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AVALIAÇÕES BIOENERGÉTICAS MITOCONDRIAIS EM TECIDOS PERMEABILIZADOS DO ZEBRAFISH (*Danio rerio*)

Recife

2018

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas da Universidade Federal de Pernambuco ï UFPE como requisito parcial para obtenção do título de Doutor em Ciências Biológicas.

Área de concentração: Biotecnologia

Orientador: Prof.Dr. Ranilson de Souza Bezerra
Co-orientadores: Profa. Dra. Ana Catarina Resende Leite
Prof. Ph. D. Ian Porto Gurgel do Amaral

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Aprovada em: 08 / 02 / 2018

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VOLPATO, Regina, 2017, p. 156.

RESUMO

Após a identificação de que a mitocôndria participa ativamente de vários processos que regulam a função e a sobrevivência celular esta organela passou a ser utilizada como janela experimental para diversas áreas do conhecimento como Fisiologia e Ecotoxicologia, por exemplo. Neste trabalho o modelo experimental zebrafish (*Danio rerio*) foi utilizado para estudo da bioenergética mitocondrial através de tecidos seletivamente permeabilizados.Neste sentido, foi demonstrado como a mitocôndria do zebrafish é uma ferramenta útil para estudos na área de toxicologia (ambiental e farmacológica), bioquímica de proteínas, respiração mitocondrial, homeostase de cálcio e geração de espécies reativas de oxigênio e nitrogênio (EROS e ERNS). Além disso, a tecido-especificidade mitocondrial entre fígado, cérebro, músculo cardíaco e músculo esquelético e os possíveis efeitos deletérios do pesticida piriproxifeno foram estudadas. Para isso, os tecidos foram permeabilizados com digitonina ou saponina eestudadoso funcionamento dos complexos respiratórios da cadeia transportadora de elétrons, a geração de espécies reativas de oxigênio e nitrogênio (utilizando H_2DCF-DA, MitoSOX e DAF-FM-DA) e captação de cálcio(com Calcium Green 5N).Como resultados observou-se que a mitocôndria do zebrafish é uma ferramenta altamente atrativa mitocondriólogos de todo mundo. Coração e músculo esquelético mostraram diferenças para o consumo de O_2pelo complexo I quando comparados com fígado e cérebro.O músculo esquelético teve o maior consumo de oxigênio para os complexos II e IV (51% e 65% maiores, respectivamente). A geração de espécies reativas (ERs) com H_2DCF-DA mostrou que fígado e coração possuem maior susceptibilidade para geração de EROS, cerca de 58% maior que o gerado por cérebro e musculo esquelético. Além disso, a geração de EROS foi Ca^{2+}dependente para todos os tecidos estudados.Cérebro, seguido pelo fígado, foi o tecido permeabilizado com maior potencial para geração de O_2Â˙Ë (96% e 68% maior que músculo cardíaco e esquelético, respectivamente). Por fim, uma exposição a pequenas doses do pesticida piriproxifeno foi capaz de afetar o controle respiratório (CR) dos complexos I e II em tecidos cerebrais.Houve um aumento na geração de ERs e este teve um efeito dose dependente para geração de O_2Â˙Ë. Em síntese, o zebrafish é uma ferramenta de baixo custo altamente atrativa para estudo da bioenergética mitocondrial e tem permitido a avaliação de propriedades tóxicas de diversos compostos, inclusive para os que possuem escopo terapêutico.

Palavras-chave**:** *Danio rerio*. Ecotoxicologia. Fisiologia comparada. Função mitocondrial. Permeabilização seletiva. Oxido nítrico.

ABSTRACT

Behind mitochondrial activity be linkage with cell survive and function, this organelle has been widely used as an experimental window to many scientific areas such as physiology and ecotoxicology. In this work the zebrafish (*Danio rerio*) experimental model was used to mitochondrial bioenergetic study through of selective permeabilized tissues. In this hand, was showed the zebrafish mitochondria such as useful tool to studies about toxicology (environmental and pharmacological), protein biochemistry, mitochondrial respiration, calcium homeostasis and reactive oxygen e nitrogen species (ROS and RNS) generation. In another hand, the mitochondrial tissue-specificity between hepatic, neuronal, muscular cardiac and muscular skeletal (SM) tissues, beyond possible pyriproxyfen side effects was studied using mitochondrial bioenergetic as physiological state translator. For this, mitochondrial respiratory complexes, ROS and RNS (using H_2DCF-DA, MitoSOX e DAF-FM-DA) and Ca^{2+} uptake (by Calcium Green 5N) was studied through of tissue selective permeabilization with digitonin or saponin. As result, zebrafish mitochondrial study is a very attractive tool due a lot of benefits that this little teleost fish introduces to mitochondriologists community around the world. Heart and SM showed differences to O_2 consume at NADH dehydrogenase level when compared with liver and brain. SM have higher O_2 consume for Succinate dehydrogenase and Cytochrome c oxidase (51 and 65% higher, respectively).Reactive species (RS) generation by H_2DCF-DA showed that liver and heart have more susceptibility to RS generation (around 58% more that brain or SM). Furthermore, RS generation was Ca^{2+}-dependent for all studied tissues. Brain, followed by liver, was permeabilized tissue with high potential to O_2Ä generation. (96% and 68% more that heart and SM, respectively).Ca^{2+} transport showed that brain tissue has high affinity to Ca^{2+} uptake. Lastly, pyriproxyfen lower concentrations affected the respiratory control (RC) to neuronal NADH dehydrogenase and Succinate dehydrogenase of respiratory chain. Pyriproxyfen side effects maximize RS generation and showed a dose-dependent effect to O_2Ä generation.As well as for nitric oxide production and calcium transport.In summary, zebrafish is a highly attractive low-cost tool for mitochondrial bioenergetics study and has viabletoxic propertiesevaluation of several compounds, including as therapeutic scope.

Keywords: Comparative physiology. *Danio rerio*. Ecotoxicology. Mitochondrial Function. Nitric oxide. Selective permeabilization.

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LISTA DE ABREVIAÇÕES E SIMBOLOS

$^1O_2$	Oxigênio singleto
4-HNE	4-hidroxinonenal
AChE	Acetilcolinesterase
ADP	Adenosina Difosfato
APAF1	Fator ativador de protease apoptótica 1
ATP	Adenosina Trifosfato
BAK	Proteína reguladora da apoptose
BAX	Proteína reguladora da apoptose
Bcl-2	Família de Proteínas Reguladoras da apoptose
BH3	Proteína ligante complementar a função apoptótica
BID	Domínio interativo agonista de morte
CCCP	Cianeto Carbonil-4- (trifluorometoxi) Fenil-hidrazona
cNOS	Óxido nítrico sintase constitutiva
CsA	Ciclosporina A
CTE	Cadeia Transportadora de Elétrons
DCF	Diclorofluorescina
Digitonina	(P-D-Galactopiranosido, (2a, 3p, 5a, 15p, 25R) -2,15-dihidroxispirostan-3-ilo O-ɓ-D-glucopiranosil- (1-> 3) -O-p-D-galactopiranosil - (1-> 2) -O- [p-D-xilopiranosil- (1-> 3)] - O-p-D-glucopiranosil- (1-> 4)
DNA	Ácido Desoxirribonucleico
DNP	2,4 dinitrofenol
dpf	dias pós fertilização
eNOS	Óxido nítrico sintase endotelial

ERNôs	Espécies Reativas de Nitrogênio
EROS	Espécies Reativas de Oxigênio
F_1	Fração insolúvel da ATP sintase localizada na membrana interna da mitocôndria
FADD	Domínio proteico FAS associado à morte
$FADH_2$	Dinucleotídeo de flavina e adenina reduzido
FDA	Agência Americana de alimentos e medicamentos
F_o	Fração solúvel da ATP sintase, que pode ser inibida pela Oligomicina
GPx	Glutationa peroxidase
GSH	Glutationa
GST	Glutationa S-transferase
H_2O	Água
H_2O_2	Peróxido de Hidrogênio
H_2S	Sulfinil nitrito
HDAC	Histona deacetilase
HHE	Hidroxihexenal
HPAôs	Hidrocarbonetos policíclicos aromáticos
hpf	horas pós fertilização
iNOS	Óxido nítrico sintase indutiva
KCN	Cianeto de potássio
LOOA	Hidroperóxidos lipídicos
MCU	Canal mitocondrial de cálcio uniporta
MDA	Malondialdeído
MeHg	Metilmercúrio

MnSOD	Superóxido dismutase manganês dependente
MPTP	Neurotoxina que afeta o receptor de dopamina
mtDNA	DNA mitocondrial
mtNOS	Óxido nítrico sintase mitocondrial
NADH	Dinucleótido de nicotinamida e adenina reduzido
nNOS	Óxido nítrico sintase neuronal
NOÅ	Óxido Nítrico
NOS	Óxido nítrico sintase
O_2	Oxigênio
O_2ÆË	Ânion Superóxido
OG	Óxido de grafeno
PBDEs	Éter difenil polibrominato
Prx3	Peroxirredoxina 3
PTP	Poro de transição de permeabilidade mitocondrial
RE	Retículo endoplasmático
Saponina	2,3,4-tri-O-benzil-p-D-xilopiranosil-(1->4)-6-desoxi-2,3-O-isopropilideno-a-L-manopiranosil- (1-> 2) -4-amino-3,6-di-O-benzil-4-desoxi-1-O - {(3p, 16a) -23,28-dioxo-3,16-bis [(trietilsilil) oxi]12-en-28-il} -p-D-galactopiranose
-SH	Grupamentos tióis
SMAC	Segundo derivado mitocondrial ativador de caspases
TBARS	Substâncias reativas ao ácido tiobarbitúrico
tBID	Forma reduzida do BID
NAD(P)+ transidrogenase	Enzima que catalisa NADPH e NAD+

TiO_2	Dióxido de titânio
TNF	Fator de necrose tumoral
TNRF1	Receptor cognitivo do fator de necrose tumoral
TPM	Transição de Permeabilidade Mitocondrial
TPTA	Fungicida acetato de trifenil
TPx	Tioredoxina peroxidase
TR	Tioredoxina redutase
TRADD	Domínio proteico TNRF associado a morte
Trx	Tiorredoxina
TSH	Tiorredoxina
UCP1	Proteína desacopladora 1
UCP2	Proteína desacopladora 2
UCP3	Proteína desacopladora 3
UCPs	Proteínas desacopladoras
UQ	Ubiquinona
UQ$\dot{A}\dot{E}$	Ânion radical ubisemiquinona
UQH_2	Ubiquinol
VDAC	Canal aniônico voltagem dependente
VDAC2	Canal aniônico voltagem dependente 2
XIAP	Proteína X Inibidora de Apoptose
zbACHE	Acetilcolinesterase cerebral do zebrafish
$q\mu H^+$	Potencial Eletroquímico de Membrana Mitocondrial
qpH	Componente químico da CTE
qΦ	Potencial Elétrico de Membrana Mitocondrial

SUMARIO

1 INTRODUÇÃO

O trabalho de Tese procurou investigar diferenças na bioenergética mitocondrial do peixe-zebra que podem ser identificadas entre vários tecidos, sendo estas investigações fracionadas em três estudos. O primeiro estudo baseou-se numa revisão sistemática sobre a função mitocondrial do zebrafish. O segundo investigou as diferenças bioenergéticas e na produção de espécies reativas de oxigênio pela mitocôndria entre o tecido neuronal, hepático e muscular cardíaco e esquelético do peixe. O terceiro objetivou estudar os possíveis efeitos deletérios de uma exposição de exemplares machos adultos do zebrafish ao piriproxifeno utilizando a atividade da acetilcolinesterase cerebrais e da integridade e bioenergética mitocondrial do cérebro como indicadores do estado fisiológico. Estes estudos baseiam-se na hipótese geral de que a mitocôndria do zebrafish é um poderoso modelo para estudos de fisiologia comparada e para avaliações toxicológicas. Sobre isso, é hipotetizado que há um padrão tecido específico para bioenergética mitocondrial, geração de espécies reativas de oxigênio e de nitrogênio no zebrafish e que pesticidas como o piriproxifeno induzem a disfunção mitocondrial por comprometer a fosforilação oxidativa e fomentar a geração de espécies reativas de oxigênio e nitrogênio

A mitocôndria é uma organela amplamente reconhecida por sua capacidade de produzir ATP via fosforilação oxidativa e por ter a habilidade de geração de calor em animais homeotérmicos por meio de um transporte de prótons na Cadeia Transportadora de Elétrons (CTE). Uma análise mais detalhada da função mitocondrial tem possibilitado a identificação de que esta organela participa ativamente de muitas atividades celulares como apoptose, necrose e autofagia (Lemasters et al., 2002), regulação de Ca^{2+} intracelular (Chernorudskiy and Zito, 2017; Vercesi et al., 1988; Vercesi et al., 2006), geração de Espécies Reativas de Oxigênio e Nitrogênio (EROS e ERNôs, respectivamente) (Figueira et al., 2013; Kowaltowski et al., 2009) e sinalização redox . Além disso, a mitocôndria é uma organela importante para biossíntese do oxido nítrico (NO) e S-nitrosilação de grupos proteicos de membrana (Leite et al., 2010).

Os estudos mitocondriais em peixes possuem os mais variados escopos, que vão desde análises toxicológicas para este grupo animal até delicados processos fisiopatológicos como distúrbios metabólicos e doenças neurodegenerativas (Bourdineaud et al., 2013; Flinn et al., 2009; Hiramitsu et al., 2014). O zebrafish, conhecido popularmente como paulistinha e cientificamente como *Danio rerio*, é o peixe modelo que mais tem contribuído para ampliar o

conhecimento sobre a bioenergética mitocondrial e suas especificidades. Dentre as vantagens que este modelo animal trouxe para o estudo da mitocôndria estão análises *in vivo* através de *probes* fluorescentes (Flinn et al., 2009; Lepiller et al., 2007; Plucinska et al., 2012; Sasagawa et al., 2016) e a facilidade para indução de doenças mitocondriais que afetam os sistema nervoso e cardiovascular (Steele et al., 2014). Além dos mesmos padrões mitocondriais encontrados nos demais modelos animais para o transporte de Ca^{2+}(Azzolin et al., 2010) e produção de EROS (Hagedorn et al., 2012; Mugoni et al., 2014).

Dentre as diversas metodologias utilizadas para estudar a função ou disfunção mitocondrial, o uso de glicosídeos como digitonina ou saponina tem possibilitado o estudo da mitocôndria através de uma permeabilização seletiva da membrana plasmática, caracterizando, assim, uma análise *in situ* da mitocôndria. Esta análise permite o acesso ao conteúdo da célula através da indução de uma perca da integridade da membrana plasmática, mas mantendo toda estrutura celular em funcionamento. E deste modo, tornando viável uma caracterização detalhada da mitocôndria em seu ambiente natural por conservar interações essenciais com outras organelas (Kuznetsov et al., 2008). Além disso, estudo *in situ* da mitocôndria requer pequenas quantidades de amostras, células ou embriões para tal finalidade o que se torna particularmente atrativo para espécies como o zebrafish. Desse modo, a presente tese tem por objetivo geral a avaliação da função mitocondrial do zebrafish através de diferentes tecidos permeabilizados (*in situ*).

1.1 OBJETIVOS

1.1.1 Objetivo Geral

Investigar possíveis alterações na bioenergética mitocondrial do zebrafish (*D. rerio*), a partir de tecidos seletivamente permeabilizados, diante comparações entre o conhecimento científico acumulado, fisiológicas e de estressores ambientais.

1.1.2 Objetivos Específicos

- Realizar um levantamento bibliográfico sobre a função mitocondrial do zebrafish;
- Verificar o uso do zebrafish como modelo para o estudo da cadeia transportadora de elétrons, de proteínas mitocondriais, da mitocôndria como marcador toxicológico e para geração de EROS;
- Buscar padrões tecido-específicos para bioenergética mitocondrial entre os tecidos neuronal, hepático, muscular cardíaco e muscular esquelético de exemplares machos adultos do zebrafish;
- Identificar uma possível tecido-especificidade para geração de EROS e captação de cálcio entre os tecidos neuronal, hepático, muscular cardíaco e muscular esquelético;
- Averiguar como o piriproxifeno pode comprometer a função neuronal do zebrafish utilizando a bioenergética mitocondrial como tradutor do estado fisiológico;

- Estimar como o piriproxifeno pode comprometer a atividade enzimática da acetilcolinesterase cerebral de exemplares machos adultos do zebrafish

2 REVISÃO DE LITERATURA

2.1 ESTRUTURA E FUNÇÃO MITOCONDRIAL

As mitocôndrias são organelas celulares de diâmetro entre 0,5 e 1µm e de 6 e 10 µm de comprimento a depender da espécie, tecido ou órgão e função fisiológica. Cada mitocôndria é estruturada por duas membranas, uma externa (permeável a todas as moléculas de 5.000 D. ou menos) e uma interna (**Figura 1**) onde localiza-se a CTE que é formada por complexos multienzimáticos e diversas proteínas. Após estas duas membranas há um espaço comumente denominado de matriz mitocondrial e é nele que as mitocôndrias conduzem os processos de oxidação na CTE, a ß-oxidação de ácidos graxos e o ciclo do ácido cítrico, mais conhecido como Ciclo de Krebs, que recebe esse nome devido à Hans Adolf Krebs que o descreveu em 1937 (Krebs and Johnson, 1937). Além disso é na matriz mitocondrial que as mitocôndrias realizam a síntese de proteínas, geração e detoxificação de espécies reativas de oxigênio (EROS) e nitrogênio (ERNôs), além de suas próprias transcrições e replicações de DNA.

Uma característica peculiar destas organelas é a presença de cristas mitocondriais, que são um sistema de microvilosidades que se originam na membrana interna e atravessam a largura da organela (**Figura 2a, 2b e 2c**). A principal função para estas microvilosidades é aumentar a área interna da mitocôndria, aumentando, assim, a quantidade de CTEs e de ATPôs sintase (**Figura 2d**). A atividade da CTE é subsidiada pelas coenzimas NADH e $FADH_2$ formadas, principalmente, durante a oxidação de ácidos graxos, carboidratos e aminoácidos. Na CTE os componentes químicos (qpH) e os componentes elétricos (q=) mitocondriais são gerados. Para isso, um eficiente fluxo de prótons H^+ gera uma poderosa força próton-motriz para o espaço intermembrana através dos complexos enzimáticos da CTE (**Figura 3**).

O potencial eletroquímico gerado (qpH + q= = qpµH^+) durante esse fluxo de prótons possibilita que a ATP sintase (**Figura 2d**) desempenhe sua função mais notável, a produção de ATP via fosforilação oxidativa, sendo esta a teoria quimiosmótica proposta por (Mitchell, 1966). Este é um dos processos vitais mais sofisticados, onde a energia química pode ser convertida em energia biológica. Interessantemente, esta produção de energia biológica é análoga entre células animais e vegetais. Portanto, o gradiente eletroquímico

transmembrânico de prótons da CTE e de seus complexos respiratórios caracteriza-se como artefato chave para o metabolismo eucariótico[1].

Figura 1: Representação esquemática de uma mitocôndria típica e da partícula submitocondrial. P e N se referem aos compartimentos positivo e negativo, respectivamente. Observe que as formas das cristas mitocondriais são altamente variáveis e que a comunicação entre as cristas e o espaço intermembrana pode ser restrita. A variedade de morfologias mitocondriais é consideravelmente mais complexa. (Traduzido de Nicholls & Ferguson, 2013).

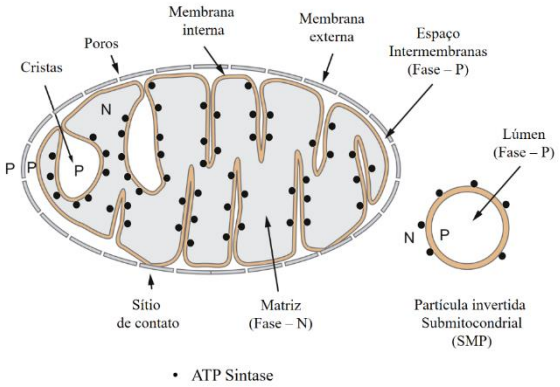
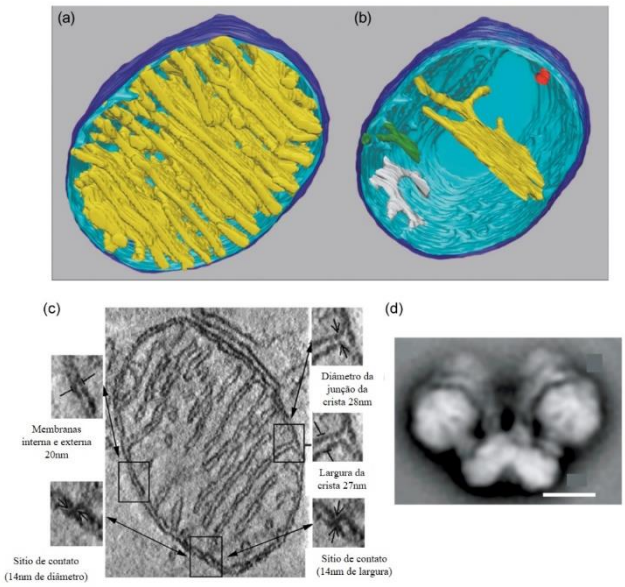
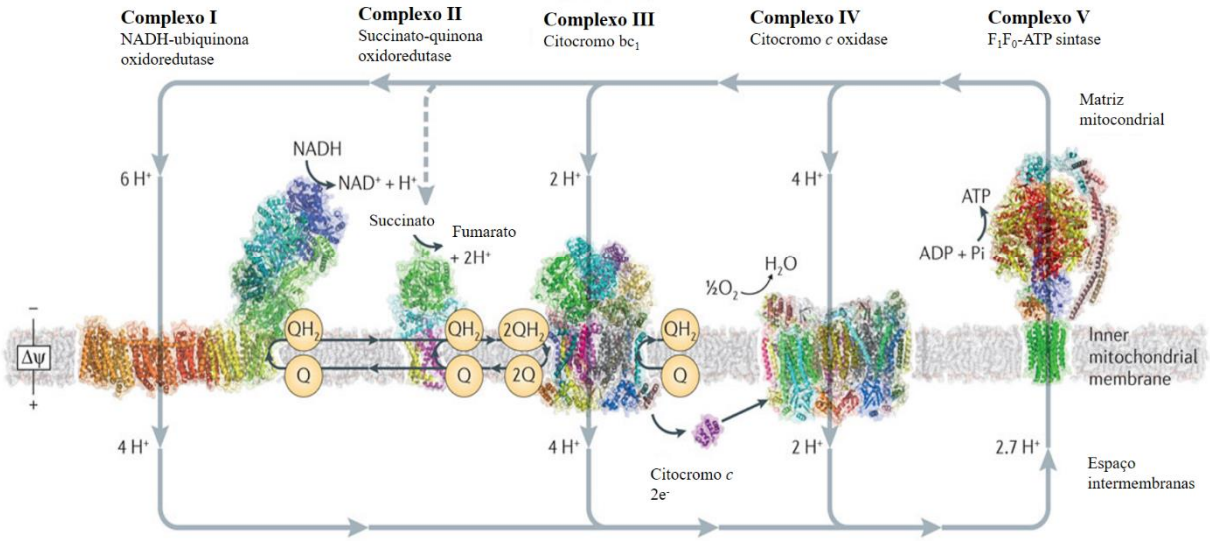


Figura 2: Ultraestrutura mitocondrial. (a) Tomografia eletrônica tridimensional de uma mitocôndria do tecido adiposo marrom. As cristas mitocondriais são mostradas em amarelo, o limite da membrana mitocondrial interna é mostrado em azul claro e a membrana mitocondrial externa é mostrada em azul escuro. (b) Representação de quatro cristas mitocondriais mostradas em cores diferentes. (c) Seção única através da mesma mitocôndria mostrando dimensões representativas das principais características estruturais. Reproduzida no livro Bioenergetics com autorização de Frey e Mannela (2000). (d) Coloração negativa da vista lateral da estrutura dimérica da ATP sintase. Reproduzida no livro Bioenergetics com autorização de Dudkina et al. (2010) (Traduzido de Nicholls & Ferguson, 2013).



[1]Evidencias recentes demonstram que eucariotos como os *Monocercomonoides sp*não possuem mitocôndrias, provavelmente como estratégia evolutiva.Karnkowska, A., V. Vacek, Z. Zubacova, S. C. Treitli, R. Petrzelkova, L. Eme, L. Novak, V. Zarsky, L. D. Barlow, E. K. Herman, P. Soukal, M. Hroudova, P. Dolezal, C. W. Stairs, A. J. Roger, M. Elias, J. B. Dacks, C. Vlcek, and V. Hampl, 2016, A Eukaryote without a Mitochondrial Organelle: Current Biology, v. 26, p. 1274-1284..

Figura 3: Cadeia transportadora de elétrons mitocondrial. A Cadeia Transportadora de Elétrons (CTE) da mitocôndria é formada por complexos enzimáticos bombeadores de prótons. O Complexo I (NADH-ubiquinona oxidoredutase). O Complexo II (Succinato oxidoredutase). O Complexo III (Citocromo bc_1) e o Complexo IV (Citocromo c oxidase). Estes complexos geram uma força próton motriz que conduz F_1F_0-ATP sintase. O transporte de elétrons entre os complexos é mediado pela Ubiquinona Q da membrana mitocondrial interna e pelo Citocromo c solúvel. O complexo 1 é o ponto de entrada para os elétrons do NADH que são utilizados para reduzir Q a Ubiquinol (QH_2). O QH_2 é posteriormente utilizado pelo complexo III para reduzir o citocromo c no espaço intermembrana. O complexo IV utiliza o citocromo C para reduzir o oxigênio molecular e é o último receptor de elétrons da CTE. Para cada molécula de NADH oxidada, 10 H^+ são translocados através da membrana da matriz para o espaço intermembrana. O Complexo II (Succinato oxidoredutase) proporciona um ponto de entrada adicional para os elétrons na CTE. (Traduzido de (Sazanov, 2015).



O complexo I, também conhecido como NADH desidrogenase ou NADH-ubiquinona oxidoredutase, é o único complexo enzimático da cadeia respiratória que catalisa a oxidação da matriz de NADH. Para cada NADH oxidado o complexo I bombeia $4H^+$ para o espaço intermembrana, estes elétrons são utilizados pela forma oxidada da coenzima Q (UQ) gerando a forma reduzida (UQH_2). Além disso, o complexo I da mitocôndria, que é amplamente reconhecido como um importante sitio para geração de EROs, recentemente tornou-se alvo para intervenção terapêutica contra infarto ou acidentes vasculares por realizar um processo delicado de ativação (A) e inativação (D do inglês *Dormant form*) em órgãos como coração e cérebro (Galkin and Moncada, 2017). Esse processo de transição A/D da NADH-ubiquinona oxiredutase é um importante mecanismo regulador da fosforilação oxidativa durante eventos de isquemia ou hipóxia.

O complexo II, ou Succinato-quinona oxidoredutase, possui quatro subunidades proteicas e um cofator, o dinucleotídeo de flavina-adenina (FAD). O complexo II é o único

que participa do ciclo de Krebs e da CTE, além de também poder contribuir para geração de EROS (Grivennikova et al., 2017). Recentemente, foi demostrado que o fluxo de elétrons entre o Complexo I e II da CTE pode ser severamente comprometido durante o câncer de pele aumentando a susceptibilidade para o estresse oxidativo mitocondrial (Mori et al., 2017) e que este complexo, é o principal complexo envolvido na reprogramação metabólica e adaptação respiratória diante estímulos e anormalidades intrínsecas ou extrínsecas como hipóxia, por exemplo. (Bezawork-Geleta et al., 2017). Além disso, pode haver um transporte reverso de elétrons do Complexo II para o Complexo I reduzindo NAD+ em NADH. Este processo gera quantidades significativas de EROS e aparenta possuir papel importante para saúde ou estabelecimento de condições patológicas (Scialò et al., 2017). O complexo III, por sua vez, é reconhecido por ser uma das principais fontes de superóxido ($O_{2}^{\cdot-}$) pela mitocondria. Já o complexo IV, ou citocromo c oxidase, encerra o transporte de elétrons através da CTE transferindo-os para o O_2 que posteriormente é reduzido a H_2O.

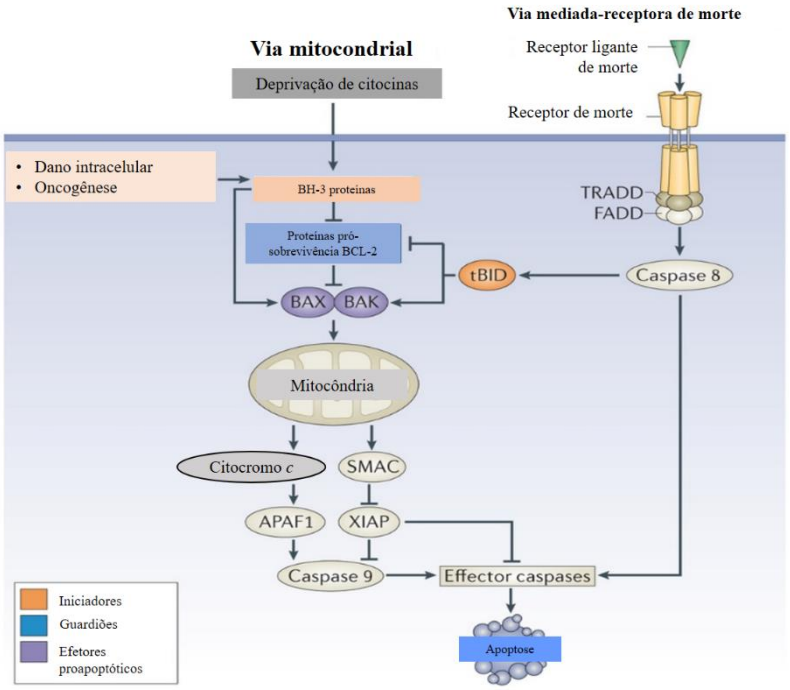
A ATP sintase utiliza a energia acumulada através do gradiente de prótons da CTE para realizar a síntese de ATP a partir ADP e do fosfato inorgânico (P_i). É importante destacar que a ATP sintase não é um componente da CTE (**Figura 3**) e pode funcionar isoladamente desde que haja H^+ no espaço intermembrana. A ATP sintase é formada por duas subunidades, uma F_O altamente hidrofóbica e mergulhada na membrana mitocondrial interna, e uma F_1 solúvel que é localizada na matriz mitocondrial. A fosforilação do ADP que ocorre na ATP sintase se dá a favor do gradiente de prótons (diferentemente do que ocorre na CTE onde os prótons são transportados contra o gradiente) através das subunidades F_O F_1.

