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Efeitos da suplementação de vitamina A nos parâmetros redox e resposta inflamatória de ratos Wistar treinados

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Orientador: Prof. Dr. Daniel Pens Gelain

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“And now that you don’t have to
be perfect, you can be good.”

John Steinbeck, East of Eden

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RESUMO

A vitamina A (VA), uma vitamina lipossolúvel obtida na dieta, exerce um papel fundamental em vários processos fisiológicos e metabólicos, como a transcrição genética e a resposta imune. É armazenada no fígado, e frequentemente utilizada como antioxidante. A ingestão de suplementos é uma prática comum para a prevenção do estresse oxidativo, especialmente um estresse induzido por exercício. Dependendo da carga, a sinergia entre exercício, equilíbrio redox e sistema imunológico pode ser prejudicial, e é possível usar a suplementação como estratégia para a prevenção de lesões. Neste estudo, investigamos o papel da suplementação de VA nos parâmetros redox e resposta inflamatória do soro, músculo esquelético e fígado de ratos Wistar adultos treinados. Durante oito semanas, os animais foram submetidos a um treino de natação 5x por semana e ingestão diária de 450 equivalentes de retinol. A VA comprometeu a capacidade antioxidante total do soro adquirida pelo exercício, sem alteração nos níveis de IL-1β e TNF-α. No músculo esquelético, a VA causou peroxidação lipídica e dano proteico sem interferir na atividade das enzimas antioxidantes; no entanto, a VA diminuiu o imunoconteúdo de SOD1 e SOD2. Além disso, a VA diminuiu o imunoconteúdo da citocina anti-inflamatório IL-10 e da chaperona HSP70, duas proteínas importantes no processo de adaptações ao exercício e prevenção de danos teciduais. No fígado, a VA também causou dano lipídico e proteico, além de inibir o aumento da expressão de HSP70. O exercício sozinho aumentou a atividade das enzimas antioxidantes, e a VA inibiu esse aumento. Ainda assim, as citocinas pró-inflamatórias IL-1β e TNF-α apresentaram níveis mais baixos e a anti-inflamatória IL-10 aumentou no grupo exercitado e suplementado com VA. Ambos os grupos exercitados apresentaram níveis mais baixos do imunoconteúdo do receptor RAGE, mostrando que a VA não afetou esse fator. Em conclusão, no músculo esquelético, a suplementação de VA causou dano oxidativo e atenuou algumas importantes adaptações positivas adquiridas com o exercício; no entanto, apesar de a VA ter causado danos oxidativos no fígado, exerceu efeitos protetores liberando mediadores pró-inflamatórios. Portanto, a suplementação com VA parece ser prejudicial para o músculo esquelético, o tecido mais recrutado durante o treino, sem prejuízo para o local onde ocorre seu armazenamento e metabolismo, o fígado.

ABSTRACT

Vitamin A (VA), a fat-soluble vitamin obtained in daily diet, exerts a fundamental role in several physiological and metabolic processes, such as gene transcription, and the immune response. It is stored in the liver, and usually applied as an antioxidant. Supplement intake is a common practice for oxidative stress prevention, especially an exercise-induced stress. Depending on the working load, exercise, redox balance, and immune system synergy can be harmful, and supplementation can be applied as a strategy for injury prevention. In this study, we investigated the role of VA supplementation on redox and immune responses in the serum, skeletal muscle and liver of adult Wistar trained rats. Over eight weeks, animals were submitted to swimming exercise training 5x/week and a VA daily intake of 450 retinol equivalents/day. VA impaired the total serum antioxidant capacity acquired by exercise, with no change in IL-1β and TNF-α levels. In skeletal muscle, VA caused lipid peroxidation and protein damage without differences in antioxidant enzyme activities; however, immunocontent analysis showed that expression of SOD1 was downregulated, and upregulation of SOD2 induced by exercise was blunted by VA. Furthermore, VA decreased anti-inflammatory IL-10 and HSP70 immunocontent, important factors for positive exercise adaptations and tissue damage prevention. In the liver, VA also caused lipid and protein damage, in addition to inhibiting the increase of HSP70 expression. Exercise alone increased the activity of antioxidant enzymes, and VA inhibited this improvement. Still, pro-inflammatory cytokines IL-1β and TNF-α showed lower levels and anti-inflammatory IL-10 was increased in the exercised group supplemented with VA. Both exercised groups had lower levels of the receptor RAGE immunocontent, showing that VA did not affect this factor. In conclusion, VA caused oxidative damage and blunted some important positive adaptations acquired with exercise in the skeletal muscle; however, even though VA caused oxidative damage in the liver, it exerted protective effects by releasing pro-inflammatory mediators. Therefore, VA supplementation appears to be detrimental to skeletal muscle, the most recruited tissue during exercise training, without harm for its storage and metabolism site, the liver.

LISTA DE ABREVIATURAS

4-HNE – 4-hidroxinonenal

AAPH – 2,2-azobis[2-amidinopropane]

AGE – produtos de glicação avançada

ALT – alanina transaminase

AST – aspartato transaminase

AUC – área sob a curva

CAT – catalase

CK – creatina quinase

DNA – ácido desoxirribonucleico

DNPH – 2,4-dinitrofenilhidrazina

DTNB – ácido 5,5-ditionitrobis 2-nitrobenzóico

EDTA – ácido etilenodiaminotetracético

EGTA – ácido etileno glicol-bis(2-aminoetileter)-N-N-N’-N’-tetraacético

ELISA – ensaio de imunoabsorção enzimática

ERO – espécies reativas de oxigênio

ET – animais exercitados

ET+VA – animais exercitados e suplementados com vitamina A

GPx – glutationa peroxidase

GSH – glutationa

GSSG – glutationa dissulfeto

HLD – lipoproteínas de alta densidade

HSP70 – proteína *heat-shock* 70

H_2O_2 – peróxido de hidrogênio

IL – interleucina

IFN-γ – interferon gama

IU – unidades internacionais

LDH – lactato desidrogenase

LDL – lipoproteínas de baixa densidade

NADPH – fosfato de dinucleotídeo de nicotinamida e adenina reduzido

NF-κB – fator nuclear kappa B

NK – células *natural killers*

PBS – tampão fosfato-salino

RAGE – receptor de produtos finais de glicação

RE – equivalentes de retinol

RIPA buffer – tampão para ensaio de radioimunoprecipitação

SDS-PAGE – técnica para separação de proteínas utilizando o detergente SDS e gel de poliacrilamida

SE – animais sedentários

SE+VA – animais sedentários e suplementados com vitamina A

SOD – superóxido dismutase

SH – sulfidril

TBARS – espécies reativas de ácido tiobarbitúrico

TCA – ácido tricloroacético

TNF-α – fator de necrose tumoral alfa

TRAP – potencial antioxidante reativo total

TTBS – tampão tris-salino com 0,01% de Tween 20

URTI – infecção do trato respiratório superior

VA – vitamina A

PARTE 1

I. INTRODUÇÃO

Exercício físico e estresse oxidativo

A prática regular de exercício físico acarreta diversos benefícios para a saúde, como a redução do risco para o desenvolvimento de doenças cardiovasculares, alguns tipos de câncer e diabetes [1,2]. Paradoxalmente, também está claro que a contração repetitiva do músculo esquelético durante o exercício causa a produção de espécies reativas de oxigênio (ERO), que em altas concentrações pode causar estresse oxidativo [3]. É estimado que, para cada 25 moléculas de oxigênio (O_2) utilizadas na respiração mitocondrial, uma ERO é formada [4].

O estresse oxidativo foi primeiro definido como “um distúrbio no equilíbrio pró e antioxidante, em favor do pró-oxidante” [5]. Apesar desta definição ser amplamente utilizada, o termo *estresse oxidativo* é muito amplo, e é indicado usá-lo quando se tem conhecimento das bases moleculares desse desequilíbrio. Na busca de definições mais acuradas, foi proposta a definição para estresse oxidativo como “a ruptura/interrupção do controle e sinalização redox da célula” [6]. O ambiente redox é determinante para o controle de várias funções celulares, como diferenciação, proliferação, migração, quiescência e morte celular [7,8]. Em sistemas biológicos, uma série de parâmetros podem caracterizar o estresse oxidativo, como: o aumento de ERO; a diminuição do potencial antioxidante total da célula; a perturbação do equilíbrio redox celular; e a detecção de dano oxidativo em componentes celulares, como lipídios, proteínas e DNA [9].

Uma das consequências do treino aeróbio de alta intensidade, como corrida, ciclismo e natação, é o aumento do VO_2, ou seja, o aumento da capacidade de consumo de oxigênio pelos tecidos. Durante o exercício, a taxa de consumo de oxigênio de todo corpo aumenta de 10-15 vezes, e nos músculos ativados esse fluxo de oxigênio pode ter um aumento de até 100 vezes [10]. No entanto, 1-5% de todo oxigênio consumido gera a o ânion superóxido (O_2^-), uma ERO com alta reatividade [11]. As ERO, como o radical hidroxila (^-OH) e peróxido

de hidrogênio (H_2O_2), surgem como subprodutos da utilização de oxigênio pelas células [12]. Já foi evidenciado que tais moléculas estimulam o aumento da produção de citocinas inflamatórias, o que pode explicar o excesso na formação das mesmas após o exercício físico intenso [13]. As ERO são altamente reativas, podendo danificar o DNA, estruturas proteicas como enzimas e receptores de membrana, além de estruturas lipídicas como as membranas celulares [14]. Níveis basais de ERO são necessárias para produção de força muscular, no entanto, a perturbação dessa homeostase por fatores como inflamação ou exercício intenso causam disfunção na contração, levando a fraqueza e fadiga muscular [15]. Portanto, para praticantes de exercício físico, o estresse oxidativo que ocorre de forma crônica pode levar a uma diminuição no desempenho, fadiga muscular, dano muscular e sintomas de *overtraining* [16].

A atividade muscular durante o exercício aumenta a produção de ERO, mas ao mesmo tempo também aumenta o sistema antioxidante endógeno. O sistema é composto por enzimas e moléculas capazes de neutralizar as ERO radicais ou não radicais, inibindo assim a oxidação e dano de lipídios, proteínas e bases nitrogenadas. Os sistemas de defesa enzimáticos são compostos por enzimas antioxidantes, como as superóxidos dismutases citosólica (CuZn-SOD ou SOD1) e mitocondrial (Mn-SOD ou SOD2), a glutationa peroxidase (GPx) e a catalase (CAT); a principal defensa não enzimática é o tiol glutationa (GSH), presente na célula em altas concentrações, que vão de 1-15 mM [3]. A enzima SOD catalisa a dismutação de O_2^- à H_2O_2; a CAT converte o H_2O_2 à água (H_2O) e oxigênio (O_2); já a GPx utiliza GSH para neutralizar hidroperóxidos [17]. As enzimas SOD, CAT e GPx são a primeira linha de defesa do organismo contra as ERO produzidas em excesso durante o exercício físico [18], e apesar do exercício aumentar drasticamente a formação de ERO, os sistemas de defesa sofrem adaptações positivas para fazer frente à esse estresse oxidativo [19].

Exercício físico e inflamação

A prática regular de exercício físico aumenta o gasto energético e, consequentemente, aumenta a queima de gordura corporal, prevenindo assim o aparecimento de sobrepeso e obesidade, duas condições que levam a processos inflamatórios crônicos [20]. O exercício físico também melhora o perfil

lipídico da circulação, diminuindo as concentrações séricas de triglicerídeos e LDL e aumentando a concentração de HDL, o que limita o desenvolvimento de aterosclerose [21]. Entre outros efeitos, a prática moderada de exercício físico mostra acarretar efeitos anti-inflamatórios para saúde. Esses efeitos parecem ocorrer devido a três principais mecanismos: a redução de gordura visceral; o aumento na produção e liberação de citocinas anti-inflamatórias do músculo esquelético (também chamadas de miocinas); e a diminuição na expressão de receptores *toll-like* em macrófagos e neutrófilos, o que reduziria a ativação e consequente produção de agentes pró-inflamatórios por essas células [20]. Apesar desses efeitos benéficos, o exercício físico quando realizado de forma intensa também pode levar a processos inflamatórios.

Dependendo do esforço realizado no exercício, um quadro de lesão muscular pode ou não ocorrer. Após o músculo esquelético ser lesionado, ele normalmente passa por estágios de degeneração, inflamação e regeneração, o que mostra que o processo inflamatório é necessário para a recuperação [22]. A prática diária de exercício físico mostra diminuir a inflamação, no entanto, caso a mesma seja constante e sem recuperação, pode levar a modificações no sistema imunológico. A resistência a infecções é dependente da efetividade desse sistema em proteger o organismo contra microrganismos patológicos. A função imune é influenciada por fatores ambientais e genéticos, e já foi observado que o exercício físico intenso exaustivo é capaz de deprimir a função imune [23]. A relação entre exercício e suscetibilidade à infecção tem sido colocada num modelo de curva em forma de “J”. Esse modelo sugere que, enquanto a prática de exercício físico moderado pode aumentar a função imune quando comparado a sedentários, quantidades excessivas de exercício físico prolongado e de alta intensidade podem prejudica-la [24]. Em um estudo realizado com 547 adultos saudáveis entre 20-70 anos, foi descrito que a prática diária de exercício físico moderado está associada com uma redução de 29% no risco de contrair infecção no trato respiratório superior (URTI) [25]. Já em atletas praticantes de treinamentos intensos, o risco de contrair URTI na semana seguinte a uma competição de corrida *endurance*, como uma maratona, aumenta entre 100-500% [26]. Sintomas como dores de garganta e outros relacionados à gripe são mais comuns em atletas do que no restante da

população. Além disso, uma vez já infectados, as gripes se mostram mais longas em atletas [27].

As respostas do sistema imunológico podem ser atribuídas tanto ao efeito agudo (apenas uma sessão) quanto ao efeito prolongado do exercício físico. Os efeitos desse tipo de treinamento envolvem várias respostas inflamatórias, como: a proliferação de células T; liberação de citocinas inflamatórias, como a TNF-α e IL-1β; citocinas anti-inflamatórias, como IL-6 e IL-10; proteínas de fase aguda, como a proteína C-reativa; células *natural killers* (NK), entre outras [28]. Também existem evidências que a produção de Interferon-gama (IFN-γ), proteína produzida pelas células T em resposta a presença de patógenos, é anulada por um período curto após a prática de exercício exaustivo, o que pode estar envolvido com a imunodepressão observada em atletas [29].

