Evaluation of the Anti-Inflammatory Effects of Creatine in Canine Chondrocytes as an in-vitro Model of Joint Inflammation

By

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Abstract:

Little is known about the anti-inflammatory activity of creatine. The aim of this study was to evaluate the anti-inflammatory effects of creatine supplements in canine chondrocytes (CnC). CnC were stimulated with IL-1β. Release of PGE2 and TNFa was measured using ELISA. Changes in oxylipin profile was assessed using HPLC/MS. Expression of COX-2 and phosphorylated NF-kB was performed using western blot. Changes in above inflammatory responses were examined following treatment with various creatine compounds including the metabolite creatinine. COX inhibitor, Rimadyl, substantially reduced PGE2 release, despite increasing both TNFa release and COX-2 expression. All creatine compounds, including creatinine, reduced PGE2, COX-2 and TNFa in stimulated CnC. In addition, all the compounds examined reduced phosphorylated NF-kB expression. The creatine compounds were also able to interfere with the production of several oxylipins in response to IL-1β. Creatine supplements may have a beneficial role in preventing inflammation within the joint and other tissues.

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Abbreviations:

MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
3-NP	3-nitropropionic acid
6,15-dk-,dh-PGF1a	6,15-diketo-,dihydro PGF1a
6k PGF1a	6-Keto Prostaglandin F1-alpha
11-HETE	11-Hydroxyeicosatetraenoic acid
12-HETE	12-Hydroxyeicosatetraenoic acid
13-HDoHE	13-Hydroxy-docosahexaenoic acid
14,15 EpETrE	14,15 epoxy-eicosatrienoic acid
16,17 EpDPE	16,17 epoxy-docosapentaenoicacid
18-HEPE	18-Hydroxy-eicosapentaenoic acid
20-HDoHE	20-Hydroxy-docosahexaenoic acid
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AdA	Adrenic acid
ALA	Alpha-linolenic acid
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis

AA	Arachidonic acid
CrC	Creatine citrate
CEE	Creatine ethyl ester
CHCL	Creatine hydrochloride
CRN	Creatinine
CK	Creatine kinase
CM	Creatine monohydrate
CrPyr	Creatine pyruvate
CT1	Creatine transporter 1
COX-2	Cyclooxygenase-2
CYP450	Cytochrome P450 epoxygenase
cPLA2	Cytosolic phospholipase A2
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ELISA	Enzyme-linked Immunosorbent Assay
ECM	Extracellular matrix
GABA	Gamma-Aminobutyric acid
GLA	Gamma-linolenic acid
GAMT	Guanidinoacetate-methyltransferase

HPLC/MS	High Performance Liquid Chromatography/ Mass Spectrometry
HD	Huntington's disease
iNOS	Inducible nitric oxide synthase
IFNa	Interferon-alpha
IL-1β	Interleukin-1 beta
LDH	Lactate dehydrogenase
AGAT	L-arginine:glycine amidinotranferase
LPS	Lipopolysaccharide
MMPs	Metalloproteinases
K_m	Michaelis constant
mPTP	Mitochondrial permeability transition pore
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
NF-kB	Nuclear factor kappa B
OA	Osteoarthritis
PD	Parkinson's disease
PCr	Phosphocreatine
PUFAs	Polyunsaturated fatty acids
CnC	Primary canine chondrocytes

PGE2	Prostaglandin E2
ROS	Reactive oxygen species
RT-PCR	Reverse Transcriptase – Polymerase Chain Reaction
SLC6	Solute carrier 6
TLR	Toll-like receptor
TNFa	Tumor necrosis factor-alpha
LA	Linoleic acid
LOX	Lipooxygenase

1. Introduction:

1.1 Creatine Synthesis and Sources:

Since the discovery of creatine in 1832 by the French chemist Michel Eugène Chevreul,[1, 2] it has been one of the most extensively studied dietary supplements. Creatine (Figure 1), is a nitrogenous guanidine compound found naturally in protein-based foods such as meat, fish, milk, and nuts. [3] In the body, creatine is found both as free creatine and as phosphocreatine (PCr). Creatine and PCr together comprise the total creatine pool. [4]

Approximately 95% of creatine is stored in skeletal muscles, mainly in the form of PCr with the remaining 5% distributed in other tissues with the highest concentrations found in the brain, liver, kidneys, and testes. [5] For the average 70 kg human, it is estimated that the total creatine content in the body is around 120 grams. The daily requirements of creatine for an average human are around 2 grams, which matches the rate of conversion of creatine and PCr to creatinine (CRN) (1.7-2 grams/day for an average human). [6] It is estimated that approximately half of the creatine found in the body is obtained exogenously through the diet (~ 1 grams daily). [7] In addition, creatine can be also synthesized de novo in the body from amino acids (~1 grams daily). The synthesis of creatine requires three amino acids, L-arginine, glycine, and L-methionine. The first and rate-limiting step of creatine synthesis occurs in the kidney and is catalyzed by the

enzyme L-arginine:glycine amidinotranferase (AGAT). This enzyme catalyzes the transfer of an amidino group from L-arginine to glycine producing L-ornithine and guanidino-acetate, the precursor of creatine. Guanidino-acetate, produced in the kidney, is then taken up by the liver where a second enzyme, guanidinoacetate-methyltransferase (GAMT), catalyzes the methylation of guanidino-acetate to produce creatine (Figure 2).

Figure 1. Creatine Structure.

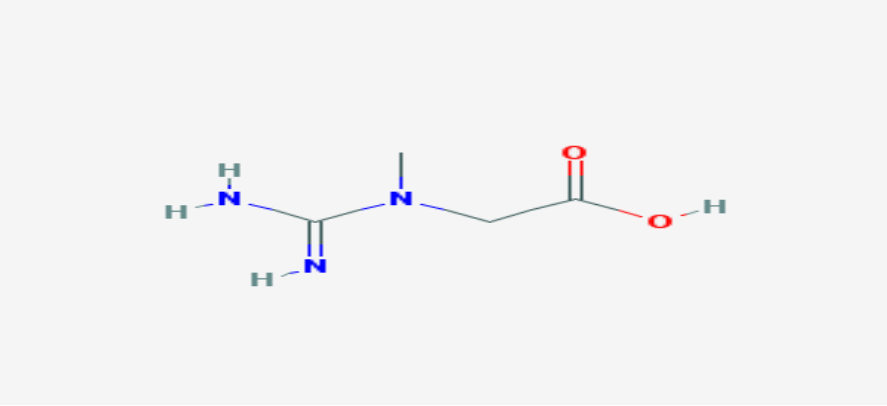
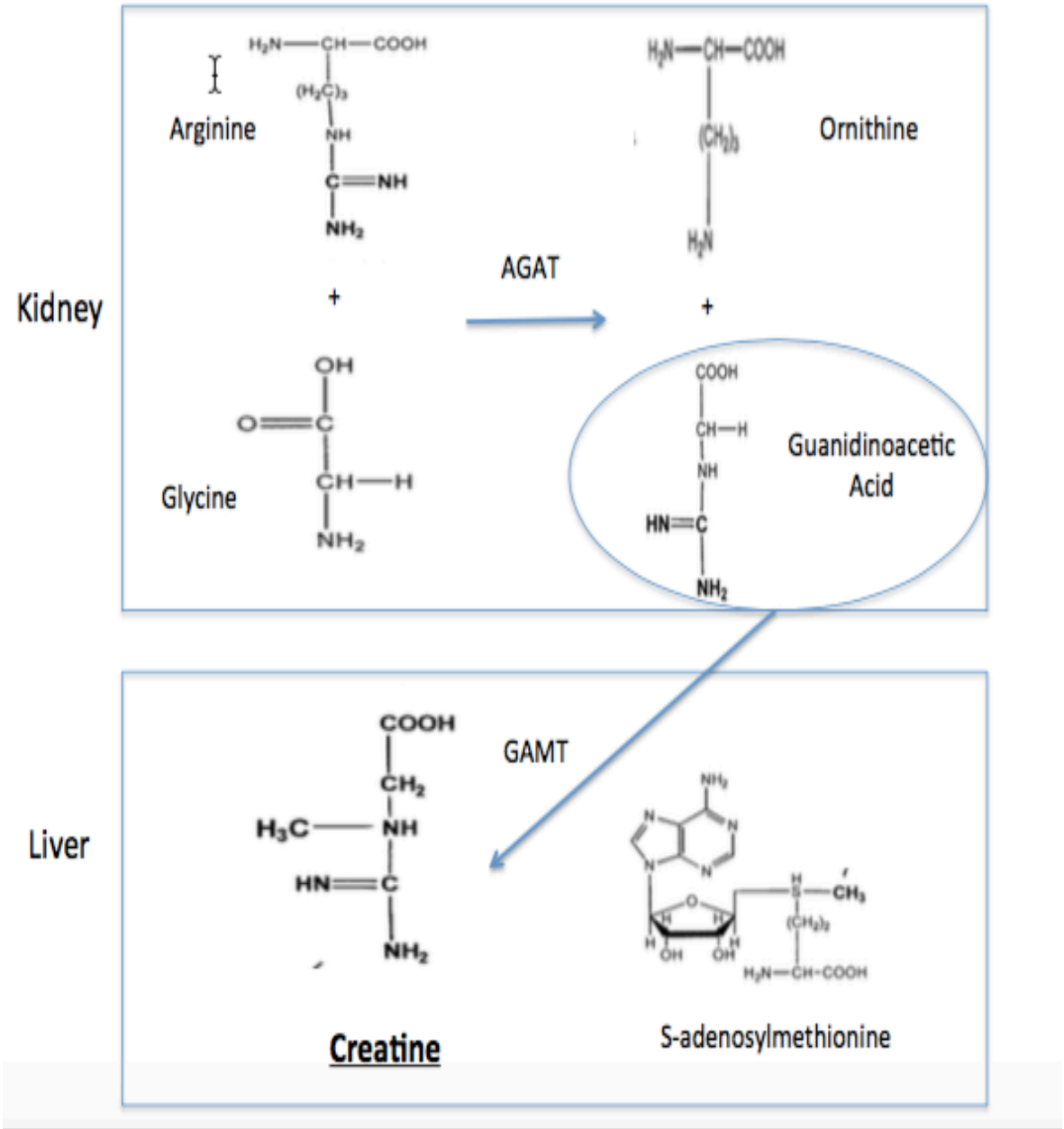


Figure 2. Endogenous creatine synthesis. [6]

AGAT: L-arginine:glycine amidinotranferase. GAMT: guanidinoacetate-methyltransferase



1.2 Creatine Transporter:

Creatine circulating in the blood is taken up by tissues with high and fluctuating energy demands through a specific plasma membrane creatine transporter (CT1). [5] CT1 belongs to the solute carrier 6 (SLC6) family of membrane transport proteins. SLC6 represents a large family of membrane transporters that facilitate the transport of many solutes including neurotransmitters and amino acids across the plasma membrane of cells. [8] The CT1 is encoded by the SLC6A8 gene (SLC6 family, subfamily: A, member: 8), which is located on chromosome Xq28. [9] At the mRNA level, CT1 has been found to be expressed predominantly in muscles, kidney, heart, brain, colon, and testes. [10-12] In addition to CT1, another creatine transporter has been identified (CT2), which is encoded by the SLC6A10 gene, located on chromosome 16p11 and is expressed in the testes only. [13] The physiological significance of this particular isoform is still not clear.

The expression of the CT1 protein and the presence of two major isoforms (70 and 55 kDa) have been identified in various tissues. These isoforms are most likely generated by alternative splicing and the functional significance of their presence is still not clear. [14] Previously, it was believed that these major isoforms are present exclusively in the plasma membranes of cells. However, recent evidence indicates

the presence of these isoforms in mitochondrial membranes in different tissues including the heart, brain, and kidneys. [14]

CT1 is a saturable sodium- and chloride- dependent transporter that transports creatine against its concentration gradient with high specificity. [9] The uptake of creatine depends on the sequential binding and co-transport of Na^+ and Cl^- ions with a coupling ratio of 2 Na^+: 1 Cl^-: 1 creatine molecule. [15] The ion concentration gradient generated by the plasma membrane Na^+/K^+ ATPase provides the driving force for creatine uptake into the cell. [15] Tissue accumulation of creatine through the CT1 is a saturable process. Michaelis constant (K_m) values of CT1 and serum creatine concentrations in different species are shown in Table 1. K_m represents the substrate concentration at which the reaction rate is half maximum. It is important to note that these K_m values are gathered from studies done on different tissues and cell types. However, based on blood concentrations of creatine, it is apparent from these Km values that CT1 is working close to saturation suggesting a possible limitation of creatine accumulation by the amount of CT1 protein in the cell membrane. [15] For example, in un-supplemented humans, the normal plasma levels of creatine range from 50 to 100 uM and with K_m values of 15-77 uM, suggesting that the transporter is working near saturation and any further increase in the plasma concentrations of creatine will most likely saturate the transporter resulting

in mostly passive diffusion pathways of entry into the cell. This fact needs to be treated cautiously, since it is known that supplementation with 5 grams of creatine, which is the lowest dose used by athletes, results in maximal plasma creatine concentrations of around 800 uM, [16] and it is most likely that these high concentrations will saturate the transporters thus limiting transporter mediated uptake as a route for entry of most creatine supplements into the cell.

Table 1. CT1 K_m values and serum creatine levels across different species. [5, 15]

Species	K_m	Serum Creatine Concentration
Human	15-77 uM	50-100 uM
Rat	22-46 uM	140 uM
Rabbit	35 uM	150 uM
Mouse	110 uM	200 uM
Bovine	188 uM	

1.2.1 Creatine Transporter Expression in The Brain:

In the brain, CT1 is expressed in the luminal and abluminal sides of the micro-capillary endothelial cells suggesting blood-brain barrier permeability of creatine is dependent on transporter activity.[16, 17] Moreover, it is also widely expressed in neurons and oligodendrocytes. However, it is not expressed in the astrocytic feet processes, which cover more than 98% of the surface of the micro-capillary endothelial cells.[17, 18] While AGAT and GAMT are expressed in almost all the cells in the brain, including neurons and astrocytes,[19, 20] it is still not known to what extent de novo synthesis of creatine in the brain contributes to the total brain creatine content. In patients with creatine transporter deficiency, caused by mutations in the SLC6A8 gene, the uptake of creatine into the brain is inhibited and despite normal functioning of AGAT and GAMT, the levels of creatine in the brain are significantly reduced leading to neurological symptoms, including intellectual disability, delays in speech, autism, and seizures.[21] This highlights the importance of peripheral and exogenously administered sources of creatine as determinants in brain levels of creatine and has fueled interest in potential benefits of creatine supplementation in the treatment of creatine deficient syndromes and neurodegenerative diseases.

1.3 Cellular Effects of Creatine:

1.3.1 Energetic Effects (The Creatine-PCr System):

The main function of creatine within the cell is to provide a readily available source of phosphate for the replenishment of Adenosine triphosphate (ATP). As ATP is considered to be the energy currency of life for cells it is important to all the biological systems within the body. [22] The creatine – PCr system is of paramount importance especially in tissues with high and fluctuating energy demands, such as the heart, brain, and muscles. [23] Energy demands and supply in those tissues are highly balanced and regulated. For instance, the rate of ATP hydrolysis can be increased significantly by cells with high energy demands within seconds, but the level of intracellular ATP remains surprisingly constant. This can be explained by the complex action of the immediately available creatine-PCr system, which represents an extremely efficient energy buffering system. [23] The levels of ATP in excitable cells, such as skeletal muscle, are usually low, ranging from 2 to 5 mM. Based on these levels of ATP and the rate of ATP hydrolysis when active, muscle contractions could only be sustained for few seconds. However, the levels of PCr in muscle cells can reach up to 22 to 35 mM, thus providing a relatively large pool of cellular phosphate for replenishing ATP. [24] Despite the fact that the levels of ATP in excitable tissues are very small, the

intracellular levels of ATP remain constant even after activation of the cell, because ATP is rapidly replenished by the use of PCr. [24]

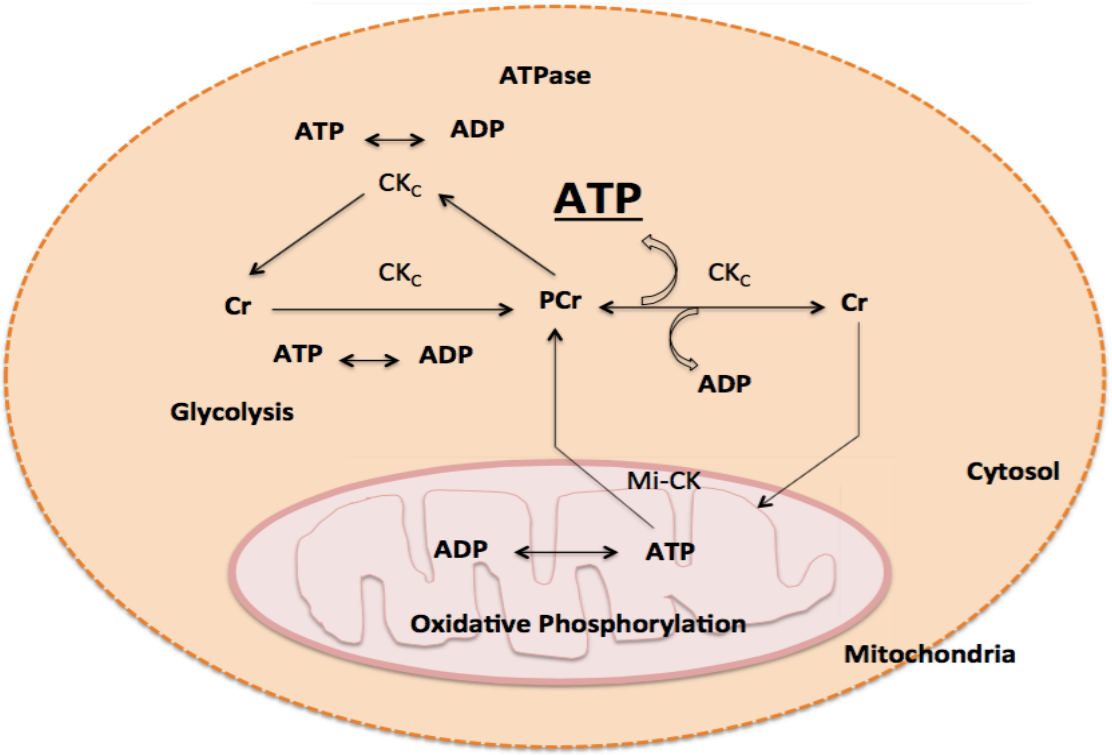
The enzyme creatine kinase (CK), which is a key enzyme in cellular energetics, catalyzes the reversible transfer of a phosphate group from PCr to Adenosine diphosphate (ADP), to regenerate ATP and Cr, [25, 26] (Figure 3). There are 2 types of CK, which are always co-expressed together in a tissue-specific manner. The first type is cytosolic CK, which consists of 2 subunits, B (brain type) or M (muscle type). The subunit combinations give rise to 3 different isoenzymes: CK-MM, CK-BB, and CK-MB. CK-MM is specific to sarcomeric muscles, whereas CK-BB is expressed in brain and some other tissues. It is thought that CK-MM is produced from CK-BB through the transitory CK-MB during development.[24] The second type of CK is mitochondrial CK, which accumulates in the mitochondria and can exist in 2 different isoenzymes, ubiquitous Mi-CK and sarcomeric Mi-CK, which is striated muscle specific.[27, 28]

