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Effect of different doses of creatine on the bone in thirty days of supplementation in mice: FT-Raman study

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Abstract. In this study, Raman spectroscopy was employed in order to provide information about the effects of different doses of creatine on bone tissue composition of phosphate apatite (960 cm^{-1}), carbonate apatite (1170 cm^{-1}) representing the mineral content and collagen matrix (amide I, 1665 cm^{-1}). The animals (27 Balb-C male) were divided into three groups ($n = 9$ per group): control (CON), supplemented with 0.5 g/kg (Cre-0.5) and with 2.0 g/kg (Cre-2.0) creatine. The experiment was carried out for thirty days. After this time, the right femur of each animal was harvested. The specimens were assessment by FT-Raman spectroscopy and in a total of 81 spectra were acquired in the medial diaphysis of the femur. The Raman data strongly suggest that only the creatine supplementation of 0.5 g/kg effective to the bone constitution. Furthermore, the present results demonstrate that creatine ingestion provokes decrease in the relative presence of carbonate in the chemical constitution of bones. The decrease in the carbonate content can be associated to a significantly bone resistance altered to several mammalians. The analysis evidenced that the mineral concentrations in the Raman spectroscopy could be a feasible method for non-invasive or minimally invasive assessment of bone tissue composition. Probably this high sensitivity can be employed to determine spectral profiles, such as wavelength of maximum absorption and maximum intensity of absorption of each wavelength, of several bone diseases.

Keywords: FT-Raman spectroscopy, femur, bone quality, creatine, nutrition supplement

1. Introduction

Several athletes and physical activity practitioners, have used creatine (Cr) (Fig. 1) supplementation because of a number of benefits that it can bring. Creatine (Cr) was identified in 1832 by Michel Eugene

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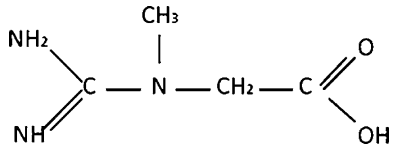

Fig. 1. Schematic representation of the chemical structure of creatine.

Chevreul, who discovered that this organic compound is a relevant component of the skeletal muscle. The name "creatine" is be associated to the Greek word that means flesh, i.e., *Kreas*. Creatine (Cr) supplementation became a popular ergogenic aid to increase exercise performance. Studies have shown several benefits of creatine supplementation, such as increased muscle power [15,34], increased fat-free mass [2,23,31] and increase strength in healthy subjects [29]. The benefits of Cr supplementation on exercise performance have been extended as a possible therapeutic agent in the treatment of disease conditions [16].

Creatine (Cr) (Fig. 1) is a non-essential dietary element occurring naturally in the human body and is partly synthesized by the kidney, pancreas and liver (approximately 1–2 g per day), and partly ingested with food (approximately 1–5 g per day), especially with meat and fish [38]. In humans, about half of the daily creatine is biosynthesized from three different aminoacids (arginine, glycine and methionine), being that the rest is taken in by alimentary sources. Cr is distributed throughout the body with 95% of Cr found in skeletal muscle [12]. Consequently, the Cr research has focused primarily on its effects on skeletal muscle, which plays an important role in rapid energy provision during muscle contraction regenerating ATP through a reversible reaction catalyzed by creatine kinase (CK) [37].

For the development and repair process of bone, osteoblasts require high energetic demand to survive, proliferate, differentiate and synthesize the extracellular matrix. Evidence suggests that the Cr–CK system also plays an energetic role in this tissue [11]. Further corroborating evidence for the importance of CK in skeleton formation is that high CK activity is noted in chondrocytes [14,17]. Moreover, it has been noted that CK activity is also required for the development of endochondral bone and is a key enzyme in the cellular energy metabolism of osteoblasts [9]. Ch'ng and Ibrahim [4] demonstrated those periods of increased energy demands in osteoblasts leaded to upregulated CK expression and allowed changes in cell shape and adherence of osteoblasts cells to bone surfaces. Likewise, it is tempting to speculate whether Cr supplementation could be used as promoter of improved bone quality. Recently, Antolic et al. [1] verified the influence of supplementation with Cr monohydrate on bone structure and function in growing rats during 8 weeks, to establish a therapeutic model. Bone mineral density (BMD) and content (BMC) femoral were assessed using dual-energy X-ray absorptiometry (DEXA) at the beginning and end of the protocol and it was demonstrated that the CR-treated rats presented greater lumbar BMD and femoral bending load at failure compared with the control group rats ($p < 0.05$). However, more studies are need to determine the influence of Cr supplementation in the biochemical composition of bones.

