The Effects of Molecular Hydrogen Supplements on Acid-Base Status and Skeletal Muscle Oxidative Metabolism during Exercise in Humans

ヒトにおける運動時の酸塩基平衡と骨格筋酸化代謝に及ぼす水素サプリメントの効果

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Abstract

Molecular Hydrogen (H_2) is an anti-apoptotic and anti-inflammatory gas without toxicity, it has multiple routes of administration, such as hydro-calcium powder (HCP), which utilizes calcium to absorb highly concentrated hydrogen. Very few research examined H_2 during sport or physical performance, however, they demonstrated no side effects of H_2 and showed a positive trend of effect on exercise and/or recovery from physical exhaustion. The research purpose was to investigate the effect of H_2 on acid-base status and skeletal muscle oxidative metabolism during exercise in humans.

Two experiments were conducted with both an aerobic and anaerobic exercise models, using a randomized, double-blind, crossover design, subjects either received HCP or H_2-depleted placebo before exercise. During which measurements of muscle deoxygenation (deoxy[Hb + Mb]) and tissue O_2 saturation (S_tO_2) was determined via time-resolved near-infrared spectroscopy (TR-NIRS) in the vastus lateralis (*VL*) and rectus femoris (*RF*). Blood gases' pH, partial pressure of CO_2 (PCO_2), and bicarbonate ion (HCO_3^-) concentrations measurements were simultaneously carried out.

Starting with the aerobic exercise model, we investigated effects of HCP during incremental exercise. Eighteen healthy, trained subjects consumed HCP (1500 mg/day, 2.544 µg/day of H_2) or placebo (1500 mg/day) for three consecutive days. They performed a cycling incremental exercise starting at 20 watts (W), increasing by 20 W/2 min until exhaustion. In addition to TR-NIRS, pulmonary gas exchange parameters including pulmonary ventilation ($\dot{V}_E$), oxygen uptake ($\dot{V}O_2$), and CO_2 output ($\dot{V}CO_2$) were measured, blood was sampled at rest and 120-, 200-, and 240-W.

At rest, HCP had a significantly lower $\dot{V}_E$, $\dot{V}O_2$, $\dot{V}CO_2$, and pH and a significantly higher HCO_3^-, PCO_2. During incremental exercise, the significant decrease in pH and greater HCO_3^- continued, and HCP increased absolute values of deoxy [Mb+Hb] at the *RF* but not the *VL* muscle. HCP induced a significantly lower O_2 delivery/utilization ratio (i.e., an increased O_2 extraction) in the *RF*, which may be due to the greater recruitment patterns of fast-twitch muscle fibers in the *RF*. Additionally, $\dot{V}_E$ was significantly lower at some work rates in HCP, the lower $\dot{V}_E$ response to pH is likely a result of HCP and H_2 antioxidants effects. Finally, no change in overall exhaustive performance time between groups was present. Therefore, we demonstrated that the implemented HCP dose for 3 days, might not have been suitable for aerobic incremental exercise performance.

Nevertheless, as HCP effect is most prominent with higher recruitment of fast-twitch muscle fibers which are dominant in anaerobic protocols, we saw potential of HCP in improving performance in high intensity anaerobic exercise. Therefore, the research proceeded to testing using an anaerobic exercise model, and we examined the acute effects of single-dose of HCP during rest, high-intensity

intermittent training (HIIT) performance, and the recovery.

Ten healthy, trained subjects received a single dose of HCP (2.544 μg of H_2) or placebo 1 h pre-exercise. They performed six bouts of 7 sec all-out pedaling (HIIT) at 7.5% of body weight separated by 40 sec active pedaling intervals, followed by a passive rest recovery period. Blood samples were collected at rest, and TR-NIRS values were measured from rest to recovery.

At rest, HCP had a significantly higher PCO_2 and HCO_3^- and a slight tendency toward acidosis. During HIIT exercise, the first HIIT bout's peak power was significantly higher in HCP vs. Placebo, and HCP had a notable effect on significantly increased deoxy[Hb + Mb] concentration during HIIT, despite no differences in heart rate response. The HCP group showed significantly greater O_2 extraction in the *VL* and microvascular (Hb) volume in the *RF* during HIIT. The HIIT exercise provided significantly improved blood flow and muscle reoxygenation states in both the *RF* and *VL* muscles during passive recovery compared to rest in both groups. The HCP supplement might exert ergogenic effects on high-intensity exercise and prove advantageous for improving anaerobic exercise performance.

In conclusion, H_2 using HCP acts on blood gas directly by inducing greater PCO_2 and HCO_3^- and by extension effecting pH. Moreover, HCP influences lower ventilation (e.g., hypoventilation) and chemosensitivity of ventilatory response to acid-base status. HCP as well impacts muscle spatial heterogeneity responses and O_2 delivery/utilization in the *RF* and *VL* muscles during exercise. Finally, HCP supplement might exhibit ergogenic effects on high intensity exercise and prove advantageous for improving anaerobic exercise performance.

Research Achievements

Papers

Alharbi, A.A.D.; Ebine, N.; Nakae, S.; Hojo, T.; Fukuoka, Y. Application of molecular hydrogen as an antioxidant in responses to ventilatory and ergogenic adjustments during incremental exercise in humans. Nutrients. 2021, 13, 459. doi:10.3390/nu13020459.

Alharbi, A.A.D.; Iwamoto, N., Ebine, N.; Nakae, S.; Hojo, T.; Fukuoka, Y. The acute effects of a single dose of molecular hydrogen supplements on responses to ergogenic adjustments during high-intensity intermittent exercise in humans. Nutrients. 2022, 14, 19. doi.org/10.3390/nu14193974.

International Conference

Alharbi, A.A.D.; Ebine, N.; Nakae, S.; Hojo, T.; Fukuoka, Y. The effects of hydrogen supplement on cycling performance during aerobic and anaerobic exercise in humans. A pilot study. NUTRITION Live Online, 2021. American Society for Nutrition June 7-10, 2021.

Conflict of Interest Statement

The author would like to disclose that this research received financial support from ENAGEGATE Inc. In addition, the company manufactured and provided both the hydro-calcium powder (HCP) supplements and placebo capsules for the experiment. However, the author would like to emphasize that they are not an employ of ENAGEGATE Inc., nor did they receive any payment from them. There was no intercession, restrictions or agreements imposed between the two parties regarding research design, results, or publications. The research was conducted in compliance with scientific research objectivity and integrity standards. In addition, all the information regarding HCP in this research was obtained through private documents provided by the manufacturing company ENAGEGATE Inc.

Table of Contents

Abstract ..... **I**

Research Achievements ..... **III**

Conflict of Interest Statement ..... **IV**

Table of Contents ..... **V**

List of Abbreviations ..... **VII**

Chapter 1. Introduction **1**

Chapter 2. General Purpose **3**

Chapter 3. Methodology **4**

3.1 Subjects 4

3.2 Supplements 4

3.3 Experimental Overview 4

3.4 Blood Testing and Analyses of Blood Metabolites 5

3.5 Measurement of Muscles Oxygenation Statues with TR-NIRS 5

Chapter 4. Aerobic Exercise: Experiment 1 ..... **7**

4.1 Experiment Rationales and Hypothesis 7

4.2 Methods & Materials 7

4.3 Result 9

4.4 Discussion 11

4.5 Conclusion 15

Chapter 5. Anaerobic Exercise: Experiment 2 ..... **16**

5.1 Experiment Rationales and Hypothesis 16

5.2 Methods & Materials 16

5.3 Result 18

5.4 Discussion 20

5.5 Conclusion 23

Chapter 6. General Conclusions **24**

Acknowledgment **25**

References **26**

Tables and Figures **33**

Appendices ..... **i**

Appendix A: HCP and Placebo Additional Information i

Appendix B: Preliminary Experimental Data from the Manufacturer iii

Appendix C: Preliminary Internal Experimental Data v

List of Abbreviations

- Anion gap with potassium: AGapK
- Anion gap: AGap
- Base excess in blood: BE (b)
- Base excess in extracellular fluid: BE (ecf)
- Bicarbonate: HCO_3^-
- Body mass index: BMI
- Body temperature and pressure, saturated: BTPS
- Calcium: Ca
- Carbon dioxide output: $\dot{V}CO_2$
- Cerebral blood flow: CBF
- Chloride: Cl
- Deoxyhemoglobin and deoxymyoglobin: deoxy[Hb + Mb]
- Heart rate: HR
- Hemoglobin: Hb/Hgb
- High intensity intermittent test: HIIT
- Hour: h
- Hydro-calcium powder: HCP
- Hydrogen: H_2
- Hydrogen-rich water: HRW
- Lactate: Lac
- Minute: min
- Oxidative stress: OS
- Oxygen saturation: SO_2
- Oxygen uptake: $\dot{V}O_2$
- Oxyhemoglobin and oxymyoglobin: oxy[Hb + Mb]
- Partial pressure of carbon dioxide: PCO_2
- Partial pressure of oxygen: PO_2
- Peak power: PP
- Potassium: K
- Reactive oxygen species: ROS
- Rectus femoris: *RF*
- Respiratory exchange ratio: R
- Second: sec
- Sodium: Na
- Standard temperature and pressure, dry: STPD
- Time-resolved near infrared spectroscopy: TR-NIRS
- Tissue oxygen saturation: S_tO_2
- Total carbon dioxide: TCO_2
- Total hemoglobin and myoglobin: total[Hb + Mb]
- Vastus lateralis: *VL*
- Ventilation: $\dot{V}_E$
- Watt: W

Chapter 1. Introduction

Nutritional supplements or nutritional ergogenic aids, such as bicarbonates and dietary antioxidants has been of great interest in sports or different types of exercise. As athletes usually engage in high-intensity exercise and exercise training, energy demands and oxygen consumption increase causing the production of reactive oxygen species (ROS) and reactive nitrogen species which increases oxidative stress (OS) (Aoki et al., 2012). OS is related to prolonged recovery and exercise-induced fatigue, and antioxidant supplementation can decrease these OS biomarkers and improve muscular performance in humans (Ostojic, 2014; Lebaron et al., 2019).

One of these powerful anti-oxidants is molecular hydrogen (H_2) or diatomic hydrogen, a tasteless, odorless, flammable gas. Which also possesses anti-apoptotic and anti-inflammatory properties without toxicity (Ohta, 2014; Ostojic, 2014; Ichihara et al., 2015). H_2 is also a minimal molecule that can quickly diffuse through the alveoli into the blood and circulate throughout the body during breathing. It can penetrate the cellular membrane and rapidly diffuse into cell organelles, playing major biological roles. Moreover, H_2 can easily pass through the blood–brain barrier where most antioxidant compounds cannot (Tian et al., 2021). H_2 possesses a selective free radical and inflammation scavenging ability, it is thought to have no effect on physiologically reactive species (e.g., H_2O_2), since it can selectively reduce hydroxyl radical and peroxynitrite (Ohsawa et al., 2007). Additionally, H_2 has minimal side effects as it is excreted by exhaling through the lungs, it has multiple routes of administration, such as oral intake of H_2-rich water (HRW), H_2 bathing, intravenous infusion of H_2-saline, and inhalation of H_2 gas.

Molecular hydrogen research has been increasing progressively, most of this research demonstrated the effects of H_2 on disease models, human diseases, treatment-associated pathologies, and pathophysiological conditions of plants (Ichihara et al., 2015). Nevertheless, the potential applications and benefits of H_2 during exercise were yet to be investigated and thus it started to gather a lot of interest lately. Even though the mechanism of its effect is still unclear, some studies demonstrated no side effects of H_2 when we ingested and showed a positive effect on exercise and recovery by increasing blood pH or decreasing lactate production (Aoki et al., 2012; Ostojic, 2014; Kawamura et al., 2020). These findings support the potential of H_2 as a possible ergogenic aid in exercise and sport performance. However, these studies mostly used H_2 in the forms of gas or infused water, which in these forms might be difficult for the whole body to effectively absorb and utilize due to the gas escaping from the water (through the gas bubbles dispersing), or from the gastrointestinal track (through belching for example) before absorption. To solve these problems, the concept of

manufacturing and utilizing HCP, that utilizes calcium extracted to absorb highly concentrated hydrogen molecules (**International Patent No. 4472022**), was established. HCP claims to differ from HRW in terms of stability that the powder remains dry longer, hydrogen molecules will remain attached in a stable manner. In addition, the timing and amount of hydrogen gas released, as HCP states to have continuous H_2 release. With HRW, the amount of H_2 that can be dissolved in the water is about 0.8 mM or 1.6 mg/L at standard ambient temperature and pressure (Ohta, 2014; Lebaron et al., 2019), and most HRW products seems to have been standardized to a hydrogen concentration of 1–1.3 mg/L (Ostojic, 2014), meanwhile 1 g of HCP contains approximately 1.7 μg of H_2. Even though HCP has a comparably smaller amount of H_2 than HRW, it promises a continuously H_2 release and possibly a better H_2 delivery as HCP releases H_2 directly in the intestinal lumen, which might make HCP a more convenient and constant method of administrating H_2 to the body. At the present time, as the more efficient method of ingesting H_2 is yet to be established, H_2 research using a stable H_2 releasing form such as HCP are inevitable.

Chapter 2. General Purpose

The purpose of this research was to investigate the effect of molecular hydrogen supplements in the form of hydro-calcium powder/capsules on acid-base status and skeletal muscle oxidative metabolism during aerobic and anaerobic exercises in humans.

To the best of my knowledge, there has been no research examining the effect of H_2 in the form of HCP during exercise in humans.

Chapter 3. Methodology

In this study, the effect of hydrogen supplements in the form of hydro-calcium powder was examined on acid-base status and skeletal muscle oxidative metabolism during exercise through double-blind, crossover, placebo-controlled trials.

3.1 Subjects

Healthy university students who meet the criterion of being athletic, male, with no preexisting medical conditions, injuries, or metabolic abnormalities. Written informed consent was obtained from all subjects after a detailed explanation of the purpose of the study, procedures, possible risks, and benefits of participation. The study conformed to the Declaration of Helsinki, and the ethical committee of Doshisha University approved all the study procedures (No. 18012).

3.2 Supplements

Supplements and placebo were provided by the manufacturing company ENAGEGATE Inc. and handed over in an indistinguishable manner to insure a double-blind design. The supplements and placebo were revealed after the completion of the data analysis and the summarizing of the results. Capsules for both HCP and placebo were identical with clear outer shells and white powder fill inside. Each capsule weighted approximately 633 mg, with 375 mg of either HCP powder for the supplement or only calcium powder for the placebo. In HCP hydrogen amount was 0.636 μg/capsule. For additional details regarding HCP and placebo, please refer to Appendix A (page i).

3.3 Experimental Overview

The subjects were familiarized with all measurement techniques prior to starting the experiment, and testing sessions were separated by an appropriate washout/rest period. On the day prior to testing, the consumption of alcohol and caffeine was prohibited, and when the exercise or training was carried out, the intensity, the timing, and duration were matched for both experiments. To avoid the effect of nutrient consumed on the exercise results, the subjects were also instructed to record and replicate their dietary intake for dinner the previous night and for breakfast before the testing. They were also instructed to have over 6 hours of sleep to ensure enough rest.

