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ESTUDO DA DISFUNÇÃO MITOCONDRIAL EM MODELOS DE DOENÇA DE PARKINSON *IN VITRO* E *IN VIVO* E PERSPECTIVAS TERAPÊUTICAS

Santa Maria, RS, Brasil
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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutora em Ciências Biológicas: Bioquímica Toxicológica.**

Orientadora: Professora Dra. Cristiane Lenz Dalla Corte

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Marie Curie

APRESENTAÇÃO

No item INTRODUÇÃO consta uma revisão sucinta da literatura sobre os temas trabalhados nesta tese.

O DESENVOLVIMENTO da tese está apresentado sob a forma de dois artigos, os quais se encontram alocados no item ARTIGOS CIENTÍFICOS.

A metodologia realizada, os resultados obtidos, discussão, conclusão e referências bibliográficas que fazem parte desta tese encontram-se nos próprios artigos e representam a íntegra deste estudo.

O item DISCUSSÃO apresenta interpretações e comentários gerais sobre os trabalhos científicos aqui incluídos.

Os itens CONCLUSÕES e PERSPECTIVAS, encontrados no final desta tese, apresentam interpretações e comentários gerais sobre a investigação desenvolvida.

As REFERÊNCIAS BIBLIOGRÁFICAS referem-se somente às citações que aparecem no item INTRODUÇÃO e DISCUSSÃO, uma vez que os artigos científicos contêm as suas próprias referências.

RESUMO

ESTUDO DA DISFUNÇÃO MITOCONDRIAL EM MODELOS DE DOENÇA DE PARKINSON *IN VITRO* E *IN VIVO* E PERSPECTIVAS TERAPÊUTICAS

AUTORA: Débora Farina Gonçalves
ORIENTADORA: Professora Dra. Cristiane Lenz Dalla Corte

A doença de Parkinson (DP) é uma doença neurodegenerativa cujos sintomas são resultado da perda neuronal progressiva, em especial de neurônios dopaminérgicos. Outra característica da DP é a disfunção mitocondrial que pode levar a um desbalanço na manutenção da energia celular. Modelos experimentais *in vitro* e *in vivo* têm sido utilizados para o estudo da DP. Nesse trabalho investigamos a bioenergética mitocondrial, e possíveis intervenções com foco na funcionalidade mitocondrial em modelos de DP *in vitro* e *in vivo*. No estudo *in vitro* utilizamos fatias de córtex, hipocampo e estriado expostas a 6-hidroxidopamina (6-OHDA), por 1 hora na concentração de 100 µM. Em fatias de córtex a 6-OHDA aumentou a resposta mitocondrial o que é demonstrado pelo aumento na fosforilação oxidativa (OXPHOS) relacionada ao complexo mitocondrial I (CI) e complexo mitocondrial II (CII), também houve aumento na atividade da lactato desidrogenase (LDH), sugerindo uma resposta adaptativa. Fatias de estriado expostas a 6-OHDA apresentaram um prejuízo mitocondrial, demonstrado pela diminuição de OXPHOS e diminuição do fluxo de elétrons relacionado à ATP sintase. Houve um aumento na produção de peróxido em fatias de hipocampo expostas à 6-OHDA, porém sem resposta adaptativa. Fatias de hipocampo, apresentaram um aumento na produção de peróxido, mas sem resposta adaptativa. A exposição simultânea das fatias a 6-OHDA e n-acetilcisteína (NAC) foi capaz de conter os efeitos da 6-OHDA sobre o fluxo de elétron em todas as regiões cerebrais testadas. No estudo *in vivo*, utilizamos moscas *Drosophila melanogaster* com deleção do gene PTEN-induced putative kinase 1 (*pink1*) como modelo de DP avaliando parâmetros relacionados a funcionalidade mitocondrial e manutenção da energia celular durante o envelhecimento nos estágios de 3, 15 e 30 dias de vida. Em nosso estudo, a perda do gene *pink1* diminuiu a porcentagem de sobrevivência das moscas e comprometeu o comportamento de escalada durante o envelhecimento. Além disso, moscas *pink1* apresentaram prejuízo na função mitocondrial o que foi demonstrado pela diminuição no fluxo de oxigênio relacionado a OXPHOS CI&CII e na transferência de elétrons ETS CI&CII em 15 e 30 dias de vida. Curiosamente, moscas *pink1* aos 15 dias de vida apresentaram atividade da citrato sintase (CS) dimunuída acompanhada de um aumento na atividade da lactato desidrogenase (LDH) e diminuição da OXPHOS CII e ETS CII. Contrariamente, houve um aumento na atividade da CS e uma diminuição da atividade da LDH em moscas *pink1* aos 30 dias de vida. Além disso, houve aumento dos níveis de peróxido em moscas *pink1* aos 15 e 30 dias de vida. Esses resultados demonstram um perfil bioenergético distinto em moscas *pink1* durante o envelhecimento sugerem uma possível troca de metabolismo energético de oxidativo para glicolítico aos 15 dias de vida. Considerando esses resultados, utilizamos um tratamento com cafeína na concentração de 0,5 mg/mL com o intuito de investigar se essa xantina com característica antioxidante teria potencial para reverter ou melhorar parâmetros relacionados à funcionalidade mitocondrial em moscas *pink1*. Os resultados obtidos demonstraram que o tratamento com cafeína em moscas *pink1* desde o estágio larval até o terceiro dia de vida adulta foi capaz de aumentar os níveis de OXPHOS CI&CII e ETS CI&CII, além de aumentar as

eficiências de acoplamento relacionadas a OXPHOS e ETS e melhorar a organização da rede mitocondrial. Sendo assim em linhas gerais, esse trabalho demonstra as alterações de funcionalidade/ adaptação mitocondrial em dois modelos distintos de DP, além de confirmar a função mitocondrial como possível alvo de ação para duas reconhecidas substâncias antioxidantes, NAC e cafeína.

Palavras-chave: 6-hidroxidopamina, cadeia transportadora de elétrons, disfunção mitocondrial, *Drosophila melanogaster, pink1*, envelhecimento, bioenergética, cafeína.

ABSTRACT

AUTHOR: Débora Farina Gonçalves
ADVISOR: Cristiane Lenz Dalla Corte

Parkinson disease (PD) is a neurodegenerative disease which symptoms are consequence of progressive neuron lost, especially dopaminergic neurons. Other feature related to PD is mitochondrial dysfunction which could target a deficit on the cellular energy maintenance. Experimental models *in vitro* and *in vivo* have been used to study PD. The aim of this work is to investigate mitochondrial bioenergetics, as well as, possible interferences with focus on mitochondrial functionality using DP models *in vitro* and *in vivo*. In the *in vitro* study we used slices of cortex, hippocampus and striatum exposed to 6-hydroxydopamine (6-OHDA), for 1 hour at concentration of 100 μM. In cortex brain slices 6-OHDA increased mitochondrial activity which is demonstrated by an increase of oxidative phosphorylation (OXPHOS) related to mitochondrial complex I (CI) and mitochondrial complex II (CII), also there was an increase on lactate dehydrogenase activity (LDH), implying an adaptative response. Striatum brain slices exposed to 6-OHDA presented a mitochondrial impairment demonstrated by decreased OXPHOS and decrease of electron flux related to ATP synthase. There was an increase in peroxide production in slices hippocampus slices exposed to 6-OHDA, but without adaptive response. The simultaneous exposure from brain slices to 6-OHDA and n-acetylcysteine (NAC) was able to counteract 6-OHDA effects on on electron flux in all brain structures analyzed. In the *in vivo* study we used *Drosophila melanogaster* with deletion of PTEN-induced putative kinase 1 (*pink 1*) gene as DP model, evaluating mitochondrial parameters and cellular energy maintenance during aging on the life stages of 3, 15 and 30 days of life. In our study *pink1* gene lost decreased survival percentual and impaired the climbing behavioral during aging. Moreover, *pink1* flies presented impairment of mitochondrial function evidenced on OXPHOS related to CI&CII and electron transfer (ETS) related to CI&CII at 15 and 30 days of life. Curiously, *pink1* flies at 15 days of life presented a decrease of citrate synthase (CS) activity following by an increase of LDH activity and decrease of OXPHOS and ETS related to CII. On the other hand, there was an increase of CS activity and a decrease of LDH activity of *pink1* flies at 30 days of life. Furthermore, there was an increase of peroxide levels in *pink1* flies at 15 and 30 days of life. These results suggest a distinct bioenergetic profile of *pink1* flies during aging, presenting as main result a possible change on energetic metabolism from oxidative to glycolytic pathway at 15 days. Due these results, we used a treatment with caffeine at concentration of 0.5 mg / mL in order to investigate if this xanthine, with antioxidant features, would have potential to reverse or improve parameters related to mitochondrial functionality in pink1 flies. Our results demonstrated that treatment with caffeine in pink 1 flies from the larval stage until 3rd day of adult life was able to increase the levels of OXPHOS CI & CII and ETS CI & CII, moreover increased the coupling efficiencies related to OXPHOS and ETS and improve the mitochondrial network organization. Therefore, this work demonstrates the mitochondrial functionality/ adaptation changes due two different models of PD, in addition, the results confirm mitochondrial function as a possible target for two recognized antioxidant substances, NAC and caffeine.

Keywords: 6-hydroxydopamine, electron transport chain, mitochondrial dysfunction, *Drosophila melanogaster*, *pink1*, aging, bioenergetics.

LISTA DE ABREVIATURAS E SIGLAS

6-OHDA- Cloridrato de 2,4,5-tri-hidroxifenetilamina ou 6-hidroxidopamina
AmR- Amplex® Red
ATP- Adenosina trifosfato
CI- Complexo I
CII- Complexo II
CS- Citrato sintase
DP- Doença de Parkinson
D- Estado fosforilado OXPHOS
E- Estado desacoplado para avaliação de ETS
ERO's- Espécies reativas de oxigênio
ETS- Sistema de transferência de elétrons
ETS CI-Linked- Sistema de transferência de elétrons dependente de CI
ETS CII-Linked- Sistema de transferência de elétrons dependente de CII
ETS CI&CII-Linked- Sistema de transferência de elétrons dependente de CI&CII
FCCP- Carbonil cianeto-p-trifluorometoxifenilhidrazona
GPx- Glutationa peroxidase
GSH- glutationa reduzida
GSSG- Glutationa oxidada
HIF1α- Fator de hipóxia
H_2O_2- Peróxido de hidrogênio
HRP- Horseradish peroxidase
JNK- c-Jun N-terminal cinase
LDH- Lactato desidrogenase
LEAK- Fluxo de elétrons independente da ATP-sintase com a utilização de inibidor específico
MAPK- proteína cinase ativada por mitogênio
MPTP- 1-metil l-4-fenil-1,2,3,6-tetraidropiridina
MPP+- 1-metil-4-fenilpiridina
O_2^-- Íon superóxido
NAC- n-acetilcisteína
OXPHOS- Fosforilação oxidativa
OXPHOS CI-Linked- Fosforilação oxidativa dependente de CI
OXPHOS CII-Linked- Fosforilação oxidativa dependente de CII
OXPHOS CI&CII-Linked- Fosforilação oxidativa dependente de CI&CII
P- Estado fosforilado com concentração saturante de ADP
PINK 1- PTEN-Induced quinase 1
Proton LEAK- Fluxo de elétrons independente da ATP-sintase sem utilização de inibidor específico
RCR- Razão de controle respiratório
ROX- Oxigênio residual
SOD- Superóxido dismutase
UAS- Upstrem activating sequence

Sumário

1. INTRODUÇÃO

A doença de Parkinson (DP) é a segunda desordem neurodegenerativa mais comum em nível mundial (SIMUNI et al., 2018). A DP é caracterizada pela perda de neurônios, especialmente neurônios dopaminérgicos e também pela formação de agregados de proteínas, destacando-se a α-sinucleína. Os agregados proteicos de α-sinucleína formam os chamados corpos de Lewy, que são estruturas fibrilares associadas a danos neuronais e à demência (REQUEJO-AGUILAR et al., 2014).

Entre os principais sintomas da DP estão as disfunções motoras como tremor e bradicinesia além de prejuízos cognitivos e de memória (GOETZ, 2011; RIEDERER et al., 2018). Os sintomas relacionados a DP já são bem descritos na literatura científica, mas as suas causas, assim como alguns mecanismos da doença, permanecem desconhecidos.

Um dos mecanismos bem descritos na DP é a disfunção mitocondrial, caracterizada, em geral, por prejuízos no sistema de transferência de elétrons (ETS) da cadeia respiratória, em especial afetando o complexo I (CI) mitocondrial (SCHAPIRA et al., 1990), o que leva a uma diminuição da fosforilação oxidativa (OXPHOS) (AMMAL KAIDERY; THOMAS, 2018; TEVES et al., 2018). O controle da qualidade e das funções normais da mitocôndria é base para a manutenção do balanço energético celular (LISOWSKI et al., 2018). Atualmente, já existem trabalhos demonstrando que além do prejuízo das funções mitocondriais a DP pode interferir em outras rotas de geração de energia celular (REQUEJO-AGUILAR et al., 2014). Observações em modelos genéticos de DP indicam uma troca de rota energética de aeróbica para glicolítica, cujo objetivo seria manter o balanço energético prejudicado pela disfunção mitocondrial (DEVINE; PLUN-FAVREAU; WOOD, 2011; REQUEJO-AGUILAR; BOLAÑOS, 2016).

A perda de neurônios dopaminérgicos em especial da via nigorestriatal também é comumente associada a DP (MASSARI et al., 2016; RIEDERER et al., 2018). Além disso, estudos já concluíram que as diferentes regiões do cérebro podem apresentar respostas diferentes em relação ao metabolismo celular em casos de doenças neurodegenerativas como a DP, assim como em casos de exposições químicas e envelhecimento. Esse fato pode ser explicado devido às diferentes funções desempenhadas pelas diferentes áreas cerebrais (PANDYA et al., 2016).

Modelos *in vitro* têm sido amplamente utilizados para o estudo dos mecanismos envolvidos na DP (FEITOSA, 2018; MORRONI et al., 2018). Análogos da dopamina, como por exemplo o cloridrato de 2,4,5-tri-hidroxifenetilamina ou 6-hidroxidopamina (6-OHDA)

(Fig.1) mimetizam os sintomas da doença. A 6-OHDA já demonstrou causar disfunção mitocondrial em estudo *in vitro* usando fatias de diferentes áreas do cérebro (MASSARI et al., 2016), entretanto existem poucos estudos com foco no efeito da 6-OHDA na bioenergética mitocondrial de diferentes áreas cerebrais. Estudos *in vitro,* capazes de descrever individualmente o metabolismo de diferentes áreas do cérebro são importantes, para a avaliação de tratamentos e medicamentos que possam ter como possível alvo o metabolismo energético.

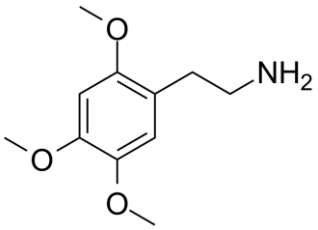

Figura 1: estrutrura molecular da 2,4,5-tri-hidroxifenetilamina

O processo de disfunção mitocondrial na DP também leva ao aumento na geração de ERO's, o que pode intensificar o dano celular e ativar vias relacionadas a apoptose (NAVARRO-YEPES et al., 2014; TRACHOOTHAM et al., 2008). Nesse sentido, a modulação da resposta mitocondrial com o intuito de diminuir a geração de ERO´s pode reduzir os danos decorrentes da disfunção mitocondrial.

Agentes naturais e sintéticos utilizados como antioxidantes tem demonstrado efeito protetor em modelos experimentais de doenças neurodegenerativas como a DP, devido ao seu efeito de diminuir a geração de ERO´s por diferentes mecanismos (ROMUK et al., 2017). A N-acetilcisteína (NAC) (Fig.2) e a cafeína (Fig.3) são substâncias com caráter antioxidante bastante utilizadas. A eficiência dessas substâncias já é demonstrada principalmente na redução de ERO's, no caso da NAC (MARTÍNEZ-BANACLOCHA, 2016) e também em mecanismos relacionados ao sistema adenosinérgico, no caso da cafeína que tem sua ação relacionada tanto à atividade antioxidante quanto a inibição de receptores de adenosina, em especial o receptor A2A (FREDHOLM; YANG; WANG, 2016).

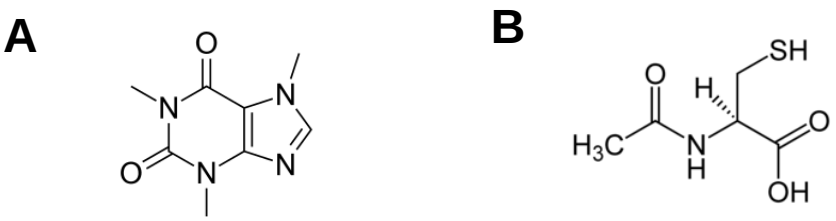

Figura 2: estrutruras moleculares da cafeína (A) e da n-acetilcisteína (B)

Tanto a cafeína quanto a NAC são utilizadas como possíveis tratamentos secundários para atenuar distúrbios relacionados a doenças neurodegenerativas, demonstrando potencial como neuroprotetores (BAI et al., 2018; RB et al., 2017).

A evolução dos estudos sobre DP também deu acesso à identificação de fatores genéticos envolvidos nessa patologia. Entre os genes relacionados com a DP podemos citar SNCA, *pink1*, LRRK2, ATP13A2, MAPT, VPS35, e DJ-1 (ARYAL; LEE, 2018). Mais especificamente o gene *pink1*, além de sua relação com a DP , também está associado ao estresse oxidativo e ao controle de qualidade da rede mitocondrial celular (GAUTIER; KITADA; SHEN, 2008; REQUEJO-AGUILAR; BOLAÑOS, 2016). Modelos experimentais *in vivo* têm ajudado a elucidar possíveis mecanismos genéticos associados a DP, entre os modelos que tem obtido sucesso nas pesquisas encontra-se a *Drosophila melanogaster*.

A *D. melanogaster*, popularmente conhecida como mosca da fruta é vista como um bom modelo para o estudo de doenças neurodegenerativas, cerca de 75% de todos os genes para doenças humanas, possuem homólogos em *D. melanogaster* (BILEN; BONINI, 2005). Além disso, a utilização da mosca da fruta oferece muitas vantagens para estudos a nível celular e molecular de patologias humanas. Igualmente a outros modelos alternativos como o nematódeo *C. elegans* e leveduras, a *D. melanogaster* apresenta um curto ciclo de vida, uma grande progênie, permite técnicas de manipulação gênica e possui anatomia e fenótipos já bem descritos pela literatura científica (MATTHEWS; KAUFMAN; GELBART, 2005).

Atualmente, a *D. melanogaster* já é utilizada como modelo para diversas doenças neurodegenerativas, como doença de Alzheimer, Huntington e DP (BILEN; BONINI, 2005). Em geral, os modelos genéticos são construídos pela expressão de proteínas relacionadas a essas doenças. Por exemplo, na doença de Huntington é comum o aparecimento de sequências proteicas poliglutamínicas, formando aglomerados de proteínas mal dobradas que se acumulam, sendo esse um dos principais marcadores moleculares da doença (KRZYSZTOŃ-RUSSJAN, 2016). É possível direcionar a formação da poliglutamina em tecidos específicos de moscas utilizando um sistema chamado GAL4/UAS o qual ativa o gene de interesse para a expressão da proteína via “upstream activating sequence” (UAS) com uma transcrição dependente de GAL-4 (BRAND; PERRIMON, 1993a).

Também é possível utilizar modelos com mutações genéticas as quais geram consequências similares à doença que se deseja estudar. Um exemplo é o modelo de DP em *D. melanogaster* caracterizado por mutações ou deleção do gene PTEN-induced putative kinase 1 (*pink 1*). Mutações nesse gene estão associadas à disfunção, fusão e fissão da rede mitocondrial, consequências também associadas com a DP (REQUEJO-AGUILAR; BOLAÑOS, 2016).

Os prejuízos na função mitocondrial causados por mutações no gene *pink1* comprometem a autofagia e a fosforilação oxidativa, aumentando os níveis de ERO´s .Como consequência disso é ativado o fator de hipóxia 1α (HIF1α) o qual leva a uma troca de rota metabólica de aeróbica para glicolítica, buscando gerar energia e manter a proliferação celular (REQUEJO-AGUILAR et al., 2014). Esse efeito de troca de rota metabólica pode ser chamado de efeito Warburg e foi primeiramente descrito em células cancerígenas (MURRAY et al., 2015). Essa troca de rota também acontece na DP como consequência da disfunção mitocondrial (DEVINE; PLUN-FAVREAU; WOOD, 2011).

Processos ligados à mitocôndria, como disfunção mitocondrial e prejuízo em vias de sinalização para a fissão e fusão da rede mitocondrial são relacionados com a DP. Em geral, a ativação ou não ativação das vias de fusão e fissão da rede mitocondrial são dependentes da regulação do potencial de membrana e dos níveis de ERO's, sendo assim o controle e/ou modulação da resposta mitocondrial pode auxiliar na descoberta de possíveis tratamentos para a DP (REQUEJO-AGUILAR; BOLAÑOS, 2016). Já foi demonstrado que a utilização de produtos naturais contendo moléculas antioxidantes pode melhorar aspectos relacionados a doenças neurodegenerativas como a DP (ABOLAJI et al., 2018).

O envelhecimento é fator primário para o desenvolvimento de doenças neurodegenerativas como a DP. Um em cada dez indivíduos acima de 65 anos apresenta algum transtorno neurodegenerativo e a prevalência de casos continua aumentando com o passar da idade (HOU et al., 2019). Estudos moleculares evidenciam que o tecido cerebral de pessoas idosas apresenta depósitos anormais de proteínas como a proteína amilóide-β e a α-sinucleína, as quais são marcadores clássicos da doença de Alzheimer e Parkinson, respectivamente.

As características moleculares e bioquímicas do processo de envelhecimento também incluem danos ao DNA e disfunção mitocondrial e portanto estudos que investigam a comunicação entre o núcleo celular e as mitocôndrias, são importantes chaves para melhor entender o metabolismo celular durante o processo de senescência (OU; BJ¨; SCHUMACHER, 2018). A maioria das marcas biológicas do envelhecimento mostra similaridade com as características associadas às doenças neurodegenerativas, portanto estudos usando modelos *in vitro* e *in vivo* de DP que buscam uma compreensão mais ampla acerca de como esses mecanismos se associam permitem um maior entendimento e podem trazer novas esperanças para o tratamento de doenças neurodegenerativas como a DP.

2. OBJETIVOS

2.1. OBJETIVO GERAL

O presente estudo tem como objetivo investigar a disfunção mitocondrial e as diferenças no metabolismo energético em modelos de DP *in vitro* e *in vivo,* assim como estudar se o uso de substâncias antioxidantes pode melhorar a funcionalidade mitocondrial nesses modelos de DP.

2.2. OBJETIVOS ESPECÍFICOS

2.2.1 Modelo de DP *in vitro* e efeitos da NAC

- Quantificar o fluxo de elétrons relacionado à respiração mitocondrial analisando razões de controle de fluxo, fluxo de elétrons relacionado à fosforilação oxidativa (OXPHOS) e sistema de transferência de elétrons (ETS).
- Quantificar o fluxo de elétrons dependente dos complexos I e II da cadeia respiratória mitocondrial, assim como a avaliar a funcionalidade dos mesmos.
- Dosar a atividade das enzimas citrato sintase e lactato desidrogenase.
- Avaliar a variação dos níveis de peróxido dependentes de diferentes estágios da respiração mitocondrial.
- Estudar os efeitos antioxidantes da NAC sobre a resposta mitocondrial relacionada à respiração mitocondrial, assim como, razões de controle de fluxo, fluxo de elétrons ligado à fosforilação oxidativa (OXPHOS) e sistema de transferência de elétrons (ETS).

2.2.2 Modelo de DP *in vivo* concomitante ao proceso de envelhecimento

- Estabelecer protocolo de respirometria de alta resolução utilizando moscas com deleção total do gene *pink1* em diferentes estágios de vida, buscando avaliar como a idade interfere na resposta mitocondrial.
- Avaliar parâmetros comportamentais relacionados com a DP em modelo de *D. melanogaster* com deleção no gene *pink1*.
- Dosar a atividade das enzimas citrato sintase e lactato desidrogenase em modelo de *D. melanogaster* com deleção no gene *pink1*em diferentes idades.

- Avaliar a variação dos níveis de peróxido dependentes de diferentes estágios da respiração mitocondrial em *D. melanogaster* com deleção no gene *pink1*em diferentes idades.

2.2.3. Modelo de DP *in vitro* e tratamento com cafeína

- Avaliar os efeitos do tratamento com cafeína nos resultados de respirometria de alta resolução em moscas com deleção do gene *pink1*.
- Estabelecer se o tratamento com cafeína altera parâmetros comportamentais em moscas com deleção do gene *pink1*.
- Analisar através de microscopia confocal se o tratamento com cafeína altera a organização da rede mitocondrial em moscas com deleção do gene *pink1*.

3. REVISÃO DA LITERATURA

3.1. Modelos experimentais de DP induzidos por toxinas

A DP assim como outras doenças neurodegenerativas é incurável e devido a característica da progressiva perda neuronal, torna os pacientes altamente debilitados. Por ser uma doença que atinge de 2 a 3% da população mundial acima de 65 anos a busca por um tratamento eficaz é um desafio com implicações tanto para os indivíduos portadores da doença, que buscam uma melhor qualidade de vida, quanto para a sociedade, visto que o aumento da expectativa de vida, consequentemente virá acompanhada pelo aumento da prevalência de casos de DP. Muitas substâncias com potencialidade neuro protetora que poderiam ser utilizadas como possível tratamento para a DP não chegam à fase de testes em humanos, apesar dos resultados pré-clínicos promissores, o que demonstra a grande complexidade dos mecanismos bioquímicos e moleculares envolvidos na DP (PONZ-SARVISE et al., 2020). Por esse motivo se torna importante reconhecer minuciosamente todos os mecanismos celulares relacionados a essa patologia para que se possa definir alvos farmacológicos com maior segurança.

Nesse sentido os diferentes modelos experimentais que mimetizam a doença se consolidam como uma boa alternativa, permitindo respostas mais específicas, como por exemplo, o estudo de áreas cerebrais isoladamente ou a interação neuronal específica, no caso de culturas celulares. Tais respostas podem ser úteis para esclarecer dúvidas ainda existentes sobre as características da patologia em humanos (LOPES et al., 2017).

Um bom modelo de DP deve reproduzir as características comuns da patologia, como a perda de neurônios dopaminérgicos da substância negra compacta e dos gânglios da base, a formação das inclusões chamadas de corpos de Lewy, o acúmulo de alfa-sinucleína, fatores genéticos como mutações nos genes *pink1* e parkin, disfunção mitocondrial entre outras (SALARI; BAGHERI, 2019). A descoberta e utilização de neurotoxinas capazes de reproduzir as características da DP no sistema nervoso central, deu início a utilização de modelos experimentais *in vivo* para a DP.

A 6-hidroxidopamina (6-OHDA) foi uma das primeiras toxinas utilizadas para desenvolver modelos *in vitro* e *in vivo* de DP. A 6-OHDA foi identificada em 1959 (TIEU, 2011), e vem sendo usada há décadas para induzir sintomas e mimetizar efeitos bioquímicos relacionados a DP. Por não ultrapassar a barreira hematoencefálica a 6-OHDA precisa ser administrada via injeção intracerebral, em modelos *in vivo* ou por administração direta no tecido cerebral, ou culturas celulares de neurônios, no caso de modelos *in vitro* (CHIA; TAN; CHAO, 2020)

A 6-OHDA exerce seus efeitos devido a sua semelhança química com a molécula de dopamina. A dopamina pertence à família das catecolaminas, que também compreende a norepinefrina e a epinefrina e é sintetizada a partir da tirosina no citoplasma do neurônio sendo em seguida transportada no interior de vesículas sinápticas (PANTIC et al., 2021). O transportador de monoaminas vesicular é regulado via gradiente de prótons e responsável pelo transporte da dopamina nos neurônios. Depois da sua administração a molécula de 6-OHDA se liga aos transportadores cerebrais dopaminérgicos sendo absorvida por esses neurônios. Uma vez dentro da célula, 6-OHDA rapidamente oxida e produz ERO's, como radicais superóxido e radicais hidroxila, levando a peroxidação lipídica e consequentemente à morte de neurônios dopaminérgicos. (BLUM et al., 2001; J et al., 1989).

A 6-OHDA também é conhecida por prejudicar a cadeia transportadora de elétrons principalmente pela inibição do complexo I (GLINKA; YOUDIM, 1995). Além disso, estudos sugerem que a neuroinflamação também faz parte do mecanismo de toxicidade da 6-OHDA (STRÖMBERG et al., 1986), análises de sequenciamento de RNA em modelos de DP induzidos por 6-OHDA em ratos identificaram um aumento na expressão de genes relacionados a neuroinflamação como IRF7 e ISG15, esses genes possuem papel importante na ativação neuroinflamatória e na neurodegeneração (LI; SUN; CHEN, 2019).

Outra substância que se relaciona com casos sintomáticos de DP é o MPTP, seus efeitos foram estudados em 1982 pelo neurocientista William Langston (LANGSTON, 2017). O MTPT por si só não é tóxico, mas como um composto lipofílico é capaz de ultrapassar a barreira hematoencefálica, no cérebro é rapidamente convertido a 1-metil-4-fenilpiridina (MPP+) (LANGSTON et al., 1984), através do sistema monoaminaoxidase (MAO) (CASTAGNOLI; CHIBA; TREVOR, 1985). O MPP+ se liga especificamente aos receptores de dopamina, causando a morte de neurônios dopaminérgicos da substância negra (RAMSAY et al., 1987). Quando captado pelos neurônios da substância negra o MPP+, torna-se altamente concentrado na mitocôndria contra um gradiente de concentração o qual é energizado pelo potencial transmembrana. Uma vez que atinge níveis tóxicos nas mitocôndrias, o MPP+ inibe o complexo I da cadeia respiratória mitocondrial, gerando consequências como aumento de ERO´s e diminuição dos níveis de ATP (CHAN et al., 1991). Após os a consolidação dos resultados relacionados aos efeitos do MPTP, essa toxina passou a ser utilizado para induzir modelos de DP *in vitro* e *in vivo.*

Outra toxina utilizada como molécula indutora de DP é a rotenona. A neurotoxicidade da rotenona à via nigroestriatal e aos neurônios dopaminérgicos foi primeiramente descrita em um estudo com modelo *in vivo* em 1985 onde os autores demonstraram uma inibição da

respiração mitocondrial induzida por piruvato-malato em preparações de mitocôndrias isoladas da região nigroestriatal e expostas a rotenona (HEIKKILA et al., 1985). A rotenona é um composto natural comumente usada em todo o mundo como inseticida (RICHARDSON et al., 2019). A ingestão de rotenona em altas doses pode causar acidose metabólica, disfunção respiratória, sintomas neurológicos e lesões no sistema cardiovascular, fígado e cérebro (PASHA; SHARMA; RAJPUT, 2005; PENG et al., 2017; SIDDIQUI et al., 2013; ZHANG et al., 2017b). A neurotoxicidade da rotenona é principalmente resultado de sua toxicidade mitocondrial, por inibição do complexo I da cadeia respiratória mitocondrial (SCHULER; CASIDA, 2001).

Mais tarde, dados experimentais consolidaram a rotenona como uma toxina padrão para o desenvolvimento de modelos de DP *in vivo* e *in vitro*. Características como a sua alta lipofilicidade (COVI et al., 2016), que permite ultrapassar a barreira hematoencefálica sem o envolvimento de um sistema de transporte garantiram a utilização da rotenona como molécula indutora para modelo experimental DP. Além disso, estudos trazem uma associação positiva entre a exposição à rotenona e o desenvolvimento de DP (MCKNIGHT; HACK, 2020). Dessa maneira a rotenona é apontada como uma toxina capaz de causar neurodegeneração, sendo bastante utilizada na indução de modelos de DP in vivo e *in vitro* a exemplo do que demonstram Kabiraj e colaboradores (2015) os quais confirmaram que a exposição a rotenona induziu apoptose em linhagens celulares de neuroblastoma (SH-SY5Y) (KABIRAJ et al., 2015).

O estudo de doenças neurodegenerativas como a DP exige a construção/comparação de diferentes modelos experimentais, visto que os mecanismos bioquímicos e moleculares relacionados a essa patologia são complexos. A construção de modelos experimentais com a utlização de toxinas que atingem o sistema nervoso central reproduzindo consequências relacionadas a DP, como perda neuronal e disfunção cognitiva vêm há muito tempo sendo utilizadas para o melhor entendimento sobre a evolução e estudo de possíveis tratamentos para a DP. Atualmente, já se tem comprovação de que fatores genéticos também estão associados às doenças neurodegenerativas (SERRANO-POZO; DAS; HYMAN, 2021; TSUANG; BIRD, 2017) e alguns genes de interesse tem sido estudados. Através de ferramentas de manipulação genética, novos modelos experimentais de DP foram criados, em geral, utilizando animais transgênicos (DAWSON; KO; DAWSON, 2010), essas novas metodologias permitem uma maior base informacional sobre características genéticas da doença e também auxiliam na busca por possíveis intervenções farmacológicas.

3.2. Fatores genéticos relacionados a DP

Nos últimos vinte anos o progresso no estudo da DP, trouxe a descoberta de características genéticas associadas ao desenvolvimento da doença. Em torno de 15% dos pacientes com DP possuem histórico prévio da doença na família e 5 a 10% apresentam uma forma monogênica da patologia com herança mendeliana (KALINDERI; BOSTANTJOPOULOU; FIDANI, 2016). Embora haja consenso de que a grande maioria dos casos de DP seja esporádica, possivelmente haja uma ligação entre variáveis genéticas e ambientais como fatores de risco para essa desordem neurológica.

De acordo com a organização "HUGO gene nomenclature committee (HGNC)", que é responsável por aprovar a nomenclatura dos genes humanos, existem, até agora, pelo menos 19 genes relacionados ao Parkinsonismo, sendo 10 desses genes autossômicos dominantes e 9 genes autossômicos recessivos. Cinco genes despertam maior interesse, pois apresentam maior número de resultados considerando as bases de estudos genéticos da DP, sendo os genes: alfa sinucleína (SNCA), LRRK2, Parkin, *pink1* e DJ-1 (KALINDERI; BOSTANTJOPOULOU; FIDANI, 2016).

O gene que codifica a proteína alfa sinucleína (SNCA) foi o primeiro definitivamente associado a DP. Esse gene está localizado nos locus PARK1 e PARK4 ao longo dos cromossomos 4q21-4q23 (POLYMEROPOULOS et al., 1996). Em 1996 se confirmou a mutação A53T relacionada ao SNCA que estava presente em famílias gregas e italianas com diagnóstico de DP autossômica dominante. Mais tarde outras mutações relacionadas ao gene SNCA foram descobertas: A53T, A30P, E46K, H50G (TRINH; FARRER, 2013). As mutações do gene SNCA são geralmente associadas ao início precoce e rápida progressão da DP, com incidência de danos cognitivos, rápida agregação proteica e demência (XIROMERISIOU et al., 2010).

Já em 2004 a descoberta de mutações associadas ao gene que expressa a proteína LRRK2, trouxeram um avanço na pesquisa sobre a ligação da DP com fatores genéticos, isso porque modificações no gene LRRK2 foram encontrados tanto em casos familiares de DP como em casos esporádicos, ampliando a ideia de que rotas genéticas comuns poderiam estar implicadas no desenvolvimento da doença (PAISÁN-RUÍZ et al., 2004). A LRRK2 é uma proteína com funções de quinase e também GTPase, atuando em múltiplos processos biológicos como transmissão neuronal, endocitose e autofagia (TRINH; FARRER, 2013).

O locus PARK8 encontra-se no cromossomo 12q12 e abriga o gene codificante de LRRK2 (PAISÁN-RUÍZ et al., 2004). Até agora 80 mutações de LRRK2 foram associadas ao Parsinsonismo autossômico dominante sendo essa a causa genética mais comum de DP. No

entanto a patogenicidade foi comprovada apenas em sete dessas mutações: R1441C, R1441G, R1441H, Y1699C, G2019S, I2020T, N1437H (BERG et al., 2005). A idade de início para o desenvolvimento de sintomas de DP em portadores de mutações em LRRK2 varia entre a quarta e a nona década de vida, sendo comparada aos casos esporádicos de DP, além disso as características patológicas são diversas, incluindo enrijecimento muscular e formação de agregados neurofibrilares. Essa diversidade sintomática e etária relacionando mutações em LRRK2 e desenvolvimento de DP conclui que essa proteína está envolvida em diversos processos celulares podendo ser crucial para múltiplas vias de sinalização importantes para o sistema nervoso central (KALINDERI; BOSTANTJOPOULOU; FIDANI, 2016; ZIMPRICH et al., 2004).

Um ano depois da descoberta sobre mutações em SNCA estarem relacionadas ao parkinsonismo, mutações em um outro gene denominado parkin foram identificadas em uma família japonesa com fenótipo de DP (ABBAS et al., 1999). Interessantemente, essa família apresentava histórico juvenil de DP, caracterizada por um genótipo autossômico recessivo. Mais tarde se descobriu que mutações no gene parkin também eram encontradas em outras etnias e estavam ligadas a manifestação de Parsinsonismo juvenil. Em torno de 50% dos pacientes que apresentam DP em idades próximas aos 25 anos e 3 a 7% dos pacientes com idade entre 30 e 45 anos apresentam mutação do gene parkin (GASSER, 2011). Até agora mais de 100 mutações relacionando parkin já foram identificadas. O gene parkin, o qual codifica para a proteína com o mesmo nome, encontra-se no locus PARK2, localizado no cromossomo 6q25.2-q27 (KITADA et al., 1998). Parkin tem papel de ubiquitina ligase E3, participando do sistema de degradação do proteassoma (SHIMURA et al., 2000). Mutações nesse gene resultam na inativação de sua função E3 ligase, assim como em falha na ubiquitinação de proteínas alvo com consequente acúmulo de proteínas que não são mais degradadas pela via ubiquitina/proteassomo. A formação desses agregados proteicos é tóxica aos neurônios, em especial neurônios da substância negra, esse fenótipo parece ser crucial no envolvimento do gene parkin com a DP (KALINDERI; BOSTANTJOPOULOU; FIDANI, 2016).

O gene parkin interage com um outro gene também já associado a DP, o gene *pink1*. O gene *pink1* codifica uma serina treonina quinase responsável por se ligar a mitocôndrias despolarizadas (PARK et al., 2006). A ação de proteína quinase resultante da codificação do gene *pink1*, funciona como um ativador de parkin exclusivo para a rede mitocondrial. O gene *pink1* codifica uma serina treonina quinase mitocondrial (PTEN-induced quinase 1), que é um importante marcador na manutenção da qualidade da rede mitocondrial, estando ligado a processos de autofagia (CHEN; DORN, 2013). A atividade da proteína derivada desse gene é

regulada pelo potencial elétrico transmembrana da mitocôndria. Em mitocôndrias despolarizadas há um acúmulo da proteína PINK1 nas membranas mitocondriais, resultando no recrutamento de parkin. A proteína parkin é uma ubiquitina ligase normalmente encontrada no citosol, envolvida na formação de autofagossomos. A formação dos autofagossomos para retirada de mitocondrias prejudicadas da rede mitocondrial inicia um processo específico de autofagia que se denomina mitofagia. A regulação do processo de mitofagia envolvendo a interação da proteína PINK1, está associada à DP (WHITWORTH; PALLANCK, 2009). Em geral deleções do gene *pink1* em modelos experimentais se associa a fenótipos de disfunção mitocondrial, agregação de mitocôndrias disfuncionais na rede mitocondrial, menor geração de ATP, aumento da produção e ERO's e desvios de rotas metabólicas caracterizadas por uma inversão entre rota oxidativa e glicolítica (COSTA; LOH; MARTINS, 2013a; PIMENTA DE CASTRO et al., 2012a; SCIALÒ et al., 2016).

Em 2004 o locus PARK6 foi mapeado no cromossomo 1p35-p36 em uma família italiana que apresentava uma forma autossômica recessiva de DP, sendo identificadas mutações "missense" e "nosense" relacionadas ao gene *pink1*. Cerca de 2 a 4% das pessoas que apresentam manifestação precoce de DP carregam mutações no gene *pink1*. O fenótipo da doença é caracterizado por uma progressão lenta com manifestações atípicas como distonia, sonolência e comorbidades psiquiátricas, incluindo transtornos de ansiedade e depressão (SAMARANCH et al., 2010).

Um outro gene também relacionado a DP é o DJ-1, localizado no cromossomo 1p36. locus PARK7 e que codifica a proteína de mesmo nome (BONIFATI et al., 2003). A DJ-1 é uma proteína multifuncional, podendo atuar como chaperona, antioxidante e regulador transcricional, sendo também sensor de estresse, apresentando expressão aumentada durante eventos de estresse oxidativo (TAKAHASHI-NIKI et al., 2017). Estudos já indicam que DJ-1 participa de uma via comum com *pink1* e parkin, sendo um regulador positivo da transcrição de PINK1 por ligar-se a ao promotor "forkhead box O3a (Foxo3a)" (REQUEJO-AGUILAR et al., 2015). Também já foi comprovado que a perda da função de DJ-1 leva a degeneração de neurônios dopaminérgicos, que é um dos sintomas da DP (DOLGACHEVA et al., 2019).

3.3. Uso de *Drosophila melanogaster* como modelo experimental de DP

Tendo em vista os genes associados a fenótipos de DP, tomou ênfase a utilização de modelos experimentais usando animais transgênicos com deleções totais ou parciais de genes de interesse. A utilização de animais com deleção genética tem objetivo de melhor entender a

influência de genes específicos em aspectos relacionados ao processo de evolução da DP, assim como testar possíveis fármacos que poderiam atenuar consequências já conhecidas da doença, como a perda neuronal, a disfunção mitocondrial, as restrições de movimento, entre outras.

Um dos modelos animais que permite fácil manipulação genética emergindo como aceitável para o estudo da DP é a *Drosophila melanogaster*. A *D. melanogaster*, comumente reconhecida como mosca da fruta começou a ser utilizada como modelo em estudos de genética desde o início do século XVIII, sendo utilizada também para estudos relacionados a bioquímica, biologia celular, fisiologia e metabolismo, além de neurologia e estudos de bioenergética (MUSSELMAN; KÜHNLEIN, 2018; RUBIN, 1988). No ano 2000 se completou o projeto genoma *D. melanogaster* o qual foi anunciado em março e se tratava do sequenciamento total do genoma de *D. melanogaster*. O projeto foi resultado de uma megaoperação, envolvendo diversos grupos de pesquisa ao redor do mundo (ADAMS, 2000). Alguns anos mais tarde o projeto genoma humano também foi finalizado e a comparação de ambas as sequências genômicas revelou altos níveis de homologia entre humanos e *D. melanogaster*. Cerca de 75% dos genes relacionados às doenças humanas tem ortólogos funcionais em *D. melanogaster*, essas descobertas ajudaram a consolidar a mosca da fruta como um bom modelo experimental para o estudo de diferentes patologias (MYERS, 2000).

Outras características fazem com que a *D. melanogaster* se apresente como um bom modelo experimental, entre elas seu curto ciclo de vida, com a produção de um grande número de ovos por ciclo (Figura 1), além do fácil manuseio e manutenção e fácil manipulação genética para a construção de cepas com mutações específicas em genes alvo (YAMAGUCHI; YOSHIDA, 2018).

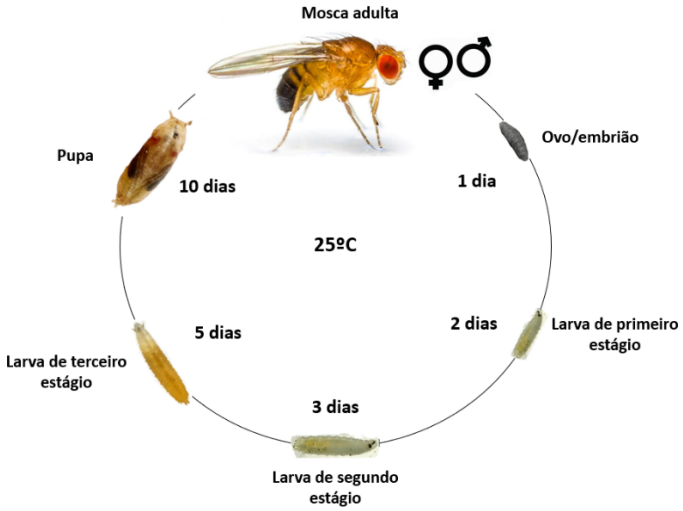

Figura 3: ciclo de vida de *Drosophila melanogaster*

O primeiro modelo genético de DP usando *D. melanogaster*, foi projetado através da super expressão da proteína humana α-sinucleína (FEANY; BENDER, 2000). A *D. melanogaster* não possui genes homólogos para a codificação da α-sinucleína e a super expressão resultou em um fenótipo com déficits na função locomotora, assim como diminuiu a sobrevivência de neurônios dopaminérgicos, caracterizando um bom modelo para o estudo de DP (RIEMENSPERGER et al., 2013).

Mais tarde a construção de cepas específicas de *D. melanogaster* com deleção de genes alvo como *pink1, lrrk2* e *DJ-1* também passou a fazer parte dos estudos de DP. O sistema Gal4/UAS (BRAND; PERRIMON, 1993b), também trouxe uma nova perspectiva para os estudos genéticos usando *D. melanogaster* com a criação de uma ferramenta de expressão gênica que permite a expressão/silenciamento de qualquer gene de interesse em qualquer tecido específico dentro da mosca. Esse sistema compreende um fator de transcrição de levedura GAL4 em um vetor de elemento P sendo possível colocar um promotor definido a montante de GAL4 e para acompanhar esse direcionador de expressão gênica, há um vetor de elemento P correspondente, pUAST, contendo as sequências de ativação a montante (Upstream Activation Sequence) às quais a proteína GAL4 pode se ligar. Essas sequências UAS são conectadas a um promotor geral e a um local de clonagem para permitir a inserção de qualquer gene de interesse. Esse sistema de expressão bipartida pode ser usado para conduzir a expressão de um gene de uma forma definida, permitindo aos pesquisadores realizar experimentos de resgate e outras manipulações genéticas com maior facilidade (HALES et al., 2015).

Tendo em vista as vantagens de manutenção associadas a uma forte base genética para a construção de cepas mutantes a *D. melanogaster* passou a ser utilizada como modelo de DP (PIENAAR; GÖTZ; FEANY, 2010). Além das vantagens já citadas que incluem, por exemplo, a possível expressão/ silenciamento de genes em neurônios específicos com a utilização do sistema Gal4/UAS, a mosca da fruta apresenta anatomia, fenótipos e comportamentos padrões já bem descritos pela literatura científica (MATTHEWS; KAUFMAN; GELBART, 2005) o que auxilia na construção de um modelo experimental para doenças neurodegenerativas como a DP.

Sendo assim, a descoberta de novas rotas metabólicas, novos genes envolvidos na DP, assim como efeitos comportamentais e possíveis estratégias farmacológicas para o tratamento dessa doença neurodegenerativa, passam pela utilização de modelos experimentais alternativos como a *D. melanogaster*, salientando a importância desses novos conhecimentos e fazendo com que a mosca da fruta se consolide como um bom modelo experimental para o estudo de várias doenças incluindo a DP.

3.4. Disfunção mitocondrial e metabolismo energético durante o envelhecimento e na DP

Em mamíferos o cérebro utiliza 75% de toda a energia oriunda das moléculas de glicose. A maior parte dessa energia é resultado das reações de oxidação completa da glicose a CO_2 utilizando a maquinaria das mitocôndrias para a produção de ATP, através do processo de fosforilação oxidativa. O ATP produzido é utilizado para dar suporte energético às transmissões sinápticas (HAVRANKOVA; ROTH; BROWNSTEIN, 1979). Por consequência da alta demanda energética do sistema nervoso central, tanto o processo de captação da glicose como os processos de oxidação dessa molécula para geração de ATP, precisam estar devidamente acoplados para garantir a eficiência de eventos de transmissão e plasticidade sináptica (COHEN; DILLIN, 2008).

O processo de envelhecimento interfere na disponibilidade de glicose para oxidação pela via mitocondrial, assim como ocasiona uma diminuição da atividade dos complexos da cadeia respiratória mitocondrial (VAN HEEMST, 2010). A disfunção mitocondrial também é uma das consequências já bem estabelecidas da DP (BEAL, 1995), nesse sentido, tanto o processo de envelhecimento como a DP parecem compartilhar marcadores comuns relacionados ao metabolismo energético, configurando processos metabólicos que podem estar interligados, e cujo conhecimento pode auxiliar no melhor entendimento tanto sobre a doença quanto sobre o processo de envelhecimento visto que ambos, em geral, ocorrem em concomitância.

As mitocôndrias tem papel fundamental na produção de energia para o sistema nervoso central, porque 90% da glicose (fonte primária de energia do cérebro) é oxidada a CO_2 com geração de ATP no cérebro utilizando o processo de fosforilação oxidativa mitocondrial (BÉLANGER; ALLAMAN; MAGISTRETTI, 2011; CUNNANE et al., 2020), por isso qualquer perda de funcionalidade mitocondrial pode afetar a função neuronal com efeitos sobre a cognição, aprendizado e memória.

O piruvato, metabólito final da glicólise entra na mitocôndria utilizando um transportador de piruvato (MCCOMMIS; FINCK, 2015), após sua entrada o piruvato é substrato para o complexo da enzima piruvato desidrogenase que o converte em acetil-CoA, substrato para a primeira reação do ciclo do ácido cítrico. O acetil-CoA gerado pela descaboxilação oxidativa do piruvato, pode reagir com o oxaloacetato e ser convertido a citrato por ação da citrato sintase, seguindo pelo ciclo do ácido cítrico até sua oxidação total para geração de ATP, mas também pode ser utilizado como um cosubstrato para a síntese de

moléculas como o N-acetilaspartato o qual é uma das moléculas mais abundantes no cérebro, sendo utilizado como um biomarcador neuronal e axonal. Durante o envelhecimento se observa uma atividade reduzida da piruvato desidrogenase, o mesmo já foi observado em tecidos derivados de autopsias cerebrais de pacientes com DP, o que poderia estar relacionado com uma menor disponibilidade de substratos, os quais fazem parte de uma via convergente que não envolve apenas geração de energia via oxidação, mas também a síntese de moléculas precursoras que são importantes para a plasticidade sináptica (MIKI et al., 2017; REX SHEU et al., 1985).

Outros substratos que participam do ciclo do ácido cítrico, também podem ser utilizados como precursores reacionais para a síntese de neurotransmissores, o α-cetoglutarato, por exemplo, é um intermediário metabólico utilizado como substrato para a reação de formação de glutamato e GABA, via glutamato desidrogenase e glutamato descarboxilase (BAK; SCHOUSBOE; WAAGEPETERSEN, 2006). Nesse sentido, uma menor geração de metabólitos derivados do ciclo do ácido cítrico, diminui também a disponibilidade de precursores importantes para a transmissão sináptica como GABA e glutamato. Um estudo utilizando ratos idosos tratados com moléculas de glicose previamente marcadas com carbonos radioativos (^{13}C) revelou que a incorporação de ^{13}C derivado de glicose assim como os níveis de neurotransmissores como glutamato, aspartato e GABA diminuem no cérebro idoso, a redução de neurotransmissores também é uma característica relacionada a doenças neurodegenerativas como a doença de Alzheimer e DP (DEDEOGLU et al., 2004; MICCHELI et al., 2003; ZHANG et al., 2017a).

Além de intermediários metabólicos, o ciclo do ácido cítrico também gera equivalentes redutores como o NADH que flui através da cadeia respiratória mitocondrial em um processo que acopla um gradiente de prótons e pH para a produção de energia convertendo ADP à ATP através da fosforilação oxidativa (SIGNES; FERNANDEZ-VIZARRA, 2018). A cadeia respiratória mitocondrial é composta por 5 complexos enzimáticos responsáveis por reações de oxirredução e transferência de elétrons (Figura. 2).

Déficits na maquinaria catalítica de transferência de elétrons das mitocôndrias caracterizados pela atividade diminuída dos complexos da cadeia respiratória são conhecidos como disfunções mitocondriais e contribuem para um processo de hipometabolismo que geralmente é acompanhado por um aumento das ERO's e diminuição dos níveis de ATP (BROOKES et al., 2004). Estudos já demonstraram uma redução significativa da atividade dos complexos mitocondriais I e IV em cérebro de macacos idosos (BOWLING et al., 1993), além disso ratos idosos apresentaram atividade mitocondrial do complexo I 30% menor quando

comparados com ratos jovens (PETROSILLO et al., 2008). A disfunção mitocondrial também é uma das consequências da DP. Em geral modelos de DP demonstram a diminuição da atividade do complexo mitocondrial I, aumento da formação de ERO's e redução da síntese de ATP (COSTA; LOH; MARTINS, 2013b; GAUTIER; KITADA; SHEN, 2008; NARENDRA et al., 2010; SUDATI et al., 2013).