Interessantemente, a eficiência e a taxa de produção de ATP pela mitocôndria se adapta à demanda energética específica de cada tecido, dependendo do conteúdo mitocondrial, quantidade de cadeias respiratórias e atividade intrínseca (Benard et al., 2006). Isto sugere que a mitocôndria é, também, uma organela de ampla plasticidade fisiológica. Outro fator que relaciona as especificidades na função mitocondrial com sua característica tecido-específica são os eventos de fusão e fissão mitocondrial. Estes fenômenos de regulação são induzidos por mudanças na disponibilidade de nutrientes ou demandas metabólicas, inclusive durante processos de doenças (van der Bliek et al., 2013; Wai and Langer, 2016). Portanto, o maquinário envolvido nos processos de fusão e fissão mitocondrial também é essencial para sobrevivência e adaptação da célula.

Deste modo é possível inferir que, além de atuar na bioenergética, a função ou disfunção mitocondrial participa ativamente de muito processos celulares. Dentre eles,

processos que regulam a sobrevivência da célula e sua morte programada por necrose, apoptose ou autofagia (Dhillon and Fenech, 2014; Lee et al., 2012; Martinou and Youle, 2011). Estes mecanismos ocorrem através de sinalizadores da família Bax e Bcl-2 que regulam parcialmente a permeabilidade da membrana externa da mitocôndria permitindo a liberação de estímulos para ativação de caspases no citosol. O mecanismo de ação das Bax e Bcl-2 envolvem a regulação de fatores considerados como pró- ou anti- apoptóticos através de uma cascata de sinais celulares (**Figura 4**). É importante destacar que a composição lipídica e a organização estrutural da membrana externa mitocondrial tem papel central na regulação da atividade da Bcl-2 (Martinou and Youle, 2011).

Figura 4: Receptores de morte celular e mitocondrial que regulam as vias para apoptose. Diversos estímulos citosólicos, incluindo estressores oncogênicos e agentes quimioterápicos, bem como vias de desenvolvimento, ativam o sinal mitocondrial que é regulado por membros da família Bcl-2. Estes estímulos ativam os membros da família BH3 (iniciadores) que inibem as proteínas pro-sobrevivência como as Bcl-2 (guardiãs) permitindo, assim, a ativação dos efetores proapoptóticos BAX e BAK que destroem a membrana mitocondrial externa. A liberação do citocromo C da mitocôndria promove a ativação da caspase 9 e da proteína APAF1 (Fator 1 de ativação de protease apoptótica) enquanto ocorre a liberação da proteína SMAC (Segundo derivado mitocondrial ativador de caspases) que bloqueiam o inibidor de caspases XIAP (Proteína Inibidora de Apoptose X). A via mediada pelo receptor de morte celular (ou extrínseca) da apoptose é ativada quando ocorre a ligação com ligantes da família do fator de necrose tumoral (TNF) (Como o ligante FAS ou TNF) envolvem seus receptores cognitivos de morte (FAS eTNFR1, respectivamente) na membrana plasmática levando a ativação da caspase 8 através da FAS-domínio proteico associado a morte (FADD) e TNRF-domínio proteico associado a morte (TRADD). O TRADD é necessário para indução da apoptose por alguns receptores de morte celular (como o TNRF1), as não por outros (como a FAS). As duas vias convergem para ativação de caspases efetoras (caspase 3, caspase 7 e caspase 6). Além disso, a forma clivada da BID (tBID) que é gerada pela proteólise mediada pela caspase 8 pode ativar a via mitocondrial para amplificar a resposta apoptótica. Esse mecanismo de amplificação é necessário para apoptose em certos tipos celulares (chamadas de células do ñtipo 2ò) como os hepatócitos, mas não para as células do ñtipo 1ò, como os timócitos. Os níveis de XIAP distinguem esses dois tipos celulares, sendo esses níveis maiores em células do tipo 2. (Traduzido de (Czabotar et al., 2014).



Uma vez que a mitocôndria contribui para homeostase celular, sua participação na miríade de condições fisiopatológicas que acometem a célula também pode ser investigada. A relação entre a disfunção mitocondrial e o envelhecimento celular, por exemplo, é rotineiramente revisada. Onde a organela em questão é acusada de ser uma reguladora chave para longevidade por modular a bioenergética celular de modo desigual a depender da idade fisiológica (Bolaños et al., 2016; Bratic and Trifunovic, 2010).

Além disso, a disfunção mitocondrial é amplamente associada a desordens neurológicas como Doença de Parkinson, Alzheimer, Huntington e Esclerose Lateral Amiotrófica (Dhillon and Fenech, 2014). Ou patologias como Dislipidemias, Desordens nutricionais e Doenças do Sistema Cardiovascular (Hiramitsu et al., 2014; Pinho et al., 2016; Vercesi et al., 2006; Walters et al., 2016). Notadamente, todas estas fisiopatologias são positivamente correlacionadas com um aumento na geração de EROS e essa condição possui um padrão tecido-específico (Tahara et al., 2009).

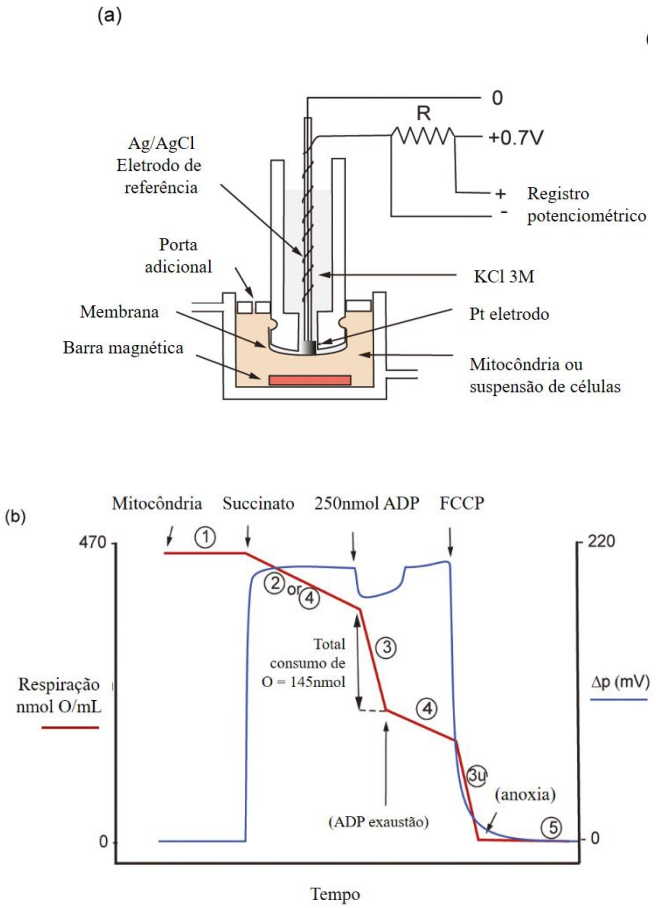
2.2 AVALIAÇÕES BIOENERGÉTICAS EM TECIDOS PERMEABILIZADOS

Uma vez comprovada que a função ou disfunção mitocondrial afeta a homeostase celular, diversas técnicas são empregadas para estimar o status mitocondrial. Os procedimentos padrão utilizados para estudar a mitocôndria utilizam, principalmente, abordagens *in vitro* ou *in vivo.* Os estudos da mitocôndria *in vitro* baseiam-se no isolamento da mitocôndria dos demais componentes celulares por centrifugação diferencial seguida por análise através de eletrodos específicos, por exemplo, o oxigrafo Clark (**Figura 5a**), que possibilita o estudo etapa por etapa da respiração mitocondrial e fosforilação oxidativa (**Figura 5b**). Esse processo vem sendo realizado com sucesso há mais de 40 anos e fornece uma base sólida de conhecimento sobre a função mitocondrial. Já os estudos *in vivo* são mais recentes e utilizam estímulo e monitoramento a embriões ou culturas de células por espectroscopia e diversas técnicas fluorescentes (Kuznetsov et al., 2008).

Contudo, ambos métodos (*in vivo* ou *in vitro*) apresentam desvantagens, como a exigência de altas concentrações de substratos ou o comprometimento de algumas propriedades da organela, a exemplo, indução da Transição de Permeabilidade Mitocondrial (TPM), ruptura das interações normais da mitocôndria com outras organelas, além de limitar a análise de efetores na função mitocondrial (Kuznetsov et al., 2008). Como demonstrado recentemente por (Dos Santos et al., 2013) ao comparar a função mitocondrial em fibras

musculares permeabilizadas e mitocôndrias isoladas do *goldfish*, os resultados da pesquisa mostraram que as fibras musculares permeabilizadas traduzem melhor o estado fisiológico do animal. Isso se torna particularmente importante quando a mitocôndria é isolada de tecidos já prejudicados por processos patológicos e pela geração de EROS (Figueira et al., 2013; Saks et al., 1998) a depender do tipo da célula e/ou tecido que a mitocôndria é isolada.

Figura 5: Eletrodo de O_2 do tipo Clark. (a) O O_2 é convertido em H_2O no eletrodo pt que é mantido 0,7V negativos com respeito ao Ag/AgCl eletrodo de referência e uma corrente que é proporcional à concentração de O_2 no meio. Uma fina membrana permeável evita que a incubação faça contato direto com o eletrodo. Como o eletrodo consome lentamente O_2, a incubação deve ser continuamente agitada para evitar que uma camada de depleção se forme na membrana. A câmara é selada, exceto por uma pequena abertura superior. O eletrodo é calibrado com um meio saturado de ar e sob condições anóxicas, após a adição de ditionito de sódio. (b) Neste experimento, a mitocôndria foi adicionada a câmara do eletrodo, seguida pela adição de Succinato como substrato. Há a presença de P_i. A respiração é lenta porque o circuito do próton não é completo pela reentrada de H^+ através da ATP sintase. Para que haja qualquer respiração é necessária a uma liberação de prótons através da membrana. Uma quantidade limitada de ADP é adicionada, permitindo que a ATP sintase sintetizasse ATP acoplada a liberação de prótons através da membrana, ñestado 3ò. Se a quantidade de ADP é conhecida, o consumo de oxigênio durante a aceleração do estado 3 da respiração pode ser quantificado, permitindo que uma razão P/O seja calculada (moles ATP sintetizado por mole Oxigênio). Porque a fuga de prótons é fortemente reduzida no estado 3, quase toda a captação de oxigênio durante o estado 3 é efetivamente utilizada para a síntese de ATP. Neste exemplo, a razão ADP/O para o substrato foi de 250/145=1,72. Observe a convenção bioenergética de se referir a "O" (i.e., ½ O_2), que é equivalente a 2eĖ Além disso, a respiração controlada, antes da adição de ADP, que é estritamente denominado "estado 2" é funcionalmente semelhante ao estado 4, e este último termo é normalmente utilizado para ambos os estados. O traçado azul se refere aos valores da *qp* durante o experimento. Traduzido de Nicholls & Ferguson, 2013.



O estudo da função mitocondrial *in situ* tem minimizado estes problemas por induzir a perda da integridade da membrana celular sem afetar funções celulares ou comprometer demais organelas (**Figura 6**). Deste modo, a mitocôndria se mantém intacta e acoplada, conservando suas interações com outras estruturas celulares (Saks et al., 1998).

Além disso, o método *in situ* permite a análise da mitocôndria em seu ambiente natural e reduz os efeitos deletérios dos métodos *in vivo* ou *in vitro*. Em contramão, as principais desvantagens dos métodos *in situ* são: o tempo de análise, que normalmente são mais longos em comparação a mitocôndrias isoladas, não distinção de diferentes subpopulações mitocondriais e a não viabilidade para análise de fatores citosólicos na mitocôndria (Kuznetsov et al., 2008). Além da impossibilidade de acesso ao metabolismo oxidativo caso a organela esteja envolvida em um processo de hibernação (Mathers and Staples, 2015).

Todavia, retomando as vantagens dos métodos *in situ*, outra propriedade muito importante destes métodos é que não são necessárias elevadas quantidades de tecidos para o estudo da mitocôndria. Esse aspecto torna-se altamente atrativo em estudos com culturas de células, animais transgênicos, em risco de extinção ou pequenos, dada a relativa escassez de amostras biológicas. Outra característica de muita valia é que fibras musculares permeabilizadas apresentam estabilidade mitocondrial por até 24 horas sob baixa temperatura[2](Kuznetsov et al., 2008; Trumbeckaite et al., 2001). Característica que amplia significativamente as possibilidades de estudo da função mitocondrial, uma vez que, através dos métodos tradicionais, as análises precisam ser realizadas quase que imediatamente após o isolamento.

Este processo de permeabilização seletiva normalmente é realizado utilizando detergentes de baixa carga iônica como saponina ou digitonina ou ainda, filipina, Ŭ-solanina ou Ŭ-tomatina. Além disso, técnicas mais sofisticadas como a permeabilização através de isótopos estáveis também podem ser empregadas (Nonnenmacher et al., 2016).

A saponina (**Figura 7A**) é um glicosídeo natural com propriedades de detergentes e surfactantes. As saponinas podem ser classificadas em esteroidais ou triterpênicas a depender da quantidade de carbonos que integra a molécula da saponina (Sudji et al., 2015).

[2]Experimento realizado com fibras musculares de coelho.

A digitonina (**Figura 7B**) também é um glicosídeo e é classificada como uma saponina esteroide. Ambos permeabilizantes possuem afinidade pelas porções polar formadas por colesterol na membrana celular levando a vesiculação e formação de poros na membrana celular (Sudji et al., 2015).

Figura 6: Esquema mostrando o princípio da análise da mitocôndria *in situ* pela permeabilização seletiva da membrana plasmática. Células antes (A) e depois do tratamento com agentes que formam complexos com colesterol da membrana celular (B). Os agentes que formam complexos com colesterol da membrana celular como saponina e digitonina interagem com as moléculas de colesterol que estão abundantemente presentes na membrana plasmática (As cabeças polares do colesterol estão associadas com as cabeças polares dos fosfolípides). Essa interação induz a perda na integridade da membrana (permeabilização) de modo que a barreira entre o espaço intracelular e o meio extracelular desaparece. O citosol, incluindo todos os seus solutos, são carreados para fora e a composição do espaço intracelular é equilibrada com o meio de incubação experimental. O conteúdo do colesterol das organelas intracelulares ou estruturas de membrana como a mitocôndria ou retículo endoplasmático é consideravelmente inferior ao encontrado nos agentes que formam complexos com colesterol da membrana celular assim as concentrações utilizadas para permeabilização da membrana celular perturba essas organelas permitindo analisar suas interações funcionais com a mitocôndria. Traduzido de Kuznetsov et al. 2008.

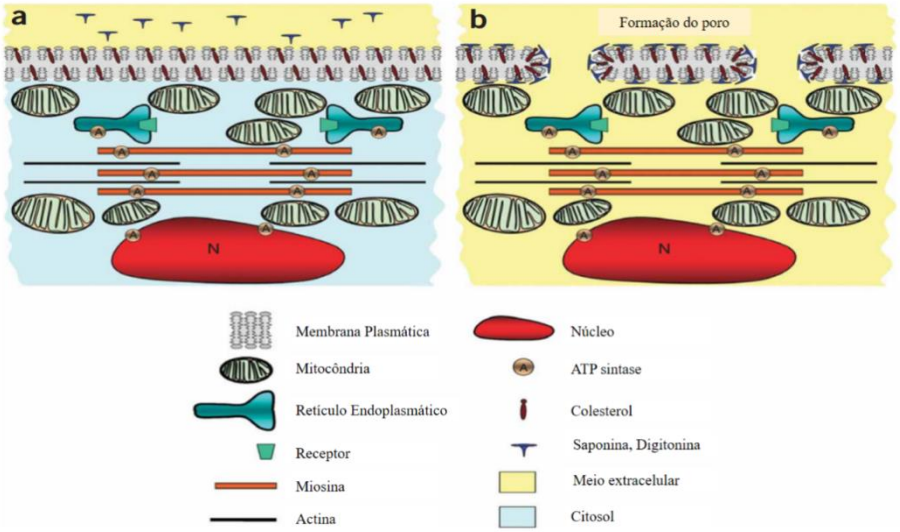
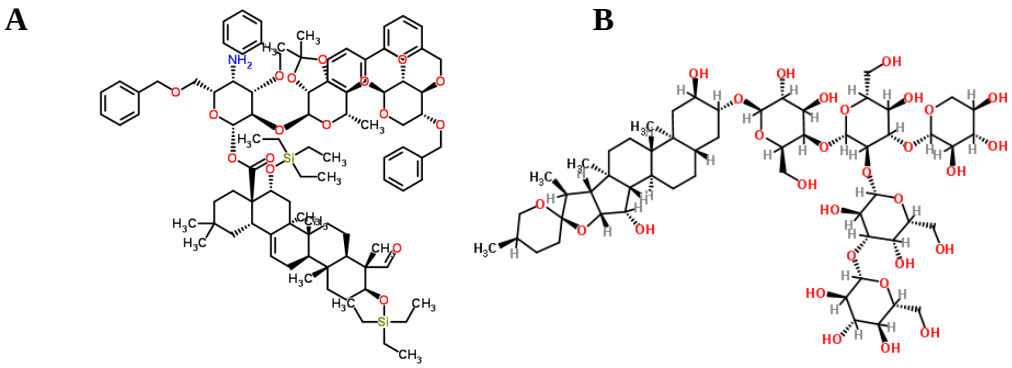


Figura 7: Estruturas químicas dos glicosídeos esteroidais saponina e digitonina. (A) Saponina e (B) Digitonina.



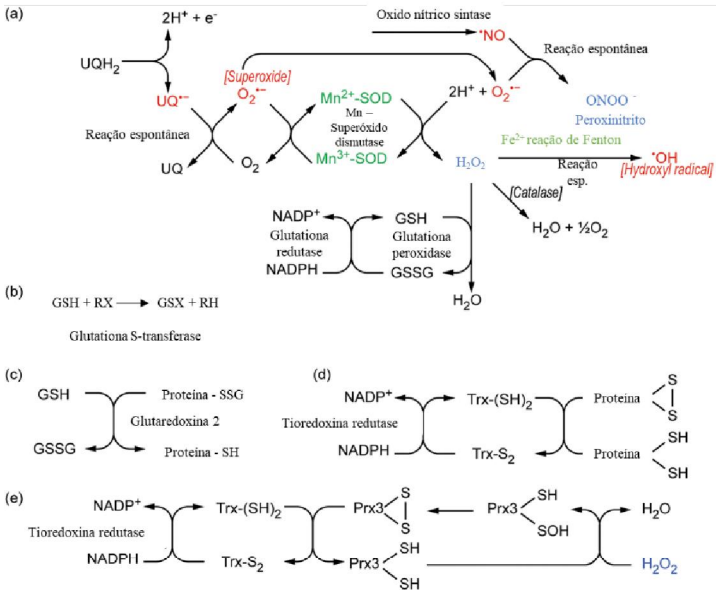
Fonte: (A) www.chemspider.com/ImageView.aspxid=58190622 (B) www.chemspider.com/ImageView.aspxid=4976216

2.3 PRODUÇÃO DE EROS E ERNôS PELA MITOCÔNDRIA

Interessantemente, a função mitocondrial localiza-se em uma linha tênue entre a regulação de processo fisiológicos, o fornecimento de energia biológica via ATP, que é essencial, e o desencadeamento de diversos processos patológicos a partir do estresse oxidativo induzido por espécies reativas de oxigênio e/ou nitrogênio. Entretanto, é importante ressaltar que o mecanismo de produção destas EROS é fisiológico e somente quando o balanço entre sua produção e degradação encontra-se desequilibrada é que as EROS tornam-se prejudiciais (Kowaltowski et al., 2009). Para isso, a mitocôndria conta com um poderoso sistema redox formado por enzimas como a Superóxido Dismutase (MnSOD), Catalase, Glutationa Peroxidase (GPx), Glutationa S-transferase (GST), Tiorredoxina Peroxidase Mitocondrial (mTPxI) e um sistema não enzimático através das glutationas.

A maior parte de EROS é gerada na CTE durante a oxidação de nutrientes à oxigênio molecular (**Figura 8**). Estima-se que 1-2% do oxigênio consumido durante a respiração fisiológica é convertido em superóxido ($O_2^{\bullet-}$) (Boveris and Chance, 1973). Portanto, é possível inferir que o oxigênio é fundamental para fosforilação oxidativa, mas potencialmente reativo para célula. Sobre isso, cada complexo da CTE possui suas próprias especificidades e susceptibilidades para geração de EROS e/ou ERNôs.

Figura 8: Geração e detoxificação das EROS mitocondriais. (a) Metabolismo do superóxido gerado pela transferência de 1eÈ da ubisemiquinona. Radicais são mostrados em vermelho e outras espécies reativas em azul. Spont = processo espontâneo. (b-e) metabolismo tiol na mitocôndria. Prx3, peroxirredoxina; Trx, tiorredoxinas. (Traduzido de Nicholls & Ferguson, 2013).



O complexo I possui dois sítios responsáveis pela produção de $O_{2Å}^{\bar{I}}$. Por muito tempo, somente o complexo III era identificado como um potencial gerador de $O_{2Å}^{\bar{I}}$ após o trabalho de Liu et al. 2001 o mononucleotídeo de flavina (FMN) do complexo I também foi identificado como uma potencial fonte de $O_{2Å}^{\bar{I}}$ através da transferência reversa de elétrons do complexo II (Liu et al., 2002). Sendo este processo dependente do gradiente de pH através da membrana mitocondrial interna (Lambert and Brand, 2004) e possuir intíma relação com o estabelecimento de condições fisiopatológicas (Scialò et al., 2017).

Há, ainda, possibilidades de geração de $O_{2Å}^{\bar{I}}$ pelo complexo II e III quando há disfunção na Ubiquinona, onde sua forma reduzida Ubiquinol (UQH_2) passa a doar elétrons ao complexo III possibilitando o surgimento de um ânion $UQ_{Å}^{\bar{I}}$, e este, por sua vez, viabiliza a geração de $O_{2Å}^{\bar{I}}$(Crofts, 2004). Entretanto o conhecimento sobre como os grupamentos heme dos citocromos podem contribuir para essa geração, ou sobre como mediadores redox podem ajustar esse processo, ainda permanecem evasivos (Figueira et al., 2013).

Já o complexo II, por muito tempo não era associado a geração de espécies reativas. Entretanto, após a identificação de que mutações genéticas levam a alterações estruturais na Succinato Desidrogenase, foi possível detectar que essas mutações podem resultar em um aumento expressivo da geração de EROS (Yankovskaya et al., 2003), com isso os conceitos sobre a geração de EROS pelo complexo II passaram a ser revisados. Atualmente a correlação entre estresse oxidativo e mutações no complexo II norteiam diversos questionamentos científicos. Especialmente, sobre as subunidades SdhA, SdhB e SdhD da succcinato desidrogenase que relacionam a oxidação do Succinato, a geração de EROS e suas contribuições para formação de tumores (Iverson et al., 2012). Além disso, uma inibição no Complexo II aumenta a geração de $O2_{Å}^{\bar{}}$pelo Ubiquinol do Complexo III (Drose et al., 2009).

Sobre isso, o complexo III é um sítio reconhecido de geração de $O_{2Å}^{\bar{}}$ e é considerado o principal *loci* de geração de espécies reativas na mitocôndria (Chen et al., 2003; West et al., 2011) uma vez que esse processo ocorre normalmente durante a oxidação de substratos do complexo I. Além disso, foi identificado que na presença de uma ubiquinona oxidada, a formação de $O_{2Å}^{\bar{}}$ no centro do Ubiquinol é estimulada (Drose and Brandt, 2008). Esse dado indica que numa reação reversa o elétron é transferido para o oxigênio pelo citocromo através da ubiquinona e não através da reação do ciclo da ubiquinona. No entanto,

este ponto ainda é matéria de debate, especialmente ao considerar possíveis modificações pós-translacionais (por exemplo, acetilação) das subunidades do complexo III (Bleier and Dröse, 2013). É importante registrar que, sob condições patológicas, a Ubiquinona pode aumentar ainda mais geração de superóxido (Crofts, 2004).

O complexo IV contribui para geração de EROS quando há inibição do seu funcionamento por monóxido de carbono, sulfeto de hidrogênio, cianeto de hidrogênio ou pelo oxido nítrico (NO) (Cooper and Brown, 2008). No que concerne à ação do NO˙ neste processo, é sabido que a inibição na síntese de NO˙ leva a um estímulo na respiração celular. Devido a isso, foi hipotetizado que o NO˙ regulasse a respiração celular e funções celulares através da inibição do complexo IV (Brown, 1995). Essas questões foram solucionadas após a identificação que altas concentrações de NO˙ e seus derivados (peroxinitrito, dióxido de nitrogênio e nitrotirosinas) causam inibição irreversível da CTE, desacoplamento mitocondrial, transição de permeabilidade e morte celular (Brown, 2001). Além disso, mutações em várias subunidades da citocromo oxidase são correlacionadas a encefalopatia, acidose e falha hepática (Diaz, 2010). Atualmente sabe-se que o NO˙ pode, através da S-nitrosilação de tiós proteicos de membrana, inibir a transição de permeabilidade e regular o metabolismo de ácidos graxos (Doulias et al., 2013; Leite et al., 2010). Além disso, mudanças nas concentrações estáveis de NO˙ podem estar envolvidas em vias de sinalização para biogênese mitocondrial em ratos diabéticos (Bombicino et al., 2017). E que a atividade a oxido nítrico sintase regula mecanismos mitocondriais homeostáticos chave para o desenvolvimento de fibras musculares (De Palma et al., 2014). Deste modo é possível inferir que diversas questões sobre o delicado papel do NO˙ na mitocôndria ainda continuam em aberto e necessitam de esclarecimentos.

Embora a geração das espécies reativas pela mitocôndria seja um mecanismo essencial para o metabolismo dada sua regulação de muitos processos celulares (Figueira et al., 2013), como citado anteriormente, diversos efeitos fisiológicos deletérios podem ser observados após a disfunção mitocondrial induzida por EROS e ERNôs, a exemplo: doenças neurodegenerativas, modificações pós-translacionais em proteínas, arteriosclerose e até mesmo o envelhecimento (Dorighello et al., 2017; Lee et al., 2012; Leite et al., 2010).

Dentre as ERNôs, o óxido nítrico é amplamente estudado por contribuir para o balanço oxidativo e para o dano a proteínas, além de ser fundamental para uma série de processos fisiológicos como vasodilatação, resposta imune e neurotransmissão (Ignarro et al., 1999). A óxido nítrico sintase (NOS) é a enzima responsável pela síntese do NO˙ Atualmente

são descritas três classes de NOS. A primeira é responsável por sintetizar a isoforma 1 chamada de nNOS (óxido nítrico neuronal).A segunda é responsável por sintetizar as isoformas 2 denominada eNOS (óxido nítrico endotelial), ambas são constitutivas (cNOS) e Ca^{2+}dependentes. A terceira isoformas, a iNOS, é indutiva e normalmente é induzida por processos inflamatórios. A existência de uma NOS mitocondrial (mtNOS) passou por profundas discussões. Muitos dos estudos que desconsideravam a existência da mtNOS falhavam na preparação das amostras para ensaios bioquímicos ou proteômicos, ou continham contaminantes celulares nos isolados de mitocôndrias. A existência da mtNOS foi comprovada por Guilivi et al. 1998 (Giulivi et al., 1998) a partir disto a mtNOS adquiriu elevada importância por possibilitar caminhos para o estudo de como o NOÅe suas formas oxidantes podem afetar a mitocôndria.

Atualmente sabe-se que os complexos respiratórios III e IV têm capacidade de metabolizar NO_2 ;e NOÅ(Basu et al., 2008) através da ação de nitrito redutases, além disso, o NOÅtem capacidade de inibir parcialmente o funcionamento do complexo IV (**Figura 9**) competindo pelo O_2 durante a respiração ou pelo processo de S-nitrosilação de grupos tióis na mitocôndria. Sendo este um dos efeitos mais reconhecidos do NOÅna mitocôndria. Contudo, outros efeitos também podem ser observados em outros locais da CTE. A exemplo da S-nitrosilação, que é uma modificação nos tióis do complexo 1 induzida pelo NOÅ A formação de S-nitrosotióis proteicos na membrana tem elevada importância para evitar a Transição de Permeabilidade Mitocondrial (TPM) (Leite et al., 2010), o que demonstra a importância desse processo para homeostase mitocondrial e celular. Deste modo, a interação biológica do NOÅ torna-se um importante marcador para doenças cardiovasculares, processos inflamatórios e neurodegeneração (Hensley et al., 1998; Kaur and Halliwell, 1994). Para isso, o NOÅreage com superóxido gerando peroxinitrito, este por sua vez, pode ser decomposto em compostos nitro-aromáticos que são utilizados como marcadores do dano oxidatido NOÅdependente (Kaur and Halliwell, 1994). A 3-nitrotirosina é um destes marcadores, contudo a relevância de nitração de tirosinas de proteínas, tanto como ñmarcadoresò ou ñmediadoresò do estresse nitrosativo, requer consideração de aspectos quantitativos, compartimentalização, reatividade e propriedades de difusão das espécies reativas de nitrogênio (ERN) envolvidas, bem como a presença ou ausência de antioxidantes e/ou redutores endógenos (Leite, 2010). Sobre isso, sabe-se que a nitração de tirosinas possui sítio especificidade e gera efeitos na estrutura e função de proteínas, sendo essas características de relevância para diversas doenças humanas

(Batthyany et al., 2017). Sendo, inclusive, utilizado como marcadores doenças cardiovasculares e arteriosclerose (Radi et al., 2002; Thomson, 2015).

