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Caffeine supplementation modulates oxidative stress markers in the liver of trained rats

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ABSTRACT

Aims: Caffeine has been widely used in sports competitions due to its ergogenic effects. Most of the studies regarding caffeine and exercise have focused on muscle and plasma adaptations, while the impact on the liver is scarcely described. The aim is to analyze the effects of caffeine and exercise training on oxidative stress markers and injury-related parameters in the liver.
Main methods: Rats were divided into sedentary/saline, sedentary/caffeine, exercise/saline, and exercise/caffeine groups. Exercise groups underwent 4 weeks of swimming training, and caffeine (6 mg/kg, p.o.) was supplemented throughout the training protocol. Injury-related liver parameters were assessed in plasma, while redox status and oxidative stress markers were measured on liver homogenates.
Key findings: Exercise training increased muscle citrate synthase activity in the muscle, while in caffeine decreased its activity in both sedentary and trained rats. Aspartate transaminase levels were increased after training, and caffeine intake suppressed this elevation ($p < 0.05$). Caffeine also diminished alanine transaminase levels in both sedentary and exercised rats ($p < 0.05$). Exercise training induced a significant increase on the activity of the enzymes superoxide dismutase and glutathione peroxidase, as an increase on thiobarbituric acid-reactive substances levels was also reached ($p < 0.05$); caffeine intake blunted these alterations. Caffeine intake also suppressed liver catalase activity in both sedentary and exercise groups ($p < 0.05$).
Significance: Our data suggest that caffeine modified the hepatic responses associated to exercise-induced oxidative stress without affecting the performance, exerting different actions according to the tissue. However, further studies are needed to better understand caffeine's role on liver under exercise training.

Introduction

Caffeine (1,3,7-trimethylxanthine) is a xanthine alkaloid compound present in many commercial beverages and medicines that acts as a potent stimulant of the central nervous system (CNS) (Tunnicliffe et al., 2008). In addition, caffeine incites skeletal muscle metabolism and therefore it has been widely used in athletic competitions due to its ergogenic effects (Kalmar and Cafarelli, 2004; Tarnopolsky, 2008; Goldstein et al., 2010). Its widespread use is allowed by the World Anti-Doping Agency's despite evidence-based data that documents its physiological and performance-enhancing effects (Tarnopolsky, 2010). In this line, it is well known that caffeine may affect substrate utilization during exercise (Tunnicliffe et al., 2008; Goldstein et al., 2010; Yang et al., 2009; Davis et al., 2003). Caffeine increases fatty acid mobilization during exercise thus decreasing glycogen reliance during performance (Ivy et al., 1979; Erickson et al., 1987; Spriet et al., 1992). These effects have been broadly linked to improvements on aerobic exercise performance due to enhanced twitch strength of both skeletal and cardiac muscles, which result in delay fatigue onset (Goldstein et al., 2010; Tarnopolsky and Cupido, 2000; Simmonds et al., 2010).

On the same line, it is well known that regular exercise training plays a protective role against lifestyle-related diseases across health status and quality of life improvements (Radak et al., 2004, 2005a,b). Accordingly, it has been stated that regular exercise training may increase the resistance to various stressors via hormesis (Radak et al., 2005a). The molecular events involved in this regulation may be linked to redox status homeostasis, an oxidative stress-related adaptive response (Radak et al., 2008; Jackson, 2008; Gomez-Cabrera et al., 2008). In fact, exercise training incites regular adaptations to the continuous presence of small stimuli such as mild amounts of reactive oxygen species (ROS). In this case, the regular stimuli can trigger the expression of antioxidant enzymes and modulates other oxidative stress markers (Radak et al., 2005a; Jackson, 2008; Gomez-Cabrera et al., 2008; Ji, 1993).

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Although growing evidence shows the beneficial effects of caffeine intake on skeletal muscle performance during exercise training, the role of caffeine in other tissues is scarcely described. Specifically, the influence of caffeine on the oxidative responses elicited by exercise training has been mostly limited to the skeletal muscle, brain and plasma samples. Therefore, considering the remarkable metabolic role of the liver during exercise, this study aimed to analyze the isolated and/or combined effects of caffeine supplementation and exercise training on oxidative stress and tissue injury-related markers.

