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Impact of a Novel Marine Algae Supplement on Inflammatory and Immune Response After High-Intensity Exercise

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IMPACT OF A NOVEL MARINE ALGAE SUPPLEMENT ON INFLAMMATORY AND IMMUNE RESPONSE AFTER HIGH-INTENSITY EXERCISE

by

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DEDICATION

I would like to dedicate this work to my husband, Jonathan, for his unwavering support, care, and understanding. Thank you for being you.

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ABSTRACT

INTRODUCTION: High-intensity exercise (HIE) can damage the musculotendon complex and impact the immune response, which may result in post-exercise inflammation and reductions in strength and performance. Sufficient rest and recovery will improve muscular resilience against future damaging bouts, however HIE with minimal durations of rest are common in athletic competitions that facilitate persistent inflammation and immune dysregulation. Marine algae-derived fucoidans (MA) have previously shown promise to mitigate pro-inflammatory and immune dysregulation responses, however it has not been investigated in the context of HIE. The purpose of this research is to investigate the safety and efficacy of MA on markers of inflammation and immune response following HIE compared to placebo (PL). It was hypothesized that MA supplementation will reduce inflammatory cytokine concentration and immune cell perturbations. It was also hypothesized that MA supplementation may facilitate exercise performance in a fatiguing HIE protocol.

METHODS: 8 male and 8 female participants (N=16) were randomized into this double-blind, placebo-controlled, counterbalanced, crossover design study and supplemented with either 1 g/d MA (*Undaria pinnatifida*) or PL for two weeks. Each supplement period was concluded with a HIE testing session, and one week of washout was allotted between the supplements. HIE testing involved one 30s-Wingate anaerobic test (WAnT) and eight 10s-WAnT intervals. Blood was drawn pre-exercise, immediately post-exercise, 30min post-exercise, and 60min post-exercise. Serum cytokine concentrations and blood biomarkers

were primary outcome measures and exercise peak power (PP) and mean power (MP) over the 30s-WAnT and total exercise session were secondary measures. A 2 (condition) x 4 (time) design with repeated measures on both factors were statistically analyzed. Significance was set at $\alpha = 0.05$.

RESULTS: Significantly lower post-HIE concentrations of inflammatory and immune cell counts were observed for MA compared to PL, including WBC, lymphocytes, CD4, CD8, IL-6, and IL-10 ($P<0.05$). No significant differences were observed in exercise performance between MA or PL for PP and MP in either the 30s-WAnT or the total exercise effort ($P>0.05$).

DISCUSSION: MA supplementation did not influence exercise performance during HIE, however it did reduce the inflammatory cytokine and immune cell response induced by HIE. There were physiological differences following HIE that allowed for the same total work with less systemic disruption. Future research is needed to determine the specific mechanism of the effect of MA under these conditions.

CONCLUSION: MA supplementation may be a beneficial strategy to mitigate acute post-HIE induced inflammation and immune dysregulation in periods where recovery time is limited.

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LIST OF ABBREVIATIONS

CD ..... Cluster of Differentiation

d ..... Cohen's *d* Effect Size

DOMS ..... Delayed-Onset Muscle Soreness

EIMD ..... Exercise-Induced Muscular Damage

HGB ..... Hemoglobin

HIE ..... High-Intensity Exercise

hr ..... Hour

HTC ..... Hematocrit

IL ..... Interleukin

MA ..... Marine Algae

MCH ..... Mean Corpuscular Hemoglobin

MCHC ..... Mean Corpuscular Hemoglobin Concentration

MCV ..... Mean Corpuscular Volume

MIE ..... Moderate-Intensity Exercise

min ..... Minutes

MPV ..... Mean Platelet Volume

PL ..... Placebo

RBC ..... Red Blood Cell

RDW ..... Red Blood Cell Distribution Width

s ..... Second

S1 ..... Supplement 1

S2 ..... Supplement 2

T1 ..... Testing Session 1

T2 ..... Testing Session 2

WAnT ..... Wingate Anaerobic Test

WBC ..... White Blood Cell

yr ..... Years

CHAPTER 1

INTRODUCTION

1.1. EXERCISE-INDUCED MUSCULAR DAMAGE

An acute bout of exercise can cause structural damage to the musculotendon complex that necessitates repair following the cessation of the session (Peake et al., 2017). The mechanical action of the myocytes can disrupt sarcomere integrity by stretching and manipulating the myofibrils beyond the overlapping filaments (Peake et al., 2017). This can lead to disrupted structural components and potential rupture of the cell walls that may result in unfavorable reductions in strength and the manifestation of inflammation and muscular soreness in the days following exercise (Allen et al., 2015; Peake et al., 2017). Exercise-induced muscular damage (EIMD) is the outcome of the working musculature and connective tissues that are systematically damaged throughout an acute bout of exercise. However, repeated bouts of exercise can induce favorable adaptions of the trained muscles following rest and recovery, which may lead to physiological and functional resilience against future EIMD (Allen et al., 2015; Antunes et al., 2019; Peake et al., 2017).

The magnitude EIMD from an acute exercise bout will determine the degree of needed repair, however this varies greatly between the exercise performed. The severity of EIMD depends on exercise-intensity, duration of exercise, and mode of exercise (Allen et al., 2015; Cerqueira et al., 2020; Paulsen et al., 2010; Peake et al., 2017). Higher intensities and longer durations of exercise will incur greater damage to the working musculature (Peake et al., 2017). Low to moderate intensities of eccentric muscular

contractions will cause EIMD, however concentric muscle contractions depend more on duration and intensity to induce EIMD (Peake et al., 2017). Many actions involve eccentric muscular actions including change-of-direction tasks performed in athletic performances and downhill running that can induce severe EIMD (Chaouachi et al., 2012; Sorichter et al., 2001).

Skeletal muscle damage will activate the immune system to help control the damage, clear cellular debris, and initiate repair of the damaged tissues (Cerqueira et al., 2020). The magnitude of immune activation correlates with the severity of EIMD, with greater leukocyte infiltration and inflammatory-related cytokine presence observed in longer durations and higher intensities (Allen et al., 2015; Cerqueira et al., 2020; Paulsen et al., 2010; Peake et al., 2017). Following high-intensity exercise (HIE), leukocytes, like neutrophils, accumulate locally in the damaged tissue where they secrete chemical messengers called cytokines (Cerqueira et al., 2020; Shaw et al., 2018). Additionally, cytotoxic T-cells become activated and can accumulate as well. These cells are identified by the cluster of differentiation (CD) membrane receptors, with CD4 cells participating in cytokine signaling and CD8 cells act to destroy cells infected with viruses (Shaw et al., 2018). Acute exercise mobilizes CD4 cells to a greater extent than CD8 cells, an effect that is exercise intensity dose-dependent (Shaw et al., 2018).

Cytokines can act in both a pro- and anti-inflammatory manner. For example, interleukin-1β (IL-1β) is pro-inflammatory and acts to recruit more leukocytes to the damaged cells (Dinarello, 2018). Other cytokines such as interleukin-10 (IL-10) act as anti-inflammatory agents to reduce leukocyte secretion of pro-inflammatory cytokines and to increase phagocytic activity of macrophages (Sabat et al., 2010). Where IL-1β

will be secreted following moderate intensity exercise (MIE) and in a dose-dependent manner, IL-10 is predominantly secreted following high-intensity exercise (Cerqueira et al., 2020) and acts to mediate the pro-inflammatory pathway (Ostrowski et al., 2000; Petersen & Pedersen, 2006).

