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Effects of Short-Term Sodium Nitrate versus Sodium Chloride Supplementation on Energy and Lipid Metabolism during High-Intensity Intermittent Exercise in Athletes

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Abstract: The aim of this study was to investigate the possible effects of chronic nitrate supplementation on the metabolites of energy metabolism during high-intensity, high-volume intermittent training (HIHVT). In this placebo-controlled double-blind study, 17 participants exercised 3 times a week on a cycle ergometer. Sodium nitrate or sodium chloride as the placebo was supplemented daily at 8.5 mg/kg body weight for 10 days. The training exercise consisted of a warm-up, a 45-min interval period, and a post-exercise period. Oxygen uptake, respiratory exchange ratio, and various parameters were measured in the venous blood and plasma. During training, the oxygen uptake and respiratory exchange ratio did not differ between the nitrate and the placebo group. Venous plasma concentrations of nitrate and nitrite were significantly increased in the nitrate group ($p < 0.001$ and $p = 0.007$, respectively). Triglyceride concentrations were significantly lower in the nitrate group than in the placebo group ($p = 0.010$). The concentration of free fatty acids in the plasma did not change upon nitrate supplementation and no significant differences were observed in the contribution of fat to energy metabolism during exercise. An increase in plasma ammonia concentration was observed in the nitrate group during and after exercise ($p = 0.048$). Metabolites of energy-rich phosphates did not differ between the nitrate and chloride groups, suggesting no improvement in efficiency through the supplemented nitrate. It was concluded that nitrate supplementation did not reduce oxygen uptake and adenosine triphosphate resynthesis by hydrolysis or through creatine kinase activity during high-intensity, high-volume intermittent exercise. Although, lipid metabolism as well as amino acid metabolism might be affected by nitrate supplementation during HIHVT.

Keywords: exercise; nitrate supplementation; free fatty acids; nitric oxide; nitrite; oxygen consumption; energy metabolism; triglyceride; ammonia

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1. Introduction

Several studies have reported that both acute and chronic supplementation with inorganic nitrate (NO_3^-) or beetroot juice (BRJ), which is known to contain NO_3^-, can improve exercise performance; yet, the underlying mechanisms are still not completely understood [1–6]. NO from endogenous and exogenous sources is a signaling molecule and has many biological functions, such as dilation of arterioles [7–9], glucose uptake [10,11], and stimulation of mitochondrial biogenesis [12]. Research also suggests that NO may improve mitochondrial efficiency by reducing the O_2 cost of adenosine triphosphate (ATP) resynthesis [13] and/or improve muscle contractile efficiency by reducing the ATP cost of muscle contraction and the ATP turnover rate from creatine phosphate (PCr) hydrolysis [14,15].

It has been shown that ingested nitrate, such as in the form of beetroot juice (BRJ), can lead to reduced oxygen cost during exercise, altered oxygen uptake kinetics [15–21], and improved exercise tolerance [15–17,20,22,23]. Studies suggest that increased NO bioavailability may also improve lipid metabolism [24,25] or reduce the utilization of muscle glycogen [26].

Most studies have investigated the effects of nitrate supplementation on single exercise tasks, and the effects of long-term nitrate supplementation on exercise training remain unknown. Some studies with sprint interval training (SIT) have reported an increase in sprint performance upon nitrate supplementation [27–29]. Yet, the meta-analysis by Wong et al. [30] revealed no changes in power during high-intensity interval training. Additionally, look at more recent review and meta-analysis papers on nitrate supplementation [31–34].

In a previous study [35], we showed a reduction in the ratio of VO_2 to power (VO_2/W), up to seven days after the end of nitrate supplementation during 3-week high-intensity, high-volume intermittent training (HIHVT). These observations suggest that supplemented inorganic nitrate may have changed the physiological adaptations to HIHVT [35]. The mechanisms leading to a reduction in VO_2 after nitrate supplementation remain unclear. In the study previously reported by Finkel et al. [35], nitrate supplementation was found to increase the synthesis of homoarginine (hArg) during HIHVT [36], suggesting the effects of nitrate supplementation on amino acid metabolism.

In addition to the reduction in VO_2/W [35], nitrate supplementation may also reduce the substrates' turnover, thus leading to an altered energy supply, possibly resulting in changes in the plasma concentrations of related parameters. We hypothesized that nitrate supplementation would be accompanied by changes in various metabolic pathways during HIHVT [35]. If VO_2 during exercise is decreased, muscle efficiency may be increased and therefore, a decrease in metabolites of ATP degradation, represented by xanthine (XA) and uric acid (UA), may be expected. We further hypothesized that lipid metabolism, represented by triglycerides (TG), free glycerol (Glyc), and free fatty acids (FFA), may be decreased with nitrate supplementation. Other changes may occur in amino acid metabolism, including arginine (Arg), creatinine, and ammonia (NH_3). In the present study, the potential effects of short-term nitrate supplementation on healthy male recreational athletes during HIHVT were investigated.

2. Materials and Methods

2.1. Participants

Originally, the study was designed for 20 healthy young male recreational athletes to be divided into two groups: the nitrate group (N group) and the placebo group (P group), each containing 10 subjects [35]. The sample size calculation was based on the main parameters, i.e., VO_2, electrolyte concentration, acid-base status, and blood volume changes [35]. It was assumed that these would be normally distributed with $\alpha = 5\%$ and $\beta = 20\%$. The calculated sample size is comparable to the number of subjects in similar studies [34,37]. Due to resources, 17 subjects participated in and completed the double-blind placebo-controlled study (Table 1). The groups did not differ from each other. Subjects with cardiovascular disease and a predisposition to collapse and breathlessness during training were excluded. None of the participants were taking any medication or smoking tobacco. All subjects were in good health condition during the experiment. The study was part of a short-term study and was approved by the local Ethics Committee of Hannover Medical School (MHH) (2015–2013) [35,36]. The participants gave their written consent. The participants were instructed to keep a nutritional diary for 1 week and to come to the laboratory healthy and rested. The nutritional diary from week 1 was reproduced for the post-supplementation tests in week 5 as reported elsewhere [35]. The participants were advised not to change their normal exercise routine.

