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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS

INTERAÇÃO DO EXERCÍCIO FÍSICO CRÔNICO E DA SUPLEMENTAÇÃO COM VITAMINA E SOBRE PARÂMETROS OXIDATIVOS MUSCULARES DE PEIXE ZEBRA *(Danio rerio)*

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SUMÁRIO

RESUMO GERAL

O exercício físico produz um aumento nas espécies reativas de oxigênio (ERO) agudamente que podem levar ao estresse oxidativo, mas cronicamente pode ter como consequência uma melhora da capacidade antioxidante. Considerando que o treinamento físico e a suplementação com vit. E podem proporcionar mudanças no estado oxidativo em diversos tecidos em peixes, este trabalho teve como objetivo estudar os efeitos do treinamento físico crônico e da suplementação com Vit. E em machos de peixes Zebra (*Danio rerio*) para determinar as adaptações do estado oxidativo da musculatura cardíaca e esquelética. Os peixes foram divididos em 4 grupos (n=30 animais/grupo): Controle (nado livre no túnel de natação por 5 minutos); VitE (suplementados com Vit. E (500 mg/kg), e que seguiram o protocolo controle); N (submetidos à natação contra a corrente, 1h/dia, 5x/semana, por 3 semanas, à velocidade máxima de 21cm/s, estabelecida progressivamente); NVitE (associação dos tratamentos dos grupos VitE e N). Metade dos peixes foram pesados no início do experimento e no final da 3a semana de experimento e finalizados por secção medular ao final da 3° semana imediatamente após o término da última sessão de treinamento para determinação de lactato no músculo esquelético. A outra metade foi finalizada como descrito 72 hs após da última sessão de treinamento na 3a semana para as análises de ERO e capacidade antioxidante (ACAP) nos tecidos cardíaco e esquelético. Os resultados obtidos demonstraram que o peso dos animais treinados foi maior ao final do experimento (N=337±13, NVit E=327±8) com relação ao peso inicial (N=305±26, NVit E=313±11). Para a musculatura esquelética os níveis de ERO foram menores com aumento de ACAP no grupo associação NVitE com relação aos demais grupos. Os níveis de ERO na musculatura cardíaca foram menores nos três grupos experimentais com relação ao grupo controle. Além disso, foi observado um aumento de ACAP no grupo NVitE com relação aos grupos C e VitE. Este trabalho conclui pela primeira vez para peixe zebra que a associação entre o exercício crônico (num protocolo de baixa intensidade) e a suplementação com Vit. E pode trazer melhorias para o estado oxidativo da musculatura cardíaca e esquelética, aumentando a capacidade antioxidante.

Palavras-chave: Alfa tocoferol; Estado oxidativo; Natação; Zebrafish

INTRODUÇÃO GERAL

A atividade física é um comportamento básico dos animais, desencadeando importantes ajustes neurais, hormonais, cardiovasculares e respiratórios (Brum et al. 2004). Esta atividade pode afetar vários componentes do sistema cardiovascular, causando hipertrofia cardíaca, aumento do débito cardíaco, hematócrito, e do conteúdo de oxigênio arterial (Brum et al. 2004, Bernardo et al. 2010).

O exercício físico caracteriza-se por uma situação que retira o organismo de sua homeostase, pois implica no aumento instantâneo da demanda energética da musculatura exercitada e, consequentemente, do organismo como um todo. Assim, para suprir a nova demanda metabólica, várias alterações fisiológicas são necessárias (Brum et al. 2004). Dentre as alterações metabólicas ocorridas durante o exercício físico contínuo a intensidade e a duração do exercício realizado tem sido bastante discutidos. Durante o exercício físico intenso e prolongado, a fadiga se relaciona, principalmente com a hipoglicemia, pois, tanto a glicose e consequentemente a oxidação de carboidratos diminui. Outro fator de destaque é a relação entre depleção de estoques de glicogênio muscular e hepático, e a resistência ao exercício, correlacionados em nível de glicogênio pré-exercício (Snyder, 1998; Tsintzas e Williams, 1998). Em intensidades de exercício mais altas e de curta duração (90% do VO^2máx), a energia exigida para a atividade física irá resultar também na produção de metabólitos (lactato, H + , fosfato Pi, ADP), cujo acúmulo no organismo irá gerar diminuição no rendimento da contração. O lactato é um metabólito resultante do metabolismo anaeróbico (Hochachka, 1986), portanto, a avaliação do lactato plasmático é uma das ferramentas utilizadas para determinar a transição do metabolismo aeróbico para o anaeróbico, e a percepção da fadiga (Bianchi et al; 1997). Como o lactato é produzido principalmente na musculatura (McArdle et al., 2011), o lactato muscular é um bom indicador de qual metabolismo está sendo empregado.

O exercício físico também pode levar ao estresse oxidativo devido ao aumento de espécies reativas de oxigênio (ERO) agudamente (Urso e Clarkson, 2003), devido ao aumento da fosforilação oxidativa em resposta ao exercício. As EROs são produzidas naturalmente em nosso organismo através de processos metabólicos oxidativos e, muitas vezes, são de extrema utilidade, como em situações em que há necessidade de

ativação do sistema imunológico como por exemplo, quando os macrófagos utilizam o peróxido de hidrogênio para destruir bactérias e outros elementos estranhos, na desintoxicação de drogas, e na produção do óxido nítrico que é extremamente importante nos processos que desencadeiam o relaxamento dos vasos sanguíneos (Moncada et al. 2001). Por outro lado, as EROs podem causar danos na camada lipídica da membrana celular (lipoperoxidação), em proteínas e no DNA (Halliwel e whiteman, 2004).

O oxigênio (O^2) que respiramos é metabolizado em nosso organismo aproximadamente 85 a 90% utilizado pela mitocôndria, através da cadeia de transporte de elétrons, e os 10 a 15% restantes são utilizados por diversas enzimas oxidases e oxigenases e também por reações químicas de oxidação diretas (Halliwell e Gutteridge, 1999). Na parte terminal da cadeia de transporte de elétrons, a enzima citocromo oxidase remove um elétron de cada uma das quatro moléculas reduzidas de citocromo c, oxidando-as, e adiciona os quatro elétrons ao O^2 para formar água (em torno de 95 a 98% dos 85 a 90% citados acima) (Halliwell e Gutteridge, 1999). Os 2 a 5% restantes são reduzidos univalentemente em metabólitos formando EROs (Halliwell e Gutteridge, 1999). As EROs são moléculas resultantes da redução ou oxidação do oxigênio (Shibata et al., 2008). Como as EROs são continuamente formadas em pequenas quantidades pelos processos normais do metabolismo, todas as células possuem mecanismos para mitigar seus efeitos agressores. Pode-se dizer que um organismo encontra-se em estresse oxidativo quando ocorre um desequilíbrio entre os sistemas pro-oxidantes e antioxidantes, de maneira que os primeiros sejam predominantes, ocasionando assim danos celulares (Sies, 1986).

