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Research Article

Creatine lysinate – part I: investigation of the toxicity and the influence on some biochemical parameters in mice

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Abstract

In our study we investigated the acute toxicity of a newly synthesized creatine lysinate as well as its effect on the biochemical parameters in mice. Creatine lysinate exerts better solubility in water (3.3%) in comparison to creatine monohydrate (1.4%) at 20 °C and it is determined as a non-toxic after intraperitoneal (LD50 – 4543 mg/kg) and oral administration (LD50 > 8000 mg/kg). Oral administration of creatine lysinate at doses of 3 g/kg/day and 6 g/kg/day for 2 weeks reduced the creatine kinase levels, which indicates muscle protection. An increased levels of liver enzymes like alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) was observed after the supplementation with creatine lysinate at both administered doses and the level of lactate was comparable both in the studied and the control group.

Keywords

Biochemistry, creatine derivatives, mice, LD50

Introduction

Creatine deserves a special place among ergogenic supplements, as it is one of the most studied and scientifically supported supplements on the market. Creatine is a naturally occurring non-protein nitrogen compound synthesized in the liver and kidney from amino acids arginine, glycine and methionine (Ribeiro et al. 2021). The storage of creatine occurs mainly in skeletal muscle, corresponding to 95%, where it remains as a free (40%) or a phosphorylated creatine (60%). Creatine is also found in the brain, liver, kidneys and testicles (Persky and Brazeau 2001; Mendes and Tirapegui 2002). In addition to creatine synthesis in the body, the food provide about 1 gram of creatine/day, mainly through the consumption of animal products, such as beef and fish (Greenhaf et al. 1994; Engelhardt et al. 1998). The average daily requirement is 2 grams/day: 1 gram from endogenous production and 1 gram obtained from the diet (Maughan et al. 2004; Calfee and Fadale 2006; Alves and Lima 2009). Creatine monohydrate (CrM) is considered as a standard for comparison to other creatine derivatives because of its well-known

physiochemical properties, high bioavailability, stability, low cost, and a large number of studies that have investigated its efficacy and safety (Kreider et al. 2017).

The mechanism of action of creatine is based on effects that trigger improvement in muscle energy metabolism, in which phosphorylated creatine has the ability to re-synthesize ATP from adenosine diphosphate, thus increasing their deposits (Ahrendt 2001; Preen et al. 2001; Kreider et al. 2017). Another beneficial effect of the creatine intake is the increase of the size of muscle fibers, as well as lean body mass, since there is increased protein synthesis and decreased catabolism. Creatine also prevents tissue damage, as it develops mechanisms of cellular membrane stabilization and ATP maintenance (Balsom et al. 2004; Gualano et al. 2010; Baracho et al. 2015). Due to these characteristics, creatine has become very popular among athletes to enhance muscle performance and muscle mass. For this purpose, creatine is usually taken at 20 g/day for one week as a loading dose and at 5–10 g/day as a maintenance dose during extended periods of training. According to numerous publications, this scheme is well tolerated and does not cause significant side effects. Slight gastrointestinal discomfort or muscle cramping were reported. The examination of liver and kidney functions of healthy athletes didn't indicate adverse effects on these organs (Mertschenk et al. 2001).

In the study where rats that have been supplemented with supraphysiological doses of creatine (5 g/kg bw/day for 1 week, thereafter 1 g/kg bw/day for 3 to 7 weeks; equivalent to 350 g/day and 70 g/day for a 70-kg adult) had higher plasma levels of alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), g-glutamyltransferase (GGT) and alkaline phosphatase (AP) and demonstrated some structural changes indicating hepatic damage. Interestingly, creatine supplementation in combination with physical activity decreased the levels of liver enzymes (Souza et al. 2009; Souza et al. 2013).

In our study we revealed a data about the toxicity of a newly synthesized creatine lysinate (CrLys) and its influence on some biochemical parameters such ASAT, ALAT, creatine kinase (CK) and lactate after 2 weeks of administration.

Materials and methods

Chemicals and reagents

CrLys (Mw – 277.32 g/mol) was synthesized and provided by Prof. Lyubomir Vezenkov from the Department of Organic Chemistry at the University of Chemical Technology and Metallurgy - Sofia (Patent for invention N 66 511, Creatine salts, LT Vezenkov, PT Angelov, LL Vezenkov, published in Bulletin 11 on 30.11.2015). The solubility of creatine lysinate in water is 3.3%. Creatine monohydrate (Biogame Co.) was used as standard for comparison. CrM (Mw –149.15 g/mol) is well-known food supplement with solubility in water about 1.4% at 20 °C (Jäger et al. 2011).