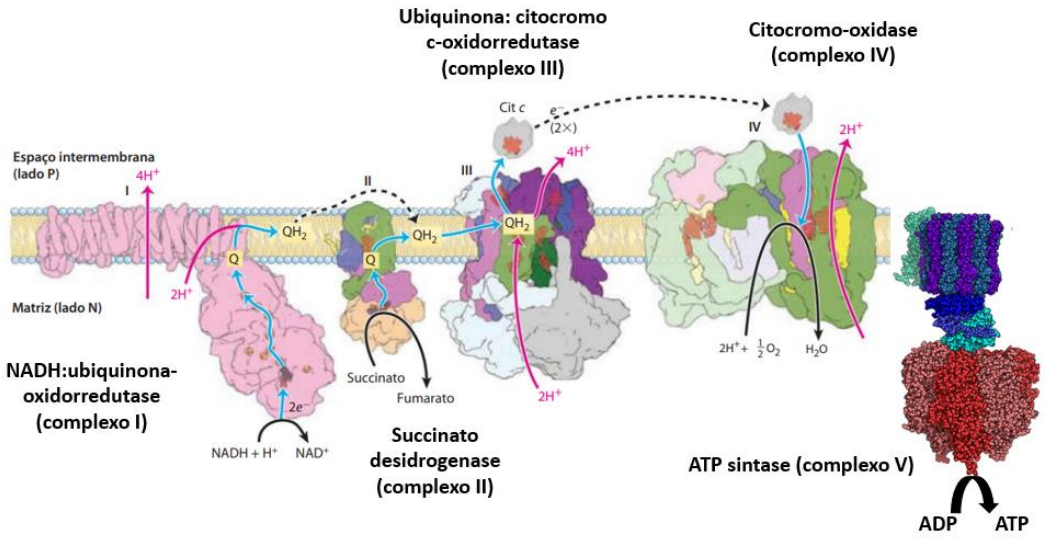

Figura 4: Complexos da cadeia respiratória mitocondrial. Adaptado do livro Princípios de Bioquímica de Lehninger [recurso eletrônico] / David L. Nelson, Michael M. Cox; [tradução: Ana Beatriz Gorini da Veiga ... et al.]; revisão técnica: Carlos Termignoni ... [et al.]. – 6. ed. – Dados eletrônicos. – Porto Alegre : Artmed, 2014.

As mitocôndrias também são as principais formadoras de peróxido de hidrogênio (H_2O_2), que é uma molécula sinalizadora de mudanças no ambiente celular (LIN; BEAL, 2006). A formação de H_2O_2 mitocondrial origina-se pela dismutação do oxigênio e embora seja uma molécula que contribui para o aumento do estresse oxidativo, uma produção controlada de H_2O_2 é capaz de regular processos de sinalização celular e transcrição nuclear (RHEE, 2006). Baixos níveis de peróxido gerados pela mitocôndria são reconhecidos por ativar a ação de fatores de crescimento como a via das MAPK. O colapso mitocondrial causado por substâncias que desfazem o gradiente de prótons mitocondrial como o FCCP, não apenas eliminam a formação de peróxido como suprimem a fosforilação e consequente inativação do receptor de tirosina quinase, mesmo na presença de insulina (STOROZHEVYKH et al., 2007). A geração de H_2O_2 é capaz de oxidar resíduos de cisteína no receptor de insulina, facilitando a fosforilação desse receptor, as moléculas de H_2O_2 também inibem por oxidação dois reguladores negativos do

receptor de insulina: tirosina fosfatase (PTP1B) e lipídeo fosfatase (PTEN) (ELCHEBLY, 1999; LOH et al., 2009).

Porém enquanto concentrações mais baixas de H_2O_2 (~ 5 μM) ativam os receptores de insulina níveis mais altos (~ 50 μM) os inativam (IWAKAMI et al., 2011). A inativação se dá devido aos altos níveis de H_2O_2 estimularem vias inibitórias como JNK e IκB quinase (IKK) (YIN; JIANG; CADENAS, 2013), que constituem mecanismo de resistência à insulina induzida por estresse e inflamação, respectivamente. O processo de regulação dos níveis de H_2O_2 é condicionada pela reação de redução dessa molécula que é convertida em H_2O, sendo esse processo dependente do ciclo das glutationas. A reação de redução das moléculas excedentes de H_2O_2 é acoplada a reação de oxidação de GSH a GSSG catalisada pela enzima glutationa peroxidase (MUYDERMAN, 2004). Tanto em neurônios quanto em astrócitos a depleção de GSH mitocondrial leva a um aumento excessivo na produção de H_2O_2 resultando em perda do potencial de membrana mitocondrial e morte celular (WÜLLNER et al., 1999).

Durante o envelhecimento ocorre naturalmente a depleção dos níveis de GSH no sistema nervoso central. Um estudo avaliando os níveis de GSH em córtex cerebral confirmou uma redução de 30% nos níveis de GSH em córtex de idosos saudáveis quando comparados com jovens saudáveis (EMIR et al., 2011). O declínio nos níveis de GSH dependente da idade também foi relacionado com a função cognitiva prejudicada durante envelhecimento. O tratamento de roedores com compostos que esgotam os níveis de GSH resultou em um prejuízo de memória e aprendizagem (CRUZ-AGUADO et al., 2001). Esses fatores podem aumentar os níveis de H_2O_2 para níveis acima do aceitável e contribuir para quadros de aumento de estresse oxidativo durante o envelhecimento.

A DP, assim como outras doenças neurodegenerativas são reconhecidas por aumentar os níveis de tanto de ERO's como H_2O_2, como de espécies reativas de nitrogênio (ISLAM, 2017) e modelos de DP induzidos por toxinas como a rotenona apresentam como consequência uma redução significativa dos níveis de GSH (WANG et al., 2020). Sendo assim é possível perceber que o envelhecimento e a DP compartilham rotas semelhantes que relacionam disfunção mitocondrial, metabolismo energético e balanço redox celular, sendo, portanto, importante estudar os efeitos da DP e do envelhecimento de forma concomitante.

3.5. Utilização de substâncias antioxidantes como estratégia farmacológica para tratamento de DP

Com o aumento da expectativa de vida em âmbito mundial, a população acima dos 65 anos também tem aumentado, o que é visto de maneira geral como um fator positivo. Mas esse aumento de uma população idosa traz consigo um aspecto negativo: o aumento do número de pessoas com desordens neurodegenerativas como DP e doença de Alzheimer (CHRISTENSEN et al., 2009). As estatísticas apontam que o número de pacientes com doença de Alzheimer chegará a 100 milhões no ano de 2050 e o número de pacientes acometidos com DP irá dobrar nas próximas décadas (BONDI; EDMONDS; SALMON, 2017; DORSEY et al., 2007). As doenças neurodegenerativas não apresentam cura e nem mesmo prevenção comprovadamente eficaz, alguns compostos se apresentam como possíveis estratégias para alívio dos sintomas e melhoria da qualidade de vida das pessoas com doenças neurodegenerativas (LIN; BEAL, 2006).

Tendo em vista esse cenário associado ao fato que as doenças neurodegenerativas como a DP são multifatoriais e geralmente associadas ao processo de envelhecimento, a mistura de compostos ou, o estudo sobre extratos de plantas e compostos naturais que possam agir sobre diferentes alvos de doenças sem muitos efeitos colaterais servindo como tratamentos secundários, podem levar a resultados promissores na melhoria da qualidade de vida da população acometida por distúrbios neurodegenerativos (ESPINOZA-FONSECA, 2006).

A N-acetil-cisteína (NAC) é um composto considerado antioxidante e geralmente utilizado para o tratamento de overdoses de paracetamol (CARVALHO et al., 2013). A NAC serve como um precursor biológico para a glutationa reestabelecendo os estoques dessa molécula redutora, permitindo a redução química da N-acetil-p-benzo-quinona imina (NAPQI) que é a molécula tóxica derivada da metabolização de altas doses de paracetamol. Em geral a NAC não apresenta efeitos adversos relevantes derivados de sua ingestão (WANG et al., 2006), resultando na sua utilização para o tratamento de outras doenças como, por exemplo, tratamento secundário para a DP e outras desordens neurodegenerativas.

Em estudo de Monti e colaboradores (2019), 42 pacientes com DP foram randomizados para receber infusões intravenosas semanais de NAC (50 mg / kg) além de doses orais (500 mg duas vezes por dia) por 3 meses. Esse estudo concluiu que o tratamento com NAC apresentou resultados positivos aumentando a ligação da dopamina ao seu receptor nos pacientes avaliados, além de melhorar significativamente os sintomas físicos da DP (MONTI et al., 2019). Um outro estudo também apresenta resultados positivos relativos a administração de NAC em pacientes

com DP confirmado pelo aumento da razão GSH/GSSG no cérebro, entretanto os autores afirmam que esse aumento de GSH não resultou na melhora de mercadores de peroxidação lipídica (COLES et al., 2018a).

Outra molécula bastante explorada como tratamento secundário para doenças neurodegenerativas é a cafeína (MCLELLAN; CALDWELL; LIEBERMAN, 2016). Essa metil xantina é reconhecida por exercer seus efeitos como antioxidante e também como antagonista dos receptores de adenosina em especial do receptor A2A (SAWYNOK, 2011). A interação da cafeína com receptores de adenosina também traz relações com o sistema dopaminérgico, o qual é um dos principais alvos da DP. Dados da literatura demonstram que antagonistas de receptores de adenosina aumentam a afinidade e sinalização de receptores dopaminérgicos (VOICULESCU et al., 2014).

Em estudos relacionados a doenças neurodegenerativas, a cafeína demonstrou ser benéfica em grupo masculino com DP, porém, em mulheres, a molécula não demonstrou o mesmo efeito por competir pelo sítio de ativação da enzima CYP1A2 que metaboliza o estrogênio. Um outro estudo demonstrou que a cafeína está associada com a idade precoce do início da doença de Huntington em ingestões maiores que 190 mg/dia, porém estudos utilizando modelos animais encontraram resultados ambíguos. Já em estudo com modelo de DP a cafeína demonstrou efeito protetor em dosagens equivalentes a 3-5 mg/kg (KOLAHDOUZAN; HAMADEH, 2017). Outros estudos demonstraram que a cafeína exerce efeitos positivos sobre a atividade mitocondrial, aumentando fluxo de oxigênio relacionado a OXPHOS no cérebro de camundongos na dose de 20 mg/Kg (GONÇALVES et al., 2020) além de aumentar o nível de aproveitamento de NADH e FADH na cadeia respiratória mitocondrial, resultando em um maior acoplamento do sistema de transferência de elétrons (MISHRA; KUMAR, 2014).

Embora os resultados sobre a utilização de moléculas antioxidantes como possível tratamento para doenças neurodegenerativas como a DP apresentem ainda pontos contraditórios, substâncias como a NAC que já é bastante utilizada como fármaco e encontra-se na lista de medicamentos essenciais da Organização Mundial da Saúde (WHO, 2015) sendo considerada um medicamento eficaz e seguro, assim como a cafeína que é amplamente consumida pela população em bebidas e alimentos (TARNOPOLSKY, 2010), despontam como estratégias secundárias que podem melhorar a qualidade de vida de pacientes com DP e outras desordens neurológicas.

3.6. Respirometria de alta resolução e o protocolo SUIT

As alterações do metabolismo celular estão relacionadas com as doenças neurodegenerativas como a DP (DEVINE; PLUN-FAVREAU; WOOD, 2011). A partir disso, se torna importante avaliar a funcionalidade mitocondrial nos modelos de doenças neurodegenerativas, visto que as mitocôndrias são o centro de transdução energética celular (OST et al., 2018). Para a avaliação da funcionalidade mitocondrial, tanto em modelos *in vivo* quanto *in vitro* em geral, são utilizados os chamados eletrodos de Clarck, capazes de medir a concentração de oxigênio do ambiente ao mesmo tempo que detectam a transferência de elétrons da cadeia respiratória mitocondrial (SCHÖPF et al., 2016a). A medição da variação do consumo de oxigênio dependente do fluxo de elétrons da cadeia respiratória mitocondrial se chama respirometria de alta resolução e é capaz de descrever a funcionalidade da cadeia respiratória mitocondrial em tempo real (PUURAND et al., 2018).

Um dos protocolos de respirometria de alta resolução adaptado para uso no respirômetro mitocondrial OROBOROS O2k® é chamado protocolo SUIT fazendo menção às iniciais das palavras derivadas da língua inglesa “substrate”, “uncoupler”, “inhibitor” e “titration” (KRUMSCHNABEL et al., 2015a). Esse protocolo consiste na titulação sequencial de substratos, inibidores e desacopladores permitindo analisar o fluxo de elétrons em pontos específicos da cadeia respiratória mitocondrial. Na tabela 1 são demonstradas as principais etapas relacionadas ao protocolo SUIT.

Tabela 1: Descrição das etapas do protocolo SUIT

ETAPAS DO PROTOCOLO SUIT	
ETAPAS LEAK E OXPHOS	***SUBSTRATOS/ INIBIDOR***
PROTON LEAK	Substratos relacionados a CI • GLUTAMATO, • MALATO, • PIRUVATO
OXPHOS-CI *****OXPHOS CI- se refere a fosforilação oxidativa dependente do complexo I com substratos específicos	• SUBSTRATOS RELACIONADOS A CI+ CONCENTRAÇÕES SATURANTES DE ADP
OXPHOS-CI&CII *****OXPHOS CI- se refere a fosforilação oxidativa dependente do complexo I e complexo II com substratos específicos	Substratos relacionados a CII • SUCCINATO + CONCENTRAÇÕES SATURANTES DE ADP

LEAK	• ADIÇÃO DE OLIGOMICINA (INIBIDOR DA ATP SINTASE)
ETAPA ETS	***DESACOPLADOR /INIBIDORES***
ETS CI&CII	• FCCP; CCP; DNP
ETS-CI *****ETS CI- se refere a transferência de elétrons dependente do complexo I na presença de inibidor específico	Inibidor relacionado a CI • ROTENONA
ETS CI&CII *****ETS CII- se refere a transferência de elétrons dependente do complexo II na presença de inibidor específico	Inibidor relacionado a CII • MALONATO
ROX	• ANTIMICINA

A etapa da respirometria de alta resolução chamada de OXPHOS oferece respostas sobre o acoplamento do processo convergente de transferência de elétrons para a produção de ATP. Nessa etapa há adição de substratos específicos dos complexos mitocondriais I e II e concentrações saturantes de ADP (GONÇALVES et al., 2020). Já as etapas proton LEAK e LEAK se referem aos estágios não acoplados da cadeia respiratória mitocondrial na ausência de desacopladores, porém com atividade reduzida ou inibida da ATP sintase, tanto pela ausência de concentrações saturantes de ADP (proton LEAK), quanto pela presença de inibidor específico da ATP sintase (LEAK) (SCHÖPF et al., 2016b). O estágio ETS compreende a transferência de elétrons através dos complexos da cadeia respiratória mitocondrial na presença de desacoplador específico, além disso através da utilização de inibidores específicos como a rotenona e o malonato é possível identificar a funcionalidade dos complexos I e II no estágio ETS.

O acoplamento entre os estágios OXPHOS e ETS na respirometria de alta resolução é capaz de oferecer respostas sobre as interações entre os estados acoplados e desacoplados da cadeia respiratória mitocondrial os quais estão relacionadas a possíveis alterações no processo bioenergético celular (GONÇALVES et al., 2019). O reconhecimento de pontos específicos de alteração, ajuda a entender como a funcionalidade mitocondrial se relaciona com os processos celulares. Por exemplo, quando há um desacoplamento da força motriz de transferência de elétrons no estágio OXPHOS, em geral, há um aumento na formação de ERO’s, as quais podem funcionar como sinalizadores para rotas celulares específicas de adaptação, ou morte celular.

Nesse sentido, a técnica de respirometria de alta resolução associada ao protocolo SUIT, em especial quando se usa o OROBOROS O2K®, pode ser considerada uma boa ferramenta para avaliação da funcionalidade mitocondrial em modelos experimentais *in vitro* e *in vivo*, permitindo entender os processos de fisiologia mitocondrial de maneira específica e em tempo real.

4. DESENVOLVIMENTO

O desenvolvimento desta tese conta com um artigo publicado e dois manuscritos ainda não publicados. O item 4.1 traz o artigo publicado na revista Neurotoxicology, volume 70, p. 1-11 DOI: doi: 10.1016/j.neuro.2018.10.005, intitulado: 6-Hydroxidopamine induces diferente mitochondrial bioenergetics response in brain regions of rat. O item 4.2 traz o manuscrito intitulado: Mitochondrial function and cellular energy maintenance during aging in a *Drosophila melanogaster* model of Parkinson Disease. Por final, o item 4.3 traz o manuscrito intitulado: Caffeine improves mitochondrial function in a *pink1* model of Parkinson disease, o qual está submetido à revista Life Sciences, respeitando as diretrizes de formatação para a submissão. O artigo e os manuscritos estão separados em seções de introdução, materiais e métodos, resultados, discussão e referências bibliográficas. Nos manuscritos, as figuras referentes aos resultados são colocadas após o item referências bibliográficas.

4.1. ARTIGO CIENTÍFICO 1: 6-Hydroxidopamine induces diferente mitochondrial bioenergetics response in brain regions of rat

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Full Length Article

6-Hydroxydopamine induces different mitochondrial bioenergetics response in brain regions of rat

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ABSTRACT

Mitochondrial dysfunction has been demonstrated to have a central role in Parkinson Disease (PD) pathophysiology. Some studies have indicated that PD causes an impairment in mitochondrial bioenergetics; however, the effects of PD on brain-region specific bioenergetics was never investigated before. This study aimed to evaluate mitochondrial bioenergetics in different rat brain structures in an *in vitro* model of PD using 6-OHDA. Rat brain slices of hippocampus, striatum, and cortex were exposed to 6-OHDA (100 μM) for 1 h and mitochondrial bioenergetic parameters, peroxide production, lactate dehydrogenase (LDH) and citrate synthase (CS) activities were analyzed. Hippocampus slices exposed to 6-OHDA presented increased peroxide production but, no mitochondrial adaptive response against 6-OHDA damage. Cortex slices exposed to 6-OHDA presented increased oxygen flux related to oxidative phosphorylation and energetic pathways exchange demonstrated by the increase in LDH activity, suggesting a mitochondrial compensatory response. Striatum slices exposed to 6-OHDA presented a decrease of oxidative phosphorylation and decrease of oxygen flux related to ATP-synthase indicating an impairment in the respiratory chain. The co-incubation of 6-OHDA with n-acetylcysteine (NAC) abolished the effects of 6-OHDA on mitochondrial function in all brain regions tested, indicating that the increased reactive oxygen species (ROS) production is responsible for the alterations observed in mitochondrial bioenergetics. The present results indicate a brain-region specific response against 6-OHDA, providing new insights into brain mitochondrial bioenergetic function in PD. These findings may contribute to the development of future therapies with a target on energy metabolism.

1. Introduction

Parkinson disease (PD) is considered the second most common neurodegenerative disorder in the world (Simuni et al., 2018). PD is characterized by loss of dopaminergic neurons and formation of protein aggregates, such as α-synuclein (Requejo-Aguilar et al., 2014), leading to motor dysfunction, and impairment of cognitive and memory functions (Goetz, 2011; Riederer et al., 2018). The pathology and symptoms of PD are well described, although its mechanisms and causes remain unclear (Goetz, 2011). One mechanism involved in PD is mitochondrial dysfunction (Ammal Kaidery and Thomas, 2018; Teves et al., 2018; Wu et al., 2018). Mutation in genes involved in mitochondrial quality control, such as PARK2 and PINK1, produce PD symptoms (Wang et al., 2011). These genes code for proteins such as PINK1 (PTEN induced kinase 1) that is a serine/threonine kinase involved in mitochondrial network quality control (Gautier et al., 2008).

Mitochondrial quality control and normal mitochondrial functionality are crucial to maintaining the cell energy balance (Lisowski et al., 2018). Investigations demonstrated that besides the impairment of mitochondrial function, PD has consequences over the energetic routes (Requejo-Aguilar et al., 2014). Observations from genetic models of the disease indicate that PD may drive an energy generation (Requejo-Aguilar and Bolaños, 2016) shift from aerobic to glycolytic route (Requejo-Aguilar et al., 2014) similar to what is observed in cancer cells (Devine et al., 2011).

Dopaminergic neurons from striatum are usually described to be affected by PD (Massari et al., 2016; Riederer et al., 2018; Zheng et al., 2018). However, the brain is constituted by different regions that are responsible for distinct functions, therefore, bioenergetics and mitochondrial response against chemical exposure, aging or

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neurodegenerative disease may differ (Pandya et al., 2016) depending on the brain region.

In vitro models of PD are valuable tools for the investigation of mechanisms involved in the disease (Feitosa et al., 2018; Morroni et al., 2018). Analogs of dopamine, such as 6-hydroxydopamine (6-OHDA), are used both as *in vitro* and *in vivo* models of PD (Hao et al., 2017; Massari et al., 2016). Exposure to 6-OHDA mimics the PD effects like dopaminergic neurons death and increased ROS production (Lehmensiek et al., 2006). 6-OHDA was the first neurotoxin employed to cause damage specifically in neurons that use catecholamines as neurotransmitter (UNGERSTEDT, 1968) being the most used toxin in experimental PD models (Blandini et al., 2008). 6-OHDA accumulates inside neurons, promoting ROS formation (Blandini et al., 2008) and selective damage of dopaminergic/catecholaminergic neurons.

6-OHDA was also demonstrated to cause brain mitochondrial dysfunction in an *in vitro* study (Massari et al., 2016). However, there is a lack of studies focusing on 6-OHDA effects on mitochondrial bioenergetics of different brain structures. Studies about the metabolic status of individual brain regions are important for the evaluation of drugs or therapies that may target energy metabolism. Therefore, this study aimed to investigate different responses against 6-OHDA damage on mitochondrial bioenergetics function. In this way, we chose three different brain structures, cortex, hippocampus and striatum of rats, to study 6-OHDA effects. These brain regions have different concentration of dopaminergic neurons, so it is possible to predict different responses to 6-OHDA mainly in mitochondria, which play a main role in cellular response against damage.

2. Materials and methods

2.1. Chemicals

2,4,5-Trihydroxyphenethylamine hydrochloride (6-OHDA), adenosine 5′-diphosphate sodium salt (ADP), pyruvic acid, antimycin A, rotenone, malonic acid and carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) were purchased from Sigma-Aldrich (St. Louis, MO). Lactate dehydrogenase (LDH) commercial kit (LDH liquiform) was purchased from Labtest® Diagnostica S.A. (Minas Gerais, Brazil). Other chemicals used in this work were purchased from local suppliers.

2.2. Animals

Male adult Wistar rats (8 weeks old, 200–250 g) from our own breeding colony were maintained in a separate animal room, with light/dark cycles of 12 h each, at a temperature of 22 ± 2 °C, with free access to food and water. This study was approved by the Ethical and Animal Welfare Committee of the Federal University of Santa Maria, Brazil, under the process number 1908310517/2017.

2.3. Preparation of cerebrocortical, hippocampal and striatal slices

Animals were killed by decapitation, the brain was removed, and the cerebral cortex, hippocampus, and striatum were dissected in ice-cold Krebs–Ringer bicarbonate buffer (KRB), this buffer is compound by 136 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 23.8 mM $NaHCO_3$, and 11 mM D-glucose. The buffer was bubbled with 95% O_2 – 5% CO_2 and established pH 7.4. Slices (0.4 mm) were prepared using a McIlwain Tissue Chopper (The Mickle Laboratory Engineering Co. ltd., England) and transferred to KRB buffer for 30 min at 37 °C to metabolic recover, before starting the experiments.

2.4. Slices exposure to 6-OHDA

In order to evaluate 6-OHDA toxic effects, slices were exposed for 1 h at 37 °C to 6-OHDA at a concentration of 100 µM diluted in KRB as described by a previous study (Massari et al., 2016).

2.5. Slices co-exposure to 6-OHDA and n-acetylcysteine (NAC)

To evaluate the effects of the concomitant exposure to NAC and 6-OHDA on high-resolution respirometry (HRR), brain slices were incubated for 1 h at 37 °C with 6-OHDA at a concentration of 100 µM diluted in KRB plus NAC at a concentration of 1 mM also diluted in KRB. The NAC concentration used in this assay was based on a previous study (Qian and Yang, 2009).

2.6. High-resolution respirometry (HRR)

The analyses were performed on O2k-system high-resolution oxygraph (Oroboros Instruments, Innsbruck, Austria). Cerebral structure slices were added to the chamber containing the respiration medium - MIR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 0.1 mg/mL fatty acid free BSA) at 37 °C. The protocol consisted of a sequential titration of multiple substrates, uncouplers and inhibitors (SUIT protocol) as described by Figs. 1A, 2A and 3A (Pesta and Gnaiger, 2011). After signal stabilization, the experimental SUIT protocol was performed by sequential addition of pyruvate (5 mM), malate (2 mM) and glutamate (10 mM); ADP (5 mM); succinate (10 mM); oligomycin (2.5 µM); carbonyl cyanide-4-(tri-fluoromethoxy) phenylhydrazone (FCCP - titrations of 0.25 µM until reaching the maximum oxygen consumption); rotenone (0.5 µM); malonate (5 mM) and antimycin (2.5 µM) (Pesta and Gnaiger, 2011; Schöpf et al., 2016). All data related to SUIT protocol were normalized by the citrate synthase activity of each sample.

2.7. Oxygen consumption related to cytochrome c oxidase

Oxygen consumption driven by cytochrome c oxidase (mitochondrial complex IV) was evaluated based on previous protocol with some adaptations (Lemieux et al., 2017). To evaluate the effects of 6-OHDA related to complex IV driven respiration, slices of hippocampus, cortex, and striatum were placed in the oroboros chambers and substrates and inhibitor were sequentially added. Glutamate (10 mM), malate (2 mM) and succinate (10 mM), and ADP (5 mM) were added to drive a coupled state, after, potassium cyanide (KCN) was used as an inhibitor in titrations of 10 µM until reaching the concentration of 100 µM. Oxygen consumption was monitored after each KCN addition.

2.8. Hydrogen peroxide (H_2O_2) production

H_2O_2 production was measured in the Oxygraph-2k (O2k, OROBOROS Instruments, Innsbruck, Austria) using the Sensor Green of the O2k-Fluo LED2-Module for fluorescence while respiration was analyzed. The H_2O_2-sensitive probe Amplex®Red was used to measure the peroxide flux (Krumschnabel et al., 2015). Around 20 mg of the cerebral structure slices (cerebrocortical, hippocampal and striatal) were placed inside oroboros chamber containing MiR05, 10 µM Amplex® Red (AmR) and 1 U/mL horseradish peroxidase (HRP). The product of the reaction between AmR and H_2O_2, catalyzed by HRP, is fluorescent, and O2k-Fluo LED2-Module is sensitive to this fluorescence difference. Through this protocol was possible to evaluate peroxide production in different steps of the HRR with the addiction of substrates and inhibitors. Experiments were performed with sequential additions of the following substrates and inhibitors: pyruvate (5 mM), malate (2.5 mM) and glutamate (10 mM); succinate (10 mM); rotenone (0.5 µM), to evaluate reverse flow in complex I, Olygomicin (2.5 µM), FCCP (0,25 µM) and malonate (5 mM), to evaluate reverse flow in complex II. All data related to peroxide production were normalized by citrate synthase activity of samples.

2.9. Enzyme activity assays and protein determination

For determination of enzyme activities and protein content, 2 mL suspension was removed from the Oxygraph-2k chamber at the end of each experiment and stored at −80 °C until further analysis. Enzyme activities were assayed at 37 °C.

Citrate synthase activity was measured at 412 nm, recording the linear reduction of 0.1 mM 5,5'dithiobis-2-nitrobenzoic acid (ε 412: 13.6 ml·cm^{-1} µmol^{-1}) in the presence of 0.10 mM acetyl-CoA, 10 mM oxalacetic acid and 0.1 M Tris/HCl, (pH 8.1) (Lemieux et al., 2017).

Lactate dehydrogenase (LDH) activity was measured at 340 nm using the commercial kit LDH liquiform (Labtest®, Diagnostica S.A., Minas Gerais, Brazil).

Protein content was determined by Bradford's test (Bradford, 1976) using serum albumin as a standard.

2.10. Statistical analysis

Statistical analysis and figures were performed using GraphPad Prism 6. Data are expressed as the Mean ± Standard Error of Mean (S.E.M.). Data were analyzed with unpaired t-test, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of 6-OHDA treatment on HRR in brain slices

The effects of 6-OHDA on HRR were analyzed in different brain regions, cortex, hippocampus, and striatum, to investigate how these brain regions are affected by the neurotoxin. HRR data have five different states (Figs. 1A, 2A and 3A): pyruvate, glutamate, malate (PMG) related to oxygen flux without ADP; oxidative phosphorylation (OXPHOS) state that is related to oxygen flux in saturated ADP concentrations; LEAK representing oxygen flux in presence of oligomycin; electron transfer system (ETS) which is related to oxygen flux in presence of FCCP as uncoupler; and oxygen residual (ROX) which is related to oxygen flux in presence of antimycin. In addition, the ratios between oxygen flux, ETS CI/ETS CI&CII and ETS CII /ETS CI&CII, clarifies the dependent oxygen flux linked to each complex (CI e CII) in ETS state.

It was observed that exposure to 6-OHDA has different effects depending on the brain structure. In the striatum, 1 h-exposure to 6-OHDA caused a significant decrease of 46% in OXPHOS CII-Linked oxygen consumption, a decreased of around 35% in OXPHOS CI&CII-Linked oxygen consumption and a decreased of 33% in oxygen consumption linked to the LEAK state (Fig. 1A). Moreover, there was a significant increase in ETS CI/ETS CI&CII ratio in striatum exposed to 6-OHDA when compare to control group (Fig. 1B). Although, there was no significant difference in striatum ETS CII /ETS CI&CII ratios between control and 6-OHDA groups (Fig. 1C).

On the other hand, hippocampus slices exposed to 6-OHDA presented a significant increase of 98% in basal oxygen flux demonstrated by PMG levels (Fig. 2A). In addition, there was a significant increase of 62,1% in oxygen consumption drive by OXPHOS CI-linked in hippocampus slices exposed to 6-OHDA. In the same way, it was possible to verify an increase in oxygen flux (about 200%) in ROX state after 6-OHDA exposure in hippocampus brain slices. The ROX state can be associated with the ROS released by mitochondria. Concerning ETS CI/ETS CI&CII and ETS CII /ETS CI&CII ratios, there was no significant difference between control and 6-OHDA groups in the hippocampus (Fig. 2B and C).

In cortex slices exposed to 6-OHDA, it was possible to verify a significant increase of 60% in OXPHOS CII-Linked oxygen consumption. In the same way, the oxygen flux increased about 87% in OXPHOS CI&CII Linked (Fig. 3A), and an increase of 49% in ETS CII-Linked in slices exposed to 6-OHDA (Fig. 3A). Besides, there was an increase in oxygen flux of 100% in the ROX state, which is associated with increased ROS production. ETS CI/ETS CI&CII and ETS CII /ETS CI&CII ratios were not significantly affected by 6-OHDA in the cortex (Fig. 3B and C).

3.2. Effects of 6-OHDA on oxygen flux related to cytochrome c oxidase in brain slices

There were no significant effects on oxygen consumption driven by mitochondrial complex IV in brain slices in response to 6-OHDA exposure. The titration with cyanide, until the final concentration of 100 µM, decreased oxygen consumption around 50%, both in control group and 6-OHDA group in striatum (Fig. 4A), hippocampus (Fig. 4B) and cortex (Fig. 4C) brain slices.

3.3. Effects of 6-OHDA on peroxide production in brain slices

Peroxide production was evaluated in brain slices using the fluorescent probe Amplex® Red. There was an increase of 280% in peroxide production in striatum brain slices exposed to 6-OHDA when compared with the control group (Fig. 5A). At the same time, striatum slices exposed to 6-OHDA presented a decrease of 54,53% in peroxide production induced by reverse flow related to CI−Q junction (Fig. 5B).

Hippocampus slice exposed to 6-OHDA presented a significant increase of 292% in peroxide production compared to the control group (Fig. 5C). However, there was no significant difference in peroxide production related to reverse flow in CI−Q junction (Fig. 5D).

Cortex brain slices exposed to 6-OHDA presented a significant increase of 227% in peroxide production (Fig. 5E). Nevertheless, 6-OHDA exposure did not induce any significant alteration in cortex slices peroxide production related to reverse flow in CI-Q junction (Fig. 5F).

The peroxide production linked to reverse flow is associated with mitochondrial peroxide release after rotenone inhibition. We aimed to demonstrate the complex I capacity of electron transfer through the Q-junction. The amount of peroxide formed after inhibition is strongly associated with the complex I capacity to carry electrons through mitochondrial respiratory chain before inhibition. For example, a decrease in peroxide formation after rotenone inhibition in slices treated with 6-OHDA indicates an impairment in CI.

3.4. Effects of 6-OHDA on citrate synthase and lactate dehydrogenase activities in brain slices

Exposure to 6-OHDA significantly increased lactate dehydrogenase activity in cortex brain slices (Fig. 6F) but, had no effect on citrate synthase activity (Fig. 6E). On the other hand, there were no significant alterations on citrate synthase and lactate dehydrogenase activities in hippocampus and striatum brain slices after 6-OHDA exposure (Fig. 6C and D and Fig. 6A and B).

3.5. Effects of co-exposure to 6-OHDA and NAC on HRR

In order to test if the increase in ROX respiration observed in HRR experiments was a result of 6-OHDA exposure, we performed an assay using NAC as an antioxidant. Brain slices were co-incubated with 6-OHDA and NAC and the HRR parameters were evaluated. Co-exposure to 6-OHDA and NAC did not cause any significant alteration in HRR parameters in striatum (Fig. 7A), hippocampus (Fig. 7B) and cortex (Fig. 7C) when compared to the control. These results support the hypothesis that the alterations caused by 6-OHDA on mitochondrial function are related to ROS production.

4. Discussion

The different regions of the brain are responsible for distinct behaviors and actions. For instance, frontal cortex is responsible to decision, planning and judgment, hippocampus is responsible to memory, emotion and learning, and striatum is responsible to movement and

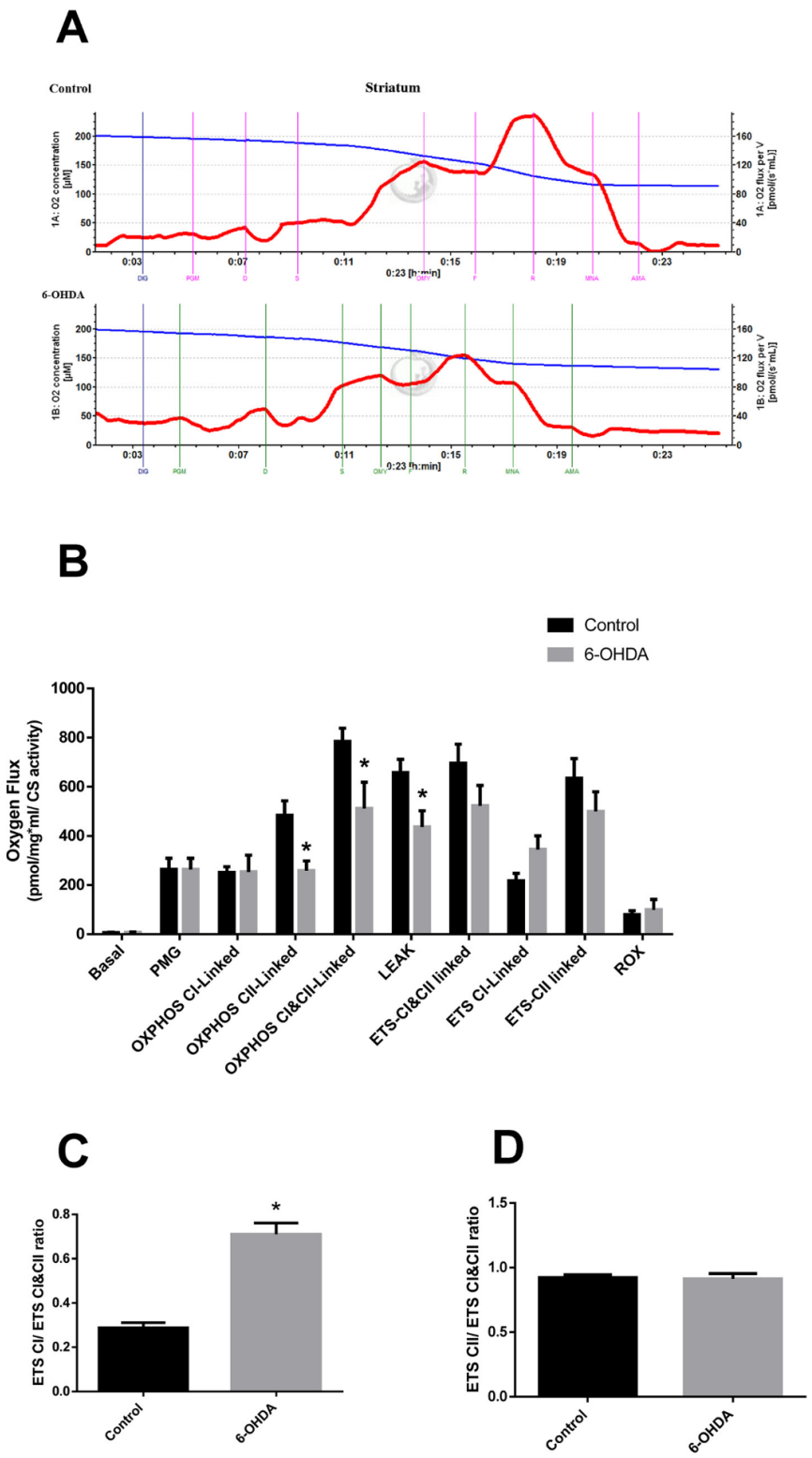

Fig. 1. Effects of 6-OHDA exposure in striatal brain slices HRR. (A) Oxygen consumption of high resolution respirometry (HRR) protocol by oroboros. Blue line represents oxygen concentration inside to oroboros chambers and red line represents electron transfer related to oxygen flux response to different substrates addition. (B) SUIT protocol result: Basal is the state without any substrates. Pyruvate, glutamate and malate were used to evaluate oxygen flux without phosphorylation (PMG). OXPHOS represents coupled states dependent on different mitochondrial substrates, pyruvate, glutamate, malate (OXPHOS CI-Linked) and succinate (OXPHOS CII-Linked and OXPHOS CI&CII-Linked) in presence of saturated ADP concentrations. LEAK is the state related to ATP-synthase inhibition by olygomicin. ETS represents maximum oxygen consumption by addition of the uncoupler FCCP (ETS CI&CII-Linked), in sequence it is demonstrated the maximum oxygen consumption related to the complex I inhibitor rotenone (ETS CI-Linked) and maximum oxygen consumption related to complex II inhibition by malonate (ETS CII-Linked), lastly, antimycin was used to inhibit complex III obtaining oxygen residual flux (ROX). (C) Ratio of values obtained in ETS CI divided by values obtained in ETS CI&CII. (D) Ratio of values obtained in ETS CII divided by values obtained in ETS CI&CII. Data are reported as mean ± S.E.M., n = 4–5. *Indicates $p < 0.05$ as compared to the control group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

cognition (Pandya et al., 2016; Zheng et al., 2018). These different functional roles played by the brain regions require appropriated energetic demands. In this way, mitochondrial metabolism and bioenergetics, may not be equal among brain regions, especially when brain is subject to impairment by drugs, aging, metabolic syndrome or neurodegenerative diseases (da Silva et al., 2012; Etchegoyen et al., 2018; Pandya et al., 2016).

Mitochondria are central regulators of energy and cell homeostasis

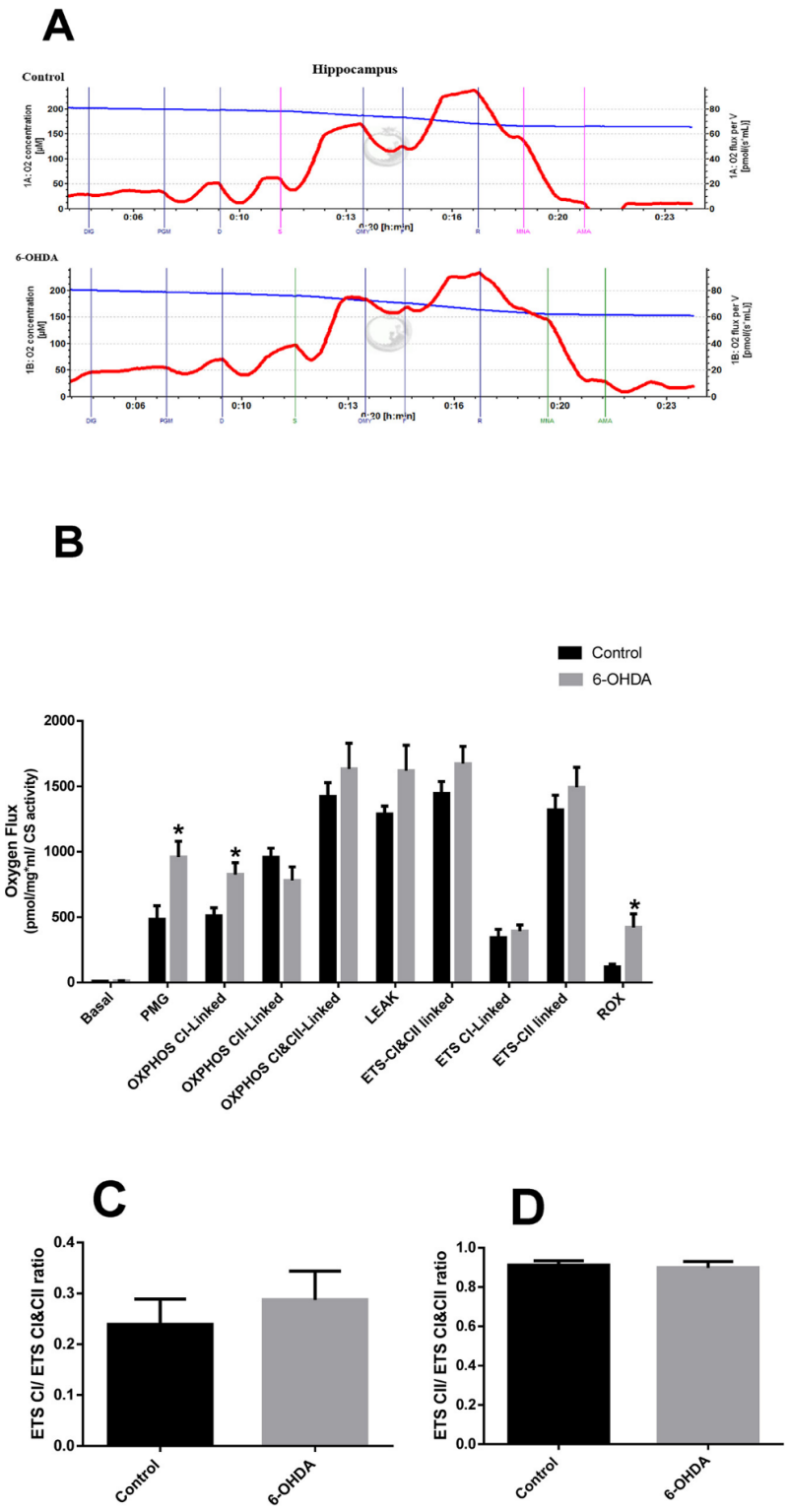

Fig. 2. Effects of 6-OHDA exposure in hippocampus brain slices HRR. (A) Oxygen consumption of high resolution respirometry (HRR) protocol by oroboros. Blue line represents oxygen concentration inside to oroboros chambers and red line represents electron transfer related to oxygen flux response to different substrates addition. (B) SUIT protocol result: Basal is the state without any substrates. Pyruvate, glutamate and malate were used to evaluate oxygen flux without phosphorylation (PMG). OXPHOS represents coupled states dependent on different mitochondrial substrates, pyruvate, glutamate, malate (OXPHOS CI-Linked) and succinate (OXPHOS CII-Linked and OXPHOS CI&CII-Linked) in presence of saturated ADP concentrations. LEAK is the state related to ATP-synthase inhibition by oligomicin. ETS represents maximum oxygen consumption by addition of the uncoupler FCCP (ETS CI&CII-Linked), in sequence it is demonstrated the maximum oxygen consumption related to the complex I inhibitor rotenone (ETS CI-Linked) and maximum oxygen consumption related to complex II inhibition by malonate (ETS CII-Linked), lastly, antimycin was used to inhibit complex III obtaining oxygen residual flux (ROX). (C) Ratio of values obtained in ETS CI divided by values obtained in ETS CI&CII. (D) Ratio of values obtained in ETS CII divided by values obtained in ETS CI&CII. Data are reported as mean ± S.E.M., n = 4–5. *Indicates $p < 0.05$ as compared to the control group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

and evidences support the critical role of mitochondrial dysfunction in neurodegenerative diseases, such as PD (Féger et al., 2002; Gautier et al., 2008; Requejo-Aguilar and Bolaños, 2016). Some studies compared PD effects in different brain regions (Massari et al., 2016; Romuk et al., 2017), although, the effects of PD on mitochondrial bioenergetics in brain regions was never investigated before. The stressors and diseases may target mitochondria energy metabolism in distinct ways with respect to different structures of the brain. Therefore, the understanding

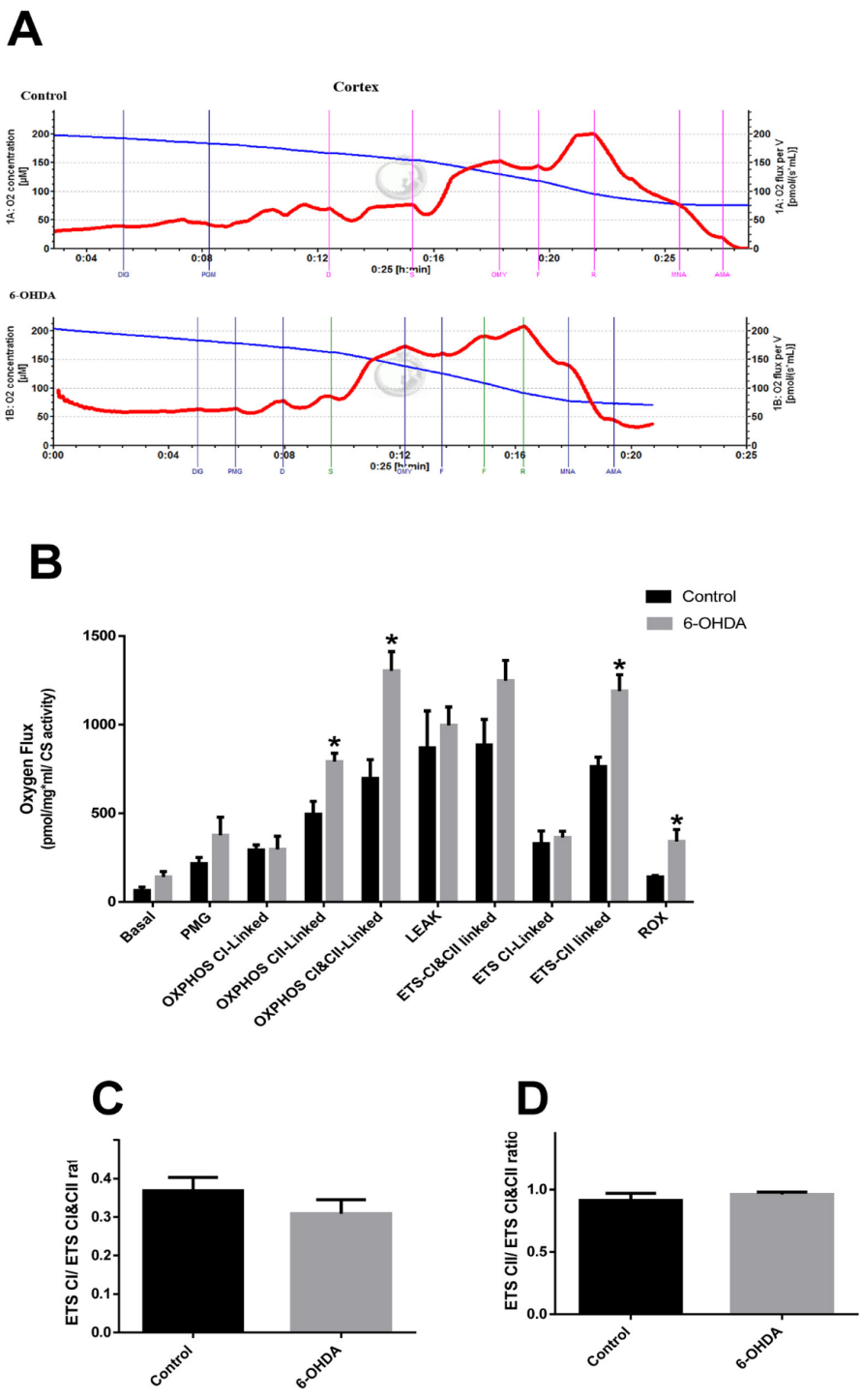

Fig. 3. Effects of 6-OHDA exposure in cortical brain slices HRR. (A) Oxygen consumption of high resolution respirometry (HRR) protocol by oroboros. Blue line represents oxygen concentration inside to oroboros chambers and red line represents electron transfer related to oxygen flux response to different substrates addition. (B) SUIT protocol result: Basal is the state without any substrates. Pyruvate, glutamate and malate were used to evaluate oxygen flux without phosphorylation (PMG). OXPHOS represents coupled states dependent on different mitochondrial substrates, pyruvate, glutamate, malate (OXPHOS CI-Linked) and succinate (OXPHOS CII-Linked and OXPHOS CI&CII-Linked) in presence of saturated ADP concentrations. LEAK is the state related to ATP-synthase inhibition by oligomicin. ETS represents maximum oxygen consumption by addition of the uncoupler FCCP (ETS CI&CII-Linked), in sequence it is demonstrated the maximum oxygen consumption related to the complex I inhibitor rotenone (ETS CI-Linked) and maximum oxygen consumption related to complex II inhibition by malonate (ETS CII-Linked), lastly, antimycin was used to inhibit complex III obtaining oxygen residual flux (ROX). (C) Ratio of values obtained in ETS CI divided by values obtained in ETS CI&CII. (D) Ratio of values obtained in ETS CII divided by values obtained in ETS CI&CII. Data are reported as mean ± S.E.M., n = 4–5. *Indicates $p < 0.05$ as compared to the control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

of the mitochondrial role in neurodegenerative diseases and how it modifies metabolic brain conditions in the different brain regions are important issues to be addressed (Bulteau et al., 2017; Karbowski, 2007).

In this work, we used an *in vitro* PD model with 6-OHDA to study the mitochondrial bioenergetics function in brain slices of cortex, hippocampus, and striatum. 6-OHDA is a neurotoxic compound molecularly similar to the neurotransmitter dopamine, which means that 6-OHDA

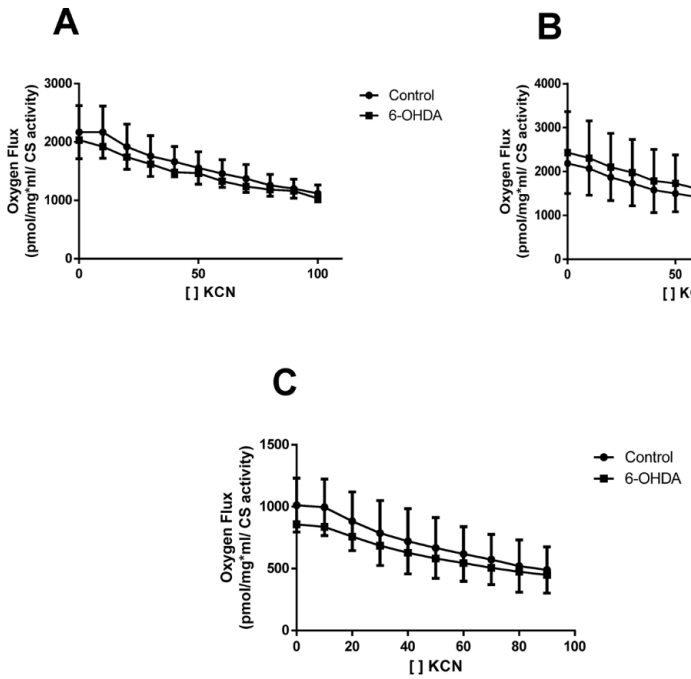

Fig. 4. Effects of 6-OHDA exposure in cytochrome c oxidase. Oxygen flux drive by cytochrome c oxidase activity in brain slices of striatum (A), hippocampus (B) and cortex (C). Potassium cyanide (KCN) was used as inhibitor of cytochrome c oxidase. We used KCN titrations of 10 μM, each value of titration in X axis corresponds to oxygen flux in Y axis. Data are reported as mean ± S.E.M of 4 different assays.

manly affects dopaminergic neurons mimicking some PD symptoms (Massari et al., 2016). Our results indicate distinct mitochondrial responses among the different brain regions due to 6-OHDA insult.

In the hippocampus it was not observed significant changes in mitochondrial functionality after 1 h of exposure to 6DA since oxygen flux in the OXPHOS CI&CII-Linked was not altered. However, 6-OHDA induced an increase in oxygen consumption related to mitochondrial proton leak, observed after PMG addition (Fig. 2A), and ROX state (Fig. 2A) which is associated with increased ROS levels. Indeed, as evidenced by the assay with Amplex® Red, hippocampus exposed to 6-OHDA presented increased peroxide production (Fig. 5C). The fact that exposure to 6-OHDA did not affect enzymatic activities of CS and LDH (Fig. 6C and D) in hippocampus slices suggest that 6-OHDA effects on hippocampus do not generate compensatory or adaptive mitochondrial response due to augmented ROS generation. When we exposed hippocampus slices to 6-OHDA associated with NAC there were no differences in mitochondrial parameters evaluated by HRR (Fig. 7B). This result indicates that 6-OHDA effects on hippocampus brain slices are mainly related to increased ROS production

Previous study demonstrated that the hippocampus is the brain structure in rats most impaired during aging (Pandya et al., 2016). Moreover, the hippocampus is important in memory formation and learning (Teixeira et al., 2018; Velazquez et al., 2018), which are abilities early affected in PD (GOETZ, 2011; Kalia and Lang, 2015). Our results corroborate these observations since the reduced mitochondrial adaptability would render the hippocampus more susceptible to ROS production and damage resulting in impaired function when treated with 6-OHDA.

