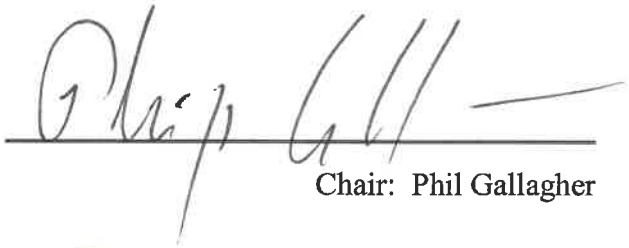
Skeletal Muscle Cytokines following Repeated Bouts of Exercise and Orange Juice

By

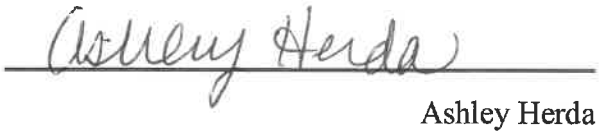
Evan Landes

Submitted to the graduate degree program in Health Sport and Exercise Science and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science in Education.



Chair: Phil Gallagher

Dawn Emerson



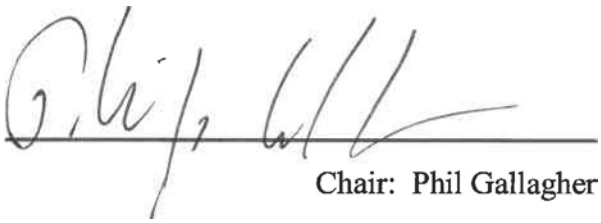
Ashley Herda

Date Defended: May 17, 2018

The thesis committee for Evan Landes

certifies that this is the approved version of the following thesis:

Skeletal Muscle Cytokines following Repeated Bouts of Exercise and Orange Juice



Chair: Phil Gallagher

Date Approved: May 24, 2018

Abstract

INTRODUCTION: The primary aim of this study is to determine if orange juice (OJ) supplementation can attenuate cytokine (IL-6 and TNF-α) levels in human skeletal muscle following exercise in the heat. We hypothesized that exercise in a hot humid environment would increase IL-6 and TNF-α levels in skeletal muscle. Secondly we hypothesized that supplementation with 100% OJ would partially attenuate the cytokine response to exercise in a similar manner to carbohydrate-electrolyte beverage (CEB) but more so than just drinking water (W) following exercise. **METHODS:** In a single blind fashion, fourteen healthy males and four healthy females, consumed either 100% OJ, W, or a CEB for eleven days. Over five of those days they performed 80 minutes of intermittent cycling (4 sets of 15min at 70% max heart rate) in an environmental chamber set at a mild, thermal temperature (30°C, 50% humidity). Muscle biopsies were taken from *vastus lateralis* on days 1, 4, 8, and 11 of the study and analyzed for the cytokines IL-6 and TNF-α via Western Blot protein immunoblotting technique. **RESULTS:** There was no main effect for either time or group for IL-6 in skeletal muscle. However, there was a group x time interaction ($p=0.018$), where the group that consumed W linearly increased from day 1 to day 11 while the OJ, and CEB groups did not change. TNF-α skeletal muscle levels were below the detection level of the imaging system and were not analyzed statistically. **DISCUSSION:** In conclusion, there was significant group times time interaction in the W group but not the CEB or OJ groups However, our data does suggest that the carbohydrate content of OJ and CEB could plausibly attenuate IL-6 increase when compared to just W.

Acknowledgements

I would like to thank my thesis committee, Dr. Phil Gallagher, Dr. Dawn Emerson, and Dr. Ashley Herda for serving on my committee and for their guidance and feedback throughout my time in the graduate program at the University of Kansas. I am especially grateful to my advisor Dr. Phil Gallagher for the time he has put into aiding me through this project. I would also like to offer a special thanks to my fellow students in the Osness Human Performance Lab, Melani Kelly, Matt Bubak, Evan Barnes, and Jake Deckert as well our undergraduate volunteers. Without their help, this project would not have been possible.

Table of Contents

Chapter I

Introduction

Background

Inflammation in the body can occur with conditions such as fever or tissue damage, and also with chronic inflammation associated with diseases such as cancer, cardiovascular disease and Type 2 diabetes (Petersen & Pedersen, 2005). Indicators of inflammation include small proteins called cytokines such as interleukin-6 (IL-6) and tumor necrosis factor- α (TNF-α). Broad ranges of tissues are capable of producing cytokines. These tissues include but are not limited to; leukocytes, skeletal muscle, adipose tissue, and endothelial cells, (C. P. Fischer, 2006). Cytokines act in the acute phase response as signaling molecules. There they aid in the regulation of immune system activity. In recent years, skeletal muscle has been found to possess the ability to function as an endocrine organ that can secrete cytokines that act in a fashion separate from the traditional acute phase response (Munoz-Canoves, Scheele, Pedersen, & Serrano, 2013).

In the acute phase response TNF-α is amongst the first to appear in the cytokine cascade (Petersen & Pedersen, 2005). When pro-inflammatory cytokines TNF-α and IL-β are released, IL-6 production is stimulated, which can then act in a pleiotropic manner (Nieman, 2000). The pro-inflammatory function of IL-6 is to induce the acute phase response proteins that aid the immune system in recognizing foreign bodies and dying cells to prevent infection. When levels of pro-inflammatory cytokines, interleukin-1 (IL-1) and TNF- α, get too high IL-6 shifts into an anti-inflammatory role by stimulating the release of IL-receptor antagonist (IL-1ra). IL-1ra, another anti-inflammatory cytokine, acts to inhibit the activity of the pro-inflammatory IL-1. This pathway is also known as the classical cytokine response. In this way these cytokines regulate inflammation and the removal and repair of damaged tissue (Nieman, 2000) .

The ability of active skeletal muscle to produce cytokines, or myokines as they are known when secreted by muscle, has become a widely accepted theory and one that appears to be independent of the classical cytokine response (Bente K. Pedersen & Febbraio, 2008). This stems from the observation that TNF-α levels do not always increase significantly with exercise while IL-6 production does increase to a significant degree (Petersen & Pedersen, 2005). Therefore, if vigorous exercise can alter cellular homeostasis enough to induce cytokine activity it then provides a prime avenue for studying exercise-induced inflammation (Brenner et al., 1999).

Researchers have also suggested that exercise induces gastrointestinal endotoxemia. Increased permeability of the gastrointestinal endothelial wall caused by reduced blood and oxygen flow during exercise can cause what is termed as “leaky gut”. A “leaky gut” enables gastrointestinal pathogens or endotoxins to cross the wall of the gastrointestinal tract and enter the bloodstream, that then leads to an acute phase response and cytokine production (Mach & Fuster-Botella, 2017). Considering the “leaky gut” theory, there is a degree of debate as to the source of cytokine production during exercise, regardless of whether the measures are from the analysis of blood, muscles, or other tissue. Although this theory presents a conflicting source of cytokines during exercise, there is a bulk of research that supports the production of cytokines via active skeletal muscle (R. L. Starkie, Hargreaves, Rolland, & Febbraio, 2005) .

Many factors can influence the degree of cytokine release and manipulation of some of those factors can lead to novel ways of reducing post-exercise inflammation. Environmental factors, specifically high heat and humidity, can significantly increase cytokine activity during and post-exercise (Cosio-Lima, Desai, Schuler, Keck, & Scheeler, 2011). Conversely, nutritional intake has shown promise in reducing markers of inflammation. Supplementation with carbohydrates and the antioxidant ascorbic acid (vitamin C) can possibly aid in attenuating cytokine activity after exercise (Christian P. Fischer et al., 2004; R. L. Starkie, Arkinstall, Koukoulas, Hawley, & Febbraio, 2001). There is little research however on the ability of ascorbic acid supplementation to attenuate cytokine activity after repeated exercise in a hot environment.

Much exercise induced cytokine literature is conducted over one or two exercise bouts, with IL-6 measures taken from blood samples. This leaves a lack of research investigating the activity of cytokines within skeletal muscle, where it can be produced, over multiple exercise sessions. Therefore, the focus of this investigation was the influence that exercise, hot environment, and carbohydrate and ascorbic acid supplementation have on IL-6 and TNF- α levels in active skeletal muscle.

Statement of the Problem

The nutritional properties of 100% orange juice (OJ) may provide health benefits to physically active individuals recovering from intense exercise. Compared to standard commercially available carbohydrate-electrolyte beverages (CEB), 100% OJ provides a similar carbohydrate profile, has less sodium, more potassium and other vitamin and minerals such as ascorbic acid, a known antioxidant. Few research studies have investigated the effects of 100% OJ on markers of inflammation following acute intense exercise in the heat or over consecutive exercise days.

Specific Aim and Hypotheses

The primary aim of this study is to determine if OJ supplementation can attenuate cytokine levels in human skeletal muscle following exercise in the heat.

Hypothesis1.1. Exercise in a hot humid environment would increase IL-6 and TNF- α levels in skeletal muscle.

Hypothesis1.2. Supplementation with 100% orange juice (OJ) (83.6 mg ascorbic acid /8 fl oz) would result in greater attenuation of increases in markers of inflammation in skeletal muscle compared to just drinking water (W) but would have similar results to supplementation with a carbohydrate-electrolyte beverage (CEB) following exercise (USDA, 2016).

Chapter II

Review of Literature

Introduction

IL-6 and TNF-α are cytokines that can be stimulated wherever inflammation or damage occurs in the body. It has also been established that vigorous exercise and can induce cytokine activity and inflammation (Brenner et al., 1999; Petersen & Pedersen, 2005). Overtime as the body adapts to training the rise in cytokines can be attenuated as regular exercise is known to decrease inflammation and protect the body against chronic inflammatory diseases (Petersen & Pedersen, 2005). Exercise bouts in environmental extremes, specifically high heat and humidity, have significant effects on cytokine activity (Cosio-Lima et al., 2011). There are a number of emerging interventions that show promise in combating inflammation following exercise in the heat. Supplementation with carbohydrates and the antioxidant ascorbic acid can aid in attenuating cytokine activity after exercise (Christian P. Fischer et al., 2004; R. L. Starkie et al., 2001). Therefore, the focus of this literature review is on the influence of exercise, hot environment, and carbohydrate and ascorbic acid supplementation on IL-6 and TNF- α levels in active skeletal muscle.

Tumor Necrosis Factor-α

TNF-α is one of the first cytokines produced in response to exercise induced injury or damage and takes part in the acute phase response. Various tissues in the body can produce this signaling protein, anywhere cell damage may occur. TNF-α can spearhead a cascade of cytokine activity that is part of the acute phase response to injury or infection and can induce the release of IL-6 (Nieman, 2000).

