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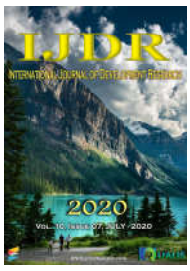

RESEARCH ARTICLE OPEN ACCESS

CREATINE SUPPLEMENTATION IN EXERCISED RATS REDUCES THE ACTION OF REACTIVE OXYGEN SPECIES

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ABSTRACT

The objective of this study was to determine the effects of creatine supplementation on the biomarkers of oxidative stress in the red blood cells from trained rats. Forty adults male Wistar rats (90 days old) were distributed into four groups during eight weeks: Control group (C) - rats feed balanced diet; Creatine control group (CCr) - rats that received 2% creatine supplementation through a balanced diet; Trained group (T) – rats that trained at an intensity equivalent to the maximal lactate steady state and Trained-supplemented group (TCr) - rats that trained at the same intensity and supplemented with 2% creatine through a balanced diet. The concentration of creatine and H_2O_2 such as theactivity of SOD, GSH-GPx, CAT, GSH and GSSG in red blood cells were evaluated. Creatine concentration increased in CCr and TCr than in other groups and H_2O_2 decreased in the CCr and TCr groups compared with the other two groups. SOD decreased in the TCr group opposed to C and CCrgrups. CAT increased in TCr group compared against C and T groups. GSH improved in TCr when compared with CCr group. These results confirm the hypothesis that creatine acts as an antioxidant, neutralizing the H_2O_2 concentrations.

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INTRODUCTION

In recent years, there has been a growing interest in the relationship between free radicals, exercise and antioxidant nutrients (De Sousa, 2017), also, some recent research has been performed to identify other relevant benefits of antioxidant supplementation in physical exercise. The regular practice of physical activities associated with a balanced diet may be an important factor in promoting good health. However, frequent or exhaustive high intensity physical exercises may increase susceptibility to injury and promote chronic fatigue and overtraining due to the high synthesis of reactive oxygen species (ROS) (Kreider, 2003; Cruzat, 2007). Therefore, supplementation with antioxidants may prolong the initiation phase or inhibit the propagation phase of ROS and reactive nitrogen species (Ernst) (Teixeira *et al.,* 2008; Bhavsar, 2009), which is observed during physical exercise (Kreider, 2003; Cruzat, 2007). Among the commercially available antioxidant supplements, creatine has received attention because it related to fighting the action of ROS.

Creatine (methyl guanidine acetic acid) - an amine nitrogen - is found in food. Although creatine is not an essential nutrient because the body's needs can be fulfilled by endogenous synthesis, it is intimately involved in human metabolism (Williams *et al.,* 2000). Creatine consists of glycine and arginine amino acids, which affected by glycine amidiltransferase, resulting in guanidinoacetate and ornithine. In turn, ornithine is affected by guanidinoacetate methyltransferase enzyme and acts as a substrate for nitric oxide synthase, resulting in the formation of nitric oxide as well as stimulating the production of free radicals, which modulates the metabolism, contractility and glucose uptake in skeletal muscle(Reid, 2001; Paddon-Jones *et al.,* 2004; Pereira and Souza Junior, 2008; Araújo and Mello, 2009). Based on studies by Vergnani*et al.* (2000) that show the fundamental antioxidant role of the amino acid arginine, which is one of the components of creatine in the removal of O_2- radicals in endothelial cells, it was hypothesized that creatine also affects cellular redox metabolism. Lawler *et al.* (2002) also reported that creatine supplementation could help reduce oxidative stress. In

their study, creatine decreased the number of •O2- radicals and peroxynitrite (•OONO-). However, lower H_2O_2 levels was not observed, suggesting that the antioxidant properties of creatine are selective and limited. More recently, Demenice and Jordão (2012) showed that creatine supplementation inhibits increases in the oxidative stress markers in the plasma and muscle of rats that participated in an acute swimming exercise. As a result, some *in vitro* data are available, showing that creatine may act as a ROS scavenger. However, few studies demonstrate the *in vivo* antioxidant capacity of creatine supplementation for oxidative stress induced by aerobic exercise. Therefore, it is interesting to speculate on the novel use of antioxidant agents, such as creatine supplementation, for inhibiting the damage caused by excess ROS. The objective of this study was to determine the effects of creatine supplementation on the biomarkers of oxidative stress in the red blood cells of trained rats.

