



## Additive anticonvulsant effects of creatine supplementation and physical exercise against pentylenetetrazol-induced seizures

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### ABSTRACT

Although physical activity and creatine supplementation have been a documented beneficial effect on neurological disorders, its implications for epilepsy are still controversial. Thus, we decided to investigate the effects of 6 weeks swimming training, creatine supplementation (300 mg/kg; p.o.) or its combination seizures and neurochemical alterations induced by pentylenetetrazol (PTZ). We found that 6 weeks of physical training or creatine supplementation decreased the duration of PTZ-induced seizures in adult male Wistar rats, as measured by cortical and hippocampal electroencephalography and behavioral analysis. Importantly, the combination between physical training and creatine supplementation had additive anticonvulsant effects, since it increased the onset latency for PTZ-induced seizures and was more effective in decrease seizure duration than physical training and creatine supplementation individually. Analysis of selected parameters of oxidative stress and antioxidant defenses in the hippocampus revealed that physical training, creatine supplementation or its combination abrogated the PTZ-elicted increase in levels of thiobarbituric acid-reactive substances (TBARS) and protein carbonylation, as well as decrease in non-protein-thiols content, catalase (CAT) and SOD activities. In addition, this protocol of physical training and creatine supplementation prevented the PTZ-induced decrease in hippocampal Na<sup>+</sup>,K<sup>+</sup>-ATPase activity. Altogether, these results suggest that protection elicited physical training and creatine supplementation of selected targets for reactive species-mediated damage decrease of neuronal excitability and consequent oxidative damage elicited by PTZ. In conclusion, the present study shows that physical training, creatine supplementation or its combination attenuated PTZ-induced seizures and oxidative damage *in vivo*, and provide evidence that combination between creatine supplementation and physical exercise may be a useful strategy in the treatment of convulsive disorders.

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### 1. Introduction

Epilepsy is a common chronic neurological condition which constitute a large group of neurological diseases with an incidence of 0.5–1% in the general population (Andrade and Minassian, 2007).

While single-drug therapy provide optimal seizure control in about 80% of all patients, seizure activity remains uncontrolled in a significant number of individuals, even with the use of combination therapy (Dichter et al., 2007). Therefore, the search for alternative therapies for epilepsy deserves further investigation. In this context, physical exercise has emerged as a therapeutical aid and a protective factor in several neurological diseases, including epilepsy (Arida et al., 2004; Radak et al., 2007; Arida et al., 2009). In fact, it has been demonstrated that physical exercise have beneficial effects in animal models of epilepsy with predictive value for screening of anticonvulsant approaches, such as the amygdala electrical kindling

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model and the pilocarpine model of temporal lobe epilepsy (Arida et al., 1998, 1999a,b; Setkowicz and Mazur, 2006). Physical exercise increases the number of electrical stimulations necessary to reach the kindled state in the amygdala electrical kindling (Arida et al., 1998). Moreover, physical training decreases the susceptibility to subsequently evoked seizures and the frequency of spontaneous recurrent seizures in the pilocarpine model of epilepsy (Arida et al., 1999a; Setkowicz and Mazur, 2006). Altogether, these results suggest that regular physical training may be a useful adjuvant in the treatment of convulsive disorders.

Among the variety of physical exercises most used in researches involving animals, treadmill running and swimming stand out (Carvalho et al., 2005; Prada et al., 2004). Although there are still doubts in regarding which exercise (swimming or treadmill running) would be the most suitable to avoid unnecessary stress to the animals, the use of swimming rats as a model of exercise presents advantages since swimming is a natural ability of the rats. In this context, studies using swimming as an animal model of training revealed the occurrence of adaptation to physical training similar to those observed in human beings (Gobatto et al., 2001a; Voltarelli et al., 2002).

Creatine ( $C_4H_9O_2N_3$ ; methyl-guanidine-acetic acid) is an endogenous guanidine compound which exogenous supplementation has been widely used as an ergogenic aid by athletes and also eventual practitioner of physical activity (Andres et al., 2008). Despite its use as an ergogenic aid, a large body of evidence has showed that creatine administration has neuroprotective and anticonvulsant properties in a variety of experimental models of neurological diseases, including seizure activity (Das et al., 2003; Royes et al., 2003; Andres et al., 2005; Magni et al., 2007; Bender et al., 2008). In addition, creatine and the more lipophylic derivates creatine-Mg-complex (acetate) and phosphocreatine-Mg-complex (acetate) increased the latency to population spike disappearance during anoxia and anoxic depolarization and decreased anoxic “bursting” in hippocampal slices (Perasso et al., 2008). Such broad range of situations in which creatine supplementation has neuroprotective actions provides good reasons for the enthusiasm to establish creatine supplementation as an adjuvant or preventive therapy for a number of neurodegenerative human diseases (Andres et al., 2008). However, the effect of the combination between creatine and physical exercise in an experimental model of seizures was not studied to date. Given this premise, we hypothesized that combination between physical exercise and creatine supplementation has additive anticonvulsant effects against PTZ-induced seizures. In addition, since it has been proposed that at least part of the neuroprotective effects of physical training and/or creatine are due to antioxidant effects (Lawler et al., 2002; Ang and Gomez-Pinilla, 2007; Radak et al., 2008a,b; Guidi et al., 2008), and that it has been demonstrated that oxidative stress facilitates the appearance and/or propagation of seizures in several models of experimental epilepsy (Frantseva et al., 2000; Gluck et al., 2000; Gupta et al., 2003; Patsoukis et al., 2004), we evaluated the effect of the combination between physical exercise and creatine on PTZ-induced electrographic, oxidative and neurochemical alterations in the rat hippocampus.

