TITLE: Ketone monoester ingestion increases post-exercise serum erythropoietin concentrations in healthy men

AUTHORS: Eric Evans[1], Jean-Philippe Walhin[1,2], Aaron Hengist[1,2], James A. Betts[1,2], David J. Dearlove[3], Javier T. Gonzalez[1,2].

AFFILIATIONS:

[1]Centre for Nutrition, Exercise and Metabolism, University of Bath, Bath, UK

[2]Department for Health, University of Bath, Bath, UK

[3]Oxford Centre for Diabetes, Endocrinology and Metabolism, Churchill Hospital, University of Oxford, Oxford, UK.

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Correspondence:

Javier T. Gonzalez

Department for Health,

University of Bath,

Bath,

BA2 7AY,

UK

Email: J.T.Gonzalez@bath.ac.uk

ABSTRACT

Intravenous ketone body infusion can increase erythropoietin (EPO) concentrations, but responses to ketone monoester ingestion post-exercise are currently unknown. The purpose of this study was to assess the effect of ketone monoester ingestion on post-exercise erythropoietin (EPO) concentrations. Nine healthy men completed two trials in a randomized, crossover design (one-week washout). During trials, participants performed a one-hour of cycling (initially alternating between 50% and 90% of maximal aerobic capacity for 2 min each interval, and then 50% and 80%, and 50% and 70% when the higher intensity was unsustainable). Participants ingested 0.8 $g \cdot kg^{-1}$ sucrose with 0.4 $g \cdot kg^{-1}$ protein immediately after exercise, and at 1, 2, and 3 hours post-exercise. During the control trial (CONTROL), no further nutrition was provided, whereas on the ketone monoester trial (KETONE), participants also ingested 0.29 $g \cdot kg^{-1}$ of the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate immediately post-exercise and at 1 and 2 hours post-exercise. Blood was sampled immediately post-exercise, every 15 min in the first hour, and hourly thereafter for 4 hours. Serum EPO concentrations increased to a greater extent in KETONE than CONTROL (time x condition interaction: $p = 0.046$). Peak serum EPO concentrations were higher with KETONE (mean ± SD: 9.0 ± 2.3 $IU \cdot L^{-1}$) compared with CONTROL (7.5 ± 1.5 $IU \cdot L^{-1}$, $p < 0.01$). Serum beta-hydroxybutyrate concentrations were also higher, and glucose concentrations lower, with KETONE *vs* CONTROL (both $p < 0.01$). In conclusion, ketone monoester ingestion increases post-exercise erythropoietin concentrations, revealing a new avenue for orally ingestible ketone monoesters to potentially alter haemoglobin mass.

INTRODUCTION

Ketone bodies are compounds derived from acetyl-CoA produced by the liver during conditions of low carbohydrate and high fatty acid availability (1). The primary ketone body is β-hydroxybutyrate, which exhibits the highest circulating concentrations and can be used as a fuel by the brain and skeletal muscle (2, 3). β-hydroxybutyrate has wide-ranging effects in human physiology, including suppression of endogenous glucose production and whole-body glycerol appearance rates, suggesting decreased hepatic glucose output and adipose tissue lipolysis (3). The metabolic effects of β-hydroxybutyrate have led to interest in developing ways of increasing circulating β-hydroxybutyrate concentrations for human health and/or performance.

One of the most effective methods to rapidly increase circulating β-hydroxybutyrate concentrations whilst circumventing the requirement of low carbohydrate or high fatty acid availability is to ingest exogenous ketone bodies. The ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate represents an ingestible method of rapidly increasing circulating ketone body concentrations without providing excess sodium (4-6). Oral ingestion of this ketone monoester can increase circulating β-hydroxybutyrate concentrations to >2.5 $mmol \cdot L^{-1}$ within an hour and recapitulates many of the metabolic effects of β-hydroxybutyrate infusion (7). However, recent evidence suggests that the potential for ketone monoesters to provide exogenous fuel during exercise is unlikely to be sufficient to impact performance (8, 9). Therefore, if ketone monoesters are to alter human performance, then it may be via mechanisms other than acting as a substrate for skeletal muscle metabolism.