Determination of acute toxicity

Lethal dose 50 (LD50) is one of the ways to measure the short-term toxic potential (acute toxicity) of the substances. LD50 values can be compared using toxicity scales. The most commonly used in practice are the Hodge & Sterner Scale and the Gosselin, Smith & Hodge Scale (Hodge and Sterner 2005). The solutions are prepared *ex tempore*. The substances were weighed on an analytical balance, and then the specified amount of saline or water were added to obtain the required concentration. The substances were administered intraperitoneally (i.p.) or orally (p.o.) in an amount of 0.1 mL/10 g body weight (b.w.) to male albino mice, line H with body weight 28–32 g, aged 8 to 9 weeks. The experiments were performed at the same time of the day in order to avoid the influence of circadian rhythms. After administration, the animals were observed for behavioral changes and lethal outcome for 7 days. During this time, they had a free access to food and water. LD50 values are presented as mg/kg b.w.

Animals and treatment

Male albino mice, line H with body weight 28–32 g were divided into five groups of six animals per 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 are divided into five groups of six animals (n=6) 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 the 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 latest experiments are subject of our next publication. The experiments were 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 Local Animal Care Ethics Committee (№ 329/01.06.2022).

Evaluation of blood biochemical parameters

After 2 weeks of administration of creatine derivatives, the blood samples were collected via decapitation in tubes containing dipotassium-ethylenediaminetetraacetic acid (K2-EDTA). For the biochemical analysis the blood was centrifuged for 5 minutes at 7000 rpms. Afterwards the

serum was analyzed on Mindray BS-120 biochemistry analyzer, counting the following parameters: ALAT, ASAT and CK. Lactate was measured with StatStrip Lactate Xpress Meter.

Statistical analysis

LD50 values were determined with the Origin computer program by the method of Litchfield and Wilcoxon (Litchfield and Wilcoxon 1949). Statistical processing of the obtained results was done with the program GraphPad Prism 6.0. The arithmetic mean and the standard errors of the arithmetic mean (SEM) were determined for the biochemistry 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 was considered statistically significant. For graphical presentation of the data, Microsoft Office Excel 2019 was used.

Results

Toxicity data

CrM and CrLys were administered i.p. and p.o. in order to establish LD50. The results for the p.o. toxicity are presented in Table 1.

Table 1. Data on the toxicity of creatine monohydrate (CrM) and creatine lysinate (CrLys) after p.o. administration.

Compound	Administered dose p.o. (mg/kg b.w.) and the survival percentage	
	8000 mg/kg	10 000 mg/kg
CrM	0/6 (100%)	0/6 (100%)
CrLys	0/6 (100%)	–

For CrLys, the signs of toxicity appeared after the i.p. administration of 4000 mg/kg (percentage of survival 75%) and the percentage of survival decreased to 26.7% at 5000 mg/kg (Fig. 1). LD50 after i.p. administration of CrLys was calculated as 4543 mg/kg b.w., and the LD50 was not reached after p.o. administration of 8000 mg/kg. With CrM, the LD50 cannot be reached after i.p. and p.o. administration of 7500 mg/kg and 10 000 mg/kg, respectively (Table 1 and Fig. 1).

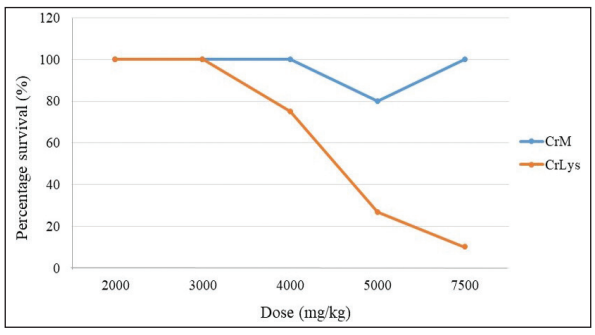

Figure 1. Data on the toxicity of creatine monohydrate (CrM) and creatine lysinate (CrLys) after i.p. administration.

Biochemical analysis after 2 weeks of administration of creatine derivatives

On Table 2 are shown the results from the biochemical analysis of the blood samples. Creatine derivatives decreased the level of CK and increased the level of ASAT and ALAT. After 2 weeks of administration, CrM at a lower dose (1.5 g/kg/day) and CrLys at a higher dose (6 g/kg/day) decreased to a greatest extent ($p \leq 0.0001$) the CK level in comparison to the control group. In the case of liver transaminases, CrM at a dose of 1.5 g/kg/day ($p \leq 0.01$) and 3 g/kg/day ($p \leq 0.05$) increased ASAT and CrLys at a dose of 3 g/kg/day ($p \leq 0.01$) increased both ASAT and ALAT after 2 weeks of supplementation in comparison to the control group.

