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

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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)-1 β and tumor necrosis factor- α , was reduced in the ET+VA group, while 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 caused oxidative damage, a controlled administration might exert anti-inflammatory effects. Further studies with higher VA doses and longer ET interventions would elucidate more the effects of the supplementation and exercise on liver parameters.

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

Introduction

Regular exercise training (ET) is recommended for the prevention and treatment of several disorders (Neufer et al. 2015), in addition to 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 the large increase in oxygen consumption, that can reach up to 10–15-fold during ET (Sen 1995), leads to the production of reactive oxygen species (ROS), high concentrations of which can cause oxidative stress and tissue damage (Chance et al. 1979). 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). An imbalance in ROS production occurs when the formation of ROS is not followed by a proper antioxidant response of the cell, and it can be caused by an increase or decrease in ROS production combined with a wide range of alterations in the activation and expression of antioxidant enzymes and/or the presence of non-enzymatic antioxidants (Banerjee et al. 2003).

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 and Kjaer 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 protein synthesis without changes in fat content, reduce hepatic blood flow, and cause a significant imbalance in ROS production (Shephard and Johnson 2015). Furthermore, in

streptozotocin-induced diabetic rats, chronic ET has been shown to prevent the impairment of hepatic redox defenses, such as decreased expression of liver antioxidant enzymes and increased ROS levels (Lima et al. 2015). Although consumption of a weight-loss diet is still the most effective treatment for non-alcoholic fatty liver disease, ET alone has been shown to be highly beneficial in treating this disease. ET reduces insulin resistance and increases the expression of genes responsible for fatty acid metabolism in this condition (Oh et al. 2014; Ordonez et al. 2015). The redox imbalance caused by exercise may lead to inflammation, wherein increased ROS levels are related to inflammatory processes (Kosmidou et al. 2002). Regular exercise can decrease the levels of inflammatory cytokines, adipokines, and other injury-related markers in the liver (Gleeson 2007). These anti-inflammatory effects appear to occur due to three main factors: reduced visceral fat, increased production and release of anti-inflammatory cytokines such as interleukin (IL)-10, and decreased expression of toll-like receptors in immune cells (Gleeson et al. 2011).

Dietary supplementation during ET is a common practice to enhance performance or to prevent/treat diseases. However, the effects of different combinations of dietary supplements and regular ET are unknown. Several natural supplements have been associated with liver injury, such as green tea extract and other herbal preparations, usnic acid, and vitamin A (VA) (Garcia-Cortes et al. 2016). VA is a fat-soluble vitamin essential for many key biological processes, including vision, embryonic development, gene transcription, and immune responses (Chapman 2012). It can be found in the form of all-*trans* retinol and retinyl esters (in foods from animal sources) and in the form of pro-vitamin A carotenoids, such as β -carotene (in foods from vegetable sources) (Blomhoff and Blomhoff 2006). The liver is one of the main sites of VA metabolism and storage, and both the hepatic cells, parenchymal

(hepatocytes) and stellate cells, participate in these processes (D'Ambrosio et al. 2011). Oral intake or administration of therapeutic doses of VA may induce hepatotoxicity through hypervitaminosis (Geubel et al. 1991). Furthermore, studies evaluating the effect of VA supplementation over oxidative damage induced by different stressors in the liver demonstrated that VA may act as an antioxidant molecule (protective), but also as pro-oxidant (leading to liver damage), and therefore the effects of VA will largely depend on the dose, mechanism of administration and the characteristics of the stressors (de Oliveira et al. 2009; Schnorr et al. 2011; Wang et al. 2014; Shimizu et al. 2017).

Thus, considering the major role of liver in VA metabolism and storage, and the wide range of effects of VA on liver oxidative stress regulation, the present work was designed to evaluate the effect of VA supplementation on parameters of ROS-mediated damage, antioxidant defense, cell stress, and pro-inflammatory modulation in the liver of rats submitted to intense swimming ET. In previous works, we observed that VA supplementation with ET impaired some exercise-acquired benefits in highly demanded tissues of trained rats, by decreasing the activity of antioxidant defenses and causing lipoperoxidation in the lungs (Gasparotto et al. 2015) and skeletal muscle (Petiz et al. 2017). Due to the role of the liver in the regulation of energetic homeostasis during exercise and also in the metabolism of VA, this study is intended to improve the understanding of the mechanisms by which VA supplementation presented potentially harmful effects when combined with ET.

