LiposoMax™ Liposomal Creatine® demonstrates superior metabolic and mitochondrial support through improved cellular absorption.

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Introduction

Cellular energy levels define the body's capacity to work and heal, and sugar is the primary fuel for this work. Converting sugar to cellular capacity to work and heal is found in the molecule adenosine triphosphate (ATP) and the chemical bonds between the phosphates of ATP hold the metabolic energy from sugar. Sugar is metabolically oxidized by over 20 enzymes in a series of reactions known as glycolysis (10 enzymes), the citric acid cycle (8 enzymes) and the electron transport chain (4 enzymes) the latter two of which consists of enzymes in the mitochondria which is where ATP is produced. This overall process is known as respiration in which electrons are removed (oxidation) from sugar to build ATP and molecular oxygen is used to manage and hold the electrons. The work of some cells in the body requires bursts of ATP production such as muscles and the brain which means these cells must have high sugar supplies to burn and plenty of oxygen to burn it with. These immediate draws on ATP supplies and demands on ATP production can overwhelm fuel supplies and without sufficient substrates from glycolysis it is more energetically favorable for metabolic enzymes to produce reactive oxygen species (ROS) rather than get the electron to oxygen and form ATP (1-3). The ROS molecules are highly reactive and either permanently or temporarily bind to and alter the structure and function of proteins, lipids and DNA through oxidation (4). Instead of oxidizing sugar, the cells begin to oxidize self-molecules which do not produce ATP but rather destroy cellular function (4-7). Even with ample metabolic supply from glycolysis, the enzymes involved in making ATP are not the most efficient and some ROS can be produced all along the way of glucose oxidation (4-7). At times of high demand and ATP use, if phosphate is not available for the enzymes of respiration and ADP builds up then it becomes more energetically favorable for ROS to form rather than to oxidize sugar metabolites or form ATP (4 – 7). Therefore, even with plenty of fuel to burn, the lack of phosphate at times of work can lead to ADP accumulation and ROS formation. To preserve as much phosphate as possible in the cell for making ATP, the molecule creatine serves as a phosphate sponge, binding phosphate from the diet and forming phosphocreatine (PCr) (8 - 11). Phosphocreatine is then a source of phosphate in the conversion of ADP to ATP in what is known as the phosphagen system in which the enzyme creatine kinase can take the phosphate from PCr and add it to ADP to form ATP (8 – 11). Therefore, increased concentrations of creatine allow cells to hold more phosphate and run less risk of ATP depletion and ROS production. In this regard PCr and the phosphagen system are a cellular buffer against phosphate unavailability during high demand. Increased availability of ATP therefore reduces ROS build up through the phosphagen system and in this way, creatine is considered an important antioxidant (8 - 11). While there is some evidence that creatine can also act directly

as an antioxidant (12), elevated creatine levels in muscle when combined with exercise have overall positive antioxidant effect through the phosphagen system (13-17). In muscle and exercise physiology, creatine is known to reduce cellular oxidative stress and ROS production at times of high metabolic demand and improve recovery (healing) from the demand leading to improve muscle mass and function (13 – 17). As noted, the role of the phosphagen system in the immune system and brain health and cognitive function has also been observed (18 – 21). Therefore, there is a great deal of interest in dietary supplementation with creatine as a phosphagen and antioxidant for health of the immune and nervous system and especially for athletes to reduce the oxidative damage from exercise and improve healing and the maintenance of muscle.

Here we show that the dietary supplement, LiposoMax™ Liposomal Creatine® is better at reducing ROS activity and oxidative stress in cells compared to standard non liposomal creatine. Liposomes are small phospholipid bilayers with aqueous interiors that can be used to deliver water soluble creatine to cells at high levels and rapidly because the liposome can fuse with cellular membrane and deliver the creatine by diffusion (22). In a model of oxidative stress, we have added hydrogen peroxide to cells in culture and measured the loss of mitochondrial enzyme function due to oxidative stress in a 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) colorimetric assay. Our results confirm that creatine protects cells from oxidative stress and that Liposomal Creatine® is statistically significantly better at protecting cells from oxidative stress and ROS production than non-liposomal creatine. Further, to determine if the enhanced protection of LiposoMax™ Liposomal Creatine® was due to improved absorption into the cell, the level of cytoplasmic creatine was measured by enzymatic conversion and colorimetric assay. We confirm that the improved biological activity of LiposoMax™ Liposomal Creatine® is likely due to its significantly nearly two-fold faster and greater absorption into cells.

