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Creatine Intranasal Administration in Vivo and in Vitro Models

A Dissertation submitted in partial satisfaction
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ABSTRACT OF THE DISSERTATION

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by

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While creatine levels in skeletal muscles can be enhanced by exogenous creatine supplementation, the elevation of creatine levels in the brain with oral creatine administration remains a challenge due to a lack of effective creatine transportation through the blood-brain barrier (BBB). As consequence, intranasal administration has been established as a method to bypass the blood-brain barrier and deliver drugs directly to the brain. In our studies, we focused on using intranasal delivery to bypass the BBB and deliver creatine to the brain.

Our first objective was to use magnetic resonance spectroscopy to measure creatine accumulation in the brain after a 14-day period of intranasal administration. The spectra showed that the brain creatine/choline ratio was significantly increased after creatine administration. This proved the accumulation of exogenous creatine in the brain.

In the second objective, we studied the effects of intranasal creatine administration on wild-type rats and evaluated the cognitive performance after administration via the Barnes maze experiments. This study demonstrated that intranasal creatine administration can improve the performance of rats in the Barnes maze. The results of biochemical assays showed that administration of creatine can increase the creatine content in the rat brain.

Despite the success of intranasal administrations, there are still drawbacks, especially when it comes to pH and solubility. To address the solubility limitation of creatine concentration per unit volume and ensure a pH value close to neutral, we assembled creatine microparticles. We tested the characteristics of these creatine microparticles, including size distribution, functional group composition, assembly rate, stability, and cytotoxicity. In vitro permeability measurements indicate creatine microparticles are permeable to human respiratory epithelial cells.

Overall, we developed methods to evaluate efficacy of intranasal creatine administration in vivo and methods to evaluate characteristics and permeability of creatine microparticles in in vitro drug delivery models. Our experiments indicate intranasal administration of creatine can improve wild type rats' cognitive performance in Barnes maze and increases creatine content in the brain. Moreover, creatine microparticle suspensions were proven to exceed the limits of creatine solubility. This approach enables the delivery of creatine intranasally beyond their solubility limits without significant cytotoxicity and alteration of cell layer integrity.

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ADP	Adenosine Diphosphate
AGAT	Arginine: glycine amidinotransferase
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
CNS	Central Nervous System
FTIR	Fourier-Transform Infrared Spectroscopy
GAA	Guanidinoacetic Acid
GAMT	Guanidinoacetate N-methyltransferase
MR	Magnetic Resonance
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
PTA	Post-Traumatic Amnesia
ROI	Region of Interest
SAMe	S-Adenosyl-L-Methionine
SEM	Scanning Electron Microscopy
TBI	Traumatic Brain Injury
TEER	Trans-Epithelial Electrical Resistance

1 Chapter 1 Background and Significance

1.1 Creatine-phosphocreatine system, and creatine synthesis

The main function of the creatine-phosphocreatine system involved in the bioenergy supply chain is to act as an energy buffer to convert and store energy (Wallimann et al., 2011) . In the creatine-phosphocreatine system, creatine kinase, an essential enzyme, is required to reversibly catalyze these two compounds (Wallimann et al., 2011). As an energy storage carrier, phosphocreatine can remove the phosphate group from the phosphocreatine, in which the latter will then be bonded to adenosine diphosphate (ADP) to generate adenosine triphosphate (ATP) (Figure 1.1) (Marques & Wyse, 2019). This occurs when energy is needed: when the heart beats, skeletal muscles contract, and neurons signal (Marques & Wyse, 2019). When phosphocreatine is converted into creatine after dephosphorylation, creatine returns to the mitochondria and rebinds a phosphate group through creatine kinase to continue the next energy cycle (Marques & Wyse, 2019). This process is called an "energy shuttle". The energy shuttle does not require oxygen to regenerate ATP (Marques & Wyse, 2019). The recovery of ADP by phosphocreatine and the subsequent generation of ATP is faster than the generation of ATP by fat oxidation, although the latter produces more ATP (Sahlin et al., 1998). Therefore, phosphocreatine is the first source used as an energy reservoir when cells need to restore ATP levels (Sahlin et al., 1998). ATP as "energy currency" can theoretically diffuse to sites where cells demand energy, but phosphocreatine could diffuse at a higher rate, being more efficient, due to its smaller molecular weight (Balestrino, 2021). As a result of the different diffusion

efficiencies, the cells use phosphocreatine more as a source of energy storage instead of directly storing large amounts of ATP.

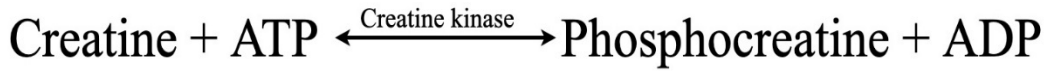

Figure 1.1 Phosphocreatine and creatine conversion. Adenosine diphosphate (ADP), Adenosine triphosphate (ATP).

Additionally, creatine can be synthesized from two amino acids, glycine, and arginine (Allen, 2012; Wyss & Kaddurah-Daouk, 2000). Most creatine is synthesized in the kidneys and liver (Figure 1.2) by the cells that express arginine: glycine amidinotransferase (AGAT) and/or guanidinoacetate N-methyltransferase (GAMT). In the liver, AGAT catalyzes guanidine group from arginine to glycine and produces L-ornithine and guanidinoacetic acid (GAA) (Allen, 2012; Wyss & Kaddurah-Daouk, 2000). Then, the GAA is methylated by S-Adenosyl-L-Methionine (SAMe) and yields creatine. This reaction mostly happens in the liver and is catalyzed by the enzyme GAMT (Allen, 2012; Wyss & Kaddurah-Daouk, 2000). After creatine has been synthesized, it will be transported through the bloodstream and accumulated in ATP-demanding tissues, such as skeletal or cardiac muscle (Wallimann & Hemmer, 1994).

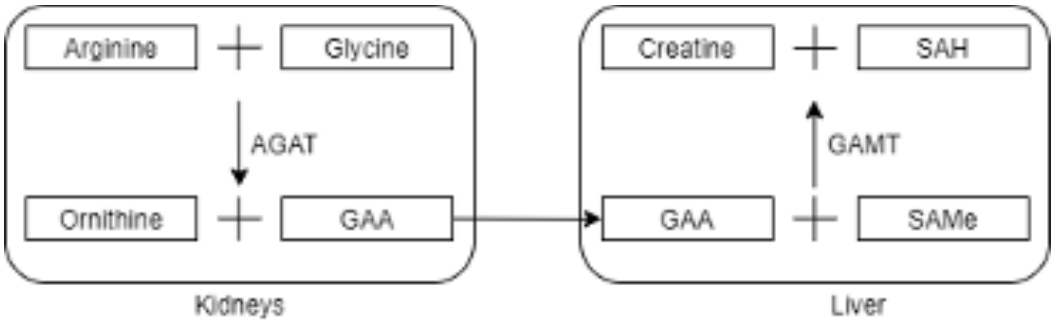

Figure 1.2 Creatine synthesis. Arginine: glycine amidinotransferase (AGAT), Guanidinoacetic acid (GAA), Guanidinoacetate N-methyltransferase (GAMT), S-Adenosyl-L-Methionine (SAMe), S-Adenosyl-l-homocysteine (SAH).

1.2 Creatine supplementation benefits besides exercise performance

Creatine, the most common sports supplement, can improve muscle mass and muscle strength (Hall et al., 2021; Hall & Trojian, 2013; Williams & Branch, 1998). Over 300 studies on creatine supplementation showed significant improvements in performance after creatine intake (Kreider, 2003). When integrated, especially in short-term, high-intensity exercise, orally administered creatine is used to its known potential (Hall et al., 2021; Hall & Trojian, 2013; Williams & Branch, 1998). Although creatine supplements have shown to improve athletic performance, there are additional benefits to its use. When heart failure or myocardial infarction occurs, cardiomyocytes cannot obtain the required oxygen and energy from the blood to maintain a normal heartbeat (Balestrino, 2021). In heart failure, ATP levels are stable or slightly decreased, but phosphocreatine levels are substantially decreased, which confirms the role of creatine and phosphocreatine in energy

shuttling (Balestrino, 2021). As the fastest way to replenish ATP, creatine phosphate can protect myocardial damage and maintain ATP levels (Balestrino, 2021).

Similarly, energy shuttling is crucial in other cases such as traumatic brain injury (TBI). Consequences from TBI include local ischemia and inflammation (Ainsley Dean et al., 2017). In this condition, brain cells cannot uptake enough ATP to maintain normal metabolism and function when blood flow is reduced (Ainsley Dean et al., 2017). The researchers found that magnetic resonance spectroscopy (MRS) spectra showed reduced levels of N-acetyl aspartate and creatine in the brains of concussion patients (Vagnozzi et al., 2013). These metabolites may take up to 45 days to return to normal levels (Vagnozzi et al., 2013). In order to combat this, doctors tried restoring brain creatine to normal levels post-concussion by providing exogenous creatine supplements (Sakellaris et al., 2006). In the study, the subjects took 0.4g/kg creatine suspension orally every day after TBI for 6 months (Sakellaris et al., 2006). They found that subjects that were treated with creatine improved several aspects after the TBI, including post-traumatic amnesia (PTA), neurophysical function, and cognitive function (Sakellaris et al., 2006).

Creatine has also been shown to have antioxidant effects that protect cells (Smith et al., 2014). Reactive oxygen species are byproducts of metabolism that are normally eliminated by cells themselves (Pizzino et al., 2017). However, if the production of reactive oxygen species and the scavenging capacity do not balance each other, oxidative stress accumulation will occur (Pizzino et al., 2017). At this point, free radicals accumulate inside the cells, which often lead to nucleus damage and cell apoptosis (Pizzino et al., 2017). In

both cell-free, human cell, and animal models, creatine has demonstrated a significant ability to scavenge reactive oxygen species. (Lawler et al., 2002; Sestili et al., 2006).

Oxidative stress is associated with many diseases, such as cancer, cardiovascular disease, respiratory disease, and neurological disease (Pizzino et al., 2017). In Huntington's disease animal models, the researchers first used malonate and 3-nitropropionic acid to induce Huntington's disease-like lesions in rats. Then they gave rats two weeks of creatine supplementation, finding significantly increased phosphocreatine levels and reduced malonate-induced striatal lesions (Matthews et al., 1998). This study showed that creatine treatment resists the reduction of phosphocreatine and ATP, as well as suppressed the oxidative damage biomarker production (Matthews et al., 1998). Besides Huntington's disease, mitochondrial damage is commonly found in other neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease (Yan et al., 2013). For example, looking into Alzheimer's disease, oral administration of creatine for 9 weeks improved spatial learning and reduced amyloid β trimers in female Alzheimer's disease mice models, in which the latter is a biomarker for Alzheimer's disease (Snow et al., 2020).

However, the benefits of creatine to Alzheimer's disease rats were not consistent. Some studies showed that creatine did not improve the learning ability of rats and had no positive effect on rats' neuronal apoptosis (Alimohammadi-Kamalabadi et al., 2016). Similar conflicting results have also been reported for oral creatine administration in other neurodegenerative diseases such as Parkinson's diseases (Matthews et al., 1999; Writing Group for the et al., 2015).

1.3 Creatine absorption after oral administration and limitation

Creatine can be absorbed through the gastrointestinal tract as evidenced by the expression of active, saturable and electrogenic Na^+ Cl^- creatine cotransporter in small intestine brush-border membrane cells (Peral et al., 2002). It was found that intestinal cells can accumulate free creatine against a concentration gradient because of creatine transporters (Peral et al., 2002). The creatine transporter is a protein that helps cells uptake creatine from the blood (Christie, 2007). Oral administration is the most common method to supplement creatine. Various studies confirmed that oral administration of creatine is able to improve creatine levels in the skeletal muscles, and exercise performance (Harris et al., 1992). However, the CNS-related improvements after creatine oral administration are still controversial.

Oral creatine supplementation to the brain creatine level is minimal, as the blood-brain barrier (BBB) blocks creatine absorption. This is because the BBB is a barrier formed by different types of cells including micro capillary endothelial cells, pericytes, and astrocytes (Braissant, 2012). These three cells tightly connect to prevent pathogens from entering the brain. Microcapillary endothelial cells are cells that form the microvasculature of the BBB (Braissant, 2012). Pericytes cover around 30% of microcapillary endothelial cells (Braissant, 2012). Astrocytes endfeet are the peripheral cells that almost completely cover the surface of microcapillary endothelial cells and pericytes (Braissant, 2012). These cells are surrounded by an extracellular matrix to prevent molecular transport. Researchers have found that the BBB microcapillary endothelial cells expressed creatine transporters (Braissant, 2012). Nevertheless, the peripheral astrocytes do not have a transporter on the

membrane, meaning that only a limited amount of creatine is transported across the BBB (Braissant, 2012) (Figure 1.3).

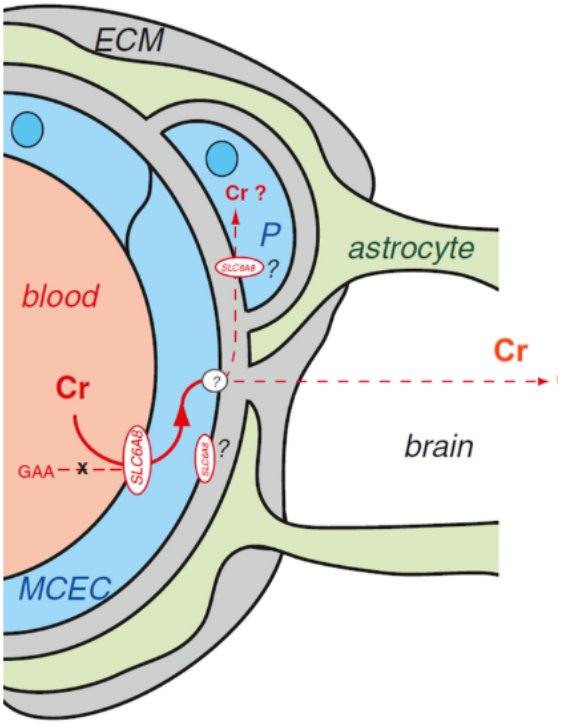

Figure 1.3 Creatine transport across the blood-brain barrier (Braissant, 2012). Creatine (Cr), Extracellular matrix (ECM), Guanidinoacetate (GAA), Microcapillary endothelial cells (MCEC), Pericyte (P), Creatine transporter (SLC6A8).

1.4 Mechanism of Intranasal administration

Intranasal administration can be a potential alternative route for drug delivery to the CNS. It is a non-invasive method of administration that patients can self-administer (Guastella et al., 2013). More importantly, intranasal administration can bypass the BBB through the olfactory pathway (Guastella et al., 2013).

Although the mechanism of the drug absorption from intranasal administration to the central nervous system is not fully understood, evidence has suggested that drugs can be absorbed by one or more routes. Drugs can be delivered through paracellular and axonal transport by olfactory pathway, the trigeminal pathway, and the paravascular pathway into the cerebrospinal fluid to enter the CNS (See Figure 1.4) (Bahadur & Pathak, 2012; Guastella et al., 2013; Weller et al., 2010). Drugs may also be absorbed through the lungs or gastrointestinal tract when they mistakenly enter into non intranasal administration target areas (Guastella et al., 2013).

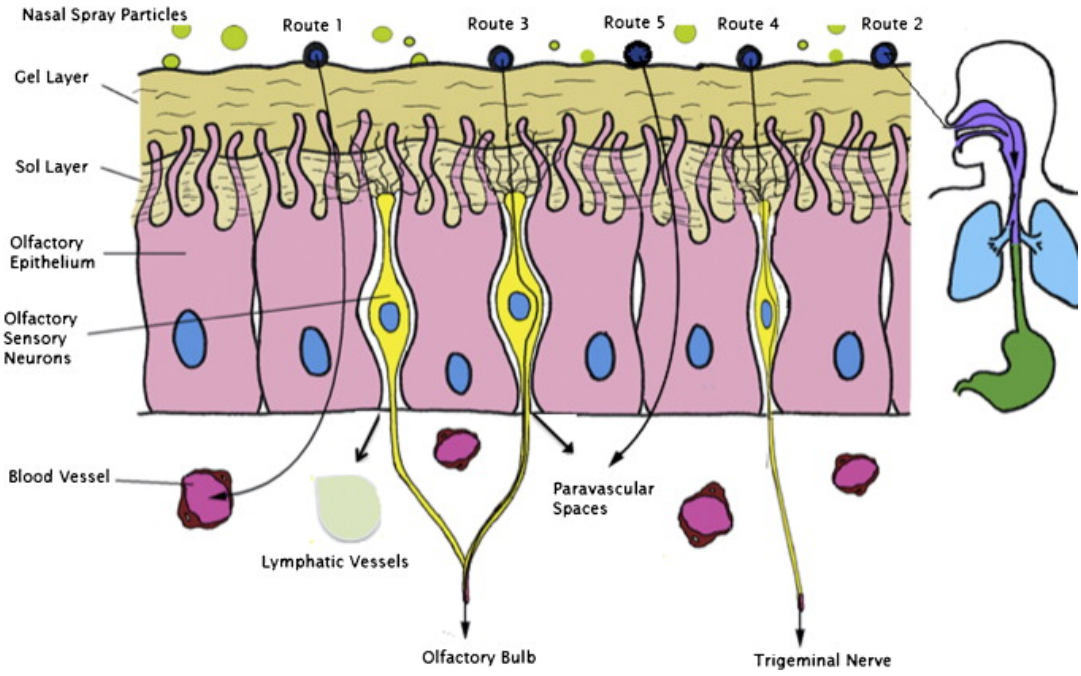

Figure 1.4 Drug Absorption Routes After Intranasal Administration (Guastella et al., 2013). Route 1 (through the nasal vasculature), Route 2 (through oral mucosa or gastrointestinal tract), Route 3 (through olfactory pathways, including surrounding lymphatic fluid), Route 4 (through trigeminal nerve pathways), Route 5 (through the paravascular spaces).

The two most important routes of absorption for intranasal administration are the olfactory pathway and the trigeminal neuralgia pathway. After the drug is deposited on the olfactory epithelium, the olfactory neurons can absorb the drug and transport the drug into the olfactory bulbs and brain (Liu et al., 2012). The trigeminal nerve has also been confirmed by various drugs as a route for drugs to enter the brain (Liu et al., 2012). For example, wheat germ agglutinin conjugated PEG-PLA nanoparticles were imaged to record the transcellular uptake of the olfactory epithelium into the olfactory bulb (Liu et al., 2012). Nanoparticles were also found in the cervical spinal cord and medulla, suggesting that nanoparticles can also be transported by the trigeminal nerve pathway (Liu et al., 2012). The fluorescence imaging results imply that the PEG-PLA nanoparticles are taken up by both the olfactory and trigeminal nerves pathway.

1.5 Factors Affecting Intranasal Administration Absorption

Lipophilic drugs, such as propranolol, pentazocine, etc., may be the most effective drugs that pass through the nasal mucosa and are absorbed (Illum, 2002). The pharmacokinetic profile of pentazocine following intranasal administration approximates the effectiveness of intravenous administration (Sankar et al., 2001). Small molecule drugs usually with a molecular weight below 1000 Da may enter the brain through the paracellular pathway (McMartin et al., 1987).

The structure of tight junctions can also be adjusted by drug adjuvants or surfactants to allow larger molecules to pass through tight junctions. With the adjuvants, the molecular weight of the drug limit can be extended to 6000 Da or above (McMartin et al., 1987).

Another strategy of adjuvants is to increase the viscosity of the drug solution (Guastella et al., 2013; Illum, 2002). High-viscosity drugs can reduce the clearance rate and prolong the contact time of the drug with the olfactory epithelium to increase the absorption rate (Grassin-Delyle et al., 2012; Illum, 2002). Moreover, some cells such as the olfactory epithelium secrete mucus with various enzymes to degrade chemicals in the nasal cavity, such as peptide-protein (Kahraman et al., 2023). Adding enzyme inhibitors to the formula can inhibit the activity of enzymes and make the drug more stable (Kahraman et al., 2023).

1.6 Thesis Contribution

The results of previous studies with oral administration of creatine suggest that oral creatine administration may require prolonged or higher doses to achieve positive efficacy due to low passage of creatine through the BBB and constant muscle absorption (Dolan et al., 2019). Our objective is to try to find an alternative oral delivery method to deliver creatine to the CNS more efficiently.

Intranasal administration of creatine has not been fully studied, and previous studies have attempted it in creatine-deficient animal models (Chen et al., 2021). Whether intranasal administration of creatine further accumulates exogenous creatine in wild-type rats is unclear. In the first study, we attempted to use MRS to measure brain creatine accumulation in rats after intranasal administration of creatine. In the second study, we administered creatine solution intranasally to rats and assessed their cognitive function, through the Barnes maze experiments. We also compared creatine accumulation in different brain structures and maze performance after oral and intranasal administration. In

the third study, we focused on optimizing the formula and increasing the creatine concentration for intranasal administration and administered creatine. At the end, our data supports that administration of creatine microparticles suspension is more effective in accumulating creatine than creatine solution.