Os antioxidantes são agentes responsáveis por combater as EROs, e com isso acabam atenuando as lesões causadas por essas EROs nas células (Gomez-Cabrera et al., 2008). Uma ampla definição de antioxidante é “qualquer substância que, presente em baixas concentrações quando comparada a do substrato oxidável, atrasa ou inibe a oxidação deste substrato de maneira eficaz” (Sies & Stahl, 1995). Esses agentes que protegem as células contra os efeitos dos radicais livres podem ser classificados em antioxidantes enzimáticos ou não-enzimáticos (Sies, 1993). Os sistemas enzimáticos são compostos por constituintes primários como as enzimas superóxido dismutase, catalase e glutationa peroxidase, e por enzimas de ação secundária, como a glutationa redutase, a glicose-6-fosfato desidrogenase e a glutationa S-transferase (Marcon, 1997). O sistema

de defesa antioxidante não-enzimático desempenha papel importante na proteção de macromoléculas contra possível dano oxidativo, principalmente no plasma que possui pouca defesa enzimática. De acordo com sua solubilidade, está dividido em dois grupos: lipossolúveis (vitamina E (alfa tocoferol), ubiquinol10, β-caroteno) e hidrossolúveis (glicose, piruvato, ácido úrico, ácido ascórbico, bilirrubina, glutationa - GSH) (Chan, 1996). Devido aos efeitos danosos das EROs, todas as células mantêm sistemas de defesas antioxidantes através de três níveis de proteção: prevenção da formação das EROs, diminuição das EROs através dos varredores (*scavengers*) de radicais livres ou reparo dos componentes celulares danificados (Sampaio, 2003). Neste trabalho foi usada a suplementação com o antioxidante vit E que constitui em uma das primeiras linhas de defesa não-enzimática dos sistemas biológicos. Atua principalmente protegendo as membranas dos compostos oxidáveis do citoplasma celular, fazendo a estabilização dos ácidos graxos insaturados e quebrando as cadeias de peróxidos ($LOO^{\cdot}$) (Yamamoto et al., 2001). A Vit. E também pode previnir a formação de hidroperóxidos lipídicos em seu estágio inicial através da doação de um átomo de hidrogênio a essa espécie reativa (Fang et al., 2002), resultando em um radical α-tocoferoxil ($TO^{\cdot}$). Esse radical será reduzido novamente à α-tocoferol (TOH) com a doação de elétrons pelo ácido ascórbico (Azzi e Stocker, 2000).

Diversos estudos da década de 80 apresentaram resultados nos quais repetidas cargas de exercício levavam a dano ou envelhecimento acelerado do músculo em indivíduos ou cobaias que se exercitavam regularmente. Entretanto Heath et al. (1981), depois de acompanhar atletas durante muitos anos, verificaram que seu potencial metabólico e sua capacidade funcional muscular não eram prejudicados. Além disso, Gutteridge et al. (1985) apontaram como possibilidade de mecanismo protetor o fato de terem encontrado incremento nos níveis de ferro e cobre no suor de atletas após o exercício, especulando que a excreção de tais metais no suor diminuiria a extensão do dano oxidativo mediado por tais metais. A partir destes estudos levantou-se a teoria de que o exercício regular pudesse promover um aumento adaptativo dos mecanismos de defesa do músculo esquelético capaz de proteger contra as lesões produzidas pelas EROs. Davies et al. (1982) propuseram que a formação de radicais livres induzida por exercício pudesse ser o estímulo inicial para a biogênese mitocondrial em uma situação de treinamento crônico. Neste sentido, Ji et al. (1992) demonstraram que em músculo esquelético uma carga isolada de trabalho exaustivo produzia um aumento de LPO e

que um aumento significativo na atividade das enzimas antioxidantes glutationa redutase (GR), Glutationa peroxidase (GPx), Superóxido Dismutase (SOD) e catalase (CAT). Ao estudar humanos, Nies et al. (1996) demonstraram a ocorrência de dano ao DNA nos leucócitos circulantes após exercício exaustivo em esteira. Neste trabalho, pela primeira vez isto foi mostrado em indivíduos treinados, mas, como a extensão do dano foi pequena, os autores sugerem que a adaptação ao treinamento de resistência aeróbia pode reduzir os efeitos das ERO, como o dano ao DNA. O trabalho de Venditti e Di Meo (1997) submeteu ratos adultos a um programa de treinamento regular com duração de um ano comprovando a hipótese de que tal treinamento prolonga a capacidade de resistência aeróbia e aumenta as defesas antioxidantes, limitando assim o dano tecidual causado por ERO. Atualmente temos diversos trabalhos mostrando alterações nas vias de sinalização, agudas e crônicas, geradas pelo exercício físico, por exemplo, captação de glicose no músculo esquelético e sensibilidade à insulina muscular (Merry e MacConell, 2012; Trewin et al., 2015), modulação dos níveis endógenos de enzimas antioxidantes (Alessio et al., 1988; Vincent et al., 2000; MacCardle et al., 2001), biogênese mitocondrial (Gomez-Cabrera et al., 2008; Strobel et al., 2011; Paulsen et al., 2014), força de contração muscular (Jackson et al., 2009; Paulsen et al., 2014) e hipertrofia muscular (Makanae et al., 2013).

Sabe-se que o excesso de ERO possuem efeitos prejudiciais que podem incluir diminuição da funcionalidade muscular, alterações histológicas e dor muscular e podem atenuar o desempenho do exercício (Mendelsohn e Larrick, 2013). Este tem sido o motivo para a suplementação de antioxidantes, entre eles a vit E, e também sobre a investigação se a suplementação de antioxidantes não enzimáticos poderiam prevenir os efeitos prejudiciais das ERO durante o exercício e assim melhorar o desempenho do exercício de resistência ((Mendelsohn e Larrick, 2013) em animais e humanos.