In addition to leukocyte secretion of cytokines, the working muscle can locally secrete cytokines such as interleukin-6 (IL-6) (Allen et al., 2015; Cerqueira et al., 2020; Febbraio & Pedersen, 2002; Ostrowski et al., 2000). IL-6 increases in concentration following exercise and is highly sensitive to intensity, modality, and duration of exercise. IL-6 is secreted following both MIE and HIE, however IL-6 is observed immediately following HIE, while it may take up to an hour following MIE to increase (Lira et al., 2017). HIE can quickly deplete the working musculature of glycogen which will stimulate IL-6 secretion to activate hepatic glycogenolysis and lipolysis (Febbraio & Pedersen, 2002; Metsios et al., 2020). This increases glucose availability for the working musculature from extracellular sources. Accumulation of exercise-induced IL-6 may further act to increase neuromuscular fatiguability during HIE (Allen et al., 2015; Scheller et al., 2011; Vargas & Marino, 2014). Chronically high levels of IL-6 are associated with glucose intolerance and insulin resistance, however the transient increase observed during exercise primarily acts to provide additional energy to the active musculature (Febbraio & Pedersen, 2002; Metsios et al., 2020; Scheller et al., 2011).

Following exercise, IL-6 initially acts in a pro-inflammatory manner to recruit more immune cells towards the damaged tissues and plays a role in sensitizing local nociceptors to augment the pain response following EIMD (Allen et al., 2015; Scheller et al., 2011). IL-6 then has a later role to help mediate the inflammatory response by

initiating production of IL-10 and acute-phase proteins in the liver, like C-reactive protein, that work to shift leukocyte production away from pro-inflammatory actions (Allen et al., 2015; Febbraio & Pedersen, 2002). In this way, IL-6 works in an "immune-responsive" manner to help facilitate inflammation following cellular damage and then activate downstream actions to help mediate the inflammatory response (Allen et al., 2015; Ostrowski et al., 2000).

Inflammation is the critical first-step of the immune response following tissue damage that causes localized swelling, redness, heat, and pain to the affected regions, which can manifest as soreness and reduced function (Peake et al., 2017; Scheller et al., 2011). Inflammatory cytokines collectively serve to recruit leukocyte transmigration to the damaged areas and influence differentiation to facilitate clearance of cellular debris (Scheller et al., 2011). The degree of EIMD and the associated magnitude of the inflammatory response and physical discomfort increases in severity with higher exercise intensities (Clarkson & Sayers, 1999). Additionally, muscular soreness peaks about 24-48 hours following damaging exercise bout (Clarkson & Sayers, 1999). Delayed-onset muscular soreness (DOMS) will result in a loss of function proportional to the amount of EIMD that may continue for several days following the exercise session (Allen et al., 2015; Peake et al., 2017).

When appropriate durations of rest follow the EIMD exercise bout, the damaged tissues will recover and improve physiological resilience against subsequent bouts of EIMD as part of chronic exercise adaptations (Allen et al., 2015; Antunes et al., 2019; Cerqueira et al., 2020; Lira et al., 2017). Muscular functioning, inflammation, and immune dysregulation caused by EIMD will typically subside after a few days following EIMD,

however the reduced muscular strength and local leukocyte accumulation has been observed up to 7-days following severe eccentric exercise events (Paulsen et al., 2010; Peake et al., 2017). This means that persistent immune dysregulation is possible during periods of increased exercise intensity with reduced rest, such as during athletic tournaments or competitions (Cerqueira et al., 2020). This warrants the need to investigate strategies that may mitigate inflammation and immune dysregulation often associated with EIMD and intense exercise during periods of reduced rest that can facilitate return to play.

1.2. SUPPLEMENTS

Roughly 40-100% of athlete use supplements to help improve performance or facilitate recovery (Garthe & Maughan, 2018). Various supplements have been examined due to their potential role with enhancing recovery or adaptation. For example, protein ingestion will independently increase muscle protein synthesis following ingestion, and it is synergistic with exercise to improve recovery and reduce the magnitude of EIMD (Jäger et al., 2017). Other supplements such as black tea extract have shown to reduce the magnitude of DOMS following 24hrs and 48hrs after an acute bout of interval-based HIE (Arent et al., 2010) and tart cherry juice may improve inflammatory response and recovery by attenuating pro-inflammatory cytokine release (Bell et al., 2015). As a result, athletes and researchers have become increasingly interested in the use of supplements as a tool to improve recovery and bolster the immune system when rest periods are limited.

Recently, marine algae (MA)-derived fucoidans have been of interest for their role surrounding inflammation and immune system regulation. Fucoidans are polysaccharides with a high molecular weight with high proportions of fucose and sulfate content (Fitton et al., 2019, 2015). Fucoidans have shown favorable anti-inflammatory and pro-immune

responses following supplementation (Berteau, 2003; Fitton et al., 2019, 2015). However, the available literature on the topic is rife with methodological inconsistencies including MA species type, sample population selection, and immunological outcomes measures. Over 40 species of fucoidans have been discovered since 1913 (Kylin, 1913), derived from either MA or echinoderms (i.e. sea cucumbers), and the various methods of extraction may fractionate the fucoidans that may further influence rate of bioactivity (Berteau, 2003). Regardless, the collective literature on fucoidan supplementation has shown a multitude of possible avenues for fucoidans to help promote positive immune system responses and to reduce systematic inflammation (Fitton et al., 2019, 2015). For example, a 4-week MA (*Undaria pinnatifida*) supplementation prior to an annual influenza vaccination resulted in an improved and sustained antibody response in elderly Japanese men and women for up to 20-weeks following the vaccination, which may demonstrate that MA has the potential to modulate the immune system in various situations. Another study found that a 4-week supplementation of MA (*Cladosiphon novae-caledoniae*) improved chronic inflammatory biomarkers in cancer patients including reductions in systemic IL-1β and IL-6 concentrations and improved quality of life (Takahashi et al., 2018). A combined Phase I and Phase II study investigated a 4-week supplementation of MA (*Fucus vesiculosis, Macrocystis pyrifera, Laminaria japonica*) in two doses, 100mg per day and 1000mg per day. Ultimately, safety and efficacy of MA to possibly influence cytokine and immune cell activity was observed at both doses with reduced basal IL-6 levels and cytotoxic T-cell activity following 28d of supplementation (Myers et al., 2011). Despite methodological differences and variations in study design, fucoidans have consistently been shown to reduce inflammatory markers and improve resilience of the immune system with no known

toxicity or adverse side-effects (Dutot et al., 2019; Fitton et al., 2019, 2015; Myers et al., 2011). Currently, most available research involving MA supplementation in humans measure alterations in basal levels of cytokines and immune cells where no inflammatory stimulus is provided. Therefore, more research is needed to elucidate whether MA may influence human cytokine and immune cell response following an acute inflammatory stimulus, such as exercise.

