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Beyond Muscles: The Untapped Potential of Creatine

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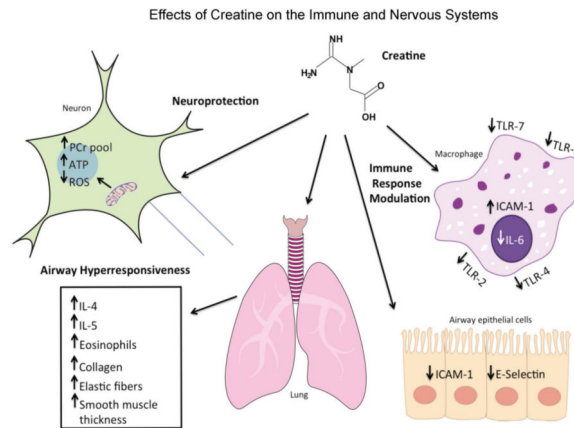
Abstract

Creatine is widely used by both elite and recreational athletes as an ergogenic aid to enhance anaerobic exercise performance. Older individuals also use creatine to prevent sarcopenia and, accordingly, may have therapeutic benefits for muscle wasting diseases. Although the effect of creatine on the musculoskeletal system has been extensively studied, less attention has been paid to its potential effects on other physiological systems. Because there is a significant pool of creatine in the brain, the utility of creatine supplementation has been examined *in vitro* as well as *in vivo* in both animal models of neurological disorders and in humans. While the data are preliminary, there is evidence to suggest that individuals with certain neurological conditions may benefit from exogenous creatine supplementation if treatment protocols can be optimized. A small number of studies that have examined the impact of creatine on the immune system have shown an alteration in soluble mediator production and the expression of molecules involved in recognizing infections, specifically toll-like receptors. Future investigations evaluating the total impact of creatine supplementation are required to better understand the benefits and risks of creatine use, particularly since there is increasing evidence that creatine may have a regulatory impact on the immune system.

Graphical Abstract

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Keywords

creatine; immune response; immunomodulation; neuroprotection

1. Introduction

Creatine is naturally synthesized in the liver, kidney, and pancreas of vertebrates from the amino acids arginine, methionine, and glycine [1³]. *In vivo*, creatine is a product of the arginine biosynthesis pathway and metabolizes to creatinine [3·4]. Individuals who eat meat and/or fish obtain approximately 1 g d⁻¹ of creatine from the diet [5], and approximately 1 g d⁻¹ is synthesized endogenously. Vegetarians have significantly lower muscle creatine stores and lower creatinine levels as compared to those who eat meat and/or fish products [6·7]. The average creatine pool for a 70 kg individual ranges from 120-140 g and approximately 2 g d⁻¹ is lost in the urine in the form of creatinine [1]. Given that daily intake and excretion are approximately equal, the most efficient way to increase creatine stores in the body is through dietary supplementation. Creatine enters the muscle cells via a sodium- and chloride-dependent creatine transporter [3·8·9] and is primarily stored in the skeletal muscle as free creatine or phosphocreatine, which is a major source of energy to the host [3·8·10].

While the majority of creatine in the body is stored in skeletal muscles [3], there is also a significant pool of creatine in the brain [11], which may provide some protection against neurological disorders and trauma. For example, several studies using animal models have shown that oral creatine supplementation provides neuroprotective effects in a variety of neurological conditions including traumatic brain injury [12], Huntington's Disease [13], amyotrophic lateral sclerosis [14], ischemia [15], and Parkinson's Disease [16]. There have also been a small number of studies that suggest that pro-inflammatory responses are reduced following creatine supplementation [17-24]. However, the mechanism of how creatine acts in modulating inflammation remains unclear, although recent work [25] demonstrating that European sea bass fed a diet supplemented with arginine (a precursor of creatine) had reduced disease resistance suggests that the mechanism may be evolutionarily conserved. The potential of creatine as an immunomodulator may have important implications for individuals with certain pro-inflammatory diseases such as arthritis.

The purpose of this review is to examine the potential of dietary creatine supplementation to modulate disease, as well as to discuss potential mechanisms of action of creatine in its ability to function as a neuroprotective or immunomodulatory agent.

2. Creatine use by athletes

Creatine was first discovered as a constituent of meat in the 1800's; however, it was not until the 1970's that it was used as a potential ergogenic aid by athletes in the Soviet Union and Eastern bloc countries, and then gained wide research interest in the 1990's [26]. The phosphagen energy system is the metabolic system that produces ATP most rapidly, as compared to glycolysis or the aerobic system [27]. Inside the cell, creatine phosphokinase catalyzes a reversible reaction between the γ -phosphate group of ATP to the guanidino group of creatine resulting in ADP and phosphocreatine (**Figure 1**). Cellular stores of phosphocreatine can reach concentrations of up to 40 mM [3·8·28-30] and is essential for replenishing ATP stores that are immediately used during high intensity exercise.

Dietary creatine supplementation increases the phosphocreatine stores in the muscles, and has been shown to enhance performance during high-intensity, short duration activities or repeated bouts of high-intensity exercise with short rest periods such as jumping, sprinting, and strength training [26·31-39]. It is estimated that 27-78% of all college athletes have used creatine supplements [40-43] and the proportion of individuals using creatine is likely much higher in athletes participating in sports such as football, track, wrestling, and soccer [26·31-34·44]. A recent study of body builders in Iran reported that creatine was the most common nutritional supplement used by men (60.8%) [35]. Initially, creatine was primarily utilized by elite athletes; but its use has become increasingly widespread among older adults, recreational athletes, and high school athletes [1·45-51].

The most widely used form of creatine by athletes is creatine monohydrate [41-43]. Oral bioavailability of creatine monohydrate is low due to the rapid conversion of creatine to creatinine in acidic environments, as would be encountered in the stomach [52·53]. While there has been little study examining how creatine crosses the intestine, the small intestine does express the Na⁺/Cl⁻ creatine transporter [54] which is also expressed in other organs including the brain, kidney, and heart [55·56]. However, some work has suggested that creatine may move across the jejunum by paracellular movement [57]. The contributions of each of these potential mechanisms of transport is unclear although it has been shown that creatine supplementation of individuals deficient in the creatine transporter does improve muscular, but not cognitive and psychiatric symptoms of the condition [58], indicating that paracellular transport may be a sufficient to increase creatine stores in the muscles.