Cytosolic CK catalyzes the transfer of high energy phosphate from PCr to ADP, functioning as a temporal energy buffer in times of high ATP turnover. [27] Moreover, some fractions of the cytosolic CK are either coupled functionally to glycolysis to replenish the PCr pool with the use of glycolytic ATP or coupled with

ATPase at sites of energy consumption, [27] (Figure 3). Beside the function of the creatine-PCr system as a temporal energy buffer, it has been suggested that it might also act as an energy shuttle or transporter, with creatine and PCr moving to and from sites of energy consumption and energy production. [24] In the mitochondria, ATP is generated by oxidative phosphorylation (site of energy production). The phosphate group of ATP is transferred to creatine by the action of mitochondrial CK forming ADP and PCr. PCr can then by exported to the cytosol and is used to regenerate ATP by the action of cytosolic CK (at sites of energy consumption), thus the system here serves as a spatial energy buffer. Creatine generated as a result of this reaction diffuses back to the mitochondria to be recharged again. [24, 27, 28]

Figure 3. Theorotical model of the creatine-PCr system. (adapted from Wallimann et al., 1998) [27]

PCr: Phophocreatine. ATP: Adenosine triphosphate. ADP: Adenosine diphosphate. CK: creatine kinase



1.3.2 Mitochondrial Membrane Stabilization:

The mitochondrial permeability transition pore (mPTP) is a pathological opening in the inner mitochondrial membrane, which is characterized by an increase in the permeability of the mitochondrial membranes to molecules up to 1500 Da. [29, 30] mPTP opening is linked to mitochondrial dysfunction because it leads to loss of the electrochemical potential, cessation of ATP synthesis, calcium release, release of pro-apoptotic proteins, apoptosis and cell death. [30] The physical interaction of mitochondrial CK, which is located in the mitochondrial inter-membrane space along the inner membrane, with proteins of both inner and outer mitochondrial membranes that contribute to mPTP formation suggests an involvement of mitochondrial CK in the regulation of the opening of mPTP. [31] It was suggested that creatine has the ability to convert mitochondrial CK from its dimeric form to the more stable octameric form that stabilizes mPTP, thus preventing its opening. [29, 32]

1.3.3 Antioxidant Effects:

One of the possible cellular effects of creatine is the direct scavenging of free radical species. [33] It was shown that creatine supplementation in cultured mammalian cells exposed to different oxidative agents exerts a dose-dependent (up

to 10 mM) antioxidant activity via a mechanism dependent on direct scavenging of reactive oxygen and nitrogen species. [33]

1.4 Creatine Supplementation:

1.4.1 Exercise Performance:

Creatine is a very popular nutritional supplement and ergogenic aid that has been used extensively by athletes and bodybuilders to enhance performance and muscle mass with an annual market of more than 400 million dollars worldwide. [34] As mentioned previously, creatine is involved in the rapid production and replenishment of ATP, thus increasing the amount of free energy available for exercise and muscle function, especially under anaerobic conditions. [35] Many studies have shown that dietary supplementation with creatine monohydrate (CM) (up to 20g/day) resulted in significant increases in total creatine, free creatine, and PCr concentrations in the muscles. [36-38] The observed increase in total creatine concentrations (free creatine + PCr) in muscles after oral creatine supplementation is correlated with increased exercise performance as measured by increased work output, strength, exercise capacity, as well as fat-free body mass, and muscle fiber size in supplemented individuals compared to placebo group. [35, 39-41]

1.4.2 Therapeutic Indications:

1.4.2.1 Neurological Conditions:

Creatine supplementation has been shown to be neuroprotective in many cellular and animal models of neurodegenerative diseases including Huntington's disease (HD), Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). The exact molecular mechanism by which creatine exerts its neuroprotective effect is not clearly understood. However, several potential mechanisms of neuroprotection of creatine have been suggested and one or more of these postulated mechanisms are likely involved in the neuroprotection observed with creatine supplementation. Potential mechanisms of neuroprotection following creatine supplementation include buffering of intracellular energy reserves, improvement of mitochondrial function, stabilization of the mitochondrial permeability transition pore, reduced excitotoxic effects, and antioxidant activity. [42-45] A summary of the neurological implications for creatine supplementation is described below.

1.4.2.1.1 Huntington's Disease:

HD is a progressive autosomal dominant inherited neurodegenerative disorder. It is characterized by degeneration of Gamma-Aminobutyric acid (GABA)-ergic

medium-sized spiny projections neurons within the striatum in the basal ganglia leading to progressive development of motor incoordination and loss of cognitive function. [46, 47] It is caused by an unstable expansion of CAG trinucleotide repeat in the huntingtin gene, which is located on chromosome 4, resulting in mutated elongated poly-glutamine stretches in the huntingtin protein. [48] There is increasing evidences suggesting a role of cellular energetics and mitochondrial dysfunction in HD. [49-55]

Matthews et al. showed that administration of 1% dietary creatine reduces striatal lesion volume produced by the mitochondrial toxin 3-nitropropionic acid (3-NP) in rats. The neurotoxin 3-NP, inhibits succinate dehydrogenase (complex II), induces striatal lesions and abnormal motor behavior that closely resemble that seen in HD patients. [56] Ferrante et al. used the transgenic R6/2 mouse to model HD. This model has CAG repeat length of 141-157 (normal < 35) and it develops a progressive neurological phenotype. At 4.5-6 weeks, these mice show loss of brain and body weight as well as neuronal intra-nuclear inclusions that are immune-positive for huntingtin. Additionally, at 9-11 weeks the mice develop many of the neurological features associated with HD including irregular gait, stereotypic movements, and resting tremors.[57] They reported that dietary supplementation with 1 or 2% creatine at 21 days of age significantly increased survival compared to the control group (9.4 and 17.4% in mice supplemented with 1 or 2% creatine,

respectively). In addition, creatine administration significantly slowed motor deterioration, reduced gross brain atrophy, and delayed striatal neuron atrophy. [57]

Due to the fact that oral creatine supplementation had produced significant neuroprotection in transgenic animal models of HD, small pilot studies were conducted to assess the safety, tolerability, and efficacy of creatine in patients with HD. Verbessem et al. conducted a double-blind placebo-controlled pilot study over a 1-year period. 41 patients ingested 5 grams of creatine or a matching placebo.[58] In the same year, Tabrizi et al. conducted an open label pilot study with 13 HD patients given 10 grams of creatine per day for 12 months.[59] In both of these studies, creatine administration was safe and well tolerated, however, no improvement in cognitive function was found. The lack of neuroprotective effect of creatine might be due to three reasons. First, the sample size in both of these studies was small and may not have been sufficiently powered to detect the relatively small differences in the treatment groups. Second, the follow up period (12 months) was short and may not have provided sufficient time for the neuroprotective effects of creatine to manifest themselves. Third, the doses used in these studies were substantially lower than the allometric dose equivalent given to the transgenic HD animal models. In most of the successful animal studies, creatine was given at doses ranging from approximately 2.5 to 5 grams per kg body

weight. Based on allometric dosing, [57, 60] this would require a 14-28 g daily dose in human studies, which is substantially larger than the 10 grams daily dose used.

Recently, the Huntington study group (HSG) published the results of PRECREST, a phase II clinical trial to assess the safety, tolerability, and efficacy of high dose creatine in patients that carried the gene mutation but were asymptomatic. They reported that high dose creatine (up to 30 grams daily) was safe and well tolerated. In addition, creatine supplementation significantly slowed that rate of cortical thinning, and reduced the change in white matter, grey matter, and basal ganglia volumes compared to the placebo group.[61] Based on these positive results, CREST-E, a large multicenter, phase III, randomized, double-blind, placebo-controlled trial of high dose creatine treatment (up to 40 grams daily) in patients with HD (stage II or III) was initiated. While the CREST-E trial was to cover a period of 3 years, it was recently terminated (October 2014) at the half-way mark due to lack of effectiveness of creatine.

1.4.2.1.2 Alzheimer's Disease:

AD is a late-onset, age-dependent, progressive neurodegenerative disease characterized by progressive cognitive decline. [44] The most important pathological

features of AD are neurofibrillary tangles, plaques rich in insoluble β-amyloid protein, cortical atrophy, and neuronal loss. [62]

So far, very few studies have been conducted to examine the neuroprotective effect of creatine in AD. Brewer and Wallimann have shown that creatine supplementation at doses between 0.125 and 2 mM protected against glutamate and β-amyloid toxicity in cultured rat hippocampal neurons.[63] The mechanism by which creatine exerts its neuroprotective effect might be partially through increased levels of ATP and PCr.[63]

1.4.2.1.3 Parkinson's Disease:

PD is a chronic, progressive, age-dependent neurodegenerative disorder. It is characterized by progressive bradykinesia, muscle rigidity, tremors, and gait abnormalities. These devastating symptoms are mainly caused by the extensive loss of the dopaminergic neurons in the substantia nigra of the brain. [64] The histological hallmark of PD is the presence of fibrillar aggregates called Lewy Bodies and the accumulation of the protein alpha-synuclein into these inclusions in neurons. [65]

Matthews et al demonstrated that creatine supplementation at doses of 0.25 to 0.1% produced dose-dependent neuroprotection against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity in mice. [66] MPTP, which is a known inhibitor of complex I activity of the mitochondrial electron transport chain, produces neurological damage that resembles that seen in patients with PD and this neurotoxin has been used as a model of PD in many studies. [67-71]

1.4.2.1.4 Amyotrophic Lateral Sclerosis:

ALS is a chronic, progressive, and usually fatal neurodegenerative disease. The terms motor neuron disease (MND) or Lou Gehrig's disease are sometimes used interchangeably with ALS. The defining feature of ALS is the progressive degeneration and loss of upper and lower motor neurons in the motor cortex of the brain, brainstem, and the spinal cord and it is characterized by muscle spasticity, rapidly progressive weakness, and atrophy. [72]

It has been reported that oral administration of 1-2% creatine in the diet resulted in a dose-dependent increase in survival of a transgenic mouse model of ALS and an improvement in motor performance compared to un-supplemented mice. [73]

1.4.2.2 Anti-Inflammatory Effects:

Recently, many investigators have shown interest in the anti-inflammatory effects of creatine supplements. Evidence in support of the anti-inflammatory effects of creatine supplementation include reduced production of inflammatory markers associated with intense exercise upon supplementation with oral creatine. [74-76] Studies by Santos et al. evaluated the effect of creatine supplementation on the levels of inflammatory markers and muscle soreness in athletes after running 30km. They found that supplementation with creatine (20 grams daily), 5 days prior to the 30 km race, significantly attenuated the increase in Prostaglandin E2 (PGE2), tumor necrosis factor-alpha (TNFa) (by 60.9% and 33.7%, respectively), and lactate dehydrogenase (LDH) compared to the placebo group. [75] Similarly, using the same creatine supplementation protocol, Bassit et al. found that creatine significantly reduced the levels of TNFa, interferon-alpha (IFNa), interleukin-1 beta (IL-1β), and PGE2 after a half-man iron competition. [74] These results suggest that creatine supplementation during a short period of five days before intense exercise may attenuate the increase in plasma levels of pro-inflammatory cytokines and PGE_2.

In 2012, Leland et al. using RAW 264.7 cells, a mouse macrophage cell line, demonstrated that creatine, as well as the metabolite CRN down-regulated the

expression of toll-like receptor 2 (TLR-2), TLR-3, TLR-4, and TLR-7. [77] TLRs are expressed by many cell types and are involved in sensing the presence of foreign antigen by recognizing distinct pathogen-associated molecular patterns (PAMPs) on the microbe. Binding of these PAMPs to TLRs leads to the generation of inflammation. [77] In spite of this, however, the mechanism by which creatine supplementation exerts its anti-inflammatory effects remains to be elucidated.

1.5 Dosing and Safety Considerations for Creatine Supplements:

CM is the standard and most commonly used form of creatine and it is the most commonly cited form of creatine in the scientific literature. Dietary supplementation with CM for improved athletic performance typically involves a loading dose of 20 grams per day over a five day period, followed by a daily maintenance dose of 3-5 grams.[5] This regiment has been reported to result in an approximately 20% increase in intramuscular creatine levels [5, 7] and to enhance strength and improve recovery time in a variety of performance conditions.[7, 36, 37] Studies suggest this dosing level is safe and well tolerated with minimal side effects. [40, 41, 78, 79, 82]

In addition to creatine supplementation to improve athletic performance there is increasing use for various therapeutic applications discussed previously. Dietary supplementation with CM for therapeutic indications typically involves daily doses that exceed 20 grams.[61] Indeed the recent HD clinical trials had a targeted daily dose of 30-40 grams. Even at these higher doses, creatine supplementation appears relatively safe and well tolerated and no significant adverse events were reported. [61] The most common adverse effects reported after 18 months of treatment were mild gastrointestinal discomfort, diarrhea and nausea (22-50% in creatine treated group compared to 3-16% in placebo). [61] Moreover, supplementation with high dose creatine had no adverse effects on renal, hepatic, or cardiovascular function.[61] In a double-blinded, placebo-controlled, 12-week study, 34 subjects were given 20 grams of CM per day for 5 days and 10 grams per day for 51 days. No significant changes in total protein, serum CRN, bilirubin and blood urea nitrogen, or in serum enzymes aspartate transaminase, alkaline phosphatase, gamma-glutamyl transpeptidase, lactate dehydrogenase, and creatine phosphokinase were reported, indicating minimal changes in clinical markers of renal and hepatic function. [80]

1.6 Disadvantages of the Current Commercially Available form of Creatine: Creatine Monohydrate:

Despite the widespread use of CM as a dietary supplement, a critical examination of the pharmacokinetic profile and the absolute oral bioavailability of CM remains undetermined. There are few studies focusing on the pharmacokinetics properties of creatine supplements and none of them report any estimated pharmacokinetic parameters. This is surprising, giving the widespread use of creatine by many athletes and the emerging indications of creatine in the treatment of many diseases. Optimization of desired therapeutic effects will be dependent on foundational knowledge of the pharmacokinetic properties.

It is known that CM has a relatively low aqueous solubility (~ 16.6 mg/ml). [81] Since it is usually given at high doses (5-25 g/day for athletes, and > 25 g/day for therapeutic applications), it is likely administered as an oral suspension. As only the solubilized creatine is available for absorption in the intestines, such a dosing practice would suggest that a portion of the creatine consumed is unabsorbed. Aside from diminished beneficial responses, the unabsorbed CM in the intestines could contribute to water retention and gastrointestinal discomfort reported in many studies with creatine supplementation. [82] Gufford et al. evaluated the permeability of CM, CRN, and creatine ethyl ester (CEE) using the human

intestinal epithelial cell line derived from a human colon carcinoma (Caco-2). Caco-2 is a well-established cell line that has been used in many studies to predict the oral absorption of many compounds. Of the three compounds examined, CM had the lowest permeability in Caco-2 monolayers. [83] The results of this study were also in agreement with the low permeability of CM and radiolabeled CM in Caco-2 monolayers reported in two other published studies. [82, 84]

These characteristics of CM suggest low and incomplete absorption and low bioavailability. In fact, Jager et al. compared the effects of CM, and two other salts of creatine, creatine citrate (CrC) and creatine pyruvate (CrPyr), in raising plasma creatine levels in 6 healthy subjects. One hour after ingestion, they found a significant increase in bioavailability of CrPyr (17%) compared to CM. [16]
Given these properties of CM, the identification of newer creatine derivatives with enhanced aqueous solubility and improved absorption is warranted.

1.7 Newer Creatine Derivatives:

To overcome the formulational disadvantages of CM, new derivatives of creatine with improved water solubility and increased oral bioavailability have been developed. Two general approaches for creatine derivatives have been taken. One

involves examination of different salt forms of creatine. These studies have led to the identification of several different salt forms of creatine with improved aqueous solubility over that of CM, [82] (Table 2). Gufford et al. compared the aqueous solubility of different creatine salts to that of CM. They reported that all the creatine salts examined were significantly more water soluble than CM. For instance, the aqueous solubility of creatine hydrochloride (CHCL) was around 709 mg/ml compared to only 16-17 mg/ml for CM. [82] Studies by Jager reported approximately 20% increase in oral absorption of CrPyr and citrate salts compared to CM,[16] while studies with CHCL indicated approximately 70% increase in oral absorption over CM in human subjects. [85] Together these studies suggest improved aqueous solubility of the creatine salts will provide advantages in dosing.