Alternatively, MA-derived fucoidans have been implicated in anti-fatigue actions in mouse musculature during a swim-to-exhaustion task with those that supplemented with MA (*Laminaria japonica*) for 21-days (Chen et al., 2014). A later study observed increased treadmill distance and an upregulation of genes to stimulate angiogenesis and mitochondrial biogenesis in mice that supplemented with MA (*Undaria pinnatifida*) for 8 weeks (Ahn et al., 2020). It is possible that upregulation of angiogenesis and mitochondrial biogenesis may have facilitated the anti-fatigue actions of the musculature in the previous study as both MA supplementation protocol were administered in the absence of exercise training (Ahn et al., 2020; Chen et al., 2014). Research performed on MA supplementation combined with exercise in humans is scarce. However, one study indicated increased fecal lysosomes, which are antimicrobial and anti-inflammatory in action, in high performance athletes following a 1-week MA (*Fucus vesiculosus, Undaria pinnatifida*) supplementation period during pre-season training camp (Cox et al., 2020). Generally, pre-season training increases exercise intensity and volume to prepare for competition. Athletes had 73% lower levels of fecal lysosomes at baseline compared to the healthy adult comparator group, but athlete lysosome count improved 43% following MA supplementation (Cox et al., 2020). Perhaps fucoidans may be able to improve inflammation and immune responses following

exercise. Ultimately, there is not much research available for human exercise performance following MA supplementation, but there is enough compelling mechanistic evidence to warrant investigating whether MA-derived fucoidans may aid in recovery from EIMD and the corresponding cascade of inflammation and immune dysregulation following HIE.

The purpose of this study is to investigate the safety and efficacy of a MA fucoidan (*Undaria pinnatifida*) supplement on markers of inflammation and immune response following an acute bout of HIE compared to a placebo. The primary hypothesis was that MA supplementation would reduce the inflammatory cytokine and immune cell response following interval-based HIE compared to placebo. Since MA may aid inflammation and inflammatory cytokines have been linked to increase fatigue during exercise (Allen et al., 2015; Scheller et al., 2011; Vargas & Marino, 2014), a secondary hypothesis was that MA supplementation may allow the participants to maintain higher power output and performance during the interval-based HIE.

CHAPTER 2

METHODS

2.1 PARTICIPANTS

An equal number of healthy, adult males and females (N=16; M=8, F=8) [*Table 1*] were recruited and randomized into either arm of this double-blind, placebo-controlled, counterbalanced cross-over design. To be eligible to participate, individuals must have been physically active, which was defined as consistent with the cumulative weekly recommended physical activity guidelines for Americans (150min-500min per week). They were instructed to not alter their weekly exercise habits throughout the course of the study. Professional athletes, collegiate athletes, competitive bodybuilders, or any individual that would have been competing at the elite category within their sport were not included.

All participants agreed to use a double-barrier method of protection during all sexual activity to reduce the risk of pregnancy and contraction of sexually transmitted infections throughout the duration of the study. Females were excluded if they were pregnant, lactating, or planning on becoming pregnant during the course of the study.

Participants were excluded if they had any current musculoskeletal injuries that would prevent them from completing the exercise protocol or if they had a history of any clinically significant cardiovascular, respiratory, renal, cerebrovascular, hematological, pulmonary, gastrointestinal, autoimmune, lymphatic, hepatobiliary, neurological, psychiatric, metabolic, or endocrine disorders including Type 1 and Type 2 diabetes.

Participants were screened to exclude blood thinning medications or supplement use, participation in another study where supplement product was consumed within 30-days of their first visit, and for any known sensitivity or allergy for the current study products.

All inclusion and exclusion criteria were reviewed by the research team and all participants signed their consent to participate that was approved by the University of South Carolina Institutional Review Board (Approval Date: 10/21/2021). This study was registered on ClincalTrials.gov (NCT05181410).

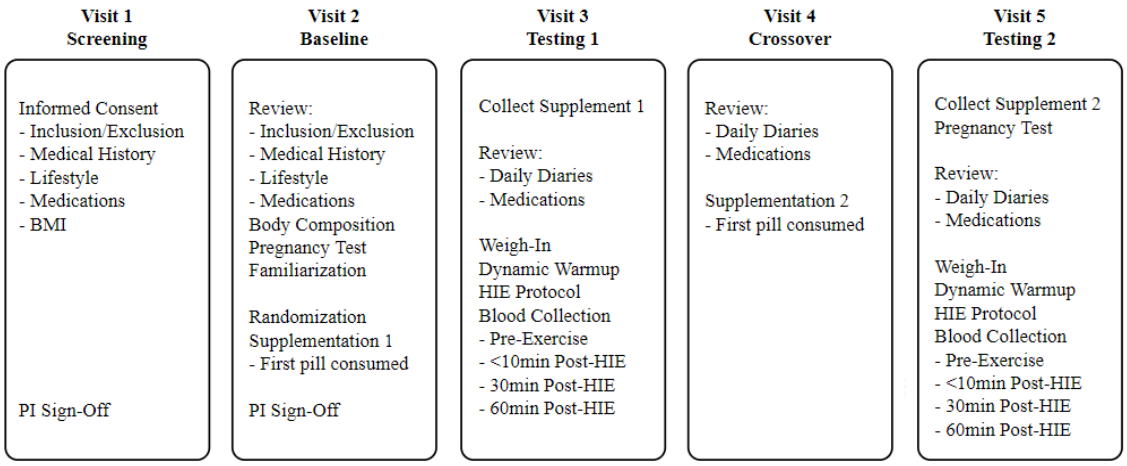

Figure 2.1 *Study Design: Visits*

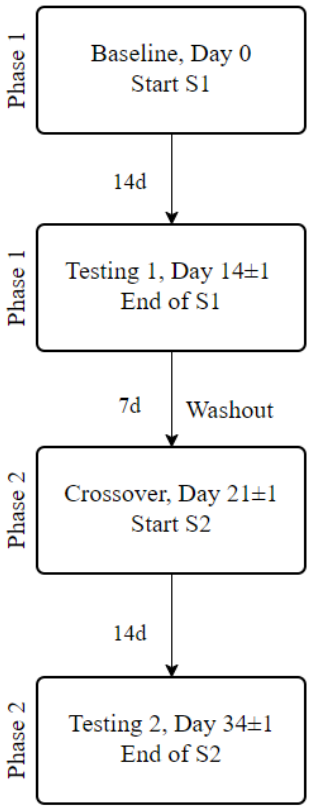

Figure 2.2 *Supplementation Flow*

2.2 STUDY DESIGN AND SUPPLEMENTATION PROTOCOL

Participants attended five visits at the Sports Science Lab at the University of South Carolina (*Columbia, SC*) over the course of the study (*Figure 1*). The investigational product was 500mg capsules containing fucoidan marine algae (MA; *Undaria pinnatifida*) produced by Maritech® (*Cambridge, Tasmania, Australia*) and the placebo (PL) was 500mg microcrystalline cellulose capsules. Capsules were identical in shape, dose, and size, however there was a slight color difference between MA and PL, so only the unblinded study personnel was in charge of distribution, handling, and compliance calculations to prevent any unintentional unblinding. The unblinded personnel did not partake in any other activities with the participants, data entry, or analysis throughout the rest of the study protocol.