TNF-α and Exercise

TNF-α has a pro-inflammatory role in exercise and is linked with muscle catabolism (Goodman, 1991). TNF-α presence in contracting muscle varies in the literature. Compared to the IL-6 response to exercise, TNF-α does not increase nearly as much but it has been shown to be present in muscle (Petersen &

Pedersen, 2005). The degree of increase may depend on modality and intensity of exercise, with greater intensities yielding greater increases. Also of note, much research presents TNF-α mRNA measures, but does not include TNF-α protein measures from biopsies. Though gene expression does not directly reflect the production of this cytokine, it can still provide insights into its role in exercise.

TNF-α activity in muscle has been investigated after 40 trained male cyclists participated in a three-week double-blind study. Over three days of the study, they cycled for 3 hours each at 57% max effort. The cyclists were split up into two groups. The experimental group consumed the antioxidant quercetin for three weeks, and the control group consumed a placebo over the same time period. Blood samples and muscle biopsy samples were collected. Analysis of the plasma cytokines after the cycling sessions showed significant increases of cytokines, including TNF-α, in the control group. There was a less significant increase in the quercetin group. TNF-α mRNA expression in biopsy tissue was significantly increased after cycling in both supplement groups (Nieman et al., 2007). This study demonstrates that significant increases in TNF-α mRNA can occur following endurance exercise.

In an opposing study, six males performed two legged knee extensor exercises at 55% of their 1 RM for 180 minutes. Blood and muscle tissue samples were obtained pre exercise and at 30, 90, and 180 minutes during the exercise trial. There was no significant increase found in TNF-α mRNA expression from the blood or muscle tissue during the exercise (A. Steensberg et al., 2002). It was concluded that there was no significant TNF-α production in skeletal muscle during this exercise. This study admittedly did not analyze TNF-α protein and did not take post measures to see if the acute phase reaction took place after exercise, which according to the previous study cited, is when TNF-α expression occurred.

The time course of TNF-α release and production should be considered when studying the effects of exercise on cytokine production. For example immediately after running a marathon no changes were observed in TNF-α mRNA expression in muscle biopsies or blood mononuclear cells, but a significant

difference was measured 2 hours post-race in TNF-α plasma concentrations (Ostrowski, Rohde, Zacho, Asp, & Pedersen, 1998).

These studies further highlight the importance of post-exercise data collection points. TNF-α mRNA significantly increased in biopsies taken immediately post and 2, 12, and 24 hours after 30 min of running at 75% max O_2 uptake, peaking around the 12 hour mark with a 4.5-fold increase from baseline. After a bout of resistance exercise consisting of 3 sets of 10 knee extensions at 70% 1RM, TNF- α mRNA was significantly increased immediately, and at 2, 4, 8, and 24 hours post, peaking in the 8 hour biopsy with a 6.3-fold increase from baseline (Louis, Raue, Yang, Jemiolo, & Trappe, 2007).

It appears that when investigating TNF-α after exercise, measures should be monitored at least 2 hours post and even up to 12 hours post-exercise. The lack of extended post-exercise measurements in some literature could partly explain the variance in findings concerning TNF-α in muscle. Further research with extended time point post-exercise biopsies and actual intramuscular TNF-α protein measures will be needed to explore this cytokine further.

TNF-α and Exercise in Heat

Exercise in hot environments also appears to alter TNF-α concentration. In a crossover study, 6 elite male cyclists (VO_2 max =66±6 mL/kg/min) took part in two training rides at 60% max wattage and 75% VO_2 max for 2.5 hours. One ride at 15˚C and 40% humidity and the other at 35˚C and 40% humidity. The two rides were separated by a week. TNF-α concentration in blood plasma did not change significantly in the neutral condition ride (15˚C) or post-ride. No significant change in TNF-α was observed during the ride in hot (35˚C) conditions but levels did increase significantly post-ride and up to 12 hours post-ride in the hot (35˚C) condition (Cosio-Lima et al., 2011). Thus, it appears that exercise in the heat and humidity increases TNF-α production post-exercise and extends the time over which it remains significantly elevated. It is worth noting that the small sample size as well as the training status of these subjects were two variables that could have affected the release of cytokines in this particular study.

Interleukin-6

IL-6 is a cytokine which can be stimulated wherever inflammation or damage occurs in the body. IL-6 acts in either a pro or anti-inflammatory manner dependent on site of production. Its main pro-inflammatory function is to induce the acute phase response, which releases acute phase proteins into the blood stream that aid the immune system in recognizing foreign bodies or dying cells to prevent infection (C. P. Fischer, 2006). As an anti-inflammatory cytokine, IL-6 stimulates the release of IL-1ra, which inhibits the pro-inflammatory IL-1. In this way, IL-6 can regulate anti-inflammatory and pro-inflammatory pathways (Nieman, 2000). IL-6 is also known as a myokine, meaning it is a cytokine that is produced by and acts on skeletal muscle (Munoz-Canoves et al., 2013). It is has pleiotropic functions and is capable of playing a hormonal role in muscle metabolism as well as effecting muscle function (Plomgaard, Penkowa, & Pedersen, 2005).

IL-6 Production during Exercise

Levels of IL-6 increase within skeletal muscles during and after prolonged training bouts and competitions such as marathon racing (C. P. Fischer, 2006; Nieman et al., 2001). An increase in intermuscular IL-6 mRNA can also occur after as little as 30 minutes of exercise (A. Steensberg et al., 2002). These increases have been reported to be anywhere from 5 fold that of circulating plasma levels, up to extreme 100-fold increases following extended endurance runs (C. P. Fischer, 2006). As for IL-6 protein, immunohistochemical analysis after 3hr of cycling at 60% VO_2 max showed that IL-6 levels in muscle tissue was significantly elevated post exercise and remained significant for 6 hours post (Penkowa, Keller, Keller, Jauffred, & Pedersen, 2003).

Adaptations to training result in a decrease in the basal plasma levels of IL-6 but significant increases in circulating levels of this cytokine are still induced after a single training bout. Despite a basal decrease of IL-6 protein in response to training, muscular levels of IL-6mRNA increase. This increase possibly leading to a heightened sensitivity to and greater capability of IL-6 protein production if needed (C. P. Fischer,

2006). During exercise, skeletal muscle can be the primary producer of IL-6. A study investigating the effects of the immune response after exercise found that circulating IL-6 protein levels rose in response to 90 minute cycling bouts at 70% VO_2 max in both a heated and control condition. However, flow cytometry demonstrated that circulating monocytes were not the source of IL-6 production in either condition. This lead the researchers to conclude that IL-6 was being released primarily from contractile skeletal muscle (R. L. Starkie et al., 2005).

As for the time course of IL-6 release, it was observed that after a 30 minute run at 75% max O_2 uptake IL-6 mRNA from skeletal muscle was increased from baseline immediately post, and at 4, 8, 12, and 24 hours post, peaking between 4 and 12 hours with up to a 187-fold increase. After 3 sets of 10 knee extensions at 70% 1RM, mRNA was significantly elevated from 4-24 hours post-exercise with a peak 791-fold increase occurring at 4 hours post (Louis et al., 2007). Regardless, this study highlights that even though these increases may be extreme, IL-6 protein levels rise much more so than TNF-α after exercise. When measuring both IL-6 mRNA and IL-6 protein from muscle biopsies post exercise it was found that IL-6 mRNA peaked immediately post exercise and leveled off by the 6 hour post biopsy. As for IL-6 protein from the same study, levels increased significantly post exercise and remained significant 1.5, 3, and 6 hours post but had decreased to non-significance by 24 hours post exercise (Penkowa et al., 2003). Another point of interest is the value of taking biopsies at post-exercise time points in order to measure the peak values of cytokine reactions to exercise. This is important because it seems that maximal IL-6 levels are often observable immediately after exercise and rapidly decrease with a half-life of 1-2 hours (B. K. Pedersen, 2000).

The skeletal muscle production of IL-6 protein during exercise is actually separate from the traditional acute phase response and is independent of TNF-α stimulation (Munoz-Canoves et al., 2013). In fact in exercise it can play an anti-inflammatory role, as mentioned, by inhibiting TNF-α and stimulating the

release of other anti-inflammatory cytokines (Petersen & Pedersen, 2005). This possibly partially explains why non-significant changes or even dampened levels of TNF-α in skeletal muscle are seen in literature.

IL-6 and Exercise Modality

Exercise modality can determine the degree to which cytokines and the immune system become active during and following physical activity. When comparing maximal anaerobic exercise (5 minutes of cycling at 90% VO_2 max), circuit training (three sets of ten at 60-70% 1-RM for bicep curl, knee extension, hamstring curl, bench press, and leg press), 2 hours of cycling at 60-65% VO_2 max, and 5 hours of rest in a randomized block design, it was found that only the 2 hours of cycling produced significant changes in plasma IL-6 levels (Brenner et al., 1999). Furthermore, after high intensity resistance activities, IL-6 may not peak until 1-1.5 hours post-exercise whereas following prolonged endurance exercise IL-6 levels can reach peak values immediately post-exercise (B. K. Pedersen, 2000). Thus, timing of measurement and exercise modality should certainly be considerations when investigating IL-6 activity.

While prolonged endurance exercise elicits the greatest response of plasma and muscular IL-6, running in particular can exhibit the greatest response in comparison to cycling, swimming, or rowing (Nieman et al., 1998). One possible explanation could be the greater amount of muscle mass utilized and that other endurance sports have little to no eccentric component that would produce greater muscle damage. Duration of exercise, regardless of modality is one of the greatest factors in determining the IL-6 response to exercise, accounting for more than 50% of variance in observed levels of IL-6 (Fischer, 2006).

IL-6 and Exercise in Heat

Research regarding IL-6 response to exercise in heat contains discrepancies as to whether or not heated environmental conditions cause an increase in IL-6 levels. As skeletal muscle produces heat with contraction, and especially as excessive external temperatures increase heat stress during exercise, heat shock factors 1 and 2 (HSF1 and HSF2) can induce production of IL-6 (Pritts et al., 2002).

