



MOISÉS SILVESTRE DE AZEVEDO MARTINS

**EFEITOS DO EXERCÍCIO FÍSICO SOBRE O METABOLISMO
E COMPORTAMENTO DE ZEBRAFISH (*Danio rerio*)**

LAVRAS-MG

2023

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Prof. Dr. Luis David Solís Murgas

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APROVADO em 05 de setembro de 2023.

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A minha mãe, por todo amor, carinho, ensinamentos e apoio durante toda minha vida.

DEDICO.

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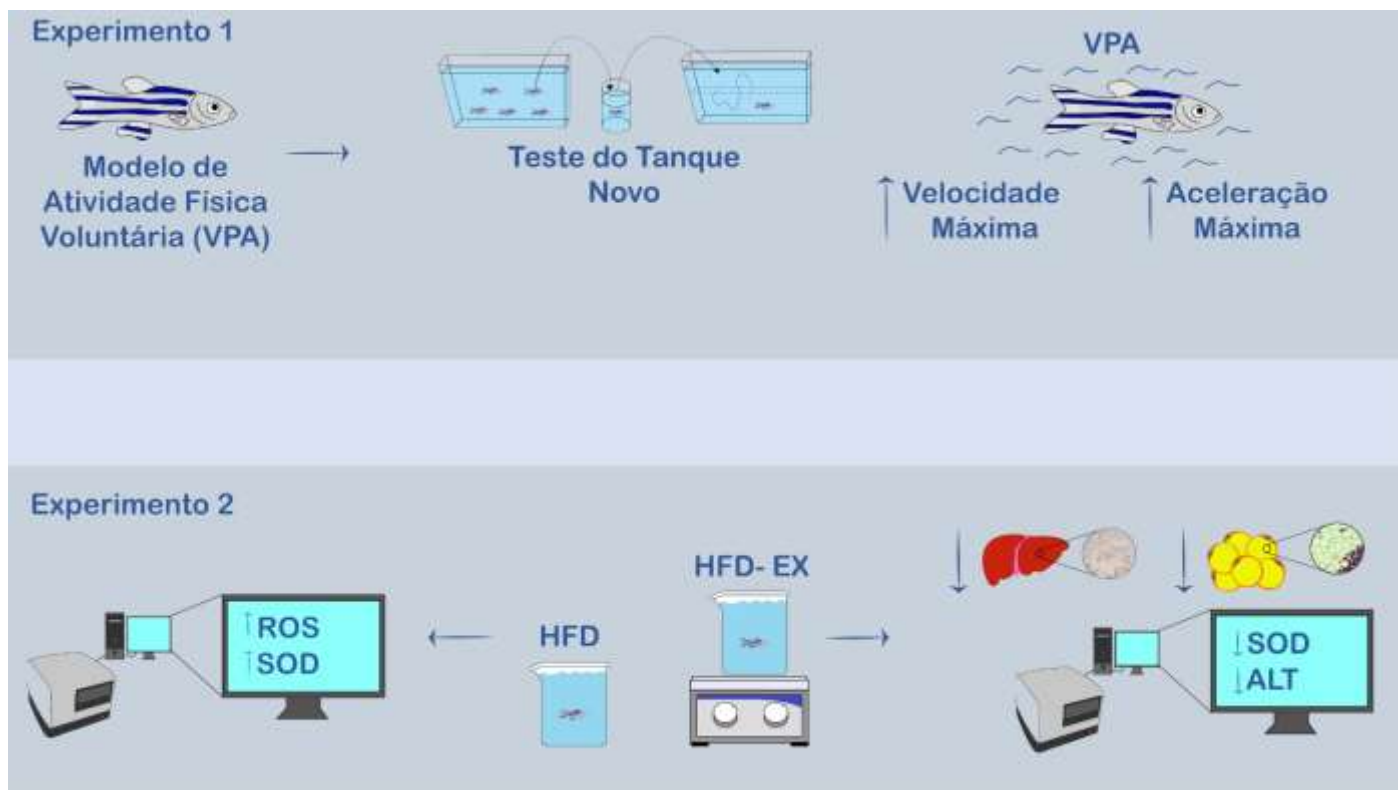
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*“Se a educação sozinha não transforma a sociedade,
sem ela tampouco a sociedade muda”*
(FREIRE, 2000).

RESUMO GRÁFICO



RESUMO

A obesidade é uma condição médica caracterizada pelo acúmulo patológico e excessivo de tecido adiposo, tanto visceral quanto subcutâneo. Essa condição acarreta significativos impactos à saúde, incluindo o desenvolvimento de doenças graves, tais como aterosclerose, diabetes mellitus tipo 2, doenças cardiovasculares, além de outras enfermidades. A partir de 40 anos já é estudada a relação entre exercício físico e estresse oxidativo, porém devido à alta complexidade da temática, ainda há grandes lacunas sobre o assunto, como as vias de sinalização e o real papel das enzimas antioxidantes durante o exercício físico principalmente quando se insere a variável obesidade. Analisando os estudos pré-existentes eles são conduzidos com seres humanos ou roedores, criando uma lacuna ainda maior sobre o assunto quando se trata do modelo Zebrafish (*Danio rerio*). Este estudo apresenta os resultados de um novo modelo de exercício físico voluntário, bem como os efeitos metabólicos de um protocolo de exercício em zebrafish alimentados com uma dieta rica em gordura. No primeiro estudo, foi criado um modelo de exercício físico voluntário (VPE) que pudesse ser aplicado em estudos de metabolismo e comportamento. 40 Zebrafish (*D. rerio*) divididos em dois grupos, foram submetidos a um sistema de VPE que consistia em dois aquários conectados por um tubo translúcido e uma câmera de vídeo na lateral para medir parâmetros de exercício físico dos animais. Os animais apresentaram maior aceleração e velocidade máxima e tiveram maior frequência de atividade no período claro. Nesse modelo de exercício físico voluntário, observou-se melhor desempenho nos testes de avaliação locomotora, não acompanhado de aumento da ansiedade ou alteração de parâmetros bioquímicos relacionados ao metabolismo lipídico. O segundo estudo teve por objetivo identificar os efeitos metabólicos de um novo modelo de exercício físico em zebrafish (*D. rerio*) alimentados com uma dieta rica em gordura. O experimento compreendeu um ensaio alimentar com duração de doze semanas, no qual as últimas quatro semanas incorporaram um protocolo de exercício físico. Esse protocolo consistiu na inserção de quinze animais em um aquário de cinco litros, onde foram submetidos a nadar a uma velocidade aproximada de 0,8 m/s, durante 30 minutos por dia. Os resultados demonstraram que o grupo alimentado com a dieta rica em gordura (HFD) apresentou um aumento significativo na produção de espécies reativas de oxigênio (ROS) e atividade da superóxido dismutase (SOD). Em contrapartida, o grupo alimentado com a dieta rica em gordura e submetido ao exercício físico (HFD-EX) mostrou uma notável redução na área adipocitária visceral, nos níveis de esteatose hepática, na atividade da superóxido dismutase (SOD) e alanina aminotransferase (ALT). Esses

resultados indicam que o exercício físico exerce um efeito positivo no contexto da obesidade e estresse oxidativo em zebrafish, fornecendo perspectivas promissoras para futuras investigações nesta área.

Palavras-chave: Obesidade. Esteatose hepática. Estresse Oxidativo. Ansiedade.

ABSTRACT

Obesity is a medical condition characterized by pathological and excessive accumulation of adipose tissue, both visceral and subcutaneous. This condition has significant impacts on health, including the development of severe diseases such as atherosclerosis, type 2 diabetes mellitus, cardiovascular diseases, and other illnesses. For over 40 years, the relationship between physical exercise and oxidative stress has been studied. However, due to the high complexity of the subject, there are still major gaps in understanding, such as the signaling pathways and the real role of antioxidant enzymes during physical exercise, especially when obesity is involved. Analyzing the existing studies, they have been conducted on humans or rodents, which creates an even larger gap when it comes to the Zebrafish (*Danio rerio*) model. This study presents the results of a new voluntary physical exercise model and the metabolic effects of an exercise protocol on zebrafish fed with a high-fat diet. In the first study, a voluntary physical exercise (VPE) model was developed to be applied in metabolism and behavior studies. 40 Zebrafish (*D. rerio*) were divided into two groups and subjected to a VPE system, consisting of two interconnected aquaria with a translucent tube and a lateral video camera to measure the animals' physical exercise parameters. The animals showed increased acceleration and maximum velocity and had higher activity frequency during the light period. In this voluntary physical exercise model, improved locomotor performance was observed, without an increase in anxiety or alterations in biochemical parameters related to lipid metabolism. The second study aimed to identify the metabolic effects of a new physical exercise model in zebrafish fed with a high-fat diet. The experiment consisted of a feeding trial lasting twelve weeks, with the last four weeks incorporating a physical exercise protocol. This protocol involved placing fifteen animals in a five-liter aquarium and having them swim at an approximate speed of 0.8 m/s for 30 minutes per day. The results demonstrated that the group fed with a high-fat diet (HFD) had a significant increase in reactive oxygen species (ROS) production and superoxide dismutase (SOD) activity. In contrast, the group fed with a high-fat diet and undergoing physical exercise (HFD-EX) showed a remarkable reduction in visceral adipocyte area, hepatic steatosis levels, superoxide dismutase (SOD) activity, and alanine aminotransferase (ALT) levels. These findings suggest that physical exercise has a positive effect on obesity and oxidative stress in zebrafish, offering promising perspectives for future investigations in this field.

Keywords: Obesity. Hepatic Steatosis. Oxidative Stress. Anxiety.

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PRIMEIRA PARTE

1 INTRODUÇÃO

A obesidade é uma comorbidade caracterizada pelo acúmulo excessivo de gordura tanto visceral como subcutâneo, que causa sérios danos à saúde (MONTALBANO et al., 2021; NAKAYAMA et al., 2020) como desenvolvimento de aterosclerose, diabetes mellitus tipo 2, doenças cardiovasculares entre outras doenças (NAKAYAMA et al., 2020). Ela é uma consequência da interação entre fatores genéticos e ambientais como uma dieta desbalanceada e falta de exercício físico (LE LAY et al., 2014; LI et al., 2019), em que há um balanço energético positivo, ou seja, a ingestão energética excede o gasto energético (MONTALBANO et al., 2021).

Indivíduos obesos demonstram marcadores indicativos de estresse oxidativo (ER), como aumento das espécies reativas de oxigênio (ROS) (KEANEY et al., 2003) e diminuição das defesas antioxidantes como superóxido dismutase (SOD), catalase (CAT), glutatona reduzida (GR) e glutatona S-transferase (GST) (BARBOSA et al., 2010; BRIEGER et al., 2012; HAYES; FLANAGAN; JOWSEY, 2005). O ER em indivíduos obesos está associado à inflamação sistêmica, proliferação de células endoteliais e apoptose (HUANG et al., 2015).

Os danos advindos da obesidade podem ser atenuados de duas formas: mudando a dieta e alterando o estilo de vida, mais precisamente inserindo o exercício físico na rotina diária (MING FOCK; KHOO, 2013). Porém, o exercício físico quando praticado até a exaustão pode causar danos celulares nos músculos, como aumento na atividade plasmática de enzimas citosólicas, devida a alta produção de ROS gerando um estresse oxidativo (GOMEZ-CABRERA; DOMENECH; VIÑA, 2008). Quando o exercício físico é realizado de maneira regular a uma intensidade moderada, também a geração de ROS, porém são contrabalanceados pelo aumento concomitante das enzimas antioxidantes, como SOD, CAT e GPX (POWERS et al., 2023; SIMIONI et al., 2018; VIÑA et al., 2000). Vale ressaltar que a geração de ROS durante o exercício físico moderado é benéfica, pois são necessárias para a concretização dos efeitos adaptativos do exercício físico (POWERS et al., 2020).

Há mais de 40 anos já é estudado a relação entre exercício físico e estresse oxidativo (TAHERKHANI et al., 2021), porém devido à alta complexidade da temática, ainda há grandes

lacunas sobre o assunto, como as vias de sinalização e o real papel das enzimas antioxidantes durante o exercício (BOUVIERE et al., 2021; POWERS et al., 2020), principalmente quando se insere a variável obesidade. Quando analisamos os estudos pré-existentes, observamos que eles são conduzidos com seres humanos ou roedores, criando uma lacuna ainda maior sobre o assunto quando se trata do modelo Zebrafish (*Danio rerio*). Com isso o estudo tem por objetivo avaliar o potencial do zebrafish (*D. rerio*) como um modelo de exercício físico voluntário e identificar os efeitos metabólicos de um protocolo de exercício físico em zebrafish (*D. rerio*) alimentados com uma dieta rica em gordura.

2 REFERENCIAL TEÓRICO

2.1 Testes comportamentais utilizados em Zebrafish (*Danio rerio*) submetidos a atividade física e ao exercício físico.

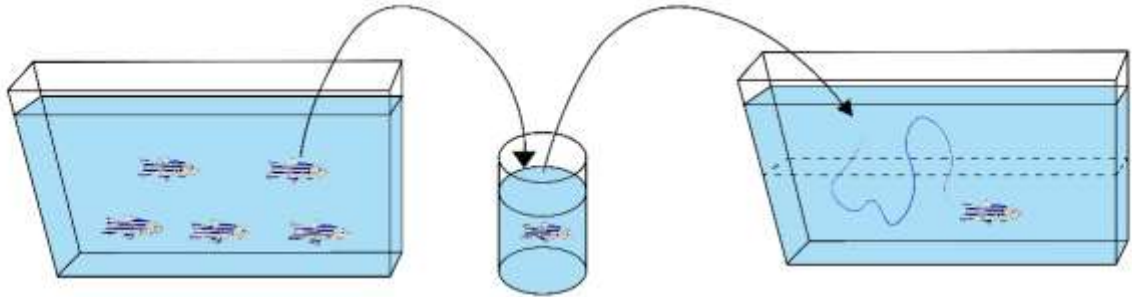
2.1.1 Teste Tanque Novo

O teste tem como premissa quantificar o comportamento de ansiedade do *Zebrafish* (EGAN et al., 2009). É um teste relativamente simples de ser aplicado que pode ser utilizado em inúmeras situações, como para avaliar o comportamento perante à uma doença, exercício (DA ROSA et al., 2017; DE MELO MARTINS et al., 2022; DEPASQUALE et al., 2022; DEPASQUALE; LERI, 2018; SARTORI et al., 2023; WANG et al., 2022) ou em qualquer outra situação que a priori se verificou interferência comportamental.

O teste de tanque novo consiste inicialmente em um pré-tratamento feito em um *backer* contendo 400ml de água com apenas um *zebrafish*, com duração de 20 min, o pré-tratamento pode ser realizado com intuito de exposição a um fármaco ou apenas para transposição do *zebrafish* até o aquário experimental. Logo em seguida o *zebrafish* é retirado do *backer* e colocado em um tanque retangular (1,5 litros) que está em uma superfície nivelada e estável, e são divididos em duas porções horizontais virtuais iguais, marcado por uma linha divisória nas paredes externas, então o comportamento de natação é registrado por um sistema de vídeo por um período de 6 minutos (CACHAT et al., 2010, 2011; YOSHIDA, 2022) (Figura 1). Vale ressaltar que as condições do teste como pH, condutividade, temperatura e amônia devem ser os mesmos das condições previa que o animal se encontrava.

Após as filmagens os parâmetros podem ser analisados manualmente por 2 observadores treinados (confiabilidade entre avaliadores >0,85), ou por um software de análise automática como o EthoVision XT® software (Noldus) ou Top-Scan (TopView Animal Behavior Analyzing System). Os parâmetros de análise são o número de vezes que o *zebrafish* entrou no topo do tanque, duração no topo/fundo, distância (m) percorrida no topo/fundo, latência para entrar no topo(s), velocidade (m/s), distância total percorrida e informações sobre padrões de movimento e crises de congelamento (frequência, duração) (CACHAT et al., 2010, 2011).

Figura 1 - Esquema explicativo do teste de tanque novo



Fonte: do autor.

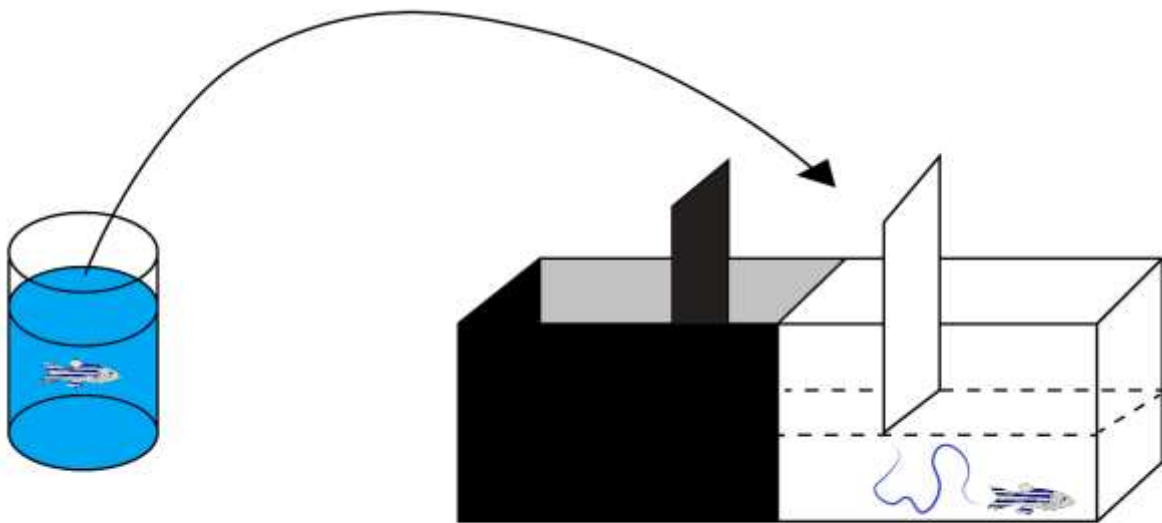
2.1.2 Teste Claro e Escuro

O teste tem como objetivo quantificar o comportamento de ansiedade do *Zebrafish* (DEPASQUALE; LERI, 2018). Assim como o teste de tanque novo esse pode ser aplicado em diversas situações, como para avaliar o comportamento frente à uma doença ou exercício (DEPASQUALE; LERI, 2018; MEZZOMO et al., 2016).

