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Case Report

Clonapure® vs Creatine Monohydrate on ATP Synthesis in a Human Neuron Cell line

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Abstract

The brain is a highly energetic complex organ, consuming 20% of total resting energy despite representing only about 2% of total body mass. Beyond sports endurance and muscle, existing evidence suggests that creatine supplementation may also improve brain function, particularly under conditions where cellular bioenergetics are compromised, such as metabolic stress, sleep deprivation, or certain diseases. We conducted a study using human dopaminergic neuronal cells to evaluate the effect of Clonapure®, versus standard creatine monohydrate supplementation. Cells were cultured in a medium containing D-galactose instead of glucose to force neurons to rely on mitochondrial ATP synthesis via oxidative phosphorylation, thereby inducing metabolic stress and decreasing energy production.

Clonapure®, even at its lowest doses, significantly enhanced neuronal ATP levels, with results notably higher than creatine monohydrate. Creatine monohydrate was not able to stimulate ATP synthesis at the same levels, even at higher concentrations. We hypothesize that Clonapure® increases energy production more efficiently due to its phosphocreatine content, which donates phosphagen immediately to support ATP synthesis, and to a possible synergistic effect between creatine monohydrate, phosphate salts and phosphocreatine. These results in compromised cells may be extrapolated to conditions demanding higher ATP levels, including altered brain energetics and mitochondrial dysfunction.

Keywords: Creatine, Clonapure®, Phosphocreatine, ATP, brain bioenergetics, SLC6A8, central nervous system, neurons, cognition.

Introduction

Creatine supplementation has been widely used to improve sports endurance and performance for many years with robust evidence behind. Its main mechanism of action is to immediately provide adenosine triphosphate (ATP) in muscle cells through phosphocreatine (PCr) in energy demands. Muscle consumes a big amount of energy via ATP.

The brain is a highly energetic complex organ, consuming 20% of total resting energy despite accounting for only about 2% of total body mass [1]. Neurons demand a constant supply of ATP for several cellular processes related to normal brain function and cognition, such as synaptic functioning, maintenance of ion gradients and neurotransmitter exocytosis [2].

The creatine transporter CrT1 (creatine transporter type 1 or SLC6A8) is a specific, sodium-dependent and active transporter [3] expressed in diverse cells that facilitates the uptake of creatine from the circulation into tissues [4,5]. Studies in rats have shown that SLC6A8 is expressed, among others, in neurons, oligodendrocytes and microcapillary endothelial cells of the blood-brain barrier (BBB), enabling creatine entry into the central

nervous system (CNS) [6]. Apart from acquiring peripheral creatine from transporters, the brain can endogenously synthesize creatine in a small percentage, such as in the liver, pancreas and kidney [7,8]. The mechanism of synthesis includes the enzyme L-arginine: glycine amidino transferase (AGAT), which uses arginine and glycine as substrates to produce guanidinoacetate. Subsequently, guanidinoacetate methyltransferase (GAMT) converts guanidinoacetate into creatine [3,6].

Because of the partial ability to cross the BBB via microcapillary endothelial cells expressing the creatine transporter SLC6A8, creatine supplementation appears to have limited effects on brain creatine levels in healthy young subjects. Some interventions in human healthy volunteers reported minimal improvements on brain creatine (5-10%) from supplementation with different dosages, such as 20 g/day for 1-4 weeks or 2-5 g/day for 8 weeks [9-11]. A review by Dolan et al. [12] analyzing the effects of supplementation with various dosages reported an increase of 3-10% in brain stores. These findings suggest that under physiological conditions, creatine uptake into the brain may be limited. However, local cellular mechanisms and differential transporter expression, such as the presence of SLC6A8 in neurons but not in astrocytes, may influence distribution and responsiveness to supplementation at the cellular level.

Nevertheless, increasing evidence suggests that creatine intake can improve brain function, primarily under certain conditions or challenges -such as sleep deprivation, stress, depression, aging, or brain injuries-, which could lead to a reduction in brain creatine stores [13-16]. Long-term and high-dose creatine supplementation has been shown to augment brain creatine levels [8] higher than threshold [17].