Materials and methods

Ethical approval

The experimental assays were conducted in accordance to national and international legislations (Brazilian College of Animal Experimentation (COBEA) and the U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals-PHS Policy). The study protocol was also approved by the Ethics Committee for Animal Research of the Universidade Federal de Santa Maria (UFSM, permit number 115/2010) before experimental set beginning.

Animals and reagents

Male Wistar rats (180–250 g) were obtained from our own breeding colony and kept in plastic boxes containing a maximum of five animals per cage. After, cages were placed in controlled environment conditions (12:12 h light–dark cycle, with onset of light phase at 7:00, 25 ± 1 °C, 55% relative humidity) with food (Guabi, Santa Maria, Brazil) and water ad libitum. Assay reagents were purchased from Sigma (St. Louis, MO, USA), and the other chemicals used in this study were of analytical grade and obtained from standard commercial suppliers.

Study design

The animals were randomly divided into four groups (n = 8): sedentary-saline (SED-SAL), sedentary-caffeine (SED-CAF), exercise-saline (EXE-SAL) and exercise-caffeine (EXE-CAF). The exercise and sedentary groups received caffeine (6 mg/kg in saline) or its vehicle by intragastric gavage (p.o.) through the experimental period.

Training protocol

For exercise training, animals were weighed and randomly assigned to the aforementioned groups. The tank used in this study was 80 cm in length, 50 cm in width, and 90 cm in depth, and the swimming training was performed in water temperature of 31 ± 1 °C (70 cm depth) between 10 and 12 h am. The training session consisted of 50 min per day and 5 days per week (Song et al., 1998). The EXE groups performed swimming training with a 5% body weight overload attached to the back to improve endurance (Lima et al., 2013; Gobatto et al., 2001). The SED groups were placed in a separate but similar tank with shallow water (5 cm) at the same temperature for 30 min, 5 days a week without the back overload. Caffeine supplementation (6 mg/kg) was administered daily throughout the training protocol (Fredholm et al., 1999).

Tissue sampling, and organs weighting

At the end of the exercise training protocol, the rats were euthanized and, liver, adrenal gland, soleus and gastrocnemius muscles were removed and weighted. A single ratio between organ and total body weights was calculated to express this data. Samples of liver and gastrocnemius muscle were quickly removed, placed on ice, and homogenized within 10 min in 10 volumes of cold Tris 10 mM (pH 7.4). Liver and muscle homogenates were centrifuged at 4000 ×*g* at 4 °C for 10 min to yield the low-speed supernatant fraction that was used for different biochemical assays in all trials. Blood samples were collected and centrifuged 1500 ×*g* for 10 min for plasma isolation in order to perform biochemical analysis.

Plasma assays

Biochemical parameters

Plasma levels of creatinine kinase (CK), aspartate transaminase (AST), alanine transaminase (ALT), triglycerides (TG), total cholesterol (TC), uric acid (UA), high density lipoprotein (HDL) and urea (UR) were estimated by standard commercially biological kits (Labtest, Lagoa Santa, Brazil).

Estimation of DNA damage

The cell death indicated by the presence of double strand DNA in the plasma (dsDNA) was measured using the PicoGreen® fluorescent assay (Ahn et al., 1996). The assay was performed according to protocol supplied by the manufacturer (Quant ItTM, Invitrogen, Brazil). The fluorescence measurements were recorded on a fluorimeter, and fluorescence emission of PicoGreen® alone (blank) and PicoGreen® with DNA were recorded at 520 nm using an excitation wavelength of 480 nm at room temperature (25 °C). The results were expressed as % of control.

Citrate synthase (CS) activity

Citrate synthase activity was determined spectrophotometrically in mixed gastrocnemius muscle and liver according to the method previously described (Srere, 1968). The enzyme activity was measured in homogenates and the amount of the complex resulting from acetyl-CoA and oxaloacetate was determined at 412 nm and 25 °C. The CS activity was expressed as percentage of control.

