ORIGINAL PAPERS

Histological Effects of Creatine Monohydrate Supplementation on Muscle Tissue in Wistar Rats

Isabela Ferreira Silveira ARRUDA, Amilton IATECOLA, Vanessa Zanoni CARVALHAES, Marcelo Rodrigues da CUNHA, Victor Augusto Ramos FERNANDES

Abstract

Creatine is a dietary supplement with the potential to stimulate the phosphocreatine pathway and protein synthesis, through the stimulation of PI3eK/AKT and mTOR responsible for the proliferation and differentiation of satellite cells, responsible for hypertrophy. The present study aimed to evaluate the morphological effects of the use of creatine monohydrate on the soleus muscle tissue of 26-month-old Wistar rats. **Methods:** Twelve Wistar rats were divided into two groups of six animals each. Group 1 was not supplemented with creatine and received a standard diet consisting of water and food. Group 2 received the same diet, but was supplemented with creatine monohydrate at a dose of 0.03 mg/kg of body weight diluted in 200 ml of drinking water for 8 weeks. **Results:** The supplementation promoted morphological and morphometric effects on the soleus muscle tissue, promoting changes in the perimeter and area of the muscles of the animals treated with the supplement. It is estimated that this supplement may promote, in addition to increasing the cross-sectional area of myocytes, increased stimulation of the protein synthesis pathway associated with PI3K/AKT.

Keywords: creatine supplementation, muscle tissue, soleum, hypertrophy, supplementation.

University Nossa Senhora do Patrocínio, Brasil

Corresponding author:
Victor Augusto Ramos FERNANDES, University Nossa Senhora do Patrocínio, Brasil
E-mail: victorramosfernandes@gmail.com

INTRODUCTION

Creatine is a naturally occurring amine synthesized endogenously by the liver, kidneys, and pancreas from the amino acids glycine, arginine, and methionine. In addition to the endogenous formation, it can also be obtained exogenously, via food, especially through the consumption of red meat and fish. The endogenous production (1g/day) added to that obtained in the diet (1g/day for an omnivorous diet) equals the rate of spontaneous degradation of creatine and creatine phosphocreatine in the form of creatinine, by a non-enzymatic reaction at the level of proximal convoluted tubules of the kidneys.[1]

About 95% of this organic pool is found in the skeletal striated musculature, and its main physiological function is to provide fast energy for short and intense efforts through the formation of phosphocreatine and ATP resynthesis. The remaining 5% is distributed in the cardiac striated muscles (heart), in blood cells (erythrocytes and leukocytes), in the testes, brain, and retina, acting in hydrogen buffering and the resynthesis of adenosine triphosphate.[2]

In the skeletal muscles, approximately (65%) is found in the form of Creatine Phosphate (CP), and the remainder (35%) is in the form of free creatine. Together, these two reserves constitute the source of energy generation of the ATP-CP System (creatine-phosphate) promoting a large amount of energy.[3]

Associated with the mentioned physiological effects, it was identified that strength training combined with creatine supplementation is capable of increasing the expression of regulatory myogenic factors (from the MRFs family, from the English myogenic regulatory factors), such as MRF4 and myogenin, partially responsible for the proliferation and differentiation of satellite cells.[4]

Added to this, creatine supplementation alters the transcription of regulatory myogenic factors, increasing protein translation through the hypertrophic pathway PI3K-AKT/PKM-mTOR[5], thus causing the proliferation and differentiation of satellite cells necessary for the hypertrophic process.[1,5,6]

Studies have shown that the role of creatine is not restricted to ergogenic effects, such as increased strength and lean mass of the user, but findings that have therapeutic potential have also been identified, mainly active in the process of neuroplasticity and anti-inflammatory.[6]

The soleus muscle is a deep muscle of the posterior region of the leg, located inferior to the gastrocnemius, sharing with it the insertion in the calcaneal bone through a common tendon (calcaneal tendon). The soleus is innervated by branches that derive from the tibial nerve, the largest branch of the sciatic nerve, with contributions from roots that derive from L4 and L5.[7]

The soleus muscle is fundamental for the gait mechanism, being this a muscle present in different phases of the movement. Strengthening this muscle contributes to improving gait biomechanics, as well as reducing the incidence of accidents that compromise the talocrural joint, such as potentially traumatic inversions.

In this sense, the present study investigated the effects of the use of creatine monohydrate on the morphometry and stereology of the soleus muscle tissue in healthy Wistar rats.

MATERIAL AND METHODS

Experimental Design

The present study was conducted at the facilities of the Jundiaí Medical School, Jundiaí, State of São Paulo, Brazil, and the animals were cared for by the animal house of the institution. This study used samples shared with other studies, optimizing the use of animals, animal welfare, and public expenditure (protocol number 490/12). This study was completed in January 2023.