The other approach to the development of improved forms of creatine for dietary supplementation involves creation of ester derivatives of creatine, [86] (Table 3). Vennerstrom and Miller suggested creatine esterification as a potential strategy to improve the oral bioavailability of CM. They were able to synthesize a series of esterified creatine derivatives, including CEE, creatine benzyl ester, creatine t-butyl ester and creatine octyl ester. [86] The lead compound, CEE was shown to have improved aqueous solubility as well as a 2-fold greater octanol partition coefficient compared to CM. *In-vitro* permeability studies demonstrated that CEE had

significantly greater permeability in Caco-2 monolayers compared to either CM or CRN. [83] The fact that the permeability of CEE was higher than that of CM is certainly consistent with its higher partition coefficient value compared to CM. These characteristics of CEE suggest improved oral bioavailability. However, the pharmacokinetic profile of CEE is still unknown. In addition to the improved physicochemical properties of CEE, this compound was also shown to exert significant neuroprotective effects against anoxic damage. [87]

Interestingly, in *in-vitro* mouse hippocampal slices, two creatine derivatives, creatine benzyl ester and phosphocreatine-Mg-complex acetate were able to increase the creatine content of the tissue. This increase was not prevented by 3-guanidinopropionic acid (GPA), a creatine transporter blocker, suggesting a crossing of the plasma membranes in a transporter-independent way. [88] This finding suggests that some creatine derivatives might cross the plasma membranes in a transporter-independent manner, possibly by passive diffusion. Phosphocreatine-Mg-complex acetate and creatine-Mg-complex acetate were also demonstrated to have significant neuroprotective effects in mouse hippocampal slices, and the former was also effective against brain ischemic damage *in-vivo*. [89, 90]

Another potential strategy to overcome the limitations of CM is the conjugation of creatine with amino acid derivatives, which have their own carrier; this strategy allows the improvement of the physicochemical and biological properties of creatine compounds and may present a potential therapeutic option for patients with creatine transporter deficiency. [91] Burov et al. have shown that creatinyl amino acids have improved aqueous solubility. Moreover, they also tested the neuroprotective activity of these compounds in vivo in different models including a model of ischemic stroke, and they reported pronounced neuroprotective activity of creatinyl amino acids suggesting a crossing of the blood brain barrier by these compounds. [92]

Table 2. Physico-chemical properties of creatine salts. [86]

Property	Monohydrate	Hemisulfate	Hydrochloride	Mesylate	Hydrogen Maleate	Pyruvate	Citrate
Molecular weight g/mol	149.1	180.2	167.6	240.3	260.2	219.2	451.1
Percent by weight creatine	87.9	72.8	78.2	54.6	50.4	59.8	58.1
Aqueous solubility 25°C mg/ml	17.1 ± 0.4	121 ± 1	709 ± 7	588 ± 8	37.4 ± 7.3	91.6 ± 7.7	44.8 ± 2.4
Ratio of aqueous solubility (relative to monhydrate)	1.0	7.1	37.9	29.6	2.2	4.8	3.0
Octanol-water LogP at 25°C	−3.5	−3.5	−3.2	−3.3	−3.8	−3.3	−3.2

Table 3. Physico-chemical properties of ester derivatives of creatine.

Property	Creatine MonoHydrate (CM)	Creatine Ethyl Ester HCl (CEE)	Creatine Benzyl Ester HCl (CE2)	Creatine t-butyl Ester HCl (CE3)	Creatine Octyl Ester HCl (CE6)
Molecular weight g/mole	149.7	195.6	257.7	223.7	279.8
Percent by weight Creatine	88	67	51	59	47
Aqueous solubility 25 ºC mg/mL	21.0 ± 1.9	970.8 ± 14.3	172 ± 26.3	389.0 ± 10.6	56.8 ± 4.8
Ratio of solubility (relative to monohydrate)	1.00	46.2	8.2	18.4	2.7
Partition coefficient 25 ºC	0.10 ± 0.02	0.21 ± 0.13	0.54 ±0.03	0.06 ± 0.07	0.05± 0.001
Ratio of Partition coefficient (relative to monohydrate)	1.00	2.01	5.31	0.61	0.43

1.8 Osteoarthritis:

Osteoarthritis (OA), also known as degenerative joint disease, is the most prevalent joint disease among the elderly and one of the leading causes of disability and morbidity around the world. [93] OA is characterized clinically by joint pain, stiffness, and functional disability and radiographically by joint space narrowing and osteophytes. [94] This disorder can damage any joint in the body, but most commonly it affects joints in the knees, hands, hip, and spine. [95]

1.8.1 Prevalence and Incidence of Osteoarthritis:

OA can be defined both clinically and radiographically and different epidemiologic studies have reported different estimates using one or both of these definitions. This is a summary of the prevalence data primarily from two US population-based studies: The Third National Health and Nutrition Examination Survey (NHANES III) and the Framingham Osteoarthritis Study. In the Framingham Osteoarthritis Study, the prevalence of age-standardized radiographic knee OA in adults (≥ 45 years) was 19.2%. [96] While in the (NHANES III), the prevalence was 37% in adults (≥ 6o years). [96] The prevalence of radiographic hand OA in the Framingham study was 27.2% overall. [96] The prevalence radiographic evidence of hip OA ranged from 7.2% to 27% depending on the age. [96]

Symptomatic OA is defined by the presence of frequent pain and stiffness in a joint with radiographic evidence of OA. The prevalence of symptomatic knee OA was 4.9% in adults (≥26 years) in the Framingham study and 12.1% in adults (≥60 years) in the NHANES III study. [96] The prevalence of symptomatic hand and hip OA in adults was 6.8% and 9.2%, respectively. However, symptomatic hand OA was more prevalent in adults (≥ 71 years) ranging from 13.4% to 26.2%. [96]

1.8.2 Normal Structure and Composition of the Articular Cartilage:

Articular cartilage is a thin layer of specialized connective tissue of diarthrodial joints (the site of junction of two bones in the body). This cartilage serves two main functions, decreasing friction during joint movement and transmitting loads to the surrounding tissues. [97] It is mainly composed of a sparse distribution of highly specialized cells called chondrocytes. The chondrocytes are the only cells present in the cartilage, and are surrounded by a dense extracellular matrix (ECM). The ECM contains water, collagen (mainly type II collagen), proteoglycans, and some non-collagenous proteins.[97]

The main function of the chondrocytes is to synthesize, maintain, and repair the ECM. The unique structure of type II collagen, consisting of regions containing 3 polypeptide chains foiled into a triple helix, provides the cartilage with tensile

strength stabilizing the matrix. [97] Collagen is also involved in restraining the osmotic swelling pressure caused by the aggregating proteoglycans, which are responsible for hydrating the articular cartilage and for maintaining its viscoelasticity. [98]

1.8.3 Pathophysiology of Osteoarthritis:

Continuous remodeling processes as a result of anabolic and catabolic events regulate the normal articular cartilage. In normal situations, chondrocytes maintain a balance between the synthesis and the degradation of the ECM. [99] However, in OA, there is an imbalance between the synthesis and destruction of the ECM with the catabolic activity overweighing the anabolic activity of the chondrocytes leading to gradual loss of the articular cartilage. [99] Catabolic events responsible for the degradation of collagen and proteoglycans in OA involve the release of the matrix metalloproteinases (MMPs) that are synthesized and released by the chondrocytes.[94] MMPs are proteolytic enzymes and the main MMPs involved in OA are collagenases, gelatinases, and stromelysins. [100] Eventually, an imbalance between MMPs and the inhibitors of MMPs lead to collagen and proteoglycan breakdown. [94] The exact reason(s) for the activation of the catabolic events within the articular cartilage and the role of the chondrocytes in the destruction of the

cartilage is not well understood. In the past, it was hypothesized that OA and damage to the articular cartilage resulted from the low metabolic rate of the chondrocytes that could not keep up with the wear and tear due to age-related mechanical loads. [100] However, this hypothesis seems unlikely, as chondrocytes have been shown to have higher metabolic rates than previously reported. [100] Furthermore, few studies reported that the rates of metabolic repair processes in chondrocytes were increased during the early course of OA. [100] These facts suggest that chondrocytes do not passively tolerate changes in the ECM, but play a central role in the development of OA. [100] In addition to damage of the articular cartilage, other tissues such as the synovial membrane and the subchondral bone have a substantial role in the progression of OA. [101] However, complicating factors such as previous joint injury, genetic predisposition, age, obesity, and malalignment of the joint [102] makes mechanistic studies of chondrocytes role in OA difficult.

1.8.4 Role of Inflammation in Osteoarthritis:

OA is no longer considered simply as a wear and tear disease with a growing body of evidence suggesting that inflammation is present in the joints of patients suffering from OA even before the development of significant radiographic changes.[103] The discovery that inflammatory mediators such as IL-1β, TNFa, IL-6,

IL-8, and prostaglandins, which are also produced and released by the chondrocytes, can actually activate and increase the release of MMPs led to the first steps of an inflammation theory. [104] Many studies have shown that the two pro-inflammatory cytokines, IL-1β and TNFa, which can be produced by chondrocytes and synovial tissues, are key players in the pathogenesis of OA. [101] For instance, the levels of these cytokines are known to be elevated in cartilage, synovial tissue, and subchondral bone in OA patients. [101] In addition, *in-vitro* studies have shown that treating human chondrocytes with IL-1β down-regulates the expression of type II collagen and aggrecan, which are major components of ECM, [105, 106] suggesting possible interference with the anabolic activity of the chondrocytes. In fact, IL-1β and TNFa stimulate the release of different MMPs by the chondrocytes leading to cartilage destruction. [107, 108] The pro-inflammatory effects of IL-1β and TNFa in OA are regulated mostly by the transcription factor nuclear factor kappa B (NF-kB).[101, 109] Under normal situations, NF-kB is held in the cytoplasm in an inactive state complexed with its inhibitor (IκBα). In response to different activators, such as IL-1β, it becomes phosphorylated and disociated from the inhibitor subunit, then it translocates to the nucleus and binds to its target genes, increasing the expression of several inflammatory genes including cyclooxygenase-2 (COX-2).[110] Indeed, Chen et al. used small interfering RNA specific for the p65 subunit, which is the active subunit of NF-kB, in a rat model of

OA and found that interfering with the expression of NF-kBp65 reduced inflammation and slowed the progression of the disease. [111]

Treating chondrocytes with IL-1β and TNFa stimulate the release of many inflammatory mediators such as COX-2, PGE2, inducible nitric oxide synthase (iNOS), and nitric oxide (NO) via the NF-kB pathway. [101] These inflammatory mediators play an important role in the development of inflammation in chondrocytes by increasing the release of many MMPs and inhibiting the synthesis of ECM. [101] These findings demonstrating a role for inflammation within the articular cartilage in OA highlights the importance of finding new disease-modifying drugs targeting the inflammatory process in OA.

1.8.5 Oxylipins:

Oxylipins are lipid mediators that are produced from polyunsaturated fatty acids (PUFAs) via oxidation. [112] Upon cell activation, PUFAs are liberated from membrane phospholipids by the action of cytosolic phospholipase A2 (cPLA2). Subsequently, these PUFAs are oxidized by one of three enzymes, COX, lipooxygenase (LOX), or cytochrome P450 epoxygenase (CYP450), leading to the formation of more than 100 different oxylipins. [113] While arachidonic acid (AA) is

the most well known PUFAs that leads to the generation of eicosanoids, oxylipins can also be generated from other PUFA. The most common PUFAs are omega-6 PUFAs such as AA, linoleic acid (LA), gamma-linolenic acid (GLA), dihomo-gamma-linolenic acid (DGLA), and adrenic acid (AdA), and omega-3 PUFAs such as alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). [112, 113] The type of oxylipins produced from a specific tissue depends on the type of PUFAs incorporated into membrane phospholipids of that particular tissue, the enzyme present to oxidize the PUFA, and the enzyme preferences for a specific PUFA, thus different tissues have different oxylipin profiles. [112] Oxylipins are found in all tissues of the body and many of them have physiologically significant and sometimes opposing functions. Of the many functions that oxylipins have, some of them are involved in inflammation, tissue repair, apoptosis, blood clotting, or pain. [112]

Although the role of prostaglandins and specifically PGE2 in the pathophysiology of OA has been subject of extensive research, the role of oxylipins is not well understood. Oxylipins are strongly implicated in inflammation; for example, AA is the substrate of pro-inflammatory mediators, while EPA and DHA are substrates of lesser inflammatory or anti-inflammatory lipid mediators. [114] Since inflammation is an important component in the pathophysiology of OA, these oxylipins might play an important role in the development of the disease. Few studies have been

conducted examining the differences in oxylipins levels between healthy controls and different models of OA. For instance, Wong et al. measured bioactive oxylipins in knee joints of a rat model of OA and compared it to healthy controls, and found that 12-Hydroxyeicosatetraenoic acid (12-HETE) was significantly higher in rats with OA. [115] 12-HETE is derived from AA by the action of LOX and is known to be a strong pro-inflammatory mediator. [112] Therefore, investigation of the changes of oxylipins in OA is important and will contribute to the understanding of the biochemical events in OA and may provide valuable biomarkers for treatment of OA.

1.8.6 Current Treatment Options:

Currently, there is no known cure for OA and medical therapy has focused on providing symptomatic relief and on maintaining joint function. Current treatment approaches can be divided into physical measures (special exercise programs), medical treatment, and surgical therapy. [94] For symptomatic relief, the patient is usually prescribed Acetaminophen and/or a second class of non-steroidal anti-inflammatory drugs (NSAIDs), the COX-2 inhibitors, mainly Celexocib. In theory selective COX-2 inhibitors are preferred over the traditional non-selective NSAIDs as they allow for the production of the constitutive prostaglandins through COX-1

and are associated with less gastrointestinal toxicity. [94] Nutraceuticals, such as glucosamine and chondroitin, have also been shown to reduce pain and stiffness of the joints in patients suffering from OA.[94] However, the results from other clinical trials report no significant effects with glucosamine and chondroitin.[94] Additional randomized clinical trials are warranted to determine beneficial effects of these dietary supplements. In addition, it has been speculated that creatine may benefit patients with knee OA accompanied with muscle weakness and poor physical function. [116] In a study conducted by Neves et al. patients with knee OA were supplemented with CM (20 grams/day for 1 week followed by 5 grams/day for 12 weeks) and were enrolled in a lower limb resistance training program. They found that CM significantly improved physical function, stiffness, and lower limb lean mass compared to placebo. [116] In contrast, CM (10 grams/day for 10 days pre-surgery to 5 grams/day for 30 days post-surgery) did not improve body composition or muscle strength when given before total knee arthroplasty in patients with OA, nor did it enhance recovery after the surgery. [117] Patients who continue to have pain and functional disability despite medical treatment are considered candidates for joint replacement surgery. [94]

2. Objectives:

While there is interest in the potential beneficial effects of creatine supplementation in OA,[116] the effects are attributed to improved muscle recovery and ability to better participate in physical rehabilitation. The anti-inflammatory activity observed by the reduction of key inflammatory markers such as, IL-1β, TNFa, and PGE2 induced by intense exercise following creatine supplements (section 1.2.1.3) [74-76] suggests a beneficial effect of the supplement in soft tissue inflammation. In addition, creatine supplementation was shown to be neuro-protective in several models of neurodegenerative diseases and part of this might by due to the anti-inflammatory properties of creatine within the brain. However, little is known regarding the mechanism of anti-inflammatory responses to creatine. Is it a product of improved cellular energy, mitochondrial stability, free radical scavenging? Likewise, to what extent are the anti-inflammatory effects of creatine, which have been documented in skeletal muscle, transferable to other cell types?

In this project, primary canine chondrocytes (CnC) derived from normal canine articular cartilage were used as an in vitro model for examining the anti-inflammatory effects of creatine compounds. As most studies with creatine have

used CM, comparison of anti-inflammatory activity of newer creatine salt forms and derivatives were examined. In addition, the metabolic breakdown product, CRN, was also examined for potential anti-inflammatory effects. Given the increasing role of inflammation within the chondrocytes in OA, these studies may provide evidence as to the potential usefulness of creatine supplementation in OA, a topic of increased clinical interest.

The objectives of this study were to:

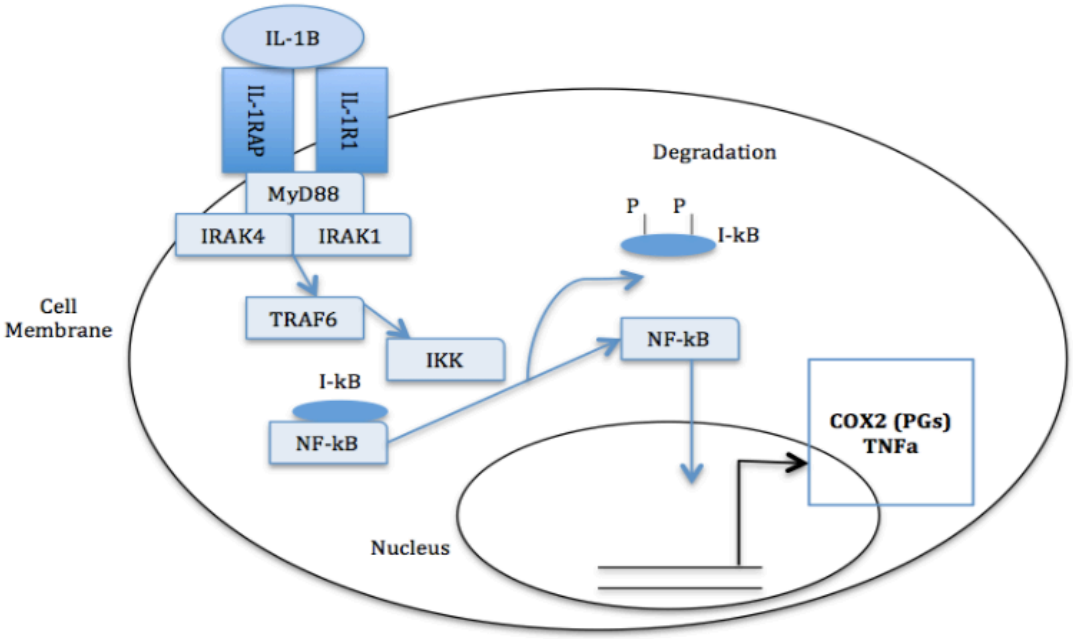
1) Examine the release of PGE2 from stimulated CnC cells in response to inflammatory stimuli (IL-1β) (Figure 4) in the presence and absence of creatine compounds, and correlations to changes in COX-2 expression.

2) Examine TNFa release and NF-kB expression in simulated CnC in response to inflammatory stimuli in the presence and absence of creatine compounds

3) Determine the extent to which anti-inflammatory effects of creatine compounds in chondrocyte model correlate with effects on oxylipins production.

Figure 4. Simplified signal transduction pathway of IL-1β. (adapted from Risbud and Shapiro. 2014) [118]

IL-1β: Interleukin-1 beta. IL-1R1: Interleukin-1 receptor type 1. IL-1RAP: Interleukin-1 receptor accesory protein. MyD88: Myeloid differentiation primary responce gene 88. TRAF6: TNF receptor-associated factor 6. I-kB: Inhibitor of kB. IKK: I-kB kinase. NF-kB: Nuclear factor kappa B. COX-2: cyclooxygenase 2. TNFa: Tumor necrosis facrot alpha.



