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Research Article

Evaluation of the Effect of Clonapure® Versus Creatine Monohydrate on ATP Levels in a C2C12 Muscle Cell Line

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Abstract

Background: Creatine is one of the most widespread dietary supplements among athletes and weightlifters. It is most commonly administered as creatine monohydrate, which transforms into phosphocreatine once within the muscle, which leads to the production of adenosine triphosphate (ATP). Clonapure® is a functional ingredient which consists of a combination of creatine monohydrate, phosphate salt and phosphocreatine. It is believed that Clonapure® has an impact on muscular ATP synthesis due to its phosphocreatine content. A comparative in vitro study was carried to evaluate the potential effect of Clonapure® on ATP synthesis versus creatine monohydrate. *Methods:* A C2C12 myoblast cell line finally differentiated into myotubes was used. Bioaviability of samples was measured at 24h through biocompatibility studies. Myotubes were also exposed to samples at different concentrations for 2h and intracellular ATP levels were evaluated througha ergular validated luminescence methos. Glucose was used as positive control. *Results:* The increase in intracellular ATP levels induced by Clonapure® was 10% higher than the caused by creatine monohydrate and glucose, which both showed similar effects in ATP synthesis. The combination of creatine-phosphate phosphate and creatine monohydrate contained in Clonapure® triggers the intracellular ATP production cascade more efficiently in muscle cells. Dose response analysis revealed that Clonapure® supplementation could boost conveniently physical activities related to endurance and performance.

Keywords

Creatine Monohydrate, Clonapure®, Phosphocreatine, ATP, Supplementation, Endurance, Exercise, Performance, Muscle Cells, Phosphogen System

1. Introduction

Creatine is one of the most widespread dietary supplements. The Office of Dietary Supplements at the National Institutes of Health, professional organizations and athletic governing bodies recognize creatine as effective and safe to improve sports endurance and performance, enhancing exercise training adaptation and diminish recovery time. [1-6]

Creatine gained popularity in the 1990s with the publication of several studies demonstrating that its supplementation en-

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hances high-intensity exercise performance by increasing phosphocreatine in muscle. During the 1992 Olympics, some athletes began using creatine supplementation. [7] Today, it is one of the most popular and extensively studied supplements among athletes and weightlifters.

It is a nitrogenous amine that can be synthesized endogenously by the liver, kidney and pancreas [8-10] from amino acids including arginine, methionine and glycine. [11] Creatine can also be obtained from exogenous sources, such as animal products like fish and red meat or through supplementation. Endogenous production is influenced and regulated by exogenous administration. [10]

Creatine most common supplemented source is creatine monohydrate (CRM). Its ability to enhance muscular performance is attributed to the phosphocreatine source, supporting the production of adenosine triphosphate (ATP), also known as phosphagen shuttle.

Once inside the muscle cells, creatine is phosphorylated to form phosphocreatine in a reversible enzymatic reaction catalyzed by creatine kinase. In this process, the phosphate group is donated by ATP, resulting in adenosine diphosphate (ADP). The reverse reaction occurs when phosphocreatine donates a phosphate group to ADP, thereby releasing ATP within the muscle cell. (Figure 1)

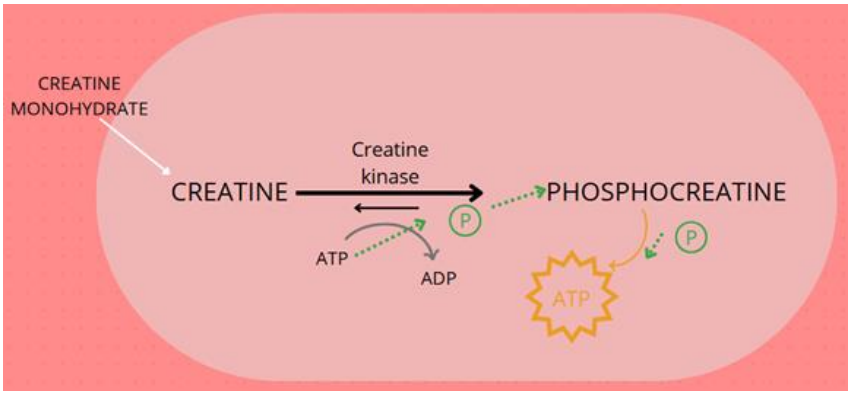

Figure 1. *Synthesis of phosphocreatine from CRM.*

Approximately 95% of total creatine stores are located in skeletal muscle, distributed by transporters across the cell membrane. [10] A small proportion is found in other tissues, such as the brain, bones and heart. [12]

The International Society of Sports Nutrition has considered creatine as the most effective supplement for increasing training-based gains in lean body mass. Several meta-analyses [13-16] have demonstrated that, compared to placebo, creatine supplementation significantly augments muscle mass, lean body mass and fat-free mass, as well as it enhances strength, even in resistance exercise training. [14, 17] Creatine supplementation rises muscle stores, therefore increasing phosphocreatine content by approximately 20-40%. [7, 17] Additionally, evidence suggests that creatine reduces the risk of injury. [18] Therefore Creatine is considered an optimal ingredient for athletes due to its capacity to increase ATP levels in muscular cells rapidly.