For example, cycling in heat (35˚C) does not significantly affect monocyte production of IL-6 but it can stimulate a 4 fold increase in plasma circulating IL-6 versus cycling in control (15˚C) suggesting an intramuscular release of the cytokine (R. L. Starkie et al., 2005). This was supported by a study in which 8 trained males cycled for 60 minutes in 20˚C (CON) and 35 ˚C (HOT) settings at 70% VO_2 max. Plasma IL-6 levels in the HOT trial were almost double those from the CON trial at 30 minutes of exercise, 60 minutes of exercise, and 30 minutes post exercise showing a significant effect for condition (Mundel, Jaime, & Jones, 2010)

In a crossover study, 6 elite male cyclists took part in 2 training rides at 60% max wattage and 75% VO_2 max for 2.5 hours. One ride took place in an environmental chamber at 15˚C, 40% humidity, the other at 35˚C, and 40% humidity. The two rides being separated by a week. IL-6 plasma levels were significantly increased post-ride in the neutral (15˚C) and hot (35˚C) conditions and remained significantly increased at 12 hours post-ride in the hot (35˚C) condition (Cosio-Lima et al., 2011). Although there was a significant main effect for time (P=0.003) there was not a significant main effect for conditions (P=0.481). Similar results were found in 7 endurance athletes after running at 90% velocity of anaerobic threshold for 60 minutes in both 18˚C/50% humidity and 28 ˚C/50% humidity. No differences in IL-6 plasmas levels were detected between the two tests, therefore environment did not influence IL6 production (Niess et al., 2003)

Furthermore, eleven recreationally trained males cycled for 1 hour in three separate conditions: hot (33 ˚C), cold (7˚C), and temperate (20˚C). Blood draws were taken pre-exercise, post-exercise, and 3 hours post-exercise to measure IL-6 plasma concentration. Again no significance was found across environmental conditions (p=0.207). Core temperatures were also taken during this study and there was no significant changes seen across conditions (E Dinan et al., 2017)

Further research is needed to elucidate whether or not environmental conditions are a consistent stimulus of IL-6 production. It should be noted that many of these studies contain small subject pools. All of the

above studies provide blood plasma data and there is a lack of research in which IL-6 in muscle tissue is examined during and after exercise in the heat.

IL-6 and Carbohydrate Supplementation

Substrate availability and in particular muscle glycogen stores affect IL-6 release. The relationship between glycogen levels and skeletal muscle IL-6 production is inverse. In other words, the more intramuscular glycogen depletion that occurs during exercise the greater the muscular production of IL-6 (Adam Steensberg et al., 2001). Furthermore, carbohydrate ingestion attenuates plasma levels of IL-6 post-exercise in both running and cycling when compared to no carbohydrate consumption (R. L. Starkie et al., 2001). In that study, intramuscular levels of IL-6 mRNA remained unchanged but due to carbohydrate ingestion, circulating plasma levels of IL-6 decreased.

This could be due to the proposed glucoregulatory function of IL-6. Cultured rat hepatocytes increased glucose release when exposed to IL-6 (Ritchie, 1990). IL-6 not only increases hepatic glucose release but can lead to the uptake of glucose via translocation of GLUT4 to cellular plasma membranes for increased insulin-stimulated glucose uptake (Munoz-Canoves et al., 2013). If blood glucose or muscle glycogen levels are not an issue after vigorous exercise, then IL-6 may not need to perform this function, providing a possible explanation for the decrease in plasma IL-6 in the Starkie et al. study mentioned previously.

This finding is confirmed in other exercise studies as well. During 2.5 hours of cycling or running subjects consumed either a 6% carbohydrate beverage every 15 minute or a placebo beverage. Blood samples were taken pre, immediately post, 1-hour post, 3 hours post, and 6 hours post-exercise. IL-6 levels in the blood plasmas were attenuated in the carbohydrate group in both the cycling and running modalities when compared to the placebo group (Nieman et al., 1998). Based on these results and the results of the above studies it would appear that supplementation with carbohydrates or adequate carbohydrate fueling helps to attenuate cytokine production and inflammation from exercise.

As mentioned, it could be that IL-6 has some glucoregulatory role in the body. IL-6 levels are higher when glycogen depletion occurs and attenuated when subjects are in a glycogen fed state. When glycogen levels become exhausted, AMP-activated protein kinase (AMPK) can trigger glucose uptake. The relationship between IL-6 and AMPK has been demonstrated but the mechanisms behind it have yet to be elucidated.

For example, increases in IL-6 correlate with increase in AMPK, which regulates cellular energy homeostasis. This correlation was made apparent when both IL-6 and AMPK levels rose similarly in subjects during a 60-min trial on a bicycle ergometer at 70% VO_2 max while in a glycogen depleted state. Measurements were taken via muscle biopsies performed pre-exercise, and at the 10 and 60 minute mark of exercise. Blood draws occurred at 10, 20, 30, 45 and 60 minutes of exercise. When this bicycle ergometer protocol was repeated a second time with subjects in a glycogen loaded state both IL-6 and AMPK increases were attenuated (MacDonald, Wojtaszewski, Pedersen, Kiens, & Richter, 2003).

This relationship has been further examined in rat skeletal muscle. Both in vitro experiments with *extensor digitorum* longus and in vivo experiments with rat gastrocnemius, significantly increased α2AMPK activity after incubation in 120ng/ml of IL-6 for 15 minutes and injection with 25ng/g of IL-6 for 60min respectively (Kelly, Gauthier, Saha, & Ruderman, 2009). Thus, they observed that when IL-6 was introduced to the muscle there was an increase in AMPK activity. The same researchers also conducted a study in which IL-6 gene knockout mice and control mice were split evenly so that half of each group remained sedentary and the other half performed a 60-minute swim. Analysis of the *gastrocnemius* found that the level of phosphorylated AMPK was significantly blunted in the knockout group after the exercise when compared to the control group (Kelly et al., 2004). It appears that during or after exercise when glycogen is needed, IL-6 could activate AMPK that would in turn promote glucose uptake by active skeletal muscle.

These findings suggest that IL-6 can play an anti-inflammatory glucoregulatory role. Furthermore, it implies that carbohydrate fueling status has an effect on exercise-induced inflammation. Proper

carbohydrate supplementation prior to and during exercise could act as a safe guard against fuel depletion via muscle glycogen regulation and further preventing inflammation and muscle damage.

IL-6 and Ascorbic Acid Supplementation

Antioxidants, specifically ascorbic acid, may blunt the release of IL-6 post-exercise. However, the results from research investigating IL-6 activity after ascorbic acid supplementation have provided mixed conclusions.

Ascorbic acid supplementation over 4 weeks resulted in no significant change in IL-6 protein or IL-6 mRNA expression in muscle biopsy but did inhibit the translocation of IL-6 from skeletal muscle into circulation and reduced plasma IL-6 by 50% (Christian P. Fischer et al., 2004).

Another study split ultra-marathoners into an experimental ascorbic acid supplementation group and a control group. The experimental group supplemented with ascorbic acid for a week prior to a 48-80km race and received a carbohydrate-ascorbic acid drink during the race. The control group did not receive anything throughout the study. After analysis, there was no significant correlation between post-race plasma ascorbic acid and immune measures including IL-6 (Nieman et al., 2002). It should be noted that the carbohydrates in the drink mix might have effected IL-6 production as well.

Similar findings came from a 4-month supplementation study that included 3 months of cycle training 5 days a week. Twenty-one healthy males were split into a vitamin supplementation group (n=11) and a placebo group (n=10). The vitamin group ingested 500mg ascorbic acid and 400 IU vitamin E tablets daily for 16 weeks. After one month of supplementation, the cycle training commenced and lasted the final 12 weeks of supplementation. One-hour cycle ergometer tests were performed at 65% max power pre and post-training. No significant differences were found in IL-6 plasma or muscle IL-6 mRNA levels between groups (Yfanti et al., 2012). After 4 months of supplementation, ascorbic acid did not have an effect on exercise-induced increases in IL-6 levels in blood or muscle.

Contrary to the previous studies, the plasma IL-6 response to 45 minutes of cycling at 70% VO_2 max was significantly blunted after 15 days of 1000mg ascorbic acid supplementation. Six healthy untrained males preformed two 42 minute cycling tests pre and post 15 days of vitamin supplementation (Vassilakopoulos et al., 2003). Training status could have played a role in these findings when compared to the literature mentioned above. This shows though that as an antioxidant ascorbic acid may be able to reduce inflammation in untrained individuals after exercise.

With multiple findings, more research will have to be conducted to determine the interaction between ascorbic acid and IL-6. Factors such as training status, timing of supplementation and dosage may alter whether or not ascorbic acid is beneficial for reducing exercise-induced inflammation.

The research reviewed here administered daily doses of 500-1000mg whereas 8 oz of 100% orange juice contains 83.6 mg of ascorbic acid (USDA, 2016). The recommended daily allowance of ascorbic acid for persons 19 and older is 90mg for males and 75mg for females (Medicine, 2000). Ingestion of 30–180 mg/day of ascorbic acid results in an absorption rate of 70%–90%. However, this rate falls to less than 50% absorption when the dosages are increased above 1 g/day (Jacob & Sotoudeh, 2002). When comparing a high dosage supplementation regime with normal ascorbic acid-rich food consumption it was observed that oral ingestion of 1.25 g/day ascorbic acid results in plasma ascorbic acid concentrations that were nearly two times higher than those produced by consuming 200–300 mg/day ascorbic acid from ascorbic acid-rich foods (Padayatty, Sun, Wang, & et al., 2004).

Chapter III

Methods

Participants

Fourteen male and four female subjects (male: age= 22 ± 3 yrs., weight= 74.16 ± 8.72 kg, height= 175.74 ± 6.76 cm; female: age= 23 ± 5 yrs., weight= 65.95 ± 13 kg, height= 169.28 ± 9.975 cm) volunteered to participate in this study. Participants were moderately endurance trained (running 3 days/week minimum) and recruited from The University of Kansas and surrounding community. Inclusion criteria was defined as having good general health, and accepted based upon the absence of cardiovascular, respiratory, and metabolic disorders; musculoskeletal disorders preventing exercise; fluid or electrolyte balance disorders; and GI or swallowing disorders. Female participants were scheduled according to menstruation to limit variances in hormone, body temperature, and the inflammatory process. It was also required that they have a VO_2max equal to or greater than 40 ml · kg-1 · min-1 for males and equal to or greater than 38 ml · kg-1 · min-1 for females (male: VO_2max= 49.99 ± 7.02; female VO_2max= 44.6 ± 7.45). This study was submitted for approval and accepted by the University Institutional Review Board for the protection of human subjects, and all participants were required to complete a health history questionnaire and sign an informed consent document.

Research Design

A randomized controlled, single blind design was utilized to determine the effects of 100% orange juice on the following dependent variables: IL-6, and TNF-α. This took place over 11 days of daily beverage supplementation and 5 consecutive days of repeated cycle ergometer bouts. Independent variables were OJ (237ml [8 oz] of 100% OJ/day), CEB (237ml of CEB/day), and W (237ml of W/day). OJ contained 83.6

mg of ascorbic acid /8 fl oz (USDA, 2016) (Figure 1). All experimental trials took place in a mild thermal environment (30°C, 50% humidity).