MATERIAL AND METHODS

Animals: Forty male Wistar rats with 90 days old selected, receiving food and water *ad libitum*. The animals were housed in collective polyethylene cages (5 animals per cage), measuring 37.0 x 31.0 x 16.0cm, under controlled temperature conditions (22ºC) and with 12h light/dark cycle. All experiments involving animals was approved by the Ethics Committee on Animal Experimentation at the Taubaté University - UNITAU, São Paulo State, Brazil (register CEEA / UNITAU n º 018/08).

Diets: The animals belonging to groups supplemented with creatine (CCr and TCr) received a balanced diet AIN-93M (Reeves *et al.,* 1993) with 13% in the stage of creatine overload during seven days, after this phase the animals maintained 2% of creatine Monohydrate up to the end of the experiment (All Chemistry, São Paulo, SP) (Araújo *et al.,* 2013).

Experimental Groups: The time of intervention from both the exercise and creatine supplementation was eight weeks, the animals were distributed into four experimental groups: 1) control (C): sedentary rats that recieved balanced diet; 2) creatine control (CCr): sedentary rats that received supplementation with with 13% in the stage of creatine overload during seven days, after this phase the animals maintained 2% of creatine through balanced diet; 3) trained (T): rats that received balanced diet and were subjected to a training protocol and, 4) supplemented trained (TCr): rats that were subjected to a training protocol and received supplementation with 13% in the stage of creatine overload during seven days, after this phase the animals maintained 2% creatine through balanced diet.

Training Protocol: The animals were subjected to the aerobic capacity test for the identification of the maximal lactate steady state (MLSS), according to Araújo*et al.,* (2013) and Manchado*et al.* (2005). The MLSS was determined before and after six weeks of the experimental period. For the determination of MSSL the animals carried out several tests of treadmill running. Each test consisted of 30 minutes of uninterrupted running. The objective of test is to determine the higher load in which the blood lactate present stabilization, i.e., difference lesser than or equal to 1.0 mmol/L between 10th and 25th minutes of exercise. The first test was performed with the load of 5% bm and variation of 0.5% to above or below, until to find the maximum load in which still occurs stabilization of blood lactate concentration during the exercise session. Blood samples collected through a puncture at the end of the tip of the tail of animal, every 5 minutes of exercise for the analyses of lactate concentrations. The blood samples (25µl), transferred into Eppendorf (1.5 ml capacity) tubes containing 50µL of sodium fluoride (1%). The blood lactate concentrations were determined in alactate analyzer (Lactimeter: YSI Model 1500 Sport, Yellow Springs, OH, USA). *Blood sampling and biochemical analyses:* At the end of the experiment, the fed and rest, the animals anesthetized with carbon dioxide and after exsanguinated; blood collected from the heart (10 ml). Immediately after collection, the blood was centrifuged three times at 2.500 rpm for five minutes in 0,05N phosphate buffer (composition, in g/L: KH_2PO_4, 1.34 and $NaHPO_4$.2H2O, 7.1) with NaCl 0.9% pH 7.4. After isolation of red blood cells, that were lysed by adding H_2O (1:1 v/v) and centrifuged at 5.000 rpm for 10 minutes twice in order to remove the remaining membranes, the pellet was discarded, and the supernatant was frozen at –70ºC for later analyses. The hemoglobin dosage of the hemolysate made using the method of Drabkin (Beutler, 1975). For measurement of serum creatine, Clark method utilized, using Jaffé reaction (Clark and Thompson, 1949). As a biomarker of oxidative stress, the concentrations of hydrogen peroxide - H_2O_2 (Amplex® UltraRed Reagent) were analyzed. As indicators of the antioxidant system, the activity of the flowing enzymes was analyzed: superoxide dismutase - SOD (commercial kit from Cayman Chemical, Ann Arbor, Michigan, EUA), glutathione peroxidase – GSH-GPx by (commercial kit from Cayman Chemical, Ann Arbor, Michigan, EUA), catalase - CAT (Aebi, 1984) and reduced glutathione – GSH and oxidized– GSSG analyzed according to Hissin and Hilf method (Hissi and Hilf, 1976). *Data analysis:* The statistical analysis of results performed with the aid of statistical packages *STATISTICA,* version 7.0. All results, we tested for normalityusing Shapiro-Wilks test, to establish the necessity of parametric statistics. The data were determined to have a normal distribution. Results expressed by mean $\pm$ standard deviation, and statistically analyzed by *Two-Way* ANOVA followed by a post-*hoc* Tukey HSD, when necessary.For all analyses, $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

It is evident that animals supplemented with creatine show a significant increase in the concentration of creatine in the serum compared to animals that did not receive the supplementation (Figure 1).