Athletes normally engage in the practice of loading that consists of ingesting 20 g d⁻¹ of creatine for five days administered over several (usually four) doses followed by 1-10 g d⁻¹ for several weeks or months [3·34]. The loading phase increases muscle stores of phosphocreatine 15 to 40% [59·60]. Minimal side effects as a result of the creatine loading phase have been reported and include cramping, nausea, fluid retention, and diarrhea [43·61]. Although it is typically recommended that individuals creatine load for 4-7 days, it has been reported that creatine uptake into muscle is greatest during the first 2 days of

loading [62]. Hultman et al. [63] have also reported that a dose of 3 g d⁻¹ for 28 days is as effective as creatine loading for increasing total muscle creatine stores. Therefore, 'slowly loading' the muscle with creatine may result in significant increases in performance and alleviate side effects that are sometimes associated with a 4-7 day loading regimen.

Very few individuals (~20% of users) reach maximal creatine saturation of their skeletal muscles (160 mmol/kg dry mass [10]), thus there is significant interest in developing formulations that have enhanced bioavailability. One currently available form of creatine, creatine ethyl ester, is reported to have a greater degree of stability and bioavailability than creatine monohydrate [53]. It is postulated that because the carboxyl group is no longer available, creatine ethyl ester is not converted to creatinine in the stomach, but can be absorbed in the intestine where the creatine ethyl ester enters the blood. Esterases in the intestinal cells and blood convert the creatine ethyl ester to creatine, which is then stored in the muscle cells as phosphocreatine. Of particular note, creatine ethyl ester is more stable than creatine monohydrate at a lower pH (as would be encountered in the stomach) [52-53]. In addition, *in vitro* studies utilizing Caco-2 cell monolayers have demonstrated increased permeability of creatine ethyl ester compared to creatine monohydrate [53]. Together, these studies [52-53] suggest that creatine ethyl ester may be more bioavailable than creatine monohydrate.

There are multiple mechanisms by which creatine functions to enhance athletic performance. As shown in **Figure 1**, creatine is the substrate for the creatine kinase reaction, resulting in the generation of phosphocreatine, which comprises 60% of the creatine in skeletal muscle (~ 60% of muscle creatine is stored as phosphocreatine and 40% as free creatine) [2]. As previously mentioned, phosphocreatine is responsible for the re-phosphorylation of ADP to ATP during bursts of high intensity movements, thus resulting in an increased availability of energy during short periods of explosive exercise [35-39-64]. As phosphocreatine levels decline due to the re-phosphorylation of ADP, phosphofructokinase production is stimulated, thereby increasing the rate of glycolysis [39]. Creatine can also function to buffer the pH changes that occur due to the accumulation of lactate and hydrogen ions by using the hydrogen ions in the creatine kinase reaction [65-66]. Individuals with creatine or phosphocreatine deficiencies due to genetic defects in proteins involved in creatine synthesis (L-arginine-glycine amidinotransferase or guanidinoacetate methyltransferase) or transport (creatine transporter [SLC6A8]) have reduced levels of ATP in the brain resulting in developmental delays and mental retardation [67-69]. While individuals with defects in creatine synthesis can be treated with exogenous creatine, no treatment exists for individuals with deficits in the creatine transporter [67-69].

3. Creatine as a mediator of neuroprotection

3.1 The mitochondrial permeability transition pore

Because of the high levels of creatine in the central nervous system [11], a considerable number of studies have focused on the potential neuroprotective effects of oral creatine supplementation in a variety of neurological conditions including traumatic brain injury (TBI) [12-70-71], Huntington's Disease (HD) [13-72-76], amyotrophic lateral sclerosis (ALS) [14-77-80], cerebral ischemia [15-81], and Parkinson's Disease (PD) [82-88]. One of

the key focuses as to how creatine may work to reduce neuropathology in the central nervous system (CNS) has been the effect of creatine on the mitochondrial permeability transition pore [14·89]. In the CNS, the mitochondrial permeability transition pore is induced in the conditions mentioned above including stroke [90], PD [91·92], HD [93], TBI [94·95], and ALS [14·96·97]. The mitochondrial permeability transition pore is also induced by reactive oxygen species (ROS) [98] and, correspondingly, ROS are released as a result of development of the pore [99]. Conversely, high ATP and ADP levels prevent the mitochondrial pore from being induced [98·100·101].

Excitotoxicity is a major cause of neuronal death that is the result of an influx of calcium into the cell as a result of glutamate receptor overactivation [102–107]. The mitochondrial permeability transition pore permits the influx of molecules smaller than 1.5kDa (including calcium), which ultimately results in altered osmotic balance in the organelle resulting in damage to the mitochondria and the release of cytochrome *c* [108]. ATP generation is also dysregulated by the development of the mitochondrial permeability transition pore as ATP synthase hydrolyzes ATP under these conditions [109]. It has been hypothesized that neuroprotection via creatine supplementation may occur via inhibition of the mitochondrial permeability transition pore by stabilizing mitochondrial creatine kinase and stimulating the production of phosphocreatine; which stabilizes energy (ATP) levels in the cell [110]. The inhibition of the mitochondrial permeability transition pore ultimately inhibits cell death from occurring by prohibiting proapoptotic proteins from being released into the cytosol [105].

