Dietary Nitrate Supplementation Is Not Helpful for Endurance Performance at Simulated Altitude Even When Combined With Intermittent Normobaric Hypoxic Training

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Introduction: Training intensity and nutrition may influence adaptations to training performed in hypoxia and consequently performance outcomes at altitude. This study investigates if performance at simulated altitude is improved to a larger extent when high-intensity interval training is performed in normobaric hypoxia and if this is potentiated when combined with chronic dietary nitrate (NO_3^-) supplementation.

Methods: Thirty endurance-trained male participants were allocated to one of three groups: hypoxia (13% F_iO_2) + NO_3^-; hypoxia + placebo; and normoxia (20.9% F_iO_2) + placebo. All performed 12 cycling sessions (eight sessions of 2*6 × 1 min at severe intensity with 1 min recovery and four sessions of 4*6*10 s all-out with 20 s recovery) during a 4-week period (three sessions/week) with supplementation administered 3–2.5 h before each session. An incremental exhaustion test, a severe intensity exercise bout to exhaustion (T_{lim}) and a 3 min all-out test (3AOT) in hypoxia ($F_iO_2 = 13\%$) with pulmonary oxygen uptake ($\dot{V}O_2$), $\dot{V}O_2$ kinetics, and changes in *vastus lateralis* local O_2 saturation (SmO_2) measured were completed by each participant before and after training.

Results: In all tests, performance improved to the same extent in hypoxia and normoxia, except for SmO_2 after T_{lim} ($p = 0.04$, $d = 0.82$) and 3AOT ($p = 0.03$, $d = 1.43$) which were lower in the two hypoxic groups compared with the normoxic one. Dietary NO_3^- supplementation did not bring any additional benefits.

Conclusion: Performance at simulated altitude was not improved to a larger extent when high-intensity interval training was undertaken in normobaric hypoxic conditions, when compared with normoxic training. Additionally, dietary NO_3^- supplementation was ineffective in further enhancing endurance performance at simulated altitude.

Keywords: nitrate, hypoxia, performance, intermittent training, endurance

INTRODUCTION

Although most sport events are held at sea level, athletes may be required to compete in hypoxic environments (Burtscher et al., 2018); e.g., some stages of the road cycling "Tour de France," mountain ultramarathon as the Hard Rock or Ultra Trail du Mont Blanc, ski mountaineering competitions, or soccer championship in Bolivia. Yet, altitude has a detrimental effect on aerobic performance (for a review see Millet et al., 2010). When acutely exposed to lower inspired oxygen (O_2) pressure (inducing a lower O_2 availability), a significant reduction of maximal oxygen uptake ($\dot{V}O_2max$) occurs (Lawler et al., 1988). In contrast, anaerobic capacity is well maintained in hypoxia (Friedmann et al., 2007). When logistically impossible to live and/or train in real altitude conditions, intermittent training in normobaric/hypobaric hypoxia (i.e., living low training high, LLTH) may represent a reliable alternative to pre-acclimatize and prepare for competitions in altitude (Chapman et al., 2013). By adding the stress of hypoxia during both aerobic or anaerobic interval training, performance at sea level in endurance athletes may not increase any more than simply perform the same training at sea level (Faiss et al., 2013a). Benefits on sea-level performance, if training stimulus would be increased (allowing higher recruitment of fast twitch fibers), are still ambiguous (Lundby et al., 2012), and presently unknown when preparing athletes for altitude performance.