Discussion

In our study, we examined the toxicity and the influence of newly synthesized CrLys on the biochemical parameters in mice after 2 weeks of administration. Our results revealed that CrLys has lower LD_{50} value in mice (4543 mg/kg after i.p. administration) than the standard CrM, but nevertheless the value is above 4000 mg/kg and CrLys cannot be classified as a toxic substance according to the Hodge and Sterner Toxicity Scale (Hodge and Sterner 2005). There is a large amount of data about the standard CrM from various tests like an Ames test, an *in vitro* micronucleus test, a mammalian cell gene mutation assay, acute dermal, p.o. and i.p. toxicity and a 28-day oral toxicity test. The Ames test had been performed with the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 100 and TA 98 and concentrations of CrM ranged from 100 to 5000 µg/plate. Testing was conducted with and without metabolic activation. Under these conditions, CrM did not result in greater than normal revertant colony counts and is therefore not mutagenic (Mertschenk et al 2001). The *in vitro* micronucleus test was performed using human peripheral lymphocytes for treatment periods of 4 and 18 hours at concentrations of CrM from 190 to 1490 µg/ml. No cytotoxic effects were observed at any of the experimental settings. In this *in vitro* experiment, CrM was considered as "not genotoxic" because wasn't observed formation of micronuclei in human lymphocytes (AlzChem Trostberg GmbH (2020) Creatine monohydrate - Creapure. GRAS notification. p. 47. https://www.fda.gov/media/143525/download).

In the acute toxicity studies performed by Mertschenk et al. (2001), groups of male and female animals (Wistar rats for oral toxicity and Swiss CD 1 mice for i.p. toxicity) received 2000 mg/kg b. w. of CrM as an aqueous solution by stomach tube or by i.p. application. All animals survived the treatment without any signs of toxic effects for the observation period of 15 days. The oral LD_{50} (rat) and the i.p. LD_{50} (mouse) were greater than 2000 mg/kg body weight. No signs of skin irritation were detected after the 4-hour skin application of 500 mg CrM on New Zealand white rabbits. The subacute toxicity trial of the same authors was

Table 2. Biochemical analysis after 2 weeks of administration of creatine derivatives. Results are presented as mean ± SEM. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$ – statistical difference between the creatine derivatives at different doses and the control group.

Group	ASAT U/L	ALAT U/L	CK U/L	Lactate, mmol/L
Control group	195.70 ± 14.72	57.65 ± 5.61	3724 ± 400.5	6.96 ± 0.69
CrM 1.5 g/kg/day	332.30 ± 29.18 **	74.10 ± 4.20	828.8 ± 157.9 ****	7.44 ± 1.02
CrM 3 g/kg/day	307.60 ± 22.91*	73.58 ± 5.59	1311 ± 138.5 ***	6.98 ± 0.60
CrLys 3 g/kg/day	360.80 ± 26.36 **	87.40 ± 5.95 **	1833 ± 419.8 **	7.24 ± 0.94
CrLys 6 g/kg/day	286 ± 19.18	60.15 ± 3.24	1086 ± 112 ****	6.15 ± 1.21

conducted with p.o. administration of CrM in increasing doses up to 2000 mg/kg body weight for 28 days in Wistar rats. The treatment was tolerated without any evident signs of toxicity and autopsy of the sacrificed animals did not reveal any remarkable macroscopic and microscopic changes. Therefore 2000 mg/kg body weight/day was considered by the authors as the No Observed (Adverse) Effect Level (NO(A)EL) (Mertschenk et al. 2001).

Ipsiroglu et al. (2001) stated that guinea pigs, mice and rats fed with 1.3 to 2 g/kg/day of CrM for a period of 2 to 8 weeks showed significant augmentation of total tissue creatine concentrations and no negative effects on body weight of the animals. Sartini et al. (2016) investigated the effects of creatine administration in pregnant rats (1 g/100 mL in drinking water from the 11-th day of the pregnancy until delivery) on the development of hippocampal neurons in the rat offspring. Indicators as survival rate, weight at birth and the size of the litter were unaffected by creatine supplementation, and no teratogenic effect was observed in the creatine-supplemented group.

