



Benfotiamine (BFT) reduces oxidative damage in muscle of endurance-trained mouse

Álison de Carvalho Gonçalves^{1*}, Renata Campos Leão¹, Fábio Lera Orsatti² and Guilherme Vannucchi Portari³

¹Laboratório de Nutrição Experimental, Instituto de Ciências da Saúde, Universidade Federal do Triângulo Mineiro, Av. Getúlio Guaritá, 159, 38025-440, Uberaba, Minas Gerais, Brazil. ²Grupo de Pesquisa em Biologia do Exercício, Instituto de Ciências da Saúde, Universidade Federal do Triângulo Mineiro, Uberaba, Minas Gerais, Brazil. ³Departamento de Nutrição, Instituto de Ciências da Saúde, Universidade Federal do Triângulo Mineiro, Uberaba, Minas Gerais, Brazil. Author for correspondence. E-mail: alisoncg88@hotmail.com

ABSTRACT. High levels of reactive oxygen species can trigger an imbalance in redox status, which generates oxidative damage in macromolecules. The present study aimed to investigate the influence of oral supplementation with benfotiamine on oxidative stress and antioxidant activity in muscle of trained mice. Twenty-five male Balb/c mice were placed in groups. Sta-Sed: standard diet and sedentary (n = 6); Ben-Sed: benfotiamine supplemented and sedentary (n = 6); Sta-Tr: standard diet and trained (n = 6); and Ben-Tr: benfotiamine supplemented and trained (n = 7). Standard diet was AIN-93 growth and supplemented diet was AIN-93 with benfotiamine (500 mg kg⁻¹). Trained mice were submitted to 6-weeks of endurance swimming training. The concentration of thiobarbituric acid reactive substances (TBARS), non-protein thiols, catalase (CAT) and superoxide dismutase activities (SOD) were analyzed in the gastrocnemius muscle. TBARS concentration was lower in the Ben-Tr group than in Ben-Sed and Sta-Tr groups. Thiol levels were higher in the Ben-Sed group than in the non-supplemented groups. CAT activity was more pronounced in both supplemented groups while SOD activity was higher in the Ben-Tr group than in the non-supplemented groups. The results show that benfotiamine supplementation is effective in enhancing antioxidant defenses and reducing oxidative damage in muscle of endurance-trained mouse.

Keywords: thiamine; antioxidant; free radicals; endurance exercise; mice.

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Introduction

Oxidative energy metabolism in the mitochondria is the main producer of reactive oxygen species (ROS) (Carocho & Ferreira, 2013; Dröse & Brandt, 2012). During exercise, oxidative processes are increased to supply the energy demand, and ROS production is consequently increased (Powers, Nelson & Hudson, 2011). The ROS can cause damage in macromolecules and trigger deleterious reaction cascades in cells if the antioxidant system cannot equilibrate the redox status. The imbalance in redox status is known as oxidative stress (Sies, Berndt & Jones, 2017).

There are studies, *ex vivo* and *in vivo*, showing the role of oxidative stress in fatigue processes (Cobley, McGlory, Morton & Close, 2011; Jendzjowsky & DeLorey, 2013; Kuwahara et al., 2010) and some studies show that exercise increases the ROS production and oxidative stress, both in humans (Miyazaki et al., 2001) and rats (Bejma & Ji, 1999). A study showed that supplementation with an antioxidant coformulation prevents the protein oxidation, enhances mitochondrial function and performance in untrained female rodents (Abadi et al., 2013). Other studies found that the supplementation vitamin C and E was sufficient to combat ROS but with loss of endogenous antioxidant adaptation (Gomez-Cabrera et al., 2008; Meier, Renga, Hoppeler, & Baum, 2013). Thus, there is no consensus about the positive effects of antioxidant supplementation on endurance performance (Draeger et al., 2014).

Some experimental studies have demonstrated the antioxidant capacity of thiamine (vitamin B1) (Gioda et al., 2010; Karachalias, Babaei-Jadidi, Rabbani, & Thornalley, 2010; Portari, Ovidio, Deminice & Jordão Jr, 2016). Aside from thiamine being efficient in transferring ions to the reactive species (Lukienko, Mel'nichenko, Zverinskii & Zabrodskaya, 2000), there are evidences that the treatment with benfotiamine, a lipophilic thiamine derivate, increased protein expression and activity of antioxidant enzymes in culture of neurological cells (Bozic et al., 2015). Thiamine diphosphate is an essential coenzyme in the pentose

phosphate pathway, which produces reduced nicotinamide adenine dinucleotide phosphate (NADPH), a molecule which transfer their H^- to highly reactive molecules and is essential for the activity of the antioxidant enzymes catalase (CAT) and glutathione peroxidase (GPx) (Stincone et al., 2017).

