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ORIGINAL PAPERS

Use of Creatine Monohydrate in MDX Mice: Morphometric and Stereological Analysis of the Diaphragm

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Abstract

Duchenne muscular dystrophy is a genetic disease that is clinically manifested by progressive muscle atrophy, followed by loss of strength, motor coordination and functional impairment. In the final stages of the disease, the patient has severe difficulty in breathing mechanics, due to the involvement of the muscles involved with the mechanics of breathing, including the diaphragm. The present study sought to identify the effects of creatine monohydrate supplementation in MDX mice on the morphology, morphometry and stereology of the striated muscle tissue of the diaphragm of these animals. The results indicate that, despite not influencing the increase in cell volume, supplementation acts in an anti-inflammatory way, reducing the progressive process of fibrosis that occurs in these animals in the face of muscle atrophy followed by the replacement of muscle parenchyma by connective tissue. in this way, supplementation provides a better condition for tissue maintenance, enabling more survival of MDX mice. Monohydrate creatine supplementation has been shown to be a complementary therapeutic alternative, especially for muscular dystrophies and severe myopathies.

Keywords: Duchenne Muscular Dystrophy, Creatine Supplementation, Diaphragm, Respiratory Muscle, Muscle Diseases.

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INTRODUCTION

Duchenne muscular dystrophy, a disease that affects male children and leads to progressive atrophy of striated skeletal muscle tissue[1-4]. It is a hereditary genetic disease and has a recessive inheritance linked to the mutation of a gene located on the short arm of the X chromosome, under abnormal conditions it is responsible for the atrophy of skeletal muscle cells, due to the absence of dystrophin, a protein that makes the connection between the myocyte sarcolemma and the extracellular matrix, which becomes progressively reduced in patients suffering from this dystrophy[5-9].

The use of creatine monohydrate has been extensively studied beyond the sports environment, mainly for rehabilitation or therapeutic purposes that affect muscle and nervous tissue[1,2]. Creatine administered in appropriate doses provides benefits such as increased strength, improved muscle endurance, reduced fatigue, balance of the hydrogen ion potential of the cell cytoplasm, neuroprotective effects, anti-inflammatory effects and potential tissue repair effects[4,8]. Recent studies show that creatine supplementation has positive effects on skeletal muscle physiology and metabolism, including increased muscle mass (hypertrophy) and improved intracellular redox. These situations could benefit patients who suffer from the progressive atrophy and chronic inflammation present in Duchenne muscular dystrophy[10-12].

The diaphragm is the main muscle responsible for respiratory mechanics, being composed of three muscle parts, sternal, costal and lumbar. The diaphragmatic movement converges with the entry of atmospheric air into the upper airways that direct this air to the pulmonary level, with the process of hematosis in the alveoli being carried out. This activity is essential for the patient's breathing, and patients with Duchenne muscular dystrophy suffer a progressive reduction in their respiratory capacity, mainly due to the atrophy of the muscle fibers of the diaphragm, leading to a lower capacity for ventilation and, therefore, a reduction in their functional activity.[13-16]

Knowing that Duchenne muscular dystrophy is characterized by reduced mechanical strength, the objectives of palliative treatment are to improve the patient's quality of life and mobility, as well as to delay the progression of respiratory muscle atrophy[17-19]. In this sense, the use of creatine monohydrate could be a complementary therapeutic alternative in the treatment of Duchenne muscular dystrophy and the investigation of this condition in the diaphragm tissue could help in conducting investigations that promote approximations between experimental and clinical research on this pathology.

In view of this, the present study aims to identify the effects of this supplement, at doses recommended by international reports[4] that do not promote renal and hepatic overload, in MDX mice, which spontaneously manifest Duchenne muscular dystrophy and progressive muscular atrophy.

MATERIAL AND METHOD

Experimental draw

In the study, 20 MDX mice (*dystrophic mice)* (n =10) and the C57/BL10 strain (n=10). The animals were organized into four groups:

- Group I: Consisting of 5 C57BL/ 10 mice, representing the control group of the study (Cos);
- Group II: Consisting of 5 C57BL/10 mice that received creatine monohydrate supplementation for sixteen weeks (CrS);
- Group III: Consisting of 5 *MDX mice* that received creatine monohydrate supplementation for sixteen weeks (CrMDX).
- Group IV: Consisting of 5 *MDX mice* that did not receive creatine monohydrate supplementation during the trial period (CoMDX).