3.2. Traumatic Brain Injury

TBI due to accidents and sports is a significant contributor of cognitive impairment to individuals in the United States and Canada [111·112]. While the primary injury results from the disruption of tissue at the time of impact, the secondary injuries occur minutes to days later. A component of the secondary injury is the result of alterations in calcium disruption that results in mitochondrial dysfunction and an inadequate supply of ATP to the neuron [113–117]. In 2000, Sullivan et al. [12] examined the ability of creatine supplementation to reduce the level of tissue damage in mice and rats in a model of TBI. Mice intraperitoneally injected with creatine for three and five days prior to TBI had reduced lesion volumes than control injected mice at day seven post injury. Similar results were observed in rats treated with creatine prior to TBI [12]. The authors then determined that rats fed a creatine-supplemented diet for seven days after TBI experienced significantly smaller cortical lesions than animals fed the control diet. Additionally, diet-based supplementation of rats for four weeks post TBI determined that there was reduced reactive oxygen intermediates production, increased ATP levels, and increased mitochondrial membrane potentials [12]. In humans, pilot data has examined both the short-term [70] and long-term [71] effect of creatine supplementation on TBI in children and adolescents. Individuals receiving 0.4 g kg⁻¹ oral creatine for 6 months following TBI had significant improvement in communication, cognition, personality/behavior, and self-care as compared to individuals who did not receive creatine. While not statistically significant, the length of stay in the intensive care unit was shortened which would result in decreased hospitalization costs [70]. A follow-up of these patients at 6 months post-injury indicated that the creatine-treated

individuals had less dizziness, headaches, and fatigue than the control patients [71]. While there appear to be no other studies that have examined the potential benefit of creatine supplementation in TBI patients, the results of these studies [70-71] are promising and future large-scale studies on the utility of creatine are warranted, particularly since no negative side-effects were noted in these reports.

3.3. Huntington's Disease

HD is an autosomal dominant, progressive neurological disorder that is ultimately fatal. The mean survival from the time of diagnosis is 15 to 20 years and symptoms include both cognitive disorders and progressive motor impairment [118]. Chemical lesioning of animals has been used to induce pathology similar to that observed in HD patients [110-119-122]. Malonate is a reversible succinate dehydrogenase inhibitor that when injected intrastrially in rats, induces neuropathology similar to that observed in HD patients [119]. Similarly, 3-nitropropionic acid (3-NPA), an irreversible succinate dehydrogenase inhibitor that is a metabolite of the fungus *Arthrimum sp.* [123], induces similar pathology to malonate [124]. Both malonate and 3-NPA impair mitochondrial respiration resulting in membrane depolarization and eventually triggering apoptotic pathways and production of ROS leading to cell death [121]. In rat models of brain lesioning with malonate or 3-NPA, oral supplementation with either creatine or cyclocreatine resulted in smaller lesion volumes in the malonate lesioning model, while only creatine was effective in reducing lesion volume in the 3-NPA model of lesion induction [120].

The mutation in HD patients is an extended (>35) CAG (glutamine) repeat [125] and disease onset and severity correlates with the repeat length [126-128]. Identification of this mutation permitted the development of a transgenic mouse model of HD with CAG repeats of 141-157 [129]. In 2000, studies by Ferrante et al. [13] demonstrated that mice fed a diet supplemented with 1-3% creatine had increased survival and reduced brain atrophy compared to controls. In addition, the mice had better rotarod performance (a measure of coordination) as compared to mice fed the control diet. An interesting finding of this study [13] was that mice fed 1% creatine had better rotarod performance than mice fed 3% creatine, suggesting that the range of effectiveness (at least in regard to motor performance in this model) is very narrow. In a similar study [73], HD transgenic mice fed a diet of 2% creatine had increased survival and reduced brain atrophy compared to control mice. Work by Dedeoglu et al. [74] examined the impact of creatine feeding on HD transgenic mice after clinical signs of HD were apparent (6 and 8 weeks of age). In these studies, rotarod performance was significantly improved compared to the performance of control mice. Brain weight was higher in mice receiving 2% oral creatine supplementation. Consistent with the increased brain weights, the supplemented mice also had less atrophy of the striatal neurons. Starting creatine supplementation at a later point in the disease (10 weeks) did not result in improvements [74], indicating that there may be an ideal window in which to begin supplementation to observe improvements in HD patients.

These studies provided the basis to test creatine in a pilot study in HD patients [76]. HD patients were given either creatine (5 g d⁻¹) or placebo for one year. Muscular and cognitive function was assessed at 6 and 12 months. No differences were observed between groups

using the Unified Huntington's Disease Rating Scale to assess cognitive and motor function and functional ability. Additional tests included a maximal incremental exercise test using a cycle ergometer and assessment of elbow muscle strength using an isokinetic dynamometer [76]. Of note, this study was small (n=26 in treatment group, n=15 in placebo) and utilized patients with stage I, II, and III HD [76]. Bender et al. [130] reported that in HD patients undergoing creatine supplementation (20 g d⁻¹ × 5 d, then 6 g d⁻¹ for remainder of the study) had a decrease in brain glutamate concentrations following 8-10 weeks of creatine ingestion, but no change on the clinical rating scales. When considering the dose of creatine fed to mice and how that would translate into humans, much higher doses of creatine (40-50 g d⁻¹) [131] would be required in humans than have been utilized in most clinical studies (5-10 g d⁻¹).

A recent study utilized higher doses of creatine (30 g d⁻¹) in a study designed to test both the safety and tolerability of creatine in patients at risk of developing HD [132]. The PRECREST (Creatine Safety and Tolerability in Premanifest HD) trial enrolled individuals who were at 50% risk of developing HD who had affected first degree relatives, as well as individuals who had undergone genetic testing and had the HD mutation. At 6 and 18 months of creatine or placebo, neuroimaging was performed to assess brain atrophy. Individuals in the creatine group had significantly less cortical and striatal atrophy compared to that observed in control group [132], suggesting that creatine may be helpful in slowing disease progression. It is important to note that this study was performed on individuals prior to the onset of clinical signs. There can be minor changes in clinical signs or brain atrophy for many years prior to a diagnosis of HD [133-136]. Together, these data suggest that creatine may be most effective in HD treatment prior to the development of clinical signs.