Moreover, creatine supplementation has demonstrated to enhance cognitive and memory performance, especially in older adults and during periods of metabolic stress, and to decrease symptoms of poor sleep in both human and animal studies [18] Increasing brain creatine content has been associated with improvements in the recovery from traumatic brain injury in both children and adults [8,18]. Additionally, creatine supplementation increased neuronal energy supply in healthy subjects [19,20].

A systematic review by Avgerinos et al. [5] provides full evidence that creatine supplementation improves reasoning, intelligence and short-term memory in older individuals. Recent studies suggest that creatine may act as a neurotransmitter (NT) in the CNS [7,21,22] as it has been detected in synaptic vesicles from the mouse brain, along with classical NT. Creatine concentrations were found to be lower than NT such as GABA and glutamate but higher than acetylcholine and serotonin (5-hydroxytryptamine) [23].

Additionally, creatine may function as a central neuromodulator, due to its interaction with various molecules, including N-methyl-D-aspartate receptor (NMDAr), Na+/K+ ATPase, serotonin receptors and postsynaptic GABA receptors, and to its participation in critical roles of central neurotransmission [24,25]. Creatine acts as an NMDAr antagonist, attenuating glutamate excitotoxicity without side effects [26,27]. Creatine as well appears to reduce the loss of GABAergic interneurons, which may contribute to neuroprotection [28]. Moreover, creatine decreases extracellular glutamate accumulation and excitotoxicity mediated by glutamate through the stimulation of synaptic uptake [29,30].

Creatine has been identified as a neuroprotective factor for dopaminergic neurons against neurotoxicity [31]. Creatine supplementation prevents defects of hippocampal neurogenesis induced by chronic stress, through modulation of the Wnt/GSK3β/β-catenin pathway [32]. Due to the requirements of ATP in cognition, we conducted a comparative study to evaluate the effect of Clonapure® (CLP) and creatine monohydrate (CRM) alone on ATP synthesis in a human neuronal cell model.

CLP is an ingredient composed of a combination of CRM, PCr (creatine-phosphate) and phosphate salt. PCr is the active form of creatine that provides ATP. CLP is believed to efficiently enhance ATP synthesis [33].

Materials and Methods

Samples

- Clonapure® (CLP) Micronized, kindly provided by Florida Human Nutrition, a Tradichem group Company
- Creatine Monohydrate (CRM) is synthetically derived, with 99.8% purity.
- Prayphos™ DCPD 308 SP FG; Dicalcium Phosphate Dihydrate (Phosphate)

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 7.0. The assay dilutions (5 mM, 1 mM, 500 µM and 100 µM) were prepared from stock solutions.

Cell line

SH-SY5Y (ECACC 94030304), a human neuroblastoma cell line, was cultured in a 1:1 mixture of Ham's F12 (Gibco™ 11765054) and EMEM (ATCC 30-2003), supplemented with 2 mM L-glutamine, 1% non-essential amino acids (NEAA), 15% fetal bovine serum (FBS) (Biowest), and 1% penicillin/streptomycin. Cultures were maintained at 37ºC in a humidified atmosphere containing 5% CO_2 under standard conditions.

For neuronal differentiation toward a cholinergic phenotype, SH-SY5Y cells were treated with 10 µM retinoic acid (RA) in medium containing 2% FBS for 6 days, with medium renewed every 2-3 days. To further induce dopaminergic-like differentiation, RA was withdrawn, and cells were subsequently exposed to 100 nM

phorbol 12 myristate 13-acetate (PMA) in medium containing 0,5% FBS for an additional 2 days.