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2 Chapter 2 A Longitudinal Study of Creatine Accumulation in the Brain Following Intranasal Creatine Administration

2.1 Abstract

Creatine is a non-essential nutrient for the body, but it is one of the most abundant metabolites in the brain. Here, the main function of creatine is to quickly replenish energy. The energy consumed by the brain accounts for about 25% of the energy produced by the body. Intranasal administration has been shown to deliver some small molecule drugs directly into the brain. Whether exogenous creatine can enter the brain via intranasal administration and accumulate in wild-type rats is unknown. The purpose of this experiment is to compare the levels of creatine in brains before and after administration by magnetic resonance spectroscopy (MRS), and to prove that creatine can enter the brain through intranasal administration. In our results, MRS showed that the ratio of creatine/choline was significantly increased after intranasal administration of creatine in rats. This suggests that creatine can enter the brain of wild-type rats through the intranasal route and that exogenous creatine can accumulate in the brain.

2.2 Introduction

Magnetic resonance spectroscopy (MRS) is a non-invasive method to detect metabolites in the body (Gujar et al., 2005; Rackayova et al., 2017). MRS is mainly used to measure metabolites and assist in the diagnosis of diseases through the content of metabolites (Gujar et al., 2005). For example, multiple studies have shown that myocardial creatine is significantly decreased in patients with myocardial infarction, dilated cardiomyopathy, or hypertrophic cardiomyopathy when measured by MRS (Bottomley et al., 2009; Nakae et al., 2003).

Although MRS can be used to measure metabolites in any part of the body, its most common use is to measure metabolites in the brain such as creatine, choline, N-acetyl aspartate (Gujar et al., 2005; Weinberg et al., 2021). Using the MRS method, doctors identified the first patient with a guanidinoacetate methyltransferase (GAMT) creatine deficiency (Stöckler et al., 1994). MRS measurement revealed an abnormally low creatine signal in the patient's brain associated with impaired body movement (Stöckler et al., 1994). A few years later creatine deficiency due to a mutation in the creatine transporter was also discovered by MRS (Salomons et al., 2001). The MRS method was taken further, evaluating the oral creatine administration treatment of patients with creatine deficiency (Bianchi et al., 2000; Rackayova et al., 2017; Stöckler et al., 1994). Creatine levels usually increase after taking creatine in patients with creatine synthase deficiency (Stöckler-Ipsiroglu et al., 2001; Stöckler et al., 1994).

Previous studies have focused on the efficacy of oral creatine administration for some conditions besides creatine deficiency, such as athletic performance and Alzheimer's

disease models (Hall & Trojian, 2013; Kreider, 2003; Snow et al., 2020; Williams & Branch, 1998). The positive effects of creatine have been demonstrated in animal disease models, but results in human clinical trials were not consistent with animal studies (Gualano et al., 2010). Oral creatine administration was found to have ineffective delivery to the brain because of the lack of creatine transporters in the blood-brain barrier region (Braissant, 2012). In general, researchers have agreed that oral administration of creatine to the brain may only be effective in specific conditions, such as hypoxia, or in patients with creatine deficiency (Dolan et al., 2019).

In recent years, other drug delivery strategies were used to deliver creatine to the brain such as intranasal administration (Chen et al., 2021). Intranasal administration allows delivery to the central nervous system (CNS) by bypassing the BBB. Different molecules can enter the CNS through intranasal administration by different mechanisms. The mechanism by which creatine enters the brain via intranasal administration is currently not well understood, as creatine can theoretically enter the CNS in combination of the olfactory pathway, the trigeminal pathway, and the paracellular pathway. One of these pathways, the olfactory pathway, the olfactory nerve directly absorbs the drug into the olfactory bulb (Liu et al., 2012; Thorne et al., 2004). In the past, through immunohistochemistry staining with immunofluorescence labeling, researchers have identified the olfactory bulb as one of the highest creatine transporter immunoreactivity areas in the brain (Mak et al., 2009). In the olfactory bulbs, the glomerular layer is connected to the olfactory nerve layer by the olfactory receptor neurons (Sinakevitch et al., 2018). The glomerular layer has been confirmed to have a strong creatine transporter immunolabeling (Mak et al., 2009). Once

creatine is absorbed by the olfactory endocytosis, it will reach the creatine transporter-rich olfactory bulb area and enter the CNS. This provides theoretical support for creatine transport to the CNS through the olfactory route.

The trigeminal pathway has also been shown to be a drug delivery route for intranasal administration (Liu et al., 2012; Thorne et al., 2004). Interestingly, trigeminal nerves, such as motor and sensory trigeminal nerves, show strong immunoreactivity creatine transport (Mak et al., 2009). This may allow creatine to travel along the trigeminal nerve into the brainstem.

In addition to the previously summarized transcellular pathways, paracellular pathways can also be considered for creatine transport. The rate of paracellular transport depends on the molecular mass. Molecules with a molecular weight below 1000 Da can be transported through paracellular pathways (McMartin et al., 1987). Creatine has a molecular mass of 131.13 Da, which is lower than the size limit of paracellular pathway transport (Galbraith et al., 2006). The low molecular weight assumes creatine may cross the olfactory epithelium via a paracellular transport pathway as well.

Although intranasal administration of creatine has been tested in creatine-deficient mice, the feasibility of exogenous creatine supplementation by intranasal administration in wild-type rats has not been demonstrated. No studies have compared changes in brain content in healthy rats before and after taking creatine. Whether intranasal administration is feasible for further brain creatine improvement in healthy rats needs to be tested. Despite the difficulties and inconsistencies shown by the oral administration studies, based on the advantages of intranasal administration and three potentially suitable routes for creatine to

enter the CNS via intranasal administration, we hypothesized that exogenous creatine could accumulate in the wild-type rat brains following intranasal administration of creatine.

In our study, we used intranasal administration to supplement wild-type rats with creatine for 14 days, and measured the creatine content before and after intranasal administration by MRS. We primarily use MRS to scan the thalamus region of the rat brain. The thalamus and striatum area was chosen because the previous study showed that thalamus had the most pronounced creatine increase in the brain after four weeks of oral creatine supplementation (Dechent et al., 1999). This finding is consistent with the results of immunofluorescence labeling experiments of creatine transporters (Mak et al., 2009). High expression of the creatine transporter in the thalamus indicates that cells in the thalamus generally have a higher energy demand or higher ATP metabolic requirements, and more importantly are able to uptake creatine more efficiently (Mak et al., 2009). Our experimental results indicate that creatine can accumulate and store in the brain of wild-type rats by intranasal administration.

2.3 Materials and methods

2.3.1 Animals and ethics

Six male Long-Evans rats weighing between 200 to 225 g from Charles River Laboratories (Wilmington, MA) were used in this study. Rats were housed in a temperature-controlled room at the University of California, Riverside (UCR) Spieth Vivarium. Rats had free access to food pellets (Purina 5001 Rodent Chow, LabDiet,

Richmond, IN) and clean water. All animal use protocols were approved by the UCR Institutional Animal Care and Use Committee.

2.3.2 Creatine administration

Based on a paper by Persky, the dosage of creatine supplementation had a loading phase followed by a maintaining phase (Persky et al., 2003). Specifically, in the loading phase (days 1-3), rats were administered 0.075 g/kg/d of creatine hydrochloride, and on days 4-14, rats were given 0.0375g/kg/d of creatine hydrochloride. For a 200g rat, the creatine hydrochloride solution concentration for intranasal administration at loading phase is 0.1 g/ml, and 0.05 g/ml for maintaining phase.

The procedure of intranasal administration followed Hanson's method with modifications (Hanson et al., 2013). Rats were anesthetized by inhalation of isoflurane (Covetrus, Inc.). Then, the anesthetized rats were transferred to a rat restrainer and placed in a supine position, which restrained the possible head movement during the intranasal administration. The restrainer can also maintain the rats' posture after the intranasal administration. With the restrainer tilted up by 45 degrees, 50 µl of solution was gently administered to the nostrils by pipette and inhaled by the rats.

2.3.3 Animal Anesthesia, Support, and Monitoring System

Six rats were used in the magnetic resonance imaging (MRI) and MRS scans. Each rat was scanned multiple times to develop the procedure of animal scans and optimize the parameters of MRI and MRS. To reduce the influence of isoflurane accumulation in the body, each rat was scanned no more than 3 hours per week. The rats were transferred from

the Spieth Hall animal vivarium room to the Center for Advanced Neuroimaging (UCR CAN) building in a dark storage box with a cage. Food, water, bite bar and bedding are provided with the cage.

Before the animal scan, the temperature for the laboratory animal water warming systems was set and pre-warmed to 37 °C. Then, the rat was transferred from the animal cage to an animal anesthesia induction box. Animal anesthesia was conducted via an MRI-compatible NRB veterinary anesthesia ventilator (DRE Company). The ventilator delivers and regulates the air flow of anesthesia gas and oxygen to the animal anesthesia induction box. The animal anesthesia induction box is transparent and allows the visibility of the rat during the anesthesia induction. Once the rat became recumbent and had no response to the toe pinch, it was transferred onto the animal warming pad and its upper front teeth were hooked to the animal bite bar. After the rat was restrained on the animal bite bar the nose cone was put on the rat's face and the valve opened to allow the rat to inhale anesthesia gas. The concentration of isoflurane was reduced to 1.5% to maintain the rat under the anesthesia. After the rat was immobilized, temperature and respiration sensors were attached to the rat before the MRS scan started. Respiration rate was monitored by an MR safe pressure pad/ respiration transducer (Biopac systems, Inc.) During the MRS scan, physiological conditions such as rectal temperature and respiratory rate of the rat were continuously recorded and monitored by the data acquisition system (Biopac systems, Inc.). The animal water warming bag was placed under the anesthetized rats and set to a constant temperature of 37°C throughout the experiment.

2.3.4 Animal magnetic resonance spectroscopy (MRS) scan

The rat was placed in an animal holder (Suzhou Medcoil Healthcare Co., Ltd) to immobilize the rat in the scanner. The animal scan was performed on a 3.0T Siemens MRI scanner and an animal coil was used in the animal scan. The region of interest (ROI) was chosen as 5 x 5 x 5 mm^3, and the ROI is large enough to include the entire rat thalamus. Thus, the field map image showed that the thalamus and striatum area had relatively higher homogeneity than other brain areas. Homogeneity is critical for the MRS spectrum signal to noise ratio. The MR scan session includes a localizer scan, field map scan, MRI T1-weighted scan, manual shimming, and single-voxel spectroscopy scan. When the scan was complete, the rat was removed from the MRI scanner. The rat was then laid on the warming pad for a few minutes and waited for rat recovery from anesthesia. Then all used animal equipment were disinfected, and the rats were transferred back to the animal vivarium room.

2.3.5 Data analysis

After MRS data was generated, the Tarquin software was used to analyze the MRS spectrum. It is an automatic software using a least-squares approach to reconstruct a MRS spectrum. The Tarquin will show fitted MRS spectra with x-axis as signal intensity and y-axis as a chemical shift. In the MRS spectra, the creatine peak shows resonance at 3.0 ppm, and the choline peak shows resonance at 3.2 ppm (Star-Lack et al., 1998). Then, the creatine and choline peak height at the position on the MRS spectrum will be used to calculate creatine/choline height ratio. The Wilcoxon signed-rank test was used to compare the difference in creatine/choline ratio signal intensity before and after administration in rats. The statistical significance level was set at $p < 0.05$.

2.4 Results

The creatine to choline ratio before and after the intranasal administration are shown below (Table 2.1). Representative MRS results are shown in Figure 2.1. Six mice were divided into three experiments in this study. The thalamus/striatum region of the rat brain was used for scanning. The data was analyzed by Wilcoxon signed-rank test and p-value is 0.016.

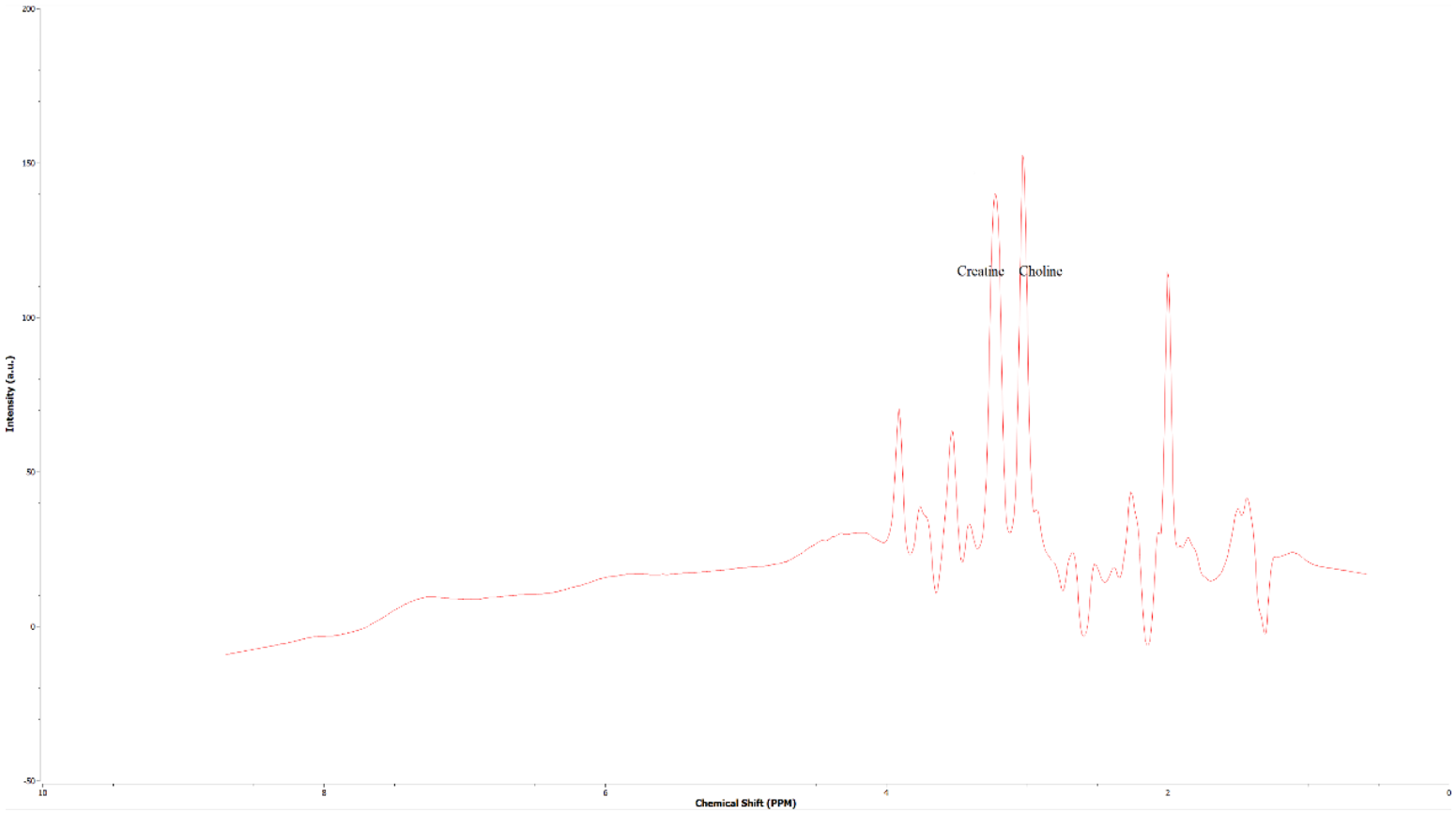

Figure 2.1 A representative MRS spectrum of Rat 1 before administration in the experiment 2. The creatine peak shows resonance at 3.0 ppm, and the choline peak shows resonance at 3.2 ppm

	Creatine/Choline Ratio before administration	Creatine/Choline Ratio after administration
Experiment 1:		
Rat1 (Thalamus/Striatum)	0.86	0.95
Experiment 2:		
Rat1 (Thalamus/Striatum)	0.91	0.97
Rat2 (Thalamus/Striatum)	0.87	1.07
Rat3 (Thalamus/Striatum)	0.75	0.98
Experiment 3:		
Rat1 (Thalamus/Striatum)	0.80	1.09
Rat2 (Thalamus/Striatum)	0.79	0.99

Table 2.1 Creatine/Choline ratio in the thalamus/striatum area before and after intranasal creatine administration.

2.5 Discussion

2.5.1 Preliminary collaborative study and limitations

Intranasal administration of creatine was initially conducted in mice. The formulation for intranasal administration was creatine monohydrate powder mixed with PBS. In this study, the creatine suspension concentration was calculated to be the same. However, Intranasal administration protocol was slightly different for this study.

There are three major difficulties in intranasal administration of creatine monohydrate suspension in mice. Mice were not anesthetized during the intranasal administration and rodent restrainers were not used in mice experiments. Instead, the researchers restrained the mice by the scruff in dorsal recumbency with their hands. The nostrils of the mice were difficult to locate, and the awake mice would try to avoid pipettes approaching their nostrils. Moreover, the mouse nostrils were too small to confirm that creatine suspension was successfully delivered into the nasal cavity.

In addition, the solubility of creatine monohydrate is lower than our target dosage concentration. We had to use a creatine monohydrate suspension for administration. During the administration, creatine monohydrate precipitated at the target concentration, failing to maintain a stable suspension. As a result, the creatine monohydrate caused pipette tips to be clogged after creatine was loaded in. In conclusion, using this method, we were not able to confirm that the mice were successfully administered creatine at the correct dosage.

Furthermore, our MRI scanner could not achieve sufficient resolution to scan mouse brains. In addition, the animal support and monitoring systems in the Imaging

Center are designed and configured for rats. MRS cannot be used to measure creatine content in the brain of mice due to technical constraints.

Subsequently, we switched the experiment subjects to rats. Since rats have a larger brain size than mice, we were able to implement MRS to conduct the longitudinal study and measure brain creatine levels. Moreover, animal anesthesia and animal restrainers were used during intranasal administration to reduce animal stress and ensure the success of drug administration. We even changed the intranasal administration formula from creatine monohydrate to creatine hydrochloride because of the low solubility. At our target dosage, creatine hydrochloride was completely soluble.

2.5.2 Rats MRS study

After 14 days of creatine administration, the creatine content in the rat brain was significantly increased compared with that before administration. This study preliminarily validated the feasibility of evaluating intranasal administration of creatine in wild-type rats by MRS. The MRS spectra indicate that creatine could accumulate in rat brains by intranasal administration. Accumulation of exogenous creatine demonstrates that creatine can enter the brain via the intranasal route. More specifically, creatine transporters in the brain are able to uptake creatine from the intranasal route into the brain cells. Consequently, the brain cells have the capability to store additional creatine.

Our study is the first longitudinal study to measure intranasally administered creatine deposition in rats using MRS. Furthermore, previous intranasal administration of

creatine was demonstrated by creatine-deficient mice depositing creatine after intranasal administration (Chen et al., 2021). Our experiments demonstrate the feasibility of intranasal administration of creatine without depleting creatine in the brain or knocking out creatine related genes.

2.5.3 Limitations and Future Work

Although this study demonstrates the measurement of creatine in the brain of rats by 3T MRI scanner and compares the difference from before and after creatine administration, it should be mentioned that there are limitations to the study. Due to the instrumental limitations of the 3T MRI scanner, the quality of the MRS spectra was not always successful. Each scan requires manual optimization by suppressing the brain water signal to enhance the spectral signals of metabolites in the brain. When manual water suppression was unsuccessful, the low signal-to-noise ratio made it impossible to distinguish between noise and metabolite peaks. Future research can be improved in the following directions. A high-field animal MRI scanner will greatly improve the quality of MRS spectra. Moreover, creatine can be measured in combination with other methods, such as biochemistry methods like creatine assay or mass spectrometry.

2.6 Conclusion

In conclusion, this study demonstrates that creatine can be delivered intranasally to the brain of wild-type rats. When comparing the MRS signal intensity of creatine in rat brains before and after 14 days of creatine intranasal administration, creatine has shown significant accumulation in rat brains.

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3 Chapter 3 Intranasal Creatine Administration Increases Brain Creatine Level and Improves Barnes Maze Performance in Rats

3.1 Abstract

While skeletal muscle creatine levels can be enhanced by exogenous creatine supplementation, the elevation of brain creatine levels with oral creatine administration remains a challenge due to a lack of effective transportation of creatine through the blood-brain barrier. Intranasal administration can bypass the blood-brain barrier and deliver drugs directly to the brain. This study purpose was to assess the effect of intranasal administration of creatine on brain creatine level and cognitive performance. Rats were randomly assigned into three groups intranasal administration group, oral administration group, and control group. The intranasal group exhibited fewer errors and shorter primary latency compared to the control and oral groups, respectively, during the acquisition phase of the Barnes maze. The intranasal group spent a higher percentage of time in the target quadrant during the probe trial compared to the control group. Biochemical measurements showed that the concentration of creatine in the olfactory bulbs, medial prefrontal cortex, and hippocampus of the rats in the intranasal group was higher than in the oral, and control groups. These results indicate that intranasal administration of creatine hydrochloride increases the creatine level in the rat's brain and improves their performance in the Barnes maze.

3.2 Introduction

Creatine is a naturally occurring chemical molecule in vertebrates that aids in energy synthesis and recycling (Rae & Bröer, 2015). The creatine-phosphocreatine system acts in concert to maintain adenosine triphosphate (ATP) homeostasis (Wyss & Kaddurah-Daouk, 2000). ATP as an 'energy currency' is necessary for cell metabolism and signaling in the ATP-phosphocreatine energy system. ATP is hydrolyzed by the loss of one of its Gamma phosphate groups to become adenosine diphosphate (ADP), which then can be used to generate energy for cell metabolism (Wallimann & Hemmer, 1994). When ATP levels fall below a certain level, phosphocreatine donates a stored phosphate group to ADP to replenish and maintain ATP at a steady level. Removing the phosphate group from the phosphocreatine will lead to the formation of creatine.