On experiment day, Subjects were instructed to come to the camps by bus or by walking in a relaxed pace. They consumed breakfast at home ≥ 6 h prior to the start of the experiment (and were

instructed to replicate it from their dietary intake report). At 3 h before the experiment, the subjects ate a small meal consisting of the Calorie Mate (four blocks, Otsuka Pharmaceutical, Tokyo, Japan) and one bottle of caffeine-free barely tea (Healthy Mineral Barley Tea, 600 mL, ITO EN, Tokyo, Japan) that was standardized for all subjects, in order to avoid hunger and minimize fluctuations in significant blood metabolic parameters (specifically blood glucose) among/within subjects.

Upon arrival to the laboratory, a short interview was conducted to confirm if the health condition was experiment appropriate. All sessions were completed in Doshisha University in a custom-made environmental chamber (LP-2.5PH-SS, NKsystem, Osaka, Japan) maintained at a temperature of 25 ˚C with 50% relative humidity with minimal external stimuli. Height and weight were measured using a digital height meter (DSN-90, Muratec-KDS, Kyoto, Japan), and a body composition meter Inner Scan V (BC-612, Tanita, Tokyo, Japan) respectively.

3.4 Blood Testing and Analyses of Blood Metabolites

A peripheral venous blood catheter was placed by a certified nurse in the subject's forearm to allow free movement of the elbow and hands during the experiment. Using a 22G indwelling needle (BD Insyte Autoguard BC, BD, Tokyo, Japan) a 0.4 ml tube line (Surplug extension tube, Terumo, Tokyo, Japan). A 1 ml blood sample was collected using a 2.5 ml small lock type syringe (Terumo syringe, Terumo, Tokyo, Japan). Between blood draws the tube was flushed with saline (Otsuka normal saline, Otsuka Pharmaceutical, Tokyo, Japan) to avoid blood clotting, and after the saline flush, a 1 ml of fluid/blood was be removed before taken a viable blood sample. The blood samples were analyzed for blood gas, electrolytes, and the metabolic profile with a portable blood analysis system (epoc®, Siemens Healthcare, Tokyo, Japan) for the determination of blood gases, acid status of pH, the partial pressure of O_2 (PO_2), the partial pressure of CO_2 (PCO_2),bicarbonate (HCO_3^-), base excess of the extracellular fluid (BE(ecf)), base excess in the blood (BE(b)), hemoglobin (Hgb), and hematocrit (Hct). The metabolic status of lactate (Lac), glucose (Glu), creatinine (Crea), and serum electrolytes including the sodium (Na^+), potassium (K^+), chloride (Cl^-) concentrations, and the Aniongap (AGap) and Aniongap with potassium (AGapK) were also measured for all subjects.

3.5 Measurement of Muscles Oxygenation Statues with TR-NIRS

The absolute values of oxygenated (oxy[Hb + Mb]) and deoxygenated (deoxy[Hb + Mb]) and the total hemoglobin and myoglobin concentration (total[Hb + Mb]) were sampled from the vastus lateralis (*VL*) and rectus femoris (*RF*) muscles of the subject's dominant leg by a time-resolved near-infrared

spectroscopy (TR-NIRS) system (C12707, Hamamatsu Photonics, Hamamatsu, Japan).

This system measures the distribution of in vivo optical path lengths, thereby enabling the determination of the absolute [Hb + Mb] concentration ($\mu mol \cdot L^{-1}$). The deoxygenation measured by the TR-NIRS was demonstrated to correlate significantly with the oxyhemoglobin saturation in both the blood and a purified-hemoglobin phantom solution (Hamaoka et al., 2000; Ijichi et al., 2005; Chin et al., 2011). The optodes were housed in an elastic flexible black thigh sleeve that prevented light penetration to the optodes and ensured that their positions were fixed during the experiment protocol without compromising the subjects' free movements, thus ensuring that the position of the optodes was fixed and invariant. The distal optodes were placed on the lower third of the *VL* and *RF* muscles parallel to the major axis of the thigh. The location of the distal optodes on the *VL* muscle was chosen to represent the single-site TR-NIRS measurement conducted in previous studies (Wilkerson et al., 2004; Ferreira et al., 2005; Ferreira, Koga and Barstow, 2007). The proximal optode pairs on the *VL* and *RF* muscles were located ~10–15 cm from the distal optode pairs. The interoptode spacing between the emitter and the receiver was 3 cm. The depth of the measured area was assumed to be approximately one-half of the distance between the emitter and the receiver, ~1.5 cm.

The skin under the probes was carefully shaved. Pen marks were made on the skin to indicate the margins of the sleeve to check for any downward sliding of the probe during cycling and for accurate probe repositioning on subsequent days. No sliding was observed in any subject at the end of each protocol. The principles of operation and the algorithms used by the equipment are described in detail elsewhere (Oda et al., 1999; Ohmae et al., 2006). Calibration of the instrument was performed before each test by measuring the response when the input and receiving fibers faced each other through a neutral-density filter in a black tube. At the end of the exercise test, pen marks were made on the subject's skin to indicate the margins of the TR-NIRS optode sleeve to reposition the probes for subsequent laboratory visits. The adipose tissue thickness (ATT) and muscle thickness were measured using B-mode ultrasound (SSD-3500SV, Hitachi-Aloka Medical, Tokyo, Japan); or (Aixplorer Multiwave™, Supersonic Imagine, Aix-en-Provence, France) with the subject at rest and seated in an upright position. To quantify the influence of ATT on dynamic changes in TR-NIRS signals, we used the ATT correction method of Bowen et al. (2013). With this method, the tissue O_2 saturation (S_tO_2) was calculated using oxy[Hb +Mb]/total[Hb + Mb].

Chapter 4. Aerobic Exercise: Experiment 1

4.1 Experiment Rationales and Hypothesis

In investigations of the relationship between muscle O_2 delivery (QO_2) and oxygen uptake ($\dot{V}O_2$), TR-NIRS was able to resolve the absolute values of [heme] chromophores (Koga et al., 2011) and provided improved mechanistic insights into the effects of HCP on muscle QO_2-to-$\dot{V}O_2$ relationships. As it is also presently unclear whether dietary HCP supplementation might alter the muscle spatial heterogeneity in muscle deoxygenation (deoxy[Hb + Mb]) kinetics (and by extension, QO_2-to-$\dot{V}O_2$ matching) that was observed during cycling exercise (Koga et al., 2007; Koga et al., 2011; Spencer et al., 2014; Fukuoka et al., 2015; Okushima et al., 2015; Okushima et al., 2016). HCP also may increase HCO_3^- and delay metabolic acidosis. And since HCP causes opposite effects at the same time, the extent of change that HCP causes in the muscle spatial heterogeneity in deoxy[Hb + Mb] kinetics at different muscle sites is not yet known.

H_2 is expected to be a potential scavenger of superoxide anions, and as it was reported that the augmented hypoxic carotid body sensory response was abolished in a group of rats pretreated with a superoxide dismutase mimetic (Peng et al., 2003; Peng and Prabhakar, 2004). Wilkerson et al. (2008) also demonstrated that the intravenous administration of a superoxide anion scavenger to anesthetized, vagotomized, and ventilated rats before their exposure to acute hypoxia abolished the long-term facilitation (LTF) of phrenic nervous activity. Given these findings in animals, one of the possibilities is that ROS are downregulated by the administration of molecular hydrogen, resulting in hypoventilation and blood hypercapnia. We, therefore, hypothesized that the administration of a strong antioxidant of molecular hydrogen would reduce the magnitude of ventilatory and deoxy[Hb + Mb] responses before and during exhaustive exercise in humans.

To test these contradictory hypotheses, we conducted the present study to determine whether an HCP supplement causes different responses to muscle deoxy[Hb + Mb] and the responses of pulmonary ventilatory and blood gas values at both rest and incremental exercise compared to placebo.

4.2 Methods & Materials

4.2.1 Subjects

Eighteen healthy males. Their mean ± standard deviation (SD) values were age, 21 ± 1 years old; height, 174.0 ± 4.4 cm; and body weight, 66.6 ± 6.3 kg. For additional details regarding subjects' recruitment criteria and participation consent please refer to Chapter 3, section 3.1.

4.2.2 Supplements Protocol

Supplements protocol was 4 capsules/day for 3 days. Subjects were instructed to consume supplements in 1 dose of 4 capsules at 21 o'clock (± 1 Hour) at the same time every day for the 3 days prior to the experiment day, amounting to a total of H_2 of 2.544 μg/day. For additional details regarding supplement and placebo composition please refer to Chapter 3, section 3.2.

4.2.3 Experimental Protocol & Measurements

For full details regarding experimental overview please refer to Chapter 3, section 3.3. Familiarization test was conducted 1–2 weeks prior to the start of the experiments, and the experiments were separated by a six- to seven-days washout/rest period.

In this experiment, each subject's pulmonary gas exchange was measured *breath-by-breath* throughout all tests as described (Fukuoka et al., 2015; Fukuoka et al., 2017; Ebine et al., 2018). The *breath-by-breath* gas exchange system (AE-310s, Minato Medical Sciences, Osaka, Japan) was calibrated according to the manufacturer's recommendation before each exercise test.

The subject breathed through a lower-resistance mouthpiece connected to a hot wire flowmeter for the measurement of inspiratory and expiratory flow and volume. Inspired and expired gases were continuously sampled from the subject's mouth, and the O_2 and CO_2 fractional concentration were measured by fast-responding paramagnetic and infrared analyzers, respectively. The gas volume and concentration signals were time-aligned to account for the time lag between the signals for the calculation of the gas exchange parameters on a breath-by-breath basis. Alveolar gas exchange variables were calculated according to the algorithms of Beaver, Lamarra and Wasserman (1981). The *breath-by-breath* $\dot{V}_E$ (BTPS), $\dot{V}O_2$ (STPD), $\dot{V}CO_2$ (STPD), gas exchange ratio (R), and end-tidal CO_2 pressure ($P_{ET}CO_2$) were determined.

The electrocardiogram (ECG), taken from a V5 lead, was monitored continuously on a wireless ECG monitor (DS-2150; Fukuda Denshi, Tokyo, Japan), and subject's heart rate (HR) was measured by beat-by-beat counting of the R-spike of the ECG taken simultaneously with the other measurements.

Finally, for full details regarding blood analysis and TR-NIRS measurements please refer to Chapter 3, section 3.4 and 3.5 respectively.

4.2.4 Exercise Protocol

The subjects performed an incremental exercise test using a cycle ergometer (75XL-III; Konami, Tokyo, Japan). The exercise started with 2 min at the workload of 20 W, after which the workload

was increased at 20 W/2 min until the subject's exhaustion, or 300 W was reached. The subjects were instructed to maintain their pedal frequency at 60 rpm throughout the exercise. When given criteria were met (e.g., a plateau or a drop in $\dot{V}O_2$, a HR > 95% of the age-predicted maximum (Fabre et al., 2013), or a respiratory exchange ratio > 1.1), the highest average value of 1-min $\dot{V}O_2$ was regarded as the individual's peak oxygen uptake (Green et al., 2009).

4.2.5 Sampling and Data Analysis

The peak values for gas exchange/ventilation were detected and then averaged for 30 sec at the peak. Similarly, the baseline (resting) values of gas exchange/ventilation were calculated as the mean value over the final 30 sec of the rest period. The output frequency of both TR-NIRS systems was set to 1 Hz and averaged post hoc to increase the signal-to-noise ratio, providing one measurement every 5 sec. The baseline of each TR-NIRS measurement was calculated as the mean value of the 30 sec prior to the start of the incremental exercise.

The absolute values of gas exchange/ventilation and absolute muscle deoxy[Hb + Mb], oxy[Hb + Mb], and total[Hb + Mb] measurements were then calculated every 20 W from 20 W to the maximal exercise for each subject. The value for each variable at each 20 W increment was calculated as the last 60 s from the initial 20 W to 240 W. Blood gas and metabolic profiles were obtained three times (at 120, 200, and 240 W). Since there were individual differences in the exhausted exercise duration despite the absence of a difference between the HCP and placebo groups, data arrangements were made at up to 240 W of each blood gas sample.

4.2.6 Statistical Analysis

All data are expressed as the mean ± SD and were analyzed using the statistical package IBM SPSS, PC program, ver. 25.0 (IBM, Tokyo, Japan). The peak values of gas exchange, overall exercise time, and mean values of parameters at rest between the HCP and placebo groups was compared using a paired t-test. The significance of differences in each variable was determined by a two-way analysis of variance (ANOVA), comparing supplements (HCP and placebo) × work rates (20–240 W). A post hoc comparison was applied by Bonferroni test for the appropriate data sets when a significant F-value was obtained. Probability (p)-values < 0.05 were considered significant.

4.3 Result

4.3.1 At Rest

The mean values of the ventilatory, acid-base, and TR-NIRS profiles are presented in Table 1. The

mean values of the ventilatory parameters displayed a significantly lower $\dot{V}_E$ (HCP: 11.8 ± 3.1 vs. placebo: 13.2 ± 3.2 L·min^{-1}, $p = 0.015$), $\dot{V}O_2$ (HCP: 355 ± 109 vs. placebo: 429 ± 136 mL·min^{-1}, $p = 0.009$), and $\dot{V}CO_2$ (HCP: 306 ± 96 vs. placebo: 364 ± 130 mL·min^{-1}, $p = 0.027$) in the HCP group compared to the placebo group, whereas no significant difference in HR or R was observed between the HCP and placebo groups.

With the blood gas status related to metabolism, the HCP group showed the following significant differences from the placebo group: lower pH (HCP: 7.356 ± 0.042 vs. placebo: 7.376 ± 0.039, $p = 0.048$) and higher PCO_2 (52.4 ± 8.3 vs. 47.4 ± 8.2 mmHg, $p = 0.026$) and HCO_3^- (29.1 ± 2.2 vs. 27.5 ± 2.6 mmol·L^{-1}, $p = 0.041$). The metabolic profile as Lac or Glu was within the standard range at rest with no significant difference between the HCP and placebo groups. The TR-NIRS profiles at the *RF* muscle revealed significantly higher deoxy[Hb + Mb] (HCP: 96 ± 22 vs. Placebo: 85 ± 23 μM, $p = 0.045$) and lower S_tO_2 (HCP: 53 ± 8 vs. Placebo: 57 ± 12%, $p = 0.028$) values, whereas the *VL* muscle showed no significant difference in either the HCP or placebo group.

4.3.2 During Incremental Exercise

All peak values of gas exchange parameters, workload, and the exhausted time were similar in the two supplement groups (Table 2). Note that the $\dot{V}_E$, $\dot{V}O_2$, $\dot{V}CO_2$, HR, and R responses increased in proportion to the increasing work rate (time effect, all $p < 0.001$, $\eta^2 = 0.917$–0.993) and only $\dot{V}_E$ showed a significant difference between the HCP and placebo groups at some work rates (interaction effect: $F_{(11,110)} = 2.206$, $p = 0.019$, $\eta^2 = 0.181$, Figure 1A). The metabolic parameters of $\dot{V}O_2$, $\dot{V}CO_2$, HR, and R were quite similar in the two conditions at all work rates (Figure 1B–E). $P_{ET}CO_2$ first increased from 20 to 120 W then decreased from 160 to 240 W despite the lack of a significant difference between the HCP and placebo groups (Figure 1F).

