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Antioxidant activity elicited by low dose of caffeine attenuates pentylenetetrazol-induced seizures and oxidative damage in rats

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ABSTRACT

Although caffeine supplementation has a beneficial effect on people with neurological disorders, its implications for oxidative damage related to seizures are not well documented. Thus the aim of this study was to investigate the effects of two weeks caffeine supplementation (6 mg/kg; p.o.) on seizures and neurochemical alterations induced by pentylenetetrazol (PTZ 60 mg/kg i.p.). Statistical analyses showed that long-term rather than single dose caffeine administration decreased the duration of PTZ-induced seizures in adult male Wistar rats as recorded by cortical electroencephalographic (EEG) and behavioral analysis. The quantification of EEG recordings also revealed that caffeine supplementation protected against a wave increase induced by PTZ. Neurochemical analyses revealed that caffeine supplementation increased glutathione (GSH) content *per se* and protected against the increase in the levels of thiobarbituric acid reactive substances (TBARS) and oxidized dichlorofluoresceine diacetate (DCFH-DA). Also, caffeine prevent the decrease in GSH content and Na^+, K^+-ATPase activity induced by PTZ. Our data also showed that the infusion of L-buthionine sulfoximine (BSO; 3.2 μmol/site *i.c.v*), an inhibitor of GSH synthesis, two days before injecting PTZ reversed the anticonvulsant effect caused by caffeine. BSO infusion also decreased GSH content and Na^+, K^+-ATPase activity. However, it increased DCFH-DA oxidation and TBARS *per se* and reversed the protective effect of caffeine. Results presented in this paper support the neuroprotective effects of low long-term caffeine exposure to epileptic damage and suggest that the increase in the cerebral GSH content caused by caffeine supplementation may provide a new therapeutic approach to the control of seizure.

1. Introduction

Epilepsy is a neurological disorder characterized by recurrent episodes of seizures due to an imbalance between cerebral excitability and inhibition with a tendency towards uncontrolled excitability (Papandreou et al., 2006). Currently, around 50 million people worldwide have active epilepsy with continuing seizures that need treatment. Despite the increasing number and variety of antiepileptic drugs, more than 30% of cases are medically intractable (Fisher and Kettl, 2005). Animal models for seizures and epilepsy have played a key role in advancing our comprehension of the ictogenesis basic mechanisms underlying epileptogenesis (Loscher, 2011). More important than understanding the mechanism of seizure is the possibility of devising novel strategies to treat epilepsy, which may also offer some additional insights in key mechanism processes. Alternative therapies such as caffeine, which reduce seizures and related brain damage, also have been explored (El Yacoubi et al., 2008; Rigoulot et al., 2003).

Caffeine (1,3,7-trimethylxanthine), which belongs to the group of purine alkaloids, is the most commonly and widely ingested psychoactive substance. Caffeine is found in beverages such as coffee, tea, and many soft drinks as well as in chocolate products and desiccated coconut (Butt and Sultan, 2011). Structurally, caffeine is similar to adenosine, an endogenous neuromodulator, and binds to adenosine receptors to act as a nonselective antagonist (Fredholm and Lindstrom, 1999). Experimental and clinical studies have indicated that chronic caffeine supplementation provides neuroprotective effects against several neurological disorders, including Alzheimer's and Parkinson's diseases by antagonism of

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adenosine receptors (Kalda et al., 2006; Maia and de Mendonca, 2002; Ross et al., 2000; Xu et al., 2006). In particular, the neuroprotection afforded by chronic caffeine consumption is mediated by the antagonism of adenosine A_{2A} receptors (for review see Cunha and Agostinho, 2010). This has been confirmed in the particular case of PTZ-induced seizures (El Yacoubi et al., 2008). However, it has been shown that a high single dose of caffeine can aggravate seizures (Boison, 2011) and cause damage to hippocampal sectors and striatum in the brain (Enns et al., 1996). The worsening effects of caffeine on seizures are thought to be due to its antagonist properties at the A_1 receptors (Dragunow, 1990). It was also demonstrated that acute versus chronic administration of an adenosine receptor ligand can result in opposite effects in a number of settings, including cognitive processes, seizures and ischaemic damage (Jacobson et al., 1996). Taken together, these experimental studies suggest that acute caffeine administration mainly targets adenosine A_1 receptors, aggravating seizures and amplifying excitotoxicity while chronic consumption of moderate doses of caffeine mainly targets adenosine A_{2A} receptors, affording neuroprotection (for review see Boison, 2011; Cunha and Agostinho, 2010).

The brain is one of the major organs that generates large amounts of reactive oxygen species (ROS). Compared with other organs, the brain is especially vulnerable to oxidative stress because of its lower antioxidant enzyme activities and high quantities of lipids with unsaturated fatty acids, which are targets of lipid peroxidation (Milder and Patel, 2012). Under normal conditions, the brain can equilibrate the generated ROS with its own antioxidant defense. In this context, glutathione (GSH) is the most abundant thiol-reducing agent that plays a critical role as a major antioxidant in the CNS (Dringen, 2000). In line with this view, a growing number of studies have demonstrated that oxidative stress facilities the appearance and/or propagation of seizures (Patsoukis et al., 2004; Shin et al., 2011). Accordingly, experimental findings from our group have demonstrated that the inhibition of some selected target for oxidative stress such as Na^+, K^+-ATPase may lead to neuronal excitability and appearance of convulsions in the PTZ model of seizure (Rambo et al., 2009; Saraiva et al., 2012; Souza et al., 2009). This is particularly important considering the fact that this ion pump plays a key role in regulating and controlling nerve excitability (Vasilets and Schwarz, 1993). Based on the hypothesis that oxidative stress is involved in epilepsy, approach's aimed at reducing such stress would be the use of neuroprotective therapy to prevent or slow down seizure progression.

Over the years, accumulating evidence has suggested a potential antioxidant role for caffeine (Aoyama et al., 2011; Noschang et al., 2009; Rossowska and Nakamoto, 1994; Varma et al., 2010). The suggestions are largely based on chemical studies showing it to be able to scavenge ROS, particularly the hydroxyl radical (OH**.**), known to be generated in the body by many physiologic reactions involving oxygen utilization (Devasagayam et al., 1996; Shi et al., 1991). Additionally, caffeine has been shown to prevent Fenton's reaction-induced oxidation of GSH (Shi et al., 1991), a major antioxidant reserve in many tissues, including the CNS. However, the effect of caffeine against seizure inducing oxidative stress has not been studied to date. Therefore, since it has been proposed that at least part of the neuroprotective effects of caffeine are due to antioxidant effects (Aoyama et al., 2011; Varma et al., 2010) and that oxidative stress facilitates the appearance and propagation of seizures in several experimental models (Shin et al., 2011; Waldbaum and Patel, 2010), our goal was to evaluate the effect of caffeine supplementation on electrographic and neurochemical alterations (characterized here by GSH and TBARS content, DCFH-DA oxidation and Na^+, K^+-ATPase activity) induced by PTZ in the cerebral cortex of rats.