Twelve Wistar rats were used. The animals were about 26 months old, when rats are considered old and already in a state of high organic fragility. The animals were divided into two groups:

- Group 1 (control, n = 10): 26-month-old Wistar rats not supplemented with creatine monohydrate;
- Group 2 (n = 10): 26-month-old Wistar rats supplemented with creatine monohydrate.

The body weight of the animals was standardized at the beginning of the experimental protocol. The animals received a solid diet and water *ad libitum* and were housed in collective cages (4 rats per cage) at a constant temperature of 23 ± 2°C under a 12-hour light/dark cycle, with lights on from 6:00a.m. to 6:00p.m. All animals were fed Labina® (standard ration for rats, Purina, Brazil). The body weight (g) of the animals was measured at the beginning and end of the experiment. The animals were sacrificed one day after the last creatine supplementation following the guidelines of the Animal Use Ethics Committee. For analysis of the variables of this study, hepatic, renal, and gastroc-

nemius muscle tissue samples were collected after anesthetic induction and sacrifice.

Euthanasia of Animals

Painless death of the animals was induced one day after the experimental protocol by intraperitoneal administration of a high dose of the anesthetic (0.3 mg/g) consisting of xylazine, ketamine, and thiopental, followed by intracardiac injection of potassium chloride (KCl). After confirmation of the animal's death, tissue samples were collected for general histological and morphometric analyses.

Creatine Supplementation

Only animals of group 2 were supplemented with creatine monohydrate at a dose of 0.3 mg/kg body weight for 8 weeks. The dose was based on other studies using supplementation of rodents[6] and corresponds to the dose regimen used in humans to obtain ergogenic effects. The supplement was administered orally, diluted in water, for 8 consecutive weeks. Control animals received only water.

Water intake was measured throughout the day and weeks in order to identify possible leaks and to evaluate the consumption of water and creatine solution. To standardize the supplementation protocol, creatine was diluted in 200 ml of filtered drinking water.

Light Microscopy and Stereology

After the supplementation period and euthanasia of the animals, the collected tissues were extracted and fixed in Bouin's solution (saturated aqueous solution of 75 ml picric acid, 25 ml formalin, and 5 ml glacial acetic acid) for 12 hours for subsequent processing and embedding in paraffin. The tissues were then washed in 70% alcohol and dehydrated in an increasing alcohol series (80% alcohol: 2 times, absolute alcohol: 3 times; 1 to 2 hours each). Next, the fragments were cleared in xylene for 1 to 2 hours until they became translucent. The fragments were embedded in paraffin and plastic polymers (Paraplast Plus, Polysciences, Niles, IL) at 56ºC for approximately 1 hour and then transferred to new paraffin at the same temperature. The specimens were carefully arranged at the bottom of the plastic cubes in order to obtain histological cross-sections. The blocks were trimmed to obtain flat surfaces and cut into 5-µm thick sections. The sections were mounted on albuminized slides, which were kept in an oven at 60ºC. After preparation, the slides were stained with hematoxylin/eosin for general morphological study and morphometric analysis using the open-access ImageJ software. The histological slides were analyzed and photographed under a Nikon Eclipse E100 (FAPESP grant 08/55521-7) equipped with the Sony DSC-W120 image acquisition system (Sony, Tokyo, Japan), prioritizing 20x, 40x and 100x objectives, at the Department of Morphology and Basic Pathology, Jundiaí Medical School.

All sections were used for quantification of cytoplasmic and nuclear volumes of liver, muscle and kidney cells. The diameters of the nuclei were measured in each sample. The choice of nuclei was random, prioritizing defined cell boundaries. Elliptical or spherical nuclei with flat sections throughout the specimens were chosen. The measurements were made with the aid of a graduated 10x eyepiece ruler coupled to a Nikon Eclipse E100 light microscope using a 100x objective. For the measurement of these structures, the eyepiece was previously calibrated with a special slide containing 0.01-mm divisions in order to transform the eyepiece units into micrometers. Based on these values, the mean nuclear volume was calculated using the following formula: $V=4/3\ \pi\ r^3$ for spherical nuclei, where r is the radius of the nucleus, and $V=4/3\ \pi\ (d/2)^2$ $D/2$ for elliptical nuclei, where d is the smallest nuclear diameter and D is the largest nuclear diameter. In addition, the fractions of volume (Vv) occupied by nucleus and cytoplasm were measured. These measurements were made with a 10x eyepiece grid reticle consisting of 100 points coupled to a Nikon Eclipse E100, also using a 100x objective. The number of points on the cell nucleus and cytoplasm was determined in four randomly defined fields. The volume fraction occupied by the nucleus in relation to the cytoplasm was calculated using the following formula: $Vv=p/P$, where Vv is the density or fraction of volume (%), p is the number of points on the nucleus, and P is the total number of points or sum of points on the nucleus and cytoplasm in the different fields. The cytoplasmic volume was calculated as the ratio between cytoplasmic Vv and nuclear Vv. The cell volume was obtained as the sum of nuclear and cytoplasmic volumes.