Suplementação e exercício físico

Além das respostas adaptativas do organismo, outros fatores, como a nutrição, podem exercer um papel importante na prevenção do estresse oxidativo e desequilíbrio imunológico causado por exercício físico [30,31]. Uma dieta saudável aliada ao exercício mantém um fenótipo anti-inflamatório nos tecidos, caracterizado por adipócitos pequenos e presença de células imunológicas com ação anti-inflamatória, como as células T do tipo regulatórias (T_{reg}) e macrófagos do tipo M2 [20]. A literatura não chega a um consenso sobre os efeitos do uso de suplementos antioxidantes na prática de exercício físico, no entanto, eles são utilizados apesar de existir pouca ou nenhuma evidência de sua eficácia. De fato, uma revisão feita com 51 estudos que reuniu relatos de mais de 10.000 atletas mostrou uma prevalência de 46% no uso de suplementação de vitaminas e minerais [32]. Entre os motivos mais reportados por usuários de suplementos do porquê utiliza-los, os mais comuns são “evitar doenças”, “recuperar de lesões” e “melhorar a dieta” [33].

Os polifenóis encontrados em frutas, plantas e vegetais demonstram potencial antioxidante na circulação, embora os mecanismos moleculares de como isso afeta o treinamento físico ainda serem desconhecidos [34]. A suplementação com vitaminas como as vitaminas C e E é muito utilizada por atletas como alternativa antioxidante, na tentativa de neutralizar a grande

formação de ERO induzida por exercício [11]. Alguns alimentos, como ácidos graxos insaturados e aminoácidos como a glutamina e arginina fornecem benefícios adicionais para pessoas imunodeprimidas [35]. Nesse sentido, a dieta se torna aliada na manutenção redox e inflamatória dos tecidos. Sabe-se que o consumo de carboidratos durante a prática de exercício físico atenua o grande aumento de catecolaminas, hormônio do crescimento, hormônio adrenocorticotrófico, cortisol e citocinas induzido pelo treino, diminuindo assim o estresse gerado [36]. Além disso, podem ser utilizados outros nutrientes chamados imunoestimulantes, como o β-caroteno, aminoácidos de cadeia ramificada e probióticos [30]. Esses dados indicam que a suplementação pode ser utilizada como estratégia para uma possível reversão do quadro de estresse oxidativo e consequente inflamação induzido por treinamento intenso.

Vitamina A

A vitamina A (VA) é um micronutriente sem valor energético e essencial para a vida. A VA é encontrada em alimentos de origem animal, como fígado, rim, peixes ricos em gordura, ovos e produtos lácteos, e também alimentos de origem vegetal, principalmente aqueles de cor alaranjada, como cenoura, batata doce, abóbora, e também brócolis e couve [37]. É uma vitamina lipossolúvel que pode ser obtida na dieta em 3 formas: *all-trans*-retinol e ésteres de retinil (origem animal), ou carotenoides com atividade pró-vitamina A, como o β-caroteno (origem vegetal). No fígado é armazenada na forma de ésteres de retinil, e nos tecidos que a utilizam a VA é oxidada por desidrogenases a ácido retinóico, a sua forma mais biologicamente ativa [38]. É necessária para o funcionamento correto de vários processos metabólicos e fisiológicos, como a visão, hematopoiese, desenvolvimento embriogênico, diferenciação celular, transcrição de alguns genes e sistema imune [39]. A VA é citada na literatura como uma molécula antioxidante [11], no entanto nenhum trabalho realmente explorou o papel da suplementação dessa vitamina nos parâmetros redox do músculo esquelético, o principal tecido mobilizado durante o exercício físico. Apesar dessa característica, a suplementação de VA tem sido associada com alguns efeitos adversos, que ao invés de agir como antioxidante, causa estresse oxidativo. Foi visto que a suplementação com palmitato de retinol induz, na

verdade, um ambiente pró-oxidante em diversos tecidos, incluindo coração, cérebro e pulmão de ratos Wistar [40-42].

A dose diária recomendada de VA para adultos é de 900 equivalentes de atividade de retinol (RAE), e a dose máxima tolerada é de 3000 RAE. Neste sistema, 1 RAE é equivalente a 1 µg de retinol, 2 µg de β-caroteno na forma de suplemento, 12 µg de β-caroteno e 24 µg de outros carotenoides. A importância do consumo adequado de VA é demonstrado pelos efeitos decorrentes da sua deficiência e excesso, que afetam principalmente o tecido epitelial, ósseo, hepático e sistema nervoso [37]. Além desses efeitos, a deficiência de VA causa uma intensa redução na resistência a infecções, o que motiva a realização de estudos investigando as bases celulares e moleculares promovidas pela VA no sistema imune [43]. A literatura apresenta muitos estudos que demonstram o papel do ácido retinóico, a forma da VA metabolicamente ativa dentro da célula, como um imunomodulador. O que tem sido observado é que, dependendo do estímulo, ele pode agir como um indutor ou supressor do sistema imune. Já foi descrita, por exemplo, uma relação direta entre a VA e a regulação na produção de dois tipos específicos de células T, as T_{reg} e as T_h17, ação essa que levaria a uma tolerância do sistema imune, envolvendo mecanismos atuantes em doenças inflamatórias e autoimunes [44]. Por outro lado, o AR demonstrou regular positivamente receptores *gut-homing* que induzem a produção de células T, estimular a produção de células B secretoras de imunoglobulinas, como a IgA, além de promover a maturação de neutrófilos. Estes fatores são favoráveis à ação do sistema imune [45]. Tais fatores reforçam o papel da VA na modulação das respostas imunológicas.

II. JUSTIFICATIVA

A literatura mostra como a prática de exercícios físicos afeta o sistema redox dos tecidos, podendo causar danos e levar a um quadro de inflamação e, consequentemente, aumentar a suscetibilidade à infecção.

A VA já mostrou ser uma molécula com potencial antioxidante e um modulador da resposta imunológica, exercendo efeitos indutores ou supressores, dependendo do estímulo recebido. Através de comprovações

obtidas em estudos que apontam como a alimentação pode ser utilizada como estratégia para modular o estresse oxidativo e manter o sistema imunológico em funcionamento ideal, é relevante que intervenções com suplementação/exercício físico sejam realizadas, a fim de esclarecer possíveis efeitos positivos para a saúde e rendimento de atletas e praticantes de exercício físico moderado à intenso. A escolha dos tecidos analisados se baseou em dois fatores: o do exercício e o da suplementação. O músculo esquelético foi escolhido por ser o tecido mais recrutado durante a prática de exercício físico; já o fígado, além de ser um órgão importante para manutenção da glicemia e metabolismo do lactato durante o exercício, é o local de armazenamento e metabolismo de retinóides no corpo. Por serem moléculas lipossolúveis, os retinóides são armazenados em lipócitos localizados no fígado. Parâmetros do soro foram analisados para fornecer uma visão geral sistêmica dos efeitos do exercício e suplementação com vitamina A.

III. OBJETIVOS

Objetivo geral

Avaliar os efeitos da suplementação oral de vitamina A, no formato de palmitato de retinol, nos parâmetros redox e resposta inflamatória no soro, músculo esquelético e fígado de ratos Wistar treinados em um protocolo de natação.

Objetivos específicos

Avaliar e quantificar em animais submetidos a 8 semanas de treinamento de natação suplementados com vitamina A:

- Parâmetros indicativos de dano oxidativo sobre proteínas e lipídios;
- Atividade e o imunoconteúdo de enzimas antioxidantes;
- Citocinas anti e pró-inflamatórias;
- Expressão diferencial de genes relacionados ao estresse físico, como a proteína HSP70 e o receptor RAGE.

IV. METODOLOGIA

Animais

Todos os experimentos deste trabalho foram aprovados pelo comitê de ética da Universidade Federal do Rio Grande do Sul (CEUA-UFRGS) sob o número de acesso 25837. Foram utilizados ratos Wistar machos (7 semanas, 205-300 g) obtidos na própria colônia do biotério localizado no Departamento de Bioquímica da UFRGS. Os animais eram mantidos em caixas plásticas com no máximo 4 animais por caixa, em uma sala com temperatura controlada (23 ± 1ºC) e ciclo claro/escuro de 12h (7h-19h), com acesso à água e comida *ad libitum*. A eutanásia foi realizada por decapitação com guilhotina, evitando interferência nos parâmetros bioquímicos do fígado pela presença de anestésicos.

Protocolo de treinamento físico

Ratos com 4 semanas de idade passaram por um período adaptativo de manipulação. Durante todo o projeto, a mesma pessoa manipulou, nadou e suplementou os animais. Ao completar 7 semanas, os animais começaram o período de adaptação na água. Na primeira semana, os animais permaneceram de 20-60 min em água rasa todos os dias. Em seguida, foram randomizados em 4 grupos: sedentários, sedentários + vitamina A, exercitados, exercitados + vitamina A. Nas primeiras duas semanas os animais começaram nadando 10 min/dia, aumentando progressivamente (10, 20, 40, 60min) para 60 min/dia. Nas duas semanas seguintes, os animais nadaram 60min/dia, 5x por semana, sem sobrecarga. Nas últimas 3 semanas, os animais nadaram com um sobrepeso anexado ao torso, que foi de 2, 4 e 6% do peso corporal. Os animais eram pesados semanalmente, e o peso utilizado para calcular o valor da sobrecarga. Ao total, o programa de natação (incluindo a adaptação) durou 8 semanas. O protocolo era realizado entre 18-20h, anterior à suplementação. A temperatura da água para o exercício era de 31 ± 1ºC. Para minimizar o estresse induzido pelo contato com a água, os animais sedentários eram colocados em um tanque com água rasa 20 min/dia, 5x por semana. Após cada sessão, todos animais eram secados com toalha e colocados de volta nas suas caixas.

Suplementação de vitamina A

Durante todo o período de 8 semanas, os animais dos grupos suplementados + vitamina A e exercitados+ vitamina A eram suplementados diariamente com 450 equivalentes de retinol (1500IU/kg/dia) do suplemento Arovit® (palmitato de retinol). O suplemento Arovit apresenta uma composição que é solúvel em água, o que permitiu usar solução salina como veículo. Os animais controle receberam apenas o veículo. A suplementação era administrada por via oral usando o método de gavagem, e era realizado posterior ao protocolo de exercício.

Coleta de tecidos

Após 24h da última sessão de exercício físico, os animais foram eutanasiados e foi realizada a coleta de tecidos. Tecidos foram imediatamente congelados a -80ºC até homogeneização para procedimentos experimentais. Mais detalhes de coleta e técnicas utilizadas estão descritas na parte 2 deste trabalho, inserido nos artigos científicos.

PARTE 2

I. RESULTADOS

CAPÍTULO I

Vitamin A oral supplementation induces oxidative stress and suppresses IL-10 and HSP70 in skeletal muscle of trained rats

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Vitamin A Oral Supplementation Induces Oxidative Stress and Suppresses IL-10 and HSP70 in Skeletal Muscle of Trained Rats

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Abstract: Exercise training intensity is the major variant that influences the relationship between exercise, redox balance, and immune response. Supplement intake is a common practice for oxidative stress prevention; the effects of vitamin A (VA) on exercise training are not yet described, even though this molecule exhibits antioxidant properties. We investigated the role of VA supplementation on redox and immune responses of adult Wistar rats subjected to swimming training. Animals were divided into four groups: sedentary, sedentary + VA, exercise training, and exercise training + VA. Over eight weeks, animals were submitted to intense swimming 5 times/week and a VA daily intake of 450 retinol equivalents/day. VA impaired the total serum antioxidant capacity acquired by exercise, with no change in interleukin-1β and tumor necrosis factor-α levels. In skeletal muscle, VA caused lipid peroxidation and protein damage without differences in antioxidant enzyme activities; however, Western blot analysis showed that expression of superoxide dismutase-1 was downregulated, and upregulation of superoxide dismutase-2 induced by exercise was blunted by VA. Furthermore, VA supplementation decreased anti-inflammatory interleukin-10 and heat shock protein 70 expression, important factors for positive exercise adaptations and tissue damage prevention. Our data showed that VA supplementation did not confer any antioxidative and/or protective effects, attenuating exercise-acquired benefits in the skeletal muscle.

Keywords: antioxidant enzymes; antioxidant supplements; exercise; cytokines; vitamin

1. Introduction

Benefits generated by regular physical exercise on human health are well known. Regular physical activity is recommended for the prevention and treatment of metabolic syndrome diseases, such as high blood pressure and type 2 diabetes [1]. Moderate to intense physical activity exerts a large influence on redox balance and immunity modulation [2,3]. Due to high oxygen demand by skeletal muscle, exercise increases the generation of reactive oxygen species (ROS), such as the superoxide anion radical, hydrogen peroxide, and the hydroxyl radical [4,5]. At physiological concentrations, ROS act as signaling molecules that lead to positive adaptations induced by exercise, such as upregulation of endogenous antioxidant defenses, skeletal muscle hypertrophy, and mitochondrial biogenesis [6–8]. When the redox imbalance intensifies towards an excessive pro-oxidant state, ROS may cause DNA damage, functional loss of protein structures, such as enzymes and membrane receptors, and structural damage of the cell lipid bilayer [9]. In athletes, chronic oxidative stress can lead to performance decline,

fatigue, muscular damage, and overtraining [10]. Furthermore, levels of inflammatory cytokines rise considerably after vigorous exercise training, which is often related to ROS overload [11]. This can lead to immune improvement or depression, and the outcome is determined by training intensity. The relationship between exercise and susceptibility to illness is described by a "J curve" concept. It suggests that, while individuals that regularly perform moderate intensity exercise improve their immune system, excessive bouts of prolonged training can impair immune function [12]. This results in responses such as release of pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β), and anti-inflammatory cytokines, such as interleukin-6 (IL-6) and interleukin-10 (IL-10) [13].

Besides the endogenous defenses, other factors like nutrition can exert a major role in the prevention of oxidative stress and immunity depression [14]. Polyphenols found in fruits, plants, and vegetables demonstrate antioxidant potential in the circulatory system, although the molecular basis of how they affect exercise training remains unclear [5]. Supplementation with vitamins is also widely used by athletes to avoid skeletal muscle injury, especially vitamin C and E [15,16]; however, these benefits remain questionable in the literature. Studies show different outcomes from the combination of vitamins C and E, such as no effect during exercise training [15,17], reduced lipid and protein damage after eccentric exercise [18,19], and decreased stress markers without antioxidant benefits [20].