A main effect of supplement was noted in the form of a lower pH (supplement effect: $F_{(1,15)} = 4.879$, $p = 0.043$, $\eta^2 = 0.245$, Figure 2A) and a higher HCO_3^- (supplement effect: $F_{(1,15)} = 5.762$, $p = 0.030$, $\eta^2 = 0.278$, Figure 2C) between the HCP and placebo groups during incremental exercise. The change in Lac was similar between the two groups, which might be due to a greater HCO_3^- value as an index of buffering capacity, which was supported by our observation of a lower AGap in the HCP group compared to the placebo group (Figure 2D).

In the *VL* muscles of the subjects, even though the deoxy[Hb + Mb] and total[Hb + Mb] increased with the work rates ($p < 0.001$, Figure 3A,B), the S_tO_2 was kept constant throughout the incremental exercise at a given work rate (time effect: $F_{(11, 121)} = 0.791$, $p = 0.648$, $\eta^2 = 0.067$, Figure 3C). The deoxy[Hb + Mb] and total[Hb + Mb] values did not differ between the HCP and placebo

groups at a given work rate. In contrast, the deoxy[Hb + Mb] value in the *RF* muscles of the subjects was significantly greater from 200 to 240 W in the HCP group compared to the placebo group (interaction effect: $F_{(11, 121)} = 2.726$, $p = 0.004$, $\eta^2 = 0.199$, Figure 3D). The total[Hb + Mb] profile in the *RF* muscle was affected by deoxy[Hb + Mb], although there was not a significant interaction between the HCP and placebo groups (interaction effect: $F_{(11, 121)} = 1.781$, $p = 0.064$, $\eta^2 = 0.139$, Figure 3E). The S_tO_2 tended to decrease with the work rates from approximately 57% to 45% (time effect: $F_{(11, 121)} = 1.469$, $p = 0.152$, $\eta^2 = 0.118$, Figure 3F) with lower mean values in the HCP group compared to the placebo group (supplement effect: $F_{(1, 11)} = 4.405$, $p = 0.060$, $\eta^2 = 0.286$). Regarding the *RF* muscle, muscle deoxygenation would be promoted by the HCP supplement, providing greater deoxy[Hb + Mb] and lower muscle S_tO_2 values.

The slope of the relationship between the $\dot{V}_E$ and deoxy[Hb + Mb] profiles was steeper in the HCP group (slope = 0.367) compared to the placebo group (slope = 0.227) (Figure 4), which means that the depressed $\dot{V}_E$ using HCP promotes working muscle deoxygenation (especially in the *RF* muscle) during incremental exercise as well as rest.

In the relationship between ROS and ventilation, we calculated the ventilatory responsiveness to pH using the mean values of $\dot{V}_E$ and pH, and the ventilatory responsiveness to pH was reduced by the intake of HCP compared to placebo (Figure 5), the regression line for the $\dot{V}_E$–pH profiles' relationship was steeper in the HCP group (slope = 0.892) than in the placebo group (slope = 0.608) as a function of the reduced chemoreflex drive to pH by the administration of HCP.

4.4 Discussion

To the best of our knowledge, the present study is the first investigation to use a form such as HCP capsules as a method of administering H_2 (instead of H_2 water or H_2 gas) and to comprehensively investigate the effects of HCP by examining a wide spectrum of acid-status and metabolic status values in addition to the working muscle deoxygenation measured by TR-NIRS. The main finding of this study was that H_2 in the form of HCP after three days of intake caused a slightly but significantly lower pH and greater PCO_2 due to hypoventilation, which might be due to the reduced ventilatory responsiveness to pH by the consumption of HCP (Figure 5). The reduction in oxidative stress with antioxidant treatment may suppress the peripheral chemoreceptor response (i.e., inhibition) more than the central chemoreceptor response (i.e., stimulation) (Zakynthinos et al., 2007). No similar observation was made thus far in previous research examining H_2. In fact, quite the opposite effect was observed, as H_2-infused water caused metabolic alkalosis, decreased the blood lactate level, and

lowered the rate of perceived exertion (Aoki et al., 2012; Ostojic, 2014; Ostojic and Stojanovic, 2014).

The existing hypoventilation can be clarified by this study's results, as the mean values of $\dot{V}_E$ at rest were significantly lower with HCP concomitant with a greater PCO_2 compared to the placebo, suggesting hypoventilation, as seen by the lower $\dot{V}O_2$ and $\dot{V}CO_2$ (Table 1). In addition, a significant difference was detected between the HCP and placebo groups, showing the presence of mitigated ventilation at rest that was apparent in the blood results of lower pH, PO_2, SO_2, Cl, and higher PCO_2 and HCO_3^- with HCP compared to the placebo (Table 1). The decreased pH was most likely caused by the primary elevation in PCO_2 as a result of suppressed ventilation (Kraut and Kurtz, 2012), and, consequently, as lower pH caused the renal retention of bicarbonate. This is a compensatory mechanism to induce acidemia to maintain the balance pH, and the end result is an increased concentration of bicarbonate and a decreased chloride concentration (Galla, 2000; Hadjikoutis and Wiles, 2001).

During incremental exercise, a significantly lower $\dot{V}_E$ at some work rates was reflected by the ventilatory analysis values (Figure 1A) demonstrating the ongoing impact of HCP on the $\dot{V}_E$ response during incremental exercise as well. Despite that, the negative effect of HCP is eliminated in $\dot{V}CO_2$ and $\dot{V}O_2$, as shown by the lack of significant changes in these parameters during exercise (Figure 1B,C); thus, the metabolic parameters increased proportionally to the work rate as a consequence of the increased respiratory and metabolic demands of the exercise (Guenette and Sheel, 2007). At peak performance, HCP also had no effect on the exhausted exercise time or peak gas exchange parameters or on the subjects' HR (Table 2). Therefore, it has not yet been demonstrated that the ingestion of molecular hydrogen is effective for peak performance in trained subjects.

As a result of the increased $\dot{V}_E$ during the progressive exercise, the Cl depletion ceased (Figure 2E) and the blood Cl was normalized (Galla, 2000). However, the changes in blood pH and HCO_3^- continued (Figure 2A,C), due to the continued hypoventilation despite the absence of significant differences in $P_{ET}CO_2$ and lactate between the HCP and placebo groups. Consequently, the decreased AGap in the HCP subjects (Figure 2D) was influenced as a result of the corrected Cl but not HCO_3^-. In addition, no significant difference in Lac was observed at rest or during exercise between the HCP and placebo groups, which might have been due to the higher HCO_3^- acting as a buffer. Therefore, judging from the acid-base status (Figure 2A–C), it is likely that HCP enhances the lactic acid buffering capacity despite hypoventilation.

In the relationship between ROS and ventilation, the ventilatory responsiveness to pH was reduced by the intake of HCP compared to placebo (Figure 5). Lee, Badr and Mateika (2009) showed that ventilatory sensitivity to hypercapnia following antioxidant administration was significantly

reduced compared to placebo in patients with sleep apnea, which we speculated was a measure of the patients' central and peripheral chemoreflex sensitivity. The reduced ventilatory responsiveness to pH or CO_2 is associated with an increase in the CO_2 reserve in the presence of changes in hypercapnic hypoventilation (Katayama et al., 2007). The long-term facilitation (LTF) was reduced in patients with sleep apnea who were given an antioxidant cocktail, and it was suggested that the release of ROS may have a role in the induction and maintenance of LTF (Lee, Badr and Mateika, 2009; Mateika and Narwani, 2009). Wilkerson et al. (2008) also demonstrated that an intravenous administration of 10 mg of a superoxide anion scavenger to anesthetized, vagotomized, and ventilated rats before their exposure to acute hypoxia abolished the LTF of phrenic nervous activity, which led to lower ventilation.

If LTF were active as a result of HCP, continued hyperventilation would be predicted, which is associated with ROS production (Cao et al., 1992; Peng et al., 2003). In fact, we observed that the HCP supplementation clearly instigated lower PO_2 and greater PCO_2 values due to hypoventilation in our subjects, and we thus speculated that these physiological phenomena are probably caused by hypoventilation due to an excessive suppression of ROS production by HCP. The neuromodulation of LTF in humans is not well understood, and few studies have attempted to tackle this issue. However, additional research is necessary to determine the mechanisms responsible for modulating LTF in humans via antioxidant administration. Given these findings in animals and humans, there is a possibility that the ROS would be downregulated by the administration of molecular hydrogen and cause hypoventilation and blood hypercapnia. We thus hypothesized that an excessive administration of an antioxidant of molecular hydrogen would reduce the magnitude of the ventilatory response that was associated with the increase in the CO_2 reserve before and during exhausted exercise in humans.

With the use of TR-NIRS during exercise, different reactions were observed in the HCP and placebo subjects depending on the muscle site, which can be attributed to the differing recruitment patterns of fast-twitch muscle fibers and slow-twitch muscle fibers in the *RF* and *VL* muscles (Chin et al., 2011; Okushima et al., 2015; Iannetta et al., 2017).

In the *RF* muscle, we observed significantly greater deoxy[Hb + Mb] at rest and higher work rates during the incremental exercise by the administration of HCP. The total[Hb + Mb] was also higher, mostly reflecting the significantly increased deoxy[Hb + Mb] between the HCP and placebo groups, with an average total increase of 12 μM. The greater deoxy[Hb + Mb] at the same work rates suggested that impairments in the $QO_2/\dot{V}O_2$ ratio necessitated higher fractional O_2 extractions to support any given change in the external work rate in this muscle. These observations suggest that the primary mechanism by which the *RF* achieved greater fractional O_2 extraction at higher work rates in

the HCP group was via elevated diffusive O_2 conductance consequent to an increased microvascular [hematocrit] (Okushima et al., 2015; Goulding et al., 2021). Indeed, the velocity of capillary red blood cells increases more with contractions in less-oxidative rat muscles (Dawson, Tyler and Hudlicka, 1987), and faster red blood cell velocity is associated with a higher capillary hematocrit (Kindig, Richardson and Poole, 2002). The *RF* muscle is more dependent on O_2 extraction for a given degree of muscle activation compared to the *VL* muscle (Dahmane et al., 2005; Goulding et al., 2021). As shown in Figure 4, the greater deoxy[Hb + Mb] concentrations as a function of ventilation in the HCP group might be attributed to increased O_2 extraction at hypoventilation that accompanies the greater recruitment patterns of fast-twitch fibers (Kume et al., 2016).

In addition, the S_tO_2 value in the *RF* muscle was lower with HCP compared to the placebo as a result of hypoventilation. Collectively, therefore, the present findings suggest that HCP exerted its greatest effects on the *RF* muscle at higher work rates where type II fiber recruitment patterns would be expected to be higher (Krustrup et al., 2004). In the *VL* muscle, the total[Hb + Mb] and deoxy[Hb + Mb] concentrations did not differ significantly between the placebo and HCP groups at any work rates. The S_tO_2 value also did not differ significantly between the two supplement groups, and this can be attributed to the relative greater recruitment patterns of slow-twitch fibers in *VL* muscle. Slow-twitch fibers have a greater number of capillaries around each fiber and show better vasodilatory dynamic control and better oxygen extraction compared to fast-twitch fibers (Dahmane et al., 2005; Iannetta et al., 2017). In particular, the *VL* muscle appears to possess a greater $QO_2/\dot{V}O_2$ ratio during exercise compared to the *RF* muscle (Koga et al., 2015; Okushima et al., 2015; Koga et al., 2017; Koga et al., 2019). It has been suggested that differences between the *VL* and *RF* muscles may emanate, in part, from higher blood flow (Kalliokoski et al., 2000; Kalliokoski et al., 2003; Heinonen et al., 2010), and a greater proportion of more highly oxidative type I fibers in the *VL* muscle (Lexell, Henriksson-Larsén and Sjöström, 1983).

In our study, the HCP supplementation provided approximately 2.5 μg/day of H_2. However, although HCP has a smaller amount of H_2 compared to H_2-rich water (Ostojic, 2014; Ostojic and Stojanovic, 2014), the presence of a compensatory respiratory response suggests an abnormal intake/absorption of H_2, thus indicating that ingesting H_2 in a form like HCP capsules provides a possibly better delivery as it releases H_2 continuously in the intestinal lumen, which might make it an appealing method of administering H_2. However, further research is warranted to test these concepts.

Finally, some study limitations must be considered when interpreting the present results, including the small number of available investigations of hydrogen during exercise. As mentioned earlier, the present study is the first to use a form like HCP as a method of administering H_2, and

further research is needed to better comprehend the working conditions and limitations of delivery methods such as HCP. A further examination of the respiratory gas and blood sampling at rest with different durations of supplementation prior to exercise and post-exercise recovery might have shed further light on the mechanisms and actions of H_2 in the form of HCP. Finally, we did not measure the level of ROS in the HCP intake. However, these limitations do not negate the important findings of this research.

4.5 Conclusion

H_2-rich calcium powder supplementation, which increased the potential for antioxidant-dependent slightly lower pH at rest, resulted in significantly lower $\dot{V}_E$ and pH status during the incremental exercise compared to placebo. The gas exchange status of $\dot{V}CO_2$ and $\dot{V}O_2$ were not affected by HCP. In addition, the HCP induced a significantly lower O_2 delivery/utilization ratio at the *RF* muscle site but not in the *VL* muscle, which may be explained by these sites possessing inherently different vascular and metabolic control properties, perhaps related to their fiber-type recruitment patterns.

Chapter 5. Anaerobic Exercise: Experiment 2

5.1 Experiment Rationales and Hypothesis

Our previous study revealed that when 1500 mg of HCP was well absorbed in subjects' intestinal tract for 3 consecutive days, it led to slight acidosis and to increases in the venous PCO_2 and HCO_3^-; in addition, the HCP significantly impacted the muscle O_2 delivery/utilization ratio in the rectus femoris (*RF*) muscle but not in the vastus lateralis (*VL*) muscle during incremental exercise, without impacting the subjects' exercise performance (Alharbi et al., 2021). Dietary HCP supplementation thus impacted the muscle spatial heterogeneity in muscle deoxygenation (deoxy[Hb + Mb]) kinetics, and, by extension, the O_2 delivery (QO_2-to-$\dot{V}O_2$ matching). And because it is possible that the effect of HCP might be more pronounced on the *RF* muscle with predominant fast-twitch-fiber recruitment (Chin et al., 2011; Okushima et al., 2015; Iannetta et al., 2017) it was speculated that an investigation of the effects of HCP during an anaerobic high-intensity intermittent training (HIIT) exercise in which fast-twitch-fiber-recruitment is dominant might reveal more about the influence of HCP on QO_2-to-$\dot{V}O_2$ matching and O_2 extraction in these muscles.

