Effects of Macro- and Micro- Nutrients on Exercise Induced Hepcidin Response in Highly-Trained Endurance Athletes

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Effects of Macro- and Micro- Nutrients on Exercise Induced Hepcidin Response in Highly-Trained Endurance Athletes

Running Title: Nutrient Composition and Hepcidin in Highly-Trained Athletes

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Abstract

Iron deficiency (ID) has ergolytic effects on athletic performance. Exercise induced inflammation impedes iron absorption in the digestive tract by up-regulating the expression of the iron regulatory protein, hepcidin. Limited research indicates the potential of specific macro- and micro- nutrients on blunting exercise induced hepcidin. Therefore, we investigated the effects of post-exercise supplementation with protein and carbohydrate (CHO) and vitamins D\textsubscript{3} and K\textsubscript{2} on the post-exercise hepcidin response. Ten highly-trained male cyclists (age: 26.9±6.4 years; VO\textsubscript{2}max: 67.4±4.4 ml/kg/min) completed four cycling sessions in a randomized, placebo-controlled, single-blinded triple crossover study. Experimental days consisted of an 8-min warm-up at 50% power output at VO\textsubscript{2}max (pVO\textsubscript{2}max), followed by 8x3 min intervals at 85% pVO\textsubscript{2}max with 1.5 min at 60% pVO\textsubscript{2}max between each interval. Blood samples were collected pre- and post- exercise, and 3-hr post-exercise. Three different drinks consisting of CHO (75g) and protein (25g) with (VPRO) or without (PRO) vitamins D\textsubscript{3} (5,000 IU) and K\textsubscript{2} (1,000 mcg), or a zero-calorie control drink (PLA) were consumed immediately after the post-exercise blood sample. Results showed that the post-exercise drinks had no significant (p ≥ 0.05) effect on any biomarker measured. There was a significant (p < 0.05) increase in hepcidin and IL-6 following intense cycling intervals in the participants. Hepcidin increased significantly (p < 0.05) from baseline (nmol/L: 9.94±8.93, 14.18±14.90, 10.44±14.62) to 3-hr post-exercise (nmol/L: 22.27±13.41, 25.44±11.91, 22.57±15.57) in VPRO, PRO and PLA respectively. Contrary to our hypothesis, the drink compositions used did not blunt the post-exercise hepcidin response in highly-trained athletes.

Key Words

Cycling; Inflammation; Iron metabolism; Protein and Carbohydrates; Vitamin D
Introduction

Iron deficiency (ID) is the most prevalent nutrient deficiency in the world (Cogswell et al. 2009; DellaValle and Haas 2014), affecting billions worldwide (McLean et al. 2009), with even higher susceptibility to this deficiency in endurance athletes (Koehler et al. 2012). Twenty to 60% of all female athletes and 4 to 50% of all male athletes (Brandy et al. 2003; Hinton 2014) suffer from ID in a given year. This deficiency can lead to deleterious effects on endurance performance and reduce the oxygen carrying capacity of the blood by up to ~30% (Woodson et al. 1978; Davies et al. 1982; Davies et al. 1984; Brownlie et al. 2004; Dellavalle and Haas 2011). Iron repletion studies in ID athletes have shown reductions in fat mass and increases in lactate threshold, improved exercise economy, and a 7.4% increase in VO\textsubscript{2}max (Hinton and Sinclair 2007; DellaValle and Haas 2014; Wachsmuth et al. 2015).

One of the mechanisms that may be responsible for the high rates of ID in endurance athletes could be, in part, due to the acute inflammation response caused by intense exercise (Varamenti et al. 2013). It has been shown that exercise (Auersperger et al. 2013), injury/illness (Auersperger et al. 2013) and/or hypoxia (Haase 2010; Liu et al. 2012) increase the inflammatory cytokine interleukin-6 (IL-6) (Nicolas et al. 2002; Wachsmuth et al. 2015). High IL-6 concentrations are correlated with the down-regulation of iron absorption in the human digestive tract (Przybyszewska and Zekanowska 2014) by up-regulating the gene expression of the iron regulatory protein hepcidin (Roecker et al. 2005). Hepcidin inhibits iron absorption (Przybyszewska and Zekanowska 2014), and has been shown to increase post-exercise, peak three to six hours later, and then slowly return to pre-exercise baseline concentrations 24 hours later (Peeling et al. 2009; Peeling et al. 2014). Furthermore, it has been shown that the fate of the stored form of iron, ferritin, is multifaceted and is tightly regulated by the size of the iron pool and demands of iron within the body (Aisen 2001). Primarily, ferritin levels below 30 mcg/L

