Caffeine prevents high-intensity exercise-induced increase in enzymatic antioxidant and Na\(^{+}\)-K\(^{+}\)-ATPase activities and reduction of anxiolytic-like behaviour in rats

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ABSTRACT

Objective: Here we investigated the impact of chronic high-intensity interval training (HIIT) and caffeine consumption on the activities of Na\(^{+}\)-K\(^{+}\)-ATPase and enzymes of the antioxidant system, as well as anxiolytic-like behaviour in the rat brain.

Methods: Animals were divided into groups: control, caffeine (4 mg/kg), caffeine (8 mg/kg), HIIT, HIIT plus caffeine (4 mg/kg) and HIIT plus caffeine (8 mg/kg). Rats were trained three times per week for 6 weeks, and caffeine was administered 30 minutes before training. We assessed the anxiolytic-like behaviour, Na\(^{+}\)-K\(^{+}\)-ATPase, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities, levels of reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) in the brain.

Results and discussion: HIIT-induced anxiolytic-like behaviour increased Na\(^{+}\)-K\(^{+}\)-ATPase and GPx activities and TBARS levels, altered the activities of SOD and CAT in different brain regions, and decreased GSH levels. Caffeine, however, elicited anxiogenic-like behaviour and blocked HIIT effects. The combination of caffeine and HIIT prevented the increase in SOD activity in the cerebral cortex and GPx activity in three brain regions. Our results show that caffeine promoted anxiogenic behaviour and prevented HIIT-induced changes in the antioxidant system and Na\(^{+}\)-K\(^{+}\)-ATPase activities.

Introduction

Healthier lifestyles are frequently associated with the regular practice of physical exercise, which disrupts cellular homeostasis by stimulating muscular activity [1]. Researchers have sought to elucidate the benefits of high-intensity interval training (HIIT), a training characterized by brief periods (around 30 seconds) of extreme stress (≥90–100% of VO\(_2\) max) intercalated with short periods of recovery [2]. HIIT exercise affects all body systems, increasing muscle glycogen levels [3], aerobic capacity and muscle hypertrophy [4], and also improves brain functions [5]. Studies show that HIIT increases the levels of brain-derived neurotrophic factor (BDNF) and insulin receptors, improving glucose uptake and metabolism [6,7,8]. Moreover, HIIT has been shown to improve blood flow in the brain [9] and activate signalling pathways that promote adult hippocampal neurogenesis [10].

Na\(^{+}\)-K\(^{+}\)-ATPase plays a key role in the maintenance of intracellular electrolyte homeostasis in virtually all tissues [11]. Reduction of Na\(^{+}\)-K\(^{+}\)-ATPase levels and activity directly impairs neurotransmitter signalling with deleterious consequences on learning and memory and increases locomotor activity and anxiety [12,13]. Impairment of Na\(^{+}\)-K\(^{+}\)-ATPase activity and/or mutations in its alpha subunits lead to neuronal dysfunction and may trigger depression, anxiety and bipolar disease [14,15,16].

Intake of ergogenic substances has become a common strategy to enhance sports performance. Caffeine is an alkaloid compound with psychostimulant effects that is mainly consumed through tea and coffee [17], and that acts as a non-selective adenosine receptor antagonist [18], with antioxidant [19,20] and neuroprotective effects [21–24]. In recent decades, athletes have used caffeine to improve their performance [25–27], and several studies have suggested that intake of 3–9 mg/kg of caffeine can slow the process of fatigue during long-term exercise, prolonging activity by 20–50% [27–30]. However, caffeine doses greater than 15 mg/kg trigger several undesired effects, including anxiety, irritability, tachycardia, nausea and seizures [31,32].

Considering that HIIT has beneficial effects on the central nervous system (CNS), we investigated the role of caffeine in combination with HIIT training in anxiolytic-like behaviour and in the activity of Na\(^{+}\)-K\(^{+}\)-ATPase and oxidative stress biomarkers in the rat brain.

Materials and methods

Animals

Male Wistar rats (100 days old; 250–280 g) from the Central Animal House of the Federal University of Santa Maria (UFSM) were maintained at a constant temperature (23 ± 1°C) on a 12-hour light/dark cycle with free access to food.
and water. All animal procedures were approved by the Animal Ethics Committee of the UFSM (077/2011).

**Protocol of HIIT training**

We used swimming as an HIIT exercise. In the first 5 days, all rats were put in shallow water (5 cm deep, 32°C) for 20 minutes before beginning the training. The objective was to give the animals time to adapt to the new environment and reduce their stress without, however, promoting adaptation to the training. After this procedure, animals were put in a circular tank (115 cm diameter, 90 cm depth) with water (32°C) for 12 minutes per day for 10 consecutive days (Table 1, Figure 1).