Statistical Analysis

Mean profiles were compared between the two groups using analysis of variance (ANOVA) as parametric test, complemented by the nonparametric Kruskal-Wallis test for pairs of each group. To compare the statistical

results after the reported treatments, one-way ANOVA with Bonferroni's post hoc test was also applied. A level of significance of at least 5% was adopted for all tests, thus assuming p <0.03 and <0.05.

Ethical Aspects

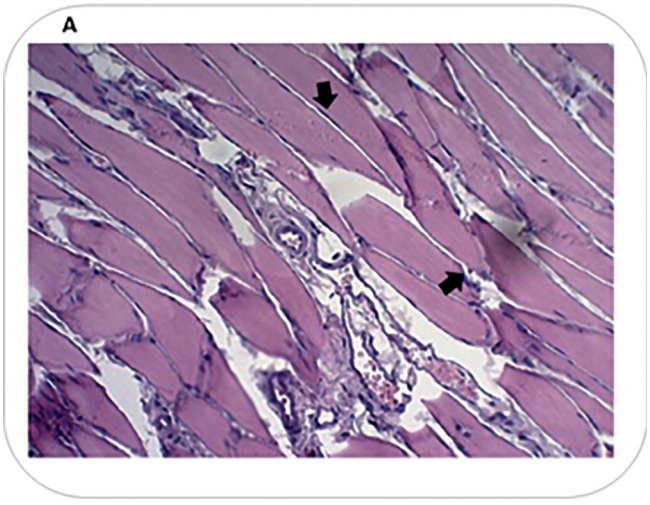
The study was approved by the Animal Use Ethics Committee of the Jundiaí Medical School (Approval number 490/2012). All animal procedures were conducted in accordance with international protocols for the care and use of experimental animals.

RESULTS

After the experimental period, as well as the extraction procedures and application of histological techniques to the soleus muscle tissues of the study animals, the morphometric analysis of the myocytes was performed, by measuring the area and perimeter, using the ImageJ software. The results are presented in tabular form and the following histological images. Subsequently, the images of the fabrics will be presented.

Chart 1: Comparison between the experimental protocol groups, showing the area and perimeter of the investigated tissue. One-way ANOVA statistical analysis, with Bonferroni post-test, assuming p<0.05.

Group	Area	Perimeter
CoS (Control)	5,22μM	2,22μM
CrS (Supplemented)	5,84μM	6,26μM



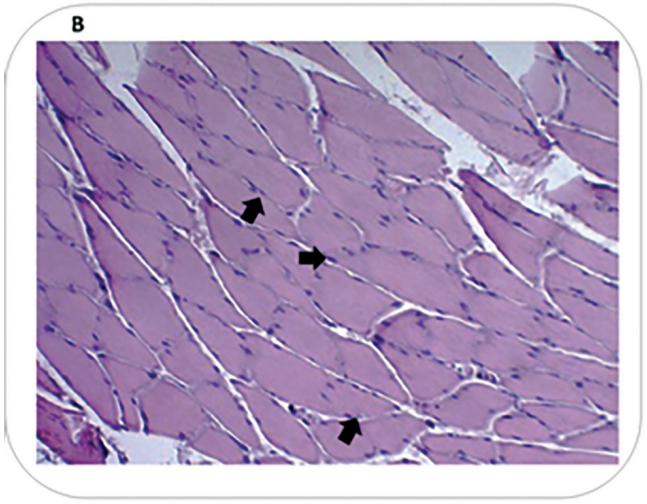


Figure 1: Stereological analysis of the soleus of wistar rats from the experimental protocol. Chart A indicates animals from the supplemented group (Group II) and chart B animals from the control group (Group I). Arrows indicate the eccentric nuclei present in this tissue.

DISCUSSION

The use of creatine monohydrate as an ergogenic supplement is widely recognized, especially in sports and rehabilitation contexts[1-4]. Previous data from the group identified that animals of the Wistar lineage, supplemented over 8 weeks, three times a week, and periodically performed resistance training with weights on their tails[5]. The results indicated that the use of amine as a supplement significantly increased the cross-sectional area of the animals that performed training and ingested the supplement, however, even the animals that only ingested the supplement had an increase in the cross-sectional area, even without the practice. of training. This factor motivated the group to investigate the use of creatine in other conditions, such as the aging process and degenerative diseases.