Raman spectroscopy is an optical tool which could permit precise information on the chemical composition of organic and inorganic samples, allowing less invasive and non-destructive qualitative and quantitative analysis [5,13]. This great potential has motivated several biological and biomedical applications of Raman spectroscopy in order to obtain biochemical characterization and diagnosis of several diseases [24]. In fact, the clinical process to assess pathological changes in tissue is currently related to the histopathology. However, the management of biopsy material and the interpretation of the respective analysis 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 patient trauma [33]. 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 to nmol/l) [30]. In spite of these difficulties, several spectroscopy techniques have been considered as basis for minimally invasive and non-destructive measuring systems [41].

In this context, Raman spectroscopy has been considered effective to assess sample information at the molecular level, and has been used on several minimally and non-invasive diagnostic applications of biological samples such as detecting toxicity of different doses of a pollutant on bacterium media [6] atherosclerosis in coronary and carotid arteries [25,32], human basocellular skin cancer [26], lactate identification in blood [27], osteoinduction in biomaterial implants [40], several bone diseases [3], bone synthesis and osteointegration after healing [20,21], and evaluating the microstructure of human cortical bone (osteon) [35]. Thus, Raman spectroscopy has been accepted by many authors as a viable tool for the study of bone mineralization [7,39] and emerges as an important complement to traditional methods for detection, quantification and imaging of local variations in the molecular structures of bone matrix and mineral [3].

Several works have proposed the use of Raman spectroscopy to obtain the mineralization and organic matrix to assess the bone quality [3,7,28,39]. At least two different aspects of the mineral component of bone affect its mechanical properties: the degree of remodeling and bone mineral density (mineral to matrix phases ratio) and the degree of crystallinity of the mineral content (related to mineral quality and carbonate/phosphate ratio) [3,8]. The chemical composition of bone tissue could be assessment by the ratios between spectral Raman bands of interest, i.e., femoral trabecular bone had a higher mineral/matrix ratio in fractured rather than in unfractured women, while iliac crest biopsies revealed a higher carbonate/phosphate ratio in cortical bone from woman who had sustained a fracture [22]. Also, bones from postmenopausal osteoporotic patients found a lower mineral/matrix ratio and higher carbonate/phosphate ratio when compared with normal patients [10]. The crystallinity, an important bone quality factor, could be associated with carbonate/phosphate ratio and indicates the extent of carbonate incorporation in the hydroxiapatite lattice [39].

Although there are many articles focuses in bone study by spectroscopy, from our best acknowledgment the study of the effect of supplement in the bone are rare. Therefore, the aim of this study was to use near-infrared FT-Raman spectroscopy to evaluate the bone tissue composition in creatine supplemented mice.

2. Material and methods

2.1. Experimental model

Twenty-seven male Balb-C, young adult mice (12 week old), were obtained from the bioterium of Campinas University (CEMIB-UNICAMP – Campinas, SP, Brazil). The mice were kept in plastic cages (10 animals/cage) in the bioterium of the Physiology and Pharmacodynamics Laboratory of the Research and Development Institute of the University of the Vale do Paraíba (São José dos Campos, SP, Brazil) with controlled temperature (22–24°C), relative humidity (40–60%) and photoperiod (12 h light–dark cycle). The rats were acclimatized to the facility, cage and standard rodent diet for two weeks before to the initiation of the experimental interventions. Moreover, all the animals had access to palletized food (Purina® lab chow) and water *ad libitum*. After four weeks the animals were randomized into one of three experimental groups ($n = 9$ per group): control group (CON), supplemented with creatine 0.5 g/kg

group (Cre-0.5) and supplemented with creatine 2.0 g/kg group (Cre-2.0). All the procedures adopted in this study were according to the laboratory animals handling and care principles recommended by the COBEA (Brazilian School of Animal Experimentation) and approved by the Ethics in Research Committee of UNIVAP (Protocol # A088/CEP/2007).

2.2. Bone samples

At aged of 18 weeks all animals were anesthetized by intramuscular administration of Xylazine (40 mg/kg) and Ketamine (50 mg/kg) and euthanized by intracardiac administration of KCl solution (10%). The right femur was harvested. All soft tissues were removed from the bones. The bones were identified snap frozen and stored in liquid nitrogen (77 K) in cryogenic vials (Nalgene®). Before the FT-Raman spectroscopy, samples were warmed up to room temperature with 0.9% physiological solution.