O peixe zebra (*Danio rerio)* (Figura 1), conhecido mundialmente como *zebrafish*, é um pequeno teleósteo de água doce pertencente à família Cyprinidae, encontrado no sul e sudeste da Ásia (Spence et al., 2008). Possui de 3 a 5cm de comprimento, é ovípara, possui tempo de vida médio entre três a cinco anos, é caracterizado por seu padrão de coloração distinto, baseado em linhas horizontais claras e escuras alternadas e hoje no Brasil é considerado um peixe ornamental (Luzardo, 2013; Ramsay et al., 2006). Por ser pequeno e de fácil manipulação, o peixe zebra tornou-se atrativo para o desenvolvimento de pesquisas, uma vez que pode ser

armazenado em grande quantidade em um espaço pequeno e com baixos custos de manutenção laboratorial (Málaga-Trillo et al., 2011; Shin e Fishman, 2002).

Figura 1: Peixe zebra. Disponível em http://www.sci-news.com/biology/article00415.html.

Dentre as diversas técnicas de cultivo de peixes, o exercício de longa duração tem se mostrado um forte coadjuvante no crescimento de diversos peixes: truta arco-íris (O*ncorhyncuss mykiss)*, salmão (*Salmo salar)*, truta-marrom (*Salmo trutta),*"striped bass" (*Morone saxatilis)*, "red sea bream" (*Pagrus major)*, "masu salmon" (*Oncorhynchus masou masou),* "japanese flounder" (*Paralichthys olivaceus*), "yellowtail" (*Seriola quinqueradiata)* (Davison, 1997; Jobling, 1994; Bugeon et al, 2003; Young & Cech Jr, 1994; Forster & Ogata, 1996; Azuma et al, 2002; Ogata & Oku, 2000; Yogata & Oku, 2000). Entretanto, o crescimento depende tanto do tipo de atividade realizada quanto da espécie exercitada e mesmo peixes que possuem pouca habilidade natatória podem ser beneficiados pelo exercício, desde que a velocidade seja adequada para sua espécie (Ogata & Oku, 2000). Quando os peixes são exercitados a velocidades ótimas, 30 a 60% da velocidade máxima que eles podem atingir, ocorre uma série de respostas positivas, como aumento da tolerância ao exercício, otimização da taxa de conversão alimentar e maior crescimento e há peixes que mesmo após a interrupção do exercício obtêm ganho de peso (Young & Cech Jr., 1994; Jobling, 1994; Davison, 1997; Yogata & Oku, 2000; Ogata & Oku, 2000; Azuma et al, 2002; Bugeon et al, 2003). Contudo, poucos estudos têm sido desenvolvidos visando definir como o treinamento de exercício em peixes, agudo ou crônico, com diferentes intensidades e velocidades de natação, podem promover alterações fisiológicas e bioquímicas nos animais conforme diferentes protocolos de treinamento.

Tendo em vista que o treinamento físico pode proporcionar alterações nos parâmetros oxidativos na musculatura cardíaca e esquelética em peixes Zebra (*Danio*

rerio), justifica-se estudar os efeitos do treinamento físico, da suplementação com Vit. E e de sua associação em peixes saudáveis para determinar esses parâmetros oxidativos da musculatura cardíaca e esquelética frente a estes estímulos.

OBJETIVO GERAL

O objetivo deste trabalho foi investigar como o treinamento físico crônico e a suplementação com vitamina E influencia na produção de lactato na musculara esquelética em parâmetros oxidativos na musculatura cardíaca e esquelética do peixe zebra (*Danio rerio*).

OBJETIVOS ESPECÍFICOS

1- Avaliar os níveis de lactato muscular em resposta ao treinamento físico crônico e com suplementação com vitamina E;
2- Determinar alterações em parâmetros oxidativos (níveis de EROs e capacidade antioxidante total contra radicais peroxil) da musculatura cardíaca e esquelética ocorridas em resposta ao treinamento físico crônico, com suplementação de vitamina E e com a associação dos dois;

Manuscrito a ser submetido à revista Zebrafish:

INTERACTION OF CHRONIC PHYSICAL EXERCISE AND SUPPLEMENTATION WITH VITAMIN E ON MUSCLE OXIDATIVE PARAMETERS OF ZEBRAFISH (*Danio rerio*)

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ABSTRACT

Considering that physical training and vitamin E (Vit E) can provide changes in oxidative status in several tissues in fish, this study aimed to analyze the effects of chronic physical training and supplementation with vitamin E on healthy male Zebrafish (*Danio rerio*). Fish were divided into 4 groups (n = 30/group): Control (C, free swimming); Vit E free swimming (VitE 500 mg/kg); N (swimming, speed of 21cm/s), NVitE (association Vit E and swimming). For skeletal musculature the ROS levels were lower with increase of ACAP in the NVitE group in relation to the other groups. ROS levels in the cardiac musculature were lower in the three experimental groups than in the control and increase in ACAP was observed in the NVitE group in relation to the C and VitE groups. This work concludes for the first time for zebrafish that the association between chronic exercise (low intensity) and VitE bring benefits to the oxidative state of the cardiac and skeletal muscles, increasing the antioxidant capacity and reducing ROS levels.

Keywords: Alpha tocopherol; Swimming; Oxidative state; Zebrafish.

1. INTRODUCTION

Physical exercise represents a stress for the organism that generates a deviation from the homeostatic state, leading to the reorganization of the physiological response of several systems (Brum et al., 2004). This reorganization of responses is divided into acute and chronic. Acute changes are the temporary changes caused by an exercise session and, on the other hand, chronic changes are the changes in the systems caused by several exercise sessions, characterizing a physical training (Brum et al., 2004). The physiological changes that the exercise promotes acutely are caused by the increase of the metabolic rate (Carmeli et al., 2000). Followed by increased production of reactive oxygen species (ROS) (Alessio et al., 1988), increased AMP (Adenosine monophosphate) (McConell et al., 2010, Lira et al., 2010) and increased cytosolic calcium (McConell et al., 2010; Baar et al., 2003; Wadley et al., 2013). These results suggest that there is a significant increase in the amount and activity of endogenous antioxidant enzymes in the skeletal muscle (McArdle et al., 2001; Alessio et al., 1988; Vincent et al. al., 2000).