2. Materials and methods

2.1. Animals and reagents

Adult male Wistar rats (270–300 g) were used in the present study. Rats were housed four to a cage. Light and temperature were controlled (12-h light/dark cycle, 22 ± 1 °C, 55% relative humidity) and rats had free access to food (Guabi, Santa Maria, Brazil) and water. All experimental protocols were designed to keep the number of animals used to a minimum as well as to keep them from suffering. All experimental protocols were conducted in accordance with national and international legislation (National Council for Control of Animal Experimentation (CONCEA) and of U.S. Public Health Service's Policy on Human Care and Use of Laboratory Animals-PHS Policy), and approved by the Ethics Committee for animal research at the Federal University of Santa Maria. Behavioral tests were conducted during the light phase of the cycle (between 10:00 AM and 4:00 PM). All reagents were purchased from Sigma (St Louis, MO, U.S.A.). Caffeine anhydrous was dissolved in water and L-buthionine sulfoximine (BSO) and PTZ were dissolved in 0.9% physiological saline.

2.2. Study design

The study design is summarized in Fig. 1 and consisted of two experiments. The experiments were as follows.

Experiment 1: in order to determine the role of caffeine on the electrographic, and neurochemical alterations in cerebral cortex of rats induced by convulsive dose of PTZ (60 mg/kg, i.p), animals were supplemented with caffeine (6 mg/kg) (Fredholm et al., 1999) or its vehicle (water) by intragastric gavage (p.o) for 15 days. In the present study, we also evaluated the participation of glutathione pathway on electroencephalographic and neurochemical alterations exerted by caffeine in this model of seizure. For this propose, a subset of animals was supplemented with caffeine (6 mg/kg p.o) for 15 days. On the days 14 and 15 of caffeine treatment, another subset of animals received intracerebroventricular infusion of L-buthionine sulfoximine (BSO; 3.2 μmol/site i.c.v), an inhibitor of GSH synthesis. The present protocol of BSO injection was choosen based on Abe et al., 2000. Twenty-four hours after the last administration of BSO, animals were injected with a convulsive dose of PTZ (60 mg/kg, i.p.) as described in Fig. 1.

Experiment 2: To evaluate if the acute caffeine administration protects against PTZ-induced electrographic seizures and neurochemical alterations, a subset of animals was treated with caffeine (6 mg/kg p.o.) 60 min before the injection of a convulsive dose of PTZ (60 mg/kg, i.p.). After a 20 min seizure evaluation, animals were sacrificed and parietal cortex removed for biochemical analyses.

2.3. Surgical procedure

For the electroencephalographic recordings (EEG), all animals were subjected to surgery. In brief, animals were anesthetized with Equithesin (1% phenobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, 11% ethanol; 3 mL/kg, i.p) and placed in a rodent stereotaxic apparatus. For the EEG recordings, two screw electrodes were placed bilaterally over the parietal cortex along with a ground lead positioned over the nasal sinus. The electrodes were connected to a multipin socket fixed to the skull with acrylic cement. For intracerebroventricular infusion of BSO (3.2 μmol/site i.c.v), a cannula was positioned on the right ventricle (coordinates relative to bregma: AP 0 mm; ML 1.5 mm; 2.5 mm from the dura) (Paxinos et al., 1980). Ceftriaxone (200 mg/kg, i.p) was administered immediately before the surgical procedure. The

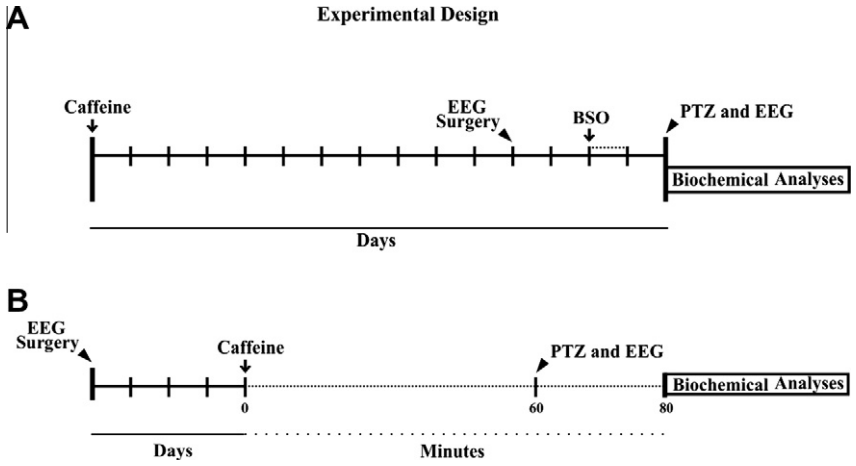

Fig. 1. Representation of experimental design. (A) Animals were treated with caffeine (6 mg/kg *p.o*) during 15 days. On the twenty day, the animals were submitted to a surgery for electrodes and/or cannula implantments. In the experiment with BSO, animal received an i.c.v infusion (3.2 umol/5 ul) on 14th and 15th day. On the 16th day animals were connected to EEG and injected with PTZ (60 mg/kg i.p) or saline 0.9% and 20 min after were killed to biochemical analyses. (B) Animals were submitted to a surgery for electrodes implantments, on 4th day they were treated with caffeine (6 mg/kg p.o.) 60 min before the PTZ (60 mg/kg, i.p.) injection. After a 20 min seizure evaluation, animals were sacrificed and parietal cortex removed for biochemical analyses.

behavioral and EEG evaluation were performed 4 days after surgery.