Figure 1

Nutritional Information

Carbohydrate Electrlyte Beverage			Orange juice		
Nutrient	Unit	8 fl oz = 248.8g	Nutrient	Unit	8 fl oz = 248.8g
Total lipid (fat)	g	0	Total lipid (fat)	g	0.3
Carbohydrates	g	14	Carbohydrates	g	28.71
Sugars, total	g	14	Sugars, total	g	20.68
Protein	g	0	Protein	g	1.69
Minerals			Minerals		
Sodium, Na	mg	100	Sodium, Na	mg	5
Potassium, K	mg	24	Potassium, K	mg	443
Vitamins			Vitamins		
Niacin	mg	4.5	Niacin	mg	0.697
Vitamin B-6	mg	0.375	Vitamin B-6	mg	0.189
Vitamin B-12	µg	0.624	Vitamin B-12	µg	0
Vitamin C, total ascorbic acid	mg	0	Vitamin C, total as	mg	83.6

(Co., 2018; USDA, 2016)

Study Design

Participants reported Day 1 for baseline testing (muscle biopsy). Participants were provided take home instructions and, 3 bottles containing 237 ml each of assigned beverage to consume on Days 1 – 3. Prior to arriving for each exercise day (Days 4 - 8), participants were instructed to consume a small meal and report euhydrated. Euhydration was defined as a plasma osmolality (Posm) < 290 mOsmols or urine specific gravity (Usg) < 1.020.6. Upon completing post-exercise measures, participants consumed 237 ml of assigned beverage and rested for 1 hr in a thermoneutral environment. Prior to leaving the laboratory on Day 8, participants were given Take Home Instructions and 3 bottles containing 237 ml each of assigned beverage to consume on Days 9 - 11. Final muscle biopsies were taken Day 11 (Figure 2).

Figure 2

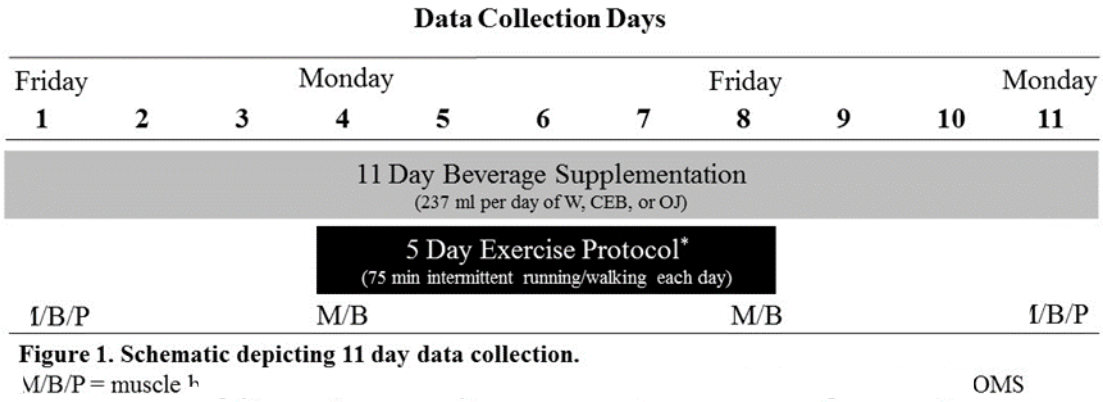


Figure 1. Schematic depicting 11 day data collection.

VO_2max Protocol

One week prior to the trial, subjects performed a graded exercise test to determine aerobic capacity or peak oxygen uptake (VO_{2max}). A one-way, non-rebreathing respiratory valve and a nose clip were utilized to collect expired oxygen and carbon dioxide. A metabolic cart (True One 2400, Parvo Medics, Sandy, UT) was used to determine respiratory exchange ratio and oxygen consumption (VO_2) values every 30 seconds while cycling (Excalibur 91900 V2.23, Lode B.V., Groningen, Netherlands). VO_{2max} was determined when subjects attained a respiratory exchange ratio value > 1.10 and/or a VO_2 increase from the previous exercise intensity of < 0.2 l/min.

5 Day Exercise Protocol

The exercise protocol was designed to induce muscle glycogen depletion and mild hypo-hydration to determine beverage effect on inflammation. On Days 4 - 8, participants performed 80 min of cycling exercise (Monark 818 E, Monark Exercise AB, Vonsbro, Sweden). The exercise consisted of a 2.5 minute

warmup and then four 15 min periods of intermittent cycling at 70% of max heart rate. In between each 15 minute interval was a 5 minute interval during which subjects could cycle at a self-paced effort. Following the last 15 minute interval was a 2.5 minute cool down. The exercise protocol was conducted in an environmental chamber set at a mild, thermal temperature (30°C, 50% humidity). PArticpants were given 1.5mk/kg body mass to drink during each of the 15 minute intervals.

Measures of Muscle Inflammation

Beverage effects on muscle inflammation was characterized using cytokines (TNF-α and IL-6). Skeletal muscle biopsies are taken Days 1, 4, 8, and 11 from the lateral aspect of the *vastus lateralis* by percutaneous needle biopsy (Bergstrom, 1962). Muscle samples were taken prior to exercise on the exercise protocol days (days 4 and 8). Samples were immediately placed in a -20°C freezer for further analysis.

Muscle Homogenization and Protein Quantification

Proteins were extracted with a cocktail of cell extraction buffer protease inhibitor (Halt Protease Inhibitor; Thermo Scientific, Waltham, MA), phosphatase inhibitor (Halt Phosphatase Inhibitor; Thermo Scientific, Waltham, MA), and phenylmethylsulfonyl fluoride (PMSF) protease inhibitor (Nunc; Nalgene International, Rochester, NY). The muscle samples were placed in the solution, ground using a mortar and pestle, and then vortexed shortly every 10 minutes for 30 minutes then centrifuged at 3000rpm for 15 minutes. Supernatant was removed and stored at -20°C until samples were measured for protein content with a BCA protein assay kit (Pierce BCA Protein Assay Kit; Pierce, Rockford, IL). (Aaron Graham, 2010)

SDS-PAGE

Laemlli sample buffer was prepared and mixed 2:1 with dithiothreitol. Muscle homogenate protein (60 μg) was mixed with sample buffer and were vortexed, boiled for 5 min, vortexed again and then loaded into poly-acrylamide gels. Gels were run at a constant 250 V, 0.04 A, and in 1 × running buffer for 60–70 min

Western Blotting

Proteins were transferred onto a PVDF membrane by wet transfer for 3 hours. The PVDF membranes were blocked for an hour using 5% milk blotto. Primary antibodies were diluted 1:10,000 in 1% milk blotto (Cell Signaling, Danvers, MA, USA). The membranes incubated overnight at 4 °C with the primary antibody in a 1% milk blotto. The following day membranes were washed in TBS-T, then membranes were incubated with a secondary antibody and anti-biotin in a 1% milk TBS-T solution (Cell Signaling, Danvers, MA, USA) at a 1:1000 dilution for an hour. After rinsing membranes in TBS-T, they were incubated in a horseradish peroxidase chemilumenescent reagent for 5 minutes (Amersham, Piscataway, NJ, USA). Membranes were then imaged with a digital imaging system (Fluorchem HD2, ProteinSimple; San Jose, CA, USA or Amersham Imager 600, Amersham). Densitometry software (ImageQuant TL v8.1; Amersham) was used to quantify pixel brightness.

Statistical Analysis

A repeated measures analysis of variance (ANOVA) test was conducted to examin the main effects for time and markers of inflammation, as well as group interactions. A 3 x 4 group x time ANOVA was used. The independent variable, the beverage group, was made up of 3 levels: OJ, W, and CEB. The dependent variables were intramuscular cytokines measures and time. Then a one way ANOVA was conducted for each beverage group to determine any significant differences between each day within a group. Significance was based on an alpha level of 0.05.

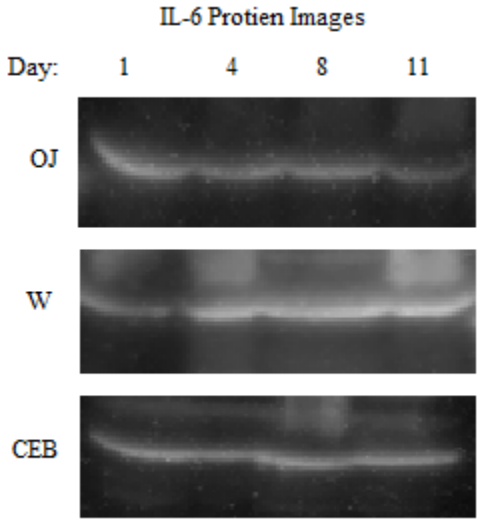
Chapter IV

Results

IL-6 Measures

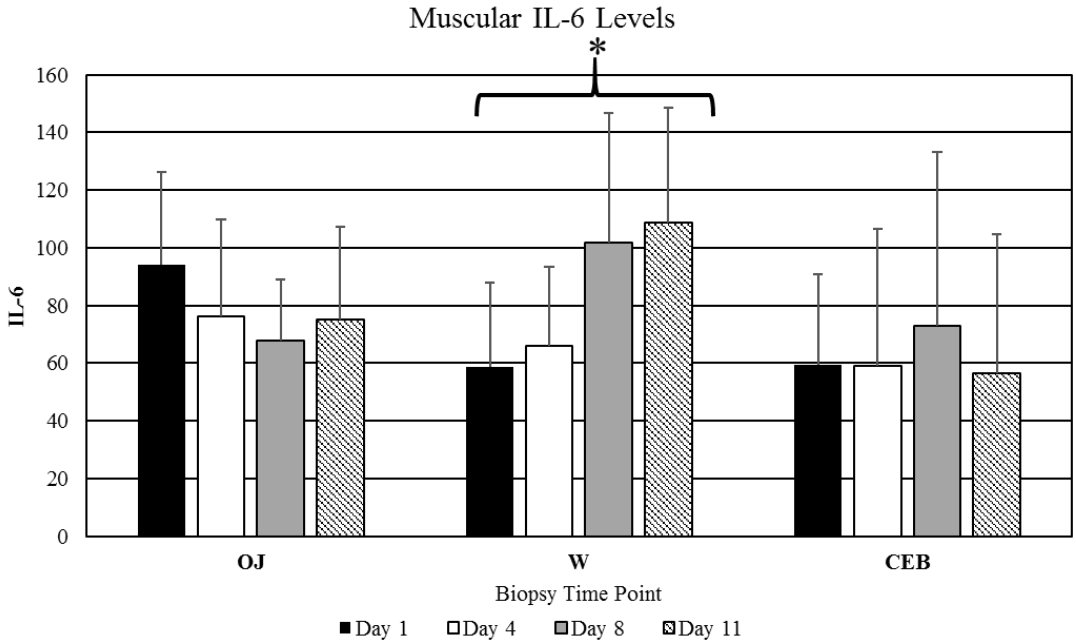
A repeated measures ANOVA was used to examine the differences in IL-6 within skeletal muscle between three groups and across four time points: OJ, W, and CEB groups, as well as time points Day 1, 4, 8, and 11 during the exercise protocol (Figure 3).

Figure 3



The was no main effect for time or group for IL-6 in skeletal muscle. However, there was a group x time interaction (p=0.018), where the IL-6 levels of the W group increased linearly from day 1 to day 11 while the OJ, and CEB groups remained unchanged (Figure 4). Post-hoc independent T-tests found a significant difference between the OJ and W group on Day 8 (p=0.028).