HCP as well increases HCO_3^- and thus might delay fatigue by increasing the extracellular buffer capacity, which might improve exercise performance (Wang et al., 2019; Calvo et al., 2021; Dalle, Koppo and Hespel, 2021). Additionally, as slight increases in PCO_2 might be associated with increased cerebral blood flow (CBF) in humans (Yoon, Zuccarello and Rapoport, 2012; Smith and Ainslie, 2017), and diminished CBF during exercise or physical activity results in a reduced motor drive to working muscles, consequently negatively affecting the tolerance to whole-body exercise and possibly performance (Kim et al., 2015; Marillier et al., 2022). Therefore, the increased levels of PCO_2 as result of HCP ingestion might further influence anaerobic exercise performance. Thus, it was hypothesized that the administration of H_2 might (*i*) impact the muscle spatial heterogeneity responses and blood gas profile and (*ii*) exert ergogenic effects on power output and performance during HIIT exercise. To test this hypothesis, the present study was conducted to determine whether HCP supplement causes different responses to muscle deoxy[Hb + Mb] and the responses of blood gas values at rest, during anaerobic HIIT exercise, and recovery compared to a placebo supplement.

5.2 Methods & Materials

5.2.1 Subjects

Ten healthy males, all were active members of the same university affiliated track and field team (age

20 ± 1 years; body weight 66.9 ± 5.9 kg; and height 172.4 ± 7.2 cm). All subjects are members of college athletic team and had been training for 100 m event for ≥ 5 years and averaged their 100 m race-record is 11.12 ± 0.38 sec. For additional details regarding subjects' recruitment criteria and participation consent please refer to Chapter 3, section 3.1.

5.2.2 Supplements Protocol

Subjects were given a single dose of four capsules of HCP or placebo 1 h before the experiment (Figure 6A), providing 2.544 μg as the total dose of H_2. For additional details regarding supplement and placebo please refer to Chapter 3 (section 3.2) and the Appendices section (appendix A, B, C).

5.2.3 Experimental Protocol & Measurements

For full details regarding experimental overview please refer to Chapter 3, section 3.3.

A familiarization test was conducted a few weeks prior to the start of the experiments. And the testing sessions were separated by a 1- to 2-weeks washout period. Each subject's HR was measured by a chest-strapped Polar H10 HR monitor (Polar Electro Oy, Kempele, Finland). For full details regarding blood analysis and TR-NIRS measurements please refer to Chapter 3, section 3.4 and 3.5 respectively.

5.2.4 Exercise Protocol

The subjects performed a HIIT exercise protocol using a cycle ergometer (Fujin-raijin, OCL, Tokyo, Japan). After 3 min of rest, the subject performed a 5 min warm-up exercise at a workload of 60 W. They then performed all-out pedaling for 7 sec at a load of 7.5% of body weight (BWT). After 5 min of rest, the subject then performed six repetitions of the 7 sec all-out pedaling at 7.5% BWT separated by 40 sec intervals of pedaling at a workload of 60 W (i.e., the HIIT protocol). This protocol was adapted to repeatedly stress the phosphocreatine (PCr)/ATP system (Lee, Lin and Cheng, 2011) and to target the recruitment of fast-twitch-dependent muscle, which is greater in short duration sprints (Wylie et al., 2016). A post-HIIT cool-down exercise was performed at a work-load of 40 W for 5 min. The recovery period was maintained for 10 min in a passive resting state (Figure 6). The observed mean HR reached approximately 80% of the age-predicted maximum HR even during this HIIT protocol (Figure 6B).

5.2.5 Sampling and Data Analysis

A 1 mL blood sample was collected at rest. The TR-NIRS and HR results were calculated as the mean value for 30 sec at sampling points, including rest and the recovery periods at the 1st min (Rec.1), 3rd min (Rec.3), 5th min (Rec.5), 7th min (Rec.7), and 10th min (Rec.10) (Figure 6). The peak values for

the TR-NIRS deoxy[Hb + Mb], total[Hb + Mb], HR, and peak power output (PP) were determined as the highest detected value during the 7 sec all-out bout for each set (Peak.1–6). The StO_2 was determined as the lowest detected value during the 7 sec all-out bouts.

5.2.6 Statistical Analysis

All data are expressed as the mean ± SD and were analyzed using the statistical package IBM SPSS, PC program, ver. 28.0 (IBM, Tokyo, Japan). Mean values of parameters between the HCP and placebo supplement groups at rest and the mean values across the six peaks of HIIT exercise were compared using a paired t-test. A two-way ANOVA for repeated measurers was employed to identify the significance of differences in variables between the HCP and placebo supplement groups at the sampling points. A *post hoc* Bonferroni test was implemented when a significant F-value was noted. Probability (p)-values < 0.05 were considered significant.

Associated metrics of effect size was calculated to further assess the result within the current study's sample size, Cohen's d was used for paired t-tests (d) and partial eta squared (η^2) within repeated measures ANOVA were calculated. Cohen's d effect sizes can be interpreted as: ≥ 0.20 (small), ≥ 0.50 (medium), and ≥ 0.80 (large) and partial eta squared (η^2) effect sizes can be interpreted as: ≥ 0.01 (small), ≥ 0.06 (medium), ≥ 0.14 (large) (Cohen, 1988). Pearson's correlation coefficient (r) for liner relationship correlation graphs were also calculated and can be interpreted as: ≥ 0.20 (weak), ≥ 0.40 (moderate), ≥ 0.70 (strong), ≥ 0.90 (very strong) (Rowntree, 1981; Overholser and Sowinski, 2008).

5.3 Result

5.3.1 At Rest

The mean values of acid-base, TR-NIRS, and HR profiles are presented in Table 3. The blood gas status values in the HCP group showed the following significant differences from the placebo group: Lower PO_2 (HCP: 31.3 ± 5.9 vs. placebo: 41.3 ± 8.7 mmHg, $t_{(6)} = -2.860$, $p = 0.029$, d = 1.081), higher PCO_2 (55.0 ± 4.7 vs. 50.7 ± 5.0 mmHg, $t_{(6)} = 3.606$, $p = 0.011$, d = 1.363) and HCO_3^- (30.5 ± 1.6 vs. 29.2 ± 1.7 mmol·L^{-1}, $t_{(6)} = 4.156$, $p = 0.006$, d = 1.571), and significantly lower SO_2%, higher BE and Hgb (all values, $p < 0.05$).

Although the pH was slightly lower in the HCP group at 7.354 ± 0.021 (placebo: 7.369 ± 0.018), the difference was not significant; however, a large effect size was present ($t_{(6)} = -2.331$, $p = 0.059$, d = 0.881). The metabolic profiles of Lac, Glu, and electrolytes were within the standard ranges at rest with no significant difference between the HCP and placebo groups. The at rest TR-NIRS

profiles of the *RF* and *VL* muscles and HR also showed no significant between-group difference.

5.3.2 HR Response during HIIT Protocol and Recovery

HR response from rest to recovery and peak values adapted in proportion to the changes in the work rate (time effect: $F_{(11, 99)} = 625.470$, $p < 0.001$, $\eta^2 = 0.986$) and showed no significant difference between HCP and placebo groups during the HIIT protocol, as illustrated in Figure 7.

5.3.3 TR-NIRS Profiles of the *RF* and *VL* Responses during the HIIT and Recovery

Overall, there were no significant differences between the HCP and placebo groups from rest to recovery in the *RF* and *VL* muscles' deoxy[Hb + Mb], total[Hb + Mb], and S_tO_2 (all interactions: $p > 0.05$, Figure 8). Table 4 presents the mean peak values of TR-NIRS profiles in the *RF* and *VL* muscles between HCP and placebo across the six peaks (mean peak.1–6) of the HIIT exercise.

In the *RF*, the mean peak deoxy[Hb + Mb] and S_tO_2 values showed no significant difference between the groups. However, the total[Hb + Mb] was significantly higher with HCP than placebo (HCP: 183 ± 1 vs. Placebo: 181 ± 1 μM, $t_{(5)} = 2.973$, $p = 0.031$, d = 1.214). In the *VL* muscle, the mean peak values of deoxy[Hb + Mb] displayed a significant difference of approximately 4 μM in deoxy[Hb + Mb] with HCP compared to placebo (HCP: 60 ± 3 μM vs. Placebo: 56 ± 3 μM, $t_{(5)} = 3.438$, $p = 0.018$, d = 1.404), which demonstrated a possibly greater O_2 extraction at peaks during the HIIT exercise in the *VL* muscle. However, no significant difference was observed in the *VL* muscle, with similar mean peak values of total[Hb + Mb] and S_tO_2 between HCP and placebo groups.

During the subjects' recovery, in both the *RF* and *VL* muscles, despite the lack of a significant difference between the supplement groups, it was observed that deoxy[Hb + Mb] decreased and S_tO_2 increased substantially during recovery compared to the baseline at rest values before the HIIT protocol. In the comparison between Rest and Rec10 in the *RF* muscle, the deoxy[Hb + Mb] following HIIT protocol was significantly lower at 24 ± 9 μM, and its decrease was approximately 30% (time effect: $F_{(11, 99)} = 66.131$, $p < 0.001$, $\eta^2 = 0.880$, Figure 8A). Similarly, the S_tO_2 for both groups was 13 ± 5% and thus significantly promoted at Rec10 (corresponding to a 23% increase from rest) compared to the rest value (time effect: $F_{(11, 99)} = 138.472$, $p < 0.001$, $\eta^2 = 0.939$, Figure 8C).

The *VL* muscle demonstrated a significantly remarkable decrease in deoxy[Hb + Mb] of 25 ± 7 μM at Rec10, and the percentage of change from rest to final recovery (at Rec.10) was an approximately 53% decrease (time effect: $F_{(11, 99)} = 76.271$, $p < 0.001$, $\eta^2 = 0.894$, Figure 8D). Moreover, the S_tO_2 for the *VL* muscle in both groups was 13 ± 4%, which is significantly increased (a 17% increase from rest) (time effect: $F_{(11, 99)} = 106.156$, $p < 0.001$, $\eta^2 = 0.922$, Figure 8F).

5.3.4 Peak Power during HIIT

The peak power showed a significant difference between HCP and placebo groups (interaction effect: $F_{(5, 45)} = 3.560$, $p = 0.009$, $\eta^2 = 0.283$). The PP increased significantly in the first bout with HCP compared to the placebo, 839 ± 112 W vs. 816 ± 108 W, $p = 0.001$. However, no significant difference was observed after that from bouts 2 to 6 (Figure 9).

5.3.5 The Relationship between ΔPCO_2, ΔHCO_3^- and ΔPP

During the HIIT protocol, a significant increase in the PP in the HCP group compared to the placebo group was observed at the 1st bout. When further examining the relationship between blood gas at rest and the PP at the 1st bout (Figure 10), a significant correlation was noted between the changes in PP at the first bout (ΔPP (HCP − Placebo)), changes in HCO_3^- at rest (ΔHCO_3^- (HCP − Placebo)) (Figure 10A), and changes in PCO_2 at rest (ΔPCO_2 (HCP − Placebo)) (Figure 10B). Higher changes in HCO_3^- and PCO_2 at rest were significantly associated with a greater PP reduction ($r = 0.771$, $p = 0.042$ and $r = 0.809$, $p = 0.027$), respectively, and the changes in pH at rest were mainly sensitive to changes in PCO_2 ($r = 0.907$, $p = 0.004$) (Figure 10C).

5.4 Discussion

This research examined the effects of the consumption of a single dose of H_2 in the form of HCP on healthy subjects' rest, HIIT performance, and recovery. The results showed that this dose of HCP significantly increased PCO_2 and HCO_3^- and showed a slight tendency toward acidosis at rest (i.e., a slightly but not significantly lowered pH). In the HCP group, the increased HCO_3^- was also reflected in the significantly increased BE, and the tendency toward acidosis in pH as a result of the increased PCO_2 could be additionally augmented as an effect of HCP's antioxidant properties and the suppression of ROS, as a reduced ventilatory sensitivity to hypercapnia following antioxidant administration has been observed in several studies (Lee, Badr and Mateika, 2009; Mateika and Narwani, 2009, Alharbi et al., 2021). However, further examination of the direct effect of HCP on ROS is needed.

HCP also had no effect on the *RF* and *VL* muscles' oxygenation status at rest. Comparably, in our previous work (Alharbi et al., 2021) when examining the intake of the same dose of HCP (1500 mg) for 3 consecutive days, a slight respiratory acidosis was detected at rest. Reduced rates of ventilation and muscle deoxygenation at rest in the *RF* muscle were also observed. However, the lack of an effect on the skeletal muscle oxygenation status in the *RF* and *VL* muscles in the present study can be attributed to the single dose of HCP and/or possibly the shorter period of ingestion and exposure

to changes in PCO_2 and PO_2 at rest.

It is possible that the significant increase in PCO_2 at rest is associated with increased cerebral blood flow, with hypercapnia resulting in dilatation of the cerebral vasculature as PCO_2, independently and in conjunction with pH, regulates the CBF (Yoon, Zuccarello and Rapoport, 2012; Smith and Ainslie, 2017). Regarding the relationship between CBF and exercise performance, it has been documented that a reduced CBF results in a reduced motor drive to working muscles, consequently reducing the tolerance to whole-body exercise and possibly performance (Kim et al., 2015; Marillier et al., 2022). The CO_2-induced CBF might thus have contributed to the change in the 1st PP performance. However, considering the effect of the HCP supplement on the averaged PP performance up to six exercise repetitions, the PCO_2-induced increase in CBF with the intake of HCP did not have an ergogenic effect on repeated HIIT, as the PCO_2 effects on CBF during exercise might be affected by the severity of hypoxia and the intensity and duration of exercise (Subudhi et al., 2008; Smith et al., 2012; Fan and Kayser, 2013).

In addition, the significantly increased HCO_3^- at rest that was observed in the present study might have further positive effects on the PP by increasing the extracellular buffer capacity, facilitating H^+ as well as lactate–ion efflux from muscles and delaying muscle fatigue during high-intensity muscle contractions (Wang et al., 2019; Calvo et al., 2021; Dalle, Koppo and Hespel, 2021).

As is clear from Figure 10A,B, the relationship between ΔPCO_2 (HCP − Placebo), ΔHCO_3^- (HCP − Placebo) and ΔPP (HCP − Placebo) is negatively correlated, with HCP improving the PP correlated to changes of approximately 2 mmHg for PCO_2 and 1.0 $mmol \cdot L^{-1}$ for HCO_3^-. Thus, the HCP supplement contributed to the improvement of the subjects' HIIT performance through slight increases in PCO_2 and HCO_3^-. Consequently, the HCP supplement resulting in this unique balance among pH, PCO_2, and HCO_3^- in the present experiment might have exerted a short ergogenic effect on the PP at the first bout at the beginning of the exercise protocol (Figure 9). However, more precise examinations of these factors are necessary to test these conjectures.

With the use of TR-NIRS during HIIT, it was observed that the mean peak values across the six peaks in the *RF* muscle's total[Hb + Mb] and the *VL* muscle's deoxy[Hb + Mb] were significantly higher in the HCP group compared to the placebo group. The increased deoxy[Hb + Mb] might suggest impairments in the matching of QO_2-to-VO_2 induced by the HCP, resulting in higher fractional O_2 extractions during the peak power exertion during HIIT in order to meet muscle demands, especially in the *VL* muscle (Alharbi et al., 2021). By contrast, the greater total[Hb + Mb] in the *RF* muscle was associated with an increased microvascular [Hb] volume (Fukuoka et al., 2015).