2.4. Seizure evaluation

Seizures were monitored in all animals by EEG recording. On the day of the experiments, each animal was transferred to an acrylic glass cage (25 × 25 × 40 cm) and allowed to adapt for 20 min before EEG recording. The rat was then connected to the lead socket in a swivel inside a Faraday's cage, and the EEG was recorded using a digital encephalographer (Neuromap EQSA260, Neuromap LTDA, Itajaú, MG, Brazil). EEG signals were amplified, filtered (0.1–70.0 Hz, band pass), digitalized (sampling rate 256 Hz), and stored in a personal computer for off-line analysis. Routinely, a 10 min baseline recording was obtained to establish an adequate control period. After baseline recording animals received an injection of saline (0.9% NaCl, 1 mL/kg, i.p) or PTZ (60 mg/kg, i.p). The animals were observed for the appearance of clonic and generalized tonic–clonic convulsive episodes for 20 min according to (Ferraro et al., 1999), who describes clonic convulsions as episodes characterized by typical partial clonic activity affecting the face, head, vibrissae, and forelimbs. Generalized convulsive episodes were considered as generalized whole-body clonus involving all four limbs and tail, rearing, and wild running and jumping, followed by sudden loss of upright posture and autonomic signs, such as hypersalivation and defecation, respectively. During the 20 min observation period, latencies for the first clonic and generalized tonic–clonic convulsions were measured. EEG recordings were visually analyzed for seizure activity, which were defined by the occurrence of the following alterations in the recording leads (McColl et al., 2003): isolated sharp waves ($\geqslant 1.5 \times$ baseline); multiple sharp waves ($\geqslant 2 \times$ baseline) in brief spindle episodes ($\geqslant 1$ s $\geqslant 5$ s); multiple sharp waves ($\geqslant 2 \times$ baseline) in long spindle episodes ($\geqslant 5$ s); spikes ($\geqslant 2 \times$ baseline) plus slow waves; multispikes ($\geqslant 2 \times$ baseline, $\geqslant 3$ spikes/complex) plus slow waves; and major seizure (repetitive spikes plus slow waves obliterating background rhythm, $\geqslant 5$ s). For quantitative analysis of EEG amplitude, we averaged EEG amplitude over the 20 min of observation.

2.5. Sample processing

Immediately after the seizure evaluation period, animals were killed by decapitation and their brains were exposed by the removal of the parietal bone. The parietal cerebral cortex (local of EEG recordings) was quickly dissected on an inverted ice-cold Petri dish and the material was stored at −80 °C for subsequent biochemical analyses. Samples were prepared according to the guidelines for each technique, as described below.

2.6. Measurement of TBARS content

Thiobarbituric Acid Reactive Substances (TBARS) content was estimated in a medium containing 0.2 ml of cerebral cortex homogenate, 0.1 mL of 8.1% SDS, 0.4 mL of acetic acid buffer (500 mM, pH 3.4), and 0.75 mL of 0.81% (TBA). The mixture was finally made up to 2 mL with type I ultrapure water and heated at 95 °C for 90 min in a water bath using a glass ball as a condenser. After cooling to room temperature, absorbance was measured in the supernatant at 532 nm (Ohkawa et al., 1979).

2.7. Isolation of rat brain mitochondria for Oxidized Diclorofluoresceine (DCFH) level determination

Rat cerebral cortex mitochondria were isolated as described by (Bhattacharya et al., 1991) with some modifications. Firstly, the cerebral cortex was quickly removed from the rat skull and homogenized in a buffer containing (in mM): 100 sucrose, 10 EDTA, 100 Tris–HCl, and 46 KCl (pH 7.4). After homogenization, the resulting suspension was centrifuged for 3 min at 2000*g* (4 °C) to obtain a low speed supernatant fraction (S1). S1 was centrifuged for 20 min at 12,000*g* (4 °C). The pellet was re-suspended in a buffer containing (in mM): 100 sucrose, 10 EDTA, 100 Tris–HCl, 46 KCl and bovine serum albumin (BSA, 0.5%; pH 7.4) and re-centrifuged for 10 min at 12,000*g* (4 °C). The supernatant was decanted and the final pellet re-suspended in a buffer containing (in mM): 70 sucrose, 0.02 EDTA, 20 Tris–HCl, 230 mannitol, 1 K_2HPO_4, to yield a protein concentration of 30–40 mg/mL.

2.8. Oxidized Diclorofluoresceine (DCFH) level determination

The levels of DCFH were determined as an index of the peroxide production by the cellular components. This experimental method of analysis is based on the deacetylation of the probe DCFH-DA and its sub-sequent oxidation by reactive species to DCFH, a highly fluorescent compound (Halliwell, 2007). Fractions of cerebral cortex mitochondria (350 μg/μl) were added to a medium containing

buffer III and DCFH-DA (1 mM). After DCFH-DA addition, the fluorescence measurement procedure started (excitation at 488 nm and emission at 525 nm, and both slit widths used were at 1.5 nm). DCFH oxidation was determined using a standard curve of DCF and results were corrected by the protein content.

2.9. GSH levels

The levels of GSH were determined fluorometrically as described by (Hissin and Hilf, 1976), using 0-phthaladehyde (OPA) as fluorophore. Briefly, cortex was homogenized in 0.1 M $HClO_4$. Homogenates were centrifuged at 2500*g* for 10 min and the low-speed supernatants were separated for measurement of GSH. Supernatant (100 μl) was incubated with 100 μl of OPA (0.1% in methanol) and 1.8 ml of 0.1 M phosphate buffer (pH 8.0) for 15 min at room temperature in the dark. Fluorescence was measured with a fluorescence spectrophotometer at excitation wavelength of 350 nm and at emission wavelength of 420 nm. GSH levels were expressed as nmol GSH/g of tissue.

2.10. Na^+, K^+-ATPase activity measurements

Assay of Na^+, K^+-ATPase activity was performed according to (Wyse et al., 2000). Briefly, the reaction medium consisted of 30 mM Tris–HCl buffer (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM MgCl2, and 50 μg of protein in the presence or absence of the Na^+, K^+-ATPase inhibitor ouabain (1 mM), in a final volume of 320 μL.

The reaction was started by the addition of adenosine triphosphate (ATP) to a final concentration of 5 mM. After 30 min at 37 °C, the reaction was stopped by the addition of 70 μL of trichloroacetic acid (TCA, 50%). Saturating substrate concentrations were used and the reaction was linear with protein and time. The amount of inorganic phosphate released was quantified by the colorimetric method described by (Fiske and Subbarow, 1927). The Na^+, K^+-ATPase activity was calculated by subtracting the ouabain-sensitive activity from the overall activity (in the absence of ouabain).

2.11. Protein determination

Protein content was measured colorimetrically by the method of (Bradford, 1976) using bovine serum albumin (1 mg/mL) as standard.

2.12. Statistical analysis

Data from *ex-vivo* total TBARS, GSH levels, DCFH and Na^+, K^+-ATPase activity determinations were analyzed by three-way ANOVA (analysis of variance) and were expressed as mean ± S.E.M. Latency to first clonic and generalized tonic-clonic seizures were analyzed by Scheirer–Ray–Hare test and expressed as median ± interquartile range. A probability of $p < 0.05$ was considered significant.