Another vitamin that has shown to be involved in the redox process is vitamin A (VA), a fat-soluble vitamin obtained from different compounds: all-*trans* retinol (considered the VA molecule), β-carotene (VA precursor), and retinyl esters (retinol esterified to other molecules, such as palmitate) [21]. It is essential to the correct functions of several metabolic and physiological processes, such as vision, hematopoiesis, embryogenic development, cell differentiation, gene transcription, and the immune system [22]. The arrangement of long chains of conjugated double bonds, common to all retinoids, allows the structure to exert ROS scavenging properties, and usually, this activity is involved in the prevention of lipid peroxidation [23–25]. However, retinol has been observed to present moderate to low antioxidant activity, and VA supplementation has been associated with some adverse effects. Our group has previously shown that oral retinyl palmitate supplementation induces, in fact, a pro-oxidant environment in several tissues, including the heart, brain, and lungs of Wistar rats [26–28]. Moreover, it was previously described that mice fed with retinyl palmitate in low doses developed aortic valve stenosis and calcification [29]. A clinical study of the effect of a combined supplement of β-carotene and retinyl palmitate on lung cancer prevention actually revealed harmful effects, as it increased the incidence of lung cancer and cardiovascular diseases in smokers and workers exposed to asbestos [30]. Reviews that address the effects of supplementation on exercise-induced oxidative stress often mention VA or its precursor β-carotene as a potential antioxidant molecule [4,31]. However, its effects on exercise training are poorly documented, and mechanisms in vivo remain unclear. Here, we evaluated the effect of VA supplementation, given in the form of retinyl palmitate, on parameters of oxidative stress and inflammation in rats subjected to exercise training, to determine if VA enhances the benefits conferred by regular exercise. The dose of choice for VA treatment was 450 retinol equivalents (RE)/day. We calculated the human equivalent doses (HED) using the dose-by-factor approach [32], with values based on the daily recommendation for adults. We considered the daily recommendation of 800 RE [33] and the fact that VA is provided in the diet as it is present in the standard chow in amounts meeting the daily requirement for this vitamin. We chose this approach to avoid hypervitaminosis or other effects caused by excessive VA intake, since higher doses of VA produce deleterious effects on the brain, lungs, and cardiovascular systems as mentioned above. This is the first study describing the effects of chronic aerobic exercise training and VA supplementation on redox and immunity parameters on skeletal muscle.

2. Materials and Methods

The Ethical Committee for Animal Experimentation of the Federal University of Rio Grande do Sul (CEUA-UFRGS) granted the approval for this project under the number 25837, and all experiments

were conducted under the National Institute of Health Guide for the Care and Use of Laboratory Animals (2011) [34]. Protocols also followed the guidelines of the Brazilian Society of Animal Science Experimentation (SBCAL). This study complied with the 3Rs principle: replacement of animals by alternatives wherever possible; reduction in the number of animals used; and refinement of experimental conditions and procedures to minimize the harm to the animals.

2.1. Animals

Thirty-two adult male Wistar rats (7 weeks old, weight 250–300 g) were provided by our breeding colony. During one week, animals were manipulated for adaptation. Animals were maintained in cages in a room with an ambient temperature of 22 ± 1 °C and a 12/12 h light/dark cycle, with access to food and water ad libitum.

2.2. Swimming Exercise Training Protocol

The training protocol lasted 8 weeks in total (Figure 1). For the first week, animals remained in shallow water for 20–60 min each day. Next, animals were randomized into four groups: sedentary (SE), sedentary supplemented with vitamin A (SE + VA), exercise training (ET), and exercise training supplemented with vitamin A (ET + VA). In the following two weeks, the swimming protocol started with 10 min/day, gradually increasing to 60 min/day. The exercise protocol was conducted between 6 and 8 pm, in a specific swimming tank for rodents with water at 31 ± 1 °C. Over the following 5 weeks, training consisted of 60 min/day, 5 days/week [35]. Once a week, animal weight values were utilized to calculate the overload (0, 2, 4, 6% body weight). To minimize water-induced stress differences between groups, sedentary animals were placed in shallow water for 20 min 5 days/week during the 8 weeks. After each session, animals were towel-dried and returned to their cages.

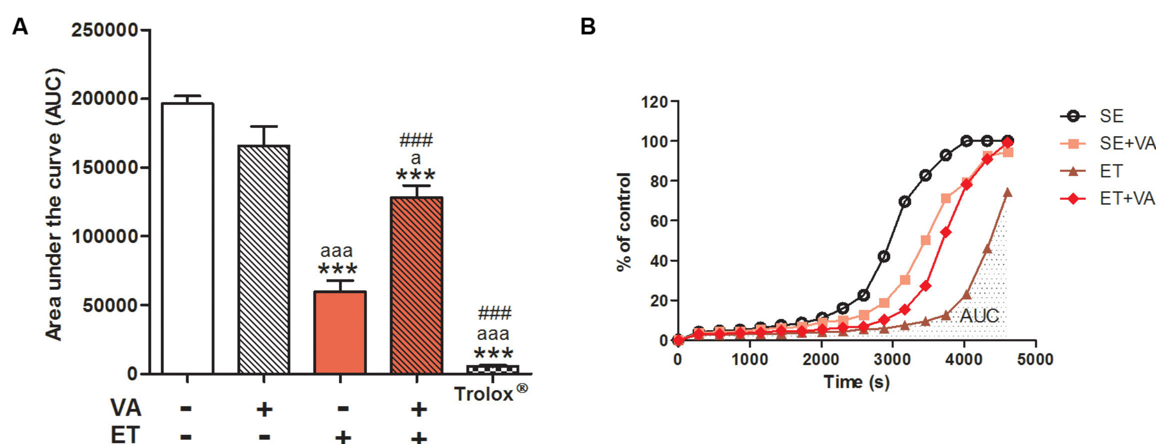

Figure 1. (**A**) Total reactive antioxidant potential (TRAP) from serum. Data presented as mean ± SEM (n = 6–8); (**B**) demonstrative reaction kinetics of TRAP. Control: peroxyl radical system that generates luminescence at a steady rate (considered 100% of free radical production). Luminescence generated by this system in the presence of samples is monitored through time. Trolox = antioxidant applied as positive control (100 µM). VA: vitamin A; ET: exercise training. *** $p < 0.001$ significant difference from sedentary group; ^{a} $p < 0.05$; ^{aaa} $p < 0.001$ significant difference from sedentary + vitamin A group; $^{###}$ $p < 0.001$ significant difference from exercise training group using one-way ANOVA followed by Tukey's post hoc test.

2.3. Vitamin A Supplementation

Throughout the protocol period (8 weeks), animals from groups SE + VA and ET + VA had a daily intake of 450 RE (1500 IU)/kg/day of retinyl palmitate (Arovit, Bayer, Rio de Janeiro, RJ, Brazil). We calculated the human equivalent doses (HED) using the dose-by-factor approach [32].

Arovit presents a water-soluble form of vitamin A, allowing use of saline as a vehicle solution. Supplementation was orally administered via gavage, in a maximum volume of 0.5 mL. Groups SE and ET received only the vehicle.

2.4. Tissue Preparation

Twenty-four hours after the last session of exercise and vitamin A supplementation animals were euthanized by decapitation. Blood samples were immediately centrifuged for serum separation. Vastus medialis skeletal muscle was removed and stored at −80 °C. For biochemical analysis, tissue was homogenized in phosphate buffer (PB) and centrifuged (3000× *g*, 10 min), and sample supernatant was used for analysis. Protein content was quantified by the Lowry method [36] using bovine serum albumin (BSA) as a standard. For Western blotting, tissue was homogenized in RIPA buffer (20 mM Tris-HCl pH 7.5; 150 mM NaCl; 1 mM ethylenediamine tetra-acetic acid (EDTA); 1 mM ethylene glycol tetra-acetic acid (EGTA); 2.5 mM sodium pyrophosphate; 1% sodium deoxycholate; 1% Tergitol-type NP-40; 1 mM β-glycerophosphate; 1 mM sodium orthovanadate; 1 µg/mL leupeptin), centrifuged, and the homogenate was added to Laemmli-buffer (62.5 mM Tris-HCl pH 6.8; 1% SDS; 10% glycerol) with 5% β-mercaptoethanol.

2.5. Serum Analysis

Serum activity of enzymes creatine kinase (CK) and lactate dehydrogenase (LDH) was measured with commercial kits (Labtest, São Paulo, Brazil). Total reactive antioxidant potential (TRAP) was determined as described in the literature [37]. The assay is based on the employment of a peroxyl radical generator (2,2-azo-bis(2-amidinopropane); AAPH) mixed with luminol, and the scavenging activity of samples prevents luminol oxidation by AAPH. The synthetic antioxidant Trolox (Acros Organics BVBA, Geel, Belgium), a vitamin E analog, was applied as a positive control at a concentration of 100 µM [38]. The antioxidant capacity of samples was recorded through 60 min and results were calculated as area under the curve (AUC). Quantitative analysis of IL-1β and IL-10 was determined by indirect ELISA using polyclonal antibodies (Abcam, Cambridge, UK). TNF-α was quantified using an ELISA sandwich kit following the manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN, USA).

2.6. Skeletal Muscle Analysis

2.6.1. Oxidative Stress Parameters

Lipid peroxidation was detected through measuring thiobarbituric acid reactive species (TBARS) levels [39]. Samples deproteinized by 10% trichloroacetic acid (TCA) were heated at 100 °C for 25 min with 0.67% thiobarbituric acid, and TBARS were quantified spectrophotometrically at a wavelength of 532 nm. Protein damage was quantified by carbonyl group detection [40]. The technique involves incubating sample proteins, previously precipitated with 20% TCA, with 2,4-dinitrophenylhydrazine (DNPH), and quantification at a wavelength of 370 nm. Thiol content was quantified in protein-containing and non-protein-containing (after acid-induced precipitation) samples through an Ellman's assay [41]. Samples were diluted in phosphate-buffered saline (PBS) and incubated with 10 mM of 5,5-dithiobis(2-nitrobenzoic) (DTNB) for 60 min at room temperature of 23 ± 1 °C. Quantification was performed using a spectrophotometer at a wavelength of 412 nm.

2.6.2. Activity of Antioxidant Enzymes

Determination of the activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) was performed using spectrophotometric kinetics. CAT (EC 1.11.1.6) activity was measured by the decrease of hydrogen peroxide (H_2O_2) followed by measurement at a UV wavelength of 240 nm [42]. SOD (EC 1.15.1.1) activity was measured indirectly by inhibition of adrenaline auto-oxidation, measured at 480 nm [43]. GPx (EC 1.11.1.9)

activity was evaluated by the decrease of nicotinamide adenine dinucleotide phosphate reduced form (NADPH) in the presence of glutathione (GSH), *tert*-butyl hydroperoxide, and glutathione reductase, measured at 340 nm [44].

2.6.3. Western Blotting

Samples from skeletal muscle (25 µg), run on an SDS-PAGE gel, were transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA) through semi-dry transference, and protein content confirmed using Ponceau S staining. After three cycles of TTBS (Tris 100 mM pH 7.5; 0.9% NaCl, and 0.1% Tween-20) washing, membranes were blocked with 5% non-fat dry milk for 1 h at room temperature. After washing, membranes were incubated with primary antibodies for SOD1, SOD2, CAT, IL-1β, IL-10, TNF-α, heat-shock protein 70 (HSP70), and β-actin for 2 h at room temperature in a 1:1000 dilution range. Secondary antibodies (anti-rabbit/mouse/goat peroxidase-linked—Cell Signaling Technology, Beverly, MA, USA) were incubated for 1 h at room temperature in a 1:2000 dilution range. Detection of immunoreactivity was performed through chemiluminescence using a Supersignal West Pico Chemiluminescent kit (Thermo Scientific, Rockford, IL, USA). Densitometry analysis was conducted with ImageJ software (version 1.50i, National Institutes of Health, Bethesda, MD, USA), and the results were expressed as ratio of protein:β-actin.

2.7. Statistics

All analyses and graphics were performed using GraphPad Prism (version 5.0, GraphPad Software Inc., San Diego, CA, USA). For comparison of four groups, one-way ANOVA followed by Tukey's post hoc test was applied, and data expressed as the mean ± standard error (SEM) or median and interquartile. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Protocol and Supplementation Effect on Total Body Weight

The ET group exhibited a significant reduction in body weight gain when compared to both sedentary groups (SE and SE + VA), probably due to the intense exercise protocol (Table 1). ET + VA group weight gain did not differ from the ET group, demonstrating that VA supplementation did not affect this parameter.

Table 1. Effects of chronic exercise training and vitamin A supplementation on total body weight.

	SE	SE + VA	ET	ET + VA
Initial Weight (g)	337.6 ± 21.9	345.8 ± 24.3	350.5 ± 25	336.1 ± 27.2
Final Weight (g)	440.4 ± 25.2	456.9 ± 25.3	403.3 ± 26.9	398 ± 31.1
Δ weight gain (g)	99.3 ± 10	101.1 ± 16	68.7 ± 11.8 ^{**,aa}	66.6 ± 16.4 ^{**,aaa}

Data presented in mean ± standard error (SEM) (n = 6–8). SE: sedentary; SE + VA: sedentary + vitamin A; ET: exercise training; ET + VA: exercise training + vitamin A. ** $p < 0.01$ and significant difference from SE group; ^{aa} $p < 0.01$; ^{aaa} $p < 0.001$ significant difference from SE + VA group using one-way ANOVA followed by Tukey's post hoc test.

3.2. Serum Results

3.2.1. Tissue Damage Markers

The cytosolic enzymes LDH and CK are expressed in myocytes, and detection of unusual activity in serum indicates tissue injury, especially skeletal muscle damage [45]. Table 2 displays the serum activity of these markers. LDH activity significantly increased in ET and ET + VA samples compared to SE, although no differences were detected between both exercised groups. CK activity did not change in exercised groups compared to SE, although the SE + VA group showed lower CK activity compared to SE.

Table 2. Effects of chronic exercise training and vitamin A supplementation on serum tissue damage markers.

	SE	SE + VA	ET	ET + VA
LDH	43.7 ± 1.18	49.5 ± 1.78	57.1 ± 0.08 *	68.7 ± 2.4 **,a
CK	315.8 ± 3.6	273.1 ± 2.8 *	304.8 ± 4.4	290.9 ± 5

Data presented in mean ± SEM (n = 6–8). LDH: lactate dehydrogenase; CK: creatine kinase. LDH and CK values expressed as U/L. SE: sedentary; SE+VA: sedentary + vitamin A; ET: exercise training; ET + VA: exercise training + vitamin A. * $p < 0.05$; ** $p < 0.01$ significant difference from SE group; [a] $p < 0.05$ significant difference from SE + VA group using one-way ANOVA followed by Tukey's post hoc test.

3.2.2. Redox Balance

The serum total antioxidant profile was assessed by the TRAP assay (Figure 1). The SE + VA group did not display a significant difference in serum antioxidant potential compared to SE. The ET group presented a high antioxidant profile, as expected since regular exercise improves endogenous antioxidant capacity [8]. However, the ET + VA group showed a significant reduction in antioxidant potential (approximately 50%), suggesting that vitamin A supplementation attenuates the antioxidant effect of exercise training on serum.

