

## Creatine supplementation in trained rats causes changes in myenteric neurons and intestinal wall morphometry

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**ABSTRACT:** Creatine is widely used by athletes as an ergogenic resource. The aim of this study was to evaluate the influence of creatine supplementation on the duodenum of rats submitted to physical training. The number and myenteric neuronal cell bodies as well mucosal and muscular tunic morphometry were evaluated. Control animals received a standard chow for 8 weeks, and the treated ones received the standard chow for 4 weeks and were later fed with the same chow but added with 2% creatine. Animals were divided in groups: sedentary, sedentary supplemented with creatine, trained and trained supplemented with creatine. The training consisted in treadmill running for 8 weeks. Duodenal samples were either processed for whole mount preparations or for paraffin embedding and hematoxylin-eosin staining for histological and morphometric studies of the mucosa, the muscular tunic and myenteric neurons. It was observed that neither creatine nor physical training alone promoted alterations in muscular tunic thickness, villus height or crypts depth, however, a reduction in these parameters was observed when both were associated. The number of myenteric neurons was unchanged, but the neuronal cell body area was reduced in trained animals but not when training and creatine was associated, suggesting a neuroprotector role of this substance.

### Introduction

Creatine (=methylguanidinoacetic acid) is an amino acid used as a nutritional supplement with large application as ergogenic substrate (Kreider *et al.*, 1998; Volek and Rawson, 2004) and a neuroprotector (Matthews *et al.*, 1999; Wyss and Schulze, 2002; Bender *et al.*, 2006). During the last years this substance has become one of the most popular nutritional supplements, aiming the improvement in physical performance during training sessions, mainly because its use reportedly increase lean muscular mass, strength, and muscular power. In

face of that, creatine intake has become popular among professional and amateur athletes (Mesa *et al.*, 2002; Branch, 2003; Volek and Rawson, 2004).

The comprehension of creatine synthesis, transport and degradation is the basis for its use as a nutritional supplement and therapeutic compound in treatment of Parkinson's and Huntington's disease, disorders that affect the mitochondria (Klivenyi *et al.*, 1999). It has also been used for the treatment of cardiac muscular atrophy rehabilitation due to disuse, as a neuroprotector in brain hypoxia (Baker-Fulco *et al.*, 2006) and even for the treatment of diabetes (Blum, 2002).

The daily creatine demand is supplied by endogenous synthesis or diet, predominantly by meat intake (Peralta and Amancio, 2002). The endogenous synthesis is down-regulated by diet and when the creatine intake is increased (Mesa *et al.*, 2002).

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Creatine is exported from liver and gastrointestinal tract to be stored in tissues such as cardiac and skeletal muscles, which contains significant creatine kinase (CK) concentrations, enzyme responsible for the conversion of creatine to phosphocreatine (PCr), a high energy compound (Garcia, 2000).

The gastrointestinal tract does not store considerable amounts of creatine, but is the first place to capture and export this substance (Peral *et al.*, 2005). Creatine is coupled to the contractile myosin ring, indicating that a high-energy PCr system is required for an appropriate motor function (Gordon and Keller, 1992). The CK abundance in mitochondria and enterocytes suggests that creatine is part of a system, which keeps temporally and/or spatially the dynamic energetic need of the epithelial cells (Keller and Gordon, 1991).

The creatine intestinal transporters are mainly associated to the apical membrane of villus enterocytes, but is absent in crypts and goblet cells (Peral *et al.*, 2005). The transporters are  $\text{Na}^+/\text{Cl}^-$  dependent and accumulate creatine against its concentration gradient (Wyss and Kaddurah-Daouk, 2000; Peral *et al.*, 2005) in a stoichiometric ratio of 2  $\text{Na}^+$ :1  $\text{Cl}^-$ : 1 creatine (Peral *et al.*, 2005). Some evidence suggests that changes in this transporter system are genetically programmed and controlled by creatine uptake (Peral *et al.*, 2005).

Occurrence of cramps (Bizzarini and De Angelis, 2004), nausea, vomits and diarrhea (Brudnak, 2004) are often related to the usual creatine consumption by athletes (Wyss and Kaddurah-Daouk, 2000; Brudnak, 2004). Regardless of it, there are no clear evidences associating creatine supplementation to deterioration of the gastrointestinal function. For this reason, the aim of this study was to verify if creatine supplementation in trained and sedentary rats affects intestinal wall morphometry as well the number and morphometry of neurons in the myenteric plexus.