Intravenous infusion of β-hydroxybutyrate (βHB) to ~5 $mmol \cdot L^{-1}$ in fasted humans over 5 hours has been shown to increase erythropoietin (EPO) concentrations by ~30% (10).

EPO is the primary regulator of erythrocyte production. Not only is EPO essentially permissive for erythropoiesis, EPO also dose-dependently stimulates erythropoiesis, acting on proliferation, differentiation, and maturation of erythrocytes (11). Emerging evidence also suggests EPO has a number of non-haemopoietic effects, including effects on inflammation, angiogenesis and skeletal muscle regeneration (12). Notably, 3 weeks of a low-carbohydrate, ketogenic diet in endurance athletes has been shown to increase haemoglobin concentration and haematocrit relative to a high-carbohydrate diet (13), and this increase in haemoglobin concentration seems to be detectable within 6 days (14). It is therefore plausible that increased EPO production by ketosis may alter haemopoiesis in athletes. However, it is unclear whether similar effects are observed from ketone monoester ingestion – a more practical approach to elevating βHB in physically active individuals or athletes. Regular exposure to exercise can itself stimulate EPO secretion (15), with the potential for a ceiling effect to limit the ability for ketone monoesters to increase EPO concentrations in physically active individuals. Furthermore, it is unclear if the oral ingestion of a ketone monoester in a context representative of free-living scenarios (e.g., with ingestion of macronutrients) would reproduce the effects of intravenous infusion in a fasted state, due to the different tissues that would be exposed to βHB depending on the mode of administration, and because feeding reduces βHB concentrations following ketone monoester ingestion (7).

The aim of this study was to reveal whether ketone monoester ingestion post-exercise increases EPO concentrations in humans. Since βHB can suppress endogenous glucose production, a secondary aim was to assess whether ketone monoester ingestion would decrease glucose concentrations during post-exercise carbohydrate feeding. It was hypothesized that ketone monoester ingestion would increase EPO concentrations to a greater extent than control and decrease glucose concentrations relative to control.

MATERIAL AND METHODS

Study design

This study was an acute, open-label, randomised, crossover laboratory-based experiment, with two experimental conditions, ketone monoester (KETONE) and control (CONTROL). An open-label approach was chosen following a lack of success with blinding during pilot testing (due to the strong taste of the ketone monoester) and on the basis that the physiological (rather than behavioural or performance) outcomes in the current study are not particularly influenced by placebo effects. Participants performed one preliminary visit to determine peak aerobic capacity ($\dot{V}O_2$peak), followed by the two experimental conditions with a washout interval of 7 days. The study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the University of Bath Research Ethics Approval Committee for Health (REACH; MSES20/21-026). Written informed consent was provided prior to participation and the manuscript has been drafted in accordance with the PRESENT 2020 checklist (16).

Participants

Participants were 9 healthy men, who participated in physical activity at a level ranging from recreational to competitive (age: 25 ± 8 y, body mass: 73 ± 12 kg, body mass index: 23.0 ± 2.3 $kg \cdot m^{-2}$, $\dot{V}O2$ peak: 55.4 ± 8.1 $mL \cdot kg^{-1} \cdot min^{-1}$; mean ± SD). Exclusion criteria included: age of <18 y or >60 y, habitual smoker within past 5 y; $\dot{V}O_2$peak <35 $mL \cdot kg^{-1} \cdot min^{-1}$, or history of uncontrollable metabolic or respiratory diseases (e.g., cardiovascular disease, diabetes, asthma).

Preliminary testing

Following measurement of body mass and height, participants completed an incremental exercise test of a bicycle ergometer (Monark 894E, Varberg, Sweden) to assess aerobic capacity. The test consisted of 3 x 3-min stages at 80, 160 and 240 W followed by 1-min stages where the intensity increased by 40 W per min until task failure. One-minute samples of expired breath were collected in the final minute of each 3-min stage, and at the point of task failure using the Douglas bag method.