3.4. Amyotrophic Lateral Sclerosis

ALS is an extremely rare (estimated incidence of 2 in 100,000 per year) [137,138] progressive neurodegenerative disease wherein 90 to 95% of the cases have no known family history [139-141]. In addition to the loss of motor neurons in the spinal cord and cortex, muscle weakness and atrophy are also hallmarks of the disease [142,143]. Proposed causes of the disease include neuroinflammation, oxidative stress, and mitochondrial dysfunction [142-144] and many have speculated that oral creatine supplementation may slow the progression of this universally fatal condition [14,77]. Hereditary causes of ALS have been linked to a mutation in copper zinc superoxide dismutase, which is responsible for reducing free radicals in the cell [139]. The identification of this mutation made it possible to develop a mouse model of hereditary ALS [145] which mimics the key pathology associated with the disease in humans, particularly the profound loss of large ventral horn neurons [139,140,142,143]. The mouse model is representative of approximately 10% of the cases of human ALS, as the cause of sporadic ALS is unknown [138,140-144]. The impact of oral creatine treatment on the transgenic mouse model of ALS was examined and it was found that creatine provided significant neuroprotection to the mice and that these mice were indistinguishable from control wildtype mice [14]. Further studies in this mouse model determined both survival and rotarod performance was enhanced in mice fed a diet supplemented with 2% creatine [77]. Human trials with ALS patients were initiated based upon the results of animal studies. In contrast to the animal studies, creatine supplementation

had no effect on survival, motor function, or respiratory function of patients [78–80]. While disappointing, it should not be surprising that the human trials did not parallel the mouse studies, since only a small percentage of the human cases have the same genetic defect found in the transgenic mice, and the trials were not specifically targeted using individuals with a mutation in copper zinc superoxide dismutase. In addition, the amount of creatine used for supplementation in the human trials was significantly less than the amount used in the mouse studies.

3.5. Cerebral Ischemia

In the United States, stroke is the fifth leading cause of death, and a significant cause of disability [146]. As a result of reports of the neuroprotective effects of creatine, Zhu et al. [15] examined the ability of creatine to provide protection in a mouse model of stroke. In these studies, animals were fed a 2% creatine diet for 4 weeks prior to occlusion of the middle cerebral artery for 2 hours. Animals were evaluated 24 hours post-reperfusion. Lesion volume and neurological impairment were significantly reduced in the creatine fed mice compared to the controls. Brain creatine and ATP levels were depressed in lesioned brain hemispheres of animals that did not receive the creatine-supplemented diets compared to levels in the contralateral hemisphere [15]. One of the main mediators of ischemic injury in the brain has been reported to be the activation of caspases [147–150]. Levels of caspase-3 activation and cytochrome *c* release in mice fed creatine were decreased compared to control fed mice [15]. The authors [15] stated that the effectiveness of creatine feeding was similar to that observed in mice and that the anti-apoptotic protein, Bcl-2, was overexpressed in neurons [148,149,151].

In a study examining the effectiveness of creatine on newborn mice with cerebral hypoxia-ischemia, carotid ligation was performed at post-natal day 10 for 25 minutes [81]. At post-natal day 20 (weaning), mice were placed in either control or creatine-fed groups. Creatine was supplemented at either 1% or 3% for 3 weeks. Creatine fed mice demonstrated increased performance on the rotarod and the Morris water maze (a measure of memory) ($p < 0.05$) as compared to control-fed animals. Additionally, mice fed a diet of 3% creatine had increased performance on both tests as compared with animals fed the 1% creatine diet [81]. Lesion size measurements were also reduced in creatine fed mice. While no clinical trials examining the impact of creatine supplementation were identified, the findings described herein suggest that it may be advisable to supplement stroke patients with creatine, in addition to the standard medical care.

3.6. Parkinson's Disease

PD is a neurodegenerative disorder characterized by progressive loss of dopaminergic neurons in the substantia nigra and the development of Lewy bodies in the brain. Many studies of PD utilize the loss of tyrosine hydroxylase-positive neurons as an indicator of the level of disease [152–157], as tyrosine hydroxylase is the rate-limiting enzyme in the generation of L-3,4-dihydroxyphenylalanine(L-DOPA) [158], the precursor to the neurotransmitter dopamine and norepinephrine [158]. Tremors, rigidity, and gait disorders are common clinical signs of the disease [159] and are correlated with the loss of

dopaminergic neurons [159]. In 2013, direct and indirect costs for the population of PD patients in the United States exceeded \$20 billion per year [160].

Systemic administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to mice [161-166] or non-human primates [167-169] is a well-accepted model of PD. Matthews et al. [16] examined the neuroprotective effects of creatine in the mouse model of MPTP-induced PD. In this study, oral creatine Nissl and tyrosine hydroxylase immunostaining demonstrated that creatine significantly reduced the level of dopaminergic neurons lost following MPTP administration. Furthermore, there was no significant difference in the neuronal counts in the substantia nigra between control (no MPTP) mice and creatine fed MPTP-lesioned mice, indicating the effectiveness of creatine as a neuroprotective agent [16]. Protection against dopamine depletion and loss of tyrosine hydroxylase positive neurons in the substantia nigra was further enhanced when co-administered with the cyclooxygenase 2 (COX-2) inhibitor rofecoxib [170]. A related study examined a combination treatment using creatine and coenzyme Q₁₀ (CoQ₁₀). In this study, α -synuclein levels (a key neuronal protein that serves as a marker for staging PD pathology) were examined [171-172] in MPTP-treated rats. In PD, α -synuclein forms aggregates that have been compared to the β -amyloid aggregates observed in Alzheimer's Disease [173]. Because α -synuclein downregulates tyrosine hydroxylase, elevated α -synuclein levels are associated with decreased dopamine levels [173]. Rats receiving the combination creatine and CoQ₁₀ treatment had reduced lesion volumes and reduced α -synuclein levels in the brain [172], as well as a reduced loss of tyrosine hydroxylase-positive neurons as compared with singly-treated rats [172].