Figure 4

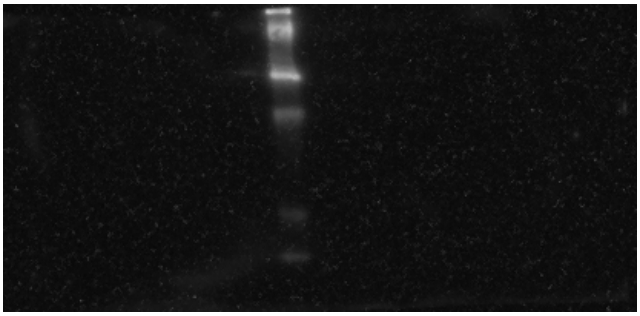


TNF- α Measures

TNF-α skeletal muscle levels were below the detection level of the imaging system and were not analyzed statistically (Figure 5).

Figure 5

TNF- α Protein Images



Chapter V

Discussion

The aim of the present study was to determine if OJ supplementation attenuates IL-6 or TNF-α levels in human skeletal muscle following repeated exercise bouts in the heat. We proposed two hypotheses; first, that exercise in a hot humid environment would increase cytokine levels in skeletal muscle and second, that supplementation with 100% OJ or a carbohydrate-electrolyte beverage following exercise would attenuate increases in cytokines in skeletal muscle in comparison to the control, water. We saw no significant difference between time points suggesting that the first hypothesis is not supported by these results. However, a significant group x time interaction was seen, supporting our second hypothesis. This interaction showed that IL-6 protein levels from individuals in the W increased linearly over the course of the study (Days1-11). Further, a significant difference was seen between OJ and W group Day 8 IL-6 measures. Meanwhile the OJ and CEB groups saw no such significant differences in IL-6 levels overtime and remained relatively the same. This suggests that consumption of supplemental beverages like OJ or CEB may attenuate increases in intramuscular IL-6.

This study also investigated TNF-α but we observed no measurable levels of TNF-α in the muscle samples of any of the three groups. These data are in agreement with previous studies showing that TNF-α is not necessary to stimulate the release of IL-6 during exercise and is often not detectable after exercise or not significantly produced or released from skeletal muscle (A. Steensberg et al., 2002). As mentioned previously, skeletal muscle production of IL-6 protein during exercise is separate from the traditional acute phase response and is independent of TNF-α stimulation (Munoz-Canoves et al., 2013). In fact, exercise and IL-6 injection are known to inhibit endo-toxin induced increases of TNF-α (R. Starkie, Ostrowski, Jauffred, Febbraio, & Pedersen, 2003).

Ascorbic acid has been suggested to attenuate IL-6 levels (Vassilakopoulos et al., 2003). However, we saw no difference between the OJ and CEB groups which is not surprising considering the mixed results of research investigating ascorbic acid supplementation. While Fischer et al. (Christian P. Fischer et al., 2004) found no significant changes in intramuscular IL-6 after supplementing with ascorbic acid, Vassilakopoulos et al. (2003) saw significant attenuation of cytokines post exercise. The latter study used high 1000mg/day dosages of ascorbic acid though, much higher the daily-recommended amount of 75-90mg and the 83.6mg present in the OJ consumed daily in the present study (Medicine, 2000). Supplementation with pills is also known to produce higher plasma levels of ascorbic acid than just a diet of high ascorbic acid foods (Padayatty et al., 2004). Therefore, the amount of ascorbic acid ingested daily in this study was not enough to boost antioxidant levels to amounts that would lead to significant attenuation of cytokine release.

The non-significant increases in cytokines post-exercise conflicts with findings from studies with similar exercise and environmental protocols (Brenner et al., 1999; Mundel et al., 2010). The non-significant difference for time or group could be attributed to the time points at which muscle biopsies were collected. Biopsies were taken from subjects pre-exercise on days 1, 4, 8, and 11. Days 4 through 8 were exercise days. Maximal IL-6 levels are often observable immediately after exercise and rapidly decrease with a half-life of 1-2 hours (B. K. Pedersen, 2000). Ostrowski et al. (1998) reported a decrease in IL-6 proteins from their peak measurement immediately post. After 3 hours of cycling at 60% VO_{2max} IL-6 protein in muscle biopsies significantly increased, peaking at 6 hours post, but decreasing below significance by 24 hours post exercise (Ostrowski et al., 1998). The measures taken in the current study occurred nearly 24 hours post exercise, which would be near a time at which IL-6 levels would be returning to resting values.

The significant interaction found for the W group and differences between the W and OJ grpups on Day 8 could be due to the carbohydrates present in the OJ and CEB beverages that are not present in W (Figure 5). Though it is widely accepted that skeletal muscle is capable of producing IL-6 during exercise it is

possible that the “leaky gut” theory contributes to inflammation during exercise. If so, the consumption of carbohydrates could play a role in decreasing the level of inflammation and cytokine production that result from vigorous exercise.

A more likely mechanism for the attenuation of the IL-6 in the OJ and CEB groups compared to W is the addition of carbohydrates. IL-6 is thought to help regulate glucose metabolism during vigorous exercise when glycogen stores begin to be depleted (Helge et al., 2003). The presence of IL-6 in cultured rat hepatocytes induces glucose release (Ritchie, 1990). After release into the bloodstream, this glucose could then be taken up by active muscle for substrate utilization. It has been observed that blood glucose and IL-6 measures have an inverse relationship (Adam Steensberg et al., 2001) . Carbohydrate ingestion attenuates plasma levels of IL-6 post-exercise compared to no carbohydrate consumption, but intramuscular IL-6 mRNA levels were not significantly affected by carbohydrate supplementation (R. L. Starkie et al., 2001; Adam Steensberg et al., 2001). The present study did not investigate gene expression but IL-6 protein content showed a greater increase in the W group compared to OJ and CEB. It could be possible that carbohydrate ingestion affects IL-6 mRNA translation or release from active skeletal muscle. As it relates to the present study, that would suggest that the ingestion of beverages that contain adequate carbohydrates like OJ and CEB help replenish glycogen stores thus dampening the increase in cytokine levels.

Limitations of this study would include the study design, which did not allow for post-exercise muscle biopsies that would have led to more insight into the effect that supplementation with these beverages had on recovery and cytokine production after cycling in the heat. Other limitations would be the measures taken. Perhaps measuring mRNA values or investigating cytokines that appear more readily would have provided more data. In order to test the hypotheses of this study further, future research is needed. Ideally using repeated bouts of exercise or a training study in which skeletal muscle biopsies are taken pre and post exercise would provide more data on IL-6 and TNF-α. Possibly cytokine mRNA and protein measures can be compared in order to gain a better sense of the viability of protein measures.

In conclusion, there was no main effect for time or group concerning intramuscular IL-6 after repeated bouts of aerobic exercise. TNF-α production was not induced in measurable amounts by the exercise stimulus in this study. The ascorbic acid content of OJ (83.6mg/8oz) alone may not be enough to negate the inflammatory effects of exercise in such a short time period (USDA, 2016). However, there was significant group x time effect in which IL-6 increased over time in the W group but did not increase in the OJ or CEB groups and a significant difference between the OJ and W groups on Day 8. This data suggests that the carbohydrate content of OJ and CEB could attenuate IL-6 increases when compared to a control (W). Replenishment of fuel has long been a standard practice post-exercise and its value is understood but another added benefit may be the reduction of inflammation.

References

Aaron Graham, Z. (2010). *Integrin Signaling at 2hr and 48hr Post-Eccentric Exercise in Heat Treated Rat Skeletal Muscle*.

Bergstrom, J. (1962). *Muscle electrolytes in man. Determined by neutron activation analysis on needle biopsy specimens* (Vol. Vol: 14: Suppl. 68).

Brenner, I. K., Natale, V. M., Vasiliou, P., Moldoveanu, A. I., Shek, P. N., & Shephard, R. J. (1999). Impact of three different types of exercise on components of the inflammatory response. *Eur J Appl Physiol Occup Physiol, 80*(5), 452-460. doi:10.1007/s004210050617

Co., C.-C. (2018). Coca-Cola Proct Facts, Orange Powerade. Retrieved from https://www.coca-colaproductfacts.com/en/products/powerade/orange/20-oz/

Cosio-Lima, L. M., Desai, B. V., Schuler, P. B., Keck, L., & Scheeler, L. (2011). A comparison of cytokine responses during prolonged cycling in normal and hot environmental conditions. *Open Access J Sports Med, 2*, 7-11. doi:10.2147/oajsm.s15980

E Dinan, N., B Zak, R., Shute, R., Laursen, T., Bubak, M., La Salle, D. T., . . . Slivka, D. (2017). *Exercise-Induced Interleukin-6 and Metabolic Responses in Hot, Temperate, and Cold Conditions* (Vol. 13).

Fischer, C. P. (2006). Interleukin-6 in acute exercise and training: what is the biological relevance? *Exerc immunol rev, 12*, 6-33.

Fischer, C. P., Hiscock, N. J., Penkowa, M., Basu, S., Vessby, B., Kallner, A., . . . Pedersen, B. K. (2004). Supplementation with vitamins C and E inhibits the release of interleukin-6 from contracting human skeletal muscle. *J Physiol, 558*(Pt 2), 633-645. doi:10.1113/jphysiol.2004.066779

Goodman, M. N. (1991). Tumor necrosis factor induces skeletal muscle protein breakdown in rats. *Am J Physiol, 260*(5 Pt 1), E727-730.

Helge, J. W., Stallknecht, B., Pedersen, B. K., Galbo, H., Kiens, B., & Richter, E. A. (2003). The effect of graded exercise on IL-6 release and glucose uptake in human skeletal muscle. *J Physiol, 546*(Pt 1), 299-305. doi:10.1113/jphysiol.2002.030437

Jacob, R. A., & Sotoudeh, G. (2002). Vitamin C function and status in chronic disease. *Nutr Clin Care, 5*(2), 66-74.

Kelly, M., Gauthier, M.-S., Saha, A. K., & Ruderman, N. B. (2009). Activation of AMP-Activated Protein Kinase by Interleukin-6 in Rat Skeletal Muscle. *Association With Changes in cAMP, Energy State, and Endogenous Fuel Mobilization, 58*(9), 1953-1960. doi:10.2337/db08-1293

Kelly, M., Keller, C., R Avilucea, P., Keller, P., Luo, Z., Xiang, X., . . . B Ruderman, N. (2004). *AMPK activity is diminished in tissues of IL-6 knockout mice: the effect of exercise* (Vol. 320).