Materials and Methods

Murine N38 neuronal cells were maintained in DMEM containing 5% FBS and incubated at 5% CO2 at 37 ºC in a water-jacketed incubator. For cell viability assays, in order to harvest cells, the plates were rinsed with serum free DMEM and then incubated with trypsin-EDTA and after the cells lifted the trypsin was neutralized with 5% serum and the cells were pelleted by centrifugation at 1000 rpm in a 10 ml conical. For assay, after harvest, the serum and phenol red were washed out of the cell pellet by centrifugation three times using 10 ml of serum-free and phenol red-free DMEM and the cells were plated at 0.5 x 10^6 cells per well in 1.0 ml of the serum- and phenol red-free DMEM. Cells were then treated with 300 μM H2O2 alone or with 300 μM H2O2 and concomitant treatment with 0.1 mM, 1.0 mM or 10 mM creatine either in the non-liposomal form or using LiposoMax™ Liposomal Creatine®. All treatments were dissolved in serum-free and phenol red-free DMEM and so the medium was the control vehicle or no treatment. To measure cell viability, after 24 hours, the medium was removed and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to scale in

the 12 well tissue cluster. 50 µl of the reaction was transferred to a 96 well plate to read OD at 570 nm. MTT is a synthetic designed substrate for the mitochondrial electron transport chain enzyme, succinate dehydrogenase, which acts on MTT to produce a purple color product that absorbs light at 570 nm. In short MTT will turn color in cells when they are not oxidatively stressed and when the cells are oxidatively stressed the succinate dehydrogenase will be damaged and be less able to convert MTT to the color product. Damaged and inhibited succinate is a sign of the loss of viability and a mechanism of toxicity and so a reduction in the purple color read at 570 nm is a measure of the loss of cell viability.

For creatine absorption into cells, harvested cells were washed three times with DMEM by centrifugation and suspended in DMEM containing an insulin, selenium and transferrin (ITS) (DMEM-ITS) supplement and seeded at 0.5 $x10^5$ cells per well in a 24 well tissue cluster plate. Cells were cultured for 36 hours until reaching $1x10^5$ cells in each well which was confirmed by counting. The wells were then rinsed with DMEM-ITS and LiposoMax™ Liposomal Creatine® and non-liposomal creatine was added at 10 mM and incubated at 37ºC in a water-jacketed CO_2 incubator. Cell lysates with absorbed creatine were then collected immediately (time 0), 1 hour, 3 hours, 6 hours and 12 hours after incubation to measure cellular levels. To measure cellular creatine cells were rinsed at the time points above three times with DMEM in the 24 well plate. The cell were then lysed by three 30-minute freeze thaw cycles at -80ºC and room temperature. The two step enzymatic reaction converted by adding creatinase to the 24 well plates to convert all creatine to sarcosine and urea. Sarcosine oxidase was then added to the wells to produce H2O2. Horseradish peroxidase (HRP) and the HRP chromogen from the Abcam creatine Assay Kit (ab65339) was then added to produce a red color. To read the optical density at 570 nm, 100 µl of the medium was transferred (and diluted if needed) to a 96 well plate and read on a Bio-Rad Microplate Reader and presented as relative creative per 10^5 cells.

Results

The results in Figure 1 show that H2O2 as expected reduced cell viability by approximately 60% (Figure 1). When co-treated with 0.1 mM creatine viability was significantly restored to approximately 70% by LiposoMax™ Liposomal Creatine® but not the non-liposomal creatine (Figure 1). Both liposomal and LiposoMax™ Liposomal Creatine® resorted viability at 1.0 mM and 10 mM, however at each concentration LiposoMax™ Liposomal Creatine® increased viability to a statistically significant greater extent than the non-liposomal and only the LiposoMax™ Liposomal Creatine® restored viability to essentially 100% and full protection from H2O2 and oxidative damage (Figure 1).

The results Figure 2 show that LiposoMax™ Liposomal Creatine® is more rapidly absorbed into cells and absorbed at approximately double the level of the non-liposomal creatine. As early as one hour LiposoMax™ Liposomal Creatine® shows a 1.5-fold increase in absorption compared to non-liposomal creatine and by 3 hours the increase in nearly doubled which is sustained over the tested 12-hour period. Indeed, the area under the curve for the 12 hours for LiposoMax™

Liposomal Creatine® is 88.25 and for the non-liposomal is only 44.65. The increase observed at all time points is statistically significant at $p \leq 0$ in an analysis on variance (ANOVA).