In vitro test systems have also demonstrated the effectiveness of creatine in a variety of culture systems [174-176]. Andres et al. [174] examined the ability of creatine to protect dopaminergic neurons from a variety of insults including serum and glucose starvation, 1-methyl-4-phenyl pyridinium ion (MPP⁺) and 6-hydroxydopamine (6-OHDA). Primary neuronal cultures from embryonic rats were treated with 5 mM creatine monohydrate and then exposed to MPP⁺ or 6-OHDA. In both instances, creatine-treated neuronal cultures were significantly protected as compared to non-creatine treated neurons. In addition, neurons that experienced serum and glucose starvation had enhanced survival when cultured with creatine [174]. Similar results have been obtained in an *in vitro* slice culture system using striatal slices from adult rats and 6-OHDA lesioning [175].

One complication of PD is the development of L-DOPA-induced dyskinesia (LID) which is characterized by abnormal involuntary movements (AIMs) and is the result of prolonged use of levodopa (L-DOPA) to manage the disease [177-178]. To study the effects of creatine on the development of LID, lesions were induced in rats using 6-OHDA and 21 days later rats were placed on either a 2% creatine-supplemented diet or a normal diet. After 32 days on the diet, LDOPA treatment began and for 3 weeks animals were observed for the development of AIMs [179]. The results showed that AIMs were significantly reduced in the rats on the creatine diet. In addition, markers associated with LID – prodynorphin and FosB/ Fos [180-183] – were reduced in the brains of mice fed a creatine-supplemented diet indicating that creatine supplementation had significant positive effects even after the development of pathology in this model [179].

On the basis of the animal and tissue culture studies, a number of clinical trials have examined the potential of creatine as a possible therapy for PD [82–88,184]. A randomized, double-blind futility trial was undertaken with 200 participants who received creatine (10 g d⁻¹), minocycline (200 mg d⁻¹), or placebo. Creatine reduced disease progression in PD patients diagnosed within 5 years and could not be rejected as futile [88]. Another study determined that creatine (20 g·d⁻¹ for 6 days, then 2 g d⁻¹ for 6 months, and then 4 g d⁻¹ for up to 2 years) reduced the level of dopaminergic therapy but did not alter PD clinical scores [87]. Long-term creatine (4 g·d⁻¹ for 2 years) has been shown to be safe in patients with PD [184], although one study associated high caffeine intake with faster progression in creatine-supplementing patients [83]. A double-blind, multicenter, long-term efficacy trial recruited 1,741 PD patients who were randomly treated with either creatine monohydrate (10 g d⁻¹) or placebo. Because no differences were noted between creatine- and placebo-treated groups after 5 years, the trial was concluded early [82]. While the studies in humans have been disappointing, the doses of creatine used in humans do not reflect those used in the mouse and rat studies, but rather doses similar to those used by athletes. Therefore, future studies in humans involving creatine should be designed to more closely utilize doses that are consistent with rodent studies.

4. Creatine as an anti-inflammatory agent

4.1. Early studies

Although the available literature is limited, there are a few reports that have supported the use of creatine as an anti-inflammatory agent [17–19,22,24,185]. Creatine was first reported as an anti-inflammatory by Madan and Khana in 1976 who tested a series of amino acids in a rat model of carrageenan-induced acute inflammation [186]. In this widely-used model for testing anti-inflammatory agents [187–194], carrageenan is injected into the paw, and a number of potent inflammatory mediators are induced both locally and systemically, with maximum edema normally occurring within 3 hours of injection, due to activation of the cyclo-oxygenase pathway [187]. Mediators induced include histamine, bradykinin, serotonin, prostaglandins, tumor necrosis factor- α (TNF- α), interleukin -1 (IL-1), and IL-6 [188–190]. Recent data suggests that carrageenan-induced inflammation is the result of activation of nuclear factor- κ B (NF- κ B) via a toll-like receptor-4 (TLR-4)-dependent mechanism [195]. Using this model, Madan and Khana [186] found that intraperitoneal injection of creatine was a potent reducer of edema [186]. As a follow-up to this initial study, they tested the efficacy of oral creatine as a mediator of acute and chronic inflammation in rats, as well as the ability of creatine to function as an analgesic [19]. In a model of carrageenan-induced acute inflammation of the foot paw, creatine suppressed inflammation as effectively as the control drug phenylbutazone [19], a nonsteroidal anti-inflammatory drug (NSAID) used in animals [196–197]. Because serotonin (also referred to as 5-HT) plays a significant role in the early stages of inflammation in the carrageenan model [189–192], the authors utilized the serotonin-induced model of paw edema to verify the utility of oral creatine as an anti-inflammatory agent [198] and found that creatine-fed animals had reduced inflammation compared to control-treated animals [198]. **Table 1** summarizes the *in vivo* animal studies that have been performed using oral creatine to impact inflammatory responses.

To ensure that the observed effect was not model-specific, an additional model of inflammation was tested. Oral creatine was found to be effective for decreasing paw swelling in a nystatin model of inflammation [19]. The nystatin model of paw edema induces chronic inflammation, which can last up to 15 days [199]. Nystatin triggers TLR-2 and induces secretion of TNF- α , IL-8, and IL-1 β [200]. Based on more recent data from our laboratory [20], we would suggest that the reduced inflammation in this model was the result of creatine reducing expression of TLR-2 *in vivo*.

To determine if oral creatine treatment could induce similar results in chronic inflammatory conditions, two animal models of arthritis were used [198]. Using a formaldehyde-induced arthritis model, formaldehyde was injected into the plantar fascia to induce a biphasic localized response, with the first phase characterized by release of serotonin, histamine, and kinins, and the second phase characterized by the release of prostaglandins [192,201–203]. The second model utilized in these experiments was the granuloma pouch model [198]. In this model, air was injected between the shoulder blades of the rat and an irritant injected into the air pouch and after 8 days, the animals were sacrificed and the exudate collected. In this experiment, 2% croton oil in a vehicle of ground nut oil was utilized as the irritant [198]. Both creatine and phenylbutazone caused a significant reduction of formaldehyde-induced arthritis and granuloma pouch weight ($p < 0.001$) [19], indicating that creatine was effective at reducing chronic inflammation. The authors [18] also examined the impact of creatinine on a variety of edema models including those induced by carrageenan, serotonin, nystatin, and formaldehyde-induced arthritis. In these studies, the authors also demonstrated that creatinine was effective at reducing edema in these models. While these studies [18,19] were purely descriptive in nature, they served as the impetus to further examine the immunomodulating properties of creatine and its alleged biologically inert breakdown product – creatinine.