When athletes perform hypoxic training, several key components of the training program need to be adjusted; i.e., planning and periodization; training intensity; recovery; fatigue monitoring and acclimatization; hydration; and nutrition (Mujika et al., 2019). One nutrient is of particular interest: inorganic nitrate (NO_3^-) and a precursor of nitric oxide (NO), which is traditionally ingested by athletes in the form of beetroot juice. Studies conducted with moderately trained participants in normoxia conditions indicate that dietary NO_3^- can improve muscle efficiency (by reducing the O_2 cost of submaximal exercise) and enhance skeletal muscle contractile function. However, others have not been able to confirm these improvements with highly trained individuals (for a review see Jones et al., 2018). The dietary practice involving the serial reduction of NO_3^- to nitrite (NO_2^-) and further to NO, a recently described pathway to generate NO (Lundberg et al., 1994), has gained popularity among athletes. Such interest results in the enhanced reduction of NO_2^- to NO in conditions of acidosis (Modin et al., 2001) and hypoxia (Castello et al., 2006), suggesting that dietary NO_3^- has the potential to improve training adaptation in hypoxic conditions.

Vanhatalo et al. (2011) showed that 0.75 L (9.3 mmol NO_3^-) of beetroot juice ingested during 24 h prior to hypoxic exposure (~14.5% O_2—3,000 m) augmented time limit for single-leg knee extension high-intensity exercise, accompanied by a reduction in muscle metabolic perturbation in healthy subjects. Similarly, it was reported that 3 (8.4 mmol/day; Kelly et al., 2014) and 6 days (5.1 mmol/day; Masschelein et al., 2012) of beetroot juice induced longer time limit at 75%Δ cycling exercise (~13.1% O_2–3,600 m) and in an incremental cycle test (~11.0% O_2— 5,200 m). The latter was also accompanied by a minor decrease

in arterial saturation (SaO_2). In contrast, trained cyclists have shown no beneficial effects on 15 km cycle time trial at ~11.0% O_2 (5,200 m) after 3 days of sodium nitrate supplementation (0.1 mmol/kg/day; Bourdillon et al., 2015).

Given the lack of consistency in performance and metabolic improvements, dietary NO_3^- supplementation could be interpreted as unnecessary to improve performance or training adaptation in hypoxic conditions. However, several factors may potentially contribute to a misinterpretation of this recommendation. It was previously suggested that extending the pre-competition supplementation period (at least up to 4 weeks) may benefit exercise performance (Wylie et al., 2016). Therefore, the short duration of NO_3^- supplementation traditionally used in previous studies (~3–6 days) may not be sufficient to elicit the full ergogenic effect of NO_3^-. Moreover, the hypoxia-induced lowering of the training stimulus (especially for high-intensity exercise that requires greater input of type II fibers), that is, paramount for the NO_3^-—NO_2^-—NO bioavailability backup system enhancement (Castello et al., 2006), may have also influenced the effects on hypoxic training adaptations and consequently, performance outcomes in hypoxic environments. However, the effects of the combination of chronic dietary NO_3^- supplementation and of high-intensity (i.e., in severe intensity domain and with repeated sprints) training in hypoxia on subsequent aerobic and anaerobic performances at altitude remain to be clearly investigated. Therefore, the aim of the present study was to investigate the influence of an extended dietary NO_3^- supplementation period (4 weeks) through beetroot juice ingestion, combined with intermittent high-intensity exercise training sessions in hypoxia (3 days/week at ~3,000 m) on the performance at simulated altitude (~3,000 m). It was hypothesized that (i) performance at simulated altitude would be improved to a larger extent when intermittent high-intensity exercise training sessions are performed in hypoxia, when compared to similar training in normoxia and (ii) that chronic dietary NO_3^- supplementation would potentiate a performance improvement, when compared to the placebo condition.

MATERIALS AND METHODS

Participants

Thirty endurance-trained male participants, regularly involved in competitive running, cycling, and/or triathlon events (4–5 times per week: ~10–15 h) in the past 5 years, and volunteered to take part in this study. Exclusion criteria were as: (i) smoking or other chronic issues; (ii) using medication or dietary supplements in the past 3 months, or during the duration of the study; and (iii) being exposed to altitude (≥2,000 m) in the past 3 months. The Ethics Committee of the University of Trás-os-Montes and Alto Douro approved this study (Reference 14A/CE/2017) and all participants provided written informed consent after being informed of all experimental procedures and possible risks associated with the experiments. All procedures complied with the ethics code of the Declaration of Helsinki.