About 95 percent of creatine is contained in skeletal muscle, while other energy-demanding tissues such as the brain and heart have around 5% of total creatine in the body (Dolan et al., 2019). Although brain creatine accounts for a small percentage of the body's total creatine, it is essential for the brain to operate properly (Dolan et al., 2019). In addition, while creatine synthases, guanidinoacetate methyltransferase (GAMT) and L-arginine: glycine amidino transferase (AGAT) are mainly express in the liver and kidneys, in situ hybridization analysis also revealed that creatine synthetases are commonly found in the brain (Braissant et al., 2001). Creatine in the brain is primarily synthesized endogenously by neurons and glial cells expressing AGAT and AGMT, rather than being synthesized and transported into the brain from the liver and kidneys (Braissant et al., 2001).

Consequently, defects in either enzyme or transporter result in a significant reduction in brain creatine compared to wild-type rats (Baroncelli et al., 2014; Baroncelli et al., 2016; Stockebrand et al., 2018). Researchers have studied animal models of different types of creatine synthetase or creatine transporter deficiency (Baroncelli et al., 2014; Choe et al., 2013; Duran-Trio et al., 2022; Schmidt et al., 2004). These animals exhibited significantly impaired cognitive abilities compared to wild-type animals. For example, creatine transporter-deficient mice showed reduced muscular mass, decreased motor function (Duran-Trio et al., 2022), and alterations in neuronal morphology and connectivity in the cerebellum (Duran-Trio et al., 2022; Stockebrand et al., 2018) and impaired performance in novel object recognition in the Y-maze, and Morris water maze tests (Baroncelli et al., 2014; Skelton et al., 2011). Furthermore, creatine transporter knockout mice exhibited impaired performance on conditioned fear tests, indicating a general cognitive impairment in the creatine transporter deficiency model (Skelton et al., 2011). AGAT deficiency also showed similar results in morphological changes in mice, usually manifested as decreased body weight and lower fat content (Choe et al., 2013).

Three types of human creatine deficiency have been identified, namely guanidinoacetate methyltransferase (GAMT)-deficient, L-arginine: glycine amidino transferase (AGAT)-deficient, and creatine transporter deficiency (Salomons et al., 2001; Stöckler-Ipsiroglu et al., 2001; Stöckler et al., 1996) These types of creatine deficiency are related to either creatine synthesis or creatine transportation and reflect the crucial role of creatine in brain energetics. Developmental delay and intellectual disability are common

symptoms associated with creatine deficiency (Salomons et al., 2001; Stöckler-Ipsiroglu et al., 2001; Stöckler et al., 1996).

Taking creatine orally with water is the most common route of creatine supplementation. Several previous investigations have established that oral creatine supplementation can increase creatine levels in skeletal muscle and improves muscular performance (Harris et al., 1992; Volek et al., 1997). A few animal studies also investigated whether oral creatine administration can elevate the brain creatine pool and facilitate brain energy recycling (Ipsiroglu et al., 2001; Mink et al., 1981). The results of behavioral or biochemical measures after the creatine administration are varied (Ipsiroglu et al., 2001; Snow et al., 2018). Some animal studies reported an increase in creatine levels after taking creatine (Ipsiroglu et al., 2001). In other experiments, although oral creatine could not be demonstrated to significantly elevate brain creatine levels, positive correlation was found between brain creatine levels and memory performance (Robinson et al., 2020). Oral creatine supplementation to increase brain creatine is thought to be ineffective due to lack of effective transportation of creatine across the blood-brain barrier (BBB). Specifically, researchers have found that astrocytes surrounding the BBB do not express the creatine transporter, and consequently only a limited amount of creatine is transported across the BBB (Braissant, 2012).

To overcome the limitation of oral supplementation, attempts were made with invasive routes of administration, including intrahippocampal injection, and intraperitoneal

injection (Perasso et al., 2003; Souza et al., 2012). These approaches are undoubtedly non-ideal and limited in spatial extent.

Intranasal administration is a potentially effective means for supplementing creatine to the brain since it does not require crossing the BBB and is not affected by the first-pass effect that is often a limitation for oral administration. Intranasal administration has been used for topical or systemic administration of medications for the treatment of various diseases (Ellerington et al., 1996; Fowler et al., 1991). Compared to oral administration, intranasal administration can bypass the BBB because the olfactory epithelium, as part of the central nervous system (CNS), is exposed to the external environment, making it possible to deliver drugs directly to the CNS. There are evidences suggesting that intranasal administration could serve as a potential therapeutic approach or treatment for neurological and neurodegenerative disorders such as migraine and Alzheimer disease (Gozes et al., 1996; Ueberall & Wenzel, 1999). Although the mechanism of the drug from the intranasal pathway to the CNS is not fully understood, evidence suggests that after the drug passes through the epithelium, it can enter the brain through the olfactory pathway and the trigeminal neural pathway (Thorne et al., 2004). In recent years, researchers have administered creatine intranasally to creatine transporter knockout mice. They demonstrated neuroprotection and reduced brain infarction compared with intraperitoneal injection after stroke(Chen et al., 2021).

In this work, our experiment aimed to explore the feasibility of elevating brain creatine level in wild type rats by intranasal administration of creatine. In addition to

assaying the creatine level in various brain structures, we assessed the cognitive benefits of creatine supplementation using the Barnes maze test. The Barnes maze test is one of the most widely used test to evaluate animal spatial memory. Compared to other animal memory tests such as the Morris water maze test, the advantage of the Barnes maze test is it causes less stress and reduced reliance on animal motor skills and avoids the use of strong aversive stimuli such as water (Harrison et al., 2009). The Barnes maze test consists of four phases: habituation, acquisition, probe trial, and reversal learning. In the Barnes maze test, the acquisition phase, and probe trial have been used to assess the spatial memory associated with the hippocampus functions(Zappa Villar et al., 2018). The Barnes maze reversal learning period has been shown to be related to the prefrontal cortex associated function, specifically the ability to learn new target box location (Vetreno & Crews, 2012). Medial prefrontal cortex involvement in reversal maze learning was also confirmed by mouse T-maze experiments (Watson & Stanton, 2009). After 14 days of dosing and performing Barnes maze test, the rats were euthanized and dissected to collect specimen of various brain structures, including olfactory bulbs, medial prefrontal cortex, and hippocampus. Our results indicate that intranasal creatine administration is effective in elevating brain creatine level and improve Barnes maze performance in wild type rats.

3.3 Materials and methods

3.3.1 Animals and ethics

Twenty-nine male Long-Evans rats weighing between 200 to 225 g from Charles River Laboratories (Wilmington, MA) were used. Rats were housed in a temperature-controlled room at the University of California, Riverside (UCR) Spieth Vivarium. Rats had free access to food pellets (Purina 5001 Rodent Chow, LabDiet, Richmond, IN) and clean water. All animal use protocols were approved by the UCR Institutional Animal Care and Use Committee.

3.3.2 Creatine administration

The rats were randomly divided into three groups (n= 9-10): an intranasal administration group, an oral administration group, and a control group. Creatine hydrochloride (Vireo Systems, Inc.) was administered to the oral group of rats via drinking water. The water consumption was measured every day to confirm that the oral and intranasal administration groups are receiving the same dose of creatine. Once the rats consumed the designated amount of creatine by water, the creatine water was replaced with pure water. Both oral and intranasal groups were administered creatine for 14 days. The dosage of creatine supplementation was based on a paper by Persky (Persky et al., 2003), with a loading phase, followed by a maintaining phase. Specifically, in the loading phase (days 1-3), rats were administered 0.075 g/kg/d of creatine hydrochloride, and on days 4-14, rats were given 0.0375g/kg/d of creatine hydrochloride. For a 200 g rat, the creatine hydrochloride solution concentration for intranasal administration at loading phase is 0.1 g/ml, and 0.05 g/ml for maintaining phase.

All rats were intranasally administered a solution, but only the intranasal group received 50 µl of creatine hydrochloride solution while the oral and control groups were administered the same volume and pH of placebo (diluted hydrochloric acid solution). All rats were administered either a creatine or placebo solution three times a day. The minimum interval between intranasal administrations was 2 hours.

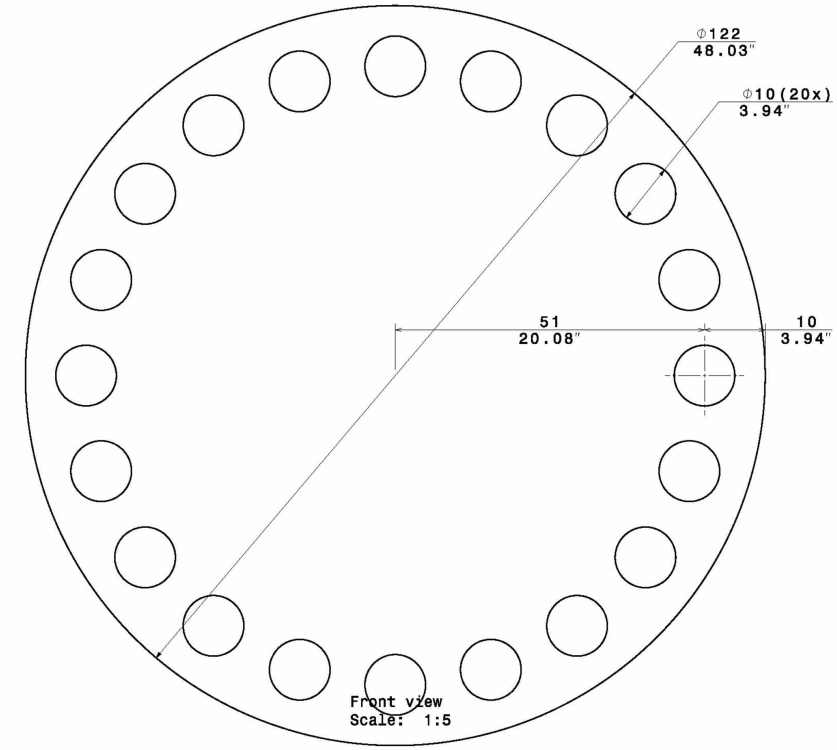
The procedure of intranasal administration followed Hanson's method with modifications (Hanson et al., 2013). Rats were anesthetized by inhalation of isoflurane (Covetrus, Inc.). The anesthetized rats were transferred to a rat restrainer, which restrained the possible head movement during the intranasal administration. The restrainer can also maintain the rats' posture after the administration. Rats were placed in a supine position. With the restrainer tilted up by 45 degrees, 50 µl of solution was gently administered to the nostrils by pipette and inhaled by the rats. Delivery was alternated between the left and right nostrils until administration was complete. After the last day of administration, rats were euthanized by isoflurane overdose. Brain tissue dissection was performed as described in previous studies (Chiu et al., 2007; Spijker, 2011). Olfactory bulbs, medial prefrontal cortex, and hippocampus were quickly dissected on a cold petri dish over ice. Then brain tissues snap-frozen in a dry ice ethanol bath, and stored at a –80°C freezer until the creatine assay measurement.

3.3.3 Barnes maze

We utilized the Barnes maze to evaluate the rats' learning ability and working memory. The memory and learning abilities of the rats can be assessed by comparing the

time spent locating the target hole and the number of errors in checking the non-target hole before finding the target hole.

The Barnes maze platform was purchased from Maze Engineers (Skokie, IL). The platform (122 cm diameter) was made of thick acrylic and had 20 circular holes (10 cm diameter) along the periphery (Figure 3.1). An opaque black target box was under one of the holes. The Barnes maze uses stimuli, such as light and noise, to motivate the animal to search for the target box located under the circular platform. There were two 120 w lights and a buzzer for creating the stimuli, and a camera was placed above the platform to record the movement of the rats. Four different shapes of visual cues were attached to black curtains around the Barnes maze platform. Four visual cues were square, triangle, parallel lines, and cross.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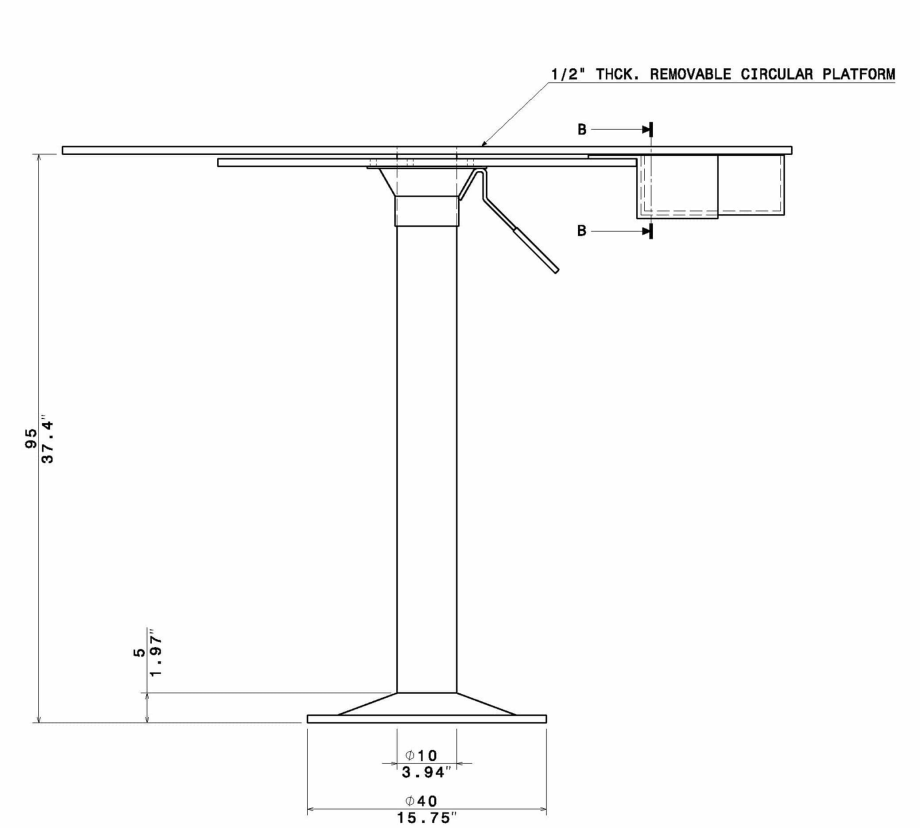
48.03"
⌀10(20x)
3.94"
51
20.08"
10
3.94"
Front view
Scale: 1:5



Bottom view
Scale: 1:5

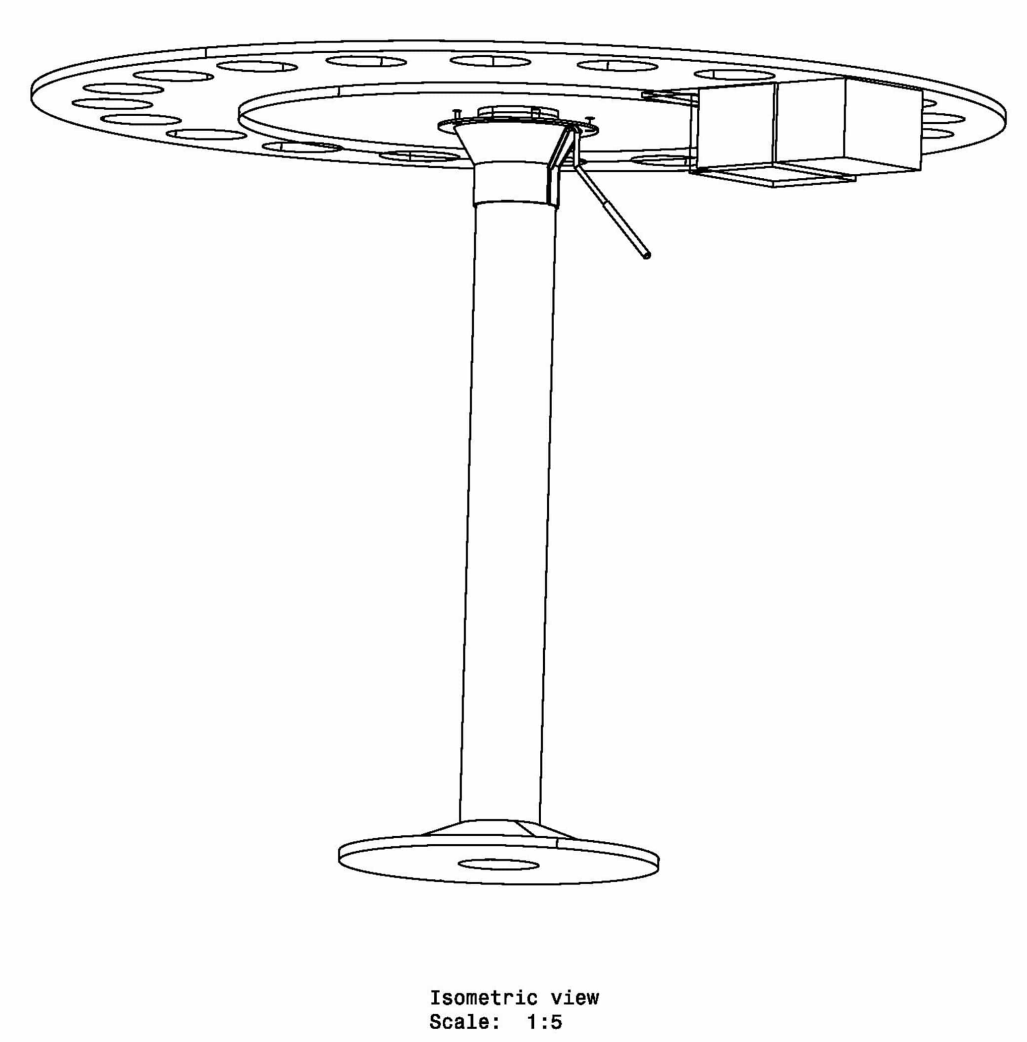


Figure 3.1 The Barnes maze platform apparatus in front view, bottom view, and isometric view. Figures are from the Barnes maze manufacture websites (*Barnes-Maze-Rat-Image-1*; *Barnes-Maze-Rat-Image-2*; *Barnes-Maze-Rat-Image-3*).

The Barnes maze protocol was adapted from a published study (Gawel et al., 2019). It included four phases: habituation (day 1), acquisition (day 2-5), probe trial (day 6), and reversal learning (day 7-8) (Figure 3.2). Habituation started 24 hours before the acquisition. Two lights above the maze were illuminated, but no buzzer sounds were included during

the habituation. Visual cues were hidden to avoid the potential learning effect in this phase. Rats were kept in a start chamber for 1 minute and then the start chamber was lifted. Rats were able to explore the maze freely for 1 minute. After 1 minute of exploration, rats were guided by gently pulling the rats' tail away from the target box. Due to their natural tendency to move in the opposite direction of being pulled, this approach guides them to enter the target box (Sunyer et al., 2007). Then rats stayed in the target box for 1 minute. The entire maze platform and target box were carefully wiped with 70% ethanol prior to the next rat habituation, and the maze platform was rotated 1- 4 holes in either clockwise or counterclockwise direction to exclude the effects of potential odor cues.

Twenty-four hours after habituation, rats were subjected to Barnes maze acquisition tests. The rats had to find the target box within three minutes. If rats entered the target box, they were allowed to stay in the box for 30 seconds. If rats failed to find the target box, they were gently guided to the target box and kept inside the box for 1 minute as described above. Two lights and a buzzer were switched on when they transferred to the maze platform from the cage, and the buzzer was turned off when they entered the target box. Each rat had two trials per day for 4 days during acquisition. The inter-trial interval was set to 20-25 minutes. After each trial, the maze platform and target box were thoroughly disinfected with 70% ethanol. The maze platform was rotated 1- 4 holes in either clockwise or counterclockwise direction, while the position of the target box remained the same.

We manually classified the search strategies employed by the rats in the acquisition phase trials into three categories, following a previous study (Gawel et al., 2019). With the

serial strategy, the rats explore clockwise or counterclockwise along the outside of the maze platform until they find the target hole and do not cross the center of the platform. With the direct strategy, the rats find the target hole on the first try or find 1-2 holes adjacent to the target hole and the total number of errors is less than 2. When the search does not fall into either serial or direct search strategies, the search is classified as belonging to the random strategy. Examples of random strategy include switching directions when searching, going through the center of a maze platform when searching.

After 4 consecutive days of acquisition trials, rats were subjected to a 3 minute probe trial. The apparatus of the probe trial was the same as the acquisition trial, except that the target box was removed from the maze platform. Each rat only had one probe trial test. After each trial, the maze platform was thoroughly disinfected with 70% ethanol. The maze platform was rotated 1- 4 holes in either clockwise or counterclockwise direction, while the position of the target box remained the same.

We performed the reversal learning trials 24 hours after the probe trial. On the last day of the Barnes maze test, the location of the target box was rotated 120 degrees counterclockwise from its original location, and rats had 3 minutes to find the target box at the rotated location. Rats had two trials for the reversal learning trials with 20-25 minutes inter-trial interval. After each trial, the maze platform and target box were thoroughly disinfected with 70% ethanol. The maze platform was rotated 1- 4 holes in either clockwise or counterclockwise direction, but the position of the target box remained the same.