4.2. Effect of creatine on the airways

In a study by Vieira et al. (2007), the authors used a mouse model of airway inflammation and remodeling to examine the impact of aerobic exercise on airway health [21]. Mice were sensitized to ovalbumin (OVA) and were exercised on a treadmill at either low or moderate intensity and showed reduced levels of eosinophils in their bronchoalveolar lavage fluids compared with non-exercisers. Additionally, reduced levels of IL-4 and IL-5, as well as decreased thickening of the airway walls were observed in the exercised animals as compared with the non-exercised animals. These findings reflect reports in humans that showed exercise improved cardiorespiratory fitness in individuals with asthma [204], and that individuals who trained at moderate intensity were more likely to demonstrate improvements in their asthma symptoms than those who exercised at a low intensity [205–210]. Based upon these data, it seems reasonable to suggest that athletes would have a lower incidence of asthma compared to the general public; however, in reality, the opposite may be true [211–214]. While there could be several factors to explain this, strenuous exercise increases the levels of T helper 2 (Th2) cytokines [215–218] along with other factors that also impact airway remodeling. While the factors that impact the severity of asthma are not completely defined, the intensity of exercise appears to be one factor, thus the casual

exerciser may experience improvements in their asthma, while elite athletes may have a deterioration of their condition.

A related study focused on the impact of creatine supplementation on allergic inflammation and airway remodeling in a mouse model of allergic asthma [24]. This investigation was based upon the observation that 1) the rate of asthma in athletes is higher than that observed in the general population; and 2) athletes are more likely to use creatine supplementation than the general population. The impact of creatine supplementation on female BALB/c mice with OVA-induced airway hyper-responsiveness was examined [24]. Under normal circumstances (that is, in the absence of creatine treatment), this particular model of airway hyper-responsiveness was characterized by a strong Th2 response in mice with high levels of IL-4, IL-5, and insulin-like growth factor-1 (IGF-1) [219]. The mice also developed thickened airway smooth muscles and collagen and elastic fiber deposition along with eosinophil infiltration of the airways. After creatine monohydrate treatment of the OVA-sensitized animals, mice experienced increased levels of IL-4, IL-5, IGF-1, eosinophil infiltration of the airways, increased smooth muscle thickness, and increased levels of collagen and elastic fibers in the lungs. Interestingly, the control group that was not OVA sensitized, but received creatine also experienced significant alterations in airway physiology and immune responses consistent with allergic inflammation [24], demonstrating that creatine, in and of itself, may be a risk factor for the development of asthma. The data also demonstrated a skewing of the immune response towards a more profound Th2-like response. As a follow-up to these studies, the impact of exercise on the creatine-induced changes in OVA-sensitized mice was examined [220]. The data demonstrated that exercise can reduce the effects of creatine in the model, since mice that completed a low intensity exercise regimen showed reduced numbers of eosinophils, numbers of IL-5 and IL-4 positive cells, and other pathological changes in the lungs indicative of airway hypo-responsiveness compared to animals that did not exercise [220]. Collectively, the data leads to the intriguing possibility that individuals with Th2-mediated diseases (i.e., allergy, asthma, lupus) could exacerbate their disease if they supplement their diet with creatine monohydrate. The results of these studies also lead to the question of whether creatine has the potential to alleviate Th1-mediated immunopathology.

The ability of creatine to impact various respiratory disease states is not limited to its effects on cells of the immune system. Ferreira et al. (2010) examined the effects of creatine supplementation on the airway epithelium in the mouse model of OVA-induced allergic asthma [221]. Creatine supplementation of the OVA-sensitized mice had decreased NF- κ B activation in epithelial cells compared to the level observed in the non-creatine supplemented, OVA-sensitized mice [221]. Since this transcription factor acts as a master regulator for the immune response [222–224], this alteration of NF- κ B activation resulted in increased expression of IL-5, CCL5, CCL2, TIMP-1, matrix metalloproteinase-9 (MMP-9), transforming growth factor- β 1 (TGF- β 1), IGF-1, EGFR, TIMP-2, and MMP-12 in epithelial cells compared to non-creatine supplemented OVA-sensitized mice. Because of these widespread alterations in immune mediators, the authors examined the levels of NF- κ B in the epithelial cells of mice [221]. These mediators are associated not only with inflammation, but also with airway remodeling. The chemokine CCL-5 is involved in recruitment of eosinophils, a key cell type in asthma, while CCL2 recruits dendritic cells, T

cells, and monocytes to the site. Together, these data provide insight into why some athletes may have a higher than normal rate of asthma than the general population, and therefore, should be cautioned before beginning a creatine supplementation program.

4.3. Impact of creatine on the innate immune system

Creatine monohydrate and creatine ethyl ester also appear to exhibit immunomodulatory potential. Leland and colleagues (2011) demonstrated that both forms of creatine down regulate expression of TLR-2, TLR-3, TLR-4, and TLR-7 in RAW 264.7 cells, a mouse macrophage cell line [20]. These TLRs interact with constituents of a wide array of pathogen-associated molecular patterns (PAMPs) including lipoteichoic acid (TLR-2), double-stranded RNA (TLR-3), bacterial lipopolysaccharide (TLR-4), and single-stranded RNA (TLR-7) [225]. As TLR down-regulation in the host should reduce the effectiveness of the innate immune response in sensing infection [226], this could result in an immunosuppressive impact and, thus, could be significant in individuals who have compromised immune systems, such as the elderly. The down regulation of TLRs by creatine may also be a mechanism by which creatine could alter PD pathogenesis, as it has been shown that α -synuclein upregulates several TLRs (TLRs-1, 2, 3, 4, and 7), as well as MyD88, a key signaling model involved in TLR activation [227]. In addition, TLR-4 antagonists have been shown to inhibit neuronal death *in vitro* in an ALS model [228].