## Material and Methods

### Animals

Male Wistar rats weighing approximately 300 g, from the Animal Resources Center of the State University of Maringá, were used in the experiments. They were housed in group cages (4 animals per cage) with free access to water and food. The animals were maintained in light/dark cycle of 12h (lights on at 6 am, off at 6 pm) and temperature of  $20^\circ \pm 2^\circ\text{C}$ . Control groups rats (sedentary and trained) were fed with a balanced

chow (Nuvilab CR1, Nuvital SA, Colombo, Brazil) for 8 weeks, while animals in the treated groups (sedentary and trained rats supplemented with creatine) were fed with the same balanced chow for 4 weeks, but the chow was supplemented with 2% of creatine monohydrate (Probiótica<sup>®</sup>, São Paulo, Brazil) for 4 additional (Mc-Millen *et al.*, 2001).

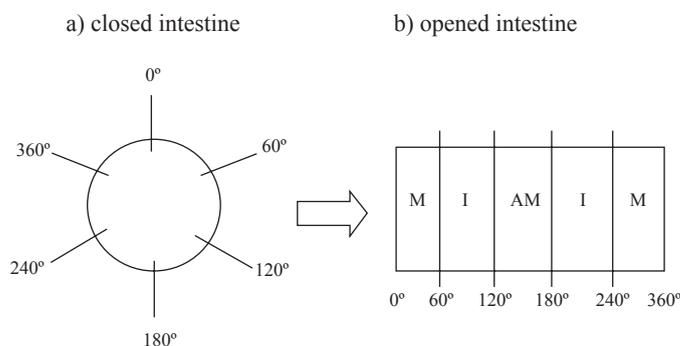
The Ethics Committee on Animal Experimentation of the State University of Maringá approved all procedures involving the use of animals.

### Experimental procedure

Animals were randomly divided into 4 groups, 4 animals each: sedentary control (S), trained control (T), sedentary supplemented with creatine (SCre) and trained supplemented with creatine (TCre). Physical training lasted 8 weeks, during which the animals were forced to run in a treadmill for 60 min, 5 times a week, according to the protocol established by Dufloth *et al.* (1997) and adapted by Negrão *et al.* (1992). Animals were sacrificed between 7:30 and 10:30h. No physical training sessions were performed on the day of sacrifice. Both body weight and food intake were recorded twice a week and water consumption three times a week throughout the experiments.

### Exercise protocol

The physical exercise consisted of a running training protocol in a programmable treadmill (Inbramed,



**FIGURE 1.** The diagram represents (a) closed intestine:  $0^\circ$  comprises the mesenteric insertion; (b) opened intestine: M corresponds to the mesenteric region, I, intermediary region and AM, anti-mesenteric region.

KT3000, Porto Alegre, Brasil), adapted to run 8 rats simultaneously, from 6:30 to 7:30 am, at an intensity of 60 to 75% of the maximum  $O_2$  uptake ( $VO_2$  max). Trained animals were submitted to an adaptation week constituted by sessions of 10 minutes/day, in which the treadmill speed ranged from 0.3 Km/h to 0.8 Km/h, in stages that lasted 2 minutes each. After this adaptation period, either the treadmill speed and session endurance were gradually increased until the animals were able to run 1 hour/day, in a average speed of 1.0 km/h during 5 days/week, for a total period of 8 weeks. Each session consisted of various stages, where speed and duration varied, with no changes on treadmill inclination.