Physical exercise is able to increase antioxidant defenses in different animal models, including fish, as observed by the increase of the activity of the enzyme superoxide dismutase in *Brycon cephalus* after training of 60 and 90 days (Oba, 2006) and by the increase in the activity of the enzyme catalase in *Cyprinus carpio* swimming for three weeks at 30 cm/s (Hackenberger et al., 2014).

Fish, like others superior vertebrates, have two antioxidant defense systems: enzymatic and non-enzymatic (Guerriero et al., 2002). They comprise the non-enzymatic defense system, endogenous antioxidants such as glutathione, uric acid, albumin among others and exogenous antioxidants such as vitamins C, E and carotenoids. This non-enzymatic antioxidant defense system plays an important role in the protection of macromolecules against possible oxidative damage (Papas, 1999; Hamre et al., 2004).

In fish and others vertebrates, vitamins are part of the first line of antioxidant defense. These are involved in maintaining several physiological processes and metabolic reactions (Martínez-Álvarez et al, 2005; Kumari and Sahoo, 2005; Lopera-Barrero and Poveda-Parra, 2009). Vitamins are organic compounds obtained primarily

through diet due to the lack of key enzymes involved in their synthesis, or the inability to produce them in sufficient amounts (Weber, 1995; Drouin et al, 2011).

Among the vitamins, vitamin E (Vit. E) is involved in the immune response and acts by protecting cell membranes from oxidizable compounds in the cell cytoplasm, stabilizing unsaturated fatty acids and breaking the peroxide chains (Yamamoto et al., 2001). In trouts the effects of diets with Vit. E were evaluated by Gatlin III et al. (1992) when levels higher than 240 mg/kg increased Vit. E concentration in tissues, decreasing lipid oxidation. Goldfarb et al. (1994) observed lower concentrations of lipid hydroperoxides and thiobarbituric acid reactive substances (TBARS) in plasma and muscle fibers of rats submitted to intense exercise and supplemented for five weeks with α-tocopherol (250 IU α-tocopherol/kg of diet). Metin et al. (2002) also found a reduction in TBARS concentration in rats supplemented with α-tocopherol (30mg/kg/day) underwent swimming for 30 minutes per day for 8 weeks of training.

About the metabolic changes that occur during continuous physical exercise, the intensity and duration of the exercise performed are important. Depending on the intensity and duration of the exercise part of the energy required for physical activity will lead to the production of metabolite (lactate, creatinine, hydrogen ions, inorganic phosphate, and compounds such as adenosine di-phosphate). Lactate is a metabolite resulting from anaerobic metabolism (Hochachka, 1986) and lactate levels indicate the metabolic pathway most used in the exercise in question. In mammals, tests have already been made that determined the anaerobic threshold (Lan), defined as the workload at which blood lactate begins to accumulate disproportionately during progressive exercises. This has been used in the prescription of training in different modalities of exercise for humans (Jones and Doust, 2000, Kinderman et al., 1979, Ribeiro et al., 2003). It was observed that the measurement of blood lactate is a good parameter to determine the intensity of exercise used, with lactate being a metabolite originated in the skeletal muscles, being also a good parameter to determine how intense the exercise, because as more lactate is found greater exercise intensity (Robergs et al., 2004). Both oxidative stressors and antioxidant molecules may have tissue-dependent responses. The cardiac musculature presents structural differences regarding the skeletal muscles, for example, it presents high volume and mitochondrial density, as well as higher O_2 consumption, resulting in a higher leakage rate of electrons from the transport chain in mitochondria and ROS production (Atalay and Sen, 1999). The antioxidant capacity in the heart tends to be limited, which makes it susceptible to tissue

damage due to oxidative stress after a period of acute exercise (Ascensão et al., 2003). However, chronic resistance training in rats is capable of inducing increased activity of Glutathione peroxidase (GPX) and Glutathione Reductase (GR) (Somani et al., 1995; Venditti and Di Meo 1996; Ramires and Ji 2001), Catalase (CAT) (Somani et al., 1995; Kim et al., 1996) and antioxidant capacity against peroxyl radicals (ACAP) (Guerreiro et al., 2016). Just as physical exercise can generate changes in the antioxidant capacity of the heart, a supplementation with an antioxidant, such as Vit. E, could bring additional benefits to these changes. In fact, in rats, a supplementation of 596 mg Vit. E per kg of feed, acts on the heart by eliminating free radicals (Azzi et al., 2003).

Considering that physical training and Vit. E can provide changes in oxidative status in several tissues in fish, this study aimed to analyze the effects of chronic physical training and supplementation with vitamin E on healthy male Zebrafish (*Danio rerio*) to determine the adaptations of the oxidative state of the cardiac and skeletal muscle face to these stimuli.

2. MATERIALS AND METHODS

2.1. ANIMAL MODEL

A total of 120 adult zebrafish (*Danio rerio*) males, 6 months old, were purchased from commercial breeders and kept under culture conditions in aquifers with a water re-circulation system, with biological filter, ultraviolet lamp (microbicide), in photoperiod of 14 light/10 dark, temperature of 28°C, pH between 6 and 7, ammonia levels lower than 0.02 mg/L and density of 5 fish per liter. Feeding with 5% of the biomass divided twice a day (Salvador, 2008), with commercial diet of the brand Alcon Basic. The ration with vit E was made in the dependencies of the Federal University of Rio Grande and is the same commercial ration of the other groups, only added with vit E. The fish weighed on average 313 ± 25 mg at the beginning of the experiment. All the procedures of the experiments were approved by the Animal Ethics Usage Committee (CEUA-FURG, register number P062/2015). The mortality of the animals was observed daily throughout the periods of acclimatization and experimentation.

2.2. SWIMMING SYSTEM

The swimming system (figure 1) was constructed based on the works of Hackbarth (2010) and Pereira (2014), being composed by an acrylic tube of 7cm in diameter and 100cm in length, connected to a system of PVC pipes. The water flow is generated by submerged pump.