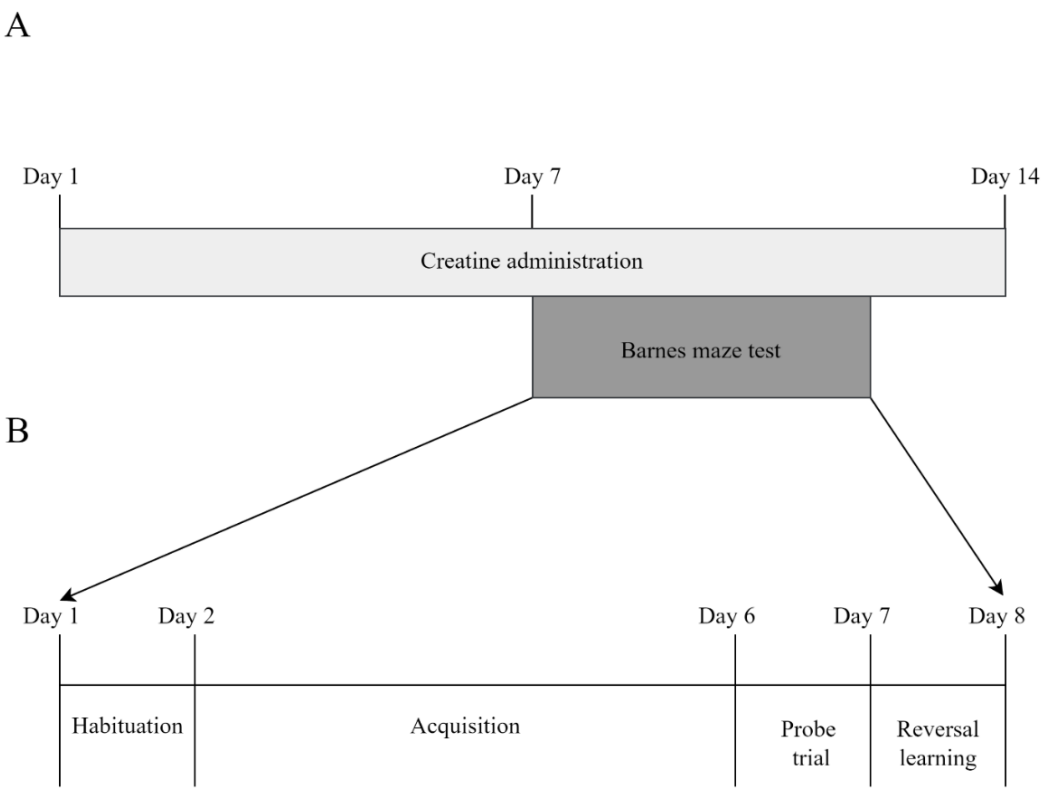


Figure 3.2 Timeline of experiment. Figure 3.1 A: creatine administration timeline. Creatine hydrochloride was administered for a total of 14 days. The Barnes maze was initiated at 7 days after creatine supplementation. Figure 3.1 B: Barnes maze test timeline. Day 1 of the Barnes maze was the habituation phase, days 2-5 were the acquisition phase, day 6 was the probe trial, and day 7 was the reversal learning phase.

3.3.4 Creatine assay

Creatine in rat medial prefrontal cortex, hippocampus, and olfactory bulbs from 29 rats were measured by creatine assay kit (Sigma Aldrich, St. Louis, MO). The biochemical analysis procedure follows the creatine assay kit (MAK079) protocol. All samples were homogenized on ice in a temperature-controlled room. The homogenates were stored at a –80°C freezer if homogenates were not analyzed by the same day. The period between homogenate preparation and creatine assay analysis was less than 2 days.

3.3.5 Data analysis

We recorded the number of errors, primary latency, and search strategy manually. The movement trajectory of rats and their percentage time in the target quadrant in the probe trial were analyzed by ezTrack from recorded videos (Pennington et al., 2019). Statistical analysis of biochemical measurements and Barnes maze primary latency, number of errors, and percentage of target regions, and regression analysis were performed using IBM SPSS statistics 27. Probe trials, reversal learning phase trials and biochemical measurements data were analyzed by one-way analysis of variance (ANOVA). The acquisition phase trials data were analyzed using two-way repeated measures ANOVA test. The number of errors data were logarithm +1 transformed and the primary latency data were logarithm transformed before the analysis. Figure 3.3 presents raw data. Both one-way ANOVA and repeated measures two-way ANOVA tests followed by post hoc Tukey's test. The statistical significance level was set at $p < 0.05$.

3.4 Results

3.4.1 Barnes Maze Acquisition Phase

During the acquisition phase of the Barnes maze test, rats had two trials per day for 4 days. We averaged and recorded primary latency and errors from eight trials. Figure 3.3 shows the Barnes maze performance of control, oral, and intranasal administration groups during the 4 days of the acquisition phase. We used Two-way repeated measures ANOVA and post hoc analysis to analyze different groups of rats' primary latency performance in the Barnes maze test. The main effect of the treatment group on the latency was statistically significant ($F(2, 26) = 9.742$, $p < 0.001$). The post hoc analysis result shows that intranasal administration group had a significantly lower primary latency than either the control or the oral administration group ($p = 0.005$, $p = 0.001$), while the difference between the control and oral administration group was not significant ($p = 0.769$) (Figure 3.3 A). Two-way repeated measures ANOVA shows the number of errors was significantly different ($F(2, 26) = 14.934$, $p < 0.001$) between groups. Tukey's post hoc test shows that the difference between the control and oral administration groups was not significant ($p = 0.965$), but the intranasal group had significantly lower number of error than either the control or the oral administration groups, respectively ($p < 0.001$, $p < 0.001$) (Figure 3.3 B).

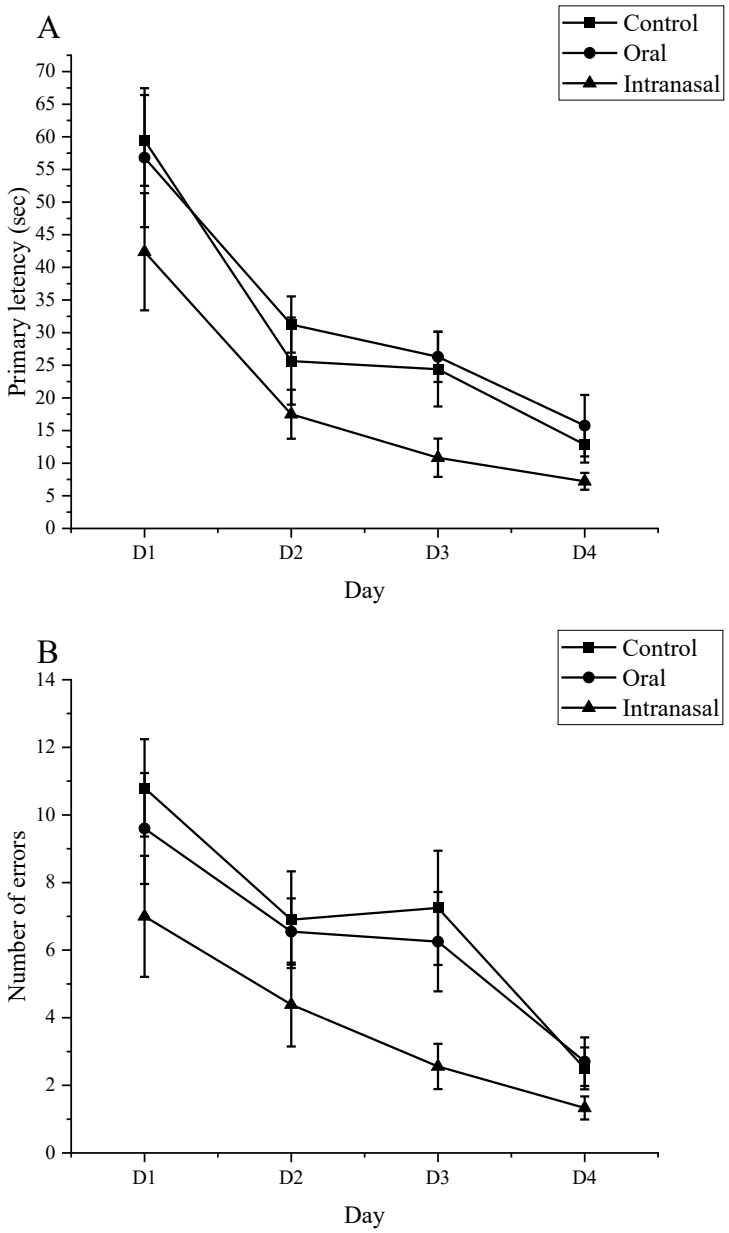


Figure 3.3 Barnes maze performance of control, oral, intranasal administration groups during the acquisition phase. Figure 3.3 A. Average primary latency. Figure 3.3 B. Average primary error. The mean value of the group and standard error of the mean for each data point are shown.

3.4.2 Barnes Maze Search Strategies

Search strategies were also evaluated manually. Rats employed three types of search strategies, random, serial, and direct, to locate the Barnes maze target box. During the acquisition phase, the frequency of using the direct search strategy was the highest in the intranasal group, and the lowest in the control group except on day 4 (Figure 3.4). On the last day of the acquisition phase of the Barnes maze experiments, the intranasal group used the highest proportion of direct search strategy (72.5%) and the lowest proportion of random strategy (5%) among the three groups (Figure 3.4). Fisher's Exact Test was used to examine the correlation between the three groups and the total number of direct strategies during the acquisition phase of the Barnes maze. The results of Fisher's Exact Test ($P = 0.028$) indicated a significant association between different groups with search strategy selection in this phase.

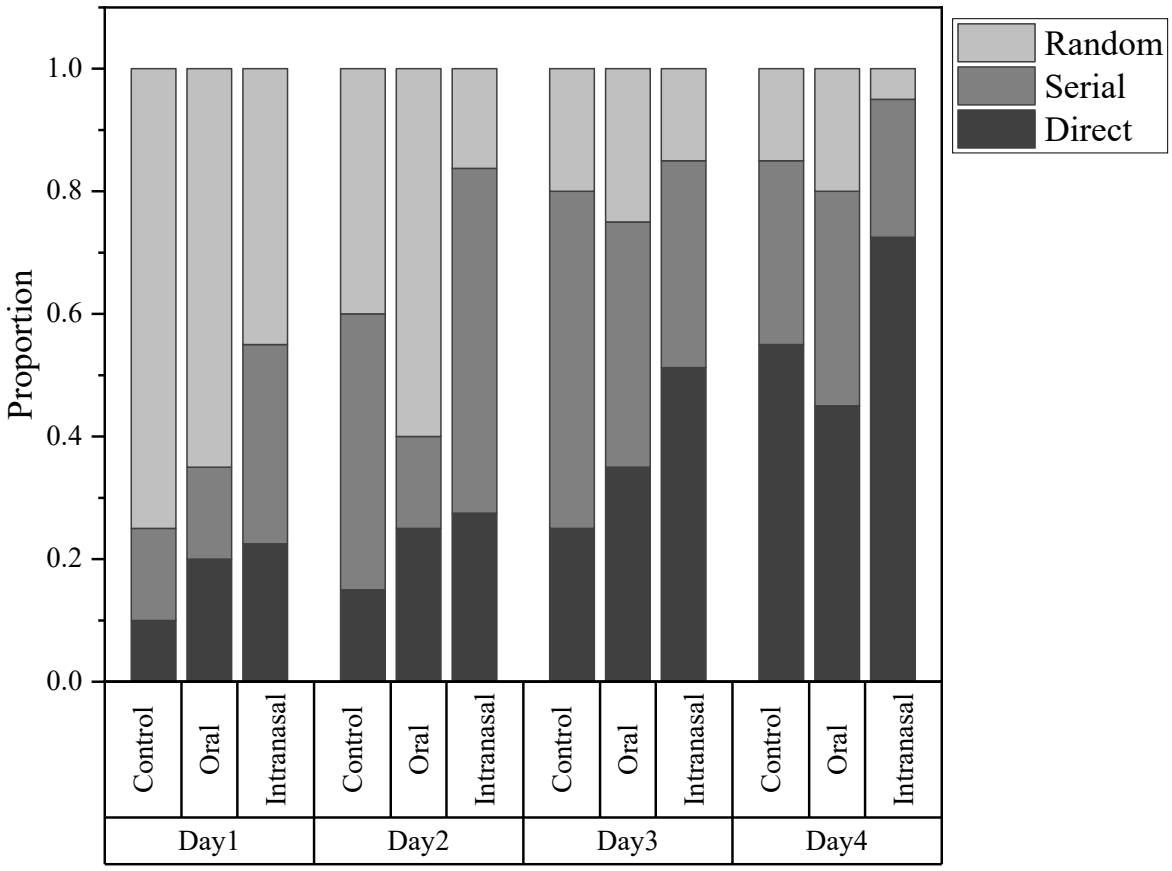
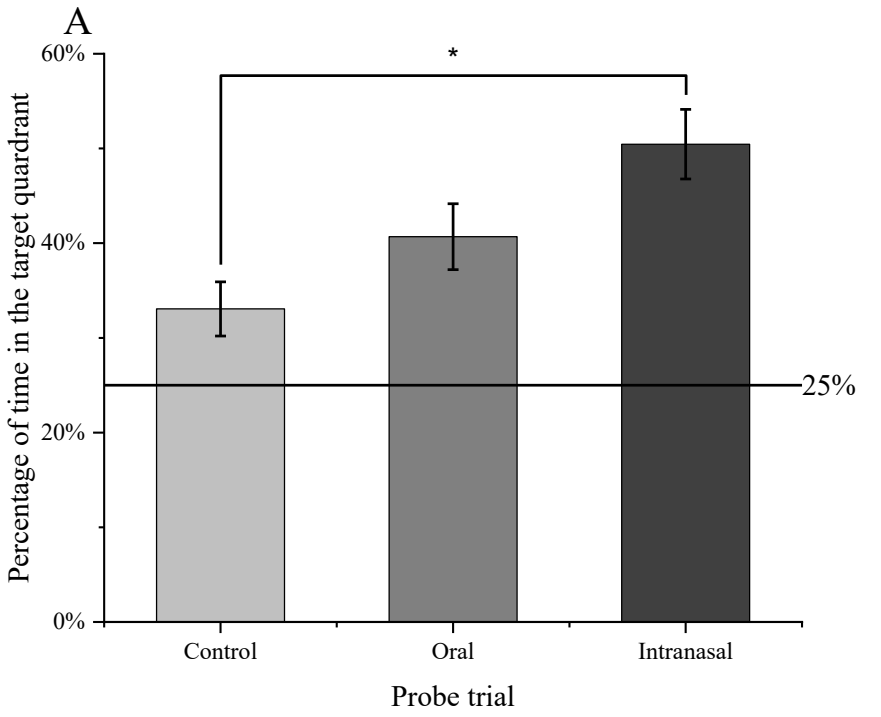


Figure 3.4 The search strategies of day 1-4 Barnes maze acquisition phase in control, oral, intranasal groups.

3.4.3 Barnes Maze Probe Trial

Probe trial experiments were conducted 24 hours after the last day of acquisition phase. The target box was removed, before the probe trial starts and each rat was free to explore the maze for 3 minutes. The rat trajectories and the percentage of time in the target quadrant were analyzed by ezTrack. Percentage of time in the target quadrant and poke

percentage of each hole are presented in Figures 3.5 A and 3.5 B. By comparing group probe trial data, it can be seen that the control group spent the lowest percentage of time while the intranasal group had the highest percentage of time in the target quadrant among the three groups. The time spent in the target quadrant in the intranasal group was significantly higher than that of the control group according to one-way ANOVA analysis with post hoc Tukey's test ($p = 0.03$). However, there was no significant difference in percentage of time in the target quadrant between the intranasal group and the oral group ($p = 0.121$), and there was no significant difference in percentage of time in the target quadrant between the oral group and the control group ($p = 0.246$) (Figure 3.5 A).


A
60%
40%
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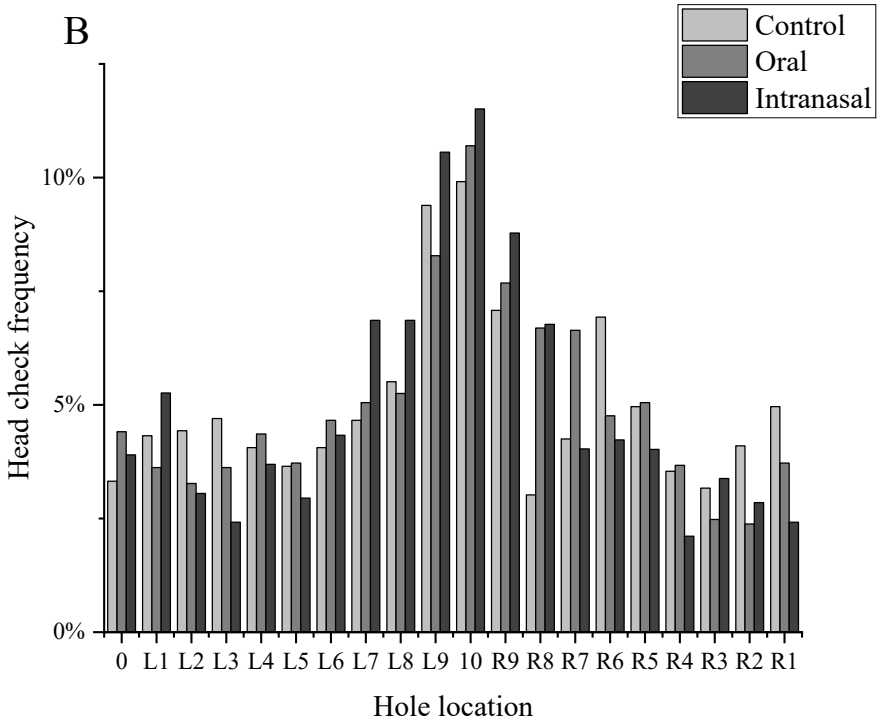
B
Control
Oral
Intranasal
10%
5%
0%
Head check frequency
0 L1 L2 L3 L4 L5 L6 L7 L8 L9 10 R9 R8 R7 R6 R5 R4 R3 R2 R1
Hole location

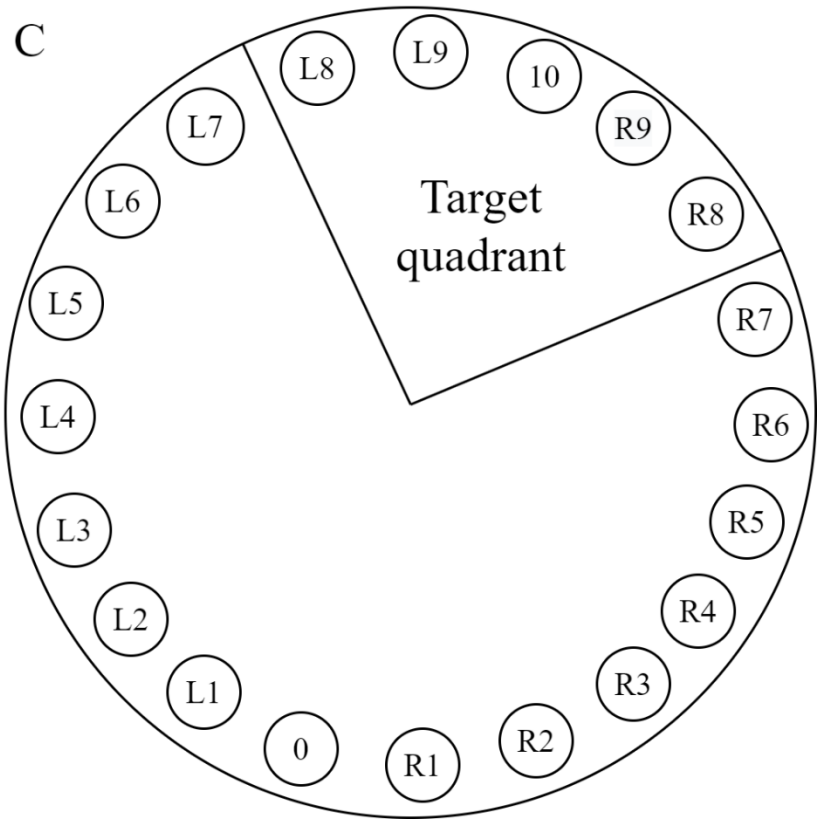

Figure 3.5 Barnes maze probe trial. Figure 3.5 A. Percentage in the target quadrant, Figure 3.5 B. Poke percentage in each hole. Target quadrant: The quadrant from the center of the platform to the two adjacent holes to the left and right of the target hole. The target quadrant accounts for 25% of the total area of the maze platform. Figure 3.5 C. The target hole was marked 10. The hole adjacent to the left of the target hole was marked L9, and the hole adjacent to the right of the target hole was marked R9. Hole opposite to the target hole was marked as 0.

3.4.4 Biochemical Analysis

Olfactory bulbs, medial prefrontal cortex, and hippocampus creatine concentration were measured using creatine assay kit (Figure 3.6) (Sigma Aldrich, St. Louis, MO). The olfactory bulbs, medial prefrontal cortex, and hippocampal creatine in the intranasal group were higher than that in the control group ($p = 0.01$, $p = 0.01$, $p < 0.01$) and the oral group ($p < 0.01$, $p = 0.036$, $p = 0.026$). The creatine in the medial prefrontal cortex and hippocampus of the rats in the oral group tended to increase, but the change was not statistically significant. Notably, the upward trend of creatine in the oral group was not found in the olfactory bulbs area.