Experimental trials

Participants reported to the physiology laboratories in the Department for Health, University of Bath following an overnight (>10 h) fast. Following a standardised warm-up (50 W for 5 min), participants completed 1 h of cycling intervals, alternating between 2 min at the W equivalent to 90% $\dot{V}O_2$peak and 2 min at the W equivalent to 50% $\dot{V}O_2$peak. If participants indicated they could not maintain 90% $\dot{V}$O2peak, then the intensity was reduced to 80% $\dot{V}$O2peak and if required, 70% $\dot{V}$O2peak. Where this occurred, participants still exercised to their volitional capacity on their subsequent trial. This protocol was based on prior work which results in task failure by ~90 mins (17), and thereby represents a demanding exercise session over 60 mins. Whilst this design was to match for relative effort rather than absolute intensity, the mean absolute intensity was within 1 W between each trial (**Table 1**). The rationale for this exercise protocol was to accumulate a large amount of high intensity work to stimulate EPO production.

Table 1. Intensities completed and testing condition in both trials.

	Mean intensity		**Testing Conditions**		
	(W)	($W \cdot kg^{-1}$)	Temperature (C°)	Humidity (%)	Pressure (mmHg)
CON	220 ± 50	3.0 ± 0.4	18.5 ± 1.5	40 ± 5	757 ± 18
KET	220 ± 50	3.0 ± 0.4	18.2 ± 1.1	41 ± 3	744 ± 6

Data presented as mean ± SD. No differences were identified between trials when comparing all measures (all $p > 0.05$).

Following exercise, a cannula was inserted into a forearm vein (within 5 min of exercise cessation) for repeated blood sampling. A blood sample was taken before participants ingested carbohydrate and protein drinks [Silverspoon Sugar Cane, Sucrose, 0.8 $g \cdot kg^{-1} \cdot h^{-1}$ (3.2 $kcal \cdot kg^{-1} \cdot h^{-1}$) MyProtein Vanilla Whey, protein hydrolysate, 0.4 $g \cdot kg^{-1} \cdot h^{-1}$ (1.6 $kcal \cdot kg^{-1} \cdot h^{-1}$)] for four hours, in line with post-exercise recovery guidelines for optimal replenishment of glycogen stores (18), with sucrose chosen due to the potential to further enhance liver glycogen repletion (17). The carbohydrate-protein drinks were ingested either with (KETONE) or without (CONTROL) a ketone monoester (KME) (R)-3-hydroxybutyl (R)-3-hydroxybutyrate [ΔG; TΔS Ltd., Oxford, UK; 0.31 $mL \cdot kg\ body\ mass^{-1} \cdot h^{-1}$ (0.29 $g \cdot kg\ body\ mass^{-1} \cdot h^{-1}$, 1.4 $kcal \cdot kg^{-1} \cdot h^{-1}$)] for the first 3 hours (**Figure 1**). The dose of ketone monoester was aimed at increase serum β-hydroxybutyrate concentrations to ~2-3 $mmol \cdot L^{-1}$ in line with prior work (6).

Blood sample processing and analysis

Blood was collected into serum separation tubes and left to clot at room temperature for 40 min prior to centrifugation (3000 *g* for 10 min at 4°C), after which serum aliquots were stored at −20°C until later analysis. Serum glucose and βHB concentrations were measured using the RX Daytona (Randox Laboratories, Crumlin, UK). Serum EPO concentrations were measured using an enzyme-linked immunosorbent assay (ab274397 Human Erythropoietin SimpleStep ELISA Kit, Abcam).