Liver homogenate assays

Catalase (CAT) activity

The CAT enzyme activity was determined according to the method proposed by Aebi (1984). The kinetic analysis of CAT was started after H_2O_2 addition and the color reaction was measured at 240 nm. Data were corrected by the protein content and expressed as percentage of control.

Superoxide dismutase (SOD) activity

The SOD enzyme activity was determined according to the method proposed by Misra and Fridovich (1972). The kinetic analysis of SOD was started after adrenaline addition, and the color reaction was measured at 480 nm. Data were corrected by the protein content and expressed as percentage of control.

Glutathione peroxidase (GPx) activity

The GPx activity was determined spectrophotometrically at 340 nm by NADPH consumption for 2 min at 30 °C (Flohé and Günzler, 1984). The reaction was initiated by adding the H_2O_2 to a final concentration of 0.4 mM. The GPx activity was determined using the molar extinction coefficient 6220 M^{-1} cm^{-1} and expressed as percentage of control.

Glutathione reductase (GR) activity

For GR activity determination, the measurements were made at 340 nm and initiated with addition of 20 mM GSSG, at 30 °C for 2 min (Carlberg and Mannervik, 1985). The GR activity was determined using the molar extinction coefficient 6220 M^{-1} cm^{-1}, corrected by the protein content, and expressed as percentage of control.

Fluorimetric assay of reduced (GSH) and oxidized glutathione (GSSG)

For measurement of GSH and GSSG levels, the method previously described by Hissin and Hilf (1976) was used (Hissin and Hilf, 1976).

The GSH and GSSG levels were determined from comparisons with a linear GSH or GSSG standard curve, respectively. The results were expressed as GSH/GSSG ratio.

Thiobarbituric acid reactive substance (TBAR) levels determination

Lipid peroxidation was estimated by measuring TBARS and expressed in terms of malondialdehyde (MDA) content according to the method of Ohkawa et al. (1979). The TBAR levels were measured at 532 nm, and the absorbance was compared with the standard curve using MDA and corrected by the protein content.

Protein determination

The protein content was determined as described previously (Lowry et al., 1951) using bovine serum albumin (BSA) as standard.

Statistical analysis

Statistical analysis was performed using two-way analysis of (ANOVA), followed by Student–Newman–Keuls test when appropriate to determine possible interactions. Data are expressed as means ± SEM. Values of $p < 0.05$ were considered significant.

Results

Total body weight and organ-to-body weight ratio

The EXE groups had a reduction in the body weight gain when compared to the SED groups as seen in Fig. 1 ($p < 0.05$). This exercise training effect was not modified by caffeine intake. Similarly, caffeine did not change the body weight of SED groups. No changes in the organ-to-body weight ratio were observed for liver, adrenal gland, soleus, or gastrocnemius muscles among the groups (Table 1).

Plasma biochemical levels

No significant differences in plasma lipid content (TC, TG, and HDL-cholesterol), urea, or uric acid were detected among the groups as presented in Table 2.

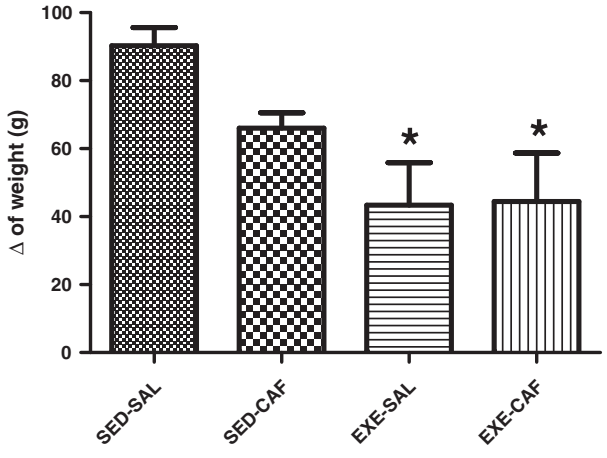

Fig. 1. Effects of exercise training and caffeine supplementation on body weight gain. Data are presented as total body weight gain after 4 weeks of exercise and expressed as means ± SEM (n = 5–8). *Denotes $p < 0.05$ when compared with the control group (SED-SAL). SED-SAL: sedentary-saline; SED-CAF: sedentary-caffeine; EXE-SAL: exercise-saline; EXE-CAF: exercise-caffeine.