#### *Sacrifices and tissue sampling*

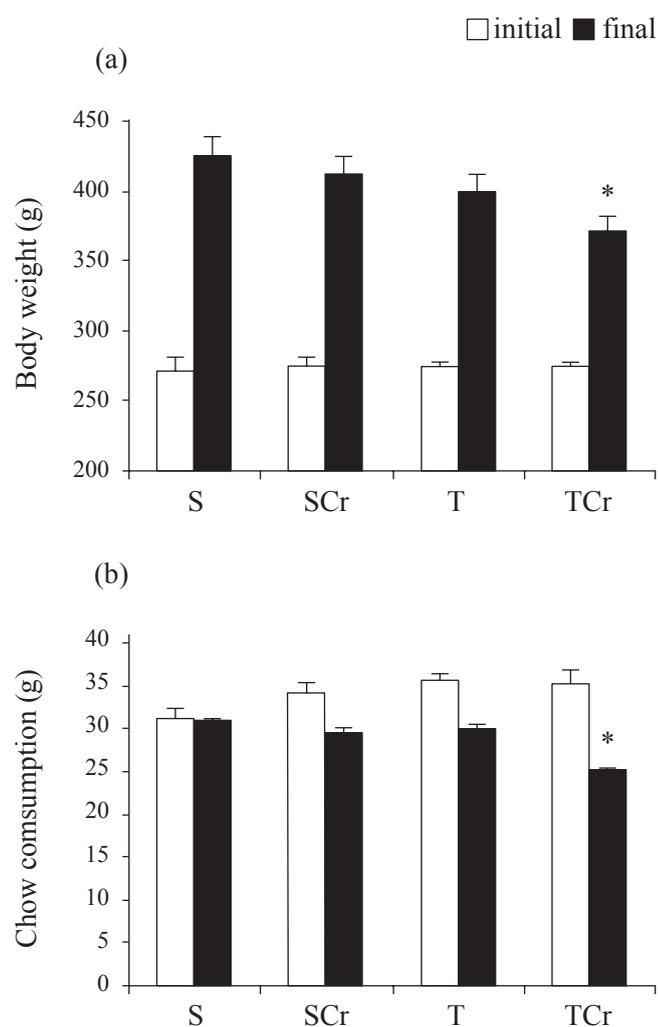
Animals were anesthetized with sodium pentobarbital (Hypnol® 3%, 4 mg/100g/ body weight, intraperitoneally) and the duodenum was excised out and divided in two segments for either whole mount preparations or for paraffin embedding and hematoxylin-eosin staining.

#### *Whole mount preparations*

After washing the intestinal lumen with physiological solution, a cotton thread was set at one end and the lumen was filled with Giemsa fix solution until obtaining an intestinal wall distension similar to that normally provoked by the intestinal content. The other end was then tighten and the whole segment was placed in the fixing solution for 24h. The segments were then opened through their mesenteric side to obtain whole mount preparations of the muscular tunic, by removing the mucosa and submucosal layers under a stereomicroscope. The muscular tunic was stained by Giemsa (Barbosa, 1978), following dehydration, diaphanization, and slides assembling for microscopic analyses. For studying the neuronal profile area, images of myenteric neurons were captured with a QCapture Pro Olympus camera (Tokyo, Japan) coupled to Olympus BX41 microscope (Tokyo, Japan) with 40x objective. The profile ( $\mu m^2$ ) of 150 cell bodies/animals were measured using Image-Pro-Plus 4 (Media Cybernetics, USA). A total of 600 neurons per group were studied. The number of myenteric neurons was determined in the intermediate region ( $60^\circ - 120^\circ$ ;  $240^\circ - 300^\circ$ ) of the intestinal circumference, considering  $0^\circ$  as the mesenteric insertion (Fig. 1). Neurons were counted in 40 randomized microscopic fields using the Olympus BX41 microscope with 40x objective. The microscopic field area was  $0.24 \text{ mm}^2$ .

#### *Histological preparations*

After removing and washing of intestinal lumen, the segments were opened through the mesenteric border and placed on a cork plate with the epithelial surface up. The samples were then fixed in Bouin fluid for 24 h and submitted to dehydration and paraffin embedding. Finally, semi-serial sections ( $4 \mu m$  thick) were stained with hematoxylin and eosin.