Table 1. Anthropometric and physical characteristics of the participants in the sodium nitrate group and the sodium chloride group (placebo group).

Variable	Nitrate Group	Placebo Group
Number	9	8
Age (y)	26.1 ± 4.4	26.5 ± 3.4
Height (cm)	184.7 ± 8.1	180.5 ± 6.2
Weight (kg)	78.3 ± 8.0	77.2 ± 10.9
$relVO_{2peak}$ (mL/min/kg)	57.3 ± 5.4	56.1 ± 8.8
$relW_{max}$ (W_{max}/kg)	4.85 ± 0.62	4.83 ± 0.91

$relVO_{2peak}$ = relative peak oxygen uptake; $relW_{max}$ = relative peak power.

2.2. Experimental Design

Each subject participated in a 3-week period of HIHVT with oral nitrate or placebo supplementation [35,38] (Figure 1). Pedaling cadence was measured, and the participants cycled with a cadence between 80 and 90 rpm. All tests for the same participants were performed at the same time of day. The participants were instructed in the use of the cycle ergometer, and individual configurations, including saddle and handlebar heights, were recorded and retained. Ambient conditions and power parameters were kept constant over time.

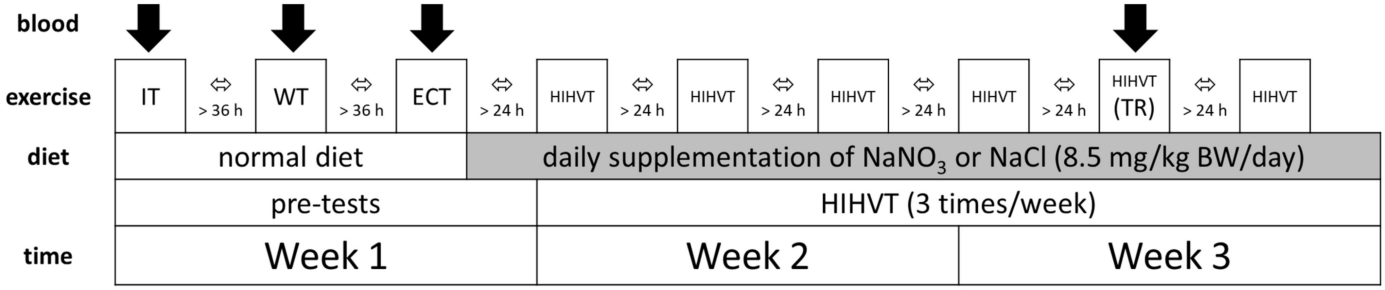


Figure 1. Overview of the time and tests of the study. Before the training period, the participants performed an incremental test (IT), a double Wingate test (WT), and an endurance capacity test at 80% relative W_{max} until exhaustion (ECT) on 3 separate days. All exercises were performed on a cycle ergometer (Excalibur Sport, Lode, Groningen, The Netherlands). Blood samples were taken during different exercises indicated by black arrows. IT = incremental test; WT = double Wingate test; ECT = endurance capacity test; TR = 5th training session; $NaNO_3$ = sodium nitrate; NaCl = sodium chloride; BW = body weight; HIHVT = high-intensity, high-volume intermittent training [39].

The IT was performed to determine the W_{max} and the peak oxygen uptake (VO_{2peak}) [40]. Both were calculated as values relative to body weight. Relative W_{max} ($relW_{max}$), relative VO_{2peak} ($relVO_{2peak}$), and weight were used to create two equally balanced groups (Table 1). The IT, WT, and ECT were performed as described elsewhere [35,40,41].

The participants remained on the ergometer after exhaustion, and recovery periods were observed for 10 min. Individual W_{max} values were used to set exercise intensity during the WT, the ECT, and the training sessions.

The participants completed intermittent training of 45 high-intensity intervals (Figure 2) 3 times per week [39,41]. The training schedule was not fixed, but the participants were encouraged to complete the training sessions evenly distributed throughout the week with at least 24 h between two training sessions. Data were collected during the 5th training session (no earlier than) the 10th supplementation day (TR).

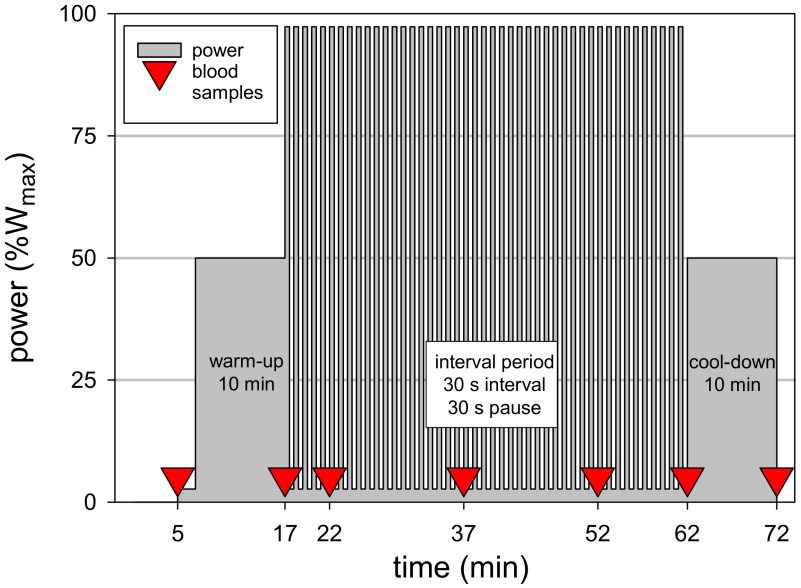


Figure 2. Schematic of the power curve during high-intensity, high-volume intermittent training (HIHVT). The HIHVT started with a 5 min rest period on the ergometer followed by 2 min at 10 W. As a warm-up, the participants pedaled for 10 min at 50%W_{max}. Then, they performed 45 intervals at W_{max} minus 10 W with an active recovery period at 10 W in between. The interval period was followed by a 10 min cool-down at 50%W_{max}. During the 5th training session on at least the 10th supplementation day, blood samples were drawn at the exercise time points as indicated by the red triangles.