Thus, results by Fernandes at al[6] identified that the use of creatine in elderly Wistar animals, aged over three years, provided an increase in the cross-sectional area of the biceps brachii and gastrocnemius, even without these animals having undergone training. force or resistance. Similarly, Op't Eijnde at al[7] (observed that creatine promotes an increase in muscle volume due to the stimulation of the mTOR protein cascade (mammalian target of rapamycin) which leads to an increase in protein synthesis and consequently stimulation of cell survival.[7-9]

Corroborating this, Fernandes at al[10] found that this bioenergetic pathway, called phosphocreatine, is of great importance for maintaining cell activity when these cells are imposed at high intensity and high energy demand. Therefore, a longer recovery time is required for this resynthesis pathway to be restored. Therefore, appropriate intervals should be greater than 60 seconds.

Studied the effects of chronic use of creatine on muscle activity, torque, and cross-sectional area of animals submitted to the rehabilitation process after lower limb muscle atrophy. The observed results showed that the animals that ingested the supplement obtained an increase in the cross-sectional area, greater torque, and functional improvement during rehabilitation when compared to the animals that did not consume high doses of creatine. Such results illustrate an important relationship between creatine and the hypertrophic process[11].

Still, when analyzing figure 1, the comparison between groups A – CoS (control group) and B – CrS (supplemented group), it is identified in the groups the increase in the cell perimeter and cytoplasmic area. The presence of the narrowing of the muscular fibers, confirms the increase of the transverse section, confirming the hypertrophic process.

These results support the hypothesis that the use of creatine may be beneficial in the hypertrophic process or degenerative diseases, mainly because it offers the possibility of increasing the cross-section, that is, increasing the hypertrophic process.

CONCLUSION

It is understood that the effects of monohydrated creatine supplementation are associated with a greater stimulation of the protein synthesis pathways and, consequently, of the hypertrophy of the evaluated tissue. However, even though the animals used in this study were aged 26 months, no harmful effects of supplement use were observed in this sample.

Declaration of Conflict of Interest

The authors declare that there is no conflict of interest in this article.

References

1. Kreider RB, Kalman DS, Antonio J, Ziegenfuss TN, Wildman R, Collins R, Candow DG, Kleiner SM, Almada AL, Lopez HL. International society of sports nutrition position stand: safety and efficacy of creatine supplementation in exercise, sport, and medicine. J Int Soc Sports Nutr. 2017. doi: 10.1186/s12970-017-0173-z.
2. Gualano B, Acquesta FM, Ugrinowitsch C, Tricoli V, Serrão JC. Effects of creatine supplementation on strength and muscle hypertrophy: currents concepts. Rev Bras Med Esporte 2010 may/jun; 16(3)

1. Fernandes VAR, Belozo FL, Conte M, Caldeira EJ. Estereologia e morfometria do tecido muscular-esquelético de animais submetidos a um programa de treinamento de força e suplementação de creatina durante 9 semanas. Revista Brasileira de Fisiologia do Exercício 2019 (no prelo).
2. Gualano B, Rawson ES, Candow DG, Chilibeck PD. Creatine supplementation in the aging population: effects on skeletal muscle, bone and brain. Amino Acids 2016 aug; 48(8): 1793-1805. doi: 10.1007/s00726-016-2239-7
3. Riesberg LA, Weed SA, McDonald TL, Eckerson JM, Drescher KM. Beyond Muscles: the untapped potential of creatine. Int Immunopharmacol 2016 aug; 37:31-42. doi: 10.1016/j.intimp.2015.12.034.
4. Fernandes VAR, Col LO, Moura EG, Matos MO, Caldeira EJ, Conte M. Treinamento de força e seus efeitos sobre a área de secção transversa e perímetro celular de miócitos do gastrocnêmio de rattus novergicus. Revista Brasileira de Nutrição Esportiva 2018.
5. Kazak L, Chouchani ET, Lu GZ, Jedrychowski MP, Bare CJ, Kumari M, et al. Genetic depletion of adipocyte creatine metabolism inhibits diet-induced thermogenesis and drives obesity. Cell Metab 2017; 26(4):660-671.
6. Op't Eijnde B, et al. Effect of oral creatine supplementation on human muscle GLUT4 protein content after immobilization. Diabetes. 2001;50(1): 18–23.
7. Braissant O, et al. Dissociation of AGAT, GAMT and SLC6A8 in CNS: relevance to creatine deficiency syndromes. Neurobiol Dis. 2010;37(2): 423–33.
8. Fernandes VAR, Delforno MC, Banov GC, et al. Renal, hepatic and muscle effects of creatine Supplementation in na older adults experimental model. Clinical Nutrition ESPEN. 2022, 48(01). Doi: https://doi.org/10.1016/j.clnesp.2021.12.020
9. Farshidfar F, Pinder MA, Myrie SB. Creatine Supplementation and Skeletal Muscle Metabolism for Building Muscle Mass- Review of the Potential Mechanisms of Action. Curr Protein Pept Sci. 2017;18(12):1273-1287. doi: 10.2174/1389203718666170606105108.