Attempting to attenuate post-exercise hepcidin release, preliminary research has focused on different nutritional interventions (Robson-Ansley et al. 2011; Sim et al. 2012; Badenhorst et al. 2014), to try to increase iron absorption and recycling by minimizing the exercise-induced inflammatory response (Ganz and Nemeth 2012). Protein ingested during exercise (Schroer et al. 2014), and protein combined with carbohydrate (CHO) after exercise (Kerasioti et al. 2013), have both been shown to downregulate IL-6 concentrations and systemic inflammatory responses immediately after and four hours post-exercise. Furthermore, large doses of carbohydrate (CHO) solution (6%, 250 mL every 15 minutes [4 mL kg\(^{-1}\)]) during a 2.5-hour run can decrease exercise-induced inflammation, specifically IL-6, by 40% (Nehlsen-Cannarella et al. 1997; Nieman et al. 1998). Despite this, four recent studies in trained endurance athletes have shown intra- or post- CHO supplementation has no effect on hepcidin following high intensity runs (Robson-Ansley et al. 2011; Sim et al. 2012; Ihalainen et al. 2014; Badenhorst et al. 2015b); and restricting CHO intake over a 24-hour period can increase susceptibility to ID by elevating baseline hepcidin concentration (Badenhorst et al. 2015a).

In addition to macronutrients, vitamin supplementation could also affect the inflammatory response to exercise. Supraphysiological doses of 50,000 IU (7,143 IU/day for three months) and 100,000 IU (single dose) of vitamin D significantly reduce hepcidin levels
(28% and 34%, respectively) in healthy individuals and patients suffering from chronic kidney disease (Bacchetta et al. 2014; Zughaier et al. 2014). At much lower dosages, 4,000 IU/day of vitamin D over 28 days in physically-active males demonstrated marked reductions in exercise induced inflammatory cytokines (Barker et al. 2013). Despite the positive findings, post-exercise inflammatory response has been reported to be different in trained versus untrained individuals (Evans et al. 1986; Schild et al. 2016), and results may be different in highly-trained athletes. No study to our knowledge has investigated this discrepancy. Additionally, vitamin K has been shown to prevent vitamin D toxicity (Dahlquist et al. 2015) and decrease IL-6 concentrations (Reddi et al. 1995; Ohsaki et al. 2006), yet a long term study using vitamin K₁ supplementation at dosages of 500 mcg/day did not demonstrate a decrease in IL-6 activity (Shea et al. 2008b). Therefore, the authors chose a vitamin K variant (vitamin K₂) that is more potent at blunting IL-6 production compared to vitamin K₁ (Reddi et al. 1995) and utilized a dose (1,000 mcg) postulated to be effective at preventing vitamin D toxicity (Dahlquist et al. 2015) for the purpose of this study.

With limited evidence that macronutrients and micronutrients may help address ID via attenuating post-exercise inflammation, we investigated the effects of a CHO and protein-rich post-exercise drink with the addition of vitamins D₃ and K₂ (VPRO) or without addition vitamins (PRO) versus a non-caloric placebo drink (PLA) on exercise-induced hepcidin response. We hypothesized that (1) both VPRO and PRO would significantly attenuate the rise of hepcidin following a bout of high intensity exercise as compared to PLA and (2) that VPRO supplementation would have a greater blunting effect on hepcidin concentration versus PRO supplementation alone.
Methods

Ethical Approval

Subjects completed a Physical Activity Readiness Questionnaire (PAR-Q) and provided written consent following a comprehensive written and explanation of the study. The University of British Columbia Clinical Research Ethics Board granted approval for the study (H15-00721).

Participants

Ten highly-trained male cyclists (age: 26.9±6.4 years; weight: 72.0±6.4 kg) with a VO$_2$max of 67.4±4.4 mL/kg/min, classified as Performance Level (PL) 4 and 5 (De Pauw et al. 2013) partook in four separate cycling sessions after passing and adhering to the following inclusion criteria: endurance athletes who were healthy, were not consuming anti-inflammatory agents on a regular basis, 18-45 years of age, and training 5-6 times a week with at least one year of training experience, with baseline serum ferritin levels of $\geq$ 30 mcg/L. Subjects also refrained from taking vitamin D, C and K, multi-vitamins 24 hours prior to experimental days and fish oil, krill oil, turmeric and serrapeptidase supplements at least 48 hours prior to experimental days. Female participants were excluded from the study due to the effects of the menstrual cycle and estradiol on hepcidin, since high estrogen and/or progesterone levels can attenuate the elevation in hepcidin levels (Yang et al. 2012; Sim et al. 2014).