**Chronic protocol for HIIT and caffeine intake**

Forty-eight rats from different litters were divided into six groups: vehicle, caffeine (4 mg/kg), HIIT, HIIT plus caffeine (4 mg/kg) and HIIT plus caffeine (8 mg/kg). The exercise groups were trained three times a week (with 48 hours of recovery between sessions) for 6 weeks, with increases in the workload corresponding to 23% of the body weight by the end of the experiment. The animals were submitted to a HIIT protocol consisting of 12 sets of swimming exercises of 25 seconds each, alternated with 35 seconds of recovery [33–35]. Non-trained groups were placed in shallow water at 32 ± 1°C for 12 minutes three times per week.

Treatment with caffeine started after the adaptation to HIIT. Caffeine was dissolved in a 0.9% saline solution (1 ml/kg) and was orally administered 30 minutes before training, 5 days per week at a dose of 4 or 8 mg/kg. The vehicle and HIIT groups received saline. Caffeine was administered for 6 weeks until 48 hours after the last training. After behavioural testing, animals were submitted to euthanasia.

**Elevated plus maze task**

Anxiolytic-like behaviour was evaluated using the elevated plus maze task [36]. The apparatus consisted of a structure 50 cm above the floor, with four arms of the same size, two of them closed (walls 40 cm) and two open. Initially, each animal was placed on the central platform of the maze in front of an open arm and was given 5 minutes to explore the apparatus. We then recorded the time spent and the number of entries in the centre, open and closed arms, the number of faecal pellets and head dips. The apparatus was thoroughly cleaned and water. All animal procedures were approved by the Animal Ethics Committee of the UFSM (077/2011).

**Sample preparation for biochemical analysis**

Cerebral cortex, hippocampus and striatum were separated and homogenized in a solution of 10 mM Tris–HCl, 0.1 mM EDTA, pH 7.4, as previously described [37,38]. After centrifugation at 1500g at 4°C for 15 minutes, the supernatant was stored at −80°C until further use.

**Na⁺-K⁺-ATPase activity**

Na⁺-K⁺-ATPase activity was measured in the supernatant as previously described [39,40]. The composition of the assay medium was 40 mM Tris–HCl buffer (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl and 6 mM MgCl₂. Enzyme activity was evaluated in the presence or absence of ouabain (4 mM). The reaction was started by adding ATP (3 mM), and after 30 minutes at 37°C, the reaction was stopped with TCA. The amount of inorganic phosphate (Pi) released was colorimetrically quantified [41]. Na⁺-K⁺-ATPase was expressed in nmol of Pi/mg of protein/min.

**Superoxide dismutase (SOD) activity**

SOD activity was measured in the supernatant with a method based on the autoxidation reaction of adrenaline to adrenochrome [42]. Results were expressed as U SOD/mg of protein. One SOD unit was defined as the enzyme amount causing a 50% inhibition of adrenaline autoxidation.

**Catalase (CAT) activity**

CAT activity was carried out as previously described [43,44]. Activity was determined by following the decomposition of H₂O₂. The specific activity was reported as units per mg protein. One unit of the enzyme is defined as 1 nmol of H₂O₂ consumed per minute.

**Glutathione peroxidase (GPx) activity**

GPx activity was measured using a commercial kit (RANSEL®; Randox Lab, Antrim, United Kingdom). GPx activity was determined in the supernatant using glutathione reductase and NADPH. This method is based on the oxidation of NADPH, indicated by a decrease in the absorbance at 340 nm. Enzymatic activity was expressed as µmol NADPH/min/mg of protein.

**Glutathione reduced (GSH) levels**

Reduced glutathione (GSH) was determined in the supernatant as previously described [45]. Results were expressed as µmol of GSH/mg of protein.

**Thiobarbituric acid reactive substances (TBARS) measurement**

TBARS levels were analyzed in the homogenate by a method previously described [46] and slightly modified [47]. The reaction mixture contained 200 μl of sample or standard (MDA, malondialdehyde 0.03 mM), 200 μl of 8.1% sodium dodecyl sulphate, 750 μl of acetic acid solution (2.5 M HCl, pH 3.5)
and 750 µl of 0.8% TBA. Reaction mixtures were heated at 95°C for 90 minutes, and the absorbance was measured at 532 nm. Results were expressed as µmol MDA/mg of protein.

**Statistical analysis**

Results were analyzed by one or two-way analysis of variance (ANOVA), followed by the Tukey post hoc test (Graph Pad Prism 5.0). Differences between groups were considered to be significant when *P* < 0.05. Data were expressed as mean ± standard error medium (SEM).