The general timeline for the study was a baseline testing visit where the first capsule of the first supplement (S1) was consumed (MA or PL), followed by a 2-week interim S1 supplementation period that ended with the first HIE testing session (T1). After the first testing session, a 1-week washout phase occurred where no supplement was consumed. After this, participants attended a cross-over visit where the first capsule of the second supplement (S2) was consumed, which was then followed by a 2-week interim S2 supplementation period that concluded with the second HIE testing session (T2). There was ±1-day allowed for each testing phase to accommodate participant schedules.

Participants were stratified for sex and randomized into either of the two groups: MA for S1 and PL for S2 (MA/PL), or PL for S1 and MA for S2 (PL/MA). They were expected to consume one capsule in the morning and one capsule in the evening with food for both interim supplementation periods. If a dose was accidentally missed, participants were instructed "to consume the missed dose as soon as possible" or "to consume two doses at their next regularly scheduled dosing". Participants consumed their first dose in front of a lab research member and were monitored for 15-minutes for any potential adverse events (i.e. allergies), and they consumed their final dose the mornings of their testing sessions (T1/T2) to prevent any premature washout of either supplement prior to the testing sessions.

Throughout the interim and washout periods, participants were instructed to not alter physical activity levels or diet, and daily supplement consumption was monitored via the online electronic data collection software Medrio (*San Francisco, California*). Participants were expected to complete a Supplement and Health Diary (SHD) per diem that collected routine times of supplement consumption and monitored any supplement or

health changes throughout the duration of the study. SHDs were reviewed daily for completion and research personnel would reach out within 24 hrs to follow-up on any potential health changes for more information, and to retrieve missing SHD information if diary entry was not completed. This information was recorded in a paper diary format and reviewed with the participant at their next visit for accuracy prior to uploading it into the Medrio software.

2.3 TESTING SESSIONS

There was one baseline testing visit and two HIE testing visits (T1, T2). At the baseline visit, participants were asked to attend the lab normally hydrated and 2 hrs fasted for body composition testing. Participants were additionally asked to not have exercised within 12 hrs and to not have consumed any alcohol, caffeine, or nicotine within 4 hrs prior to the baseline visit. Height and initial weight were obtained on a stadiometer and calibrated scale (*Health-o-meter Professional, Pelstar LLC, Alsip, IL, USA*) and body composition assessment was performed via air displacement plethysmography (*BodPod, Cosmed, Rome, Italy*). Participants were asked to void their bladder prior to BodPod measurements and female participants underwent a urine pregnancy test (*First Response Pregnancy Test, Church & Dwight, Ewing Township, NJ, USA*) to confirm a negative pregnancy status. Finally, participants underwent a brief familiarization session with the cycle ergometer (*Velotron Racer-Mate, Seattle, WA, USA*) that was used for both testing sessions and provided a detailed description of the HIE protocol and expectations.

T1 and T2 were performed at the same time of day (<1 hr difference) to help control for individual diurnal variations. Participants were required to arrive at the lab well-hydrated, to have abstained from exercise within 24 hrs to avoid artificial elevation of blood inflammation markers, and to have refrained from any alcohol, caffeine, or nicotine use within 12 hrs. Further, participants were required to consume a meal 2-4 hrs prior to the testing session but did not consume anything else except water within 2-hrs prior to the testing session. 24-hr dietary food logs were collected for food intake the day prior to T1. These food logs were sent to the participants prior to T2 and participants were asked to replicate the diet as closely as possible, which was verified by the administration of a second 24-hr food log prior to T2. A second pregnancy test was administered to female participants at T2 before the initiation of blood draws and the exercise protocol to verify a negative pregnancy status for the duration of the study. Same-day participant mass was obtained prior to HIE protocol to update programming of the equipment software (*Velotron, Wingate Version 1.0.2*). Following the completion of the above events, the first blood draw (pre-test; see section 2.5 Blood Collection) was obtained and the participant initiated the HIE protocol (2.4 HIE Protocol).

2.4 HIGH-INTENSITY EXERCISE PROTOCOL

All participants underwent a standardized 7 min dynamic warm-up then pedaled the cycle ergometer for 5 min at a self-selected cadence at 75 W. Participants then completed a 3 min rest while seated on the cycle ergometer. Velotron Wingate software was used to create a new client profile with the same-day body mass that was recorded prior to the dynamic warm-up. The protocol for the Wingate Anaerobic Tests (WAnT) was derived from previous research with resistance set at 0.10 kP/kg body mass (Arent et

al., 2010). The testing protocol included one 30-second WAnT followed by a 5 min rest, then eight 10 s WAnT intervals interspersed with 2 min rest periods. This HIE protocol was used to determine mean power (MP) and peak power (PP) during the during the 30s-WAnT, and when averaged over the total exercise time (30s-WAnT + 8, 10s-WAnTs).

2.5 BLOOD COLLECTION

Blood was collected at four time points on T1 and T2: prior to the initiation of any exercise (timepoint A), within 10 min following HIE (timepoint B), 30 min post-exercise (timepoint C), and 60 min post-exercise (timepoint D). The first blood draws occurred following a 10-minute rest in a seated position. A venous catheter (*Becton, Dickinson, and Company, Franklin Lakes, NJ*) was inserted into an antecubital vein at the pre-exercise blood draw and flushed with 5mL saline (*Becton, Dickinson, and Company, Franklin Lakes, NJ*) and was used for all 4 blood draws. If an issue with catheter insertion occurred (i.e. small/elusive vasculature, lack of blood flash), a single draw of blood was performed and the catheter was inserted into an alternate antecubital vein on the same arm or on the opposite arm at the next blood draw (immediately following exercise). Any subsequent issues with catheter use would result in individual blood draws at the prescribed times. One participant (female) was excluded from blood analysis due to missed blood samples; however, they were included in HIE power analysis.

Approximately 23mL of blood was drawn at every timepoint for approximately 92mL of blood drawn per testing session. The first 3mL of blood drawn through the catheter was discarded. The next 10mL were collected into a serum separator tube (SST; *Becton, Dickinson & Co., Franklin Lakes, NJ, USA*) then two dipotassium ethylenediaminetetraacetic acid tubes (K2EDTA; *Becton, Dickinson & Co., Franklin*

Lakes, NJ, USA) were filled with 5mL of blood each. All tubes were gently inverted 8-12 times immediately following collection. The K2EDTA tubes were sent to a CLIA-certified laboratory (Bio-Reference Laboratories, Inc.; *Elmwood Park, NJ, USA*) for whole blood and immune system analysis within 24 hrs of collection. Blood composition analysis included total white blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), RBC distribution width (RDW), platelet count, and mean platelet volume (MPV). The immune marker panel included leukocyte concentrations of neutrophils, lymphocytes, monocytes, eosinophils, and basophils. The panel also included the concentrations of T-helper cells (CD4) and T-suppressor cells (CD8), and the CD4:CD8 ratio.