Sample Size Determination

A sample size of n=10 was determined using data from (Badenhorst et al. 2014). In this study, 10 well-trained male endurance athletes completed two 8x3 minute interval running sessions at 85% of their maximal aerobic velocity ($v$VO$_2$max) on a motorized treadmill before being randomly allocated to a three hour recovery period in a hypoxic (~2,900 m above sea level) or
normoxic environment. Based on the differences of mean and standard deviations between baseline measurements and three hours post-exercise for the hypoxic intervention on hepcidin-25 (2.18±1.26 nM), an effect size of (dz=1.73) and a minimum sample size of n=9 subjects were needed.

Research Design

A randomized, placebo-controlled, single-blinded triple crossover design was used. A 48-hour washout period was used and each session took place at the same time of day. Subjects recorded their diet and activity the day prior to the experimental visits and fasted for 10 hours overnight before reporting to the laboratory. To maximize ecological validity, subjects consumed a standardized meal 60 minutes prior to arrival and adhered to a supplement protocol 48- and 24-hours prior to experimental days, described earlier. The standardized meal consisted of: 3 packs of granola bars (Nature Valley, Crunchy – Honey & Oats; Minneapolis, MN, US) with one pack containing 191 kcal, 7g of fat, 29g of CHO, 3g of protein and 1.44 mg of iron (Total for 3 pack: 573 kcal, 21g of fat, 87g of CHO, 9g of protein and 4.32 mg of iron). Subjects chose how much of the bars to consume, but were required to replicate their intake for subsequent visits.

Screening Day

Prior to familiarization (Visit 1) subjects were assessed for baseline serum ferritin and serum vitamin D, in order to ensure that their ferritin levels were ≥ 30 mcg/L as baseline hepcidin, and subsequent release, is reported to be lower in ID subjects (Auersperger et al. 2013; Peeling et al. 2014). Furthermore, it has been suggested that ferritin levels below 30 mcg/L in athletes are low (or ID) and should be the standard cut off going forth in this specific population (Clenin et al. 2015).
Visit 1, Familiarization Day

Visit 1 consisted of equipment familiarization and an incremental cycle exercise test (Wilkie et al. 2015) on an electronically-braked ergometer (Wahoo KICKR, Wahoo Fitness, Atlanta, GA, US). These results were used to determine the cycling power output (W) at percent (%) VO₂max (pVO₂max) for subsequent visits. Subjects then performed a familiarization to the exercise challenge, consisting of an 8-minute warm-up (cycle) at 50% power output (Watts) at VO₂max (pVO₂max), followed by 2 x 3 minute intervals at 85% pVO₂max. Each interval was separated by a 1.5 minute of active recovery at 60% pVO₂max.

Incremental Exercise Test (IET)

Participants performed a 10-minute self-selected warm-up protocol on their own bike on a cycle trainer (Wahoo KICKR, Wahoo Fitness, Atlanta, GA, US). Upon completion of the warm-up, the IET began immediately. Subjects were required to pedal at a cadence of ≥ 60 revolutions per minute (rpm) and work rate began at 0 Watts (W) (Wilkie et al. 2015). Work rate increased by 30 W every minute until volitional exhaustion. Subjects were fitted with a heart rate monitor (Wahoo TICKR, Wahoo Fitness, Atlanta, GA, US) and a face-mask (Oro-Nasal 74550 V2 Mask, Hans Rudolph, Shawnee, KS) connected to a two-way non-rebreathing valve (2700 T-shape, Hans Rudolph; Shawnee, KS) attached to a metabolic cart (TrueOne, Parvo Medics; Sandy, UT) in order to assess VO₂kinetics throughout the IET. The subjects’ VO₂max was then utilized to determine cycling power output (W) at percent (%) VO₂max (pVO₂max) for subsequent cycling tests.

Visits 2 to 4

Venous blood samples were collected upon arrival, immediately post-exercise, and three hours post-exercise. Body mass (kg) was recorded both pre- and post- exercise (599KL Digital Scale,
Health O Meter ® Professional Scales; McCook IL, US). Subjects were allowed to drink water *ad libitum* during all trials. HR was collected throughout (Wahoo TICKR, Wahoo Fitness, Atlanta, GA, US) and rating of perceived exertion (RPE) was collected at 3-minute intervals using the Borg perceptual scale (Borg 1981; Borg 1998). After an 8-minute cycle warm-up at 50% \( p\text{VO}_2\text{max} \), subjects began the cycle test of 8x3 minute intervals at 85% \( p\text{VO}_2\text{max} \) with a 1.5-minute active recovery (60% \( p\text{VO}_2\text{max} \)) separating the bouts. Following the final interval a 8-minute cool-down at 50% \( p\text{VO}_2\text{max} \) was undertaken and followed by a blood sample. Immediately following the blood sample, subjects consumed one of the three randomized experimental recovery. Participants then rested in the laboratory for three hours, a venous blood sample was collected, and then subjects departed.