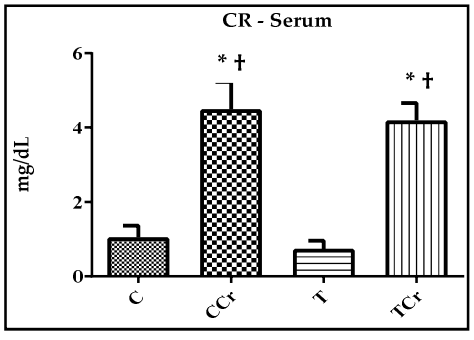

Figure 1. Concentration of creatine (CR) in the serum of animals at the end of the experiment. Results expressed by mean + SD of 10 animals per group. TCr = Trained Creatine; T = Trained; CCr = Control Creatine; C = Control not Trained. * different C; † different T

The Figure 2 shows the values of the concentration of hydrogen peroxide (H_2O_2) in red blood cells of animals in the end of the experiment. There was a significant decrease in concentrations of H_2O_2 in animals of supplemented groups (CCr and TCr) compared to animals of not supplemented groups (C and T).

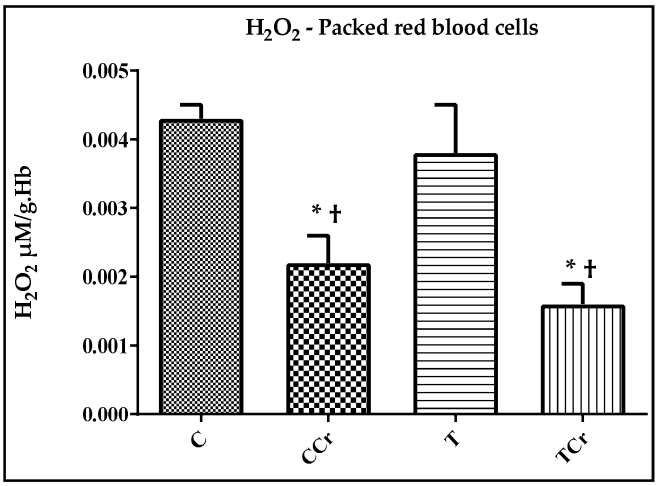

Figure 2. Concentration of hydrogen peroxide (H_2O_2) in red blood cells of animals in the end of the experiment. Results expressed by mean + SD of 10 animals per group. * different C; † different T