Statistical analysis

The primary outcome for the current study was the incremental area under the curve (iAUC) for serum EPO concentrations. The sample size was based on prior data where it was reported that intravenous infusion of βHB increased EPO concentrations from 7.6 ± 1.0 to 9.9 ± 1.1 $IU \cdot L^{-1}$ (10), which equates to an effect size (cohens *d*) of 2.19. With this

effect size, and an alpha-level of 0.05, 6 participants should provide more than 95% power with a two-tailed *t*-test. It was decided to aim for 10 participants to be conservative with the statistics and to account for potential dropout. One participant dropped out due to scheduling issues and therefore the final n = 9. Data were analysed using GraphPad Prism v9 (GraphPad Software, San Diego, CA, USA). Time series data were converted into incremental area under the curve (iAUC) using the Time Series Response Analyzer (19). Prior to analysis, paired differences were checked for normality by visual inspection of Q-Q plots and the Shapiro-Wilk test. Where there was no evidence of non-normal distribution, data were expressed as mean ± SD in text and mean ± 95% confidence intervals (CI) in figures. Where evidence of non-normal distribution was detected (i.e., in the glucose data), data were expressed as medians ± interquartile range. Time series data were analysed by two-way, repeated measures ANOVA (time x condition). Where interaction effects were detected, *post-hoc* tests were adjusted using the Holm-Sidak method to account for multiple comparisons. Summary data were analysed by two-tailed, paired t-tests. Statistical significance was accepted when $p \leq 0.05$.

RESULTS

Pre-ingestion of the recovery drinks, no differences were detected between trials in either serum βHB (**Figure 2A**; p = 0.75) glucose (**Figure 2B**; p = 0.07) or EPO concentrations (**Figure 3A**; p = 0.72). Due to one participant displaying a markedly higher (7.15 $mmol \cdot L^{-1}$) baseline glucose concentration in the CONTROL trial, a sensitivity analysis was performed by analysis with and without this participant.

Following ingestion of each dose of ketone monoester, serum βHB concentrations rose by ~1 $mmol \cdot L^{-1}$, reaching a peak of 3.2 ± 0.5 $mmol \cdot L^{-1}$ at 180 min, whereas serum βHB concentrations remained negligible with CONTROL (**Figure 2A**); time x condition

interaction: $p < 0.0001$. Serum glucose concentrations rose following ingestion of the first carbohydrate-protein drinks (time effect: $p < 0.0001$), and over the duration of recovery. However, serum glucose concentrations were lower with KETONE vs. CONTROL (condition effect: $p = 0.002$; **Figure 2B**), although no interaction effect was detected (time x condition interaction: $p = 0.76$). The removal of the participant with a high baseline glucose concentration in the CONTROL trial did not impact the overall inference (condition effect: $p = 0.005$).

Serum EPO concentrations rose throughout the recovery period (time effect: $p < 0.0001$), to a greater extent in KETONE vs. CONTROL (time x condition interaction: $p = 0.046$; **Figure 3A**). At the end of the recovery period, serum EPO concentrations were ~20% higher with KETONE vs CONTROL ($p < 0.01$). The iAUC for serum EPO concentrations was ~3-fold higher with KETONE vs CONTROL ($p = 0.03$; **Figure 3B**). Sensitivity analysis by removal of the participant displaying the largest response made little difference to the interpretation of the EPO response (Cohen's $d = 0.85$ *vs* 0.81 with, *vs* without this participant).

DISCUSSION

This study is the first to investigate whether ingestion of a ketone monoester post-exercise can increase serum EPO concentrations in humans, providing clear evidence in support of that hypothesis. Furthermore, ketone monoester ingestion lowered glucose concentrations during post-exercise recovery, during which large amounts of carbohydrate and protein were ingested in accordance with guidelines for rapid recovery from exercise.

Ketone monoesters were developed to rapidly increase circulating βHB concentrations without the need to deplete carbohydrate availability or increase fatty acid availability.

Whilst these supplements have been examined in exercise studies, much of the prior research has been centred on the potential for ketone monoesters to provide additional exogenous fuel for skeletal muscle and/or the brain (7-9). The data in the current study demonstrate that ketone monoesters may have potential alter physiology via mechanisms completely distinct from the provision of metabolic substrate for skeletal muscle and the brain. Specifically, the current data suggest that ketone monoesters may have potential to increase red cell mass via increases in EPO. The mechanisms by which βHB may increase EPO remain unknown, but may include histone acetylation, since βHB at physiological concentrations (~1.2 mmol·L-1) can induce 5-fold increases in histone H3 acetylation in kidneys of mice and subsequently provide protection against oxidative stress (20). Furthermore, increases in EPO by exposing cell lines to hypoxia is associated with histone H3 acetylation (21). Furthermore research is needed to establish whether histone acetylation and other putative mechanisms may explain the increase in EPO with ketone monoester ingestion.