Considering these evidences, it is possible that benfotiamine supplementation increases the levels of thiamin and its esters in the muscles, and improves antioxidant systems, giving and receiving electrons or enhancing the activity of antioxidant pathways. Thus, the present study aimed to investigate the influence of oral supplementation with benfotiamine on markers of oxidative stress and antioxidant activity in muscle of endurance trained mice.

Material and methods

Animals

Twenty-five 6-week-old Balb/c male mice were housed individually in plastic cages, in inverted circadian cycle (12-h dark/light), in $22 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ humidity with feeding and tap water *ad libitum*. The animals were placed into 4 groups according to the proposed intervention. The standard diet and sedentary group (Sta-Sed) received food without benfotiamine and did not train. The benfotiamine and sedentary group (Ben-Sed) received food supplemented with benfotiamine and did not train. The standard diet and trained group (Sta-Tr) received food without benfotiamine and trained swimming. The benfotiamine and trained group (Ben-Tr) received food supplemented with benfotiamine and trained swimming. Mice received isocaloric diets, AIN-93 growth standard rodents' diet (AIN-93G) (Reeves, Nielsen & Fahey, 1993) or AIN-93G supplemented with benfotiamine (500 mg kg^{-1}).

The experimental protocol lasted 7-weeks. The first week was destined to adjustment to laboratory environment and isolated cages. The training protocol started in the second week and supplemented food was offered from the third week onwards. The experiment protocol had previous authorization of Ethic Committee of Animal Use of Federal University of Triangulo Mineiro, under protocol number 343/2015.

Training protocol

Animals swam in groups, in a plastic container of 40 cm diameter and 60 cm height, with tap water depth of 40 cm, maintained at 32°C ($\pm 1^\circ\text{C}$) controlled by heater with automatic thermostat (HOPAR SA-333 Zhong Shan, China) (Chen, Huang, Chiu, Chang & Huang, 2014).

The first training week was an adaptation period, starting with exposure to shallow water in first day and finished with a 60-min swim on the fifth day. In the second week, animals trained with a metal weight fixed to tail relative to 1% of body weight, during 60-min/session in 5 session/week. The overload was 2% of body weight in the following four weeks, with the same duration and frequency of training. The sedentary groups were exposed to shallow water with similar frequency and duration of the training protocol of the trained groups (Chen et al., 2014).

Tissue preparation

The animals were euthanized by cardiac puncture exsanguination after being anesthetized with ketamine/xilazine 24-h after last training session. Left gastrocnemius muscle was excised and homogenized with phosphate buffer pH 7.8 ($1:20 \text{ w vol.}^{-1}$). The content of oxidative damage biomarkers and antioxidant enzymes activity were measured in muscle homogenates. The results were expressed in relation to total protein content, determined using commercial kit (Labtest, São Paulo, Brazil) according to the manufacturer's instructions.

Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) concentration in muscle homogenate was evaluated as a marker of lipid peroxidation. One milliliter of methanol/chloroform (1:2) was added to $600 \mu\text{L}$ of homogenate muscle and TCA 10%. After centrifugation, the aqueous phase was used to determine TBARS. $50 \mu\text{L}$ of reagent 2,5% thiobarbituric acid (TBA)/83% TCA/HCl 2N was added to $250 \mu\text{L}$ of supernatant. After 20-min of incubation in 100°C , $300 \mu\text{L}$ of butanol was added and the mixture was centrifuged. The supernatant absorbance was read with a spectrophotometer at a wavelength of 535 nm (Grintzalis, Zisimopoulos, Grune, Weber, & Georgiou, 2013). The TBARS concentration was determined using an equation of the calibration curve obtained by similar reaction using commercial malondialdehyde solution.

