

Creatine lysinate – part II: effects on the motor coordination and muscle hypertrophy in mice

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Abstract

In the current study, we investigated the effect of creatine monohydrate (CrM) and newly synthesized creatine lysinate (CrLys) in tail suspension (TST) and rotarod tests and their influence on the histology of the skeletal muscles. In the TST, a slight decrease in the immobility time from the 1st to the 3rd week was observed in the group treated with CrM at a dose of 1.5 g/kg/day and CrLys at a dose of 6 g/kg/day. The rotarod test revealed that CrM (1.5 g/kg/day) and CrLys (3 g/kg/day) lead to a significant improvement in motor coordination in the 3rd week. The results from histology showed an increase in the muscle fiber diameter of soleus muscle in animals treated with CrM (3 g/kg/day) and CrLys (6 g/kg/day). The results showed that supplementation with creatine derivatives appears to be a generally effective nutritional ergogenic aid for an improvement of physical performance.

Keywords

creatine derivatives, mice, rotarod, soleus muscle, tail suspension test

Introduction

Creatine is one of the most popular and widely investigated natural supplements. It has been shown that creatine supplementation increases strength, muscle mass, and muscle morphology in combination with training and more than training alone (Cooper et al. 2012). One of the most important physiological functions of creatine is related to the maintenance of energy levels in the muscles and the brain (Wyss and Schulze 2002). Creatine increased the synthesis of adenosine triphosphate (ATP), which is the major source of energy, and has been primarily known as a supplement for an increase of muscle power and improvement of sports performance (Benzi 2000). Creatine intake lead to the production of phosphocreatine (PC), which

is attached to a phosphate molecule, partially forms the ATP-PC energy system and free creatine, and increases the stores of creatine in the muscle to provide more immediate energy (Juhn and Tarnopolsky 1998; Williams and Branch 1998; Maughan et al. 2004). Short-term creatine supplementation (e.g. 20 g/day for 5–7 days) has been reported to increase total creatine content by 10–30% and phosphocreatine stores by 10–40% (Kreider 2003).

There are data that creatine from food sources such as meat and fish has the potential of an effective natural antidepressant. Creatine intake had an effective role in learning, memory, emotional, and cognitive function (Rae et al. 2003). Creatine supplementation improved depressive-like behavior in animal models (Allen et al. 2010; Allen et al. 2012; Cunha et al. 2012) as well as the Hamilton

Depression Rating Scale (HAM-D) score in humans (Roitman et al. 2007). The combined treatment with creatine and antidepressants was effective in patients with depression. Intake of both creatine and escitalopram has been shown to improve depression symptoms (Lyoo et al. 2012). Creatine administration increases creatine concentrations and thus the production of neurotransmitters and PC in the brain (Ferrante et al. 2000). This has both neurological and hormonal effects on the body and makes the brain less susceptible to experiencing depression. Supplementation with creatine, similar to the effect of exercise, has also been shown to have neuroprotective effects in the brain, including the delay in the progression of Huntington's, Alzheimer's and Parkinson's diseases (Adihetty and Beal 2008). Ahn et al. (2016) stated that treatment with either creatine or exercise is effective as an antidepressant, but the combination of creatine and exercise has a synergic effect, which is a more effective strategy than either treatment by itself.

Physical performance is a complex concept including the properties of skeletal muscles, substrate and energy supply, nervous and humoral regulation, as well as the neuropsychic properties and motivation of the person, quantitatively expressed in the volume and (or) intensity of the produced mechanical work (Sonkin 2010). Supplementation with creatine monohydrate (CrM) has been shown to promote greater gains in muscle mass and strength during resistance exercise (RE) training compared with placebo-treated groups (Kreider 2003). These beneficial effects are thought to occur via the accumulation of Cr in the skeletal muscles (Harris et al. 1992). The uptake of Cr by muscle seems to stimulate transcription factors that regulate contractile protein synthesis (Willoughby and Rosene 2001) and/or increase PC availability (Harris et al. 1992), which is thought to promote greater work capacity and strength improvements during training (Rawson and Volek 2003). The majority of data from longitudinal studies supported the theory that protein intake before and/or after RE will improve the chronic adaptations desired from training (muscle hypertrophy and strength) (Esmarck et al. 2001; Chromiak et al. 2004; Rankin et al. 2004; Andersen et al. 2005).

Ferrante et al. (2000) demonstrated that creatine supplementation significantly improved survival, slowed the development of brain atrophy, and delayed the atrophy of striatal neurons and the formation of huntingtin-positive aggregates in R6/2 mice. Body weight and motor performance on the rotarod test were significantly improved in R6/2 mice receiving creatine, whereas the onset of diabetes was delayed. The percent increase in rotarod performance reported by these authors wasn't dose-dependent and for 1, 2, and 3% creatine was 25, 33, and 6.5%, respectively.