An investigation into the anti-inflammatory effects of creatine on pulmonary epithelial cells *in vitro* was performed to determine whether creatine altered intercellular adhesion molecule-1 (ICAM-1) and E-selectin expression on the cells [17]. Human pulmonary cells exposed to 0.05 mM creatine had levels of ICAM-1 and E-selectin expression on their surface similar to untreated control cells. In response to the stimulus TNF- α [17], a cytokine that is induced early in an immune response [229], high levels of ICAM-1 and E-selectin were observed [17]. As the levels of creatine increased, the levels of ICAM-1 and E-selectin decreased to control levels at a concentration of 5 mM creatine [17]. In addition to altered surface molecule expression, the study also found that there was a decrease in endothelial permeability and the adhesion of neutrophils to the endothelial cells [17], suggesting that neutrophil extravasation may be affected by creatine use, as adhesion is a key step in this process [230]. These findings would suggest that the immune response may be dampened in the presence of high levels of creatine, despite the presence of pro-inflammatory processes (that is, the production of TNF- α). This effect may be cell type specific, as data from our laboratory demonstrated that when mouse macrophages (RAW 264.7) were incubated with 10 mM creatine monohydrate there was an upregulation of ICAM-1 on the cells after 24 or 48 hours (**Figure 2A-D**). However, it is important to note that our studies did not expose the macrophages to TNF- α after creatine incubation. Given that ICAM-1 is an integral molecule for antigen presentation, alterations in levels of this protein on antigen presenting cells may indicate that there are reduced or enhanced ability of cells to present antigen to T cells in the presence of creatine.

Because cytokines play a key role in both enhancing and downregulating immune responses, we examined whether creatine pretreatment of mouse macrophages altered production of IL-6, a pro-inflammatory mediator that has a key role in the production of acute phase

proteins. As shown in **Figure 3A-D**, reduced immunoreactivity was observed following incubation of macrophages with 10 mM creatine for 24 or 48 hours compared to control-treated cells. The reduction production in this cytokine could be interpreted to that the initial phases of the immune response could be dampened in individuals using creatine. The mechanism by which creatine was able to downregulate receptors, adhesion molecules, and pro-inflammatory cytokines in certain cell types while having the alternative occurring in asthma-induced models is not yet understood. However, it was hypothesized that creatine affected the NF- κ B signaling pathway [20·221·231] causing the production of cytokines, receptors, and growth factors to be increased or decreased. Down regulation of pro-inflammatory cytokines by creatine may also contribute to some the neuroprotective effects that have been observed, as inflammation is a common feature of many CNS diseases, including ALS [232] and PD [233·234].

4.4. Antioxidant properties of creatine

Since oxidants have the ability to negatively impact muscle fatigue and growth, Lawler et al. (2002) [235] examined whether creatine had direct antioxidant activity *in vitro*. To test this possibility, the effect of creatine on five different ROS was examined in a cell-free system. The ROS studied were hydrogen peroxide, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS⁺) cation radical, peroxynitrate, lipid peroxides (*tert*-butyl hydroperoxide) and superoxide anions. The findings showed that 40 mM creatine significantly ($p < 0.05$) reduced levels of ABTS⁺, superoxide anions, and peroxynitrate, but not hydrogen peroxide and lipid peroxides [235].

In a related study [236] that used a lower level of creatine (0.1-10 mM) to examine the antioxidant activity of creatine on *in vitro* cultured mouse and human cells after oxidative injury, it was found that when human promonocyte cells (U937 cells) were incubated with creatine for 2 hours and then treated with hydrogen peroxide, *tert*-butyl hydroperoxide, or peroxynitrate, the creatine-treated U937 had increased viability when compared to the control treated cells [236]. Additional cell lines (C2C12 [murine myoblasts]; HUVEC [human endothelial]) were also tested in a similar manner. Creatine reduced cell death following exposure to both hydrogen peroxide and *tert*-butyl hydroperoxide [236]. However, unlike the U937 cells, C2C12 and HUVEC cells required extended preincubation with creatine (24 hours) to provide maximal cell survival, indicating that all cell types do not respond similarly [236].

To determine whether creatine could protect DNA from ROS-mediated damage, Guidi et al. [237] used a system that generated superoxide anions, hydroxyl radicals, and hydrogen peroxide in the presence of mitochondrial and nuclear DNA. Using a PCR-based assay to identify the level of DNA damage, the authors found that there were increased levels of protection of the DNA in creatine-treated, hydrogen peroxide exposed mitochondrial DNA compared to control-treated, hydrogen peroxide exposed DNA. The levels of protection that creatine afforded approached the levels observed in control DNA that was not exposed to ROS [237]. Further studies utilizing HUVEC cells that were pretreated with creatine for 24 hours then exposed to hydrogen peroxide demonstrated that creatine treated cells had increased viability compared to control cells, and that creatine pretreatment also protected

mitochondrial DNA from significant damage compared to the control cells [237]. In addition, the nuclear DNA experienced less damage than the mitochondrial DNA in both control and creatine-treated cells, however, the differences between treatments were not statistically significant. In total, these studies [235–237] suggest that creatine has direct antioxidant activity and that both cell viability and the levels of DNA damage are positively affected by exposure to creatine.

5. Conclusions

Creatine has been used by athletes as an ergogenic aid since the 1970s. Herein, we describe potential uses for creatine in the clinical setting. Of note, creatine is extremely inexpensive compared to most neuroprotective agents and immunomodulatory drugs. It is critical that future studies in humans examining the neuroprotective properties of creatine are designed to ensure that participants are ingesting doses that parallel the doses used in rodent studies. Furthermore, it is of great interest to continue to explore the potential of this ergogenic supplement in animal models of disease to better understand the mechanism(s) of action of creatine *in vivo*. Given creatine's impact on the immune system studies in models of autoimmune disease may be particularly applicable.