3.2.3. Inflammation Markers

Levels of pro-inflammatory cytokines IL-1β and TNF-α were significantly higher in ET and ET + VA groups when compared to SE, but these groups displayed no differences between each other, indicating that VA supplementation has no effect on modulation of IL-1β and TNF-α by ET (Figure 2A,B). Interestingly, VA supplementation in sedentary animals (SE + VA) induced a significant increase in serum TNF-α (Figure 2B). Levels of the anti-inflammatory cytokine IL-10 did not increase in any group, and the SE + VA group exhibited a significant decrease in IL-10 compared to SE (Figure 2C).

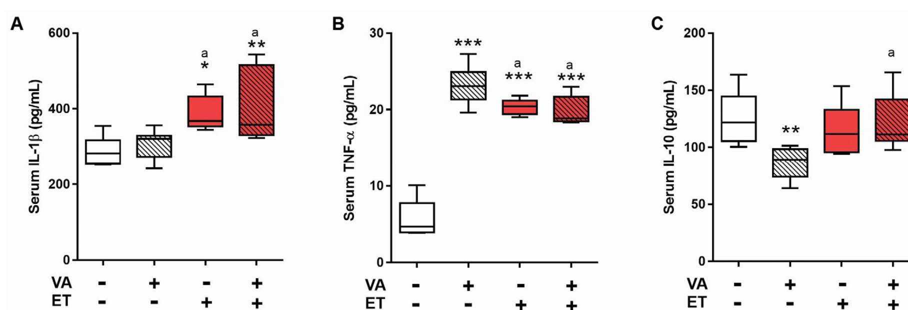

Figure 2. Levels of cytokines detected in serum by ELISA. Data presented as box (median) and whiskers (interquartile interval) diagram (n = 6–8). (**A**) Interleukin-1β; (**B**) Tumor necrosis factor-α; and (**C**) Interleukin-10. VA: vitamin A; ET: exercise training. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ significant difference from sedentary group; [a] $p < 0.05$ significant difference from sedentary + vitamin A group using one-way ANOVA followed by Tukey's post hoc test.

3.3. *Skeletal Muscle*

3.3.1. Oxidative Stress Markers

We investigated the effect of vitamin A on skeletal muscle, as this is the tissue with the highest oxidative and stress-related demands during exercise training [46]. Muscle lipoperoxidation levels (Figure 3A) and protein carbonylation (Figure 3B) were increased in the ET + VA group, with a significant difference compared to the other three groups. These results indicate that VA supplementation causes oxidative damage to lipids and proteins in skeletal muscle of animals subjected to ET. Regarding sulfhydryl group content, the ET group exhibited a significant decrease in total thiol

content (Figure 3C), which was significantly reversed in the ET + VA group. This result indicates that animals receiving VA supplementation and subjected to ET display an increased content of proteins with reduced thiol groups. Non-protein sulfhydryl levels did not show a significant difference between groups (Figure 3D).

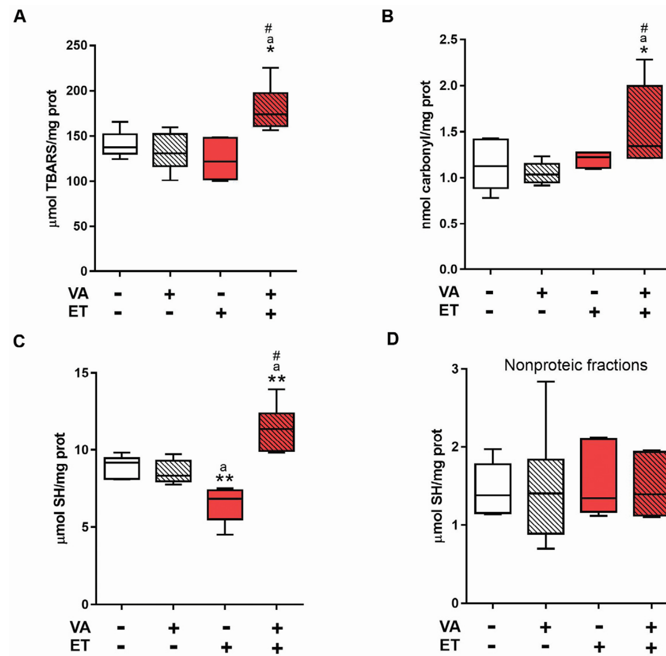

Figure 3. Effects of exercise and vitamin A supplementation on skeletal muscle oxidative damage markers. Data presented as box (median) and whiskers (interquartile interval) diagram (n = 6–8). (**A**) lipid peroxidation; (**B**) protein carbonylation; and (**C**,**D**) sulfhydryl group content. VA: vitamin A; ET: exercise training. * $p < 0.05$; ** $p < 0.01$ significant difference from sedentary group; [a] $p < 0.01$ significant difference from sedentary + vitamin A group; # $p < 0.05$ significant difference from exercise training group using one-way ANOVA followed by Tukey's post hoc test.

3.3.2. Antioxidant Enzyme Activity

SOD activity did not present differences between groups (Figure 4A). CAT activity only differed in the group SE + VA, with a significant increase compared to ET and ET + VA (Figure 4B). On the other hand, GPx activity was significantly higher in SE + VA and ET groups compared to SE (Figure 4C).

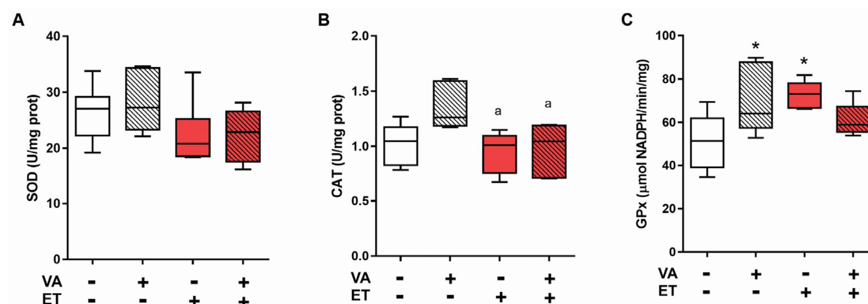

Figure 4. Effects of exercise and vitamin A supplementation on skeletal muscle antioxidant enzyme activity. Data presented as box (median) and whiskers (interquartile interval) diagram (n = 6–8). (**A**) Superoxide dismutase; (**B**) Catalase; and (**C**) Glutathione Peroxidase. VA: vitamin A; ET: exercise training. * $p < 0.05$ significant difference from sedentary group; [a] $p < 0.05$ significant difference from sedentary + vitamin A group using one-way ANOVA followed by Tukey's post hoc test.

3.3.3. Antioxidant Enzyme Content Evaluated Using Western Blotting

With no alteration in SOD and CAT activities, the next step was to determine through Western blotting the content of enzymes CuZnSOD (SOD1), the isoform present in cell cytoplasm, MnSOD (SOD2), the isoform located within the mitochondria [47], and CAT. Exercise training by itself did not increase the content of SOD1 in skeletal muscle (Figure 5A). However, the ET + VA group displayed significantly lower levels of SOD1 compared to SE and ET groups. SOD2 content (Figure 5B) increased significantly in the ET group compared to SE and ET + VA groups, though supplementation reversed this increase, as SOD2 content in the ET + VA group was significantly lower than in the ET group. CAT content in skeletal muscle (Figure 5C) decreased significantly in both exercise training groups compared to SE and SE + VA groups.

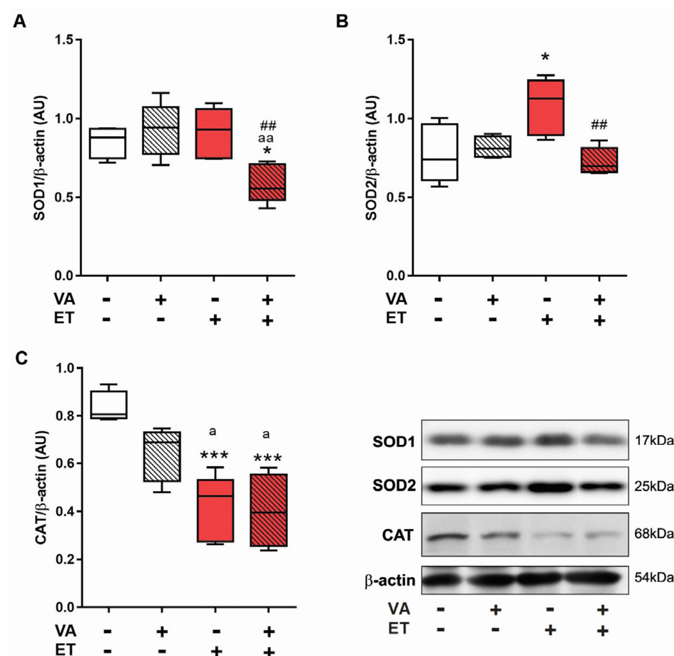

Figure 5. Effects of exercise and vitamin A supplementation on skeletal muscle antioxidant content. Data presented as box (median) and whiskers (interquartile interval) diagram ($n = 6$). (**A**) Superoxide dismutase-1; (**B**) Superoxide dismutase-2; and (**C**) Catalase content. VA: vitamin A; ET: exercise training. * $p < 0.05$; *** $p < 0.001$ significant difference from sedentary group; ^{a} $p < 0.05$; ^{aa} $p < 0.01$ significant difference from sedentary + vitamin A group; $^{\#\#}$ $p < 0.01$ significant difference from exercise training group using one-way ANOVA followed by Tukey's post hoc test. Representative Western blots are shown.

3.3.4. Inflammation Marker Content Evaluated Using Western Blotting

Taking into consideration skeletal muscle oxidative damage results, we next evaluated the levels of inflammatory and stress markers IL-1β, TNF-α, IL-10, and HSP70. IL-1β values did not differ between groups (Figure 6A). TNF-α content increased in SE + VA and ET groups compared to SE (Figure 6B). IL-10 increased significantly in the ET group compared to SE and ET + VA groups (Figure 6C). HSP70 content was significantly lower in both vitamin A groups compared to the ET group, which had increased HSP70 content compared to SE (Figure 6D).

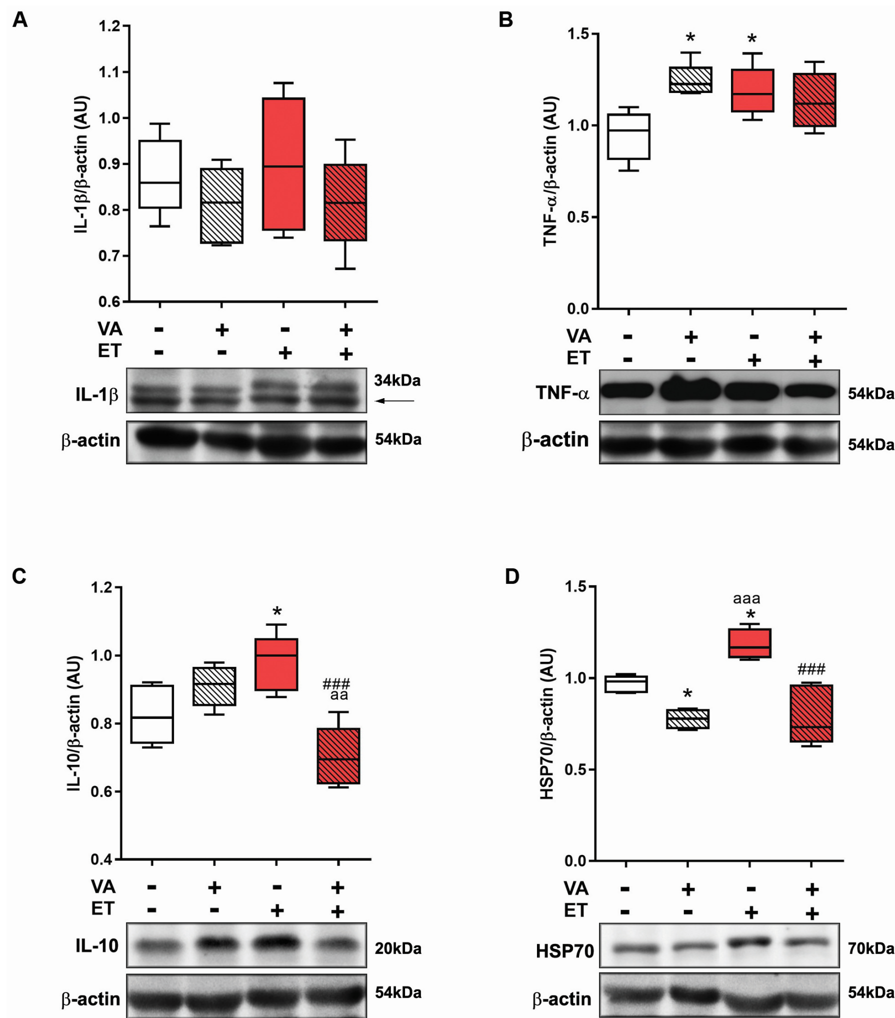

Figure 6. Effects of exercise and vitamin A supplementation on skeletal muscle inflammation marker content. Data presented as box (median) and whiskers (interquartile interval) diagram (n = 6). (**A**) Interleukin-1β; (**B**) Tumor necrosis factor-α; (**C**) Interleukin-10; and (**D**) Heat shock protein 70 content. * $p < 0.05$ significant difference from SE group; ^{aa} $p < 0.01$; ^{aaa} $p < 0.001$ significant difference from SE + VA group; $^{###}$ $p < 0.001$ significant difference from ET group using one-way ANOVA followed by Tukey's post hoc test. Representative Western blots are shown.

4. Discussion

Exercised animals exhibited a plateau in body weight gain, without influence from VA supplementation. This result is corroborated in the literature, as swimming exercise training with overload stabilizes weight gain in rats [35]. To assess tissue damage in serum, we measured the enzymatic activity of CK and LDH. When exercise intensity surpasses the capacity of muscle cell metabolism, membrane permeability increases and enzymes present in the cytosol leak into the extracellular environment [48]. In our study, the levels of LDH increased in both exercised groups, with no difference between them (Table 2). Regarding CK release, no differences between groups were detected. One explanation for this result is the timing of sample collection; the literature reports that, even with intense exercise, high levels of CK may not be detected when sample collection occurs after 24 h following the last training session [49]. TRAP serum results revealed that the ET group had higher antioxidant capacity compared to both sedentary groups, increasing more than twofold (Figure 1A), which agrees with the literature [50]. On the other hand, although the ET + VA group also showed a difference from SE, its antioxidant capacity was significantly lower compared to the

ET group, indicating that VA impaired the total antioxidant capacity improvement resultant from the exercise itself. Cooperation among antioxidants in the blood circulation is conducted through redox reactions—for example, the ability of erythrocytes to regenerate ascorbic acid to ascorbate through ferricyanide reduction [51]. As the chemical structure of vitamin A is known to have an effect on redox reactions [24], it may have acted in a non-beneficial direction, reducing the total antioxidant capacity acquired with exercise training.