Our previous study revealed data about the toxicity of newly synthesized creatine lysinate (CrLys) and its effect on biochemical parameters like aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), creatine kinase and lactate. Our results showed that CrLys is practically non-toxic in mice after intraperitoneal (i.p.) and peroral (p.o.) administration which makes it appropriate for further pharmacological investigations. Based on the available data for the effect of CrM, we performed tail

suspension test, rotarod test and histology of soleus muscle after supplementation with creatine derivatives in mice to observe the changes in depressive-like behavior, motor coordination and muscle structure.

Materials and methods

Animals and treatment

Male albino mice, line H with body weight 28–32 g were divided into five groups of six animals in each group (n = 6). Food and water were available ad libitum. During the whole experiment, the animals were maintained at room temperature 22 ± 3 °C, humidity 30%, lighting schedule 12 h light/dark cycle. Experiments were performed during the light part of the cycle. The animals were divided into the following groups depending on the administered substances and their doses:

- 1st group – control animals that received only drinking water;
- 2nd group – animals that received CrM at a dose of 1.5 g/kg/day (CrM 1.5 g/kg/day);
- 3rd group – animals that received CrM at a dose of 3 g/kg/day (CrM 3 g/kg/day);
- 4th group – that received CrLys at a dose of 3 g/kg/day (CrLys 3 g/kg/day);
- 5th group – animals that received CrLys at a dose of 6 g/kg/day (CrLys 6 g/kg/day).

All substances were dissolved and administered to the experimental animals for 2 weeks with drinking water. On the 1st, 7th and 14th days were performed tail suspension and rotarod tests and at the end of the experiment histological evaluation of the soleus muscle was performed. The experiment was conducted in accordance with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes and approved by the Bulgarian Food Safety Agency (№ 329/01.06.2022).

Tail suspension test (TST)

The tail suspension apparatus consists of plastic enclosures (20 × 25 × 40 cm) separated by an opaque partition and two animals could be tested at the same time. Following the procedure of Castagne et al. (2009) with little modifications, each mouse was suspended by the tail 20 cm above the floor of the chamber using adhesive tape placed < 1 cm from the tip of the tail. The behavior was observed for 6 min and the last 4 min was recorded. Immobility time was defined as hanging passively without any movement of the head or paws. TSTs were performed on the 1st, 7th and 14th day, to escape exhaustion in mice.

Rotarod test

The rotarod test is used for an evaluation of motor performance in mice and rats and for the assessment of potential

therapeutic approaches. The rotarod apparatus is composed of a rotating cylinder, divided into different compartments with walls, one for each animal. Mice were placed on the rod of the apparatus (Ugo Basile, RotaRod 47 600) in an accelerating mode from 4 to 40 rpm (acceleration time 30 s) and the latency to fall was recorded. Each animal had three consecutive trials with an inter-trial interval of 15 minutes. The maximum duration of every trial was 300 seconds. The average time of the three consecutive trials was used for analysis (Luh et al. 2017). Rotarod tests were performed on the 1st, 7th and 14th days, to escape exhaustion in mice.

Preparation of tissue sample and histological evaluation of muscle hypertrophy

Immediately after collecting the blood samples for biochemical analysis (our previous publication), the hind limbs of mice were quickly dissected to retrieve soleus muscle. The gastrocnemius muscle was removed and tissue blocks containing the entire soleus muscle were obtained for examination. Muscle samples from each group were carefully separated and washed with saline and then fixed in a 10% phosphate-buffered formaldehyde solution for 24 hours. Following the process of fixation, the muscles were routinely processed for paraffin embedding. Blocks were cut into approximately 6 μm thick sections using a sliding microtome. The sections were dewaxed, hydrated and sequentially incubated in solutions of hematoxylin (to color the nuclei purple) and eosin (to color the cytoplasm). Three cross-sections from the proximal, middle and distal parts of both hind limbs of each animal were examined and recorded using a Nikon Eclipse 80i light microscope at $\times 20$ magnification.

Statistical analysis

Statistical processing of the obtained results was done with the program GraphPad Prism 6.0. The arithmetic means and the standard errors of the arithmetic mean (SEM) were determined for all data. A statistically significant difference between the compared means was checked using the One-way ANOVA and the Tukey test. A p-value of 0.05 or lower between the groups was considered statistically significant. For the graphical presentation of data, GraphPad Prism 6.0 software was used. The evaluation of muscle hypertrophy was performed manually by measuring the mean value of minimum and maximum Feret's diameters of the muscle fibers using Nikon NIS-Elements software.