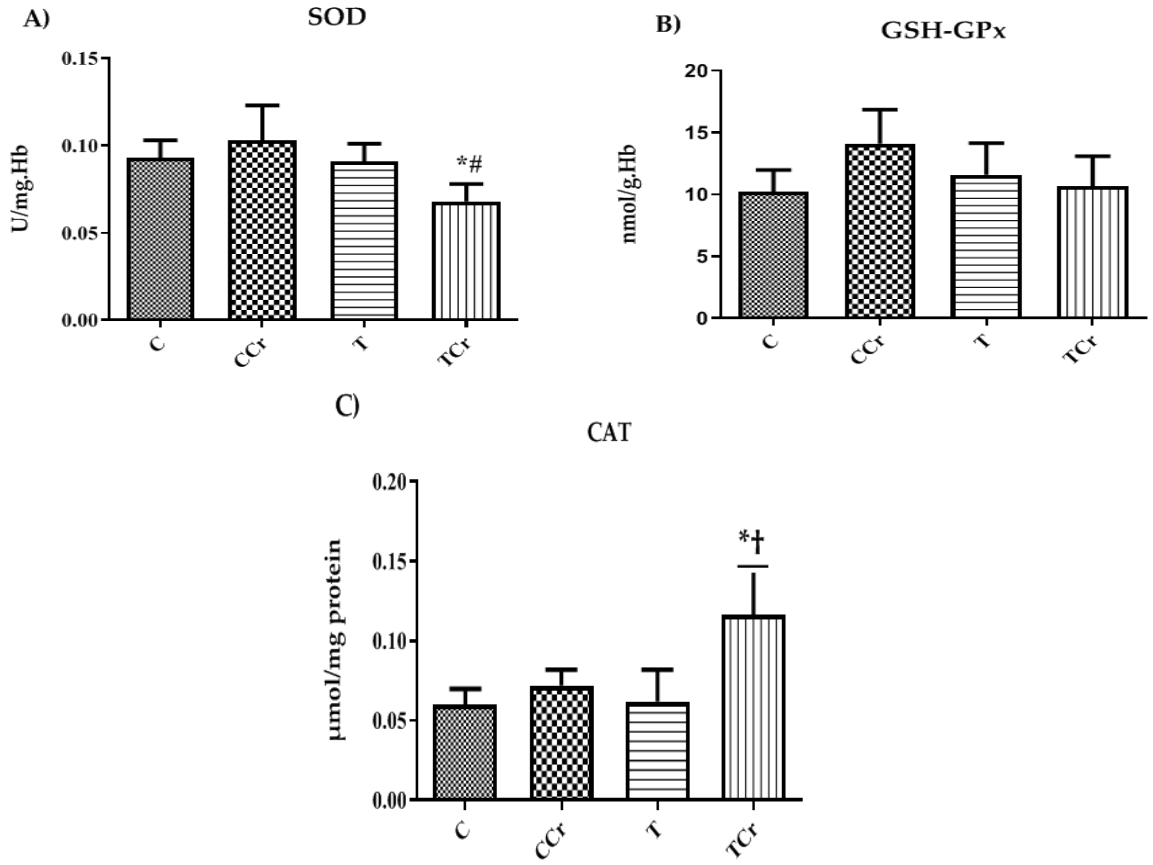

Figure 3. A) Activity of the enzyme superoxide dismutase (SOD); B) glutathione peroxidase (GSH-GPx) and C) catalase (CAT) in red blood cells of animals at the end of the experiment. Results expressed by mean + SD of 10 animals per group. TCr = Trained Creatine; T = Trained; CCr = Control Creatine; C = Control not Trained. * different C; ** different CCr and C; # different CCr; † different T

The Figure 3 shows the values related to the activity of superoxide dismutase (SOD) in red blood cells of animals at the end of the experiment (Figure 3A). Animals in the group TCr showed a decrease in SOD activity in red blood cells in relation to animals of group C and CCr. The figure 3B shows the values of the activity of the enzyme glutathione peroxidase (GSH-GPx) in red blood cells of rats at the end of the experiment. There was no significant difference between groups. The values of the activity of the enzyme catalase (CAT) in red blood cells at the end of the experiment in the tissues analyzed were higher in animals of group TCr in relation to animals of groups CCr and T (Figure 3C).

The Figure 4 shows the values of Reduced Glutathione (GSH), Oxidized Glutathione (GSSG) and Ratio between Reduced Glutathione and Oxidized Glutathione (GSH/GSSG) in red blood cells of rats at the end of the experiment. Activity of GSH showed an increase in the animals of group TCr in relation to animals of group CCr. However, the GSSG showed differ significantly in animals of supplemented groups (TCr and CCr) in relation to animals of groups C and T ($p.<0.05$). Numerous studies suggest that creatine supplementation and regular can affect the antioxidant capacity, which protects cells against the deleterious effects of oxidative stress and prevents subsequent cellular damage.