On the other side, cortex slices exposed to 6-OHDA presented mitochondrial compensatory effects or adaptation perhaps as an attempt to generate energy. This hypothesis is sustained by results of oxygen flux related to oxidative phosphorylation since, after 1 hour of exposure to 6-OHDA, brain cortex slices presented an increase in oxygen flux through OXPHOS CI&CII-Linked and ETS CII-Linked (Fig. 3A). Additionally, there was a significant increase in cortical LDH activity after exposure to of 6-OHDA (Fig. 6F), reinforcing the idea of mitochondrial adaptation to increase energy generation. Lactate is an important energy source during ischemia and has been considered necessary for neuronal signaling and plasticity (Magistretti and Allaman, 2018).

The association of 6-OHDA and NAC in cortex brain slices prevented 6-OHDA effects on HRR parameters (Fig. 7C), supporting the idea that in cortex, ROS production is capable to initiate a survival signaling pathway. An increase in ROS production can lead to activation of several survival pathways, one of them is related to activation of the hypoxia-inducible factor (HIF) which is an important transcription factor that regulates cellular metabolism and cell survival under hypoxic stress (Trachootham et al., 2008). HIF plays a major role in metabolic energy source by exchanging glucose metabolites from mitochondrial respiration to cytosolic glycolysis. This metabolic exchange to the glycolytic pathway increases lactate levels (Lu et al., 2002). It is possible that in the cortex, after 1 h of 6-OHDA exposure, ROS levels increase could activate HIF explaining, in this way, the increase in LDH levels.

Based on our evidences and other studies (Devine et al., 2011; Requejo-Aguilar et al., 2014; Requejo-Aguilar and Bolaños, 2016), we believed that cortex slices exposed to 6-OHDA activates a mitochondrial compensatory effect to generate energy which is demonstrated by the increase in oxygen flux and by the exchange to other energy sources, such as lactate, demonstrated by increased LDH activity.

Moreover, the exchange of metabolic pathways to generate energy has been demonstrated in diseases such as PD (Requejo-Aguilar and Bolaños, 2016; Wang et al., 2011). The energetic pathway exchange of mitochondrial oxidative phosphorylation to a glycolytic energy source (Requejo-Aguilar et al., 2014) was previously described in PD and in cancer cells (Devine et al., 2011). Previous study indicated that in a normal situation there is a minimal mitochondrial energetic response difference among different brain regions isolated from rat brain (Sauerbeck et al., 2011). Contrariwise, other study demonstrated differences in regional brain metabolism, with evidence that the cortex seems to spend more energy than others brain regions (Karbowski, 2007). Moreover, the highest levels of aerobic glycolysis in the normal human brain reside in cortical systems. The explanation for this different metabolism has been associated with the cortex role of organization of the brain functions (Vaishnavi et al., 2010).

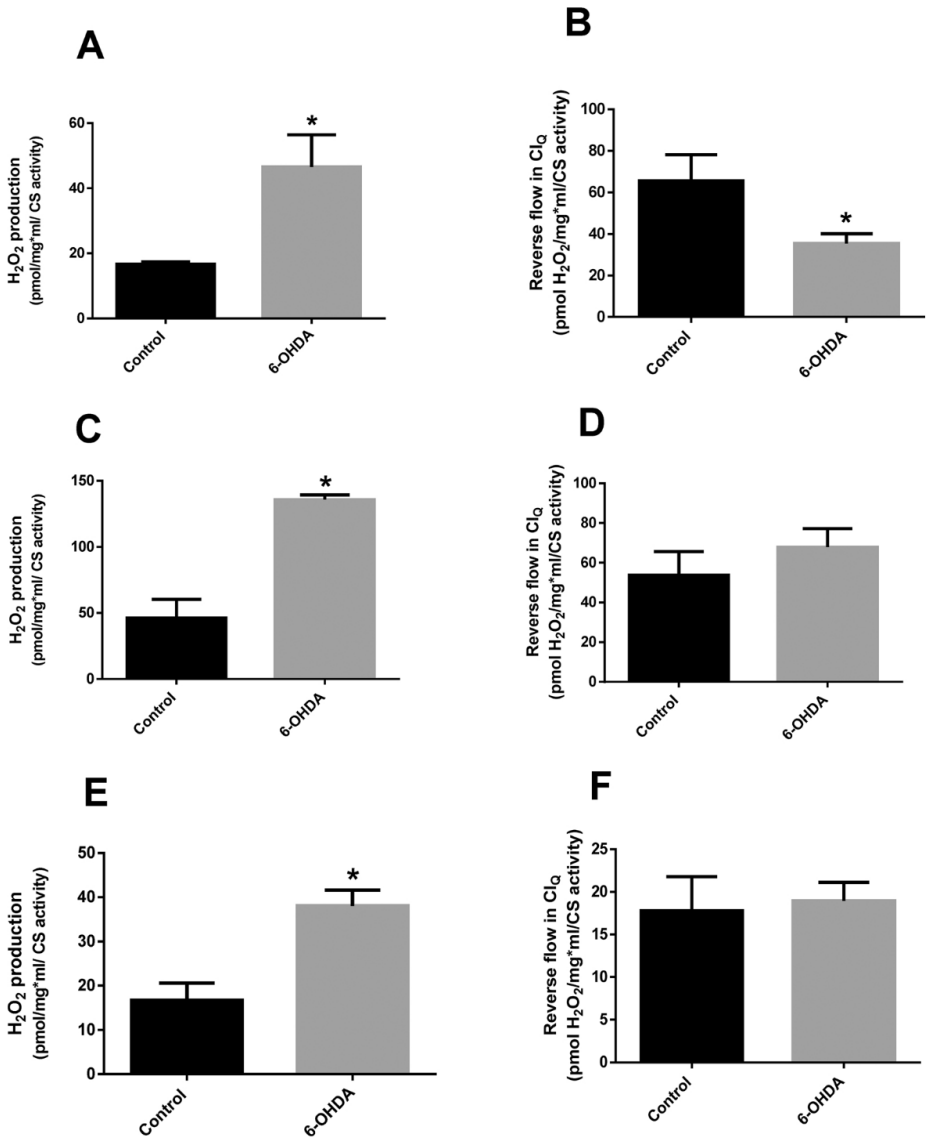

Fig. 5. Effects of 6-OHDA in brain slices peroxide production. Endogenous peroxide production, without substrates addition in striatum (A), hippocampus (C) and cortex (E) brain slices. Peroxide production by reverse flow related to CI−Q junction in striatum (B), hippocampus (D) and cortex (F) brain slices, determined by addition of substrates related to complex I (pyruvate, glutamate and malate) and complex II (succinate) and CI inhibitor rotenone. Data are reported as mean ± S.E.M., n = 4. *Indicates $p < 0.05$ as compared to the control group.

Furthermore, cortical slices exposed to 6-OHDA presented an increase in peroxide production (Fig. 5E) when compared to the control group. This increase in peroxide production in cortex after exposure to 6-OHDA was less pronounced than other brain regions tested in this work. According to other studies, ROS may promote cellular adaptations to stress conditions, by the regulation of oxidative metabolism supporting cell survival (Gutteridge and Halliwell, 2018; Makrecka-Kuka et al., 2015; Radak et al., 2016).

Even though 6-OHDA exposure increased the peroxide production in the striatum (Fig. 5A), which is in accordance with other studies (Ammal Kaidery and Thomas, 2018; Li et al., 2014; Massari et al., 2016), striatum showed a different response when compared to cortex. Different from cortex, it seems that striatum does not present any mitochondrial adaptation response against 6-OHDA damage. The increase of peroxide production in striatum slices treated with 6-OHDA was 280% higher than control (Fig. 5A). We believe that this high peroxide production in striatum was able to cause mitochondrial damage instead to mitochondrial adaptation.

Moreover, 6-OHDA exposure in striatum also induced a significant decrease in reverse flow linked to CI-Q junction after rotenone addition (Fig. 5B). This decrease in peroxide levels related to reverse flow may indicate that the general increase in peroxide production (Fig. 5A) was not caused by reverse electron flow in CI-Q junction. We believe that the amount of peroxide production in the striatum after 6-OHDA exposure could be linked to extramitochondrial cellular compartments. It was demonstrated previously that mitochondrial and cytosolic ROS formation have opposing effects on lifespan, the mitochondrial ROS formation was able to increase lifespan in Caenorhabditis elegans probably by activating cellular survival pathways, on the other hand, cytosolic ROS formation did not present the same effect in lifespan,

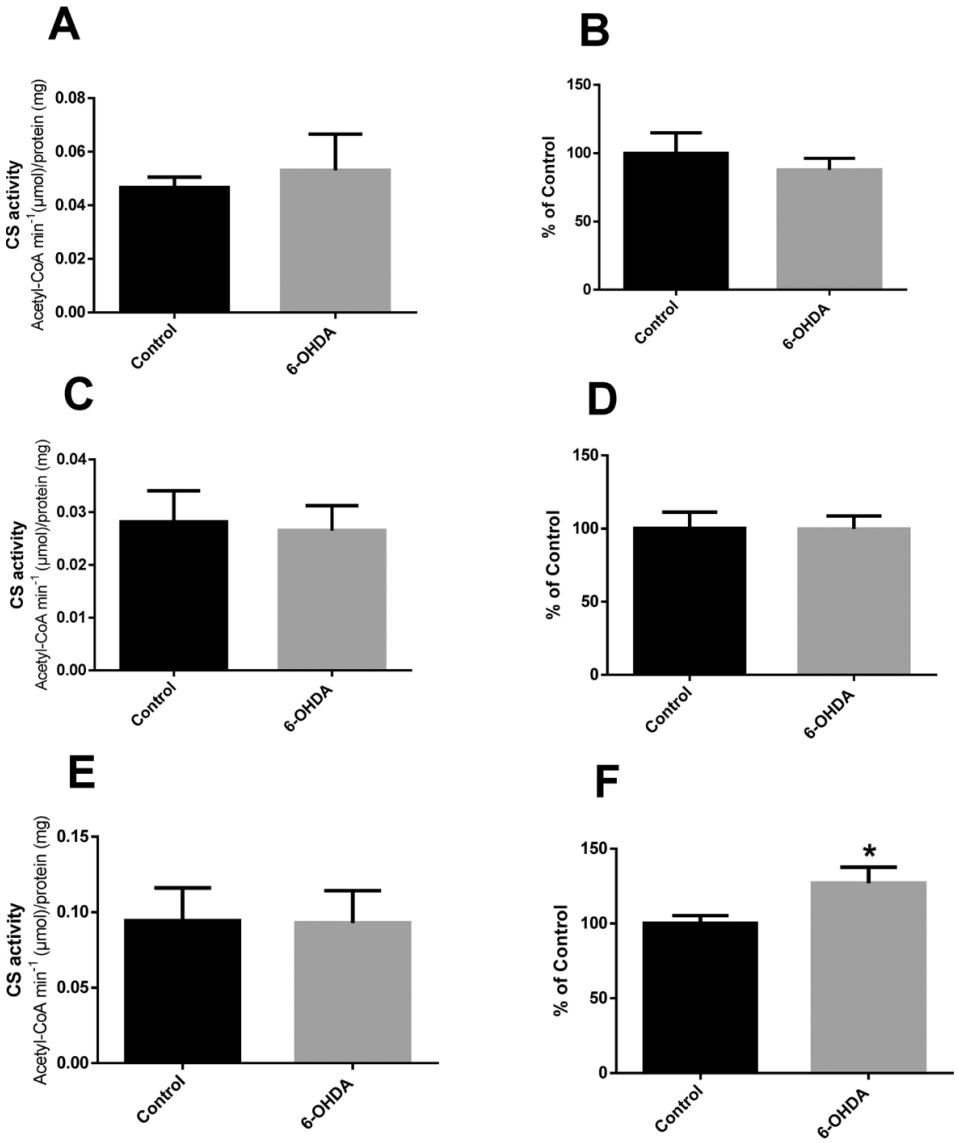

Fig. 6. Effects of 6-hydroxydopamine (6-OHDA) exposure in citrate synthase activity of striatal (A), hippocampus (C) and cortex (E) brain slices and, in lactate dehydrogenase activity of striatum (B), hippocampus (D) and cortex (F). Data are reported as mean ± S.E.M., n = 7.

(Schaar et al., 2015).

The striatum is normally the brain region more affected by 6-OHDA due to the higher number of dopaminergic neurons which are the main targets of this neurotoxin. The same occurs in PD, with striatum being the brain region most affected by the loss of dopaminergic neurons (Féger et al., 2002; Hawlitschka and Wree, 2018). In agreement with this, our results showed that striatum slices exposed to 6-OHDA presented a decrease in oxygen flux related OXPHOS CI&CII-Linked (Fig. 1) and oxygen flux related to ATP-synthase represented by the LEAK state (Fig. 1) indicating a decrease in mitochondrial functionality. Additionally, the ETS ratio evidenced an increase in CI participation on ETS state in the striatum after 6-OHDA exposure. We believe that this result is related to a decrease in mitochondrial excess capacity. Co-exposure with NAC abolished 6-OHDA effects in striatal slices most probably due to NAC antioxidant properties (Fig. 7A). At the same time, 6-OHDA exposure did not cause alterations in CS and LDH activities (Fig. 6A and B) in striatum, suggesting that this brain region cannot adapt to 6-OHDA damage.

Similarly, another work (Singh et al., 2010) demonstrated that striatal neurons exposed to 3-nitropropionic acid showed a higher mitochondrial vulnerability than cortical neurons, indicating a different energetic response against injury. Additionally, it was suggested that in neurodegenerative disease, specific nervous system components or brain regions appear to be more susceptible to the pathological process triggering. Such specific susceptibility of the brain regions to different kinds of injury is related to cell metabolism and differences in mitochondrial capacity to produce energy (Dubinsky, 2009)

5. Conclusion

In conclusion, our study demonstrated the different response related to mitochondrial bioenergetics in distinct brain regions after exposure to 6-OHDA *in vitro*. Brain regions have different metabolism and respond in different ways to 6-OHDA toxicity. The findings of this work are important to understand how mitochondrial function in the different brain regions is affected in a model of PD induced by 6-OHDA,

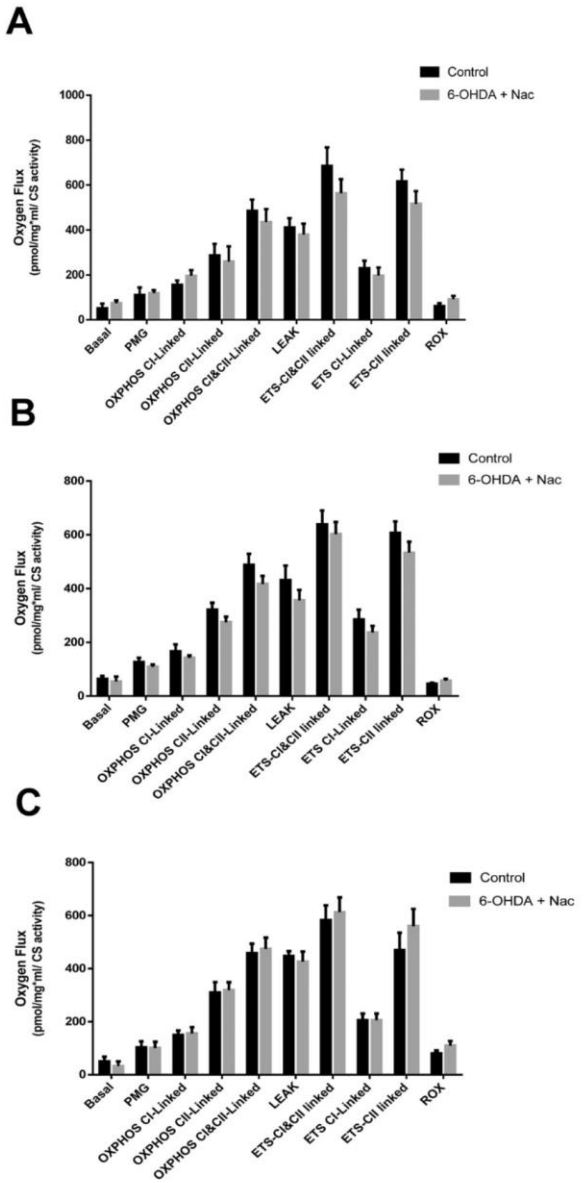

Fig. 7. Effects of 6-OHDA and NAC co-exposure in striatum (A) hippocampus (B) and cortex (C) brain slices HRR. SUIT protocol result: Basal is the state without any substrates. Pyruvate, glutamate and malate were used to evaluate oxygen flux without phosphorylation (PMG). OXPHOS represents coupled states dependent on different mitochondrial substrates, pyruvate, glutamate, malate (OXPHOS CI-Linked) and succinate (OXPHOS CII-Linked and OXPHOS CI&CII-Linked) in presence of saturated ADP concentrations. LEAK is the state related to ATP-synthase inhibition by oligomicin. ETS represents maximum oxygen consumption by addition of the uncoupler FCCP (ETS CI&CII-Linked), in sequence it is demonstrated the maximum oxygen consumption related to the complex I inhibitor rotenone (ETS CI-Linked) and maximum oxygen consumption related to complex II inhibition by malonate (ETS CII-Linked), lastly, antimycin was used to inhibit complex III obtaining oxygen residual flux (ROX). Data are reported as mean ± S.E.M., n =. 4–5.

expanding the knowledge about the involvement of mitochondrial brain metabolism in experimental PD models.

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References

Ammal Kaidery, N., Thomas, B., 2018. Current perspective of mitochondrial biology in Parkinson's disease. Neurochem. Int. 117 (14 march) 91-117.
Blandini, F., Armentero, M.-T., Martignoni, E., 2008. The 6-hydroxydopamine model: news from the past. Parkinsonism Relat. Disord. 14 (Suppl. 2), S124–9.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254 7 maio.
Bulteau, A.-L., et al., 2017. Dysfunction of mitochondrial Lon protease and identification of oxidized protein in mouse brain following exposure to MPTP: implications for Parkinson disease. Free Radic. Biol. Med. 108 (30 July), 236–246.
Da Silva, M.H., et al., 2012. Acute brain damage induced by acetaminophen in mice: effect of diphenyl diselenide on oxidative stress and mitochondrial dysfunction. Neurotox. Res. 21 (3), 334–344 abr.
Devine, M.J., Plun-Favreau, H., Wood, N.W., 2011. Parkinson's disease and cancer: two wars, one front. Nat. Rev. Cancer 11 (11), 812–823 24 out.
Dubinsky, J.M., 2009. Heterogeneity of nervous system mitochondria: location, location, location!. Exp. Neurol. 218 (2), 293–307 ago.
Etchegoyen, M., et al., 2018. Metabolic syndrome and neuroprotection. Front. Neurosci. 12, 196 20 abr.
Féger, J., et al., 2002. Experimental models of Parkinson's disease. Ann. Pharm. Fr. 60 (january 1), 3–21.
Feitosa, C.M., et al., 2018. Determination of parameters of oxidative stress in vitro models of neurodegenerative diseases-a review. Curr. Clin. Pharmacol. 13 (1 March).
Gautier, C.A., Kitada, T., Shen, J., 2008. Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. Proc. Natl. Acad. Sci. 105 (32), 11364–11369 12 ago.
Goetz, C.G., 2011. The history of Parkinson's disease: early clinical descriptions and neurological therapies. Cold Spring Harb. Perspect. Med. 1 (1), 1–15 a008862, 1 set.
Gutteridge, J.M.C., Halliwell, B., 2018. Oxidative stress, redox stress or redox success? Biochem. Biophys. Res. Commun. 502 (2), 183–186 9 maio.
Hao, X.-M., et al., 2017. Neuroprotective effect of α-mangostin on mitochondrial dysfunction and α-synuclein aggregation in rotenone-induced model of Parkinson's disease in differentiated SH-SY5Y cells. J. Asian Nat. Prod. Res. 19 (8), 833–845 3 ago.
Hawlitschka, A., Wree, A., 2018. Experimental intrastriatal applications of botulinum neurotoxin-A: a review. Int. J. Mol. Sci. 19 (5) 1392 7 maio.
Kalia, L.V., Lang, A.E., 2015. Parkinson's disease. Lancet 386 (9996), 896–912 29 ago.
Karbowski, J., 2007. Global and regional brain metabolic scaling and its functional consequences. BMC Biol. 5 (1), 18 9 maio.
Krumschnabel, G., et al., 2015. Simultaneous high-resolution measurement of mitochondrial respiration and hydrogen peroxide production. Methods Mol. Biol. (Clifton, N.J.) 1264, 245–261 s.l: s.n.
Lehmensiek, V., et al., 2006. Dopamine transporter-mediated cytotoxicity of 6-hydroxydopamine in vitro depends on expression of mutant alpha-synucleins related to Parkinson's disease. Neurochem. Int. 48 (5), 329–340 abr.
Lemieux, H., et al., 2017. Impairment of mitochondrial respiratory function as an early biomarker of apoptosis induced by growth factor removal. bioRxiv(18 June), 151480.
Li, D.-W., et al., 2014. Guanosine exerts neuroprotective effects by reversing mitochondrial dysfunction in a cellular model of Parkinson's disease. Int. J. Mol. Med. 34 (5), 1358–1364 ago.
Lisowski, P., et al., 2018. Mitochondria and the dynamic control of stem cell homeostasis. EMBO Rep. 16, e45432 abr.
Lu, H., Forbes, R.A., Verma, A., 2002. Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. J. Biol. Chem. 277 (26), 23111–23115 28 jun.
Magistretti, P.J., Allaman, I., 2018. Lactate in the brain: from metabolic end-product to signalling molecule. Nat. Rev. Neurosci. 19 (4), 235–249 8 abr.
Makrecka-Kuka, M., Krumschnabel, G., Gnaiger, E., 2015. High-resolution respirometry for simultaneous measurement of oxygen and hydrogen peroxide fluxes in permeabilized cells, tissue homogenate and isolated mitochondria. Biomolecules 5 (4), 1319–1338 29 jun.
Massari, C.M., et al., 2016. In vitro 6-hydroxydopamine-induced toxicity in striatal, cerebrocortical and hippocampal slices is attenuated by atorvastatin and MK-801.

Toxicol. Vitro 37, 162–168 dez.
Morroni, F., et al., 2018. Comparison of adaptive neuroprotective mechanisms of sulforaphane and its interconversion product erucin in *in vitro* and *in vivo* models of Parkinson's disease. J. Agric. Food Chem. 66 (January (4)), 856–865.
Pandya, J.D., et al., 2016. Age- and brain region-specific differences in mitochondrial bioenergetics in Brown Norway rats. Neurobiol. Aging 42 (1 june), 25–34.
Pesta, D., Gnaiger, E., 2011. High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. Methods Mol. Biol. 810 (3 October), 25–58.
Qian, H., Yang, Y., 2009. Neuron differentiation and neuritogenesis stimulated by N-acetylcysteine (NAC). Acta Pharmacol. Sin. 30 (july 7), 907–912.
Radak, Z., et al., 2016. Physical exercise, reactive oxygen species and neuroprotection. Free Radic. Biol. Med. 98, 187–196 set.
Requejo-Aguilar, R., et al., 2014. PINK1 deficiency sustains cell proliferation by reprogramming glucose metabolism through HIF1. Nat. Commun. 5 (july), 4514–4524.
Requejo-Aguilar, R., Bolaños, J.P., 2016. Mitochondrial control of cell bioenergetics in Parkinson's disease. Free Radic. Biol. Med. 100 (november), 123–137.
Riederer, P., et al., 2018. Lateralisation in Parkinson disease. Cell Tissue Res. 14 abr.
Romuk, E.B., et al., 2017. The evaluation of the changes in enzymatic antioxidant reserves and lipid peroxidation in chosen parts of the brain in an animal model of Parkinson disease. Adv. Clin. Exp. Med. 26 (6), 953–959 set.
Sauerbeck, A., et al., 2011. Analysis of regional brain mitochondrial bioenergetics and susceptibility to mitochondrial inhibition utilizing a microplate based system. J. Neurosci. Methods 198 (1), 36–43 15 maio.
Schaar, C.E., et al., 2015. Mitochondrial and cytoplasmic ROS have opposing effects on lifespan. PLoS Genet. 11 (2), e1004972 11 fev.
Schöpf, B., et al., 2016. Oxidative phosphorylation and mitochondrial function differ between human prostate tissue and cultured cells. FEBS J. 283 (june 11), 2181–2196.
Simuni, T., et al., 2018. Longitudinal change of clinical and biological measures in early Parkinson's disease: parkinson's progression markers initiative cohort. Mov. Disord. 33 (March (5)), 771–782.
Singh, S., et al., 2010. Brain region specificity of 3-nitropropionic acid-induced vulnerability of neurons involves cytochrome c oxidase. Neurochem. Int. 57 (3), 297–305 out.
Teixeira, C.M., et al., 2018. Hippocampal 5-HT input regulates memory formation and schaffer collateral excitation. Neuron 98, 992–1004 8 maio.
Teves, J.M.Y., et al., 2018. Parkinson's disease skin fibroblasts display signature alterations in growth, redox homeostasis, mitochondrial function, and autophagy. Front. Neurosci. 11 (12 January), 737.
Trachootham, D., et al., 2008. Redox regulation of cell survival. Antioxid. Redox Signal. 10 (8), 1343–1374 ago.
Ungerstedt, U., 1968. 6-hydroxy-dopamine induced degeneration of central monoamine neurons. Eur. J. Pharmacol. 5 (1), 107–110 1 dez.
Vaishnavi, S.N., et al., 2010. Regional aerobic glycolysis in the human brain. Proc. Natl. Acad. Sci. 107 (41), 17757–17762 12 out.
Velazquez, R., et al., 2018. Acute tau knockdown in the hippocampus of adult mice causes learning and memory deficits. Aging Cell e12775 10 maio.
Wang, H.-L., et al., 2011. PARK6 PINK1 mutants are defective in maintaining mitochondrial membrane potential and inhibiting ROS formation of substantia nigra dopaminergic neurons. Biochim. Biophys. Acta 1812 (june 6), 674–684.
Wu, H.-M., et al., 2018. Mitochondrial DNA variants modulate genetic susceptibility to Parkinson's disease in Han Chinese. Neurobiol. Dis. 114 (june), 17–23.
Zheng, X., et al., 2018. A Comparative study for striatal-direct and -indirect pathway neurons to DA depletion-induced lesion in a PD rat model. Neurochem. Int. 16 abr.

4.2. MANUSCRITO 1: Mitochondrial function and cellular energy maintenance during aging in a *Drosophila melanogaster* model of Parkinson Disease

Mitochondrial function and cellular energy maintenance during aging in a *Drosophila melanogaster* model of Parkinson Disease

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Abstract: Parkinson's disease (PD) is a common neurodegenerative disease characterized by movement disorders, as well as loss of dopaminergic neurons. Moreover, genes affecting mitochondrial function such as SNCA, Parkin, PINK1, DJ-1 and LRRK2, were demonstrated to be associated with PD and other neurodegenerative disease. Additionally, mitochondrial dysfunction and cellular energy imbalance are common marker found in PD. In this study, we used *pink1* null mutants of *Drosophila melanogaster* as Parkinson's disease model, to investigate how the energetic pathways and mitochondrial functions changes during aging in a PD model. In our study the loss of pink1 gene decreased survival percent and decreased climbing index during aging in *pink1* flies. Furthermore, there was an impairment in mitochondrial function demonstrated by a decrease in OXPHOS CI&CII-Linked and ETS CI&CII-Linked in *pink1* flies on 3, 15 and 30 days of life. Interestingly, OXPHOS CII-Linked and ETS CII-Linked presented a decrease only on 15 day of life in *pink1* flies. Moreover, there was an increase in peroxide (H_2O_2) levels in *pink1* flies on 15 and 30 days of life. Loss of pink1 gene also decreased activity of citrate synthase (CS) and increase activity of lactate dehydrogenase (LDH) in *pink1* flies on 15 days of life, otherwise, there was an increase on CS activity and a decrease of LDH activity on 30 day of life. Our results demonstrated a metabolic shift on ATP production in *pink1* flies which change from oxidative to glycolytic pathways at 15^{th} days of age.

Keywords: aging; cellular bioenergetics; mitochondrial function; PINK1

1. Introduction

First described in 1862 by James Parkinson in his essay describing cases of "shaking palsy" [1], Parkinson's disease (PD) was considered only a sporadic disease related mainly to aging process. The PD is the second most common neurodegenerative disease in the world affecting 2-3% of the population over 65 years of age [2]. PD is characterized by movement disorders as bradykinesia, as well neuronal loss mainly of dopaminergic neurons in substantia nigra [3], [4]. Accumulation of unfolded protein as α-synuclein which form cellular aggregates inclusions in neurons, also are considered a neuropathological feature of PD [5].

The clinical diagnosis of PD relies on motor symptoms and neurological impairment but, there are multiple pathways and several molecular mechanisms behind these pathophysiological symptoms [2], [6]. The cellular metabolic dysfunction characterized by the impairment of the cellular energy balance is one of the mechanisms involved in PD [7]. Usually, the energy imbalance is linked to mitochondrial dysfunction which is also a marker of this neurological disorder [3], [8], [9].

The initial link between mitochondrial dysfunction and PD was found in the 1980s when recreational drug users presented symptoms related to PD after exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is metabolized to MPP+, a mitochondrial complex I inhibitor [11]. Further studies confirmed that consequences of mitochondrial disorders as increase of oxidative stress, dysfunctions in mitochondrial OXPHOS rates, and inhibition of mitochondrial protein complexes would be associated to PD [7], [10]. The mitochondrial dysfunction has been observed in familial and sporadic forms of PD, as well as, in toxin-induced PD models [10], [11].

Moreover, novel studies have demonstrated that many genetic mutations are related with both familial and sporadic forms of this disease [12]. Recently, mutations in genes as SNCA, Parkin, PINK1, DJ-1 and LRRK2, were associated with PD [13]. One important gene in PD is pink1 [14]–[18]. This gene encodes the PTEN-induced putative kinase 1 that plays a crucial role on removal of impaired mitochondria from the mitochondrial network in a process recognized as mitophagy [19]. PINK1 recognizes loss of mitochondrial membrane potential and activates parkin, which is an E3 ubiquitin ligase recruited to dysfunctional mitochondria, leading to mitochondrial recycling through phagocytosis [14], [19], [20].

In addition, pink1 gene is related to energetic metabolism, some studies point out a shift in glucose metabolism as consequence of pink1 loss [21]. This metabolism imbalance is supported by hypoxia-inducible factor-1α (HIF1α) which is triggered by reactive oxygen

species (ROS) formed as consequence of mitochondrial dysfunction. The HIF1α stimulates glycolysis in the absence of pink1, increasing lactate amount and leading to cell proliferation, an effect similar to Warburg effect which is common in cancer cells [21], [22].

Whereas is important to recognize molecular pathways involved in PD, *pink1* mutants have been used as experimental models to study this neurological disorder [16], [18]. *Drosophila melanogaster pink1* mutant is described as a reliable model for PD studies [23]–[25], using flies were possible to identify a large number of genes that modify aggregation and toxicity in PD [26]. The easy genetic manipulation and standardized behavior are points which support use flies as model for PD [27].

In this study we used *D. melanogaster pink1* mutants in different life stages to investigate changes in energetic pathways and mitochondrial functions during aging, even as, consequences of these processes in a PD model.

2. Materials and methods

Drosophila melanogaster strain

Fly stocks were maintained on standard BDSC Cornmeal Food (yeast, soy flour, yellow cornmeal, agar, light corn syrup) at 25°C with a 12 h: 12 h light/dark cycle. The strains used were w1118 (white) and Dmel\Pink1B9-FM7c (flyBase ID: FBgn0029891) Bloomington *Drosophila* stock center. All experiments were performed using only males of each strain in different life stages (3, 15 and 30 days of age).

Chemicals

Adenosine 5′-diphosphate sodium salt (ADP), pyruvic acid (P), proline (Pr), antimycin A (AmA), rotenone (R), malonic acid (MNA), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and acetyl coenzyme A sodium salt were purchased from Sigma-Aldrich (St. Louis, MO). Lactate dehydrogenase (LDH) commercial kit (LDH liquiform) was purchased from Labtest® Diagnostica S.A. (Minas Gerais, Brazil). Other chemicals used in this work were purchased from local suppliers.

Survival Curve

The survival curve compared *pink1(-/-)* flies with w1118 which are used as control. Flies with 3 days of life were transferred to vials with standard medium, around 15 flies per vial.

Individuals were transferred every 48 h to a new vial containing fresh medium. Mortality was recorded every day until death of all flies and the experiments were repeated at least 3 times.

Climbing Assay

Locomotor ability was determined by climbing assay as previous described [28] with some modifications. Briefly, for each assay 10 to 15 flies were placed in vertical glass columns (length, 25 cm; diameter, 1.5 cm). After, flies were tapped to the bottom of the column and the number of flies able to climb up in the column over 10 sec was recorded. The assays were repeated 5 times at 1-min intervals with each group of flies and results were expressed as the mean of the 5 repetitions. The climbing ability of control and mutant flies was determined as the average of the height reached by each fly after 10 sec.

High Resolution Respirometry (HRR)

The analyses were performed on O2k-system high-resolution oxygraph (Oroboros Instruments, Innsbruck, Austria). Two flies were homogenized in MIR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 0.1 mg/mL fatty acid free BSA) and added to the chamber containing the respiration medium - MIR05 at 25ºC as previous described [15]. The protocol consisted of a sequential titration of multiple substrates, uncouplers and inhibitors (SUIT protocol) [29]. After signal stabilization, the experimental SUIT protocol was performed by sequential addition of pyruvate (5 mM), malate (2 mM) and proline (10 mM); ADP (5 mM); succinate (10 mM); oligomycin (2.5 μM); carbonyl cyanide-4-(tri-fluoromethoxy) phenylhydrazone (FCCP - titrations of 0.25 μM until reaching the maximum oxygen consumption); rotenone (0.5 μM); malonate (5 mM) and antimycin (2.5 μM) [29], [30]. On the evaluation of HRR the OXPHOS CI&CII is the mitochondrial oxygen flux in presence of substrates related to complexes I (pyruvate, proline, malate) and II (succinate) and saturated ADP concentration. The OXPHOS CI is the result of mitochondrial function in presence of proline, malate and pyruvate as substrates along with saturated ADP concentration. The OXPHOS related to CII is defined as mitochondrial function in presence of succinate as substrate and saturated ADP concentration. The values of free OXPHOS are obtained subtracting values of electron flux from OXPHOS CI&CII-Linked by values of electron flux after OMY addiction. The ETS state is the mitochondrial respiration step in which electron transfer are linked to an uncoupler effect. ETS CI is ETS oxygen flux in presence of FCCP and rotenone as inhibitor of complex I and ETS CII is the respiration step in presence of FCCP and malonate as inhibitor of complex II.

Mitochondrial respiratory control factors and respiratory control ratio

Mitochondrial flux control ratios are calculated with focus on metabolic fluxes as an indirect estimation of mitochondrial coupling efficiency [31]. Mitochondrial respiratory control ratio and respiratory control factors were calculated using values of oxygen flux obtained by HRR, to this measurements letter L= electron flux from LEAK state, letter P = electron flux from OXPHOS CI&CII-Linked and letter E = electron flux from ETS CI&CII-linked. We calculated OXPHOS coupling efficiency: 1-(L/P), ETS coupling efficiency: 1-(L/E).

H_2O_2 production

H_2O_2 production was measured in the Oxygraph-2k (O2k, OROBOROS Instruments, Innsbruck, Austria) using the Sensor Green of the O2k-Fluo LED2-Module for fluorescence while respiration was analyzed. The H_2O_2-sensitive probe Amplex®Red was used to measure the peroxide flux [32]. Two flies were homogenized in MiR05 and placed inside oroboros chamber containing MiR05, 10 μM Amplex® Red (AmR) and 1 U/mL horseradish peroxidase (HRP). The product of the reaction between AmR and H_2O_2, catalyzed by HRP, is fluorescent, and O2k-Fluo LED2-Module is sensitive to this fluorescence difference. Through this protocol was possible to evaluate peroxide production in different steps of the HRR with the addition of substrates and inhibitors. Experiments were performed with sequential additions of the following substrates and inhibitors: pyruvate (5 mM), malate (2.5 mM) and proline (10 mM); succinate (10 mM), to evaluate peroxide production by complex I and II, and olygomicin (2.5 μM) to evaluate peroxide production linked to ATP-synthase activity.

Enzyme activity assays and protein determination

Five flies were homogenized in 350 μL of cold TFK buffer 50 mM, pH 7,5 and centrifuged for 10 min at 13.000 rpm and 4 °C, then, supernatant was used to measure enzyme activities. Enzyme activities were assayed at 37 °C. Results were corrected by protein content of each sample.

Citrate synthase activity was measured at 412 nm, recording the linear reduction of 0.1 mM 5,5'dithiobis-2-nitrobenzoic acid (ε 412: 13.6 $ml \cdot cm^{-1} \cdot \mu mol^{-1}$) in the presence of 0.10 mM acetyl-CoA, 10 mM oxalacetic acid and 0.1 M Tris/HCl, (pH 8.1) [33].

Lactate dehydrogenase (LDH) activity was measured at 340 nm using the commercial kit LDH liquiform (Labtest®, Diagnostica S.A., Minas Gerais, Brazil).

Protein content was determined by Bradford's test [34] using serum albumin as a standard.

ATP measurements

The ATP measurements were performed according protocol previously described with some modifications [35]. Three male flies were homogenized in guanidine-HCl extraction buffer (100 mM Tris and 4 mM EDTA, pH 7.8). Homogenized samples were frozen in liquid nitrogen and boiled for 5 min. After centrifugation at 14000 r/min for 10 min, the supernatant was mixed with a luminescent solution (ATP determination kit A22066 - Molecular probes^{TM} by Invitrogen detection technologies). Luminescence was measured on plate luminescence reader. Relative ATP levels were calculated as the luminescence corrected by a standard curve of ATP and divided by the total protein concentration, as determined by the Bradford method.

Statistical analysis

Statistical analysis and figures were performed using GraphPad Prism 6. Data are expressed as the mean ± standard error of mean (S.E.M.). Data for survival assay was analyzed by Log-rank (Mantel-Cox) test. The other tests were analyzed using two-way ANOVA with Tukey as post test. Results were considered statistically significant when $p<0.05$

3. Results

Loss of pink1 gene decreased survival and impaired locomotor system during aging in D. melanogaster

Figure 1A demonstrates a significant decrease in survival percentage of *pink1* flies when compared with *white* flies used as control. On 20th day, a death rate around 50% was found for *pink1* flies, while *white* flies reached 50% of death around 30th day. Moreover, *white* flies presented a higher survival average when compared with *pink1* flies which presented a total mortality of individuals around the 40th day, while *white* flies presented total mortality of individuals around 70th day. The loss of *pink1* also promoted locomotor impairment, since is possible to verify a significant decrease in climbing index comparing *pink1* and *white* flies. The *pink1* flies presented decrease in climbing index (Fig. 1B) with significant difference observed only in *Pink1* flies with 30 day.

Mitochondrial electron flux related to OXPHOS capacity is reduced by loss of pink1 gene in D. melanogaster

The results of OXPHOS process driven by different mitochondrial complexes is demonstrates in Figure 2. There is a significant decrease in OXPHOS CI-Linked in *pink1* flies comparing with *white* flies with 3, 15 and 30 days of age (Fig. 2A). Moreover, a significant decrease in OXPHOS CII-Linked is observed in *pink1* flies comparing with *white* flies with 15 days of age; however, *pink1* flies with 3 and 30 days of life (Fig. 2B) don't have significant difference in OXPHOS CII-Linked when compared to *white* flies. Lastly, the results show a significant decrease in OXPHOS CI&CII-Linked for *pink1* flies with 3, 15 and 30 days comparing with *white* flies (Fig. 2C). Together these results suggest that electron flux driven by OXPHOS CI-Linked is tightly affected by loss of *pink1* gene. Moreover, complex I seems to be the main electron source in mitochondria overall OXPHOS process, while mitochondrial electron flux driven by OXPHOS CII-Linked although affected by loss of *pink1* is also affect by aging.

Loss of pink1 gene and aging are linked with decrease in free OXPHOS and increase of complex II activity in D. melanogaster

There is a significant increase of free OXPHOS in *white* flies during aging and this increase seems directly related to aging (Fig. 2D) since *white* flies with 15 and 30 days of life presented a significant increase of free OXPHOS when compared with *white* flies with 3 days of life. On the other hand, *pink1* flies didn´t present any significant difference in free OXPHOS during aging (Fig. 2D). In order to evaluate the contributions of mitochondrial complexes I and II related to overall OXPHOS we calculated the following reasons: OXPHOS (CI/CI&CII-Linked) and OXPHOS (CII/CI&CII-Linked). Our results demonstrated a decrease of OXPHOS electron flux attributed to CI in *pink1* flies when compared with *white* flies and this decrease remained with aging (Fig. 2E). Contrariwise, we verify an increase of OXPHOS electron transfer attributed to complex II in *pink1* flies when compared with *white* flies (Fig. 2F).

Loss of pink1 gene impaired the general mitochondrial ETS capacity in D. melanogaster

The ETS state is the mitochondrial respiration step in which electron transfer are linked to an uncoupler. Our results demonstrate a significant decrease in ETS CI&CII-Linked capacity for *pink1* flies comparing with *white* flies with 3, 15 and 30 (Fig. 3A) days of age. Similar result occurs on ETS related to complex I (ETS CI-Linked), our results demonstrate a significant decrease in ETS CI-Linked capacity in *pink1* flies comparing with *white* flies with 3, 15 and 30 (Fig. 3B) days of age. Lastly, in the same way as for OXPHOS CII-Linked, there was a significant decrease in ETS CII-Linked comparing *pink1* and *white* flies in age 15, which was

not observed in flies with 3 and 30 days of age (Fig. 3C). To evaluate proportional electron transfer from CI and CII we have made the following ratio ETS CI/CI&CII-Linked and ETS CII/CI&CII-Linked. In ETS CI/CI&CII-Linked there was no significant difference neither comparing *pink1* flies and *white* flies or during aging process. On the other hand, there was a significant increase in ETS CII/CI&CII-Linked comparing *pink1* flies with *white* flies mainly on 15^{th} and 30^{th} days of age.

Effects by loss of pink1 gene and aging on OXPHOS and ETS mitochondrial coupling efficiency and in D. melanogaster

Mitochondrial OXPHOS coupling efficiency indicates how mitochondrial electron flux is coupled to oxidative phosphorylation. Our results demonstrated that there is a significant decrease of OXPHOS coupling efficiency in *pink1* flies compared with *white* flies on day 15^{th} of age, however the same didn't occur at 3 and 30 days of age (Fig. 4A). Mitochondrial ETS coupling efficiency indicate how mitochondrial electron flux are coupled considering the maximum electron transfer system through mitochondrial complexes. Our results demonstrate that in the same way as for OXPHOS coupling efficiency, ETS coupling efficiency decreased in *pink1* flies compared with *white* flies on day 15^{th}, however there is no significant difference at 3 and 30 days of age (Fig 4B).

Loss of pink1 gene increased mitochondrial H_2O_2 production

Measurement of H_2O_2 production in different HRR steps was evaluated with the probe Amplex®Red. During HRR assay, there was no statistically significant increase in H_2O_2 production related to mitochondrial electron transfer driven by OXPHOS CI-Linked comparing *pink1* and *white* flies with 3 days of age (Fig. 5A). It is possible to verify that *pink1* flies with 15 days of age presented a higher increase in H_2O_2 production related to mitochondrial electron transfer driven by OXPHOS CI-Linked when compared with *white* flies at the same age (Fig. 5A), while flies with 30 days of age did not present an increase of H_2O_2 production when compared with *white* flies at the same age (Fig. 5A). At the same way, H_2O_2 production related to mitochondrial electron transfer driven by OXPHOS CI&CII-Linked presented a significant increase in *pink1* flies with 15 days when compared with *white* flies at the same age (Fig. 5B), the increase in H_2O_2 production by OXPHOS CI&CII-Linked was observed also in *pink1* flies with 30 days of life when compared with *white* flies at the same age (Fig. 5B). There was an increase in H_2O_2 production during LEAK state in *pink1* flies mainly on 3 and 15 days of life but also on 30 days of life comparing with *white* flies (Fig. 5C), however this increase of H_2O_2

production during LEAK stage did not present statistic significant difference from control groups.

Enzyme activities involved in glucose metabolism are dependent of both, aging and pink1

Activities of citrate synthase (CS) and lactate dehydrogenase (LDH) were measured since these enzymes are involved in aerobic and anaerobic glucose metabolism. CS activity was significantly decreased in *pink1* flies with 15 days of age and there was a significant increase of CS activity in *pink1* flies with 30 days of age comparing to *white* flies (Fig. 6A). In contrast, *pink1* flies with 15 days presented an increase in LDH activity while *pink1* flies with 30 days exhibited a significant decrease of LDH activity compared with *white* flies at the same age (Fig. 6B). There is no significant difference in LDH and CS activity comparing *pink1* flies and white flies with 3 days of life (Fig. 6B).

Loss of pink1 gene change ATP levels during aging

Regarding ATP levels (Fig.7) was possible verify a significant increase in *pink1* flies with 15 days of age compared to control flies, otherwise, *pink1* flies at 30th day of age presented a significant decrease of ATP levels when compared to control flies, while *pink1* flies at 3rd day of age did not present significant difference on ATP levels compared to control flies.

4. Discussion

The *pink1* gene has a central role to maintain healthy mitochondrial population, degrading damaged mitochondria through mitophagy process in association with ubiquitin ligase Parkin [36]. PINK1 loss is involved in familial early-onset Parkinson's disease [1]. In this work, our results demonstrated a significant decrease of survival percentual comparing *pink1* flies and controls (Fig. 1A), this found is consistent with other works using *pink1* as model to PD [35], [37].

Different from other works using *pink1* flies our results of climbing assay did not present any difference comparing *pink1* flies and controls on 3th and 15th days of age (Fig. 1B), a significant climbing behavior impairment was observed only on day 30th (Fig. 1B). These results are in contrast to other works which demonstrate climbing reduction from day 15th [35], and from day 3th of age [38] in *pink1* flies. On the other hand, other works showed an increase of climbing activity in *pink1* flies during aging [39]. Due these contradictory results, we believe

that climbing assay has limitations when measuring flies locomotor activity. Aggarwal and collaborators (2019) also presented concern about the limitation of climbing protocol. According to these authors, physical agitation is liable to incorporate undesirable behavior and phenotypes in a fly's innate climbing response and mask subtle phenotypes so, even this assay has been extensively used to identify gross locomotor defects [40], the physical agitation of flies can be an aggressive way to induce locomotion.

Since mitochondria quality control is close related to pink1 gene, we evaluated mitochondrial function in *pink1* flies during aging. We observed a significant decrease of oxygen flux in both stages OXPHOS and ETS (Fig. 2 and 3) dependent from CI and CI&CII (Fig. 2A, 2C, 3A and 3B). The reduction of oxygen flux is targeted mainly by CI which presented the highest impairment on oxygen flux with a large reduction in *pink1* flies at 3^{th}, 15^{th} and 30^{th} days of age (Fig. 2A and 3B). This result is consistent with other works which already demonstrated a reduced activity of CI in *pink1* model of PD [37], [38], [41] and also during aging [42]. The impairment on CI is closely related with PD, and classical CI inhibitors as rotenone, paraquat and 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) are used as environmental factors which generate oxidative stress in flies models to reproduce PD [16]. The control flies presented an increase of OXPHOS related to CI (Fig. 2A), OXPHOS related to CI&CII (Fig. 2C) and free OXPHOS (Fig. 2 D) during aging. These results are contrary to studies which demonstrated a decrease of mitochondrial function during aging [43], however this increase of OXPHOS state seems not to be related to a significant increase of ATP production (Fig 7) demonstrating that the increase of OXPHOS are not coupled to more energy generation as ATP. So, we conclude that even with increase of oxygen flux related to OXPHOS there is no increase of mitochondrial functionality on control flies during aging.

Interestingly, evaluating the coupled and uncoupled state linked to CII (Fig. 2B and Fig. 3C) *pink1* flies at 15^{th} day, presented a significant decrease of oxygen flux compared to control flies, different from *pink1* flies at 3^{th} and 30^{th} days of age. Other works demonstrated that complex II activity have a compensatory role during mitochondrial dysfunction due to regulation between oxidative and glycolytic pathways to ATP production [44]. Janowska and collaborators (2016), demonstrated that carbamate-induced mitochondrial CI dysfunction can be alleviated with an increase of oxygen flux by mitochondrial CII, increasing ATP production by oxidative pathway. The role of CII mediating aerobic instead glycolytic metabolism also was observed during re-oxygenation following hypoxia [45]. Zhu and collaborators (2020)

demonstrated that an increase of oxygen consumption rate related to mitochondrial CII is important during hypoxia recover to ensure the mitochondrial re-oxygenation in cardiac cells.

We believe that in *pink1* flies at 15th days of age, the reduction of oxygen flux related to CII in both OXPHOS and ETS state, could be related to a decrease of oxidative glucose metabolism followed by an increase of glycolytic glucose metabolism, characterizing a shift on metabolism for ATP generation with higher glycolytic pathway activity instead mitochondrial oxidative pathway. Our hypothesis also is supported by results of OXPHOS coupling efficiency (Fig. 4A) and ETS coupling efficiency (Fig. 4B) where is possible to verify a reduction in *pink1* flies at 15th day of age compared to control group, different from *pink1* flies at 3th and 30th days of age. The coupling efficiencies in both states OXPHOS and ETS demonstrate an estimative of how much electron transfer are coupled with mitochondrial protonmotive force to phosphorylation of ADP to ATP [29]. Coupling efficiency is calculated by division of LEAK values per oxygen flux (OXPHOS and ETS) values, the LEAK stage can be understood as an estimate of intrinsic uncoupling without addition of an experimental uncoupler [46].

In order to better understand metabolism differences in *pink1* flies, we evaluate the activity of two main enzymes: citrate synthase, involved in oxidative ATP production and lactate dehydrogenase, involved in glycolytic ATP production. Our results demonstrate that *pink1* flies at 30th day of age presented an increase of citrate synthase activity (Fig. 6A) at the same levels of control group at 15th days of age following by a higher decrease of lactate dehydrogenase activity (Fig. 6B).

The increase of citrate synthase activity at 30th day of age seems not be related to a higher mitochondrial capacity whereas ATP production is not increased (Fig 7), contrariwise *pink1* flies on 30th day of age presented a reduction on ATP levels when compared with control group. Barken and Peleg (2017) highlighted that even the aging process is associated with a total decline of mitochondrial activity, this fact is contradictory with studies which demonstrate a life extension caused by lower metabolic rates [47], according to the authors a progressive, monophasic model of total reduction of mitochondrial functionality during aging may be oversimplified. Other explanation that supports the increase of citrate synthase activity in *pink1* flies on 30th day of age could be related to the mitochondrial accumulation on mitochondrial network. The pink1 gene accumulates on damaged mitochondria, promote their segregation from the mitochondrial network [48], the loss of this gene causes a great accumulation of damaged mitochondrial and this process is enhanced by aging [49]. Considering this fact, the

pink1 flies have a great number of mitochondria, explaining the increase of citrate synthase activity, however these organelles are not be able to use the substrates to produce energy since these mitochondria are dysfunctional, leading to a reduced of ATP content.

Indeed, the most interesting, was the confirmation about shift of glucose metabolism in *pink1* flies at 15th of age. The results of enzymes activities demonstrated a possible increase of lactate production marked by a significant increase of lactate dehydrogenase activity (Fig. 6A) followed by a possible lower activity of Krebs cycle marked by reduction of CS activity (Fig. 6B). Requejo-Aguilar and collaborators (2014) demonstrated that loss of *pink1* stimulates an anaerobic glucose metabolism in mice, with reduction of oxidative metabolism due mitochondrial impairment. This shift between oxidative and anaerobic glucose metabolism requires hypoxia-inducible factor α (HIF1- α). The stabilization of HIF1-α is supported by mitochondrial ROS, regulating the ATP production by glucose reprograming [21].

Since the increase of ROS is an important consequence of CI impairment [41] as well as an important mediator which support HIF1- α stabilization, we assayed H_2O_2 levels in order to evaluate the redox balance in *pink1* flies during aging. Our results show an increase in H_2O_2 production during aging in *pink1* flies (Fig. 5A, 5B and 5C) specially in flies with 15 days old where this increase present significant levels, supporting our idea that there is an increase of glycolytic pathway to ATP production on *pink1* flies on 15th days of age.

The shift of glucose metabolism between aerobic and anaerobic pathways with consequences in ATP production was also described in cancer cells and is called Warburg effect. Recently, it was proved that cancer and PD have common metabolic markers and common genes (including pink1) involved in both diseases [6]. Some studies have demonstrated the ROS interference on the shift of oxidative from glycolytic metabolism. Weiping and collaborators (2019) demonstrated that inhibition of ROS in SIRT6-upregulated cells could rescue activation of the Warburg effect [50]. These results are supported by other work which demonstrate the relation between control of mitochondrial ROS and stabilization of HIF-1α [51].

Interestingly, *pink1* flies with 15th days of age presented a significant increase on ATP levels (Fig. 7) when compared to control flies at the same age, demonstrating an altered glucose metabolism. The aerobic glycolysis is recognized as a faster pathway used to produce great

amounts of ATP. This pathway presents central role in supporting cell growth, many cells use aerobic glycolysis during rapid proliferation [52].