O peroxinitrito (ONOO $^{-}$) é derivado de uma reação entre O_2Å^{-} e NOÅ e é considerada a mais lesiva ERN. Afinal, a formação do ONOO^{-} é favorecida dentro da célula porque a constante de reação do O2Å com o NOÅ(0,7-1,9x10^{10} $M^{-1}s^{-1}$) é maior que do O_2Å^{-} com a superóxido dismutase (SOD, 1-2x10^{9} $M^{-1}s^{-1}$) (Leite, 2010) havendo participação de vários sítios da cadeia respiratória nesse processo. Foi identificado recentemente que uma redução na ação da NADPH 2 Oxidase inibe a formação de ONOO^{-} (Zielonka et al., 2016).

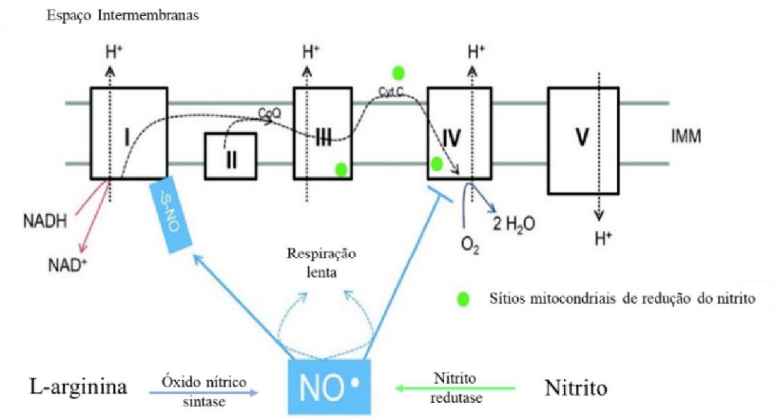
Uma vez produzido, ONOO $^{-}$ é altamente oxidante e conduz ao dano oxidativo por afetar a fosforilação oxidativa inibindo o funcionamento do complexo I, IV (aumentando a K*m* por oxigênio da Citocromo Oxidase) e da ATP sintase, além de prejudicar a homeostase do cálcio (Brown and Borutaite, 2004; Cooper et al., 2003). Os efeitos fisiológicos registrados dessa inibição têm relação com doenças como Alzheimer, artrite, arteriosclerose, isquemia-reperfusão e degeneração vascular (Federico et al., 2012; Radi et al., 2002; Squadrito and Pryor, 1998). Além de ativar a matriz de metaloproteínases e inativação de antiproteases (Sugiura and Ichinose, 2011) que são fundamentais em respostas a processos inflamatórios.

Esses efeitos adquirem ainda mais importância quando observa-se que o tempo de vida livre do ONOO $^{-}$ varia entre t ½ = 3 -5 ms dentro da mitocôndria (Radi et al., 2002). Assim, mesmo com um tempo de vida muito curto este oxidante tem capacidade de induzir disfunção mitocondrial e levar a uma série de condições patológicas. A exemplo da associação do ONOO $^{-}$ com sulfeto de hidrogênio que leva a formação de um novo doador para NOÅ o *sulfinyl nitrite* (H_2S), que pode tornar-se um mediador de grande importância para química do peroxinitrito após condições inflamatórias (Filipovic et al., 2012). Essa característica possibilita abordagens farmacológicas específicas para este doador do NOÅ contra doenças relacionadas a processos inflamatórios.

O processo de peroxidação lipídica leva ao desgaste dos lipídeos, especialmente na membrana celular, e também é um importante indicativo do dano oxidativo. A peroxidação lipídica pode ocorrer em tecidos, fluidos e lipoproteínas. Os produtos da peroxidação lipídica mais estudados são os hidroperóxidos lipídicos (LOOH), aldeídos (malondialdeído [MDA], hidroxihexenal [HHE] e 4-hidroxinonenal [4-HNE]), os fosfolípides oxidados e os isoprostanos. Sendo estes últimos os principais produtos da peroxidação lipídica. Todos estes

compostos citados anteriormente têm alta capacidade para alterar a permeabilidade e integridade das membranas celulares levando ao dano oxidativo.

Figura 9: Inibição da respiração mitocondrial pelo NO. A inibição da respiração mitocondrial pelo NO ao nível do complexo IV é reversível e competitiva com o oxigênio. Através da S-nitrosilação do complexo I da cadeia transportadora de elétrons é que ocorre a inibição parcial da atividade deste complexo retardando a respiração mitocondrial. Essa inibição pode ter efeitos positivos na homeostase do estado mitocondrial (Traduzido de (Figueira et al., 2013).



Os hidroperóxidos lipídicos são considerados os produtos primários da peroxidação lipídica e podem ser formados após a oxidação de ácidos graxos insaturados. Sua ação oxidante, além de causar danos a proteínas e ao DNA, tem sido relacionada a diversas condições patológicas, como hipertensão, Parkinson e artrite (Altay et al., 2015; de Farias et al., 2016; Miura, 2015; Yavuzer et al., 2016). A formação do reativo $^1O_2$ a partir de LOOH é amplamente estudado dada a elevada citotoxicidade deste oxigênio singleto (Miyamoto and Di Mascio, 2014).

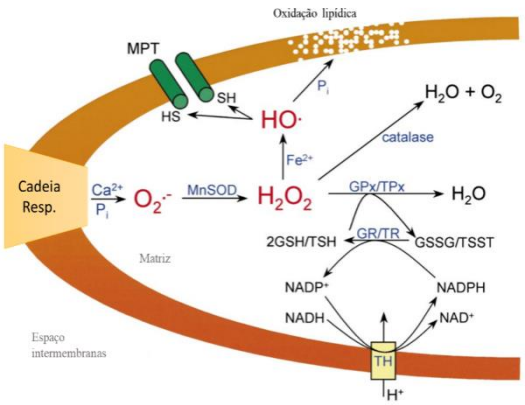
Os aldeídos podem ser associados a danos oxidativo, caso haja falhas nas defesas antioxidantes. A exemplo, o 4-HNE que induz a produção de EROS e formação de defesas antioxidantes (Landar et al., 2006). Através da avaliação das Substancias Reativas ao Ácido Tiobarbitúrico (TBARS) o malondialdeído (MDA) é amplamente estudado como marcador para peroxidação lipídica, principalmente devido ao baixo custo e rapidez desta metodologia. Entretanto, a baixa especificidade e a quantidade de compostos que reagem com o TBA tem minimizado as possibilidades de uso desta metodologia como ferramenta precisa para avaliação da peroxidação lipídica. Portanto, o uso do TBA para estimar a peroxidação lipídica se torna dependente de técnicas para purificação prévia da amostra, por HPLC, por exemplo.

Os isoprostanos, que também são produtos da peroxidação lipídica, podem ser formados após a oxidação de ácidos graxos poli-insaturados como o ácido linoleico, ácido eicosapentaenoico e ácido docosaexaenoico, além do ácido araquidônico. Atualmente é

amplamente aceito que os isoprostanos são os marcadores ideais para peroxidação lipídica (Halliwell and Lee, 2010).

Recentemente, isoprostanos como F2-isoprostano e F4-neuroprostano foram testados como marcadores para peroxidação lipídica no zebrafish (Nik et al., 2014). O estudo demonstrou que existem alterações significativas entre eles, contudo ambos contribuem para peroxidação lipídica no cérebro deste modelo animal. Padrões semelhantes também são observados após a quantificação *in vivo* da produção do F3-isoprostano do *Caenorhabditis elegans* (Labuschagne et al., 2013), o que demonstra como a produção de isoprostanos contribui para o processo de peroxidação lipídica e consequente dano oxidativo, principalmente quando se observa que a produção de EROS pode estar associada a interação de lipídeos com a mitocôndria (Landar et al., 2006). Contudo, existe um fosfolipídio mitocondrial (cardiolipina) que pode ser considerado um marcador de ligação entre os oxidantes gerados pela mitocôndria com a peroxidação lipídica (Figueira et al., 2013). Este fenômeno foi demonstrado recentemente por Lokhmatikov et al 2016 (Lokhmatikov et al., 2016). Além disso, um preciso mecanismo de peroxidação lipídica pode ser induzido diretamente pela mitocôndria quando há presença de concentrações elevadas de Fe^{2+} na matriz mitocondrial. Nestas condições o Fe^{2+} pode reagir com o H_2O_2 (Reação de Fenton) induzindo a peroxidação lipídica (**Figura 10**).

Figura 10: Acúmulo de EROS leva a TPM e peroxidação lipídica. A cadeia respiratória, inserida na membrana mitocondrial interna, gera, constantemente, pequenas quantidades de O_2Å. Esse radical é normalmente removido pela Mn-superóxido dismutase (MnSOD), que promove a geração de H_2O_2. H_2O_2 é reduzido à H_2O pela GPx, TPx ou catalase (em mitocôndrias de coração e fígado). Glutationa (GSH), oxidada pela glutationa peroxidase GPx, e tiorredoxinas (TSH), oxidada pela tiorredoxinas peroxidase (TPx), são reconstituídos pela glutationa e tiorredoxinas redutases (GR e TR), que utilizam o NADPH como doador de elétrons. NADH, que está disponível em quantidades reguladas pela respiração reduz o NADP+, utilizando a NADP transidrogenase (TH). Quando a geração de O_2Å aumenta na presença de Ca2+ e P_i, e/ou as vias de remoção do H_2O_2 estão deficientes, o H_2O_2 acumula e, na presença de Fe^{2+}, gera o radical HOÅ, que é altamente reativo. HOÅ oxida grupamentos tióis (-SH), levando a abertura do poro de transição de permeabilidade. Alternativamente, HOÅ também pode promover a permeabilização da membrana através da oxidação lipídica, um processo que é fortemente estimulado pelo P_i (Traduzido de (Kowaltowski et al., 2001).



A peroxidação lipídica promove o *swelling* mitocondrial, além de permitir a liberação do citocromo C para o citosol e prejudicar a fosforilação oxidativa (Kowaltowski et al., 2001). Portanto, a peroxidação lipídica é um fenômeno irreversível e por isso torna-se potencialmente mais letal que a Transição de Permeabilidade Mitocondrial (TPM).

A TPM é um processo no qual a mitocôndria perde a integridade da membrana externa através de uma permeabilização não seletiva por estímulos externos (por exemplo, Ca^{2+}) ou internos (condições de estresse oxidativo). Interessantemente, a TPM pode ser revertida após seu início (Castilho et al., 1996) desde que detectada com rapidez. Esse processo foi demonstrado inicialmente em corações de ratos sob condições de isquemia na presença de concentrações sub-micromolares de Ciclosporina A (CsA) (Halestrap et al., 1997). Atualmente, a CsA é considerada um inibidor clássico da abertura do Poro de Transição de Permeabilidade Mitocondrial (PTPM), e seus mecanismos de ação envolvem ligações com a enzima ciclofilina-D na membrana mitocondrial interna.

Em síntese reconhece-se que a mitocôndria possui relação direta com estresse oxidativo seja pela geração direta de EROS ou ERNôs, ou por sua afinidade com a peroxidação lipídica, mesmo que em condições fisiológicas normais. Assim sendo, o estresse oxidativo regulado pela mitocôndria adquire caráter multidisciplinar devido às suas relações patológicas nos mais variados sistemas fisiológicos.

2.4 CAPTAÇÃO DE CA^{2+} PELA MITOCÔNDRIA

O estudo do papel do Ca^{2+} como controlador de eventos fisiológicos teve início a mais de um século atrás, na oportunidade, (Ringer, 1883) estudou como componentes inorgânicos podem influenciar a contração ventricular. Anos depois, (DeLuca and Engstrom, 1961) conseguiram demonstrar que mitocôndrias de rins de ratos energizadas podem acumular cálcio, afetando a fosforilação oxidativa.

O influxo de Ca^{2+} do citosol para mitocôndria é relativamente rápido e altamente condicionado ao gradiente eletroquímico da matriz mitocondrial que geralmente é estimado entre 150 e 200mV. Esse influxo é dependente de canais altamente especializados, a exemplo dos Canais Iônicos Dependentes de Voltagem (VDAC). Estes canais são localizados em abundancia na membrana mitocondrial externa e realizam uma rápida captação de cálcio condicionada ao q= . É importante ressaltar que as propriedades voltagem dependentes dos

VDACôs são influenciadas também por interações com proteínas e metabólitos (Rizzuto et al., 2012). Além disso, (Griffiths et al., 1998) propuseram que o Ca^{2+} também pode captado pelos trocadores de Na^{+}-Ca^{2+} através de um mecanismo reverso quando as concentrações de Ca^{2+} no citoplasma forem elevadas. Todavia, os mecanismos de reequilíbrio através dos trocadores de Na^{+}-Ca^{2+} não são tão rápidos (Duchen, 2000), tampouco são encontrados em todos os tipos de células. Assim, cria-se um relativo platô de elevadas concentrações de Ca^{2+} na matriz mitocondrial.

Além dos VDACôs e dos trocadores de Na^{+}-Ca^{2+}, outro canal de Ca^{2+} tem sido extensivamente estudado pela sua elevada seletividade para o transporte de Ca^{2+}. O Canal Mitocondrial Uniporta (MCU) foi caracterizado quanto a sua capacidade cinética e termodinâmica, além de seus possíveis inibidores específicos (Docampo and Vercesi, 1989; Huang et al., 2013; Kirichok et al., 2004). O MCU tem sua função mediada por dois domínios transmembrana de 40kDa cada, sendo estes fundamentais para seu correto funcionamento (De Stefani et al., 2011), inclusive para correta ação de inibidores específicos do MCU, como o Rutênio Red. O nome uniporter é devido à ausência de informações sobre outros íons que podem ser transportados através do MCU.

Uma vez na matriz mitocondrial, o cálcio possui duas vias principais, uma direta para os trocadores de Na^{+}-Ca^{2+} e outra via para as desidrogenases do ciclo de Krebs (Piruvato desidrogenase Ca^{2+} fosfato dependente, Isocitrato desidrogenase e Ŭ-cetoglutarato desidrogenase, ambas dependentes diretas de Ca^{2+}) o que favorece a produção de ATP e leva a um perda no qp= . Esse aumento pode levar a uma desregulação no ciclo de Krebs, que sob condições debilitantes pode favorecer condições celulares patofisiológicas (Duchen, 2000).

Após essa identificação os possíveis efeitos positivos ou negativos da captação de Ca^{2+} foram, e ainda são, alvo de diversas investigações. Dentre elas, a hipótese de que o Ca^{2+} afetasse a respiração celular e o funcionamento da CTE (Lehninger et al., 1978) rapidamente ganhou força e, atualmente, condições fisiopatológicas como Parkinson, Alzheimer, Diabetes, dentre outras, aparentam estar positivamente correlacionadas com alterações da homeostase de Ca^{2+}. Estes fenômenos também estão correlacionados a abertura do Poro de Transição de Permeabilidade (PTP) (**Figura 11**). A abertura do PTP foi considerada, inicialmente, como uma etapa irreversível para apoptose por permitir a liberação do Citocromo c e procaspases para o meio extramitocondrial (Crompton , 1999). Ultimamente é aceito que além da abertura do PTP ser reversível em condições iniciais, o PTP tem papel importante para efluxo de Ca^{2+}, essa noção pode ser de particular relevância em condições patofisiológias (Rizzuto et al.,

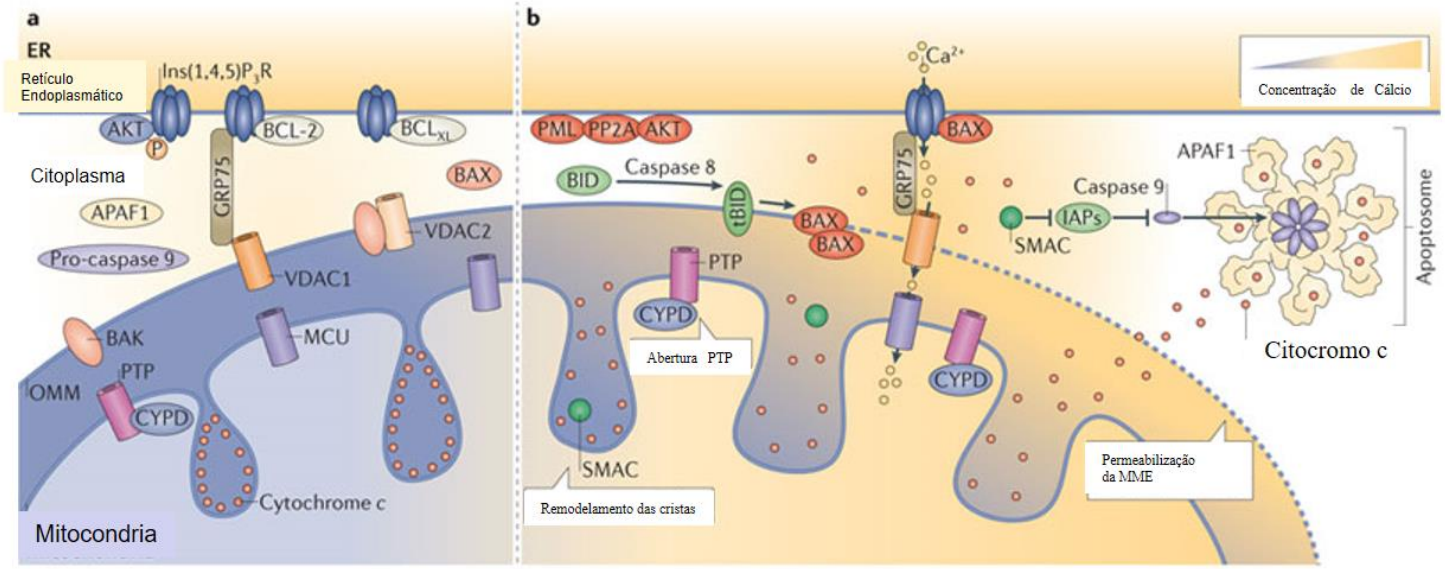
2012). Nestas situações, a abertura prolongada do PTP afeta o qp= conduzindo a uma completa falha na função mitocondrial e consequente morte celular.

Além a mitocôndria, é sabido que o retículo endoplasmático RE também é um importante regulador das concentrações de cálcio no meio intracelular. Havendo, inclusive, uma network altamente especializada entre estas duas organelas. Além dos componentes mitocondriais, trocadores de Na^{+}-Ca^{2+}, VDACôs e MCU, estão envolvidos no tamponamento de Ca^{2+} entre o RE e a mitocôndria uma série de componentes. A exemplo, a GRP75 (75kDa proteína regulada pela glicose), que é uma chaperona que faz uma importante regulação entre a ação dos VDACôs e abertura dos Ins(1,4,5)P_3Rs no RE. O Ins(1,4,5)P_3 é um dos principais canais envolvidos na regulação de Ca^{2+} no RE. Além destes canais, recentemente foi identificado que a Mitofusina 2 (MFN2), que é um componente do maquinário envolvido nos processos de fusão e fissão mitocondrial, está associado com a formação de sítios de contato entre a mitocôndria e o RE (de Brito et al. 2008). O que demonstra que o Ca^{2+} também pode ter envolvimento com processos de fusão e fissão mitocondrial.

Sobre isso, (Rizzuto et al., 1998) hipotetizou que a captação de cálcio mitocondrial era facilitada pela organização estrutural da célula e da organela. Presentemente é aceito que a capacidade de tamponamento mitocondrial contribui para o acúmulo gradual de elevadas concentrações de Ca^{2+} em domínios subcelulares definidos (Rizzuto et al., 2012). O tamponamento espacial realizado pela mitocondria adquire ampla funcionalidade a depender do tipo celular e das exigências fisiológicas de concentrações de Ca^{2+}, a exemplo a neurotransmissão que é dependente de Ca^{2+}.

Em síntese, é consensual que o cálcio é um importante sinalizador intracelular por estar envolvido na regulação de processos moleculares como, por exemplo, a apoptose regulada pela mitocôndria (**Figura 11**), necrose, autofagia, metabolismo, secreção e sinalização celular. Todos estes tópicos foram revisados por (Rizzuto et al., 2012).

Figura 11. Sinais mitocondriais de Ca^{2+} na regulação dos padrões de morte celular. A) Condições que previnem a massiva transferência de Ca^{2+} do retículo endoplasmático (RE) para a mitocôndria através de canais de Ca^{2+} como o Inositol-1,4,5- Trisfosfato (Ins (1,4,5) P3) receptor (Ins (1,4,5) P_3R), Canal Aniônico Voltagem Dependente (VDAC) e mitocondrial Ca^{2+} Uniporter (MCU) que geralmente protegem as células da morte celular por necrose (não mostrada)e apoptose. Essas condições incluem a superexpressão de proteínas anti-apoptóticas como BCL-2 and BCL-X, inibição pelo VDAC2 de proteínas pro-apoptóticas como a BAK ou quinases como a AKT que promovem a sobrevivência celular. Nestas condições pro-sobrevivência o efetor apoptótico caspase 9 permanece em sua forma não clivada de pro-caspase 9, o fator 1 de ativação de protease apoptótica (APAF1) existe na forma inativa e o cofator para caspases, citocromo C, permanece estocado nas cristas da membrana mitocondrial interna. Além disso, a falta das proteínas pro-apoptóticas BAX e BAK (antagonista da BCL-2) resultam na redução na liberação de Ca^{2+} pelo RE. **B)** Seguindo o estímulo apoptótico, o domínio agonista da morte, de interação com BH3 é clivado para forma de BID (tBID). O tBID se liga a membrana mitocondrial externa onde ativa a BAX e a, antagonista a BCL-2, BAK. A BAK ativada neutraliza os efeitos da BCL ï 2 na Ins (1,4,5) P_3R e passa a carrear Ca^{2+} do RE. Todavia BAK e BAK oligomerização promovem a permeabilização da membrana mitocondrial externa. Isso leva a um remodelamento nas cristas da membrana mitocondrial interna e abertura do Poro de Transição de Permeabilidade Mitocondrial (PTP). Além disso, cofatores como o Citocromo c e caspases são liberados paro o citosol e passam a interagir com a proteína citosolica APAF1 para formar o apopsoma. Subsequentemente, as pro-caspases 9 são recrutadas para o apopsoma onde ele é ativado e desencadeia uma cascata proteolítica que, por marcadores específicos, resulta em apoptose. O SMAC- DIABLO é liberado da mitocôndria de uma forma caspase-dependente e promove a apoptose por se ligar ao inibidor de proteínas apoptóticas IAPs permitindo a ativação da pro-caspase 9. Além disso, o pool de células leucemia promeolíticas (PML) localizadas no RE regulam positivamente a apoptose por regular os níveis de fosforilação na Ins (1,4,5) P_3R através da AKT quinase e da proteína fosfatase 2A (PP2A). GRP75, proteína regulada por glicose de 75 kDa; MCU, Mitocondrial Ca^{2+} Uniporta; VDAC, Canal Aniônico Voltagem Dependente; CYPD, ciclofilina D. (Traduzido de (Rizzuto et al., 2012).



2.5 ZEBRAFISH COMO MODELO BIOLÓGICO

O zebrafish (*Danio rerio*) é um pequeno teleósteo originário da Índia. Apresenta dimorfismo sexual, os machos são notadamente mais esguios e escuros que as fêmeas. O animal possui somente uma nadadeira dorsal e duas nadadeiras pélvicas, além da nadadeira caudal. O padrão alternado de listras escuras e claras é marcante em animais selvagens e se estendem até as nadadeiras pélvicas e caudal. Este pequeno peixe emergiu na última década como um modelo experimental alternativo e rapidamente obteve o título de vertebrado canônico devido suas similaridades com a classe *Mammalia*(Rubinstein, 2003). O interesse científico neste pequeno teleósteo adquiriu amplitude a partir da embriologia, especialmente após a descrição de todos os estágios de desenvolvimento embrionário (Kimmel et al., 1995) onde a transparência do embrião, a quantidade de ovos fertilizados durante cada evento reprodutivo, o rápido desenvolvimento e progênese, além da facilidade na manipulação dos embriões, possibilitou o surgimento de uma ferramenta poderosa para replicação de experimentos em condições laboratoriais.

A similaridade de, aproximadamente, 70% entre o genoma de referência humano e do zebrafish, aliado a presença de 82% de genes ortólogos ligados a doenças humanas, tem maximizado, ainda mais, a miríade de possibilidades para abordagens experimentais utilizando este pequeno teleósteo. Sobretudo, as que conduzem a alterações fenotípicas que, por sua vez, são facilmente identificadas nos embriões transparentes. Afinal, a transparência dos embriões possibilita um monitoramento *in vivo* para diversas condições experimentais. Além do mais, o *D. rerio* não necessita de amplos biotérios dado seu pequeno tamanho corpóreo, apresenta baixos custos para aquisição e manutenção e ainda possui alta aceitabilidade alimentar. Deste modo, ozebrafish tem abandonado sua posição de modelo experimental emergente e torna-se um instrumento atrativo e sólido para ciência. Como pode ser confirmado pelo salto no número de pesquisas que utilizam este animal em experimentos (**Figura 12**) e pela demonstração das áreas do conhecimento que tem progredido com o auxílio do zebrafish (**Tabela 1**) onde percebe-se uma constante atualização dos tópicos inerentes a cada área do conhecimento.

Interessantemente, uma notável janela experimental surgiu após o zebrafishser utilizado por mitocondriólogos. A função mitocondrial do zebrafish é similar aos demais modelos experimentais e ao homem envolvendo eventos análogos para fosforilação oxidativa, metabolismo, apoptose, captação de cálcio e para doenças relacionadas a disfunção mitocondrial. De modo que soma-se ao currículo do Zebrafish o seu uso como modelo sistêmico para estudo da biologia mitocondrial e doenças relacionadas (Steele et al., 2014).

Figura 12. Crescimento no número de trabalhos científicos que utilizam o zebrafish como modelo experimental. Os dados foram coletados no *Web of Science* utilizando os tópicos Zebrafish ou *Danio rerio* ou Zebra fish baseado no trabalho de (Kinth et al., 2013). (A) Representa o crescimento estratificado desde 1956 até 2017 registrado na base de dados. (B) Representa a evolução total no número de artigos registrados através dos mecanismos de busca supracitados.

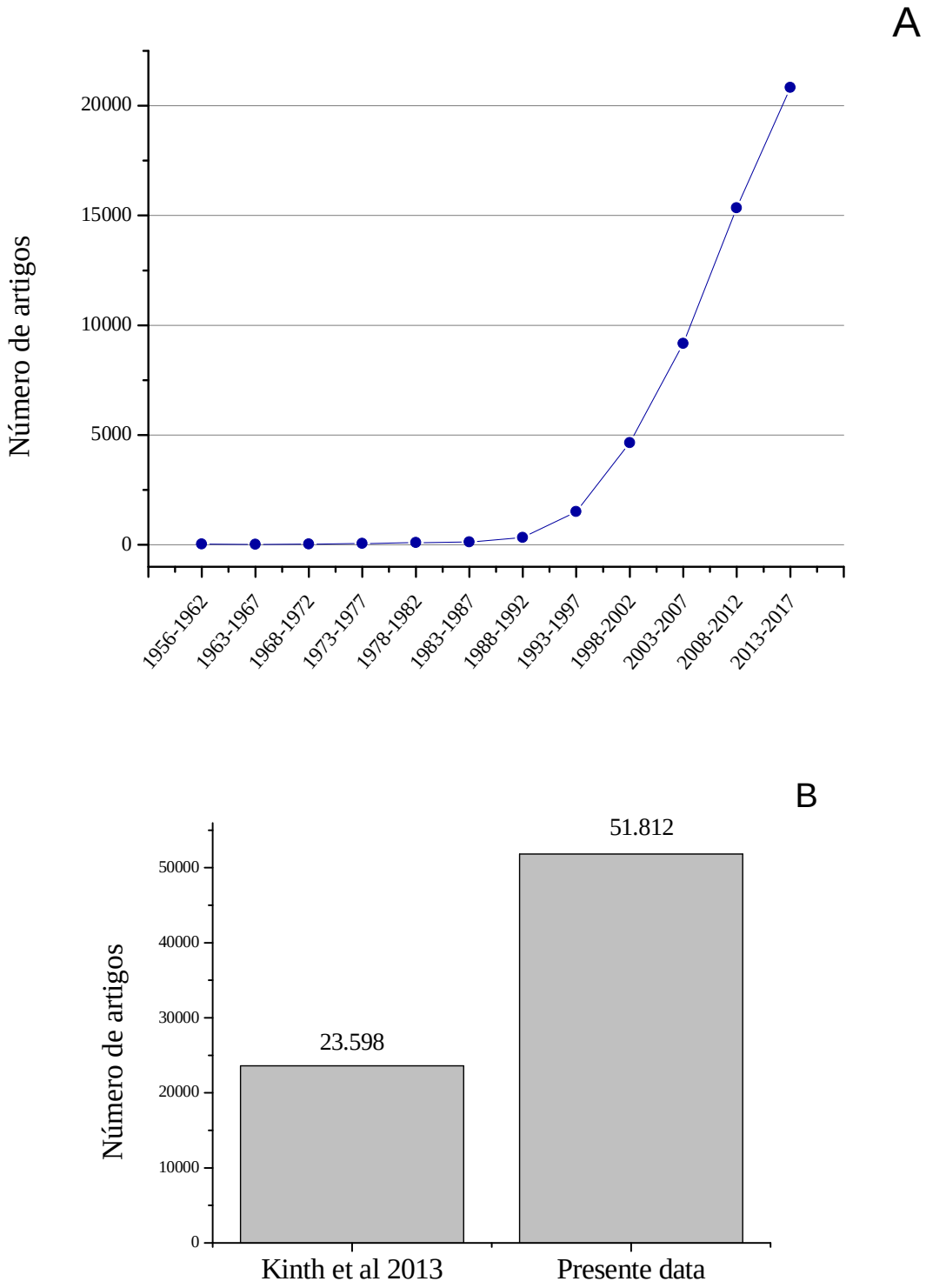


Tabela 1. Áreas do conhecimento que utilizam o zebrafish como modelo biológico.