Table 1
Effects of swimming training and caffeine on relative organ/body weight. The data are expressed as means ± SEM (n = 5–8) and presented as the ratio between tissue and total rat weight.

	SED-SAL	SED-CAF	EXE-SAL	EXE-CAF
Liver (10^{-2})	3.50 ± 0.34	3.03 ± 0.14	3.278 ± 0.11	3.275 ± 0.13
Adrenal (10^{-4})	2.58 ± 0.26	2.09 ± 0.10	1.99 ± 0.14	2.19 ± 0.16
Soleus (10^{-4})	8.60 ± 0.91	9.63 ± 1.11	11.13 ± 1.14	11.079 ± 1.06
Gastrocnemius (10^{-3})	4.79 ± 0.36	5.10 ± 0.31	5.48 ± 0.25	5.34 ± 0.27

SED-SAL: sedentary-saline; SED-CAF: sedentary-caffeine; EXE-SAL: exercise-saline; EXE-CAF: exercise-caffeine.

Muscle and liver citrate synthase (CS) activity

Statistical analysis showed that CS activity was higher among EXE groups in the gastrocnemius muscle (Fig. 2a). On the opposite, liver CS activity decreased with caffeine supplementation on both SED and EXE groups (Fig. 2b, $p < 0.05$).

Injury-related markers

Table 3 depicts the results of tissue injury-related markers in plasma. No differences were found for DNA PicoGreen® among the groups. Conversely, exercise training induced an increase in plasma AST and CK levels when compared to SED groups ($p < 0.05$). Caffeine intake restored the AST levels modified by exercise and diminished ALT levels in both SED/CAF and EXE/CAF rats. Caffeine intake did not alter plasma CK levels in EXE or SED rats.

Hepatic markers

Antioxidant enzyme activities

The liver antioxidant enzyme activities are summarized in Fig. 3. Caffeine intake reduced CAT activity in both SED/CAF and EXE/CAF groups (Fig. 3a, $p < 0.05$). Exercise training caused an increase in SOD and GPx activities when compared with SED/SAL rats ($p < 0.05$). These effects induced by exercise on antioxidant enzymes were blunted by caffeine intake (Fig. 3b and c, $p < 0.05$). No differences among the groups were observed regarding GR activity (data not shown).

Oxidative stress parameters

Fig. 4 presents the results of the oxidative stress markers in liver homogenates. No significant differences in the GSH/GSSG ratio were found among the groups (Fig. 4a). The levels of TBARS were significantly augmented in EXE/SAL group compared to SED/SAL rats (Fig. 4b, $p < 0.05$). This effect was suppressed by caffeine intake in EXE/CAF rats.

Discussion

In the present study, swimming training increased muscle CS activity, plasma CK and AST levels and decreased the weight gain of rats. Exercise training also induced changes on the antioxidant status and oxidative markers in the liver, specifically represented by lipid peroxidation and SOD and GPx activities increased. Most effects linked to

Table 2
Effects of swimming training and caffeine treatment on biochemical serum parameters. The data are expressed as means ± SEM (n = 5–8).

	SED-SAL	SED-CAF	EXE-SAL	EXE-CAF
TC (UI)	1.55 ± 0.07	1.41 ± 0.11	1.51 ± 0.09	1.55 ± 0.07
TG (UI)	0.61 ± 0.06	0.64 ± 0.08	0.61 ± 0.03	0.69 ± 0.09
HDL (UI)	2.96 ± 0.48	2.95 ± 0.25	2.85 ± 0.15	2.76 ± 0.24
UR (nmol/L)	8.11 ± 0.90	8.34 ± 1.14	7.64 ± 0.84	8.09 ± 0.48
UA (μmol/L)	79.90 ± 17.90	53.98 ± 5.00	55.22 ± 5.73	65.70 ± 6.25

Total cholesterol (TC); triglycerides (TG); high-density lipoprotein (HDL); urea (UR); uric acid (UA). SED-SAL: sedentary-saline; SED-CAF: sedentary-caffeine; EXE-SAL: exercise-saline; EXE-CAF: exercise-caffeine.