The mitochondrial impairment resulted from PINK1 loss triggers the metabolic shift from aerobic to glycolytic pathway. Our results demonstrate that mitochondrial bioenergetics is altered during aging in *pink1* flies and the mainly alteration occur on 15^{th} days of age, at this time is possible to verify a marked change on metabolic ratio followed by an increase of ATP production. The same does not occur at 3^{th} and 30^{th} days of age. Together, the results demonstrate that after metabolic shift from aerobic glycolysis it is not possible to recover from aerobic metabolism, even *pink1* flies on 30^{th} days of age presented an increase of citrate synthase activity, this is not linked with an increase of ATP production, demonstrating a failure on energy balance regulation during aging.

5. Conclusions

For the first time we demonstrated a metabolic shift caused by PINK1 loss in flies likewise occurs in *pink1* mice [21], [22]. In *pink1* flies, the metabolic change occurs at 15^{th} days of age. Since mitochondrial dysfunction is a common feature of neurodegenerative diseases as PD, the energy regulation could be very important target to better understand the cellular processes and find possible pharmacological interventions. These results help us to understand the differences on energy balance in *pink1* flies, a widely used model to PD studies. With our results we reinforce that *D. melanogaster* could be a good experimental model to study aspects of bioenergetics, cellular energy maintenance and mitochondrial function beyond genetics parameters.

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7. References

[1] C. Pirkevi, S. Lesage, A. Brice, and A. Nazli BasÌ§ak, “From Genes to Proteins in Mendelian Parkinson’s Disease: An Overview,” *Anat. Rec. Adv. Integr. Anat. Evol. Biol.*, vol. 292, no. 12, pp. 1893–1901, Dec. 2009, doi: 10.1002/ar.20968.

[2] W. Poewe *et al.*, “Parkinson disease,” *Nat. Rev. Dis. Prim.*, vol. 3, no. 1, p. 17013, Dec. 2017, doi: 10.1038/nrdp.2017.13.

[3] M. F. Beal, “Aging, energy, and oxidative stress in neurodegenerative diseases,” *Ann. Neurol.*, vol. 38, no. 3, pp. 357–366, Sep. 1995, doi: 10.1002/ana.410380304.

[4] C. G. Goetz, “The history of Parkinson’s disease: early clinical descriptions and neurological therapies.,” *Cold Spring Harb. Perspect. Med.*, vol. 1, no. 1, p. a008862, Sep. 2011, doi: 10.1101/cshperspect.a008862.

[5] N. L.-G. Del Rey *et al.*, “Advances in Parkinson’s Disease: 200 Years Later,” *Front. Neuroanat.*, vol. 12, p. 113, Dec. 2018, doi: 10.3389/fnana.2018.00113.

[6] M. J. Devine, H. Plun-Favreau, and N. W. Wood, “Parkinson’s disease and cancer: two wars, one front.,” *Nat. Rev. Cancer*, vol. 11, no. 11, pp. 812–23, Oct. 2011, doi: 10.1038/nrc3150.

[7] A. Anandhan *et al.*, “Metabolic Dysfunction in Parkinson’s Disease: Bioenergetics, Redox Homeostasis and Central Carbon Metabolism.,” *Brain Res. Bull.*, vol. 133, pp. 12–30, Jul. 2017, doi: 10.1016/j.brainresbull.2017.03.009.

[8] X.-M. Hao, L.-D. Li, C.-L. Duan, and Y.-J. Li, “Neuroprotective effect of α-mangostin on mitochondrial dysfunction and α-synuclein aggregation in rotenone-induced model of Parkinson’s disease in differentiated SH-SY5Y cells,” *J. Asian Nat. Prod. Res.*, vol. 19, no. 8, pp. 833–845, Aug. 2017, doi: 10.1080/10286020.2017.1339349.

[9] C. A. Gautier, T. Kitada, and J. Shen, “Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress,” *Proc. Natl. Acad. Sci.*, vol. 105, no. 32, pp. 11364–11369, Aug. 2008, doi: 10.1073/pnas.0802076105.

[10] D. F. Gonçalves *et al.*, “6-Hydroxydopamine induces different mitochondrial bioenergetics response in brain regions of rat.,” *Neurotoxicology*, vol. 70, pp. 1–11, 2019, doi: 10.1016/j.neuro.2018.10.005.

[11] C. M. Massari, A. A. Castro, T. Dal-Cim, D. Lanznaster, and C. I. Tasca, “In vitro 6-hydroxydopamine-induced toxicity in striatal, cerebrocortical and hippocampal slices is attenuated by atorvastatin and MK-801,” *Toxicol. Vitr.*, vol. 37, pp. 162–168, Dec. 2016, doi: 10.1016/j.tiv.2016.09.015.

[12] C. Klein and K. Lohmann-Hedrich, “Impact of recent genetic findings in Parkinson??s disease,” *Curr. Opin. Neurol.*, vol. 20, no. 4, pp. 453–464, Aug. 2007, doi: 10.1097/WCO.0b013e3281e6692b.

[13] Y. Sai, Z. Zou, K. Peng, and Z. Dong, “The Parkinson’s disease-related genes act in mitochondrial homeostasis,” *Neurosci. Biobehav. Rev.*, vol. 36, no. 9, pp. 2034–2043, Oct. 2012, doi: 10.1016/J.NEUBIOREV.2012.06.007.

[14] A. N. Murphy, “In a flurry of PINK, mitochondrial bioenergetics takes a leading role in Parkinson’s disease,” *EMBO Mol. Med.*, vol. 1, no. 2, pp. 81–84, May 2009, doi: 10.1002/emmm.200900020.

[15] A. C. Costa, S. H. Y. Loh, and L. M. Martins, “Drosophila Trap1 protects against mitochondrial dysfunction in a PINK1/parkin model of Parkinson’s disease,” *Cell Death Dis.*, vol. 4, no. 1, pp. e467–e467, Jan. 2013, doi: 10.1038/cddis.2012.205.

[16] B. Aryal and Y. Lee, “Disease model organism for Parkinson Disease: Drosophila melanogaster.,” *BMB Rep.*, Dec. 2018, Accessed: Jan. 07, 2019. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/30545438.

[17] I. Pimenta de Castro *et al.*, “Genetic analysis of mitochondrial protein misfolding in Drosophila melanogaster,” *Cell Death Differ.*, vol. 19, no. 8, pp. 1308–1316, Aug. 2012, doi: 10.1038/cdd.2012.5.

[18] R. B. Creed and M. S. Goldberg, “New Developments in Genetic rat models of Parkinson’s Disease.,” *Mov. Disord.*, vol. 33, no. 5, pp. 717–729, 2018, doi: 10.1002/mds.27296.

[19] Y. Chen and G. W. Dorn, “PINK1-Phosphorylated Mitofusin 2 Is a Parkin Receptor for Culling Damaged Mitochondria,” *Science (80-.).*, vol. 340, no. 6131, pp. 471–475, Apr. 2013, doi: 10.1126/science.1231031.

[20] D. P. Narendra *et al.*, “PINK1 is selectively stabilized on impaired mitochondria to activate Parkin.,” *PLoS Biol.*, vol. 8, no. 1, p. e1000298, Jan. 2010, doi: 10.1371/journal.pbio.1000298.

[21] R. Requejo-Aguilar, I. Lopez-Fabuel, E. Fernandez, L. M. Martins, A. Almeida, and J. P. Bolaños, “PINK1 deficiency sustains cell proliferation by reprogramming glucose metabolism through HIF1.,” *Nat. Commun.*, vol. 5, p. 4514, Jul. 2014, doi: 10.1038/ncomms5514.

[22] R. Requejo-Aguilar and J. P. Bolaños, “Mitochondrial control of cell bioenergetics in Parkinson’s disease,” *Free Radic. Biol. Med.*, vol. 100, pp. 123–137, Nov. 2016, doi: 10.1016/j.freeradbiomed.2016.04.012.

[23] M. Ayajuddin *et al.*, “Parkinson’s Disease: Insights from Drosophila Model,” in *Drosophila melanogaster - Model for Recent Advances in Genetics and Therapeutics*, InTech, 2018.

[24] I. S. Pienaar, J. Götz, and M. B. Feany, “Parkinson’s disease: insights from non-traditional model organisms.,” *Prog. Neurobiol.*, vol. 92, no. 4, pp. 558–71, Dec. 2010, doi: 10.1016/j.pneurobio.2010.09.001.

[25] Y. Xiong and J. Yu, “Modeling Parkinson’s Disease in Drosophila: What Have We Learned for Dominant Traits?,” *Front. Neurol.*, vol. 9, p. 228, 2018, doi: 10.3389/fneur.2018.00228.

[26] T. J. van Ham, R. Breitling, M. A. Swertz, and E. A. A. Nollen, “Neurodegenerative diseases: Lessons from genome-wide screens in small model organisms.,” *EMBO Mol. Med.*, vol. 1, no. 8–9, pp. 360–70, Nov. 2009, doi: 10.1002/emmm.200900051.

[27] F. Hirth, “Drosophila melanogaster in the Study of Human Neurodegeneration,” *CNS Neurol. Disord. - Drug Targets*, vol. 9, no. 4, pp. 504–523, Aug. 2010, doi: 10.2174/187152710791556104.

[28] M. Jimenez-Del-Rio, C. Guzman-Martinez, and C. Velez-Pardo, “The Effects of Polyphenols on Survival and Locomotor Activity in Drosophila melanogaster Exposed to Iron and Paraquat,” *Neurochem. Res.*, vol. 35, no. 2, pp. 227–238, Feb. 2010, doi: 10.1007/s11064-009-0046-1.

[29] D. Pesta and E. Gnaiger, “High-Resolution Respirometry: OXPHOS Protocols for Human Cells and Permeabilized Fibers from Small Biopsies of Human Muscle,” 2012, pp. 25–58.

[30] B. Schöpf *et al.*, “Oxidative phosphorylation and mitochondrial function differ between human prostate tissue and cultured cells,” *FEBS J.*, vol. 283, no. 11, pp. 2181–2196, Jun. 2016, doi: 10.1111/febs.13733.

[31] J. Burtscher, L. Zangrandi, C. Schwarzer, and E. Gnaiger, “Differences in mitochondrial function in homogenated samples from healthy and epileptic specific brain tissues revealed by high-resolution respirometry,” *Mitochondrion*, vol. 25, pp. 104–112, Nov. 2015, doi: 10.1016/j.mito.2015.10.007.

[32] G. Krumschnabel *et al.*, “Simultaneous High-Resolution Measurement of Mitochondrial Respiration and Hydrogen Peroxide Production,” in *Methods in molecular biology (Clifton, N.J.)*, vol. 1264, 2015, pp. 245–261.

[33] H. Lemieux, P. Subarsky, C. Doblander, M. Wurm, J. Troppmair, and E. Gnaiger, “Impairment of mitochondrial respiratory function as an early biomarker of apoptosis

induced by growth factor removal," *bioRxiv*, p. 151480, Jun. 2017, doi: 10.1101/151480.

[34] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.," *Anal. Biochem.*, vol. 72, pp. 248–54, May 1976, Accessed: Jul. 09, 2014. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/942051.

[35] M. Liu *et al.*, "Ginseng protein protects against mitochondrial dysfunction and neurodegeneration by inducing mitochondrial unfolded protein response in Drosophila melanogaster PINK1 model of Parkinson's disease," *J. Ethnopharmacol.*, vol. 247, Jan. 2020, doi: 10.1016/j.jep.2019.112213.

[36] B. Bingol and M. Sheng, "Mechanisms of mitophagy: PINK1, Parkin, USP30 and beyond," *Free Radical Biology and Medicine*, vol. 100. Elsevier Inc., pp. 210–222, Nov. 01, 2016, doi: 10.1016/j.freeradbiomed.2016.04.015.

[37] A. C. Costa, S. H. Y. Loh, and L. M. Martins, "Drosophila Trap1 protects against mitochondrial dysfunction in a PINK1/parkin model of Parkinson's disease.," *Cell Death Dis.*, vol. 4, no. 1, p. e467, Jan. 2013, doi: 10.1038/cddis.2012.205.

[38] D. Senyilmaz *et al.*, "Regulation of mitochondrial morphology and function by stearoylation of TFR1," *Nature*, vol. 525, no. 7567, pp. 124–128, Sep. 2015, doi: 10.1038/nature14601.

[39] S. Poddighe *et al.*, "Mucuna pruriens (Velvet bean) Rescues Motor, Olfactory, Mitochondrial and Synaptic Impairment in PINK1 B9 Drosophila melanogaster Genetic Model of Parkinson's Disease," 2014, doi: 10.1371/journal.pone.0110802.

[40] A. Aggarwal, H. Reichert, and K. Vijayraghavan, "A locomotor assay reveals deficits in heterozygous Parkinson's disease model and proprioceptive mutants in adult Drosophila," doi: 10.1073/pnas.1807456116/-/DCSupplemental.

[41] F. Scialò *et al.*, "Mitochondrial ROS Produced via Reverse Electron Transport Extend Animal Lifespan," *Cell Metab.*, vol. 23, no. 4, pp. 725–734, Apr. 2016, doi: 10.1016/j.cmet.2016.03.009.

[42] D. A. Stroud *et al.*, "Accessory subunits are integral for assembly and function of human mitochondrial complex i," *Nature*, vol. 538, no. 7623, pp. 123–126, Sep. 2016, doi: 10.1038/nature19754.

[43] S. K, S. E, and V. L, "Mitochondria, Oxidative Stress and the Kynurenine System, with a Focus on Ageing and Neuroprotection," *Molecules*, vol. 23, no. 1, 2018, doi: 10.3390/MOLECULES23010191.

[44] J. I. Janowska *et al.*, “Mitochondrial respiratory chain complex I dysfunction induced by N-methyl carbamate ex vivo can be alleviated with a cell-permeable succinate prodrug,” *Toxicol. Vitr.*, vol. 65, Jun. 2020, doi: 10.1016/j.tiv.2020.104794.

[45] S. C. Zhu *et al.*, “Cardiac complex II activity is enhanced by fat and mediates greater mitochondrial oxygen consumption following hypoxic re-oxygenation,” *Pflugers Arch. Eur. J. Physiol.*, vol. 472, no. 3, pp. 367–374, Mar. 2020, doi: 10.1007/s00424-020-02355-8.

[46] C. Doerrier, L. F. Garcia-Souza, G. Krumschnabel, Y. Wohlfarter, A. T. Mészáros, and E. Gnaiger, “High-resolution fluorespirometry and oxphos protocols for human cells, permeabilized fibers from small biopsies of muscle, and isolated mitochondria,” in *Methods in Molecular Biology*, vol. 1782, Humana Press Inc., 2018, pp. 31–70.

[47] D. J. Baker and S. Peleg, “Biphasic Modeling of Mitochondrial Metabolism Dysregulation during Aging,” *Trends Biochem. Sci.*, vol. 42, no. 9, pp. 702–711, Sep. 2017, doi: 10.1016/j.tibs.2017.06.005.

[48] G. Ashrafi and T. L. Schwarz, “The pathways of mitophagy for quality control and clearance of mitochondria,” *Cell Death Differ.*, vol. 20, no. 1, pp. 31–42, Jan. 2013, doi: 10.1038/cdd.2012.81.

[49] S. R and S. A, “The role of mitochondrial ROS in the aging brain,” *FEBS Lett.*, vol. 592, no. 5, pp. 743–758, Mar. 2018, doi: 10.1002/1873-3468.12902.

[50] W. Yu, Z. Yang, R. Huang, Z. Min, and M. Ye, “SIRT6 promotes the Warburg effect of papillary thyroid cancer cell BCPAP through reactive oxygen species,” *Onco. Targets. Ther.*, vol. Volume 12, pp. 2861–2868, Apr. 2019, doi: 10.2147/OTT.S194256.

[51] H. Lu, R. A. Forbes, and A. Verma, “Hypoxia-inducible Factor 1 Activation by Aerobic Glycolysis Implicates the Warburg Effect in Carcinogenesis,” *J. Biol. Chem.*, vol. 277, no. 26, pp. 23111–23115, Jun. 2002, doi: 10.1074/jbc.M202487200.

[52] S. Y. Lunt and M. G. Vander Heiden, “Aerobic Glycolysis: Meeting the Metabolic Requirements of Cell Proliferation,” *Annu. Rev. Cell Dev. Biol.*, vol. 27, no. 1, pp. 441–464, Nov. 2011, doi: 10.1146/annurev-cellbio-092910-154237.

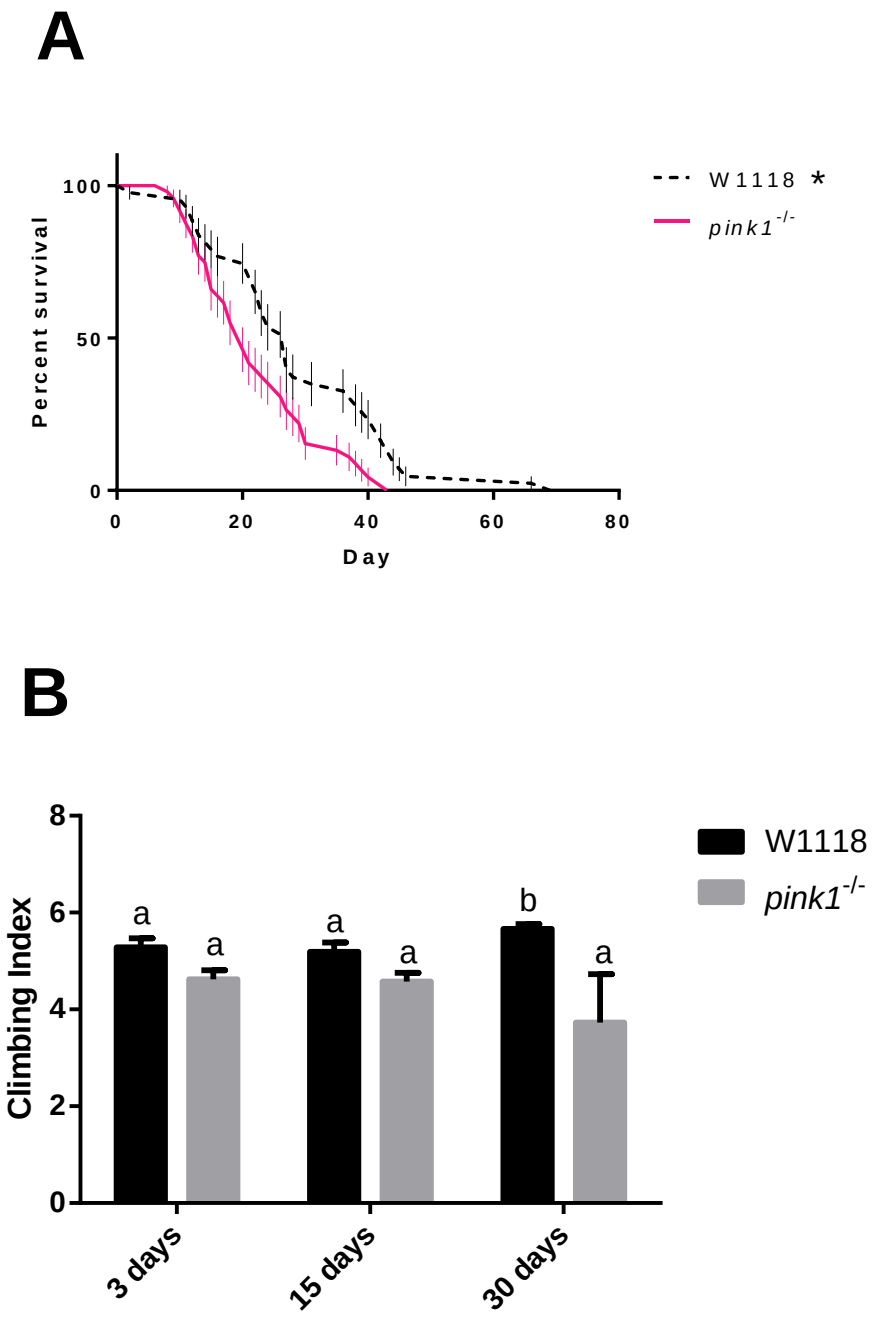

Figure 1: Survival rate and locomotor ability. (A) Survival rate of *white* flies compared to *pink1*$^{-/-}$ flies. Data are presented as mean ± S.E.M of 3 different assays, at least, and 50 flies for each group. (B) Locomotor ability evaluated by climbing index, comparing *white* flies with *pink1*$^{-/-}$ flies at age 3, 15 and 30 days. Data are presented as mean ± S.E.M of 8 to 10 different assays and a total of 75 to 100 flies for each group. * Represent significant difference ($p<0.05$). Different letters indicate statistical difference among groups ($p<0.05$).

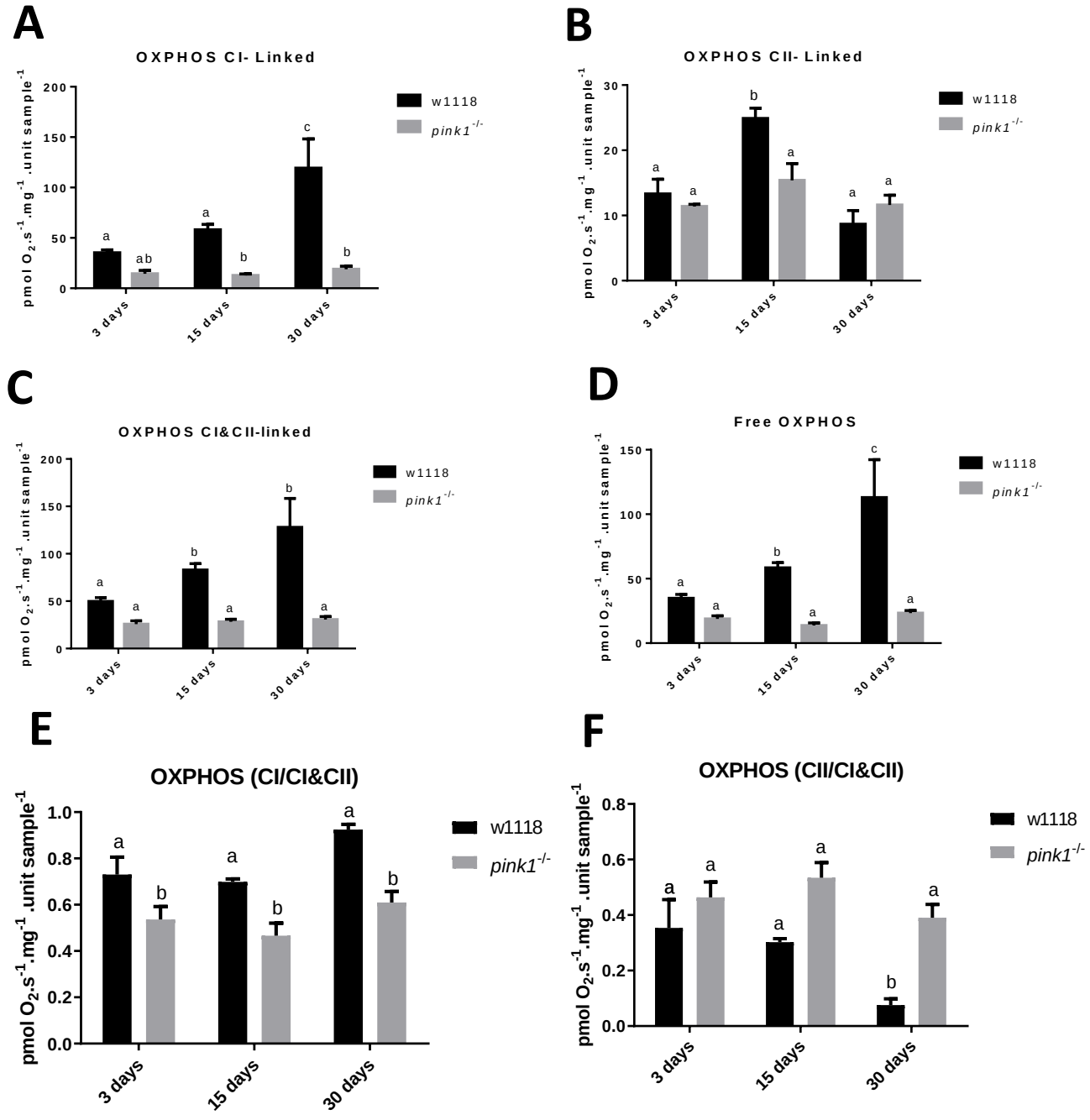

Figure 2: High resolution respirometry comparing *white* and *pink1*$^{-/-}$ flies with 3, 15 and 30 days of age. Oxygen flux during OXPHOS state which is mitochondrial coupled state dependent on different mitochondrial substrates in presence of saturated ADP concentrations (A) Substrates: proline, pyruvate, malate (OXPHOS CI-Linked) (B) Substrate: succinate (OXPHOS CII-Linked) and (C) Substrates: proline, pyruvate, malate and succinate (OXPHOS CI&CII-Linked). (D) Oxygen flux representing free OXPHOS. (E) Ratio between values of OXPHOS CI-Linked and values of OXPHOS CI&CII-Linked. (F) Ratio between values of OXPHOS CII-Linked and values of OXPHOS CI&CII-Linked. Results are presented as mean ± S.E.M. n=4-5. Different letters indicate statistical difference among groups ($p<0.05$).

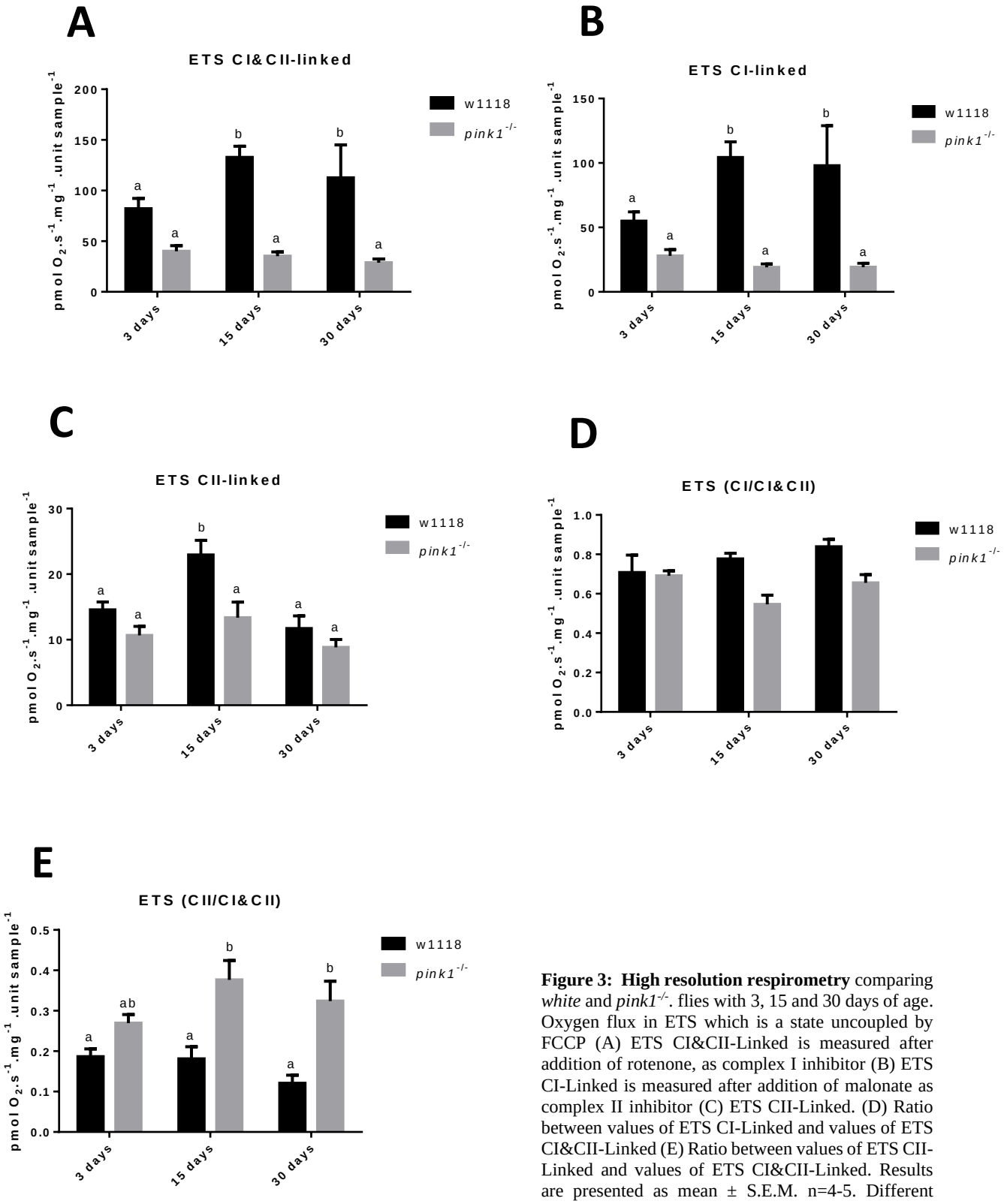

Figure 3: High resolution respirometry comparing *white* and *pink1*$^{-/-}$. flies with 3, 15 and 30 days of age. Oxygen flux in ETS which is a state uncoupled by FCCP (A) ETS CI&CII-Linked is measured after addition of rotenone, as complex I inhibitor (B) ETS CI-Linked is measured after addition of malonate as complex II inhibitor (C) ETS CII-Linked. (D) Ratio between values of ETS CI-Linked and values of ETS CI&CII-Linked (E) Ratio between values of ETS CII-Linked and values of ETS CI&CII-Linked. Results are presented as mean ± S.E.M. n=4-5. Different letters indicate statistical difference among groups. ($p<0.05$).

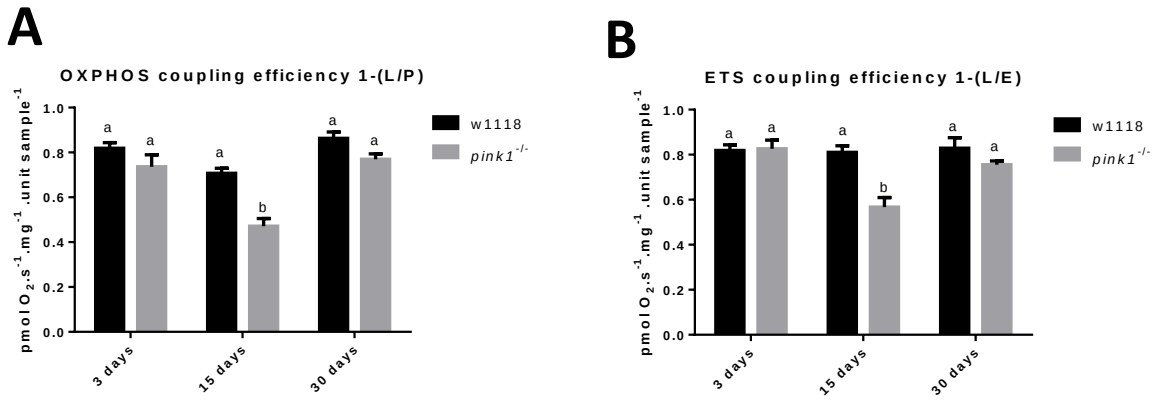

Figure 4: Mitochondrial respiratory control factors of *white* and *pink1*$^{-/-}$ flies with 3, 15 and 30 days of age. Results are presented as mean ± S.E.M. n=4-5. Different letters indicate statistical difference among groups ($p<0.05$).

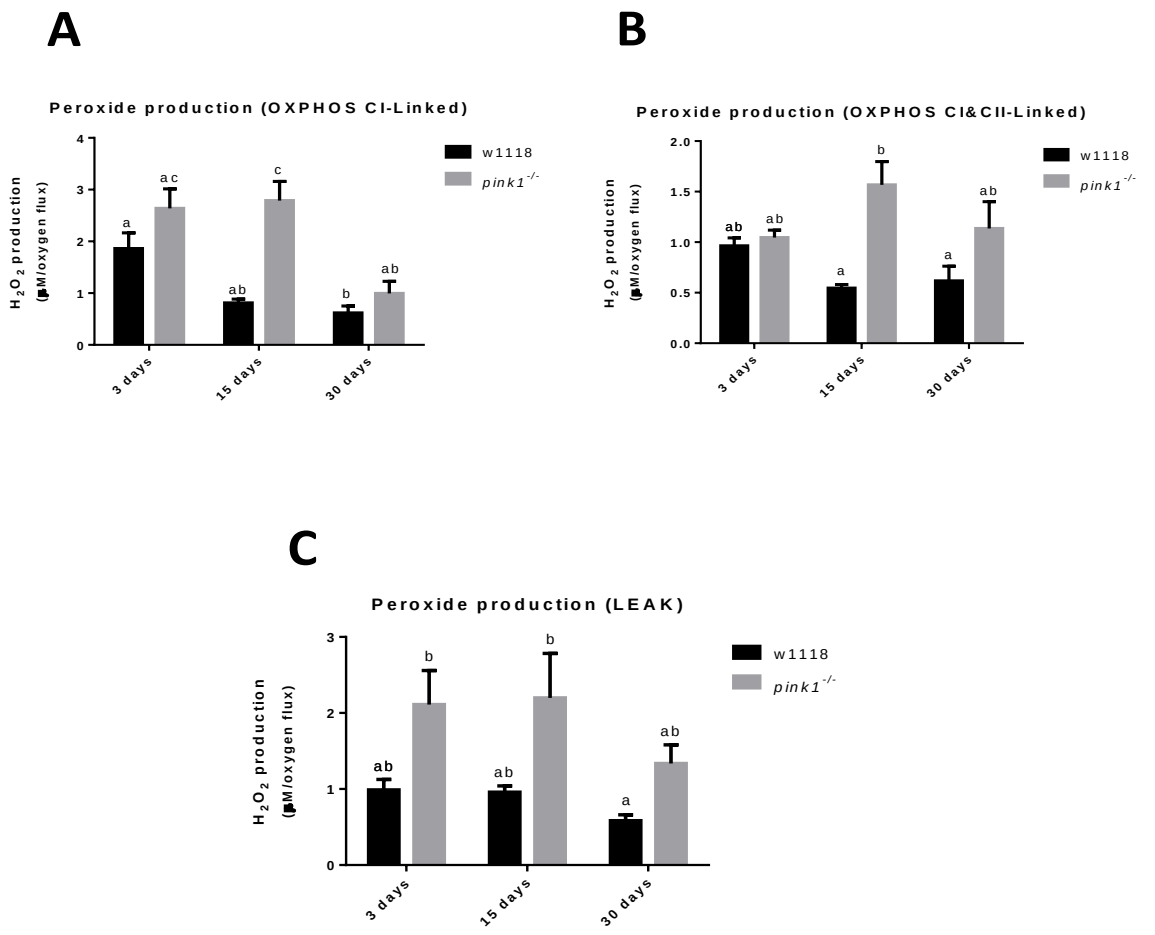

Figure 5: Peroxide production by different mitochondrial respiration steps in white and pink-/- flies with 3, 15 and 30 days of age. Data demonstrate peroxide production during OXPHOS state which are mitochondrial coupled state dependent on different mitochondrial substrates: (A) proline, pyruvate, malate-OXPHOS CI-Linked and (B) succinate OXPHOS CI&CII-Linked in presence of saturated ADP concentrations and (C) peroxide production in presence of oligomycin used as ATP synthase inhibitor. Results are presented as mean ± S.E.M. n=4-5. Different letters indicate statistical difference among groups ($p<0.05$).

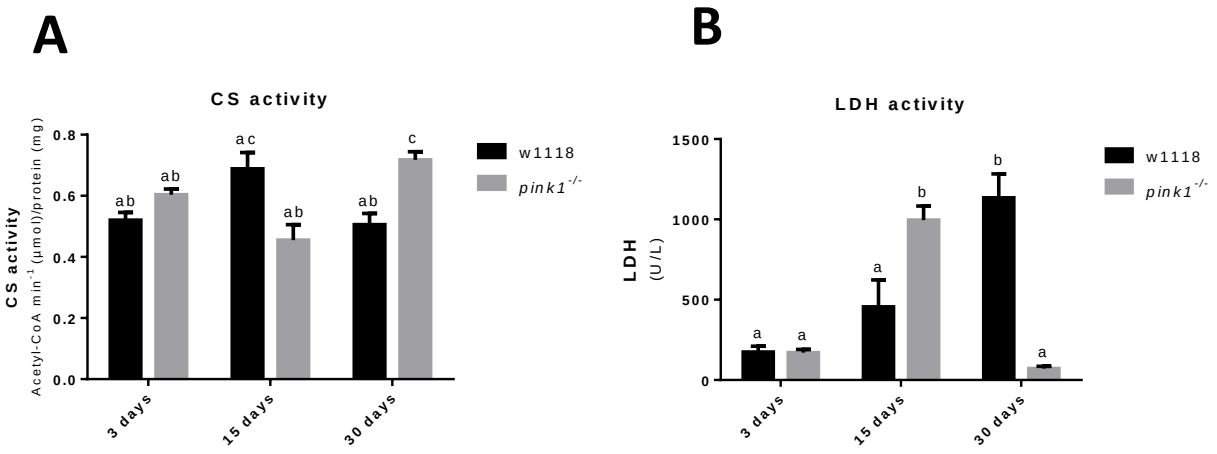

Figure 6: Enzyme activities of *white* flies and *pink1*$^{-/-}$ flies with 3, 15 and 30 days of age. (A) Citrate synthase activity. (B) Lactate dehydrogenase activity. Results are presented as mean ± S.E.M of five to seven independent assays. Means for a variable with superscripts without common letters differ ($p<0.05$).

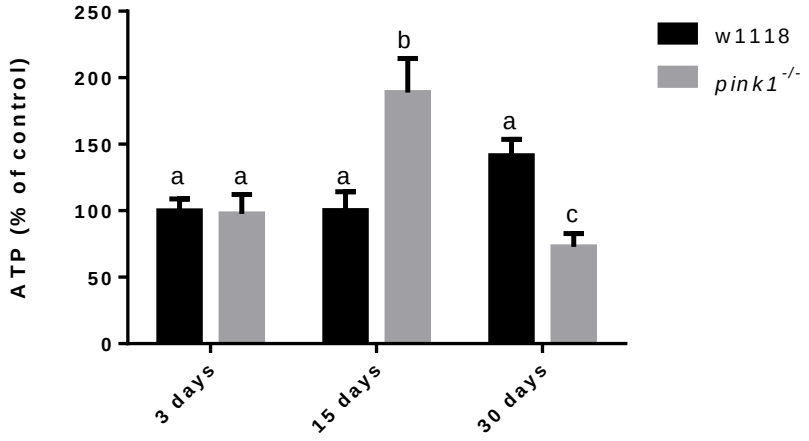

Figure 7: ATP content in *white* and *pink1*$^{-/-}$ flies with 3, 15 and 30 days of age. Results are presented as mean ± S.E.M of 4-5 independent assays. Different letters indicate statistical difference among groups ($p<0.05$).

4.3. MANUSCRITO 2: Caffeine improves mitochondrial function in a *pink1* model of Parkinson disease

Caffeine improves mitochondrial function in a *pink1* model of Parkinson disease

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Abstract:

Parkinson's disease (PD) is a neurodegenerative disorder associated to neuronal loss and motor impairment. The major consequences related to PD are linked to decrease of dopaminergic neurons and abnormal levels of unfolded α-synuclein. Other feature of PD is mitochondrial dysfunction since genes involved in mitochondrial quality control, such as SNCA, Parkin, PINK1, DJ-1 and LRRK2, were demonstrated to be associated with PD. Natural compounds like caffeine have been proposed as secondary treatment to relief PD symptoms. Caffeine is a methylxanthine considered an antioxidant molecule and used as adjuvant treatment of neurodegenerative diseases. In this study we used *pink1* null mutants of *Drosophila melanogaster* as model of a Parkinson's disease to investigate if caffeine treatment could improve mitochondrial functions. Our results demonstrated that caffeine treatment improved parameters related to oxidative phosphorylation (OXPHOS) in *pink1* flies, increasing the mitochondrial oxygen flux in an average of 90% compared to untreated *pink1* flies. The same occurs on mitochondrial electron transfer system (ETS), the treatment with caffeine increased ETS in a range of 80% comparing with no treated *pink1* flies. Moreover, caffeine treatment increased OXPHOS coupling efficiency and mitochondrial respiratory control ratio (RCR) in *pink1* flies. Furthermore, *pink1* flies treated with caffeine presented a better organization of

mitochondrial network on muscle thorax of *pink1* flies. Together these results demonstrated that caffeine treatment improve parameters related to mitochondrial function in *pink1* flies.
Keywords: Pink1; bioenergetics; high resolution respirometry; xanthine.

1. Introduction

PD is affects 2-3% of the population over 65 years of age [1]. This neurodegenerative condition is characterized by movement disorders as bradykinesia, and neuronal loss which are consequence of accumulation of unfolded protein α-synuclein it forming cellular aggregates inclusions in neurons [2,3].

There are multiple pathways and molecular mechanisms behind PD symptoms [1,4], among them the cellular metabolic dysfunction, characterized by the impairment of the cellular energy balance [5]. Usually, the energy imbalance is linked to mitochondrial dysfunction which is also a marker of this neurological disorder [2,6,7].

In the 1980s when recreational drug users presented symptoms related to PD after exposition to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is metabolized to MPP+, a mitochondrial complex I inhibitor [11], this fact close relate mitochondrial function to PD. Further studies confirmed that consequences of mitochondrial disorders as increase of oxidative stress, dysfunctions in mitochondrial OXPHOS rates, and inhibition of mitochondrial protein complexes would be associated to PD [5,8].

Recently, mutations in genes such as alpha-synuclein, Parkin, pink1, dj-1 and lrrk2, also have been associated with phenotypes of this PD [9][10]. One important gene in PD is pink1 [11–15]. This gene encodes the PTEN-induced putative kinase 1 that plays a crucial role on removal of impaired mitochondria from the mitochondrial network in a process recognized as mitophagy [16]. PINK1 recognizes loss of mitochondrial membrane potential and activates parkin, which is an E3 ubiquitin ligase recruited to dysfunctional mitochondria, leading to mitochondrial recycling [11,16,17].

On this context's caffeine, a methylxanthine alkaloid compound present in several beverages (coffee, tea, energy drinks), food (chocolate, desserts) and medicines, has long being considered as an antioxidant molecule and has been used as a possible adjuvant treatment of neurodegenerative diseases, including PD [18,19].

Studies using caffeine as PD treatment presented good results and bring this molecule as a promising adjuvant pharmacological treatment [18]. The mechanisms of caffeine action on PD

remains unclear, although, it could be related to caffeine capacity to antagonize adenosine receptors, specially A2A receptors [20]. Indeed, inhibitors of A2A receptors has been used as a target for symptomatic therapies aimed at ameliorating motor deficits in PD [21]. Likewise, caffeine is recognized to improve mitochondrial function [22], increase mitochondrial activity related to OXPHOS [23], in addition to increase mitochondrial biogenesis [24] and improve mitochondrial substrates oxidation [25].

Up to now, caffeine was used as treatment in several studies with different models of PD [26], however there are no studies demonstrating the effects of caffeine in a *pink1* PD model. Here, we aimed to understand the effects of caffeine, mainly related to mitochondrial functionality in a *Drosophila melanogaster* model with deletion of pink1 gene.

2. Materials and methods

Drosophila melanogaster strain and caffeine treatment

Fly stocks were maintained on standard BDSC Cornmeal Food (yeast, soy flour, yellow cornmeal, agar, light corn syrup) at 25°C with a 12 h: 12 h light/dark cycle. The strains used were W1118 (white), Dmel\Pink1B9-FM7c (flyBase ID: FBgn0029891) from Bloomington *Drosophila* stock center and mef2-GAL4,UAS-mitoGFP donated by University of São Paulo-BR. All experiments were performed using females of each crossing as control and males as treated flies. The treatment of *D. melanogaster* was made from 1st stage larvae until adult in BDSC culture medium containing caffeine at concentration of 0,5 mg/mL, and experiments were performed with 3-day-old flies [27].

Drosophila Hybridization

Firstly, the Dmel\Pink1B9-FM7c virgin *Drosophila* was crossed with W1118 male *Drosophila*, and the F1 male *Drosophila* was obtained, with genotype pink1-/y, while females obtained in the F1 presented genotype pink1-/+ and was used as control group.

In order to obtain *pink1* flies with mitochondria from thorax muscle marked with GFP, we crossed virgin *Drosophila* of Dmel\Pink1B9-FM7c strain with males UAS-GFP.S65T; Mef2-Gal4, and the F1 male *Drosophila* was obtained with genotype pink1-/y;mef2-GAL4,UAS-mitoGFP, while females obtained in the F1 presented genotype pink1-/+;mef2-GAL4,UAS-mitoGFP and was used as control group.

High resolution respirometry (HRR)

The analyses were performed on O2k-system high-resolution oxygraph (Oroboros Instruments, Innsbruck, Austria). Two flies were homogenized in MIR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 0.1 mg/mL fatty acid free BSA) and added to the chamber containing the respiration medium - MIR05 at 25ºC as previous described [28]. The protocol consisted of a sequential titration of multiple substrates, uncouplers and inhibitors (SUIT protocol) [29]. After signal stabilization, the experimental SUIT protocol was performed by sequential addition of pyruvate (5 mM), malate (2 mM) and proline (10 mM); ADP (5 mM); succinate (10 mM); oligomycin (2.5 μM); carbonyl cyanide-4-(tri-fluoromethoxy) phenylhydrazone (FCCP - titrations of 0.25 μM until reaching the maximum oxygen consumption); rotenone (0.5 μM); malonate (5 mM) and antimycin (2.5 μM) [29,30]. On the evaluation of HRR the OXPHOS CI&CII is the mitochondrial oxygen flux in presence of substrates related to complexes I (pyruvate, proline, malate) and II (succinate) and saturated ADP concentration. The OXPHOS CI is the result of mitochondrial function in presence of proline, malate and pyruvate as substrates along with saturated ADP concentration. The OXPHOS related to CII is defined as mitochondrial function in presence of succinate as substrate and saturated ADP concentration. The values of free OXPHOS are obtained subtracting values of electron flux from OXPHOS CI&CII-Linked by values of electron flux after OMY addiction. The ETS state is the mitochondrial respiration step in which electron transfer are linked to an uncoupler effect, we used FCCP as uncoupler. ETS CI is ETS oxygen flux in presence of FCCP and rotenone as inhibitor of complex I and ETS CII is the respiration step in presence of FCCP and malonate as inhibitor of complex II.

Mitochondrial respiratory control factors and respiratory control ratio

Mitochondrial flux control ratios are calculated with focus on metabolic fluxes as an indirect estimation of mitochondrial coupling efficiency [31]. Mitochondrial respiratory control factors were calculated using values of oxygen flux obtained by HRR, to this measurements letter L= electron flux from LEAK state, letter P= electron flux from OXPHOS CI&CII-Linked and letter E= electron flux from ETS CI&CII-linked. We used the following formulae to calculated OXPHOS coupling efficiency: 1-(L/P), ETS coupling efficiency: 1-(L/E) and respiratory control ratio (RCR): P/L

Confocal images

The thorax of flies of mito-GFP line with the following genotypes *pink1*-/y;mef2-GAL4,UAS-mitoGFP and *pink1*-/+;mef2-GAL4,UAS-mitoGFP (control), were dissected using phosphate buffered saline with Tween-20 (PBST) at 0,1% and then transferred to paraformaldehyde at 4% and incubated for 20 minutes after, the structures was washed with PBST 0,1% and put on a pre-prepared glass slide and covered with a coverslip fixed with glycerol 80% diluted in PBS. Fluorescence images was acquired with an OLYMPUS® FLUOVIEW FV10i Confocal Microscope in controlled temperature room (25°C). The intensity of fluorescence was analyzed using ImageJ and expressed by total sum of pixels.

Climbing behavior

Locomotor ability was determined by climbing assay as previous described [32] with some modifications. Briefly, for each assay 10 to 15 flies were placed in vertical glass columns (length, 25 cm; diameter, 1.5 cm). After, flies were tapped to the bottom of the column and the number of flies able to climb up in the column over 10 sec was recorded. The assays were repeated 5 times at 1-min intervals with each group of flies and results were expressed as the mean of the 5 repetitions. The climbing ability of control and mutant flies was determined as the average of the height reached by each fly after 10 sec.

Statistical Analysis

Statistical analysis and figures were performed using GraphPad Prism 6. Data are expressed as the Mean + Standard Error of Mean (S.E.M.). Data was analyzed by one way ANOVA with Tukey's pos test. Results were considered statistically significant when $p<0.05$.

3. Results

3.1. Caffeine improves mitochondrial function in *pink1* flies

On the evaluation of oxygen flux related to OXPHOS CI&CII was possible to verify a significant decrease of this parameter on *pink1* flies when compared with control group, on the other hand, the treatment with caffeine increases the oxygen flux related to OXPHOS CI&CII in *pink1* flies (Fig. 1A). The *pink1* flies treated with caffeine did not present statistic difference from both control and *pink1* group. Although caffeine was not able to return OXPHOS CI&CII levels in *pink1* flies to the same levels of control, caffeine induced an increase of 90% to 100%

in OXPHOS CI&CII comparing to *pink1* flies. Control flies treated with caffeine didn't present significant difference from control flies on oxygen flux related OXPHOS CI&CII.

Similar effect was observed on the oxygen flux related to OXPHOS CI (Fig. 2A). The *pink1* flies presented a significant decrease of oxygen flux related to CI compared to control group, while *pink1* flies treated with caffeine did not present statistical difference from both control and *pink1* group. Flies treated with caffeine presented increased levels of oxygen flux on OXPHOS CI-linked which was 90% higher than oxygen flux on *pink1* flies. Control flies treated with caffeine didn't present significant difference from control flies on OXPHOS oxygen flux related to CI. On the evaluation of OXPHOS CII (Fig.1C) there is no differences among control and *pink1* flies treated or not with caffeine.

During mitochondrial respiration process measured by HRR, free OXPHOS is the electron transfer after addition of oligomycin (OMY) which is used as ATP synthase inhibitor. The *pink1* flies group presented a significant decrease of free OXPHOS compared with control group, and the treatment with caffeine caused a 90 % increased in oxygen flux related to free OXPHOS (Fig. 1D) in *pink1* flies compared to *pink1* flies not treated. Control flies treated with caffeine didn't present significant difference compared to control flies on OXPHOS oxygen flux related to free OXPHOS.

On the evaluation of oxygen flux related to ETS it is possible to verify a significant decrease in *pink1* flies compared to control flies (Fig. 2B), on the other hand caffeine treatment increased ETS CI&CII (Fig. 2B) in *pink1* flies. The *pink 1* flies treated with caffeine presented an oxygen flux related to ETS CI&CII 80% higher than *pink1* flies. Control flies treated with caffeine didn't present significant difference compared to control flies on ETS CI&CII oxygen flux. On ETS CI (Fig. 2A), also there was a significant decrease in *pink1* flies compared to control flies, while *pink1* flies treated with caffeine did not present statistic difference from both control and *pink1* group. In the oxygen flux related to ETS CII (Fig. 2 C), is possible verify the same effect, a decrease of oxygen flux related to ETS CII in *pink1* flies compared to control flies while *pink1* flies treated with caffeine did not present statistic difference from both control and *pink1* group. The *pink1* flies treated with caffeine presented an oxygen flux related to CII around 60% higher than untreated *pink1* flies. Control flies treated with caffeine didn't present significant difference on ETS CII oxygen flux compared to untreated control flies.

3.2.Caffeine improves mitochondrial quality control in *pink1* flies

Considering mitochondrial efficiency, the treatment with caffeine increased OXPHOS coupling efficiency (Fig. 3A) in *pink1* flies, while in control flies, the treatment with caffeine did not alter OXPHOS coupling efficiency (Fig. 3A). The treatment with caffeine did not increase the ETS coupling efficiency neither in *pink1* nor in control flies (Fig. 3B). Regarding RCR, *pink1* flies presented a significant decrease in RCR when compared with control group. The treatment with caffeine significantly increases this ratio in *pink1* flies (Fig. 3C), although, in control flies caffeine treatment did not alter the RCR.

3.3.Caffeine improves mitochondria network organization and don't alter climbing index in *pink1* flies

The confocal images from muscle mitochondrial network demonstrated a better organization of mitochondrial network in control flies compared to *pink1* flies (Fig. 4A). Caffeine treatment didn't affect muscle mitochondrial network in control flies but seems to improve this parameter in *pink1* flies (Fig. 4A). On the image fluorescence quantification, we don't observe significant differences due caffeine treatment (data not shown). The treatment with caffeine did not alter the climbing index in both *pink1* or control flies (Fig 4B).

4. Discussion

The neuroprotective effects of caffeine have attracted attention in the field of neurodegenerative diseases [33]. Caffeine is largely recognized by its antioxidant effects [34,35] and as antagonist molecule of adenosine receptors [20]. Caffeine actions on central nervous system make this molecule a possible secondary treatment for neurological disorders as PD [18]. Costa and collaborators (2010) in a meta-analyze study confirmed an inverse association between caffeine intake and the risk of PD despite the authors concern about heterogeneity and conflict among evaluated results [36]. Due to the several mechanisms associated with caffeine effects [37] the proposal of this molecule as a secondary treatment for PD require further investigation.

One recognized effect of caffeine is related to mitochondrial function, caffeine was able to increase mitochondrial activity [38], increase mitochondrial biogenesis [24] and improve mitochondrial NAD^+/ FAD^+ ratio linked to state 3 respiration [25]. Since mitochondrial dysfunction is considered a feature of PD [39], here we investigated at the first time the effects of caffeine related to mitochondrial function in a model of PD using *pink1 D. melanogaster*.