In spite of these relevant findings and benefits on sports, CRM supplementation did not show significant benefits on some intermittent sports as volleyball, in which active phases last seconds during intervals. [19-21]

Creatine is safe and well-tolerated. Not serious side effects have been occasionally reported, including gastrointestinal issues and muscle cramping. Creatine does not affect general health markers or renal function. [17, 22] Few cases of liver side effects has been described. where high doses of creatine combined with other dietary supplements was administrated. [23] At regular dosages, no adverse events on liver function have been reported. [10]

The effective dosage for CRM studied includes an initial loading phase lasting 5 to 7 days, which is believed to rapidly increase and saturate intramuscular stores of total creatine and phosphocreatine. This loading phase involves daily doses of 20 g. Following this initial phase, a maintenance phase is implemented, which consists of a dose of 0.03 g/kg per day. [10]

Clonapure® (CLP) is a functional ingredient which consists of a combination of CRM, phosphate salt and phosphocreatine (creatine-phosphate). Potentially, CLP can be effective from the first dose, which is attributed to its phosphocreatine content, acting directly in the muscles and boosting the muscle phosphogen system where creatine and ATp are directly involved.

A comparative study was conducted to evaluate the potential effect of CLP vs CRM on ATP synthesis on a C2C12 muscle cell line.

2. Materials and Methods

2.1. Cell Line

The C2C12 cell line, a widely used mouse myoblast cell line, plays a crucial role in understanding energy metabolism

and muscle differentiation. These cells are known for their ability to differentiate into myotubes and are often used to study ATP production and regulation. Myotubes are the basic unit of skeletal muscle and play a crucial role in muscle function. C2C12 cells are frequently used as a model system to study muscle development, differentiation, and the impact of various factors on energy metabolism and ATP production.

The C2C12 (ATCC, CRL-1772) mouse myoblast cell line was cultured in DMEM High Glucose with L-glutamine and sodium pyruvate (Capricorn). The medium was supplemented with 10% foetal bovine serum (FBS) (Biowest) and 1% penicillin/streptomycin. Cells were maintained at 37 ℃ in a humidified atmosphere with 5% CO_2, following standard protocols.

To induce differentiation into myotubes, C2C12 cells were cultured in differentiation medium: DMEM supplemented with 1% FBS and 1% penicillin/streptomycin. Upon reaching 80-90% confluence, the serum concentration was reduced to 1% FBS and cells were maintained in this medium for 5 days, with medium renewal every 48 hours.

2.2. Samples

Three different samples were measured:

1. Clonapure® (CLP) was kindly provided by Florida Human Nutrition, a Tradichem group Company.
2. Creatine monohydrate (CRM), synthetically derived and with 99.8% purity.
3. Phosphate (Dicalcium phosphate dihydrate): Prayphos™ DCPD 308 SP FG.

Stock solutions were prepared by dissolving the samples in culture medium and filtering them through a 0,2 µm filter prior to the use. The pH was adjusted to 7. The assay dilutions (1 mM, 500 µM and 100 µM) were prepared from the stock solutions.

2.3. Studies of Biocompatibility

Viability of samples was evaluated in C2C12 cell line. Cells were seeded in 96-well plates and incubated with 6 different concentrations of the samples at 37 ℃ and 5% CO_2. DMSO was included as a positive control of death. Cell viability was evaluated after 24 hours of incubation using a fluorometric assay with AlamarBlue™ Cell Viability Reagent (LifeTechnologies). Fluorescence was measured at λ excitation = 540 nm and λ emission = 590 nm using a Fluoroskan FL plate spectrofluorimeter. Viability was calculated with the following formula:

$$\% \text{ Viability} = (\text{Fluorescence units' sample} / \text{Fluorescence units' control}) \times 100$$

Samples are considered biocompatible when there is a minimum of cellular viability of 80%.

At 24h, two assays were conducted to measure percentage of cell viability versus control cells.

2.4. Studies of Intracellular ATP Levels

Intracellular ATP levels were measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega), following the manufacturer's instructions. Briefly, C2C12 were seeded in a 96-well plate. After myogenic differentiation, myotubes were exposed to different concentrations of the test compounds (500 µM and 1 mM) for 2 hours. Following treatment, the plate was equilibrated to room temperature for 30 minutes. An equal volume of CellTiter-Glo Reagent was then added to each well, and the content was mixed for 2 minutes on an orbital shaker to ensure cell lysis. Luminescence was subsequently measured using a luminometer (Fluoroskan FL (Thermo)), and the values were normalized to the untreated control, which was set as 1. Glucose 20 mM was used as positive control.