Louis, E., Raue, U., Yang, Y., Jemiolo, B., & Trappe, S. (2007). Time course of proteolytic, cytokine, and myostatin gene expression after acute exercise in human skeletal muscle. *J Appl Physiol (1985), 103*(5), 1744-1751. doi:10.1152/japplphysiol.00679.2007

MacDonald, C., Wojtaszewski, J. F., Pedersen, B. K., Kiens, B., & Richter, E. A. (2003). Interleukin-6 release from human skeletal muscle during exercise: relation to AMPK activity. *J Appl Physiol (1985), 95*(6), 2273-2277. doi:10.1152/japplphysiol.00242.2003

Mach, N., & Fuster-Botella, D. (2017). Endurance exercise and gut microbiota: A review. *Journal of Sport and Health Science, 6*(2), 179-197. doi:https://doi.org/10.1016/j.jshs.2016.05.001

Medicine, I. o. (2000). *Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids*. Washington, DC: The National Academies Press.

Mundel, T., Jaime, P. C., & Jones, D. (2010). *Exercise, Heat Stress and the Interleukin-6 Response: Support for Temperature-Mediated Neuroendocrine Regulatory Mechanisms* (Vol. 14).

Munoz-Canoves, P., Scheele, C., Pedersen, B. K., & Serrano, A. L. (2013). Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword? *Febs j, 280*(17), 4131-4148. doi:10.1111/febs.12338

Nieman, D. C. (2000). Special feature for the Olympics: effects of exercise on the immune system: exercise effects on systemic immunity. *Immunol Cell Biol, 78*(5), 496-501. doi:10.1111/j.1440-1711.2000.t01-5-.x

Nieman, D. C., Henson, D. A., Davis, J. M., Angela Murphy, E., Jenkins, D. P., Gross, S. J., . . . Mayer, E. P. (2007). Quercetin's influence on exercise-induced changes in plasma cytokines and muscle and leukocyte cytokine mRNA. *J Appl Physiol (1985), 103*(5), 1728-1735. doi:10.1152/japplphysiol.00707.2007

Nieman, D. C., Henson, D. A., McAnulty, S. R., McAnulty, L., Swick, N. S., Utter, A. C., . . . Morrow, J. D. (2002). Influence of vitamin C supplementation on oxidative and immune changes after an ultramarathon. *J Appl Physiol (1985), 92*(5), 1970-1977. doi:10.1152/japplphysiol.00961.2001

Nieman, D. C., Henson, D. A., Smith, L. L., Utter, A. C., Vinci, D. M., Davis, J. M., . . . Shute, M. (2001). Cytokine changes after a marathon race. *J Appl Physiol (1985), 91*(1), 109-114.

Nieman, D. C., Nehlsen-Cannarella, S. L., Fagoaga, O. R., Henson, D. A., Utter, A., Davis, J. M., . . . Butterworth, D. E. (1998). Influence of mode and carbohydrate on the cytokine response to heavy exertion. *Med Sci Sports Exerc, 30*(5), 671-678.

Niess, A. M., Fehrenbach, E., Lehmann, R., Opavsky, L., Jesse, M., Northoff, H., & Dickhuth, H.-H. (2003). Impact of elevated ambient temperatures on the acute immune response to intensive endurance exercise. *European Journal of Applied Physiology, 89*(3), 344-351. doi:10.1007/s00421-003-0809-3

Ostrowski, K., Rohde, T., Zacho, M., Asp, S., & Pedersen, B. K. (1998). Evidence that interleukin-6 is produced in human skeletal muscle during prolonged running. *J Physiol, 508*(Pt 3), 949-953. doi:10.1111/j.1469-7793.1998.949bp.x

Padayatty, S. J., Sun, H., Wang, Y., & et al. (2004). Vitamin c pharmacokinetics: Implications for oral and intravenous use. *Annals of Internal Medicine, 140*(7), 533-537. doi:10.7326/0003-4819-140-7-200404060-00010

Pedersen, B. K. (2000). Special feature for the Olympics: effects of exercise on the immune system: exercise and cytokines. *Immunol Cell Biol, 78*(5), 532-535. doi:10.1111/j.1440-1711.2000.t01-11-.x

Pedersen, B. K., & Febbraio, M. A. (2008). Muscle as an Endocrine Organ: Focus on Muscle-Derived Interleukin-6. *Physiological Reviews, 88*(4), 1379-1406. doi:10.1152/physrev.90100.2007

Penkowa, M., Keller, C., Keller, P., Jauffred, S., & Pedersen, B. K. (2003). Immunohistochemical detection of interleukin-6 in human skeletal muscle fibers following exercise. *Faseb j, 17*(14), 2166-2168. doi:10.1096/fj.03-0311fje

Petersen, A. M., & Pedersen, B. K. (2005). The anti-inflammatory effect of exercise. *J Appl Physiol (1985), 98*(4), 1154-1162. doi:10.1152/japplphysiol.00164.2004

Plomgaard, P., Penkowa, M., & Pedersen, B. K. (2005). Fiber type specific expression of TNF-alpha, IL-6 and IL-18 in human skeletal muscles. *Exerc immunol rev, 11*, 53-63.

Pritts, T. A., Hungness, E. S., Hershko, D. D., Robb, B. W., Sun, X., Luo, G. J., . . . Hasselgren, P. O. (2002). Proteasome inhibitors induce heat shock response and increase IL-6 expression in human intestinal epithelial cells. *Am J Physiol Regul Integr Comp Physiol, 282*(4), R1016-1026. doi:10.1152/ajpregu.00492.2001

Ritchie, D. G. (1990). Interleukin 6 stimulates hepatic glucose release from prelabeled glycogen pools. *Am J Physiol, 258*(1 Pt 1), E57-64.

Starkie, R., Ostrowski, S. R., Jauffred, S., Febbraio, M., & Pedersen, B. K. (2003). Exercise and IL-6 infusion inhibit endotoxin-induced TNF-α production in humans. *The FASEB Journal, 17*(8), 884-886. doi:10.1096/fj.02-0670fje

Starkie, R. L., Arkinstall, M. J., Koukoulas, I., Hawley, J. A., & Febbraio, M. A. (2001). Carbohydrate ingestion attenuates the increase in plasma interleukin-6, but not skeletal muscle interleukin-6 mRNA, during exercise in humans. *J Physiol, 533*(Pt 2), 585-591.

Starkie, R. L., Hargreaves, M., Rolland, J., & Febbraio, M. A. (2005). Heat stress, cytokines, and the immune response to exercise. *Brain Behav Immun, 19*(5), 404-412. doi:10.1016/j.bbi.2005.03.005

Steensberg, A., Febbraio, M. A., Osada, T., Schjerling, P., van Hall, G., Saltin, B., & Pedersen, B. K. (2001). Interleukin-6 production in contracting human skeletal muscle is influenced by pre-exercise muscle glycogen content. *J Physiol, 537*(Pt 2), 633-639. doi:10.1111/j.1469-7793.2001.00633.x

Steensberg, A., Keller, C., Starkie, R. L., Osada, T., Febbraio, M. A., & Pedersen, B. K. (2002). IL-6 and TNF-alpha expression in, and release from, contracting human skeletal muscle. *Am J Physiol Endocrinol Metab, 283*(6), E1272-1278. doi:10.1152/ajpendo.00255.2002

USDA. (2016). National Nutrient Database for Standard Reference Release 28. Retrieved 1/9/2017 https://ndb.nal.usda.gov/ndb/foods

Vassilakopoulos, T., Karatza, M.-H., Katsaounou, P., Kollintza, A., Zakynthinos, S., & Roussos, C. (2003). Antioxidants attenuate the plasma cytokine response to exercise in humans. *Journal of Applied Physiology, 94*(3), 1025-1032. doi:10.1152/japplphysiol.00735.2002

Yfanti, C., Fischer, C. P., Nielsen, S., Akerstrom, T., Nielsen, A. R., Veskoukis, A. S., . . . Pedersen, B. K. (2012). Role of vitamin C and E supplementation on IL-6 in response to training. *J Appl Physiol (1985), 112*(6), 990-1000. doi:10.1152/japplphysiol.01027.2010

Appendices

Appendix A: Informed Consent

Appendix B: Health History Questionnaire

Appendix A: Informed Consent

INFORMED CONSENT TO PARTICIPATE IN RESEARCH STUDY

Examination of 100% Orange Juice on Rehydration and Recovery following Repeated Endurance Exercise

INTRODUCTION

The Department of Health, Sport, and Exercise Sciences at the University of Kansas supports the practice of protection for human subjects participating in research. The following information is provided for you to decide whether you wish to participate in the present study. You may refuse to sign this form and not participate in this study. You should be aware that even if you agree to participate, you are free to withdraw at any time. If you do withdraw from this study, it will not affect your relationship with this unit, the services it may provide to you, or the University of Kansas.

STUDY PURPOSE

The purpose of this study is to determine how 3 different fluids effect hydration status, muscle recovery, and subjective measures in exercising persons. This study is funded by the Florida Department of Citrus.

PROCEDURES

You are being invited to participate in this study because you are between the ages of 18 and 35 years old, complete endurance exercise at least 3 days a week, and are in good general health. If you agree to participate, you will be participating with approximately 30 other people.

Study Participation

After signing the informed consent form, you will complete a Health and Injury History Questionnaire to determine inclusion or exclusion into the study. You cannot participate in this study if you have cardiovascular, respiratory, metabolic, or bleeding disorders; muscle disorders preventing exercise; fluid or electrolyte disorders; and/or gastrointestinal or swallowing disorders. We will take a hydrated weight to use as a baseline throughout the data collection. Before inclusion into the study you will complete a $\dot{V}O_2max$ tests to measure your endurance capacity. If included in the study, you will be randomly assigned to complete 1 experimental trial (water, carbohydrate electrolyte beverage, or orange juice). You will be unaware of which trial you are completing. The trial will take 11 days. You will be given a beverage to drink for each of the 11 days. On 5 days you will exercise for 75 minutes and rest for 1 hour after exercise. Total time to complete each exercise day is approximately 3 hours. On 4 days you will have blood and a muscle biopsy taken. While participating in the study, we ask that you maintain your normal levels of physical activity, avoid changing your caffeine consumption, and avoid drinking alcohol. All exercise sessions will be scheduled in the morning.

Experimental Trials

All beverages will be given in bottles that are covered so you cannot see what is inside. On non-exercise days (Days 1-3 and 9-11) you will consume one bottle in the morning. On exercise days (Days 4-8) you will consume the beverage after exercising.

Water (W). Each bottle will contain 237 ml (approximately 8 oz or 1 cup) of orange flavored

Carbohydrate-electrolyte beverage (CEB). Each bottle will contain 237 ml (approximately 8 oz or 1 cup) of a commonly available CEB.

Orange Juice (OJ). Each bottle will contain 237 ml (approximately 8 oz or 1 cup) of 100% OJ.

Instruments and Protocols

V̇O2max Test. The V̇O2max test is a graded exercise test to determine aerobic capacity or peak oxygen uptake. While cycling on a stationary bike you will wear a mask and a nose clip to collect expired oxygen and carbon dioxide. To be included in the study you must have a V̇O2max > 40 ml/kg/min.