3. Results

Fig. 2 shows the effect of a two week caffeine supplementation (6 mg/kg) on behavioral seizures induced by PTZ (60 mg/kg). Statistical analyses revealed that the caffeine treatment did not alter the latency periods for the first myoclonic jerk [$U = 24$; $p > 0.05$ Fig. 2A] or first generalized tonic-clonic seizures [$U = 24$; $p > 0.05$; Fig. 2B]. However, it decreased the time spent in generalized tonic–clonic seizure [$U = 10$; $p < 0.05$; Fig. 2C] induced by the convulsive dose of PTZ. The behavior repertoire observed after PTZ injection occurred concomitantly with electrographically recorded seizures: myoclonic jerks were characterized by multiple sharp waves in brief spindle episodes, whereas generalized seizures were characterized by the appearance of 2–3 Hz high-amplitude activity (Fig. 4A and B, E and F). The quantification of electroencephalographic wave amplitude revealed that all groups increased EEG amplitude after PTZ administration [$F(1,13) = 23{,}93$; $p < 0.05$, Fig. 2J]. However, caffeine atenuates the increase in wave amplitude after the injection of PTZ (60 mg/kg; i.p) [$F(1,13) = 5{,}98$; $p < 0.05$].

Considering that the oxidative stress facilitates the appearance and/or propagation of seizures in several models of epilepsy (Rambo et al., 2009; Souza et al., 2009) and that caffeine has been shown antioxidant effects (Aoyama et al., 2011; Varma et al., 2010), we decided to investigate the effects of caffeine supplementation on oxidative stress induced by PTZ, characterized here by DCFH-DA oxidation, TBARS content, and GSH levels in cerebral cortex of rats. The results presented in this report revealed that caffeine supplementation increased GSH content [$F(1,27) = 5.54$; $p < 0.05$; Fig. 3A] *per se* and protected against PTZ-induced GSH decrease [$F(1,27) = 5.54$; $p < 0.05$; Fig. 3A]. In addition, statistical analyses revealed that caffeine supplementation prevented against PTZ-induced DCFH-DA oxidation [$F(1,827) = 4.28$; $p < 0.05$ Fig. 3B] and TBARS content increase [$F(1,27) = 4.56$; $p < 0.05$ Fig. 3C]. The caffeine supplementation also protected against PTZ-induced Na^+, K^+-ATPase activity inhibition [$F(1,27) = 8.76$; $p < 0.01$].

In the present study, we also evaluated the participation of glutathione pathway on electroencephalographic and neurochemical alterations exerted by caffeine in this model of seizure. Behavioral and EEG recordings revealed that caffeine supplementation decreased the time spent in generalized tonic–clonic seizures induced by PTZ ($U = 8{,}15$; $p < 0.05$, Fig. 4E) and that infusion of BSO (3.2 μmol/5 μl i.c.v) 2 days before PTZ injection reverted the anticonvulsant effect elicited by caffeine ($U = 8{,}15$; $p < 0{,}05$, Fig. 4E). The quantification of electroencephalographic wave amplitude revealed that the infusion of BSO altered the effect exerted by caffeine supplementation characterized here by EEG wave amplitude increase after PTZ injection ($F(1,26)$=4.52; $p < 0.05$ Fig. 4L). Neurochemical analyses also revealed that the BSO (3.2 μmol/5 μl i.c.v) infusion decreased GSH content [$F(1,54) = 26.73$; $p < 0.01$ Fig. 5A] and Na^+, K^+-ATPase activity [$F(1,54) = 9.15$; $p < 0.01$ Fig. 5D], whereas DCFH-DA oxidation [$F(1,54) = 5.18$; $p < 0.01$] and TBARS content *per se* [$F(1,54) = 18{,}85$; $p < 0.01$ Fig. 5D] were increased. In addition, BSO reverted the protective effect exerted by caffeine against PTZ-induced GSH decrease [$F(1,54) = 11.54$; $p < 0.05$], Na^+, K^+-ATPase activity inhibition [$F(1,54) = 13.43$; $p < 0.05$], as well as DCFH-DA oxidation [$F(1,54) = 13.03$; $p < 0.05$] and TBARS increase [$F(1,54) = 16.93$; $p < 0{,}05$].

In the present study we investigated the role of the acute caffeine administration on PTZ-induced electrographic seizures as well as neurochemical alterations. Fig. 6 shows that the acute caffeine administration one hour before PTZ administration (6 mg/kg) had no effect on the latency periods for the first myoclonic jerk [$U = 10.5$; $p > 0.05$], the first generalized tonic-clonic seizure [$U = 9.5$; $p > 0.05$] or the time spent in generalized tonic–clonic seizure [$U = 11$; $p > 0.05$]. Behavioral seizures were accompanied by EEG recording observed after PTZ injection. EEG recordings revealed that PTZ treatment increase EEG amplitude [$F(1,11) = 21{,}48$; $p < 0.05$] the acute caffeine administration had no effect on wave amplitude increase elicited by PTZ (Fig. 6J). Accordingly, neurochemical analyses revealed that the acute caffeine administration did not protect against the increase of DCFH-DA oxidation [$F(1,25) = 0.14$; $p > 0.05$ Fig. 7B], TBARS content [$F(1,25) = 0.05$; $p > 0.05$, Fig. 7C], as well as Na^+, K^+-ATPase activity inhibition [$F(1,25) = 0.38$; $p > 0.05$ Fig. 7D] and GSH level

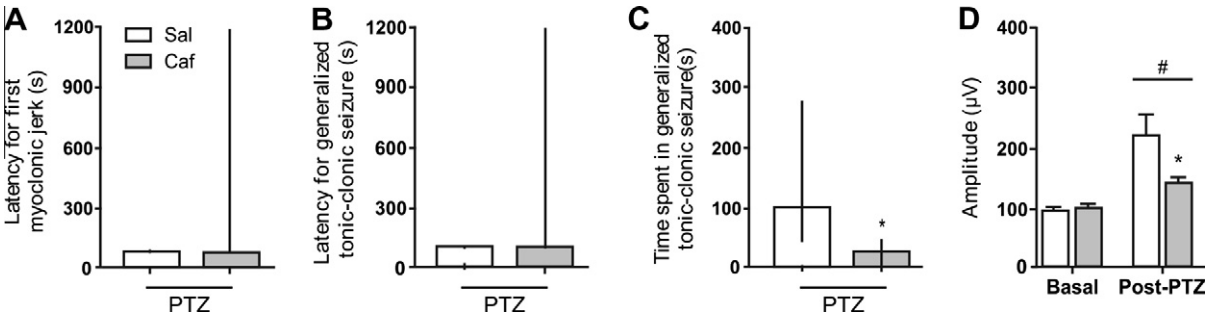

Fig. 2. Effect of long-term caffeine administration (6 mg/kg, p.o.) on the convulsive behavior induced by PTZ (60 mg/kg, i.p.). (A) Latency for first clonic seizure; (B) latency for generalized tonic-clonic seizure; (C) time spent in generalized tonic-clonic seizure; (D) wave amplitude quantification. Data are presented as median and interquartile range Mann–Whitney test (A–C) and data are presented as the mean ± S.E.M One-Way Anova $^{*}p < 0.05$ compared with PTZ-treated group, $^{\#}p < 0.05$ compared with basal period ($n = 7–8$).