At a contact time of 2h, two assays were conducted to measure ATP levels in myotubes versus control cells.

2.5. Statistical Analysis

Results were expressed as the mean and standard error of the mean (SEM) from two independent experiments, each performed in duplicate in both studies. Statistical analysis was carried out using Student's t-test to compare each treatment condition against the untreated control. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Biocompatibility

In order to evaluate the toxicity of the samples in C2C12 cells, a biocompatibility assay was carried out. To consider the sample as not-toxic, 80% of cellular viability was the minimum necessary. All samples at any range concentration maintained the cellular viability above 80% at 24h of incubation, confirming their biocompatibility under the conditions used, showing the lack of toxicity of CRM and CLP at the range of concentrations tested on C2C12 muscle cells.

3.2. Intracellular ATP Levels

Relative ATP levels in cells treated with the different samples and untreated cells were measured. D-glucose (20 mM) and phosphate were included as positive controls.

An increased ATP response was observed at shorter exposure times; therefore, a contact time of 2 hours was established for analysis. Because lack of cytotoxicity was observed at 24h (Figure 2), the cellular viability remained unaffected at the contact time of 2h.

Results showed that CLP significantly increase intracellular ATP levels at concentrations of 500 µM and 1 mM, more efficiently than CRM. Phosphate was used as control, revealing a modarate increase on ATP levels.

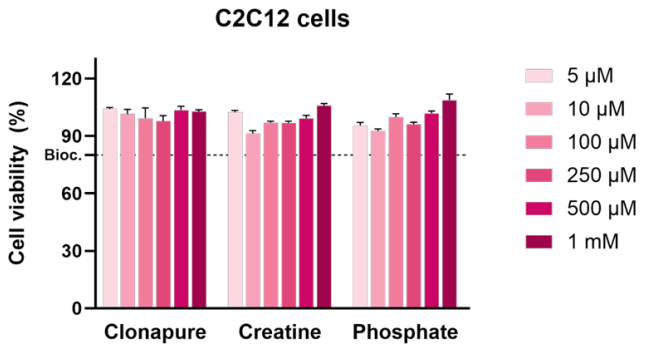

Figure 2. *Biocompatibility assay results at 24h.*

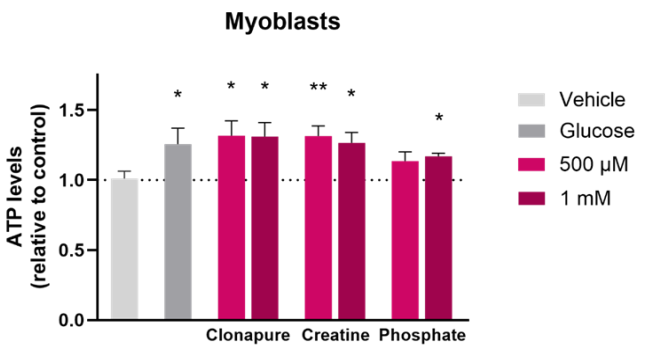

Figure 3. *Relative ATP levels in C2C12 myotubes after treatment with CLP, CRM and phosphate at two concentrations (500 µM and 1 mM). D-glucose was used as a positive control. The dotted line represents the mean ATP level of the untreated control (vehicle). Data are expressed as mean ±SEM. *p < 0.05.*

Table 1. *Relative ATP levels at different concentrations (4 assays conducted) (CLP).*

Concentration	Relative levels of ATP at 2h (CLP)	Mean	Sd
500 µM	1.245		
500 µM	1.631		
500 µM	1.190		
500 µM	1.200	1.317	0.2110
1 mM	1.513		
1 mM	1.446		
1 mM	1.15		
1 mM	1.13	1.310	0.1981

Table 2. *Relative ATP levels at different concentrations (4 assays conducted) (CRM).*

Concentration	Relative levels of ATP at 2h (CRM)	Mean	Sd
500 µM	1.304		
500 µM	1.517		
500 µM	1.17		
500 µM	1.26	1.313	0.1471
1 mM	1.353		
1 mM	1.426		
1 mM	1.17		
1 mM	1.11	1.265	0.1491

At 500 µM and 1mM concentrations CRM increases ATP levels by a similar percentage as glucose. Interestingly, CLP exceeds ATP levels, showing a 10% increase compared to CRM and glucose.

3.3. Dose Response

The dose–response relationship, or exposure–response relationship, describes the response of an organism caused by differing levels of exposure (or doses) to a stimulus or stressor (usually a chemical) after a certain exposure time. [24]

Figure 4. represents muscle cell response related to ATP synthesis after the exposure to CRM and creatines from CLP.