O teste claro e escuro consiste inicialmente em transferir o *zebrafish* para um *backer* contendo 300ml de água tratada por 5 minutos. Em seguida o *zebrafish* é transferido para um aquário retangular ($15 \times 10 \times 45$ cm) (10 cm de água) dividido em duas áreas com o mesmo tamanho denominadas claro e escuro, e uma área central contendo 5cm cercada por 2 portas denominada área neutra. A área clara deve conter uma lâmpada de 75W, localizada a 1,80m acima do aquário, com o objetivo de manter a iluminação uniforme e constante no aquário, a luz a essa distância tende a produzir em média 975 lux. Já a região escura deve estar completamente tampada sem produzir nenhuma iluminação na região (FONTANA; ALNASSAR; PARKER, 2022; MAGNO et al., 2015; MAXIMINO et al., 2010) (Figura 2).

O *zebrafish* deve ficar 300 segundos na área neutra para se familiarizar com o ambiente, em seguida a câmera de filmagem é ligada e as portas são removidas, permitindo que o *zebrafish* explore livremente o aquário por 900 segundos. Os parâmetros comumente analisados são: tempo total que o animal permanece na área escura, latência para explorar o lado oposto pela primeira vez e o número de travessias. O tempo no escuro indica comportamento ansioso, a primeira latência é uma medida de impulsividade ao explorar novos ambientes e o número de travessias é uma medida de atividade motora (MAGNO et al., 2015; MAXIMINO et al., 2010). Esses parâmetros podem ser analisados manualmente por um avaliador experiente ou de forma automática por softwares como Ethovision, EthoLog, JWatcher e X-Plo-Rat (MAXIMINO et al., 2010).

Figura 2 - Esquema explicativo do teste claro e escuro



Fonte: do autor.

2.1.3 Teste de Campo aberto

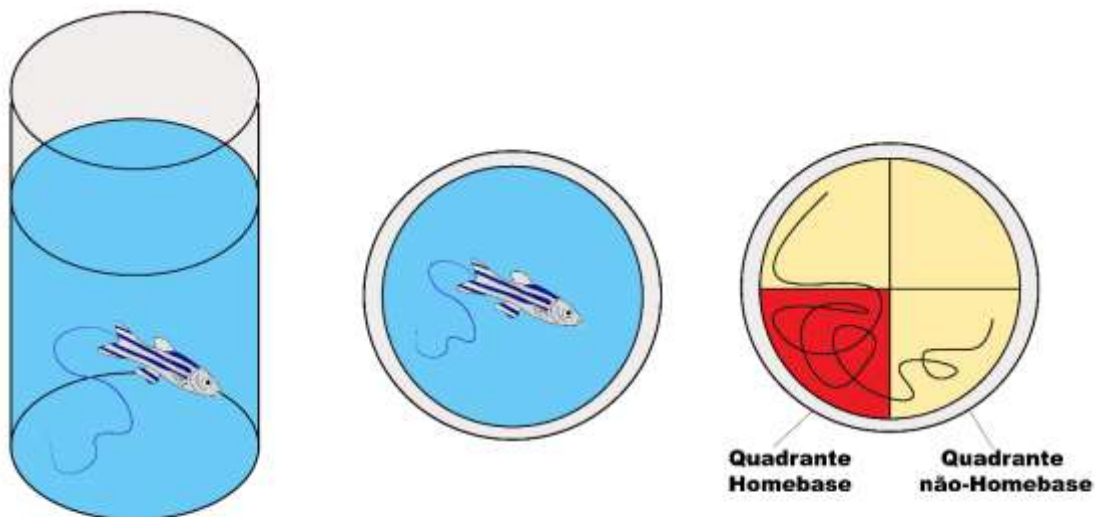
O teste de campo aberto com *zebrafish* conceitualmente é semelhante ao teste de campo aberto com roedores (CHOLERIS et al., 2001; GROSSMAN et al., 2010; STEWART et al., 2010a). O teste tem como premissa quantificar o comportamento de ansiedade do *Zebrafish* (CACHAT et al., 2013; GROSSMAN et al., 2010; STEWART et al., 2010b), podendo ser aplicado

em diversas situações, como para avaliar o comportamento frente à exposição a uma droga ou exercício (DEPASQUALE et al., 2022; GROSSMAN et al., 2010).

O teste pode variar no tipo de aquário que será utilizado, tamanho e quantidade de água no aquário (CACHAT et al., 2013; DEPASQUALE et al., 2022; GROSSMAN et al., 2010; STEWART et al., 2010b), vamos nos basear no método mais utilizado que consiste em um cilindro plástico de cor branca (21 cm de diâmetro, 24 cm de altura) cheio de água até uma altura de 12 centímetros. Inicialmente o *zebrafish* deve ser pré-exposto em um *backer* de 1 litro por 20 minutos, logo em seguida o peixe é colocado no centro do tubo e filmado durante 6 minutos, por uma câmera fixada em um tripe de modo a não produzir sombra nos tubos (CACHAT et al., 2013; GROSSMAN et al., 2010) (Figura 3).

Os parâmetros que podem ser avaliados através desse teste são: Tempo gasto (s), distância percorrida (m), velocidade média (m/s), sinuosidade ($^{\circ}/m$), número de visitas nas zonas centrais e periféricas pré-definidas, comparação entre preferência por quadrante *Homebase* e não-*Homebase*, tigmotaxia pela proporção centro/periferia (CACHAT et al., 2013; GROSSMAN et al., 2010). Todos os parâmetros podem ser analisados de forma automatizada por software como EthoVision XT® (Noldus) ou de forma manual por 3 observadores experientes (confiabilidade entre avaliadores $>0,85$) (CACHAT et al., 2013).

Figura 3 - Esquema explicativo do teste de campo aberto



Fonte: do autor.

2.1.4 Teste de Cardume

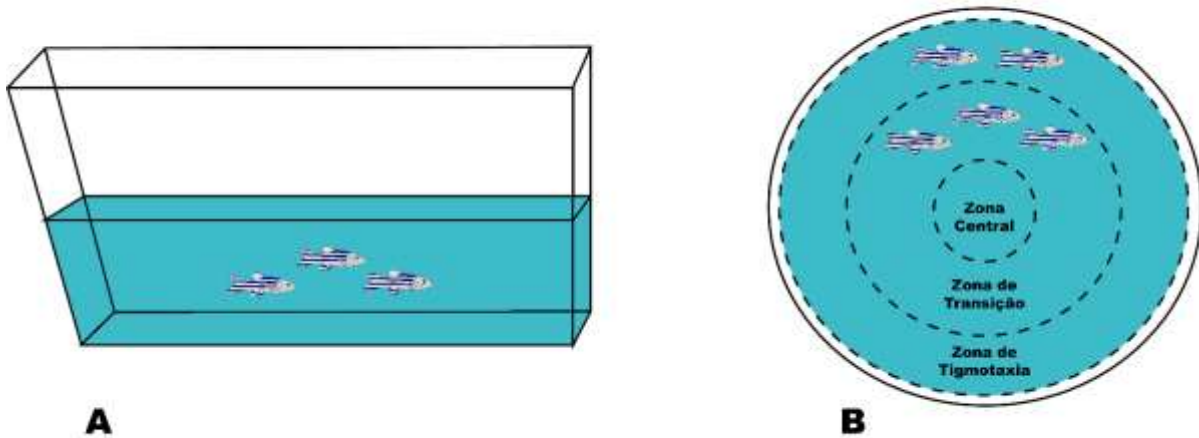
A natação em cardume é de extrema importância para o zebrafish, facilita a localização de potenciais parceiros e fornecem vantagens hemodinâmicas. Peixes em cardume tem proporcionalmente mais fontes de informação social, além disso, a transferência de informações em cardumes pode ser facilitada levando a uma melhor utilização das informações disponíveis sobre, por exemplo, predadores locais e distribuições de alimentos (MILLER; GERLAI, 2011).

O teste de cardume tem como premissa avaliar o comportamento social do *Zebrafish*, tendo relatos de sua utilização em diferentes objetivos, como avaliar o efeito de um composto alucinógeno, ansiedade e exercício no comportamento social (CACHAT et al., 2013; GROSSMAN et al., 2010; JOHNSON et al., 2023).

O teste tem duas metodologias de aplicação, a primeira consiste na exposição de 8 *zebrafish* a um aquário (4 L, 10 x 20 x 20 cm), com profundidade de água de 2 cm, por 6 minutos, analisando 8 capturas de tela feitas a cada 20 segundos. Cada captura de tela deve ser filmada e analisada manualmente por observadores treinados, o parâmetro analisado é a distância (cm) entre cada peixe do grupo e, em seguida, realiza-se o cálculo da média desses dados para obter uma distância média entre peixes por captura de tela (CACHAT et al., 2013; GROSSMAN et al., 2010; PHAM et al., 2012; WANG et al., 2022) (Figura 4A).

A segunda metodologia consiste na exposição de 5 *zebrafish* em um cilindro plástico na cor branca (35,0 centímetros de diâmetro) com uma profundidade de água de 5 centímetros por 15 minutos. Após as filmagens, em um programa de análise de vídeo como o EthoVision XT® (Noldus), deve-se criar 3 zonas denominadas zona tigmotática, zona de transição e zona central (Figura 4B), a fim de analisar os seguintes parâmetros: distância interindividual, distância do vizinho mais próximo, velocidade média do cardume a partir da soma das velocidades medias de cada peixe e dividido pelo total de peixes no cardume, duração acumulativa média do tempo gasto imóvel calculada para cada peixe e então dividida pelo número total de peixes no cardume para determinar a imobilidade média do cardume (JOHNSON et al., 2023; NUNES et al., 2017).

Figura 4 - Esquema explicativo do teste do cardume



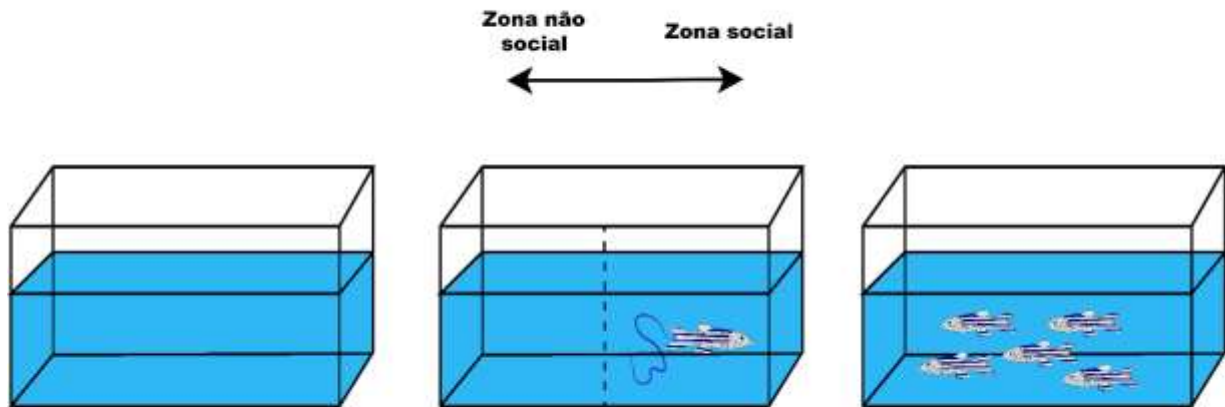
Fonte: do autor.

2.1.5 Teste de Interação Social

O protocolo de interação social tem como premissa a exposição concomitante do *zebrafish* a um aquário vazio e a um aquário contendo um grupo de peixes, com o objetivo de avaliar a preferência dos animais, pois o *zebrafish* frente a um cardume de peixes da sua espécie reflete manifestações comportamentais relacionadas às preferências sociais (RESENDE; SOCCOL, 2015).

O teste consiste em um tanque experimental (30 × 15 × 10 centímetros) no meio, e nas laterais um tanque vazio e outro tanque idêntico contendo 15 *zebrafish* que são denominados peixes estímulo. Inicialmente um *zebrafish* é colocado no tanque experimental para aclimação por 60 segundos, em seguida inicia-se a gravação em vídeo durante 5 minutos para posterior análise no software EthoVision XT® (Noldus) ou software similar. Para quantificar a preferência do *zebrafish* entre o lado peixe estímulo e o lado tanque vazio, o tanque experimental deve ser dividido virtualmente em duas zonas iguais denominadas zona social e zona não social (Figura 5). O parâmetro quantitativo do teste é o tempo que o *zebrafish* gastou em cada zona durante 5 minutos (DE MELO MARTINS et al., 2022; GERLAI et al., 2000; NABINGER et al., 2018).

Figura 5 - Esquema explicativo do teste de interação social



Fonte: do autor.

2.1.6 Teste de evitação inibitória

O aparato utilizado nesse teste é composto por um tanque (18 x 9 x 7 cm) dividido em dois hemisférios iguais denominados escuro e branco, separados por uma porta deslizante (9 x 7 cm). O nível da água utilizada no tanque deve ser de 3 centímetros e a divisória que separa os hemisférios deve ser elevada 1 centímetro em relação ao fundo do tanque, assim permitindo que o *zebrafish* nade livremente para o outro lado do tanque. Dois eletrodos que se estendem pela parede do aquário devem ser colocados ao fundo do hemisfério escuro e conectados a um estimulador de 8 volts, para que, se necessário o avaliador administre um choque com uma voltagem de $3 \pm 0,2$ volts AC (BLANK et al., 2009; DE MELO MARTINS et al., 2022; NABINGER et al., 2018)(Figura 6).

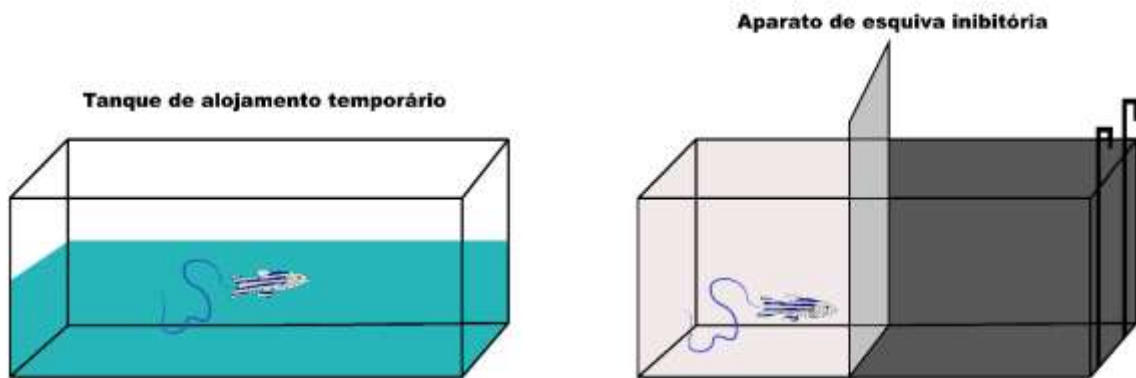
A primeira fase do teste corresponde ao treinamento que consistem em colocar um *zebrafish* no hemisfério branco com a divisória fechada por um período de 1 minuto para a familiarização do peixe com o novo ambiente, após esse período a divisória deve ser levantada a uma altura de 1

centímetro, permitindo que o *zebrafish* passe para o hemisfério escuro, assim que todo seu corpo estiver no hemisfério escuro a divisória deve ser fechada e um choque elétrico pulsado deve ser administrado por 5 segundos. O peixe então é removido do aparato e colocado em um tanque de alojamento temporário (BLANK et al., 2009; DE MELO MARTINS et al., 2022; NABINGER et al., 2018) (Figura 6).

A segunda fase corresponde ao teste propriamente dito, onde será repetido o protocolo de treinamento, com exceção da familiarização, nenhum choque será administrado e o peixe será removido imediatamente do compartimento escuro. O parâmetro analisado após o teste é a latência para entrar completamente no hemisfério escuro, utilizado como um índice de retenção de memória (BLANK et al., 2009; DE MELO MARTINS et al., 2022; NABINGER et al., 2018).

Ao final do teste o *zebrafish* deve ser monitorado para avaliar possíveis efeitos negativos do choque elétrico, como alteração no desempenho na natação, orientação corporal, lesão externa ou aumento da suscetibilidade a doenças nos dias seguintes ao teste (BLANK et al., 2009).

Figura 6 - Esquema explicativo do teste de evitação inibitória



Fonte: do autor.