The exact profile of cytokines released in response to exercise depends on the particular aspects of training, such as type, intensity, and duration [13]; nutritional issues [14,52]; and blood flow [53]. Results from this study showed increased levels of IL-1β in both exercise groups (ET and ET + VA) and increased TNF-α in all groups when compared to SE (Figure 2A,B). IL-1β and TNF-α are substantially present after long endurance bouts of exercise [53], as was applied in this study. The increase in TNF-α (Figure 2B) and the decrease in anti-inflammatory IL-10 (Figure 2C) observed in the SE + VA group indicates that vitamin A alone affected the immune response. Cytokines work synergistically to regulate the inflammatory cascade, and these results suggest that vitamin A by itself held up the basal inflammatory response in sedentary animals. Modulation of levels of circulatory cytokines by exercise or diet supplementation may take place due to changes in the inflammatory state of a variety of tissues, including adipose and liver tissues [3]. Here, we observed that muscle cytokine levels varied in response to exercise and VA supplementation, suggesting that the modulation of circulatory cytokines is influenced by cytokine production in muscle. This is further discussed below.

Serum findings indicated that vitamin A did not have any protective or beneficial effects during or following exercise; taking this into consideration, we decided to evaluate the skeletal muscle, the tissue most under demand and affected by exercise training. For oxidative stress analysis, TBARS, carbonylation, and sulfhydryl residues were analyzed (Figure 3) [9]. The ET + VA group displayed significant increased lipid and protein damage, which did not happen in the ET group. Previous studies investigating the effects of supplementation on exercise showed no effect of vitamins in preventing oxidative damage [16,17]. Vitamin A, by its structure and potential free radical quenching action, apparently induced more oxidative stress in the skeletal muscle, leading to tissue damage. Regarding proteins that were oxidatively modified, the ET group exhibited a significant decrease in total thiol content, likely indicating elevated levels of glutathione disulfide (GSSG). GSSG is often employed as a sign of system's response to oxidative stress, as its detection indicates that GSH groups are being actively involved in redox reactions [54]. Moreover, when tissue goes through intense oxidative stress, as provided by high-intensity exercise training, depletion of GSH within the cell is commonly observed [55]. Indeed, GPx activity increased in the ET group (Figure 4C). The ET + VA group showed higher levels of total thiol content, although TRAP assay results indicate that this group had lower serum antioxidant capacity. However, TRAP evaluates total antioxidant capacity, which, in the serum, is not exclusively comprised of thiols, but also phenols, ascorbic acid, and uric acid, among others [37]. Activity of the antioxidant enzymes SOD and CAT was also measured in skeletal muscle (Figure 4A,B), and activity did not show any difference between SE and exercised groups. In another study, a moderate swimming exercise protocol also displayed no difference in SOD activity in the skeletal muscle [56]. High-intensity exercise, like the activity performed in our study, induced no difference in aorta CAT activity, although SOD activity was greater in the exercised group [50]. Furthermore, in this study, tissue collection was performed 24 h after the last bout of exercise training. Some studies collect samples up to 2 h after the last bout, when the antioxidant system is working at its maximum and differences in enzymatic activity can be easily detected [57].

In order to clarify whether upregulation of endogenous antioxidant enzymes did occur, we performed Western blotting for SOD1 (Cu-ZnSOD), the isoform localized on cell cytosol; SOD2 (MnSOD), the isoform localized inside cell mitochondria [58,59]; and CAT within skeletal muscle (Figure 5). The expression of SOD1 did not change with exercise only; however, the ET + VA group presented a lower content of SOD1, which may be behind the elevated levels of oxidative tissue damage seen in this group. SOD2 content increased in the ET group, with a significant difference compared

to SE and ET + VA groups. Studies with animals exposed to chronic exercise training showed an upregulation of mitochondrial SOD and GPx when compared to sedentary animals, thus presenting lower oxidative stress [55]. In this study, SOD2 content was higher only in the ET group. This may be one explanation for the lack of enhanced SOD activity, as specifically in this tissue, SOD2 only contributes 15%–35% of all SOD cell content [60]. CAT expression was lower in both exercise groups compared to the SE group, justifying the lower enzyme activity results. Literature findings regarding skeletal muscle CAT are controversial, as there is no consensus on the true effect of chronic exercise training. Some studies reported increased [61], decreased [6,62], or unchanged [63] CAT activity in response to exercise. Furthermore, ET and ET + VA groups were not different regarding CAT activity or expression, indicating no effect of vitamin A on the response of CAT to chronic exercise training. Studies addressing the effects of vitamin A supplementation and exercise training are rare, although VA is frequently associated with oxidative stress prevention. A recent study from 2016 evaluated the effects of four weeks of VA supplementation and changes in circulatory redox parameters in healthy young male subjects [64]. Using a daily dose of 30,000 RE (considered very high), parameters including lipid peroxidation, NO production, GSH levels, and antioxidant enzyme activity showed no difference from non-supplemented subjects. However, this study was conducted with sedentary individuals, and the training protocol was performed once a week over four weeks, using a protocol of exhaustion, different from a daily 60 min moderate to high-intensity exercise such as swimming training. Our study shows that VA supplementation causes oxidative stress in trained animals, and human studies using the same models will clarify the reproducibility of these results. Besides this study, no other works on the effects of VA on exercise training have been performed so far.

Regular physical activity combined with a healthy diet is known to maintain a tissue anti-inflammatory phenotype [3]. Cytokines IL-1β, IL-10, and TNF-α are expressed in skeletal muscle and are increased upon exercise training; furthermore, both interleukins respond to a TNF-α stimulus [65]. While IL-10 acts as a highly effective anti-inflammatory agent, inhibiting the expression of pro-inflammation mediators [3], IL-1β induces pro-inflammatory events, and is related to pain susceptibility [66]. TNF-α was increased in the skeletal muscles of rats in the SE + VA and ET groups; however, this did not affect IL-1β protein content in any group (Figure 6). Interestingly, IL-10 levels increased only in the ET group, with a statistical difference from SE + VA and ET + VA. Exercise training may raise the levels of TNF-α, but adaptive responses, such as a greater expression of anti-inflammatory cytokines, also occur. This was not true in the ET + VA group, indicating that VA impaired this beneficial aspect of exercise training. In exercise training studies, it is usual to measure circulatory markers for stress, but the circulatory profile may be influenced by tissues other than muscles, which could complicate the interpretation of the results. One example is adipose tissue, which is very important when it comes to exercise training [67]. Obesity leads to an increase in circulatory pro-inflammatory cytokines by stimulating a pro-inflammatory state in adipose tissue, and healthy habits tend to prevent this by inhibiting the inflammation in this tissue [3]. The circulatory cytokine levels may indicate that inflammation is occurring in some tissues, and analyzing cytokine levels in specific tissues helps to clarify their origin.

Finally, Western blotting for HSP70 revealed differences between all groups, with upregulation of protein expression in the ET group and downregulation in vitamin A groups SE + VA and ET + VA. HSP70 is one component of a stress protein family that has increased expression as a cellular defense strategy. The literature describes that basal HSP70 expression occurs in athletes as well as healthy subjects, with no difference in quantity between type I and type II muscle fibers [68]. During exercise training, the cell environment undergoes changes in homeostasis, such as redox imbalance, high temperature, hypoxia, and glucose depletion. This kind of stress enhances tissue HSP70 levels, providing a cytoprotective effect [69] that includes prevention of oxidative damage and repair of proteins damaged by muscle contractions [70]. Our study revealed that vitamin A supplementation induced tissue oxidative damage and downregulation of endogenous antioxidant defenses in skeletal muscle of trained rats, which is likely to be related to suppression of HSP70 expression. Furthermore,

VA combined with chronic exercise training inhibited the increase of IL-10 in skeletal muscle levels, blunting the anti-inflammatory cytokine response caused by exercise. Other studies have described that antioxidant supplementation impairs HSP70 synthesis induced by exercise [71], which leads us to believe that redox-dependent mechanisms are responsible for HSP70 downregulation, since VA is also considered an antioxidant molecule. The VA dose utilized in this study was based on the daily recommendation for human adult daily ingestion and considered the fact that the standard laboratory food provided to the animals contained a mix of vitamins including VA at a dose to fulfill the daily requirement for this vitamin. Higher or lower doses may show different effects than the ones presented here. Higher doses would probably provoke more tissue damage and inflammation as observed previously [26–28], and lower doses may show no effect at all, as many of the effects observed here could be considered mild. Exercise training itself is already a major source of ROS production, hence higher doses of VA were not considered for this study, as a synergistic pro-oxidant effect could take place, since excessive doses cause oxidative stress. The combination of VA intake from supplementation and food applied here is very likely to exceed the daily recommended amount, as often occurs with regular intake of diet supplements, but does not characterize hypervitaminosis, since it is below the tolerable upper intake level of 3000 RE/day for VA and acute toxic effects were not observed.

5. Conclusions

In conclusion, our results show that, despite its antioxidant status, vitamin A supplementation induces the release of stress markers, redox imbalance in serum, tissue damage, impaired antioxidant capacity, and inflammation in the skeletal muscle, probably due to inhibition of HSP70 expression in trained Wistar rats.

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CAPÍTULO II

Role of vitamin A oral supplementation on oxidative stress and inflammatory response in the liver of trained rats

Artigo em *major revisions* no periódico *Applied Physiology, Nutrition and Metabolism*

1 **Role of vitamin A oral supplementation on oxidative stress and inflammatory**

2 **response in the liver of trained rats**

3

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ABSTRACT

The use of dietary supplements to enhance the benefit of exercise training is a common practice. The liver is the organ where all substances are metabolized, and certain supplements have been associated with liver injury. Vitamin A (VA), a liposoluble vitamin stored in the liver, is commonly used as an antioxidant supplement. Here, we evaluated the effect of chronic VA supplementation on oxidative damage and stress parameters in trained rats. Animals were divided into the following groups: sedentary (SE), sedentary/VA (SE+VA), exercise training (ET), and exercise training/VA (ET+VA). During 8 weeks, animals were subjected to swimming (0, 2, 4, 6% body weight) 5 days/week and a VA daily intake of 450 retinol equivalents/day. Parameters were evaluated by enzymatic activity analysis, ELISA, and western blotting. VA caused liver lipid peroxidation and protein damage in exercised rats, and inhibited the increase in HSP70 expression acquired with exercise alone. ET group showed higher levels of antioxidant enzyme activity, and VA inhibited this adaptation. Expression of the pro-inflammatory cytokines, interleukin (IL)-1β and tumor necrosis factor-α, was reduced in the ET+VA group, whereasile the anti-inflammatory cytokine, IL-10, was increased. Western blotting showed that both exercised groups had lower levels of the receptor for advanced glycation end products (RAGE), suggesting that VA did not affect this receptor. Our study demonstrated that although VA influences some redox parameters, it might exert a protective effect on the production of pro-inflammatory mediators in the liver, suggesting that controlled administration of VA during some types of exercise may be beneficial.

Keywords: antioxidant enzymes; exercise; cytokines; liver; reactive oxygen species; vitamin A; western blotting; Wistar rat model

Introduction

Regular physical activity is recommended for the prevention and treatment of metabolic disorders such as metabolic syndrome, characterized by high blood pressure and insulin resistance (Neufer et al. 2015). In addition, physical activity helps maintain an anti-inflammatory state, characterized by small adipocyte size and the presence of anti-inflammatory cell types such as regulatory T cells and M2-type macrophages (Gleeson et al. 2011). Paradoxically, it is also clear that repetitive contraction of skeletal muscle during exercise training (ET) leads to the production of reactive oxygen species (ROS), high concentrations of which can cause oxidative stress and tissue damage (Chance et al. 1979). During exercise, whole body oxygen consumption can increase 10–15-fold, and can reach up to 100 fold in activated muscles (Sen 1995). Oxidative stress is characterized by the disruption of redox signaling control within the cell (Jones 2006), which can have an impact on several cellular functions such as cell differentiation, proliferation, migration, quiescence, and death (Sarsour et al. 2009; Kunzler et al. 2016). Although skeletal muscle is the most employed tissue during exercise, the function of different organs, such as the stomach, heart, brain, and liver, is also affected (Cakir et al. 2010).

The liver is one of the most metabolically challenged organs during ET, mainly owing to its key role in the removal of lactate from circulation for gluconeogenesis and maintenance of blood glucose levels (Brooks 1986; Coker et al. 2005). The liver is a major regulator of energy metabolism at a systemic level, and maintaining its function is crucial to sustain the performance of other organs and tissues during ET. Acute or chronic exercise can affect liver function. An acute bout of exercise can increase hepatic

73 protein synthesis without changes in fat content, reduce hepatic blood flow, and cause a
74 significant imbalance in ROS production (Shephard et al. 2015). Furthermore, in
75 streptozotocin-induced diabetic rats, chronic ET has been shown to prevent the
76 impairment of hepatic redox defenses, such as decreased expression of liver antioxidant
77 enzymes and increased ROS levels (Lima et al. 2015). Although consumption of a
78 weight-loss diet is still the most effective treatment for non-alcoholic fatty liver disease,
79 exercise alone has been shown to be highly beneficial in treating this disease. ET
80 reduces insulin resistance and increases the expression of genes responsible for fatty
81 acid metabolism in this condition (Oh et al. 2014; Ordonez et al. 2015). The redox
82 imbalance caused by exercise may lead to inflammation, wherein increased ROS levels
83 are related to inflammatory processes (Kosmidou et al. 2002). Regular exercise can
84 decrease the levels of inflammatory cytokines, adipokines, and other injury-related
85 markers in the liver (Gleeson 2007). These anti-inflammatory effects appear to occur
86 due to three main factors: reduced visceral fat, increased production and release of anti-
87 inflammatory cytokines such as interleukin (IL)-10, and decreased expression of toll-
88 like receptors in immune cells (Gleeson, Bishop et al. 2011).