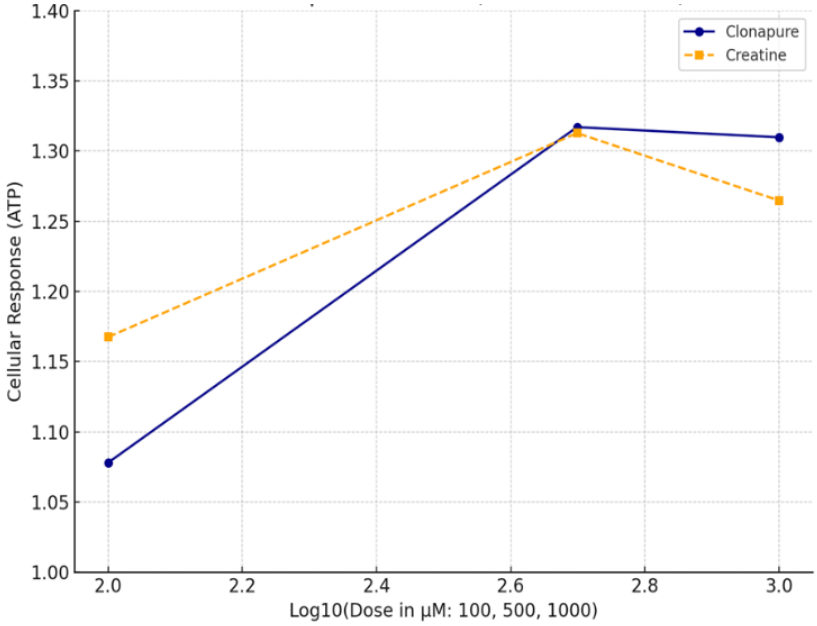

Figure 4. *Dose response plot.*

4. Discussion

Creatine is considered as a safe and effective supplement for all ages. [1, 7] A systematic review and meta-analysis by Desai et al. (2024) reported that creatine supplementation augments muscle mass induced by resistance training, reduces body fat percentage and increases cellular water content, leading to an increased expression of genes involved in protein synthesis, potentially contributing to lean body mass growth over time. [16, 25] Similarly, Burke et al. demonstrated that creatine supplementation promotes skeletal muscle hypertrophy when combined with resistance training, with similar effects observed in both younger and older adults. [26] Additionally, Mielgo-Ayuso et al. showed that creatine supplementation, at recommended doses, leads to significant improvements in anaerobic performance. [27]

Beyond sports endurance and performance, creatine has also demonstrated to ameliorate and decrease the risk of diverse health conditions. Evidence showed enhancements in female reproductive health, reducing the risk of reproductive disorders. [28] Additional studies suggested that creatine may reduce the risk of cardiovascular disease and diverse types of cancer. [29-33]

Creatine supplementation has also shown positive effects on preventing bone loss, enhancing functional capacity of osteoarthritis and fibromyalgia [12, 34-36] and improving cognitive and brain function, reducing mental fatigue. [37] Additionally, creatine has also been proven to serve as an antioxidant. [30, 38]

CLP is an ingredient containing two different forms of creatine plus and phosphate salt. CLP increases ATP production in muscular cells in a significantly larger percentage compared to CRM at 1mM (Figure 3. ($p< 0,05$)). Moreover, data obtained from the dose-response plot reveal that the sum of creatines contained in CLP exerts a similar effect on ATP synthesis compared to CRM. Therefore, CLP appears to be significantly more effective in the production of ATP in muscle cells. Consequently, CLP can participate more efficiently on the physiology of endurance and performance. Further human interventions are needed to confirm this data.

The combination of creatine-phosphate and CRM triggers intracellular ATP production effectively. Creatine phosphate potentially stimulates ATP production in muscle cells, while CRM can be considered a reservoir of creatine that is posteriorly converted into creatine phosphate es. This might be due to the combination of creatine monohydrate, phosphate, phosphocreatine, which could exert a synergic effect at specific concentrations. Dose-response analysis (Figure 4) show that CLP has a more efficient effect on the muscle cells phospagen system providing a rapid source of energy by utlizing creatine phosphate to replenish ATP fueling muscle contractions.

Abbreviations

CRM	Creatine Monohydrate
CLP	Clonapure

Author Contributions

Tamara Lapena-Luzon: Methodology, Investigation, Data curation, validation

Alejandra Hernandez-Bueno: Formal analysis, writing, validation

Lidia Tomas-Cobos: Data curation, Methodology, Validation

Patricia Moreno-Puente: Conceptualization, Formal Analysis, Methodology

Sandra Garcia-Benlloch: Investigation

Laura Soriano Romani: Investigation

José Angel Maranon: Conceptualization, Formal Analysis, Methodology, writing, validation

Data Availability Statement

The data is available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

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