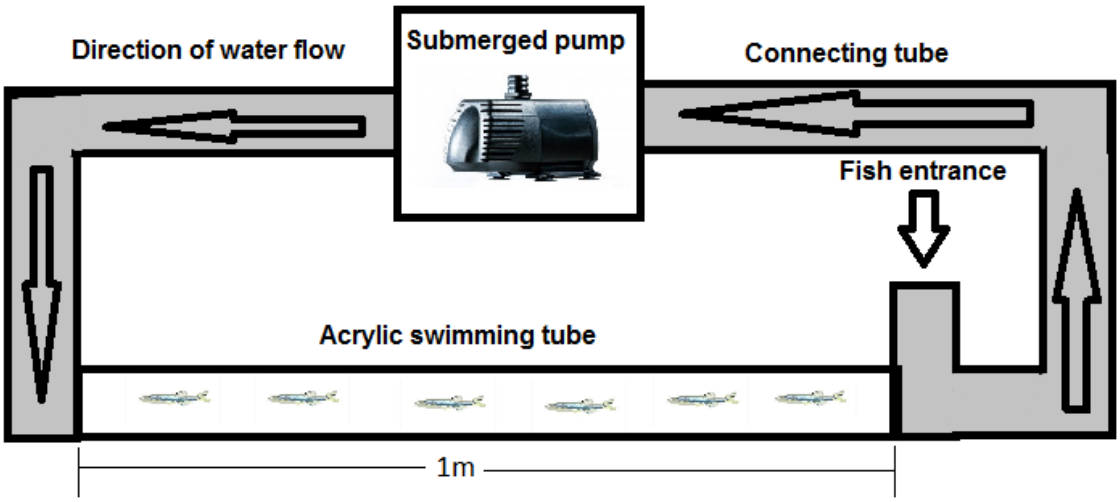

Figure 1 - Scheme of the swimming system.

2.3. EXPERIMENTAL DESIGN

The 120 fish were randomly divided into four experimental groups (n= 30/group).

Control (C): were placed in the swimming tunnel for 60 minutes, in free swimming, simulating the stress of manipulation and confinement in the swimming tunnel; **Supplementation with Vit.E (VitE):** were supplemented with vitamin E from the sixth month of age with a dose of 500 mg/kg (Miller et al., 2012), and at 7 months-old followed the protocol of the control group, if supplementation was continued during the swimming protocol;

Chronic swimming training (N): 7-month-old fish were submitted to swimming against the current, 1h/day, 5x/week, at a maximum velocity of 21cm/s, progressively established according to the training regime presented in figure 2. The Swimming consisted of a week of progressive acclimatization, with the increment of the speed of the flow gradually day by day until arriving at the maximum speed supported by the

fish. In the subsequent two weeks the protocol consisted of chronic swimming training at the maximum speed supported by the fish when the fish swam 1h/day, 5 days/week, at maximum speed.

Chronic swimming training supplemented with Vit. E (NVitE): In this group the animals were supplemented according to the Vit. E group and exercised according to group N.

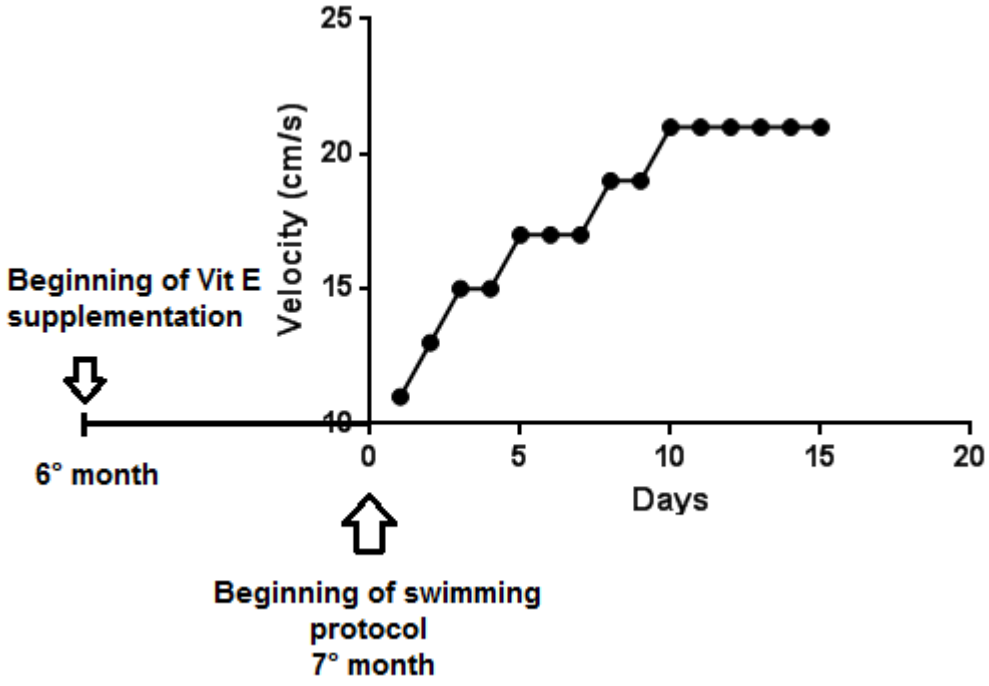

Figure 2 - Diagram of the experimental design of zebrafish supplementation with Vit. E for 30 days, and a subsequent 21-day of chronic swimming training protocol of progressive swim speed (cm/s).

2.4. BIOLOGICAL SAMPLES

At the beginning of the first week of training protocol and at the end of the experiment (third week of training) the fishes were weighed and finalized by the medullary section. Immediately after the end of the last training session (third week), the dissection was performed to remove the the epaxial and hypoaxial skeletal muscle to quantify lactate levels and mass increment, to determine lactate levels immediately after the last exercise session. Complementarily 72 hours after the last training session, heart and muscle were dissected for analysis of ROS and total antioxidant capacity (ACAP).

2.5. PROTEIN AND LACTATE DETERMINATION

Fish skeletal muscle samples from each group (N = 8) were weighed 101 ± 11mg and homogenized in a mixture of 1mg of tissue to 9ml of phosphate buffer (100mM KH_2PO_4, 100mM K_2HPO_4, 1mM EDTA; 10 mM PMSF, pH 7.2). The samples were homogenized, centrifuged for 20 min at 10,000 x g and held at 4 °C. The supernatant was removed and a 15 µl aliquot was used to measure the total protein content using Biuret method protein assay kit (colorimetric test, 550nm) (Invitrogen, Brazil), following the manufacturer's recommendations. The resulting values were normalized by the weight of the sample and used in the following analyzes in millimol per gram of muscle tissue ($mmol.g^{-1}$) for lactate. Lactate content (enzymatic UV test, 340 nm) was measured using commercial reagent kits (Bioclin) by spectrophotometry using a microplate reader (EL-x808IU, BioTek Instruments). The results were normalized by the protein content of the sample.