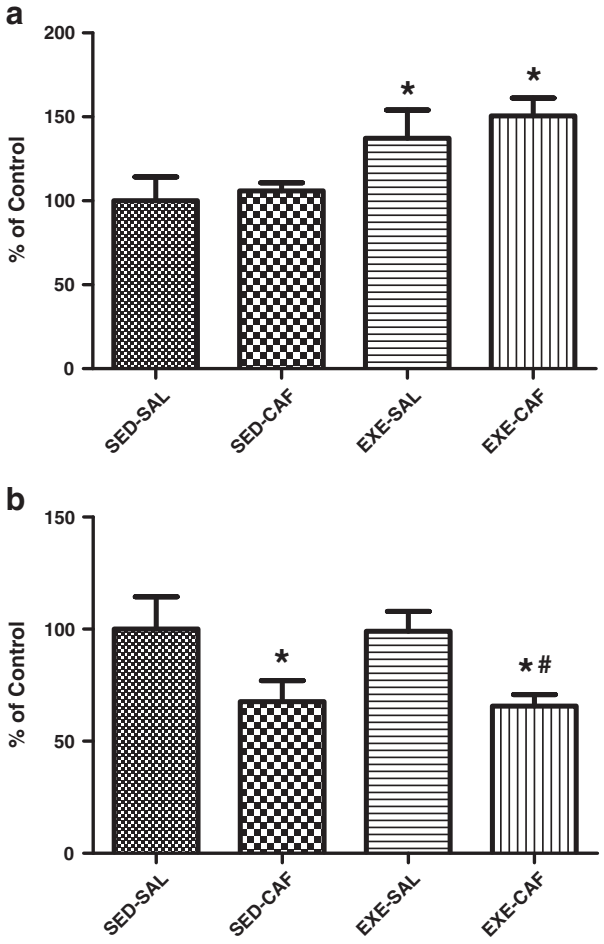

Fig. 2. Effects of exercise training and caffeine supplementation on muscle (a) and liver (b) citrate synthase activity. The data are presented as percentage of control and expressed as means ± SEM (n = 5–8). *Denotes $p < 0.05$ when compared with the control group (SED-SAL). #Denotes $p < 0.05$ when compared with the EXE-SAL group. SED-SAL: sedentary-saline; SED-CAF: sedentary-caffeine; EXE-SAL: exercise-saline; EXE-CAF: exercise-caffeine.

exercise training were modified by caffeine intake. Caffeine decreased CS activity in both SED/CAF and EXE/CAF groups in the liver.

Exercise trained rats exhibited a plateau in body weight gain during the 4-week training period. This finding corroborates previous data indicating that swimming training stabilizes the body weights of rats (Lima et al., 2013; Ravi Kiran et al., 2006; Clavel et al., 2002). This plateau in body weight may be related to the intensity of the swimming protocol applied in this study considering it has been long stated that intense training influences body weight in rats (Lima et al., 2013). Despite the well-described caffeine effects on the metabolism, the CAF groups did not show differences compared to SAL groups.

Table 3
Effects of swimming training and caffeine treatment on tissue injury-related marker levels. The data are expressed as means ± SEM (n = 5–8) comparing to control group (%).

	SED-SAL	SED-CAF	EXE-SAL	EXE-CAF
AST	100.00 ± 5.83	91.49 ± 7.84	132.66 ± 10.76*	104.19 ± 5.46#
ALT	100.00 ± 10.62	63.29 ± 3.55*	84.51 ± 9.13	66.22 ± 6.20*#
DNA	100.00 ± 4.54	102.60 ± 4.26	102.34 ± 3.91	95.02 ± 3.45
CK	100.0 ± 10.84	140.6 ± 23.86	225.41 ± 46.11*	233.3 ± 40.49*

Aspartate transaminase (AST); and alanine transaminase (ALT); creatine kinase (CK). SED-SAL: sedentary-saline; SED-CAF: sedentary-caffeine; EXE-SAL: exercise-saline; EXE-CAF: exercise-caffeine.
* Denotes $p < 0.05$ when compared with the control group (SED-SAL).
# Denotes $p < 0.05$ when compared with the EXE-SAL group.