Study Design

This randomized, single-blind, placebo-controlled, and independent group study was carried out in a laboratory at sea level (Porto's Exercise Medical Center, Portugal), which allows controlling for humidity, temperature, altitude, and % O_2 availability. This chamber reduced the fraction of O_2 in the inspired air by diluting it with extra nitrogen (normobaric hypoxic system: b-Cat®). The oxygenation level in the chamber was controlled by a specific O_2 sensor placed inside the chamber and remained stable in each training session/test. All participants had previously been involved with research in our laboratory and were familiar with the equipment and testing protocols implemented, thus familiarization sessions were not required. Consequently, $\dot{V}O_2max$ values were already known, and participants were randomly assigned to one of three experimental groups (HNO, HPL, and CON) with similar distributions for $\dot{V}O_2max$, age, body mass, and height (55.1 ± 5.4, 57.3 ± 8.3, and $51.9 \pm 7.9\,ml \cdot kg^{-1} \cdot min^{-1}$; 36.1 ± 4.6, 37.4 ± 5.7, and 34.9 ± 8.4 years old; 71.0 ± 7.7, 70.2 ± 7.3, and 73.2 ± 9.5 kg; and 175.7 ± 7.1, 173.3 ± 6.8, and 175.3 ± 6.9 cm, for HNO, HPL, and CON groups, respectively). Participants performed 12 high-intensity interval-training (HIIT) sessions during a 4-week period (three sessions/week, 48 h in-between each session), in one of three experimental groups: (i) HNO: high-intensity exercise training sessions in normobaric hypoxia ($F_iO_2 = \sim13\%$, ~3,000 m) with NO_3^- supplement; (ii) HPL: high-intensity exercise training sessions in normobaric hypoxia ($F_iO_2 = \sim13\%$, ~3,000 m) with placebo supplement, and (iii) CON: high-intensity exercise training sessions in normoxia ($F_iO_2 = 20.9\%$) with placebo supplement.

Participants were instructed to maintain their normal daily activity throughout the 4-week period, and only an extra training session peer week was allowed (except for week 4). Also, they were advised to maintain their normal diet although they received a list of foods rich in NO_3^- to be avoided (e.g., leafy green vegetables, beetroot, and processed meats) to isolate supplemented NO_3^- as a cause of any potential beneficial effect. Moreover, participants were asked to abstain from the use of any chewing gum and antibacterial mouthwash products, as these have been shown to inhibit the conversion of NO_3^- to NO_2^- by bacteria in the oral cavity. A food diary was used to ensure adherence to the intervention.

In the week before (pre-intervention) and after (post-intervention) the 4-week exposure, participants performed an incremental, continuous test to exhaustion (INC), an exercise transition from rest to severe intensity to exhaustion (T_{lim}), and a 3 min all-out test (3AOT), in a cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands) in normobaric hypoxia ($F_iO_2 = \sim13\%$, ~3,000 m).

Training Intervention

From the 12 HIIT sessions, in each week, two sessions of short aerobic intervals (HIT; first- and third-week's sessions) and one session of repeated-sprint training (RST; second week's session) were performed on the cycle ergometer. A warm-up of 10 min at 100 W + 4 min at 50% of power associated with $\dot{V}O_2max$ (p $\dot{V}O_2max$) and a cool-down period of 15 min of active recovery at 100 W were completed in each session. The main sets for HIT were as follows: two sets of 6×1 min at 90%Δ (90% between $\dot{V}O_2max$ and the first ventilatory threshold), with 1 min active recovery between repetitions and 3 min between sets, both at 50% of p $\dot{V}O_2max$ (short intervals). The main sets were for RST: four sets of 6×10 s all-out, with 20 s active recovery and 3 min between sets, both at 50% of p $\dot{V}O_2max$ (repeated-sprint training). The number of repetitions was increased from 6 (first and second weeks) to 7 (third and fourth weeks) in both sessions. The training intensities used were relative to specific p $\dot{V}O_2max$ (assessed in hypoxia for HNO and HPL, and in normoxia for CON group). For the total duration of all training sessions (ranging from 52 to 57 min), participants always remained inside the chamber. For the oxygenation level in the environmental chamber to remain stable, no one was allowed in or out of the chamber during the training sessions.