2.6. DETERMINATION ROS LEVELS AND ACAP

The determination of myocardial ROS and skeletal muscle contents was performed following the methodology of Ferreira-Cravo et al. (2007) using the reduction reaction of H_2DCF-DA (Diacetate of 2 ', 7' dichloroflurescein), which in the presence of ROS generates a fluorochrome, detected using wavelengths of 488 to 525 nm for excitation and emission, respectively. The readings were performed in fluorimeter (Victor 2, Perkin Elmer) with microplate reader. The antioxidant capacity against myocardial and skeletal muscle peroxyl radicals was determined according to the method described by Amado et al. (2009) using the thermal decomposition of ABAP (2,2'-azobis-2-methylpropionamidine dihydrochloride). This methodology is similar to the determination of reactive oxygen species (ROS), but in this case the production of the reactive species is increased with the presence of ABAP. The antioxidant capacity against peroxyl radicals is calculated by the relative area through the difference between the area of the EROs curve without and in the presence of ABAP, divided by the EROs area without ABAP.

2.7. STATISTICAL ANALYSIS

Parametric data were analyzed using Analysis of Variance (ANOVA) followed by Tukey HSD post-test, and non-parametric tests using the Kruskal-Wallis test, both

with a significance level of 5% (P <0.05). Data are reported as means ± SD. We also used Student's t test with significance level of 5% (P <0.05) for comparisons between 1st and 3rd week of training body weight in the same experimental group.

3. RESULTS

3.1. ANIMALS WEIGHT

The weight of the trained animals was significantly higher (P <0.05) at the end of the experiment in relation to the initial weight, except for the control and VitE groups, which showed no significant difference. There was also no significant difference in weight between the experimental groups (Table 1). The percentage of mortality observed during the acclimation and experimentation periods was: C: 23%, N: 30%, VitE: 20%, NVitE: 25%.

Groups	Mean initial weight (mg)	Mean final weight (mg)
C	**316 ± 44[a]**	**322 ± 26[a]**
N	**305 ± 26[a]**	**337 ± 13[a]***
VitE	**317 ± 5[a]**	**317 ± 5[a]**
NVitE	**313 ± 11[a]**	**327 ± 8[a]***

Table 1 - Average initial and final weight (mg). C - control fish; N - fish that have been subjected to swimming; VitE - fish that only had supplementation with vitamin E; NVitE - fish that had Vit. E supplementation and were subjected to swimming. * Asterisk represents significant difference in the same group between initial and final weight. Different letters represent a significant difference between the groups (P <0.05).

3.2. MUSCLE LACTATE

Lactate in the zebrafish skeletal muscle did not vary between the experimental groups (Figure 3).

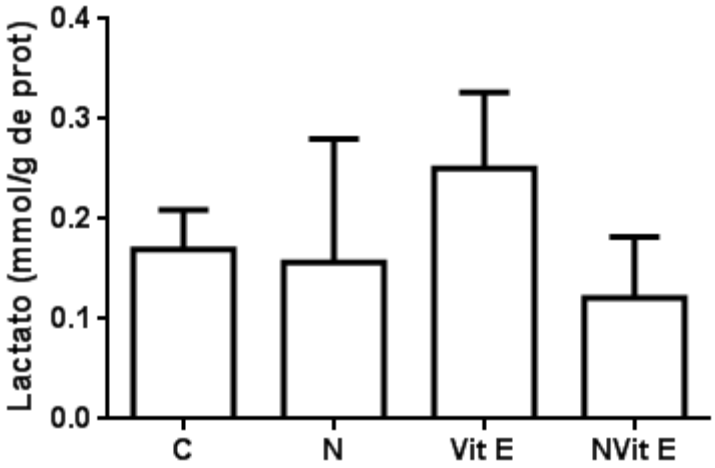

Figure 3 - Muscle skeletal lactate levels (mmol/g proteins) after 3 weeks of training in male zebrafish. C- control fish; N - fish that have been subjected to swimming; VitE - fish that only had supplementation with vitamin E; NVitE - fish that had vitamin E supplementation and were subjected to swimming.

3.3. OXIDATIVE PARAMETERS IN SKELETAL MUSCULATURE

The levels of ROS were significantly lower (P <0.05) in the NVitE group compared to the other groups (Figure 4A). There was also a significant increase (P <0.05) in ACAP observed in the NVitE group in relation to the other groups (Figure 4B). The N and VitE groups had ERO and ACAP levels similar to the control (P> 0.05).

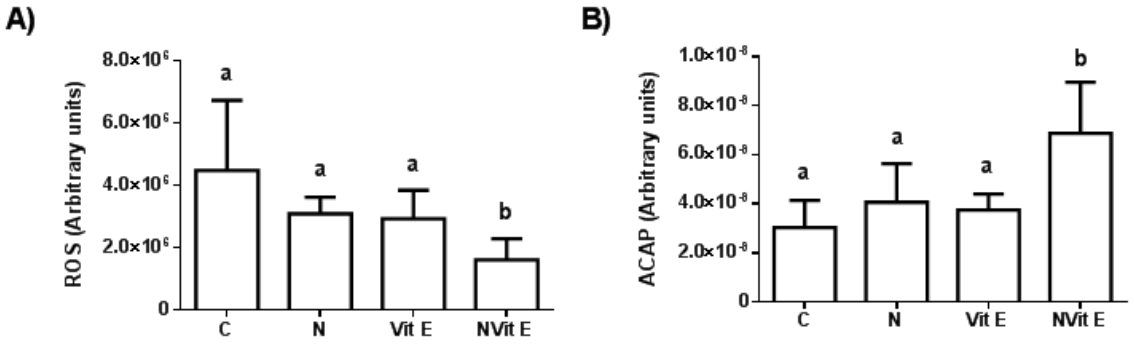

Figure 4 - (A) Levels of reactive oxygen species (ROS) and (B) antioxidant capacity against peroxyl radicals (ACAP) in zebrafish skeletal muscle after training protocol and supplementation with vitamin E. C- control fish; N - fish that have been subjected to swimming; VitE - fish that only had supplementation with vitamin E; NVitE - fish that

had vitamin E supplementation and were subjected to swimming. Different letters represent a significant difference between the groups (P <0.05).