All animals were in the four-week age group and had body mass duly standardized for the start of the experimental protocol (503.3g on average). The animals received a solid diet and water *ad libitum*, and were kept in collective cages (5 mice of the same species per cage) at a constant temperature of 23±20C, cycle of 12 hours light/12 hours dark, with lights on from 06:00 h to 18:00 h. All animals were fed Labina® (a standard rat chow diet supplied by Purina, Brazil). The body weights (g) of the animals were checked at the beginning and at the end of the experiment. The animals were sacrificed one day after the last creatine supplementation, following the recommendations standardized by the ethics committee for animal use. The samples needed for analysis of the variables in this study (muscle tissue) were collected after anesthetic induction and sacrifice.

Painless death induced in animals

The animals had painless death induced one day after the experimental protocol, through a high dosage (0.3 mg/100gr) of anesthetic via intraperitoneal solution composed of Xylazine, Ketamine and Thiopental. Subsequently, pneumothorax was induced in the animal. After confirming the death of the animals, the diaphragms were extracted for morphometric and stereological analysis. Ethical aspects

The present study was approved by the Animal Use Ethics Committee under process 19/2021.

Creatine Supplementation

Only the animals in groups II and III received Creatine Monohydrate supplementation, at a dosage of 0.3mg per kg of body mass for 16 weeks. Dosages were based on other studies using supplementation in rodents[6,7] and which are equivalent to the dosage regimen used in humans to obtain ergogenic effects[13]. Supplementation was administered by gavage, as proposed by Souza[14] using an oroesophageal probe (1 mm in diameter; 3 cm in length) adapted to a 3 ml syringe, with water as the infusion vehicle. The animals in groups II and III received creatine supplementation on Mondays, Wednesdays and Fridays in the morning, between 06:00 and 10:00. The animals in the other groups were submitted to the same gavage process, however only water was instilled.

Light Microscopy and Stereology

After the period of experimentation and euthanasia of the animals, the muscular tissues of the diaphragm were extracted and these were then fixed in Bouin (Saturated aqueous solution of picric acid - 75 ml, formaldehyde - 25 ml, glacial acetic acid - 5 ml) for 12 hours for further processing and embedding in paraffin.

The tissues were then washed in 70% alcohol and subjected to dehydration in an increasing series of alcohols (80% alcohol - 2 times, absolute alcohol - 3 times; 1 to 2 hours each) for inclusion. After that, the fragments were cleared in xylene for 1 to 2 hours until they became translucent. The fragments were then embedded in paraffin and plastic polymers (Paraplast Plus, Polyscience, Niles, IL) at 56°C for approximately 1 hour and then transferred to new paraffin at the same temperature.

Tissues were carefully arranged at the bottom of plastic vats, with a view to obtaining cross-sectional histological sections. The blocks were trimmed to obtain flat surfaces and sectioned to five micrometers thick. Then the fragments were placed on albuminized slides and taken to the oven at 60°C. After preparing the sections, the slides were stained with hematoxylin/eosin (HE) for general morphological study. After obtaining the histological slides, they were analyzed and photographed, prioritizing the 10x and 40x objectives.

All these sections were used for morphometric and stereological analyzes of the extracted tissues.

Statistical analysis

Bonferroni Test involving the pairs of each group, representing the non-parametric test. The entire study was performed with at least 5% significance.

RESULTS

After the experimental protocol period of 16 weeks, the animals' diaphragm muscle tissue was collected, which were cut and stained. The stains applied were hematoxylin and eosin and Masson's trichrome, for analysis of inflammatory infiltrates, myocyte morphometry and identification of fibrosis in the local tissue.

Figure 1 indicates the degeneration of muscle tissue in animals of the MDX lineage, image A being animals without creatine treatment, while B with food supplement treatment. Inflammatory progress is more extensive in MDX animals from the untreated group when compared to the treated group. Furthermore, greater local fibrosis is observed with the presence of more fat cells in the region. Centralized nuclei, an unusual aspect for this type of tissue, are observed in both experimental groups.