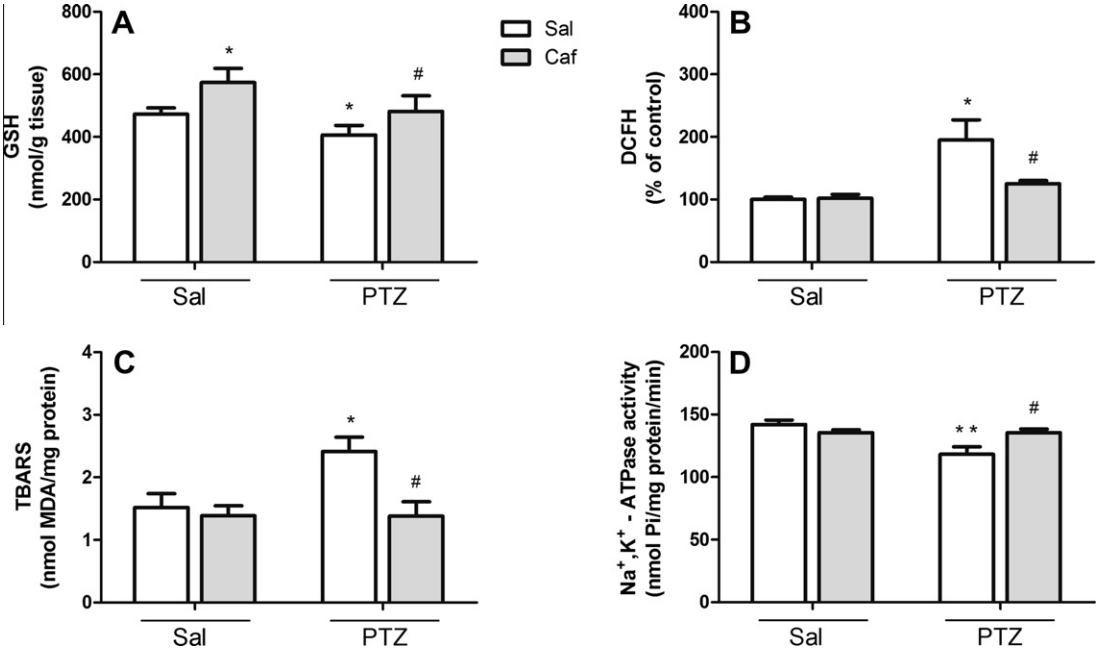

Fig. 3. Effect of long-term caffeine administration (6 mg/kg, p.o.) on the oxidative damage induced by PTZ (60 mg/kg, i.p.). The effect of caffeine and PTZ on GSH content (A), DCFH oxidation (B), TBARS content (C) and Na^{+}, K^{+}, ATPase activity. Data are presented as the mean ± S.E.M Two-Way Anova $^{*}p < 0.05$ compared with vehicle-treated group, $^{**}p < 0.01$ compared with vehicle-treated group, $^{\#}p < 0.05$ compared with PTZ-treated group ($n = 7–8$).

decrease [$F(1,25) = 0.46$; $p > 0.05$, Fig. 7A] induced by the injection of convulsive dose of PTZ.

4. Discussion

The results presented in this report revealed that long-term caffeine administration (6 mg/kg) attenuates EEG alterations and decreases generalized tonic–clonic seizures induced by PTZ. Furthermore, our data revealed that caffeine supplementation increases GSH content *per se* and the infusion of BSO (an inhibitor of GSH synthesis) reverts the protective effect of caffeine against toxicity elicited by PTZ characterized here by EEG seizures, Na^{+}, K^{+}-ATPase activity inhibition, GSH decrease, increased lipid peroxidation, and DCFH-DA oxidation.

The results presented in this paper also showed that acute caffeine administration (6 mg/kg) had no effect on seizures and did not protect against the increase of oxidative stress and Na^{+}, K^{+}-ATPase activity inhibition induced by injection of PTZ. These experimental data reinforce the idea that adaptive long-lasting neurochemical and behavioral responses are usually different from the acute drug effect (Hughes and Beveridge, 1990; Lopez et al., 1989; Tchekalarova et al., 2009). Furthermore, the protection exerted by caffeine supplementation on the epileptic activity and neurochemical alterations induced by PTZ is of particular interest because PTZ-induced seizure is an important model of myoclonic and generalized tonic–clonic seizures, which is used in routine testing for screening anticonvulsants (Swinyard et al., 1987).

Caffeine is one of the most favorable psycho stimulant in beverages or foods for motor activation, mood changes, information processing, and cognitive performance (Fredholm et al., 1999). Considering that caffeine is structurally similar to adenosine, an endogenous inhibitory neuromodulator, most of the studies have suggested that caffeine has neuroprotective effects as an adenosine receptor antagonist (Chen et al., 2001; Dall'Igna et al., 2003; Nakaso et al., 2008). Although epidemiological studies have indicated that caffeine consumption is negatively correlated with the incidence of some neurological diseases (Ascherio et al., 2001; Lindsay et al., 2002; Maia and de Mendonca, 2002), anecdotally, caffeinated beverages are "known" to lower seizure thresholds in patients with epilepsy (Kaufmann et al., 2003). However, due to the lack of well-designed, randomized, and placebo-controlled