Non-protein thiols

The non-protein thiols content was analyzed by colorimetric method, using the reaction of sulfhydryl group with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) with muscle homogenate supernatant aliquot. After homogenate deproteinization using 10% TCA, 200 μ L of 0.2 M Tris -0.02M EDTA, 300 μ L of DTNB and 1.6 mL of methanol were added to 100 μ L muscle homogenate supernatant. After an incubation of 15-min, the absorbance was read with a spectrophotometer set a wavelength of 412 nm. The non-protein thiols concentration was calculated using the molar extinction coefficient of 13.100 M⁻¹cm⁻¹ (Sedlak & Lindsay, 1968).

Superoxide dismutase activity

SOD activity was determined by the ability of the enzyme to inhibit the pyrogallol autoxidation following a method before described by Marklund and Marklund (1974). Fifty microliters of homogenate were added to 1 mL of 50 mM Tris-Cl /1 mM EDTA pH 8.2 buffer. Then 1 mL of 0.2 mM pyrogallol/50 mM potassium-phosphate buffer pH 6.5 was added and immediately read with the spectrophotometer set a 420 nm. One unit of SOD activity was defined as the SOD amount capable of inhibiting 50% of pyrogallol autoxidation.

Catalase activity

The CAT activity was determined by the ability of the enzyme to inhibit the dichromate/acetic acid reduction to chromic acetate after addition of H₂O₂. One milliliter of 65 mM H₂O₂ / 50mmol L⁻¹ sodium, potassium-phosphate buffer was added to 100 μ L of homogenate. After incubation for 3-min at 37°C, 2 mL of dichromate/acetic acid was added. In the control tube, distilled water was substituted for H₂O₂. In the standard tube, the homogenate was substituted for distilled water. Blank tube was a solution of 1.1 mL of distilled water and 2 mL of dichromate/acetic acid. The tubes were then incubated for 10-min. at 100°C. After a brief centrifugation, the chromic acetate produced was measured colorimetrically at 570 nm (Hadwan, 2016).

Statistical analysis

The data are presented as mean \pm standard deviation. The results were analyzed using the software SPSS 20.0. To check the variances' equality and data distribution, Levene's test and Shapiro-Wilk's test, respectively, were applied. Student's t test for independent samples was used to compare the benfotiamine intake. Two-way analyses of variance (ANOVA) for repeated measures was used to compare body weight evolution. The results of the biochemical analysis and food consumption were compared by ANOVA one-way. Post-hoc Bonferroni's test was applied. A significance level of 95% ($p < 0.05$) was adopted.

Results

The mean body weight was not different among the four experimental groups throughout experiment (Figure 1).

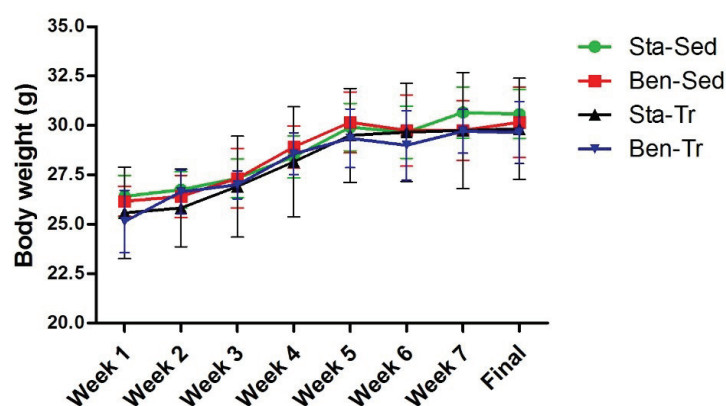


Figure 1. Weekly body weight of animals during the trial period.

Source: Author's own figure

The sedentary groups showed similar food intake. However, trained mice ingested less food than Sta-Sed animal ($p < 0.05$). The Sta-Sed, Ben-Sed, Sta-Tr and Ben-Tr groups ingested 4.47 ± 0.27 g day⁻¹, 4.33 ± 0.25 g day⁻¹, 4.24 ± 0.30 g day⁻¹ and 4.17 ± 0.18 g day⁻¹ respectively. The Ben-Sed group showed no difference in relation to any of the other groups. (Figure 2A). The benfotiamine consumption showed no difference between Ben-Sed and Ben-Tr groups (2.16 ± 0.12 mg day⁻¹ versus 2.09 ± 0.09 mg day⁻¹) (Figure 2B).