Our results demonstrate an increase of oxygen flux related to all steps linked to OXPHOS mitochondrial respiration after caffeine treatment. Regarding OXPHOS CI&II (Fig. 1A), OXPHOS CI (Fig. 1B), OXPHOS CII (Fig. 1C) and free OXPHOS (Fig. 1D), *pink1* flies treated with caffeine presented an increase of oxygen flux around 90 % higher than untreated *pink1* flies. Our research group previously demonstrated that caffeine increases mitochondrial bioenergetics response in brain of rats by the enhancement of the oxidative phosphorylation [22].

Moreover, *pink1* gene is not related only with PD, but also with other diseases as cancer [4]. The *pink1* loss was demonstrated to induce the Warburg effect, which is a metabolic exchange with increase ATP production by glycolytic pathway instead mitochondrial OXPHOS [40]. The Warburg effect is recognized as a metabolic marker of both cancer and *pink1* loss [4,28,41].

Jianrong and collaborators (2015) demonstrated the importance of glycolysis coupled to mitochondrial tricarboxylic acid (TCA) cycle by OXPHOS process. According to the authors, the stimulation of OXPHOS in cancer cells raises oxidative stress and restores cells sensitivity to anoikis which is a specific type of normal cell death, suggesting OXPHOS as an anti-metastasis mechanism [42]**.** So, the increase of OXPHOS could improve the cell metabolism and reduce exacerbated cellular proliferation during cancer. Since *pink1* loss is involved in similar metabolic effects of that observed in cancer [28], we believe that the capacity of caffeine treatment in increase OXPHOS could be related to a better use of substrates to generate energy by OXPHOS process inside the mitochondria. According to Mishra and Kumar (2014), a chronic treatment with caffeine was able to increase the ratio of NAD^+/ FAD^+ linked to mitochondrial OXPHOS in brain of rats in a Hungtington disease model. Moreover, caffeine was also demonstrated to reduce toxicity caused by malonic acid (a classical inhibitor of mitochondrial complex II) in *a D. melanogaster* model of neurotoxicity [34]. The significant increase of OXPHOS coupling efficiency (Fig. 4A) and RCR (Fig. 4C) in *pink1* flies treated with caffeine also support the effect of this molecule on the improvement of mitochondrial function by raising mitochondrial OXPHOS process.

Caffeine effects on mitochondrial oxygen flux in ETS state are in agreement with our findings related to oxygen flux in OXPHOS state. Although treatment with caffeine increases ETS CI&CII, ETS CI and ETS CII in *pink1* flies in a range of 80% compared to untreated *pink1* flies, caffeine treatment didn't affect significantly ETS coupling efficiency (Fig. 3B). The potential of caffeine to increase mitochondrial complexes activity have been previously demonstrated, Freddo and collaborators (2021) showed that caffeine increases activity of

mitochondrial complexes II, III and IV in a zebrafish model of neurotoxicity [43]. However, there are no reports about caffeine effects on mitochondrial ETS or mitochondrial complexes activity specifically in *D. melanogaster*. So far, studies about caffeine effects on *D. melanogaster* are related to increase of intracellular cAMP [44], interference on the sleep-wake activity [45] and cytochrome P450-dependent metabolism [46]. Furthermore, it was previously demonstrated that supplementation with 0.05% of caffeine was able to increase catalase activity and GSH levels in adult flies but it was not able to extend the lifespan *in D. melanogaster* [47].

Therefore, our results show for the first time the effect of caffeine to improve mitochondrial ETS and OXPHOS on *pink1* flies, demonstrating that caffeine present significant effects on mitochondrial functions in a *D. melanogaster* model of PD. Further, confocal images of muscle mito-GFP fluorescence demonstrated a better organization of mitochondrial network in *pink1* flies treated with caffeine (Fig. 4A). Nevertheless, we believe that the mitochondrial improvement of caffeine treatment in *pink1*flies even was not able to restore the normal cellular processes could delay deficits caused by deletion of *pink1* gene, bringing this molecule as a good secondary treatment to increase the health span and delay cell death processes.

Caffeine has been studied as a promising molecule with possible clinic application, mainly on the field of neurodegenerative diseases [18,36,48]. At low doses caffeine don't present toxicity [49–51], moreover, this molecule is largely consumed by people in food and beverages [52,53]. Our results demonstrated that caffeine could be helpful as a coadjuvant treatment for PD, due the increase of mitochondrial OXPHOS and ETS processes which is closely related with the improvement of mitochondrial function.

5. Conclusion

According to our results treatment with caffeine at 0.5 mg/mL from larvae until adult, promote improvement of mitochondrial function in *pink1* flies. We demonstrate for the first time the effects of caffeine on mitochondrial parameters in the PD model using *pink1* flies. These results are in agreement with other studies that propose caffeine as a promise molecule to PD treatment [26,36,48].

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7. References

[1] W. Poewe, K. Seppi, C.M. Tanner, G.M. Halliday, P. Brundin, J. Volkmann, A.-E. Schrag, A.E. Lang, Parkinson disease, Nat. Rev. Dis. Prim. 3 (2017) 17013. https://doi.org/10.1038/nrdp.2017.13.

[2] M.F. Beal, Aging, energy, and oxidative stress in neurodegenerative diseases, Ann. Neurol. 38 (1995) 357–366. https://doi.org/10.1002/ana.410380304.

[3] C.G. Goetz, The history of Parkinson's disease: early clinical descriptions and neurological therapies., Cold Spring Harb. Perspect. Med. 1 (2011) a008862. https://doi.org/10.1101/cshperspect.a008862.

[4] M.J. Devine, H. Plun-Favreau, N.W. Wood, Parkinson's disease and cancer: two wars, one front., Nat. Rev. Cancer. 11 (2011) 812–23. https://doi.org/10.1038/nrc3150.

[5] A. Anandhan, M.S. Jacome, S. Lei, P. Hernandez-Franco, A. Pappa, M.I. Panayiotidis, R. Powers, R. Franco, Metabolic Dysfunction in Parkinson's Disease: Bioenergetics, Redox Homeostasis and Central Carbon Metabolism., Brain Res. Bull. 133 (2017) 12–30. https://doi.org/10.1016/j.brainresbull.2017.03.009.

[6] X.-M. Hao, L.-D. Li, C.-L. Duan, Y.-J. Li, Neuroprotective effect of α-mangostin on mitochondrial dysfunction and α-synuclein aggregation in rotenone-induced model of Parkinson's disease in differentiated SH-SY5Y cells, J. Asian Nat. Prod. Res. 19 (2017) 833–845. https://doi.org/10.1080/10286020.2017.1339349.

[7] C.A. Gautier, T. Kitada, J. Shen, Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress, Proc. Natl. Acad. Sci. 105 (2008) 11364–11369. https://doi.org/10.1073/pnas.0802076105.

[8] D.F. Gonçalves, A.A. Courtes, D.D. Hartmann, P.C. da Rosa, D.M. Oliveira, F.A.A. Soares, C.L. Dalla Corte, 6-Hydroxydopamine induces different mitochondrial bioenergetics response in brain regions of rat., Neurotoxicology. 70 (2019) 1–11. https://doi.org/10.1016/j.neuro.2018.10.005.

[9] C. Klein, K. Lohmann-Hedrich, Impact of recent genetic findings in Parkinson??s disease, Curr. Opin. Neurol. 20 (2007) 453–464. https://doi.org/10.1097/WCO.0b013e3281e6692b.

[10] Y. Sai, Z. Zou, K. Peng, Z. Dong, The Parkinson's disease-related genes act in

mitochondrial homeostasis, Neurosci. Biobehav. Rev. 36 (2012) 2034–2043. https://doi.org/10.1016/J.NEUBIOREV.2012.06.007.

[11] A.N. Murphy, In a flurry of PINK, mitochondrial bioenergetics takes a leading role in Parkinson's disease, EMBO Mol. Med. 1 (2009) 81–84. https://doi.org/10.1002/emmm.200900020.

[12] A.C. Costa, S.H.Y. Loh, L.M. Martins, Drosophila Trap1 protects against mitochondrial dysfunction in a PINK1/parkin model of Parkinson's disease, Cell Death Dis. 4 (2013) e467–e467. https://doi.org/10.1038/cddis.2012.205.

[13] B. Aryal, Y. Lee, Disease model organism for Parkinson Disease: Drosophila melanogaster., BMB Rep. (2018). http://www.ncbi.nlm.nih.gov/pubmed/30545438 (accessed January 7, 2019).

[14] I. Pimenta de Castro, A.C. Costa, D. Lam, R. Tufi, V. Fedele, N. Moisoi, D. Dinsdale, E. Deas, S.H.Y. Loh, L.M. Martins, Genetic analysis of mitochondrial protein misfolding in Drosophila melanogaster, Cell Death Differ. 19 (2012) 1308–1316. https://doi.org/10.1038/cdd.2012.5.

[15] R.B. Creed, M.S. Goldberg, New Developments in Genetic rat models of Parkinson's Disease., Mov. Disord. 33 (2018) 717–729. https://doi.org/10.1002/mds.27296.

[16] Y. Chen, G.W. Dorn, PINK1-Phosphorylated Mitofusin 2 Is a Parkin Receptor for Culling Damaged Mitochondria, Science (80-.). 340 (2013) 471–475. https://doi.org/10.1126/science.1231031.

[17] D.P. Narendra, S.M. Jin, A. Tanaka, D.-F. Suen, C.A. Gautier, J. Shen, M.R. Cookson, R.J. Youle, PINK1 is selectively stabilized on impaired mitochondria to activate Parkin., PLoS Biol. 8 (2010) e1000298. https://doi.org/10.1371/journal.pbio.1000298.

[18] X. Ren, J.-F. Chen, Caffeine and Parkinson's Disease: Multiple Benefits and Emerging Mechanisms, Front. Neurosci. 14 (2020). https://doi.org/10.3389/fnins.2020.602697.

[19] R.B. Postuma, J. Anang, A. Pelletier, L. Joseph, M. Moscovich, D. Grimes, S. Furtado, R.P. Munhoz, S. Appel-Cresswell, A. Moro, A. Borys, D. Hobson, A.E. Lang, Caffeine as symptomatic treatment for Parkinson disease (Café-PD), Neurology. 89 (2017) 1795–1803. https://doi.org/10.1212/WNL.0000000000004568.

[20] J.A. Ribeiro, A.M. Sebastião, Caffeine and Adenosine, J. Alzheimer's Dis. 20 (2010) S3–S15. https://doi.org/10.3233/JAD-2010-1379.

[21] A. Pinna, M. Serra, M. Morelli, N. Simola, Role of adenosine A2A receptors in motor control: relevance to Parkinson's disease and dyskinesia, J. Neural Transm. 125 (2018) 1273–1286. https://doi.org/10.1007/s00702-018-1848-6.

[22] D.F. Gonçalves, C.C. Tassi, G.P. Amaral, S.T. Stefanello, C.L. Dalla Corte, F.A. Soares, T. Posser, J.L. Franco,, N.R. Carvalho, Effects of caffeine on brain antioxidant status and mitochondrial respiration in acetaminophen-intoxicated mice, Toxicol. Res. (Camb). 9 (2020) 726–734. https://doi.org/10.1093/toxres/tfaa075.

[23] D.F. Gonçalves, N.R. de Carvalho, M.B. Leite, A.A. Courtes, D.D. Hartmann, S.T. Stefanello, I.K. da Silva, J.L. Franco, F.A.A. Soares, C.L. Dalla Corte, Caffeine and acetaminophen association: Effects on mitochondrial bioenergetics, Life Sci. 193 (2018) 234–241. https://doi.org/10.1016/j.lfs.2017.10.039.

[24] R.A. Vaughan, R. Vaughan, M. Randi Garcia-Smith, K.A. Marco Bisoffi, C.A. Kristina Trujillo, Carole A. Conn, Effects of Caffeine on Metabolism and Mitochondria Biogenesis in Rhabdomyosarcoma Cells Compared with 2,4-Dinitrophenol, Nutr. Metab. Insights. 5 (2012) 59. https://doi.org/10.4137/NMI.S10233.

[25] J. Mishra, A. Kumar, Improvement of mitochondrial NAD+/FAD+-linked state-3 respiration by caffeine attenuates quinolinic acid induced motor impairment in rats: Implications in Huntington's disease, Pharmacol. Reports. 66 (2014) 1148–1155. https://doi.org/10.1016/j.pharep.2014.07.006.

[26] G. Schepici, S. Silvestro, P. Bramanti, E. Mazzon, Caffeine: An Overview of Its Beneficial Effects in Experimental Models and Clinical Trials of Parkinson's Disease, Int. J. Mol. Sci. 21 (2020) 4766. https://doi.org/10.3390/ijms21134766.

[27] E.S. Keebaugh, J.H. Park, C. Su, R. Yamada, W.W. Ja, Nutrition influences caffeine-mediated sleep loss in drosophila, Sleep. 40 (2017). https://doi.org/10.1093/sleep/zsx146.

[28] A.C. Costa, S.H.Y. Loh, L.M. Martins, Drosophila Trap1 protects against mitochondrial dysfunction in a PINK1/parkin model of Parkinson's disease., Cell Death Dis. 4 (2013) e467. https://doi.org/10.1038/cddis.2012.205.

[29] D. Pesta, E. Gnaiger, High-Resolution Respirometry: OXPHOS Protocols for Human Cells and Permeabilized Fibers from Small Biopsies of Human Muscle, in: 2012: pp. 25–58. https://doi.org/10.1007/978-1-61779-382-0_3.

[30] B. Schöpf, G. Schäfer, A. Weber, H. Talasz, I.E. Eder, H. Klocker, E. Gnaiger, Oxidative phosphorylation and mitochondrial function differ between human prostate tissue and cultured cells, FEBS J. 283 (2016) 2181–2196. https://doi.org/10.1111/febs.13733.

[31] J. Burtscher, L. Zangrandi, C. Schwarzer, E. Gnaiger, Differences in mitochondrial function in homogenated samples from healthy and epileptic specific brain tissues

revealed by high-resolution respirometry, Mitochondrion. 25 (2015) 104–112. https://doi.org/10.1016/J.MITO.2015.10.007.

[32] M. Jimenez-Del-Rio, C. Guzman-Martinez, C. Velez-Pardo, The Effects of Polyphenols on Survival and Locomotor Activity in Drosophila melanogaster Exposed to Iron and Paraquat, Neurochem. Res. 35 (2010) 227–238. https://doi.org/10.1007/s11064-009-0046-1.

[33] M. Kolahdouzan, M.J. Hamadeh, The neuroprotective effects of caffeine in neurodegenerative diseases, CNS Neurosci. Ther. 23 (2017) 272–290. https://doi.org/10.1111/cns.12684.

[34] J.L. Portela, M.C. Bianchini, D.H. Roos, D.S. de Ávila, R.L. Puntel, Caffeic acid and caffeine attenuate toxicity associated with malonic or methylmalonic acid exposure in Drosophila melanogaster, Naunyn. Schmiedebergs. Arch. Pharmacol. 394 (2021) 227–240. https://doi.org/10.1007/s00210-020-01974-3.

[35] T.P. Devasagayam, J.P. Kamat, H. Mohan, P.C. Kesavan, Caffeine as an antioxidant: inhibition of lipid peroxidation induced by reactive oxygen species., Biochim. Biophys. Acta. 1282 (1996) 63–70. http://www.ncbi.nlm.nih.gov/pubmed/8679661 (accessed June 13, 2016).

[36] J. Costa, N. Lunet, C. Santos, J. Santos, A. Vaz-Carneiro, Caffeine Exposure and the Risk of Parkinson's Disease: A Systematic Review and Meta-Analysis of Observational Studiess, J. Alzheimer's Dis. 20 (2010) S221–S238. https://doi.org/10.3233/JAD-2010-091525.

[37] A. Nehlig, Effects of coffee/caffeine on brain health and disease: What should I tell my patients?, Pract. Neurol. 16 (2016) 89–95. https://doi.org/10.1136/practneurol-2015-001162.

[38] N. Dragicevic, V. Delic, C. Cao, N. Copes, X. Lin, M. Mamcarz, L. Wang, G.W. Arendash, P.C. Bradshaw, Caffeine increases mitochondrial function and blocks melatonin signaling to mitochondria in Alzheimer's mice and cells., Neuropharmacology. 63 (2012) 1368–79. https://doi.org/10.1016/j.neuropharm.2012.08.018.

[39] P.I. Moreira, X. Zhu, X. Wang, H.-G. Lee, A. Nunomura, R.B. Petersen, G. Perry, M.A. Smith, Mitochondria: a therapeutic target in neurodegeneration., Biochim. Biophys. Acta. 1802 (2010) 212–20. https://doi.org/10.1016/j.bbadis.2009.10.007.

[40] A.C. Costa, S.H. Loh, L.M. Martins, Drosophila Trap1 protects against mitochondrial dysfunction in a PINK1/parkin model of Parkinson's disease., Cell Death Dis. 4

(2013). https://doi.org/10.1038/cddis.2012.205.

[41] W.X. Zong, J.D. Rabinowitz, E. White, Mitochondria and Cancer, Mol. Cell. 61 (2016) 667–676. https://doi.org/10.1016/j.molcel.2016.02.011.

[42] J. Lu, M. Tan, Q. Cai, The Warburg effect in tumor progression: mitochondrial oxidative metabolism as an anti-metastasis mechanism., Cancer Lett. 356 (2015) 156–64. https://doi.org/10.1016/j.canlet.2014.04.001.

[43] N. Freddo, S.M. Soares, M. Fortuna, A. Pompermaier, A.C.C. Varela, V.C. Maffi, M.T. Mozzato, H.H. de Alcantara Barcellos, G. Koakoski, L.J.G. Barcellos, L.G. Rossato-Grando, Stimulants cocktail: Methylphenidate plus caffeine impairs memory and cognition and alters mitochondrial and oxidative status, Prog. Neuro-Psychopharmacology Biol. Psychiatry. 106 (2021) 110069. https://doi.org/10.1016/j.pnpbp.2020.110069.

[44] S. Bhaskara, M.B. Chandrasekharan, R. Ganguly, Caffeine induction of Cyp6a2 and Cyp6a8 genes of Drosophila melanogaster is modulated by cAMP and D-JUN protein levels, Gene. 415 (2008) 49–59. https://doi.org/10.1016/j.gene.2008.02.017.

[45] F.J. Lin, Effect of taurine and caffeine on sleep–wake activity in Drosophila melanogaster, Nat. Sci. Sleep. (2010) 221. https://doi.org/10.2147/NSS.S13034.

[46] A. Coelho, S. Fraichard, G. Le Goff, P. Faure, Y. Artur, J.-F. Ferveur, J.-M. Heydel, Cytochrome P450-Dependent Metabolism of Caffeine in Drosophila melanogaster, PLoS One. 10 (2015) e0117328. https://doi.org/10.1371/journal.pone.0117328.

[47] H.J. Suh, B. Shin, S.-H. Han, M.J. Woo, K.-B. Hong, Behavioral Changes and Survival in Drosophila melanogaster: Effects of Ascorbic Acid, Taurine, and Caffeine., Biol. Pharm. Bull. 40 (2017) 1873–1882. https://doi.org/10.1248/bpb.b17-00321.

[48] E. Tellone, A. Galtieri, A. Russo, S. Ficarra, Protective Effects of the Caffeine Against Neurodegenerative Diseases., Curr. Med. Chem. 26 (2019) 5137–5151. https://doi.org/10.2174/0929867324666171009104040.

[49] T.M. McLellan, J.A. Caldwell, H.R. Lieberman, A review of caffeine's effects on cognitive, physical and occupational performance, Neurosci. Biobehav. Rev. 71 (2016) 294–312. https://doi.org/10.1016/j.neubiorev.2016.09.001.

[50] B.B. Fredholm, J. Yang, Y. Wang, Low, but not high, dose caffeine is a readily available probe for adenosine actions, Mol. Aspects Med. (2016). https://doi.org/10.1016/j.mam.2016.11.011.

[51] D. Wikoff, B.T. Welsh, R. Henderson, G.P. Brorby, J. Britt, E. Myers, J. Goldberger,

H.R. Lieberman, C. O'Brien, J. Peck, M. Tenenbein, C. Weaver, S. Harvey, J. Urban, C. Doepker, Systematic review of the potential adverse effects of caffeine consumption in healthy adults, pregnant women, adolescents, and children, Food Chem. Toxicol. 109 (2017) 585–648. https://doi.org/10.1016/j.fct.2017.04.002.

[52] M.H. Madeira, R. Boia, A.F. Ambrósio, A.R. Santiago, Having a Coffee Break: The Impact of Caffeine Consumption on Microglia-Mediated Inflammation in Neurodegenerative Diseases, Mediators Inflamm. 2017 (2017) 1–12. https://doi.org/10.1155/2017/4761081.

[53] P. Muriel, J. Arauz, Coffee and liver diseases, Fitoterapia. 81 (2010) 297–305. https://doi.org/10.1016/j.fitote.2009.10.003.

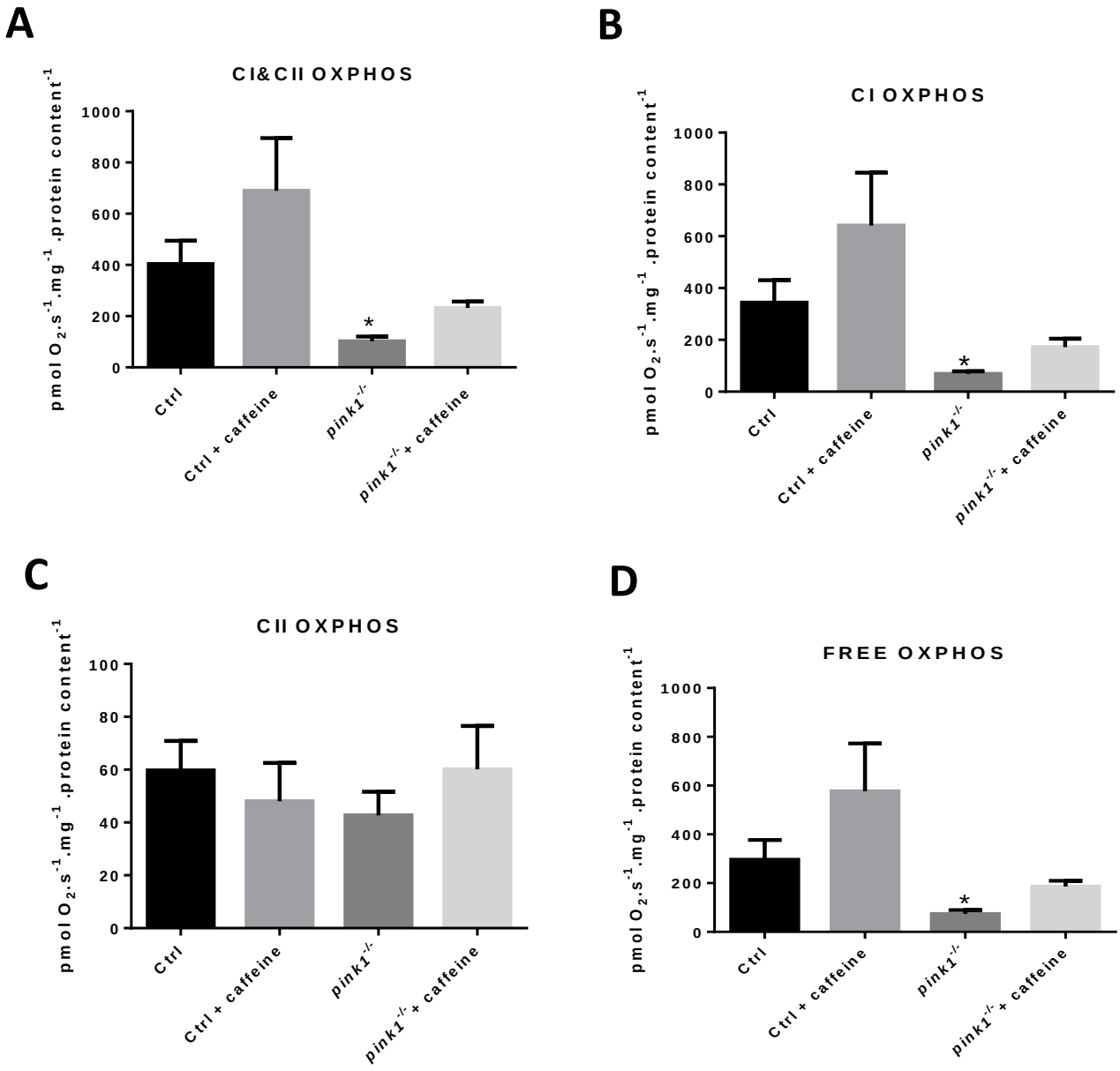

Fig. 1: High resolution respirometry on OXPHOS state from pink1-/+ (ctrl) and pink1-/y flies treated or not with caffeine. (A) Oxygen flux related to OXPHOS linked to mitochondrial complex I and II. (B) Oxygen flux related to OXPHOS linked to mitochondrial complex I (C) Oxygen flux related to OXPHOS linked to mitochondrial complex II. (D) Oxygen flux related to OXPHOS linked to mitochondrial complex I (D) Oxygen flux related to free OXPHOS. Statistical analyses using one-way ANOVA with Tukey's pos-test. * Represents significant difference from control group. Significant difference: $p<0,05$, n= 4-7.

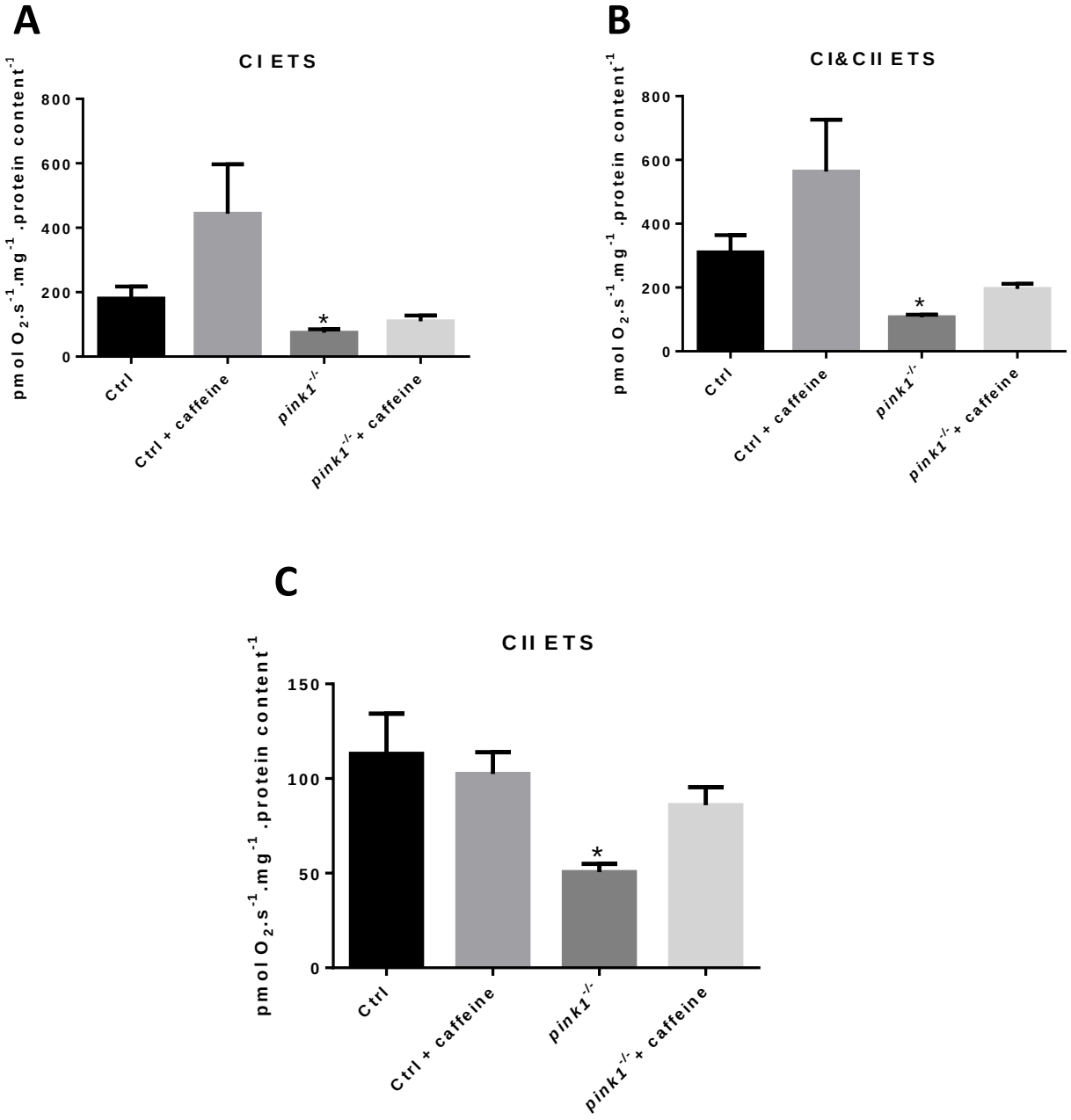

Fig. 2: High resolution respirometry on ETS state from pink1-/+ (ctrl) and pink1-/y flies treated or not with caffeine. (A) Oxygen flux related to ETS linked to mitochondrial complex I and II. (B) Oxygen flux related to ETS linked to mitochondrial complex I (C) Oxygen flux related to ETS linked to mitochondrial complex II. Statistical analyses using one-way ANOVA with Tukey's pos-test. * Represents significant difference from control group. Significant difference: $p<0,05$, n= 4-7.

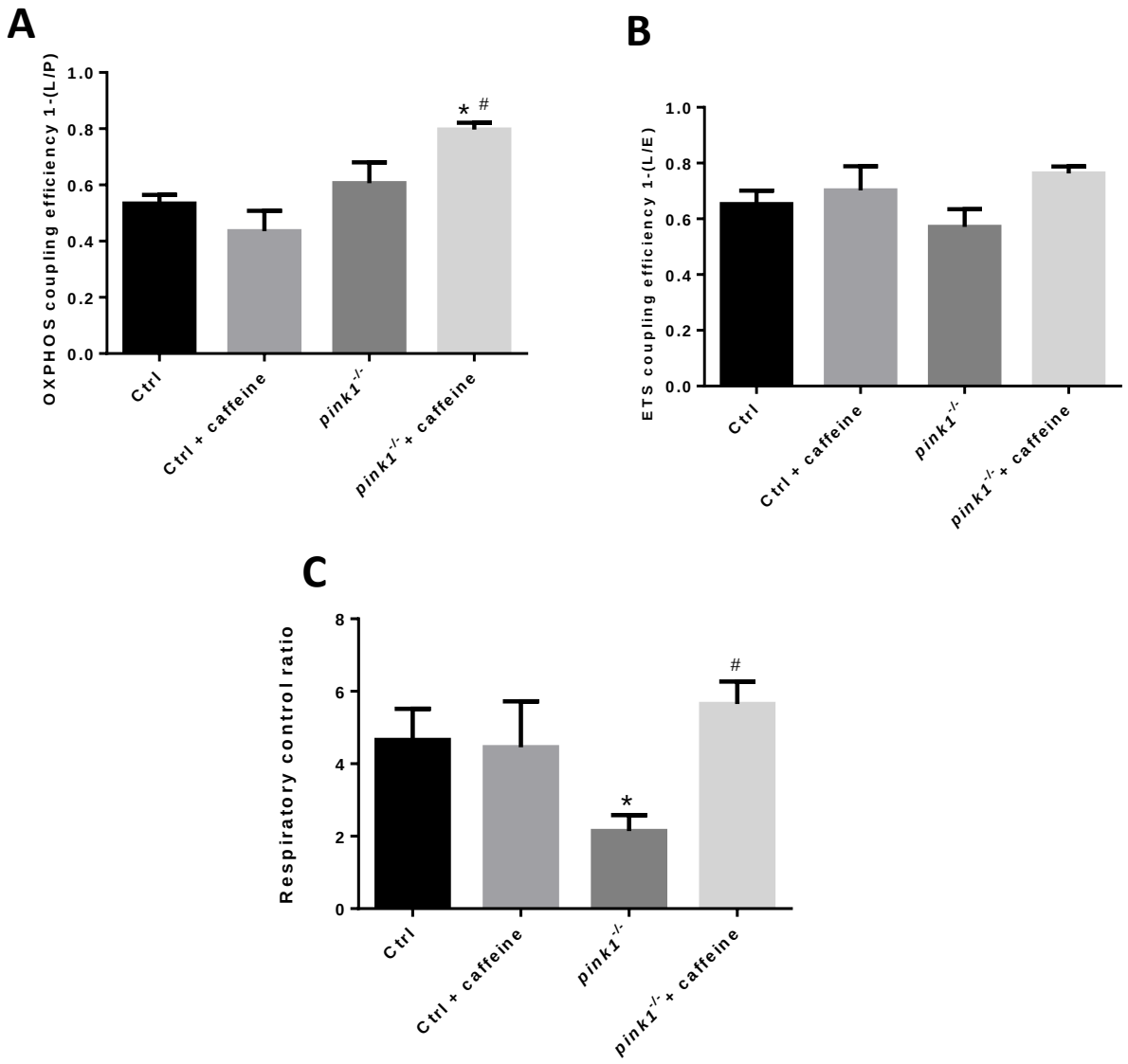

Fig. 3: Mitochondrial coupling efficiencies and respiratory control ratio in pink1-/+ (ctrl) and pink1-/y flies treated or not with caffeine. (A) OXPHOS coupling efficiency (B) ETS coupling efficiency. (C) Respiratory control ratio. Statistical analyses using one-way ANOVA with Tukey's pos test. * Represent significant difference from control group. Significant difference $p<0,05$, n=4-7

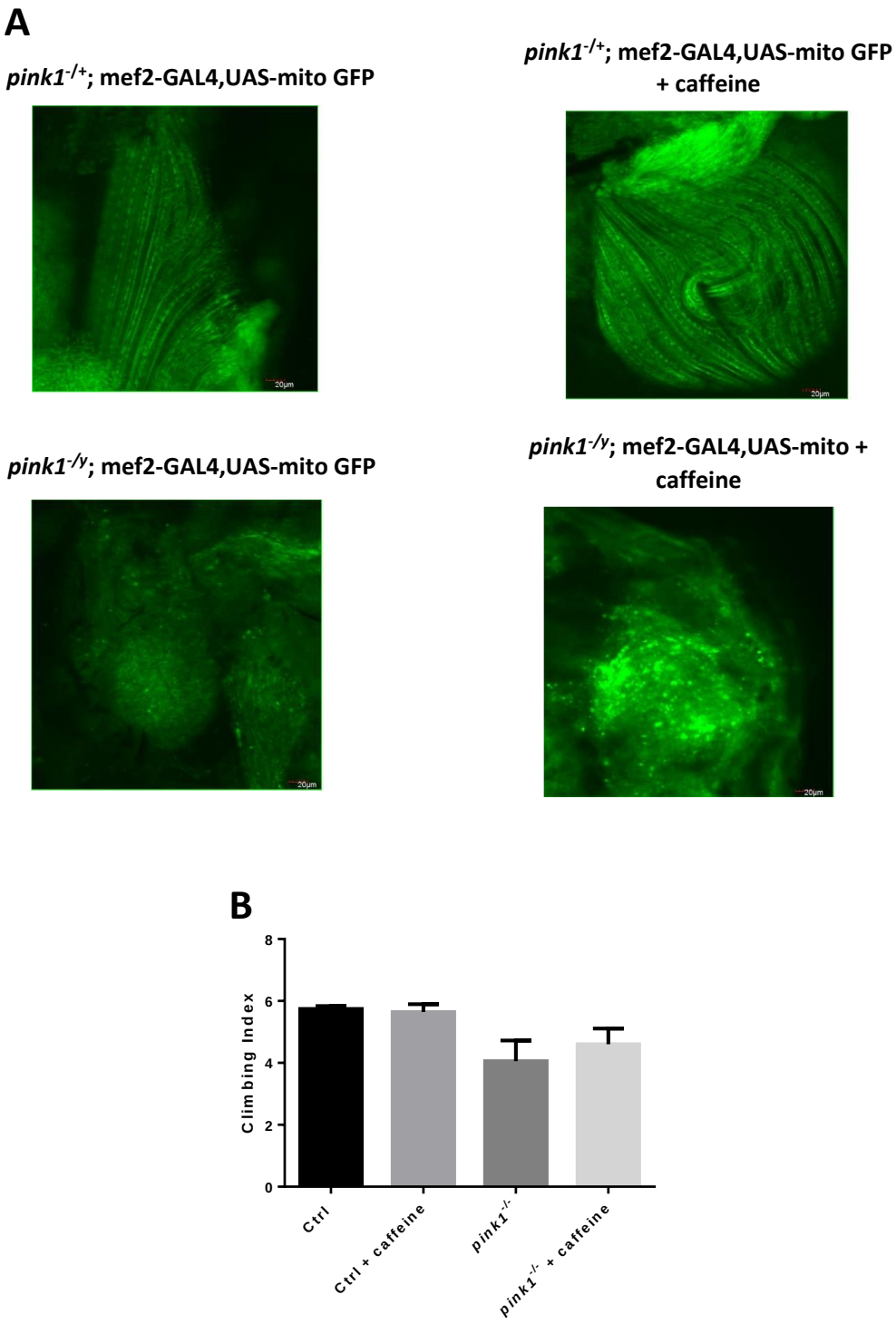

Fig. 4: Confocal images from pink1-/+; mef2-GAL4,UAS-mitoGFP (ctrl) and pink1-/y;mef2-GAL4,UAS-mitoGFP flies treated or not with caffeine. Climbing index from pink1-/+ (ctrl) and pink1-/y flies treated or not treated with caffeine. (A) Images from muscle thorax with mitochondria marked with GFP. (B) Climbing index, flies per group. Statistical analyses using one-way ANOVA with Tukey's pos-test. n= 20 to 50

5. DISCUSSÃO

Os resultados dessa tese trazem elucidações sobre bioenergética e funcionalidade mitocondrial em dois modelos distintos de DP um *in vitro,* utilizando a toxina 6-OHDA e outro *in vivo* utilizando *D. melanogaster* com deleção do gene pink1. O principal objetivo foi entender parâmetros relacionados a bioenergética e funcionalidade mitocondrial em diferentes áreas cerebrais, assim como estudar o processo de envelhecimento dentro de um modelo de DP, além de testar em ambos os modelos moléculas já reconhecidas pelo seu potencial antioxidante. Nessa seção discutiremos de maneira geral os resultados de maior relevância a cada trabalho, estabelecendo as possíveis relações entre eles.

Em linhas gerais os resultados do modelo de DP *in vitro* comprovaram que diferentes áreas do cérebro, respondem de forma diferente ao tratamento com a 6-OHDA. Quando avaliados parâmetros relacionados a bioenergética e funcionalidade mitocondrial. Em fatias de hipocampo de ratos expostas a 6-OHDA na concentração de 100 µM por 1 hora não foi possível perceber modificações na funcionalidade mitocondrial, entretanto houve um aumento na produção de H_2O_2. Também não foi possível perceber modificações relacionadas às atividades enzimáticas de CS e LDH. O hipocampo é a estrurura mais atingida pelo processo de envelhecimento (PANDYA et al., 2016), assim como já foi demonstrado ser a primeira estrutura com perda de funcionalidade durante a progressão de doenças neurodegenerativas como a DP (TEIXEIRA et al., 2018; VELAZQUEZ et al., 2018). De acordo com nossos resultados, o hipocampo não apresenta adaptação relacionada à funcionalidade mitocondrial após a exposição com 6-OHDA, o que torna essa região cerebral mais sucetível ao dano causado pelo aumento da produção de ERO's.

Quando avaliamos o efeito da exposição de fatias de córtex cerebral a 6-OHDA na concentração de 100 µM por 1 hora, foi possível perceber um resultado diferente. No córtex a exposição com 6-OHDA causou um efeito mitocondrial compensatório sustentado pela demonstração de aumento do fluxo de oxigênio relacionado a OXPHOS CI&CII, assim como aumento do fluxo de oxigênio relacionado a ETS-CII e aumento na atividade da enzima LDH, resultado que poderia estar relacionado com um aumento nos níveis de lactato. Quando utilizamos a NAC na concentração de 1 mM em coexposição com a 6-OHDA, aboliu-se os efeitos da 6-OHDA relacionados ao fluxo de oxigênio dependente tanto de OXPHOS quanto de ETS em córtex. Esse resultado sugere que a produção de ERO's no córtex é capaz de ativar uma via de sinalização de sobrevivência celular, podendo esta via estar relacionada com a ativação de HIF1-α. O HIF1-α é um importante fator de transcrição que regula o metabolismo,

mantendo os níveis de ATP pela conversão entre a rota de glicólise aeróbica e a rota de glicólise anaeróbica, esse processo de inversão de rotas se dá diante de situações de estresse ou falta de oxigênio (LU; FORBES; VERMA, 2002; TRACHOOTHAM et al., 2008).

Em fatias de estriado expostas a 6-OHDA na concentração de 100 µM por 1 hora foi possível perceber uma redução significativa do fluxo de elétrons relacionado a OXPHOS CI&CII, acompanhado de um grande aumento na produção de H_2O_2. Diferentemente do cortex a produção de ERO's causada pela exposição da 6-OHDA em estriado não produziu nenhum efeito adaptativo ou de troca de rota para a produção de energia. A coexposição de 6-OHDA e NAC em fatias de estriado recuperou os parâmetros relacionados a respirometria mitocondrial para os mesmos níveis de controle, demonstrando uma possível ação antioxidante e protetora dessa molécula. A região do estriado, assim como a substância negra cerebral são as regiões mais afetadas pela toxicidade da 6-OHDA devido ao grande número de neurônios dopaminérgicos os quais são centros de ligação para a 6-OHDA devido a sua estrutura química ser semelhante a dopamina, neurotransmissor que em situação normal seria o ligante dos neurônios dopaminérgicos (FÉGER et al., 2002; HAWLITSCHKA; WREE, 2018).

De acordo com nosso resultados de modelo *in vitro* de DP diferentes regiões do cérebro têm metabolismo diferente e respondem de maneiras diferentes à toxicidade da 6-OHDA, além disso os efeitos da exposição a 6-OHDA são perdidos na presença de NAC na concentração de 1mM. Outros estudos já demonstraram que áreas cerebrais distintas podem apresentar diferentes respostas em condições como: síndrome metabólica ou envelhecimento (DA SILVA et al., 2012; ETCHEGOYEN et al., 2018; PANDYA et al., 2016).

A partir dos resultados em modelo *in vitro* de DP os quais demonstraram importantes particularidades relacionadas ao funcionamento mitocondrial de diferentes áreas cerebrais, buscamos entender como esse processo se dá em um modelo *in vivo*. Para isso, utilizamos um modelo experimental de *D. melanogaster* com deleção do gene *pink1*.

Tendo em vista que a DP, em geral ocorre concomitante com o processo de envelhecimento procuramos entender como a DP e o envelhecimento dentro de suas particularidades se relacionam com a bioenergética mitocondrial. Para isso avaliamos parâmetros relacionados a bioenergética em *D. melanogaster* com deleção do gene *pink1* em três tempos diferentes: adultas jovens (3 dias), de meia-idade (15 dias) e velhas (30 dias).

O gene *pink1* é responsável por codificar um serina treonina quinase mitocondrial (PTEN-induced quinase 1), cuja ação é importante na manutenção da qualidade da rede mitocondrial, estando ligado a processos de autofagia (CHEN; DORN, 2013). A atividade de *pink1* é regulada pelo potencial elétrico transmembrana da mitocôndria. Em mitocôndrias

despolarizadas há um acúmulo da proteína PINK1 nas membranas mitocondriais, resultando no recrutamento de Parkin, uma ubiquitina ligase normalmente encontrada no citosol, envolvida na formação de autofagossomos. A formação dos autofagossomos inicia o processo de mitofagia, definido como a retirada seletiva de mitocôndrias danificadas da rede mitocondrial, a regulação do processo de mitofagia envolvendo essa interação, está associada à DP (LAZAROU et al., 2013; NARENDRA et al., 2010).

Nas moscas mutantes *pink1* com 3 dias de vida não foi possível observar diferenças significativas na atividade mitocondrial visto que não há redução significativa no fluxo de elétrons associado à fosforilação oxidativa, representada por OXPHOS-CI e OXPHOSCI&CII. Também não observamos uma diminuição significativa no fluxo de elétrons dependente ETS-CI. Nossos resultados destoam de outros dados da literatura científica que demonstram uma diminuição significativa da funcionalidade mitocondrial relacionada a OXPHOS e ETS já no terceiro dia de vida adulta de *D. melanogaster* (COSTA; LOH; MARTINS, 2013c; MURPHY, 2009; NARENDRA et al., 2010).

Um resultado diferente se deu quando avaliamos moscas *pink1* com 15 dias de vida já que, observamos uma diminuição significativa do fluxo de oxigênio relacionado a OXPHOS CI&CII e também uma redução significativa no fluxo de oxigênio relacionado ao estado ETS-CI&CII e ETS-CI. Esses resultados foram acompanhados por redução tanto na eficiência de acoplamento no estado OXPHOS quanto na eficiência de acoplamento no estado ETS. Por outro lado, houve um aumento significativo dos níveis de ATP, o que é bastante curisoso quando associado às menores taxas de fluxo de oxigênio no estado OXPHOS. Avaliamos então as atividades das enzimas CS e LDH e percebemos uma diminuição da atividade da CS acompanhada de um aumento da atividade da LDH em moscas *pink1*. Esses resultados demonstram uma possível troca de rota energética para a produção de ATP, passando de uma rota oxidativa para uma rota glicolítica.

Já em moscas *pink1* com 30 dias de vida foi possível perceber uma diminuição geral do fluxo de oxigênio relacionado aos estados OXPHOS e ETS acompanhado de um aumento na atividade CS. O aumento na atividade da CS se mostrou um resultado interessante visto que essa enzima está diretamente relacionada com o conteúdo mitocondrial. Entretanto, o aumento da atividade da CS em moscas *pink1* parece não estar relacionado a uma maior capacidade mitocondrial já que a produção de ATP não é aumentada, pelo contrário, as moscas *pink1* no 30º dia de idade apresentaram redução significativa nos níveis de ATP.

Mesmo o processo de envelhecimento estando associado a um declínio total da atividade mitocondrial, esse fato é contraditório com estudos que demonstram uma extensão de

vida causada por taxas metabólicas mais baixas (BAKER; PELEG, 2017), sendo assim, um modelo progressivo e monofásico de redução total da funcionalidade mitocondrial durante o envelhecimento pode ser simplificado demais. Outra explicação que sustenta o aumento da atividade da CS em moscas *pink1* com 30 dias de idade pode estar relacionada ao acúmulo de mitocôndrias na rede mitocondrial. A perda do gene *pink1* causa um grande acúmulo de mitocôndrias danificadas e esse processo é potencializado pelo envelhecimento (ASHRAFI; SCHWARZ, 2013). Diante desse fato, as moscas *pink1* com 30 dias provavelmente possuem um grande número de mitocôndrias, o que explicaria o aumento da atividade da CS, porém essas organelas não são capazes de utilizar os substratos para produzir energia, pois essas mitocôndrias são disfuncionais, levando à redução do conteúdo de ATP.

Por fim tratamos as moscas *pink1* com cafeína na concentração de 0,5 mg/mL desde o estágio larval 1 até o terceiro dia de vida adulta. Nossos resultados demonstraram um aumento do fluxo de oxigênio relacionado a OXPHOS nas moscas *pink1* tratadas com cafeína esse aumento de fluxo de oxigênio foi 90% maior quando comparado a moscas *pink1* sem tratamento com cafeína. Nosso grupo de pesquisa já havia demonstrado anteriormente que a cafeína aumenta a resposta bioenergética mitocondrial no cérebro de ratos pelo aumento de OXPHOS (GONÇALVES et al., 2020). O aumento do fluxo de oxigênio ligado a OXPHOS causado pelo efeito do tratamento com a cafeína em moscas *pink1* se mostra um resultado importante. Já foi demonstrado que a estimulação de OXPHOS em células cancerosas aumenta o estresse oxidativo e restaura a sensibilidade das células ao “anoikis”, que é um tipo específico de morte celular normal, sugerindo OXPHOS como um mecanismo antimetástase (LU; TAN; CAI, 2015). Portanto, o aumento de OXPHOS poderia melhorar o metabolismo celular e reduzir a proliferação celular exacerbada durante o câncer. Como a perda do gene *pink1* está envolvida em efeitos metabólicos semelhantes aos observados no câncer (COSTA; LOH; MARTINS, 2013a), acreditamos que o fato do tratamento com cafeína aumentar OXPHOS pode estar relacionada a um melhor aproveitamento de substratos para gerar energia utilizando a via mitocondrial. Além disso a disfunção mitocondrial caracterizada como a diminuição do fluxo de oxigênio dependente de OXPHOS é um marcador para doenças neurodegenerativas com Alzheimer e DP (DIAS et al., 2014; FIGUEIRA et al., 2013; MOREIRA et al., 2010), demonstrando um desacoplamento do fluxo de elétrons para a produção de ATP. Sendo assim, aumenta o interesse por compostos que sejam capazes de melhorar o processo de OXPHOS, assim como a cafeína demonstra fazer em nossos resultados. Compostos com essas características poderiam trazer resultados benéficos, como melhor acoplamento da cadeia

respiratoria mitocondrial, podendo servir como possíveis tratamentos secundários para doenças neurodegenerativas.

O tratamento com cafeína em moscas *pink1* também aumentou o fluxo de oxigênio relacionado a ETS em uma taxa em torno de 80% quando comparado com moscas *pink1* não tratadas. Freddo e colaboradores (2021) já mostraram que a cafeína aumenta a atividade dos complexos mitocondriais II, III e IV em um modelo de neurotoxicidade utilizando peixe zebra como modelo experimental (FREDDO et al., 2021). No entanto, não há relatos sobre os efeitos da cafeína na ETS mitocondrial ou na atividade dos complexos mitocondriais especificamente em *D. melanogaster*. Até o momento, estudos sobre os efeitos da cafeína em *D. melanogaster* estão relacionados ao aumento do AMPc intracelular (BHASKARA; CHANDRASEKHARAN; GANGULY, 2008), interferência na atividade sono-vigília (LIN, 2010) e metabolismo dependente do citocromo P450 (COELHO et al., 2015). Além disso, foi demonstrado anteriormente que a suplementação com 0,05% de cafeína foi capaz de aumentar a atividade da catalase e os níveis de GSH em moscas adultas, mas não foi capaz de estender a vida útil em *D. melanogaster* (SUH et al., 2017).

Avaliando os resultados obtidos nos diferentes modelos experimentais, inferimos que existem especificidades importantes que devem ser consideradas para estudos de funcionalidade mitocondrial e bioenergética em modelos de DP. Percebemos que tanto em fatias de córtex expostas a 6-OHDA, assim como em moscas com deleção do gene *pink1* aos 15 dias de vida, houve uma resposta compensatória na produção de energia. Destacamos a participação do lactato como substrato chave de uma possível troca de rota de aeróbica para glicolítica, visando a geração de ATP. O lactato é uma importante fonte de energia durante eventos isquêmicos e considerado necessário para eventos de transmissão e plasticidade sináptica (MAGISTRETTI; ALLAMAN, 2018).

Além disso, é provável que tanto o evento de adaptação mitocondrial ocorrida no cortex após exposição a 6-OHDA, assim como o efeito observado pela deleção do gene pink1 em moscas com 15 dias de vida, seja mediado pelo fator HIF1-α. Já foi comprovado que em ratos, a deleção do gene *pink1* promove a troca de rota metabólica resultando em aumento de lactato, aumento da atividade da lactato desidrogenase e aumento da expressão de HIF1-α, esse mecanismo é ativado devido a redução da fosforilação oxidativa para a produção de ATP em eventos de disfunção mitocondrial (REQUEJO-AGUILAR et al., 2014). Pela primeira vez demonstramos que o mesmo efeito ocorre em *D. melanogaster* com deleção do gene *pink1* e curiosamente apenas no 15º dia de vida.

Outro fator importante a ser considerado são os efeitos exercidos tanto pela NAC, quanto pela cafeína nos diferentes modelos estudados. Ambas as substâncias são reconhecidas como antioxidantes (COLES et al., 2018b; ZEIDÁN-CHULIÁ et al., 2013; ZHANG et al., 2012) e interferiram diretamente em parâmetros mitocondriais. Nas fatias de cortex, hipocampo e estriado de cérebro de ratos a coexposição de 1mM de NAC juntamente com a 6-OHDA durante 1 hora extinguiu efeitos relacionados a funcionalidade mitocondrial causados pela exposição de 6-OHDA. Já em moscas, o tratamento com 0,5 mg/mL de cafeína em moscas com deleção do gene *pink1* desde o estágio larval 1 até o terceiro dia de vida adulta, demonstrou interferir positivamente e de maneira direta na funcionalidade mitocondrial, tanto em OXPHOS quanto em ETS.

A ativação de OXPHOS é essencial para o processo acoplado de produção de energia, assim como a inibição desse processo leva a uma maior produção de ERO's, aumentando a tendência de apoptose celular (YAN; WANG; ZHU, 2013). Procaccio e colaboradores, (2014) destacam as mitocôndrias como bons alvos para o desenho de drogas relacionadas a doenças neurodegenerativas. Segundo os autores, substâncias capazes de aumentar OXPHOS assim, como ter atividade "scavenger" contra ERO's ou ainda aumentar o processo de biogênese mitocondrial poderiam ser boas alternativas de tratamento coadjuvante para doenças como Parkinson, Huntington e Alzheimer (PROCACCIO et al., 2014). Considerando as afirmações desse estudo, correlacionamos que tanto a cafeína quando a NAC, devido aos seus efeitos sobre a funcionalidade mitocondrial, poderiam contribuir para a melhoria de quadros de doenças neurodegenerativas incluindo a DP.