In our study after 2 weeks of administration, some of the creatine derivatives (CrM 1.5 g/kg/day, CrM 3 g/kg/day and CrLys 3 g/kg/day) showed increased levels of ASAT when compared with the control group. There is also an elevation in another transaminase – ALAT, especially in the group treated with CrLys 3 g/kg/day in comparison to the control group. Furthermore, there were significant lower levels of CK in the groups treated with creatine derivatives at both doses in comparison to the control group.

Souza et al. (2009) evaluated the effects of creatine supplementation on kidney and liver in sedentary and exercised rats. They demonstrated that at eight weeks, the creatine group showed increased levels of ALAT, ASAT, GGT and AP also when compared with all others groups. The creatine group also demonstrated some structural alterations that indicate renal and hepatic damage at four and eight weeks, respectively. Based on these results, they suggest that long-term creatine supplementation (4–8 weeks) may adversely affect kidney and liver structure and function of sedentary but not of exercised rats. The presence of CK in the blood is generally considered to be an indirect marker of muscle damage, especially for the diagnosis of medical conditions such as myocardial infarction, muscular dystrophy and brain disease or after exercise. Elevated serum CK levels are still closely associated with cell damage, muscle cell destruction or disease. Our results revealed that there were significant lower levels of CK in the groups treated with creatine derivatives in comparison to the control group. Cooke et al. (2009) found that there was significantly higher muscle strength after creatine supplementation during recovery from a muscle damaging exercise session. While this may be due in part to a faster muscle growth during the recovery period, significantly lower plasma CK activity in the days after injury is indicative of lower muscle damage.

In our study, the blood lactate concentration of the groups treated with creatine derivatives didn't change significantly in comparison to the control group. The lowest lactate level was registered in the group, supplemented with CrLys at a dose of 6 g/kg/day – 6.15 vs. 6.96 mmol/L in the control group. Ceddia and Sweeney (2004) supplemented the incubation medium of L6 rat skeletal muscle cells with 0.5 mM l^{-1} of creatine, which corresponds to the concentration reached in humans after a single oral dose of 5 g creatine. According to their protocol, in the presence of insulin, the L6 myoblasts induced a significant increase in lactate production compared to control cells. They concluded that creatine supplementation did not alter the insulin-stimulated lactate production by the cells but reduced the basal production of lactate by ~42%.

Conclusion

The major finding that can be reported from this research is related to the low toxicity of a newly synthesized CrLys. After 2 weeks of administration of creatine derivatives, an increase in the levels of liver transaminases and decreased level of creatine kinase were detected. Our next article will be focused on the effect of newly synthesized creatine derivatives on the tail suspension test and rotarod test and influence on the histology of the skeletal muscles.

Authors' contributions

Conceptualization, I.K. and N.D.; methodology, I.K., N.D.; investigation, I.K., B.L., L.M., I.I. and D. T.; writing—original draft preparation, I.K.; writing—review and editing, I.K., N.D.; 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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References

Ahrendt DM (2001) Ergogenic aids: counseling the athlete. American family physician 63(5): 913–922. [PMID: 11261867]

Alves C, Lima RVB (2009) Dietary supplement use by adolescents. Jornal de Pediatria 85(4): 287–94. https://doi.org/10.1590/S0021-75572009000400004

AlzChem Trostberg GmbH (2020) Creatine monohydrate - Creapure. GRAS notification. p. 47. https://www.fda.gov/media/143525/download

Balsom PD, Söderlund K, Ekblom B (1994) Creatine in humans with special reference to creatine supplementation. Sports medicine (Auckland, N.Z.) 18(4): 268–280. https://doi.org/10.2165/00007256-199418040-00005

Baracho NC, Castro LP, Borges Nda C, Laira PB (2015) Study of renal and hepatic toxicity in rats supplemented with creatine. Acta Cirurgica Brasileira 30(5): 313–318. https://doi.org/10.1590/S0102-865020150050000002

Calfee R, Fadale P (2006) Popular ergogenic drugs and supplements in young athletes. Pediatrics 117(3): e577–e589. https://doi.org/10.1542/peds.2005-1429

Ceddia RB, Sweeney G (2004) Creatine supplementation increases glucose oxidation and AMPK phosphorylation and reduces lactate production in L6 rat skeletal muscle cells. The Journal of physiology 555(Pt 2): 409–421. https://doi.org/10.1113/jphysiol.2003.056291

Cooke MB, Rybalka E, Williams AD, Cribb PJ, Hayes A (2009) Creatine supplementation enhances muscle force recovery after eccentrically-induced muscle damage in healthy individuals. Journal of the International Society of Sports Nutrition 6: 13. https://doi.org/10.1186/1550-2783-6-13

