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Creatine Supplementation in Wistar Rats Submitted to Physical Tests of Swimming: Evaluation of the Physiological Effects through Raman Spectroscopy

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Research Article

ABSTRACT

Creatine is a nitrogenous organic acid that naturally occurs in vertebrates. This compound is associated to energy supply to muscles, being that its biological synthesis occurs mainly in liver and is related to the aminoacids glycine, arginine and ornitine. Several studies have shown the benefits of creatine supplementation, such as higher muscle power, increased fat-free mass, and strength in healthy subjects. Besides, it can be applied as therapeutic agent for some diseases. In this work, the physical condition of Wistar rats that received creatine supplementation is compared with individuals without any supplementation. For this evaluation, swimming tests were developed in order to elucidate the physical condition of animals in different nutritional conditions. Indeed, swimming is considered one of the most complete exercises, since the practice of this physical activity involves great increase in the aerobic and anaerobic capability.

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Raman spectroscopy was employed to analyse the biochemical profile of the biological tissues obtained from the animals evaluated in the experiments. The present results allow to infer that the association of these two factors, i.e., the addition of creatine supplementation together with physical activity, propitiates a completely different physiological effect when compared with the isolated action of each one of these factors (creatine supplementation and physical activity). In fact, these factors seem to act in synergy, favoring a more accentuated physiological response. In fact, the data obtained in this work denotes that the physical activity, as well as the creatine supplementation, when used independently, does not provoke a very significant physiological effect. Raman spectroscopy was able to analyze important biological indicators, such as protein concentration in blood and intensity of metabolic activity, being an interesting option to similar biophysical evaluations.

Keywords: Creatine Supplementation; Swimming; Raman Spectroscopy; Wistar rats; Physical tests; Physiological effects;

1. INTRODUCTION

Creatine (Cr) is an amine denominated methylguanidine acetic acid (Figure 1) identified in 1832 by Michel Eugene Chevreul, who discovered that this organic compound is a relevant component of the skeletal muscle. The relevance of Cr to the physiology of mammals was demonstrated initially by Hunter in 1928 (Branch et al., 2000). Its biological synthesis is associated to the aminoacids glycine, arginine and ornitine, occurring mainly in liver (Walker, 1979).

The name "creatine" is associated to the Greek word that means flesh, i.e., *Kreas*. Cr supplementation became a popular ergogenic aid to increase exercise performance. Studies have shown several benefits of creatine supplementation, such as increased muscle power, increased fat-free mass, and increase strength in healthy subjects (Vieira et al., 2008). The benefits of Cr supplementation on exercise performance have been extended as a possible therapeutic agent in the treatment of disease conditions. In fact, several data from literature have demonstrated positive effects regarding medical results (Vieira et al., 2008).

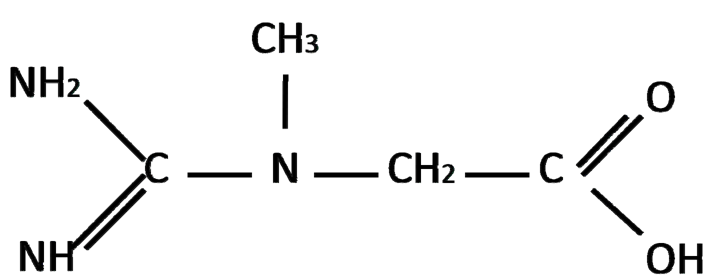

Fig. 1. Schematic representation of the chemical structure of creatine

Raman Raman spectroscopy is a very versatile instrumental technique for the analysis of biological materials (Moreira et al., 2008). This spectroscopic tool of vibrational characterization is well known as a resource of great number of structural details, allowing qualitative, quantitative and physicochemical characterization (Cleveland et al., 2007; Daniel et al., 2008; Drapper et al., 2005; Freeman et al., 2001; Funanage et al., 1992; Gadeleta et al., 2000; Geber et al., 2005; Greenhalf et al., 2001; Hanlon et al., 2000). Furthermore, Raman spectroscopy allows less invasive and nondestructive biological analysis, which can be optimized for biochemical characterization and diagnosis of several diseases (Moreira et al., 2008).

In fact, the assessment of pathological changes in tissue is currently performed by histopathological analysis. However, the management of biopsy material and the determination of the kind of correlation with the physiological profile are not trivial. The clinical characterization based on these analyses could lead to diagnostic delay and to the possibility of taking an unrepresentative sample. Furthermore, this clinical procedure presents high cost and provokes significant trauma to the patient. Actually, biological samples present significant issues to be addressed, which are associated to their heterogeneous characteristics. Besides, most components are present in low physiological concentration in body fluids (in order of mmol L^{-1} to nmol L^{-1}), which denotes the necessity of more efficient analytical methodologies to contribute for the biochemical characterization and diagnosis of several clinical cases. In this way, several spectroscopy techniques have been considered as basis for minimally invasive and non-destructive measuring systems, becoming the employment of these instrumental analytical tools a very interesting and auspicious medical alternative.