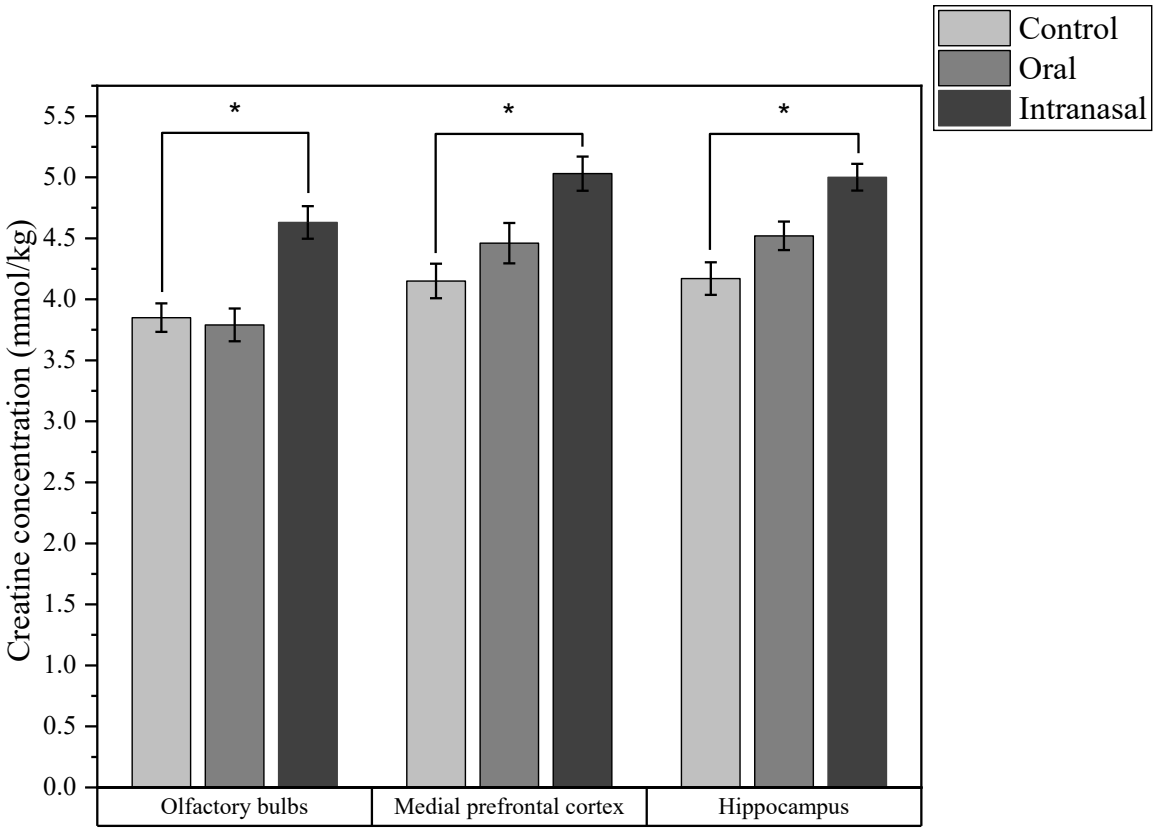

Figure 3.6 Biochemical analysis of levels of creatine in olfactory bulbs, medial prefrontal cortex, and hippocampus. The mean value of the group and standard error of the mean for each data point are shown.

3.4.5 Regression Analysis

Regression analysis was applied between percentage of time in the target quadrant during the probe trial and the concentration of creatine in the hippocampus (Figure 3.7). Creatine concentration in the hippocampus was positively correlated with percentage of time in the target quadrant ($r = 0.65$, $p < 0.001$)

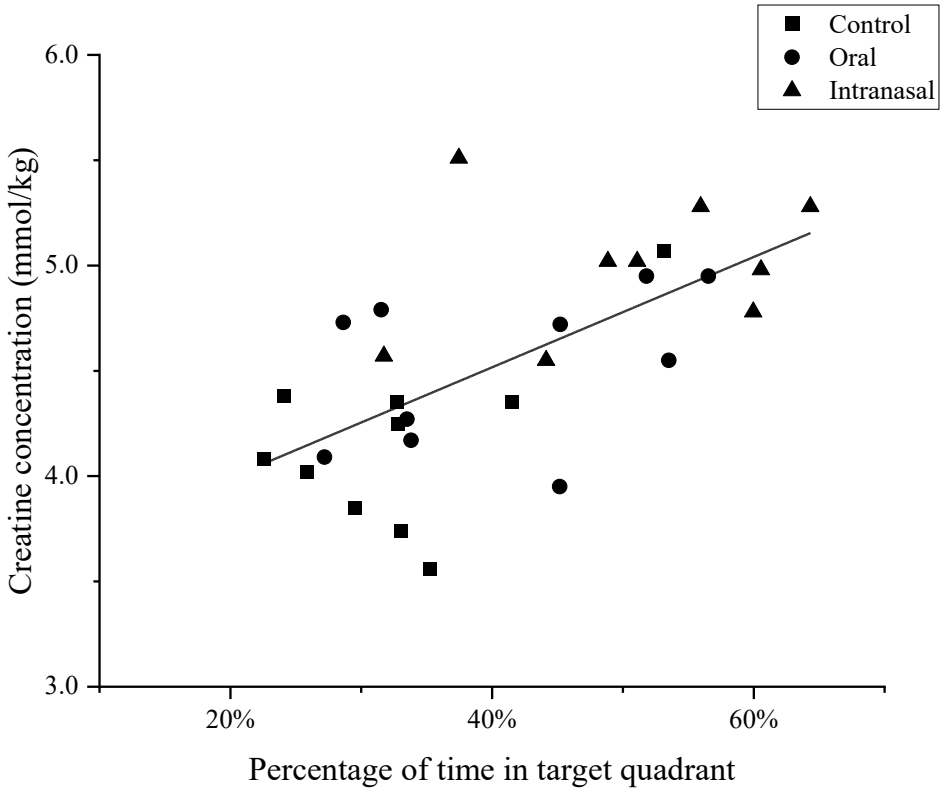

Figure 3.7 Correlation between creatine concentration in the hippocampus with percentage of time in the target quadrant in probe trial.

3.5 Discussion

As a part of the rapid energy supply system, creatine is considered necessary for the normal functioning of the brain. Oral creatine supplementation to the brain is thought to be inefficient because of the lack of effective transportation across the BBB (Braissant, 2012). Intranasal administration is used as an alternative route of oral administration, delivering drugs directly to the brain by bypassing the BBB. The baseline level of creatine in the brain was obtained by eating creatine free diet from the control group of rats. After fourteen days of intranasal or oral creatine administration, the rats were euthanized, and creatine concentration in brain regions were measured using a creatine assay kit. Creatine in the olfactory bulbs was measured because the olfactory bulbs are considered to be the area that the intranasal administration route must pass through. In addition, medial prefrontal cortex and hippocampus creatine was assessed since both structures are known to be involved in cognitive function and memory (Hoover & Vertes, 2007; Jarrard, 1993). The memory process consumes brain energy reserves, and creatine supplementation can improve memory by accelerating energy circulation and increasing 'energy pool' capacity.

3.5.1 Biochemical Analysis

In the brain tissue measurements, the olfactory bulbs, medial prefrontal cortex, and hippocampus were found to have significantly increased creatine levels in the intranasal group compared to the control and oral groups. We also found that the creatine content in the medial prefrontal cortex and hippocampus of the rats in the oral creatine administration group showed an increase in creatine level, although the difference was not statistically

significant, suggesting that oral creatine administration could increase brain creatine levels. However, according to biochemical assay results, fourteen days of oral creatine supplementation at the dosage was not enough to achieve a significant improvement in creatine level. Accumulation of creatine through oral administration in the brain may require repeated dosing over several weeks, which is consistent with previous studies (Perasso et al., 2003). Interestingly, comparing the oral group with the control group of rats, creatine in the olfactory bulbs did not show an upward trend, while there is a clear elevation of creatine level in the olfactory bulbs with intranasal administration. This observation indicates that changes in brain creatine level by intranasal creatine administration are transported to the brain through the nasal pathway, rather than the gastrointestinal pathways.

3.5.2 Barnes Maze Performance

We also used the Barnes maze to assess changes in cognition and memory after creatine supplementation. Contrasting the mean values of primary latency and the number of errors on the first day and fourth day of acquisition trials, all three groups of rats showed significant improvement over time in the acquisition phase, reflecting the learning effects in the groups. Differences between the groups resulted from differences in learning and memory. Intranasal administration of creatine improved spatial memory in rats as reflected in primary latency and the number of errors in acquisition phase. Compared to the control group, no significant improvement in memory was observed after oral administration.

During the probe trial, all three groups spent more than 25% of the time in the target quadrant, which indicates that the three groups of rats remembered the target box location. The target quadrant accounts for 25% of the total maze platform area. If rats spent more than 25% of the time in the target quadrant, we conclude that rats were more likely to remember the location of the target box than to explore the maze platform randomly. We observed that the intranasal group spent a significantly higher percentage of time in the target quadrant compared to the control group, suggesting that creatine has an improved effect on short-term memory. However, the time spent in the target quadrant was not significantly improved in the intranasal administration group compared to the oral administration group. Comparing the Barnes maze probe trial data and hippocampal creatine biochemical analysis, we found that there was a significant positive correlation between hippocampal creatine content and the proportion of time spent in the target region during the probe trial.

In the reversal learning experiment, we found no improvement in the number of errors or primary latency in the oral or intranasal groups compared to the control. This may be due to the absence of repeated measures of reversal learning in consecutive days.

3.5.3 Limitations and Future Work

Although we found that creatine was elevated in different parts of the rat brain following intranasal creatine administration, the mechanism by which creatine passes through the nasal mucosa remains unclear. Based on literature, creatine can only pass through the nasal mucosal cells through the transcellular pathway with low efficiency due

to its hydrophilicity. Paracellular pathways can be used for small molecules like creatine, as the paracellular pathway is suitable for molecules with molecular weights less than 1000 Da (McMartin et al., 1987). Investigating how creatine bypasses BBB and enters the brain via intranasal administration remains to be further studied.

In addition to improving cognition and memory, creatine supplementation may have neuroprotective effects on neurodegenerative disease. In animal model experiments, oral creatine showed a neural protective effect in Parkinson's disease and Huntington's disease (Andreassen et al., 2001; Matthews et al., 1999). Whether intranasal creatine administration provides greater therapeutic effects in diseases remains unknown. Therefore, exploring creatine intranasal administration as a potential therapeutic approach could be a future research direction.

3.6 Conclusion

In conclusion, we have demonstrated that intranasal creatine administration increases brain creatine levels and improves cognitive performance in rats. The effects of intranasal administration are significantly higher than those of oral administration. Our results point to a more effective means of creatine supplementation to the brain.

3.7 Reference

Andreassen, O. A., Dedeoglu, A., Ferrante, R. J., Jenkins, B. G., Ferrante, K. L., Thomas, M., Friedlich, A., Browne, S. E., Schilling, G., & Borchelt, D. R. (2001). Creatine increases survival and delays motor symptoms in a transgenic animal model of Huntington's disease. *Neurobiology of disease*, *8*(3), 479-491.

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Barnes-Maze-Rat-Image-2. https://conductscience.com/maze/wp-content/uploads/2015/09/Barnes-Maze-Rat-Image-2.jpg

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4 Chapter 4 Creatine Microparticles Assembly, Characterization Measurement, and in Vitro Delivery Study by Human Epithelial Cells

4.1 Abstract

Creatine has been administered by various methods other than oral administration: intracerebral injection, intraperitoneal injection, and intranasal administration. Although Intracerebral injections significantly increases creatine levels in the brain, due to its invasiveness, it may bring other potential risks such as infection. In other administration methods such as creatine intraperitoneal injection, creatine rapidly increases its concentration in the systemic circulation. Although this is the case, the entry of creatine from the systemic circulation into the brain remains inefficient due to the lack of creatine transporters at the blood-brain barrier region. In intranasal studies, creatine has been shown to accumulate in the brain through the olfactory pathway. Due to the limited aqueous solubility of creatine and the volume limitation for intranasal administration, the main limitation is the concentration of creatine administered. This study changed the formulation, using a suspension to increase the concentration of creatine per unit volume. Instead of directly using creatine powder, we assembled creatine microparticles for studying in vitro drug delivery. Compared with creatine powder, the size of creatine particles is significantly smaller, which makes it easier to distribute the suspension of creatine evenly. As an

additional benefit, creatine microparticles have a high assembly. In the in vitro model, the creatine microparticle suspension can safely pass through the Calu-3 cell layer without disrupting the cell barrier function. Creatine suspension administration has been shown to be more effective than creatine solution administration.

4.2 Introduction

Creatine, an important component of energy cycling, is primarily stored in organs, where energy demands fluctuate (Wallimann et al., 1992). As one of the most abundant metabolites in the brain, creatine was identified by magnetic resonance spectroscopy alongside N-acetyl aspartate, choline, and other metabolites decades ago (Christiansen et al., 1993; Kreis et al., 1993). Studies have indicated that creatine can be synthesized in the brain cells, expressing arginine: glycine amidinotransferase (AGAT) and guanidinoacetate N-methyltransferase (GAMT) (Hanna-El-Daher & Braissant, 2016). Creatine has been shown to be necessary for normal brain function as it provides energy for cognitive functions (Forbes et al., 2022). Intellectual disability due to lack of creatine in the brain is one of the prominent symptoms of all kinds of creatine deficiencies (Schulze, 2013). With orally administered creatine, supplementation to the brain has been effective, however inefficient. Most of the orally administered creatine ends up in the skeletal muscles, improving muscle creatine levels and exercise performance as consequence (Harris et al., 1992; Volek et al., 1999). Despite creatine's effectiveness in skeletal muscles, improvements in brain function from creatine supplementation may require larger doses and longer periods of time due to the blood-brain barrier (BBB) (Dolan et al., 2019).

The BBB selectively blocks permeable compounds and nutrients from the blood to the brain (Luissint et al., 2012). Diseases of the central nervous system (CNS) are difficult to treat mainly due to the lack of effective drug delivery methods to penetrate the BBB. The barrier effect of the BBB is mainly achieved through a low rate of transcytosis vesicles

and tight junctions (Kniesel & Wolburg, 2000). Specifically, tight junctions of the BBB are non-permeable for macromolecules, drugs, and 98% of small molecules (Pardridge, 2005). These mechanisms limit drug entry into the CNS from both transcellular and paracellular pathways.

Different approaches have been developed to enhance the delivery of drugs to the CNS or to treat CNS diseases, such as intracerebroventricular injection (ICV) and intracerebral implants. ICV has been applied to neurodegenerative disorder subjects including Alzheimer's disease, amyotrophic lateral sclerosis (ALS), and progressive multiple sclerosis (MS-P) (Atkinson, 2017; Duma et al., 2019). In a study using ICV treatment, a majority of subjects showed improved cognitive performance in the Alzheimer's disease group and improved conditions in the MS-P group (Duma et al., 2019). However, in another phase 1 clinical trial of ICV, subjects reported varying degrees of side effects, including headaches, nausea, and vomiting (Atkinson, 2017; Kim et al., 2021). In an article discussing the safety of intracranial implantation, problems similar to ICV are also pointed out, including infection, bleeding (Bullard et al., 2020). In addition, after long-term implantation, pulse generator failure, as well as lead displacement and fracture problems are all limitations of intracranial implantation (Bullard et al., 2020). Although these methods are effective for treating CNS diseases, they are considered invasive and could potentially cause infection or allow pathogens to enter the brain.

A noninvasive method to deliver drugs to the brain is through the focused ultrasound-induced BBB opening method (Chen et al., 2019). Studies have proven that the focused ultrasound-induced BBB opening reduces β-Amyloid plaque in the early

Alzheimer's disease ultrasound treatment group (D'Haese et al., 2020). Although this method is noninvasive, by opening the BBB with this procedure, there are potential tissue lesions and microbleeds that need to be considered (Meng et al., 2019). To avoid these risks, intranasal administration can be used as drug delivery method that is noninvasive to the subject, while avoiding disruption to the barrier function of the blood-brain barrier.

In general, small, and lipophilic drugs are more likely to pass through the nasal mucosa without drug manipulation (McMartin et al., 1987). However, intranasal administration of macromolecular hydrophilic drugs is difficult with low permeability to the olfactory epithelium by transcellular pathway (Ozsoy et al., 2009). In terms of paracellular pathway, researchers have found a relationship between the molecular weight of drugs and the absorption rate through intranasal administration. It was found that the higher the molecular weight, the lower the absorption rate (Romeo et al., 1998). Another limitation of intranasal administration is the lack of therapeutic effect due to the volume limitations of intranasal drugs (Ozsoy et al., 2009). For example, the maximum volume of a single nostril utilizing intranasal administration should not exceed 0.5 - 1 ml, otherwise the drug solution will come out of the nostrils (Wolfe & Bernstone, 2004). In the past creatine solution has been administered intranasally in creatine deficient animal models and has shown reduced lesions after strokes and brain creatine accumulation (Chen et al., 2021). Another study indicated intranasal administered creatine hydrochloride solution can increase rat brain creatinine level and improve rats' Barnes maze performance during the acquisition phase and probe trial (Chen & Hu, 2023).

In both studies, creatine dosing was limited within the solubility range. Increasing temperature or lowering pH can further increase creatine solubility (Jäger et al., 2011). According to a previous study, the solubility of creatine has a nearly linear relationship with temperature (Jäger et al., 2011). Creatine can only dissolve 14 mg/ml in water at 20 °C, while in 60°C water, the solubility of creatine is 45 mg/ml (Jäger et al., 2011).

Both temperature and pH strategies have their own limitations in intranasal administration. Although heating the drug with solvent is helpful for drug solubility, the method of heating is difficult to operate. After the drug solution leaves the intranasal administration device, typically a nasal sprayer, the temperature of the intranasal drug will begin to drop continuously to ambient temperature. Moreover, it is also difficult to keep the temperature constant during intranasal administration. In a study recording the nasal cavity temperature while breathing, the average temperature range of the nasal mucosa was measured to have a significant difference between inspiration and expiration (Lindemann et al., 2002). In addition to breathing changing the temperature of the nasal mucosa, the temperature inside the nasal cavity is also different (Yu et al., 2014). The intranasal temperature shows an increasing trend from the nostrils to the nasopharynx (Yu et al., 2014). Unstable environmental temperatures like this make intranasal solutions difficult to maintain constant temperature during drug delivery and absorption phase. Furthermore, the high temperature and strong acidity of the intranasal solution may cause potential nasal irritation during administration.

In contrast, intranasal suspension delivery can effectively address the limitations of drug concentration in intranasal solution administration. Using a suspension allows a

higher concentration of drug to be administered in the same unit volume and reach a higher bioavailability. In addition, suspension formulation can also be optimized by adding an absorption enhancer to facilitate absorption or a thickener to prolong the exposure time of the drug on the nasal mucosa while reducing the clearance effect.

To overcome the limitation of creatine solubility and improve the efficiency of drug delivery, creatine powders were micronized by transient emulsions self-assembly method (Liu et al., 2021). The creatine microparticles were then imaged using a scanning electron microscope (SEM). The average size and size distribution of creatine microparticles were calculated and compared with laboratory grade creatine and commercially available creatine monohydrate. In addition, the Fourier-transform infrared spectroscopy (FTIR) spectrum confirmed that the creatine microparticle chemical structure and functional groups were the same as pure creatine. According to the results of the creatine assay kit, the assembly rate and stability of creatine microparticles were also determined.

Calu-3 cell lines have been evaluated and applied in multiple in vitro intranasal administration and drug delivery studies (Sibinovska et al., 2022; Zhang et al., 2016). In this study, Calu-3 cells were used to assess the toxicity of creatine microparticles as well as used in in vitro permeability experiments. Specifically, the cytotoxicity induced by creatine microparticles was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The MTT is reduced into insoluble purple formazan crystals by the mitochondria in living cells, dead cell mitochondrial reductase is inactive and does not produce formazan crystals (Kumar et al., 2018). MTT assay evaluates drug cytotoxicity in multiple in vitro studies focusing on intranasal administration (Matilainen

et al., 2008; Ye et al., 2013; Zhang et al., 2016). The toxicity of creatine microparticles was measured at different concentrations in this study. The creatine microparticle concentration used during the MTT assay were 1, 10, 20, 40, 80, 120 mg/ml. The cytotoxicity of creatine solutions was also measured, using a creatine concentration of 1 and 10 mg/ml. In this condition, creatine microparticles were completely dissolved in the cell medium. When the creatine concentration reaches 14 mg/ml and higher, the creatine concentration exceeds its solubility, and undissolved creatine microparticles form a creatine suspension, starting from 20 mg/ml, the creatine concentration is 1.4 – 8.6 times higher than the solubility of creatine at room temperature.

In the in vitro creatine microparticle delivery experiment, Calu-3 cells were cultured on the apical side of transwell inserts for 21 days and later used to measure creatine microparticles delivery across the epithelial cell layer. In order to evaluate the cell coverage and integrity of the Calu-3 cell layer, we assembled the transepithelial/endothelial electrical resistance (TEER) system by a published method (Theile et al., 2019). In this study, inserts with recorded cell resistances above 600 Ω were considered achieved by intact cell layers. The creatine microparticles were then measured through the Calu-3 cell layer using a creatine assay kit. The effect of absorption enhancer on creatine delivery was compared by an additional experimental group using an absorption enhancer Polyvinylpyrrolidone (PVP). In a previous study comparing the penetration of different absorption enhancer formulations to the sheep nasal mucosa, PVP showed an increase in drug permeation to the nasal mucosa (Karasulu et al., 2008). In another in vivo animal intranasal administration, it was proved that PVP had a similar enhancing effect (Ogawa et al., 2021).