Red cell mass displays a strong correlation with aerobic capacity, which is unsurprising given the potential for the oxygen carrying capacity of the blood to dictate peak oxygen uptake. EPO is the primary regulator of red blood cell production, and recombinant EPO injections can increase red cell mass by 10% in 5 weeks (22). Furthermore, supplementation of nutritional compounds can increase EPO concentrations and red cell mass. For example, cobalt supplementation has been shown to acutely increase EPO concentrations from ~7.5 to ~10.7 $IU \cdot L^{-1}$ (23) and, when supplemented over a 3-week period, can increase haemoglobin mass by >10% (24) – roughly equivalent to what can be achieved with moderate altitude exposure over a similar timeframe (25). It is therefore notable that the magnitude of acute increase in EPO in the current study (~21%) is comparable to both cobalt supplementation (~22%) and hypoxia equivalent to 2000 m

altitude (20%). Further work is needed to confirm if the acute EPO response to ketone monoester ingestion does indeed translate into increased red cell mass and aerobic capacity.

The current study also demonstrated that ketone monoester ingestion lowers serum glucose concentrations during post-exercise recovery when large amounts of carbohydrate and protein were being ingested. βHB is known to suppress endogenous glucose production (3), and ketone monoesters have been demonstrated to increase post-exercise whole-body glucose disposal during a hyperglycaemic clamp (4), although effects on muscle glycogen resynthesis are equivocal (4, 5). It would be expected that the high carbohydrate and protein ingestion would produce hyperinsulinemia, which could have maximally suppressed endogenous glucose production even in the absence of ketone monoester ingestion. However the addition of protein to carbohydrate ingestion during post-exercise recovery can increase arterial glucagon concentrations by ~50 $pg \cdot mL^{-1}$ compared to carbohydrate ingestion alone (26). This difference in glucagon has been demonstrated to potently counteract the effects of insulin on liver glucose metabolism. Only a 35 $pg \cdot mL^{-1}$ increase in glucacon concentrations is sufficient to suppress hepatic glycogen synthesis by ~40%, even under hyperinsulinemia (~192 $pmol \cdot L^{-1}$) (27). It is, therefore, entirely possible that under the paradigm of post-exercise protein-carbohydrate feeding, that endogenous glucose production is not maximally suppressed, allowing for the opportunity for βHB to further suppress endogenous glucose production. Taken together, it is likely that the lower glucose concentrations are due to lower hepatic glucose output and/or increased hepatic (and/or skeletal muscle) glucose uptake. This may have implications for post-exercise liver glycogen recovery, which could be assessed in future studies with the application of ^{13}C nuclear magnetic resonance spectroscopy.

A limitation of the current study is the lack of haematological outcomes such as haemoglobin concentration, haematocrit, reticulocyte count, ferritin concentrations etc., to establish whether the observed EPO response translates into changes in haemopoiesis. The reason these outcomes were not determined in the present study was due to the lack of information a priori as to whether ketone monoester ingestion would increase EPO concentrations and as such, it was deemed inappropriate to require participants to provide blood with the diet and lifestyle control, over the necessary timeframe to detect changes in such parameters (i.e., 7-14 days) (22). The study was therefore designed with the primary aim to assess the acute EPO response to ketone monoester ingestion, and future studies are required to examine the haematological responses over days to weeks.

In conclusion, ingestion of ketone monoesters during post-exercise recovery increases serum EPO concentrations in humans, when following current best-practice nutrition guidelines for rapid glycogen replenishment. In addition, ketone monoester ingestion lowered serum glucose concentrations during post-exercise recovery. These data reveal new applications of ketone monoesters in human health and performance separate from their role as exogenous fuels, such as to increase aerobic capacity via erythropoiesis or other EPO-related effects.