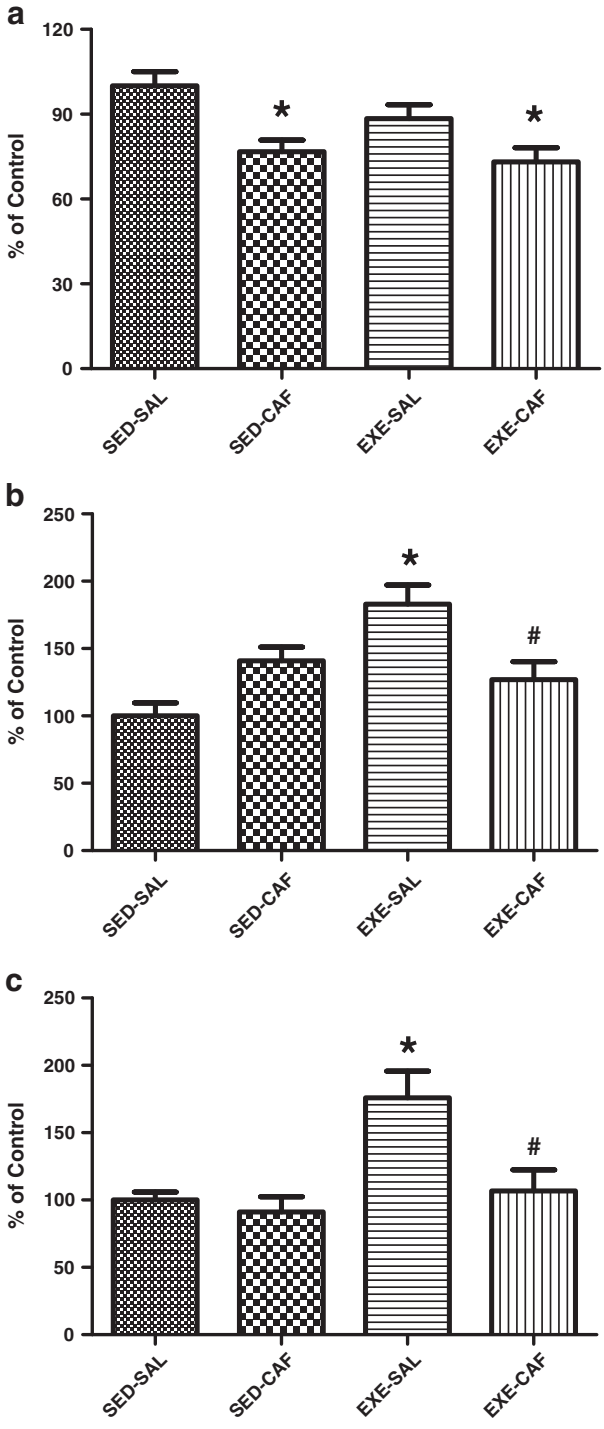

Fig. 3. Effects of exercise training and caffeine supplementation on liver catalase (a), superoxide dismutase (b), and glutathione peroxidase (c) activities. The data are presented as means ± SEM (n = 5–8) and expressed as percentage of control. *Denotes $p < 0.05$ when compared with the control group (SED-SAL). #Denotes $p < 0.05$ when compared with EXE-SAL group. SED-SAL: sedentary-saline; SED-CAF: sedentary-caffeine; EXE-SAL: exercise-saline; EXE-CAF: exercise-caffeine.