SST vials were allowed to stand for 30 min following collection and inversion, prior to processing where vials were centrifuged for 15-minutes at 3300xg. Serum was then pipetted into aliquot tubes and stored at -80^{o}C until cytokine analysis of IL-1β, IL-6, and IL-10. Cytokine analysis was performed following the collection of all samples using the commercially available magnetic-bead assay kits (*Human TH17 Multiplex Assay, EMD Millipore Corporation, Burlington, MA, USA*) and a magnetic multiplex analyzer (*MAGPIX, Luminex, Austin, TX, USA)* with the coefficient of variation (CV) between 6.7 – 8.4%. Blood biomarkers were adjusted to account for plasma volume shifts during exercise using the hemoglobin and hematocrit adjustment factor method described in Greenleaf, et al., (1979) (Greenleaf et al., 1979).

2.5 STATISTICAL ANALYSIS

Primary outcome measures were assessed via a mixed-model approach in a 2 (condition) x 4 (time) design with repeated measures on both factors. Post-hoc analyses were run when an main effect or interaction effect reached significance. Secondary outcomes, average peak power (PP) and average mean power (MP), were assessed between conditions for the 30s-WAnT and for total exercise duration (30s-WAnT + 8, 10s WAnT intervals [Total PP and Total MP]). Supplementation order was used as a covariate in all models. Effect size was calculated with Cohen's D (small=0.2; medium=0.5; large=0.8). All effect sizes (*d*) are compared to timepoint A. Significance was set at $\alpha = 0.05$. Statistical analysis was performed using the statistical software R (*Version 4.2.0*).

CHAPTER 3

RESULTS

3.1. PARTICIPANTS

Table 3.1

Participant Descriptive Data.

	Sample (N=16)	
	Female (n=8)	Male (n=8)
Age (yrs)	21.7 ± 3.5	20.7 ± 1.8
Height (cm)	163.1 ± 6.8	181.1 ± 6.4
Body Mass (kg)	58.8 ± 9.1	83.0 ± 11.9
Body Fat %	24.2 ± 4.3	13.0 ± 7.7

Data is presented as mean ± standard deviation.

One male participant was excluded from exercise and blood analysis due to low supplementation compliance (73.1%). One female participant was excluded from blood analysis due to missing blood samples but was included in exercise analysis. Excluding the low compliance participant, average supplement compliance was 97.5 ± 5.3% and SHD completion was 100%. All female participants tested negative for pregnancy throughout the study.

3.2 PERFORMANCE

Table 3.2

HIE Peak Power and Mean Power by Condition.

	30s PP (W)	30s MP (W)	Average PP (W)	Average MP (W)
PL	1013 ± 308	614 ± 192	1078 ± 323	684 ± 192
MA	1054 ± 339	612 ± 178	1086 ± 347	690 ± 202

Data is presented as mean ± standard deviation.

Average peak power (PP) and average mean power (MP) did not differ between MA and PL supplementation on either the 30s WAnT or PP and MP for the total exercise session ($P>0.05$).

3.3 BLOOD ANALYSIS

3.3.1 WHOLE BLOOD ANALYSIS

Table 3.3

Complete Blood Count Analysis Following High-Intensity Exercise.

		PL	*d*	MA	*d*
WBC**	A	6.5 ± 1.6		6.4 ± 1.3	
(1000/uL)	B#	10.2 ± 2.9	1.58	9.3 ± 1.9	1.78
	C	7.4 ± 2.3	0.45	6.6 ± 1.5	0.14
	D#	7.9 ± 2.9	0.60	7.3 ± 2.2	0.50
RBC**	A	4.8 ± 0.4		4.6 ± 0.5	
(1000000/uL)	B#	4.4 ± 0.5	-0.88	4.2 ± 0.6	-0.72
	C	4.9 ± 0.4	0.25	4.6 ± 0.5	0.00
	D	4.8 ± 0.6	-0.00	4.6 ± 0.6	0.00
MCV**	A	90.0 ± 2.6		90.0 ± 2.8	
(fL)	B#	81.0 ± 11.2	-1.11	75.4 ± 8.1	-2.41
	C	93.4 ± 7.7	0.59	89.0 ± 5.0	-0.25
	D	95.7 ± 11.9	0.66	90.4 ± 8.8	0.06
MCH**	A	30.0 ± 1.2		30.1 ± 1.2	
(pg)	B#	27.0 ± 4.0	-1.02	24.8 ± 2.9	-2.39
	C	31.2 ± 2.6	0.59	29.6 ± 1.9	-0.31
	D	32.0 ± 4.1	0.66	30.0 ± 3.2	-0.04
MCHC**	A	33.3 ± 0.8		33.5 ± 0.7	
(g/dL)	B#	29.7 ± 4.0	-1.25	27.2 ± 3.4	-2.57
	C	34.6 ± 2.6	0.68	32.7 ± 2.3	-0.47
	D	35.5 ± 4.1	0.74	33.1 ± 3.4	-0.16

		PL	*d*	MA	*d*
RDW**	A	12.8 ± 0.7		12.7 ± 0.6	
(%)	B#	11.3 ± 1.4	-1.36	10.6 ± 1.6	-1.74
	C	13.2 ± 1.1	0.43	12.5 ± 1.1	-0.23
	D	13.6 ± 1.1	0.87	12.7 ± 1.2	0.00
Platelets**	A	259.7 ± 64.0		248.5 ± 40.5	
(1000/uL)	B#	313.0 ± 89.7	0.68	277.9 ± 61.2	0.57
	C	269.5 ± 64.6	0.15	242.0 ± 46.8	-0.15
	D	254.1 ± 83.9	-0.08	234.0 ± 47.8	-0.33
MPV**	A	10.7 ± 0.9		10.8 ± 0.6	
(fL)	B#	9.9 ± 1.5	-0.65	9.2 ± 1.3	-1.58
	C	11.4 ± 1.2	0.66	10.9 ± 1.0	0.12
	D#	11.7 ± 1.9	0.67	10.9 ± 1.3	0.10

Data is presented as adjusted mean ± standard deviation. All effect sizes (*d*) are compared to timepoint A.

#: Indicates a difference from timepoint A ($P<0.05$). **: Indicates differences between conditions ($P<0.05$).

For all variables, there was a significant condition effect ($P<0.05$). Post-hoc analysis revealed MA values were significantly lower than PL for white blood cells (WBC; $P=0.0157$), red blood cells (RBC; $P<0.0001$), mean corpuscular volume (MCV; $P=0.0010$), mean corpuscular hemoglobin (MCH; $P=0.006$), MCH concentration (MCHC; $P=0.0004$), RBC distribution width (RDW; $P=0.0015$), platelet count ($P=0.0002$), and mean platelet volume (MPV; $p=0.0036$).

For all variables there was a significant time effect ($P<0.05$). Post-hoc analysis revealed that timepoint B had significantly higher values for WBC ($P<0.0001$) and platelet count ($P<0.0001$) than timepoint A for both supplementation conditions. Post-hoc analysis also revealed lower values for PL and MA at timepoint B than timepoint A for RBC ($P<0.0001$), MCV ($P<0.0001$), MCH ($P<0.0001$), MCHC ($P<0.0001$), RDW ($P<0.0001$), and MPV ($P<0.0001$). Additionally, the post-hoc analysis revealed that for variables WBC ($P=0.0064$) and MPV ($P=0.0215$), timepoint D had significantly greater concentrations for MA and PL than timepoint A.