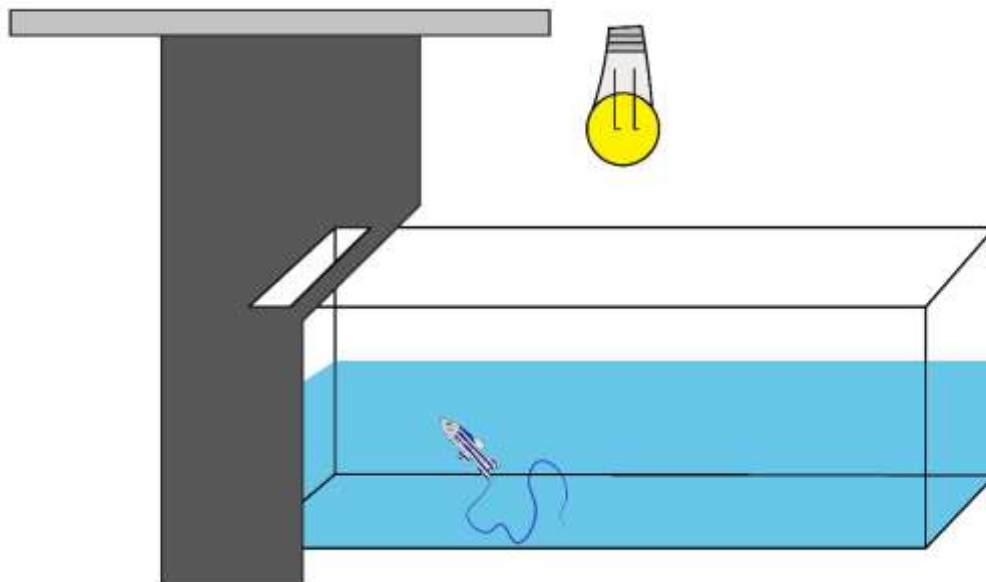
2.1.7 Estimulo condicionado

O teste de estímulo condicionado com *zebrafish* tem o mesmo princípio do teste aplicado a roedores (BROOKS, 2021; CATLOW et al., 2013) que é avaliar a aprendizagem através de um estímulo condicionado como alimentação ou luminosidade. O aparato consiste em um tanque (40 × 25 × 20 cm) isolado química, acústica e visualmente, contendo 15 litros de água. O tanque deve estar atrás de uma cortina escura, com uma pequena abertura (25 × 2 cm) opaca na região superior esquerda para realizar a alimentação e uma lâmpada centralizada no aquário (910 ± 2 lux) (Figura 7). A alimentação e iluminação devem ser utilizados como estímulos condicionados e incondicionados, respectivamente (LUCHIARI; CHACON, 2013). Lembrando que os parâmetros como pH, condutividade, amônia, temperatura e iluminação devem ser os mesmos da condição inicial dos peixes.

O teste consiste em colocar um *zebrafish* no aparato de estímulo condicionado e oferecer a alimentação uma vez por dia, após um estímulo luminoso de 10 segundos, a alimentação deve ser oferecida por meio de um tubo conectado ao orifício da cortina, assim evitando que o *zebrafish* associe o pesquisador a estímulos incondicionados. O comportamento do peixe deve ser registrado diariamente por um período de 2 minutos e 10 segundos, durante 8 dias consecutivos. O registro deve ser realizado 1 minuto antes e após o estímulo condicionado (LUCHIARI; CHACON, 2013).

Os parâmetros que podem ser analisados com o teste são: A área de dispersão ocupada durante 1 minuto antes e após o estímulo luminoso, utilizada para estimar a atividade do peixe. A distância média, através da distância do peixe até a área de alimentação a cada 10 segundos. E o índice de aproximação, calculado através da distância da área de alimentação após o estímulo luminoso menos a distância da área de alimentação antes do estímulo luminoso. A aprendizagem nesse teste é inferida a partir desse índice, quanto mais negativo os valores, maior foi a aproximação do peixe da área de alimentação.

Figura 7 - Esquema explicativo do teste de estímulo condicionado



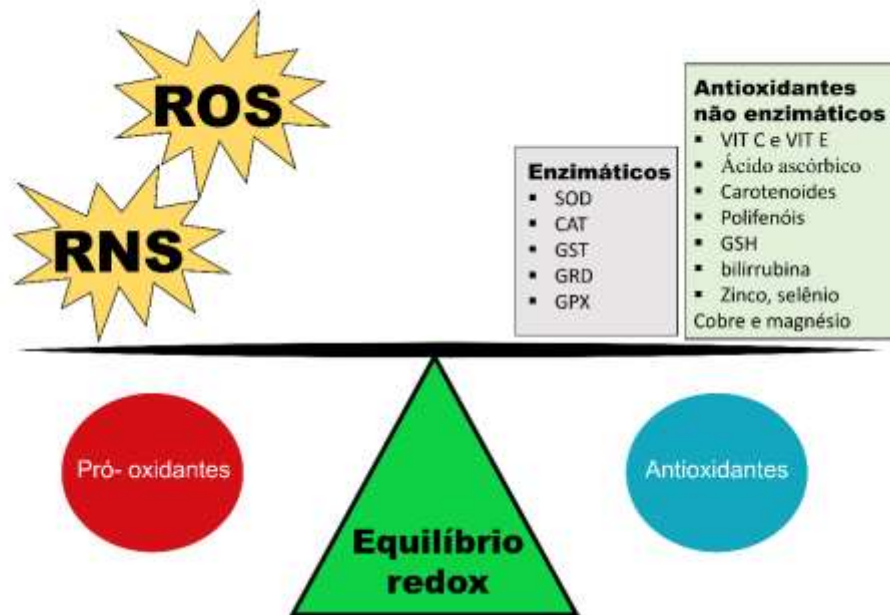
Fonte: do autor.

2.2 Estresse oxidativo e seus biomarcadores enzimáticos.

Estresse oxidativo pode ser definido como um desequilíbrio entre fatores pró-oxidantes e antioxidantes, em favor da geração excessiva de radicais livres ou em detrimento da velocidade de remoção desses, que resulta em dano macromolecular e interrupção da sinalização e controle redox (BARBOSA et al., 2010; JONES, 2008; PISOSCHI; POP, 2015). Esse processo causa oxidação de biomoléculas, com perda de suas funções fisiológicas e/ou desequilíbrio homeostático, a cronicidade desse processo pode contribuir com o surgimento de inúmeras patologias crônicas não transmissíveis como câncer, aterosclerose, diabetes e obesidade (BARBOSA et al., 2010).

O equilíbrio desses fatores pró-oxidantes pode acontecer de várias formas como pelas defesas enzimáticas através das enzimas superóxido dismutase (SOD), catalase (CAT), via da glutatona como glutatona redutase (GR), glutatona S-transferases (GST) e glutatona peroxidase (GPX) e por antioxidantes não enzimáticos como o ácido ascórbico, GSH, bilirrubina, (vitamina C), vitamina E, carotenoides, polifenóis, zinco, selênio, cobre e magnésio (BARBOSA et al., 2010; BRIEGER et al., 2012; HAYES; FLANAGAN; JOWSEY, 2005) (Figura 8).

Figura 8 - Geração e degradação de espécies reativas contribuindo para o balanço oxidativo.



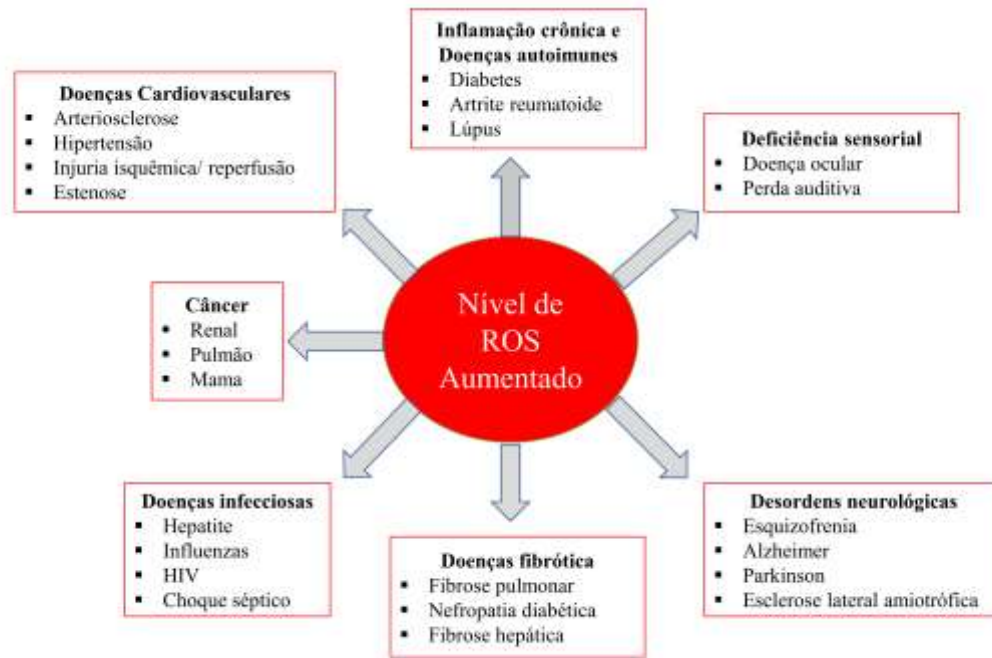
Fonte: Do autor.

2.2.1 Espécies reativas de oxigênio (ROS).

A oxidação faz parte da vida aeróbica no metabolismo dos seres vivos, assim, os radicais livres como espécies reativas de oxigênio (ROS) e espécies reativas de nitrogênio (RNS) são produzidas através da respiração aeróbica e da oxidação do substrato de forma natural ou por alguma disfunção biológica no organismo (BARREIROS; DAVID; DAVID, 2006; BRIEGER et al., 2012; MATÉS; PÉREZ-GÓMEZ; DE CASTRO, 1999).

As ROS têm um papel crucial nos processos fisiológicos e fisiopatológicos dos animais, estando envolvidos em grande parte das doenças da atualidade (Figura 9). Todos os tipos de ROS, como os radicais hidroxila (OH^{\bullet}), ânions superóxido ($\text{O}_2^{\bullet-}$) e peróxido de hidrogênio (H_2O_2), possuem elétrons de valência desemparelhados ou ligações instáveis. Em altas concentrações as ROS reagem com carboidratos, ácidos nucleicos, proteínas e lipídios, causando destruição ou alterações funcionais irreversíveis (BRIEGER et al., 2012; HURST et al., 1997; JORNOT; PETERSEN; JUNOD, 1998; MATÉS; PÉREZ-GÓMEZ; DE CASTRO, 1999; MILLS et al., 1998; SASSETTI; CLAUSEN; LARAIA, 2021).

Figura 9 - Contribuição das ROS em patologias.

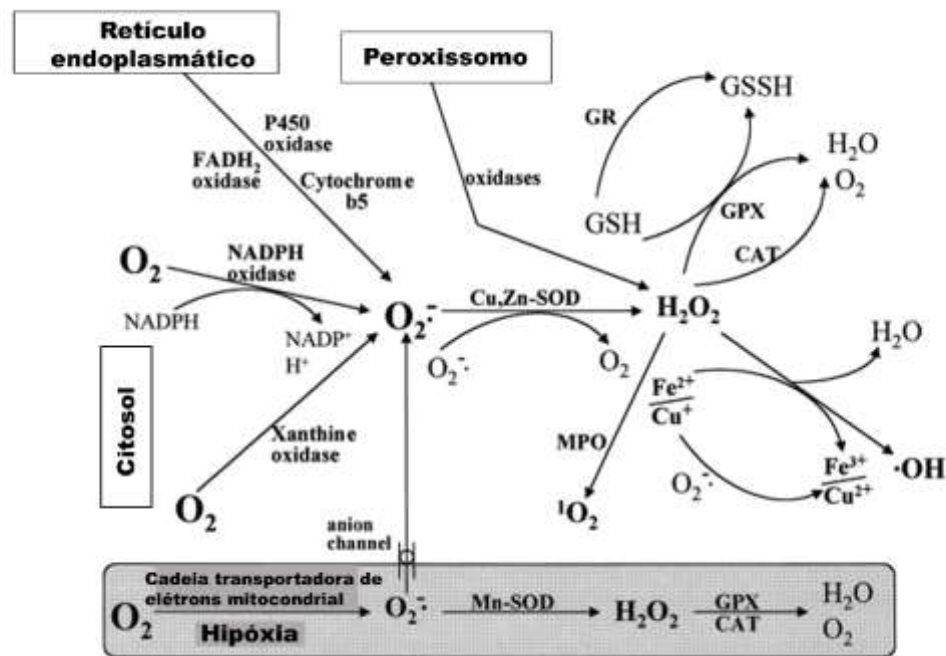


Fonte: adaptado de BRIEGER et al., (2012).

As principais ROS podem ser distribuídas em dois grandes grupos, os radicalares: superóxido ($O_2^{\cdot-}$), hidroxila (HO^{\cdot}), peroxila (ROO^{\cdot}) e alcoxila (RO^{\cdot}); e os não-radicalares: oxigênio, peróxido de hidrogênio (H_2O_2) e ácido hipocloroso ($HOCl$). Já entre as RNS temos óxido nítrico (NO^{\cdot}), óxido nitroso (N_2O_3), ácido nitroso (HNO_2), nitritos (NO_2^-), nitratos (NO_3^-) e peroxinitritos ($ONOO^-$) (BARREIROS; DAVID; DAVID, 2006; SASSETTI; CLAUSEN; LARAIA, 2021).

O radical HO^{\cdot} é o mais deletério ao organismo, ele é formado por dois mecanismos, reação de peróxido de hidrogênio com metais de transição e homólise da água por exposição à radiação ionizante (BARREIROS; DAVID; DAVID, 2006; HALLIWELL; GUTTERIDGE; CROSS, 1992). O peróxido de hidrogênio de forma isolada é praticamente inofensivo, mas se difunde com facilidade através das membranas celulares, devido ao fato de as células possuírem metais de transição como Cu^{1+} , Fe^{2+} e Zn , porém o metal ferro tem uma maior importância por sua alta biodisponibilidade no organismo. A reação do metal ferro em junção com o peróxido de hidrogênio é conhecida como reação de Fenton, responsável pela formação de radicais hidroxila (BARREIROS; DAVID; DAVID, 2006; MATÉS; PÉREZ-GÓMEZ; DE CASTRO, 1999) como podemos ver no esquema da figura 10.

Figura 10 - Geração de espécies reativas de oxigênio



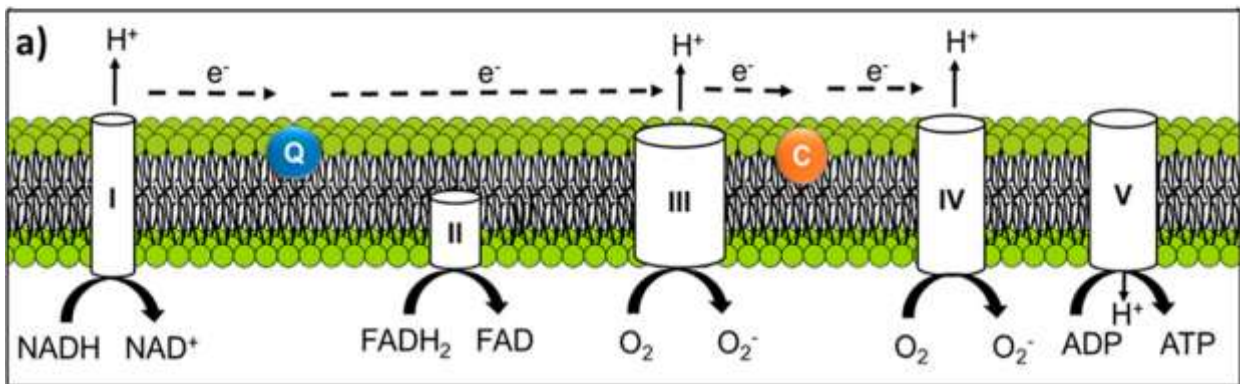
Legenda: Durante a hipóxia, o $O_2^{\cdot-}$ pode ser degradado na mitocôndria por Mn-SOD ou, se atingir o citosol, por Cu, Zn-SOD. No retículo endoplasmático, NADPH-citocromo P450 redutase pode vaziar elétrons para O_2 gerando $O_2^{\cdot-}$. $FADH_2$ e citocromo b5 também podem contribuir para este sistema. Dentro dos peroxissomos, localizam-se enzimas que produzem H_2O_2 sem a intermediação de $O_2^{\cdot-}$. Ao contrário do $O_2^{\cdot-}$, H_2O_2 é capaz de atravessar as membranas celulares e dentro das células pode reagir com Fe^{2+} ou Cu^+ para formar $HO\cdot$ via reação de Fenton. GR = glutationa redutase; MPO = mieloperoxidase; 1O_2 = oxigênio singlete

Fonte: MATÉS, PÉREZ-GÓMEZ e DE CASTRO (1999).

O local com maior produção de ROS é a mitocôndria (MUNTEAN et al., 2016), mais especificamente na cadeia transportadora de elétrons mitocondrial da membrana interna da mitocôndria, através da fosforilação oxidativa gerando adenosina trifosfato (ATP) (LI et al., 2013; SASSETTI; CLAUSEN; LARAIA, 2021). O processo acontece inicialmente pela captação de elétrons do NADH (complexo I) ou $FADH_2$ (complexo II), que são produzidos no ciclo do ácido tricarboxílico, e transferidos pela cadeia transportadora de elétrons mitocondrial para o complexo IV, que em condições fisiológicas produzira água pela redução do O_2 . Em condições adversas quando a redução não prossegue até a formação da água e termina antes da formação do $O_2^{\cdot-}$ temos

a produção das ROS. O potencial de membrana mitocondrial ($\Delta\psi_m$) possibilita a geração de ATP pela ação da ATP sintase no complexo V com redirecionamento dos prótons de volta à matriz (Figura 11) (SASSETTI; CLAUSEN; LARAIA, 2021).

