(D)** Optimal enzyme concentration and **(E)** optimal dissociation time of brain tissue using Trypsin-EDTA. All data were presented by mean ± S.D. and were analyzed by unpaired t test for (A) and one-way ANOVA followed by Tukey *post hoc* test for (B), (C), (D) and (E). $p \leq 0.05$ was considered significantly.

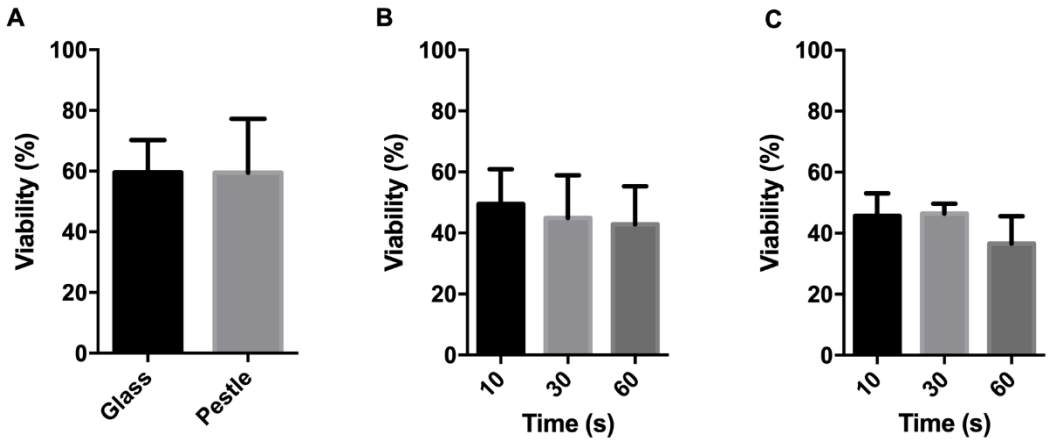

Figure 2. Cellular viability of zebrafish brain tissue dissociated with different methods using lactate dehydrogenase activity. (A) brain mechanically dissociated with glass pasteur and microtube pestle. **(B)** Optimal enzyme time with collagenase IV at the respective optimal enzyme's dissociation concentration of 0.1 mg/mL and **(C)** trypsin-EDTA at the respective optimal enzyme's dissociation concentration of 0.01%. All data were presented by mean ± S.D. and were analyzed by unpaired t test for (A) and one-way ANOVA followed by Tukey *post hoc* test for (B) and (C). $p \leq 0.05$ was considered significantly.

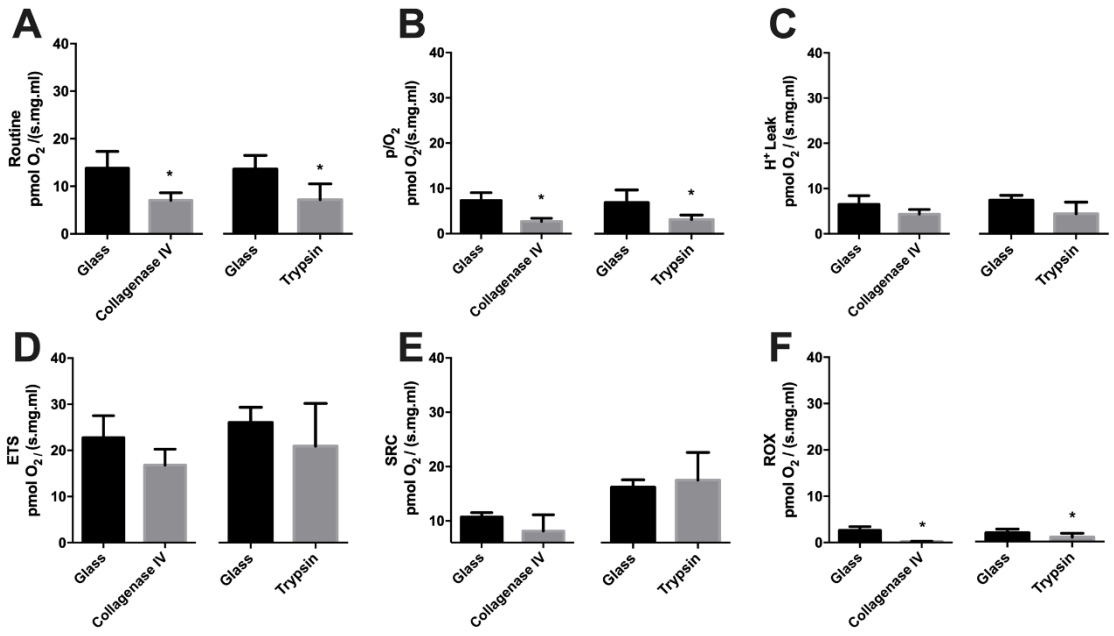

Figure 3. Respiration of intact cells from zebrafish brain dissociated mechanically (black bars) or with enzymes (grey bars). (A) Routine respiration, **(B)** ATP-linked respiration, **(C)** proton leak, **(D)** maximal respiration, **(E)** spare respiratory capacity, and **(F)** non-mitochondrial respiration. Data were presented by mean ± S.D. and were analyzed by unpaired t test. $p \leq 0.05$ was considered significantly.