In the present work, the physical condition of Wistar rats that received creatine supplementation is compared with individuals without any supplementation. For this evaluation, swimming tests were developed in order to elucidate the physical condition of animals in different nutritional conditions. Indeed, swimming is considered one of the most complete exercises, since the practice of this physical activity involves great increase aerobic and anaerobic capability. In addition, the presence of metallic plates upon the individuals during the exercise accentuates the intensity of the physical activity, becoming this trial a very significant methodology to determine the creatine effect on the organic capability of mammals. In this way, this kind of test can be an interesting reference to analyze the physiological effect of creatine supplementation, since this trial requires high intensity of activity of great number of muscles as well as the circulatory system.

2. MATERIALS AND METHODS

2.1 ENVIRONMENTAL CONDITIONS

The experiments were performed in the Laboratory of Physiology of the Experimental Exercise of the Institute of Research and Development II (IP&DII) of Universidade do Vale do Paraíba (Univap), in São José dos Campos, São Paulo, Brazil.

2.2 SUBJECTS

The experimental sample was composed by 36 male Wistar rats (*Rattus norvegicus,* of the albino variation). These animals belonged to the Central Biotherium of IP&DII of Univap. The animals were submitted to a period of one week of adaptation in the local Biotherium of the Laboratory of Physiology of the Experimental Exercise. In this week, in the first three days, the rats were treated with anthelmintic (15 mL of ricobendazole (*Orurofino*) solubilized in 5 L of water). The rats were maintained in a standard condition of temperature (22-25°C), with relative humidity (40-60%) and cycle of 12 hours with light/without light, being feed with ration *Labcil* and water. After the adaptation period, the animals were randomized and divided in groups of 5 individuals by box of contention.

2.3 ADAPTATION TO SWIMMING

After the adaptation to the local biotherium, all rats were submitted to a period of adaptation to the swimming in order to reduce the stress of this physical activity, without promoting adaptations in the training (Voltarelli et al., 2002). This process of adaptation was performed in a tank of asbestos with capability to 250 L of water, maintained in a temperature of 34±1°C controlled by a thermostat. The adaptation procedure was constituted by crawl swimming without charge, 10-30 minutes by day, during 5 days. This training occurred between 8 and 12 hours a.m. during the first week. In the second week, the animals swan during 30 minutes without charge in the 4 first days and, in the fifth day, the charge test was developed. The swimming in group was utilized because this procedure becomes the exercise a more vigorous physical activity, when compared with the individual swimming, as function of some factors, such as mutual stimulation. Subsequently to the adaptation procedure, the animals already had a time of life of 60-65 days and an adequate corporal mass (237.77±4.6 grams), when compared to their physical conditions in the initiation of the training protocol.

2.4 EXPERIMENTAL GROUPS

After the period of adaptation to the swimming, the rats were distributed in 4 experimental groups:

1) Control Group (CON), constituted by 9 sedentary rats;
2) Group trained with 70% of the test of maximum charge (NAT), which is constituted by 9 trained rats;
3) Group without training supplemented with creatine (CRE), which is constituted by 9 sedentary rats;
4) Group trained with 70% of the test of maximum charge and supplemented with creatine (CRE_NAT), which is constituted by 9 trained rats.

2.5 DETERMINATION OF THE MAXIMUM CHARGE

After the delimitation of the experimental groups, all animals were submitted to the test of maximum charge, in agreement with Osorio and co-workers (Osorio et al., 2003 a, b). This test is developed in a cylindrical pool (50 cm x 45 cm), which is constituted by transparent acrylic, with temperature of 35±1°C. The test of maximum charge was performed individually, i. e., each animal was analyzed isolatedly. Aluminum plates were employed as charge, being that they were positioned in a clip fixed to an elastic line, which was disposed of a comfortable form together to the thorax of each rat (Voltarelli et al., 2002)

Initially, the corporal mass of the individuals was determined in order to calculate the charge of work to each animal, which was increased in intervals of 3 minutes through the addition of the metallic plates in the clip fixed to the elastic line. The mass of the metallic plate corresponded to values relative to the total corporal mass of each individual (1%; 2%; 3% etc.), until the condition of exhaustion, which corresponds to the maximum charge. The exhaustion is determined by the impossibility of the animal to keep itself under the water surface for a time interval of 8-10 seconds (Osorio et al., 2003 a, b).