Regarding muscle deoxygenation in the both *RF* and *VL* muscles, it was noted that during

the subjects' recovery the deoxy[Hb + Mb] decreased and the S_tO_2 increased substantially compared to the baseline at-rest values with the prescribed HIIT protocol, despite the lack of a significant difference between the HCP and placebo supplement groups. In the comparison between Rest and Rec10, the deoxy[Hb + Mb] value following HIIT exercise was significantly lower by 24 ± 9 μM (~30% decrease) in the *RF* and by 25 ± 7 μM (~53% decrease) in the *VL*. Similarly, the S_tO_2 for both supplement groups was promoted at 10 min (Rec10) of passive recovery by 13 ± 5% (~23% increase) in the *RF* and 13 ± 4% (~17% increase) in the *VL* (Figure 8). These results demonstrated an abrupt increase in the regional muscle blood flow that was reflected by the tissue oxygen saturation exceeding the resting levels after the HIIT.

As there is a notable difference in the rate of oxygenation among muscle sites during recovery, it has been suggested that differences between the *RF* and *VL* muscles may originate, in part, from higher muscle blood flow (Kalliokoski et al., 2000; Kalliokoski et al., 2003; Heinonen et al., 2010), and a greater proportion of more highly oxidative type I fibers in the *VL* muscle (Lexell, Henriksson-Larsén and Sjöström, 1983). Additionally, judging by the continuing changes observed in the deoxy[Hb + Mb] and S_tO_2 values form Rec.1 to Rec.10, it can be assumed that a further increase in the reoxygenation of the *RF* and *VL* muscles would be present after the 10 min of passive recovery in this study.

The first observations regarding muscle deoxygenation kinetics suggested that the HIIT protocol has the advantages of improving the local blood flow and oxygen supply into the working skeletal muscles. Muscle gas exchange is facilitated when the functional cross-sectional area of capillaries is increased, as the vasodilation of small arterioles enhances the functional capillary density (increased number of perfused capillaries), which shortens the diffusion distance for oxygen and other substrates from and to the muscles (Korthuis, 2011; Stöcker et al., 2016).

Moreover, greater local muscle perfusion is associated with enhanced oxidative metabolism, such as fatty acid oxidation (Stöcker et al., 2016). The HIIT protocol employed in the experiment might therefore prove to be a quite advantageous tool in training and improving subsequently exercise performance.

Some study limitations must be considered when interpreting the present results, including the small number of available investigations of hydrogen's effects prior to and during exercise in healthy subjects. There have been few investigations of the effects and mechanisms of a unique method of delivering H_2 (such as HCP supplementation), and further research is needed to better comprehend the working conditions and limitations of HCP. In addition, this study did not measure the ventilatory response or provide ROS value changes, and these variables might have provided a better understating

of the acute effects of HCP intake during rest and exercise. Finally, as a large individual variation occurs in HCP experiments affecting the acid-base balance, further examinations of the optimal dosage and intake protocols should be considered. However, these limitations do not negate the important findings of this study.

5.5 Conclusion

The results of this study demonstrated that the ingestion of an H_2-rich calcium powder supplement increased the subjects' PCO_2 and HCO_3^- concentrations at rest and improved the peak power in the 1st bout of the HIIT protocol. The HCP had no notable effect on the subjects' heart rate or muscle O_2 at rest or recovery; however, the HCP group did exhibit significantly greater O_2 extraction and microvascular [Hb] volume in their working muscles during the HIIT exercise. The HIIT protocol resulted in improved muscle reoxygenation and may have improved the local muscle perfusion during recovery compared to rest.

These findings suggest that the use of an HCP supplement might produce short ergogenic effects on high-intensity exercise and prove advantageous for improving anaerobic exercise performance.

Chapter 6. General Conclusions

H_2 using HCP acts on blood gas directly by inducing greater PCO_2 and HCO_3^- and by extension effecting pH. Moreover, it influences lower ventilation (e.g., hypoventilation) and chemosensitivity of ventilatory response to acid-base status. HCP impacts muscle spatial heterogeneity responses and O_2 delivery/utilization balancing in the *RF* and *VL* muscles during exercise. Additionally, HCP supplement might exhibit short ergogenic effects in high intensity exercise protocols and prove advantageous for improving anaerobic exercise performance.

However, HCP's effect might be correlated to intake dose and ingestion/exposer time to the supplement, and individual variation to the HCP supplements largely occurred, which should be consider in future research and when using and prescribing HCP.

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Tables and Figures

Table 1 mean values of ventilatory, acid -base, and TR -NIRS profiles at rest between HCP and Placebo

	HCP	Placebo	*P* value
Metabolic gas exchange			
$\dot{V}_E$ ($L \cdot min^{-1}$)	11.8 ± 3.1	13.2 ± 3.2	0.015*
$\dot{V}O_2$ ($mL \cdot min^{-1}$)	355 ± 109	429 ± 136	0.009**
$\dot{V}CO_2$ ($mL \cdot min^{-1}$)	306 ± 96	364 ± 130	0.027*
HR ($beats \cdot min^{-1}$)	75 ± 12	79 ± 15	0.141
R	0.9 ± 0.1	0.8 ± 0.1	0.216
Blood gas			
pH	7.356 ± 0.042	7.376 ± 0.039	0.048*
PO_2 (mmHg)	43.9 ± 19.3	51.0 ± 17.8	0.107
PCO_2 (mmHg)	52.4 ± 8.3	47.4 ± 8.2	0.026*
HCO_3^- ($mmol \cdot L^{-1}$)	29.1 ± 2.2	27.5 ± 2.6	0.041*
SO_2 (%)	66.8 ± 25.2	76.9 ± 19.7	0.051
BE(ecf) ($mmol \cdot L^{-1}$)	3.6 ± 1.9	2.4 ± 2.3	0.071
BE(b) ($mmol \cdot L^{-1}$)	2.3 ± 1.3	1.6 ± 1.7	0.154
TCO_2 ($mmol \cdot L^{-1}$)	30.7 ± 2.5	29.0 ± 2.8	0.041*
Hct (%)	46 ± 2	46 ± 3	0.328
Hgb ($g \cdot dL^{-1}$)	15.8 ± 0.8	15.6 ± 1.1	0.313
Electrolytes			
Na ($mmol \cdot L^{-1}$)	141 ± 2	141 ± 2	0.069
K ($mmol \cdot L^{-1}$)	3.8 ± 0.3	4.0 ± 0.2	0.062
Ca ($mmol \cdot L^{-1}$)	1.26 ± 0.04	1.25 ± 0.05	0.471
Cl ($mmol \cdot L^{-1}$)	105 ± 2	106 ± 2	0.011**
AGap ($mmol \cdot L^{-1}$)	7 ± 2	7 ± 2	0.444
AGapK ($mmol \cdot L^{-1}$)	11 ± 2	11 ± 2	0.365
Metabolic status			
Lactate ($mmol \cdot L^{-1}$)	1.13 ± 0.42	1.28 ± 0.61	0.312
Glucose ($mg \cdot dL^{-1}$)	98 ± 14	104 ± 21	0.165
Creatinine ($mg \cdot dL^{-1}$)	0.96 ± 0.14	0.92 ± 0.16	0.212
TR-NIRS in the *RF*			
Total[Hb + Mb] (μM)	206 ± 48	201 ± 37	0.469
Deoxy[Hb + Mb] (μM)	96 ± 22	85 ± 23	0.045*
S_tO_2 (%)	53 ± 8	57 ± 12	0.028*
TR-NIRS in the *VL*			
Total[Hb + Mb] (μM)	200 ± 37	212 ± 37	0.164
Deoxy[Hb + Mb] (μM)	76 ± 20	76 ± 22	0.877
S_tO_2 (%)	61 ± 12	63 ± 12	0.222

Data are shown as mean ± standard deviation (SD).

Significant difference between HCP and Placebo (* $p < 0.05$, ** $p < 0.01$).

Table 2 peak values of gas exchange parameters, workload, and the exhausted time during incremental exercise between HCP and Placebo

	HCP	Placebo	*P* value
Metabolic gas exchange			
$\dot{V}_E$ (L·min^{-1})	115.2 ± 24.3	114.7 ± 28.8	0.918
$\dot{V}O_2$ (mL·min^{-1})	3119 ± 423	3141 ± 546	0.716
$\dot{V}CO_2$ (mL·min^{-1})	3496 ± 576	3528 ± 702	0.721
HR (beats·min$_{-1}$)	178 ± 7	179 ± 8	0.383
R	1.1 ± 0.1	1.1 ± 0.1	0.859
Workload (watts)	270 ± 34	272 ± 29	0.430
Exhausted time (min)	26.8 ± 4.0	26.9 ± 3.9	0.701

Data are shown as mean ± standard deviation (SD).

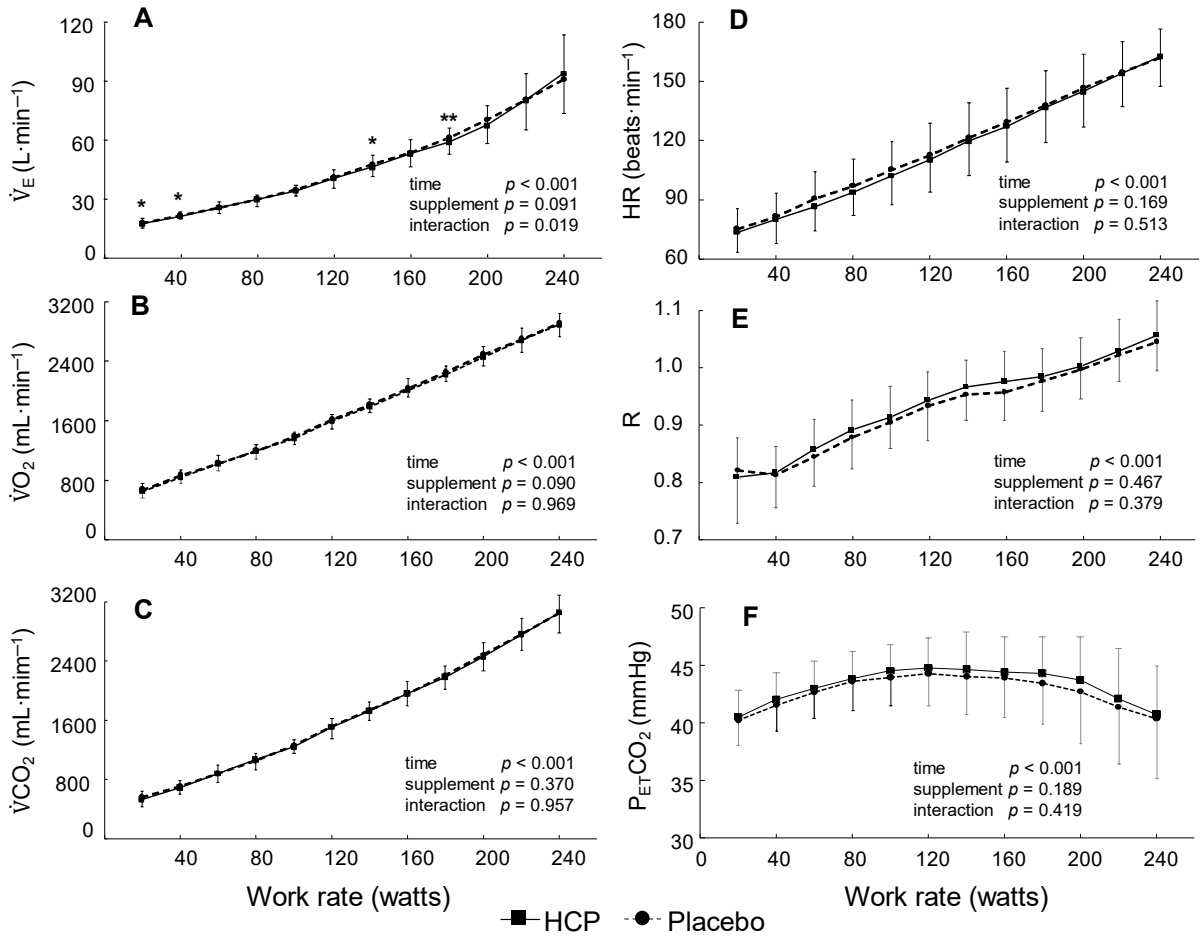


Figure 1. Group mean values of pulmonary ventilation ($\dot{V}_E$) (**A**), O_2 uptake ($\dot{V}O_2$) (**B**), CO_2 output ($\dot{V}CO_2$) (**C**), heart rate (HR) (**D**), gas exchange ratio (R) (**E**), and end-tidal CO_2 pressure ($P_{ET}CO_2$) (**F**) versus the power output during the cycle exercise in the HCP and placebo groups. Error bars: SD. The significant difference between HCP and placebo at higher work rates is shown. * $p < 0.05$, ** $p < 0.01$.

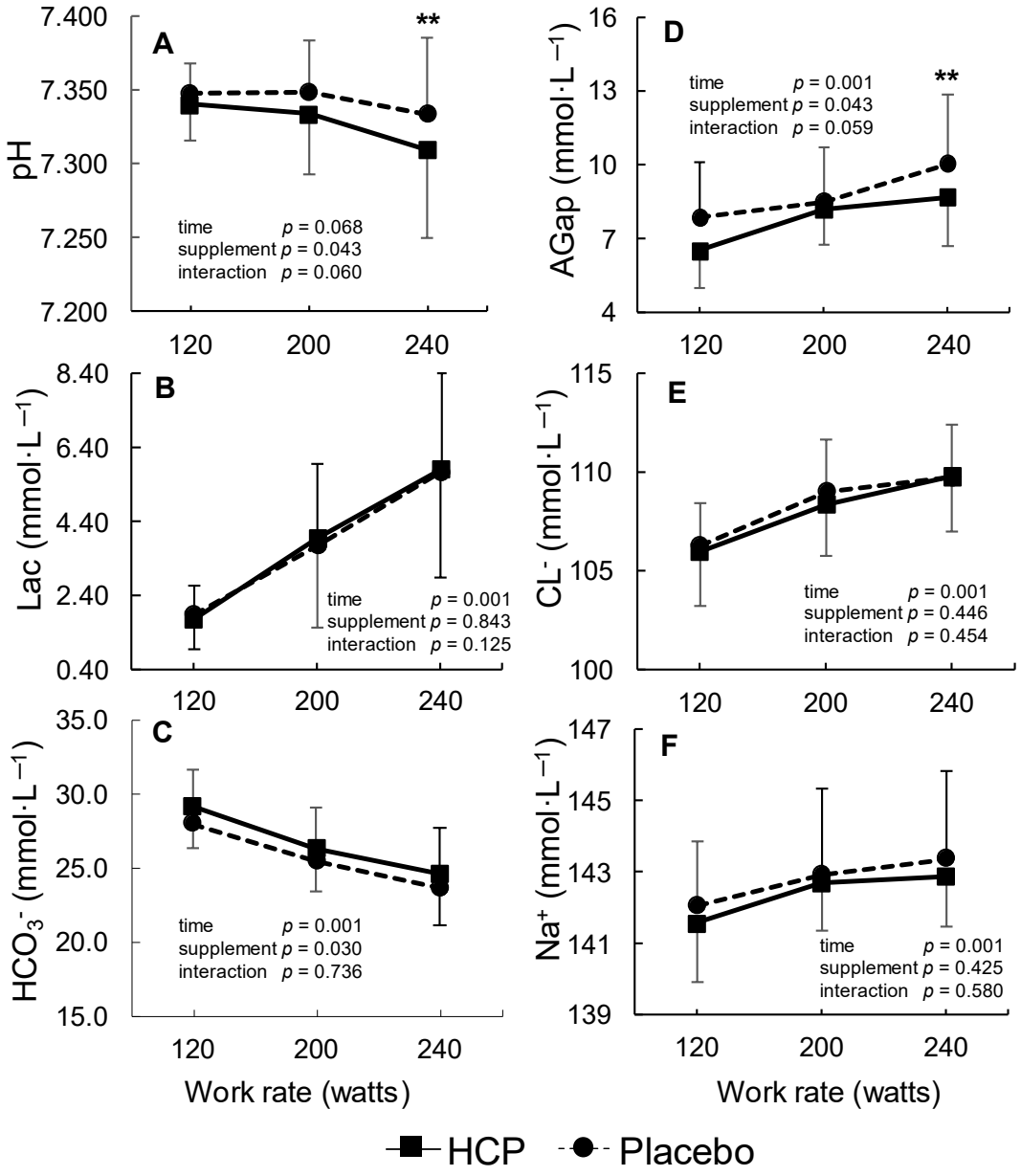


Figure 2. Group mean values of peripheral venous blood pH (**A**), lactate (Lac) (**B**), bicarbonate (HCO_3^-) (**C**), anion gap (AGap) (**D**), chloride (Cl^-) (**E**), and sodium (Na^+) (**F**) with the function of different work rates during the cycle exercise in the HCP and placebo groups. Error bars: SD. Significant difference between HCP and placebo at higher work rates. ** $p < 0.01$.