3.4. OXIDATIVE PARAMETERS IN HEART MUSCULATURE

ROS levels in the cardiac musculature were significantly lower in the three experimental groups (P <0.05) than in the control group, with no significant difference between them (P> 0.05) (Figure 5A). A significant increase (P <0.05) of ACAP in the NVitE group was observed in relation to groups C and VitE (Figure 5B).

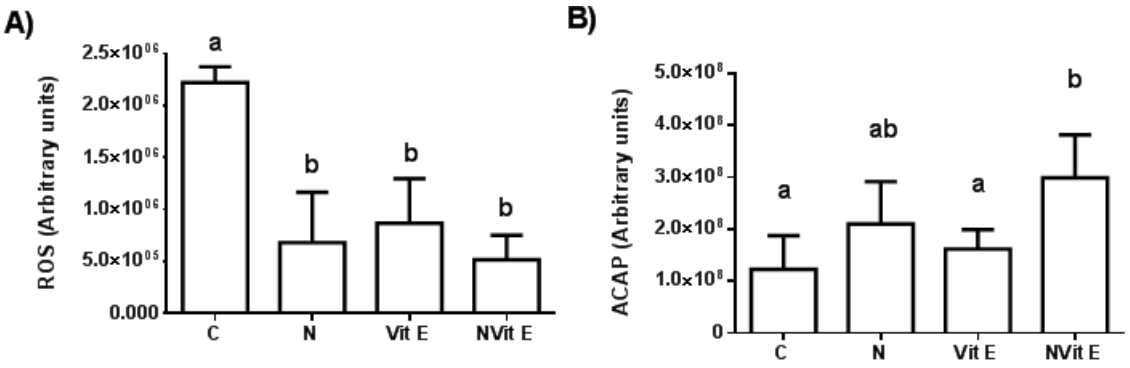

Figure 5 - (A) Reactive oxygen species and (B) antioxidant capacity against peroxyl radicals in the cardiac musculature. C- control fish; N - fish that have been subjected to swimming; VitE - fish that only had supplementation with vitamin E; NVitE - fish that had vitamin E supplementation and were subjected to swimming. Different letters represent significant differences between groups (P <0.05).

4. DISCUSSION

It is known that when fish are exercised at optimum speeds they usually present larger weights than non-exercised fish (Ogata and Oku, 2000; Bugeon et al., 2003). In the study of Hackbart (2004), the *Brycon* (*Brycon amazonicus*) weight gain was verified after the physical exercise of low intensity for 37 and 72 days. Another study (Palstra et al., 2010), zebrafish exercised 6h/day, 5 days/week for 4 weeks at a rate that increased slowly to 39.6cm/s increased their weight considerably both in relation to the initial measures and control group. In this study, we also observed an increase in the final

weight significant in relation to the initial weight in the groups exercised and exercised with Vit. E supplementation, but without differences between the groups not exercised.

According to Robergs et al. (2004) muscle lactate its a good parameter to determine exercise intensity, and our results demonstrating that the protocol of chronic physical exercise was of low intensity, due to no significant difference in lactate levels between the four groups. Several studies have shown lactate values in fish with a great deal of divergence in terms of values. For example, we have total body lactate levels of zebrafish 4 μmol/g (Almeida et al., 2013) and 11 μmol/g (Thomas et al., 2013) in untrained fish. For the skeletal muscles the values also vary between 2.1 ± 0.1 μmol/g lactate in *Carassius carassius* and 1.7 ± 0.3 μmol/g lactate in *Cyprinus carpio* (Genz et al., 2013), until Of 32.9 ± 2.7 mmol/g lactate in *Oreochromis mossambicus* (Vijayavel et al., 2006), all measured for untrained animals. There are no reports in the literature on fish that correlate lactate with the intensity of exercise training, being this a pioneering study for this assessment for zebrafish. In other animal models, however, this relationship has been studied, as in Voltarelli et al. 2002, Manchado et al. 2006 and Guerreiro et al. 2016 all done in rats, where blood lactate increased as the intensity increased.

The physical exercise load in the present study (21 cm/s) was of low intensity and this may explain the absence of significant difference between the control group and the groups with physical exercise in relation to the determination of ROS and ACAP in the skeletal muscles. Different results were found in the literature, for example in the study of Hackenberg et al. (2014) who submitted common carp (*Cyprinus carpio*) to the exercise for 24 h/day for 3 weeks at speeds of 10 cm.s-1, 30 cm.s-1 and 80 cm.s-1 (0.44 BL. S-1, 1.33 BL.s-1, 3.55 BL.s-1) and found an increase in catalase enzyme activity at the rates of 1.33 BL.s-1, 3.55 BL.s- 1, also showed increase of carbonyl groups (measure of protein oxidation) at the rate of 3.55 BL.s-1. Already, Tromm et al. (2014) found a decrease in ROS, an increase in the enzyme superoxide dismutase activity, a decrease in lipid peroxidation and protein oxidation in the skeletal muscle in rats trained for 6 weeks at 60% intensity maximal oxygen consumption (moderate intensity) for 45 minutes per day. This divergence is probably due to the intensity and the time of the exercise, because despite the work of Hackenberg et al. (2014) being at a lower intensity the animals performed the exercise protocol 24h/day, whereas in the work of Tromm et al. (2014) was an exercise at a moderate intensity but less time per exercise session.

Also the differences of species tested may result in these discrepancies, since animals of distinct taxonomic classes may have different physiological responses to the same stressor (Marcon, 1997).

It can be verified in the literature that physical exercise chronically prevents the increase of lipid peroxidation (Venditti and Di Meo, 1996), induces the increase of the endogenous antioxidant capacity of the tissue (McArdle et al., 2001; Maruhashi et al., 2007) and decrease in ROS (Molnar et al., 2006). For zebrafish, it is observed for the first time that both skeletal muscle (in the association of exercise with Vitamin E) and cardiac musculature (during exercise, Vit. E supplementation, and association) respond to chronic training with ROS reduction. This reduction in both the skeletal and cardiac muscles is accompanied by increased antioxidant capacity against peroxyl radicals (ACAP) in the NVitE. group.