*Post-exercise Recovery Drink Consumption*

The beverages looked, smelled, and tasted similarly based on subject feedback while consuming. They were mixed in identical opaque bottles and consisted of the following ingredients:

1) **PLA**: non-nitrogenous, zero calorie control drink (artificial flavour, sweetener [Kraft Foods, Crystal Light; Northfield, IL, US] and water [550 mL]).

2) **VPRO**: 75g of CHO (Cytocarb\(^2\), CytoMax - CytoSport; Benicia, California, US), 25g of protein (Whey Protein Isolate – Vanilla - Restore, EXOS Fuel; Sandpoint, ID, US), 10 droplets of a vitamin D complex (Vitamin D + K Complex, EXOS Fuel; Sandpoint, Idaho, US) containing 5,000 IU of vitamin D\(_3\) and 1,000 mcg of vitamin K\(_2\) (as menatetrenone), artificial flavour, sweetener (same as PLA) and water (550 mL).

3) **PRO**: 75g of CHO (Cytocarb\(^2\), CytoMax - CytoSport; Benicia, California, US), 25g of protein (Whey Protein Isolate – Vanilla - Restore, EXOS Fuel; Sandpoint, ID, US), artificial flavour, sweetener (same as PLA) and water (550 mL).
Laboratory Procedures

Blood Collection

Blood sampling procedures mimicked that of previous literature (Badenhorst et al. 2014; Peeling et al. 2014; Badenhorst et al. 2015b; Badenhorst et al. 2015a). All venous blood samples were taken after the subject rested in a seated position for five minutes with a 21-gauge needle into two 5 mL SST Gel separator tubes, a 3 mL SST Gel separator tube, a 4.5 mL PST Gel separator tube, and a 3 mL EDTA tube. Immediately following blood collection, the fresh samples were taken to the University of British Columbia Hospital laboratory to perform a complete blood count (CBC), serum iron and serum ferritin within a 24 hour period. The remaining samples were allowed to clot for 60 minutes at room temperature and then centrifuged at 10°C and 1,500 G for 10 minutes. The centrifuged samples were separated into 1 mL aliquots and stored at -80°C.

Blood Analysis

The remaining frozen serum samples were used to measure the bioactive form of hepcidin, hepcidin-25 via ELISA, and interleukin-6 (IL-6). Serum IL-6 was analyzed using a commercially-available ELISA (Quantikine HS, R&D Systems; Minneapolis, Minnesota, USA) with a sensitivity of 0.11 pg/mL and range of 0.2-10 pg/mL. Hepcidin-25 was assessed using a commercially-available ELISA (Quantikine HS, R&D Systems; Minneapolis, Minnesota, USA) with a sensitivity of 3.81 pg/mL and a detection range of 15-1,000 pg/mL. Coefficient of variation (CV) for IL-6 and hepcidin-25 were <5%.

Statistical Analysis

Results were analyzed with SPSS (IBM North America, New York, NY). Data and results were reported as mean ± SD. A 3 X 3 repeated measures analysis of variance (supplement type X
time) for each of hepcidin-25, Hb, IL-6, serum ferritin and serum iron was performed. Paired sample t-tests were performed to determine significance between time points within-treatments. A multivariate general linear model was performed in order to determine if starting baseline vitamin D measurements had a significant effect on individual post-exercise hepcidin responses to the VPRO drink only. The data that did not pass normality (hepcidin-25, IL-6, and serum ferritin) were transformed using the natural logarithm. Data that were statistically analyzed utilizing log-transformed data are presented as mean ± SD of non-transformed data within tables and results. F-ratios will be found significant at $p \leq 0.05$.

Results

Subject Characteristics

Subject characteristics and VO$_2$max results are presented above in the methods. Subjects had serum ferritin levels of 109±77 mcg/L and serum vitamin D levels of 75±28 nmol/L at baseline.

Physiological Responses

Mean HR, RPE, energy expenditure (Vogt et al. 2005; Haakonsen et al. 2013), pre- and post-exercise body mass and power output for the interval cycling trials are presented in Table 1.