The EXE groups exhibited higher CK levels, potentially indicating exercise-related damage caused by intense swimming training. In this regard, there is evidence that high CK levels lead to oxidative damage and increased lipid peroxidation, which was also seen in this study through increased TBAR levels on the EXE/SAL group (Itoh et al., 2000; Jiménez et al., 2001; Márquez et al., 2001; Zajac et al., 2001). On the same line, muscle CS activity was higher in the trained groups, supporting previous reports on favorable adaptations of the aerobic

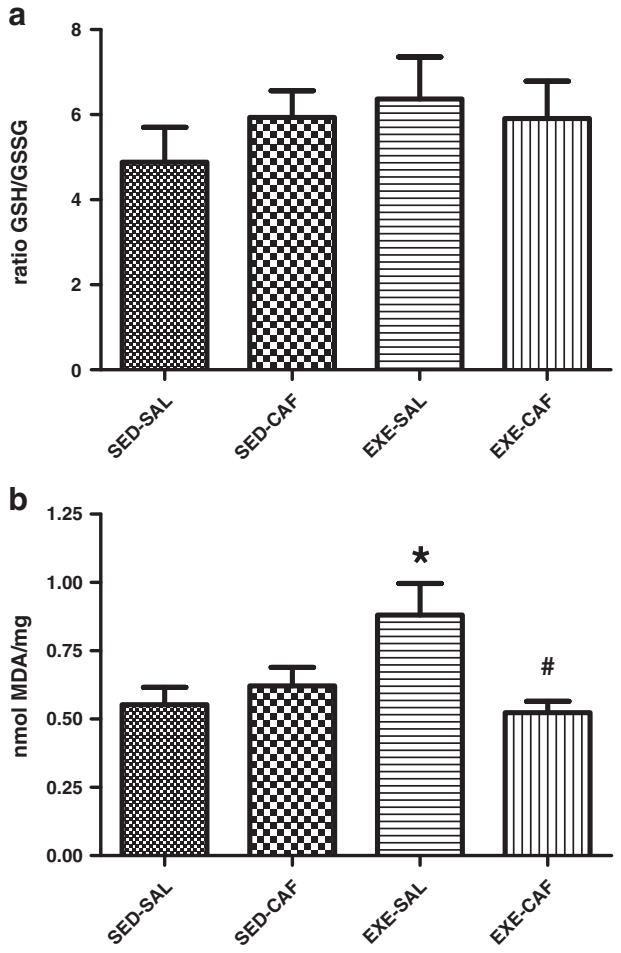

Fig. 4. Effects of exercise and caffeine on liver GSH/GSSG ratio (a) and TBAR levels (b). The data are represented as means ± SEM (n = 5–8). *Denotes $p < 0.05$ when compared with the control group (SED-SAL). #Denotes $p < 0.05$ when compared with EXE-SAL group. SED-SAL: sedentary-saline; SED-CAF: sedentary-caffeine; EXE-SAL: exercise-saline; EXE-CAF: exercise-caffeine.

metabolism to regular training (Jackson, 2008; Gomez-Cabrera et al., 2008; Ji, 1993; Ristow and Schmeisser, 2011; Ji et al., 2006). However, our results showed that the liver CS activity was decreased in CAF groups, which may indicate a differential caffeine modulation according to the analyzed tissue. The decreased liver CS activity was accompanied by a similar tissue injury-related marker modulation. AST and ALT are useful plasmatic parameters of hepatic injury that are associated with liver toxicity (Qin et al., 2007; Ozer et al., 2008; de David et al., 2011). In this study, increases in AST levels among EXE/SAL rats were observed. This is in agreement with previous studies that suggest exhaustive exercise may increase AST and ALT activities (Bowers et al., 1978). Interestingly, caffeine supplementation protected the liver from increased AST levels induced by swimming training, and reduced ALT levels in SED/SAL animals. In this sense, ALT levels showed a similar trend as CS activity in the liver, indicating a decrease on liver metabolism through caffeine supplementation. These are remarkable findings and corroborate previous studies showing that caffeine may prevent increases in plasma AST and ALT and thus decrease the risk of chronic liver damage (Honjo et al., 2001; Ruhl and Everhart, 2005; Tanaka et al., 1998; Cadden et al., 2007). These data suggest that exercise-induced liver adaptations may be modified by caffeine supplementation on a tissue-specific trend.