## 2. Materials and methods

### 2.1. Animal and reagents

In the present study we used adult male Wistar rats, weighing 250–300 g at the beginning and 400–450 g at the end of the experimental period. Animals were maintained in a controlled environment (12:12 h light-dark cycle,  $24 \pm 1^\circ\text{C}$ , 55% relative humidity) with free access to food (Guabi, Santa Maria, Brazil) and water. All experiments were conducted in conformance with the policy statement of the American College of Sports Medicine and Official Government Ethics guidelines and were approved by the University Ethics Committee. All efforts were made to reduce the number of animals used, as well as to minimize their suffering.

All the reagents used in the present study were purchased from Sigma (St. Louis, MO).

### 2.2. Adaptation to the water

One week before the beginning of the experiment, all animals were adapted to the water. The adaptation procedure consisted of keeping the animals in shallow water (5 cm in depth) at  $32^\circ\text{C}$  between 9:00 and 11:00 a.m. The purpose of the adaptation was to reduce stress without promoting a physical training adaptation.

### 2.3. Creatine supplementation, training protocol and lactate threshold assay

In order to evaluate the combination between physical exercise and creatine on the electrographic, oxidative and neurochemical alterations in hippocampus of rats induced by systemic administration of subeffective or fully convulsive doses of PTZ animals were supplemented with creatine (300 mg/kg) (Magni et al., 2007) or its vehicle (0.1% carboxymethylcellulose) by intragastric gavage 45 min before every swimming training. The swimming training period lasted 5 weeks and consisted of 60-min daily sessions five times per week. Swimming was always performed in water at a temperature of  $32^\circ\text{C}$  between 9:00 and 11:00 a.m. During the first week of training, all animals underwent a swimming adaptation period without weights, as described above. After the swimming adaptation period, the rats were subjected to swimming training with working load (5% of body weight) for improvement of endurance exercise capacity (Gobatto et al., 2001a,b). At the same time of the training session, control rats were placed in a separate tank with shallow water (5 cm in depth) at  $32^\circ\text{C}$ , 5 days/week without the workload. Creatine supplementation, control and trained animals were randomly assigned in groups, as follows:

Experimental group	n
Vehicle/control rats	10
Vehicle/control rats/PTZ (30 mg/kg, i.p.)	8
Vehicle/control rats/PTZ (60 mg/kg, i.p.)	8
Creatine suppl. (300 mg/kg, p.o.)/control rats	8
Creatine suppl. (300 mg/kg, p.o.)/control rats/PTZ (30 mg/kg, i.p.)	8
Creatine suppl. (300 mg/kg, p.o.)/control rats/PTZ (60 mg/kg, i.p.)	8
Vehicle/trained rats	8
Vehicle/trained rats/PTZ (30 mg/kg, i.p.)	8
Vehicle/trained rats/PTZ (60 mg/kg, i.p.)	8
Creatine suppl. (300 mg/kg, p.o.)/trained rats	8
Creatine suppl. (300 mg/kg, p.o.)/trained rats/PTZ (30 mg/kg, i.p.)	8
Creatine suppl. (300 mg/kg, p.o.)/trained rats/PTZ (60 mg/kg, i.p.)	8

On the first day after the fifth week of training, a standard test protocol was used to determine the lactate threshold (LT) in vehicle/control ( $n = 6$ ), vehicle/trained ( $n = 6$ ); creatine/control ( $n = 6$ ), creatine/trained rats ( $n = 6$ ). The LT test was carried out according to the protocol described by (Marquezi et al., 2003) and consisted of swimming exercises with progressive overload through weights attached to the animal's tail, corresponding to 4%, 5%, 6%, 7%, and 8% of the body weight of each animal for 3-min periods, separated by 1-min resting periods. During the resting periods, 25  $\mu\text{l}$  blood samples were collected from the tail vein into heparinized capillary tubes for determination of lactate concentration (Voltarelli et al., 2002). The LT for each animal was calculated based on the point of inflection of the graph when plotting lactate concentration against the corresponding exercise workload.

### 2.4. Surgical procedures

For the electroencephalographical recordings, all animals were subjected to surgery 24 h after the last session of training. In brief, rats were deeply 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. Two screw electrodes were placed bilaterally over the parietal cortex (coordinates relative to bregma: AP  $-4.5\text{ mm}$  and L  $2.5\text{ mm}$ ), along with a ground lead positioned over the nasal sinus. Bipolar nichrome wire Teflon-insulated depth electrodes (100  $\mu\text{m}$  in diameter) were implanted 1 mm above the CA1 region of the dorsal hippocampus (coordinates relative to bregma: AP  $4\text{ mm}$ , ML  $3\text{ mm}$  and V  $2\text{ mm}$ ) (Paxinos, 1986). All electrodes were connected to a multipin socket fixed to the skull with acrylic cement. All experiments were performed 5 days after surgery.