***Insert Table 1 Here***

Serum Interleukin-6

There was no significant difference in baseline measurements of IL-6 across conditions. There was a significant effect over time, but no significant condition effect on IL-6 (see Table 2).

Hepcidin-25 & Baseline Vitamin D

There was a significant effect of time, but not treatment (drink) on hepcidin-25 (see Table 2). There was a significant increase from baseline to post-exercise in VPRO and PRO, but no
significant increase in PLA. There was a significant increase in hepcidin-25 from baseline and post-exercise to three hours post-exercise for all conditions. Furthermore, there was no order effect for hepcidin values based on visit day. Starting vitamin D levels (74±28 nmol/L, MIN = 34 nmol/L, MAX = 132 nmol/L) did not affect hepcidin-25 response in the VPRO condition.

*Iron Parameters*

There was a significant time effect on serum iron and serum ferritin for all conditions (see Table 2). There was no condition effect on serum iron or log transformed serum ferritin.

*Hemoglobin & Hematocrit*

Significant time effects were recorded for Hb and hematocrit. No significant difference was observed for either Hb or hematocrit between VPRO, PRO and PLA (see Table 2).

***Insert Table 2 Here [Landscape or Own Page if Possible]***

*Discussion*

The present study examined the effects of a post-exercise drink consisting of whey protein isolate and carbohydrates (CHO) with vitamins D₃ and K₂ (VPRO) or without vitamins (PRO) on the acute post-exercise hepcidin response in highly-trained athletes. The results from the investigation demonstrate that following a fatiguing interval-based cycle, subjects experienced a significant time-dependent increase in hepcidin, IL-6, Hb, hematocrit and iron biomarkers independent of the post-exercise drink composition (Table 2). Contrary to our hypothesis, VPRO, PRO or PLA post-exercise drink compositions had no significant effect on any biomarker measured and is not likely to impact iron malabsorption due to post-exercise hepcidin release.
The hepcidin levels in the current study mimic those of previous literature, where the highest levels were seen concurrent with peak levels of IL-6 (Kemna et al. 2005). IL-6 has been reported to drastically increase five- to 100-fold after exercise (Ostrowski et al. 1999; Peeling et al. 2009), and peak levels are attained immediately following the cessation of exercise (Pedersen 2000). Our results demonstrated that IL-6 significantly increased from baseline to post-exercise in each condition (see Table 2), with no significant differences between the drinks. These findings could suggest that the exercise intensity and muscle glycogen content were similar between trials since both can alter IL-6 concentration (Ivy et al. 1988; Keller et al. 2001; Steensberg et al. 2001).

Furthermore, a limited number of studies on the effects of peri-workout consumption of both protein and CHO on inflammatory markers (such as IL-6) remain equivocal. Participants given either a 6% CHO solution or a 4:1 CHO-protein solution during a cycling session saw no significant differences in IL-6 concentrations between conditions (Cosio-Lima et al. 2012). Likewise, the ingestion of a high protein (0.7 g kg\(^{-1}\) hour\(^{-1}\)), moderate CHO (0.26 g kg\(^{-1}\) hour\(^{-1}\)) or a low protein (0.1 g kg\(^{-1}\) hour\(^{-1}\)), high CHO (2.1 g kg\(^{-1}\) hour\(^{-1}\)) beverage had no meaningful effect on IL-6 (Rowlands et al. 2008). Our results correspond with the findings of these two studies, since the PRO and VPRO drinks had no significant effect on IL-6 or hepcidin when compared to the PLA drink. However, Kerasioti et al (2013) demonstrated a significant 50% decrease in IL-6 concentrations when subjects consumed a high protein, moderate CHO cake (Total Intake, Protein: 23.92 g and CHO: 82.8 g, Calories: 426.9) compared to a low protein, high CHO (Total Intake, Protein: 9.2 g and CHO: 101.2 g, Calories: 441.6) cake during a four-hour recovery period after an exhaustive cycling session. Furthermore, it has been demonstrated that the consumption of 45g hour\(^{-1}\) of protein significantly decreases IL-6 concentrations immediately post-exercise, when compared to a placebo drink in individuals cycling at a constant
load for 120 minutes (Schroer et al. 2014). Contrary to Kerasioti et al (2013) and Schroer et al (2014), our results are in line with findings by Corsio-Lima et al (2012) and Rowlands et al (2008), where the protein and CHO dosages of the PRO and VPRO drinks had no significant effect on IL-6 levels when compared to the PLA drink.