The progression of the connective tissue in the diaphragm of animals from the MDX groups is evidenced by the Masson's Trichomium technique, in figure 2. This accentuated condition in the animals from the MDX group is shown by an extensive band (in blue) of fibrous tissue that invades the muscle. Physiologically, this aspect greatly reduces the contractile capacity of the diaphragm in these animals. Supplemented animals have local fibrosis to a lesser extent and with well-defined margins.

Graph 1: description of the morphometry of the myocyte sarcoplasma present in the diaphragm of the animals in the experimental protocol groups. ANOVA one statistical treatment way, with post Bonferroni test, assuming $p<0.05$.

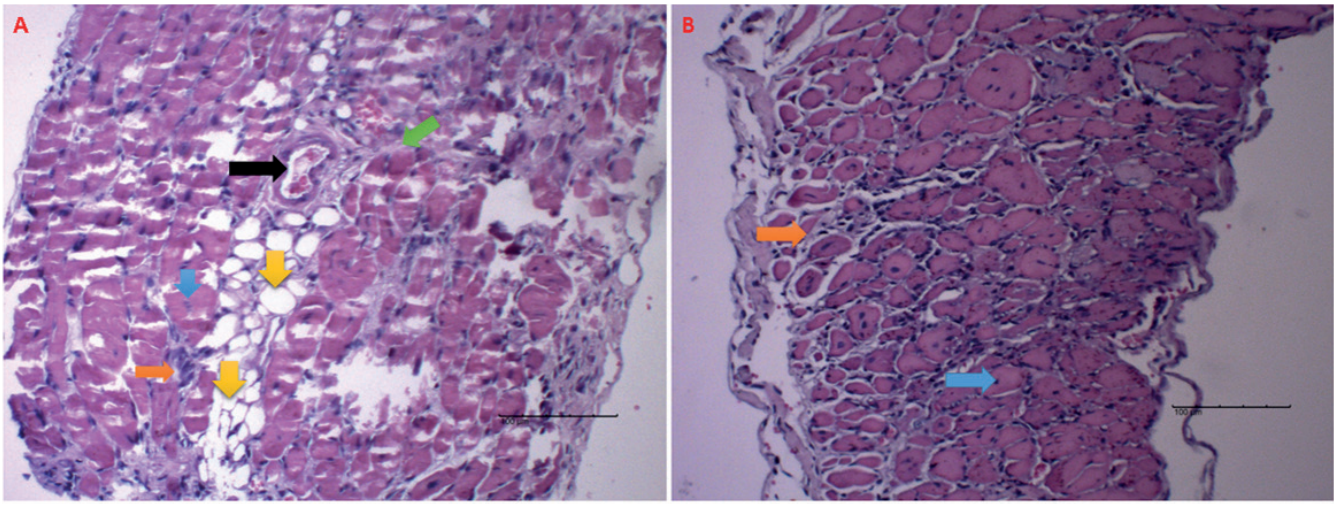


Figure 1. Section of the diaphragm of animals from the MDX (A) and MDX groups with creatine supplementation (B) for 16 weeks at a dosage of 0.3g X kilogram of body mass. The black arrow indicates a vessel undergoing hyaline degeneration, with a perimeter rich in mononuclear leukocytes and fibrosis (green arrow). The blue arrow indicates centralized nuclei, an atypical appearance in skeletal muscle tissue. Yellow arrows show fat cells at the site. Orange arrow indicates regional inflammatory infiltration. Observation in 10x objective (H/E).