Engelhardt M, Neumann G, Berbalk A, Reuter I (1998) Creatine supplementation in endurance sports. Medicine and Science in Sports and Exercise 30(7): 1123–1129. https://doi.org/10.1097/00005768-199807000-00016

Greenhaf PL, Bodin K, Soderlund K, Hultman (1994) Effect of oral creatine supplementation on skeletal muscle phosphocreatine resynthesis. The American journal of physiology 266(5 Pt 1): E725–E730. https://doi.org/10.1152/ajpendo.1994.266.5.E725

Gualano B, Acquesta FM, Ugrinowitsch C, Tricoli V, Serrão JC, Lancha Junior AH (2010) Effects of creatine supplementation on strength and muscle hypertrophy: current concepts. Revista Brasileira de Medicina do Esporte 2010 16(3): 219–23. https://doi.org/10.1590/S1517-86922010000300013

Hodge A, Sterner B (2005) Toxicity Classes. In: Canadian Center for Occupational Health and Safety.

Ipsiroglu OS, Stromberger C, Ilas J, Höger H, Mühl A, Stöckler-Ipsiroglu S (2001) Changes of tissue creatine concentrations upon oral supplementation of creatine monohydrate in various animal species. Life sciences 69(15):1805–1815. https://doi.org/10.1016/s0024-3205(01)01268-1

Jäger R, Purpura M, Shao A, Inoue T, Kreider RB (2011) Analysis of the efficacy, safety, and regulatory status of novel forms of creatine. Amino Acids 40(5): 1369–1383. https://doi.org/10.1007/s00726-011-0874-6

Kreider RB, Kalman DS, Antonio J, Ziegenfuss TN, Wildman R, Collins R, Candow DG, Kleiner SM, Almada AL, Lopez HL (2017) International Society of Sports Nutrition position stand: safety and efficacy of creatine supplementation in exercise, sport, and medicine. Journal of the International Society of Sports Nutrition 14: 18. https://doi.org/10.1186/s12970-017-0173-z

Litchfield Jr JT, Wilcoxon F (1949) A simplified method of evaluating dose-effect experiments. The Journal of pharmacology and experimental therapeutics 96(2): 99–113. [PMID: 18152921]

Maughan RJ, King DS, Lea T (2004) Dietary supplements. Journal of Sports Sciences 22: 95–113. https://doi.org/10.1080/0264041031000140581

Mendes RR, Tirapegui J (2002) Creatine: the nutritional supplement for exercise - current concepts. Archivos Latinoamericanos de Nutrición 52(2): 117–27. [PMID: 12184144]

Mertschenk B, Gloxhuber C, Wallimann T (2001) Health assessment of creatine as dietary supplement. Deutsche Lebensmittel-Rundschau 97(7):250–257.

Persky AM, Brazeau GA (2001) Clinical pharmacology of the dietary supplement creatine monohydrate. Pharmacological reviews 53(2): 161–76. [PMID: 11356982]

Preen D, Dawson B, Goodman C, Lawrence S, Beilby J, Ching S (2001) Effect of creatine loading on long-term sprint exercise performance and metabolism. Medicine and science in sports and exercise 33(5): 814–821. https://doi.org/10.1097/00005768-200105000-00022

Ribeiro F, Longobardi I, Perim P, Duarte B, Ferreira P, Gualano B, Roschel H, Saunders B (2021) Timing of creatine supplementation around exercise: A real concern? Nutrients 13(8): 2844. https://doi.org/10.3390/nu13082844

Sartini S, Lattanzi D, Ambrogini P, Di Palma M, Galati C, Savelli D, Polidori E, Calcabrini C, Rocchi MB, Sestili P, Cuppini R (2016) Maternal creatine supplementation affects the morpho-functional development of hippocampal neurons in rat offspring. Neuroscience 312: 120–129. https://doi.org/10.1016/j.neuroscience.2015.11.017

Souza RA, Miranda H, Xavier M, Lazo-Osorio R A, Gouvea HA, Cogo JC, Vieira RP, Ribeiro W (2009) Effects of high-dose creatine supplementation on kidney and liver responses in sedentary and exercised rats, Journal of sports science & medicine 8(4): 672–681.

Souza WM, Heck TG, Wronski EC, Ulbrich AZ, Boff E (2013) Effects of creatine supplementation on biomarkers of hepatic and renal function in young trained rats. Toxicology mechanisms and methods 23: 697–701. https://doi.org/10.3109/15376516.2013.843108