2.3. Supplementation

Starting the day after the last test of week 1, the participants consumed the same dose of 8.5 mg/kg body weight/day of $NaNO_3$ (N group), equivalent to 0.14 mmol NO_3^-/kg body weight/day, or NaCl as a placebo (P group) (both Merck, Darmstadt, Germany). All participants were instructed to dissolve the supplements ($NaNO_3$ or NaCl) in 250 to 500 mL of tap water and to consume the solution distributed throughout the day. No other restrictions such as diet, use of mouthwash, or caffeine intake were made in order not to interfere with the individual lifestyle behavior or the training conditions.

2.4. Measurements

Spirometry was performed after calibration of the instrument (Metalizer 3B, Cortex, Leipzig, Germany) as described elsewhere [35]. During TR, breathing frequency (BF), ventilation (VE), end-tidal carbon dioxide ($petCO_2$), and respiratory exchange ratio (RER) were taken as mean values of 120 s. Lactate (Lac_a) and glucose (Glu_a) concentrations were measured (Elacure M Hote Créme, Carinopharm GmbH, Elze, Germany; Biosen S line, EKF Diagnostic, Barleben, Germany) in arterialized (a) and heparinized venous (v) blood samples during IT and TR. Venous (v) blood samples were collected at the same time as arterialized blood samples, except at 2 min at 10 W. For HCT, venous blood was collected in heparinized microhematocrit tubes (Brand GmbH & Co KG, Wertheim, Germany), immediately centrifuged, and analyzed.

The blood samples were analyzed with ABL 825 flex (Radiometer, Copenhagen, Denmark) to determine the concentrations of glucose (Glu_v), lactate (Lac_v), sodium (Na_v), and potassium (K_v), Hb, and pH. For the determination of the total protein concentration, venous blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA, S-monovette, Sarstedt, Germany). All tubes were centrifuged to obtain plasma. Protein and NH_3 concentrations were measured immediately after the end of the test. The biuret method (E. Merck, Darmstadt, Germany) was used to determine total protein concentration. NH_3 was measured using an enzymatic kit (Roche Diagnostics, Mannheim,

Germany) and an Ammonia/Ethanol/CO_2 Normal Control (Roche Diagnostics, Mannheim, Germany). NO_3^-, NO_2^-, CRN, UA, and XA were measured simultaneously in plasma (stored at −80 °C) by gas chromatography-mass spectrometry (GC-MS, Dreieich, Germany) as described elsewhere with minor modifications [42,43]. The concentrations of NO_3^- and NO_2^- in the tap water used in the study to prepare the drinking solutions of NO_3^- and chloride were about 5 µM and 1 µM, respectively, as measured by GC-MS [43].

An enzymatic colorimetric technique (NEFA-C test kit, Wako Chemicals, Neuss, Germany) was used for the quantitative determination of non-esterified FFA in plasma. The values were reported as the sum of FFA. To determine Glyc and TG in plasma, ultraviolet (UV) test kit no. 148270 (Boehringer, Mannheim, Germany) was used. The DiaSys ST FS standard and Wako Chemicals GmbH (Neuss, Germany) were used for calibration.

2.5. Data Analysis, Presentation, and Statistics

The results are presented as the mean ± standard deviation. Statistical analysis was performed using two-way repeated measures ANOVA (SigmaPlot 14.0, Systat Software, San Jose, CA, USA). Missing data points were automatically estimated with least-squares means using a general linear model. When interactions were detected, the results were further explored with the Holm–Sidak test to examine changes over the course of the exercise. Pearson's product–moment correlation coefficient was used to determine correlations. A two-tailed p-value < 0.05 was considered significant.

3. Results

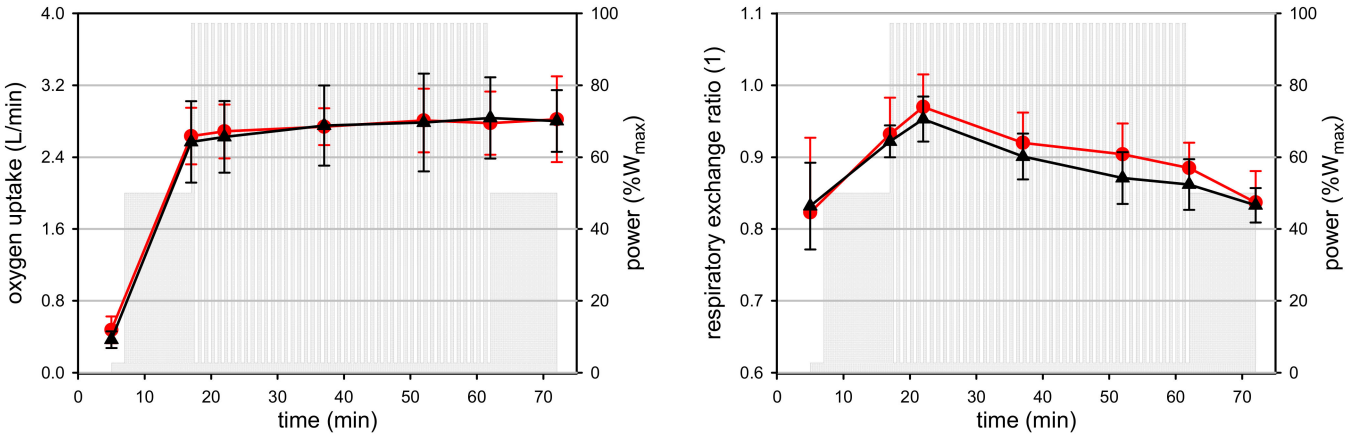
There were significant differences in the plasma concentrations of NO_3^- ($p < 0.001$) and NO_2^- ($p = 0.007$) between the groups during training (Table 2). The plasma concentrations of NO_3^- ($p = 0.084$) and NO_2^- ($p = 0.647$) did not change significantly over time. VO_2, RER, BF, and VE were not different between the groups (VO_2: $p = 0.916$; RER: $p = 0.409$; Figure 3) (BF: $p = 0.562$; VE: $p = 0.607$; Table 3) and no group×time interaction was observed (BF: $p = 0.989$; VE: $p = 0.711$; VO_2: $p = 0.837$; RER: $p = 0.753$).

Table 2. Plasma concentrations of nitrate and nitrite during the 5th training session in the nitrate group (N) and the placebo group (P).