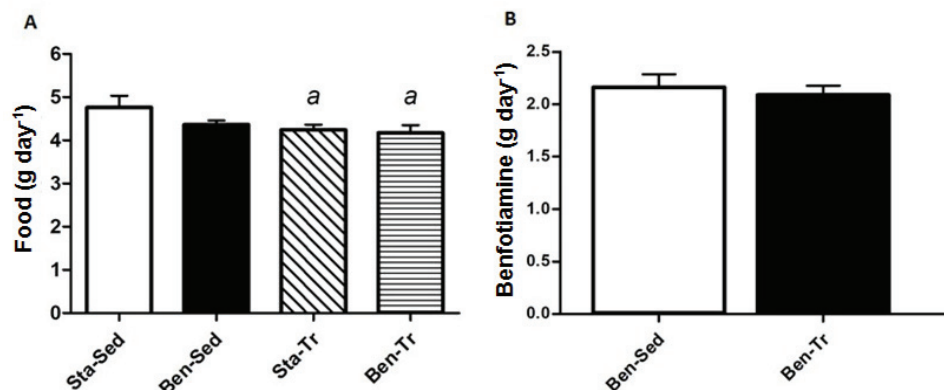


Figure 2. Food and benfotiamine intake; A: Mean food consumption; B: Mean benfotiamine intake in supplemented groups. a= $p < 0.05$ in relation to Sta-Sed.

Source: Author's own figure

The activity of SOD was different only in the Ben-Tr group (21.35 ± 4.39 U.100 mg Pt⁻¹) compared with Sta-Sed (14.01 ± 1.45 U.100 mg Pt⁻¹) and Sta-Tr (12.22 ± 2.95 U.100 mg Pt⁻¹) ($p < 0.05$). The SOD activity of Ben-Sed animals was 16.7 ± 2.92 U.100 mg Pt⁻¹ (Figure 3A).

Catalase activity of Ben-Sed group was higher than Sta-Sed (231.8 ± 41.02 U.100mg Pt-1 versus 144.4 ± 23.96 U.100 mg Pt-1, $p < 0.05$). The CAT activity of Ben-Tr group (230.9 ± 57.63 U.100 mg Pt-1) was more active than Sta-Sed (231.8 ± 41.02 U.100 mg Pt-1) and Sta-Tr (161.5 ± 36.8 U.100 mg Pt-1) ($p < 0.05$) (Figure 3B).

The Ben-Sed group showed higher non-protein thiols concentration (393.0 ± 84.91 nM mg Pt⁻¹) than Sta-Sed (260.1 ± 64.89 nM mg Pt⁻¹) and Sta-Tr (253.9 ± 80.64 nM mg Pt⁻¹) ($p < 0.05$). The non-protein thiols concentration of Ben-Tr was 358.5 ± 57.38 nM mg Pt⁻¹, and there was no difference compared to the other groups (Figure 3C).

The TBARS concentrations of Sta-Sed, Ben-Sed, Sta-Tr and Ben-Tr groups were 17.9 ± 4.22 nM mg Pt⁻¹, 20.09 ± 5.15 nM mg Pt⁻¹, 23.56 ± 4.73 nM mg Pt⁻¹ and 12.4 ± 3.73 nM mg Pt⁻¹ respectively. The TBARS concentration in Ben-Tr group was lower than in Sta-Sed and Sta-Tr groups ($p < 0.05$) (Figure 3D).

Discussion

The present study aimed to investigate the role of benfotiamine in exercise-induced oxidative damage. Thus, mice received standard AIN-93 diet or AIN-93 diet supplemented with benfotiamine. However, the diets were isocaloric with few differences between activity was higher than non-supplemented groups, suggesting that SOD activity in gastrocnemius muscle was increased in response to benfotiamine supplementation. This data shows the effect of benfotiamine on SOD efficiency, in agreement with other studies that showed positive effects of thiamine analogous in SOD activity and gene expression (Bozic et al., 2015; Vidhya, Renjugopal & Indira 2013). In addition, the results of this study suggest that there was a combined effect of exercise and benfotiamine supplementation, since the Ben-Sed group did not show an increase in SOD activity, unlike the Ben-Tr. In fact, a study showed that 3-months of moderate-intensity endurance cycling training enhanced muscle antioxidant systems in humans previously sedentary (Samjoo, Safdar, Hamadeh, Raha & Tarnopolsky, 2013).