Materials and Methods

Ethics

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

Animals

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

Study design

Animals were randomized into four groups: sedentary (SE), sedentary supplemented with VA (SE+VA), exercise training (ET), and exercise training supplemented with VA (ET+VA). Throughout the 8 weeks, the exercised and sedentary groups were administered 450 retinol equivalents (RE) (1500 IU)/kg/day of retinyl palmitate (Arovit[®]; Bayer, Rio de Janeiro, RJ, Brazil) or its vehicle (saline) daily by intragastric gavage. The chosen dose of 450 RE was calculated based on the human equivalent dose (HED), using the dose-by-factor approach (Nair et al. 2016). The exercised groups were subjected to a protocol of 60 min of swimming 5 days/week, while the sedentary groups

remained in shallow water for 20 min 5 days/week. ET and supplementation protocols were performed between 6–8 pm.

Exercise training protocol

The training protocol lasted 8 weeks in total. It was conducted between 6 and 8 pm, in a swimming tank for rodents with water at 31 ± 1 °C. During the first week, all animals remained in shallow water for 20–60 min each day. For the next 2 weeks, training started with 10 min/day and progressed to 60 min/day. Over the following 5 weeks, training consisted of 60 min/day, 5 days/week, with an overload attached to the animal's torso, progressing each week (0, 2, 4, 6% body weight) (Gobatto et al. 2001). Animals were weighed once a week and the values were utilized to calculate the overload. After each session, animals were towel-dried and returned to their cages.

Tissue sampling

After a 24 h interval from the last exercise bout and VA supplementation, animals were euthanized by decapitation, and their blood and liver tissue samples were collected. Blood samples were centrifuged at $1500 \times g$ for 10 min for serum isolation. Tissue samples were homogenized in phosphate buffer (PB) and centrifuged ($3000 \times g$, 10 min), and sample supernatants were used for analysis. Protein content was quantified by the Lowry method (Lowry et al. 1951) using bovine serum albumin as standard. For western blotting, the tissue was homogenized in RIPA buffer (20 mM Tris-HCl pH 7.5; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 2.5 mM sodium pyrophosphate; 1% sodium deoxycholate; 1% Tergitol-type NP-40; 1 mM β -glycerophosphate; 1 mM sodium orthovanadate; 1 μ g/mL leupeptin) and centrifuged, following which the

homogenate was added to Laemmli-buffer (62.5 mM Tris-HCl pH 6.8; 1% SDS; 10% glycerol) with 10% β -mercaptoethanol.

Plasma assays

Biochemical parameters

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

Liver homogenate assays

Redox parameters

Thiobarbituric acid reactive species (TBARS) levels in the liver samples were quantified as an index of lipid peroxidation (Draper and Hadley 1990). First, samples were deproteinized by 10% trichloroacetic acid (TCA), followed by heating at 100 °C for 25 min with 0.67% thiobarbituric acid. TBARS levels were then quantified spectrophotometrically at a wavelength of 532 nm. Oxidative damage to proteins was quantified by detection of carbonyl groups (Levine et al. 1990), which involved incubation of sample proteins, previously precipitated with 20% TCA, with 2,4-dinitrophenylhydrazine (DNPH), followed by spectrophotometric quantification at 370 nm. Nitrotyrosine content was detected by an indirect enzyme-linked immunosorbent assay (ELISA) using a polyclonal antibody (Abcam, Cambridge, UK). Quantification of antioxidant enzyme activity was performed through kinetic spectrophotometric assays. Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined as the inhibition of superoxide anion-dependent adrenaline auto-oxidation in the presence of the liver

sample at 480 nm (Misra and Fridovich 1972). Catalase (CAT; EC 1.11.1.6) activity was measured as the decrease in hydrogen peroxide (H_2O_2) absorbance in the presence of the liver sample at 240 nm (Aebi 1984).

ELISA

Indirect ELISA assay was performed using antibodies to detect the pro-inflammatory cytokines [IL-1 β , tumor necrosis factor- α (TNF- α), and IL-6], the anti-inflammatory cytokine (IL-10), the advanced glycation end products (AGEs), 4-hydroxynonenal (4-HNE), and carboxymethyl lysine (all the six antibodies were purchased from Abcam, Cambridge, UK). The liver tissue samples homogenized in PB were placed in a specific round-bottom plate and left overnight for sample adherence. They were then incubated overnight with primary antibody in a 1:1000 dilution range, followed by incubation with secondary antibody for 2 h in a 1:2000 dilution range. Between every step, the plate was washed three times with PB in 0.05% Tween-20, and all incubations were conducted at 4 °C under constant agitation (45 rpm). Immunoreactivity was detected with a colorimetric assay using the TMB Chromogen solution for ELISA (Thermo Fisher Scientific, Rockford, USA).