Exercise Protocol. The exercise protocol is designed to induce muscle glycogen depletion and mild dehydration. On Days 4-8, participants will perform 80 min of exercise each day. The exercise starts with a 2.5 min warm up, then four 15 min periods of cycling at 80% V̇O2max. Each 15 min period is separated by 5 min of cycling at a lower intensity. The exercise ends with a 2.5 min cool down. To achieve 1-2% dehydration, you will be provided water in the amount of 1.5 ml/kg of body weight every 15 minutes. Exercise will be conducted in an environmental chamber set at a mild, thermal temperature (86°F, 50% humidity).

Blood Measures. To obtain blood we will insert a needle into a vein in your arm. On Days 1 and 11 we will use one single needle stick to take 2 6 ml tubes (approximately 2.5 tsp). On each of Days 4-8 a single needle stick will be done 3 times: before and after exercise and after the 1 hour rest. For each blood draw we will take 2 6 ml tubes for a total of 6 tubes (approximately 2.5 Tbsp) a day. The total blood taken on Days 4-8 is approximately 12.5 Tbsp, about 1/3rd of what is taken when donating blood.

We will use blood to measure plasma osmolality, electrolytes (sodium, potassium, calcium, and chloride), inflammatory markers (cytokines and C-reactive protein), muscle damage (phosphocreatine kinase), and oxidative stress.

Urine Measures. On exercise days urine will be used to determine hydration status. You will use a private restroom to collect the urine sample. Using a urine cup, you will provide 1 sample upon arriving to the laboratory and one sample after exercise. During the 1 hour rest period you will collect urine into a urine container to measure volume. The cup will be labeled with your participant number. We will measure urine for specific gravity and osmolality.

Body Weight. A body composition scale will be used to determine your nude weight to characterize hydration. Once you arrive to the laboratory, you will be asked to void all urine. The scale will be set up in a private area where you will be asked to remove all clothing and step on the scale. A second body weight will be taken immediately after exercise and after the 1 hour rest.

Muscle Biopsies. Four skeletal muscle biopsies will be taken during this study (on days 1, 4, 8, and 11). Each muscle sample (approximately the size of a pea or ~100 mg) will be obtained from the lateral aspect

of your thigh using the percutaneous needle biopsy technique. The biopsies will be performed on alternate legs (ie, right leg for day 1 and 8, and left leg for day 4 and 11). The procedure is overseen by Jeff Burns, M.D., who is a medical doctor in Neurology at the KU Medical Center in Kansas City, Kansas. Dr. Burns supervises the procedure, but will not be physically present for the biopsies. Each biopsy should take approximately 20 minutes.

For the biopsy procedure, the following will occur:

1. You will be placed on an examination table lying down on your back (supine).
2. Your skin will be thoroughly cleaned with antiseptic solution (Betadine) around the sampling site. If you are allergic to Betadine, an alternative antiseptic solution will be used to clean the skin.
3. A small amount (3 ml or 3 cc) of a local anesthetic (2% Lidocaine) will be injected into the tissue under the skin around the site to be sampled. If you are allergic to the local anesthetic, or have had allergic reactions to other anesthetics (ie, Novocain) then you will be disqualified from the study.
4. Following a minimum of 5 minutes to ensure adequate time for the agent to take effect, a small incision (1 cm) will then be made in the skin and the biopsy needle inserted into the middle of the muscle (muscle belly) at a depth of 3 cm (about 1 inch). During this time that the sample is being taken (about 5 seconds) you may feel some deep pressure and cramping that will be moderately painful.
5. Following the biopsy procedure, firm and constant pressure will be placed on the wound to stop the bleeding. The incision site will be closed with a steri-strip, covered with a large Band-Aid and the site compressed using a 10 cm stirp of sterile elastic foam tape for a 24 hr period.

Thirst. On exercise days we measure your perceived thirst using a 9-point scale. The scale ranges from 1 (not thirsty at all) to 5 (moderately thirst) to 9 (very, very thirsty). You will be asked to rate your thirst pre- and 15, 30, 45, and 60 minutes post drinking your assigned beverage.

Palatability. Palatability will also be measured immediately after drinking the beverage. This questionnaire uses a 9-point scale, ranging from 1 (dislike extremely) to 5 (neither like nor dislike) to 9 (like extremely). Questions include overall beverage, flavor, sweetness, saltiness, and tartness.

Gastrointestinal Symptom Index. Gastrointestinal discomfort will be measured before exercise, every 15 minutes during exercise, immediately after exercise, and 1 hour after exercise. Questions include upper abdominal problems (belching, reflux, bloating, etc.), lower abdominal problems (cramping, diarrhea, pain, etc.), and systemic problems (dizziness, headache, etc.).

Profile of Mood States. This questionnaire measures your mood disturbance and is taken by computer online. There are 35 items on a 5-point scale (0 = not at all to 4 = extremely) related to feelings of anger, hostility, confusion, bewilderment, depression, dejection, fatigue, inertia, tension, anxiety, vigor, and activity. You will complete this questionnaire on the first and last days of the trial as well as before and after exercise.

Rate of Perceived Exertion (RPE). The Borg Scale is used to measure your RPE before, every 15 minutes during, and immediately after exercise.

Cardiovascular. To ensure you remain at safe limits and determine effects on cardiovascular function, your heart rate will be continuously monitored during exercise using a strap worn around your chest. In addition, blood pressure will be measured every 15 minutes during exercise.

Core Temperature. To assess thermoregulatory strain and ensure you remain at safe limits, core temperature is recorded continuously throughout exercise using a rectal temperature probe. You will be

asked to insert the flexible probe ~ 4 inches (10 cm). Appendix A includes additional information on the rectal temperature probe. You will not be allowed to exceed a core temperature greater than 104°F and we will monitor your temperature after exercise until you return to your normal temperature.

Diet and Physical Activity. On each day of the trial you are asked to complete a diet and activity log. We ask that you maintain similar diet and activity habits during the 11 days, but that you avoid orange juice and other citrus food and beverages. We ask you to avoid alcohol during the trial. We also ask that you do not complete strenuous activity at least 2 days before the start of your trial and that you do not complete additional exercise outside the trial or take any anti-inflammatory or pain medications (for example, Aleve, Tylenol, aspirin, etc).

Procedures

Data Collection (Figure 1). You will report Day 1 for baseline testing which includes a blood draw, muscle biopsy, and completing the profile of mood states (POMS) survey. We will provide you with *Take Home Instructions* and 3 bottles containing 8 oz (237 ml) each of your assigned beverage. You will consume 1 bottle on Days 1-3. Before arriving for each exercise day (Days 4-8), you should eat a small meal and drink at least 8 oz of water. Before beginning the exercise protocol you must be hydrated. Urine, body weight and all survey measures are taken before exercise, after exercise, and 1 hour after exercise. Blood measures and muscle biopsies are taken on Days 4 and 8. Gastrointestinal discomfort symptoms, RPE, heart rate, blood pressure, and core temperature will be assessed during exercise. Once you finish exercise you will be given 1 bottle with 8 oz of your assigned beverage to drink. Immediately after drinking your beverage you will complete the palatability survey. You will rest for 1 hour in an ambient environment. During the 1 hour you will complete the thirst survey. Prior to leaving the laboratory on Day 8 (final exercise day), we will give you *Take Home Instructions* and 3 bottles containing 8 oz each of your assigned beverage to consume on Days 9-11. Final muscle biopsies, blood, and POMS are taken Day 11.

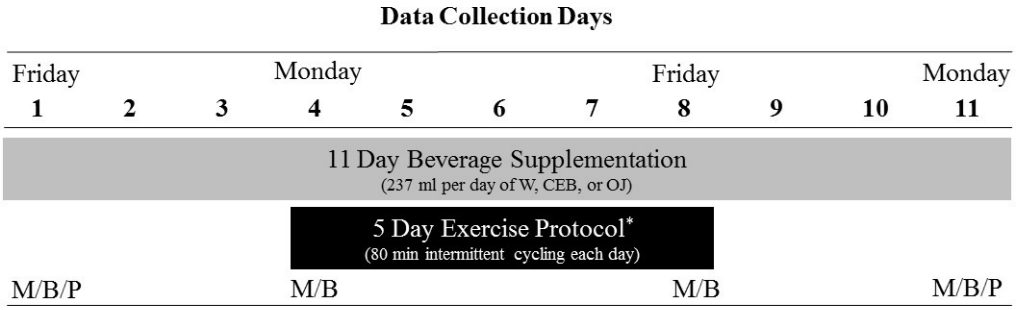


Figure 1. Schematic depicting 11 day data collection.
M/B/P = muscle biopsy, blood (inflammatory, oxidative stress, and muscle damage markers), POMS
*Measures during each exercise protocol: thirst, GI distress, palatability, POMS, hydration, electrolytes, Tc, HR, BP
Abbreviations: BP = blood pressure; CEB = carbohydrate electrolyte beverage; GI = gastrointestinal; HR = heart rate; OJ = orange juice; Tc = core temperature; POMS = Profile of Mood States; W = water

RISKS

You may experience one or more of the risks indicated from being in the study. In addition to these, there may be other unknown risks, or risks we did not anticipate, associated with participation in this study.

The following risks may occur due to the muscle biopsy:

1. The local anesthetic will result in a slight burning sensation, lasting approximately 5 seconds.
2. There is an extremely low risk of allergic reaction to the local injection (1 in 1 million).

3. Nausea, dizziness, and fainting can occur in remote cases (1 in 100)
4. There is a minimal risk of infection (1 in 1,000) and irritation. The use of aseptic techniques, careful cleaning of the skin, and keeping the area dry will minimize the risk of infection.
5. There is a minimal risk of bruising (1 in 100). Applying a compression bandage on the muscle biopsy site for 24 hrs following will minimize bruising.
6. In rare instances (1 in 200), some motor nerves may be damaged which may cause local muscle atrophy (decrease in the size of muscle fibers, with a small dimple on the skin).
7. There is likely to be a small scar (1/2 inch in length) where the incision for the muscle biopsy is performed. The scare usually dissipates over a period of 6-12 months at which time the scaring is very modest.

The following risks may occur due to the blood draw:

1. It is likely to experience mild pain or discomfort when having blood drawn, although we do not anticipate additional blood draws than what is require for normal treatment of care.
2. Bruising at the site in your arm where the needle is inserted is less likely/common to occur.
3. In rare cases you may develop a hematoma (localized swelling filled with blood) at the site in your arm where the needle was inserted.