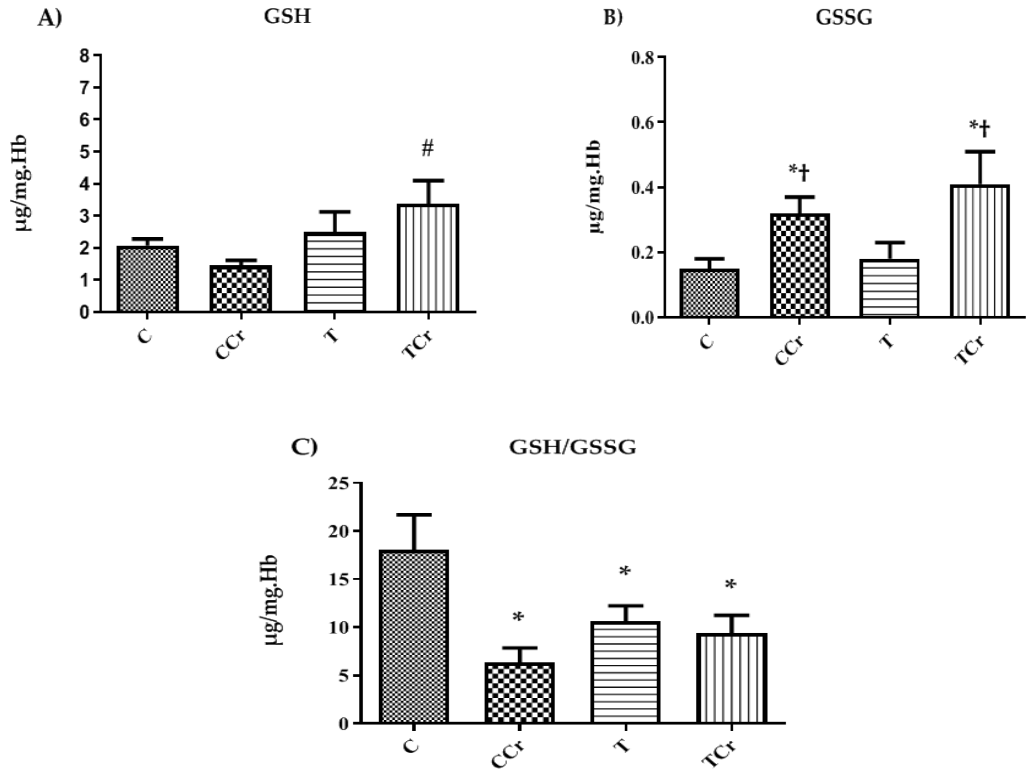

Figure 4. Reduced glutathione oxidized glutathione and reduced glutathione/oxidized glutathione ratio in red blood cells of animals at the end of the experiment. Results expressed by mean + SD of 10 animals per group. TCr = Trained Creatine; T = Trained; CCr = Control Creatine; C = Control not Trained. * different C; # different CCr; † different T.

In this regard, the present study aimed to verify the possible antioxidant effect of creatine in vivo. Our results agree with findings in the literature on the potential effect of creatine in removing ROS (Guidi*et al.,* 2008; Fimognari*et al.,* 2009). The concentrations of H_2O_2 analyzed in the study significantly decreased in animals in sedentary and trained groups that received creatine (CCr and TCr) compared with in other groups (C and T). It is worth mentioning that creatine supplementation appears to exert a selective effect on the neutralization of ROS (Pereira and Souza Junior, 2008). Demenice and Jordão (2001) showed that supplementation whit creatine decreases the concentration of H_2O_2 in rats that performance acute exercise. Guimarães Ferreira *et al.* (2012) reported that creatine supplementation decreases the ROS content in skeletal muscle, which is possibly due to the direct action of the creatine molecule in neutralizing the superoxide radical. Thus, the direct antioxidant effect of creatine seems to be limited to certain types of free radicals or reactive oxygen species. Although lower production of ROS being enough to provide a safe cellular environment due to the otherwise high potential toxicity of oxygen and its use by aerobic organisms, a variety of antioxidant systems must also be sufficiently equipped to protect cells from the damaging effect of ROS (Ferreira *et al.,* 2007; Lambertucci*et al.,* 2007; Hamid *et al.,* 2011). The activities of superoxide dismutase – SOD, glutathione peroxidase – GSH-GPx, catalase – CAT as well as the oxidized glutathione – GSH and reduced glutathione – GSSG amounts and the ratio between the two – GSH-GSSG analyzed.

Despite the lack of change in the activity of GSH-GPx and decreased SOD activity in animals in the TCr group and GSH/GSSG ratio in the CCr, T and TCr animals, creatine supplementation appears to act directly and indirectly on antioxidant enzymes. In the present study, the animals that trained and supplemented with creatine had a significant increase in the CAT activity and GSH levels, suggesting that creatine could be up regulating the activity of these enzymes. Recently, Young *et al.* (2010) reported that creatine can adjust the system redox thiol, of which GSH is an important component. Demenice and Jordão (2011) reported that rats that were supplemented with creatine had increased GSH and total antioxidant capacity (FRAP). There is controversy over whether creatine supplementation, in combination with physical training, increases the activity of the antioxidant system enzymes. Some authors reported an increase in the activity of antioxidant enzymes (SOD, GSH-GPx, CAT and GSH) in serum and skeletal muscle induced by physical training (Pereira *et al.,* 1994; Smolka*et al.,* 2000), but others have found no significant changes in the activity of these enzymes (Deminece and Jordão, 2012; Guimarães – Ferreira *et al.,* 2012). The fact that SOD, GSH-GPx and the GSH/GSSG ratio analyzed in this study do not change, along with a decrease in their activities, indicates that creatine may exert a sparing effect, i.e., creatine may neutralize ROS. As a result, there is a readjustment of the antioxidant system. This hypothesis is based on the fact the numerous studies on antioxidant supplementation of vitamin E, claim that it inhibits the action of antioxidant enzymes (Hamid *et al.,* 2011; Asha and Ravikiran, 2004).

Conclusions

In this study, the trained animals that supplemented with creatine had affected antioxidant enzyme activity. These results confirm the hypothesis that creatine may have an antioxidant action on the H_2O_2 concentrations, thus neutralizing their effects.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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In addition, the GSH/GSSG ratio showed a decrease in values in the animals of groups CCr, T and TCr in relation to animals of group C.

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