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Authorship: J.T.G., J.A.B., E.E., and D.J.D. conceptualized and designed the project. J.T.G., J.P.W., E.E., and A.H., collected the data. J.T.G. and E.E. analyzed the data.

J.T.G. and E.E. wrote the initial draft, all authors contributed to reviewing and editing the final manuscript.

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FIGURE LEGENDS

Figure 1. Schematic of the main trial days. Nine healthy males completed a 1-hour but of cycling intervals followed by a 4-hour recovery period. During recovery, participants ingested 0.8 g/kg of carbohydrate (CHO) and 0.4 g/kg of protein (PRO) at 0, 1, 2 and 3 hours of recovery with or without 0.29 g/kg of ketone monoester (KET) ingested at 0, 1 and 2 hours of recovery. Red droplets represent blood sampling timepoints.

Figure 2. Serum beta-hydroxybutyrate (A) and glucose (B) concentrations during post-exercise recovery with ingestion of carbohydrate plus protein, either with (KETONE), or without (CONTROL) the addition of a ketone monoester (KETONE) in healthy men. Data are mean ± 95%CI for panel A and median ± interquartile range for panel B, $n = 9$. *$p < 0.05$ for KETONE vs CONTROL.

Figure 3. Serum erythropoietin (EPO) concentration (A) and incremental area under the curve (B) during post-exercise recovery with ingestion of carbohydrate plus protein, either with (KETONE), or without (CONTROL) the addition of a ketone monoester (KETONE) in healthy men. Data are means ± 95%CI, $n = 9$. *$p < 0.05$ for KETONE vs CONTROL.

Table 1. Intensities completed and testing condition in both trials.