Figura 11 - Estrutura e processos de formação de ROS da cadeia transportadora de elétrons mitocondrial

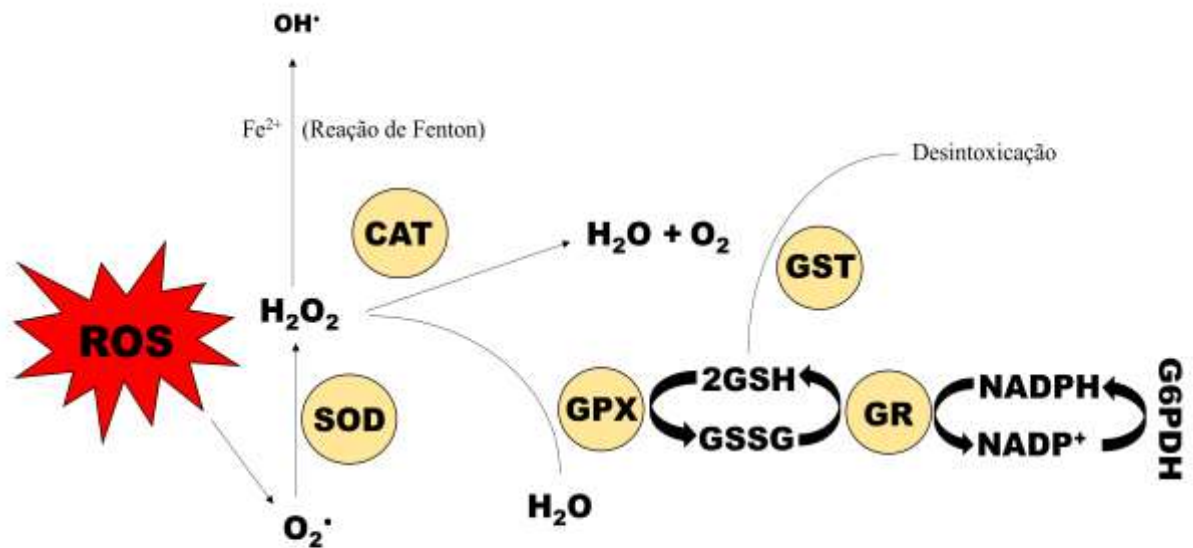


Fonte: SASSETTI, CLAUSEN e LARAIA (2021).

2.2.2 Superóxido dismutase (SOD)

A SOD é a enzima antioxidante que catalisa a dismutação do ânion superóxido altamente reativo para O_2 e para espécie menos reativa H_2O_2 . O peróxido de hidrogênio pode ser decomposto pelas enzimas CAT ou GPX (MATÉS; PÉREZ-GÓMEZ; DE CASTRO, 1999) (Figura 12).

Figura 12 - Atividade antioxidante por ação enzimática.



Fonte: Do autor.

Em seres humanos e zebrafish, existem três formas de SOD, Cu/Zn-SOD citosólica, Mn-SOD mitocondrial e SOD extracelular (EC-SOD) (BARBOSA et al., 2010; BEAUCHAMP; FRIDOVICH, 1971; KEN et al., 2003; LIN et al., 2009; MATÉS; PÉREZ-GÓMEZ; DE CASTRO, 1999; MATTHIESEN et al., 2021).

Mn-SOD (SOD 2) é um homotetrâmero (96 kDa) que contém um átomo de manganês por subunidade que cicla de Manganês (III) para Manganês (II) e retorna para Manganês (III) durante a dismutação do radical $\text{O}_2^{\cdot-}$ (ANN MACMILLAN-CROW; CROW; THOMPSON, 1998). A Mn-SOD demonstrou ser fortemente induzida por citocinas, porém moderadamente influenciada por fatores oxidantes (STRALIN; MARKLUND, 1994). Sua importância biológica pode ser demonstrada em outros modelos, pelas seguintes observações, a supressão do gene Mn-SOD em *Saccharomyces cerevisiae* aumenta sua sensibilidade ao oxigênio (VAN LOON; PESOLD-HURT; SCHATZ, 1986), já em camundongos *knock-out* para Mn-SOD resulta em cardiomiopatia dilatada e letalidade neonatal (LI et al., 1995), o fator de necrose tumoral induz seletivamente Mn-SOD, mas não Cu/Zn-SOD, CAT ou GPX mRNA em vários tecidos de roedores e modelos in vitro (HACHIYA et al., 1997; KIZAKI et al., 1993).

A Cu/Zn-SOD (SOD 1) é considerada uma enzima importante na neutralização da toxicidade mediada por radicais de oxigênio (KEN et al., 2003), sua estrutura é monomérica com duas subunidades idênticas de 32 kDa. Cada subunidade contém um aglomerado metálico, com o sítio ativo constituído por um átomo de Cu e um átomo de Zn ligados por um resíduo de histamina (MATÉS; PÉREZ-GÓMEZ; DE CASTRO, 1999; STROPPOLO et al., 1998). Acreditasse Cu/Zn-SOD tem um papel de importância na primeira linha de defesa antioxidante (MATÉS; PÉREZ-GÓMEZ; DE CASTRO, 1999).

A EC-SOD (SOD 3) é uma glicoproteína secretora, tetramérica, contendo Cu e Zn, com alta afinidade por certos glicosaminoglicanos, como heparina e sulfato de heparano (MATÉS; PÉREZ-GÓMEZ; DE CASTRO, 1999). A EC-SOD pode ser encontrada em fluidos extracelulares e espaços intersticiais dos tecidos, sendo o tipo com maior atividade de SOD no plasma, líquido sinovial e linfa (SANDSTRÖM et al., 1994). Ao contrário das outras SODs ela não é induzida por respostas de células individuais a fatores oxidantes, sua regulação é coordenada por citocinas (MATÉS; PÉREZ-GÓMEZ; DE CASTRO, 1999).

2.2.3 Catalase (CAT)

A CAT é uma enzima tetramérica que consiste em quatro subunidades idênticas de 60 kDa que contém um único grupo ferriprotoporfirina por subunidade e tem uma massa molecular entre 200 e 340 kDa (GLORIEUX; CALDERON, 2017; MATÉS; PÉREZ-GÓMEZ; DE CASTRO, 1999). A função primordial da CAT é a dismutação do H_2O_2 em H_2O e O_2 (figura. 13), importante função na defesa celular contra danos oxidativo gerados pelo H_2O_2 (GLORIEUX; CALDERON, 2017). O ânion peróxido de hidrogênio não é tóxico apenas pela função de formar outras ROS, como o radical OH^\bullet através da reação de fenton (FENTON, 1894) (Figura 12), ele também atua como segundo mensageiro, participando em vários processos biológicos, incluindo alterações da morfologia, proliferação, sinalização de NF- κ B, apoptose, dentre outros (SIES; CHANCE, 1970). Além da atividade primordial (dismutação do H_2O_2) a CAT reage com pequenos doadores de H como metanol, etanol, ácido fórmico ou fenóis, decompondo-os através da atividade peroxidática (GLORIEUX; CALDERON, 2017; MATÉS; PÉREZ-GÓMEZ; DE CASTRO, 1999).

2.2.4 Glutationa (GPX, GST e GR)

A Glutationa peroxidase (80 kDa) catalisa a redução de hidroperóxidos usando a GSH, assim protegendo as células de danos oxidativos. Embora a GPX compartilhe o substrato peróxido

de hidrogênio com a catalase (Figura 12), ela pode reagir efetivamente com lipídios e outros hidroperóxidos orgânicos, sendo a principal fonte de proteção contra baixos níveis de estresse oxidativo (MATÉS; PÉREZ-GÓMEZ; DE CASTRO, 1999).

Existem cinco isoenzimas GPX encontradas em mamíferos (MATÉS; PÉREZ-GÓMEZ; DE CASTRO, 1999), porém no zebrafish encontramos relatos de apenas duas GPX₁ e GPX₄ (CSETTA et al., 2021; RONG et al., 2017). Os níveis de cada isoforma de GPX varia dependendo do tecido. A GPX citosólica e mitocondrial (GPX₁) está predominantemente presente em eritrócitos, rins e fígado, ela reduz o peróxido de hidrogênio em detrimento da glutathiona (figura. 13). Já a GPX₄ está localizada tanto no citosol quanto na fração da membrana e pode ser encontrada em vários tecidos, como células epiteliais renais e testículos, ela pode reduzir diretamente os hidroperóxidos de fosfolipídeos, hidroperóxidos de ácidos graxos e hidroperóxidos de colesterol que são produzidos nas membranas peroxidadas e lipoproteínas oxidadas (IMAI et al., 1998; MATÉS; PÉREZ-GÓMEZ; DE CASTRO, 1999).

A Glutathiona S- transferase é uma enzima que catalisa o ataque nucleofílico pela glutathiona reduzida (GSH) em compostos apolares que contêm um átomo eletrofílico de carbono, nitrogênio ou enxofre, o estudo dessa enzima é de grande interesse na área medica e toxicológica, pois as GSTs metabolizam agentes quimioterápicos, inseticidas, herbicidas, carcinógenos e subprodutos do estresse oxidativo (HAYES; FLANAGAN; JOWSEY, 2005). Existem três grandes famílias de proteínas que exibem a atividade de glutathiona S- transferase, a GST mitocondrial e citosólica que compreendem enzimas solúveis e a GST microsomal referida como proteínas associadas à membrana no metabolismo de eicosanóides e glutathiona (MAPEG) (LADNER et al., 2004), as GST mitocondrial e citosólica compartilham algumas semelhanças em suas dobras tridimensionais, mas não compartilham semelhança com a MAPEG. No entanto as três famílias catalisam a junção de GSH com 1-cloro-2,4-dinitrobenzeno (CDNB) (HAYES; FLANAGAN; JOWSEY, 2005).

As GSTs podem reduzir os níveis de hidroperóxidos de colesterol e hidroperóxidos de ácidos graxos (HIRATSUKA et al., 1997; PRABHU et al., 2004). A redução de fosfolipídeos, ácidos graxos e hidroperóxidos de colesterol reduz a formação de epóxidos e carbonilas reativas decorrentes da oxidação de membranas (HAYES; FLANAGAN; JOWSEY, 2005). Entre os produtos finais da peroxidação lipídica, as GSTs conjugam GSH com 2-alquenais acroleína, crotonaldeído e 4-hidroxi-2-alquenais (HAYES; MCLELLAN, 2009; HUBATSCH;

RIDDERSTRÖM; MANNERVIK, 1998). As GSTs também catalisam a conjugação de colesterol-5,6-óxido, ácido epóxi-eicosatrienóico e ácido 9,10-epoxiesteárico com GSH (HAYES; PULFORD, 2008). Esses achados indicam que a GST juntamente com outras enzimas antioxidantes como SOD e CAT, fornecem proteção as células contra uma série de agentes nocivos produzidos durante o dano oxidativo as membranas (HAYES; FLANAGAN; JOWSEY, 2005).

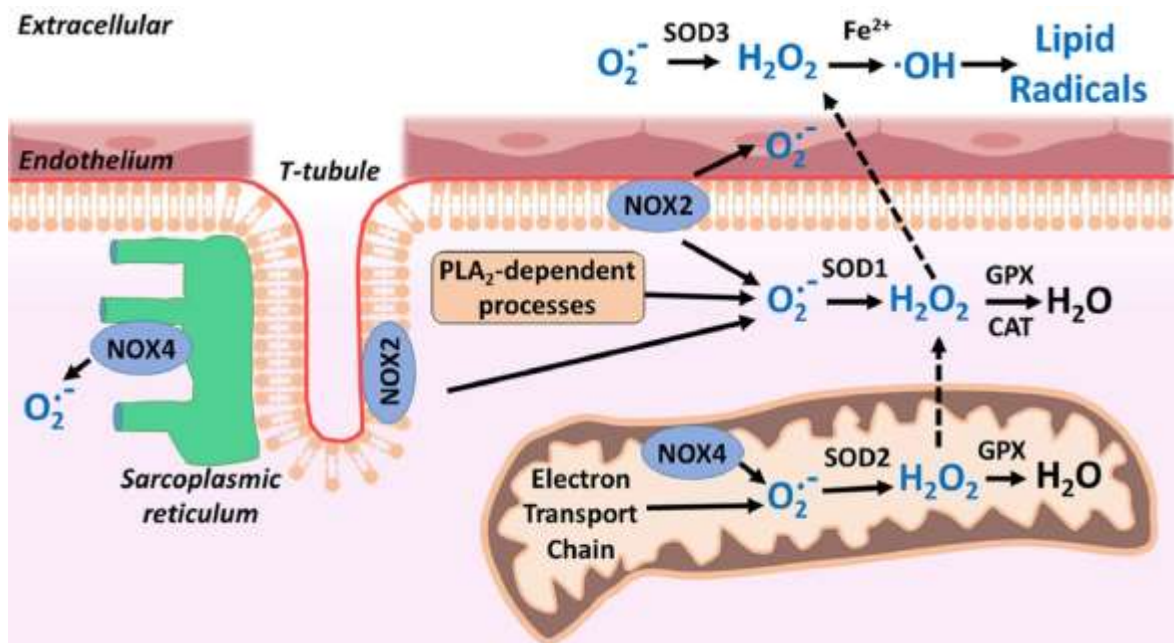
A Glutathione redutase tem como função primordial reciclar a glutathione oxidada de volta a sua forma reduzida (Figura. 13). A GR contém domínios altamente conservados, incluindo dois domínios de dobra de *Rossmann* (COUTO; WOOD; BARBER, 2016), onde um domínio se liga ao dinucleotídeo de flavina adenina (FAD) e fosfato de dinucleotídeo de nicotinamida adenina reduzido (NAPDH) já o outro é um domínio de dimerização de interface (COUTO; WOOD; BARBER, 2016). A GR tende a se acumular em regiões celulares com alto fluxo de elétrons, onde são geradas as ROS. Ela pode ser encontrada principalmente no citoplasma e dentro de organelas, como no núcleo e nas mitocôndrias (COUTO et al., 2013; COUTO; WOOD; BARBER, 2016; OUTTEN; CULOTTA, 2004).

2.2.5 Exercício Físico e estresse oxidativo

Há evidências científicas suficientes para deixar claro que o exercício físico dependente do volume e intensidade aumenta a produção de ROS em tecidos biológicos como músculo estriado esquelético e cardíaco (BEJMA; RAMIRES; JI, 2000; BOUVIERE et al., 2021; DAVIES et al., 1982; GOMEZ-CABRERA et al., 2006; GOMEZ-CABRERA; DOMENECH; VIÑA, 2008; SASTRE et al., 1992). O exercício físico quando praticado até a exaustão, especialmente de maneira esporádica, causa danos estruturais às células musculares ou reações inflamatórias dentro dos músculos, como aumento na atividade plasmática de enzimas citosólicas, esses danos ocorrem devido à alta produção de radicais livres podendo gerar estresse oxidativo (GOMEZ-CABRERA; DOMENECH; VIÑA, 2008). A pesquisa de VIÑA et al., (2000) demonstrou que exercícios físicos moderados causam aumento na produção de ROS, mas são contrabalanceados pelo aumento concomitante das enzimas antioxidantes, como SOD, CAT e GPX (POWERS et al., 2023; SIMIONI et al., 2018) (Figura 13). Assim, é importante distinguir entre exercício físico até a exaustão, que causa estresse oxidativo e exercício físico moderado, que não é acompanhado de estresse oxidativo (GOMEZ-CABRERA et al., 2006; GOMEZ-CABRERA; DOMENECH; VIÑA, 2008).

Embora as ROS possam ser produzidas em uma variedade de tecidos durante o exercício físico (POWERS et al., 2020), foi estabelecido que o músculo esquelético é a fonte predominante de produção de ROS durante o exercício físico (JACKSON; VASILAKI; MCARDLE, 2016). As fontes mais relevantes no músculo esquelético durante o exercício são as mitocôndrias, xantina oxidase (XO), fosfolipase A₂ (PLA₂) e (3) NADPH oxidases (NOX2 e NOX4) presentes em quatro locais dentro das fibras: mitocôndrias, sarcolema, retículo sarcoplasmático e túbulos T (BOUVIERE et al., 2021; GOMEZ-CABRERA et al., 2010; GOMEZ-CABRERA; DOMENECH; VIÑA, 2008; POWERS et al., 2020, 2023; SAKELLARIOU et al., 2013) (figura 13). Enquanto os ânions superóxido são gerados pelas mitocôndrias e as xantinas oxidases como um subproduto secundário da fosforilação oxidativa e do metabolismo das purinas, respectivamente, as enzimas NADPH oxidase são exclusivamente dedicadas à geração de superóxido e peróxido de hidrogênio (BOUVIERE et al., 2021) (Figuras 13 e 14).

Figura 13 - Locais potenciais de produção de ROS na contração dos músculos esqueléticos e sua eliminação por enzimas antioxidantes.