3. Materials and Methods:

3.1 Materials:

CnC derived from normal canine articular cartilage, cell culture medium, and sub-culturing reagents were purchased from Cell Applications, Inc. (San Diego, CA, USA). Cell culture flasks, plates, and dishes were obtained from either Corning Incorporated (Corning, NY, USA) or from Thermo Scientific (Waltham, MA, USA). Recombinant canine IL-1 β was purchased from R&D Systems (Minneapolis, MN, USA). CRN and Rimadyl were obtained from Acros Organics (Geel, Belgium) and Fluka Analytical (St. Gallen, Switzerland), respectively. PureLink ® RNA Mini Kit and the QuantiTect Probe ® RT PCR Kit were purchased from Ambion (Foster city, CA, USA) and Qiagen (Cambridge, MA, USA), respectively. Canine TNF-alpha ELISA kit and Prostaglandin E2 Parameter Assay kit were purchased from Raybiotech, Inc. (Norcross, GA, USA) and R&D Systems, respectively. Pierce protease inhibitor mini tablets and the Pierce bicinchoninic acid (BCA) protein assay kit were obtained from Pierce Biotechnology (Rockford, IL, USA). PageRuler ™ Plus Prestained protein ladder, 10 to 250 kDa was obtained from Thermo Scientific. Immuno-blot PVDF membrane was purchased from Bio-Rad Laboratories (Mississauga, Ontario,

Canada). Primary rabbit polyclonal antibody for COX-2 was obtained from Abcam (Cambridge, UK). Mouse monoclonal antibody for B-actin was purchased from Sigma Aldrich Chemical Company (St Louis, MO, USA). The secondary HRP-linked antibodies were purchased from Novus Biologicals (Littleton, CO, USA). All other reagents and chemicals were purchased from Sigma Aldrich Chemical Company.

3.2 Cell Culture:

Primary CnC were received in an ampule of > 500.000 cryopreseved cells at passage 1 and were cultured according to the supplier's instructions. Briefly, cells were plated in a T-75 flask and grown in CnC complete medium. Upon reaching confluence, they were further passaged at a seeding density of 8000-15000 cells/cm^2. Cells were maintained in a humidified environment at 37°C with 5% CO_2, with media replacement every other day until the cells reach 60-70% confluence and every day upon reaching confluency. All experiments were performed on confluent monolayers (passage number 4).

3.3 Cell Viability Studies:

CnC were seeded in 96-well plates and treated with culture media containing IL-1β (10 ng/ml) in the presence or absence of CM, CEE, CRN, CHCL, or Glucosamine (10-1000 uM) or with Rimadyl (1-100 uM). Cells were exposed to these treatments for 72 hours. Additional cells treated with culture media alone were used as a control. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After 72 hours, 25 ul of the MTT substrate (5mg/ml in phosphate-buffered saline (PBS)) were added to each well and incubated for 3 hours at 37°C. Viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance maximum near 570 nm. After the addition of the MTT substrate, dimethylsulfoxide (DMSO) was added to solubilize the cells and the resulting absorbance of the formazan product in the cell lysates (presumably directly proportional to the number of viable cells) was measured at a wavelength of 570 nm using a Synergy HT plate reader (BioTek, Winooski, VT, USA). Cell viability was expressed as a percentage of the absorbance levels observed in the control cells (media alone).

3.4 Analysis of Creatine Transporter mRNA Expression Using Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR) and Gel Electrophoresis:

Primary CnC were seeded in 100 mm cell culture dishes. After 24 hours, cells were harvested and total RNA was obtained using PureLink ® RNA Mini Kit according to the manufacturer's instructions. The concentrations of RNA samples were determined using Eppendorf BioPhotometer Plus spectophotometer (Eppendorf, Hamburg, Germany). Total RNA samples were stored at - 80°C until needed. Reverse transcription and amplification of cDNA was carried out using QuantiTect Probe ® RT PCR Kit. Each RT reaction contained 2 ug of total RNA. RT-PCR reactions were carried out in a PTC-100 Programmable Thermal Controller (MJ Research Inc., St. Bruno, Quebec, Canada). The cyclic conditions are shown in Table 4.

Table 4: Cycling conditions for RT-PCR

Step	Time	Temperature
Reverse transcription	15 minutes	50°C
PCR initial heat activation	2 minutes	95 °C
Denaturing	15 seconds	95 °C
Combined annealing/extension	60 seconds	60 °C
Number of cycles: 45		

The primers for creatine transporter were designed using NCBI/Primer-Blast and were synthesized by Invitrogen (Ontario, Canada), Table 5.

Table 5: Primers sequences used for RT-PCR

Primer	Forward Primer	Reverse Primer	Product length
Canine Creatine transporter (SLC6A8)	TCCTGATCGCCCTAGTTGGA	GTAGCCCAGGCCTTTGAACA	122

Reactions products were separated by electrophoresis on 2% agarose gel along with a 300 bp DNA molecular weight marker. The bands were visualized under UV light exposure. The predicted size of the PCR product was 122 bp.

3.5 Measuring the Levels of TNFa and PGE2 Using Enzyme-linked Immunosorbent Assay (ELISA):

Confluent monolayers of primary CnC were grown on 96-well plates and treated with culture media containing IL-1β (10 ng/ml) with or without one of these 6 different compounds: CM, CEE, CRN, CHCL, or Glucosamine at three concentrations, 10, 100, or 1000 uM, or with Rimadyl at 1, 10, 100 uM. Additional cells were treated with culture media alone. After treatment, cells were placed in a humidified CO_2 incubator maintained at 37 °C. Samples from culture media were collected at various time points (4-72 hours) and stored at – 80 °C until further analysis.

Collected media were analyzed using commercially available ELISA kits for the appearance of the inflammatory mediators, TNFa and PGE2. ELISA assays were performed as detailed in the manufacturer's protocol and the optical density was determined using Synergy HT plate reader set at 450 nm.

3.6 Western Blot Analysis and Quantification of COX-2 Expression in Whole Cell Lysates:

Confluent monolayers of primary CnC grown on 100 mm petri dishes were treated with culture media containing IL-1β (10 ng/ml) alone or with one of the following compounds: CM, CEE, CRN, CHCL, Glucosamine at 1000 uM, or with Rimadyl 100 uM. Additional cells were treated with culture media alone. After treatment, cells were placed in a humidified CO_2 incubator maintained at 37°C for 48 hours. After the 48 hours treatment period, cell media were aspirated and the cells were washed twice with ice-cold PBS. Cells were lysed with 100 ul RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) supplemented with a protease inhibitor cocktail. A sonication step was performed before centrifugation at 15,000 rpm for 10 minutes at 4°C. Total cell protein supernatant was collected and the pellet was discarded. Protein concentration was measured using Pierce BCA protein assay kit according to the manufacturer's protocol and the optical density was determined using Synergy HT plate reader set at 562 nm. Total protein in each sample was determined based on a bovine serum albumin (BSA) standard. Total cell protein samples were prepared for electrophoresis by adding Laemmli loading buffer to 20 ug of total protein in a 1:1 ratio. Samples were then denatured by heating at 95°C for 5 minutes. Equal

amounts of protein (20 ug) and the molecular weight marker were loaded and separated by electrophoresis on a 10% polyacrylamide gel run at 120 V at room temperature. The protein bands were transferred to an immunoblot PVDF membrane by applying a constant current of 200 mA for 2 hours at 4°C. Subsequently, membranes were washed with 5% (w/v) non-fat skim milk in tris-buffered saline with 0.1% tween-20 (TBS-T) to block non-specific binding sites under constant agitation for 1 hour at room temperature. Membranes were then incubated with rabbit polyclonal primary antibody for COX-2 (1ug/ml) over night at 4°C. After washing the membranes for 3 times, they were incubated with mouse anti-rabbit immunoglobulin G-horse radish peroxidase (IgG-HRP) secondary antibody, diluted to 1:10,000 in blocking buffer for 1 hour at room temperature. After washing the membranes, the specific bands were visualized using a chemiluminescence kit (Bio-Rad, Mississauga, Ontario, Ca) and densitometry analysis was performed using the Bio-Rad ChemiDoc MP imaging system and ImageLab 5.2.1 build 11 software. Values for each lane were normalized to β-actin. After stripping the membranes using a re-blot solution (Chemicon International, Billerica, MA, USA), the membranes were blocked for 10 minutes and then incubated with mouse monoclonal primary antibody for β-actin (0.5 ug/ml) for 2 hours at room temperature. After washing, they were incubated with rabbit anti-mouse IgG-HRP for 1 hour, followed by visualizing and quantification

of the bands. COX-2 expression in each lane was quantified by taking the volume (intensity) of the band corresponding to COX-2 (69-kDa) and dividing that by the volume (intensity) of the band corresponding to actin (42-kDa) found in the same lane.

3.7 Western Blot Analysis and Quantification of Phosphorylated-NF-kB Expression in Whole Cell Lysates:

Treatment of cells and total cell protein isolation were performed as described in section 3.6. Protease and phosphatase inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA) was added to the whole cell lysate to protect the phosphorylated-NF-kB from degradation by endogenous proteases and phosphatases released during protein extraction and purification. Next, 30 μg of sample protein were separated by electrophoresis and western blot analysis was performed as previously described in section 3.6. Antibodies used were primary phosphorylated-NF-kB p65 pSer536 antibody (1:1000 dilution in 5% (w/v) non-fat skim milk in TBS-T) (ThermoFisher Scientific, Waltham, MA, USA). Secondary antibody was rabbit anti-mouse IgG-HRP (1:10,000 dilution) (Novus Biologicals, Littleton, CO, USA).

Phosphorylated-NF-kB expression in each lane was quantified by taking the volume (intensity) of the band corresponding to Phosphorylated-NF-kB (69-kDa) and dividing that by the volume (intensity) of the band corresponding to actin (42-kDa) found in the same lane.

3.8 Oxylipin Analysis Using High Performance Liquid Chromatography/ Mass Spectrometry (HPLC/MS/MS) (These studies were performed in Dr. Harold Aukema's laboratory by Ms. Tanja Winter):

Confluent monolayers of primary CnC were grown on 6-well plates and treated with culture media containing IL-1β (10 ng/ml) with or without one of the following compounds: 1000 uM of CM, CEE, CRN or CHCL, or with 100 uM of Rimadyl. Additional cells were treated with culture media alone. After treatment, cells were placed in a humidified CO_2 incubator maintained at 37 °C. After 24 hours, samples (3 ml) from culture media were collected and antioxidant solution [0.2 mg/mL BHT, 0.2 mg/mL EDTA, 2 mg/mL triphenylphosphine, 2 mg/mL indomethacin in methanol/ethanol/water (2:1:1, v/v/v)] was added to each sample to protect it from degradation, and the samples were stored at – 80 °C until further analysis.

Oxylipin concentrations were determined in each sample as described by Aukema

et al. [119] Briefly, after adding 10 ng of deuterated internal standards (Cayman Chemical, MI, USA), samples were adjusted to pH < 3. Solid phase extraction (SPE) was performed with Strata-X SPE columns (Phenomenex, CA, USA) that were preconditioned with methanol and pH 3 water, loaded with sample, rinsed with 10 % methanol, and eluted with methanol. Samples were dried and resuspended in solvent for analysis by HPLC/MS/MS (API 4000; Sciex, ON, Canada). Quantification of oxylipins was determined using the stable isotope dilution method. Dose response curves were run to determine detector response factors, which were applied to all oxylipins.

3.9 Statistical Analysis:

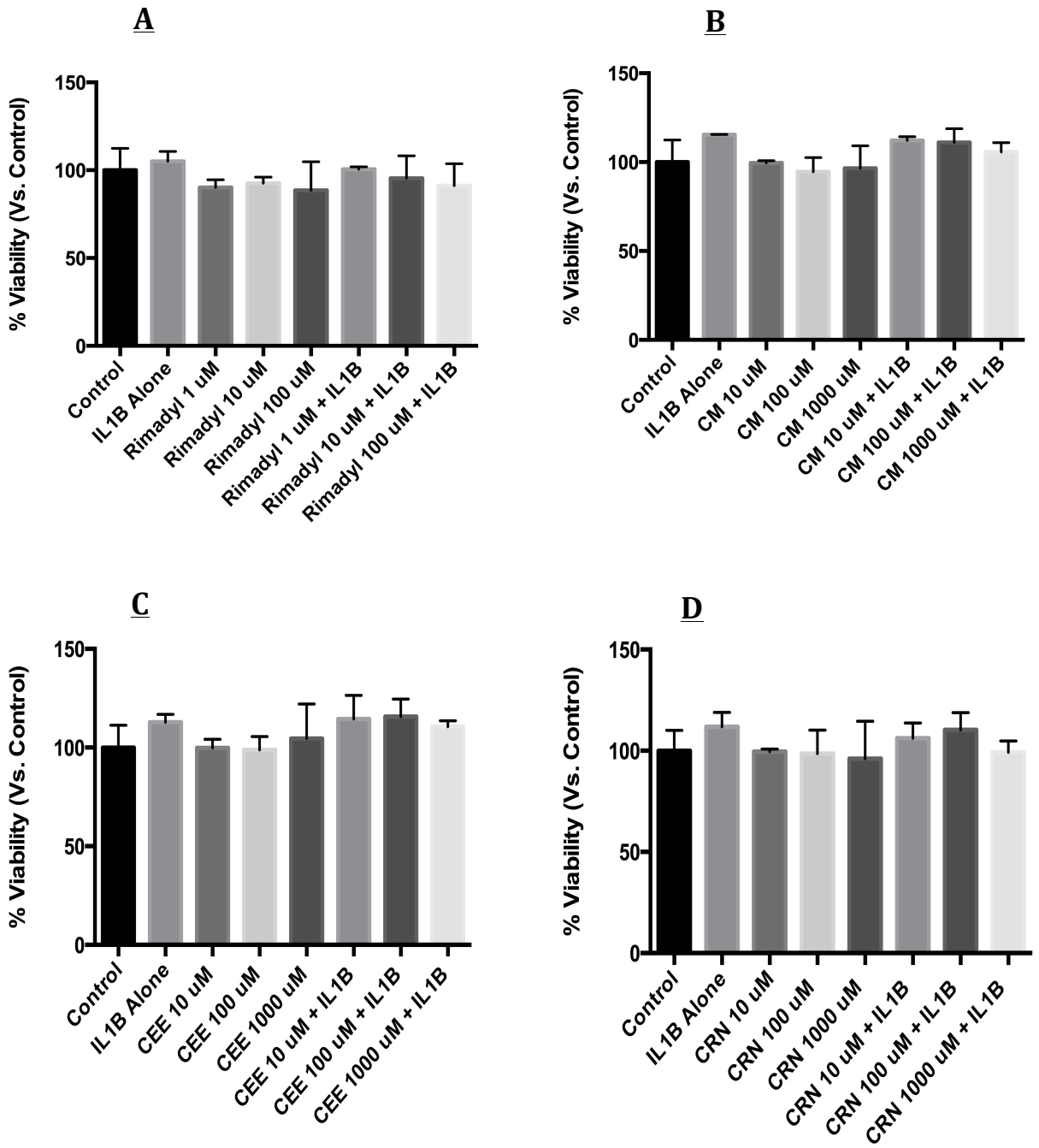
All data were expressed as mean ± standard error of the mean (SEM). Comparison among the groups of data were evaluated by either one-way ANOVA (if only 1 variable was present) or two-way ANOVA (if more than 1 variable was present) using Fisher's least significant difference test for multiple comparisons of the means. The tests were performed using GraphPad Prism, version 6 software (San Diego, CA, USA) where p values less than 0.05 were considered significant.

4. Results:

4.1 Effects of IL-1β Stimulation and Various Treatments on Canine Chondrocyte Viability:

To determine the effects of the various test compounds on cell viability, the MTT cytotoxicity assay was employed. In these experiments, the viability of primary cultured CnC was evaluated after 72 hours incubation with three different concentrations of the test compounds. No toxicity was observed in primary cultured CnC, even at the highest concentrations of the tested compounds examined compared to the control cells that received culture media only (Figure 5).

Figure 5. MTT assay to assess CnC viability after treatment with 3 different concentrations of (A) Rimadyl (B) CM (C) CEE (D), CRN (E) CHCL (F) Glucosamine with or without IL-1β for 72 hours. All data were expressed as mean ± SEM of four samples per treatment group. No differences were found between treatment groups by ANOVA.