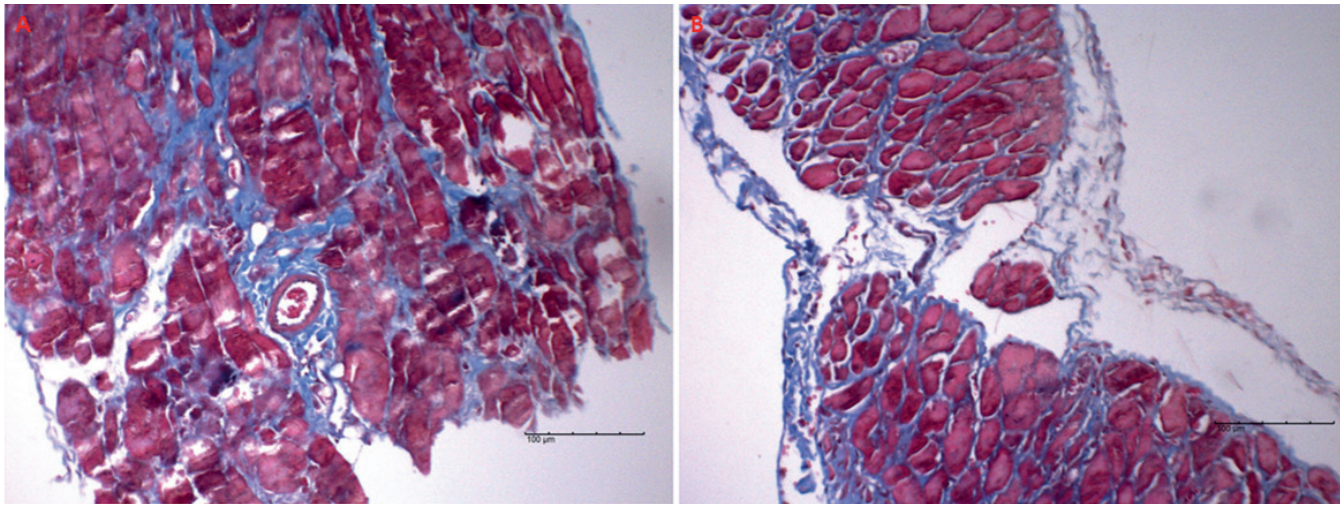


Figure 2. Section of the diaphragm of animals from the MDX (A) and MDX groups with creatine supplementation (B) for 16 weeks at a dosage of 0.3g X kilogram of body mass. In blue, the local connective tissue can be seen, which is more pronounced in the animals in the MDX group, when compared to the animals in the MDX group with creatine supplementation. Image A indicates local fibrosis with extension. Observation in a 10x objective (Masson's Trichrome).

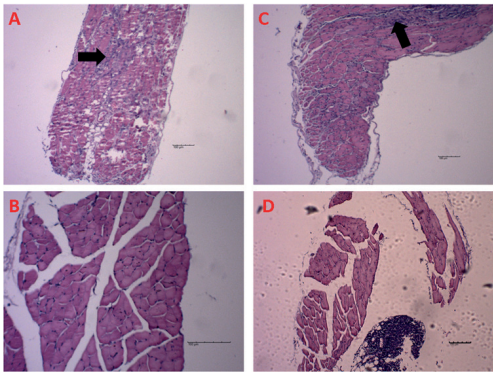
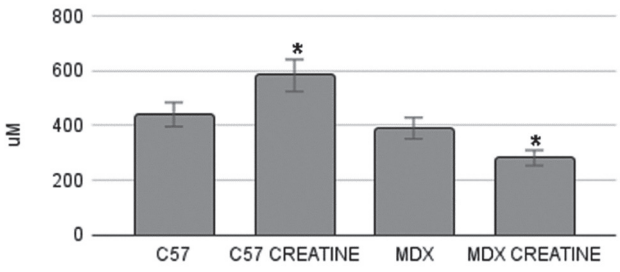


Figure 3. Comparison of diaphragm muscle tissue from animals in the experimental protocol groups. A shows the untreated MDX group. B indicates C57 animals without creatine supplementation. C indicates MDX animals with creatine supplementation and D C57 animals supplemented. All groups that were supplemented had standardized dosages per 0.3g X kilogram of body mass. Black arrow shows areas of inflammatory infiltration in muscle tissue. 10X objectives, material stained in H/E.



Graph 1. Description of the morphometry of the myocyte sarcoplasma present in the diaphragm of the animals in the experimental protocol groups. ANOVA one statistical treatment way, with post Bonferroni test, assuming $p<0.05$.

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 25 days, three times a week, and that 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.