Área do conhecimento		Ano*	Referência
Biologia Geral	Evolução	1999	(Metscher and Ahlberg, 1999)
	Biologia comportamental	2006	(Miklosi and Andrew, 2006)
	Biologia do desenvolvimento	1993 - 2017	(Meyer et al., 1993; Phan et al., 2017)
	Hematopoese	2010 - 2015	(Ellett and Lieschke, 2010; Rasighaemi et al., 2015)
Bioquímica	Metabolismo e Bioenergética	2007 - 2014	(Elo et al., 2007; Steele et al., 2014)
	Enzimologia	2010 - 2016	(Koenig et al., 2016; Liu et al., 2010)
	Química de macromoléculas	2017	(Shull et al., 2017)
Farmacologia	Caracterização de drogas	2002 - 2017	(Kithcart and MacRae, 2017; Langheinrich et al., 2002)
	Ensaios pré-clínicos	2004 - 2017	(Lee et al., 2017; Parng et al., 2002)
	Descobrimento de drogas	2007 - 2017	(Bootorabi et al., 2017; Kari et al., 2007)
	Abuso de drogas	2006 - 2017	(Brock et al., 2017; Ninkovic and Bally-Cuif, 2006)
Fisiologia	Fisiologia comparada	2002 - 2017	(Briggs, 2002; Duan et al., 2017)
	Fisiologia de órgãos e sistemas	2003 - 2013	(Bassett and Currie, 2003; Gemberling et al., 2013)
	Imagem 4D	2017	(Siegerist et al., 2017)
Genética	Genética geral	1996 - 2017	(Howe et al., 2017; Lele and Krone, 1996)
	Genética humana e médica	1999 - 2017	(Posner et al., 2017; Zon, 1999)
Educação	Métodos e técnicas de ensino	2009	(Bagatto, 2009)
Medicina	Imunologia	2008 - 2013	(Henry et al., 2013; van der Sar et al., 2006)
	Cardiologia	2011 - 2017	(Bakkers, 2011; Gut et al., 2017)
	Infectologia	2008 - 2017	(Neely, 2017; Sullivan and Kim, 2008)
	Patologia	2000 - 2016	(Barut and Zon, 2000; Bellipanni et al., 2016)
	Nefrologia	2008 - 2017	(Morales and Wingert, 2017; Wingert and Davidson, 2008)
	Neurologia	2001 - 2017	(Bilotta and Saszik, 2001; Fulcher et al., 2017)
	Angiologia	2008 - 2014	(Cao et al., 2008; Fang et al., 2014)
	Oftalmologia	2004 - 2017	(Glass and Dahm, 2004; Posner et al., 2017)
	Cancerologia	2010 - 2017	(Mione and Trede, 2010; Shull et al., 2017)
	Endocrinologia	2007 - 2017	(Elo et al., 2007; Zada et al., 2017)

	Dermatologia	2013 - 2017	(Bootorabi et al., 2017; Richardson et al., 2013)
	Ortopedia	2016	(Carnovali et al., 2016)
Microbiologia	Bacteriologia	2003 - 2017	(Neely, 2017; Prouty et al., 2003)
Morfologia	Embriologia	2002 - 2005	(Berman et al., 2005; Whitfield, 2002)
	Citologia e Biologia Celular	2010	(Eimon and Ashkenazi, 2010)
Nutrição	Nutrigenômica	2011	(Ulloa et al., 2011)
Psicologia	Psicologia experimental	2017	(Fulcher et al., 2017)
Recursos pesqueiros	Aquicultura	2006 - 2014	(Dahm and Geisler, 2006; Ulloa et al., 2014)
Toxicologia	Toxicologia geral	1996 - 2016	(Lele and Krone, 1996; Tran et al., 2016)
	Neurotoxicologia	2006 - 2017	(Pittman, 2017; Ton et al., 2006)
Zoologia	Comportamento animal	2010 - 2017	(Filby et al., 2010; Fulcher et al., 2017)

*Dados coletados até outubro de 2017.

3 RESULTADOS

3.1 ARTIGO 1: A OVERVIEW ABOUT THE USEFUL ZEBRAFISH MITOCHONDRIA

TITLE PAGE

An overview about the usefulness zebrafish mitochondria

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ABSTRACT

Zebrafish mitochondrial study have start before of 90 decades and today support with effectiveness part of scientific advances about the mitochondria function. This study was drawn to synthetize peculiarities and mitochondrial general advances acquired with this animal model. Zebrafish mitochondria inclusion in scientific studies were initiated with structural analyzes after exposures to chemical compounds. In this hand, exist the substantial sign to zebrafish model use to understand how chemicals impair mitochondrial bioenergetics. Similar predictions are employed for linkage zebrafish, mitochondria and ion homeostasis. Thus, fish have sophisticated mechanisms to ionic regulation due to constant variations in the water salts concentrations. Especially to Ca^{2+} uptake once the animal has the same mammalian microdomains MCU, VDAC, IP_3, ECaC, TRVP channels for Ca^{2+} transport/homeostasis. Moreover, this little model was support the overall knowledge about how specific proteins regulate mitochondrial signaling and function. Inexorably, biological ROS generation understanding advance keep new perspectives with zebrafish embryos. A new age for mitochondria study was unlocked after zebrafish model inclusion on mitochondrial bioenergetics researches.

Keywords: Mitochondrial function. *Danio rerio*. Toxicity. Oxidative stress. Redox signaling. ROS generation.

1. INTRODUCTION

Zebrafish use to scientific approaches have a substantial increase after meticulous description of embryonic development and completed sequence of reference genome, include human similarities (Howe et al., 2013; Kimmel et al., 1995). Multidisciplinary approaches between knowledge areas, using zebrafish as model, have solved old technique/scientific problems (such as the gap between cells culture and mammalian models) through to easy monitoring of target genes and proteins or metabolic diseases development on transparent embryos. Subsequently, a lot of knowledge areas that use zebrafish as a model is profoundly expensive and provides substantial scientific advances, especially to developmental biology and biochemistry/molecular biology (Kinth et al., 2013).

In this view, biochemistry/molecular investigations have been employed the mitochondria as instrument to understand multiples pathways about biological process. Indeed, mitochondria has long been recognized as a key organelle for a variety of cellular functions, more than ATP synthesis, such as redox signaling, ROS generation, Ca^{2+}homeostasis and cell death. Part of these mitochondrial phenomena have become even more important after linkage of these conditions with cellular networks. In addition,mitochondria it is involved in several pathophysiological conditions where mitochondrial dysfunction leads to a wide range ofdiseases and, in some cases, the diseases cause mitochondrial dysfunctions(Vercesi and Oliveira, 2017). Thus, the orchestrated association of zebrafish with mitochondrial studies has converged on a perfect match between two useful tools of the biological sciences.

Zebrafish mitochondria structure and function analyses take start with toxicological scope (Braunbeck et al., 1990) and today was employed to understand the interplay between mitochondrial status and Ca^{2+} uptake (Azzolin et al., 2010), apoptosis (Chiu et al., 2010), necrosis (Wu et al., 2008b), neuropathological diseases (Soman et al., 2017) or

ROS generation (Palanisamy et al., 2017), especially with the goal to *in vivo/real-time*monitoring. The present work was designing to provide an overview about mitochondrial study based only at zebrafish mitochondria functional aspects.

2. ZEBRAFISH MITOCHONDRIA STATE-OF-THE-ART

a. Zebrafish mitochondria as toxicity marker

Early researches that study the zebrafish mitochondria have a toxicological scope. On this occasion, zebrafish embryos were exposed to herbicide additive chloroaniline and to fungicide triphenyltin acetate (TPTA). These exposures resulted in atypical mitochondria, especially in the mitochondrial matrix, which presented distensive, flocculated and with several stratified inclusions as a possible response to the cellular stress of exposure to chemical agents (Braunbeck et al., 1990; Burkhardt-Holm et al., 1999; Oulmi and Braunbeck, 1996; Strmac and Braunbeck, 1999). Furthermore, cytopathological characteristics were also found in liver mitochondria of zebrafish when exposed to herbicide atrazine(Braunbeck et al., 1992). Together these papers are the pioneers to analysis mitochondrial structure and showed that fungicides, herbicides and their secondary metabolites are actively damaging to the zebrafish mitochondria. This point was the practical demonstration that some chemical compounds have deleterious effects on the mitochondrial structure of non-target organisms like zebrafish andenabled the development of numerous hypotheses are derive/evolved/tested from these observations. Such as, the possibility to real-time monitoring of deleterious effects on transparent embryos or about how alterations on mitochondrial conformation leads to physiological dysfunction.

Metals, such asorganometallic cation methylmercury (MeHg), have negative effects on skeletal muscle because modify mitochondrial cristae conformation, this feature maximizes the probability to apoptotic event in the celldue to the possibility of interference in the respiratory chain(Ribeiro et al., 2008). This idea was elucidated by the identification that

MeHg affects the synthesis of ATP by strongly inhibition of oxidativephosphorylation (Cambier et al., 2009). Interestingly, MeHg does not compromise state 4, which suggests the induction of a defect in ATP synthase. The connection of these deleterious effects with recently identification that MeHg affects the pyruvate transport in the human neuroblastoma cells (Lee et al., 2016) drives to open questions about to specific and irreversibleMeHg involvement in ROS generation and oxidative stress. Specially, when an up regulation of genes, atp2b3a, atp2b3b, and slc8a2b, encoding for calcium transporters and a repression of the glutathione peroxidase gene gpx1have been reported after MeHg exposure (Cambier et al., 2009). Recently, the question about the possibility of oxidative stress development on zebrafish by MeHg was registered (Strungaru et al., 2018). In this research, the oxidative stress is a fast-deleterious way to physiological damage on this vertebrate model.

Additional questions about toxins and drugs impact on the zebrafish mitochondria showed how genetic mutations are caused by chemical compounds. Embryos exposures to PAH (Polycyclic Aromatic Hydrocarbons) reveals a mitochondrial DNA copies damage (Kim et al., 2014). About this, Kopeika et al.2005 showed a significant increase in the frequency of mutations in the mtDNA of blastomeric cells when the zebrafish eggs submitted to cryopreservation (Kopeika et al., 2005). Similar mtDNA damage conditions were observed by Doganlar et al., 2016 after exposure to heavy metals like As^{+} and Cd^{+} that leads to mitochondrial dysfunction and oxidative stress (Doganlar et al., 2016). Subsequently, it becomes consensual to suggest that zebrafish mtDNA be considered a universal zebrafish biomarker for exposures to toxic components.

In this way, alterations in the Cytochrome P4501a gene expression were observed when zebrafish embryos are exposed to dioxins (Alexeyenko et al., 2010) and, today, an look at Cytochrome P450 status has emerged as a important way to estimate detox and toxic possible effects. An interesting study also measured how MPTP (1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine)neurotoxin modify the dopamine transporter expressionin the mitochondria affecting the development of dopaminergic neurons(Noble et al., 2015). Everywhere the mitochondrial biogenesis and organizationtogether were affected in embryos when their parents were exposed to concentrations of sexual hormones(Santos et al., 2007). These exposures also minimized egg production and fertilization. These observations show that mitochondria may play a relevant role during phenomena of intoxication, detoxification and embryonic development.

In the presence of flame retardants such as diphenyl ether polybrominated (PBDEs) Van Boxtel et al. 2008 show that this compound acts as a uncoupler of the oxidative phosphorylation and strongly inhibits complex II of electron transport chain(Van Boxtel et al., 2008). This profile is also identified during viral infections in embryos. Viral infections by Beta-nodavirus B2 protein inhibit this respiratory complex associated with H_2O_2 production, resulting in mitochondrial fission and apoptosis(Su et al., 2014). A similar mechanism is also observed by nitro-derivative 2,4 dinitrophenol (DNP)which induces mitochondrial dysfunction through reduced production and ATP levels by impairing oxidative phosphorylation(Bestman et al., 2015). Indeed, DNP is one of the most well-known uncoupling agents in the respiratory chain.

An exhibition 72-96 hpf to Tris(2,3-dibromopropyl) isocyanurate(TBC) drives to defects in swim bladder inflation probably due to mitochondrial damage(Li et al., 2011). It is important to mention that TBC is an emerging persistent organic pollutant and universally employed to flame retardant. Atypical mitochondria were observed when zebrafish were exposed to cyanobacteria toxin microcystin-LR (MC-LR). It was observed mitochondrial and endoplasmic reticulum swelling, in addition to an alteration in the activities of liver phosphatase protein(Wang et al., 2010). Due to the concomitant swelling of endoplasmic reticulum and mitochondria, probably all microdomain for calcium homeostasis are being

compromised. Indeed, MC-LR disrupts Ca^{2+}homeostasis in neurons by releasing Ca^{2+} from intracellular stores(Cai et al., 2015). Interestingly, MC-LRs have also a reproductive toxicity related to an increase in ROS production and mitochondrial dysfunction(Chen et al., 2016a).

An important warning emerged after demonstrating that particles composed of titanium dioxide (TiO_2), such as rutile, between 80-100nm penetrate the mitochondrial matrix of zebrafish, corroborating with the toxic effects of compounds based on TiO2, therefore this mineral has potential to compromises fish embryogenesis(Yeo and Kang, 2012). Zebrafish embryogenesis has also been affected by graphene oxide (GO), which is a compound based on carbon particles and has been extensively used given its medical and biological properties.GO penetrates the zebrafish embryo easily causing mitochondrial damage and inducing ROS production and leading to an, increased oxidative stress(Chen et al., 2016b). Although OG has recently emerged as a therapeutic approach, its effect on mitochondrial functioning demonstrates health risks. These risks may be still maximized under physiopathological conditions.

Related questions were also observed after exposure of 12dpf embryos to cisplatin (which is one of the most used chemotherapeutic agents in the world). This exposure leads to widespread injury to the mitochondria, especially about swelling, electrical density and damage to the mitochondrial cristae (Giari et al., 2012). Remarkably, this antineoplastic and cytotoxic agent has been widely used, including with the authorization of the FDA for antitumor use in chemotherapeutic approaches.On the other hand, administration of this compound associated with protective radio-and neuro-protective agents such as KR-22332 or Edaravone has minimized apoptosis, changes in Mitochondrial Membrane Permeability (MMP) and ROS production (Hong et al., 2013; Shin et al., 2013).

Drug toxicity was also evaluated using opioids such as tramadol hydrochloride. Zebrafish mitochondria present serious morphological changes when exposed to tramadol

(Zhuo et al., 2012). Since tramadol is a widely used analgesic for the general population, this finding corroborates the discussions about the advantages of including mitochondrial bioenergetic evaluations during preclinical research phases especially when match with simply models like zebrafish.As a therapeutic approach the radioprotectant and fluorescence probe ZMJ214 was recently synthesized for the real-time monitoring of MMP in live zebrafish embryos(Gibert et al., 2013a; Gibert et al., 2013b; Sasagawa et al., 2016). This advancement makes it possible to more accurately identify toxins, drugs or genes involvied in the mitochondrial dysfunction. In addition ebables the practical demonstration that mitochondrial function specific markers can be used on preclinical research.

Mitotoxic and physiological effects of dietary components offered to zebrafish urgently require more information. About this, exist an urgency of a standardization in the diets that are offered to this animal, especially in view of the possibility of masking scientific results(Penglase et al., 2012). In this way, the evaluation of dietaryMeHg and gold nanoparticles,both with high capacity to accumulate by the food chain, showed that at environmental doses or dietary level MeHg and gold nanoparticles has potential to impair the zebrafishtissue mitochondrial respiration(Bourdineaud et al., 2013; Cambier et al., 2009; Ribeiro et al., 2008).

To date, it has been known that after *D. rerio* obesity induction with a rich fatty acids diet and followed to treatment with the antioxidant eriocitrin, an improvement in the hepatic steatosis has been seen induced by the diet and also an activation of mitochondrial biogenesisgenes (Hiramitsu et al., 2014). This result corroborates with the interpretation of a mitochondrial reprogramming for physiological homeostasis in the face of induced obesity.Interestingly, the FABP3 protein that binds to fatty acids and is highly involved in mitochondrial regulated apoptosis, a FABP3 knockout leads to apoptosis, due to an increase in ROS production and reduction in the number of mtDNA, resulting in serious cardiac

dysfunction(Liu et al., 2013). In addition, it has recently been identified that fatty acids present in the diet may be linkage with mitochondrial phospholipid membrane compositionand the mitochondrial gene expression, those points have influence on animal growth(Betancor et al., 2015).

Pertinently, when zebrafish are fed clofibrate (which is a drug widely used to increase fatty acid secretion via lipases activation), there leads to a considerable increase in the number of mitochondria and peroxisomes in the liver and heart, respectively. This component is also optimized for mRNA transcripts for COX1 (Venkatachalam et al., 2012). Showing a reprogrammation to make ϐ-oxidation of fatty acids more effective. From the results obtained by this author it is possible to affirm that zebrafish is responsive to clofibrate, which is not observed for other fish to date.

Therefore, zebrafish could be consolidated as an animal model for toxic impact on mitochondrial bioenergetics evaluation (Bourdineaud et al., 2013) because it allows, in a more concise way, the identification of how chemical agents can inhibit respiratory complexes, affect oxidative phosphorylation and lead to mitochondrial oxidative stress. As for the pathophysiological effects of dietary components, there is still a long way to go, however, after the indication that zebrafish may be a model organism for nutrition, nutrigenomics, lipid metabolism and diseases such as arteriosclerosis (Fang et al., 2014; Ulloa et al., 2011; Ulloa et al., 2014) there is a prospect of further advances in these areas.

b. Mitochondrial proteins

Several proteins linked zebrafish mitochondrial function were subjected to experimental manipulations, especially in embryos, which enabled a more detailed investigation of functions, knockouts and protein isoforms through *in vivo* monitoring. For example, the mitochondrial transition permeability MTP which is a mechanisms inherent to apoptosis and which can also be investigated in zebrafish. The disrupting on ϕ= *m* is observed

177 at when there is a knockout of proteins, such as viral protein AVP3 (antiviral protein 3),
178 which leads to mitochondrial membrane potential loss and activation of caspase-9 and
179 caspase-2 in zebrafish and mouse cells (Chiu et al., 2010). For this, the specific viral genome-
180 encoded protein is involved on Bad way to apoptosis.

181 Crucial to apoptosis mediated by mitochondrial Permeability Transition Pore
182 (PTP) was first studied by Azzolin et al. 2010 in zebrafish model. Theses authors show that
183 the PTP opening is a Ca^{2+} dependent mechanism in zebrafish, just as in other animal models,
184 and signals for use of this model to study PTP (Azzolin et al., 2010). Remarkably, was
185 identified that PP2Cm (phosphatase protein) plays a critical role in cell death by playing a key
186 opening of PTP (Lu et al., 2007).Indeed, it is known that the release of proapoptotic factors in
187 the intermembrane space is conditioned by the opening of mitochondrial PTP. In this view,
188 several pro-apoptotic pathways (e.g. Bax and Bad) have also been investigated using this
189 animal mode l(Antonsson, 2001; Hsieh et al., 2003). As well as pre-proteins and
190 intermembrane transporters, such aspreprotein translocase of the inner membrane of
191 mitochondria(Tim50), which acts on the translocation of internal and external mitochondrial
192 membrane proteins (Guo et al., 2004; Yamamoto et al., 2002).

193 The Bcl-2 family of proteins had part of their mechanisms of action investigated
194 using this animal model when two proteins homologous to zlBLP1 and zfBcl-xL were cloned
195 and characterized (Chen et al., 2001; Wu et al., 2008a). These proteins have high similarity
196 with human Bcl-XL and play a key role to fish embryogenesis. The study of another protein
197 homologous to BcL-2, NRZ, has identified that this protein acts on the dynamics of
198 cytoskeletal movements by regulating the Ca^{2+} flux between the endoplasmic reticulum and
199 the mitochondria (Popgeorgiev et al., 2011). This is probably intimately involved with
200 zebrafish gastrula morphogenesis (Prudent et al., 2013b). New points about this was added
201 after precise description of wild-type Nrz, but not Nrz with phosphomimetic mutations,

interacted with the IP3 binding domain of IP3R1, inhibited binding of IP_3 to IP_3R_1(Bonneau et al., 2014). This characteristic can be correlated with events of cell death by importance of the cytoskeleton for maintenance of basic metabolic functions and due to the importance of intracellular Ca^{2+} homeostasis for mitochondrial function. Moreover, NRZ controls development during somitogenesis and gastrulation via apoptosis-dependent and -independent mechanisms(Arnaud et al., 2006).

Uncoupling proteins (UCP) are a sequence of proteins attached on inner mitochondrial membrane that takes key roles for mitochondrial functionality. UCPs presence and function on ectodermal vertebrates such as zebrafish needs more information. Primarily, for UCP1 because this protein is routinely associated with thermoregulation in mammals,nevertheless ectothermic is main fish characteristics. Interestingly, mammalian UPC1 was a match of 94% in homologues for zebrafish mRNA UCP1 (Stuart et al., 2001). This information linkage with UCP2 and UCP3 identifications in fish such as zebrafish and carp (Stuart et al., 1999) has corroborated the accepted theory that these UCPs participate in various mitochondrial regulation processes e.g. redox signaling.

The mitochondrial proliferation and differentiation at stages of the metameric development was elucidated by (Wanga et al., 2001)which provided the basis for future research on mitochondrial dynamics in these stages. Recently (Masuda et al., 2016) showed that ES1 protein is one of key factors contributing to mitochondrial proliferation and differentiation in zebrafish, ES1 contributes to the formation of mega-mitochondrianecessary for the rapid embryonic development of fish. Beyond ES1, GTPases activity appears be essentially. The zebrafish GTPases are essentials to regulate morphogenesis, movement and mitochondrial distribution (Reis et al., 2009). These functions are supported by mitochondrial Miro/Rhot proteins which are located in the outer mitochondrial membrane and are fundamental for the mitochondrial stationary and mobile phases, the genes of the Miro / Rhot

proteins (Rhot1a, Rhot1b and Rhot2) bound to GTPases are orthologs between humans and zebrafish (Hollister et al., 2016). Beyond all three isoforms identified, this demonstrated a dose dependent effect for body axis elongation. Thus, are fundamental for vertebrate development.

Through zebrafish embryos the interaction between mitochondria, actin and tubulin filaments and probable mitochondrial stressors during biological sample preservation steps was investigated. As a result, it was obtained that cryopreservation compromised ATP levels and mitochondrial structure (Zampolla et al., 2011b). These are important because most of the methodologies employed to evaluate the success of cryopreservation strategies are based only on cell survival. Thereby, (Zampolla et al., 2009) investigate the preservation strategies effects and reported that the use of high concentrations of methanol and Me2SO during the cryopreservation of ovarian follicles of zebrafish affects mtDNA number of copies, the mitochondrial hexagonal arrangement, the membrane potential and ATP levels, as well as the ADP/ATP ratio. These damages can be easily reversed by the removal of methanol and subsequent follicular maturation (Spikings et al., 2012). In conclusion, detecting changes in mitochondrial membrane potential may become an effective method for estimating the viability of cryopreservation protocols.

Considering that cryopreservation has impacts on mitochondria and the distribution of cytoskeletal proteins (Zampolla et al., 2011a; Zampolla et al., 2011b). This point may partially explain the failure of some cryopreservation methodologies. Indeed,these results produce evidence that after mitochondrial bioenergetic and morphology impairment the biological sample viability is strongly reduce.

The zebrafish has also contributed to maximize the match between mitochondrial dysfunction and neurodegenerative proteins.Parkinson's disease affects the functioning of mitochondrial complex I in disease-induced embryos (Flinn et al., 2009). Furthermore, it has

recently been identified, also on this animal model, that histone deacetylases (HDACs) (which were long reported only in the cell nucleus) are key enzymes during neurodegeneration process in Parkinson's disease by carries important neuroprotective role. Specific pharmacological approaches to these HDAC's can minimize mitotoxic effects of neurotoxin 1-methyl-4-phenylpyridinium (MPP+) (Pinho et al., 2016).

Another disease that had part of its mechanisms of action elucidated using this animal model was Alzheimer's disease. The relationship between TAU protein (who is responsible for stabilizing microtubules) and mitochondrial distribution showed a way to connection with Alzheimer's. Through of the transgenic named Mitofish(Plucinska et al., 2012)showed how TAU is important for mitochondrial distribution and functionalization, this can be proving the relationship between mitochondria and neurogenerative diseases also in this animal model. The transgenic was named for enabling a rapid and sensitive technique for the ñ*in vivo*ò mitochondria study. The results obtained by the study, besides making the relations between TAU and neuropathies even more universal, opened, for the first time, the possibility of specificmarkers study to these pathologies using mitochondrial regulation as a marker in alive zebrafish. After all, it was the first time that mitochondrial dynamics can be monitored in real time in fish neurons.

A notable contribution of zebrafish to the study of mitochondria was the description, also in real time of mitochondrial fusion and fission process.Zebrafish embryos exposed to Valinomycin, FCCP and Staurosporine (Kim et al., 2008) creates a pathway to induce apoptosis and changes in mitochondrial physiology. The results obtained encourage the use of embryos to study drugs that can modulate apoptosis in real time.As demonstrated later by fluorescence probes (GFPand GFP-OPT) that make it possible to more accurately study apoptosis and FHZ probe which facilitated the *in vivo* identification of mechanisms inherent to OH and hypochlorous acid production (Nasu et al., 2016; Zhang et al., 2016).

c. Mitochondrial respiration

Cellular respiration is an event intrinsic to aerobic organisms and can occur, in synthesis, by two routes. A non-mitochondrial way where oxygen consumption may arise from many sources such as peroxisomes or from plasma membrane NADPH oxidase activity (Stackley et al., 2011). Or by the mitochondrial pathway that is widely studied and occurs through oxidative phosphorylation in the electric respiratory chain located in the inner mitochondrial membrane.

To date, zebrafish mitochondrial respiration has been studied step by step using Clark-type and OX1LP polarographic electrodes e.g. (Bourdineaud et al., 2013; Mendelsohn et al., 2008), spectrophotometers e.g. (Flinn et al., 2009) and XF24 SeaHorse Cellular Extra Flow Analyzer e.g. (Stackley et al., 2011). All these methods present variations in sensitivity and/or rate of transfer even though they are widely validated by the scientific community.

The step-by-step study of each respiratory complex has shown that specific inhibitors for Complex I and II (Rotenone and 3NP) have a greater teratogenic potential even as the inhibitors mixotiazol and antimycin A for complex II I(Pinho et al., 2013). When there is acute inhibition of the I complex with Rotenone, this inhibition leads to developmental abnormalities, in addition to heart failure and ATP depletion (Pinho et al., 2013). Complex III activity and its subunits is crucial for the process zebrafish angiogenesis, its suppression through drugs or genetic manipulation leads to complications in vascular endothelial growth factor, hypoglycemia, lactic acidosis and other human disorders (Cho et al., 2013; de Lonlay et al., 2001).

An inhibition of cytochrome c oxidase COX (mitochondrial complex IV) results in defects in endothelial tissue and cardiac failure, as well as increase apoptosis in the parietal lobe and neuronal tubules of zebrafish affecting motor neurons, as a result motility

defects(Baden et al., 2007). These specific responses enabled the use of COX deficiency in this animal model as a promising tool for the study of human diseases linked to mitochondrial complex IV. Additional information for the activity of this enzymatic complex is observed after cold acclimatization. Processes of acclimatization to the cold lead to an increase in the activity of enzymes related to mitochondrial activity, besides inducing biogenesis and mitochondrial proliferation in fish(Bremer and Moyes, 2011). Nevertheless, (Duggan et al., 2011) did not identify differences in COX activity when zebrafish was acclimatized to heat or cold. This result becomes relevant, observing that, under the same experimental conditions, the fish *Chrosomus eos* and *Carrasius auratus* presented significant differences in the COX activity. Thus, it becomes possible to hypothesize that the zebrafish may, metabolically, have some compensatory mechanism.

Interestingly, anoxia conditions in zebrafish embryos have similar CTE impact and ATP synthase than specific inhibitors and uncouplers such as KCN, Oligomycin or CCCP (Mendelsohn et al., 2008). Indeed, CCCP and Oligomycin has direct correlation with mitochondrial membrane potential loss in *in vivo* experiments (Sasagawa et al., 2016).The plasticity of zebrafish muscle tissue has made viable the development of consistent hypotheses about how high mitochondrial density is a link between capillarization, muscle fibers and bioenergetic demand(Bagatto et al., 2001). In this work, the authors highlighted these issues and elaborated theories about changes in cellular organelles, oxidative capacity and oxygen transport in muscle tissues under the perspective of exercise physiology.These results became more important after identifying that the major protein involved in the transport of oxygen in neuronal tissue, neuroglobin, works similarly between zebrafish and mammals(Fuchs et al., 2004). Thus, mitochondria stay at evidence in this context. Indeed, cells rich in mitochondria are recognized for oxygen use with highly efficiency.

The role of proteins in the stability and function of respiratory complexes has also been studied through this animal model.For example, metalloendopeptidase OMA1, which has the function of stabilizing respiratory supercomplexes. OMA1 knockout results in respiratory decline and failure in mitochondrial bioenergetics correlated with morphological defects in the heart and eyes of zebrafish (Bohovych et al., 2015). This feature may be associated with some phenotypes of human diseases. Thus, individual study of each zebrafish mitochondrial respiratory complex function can be an attractive tool for human pathophysiological conditions exploitation.

Zebrafish diets enrichment with gold particles has shown that these compounds tend to accumulate in the brain and muscle affecting the respiratory states 3 and 4, respectively (Bourdineaud et al., 2013). This feature has seriously impaired on cellular energy supply and can be an inducer of ROS generation.

d. Ca^{2+} homeostasis

Fish intracellular Ca^{2+} homeostasis with emphasis on zebrafish experimental model has shown peculiarities to ionic regulation. Especially because fish are subject to variations in environmental concentrations of salts and these oscillations force these animals to develop survival strategies against ionic and osmotic gradients in aquatic environments. Therefore, internal homeostasis for these animals may be different in comparison to the other vertebrates or support the elucidation of delicate mechanisms involved ions transport between cellular organelles.