Biocompatibility studies

Viability of samples was evaluated in SY-SY5Y cell line. Cells were seeded in 96-well plates and incubated with 6 different concentrations of the samples at 37ºC and 5% CO2. 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 (Life Technologies). Fluorescence was measured at λ excitation = 540 nm and λ emission = 590 nm using a Fluoroskan FL plate spectrofluorometer. Viability was calculated with the following formula:

% Viability = (Fluorescence units' sample / Fluorescence units' control) x 100

Intracellular ATP levels

Intracellular ATP levels were measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega), according to the manufacturer's instructions. SH-SY5Y cells were seeded in 96-well plates and subjected to neuronal differentiation. Cells were first cultivated in a glucose medium; subsequently, cells were incubated for 24 hours in a glucose-deprived medium (DMEM without glucose, supplemented with 10 mM D-galactose, 0,5% FBS, and 1% penicillin/streptomycin) to force cells to rely on mitochondrial oxidative phosphorylation for ATP production [35–38].

Following the deprivation period, cells were exposed to different concentrations of the test compounds (5 mM, 1 mM, 500 µM and 100 µM) for 2 hours. After 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, and the values were normalized to the untreated control, which was set as 1.

Statistical Analysis

Results were expressed as the mean and standard error of the mean (SEM) from two independent experiments; each performed in at least duplicate. 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.

Results

Biocompatibility studies on SH-SY5Y cells

In order to evaluate the toxicity of samples in SH-SY5Y cells for the next ATP studies, a biocompatibility assay was carried out. 80% of viability was established as the minimum necessary for considering the sample non-toxic. Figure 1 shows the results of the biocompatibility assay at 24h of incubation.

The results indicate that all three APIs (CLP, CRM and Phosphate) maintained cell viability above the 80% threshold across all tested concentrations, confirming biocompatibility under the conditions used.

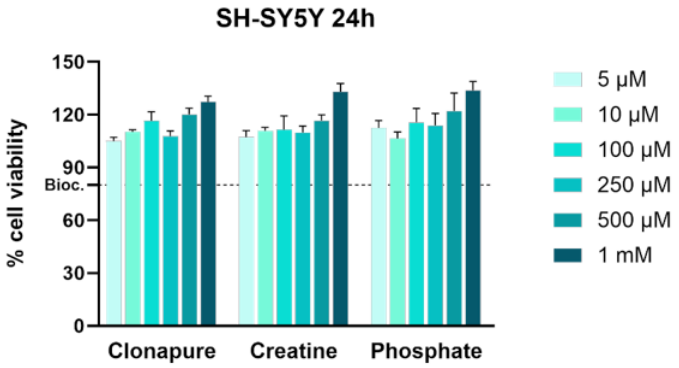


Figure 1: Biocompatibility assay results at 24h. Untreated cells were set as 100% viability. The dashed line indicates the 80% viability threshold, commonly used to define acceptable biocompatibility.

Intracellular ATP levels

Figure 2 shows the relative ATP levels in dopaminergic-like SH-SY5Y cells treated with the APIs at three different concentrations, compared to untreated cells (vehicle).

In the glucose medium, there were no changes in ATP levels. A glucose-free medium was evaluated to observe possible improvements in cell response. Gohil et al. have shown that cells grown in a galactose-containing medium maximize mitochondrial ATP production via mitochondrial respiration in a larger fraction for ATP synthesis [34]. Slow oxidation of galactose to pyruvate does not produce sufficient ATP, thereby promoting cells to rely on oxidative phosphorylation (OXPHOS) to generate ATP to maintain cellular homeostasis.

In this model, CLP significantly increased intracellular ATP synthesis at 100 µM and 500 µM. At the highest tested concentration, slight ATP synthesis was reported, possibly due to a saturation phenomenon or feedback regulatory mechanisms. Notably, CLP elicited a more consistent ATP-boosting effect than CRM alone, which only enhanced ATP synthesis levels significantly at 500 µM.

The ATP synthesis induced by CLP at 100 µM was 11% ($p < 0.05$) higher than basal levels, significantly greater compared to CRM, which increased ATP synthesis by 2.7% relative to basal (Figure 3). In contrast, phosphate did not produce any measurable increase in ATP levels at any of the concentrations tested, confirming that phosphate salts alone do not enhance ATP synthesis.