Time Point	Group	Nitrate (µM)	Nitrite (µM)
Rest	N	234.3 ± 66.7 *	1.51 ± 0.39 *
	P	61.7 ± 50.4	1.16 ± 0.22
Warm-up	N	244.4 ± 78.5 *	1.56 ± 0.29 *
	P	70.1 ± 42.0	1.25 ± 0.44
5th interval	N	246.4 ± 74.6 *	1.57 ± 0.28 *
	P	64.6 ± 46.7	1.21 ± 0.27
20th interval	N	251.3 ± 71.2 *	1.60 ± 0.24 *
	P	68.3 ± 53.1	1.13 ± 0.26
35th interval	N	254.3 ± 71.7 *	1.50 ± 0.21 *
	P	65.8 ± 46.1	1.12 ± 0.24
45th interval	N	249.2 ± 68.6 *	1.40 ± 0.23
	P	71.4 ± 44.9	1.21 ± 0.28
Cool-down	N	256.1 ± 89.9 *	1.54 ± 0.36 *
	P	67.6 ± 47.4	1.15 ± 0.20

* Denotes significant differences compared to the placebo group.

The concentration of lactate in arterialized and venous blood changed over time ($p < 0.001$), but no differences were found (Lac_a: $p = 0.954$; Lac_v: $p = 0.839$). The concentration of glucose in arterialized and venous blood changed over time ($p < 0.001$), but for Glu_v, there was group × time interaction during TR ($p = 0.002$). Post hoc tests revealed no significant differences between the groups (Table 3).



(**a**) (**b**)

Figure 3. Oxygen uptake (**a**) and respiratory exchange ratio (**b**) (left, *y*-axis) and power (right, *y*-axis) during TR in the nitrate group (red) and the placebo group (black); no significant differences were observed between the groups.

Table 3. Summary of ventilation, carbon dioxide, pH, lactate, and glucose values in the nitrate group (N) and in the placebo group (P) at the indicated time points.

Time Point	Group	VE (L/min)	$petCO_2$ (mmHg)	pH_v (1)	Lac_v (mM)	Glu_v (mM)
Rest	N	14.7 ± 5.0	35.9 ± 1.9	7.36 ± 0.02	1.04 ± 0.25	4.55 ± 0.71
	P	13.1 ± 3.1	32.6 ± 3.1	7.34 ± 0.03	1.25 ± 0.48	4.36 ± 0.62
Warm-up	N	66.1 ± 10.3	43.2 ± 4.5	7.33 ± 0.03	2.31 ± 0.49	3.93 ± 0.59
	P	68.8 ± 14.8	39.8 ± 2.9	7.32 ± 0.03	2.43 ± 0.58	3.83 ± 0.66
5th interval	N	74.2 ± 12.1	41.2 ± 5.8	7.33 ± 0.03	4.03 ± 0.60	3.94 ± 0.61
	P	76.8 ± 17.9	38.0 ± 3.7	7.33 ± 0.03	3.89 ± 0.89	3.89 ± 0.78
20th interval	N	79.4 ± 13.6	37.8 ± 6.2	7.35 ± 0.05	5.08 ± 0.90	4.23 ± 0.64
	P	83.3 ± 21.6	35.2 ± 4.0	7.34 ± 0.03	5.10 ± 1.29	4.67 ± 0.44
35th interval	N	84.8 ± 17.8	36.7 ± 6.3	7.35 ± 0.04	5.46 ± 0.95	4.40 ± 0.64
	P	88.7 ± 21.9	32.8 ± 3.7	7.35 ± 0.04	5.57 ± 1.34	4.90 ± 0.45
45th interval	N	85.0 ± 14.4	35.3 ± 7.1	7.36 ± 0.04	5.76 ± 0.98	4.40 ± 0.57
	P	91.8 ± 23.4	32.4 ± 4.7	7.36 ± 0.04	5.85 ± 1.49	4.93 ± 0.63
Cool-down	N	78.9 ± 17.8	37.1 ± 5.7	7.39 ± 0.05	3.86 ± 0.95	4.23 ± 0.60
	P	86.3 ± 20.0	34.4 ± 3.9	7.36 ± 0.08	4.34 ± 1.55	4.73 ± 0.81

VE = ventilation; petCO2 = partial pressure of end-tidal carbon dioxide; Lac_v = venous lactate concentration; Glu_v = venous glucose concentration. No significant differences were observed between the groups.

No differences were found between groups in the concentrations of Na_v ($p = 0.293$), K_v ($p = 0.180$), and Hb ($p = 0.094$) or in HCT ($p = 0.802$) (Table 4).

The plasma concentrations of CRN, XA, NH_3, and total protein are shown in Figure 4. The concentrations of all analytes increased over time ($p < 0.001$). For CRN (group: $p = 0.741$, group × time: $p = 0.981$) and XA concentration (group: $p = 0.718$, group × time: $p = 0.345$), no interaction or differences between the groups were found. For NH_3, there was significant group × time interaction ($p = 0.026$). At the end of TR, the NH_3 concentration was higher in the N group compared to the P group (post hoc, $p = 0.034$). The plasma protein concentration changed significantly during TR ($p < 0.001$), but no interaction ($p = 0.110$) was observed. There were no differences between the groups ($p = 0.527$). The plasma UA concentration was not different between the groups ($p = 0.794$). UA changed over time ($p < 0.001$), but no interaction was found ($p = 0.304$).

Table 4. Summary of sodium, potassium, hematocrit, and hemoglobin in the nitrate group (N) and in the placebo group (P) at the indicated time points.