Cell layer integrity after creatine delivery experiment was assessed by several methods. First, changes in the TEER values of the control group and the creatine treatment group were calculated and compared. Secondly, the permeability of Lucifer yellow after the creatine administration was measured. Lucifer yellow is a fluorescence dye that has been used to assess cell layer barrier function in previous studies (Au - Zhao et al., 2019; Frost et al., 2019). If the cell layer or tight junction structure is damaged by the creatine microparticles, it will lead to an increasing permeability to Lucifer yellow. Then, the nuclei and actin filaments were stained by Hoechst and phalloidin accordingly, and cell images were taken by a fluorescence microscope. Fluorescent images can visualize cell layer and detect cell layer damage macroscopically.

Our experiments indicate that creatine microparticles are significantly smaller than creatine and creatine monohydrate. Creatine microparticles showed no significant cytotoxicity for 1 hour treatment and are permeable to the Calu-3 cell layer. Creatine microparticles has high assembly rate and stability. In vitro creatine administration found that administration of creatine suspension is more efficient than creatine solution and does not disrupt the barrier function of the cell layer. This study would validate a method to increase creatine concentration above the solubility for future intranasal creatine administration.

4.3 Materials and Methods

4.3.1 Materials

Creatine and Creatine assay kit were from Sigma-Aldrich. Polyvinylpyrrolidone (PVP) was brought from Tokyo Chemical Industry. Dimethyl Sulfoxide (DMSO), 1-butanol was from Fisher Chemical, Eagle's Minimum Essential Medium (EMEM) was purchased from American Type Culture Collection. The phosphate-buffered saline (PBS), penicillin and streptomycin, 0.25% Trypsin, 96-well plates, T-75 cm^2 cell culture flasks and 12 wells Transwell plates with 12 mm diameter 0.4 μm pore size polycarbonate membrane inserts were from Corning. Hanks' balanced salt solution (HBSS) and fetal bovine serum were purchased from Gibco. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Tocris Bioscience and phalloidin 488 was from Cell Signaling Technology. Triton X-100 were from Ricca accordingly. Hoechst 33342 was from Thermo Fisher and Lucifer yellow was from Biotium.

4.3.2 Creatine Microparticle Assembly

A self-assembly transient emulsion method from a previous published paper was applied to creatine microparticle production (Liu et al., 2021). First, creatine powder was dissolved in a 5% W/V PVP solution at a concentration of 10mg/ml. Next, when the creatine powder was completely dissolved in the PVP solution, then added it to 1-butanol in a 1:10 ratio. We then used an ultrasonic bath to disperse creatine solution evenly and assembled it into creatine microparticles. After the creatine microparticles were collected from the 1-butanol using a centrifuge, the 1-butanol supernatant was removed. Following

that, creatine microparticles were washed with a freshly made creatine solution, removing 1-butanol residual, and then dried in a vacuum desiccator at room temperature.

4.3.3 Fourier-transform Infrared Spectroscopy (FTIR)

FTIR wavenumber from 4000-400^{-1} was performed and compared between creatine microparticles and pure creatine by Thermo Fisher Scientific Nicolet iS10. The spectra of the samples were reconstructed and visualized using.

4.3.4 Scanning Electron Microscope (SEM)

SEM was performed to measure creatine microparticle size distribution. The size of creatine microparticles, creatine and creatine monohydrate were determined from TESCAN Vega3 SBH. The SEM and bright field images were imported into ImageJ, and the size distribution was calculated. 30 samples in each image were used to calculate the average size and standard deviation. To avoid bias in particle selection, the particle size was selected and measured by a researcher blinded to the sample condition.

To interpret SEM images, we used Feret's diameter to read and calculate the average size of creatine particles. Feret's diameter is often used in analysis for determining the average size of a sample, be it pore size, particles, or fibers. Feret's diameter can be explained as the size measurement of an object along a specified direction; for the case of the projection of a 3D object on a 2D plane, it is defined as the distance between two

parallel tangents (Yolanda & Nandiyanto, 2022). The Feret's diameter equation is described by the following:

$$D_{\text{ferret}} = \frac{D_{max} + D_{min}}{2}$$

Feret diameter was calculated similar to the figure below. The illustration of D_{max} and D_{min} is shown in Figure 4.1.

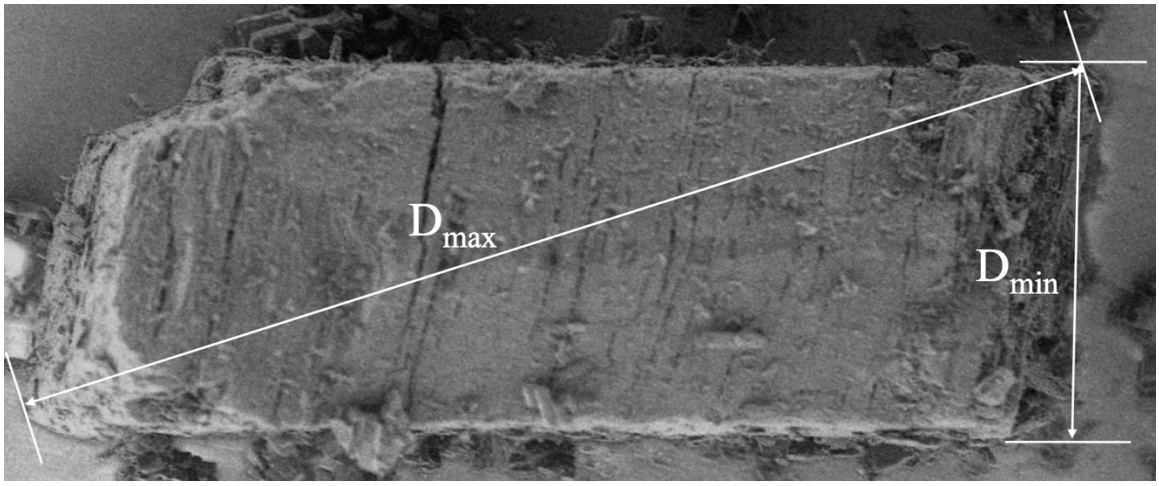


Figure 4.1 Illustration of Feret's diameter measurement. Creatine powder is shown in the image. The image was taken by Hitachi TM4000PlusE II at 100 X magnification.

4.3.5 Creatine Microparticles Assembly Rate and Stability

A creatine assay kit was used to assess the total creatine content in the microparticles assembled from 100 ul of creatine solution. To calculate the assembly rate, we compared it to a stock creatine solution of the same volume. Stability was also measured

by comparing creatine content from 1 day, 1 month, and 6 months of creatine microparticles samples.

4.3.6 Calu-3 Cell Culture Protocol

Calu-3 cells were cultured in 25 or 75 cm^2 cell culture flasks at a 37°C incubator with 5% CO_2. The cell complete culture medium was made from EMEM, with 1% penicillin and streptomycin, and 10% fetal bovine serum. The cell medium was replaced every 2-3 days. Cells were passaged before they reached full confluency every week and split at a 1:5 ratio during cell passaging.

Calu-3 cells were trypsinized from T-75 cell culture flasks by 0.025% Trypsin then seeded on prewarmed 12-well transwell plates at a density of 5×10^5 cells per insert. Cells were cultured on transwell inserts for a total of 21 days. Starting on day 3, the cell culture medium was changed every other day with a warmed cell culture medium. Dead cells were washed by warmed PBS before adding fresh cell culture medium. 500 µl of complete cell culture medium was added to the apical side of transwell inserts and 1500 µl of complete cell culture medium was added to the basolateral side of transwell inserts.

4.3.7 Cell Viability Assay

Cell viability was measured by MTT assay after creatine microparticle treatment. MTT powder was dissolved in PBS solution at 1 mg/ml concentration and stored at -20°C until use. Calu-3 cells were seeded in 96 well plates at the density of 1×10^4 cells per well.

Then, 96 well plates with Calu-3 cells were incubated 24 hours before creatine treatment. Calu-3 were treated with 1, 10, 20, 40, 80, and 120 mg/ml of creatine microparticles in a complete cell culture medium for 1, 2, and 4 hours. To each well, 100 µl of creatine microparticles solution or suspension was added. After treatment, creatine microparticles were removed and washed by PBS, then 110 µl premixed MTT solution with complete cell culture medium at a ratio of 1:10 was added to each well. Cells were incubated in a cell culture incubator for 4.5 hours at 37°C in the following step. After the incubation, the MTT solution was carefully aspirated and 100 µl of DMSO was added to each well. DMSO was also added to blank wells at a volume of 100 µl to measure the blank well absorbance value. To facilitate the dissolution of formazan crystals, the 96-well plate was incubated at 100 rpm for 15 minutes in a 37°C shaker until formazan crystals were fully dissolved. Following this, the absorbance of the formazan was measured by a spectrophotometer at a wavelength of 570 nm. The cell viability was calculated using the following equation:

$$Cell\ viability = 100\% * \left(\frac{Experimental\ group\ reading - blank\ control\ reading}{Control\ group\ reading - blank\ control\ reading}\right)$$

4.3.8 Trans-Epithelial Electrical Resistance (TEER) Measurement

The TEER system was assembled based on a published paper (Theile et al., 2019). TEER was measured every four days starting from day 9 after Calu-3 cells were seeded on the transwell insert. TEER were measured five times throughout the transwell study on

days 9, 13, 17, and before and after creatine treatment for day 21. TEER for each insert was measured from three different sides. The average and standard error of the mean was calculated and recorded by each insert. Only inserts with a resistance value greater than 600 Ω were used to measure creatine permeability. Background electrical resistance value was measured from empty inserts without cells while total electrical resistance value was measured from the inserts with cells. The growth area of each insert is 1.12 cm^2. The TEER calculation formula is shown below (Srinivasan et al., 2015).

$$Cell\ resistance \frac{\Omega}{cm^2} = (Resistance_{total} - Resistance_{blank}) \times\ 1.12\ cm^2$$

4.3.9 Creatine Microparticles Delivery Measurement

Calu-3 cells were cultured 21 days on the transwell inserts before the creatine administration. Different concentrations of creatine solutions or suspensions were added to Calu-3 cells cultured in transwell inserts on day 21. 10, 20, 40 mg/ml of creatine microparticles, or 40 mg/ml creatine microparticles with 5% w/v PVP solution were used in this permeability study. 500 µl of creatine solution or creatine microparticles suspension in HBSS buffer was added to the apical side of transwell inserts while 1 ml of HBSS was added to the basolateral side. Cells were treated with creatine microparticles at different concentrations in a 37°C incubator with 5% CO_2 for 1 hour, then HBSS samples from the basolateral side were collected for creatine assay measurement.

4.3.10 Creatine Permeability Assay

Creatine was measured according to the instructions from the creatine assay kit. The standard curve was generated from the creatine standard included in the kit. Samples were diluted by creatine assay buffer to fit creatine assay measurement range, and then each sample measured in duplicate. 50 µl of diluted samples with 50 µl of creatine assay master mix were cultured in a 37°C incubator for 1 hour. The absorbance of the sample was measured by a spectrophotometer at wavelength 570 nm.

4.3.11 Post-Treatment Analysis for Cell Layer Integrity by Lucifer yellow Permeability Assay

Lucifer yellow was applied to both transwell inserts after creatine microparticle permeability tests as well as blank inserts. For each insert, Lucifer yellow was diluted into HBSS buffer at concentrations of 100 nM. 500 µl of Lucifer yellow solution was added to the apical side of the transwell inserts while 1 ml of HBSS was added to the basolateral side. The plate was then incubated in a 37°C incubator for 1 hour. Following this, HBSS from the basolateral side was collected to measure fluorescence intensity. The standard curve was generated from diluted stock Lucifer yellow solution. All samples were measured in duplicate.

4.3.12 Fluorescent Microscopy

After completing the post-treatment procedure, 4% PFA with 0.25% Triton X-100 was added to the apical side of the insert at room temperature for 30 minutes and washed

with PBS two times. Phalloidin and Hoechst was used to stain actin filaments and the nucleus. Cells were incubated with Phalloidin overnight at 4°C and incubated with Hoechst at room temperature for 20 minutes, fluorescence stains were washed twice with PBS. Transwell inserts were mounted between a coverslip and a glass slide before the imaging. Cell layers faced toward the oil immersed objective lens during the imaging. A Leica fluorescence microscope was used with the GPF channel for phalloidin stain imaging and the DAPI channel for Hoechst stain imaging.

4.4 Results

4.4.1 FTIR Spectra

In comparing the spectra of the pure creatine and creatine microparticles samples, creatine microparticles FTIR spectra exhibited the similar intensity and same characteristic peaks as pure creatine (Figure 4.2).

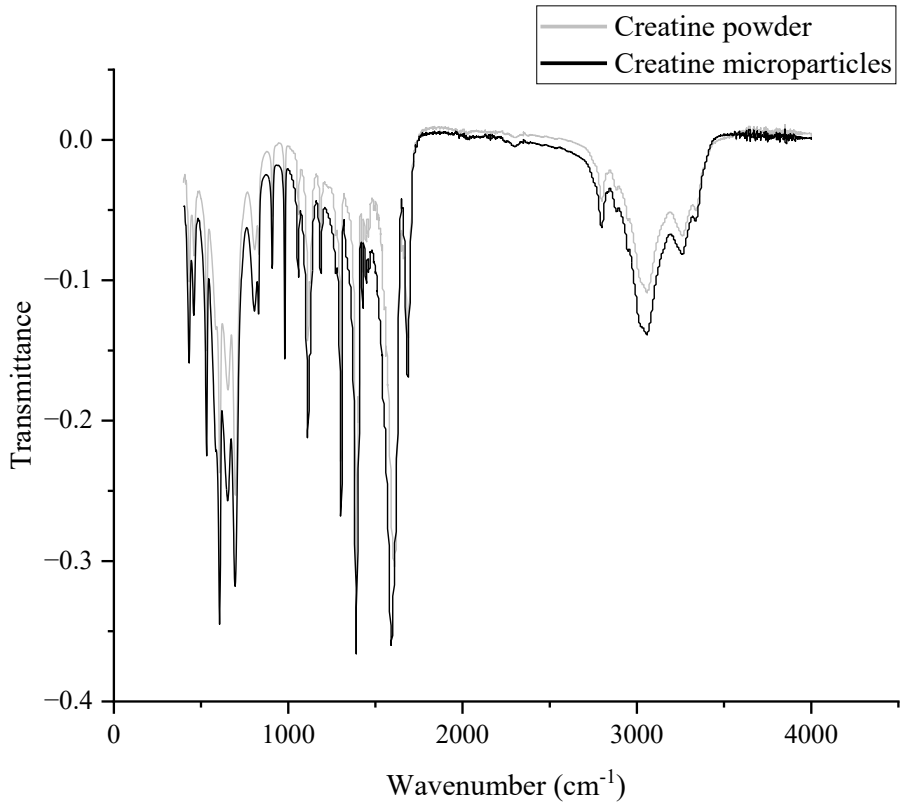


Figure 4.2 Comparison of FTIR spectra of creatine microparticles and pure creatine.

4.4.2 SEM Images

The average size, and size distribution was calculated by a researcher who was blinded to the sample's condition. 30 samples from each condition were randomly picked by the researcher using ImageJ. The observed average size of creatine microparticles is noted to be 3.44 μm. Creatine monohydrate was observed to have an average size of 120.72

μm, while creatine powder exhibited an observed average size of 118.8 μm (Figure 4.3). In the creatine samples, we observed many small powder fragments which were not counted as creatine samples. The same situation was found with creatine monohydrate samples as well. One-way ANOVA test results showed significant differences among the three groups of samples ($F = 87.110$, $p < 0.001$). The creatine microparticles was confirmed significantly smaller than creatine and creatine monohydrate by Tukey's post-hoc analysis ($p < 0.01$), while no difference was found between creatine monohydrate and creatine groups ($p = 0.981$). The SEM images of samples are shown in Figure 4.4. Size distribution of the samples are shown in Figure 4.5.

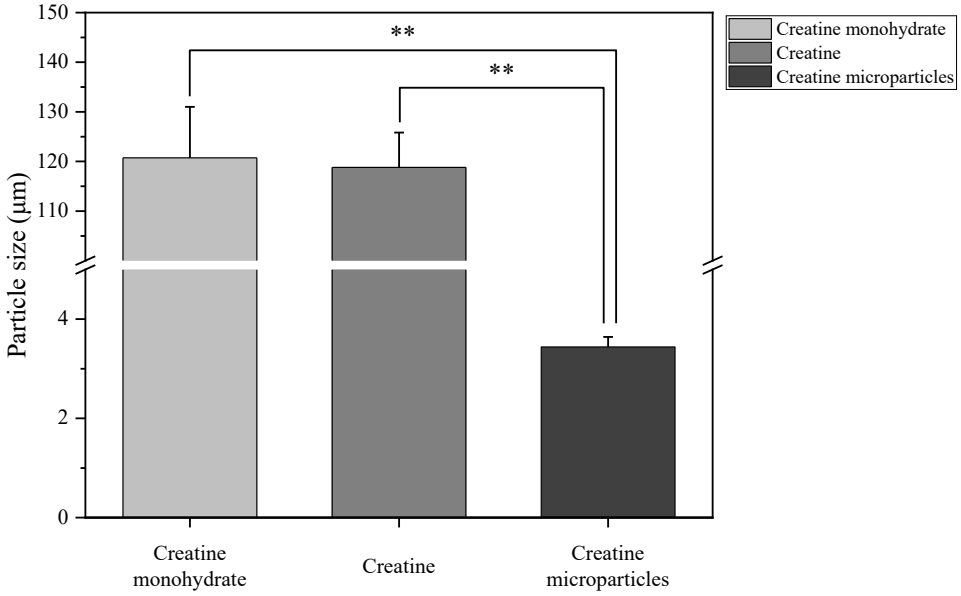


Figure 4.3 Particle size of creatine monohydrate, creatine and creatine microparticles. The mean value of the group and standard error of the mean are shown.

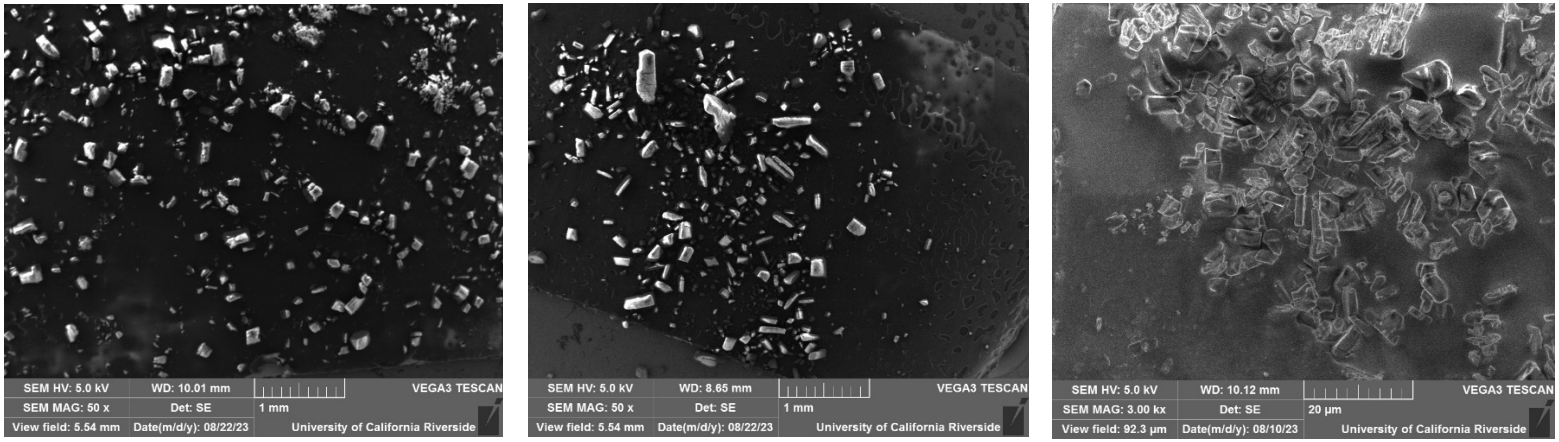


Figure 4.4 Representative SEM images. Left: creatine monohydrate, Middle: creatine, Right: creatine microparticles.

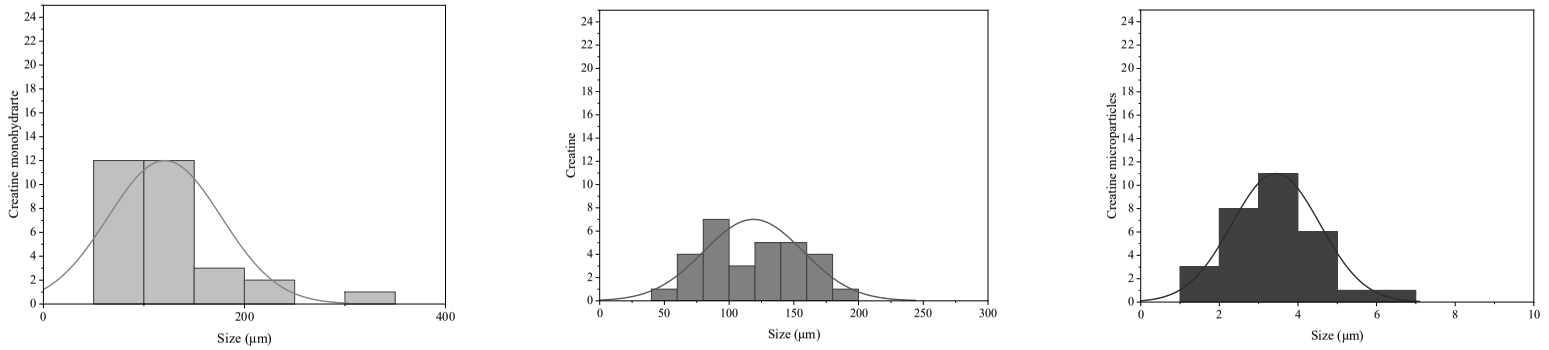


Figure 4.5 Size distribution of creatine monohydrate, creatine, and creatine microparticles. Left: creatine monohydrate, middle: B creatine, and right creatine microparticles.