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Highlights

- Creatine increases airway hyperresponsiveness in mice.
- Soluble mediator production, such as IL-4, IL-5, and IL-6, is altered by creatine.
- ICAM-1, E-selectin, and TLR expression are impacted by creatine exposure.
- Creatine has neuroprotective and antioxidative effects in mice.
- Inadequate creatine dose may explain clinical trial failure in humans.

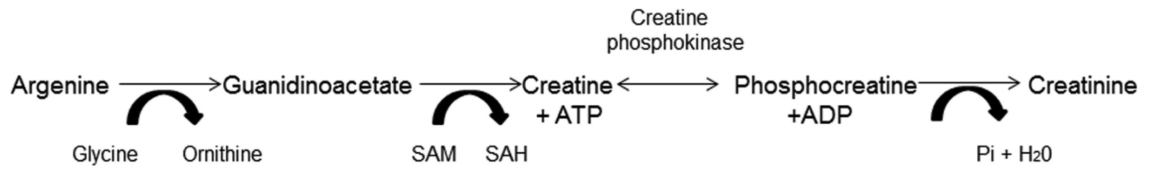


Figure 1. The arginine biosynthesis pathway

Creatine is generated as part of the arginine biosynthesis pathway. Creatine and phosphocreatine exist in equilibrium in the cell. Creatine phosphokinase catalyzes the reaction between creatine and phosphocreatine, which results in energy (ATP) generation. Hydrolysis of phosphocreatine yields the end product of the pathway, creatinine. SAM, S-adenosyl-methionine; SAH, S-adenosyl-homocysteine

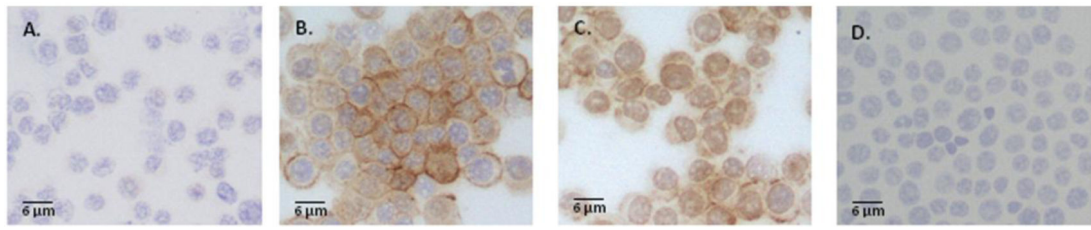


Figure 2. ICAM-1 expression is upregulated in mouse macrophages following exposure to 10 mM creatine monohydrate

Immunohistochemical staining revealed an increase in ICAM-1 signal after incubation with 10 mM creatine monohydrate for 24 (B) or 48 (C) hours. Control (A) cells were incubated with culture media alone. Non-specific staining controls received rat IgG (isotype control) in lieu of primary antibody (D). Immunohistochemical staining for ICAM-1 was performed using the Avidin Biotin Complex (ABC) method (Vector Laboratories) as previously described [238] using an antibody to mouse ICAM-1 (BD Pharmingen). Development was performed using the AEC Substrate Kit (Vector Laboratories). Positive staining is indicated by the brown reaction product.

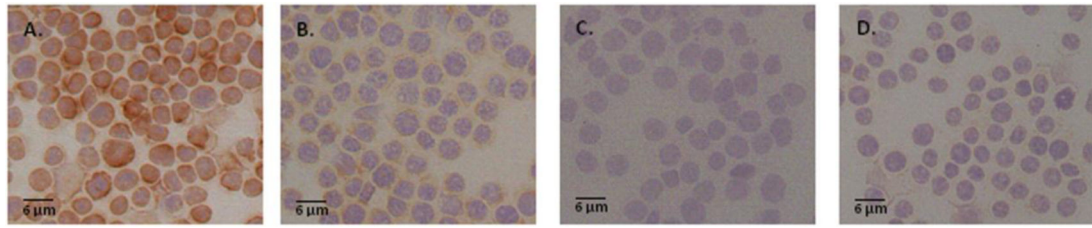


Figure 3. IL-6 expression is downregulated by mouse macrophages following exposure to 10 mM creatine monohydrate

Immunohistochemical staining demonstrated a decrease in IL-6 expression following incubation with 10 mM creatine monohydrate for 24 (B) or 48 (C) hours. Control (A) cells were incubated with media alone. Non-specific staining controls received rat IgG (isotype control) in lieu of primary antibody (D). Immunohistochemical staining for IL-6 was performed using the Avidin Biotin Complex (ABC) method (Vector Laboratories) as previously described [238] using an antibody to mouse IL-6 (BD Pharmingen). Development was performed using the AEC Substrate Kit (Vector Laboratories). Positive staining is indicated by the brown reaction product.

Table 1Summary of *in vivo* studies examining the effects of oral creatine on inflammation

Species	Model	Dose	Results	Reference
Rat	Carrageenan-induced edema	50-500 mg kg ⁻¹	Reduced swelling	[19]
	Nystatin-induced edema	500 mg kg ⁻¹	Reduced swelling	
	5-HT-induced edema	500 mg kg ⁻¹	Reduced paw volume	
	Formaldehyde-induced arthritis	100-500 mg kg ⁻¹	Reduced paw volume	
	Granuloma pouch	100 mg kg ⁻¹ ×7d	Reduced exudate, reduced pouch weight	
Mouse	OVA sensitization	0.5 g kg ⁻¹ d ⁻¹	Increased allergic inflammation, airway responsiveness, airway remodeling	[24]
Mouse	OVA sensitization ± exercise	0.5 g kg ⁻¹ d ⁻¹	Low intensity exercise reduced negative effects of creatine	[220]
Mouse	OVA sensitization	0.5 g kg ⁻¹ d ⁻¹	Increased allergic inflammation and airway remodeling	[221]

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