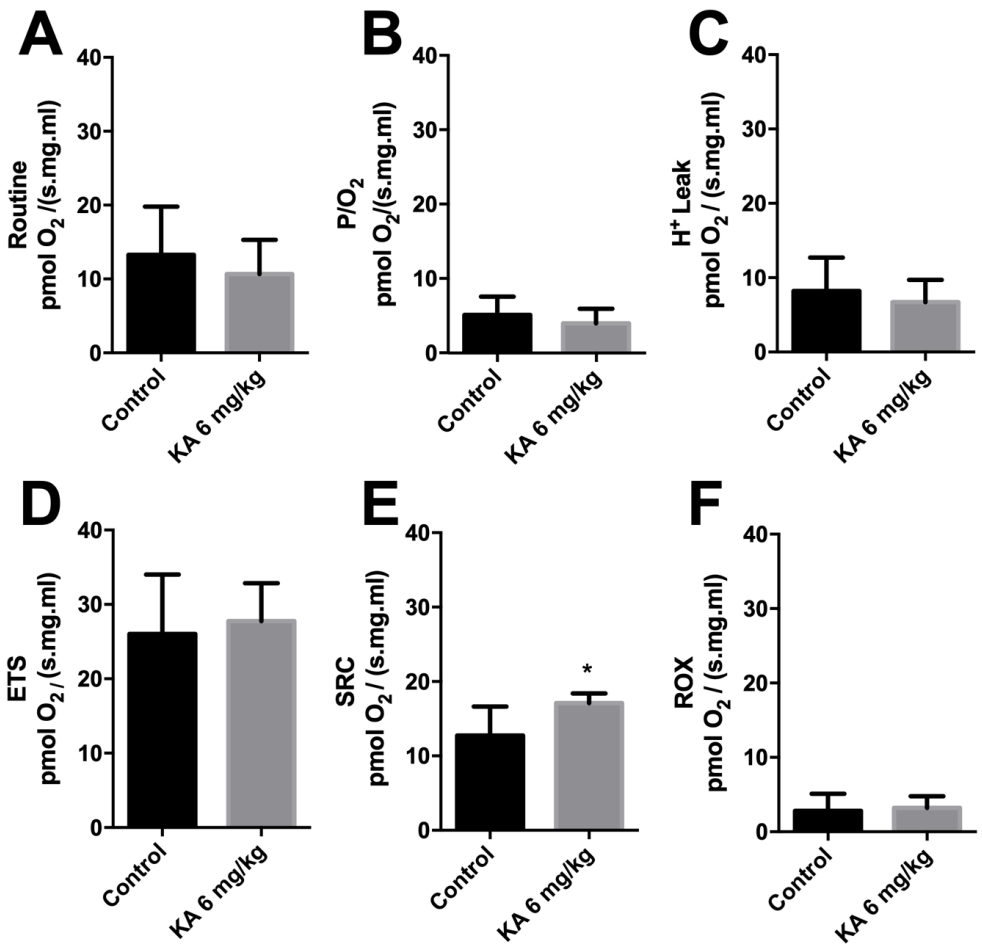

Figura 4. Effects of kainic acid-induced *status epilepticus* on mitochondrial respiration (control group: black bars; kainic acid group; grey bars). (A) Routine respiration, **(B)** ATP-linked respiration, **(C)** proton leak, **(D)** maximal respiration, **(E)** spare respiratory capacity, and **(F)** non-mitochondrial respiration. Data were presented by mean ± S.D. and were analyzed by unpaired t test. $p \leq 0.05$ was considered significantly.