89 Dietary supplementation during ET is a common practice to enhance
90 performance or to prevent/treat diseases. However, the effects of different combinations
91 of dietary supplements and regular ET are unknown. Several natural supplements have
92 been associated with liver injury, such as green tea extract and other herbal
93 preparations, usnic acid, and vitamin A (VA) (Garcia-Cortes et al. 2016). VA is a fat-
94 soluble vitamin required for many key biological processes, including visual cycle,
95 embryonic development, gene transcription, and immune responses (Chapman 2012). It
96 can be found in the form of all-*trans* retinol and retinyl esters (in foods from animal
97 sources) and in the form of pro-vitamin A carotenoids, such as β-carotene (in foods

98 from vegetable sources) (Blomhoff et al. 2006). The liver is one of the main sites of VA
99 metabolism and storage, and both the hepatic cells, parenchymal (hepatocytes) and
100 stellate cells, participate in these processes. The role of these two cell types is different
101 during VA metabolism; while the hepatocytes are responsible for the uptake and
102 processing of VA, the stellate cells store the retinoids in the liver (D'Ambrosio et al.
103 2011). However, oral intake or administration of therapeutic doses of VA may induce
104 hepatotoxicity through hypervitaminosis (Geubel et al. 1991).

105 VA is an essential micronutrient that has its metabolism and storage occurring in
106 the liver, and hence, it is important to study the effects of VA supplementation on the
107 modulation of oxidative stress and inflammatory parameters in the liver. However,
108 studies evaluating the combined effects of VA supplementation and ET are lacking.
109 Here, we evaluated the effects of chronic (8 weeks) VA supplementation and intense
110 swimming ET on parameters, such as ROS-mediated damage, antioxidant defense, cell
111 stress, and pro-inflammatory modulation in rats.

112

113 Materials and Methods

114 Ethics

115 All experimental assays in this work were conducted in accordance with the National
116 Institute of Health Guide for the Care and Use of Laboratory Animals (2011) and
117 followed the guidelines of the Brazilian Society of Animal Science Experimentation
118 (SBCAL). Before beginning the research project, it was approved by the Ethical
119 Committee for Animal Experimentation of the Federal University of Rio Grande do Sul
120 (CEUA-UFRGS) under the accession number 25837.

121

122 **Animals**

123 Male Wistar rats (7-week-old; weight: 250–300 g) were obtained from our own
124 breeding colony at the Federal University of Rio Grande do Sul and kept in plastic
125 cages, with a maximum of 4 animals per cage. Animals were maintained in a room with
126 an ambient temperature of 23 ± 1 ºC and 12 h light-dark cycle (7am-7pm), with *ad*
127 *libitum* access to food and water.

128

129 **Study design**

130 Animals were randomized into four groups: sedentary (SE), sedentary supplemented
131 with VA (SE+VA), exercise training (ET), and exercise training supplemented with VA
132 (ET+VA). Throughout the 8 weeks, the exercised and sedentary groups were
133 administered 450 retinol equivalents (RE) (1500 IU)/kg/day of retinyl palmitate
134 (Arovit®; Bayer, Rio de Janeiro, RJ, Brazil) or its vehicle (saline) daily by intragastric
135 gavage. The chosen dose of 450 RE was calculated based on the human equivalent dose
136 (HED), using the dose-by-factor approach (Nair et al. 2016). The exercised groups were
137 subjected to a protocol of 60 min of swimming 5 days/week, while the sedentary groups
138 remained in shallow water for 20 min 5 days/week. ET and supplementation protocols
139 were performed between 6-8 pm.

140

141 **Exercise training protocol**

142 The training protocol lasted 8 weeks in total. It was conducted between 6 and 8 pm, in a
143 swimming tank for rodents with water at 31 ± 1 ºC. During the first week, all animals
144 remained in shallow water for 20–60 min each day. For the next 2 weeks, training
145 started with 10 min/day and progressed to 60 min/day. Over the following 5 weeks,

146 training consisted of 60 min/day, 5 days/week, with an overload attached to the animal's
147 torso, progressing each week (0, 2, 4, 6% body weight) (Gobatto et al. 2001). Animals
148 were weighed once a week and the values were utilized to calculate the overload. After
149 each session, animals were towel-dried and returned to their cages.

150

151 **Tissue sampling**

152 After a 24 h interval from the last exercise bout and VA supplementation, animals were
153 euthanized by decapitation, and their blood and liver tissue samples were collected.
154 Blood samples were centrifuged at 1500 × *g* for 10 min for serum isolation. Tissue
155 samples were homogenized in phosphate buffer (PB) and centrifuged (3000 × *g*, 10
156 min), and sample supernatants were used for analysis. Protein content was quantified by
157 the Lowry method (Lowry et al. 1951) using bovine serum albumin as standard. For
158 western blotting, the tissue was homogenized in RIPA buffer (20 mM Tris-HCl pH 7.5;
159 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 2.5 mM sodium pyrophosphate; 1%
160 sodium deoxycholate; 1% Tergitol-type NP-40; 1 mM β-glycerophosphate; 1 mM
161 sodium orthovanadate; 1 µg/mL leupeptin) and centrifuged, following which the
162 homogenate was added to Laemmli-buffer (62.5 mM Tris-HCl pH 6.8; 1% SDS; 10%
163 glycerol) with 10% β-mercaptoethanol.

164

165 **Plasma assays**

166 **Biochemical parameters**

167 Serum levels of aspartate transaminase (AST) and alanine transaminase (ALT) were
168 evaluated using standard commercially available biological kits following the
169 manufacturer's instructions (Labtest, São Paulo, Brazil).

170

171 **Liver homogenate assays**

172 **Redox parameters**

173 Thiobarbituric acid reactive species (TBARS) levels in the liver samples were
174 quantified as an index of lipid peroxidation (Draper et al. 1990). First, samples were
175 deproteinized by 10% trichloroacetic acid (TCA), followed by heating at 100 ºC for 25
176 min with 0.67% thiobarbituric acid. TBARS levels were then quantified
177 spectrophotometrically at a wavelength of 532 nm. Oxidative damage to proteins was
178 quantified by detection of carbonyl groups (Levine et al. 1990), which involved
179 incubation of sample proteins, previously precipitated with 20% TCA, with 2,4-
180 dinitrophenylhydrazine (DNPH), followed by spectrophotometric quantification at 370
181 nm. Nitrotyrosine content was detected by an indirect enzyme-linked immunosorbent
182 assay (ELISA) using a polyclonal antibody (Abcam, Cambridge, UK). Quantification of
183 antioxidant enzyme activity was performed through kinetic spectrophotometric assays.
184 Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined as the inhibition of
185 superoxide anion-dependent adrenaline auto-oxidation in the presence of the liver
186 sample at 480 nm (Misra et al. 1972). Catalase (CAT; EC 1.11.1.6) activity was
187 measured as the decrease in hydrogen peroxide (H_2O_2) absorbance in the presence of
188 the liver sample at 240 nm (Aebi 1984).

189

190 **ELISA**

191 Indirect ELISA assay was performed using antibodies to detect the pro-inflammatory
192 cytokines [IL-1β, tumor necrosis factor-α (TNF-α), and IL-6], the anti-inflammatory
193 cytokine (IL-10), the advanced glycation end products (AGEs), 4-hydroxynonenal (4-

194 HNE), and carboxymethyl lysine (all the six antibodies were purchased from Abcam,
195 Cambridge, UK). The liver tissue samples homogenized in PB were placed in a specific
196 round-bottom plate and left overnight for sample adherence. They were then incubated
197 overnight with primary antibody in a 1:1000 dilution range, followed by incubation with
198 secondary antibody for 2 h in a 1:2000 dilution range. Between every step, the plate was
199 washed three times with PB in 0.05% Tween-20, and all incubations were conducted at
200 4 ºC under constant agitation (45 rpm). Immunoreactivity was detected with a
201 colorimetric assay using the TMB Chromogen solution for ELISA (Thermo Fisher
202 Scientific, Rockford, USA).
203
204 **Western blotting**
205 After subjecting the liver samples (20 μg) homogenized in RIPA buffer with 10% β-
206 mercaptoethanol to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-
207 PAGE), transference was achieved by a semi-dry transfer of proteins to a nitrocellulose
208 membrane (Millipore, Bedford). Protein content was then determined by Ponceau S
209 staining. Membranes containing the sample proteins were washed using Tris-Tween-
210 Buffer-Saline (TTBS - 100 mM Tris, pH 7.5; 0.9% NaCl; 0.1% Tween-20) for posterior
211 blocking with 5% non-fat dry milk for 1 h (room temperature). After TTBS washes,
212 membranes were incubated with primary antibodies (1:1000 dilution) against HSP70
213 (Cell Signaling Technology, Beverly, USA) and the receptor for AGEs (RAGE) and β-
214 actin (Sigma Chemical, St. Louis, USA) for 2 h at room temperature, followed by
215 incubation with anti-rabbit/mouse horseradish peroxidase-linked secondary antibodies
216 (1:2000 dilution; Cell Signaling Technology, Beverly, USA) for 1 h at room
217 temperature. Immunoreactivity was detected through chemiluminescence using the
218 SuperSignal West Pico Chemiluminescent kit (Thermo Scientific, Rockford, USA).

219 Densitometry analysis was conducted with ImageJ software, and results were expressed
220 as the ratio of target protein/β-actin.

221

222 **Statistical analysis**

223 Statistical analysis was performed by analysis of variance (ANOVA) followed by
224 Bonferroni's test for average comparison, using GraphPad Prism version 5.0 (GraphPad
225 Software Inc, San Diego, USA). The data are expressed as mean ± standard error of
226 mean (SEM) and values were considered significant at $p < 0.05$.

227

228 **Results**

229 **Serum ALT and AST levels**

230 ALT and AST are enzymes expressed in the liver, and their serum activities indicate the
231 degree of liver tissue damage. ALT activity (Fig. 1A) was significantly enhanced in
232 both ET and ET+VA groups, compared to that in both the sedentary groups. AST (Fig.
233 1B) had lower activity in both the exercised groups compared to that in the SE group.
234 These results showed that ET and VA supplementation both have an impact on liver
235 function.

236

237 **ROS-mediated damage and stress parameters**

238 We analyzed the oxidative damage biomarkers and antioxidant enzyme activities in the
239 liver tissue. The ET+VA group showed increased lipoperoxidation compared to other
240 groups (Fig. 2A). This effect was confirmed by the assessment of levels of 4-HNE, an
241 end product of lipid peroxidation chain reaction (Fig. 2B). Analysis of oxidative protein

242 damage showed decreased carbonyl formation in the SE+VA group compared to that in
243 the SE group; however, carbonyl formation in the other groups was not significantly
244 affected (Fig. 2C). Nitrotyrosine accumulation, on the other hand, was increased in the
245 ET group, and VA supplementation did not change this effect (Fig. 2D). AGE
246 formation, which is increased in several conditions related to oxidative/nitrosative stress
247 and metabolic dysfunction (such as diabetes), was decreased by VA supplementation
248 itself and in ET and ET+VA groups (Fig. 2E). Finally, the levels of the chaperone
249 protein, HSP70, which is induced under conditions of cellular stress, were evaluated by
250 western blotting. ET enhanced HSP70 levels in the liver, but VA supplementation
251 inhibited this effect (Fig. 2F).

252

253 **Antioxidant enzyme activities**

254 The activities of the antioxidant enzymes, SOD and CAT, were enhanced in response to
255 activation of endogenous ROS production. SOD (Fig. 3A) and CAT (Fig. 3B) activities
256 were both increased in the ET group; however, VA supplementation inhibited this
257 effect.

258

259 **Modulation of inflammatory parameters**

260 Next, we measured cytokine levels to evaluate the effect of VA supplementation on the
261 pro-inflammatory stimulation caused by ET. IL-1β levels were increased in the ET
262 group, and VA supplementation inhibited this effect (Fig. 4A). Basal levels of liver
263 TNF-α were unaffected in the ET group; however, the ET+VA group presented
264 decreased TNF-α levels compared to all other groups (Fig. 4B). The pro-inflammatory
265 cytokine, IL-6, is also an anti-inflammatory myokine, which is generally stimulated

266 during muscle contraction and ET. Levels of IL-6 in the liver were increased in the
267 SE+VA and ET groups, compared to SE group; however, this effect was inhibited in the
268 ET+VA group (Fig. 4C). The levels of the anti-inflammatory cytokine, IL-10, were
269 enhanced in the ET group as compared with that in the SE group, and the combination
270 of ET and VA further stimulated this effect (Fig. 4D). Finally, since increased RAGE
271 levels have been associated with chronic pro-inflammatory conditions (Wautier et al.
272 2016), their levels were evaluated. Although RAGE levels were increased by VA
273 supplementation alone, the ET and ET+VA groups demonstrated a significant decrease
274 in RAGE levels compared to the SE and SE+VA groups, respectively (Fig. 4E).

275

276 **Discussion**

277 While ET increased ALT and decreased AST levels, VA supplementation did not have
278 an influence on these parameters. ALT and AST are well-known serum markers used to
279 assess and monitor liver damage. In athletes, however, these enzymes may originate
280 from different tissues; while ALT comes mainly from the liver, AST can be linked to
281 muscle cell leakage (Banfi et al. 2012). Indeed, increased levels of ALT and AST are a
282 common response to intense ET (Kayatekin et al. 2002; Koury et al. 2016).

283 It is well established that vigorous exercise increases ROS production and
284 antioxidant activity due to the enhanced oxygen demands of the tissue and considerable
285 increase in blood flow (Finaud et al. 2006). As a consequence, oxidative damage
286 resulting from long-term ET leads to an antioxidant adaptation (Leeuwenburgh et al.
287 1994; Abruzzo et al. 2013; Radak et al. 2013). The liver response to exercise may vary,
288 training intensity being the major factor that determines oxidative damage and
289 antioxidant adaptation. VA presents variable redox-active properties in biological

290 systems, and usually, its effects are related to prevention of oxidative damage
291 (Ozhogina et al. 1995; Powers et al. 1999; Schroder et al. 2001). However, as a
292 lipophilic compound, VA can easily interact with membrane lipids, and the conjugated
293 double bonds present in its structure can facilitate the formation of conjugated dienes
294 during lipid peroxidation chain reactions, if concentrations are high (Halliwell 2006). In
295 our study, the overall effect of VA on tissue redox activity and antioxidant defense
296 varied according to the properties of each tissue fraction. Animals subjected to intense
297 swimming ET showed basal levels of lipid peroxidation products (TBARS and 4-HNE)
298 and protein carbonyls, but VA supplementation increased lipid damage. On the other
299 hand, nitrotyrosine formation, a hallmark of peroxynitrite-mediated protein damage,
300 was increased in the ET group, and VA supplementation could not inhibit this effect.
301 These results may be explained by the differential effects of ET and VA on ROS
302 production in different cell compartments. The effect of VA supplementation on
303 antioxidant enzyme activity supportss this hypothesis.