Results

On Fig. 1 are presented the results from the TST aimed to assess the antidepressant activity. Reduced immobility time is an indicator of a potential antidepressant activity of the substance.

Between the first and third week, there was a slight decrease in the immobility time in the groups treated with CrM 1.5 g/kg/day (from 125.2 ± 29 to 99 ± 15 s, data represents means and SEMs) and CrLys 6 g/kg/day (from

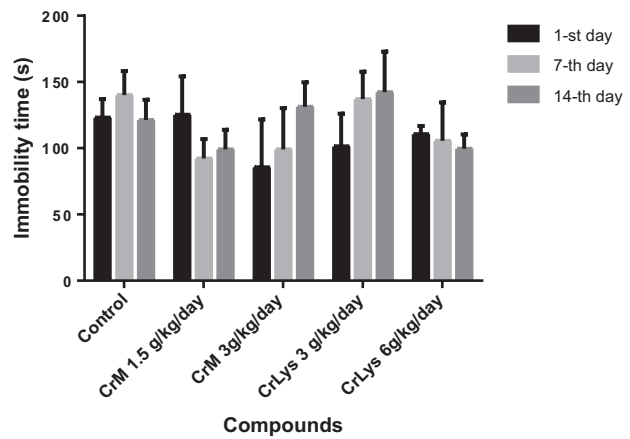


Figure 1. Effect of creatine derivatives on immobility time in the TST. The results are presented as arithmetic mean (mean) \pm SEM.

110.5 ± 6.3 s to 99.5 ± 11 s, data represents means and SEMs). In the groups receiving CrM 3 g/kg/day and CrLys 3 g/kg/day, the time of immobility gradually increased. The results of the creatine derivatives were comparable to those of the control group and didn't show significant antidepressant or pro-depressant effects at these doses for 2 weeks.

The effects of oral administration of creatine for 2 weeks on rotarod performance are shown in Fig. 2. Oral administration of 1.5 g/kg/day CrM significantly improved motor coordination and physical endurance in the 3rd week in contrast to the unsupplemented control group (1.5 g/kg/day CrM, 268 ± 47 s; unsupplemented, 107 ± 12 s, $p \leq 0.01$, data represents means and SEMs). Supplementation with CrM 1.5 g/kg/day resulted in significant motor improvement from the 1st to 3rd week (1st week, 45 ± 4 sec; 3rd week, 268 ± 47 s, $p \leq 0.0001$, data represents means and SEMs). In the group treated with the newly synthesized CrLys at a lower dose (3 g/kg/day), there was also an improvement in motor coordination between the 1st and 3rd week of supplementa-

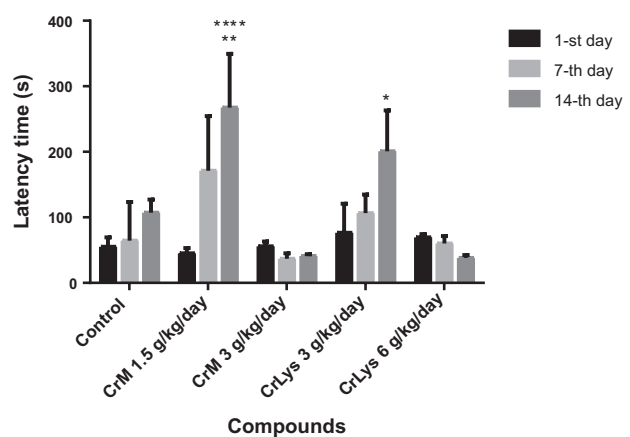


Figure 2. Effect of creatine derivatives on the motor coordination in the rotarod test in mice. The results are presented as arithmetic mean (mean) \pm SEM. Statistically significant differences: * $p \leq 0.05$ – between the 1st and 3rd week in the group treated with CrLys 3 g/kg/day; ** $p \leq 0.01$ – between the 3rd weeks in the control group and CrM 1.5 g/kg/day; **** $p \leq 0.0001$ – between the 1st and 3rd week in the group treated with CrM 1.5 g/kg/day and between the 3rd weeks in CrM 1.5 g/kg/day and the group receiving CrM 3 g/kg/day.

tion (1st week, 77 ± 25 s; 3rd week, 200 ± 36 s, $p \leq 0.05$, data represents means and SEMs). Oral supplementation with higher doses, CrM 3 g/kg/day and CrLys 6 g/kg/day respectively, had no significant effect on the rotarod performance.