Legenda: CAT= catalase; GPX= glutatona peroxidase; H₂O₂= peróxido de hidrogênio; NOX= NADPH oxidase; O₂^{·-}= superóxido; ·OH= Radical hidroxila; PLA₂= Fosfolipase A₂; SOD= superóxido dismutase.

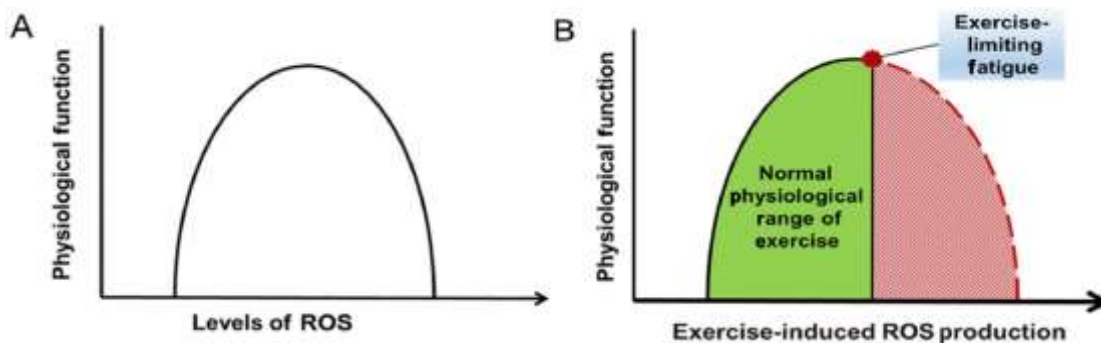
Fonte: POWERS et al. (2020).

Figura 14 - Visão geral dos mecanismos de geração de ROS após o exercício.

Acredita-se que a regulação redox ajustada seja necessária para alcançar as adaptações fisiológicas induzidas pelo exercício físico, fatores externos, como a suplementação com antioxidantes, podem prejudicar as melhorias musculares induzidas pelo exercício físico em roedores e humanos (BRAAKHUIS; HOPKINS; LOWE, 2014; GOMEZ-CABRERA et al., 2008). Além da capacidade máxima do exercício, outra resposta desejável é a melhora da captação muscular de glicose (SYLOW et al., 2016), já há relatos na literatura que a suplementação com vitaminas C e E impedem a melhora na sensibilidade à insulina de jovens pré-treinados, seguida pela expressão reduzida de PPAR α , PGC-1 α e PGC-1 β (RISTOW et al., 2009).

Por fim se a formação de ROS induzida pelo exercício é prejudicial ou benéfica para a saúde, depende do equilíbrio entre os níveis de produção de ROS gerada pelo exercício e o acionamento de defesas antioxidantes celulares como SOD, CAT e GPX para proteger as células contra um desafio oxidante (POWERS et al., 2020, 2023). Várias revisões já concluíram que o exercício físico regular não resulta em estresse oxidativo crônico nos músculos ativos (DE SOUSA et al., 2017; DI MEO; NAPOLITANO; VENDITTI, 2019; NOCELLA et al., 2019; RADAK et al., 2017), essa conclusão é apoiada pelo conceito de hormese induzida por exercício, ou seja, uma curva de dose resposta, em que aumento transitório de ROS em baixos níveis fornece um efeito adaptativo benéfico as células, enquanto uma dose crônica e/ou alta do ROS resulta em danos às células (POWERS et al., 2020) (Figura 15).

Figura 15 - Conceito de hormese aplicado ao exercício físico e estresse oxidativo.



Fonte: POWERS et al. (2020).

2.2.5.1 Influência da dieta na interação entre o exercício físico e o estresse oxidativo

A dieta é um fator importante para manutenção da homeostase animal, tanto de humanos, como de roedores e peixes (BIRNIE-GAUVIN et al., 2017; KITABCHI et al., 2013; LAN et al.,

2022; SUN et al., 2020). O efeito do estresse oxidativo induzido pela dieta está relacionado ao tipo de macronutriente consumido e sua quantidade absoluta, ambos aspectos contribuem para indução de um estresse oxidativo e podem favorecer aparecimento de doenças metabólicas como obesidade e diabetes (TAN; NORHAIZAN; LIEW, 2018). Em uma via contrária temos a dieta como fator antioxidante, como a ingestão de alimentos ricos em vitaminas C e E, bilirrubina, carotenoides, polifenóis, zinco, selênio, cobre e magnésio (ATALAY; LAPPALAINEN; SEN, 2006; BARBOSA et al., 2010; BRIEGER et al., 2012; URSO; CLARKSON, 2003). A ingestão alimentos ricos em antioxidantes é realizada com intuito de suprimir o “dano” oxidativo causado pelo exercício físico extenuante (PANZA et al., 2016, 2008; ZENG et al., 2021), pois durante o exercício físico regular essa suplementação pode suprimir as adaptações advindas do exercício físico, causando prejuízos invés de benefícios (BOUVIERE et al., 2021; BRADY; BRADY; ULLREY, 1979; RISTOW et al., 2009; SHARMAN; DOWN; SEN, 1971).

Dentre os macronutrientes que podem causar estresse oxidativo se ingeridos em quantidade exacerbada estão os carboidratos dietéticos, quando consumidos a longo prazo podem causar inflamação crônica (DI PENTA et al., 2013; WIEGMAN et al., 2015), aumentando ainda mais o acúmulo de citocinas pró-inflamatórias e a produção de ROS. Uma dieta com alto índice glicêmico está associada com o surgimento de doenças como diabetes, obesidade, câncer e doença cardíaca coronária (SCHWINGSHACKL; HOFFMANN, 2013; TURATI et al., 2015), além disso a alta ingestão de grãos de carboidratos refinados também foi associada positivamente aos níveis de glicose no sangue e triglicerídeos em jejum e negativamente associada ao colesterol de lipoproteína de alta densidade (HDL), indicando que uma dieta com alto índice glicêmico pode afetar negativamente a saúde (RADHIKA et al., 2009; SONG et al., 2014; TAN; NORHAIZAN; LIEW, 2018).

Uma dieta rica em carboidratos fará com que mais substratos entrem na respiração mitocondrial (TAN; NORHAIZAN; LIEW, 2018) e aumentará o número de elétrons doados para a cadeia de transporte de elétrons mitocondrial (BROWNLEE, 2001). Ao atingir uma tensão limite, os elétrons extras podem retornar ao complexo III com mais doações para o oxigênio molecular, que produz altos níveis de superóxido (TAN; NORHAIZAN; LIEW, 2018).

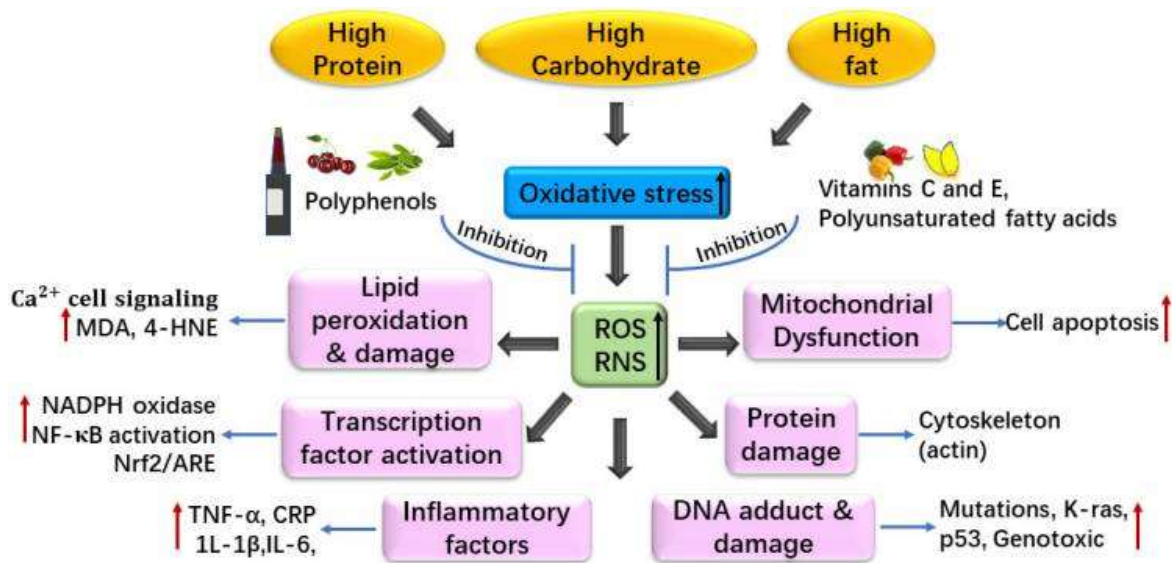
Outro macronutriente que pode causar estresse oxidativo se ingerido em quantidade exacerbada é a proteína de origem animal mais especificamente a carne vermelha. A ingestão

excessiva dessas proteínas pode aumentar a produção de espécies reativas de oxigênio na glândula digestiva, prejudicando a oxidação de aminoácidos no sistema digestivo e causando instabilidade das enzimas antioxidantes (TAN; NORHAIZAN, 2021). Vários produtos do metabolismo são formados, como sulfeto de hidrogênio (H_2S) e amônia NH_3 , compostos conhecidos por induzir toxicidade na mucosa intestinal durante a fermentação de proteínas em excesso (SCOTT et al., 2013).

Por fim uma dieta com alto teor de gordura irá produzir um alto teor de ácido graxos livres (AGL), aumentando os quilomícrons no intestino que são absorvidos pelo intestino, podendo entrar na β -oxidação ou esterificação em bases triglicerídeas (TG). Os TG se acumulam dentro das células hepáticas ou produzem lipoproteínas de muito baixa densidade (VLDL) que são transformadas em LDL (COLCA et al., 2017). Uma quantidade elevada de LDL pode formar LDL oxidado (Ox-LDL) no sangue, que é endocitado por macrófagos e depois se transforma em células espumosas, podendo formar placas através da agregação de células endoteliais arteriais levando a doenças cardiovasculares (FERENCE et al., 2017). A estimulação da β -oxidação mitocondrial de AGL causada pelo excesso de acúmulo de gordura, leva a um aumento excessivo no fluxo de elétrons da citocromo c oxidase, fazendo que aumente o acúmulo de ROS nas células (KOO; KANG, 2019). As mitocôndrias são uma das principais fontes de ROS nas células, que oxidam os lipídios insaturados e causam a peroxidação lipídica (JIANG; LIU; LI, 2021). A produção de ROS estimuladas por dietas com excesso de gordura pode desencadear sinais pró-inflamatórios e fatores de transcrição NF- κ B, levando a ativação de moléculas pró-inflamatórias dependentes de NF- κ B como IL-1 e TNF- α (DALVI et al., 2017; JIANG; LIU; LI, 2021).

Todas as dietas com consumo excessivo de macronutrientes (proteínas, carboidratos e gordura) não afetam apenas o nível de estresse oxidativo, mas também têm um impacto adverso na saúde devido ao excesso de radicais livres (ROS e RNS) (JIANG; LIU; LI, 2021). Os antioxidantes enzimáticos e exógenos (polifenóis, vitamina C e E, dentre outros), podem neutralizar as ROS e desempenhar um papel importante na prevenção do dano celular oxidativo induzido pelos radicais livres, intervenções no estilo de vida estabelecem uma relação bidirecional entre alimentação e saúde (JIANG; LIU; LI, 2021; LOBO et al., 2010) (Figura 16).

Figura 16 - Associações entre dieta e estresse oxidativo.



Fonte: JIANG, LIU e LI (2021).

Dentre os vários micronutrientes, temos alguns que são conhecidos por suas propriedades antioxidantes como vitamina E e C, selênio, taurina, dentre outros. A suplementação com vitamina E combinada com vitamina C é comumente utilizada por praticantes de exercício físico, devido ao seu efeito antioxidante combinado (PAULSEN et al., 2014), em peixes também já tem relatos dos seus efeitos antioxidantes (PADURARU et al., 2021; ROBEA et al., 2020). A vitamina E é um poderoso antioxidante solúvel em gordura que inclui quatro tocoferóis e quatro tocotrienóis com α -tocoferol, ela é capaz de doar átomos de hidrogênio para radicais livres, incluindo radicais OH^\bullet e $\text{O}_2^{\bullet-}$, transformando-os em uma forma mais estável atenuando a peroxidação lipídica. A vitamina C, uma vitamina hidrossolúvel, também tem um efeito protetor contra a produção de radicais livre. Ambas as vitaminas trabalham de forma conjunta, a vitamina C ajuda a reciclar a vitamina E de volta a um estado reduzido permitindo que continue a oxidar os radicais livre (HIGGINS; IZADI; KAVIANI, 2020).

O micronutriente selênio é um componente essencial das GPXs e tioredoxina redutases entre vários outros, estabelecendo um papel importante na proteção celular contra o estresse oxidativo (RAYMAN, 2000), como relatado por FONTAGNÉ-DICHARRY et al., (2015) que demonstraram o poder antioxidante do selênio em *Oncorhynchus mykiss* suplementados com dietas à base de vegetais suplementados com selênio. RIDER et al., (2009) descobriram que *Oncorhynchus mykiss* suplementados com levedura de selênio tiveram um aumento na glutathione reduzida oxidada em comparação com peixes alimentados com selenito de sódio ou dietas controle.

A Taurina é conhecida por seu papel na proteção do organismo contra o estresse oxidativo. O efeito antioxidante da taurina parte da premissa que o impedimento do desvio de elétrons da cadeia transportadora de elétrons (CTE) para o aceptor de O_2 formado no processo do anión $O_2^{\cdot-}$, aumentando a atividade da CTE e protegendo as mitocôndrias contra a geração excessiva de aníons $O_2^{\cdot-}$ (JONG; AZUMA; SCHAFFER, 2012). É aceito pela comunidade científica que diminuir o fluxo de elétrons através da CTE pode desviar elétrons dos complexos I-III para um aceptor alternativo, como o oxigênio, sabendo que os complexos I-III são considerados fontes primárias para geração de $O_2^{\cdot-}$ na mitocôndria (CECCOTTI et al., 2019), foi documentado que a deficiência de Taurina compromete as atividades celulares (JONG; AZUMA; SCHAFFER, 2012). O estudo de (CECCOTTI et al., 2019) comprovou o efeito da Taurina durante um estresse oxidativo induzido por exercício físico em *Centropomus undecimalis*. Os autores identificaram uma diminuição significativa na expressão de catalase e glutathiona peroxidase no musculo e fígado dos animais.

3 CONSIDERAÇÕES FINAIS

A obesidade é uma comorbidade caracterizada pelo acúmulo excessivo de gordura, tanto visceral quanto subcutânea, resultando em significativos impactos na saúde, como distúrbios no metabolismo lipídico e aumento do estresse oxidativo. O zebrafish tem sido amplamente utilizado como modelo em estudos biomédicos e, cada vez mais, tem se consolidado como uma ferramenta relevante para investigações sobre obesidade e suas alterações metabólicas. Uma abordagem não farmacológica para o tratamento da obesidade é a mudança no estilo de vida, especificamente a inclusão de exercício físico na rotina diária. Embora alguns estudos tenham explorado a relação entre exercício físico e zebrafish, ainda não existem na literatura pesquisas que tenham investigado a relação entre obesidade, exercício físico e estresse oxidativo no modelo zebrafish.

Portanto, este estudo representa o primeiro esforço em fornecer uma base científica sólida sobre os efeitos do exercício físico nos parâmetros histológicos, bioquímicos e estresse oxidativo em zebrafish obesos, apresentando novas perspectivas que podem ser úteis para pesquisas futuras nesta área.

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SEGUNDA PARTE

ARTIGO 1 - The Zebrafish (*Danio rerio*) as a Model for Studying Voluntary Physical Exercise and its Effects on Behavior and Metabolism

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The Zebrafish (*Danio rerio*) as a Model for Studying Voluntary Physical Exercise and its Effects on Behavior and Metabolism

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HIGHLIGHTS

- The proposed voluntary physical exercise model does not cause stress in zebrafish.
- The zebrafish in the light period voluntarily chooses to frequent areas with faster water.
- The animals in the VPE group showed a higher maximum acceleration and speed during the locomotion test.

Abstract: The objective of this study was to develop a viable and low-cost model of voluntary physical exercise that could be applied to studies on metabolism and behavior. 40 male zebrafish (*Danio rerio*) were studied, divided into two groups: control and voluntary physical exercise. The model consisted of two aquariums connected by a translucent tube and a video camera on the side to measure physical exercise parameters of the animals. The animals showed higher acceleration and maximum speed and had a higher frequency of activity in the light period. In this model of voluntary physical exercise, we observed better performance in locomotor assessment tests, which was not accompanied by increased anxiety or changes in biochemical parameters related to lipid metabolism. Zebrafish responded positively to voluntary physical exercise and this model appears to be a good option for further studies.

Keywords: fish; novel tank test; locomotor activity.

INTRODUCTION

Zebrafish (*Danio rerio*) is a well-established animal model for several research fields, such as genetics, developmental biology, human diseases, and ecotoxicology [1-5]. It has a low maintenance cost, high reproduction rate, short generation time (approximately 3 months), and large number of eggs per spawning, allowing many animals to be studied at once [6]. Because zebrafish has high genetic, anatomical, and physiological homology with mammals, it has great potential for the development of exercise-related models.