304 The levels of both the antioxidant enzymes, SOD and CAT, were increased in
305 the ET group, which is in accordance with the positive adaptations that occur as a
306 consequence of ET. SOD and CAT activities are stimulated by increased substrate
307 availability. Hence, when superoxide and hydrogen peroxide production are stimulated,
308 their activities are increased. SOD is present in the mitochondria and the cytosol, and its
309 activity is stimulated by superoxide production resulting from an increased demand for
310 mitochondrial activity during intense exercise (Finaud, Lac et al. 2006; Myburgh 2014).
311 The effects of chronic ET in the liver includes increased SOD activity and reduced lipid
312 damage (da Silva et al. 2009), improved activity of the antioxidant enzyme glutathione
313 peroxidase (Barcelos et al. 2014), and increased mitochondrial biogenesis and citrate
314 synthase activity (Santos-Alves et al. 2015), all of which contribute to adaptation to

315 increased ROS production and elevated aerobic demand for ATP synthesis. Increased
316 reactive species production and redox imbalance result in upregulation of tissue defense,
317 in order to cope with the adverse conditions (Banerjee et al. 2003). Supplementation
318 with VA blocked the activation of SOD and CAT caused by ET, indicating that VA
319 inhibited reactive species production, and thus SOD and CAT activities decreased due
320 to reduced substrate availability. VA (β-carotene) has previously been shown to have
321 scavenging activity on several ROS, such as superoxide and peroxyl radicals (Yu 1994),
322 which could have caused the decreased SOD activity in the ET+VA group. However,
323 increased lipid damage and nitrotyrosine levels in the ET+VA group indicate that VA
324 modulates SOD and CAT activity, but does not inhibit reactive species production.

325 ET is often associated with the prevention and treatment of lifestyle-related
326 diseases (Neufer, Bamman et al. 2015). This includes liver diseases; fat accumulation in
327 the liver can lead to non-alcoholic fatty liver disease, and previous studies have reported
328 that chronic aerobic exercise reduces this effect (Batatinha et al. 2016). Inflammation
329 plays a key role in the development of this adverse liver condition (Nov et al. 2013).
330 After intense ET, the levels of inflammatory cytokines rise significantly, and this
331 response is often related to reactive species overload (Kosmidou, Vassilakopoulos et al.
332 2002). This effect may be followed by a compensatory response to increased production
333 of anti-inflammatory mediators and activation of antioxidant enzymes. Here, we
334 observed that VA supplementation combined with ET reduced the tissue levels of the
335 pro-inflammatory cytokines, TNF-α and IL-1β. It has been shown that ET does not
336 affect the TNF-α expression levels in the liver, which is consistent with our results (E et
337 al. 2013). One transcription factor that is associated with oxidative stress and
338 inflammation is NF-κB (nuclear factor kappa-B), and it is also responsible for the
339 regulation of TNF-α and IL-1β expression (Barnes et al. 1997). It has already been

340 described that retinoic acid, the most active metabolic form of VA, can disrupt the
341 nuclear translocation of NF-κB under inflammatory situations (Wang et al. 2015).
342 Indeed, the NF-κB pathway is among the most relevant signaling pathways in liver
343 inflammation (He et al. 2011). Therefore, VA supplementation may inhibit the
344 activation of NF-κB, resulting in blunted pro-inflammatory cytokine release in response
345 to intense exercise. Furthermore, the increase in IL-1β and IL-6 levels caused by ET or
346 VA alone, were inhibited when combined together. Similar effects have been observed
347 in rats supplemented with caffeine and subjected to swimming ET (Cechella et al.
348 2014). IL-6 is considered a myokine and promotes anti-inflammatory actions in the
349 muscle, as opposed to its pro-inflammatory actions in the liver and other tissues (Ost et
350 al. 2016). In muscle cells, IL-6 is responsible for increasing fat oxidation and
351 stimulating glucose uptake by insulin; besides, it has been shown that IL-6 deficient
352 mice have reduced capacity to regenerate their livers (El-Kadre et al. 2013). It is
353 possible that ET enhances IL-6 levels in the liver as well as other tissues, where it
354 promotes the inflammatory responses. Furthermore, the effect of ET on IL-10, a very
355 effective anti-inflammatory cytokine, indicates that the pro-inflammatory effect of
356 exercise on the liver is accompanied by the activation of anti-inflammatory response,
357 and in combination with VA supplementation, this effect is further enhanced. We also
358 evaluated the levels of RAGE, a multi-ligand receptor associated with inflammation in
359 chronic diseases (Bohlooli et al. 2014; Schmidt 2015). Interestingly, SE+VA group
360 showed enhanced RAGE levels, but both the ET and ET+VA groups showed decreased
361 levels of RAGE. This is in agreement with the decrease in the levels of carboxymethyl
362 lysine, a key AGE that arise from non-enzymatic oxidative reactions between
363 carbohydrates and proteins (Gaens et al. 2014), observed in all groups, as the regulation
364 of this receptor depends on ligand availability. Thus, considering these effects, ET

365 combined with VA supplementation induces an overall protective effect on pro-
366 inflammatory activation induced by ET.

367 It is known that physical exercise increases the expression of proteins from the
368 heat-shock family (Qu et al. 2015). Such a response generally protects cells from stress
369 conditions caused by ET, such as redox imbalance, elevated body temperature, hypoxia,
370 and glucose depletion (Krause et al. 2015). In the liver, a single bout of exercise is
371 capable of enhancing the synthesis of HSP70 family proteins by 2-fold (Gonzalez et al.
372 2004). Our ET protocol significantly increased liver HSP70 expression. A number of
373 liver diseases show impaired HSP70 expression due to a decrease in heat-shock
374 transcription factor-1 (Qu, Jia et al. 2015), suggesting that ET may contribute to
375 restoring basal levels of this protein. VA supplementation hindered the effect of ET on
376 HSP70 levels, which may explain why the increase in nitrotyrosine by ET was not
377 inhibited by VA supplementation, as HSP70 normally acts to prevent protein damage
378 (Banerjee, Mandal et al. 2003). Besides, as VA supplementation blocks the SOD and
379 CAT activation caused by ET, the antioxidant response against ROS is impaired, which
380 is in agreement with the increase in lipid damage and nitrotyrosine formation. In this
381 context, despite the protective effect of inflammatory mediators, VA supplementation
382 seems to impair antioxidant defense in the liver, thus contributing to the increase in
383 oxidative lipid and protein damage during exercise.

384 The dose of choice for VA treatment in this study was based on the daily
385 recommended value of 800 RE for human adults (Institute of Medicine Panel on 2001)
386 applying the HED, using the dose-by-factor approach (Nair and Jacob 2016). The food
387 provided to the animals already contains VA at a dose that fulfills their daily
388 recommendation; therefore, combination with daily gavage supplementation is very

389 likely to extrapolate the daily requirements for this vitamin. VA influences several
390 metabolic processes (Chapman 2012), and its deficiency or excess may show very
391 different effects. As described in the literature, higher doses would probably increase
392 tissue damage (Pasquali et al. 2009; Gasparotto et al. 2015; Schnorr et al. 2015; Petiz et
393 al. 2017), and lower doses may show no effect at all. Both VA and ET are potentially
394 pro-oxidant to tissues when applied in excess, and we wanted to avoid potential harmful
395 effects by using higher doses of this vitamin. Nonetheless, the dose of 450 RE is below
396 the tolerable VA daily upper intake level of 3000 RE, and the effects observed here
397 could be considered mild.

398

399 **Conclusion**

400 VA, which has antioxidant properties and is often consumed in supplements, increased
401 oxidative damage in the rat liver tissue. However, considering the lack of pro-
402 inflammatory markers, a dose of 450 RE did not appear to cause liver injury. This
403 suggests that controlled administration of VA for some types of exercise may be
404 beneficial, as it appears to reverse the release of pro-inflammatory mediators.
405 Monitoring of oxidative stress markers during VA supplementation is recommended.

406

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416

417 **Conflict of Interest statement**

418 The authors declare that there are no conflict of interest regarding this study.

419

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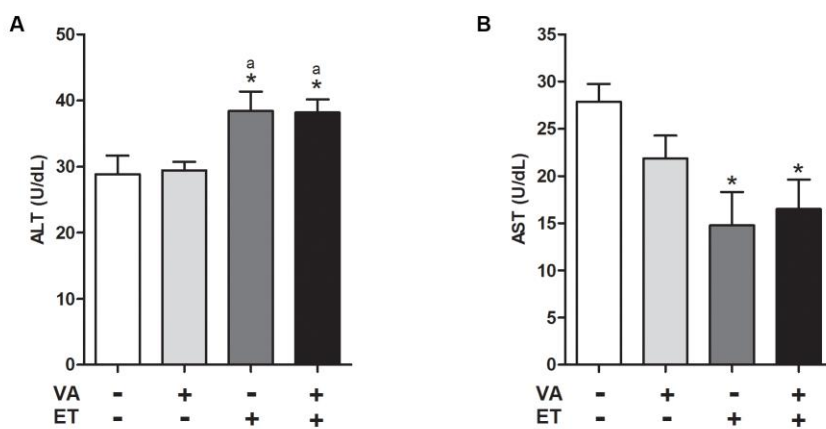
618 **Figures**



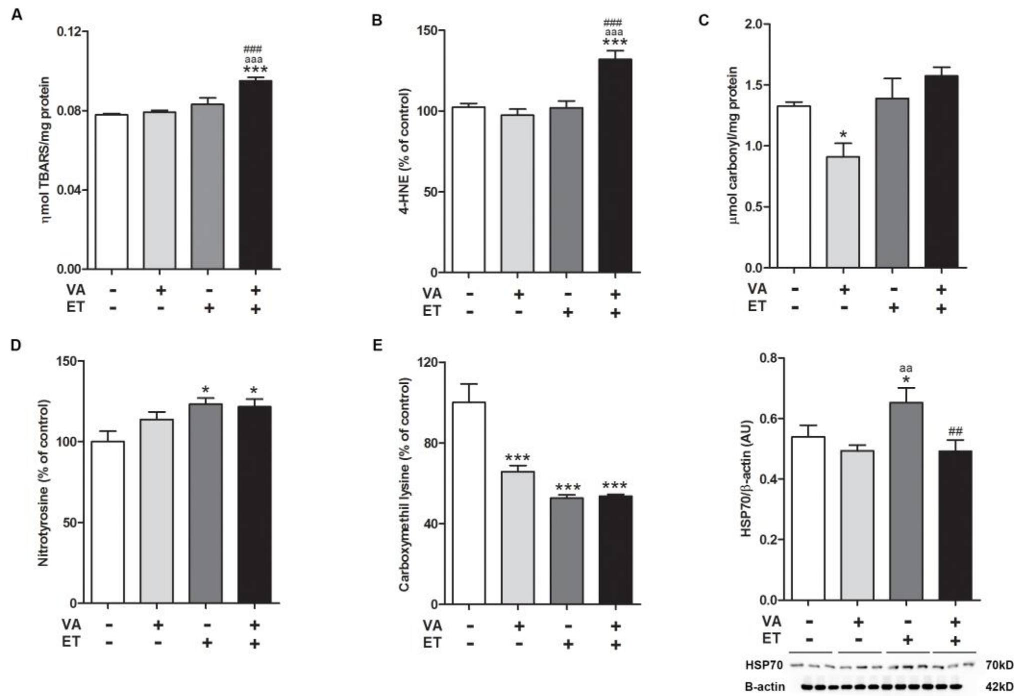
619

620 **Fig 1.** Effects of exercise training and vitamin A supplementation on serum levels of
621 hepatic enzymes ALT (A) and AST (B). Data presented as main ± SEM (n=6-8). *

622 p<0.05 significant difference from SE group. [a] p<0.05 significant difference from

623 SE+VA group using one-way ANOVA followed by Bonferroni's posthoc test.

624



625

626 **Fig 2.** Effects of exercise training and vitamin A supplementation on liver oxidative

627 stress markers and stress parameters. TBARS (A), 4-HNE (B), carbonyl (C),

628 nitrotyrosine (D), carboxymethyl lysine (E), HSP70 (F). Data presented as main ± SEM

629 (n=6-8). * p<0.05 *** p<0.001 significant difference from SE group. [aa] p<0.01 [aaa]

630 p<0.001 significant difference from SE+VA group. [##] p<0.01 [###] p<0.001 significant

631 difference from ET group using one-way ANOVA followed by Bonferroni's posthoc

632 test.

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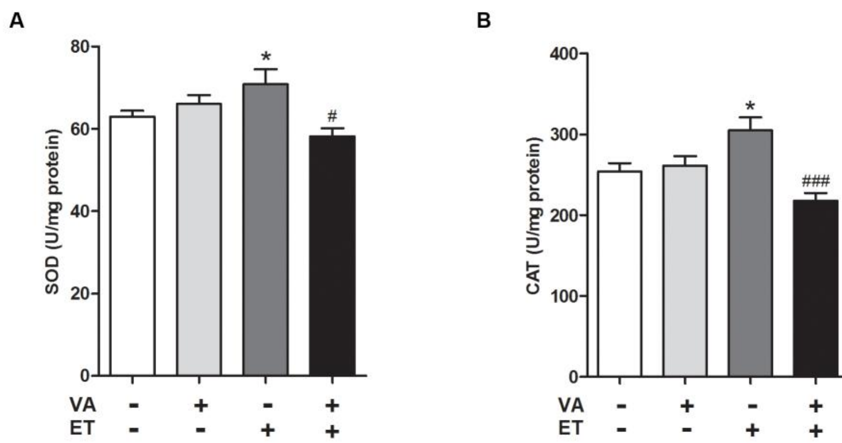

637

638 **Fig 3.** Effects of exercise training and vitamin A supplementation on the activity of liver

639 antioxidant enzymes SOD (A) and CAT (B). Data presented as main ± SEM (n=6-8). *

640 $p<0.05$ significant difference from SE group. # $p<0.05$ ### $p<0.001$ significant difference

641 from ET group using one-way ANOVA followed by Bonferroni's posthoc test.