4.4.3 Creatine Microparticles Assembly Rate and Stability

The assembly rate of microparticles was measured by creatine assay (Figure 4.6). The assembly rate of creatine microparticles was 96%.

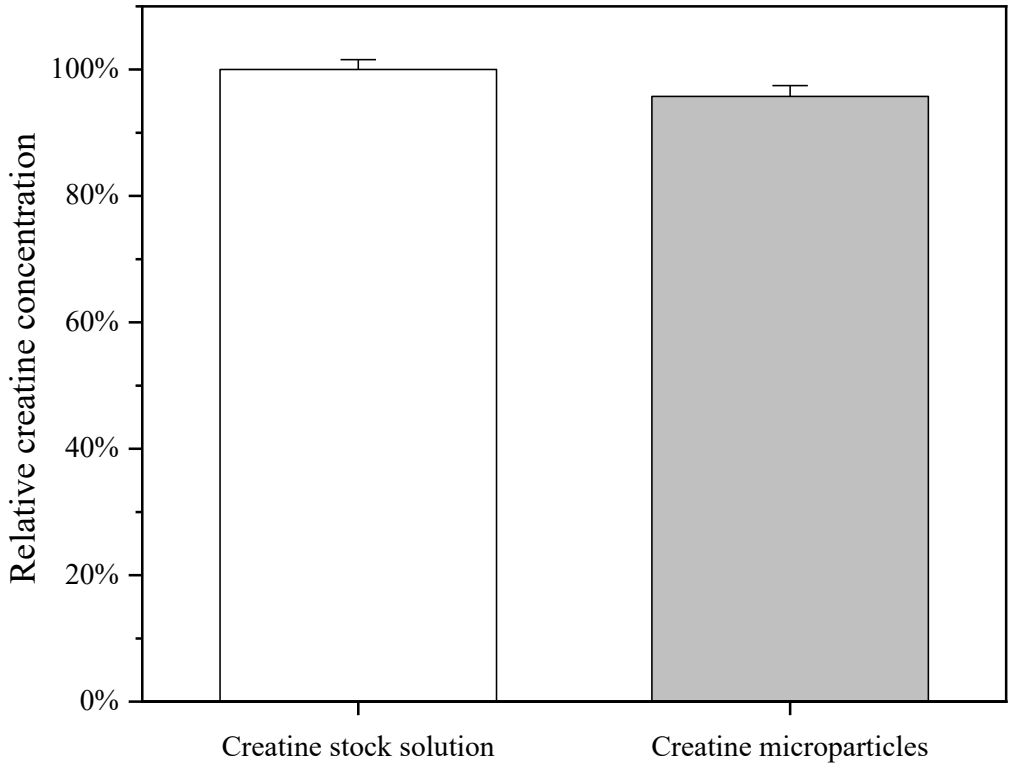


Figure 4.6 Creatine microparticles assembly rate measurement. The mean value of the group and standard error of the mean are shown.

Stability was also measured from three batches of creatine microparticle samples. The creatine microparticles were stored in a dry and room temperature condition for 1 day, 1 month and 6 months. Creatine content in samples of creatine microparticles 1 day, 1 month, and 6 months after assembly are shown in Figure 4.7. Concentrations are shown

after the dilution. The creatine content in creatine microparticle samples only dropped by 2.02% and 3.71% after one month and six months.

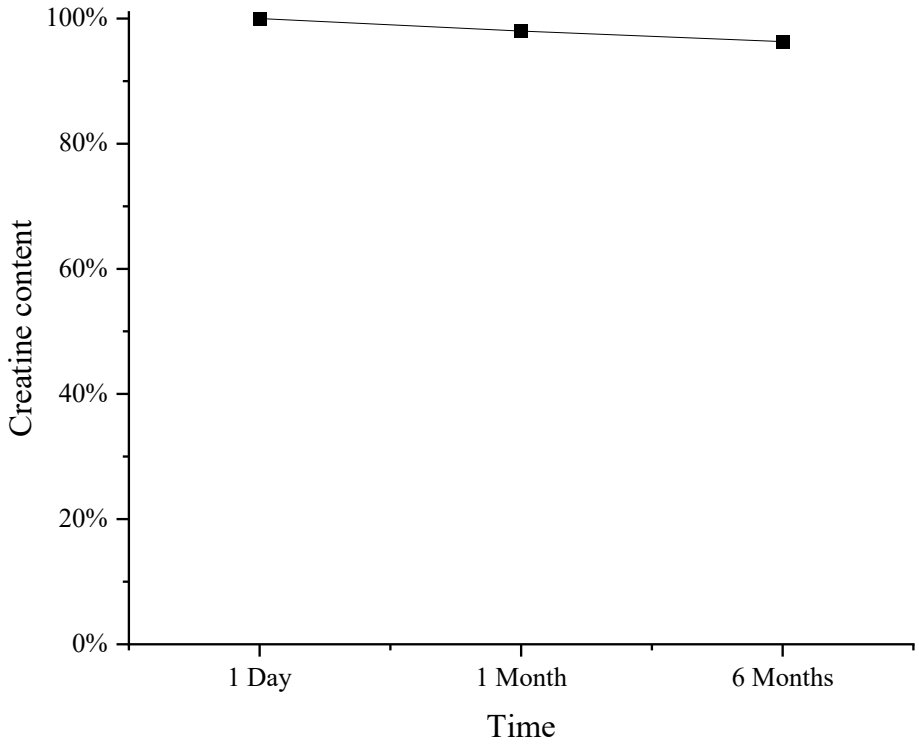


Figure 4.7 Creatine content in creatine microparticles over time.

4.4.4 Cell Viability Assay

MTT results are shown in the Figure 4.8 where cells were treated at different creatine concentrations for different durations. A one-way ANOVA test showed no significant difference between control and creatine treatment groups after 1 hour creatine treatment ($F = 1.312$, $p = 0.27$). Cell viability of the creatine treated groups after 2 hours

of treatment was significantly different with the control group. Specifically, the one-way ANOVA test showed significant differences between groups ($F = 5.728$, $p < 0.01$). Post hoc Dunnett t tests showed $p = 0.02$ in the 80 mg/ml group, and $p < 0.001$ in the 120 mg/ml group. No significant differences were found between the lower creatine concentration treatment groups (1, 10, 20, and 40 mg/ml) with the control group. In the 4 hours creatine treatment, some creatine treatment groups showed a lower cell viability compared to the control group ($F = 10.417$, $p < 0.01$). The post hoc Dunnett t-test showed a significant difference in 40, 80, and 120 mg/ml groups, when compared with the control group ($p = 0.002$, $p < 0.001$, $p < 0.001$).

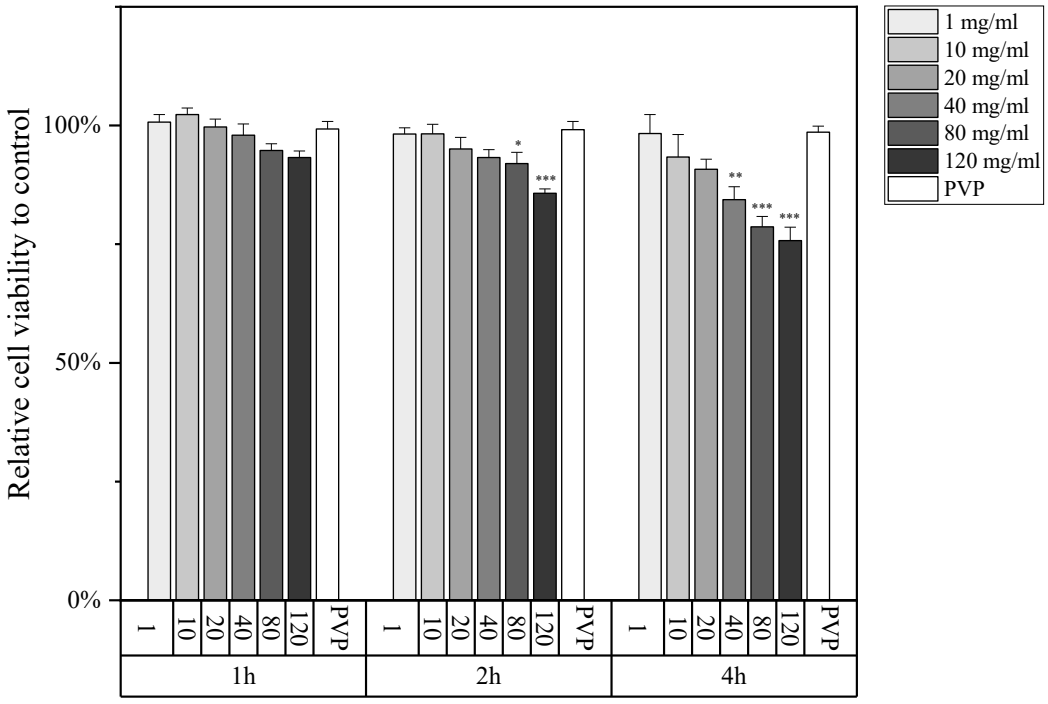
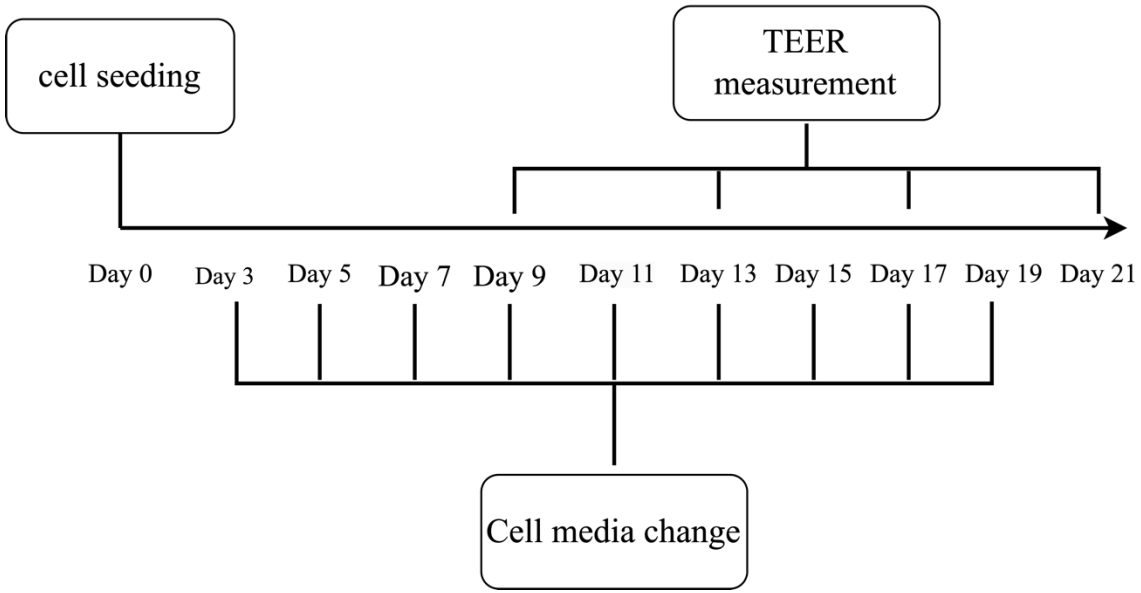


Figure 4.8 Cell viability after various creatine microparticles concentration and duration of creatine treatment. The unit for the concentration of creatine microparticles is mg/ml. The mean value of the group and standard error of the mean are shown.

4.4.5 Transwell Study and TEER measurement

Transwell plates were used as the in vitro model for the creatine microparticle delivery experiment. Calu-3 were seeded on the apical side of transwell inserts and cultured in them for 21 days. The timeline of cell culture before the creatine delivery experiment is shown as Figure 4.9 A. The creatine delivery experiment and post experiment evaluation workflow are shown on Figure 4.9 B.

A.


cell seeding
TEER measurement
Day 0
Day 3
Day 5
Day 7
Day 9
Day 11
Day 13
Day 15
Day 17
Day 19
Day 21
Cell media change

B.

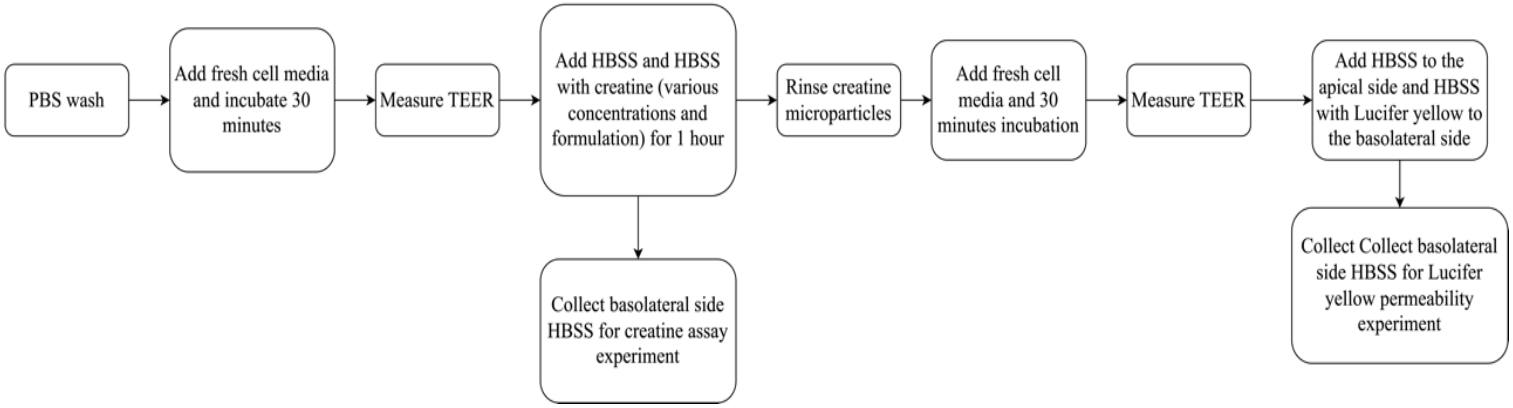


Figure 4.9 Calu-3 cell culture and transwell experiment. Figure 4.9 A. Timeline for the transwell plate experiment. Figure 4.9 B. Workflow for in vitro creatine microparticles permeability and post treatment evaluations.

Cells confluency and morphology cannot be observed directly because the transwell plate inserts are made of polycarbonate and are only translucent to light. TEER was used to measure the cell layer integrity. TEER is a well-accepted indirect method for assessing cellular integrity (Theile et al., 2019). The first TEER was measured 9 days after the Calu-3 cells were seeded on the transwell inserts, and measurement repeated every 4 days. In total, TEER was recorded four times during the cell culture period. The mean TEER value and standard error of the mean is shown on the figure 4.10. The cell layer reached 600 Ω at about 14 days after seeding and reached a plateau phase after 17 days.

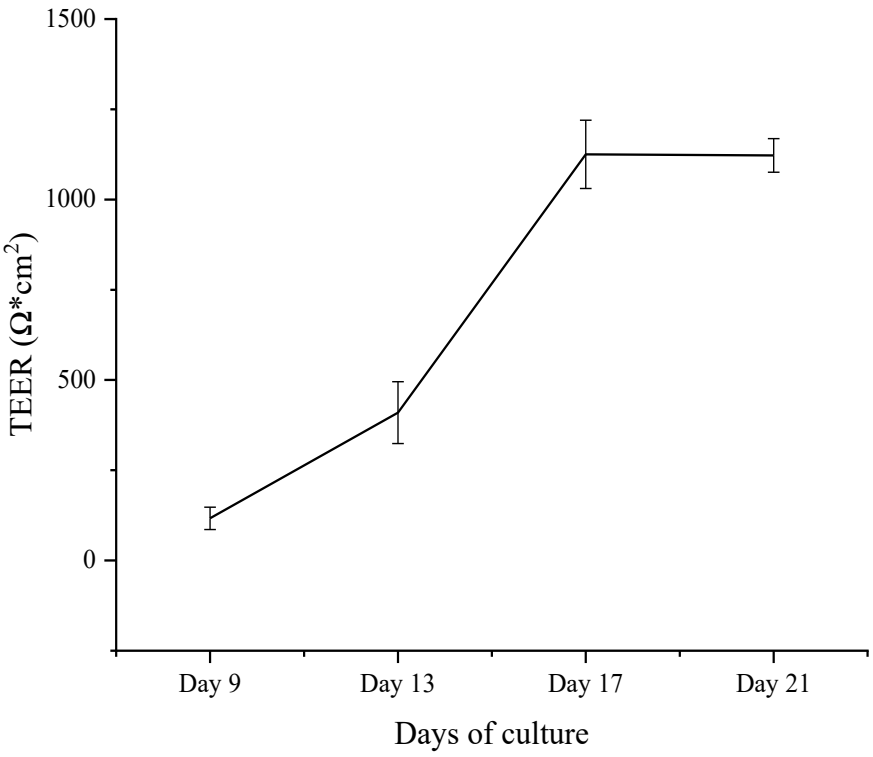


Figure 4.10 TEER measurements of Calu-3 cells overtime. The reference line is shown as 600Ω, which is considered to be the cell layer reached to the intact layer in this study. The mean value of the group and standard error of the mean for each data point are shown.

The TEER readings have been used to compare before and after creatine treatment in determining the potential damage caused by creatine microparticles. Figure 4.11 shows TEER changes in fold after creatine treatment. TEER fold change data between groups was analyzed by one-way ANOVA. The results from one-way ANOVA showed significantly different fold changes between different groups ($F = 2.37$, $p < 0.001$). Post hoc results indicate the 40 mg/ml creatine microparticles with PVP group has a significant lower TEER compare with the control group $p < 0.001$, while 10 mg/ml, 20 mg/ml, 40 mg/ml had no different with the control group ($p = 0.932$, $p = 0.914$, $p = 0.293$).

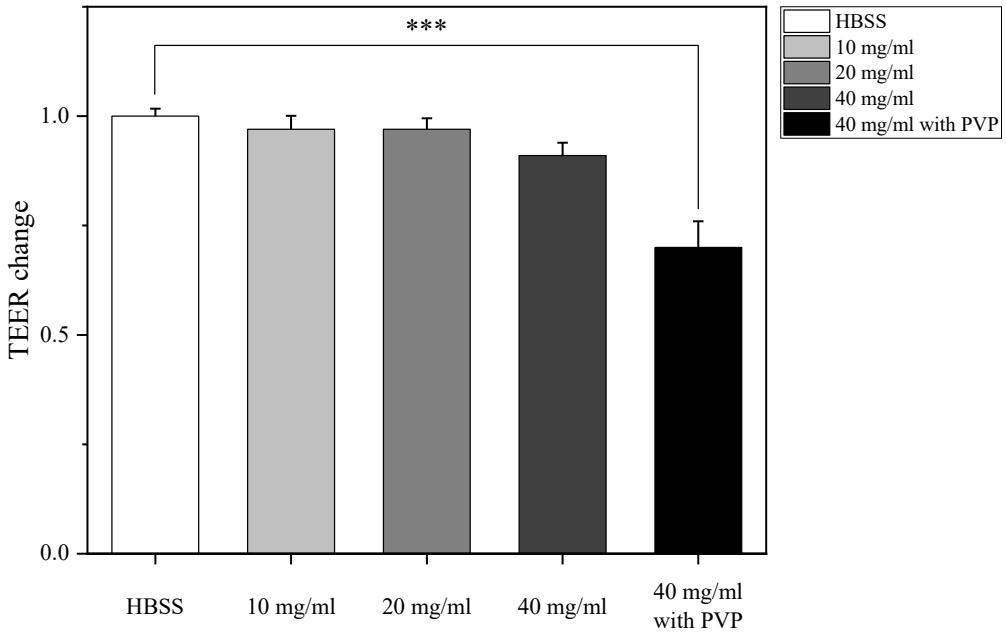


Figure 4.11 TEER changes in fold after creatine treatment. The average TEER change in HBSS group was normalized to 1. The mean value of the group and standard error of the mean are shown.

4.4.6 Lucifer yellow permeability

After creatine treatment, the relative permeability of lucifer yellow for the creatine microparticles group permeability was compared to the blank inserts without cells. Figure 4.12 showed the Lucifer yellow permeability on the control and the creatine microparticles group. The mean value of the group and standard error of the mean are shown. The permeability of Lucifer yellow showed an increasing trend with the creatine microparticles concentration. However, one-way ANOVA test was conducted to analyze relative permeability data. No significant difference was found between the control and creatine microparticles treated groups ($F= 0.382$, $p = 0.819$).