No significant time by condition interactions were observed for any of the complete blood count variables ($P>0.05$).

3.3.2 IMMUNE SYSTEM ANALYSIS

Table 3.4

Immune System Analysis Following HIE.

		PL	*d*	MA	*d*
Neutrophils	A	3.6 ± 1.2		3.6 ± 1.0	
(1000/uL)	B[#]	4.8 ± 1.8	0.78	4.2 ± 1.6	0.45
	C	4.5 ± 1.8	0.59	4.1 ± 1.2	0.45
	D[#]	5.5 ± 2.4	1.00	5.0 ± 1.9	0.92
Lymphocytes[**]	A	2.2 ± 0.8		2.1 ± 0.5	
(1000/uL)	B[#]	4.4 ± 1.7	1.66	3.7 ± 1.4	1.52
	C	2.1 ± 0.9	-0.12	1.9 ± 0.6	-0.36
	D[#]	1.7 ± 0.6	-0.71	1.6 ± 0.7	-0.82
Monocytes	A	0.5 ± 0.1		0.5 ± 0.1	
(1000/uL)	B[#]	0.7 ± 0.2	1.26	0.7 ± 0.3	0.89
	C	0.6 ± 0.1	1.00	0.5 ± 0.1	0.00
	D	0.6 ± 0.1	1.00	0.5 ± 0.2	0.00
Eosinophils	A	0.1 ± 0.1		0.1 ± 0.1	
(1000/uL)	B[#]	0.2 ± 0.1	1.00	0.1 ± 0.1	0.00
	C	0.1 ± 0.1	0.00	0.1 ± 0.1	0.00
	D	0.1 ± 0.1	0.00	0.1 ± 0.1	0.00
Basophils[*#]	A	0.0 ± 0.0		0.0 ± 0.0	
(1000/uL)	B[*#]	0.1 ± 0.0	1.24	0.1 ± 0.0	0.92
	C	0.1 ± 0.0	0.24	0.0 ± 0.0	0.41
	D	0.0 ± 0.0	-0.37	0.0 ± 0.0	0.27

Data is presented as adjusted mean ± standard deviation. All effect sizes (*d*) are compared to timepoint A.

*#: Indicates a significant time by condition interaction (P<0.05). #: Indicates a difference from timepoint A (P<0.05). **: Indicates differences between conditions (P<0.05).

A significant condition effect was only observed for lymphocytes concentration between PL and MA (P=0.0211). Post-hoc analysis revealed that lymphocyte concentrations for MA was lower than lymphocyte concentrations for PL (P<0.0001).

Significant time effects were observed for all variables (P>0.05). Post-hoc analysis revealed that timepoint B had significantly higher concentrations than timepoint A for all variables: neutrophils (P=0.0104), lymphocytes (P<0.0001), monocytes (P<0.0001), eosinophils (P=0.0003), and basophils (P=0.0003). Additionally, post-hoc analysis indicated higher neutrophil concentrations at timepoint D than A (P<0.0001). Lymphocyte concentrations declined at timepoint D compared to A (P=0.0099). No other time effects were observed (P>0.05).

A significant time x condition interaction effect was observed for basophil concentration (P=0.0052). Post-hoc analysis revealed that MA had a lower basophil concentration than PL at timepoint B (P=0.0005). No other significant condition x time interaction effects were seen for the immune system variables (P>0.05).

a. CD4 Concentration

b. CD8 Concentration

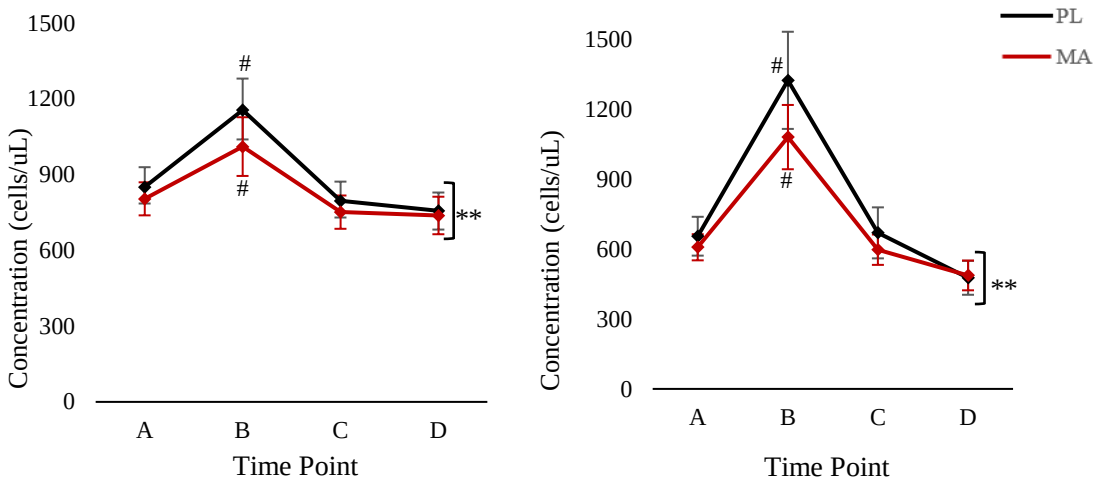

Figure 3.1 *CD4 and CD8 Concentrations Following HIE.* #: Indicates a difference from timepoint A (P<0.05). **: Indicates a condition main effect (P<0.05).

A significant condition effect was observed for CD4 concentrations between PL and MA (P=0.0193). Post-hoc analysis revealed that CD4 concentrations for MA were lower than CD4 concentrations for PL (P=0.0193). A significant time effect was also observed for CD4 concentrations (P<0.0001) and the post-hoc analysis revealed higher CD4 concentrations for both PL and MA at timepoint B compared to A (d_{MA}=0.59; d_{PL}=0.78; P<0.0001). No significant interaction effects were observed (P=0.5914).

A significant condition effect was observed for CD8 concentrations between PL and MA (P=0.0426). Post-hoc analysis revealed that CD8 concentrations for MA were significantly lower than CD8 concentrations for PL (P=0.0426). A significant time effect was also observed for CD8 concentrations (P<0.0001) and post-hoc analysis revealed CD8 concentrations increased at timepoint B for both PL and MA (d_{MA}=1.20; d_{PL}=1.13; P<0.0001). No significant interaction effects were observed (P=0.3732).