Western blotting

After subjecting the liver samples (20 μg) homogenized in RIPA buffer with 10% β -mercaptoethanol to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transference was achieved by a semi-dry transfer of proteins to a nitrocellulose membrane (Millipore, Bedford). Protein content was then determined by Ponceau S staining. Membranes containing the sample proteins were washed using Tris-Tween-

Buffer-Saline (TTBS - 100 mM Tris, pH 7.5; 0.9% NaCl; 0.1% Tween-20) for posterior blocking with 5% non-fat dry milk for 1 h (room temperature). After TTBS washes, membranes were incubated with primary antibodies (1:1000 dilution) against HSP70 (Cell Signaling Technology, Beverly, USA) and the receptor for AGEs (RAGE) and β -actin (Sigma Chemical, St. Louis, USA) for 2 h at room temperature, followed by incubation with anti-rabbit/mouse horseradish peroxidase-linked secondary antibodies (1:2000 dilution; Cell Signaling Technology, Beverly, USA) for 1 h at room temperature. Immunoreactivity was detected through chemiluminescence using the SuperSignal West Pico Chemiluminescent kit (Thermo Scientific, Rockford, USA). Densitometry analysis was conducted with ImageJ software, and results were expressed as the ratio of target protein/ β -actin.

Statistical analysis

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

Results

Serum ALT and AST levels

ALT and AST are enzymes expressed in the liver, and their serum activities indicate the degree of liver tissue damage. ALT activity (Fig. 1A) was significantly enhanced in both ET and ET+VA groups, compared to that in both the sedentary groups. AST (Fig. 1B) had lower activity in both the exercised groups compared to that in the SE group.

These results showed that ET and VA supplementation both have an impact on liver function.

ROS-mediated damage and stress parameters

We analyzed the oxidative damage biomarkers and antioxidant enzyme activities in the liver tissue. The ET+VA group showed increased lipoperoxidation compared to other groups (Fig. 2A). This effect was confirmed by the assessment of levels of 4-HNE, an end product of lipid peroxidation chain reaction (Fig. 2B). Analysis of oxidative protein damage showed decreased carbonyl formation in the SE+VA group compared to that in the SE group; however, carbonyl formation in the other groups was not significantly affected (Fig. 2C). Nitrotyrosine accumulation, on the other hand, was increased in the ET group, and VA supplementation did not change this effect (Fig. 2D). AGE formation, which is increased in several conditions related to oxidative/nitrosative stress and metabolic dysfunction (such as diabetes), was decreased by VA supplementation itself and in ET and ET+VA groups (Fig. 2E). Finally, the levels of the chaperone protein, HSP70, which is induced under conditions of cellular stress, were evaluated by western blotting. ET enhanced HSP70 levels in the liver, but VA supplementation inhibited this effect (Fig. 2F).

Antioxidant enzyme activities

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

Modulation of inflammatory parameters

Next, we measured cytokine levels to evaluate the effect of VA supplementation on the pro-inflammatory stimulation caused by ET. IL-1 β levels were increased in the ET group, and VA supplementation inhibited this effect (Fig. 4A). Basal levels of liver TNF- α were unaffected in the ET group; however, the ET+VA group presented decreased TNF- α levels compared to all other groups (Fig. 4B). The pro-inflammatory cytokine, IL-6, is also an anti-inflammatory myokine, which is generally stimulated during muscle contraction and ET. Levels of IL-6 in the liver were increased in the SE+VA and ET groups, compared to SE group; however, this effect was inhibited in the ET+VA group (Fig. 4C). The levels of the anti-inflammatory cytokine, IL-10, were enhanced in the ET group as compared with that in the SE group, and the combination of ET and VA further stimulated this effect (Fig. 4D). Finally, since increased RAGE levels have been associated with chronic pro-inflammatory conditions (Wautier et al. 2016), their levels were evaluated. Although RAGE levels were increased by VA supplementation alone, the ET and ET+VA groups demonstrated a significant decrease in RAGE levels compared to the SE and SE+VA groups, respectively (Fig. 4E).