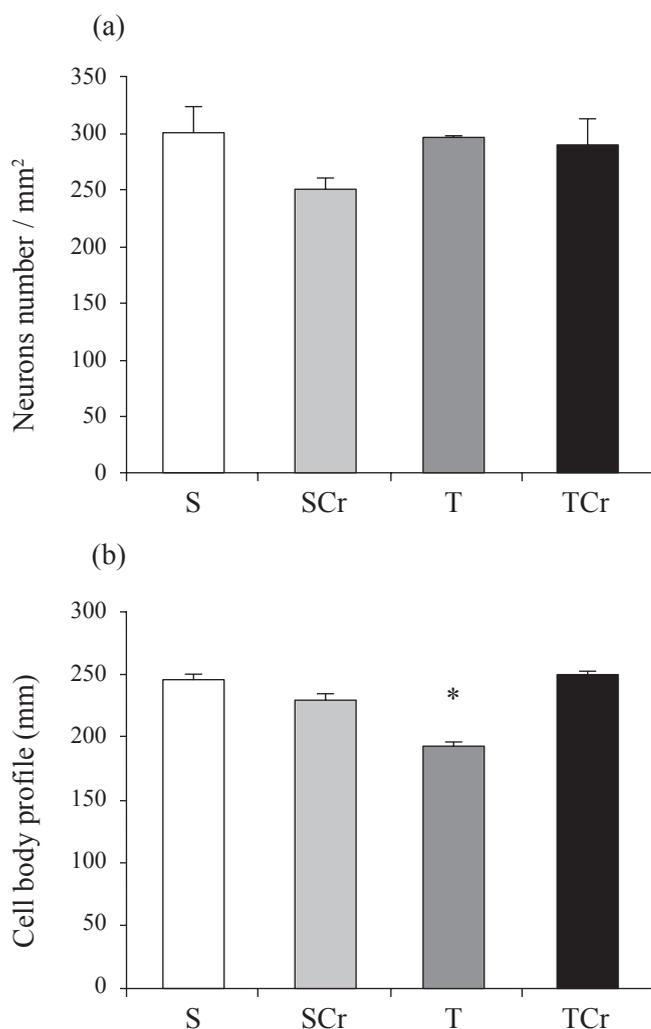
**FIGURE 2.** Initial and final (a) body weight (g) and (b) chow consumption (g) in group of sedentary animals (S), sedentary animals supplemented with creatine (SCr), trained animals (T) animals trained and supplemented with creatine (TCr). The asterisk indicates statistically significant differences when compared to the S group. Values were expressed as mean  $\pm$  SE (n=8), using ANOVA and Tukey's post test.

### Intestinal wall morphometry

Morphometric analyses were performed with a ruler coupled to the 40x objective using the Olympus BX41 microscope. Crypts depth and villus height were determined in longitudinal cuts. The measurements were done on 40 villi from 40 crypts per animal. Muscular tunic thickness was determined in 8 sections per animal at 5 different places (a total of 40 measurements/animal). Results were expressed in micrometers.

### Statistical analysis

The statistical analysis was performed using the GraphPad Prism<sup>®</sup> 3.0 program. Data are presented as



**FIGURE 3.** (a) Myenteric neurons number /mm<sup>2</sup> in the duodenal intermediate region and (b) neuronal profile area (μm<sup>2</sup>). Abbreviations as in Figure 2. Values were expressed as mean ± SE (n=4), using ANOVA and Tukey's post test. The asterisk indicates statistically significant differences when compared to S and TCr groups.

mean ± standard error. A two-way ANOVA was performed for all morphometric parameters and the Tukey test was employed as post-test to multiple comparisons between the groups. For animal weight analysis, the Student *t* test was used for pair-wise comparisons. In all cases, significance level was set at  $P < 0.05$ .

## Results

### Weight, water and chow consumption

Animals from all groups gained weight during the whole experimental period. Animals from the TCr group presented the lower gain in weight at the end of the experiment (Fig. 2a). The weight loss was estimated in 13% in relation to the S group ( $p < 0.05$ ), 6% in relation to T group and 3% in relation to SCr group. This was associated with a significant reduction in food intake by animals in the TCr group as compared with the S group (Fig. 2b). No differences in water consumption were observed between any of the groups studied.