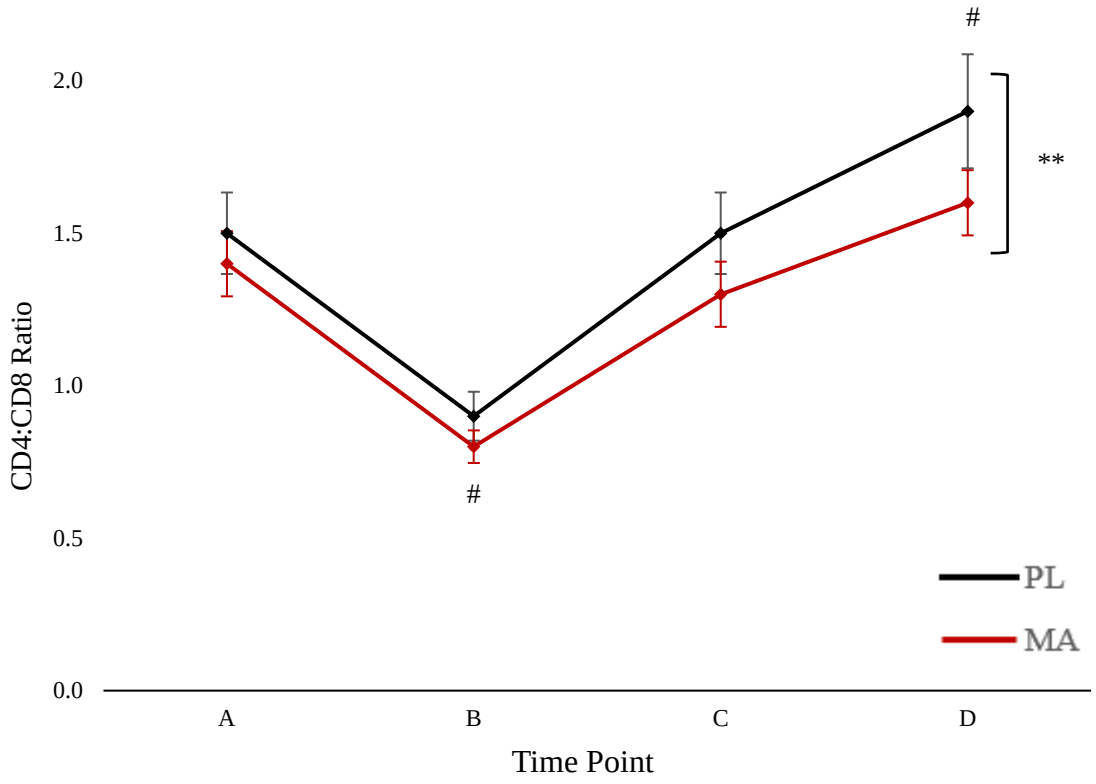

Figure 3.2 *CD4:CD8 Ratio Following HIE.* #: Indicates a difference from timepoint A (P<0.05). **: Indicates a condition main effect (P<0.05).

A significant condition main effect was observed in the CD4:CD8 ratio (P=0.0031) and post-hoc analysis revealed that the CD4:CD8 ratio was lower for MA than PL conditions (P=0.0031). A significant time effect was observed (P<0.0001) and post-hoc analysis revealed that timepoint B had a significantly lower CD4:CD8 ratio for both conditions than A (d_{MA}= -1.90; d_{PL}= -1.46; P<0.0001) and timepoint D had a significantly higher CD4:CD8 ratio for both conditions than A (d_{MA}= 0.50; d_{PL}= 0.66; P<0.0001).

3.3.3 CYTOKINES

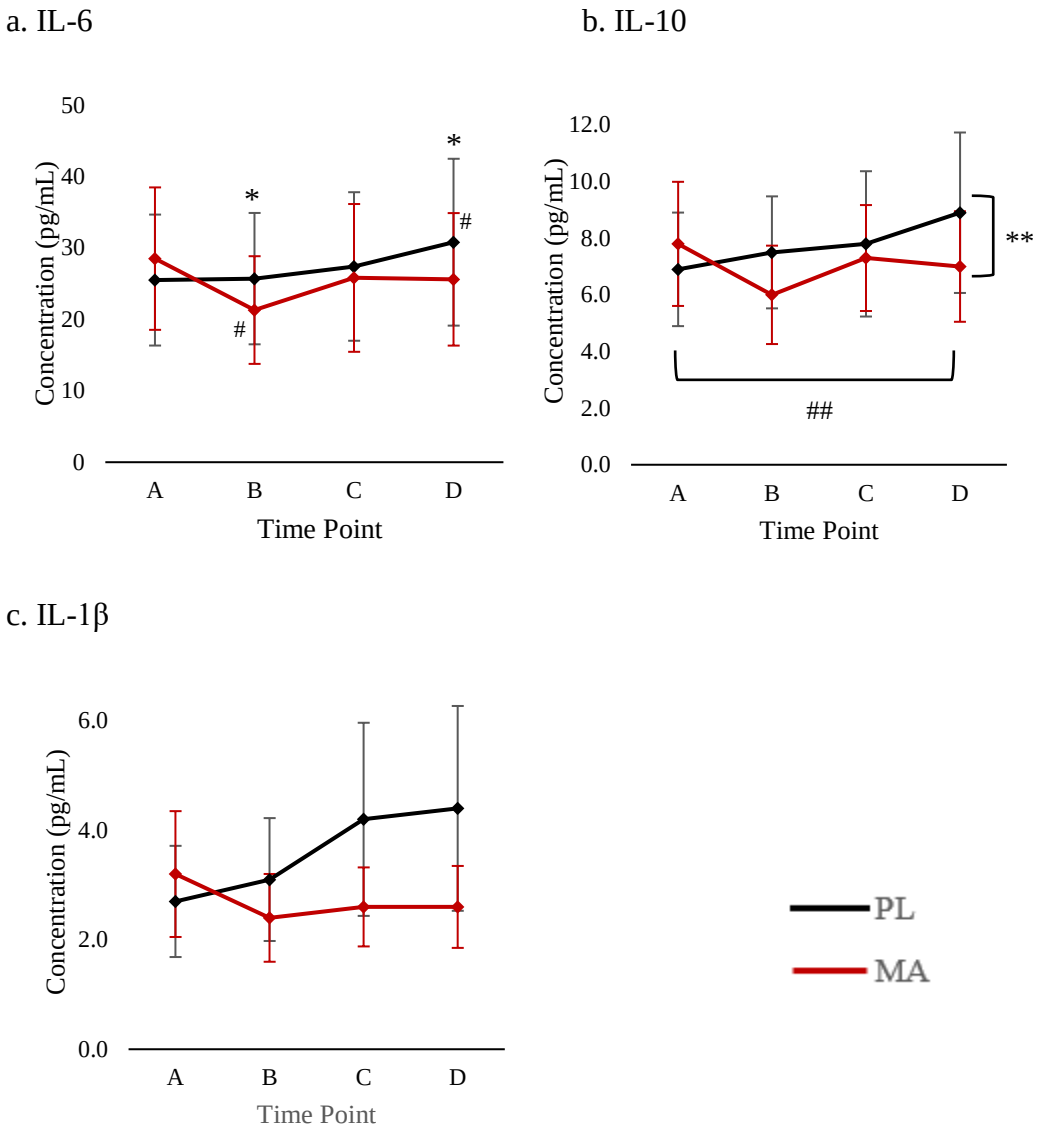

Figure 3.3 *Cytokine Concentrations Following HIE*. #: Indicates a difference from timepoint A (P<0.05). ##: Indicates a time main effect. *: Indicates differences between conditions (P<0.05). ** Indicates a condition main effect.

A significant condition main effect was observed for IL-6 concentrations between PL and MA (P=0.0313). A significant time main effect was observed for IL-6 (P=0.0131) for both conditions. Post-hoc analysis observed lower IL-6 at timepoint B for MA (d_{MA}= -0.22; d_{PL}= 0.01; P=0.0059) and higher IL-6 at timepoint D for placebo (d_{MA}= -0.08; d_{PL}= 0.13; P=0.0305).