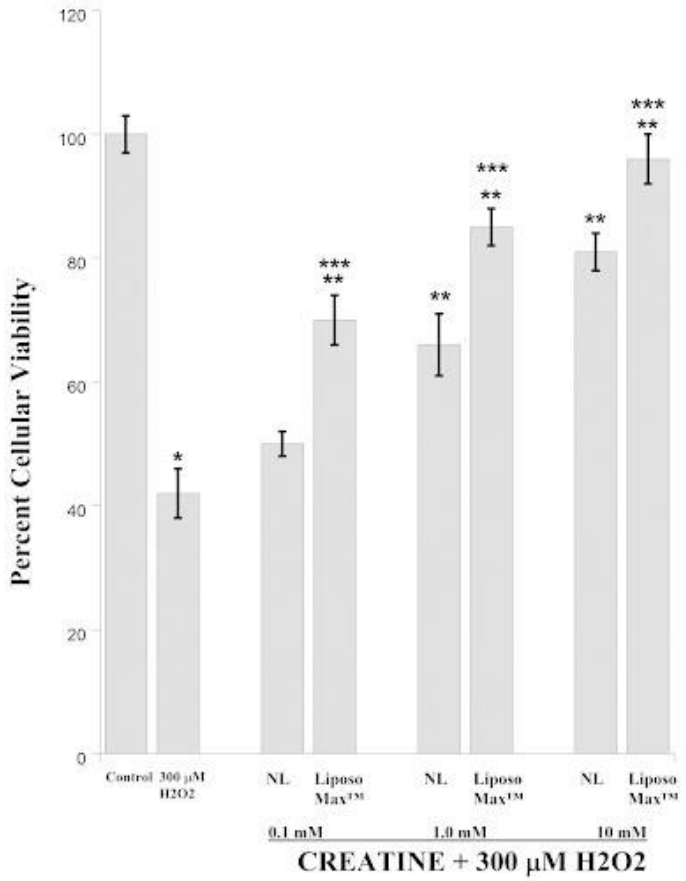

Figure 1. LiposoMax™ Liposomal Creatine® protects cells from H2O2-induced ROS reduced succinate dehydrogenase activity (MTT assay). Murine neuronal N38 cells were treated with untreated (control) or treated with 300 mM H2O2 alone or concomitantly with non-liposomal (NL) or LiposoMax™ Liposomal Creatine® at 0.1, 1.0 and 10 mM. The treatments were dissolved in medium, so the control was no treatment. The experiment was run in triplicate wells and the mean viability for each treatment groups was determined and subjected to a statistical analysis of variance (ANOVA). The single asterisk (*) shows statistically significant loss of viability with H2O2 treatment. The double asterisk (**) shows statistically significant increase in viability from the H2O2 treatment. The triple asterisk (***) shows statistically significant increase in the LiposoMax™ Liposomal Creatine® compared to NL at the same concentration. All statistically significant results are at 95% confidence with a p value of ≤ 0.5.