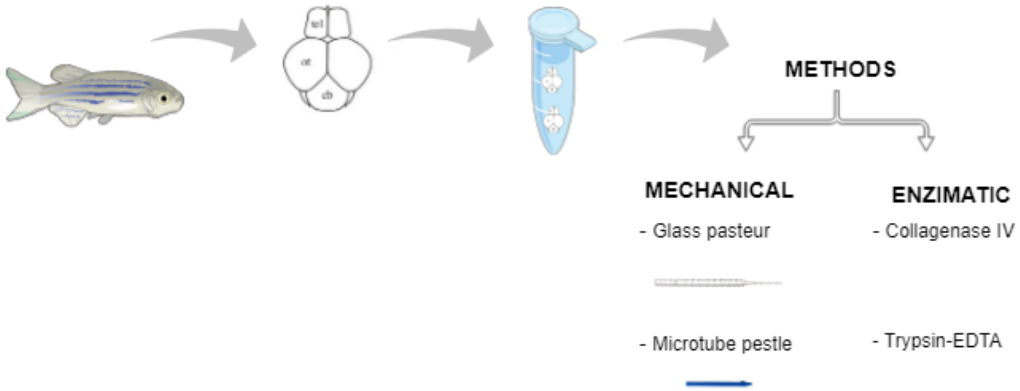

Figure S1. Methods used to dissociate zebrafish brain tissue. Two zebrafish brains were dissected, transferred to a microtube with 1 mL volume (HBSS only or HBSS with enzymes Collagenase IV or trypsin-EDTA, according to the protocol). They were exposed to four different protocols: mechanical dissociation with glass Pasteur pipette, mechanical dissociation with microtube pestle, enzymatic dissociation with collagenase IV or Trypsin-EDTA.

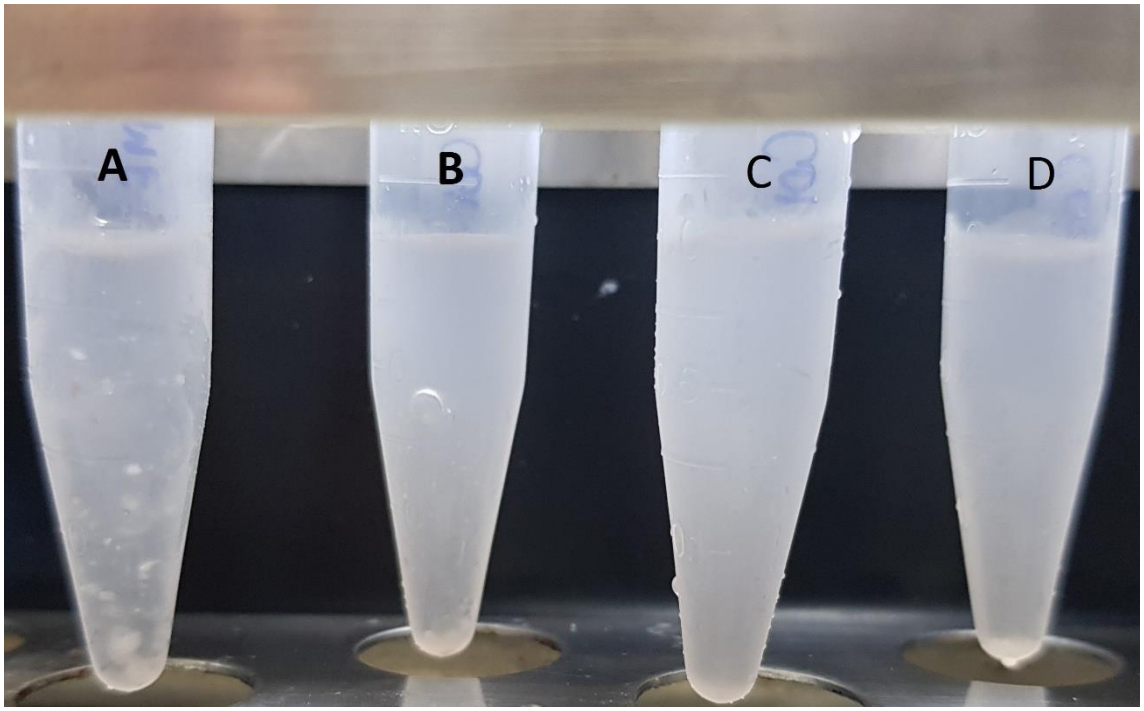

Figure S2. Quality of cell mixture after brain homogenization. Undissociated cell clumps after 30 minutes of exposure to **(A)** nonenzymatic buffer, only mechanical dissociation with glass pasteur pipette **(B)** collagenase IV 0.1 mg/mL **(C)** collagenase IV 0.5 mg/mL **(D)** collagenase IV 1 mg/mL.

PARTE III

DISCUSSÃO

Diversas vantagens já foram descritas para a utilização do peixe-zebra como um modelo bastante atrativo no campo das ciências biomédicas. A sua utilização vem aumentando de maneira significativa, contribuindo para a consolidação deste teleósteo como uma alternativa para estudos envolvendo desordens do SNC. No entanto, uma melhor compreensão da sua fisiologia e bioquímica se faz necessária. Nesse trabalho demonstramos pela primeira vez na literatura, o perfil respiratório do sistema nervoso central do peixe-zebra adulto.