### 2.5. Seizure evaluation

Seizures were monitored in all animals by electroencephalographic recordings. At the day of the experiments, each animal was transferred to a Plexiglas cage (25 cm  $\times$  25 cm  $\times$  40 cm) and habituated 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, Neurotec LTDA, Brazil). EEG signals were amplified, filtered (0.1–70.0 Hz, bandpass), digitized (sampling rate 256 Hz) and stored in a PC for off-line analysis.

Routinely, a 10 min baseline recording was obtained to establish an adequate control period. After baseline recording, control and trained animals, supplemented or not with creatine, were injected with vehicle (0.1% carboxymethylcellulose) or PTZ (30 or 60 mg/kg, i.p.). The animals were observed for the appearance of generalized tonic-clonic convulsive episodes for 20 min according to (Ferraro et al., 1999), who describes generalized convulsive episodes as generalized whole-body clonus involving all four limbs and tail, rearing, wild running and jumping, followed by sudden loss of upright posture and autonomic signs, such as hypersalivation and defecation. PTZ-induced generalized convulsions typically lasted between 30 and 60 s, and were followed by a quiescent period. During the 20-min observation period, the latency for generalized tonic-clonic convulsions was 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 ( $\geq 1.5 \times$  baseline); multiple sharp waves ( $\geq 2 \times$  baseline) in brief spindle episodes ( $\geq 1$  s,  $\geq 5$  s); multiple sharp waves ( $\geq 2 \times$  baseline) in long spindle episodes ( $\geq 5$  s); spikes ( $\geq 2 \times$  baseline) plus slow waves; multispikes ( $\geq 2 \times$  baseline,  $\geq 3$  spikes/complex) plus slow waves; major seizure (repetitive spikes plus slow waves obliterating background rhythm,  $\geq 5$  s).

#### 2.6. Tissue processing for neurochemical analyses

Immediately after the behavioral and electroencephalographical evaluation, animals were sacrificed by decapitation and had their brain exposed by the removal of the parietal bone. Hippocampi were rapidly dissected on an inverted ice-cold Petri dish. The right side was homogenized in cold 10 mM Tris-HCl buffer (pH 7.4) and used for determination of carbonyl content and  $\text{Na}^+,\text{K}^+$ -ATPase activity. The left side was placed in ice-cold Krebs Cerebral Hensleit buffer containing 124 mM NaCl, 5 mM KCl, 1.2 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 23 mM  $\text{NaHCO}_3$ , 3 mM HEPES and 10 mM D-glucose equilibrated with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  (pH 7.4) and hippocampal slices (0.4 mm) were obtained using a McIlwain tissue chopper. Slices were used for determination of non-protein-thiols and TBARS levels and superoxide dismutase and catalase activities.

#### 2.7. Non-protein-thiols (NPS) levels

Levels of NPS in slice of hippocampus were determined according to Ellman and Lysko (1967), in the presence of 50 mM Tris-Cl, pH 7.4. Incubation at 37 °C was initiated by the addition of the thiol compounds. Aliquots of the reaction mixture (100  $\mu$ l) were checked for the amount of –SH groups at 412 nm after 90–120 min of addition of color reagent 5'5'-dithio-bis (2-nitrobenzoic) acid (DTNB).

#### 2.8. Measurement of the protein carbonyl

Protein carbonyl content was determined by the colorimetric method described by Levine et al. (1990), adapted for brain tissue (Oliveira et al., 2004).

#### 2.9. Measurement of TBARS content

TBARS assay, slices from hippocampus were homogenized in ultra-pure water and mixed with the TBA reagent (15% of trichloroacetic acid, 0.375% of thiobarbituric acid and 2.5% v/v of HCl). After 30 min of incubation, samples were centrifuged (3000  $\times g$ , 15 min) and then TBARS levels were measured at 532 nm (Rios and Santamaria, 1991).

#### 2.10. Superoxide dismutase (SOD) and catalase (CAT) activity

In order to determine SOD and CAT activity, slices from hippocampus were homogenized in 40 volumes (w/v) with Tris-HCl 10 mM (pH 7.4) and enzyme activities were performed according to the methods of Misra and Fridovich (1972) and Aebi (1984), respectively. SOD activity was expressed as units/g of protein and CAT activity was expressed in units (1 U decomposes 1  $\mu\text{mol}$  of  $\text{H}_2\text{O}_2$  per minute at pH 7.0 at 25 °C).

#### 2.11. $\text{Na}^+,\text{K}^+$ -ATPase activity measurements

$\text{Na}^+,\text{K}^+$ -ATPase activity measurements were performed in the same homogenate used for determination of the protein carbonyl content. The enzyme assay was performed according to Wyse et al. (2000).

#### 2.12. Protein determination

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

#### 2.13. Statistics analysis

Statistical analyses were carried out by one- or two-way analysis of variance (ANOVA). Values of  $F$  are only presented if  $P < 0.05$ . Post hoc analyses were carried out, when appropriate, by the Student-Newman-Keuls test. All data are expressed as mean  $\pm$  S.E.M.