Discussion

While ET increased ALT and decreased AST levels, VA supplementation did not have an influence on these parameters. ALT and AST are well-known serum markers used to assess and monitor liver damage. In athletes, however, these enzymes may originate from different tissues; while ALT comes mainly from the liver, AST can be linked to

muscle cell leakage (Banfi et al. 2012). Indeed, increased levels of ALT and AST are a common response to intense ET (Kayatekin et al. 2002; Koury et al. 2016).

It is well established that vigorous exercise increases ROS production and antioxidant activity due to the enhanced oxygen demands of the tissue and considerable increase in blood flow (Finaud et al. 2006). As a consequence, oxidative damage resulting from long-term ET leads to an antioxidant adaptation in tissues such as skeletal muscle, liver and also the DNA (Abruzzo et al. 2013; Radak et al. 2013; Pillon Barcelos et al. 2017). The liver response to exercise may vary, training intensity being the major factor that determines oxidative damage and antioxidant adaptation. VA presents variable redox-active properties in biological systems, and usually, its effects are related to prevention of oxidative damage (Ozhogina and Kasaikina 1995; Powers and Lennon 1999; Schroder et al. 2001). However, as a lipophilic compound, VA can easily interact with membrane lipids, and the conjugated double bonds present in its structure can facilitate the formation of conjugated dienes during lipid peroxidation chain reactions, if concentrations are high (Halliwell 2006). In our study, the overall effect of VA on tissue redox activity and antioxidant defense varied according to the properties of each tissue fraction. Animals subjected to intense swimming ET showed basal levels of lipid peroxidation products (TBARS and 4-HNE) and protein carbonyls, but VA supplementation increased lipid damage. On the other hand, nitrotyrosine formation, a hallmark of peroxynitrite-mediated protein damage, was increased in the ET group, and VA supplementation could not inhibit this effect. These results may be explained by the differential effects of ET and VA on ROS production in different cell compartments. The effect of VA supplementation on antioxidant enzyme activity supports this hypothesis.

The levels of both the antioxidant enzymes, SOD and CAT, were increased in the ET group, which is in accordance with the positive adaptations that occur as a consequence of ET. SOD and CAT activities are stimulated by increased substrate availability. Hence, when superoxide and hydrogen peroxide production is stimulated, their activities are increased. SOD is present in the mitochondria and the cytosol, and its activity is stimulated by superoxide production resulting from an increased demand for mitochondrial activity during intense exercise (Finaud et al. 2006; Myburgh 2014). The effects of chronic ET in the liver includes increased SOD activity and reduced lipid damage (da Silva et al. 2009), improved activity of the antioxidant enzyme glutathione peroxidase (Barcelos et al. 2014), and increased mitochondrial biogenesis and citrate synthase activity (Santos-Alves et al. 2015), all of which contribute to adaptation to increased ROS production and elevated aerobic demand for ATP synthesis. Increased reactive species production and redox imbalance result in upregulation of tissue defense, in order to cope with the adverse conditions (Banerjee et al. 2003). Supplementation with VA blocked the activation of SOD and CAT caused by ET, indicating that VA inhibited reactive species production, and thus SOD and CAT activities decreased due to reduced substrate availability. VA (β -carotene) has previously been shown to have scavenging activity on several ROS, such as superoxide and peroxy radicals (Yu 1994), which could have caused the decreased SOD activity in the ET+VA group. However, increased lipid damage and nitrotyrosine levels in the ET+VA group indicate that VA modulates SOD and CAT activity, but does not inhibit reactive species production.

ET is often associated with the prevention and treatment of lifestyle-related diseases (Neufer et al. 2015). This includes liver diseases; fat accumulation in the liver can lead to non-alcoholic fatty liver disease, and previous studies have reported that chronic aerobic exercise reduces this effect (Batatinha et al. 2016). Inflammation plays a