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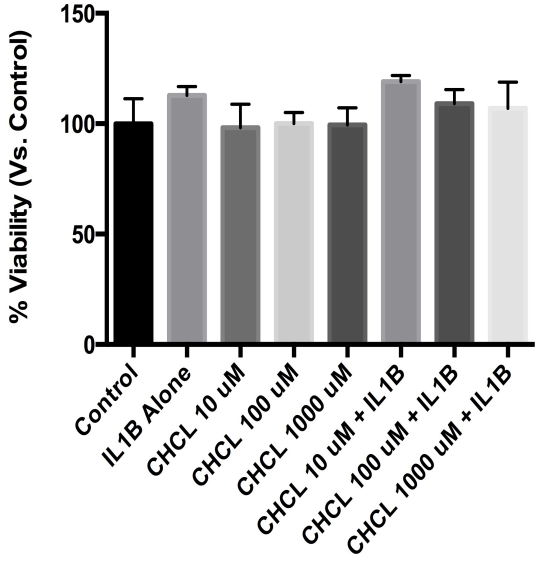

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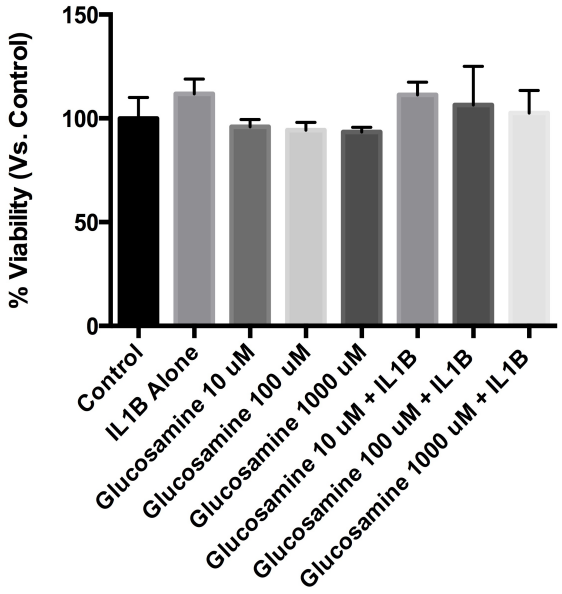
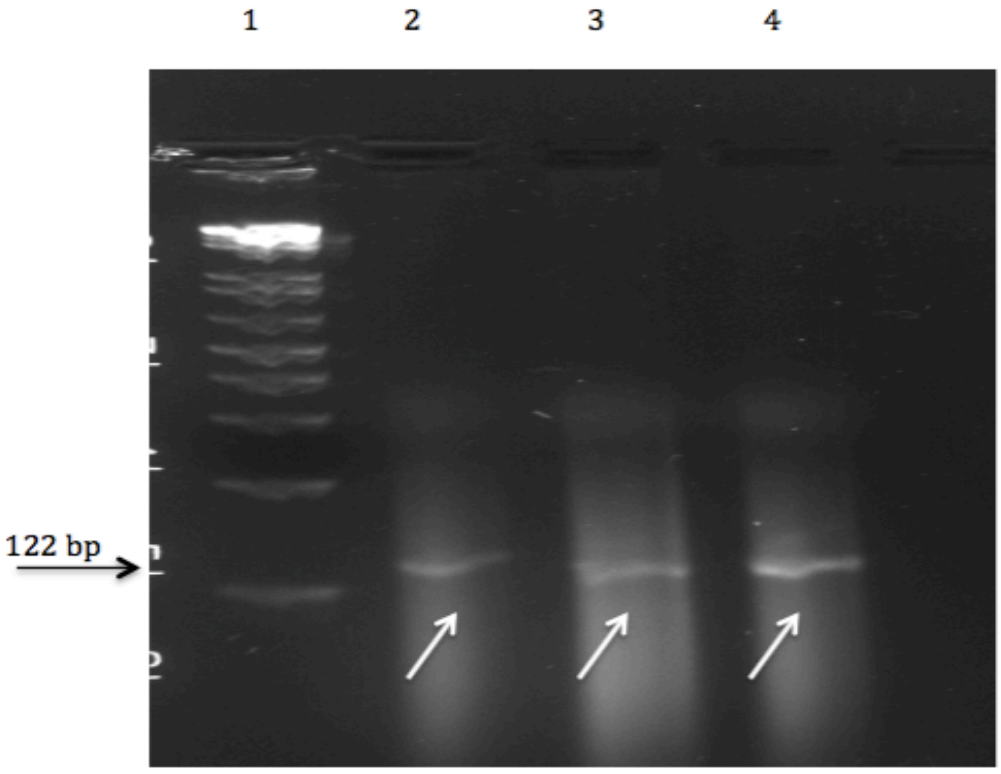


4.2 Verification of Creatine Transporter mRNA Expression in Canine Chondrocytes:

The expression of the creatine transporter was examined at the mRNA level in CnC using the RT-PCR approach in order to confirm the presence of creatine transporter in CnC. Creatine transporter gene expression in CnC was detected in three different cell culture preparations, appearing as a distinct band at the predicted size (122 bp), Figure 6.

Figure 6. Verification of Creatine Transporter mRNA Expression using RT-PCR in CnC lysates.

Lane 1: DNA ladder; Lane 2, 3, and 4: Three different samples from CnC. Arrows indicate bands of interest.



4.3 PGE2 Release from Primary CnC Monolayers Under Control and Stimulated Conditions:

The levels of PGE2 in cell culture media of primary cultured CnC at various time points were measured using ELISA. Exposure to the pro-inflammtory cytokine, IL-1β (10ng/ml), produced a time-dependent increase in PGE2 release from CnC compared to the untreated control cells. Treatment of CnC with the COX inhibitor, Rimadyl (1-100 uM), substantially attenuated the release of PGE2 induced by IL-1β. The reductions in PGE2 release were observed as early as 8 hours following Rimadyl and at all three concentrations examined, Figure 7 (A-C). In contrast, incubation of CnC with CM resulted in a significant decrease in PGE2 concentrations from the positive control (IL-1β alone) at 8 and 48 hours (33% to 56%), Figure 8 (A-C). Interestingly, CEE-treated cells, at all the examined concentrations, yielded significantly decreased PGE2 concentrations compared to IL-1β positive controls after 8 hours (33%-73%). In addition, treating the cells with 1000 uM CEE also resulted in decrease in PGE2 release after 48 hours, Figure 9 (A-C). Similarly, when CnC were treated with CRN, PGE2 concentrations were significantly reduced after 8 and 48 hours (27-57%), Figure 10 (A-C). Consistent with the trend observed with CM, CEE, and CRN, both CHCL and Glucosamine were able to reduce the levels of PGE2 at the same time points by approximately 50%-55%, Figure 11 (A-C) and Figure 12 (A-C), respectively. Although all

creatine compounds were able to reduce PGE2 release, the level of reduction was not as great as Rimadyl.

Figure 7. PGE2 release from stimulated cells after treatment with Rimadyl (1-100 uM) at 4, 8 (A), 24, 48 (B), and 72 hours (C). Values were expressed as mean ± SEM of four samples per treatment group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to IL-1β treatment group (two-way ANOVA).

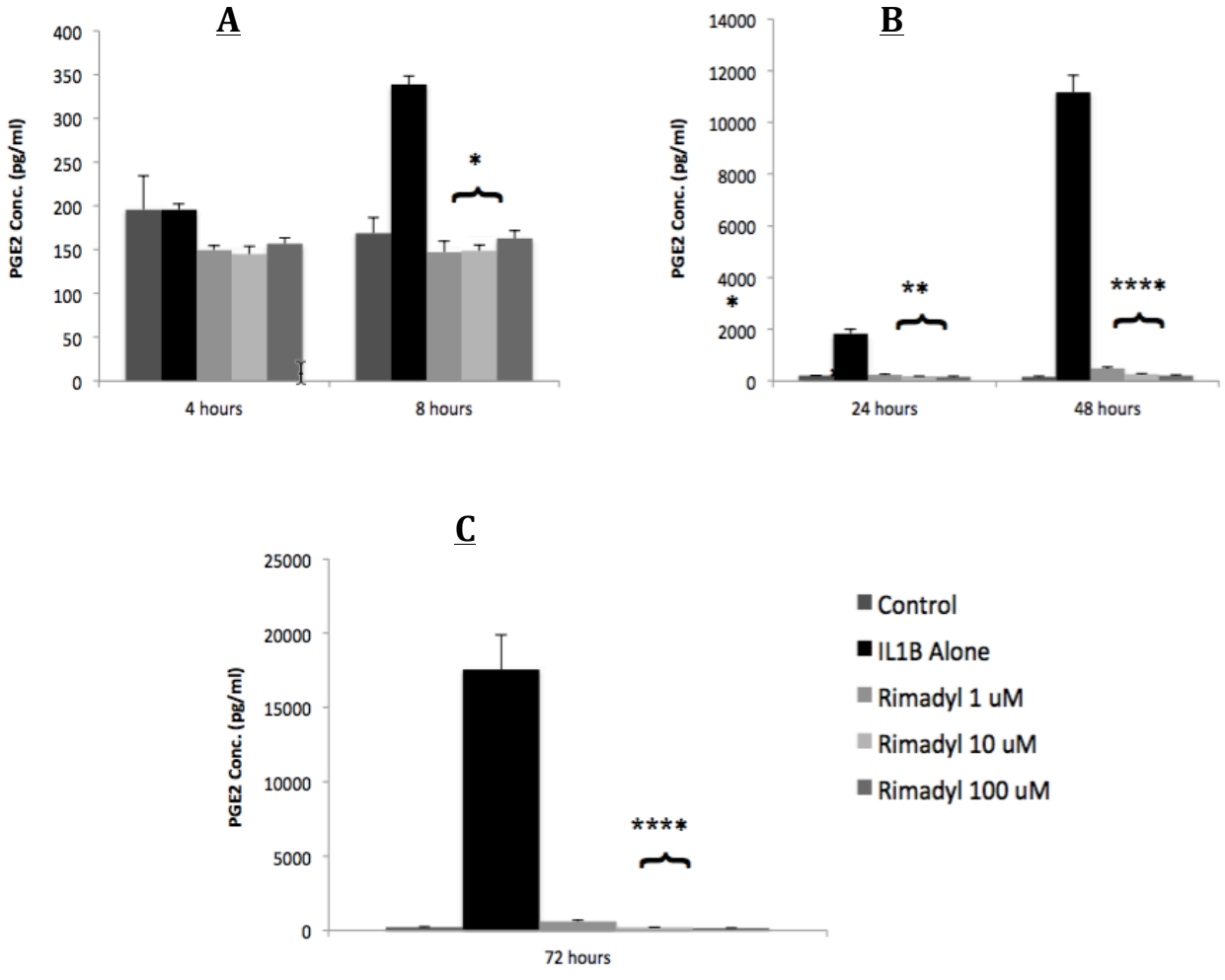


Figure 8. PGE2 release from stimulated cells after treatment with CM (10-1000 uM) at 4, 8 (A), 24, 48 (B), and 72 hours (C). Values were expressed as mean ± SEM of four samples per treatment group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to IL-1β treatment group (two-way ANOVA).

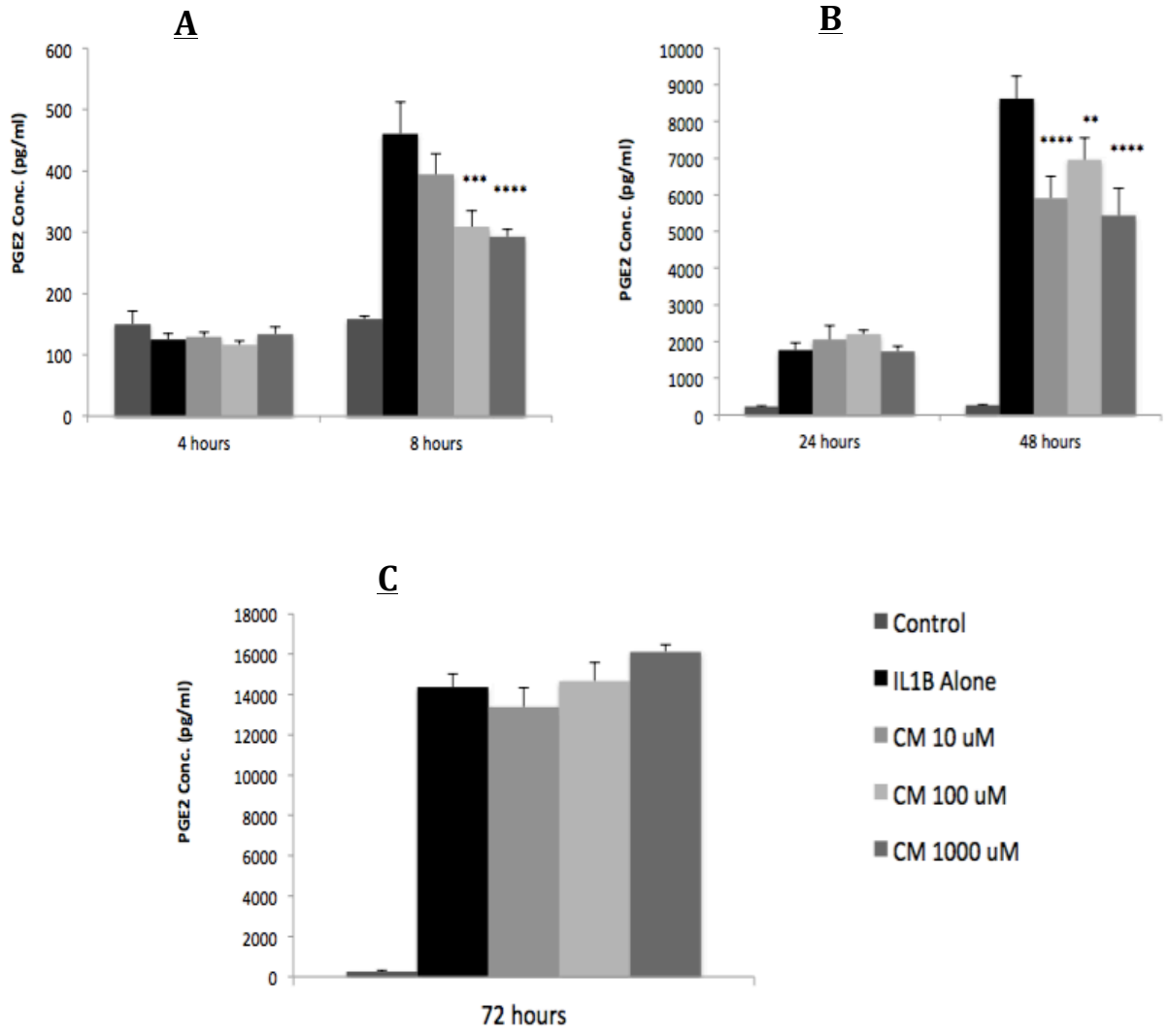


Figure 9. PGE2 release from stimulated cells after treatment with CEE (10-1000 uM) at 4, 8 (A), 24, 48 (B), and 72 hours (C). Values were expressed as mean ± SEM of four samples per treatment group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to IL-1β treatment group (two-way ANOVA)

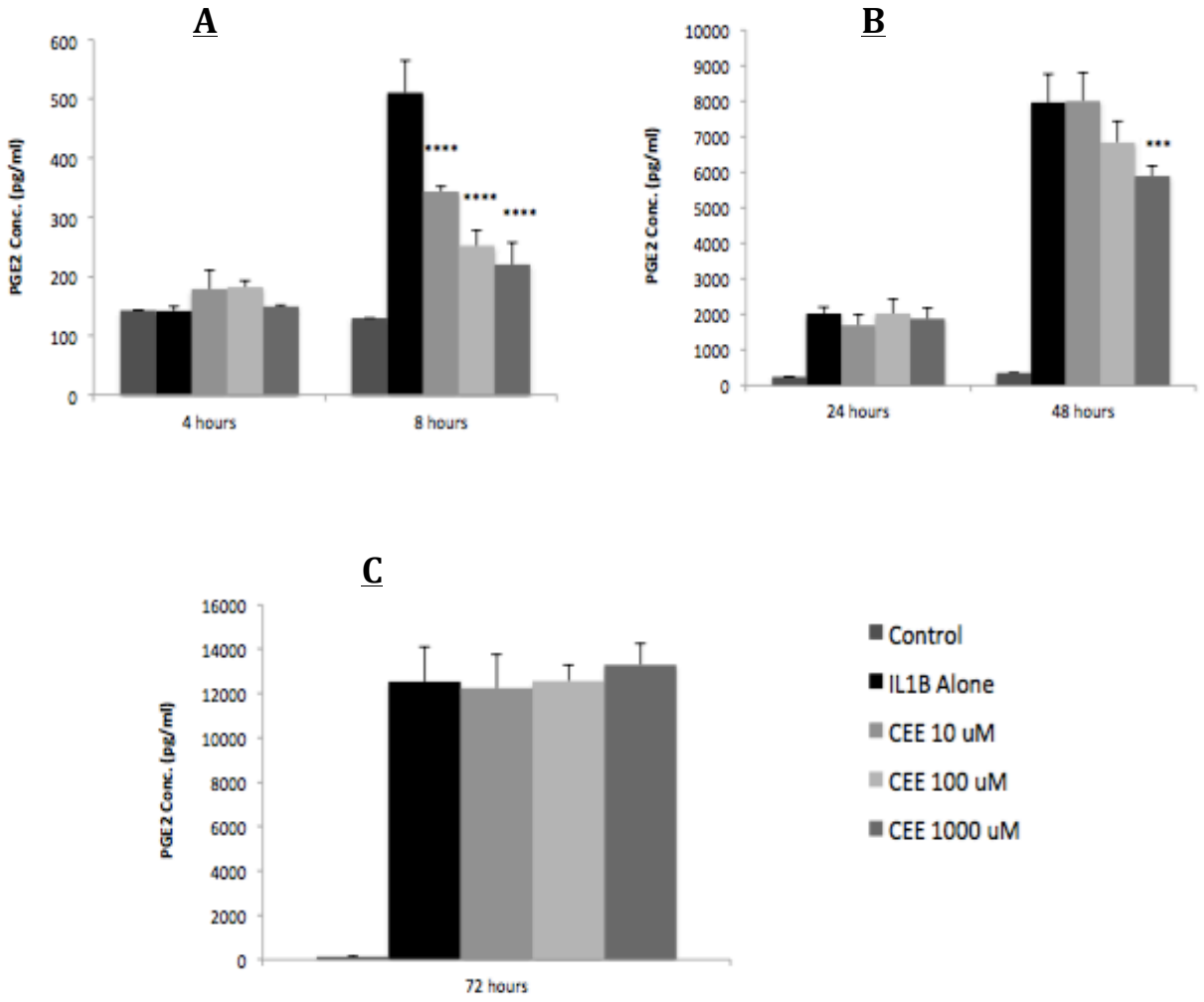


Figure 10. PGE2 release from stimulated cells after treatment with CRN (10-1000 uM) at 4, 8 (A), 24, 48 (B), and 72 hours (C). Values were expressed as mean ± SEM of four samples per treatment group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to IL-1β treatment group (two-way ANOVA)