Sendo assim é possível delinear pontos de compatibilidade importantes entre um modelo transgênico de DP e um modelo induzido por 6-OHDA. Essa convergência se apresenta tanto na questão de adaptação mitocondrial para a produção de energia, quanto na resposta direta de substâncias antioxidantes em parâmetros relacionados a respirometria de alta resolução. Tendo em vista que a disfunção mitocondrial já é um efeito reconhecido e associado a DP (MASSARI et al., 2016; PIMENTA DE CASTRO et al., 2012b), as convergências entre modelos experimentais podem ajudar no entendimento sobre esse processo. O que fica claro é que há um efeito mitocondrial compensatório, que parece ser acionado para suprir uma demanda energética celular e que pode ser ativado tanto pela exposição a 6-OHDA como pela deficiência genética sendo um elo comum relacionado a bioenergética que aproxima modelos distintos de DP.

6. CONCLUSÃO

6.1 Conclusões específicas

6.1.1 Modelo *in vitro* com exposição a 6-OHDA e efeitos da NAC

No modelo de DP in vitro induzido pela exposição de diferentes áreas cerebrais à 6-OHDA na concentração de 100 µM, concluímos a região cortical parece adptar-se ao dano causado pela 6-OHDA o que é demonstrado principalmente pelo aumento de OXPHOS CI&CII e da atividade da LDH. A região do hipocampo, apresenta aumento de OXPHOS-CI, porém tal aumento não reflete adaptação para a produção de ATP. A região do estriado demonstrou maior prejuízo frente à exposição a 6-OHDA, tendo uma atividade mitocondrial reduzida em OXPHOS e ETS dependentes de CI&CII com um grande aumento de ERO's. A coexposição de 6-OHDA e NAC na concentração de 1mM extiguiu os efeitos da 6-OHDA em todas as áreas cerebrais. Os efeitos relacionados a funcionalidade mitocondrial causado pela exposição a 6-OHDA em córtex, hipocampo e estriado são resumidos na figura 5.

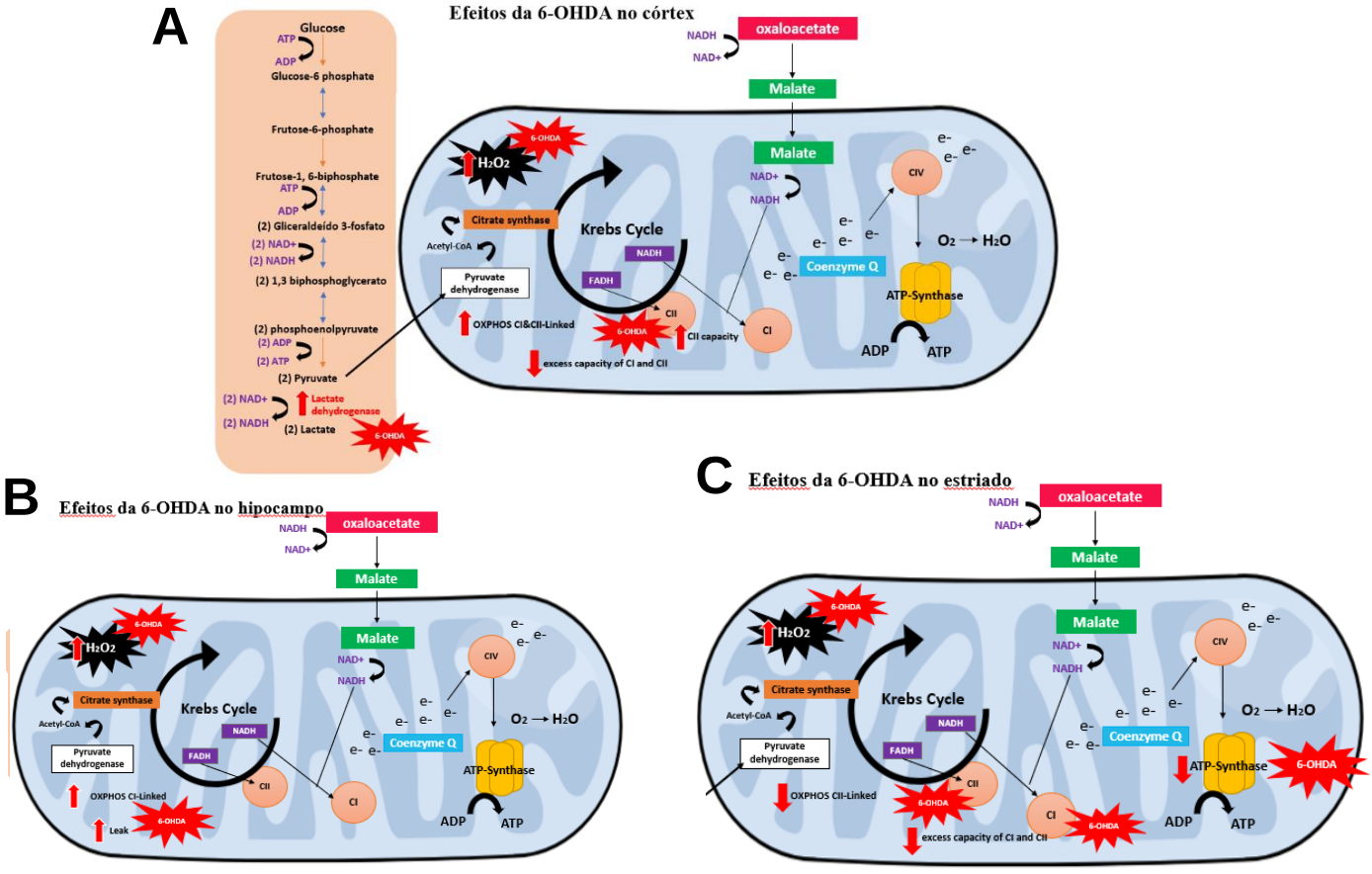

Figura 5. Efeitos da 6-OHDA na funcionalidade mitocondrial em fatias (A) córtex, (B) hipocampo e (C) estriado

6.1.2 Modelo de DP *in vivo* associado ao envelhecimento

No modelo de DP *in vivo* utilizando *D. melanogaster* com deleção do gene *pink1* observamos modificações na bioenergética mitocondrial durante o envelhecimento. Destacamos o aumento da produção de ATP aos 15 dias de vida, o qual foi acompanhado de uma redução da eficiência de acoplamento da cadeia respiratória mitocondrial dependente de OXPHOS e ETS com aumento da atividade da lactato desidrogenase e redução da atividade do citrato sintase. Associamos o aumento da produção de ATP nas moscas com 15 dias de vida com uma troca de rota energética, priorizando a rota glicolítica em detrimento da rota oxidativa para a produção de ATP. A troca de rota energética com potencialização da rota glicolítica para a produção de ATP, já foi demonstrada em modelos de DP utilizando roedores com deleção do gene *pink1*.

6.1.3 Modelo de DP in vivo e efeitos da cafeína

O tratamento de *D. melanogaster* com deleção do gene *pink1* com 0,5 mg/mL de cafeína foi capaz de aumentar o fluxo de elétrons relacionado a OXPHOS e ETS dependentes de CI&CII, além de aumentar a eficiência de acoplamento OXPHOS e a razão de controle respiratório mitocondrial, demonstrando uma melhora do fluxo de elétrons da cadeia respiratória. Além disso, vale salientar como conclusão dessa tese que pela primeira vez demonstramos os efeitos da cafeína associados a um modelo de DP com deleção do gene *pink1*.

6.2 Conclusão geral

Em nossos modelos de DP *in vivo* e *in vitro* pontuamos diferenças relacionadas a funcionalidade mitocondrial as quais se relacionam diretamente com fatores genéticos, diferenças fisiológicas e processo de envelhecimento. A DP é a segunda desordem neurodegenerativa mais comum a nível mundial (KALIA; LANG, 2015) e sua complexidade se acentua pelo fato de se caracterizar como uma patologia que, de forma geral, se dá em concomitância com o envelhecimento. Nesse sentido, os estudos de especificidades relacionadas a funcionalidade mitocondrial em diferentes modelos de DP e em associação com o envelhecimento ajudam a entender melhor ambos os processos e os possíveis efeitos sinérgicos dos mesmos.

Salientamos a importância de se reconhecer as particularidades inerentes aos processos metabólicos e como são afetados por diferentes variáveis. Através de nossos resultados demonstramos que a regulação desses processos pode ser útil como possível alvo farmacológico no tratamento de DP.

Por fim apresentamos a cafeína e a NAC como interferentes no processo de funcionalidade mitocondrial em diferentes modelos de DP. No caso da cafeína aumentando os níveis de fluxo de oxigênio dependentes de OXPHOS e ETS e no caso da NAC impedindo os danos causados pela exposição a 6-OHDA, provavelmente pela sua ação antioxidante. Esses resultados afirmam a possível utilização desses compostos como tratamentos secundários para a DP, já que não existe uma cura para essa doença e o que se busca é a melhoria da qualidade de vida dos pacientes.

7. PERSPECTIVAS

Como uma das principais perspectivas desse trabalho trazemos o tratamento com cafeína em períodos mais longos da vida de *D. melanogaster* com deleção do gene *pink1*, por exemplo, um tratamento que se inicie no estado larval até o décimo quinto, ou trigésimo dia de vida das moscas, com o objetivo de entender quais os efeitos a longo prazo causados pela cafeína e como o envelhecimento poderia interferir nesse processo. Uma outra perspectiva seria a utilização da NAC como composto de tratamento em modelo de DP em *D. melanogaster*, já que essa molécula demonstrou bons resultados em modelo *in vitro* de DP.

8. REFERÊNCIAS

ABBAS, N. et al. A Wide Variety of Mutations in the Parkin Gene Are Responsible for Autosomal Recessive Parkinsonism in Europe. **Human Molecular Genetics**, v. 8, n. 4, p. 567–574, 1 abr. 1999.

ABETE-LUZI, P.; EISENMANN, D. M. **Regulation of C. elegans L4 cuticle collagen genes by the heterochronic protein LIN-29Genesis**John Wiley and Sons Inc., , 1 maio 2018. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/29604168/>. Acesso em: 8 dez. 2020

ABOLAJI, A. O. et al. Resveratrol prolongs lifespan and improves 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced oxidative damage and behavioural deficits in Drosophila melanogaster. **Biochemical and Biophysical Research Communications**, v. 503, n. 2, p. 1042–1048, 5 set. 2018.

ADAMS, M. D. The Genome Sequence of Drosophila melanogaster. **Science**, v. 287, n. 5461, p. 2185–2195, 24 mar. 2000.

AMMAL KAIDERY, N.; THOMAS, B. Current perspective of mitochondrial biology in Parkinson's disease. **Neurochemistry International**, 14 mar. 2018.

ARYAL, B.; LEE, Y. Disease model organism for Parkinson Disease: Drosophila melanogaster. **BMB reports**, 14 dez. 2018.

ASHRAFI, G.; SCHWARZ, T. L. The pathways of mitophagy for quality control and clearance of mitochondria. **Cell Death & Differentiation**, v. 20, n. 1, p. 31–42, 29 jan. 2013.

BAI, X. X. et al. Downregulation of metabotropic glutamate receptor 5 inhibits hepatoma development in a neurotoxin rotenone-induced Parkinson's disease model. **Toxicology Letters**, v. 288, p. 71–81, 15 maio 2018.

BAK, L. K.; SCHOUSBOE, A.; WAAGEPETERSEN, H. S. The glutamate/GABA-glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer. **Journal of Neurochemistry**, v. 98, n. 3, p. 641–653, ago. 2006.

BAKER, D. J.; PELEG, S. Biphasic Modeling of Mitochondrial Metabolism Dysregulation during Aging. **Trends in Biochemical Sciences**, v. 42, n. 9, p. 702–711, set. 2017.

BEAL, M. F. Aging, energy, and oxidative stress in neurodegenerative diseases. **Annals of Neurology**, v. 38, n. 3, p. 357–366, set. 1995.

BÉLANGER, M.; ALLAMAN, I.; MAGISTRETTI, P. J. Brain Energy Metabolism: Focus on Astrocyte-Neuron Metabolic Cooperation. **Cell Metabolism**, v. 14, n. 6, p. 724–738, dez. 2011.

BERG, D. et al. Type and frequency of mutations in the LRRK2 gene in familial and sporadic Parkinson's disease*. **Brain : a journal of neurology**, v. 128, n. Pt 12, p. 3000–11, dez. 2005.

BHASKARA, S.; CHANDRASEKHARAN, M. B.; GANGULY, R. Caffeine induction of Cyp6a2 and Cyp6a8 genes of Drosophila melanogaster is modulated by cAMP and D-JUN protein levels. **Gene**, v. 415, n. 1–2, p. 49–59, maio 2008.

BILEN, J.; BONINI, N. M. *Drosophila* as a Model for Human Neurodegenerative Disease. **Annual Review of Genetics**, v. 39, n. 1, p. 153–171, dez. 2005.

BLUM, D. et al. Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. **Progress in Neurobiology**, v. 65, n. 2, p. 135–172, out. 2001.

BONDI, M. W.; EDMONDS, E. C.; SALMON, D. P. Alzheimer's Disease: Past, Present, and Future. **Journal of the International Neuropsychological Society**, v. 23, n. 9–10, p. 818–831, 4 out. 2017.

BONIFATI, V. et al. DJ-1(PARK7), a novel gene for autosomal recessive, early onset parkinsonism. **Neurological Sciences**, v. 24, n. 3, p. 159–160, out. 2003.

BOWLING, A. C. et al. Age-Dependent Impairment of Mitochondrial Function in Primate Brain. **Journal of Neurochemistry**, v. 60, n. 5, p. 1964–1967, maio 1993.

BRAND, A. H.; PERRIMON, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. **Development (Cambridge, England)**, v. 118, n. 2, p. 401–15, jun. 1993a.

BRAND, A. H.; PERRIMON, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. **Development (Cambridge, England)**, v. 118, n. 2, p. 401–15, jun. 1993b.

BRATIC, A. et al. The bicoid stability factor controls polyadenylation and expression of specific mitochondrial mRNAs in Drosophila melanogaster. **PLoS Genetics**, v. 7, n. 10, out. 2011.

BRATIC, A. et al. Complementation between polymerase- and exonuclease-deficient mitochondrial DNA polymerase mutants in genomically engineered flies. **Nature Communications**, v. 6, 10 nov. 2015.

BROOKES, P. S. et al. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. **American Journal of Physiology-Cell Physiology**, v. 287, n. 4, p. C817–C833, out. 2004.

BURTSCHER, J. et al. Differences in mitochondrial function in homogenated samples from healthy and epileptic specific brain tissues revealed by high-resolution respirometry.

Mitochondrion, v. 25, p. 104–112, 1 nov. 2015.

CAI, R. et al. Enhancing glycolysis attenuates Parkinson's disease progression in models and clinical databases. **Journal of Clinical Investigation**, v. 129, n. 10, p. 4539–4549, 1 out. 2019.

CARVALHO, N. R. et al. New therapeutic approach: diphenyl diselenide reduces mitochondrial dysfunction in acetaminophen-induced acute liver failure. **PloS one**, v. 8, n. 12, p. e81961, jan. 2013.

CASTAGNOLI, N.; CHIBA, K.; TREVOR, A. J. V. Potential bioactivation pathways for the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). **Life Sciences**, v. 36, n. 3, p. 225–230, jan. 1985.

CHAMPIGNY, C. M. et al. Omega-3 monoacylglyceride effects on longevity, mitochondrial metabolism and oxidative stress: Insights from Drosophila melanogaster. **Marine Drugs**, v. 16, n. 11, 16 nov. 2018.

CHAN, P. et al. Rapid ATP Loss Caused by 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine in Mouse Brain. **Journal of Neurochemistry**, v. 57, n. 1, p. 348–351, jul. 1991.

CHEN, Y.; DORN, G. W. PINK1-Phosphorylated Mitofusin 2 Is a Parkin Receptor for Culling Damaged Mitochondria. **Science**, v. 340, n. 6131, p. 471–475, 26 abr. 2013.

CHIA, S. J.; TAN, E.-K.; CHAO, Y.-X. Molecular Sciences Historical Perspective: Models of Parkinson's Disease. [s.d.].

CHRISTENSEN, K. et al. Ageing populations: the challenges ahead. **The Lancet**, v. 374, n. 9696, p. 1196–1208, out. 2009.

COELHO, A. et al. Cytochrome P450-Dependent Metabolism of Caffeine in Drosophila melanogaster. **PLOS ONE**, v. 10, n. 2, p. e0117328, 11 fev. 2015.

COHEN, E.; DILLIN, A. The insulin paradox: aging, proteotoxicity and neurodegeneration. **Nature Reviews Neuroscience**, v. 9, n. 10, p. 759–767, 4 out. 2008.

COLES, L. D. et al. Repeated-Dose Oral N-Acetylcysteine in Parkinson's Disease: Pharmacokinetics and Effect on Brain Glutathione and Oxidative Stress. **The Journal of Clinical Pharmacology**, v. 58, n. 2, p. 158–167, fev. 2018a.

COLES, L. D. et al. Repeated-Dose Oral N-Acetylcysteine in Parkinson's Disease: Pharmacokinetics and Effect on Brain Glutathione and Oxidative Stress. **The Journal of Clinical Pharmacology**, v. 58, n. 2, p. 158–167, fev. 2018b.

CORMIER, R. P. J. et al. Dynamic mitochondrial responses to a high-fat diet in Drosophila melanogaster. **Scientific Reports**, v. 9, n. 1, 1 dez. 2019.

CORREA, C. C. et al. Mitochondrial DNA variants influence mitochondrial bioenergetics in

Drosophila melanogaster. **Mitochondrion**, v. 12, n. 4, p. 459–464, jul. 2012.

COSTA, A. C.; LOH, S. H.; MARTINS, L. M. Drosophila Trap1 protects against mitochondrial dysfunction in a PINK1/parkin model of Parkinson's disease. **Cell death & disease**, v. 4, n. 1, 2013a.

COSTA, A. C.; LOH, S. H. Y.; MARTINS, L. M. Drosophila Trap1 protects against mitochondrial dysfunction in a PINK1/parkin model of Parkinson's disease. **Cell death & disease**, v. 4, n. 1, p. e467, 17 jan. 2013b.

COSTA, A. C.; LOH, S. H. Y.; MARTINS, L. M. Drosophila Trap1 protects against mitochondrial dysfunction in a PINK1/parkin model of Parkinson's disease. **Cell Death & Disease**, v. 4, n. 1, p. e467–e467, 17 jan. 2013c.

COVI, J. A. et al. Rotenone Decreases Hatching Success in Brine Shrimp Embryos by Blocking Development: Implications for Zooplankton Egg Banks. **PLOS ONE**, v. 11, n. 9, p. e0163231, 21 set. 2016.

CRUZ-AGUADO, R. et al. Behavioral and biochemical effects of glutathione depletion in the rat brain. **Brain Research Bulletin**, v. 55, n. 3, p. 327–333, jun. 2001.

CRUZ, L. C. et al. Honey protects against wings posture error and molecular changes related to mitochondrial pathways induced by hypoxia/reoxygenation in adult Drosophila melanogaster. **Chemico-biological interactions**, v. 291, p. 245–252, 1 ago. 2018a.

CRUZ, L. C. et al. Honey protects against wings posture error and molecular changes related to mitochondrial pathways induced by hypoxia/reoxygenation in adult Drosophila melanogaster. **Chemico-Biological Interactions**, v. 291, p. 245–252, 1 ago. 2018b.

CUNNANE, S. C. et al. Brain energy rescue: an emerging therapeutic concept for neurodegenerative disorders of ageing. **Nature Reviews Drug Discovery**, v. 19, n. 9, p. 609–633, 24 set. 2020.

DA SILVA, M. H. et al. Acute brain damage induced by acetaminophen in mice: effect of diphenyl diselenide on oxidative stress and mitochondrial dysfunction. **Neurotoxicity research**, v. 21, n. 3, p. 334–44, abr. 2012.

DAWSON, T. M.; KO, H. S.; DAWSON, V. L. Genetic Animal Models of Parkinson's Disease. **Neuron**, v. 66, n. 5, p. 646–661, jun. 2010.

DEDEOGLU, A. et al. Magnetic resonance spectroscopic analysis of Alzheimer's disease mouse brain that express mutant human APP shows altered neurochemical profile. **Brain Research**, v. 1012, n. 1–2, p. 60–65, jun. 2004.

DEVINE, M. J.; PLUN-FAVREAU, H.; WOOD, N. W. Parkinson's disease and cancer: two wars, one front. **Nature reviews. Cancer**, v. 11, n. 11, p. 812–23, 24 out. 2011.

DIAS, C. et al. Evaluation of Mitochondrial Function in the CNS of Rodent Models of Alzheimer's Disease - High Resolution Respirometry Applied to Acute Hippocampal Slices. **Free radical biology & medicine**, v. 75 Suppl 1, p. S37, out. 2014.

DILBERGER, B. et al. Mitochondrial Oxidative Stress Impairs Energy Metabolism and Reduces Stress Resistance and Longevity of C. elegans. **Oxidative Medicine and Cellular Longevity**, v. 2019, 2019a.

DILBERGER, B. et al. Polyphenols and metabolites enhance survival in rodents and nematodes—impact of mitochondria. **Nutrients**, v. 11, n. 8, 1 ago. 2019b.

DOERRIER, C. et al. High-resolution fluorespirometry and oxphos protocols for human cells, permeabilized fibers from small biopsies of muscle, and isolated mitochondria. In: **Methods in Molecular Biology**. [s.l.] Humana Press Inc., 2018. v. 1782p. 31–70.

DOLGACHEVA, L. P. et al. Role of DJ-1 in the mechanism of pathogenesis of Parkinson's disease. **Journal of Bioenergetics and Biomembranes**, v. 51, n. 3, p. 175–188, 3 jun. 2019.

DORSEY, E. R. et al. Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030. **Neurology**, v. 68, n. 5, p. 384–386, 30 jan. 2007.

E, G. Capacity of Oxidative Phosphorylation in Human Skeletal Muscle: New Perspectives of Mitochondrial Physiology. **The international journal of biochemistry & cell biology**, v. 41, n. 10, 2009.

EDERER, K. A. et al. Age-and genotype-specific effects of the angiotensin-converting enzyme inhibitor lisinopril on mitochondrial and metabolic parameters in drosophila melanogaster. **International Journal of Molecular Sciences**, v. 19, n. 11, 1 nov. 2018.

ELCHEBLY, M. Increased Insulin Sensitivity and Obesity Resistance in Mice Lacking the Protein Tyrosine Phosphatase-1B Gene. **Science**, v. 283, n. 5407, p. 1544–1548, 5 mar. 1999.

EMIR, U. E. et al. Noninvasive quantification of ascorbate and glutathione concentration in the elderly human brain. **NMR in Biomedicine**, v. 24, n. 7, p. 888–894, ago. 2011.

ESPINOZA-FONSECA, L. M. The benefits of the multi-target approach in drug design and discovery. **Bioorganic & Medicinal Chemistry**, v. 14, n. 4, p. 896–897, fev. 2006.

ETCHEGOYEN, M. et al. Metabolic Syndrome and Neuroprotection. **Frontiers in Neuroscience**, v. 12, p. 196, 20 abr. 2018.

FEANY, M. B.; BENDER, W. W. A Drosophila model of Parkinson's disease. **Nature**, v. 404, n. 6776, p. 394–398, mar. 2000.

FÉGER, J. et al. [Experimental models of Parkinson's disease]. **Annales pharmaceutiques francaises**, v. 60, n. 1, p. 3–21, jan. 2002.

FEITOSA, C. M. Determination of parameters of oxidative stress in vitro models of

neurodegenerative diseases-A review. **Current Clinical Pharmacology**, v. 13, 1 mar. 2018.

FIGUEIRA, T. R. et al. Mitochondria as a source of reactive oxygen and nitrogen species: from molecular mechanisms to human health. **Antioxidants & redox signaling**, v. 18, n. 16, p. 2029–74, 2013.

FREDDO, N. et al. Stimulants cocktail: Methylphenidate plus caffeine impairs memory and cognition and alters mitochondrial and oxidative status. **Progress in Neuro-Psychopharmacology and Biological Psychiatry**, v. 106, p. 110069, mar. 2021.

FREDHOLM, B. B.; YANG, J.; WANG, Y. Low, but not high, dose caffeine is a readily available probe for adenosine actions. **Molecular Aspects of Medicine**, 30 nov. 2016.

GASSER, T. Genetic basis of Parkinson's disease: inheritance, penetrance, and expression. **The Application of Clinical Genetics**, p. 67, jun. 2011.

GAUTIER, C. A.; KITADA, T.; SHEN, J. Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. **Proceedings of the National Academy of Sciences**, v. 105, n. 32, p. 11364–11369, 12 ago. 2008.

GERISCH, B. et al. HLH-30/TFEB Is a Master Regulator of Reproductive Quiescence. **Developmental Cell**, v. 53, n. 3, p. 316- 329.e5, 4 maio 2020.

GLINKA, Y. Y.; YOUDIM, M. B. H. Inhibition of mitochondrial complexes I and IV by 6-hydroxydopamine. **European Journal of Pharmacology: Environmental Toxicology and Pharmacology**, v. 292, n. 3–4, p. 329–332, mar. 1995.

GOETZ, C. G. The history of Parkinson's disease: early clinical descriptions and neurological therapies. **Cold Spring Harbor perspectives in medicine**, v. 1, n. 1, p. a008862, 1 set. 2011.

GONÇALVES, D. F. et al. 6-Hydroxydopamine induces different mitochondrial bioenergetics response in brain regions of rat. **Neurotoxicology**, v. 70, p. 1–11, 2019.

GONÇALVES, D. F. et al. Effects of caffeine on brain antioxidant status and mitochondrial respiration in acetaminophen-intoxicated mice. **Toxicology Research**, v. 9, n. 5, p. 726–734, 29 out. 2020.

GUBERT, P. et al. Metabolic effects of manganese in the nematode Caenorhabditis elegans through DAergic pathway and transcription factors activation. **NeuroToxicology**, v. 67, p. 65–72, 1 jul. 2018.

GUITART, T. et al. New aminoacyl-tRNA synthetase-like protein in insecta with an essential mitochondrial function. **Journal of Biological Chemistry**, v. 285, n. 49, p. 38157–38166, 3 dez. 2010.

GUITART, T. et al. Human mitochondrial disease-like symptoms caused by a reduced tRNA aminoacylation activity in flies. **Nucleic Acids Research**, v. 41, n. 13, p. 6595–6608, jul.

2013.

GURURAJA RAO, S. et al. BKCa (Slo) Channel Regulates Mitochondrial Function and Lifespan in Drosophila melanogaster. **Cells**, v. 8, n. 9, 21 ago. 2019.

HALES, K. G. et al. Genetics on the Fly: A Primer on the Drosophila Model System. **Genetics**, v. 201, n. 3, p. 815–842, 1 nov. 2015.

HAVRANKOVA, J.; ROTH, J.; BROWNSTEIN, M. J. Concentrations of Insulin and of Insulin Receptors in the Brain are Independent of Peripheral Insulin Levels. **Journal of Clinical Investigation**, v. 64, n. 2, p. 636–642, 1 ago. 1979.

HAWLITSCHKA, A.; WREE, A. Experimental Intrastriatal Applications of Botulinum Neurotoxin-A: A Review. **International Journal of Molecular Sciences**, v. 19, n. 5, p. 1392, 7 maio 2018.

HEIKKILA, R. E. et al. Dopaminergic toxicity of rotenone and the 1-methyl-4-phenylpyridinium ion after their stereotaxic administration to rats: Implication for the mechanism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity. **Neuroscience Letters**, v. 62, n. 3, p. 389–394, dez. 1985.

HOU, Y. et al. **Ageing as a risk factor for neurodegenerative diseaseNature Reviews Neurology**Nature Publishing Group, , 1 out. 2019.

ISLAM, M. T. Oxidative stress and mitochondrial dysfunction-linked neurodegenerative disorders. **Neurological research**, v. 39, n. 1, p. 73–82, jan. 2017.

IWAKAMI, S. et al. Concentration-dependent Dual Effects of Hydrogen Peroxide on Insulin Signal Transduction in H4IIEC Hepatocytes. **PLoS ONE**, v. 6, n. 11, p. e27401, 15 nov. 2011.

J, L. et al. Selective lesion of central dopamine or noradrenaline neuron systems in the neonatal rat: motor behavior and monoamine alterations at adult stage. **Behavioural brain research**, v. 33, n. 3, p. 267–277, 1 jul. 1989.

J, W.; A, N. Mitochondrial Dynamics and Mitochondrial Dysfunction in Diabetes. **Acta medica Okayama**, v. 70, n. 3, 2016.

JUMBO-LUCIONI, P. et al. Nuclear genomic control of naturally occurring variation in mitochondrial function in Drosophila melanogaster. **BMC Genomics**, v. 13, n. 1, 22 nov. 2012.

K, T. A guide to neurotoxic animal models of Parkinson's disease. **Cold Spring Harbor perspectives in medicine**, v. 1, n. 1, set. 2011.

KABIRAJ, P. et al. The Neuroprotective Role of Ferrostatin-1 Under Rotenone-Induced Oxidative Stress in Dopaminergic Neuroblastoma Cells. **The Protein Journal**, v. 34, n. 5, p.

349–358, 18 out. 2015.

KALIA, L. V; LANG, A. E. Parkinson's disease. **The Lancet**, v. 386, n. 9996, p. 896–912, 29 ago. 2015.

KALINDERI, K.; BOSTANTJOPOULOU, S.; FIDANI, L. The genetic background of Parkinson's disease: current progress and future prospects. **Acta Neurologica Scandinavica**, v. 134, n. 5, p. 314–326, nov. 2016.

KASSAHUN, H. et al. Constitutive MAP-kinase activation suppresses germline apoptosis in NTH-1 DNA glycosylase deficient C. elegans. **DNA Repair**, v. 61, p. 46–55, 1 jan. 2018.

KITADA, T. et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. **Nature**, v. 392, n. 6676, p. 605–608, abr. 1998.

KOLAHDOUZAN, M.; HAMADEH, M. J. The neuroprotective effects of caffeine in neurodegenerative diseases. **CNS Neuroscience & Therapeutics**, v. 23, n. 4, p. 272–290, abr. 2017.

KRUMSCHNABEL, G. et al. Simultaneous High-Resolution Measurement of Mitochondrial Respiration and Hydrogen Peroxide Production. In: **Methods in molecular biology (Clifton, N.J.)**. [s.l: s.n.]. v. 1264p. 245–261.

KRUMSCHNABEL, G. et al. Simultaneous High-Resolution Measurement of Mitochondrial Respiration and Hydrogen Peroxide Production. In: [s.l: s.n.]. p. 245–261.

KRZYSZTOŃ-RUSSJAN, J. Pathophysiology and molecular basis of selected metabolic abnormalities in Huntington's disease. **Postepy higieny i medycyny doswiadczalnej (Online)**, v. 70, n. 0, p. 1331–1342, 30 dez. 2016.

LANGSTON, J. W. et al. 1-Methyl-4-phenylpyridinium ion (MPP+): Identification of a metabolite of MPTP, a toxin selective to the substantia nigra. **Neuroscience Letters**, v. 48, n. 1, p. 87–92, jul. 1984.

LANGSTON, J. W. The MPTP Story. **Journal of Parkinson's Disease**, v. 7, n. s1, p. S11–S19, 6 mar. 2017.

LAZAROU, M. et al. PINK1 drives Parkin self-association and HECT-like E3 activity upstream of mitochondrial binding. **The Journal of cell biology**, v. 200, n. 2, p. 163–72, 21 jan. 2013.

LEÃO, M. B. et al. Toxicological evaluation of the herbicide Palace® in Drosophila melanogaster. **Journal of Toxicology and Environmental Health - Part A: Current Issues**, v. 82, n. 22, p. 1172–1185, 17 nov. 2019.

LEMIEUX, H.; BLIER, P. U.; GNAIGER, E. Remodeling pathway control of mitochondrial respiratory capacity by temperature in mouse heart: electron flow through the Q-junction in

permeabilized fibers. **Scientific Reports**, v. 7, n. 1, p. 2840, 6 dez. 2017.

LI, J.; SUN, Y.; CHEN, J. Transcriptome sequencing in a 6-hydroxydopamine rat model of Parkinson's disease. **Genes & Genetic Systems**, v. 94, n. 2, p. 61–69, 1 abr. 2019.

LIN, F. J. Effect of taurine and caffeine on sleep–wake activity in Drosophila melanogaster. **Nature and Science of Sleep**, p. 221, set. 2010.

LIN, M. T.; BEAL, M. F. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. **Nature**, v. 443, n. 7113, p. 787–795, 18 out. 2006.

LISOWSKI, P. et al. Mitochondria and the dynamic control of stem cell homeostasis. **EMBO reports**, p. e45432, 16 abr. 2018.

LIU, M. et al. Ginseng protein protects against mitochondrial dysfunction and neurodegeneration by inducing mitochondrial unfolded protein response in Drosophila melanogaster PINK1 model of Parkinson's disease. **Journal of Ethnopharmacology**, v. 247, 30 jan. 2020.

LOH, K. et al. Reactive Oxygen Species Enhance Insulin Sensitivity. **Cell Metabolism**, v. 10, n. 4, p. 260–272, out. 2009.

LOPES, F. M. et al. Mimicking Parkinson's Disease in a Dish: Merits and Pitfalls of the Most Commonly used Dopaminergic In Vitro Models. **NeuroMolecular Medicine**, v. 19, 2017.

LU, H.; FORBES, R. A.; VERMA, A. Hypoxia-inducible Factor 1 Activation by Aerobic Glycolysis Implicates the Warburg Effect in Carcinogenesis. **Journal of Biological Chemistry**, v. 277, n. 26, p. 23111–23115, jun. 2002.

LU, J.; TAN, M.; CAI, Q. The Warburg effect in tumor progression: mitochondrial oxidative metabolism as an anti-metastasis mechanism. **Cancer letters**, v. 356, n. 2 Pt A, p. 156–64, 28 jan. 2015.

MACCHI, M. et al. The Drosophila inner-membrane protein PMI controls crista biogenesis and mitochondrial diameter. **Journal of Cell Science**, v. 126, n. 3, p. 814–824, fev. 2013.

MACEDO, F. et al. Lipase-like 5 enzyme controls mitochondrial activity in response to starvation in Caenorhabditis elegans. **Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids**, v. 1865, n. 2, 1 fev. 2020a.

MACEDO, F. et al. Lifespan-extending interventions enhance lipid-supported mitochondrial respiration in Caenorhabditis elegans. **FASEB Journal**, v. 34, n. 8, p. 9972–9981, 1 ago. 2020b.

MACEDO, F. et al. Lifespan-extending interventions enhance lipid-supported mitochondrial respiration in *Caenorhabditis elegans*. **The FASEB Journal**, v. 34, n. 8, p. 9972–9981, 1 ago. 2020c.

MAGISTRETTI, P. J.; ALLAMAN, I. Lactate in the brain: from metabolic end-product to signalling molecule. **Nature reviews. Neuroscience**, v. 19, n. 4, p. 235–249, 8 abr. 2018.

MARTÍNEZ-BANACLOCHA, M. Cysteine Network (CYSTEINET) Dysregulation in Parkinson's Disease: Role of N-acetylcysteine. **Current drug metabolism**, v. 17, n. 4, p. 368–85, 2016.

MASSARI, C. M. et al. In vitro 6-hydroxydopamine-induced toxicity in striatal, cerebrocortical and hippocampal slices is attenuated by atorvastatin and MK-801. **Toxicology in Vitro**, v. 37, p. 162–168, dez. 2016.

MATTHEWS, K. A.; KAUFMAN, T. C.; GELBART, W. M. Research resources for Drosophila: the expanding universe. **Nature reviews. Genetics**, v. 6, n. 3, p. 179–93, mar. 2005.

MCCOMMIS, K. S.; FINCK, B. N. Mitochondrial pyruvate transport: a historical perspective and future research directions. **Biochemical Journal**, v. 466, n. 3, p. 443–454, 15 mar. 2015.

MCKNIGHT, S.; HACK, N. Toxin-Induced Parkinsonism. **Neurologic clinics**, v. 38, n. 4, p. 853–865, 2020.

MCLELLAN, T. M.; CALDWELL, J. A.; LIEBERMAN, H. R. A review of caffeine's effects on cognitive, physical and occupational performance. **Neuroscience & Biobehavioral Reviews**, v. 71, p. 294–312, dez. 2016.

MICCHELI, A. et al. [1-13C]Glucose entry in neuronal and astrocytic intermediary metabolism of aged rats. **Brain Research**, v. 966, n. 1, p. 116–125, mar. 2003.

MIKI, Y. et al. Alteration of mitochondrial protein PDHA1 in Lewy body disease and PARK14. **Biochemical and Biophysical Research Communications**, v. 489, n. 4, p. 439–444, ago. 2017.

MISHRA, J.; KUMAR, A. Improvement of mitochondrial NAD+/FAD+-linked state-3 respiration by caffeine attenuates quinolinic acid induced motor impairment in rats: Implications in Huntington's disease. **Pharmacological Reports**, v. 66, n. 6, p. 1148–1155, dez. 2014.

MONTI, D. A. et al. N-Acetyl Cysteine Is Associated With Dopaminergic Improvement in Parkinson's Disease. **Clinical Pharmacology & Therapeutics**, v. 106, n. 4, p. 884–890, 17 out. 2019.

MOREIRA, P. I. et al. Mitochondria: a therapeutic target in neurodegeneration. **Biochimica et biophysica acta**, v. 1802, n. 1, p. 212–20, jan. 2010.

MORRONI, F. et al. Comparison of Adaptive Neuroprotective Mechanisms of Sulforaphane and its Interconversion Product Erucin in *in Vitro* and *in Vivo* Models of Parkinson's Disease.

Journal of Agricultural and Food Chemistry, v. 66, n. 4, p. 856–865, 31 jan. 2018.

MURPHY, A. N. In a flurry of PINK, mitochondrial bioenergetics takes a leading role in Parkinson's disease. **EMBO Molecular Medicine**, v. 1, n. 2, p. 81–84, maio 2009.

MURRAY, T. V. A. et al. gavageNADPH oxidase 4 regulates homocysteine metabolism and protects against acetaminophen-induced liver damage in mice. **Free radical biology & medicine**, 13 out. 2015.

MUSSELMAN, L. P.; KÜHNLEIN, R. P. **Drosophila as a model to study obesity and metabolic diseaseJournal of Experimental Biology**Company of Biologists Ltd, , 1 mar. 2018. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/29514880/>. Acesso em: 27 out. 2020

MUYDERMAN, H. Highly Selective and Prolonged Depletion of Mitochondrial Glutathione in Astrocytes Markedly Increases Sensitivity to Peroxynitrite. **Journal of Neuroscience**, v. 24, n. 37, p. 8019–8028, 15 set. 2004.

MYERS, E. W. A Whole-Genome Assembly of Drosophila. **Science**, v. 287, n. 5461, p. 2196–2204, 24 mar. 2000.

NARENDRA, D. P. et al. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. **PLoS biology**, v. 8, n. 1, p. e1000298, 26 jan. 2010.

NAVARRO-YEPES, J. et al. Oxidative Stress, Redox Signaling, and Autophagy: Cell Death *Versus* Survival. **Antioxidants & Redox Signaling**, v. 21, n. 1, p. 66–85, 1 jul. 2014.

NEVES, A.; BUSSO, C.; GÖNCZY, P. Cellular hallmarks reveal restricted aerobic metabolism at thermal limits. **eLife**, v. 4, n. MAY, 1 maio 2015.

OJUKA, E. et al. **Measurement of β-oxidation capacity of biological samples by respirometry: A review of principles and substratesAmerican Journal of Physiology - Endocrinology and Metabolism**American Physiological Society, , 1 maio 2016. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/26908505/>. Acesso em: 21 dez. 2020

OST, M. et al. **Analysis of mitochondrial respiratory function in tissue biopsies and blood cellsCurrent opinion in clinical nutrition and metabolic care**NLM (Medline), , 1 set. 2018. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/29939971/>. Acesso em: 9 out. 2020

OU, H.-L.; BJ¨, B.; SCHUMACHER, B. **Review Series HEMATOLOGIC DISEASE AT OLDER AGE DNA damage responses and p53 in the aging process Institute for Genome Stability in Aging and Disease, Medical Faculty, 2 Cologne Excellence Cluster for Cellular Stress Responses in Aging-Associated Diseases**. [s.l: s.n.]. Disponível em: <http://ashpublications.org/blood/article-pdf/131/5/488/1465962/blood746396.pdf>.

PAISÁN-RUÍZ, C. et al. Cloning of the Gene Containing Mutations that Cause PARK8-

Linked Parkinson’s Disease. **Neuron**, v. 44, n. 4, p. 595–600, nov. 2004.

PANDYA, J. D. et al. Age- and brain region-specific differences in mitochondrial bioenergetics in Brown Norway rats. **Neurobiology of Aging**, v. 42, p. 25–34, 1 jun. 2016.

PANTIC, I. et al. Oxidopamine and oxidative stress: Recent advances in experimental physiology and pharmacology. **Chemico-Biological Interactions**, v. 336, p. 109380, fev. 2021.

PARK, J. et al. Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. **Nature**, v. 441, n. 7097, p. 1157–1161, 3 jun. 2006.

PASHA, M. K.; SHARMA, R. K.; RAJPUT, A. H. Increased myocardial N-myristoyltransferase activity in rotenone model of Parkinsonism. **International journal of molecular medicine**, v. 15, n. 6, p. 987–91, jun. 2005.

PENG, K. et al. The Interaction of Mitochondrial Biogenesis and Fission/Fusion Mediated by PGC-1α Regulates Rotenone-Induced Dopaminergic Neurotoxicity. **Molecular Neurobiology**, v. 54, n. 5, p. 3783–3797, 7 jul. 2017.

PEROCCHI, F. et al. Assessing systems properties of yeast mitochondria through an interaction map of the organelle. **PLoS Genetics**, v. 2, n. 10, p. 1612–1624, out. 2006.

PESTA, D.; GNAIGER, E. High-Resolution Respirometry: OXPHOS Protocols for Human Cells and Permeabilized Fibers from Small Biopsies of Human Muscle. In: [s.l: s.n.]. p. 25–58.

PETROSILLO, G. et al. Mitochondrial dysfunction in rat brain with agingInvolvement of complex I, reactive oxygen species and cardiolipin. **Neurochemistry International**, v. 53, n. 5, p. 126–131, nov. 2008.

PICHAUD, N. et al. Thermal sensitivity of mitochondrial functions in permeabilized muscle fibers from two populations of Drosophila simulans with divergent mitotypes. **American Journal of Physiology - Regulatory Integrative and Comparative Physiology**, v. 301, n. 1, jul. 2011.

PICHAUD, N. et al. Age Dependent Dysfunction of Mitochondrial and ROS Metabolism Induced by Mitonuclear Mismatch. **Frontiers in Genetics**, v. 10, n. FEB, p. 130, 20 fev. 2019.

PIENAAR, I. S.; GÖTZ, J.; FEANY, M. B. Parkinson’s disease: insights from non-traditional model organisms. **Progress in neurobiology**, v. 92, n. 4, p. 558–71, dez. 2010.

PIMENTA DE CASTRO, I. et al. Genetic analysis of mitochondrial protein misfolding in Drosophila melanogaster. **Cell Death and Differentiation**, v. 19, n. 8, p. 1308–1316, ago. 2012a.

PIMENTA DE CASTRO, I. et al. Genetic analysis of mitochondrial protein misfolding in Drosophila melanogaster. **Cell Death & Differentiation**, v. 19, n. 8, p. 1308–1316, 3 ago. 2012b.

POLYMEROPOULOS, M. H. et al. Mapping of a Gene for Parkinson's Disease to Chromosome 4q21-q23. **Science**, v. 274, n. 5290, p. 1197–1199, 15 nov. 1996.

PONZ-SARVISE, M. et al. In vitro Models of Neurodegenerative Diseases. 2020.

PROCACCIO, V. et al. Perspectives of drug-based neuroprotection targeting mitochondria. **Revue Neurologique**, v. 170, n. 5, p. 390–400, maio 2014.

PUURAND, M. et al. **Intracellular energy-transfer networks and high-resolution respirometry: A convenient approach for studying their functionInternational Journal of Molecular Sciences**MDPI AG, , 1 out. 2018. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/30261663/>. Acesso em: 26 out. 2020

RAMSAY, R. R. et al. The inhibition site of MPP+, the neurotoxic bioactivation product of 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine is near the Q-binding site of NADH dehydrogenase. **Archives of Biochemistry and Biophysics**, v. 259, n. 2, p. 645–649, dez. 1987.

RB, P. et al. Caffeine as symptomatic treatment for Parkinson disease (Café-PD): A randomized trial. **Neurology**, v. 89, n. 17, p. 1795–1803, 24 out. 2017.

REQUEJO-AGUILAR, R. et al. PINK1 deficiency sustains cell proliferation by reprogramming glucose metabolism through HIF1. **Nature communications**, v. 5, p. 4514, 24 jul. 2014.

REQUEJO-AGUILAR, R. et al. DJ1 represses glycolysis and cell proliferation by transcriptionally up-regulating pink1. **Biochemical Journal**, v. 467, n. 2, p. 303–310, 15 abr. 2015.

REQUEJO-AGUILAR, R.; BOLAÑOS, J. P. Mitochondrial control of cell bioenergetics in Parkinson's disease. **Free Radical Biology and Medicine**, v. 100, p. 123–137, nov. 2016.

REX SHEU, K.-F. et al. An immunochemical study of the pyruvate dehydrogenase deficit in Alzheimer's disease brain. **Annals of Neurology**, v. 17, n. 5, p. 444–449, maio 1985.

RHEE, S. G. CELL SIGNALING: H2O2, a Necessary Evil for Cell Signaling. **Science**, v. 312, n. 5782, p. 1882–1883, 30 jun. 2006.

RICHARDSON, J. R. et al. Neurotoxicity of pesticides. **Acta Neuropathologica**, v. 138, n. 3, p. 343–362, 13 set. 2019.

RIEDERER, P. et al. Lateralisation in Parkinson disease. **Cell and Tissue Research**, 14 abr. 2018.

RIEMENSPERGER, T. et al. A Single Dopamine Pathway Underlies Progressive Locomotor Deficits in a Drosophila Model of Parkinson Disease. **Cell Reports**, v. 5, n. 4, p. 952–960, nov. 2013.

RODRIGUES, A. P. C. et al. Developmental arrest in Drosophila melanogaster caused by mitochondrial DNA replication defects cannot be rescued by the alternative oxidase. **Scientific Reports**, v. 8, n. 1, 1 dez. 2018a.

RODRIGUES, N. R. et al. Short-term sleep deprivation with exposure to nocturnal light alters mitochondrial bioenergetics in Drosophila. **Free Radical Biology and Medicine**, v. 120, p. 395–406, 20 maio 2018b.

ROMUK, E. B. et al. The evaluation of the changes in enzymatic antioxidant reserves and lipid peroxidation in chosen parts of the brain in an animal model of Parkinson disease. **Advances in clinical and experimental medicine : official organ Wroclaw Medical University**, v. 26, n. 6, p. 953–959, set. 2017.

ROVENKO, B. M. et al. Restriction of glucose and fructose causes mild oxidative stress independently of mitochondrial activity and reactive oxygen species in drosophila melanogaster. **Comparative Biochemistry and Physiology -Part A : Molecular and Integrative Physiology**, v. 187, p. 27–39, 1 set. 2015.

RUBIN, G. M. Drosophila melanogaster as an experimental organism. **Science**, v. 240, n. 4858, p. 1453–1459, 1988.

SALARI, S.; BAGHERI, M. In Vivo, In Vitro and Pharmacologic Models of Parkinson's Disease. **Physiol. Res**, v. 68, p. 17–24, 2019.

SAMARANCH, L. et al. PINK1-linked parkinsonism is associated with Lewy body pathology. **Brain**, v. 133, n. 4, p. 1128–1142, abr. 2010.

SAWYNOK, J. Caffeine and pain. **Pain**, v. 152, n. 4, p. 726–9, abr. 2011.

SCHAPIRA, A. H. V. et al. Mitochondrial Complex I Deficiency in Parkinson's Disease. **Journal of Neurochemistry**, v. 54, n. 3, p. 823–827, mar. 1990.

SCHÖPF, B. et al. Oxidative phosphorylation and mitochondrial function differ between human prostate tissue and cultured cells. **The FEBS Journal**, v. 283, n. 11, p. 2181–2196, jun. 2016a.

SCHÖPF, B. et al. Oxidative phosphorylation and mitochondrial function differ between human prostate tissue and cultured cells. **The FEBS journal**, v. 283, n. 11, p. 2181–96, jun. 2016b.

SCHÖPF, B. et al. Oxidative phosphorylation and mitochondrial function differ between human prostate tissue and cultured cells. **The FEBS journal**, v. 283, n. 11, p. 2181–96, jun.

2016c.
SCHULER, F.; CASIDA, J. E. The insecticide target in the PSST subunit of complex I. **Pest management science**, v. 57, n. 10, p. 932–40, out. 2001.
SCIALO, F. et al. Practical recommendations for the use of the geneswitch Gal4 system to knock-down genes in drosophila melanogaster. **PLoS ONE**, v. 11, n. 8, 1 ago. 2016.
SCIALÒ, F. et al. Mitochondrial ROS Produced via Reverse Electron Transport Extend Animal Lifespan. **Cell Metabolism**, v. 23, n. 4, p. 725–734, 12 abr. 2016.
SERRANO-POZO, A.; DAS, S.; HYMAN, B. T. APOE and Alzheimer's disease: advances in genetics, pathophysiology, and therapeutic approaches. **The Lancet Neurology**, v. 20, n. 1, p. 68–80, jan. 2021.
SHIMURA, H. et al. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. **Nature Genetics**, v. 25, n. 3, p. 302–305, jul. 2000.
SIDDIQUI, M. A. et al. Rotenone-induced oxidative stress and apoptosis in human liver HepG2 cells. **Molecular and Cellular Biochemistry**, v. 384, n. 1–2, p. 59–69, 21 dez. 2013.
SIGNES, A.; FERNANDEZ-VIZARRA, E. Assembly of mammalian oxidative phosphorylation complexes I–V and supercomplexes. **Essays in Biochemistry**, v. 62, n. 3, p. 255–270, 20 jul. 2018.
SIMARD, C. et al. Metabolic characterization and consequences of mitochondrial pyruvate carrier deficiency in drosophila melanogaster. **Metabolites**, v. 10, n. 9, p. 1–16, 1 set. 2020.
SIMARD, C. J. et al. Measurement of mitochondrial oxygen consumption in permeabilized fibers of drosophila using minimal amounts of tissue. **Journal of Visualized Experiments**, v. 2018, n. 134, 7 abr. 2018.
SIMUNI, T. et al. Longitudinal change of clinical and biological measures in early Parkinson's disease: Parkinson's progression markers initiative cohort. **Movement Disorders**, 23 mar. 2018.
STEFANATOS, R. et al. Dj-1β regulates oxidative stress, insulin-like signaling and development in Drosophila melanogaster. **Cell Cycle**, v. 11, n. 20, p. 3876–3886, 15 out. 2012.
STOROZHEVYKH, T. P. et al. Mitochondrial respiratory chain is involved in insulin-stimulated hydrogen peroxide production and plays an integral role in insulin receptor autophosphorylation in neurons. **BMC Neuroscience**, v. 8, n. 1, p. 84, 2007.
STRÖMBERG, I. et al. Astrocyte responses to dopaminergic denervations by 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine as evidenced by glial fibrillary acidic protein immunohistochemistry. **Brain Research Bulletin**, v. 17, n. 2, p. 225–

236, ago. 1986.

SUDATI, J. H. et al. Valeriana officinalis attenuates the rotenone-induced toxicity in drosophila melanogaster. **NeuroToxicology**, v. 37, p. 118–126, 2013.

SUH, H. J. et al. Behavioral Changes and Survival in Drosophila melanogaster: Effects of Ascorbic Acid, Taurine, and Caffeine. **Biological & pharmaceutical bulletin**, v. 40, n. 11, p. 1873–1882, 2017.

SYRJÄNEN, L. et al. β carbonic anhydrase is required for female fertility in Drosophila melanogaster. **Frontiers in Zoology**, v. 12, n. 1, 22 ago. 2015.

TAFERNER, A. et al. FAH domain containing protein 1 (FAHD-1) Is required for mitochondrial function and locomotion activity in C. elegans. **PLoS ONE**, v. 10, n. 8, 12 ago. 2015.

TAKAHASHI-NIKI, K. et al. Transcriptional Regulation of DJ-1. **Advances in experimental medicine and biology**, v. 1037, p. 89–95, 2017.

TARNOPOLSKY, M. A. Caffeine and creatine use in sport. **Annals of nutrition & metabolism**, v. 57 Suppl 2, p. 1–8, 2010.

TEIXEIRA, C. M. et al. Hippocampal 5-HT Input Regulates Memory Formation and Schaffer Collateral Excitation. **Neuron**, 8 maio 2018.

TEULIER, L. et al. Consequences of electroshock-induced narcosis in fish muscle: from mitochondria to swim performance. **Journal of Fish Biology**, v. 92, n. 6, p. 1805–1818, 2018.

TEVES, J. M. Y. et al. Parkinson's Disease Skin Fibroblasts Display Signature Alterations in Growth, Redox Homeostasis, Mitochondrial Function, and Autophagy. **Frontiers in Neuroscience**, v. 11, p. 737, 12 jan. 2018.

THOMPSON, K. et al. OXA 1L mutations cause mitochondrial encephalopathy and a combined oxidative phosphorylation defect . **EMBO Molecular Medicine**, v. 10, n. 11, nov. 2018.

TRACHOOTHAM, D. et al. Redox regulation of cell survival. **Antioxidants & redox signaling**, v. 10, n. 8, p. 1343–74, ago. 2008.