### 3. Results

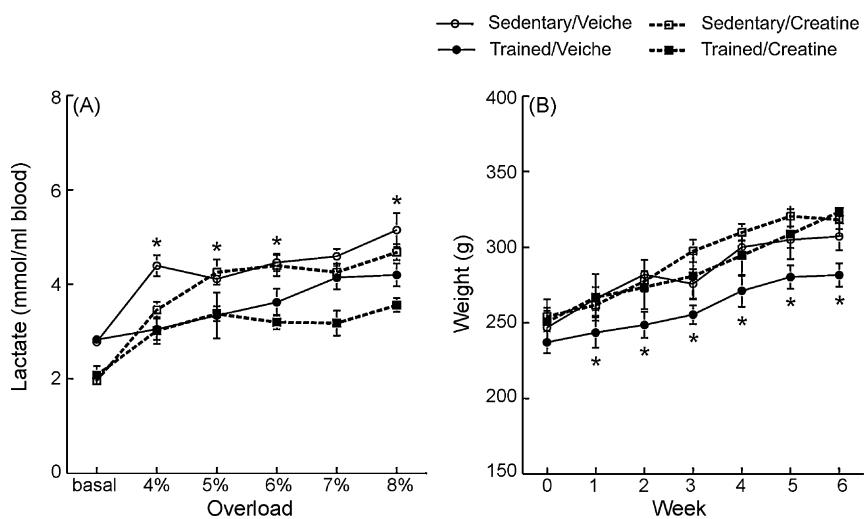
Fig. 1 shows the effect of physical training, creatine supplementation or its combination on lactate threshold and body weight. We found a clear stabilization of the blood lactate concentration in trained and trained/creatinine supplemented rats when compared with control groups in the lactate threshold test [ $F(3,19) = 6.39$ ;  $P < 0.04$ ; Fig. 1A]. In addition, statistical analysis revealed a significant decrease in total body weight in trained versus control rats along the 6 weeks of swimming training [ $F(3,19) = 15.19$ ;  $P < 0.05$ ; Fig. 1B].

The effect of physical training, creatine supplementation or its combination on PTZ-induced electrographic alteration and convulsive behavior is shown in Fig. 2. As depicted in the representative EEG recordings, PTZ injection at a fully convulsant dose (60 mg/kg) caused the appearance of multispikes plus slow waves and major seizure activity characterized by the appearance of 2–3 Hz high-amplitude activity in the recording leads (Fig. 2A). In contrast, administration of subeffective doses of PTZ (30 mg/kg) induced the appearance of an EEG pattern characterized by multiple sharp waves in brief spindle episodes, which correlated with hypoactivity and tremor (Andre et al., 1998) (data not shown). In addition, statistical analyses revealed that physical training or creatine supplementation alone had no effect on the latency to PTZ-induced seizures (Fig. 2E). However, the combination between physical training and creatine supplementation significantly increased seizure latency by approximately 400% [ $F(1,59) = 42.59$ ;  $P < 0.05$ ; Fig. 2E], showing that this combination has additive anticonvulsant effects on PTZ-induced seizure latency. Conversely, physical training, creatine supplementation or its combination decreased the time spent in convulsions [ $F(1,59) = 9.04$ ;  $P < 0.05$ ; Fig. 2F]. In addition, post hoc analysis revealed that combination between physical training and creatine supplementation was more effective than exercise or creatine alone regarding the time spent in convulsions.

Considering that reactive species have been implicated in PTZ-induced convulsive behavior and that adaptive responses to physical training and creatine supplementation include an increase in antioxidant defenses and a decrease in radical leaking during oxidative phosphorylation (Lawler et al., 2002; Packer and Cadena, 2007), several parameters that indicate the antioxidant status and oxidative stress levels were determined in the rat hippocampus.

The effect of physical training, creatine supplementation or its combination on PTZ-induced lipid peroxidation and protein carbonylation is shown in Fig. 3A and 3B. Statistical analysis showed that PTZ (60 mg/kg) elicited an increase in TBARS content in control rats [ $F(1,96) = 3.45$ ;  $P < 0.05$ ], and that physical training [ $F(1,96) = 2.16$ ;  $P < 0.05$ ] or creatine supplementation [ $F(1,96) = 1.36$ ;  $P < 0.05$ ] prevented such effect. In addition, the combination between creatine supplementation and physical training also decreased PTZ-induced increase TBARS content, being more effective than physical training alone [ $F(1,96) = 1.24$ ;  $P < 0.05$ ]. We also found that injection of PTZ (60 mg/kg) increased protein carbonylation levels in the hippocampus of control rats [ $F(1,96) = 4.13$ ;  $P < 0.01$ ; Fig. 3B], and that physical training [ $F(1,96) = 1.09$ ;  $P < 0.05$ ], creatine supplementation [ $F(1,96) = 1.19$ ;  $P < 0.05$ ] or its combination protected against such increase [ $F(1,96) = 1.07$ ;  $P < 0.05$ ; Fig. 3B].