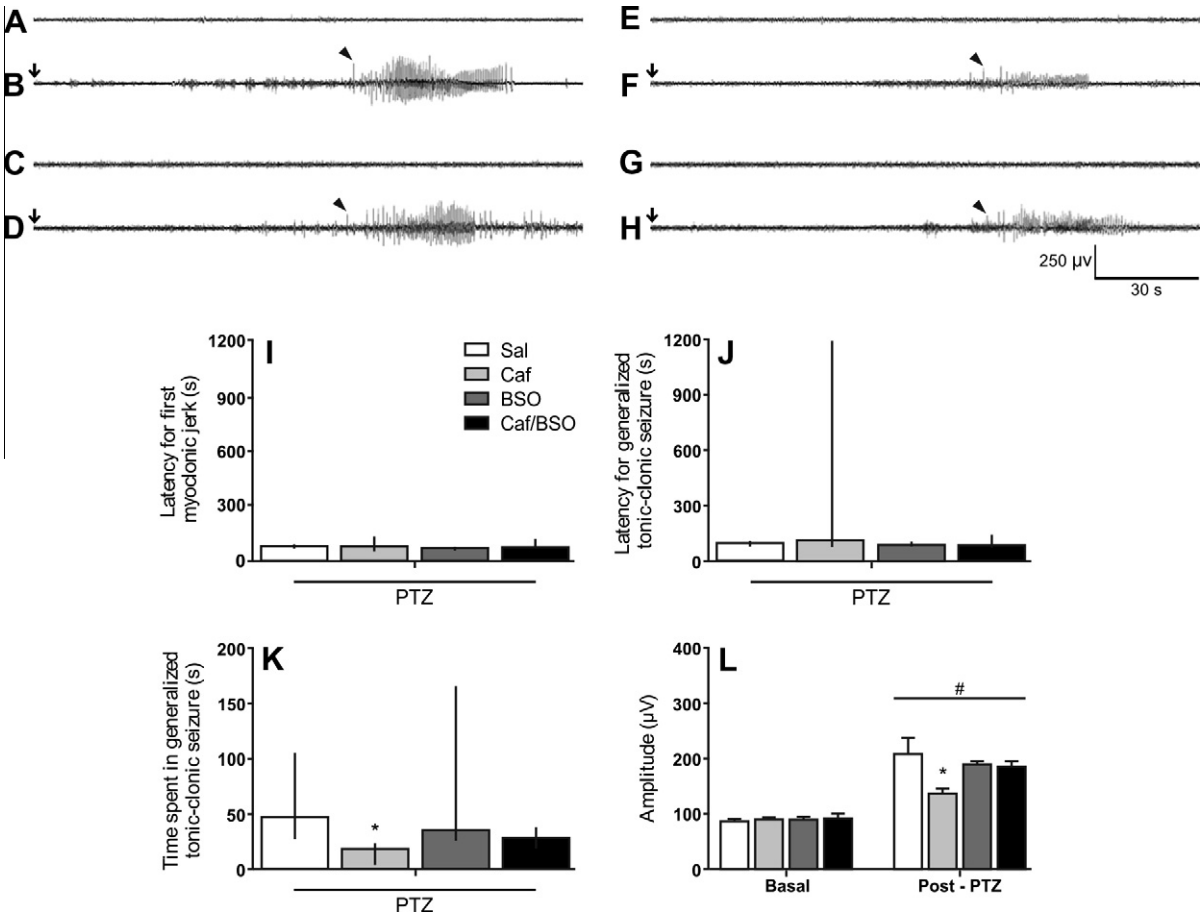

Fig. 4. Effect of BSO on the neuroprotective effect of long-term caffeine administration (6 mg/kg p.o) against behavioral and electroencephalographic seizures induced by PTZ (60 mg/kg, i.p.). Representative electroencephalographic recordings of animals treated with Vehicle (A and B), BSO (C and D) caffeine (E and F) and caffeine plus BSO (G and H) after PTZ injection (B–D – F and H). Arrows indicate PTZ injection; arrowheads indicate the first clonic seizure. Data from (I) Latency for first clonic seizure; (J) latency for generalized tonic-clonic seizure; (K) time spent in generalized tonic-clonic seizure are presented as median and interquartile range. Data from (L) wave amplitude quantification are presented as the mean ± S.E.M. $^{*}p < 0.05$ compared with PTZ-treated group $^{\#}p < 0.05$ compared with basal period (Scheirer–ray–hare and Three-way Anova test, $n = 7 = 8$).

clinical trials, this concept has been challenged (Asadi-Pooya et al., 2008). While clinical trials have demonstrated that higher doses of rolofylline, the antagonist of adenosine receptor (A_{1R}) induces seizures in patients with renal failure (Cotter et al., 2008), another study with 116,363 women revealed that caffeine ingestion, in doses without evident benefit (mean 437 mg/d), was not associated with an increased risk of epilepsy (Dworetzky et al., 2010). It is important to note that the pro and/or anticonvulsant effect elicited by methylxanthines are based on the antagonism of the brain's endogenous adenosine-based seizure control system. Whereas inhibition of A_{1Rs} by methylxanthines can directly contribute to ictogenesis and seizure spread, under certain conditions methylxanthines can also contribute to seizure suppression (Boison, 2011). In this context, the understanding of the mechanisms involved in the caffeine-related control of seizure is important since caffeine holds the second position in consumption among all beverages followed by water, and people from all over the world consume approximately 500 billion cups of coffee annually (Butt and Sultan, 2011).

In experimental animals the caffeine administration at doses of 0.3 g/L per day over a period of two weeks (resulting in plasma levels of caffeine in the range of 6 to 14 μM corresponding to chronic caffeine usage in humans) reduced NMDA, bicuculline and PTZ-induced seizures in mice in the absence of changes in A_1, A_{2A}, or $GABA_A$ receptors (Georgiev et al., 1993). Considering that chronic but not acute caffeine administration attenuates EEG seizures elicited by PTZ, we suggest that this effect could be modulated by A_{2A} antagonism since there are studies showing that A_{2A} antagonism attenuates ROS related cell damage (Behan and Stone, 2002; Leite et al., 2011). In agreement with this view previous studies have showed that both caffeine (Prasanthi et al., 2010) and especially adenosine receptors can control the formation of free radicals (Ribé et al., 2008; Gołembiowska and Dziubina, 2012), namely mitochondria dysfunction leading to the formation of free radicals (Yang et al., 2011; Tamura et al., 2012), as well as impact of free radicals on brain function (Agostinho et al., 2000; Rego et al., 2000; Behan and Stone, 2002; Almeida et al., 2003; Fatokun et al., 2007). Additionally, previous studies have shown that caffeine and adenosine receptors can control glutathione metabolism (e.g. Zhang et al., 2005; Conte et al., 2009; Aoyama et al., 2011). It is important to note that, recent observations have demonstrated that caffeine also acts as an antioxidant (Leon-Carmona and Galano, 2011). This idea is largely based on chemical studies showing it to be able to scavenge ROS, particularly the hydroxyl radical (OH**.**) *in vitro* (Devasagayam et al., 1996; Gomez-Ruiz et al., 2007). The interaction of OH**.** with caffeine results in its oxidative de-methylation generating partially N-methylated xanthines such as theobromine, paraxanthine, and theophylline (Chung and Cha, 1997; Stadler et al., 1996). In addition, studies have shown that the antioxidant effect of