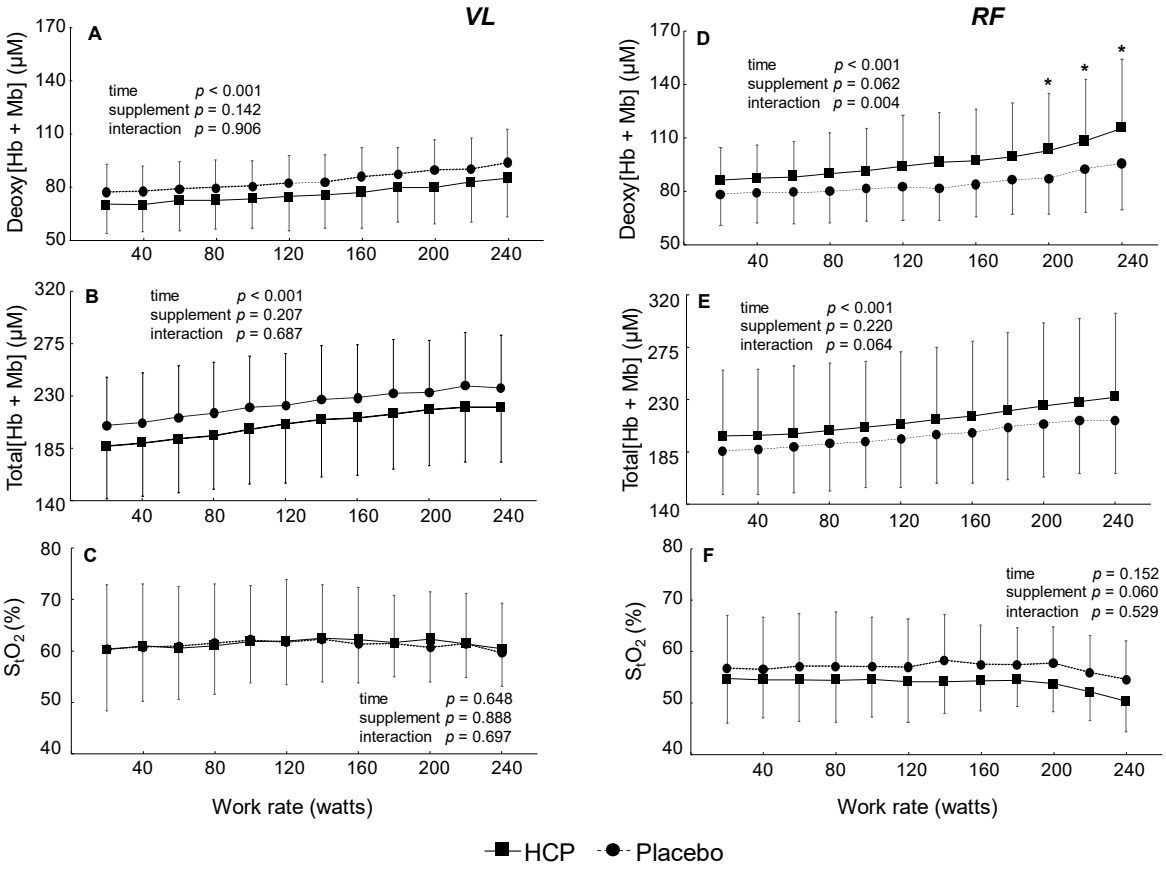


Figure 3. Group mean values for the vastus lateralis (*VL*) muscle deoxygenated hemoglobin and myoglobin concentration (deoxy[Hb + Mb]) (**A**), total hemoglobin and myoglobin concentration (total[Hb + Mb]) (**B**), tissue O_2 saturation (S_tO_2) (**C**), and rectus femoris (*RF*) muscle deoxy[Hb + Mb] (**D**), total[Hb + Mb] (**E**), and S_tO_2 (**F**) versus the power output during cycle exercise in the HCP and placebo groups. Error bars: SD. Significant difference between HCP and placebo at higher work rates. * $p < 0.05$.

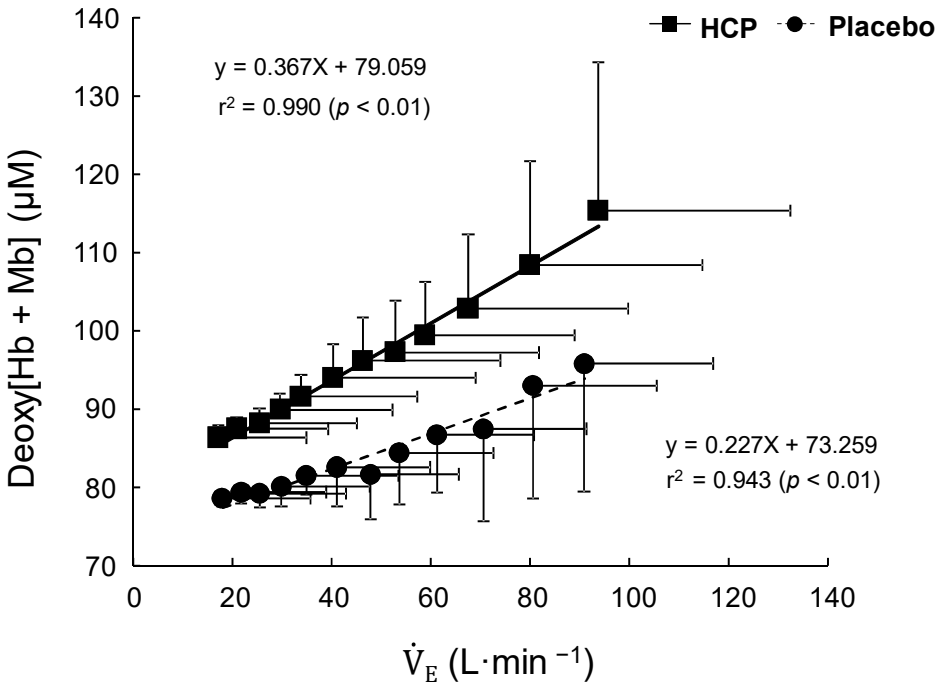


Figure 4. The relationship between ventilation ($\dot{V}_E$) and the rectus femoris (*RF*) muscle deoxygenated hemoglobin and myoglobin concentration (deoxy[Hb + Mb]) profiles during cycle exercise in the HCP and placebo groups. Note that the regression line for $\dot{V}_E$–deoxy[Hb + Mb] profiles' relationship was steeper in the HCP group (slope = 0.367) than in the placebo group (slope = 0.227). Error bars: SD.

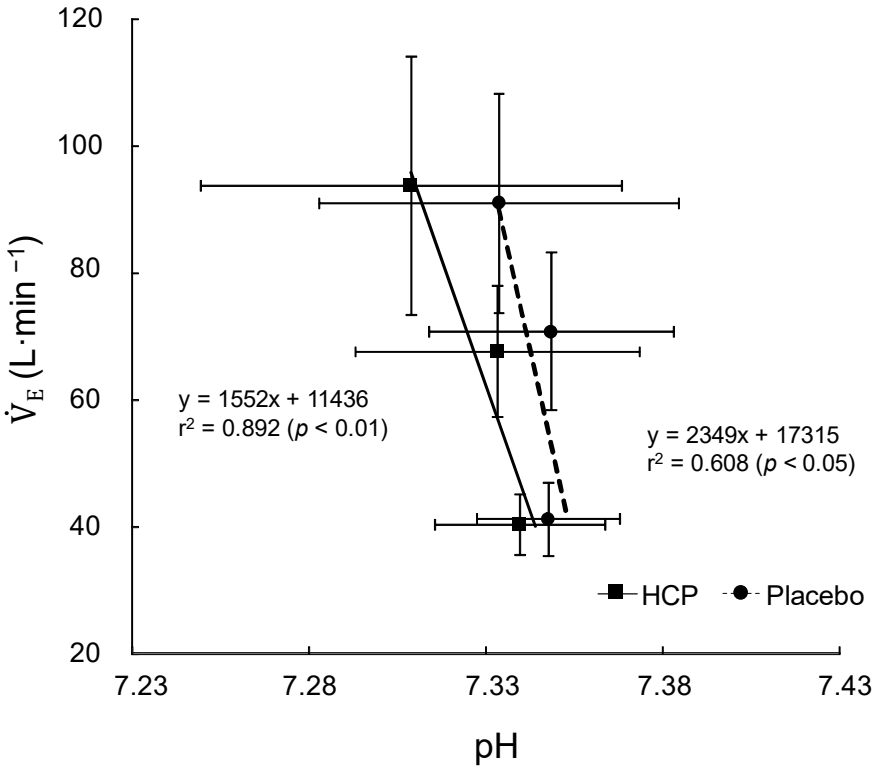


Figure 5. The relationship between ventilation ($\dot{V}_E$) and pH profiles during the cycle exercise between the HCP and placebo group. Note that the regression line for the $\dot{V}_E$–pH profiles' relationship was steeper in the HCP group (slope = 0.892) than in the placebo group (slope = 0.608) as a function of the reduced chemoreflex drive to pH by the administration of HCP. Error bars: SD.

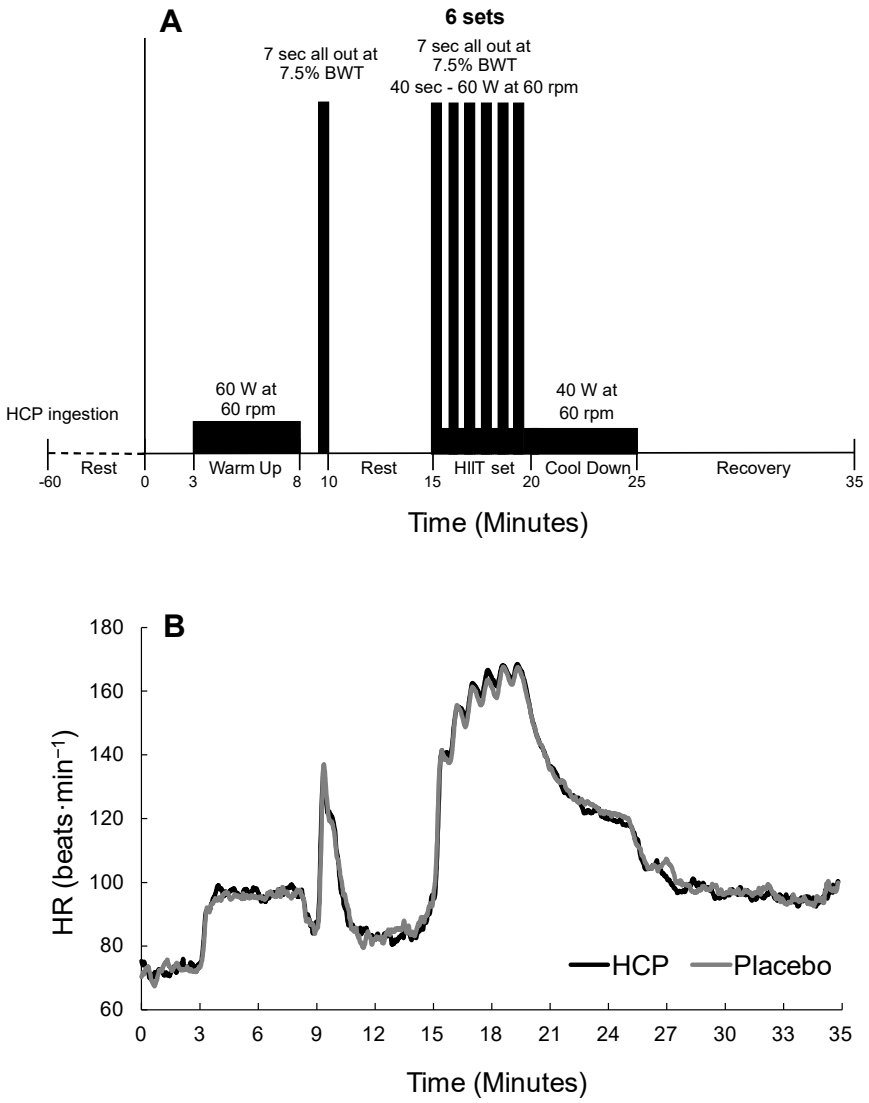


Figure 6. Experimental flow protocol at rest, warm-up, high-intensity intermittent training (HIIT), cool down, and passive recovery (**A**), and the heart rate (HR) response during rest, HITT, and recovery (**B**) in the HCP and placebo groups. HR reflecting the exercise intensity reached values near maximal intensity during the HIIT protocol.