The role of Ca^{2+}, Na^{+} and K^{+} ions in the experimental model has allowed the identification of interesting regulation ways, for example the participation of fish mitochondrial channels Na^{+}/K^{+} inneuronal and epithelial innervation(Jonz and Nurse, 2006)or that the mitochondria iskey calcium storageduring oogenesis process (Golpour et al., 2016).

In addition, cortisol stimulates Ca^{2+} uptake in zebrafish when reverse effect is observed for mammals(Lin et al., 2011). About this, Esterberg et al. 2014 discuss around an efficient Ca^{2+} flow between endoplasmic reticulum (ER) and mitochondria through IP3 channels. These ER channels regulate mitochondrial activity under various physiological conditions leading to an increase toxins susceptibility because of Ca^{2+} uptakeincreased(Esterberg et al., 2014). This demonstrates that the cost for cellular homeostasis can be high, especially for fish mitochondria that may be easily depolarized by Ca^{2+} ions.In addition, Na^{+} and Ca^{2+} epithelial channels study has showed a key mechanism aboutextracellular calcium transport to cytoplasm and deleterious possible effects of ionic transport(Esaki et al., 2007; Liao et al., 2007; Pan et al., 2005). Therefore, the lot of these characteristics encouraged zebrafish use as a model for ion regulationthrough of Calcium Epithelial Channel (ECaC), Anionic Voltage Dependent Channels (VDACôs), Transient receptor vanilloid types (TRVP) or the Mitochondrial Calcium Uniporter (MCU) (Prudent et al., 2013a; Prudent et al., 2013b; Shimizu et al., 2015; Tseng et al., 2009; Vanoevelen et al., 2011).

Zebrafish VDACs permit that Ca^{2+} high concentrations flowto mitochondria as a mechanism that stimulatesATP consumption necessary for muscle contraction, and consequently, swimming(Mizuno et al., 2013). An equivalent way was observed for cardiac muscle.Changes in VDAC-2 lead to cardiac arrhythmia in zebrafish embryos by mitochondrial exacerbated Ca^{2+}uptake(Shimizu et al., 2015). These findings show that calcium uptake to VDAC-way is also physiologically essential for zebrafish.

The zebrafish MCU acts on notochord formation through extension movements during gastrulation(Prudent et al., 2013b)as well playing a physiological key role by regulating calcium pulses in the cytoplasm(Samanta et al., 2014). Recently, MCU has been described as a neuroprotective agent in neuropathological conditions such as Parkinson's disease induced in zebrafish(Soman et al., 2017).

It was also through zebrafish that it was identified that Ca^{2+} influx plays a key role during axonal degeneration, especially when there is alteration in the redox state of neuronal cells(O'Donnell et al., 2013; Vargas et al., 2015). These observations were reinforced when V-ATPase subunit A gene (atp6v1a) was knocked and this inactivation affected the Ca^{2+} and Na^{+} balance, resulting in growth retardation and brainstem deformations possibly linked to neuronal and epithelial innervation(Horng et al., 2007). Indeed, it is known that these phosphatases are fundamental for energy generation and that deficits in mitochondrial bioenergetics can compromise growth. In this way, Ca^{2+} also has a direct relationship with the opening of the mitochondrial Permeability Transition Pore (PTP). The PTP formation similarly occurs in zebrafish including the same responses to action of classical PTP inhibitors such as cyclosporine A and ruthenium red or for the key modulators e.g. voltage, pH, ubiquinone, dithiol oxidants and crosslinkers, ligands of the adeninenucleotide translocator or arachidonic acid(Azzolin et al., 2010). The PTP can be used to specific therapeutic approaches may be based on the discovery of drugs that have mitochondria and PTP as a target, for example UCMD (Ulrich's Congenital Muscular Dystrophy), which could be attenuated by treatment with Ciclosporin A (CsA) on zebrafish model (Telfer et al., 2010). In addition, new potent PTP inhibitors, such as Diarylisoxazole-3-carboxamides, have demonstrated inhibitory activity against mitochondrial swelling and, consequently, PTP opening (Roy et al., 2015) or has made possible *in vivo* visualization of phenomena linked to loss of MMP embryos (Sasagawa et al., 2016).

e. Oxidative stress and fluorogenic probes

Differences in oxidative stress and lipid peroxidation in zebrafish brain are observed depending of methodology used for ROS generation evaluation(Nik et al., 2014). These differences are also observed when comparing maturation and aging, mostly about mitochondrial phospholipids content and superoxide dismutase activity (Almaida-Pagan et al.,

2014). Indeed, zebrafish mitochondria feel deep remodeling during development or under pathophysiological conditions(Zhang et al., 2015).

Therefore, estimating ROS and RNS by zebrafish mitochondria as a possible indicative of oxidative stress is a mechanism that requires a caution adjustment between physiological state and methodological procedure, especially when this animal model is used as a pre-clinical stage. In addition, fish transgenic cellsrespond as rapidly like human cells to ROS generation by dichlorofluorescein (DCF) oxidation (Carvan Iii et al., 2001). The results obtained supply more elaborated questions about the possibilities of zebrafish use as an environmental sentinel. Moreover, introduced questions about linkage between redox state, oxidative stress and apoptosis in this model and can be reinforce similarities about mitochondrial dynamics between the zebrafish and other animal models.

Interestingly, the carotenoids inclusion in diets for animals deficient in enzymes to carotenoid cleavage has led to oxidative stress(Amengual et al., 2011). This demonstrates that these compounds widely recognized as natural antioxidants have your activity conditioned by specific enzymes catalysis and/or absence of these enzymes has feedback-negative to usual purpose. In zebrafishthe absence of BCDO2 enzyme, responsibly to cleave carotenoids, cause mitochondrial oxidative stress by inducing cytochrome c release, proteolytic activation of caspase 3 resulting in apoptosis.

The mitochondrial potential for ROS production is higher in Growth Hormone transgenic zebrafish, these animals present higher oxygen consumption compared to the wild type (Rosa et al., 2008). The ROS production and consequent mitochondrial damage is a major deleterious factor in the quality of fish sperm(Cabrita et al., 2014).

Recently zebrafish has become an attractive tool in the search for new synthetic components to avoid mitochondrial dysfunction and suppression of ROS generation during treatment with chemotherapeutic agents, such as RK22332, which has minimized ROS

generation in non-target tissues by minimizes the mitochondrial membrane potential loss in zebrafish, rat and HEI-OC1 human cell lines(Shin et al., 2013). This corroborates the premise that zebrafish mitochondria is also an important marker to oxidative stress.On this,(Su et al., 2014) studied the role of mitochondria during free radicals production during betanodavirus infections, comparing fish cells, zebrafish embryos and human cells. The study identified that betanodavirus induces hydrogen peroxide production and activates GTPases like dynamins production.

It was through this animal model that it was discovered how the mitochondrial enzymes encoded by the immunoresponsive gene 1 (GIR1) play a key role of connection between immunology, cellular metabolism and mitochondrial mediated infection(Hall et al., 2013). In fact, ROS bactericidal activity produced by mitochondria was reported as an antimicrobial agent in bacterial infections(Tavares et al., 2011).

Concerning the production of RNS, zebrafish has recently assisted in the development of fluorescent probes such as 4-MB for the in vivo monitoring of ONOOĖ production by mitochondria(Palanisamy et al., 2017). This new tool is highly attractive because ONOOĖ action, although short-lived, as a powerful oxidant with potential to cause damage to organelles and -SH proteins groups. As nitric oxide production the advances are greater. Mainly after the identification that NOÅis also a zebrafish powerful vasodilator and dependent do nitric oxide synthase (Fritsche et al., 2000; Jensen, 2007). In this way, nitric oxide synthase activity affects the behavior of the locomotor system during embryogenesis(Murcia et al., 2016) and is also related to the prevention of cardiac and vascular anomalies during development(Sykes et al., 2016)besides being powerful controller in the breathing of adult specimens(Porteus et al., 2015). Therefore, pharmacological and toxicological approaches to nitric oxide production arevery attractive. Mostly after

448 chromatographic techniques use to detect NO Åeneration by zebrafish embryos(Leite et al.,
449 2012).

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456

3. FUTURE PERSPECTIVES

This small teleost has provided good opportunities to investigate specific modulators of mitochondrial function.More than that, new research horizons have been visualized by perfect match between transparent embryos and fluorescent probes or proteins knockout. Indeed, *real-time* ROS generation, precise identification of embryonic defects resultfrom mitochondrial proteins knockout or teratogenic effects of chemical components are just some of the examples that underlie these outcomes.In this way, the zebrafish mitochondria as toxicity marker growth force be for evaluate the negative effect of chemical compounds on non-target organisms or to screen drugs in clinical trials. Easily to get an optimistic perspective to better understand the delicate pathways that link mitochondrial function with the health/disease balance.

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3.2 ARTIGO 2: ZEBRAFISH INTEGRATIVE PHYSIOLOGY: MITOCHONDRIAL BIOENERGETICS MEASUREMENTS ON PERMEABILIZED TISSUES

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TITLE PAGE

Zebrafish integrative physiology: mitochondrial bioenergetics measurements on permeabilized tissues.

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ABSTRACT *(100-250 words)*

Zebrafish has emerged in the past decade as an alternative vertebrate model. Unquestionably today this little teleost is a powerful tool for a myriad of experimental conditions, especially for bioenergetic studies. However, it is still necessary understand some mitochondrial specificities in this animal model. Here we report that zebrafish tissues have bioenergetic tissue-specific pattern to Heart, Skeletal Muscle (SM), Brain and Liver. Mitochondrial respiration is interestingly different among the tissues studied. At the respiratory chain level, Cytochrome c oxidase had the highest O_2 consumption for all tissues (10-fold compared to another respiratory complexes). Brain and Liver had no differences during ADP-induced phosphorylation, however compared with permeabilized skeletal and cardiac muscle significant differences were observed. Succinate ubiquinone oxidoreductase respiration was 3-fold higher to skeletal muscle. Liver was quickly damaged during H_2DCF-DA oxidation, nevertheless cardiac fibers was equally responsive to ROS generation after 40 min exposure to H_2DCF-DA. Specific mitochondrial O_2ÅË generation with MitoSOXÊ showed that Liver and Brain are more susceptible to damage of this reactive oxygen specie. In this way, cardiac fibers generated minimal amounts of O_2ÅË ROS generation for all permeabilized tissues was Ca^{2+}-dependent. Moreover, SM have more Ca^{2+} uptake between all studied tissues. On another hand, Liver has lower Ca^{2+} uptake and high release. Liver and Brain showed high susceptibility to NO biosynthesis detected by DAF-FM-DA. This study demonstrated a mitochondrial specific tool for zebrafish cardiac, muscular, neuronal and hepatic permeabilized tissues.

Key words: Tissue-specific pattern. Mitochondrial function. ROS and NOĘgeneration. *Danio rerio*. Nitric oxide.

Highlights - Zebrafish mitochondrial bioenergetics has tissue- and site- specific pattern;

- Adult Liver and Heart was higher susceptibility to ROS damage;

- Brain and Liver superoxide generation are equally.

INTRODUCTION

Scientific approaches using alternative experimental models has made possible a reduction in traditional animal use, for example rodents. Zebrafish (*Danio rerio*) alternative model use in scientific research has grown rapidly in the last decades. The main reasons for this are high presence of human disease-related genes and complete sequencing of zebrafish reference genome (Howe et al., 2013) further your biological proprieties such as easy maintenance, fast life cycle, transparence of early-life stages and *in vivo* approaches. Certainly, itôs also a useful model to cell metabolism (Santoro, 2014), drug discovery (MacRae and Peterson, 2015), traditional medicine (Littleton and Hove, 2013), developmental biology (Amaral and Johnston, 2011) and aquaculture nutrition (Ulloa et al., 2014) and to many pathological processes such as dyslipidemia, arteriosclerosis and angiogenesis (Fang et al., 2014),

Zebrafish mitochondrial bioenergetics investigation had start with toxicological scope (Braunbeck et al., 1990). Today many crucial mitochondrial processes have been studied through this animal model such as Mitochondrial Permeability Transition (MPT), calcium uptake (Azzolin et al., 2010; Prudent et al., 2013), environmental impact of pollutants (Bourdineaud et al., 2013), regulation process to apoptosis (Popgeorgiev et al., 2011) and mitochondrial neuronal-related diseases (Fett et al., 2010). Such zebrafish may be considered ideal systemic model for the study of mitochondria (Steele et al., 2014). After all, will provide an opportunity for knowledge expansion about diseases related to mitochondria.

Really, mitochondria is a key organelle for many metabolic and pathophysiological processes. However, mitochondrial functions may be have tissue-specific pattern and this feature may underestimate many debilitating conditions or mask metabolic compensations affecting oxidative phosphorylation, Ca^{2+} homeostasis, ROS generation or

nitric oxide biosynthesis. All processes regulate physiologic and critical pathways. For example apoptosis and necrosis (Popgeorgiev et al., 2011), cell proliferation and differentiation (Leite et al., 2012), neuronal dysfunction (Fett et al., 2010) or fish thermic acclimation (Dos Santos et al., 2013).

Mitochondrial tissue-specific was reported to five mammalian tissues (Fernandez-Vizarra et al., 2011) insects like *Drosophila melanogaster*, *Bombyx mori* and *Locusta migratoria*(Sugahara et al., 2017) and fishes such as southern catfish (*Silirus meridionalis*) (Yan and Xie, 2015), brown trout (*Salmo trutta*) (Salin et al., 2016) or *Fundulus heteroclitus*(Chung et al., 2017). Zebrafish looking around this drive questions for need to identify mitochondrial tissue-specific patters. Especially due scientific relevance of this animal model and relative lack of information about mitochondrial status in young adults.

In the present study, zebrafish mitochondrial bioenergetics measurements were carried at permeabilized tissues (Heart, Liver, Brain and Skeletal Muscle) were examined through respiratory rate, Ca^{2+} uptake, ROS generation and nitric oxide biosynthesis.

MATERIALS AND METHODS

Animal Care

Adult male zebrafish *D. rerio* (228±0.25mg) were obtained from Centro de Biotecnologia of Universidade Federal da Paraiba and acclimatized for 4 weeks in Universidade Federal de Pernambuco before experimental technics. Were kept at 26.28 ± 0.7 ºC under 14h light: 10h dark cycle and physicochemical parameters of water were monitored routinely with YSI556 MPS Multiprobe System (Supplementary data). Fish were fed twice a day with D-50 plus by Tropical® until satiety. All protocols and methods applied to fishes was approved by Comitê de Ética e Uso Animal of Universidade Federal de Pernambuco (Process 23076.029059/2016-77).

Setup reagents

Reagents used in the present study were purchased from Sigma-Aldrich Merck KGaA (Darmstadt, Germany/Brazil affiliate). Exceptions are Calcium Green 5N, MitoSOX and 2',7'-dichloro fluorescin diacetate (H_2DCFH-DA) purchase from Molecular Probes.

Tissues permeabilization

Samples of zebrafish tissues are collected after MS-222 anesthesia followed mechanical decapitation. After kill whole tissue was immersed ice-col buffered solution. Saponin 50 µg/mL (56µM) + 10 mM Ca-EGTA buffer, 20 mM imidazole, 20 mM taurine, 49 mM K-MES, 3 mM K_2HPO_4, 9.5 mM $MgCl_2.6H_2O$, 5.7 mM ATP, 15 mM phosphocreatine, 1 µM leupeptin, pH 7.1 was used to heart and skeletal muscle permeabilization (Kuznetsov et al., 2008) with minor modifications. Liver and brain are permeabilized using digitonin 10 $\mu g/mL^{-1}$ (50µM) following (Kuznetsov et al., 2008) in Clark oxygraph chamber (Hansatech - United Kingdom).

Respiration measurements

The experiments were carried at 24ºC with continuous magnetic stirring at Clark electrode oxygraph. Two buffers were used in the present study. Medium A containing 125 mM sucrose, 10 mM HEPES, 2 mM K_2HPO_4, 65 mM KCl and 1 mM $MgCl_2$, pH 7.2 (Leite et al., 2010). Medium B consists of 0.5mM EGTA, 3mM $MgCl_2.6H_2O$, 20mM taurine, 10mM K_2HPO_4, 20mM HEPES, 60mM potassium-lactobionate, 110mM mannitol, 0.3mM, dithiothreitol, BSA 1 g $liter^{-1}$, pH 7.1 (Kuznetsov et al., 2008). The substrates to each complex of respiratory chain are described in the legends of the figures and/or tables.

ROS production by H_2DCFH-DA

General ROS production was monitorated by 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFH-DA) and its carried fluorometrically with spectrofluorometer FP-6300 (Jasco Corporation - Brazil) with permeabilized tissues in the presence of 5 mM complex I mixture (pyruvate, malate, glutamate and Ŭ-ketoglutarate acid). The þuorescence was observed under a 488 and 525 nm excitation and emission wavelengths, respectively and 2.5 nm slit widths. H_2DCFH-DA 25µM were incubated at 24 ºC with medium A for 40 min with continuous magnetic stirring. A calibration curve was obtained with dichlorofluorescein (DCF). Results were expressed in fluorescence units (FU).

mtROS superoxide generation

Mitochondrial-targeted probe MitoSOX [3,8-phenanthridinediamine, 5-(6Ŋj triphenylphosphoniumhexyl)-5,6-dihydro-6-phenyl] was used to evaluate Mitochondrial O_2Å production. MitoSox 5 µM were incubated in medium A for 40 min at 28º C at continuous magnetic stirring with spectrofluorometer FP-6300 (Jasco Corporation ï Brazil) an excitation and emission wavelengths of 510 and 580 nm (following manufacturer instructions - Molecular Probes) and 5 nm slit widths. Antimycin A 12 µM was used to stimulated superoxide production at Complex III level. Results were expressed in fluorescence units (F.U.).

Zebrafish mitochondrial Ca^{2+} uptake

Extramitochondrial Ca^{2+} uptake was measured through 1µM Ca^{2+} Green 5N probe suspended in medium A with 5 mM pyruvate, malate, glutamate and Ŭ-ketoglutarate acid as substrate. Excitation/emission wavelengths and slit widths was 488/525 nm and 5 nm, respectively at 28 ºC. Digitonin 50µM was used for permeabilize liver and brain (Huang et al., 2013). Results were expressed in fluorescence units (F.U.).

Nitric oxide production

NO release was estimated with 5 µM DAF-FM-DA an 28º C at 495 nm excitation and 515nm emission wavelengths at continuous magnetic stirring with spectrofluorometer FP-6300 (Jasco Corporation - Brazil). In all tests 1 µM catalase and 1 µM SOD were added to minimize $O_2^{\bullet-}$ and H_2O_2 to lead out the possibility of $ONOO^-$ generation that will decrease NO detection as consequence. Influence of $NO^{\bullet}$ donor, SNAP use as a positive control, as well L-NAME as $NO^{\bullet}$ inhibitor) to all permeabilized zebrafish tissues. These experiments were carried following (Leite et al., 2010; Pinard and Robitaille, 2008).

Statistical analyzes

All experiments were analysed with one-way ANOVA and Tukey pos-hoc test after Kolmorov-Smirnov normal verification. Student-*t* test were used to only two means comparations. To H_2DCFH-DA and DAF-FM-DA test a calibrated curve was obtained. Results were considered significant starting $p<0.05$.

RESULTS

Mitochondrial respiration

Zebrafish permeabilized tissues basal respiration showed differences at Complex IV_3 (ADP presence) between Heart and SM. Heart respiratory rates was -41%, -35% and -48% for Liver, Brain and SM, respectively (n=4, p=0.03 to SM). However, no difference is observed to RC for any tissues considered (Table 1). SM has higher respiration rate (ADP presence) at succinate dehydrogenase level, $p<0.001$ (+54,7%, +48,9%, +50,9% compared to Heart, Brain and Liver, respectively). Heart is the chief organ presenting differences between Complexes I and II V_3 ($p=0.002$). SM oligomycin inhibition to Complex I and II was <29% and <40% when compared to another tissue, respectively. Similarly, SM showed CCCP maximal respiratory rate +32% when compared to Heart. It should be noted that any tissue showed differences at RC level.

SM has highest activity for Cytochrome c oxidase activity +41,7%, +28,4% and +34% compared to Liver, Brain and Heart respectively, p=0.0001 (15.572 ± 0.26 nmolO_2/mL) while Heart was < -76.5% respiratory rate to Complex III. All tissues showed differences between complex III and IV. Mitochondrial respiratory chain inhibition with KCN was difference to hepatic and neuronal tissues compared with muscular cardiac and skeletal.

ROS production

ROS general production through H_2DCF-DA measurement was different to Liver and heart ($p<0.001$) (Figure 1A). Cardiac fibers presented higher ROS production by DCF detection. Liver has 95.71 ± 0.36% of total ROS production compared to heart. Brain and SM showed lower susceptibility to H_2DCFH-DA damage. The relative fluorescence presented for these tissues by DCF was 45.54 ± 3.10 and 34.93 ± 1.26 FUs, respectively. Interestingly, at 16 min only Liver have a different total ROS production compared to other tissues (p=0.0001).

ROS generation was Ca^{2+} dependent for all permeabilized tissues. EGTA or Ca^{2+} pulses affected Ca^{2+} intracellular homeostasis minimizing or leading to ROS generation. Zebrafish brain increased 47.3% ROS after Ca influx (Figure 1B). Similarly, Liver have an increase of 37.7% with Ca^{2+} pulse (Figure 1C). SM Ca^{2+} pulses have a powerful effect on ROS generation (59.8% more compared with basal state). EGTA addition in the brain and liver minimize H_2DCFH-DA damage, however no statistically difference were observed (p=0.168). Effects of EGTA pulses are strongly defined in the Heart and SM (Figure 1D and 1E). At heart level EGTA minimizesignificantly ROS generation ($p<0.0001$). This also was observed to SM with a 24.5% reduction in ROS generation.

Mitochondrial superoxide production by MitoSOXÊ Red showed a slightly different situation. Brain, followed by Liver, was the permeabilized tissue that produced a higher concentration of superoxide (Figure 2). Heart O_2Å production was 96.3 ± 0.73% lower

than that observed by brain. The relative fluorescence detected for SM was 3.98 ± 0.54 units. This result is 67.7% lower that observed by permeabilized brain. Liver is the second tissue to be more susceptible to ROS generation by zebrafish permeabilized tissues.

Mitochondrial Ca^{2+} uptake

Skeletal muscle and heart has a pronounced affinity to Ca^{2+} uptake between all studied tissues (Figure 3E). In this way, SM have approximately 40% more capacity to Ca^{2+} uptake than any other permeabilized tissue (p=0.0001). Liver have lower Ca^{2+} uptake (95% lower than SM). All muscular tissues showed a fast uptake while Liver and Brain have a slower uptake. The mitochondrial Ca^{2+} release was easily detected in Liver and Brain. Heart have a smaller Ca^{2+} release (p=0.0001) compared to the others tissues. Most of the calcium collected is mitochondrial in the Liver and brain. Although heart has high uptake, it has been observed low release of calcium by cardiac mitochondria.

NO production

Liver and Brain showed high NO biosynthesis susceptibility while Heart and SM are equally (Figure 17A). In this way, brain have 27.9% lower NO production compared to Liver. This difference is 63.5% to SM. All tissues showed a response to SNAP (NO donor) or L-NAME (NOS inhibitor). SNAP have more influence on Liver and Heart NO production compared with control (p<0.05). Brain NO biosynthesis in the presence of SNAP was different when compared to control (p=0.01) or L-NAME (p=0.004).

DISCUSSION

The differences found between tissues probably show how mitochondria, through oxidative phosphorylation, play different roles for systemic homeostasis. Statistical analysis of V_3 at Complex I level show differences to heart and skeletal muscle level (Table 1). Thus, although skeletal and cardiac muscles of zebrafish have the same mechanisms of myofibrils

contraction (Iorga et al., 2011) where mitochondria works particularly different. This evidence corroborates and enhances a mitochondrial density / tissue-specific pattern identification for zebrafish larvae muscle (Pelster et al., 2003). This feature is possibly due to the muscle contraction nature that requires a slightly mitochondrial different profile to ADP use at respiratory chain. Interestingly, Brain and Liver have no differences during oxidative phosphorylation at NADH dehydrogenase level ($p=0.97$). Thus, even though there are substantial differences in the physiology of each tissue, mitochondrial function appears to be equally.

Succinate ubiquinone oxidoreductase V_3 (usually known as Complex II) have differences when compared with V_3 NADH dehydrogenase. A higher oxygen consumption was observed for all tissues, especially for muscle. This result showed a importance this respiratory component to zebrafish metabolism. About this, complex II inhibition with 3NP induced more developmental abnormalities in zebrafish embryos than complex I or III inhibition (Pinho et al., 2013). Furthermore, Succinate ubiquinone oxidoreductase was recently revised such as possible site for ROS generation (Grivennikova et al., 2017). Thus, the observation of complex II activity allows to hypothesize that this respiratory complex has high physiological importance for zebrafish due fast O_2 consumption.

Oxygen consumption by Cytochrome c reductasewas similar between Brain, Liver and SM. Heart had at these moment a consumption 4-fold lower than the other tissues. This result deserves attention because Complex III also contributing to the generation of electrochemical potential, is an important site-specific for ROS generation and its inhibition leads a direct to normal-to-dead transition in zebrafish embryos (Chen et al., 2003; Figueira et al., 2013; Pinho et al., 2013; Tahara et al., 2009). This physiological basal response increases the susceptibility of this tissue to ROS generation. Indeed, we found that zebrafish cardiac

muscle is one tissues that generation ROS at higher levels when compared to the other tissues (Figure 8).

Cytochrome c oxidase COX (also knower as Complex IV) O_2 consumption was strongly different when compared to all permeabilized tissues. This same pattern has already been observed especially for the permeabilized Brain (Bourdineaud et al., 2013). Additionally, COX deficiency induced using morpholinos to reduce expression of Cox showed tissue-specific responses in zebrafish embryos (Baden et al., 2007). In this way, complex IV activity in the zebrafish may become an attractive tool for the study of pathological disorders related to this process given the apparent importance of this complex to the respiratory chain.

Heart lower mitochondrial activity at COX level observed in the present study diverges from the accordance to the fact that in mammals the heart is the organ that consumes most energy per mass (Goffart et a. 2004). Here, zebrafish skeletal muscle appears the most mitochondrial actives and consequently consumes most energy.

Tissue-specific pattern for ROS generation was reported to five rat tissues and side-to-side comparison reveal that complexes I and III are important sites to ROS generation (Tahara et al., 2009). Thus, these two respiratory complexes functioning can regulate many pathophysiological processes. Like this zebrafish NADH dehydrogenase and cytochrome c redectasedysfunction leads to neuronal dysfunction and impairments to angiogenesis (Cho et al., 2013; Flinn et al., 2009).

ROS generation observed was tissue and Ca^{2+} dependent for all permeabilized tissues. Interestingly, Liver showed quickly and higher susceptibility to ROS generate. In this sense, fish liver can be an important marker for waterborne pollutants. In this context, zebrafish liver accumulated the highest cadmium concentration and is highly responsive to

hepatotoxins (Gonzalez et al., 2006; Wang et al., 2010). However, cardiac muscle can become equally important for ROS generation. This becomes important because of two factors. First, mitochondrial and cardiovascular dysfunction affects zebrafish development (Pinho et al., 2013), second is that zebrafish cardiovascular tissue is an important site for H_2O_2 generation (Panieri et al., 2017) which suggests that this may be main forms of ROS generated in this tissue. Certainly, superoxide generation for this permeabilized tissue ir very low (Figure 2).

To prove that the ROS generation observed with unspecific probe H_2DCF-DA was really became trough mitochondria we used MitoSox. Our results showed that Liver and Brain permeabilized tissues has more susceptibility O_2Å generation. This is particularly interesting due physiological role of these tissues. In this way, neuronal cells have high susceptibly to damage and hepatic tissue is recognized by high metabolic activity. Therefore, the combination of these characteristics with the high relation between superoxide generation and apoptosisleads to high probability of pathophysiological conditions development (Kudin et al., 2004; Schweikl et al., 2017). Moreover, any factor that stimulates the production of superoxide by tissues that do not produce much O_2ÅËbecomes a highly a deleterious factor.

Recently was demonstrated that neuronal superoxide generation is related to nitric oxide synthase (Ihara et al., 2017). Thereby searching for NOĘtissue-specific patterns can enhance NOĘphysiological roles, especially because of its relationship with oxidative stress. Here we found that hepatic tissue is a key to NOĘbiosynthesis (Figure 4A). About this, Liver play important roles in the hepato- physiology and pathophysiology (Iwakiri and Kim, 2015). Thus, zebrafish Liver may be a useful window to understand the hepatic diseases development. Specially when we found SNAP increases NOĘand can be employed as a modulator for hepatopathology.

In other hand, we found that Brain also has higher NO production. Once NO is deep involved on neuronal signaling and this is a well-known wayfor neurodegeneration and neuropathic pain (Mukherjee et al., 2014). Zebrafish nNOS activity also drives to viable Brain use to study of neuronal diseases.

CONCLUSION

Permeabilized zebrafish tissues showed that mitochondrial function is essentially different. Certainly, mitochondrial machinery has relevant compensatory strategies for each tissue depending to their function and metabolic activity. This feature does not limit the normal metabolic pathway but also stress conditions in the event of cellular damage. Thus, more caution is required during the experimental design in studies with mitochondrial approaches using adult zebrafish experimental model.

Acknowledgements

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Table 1. Zebrafish mitochondrial respiration rate to complexes I and II. Mitochondrial respiration in presence of 5mM NADH substrates (pyruvate, malate, glutamate and Ŭ-ketoglutarate) and 5 mM succinate + 2 µM rotenone to Succinate dehydrogenase. Values expressed as mean ± SE (nmol O_2/mg/min) except to Respiratory Control (RC). n=4. [a and b] Are V_3 NADH dehydrogenase statistical groups p=0.03. [c and d] is V_4 Succinate dehydrogenase statistical groups (p<0.01). *p=0.03. ** p<0.01. # p<0.001. V_3 = ADP phosphorilation. V_4 = Oligomycin inhibiton. V_{FCCP} = Unclouped state.