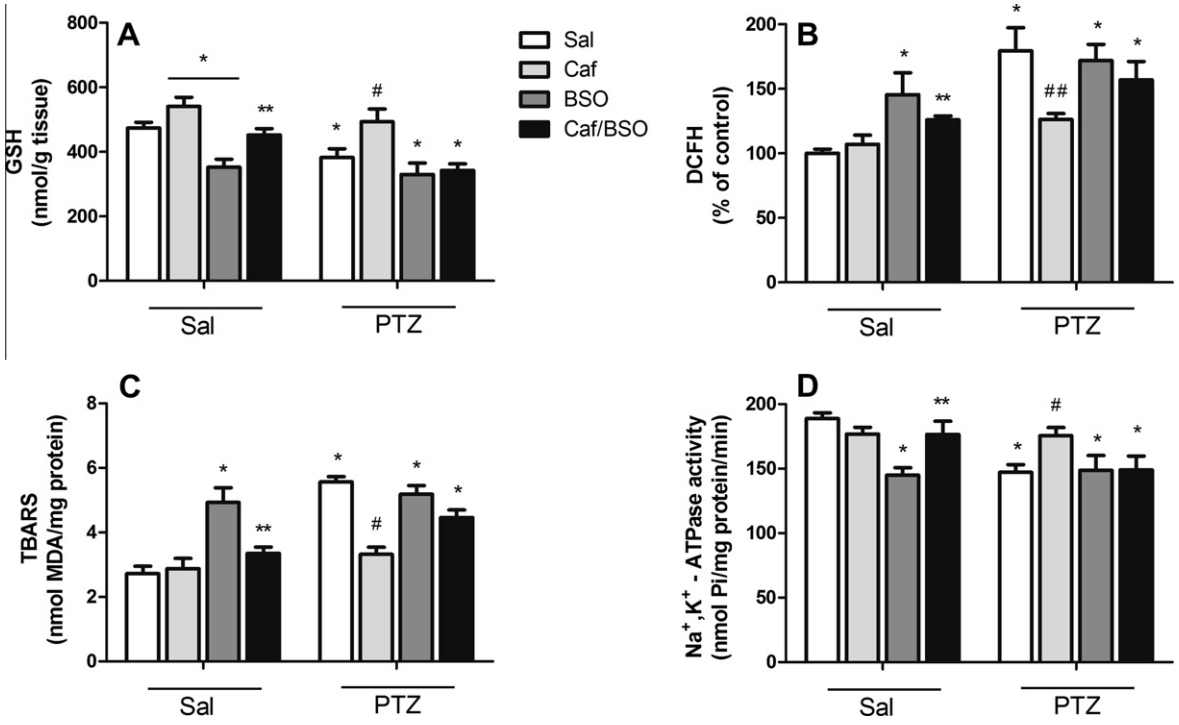

Fig. 5. Effect of BSO on the neuroprotective effect of long-term caffeine administration (6 mg/kg p.o) against oxidative damage induced by PTZ (60 mg/kg, i.p.). The effect of caffeine and PTZ on GSH content (A), DCFH oxidation (B), TBARS content (C) and Na^+, K^+, ATPase activity. Data are presented as the mean ± S.E.M Three-Way Anova $^{*}p < 0.05$ compared with vehicle treated group $^{**}p < 0.05$ compared with BSO treated group, $^{\#}p < 0.05$ compared with PTZ-treated group ($n = 6–8$).

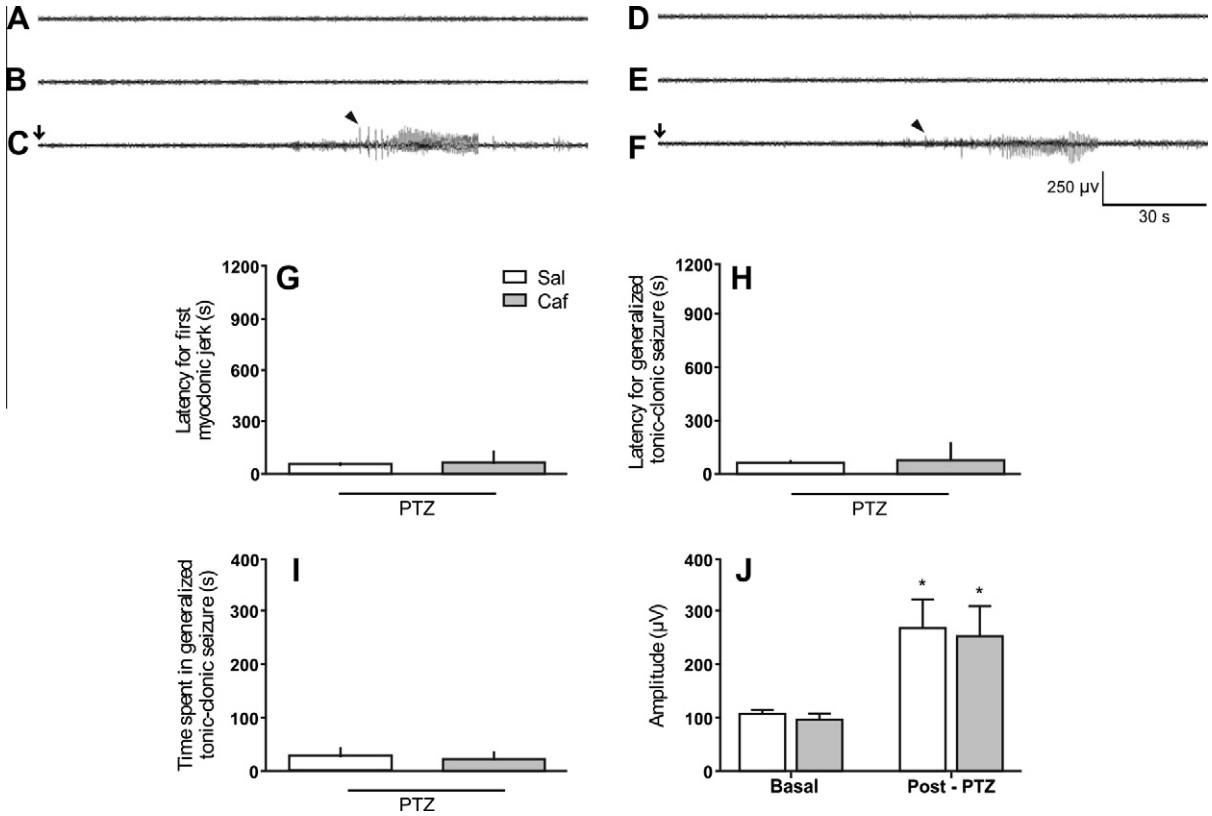

Fig. 6. Effect of acute caffeine administration (6 mg/kg, p.o.) against behavioral and electroencephalographic seizures induced by PTZ (60 mg/kg, i.p.). Representative electroencephalographic recordings of animals treated with Vehicle (A–C) or caffeine after PTZ injection (C and F), (A and D) basal period (B and E) vehicle or caffeine treatment before PTZ administration. Arrows indicate PTZ injection; arrowheads indicate the first clonic seizure. Data from (G) Latency for first clonic seizure; (H) latency for generalized tonic-clonic seizure; (I) time spent in generalized tonic-clonic seizure are presented as median and interquartile range. Data from (J) wave amplitude quantification are presented as the mean ± S.E.M. $^{*}p < 0.05$ compared with PTZ-treated group $^{\#}p < 0.05$ compared with caffeine-treated group (Mann–Whitney test and One-way Anova test, $n = 7 = 8$).