Time Point	Group	Na_v (mM)	K_v (mM)	HCT (%)	Hb (g/dL)
Rest	N	140.5 ± 1.6	3.96 ± 0.24	44.2 ± 2.2	15.9 ± 1.2
	P	140.3 ± 1.2	4.14 ± 0.21	44.0 ± 3.4	15.2 ± 0.9
Warm-up	N	142.2 ± 1.5	4.45 ± 0.28	45.1 ± 2.5	16.4 ± 1.2
	P	141.8 ± 1.2	4.54 ± 0.25	44.7 ± 3.1	15.5 ± 1.0
5th interval	N	142.7 ± 1.5	4.41 ± 0.16	45.4 ± 2.5	16.5 ± 1.1
	P	142.0 ± 1.1	4.56 ± 0.23	45.0 ± 3.1	15.6 ± 1.0
20th interval	N	142.9 ± 1.3	4.49 ± 0.21	45.7 ± 2.5	16.6 ± 1.0
	P	142.2 ± 0.8	4.62 ± 0.23	45.6 ± 3.2	15.6 ± 1.0
35th interval	N	143.3 ± 1.3	4.56 ± 0.16	45.8 ± 2.4	16.7 ± 1.1
	P	142.4 ± 0.8	4.71 ± 0.28	45.4 ± 3.5	15.7 ± 1.0
45th interval	N	143.4 ± 1.6	4.63 ± 0.25	45.8 ± 2.4	16.7 ± 1.1
	P	142.8 ± 0.7	4.80 ± 0.32	45.2 ± 3.6	15.7 ± 1.1
Cool-down	N	142.9 ± 1.9	4.88 ± 0.22	45.1 ± 2.4	16.6 ± 1.1
	P	142.4 ± 0.8	5.06 ± 0.36	44.8 ± 3.7	15.7 ± 1.1

Na_v = venous sodium concentration; K_v = venous potassium concentration; HCT = hematocrit; Hb = venous hemoglobin concentration; no significant differences between the groups.

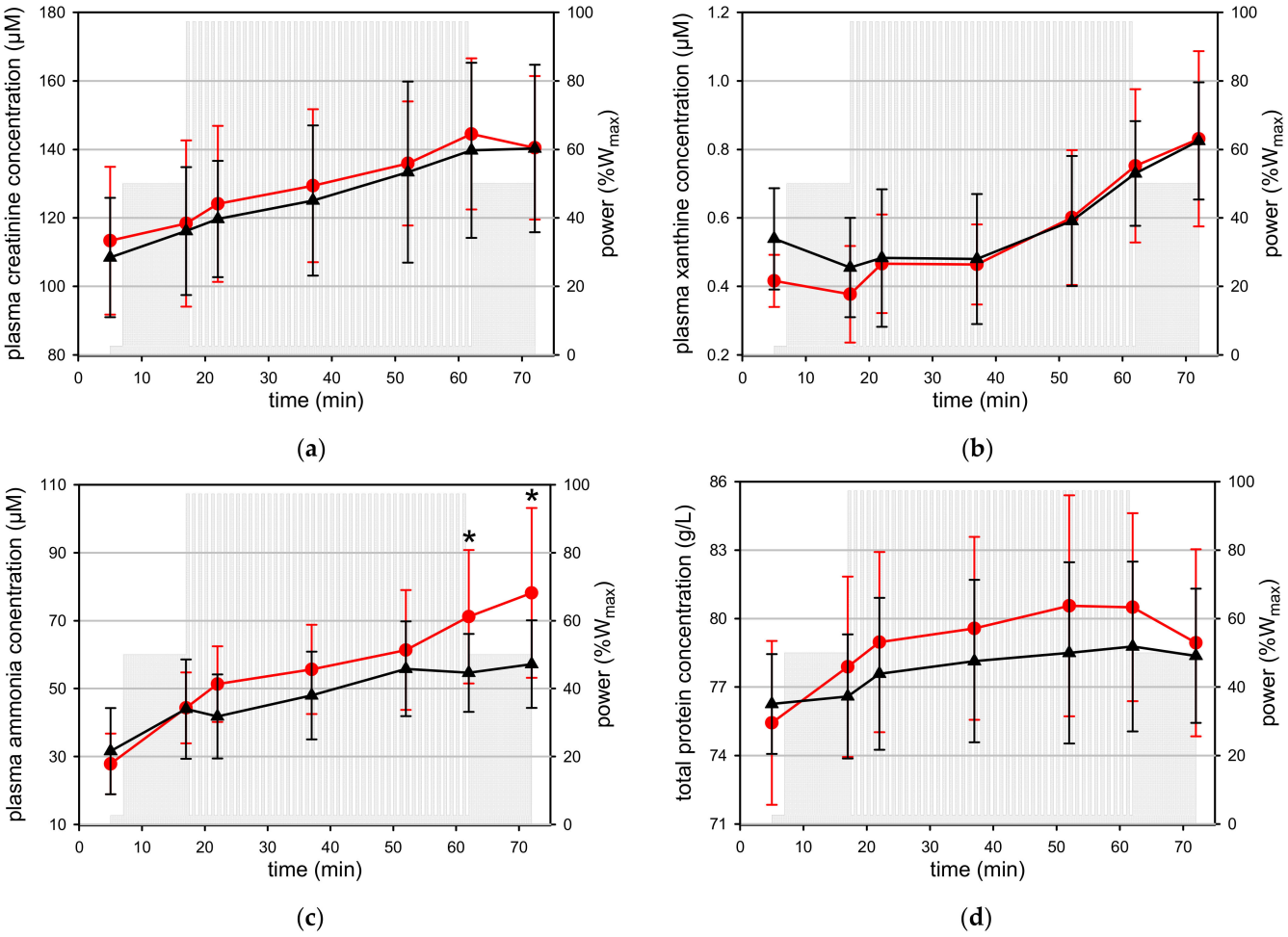


Figure 4. Concentration of metabolites in plasma (left, *y*-axis) and power (right, *y*-axis) during the 5th session of high-intensity, high-volume intermittent training: (**a**) creatinine (CRN); (**b**) xanthine (XA); (**c**) group×time interaction for ammonia (NH_3); (**d**) total protein concentration. * = significantly different between the groups.

Since no differences in CRN, XA, and UA concentrations were found, correlations were calculated for the whole group (n = 17). Plasma CRN concentrations correlated positively with XA ($p = 0.035$; r = 0.195) and UA ($p < 0.001$; r = 0.357) at all time points. A correlation was also found between UA and XA ($p = 0.018$; r = 0.219).