Any type of exercise is beneficial to health [7-13], but given the need to evaluate physical performance and the effects of exercise on diseases, it was necessary to develop models of aerobic [14, 15] and sprint exercise training [16]. A voluntary physical exercise (VPE) model for zebrafish has not yet been described, although such model is already widespread in rodents [17-21]. Using zebrafish as a model of VPE would offer all the advantages that the model has while allowing the study of VPE-stimulating drugs and their possible application to metabolic diseases. Thus, the objective of the present study was to evaluate the potential of zebrafish as a viable and low-cost VPE model that could be applied to studies on metabolism and behavior.

MATERIAL AND METHODS

Ethics statement

All experimental procedures were performed at the Central Animal Facility of Federal University of Lavras (Lavras, Minas Gerais, Brazil) and were approved by the animal research ethics committee of Federal University of Lavras (protocol 042/2019).

Experimental animals

Forty male zebrafish (*Danio rerio*) with an aged 12 months, mean weight of 0.562 ± 0.135 g, kept under a 14 h light:10 h dark photoperiod, were used. The water quality parameters (temperature, pH, and ammonia concentration) were monitored daily and kept within the ranges recommended for the species. The animals were randomly divided into two groups: VPE ($n = 20$) and control (CT) ($n = 20$).

Voluntary physical exercise system

The VPE system was adapted from McDonald and coauthors (2007) [22]. The system aimed to measure the amount of VPE using video monitoring equipment, where the maximum number of animals per hour in the tank with water flow was quantified. It consisted of two translucent tanks (22.5 cm height \times 33 cm length \times 23 cm width) connected by a translucent plastic tube (60 cm length \times 5cm) centered at 8 cm from the bottom of the tanks. This connecting tube was adjusted to the same height so that no current was produced inside the tube. The cross-sectional area of the tube was large enough to allow the zebrafish to move from one tank to the other.

The animals in the VPE group were acclimated to the system for 5 days before starting the experiment, during which the water flow simulator remained off. On the first day of the experiment, all animals were allocated to tank A, where there was no water flow-generating mechanism. Then the water flow simulator in tank B was connected to the system where the animals of the VPE group were located. Thus, the fish that opted for VPE passed through the tube to reach tank B. The water flow in tank B was generated with a pump (flow rate 2500 L/h). The water flow was restricted to this tank, as there was no water flow in the tube or in tank A. The time of day when the zebrafish preferred to perform physical activity was recorded using a high-definition infrared monitoring system (Giga Security, Brazil).

The animals in the CT group were kept in an identical system, but the water flow was kept off throughout the experiment; thus, in the system where the CT animals were kept, both tank A and tank B had the same characteristics (Figure 1).

The zebrafish were fed commercial feed (45% crude protein; Alcon, Brazil). The fish were fed three times a day to apparent satiety.

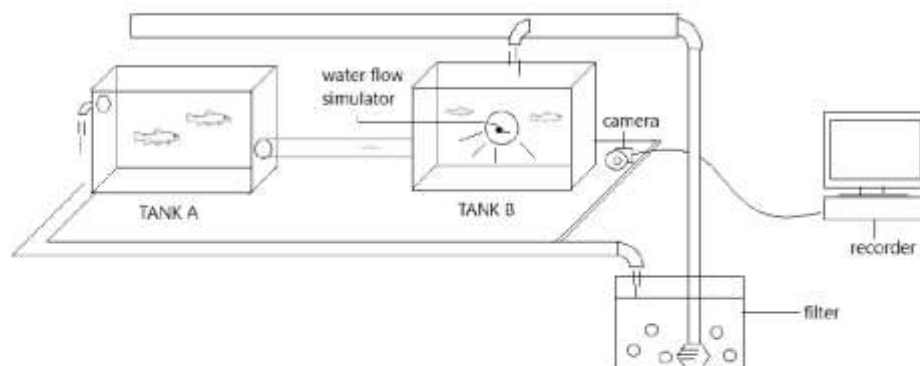


Figure 1. Model of voluntary physical exercise.

Locomotion in a regular tank

The locomotion protocol in still water was adapted from Blazina and coauthors (2013) [23] consisting of, different fish with the same characteristics were placed in a rectangular tank (27.5 x 14 x 10.5 cm, length x height x width) with 2 L of treated water. Fish locomotion was video recorded for 6 min and the Maximum speed (cm/s), Maximum acceleration and (cm/s), were automatically analyzed by the EthoVision XT® software (Noldus).

Euthanasia and biochemical parameters

At the end of the experiment, the animals were anesthetized with benzocaine (250 mg L⁻¹) [24] and euthanized. Blood was collected according to Carneiro and coauthors (2020) [25], and blood glucose levels were immediately measured using a portable glucometer (Accu-Check, Roche Diagnostics, Rotkreuz, Switzerland). Total cholesterol (TCHO), triglycerides (TG), and lactate dehydrogenase (LDH) were analyzed according to the protocol described by Sancho and coauthors (2009) [26] using the following kits: Bioclin total cholesterol (Ref. K083), LDH Bioclin (Ref. K014-2), and BioTecnica triglycerides (Ref. 10.010.00), respectively.

Analysis of cortisol

Cortisol was analyzed in five fish from each group using the extraction protocol proposed by Canavello and coauthors (2011) [27] and quantification by enzyme-linked immunosorbent assay (ELISA) (Monobind Inc., USA).

Histological analysis

Five fish were fixed whole in 10% formaldehyde aqueous solution for at least 48 h. Standard histological processing was then performed [28], and the fish were embedded in paraffin to cut 4- μ m-thick sections in a manual microtome (Lupetec, MRP2015, Brazil). The slides were then stained with hematoxylin-eosin. The images were obtained using a light microscope (Motic, USA) coupled to an image capture system (Moticam 3+, USA). The visceral adipose tissue was identified, and from 15 nonsequential fields, the area of the adipocytes was measured, as described in Virote and coauthors (2020) [28].

Novel tank test

At the end of the experimental period, the novel tank test was performed with all animals, according to the protocol of Cachat and coauthors (2011) [29]. The parameters time spent on bottom (s), time spent on top (s), distance traveled at the top (cm), distance traveled on the bottom (cm), frequency of top entries from bottom, frequency of bottom entries from top, stillness duration (s) were analyzed using EthoVision XT® software (Noldus).

Statistical analysis

The data are presented through descriptive statistics (mean, median, and standard deviation). The normality and homogeneity of variances were evaluated by the Shapiro-Wilk and Levene tests. Statistical comparisons between two means were performed by Student's t-test and the Mann-Whitney U test. A p-value <0.05 was considered statistically significant (Prism 7.04, GraphPad Software, La Jolla, CA, USA)..

RESULTS AND DISCUSSION

In recent decades, zebrafish has been used in diverse areas of biomedical research, so it is important to develop new models involving this species, as well as to understand its responses to new methods. To date, this is the first study to propose a model of VPE specific to zebrafish.

In rodents, the preference for the activity is linked to VPE being highly rewarding [30], and according to Palstra and coauthors (2010) [15], that could be extrapolated the zebrafish. But the results of the present study showed that zebrafish tended to remain in the tank without water flow (Figure 2A). This can be explained by the zebrafish take a prefer still or slow moving water [31]. The time the fish stayed in the tank with water flow was higher in the light period (Figure 2B), which result can be explained by the higher activity of zebrafish in the daytime [32].

The animals in the VPE group showed a higher maximum acceleration and speed during the test locomotion in a regular tank (Figures 2C and 2D). Similar results were found by Gilbert and coauthors (2013) [33], who evaluated the effect of intermittent exercise on zebrafish performance using the critical velocity method and found that it improved the maximum endurance and sprint swimming speeds in young and middle-aged fish. McDonald and coauthors (2007) [22] evaluated the performance of rainbow trout (*Oncorhynchus mykiss*) with the critical velocity method after the animals had passed through the VPE system and reported an improvement in sprint performance and fatigue threshold. The results observed may be related to the improvement in aerobic capacity, causing an increase in mitochondrial and vascular density, increased ventilation and blood ejection volume, and better recruitment of oxidative muscle fibers [34-36].

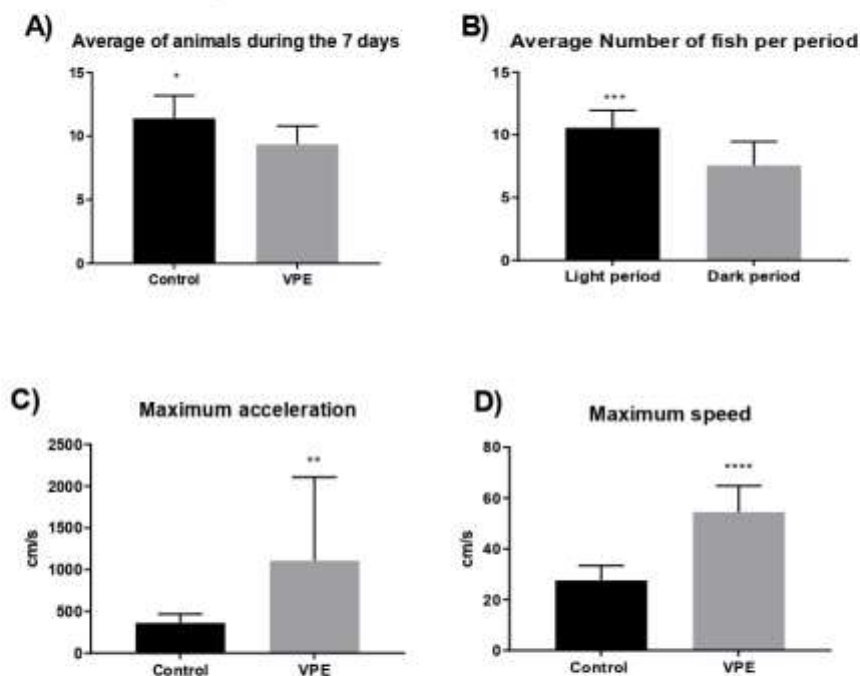


Figure 2. (A) Preference for voluntary physical exercise over time ($p= 0.0359^*$). (B) Preference for voluntary physical exercise throughout the photoperiod ($p= 0.0005^{***}$). (C) maximum acceleration comparison ($p= 0.0095^{**}$). (D) Maximum speed comparison ($p<0.0001^{****}$). Data are mean \pm SD.

We wanted to determine whether the model was of VPE and not stress, since in mammals, VPE levels are measured individually, not in groups as done in the present study. The responses to stress can be divided into three categories [37, 38]. The primary response corresponds to increased levels of plasma corticosteroids, specifically cortisol; to be considered a stressful level, it should be above 30-40 ng/ml [37]. We evaluated the primary responses and found that the animals were not in a state of stress, but there was an increase in the cortisol levels in the VPE group (Table 1). This variation may be related to non-stressors [37], such as increased cortisol for greater stimulation of gluconeogenesis to meet the energy demands of the new activity and to increase protein synthesis [39]. Secondary stress responses include numerous metabolic, hematological, hydromineral, and structural variables [37]. We evaluated two parameters, blood glucose and LDH (Table 1), but these did not change with VPE. The secondary stress response does not depend on the primary stress response, as Pickering and coauthors (1982) [40] found that cortisol and LDH in brown trout subjected to 2 min of handling returned to their resting levels within 4 h, but the peak blood glucose occurred at 4 h. Tertiary stress responses are related to the fish as a whole, i.e., to its growth rate, metabolic rate, thermal tolerance, reproductive capacity, and others [37].

Table 1. Behavioral effects after 7 days of exposure to a voluntary physical exercise system. Measures 3, 7 and 8 are expressed as mean \pm SD, and measures 1, 2, 4, 5, and 6 are expressed as median.

Measures	Control	VPE	P-value
1. Time spent on bottom (s)	350.2	352.9	0.911
2. Time spent on top (s)	0.000	0.2338	0.603
3. Distance traveled on the top (cm)	3.928 \pm 0.13	3.565 \pm 0.22	0.176
4. Distance traveled on the bottom (cm)	0.0000	0.0003	0.665
5. Frequency of top entries for bottom	0	0	0.869
6. Frequency of bottom entries for top	0	1	0.898
7. Stillness duration (s)	182.2 \pm 30.6	175 \pm 29.31	0.865
8. Total distance traveled (cm)	1154 \pm 259.4	1168 \pm 242.4	0.969

To determine the anxiety levels of the animals, we used the novel tank test because it is a well-established test [29]. We did not observe significant differences in variables related to anxiety behavior between the groups. The combined evaluation of primary, secondary, and tertiary stress responses indicates that the proposed model does not cause stress in zebrafish (Table 2).

Last, we evaluated whether exposure to the VPE system would alter factors related to lipid metabolism, as occurs in rodents [41, 42], but no differences were found in TCHO, TG, or adipocyte area (Table 1). The nonobservance of effects in the VPE group may be because the animals did not have any previous metabolic alteration. Studies of rodents typically use longer periods and animals with some pathology, such as obesity, or some metabolic syndrome-related comorbidity [43-47].

Table 2. Effects of 7 days of exposure to a voluntary physical exercise system on biochemical, hormonal, and visceral adipocyte area variables.

Variables	Control (Mean \pm SD)	VPE (Mean \pm SD)	P-value
TCHO (mg/dL)	50.084 \pm 1.213	53.034 \pm 3.706	0.129
TG (mg/dL)	129.392 \pm 9.947	126.675 \pm 6.263	0.619
LDH (U/L)	19.942 \pm 6.499	17.535 \pm 5.981	0.605
Blood glucose (mg/dL)	54.428 \pm 17.643	45.125 \pm 17.422	0.323
Cortisol (ng/g body weight)	3,765 \pm 0,4725	13,64 \pm 1,921	0.001**
Adipocyte area (μm^2)	1674.368 \pm 935.015	2278.844 \pm 442.505	0.227

CONCLUSION

Zebrafish responded positively to voluntary physical exercise and this model appears to be a good option for further studies.

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ARTIGO 2 - Metabolic Effects of Physical Exercise in Zebrafish (*Danio rerio*) Fed with a High-Fat Diet

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Metabolic Effects of Physical Exercise in Zebrafish (*Danio rerio*) Fed with a High-Fat Diet

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Abstract

The present study aimed to identify the metabolic effects of a novel physical exercise model in zebrafish (*D. rerio*) fed with a high-fat diet. The experiment spanned twelve weeks, comprising a feeding trial in which the last four weeks incorporated a physical exercise protocol. This protocol involved placing fifteen animals in a five-liter aquarium, where they were subjected to swimming at an approximate speed of 0.8 m/s for 30 minutes daily. Throughout the research, histological analyses of visceral, subcutaneous, and hepatic adipose tissues were conducted, along with biochemical analyses covering total cholesterol and its fractions, triglycerides, glucose, lactate, and Alanine Aminotransferase (ALT). Additionally, oxidative stress markers such as reactive oxygen species (ROS), superoxide dismutase (SOD), catalase, and formation of thiobarbituric acid-reactive substances were investigated. The results obtained revealed that the group fed with the high-fat diet exhibited a significant increase in ROS production and SOD activity. In contrast, the group receiving the high-fat diet and subjected to physical exercise demonstrated a notable reduction in visceral adipocyte area, hepatic steatosis levels, ALT levels, and SOD activity. These findings indicate that physical exercise exerts a positive effect in the context of obesity and oxidative stress in zebrafish, providing promising perspectives for future investigations in this field.

Keywords: Oxidative stress. Obesity. Hepatic steatosis. Reactive oxygen species.

1 INTRODUCTION

Obesity is a condition characterized by the excessive accumulation of adipose tissue, both visceral and subcutaneous [1]. This condition has serious health implications, such as the development of diseases like atherosclerosis, type 2 diabetes mellitus, and cardiovascular diseases, among others [2]. The etiology of obesity is multifactorial, resulting from a complex interaction between genetic and environmental factors. Among the environmental factors, the consumption of an unbalanced diet and lack of physical exercise play significant roles in this scenario [3,4]. Consequently, a positive energy imbalance occurs, where caloric intake exceeds energy expenditure, directly contributing to the development of obesity [1].

Obesity is associated with severe cellular damage, mainly attributed to an exacerbated inflammatory state and high production of free radicals, such as hydroxyl and superoxide radicals [5]. Under normal homeostatic conditions, these free radicals are effectively neutralized by antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GR), and glutathione S-transferase (GST) [6–8]. However, in obese organisms, the capacity of antioxidant enzymes is insufficient to counteract the high rate of free radicals, resulting in a redox imbalance that promotes lipid peroxidation [5].

Mitigating the damage caused by obesity can be achieved through two main strategies: dietary modifications and adopting a lifestyle that includes regular physical exercise [9]. Nevertheless, sporadic physical exercise to exhaustion can lead to muscular cell damage, as evidenced by an increase in plasma levels of cytosolic enzymes. This phenomenon is a result of high free radical production, culminating in oxidative stress [10]. On the other hand, when physical exercise is regularly practiced at moderate intensity, the generation of free radicals, such as reactive oxygen species (ROS), is counterbalanced by the simultaneous increase in antioxidant enzymes, such as SOD, CAT, and GPX [11–13]. It is important to emphasize that the production of ROS during moderate physical exercise is beneficial since these reactive species are essential for inducing the adaptive effects provided by physical exercise [14].