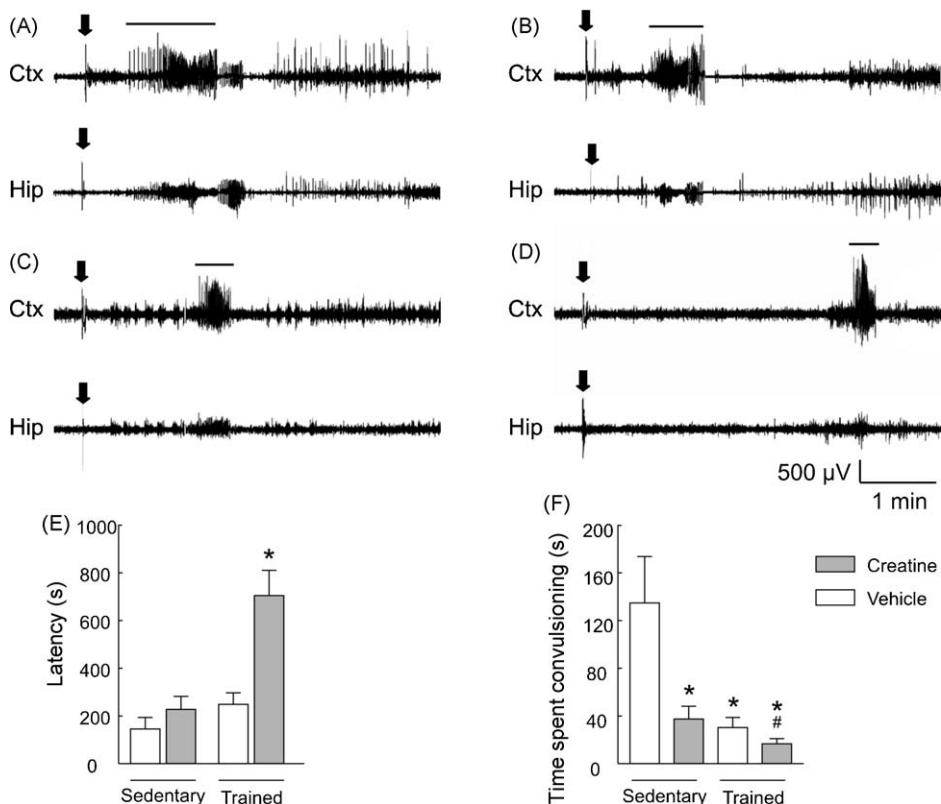
Fig. 4A and B shows the effect of physical training and creatine supplementation on CAT and SOD activities, respectively. Statistical analysis showed that the injection of PTZ (60 mg/kg) induced a significant decrease in catalase activity [ $F(1,96) = 8.08$ ;  $P < 0.05$ ], and that physical training [ $F(1,96) = 2.04$ ;  $P < 0.05$ ], creatine supplementation [ $F(1,96) = 5.15$ ;  $P < 0.05$ ], or its combination protected against such decrease [ $F(1,96) = 2.24$ ;  $P < 0.05$ ; Fig. 4A].



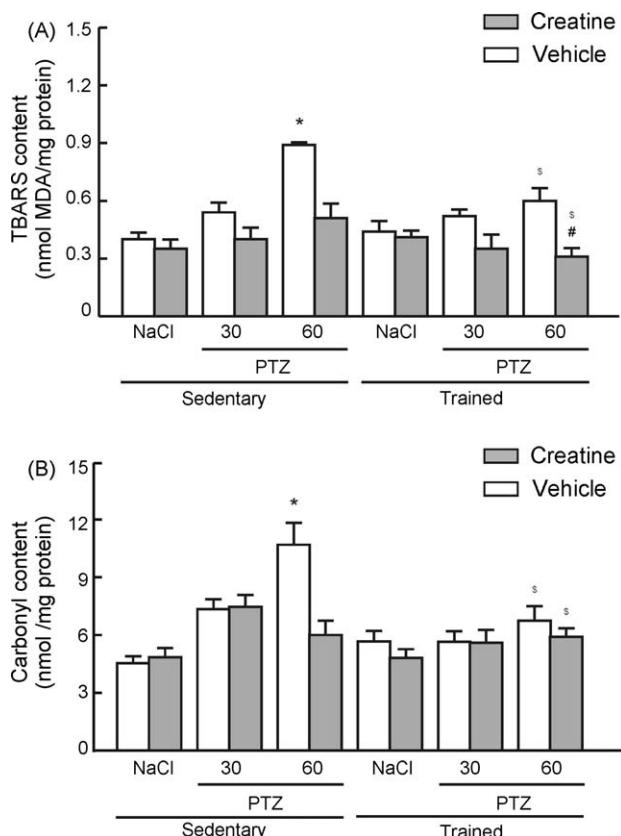
**Fig. 1.** Effect of creatine supplementation and swimming training on lactate threshold assay (A) and body weight (B). \* $P < 0.05$  compared with control/vehicle group ( $F$  test for simple effect). Data mean + S.E.M. for  $n = 6$  in each group.

In addition, creatine supplementation in control and trained rats blunted the decrease in SOD activity induced by administration of the fully convulsive dose of PTZ (60 mg/kg) [ $F(1,96) = 3.81$ ;  $P < 0.05$ ; Fig. 4B]. Interestingly, we found that physical training alone [ $F(1,96) = 3.87$ ;  $P < 0.05$ ] or in combination with creatine supplementation [ $F(1,96) = 2.14$ ;  $P < 0.05$ ] increased SOD activity when compared with control rats (Fig. 4B).