The determination of maximum charge, which is supported by each animal during the test, allows the adjustment of the relative charge of work to the physical training. This value of maximum charge can be 50%, 60%, 70%, 80% or 90% of this maximum charge. The test of maximum charge was repeated periodically (time intervals of 15 days) to propitiate the readjustment of the charges.

2.6 PHYSICAL TRAINING

The training of the groups was performed during 5 days/week for 8 weeks, in the morning period (between 8:00 and 12:00 hours), in agreement with the process of adaptation, constituting 10 weeks of swimming. Each session was developed during 30 minutes. The charge supported by the animals during the training consisted in the total mass of plates of plumb used to “fishing” involved by an adhesive tape, which is fixed in an elastic line disposed in a comfortable manner on the thorax of the rat (Voltarelli et al., 2002).

The experimental groups NAT and CRE_NAT were submitted to a process of adaptation to the charge. This process consisted of 10 minutes in the first day of training, being that this interval of time was increased 5 minutes by day until reaching 30 minutes in the fifth day, during the first week. In the second week, the animals of these two groups swan for 30 minutes in the 4 first days, being that, in the fifth day, the test of charge was performed. After the end of each session of swimming, the elastic line with the metallic plates was removed and, subsequently, the rats were disposed in a box of contention by 30 minutes. It is important to register that the base of this box has a grid that allows the rats to be dried quickly.

During the experiment, all the animals remained in the same laboratory and were submitted to the same environmental conditions. It is important to register that all the animals, independently of the group, were submitted to the same previous procedures, which are the period of adaptation and the test of maximum charge.

2.7 CONTROL OF THE CORPORAL MASS

A digital scale was used to obtain the corporal mass of the animals during the experiment (Bel Engineering) with precision of three decimal digits. The measurements of mass were realized between intervals of 15 days, before the training (between 7:00 and 8:00 hours).

2.8 OBSERVATION OF THE ANIMALS

Observations and registers of the behaviour of the animals were performed all days, mainly regarding the conditions presented by the skin and the fur.

2.9 EUTHANASIA OF THE ANIMALS

The euthanasia was performed in a morning, after two days of the last session of training in order to attenuate the acute effects of the exercise. The methodology employed was the decapitation (Osorio et al., 2003 a, b). Subsequently, the total blood (4-5 mL) was collected in two tubes: a test tube (*Vacutainer* – 10 mL) containing anticoagulant (heparine) and a second test tube (10mL) without anticoagulant (dry). Both tubes were maintained under refrigeration with ice. After this procedure, the tubes were centrifuged (3000 rpm) during 10 minutes, at room temperature, obtaining plasma and serum, respectively. Plasma and serum were frozen at -40ºC (Radak, 2000), in the freezer located in the Laboratory of Preparation of Samples of IP&DII of Univap. The heart was also isolated and maintained at -40ºC to posterior analysis focused on the possible occurrence of cardiac hypertrophy.

2.10 STATISTICS ANALYSIS

A statistic approach suitable to the experimental characterization proposed in the present work was employed, which permit to observe the possible isolated or associated effects of the exercise in the different intensities (50%, 60%, 70%, 80% and 90% of the maximum charge). The data of the samples were submitted to analysis of variance (ANOVA). Subsequently, the Test of *Tukey* was performed in the cases that this methodology was considered necessary. The statistic analysis were conduced utilizing the program SPSS (version 17.0). The results were registered with the media ± standard deviation (media ± D.P.), being that the values of $p \leq 0.05$ were considered as statistically significant.

3. RESULTS AND DISCUSSION

Figure 2 demonstrates that the Carbonate/Phosphate ratio is very lower in the CRE_NAT group when compared to the control group.