Corroborating with the results found here of ACAP increase in the skeletal and cardiac musculature of trained and supplemented zebrafish Corroborating with the results found here of ACAP increase skeletal and cardiac musculature in the exercise association group with vit E in zebrafish, the work of Metin et al. (2002) found a decrease in the levels of LPO (lipid peroxidation) in the skeletal muscle, in rats supplemented with vitamin E (30 mg / kg / day) after 8 weeks of swimming. Contrary to this, in the study of Yfanti et al. (2012) with humans, the combination of 16-week supplementation with vitamin C (500mg) and E (596mg) during a 12-week cycle exercise protocol was shown to have no effect on CAT, GPX, and still observed a Increased LPO. Similarly, Strobel et al. (2011) demonstrated that treadmill exercise in rats supplemented with 1490 mg/kg of vitamin E did not find significant differences in the antioxidant enzyme GPX, and also increased the levels of LPO. These differences in results may be due to the difference in supplementation, it was seen that high doses of Vit. E could have pro-oxidative effects and thereby increase oxidative stress (Kang et al., 2013). The dose of Vit. E used in this study is within the recommended dose (500 mg/kg of diet), while the dose used in the work of Strobel et al. (2011) (1490 mg/kg of feed) is exceeding the prescribed limits. The different results may also be related to the type of exercise and different levels of intensity.

The relation between exercise and ROS production can be seen in a review by Mankowski et al. (2016) who found, in both rat and human studies, an increase in ROS

induced by acute physical exercise. This plays a key role in the stimulation of signaling pathways of antioxidant enzymes (GPX, CAT, SOD) and other signaling pathways. Adapting the moderate production of ROS during exercise allows a more efficient elimination of ROS, increases mitochondrial capacity and efficiency, preventing the deleterious overproduction of ROS. These authors declare that to date, the suppressive and supplementary role of exogenous antioxidant supplementation is not clear by impairing or ameliorating these adaptive effects. For zebrafish in this work, this association may improve the adaptive effects since there was an increase of ACAP and consequently a decrease of ROS in both skeletal and cardiac muscle.

In the heart, despite the Vit. E and perform the same function as in the skeletal muscles, eliminating liposoluble free radicals (Azzi et al., 2003), we have a similar decrease of ROS for the three experimental groups (N, VitE and NVitE), showing that both exercise and Vit. E are reducing ROS levels, but without a synergism between them. On the other hand, the antioxidant capacity of the cardiac musculature presented the same skeletal muscle response pattern, that is, an increase in antioxidant capacity only in the association of exercise with Vit. E. So, cardiac muscle must have decreasing ROS levels to another pathway then those of peroxidation, at least in N and VitE groups, for example Vit. E can be acting as an scavanger and chronic exercise may stimulating redox signaling that actives other signaling ways to prevent ROS generation in a cardiac preconditioning mechanism (for details of redox signaling see the review Pagliaro & Pena, 2015 and Tullio et al., 2013) Corroborating with this work we have the study of Metin et al. (2002) made in rats, where there was a decrease of LPO in the groups with Vit. E (30mg/kg/day) and in the association of Vit. E and exercise. For the isolated effects of physical exercise on the myocardium, divergent results are presented in the literature. Bo et al. (2008) determined a reduction of ROS in cardiac tissue following chronic physical exercise in rats exercising on treadmill at a rate of $15 m.min^{-1}$ for 1 hour per day. In the work of Guerreiro et al. (2016) even without observing a reduction of myocardial ROS in rats trained by 60-minute swimming per day for 16 weeks found an increase in ACAP in low-intensity exercise and a reduction of ACAP in moderate to high exercise heart tissue intensity. Our results are consistent with the results found in Bo et al. (2008), where a reduction of ROS in the myocardium after chronic physical exercise was found, and partially with the results of Guerreiro et al. (2016), who also observed an increase in ACAP in the myocardium after chronic

training. We saw in this study that the myocardium demonstrates a differentiated response to both stimuli (chronic exercise and Vit. E supplementation), appearing a greater susceptibility to the reduction of ROS, even though the antioxidant capacity was improved only in the association.

This work concludes for the first time for zebrafish that the association between chronic exercise and vitamin E supplementation may bring benefits to the oxidative state of the cardiac and skeletal muscles by increasing antioxidant capacity and reducing ROS levels even with a protocol of low intensity. Such results may still be an interesting approach in order to benefit swimming protocols in aquaculture practices if demonstrated for other fish species.

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DISCUSSÃO GERAL

O exercício físico (21 dias) de baixa intensidade (21cm/s) em peixe zebra não promove cronicamente alterações tanto nas espécies reativas de oxigênio como na capacidade antioxidante contra radical peroxil da musculatura esquelética, levando ao ganho de peso corporal, parte dos benefícios clássicos do exercício quando realizado com intensidade controlada e habituação progressiva. A vitamina E suplementada cronicamente por aproxidamente 2 meses, também não provocou alterações no estado oxidativo do musculo esquelético de zebrafish, nem alterou o peso corporal. Entretanto, a interação destes dois estímulos na musculatura esquelética pode trazer benefícios para o estado oxidativo da musculatura cardíaca e esquelética, aumentando a capacidade antioxidante e reduzindo os níveis de EROs, mesmo com um protocolo de baixa intensidade.

Já a musculatura cardíaca do peixe zebra apresenta uma diminuição nos níveis de ERO pelo exercício crônico, pela suplementação crônica de vitamina E e pela interação (ainda que sem um significativo efeito sinérgico dos dois estímulos) e na interação ainda há uma elevação da capacidade antioxidante contra radicais peroxil demonstrando que o estado oxidativo do miocárdio responde diferentemente aos estímulos quando comparado ao músculo esquelético. Esta diferença pode significar que no coração outras formas de ação antioxidante estão sendo elicitadas pelo exercício e pela vitamina E isoladamente, por exemplo Vit. E pode combater as ERO diretamente por sua ação *scavanger* dos vários radicais, sem que isso altere a capacidade antioxidante testada contra o radical peroxyl. O exercício pode diminuir as ERO através de sinalização redox, isto é, produzir ERO em menor quantidade devido ao estímulo inicial dos radicais em enzimas que estabilizem e/ou reduzam a produção mitocontrial de ERO, sem alterar também a capacidade antioxidante contra peróxidos.

Tais resultados podem ainda ser uma abordagem interessante a fim de beneficiar protocolos de natação em práticas de aquicultura se demonstrados para outras espécies de peixes. Trabalhos futuros ainda precisam ser feitos de modo a definir se esses benefícios serão conseguidos em outras intensidades e com outros modelos animais, bem como algumas análises (LPO e também algumas enzimas antioxidantes) para determinar o estado oxidativo completo.

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