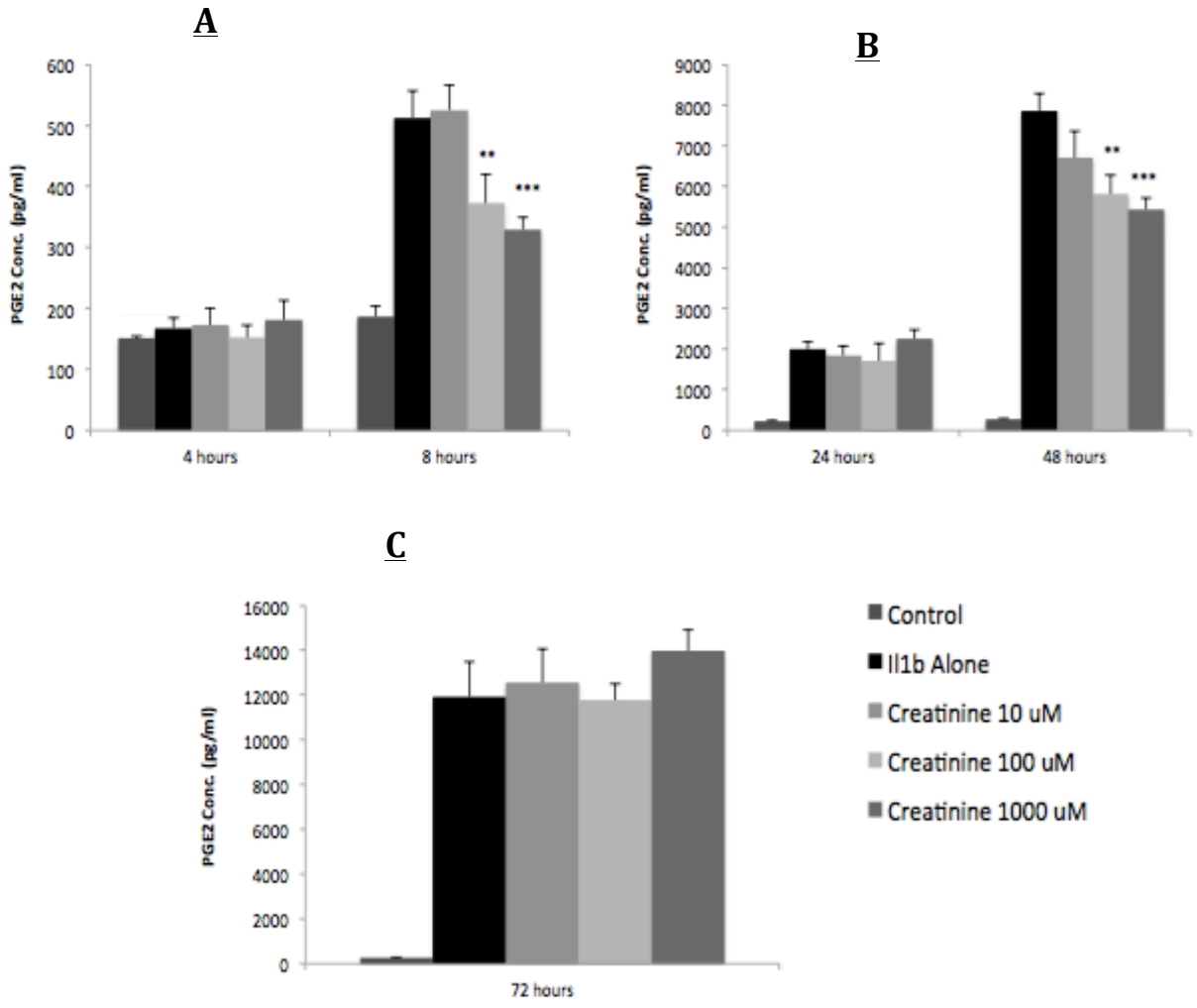


Figure 11. PGE2 release from stimulated cells after treatment with CHCL (10-1000 uM) at 4, 8 (A), 24, 48 (B), and 72 hours (C). Values were expressed as mean ± SEM of four samples per treatment group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to IL-1β treatment group (two-way ANOVA)

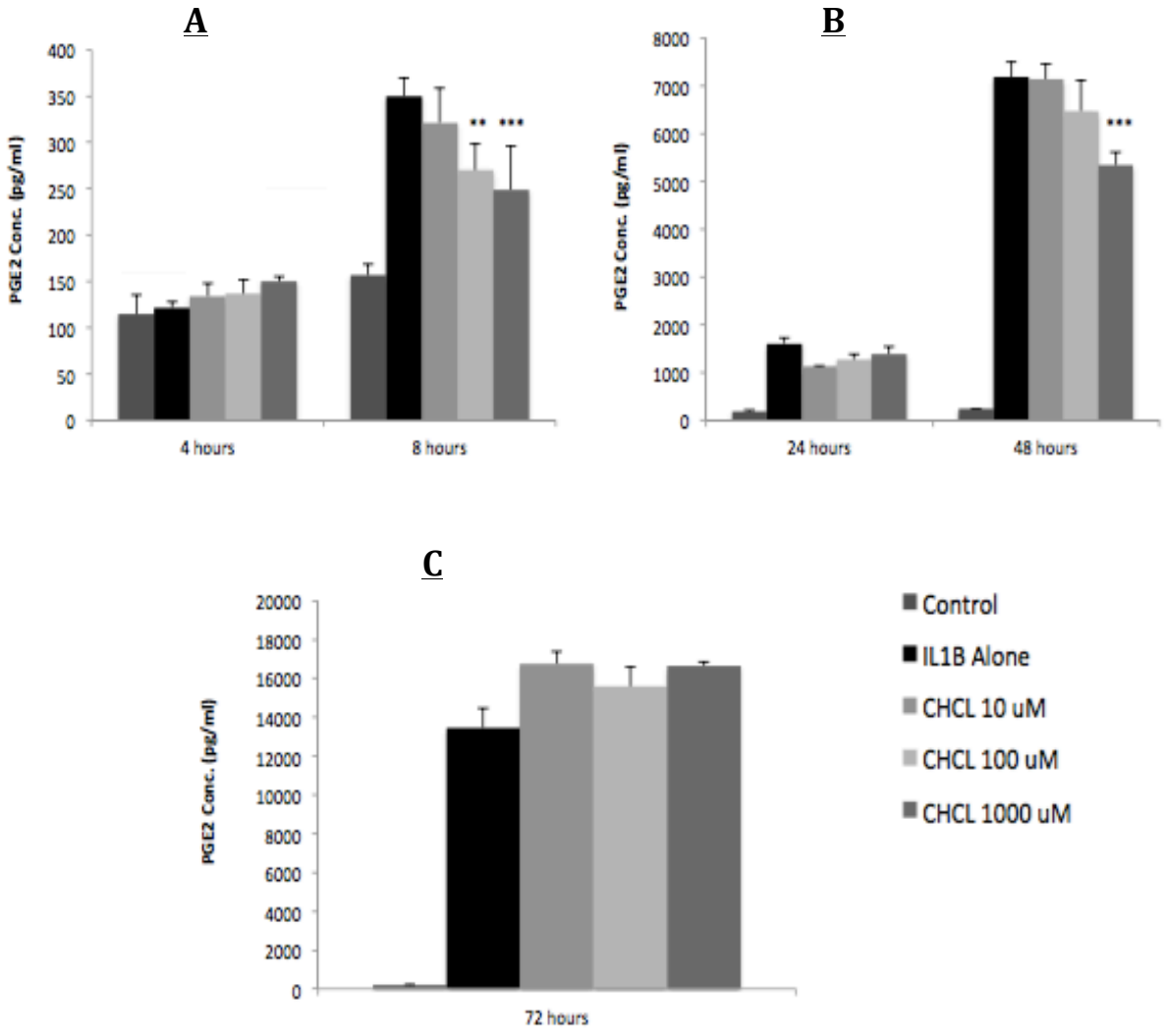
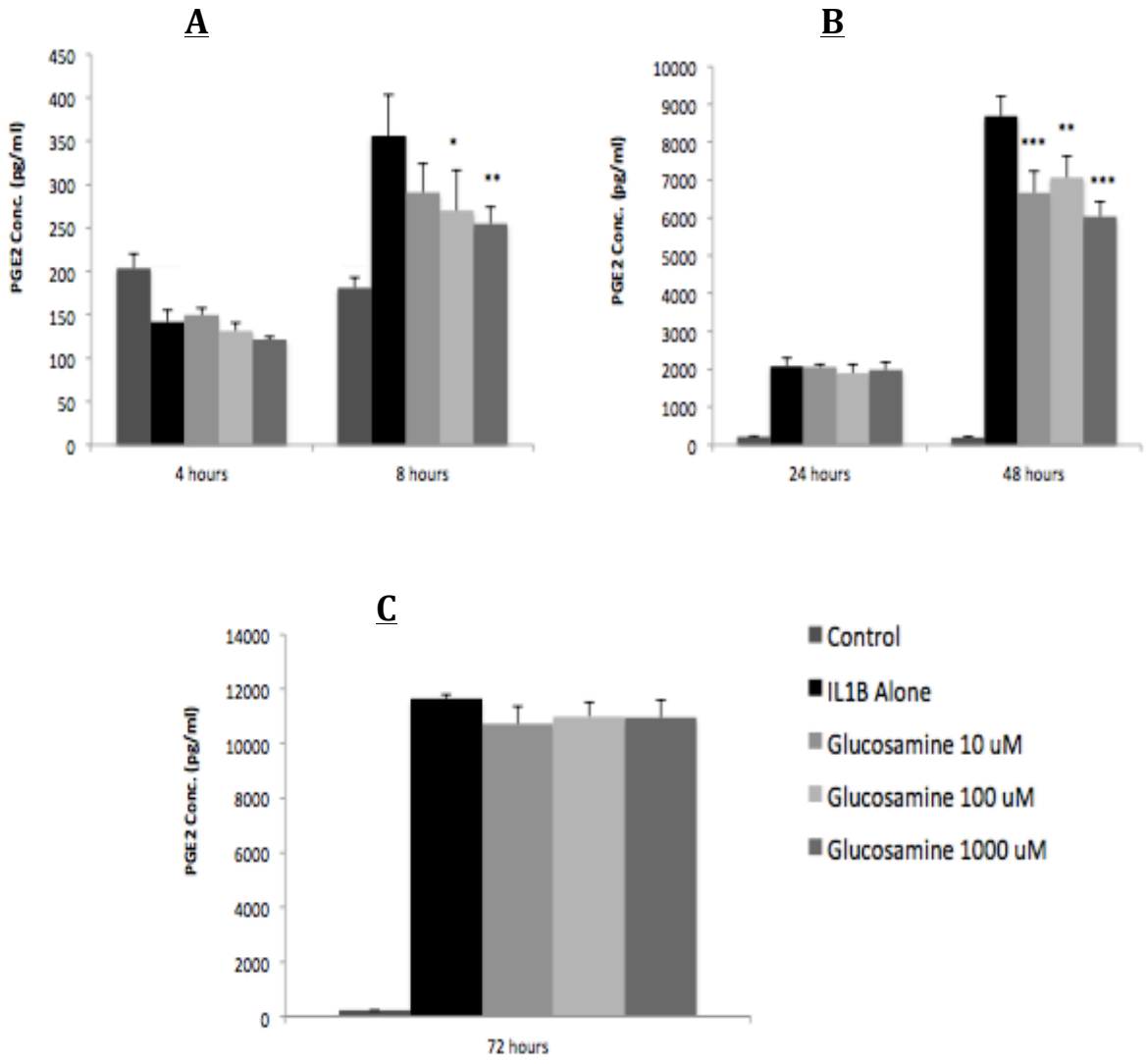


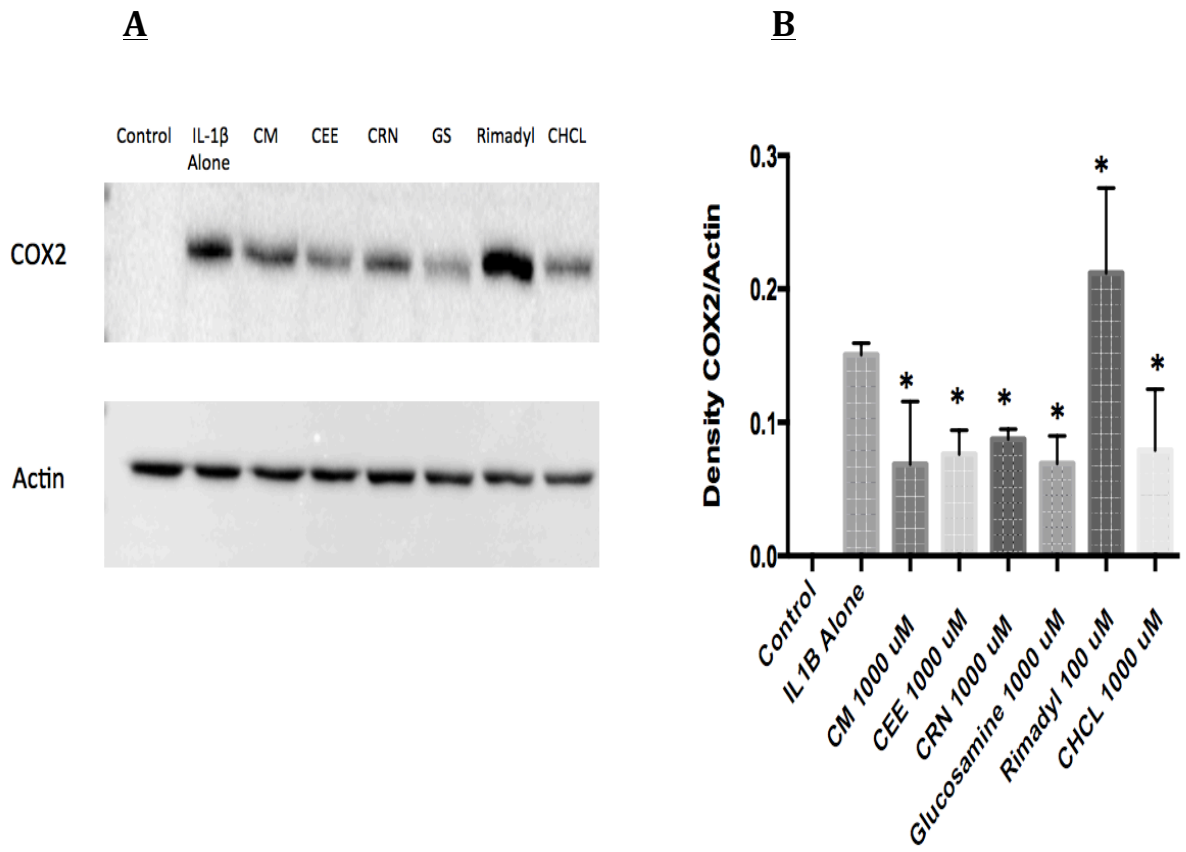
Figure 12. PGE2 release from stimulated cells after treatment with glucosamine (10-1000 uM) at 4, 8 (A), 24, 48 (B), and 72 hours (C). Values were expressed as mean ± SEM of four samples per treatment group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to IL-1β treatment group (two-way ANOVA)



4.4 COX-2 Expression in Primary Cultured CnC Under Control and Stimulated Conditions:

To determine whether the effects of creatine compounds on PGE2 release were due to reduced production, COX-2 expression was examined. As shown in Figure 13, treatment of CnC with IL-1β alone (10 ng/ml) induced the expression of COX-2 significantly. Surprisingly, treating CnC with Rimadyl (100 uM), a COX-2 inhibitor, increased the expression of COX-2 by approximately 40% compared to the positive control (IL-1β alone). In contrast, all creatine compounds, CRN, and Glucosamine reduced the expression of COX-2 by approximately 43% to 55% compared to the IL-1β treatment group, Figure 13.

Figure 13. Western blot analysis of COX-2 in stimulated cells after 48 hours under the various treatment conditions. Panel A: representative blot for COX-2 and beta-actin expression in stimulated cells after 48 hours. Panel B: Densitometry analysis of the western blot. Values were expressed as mean ± SEM of three samples per treatment group. *p<0.05 compared to IL-1β treatment group (one-way ANOVA)

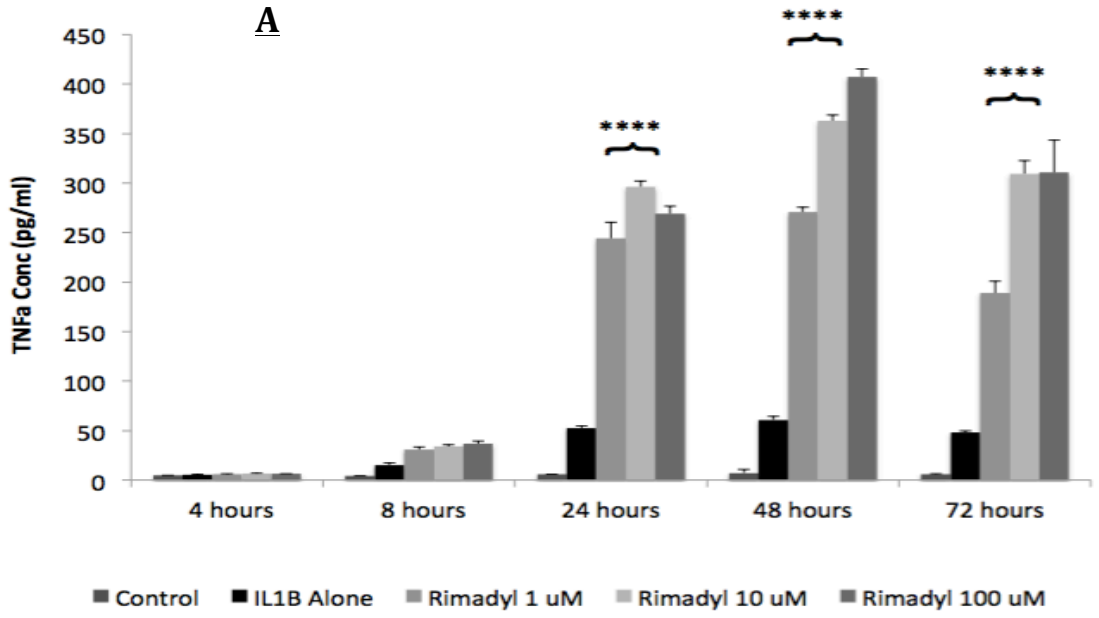


4.5 TNFa Release from Primary Cultured CnC under Control and Stimulated Conditions:

The effects of the various treatment conditions on the release of TNFa from stimulated cells were examined using ELISA. Exposure to IL-1β (10 ng/ml) alone resulted in a time-dependent increase in TNFa levels in primary cultured CnC compared to the untreated control cells, with TNFa reaching its peak at 48 hours. Consistent with the increased COX-2 expression in cells treated with Rimadyl, TNFa release was also increased at all concentrations examined (approximately 500%) compared to IL-1β alone, Figure 14.A. In contrast, all the creatine compounds examined reduced the concentrations of TNFa in culture media from primary cultured CnC compared to IL-1β alone. Treatment of IL-1β with CM resulted in a significant decrease in TNFa levels in the culture media at 24 and 48 hours, ranging from 27% to 44% compared to IL-1β alone, Figure 14.B. Interestingly, culture media taken from cells treated with CEE had the greatest reduction of TNFa (up to 62% compared to positive control). Compared to the effects observed with CM, CEE was able to reduce the levels of TNFa for a longer period of time and at lower concentrations (10 uM of CEE vs 1000 uM CM), Figure 14.C. In addition, CRN at the highest concentration examined (1000 uM) was able to reduce the levels of TNFa in media (up to 37%) compared to positive control, Figure 14.D. Similarly, samples from cells incubated with CHCl or

Glucosamine had lower levels of TNFa at different time points compared to positive control (approximately 55% reduction), Figure 14.E and Figure 14.F, respectively.