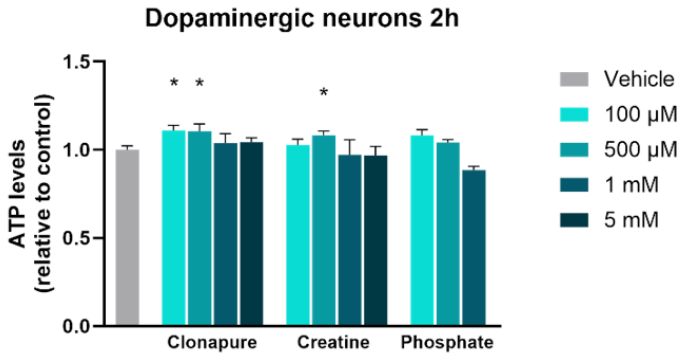
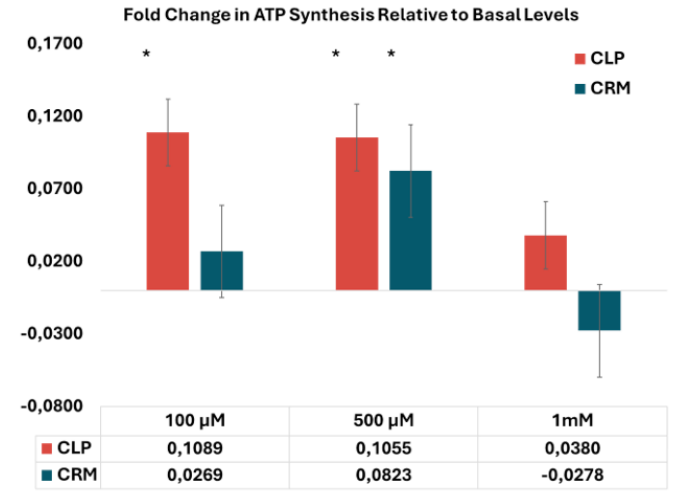


Figure 2: Relative ATP levels in dopaminergic-like SH-SY5Y cells after treatment with Clonapure®, creatine and phosphate at four concentrations (100 µM, 500 µM, 1 mM and 5 mM). Data are expressed as mean ± SEM. *p < 0.05.



	100 µM	500 µM	1mM
CLP	0,1089	0,1055	0,0380
CRM	0,0269	0,0823	-0,0278

Figure 3: Percentage of ATP synthesis in dopaminergic-like SH-SY5Y cells treated with the APIs at three different concentrations, relative to untreated cells (considered as basal levels).

Discussion

There is enough evidence that creatine supplementation has promising effects on muscle cells. Beyond muscle, creatine has also been shown to support brain processes, with proven neuroprotective effects both *In Vitro* and *In Vivo* [18].

Dietary PCr supplementation may influence brain health by supporting energy homeostasis; however, its direct effects on the BBB remain complex and not yet fully understood. While creatine, a component of PCr, is known to cross the BBB with difficulty, its accumulation in the brain after systemic administration is still being researched. Although creatine can be synthesized within the brain, supplementation may be particularly beneficial in conditions involving impaired synthesis or transport function.

Moreover, creatine supplementation is believed to be potentially beneficial for diverse clinical conditions, including neurodegenerative diseases. This is due to the effect that creatine may have in oxidative stress and mitochondrial damage [8] Existing and increasing evidence shows that creatine ameliorates the mitochondrial dysfunction commonly observed in neurodegenerative diseases [5].

Mitochondrial dysfunction can refer to any alteration of normal mitochondrial activity, particularly in ATP production, which is reduced [39]. Among the causes of mitochondrial dysfunction are overexpression of reactive oxygen species (ROS), hypoxia, or alteration of calcium homeostasis. As creatine supplementation increases PCr and subsequently provides ATP quickly, it might mitigate energy deficits associated with mitochondrial dysfunction.