2.3. FT-Raman system

The FT-Raman spectrometer (RFS 100/S® – Bruker Inc., Karlsruhe, Germany) with a germanium detector cooled by liquid nitrogen was used to collect the data. The samples were excited by an air cooled Nd:YAG laser ($\lambda = 1064.1$ nm). The power of the Nd:YAG laser incident at the sample was 250 mW. The spectral resolution was set to 4 cm^{-1} and for each specimen three different region were analyzed with 300 scans totalizing 27 spectra per group. Before the spectra treatment, the average of the three spectra per specimen for each period were performed, resulting into 81 spectra. For the qualitative and semi-quantitative spectral analysis, the average spectra were baseline corrected and then normalized to the 960 cm^{-1} peak [18,19,36]. The changes of organic and inorganic enamel components were analyzed by comparing the integrated areas of the Raman peaks centered at 960 cm^{-1} (p1), 1071 cm^{-1} (p2), to the peak at 1665 cm^{-1} (p3). The integrated areas of the peaks were calculated by the Microcal Origin 7.5® (Microcal Software, Inc., Northampton, MA, USA).

2.4. Statistical analysis

The measurements obtained from the integrated area under the Raman peaks (p1–p5) were statistically analyzed using Instat software (GraphPad Software, Inc., San Diego, CA, USA). The one-way ANOVA test at a 95% confidence level and the Tukey–Kramer multiple comparisons test were applied to test the significance of the relative area evaluation between the normal and supplement bone data. Statistical analyses were initially performed using the difference between normal and treated values of the relative area. Comparisons between the groups were also performed considering only the treated values.

3. Results and discussion

Figure 2 presents the Raman spectral profile of creatine, being that Fig. 3 demonstrates the carbonate/phosphate ratio of the control group as well as of the groups treated with creatine supplements. It is possible to infer that the relative concentration of carbonate in the bones decreased significantly with a higher ingestion of creatine by the animals. However, it is interesting to note that the carbonate/phosphate ratio is higher to the animals treated with supplement that has higher quantity of creatine, allowing to infer that the absorption of creatine is not directly proportional to the creatine quantity administrated to the individuals. Possibly, the influence of creatine ingestion in the carbonate/phosphate ratio is limited,

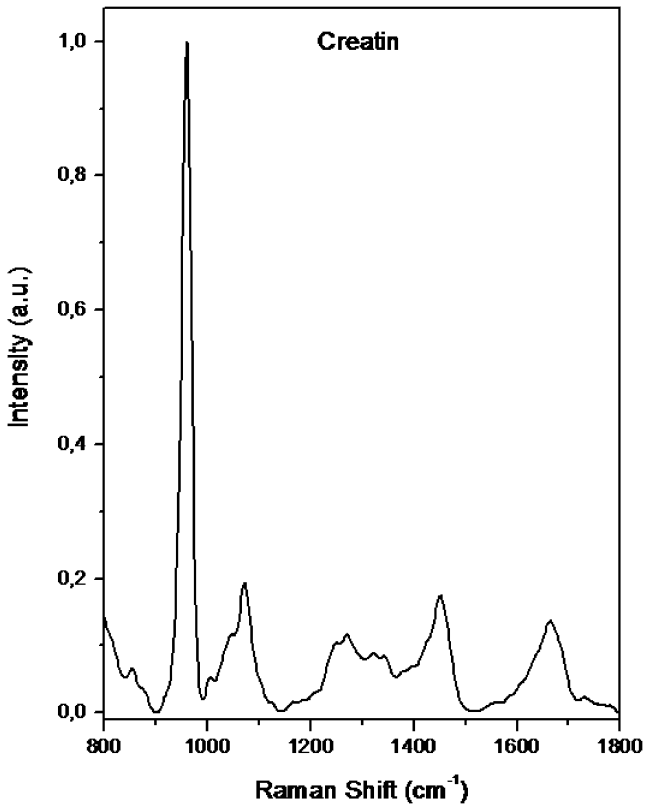

Fig. 2. Raman spectral profile of creatine.

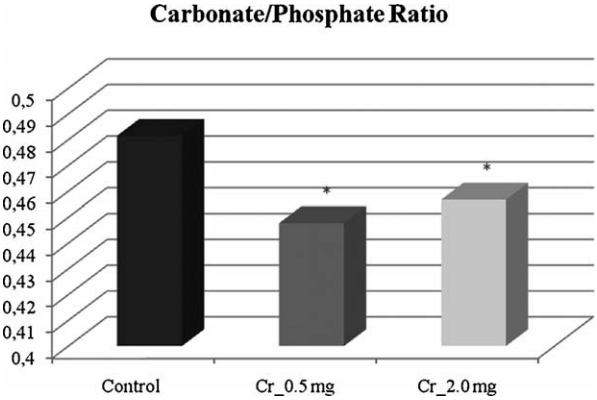

Fig. 3. *Significant difference ($p < 0.05$) for control group; control – control group; Cr_0.5 mg – supplemented with creatine 0.5 g/kg group; Cr_2.0 mg – supplemented with creatine 2.0 g/kg group.

being that 0.5 g/kg would represent an optimum administration in order to promote a more effective repercussion in the bones.