These findings could potentially be related to the timing of the blood sample and/or the timing of nutrient intake. Kerasioti and colleagues (2013) reported that IL-6 was significantly different four hours after the cessation of exercise, which also corresponds with a potential peak in hepcidin concentration identified afterwards (Peeling et al. 2009; Peeling et al. 2014). Thus, we may have seen a significant difference between conditions if we had measured IL-6 and hepcidin levels four hours and not three hours post-exercise. Additionally, Schroer et al (2014) utilized a peri-workout feeding protocol, where subjects consumed 45 g of protein per hour over a two-hour cycling session. Although they saw a marked reduction in metabolic and oxidative stress, performance in the 30km time trial was impaired by 2% (Schroer et al. 2014). This finding of a performance decrement warrants caution with this strategy if attempting to optimize performance.

In addition, muscle glycogen depletion can significantly augment IL-6 concentration produced by skeletal muscle tissue (Keller et al. 2001; Steensberg et al. 2001), and both intensity and duration of exercise are important factors to determine the rate of muscle glycogen utilization (Ivy et al. 1988). It may take upwards to 2-3 hours of exercise at 70-80% VO$_{2\text{max}}$ in order to fully deplete muscle glycogen stores (Coyle et al. 1983) which can lead to a $\sim$40 to $\sim$60-fold increase in IL-6 gene activation (Keller et al. 2001). The meal containing $\sim$1.2 g CHO kg$^{-1}$ consumed one hour prior to our high-intensity exercise trial could have served to maximize carbohydrate stores and minimized muscle and liver glycogen depletion (Ivy 2004; Burke 2010).
Furthermore, elite endurance athletes commonly consume diets rich in CHO (8 to 11 g/kg = 576.24±51.52 to 792.33±70.84) (Burke 2001; Jeukendrup et al. 2005; Vogt et al. 2005; Robins 2007; Jeukendrup 2011). Thus, the pre-exercise high CHO meal and habitually CHO heavy diets could have potentially attenuated the magnitude of IL-6 responses from post-exercise to three hours post-exercise and negated the effect of VPRO and PRO beverages. Additionally, with the highly-trained athletes used in the study, the chronic adaptations they acquire from both resistance (Calle and Fernandez 2010) and endurance (Farney et al. 2012) training leads to a marked reduction in reactive oxygen species and a subsequent decrease anti- and pro-inflammatory cytokines following a bout of high intensity exercise. Lastly, the final blood sample at three hours post-exercise may have been too soon, thus missing a peak in hepcidin and/or inflammation occurring 1-3 hours later (Peeling et al. 2009; Kerasioti et al. 2013; Peeling et al. 2014; Schroer et al. 2014). This combination of factors could possibly explain the current findings.

Significant increases in serum ferritin and serum iron were reported from baseline to post-exercise in the current study (Table 2), which has been commonly observed in prior studies (Peeling et al. 2008; Peeling et al. 2009; Sim et al. 2012; Marc et al. 2013; Badenhorst et al. 2014; Peeling et al. 2014; Badenhorst et al. 2015a). Exercise-induced inflammation has a robust effect on the reticuloendothelial system, which is comprised of monocytes, macrophages and precursor cells (Knutson and Wessling-Resnick 2003) contributing to iron recycling and storage. As erythrocytes become damaged, the cell membrane containing stored ferritin becomes impaired thus causing a leakage into circulating plasma (Pattini et al. 1990). This leads to an increase in ferritin reuptake and reticuloendothelial cells then recycle ferritin via the spleen, liver and bone marrow (Knutson and Wessling-Resnick 2003). In addition to the above, exercise-
induced dehydration has been shown to decrease plasma volume which subsequently increases the concentration of circulating proteins (Jimenez et al. 1999; Reljic et al. 2013). Body mass decreased by 0.6±0.4 kg (~500-600 mL of body water or 1.0±1.0%) during each experimental visit, indicating that there may have been some hemo-concentration. However, since we did not monitor fluid intake during and after the bouts, we cannot fully quantify the effects of hydration on the biomarkers (Maughan and Shirreffs 2010).

There was a significant decrease in serum ferritin, Hb and hematocrit seen three hours post-exercise (Table 2) which could be attributed to the reticuloendothelial system clearance mechanism of both serum ferritin and free Hb from the circulating plasma, and thus, increasing ferritin stores in the liver (Nemeth et al. 2004; Nemeth and Ganz 2006) or the impact of hemo-dilution / rehydration. Furthermore, elevated levels of hepcidin have been shown to prevent iron recycling (and ferritin release) by macrophages (Nemeth et al. 2004), which could also contribute to the marked decrease in serum iron and serum ferritin levels three hours post-exercise in the current study for VPRO, PRO and PLA.