To characterize lipid metabolism, plasma concentrations of FFA, Glyc, and TG were measured, in addition to RER (Table 5). During TR, FFA ($p = 0.497$) and Glyc ($p = 0.486$) did not differ between the supplementation and placebo groups, but both increased significantly during the interval phase ($p < 0.001$). Significant main effects were found for TG concentration (group: $p = 0.010$; time: $p = 0.032$). Post hoc tests revealed significant differences for TG between N and P at six of the seven time points (5′IT: $p = 0.051$; others: $p \leq 0.029$). There was a significant difference between TG at the end of warm-up and at the end of cool-down ($p = 0.002$). No group×time interactions were found for the lipid metabolites (FFA: $p = 0.881$; Glyc: $p = 0.810$; TG: $p = 0.416$).

Table 5. Summary of the plasma concentrations of free fatty acids, free glycerol, and triglycerides in the N and P groups at the indicated time points in the training test.

Time	Group	FFA (mM)	Glyc (mM)	TG (mM)
Baseline WT [1]	N	0.29 ± 0.33	0.09 ± 0.09	1.10 ± 0.48
	P	0.33 ± 0.28	0.08 ± 0.04	1.23 ± 0.78
Baseline ECT [1]	N	0.29 ± 0.30	0.05 ± 0.04	1.14 ± 0.52
	P	0.23 ± 0.15	0.05 ± 0.02	0.99 ± 0.61
Rest	N	0.19 ± 0.16	0.06 ± 0.02	0.77 ± 0.30 *
	P	0.27 ± 0.19	0.07 ± 0.03	1.32 ± 0.59
5th interval	N	0.15 ± 0.06	0.08 ± 0.03	0.78 ± 0.35
	P	0.18 ± 0.13	0.08 ± 0.02	1.49 ± 0.64
20th interval	N	0.16 ± 0.05	0.09 ± 0.02	0.83 ± 0.28 *
	P	0.20 ± 0.15	0.10 ± 0.02	1.32 ± 0.65
35th interval	N	0.18 ± 0.05	0.11 ± 0.04	0.74 ± 0.26 *
	P	0.20 ± 0.13	0.12 ± 0.03	1.47 ± 0.65
45th interval	N	0.20 ± 0.08	0.13 ± 0.06	0.70 ± 0.21 *
	P	0.24 ± 0.17	0.14 ± 0.05	1.49 ± 0.76
Cool-down	N	0.23 ± 0.10	0.14 ± 0.07	0.69 ± 0.21 *
	P	0.26 ± 0.17	0.17 ± 0.05	1.32 ± 0.67

[1] = Resting values in tests before supplementation; FFA = free fatty acids; Glyc = free glycerol; TG = triglycerides. * Denotes significant differences compared to the placebo group.

4. Discussion

Nitrate supplementation, as a salt or as a BRJ constituent, can increase physical performance [1–6,31–34], which is mainly attributed to NO formed from NO_3^- through the NO_3^-/NO_2^- cycle. In the present study, the effects of chronic nitrate supplementation on the metabolites of energy metabolism during high-intensity, high-volume intermittent training (HIHVT) were investigated. Venous plasma NO_3^- and NO_2^- concentrations were significantly higher during at least 10 days of nitrate supplementation. During HIHVT, differences in TG and NH_3 were found between the two supplementation groups. Contrary to the prevailing hypothesis of NO_3^- inducing a reduction in VO_2 during exercise in the participants supplemented with NO_3^- [6,15–21], no differences in VO_2 were found between the N and P groups in the present study.

4.1. Methodological Considerations—Nitrate Dosage, Oxygen Uptake, and Measurements

The total amount of NO_3^- given to the participants in the present study (0.14 mmol NO_3^-/kg body weight/day) was similar to that reported in other studies [13]. Recently, experts have recommended an acute bolus of 5–9 mmol [44] or 8–16 mmol NO_3^- [45] to be taken 2–4 h before exercise. The dose of $NaNO_3$ used in this study is within the

recommended range [44,45]. Even at lower doses, some authors have observed acute effects of NO_3^- on VO_2 during submaximal exercise [16,17,20].

No such effects were observed in the present study. These differences may be, at least in part, due to the use of different types of nitrate supplementation, for example as an aqueous solution of pure chemical $NaNO_3$ (present study) or as BRJ (in other studies) [46]. This is noteworthy because BRJ may contain antioxidants, including polyphenols, which are known to increase NO_2^- reduction to NO in the gut and the brain [46,47]. In addition, other not yet known components of BRJ may exert additional NO independent effects on physical performance. In a meta-analysis, both supplementations induced similar effects on VO_2 during moderate (<60%VO_{2peak}) and heavy (60–80%VO_{2peak}) intensity exercise [6]. However, the number of studies included in this meta-analysis is relatively small and the result may be biased.

Recent studies have found that NO_3^- can be stored not only in the blood but also in the skeletal muscle [48–51]. This NO_3^- reservoir plays an important role during intense or prolonged exercise. In the present study, the concentration of NO_3^- and NO_2^- was measured only in plasma. Therefore, it is not possible to differentiate between the effects of NO_3^- stored in the skeletal muscle or blood.

Porcelli et al. [52] showed that the decrease in VO_2 at moderate intensity due to nitrate supplementation is negatively correlated with VO_{2peak}. Highly trained individuals ($VO_{2peak} \geq 60$ mL/kg/min) may not experience ergogenic effects from inorganic nitrate supplementation [52]. Studies on elite athletes have supported these findings [53–55]. In the current study, the mean VO_{2peak} of both groups was at a moderate level ($VO_{2peak} < 60$ mL/min/kg); therefore, a moderate effect on VO_2 could be expected [52].

Consciously, no dietary restrictions were imposed to avoid interfering with food intake behavior to achieve normal training conditions. Therefore, the amounts of totally taken nitrate, chloride, and sodium during the entire study are unknown and could vary between the participants. Since supplemented nitrate is chronically well above normal dietary intake and there is little variability in plasma concentrations of these electrolytes, it is assumed that these factors did not play a significant role in this study. This is underlined by the fact that the NO_3^- and NO_2^- concentrations were significantly enhanced in the venous blood.

Because food intake was not prescribed and only recorded during week 1, it is possible that the participants changed their diet during the study. Changes in dietary intake should have affected RER and lactate concentrations during steady-state phases during rest and warm-up [40]. As no differences were found, it is assumed that dietary habits did not influence the results in this study.