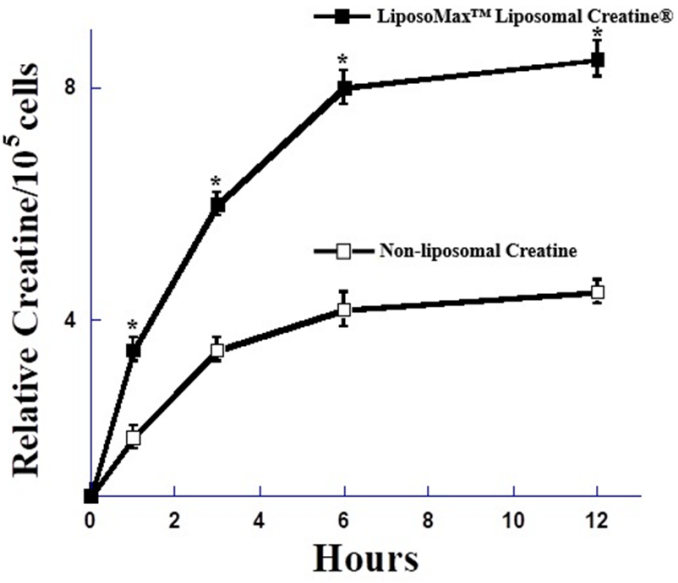

Figure 2. LiposoMax™ Liposomal Creatine® is more rapidly absorbed by cells than Non-liposomal Creatine. Murine hypothalamic cells (N38) were harvested and washed in serum free-DMEM and then seeded in wells of a 24 well tissue cluster plat in a growth medium of DMEM supplemented with insulin, transferrin and selenium (DMEM-ITS). The cells were cultured for 36 hours to a density of 10^5 cells per well and then treated with 10 mM LiposoMax™ Liposomal Creatine® (closed squares) or Non-liposomal Creatine (open squares). After treatment absorption was measured immediately (0) or at 1, 3, 6 and 12 hours post treatment by rinsing the well free of residual medium and creatine three times and lysing cells in situ by freeze thaw cycles. In a two-step process the creatine in each well was enzymatically converted hydrogen peroxide which was then detected using the chromogen substrate from the Abcam Creatine Assay Kit (ab65339) to generate red color (λmax = 570 nm). The material was transferred to a 96 well plate and the relative creatine absorbed by the cells was measured and plotted. The assay was performed in triplicate, and the error bars are the standard error of the mean (SEM). The asterisks indicate statistically significant differences in ANOVA ($p < 0.05$) between LiposoMax™ Liposomal Creatine® and Non-liposomal Creatine absorption into cells at all tested time points with and a doubling of the area under the curve (44.65 versus 82.25).

Discussion and Conclusions

The phosphagen system relies on creative availability to protect all cells of the body from oxidative stress. However, the phosphagen system is particularly vital in muscle, brain and immune system. Cellular creatine levels vary between tissue but is highest in skeletal muscle and immune system cells at approximately 20 – 50 mM and nearly one tenth the amount in brain and neurons at 5 - 20 mM and in most cells of the body only at 1 – 3 mM (23). While creatine has been shown to be an antioxidant and beneficial to all organs systems in the body and overall reduction of oxidative stress, the value of PCr and the phosphagen system in protecting muscle from damage from high demand and facilitating muscle healing and maintenance has been extensively demonstrated and supported by clinical studies which show that dietary creatine supplementation improves muscle function and maintenance not only in aging (15) but also for athletic training and in elite athletes where improvements in performance have been observed (24 - 27). Therefore, increasing cellular pools of creatine will benefit not only overall health but will protect the immune system from oxidative stress, improve cognitive and muscle function in aging and help all athletic endeavors from personal fitness to professional training reach peak performance. LiposoMax™ Liposomal Creatine® provides an innovative solution to effectively increase intracellular creatine and its antioxidant activity. As demonstrated in Figure 1, LiposoMax™ Liposomal Creatine® significantly enhances cellular protection compared to conventional, non-liposomal creatine. Specifically, it outperformed standard creatine in shielding neuronal cells from oxidative damage induced by hydrogen peroxide. The ability of LiposoMax™ Liposomal Creatine® to quickly affect cell behavior and more rapid response is an indication of its better absorption and better avidity for the receptor cell surface and peripheral sites and its improved bioavailability which facilitates faster and more efficient cellular uptake by bypassing typical transporter limitations. As demonstrated in Figure 2, LiposoMax™ Liposomal Creatine® significantly outperformed non-liposomal creatine in more rapidly absorbed at all time points. These data show that LiposoMax™ Liposomal Creatine® has superior cellular protection, because the LiposoMax™ Liposomal Creatine® significantly outperformed non-liposomal creatine in protecting neuronal cells from oxidative damage caused by hydrogen peroxide. These data also show the full viability restoration – At 10 mM concentration, only LiposoMax™ Liposomal Creatine® was able to restore 100% cellular viability, as measured by mitochondrial enzyme function (MTT assay). These data also show more rapid and efficient cellular delivery, because LiposoMax™ Liposomal Creatine® deliver creatine directly into cells, bypassing transporter limitations and allowing for immediate action. These data show that LiposoMax™ Liposomal Creatine® offers dual benefits, enhancing ATP regeneration while simultaneously reducing reactive oxygen species (ROS) accumulation. These effects support healthier energy metabolism across muscle, brain, and immune cells, contributing to improved performance, resilience, and recovery. Here were show that LiposoMax™ Liposomal Creatine®, when compared to non-liposomal creatine, offers significantly improved metabolic benefits and antioxidant protection (Figure 1) and that it does so through significantly more rapid and increased total absorption into cells (Figure 2).

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