TRINH, J.; FARRER, M. Advances in the genetics of Parkinson disease. **Nature Reviews Neurology**, v. 9, n. 8, p. 445–454, 16 ago. 2013.

TSUANG, D. W.; BIRD, T. D. Genetic factors in neurodegenerative diseases. **American Journal of Medical Genetics Part B: Neuropsychiatric Genetics**, v. 174, n. 1, p. 3–4, jan. 2017.

ULGHERAIT, M. et al. Circadian regulation of mitochondrial uncoupling and lifespan.

Nature Communications, v. 11, n. 1, 1 dez. 2020.

VAN HEEMST, D. Insulin, IGF-1 and longevity. **Aging and disease**, v. 1, n. 2, p. 147–57, out. 2010.

VELAZQUEZ, R. et al. Acute tau knockdown in the hippocampus of adult mice causes learning and memory deficits. **Aging Cell**, p. e12775, 10 maio 2018.

VM, N.; MA, F. History of research on C. elegans and other free-living nematodes as model organisms. **WormBook**, v. 2017, p. 1–84, 7 set. 2017.

VOICULESCU, M. et al. Molecular and pharmacodynamic interactions between caffeine and dopaminergic system. **Journal of medicine and life**, v. 7 Spec No., p. 30–8, 2014.

WANG, A. et al. A dual effect of N-acetylcysteine on acute ethanol-induced liver damage in mice. **Hepatology Research**, v. 34, n. 3, p. 199–206, mar. 2006.

WANG, T. et al. Neuroprotective effects of Danshensu on rotenone-induced Parkinson's disease models in vitro and in vivo. **BMC Complementary Medicine and Therapies**, v. 20, n. 1, p. 20, 23 dez. 2020.

WEISZ, E. D. et al. Loss of Drosophila FMRP leads to alterations in energy metabolism and mitochondrial function. **Human Molecular Genetics**, v. 27, n. 1, p. 95–106, 1 jan. 2018.

WHITWORTH, A. J.; PALLANCK, L. J. The PINK1/Parkin pathway: a mitochondrial quality control system? **Journal of Bioenergetics and Biomembranes**, v. 41, n. 6, p. 499–503, 5 dez. 2009.

WHO. 19th WHO Model List of Essential Medicines. **Http://Www.Who.Int/Medicines/Publications/Essentialmedicines/En/**, n. April, p. 1–51, 2015.

WREDENBERG, A. et al. MTERF3 Regulates Mitochondrial Ribosome Biogenesis in Invertebrates and Mammals. **PLoS Genetics**, v. 9, n. 1, jan. 2013.

WÜLLNER, U. et al. Glutathione depletion and neuronal cell death: the role of reactive oxygen intermediates and mitochondrial function. **Brain Research**, v. 826, n. 1, p. 53–62, abr. 1999.

XIROMERISIOU, G. et al. Genetic basis of Parkinson disease. **Neurosurgical Focus**, v. 28, n. 1, p. E7, jan. 2010.

YAMAGUCHI, M.; YOSHIDA, H. Drosophila as a Model Organism. In: [s.l: s.n.]. p. 1–10.

YAN, M. H.; WANG, X.; ZHU, X. Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease. **Free Radical Biology and Medicine**, v. 62, p. 90–101, set. 2013.

YIN, F.; JIANG, T.; CADENAS, E. Metabolic triad in brain aging: mitochondria,

insulin/IGF-1 signalling and JNK signalling. **Biochemical Society Transactions**, v. 41, n. 1, p. 101–105, 1 fev. 2013.

ZEIDÁN-CHULIÁ, F. et al. Major components of energy drinks (caffeine, taurine, and guarana) exert cytotoxic effects on human neuronal SH-SY5Y cells by decreasing reactive oxygen species production. **Oxidative medicine and cellular longevity**, v. 2013, p. 791795, 2013.

ZHANG, H. et al. Glutathione-dependent reductive stress triggers mitochondrial oxidation and cytotoxicity. **The FASEB Journal**, v. 26, n. 4, p. 1442–1451, 2012.

ZHANG, Y. et al. Regulation of glutamate transporter trafficking by Nedd4-2 in a Parkinson's disease model. **Cell Death & Disease**, v. 8, n. 2, p. e2574–e2574, 2 fev. 2017a.

ZHANG, Z. et al. Acute action of rotenone on excitability of catecholaminergic neurons in rostral ventrolateral medulla. **Brain Research Bulletin**, v. 134, p. 151–161, set. 2017b.

ZIMPRICH, A. et al. Mutations in LRRK2 Cause Autosomal-Dominant Parkinsonism with Pleomorphic Pathology. **Neuron**, v. 44, n. 4, p. 601–607, nov. 2004.

ZONG, W. X.; RABINOWITZ, J. D.; WHITE, E. **Mitochondria and CancerMolecular Cell**Cell Press, , 3 mar. 2016. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/26942671/>. Acesso em: 19 nov. 2020

9. ANEXO I: Assessment of mitochondrial function in *Drosophila melanogaster* and *Caenoharbiditis elegans* model organisms: Protocols in respirometry.

Assessment of mitochondrial function in *Drosophila melanogaster* and *Caenorhabditis elegans* model organisms: Protocols in respirometry

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Abstract: The present work describes protocols of respirometry in *Drosophila melanogaster* and *Caenorhabditis elegans*. Respirometry techniques can offer responses about general functions of mitochondria network, as well as, allow the evaluation of specific points of mitochondrial respiratory chain. There are detailed and already established protocols describing sample preparation from mammals to measure mitochondrial function through respirometry assays. On the other hand, there is a lack of protocols to access mitochondrial respirometry in alternative model organisms. Such protocols present important differences related to specificity of each organism and for some organism models the protocols are scarce or don´t present sufficient details. Currently, alternative model organisms have been used in research works involving mitochondrial function and became valuable in bioenergetics studies. In view of the wide variety of protocols in literature, we discuss here different methodologies used for evaluation of mitochondrial respirometry in *Caenorhabditis elegans, and Drosophila melanogaster,* and demonstrated alternatives to obtain a suitable sample preparation to mitochondrial respiration.

Keywords: mitochondrial function, high resolution respirometry; oxygen consumption rate, non-mammal model organisms.

1. Introduction

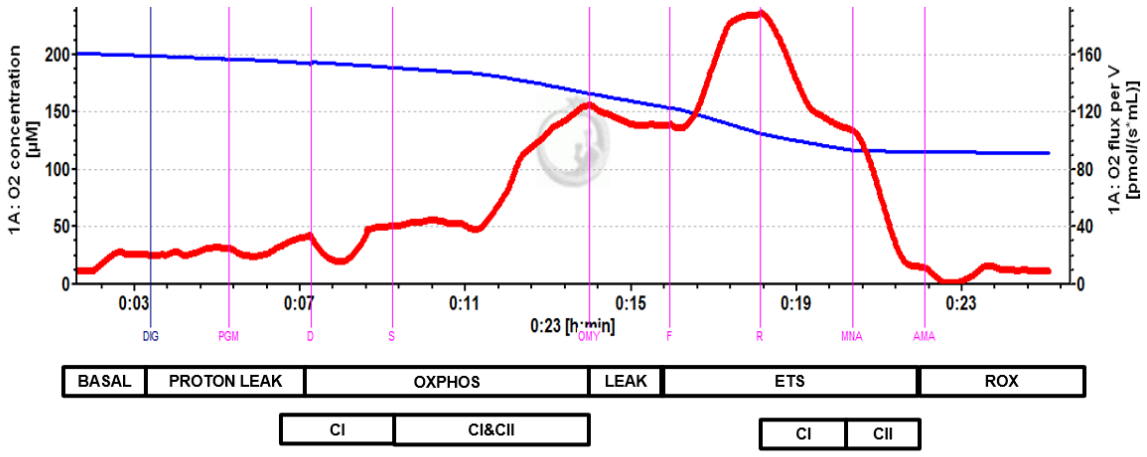
Several pathological conditions have mitochondrial dysfunction as a common biochemistry marker. Exposure to toxic substances, metabolism disorders as Diabetes mellitus (J; A, 2016), as well as neurodegenerative diseases such as Parkinson and Alzheimer (GAUTIER; KITADA; SHEN, 2008; MOREIRA et al., 2010) and cellular disturbs like cancer (ZONG; RABINOWITZ; WHITE, 2016) are some of the disorders which present disrupt energy metabolism and have as important consequence the impairment of mitochondrial functions, marked specially by uncouple of electron transfer system (ETS)/ oxidative phosphorylation (OXPHOS) processes, decreasing ATP levels and increasing ROS formation. Since mitochondria are organelles which control, not only energy generation, but also cellular processes involved in cell survival and death, approaches to evaluated mitochondrial function can offer important answers about cellular conditions in pathological and physiological processes.

In general, the major tools to evaluate mitochondrial function, oxygen rates and bioenergetics in *ex vivo* and *in vitro* samples use Seahorse extracellular flux analyzer® and Oroboros O2k® (OST et al., 2018). While Seahorse uses label-free sensors to detect extracellular changes in these analytes in order to measure rates of cellular respiration, Oroboros are based on Clark-type electrodes that measures ambient oxygen concentration. The label-free sensors of Seahorse can offer detailed results of oxygen consumption rate (OCR), glycolysis, and ATP production using less sample and optimizing the process with a 96-well microplate, while Clark-type electrodes of Oroboros allow measure of the electron transfer system associated with oxygen consumption in specific points of ETS and OXPHOS processes by high resolution respirometry (HRR).The mitochondrial function evaluation by respirometry techniques enable to work with different kinds of samples and depict the functionality of mitochondrial network in a real time (PUURAND et al., 2018). Generally, using appropriated concentrations of substrates and inhibitors of mitochondrial respiratory chain is possible to obtain responses related to ETS associated with coupling process which are essential to understand energy balance and ROS formation (KRUMSCHNABEL et al., 2015a; LEMIEUX; BLIER; GNAIGER, 2017).

1.1. The substrate-uncoupler-inhibitor titration (SUIT)

One protocol to evaluate oxygen consumption consists in a multiple substrate-uncoupler-inhibitor titration (SUIT). This protocol allows a stepwise modulation of coupling control and electron transfer pathway states (DOERRIER et al., 2018; E, 2009). Coupling process are obtained in presence of specific substrates for each mitochondrial complex and saturated ADP concentration leading a convergent electron flux to OXPHOS (BURTSCHER et al., 2015; RODRIGUES et al., 2018b). On the other hand, total mitochondrial capacity to pump electrons trough mitochondrial membranes (ETS) is obtained in presence of an uncoupler as FCCP (KRUMSCHNABEL et al., 2015b). Using this approach is possible to evaluate the coupling efficiency between mitochondrial ETS and OXPHOS processes and also identify mitochondrial complexes activity during these processes.

Figure 1: SUIT protocol demonstrating the main points of addition of substrates and inhibitors leading to OXPHOS and ETS



and Leak states. Firstly, specific substrates (glutamate, succinate, ADP) are added, then oligomycin, leading to leak state, recognized as the state without activity of ATP synthase, after an uncoupler (FCCP), and lastly, specific inhibitors to mitochondrial protein complexes I, II and III (rotenone, malonate and antimycin) (PESTA; GNAIGER, 2012).

The coupling between OXPHOS and ETS offer responses about metabolic interactions in mitochondrial network which are related to ATP generation (GONÇALVES et al., 2019; SCHÖPF et al., 2016c). Further, SUIT protocol offers answers about the functioning of particular sites of mitochondria network. Specific inhibitors as rotenone (CI-inhibitor) and malonate (CII-inhibitor) allow better understanding of functionality of each complex on ETS process. Lastly, it is possible to measure the residual oxygen consumption (ROX) by using antimycin, an inhibitor of mitochondrial complex III, in SUIT protocol (PESTA; GNAIGER, 2012).

There is a variety of protocols in respirometry and sample preparations in literature, including protocols for organisms models such as *Drosophila melanogaster*, *Caenorhabditis elegans*, *Danio rerio* and *Saccharomyces cerevisiae* (COSTA; LOH; MARTINS, 2013b; DILBERGER et al., 2019a; PEROCCHI et al., 2006; TEULIER et al., 2018). Organism models alternative to mammals have been used in research involving mitochondrial function and became valuable to study bioenergetics by advantages as: low cost, short life cycle, easy genetic manipulation comparing to mammals, beside minor ethical implications. In view of the wide variety of protocols in respirometry studies in this work we discuss the methodologies for evaluation of mitochondrial respirometry and propose suitable protocols of sample preparation in *Drosophila melanogaster* and *Caenorhabditis elegans*.

2. Drosophila melanogaster

Drosophila melanogaster, common recognized as fruit fly, has been used in studies of genetics since the early 1900s. Currently, *D. melanogaster* is used not only as a model to study classical and molecular genetics but also, in biochemistry, cell biology, and physiology in order to study diseases, molecular pathways, developmental biology, neurobiology, metabolic diseases and cellular bioenergetics (MUSSELMAN; KÜHNLEIN, 2018; RUBIN, 1988). The small size, short life cycle and easy genetic manipulation are features that make *Drosophila* a good experimental model.

Likewise, Drosophila use increased as an experimental model for different diseases. There is an increase on protocols to evaluated specific biochemistry pathways in this model. Different samples may be prepared from flies to assess mitochondrial functions trough respirometry, such as, isolated muscle from thorax, fat body, permeabilized larvae, entire flies or isolated mitochondria (LIU et al., 2020; RODRIGUES et al., 2018a; SIMARD et al., 2020; ULGHERAIT et al., 2020).

Some chemical or genetics models for diseases in *Drosophila melanogaster* are recognized to cause harm to thorax/wings muscle function, which are probably related with alterations in mitochondrial function of this tissue. There are different protocols describing sample preparation from thorax and evaluating the mitochondrial muscle function, using permeabilized muscles fibers from thorax (PICHAUD et al., 2019), or homogenization/permeabilization of entire thorax (CHAMPIGNY et al., 2018; WEISZ et al., 2018), for example.

Procedures for SUIT protocol in Oroboros present results using isolated muscle from only 3 thoraxes for each measurement of oxygen consumption in different genotypes of

Drosophila melanogaster (PICHAUD et al., 2019). Results using 3 thoraxes presented an oxygen flux around 400 pmol per tissue milligram considering the mitochondrial coupling during OXPHOS state for flies with 15 days of life. The authors also performed the oxygen consumption using flies with 30 days of age; however, there is no significant difference on mitochondrial oxygen flux by aging considering mitochondrial coupling state. In a similar protocol, Simard and collaborators (2018) described the isolation of 6 (six) thorax muscle of flies (SIMARD et al., 2018) obtaining an oxygen consumption around 410 pmol per tissue milligram, considering mitochondrial coupling state during OXPHOS. Moreover, the authors emphasized that this method was developed to overcome different problems related to the protocols using mitochondrial isolations in terms of duration and number of individuals required. They highlighted that according to their protocol, only three thoraxes are sufficient to display optimal results in respirometry assays. In general, the permeabilization of fly's thoraxes use saponin to open the muscle fibers and allowed the mitochondrial substrates intake and also use "BIOPS relaxing solution" which is a common buffer used for isolation of muscle fibers from mammals (CHAMPIGNY et al., 2018; PICHAUD et al., 2019).

The substrates used to measure oxygen consumption in thorax preparations from flies are similar to those used to evaluate oxygen consumption in isolated mitochondria, although there is some differences in concentrations, it is possible to find concentrations of pyruvate and succinate of 5 mM and 10 mM, respectively (WEISZ et al., 2018), while other work use pyruvate 10 mM and succinate 20 mM (CORMIER et al., 2019). Some specific substrates as proline also are used to evaluated flies' mitochondrial functions. Proline is used because flies and other insects have a specific mitochondrial shuttle for this molecule, so in most works related to oxygen mitochondrial consumption in flies, glutamate is replaced by proline (SIMARD et al., 2020). In order to support the phosphorylation process, in generally, protocols using fly's thoraxes use 5mM of ADP (CHAMPIGNY et al., 2018; PICHAUD et al., 2011, 2019), although the most important about ADP concentration is the saturation of this substrate, allowing maximum phosphorylation of mitochondrial respiratory chain.

In order to work with isolated mitochondria from flies, the sample preparation require a large number of flies which could be a disadvantage, some genetic modifications could reduce the fly's lifespan and consequently the number of individuals in the population. The common protocols to isolate mitochondria from flies use around 40 to 50 individuals and the process is done by differential centrifugation (GURURAJA RAO et al., 2019; RODRIGUES et al., 2018b). Beyond the high number of flies, the mitochondrial isolation protocol present other disadvantage: the selection of good mitochondria from mitochondrial network. The process of

differential centrifugation removes impaired membranes and organelles, selecting only organelles with entire membranes, which could interfere negatively on evaluation of mitochondrial function in entire mitochondrial network, since impaired mitochondria are removed from the network.

The use of isolated mitochondria also requires the correction of sample amount. In protocols using *Drosophila melanogaster*, we find this normalization by protein content of sample (CORREA et al., 2012; CRUZ et al., 2018a; EDERER et al., 2018; JUMBO-LUCIONI et al., 2012), and mitocondrial DNA quantification (GUITART et al., 2013). The activity of citrate synthase can also be used to correct mitochondrial sample amount since the activity of this enzyme are related to the quantity of mitochondria in mitochondria network (CAI et al., 2019).

Other alternative method to measure mitochondrial function in *Drosophila melanogaster* uses entire flies. In general, these protocols are simple: flies are homogenized in an appropriated buffer and immediately start the analyses. There are different protocols with different number of flies, some used 40 flies (ROVENKO et al., 2015; SCIALÒ et al., 2016), other use 20 flies (SYRJÄNEN et al., 2015) and other works demonstrate results using homogenate from two or three flies only (COSTA; LOH; MARTINS, 2013b; LEÃO et al., 2019; LIU et al., 2020; MACCHI et al., 2013). The homogenization buffer is used during the experimental procedure of mitochondrial evaluation. In general, the homogenization and mitochondrial evaluation use buffers like as MIR05 which is composed by 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 0.1 mg/mL fatty acid free BSA at pH=7,0 (LEÃO et al., 2019). The correction of sample quantity is made by protein content (ROVENKO et al., 2015; SCIALÒ et al., 2016), activity of citrate synthase (THOMPSON et al., 2018), or, in case of a low number of flies, by the sample unit (number of flies) (COSTA; LOH; MARTINS, 2013b; LEÃO et al., 2019). Larvae from flies can also be used to evaluate mitochondrial function, the protocols to sample preparation include permeabilization, or mechanical homogenization. In general, protocols use third instar larvae which are dissected, eliminating the internal organs and the hemolymph, to obtain the larval body wall, which is mechanically homogenized in isolation buffer (RODRIGUES et al., 2018a). Otherwise, third instar larvae are permeabilized using digitonin to open cell membranes (GUITART et al., 2010). There are protocols describing dissection of 3 to 7 larvae per sample (WREDENBERG et al., 2013) and another that use 10 larvae per sample (BRATIC et al., 2011) with normalization of sample quantity by protein content (BRATIC et al., 2011).

2.1. Sample preparation to mitochondrial respirometry using two adult *D. melanogaster*

According to our results, two homogenized flies is sufficient to measure oxygen flux by clark electrode (OROBOROS ®) using SUIT protocol (Figure 6).

Materials

- Two flies
- MiR05 buffer
- Eppendorf tube (1,5 mL)
- Pistil to homogenization
- Micropipette
- Hamilton microsyringes

Sample preparation

Anesthetize flies in ice cold and after transfer to an Eppendorf tube (1,5 mL), add 500 µL MiR05 buffer and carefully homogenize with a pistil for 1 minute ever in ice cold until get a homogeneous sample.

2.2. Running a OROBOROS respirometry assay using two homogenized flies

This protocol will detail the process of the running in an OROBOROS chamber with the titrations used in the SUIT assay. We optimized the assay to OROBOROS platform; however, it could be adjusted to work with other respirometry platforms like Seahorse XF96 system.

Materials

- Homogenate of flies according to protocol described before
- Pyruvate (Sigma, P2256) diluted in H_2O pH=7 neutralization with KOH 5N
- Proline (Sigma, P3350000) diluted in H_2O pH=7
- Malate (Sigma, M1000) diluted in H_2O
- ADP (Sigma, 117105) diluted in H_2O, neutralization with KOH 5N
- Succinate (Sigma, S2378) diluted in H_2O, neutralization with HCl 1N
- Oligomycin (Sigma, O4876) diluted in EtOH
- FCCP (Sigma, C2920) diluted in EtOH
- Rotenone (Sigma, R8875) diluted in EtOH
- Malonate (Sigma, M129-6) diluted in H_2O

- Antimycin (Sigma, A8674) diluted in EtOH
- Oroboros O2k®
- OROBOROS Dat.Lab version 7.4.0. 4

2.3. Start the protocol: OROBOROS chamber calibration

The oroboros chamber have a total volume of approximately 2mL. The calibration is necessary to ensure that oxygen flux range 0 pmol in order to start the assay. To start the calibration, add 2 mL of MiR05 on chamber and cover the chamber with the stopper without leaving any air bubbles, then pull on the stopper carefully in order to form a single

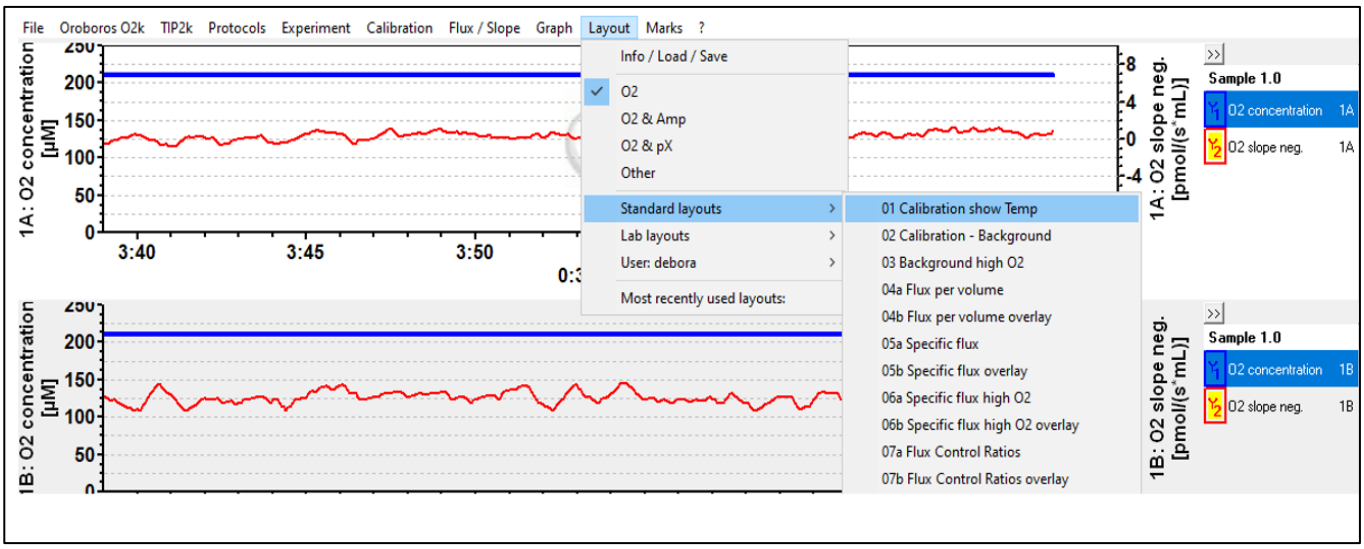


Figure 2: Demonstration of layouts finds on program OROBOROS Dat.Lab version 7.4.0. 4

air bubble, start the program and then go to the "layout" and choose the first option: "calibration show temp" as described in figure 2.

The program will redirect to the layout of calibration, wait until the red line present a straight pattern with points around 0 pmol and then select two points: one for chamber A and other for chamber B (Fig. 3). It is important that selected points have oxygen flux (red line) around 0.

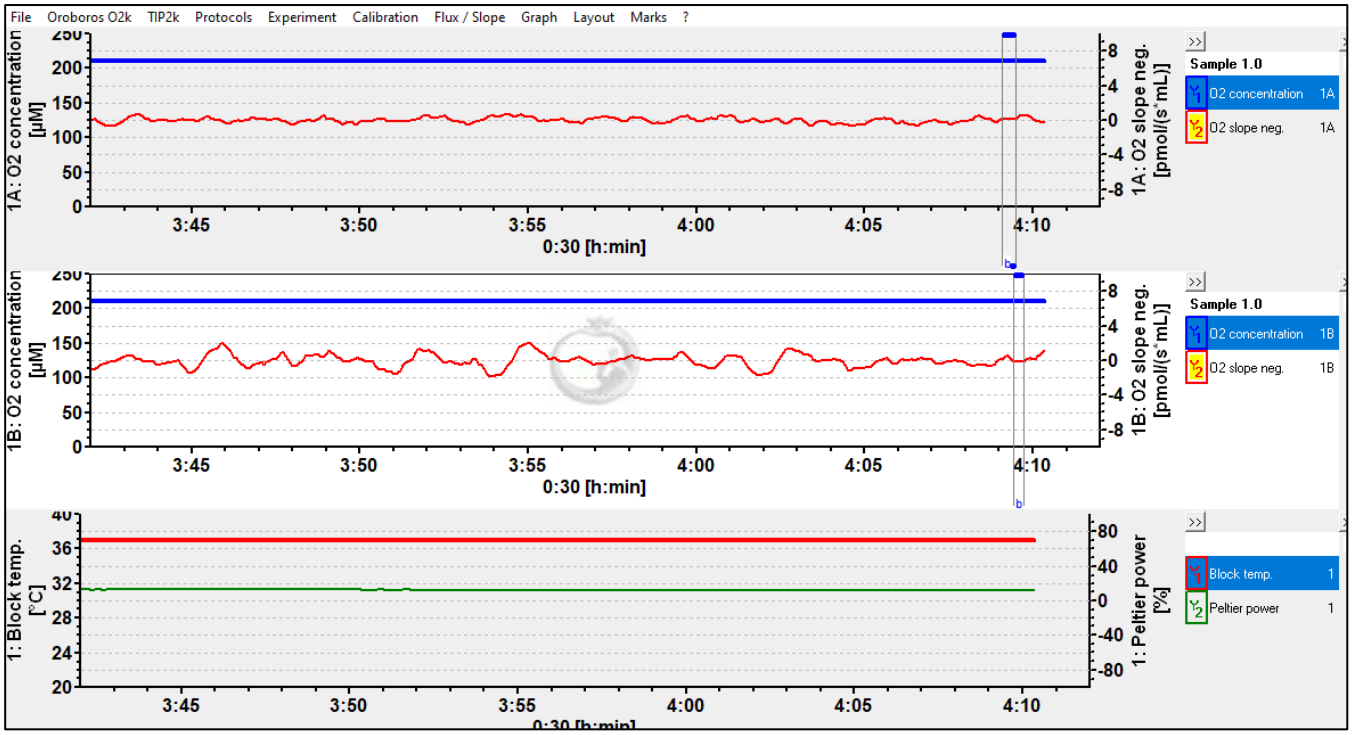


Figure 3: Demonstration of marked points to calibration of chambers on program OROBOROS Dat.Lab version 7.4.0. 4

Then, mark the points of both chambers, select “O2 concentration” on the right of the screen, select the point which you marked and copy the values of temperature and barometric pressure related to the point, then, paste these values to the boxes bellow, as demonstrated in Figure 4. Next, click on “calibrate and copy to clipboard” at the bottom right of the screen (Fig. 4). Don’t forget to make this process for both chambers. After calibration process the OROBOROS will be ready to start the assay. The program will redirect to the initial screen, so you can remove the stoppers in order to place the samples on chambers and start the HRR protocol.

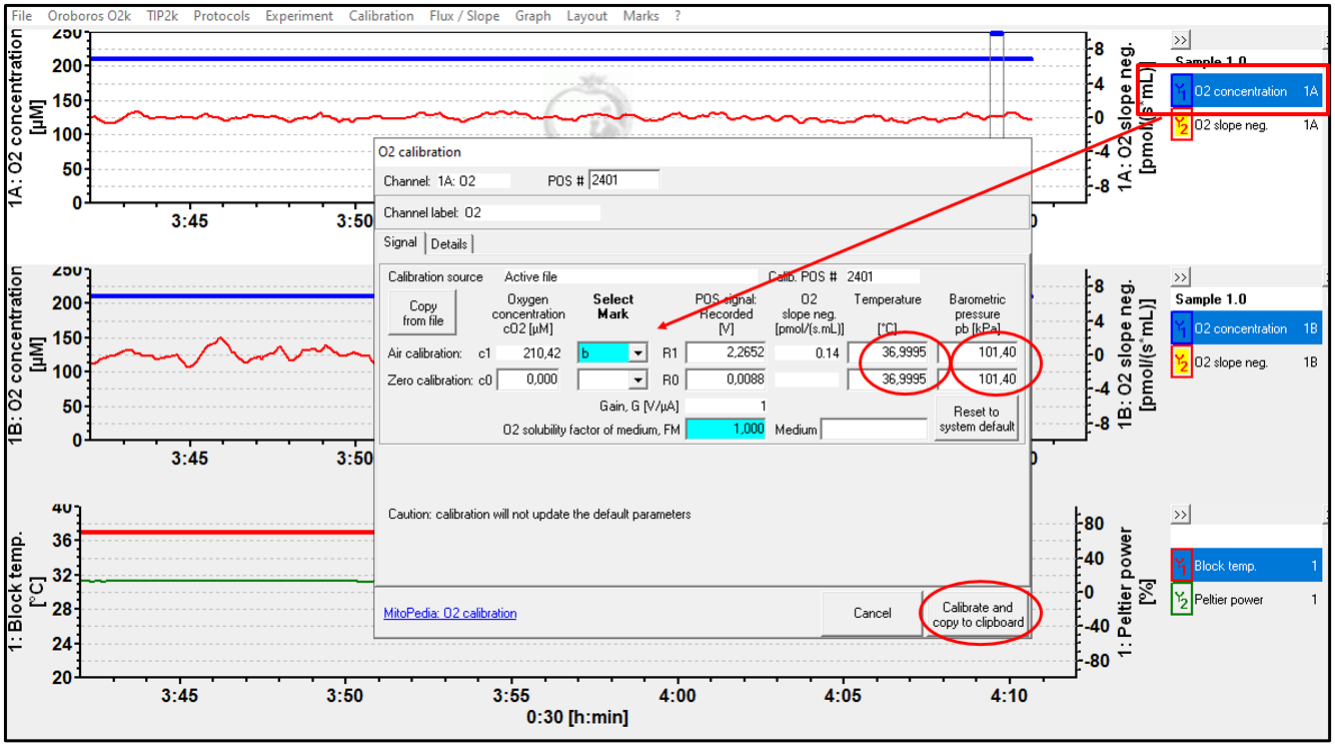


Figure 4: Steps of calibration of chambers on program OROBOROS Dat.Lab version 7.4.0. 4

2.4. OROBOROS SUIT protocol

After calibration, remove the stoppers and open the chambers, put the two homogenized flies on chambers using a micropipette, place the stoppers on chamber with attention in order to not create air bubbles. Then, start the program, which will redirect to a screen called “sample” (Fig. 5), where should be written details about the sample and assay. Define the field “unit” as “unit”, you could choose other units as “million cells” or “mg”. On the field concentration define “1 per mL” considering that we are using two flies and the volume of chamber is 2 mL. In here, HRR protocol was corrected by the number of flies since it was used only males to the assay and the protein amount as well as weight among samples didn’t present significant differences. However, you can correct the sample by weight or protein amount or other suitable method. After the signal stabilization of oxygen flux (red line) start the assay with the titration of substrates, inhibitors and uncouplers.

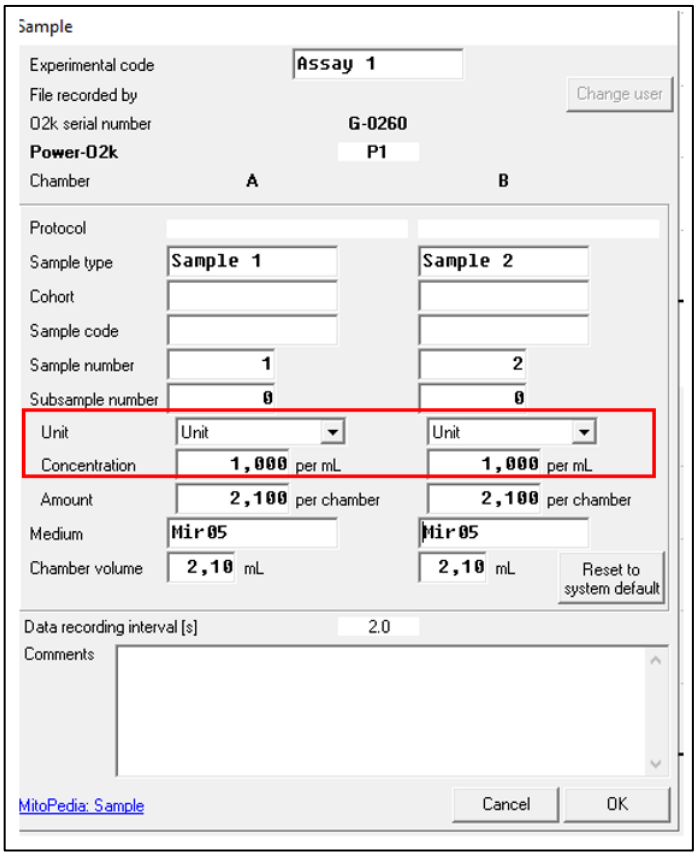


Figure 5: Demonstration of sample definitions on program OROBOROS Dat.Lab version 7.4.0. 4

Start the HRR protocol at the temperature of 25ºC with the substrates, pyruvate (5 mM), malate (2 mM) and proline (10 mM), wait until the oxygen flux increase and stabilize as demonstrated in Figure 6A and 6B. In order to mark the event of each reagent addition press the key F5 on the keyboard and you will be directed to a page where is possible to insert the name of each reagent. Add ADP (5mM) to coupling the mitochondria respiratory chain. Add ADP until the stabilization of oxygen flux (red line). Then, add succinate (10 mM), also until the stabilization of the oxygen flux. Add oligomycin (2.5 µM) due the effect of ATP synthase inhibition caused by oligomycin you will see a decrease of oxygen flux (Figure 6A and 6B), wait until the stabilization of the red line and uncouple the mitochondrial electron transfer using carbonyl cyanide-4-(tri-fluoromethoxy) phenylhydrazone (FCCP) with titrations of 0.25 µM, until reaching the maximum oxygen consumption demonstrated by the red line stabilization. Lastly, add the inhibitors of complex I: rotenone (0.5 µM), complex II: malonate (5 mM) and cytochrome c reductase: antimycin (2.5 µM). Observe a decrease of oxygen flux (Figure 6A and 6B) caused by the inhibition of electron transfer system. In the Figure 6 it is described all steps of HRR using two flies homogenate in MiR05 one of them using w1118 flies (Figure 6A) and other using pink1 null mutants flies (Figure 6B). It is possible to verify that the protocol

used is suitable to observe differences in respirometry associated with loss of pink1 gene in *D. melanogaster*.

2.5. Result from the protocol using two homogenized flies as sample

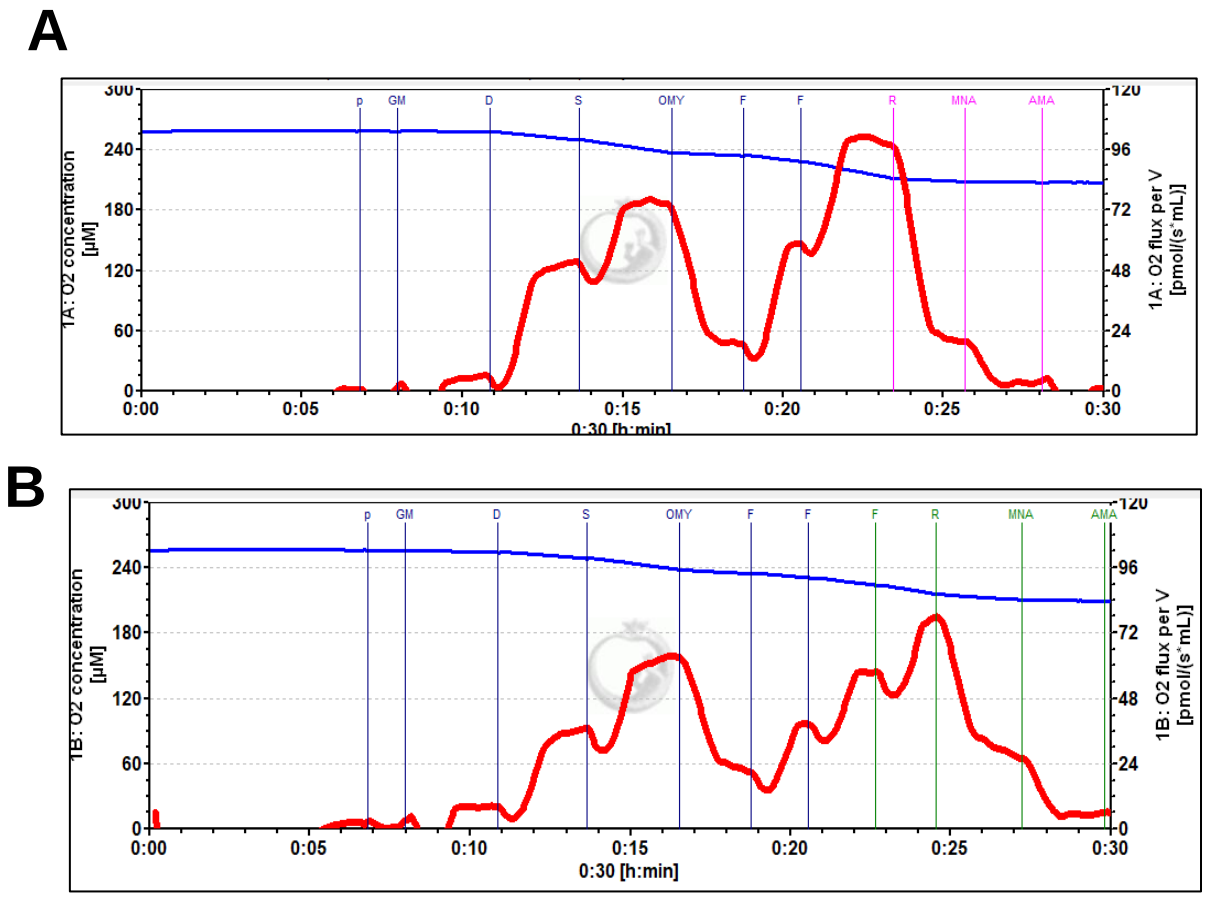


Figure 6: Images from results of high resolution respirometry demonstrating all steps of SUIT protocol using two whole homogenized flies as sample. The following reagents were added on chamber in sequence: pyruvate, proline and malate, saturated concentrations of ADP, succinate, oligomycin, FCCP, rotenone, malonate and antimycin. The red line represents electron transfer related to each step of mitochondrial respiration and the blue line is the amount of oxygen inside to the chamber in control flies (A) and null mutant flies to pink1 gene (B).

Table 1: Comparative table showing differences among protocols and number of flies related to mitochondrial oxygen flux in coupled state. The values of oxygen flux were evaluated considering control groups of each assay.

Drosophila melanogaster				
Whole fly homogenate				
Reference	**Number of flies**	**Buffer**	**Sample correction**	**Oxygen flux on coupled state**
(SCIALO et al., 2016)	20-40 flies	MIR05	Protein content	100 pmol $O_2 min^{-1}.mg^{-1}$

(LIU et al., 2020)	3 adult male flies	MIR05	Unit of sample	30 to 50 nmol/s (Oxygen flux per fly)
(COSTA; LOH; MARTINS, 2013a)	2 flies	MIR05	Unit of sample	150 nmol/s (Oxygen flux per fly)
(SYRJÄNEN et al., 2015)	20 flies	MIR05	Protein content	6.000 to 10.000 pmol O_2.mg protein^{-1}. s^{-1}
Isolated mitochondria				
Reference	**Number of flies**	**Buffer**	**Sample correction**	**Oxygen flux on coupled state**
(STEFANATOS et al., 2012)	150-200 flies	MIR05	Protein content	15 nmol O_2 .(s. mg.mt. protein)$^{-1}$
(CRUZ et al., 2018)	50 flies	-	Protein content	7000 pmol.(s.mg.protein)$^{-1}$
(WEISZ et al., 2018)	50 fly thoraxes	MIR05	Mitochondrial protein concentration	0,4 pmol.s^{-1} .(µg mitochondrial protein)$^{-1}$ Oxygen flow
Permeabilized larvae				
Reference	**Number of larvae**	**Buffer**	**Sample correction / substance used to permeabilization**	**Oxygen flux on coupled state**
(GUITART et al., 2010)	4–8 larvae	_	mtDNA copy number / digitonin	40 pmolO_2 .mg^{-1}.s^{-1}.(mt DNA rate)$^{-1}$
(BRATIC et al., 2015)	10 third-instar larvae	MIR05	Protein content / digitonin	200 pmol.min^{-1} per µg protein
Permeabilized thoraxes				
Reference	**Number of thoraxes**	**Buffer**	**Sample correction / substance used to permeabilization**	**Oxygen flux on coupled state**
(SIMARD et al., 2018)	6 male thoraxes	MIR 05	Dry weight / saponin	420 pmol.s^{-1}.mg^{-1}.tissue
(PICHAUD et al., 2019)	3 thoraxes		- / saponin	400 pmol.s^{-1}.mg^{-1}.tissue

3. Caenorhabditis elegans

Caenorhabditis elegans is a free life nematode which became as a powerful organism model to study genetics and development. The use of *C. elegans* in biological studies started on mid 1960s when Sydney Brenner inserted this specie as organism model. The major interests in using *C. elegans* as organism model are mainly related with features such as: rapid development and aging, easy cultivation and genetic tractability.

Today, is possible to use *C. elegans* as experimental model of different diseases mainly in order to identify genetic pathways due to easy genetic manipulation of this organism (VM; MA, 2017). The *C. elegans* is used as model of different pathologies so, methods to evaluate mitochondrial dysfunctions and energetic imbalance in this model become an important approach. The majority of protocols to evaluate mitochondrial oxygen consumption on *C.*

elegans are related to whole worm. Generally, it is evaluated oxygen consume by worms in a determined time scale. For example, in a protocol using 100 worms the oxygen consumption measured with a Clark electrode was 10 pmol/sec without use of substrate or inhibitor of mitochondrial respiratory chain (GUBERT et al., 2018). A different protocol described the use of 400 worms obtaining around 250 pmol O_2/second/worm as variation of oxygen consumption (KASSAHUN et al., 2018).

Another protocol described the use of 1,000 worms 1-day old adult per sample, dividing animals in two groups: incubated or not with 20 µM N,N-dicyclohexylcarbodiimide (DCCD) for 90 min before transfer to the respirometer chamber. So, oxygen consumption rates were monitored for 5 min at 25°C. The DCCD is an inhibitor of the mitochondrial inner membrane anion channel and was used to evaluate the quality of mitochondrial coupling. Results of this protocol depict oxygen consumption around 12 nmol $O_2 \cdot min^{-1}$ 1000 $worms^{-1}$ from control group. In wild worms previously treated with DCCD the oxygen rate decreased around 2 or 3 nmol $O2 \cdot min^{-1}$ 1000 $worms^{-1}$ (MACEDO et al., 2020b). In general, the buffer used to evaluated oxygen consumption using whole worms is M9 which is a common buffer used for *C. elegans* assays which is composed by 0,01M KH_2PO_4, 0,04M Na_2HPO_4, 0,085 M NaCl and 1 ml of $MgSO_4$ solution of 1 M, in H_2O to 1000 mL (GUBERT et al., 2018).

One special feature of *C. elegans* is its dermic cuticle, an outer covering of the nematode which is synthesized five times during the worm's life by the underlying hypodermis. This cuticle is resistant and protects worms against environment (ABETE-LUZI; EISENMANN, 2018). On the other hand, the cuticle also hinders the entry of substrates or other substances on *C. elegans* body, therefore due to the low absorption capacity there is no effect related to exposure of whole worms with substrates, inhibitors or uncouplers of mitochondrial respiratory chain during high resolution respirometry.

Protocols describing respirometry assays with *C. elegans* using isolated mitochondria are scarce. One limitation to mitochondrial isolation is the high number of worms necessary to perform the protocol. Macedo et al. (2020a) described a protocol using 400,000 or 800,000 worms for mitochondria isolation, obtaining an oxygen consumption on coupled state around 50 $nmol.min^{-1}$mg.protein in control groups (MACEDO et al., 2020a). They used 0.45 mg protein for complex I - or II -dependent respiration and 0.90 mg protein for β-oxidation-dependent respiration in 1.8 mL respiration buffer (120 mM KCl, 25 mM sucrose, 5 mM MgCl 2, 5 mM KH 2PO 4, 1 mM EGTA, 10 mM HEPES, 1 mg/mL fatty acid -free BSA, pH 7.35 at 25°C). Other protocol described the mitochondrial isolation from 5,000 to 10,000 worms per group (DILBERGER et al., 2019a), obtaining an oxygen consumption around 10 pmol/(s*IU

(mg/mL protein)) on coupled state with saturated ADP concentration. In this protocol results are corrected by activity of citrate synthase and expressed as IU (mg/mL protein) (DILBERGER et al., 2019a). Usually, the results from isolated mitochondria from *C. elegans* are normalized by protein content or activity of citrate synthase (DILBERGER et al., 2019a, 2019b; MACEDO et al., 2020a, 2020b).

C. elegans embryos have also been used in respirometry assays. A previous work used 25,000 embryos as sample to measure oxygen consumption. The results of oxygen consumption were expressed as logarithm of O_2 flow per volume in embryos at different temperatures from 9˚C to 28˚C, presenting an increase of oxygen flow directly dependent of the increase in temperature (NEVES; BUSSO; GÖNCZY, 2015).

3.1. Sample preparation to mitochondrial respirometry using 20,000 L4 *C. elegans*

According to our results, the homogenization of *C. elegans* in MiR05 produced the best results of oxygen flux by clarck electrode (OROBOROS ®) using SUIT protocol (Figure 7C).

Materials

- 20,000 worms at L4 larval stage
- M9 buffer
- MiR05 buffer
- Eppendorf tube (1,5 mL)
- Sonicator (ultrasonic processor UP100H with sonotrode MS1 (Hielscher Ultrasound Technology)
- Micropipette
- Hamilton microsyringes

Sample preparation

We tested three different methods to obtain an enriched mitochondria sample from *C. elegans*, in order to know which of them would be more suitable for a respirometry in clarck electrode (OROBOROS®). In all assays we used 20,000 worms, first we washed the worms 4 times in 500 µL of M9 buffer using a 1.5 mL eppendorfs tube, to remove residual bacteria, and then M9 buffer was removed, remaining only a pellet formed by worms. After that, we homogenized the pellet in three different solutions: using 500 µL of water (Figure 7A), 500 µL of M9 buffer (Figure 7B), or 500 µL of MiR05 buffer (Figure 7C). The homogenization/ lysis was made during three minutes with a time space of 20 seconds between each cycle, using ultrasonic sonicator.

3.2. Running a OROBOROS respirometry assay using enriched mitochondrial sample from *C. elegans*

This protocol will detail the process of a running in an OROBOROS chamber with the titrations using a SUIT protocol. We optimized the assay to OROBOROS platform; however, it could be adjusted to work with other respirometry platforms like Seahorse XF96 system.

Materials

- Enriched mitochondria sample from *C. elegans* according to protocols described before
- Glutamate (Sigma, G1626) diluted in H_2O pH=7 neutralization with KOH 5N
- Proline (Sigma, P3350000) diluted in H_2O pH=7
- Malate (Sigma, M1000) diluted in H_2O
- ADP (Sigma, 117105) diluted in H_2O, neutralization with KOH 5N
- Succinate (Sigma, S2378) diluted in H_2O, neutralization with HCl 1N
- Oligomycin (Sigma, O4876) diluted in EtOH
- FCCP (Sigma, C2920) diluted in EtOH
- Rotenone (Sigma, R8875) diluted in EtOH
- Malonate (Sigma, M129-6) diluted in H_2O
- Antimycin (Sigma, A8674) diluted in EtOH
- Oroboros O2k®
- OROBOROS Dat.Lab version 7.4.0. 4

3.3. OROBOROS SUIT protocol

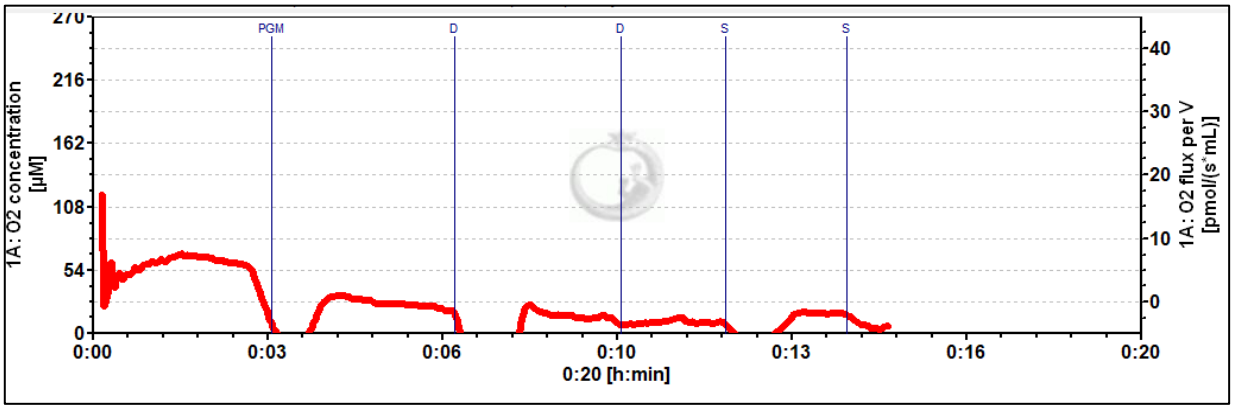
First is necessary to calibrate the oroboros chambers, the process of calibration was already described on the section 2.3. After, using a micropipette, place the homogenized sample on chambers. We used 500 µL of sample, corrected by protein content (mg/mL). On the box "sample" demonstrated in the Figure 5, correct the unit to "mg" and in the box bellow place the protein content of each sample. Next, cover the chambers with the stoppers and be careful to not form air bubbles inside to chamber. Start the HRR protocol at the temperature of 20 ºC with the substrates, pyruvate (5 mM), malate (2 mM) and proline (10 mM), wait until the oxygen flux increase and stabilize as demonstrated in Figure 7C. Then, add ADP (5mM) to coupling the mitochondria respiratory chain. Add ADP until the stabilization of oxygen flux (red line). After, add succinate (10 mM) also until the stabilization of the oxygen flux. Then, add oligomycin (2.5 µM) due the effect of ATP synthase inhibition caused by oligomycin it is

possible to see a decrease of oxygen flux (Figure 7C), wait until the stabilization of the red line and add carbonyl cyanide-4-(tri-fluoromethoxy) phenylhydrazone (FCCP) with titrations of 0.25 µM until reaching the maximum oxygen consumption to induce uncoupling of mitochondrial electron transfer. Lastly add the inhibitors of complex I: rotenone (0.5 µM), complex II: malonate (5 mM) and cytochrome c reductase: antimycin (2.5 µM), and observe a decrease of oxygen flux caused by the inhibition of electron transfer system.

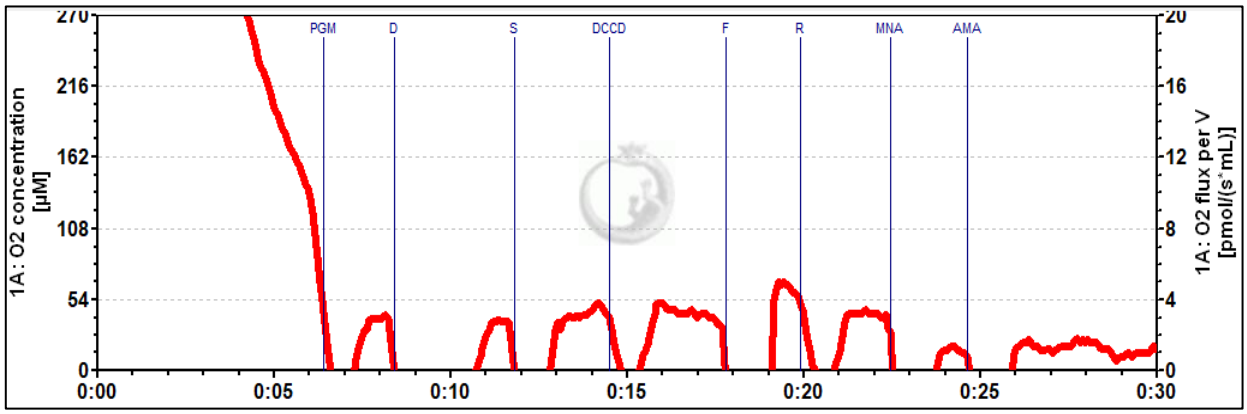
In the Figure 7 it is demonstrated the results from three different protocols. In the Figure 7A worms are homogenized in water, in the Figure 7B the enrichment mitochondrial sample was obtained with homogenization in M9 buffer and in the Figure 7C we have a representation of HRR protocol with worms homogenized in MiR05 buffer. On the three protocols we have the same number of worms and the same previous preparation, with the same time of homogenization, however is possible to verify that protocol using MiR05 buffer to homogenization (Figure 7C) produced better results comparing with the results from water (Fig. 7A) or M9 buffer (Fig. 7B) protocols. In samples homogenized with MiR05 is possible to see a better response to the substrates, uncouplers and inhibitors, what is demonstrated by bigger values of oxygen flux (red line) and a reduction of oxygen concentration inside to the chamber (blue line). Usually, the methods describing the preparation of mitochondria enriched sample from *C. elegans* use M9 as buffer to homogenization, however, according to our results, the use of MiR05 buffer to prepare mitochondrial sample present better results on the high resolution respirometry protocol using a Clarck electrode.