key role in the development of this adverse liver condition (Nov et al. 2013). After intense ET, the levels of inflammatory cytokines rise significantly, and this response is often related to reactive species overload (Kosmidou et al. 2002). This effect may be followed by a compensatory response to increased production of anti-inflammatory mediators and activation of antioxidant enzymes. Here, we observed that VA supplementation combined with ET reduced the tissue levels of the pro-inflammatory cytokines, TNF- α and IL-1 β . It has been shown that ET does not affect the TNF- α expression levels in the liver, which is consistent with our results (E et al. 2013). One transcription factor that is associated with oxidative stress and inflammation is NF- κ B (nuclear factor kappa-B), and it is also responsible for the regulation of TNF- α and IL-1 β expression (Barnes and Karin 1997). It has already been described that retinoic acid, the most active metabolic form of VA, can disrupt the nuclear translocation of NF- κ B under inflammatory situations (Wang et al. 2015). Indeed, the NF- κ B pathway is among the most relevant signaling pathways in liver inflammation (He and Karin 2011). Therefore, VA supplementation may inhibit the activation of NF- κ B, resulting in blunted pro-inflammatory cytokine release in response to intense exercise. Furthermore, the increase in IL-1 β and IL-6 levels caused by ET or VA alone were inhibited when combined together. Similar effects have been observed in rats supplemented with caffeine and subjected to swimming ET (Cechella et al. 2014). IL-6 is considered a myokine and promotes anti-inflammatory actions in the muscle, as opposed to its pro-inflammatory actions in the liver and other tissues (Ost et al. 2016). In muscle cells, IL-6 is responsible for increasing fat oxidation and stimulating glucose uptake by insulin; besides, it has been shown that IL-6 deficient mice have reduced capacity to regenerate their liver (El-Kadre and Tinoco 2013). It is possible that ET enhances IL-6 levels in the liver as well as other tissues, where it promotes the inflammatory responses.

Furthermore, the effect of ET on IL-10, a very effective anti-inflammatory cytokine, indicates that the pro-inflammatory effect of exercise on the liver is accompanied by the activation of anti-inflammatory response, and in combination with VA supplementation, this effect is further enhanced. We also evaluated the levels of RAGE, a multi-ligand receptor associated with inflammation in chronic diseases (Bohloli et al. 2014; Schmidt 2015). Interestingly, SE+VA group showed enhanced RAGE levels, but both the ET and ET+VA groups showed decreased levels of RAGE. This is in agreement with the decrease in the levels of carboxymethyl-lysine, a key AGE that arises from non-enzymatic oxidative reactions between carbohydrates and proteins (Gaens et al. 2014), observed in all groups, as the regulation of this receptor depends on ligand availability. Thus, considering these effects, ET combined with VA supplementation induces an overall positive effect on pro-inflammatory activation induced by ET. It is important to evaluate several parameters when studying oxidative stress and inflammation, and interpreting one isolated parameter may lead to different conclusions. For example, VA is known to increase the production of superoxide anion (Murata and Kawanishi 2000), which could be responsible for the lower CAT activity observed in the ET+VA group, since high concentrations of superoxide can lead to CAT inhibition (Kono and Fridovich 1982; Pasquali et al. 2009). Also, oxidative stress and inflammation relationship goes both ways. During the inflammatory process phagocytes produce a large amount of ROS, for the purpose of eliminating the pathogen (Fialkow et al. 2007). On the other hand, high levels of hydrogen peroxide, ROS derivative from the dismutation of superoxide anion, can induce inflammation through NF- κ B activation (Barnes and Karin 1997). Redox parameters alone can mislead the interpretation of results, hence the importance of evaluating, in this case, inflammatory parameters related to ET response.

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

The dose of choice for VA treatment in this study was based on the daily recommended value of 800 RE for human adults (Institute of Medicine Panel on Micronutrients 2001) applying the HED, using the dose-by-factor approach (Nair and Jacob 2016). The food provided to the animals already contains VA at a dose that fulfills their daily recommendation; therefore, the combination with daily gavage supplementation is very likely to extrapolate the daily requirements for this vitamin. VA influences several metabolic processes (Chapman 2012), and its deficiency or excess may show very different effects. As described in the literature, higher doses would

probably increase tissue damage (Pasquali et al. 2009; Gasparotto et al. 2015; Schnorr et al. 2015; Petiz et al. 2017), and lower doses may show no effect at all. Both VA and ET are potentially pro-oxidant to tissues when applied in excess, and we wanted to avoid potential harmful effects by using higher doses of this vitamin. Nonetheless, the dose of 450 RE is below the tolerable VA daily upper intake level of 3000 RE, and the effects observed here could be considered mild.