Significant condition main effects for IL-10 were observed between PL and MA (P=0.0430). Post-hoc analysis revealed IL-10 was lower in MA than PL (P=0.0430). Significant time effect was observed for both PL and MA (P=0.0249). Post-hoc analysis revealed no significant differences between timepoints (P>0.5). No significant interaction effect was observed (p>0.05).

No significant IL-1β condition (P=0.0604), time (P=0.7147), or interaction effects were observed (P=0.4772).

CHAPTER 4

DISCUSSION

Peak power and mean power output during a high intensity exercise bout were similar following two-weeks of supplementation with MA or placebo. Despite similar workloads and power outputs, notable differences were observed in inflammatory cytokine and circulating immune cells following the one-hour recovery period with the MA supplementation condition compared to the PL condition. The immune system analysis showed reduced total WBC, lymphocyte, CD4, and CD8 concentrations following the high intensity exercise bout with MA supplementation. The cytokine response was additionally altered during the recovery period when supplementing with MA with lowered IL-6 and IL-10 concentrations following exercise compared to PL supplementation.

The results of this study support the hypothesis that MA supplementation may reduce post-exercise cytokine and immune cell perturbations. However, the mechanisms behind the potential effects observed from MA supplementation cannot be elucidated by this study. IL-6 production is proportional to exercise intensity (Cerqueira et al., 2020; Febbraio & Pedersen, 2002; Ostrowski et al., 2000) and typically peaks immediately following HIE and within 1hr for MIE (Lira et al., 2017). The observations that MA supplementation significantly reduced IL-6 levels post-exercise compared to pre-exercise and maintained lower overall IL-6 levels for the following 60min may demonstrate an improved capacity of the musculature to manage energy levels during HIE and may provide

the opportunity for improved recovery following exercise (Cerqueira et al., 2020). In addition, IL-6 immediately acts in a pro-inflammatory manner and later helps mediate the inflammatory response initiating the production of anti-inflammatory cytokines, like IL-10 (Allen et al., 2015; Scheller et al., 2011). As lower IL-6 concentrations were observed following HIE in MA compared to PL, IL-10 concentrations were subsequentially lowered for MA than PL. No significant difference was observed for IL-1β between MA and PL conditions; however, MA showed a trend of reduced concentration following HIE.

The lowered inflammatory cytokine response immediately following HIE could be related to the significantly lower concentrations of WBC, specifically CD4 and CD8 cells, observed with MA supplementation. Since CD4 cells are cytokine-signaling T-cells (Shaw et al., 2018), a lowered concentration of these WBC would help attenuate or mitigate the immediate cytokine response. CD8 cells relate to destruction of damaged or infected cells (Shaw et al., 2018), so the observed reduction in concentration could indicate less EIMD for the MA supplementation group only. To further support this notion, the CD4:CD8 ratio was significantly reduced at all timepoints for MA compared to placebo, which could indicate less need to signal pro-inflammatory response (CD4). Alterations in inflammation response were not measured in this study, so inferences regarding inflammation are derived from the measured cytokine and immune cell response following HIE. The addition of macrophages to our blood biomarker profile may have strengthened our assessment of the inflammatory response. However, as macrophages typically do no circulate in the blood and instead reside locally in tissues to facilitate debris clearance following damage (Allen et al., 2015; Woods et al., 2000), the need for muscle biopsies limits the feasibility of using this as a marker of inflammation and muscle damage.

The secondary hypothesis of improved exercise performance was not supported as no significant PP or MP differences were observed between conditions. However, for the same amount of workload, there were reductions in post-HIE cytokine and immune cell response when supplemented with MA compared to a PL. Consistently lowered inflammatory markers and improved immune health have been demonstrated in previous research in healthy (Myers et al., 2011; Negishi et al., 2013), clinical (Takahashi et al., 2018), and athletic (Cox et al., 2020) populations. Therefore, MA supplementation may not act as a direct ergogenic aid to improve performance during HIE; however, the consistently lowered pro-inflammatory and immune response following supplementation and as observed following HIE in the present research may facilitate a faster return to play during repeated bouts of exercise due to less immune physiological immune.

To the authors knowledge, this is the first study using MA supplementation as a strategy to impact cytokine and immune cell response following an acute bout of HIE in a human model using a trained population. In addition, a major strength of the present research is the double-blind, placebo-controlled, counterbalanced, crossover design. All participants partook in both arms of the study with identical procedures for S1/S2 and T1/T2. Additionally, T1/T2 controlled for time of day to account for diurnal rhythms, required no MIE or HIE within 24hrs of the testing sessions to avoid superficial inflations of biomarkers, and diet was matched for each participant in the 24hrs leading into the testing sessions. Additionally, supplementation order was used as a covariate in order to mitigate the effects of HIE protocol between T1 and T2.

However, no study is without limitations. First, this research investigated a single acute bout of HIE after two weeks of supplementation. Therefore, chronic adaptations and performance changes with supplementation cannot be determined. Future research with longer supplementation periods is warranted to determine effects on HIE performance and adaptation. Additionally, since the current research investigated the recovery period during a single HIE bout, future research should investigate if MA supplementation could be applicable to improve recovery status over multiple HIE bouts when rest periods are limited. In addition, the sample size consisted of mostly college-aged students, many of whom are involved with ROTC. Future research may look at the inflammatory and immune system response following HIE for participants with a more diverse range of fitness levels. Outside workload and diet also were not controlled for beyond the testing sessions, which may influence internal validity of the study. Future research should investigate MA supplementation in a larger, more diverse population and account for outside diet and workload variability.

Another limitation of the current study is the absence of blood biomarker collection beyond 60min following HIE as some immune variables may not show significant changes until 24hr or longer following HIE. Importantly, the degree of muscle damage following the HIE bout could not be ascertained by the peripheral inflammatory and immune blood biomarkers assessed in this study. Although this HIE protocol was used previously shown to induce DOMS and oxidative stress after exercise (Arent et al., 2010), markers of muscle damage such as creatine kinase or subjective ratings of DOMS were not measured in the current study and may have helped assess how MA may influence EIMD and recovery status. The lack of inflammatory and immune blood biomarkers beyond the 60min window,

lack of local tissue physiological analysis, and the lack of objective and subjective muscle damage markers limits the ability of the present research to assess the degree of EIMD derived from the current protocol. Future research should investigate longer blood collection protocols and local tissue responses following HIE to observe the full range of biomarker changes and better understand how HIE influenced EIMD. Despite these limitations, differences between conditions were still observed and point to an attenuation in the cytokine and circulating immune cell response following supplementation with MA.

In conclusion, while performance was maintained in both the MA and PL conditions, MA significantly lowered cytokine and immune cell response following HIE. With the same level of workload, less physiological disruption occurred. As a result, MA supplementation has potential as an acute supplementation strategy to reduce the cytokine and immune cell response following an acute bout of HIE. However, future research is needed to understand the specific mechanism of the presumed beneficial effects from MA supplementation. Practical application of MA supplementation may reduce inflammation and allow for a quicker return to play during competitions and athletic tournaments where recovery between HIE is limited.

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