Considering that alterations in the redox state of regulatory sulphydryl groups in selected targets, such as  $\text{Na}^+,\text{K}^+$ -ATPase (Morel et al., 1998) increases cellular excitability and facilitates the appearance or propagation of convulsions (Ames, 2000), we investigated the effect physical training, creatine supplementation or its combination on NPS content and  $\text{Na}^+,\text{K}^+$ -ATPase activity. Statistical analysis revealed that physical training [ $F(1,96) = 13.37$ ;



**Fig. 2.** Representative electroencephalographic recordings demonstrate the typical EEG recordings variations in hippocampus and cerebral cortex after administration of PTZ (60 mg/kg, i.p.) in control (A) and physical training group (B). EEG recording confirmed that either physical training (B) or creatine supplementation (C) or its combination (D) reduced of electrographical seizure activity. The arrow indicates PTZ (60 mg/kg, i.p.) administration. The top black bar indicates the EEG pattern associated with generalized tonic-clonic seizures. Calibration bars 500  $\mu\text{V}$  and 1 min. (E and F) The effect of physical training and creatine supplementation on latency (E) and duration (F) of generalized tonic-clonic convulsions induced by PTZ (60 mg/kg, i.p.). \* $P < 0.05$  compared with control-PTZ group. # $P < 0.05$  compared to trained-PTZ group. Data mean + S.E.M. for  $n = 8$ –10 in each group.



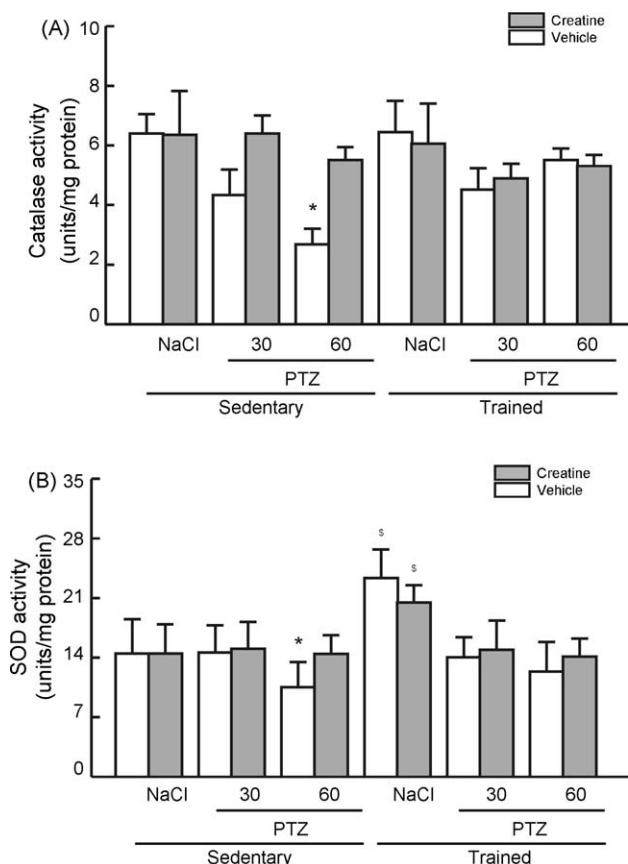
**Fig. 3.** Effect of swimming training and creatine supplementation on TBARS (A) and protein carbonyl (B) content after saline (0.9% NaCl, 1 ml/kg, i.p.) or PTZ (30 and 60 mg/kg, i.p.) injection. \*P < 0.05 compared with vehicle-control group. <sup>s</sup>P < 0.05 compared with control-PTZ group (60 mg/kg, i.p.). #P < 0.05 compared to trained-PTZ group (60 mg/kg, i.p.). Data mean + S.E.M. for n = 8–10 in each group.

P < 0.05], creatine supplementation [F(1,96) = 10.00; P < 0.05], or its combination [F(1,96) = 9.10; P < 0.05; Fig. 5A] increased NPS content *per se*. In addition, both physical training [F(1,96) = 3.25; P < 0.05], creatine supplementation [F(1,96) = 2.05; P < 0.05], or its combination [F(1,96) = 2.17; P < 0.05] protected against decrease in NPS content induced by PTZ (60 mg/kg). Statistical analysis also showed that administration of subeffective or fully convulsant doses of PTZ decreased Na<sup>+</sup>,K<sup>+</sup>-ATPase activity in the rat hippocampus [F(2,96) = 8.08; P < 0.05; Fig. 5B], and that physical training, creatine supplementation, or its combination blunted this effect.

#### 4. Discussion

In the present study we showed that physical training, creatine supplementation, or its combination increased the onset latency and decreased the duration of PTZ-induced generalized tonic-clonic seizures. Importantly, we found that the combination between physical training and creatine supplementation had additive anticonvulsant effects. Furthermore, physical training, creatine supplementation, or its combination abrogated PTZ-elicited increase in TBARS content and protein carbonylation levels, and decrease in NPS content CAT and SOD activities. In addition, physical training, creatine supplementation, or its combination blunted the PTZ-induced decrease in Na<sup>+</sup>,K<sup>+</sup>-ATPase activity.