Participants were instructed to keep their cadence between 70 and 90 rpm in HIIT's short intervals, but in the RST sessions, the cadence was not fixed (isokinetic operation mode of the ergometer) and therefore, encouragement was given to perform at their maximal effort. In addition to the three supervised high-intensity training sessions, participants could perform one extra training session (that occurred on weekend days, in every case) below the moderate intensity domain (~60 min) in weeks 1–3 at normoxic conditions. No extra training session was allowed in week 4 to eliminate acute physiological effects.

Supplementation

Supplements were ingested 2.5–3 h prior to each session. NO_3^- was administered in the form of beetroot juice, containing 400 mg of a powdered standardized beetroot extract (containing 2% of NO_3^-, ~8.4 mmol, according to the manufacturer label) dissolved in 150 ml of water (Sabeet®, Sabinsa Corporation). An equivalent volume of black currant juice, with negligible NO_3^- content, was served as a control drink. Both supplements were similar in appearance and taste. Supplementation was extended until post-intervention period (ingested 2.5–3 h prior each performance test).

Performance Tests

In INC test, which was always conducted first, both $\dot{V}O_2max$ and p $\dot{V}O_2max$ were assessed. This test consisted of a 2-min step duration with 30 W increments between steps with a self-selected cadence between 70 and 90 rpm. Time sustained was determined through the T_{lim} test and performed at 80%Δ (i.e., first ventilatory threshold—VT_1, plus 80% of the difference between VT_1 and $\dot{V}O_2max$). The test ended when the cadence could no longer be maintained within 10 rpm of the preferred cadence for >5 s. Critical power (CP) and the curvature constant (W′) were both assessed during the 3AOT test as previously described (Sousa et al., 2021). In this test, during the last 5 s of the baseline period, subjects were asked to increase their cadence to approximately 110 rpm. The braking resistance for the pedals to move was set (using the linear mode of the ergometer) so that

the subjects would attain the power output halfway between $\dot{V}O_2max$ and the VT_1 (i.e., $VT_1+50\%\Delta$) on reaching their preferred cadence (linear factor = power/cadence squared).

In both T_{lim} and 3AOT tests (performed randomly), a standard 5 min warm-up exercise at 50% of p$\dot{V}O_2max$, followed by a 5-min passive rest, was performed. In all three tests, a standard 3-min period of unloaded baseline pedaling (8 W) at each participant's preferred cadence, preceded each test. Verbal encouragement was given to motivate the participants to perform their best effort in the INC and 3AOT tests protocols and for as long as possible during the T_{lim} one. All tests were performed at room temperature (18–20°C) and 40–50% relative humidity (standardized and at the same time of the day (±2 h) for each participant to minimize an impact of the circadian variation on performance).

Experimental Measures

During each test, respiratory and pulmonary gas-exchange variables were continuously measured in a breath-by-breath mode (K5, Cosmed, Italy). The gas analyzer was calibrated before each test with gases of known concentration (16% O_2 and 5% CO_2) and the turbine volume transducer calibrated with a 3-L syringe, according to the manufacturer's instructions. $\dot{V}O_2max$ (measured as the average of breaths over the last 30 s) was defined in one of the following cases: a plateau in $\dot{V}O_2$ despite an increase in power, [La-] ≥ 8 mmol·L^{-1}, respiratory exchange ratio ≥ 1.0, HR >90% of [220—age], or a volitional exhaustion (controlled visually and case-by-case). p$\dot{V}O_2max$ was estimated as the power corresponding to the first stage of the INC test that elicited $\dot{V}O_2max$. If a plateau of less than 2.1 ml·min^{-1}·kg^{-1} could not be observed, the p$\dot{V}O_2max$ was calculated as previously described (Kuipers et al., 1985). VT_1 and VT_2 were determined as previously suggested (Sousa et al., 2021). The percentage of power, $\dot{V}O_2$ and HR relative to p$\dot{V}O_2max$, $\dot{V}O_2max$, and HRmax associated to both VT_1 and VT_2 (VT_1-W, VT_1-O_2, VT_1-HR, VT_2-W, VT_2-O_2, and VT_2-HR) were considered for analysis.