Thus, results by Fernandes et al (2022)[1] 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, Farshidfar, Pinder and Myrier[3] 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. In this sense, the present study sought to identify the effects of this supplement, at doses recommended by international reports[4] that do not promote renal and hepatic overload, in MDX mice, which spontaneously manifest Duchenne muscular dystrophy and progressive muscular atrophy.

Duchenne muscular dystrophy is a hereditary disease manifested by the mutation of the X chromosome in its short arm, which leads to a severe reduction in the synthesis of the dystrophin protein, which serves to anchor the sarcolemma of muscle cells to the extracellular matrix[7-12]. Thus, by expressing reduced levels of this protein, the muscles of MDX animals become progressively atrophied[13], mainly the lower limb musculature, progressing to the upper limbs and finally respiratory muscles, among the latter, the diaphragm[14-16].

CoMDX group had a higher incidence of fat infiltration in the muscle tissue of the diaphragm, as evidenced by figure 1. This fat infiltration is manifested in conditions of severe metabolic alteration, as it occurs in the context of the progression of Duchenne muscular dystrophy[1,17]. However, in figure 1 it is possible to identify regions of muscle tissue with myocytes already in a condition of cloudy swelling in frame A (which refers to a section of the CoMDX group). This condition of turbid swelling usually manifests itself in the face of a cellular injury, being an important anatomopathological characteristic to be considered in the study of the tissue[18-23].

The presence of inflammatory infiltrates is identified in the CoMDX and CrMDX groups, which are not presented in the CoS and CrS groups. This presence of infiltration of mononuclear and polymorphonuclear leukocytes is more evident in the CoMDX group, with an extensive area of local fibrosis even being present in this group, indicated in figure 2 by the exacerbated presence of collagen between the muscle fibers in frame A of the figure.

Duchenne muscular dystrophy is characterized by progressive muscular atrophy[16-18], with recruitment of mononuclear and polymorphonucleated leukocytes to the atrophic site, which help in tissue repair and progressive replacement of parenchyma by stroma[19,23]. In this sense, the animals in the CrMDX group had a smaller area of inflammatory infiltration, with less extension. Together with this, a less evident tissue fibrosis, with preservation of the endomysium in a condition similar to the CoS group (control).

It is known that creatine monohydrate is capable of helping to maintain intracellular redox because it is an important hydrogen buffer and thus reduces reactive oxygen species originating from metabolic processes[1,3,4]. In this scenario, supplementation contributes to preserving the local tissue, reducing inflammatory progress, by attenuating reactive species. Thus, the sup-

plemented animals, in line with what is described in the literature[1-4], have better preserved muscle tissue, with less evident local fibrosis, indicating that the inflammatory process is not chronic, as observed in animals from the CoMDX group .

In view of the results observed in Graph 1, on the morphometric analysis between the groups, it was identified that the groups of C57BL/10 animals (CoS and CrS) presented a very similar sarcoplasm area between the groups. However, when analyzing the groups of MDX animals (CoMDX and CrMDX) it is verified that the sarcoplasm is substantially reduced, being in agreement with the notes in the literature[16,18,23,24]. Thus, it was found that, although the animals in the CrMDX group showed muscle cell atrophy, they maintained a tissue environment with fewer inflammatory processes and a lower frequency of tissue fibrosis.

These results support the hypothesis that the use of creatine may be beneficial in the complementary treatment of Duchenne muscular dystrophy, mainly because it offers the possibility of reducing the chronic inflammatory process characteristic of this pathology, together with this, reducing the progression of tissue fibrosis[1,4,23,25]. However, the cell volume of myocytes was not maintained, despite sustaining an improved stereological condition in the animals from the CrMDX group of this study.

CONCLUSION

The present study aimed to identify the effects of creatine monohydrate supplementation in MDX mice, which spontaneously manifest Duchenne muscular dystrophy and progressive muscular atrophy. The observed results identified that the sarcoplasmic area of the animals did not differ statistically between the experimental protocol groups, however, the use of creatine reduced the tissue inflammatory process as well as attenuated the presence of tissue fibrosis in the diaphragm of the treated animals.

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