Hydrogen peroxide is a ROS very harmful to macromolecules, and the reduced glutathione (GSH) and CAT are the main scavengers of these molecules (Marí, Morales, Colell, García-Ruiz & Fernández-Checa, 2009). In the present study, GSH are represented by non-protein thiols (Dickinson & Forman, 2002). The results showed that muscle thiols concentration was different only between Sta-Tr and Ben-Sed groups.

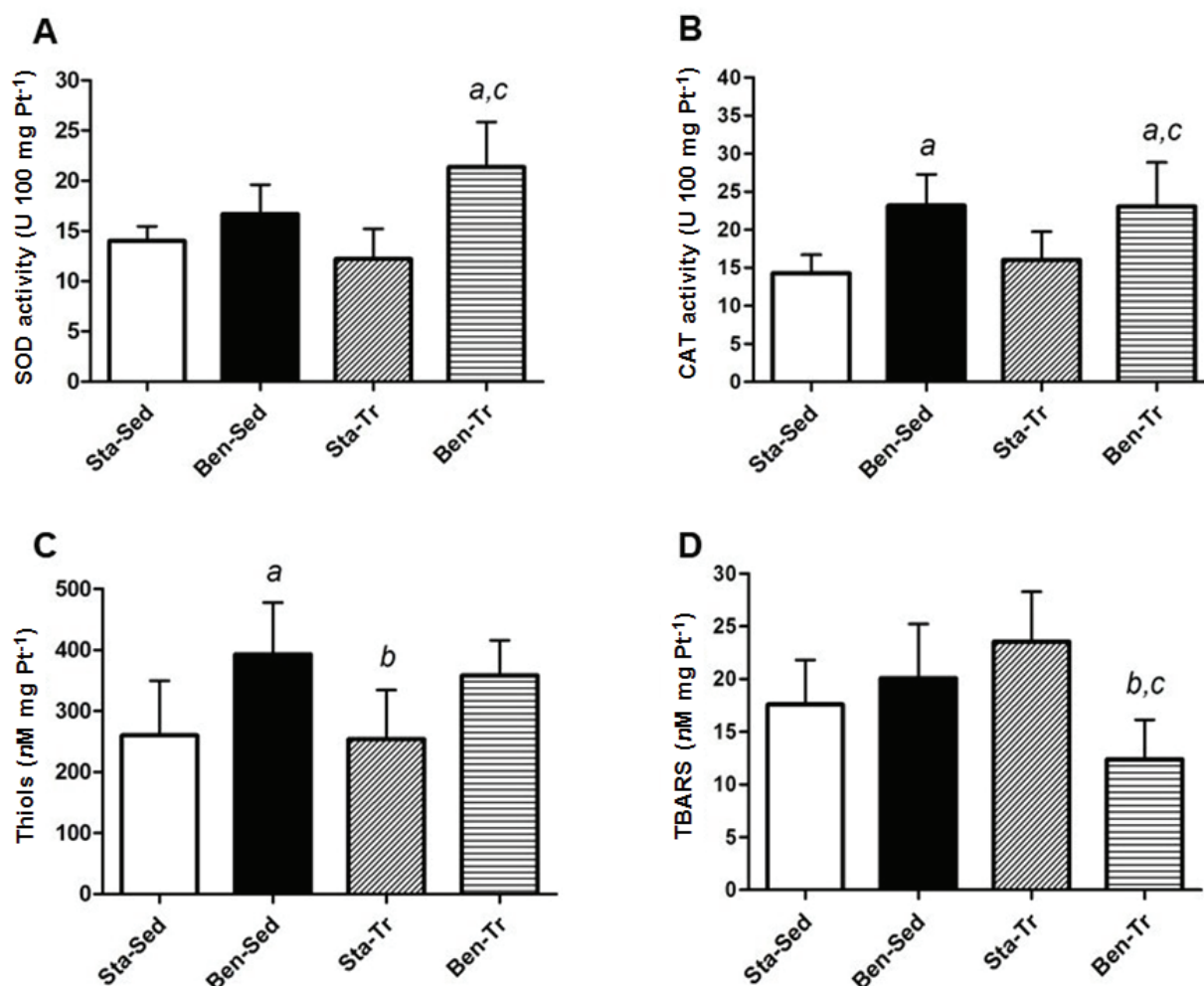


Figure 3. Antioxidant and oxidative damage markers. A: Superoxide dismutase activity; B: Catalase activity; C: non-protein thiols concentration; D: Thiobarbituric acid reactive substances (TBARS) concentration. a = $p < 0.05$ in relation to Sta-Sed; b = $p < 0.05$ in relation to Ben-Sed; c = $p < 0.05$ in relation to Sta-Tr.