Table 3 mean values of blood gas, electrolytes, metabolic parameters, TR-NIRS and heart rate profiles at rest between HCP and Placebo

	HCP	Placebo	*P* value	Effect size[a]
Blood gas				
pH	7.354 ± 0.021	7.369 ± 0.018	0.059	0.881
PO_2 (mmHg)	31.3 ± 5.9	41.3 ± 8.7	0.029*	1.081
PCO_2 (mmHg)	55.0 ± 4.7	50.7 ± 5.0	0.011**	1.363
HCO_3^- (mmol·L^{-1})	30.5 ± 1.6	29.2 ± 1.7	0.006**	1.571
SO_2 (%)	54.5 ± 13.4	71.7 ± 12.6	0.022*	1.158
BE(ecf) (mmol·L^{-1})	5.0 ± 1.5	3.8 ± 1.4	0.006**	1.555
BE(b) (mmol·L^{-1})	3.2 ± 1.1	2.6 ± 0.9	0.034*	1.034
TCO_2 (mmol·L^{-1})	32.3 ± 1.7	30.7 ± 1.8	0.004**	1.699
Hct (%)	50 ± 4	48 ± 4	0.052	0.915
Hgb (g·dL^{-1})	17.0 ± 1.4	16.2 ± 1.5	0.033*	1.038
Electrolytes				
Na (mmol·L^{-1})	141 ± 1	141 ± 1	1.000	0.000
K (mmol·L^{-1})	4.6 ± 0.4	4.6 ± 0.9	1.000	0.000
Ca (mmol·L^{-1})	1.30 ± 0.03	1.28 ± 0.05	0.119	0.688
Cl (mmol·L^{-1})	103 ± 1	103 ± 2	0.078	0.802
AGap (mmol·L^{-1})	8 ± 1	9 ± 2	0.253	0.477
AGapK (mmol·L^{-1})	13 ± 1	13 ± 1	0.289	0.439
Metabolic status				
Lactate (mmol·L^{-1})	0.95 ± 0.18	1.03 ± 0.27	0.388	0.351
Glucose (mg·dL^{-1})	92 ± 6	94 ± 9	0.512	0.264
Creatinine (mg·dL^{-1})	0.94 ± 0.03	0.93 ± 0.05	0.488	0.279
TR-NIRS in the *RF*				
Total[Hb + Mb] (µM)	172 ± 23	173 ± 25	0.805	0.080
Deoxy[Hb + Mb] (µM)	77 ± 12	77 ± 13	0.913	0.036
S_tO_2 (%)	55 ± 4	55 ± 4	0.987	0.005
TR-NIRS in the *VL*				
Total[Hb + Mb] (µM)	185 ± 15	194 ± 16	0.084	0.613
Deoxy[Hb + Mb] (µM)	47 ± 8	47 ± 7	0.925	0.031
S_tO_2 (%)	75 ± 3	76 ± 4	0.445	0.253
Heart Rate (beats·min^{-1})	69 ± 9	69 ± 7	0.912	0.036

Data are shown as mean ± standard deviation (SD).

Significant difference between HCP and Placebo (* $p < 0.05$, ** $p < 0.01$).

[a] Effect size (Cohen's d): ≥ 0.20 small effect, ≥ 0.50 medium effect, ≥ 0.80 large effect.

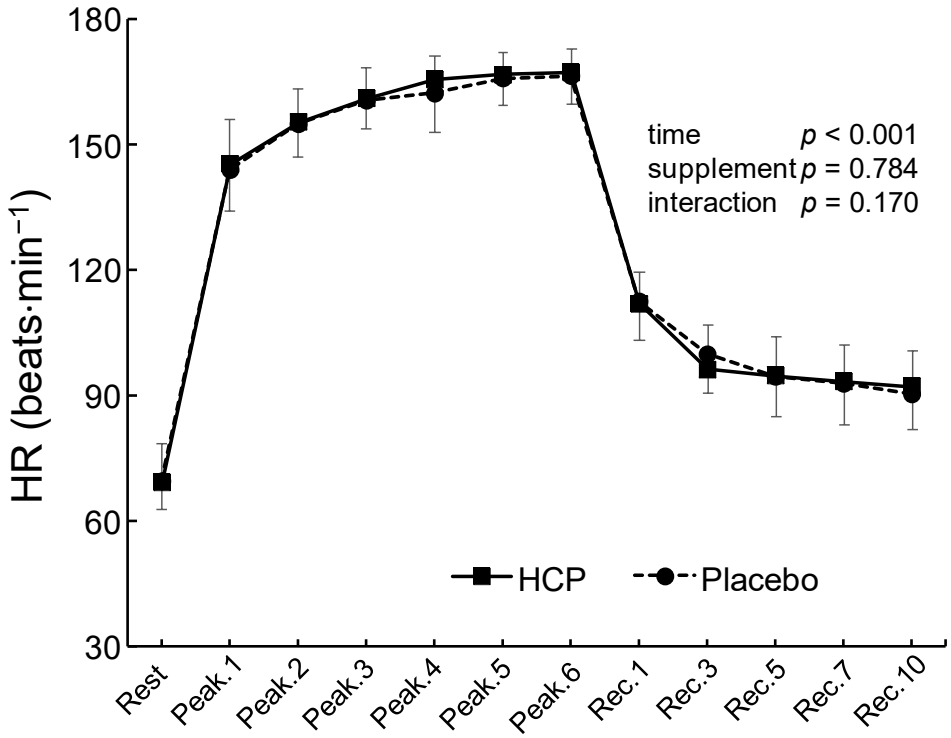


Figure 7. The subjects' heart rate (HR) response at rest and the peak values during the 7 sec all-out bout for each set (Peak.1–6) and the recovery period (Rec.1–10) between the HCP and placebo groups. Error bars: SD. The HR showed no significant difference between the groups.

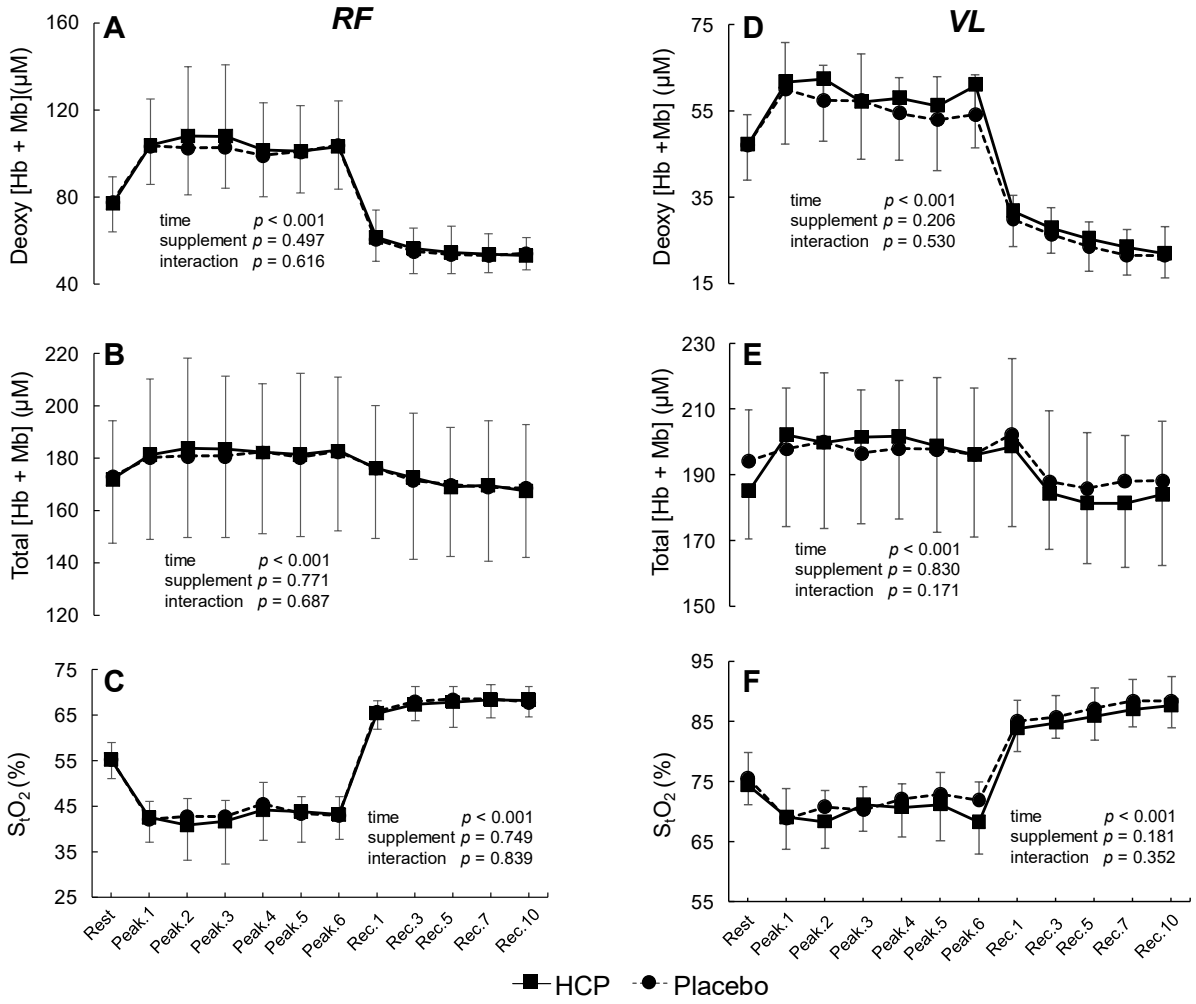


Figure 8. Group mean values for the rectus femoris (*RF*) muscle deoxygenated hemoglobin and myoglobin concentration (deoxy[Hb + Mb]) (**A**), the total hemoglobin and myoglobin concentration (total[Hb + Mb]) (**B**), and tissue O_2 saturation (S_tO_2) (**C**). The vastus lateralis (*VL*) muscle deoxy[Hb + Mb] (**D**), total[Hb + Mb] (**E**), and S_tO_2 (**F**) at rest, peak values during the 7 sec all-out bout for each set (Peak.1–6), and the recovery period (Rec.1–10) in the HCP and placebo groups. Error bars: SD. Overall values in the *RF* and *VL* muscles showed no significant difference between the groups.

Table 4 mean values of TR-NIRS profiles in the *RF* and *VL* muscles between HCP and Placebo across the 6 peaks of the HIIT protocol

	HCP	Placebo	*P* value	Effect size[a]
TR-NIRS in the *RF*				
Total[Hb + Mb] (µM)	183 ± 1	181 ± 1	0.031*	1.214
Deoxy[Hb + Mb] (µM)	104 ± 3	102 ± 2	0.092	0.851
S_tO_2 (%)	43 ± 1	43 ± 1	0.272	0.504
TR-NIRS in the *VL*				
Total[Hb + Mb] (µM)	200 ± 2	198 ± 1	0.054	1.021
Deoxy[Hb + Mb] (µM)	60 ± 3	56 ± 3	0.018*	1.404
S_tO_2 (%)	70 ± 1	71 ± 1	0.115	0.778

Data are shown as mean ± standard deviation (SD).

Significant difference between HCP and Placebo (* $p < 0.05$).

[a] Effect size (Cohen's d): ≥ 0.20 small effect, ≥ 0.50 medium effect, ≥ 0.80 large effect.

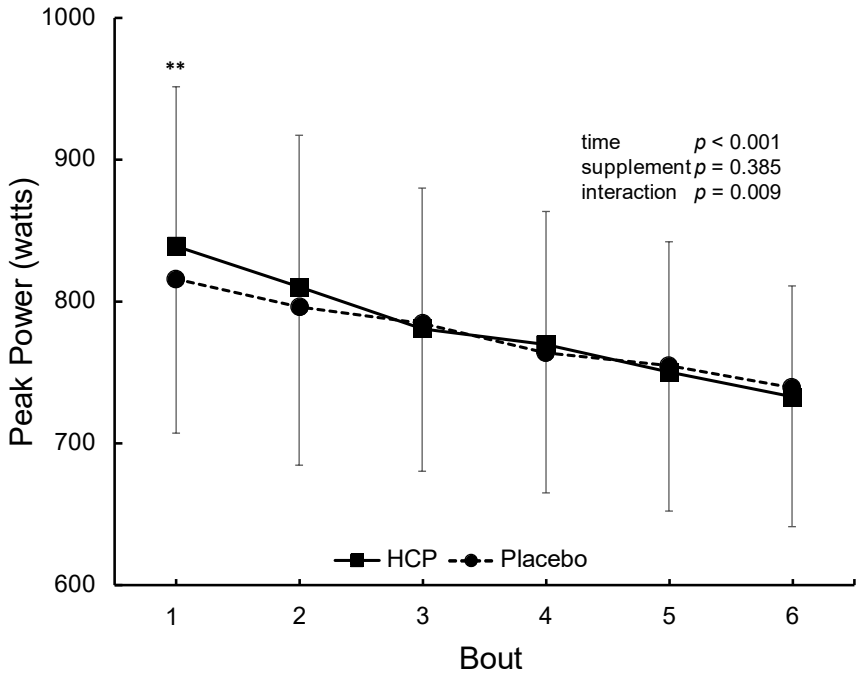


Figure 9. Peak power (PP) output in the 7 sec all-out bout during the six repetitions of the HIIT exercise in the HCP and placebo groups. Error bars: SD. ** $p < 0.01$. The PP increased significantly in the 1st bout with HCP compared to the placebo, but no significant difference occurred after that from bouts 2 to 6.

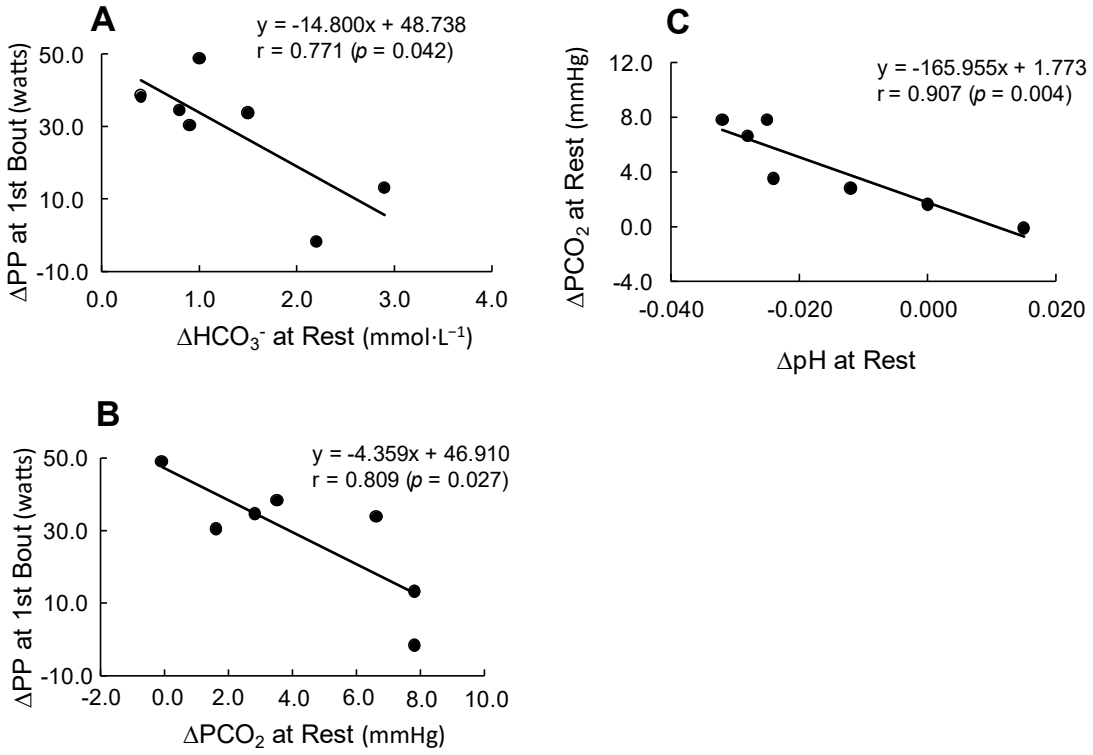


Figure 10. The relationship between changes in the peak power (PP) at the 1st bout (Δ PP (HCP − Placebo)) and changes in the HCO_3^- value at rest (ΔHCO_3^- (HCP − Placebo)) (**A**), the ΔPP at the 1st bout and changes in the PCO_2 at rest (ΔPCO_2 (HCP − Placebo)) (**B**), and the ΔPCO_2 at rest in relation to the changes to pH at rest (ΔpH (HCP − Placebo)) (**C**) in the HCP group. Note that the sample size was 7 subjects. Higher changes in ΔHCO_3^- and ΔPCO_2 at rest were significantly negatively correlated with ΔPP at the 1st bout (r = 0.771, p = 0.042 and r = 0.809, p = 0.027, respectively), and the change in pH at rest was sensitive mainly to changes in PCO_2 (r = 0.907, p = 0.004).