Zebrafish (*Danio rerio*) has emerged as a model organism in research addressing various metabolic alterations, including diabetes, obesity, and their resulting oxidative stress effects

[15,16]. Its advantages, such as low maintenance costs, high reproductive rate, short generation time (approximately 3 months), large number of eggs per spawn, and, most importantly, high genetic, anatomical, and physiological homology with mammals, make it a highly attractive model [17,18]. These characteristics have established zebrafish as a potential model for exercise-related studies, as demonstrated in the works of (HEINKELE et al., 2021; MARTINS et al., 2023). However, despite the growing interest in using zebrafish in exercise-related research, the effects of exercise-induced oxidative stress in zebrafish fed a high-fat diet have not been adequately elucidated. Therefore, this study aimed to identify the metabolic effects of a novel physical exercise model in zebrafish (*D. rerio*) fed a high-fat diet.

2 MATERIAL AND METHODS

2.1 Ethical aspects

All experimental procedures were conducted at the Central Animal Facility of the Federal University of Lavras (Lavras, Minas Gerais, Brazil) and were approved by the Institutional Animal Ethics Committee of the Federal University of Lavras (protocol 042/2019).

2.2 Fish

Male zebrafish (*D. rerio*) at five months post-fertilization (weight 0.381 ± 0.01 g) were housed in polycarbonate aquaria ($11.5 \text{ cm} \times 34.5 \text{ cm} \times 15.5 \text{ cm}$) connected to a recirculating water system (Hydrus ZEB-40, Alesco, SP, BRA) with automated control of temperature, pH, and conductivity. The animals were fed with pelleted commercial food containing 43% crude protein and 6.5% crude fat (Ovo•Vit®, Chorzów, Poland) during a 10-day acclimation period before the onset of obesity induction. A 14:10h light-dark cycle was maintained throughout the experimental period. Water quality parameters, including temperature, pH, and conductivity, were measured daily, with mean values of $28.07 \pm 0.94^\circ\text{C}$, 8.03 ± 0.11 , and $0.522 \pm 1.120 \text{ mS}$, respectively. Total ammonia ($0.03 \pm 0.02 \text{ mg/L}$) was measured weekly using a digital photometer (AT 100 PB, Alfakit, SC, Brazil).

2.3 Experimental design

Zebrafish were randomly divided into two groups ($n=90/\text{group}$): the Control group and the High-Fat Diet (HFD) group, each with six replicated tanks (15 fish per tank). The fish were fed for 12 weeks at 4% of their body weight, four times a day (at 7 AM, 10 AM, 1 PM, and 4 PM), with the amount of feed adjusted weekly according to the animals' weight. After 8 weeks of the control

or HFD diet, a physical exercise protocol was introduced, and thus the animals were re-divided into four groups: Control diet, Control diet + exercise (Control-EX), HFD, and HFD + exercise (HFD-EX), each with three replicated tanks (15 fish per tank).

The semi-purified diets were formulated with different fat levels (Table 1) using the feed formulation software SuperCrac 6.1 (TD Software, Viçosa, MG, Brazil). The ingredients were carefully mixed and moistened with approximately 40% water and then transformed into pellets using a meat grinder. The pellets were dried in a forced ventilation oven at 55°C for 24 hours, and subsequently cut to a size of approximately 0.5mm.

The proximate composition of the diets (Table 1) was analyzed according to AOAC methodologies (2005) for crude protein (Method 968.05), ether extract (Method No. 960.39), moisture (Method 930.15), and ash (Method 942.05). Gross energy was determined using a Calorimeter (Model-IKA C5000).

Table 1. Ingredients and proximate composition of the control and HFD diets.

Ingredients (%)	Diets	
	Control ^a	HFD ^b
Egg yolk	10.40	28.57
Albumin	31.12	23.61
Corn starch	29.95	21.28
Fish meal	10.00	10.00
Gelatin	6.00	6.00
Soybean oil	2.00	5.00
Bicalcium phosphate	3.00	3.00
Cellulose	6.00	1.00
Vitamin premix ¹	1.00	1.00
Salt	0.50	0.50
BHT ²	0.02	0.02
Centesimal composition ³		

Gross energy (kcal/kg)	4.630	4.889
Ether extract (%)	8,32	19.77
Protein (%)	40.02	39.31
Ash (%)	6.65	6.20
Moisture (%)	5.44	6.03
Dry matter (%)	94.56	93.97

Control diet ^a. High-fat diet ^b. Guaranteed levels of vitamin and mineral supplementation per kilogram of product: vit. A = 1200,000 IU; vit. D3 = 200,000 IU; vit. E = 12,000 mg; vit. K3 = 2400 mg; vit. B1 = 4800 mg; vit. B2 = 4800 mg; vit. B6 = 4000 mg; vit. B12 = 4800 mg; folic acid = 1200 mg; calcium pantothenate = 12,000 mg; vit. C = 48,000 mg; biotin = 48 mg; choline = 65,000 mg; niacin = 24,000 mg; Fe = 10,000 mg; Cu = 6000 mg; Mn = 4000 mg; Zn = 6000 mg; I = 20 mg; Co = 2 mg; Se = 20 mg. ² BHT: butyl hydroxy toluene. ³ The diets were analyzed by the Animal Research Laboratory (ARL) (Faculty of Animal Science and Veterinary Medicine, Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil).

2.4 Physical exercise protocol

The exercise protocol was adapted from BOSKOVIC et al. (2018) [21]. In this protocol, 15 fish were placed in a 5 L glass beaker (external diameter of 170 mm) containing a 60x10 mm magnetic stir bar. The beaker was filled with 4 L of water from the recirculating system and placed on a magnetic stirrer (Fisatom, model 754A, BR). The fish were exposed to 30 minutes of exercise per day for 30 days. The magnetic stirrer was programmed to create a flow of approximately ~0.8 m/s when turned on.

To quantify the hydrodynamic velocity experienced by the zebrafish, a plastic fragment was anchored to allow free rotation on the water surface in the beaker. The rotation frequency (rpm) of this fragment was then recorded, serving as a direct indicator of water velocity. This value was subsequently converted to meters per second - m/s through appropriate calculations, thus enabling a more precise quantification of the swimming conditions faced by the fish.

Zebrafish in the sedentary group underwent the same procedure as the exercise group, except that the magnetic stirrer was kept off, generating no flow in the beaker.

2.5 Biometric parameters

At the eighth week and at the end of the experiment, the fish were captured using a net, gently dried with paper towels, weighed (using a Shimadzu analytical balance, 220g, 0.0001g), and immediately returned to the water. The standard length was measured using a caliper, measuring

from the tip of the snout to the end of the caudal peduncle. The body mass index (BMI) was determined for each fish using the following formula:

$$\text{BMI} = \text{weight (mg)} / \text{length (cm)}^2$$

2.6 Histological analyzes

For histological analysis, three whole zebrafish per replicate (n=9/group) were fixed in 10% buffered formalin, embedded in paraffin, and sectioned using a microtome. Subcutaneous and visceral region sections were stained with hematoxylin-eosin. Microscopic images (Motic, USA; Moticom 3+, USA) were obtained, and the cell area of each animal's adipocytes was manually measured using ImageJ software (National Institutes of Health) [22].

For cryostat sections, three livers per replicate (n=9/group) were dissected and immediately fixed for 24 hours in 10% buffered formalin at -4 °C. Subsequently, the livers were cryoprotected in solutions with increasing concentrations of sucrose (10, 20, and 30%) at -4°C. They were then embedded in Tissue-Plus O.C.T (Tissue-Plus™, Thermo Fisher, Houston, TX), frozen by immersion in P.A. N-hexane, and immersed in liquid nitrogen. Sections of 6 µm thickness were obtained using a cryostat (CM 1850-3-1; Leica Microsystems). The sections were then submerged in P.A. propylene glycol for two minutes and stained with Oil-Red-O.

2.7 Biochemical parameters

At the end of the experiment, the animals were anesthetized with benzocaine immersion and euthanized (250 mg L⁻¹) [23]. Blood from three animals per replicate was collected following the protocol by Carneiro et al. (2020) [24], and glucose levels were immediately measured using a portable glucometer (Accu-Check, Roche Diagnostics, Rotkreuz, Switzerland).

For the remaining biochemical analyses, homogenates were prepared with a pool of 3 animals per repetition according to the protocol described by Sancho et al. (2009) [25]. Each pool was homogenized with five volumes of 0.1 M phosphate buffer (pH 7.2 at 0°C). Subsequently, the homogenates were centrifuged at 5500 rpm (4°C) for 30 minutes, and the resulting supernatants were diluted five times in buffer and frozen at -80°C for subsequent analyses.

Biochemical determinations of total cholesterol (TCHO) (Bioclin, Ref. K083), low-density lipoprotein (LDL) (Bioclin, Ref. K015-1), high-density lipoprotein (HDL) (Bioclin, Ref. K015-1), triglycerides (TG) (Bioclin, Ref. K117-2), lactate (BioTécnica, Ref. 10.018.00), and alanine aminotransferase (ALT) (Kovalent, Ref. MS 80115310051) were performed following the manufacturer's instructions.

2.8 Oxidative Stress Evaluation

A pool of three animals per repetition was homogenized in phosphate buffer (0.1M, pH 7) at a ratio of 1:5 (weight/volume) and centrifuged at 5500rpm for 30 minutes at 4°C. The supernatant was used to assess the formation of reactive oxygen species (ROS), the activity of the enzymes superoxide dismutase (SOD), catalase (CAT), and the formation of thiobarbituric acid reactive substances (TBARS).

The ROS content was measured using the fluorescence probe 2,7-dichlorofluorescein diacetate (DCFH-DA; Sigma Aldrich, St. Louis, USA). The DCFH-DA solution (10 μ M) was prepared by diluting DCFH-DA in 50 mM phosphate buffer (pH 7.2). The quantification of ROS was determined by the conversion of DCFH-DA into dichlorofluorescein (DCF) with a fluorescence wavelength of 485/530 nm (excitation/emission) using a microplate reader spectrophotometer (Infinite 200 PRO, Tecan, Männedorf, Switzerland) [26].

The SOD activity was measured based on the auto-oxidation of pyrogallol, with an absorbance of 560nm [27]. The CAT activity was measured at 240nm (AEBI, 1984), where a decrease in absorbance at 240nm indicates the degradation of hydrogen peroxide into oxygen and water. The absorbance readings were taken every 15 seconds for 3 minutes. Lipid peroxidation was determined by measuring the formation of TBARS as described by Buege & Aust (1978) [28]. Malondialdehyde (MDA), formed from the degradation of polyunsaturated fatty acids, was used as an index to determine the extent of lipid peroxidation, considering its reaction with thiobarbituric acid, which forms a reddish solution with an absorbance at 535 nm.

All analyses were performed in triplicate, and the results were normalized according to the total protein values, determined using the Bradford method [29]. SOD, CAT, and TBARS readings were taken using a microplate reader spectrophotometer (Multiskan Go, Thermo Scientific, Waltham, Massachusetts, USA).

2.9 Statistical analysis

The data was presented using descriptive statistics (mean \pm standard deviation). Normality and homogeneity of variances were assessed using the Shapiro-Wilk and Levene tests, respectively. Statistical analyses were performed using the Student's t-test and One-way ANOVA (with post hoc Tukey analysis). A significance level of $p < 0.05$ was considered statistically significant (Prism 9.02, GraphPad Software, La Jolla, CA, USA).

3 RESULTS

3.1 Biometric parameters

At the end of the eighth week of the feeding period, a significant increase in body weight was observed in the HFD group compared to the control group (Fig 1A). Upon implementing the exercise protocol, the fish belonging to the HFD-EX group showed a significant increase in weight compared to the Control and Control-EX groups. Furthermore, the HFD group, subjected to a high-fat diet, also exhibited a significantly higher weight compared to the Control-EX group (Fig 1B).

When comparing weight gain between the eighth and twelfth weeks, a reduction was observed in the Control-EX group compared to the Control, HFD, and HFD-EX groups (Fig 1C). Concerning BMI, the HFD group showed significantly higher values compared to the Control-EX group (Fig 1D). Additionally, the standard length was significantly greater in the HFD-EX group when compared to the Control and Control-EX groups (Fig 1E).

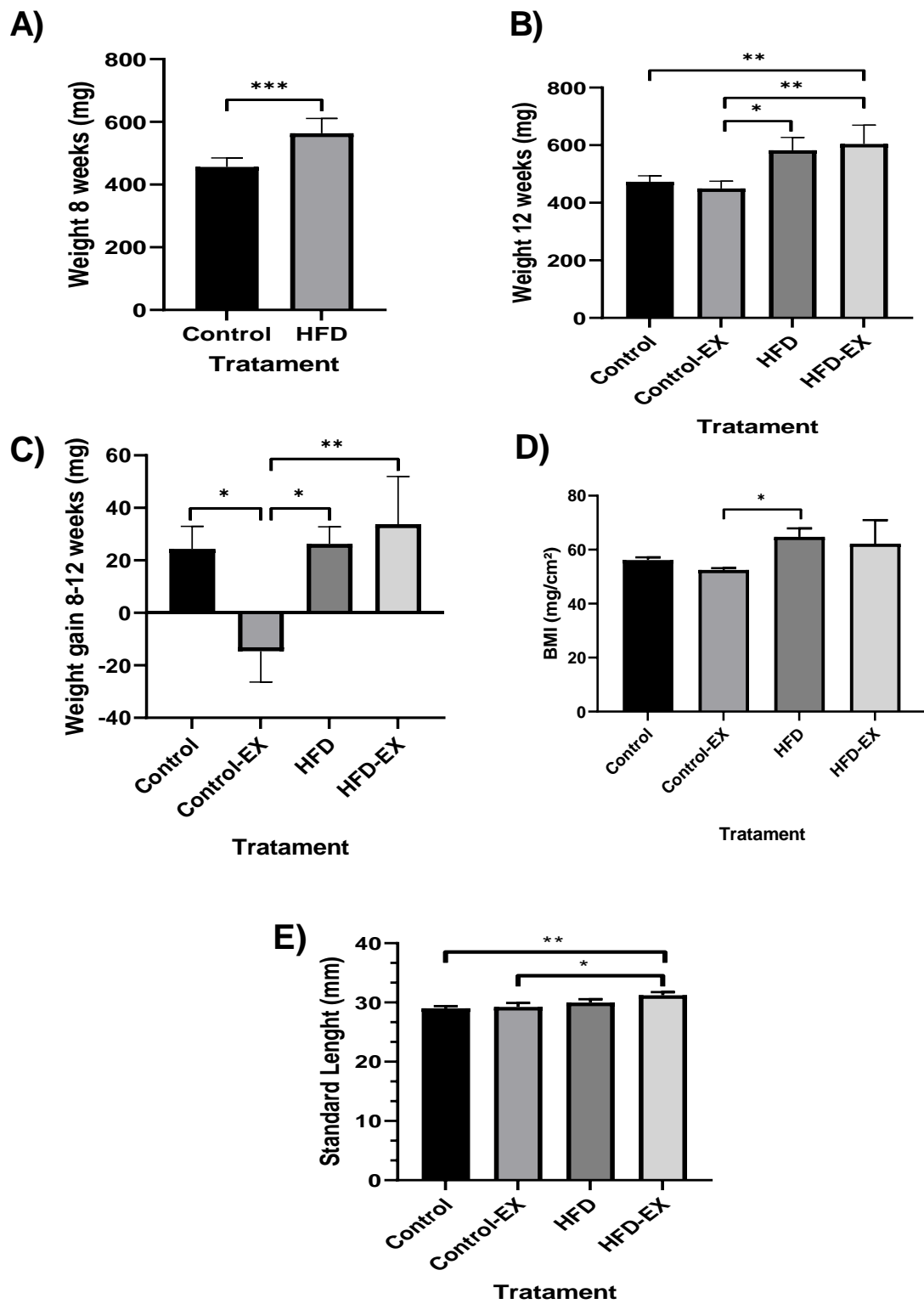


Fig 1. Biometric parameters after high-fat diet-induced obesity and the effects of exercise in zebrafish were evaluated as follows: (A) Animal weight after 8 weeks of feeding, (B) diet effects after 12 weeks of feeding and physical exercise on body weight, (C) weight gain after 4 weeks of exercise, (D) body mass index (BMI) after 12 weeks of feeding and physical exercise, (E) standard length after 12 weeks of feeding and physical exercise. The data are presented as mean \pm SD. Statistical differences between groups are denoted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (T test and one-way ANOVA followed by Tukey's post hoc test).

3.2 Body fat and hepatic lipid accumulation

The area of adipocytes located in the visceral compartment was significantly larger in the HFD group compared to the HFD-EX, Control, and Control-EX groups (Fig 2A and C). A significant difference in the area of adipocytes located in the subcutaneous compartment was observed between the HFD group and the Control and Control-EX groups (Fig 2B and C). Figure 2D qualitatively illustrates the presence of markedly increased hepatic fat accumulation in the HFD group compared to the other groups.