Source: Author's own figure

The higher thiols level in Ben-Sed group suggests that benfotiamine supplementation has increased antioxidant concentration, which was confirmed by lower thiols concentration in Sta-Sed and Sta-Tr groups. Benfotiamine could have prevented the GSH consumption, due to its direct antioxidative capacity (Schmid, Stopper, Heidland & Schupp, 2008) and the direct antioxidant role of thiamine (Lukienko et al., 2000). In addition, the pentose phosphate pathway is responsible for GSH recycling from GSSG (Lu, 2009), contributing to increase in non-protein thiols concentration in muscle of supplemented group.

Both supplemented groups presented higher CAT activity than the groups fed with standard food. This result can be related to an increase in thiamine diphosphate (TDP) levels in muscle tissue, since TDP is a coenzyme in the pentose phosphate pathway (PPP). The PPP produces NADPH, a molecule that plays a role in resynthesizing a biologically active form of CAT, the Compound I (Kirkman, Galiano, & Gaetani, 1987).

The increase in enzyme activity and thiols concentration can be related to molecular factors. The increase in TDP levels is capable of hampering the activity of p53 protein (McLure, Takagi & Kastan, 2004). The p53 protein inhibits gene expression of antioxidant enzymes (Drane, Bravard, Bouvard & May, 2001). In confirmation, a study showed that the translocation of the p53 gene to mitochondria promotes MnSOD inactivation (Zhao et al., 2005). Therefore, benfotiamine supplementation could have reduced the p53 action, increasing the gene expression of SOD, CAT and GPx, and consequently, their activities.

TBARS was used as a tissue damage biomarker. The muscle TBARS concentration was lower in the Ben-Tr group than in Ben-Sed and Sta-Tr group. This data suggests that benfotiamine supplementation is an efficient strategy to prevent lipid peroxidation in trained mice. Indeed, a study showed that benfotiamine supplementation reduces serum TBARS concentration in diabetic humans after a meal with advanced glycation end products (Stirban et al., 2006). Lipid peroxidation mainly affects cell membranes. Thus,

increase in TBARS levels indicates significant damage to the cell, that could harm the cell and/or organelles function and advance apoptosis process. If the damage occurs in mitochondria, energy production will be impaired, which can harm exercise performance and training adaptations (Anderson, Katunga & Willis, 2012).

Despite benfotiamine supplementation decreasing oxidative damage and increasing antioxidant defenses in trained mice, it is not possible to affirm that exercise endurance performance is enhanced. In fact, some studies report an increase in ROS production during exercise (Cobley et al., 2011; Kuwahara et al., 2010) and positive effects of antioxidant supplementation on endurance performance (Abadi et al., 2013). However, evidences about antioxidant supplementation and endurance performance are controversial. There is evidence demonstrating an important decrease in fatigue resistance and expression of molecules related to mitochondrial biogenesis in trained mice treated with vitamin C (Gomez-Cabrera et al., 2008). On the other hand, a study found no changes in endurance performance of men that ingested vitamin C and E, with significant antioxidant effect (Yfanti et al., 2010). Possibly, further investigations could find more and stronger evidences about the antioxidant role of benfotiamine during training period and how thiamine and its esters could interfere in endurance adaptation mechanisms.

Conclusion

The present study found evidences that benfotiamine supplementation is an efficient strategy to enhance the main antioxidant defenses and diminish oxidative damage in gastrocnemius muscle of endurance-trained mice. Despite benfotiamine supplementation enhancing the redox status in muscle, these results do not allow to sustain that endurance performance of mice subject to swimming training can be improved with supplementation, and we suggest further research to clarify this question. However, benfotiamine supplementation could be prescribed to endurance athletes and people who engage in endurance training programs aiming to prevent muscle oxidative damage.

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