### Myenteric neurons number and neuronal cell body area

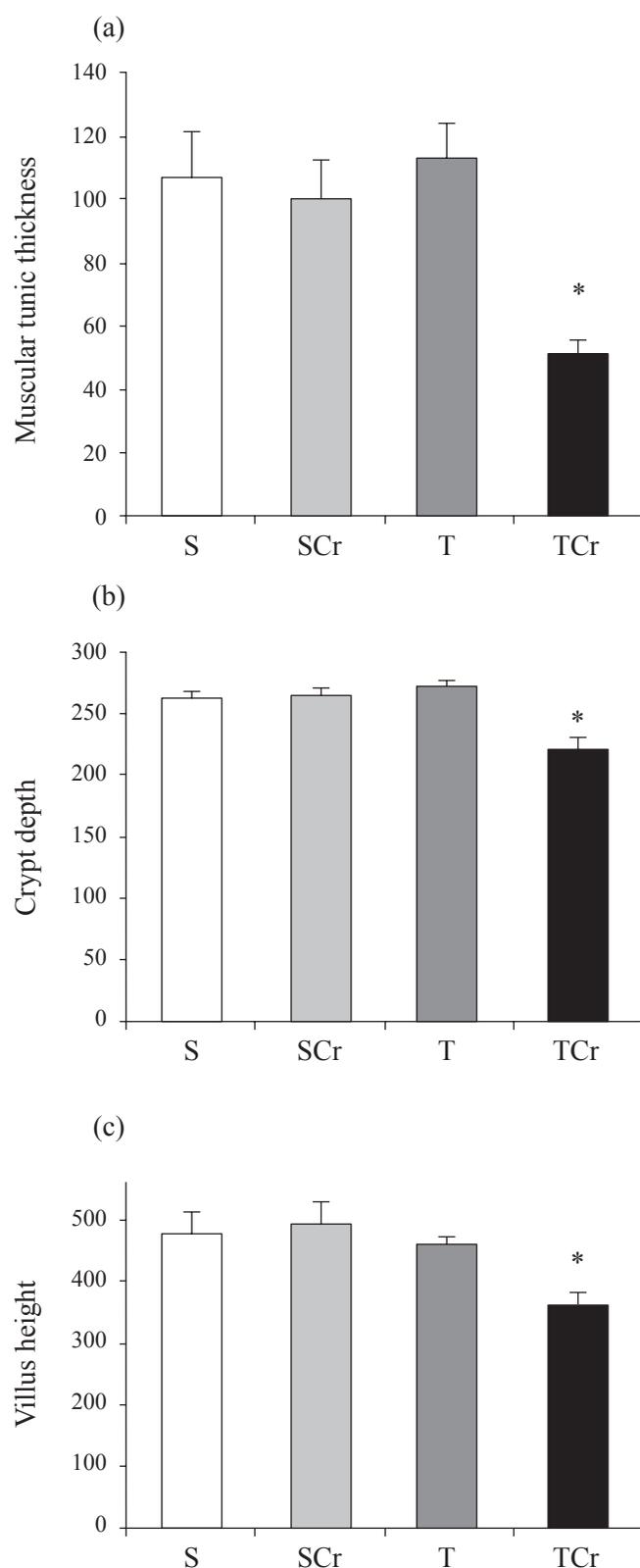
The number of myenteric neurons remained unchanged in all studied groups as shown in Figure 3a significant reduction ( $p < 0.01$ ) on neuronal profile area was observed, however, in animals from group T ( $193.0 \pm 3.3 \mu\text{m}^2$ ) when compared to either TCr animals ( $248.4 \pm 3.9 \mu\text{m}^2$ ) or S animals ( $246.0 \pm 4.6 \mu\text{m}^2$ ) (Fig. 3b).

### Muscular tunic, crypts and villus morphometry

Figures 4a, 4b and 4c show the measurements of the muscular tunic (μm), crypts depth (μm) and villus height (μm) respectively, of different studied groups. A significant reduction in all three parameters was observed when physical training and creatine supplementation were associated (TCr group), as compared with both the trained and supplemented groups.

## Discussion

Our study showed for the first time that moderate physical training associated with creatine supplementation promoted a reduction on duodenal muscular and mucosal tunics. It was also shown that physical training promoted a significant reduction in the body of myenteric neurons of this part of the intestine.



**FIGURE 4.** Effect of the creatine supplementation on duodenal (a) muscular tunic thickness ( $\mu\text{m}$ ) (b) cryptal depth ( $\mu\text{m}$ ) and (c) villus height ( $\mu\text{m}$ ). Abbreviations as in Figure 2. Values were expressed as mean  $\pm$  SE ( $n=4$ ); \* $p < 0.05$  when compared to SCr and T groups, using ANOVA and Tukey's post test.

Water consumption did not vary during the experimental period. On the other hand, there was a significant reduction in the final chow consumption in TCr group. Animals from this group have also presented the lowest weight gain in the end of the experiment. Creatine supplementation in trained animals was able to reduce the weight gain in 13%, the training itself had just reduced 6% and supplementation alone 3%, in comparison to sedentary group. Animals from all studied groups gained weight with time. However, animals from TCr group, the final mean weight was lower ( $p < 0.05$ ) when compared to T and SCr.