Figure 14. TNFa release from stimulated cells after treatment with (A) Rimadyl (1-100 uM), (B) CM (10-1000 uM), (C) CEE (10-1000 uM), (D) CRN (10-1000 uM), (E) CHCL (10-1000 uM), (F) or Glucosamine (10-1000 uM). Values were expressed as mean ± SEM of four samples per treatment group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to IL-1β treatment group (two-way ANOVA)



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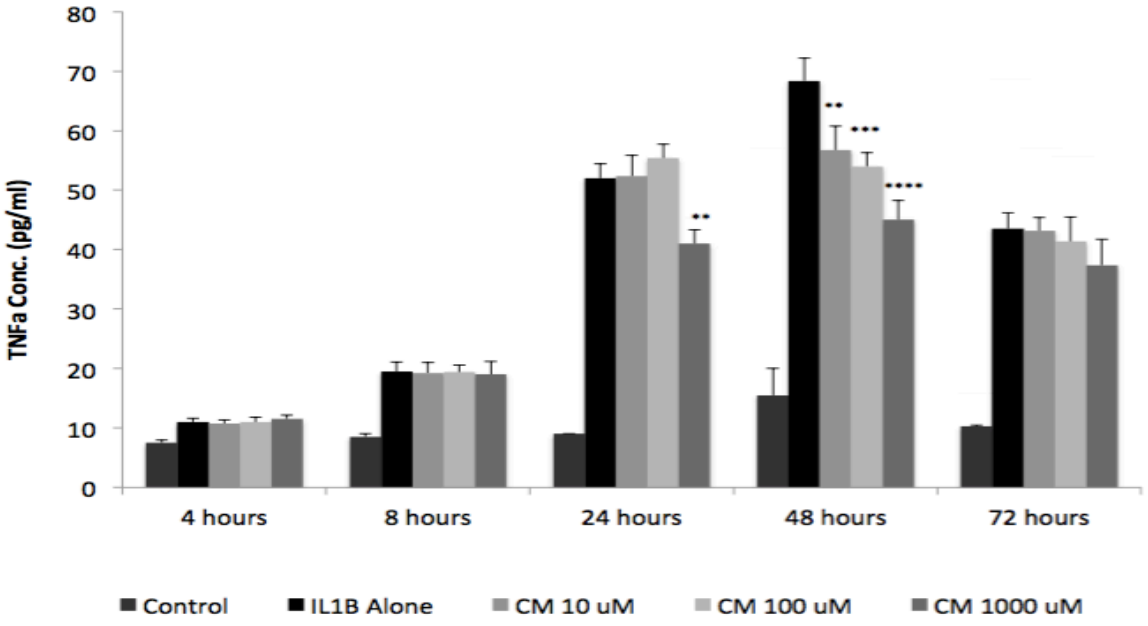
TNFa Conc. (pg/ml)
80
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10
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4 hours
8 hours
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48 hours
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**
**
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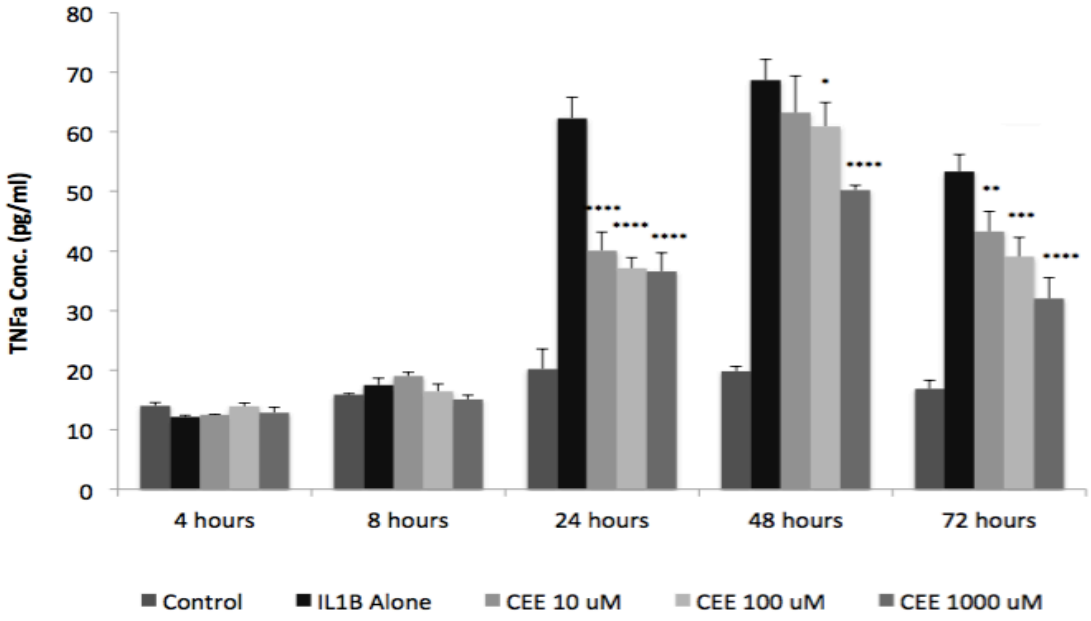
Control
IL1B Alone
CM 10 uM
CM 100 uM
CM 1000 uM

C


TNFa Conc. (pg/ml)
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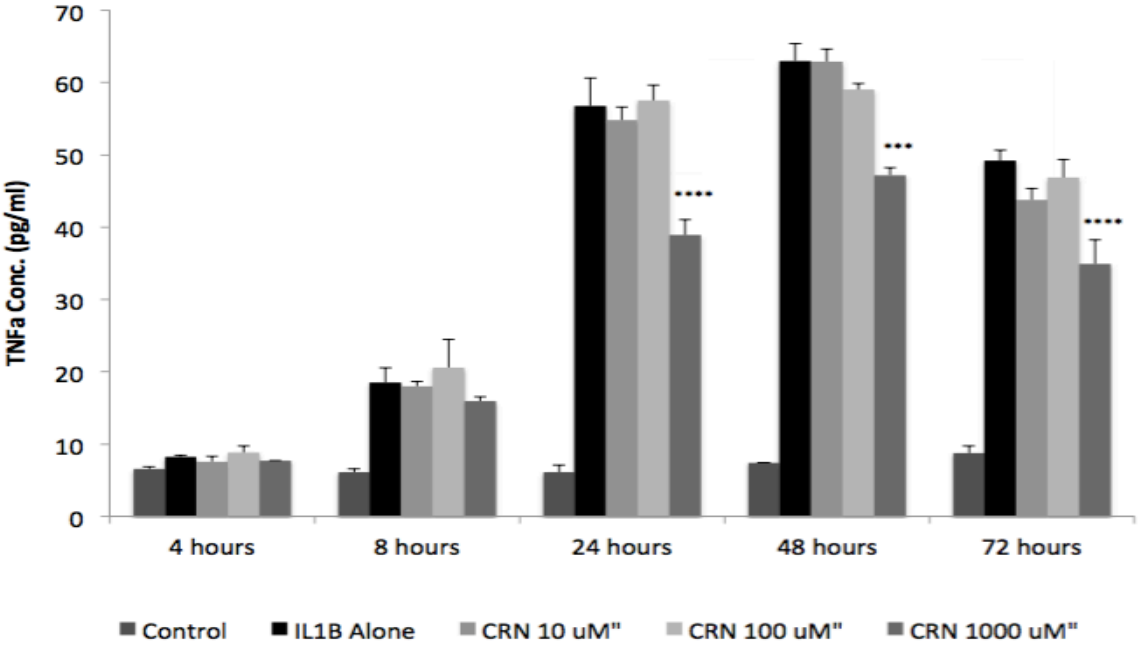
Control
IL1B Alone
CEE 10 uM
CEE 100 uM
CEE 1000 uM

D


TNFa Conc. (pg/ml)
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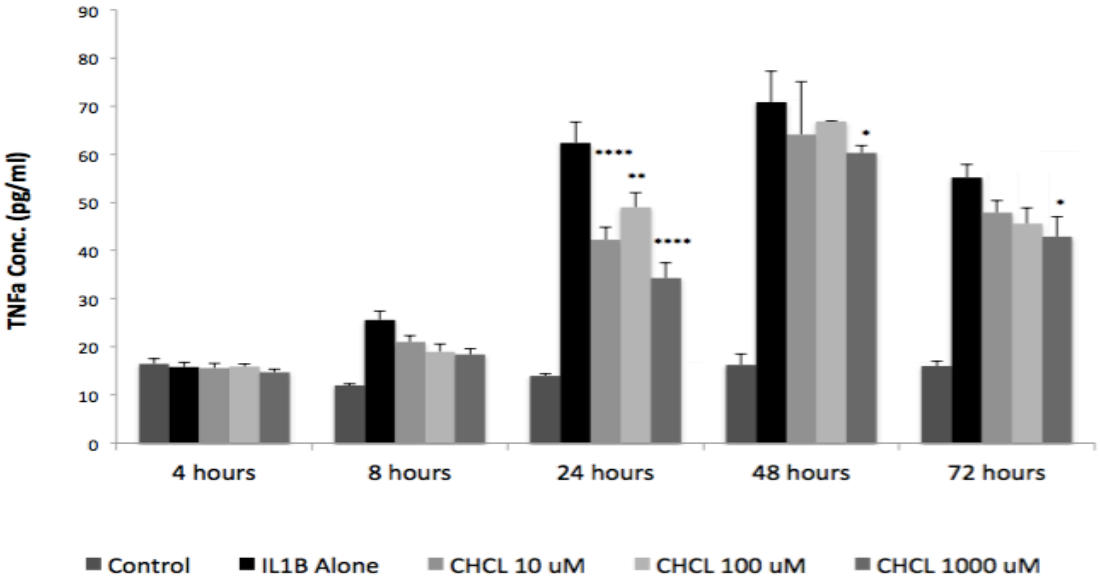
Control
IL1B Alone
CRN 10 uM"
CRN 100 uM"
CRN 1000 uM"

E


TNFa Conc. (pg/ml)
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70
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30
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4 hours
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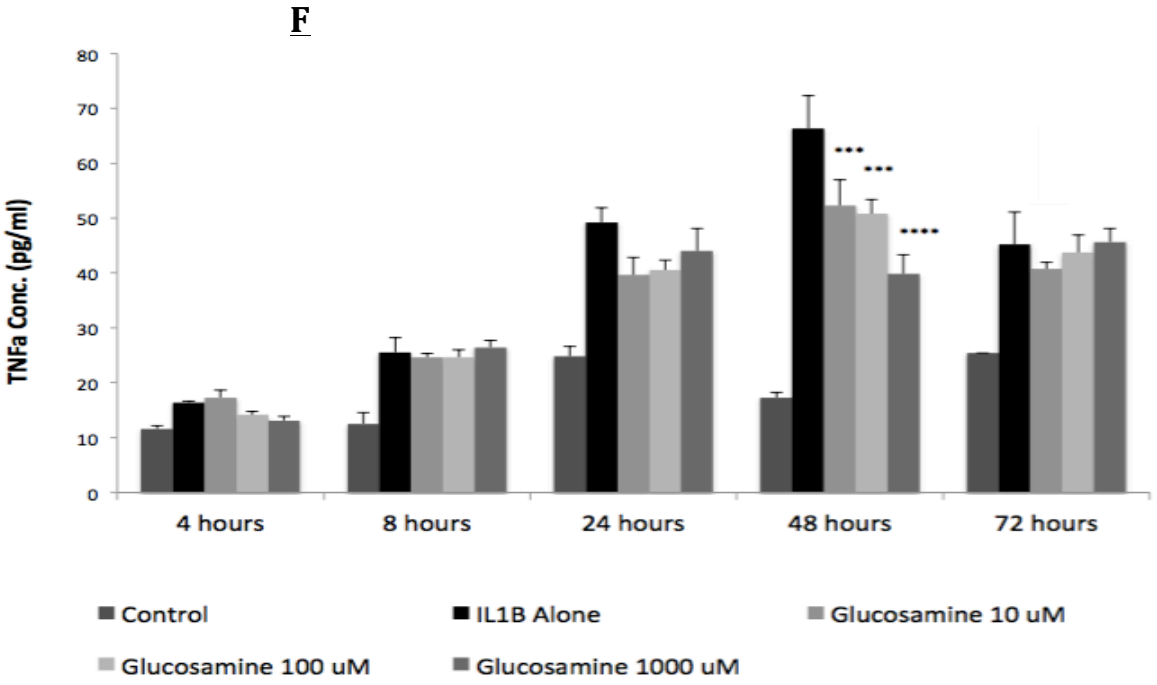
24 hours
48 hours
72 hours
Control
IL1B Alone
CHCL 10 uM
CHCL 100 uM
CHCL 1000 uM



4.6 Oxylipin Analysis:

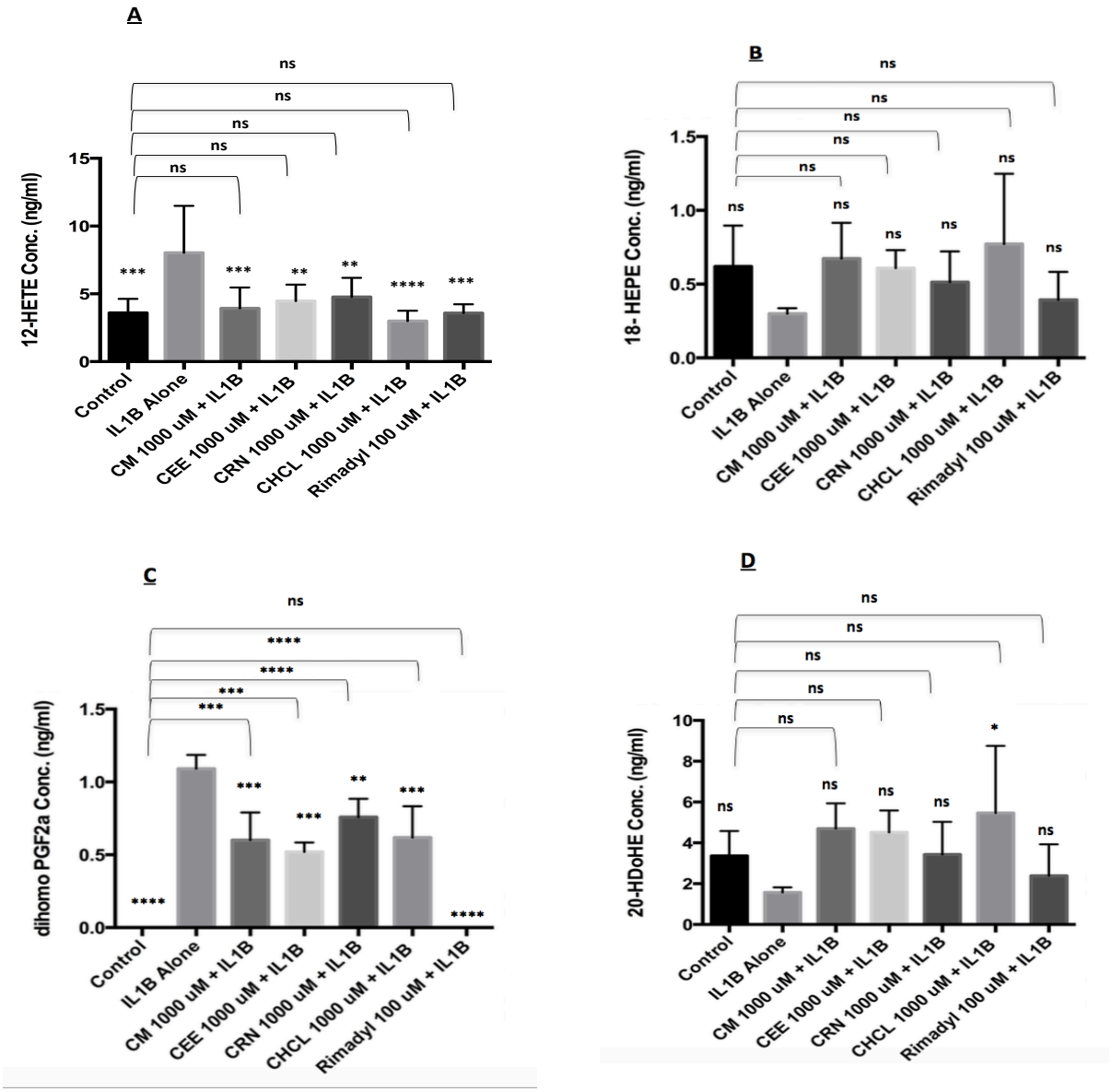
Of the 135 oxylipins that were analyzed, 57 and 55 were present at a level of ≥ 0.1 ng/mL in media taken from control cells and IL-1β stimulated cells, respectively. Of these oxylipins, the levels of 14 oxylipins were significantly changed (≥ 2 fold) after stimulating the cells with IL-1β for 24 hours compared to control cells (Table 6). Specifically, when incubated with IL-1β, the levels of 10 out of the 14 oxylipins

were increased. The oxylipins that were increased after treatment with IL-1β were PGE2, 11bPGE2, 6-Keto Prostaglandin F1-alpha (6k PGF1a), 11-hydroxy-eicosatetraenoic acid (11-HETE), 12-HETE, 13-hydroxy-docosahexaenoic acid (13-HDoHE), d17- 6k PGF1a, 6,15-diketo-,dihydro PGF1a (6,15-dk-,dh-PGF1a), dihomo PGF2a and PGE1. All of the oxylipins that were elevated following IL-1β treatment were formed via COX and LOX pathways (Table 6). In contrast, the levels of 14,15 epoxy-eicosatrienoic acid (14,15 EpETrE), 20-hydroxy-docosahexaenoic acid (20-HDoHE), 16,17 epoxy-docosapentaenoicacid (16,17 EpDPE), and 18-hydroxy-eicosapentaenoic acid (18-HEPE) were substantially reduced following IL-1β stimulation. All the oxylipins showing reduction following IL-1β were derived from the CYP pathway (Table 6). When cells were co-treated with CM, CEE, CHCl, CRN, or Rimadyl for 24 hours, the levels of 4 oxylipins of those 14 that were affected with IL-1β stimulation, changed (increased or decreased) compared to IL-1β stimulated cells, Figure 15 (A-C). These oxylipins are 12-HETE, 18-HEPE, 20-HDoHE, and dihomo PGF2a.