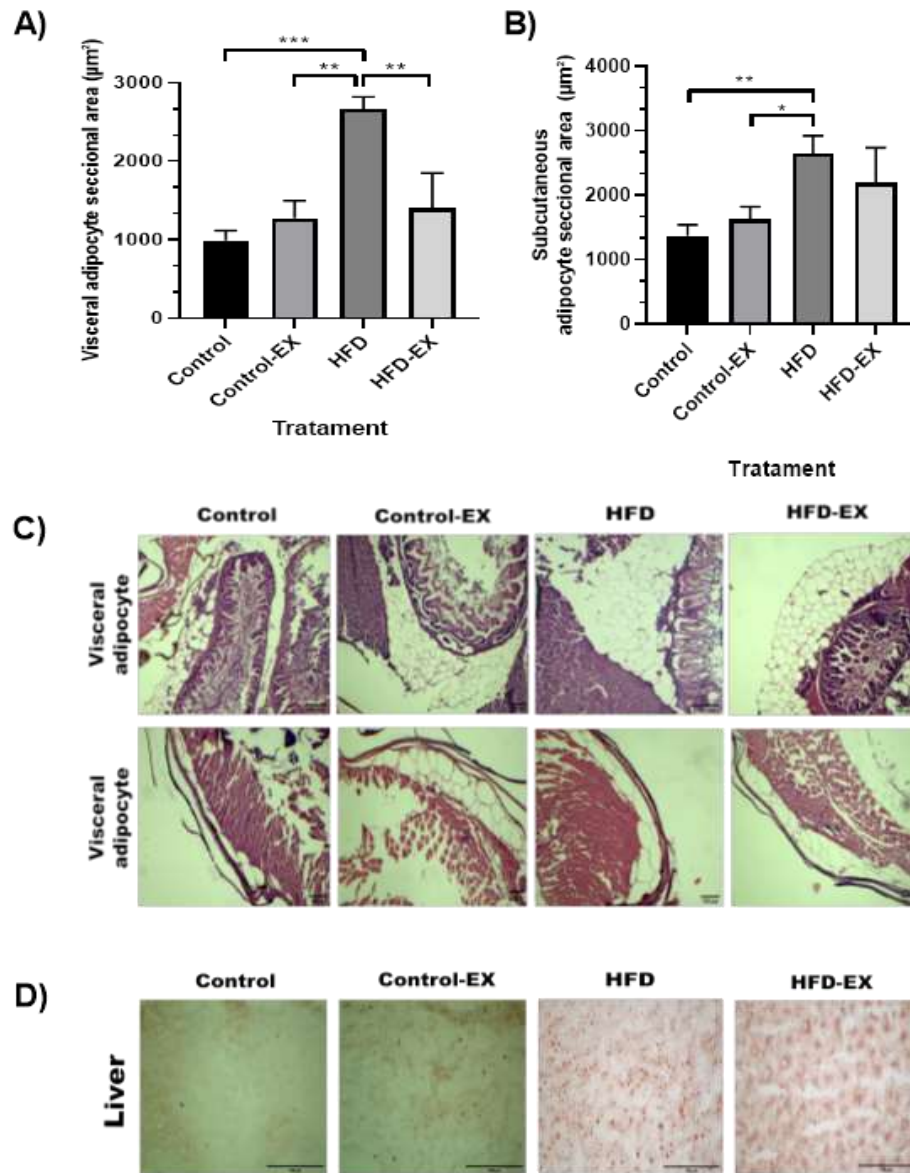


Fig 2. Effect of physical exercise on high-fat diet-induced body fat accumulation in zebrafish. (A) Effect of diet and physical exercise on visceral adipocyte area, (B) Effect of diet and physical exercise on subcutaneous adipocyte area, (C) Representative images of visceral and subcutaneous fat distribution, (D) Liver fat distribution. The data are presented as mean \pm SD. Statistical differences between groups are denoted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (ANOVA followed by Tukey's post hoc test).

3.3 Biochemical parameters

A significantly higher AST activity was observed in the HFD group compared to the HFD-EX, Control, and Control-EX groups (Table 2). No significant differences were observed in the parameters TCHO, HDL, LDL, TG, Lactate, and glucose in any of the groups (Table 2).

Table 2. Effect of diets and physical exercise on biochemical and enzymatic parameters related to metabolism.

Tratament	Metabolites ¹				P-value
	Control	Control-EX	HFD	HFD-EX	
TCHO ^a	68.04 ± 5.24	65.71 ± 4.69	69.66 ± 1.90	70.32 ± 2.54	0.505
HDL ^b	36.87 ± 0.63	35.21 ± 0.11	35.62 ± 2.28	36.04 ± 0.52	0.433
LDL ^c	12.53 ± 5.80	15.24 ± 2.54	15.06 ± 5.25	20.14 ± 7.54	0.451
TG ^d	144.7 ± 16.40	160.2 ± 17.27	157.4 ± 5.13	176.5 ± 20.12	0.185
Lactate ^e	14.61 ± 4.27	12.16 ± 1.87	12.10 ± 1.45	13.15 ± 1.60	0.617
Glucose ^f	33.11 ± 5.27	39.56 ± 10.87	40.22 ± 2.58	43.11 ± 9.76	0.499
ALT ^g	20.56 ± 1.16	21.35 ± 1.33	40.08 ± 0.52	19.76 ± 0.74	<0.001

¹ The data are presented as mean ± SD. ^a Total cholesterol (mg/dl). ^b High-density lipoprotein (mg/dl). ^c Low-density lipoprotein (mg/dl). ^d Triglycerides (mg/dl). ^e Lactate (mg/dl). ^f Glucose (mg/dl). ^g Alanine Aminotransferase (U/L). Statistical significance is denoted as ****p<0.001 (ANOVA followed by Tukey's post hoc test).

3.4 Oxidative Stress

A significantly higher production of ROS was observed in the HFD group compared to the other groups (Fig. 3A). Additionally, the production of ROS in the HFD-EX group was significantly lower than in the Control-EX group (Fig 3A). However, the HFD-EX group exhibited significantly higher ROS production compared to the Control group (Fig 3A). There was a significant reduction in ROS production in the Control group when compared to the Control-EX group (Fig 3A).

An increase in SOD activity was found in the HFD group compared to the other groups (Fig 3B). A significant reduction in SOD activity was observed in the HFD-EX group compared to the

other groups (Fig. 3B). The CAT activity and TBARS levels did not show significant differences among the experimental groups (Fig 3C and D).

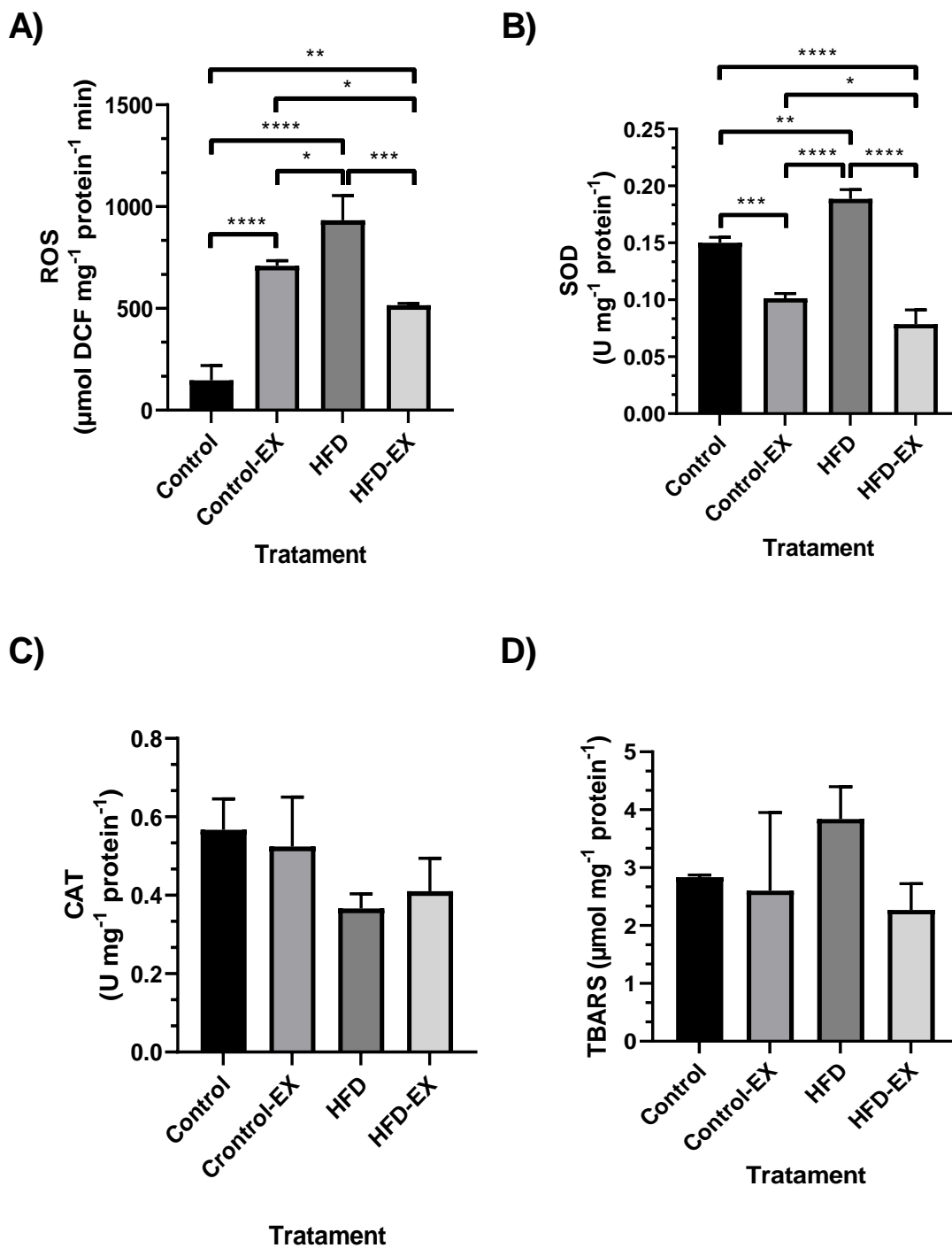


Fig 3. Effect of physical exercise and diet on the production of (A) reactive oxygen species (ROS), (B) superoxide dismutase (SOD) activity, (C) catalase (CAT) activity, and (D) thiobarbituric acid reactive substances (TBARS). The data are presented as mean \pm SD. Statistical differences between groups are denoted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.000$ (ANOVA followed by Tukey's post hoc test).

4 DISCUSSION

Obesity is characterized by a positive energy balance, leading to excessive accumulation of body fat in subcutaneous and visceral regions, consequently causing weight gain [1]. The present study demonstrated a significant increase in body weight after 8 weeks of high-fat diet consumption. These findings align with the results of LANDGRAF et al., (2017) [22] and PICOLO et al., (2021) [30], both of which identified that a high-fat diet leads to weight gain in zebrafish, inducing an obese phenotype.

Physical exercise represents a non-pharmacological approach in the treatment of obesity, as it increases energy expenditure, generating a caloric deficit and thus contributing to the reduction of body weight [31]. Although physical exercise at a flow rate of ~ 0.8 m/s for 30 minutes daily has been shown to increase the metabolic rate of zebrafish by three to four times [32], the implementation of this exercise program for four weeks did not result in a decrease in body weight, weight gain, or body mass index (BMI) in the HFD-EX group compared to the HFD group. These results suggest that while physical exercise plays a relevant role in reducing body weight, its effectiveness may be limited in the absence of a balanced diet [33].

However, the effect of physical exercise was observed in the control-ex group, where there was a significant decrease in body weight, weight gain, and BMI. The HFD-EX group, on the other hand, showed an increase in standard length compared to the control groups, supporting the finding that teleosts subjected to physical exercise tend to develop greater muscle mass and, consequently, higher growth rates [34]. This factor, combined with the high energy availability in the HFD-EX group, resulted in a greater standard length in this group.

Different studies addressing HFD-induced obesity in zebrafish provide evidence that the behavior of metabolic and histological variables is comparable to mammals [22,35]. Adipocyte hypertrophy and the presence of hepatic steatosis are conditions present in both obese mammals and zebrafish [22,36]. We demonstrated that the physical exercise protocol in obese zebrafish was

able to decrease the visceral adipocyte area and the degree of hepatic steatosis, which can be attributed to the increased lipolysis during exercise due to higher energy demand [37].

A factor that could potentially contribute to the reduction of hepatic steatosis is the upregulation of lipogenesis genes (*acaca*, *fasn*, *srebf1*, and *pparg*) and the expression of β -oxidation genes (*pgc1 α* , *pparab*, *acox1*, and *cpt1a*) as demonstrated in the study by ZOU et al., (2021) [38]. On the other hand, exercise was not able to generate a decrease in subcutaneous adipose tissue, which may have been due to the intensity of the exercise not being sufficient to lead to a reduction in subcutaneous tissue. Similar results were reported by SANTOS et al., (2017) [39], who found that low swimming activity (approximately 1.35 body length per second) during physical exercise in pacu increased the density of ventral subcutaneous adipocytes by 20%.

The increase in the biochemical parameters TCHO, HDL, LDL, TG, and glucose is frequently associated with obesity [40], as obesity and hyperlipidemia are major risk factors for cardiovascular diseases [41] and the development of type II diabetes mellitus [40]. However, no significant differences were observed in the aforementioned biochemical variables, which could be explained by the possibility of zebrafish presenting a metabolically healthy obesity phenotype, as observed in the study by LANDGRAF et al., (2017) [22]. The evaluation of ALT enzyme is recommended as an initial screening for hepatic steatosis in obese individuals [42], while LDH evaluation is used to indicate liver damage [43]. The elevation of plasma ALT is mainly due to insulin resistance in adipose tissue and hepatic triglyceride content [44], as well as the fact that approximately 50-70% of ingested glucose is converted to lactate in adipose tissue [45]. We assessed the levels of ALT and lactate in the animals and found no differences in lactate levels. However, we identified a significant decrease in ALT levels in the HFD-EX group. This decrease could be attributed to the reduction of lipid content in the liver and the prevention of hepatic damage due to the antioxidant potential of physical exercise [46]. A similar result was found in the study by THOMPSON et al., (2010) [47], where they reported that 6 months of aerobic exercise significantly reduced serum ALT levels in men.

The high fat deposition in obesity pathology is closely related to redox imbalance, leading to oxidative stress (OS) [48]. In other words, obese individuals present elevated levels of reactive oxygen species (ROS) and decreased antioxidant defenses, such as the enzymes SOD and CAT [49–51]. This OS is attributed to the excess adipose tissue, where adipocytes and pre-adipocytes

act as sources of inflammatory cytokines such as TNF- α , IL-1, and IL-6, which stimulate the production of ROS and RNS by macrophages and monocytes [52]. Physical exercise is beneficial for health independently of changes related to body weight [53]. In mammals, exercise reduces ROS production and inflammation [54] by increasing levels of anti-inflammatory cytokines such as IL-1 and IL-10, while reducing the generation of the pro-inflammatory cytokine TNF- α [55,56]. Therefore, physical exercise appears to be a promising antioxidant and anti-inflammatory strategy to prevent complications arising from obesity [48].

In the present study, we observed that regular physical exercise reduced ROS levels in animals fed an HFD. On the other hand, animals in the control diet group subjected to physical exercise showed an increase in ROS levels compared to the control group. Physical exercise is an important factor in reducing ROS production in obese individuals [57]. However, in non-obese individuals, strenuous physical exercise may lead to an increase in ROS production, especially in the muscle. Nevertheless, this exercise-induced increase in ROS is considered beneficial and can contribute to the adaptive effects of exercise [14].

The antioxidant enzymes SOD and CAT play a crucial role in cellular defenses by removing superoxide anions ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). A decrease in the activity of these enzymes can lead to an excessive availability of $O_2^{\bullet-}$ and H_2O_2 , which subsequently generate hydroxyl radicals (HO^{\bullet}), resulting in lipid peroxidation and modification of membrane proteins [58,59]. The increase in SOD activity is proportional to the increase in ROS production [60]. In our study, we demonstrated that SOD activity decreased in the exercise groups, as moderate exercise maintains a balance between pro-oxidant and antioxidant factors [11]. Although no significant differences were observed in CAT activity, there was a trend of decrease in its levels in the obese groups. The reduction in CAT activity may be attributed to its rapid consumption in combating free radicals during obesity [61].

Lipid peroxidation (LP) is a common phenomenon in obesity. MDA is one of the end products of LP and can be used as a marker of tissue oxidative stress [49]. We did not find significant differences in TBARS levels. However, we observed a trend towards a decrease in the HFD group that underwent physical exercise, although this trend did not reach statistical significance. Similar results were observed in the studies by PHILLIPS et al. (2012) [62] and SHIN et al. (2008) [63], where a decrease in TBARS, total PEROX, and MDA was noted after exercise

intervention. This trend of decrease may have occurred due to increases in maximal oxygen consumption and lean body mass and/or reductions in total body fat [49,64].

5 CONCLUSION

The study showed that zebrafish is a potential model for physical exercise studies. Physical exercise is an important non-pharmacological treatment for obesity, as demonstrated in the model organism, particularly in reducing oxidative stress levels and hepatic steatosis. It is worth noting that further studies are needed to examine the metabolic effects of physical exercise in zebrafish fed a high-fat diet.

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Declaration of competing interest

The authors declare no conflicts of interest.

Author contribution statement

Martins, M.S.A. Conceptualization; Formal analysis; Investigation; Methodology; Resources; Validation; Visualization; Roles/Writing - original draft; Writing review & editing. **Carneiro, W.F.** Investigation; Methodology; Formal analysis & Writing original draft, Writing review & editing. **Monteiro, K.S.** Investigation; Methodology; Formal analysis; Visualization. **Souza, S.P.** Investigation; Methodology; Formal analysis; Visualization. **Vianna, A.R.C.B.** Resources; Validation; Visualization; Roles/Writing - original draft; Writing review & editing. **Murgas, L.D.S.** Conceptualization; Resources; Project administration; Supervision; Writing original draft; Writing review & editing.

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Graphical abstract