Since the values relevant for fluid shifts did not differ, it was assumed that there was no influence of nitrate supplementation in fluid shifts between the groups [39].

The concentration of NO_2^- in the venous plasma in this study is higher than that reported in other studies using chemiluminescence [13,15,22,56], but it is very close to that measured in other studies using GC-MS [57–59] and HPLC [60]. The differences are most likely of a methodological nature.

4.2. Oxygen Uptake

Supplementation with nitrate can decrease VO_2 during moderate- and high-intensity exercise [6]. The effects of ingested NO_3^- on HIHVT have not been fully investigated. Essén and Kaijser [61] showed that intermittent exercise with 15/15 intervals (15 s work and 15 s rest) results in a similar VO_2 and metabolic response compared to continuous exercise at identical mean power [61,62]. Eigendorf et al. [63] found no differences in VO_2 and metabolites of lipid metabolism between HIHVT with 30/30 intervals and continuous training with a matched mean intensity [63]. Bailey et al. [15,16] showed a decrease in the primary amplitude of VO_2 after nitrate supplementation but no differences in the mean response time between the nitrate and placebo groups [15,16]. These findings suggest an alteration in VO_2 kinetics due to nitrate supplementation. Therefore, it is hypothesized that reduced VO_2 could also be demonstrated during intermittent exercise with 30/30 intervals.

In preliminary experiments with a cross-over design, nine participants were supplemented with nitrate prior to HIHVT with 30/30 intervals. Although the nitrate supplementation was higher than in the present study (10 mg/kg body weight), the VO_2 did not change during exercise. Studies with SIT and nitrate supplementation showed a decrease in VO_2 at submaximal intensities after the exercise period but not during the interval period [28,64,65]. Some studies with intermittent high-intensity tests have reported improved performance after NO_3^- supplementation [27,56,66] but without effects on VO_2 [67]. The results of the present study confirm previous research. Therefore, it is concluded that elevated NO bioavailability does not reduce VO_2 during HIHVT with the intensity in the present study.

4.3. Glucose Metabolism and Respiratory Exchange Ratio

Supplemented nitrate has been shown to have a moderate but significant effect on glucose and lipid metabolism in rats [11,25,68,69]. We hypothesized that supplemented nitrate would lead to changes in blood glucose concentrations in humans. No significant differences in venous glucose concentration were found in the present study, but there was group×time interaction. After 45 min of high-intensity, high-volume intermittent exercise, there was non-significant Glu_v reduction in the N group (uncorrected $p = 0.089$). This would be consistent with other studies, which have shown that nitrite-derived reactive species can increase glucose uptake in mice and rats by activating adenosine monophosphate-dependent kinase (AMPK) and subsequent translocation of GLUT4 [70–72]. However, resting Glu_v was not reduced in the N group, although the plasma concentrations of NO_3^- and NO_2^- were significantly higher. Lactate concentrations in venous and arterialized blood were unchanged. Regarding RER, no significant differences were found between the two groups in the present study. RER may be influenced by increased respiration, specifically through an increase in the exhalation of CO_2. However, no differences in VE, BF, and $petCO_2$ were found between the groups. These results are similar to those observed in other studies [15,16,18,21,73], although there are studies showing that RER tended to increase [15,74] or increased significantly [13] after nitrate supplementation. An increased RER would suggest a slight shift towards carbohydrate utilization and/or amino acid combustion during exercise. Due to increased plasma and muscle tissue concentrations, the transport of glucose into the skeletal muscle and organs may be enhanced [11,75]. Therefore, it could be plausible that dietary nitrate influences glucose metabolism during HIHVT. However, this effect appears to be very small and may be restricted to tissues other than working muscle, and possible changes in Glu_v and RER are not significant in the present study.

4.4. Lipid Metabolism

To further assess energy supply, metabolites of lipid metabolism were examined. HIHVT [41] as well as other endurance exercises have been shown to increase lipolysis capacity, fatty acid uptake, and fatty acid oxidation [76–78]. Talanian et al. [79] showed an increase in fat oxidation of approximately 36% during exercise in women after seven units of high-intensity interval training [79]. Nitrate supplementation has also been shown to increase fatty acid oxidation in the muscle tissue of rodents [80] and decrease TG concentrations in plasma in mice [69]. Therefore, significant effects on lipid metabolism through HIHVT under nitrate supplementation were expected.

After the 3-week training period with HIHVT and nitrate supplementation, as published elsewhere, no changes in RER were found during ECT in either the supplementation or placebo group [35]. Thus, in this study, there did not appear to be any adaptations in fat metabolism with respect to energy supply. However, other studies using the same training protocol have found a reduced RER after 3 weeks [41,81]. Therefore, the lack of training effects in the present study was not related to nitrate supplementation. Since the $relVO_{2peak}$ during IT in the present study was approximately 12% higher than that reported by Frische [41] and Maassen [81], the missing adaptation to training could be due to a

higher training level. Further research is needed to investigate possible changes in lipid metabolism with nitrate supplementation.

Although in the present study, no differences were found between the groups in RER, Glyc, and FFA, mean plasma TG concentrations at rest and during intermittent exercise decreased by approximately 37% in the N group compared to the P group. Plasma TG concentrations are influenced by dietary habits, and large inter- and intra-participant differences have been observed [76,82,83]. As no changes in dietary behavior were observed in the present study and no differences in baseline measurements were found between the two groups, it is suggested that chronic NO_3^- supplementation may reduce plasma TG concentration.