3.4. Results from the different protocols to prepare mitochondrial enriched sample from *C. elegans*

A



B



C

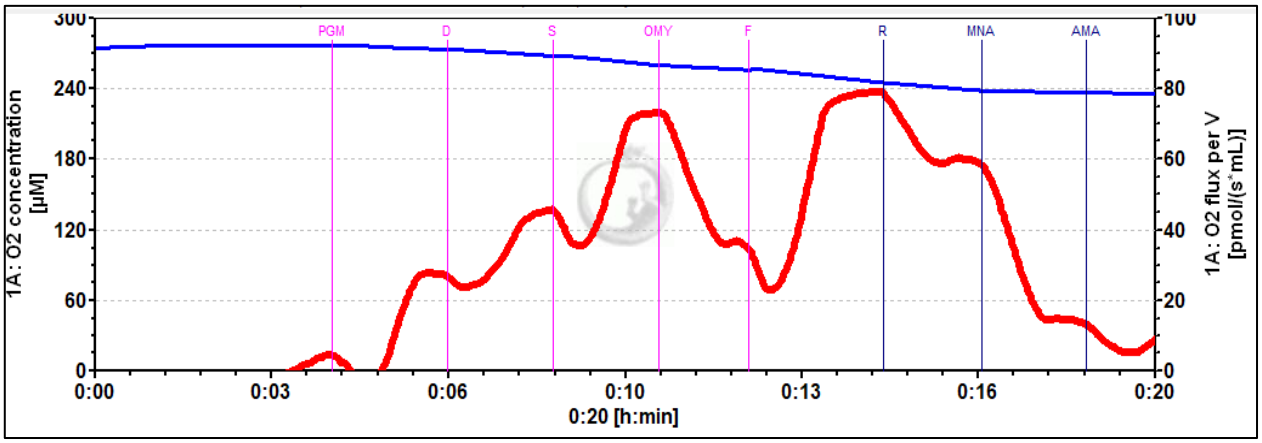


Figure 7: Images from high resolution respirometry demonstrating all steps of SUIT protocol using enriched mitochondria sample from worms. The following reagents were added on chamber in sequence: pyruvate, glutamate and malate, saturated concentrations of ADP, succinate, oligomycin, FCCP, rotenone, malonate and antimycin. The red line represents electron transfer related to each step of mitochondrial respiration and the blue line is the amount of oxygen inside to the chamber in worms homogenized using water (A) worms homogenized using M9 buffer (B) and worms homogenized using MiR05 buffer (C).

Table 2: Comparative table showing differences among protocols and number of worms related to mitochondrial oxygen flux in coupled state. The values of oxygen flux were evaluated considering control groups of each assay.

Caenorhabditis elegans			
Entire worms			
Reference	**Number of adult worms**	**Time**	**Oxygen consumption rate (control groups)**
(KASSAHUN et al., 2018)	400 worms	5 minutes	250 pmol O_2/second/worm
(TAFERNER et al., 2015)	300 worms	15 minutes	0,07 pmol/sec/worm
(GERISCH et al., 2020)	2,000 worms	30 min	Demonstrating as percent of control
Isolated Mitochondria			
Reference	**Number of adult worms**	**Sample correction**	**Oxygen consumption rate (control groups)**
(DILBERGER et al., 2019a, 2019b)	5,000 to 10,000	activity of citrate synthase	10 pmol/(s*IU (mg/mL protein))
(MACEDO et al., 2020a, 2020b)	400,000 or 800,000	Protein content	50 nmol.min^{-1}mg.protein

4. Conclusion

High resolution respirometry is an invaluable method to the study of mitochondrial function in physiological and pathological conditions (OJUKA et al., 2016). In the same way, studies using simple model organisms led to many scientific discoveries related to different disorders such as neurodegenerative diseases, cancer and diabetes (ZONG; RABINOWITZ; WHITE, 2016). Considering the pivotal role of mitochondria in the control of different cellular processes, it is important to highlight the appropriate protocols to study mitochondrial functions in the different model organisms. Each experimental model has specificities and limitations which requires adaptation in the methods and sample preparation to obtain reliable and useful results in mitochondrial respirometry assays. In this article we relate different protocols for mitochondrial respirometry in *Caenorhabditis elegans and Drosophila melanogaster* and demonstrated protocols tested by our group with the aim to help researchers to compare and choose the most proper according to their experimental design.

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6. References

ABBAS, N. et al. A Wide Variety of Mutations in the Parkin Gene Are Responsible for Autosomal Recessive Parkinsonism in Europe. **Human Molecular Genetics**, v. 8, n. 4, p. 567–574, 1 abr. 1999.

ABETE-LUZI, P.; EISENMANN, D. M. **Regulation of C. elegans L4 cuticle collagen genes by the heterochronic protein LIN-29Genesis**John Wiley and Sons Inc., , 1 maio 2018. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/29604168/>. Acesso em: 8 dez. 2020

ABOLAJI, A. O. et al. Resveratrol prolongs lifespan and improves 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced oxidative damage and behavioural deficits in Drosophila melanogaster. **Biochemical and Biophysical Research Communications**, v. 503, n. 2, p. 1042–1048, 5 set. 2018.

ADAMS, M. D. The Genome Sequence of Drosophila melanogaster. **Science**, v. 287, n. 5461, p. 2185–2195, 24 mar. 2000.

AMMAL KAIDERY, N.; THOMAS, B. Current perspective of mitochondrial biology in Parkinson's disease. **Neurochemistry International**, 14 mar. 2018.

ARYAL, B.; LEE, Y. Disease model organism for Parkinson Disease: Drosophila melanogaster. **BMB reports**, 14 dez. 2018.

ASHRAFI, G.; SCHWARZ, T. L. The pathways of mitophagy for quality control and clearance of mitochondria. **Cell Death & Differentiation**, v. 20, n. 1, p. 31–42, 29 jan. 2013.

BAI, X. X. et al. Downregulation of metabotropic glutamate receptor 5 inhibits hepatoma development in a neurotoxin rotenone-induced Parkinson's disease model. **Toxicology Letters**, v. 288, p. 71–81, 15 maio 2018.

BAK, L. K.; SCHOUSBOE, A.; WAAGEPETERSEN, H. S. The glutamate/GABA-glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer. **Journal of Neurochemistry**, v. 98, n. 3, p. 641–653, ago. 2006.

BAKER, D. J.; PELEG, S. Biphasic Modeling of Mitochondrial Metabolism Dysregulation during Aging. **Trends in Biochemical Sciences**, v. 42, n. 9, p. 702–711, set. 2017.

BEAL, M. F. Aging, energy, and oxidative stress in neurodegenerative diseases. **Annals of Neurology**, v. 38, n. 3, p. 357–366, set. 1995.

BÉLANGER, M.; ALLAMAN, I.; MAGISTRETTI, P. J. Brain Energy Metabolism: Focus on Astrocyte-Neuron Metabolic Cooperation. **Cell Metabolism**, v. 14, n. 6, p. 724–738, dez. 2011.

BERG, D. et al. Type and frequency of mutations in the LRRK2 gene in familial and sporadic Parkinson's disease*. **Brain : a journal of neurology**, v. 128, n. Pt 12, p. 3000–11, dez. 2005.

BHASKARA, S.; CHANDRASEKHARAN, M. B.; GANGULY, R. Caffeine induction of Cyp6a2 and Cyp6a8 genes of Drosophila melanogaster is modulated by cAMP and D-JUN protein levels. **Gene**, v. 415, n. 1–2, p. 49–59, maio 2008.

BILEN, J.; BONINI, N. M. *Drosophila* as a Model for Human Neurodegenerative Disease.

Annual Review of Genetics, v. 39, n. 1, p. 153–171, dez. 2005.
BLUM, D. et al. Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. **Progress in Neurobiology**, v. 65, n. 2, p. 135–172, out. 2001.
BONDI, M. W.; EDMONDS, E. C.; SALMON, D. P. Alzheimer's Disease: Past, Present, and Future. **Journal of the International Neuropsychological Society**, v. 23, n. 9–10, p. 818–831, 4 out. 2017.
BONIFATI, V. et al. DJ-1(PARK7), a novel gene for autosomal recessive, early onset parkinsonism. **Neurological Sciences**, v. 24, n. 3, p. 159–160, out. 2003.
BOWLING, A. C. et al. Age-Dependent Impairment of Mitochondrial Function in Primate Brain. **Journal of Neurochemistry**, v. 60, n. 5, p. 1964–1967, maio 1993.
BRAND, A. H.; PERRIMON, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. **Development (Cambridge, England)**, v. 118, n. 2, p. 401–15, jun. 1993a.
BRAND, A. H.; PERRIMON, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. **Development (Cambridge, England)**, v. 118, n. 2, p. 401–15, jun. 1993b.
BRATIC, A. et al. The bicoid stability factor controls polyadenylation and expression of specific mitochondrial mRNAs in Drosophila melanogaster. **PLoS Genetics**, v. 7, n. 10, out. 2011.
BRATIC, A. et al. Complementation between polymerase- and exonuclease-deficient mitochondrial DNA polymerase mutants in genomically engineered flies. **Nature Communications**, v. 6, 10 nov. 2015.
BROOKES, P. S. et al. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. **American Journal of Physiology-Cell Physiology**, v. 287, n. 4, p. C817–C833, out. 2004.
BURTSCHER, J. et al. Differences in mitochondrial function in homogenated samples from healthy and epileptic specific brain tissues revealed by high-resolution respirometry. **Mitochondrion**, v. 25, p. 104–112, 1 nov. 2015.
CAI, R. et al. Enhancing glycolysis attenuates Parkinson's disease progression in models and clinical databases. **Journal of Clinical Investigation**, v. 129, n. 10, p. 4539–4549, 1 out. 2019.
CARVALHO, N. R. et al. New therapeutic approach: diphenyl diselenide reduces mitochondrial dysfunction in acetaminophen-induced acute liver failure. **PloS one**, v. 8, n. 12, p. e81961, jan. 2013.
CASTAGNOLI, N.; CHIBA, K.; TREVOR, A. J. V. Potential bioactivation pathways for the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). **Life Sciences**, v. 36, n. 3, p. 225–230, jan. 1985.
CHAMPIGNY, C. M. et al. Omega-3 monoacylglyceride effects on longevity, mitochondrial metabolism and oxidative stress: Insights from Drosophila melanogaster. **Marine Drugs**, v. 16, n. 11, 16 nov. 2018.
CHAN, P. et al. Rapid ATP Loss Caused by 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine in Mouse Brain. **Journal of Neurochemistry**, v. 57, n. 1, p. 348–351, jul. 1991.
CHEN, Y.; DORN, G. W. PINK1-Phosphorylated Mitofusin 2 Is a Parkin Receptor for Culling Damaged Mitochondria. **Science**, v. 340, n. 6131, p. 471–475, 26 abr. 2013.
CHIA, S. J.; TAN, E.-K.; CHAO, Y.-X. Molecular Sciences Historical Perspective: Models of Parkinson's Disease. [s.d.].
CHRISTENSEN, K. et al. Ageing populations: the challenges ahead. **The Lancet**, v. 374, n. 9696, p. 1196–1208, out. 2009.
COELHO, A. et al. Cytochrome P450-Dependent Metabolism of Caffeine in Drosophila melanogaster. **PLOS ONE**, v. 10, n. 2, p. e0117328, 11 fev. 2015.

COHEN, E.; DILLIN, A. The insulin paradox: aging, proteotoxicity and neurodegeneration. **Nature Reviews Neuroscience**, v. 9, n. 10, p. 759–767, 4 out. 2008.
COLES, L. D. et al. Repeated-Dose Oral N-Acetylcysteine in Parkinson's Disease: Pharmacokinetics and Effect on Brain Glutathione and Oxidative Stress. **The Journal of Clinical Pharmacology**, v. 58, n. 2, p. 158–167, fev. 2018a.
COLES, L. D. et al. Repeated-Dose Oral N-Acetylcysteine in Parkinson's Disease: Pharmacokinetics and Effect on Brain Glutathione and Oxidative Stress. **The Journal of Clinical Pharmacology**, v. 58, n. 2, p. 158–167, fev. 2018b.
CORMIER, R. P. J. et al. Dynamic mitochondrial responses to a high-fat diet in Drosophila melanogaster. **Scientific Reports**, v. 9, n. 1, 1 dez. 2019.
CORREA, C. C. et al. Mitochondrial DNA variants influence mitochondrial bioenergetics in Drosophila melanogaster. **Mitochondrion**, v. 12, n. 4, p. 459–464, jul. 2012.
COSTA, A. C.; LOH, S. H.; MARTINS, L. M. Drosophila Trap1 protects against mitochondrial dysfunction in a PINK1/parkin model of Parkinson's disease. **Cell death & disease**, v. 4, n. 1, 2013a.
COSTA, A. C.; LOH, S. H. Y.; MARTINS, L. M. Drosophila Trap1 protects against mitochondrial dysfunction in a PINK1/parkin model of Parkinson's disease. **Cell death & disease**, v. 4, n. 1, p. e467, 17 jan. 2013b.
COSTA, A. C.; LOH, S. H. Y.; MARTINS, L. M. Drosophila Trap1 protects against mitochondrial dysfunction in a PINK1/parkin model of Parkinson's disease. **Cell Death & Disease**, v. 4, n. 1, p. e467–e467, 17 jan. 2013c.
COVI, J. A. et al. Rotenone Decreases Hatching Success in Brine Shrimp Embryos by Blocking Development: Implications for Zooplankton Egg Banks. **PLOS ONE**, v. 11, n. 9, p. e0163231, 21 set. 2016.
CRUZ-AGUADO, R. et al. Behavioral and biochemical effects of glutathione depletion in the rat brain. **Brain Research Bulletin**, v. 55, n. 3, p. 327–333, jun. 2001.
CRUZ, L. C. et al. Honey protects against wings posture error and molecular changes related to mitochondrial pathways induced by hypoxia/reoxygenation in adult Drosophila melanogaster. **Chemico-biological interactions**, v. 291, p. 245–252, 1 ago. 2018a.
CRUZ, L. C. et al. Honey protects against wings posture error and molecular changes related to mitochondrial pathways induced by hypoxia/reoxygenation in adult Drosophila melanogaster. **Chemico-Biological Interactions**, v. 291, p. 245–252, 1 ago. 2018b.
CUNNANE, S. C. et al. Brain energy rescue: an emerging therapeutic concept for neurodegenerative disorders of ageing. **Nature Reviews Drug Discovery**, v. 19, n. 9, p. 609–633, 24 set. 2020.
DA SILVA, M. H. et al. Acute brain damage induced by acetaminophen in mice: effect of diphenyl diselenide on oxidative stress and mitochondrial dysfunction. **Neurotoxicity research**, v. 21, n. 3, p. 334–44, abr. 2012.
DAWSON, T. M.; KO, H. S.; DAWSON, V. L. Genetic Animal Models of Parkinson's Disease. **Neuron**, v. 66, n. 5, p. 646–661, jun. 2010.
DEDEOGLU, A. et al. Magnetic resonance spectroscopic analysis of Alzheimer's disease mouse brain that express mutant human APP shows altered neurochemical profile. **Brain Research**, v. 1012, n. 1–2, p. 60–65, jun. 2004.
DEVINE, M. J.; PLUN-FAVREAU, H.; WOOD, N. W. Parkinson's disease and cancer: two wars, one front. **Nature reviews. Cancer**, v. 11, n. 11, p. 812–23, 24 out. 2011.
DIAS, C. et al. Evaluation of Mitochondrial Function in the CNS of Rodent Models of Alzheimer's Disease - High Resolution Respirometry Applied to Acute Hippocampal Slices. **Free radical biology & medicine**, v. 75 Suppl 1, p. S37, out. 2014.
DILBERGER, B. et al. Mitochondrial Oxidative Stress Impairs Energy Metabolism and Reduces Stress Resistance and Longevity of C. elegans. **Oxidative Medicine and Cellular**

Longevity, v. 2019, 2019a.
DILBERGER, B. et al. Polyphenols and metabolites enhance survival in rodents and nematodes—impact of mitochondria. **Nutrients**, v. 11, n. 8, 1 ago. 2019b.
DOERRIER, C. et al. High-resolution fluorespirometry and oxphos protocols for human cells, permeabilized fibers from small biopsies of muscle, and isolated mitochondria. In: **Methods in Molecular Biology**. [s.l.] Humana Press Inc., 2018. v. 1782p. 31–70.
DOLGACHEVA, L. P. et al. Role of DJ-1 in the mechanism of pathogenesis of Parkinson's disease. **Journal of Bioenergetics and Biomembranes**, v. 51, n. 3, p. 175–188, 3 jun. 2019.
DORSEY, E. R. et al. Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030. **Neurology**, v. 68, n. 5, p. 384–386, 30 jan. 2007.
E, G. Capacity of Oxidative Phosphorylation in Human Skeletal Muscle: New Perspectives of Mitochondrial Physiology. **The international journal of biochemistry & cell biology**, v. 41, n. 10, 2009.
EDERER, K. A. et al. Age-and genotype-specific effects of the angiotensin-converting enzyme inhibitor lisinopril on mitochondrial and metabolic parameters in drosophila melanogaster. **International Journal of Molecular Sciences**, v. 19, n. 11, 1 nov. 2018.
ELCHEBLY, M. Increased Insulin Sensitivity and Obesity Resistance in Mice Lacking the Protein Tyrosine Phosphatase-1B Gene. **Science**, v. 283, n. 5407, p. 1544–1548, 5 mar. 1999.
EMIR, U. E. et al. Noninvasive quantification of ascorbate and glutathione concentration in the elderly human brain. **NMR in Biomedicine**, v. 24, n. 7, p. 888–894, ago. 2011.
ESPINOZA-FONSECA, L. M. The benefits of the multi-target approach in drug design and discovery. **Bioorganic & Medicinal Chemistry**, v. 14, n. 4, p. 896–897, fev. 2006.
ETCHEGOYEN, M. et al. Metabolic Syndrome and Neuroprotection. **Frontiers in Neuroscience**, v. 12, p. 196, 20 abr. 2018.
FEANY, M. B.; BENDER, W. W. A Drosophila model of Parkinson's disease. **Nature**, v. 404, n. 6776, p. 394–398, mar. 2000.
FÉGER, J. et al. [Experimental models of Parkinson's disease]. **Annales pharmaceutiques francaises**, v. 60, n. 1, p. 3–21, jan. 2002.
FEITOSA, C. M. Determination of parameters of oxidative stress in vitro models of neurodegenerative diseases-A review. **Current Clinical Pharmacology**, v. 13, 1 mar. 2018.
FIGUEIRA, T. R. et al. Mitochondria as a source of reactive oxygen and nitrogen species: from molecular mechanisms to human health. **Antioxidants & redox signaling**, v. 18, n. 16, p. 2029–74, 2013.
FREDDO, N. et al. Stimulants cocktail: Methylphenidate plus caffeine impairs memory and cognition and alters mitochondrial and oxidative status. **Progress in Neuro-Psychopharmacology and Biological Psychiatry**, v. 106, p. 110069, mar. 2021.
FREDHOLM, B. B.; YANG, J.; WANG, Y. Low, but not high, dose caffeine is a readily available probe for adenosine actions. **Molecular Aspects of Medicine**, 30 nov. 2016.
GASSER, T. Genetic basis of Parkinson's disease: inheritance, penetrance, and expression. **The Application of Clinical Genetics**, p. 67, jun. 2011.
GAUTIER, C. A.; KITADA, T.; SHEN, J. Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. **Proceedings of the National Academy of Sciences**, v. 105, n. 32, p. 11364–11369, 12 ago. 2008.
GERISCH, B. et al. HLH-30/TFEB Is a Master Regulator of Reproductive Quiescence. **Developmental Cell**, v. 53, n. 3, p. 316- 329.e5, 4 maio 2020.
GLINKA, Y. Y.; YOUDIM, M. B. H. Inhibition of mitochondrial complexes I and IV by 6-hydroxydopamine. **European Journal of Pharmacology: Environmental Toxicology and Pharmacology**, v. 292, n. 3–4, p. 329–332, mar. 1995.
GOETZ, C. G. The history of Parkinson's disease: early clinical descriptions and neurological therapies. **Cold Spring Harbor perspectives in medicine**, v. 1, n. 1, p. a008862, 1 set. 2011.

GONÇALVES, D. F. et al. 6-Hydroxydopamine induces different mitochondrial bioenergetics response in brain regions of rat. **Neurotoxicology**, v. 70, p. 1–11, 2019.
GONÇALVES, D. F. et al. Effects of caffeine on brain antioxidant status and mitochondrial respiration in acetaminophen-intoxicated mice. **Toxicology Research**, v. 9, n. 5, p. 726–734, 29 out. 2020.
GUBERT, P. et al. Metabolic effects of manganese in the nematode Caenorhabditis elegans through DAergic pathway and transcription factors activation. **NeuroToxicology**, v. 67, p. 65–72, 1 jul. 2018.
GUITART, T. et al. New aminoacyl-tRNA synthetase-like protein in insecta with an essential mitochondrial function. **Journal of Biological Chemistry**, v. 285, n. 49, p. 38157–38166, 3 dez. 2010.
GUITART, T. et al. Human mitochondrial disease-like symptoms caused by a reduced tRNA aminoacylation activity in flies. **Nucleic Acids Research**, v. 41, n. 13, p. 6595–6608, jul. 2013.
GURURAJA RAO, S. et al. BKCa (Slo) Channel Regulates Mitochondrial Function and Lifespan in Drosophila melanogaster. **Cells**, v. 8, n. 9, 21 ago. 2019.
HALES, K. G. et al. Genetics on the Fly: A Primer on the Drosophila Model System. **Genetics**, v. 201, n. 3, p. 815–842, 1 nov. 2015.
HAVRANKOVA, J.; ROTH, J.; BROWNSTEIN, M. J. Concentrations of Insulin and of Insulin Receptors in the Brain are Independent of Peripheral Insulin Levels. **Journal of Clinical Investigation**, v. 64, n. 2, p. 636–642, 1 ago. 1979.
HAWLITSCHKA, A.; WREE, A. Experimental Intrastriatal Applications of Botulinum Neurotoxin-A: A Review. **International Journal of Molecular Sciences**, v. 19, n. 5, p. 1392, 7 maio 2018.
HEIKKILA, R. E. et al. Dopaminergic toxicity of rotenone and the 1-methyl-4-phenylpyridinium ion after their stereotaxic administration to rats: Implication for the mechanism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity. **Neuroscience Letters**, v. 62, n. 3, p. 389–394, dez. 1985.
HOU, Y. et al. **Ageing as a risk factor for neurodegenerative diseaseNature Reviews Neurology**Nature Publishing Group, , 1 out. 2019.
ISLAM, M. T. Oxidative stress and mitochondrial dysfunction-linked neurodegenerative disorders. **Neurological research**, v. 39, n. 1, p. 73–82, jan. 2017.
IWAKAMI, S. et al. Concentration-dependent Dual Effects of Hydrogen Peroxide on Insulin Signal Transduction in H4IIEC Hepatocytes. **PLoS ONE**, v. 6, n. 11, p. e27401, 15 nov. 2011.
J, L. et al. Selective lesion of central dopamine or noradrenaline neuron systems in the neonatal rat: motor behavior and monoamine alterations at adult stage. **Behavioural brain research**, v. 33, n. 3, p. 267–277, 1 jul. 1989.
J, W.; A, N. Mitochondrial Dynamics and Mitochondrial Dysfunction in Diabetes. **Acta medica Okayama**, v. 70, n. 3, 2016.
JUMBO-LUCIONI, P. et al. Nuclear genomic control of naturally occurring variation in mitochondrial function in Drosophila melanogaster. **BMC Genomics**, v. 13, n. 1, 22 nov. 2012.
K, T. A guide to neurotoxic animal models of Parkinson's disease. **Cold Spring Harbor perspectives in medicine**, v. 1, n. 1, set. 2011.
KABIRAJ, P. et al. The Neuroprotective Role of Ferrostatin-1 Under Rotenone-Induced Oxidative Stress in Dopaminergic Neuroblastoma Cells. **The Protein Journal**, v. 34, n. 5, p. 349–358, 18 out. 2015.
KALIA, L. V; LANG, A. E. Parkinson's disease. **The Lancet**, v. 386, n. 9996, p. 896–912, 29 ago. 2015.

KALINDERI, K.; BOSTANTJOPOULOU, S.; FIDANI, L. The genetic background of Parkinson's disease: current progress and future prospects. **Acta Neurologica Scandinavica**, v. 134, n. 5, p. 314–326, nov. 2016.
KASSAHUN, H. et al. Constitutive MAP-kinase activation suppresses germline apoptosis in NTH-1 DNA glycosylase deficient C. elegans. **DNA Repair**, v. 61, p. 46–55, 1 jan. 2018.
KITADA, T. et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. **Nature**, v. 392, n. 6676, p. 605–608, abr. 1998.
KOLAHDOUZAN, M.; HAMADEH, M. J. The neuroprotective effects of caffeine in neurodegenerative diseases. **CNS Neuroscience & Therapeutics**, v. 23, n. 4, p. 272–290, abr. 2017.
KRUMSCHNABEL, G. et al. Simultaneous High-Resolution Measurement of Mitochondrial Respiration and Hydrogen Peroxide Production. In: **Methods in molecular biology (Clifton, N.J.)**. [s.l: s.n.]. v. 1264p. 245–261.
KRUMSCHNABEL, G. et al. Simultaneous High-Resolution Measurement of Mitochondrial Respiration and Hydrogen Peroxide Production. In: [s.l: s.n.]. p. 245–261.
KRZYSZTOŃ-RUSSJAN, J. Pathophysiology and molecular basis of selected metabolic abnormalities in Huntington's disease. **Postepy higieny i medycyny doswiadczalnej (Online)**, v. 70, n. 0, p. 1331–1342, 30 dez. 2016.
LANGSTON, J. W. et al. 1-Methyl-4-phenylpyridinium ion (MPP+): Identification of a metabolite of MPTP, a toxin selective to the substantia nigra. **Neuroscience Letters**, v. 48, n. 1, p. 87–92, jul. 1984.
LANGSTON, J. W. The MPTP Story. **Journal of Parkinson's Disease**, v. 7, n. s1, p. S11–S19, 6 mar. 2017.
LAZAROU, M. et al. PINK1 drives Parkin self-association and HECT-like E3 activity upstream of mitochondrial binding. **The Journal of cell biology**, v. 200, n. 2, p. 163–72, 21 jan. 2013.
LEÃO, M. B. et al. Toxicological evaluation of the herbicide Palace® in Drosophila melanogaster. **Journal of Toxicology and Environmental Health - Part A: Current Issues**, v. 82, n. 22, p. 1172–1185, 17 nov. 2019.
LEMIEUX, H.; BLIER, P. U.; GNAIGER, E. Remodeling pathway control of mitochondrial respiratory capacity by temperature in mouse heart: electron flow through the Q-junction in permeabilized fibers. **Scientific Reports**, v. 7, n. 1, p. 2840, 6 dez. 2017.
LI, J.; SUN, Y.; CHEN, J. Transcriptome sequencing in a 6-hydroxydopamine rat model of Parkinson's disease. **Genes & Genetic Systems**, v. 94, n. 2, p. 61–69, 1 abr. 2019.
LIN, F. J. Effect of taurine and caffeine on sleep–wake activity in Drosophila melanogaster. **Nature and Science of Sleep**, p. 221, set. 2010.
LIN, M. T.; BEAL, M. F. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. **Nature**, v. 443, n. 7113, p. 787–795, 18 out. 2006.
LISOWSKI, P. et al. Mitochondria and the dynamic control of stem cell homeostasis. **EMBO reports**, p. e45432, 16 abr. 2018.
LIU, M. et al. Ginseng protein protects against mitochondrial dysfunction and neurodegeneration by inducing mitochondrial unfolded protein response in Drosophila melanogaster PINK1 model of Parkinson's disease. **Journal of Ethnopharmacology**, v. 247, 30 jan. 2020.
LOH, K. et al. Reactive Oxygen Species Enhance Insulin Sensitivity. **Cell Metabolism**, v. 10, n. 4, p. 260–272, out. 2009.
LOPES, F. M. et al. Mimicking Parkinson's Disease in a Dish: Merits and Pitfalls of the Most Commonly used Dopaminergic In Vitro Models. **NeuroMolecular Medicine**, v. 19, 2017.
LU, H.; FORBES, R. A.; VERMA, A. Hypoxia-inducible Factor 1 Activation by Aerobic Glycolysis Implicates the Warburg Effect in Carcinogenesis. **Journal of Biological**

Chemistry, v. 277, n. 26, p. 23111–23115, jun. 2002.
LU, J.; TAN, M.; CAI, Q. The Warburg effect in tumor progression: mitochondrial oxidative metabolism as an anti-metastasis mechanism. **Cancer letters**, v. 356, n. 2 Pt A, p. 156–64, 28 jan. 2015.
MACCHI, M. et al. The Drosophila inner-membrane protein PMI controls crista biogenesis and mitochondrial diameter. **Journal of Cell Science**, v. 126, n. 3, p. 814–824, fev. 2013.
MACEDO, F. et al. Lipase-like 5 enzyme controls mitochondrial activity in response to starvation in Caenorhabditis elegans. **Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids**, v. 1865, n. 2, 1 fev. 2020a.
MACEDO, F. et al. Lifespan-extending interventions enhance lipid-supported mitochondrial respiration in Caenorhabditis elegans. **FASEB Journal**, v. 34, n. 8, p. 9972–9981, 1 ago. 2020b.
MACEDO, F. et al. Lifespan-extending interventions enhance lipid-supported mitochondrial respiration in *Caenorhabditis elegans*. **The FASEB Journal**, v. 34, n. 8, p. 9972–9981, 1 ago. 2020c.
MAGISTRETTI, P. J.; ALLAMAN, I. Lactate in the brain: from metabolic end-product to signalling molecule. **Nature reviews. Neuroscience**, v. 19, n. 4, p. 235–249, 8 abr. 2018.
MARTÍNEZ-BANACLOCHA, M. Cysteine Network (CYSTEINET) Dysregulation in Parkinson's Disease: Role of N-acetylcysteine. **Current drug metabolism**, v. 17, n. 4, p. 368–85, 2016.
MASSARI, C. M. et al. In vitro 6-hydroxydopamine-induced toxicity in striatal, cerebrocortical and hippocampal slices is attenuated by atorvastatin and MK-801. **Toxicology in Vitro**, v. 37, p. 162–168, dez. 2016.
MATTHEWS, K. A.; KAUFMAN, T. C.; GELBART, W. M. Research resources for Drosophila: the expanding universe. **Nature reviews. Genetics**, v. 6, n. 3, p. 179–93, mar. 2005.
MCCOMMIS, K. S.; FINCK, B. N. Mitochondrial pyruvate transport: a historical perspective and future research directions. **Biochemical Journal**, v. 466, n. 3, p. 443–454, 15 mar. 2015.
MCKNIGHT, S.; HACK, N. Toxin-Induced Parkinsonism. **Neurologic clinics**, v. 38, n. 4, p. 853–865, 2020.
MCLELLAN, T. M.; CALDWELL, J. A.; LIEBERMAN, H. R. A review of caffeine's effects on cognitive, physical and occupational performance. **Neuroscience & Biobehavioral Reviews**, v. 71, p. 294–312, dez. 2016.
MICCHELI, A. et al. [1-13C]Glucose entry in neuronal and astrocytic intermediary metabolism of aged rats. **Brain Research**, v. 966, n. 1, p. 116–125, mar. 2003.
MIKI, Y. et al. Alteration of mitochondrial protein PDHA1 in Lewy body disease and PARK14. **Biochemical and Biophysical Research Communications**, v. 489, n. 4, p. 439–444, ago. 2017.
MISHRA, J.; KUMAR, A. Improvement of mitochondrial NAD+/FAD+-linked state-3 respiration by caffeine attenuates quinolinic acid induced motor impairment in rats: Implications in Huntington's disease. **Pharmacological Reports**, v. 66, n. 6, p. 1148–1155, dez. 2014.
MONTI, D. A. et al. N-Acetyl Cysteine Is Associated With Dopaminergic Improvement in Parkinson's Disease. **Clinical Pharmacology & Therapeutics**, v. 106, n. 4, p. 884–890, 17 out. 2019.
MOREIRA, P. I. et al. Mitochondria: a therapeutic target in neurodegeneration. **Biochimica et biophysica acta**, v. 1802, n. 1, p. 212–20, jan. 2010.
MORRONI, F. et al. Comparison of Adaptive Neuroprotective Mechanisms of Sulforaphane and its Interconversion Product Erucin in *in Vitro* and *in Vivo* Models of Parkinson's Disease. **Journal of Agricultural and Food Chemistry**, v. 66, n. 4, p. 856–865, 31 jan. 2018.

MURPHY, A. N. In a flurry of PINK, mitochondrial bioenergetics takes a leading role in Parkinson's disease. **EMBO Molecular Medicine**, v. 1, n. 2, p. 81–84, maio 2009.
MURRAY, T. V. A. et al. gavageNADPH oxidase 4 regulates homocysteine metabolism and protects against acetaminophen-induced liver damage in mice. **Free radical biology & medicine**, 13 out. 2015.
MUSSELMAN, L. P.; KÜHNLEIN, R. P. **Drosophila as a model to study obesity and metabolic diseaseJournal of Experimental Biology**Company of Biologists Ltd, , 1 mar. 2018. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/29514880/>. Acesso em: 27 out. 2020
MUYDERMAN, H. Highly Selective and Prolonged Depletion of Mitochondrial Glutathione in Astrocytes Markedly Increases Sensitivity to Peroxynitrite. **Journal of Neuroscience**, v. 24, n. 37, p. 8019–8028, 15 set. 2004.
MYERS, E. W. A Whole-Genome Assembly of Drosophila. **Science**, v. 287, n. 5461, p. 2196–2204, 24 mar. 2000.
NARENDRA, D. P. et al. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. **PLoS biology**, v. 8, n. 1, p. e1000298, 26 jan. 2010.
NAVARRO-YEPES, J. et al. Oxidative Stress, Redox Signaling, and Autophagy: Cell Death *Versus* Survival. **Antioxidants & Redox Signaling**, v. 21, n. 1, p. 66–85, 1 jul. 2014.
NEVES, A.; BUSSO, C.; GÖNCZY, P. Cellular hallmarks reveal restricted aerobic metabolism at thermal limits. **eLife**, v. 4, n. MAY, 1 maio 2015.
OJUKA, E. et al. **Measurement of β-oxidation capacity of biological samples by respirometry: A review of principles and substratesAmerican Journal of Physiology - Endocrinology and Metabolism**American Physiological Society, , 1 maio 2016. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/26908505/>. Acesso em: 21 dez. 2020
OST, M. et al. **Analysis of mitochondrial respiratory function in tissue biopsies and blood cellsCurrent opinion in clinical nutrition and metabolic care**NLM (Medline), , 1 set. 2018. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/29939971/>. Acesso em: 9 out. 2020
OU, H.-L.; BJ¨, B.; SCHUMACHER, B. **Review Series HEMATOLOGIC DISEASE AT OLDER AGE DNA damage responses and p53 in the aging process Institute for Genome Stability in Aging and Disease, Medical Faculty, 2 Cologne Excellence Cluster for Cellular Stress Responses in Aging-Associated Diseases**. [s.l: s.n.]. Disponível em: <http://ashpublications.org/blood/article-pdf/131/5/488/1465962/blood746396.pdf>.
PAISÁN-RUÍZ, C. et al. Cloning of the Gene Containing Mutations that Cause PARK8-Linked Parkinson's Disease. **Neuron**, v. 44, n. 4, p. 595–600, nov. 2004.
PANDYA, J. D. et al. Age- and brain region-specific differences in mitochondrial bioenergetics in Brown Norway rats. **Neurobiology of Aging**, v. 42, p. 25–34, 1 jun. 2016.
PANTIC, I. et al. Oxidopamine and oxidative stress: Recent advances in experimental physiology and pharmacology. **Chemico-Biological Interactions**, v. 336, p. 109380, fev. 2021.
PARK, J. et al. Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. **Nature**, v. 441, n. 7097, p. 1157–1161, 3 jun. 2006.
PASHA, M. K.; SHARMA, R. K.; RAJPUT, A. H. Increased myocardial N-myristoyltransferase activity in rotenone model of Parkinsonism. **International journal of molecular medicine**, v. 15, n. 6, p. 987–91, jun. 2005.
PENG, K. et al. The Interaction of Mitochondrial Biogenesis and Fission/Fusion Mediated by PGC-1α Regulates Rotenone-Induced Dopaminergic Neurotoxicity. **Molecular Neurobiology**, v. 54, n. 5, p. 3783–3797, 7 jul. 2017.
PEROCCHI, F. et al. Assessing systems properties of yeast mitochondria through an interaction map of the organelle. **PLoS Genetics**, v. 2, n. 10, p. 1612–1624, out. 2006.
PESTA, D.; GNAIGER, E. High-Resolution Respirometry: OXPHOS Protocols for Human

Cells and Permeabilized Fibers from Small Biopsies of Human Muscle. In: [s.l: s.n.]. p. 25–58.
PETROSILLO, G. et al. Mitochondrial dysfunction in rat brain with agingInvolvement of complex I, reactive oxygen species and cardiolipin. **Neurochemistry International**, v. 53, n. 5, p. 126–131, nov. 2008.
PICHAUD, N. et al. Thermal sensitivity of mitochondrial functions in permeabilized muscle fibers from two populations of Drosophila simulans with divergent mitotypes. **American Journal of Physiology - Regulatory Integrative and Comparative Physiology**, v. 301, n. 1, jul. 2011.
PICHAUD, N. et al. Age Dependent Dysfunction of Mitochondrial and ROS Metabolism Induced by Mitonuclear Mismatch. **Frontiers in Genetics**, v. 10, n. FEB, p. 130, 20 fev. 2019.
PIENAAR, I. S.; GÖTZ, J.; FEANY, M. B. Parkinson's disease: insights from non-traditional model organisms. **Progress in neurobiology**, v. 92, n. 4, p. 558–71, dez. 2010.
PIMENTA DE CASTRO, I. et al. Genetic analysis of mitochondrial protein misfolding in Drosophila melanogaster. **Cell Death and Differentiation**, v. 19, n. 8, p. 1308–1316, ago. 2012a.
PIMENTA DE CASTRO, I. et al. Genetic analysis of mitochondrial protein misfolding in Drosophila melanogaster. **Cell Death & Differentiation**, v. 19, n. 8, p. 1308–1316, 3 ago. 2012b.
POLYMEROPOULOS, M. H. et al. Mapping of a Gene for Parkinson's Disease to Chromosome 4q21-q23. **Science**, v. 274, n. 5290, p. 1197–1199, 15 nov. 1996.
PONZ-SARVISE, M. et al. In vitro Models of Neurodegenerative Diseases. 2020.
PROCACCIO, V. et al. Perspectives of drug-based neuroprotection targeting mitochondria. **Revue Neurologique**, v. 170, n. 5, p. 390–400, maio 2014.
PUURAND, M. et al. **Intracellular energy-transfer networks and high-resolution respirometry: A convenient approach for studying their functionInternational Journal of Molecular Sciences**MDPI AG, , 1 out. 2018. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/30261663/>. Acesso em: 26 out. 2020
RAMSAY, R. R. et al. The inhibition site of MPP+, the neurotoxic bioactivation product of 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine is near the Q-binding site of NADH dehydrogenase. **Archives of Biochemistry and Biophysics**, v. 259, n. 2, p. 645–649, dez. 1987.
RB, P. et al. Caffeine as symptomatic treatment for Parkinson disease (Café-PD): A randomized trial. **Neurology**, v. 89, n. 17, p. 1795–1803, 24 out. 2017.
REQUEJO-AGUILAR, R. et al. PINK1 deficiency sustains cell proliferation by reprogramming glucose metabolism through HIF1. **Nature communications**, v. 5, p. 4514, 24 jul. 2014.
REQUEJO-AGUILAR, R. et al. DJ1 represses glycolysis and cell proliferation by transcriptionally up-regulating pink1. **Biochemical Journal**, v. 467, n. 2, p. 303–310, 15 abr. 2015.
REQUEJO-AGUILAR, R.; BOLAÑOS, J. P. Mitochondrial control of cell bioenergetics in Parkinson's disease. **Free Radical Biology and Medicine**, v. 100, p. 123–137, nov. 2016.
REX SHEU, K.-F. et al. An immunochemical study of the pyruvate dehydrogenase deficit in Alzheimer's disease brain. **Annals of Neurology**, v. 17, n. 5, p. 444–449, maio 1985.
RHEE, S. G. CELL SIGNALING: H2O2, a Necessary Evil for Cell Signaling. **Science**, v. 312, n. 5782, p. 1882–1883, 30 jun. 2006.
RICHARDSON, J. R. et al. Neurotoxicity of pesticides. **Acta Neuropathologica**, v. 138, n. 3, p. 343–362, 13 set. 2019.
RIEDERER, P. et al. Lateralisation in Parkinson disease. **Cell and Tissue Research**, 14 abr.

2018.
RIEMENSPERGER, T. et al. A Single Dopamine Pathway Underlies Progressive Locomotor Deficits in a Drosophila Model of Parkinson Disease. **Cell Reports**, v. 5, n. 4, p. 952–960, nov. 2013.
RODRIGUES, A. P. C. et al. Developmental arrest in Drosophila melanogaster caused by mitochondrial DNA replication defects cannot be rescued by the alternative oxidase. **Scientific Reports**, v. 8, n. 1, 1 dez. 2018a.
RODRIGUES, N. R. et al. Short-term sleep deprivation with exposure to nocturnal light alters mitochondrial bioenergetics in Drosophila. **Free Radical Biology and Medicine**, v. 120, p. 395–406, 20 maio 2018b.
ROMUK, E. B. et al. The evaluation of the changes in enzymatic antioxidant reserves and lipid peroxidation in chosen parts of the brain in an animal model of Parkinson disease. **Advances in clinical and experimental medicine : official organ Wroclaw Medical University**, v. 26, n. 6, p. 953–959, set. 2017.
ROVENKO, B. M. et al. Restriction of glucose and fructose causes mild oxidative stress independently of mitochondrial activity and reactive oxygen species in drosophila melanogaster. **Comparative Biochemistry and Physiology -Part A : Molecular and Integrative Physiology**, v. 187, p. 27–39, 1 set. 2015.
RUBIN, G. M. Drosophila melanogaster as an experimental organism. **Science**, v. 240, n. 4858, p. 1453–1459, 1988.
SALARI, S.; BAGHERI, M. In Vivo, In Vitro and Pharmacologic Models of Parkinson's Disease. **Physiol. Res**, v. 68, p. 17–24, 2019.
SAMARANCH, L. et al. PINK1-linked parkinsonism is associated with Lewy body pathology. **Brain**, v. 133, n. 4, p. 1128–1142, abr. 2010.
SAWYNOK, J. Caffeine and pain. **Pain**, v. 152, n. 4, p. 726–9, abr. 2011.
SCHAPIRA, A. H. V. et al. Mitochondrial Complex I Deficiency in Parkinson's Disease. **Journal of Neurochemistry**, v. 54, n. 3, p. 823–827, mar. 1990.
SCHÖPF, B. et al. Oxidative phosphorylation and mitochondrial function differ between human prostate tissue and cultured cells. **The FEBS Journal**, v. 283, n. 11, p. 2181–2196, jun. 2016a.
SCHÖPF, B. et al. Oxidative phosphorylation and mitochondrial function differ between human prostate tissue and cultured cells. **The FEBS journal**, v. 283, n. 11, p. 2181–96, jun. 2016b.
SCHÖPF, B. et al. Oxidative phosphorylation and mitochondrial function differ between human prostate tissue and cultured cells. **The FEBS journal**, v. 283, n. 11, p. 2181–96, jun. 2016c.
SCHULER, F.; CASIDA, J. E. The insecticide target in the PSST subunit of complex I. **Pest management science**, v. 57, n. 10, p. 932–40, out. 2001.
SCIALO, F. et al. Practical recommendations for the use of the geneswitch Gal4 system to knock-down genes in drosophila melanogaster. **PLoS ONE**, v. 11, n. 8, 1 ago. 2016.
SCIALÒ, F. et al. Mitochondrial ROS Produced via Reverse Electron Transport Extend Animal Lifespan. **Cell Metabolism**, v. 23, n. 4, p. 725–734, 12 abr. 2016.
SERRANO-POZO, A.; DAS, S.; HYMAN, B. T. APOE and Alzheimer's disease: advances in genetics, pathophysiology, and therapeutic approaches. **The Lancet Neurology**, v. 20, n. 1, p. 68–80, jan. 2021.
SHIMURA, H. et al. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. **Nature Genetics**, v. 25, n. 3, p. 302–305, jul. 2000.
SIDDIQUI, M. A. et al. Rotenone-induced oxidative stress and apoptosis in human liver HepG2 cells. **Molecular and Cellular Biochemistry**, v. 384, n. 1–2, p. 59–69, 21 dez. 2013.
SIGNES, A.; FERNANDEZ-VIZARRA, E. Assembly of mammalian oxidative

phosphorylation complexes I–V and supercomplexes. **Essays in Biochemistry**, v. 62, n. 3, p. 255–270, 20 jul. 2018.
SIMARD, C. et al. Metabolic characterization and consequences of mitochondrial pyruvate carrier deficiency in drosophila melanogaster. **Metabolites**, v. 10, n. 9, p. 1–16, 1 set. 2020.
SIMARD, C. J. et al. Measurement of mitochondrial oxygen consumption in permeabilized fibers of drosophila using minimal amounts of tissue. **Journal of Visualized Experiments**, v. 2018, n. 134, 7 abr. 2018.
SIMUNI, T. et al. Longitudinal change of clinical and biological measures in early Parkinson's disease: Parkinson's progression markers initiative cohort. **Movement Disorders**, 23 mar. 2018.
STEFANATOS, R. et al. Dj-1β regulates oxidative stress, insulin-like signaling and development in Drosophila melanogaster. **Cell Cycle**, v. 11, n. 20, p. 3876–3886, 15 out. 2012.
STOROZHEVYKH, T. P. et al. Mitochondrial respiratory chain is involved in insulin-stimulated hydrogen peroxide production and plays an integral role in insulin receptor autophosphorylation in neurons. **BMC Neuroscience**, v. 8, n. 1, p. 84, 2007.
STRÖMBERG, I. et al. Astrocyte responses to dopaminergic denervations by 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine as evidenced by glial fibrillary acidic protein immunohistochemistry. **Brain Research Bulletin**, v. 17, n. 2, p. 225–236, ago. 1986.
SUDATI, J. H. et al. Valeriana officinalis attenuates the rotenone-induced toxicity in drosophila melanogaster. **NeuroToxicology**, v. 37, p. 118–126, 2013.
SUH, H. J. et al. Behavioral Changes and Survival in Drosophila melanogaster: Effects of Ascorbic Acid, Taurine, and Caffeine. **Biological & pharmaceutical bulletin**, v. 40, n. 11, p. 1873–1882, 2017.
SYRJÄNEN, L. et al. β carbonic anhydrase is required for female fertility in Drosophila melanogaster. **Frontiers in Zoology**, v. 12, n. 1, 22 ago. 2015.
TAFERNER, A. et al. FAH domain containing protein 1 (FAHD-1) Is required for mitochondrial function and locomotion activity in C. elegans. **PLoS ONE**, v. 10, n. 8, 12 ago. 2015.
TAKAHASHI-NIKI, K. et al. Transcriptional Regulation of DJ-1. **Advances in experimental medicine and biology**, v. 1037, p. 89–95, 2017.
TARNOPOLSKY, M. A. Caffeine and creatine use in sport. **Annals of nutrition & metabolism**, v. 57 Suppl 2, p. 1–8, 2010.
TEIXEIRA, C. M. et al. Hippocampal 5-HT Input Regulates Memory Formation and Schaffer Collateral Excitation. **Neuron**, 8 maio 2018.
TEULIER, L. et al. Consequences of electroshock-induced narcosis in fish muscle: from mitochondria to swim performance. **Journal of Fish Biology**, v. 92, n. 6, p. 1805–1818, 2018.
TEVES, J. M. Y. et al. Parkinson's Disease Skin Fibroblasts Display Signature Alterations in Growth, Redox Homeostasis, Mitochondrial Function, and Autophagy. **Frontiers in Neuroscience**, v. 11, p. 737, 12 jan. 2018.
THOMPSON, K. et al. OXA 1L mutations cause mitochondrial encephalopathy and a combined oxidative phosphorylation defect . **EMBO Molecular Medicine**, v. 10, n. 11, nov. 2018.
TRACHOOTHAM, D. et al. Redox regulation of cell survival. **Antioxidants & redox signaling**, v. 10, n. 8, p. 1343–74, ago. 2008.
TRINH, J.; FARRER, M. Advances in the genetics of Parkinson disease. **Nature Reviews Neurology**, v. 9, n. 8, p. 445–454, 16 ago. 2013.
TSUANG, D. W.; BIRD, T. D. Genetic factors in neurodegenerative diseases. **American**

Journal of Medical Genetics Part B: Neuropsychiatric Genetics, v. 174, n. 1, p. 3–4, jan. 2017.
ULGHERAIT, M. et al. Circadian regulation of mitochondrial uncoupling and lifespan. **Nature Communications**, v. 11, n. 1, 1 dez. 2020.
VAN HEEMST, D. Insulin, IGF-1 and longevity. **Aging and disease**, v. 1, n. 2, p. 147–57, out. 2010.
VELAZQUEZ, R. et al. Acute tau knockdown in the hippocampus of adult mice causes learning and memory deficits. **Aging Cell**, p. e12775, 10 maio 2018.
VM, N.; MA, F. History of research on C. elegans and other free-living nematodes as model organisms. **WormBook**, v. 2017, p. 1–84, 7 set. 2017.
VOICULESCU, M. et al. Molecular and pharmacodynamic interactions between caffeine and dopaminergic system. **Journal of medicine and life**, v. 7 Spec No., p. 30–8, 2014.
WANG, A. et al. A dual effect of N-acetylcysteine on acute ethanol-induced liver damage in mice. **Hepatology Research**, v. 34, n. 3, p. 199–206, mar. 2006.
WANG, T. et al. Neuroprotective effects of Danshensu on rotenone-induced Parkinson's disease models in vitro and in vivo. **BMC Complementary Medicine and Therapies**, v. 20, n. 1, p. 20, 23 dez. 2020.
WEISZ, E. D. et al. Loss of Drosophila FMRP leads to alterations in energy metabolism and mitochondrial function. **Human Molecular Genetics**, v. 27, n. 1, p. 95–106, 1 jan. 2018.
WHITWORTH, A. J.; PALLANCK, L. J. The PINK1/Parkin pathway: a mitochondrial quality control system? **Journal of Bioenergetics and Biomembranes**, v. 41, n. 6, p. 499–503, 5 dez. 2009.
WHO. 19th WHO Model List of Essential Medicines. **Http://Www.Who.Int/Medicines/Publications/Essentialmedicines/En/**, n. April, p. 1–51, 2015.
WREDENBERG, A. et al. MTERF3 Regulates Mitochondrial Ribosome Biogenesis in Invertebrates and Mammals. **PLoS Genetics**, v. 9, n. 1, jan. 2013.
WÜLLNER, U. et al. Glutathione depletion and neuronal cell death: the role of reactive oxygen intermediates and mitochondrial function. **Brain Research**, v. 826, n. 1, p. 53–62, abr. 1999.
XIROMERISIOU, G. et al. Genetic basis of Parkinson disease. **Neurosurgical Focus**, v. 28, n. 1, p. E7, jan. 2010.
YAMAGUCHI, M.; YOSHIDA, H. Drosophila as a Model Organism. In: [s.l: s.n.]. p. 1–10.
YAN, M. H.; WANG, X.; ZHU, X. Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease. **Free Radical Biology and Medicine**, v. 62, p. 90–101, set. 2013.
YIN, F.; JIANG, T.; CADENAS, E. Metabolic triad in brain aging: mitochondria, insulin/IGF-1 signalling and JNK signalling. **Biochemical Society Transactions**, v. 41, n. 1, p. 101–105, 1 fev. 2013.
ZEIDÁN-CHULIÁ, F. et al. Major components of energy drinks (caffeine, taurine, and guarana) exert cytotoxic effects on human neuronal SH-SY5Y cells by decreasing reactive oxygen species production. **Oxidative medicine and cellular longevity**, v. 2013, p. 791795, 2013.
ZHANG, H. et al. Glutathione-dependent reductive stress triggers mitochondrial oxidation and cytotoxicity. **The FASEB Journal**, v. 26, n. 4, p. 1442–1451, 2012.
ZHANG, Y. et al. Regulation of glutamate transporter trafficking by Nedd4-2 in a Parkinson's disease model. **Cell Death & Disease**, v. 8, n. 2, p. e2574–e2574, 2 fev. 2017a.
ZHANG, Z. et al. Acute action of rotenone on excitability of catecholaminergic neurons in rostral ventrolateral medulla. **Brain Research Bulletin**, v. 134, p. 151–161, set. 2017b.
ZIMPRICH, A. et al. Mutations in LRRK2 Cause Autosomal-Dominant Parkinsonism with

Pleomorphic Pathology. **Neuron**, v. 44, n. 4, p. 601–607, nov. 2004.
ZONG, W. X.; RABINOWITZ, J. D.; WHITE, E. **Mitochondria and CancerMolecular Cell**Cell Press, , 3 mar. 2016. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/26942671/>. Acesso em: 19 nov. 2020