Figure 4 demonstrates that the relative concentration of phosphate has a slight increase with nutrition supplemented with creatine 0.5 g/kg, while the relative presence of phosphate in animals that received creatine 2.0 g/kg is practically the same of the control group (a very small decrease). This data reinforce

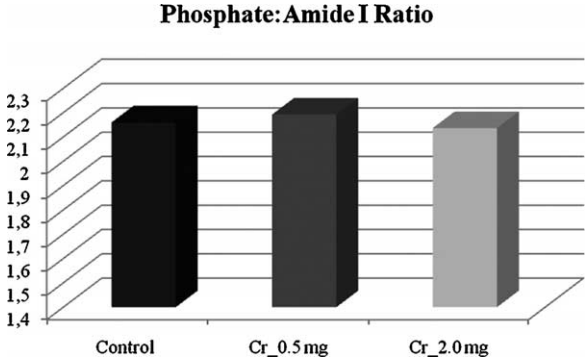

Fig. 4. Control – control group; Cr_0.5 mg – supplemented with creatine 0.5 g/kg group; Cr_2.0 mg – supplemented with creatine 2.0 g/kg group.

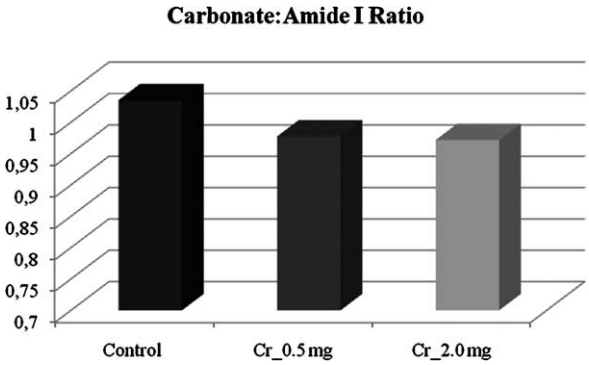

Fig. 5. Control – control group; Cr_0.5 mg – supplemented with creatine 0.5 g/kg group; Cr_2.0 mg – supplemented with creatine 2.0 g/kg group.

the analysis related to the Fig. 3. In fact, this apparently contradictory result could be associated to two factors. Firstly, the excessive creatine supplement administration would generate an opposite effect as function of a biochemical reaction of the organism, i.e., the physiological response to the supplement excess would increase significantly the metabolism of creatine, implying in a practically insignificant effect on the bone constitution. In this way, the excessive creatine supplementation does not seem to affect representatively the phosphate concentration in bones. A second factor could also be associated to the results obtained in the present work. In fact, considering the data presented in Fig. 3, the experimental data, indirectly, suggest that the decrease in the carbonate/phosphate ratio could also be related by a significant decrease in the carbonate concentration in the bones.

Figure 5 presents the relative concentration of carbonate to the amide groups showing that the control group presents a higher relative concentration of carbonate in relation to the amide groups, when compared with the two groups with alimentation enriched with creatine supplementation. This results corroborates the proposal inferred from the analysis of Fig. 4 that the decrease in carbonate/phosphate ratio is also provoked by the carbonate decrease and not only by the increase of phosphate concentration.

The analysis of the present data allows to infer that Raman spectroscopy is a suitable instrumental tool in order to be applied in the analysis of chemical constitution evaluation of bones. Actually, the well established assignment of Raman peaks permits a very sensitive study, involving qualitative and quantitative determinations. This potential can be identified firstly through the significant isolation between

the Raman signals assigned to each group evaluated in the present work. In fact, the attribution of the vibrational peaks of interest in the bone evaluation involves wavelengths quite different, i.e., mutually separated peaks. In this way, it is possible a complete investigation around all Raman peaks related to the chemical composition of the bones.

4. Conclusions

The present data strongly suggest that creatine supplementation needs to be controlated in order to promote effective results on the bone constitution. Indeed, an excessive administration would not generate a higher presence of phosphate in the bones, in opposite of an limited administration of creatine. Moreover, the ingestion of creatine supplement tends to decrease the relative presence of carbonate in the chemical constitution of bones, which can be associated to a significantly altered bone resistance to several mammalians. Furthermore, this fact can be associated to a more intense metabolism, which can alter the carbonate concentration as function of several biochemical processes.

The present article is a very original contribution focused on the biomedical applications of Raman spectroscopy, especially involving the influence of alimentation on the constitution of bones. Probably, this high sensitivity can be employed to determine spectral profiles, such as wavelength of maximum absorption and maximum intensity of absorption of each wavelength, of several bone diseases.

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