642

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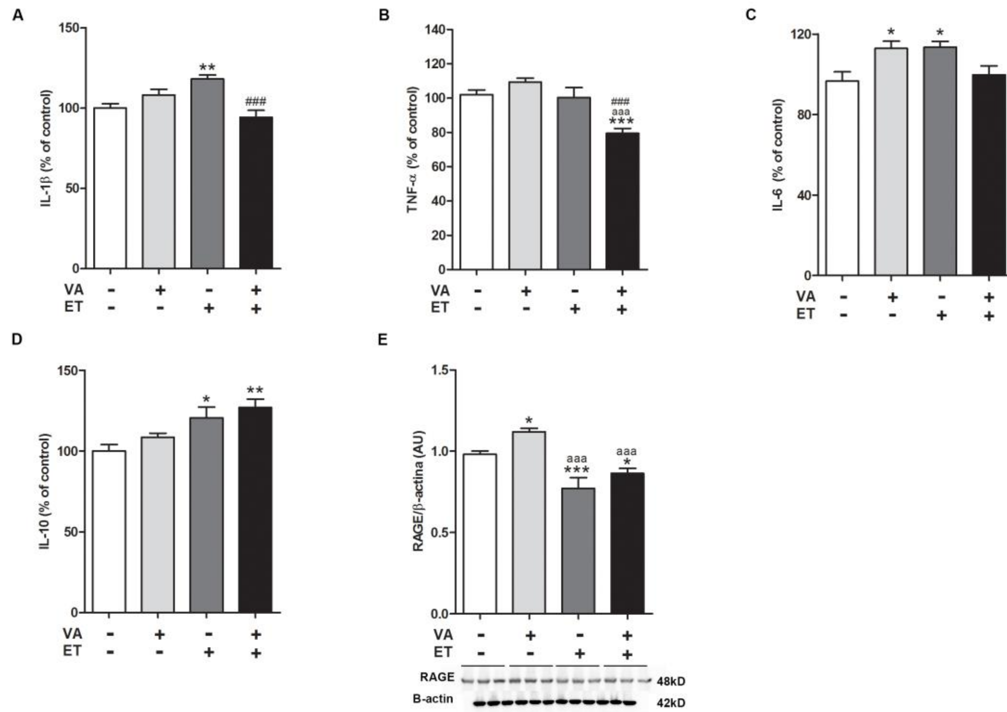

645

646 **Fig 4.** Effects of exercise training and vitamin A supplementation on liver inflammation

647 parameters. IL-1β (A), TNF-α (B), IL-6 (C), IL-10 (D), RAGE (E). Data presented as

648 main ± SEM (n=6-8). * $p<0.05$ ** $p<0.01$ *** $p<0.001$ significant difference from SE

649 group. [aaa] $p<0.001$ significant difference from SE+VA group. [###] $p<0.001$ significant

650 difference from ET group using one-way ANOVA followed by Bonferroni's posthoc

651 test.

652

653

PARTE 3

I. DISCUSSÃO

A prática de exercício físico, além de aumentar a aptidão dos músculos de sustentar mais carga e repetições de trabalho, aumenta também a capacidade de transporte e consumo de oxigênio pelos tecidos [46]. Isso não ocorre apenas devido ao desenvolvimento de um fenótipo muscular mais resistente à fadiga e efetivo na troca e utilização de fibras musculares. O aumento do consumo de oxigênio expõe o músculo esquelético a altas concentrações de ERO, que apesar de possuir um potencial danoso, funcionam como moléculas sinalizadoras no ambiente celular, induzindo adaptações positivas ao exercício [19]. Essas mudanças podem ser observadas em um aspecto amplo, como por exemplo os efeitos positivos da prática de exercício na prevenção e tratamento de doenças associadas a síndrome metabólica; melhorias nas performances cognitiva e cardiovascular; e a diminuição da incidência de doenças como a osteoporose, sarcopenia e doenças cardiorrespiratórias [47]. Em um nível molecular, o exercício físico traz adaptações como o aumento das defesas antioxidantes endógenas, hipertrofia muscular e biogênese mitocondrial [48]. Alguns fatores relacionados ao aumento de ERO durante o exercício físico ainda não são completamente claros; vários mecanismos já foram descritos, mas o quanto cada um deles contribui para o desequilíbrio redox pós-exercício ainda não é totalmente esclarecido. Os mecanismos mais conhecidos são: o vazamento de elétrons durante o funcionamento acelerado da cadeia respiratória; o aumento da atividade da enzima xantina oxidase ativada por aumento do fluxo sanguíneo; elevada atividade da enzima NADPH oxidase; e a auto oxidação de catecolaminas [18].

Em ambos tecidos avaliados nesse estudo, músculo esquelético e fígado, a suplementação de VA causou danos oxidativos a proteínas e lipídios em animais exercitados. No entanto, o registro da atividade das enzimas antioxidantes SOD e CAT nesses tecidos se mostrou diferente. Enquanto no músculo esquelético a atividade da SOD não mostrou diferença e a atividade da

CAT mostrou-se diminuída em ambos grupos exercitados, no fígado o exercício físico sozinho aumentou a atividade de ambas enzimas, e a suplementação de VA inibiu esse aumento. O fígado é um dos tecidos com maior atividade da enzima CAT [49], que decompõe o peróxido de hidrogênio a água e oxigênio. Se o dano hepático gerado por ERO for limitado a uma área, a liberação de CAT para sanar este dano é efetiva, mesmo quando a produção de ERO é alta [50]. Por ser um tecido rico em mitocôndrias, e, portanto, suscetível ao estresse oxidativo quando a demanda de oxigênio é alta, o músculo esquelético possui um sistema antioxidante altamente interligado, composto por antioxidantes enzimáticos e não enzimáticos. Ao contrário do que é descrito para o fígado, o papel do exercício físico na expressão e atividade da CAT no músculo esquelético é dúbio, uma vez que não existe um consenso na literatura [9]. Além disso, a suplementação de VA já mostrou diminuir o imunoconteúdo de CAT no pulmão de ratos Wistar [42]. Neste estudo, o imunoconteúdo de CAT foi avaliado apenas no músculo esquelético, e se mostrou diminuído pela suplementação de VA e exercício. No entanto, na avaliação da atividade enzimática, ambos músculo e fígado exibiram menor atividade de CAT no grupo de exercício e VA, mostrando que a interferência da VA na expressão/atividade desta enzima parece ocorrer em vários tecidos.

Após a descoberta de como as ERO podem ser potentes fatores de sinalização celular, o uso de antioxidantes na prática de exercício físico foi questionado, visto que a sua utilização interfere no status redox da célula. De fato, já foi descrito que o uso prolongado de vitamina C e E, frequentemente utilizados como antioxidantes, impede o aumento da sensibilidade à insulina consequente de exercício físico [51], efeito extremamente benéfico para diabéticos e também para sujeitos saudáveis. A suplementação de vitamina E feita por atletas é uma prática comum, visto que os benefícios do seu uso são comprovados na literatura; no entanto, esses benefícios parecem ser limitados à prevenção na oxidação de LDL, que em grande quantidade leva a aterosclerose [52]. A VA, por ser uma molécula com potencial ação de redução/oxidação, se mostra importante no âmbito do desequilíbrio redox gerado por exercício; no entanto, pouquíssimos estudos foram realizados para esclarecer os mecanismos de ação dessa molécula no exercício físico. No nosso

estudo, a VA prejudicou a adaptação positiva induzida por exercício no músculo esquelético, por exemplo, diminuindo a expressão de SOD2, IL-10, e HSP70. Apesar de possuírem diferentes papéis, os efeitos dessas proteínas estão interligados, uma vez que a SOD2 é uma das mais importantes enzimas antioxidantes mitocondriais, a IL-10 é uma potente citocina anti-inflamatória [28] e a HSP70 uma proteína envolvida com processos de defesa celular, incluindo prevenção de estresse oxidativo e reparo de proteínas danificadas por repetitivas contrações musculares [53].

A relação entre estresse oxidativo e inflamação é muito discutida na literatura. A inflamação é considerada um conjunto de reações complexas num tecido vascularizado em resposta à um estímulo que pode ser tanto endógeno quando exógeno. O objetivo desse processo é livrar o organismo do fator que iniciou a resposta inflamatória, bem como as consequências dessa inflamação. No entanto, respostas exageradas ou irregulares podem prolongar o processo inflamatório, induzindo dano tecidual, além de ser a causa de muitas doenças crônicas [54]. No início da inflamação o dano é detectado pelo padrão de reconhecimento de receptores, como os receptores *toll-like* (TLR), *NOD-like* (NLR) e o receptor para produtos de glicação avançada (RAGE, do inglês *receptor for advanced glycation end products*). Esses receptores são ativados quando ligados a moléculas específicas, ativas por dano ou patógenos [55,56]. Isso gera uma relação direta da inflamação com o estresse oxidativo, uma vez que algumas dessas moléculas são subprodutos do dano oxidativo causado por ERO. Por exemplo, o aldeído 4-hidroxinonenal, produto de glicação avançada reconhecido pelo receptor RAGE, é formado durante a reação em cadeia da peroxidação lipídica [57]. A inflamação crônica está ligada ao desenvolvimento de várias doenças, como diabetes, hipertensão, câncer, além de doenças neurodegenerativas como Parkinson e Alzheimer [20,58]. Não há dúvidas no papel da inflamação no desenvolvimento de patologias, no entanto, estudos epidemiológicos e experimentais sugerem fortemente que o estresse oxidativo contribui significativamente para o aparecimento de diversas doenças [59]. Durante o processo inflamatório as células fagocitárias que são ativadas, como os neutrófilos e macrófagos, produzem grandes quantidades de ERO para eliminar o agente patógeno [60]. No entanto, do mesmo jeito que o processo

inflamatório pode gerar ERO, o aumento da concentração de ERO também pode gerar um processo inflamatório. O peróxido de hidrogênio, ERO subproduto da dismutação do ânion superóxido, pode induzir inflamação através da ativação do fator de transcrição NF-κB, que por sua vez, regula a expressão de citocinas, quimiocinas e enzimas pró-inflamatórias, moléculas de adesão e receptores [61]. Entre as citocinas que tem sua expressão regulada pelo NF-κB estão as pró-inflamatórias IL-1β e TNF-α, ambas avaliadas no músculo esquelético e no fígado neste estudo. No músculo, IL-1β não mostrou diferença significativa entre todos os grupos, e o TNF-α aumentou significativamente no grupo exercitado, o que não ocorreu para o grupo exercício e VA. Já no fígado, IL-1β e TNF-α tiverem seus níveis elevados no grupo exercitado, e no grupo exercitado e suplementado com VA, esse aumento foi revertido. Com esses dados, pode-se sugerir que a suplementação de VA poderia estar influenciando a ativação de NF-κB; de fato, a literatura relata que o ácido retinóico, molécula biologicamente ativa da VA na célula, pode interromper a translocação do NF-κB para o núcleo, em condições de inflamação [62]. Uma perspectiva interessante seria analisar os níveis nucleares do fator de trancrição NF-κB nos animais exercitados e suplementados com VA.

Em relação ao exercício, devido à alta demanda de oxigênio pelos tecidos durante o mesmo, muitos processos redox são ativados, e alguns deles estão envolvidos diretamente no sentido ERO-inflamação, como o aumento da atividade da NADPH oxidase e xantina oxidase e aumento da atividade mitocondrial [63]. Nesse trabalho foram avaliados parâmetros do soro, músculo esquelético e fígado. Na circulação a VA diminuiu o poder total antioxidante presente, e no músculo e no fígado houve dano oxidativo nos animais treinados suplementados com VA, no entanto, no fígado houve aumento da citocina anti-inflamatória IL-10 e a diminuição das pró-inflamatórias, indicando que a VA não provocou um ambiente favorável à inflamação como foi observado no músculo, onde a VA inibiu o aumento de IL-10 induzida por exercício. Esse é um dado interessante pois o fígado é o principal local de metabolismo e armazenamento de VA [64], o que indica que a VA mostrou-se prejudicial às adaptações do músculo esquelético ao exercício, mas isso não se reproduziu no fígado.

O potencial antioxidante de uma molécula pode derivar de grupamentos específicos que tem atividade de oxidação/redução e como esses grupamentos estão arranjados espacialmente. A VA possui uma longa cadeia de ligações duplas conjugadas, o que é comum a todos retinóides e permite a atividade *scavenging* observada nestes compostos, como para o ânion superóxido e o radical peroxil [65]. O mais descrito na literatura é a atividade antioxidante dos carotenoides, como o β-caroteno. Já foi descrito que o β-caroteno age como um interceptador de ERO, e que essa ação está envolvida com a oxidação de ácidos graxos poli-insaturados e seus ésteres – fazendo do β-caroteno uma molécula com provável envolvimento em processos de peroxidação lipídica [66]. Os estudos que avaliam o potencial antioxidante de moléculas são frequentemente realizados *in vitro,* ou seja, sem a interferência de um sistema. O nosso trabalho foi realizado num modelo *in vivo,* com fatores sinergéticos que devem ser levados em consideração. Fatores como idade, duração e intensidade do exercício e concentração da suplementação são fatores variáveis que podem levar a resultados diferentes. Para o nosso modelo, a VA mostrou causar dano oxidativo, como o dano proteico e lipídico. O exercício em si já produz sinalização redox para adaptações, e a VA parece de alguma uma forma ter interrompido e/ou prejudicado essa cadeia de reações. Um dos controles do estudo, o do animal sedentário suplementado com VA, é muito importante nesse aspecto. Ele demonstra que a VA sozinha não causou danos, nos levando a acreditar que o dano observado deriva da influência da VA em processos fisiológicos que ocorrem especificamente durante o exercício.

Outro aspecto importante a se considerar é a dose de VA utilizada no estudo. Estudos prévios do nosso grupo observaram danos relacionados a suplementação de VA, como no pulmão [42], e no cérebro [41]. Isso pode estar relacionado também a altas doses de suplementação. Nesse estudo, a estratégia para calcular o a dose de VA foi relacionar aproximadamente à dose utilizada em suplementos alimentares farmacêuticos. Normalmente, a dose é pelo menos 100% da recomendação diária; nós utilizamos uma dose um pouco menor, uma vez que a ração consumida pelos animais ao longo da intervenção já contém uma quantidade de VA. A estratégia foi não utilizar uma dose muito

alta, que poderia causar um desequilíbrio redox grande e ficaria pouco comparável com situações reais.

Por fim, uma das limitações do estudo foi a falta de testes que comprovem a intensidade do exercício. O ideal seria a utilização de métodos para estabelecer intensidade, como a medição de lactato sanguíneo [67]. Nesse trabalho, dois aspectos observados – as adaptações ao exercício já descritas na literatura e o peso menor dos animais treinados – indicam a eficiência do protocolo de exercício físico, nesse caso, de natação com sobrepeso.

II. CONCLUSÃO

Em conclusão com base nos resultados apresentados nesse trabalho, a VA induziu a liberação de marcadores de stress e desequilíbrio redox na circulação, dano oxidativo e diminuição da atividade das defesas antioxidantes endógenas no músculo esquelético e no fígado. No entanto, isso gerou inflamação apenas no músculo, mostrando que a mobilização de transporte da VA que ocorre no fígado durante o exercício físico não gerou um processo inflamatório nesse tecido. Assim, a VA parece ter um papel negativo para praticantes de exercício físico, pois mostrou prejudicar fatores de adaptação positiva induzidas por exercício no músculo esquelético.

III. PERSPECTIVAS

Com base nos resultados deste trabalho, as perspectivas futuras são:

- Avaliar como a suplementação de vitamina A e exercício físico afeta outros tecidos;
- Avaliar se os efeitos observados neste estudo refletem no rendimento do exercício, suplementando humanos e analisando o efeito da VA no rendimento do esforço físico.

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