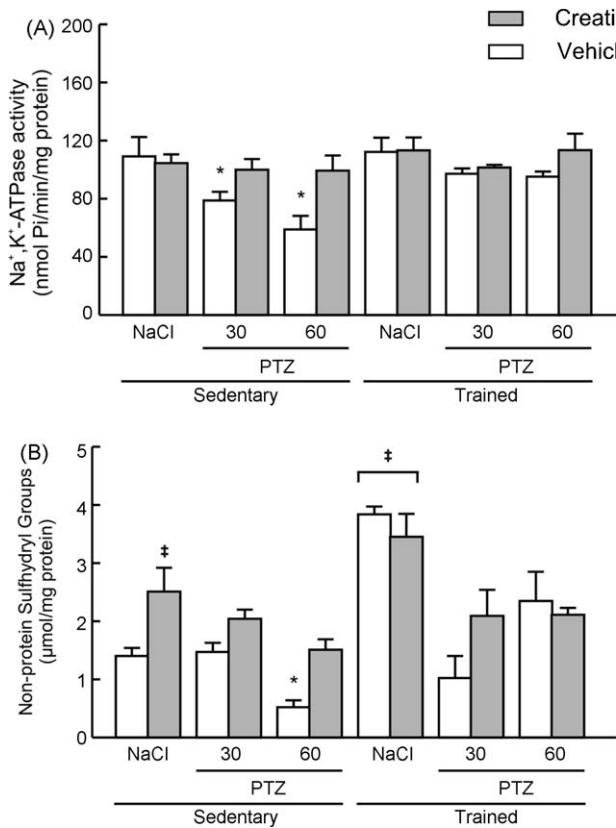
In the last decade a growing number of studies have proposed that physical training or creatine supplementation may be useful adjuvant therapies in a variety of experimental models of neurological diseases, including seizure activity (Das et al.,



**Fig. 4.** Effect of physical training and creatine supplementation on catalase (CAT) (A) and superoxide dismutase (SOD) (B) activity after saline (0.9% NaCl, 1 ml/kg, i.p.) or PTZ (30 and 60 mg/kg, i.p.) injection. Data are mean + S.E.M. for n = 8–10 in each group. <sup>s</sup>P < 0.05 compared to vehicle-control group. \*P < 0.05 compared with creatine-PTZ group (60 mg/kg, i.p.). Data mean + S.E.M. for n = 8–10 in each group.

2003; Royes et al., 2003; Andres et al., 2005; Magni et al., 2007; Bender et al., 2008; Souza et al., 2009; Arida et al., 2009). Nevertheless, the effect of the combination between physical exercise and creatine supplementation on seizure activity has not been evaluated to date. Therefore, the currently reported additive anticonvulsant effects of physical training and creatine supplementation on seizure activity induced by PTZ are of particular interest because support the idea that this combination may constitute a more effective approach as adjuvant aid in the treatment of convulsive disorders. Moreover, while physical exercise has potential as adjuvant therapy for seizure control, it is interesting to point out that the well-known positive effects of low level of physical activity on quality of life may constitute an additional attractive. In this context, concerns about increased seizure frequency and the potential for injury have led a significant number of patients with epilepsy believe that physical exercise increases likelihood of a seizure (Steinbock et al., 1996). This is likely the reason that epileptic patients lead control lives with a greater body weight, significant poorer muscle strength and respiratory capacity than people taking part in exercises from time to time (Jalava and Sillanpaa, 1997).

Though treadmill running is mentioned as the most used exercise type in experiments with animals (Daggan et al., 2000; Bennell et al., 2002; Carvalho et al., 2005), the use of swimming rats physical exercise model presents advantages over treadmill running. For instance, swimming is a natural ability of the rat, and this avoids the selection of the animals, which is necessary in experimental protocols using treadmill running (Arida et al., 1999a). Moreover, the difficulty for velocity maintenance and



**Fig. 5.** Effect of physical training and creatine supplementation on  $\text{Na}^+,\text{K}^+$ -ATPase activity (A) and non-protein sulfhydryl groups (NPS) (B) after saline (0.9% NaCl, 1 ml/kg, i.p.) or PTZ (30 and 60 mg/kg, i.p.) injection. Data are mean  $\pm$  S.E.M. for  $n=8$ –10 in each group.  $^{\dagger}P<0.05$  compared to vehicle-control group.  $^{*}P<0.05$  compared with creatine-PTZ group (30 and 60 mg/kg, i.p.). Data mean  $\pm$  S.E.M. for  $n=8$ –10 in each group.

presence of electric stimulus as stress factor are additional pitfalls of the treadmill running (Gobatto et al., 2001a). Despite such methodological differences, both swimming and treadmill running seems to be good models for investigate the effects of physical training on laboratory animals. In this context, we found a clear stabilization of the blood lactate concentration in trained versus control rats in the lactate threshold test. Since previous studies have suggested that stabilization of blood lactate in trained animals is due to muscle aerobic adaptations leading to lower lactate production and/or increased blood lactate removal for the same relative and absolute workload (Jones and Carter, 2000; Gladden, 2000; Donovan and Pagliassotti, 2000), our results are in agreement with the idea that swimming training is an effective protocol to induce muscle aerobic adaptations in rats, similar to those observed in human beings (Gobatto et al., 2001a; Voltarelli et al., 2002).

In apparent contrast to our results, Mikati et al. (2004) found that creatine supplementation did not prevent kainate-induced spontaneous recurrent seizures, increased aggressivity on the handling test and hippocampal histologic injury, suggesting that creatine-elicited anticonvulsant effects may differ depending on convulsant agent, doses, administration routes, animal species and parameters evaluated. For instance, in the present study, creatine supplementation had no effect on the onset latency, but decreased the time spent in for PTZ-induced seizures. Therefore, further studies are necessary to determine the specific types of seizures in which creatine supplementation and physical training could be an improved therapeutic measure.