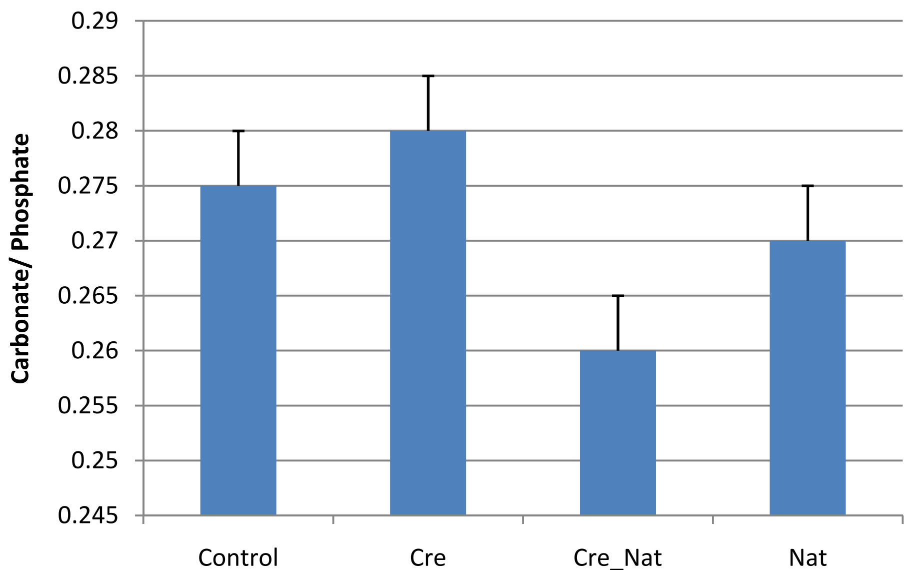

Fig. 2. Carbonate/Phosphate ratio obtained from plasma analysis by Raman Spectra, which involve control and supplied Wistar rat groups

(Control Group - constituted by 9 sedentary rats; Cre Group without training supplemented with creatine, which is constituted by 9 sedentary rats; Cre_Nat Group trained with 70% of the test of maximum charge and supplemented with creatine, which is constituted by 9 trained rats; Nat Group trained with 70% of the test of maximum charge, which is constituted by 9 trained rats;)

This data, in comparison with the values observed in CRE and NAT groups, demonstrated a very interesting fact, which is the synergic interaction between the creatine supplementation and the physical activities. Indeed, NAT group presents a lower carbonate/phosphate ratio than the control group, while CRE group has higher carbonate/phosphate ratio than the control group. However, the CRE-NAT group present a much lower carbonate/phosphate ratio, allowing to infer that the association of these two factors (addition of creatine supplementation and physical activity) propitiates a quite different physiological effect when compared with the isolated action of each one of these factors (creatine supplementation and physical activity).

Figure 3 demonstrates, in agreement with data presented in Figure 2, that the association between creatine supplementation and physical activity causes a quite distinct effect upon the physical condition of the mammals. Interestingly, the phosphate/Amide I ratio of the CRE_NAT group presents an intermediary value between CRE group, which is the lowest value, while the NAT group is the highest phosphate/Amide I ratio.

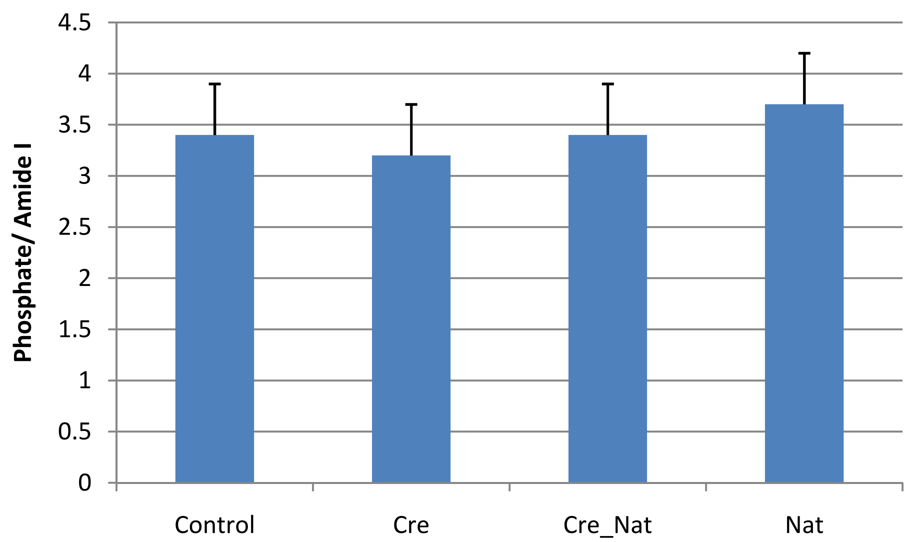

Fig. 3. Phosphate/Amide I ratio obtained from plasma analysis by Raman spectra, which involve control and supplied Wistar rat groups

(Control Group - constituted by 9 sedentary rats; Cre Group without training supplemented with creatine, which is constituted by 9 sedentary rats; Cre_Nat Group trained with 70% of the test of maximum charge and supplemented with creatine, which is constituted by 9 trained rats; Nat Group trained with 70% of the test of maximum charge, which is constituted by 9 trained rats;)

Figure 4 presents values of Carbonate/Amide I ratio, which corroborates an important data registered in Figure 3. In fact, Figure 4 demonstrates that the NAT group presents a much higher carbonate/Amide I ratio when compared with the other three groups, denoting that the isolated physical activity cannot increase significantly the presence of proteins in the mammals. The same interpretation can be inferred through the data demonstrated in the Figure 3, which presents the highest intensity of phosphate/Amide I ratio to the NAT group.