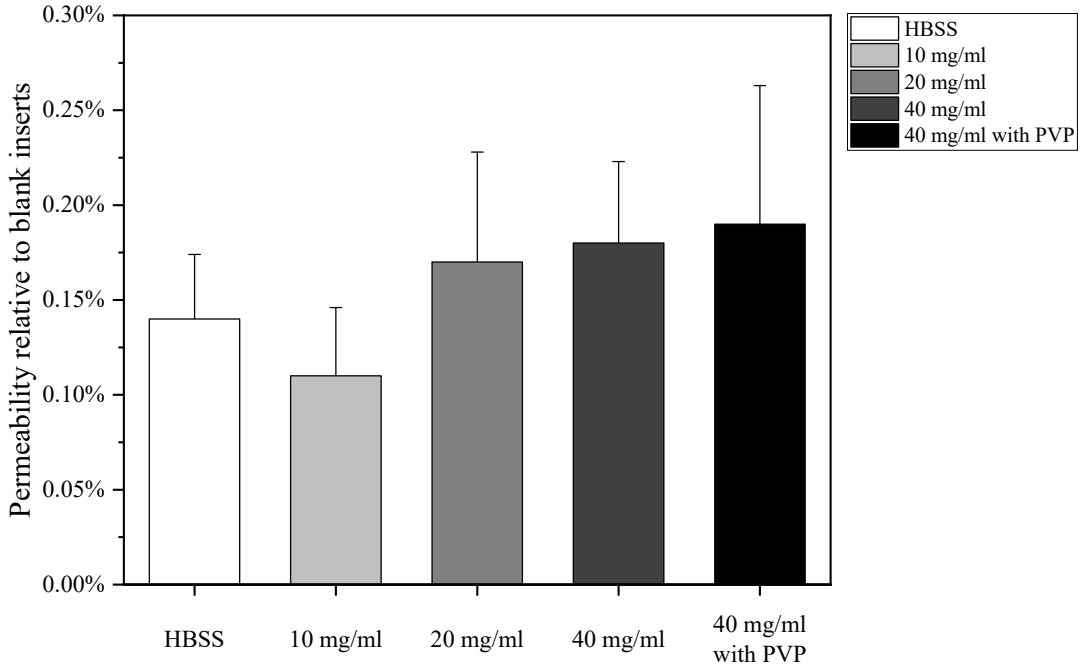


Figure 4.12 Lucifer yellow permeability on the control and the creatine microparticles group. The mean value of the group and standard error of the mean are shown.

4.4.7 Fluorescent Microscopy Images

Representative cell images in each group are shown in Figure 4.13. No cell layer damage caused by creatine microparticles were observed under the microscope. From the fluorescent images, Calu-3 cell layers have a similar cell morphology and remained confluent cell layer in different treatment conditions.

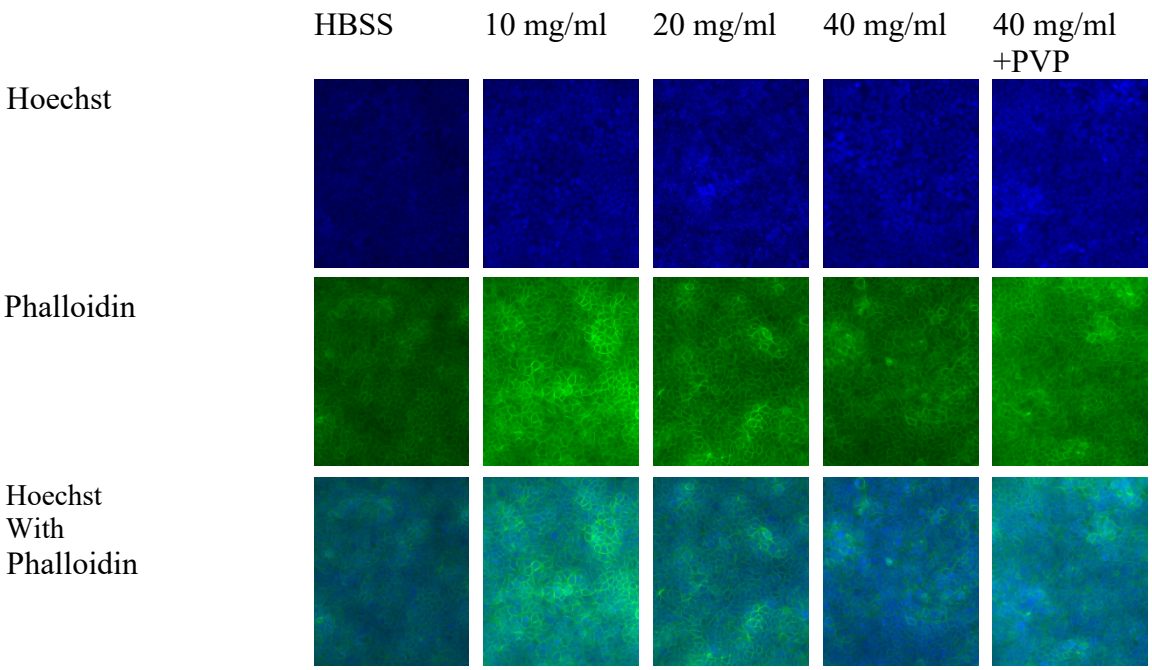


Figure 4.13 Fluorescent images of cell layer on transwell inserts after creatine treatment.

4.4.8 Creatine Assay

In this study, creatine concentration was measured by creatine assay. One hour after creatine microparticles treatment, the basolateral side of HBSS buffer was collected. Figure 4.14 shows creatine concentration on the basolateral side of transwell inserts after different treatment concentrations. The one-way ANOVA showed significant difference was found between treatment groups ($F = 46.007$, $p < 0.001$). Post-hoc Tukey test results showed significant difference on both 10 and 20 mg/ml groups with 40 mg/ml ($p < 0.001$, $p < 0.001$) and 40 mg/ml with PVP groups ($p < 0.001$, $p < 0.001$). No statistical difference was found between 10 and 20 mg/ml groups ($p = 0.324$). Also, the difference between 40mg/ml and 40 mg/ml with the PVP group was significant ($p = 0.002$).

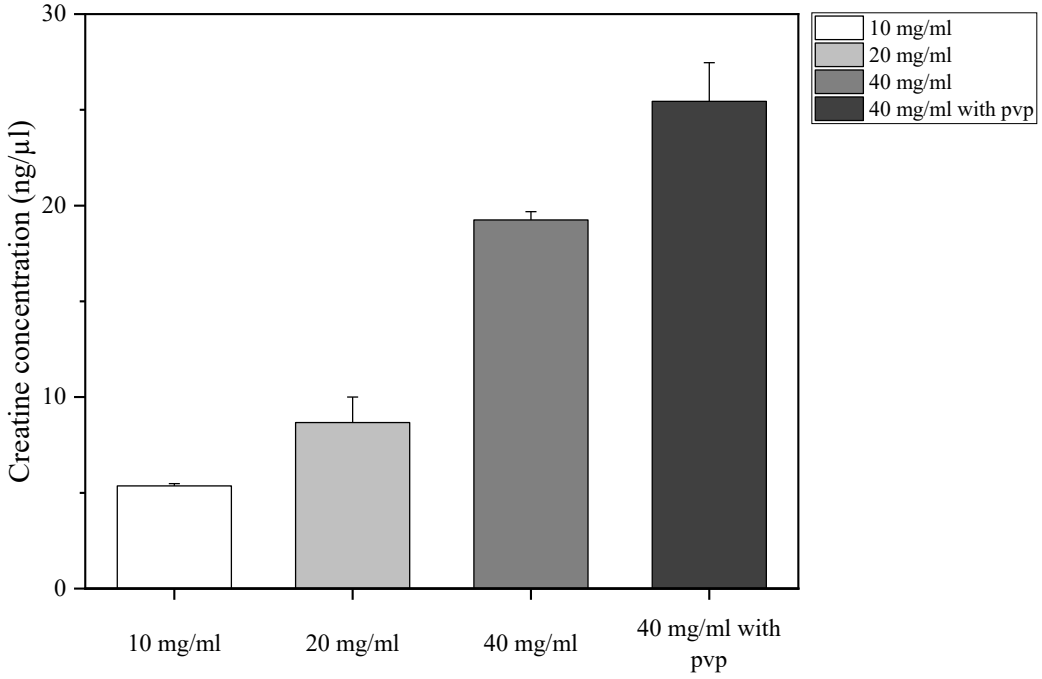


Figure 4.14 Creatine concentration from the basolateral side of transwell inserts. The mean value of the group and standard error of the mean are shown.

4.5 Discussion

Intranasal administration has the advantage of delivering drugs directly to the CNS, but at the same time, it has a restrictive element: limitation of administration volume. Intranasal administration of creatine is still relatively new compared to other routes of administration. Studies of intranasal creatine administration have only been reported in recent years and the specific mechanism of intranasal administration is still unclear. In the reported studies, intranasal administration of creatine was administered with creatine

solution as a carrier (Chen et al., 2021; Chen & Hu, 2023). The purpose of our study is to find an approach to load more creatine within the same volume, and measure whether higher concentrations of creatine can lead to more creatine passing through the cell layer barrier. Making creatine suspension can solve the limitation of creatine concentration per unit volume. However, traditional creatine is difficult to disperse into a relatively stable suspension due to its large particle size. Additionally, creatine suspensions typically stratify within ten seconds to form a creatine precipitate and clog the pipette tip, making it difficult to accurately administer the complete dose. We assembled creatine microparticles to make a creatine suspension and applied it to in vitro delivery transwell experiment.

4.5.1 Creatine Microparticles Characterization Measurement

The creatine microparticle was assembled by transient emulsion approach. By this method, creatine microparticles were assembled. The average particles size of laboratory grade creatine, commercially available creatine monohydrate and creatine microparticles were compared through SEM. In our tests, the creatine was 118.8 μm, the creatine monohydrate was 120.72 μm, and the creatine microparticles was 3.44 μm. The ANOVA test confirmed that creatine microparticle size is smaller than two other groups ($p < 0.01$). In addition, the functional groups were compared between pure creatine and creatine microparticles. FTIR spectra showed that creatine microparticles and creatine have the same functional groups. The creatine assay experiment indicates that the assembly rate of creatine microparticles is higher than 96%. The concentration of creatine microparticles

decreased only about 3% after 6 months. Together, FTIR and creatine reagent test results confirm that creatine microparticles are chemically identical to pure creatine.

The MTT assay was conducted to measure the viability of cells after culturing with creatine microparticles. The MTT assay results showed no significant cytotoxicity in 1-120 mg/ml creatine microparticles after 1 hour treatment ($p = 0.27$). The solubility of creatine at room temperature is 14 mg/ml. The creatine concentration from 20 - 120 mg/ml is 1.5 - 8.6 times higher than creatine solubility. Cytotoxicity was observed after two hours of incubation in the 80 and 120 mg/ml creatine treatment group ($P = 0.02$, $P < 0.01$). After four hours of creatine microparticles treatment cell viability dropped in the 40, 80, and 120 mg/ml creatine microparticles groups ($P = 0.002$, $P < 0.001$, $P < 0.001$). However, at the highest creatine concentration in this study, cell viability was still above 75% after four hours of treatment. PVP as an absorption enhancer was shown to be safe and showed no significant impact on cell viability after 1 - 4 hours of co-incubation period.

The results of creatine microparticles characterization measurement experiments shows that creatine particles have high assembly efficiency, smaller particle size, and no obvious cytotoxicity after one hour treatment to the cells.

4.5.2 Creatine Permeability, and Post Analysis

Before the in vitro creatine microparticle permeability experiments, Calu-3 cells were cultured in transwell plates for 21 days. According to the TEER readings, cells successfully reached an intact cell layer on the transwell inserts within 21 days. TEER

readings were also recorded before and after creatine treatment. Compared with the control group, groups treated with creatine microparticle suspension did not show a significant decrease in TEER value after after 10 mg/ml, 20 mg/ml, and 40 mg/ml creatine administration ($p = 0.0932$, $p = 0.0914$, $p = 0.0293$), which is consistent with MTT results. However, the group with absorption enhancer PVP showed significantly lower TEER compared to the control group ($P < 0.001$).

In the Lucifer yellow permeability test, there was no difference between HBSS and the creatine treated groups including the PVP group ($p = 0.819$). Although the creatine treated groups slightly increased cell layer permeability, the difference was not significant. By staining with different fluorescent dyes, the integrity of the cell layer, and the morphology of cells on the transwell inserts through fluorescence microscopy was observed and evaluated. Both experimental and control groups had intact cell layers, which is consistent with the Lucifer yellow permeability results.

The creatine content at the basolateral side of the transwell inserts were measured using creatine assay. The results showed that 40 mg/ml creatine microparticles suspension group had a significantly higher creatine content compared with creatine solution administration. This proves that the same volume of creatine suspension can deliver more creatine through the cell layer than creatine solution at 10 mg/ml concentration ($P < 0.001$). In addition, by adding PVP to the creatine suspension formula, we observed a further increase in creatine permeability indicating that PVP can significantly increase creatine suspension delivery efficiency ($p = 0.002$).

4.5.3 Limitations and Future Work

It is still unclear whether the results of different methods for measuring cell layer integrity are inconsistent in the creatine microparticles with the PVP group. The TEER reading dropped after creatine treatment but showed no difference from the Lucifer yellow permeability and cell layer images. The similar question about enhancing effect of PVP has also raised by a previous published study.

Future research can be continued from several aspects. The passive transport of intranasal administration drugs is related to particle size, meaning smaller particles are more efficient passage through the cell layer. Based on this idea, creatine particle size can be further reduced for more efficient drug delivery. Moreover, further improvement of creatine microparticle permeability can be achieved by the combination with other absorption enhancers in the future.

4.6 Conclusion

In conclusion, we successfully assembled creatine microparticles with smaller particle sizes while having a uniform distribution. This is the first study to apply the transient emulsion method to creatine and successfully reduce its particle size. In addition, the transient emulsion method also has a high assembly rate when applied to creatine. The results of creatine microparticles in FTIR characterization measurements showed a consistent functional group composition as pure creatine. In the cytotoxicity and viability experiments, creatine microparticles treated groups showed no obvious cytotoxicity after 1 hour treatment, indicating it is safe to apply in vitro. The results of in vitro permeability

experiments show that the administration of creatine microparticle suspension is as safe as the administration of creatine solution, and more efficient. This means that creatine microparticle suspension administration can deliver more creatine across cell layers with the same volume administered.

The combination of creatine microparticles and suspension through intranasal administration has a higher potential for treating creatine-related diseases or energy-related diseases in the CNS. Our microparticle assembly method can also be used for commercial creatine production due to smaller particles, larger surface area, and higher dissolution rate compared to traditional creatine powder.

4.7 Reference

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5 Chapter 5 Conclusions and Future Work

5.1 Overview

This study mainly focused on the feasibility and efficacy of intranasal administration of creatine in vitro and in vivo. In the first study, the magnetic resonance spectroscopy (MRS) spectra indicated exogenous creatine deposition in the rat brain after creatine intranasal administration. In the second study, we focused on the efficacy of creatine intranasal administration, and proved that intranasal administration of creatine can effectively improve the animal maze performance while additionally proving that creatine was supplemented in the brain. We conducted biochemical analysis to measure creatine accumulation and compared creatine content after different administration methods with the control group. In the third study, we introduced the creatine microparticle assembly method and measured creatine microparticles related characteristics. The method of microparticle assembly was learned from the previous published study (Liu et al., 2021). Then 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell viability experiments were conducted to confirm that no significant cytotoxicity was observed in the creatine solution and suspension at 1.4 to 8.6 times higher than the creatine's solubility. The transwell experiment measured the permeability of creatine microparticles, and we attempted to use surfactants to further improve the creatine microparticle permeability. We applied several methods to demonstrate that creatine microparticle administration can pass through the cell layers without impairing the barrier function of the cell layer. Creatine microparticles were proven to increase the efficiency of creatine delivery.

5.2 Summary of Research Contributions and Implications

We have demonstrated through several experiments that creatine can be delivered into the brain by intranasal administration. In the three experiments, longitudinal real-time measurement, biochemical analysis after the creatine administration, and administration of creatine microparticles to respiratory epithelial cells in vitro we proved the deposition of exogenous creatine in the brain and the cognitive performance of rats was improved after intranasal administration.

In the first study, we measured the signal ratio of creatine and choline in the rat brains before administration as a baseline level. Then after 14 days of administration, the creatine and choline signal ratios in the same brain regions were measured by the MRS with the same protocol. The results of animal MRS experiments found that the signal of creatine increased after creatine administration. Our results support that intranasal administration of creatine is effective in wild-type rats.

In the animal intranasal administration experiments, we compared the administration efficiency of two different administration methods: oral administration and intranasal administration. The control group was used to measure the creatine in the brain of the rats as a reference value to compare the creatine levels after oral and intranasal administration of creatine. Our results indicate that the intranasal group showed the highest creatine concentration among the three groups in olfactory bulbs, medial prefrontal cortex and the hippocampus. The oral creatine administration group showed a non-significant increasing trend in the medial prefrontal cortex and hippocampus, but not in the olfactory

bulbs area. These biochemical data indicate that intranasal administration is a more effective method of supplementing brain creatine. The results of our oral administration also support the conclusion of the previous studies that the improvement of brain creatine caused by oral administration may require a larger dose or a longer administration period (Dolan et al., 2019). In addition to biochemical measurements, we also performed a 7-day Barnes maze experiment to compare the effects of different administration methods on cognitive function. The Barnes maze data proved creatine intranasal administration can improve the cognitive performance of rats. More specifically, the intranasal creatine administration group of rats showed a shortened primary latency and less error in the acquisition phase of Barnes maze experiments. In the probe trial, the intranasal group spent the highest percentage of time in the target quadrant. The oral group also showed a slight but not significant improvement. This study demonstrates the cognitive benefits of intranasal administration of creatine in wild-type rats.

The goal of the third study is to find a method to safely increase the concentration of creatine per unit volume for intranasal administration. We are the first study in using creatine microparticles suspension for intranasal administration. Characterization measurements showed creatine microparticles have the same functional groups as pure creatine. Moreover, by measuring the creatine in the creatine microparticles samples, we concluded that the creatine microparticles had a high assembly rate. Compared to the laboratory grade creatine and commercially available creatine monohydrate, our creatine microparticles were significantly smaller than others. Creatine suspension administration is proved to be safe in vitro and permeable to human epithelial cells. In vitro drug delivery

experiments, the creatine microparticles suspension had a higher efficacy than creatine solution administration, and the integrity of the cell layer after administration was verified to be intact. Creatine assay data also successfully demonstrated that the creatine diffusion can be further improved by adding PVP.

5.3 Limitations and Future Directions

Our work has some limitations that we need to be aware of and some shortcomings that could be addressed in the future. At the molecular level, the mechanism of creatine permeating the nasal mucosa is still unclear. Furthermore, the formulation of intranasal administration could be further optimized. Creatine hydrochloride solutions, for example, are known to be acidic and potentially irritating to the nasal mucosa. Although, this limitation was addressed in the third study, where the creatine microparticle suspension is neutral. In addition to improving the creatine permeability, the combination of different surfactants to prolong the absorption time and reduce the clearance effect also needs to be investigated before the in vivo experiment.

Once the optimized formulation is determined, the toxicity of long-term intranasal administration of creatine in animals needs to be evaluated prior to human clinical trials. If long-term intranasal administration of creatine can be proven to be safe on animal models, intranasal administration of creatine can be evaluated as a treatment to creatine-related mutation diseases, such as creatine deficiency syndrome. Although MRS showed that oral administration can increase creatine levels in patients with creatine synthase deficiency, intranasal administration may more rapidly replenish creatine in the brain.

The clinical trials of creatine oral administration for neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Amyotrophic lateral sclerosis have been tested, but administering creatine intranasally can be applied and compared with previous findings (AliMohammadi et al., 2015; Allen, 2012; Gualano et al., 2010; Writing Group for the et al., 2015). Some studies have shown improvement in the symptoms of these diseases after oral administration of creatine, but due to the inefficiency of oral administration, it is not clear whether intranasal administration can improve the efficiency and bioavailability to achieve greater relief of symptoms, or even delay the progression of the disease (Dolan et al., 2019).

The effects of intranasal administration of creatine on mood is also worth studying, as psychiatric disorders are often associated with disturbances in brain energy (Allen, 2012). Creatine supplementation to replenish the energy supply of the brain is considered a potential treatment for the improvement of psychiatric disorders (Allen, 2012).

Intranasal administration may have more significant benefits for acute diseases such as strokes and traumatic brain injury (TBI) because of direct delivery of drugs to the brain. Insufficient cellular energy supply and accumulation of ROS are also signs of these acute brain diseases (Forbes et al., 2022). The intranasal route is suitable for such emergencies, as animal studies proves that it usually only takes 5 minutes for the drug to enter the CNS by this path (Crowe & Hsu, 2022). Creatine, as the substrate for the fastest recovery of ATP, has also been proven to have an antioxidant effect (Smith et al., 2014).

The combination of creatine and intranasal administration can provide an emergency energy supply for brain cells.

In addition to the above-mentioned diseases, the effect of intranasal administration of creatine on healthy people merits researchers' attention. Oral creatine has been shown to have a more pronounced effect in certain situations, such as mental fatigue and hypoxia (Dolan et al., 2019). According to previous studies, cognitive function improvements after oral administration of creatine was inconsistent (Avgerinos et al., 2018; Dolan et al., 2019; Prokopidis et al., 2023; Roschel et al., 2021). Whether healthy people can improve cognitive function by providing exogenous creatine to the brain still needs to be investigated. Intranasal administration of creatine is a more direct-acting administration method to the brain. This improvement has inspired a new path of creatine administration, which solves the limitations of oral creatine administration.

In general, creatine, as a metabolite involved in brain energy supply, has potentially beneficial effects related to human health.

5.4 Reference

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