	Complex I (NADH dehydrogenase)				**Complex II (Succinate dehydrogenase)**			
	RC	V_3	V_4	V_{FCCP}	RC	V_3	V_4	V_{FCCP}
Liver	1.99 ± 0.09	2.66 ± 0.06[a,b]	1.36 ± 0.07	1.36 ± 0.14	2.41 ± 0.08	3.67 ± 0.17	1.55 ± 0.10[c]	2.34 ± 0.04
Brain	2.56 ± 0.21	2.42 ± 0.23[a,b]	1.05 ± 0.14	1.05 ± 0.14	2.05 ± 0.04	3.82 ± 0.02	1.87 ± 0.04[c]	2.27 ± 0.06
Heart	1.20 ± 0.15	1.56 ± 0.10[b]*	1.11 ± 0.05	1.52 ± 0.13	1.33 ± 0.05	3.39 ± 0.20	2.55 ± 0.12[c]	3.56 ± 0.18
SM	1.98 ± 0.05	2.96 ± 0.12[a]	1.57 ± 0.08	1.79 ± 0.08	2.14 ± 0.12	7.48 ± 0.54[#]	4.28 ± 0.34[d]**	5.26 ± 0.34

Table 2. Complex III and IV oxidations by adults zebrafish. Zebrafish mitochondrial respiration with Antymicin A 12µM, TMPD 0.5mM + Asc 2µM and 1mM KCN to complex III and IV. Values expressed as mean ± SE (nmol O_2/mg/min) n=4. * and # represent a statistical difference between permeabilized tissues p=0.01, # p<0.001, respectively. **p<0.05.

	AA	**TMPD + Asc**	**KCN**
Liver	2.404 ± 0.02	9.078 ± 0.15	3.432 ± 0.11
Brain	2.117 ± 0.04	11.145 ± 0.34	3.673 ± 0.08
Heart	0.537 ± 0.26[*]	10.271 ± 0.34	6.301 ± 0.16 [**]
SM	2.285 ± 0.05	15.572 ± 0.26 [#]	7.260 ± 0.20 [**]

Figure 1. Oxidation of 2',7'-dichloro fluorescin diacetate (H_2DCFH-DA) by Zebrafish tissues. ROS generation by Zebrafish tissues supplemented with 5mM of Complex I substrates. (**A**) Comparative ROS generation by different tissues (* p<0.001). B, C,D and E were performed with 10 µM Ca^{2+} and EGTA to act to as Ca^{2+} chelator. (**B**) H_2DCFH-DA oxidation by Brain Zebrafish (* p<0,001). (**C**) Liver H_2DCFH-DA oxidation + 10 µM de Ca^{+2} ((*p<0.009). (**D**) HeartH_2DCFH-DA oxidation (* p=0.000, **p<0.001). (**E**) Skeletal Muscle H_2DCFH-DA oxidation (* p<0.02, ** p<0.01). # p=0.0001. Values are given as means ± SE (n=3).

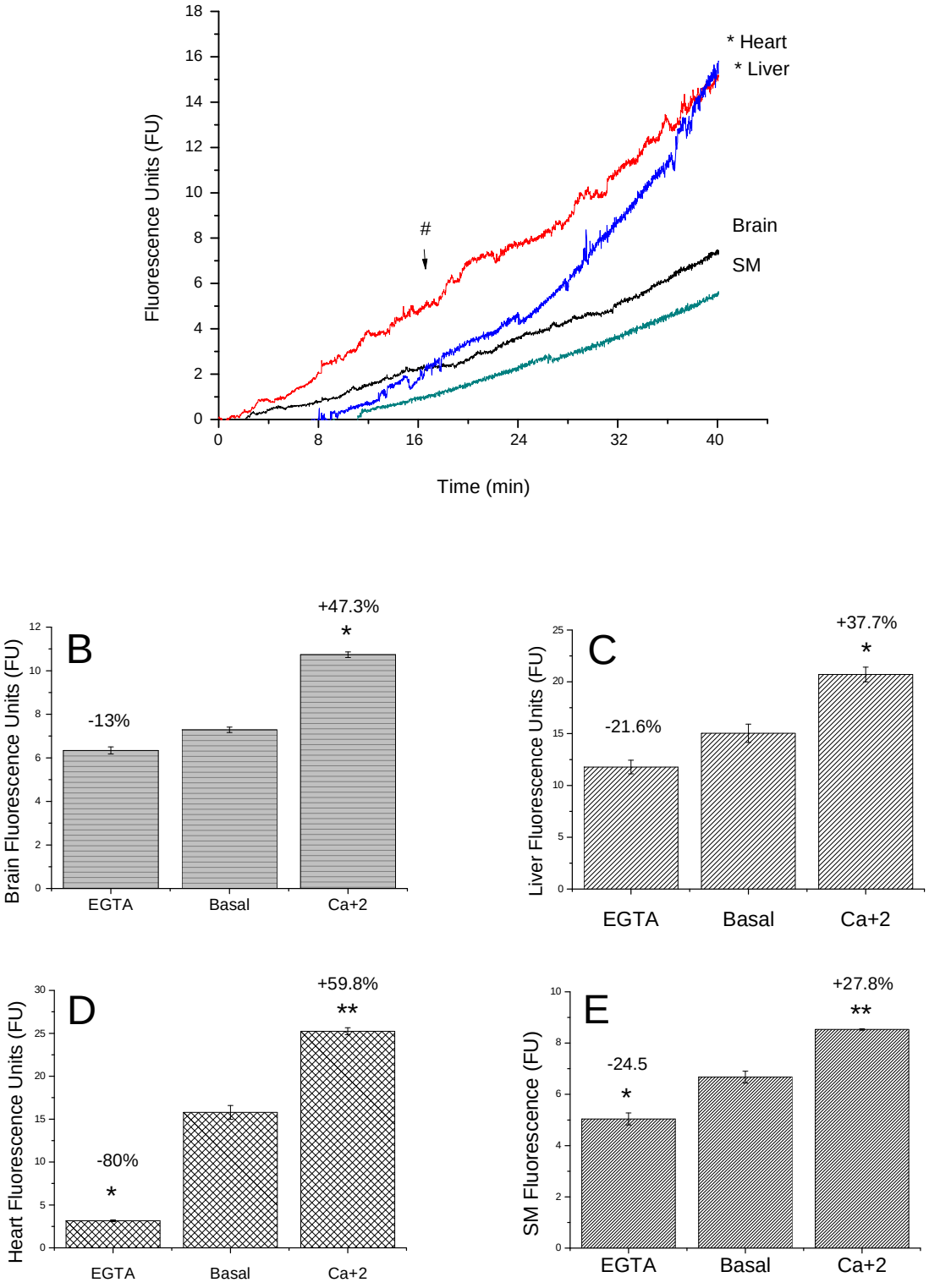


Figure 2. Superoxide production by MitoSOXÊ Red monitoring. Mitochondrial superoxide generation as determined under basal conditions of each zebrafish tissue during 40 min at 28ºC using MitoSOXÊ Red Mitochondrial Superoxide Indicator (5 µM) in the presence of 5 mM complex I mixture (pyruvate, malate, glutamate and Ŭ-ketoglutarate acid). Liver and Brain tissues has higher O_2Å generation *($p<0.001$) ** ($p=0.0001$). Results are expressed as means fluorescence ± SE (n=3).

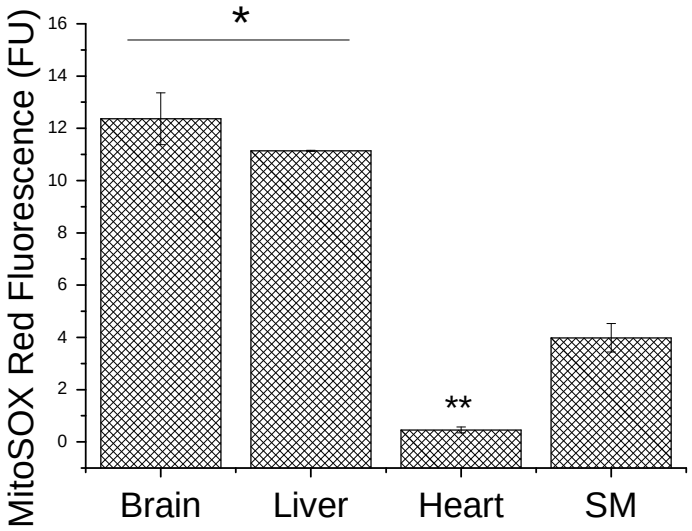


Figure 3 ï Ca^{2+} Green uptake by Zebrafish permeabilized tissues. All experiments were lead in the medium A containing 5mM of mix Complex I substrates and 1µM Calcium Green 5-N. Brain and liver were permeabilized with 50µM digitonin. Heart and SM were permeabilized as previously described. One pulse of 0.5µM FCCP were added in all experiments. Panel A show permeabilized Liver Ca^{2+} uptake. Panel B show permeabilized brain Ca^{2+} uptake. C and D showed permeabilized Heart and Skeletal Muscle (SM) respectively. Panel E show a comparative difference between zebrafish permeabilized tissues to Ca^{2+} Green uptake and Ca^{2+} release (n=5).

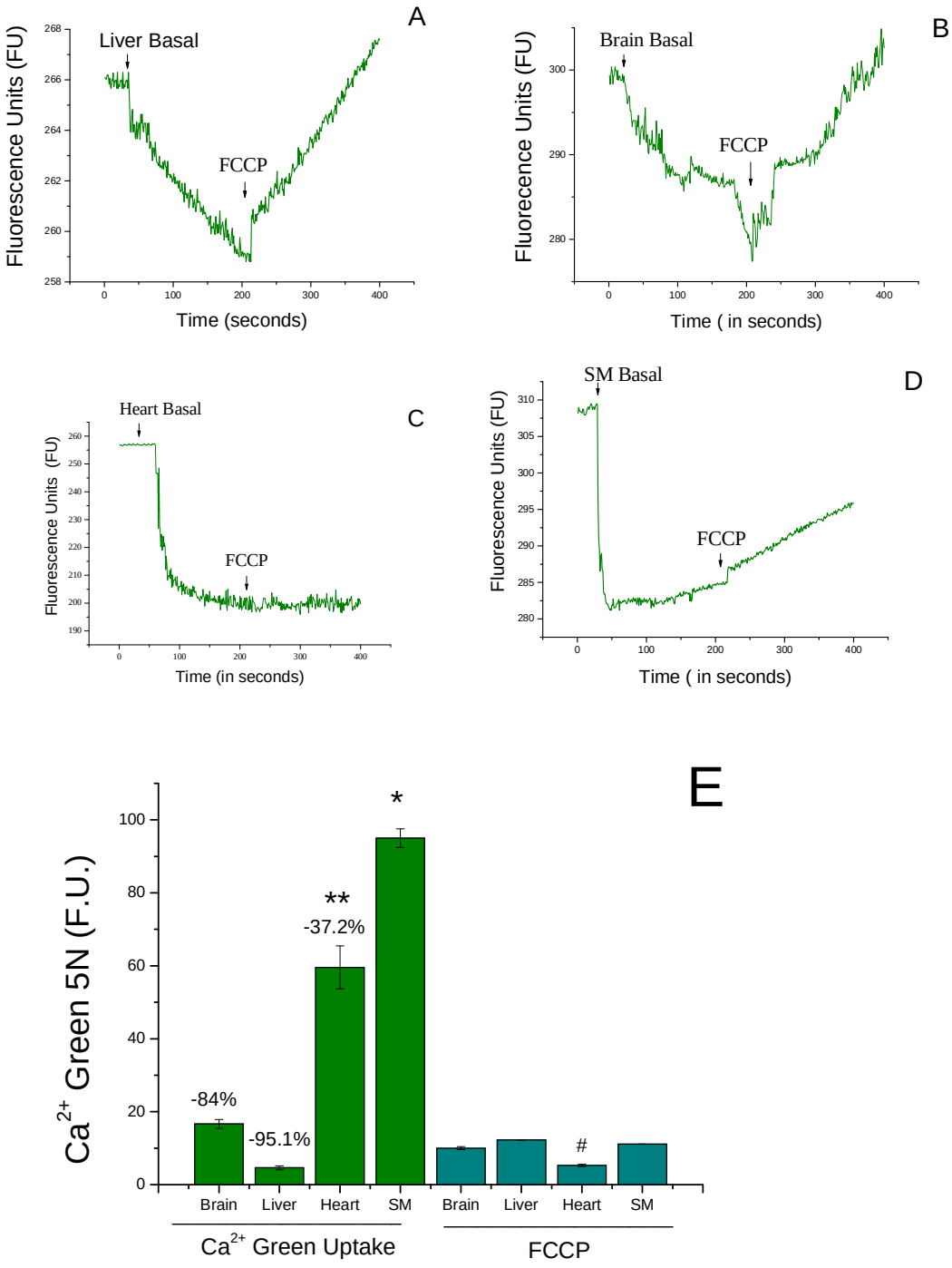
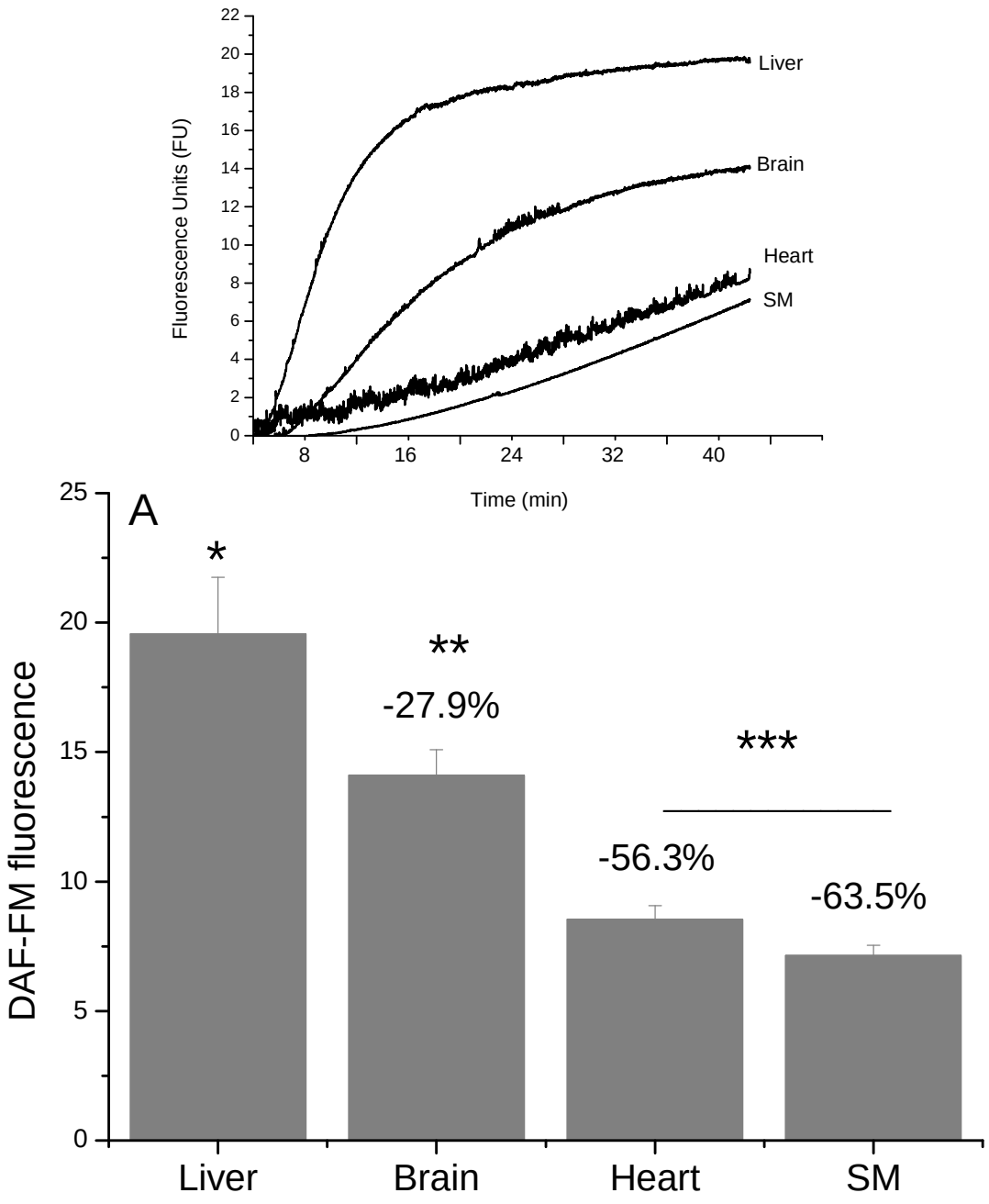


Figure 4 - Nitric oxide production by zebrafish permeabilized tissues. $NO^{Å}$ production was continuously monitored with 5µM DAF-FM added 1µM catalase and 1µM SOD. Panel **A**: Represent basal $NO^{Å}$ production (only DAF-FM was applied. Liver (19.55±2.19), Brain (14.09±1.00), Heart (8.53±0.54) and SM (7.13±0.40) *p=0.003 ** p=0.001 and ***p=0.001. 2 µM SNAP increases NOS activity while 50 µM L-LAME partially inhibit to B, C and D. Panel **B**: Represent Brain $NO^{Å}$ production. *p=0.01. Panel **C**: Represent Liver $NO^{Å}$ production. *p=0.03. Panel **D**: Represent Heart $NO^{Å}$ production. *p=0.03. Panel **E**: Represent SM $NO^{Å}$ production(n=4).



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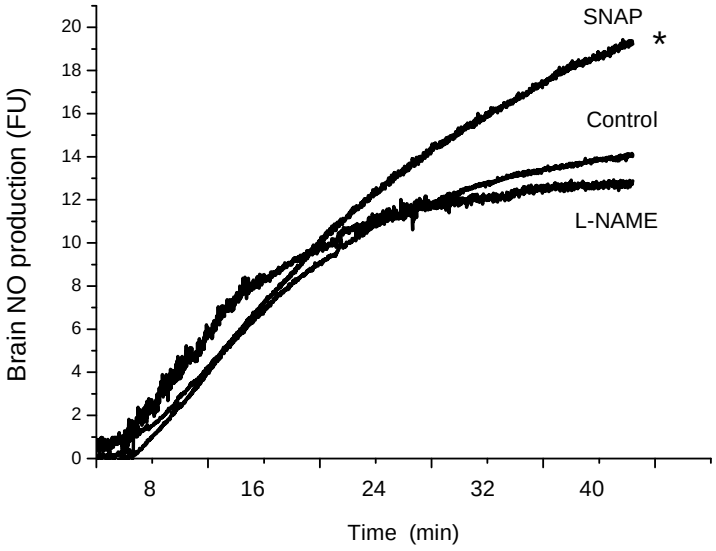
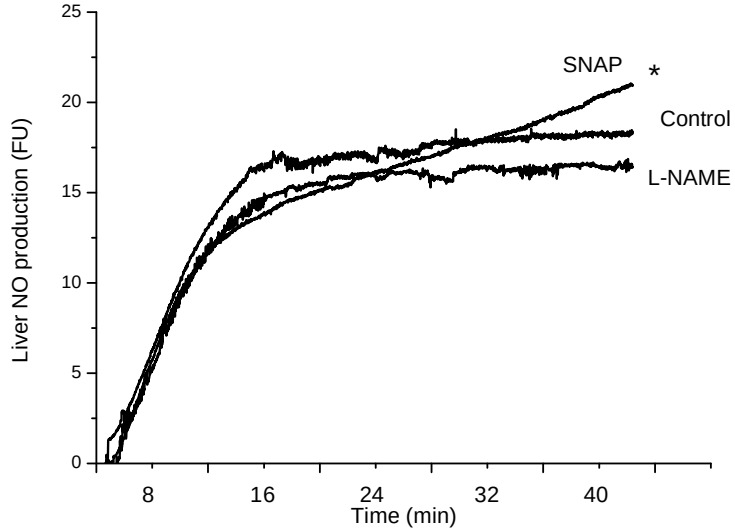

SNAP
*
Control
L-NAME
Brain NO production (FU)
0
2
4
6
8
10
12
14
16
18
20
8
16
24
32
40
Time (min)

C


SNAP
*
Control
L-NAME
Liver NO production (FU)
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10
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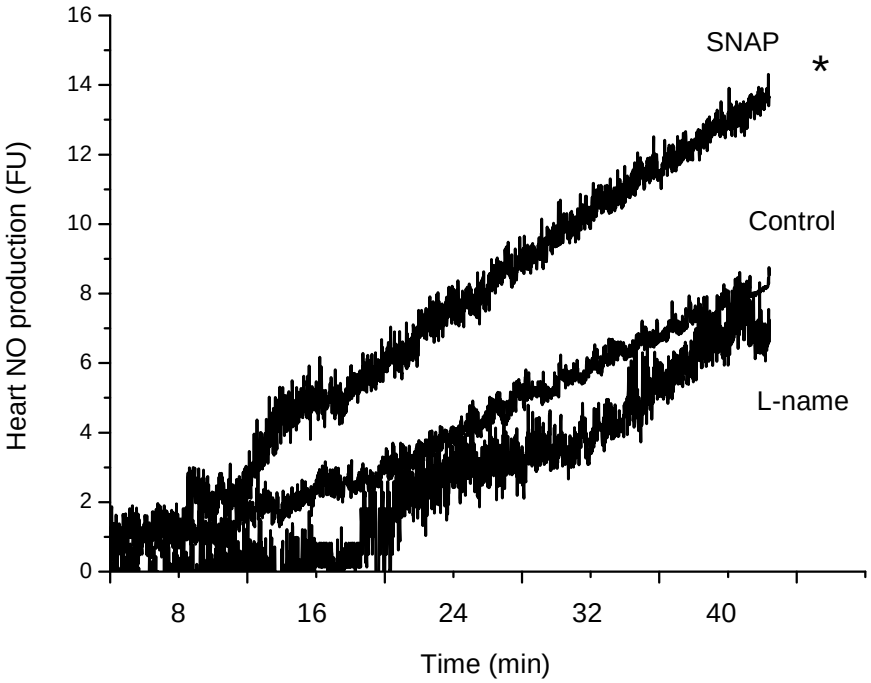
Time (min)

D


Heart NO production (FU)
16
14
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Time (min)
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Control
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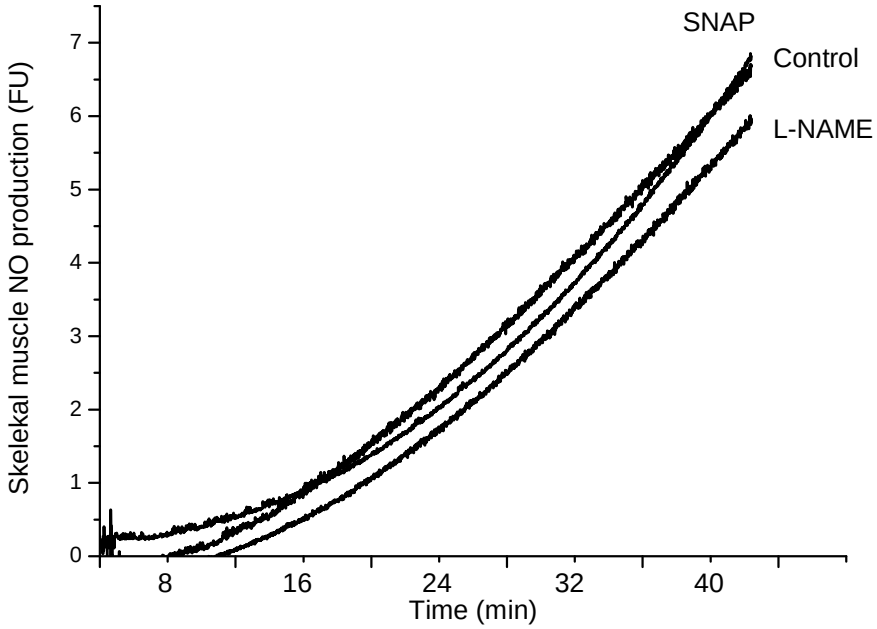
Skelekal muscle NO production (FU)
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Time (min)
SNAP
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Figure 5 - GRAPHICAL ABSTRACT

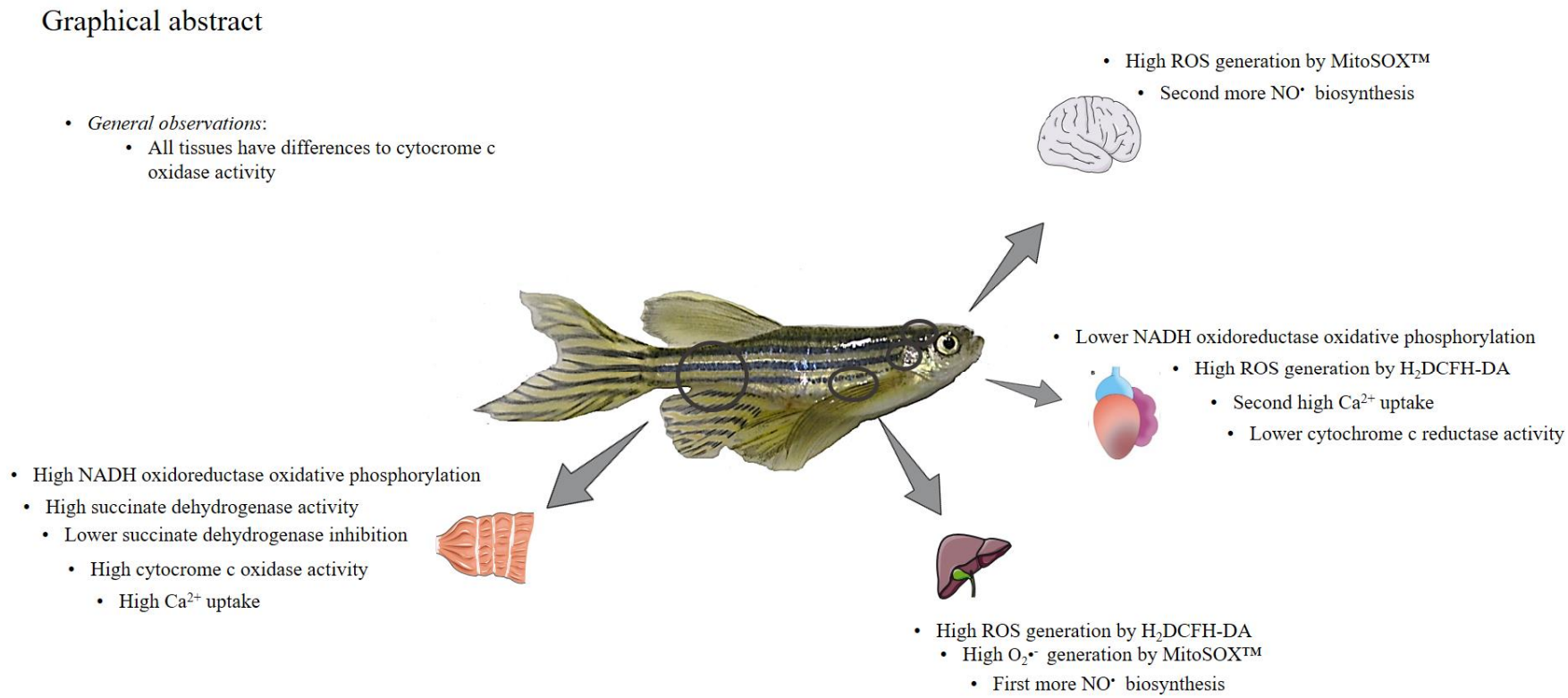

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Table 3 - Supplementary material 1.

	Tanks	Recirculation system
Temperature (ºC)	26.3±0.7	26.2±0.7
Conductivity	0.313±0.1	0.283±0.1
Dissolved solids	0.203±0.1	0.180±0.1
Salinity	0.146±0.1	0.131±0.0
Dissolved oxygen	65.078±8.3	71.044±6.5
pH	7.540±0.1	7.433±0.2

3.3 ARTIGO 3: PYRIPROXYFEN SIDE EFFECTS ON ZEBRAFISH BRAIN MITOCHONDRIA AND ACETYLCHOLINESTERASES

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TITLE PAGE

Pyriproxyfen side effects on zebrafish brain mitochondria and acetylcholinesterases.

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Abstract (***max 400 words***)

Pyriproxyfen is a common insecticide which has bio-mimetic juvenile hormone mechanism. Although there are worldwide use the pyriproxyfen side effects on non-target organisms drives to possible exposure risk. The goal of this paper is show zebrafish metabolic and synaptic impairments by low concentrations of pyriproxyfen using mitochondrial and enzymatic indicators. Zebrafish male adults were exposed to 0.001, 0.01 and 0.1 µg/mL pyriproxyfen concentrations for 16h. Subsequently, i*n vitro* exposition to 0.0001 ï 10 µM pyriproxyfen was performed to brain acetylcholinesterase assay. Mitochondrial respiratory chain was assessed by selective permeabilization with 50 µM digitonin followed by mitochondrial respiratory complexes I-IV evaluation. Reactive oxigen species generation was estimated using H_2DCF-DA. Subsequently mitochondrial specific $O_2ÅË$ generation was monitored with MitoSOX Red. Permeabilized tissue Ca^{2+} transport was monitored by Calcium GreenÊ 5N. Nitric oxide synthase (NOS) activity was estimated with DAF-FM-DA. Brain acetylcholinesterase showed IC_{20} with 0.33 µM pyriproxyfen. To mitochondrial respiratory chain NADH dehydrogenase (Complex I) and Succinate dehydrogenase (Complex II) the respiratory control (RC) decrease for all pyriproxyfen concentrations tested. ROS detection by H_2DCF-DA was an increase around 40% for all concentrations studied. Nevertheless, MitoSOX as a more precise probe, showed a $O_2ÅË$generation by dose-dependent effect. Brain tissue lost 50% of Ca^{2+} uptake capacity by 0.1 µg/mL pyriproxyfen concentration. In this hand, Ca^{2+} release showed a clear mitochondrial impairment by all lower pyriproxyfen expositions. Thus, the Ca^{2+} transport imbalance caused by pyriproxyfen may a new model of action. Discrete changes in NOS activity were observed after exposure to pyriproxyfen, especially to 0.1 µg/mL pyriproxyfen concentration. The set of these results drives that pyriproxyfen affects the electron transport chain correct functioning, ROS generation and calcium homeostasis on zebrafish. Considering high similarities between this animal model and the human, more caution is needed during the insecticide use to urban or agricultural pests control.