In this way, the higher physical activity increases the Phosphate/Amide I and Carbonate/Amide I ratios probably as function of the increase of physiological metabolism, which is, at least, partially responsible by the increase in the concentration of phosphate and carbonate in the biological medium. In this context, the comparative analysis between the NAT and CRE-NAT groups allows to infer that the lower values observed to the CRE-NAT group regarding the Phosphate/Amide I (Figure 3) and Carbonate/Amide I (Figure 4) ratios denote the elevated presence of protein in the plasma and serum of the rats supplemented with creatine.

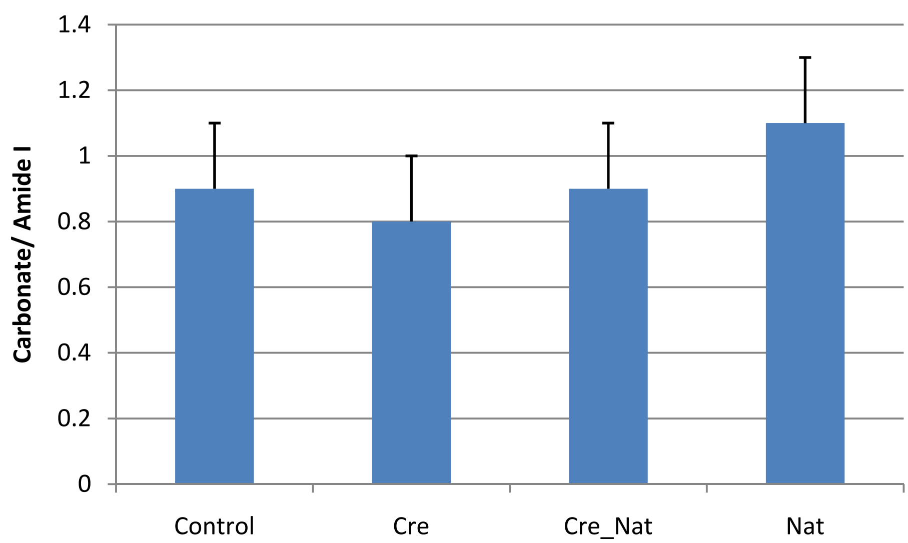

Fig. 4. Carbonate/Amide I ratio obtained from plasma analysis by Raman spectra, which involve control and supplied Wistar rat groups

(Control Group - constituted by 9 sedentary rats; Cre Group without training supplemented with creatine, which is constituted by 9 sedentary rats; Cre_Nat Group trained with 70% of the test of maximum charge and supplemented with creatine, which is constituted by 9 trained rats; Nat Group trained with 70% of the test of maximum charge, which is constituted by 9 trained rats;)

The high concentration of phosphate and carbonate in the plasma and serum suggests a higher metabolic activity, which should be associated to a higher development of the aerobic and anaerobic capabilities. Probably, the supplementation with creatine must be a very important factor of development of the physical activity, mainly regarding the anaerobic capability. Really, this anaerobic up grade would be significantly related to the musculoskeletal system, including the hypertrophy of muscles and bones. Furthermore, the improvement in the anaerobic performance probably furnishes indirectly a significant support to the aerobic development, since that the animals can overcome small time intervals of submersion with less difficulty when compared with individuals with lower anaerobic condition.

4. CONCLUSIONS

The present results allow inferring that the association of these two factors, i.e., the addition of creatine supplementation together with physical activity, propitiates a completely different physiological effect when compared with the isolated action of each one of these factors (creatine supplementation and physical activity). In fact, these factors seem to act in sinergy, favoring more accentuated physiological responses. In fact, the physical activity, as well as the creatine supplementation, when used independently, did not provoke a significant physiological effect, such as the presence of proteins in the blood and the metabolic activity. The present work denotes the efficiency of the respective training methodology, which associates creatine supplementation and physical activities, including aerobic and anaerobic exercises. The relation between relevant biological fingerprints, such as phosphate, carbonate and amide, which is an indicative of the presence of proteins, consists in an accessible methodology to several laboratories to evaluate the efficacy of the administration of food supplements and medicines. Furthermore, this work demonstrates the efficacy of Raman spectroscopy as an able tool to evaluate the alteration in the concentration of relevant biochemical indicators, being an interesting analytical alternative to this kind of biophysical and biomedical applications.

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