Os protocolos para respirometria de alta resolução fornecem informações relevantes sobre a função e a disfunção mitocondriais. Existem diferentes abordagens para avaliar a capacidade respiratória em mitocôndrias, e neste trabalho, realizamos essa caracterização da capacidade respiratória utilizando o protocolo de células intactas. Portanto, nosso objetivo foi descrever, sob condições fisiológicas, a respiração mitocondrial do cérebro de peixe-zebra adulto resultante de diferentes métodos mecânicos e enzimáticos para dissociar o tecido. Após a caracterização da respiração mitocondrial, exploramos a capacidade respiratória mitocondrial em condições patológicas, como na indução de crises epilépticas induzida pelo agente químico ácido caínico.

Inicialmente, avaliamos a viabilidade das células do SNC em diferentes protocolos de dissociação de tecido, tais como: dissociação mecânica com pipeta Pasteur de vidro; dissociação mecânica com pistilo para microtubos; digestão enzimática com colagenase IV; e digestão enzimática com tripsina. Foram testadas diferentes concentrações de enzima em diferentes tempos de incubação, nos quais foram medidos a viabilidade celular através das técnicas de incoporação de azul de tripan e atividade extracelular da enzima lactato desidrogenase.

Diversos métodos já são utilizados para dissociação tecidual cerebral de diferentes organismos, e esses incluem dissociação mecânica, digestão enzimática ou combinação de ambos (Volovitz et al., 2016). Ao avaliarmos a viabilidade das diferentes metodologias testadas, os resultados foram muito similares para ambos os métodos de dissociação mecânica. No entanto, o tecido dissociado com pipeta Pasteur de vidro apresentou-se mais homogêneo quando comparado ao pistilo para microtubos, bem como uma menor variabilidade nos dados obtidos (coeficiente de variação de 18,63% para

dissociação com Pasteur de vidro contra 53,06 para pitilo para microtubos). A dissociação mecânica deve ser realizada de maneira suave, evitando a formação de agregados de tecido não-dissociado, de forma a resultar em uma suspensão de célula viáveis (Brewer e Torricelli, 2007). Quanto às dissociações enzimáticas, de acordo com a viabilidade celular observada, selecionamos as menores concentrações de colagenase IV (0,1 mg/mL) e tripsina-EDTA (0,01%), ambas no menor tempo de incubação: 10 min.

Sendo assim, descrevemos o perfil respiratório do tecido cerebral do peixe-zebra adulto resultante dos protocolos que apresentaram maior viabilidade celular: dissociação mecânica com Pasteur de vidro, colagenase IV (0,1 mg/mL) e tripsina-EDTA (0,01%) durante 10 min. As diferenças encontradas na respiração de Rotina, P/O2 e ROX no presente estudo podem ser explicadas por algumas variações metodológicas.

Primeiramente, a temperatura relatada na literatura como ideal para manutenção do peixe-zebra é de 28,5° C (Reed e Jennings, 2011). Como a temperatura ideal para incubação de ambas as enzimas, colagenase IV e tripsina-EDTA, é de 37 ° C, é possível que esta variação de temperatura possa provocar alterações no metabolismo celular e na estabilidade das membranas celulares, levando a uma redução nos índices respirométricos. Em segundo lugar, em nosso trabalho os dados de consumo de oxigênio foram normalizados pelo peso úmido do tecido cerebral amostrado, conforme protocolos da literatura (Brand e Nicholls, 2011). Assim, visto que na digestão enzimática temos um passo adicional no protocolo, qual seja, uma centrifugação para sedimentação das células em suspensão, é possível que tenha ocorrido uma diminuição da função mitocondrial durante este processo (uma vez que não há diferenças na viabilidade celular medida por azul de tripan e atividade da lactato desidrogenase). Esta diminuição não ocorreria no protocolo de dissociação mecânica visto que o tecido é rapidamente dissociado e adicionado à câmara do oxígrafo.

Por último, analisamos o perfil respiratório do peixe-zebra frente a crises epilépticas prologadas induzidas pelo ácido caínico através da técnica de respirometria de alta resolução. Encontramos diferença significativa na capacidade de reserva, os demais parâmetros permaneceram inalterados, como descrito previamente em outros modelos de crises induzidas por agentes químicos proconvulsivos.

CONCLUSÃO

Nesse estudo, descrevemos diferentes métodos para dissociar o tecido cerebral de peixe-zebra tendo como objetivo obter células viáveis para aplicar na técnica de respirometria de alta resolução. Dentre os métodos de dissociação avaliados, a dissociação mecânica com a pipeta Pasteur de vidro foi o método que apresentou resultados satisfatórios, tanto de viabilidade celular quanto na caracterização dos parâmetros de respirometria. Sendo assim, considerando as limitações da técnica citadas previamente, o protocolo de dissociação tecidual descrito no presente trabalho poderá ser utilizado para avaliar a função e disfunção mitocondrial do cérebro de peixe-zebra adulto.

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