Alterations in mitochondrial dynamics, including defects in mitochondrial trafficking and fusion/fission [40] are implicated in the pathogenesis of neurodegenerative diseases, such as Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) [41]. Additionally, mitochondrial DNA mutations, respiratory chain-deficient cells and other mitochondrial abnormalities also exist in age-related neurodegenerative diseases, such as AD [15,42,43].

Creatine participates in the stabilization of energy metabolism and has demonstrated neuroprotective effects in diseases such as PD, HD and ALS, due to its ability to improve mitochondrial deficits associated with each condition [44]. Furthermore, a study showed that creatine is neuroprotective against toxin-induced lesions, that it might stabilize mitochondrial creatine kinase, and that creatine supplementation may reduce susceptibility to mitochondrial-mediated apoptosis [44]. Creatine kinase activity participates in regulating mitochondrial respiration [45]. It is well known that factors such as stress, hypoxia, mental fatigue or sleep deprivation alter brain energetics, leading to a decrease in ATP. These conditions can influence the efficacy of creatine supplementation in increasing brain creatine content [18].

Additionally, several mental health disorders have been characterized to have abnormalities in brain bioenergetics, associated with low creatine levels in specific regions of the brain [46]. There is existing evidence supporting the use of creatine in the treatment of depression, as research have demonstrated improvements in related symptoms [47-49]. Limited research also suggests that creatine levels may be decreased in various regions of the brain in individuals with generalized anxiety disorder and post-traumatic stress disorder. Nevertheless, further investigation is needed to evaluate the use of creatine supplementation in these patients [50,51]. Creatine supplementation has also been shown to play a protective role and ameliorate symptoms of concussion and traumatic brain injury [52,53], evidence indicating that these benefits may be mediated via mitochondrial processes, including

the sustainment of membrane potential, reduction of ROS and calcium and maintenance of ATP concentrations [8].

As mentioned before, sleep deprivation is well established to alter brain bioenergetics and affect cognitive function. In this context, creatine supplementation appears to enhance cognitive function compared to placebo, although efficacy is still limited [8]. Turner et al. examined the effects of oral CRM supplementation on neurophysiological function during acute oxygen deprivation in healthy young adults, demonstrating its effectiveness in the restoration of cognitive decline associated with hypoxia. In this study, CRM is believed to provide an abundant pool of PCr as a source of energy, balancing the ATP-generation/ATP-consumption ratio within the cell. Creatine may enhance anaerobic energy processes by prolonging the transfer of high-energy phosphates to neurons when glycolysis is compromised by hypoxia. This mechanism may help maintain ATP levels, preventing the typical decline observed during oxygen deprivation [16].

Overall, growing evidence shows that creatine supplementation has a positive effect on cognition, particularly when brain bioenergetics are compromised, such as in situations of stress or sleep deprivation. In the presence of glucose, many cancerous cells inhibit OXPHOS and promote glycolysis, even when oxygen is available [54]. In our study, glucose was replaced with D-galactose, forcing dopaminergic neurons to rely exclusively on mitochondrial ATP production via OXPHOS, a slower and less efficient pathway. Under these conditions, cells become more sensitive to molecules that affect mitochondria, and they experience metabolic stress. Dopaminergic neurons are particularly vulnerable to oxidative stress and mitochondrial dysfunction, both affecting ATP production. Ultimately, it leads to cell failure.

Under these conditions, CLP showed greater improvements in dopaminergic neuron cells treated with D-galactose instead of glucose, compensating for the loss of energy in a stress situation. We hypothesize that, in situations in which ATP production is depleted or modified, CLP enhances mitochondrial energy production due to its PCr content, which donates a phosphate group immediately to boost ATP synthesis.

Additionally, we found that the lowest dose of CLP tested (100µM) led to a significant increase in ATP levels in neuronal cells, approximately 11% above baseline ($p < 0.05$). In contrast, CRM alone, even at its most effective concentration (500µM), did not stimulate ATP synthesis as effectively as CLP. This corresponds to an estimated bioavailable effective dose of 13 mg for CLP versus 65.57mg/L for CRM in neurons. Low doses are especially relevant for brain-targeted creatine supplementation, given the limited expression of CT1 at the BBB, and the downregulation of both CT1 and the endogenous creatine synthesis. These factors may reduce the efficacy or contribute to resistance over time, as well as the need to minimize systemic side effects.