Keywords: Pesticides. Neuronal mitochondria toxicity. Oxidative stress. Ca^{2+} transport. Reactive oxygen species. Nitric oxide.

Highlights

- A multilevel approach to pyriproxyfen neurotoxicity was performed;
- Zebrafish brain acetylcholinesterase showed a IC_{20} with 0.33 µM pyriproxyfen;
- Mitochondrial coupling efficiency at succinate dehydrogenase level was disrupting by lower concentrations of pyriproxyfen;
- Brain O_2ÅË production by cytochrome c oxidoreductase inhibition with antimycin A showed a dose-dependent to pyriproxyfen;
- Calcium transport changes at the cytosolic and mitochondrial levels after insecticide exposure were found;
- Pyriproxyfen no show a NOS activity as major route to cellular impairment.

1. INTRODUCTION

Pyriproxyfen is a pesticide with broad spectrum mechanism. Our uses include antiparasitic drugs to petôs products, home and agricultural pest control. Biologically, the pathway to pyriproxyfen works involved a bio-mimetic juvenile hormone mechanism, consequently, disturb embryogenesis and adults emergence is related. To insects, the juvenile hormone plays a key role to metamorphosis, sex differentiation, courtship, locomotor system, behavior and central nervous system (CNS) (Baumann et al., 2017). Thus, is a very useful tool to minimizes mainly crop losses or to reduce tropical arboviruses vectors. Some researches demonstrated that pyriproxyfen may produce adverse effects on the aquatic ecosystem (Vieira Santos et al., 2017). In this way, several health risks are computed to pesticides (Singh et al., 2018). Particularly, pyriproxyfen residue contaminated food and represent a possible dietary risk factor (Du et al., 2017; Du et al., 2018). Recently, a delicate discussion about a possible linkage between pyriproxyfen and microcephaly cases acquires notability on media and scientific forums. This is because pyriproxyfen has been reported by partially endocrine disruption, besides presenting dietary risk and developmental toxicity (Bayoumi et al., 2003; European Food Safety, 2009; Linton et al., 2009). Likewise, due pyriproxyfen react with retinoic acid, a regulatory component to CNS development and maybe lead to same disturbs. Actually, the discussions about if pyriproxyfen not causes (Dzieciolowska et al., 2017) or has correlation with microcephaly (Parens et al., 2017) remain open.

Zebrafish model made possible a precise evaluation of side effects at aquatic toxicological level. About this, zebrafish conquest the tittle of model to analyzing environmental pollutants impact (Bourdineaud et al., 2013). Especially when consider mitochondrial bioenergetics, acetylcholinesterase, CNS development, neurobehavior, toxicokinetics and toxicodynamics studies (Altenhofen et al., 2017; Bourdineaud et al., 2013;

Nishimura et al., 2016). A multilevel accomplishment about these features represents a useful strategy to ameliorate the knowledge about chemicals damage to many organisms and physiological conditions. Thereby, brain acetylcholinesterase is a well-known biological marker to estimate toxic impacts. Through its ability to hydrolyze acetylcholine this enzyme promote environmental/toxicologic measurement with less time-consuming, lower costs and high sensibility (Assis et al., 2010). Zebrafish acetylcholinesterase combines the precision of enzyme with sophisticated features of a new animal model.

Complementarily, zebrafish mitochondria bring up to current discussions all the myriad of biological process that it regulates. Notably, this little teleost also has tittle of model to mitochondrial bioenergetic and diseases related study (Steele et al., 2014). Its well known that mitochondria has long been recognized not only by ATP supply. This organelle is deep involved on sophisticated cellular process such as redox signaling, Ca^{2+} homeostasis, ROS generation, oxidative stress, cell death and epigenetic crosstalk (Cowie et al., 2017; Esterberg et al., 2014; Huang et al., 2013; Murphy, 2009; Weinhouse, 2017). Therefore, mitochondria is employed as a physiological marker of species resilience to environmental changes or stressors (Bourdineaud et al., 2013; Jayasundara, 2017). The correlation between mitochondrial function and pesticides side effects has viable an exact identification of just how these chemical compounds injured cell function and subsequently, all biological system. Pesticides like methylparathion, carbofuran, metolachlor are reported by mitochondrial respiratory chain and respiratory control (RC) impairment as well loss on mitochondrial membrane potential (Akbar et al., 2012; Pereira et al., 2009).

Intrinsically, mitochondrial respiratory chain is a key to reactive specie (RS) generation (Murphy, 2009) include lipid peroxidation, reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation. Pathophysiological, physiological or from environmental stressors triggered ROS status. In this way, ROS generation and calcium

homeostasis are process very closed. Indeed, increasing evidences suggests a cellular damage due to mutual interplay between calcium and ROS generation (Görlach et al., 2015).

ROS generation by pesticides exposure has been reported (Akbar et al., 2012; Jin et al., 2010). However, understand pesticides side effects on intracellular calcium homeostasis urgently needs of more information. In another hand, the match between nitric oxide (NO) and pesticides effects has quickly responses because pesticides like rotenone are employed to neuronal diseases establishment and is well-know that NO have a deep involvement with Parkinson, Alzheimer and Sclerosis (Joern et al., 2010).

Summarily, this work intends to show the probable pyriproxyfen side effects to neuronal tissues at acetylcholinesterase and mitochondrial levels.

2. MATERIAL AND METHODS

2.1. Animal husbandry and pyriproxyfen exposition

Zebrafish male adults were purchase at Recife ï Brazil and acclimated to laboratory conditions for one month before experimental techniques. In this place, the animals were arbitrarily divided in fifteen flow-through tanks. Fishes were fed twice per day *ad libitum* with D-50 plus diet (Tropical®) and submitted to 14h light: 10h dark cycle at 27ºC. All animal care and experimental techniques was approved by Comitê de Ética e Uso Animal of Universidade Federal de Pernambuco, Pernambuco ï Brazil (Process 23076.031986/2017-38) following procedures of the Conselho Nacional de Controle de Experimentação Animal (CONCEA). For pesticide exposition four male zebrafish were exposed to 0.001, 0.01 and 0.1 µg/mL pyriproxyfen concentrations for 16h (0.01 µg/mL or 0.01 mg/L is a limit concentration for use by World Health Organization [WHO] recommendation) After this, the animals were individually anesthetized with MS-222 (Tricaine methanesulfonate) and neck-breaking decapitation was carried. Brain tissues were collected and immediately placed on ice.

Most reagents employed on this paper were purchase of Sigma-Aldrich Merck KGaA (Darmstadt, Germany/Brazil affiliate). However, MitoSOXÊ and Calcium Green 5N were purchase with ThermoFisher Scientific Inc/Brazil affiliate.

2.2. Acetylcholinesterase assay

Zebrafish brain tissues (20mg/mL) were homogenized at with 0.5 mol/L Tris-HCl buffer, pH 8.0. The homogenate obtained was centrifuged at 1000x*g* 4ºC for 10 min (Assis et al., 2012). The supernatant was employed to acetylcholinesterase assay. Protein content was estimated with bicinchoninic acid (Smith et al., 1985). Enzyme assay was carried with microplate spectrophotometer (Bio-Rad xMarkê - EUA) at 405nm wavelength. At this time zebrafish brain

crude extract (20µL) was incubated with a range of 0.0001 ï 10 µM pyriproxyfen for one hour. Subsequently 200 µl of 0.25 mM DTNB diluted in 0.5 M Tris-HCl pH 7.4 was added. To acetylcholinesterase reaction was utilized 62 mM acetylthiocholine as substrate. The pyriproxyfen concentration to inhibit 20 or 50% of enzymatic activity was calculated following inhibition constant (Ki) based on (Yung-Chi and Prusoff, 1973) In this way, a unit of activity (U) was defined as the amount of enzyme capable of hydrolyzing 1 µmol of substrate per minute (Assis et al., 2010).

2.3. Neuronal tissue permeabilization and mitochondrial respiration

Neuronal zebrafish tissue (10 mg) was permeabilized using 10µg/mL (50µM) digitonin following (Kuznetsov et al., 2008). The measurement of mitochondrial oxygen consume was monitored polarographically using a Clark oxygen electrode (Hansatech ï United Kingdom). A standard buffer was used containing 125 mM sucrose, 65 mM KCl, 2 mM inorganic phosphate, 1 mM magnesium chloride, 10 mM Hepes buffer (pH 7.2) following (Leite et al., 2010) and 1 mg/mL BSA to binds fatty acids. NADH dehydrogenase (Complex I) was evaluated using 5 mM of pyruvate, malate, glutamate and Ŭ-ketoglurate as substrate. Succinate dehydrogenase (Complex II) have 5 mM succinate + 1 µM rotenone as substrate. Rotenone was necessary to prevent reverse electron flow. Cytochrome c oxidoreductase (Complex III) and Cytochrome c reductase (Complex IV) has Antimycin A and TMPD + Ascorbate as substrate, respectively.

2.4. Reactive species generation by H_2DCF-DA

Reactive species generation was monitored continuously for 40 min by enzymatic esterification of 25µM H_2DCF-DA on standard buffer previously described. This experiment was carried at Jasco spectrofluorometer FP-6300 (Jasco Corporation ï Brazil) at 28ºC and 488 and 525 wavelengths to excitation/emission, respectively and 2.5 nm slit widths. As substrate was used 5

mM pyruvate, malate, glutamate and Ŭ-ketoglutarate. A calibration curve was obtained using dichlorofluorescein (DCF), the product of H_2DCF-DA oxidation by ROS.

2.5. Mitochondrial superoxide generation

To specific mitochondrial superoxide (O_2Å) generation assessment was employed MitoSoxÊ . Digitonin 50 µM was used to permeabilize zebrafish brain. This test was performed at 28 ºC and 510/excitation, 580/emission wavelengths for 40 min under continuous magnetic stirring and 5 nm slit widths. The effect of 5µM MitoSox was monitored with Jasco spectrofluorometer FP-6300 (Jasco Corporation ï Brazil). Ubiquinol: cytochrome c oxidoreductase inhibitor (12 µM Antimycin A) was added to stimulated superoxide production. Results are expressed in fluorescence units (FU).

2.6. Ca^{2+} transport

Ca^{2+} uptake and release by brain permeabilized tissue was carried using 1µM Calcium GreenÊ -5N, Hexapotassium Salt. Brain tissue was permeabilized with 50 µM digitonin following (Huang et al., 2013) suspended on standard buffer at 28 ºC with an excitation and emission wavelengths of 488/525 respectively and 5 nm slit widths on Jasco spectrofluorometer FP-6300 (Jasco Corporation ï Brazil). Mitochondria was energized with 5 mM pyruvate, malate, glutamate and Ŭ-ketoglutarate. Mitochondrial membrane complete disruption, leading to Ca^{2+} release was performed with the addition of 1 µM CCCP at 200 seconds.

2.7. Nitric oxide production

Nitric oxide brain generantio was estimated with 5 µM DAF-FM-DA at 495 nm and 515 nm filters and 2.5 slit widths at Jasco spectrofluorometer FP-6300 (Jasco Corporation ï Brazil). This test also was carried at 28ºC under moderated agitation. To minimize the probably interference

of O_2Ãand H_2O_2 generation, were added to the standard buffer 1µM catalase and 1 µM superoxide dismutase. Calibration curve was obtained with SNAP controlled additions a NOĘdonor

2.8. Statistical analyses

After Kolmorov-Smirnov normal check, all results were analysed with one-way ANOVA with Tukey pos-hoc test. Student-*t* test were used to only two means comparations. To H_2DCFH-DA and DAF-FM-DA assays a calibrated curve were obtained, and the results was applied to line equation. Results were considered significant starting $p<0.05$.

3. RESULTS

Pyriproxyfen has an able of decrease 20% (IC_{20}) of zebrafish brain acetylcholinesterase activity with 0.33 µM. Although controversial, the IC_{20} use to safe limits establishing of increase especially for acetylcholinesterase assays (Araújo et al., 2016; Wang et al., 2010). Furthermore, Food and Agriculture Organization (FAO) guidelines registered the importance of IC_{20} estimative for food security, for example (FAO, 2007). Exposures at low pyriproxyfen concentrations have a rapid enzymatic inhibitory effect as shown Figure 1.

Phosphorylation at NADH dehydrogenase (Complex I) level appears slightly lower after pyriproxyfen exposure when compared to the control. Indeed, this reduction is of approximately 15%. Likewise, phosphorylation inhibition with oligomycin showed no significant difference although have an approximately change of +34% compared to control ($p>0.05$). At this time, be a probable electron flux increase after a lower exposure to pesticide. CCCP maximal respiration exhibited an approximately decrease of -19% after any pyriproxyfen exposure. The key impairment of pyriproxyfen for NADH dehydrogenase was observed after RC calculation (rate between state 3 [ADP presence] and 4 [Oligomycin inhibition] of respiratory rate) where we found -28.1% and -37% of RC to 0.001 µg/mL and 0.01/0.1 µg/mL pyriproxyfen compared to control, respectively $p<0.04$(Table 1). Interestingly, Succinate Dehydrogenase (Complex II) CCCP maximal respiration was no detected for all animals that were exposed to insecticide concentration (Table 1). This shows a possible damage to respiratory chain coupling. Equally to observed with NADH dehydrogenase (Complex I), pyriproxyfen have a key impairment of -34%, -37% and -43% to Succinate Dehydrogenase RC to pesticide concentrations tested ($p<0.001$).

Subsequently, pyriproxyfen appears compromise cytochrome c oxidoreductase activity as show Table 2. However, no statistically differences were detected ($p=0.06$) between control and 0.1 µg/mL pyriproxyfen. This represent a RC reduction of 26% on our

O_2consumption, thus, concludes that exist a little more susceptibility to inhibition with classical antimycin A. To cytochrome c reductase, the differences are more clear wherever control and 0.1 µg/mL pyriproxyfen has difference p=0.002 an increase of +19% to O_2 consumption.

ROS yield was stimulated after insecticide exposure. To a range of 0.001 ï 0.1 µg/mL ROS have an increase around 40 ± 6.7% (mean ± SD) to each pyriproxyfen concentration p<0.01 by H_2DCH-DA measurement (Figure 2). In a corroborative way, MitoSOX showed that mitochondrial superoxide generation (O_2ÅË) by cytochrome c oxidoreductase inhibition showed a dose-dependent effect p<0.001 as shown in Figure 3.

Calcium transport appears progressively changes by pyriproxyfen exposure. After selective permeabilization with 50 µM digitonin (Huang et al., 2013) and mitochondrial energization with 5 mM pyruvate, malate, glutamate and Ŭ-ketoglutarate, brain tissues show a Ca^{2+} uptake. The mitochondrial uncoupler carbonylcyanide 3-chlorophenylhydrazone (CCCP) addition have effect only to control group (Figure 4B and 4C) while insecticide groups showed no fluorescence changes when CCCP is present. Remarkably, 0.1 µg/mL pyriproxyfen significantly reduce Ca^{2+} uptake ability p=0.001 (decrease around 50% when compared to control) (Figure 4A). Already mitochondrial Ca^{2+} release was profoundly affected by pyriproxyfen. The figure 4B show that lower pyriproxyfen concentrations has strongly negative effect at mitochondrial Ca^{2+} release p=0.001. It is 27.97 ± 1.8 FU (mean ± SE) compared to approximately 3.50 ± 0.6 FU (mean ± SD). In both cases, the Ca^{2+} release is profoundly reduced around 80%.

Nitric oxide production was very similar between tested situations as shown Figure 5. However, 0.1 µg/mL pyriproxyfen concentration discreetly NOĘbiosynthesis when compared to control p=0.035. In this hand, this result shows a possibility that pyriproxyfen

284 does not have the NOS activity as a major route of cellular damage, nevertheless shows that
285 deleterious physiological features may be correlated with crucial NOS activity.

286 **4. DISCUSSION**

287 The pyriproxyfen is a common insecticide with bio-mimetic juvenile hormone
288 way to action. Although it has little effect on larval mortality, its effect is analogous to
289 juvenile hormone, thus rapidly prevents adults emergence. Thus, insect maturation is severely
290 compromised. Therefore, it is an attractive tool for pest management. In this way, many
291 insects have hormone regulation on neuroendocrine system. Especially, insects like *Aedes*
292 *aegypt* (an important diseases vector present in developing countries) that have the *corpus*
293 *allatum* connected with the brain tissue. This is a perfect match between pyriproxyfen action
294 and neuroendocrine system of target organisms. Thus, this work was designed to check
295 possible pyriproxyfen side effects on brain tissues of non-target organisms. For this work,
296 brain acetylcholinesterase and mitochondria were used to evaluated possible neuronal
297 damages.

298 Pesticide effects on cholinesterases is a well-known useful tool to estimate side
299 effects on non-target organisms from fishes to human neuronal toxicity. This is because
300 acetylcholinesterase is a key enzyme for hydrolyzing acetylcholine during cholinergic
301 synapses. In this way, pesticides like tebuconazole, imazalil, chlorpyrifos, atrazine and
302 malathion has been reported to inhibit expression and activity of zebrafish AChE (Altenhofen
303 et al., 2017; Jeon et al., 2016; Jin et al., 2016; Liu et al., 2016) and rats (Abdel-Salam et al.,
304 2017). Once it was found in the present research that pyriproxyfen showed a AChE inhibitory
305 effect with 0.33 µM (IC_{20}) its potential for neuronal impairment was registered. This result
306 show, in first hand, that pyriproxyfen affects directly fish AChE activity. Zebrafish
307 pyriproxyfen IC_{20} with 0.33 µM is very similar to IC_{20} of pesticides such as dichlorvos to
308 *Parachromis managuensis*(IC_{20} at 0.28 µM) (Araújo et al., 2016), diazinon to human (IC_{20} at

309 0.39 µM) (Linhares et al., 2013) and TEPP to *Cichla ocellaris* (IC_{20} at 0.32 µM) (Silva et al.,
310 2013). All pesticides well-known by numerous deleterious effects on non-target organisms.
311 Furthermore, possible side effects of pyriproxyfen also may compromise other physiological
312 processes. Indeed, recent identification that acetylcholinesterase also has non-neuronal and
313 non-esterase role during embryogenesis (Pickett et al., 2017) added new points about the
314 possible range of negative effects related with this enzyme inhibition. These finds suggest a
315 correlation between AChE activity and behavioral or metabolic abnormalities.

316 Following thus, mitochondrial assays triggered fresh points about metabolic
317 impairments as result of chemicals exposure. Especially to biological resilience against these
318 environmental stressors. Indeed, mitochondrial bioenergetics support the precise detection of
319 pesticide damages to fish embryos and adults tissues such as brain and liver (Cowie et al.,
320 2017; Jin et al., 2011; Raftery et al., 2017). Therefore, the mitochondrial framework plays a
321 recognize adjusted machinery (include redox system, ROS and NOS signals, mitochondrial
322 plasticity or calcium uptake) to maintain cell homeostasis and survivor. However, responses
323 of this adjusted machinery are dependent to biological capacity of support mitochondrial
324 health.

325 This work shows that zebrafish mitochondrial damages by pyriproxyfen had as a
326 target NADH dehydrogenase and Succinate dehydrogenase RC impairment. This result is
327 very similar to that observed for mitochondrial RC damage at Succinate dehydrogenase level
328 on *Helicoverpa armigera* by pesticides methylparathion and carbofuran (Akbar et al., 2012).
329 Dual important points should be discussed with this similarity, first, *H. armigera* is a common
330 pest that has harmed many crops around the world, thus, many pesticides are designed to
331 control these invertebrates. However, clearly this is not zebrafish case, a small teleost with so
332 many similarities with the human. This proves that pesticide side effects go beyond the target
333 organisms. The second point is that RC impairments indicate a possible mitochondrial

coupling damage by pesticides (Akbar et al., 2012; Pereira et al., 2009). Which was exactly observed in the present study for succinate dehydrogenase using CCCP as a uncoupler. In addition, another study performed in this same line showed that RC decrease in a pathway to physiological impairment by herbicide metolachlor on rat liver mitochondria (Pereira et al., 2009). Thus, pyriproxyfen has high likelihood of unfeasible vital cellular functions leading to damage in ATP synthesis and electron transport chain flux. This have negative effects at physiological level. Furthermore, results obtained to cytochrome c oxidase shown that pyriproxyfen increase the O_2 consumption. This can be serious effects at ROS generation or to mitochondrial supercomplexes assembly development.

Subsequently, is well-know that mitochondria and your respiratory chain is a key organelle/structure to ROS generation (Murphy, 2009). Pesticides like profenofos, methylparathion, carbofuran, glyphosate, flusilazole, imazalil and atrazine have been reported to triggered ROS generation (Akbar et al., 2012; Heusinkveld et al., 2013; Jin et al., 2010; Lu et al., 2017; Sulukan et al., 2017). Therefore, in the present study pyriproxyfen increased ROS generation as was verified using two different probes: H_2DCF-DA and MitoSOX. First, although H_2DCF is an unspecific ROS indicator, it is a recognized method for detecting ROS in a ñglobalò way. ROS increase after pyriproxyfen treatment can lead to oxidative stress if pesticide also compromises the redox system. Indeed, this was observed with methylparathion and carbofuran that compromises glutathione reductase (GR) activity (Akbar et al., 2012), chlorpyrifos to catalase (CAT) (Jeon et al., 2016), roundup to reduced glutathione (GSH) (Cavalli et al., 2013) or profenofos to superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Lu et al., 2017). These observations can lead to serious implications because ROS may influence mtDNA methylation and, consequently, epigenetic changes (Iacobazzi et al., 2013; Weinhouse, 2017).

Furthermore, in this work MitoSOX assay, that is a specific way mitochondrial O_2ÅË showed that pyriproxyfen has a dose-dependent effect to mitochondrial O_2ÅËgeneration (Figure 3). About this, O_2ÅËhave the NADH dehydrogenase, Succinate dehydrogenase and Cytochrome c reductase as probably superoxide sources. Conversely, O_2ÅË is an oxygen radical with high damage potential and universally correlated to physiological and pathophysiological conditions, consequently dose-dependent pyriproxyfen effect drives to a high health risk potential to non-target organisms, especially when, at fisrt hand, relationship between specific O_2ÅË generation and pesticide exposure is rare and, at second hand, pesticides like rotenone and paraquat are extensively employed to neuro diseases/toxicity inducement (Nisticò et al., 2011). In this way, beyond rotenone and paraquat that are well-know tools to neuronal human diseases study, the pesticides endosulfan, zineb and 1-methyl-4-phenylpyridinium (MPP^+) (Jia and Misra, 2007; Nisticò et al., 2011; Rodriguez-Rocha et al., 2013) are examples that leads to high neuronal mitochondria impairment.

A mechanism usually related to ROS generation is Ca^{2+} transport. This happens because once the levels are high than 10 µM on the physiological concentration found to mitochondrial matrix Ca^{2+} has two pathways: One for the mitochondrial channels such as MCU, Letm1 or NCXL. Another, direct for TCA-cycle dehydrogenases disturbing the correct respiratory chain operation (Rizzuto et al., 2012; Santo-Domingo and Demaurex, 2010). Moreover, Ca^{2+} homeostasis also is conjugated between mitochondria and endoplasmic reticulum (ER). About this, it is know that IP_3 channels works with mitochondria to Ca^{2+} homeostasis and increase toxins susceptibility as a consequence (Esterberg et al., 2014). Thus, Ca^{2+} uptake observed in Figure 4A also may be from ER. In the present study, pyriproxyfen decrease Ca^{2+} uptake capacity at 0.1 µg/mL to ï49% (p=0.001).It is important to remember that this pyriproxyfen concentration is only slightly above the maximum recommended. This result may represent loss of mitochondrial membrane potential, cristae remodeling, swelling

383 or ER damage. However, pyriproxyfen exposure also disturbs Ca^{2+} release as show figure 4B.
384 So evidence of mitochondrial damage appears because CCCP is a uncoupler design to
385 mitochondria and this effect was found as shown evident on figure 4C. Indeed, pyriproxyfen
386 negative effect to mitochondrial Ca^{2+} homeostasis were related to carbofuran, azole and
387 roundup, that corroborates with our findings. These pesticides stimulate Ca^{2+} overload,
388 increase intracellular Ca^{2+} levels through voltage channels and inhibit CYP and VDACs,
389 (Cavalli et al., 2013; Heusinkveld et al., 2013; Kamboj and Sandhir, 2007). These finds
390 encourage more researches to evaluate pyriproxyfen effects to Ca^{2+} homeostasis, specially at
391 brain tissues because Ca^{2+} plays a fundamental role to neuronal fitness and function.

392 To brain tissues NOĘ have a key messenger role, nevertheless, also has deep
393 involvement on neuronal proliferation, survival and differentiation (Joern et al., 2010). The
394 relationship between pesticides exposure and NOĘ generation needs ungently of more
395 highlights. Interestingly, here NOS activity show slight changes in the present of
396 pyriproxyfen, corroborating with another finds that pesticides like malathion also affect NOS
397 (Abdel-Salam et al., 2017). Furthermore, NOĘ can likely with a reactive nitrogen species
398 (RNS) generation and cancer types (Korde Choudhari et al., 2013). However, the probability
399 to occurs here is diminish due lower cytochrome c oxidase inhibition. Here NOĘ biosynthesis
400 has a pyriproxyfen dose-dependence (Figure 5) possible side effects for brain tissues are
401 unclear, especially considering neuronal diseases development on non-target organisms.
402

5. CONCLUSIONS

Considering zebrafish universality as model and its relationships with all other animals model (include human), the present finds drives to illuminate pyriproxyfen road. Remarkably, individual analysis of each performed tests suggests a possible injury to decisive physiological processes. Thus, possibility of pathological conditions development may be growth after pyriproxyfen exposure. These points can be acquiring strong side effects that leads to establishment of pathophysiological conditions. Therefore, these combined findings suggest high damage potential on neuronal tissue of non-target organisms. It first, acetylcholinesterase showed synaptic impairment which has serious implications for the neuronal functioning and locomotor system. Next, mitochondrial analyses allow that pyriproxyfen induce a cascade side effect: once the respiratory chain has been disturbed, RS/ROS/RNS generation increases, this may also be due to Ca^{2+} imbalance. Indeed reactive oxygen species generation and calcium homeostasis has a perfect adjustment. Summarily, due pyriproxyfen side effects to neuronal system, more caution is needed to use, specifically, indiscriminate use.

419 **Figure 1. *In vitro* zebrafish acetylcholinesterase inhibition by pyriproxyfen.** Brain
420 acetylcholinesterase incubation in the presence of pyriproxyfen was performed at 25 ºC for
421 one hour. r^2 represent a linear regression. IC_{20} is an inhibitory concentration of 20% of brain
422 acetylcholinesterase by insecticide exposure. zbAChE is zebrafish brain acetylcholinesterase.
423 Values are enzymatic relative activity (%) (n=4).

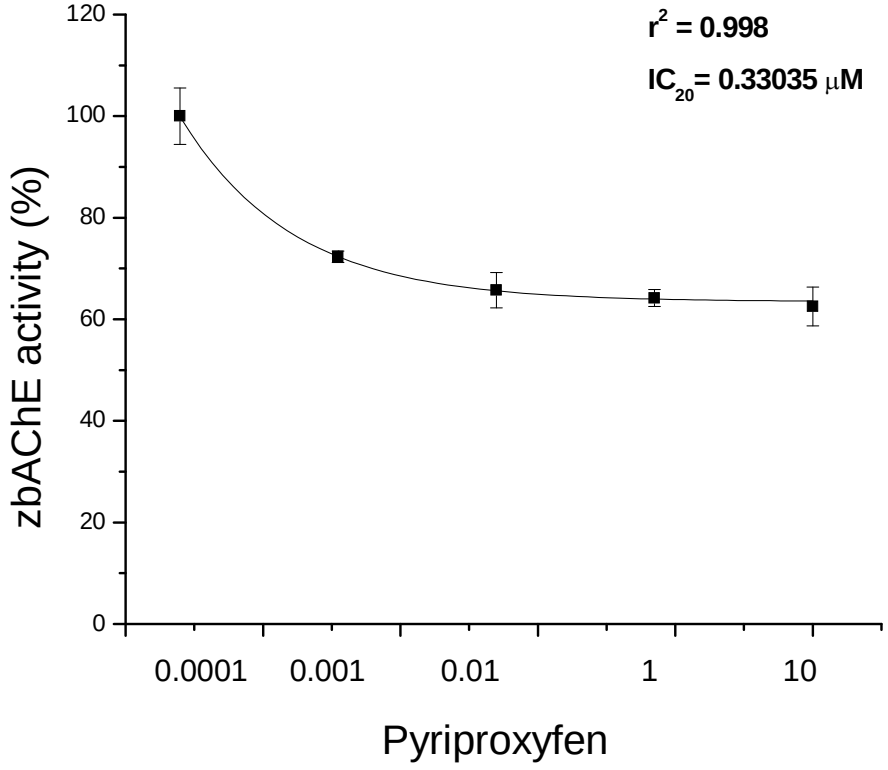

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Table 1. Pyriproxyfen effects on brain mitochondrial respiration to complexes I and II. To NADH Dehydrogenase *State 2* is mix of 5mM NADH substrates (pyruvate, malate, glutamate and Ǔ-ketoglutarate). *State 3* is ADP phosphorylation. *State 4* is Oligomycin phosphorylation inhibition. *CCCP* is maximal respiration. *RC* is Respiratory Control Ratio. To Succinate Dehydrogenase *State 2* is Succinate + Rotenone. Values at $nmolO_2/mL$ and are means and (SE) of four independent experiments performed in duplicate. Except to RC that is a rate.