Appendices

Appendix A: HCP and Placebo Additional Information

A.1 HCP and placebo composition per 1 capsule

The detailed composition of HCP and placebo was provided by the manufacturer ENAGEGATE Inc. and is listed below.

[Main raw material]

HCP: Calcium powder infused with hydrogen gas.

Placebo: Calcium powder (H_2 gas not adsorbed).

Calcium powder amount: 375 mg (compounding ratio: 65.79%).

[Secondary raw materials]

・Potato starch (amount: 162.5 mg, compounding ratio: 28.51%).

・Sucrose ester (amount: 25 mg, compounding ratio: 4.39%).

・Silicon dioxide (amount: 7.5 mg, compounding ratio: 1.32%).

A.2 HCP and placebo hydrogen gas analysis data

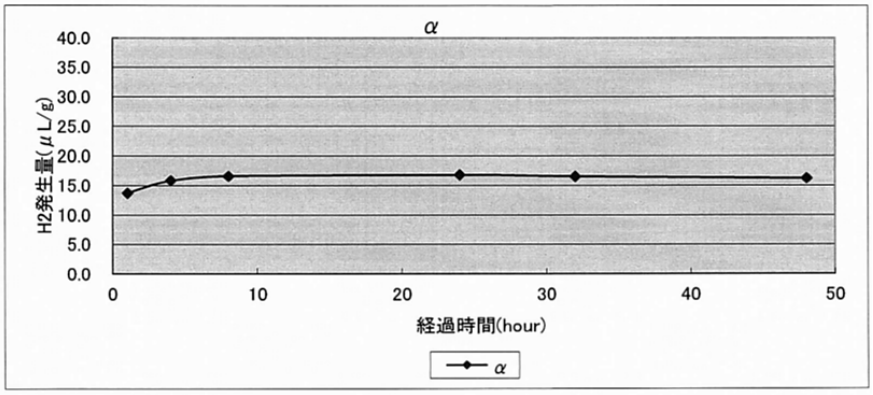
The manufactured HCP and placebo lots that was used in the experiments was additionally analyzed blindly after the end of the experiments by the manufacturer ENAGEGATE Inc. using a third-party laboratory to further confirm the presence and amount of H_2 gas released using gas chromatography.

The testing method consisted of dissolving 3.0 g of the sample powder (HCP or placebo) in 15 mL of deionized water which were placed in a 32-m thick gas container and quickly sealed at standard room temperature. Gas measurements for was performed 6 times at 1 hour, 4 hours, 8 hours, 24 hours, 32 hours, and 48 hours. H_2 concentration was then calculated per 1 g of sample powder and showed adequate H_2 gas release from HCP (Figure A1) and no significant gas release from placebo (Figure A2).

1

単位:μL/g

経過時間(h)	カルシウムパウダー α
1	13.7
4	15.9
8	16.6
24	16.8
32	16.5
48	16.2



2

単位:μL/g

経過時間(h)	カルシウムパウダー β
1	0.02
4	0.02
8	0.03
24	0.03
32	0.03
48	0.03

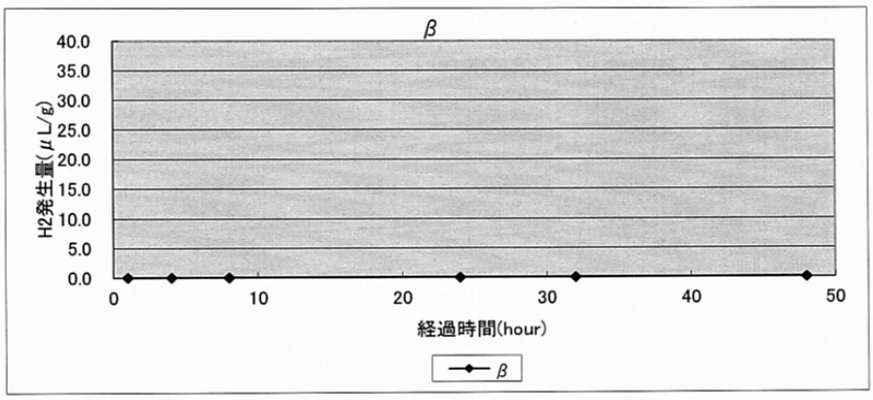


Figure A. Hydrogen gas release (μL/g) from 1 g of HCP (in graph is labeled as α) (**1**), 1 g of placebo (in graph is labeled as β) (**2**) overtime measured using gas chromatography. Figure was retrieved from analysis carried out by third-organization laboratory and documents were provided by the manufacturer ENAGEGATE Inc. in June 2022.

Appendix B: Preliminary Experimental Data from the Manufacturer

Currently no sufficient evidence or large-scale experimental data are available showing HCP trend of effect or the rection time needed to detect changes caused by the supplement in the human body. However, small scale experimental data are available and as they might provide more context to the effects and work mechanisms of HCP they were shared in this appendix.

Peak H_2 gas detected in breath post HCP intake

A small trial was carried out by ENAGEGATE Inc. using a third-organization laboratory, they tested exhaled breath H_2 after HCP ingestion in an effort to estimate and observe adequate H_2 absorption rate in the human body. The small trial consisted of the subject consuming 6 g HCP following a 12 hour fast. Two types of capsules coating were used (normal vs. enteric coating) and following ingestion exhaled air was sampled and collected in an aluminum collection bag for analysis. The collected samples were then measured for H_2 concentration 24-hour post collection on the following day.

Our main focus from the results of this experiment was that peak concentration of exhaled H_2 in breath was detected approximately 60–65 minutes post ingestion with the standard coating HCP supplement capsules (Figure B).

These preliminary results coupled with the results for peak changes in blood gas post HCP ingestion in our internal preliminary experiments (appendix C) contributed to our choice for the single short dose HCP experiment (anaerobic experiment) to examine supplement effects following 1 hour of HCP intake.

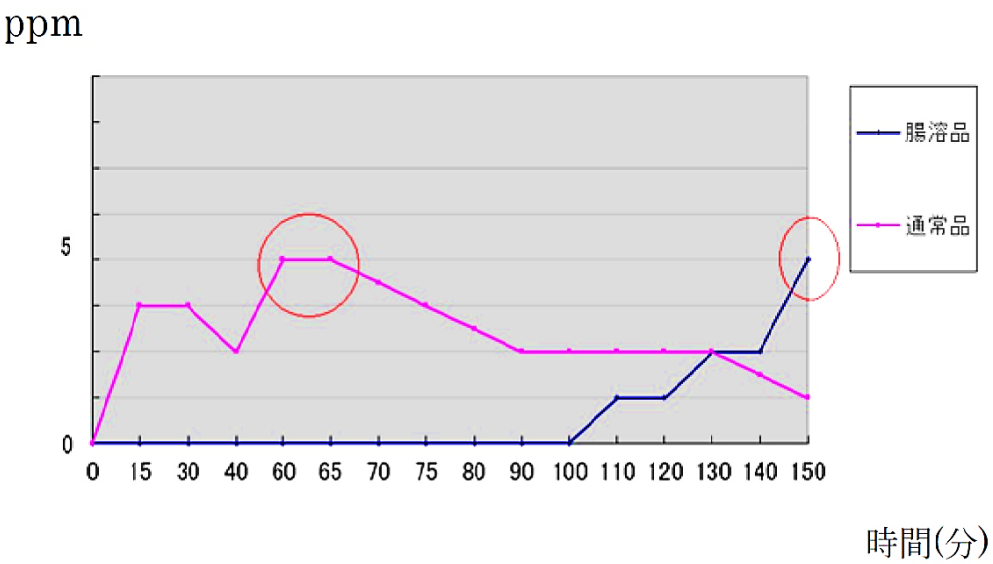


Figure B. Exhaled breath H_2 gas concentration following the intake of 6 g of HCP capsules (pink line represents normal coating HCP capsules) overtime. Note that peak breath H_2 was detected 60–65 minutes following standard HCP capsules ingestion. Figure was retrieved from analysis carried out by third-organization laboratory in 2015 and documents were provided by the manufacturer ENAGEGATE Inc. in 2021.

Appendix C: Preliminary Internal Experimental Data

HCP possible trend of effect and reaction time

Limited preliminary experiment examined the different reactions and trend of effects for HCP, the results of these trials might prove intriguing and beneficial for future research considerations, however, due to the limitation of these trials their results will be shared here in this appendix.

There are difficulties in estimating and generalizing an optimal intake time and dose for H_2 supplements a result of the unique nature of HCP and the inherited differences between individuals' response to supplement ingestion, since multiple factors might possibly affect the reaction time and the initial reaction trend (this could also be noted in the data shared below), which might include gender, age, and athletic statues among other possibilities. As H_2 effects might involve the body antioxidant system and ROS production statues of the subject as was hypothesized in the findings of this research (please refer to Chapter 4 for further details).

A trial conducted in the undergraduate department in Ebine laboratories by Yoshida and Uemura (2020) tested the trend of effect of the consumption of 4 capsules of HCP on blood gas changes during rest. After baseline measurements, the subject consumed the capsules and blood pH was sampled over time up to 7 hours, it was noted that significant changes in pH occurred 60–120 minutes post intake with the peak around 90 minutes before pH started returning closer to baseline levels (Figure C1). At 130 minutes post HCP intake a test meal (Calorie Mate, four blocks, Otsuka Pharmaceutical, Tokyo, Japan) and 500 ml of water was then introduced which contributed to the higher changes in pH after the 120 minutes blood sampling point.

Additional preliminary data collected by us prior to the anaerobic experiment is shared in Figure C2, where we compared the effect of the intake of 4 capsules of HCP with an athletic male subject (Subject.2) and a non- athletic female subject (Subject.3). It was noted that peak changes in blood pH occurred 60–90 minutes post intake and that different initial reactions trends were present between subjects most likely as a result of the large differences in physiological and physical conditions between the subjects and possibly their antioxidant capacity.

In the end it was inferred from these preliminary results that peak levels of H_2 (Appendix B, Figure B1) and blood gas changes (Figure C1, C2) following short term intake of HCP, might be most prevalent within 1–2 hours after intake, which was reflected in our experimental design choice of examining the effect of a single dose of HCP 1 hour prior to anaerobic exercise (Chapter 5). Additional testing and exploration with larger more diverse population samples and different exposers duration to H_2 and HCP intake should be considered in future research.

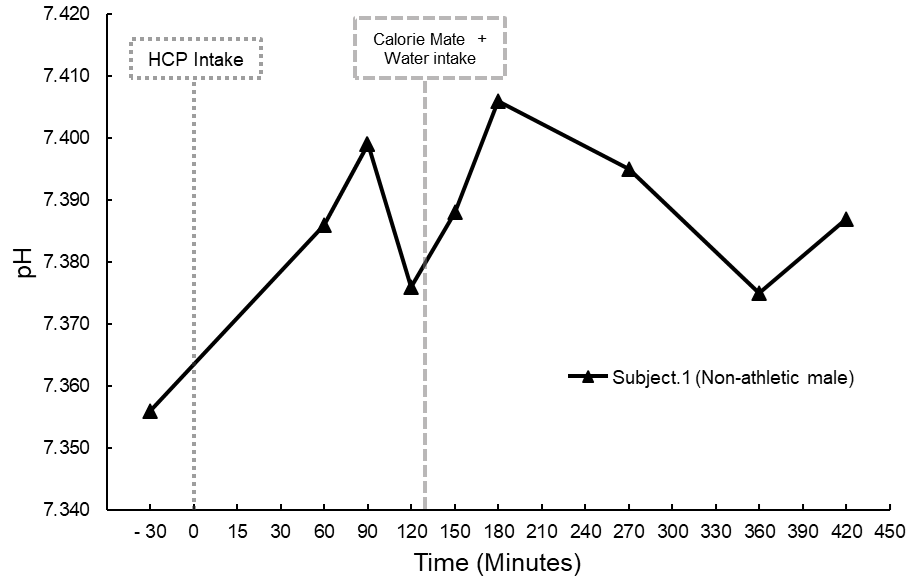


Figure C1. Blood pH changes following the intake of 4 capsules of HCP overtime. Note that peak changes in pH up to 120 minutes, without additional stimuli (food or water consumption), was detected approximately 90 minutes following intake. Figure was recreated from data obtained from Yoshida and Uemura (2020).

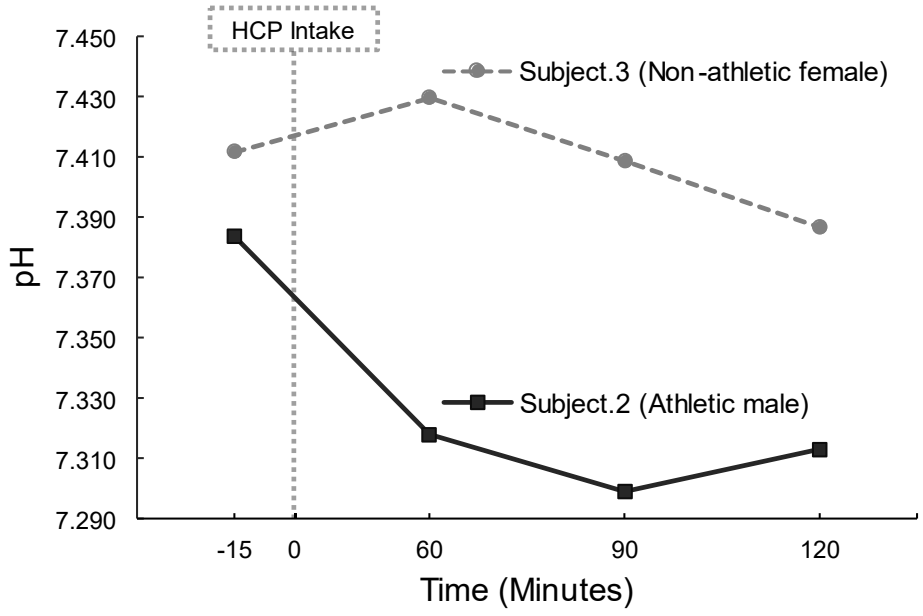


Figure C2. Blood pH changes following the intake of 4 capsules of HCP overtime. Note that peak changes in pH occurred 60–90 minutes post intake and that different initial reactions trend was noted between subjects.