To the best of our knowledge, this is the first study that investigates the isolated and combined effects of caffeine on the liver redox status homeostasis of sedentary and trained rats. Regular exercise imposes a mild beneficial ROS production, which increases organic resistance to various diseases (Radak et al., 2004, 2005a,b). This recent hypothesis has been extended to the ROS-generating effects of exercise (Radak et al., 2005a; Ji et al., 2006). Regular exercise appears to exert a dual effect: (1) the generation of oxidative stress and, consequently, (2) increased antioxidant enzyme activities that minimize the deleterious effects of these oxidants (Liu et al., 2000; Ji, 1996). Unlike acute and exhaustive exercises which allow insufficient recovery for biochemical adaptations, these antioxidant modulations have been observed during regular exercise training such as the swimming training herein described (Fukai et al., 2000; Rush et al., 2003). In our experimental protocol, swimming training induced compensatory responses regarding oxidative stress. Specifically, SOD and GPx activities were enhanced probably due to increased TBAR content in the EXE/SAL group. Similarly, the increase on GPx activity following vigorous exercise has been suggested as an adaptation to efficiently eliminate exercise-related ROS production and to minimize ROS-mediated damage (Oh-ishi et al., 1997). Therefore, it seems that TBAR increases were compensated by equal increases on SOD and GPx activities in order to maintain redox status. Nevertheless, EXE/CAF presented similar redox status when comparing to SED groups, indicating the antioxidant role of caffeine. Interestingly, this antioxidant activity of caffeine in the liver did not influence the muscle aerobic capacity herein assayed across the CS activity.

Accumulating evidence has suggested a potential antioxidant role for caffeine (Aoyama et al., 2011; Rossowska and Nakamoto, 1994; Zeidán-Chuliá et al., 2013). Chemical studies have proposed a ROS scavenging role for caffeine, particularly the hydroxyl radical (OH·) (Shi et al., 1991; Devasagayam et al., 1996). The beneficial effects of caffeine are usually attributed to its major metabolites 1-methylxanthine and methyluric acid, which are highly effective antioxidants (Lee, 2000). Herein, we verify that caffeine intake blunted exercise-induced increases in TBARS, SOD, and GPx in the liver. These effects were accompanied by decreases on CS activity in the liver of CAF groups, which indicates a decreased aerobic metabolism. In fact, epidemiologic studies have demonstrated that caffeine intake is associated with reduced levels of oxidative stress biomarkers (Ofluoglu et al., 2009); however, a growing number of evidence has indicated the deleterious effects of the antioxidant treatment to performance. In exercise training it has been described that an antioxidant supplementation may prevent the expected adaptations associated with regular training in the muscle (Jackson, 2008; Gomez-Cabrera et al., 2008). In this study, we observed a similar association with caffeine supplementation during regular exercise training which blunted antioxidant adaptations in the liver. Although caffeine have exhibited antioxidant properties in liver redox status, the aerobic training performance markers (muscle CS activity) did not changed by caffeine administration in our experimental protocol. Moreover, caffeine supplementation significantly reduced the liver CS activity, showing that in this specific tissue caffeine may influence cell bioenergetics and reduce its metabolism. Interestingly, this decrease in liver metabolism did not affect exercise performance, considering both EXE groups presented similar CS activity in the muscle. Moreover, EXE/CAF group showed the same aerobic capacity as EXE/SAL in spite of redox status and injury-related markers decreases, indicating that the liver was protected by caffeine supplementation without performance impairment.

Conclusion

In addition to previous findings in skeletal muscle, brain and plasma, our results suggest that exercise training induced liver adaptations associated to oxidative stress. Caffeine, which has antioxidant properties and is widely used in athletic competitions, blunted these exercise-related responses and decreased the liver CS activity. Thus, this study points out the differential antioxidant role of caffeine in the liver without performance deterioration. However, further investigations to undercover the role of caffeine under exercise training on the liver are still needed.

Conflict of interest

The authors declare that there are no conflicts of interest.

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