*p<0.04
**p<0.001

Complex I (NADH Dehydrogenase)

State	***Control***	***Pyriproxyfen exposed***		
		0.001µg/mL	0.01 µg/mL	0.1 µg/mL
State 2	6.86 ± 1.06	6.83 ±1.38	6.73 ± 1.25	9.11 ± 0.70
State 3	6.97 ± 0.65	6.21 ± 0.61	5.84 ± 0.75	6.42 ± 0.53
State 4	2.51 ± 0.38	3.12 ± 0.46	3.31 ± 0.24	3.67 ± 0.92
CCCP	3.39 ± 0.70	2.77 ± 0.27	2.85 ± 0.12	2.53 ± 1.05
RC	2.81 ± 0.37*	2.02 ± 0.40	1.76 ± 0.14	1.76 ± 0.46

Complex II (Succinate Dehydrogenase)

State	***Control***	***Pyriproxyfen exposed***		
		0.001µg/mL	0.01 µg/mL	0.1 µg/mL
State 2	6.84 ± 0.40	6.58 ± 0.74	7.00 ± 0.70	5.35 ± 0.51
State 3	7.61 ± 0.52	6.34 ± 0.58	6.26 ± 0.44	5.88 ± 2.57
State 4	2.54 ± 0.66	3.25 ± 1.15	4.14 ± 1.82	2.54 ± 0.63
CCCP	2.56 ± 0.45	-	-	-
RC	2.99 ± 0.08**	1.95 ± 0.23	1.88 ± 0.35	1.69 ± 0.32

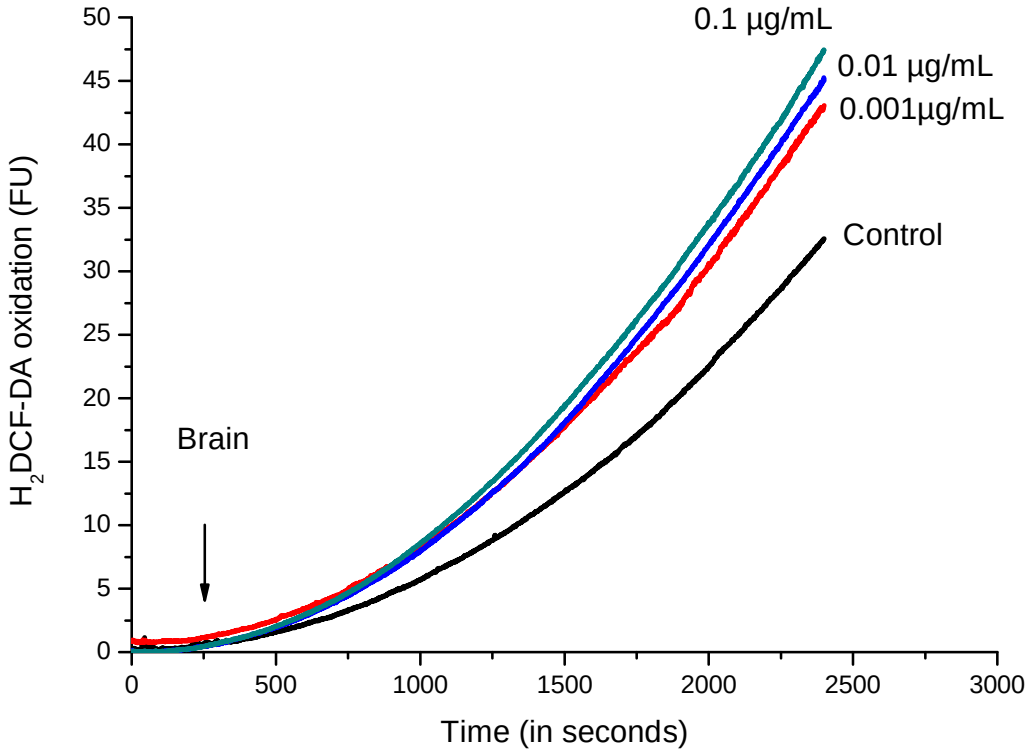
Table 2. Pyriproxyfen effects on brain mitochondrial respiration to complexes III and IV. Antimycin A was represented to AA at 12 µM. TMPD is N,N,N',N'-Tetramethyl-p-phenylenediamine at 0.5mM and Asc is Ascorbate at 12 µM. KCN is Potassium cyanide at 1 µM. Values expressed as mean ± SD (nmol O_2/mg/min).

#p=0.002

a p=0.06

		Pyriproxyfen exposed		
	Control			
		0.001µg/mL	0.01 µg/mL	0.1 µg/mL
AA	3.76 ± 0.70	3.42 ± 0.33	2.93 ± 0.25	2.77 ± 0.55[a]
TMPD + Asc	10.21 ± 0.74	9.70 ± 0.88	9.31 ± 1.07	12.11 ± 0.73[#]
KCN	2.82 ± 0.57	2.18 ± 0.48	3.02 ± 0.61	3.21 ± 0.84

Figure 2. Reactive oxygen species detection with H_2DCF-DA by zebrafish brain. H_2DCF-DA 25 µM was incubated with permeabilized zebrafish brain for 40 min at 28 ºC. This experiment was carried with 5 mM pyruvate, malate, glutamate and Ŭ-ketoglutarate as substrate. Values are means ± SD (n=4). $p<0.01$.



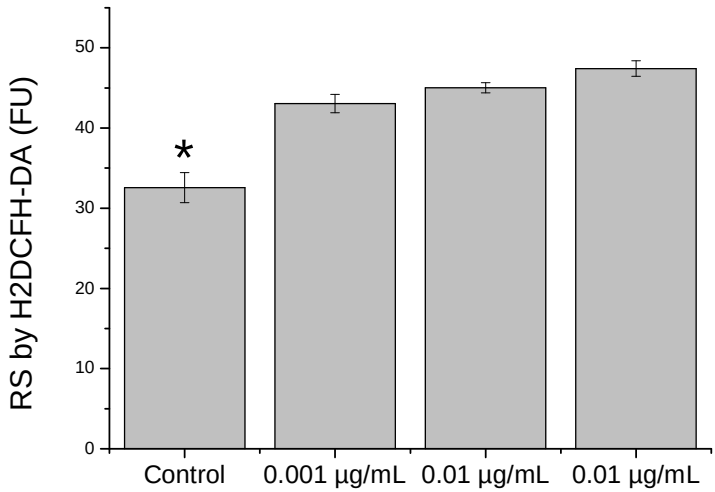


Figure 3. Superoxide production by MitoSOXÊ Red Monitoring. Mitochondrial superoxide generation as determined during 40 min at 28ºC using 5 µM MitoSOXÊ Red Mitochondrial Superoxide Indicator in the presence of 5 mM complex I mixture (pyruvate, malate, glutamate and Ŭ-ketoglutarate acid). * $p<0.001$. Results are expressed as means fluorescence ± SE (n=4). a,b,c are different statistical groups.

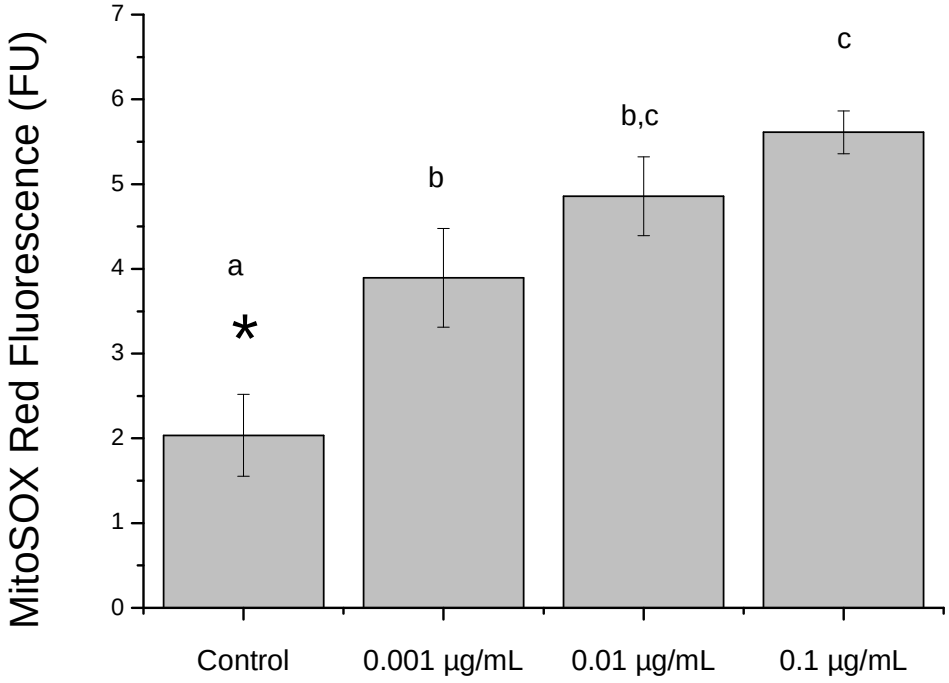
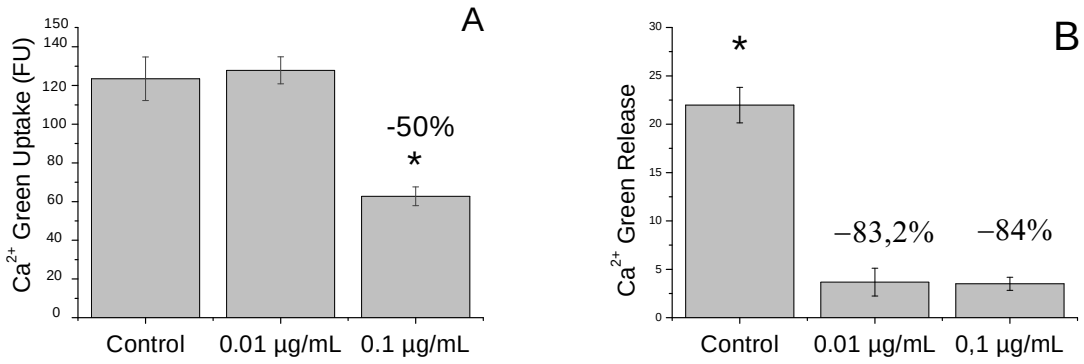
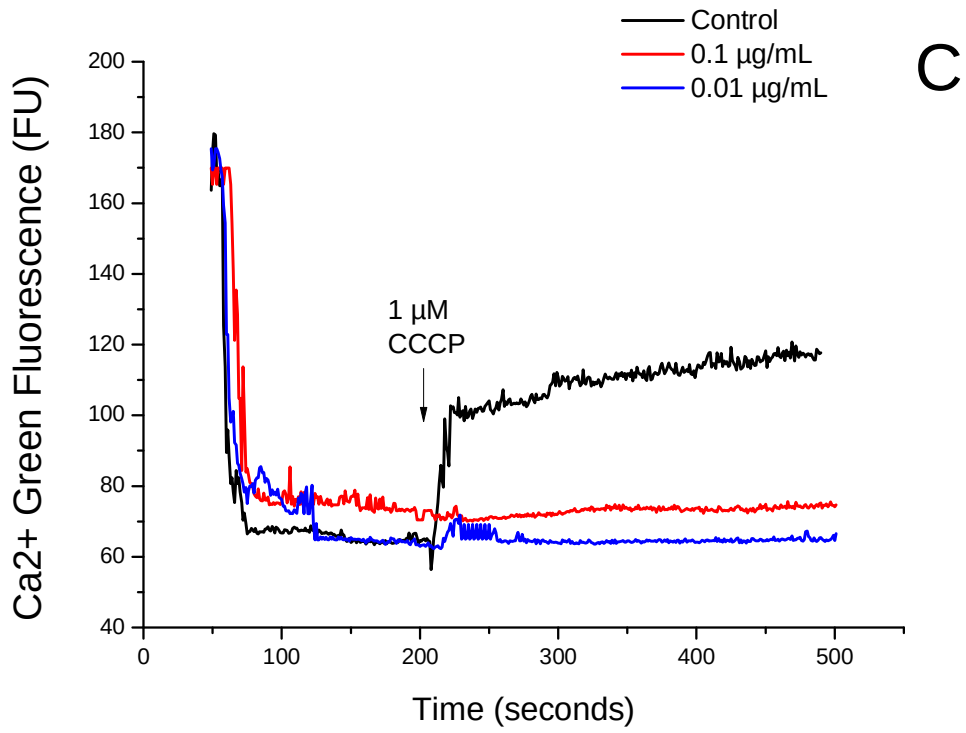


Figure 4. Ca^{2+} transport by zebrafish permeabilized brain. All experiments were carried with respiratory buffer containing 5mM of mix Complex I substrates and 1µM Calcium Green 5-N. Brain permeabilized with 50µM digitonin. (A) Ca^{2+} uptake. (B) Mitochondrial Ca^{2+} release by 1 µM CCCP addition. *p=0.001. (C) Representative Ca^{2+} transport. A fluorescence decrease shows a Ca^{2+} uptake and an increase indicate a Ca^{2+} release (n=4).





474 **Figure 5. Nitric oxide production by brain tissue.** NO[Å] production was continuously
475 monitored in all cases with 5µM DAF-FM suspended on respiratory buffer added 1µM
476 catalase and 1µM SOD. Brain permeabilization was carried like out previous describe. The
477 experiment was performed with a mixture of pyruvate, malate, glutamate and Ŭ-ketoglutarate
478 acid as substrate. *p=0.03. (n=4).

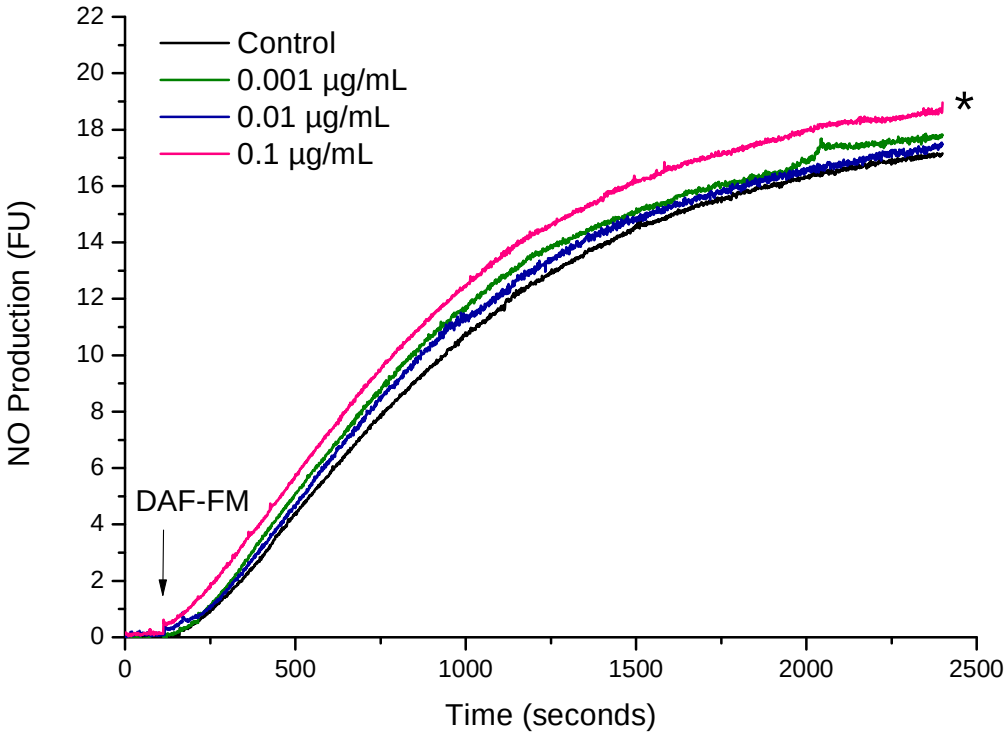


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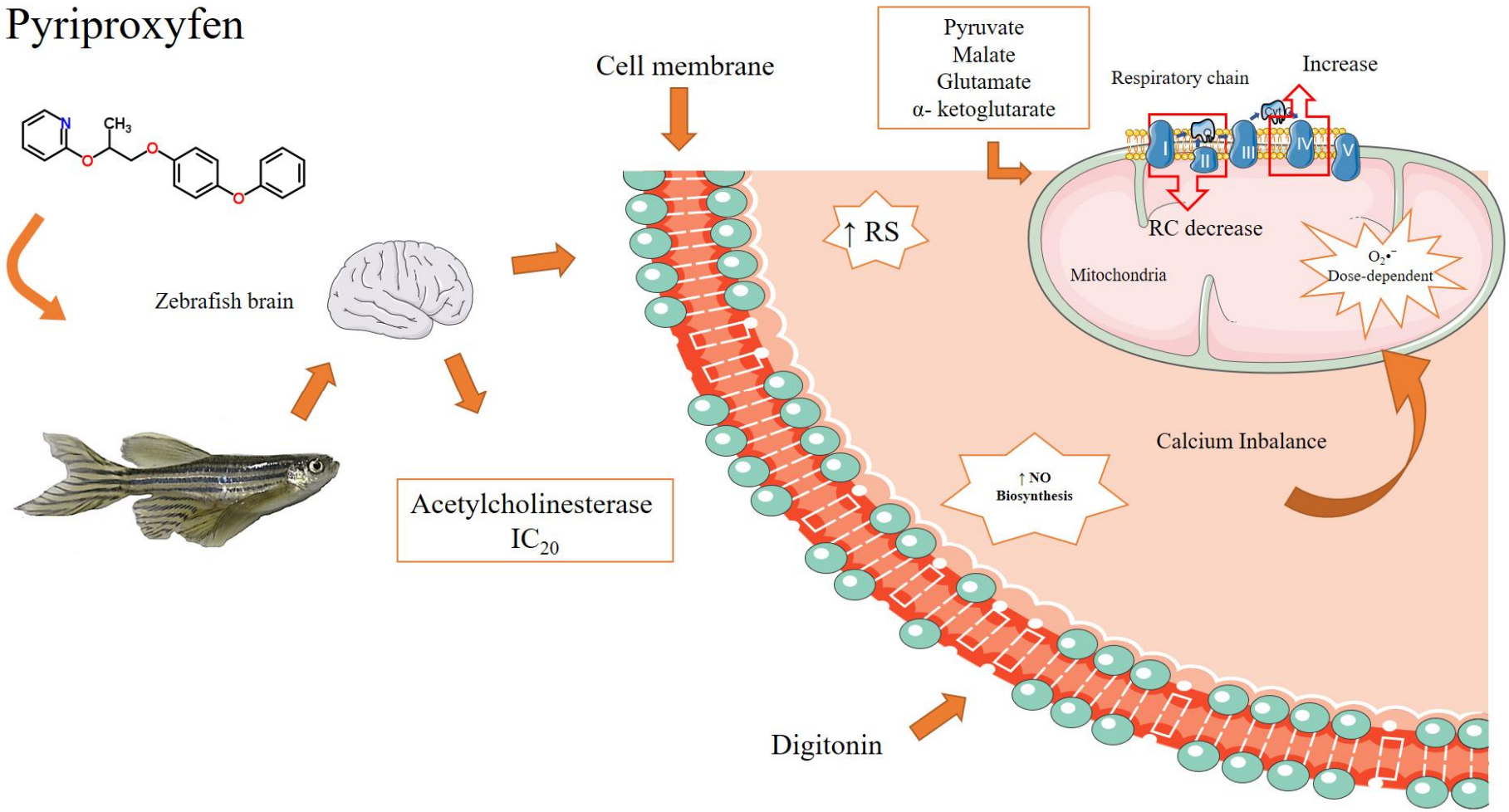
Figure 6 - GRAPHICAL ABSTRACT



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4 CONSIDERAÇÕES FINAIS

Tomando como base o conhecimento científico acumulado sobre o zebrafish como modelo para estudo da bioenergética mitocondrial é possível admitir que este pequeno teleósteo apresentou novas, e maximizou antigas, estratégias para estudo da mitocôndria e de seus moduladores. De fato, seu emprego como ferramenta metodológica para estudos científicos tem possibilitado a expansão do conhecimento para as mais variadas áreas. Sendo tal característica vital para avanços biotecnológicos e na medicina moderna. É importante destacar que com os resultados obtidos pelo presente trabalho ficou claro que exemplares adultos do zebrafish possuem um padrão tecido-específico para funcionamento da mitocôndria. Isto pode ser crucial para elaboração de delineamentos experimentais, bem como para o estudo dos efeitos de nocautes gênicos e de estressores ambientais/químicos. Sobre isso, aqui também foi reportado que pequenas doses do piriproxifeno comprometem a função mitocondrial. representa, claramente, a necessidade de um controle mais preciso sobre a utilização destes compostos químicos. Afinal, o comprometimento da correta fisiologia da mitocôndria desencadeia uma série de processos que podem levar a situações de débito bioenergético e de geração de radicais livres.

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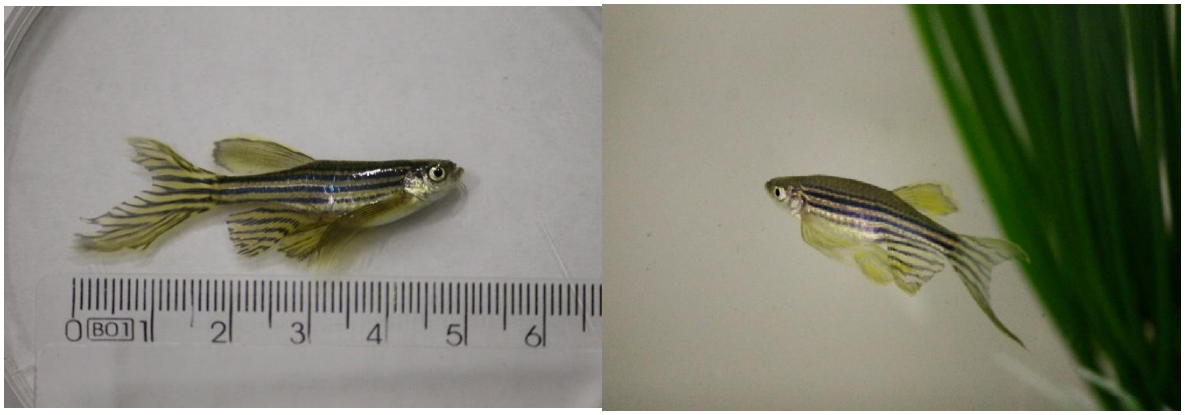
APÊNDICE A - EXEMPLARES ADULTOS DO ZEBRAFISH

Exemplar adulto do zebrafish anestesiado com MS-222. (A) Quando adulto os animais além de apresentares dimorfismo sexual podem atingir até 6 cm de comprimento. (B) Exemplar em nado no aquário. Fotos: Amália Ferreira



Exemplo dos aquários utilizados durante os ensaios. Ao todos, por aquário, foram distribuídos 20 peixes aleatoriamente e monitorados por 30 dias antes das técnicas experimentais. Foto: Amália Ferreira.

ANEXO A - CARTA DE APROVAÇÃO DO COMITÊ DE ÉTICA E USO ANIMAL

Universidade Federal de Pernambuco
Centro de Biociências
Av. Prof. Nelson Chaves, s/n
50670-420 / Recife - PE - Brasil
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Recife, 14 de outubro de 2016.

Ofício nº 104/16

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof. Ranilson de Souza Bezerra**
Departamento de Bioquímica
Centro de Biociências
Universidade Federal de Pernambuco
Processo nº 23076.029059/2016-77

Certificamos que a proposta intitulada **"Fisiologia integrada do zebrafish (*Danio rerio*): avaliações bioenergéticas mitocondriais em tecidos permeabilizados"**, registrada com o nº **23076.029059/2016-77**, sob a responsabilidade de Prof. **Ranilson de Souza Bezerra** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo CONSELHO NACIONAL DE CONTROLE DE EXPERIMENTAÇÃO ANIMAL (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE PERNAMBUCO (UFPE), em reunião de 28/09/2016.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	Até 12/2016
Espécie/linhagem/raça	Peixes *Danio rerio*
Nº de animais	80
Peso/Idade	1g/adultos
Sexo	Machos e fêmeas
Origem	Casas de aquários de Recife – PE

Atenciosamente,

Prof. Dr. Pedro V. Carelli
Presidente da CEUA / CCB - UFPE
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Recife, 04 de outubro de 2017.

Ofício nº 96/17

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof. Ranílson de Souza Bezerra**
Departamento de Bioquímica
Centro de Biociências
Universidade Federal de Pernambuco
Processo **nº 23076.031986/2017-38**

Certificamos que a proposta intitulada ñ**Efeitos do piriproxifeno sobre a bioenergética mitocondrial e atividade da acetilcolinesterase cerebral de zebrafish (*Danio rerio*)**ò, registrada com o **nº 23076.031986/2017-38** sob a responsabilidade de **Prof. Ranílson de Souza Bezerra** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo CONSELHO NACIONAL DE CONTROLE DE EXPERIMENTAÇÃO ANIMAL (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE PERNAMBUCO (UFPE), em reunião de 04/10/2017.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	05/10/2017 a 30/01/2018
Espécie/ linhagem/raça	Peixes *Danio rerio* (zebrafish)
Nº de animais	100
Peso/Idade	200mg/Adultos
Sexo	Machos e fêmeas
Origem	Recife Aquários

Atenciosamente,

Prof. Dr. Pedro V. Carelli
Presidente da CEUA / CCB - UFPE
SIAPE 1801584

ANEXO B - NORMAS DAS REVISTAS QUE OS ARTIGOS SERÃO SUBMETIDOS

ZEBRAFISH JOURNAL (F1 2.242)

Zebrafish is a bi-monthly peer-reviewed journal focusing on research using zebrafish and other aquarium species including medaka, Fugu and Xiphophorus as models for studies of vertebrate development, toxicology and human disease. The Journal will serve as a forum for papers discussing research on comparative genomics and evolution, the genetic analysis of embryogenesis and disease and the cellular and molecular mechanisms of cell growth, differentiation and gene expression in these model species. The Journal will also publish papers describing novel methods, tools and experimental approaches using aquarium models.

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(1) a title page containing the full title of the paper, the names and affiliations of all of the authors, as well as the mailing address, telephone number, fax number, and e-mail address of the corresponding author; (2) an *Abstract* of 200 words; (3) a concise, clearly worded and constructed *Introduction*; (4) *Materials and Methods* containing sufficient detail to allow for reproduction; (5) a *Results* section; (6) a *Discussion* involving interpretation of the results including their relationship to the existing body of knowledge in the field; (7) *Acknowledgments* ; and (8) *References.* Please follow the protocol described herein to avoid delay in publication. Authors should note that manuscripts may be edited if needed to ensure clear grammatical English usage.

TechnoFish Submissions

Previews: Previews are designed to alert readers to important generally useful technical advances or valuable transgenic lines that they might otherwise miss, either because they are published as part of a larger research paper, or because they are published as a methods paper in a journal that zebrafish scientists would not typically view. Authors who feel that this is appropriate for their work will submit a short (150 word or less) description, written in the third person, which highlights why their method or transgenic line is of general interest. A figure should be included to draw interest, which is attractive in both black and white (for the printed version) and in color (for the online version). The submission will be rapidly reviewed to ensure that the technology is of general interest and is clearly described. There will be no publication charge for Previews. By submitting this, authors are also agreeing that all relevant plasmids, transgenic lines, etc., will be made available to all who request it. Please submit these directly to David Kimelman (kimelman@uw.edu).

Methods: These papers are for brief descriptions of new methods, reagents, or transgenic lines that will be of widespread use in the zebrafish community, and should be 750 words or less, plus a brief abstract (100 words or less). They are intended to beone printed page with

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***Journal citation*:**
Parng C, Seng WL, Semino C, McGrath P. Zebrafish: A Preclinical Model for Drug Screening. Assay Drug Devel Technol 2002;1:41-48.

***Book citation*:**
Nusslein-Volhard C: Zebrafish: A Practical Approach. Oxford University Press, Oxford, England, 2002.

***Chapter in edited book*:**
Macdonald R: Zebrafish immunohistochemistry. In Molecular Methods in Developmental Biology: Xenopus and Zebrafish. Guille M and Guille M, (eds), pp. 77ï 88, Humana Press, Totowa, NJ, 1999.

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BBA BIOENERGETICS

AUTHOR INFORMATION PACK

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DESCRIPTION

BBA Bioenergetics covers the area of **biological membranes** involved in energy transfer and conversion. In particular, it focuses on the structures obtained by X-ray crystallography and other approaches, and molecular mechanisms of the components of **photosynthesis**, mitochondrial and bacterial **respiration**, oxidative phosphorylation, motility and transport. It spans applications of **structural biology**, molecular modeling, spectroscopy and biophysics in these systems, through bioenergeticaspectsofmitochondrialbiologyincludingbiomedicineaspectsof**energymetabolism** in **mitochondrial disorders**, neurodegenerative diseases like Parkinson's and Alzheimer's, **aging**, diabetes and even**cancer**.

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AUDIENCE

Biochemists, biophysicists, cell biologists and biomedical scientist interested in the structures, mechanisms and consequences of cellular energy metabolism.

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Ultrafast processes, electron transfer, ligand dynamics, heme proteins

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chloroplast biogenesis; chloroplast gene expression; photosynthetic protein complex assembly; protein degradation; retrograde signalling

Volker Zickermann, Goethe-Universität Frankfurt, Frankfurt am Main, Germany

respiratory complex I, structural biology, redox-linked proton translocation, assembly, mitochondrial diseases

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BBA Bioenergetics covers the area of biological **membranes** involved in energy transfer and conversion. In particular, it focuses on the structures obtained by X-ray crystallography and other approaches, and molecular mechanisms of the components of **photosynthesis**, mitochondrial and bacterial **respiration**, oxidative phosphorylation, motility and transport. It spans applications of **structural biology**, molecular modeling, spectroscopy and biophysics in these systems, through bioenergetic aspects of mitochondrial biology including biomedicine aspects of energy metabolism in **mitochondrial disorders**, neurodegenerative diseases like Parkinson's and Alzheimer's, **aging**, diabetes and even**cancer**.

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INTRODUCTION

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2. ReviewArticles
3. ShortCommunications
4. Letters to theEditor

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