**Results and discussion**

**HIIT induces anxiolytic-like behaviour and caffeine blocks this effect**

Anxiety disorders are the most common mental illness in the general population, with a prevalence of approximately 25%
Clinical symptoms are often accompanied by cognitive impairment, suggesting that interactions between the affective state and cognition may underlie the debilitating nature of pathological anxiety [50]. Physical activity has been proposed as an alternative to improve mental health due to its beneficial effects on anxiety, depression and cognition [50,51]. Our results show that chronic HIIT induced an anxiolytic-like behaviour in rats, as it decreased the number of entries in the closed arms ($F_{2,49} = 3.921$, $P < 0.001$, Figure 2(A)) and increased the number of entries in the open arms ($F_{2,49} = 3.799$, $P < 0.001$, Figure 2(B)) of the elevated plus maze. Caffeine per se (4 and 8 mg/kg) did not change the number of entries in both the open and closed arms but prevented HIIT-induced anxiolytic-like behaviour (Figure 2(A,B)). Furthermore, both doses of caffeine (without HIIT) increased the time spent in the closed arm ($F_{2,49} = 2.048$, $P < 0.001$, Figure 2(C)) and reduced that in the open arms ($F_{2,49} = 0.3178$, $P < 0.001$, Figure 2(D)). HIIT prevented caffeine-induced anxiogenic-like behaviour induced by increasing the time spent in the open arms (Figure 2(D)).

The anxiogenic behaviour induced by caffeine (either 4 or 8 mg/kg) without HIIT was also demonstrated by a reduction in the time spent in the centre ($F_{2,49} = 1.713$, $P > 0.001$, Figure 2(E)). No significant differences were observed between groups in the number of crossings between the arms ($F_{2,49} = 0.222$, $P > 0.05$, Figure 2(F)) and in head dips between groups ($F_{2,49} = 0.2360$, $P > 0.05$, Figure 3(A)). Caffeine (4 mg/kg) increased the number of faecal pellets. However, when caffeine was associated with exercise, this effect was not observed ($F_{2,49} = 3.464$, $P < 0.05$, Figure 3(B)). Caffeine intake showed an opposite effect to HIIT by inducing an anxiogenic-like behaviour, blocking the beneficial effects of HIIT in anxiety. Our findings are in line with previous studies showing the psychostimulant effect of caffeine in anxiety [52–54].

### Caffeine prevents the increase in Na⁺-K⁺-ATPase activity induced by HIIT

Na⁺-K⁺-ATPase activity is inhibited in various neuropathological conditions [14,55,56]. Indeed, impairment of ion homeostasis, triggered by reduced Na⁺-K⁺-ATPase activity, is found in patients with depression [57] and the inhibitor ouabain-induced symptoms of bipolar disorder in rats [58]. Reduced Na⁺-K⁺-ATPase activity has also been associated with increased anxiety [13,59]. In our study, HIIT increased Na⁺-K⁺-ATPase activity in the cerebral cortex ($F_{2,24} = 7.252$, $P < 0.001$, Figure 4(A)), hippocampus ($F_{2,24} = 5.282$, $P < 0.01$, Figure 4(B)) and striatum ($F_{2,24} = 0.1093$, $P < 0.01$, Figure 4(C)), whereas caffeine (8 mg/kg) prevented this effect in the cerebral cortex and caffeine (either 4 or 8 mg/kg) prevent this effect in the hippocampus.

### Chronic HIIT alters SOD and CAT activity

Considering that brain is highly sensitive to reactive oxygen species (ROS) and that Na⁺-K⁺-ATPase activity is...
known to be affected by the redox state of the cell [40], we analyzed the activity of key antioxidant enzymes. HIIT increased SOD activity in the cortex (F<sub>2,39</sub> = 6.89, P < 0.01, Figure 5(A)) and both caffeine doses prevented this effect. However, HIIT decreased SOD activity in the hippocampus (F<sub>2,39</sub> = 0.7495, P < 0.001, Figure 5(B)) and striatum (F<sub>2,39</sub> = 0.7495, P < 0.001, Figure 5(C)). HIIT also increased the CAT activity in the cerebral cortex (F<sub>2,39</sub> = 5.138, P < 0.001, Figure 5(D)) but reduced it in the hippocampus (F<sub>2,39</sub> = 1.207, P < 0.01, Figure 5(E)), and caffeine was not able to prevent these effects. No significant differences were observed in the striatum among groups (F<sub>2,39</sub> = 0.460, P > 0.05, Figure 5(F)). Studies have shown that caffeine intake provides neuroprotection against several disorders such as Alzheimer’s and Parkinson’s diseases [60–62]. This may be due to A<sub>2A</sub> receptor modulation, as previous work shows that blocking these receptors reduces ROS production and cell death [63,64].