Long-term supplementation may suppress endogenous creatine synthesis via end-product inhibition, as the expression of L-arginine: glycine amidino transferase (AGAT), the rate-limiting enzyme for creatine biosynthesis in the brain, is downregulated in the presence of high intracellular creatine levels. This feedback mechanism helps maintain cellular energy homeostasis and prevents overaccumulation [18,55]. Limited CT1 expression at the blood–brain barrier often requires high creatine doses to achieve detectable increases in brain creatine levels. Current clinical studies show wide variability in dosing regimens, ranging from 5 g/day to 20 g/day [3,8], highlighting the need for greater consistency and deeper understanding of the optimal dosing strategy for brain-targeted effects. This is critical for the effective design of neuroprotective or cognitive-enhancement interventions using creatine.

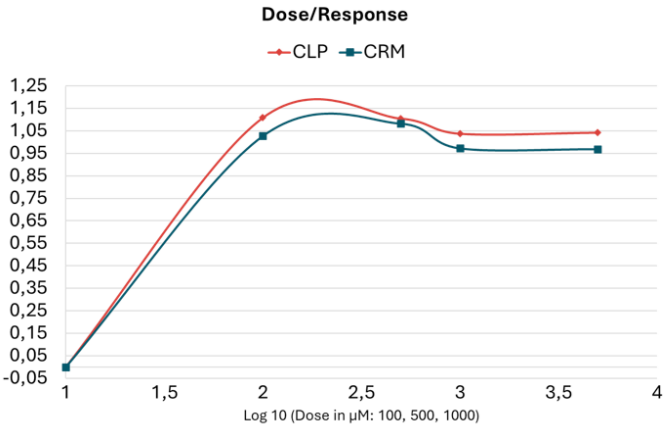


Figure 4: Dose-response plot.

The dose–response analysis (Figure 4) reveals that CLP exhibits a more efficient activation, at all and any tested dosage of the phosphagen system in dopaminergic neuron cells, enabling faster ATP replenishment through the use of PCr. CRM administration reached a higher saturation threshold at 500 µM, compared to CLP. From this concentration, ATP levels started to decline. Notably, stimulation of ATP synthesis remained more stable among the increasing concentrations of CLP, suggesting an immediate and sustained energy-supplying effect. This enhanced stability may be attributed to the synergistic combination of CRM, creatine phosphate, and phosphate salt within the CLP formulation.

PCr resynthesise is tightly linked to the efficiency of aerobic metabolism. In the neuronal system, enhanced mitochondrial function and PCr availability could contribute to greater cognitive resilience and attentional capacity under stress. Additionally, reduced intracellular acidification may create a neuroprotective environment, as excessive acidosis disrupts synaptic transmission and promotes excitotoxicity. Extracellular acidification has been shown to impair neurotransmission and induce neuronal

dysfunction and toxicity through H^+ and lactate accumulation [56,57] Moreover, enhanced aerobic metabolism may facilitate greater neuronal plasticity and synaptic adaptation. These effects could underlie some of the cognitive benefits associated with sustained PCr availability and efficient ATP buffering in the brain.

In this context, our findings highlight the potential of CLP to support brain bioenergetics under conditions favoring oxidative phosphorylation. These results obtained in compromised and metabolic stressed cells may be extrapolated to conditions with altered brain energetics and mitochondrial dysfunction, such as stress situations, anxiety, sleep deprivation, hypoxia or certain diseases mentioned above. Our data suggest that CLP supports the maintenance of brain creatine levels more efficiently than CRM alone, by preserving PCr levels and enhancing ATP buffering capacity. CLP may contribute to improved neuronal plasticity and synaptic adaptation, key processes underlying cognitive performance.

Further *In Vivo* studies are required to confirm the physiological relevance of these effects and to determine the therapeutic potential of CLP in brain health and cognitive performance.

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