Vitamin D supplementation in healthy individuals (Bacchetta et al. 2014; Smith et al. 2016) and individuals suffering from chronic kidney disease (Zughaier et al. 2014) has led to a marked decrease in hepcidin response following acute and chronic dosing. Doses 20- and 50-fold higher than that used in our study have shown a 34% and 73% reduction in hepcidin concentrations 24 hours after supplementation, respectively (Bacchetta et al. 2014; Smith et al. 2016). In chronic kidney disease patients, 7,143 IU/day of vitamin D₃ for three months decreased hepcidin concentrations by 28% (Zughaier et al. 2014). Furthermore, Barker and his colleagues (2013) showed that 4,000 IU/day of vitamin D over 28 days significantly decreased inflammatory biomarkers in moderately active adult males at 24, 48, 72 and 168 hours following the exercise
bouts. The current study looked at the acute effects of vitamin D\textsubscript{3} and K\textsubscript{2} supplementation within a three-hour time frame and at a much lower dose. No differences in IL-6 or hepcidin concentrations were found for VPRO drink when compared to PRO or PLA. Thus, lower dose or the acute time frame could have accounted for the lack of effect of these supplements in the current study. A longer time course, or chronic supplementation might have been necessary to detect a significant effect.

In the current study, baseline values of vitamin D had no significant effect on post-exercise hepcidin response in the VPRO condition. However, only 10% of our subjects were deficient in 25(OH)D, whereas 41% of Canadian males in this age group are deficient (Canada 2015). These more favourable values in the study sample could be related to dietary factors or to the time of year when data collection occurred (the end of the outdoor cycling season). Thus, these higher 25(OH)D concentrations seen in our study population could have mitigated the effects of supplemental vitamin D.

Lastly, vitamin K supplementation has been shown to blunt the increase inflammatory cytokine IL-6 in \textit{in vitro} studies in rat models, isolated cell cultures and obese humans (Reddi et al. 1995; Ohsaki et al. 2006; Shea et al. 2008a; Shea et al. 2008b). Our results did not show any significant difference in IL-6 or hepcidin when vitamin K\textsubscript{2} was combined with vitamin D\textsubscript{3} supplementation. Thus, extrapolation to healthy and highly fit males may not be warranted based on the findings of the study (Tappy et al. 1994; Reagan-Shaw et al. 2008; Sharma and McNeill 2009).

**Strengths and Limitations**

The current study has multiple strengths and limitations. The triple cross-over nature allowed us to mitigate the discrepancies commonly seen between individuals. Additionally, the study was
adequately powered *a priori*, substantiating the negative finding of the current study. Furthermore, the athletic profile of the subjects makes the findings of this study highly applicable to the highly-trained population, indicating that the timing and constituents of the post-exercise drinks may not help athletes enhance the absorption of iron zero to three hours following the cessation of intense training sessions. A limitation of this study was that it was only performed on male participants, since the menstrual cycle and estradiol affect the post-exercise hepcidin response (Sim et al. 2014). Secondly, greater control over diet and daily activity during the 24-hour period prior to the visits could have reduced confounding variables in our data. Instead, our subjects might have started the study with slightly depleted or fully replenished glycogen stores, especially due to the CHO-dense pre-workout meal, subsequently affecting the post-exercise inflammatory response. Lastly, although subjective reports indicated that the subjects were blinded to the beverage they consumed at each trial, this was not systematically measured, and thus they may not have been fully blinded.

**Conclusions**

The consumption of a CHO and protein-rich drink with or without the addition of vitamin D$_3$ and K$_2$ had no significant effects on hepcidin-25, IL-6, Hb, hematocrit, serum ferritin or serum iron in the present study. This may be due to a combination of the very high level of fitness of the subjects or the fact that they were fed a high CHO meal prior to each trial. Nevertheless, hepcidin and IL-6 response in the current study were characteristic of normal post-exercise inflammatory responses in trained individuals (Ward and Kaplan 2012; Wachsmuth et al. 2015). Thus, regardless of the interventions used in the current study, individuals could still be susceptible to iron malabsorption post-exercise. Future research needs to revisit the effectiveness
of peri-workout consumption, the constituents and dose of macro- and micro- nutrient protocols from both an acute and chronic standpoint.

Conflict of Interest

All authors declare no conflict of interest.