Figure 5. Effects of chronic high-intensity interval training (HIIT) and caffeine (4 and 8 mg/kg) in superoxide dismutase (SOD) and catalase (CAT) activities. SOD activity in the cortex (A), hippocampus (B) and striatum (C). CAT activity in the cortex (D), hippocampus (E) and striatum (F). Data are expressed as mean ± SEM. * Indicates significant difference compared to the vehicle group. # indicates significant difference compared to the HIIT group (ANOVA one-way followed by post hoc Tukey, n = 6–8).

Figure 6. Effects of chronic high-intensity interval training (HIIT) and caffeine (4 and 8 mg/kg) in glutathione peroxidase (GPx) activity and in reduced glutathione levels (GSH). GPx activity in the cortex (A), hippocampus (B) and striatum (C). GSH levels in the cortex (D), hippocampus (E) and striatum (F). Data are expressed as mean ± SEM. * Indicates significant difference compared to the vehicle group. # indicates significant difference compared to the HIIT group (ANOVA one-way followed by post hoc Tukey, n = 6–8).
Caffeine prevents HIIT-induced changes in GPx activity

HIIT increased GPx activity in the cerebral cortex ($F_{2,39} = 16.64, P < 0.0001$), hippocampus ($F_{2,39} = 10.12, P < 0.001$) and striatum ($F_{2,39} = 5.679, P < 0.001$) and caffeine (8 mg/kg but not 4 mg/kg) prevented this effect in the cerebral cortex and hippocampus (Figure 6(A–C)). HIIT reduced the GSH content in the hippocampus, which caffeine did not restore ($F_{2,39} = 3.245, P < 0.0001$, Figure 6(E)). There were no significant differences between groups in the cerebral cortex ($F_{2,39} = 4.967, P > 0.05$) and striatum ($F_{2,39} = 0.8749, P > 0.05$) (Figure 6(D,F)).

Caffeine prevents HIIT-induced increases in TBARS levels

HIIT increased TBARS levels in the cerebral cortex ($F_{2,36} = 8.8416, P < 0.001$), hippocampus ($F_{2,36} = 5.383, P < 0.01$) and striatum ($F_{2,36} = 9.704, P < 0.01$). Caffeine (4 and 8 mg/kg) was able to prevent this alteration only in the cerebral cortex and hippocampus (Figure 7(A–C)).

Neurochemical and behavioural changes induced by chronic HIIT and caffeine intake

HIIT decreased anxiety and increased the activity of antioxidant enzymes, we observed an increase in TBARS levels, which suggests lipoperoxidation. In the brain, the polyunsaturated fatty acid high content and increased oxygen use account for the susceptibility to free radical damage. Chronic HIIT induces an adaptive response in the brain redox system by stimulating the activity of the antioxidant enzymes SOD, CAT and GPx. It is possible that HIIT-induced increase in cerebral blood flow leads to an increase in tissue oxygen supply and glycolytic metabolism, resulting in elevated production of ROS in the mitochondria. Physical exercise also induces an increase in the number of mitochondria, thereby promoting ROS production. In this context, improvement of the antioxidant enzyme activity can be an adaptive response induced by HIIT. Also, previous work has shown that HIIT increases BDNF and GDNF in the rat brain, which is associated with higher concentrations of H$_2$O$_2$ and TNF-α, produced during exercise [65]. After HIIT, an increase in the hippocampal TNFα and BDNF levels have also been reported as being associated with oxidative stress, especially H$_2$O$_2$ levels, and the hypoxic condition during exercise [65,66]. These reports can explain the neurochemical alterations in the enzymatic activity of the antioxidant system, which may be a response to increased H$_2$O$_2$ production. Since caffeine is a neuroprotective and antioxidant molecule, chronic caffeine intake prevented ROS production and the adaptive response of the redox system induced by chronic HIIT. It is interesting to note that different responses in the redox state in the brain regions can be associated with: (i) the regional differences observed in the population of glutamatergic neurons, especially of the NMDAR type, since an imbalance in the Ca$^+$ currents evoked by these receptors induce a series of neurotoxic events; (ii) the distribution across the nervous system may also be important because hydrophilic compounds, such as ascorbic acid, present rapid and widespread distributions in the CNS in rodent models, including substantial penetration into brain parenchyma, as well as cerebrospinal fluid bordering regions [67]. Moreover, in the rat brain, the cerebral cortex has a lower basal level of activity of some antioxidant enzymes, such as SOD, CAT and GPx [68,69].

We conclude that HIIT induces changes in antioxidant enzymes and increases Na$^+$-K$^+$-ATPase activity and anxiolytic-like behaviour (Figure 8). It is not clear whether caffeine has a beneficial or harmful role when combined with HIIT.
since it prevented the effects induced by physical exercise. Differences in metabolism, diet, the frequency of caffeine intake and type of exercise are factors that can determine how each individual will react to combinations of caffeine and HIIT.

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Disclosure statement
No potential conflict of interest was reported by the authors.

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