During exercise, plasma TG may serve as an energy substrate but is thought to be less important [77]. However, in trained participants, intramuscular triglycerides (IMTG) account for a significant proportion of energy requirements [62,77]. In particular, type IIa muscle fibers have a lower capacity for plasma FFA consumption. They are more likely to derive FFA from IMTG. If IMTG is hydrolyzed, it is possible that FFA is immediately oxidized in muscle mitochondria and does not enter the bloodstream [78]. Something similar may happen with Glyc. Elia et al. [84] discussed that whole-body lipolysis assessed by Glyc may remain underestimated, as Glyc released from intramuscular lipolysis may be directly oxidized and reutilized in muscle [84,85]. This could be an explanation for changes in lipid metabolism in the absence of changes in plasma concentrations of Glyc and FFA. If plasma TG serves to replenish IMTG under the present circumstances, then nitrate supplementation may enhance this effect on muscle lipid metabolism. This seems plausible, as studies have shown that NO_3^- induces an increase in lipoprotein lipase (LPL) activity, which may serve to replenish IMTG stores and intramuscular glycogen [86,87]. LPL is active in muscle cells during and after exercise and is inactive in adipose tissue [88]. This would be consistent with the lower plasma TG concentrations during exercise in the present study. However, it does not fully explain why baseline TG levels were already lower in the nitrate group. Studies have shown that LPL overexpression in transgenic mice reduces plasma TG and increases high-density lipoprotein cholesterol (HDL-C) concentrations [89,90]. Reduced plasma TG concentrations may have implications for health and the prevention of cardiovascular disease [91,92], although the effect of chronic nitrate supplementation on lipid metabolism is not yet fully understood. Considering that the RER as well as the plasma FFA and Glyc concentrations were not different between the groups, the findings in this study do not seem to include changes in substrate turnover respective to energy supply.

4.5. Metabolites of Energy-Rich Phosphates

In the present study, the energy-rich phosphates ATP and PCr were not measured. Instead, CRN, a metabolite of creatine and PCr, as well as XA and UA, metabolites of purine metabolism, were measured in plasma.

Studies suggest that hypoxanthine, a product of ATP breakdown, can be used as a marker of muscle adenine nucleotide degradation [93–95]. Hypoxanthine is converted to XA and then to UA by xanthine oxidase [96–98]. Therefore, XA and UA may also be used as markers of adenine nucleotide degradation, if the activity of xanthine oxidase is not inhibited [96,98]. Since no differences in the plasma concentrations of XA and UA were found between the two groups in the current study, it can be assumed that the resynthesis of ATP with adenosine diphosphate (ADP) and adenosine monophosphate (AMP) has not changed.

CRN occurs spontaneously from creatine or PCr [99]. In vivo, the reaction to CRN is irreversible and dependent on pH and temperature [99,100]. Therefore, CRN production should be increased during highly intensive exercise [101]. The correlation of CRN with XA and UA found in the present study suggests that CRN could be used as an indicator of PCr and ATP breakdown. However, this needs to be further investigated at different intensities.

If muscle efficiency increases due to nitrate supplementation and PCr breakdown decreases during exercise [14,15], ATP hydrolysis would decrease. Therefore, the concen-

trations of ADP [15] and AMP in the working muscle tissue as well as the concentration of CRN would decrease with nitrate supplementation.

In the current study, no differences in CRN were found between N and P. The storage capacity of PCr should not be significantly increased after four additional training sessions [102,103]. Therefore, it can be assumed that creatine metabolism is not altered by the present nitrate supplementation during HIHVT. This would be contrary to Bailey et al. [15], who showed a reduced PCr amplitude in the skeletal muscle by phosphorus magnetic resonance spectroscopy after nitrate supplementation [15].

4.6. Amino Acid Metabolism—Ammonia

NH_3 can be generated from adenine nucleotides, but since no changes in XA, UA, and CRN were observed in the present study, the increase in plasma NH_3 in the N group may be indicative of increased amino acid degradation or reduced depletion of NH_3 [93,104,105]. In humans, radiolabeled NO_3^- has been found to be metabolized to radiolabeled urea [106]. Since urea is further catabolized to NH_3 by urease, it is possible that supplemented nitrate was metabolized to NH_3, albeit to a small degree [106,107].

NH_3 can also result as a product of amino acid metabolism. In the present study, an increase in plasma NH_3 concentration was observed during exercise, particularly in the late phase of training. An increase in NH_3 has also been observed in preliminary experiments and in another study with 30/30 intervals [63]. Furthermore, NH_3 accumulation during exercise has been found to be higher during training with 30/30 intervals than during sprint interval training or continuous exercise [63]. The authors suggest that glycogen depletion is higher in 30/30 intervals than in sprint interval training or continuous exercise. This is consistent with studies showing a greater increase in NH_3 accumulation with a lower glycogen concentration during exercise [108,109]. Since higher concentrations of NO_3^- and NO_2^- in muscle and plasma may increase muscle glucose uptake [11,13], there could be greater glycogen depletion during HIHVT and therefore increased amino acid deamination. On the other hand, in a muscle biopsy, Tan et al. [26] found a tendency for a sparing of muscle glycogen and PCr after nitrate supplementation during prolonged moderate-intensity exercise [26].

The total protein concentration in the venous plasma was not different between the N and P groups in the present study. This may indicate that there was no change in amino acid metabolism between the two groups during training. It is possible that protein synthesis is independent of NO_3^- or it cannot be observed during the short training phase.

It is worth mentioning that the plasma concentration of the non-proteinogenic amino acid hArg increased after nitrate supplementation [38], strongly suggesting altered amino acid metabolism by the supplemented nitrate. Recent studies suggest that higher blood and urine hArg concentrations are associated with reduced renal and cardiovascular morbidity [110–114]. Whether nitrate supplementation may enhance exercise performance via hArg remains to be investigated. Furthermore, studies have found that dietary nitrate from vegetables may have a protective role in cardiovascular disease and muscle function [115–118]. Since even a small chronic amount of supplemented nitrate can already increase stored NO_3^- in muscle and blood [48], daily consumption of a few handfuls of nitrate-rich vegetables may have health-promoting effects [115,116].

5. Conclusions

In the present study, short-term nitrate supplementation was found to be associated with changes in lipid metabolism in healthy recreational athletes during HIHVT. Lower plasma triglyceride concentrations at rest and during HIHVT suggest that lipid metabolism was altered during 10 days of supplementation without affecting the contribution of fat to the energy supply during exercise. In addition, no significant differences in free fatty acids and free glycerol were found between the nitrate and placebo groups. Nitrate supplementation had no effect on the metabolites of energy-rich phosphates. Therefore, it is assumed that nitrate supplementation did not alter ATP resynthesis by hydrolysis

or creatine kinase. Plasma ammonia accumulation was higher in the participants who consumed sodium nitrate, which may be an indicator of increased deamination. Nitrate supplementation was not associated with lower oxygen uptake during HIHVT.

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