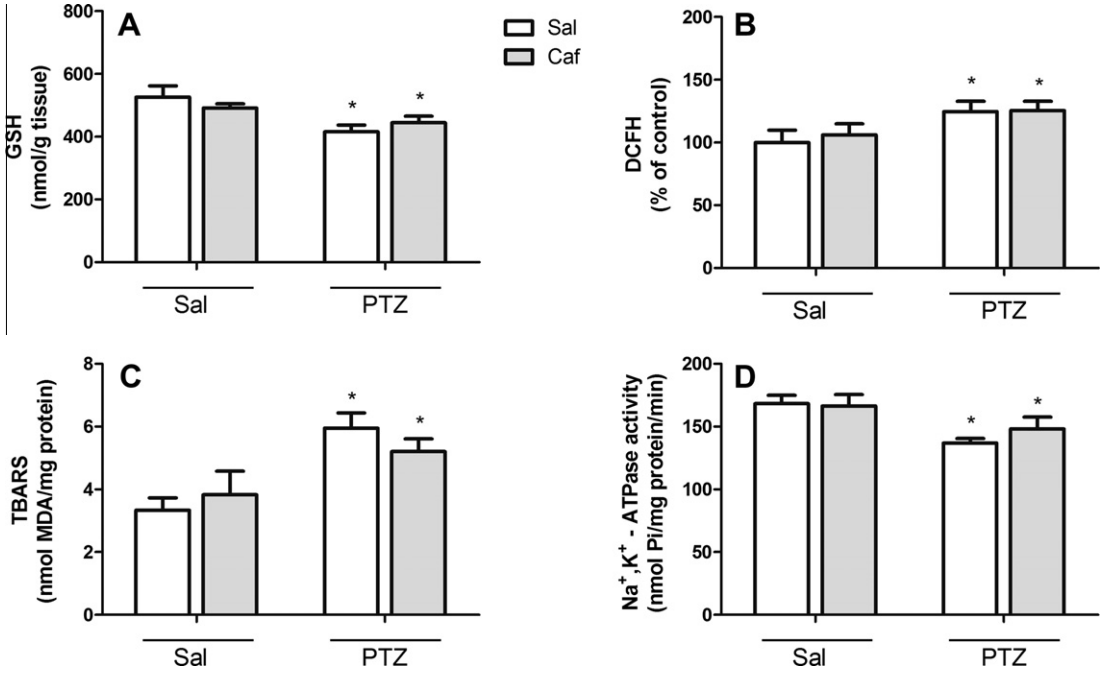

Fig. 7. Effect of acute caffeine administration (6 mg/kg, p.o.) on the oxidative damage induced by PTZ (60 mg/kg, i.p.). The effect of caffeine and PTZ on GSH content (A), DCFH oxidation (B), TBARS content (C) and Na^+, K^+, ATPase activity. Data are presented as the mean ± S.E.M Two-Way Anova $^*p < 0.05$ compared with vehicle-treated group ($n = 7–8$).

caffeine is similar to that of glutathione and higher than that of ascorbic acid (Devasagayam et al., 1996).

Studies clearly show that the effect of neuroprotection afforded by chronic caffeine consumption in animal models of health disorders associated with ROS generation such as Alzheimer's disease (Dall'Igna et al., 2003; Canas et al., 2009) and Parkinson's disease (Chen et al., 2001; Pierri et al., 2005) is mediated by adenosine A_{2A} receptors controlling synaptic dysfunction and neuroinflammation. Whether it involves a control of the formation of free radicals needs to be better clarified (Prasanthi et al., 2010; Rosso et al., 2008).

In the present study, the occurrence of DCFH-DA oxidation, TBARS increase, decrease in GSH content, and Na^+, K^+-ATPase activity inhibition after PTZ injection suggests that epileptic seizures elicited by this convulsant agent were accompanied by an increase of oxidative stress. In addition, the increase of ROS production attacks the unsaturated bonds of membrane fatty acids leading to an autocatalytic process called membrane lipid peroxidation, which may impair the function of several membrane transport proteins including Na^+, K^+-ATP-ase (Marnett, 2002). Thus, the alteration in the redox state of regulatory sulfhydryl groups in selected targets such as Na^+, K^+-ATPase activity might increase cellular excitability (Boldyrev et al., 2003; Franzon et al., 2003; Morelli et al., 2005). GSH is the major determinant of the cellular redox state (Haddad and Harb, 2005), in fact, the depletion of GSH results in the inhibition of the Na^+, K^+-ATPase activity (Petrushanko et al., 2006) and increase of lipid peroxidation in models of seizures induced by PTZ (Kumar and Gandhimathi, 2010). Furthermore, it has been demonstrated that the intracerebroventricularly administered GSH inhibited PTZ induced convulsions in mice (Abe et al., 2000) and protected against seizure episodes induced by diphenyl diselenide in rat pups by reducing oxidative stress (Prigol et al., 2011).

In line with this view, results presented in this report also revealed that caffeine supplementation increased GSH content *per se*. Considering that caffeine supplementation leads to the development of compensatory responses to oxidative stress induced by the experimental model of Alzheimer's and Parkinson's diseases (Nobre et al., 2010; Rosso et al., 2008) and GSH protects against free radical-induced Na^+, K^+-ATPase inhibition (Tsakiris et al., 2000), we suggest that the increase of antioxidant defenses (GSH) in this protocol of caffeine supplementation may protect against Na^+, K^+-ATPase inhibition induced by PTZ. In fact, the infusion of BSO (a GSH inhibitor synthesis) decreased GSH content, Na^+, K^+-ATPase activity and increased DCFH-DA oxidation *per se* as well as reverted the protective effect exerted by caffeine against PTZ-induced EEG seizures.

In conclusion, the present study reports that increased GSH levels and control of oxidative damage could be the putative effects by which the chronic consumption of caffeine affords its neuroprotective effects against convulsive behavior and excitotoxic damage induced by PTZ *in vivo*. Although further studies are necessary to determine the mechanisms involved in this protective action exerted by caffeine, these experimental findings suggest that the administration of low doses of caffeine may be a new therapeutic approach to control acute and chronic excitotoxicity including seizure activity.

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