Regarding the mechanisms underlying the presently reported anticonvulsant effects of physical exercise, creatine supplementa-

tion or its combination, our results suggest it may involve antioxidant mechanisms, since it has been demonstrated that oxidative stress facilitates the appearance and/or propagation of seizures in several models of experimental epilepsy (Frantseva et al., 2000; Gluck et al., 2000; Gupta et al., 2003; Patsoukis et al., 2004, 2005; Oliveira et al., 2004; Ribeiro et al., 2005; Fighera et al., 2006). Accordingly, we showed that physical training and creatine supplementation increased the NPS content and SOD activity *per se*, and were effective against  $\text{Na}^+,\text{K}^+$ -ATPase inhibition elicited by increasing doses of PTZ (Souza et al., 2009). In addition, combination between physical training and creatine supplementation abrogated PTZ-induced increase in TBARS and protein carbonyl levels, decrease in NPS content and CAT and SOD activities. In line with this view, it has been demonstrated that creatine supplementation and regular exercise lead to the development of compensatory responses to oxidative stress (Salo et al., 1991; Viguie et al., 1993; Leeuwenburgh and Heinecke, 2001; Lawler et al., 2002; Royes et al., 2006). In fact, previous studies that have demonstrated a significant selectivity regulation of antioxidant enzymes activity induced by physical exercise (Radak et al., 2007) and direct scavenging of ROS generation elicited by creatine (Sestili et al., 2006). In line of this view, a considerable body of evidence has suggested that exercise-induced production of ROS plays a role in the induction of antioxidants, DNA repair and protein degrading enzymes, resulting in decreased incidence of oxidative stress (Navarro et al., 2004; Radak et al., 2006; Boveris and Navarro, 2008). Additionally, experimental findings have demonstrated that creatine provides selective antioxidant role by prevent the opening of the mitochondria permeability transition pore, which ultimately increases  $\text{Ca}^{2+}$  and ROS intramitochondrial levels and causes excitotoxic and apoptotic cell death in several neurodegenerative (O'Gorman et al., 1997; Leist and Nicotera, 1998; Brewer and Wallimann, 2000; Sakellaris et al., 2006).

Since there are evidence that some selected targets for oxidative stress, such as  $\text{Na}^+,\text{K}^+$ -ATPase might lead to enhance of neuronal excitability and appearance of convulsions, the currently reported PTZ-induced increase in TBARS and protein carbonyl content, as well as decrease in NPS content and CAT and SOD activities may be interpreted either as responsible for decrease in  $\text{Na}^+,\text{K}^+$ -ATPase as well as a consequence of the decreased  $\text{Na}^+,\text{K}^+$ -ATPase activity and/or simultaneous events that synergically contribute to the neuronal excitability elicited by this convulsant agent. However, we cannot rule out other mechanisms which might be used to explain the currently reported anticonvulsant effects of physical training and/or creatine supplementation. For instance, the neuroprotective effect exerted by creatine in several neurodegenerative processes involves buffering of intracellular energy, preventing the increase of  $\text{Ca}^{2+}$  and ROS intramitochondrial levels which leads to excitotoxic cell death (O'Gorman et al., 1997; Leist and Nicotera, 1998; Dolder et al., 2003; Klivenyi et al., 2003, 2004; Andres et al., 2005). Moreover, in light of the role of steroid hormones in the correlation of physical exercise and convulsive activity, it has been demonstrated that the  $3\alpha$ -reduced metabolites of testosterone ( $5\alpha$ -dihydrotestosterone and  $3\alpha$ -androstanediol) had powerful protective activity against seizures induced by several GABA<sub>A</sub> receptor antagonists, pilocarpine and maximal electroshock model (Kaminski et al., 2004; Reddy and Rogawski, 2002). In intravenous PTZ test, the  $3\alpha$ -androstanediol and  $5\alpha$ -androstanediol cause a dose-dependent elevation of seizure threshold (Reddy, 2004) suggesting that anticonvulsant effect exerted by these metabolites correspond to its ability to potentiate the GABA<sub>A</sub> receptor-mediated inhibition (Reddy, 2008). In addition, since it has been shown that physical training upregulates the glutamate transporter EAAT-1 in the rodent brain (Molteni et al., 2002), and that increased expression of this excitatory aminoacid carrier is a critical phenomenon underlying

the anticonvulsant effects of the broad spectrum antiepileptic drug valproic acid (Aguirre et al., 2008), it is plausible that exercise-induced upregulation of EAAT-1 be involved in its anticonvulsant effects.

One last point to comment is that our protocol of swimming training decreased total body weight in trained versus control rats along the 6 weeks of training. Regarding such difference in body weight between control and trained rats, we think that it may be explained by changes in body composition. For instance, a decrease in subcutaneous adipose tissue of trained rats may explain why body mass was smaller in this group (Petridou et al., 2005). However, we have not determined body composition in the present study, and therefore this explanation remains speculative in nature, and further studies are necessary to determine the mechanisms involved.

In conclusion, the present study showed that physical training, creatine supplementation or its combination attenuated PTZ-induced seizures and oxidative damage *in vivo*. Importantly, physical training and creatine supplementation combination had additive anticonvulsant effects. Our data provide evidence about the anticonvulsant and antioxidant potential of the combination between creatine supplementation and physical exercise.

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