Histological evaluation of muscle hypertrophy

The diameter of the muscle fibers of the soleus muscle was measured to determine whether hypertrophy occurred in different groups of experimental animals. We found that, in animals treated with CrM at a dose of 3 g/kg/day and CrLys at a dose of 6 g/kg/day ($p \leq 0.05$), there is an increase in muscle fiber diameter (Figs 3, 4). Similar data on humans and animals have been described in the literature. Moura et al. (2002) noticed that the supplementation of CrM did not modify the cross-sectional area of the hind limb muscle fibers. However, the combination of creatine supplementation with exercise had an additive effect on increasing the cross-sectional areas of type I, IIA and IIB fibers of hind limb muscles in Wistar rats.

Discussion

In our study, we investigated the effect of newly synthesized creatine derivative CrLys after 2 weeks of administration in the TST, rotarod test and examined the morphology of the soleus muscle. In the TST, we observed a gradual decrease in the immobility time only in the group treated with CrM 1.5 g/kg/day and CrLys 6 g/kg/day without a significant difference with the control group. Ahn et al. (2016) investigated the effects of 4-week supplementation with 4% w/w CrM in the chow with or without exercise on antidepressant behavior and raphe 5-HT expression in a chronic mild stress-induced model of depression in mice. The authors stated that both the stress-creatine group and stress-exercise groups had significantly increased 5-HT positive cells compared to the stress control group in the dorsal raphe nucleus. Further, the stress-creatine-exercise group showed a synergistic effect greater than in stress-creatine and stress-exercise groups. These results suggest that the combination of creatine and exercise would produce an improved antidepressant effect compared to both applied separately (Ahn et al. 2016). Cunha et al. also revealed the antidepressant-like effect of oral creatine in the TST and forced swimming test in rodents (Cunha et al. 2012; Cunha et al. 2013a; Cunha et al. 2013b). In another study of these authors, they demonstrated that creatine administered by intracerebroventricular injection showed an antidepressant-like effect in the TST, suggesting the central antidepressant-like effect of creatine (Cunha et al. 2013a). A single systemic administration of creatine was reported to increase the content of creatine and phosphocreatine in the striatum and creatine kinase activity in the brain of rats (Royes et al. 2006).

In the rotarod test our results indicated improved motor coordination after 2 weeks in the groups treated with CrM 1.5 g/kg/day and CrLys 3 g/kg/day in comparison to their first administration. Iqbal et al. (2015) also stated that mice

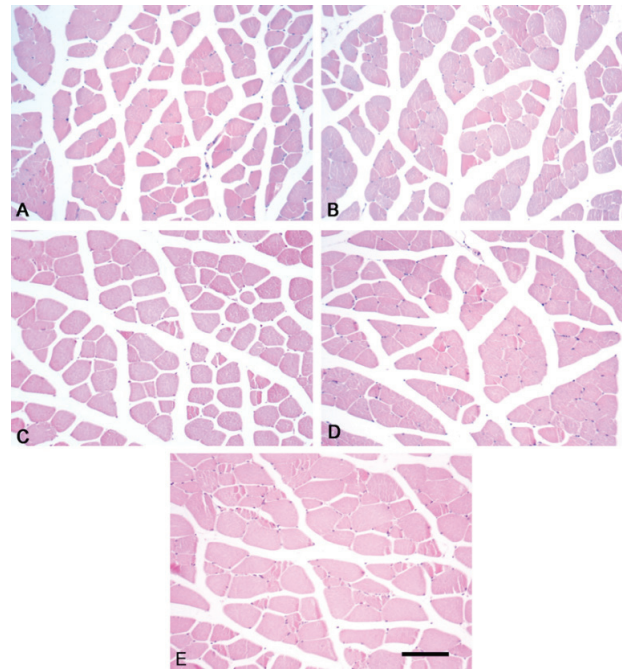


Figure 3. Representative images of hematoxylin and eosin-stained cross sections of the soleus muscle from different groups. Cross-sectioned basophilic nuclei (purple dots) are visible on the periphery of the myofibers. A small number of elongated fibroblastic nuclei between muscle fibers are also visible. (A). Photomicrograph of muscle fibers with regular diameter - control group. (B) Photomicrograph of muscle fibers with moderate diameter - animals treated with CrM 1.5 g/kg/day. (C) Photomicrograph of muscle fibers with larger diameter - animals treated with CrM 3 g/kg/day. (D) Photomicrograph of muscle fibers with moderate diameter - animals treated with CrLys 3 g/kg/day. (E) Photomicrograph of muscle fibers with the largest diameter - animals treated with CrLys 6 g/kg/day. Scale bar: 100 μ m.