Studies have shown that chronic exercise has an important effect over alimentary behavior, stimulating the release of anorexic cytokines (Valsamakis *et al.*, 2004). This effect was not evident in our study since food intake was not different between S and T groups, but the association of aerobic activity with 2% creatine reduced food intake in 16%. However the decrease in food intake was not different among the studied groups when values were corrected on a 100g of body weight basis.

Previous studies, using the same experimental model, showed that endurance exercise training leads to a decrease in fat deposits, without any effect of creatine supplementation (Proença *et al.*, 2007). This is related to the fact that exercise per se is efficient to mobilize the fat reserves in submaximal intensities independently from creatine. However, in a preceding study, Wistar rats submitted to a treadmill running training during 10 weeks and supplemented with 2% creatine, presented higher lean mass and lower fat mass than creatine exercised or control groups. The authors inferred that supplementation in combination with exercise increased the proportion of lean mass more than exercise or creatine alone (Ferreira *et al.*, 2005).

The gastrointestinal tract represents the first barrier for creatine to reach its target tissues. However, there is a lack of information regarding creatine effects on intestinal function and morphology. Creatine absorption may be determined by its physicochemical properties, as well as blood flow (Orsenigo *et al.*, 2005; McCall and Persky, 2007). As creatine is structurally similar to basic aminoacids, it can enter to systemic circulation through amino acids transporters, peptides transporters or specialized transporters. The absence of creatine increase in feces, after supplementation, has indicated that it is totally absorbed by intestinal lumen (Conway and Clark, 1996).

In this study, we have shown that moderate physical training associated with creatine supplementation

promotes reduction in muscular tunic thickness, villus height and crypts depth in the duodenum. It is possible that the higher energetic demand provoked by exercise and the increased creatine availability in the intestinal lumen promoted a saturation of the creatine transporters of enterocytes, which could increase energetic availability, leading to a downregulation of proliferation of cryptal cells, thus reducing cryptal depth reduction and villus height.

The interaction between creatine and physical training was also reflected in the smooth muscle since a reduction in muscular tunic thickness was observed. Neither creatine nor exercise itself provoked changes in intestinal wall morphometry.

The myenteric plexus neurons were evaluated considering the density and cell body area. The neurons number did not change; however, the trained group presented a significant reduction (21%) in cell body area. The decrease of the neuronal profile could have been an effect of the decreasing in the nutrient availability toward neurons. The concentration of proteins in tissues is easily affected by nutritional alterations such as changes in quality and quantity of proteins in diet. These changes can directly reflect protein synthesis and ribosomal profile of the rough endoplasmic reticulum, especially in liver and muscles (Goldspink *et al.*, 1984; Lewis *et al.*, 1984). Experimental studies showed the suppression of protein synthesis in both liver and intestine after exercise in rats (Hayase and Yokogoshi, 1992).

It has been estimated that the energy cost for the maintenance of intestinal epithelium and accessory structures represents approximately 20% of the total energy consumed by the animal (McBride and Kelly, 1990). During physical activity the blood flow in gastrointestinal tract can decrease up to 80% (Rowell *et al.*, 1964) depending on the intensity of exercise. The redirection of blood flow to muscles and lungs has been associated to cramps and diarrhea (Peters *et al.*, 2000).

The animals submitted to treadmill running and supplemented with creatine, showed neuronal areas similar to those found in sedentary ones. This result suggests a neurotrophic role for creatine, which is a substrate for creatine kinase (CK), thus increasing phosphocreatine levels, and phosphocreatine in conjunction with the creatine kinase isoenzyme system acts as a potent intracellular energy buffer (Hausmann *et al.*, 2002). Oral creatine supplementation has been shown to elevate phosphocreatine content in the brain, leading to neuroprotective effects (Hausmann *et al.*, 2002) through mechanisms involving maintenance of

mitochondrial membrane potential, decreased reactive oxygen species, decreased mitochondrial calcium levels, and preservation of ATP levels (Sullivan *et al.*, 2000). The enteric nervous system is similar to the central nervous system in many aspects (Sternini, 1998) and thus may need a large amount of ATP to maintain its metabolic activities.

This work demonstrated that creatine consumption associated to moderate exercise provoked alterations in the architecture of the intestinal wall and myenteric neurons area of Wistar rats. However, such changes were not associated to any obvious alterations of intestinal function.

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