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The authors would like to thank the subjects who partook in this study. We would also like to thank Dr. Ted Steiner and the impeccable work performed by May Wong who ran the ELISA’s for hepcidin-25 and interleukin-6.

Compliance with Ethical Standards

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Table 1. Mean ± SD heart rate (HR), energy expenditure (Kcal), rating of perceived exertion (RPE), power output (Watts) and pre- and post-exercise mass in the whey protein isolate + carbohydrates drink (PRO), whey protein isolate + carbohydrates + vitamins D₃ and K₂ drink (VPRO) and non-nitrogenous zero calorie control drink (PLA) conditions.

<table>
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<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>PRO</td>
<td>169 ± 9</td>
<td>269 ± 33</td>
<td>3.7 ± 0.3</td>
<td>942 ± 117</td>
<td>427 ± 43</td>
<td>8.7 ± 1.3</td>
<td>72.2 ± 6.2</td>
<td>71.7 ± 6.2</td>
<td>-0.58 ± 0.38</td>
</tr>
<tr>
<td>VPRO</td>
<td>170 ± 11</td>
<td>278 ± 31</td>
<td>3.8 ± 0.3</td>
<td>971 ± 107</td>
<td>430 ± 46</td>
<td>8.6 ± 0.9</td>
<td>72.3 ± 6.6</td>
<td>71.7 ± 6.4</td>
<td>-0.60 ± 0.36</td>
</tr>
<tr>
<td>PLA</td>
<td>169 ± 10</td>
<td>270 ± 31</td>
<td>3.8 ± 0.3</td>
<td>946 ± 109</td>
<td>427 ± 55</td>
<td>9.0 ± 1.3</td>
<td>72.1 ± 6.4</td>
<td>71.6 ± 6.4</td>
<td>-0.51 ± 0.29</td>
</tr>
</tbody>
</table>

Note: Results are presented as (mean ± SD). § = Significant difference (p < 0.05) from Baseline to Post-exercise. SD, standard deviation; BPM, beats per minute; kg, kilograms; Kcal, kilocalories; VPRO, whey protein isolate + carbohydrates + vitamins D₃ and K₂ drink; PRO whey protein isolate + carbohydrates drink, PLA, non-nitrogenous zero calorie control drink.
Table 2. Mean ± SD for interleukin-6, hepcidin-25, serum iron, serum ferritin, hemoglobin and hematocrit levels at baseline, post-exercise, and three hours post-exercise in the whey protein isolate + carbohydrates drink (PRO), whey protein isolate + carbohydrates + vitamins D₃ and K₂ drink (VPRO) and non-nitrogenous zero calorie control drink (PLA) conditions.

<table>
<thead>
<tr>
<th>Blood Biomarker</th>
<th>PRO</th>
<th>VPRO</th>
<th>PLA</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Post-exercise</td>
<td>3 hr Post-exercise</td>
</tr>
<tr>
<td>Serum Iron (mcg mol L⁻¹)</td>
<td>21.30 ± 5.08</td>
<td>23.00 ± 5.39 §</td>
<td>18.80 ± 5.22 *Δ</td>
</tr>
<tr>
<td>Serum Ferritin (mcg L⁻¹)</td>
<td>101.10 ± 85.17</td>
<td>108.30 ± 85.38 §</td>
<td>104.20 ± 84.27 *Δ</td>
</tr>
<tr>
<td>Serum IL-6 (pg mL⁻¹)</td>
<td>0.92 ± 1.70</td>
<td>3.79 ± 2.94 §</td>
<td>1.64 ± 1.98 *Δ</td>
</tr>
<tr>
<td>Hemoglobin (mg L⁻¹)</td>
<td>151.50 ± 8.18</td>
<td>154.40 ± 7.68 §</td>
<td>150.44 ± 10.36 *</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.43 ± 0.02</td>
<td>0.44 ± 0.02 §</td>
<td>0.43 ± 0.02 *</td>
</tr>
</tbody>
</table>

Note: Results are presented as (mean ± SD). § = Significant difference (p < 0.05) from Baseline to Post-exercise, * = Significant difference (p < 0.05) from Post-exercise to 3 hr Post-exercise, Δ = Significant difference (p < 0.05) from Baseline to 3 hr Post-exercise. SD, standard deviation; hr, hour; mcg, micrograms; mol, mole; L, liter; mL, milliliter; pg, pictograms; VPRO, whey protein isolate + carbohydrates + vitamins D₃ and K₂ drink; PRO, whey protein isolate + carbohydrates drink; PLA, non-nitrogenous zero calorie control drink.