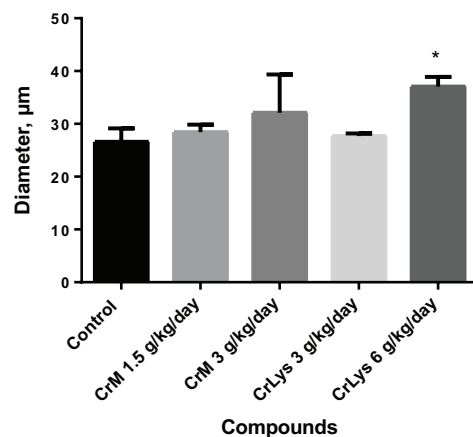


Figure 4. Diameter of muscle fibers of m. soleus in experimental animals. The results are presented as a mean value of minimum and maximum Feret's diameter \pm SEM. * $p \leq 0.05$ - comparison between the group treated with CrLys 6 g/kg/day and the control group.

supplemented with 2% Cr held longer on a rotarod than those fed on a normal diet, indicating that Cr supplementation enhanced muscular power and coordination. Kreider (2003) reviewed the available data that have examined the potential ergogenic potential of creatine supplementation

on exercise performance and also on muscle physiology and/or exercise capacity in healthy, trained, and various diseased populations. Short-term (5–7 days) creatine supplementation (e.g. 20 g/day) has typically been reported to increase total creatine content by 10–30% and phosphocreatine stores by 10–40%. Moreover, creatine supplementation during training has been reported to enhance a significantly greater increase in strength, and performance mostly in high-intensity exercise tasks. The prevalence of scientific evidence indicated that supplementation with creatine seems to be an effective nutritional ergogenic aid for a variety of exercise tasks in the athletic and clinical populations.

Ferrante et al. (2000) investigated whether creatine could exert neuroprotective effects in a transgenic mouse model of Huntington's disease. They found that creatine dose-dependently improved survival in these mice. Creatine administration resulted in improved rotarod performance and reduced weight loss in the R6/2 mice. Dietary supplementation with 1% creatine resulted in significant motor improvement from 5 to 10 weeks. 2% creatine was most efficacious in the rotarod, and 1% creatine was more effective than 3% creatine. These results from the rotarod paradigm are comparable with those from our study which indicated improvement in the equilibrium and motor performance when mice are supplemented with lower doses of CrM and CrLys in comparison to the higher doses.

Wu et al. (2022) reviewed clinical trials from 2012 to 2021 regarding creatine supplementation and muscle growth. Creatine increased muscle strength, sport performance, and muscle hypertrophy in all healthy young populations, even in those who were untrained. The data revealed that creatine supplementation appears to be more effective for muscle growth in healthy young subjects than in other populations. Cribb and Hayes (2006) find that after 10 weeks of training, supplementation with protein/creatine monohydrate/glucose immediately before and after each workout resulted in significantly greater improvements in resistant training and body composition in males compared to a group who had consumed the same supplement outside of the workout period (before breakfast and late evening) each training day. The authors stated that the group that consumed their supplement just before commencing and straight after finishing their workout, demonstrated significantly greater increases in muscle mass, hypertrophy of the type-IIa and IIx fibers, and contractile protein. This finding confirmed the improvements in body composition

with resistant training and dietary supplementation with hypertrophy responses at the cellular (fiber-specific hypertrophy) and subcellular levels (contractile protein content).

Conclusion

The results from our study revealed that after 2 weeks of administration, the newly synthesized CrLys at dose 6 g/kg/day and CrM at a dose 1.5 g/kg/day lead to a slight decrease in the immobility time in TST. On the other hand, supplementation with CrM at a dose 1.5 g/kg/day and CrLys at a dose 3 g/kg/day improved gradually the motor coordination in the rotarod test. The histological examination of soleus muscle after 2 weeks of supplementation revealed a dose-dependent increase in muscle fiber diameter in the groups treated with CrM at a dose 3 g/kg/day and CrLys at a dose 6 g/kg/day. These results provide further evidence that creatine and its derivatives improved physical endurance, motor coordination and increased muscle fiber diameter at different doses. Further investigations can elucidate the additional benefits of supplementation with the well-known creatine monohydrate and newly synthesized creatine derivatives.

Authors' contributions

Conceptualization, I.K. and N.D.; methodology, I.K., N.D.; investigation, I.K., B.L., D.T, L.M., I.I.; writing—original draft preparation, I.K.; writing—review and editing, I.K., N.D., B.L.; visualization, I.K.; supervision, N.D.; project administration, I.K.; funding acquisition, I.K. All authors have read and agreed to the published version of the manuscript.

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