Conclusion

VA, a redox-active molecule with known antioxidant properties, caused oxidative damage in the liver of trained rats. It increased lipid peroxidation and blunted the increased activity of antioxidant enzymes SOD and CAT caused by exercise alone. Even with this outcome, pro-inflammatory mediators (IL-1 β and TNF- α) decreased and anti-inflammatory (IL-10) increased in the group of trained rats supplemented with VA. This shows VA acting in an anti-inflammatory direction, regardless of the oxidative damage observed. Considering these effects, the dose of 450 RE did not appear to cause liver injury, and suggests that controlled administration of VA for some types of exercise may imply anti-inflammatory properties. This was a low dosage of VA intake, and higher doses would probably increase the oxidative damage observed and modify the inflammatory response. Knowing that both VA and exercise are redox-modulators, it is important to conduct more studies with higher doses of VA and long-term exercised interventions, to determinate if the VA supplementation combined with exercise cause positive or negative effects.

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Conflict of Interest statement

The authors declare no conflict of interest regarding this study.

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Captions for figures

Fig 1. Effects of exercise training and vitamin A supplementation on serum levels of hepatic enzymes ALT (A) and AST (B). Data presented as main $\pm$ SEM (n=6-8). * p<0.05 significant difference from SE group. ^a p<0.05 significant difference from SE+VA group using one-way ANOVA followed by Bonferroni's posthoc test.

Fig 2. Effects of exercise training and vitamin A supplementation on liver oxidative stress markers and stress parameters. TBARS (A), 4-HNE (B), carbonyl (C), nitrotyrosine (D), carboxymethyl lysine (E), HSP70 (F). Data presented as main $\pm$ SEM (n=6-8). * p<0.05 *** p<0.001 significant difference from SE group. ^{aa} p<0.01 ^{aaa} p<0.001 significant difference from SE+VA group. ^{##} p<0.01 ^{###} p<0.001 significant difference from ET group using one-way ANOVA followed by Bonferroni's posthoc test.

Fig 3. Effects of exercise training and vitamin A supplementation on the activity of liver antioxidant enzymes SOD (A) and CAT (B). Data presented as mean $\pm$ SEM (n=6-8). * p<0.05 significant difference from SE group. # p<0.05 ### p<0.001 significant difference from ET group using one-way ANOVA followed by Bonferroni's posthoc test.

Fig 4. Effects of exercise training and vitamin A supplementation on liver inflammation parameters. IL-1 β (A), TNF- α (B), IL-6 (C), IL-10 (D), RAGE (E). Data presented as mean $\pm$ SEM (n=6-8). * p<0.05 ** p<0.01 *** p<0.001 significant difference from SE group. ^{aaa} p<0.001 significant difference from SE+VA group. ^{###} p<0.001 significant difference from ET group using one-way ANOVA followed by Bonferroni's posthoc test.

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Fig 1. Effects of exercise training and vitamin A supplementation on serum levels of hepatic enzymes ALT (A) and AST (B). Data presented as main $\pm$ SEM (n=6-8). * $p < 0.05$ significant difference from SE group. a $p < 0.05$ significant difference from SE+VA group using one-way ANOVA followed by Bonferroni's posthoc test.

59x27mm (300 x 300 DPI)

Fig 2. Effects of exercise training and vitamin A supplementation on liver oxidative stress markers and stress parameters. TBAARS (A), 4-HNE (B), carbonyl (C), nitrotyrosine (D), carboxymethyl lysine (E), HSP70 (F). Data presented as mean $\pm$ SEM (n=6-8). * $p < 0.05$ *** $p < 0.001$ significant difference from SE group. aa $p < 0.01$ aaa $p < 0.001$ significant difference from SE+VA group. ## $p < 0.01$ ### $p < 0.001$ significant difference from ET group using one-way ANOVA followed by Bonferroni's posthoc test.

149x112mm (300 x 300 DPI)

Fig 3. Effects of exercise training and vitamin A supplementation on the activity of liver antioxidant enzymes SOD (A) and CAT (B). Data presented as mean $\pm$ SEM (n=6-8). * $p<0.05$ significant difference from SE group. # $p<0.05$ ### $p<0.001$ significant difference from ET group using one-way ANOVA followed by Bonferroni's posthoc test.

59x27mm (300 x 300 DPI)

Fig 4. Effects of exercise training and vitamin A supplementation on liver inflammation parameters. IL-1 β (A), TNF- α (B), IL-6 (C), IL-10 (D), RAGE (E). Data presented as mean $\pm$ SEM (n=6-8). * p<0.05 ** p<0.01 *** p<0.001 significant difference from SE group. aaa p<0.001 significant difference from SE+VA group. ### p<0.001 significant difference from ET group using one-way ANOVA followed by Bonferroni's posthoc test.

139x97mm (300 x 300 DPI)