All beverages are FDA approved and ingredient amounts are minimal. You may experience any of the following minimal risks associated with ingredients used to preserve and sweeten beverages:

Ingredient	Potential risks
Acesulfame potassium	Cancer from long-term use
Calcium disodium EDTA	Reduce antibiotic medication effects; if taken in excessive amounts low blood sugar, low blood pressure, kidney failure, and seizures
Calcium chloride	Irritation of nostrils, mouth, throat, lips, eyelids, and ears; gastrointestinal discomfort
Citric acid/sodium citrate	Allergic reactions; gastrointestinal discomfort; increase urine volume
Dextrose	Weight gain, diabetes, cardiovascular disease, certain cancers, and lowered immunity
Gum acacia	Allergic reactions; increased cholesterol; gastrointestinal distress
Glycerol ester of rosin	Allergic reactions
Magnesium sulfate	Thirst, gastrointestinal discomfort, and allergic reactions
Magnesium chloride	Allergic reactions; diarrhea
Monopotassium phosphate	Allergic reactions; kidney and cardiovascular dysfunction
Niacin	Allergic reactions; gastrointestinal discomfort; flu like symptoms
Potassium benzoate	Mild irritation to skin, eyes, and mucus membranes; in some beverages converts to benzene (a carcinogen)
Potassium sorbate	Allergic reactions; nasal congestion; and gastrointestinal discomfort
Sodium benzoate	Increase hyperactivity in individuals with attention deficit disorder
Sodium phosphate	Gastrointestinal discomfort and allergic reactions
Sucralose	Weight gain and diabetes
Vitamin A	Dizziness, headache, nausea, skin irritation, liver damage, and joint/bone pain with chronic excess use
Vitamin B3 (niacin)	Allergic reactions; gastrointestinal discomfort; flu like symptoms
Vitamin B6	Gastrointestinal discomfort; brain and nerve problems with long-term use
Vitamin B12	Allergic reactions; gastrointestinal distress

Vitamin C (ascorbic acid)	Extremely high doses may cause redness, warmth, or flushing of skin; headache; gastrointestinal discomfort
Vitamin E	Complicate existing cardiovascular disease or diabetes
Yellow 5	Allergic reactions; negative interaction with aspirin and aspirin hypersensitive individuals
Yellow 6	Allergic reactions; gastrointestinal distress; carcinogenic; increase ADHD

BENEFITS

As a result of participating in this study you will be provided your V̇O2max. We will also calculate and provide you your sweat rate during exercise by using your body weight and fluid consumed.

PAYMENT TO PARTICIPANTS

Participants will receive $25 for completing Days 1-4, $75 for completing Days 5-8, and $100 for completing Days 9-11. Investigators will ask for your social security number in order to comply with federal and state tax and accounting regulations.

PARTICIPANT CONFIDENTIALITY

Your name or any other identifiable information will not be associated in any publication or presentation. All information collected about you or with the research findings from this study will be coded with a study number. Only the primary investigators will have access to documents with your identifiable information and documents will be kept in a locked cabinet in a locked office. No identifiable information about you will be shared unless (a) it is required by law or university policy, or (b) you give written permission.

REFUSAL TO SIGN CONSENT AND AUTHORIZATION

You are not required to sign this Consent and Authorization form and you may refuse to do so without affecting your right to any services you are receiving or may receive from the University of Kansas or to participate in any programs or events of the University of Kansas. However, if you refuse to sign, you cannot participate in this study.

CANCELLING THIS CONSENT AND AUTHORIZATION

You may withdraw your consent to participate in this study at any time. You also have the right to cancel your permission to use and disclose further information collected about you, in writing, at any time, by sending your written request to: Dawn Emerson or Philip Gallagher (contact information below). If you cancel permission to use your information, the researchers will stop collecting additional information about you. However, the research team may use and disclose information that was gathered before they received your cancellation, as described above.

QUESTIONS ABOUT PARTICIPATION

Questions about procedures should be directed to the researchers listed at the end of this consent form.

PARTICIPANT CERTIFICATION

I have read this Consent and Authorization form. I had the opportunity to ask and I received answers to any questions I had regarding the study. I understand that if I have any additional questions about my rights as a research participant, I may call (785) 864-7429 or (785) 864-7385, or write the Human Subjects Committee Lawrence Campus (HSCL), University of Kansas, 2385 Irving Hill Road, Lawrence, Kansas 66045-7568, or email irb@ku.edu.

I agree to take part in this study as a research participant. By my signature I affirm that I am at least 18 years old and that I have received a copy of this Consent and Authorization form.

_____ _____

Participant's Printed Name Date

_____

Participant's Signature

RESEARCHER CONTACT INFORMATION

Dawn M. Emerson, PhD, ATC
Principal Investigator
Department of Health, Sport, and Exercise Sciences
Robinson Center, Room 101E
University of Kansas
Lawrence, KS 66045
785-864-0709
dawn.emerson@ku.edu

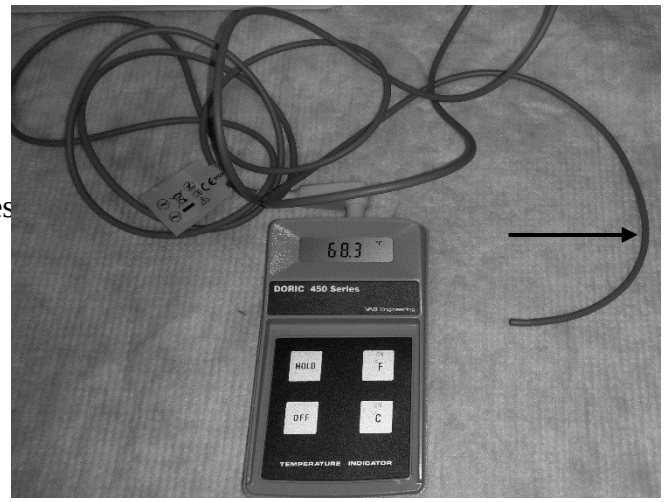
Philip Gallagher, PhD
Principal Investigator
Department of Health, Sport, and Exercise Sciences
Robinson Center, Room 101DJ
University of Kansas
Lawrence, KS 66045
785-864-0772
philku@ku.edu

Melani Kelly, MS, ATC
Co-Investigator
Robinson Center, Room 101
melani.kelly@ku.edu

Evan Landes
Co-Investigator
Robinson Center, Room 101
e879l604@ku.edu

RECTAL TEMPERATURE ASSESSMENT

The picture to the right shows the temperature monitor and flexible probe. The probe is approximately 0.1 inches (3 mm) in diameter. The entire probe length is 3 feet. You will insert the probe ~ 4 inches, about where the black arrow indicates.



The probe will remain in place throughout the biking with the monitor resting nearby, similar to the picture below.

<u>To insert the rectal probe:</u>

1. You will be given a clean probe with a piece of tape to indicate the 4 inch mark.
2. You will go to a private bathroom.
3. The probe should be inserted 4 inches past the anal sphincter. This is best done with one knee and hip slightly flexed forward. You should also try to relax as much as possible.
4. If you meet resistance, stop, remove the probe and try again.

<u>To remove:</u>

5. Once your core temperature returns to normal during the rest period, you may go to the bathroom and remove the probe. To remove, simply pull the probe out gently.
6. Wipe the probe off with tissue and discard the tissue.
7. Return the probe to a research assistant.

There are no known serious risks associated with using the rectal temperature probe. You may experience slight discomfort or pressure, but at no point should using the probe be painful.

Appendix B: Health History Questionnaire

HEALTH AND HEALTH AND INJURY HISTORY QUESTIONNAIRE

INSTRUCTIONS: Complete the following questions the best of your knowledge/ability. Let the investigator know if you need further explanation.

Part 1. Participant Information

Name: _____ Age: _____ Participant #: _____

Part 2. Physical Activity Readiness Questionnaire© (Canadian Society for Exercise Physiology)

		Yes	No
1.	Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?	☐	☐
2.	Do you lose your balance because of dizziness or do you ever lose consciousness?	☐	☐
3.	Is your doctor currently prescribing drugs (for example, water pills) for blood pressure or a heart condition?	☐	☐

Part 3. Medical History Explain "yes" answers below. (American Medical Society for Sports Medicine, American Orthopedic Society for Sports Medicine, American Heart Association, and American College of Sports Medicine)

		Yes	No
1.	Have you had a medical illness or injury since your last check up or sports physical?	☐	☐
2.	Do you have an ongoing chronic illness? _____	☐	☐
3.	Have you ever passed out during or after exercise? _____	☐	☐
4.	Have you ever been dizzy or fainted during or after exercise? _____	☐	☐
5.	Have you ever had pain, discomfort, tightness, or pressure in your chest during __ or after exercise?	☐	☐
6.	Have you ever had racing of your heart or skipped heartbeats?	☐	☐
7.	Have you had high blood pressure or high cholesterol?	☐	☐

8. Have you ever been told you have a heart murmur?
   □ □

9. Has a physician ever denied or restricted your participation for sports or for any heart
   □ □
   problems?

10. Have you ever had an unexplained seizure?
    □ □

11. Have you ever had numbness or tingling in your arms, hands, legs, or feet?
    □ □

12. Have you ever become ill from exercising in the heat?
    □ □

13. Do you cough, wheeze, or have trouble breathing during or after activity?
    □ □

14. Do you have diabetes?
    □ □

15. Do you have asthma or other lung disease?
    □ □

16. Do you smoke?
    □ □

17. Do you have or think you may have any bleeding disorders?
    □ □

18. Do you have, or think you may have, any of the following conditions? *Check all that apply.*
    ___ Nausea, vomiting, or other gastrointestinal disorders
    ___ Obstructive disease of the gastrointestinal tract, such as diverticulitis or inflammatory bowel disease
    ___ A history of disorders or impairment swallowing or of the gag reflex
    ___ Any gastrointestinal surgery

Explain "Yes" answers here:
_____
_____
_____
_____
_____

20. Do you get frequent muscle cramps when exercising?
    □ Yes □ No
21. Do you or someone in your family have sickle cell trait or disease?
    □ Yes □ No
22. Have you ever experienced an exertional heat stroke or other significant heat illness?
    □ Yes □ No

Explain “Yes” answer here:

_____
_____
_____
_____
_____
_____

23. Are you allergic to any medications?

□ Yes □ No

Explain “Yes” answers here:

_____
_____
_____

24. Are you currently taking any ***prescription*** medications, pills, or an inhaler?

□ Yes □ No

For example: Albuterol, Prednisone, Naproxen, etc.

Explain “Yes” answers here:

_____
_____
_____

25. Are you currently and regularly taking any ***non-prescription*** (over-the-counter)

□ Yes □ No

medications or pills? For example: Bayer, Prilosec, NyQuil, etc. _____

Explain “Yes” answers here:

_____
_____
_____

26. Are you currently and regularly taking any ***vitamins, supplements, or other substances***?

□ Yes □ No

For example: multi-vitamin, iron, diet pills, energy drinks, protein powder, etc.

Explain “Yes” answers here:

_____
_____
_____

FEMALES ONLY

27. Is your menstrual cycle regular?

□ Yes □ No

28. Are you on birth control?

□ Yes □ No

29. Are you currently on your period?

□ Yes □ No

30. What is the date of your last period? _____

I hereby state that, to the best of my knowledge, my answers to the above questions are complete and correct.

_____ _____ _____

Signature of Participant Printed Name of Participant Date