Table 6. Levels of oxylipins that were affected after IL-1β stimulation. Values were expressed as the mean ± SEM of four samples per treatment group. *<LOQ: below levels of quantification.

Compound	Amount of Oxylipin Released From Control Group (ng/ml)	Amount of Oxylipin Released From IL-1β Group (ng/ml)	Effect of IL-1β on the Amount of Oxylipin (fold change)	Fatty Acid	Pathway
PGE2	0.53 ± 0.06	12.59 ± 0.23	↑ 23.3	AA	COX
11bPGE2	0.60 ± 0.17	19.08 ± 0.07	↑ 31.6	AA	COX
6k PGF1a	0.44 ± 0.02	4.21 ± 0.01	↑ 9.4	AA	COX
11-HETE	1.39 ± 0.25	3.34 ± 0.34	↑ 2.4	AA	non-enz/COX /LOX
12-HETE	3.39 ± 0.51	8.03 ± 1.72	↑ 2.4	AA	LOX
14,15 EpETrE	0.12 ± 0.03	0.05 ± 0.01	↓ 2.1	AA	CYP
13-HDoHE	2.68 ± 0.13	5.71 ± 0.01	↑ 2.1	DHA	LOX
20-HDoHE	3.35 ± 0.86	1.57 ± 0.17	↓ 2.1	DHA	non-enz/CYP
16,17 EpDPE	0.63 ± 0.21	0.26 ± 0.03	↓ 2.4	DHA	CYP
18-HEPE	0.61 ± 0.19	0.29 ± 0.02	↓ 2.1	EPA	CYP
d17 6k PGF1a	<LOQ*	0.13 ± 0.001	↑	EPA	COX
6,15-dk-,dh-PGF1a	<LOQ*	0.17 ± 0.03	↑	DγLA	COX
dihomo PGF2a	<LOQ*	1.03 ± 0.01	↑	ADA	COX
PGE1	0.07 ± 0.01	2.48 ± 0.03	↑ 34.1	DγLA	COX

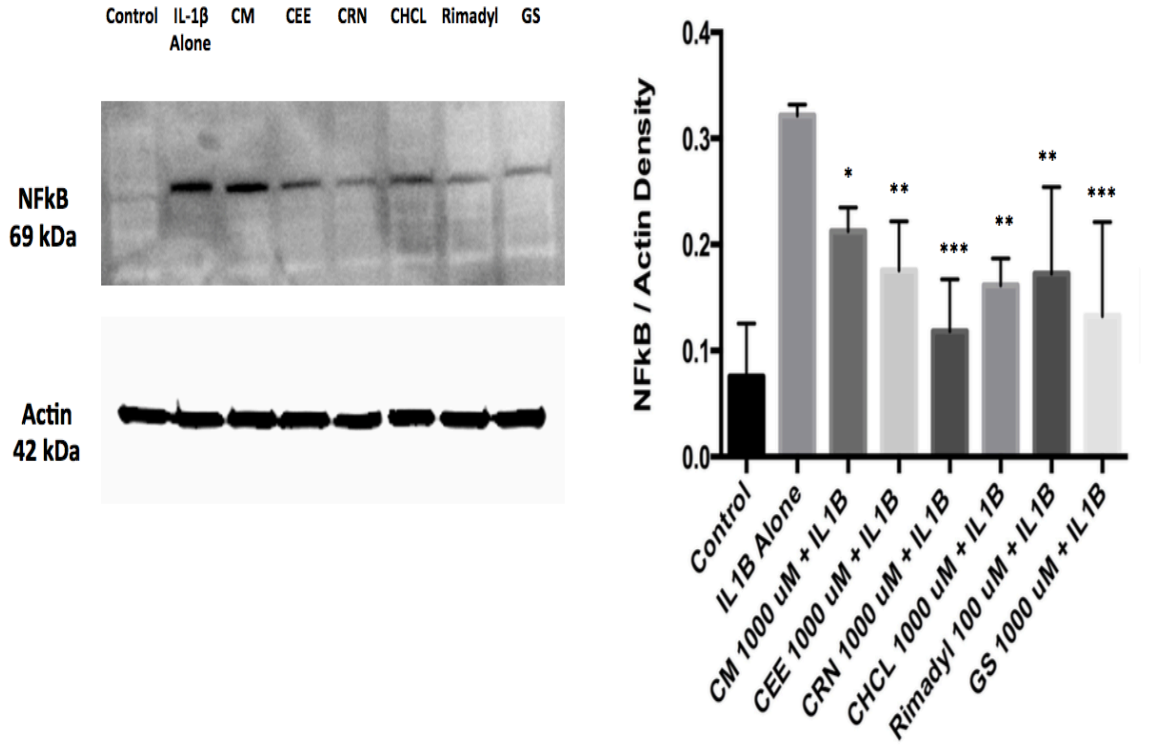
Figure 15. Levels of (A) 12-HETE, (B) 18-HEPE, (C) 20-HDoHE, (D) dihomo PGF2a in stimulated cells after 24 hours under the various treatment conditions. Values were expressed as mean ± SEM of four samples per treatment group. Asterisks above the bars indicate significance compared to IL-1β treatment group; brackets indicate significance compared to control (un-treated group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns = non significant (one-way ANOVA).



4.7 Phosphorylated-NF-kB Expression in Primary Cultured CnC Under Control and Stimulated Conditions:

Next, we wanted to determine whether the anti-inflammatory effects seen with creatine supplements are due to inhibition of the activation of the transcription factor NF-kB. To do that, western blot was performed to analyze and quantify the expression of the phosphorylated form of NF-kB after treating CnC with the various treatment conditions. As shown in Figure 16, treating the cells with IL-1β alone (10ng/ml) for 1 hour induced the expression of phosphorylated-NF-kB significantly compared to untreated control cells. Interestingly, all the compounds examined significantly reduced the expression of phosphorylated-NF-kB compared to the positive control, with the highest reduction seen with CRN (~ 63%), Figure 16.

Figure 16. Western blot analysis of phosphorylated-NF-kB in stimulated cells after 1 hour under the various treatment conditions. Panel A: representative blot for phosphorylated-NF-kB and beta-actin expression in stimulated cells after 1 hour. Panel B: Densitometry analysis of the western blot. Values were expressed as mean ± SEM of three samples per treatment group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to IL-1β treatment group.



5. Discussion:

Creatine, most commonly in the form of monohydrate, is widely used by athletes as a dietary supplement to improve performance and to increase strength and muscle mass. [5] In addition, creatine supplementation was shown to be neuroprotective in several models of neurodegenerative diseases. Potential mechanisms of action for creatine supplements include improving cellular energy, mitochondrial stability, free radical scavenging, and anti-inflammatory effects.[4] Recently, it has been shown in human studies that CM can significantly reduce the plasma levels of some key pro-inflammatory cytokines, such as IL-1β, TNFa, and PGE2 after strenuous exercise. [74, 75] However, little is known regarding the mechanism of this anti-inflammatory response to creatine. In the present study, the main objective was to examine and compare the anti-inflammatory properties of CM, and newer creatine salt forms and derivatives, in an *in-vitro* model of OA. In addition, the metabolic breakdown product, CRN, was also examined for potential anti-inflammatory effects.

In this project, primary CnC derived from normal canine articular cartilage were used as an *in-vitro* model of OA for examining the anti-inflammatory effects of creatine compounds. Primary CnC are considered a well-established *in-vitro* model to study the inflammatory mechanisms of OA and they have been used in many

studies to evaluate potential effectiveness of therapeutic agents. For example, they have been used in veterinary medicine to understand the inflammatory pathways involved and to find potential therapeutic agents for the treatment of OA in dogs. [120, 121] In addition, they were also used to screen for natural products that could be of importance in reducing inflammation in OA. [110, 122] Of importance for the current study is the ability of cultured chondrocytes to activate inflammatory pathways following exposure to IL-1β, a key inflammatory mediator in the pathogenesis of OA. [110, 121-123]

Prior to examining the anti-inflammatory effects of the various creatine compounds, studies were carried out to determine the suitability of the CnC culture model. These studies involved evaluation of potential cellular toxicity to the various treatment regimens, as well as verification of a creatine transporter presence in the cells. The results of the toxicity studies indicated that none of the tested compounds produced toxicity within the concentrations examined. This indicates that the reduction of inflammatory mediators observed in this study was not due to cell toxicity. The concentrations of creatine compounds used in this study reflect those achieved in human blood after administration of a 4.4 gram isomolar equivalent of various creatine supplements. [16] Given that most creatine supplements are taken at doses exceeding 4.4 grams per day, the concentration

range examined for the creatine compounds would appear to be realistically achievable.

Since creatine is primarily synthesized in the liver and kidney, efficient utilization in most cells requires creatine to be transported from tissues of synthesis to tissues of utilization. [5] It is transported via a specific plasma membrane creatine transporter (CT1). [5] In this study, the expression of CT1 was examined at the mRNA level in CnC using the RT-PCR approach in order to confirm that these cells were a suitable *in-vitro* model for studying the anti-inflammatory effects of creatine compounds. These studies demonstrating the expression of CT1 at the mRNA levels represent the first reports of CT1 expression in chondrocytes and suggest that these cells would be expected to have the ability to take up creatine.

We hypothesized that the creatine compounds would have an anti-inflammatory effect in the CnC model following IL-1β stimulation. This was initially confirmed using PGE2 release from CnC as an indicator of inflammatory response. The focus on PGE2 is based on its importance as a key inflammatory mediator in the pathogenesis of OA. Clinically, studies report elevated levels of PGE2 in the cartilage and synovial fluid from patients with OA. [124] At the cellular level, PGE2 has been found to inhibit proteoglycan synthesis and increase ECM degradation in

OA chondrocytes. [124]

In the present studies, all the creatine compounds examined displayed some ability to reduce PGE2 release into the culture media. The effects were both time and concentration dependent with CM, CEE, CHCL, CRN, and Glucosamine significantly decreasing the concentrations of PGE2 at 8 and 48 hours post stimulation. The reason(s) for the biphasic effects observed with the creatine compounds is not known. It could be that these compounds impact on PGE2 production through multiple pathways and thus different exposure times result in different effects on PGE2 release. In contrast, Rimadyl, was able to substantially reduce PGE2 release at all the time points examined. These findings are consistent with the ability of Rimadyl to inhibit COX-2, which would result in reductions in PGE production in the cell.

The increased PGE2 found in chondrocytes from human cartilage of patients suffering from OA appears to be associated with increased expression of COX-2. [125] In addition, COX-2 expression is influenced by transcription factors activated through IL-1β and TNFa. [124, 126] In the present study, the expression of COX-2 was significantly increased after stimulating the cells with IL-1β compared to control cells. Furthermore, all the tested compounds were able to decrease COX-2 expression at 48 hours except Rimadyl, which increased its expression

significantly. A similar effect was observed when examining TNFa release in the media, with all the tested compounds (except Rimadyl) significantly reducing the release of TNFa.

The fact that Rimadyl increased the expression and release of COX-2 and TNFa is consistent with findings of other studies concerned with the effects of COX-2 inhibitors. Page et al., showed that NSAIDs, specifically Celecoxib and Rofecoxib, which are COX-2-specific NSAIDs, and Diclofenac, which is a COX-1-and COX-2-bispecific NSAID, significantly increased the spontaneous release of TNFa in arthritic human synovial membrane cultures. [127] The same increase of TNFa with NSAIDs was observed in primary human monocytes after stimulating the cells with lipopolysaccharide (LPS). [127] These findings might explain the ability of NSAIDs to relief pain and tenderness and their lack of disease modifying anti-rheumatic activity, as increased TNFa release would be expected to exacerbate the inflammation and the pathologic processes in arthritic patients.

As the inflammatory effects of TNFa are associated with activation of NF-kB, the effects of the creatine compounds on NF-kB expression were also examined. As expected given the effects of the various creatine compounds on TNFa release, there was also a reduction in NF-kB expression observed compared to IL-1β

stimulation alone. The fact that Rimadyl was also able to decrease its expression might be due to the early time point that we chose for this study. Looking at later time points may reveal an increase in NF-kB expression with Rimadyl.

Although the role of prostaglandins and specifically PGE2 in the pathophysiology of OA has been subject of extensive research, the role of oxylipins is not well understood. Since inflammation is an important component in the pathophysiology of OA, changes in these oxylipins in response to inflammation in the CnC might provide important insight into the development of OA as well as provide potential biomarkers for treatment response.

In the present study, the production of oxylipins in CnC was examined in response to IL-1β. We showed that incubating the cells with IL-1β increases the levels of 10 oxylipins formed via COX and LOX pathways and decreases the levels of 4 oxylipins derived from the CYP pathway. The COX oxylipin present at the highest level upon incubation with IL-1β was the pro-inflammatory PGE2. Although present in smaller concentrations, there were several other COX derived oxylipins that showed substantial elevations (up to 32-fold) following IL-1β exposure. The role of these remaining oxylipins in OA and inflammation remains to be determined. Two of the oxylipins that were increased after IL-1β stimulation were 11-HETE and 12-HETE. Both of these oxylipins are pro-inflammatory, derived via

LOX pathway from AA, [112] and are strongly associated with inflammation. [112] For instance, Wong et al., measured various bioactive oxylipins in knee joints of a rat model of OA and compared it to healthy controls and found that 12-HETE were significantly higher in rats with OA. [115] In contrast, stimulating the cells with IL-1β decreased the levels of some anti-inflammatory oxylipins derived via CYP pathway including 18-HEPE. [112] 18-HEPE has the ability to decrease LPS-induced TNFa secretion in the murine macrophage cell line. [128]

Treating the cells with creatine compounds reversed some of the effects of IL-1β. Notably, the creatine compounds significantly decreased levels of the pro-inflammatory oxylipin, 12-HETE, and increased levels of 18-HEPE, an anti-inflammatory oxylipin. Treatment of IL-1β stimulated cells with the creatine compounds also effected the production and release of 20-HDoHE and dihomo PGF2a. However, the significance of those oxylipins in OA is not currently known.

In general, all the creatine compounds examined displayed anti-inflammatory activity, suggesting a potential beneficial role for this supplement in OA. There were subtle differences in response to the various creatine compounds with CEE and CRN having longer durations of activity compared to the responses observed with CM. In addition, CRN was effective only at the highest concentration (1000

uM) compared with CEE, which was effective at reducing PGE2 and TNFa release even at the lowest concentration examined (10 uM). These differences in anti-inflammatory effects might be due to the amount of each agent that enters into the cell. While CM and CHCl would most likely be dependent on transporter based entry into the cells, the increased lipophilicity of CEE would be expected to have better permeability than the other creatine salts examined. [82, 83] Higher permeability and greater cell accumulation of CEE would potentially explain the increased anti-inflammatory activity observed at lower concentrations compared to the other creatine compounds.

The observation that CRN also had anti-inflammatory properties was unexpected since this compound has been considered to be an inactive metabolite of creatine, which is excreted in the urine. [28] The finding of anti-inflammatory effects of CRN in the present study is consistent with the findings of Leland et al. They reported short-term exposure (10-60 minutes) of a mouse macrophage cell line (RAW 264.7) to 100 uM of CRN significantly down-regulated TLR2, TLR3, TLR4, and TLR7 mRNA levels in the cells suggesting that CRN may have immunosuppressive properties. [77] As CEE has been reported to hydrolyze rapidly to CRN in neutral aqueous pH conditions, the anti-inflammatory effects observed with CEE might actually be due to more efficient cellular delivery of CRN.

The exact mechanism(s) by which the creatine compounds produce their anti-inflammatory effects are not currently known. The results of this study suggest that creatine compounds can affect multiple pathways in the process of inflammation. Creatine can interfere with NF-kB and TNFa release leading to reduction in COX-2 expression and PGE2 release. It also altered the oxylipin profile of the canine chondrocytes following inflammatory stimuli. However, further research is needed to elucidate the exact mechanism of action of creatine compounds. The fact that the creatine compounds affected multiple pathways in the process of inflammation suggests that the anti-inflammatory properties of creatine may be a result of the beneficial effects of creatine on the mitochondria and its antioxidant properties. There is a growing body of evidence suggesting interplay between inflammation and mitochondrial dysfunction in OA. For example, Kim et al. have shown that pro-inflammatory cytokines such as IL-1β and TNFa disturb normal mitochondrial function in primary human osteoarthritic chondrocytes cultures by inducing mtDNA damage, decreasing energy production, and increasing reactive oxygen species (ROS) production. [129] In addition, it was shown that mitochondrial dysfunction induced by commonly used inhibitors such as Oligomycin and Antimycin A, which inhibit mitochondrial respiratory chain complexes V and III, respectively, increases inflammatory responsiveness to IL-1β and TNFa (increases

IL-8, COX-2 and PGE2 production) in normal human chondrocytes. [130] This suggests that any therapeutic intervention that has the ability to stabilize and improve mitochondrial function could also decrease the inflammation observed in OA.

6. Conclusions:

The main findings of this study are:

- Creatine compounds significantly reduce cellular mediators of inflammation induced by IL-1β activation in primary CnC as an *in-vitro* model of OA.
- CRN, often considered to be an inactive metabolite of creatine, also had anti-inflammatory properties
- The anti-inflammatory properties of creatine compounds appear to be mediated through its interference with NF-kB expression.
- Creatine compounds in addition to the metabolite CRN can interfere with the production of some of the oxylipins in response to inflammatory stimuli (decreasing pro-inflammatory and increasing anti-inflammatory mediators).
- As these same cells are involved in joint-related inflammation (i.e. arthritis), creatine based dietary supplements may have a beneficial role in preventing inflammation within the joint and other tissues.

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