	Mean intensity		Testing Conditions		
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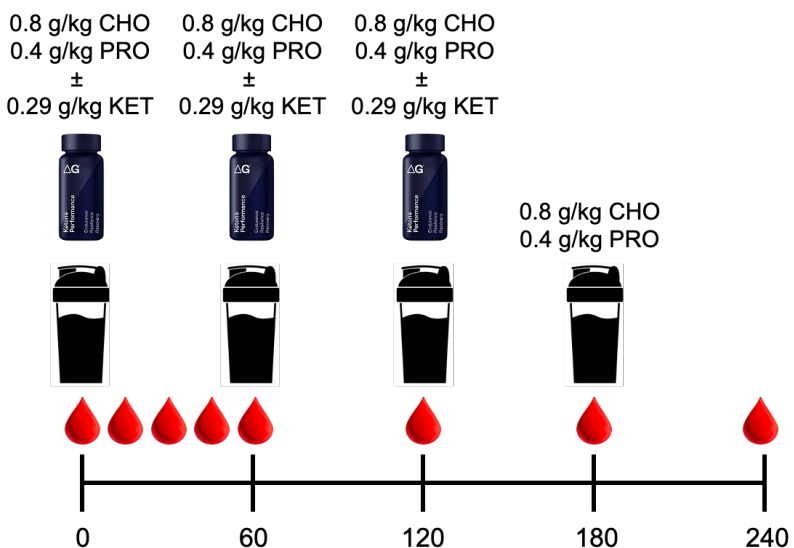
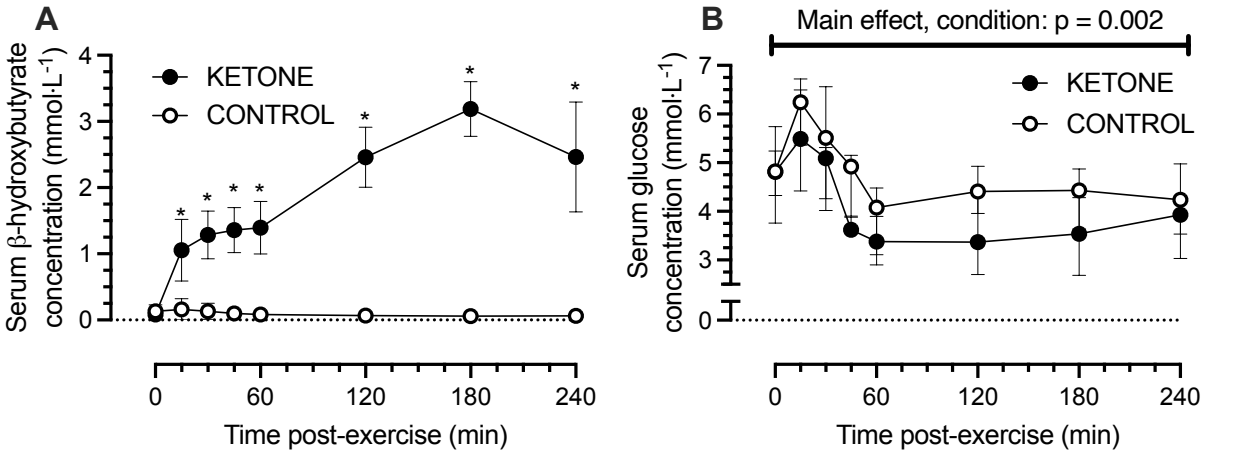
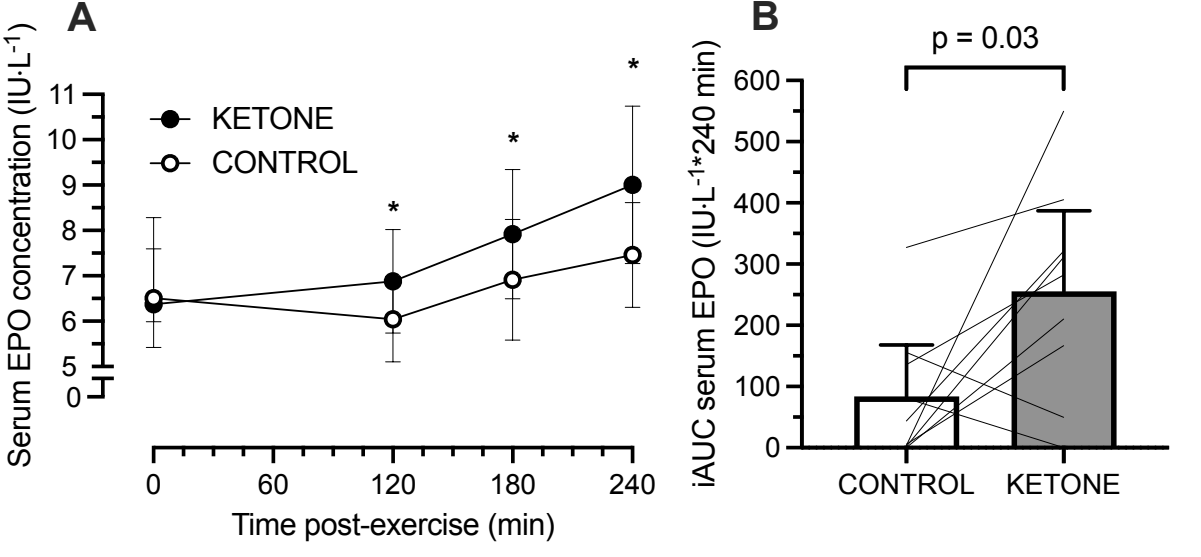
Data presented as mean ± SD. No differences were identified between trials when comparing all measures (all $p > 0.05$).

n = 9 healthy males
1-h cycling intervals:
(2 min @ 90% VO_2peak; 2 min @ 50% VO_2peak)




A
Serum β-hydroxybutyrate concentration (mmol·L-1)
KETONE
CONTROL
0
1
2
3
4
*
0
60
120
180
240
Time post-exercise (min)
B
Main effect, condition: p = 0.002
Serum glucose concentration (mmol·L-1)
KETONE
CONTROL
0
3
4
5
6
7
0
60
120
180
240
Time post-exercise (min)


A
Serum EPO concentration (IU·L-1)
11
10
9
8
7
6
5
0
KETONE
CONTROL
*
*
*
0
60
120
180
240
Time post-exercise (min)
B
p = 0.03
iAUC serum EPO (IU·L-1*240 min)
600
500
400
300
200
100
0
CONTROL
KETONE