During both T_{lim} and 3AOT tests, changes in local O_2 saturation (SmO_2), which represents the balance between O_2 delivery and extraction by the muscle, and in total hemoglobin (THb), an indicator of local blood volume, were assessed in the capillaries through a near-infrared spectroscopy (NIRS) monitor (Moxy monitor, Fortiori Design, Minnesota, United States). The Moxy monitor was positioned on the participant's dominant leg *vastus lateralis* muscle, halfway between the greater trochanter and lateral epicondyle of the femur, perpendicular to the muscle fiber orientation. This muscle was chosen as it is part of the knee extensor group which is the primary contributor to force generation in the crank of the bicycle during down stroke of the pedal. Prior to placement, this area was trimmed with an electric razor and cleaned with alcohol swabs. Moxy was secured with a light shield and black athletic tape to block ambient near-infra red light from interfering with the detectors. Its exact position was recorded and replicated in all tests (pre-and post-intervention periods). As suggested, skinfold thickness at the site of the Moxy placement was measured using a skinfold caliper (Harpenden Ltd.) to ensure that the skinfold

thickness was <1/2 the distance between the emitter and the detector (25 mm).

Capillary blood samples (5 μl) were collected from the earlobe for whole lactate concentration ([La⁻]) determination before exercise (at rest) and immediately at the end of each test, during the 1st, 3rd, 5th, and 7th minute of the recovery period until maximal ([La⁻]$_{max}$: for INC test) and peak values ([La⁻]$_{peak}$: T_{lim} and 3AOT tests) were reached (Lactate Pro2, Arkay, Inc., Kyoto, Japan).

Data Analysis

As the gas analyzer did not measure directly F_iO_2, the corrected F_iO_2 (~13%) was inputted at the end of each test. $\dot{V}O_2$ errant breaths (e.g., caused by swallowing, coughing, and signal interruptions) were omitted from the $\dot{V}O_2$ analysis by including only those that were between $\dot{V}O_2$ mean ± 4 SDs. After this process, the breath-by-breath data were used for $\dot{V}O_2$ kinetic analysis in the T_{lim} test. The first 20 s of data after the onset of exercise (cardio-dynamic phase) was not considered for model analysis. To allow comparison of the $\dot{V}O_2$ response, data were modeled using both a mono (Equation 1) and a double exponential approach (Equation 2) to isolate a possible $\dot{V}O_2$ fast component response.

$$VO_2(t) = A_0 + A_1 * \left(1 - \exp^{(-t/\tau_1)}\right) \tag{1}$$

$$VO_2(t) = A_0 + A_1 * \left(1 - \exp^{(-t/\tau_2)}\right) + A_2 * \left(1 - \exp^{(-t/\tau_2)}\right), \tag{2}$$

where $VO_2(t)$ represents the relative VO_2 at the time, t, A_0 is the VO_2 at rest (ml·kg^{-1}·min^{-1}), and A_1 and A_2 (ml·kg^{-1}·min^{-1}) and τ_1 and τ_2 (s) are the amplitudes and time constants of the fast and slow VO_2 components, respectively. A_1 was used to determine the gain ($G = A_1$/power) of the primary component. To estimate VO_2 kinetics parameters, equations were fitted to the exercise data (GraphPad Prism, GraphPad Software, La Jolla, CA, United States) by minimizing the sum of the mean squares of the differences between modeled and measured VO_2 values.

Initially, the raw SmO_2 and [THb] data were treated using a smooth spline filter to reduce the noise created by movement and data presented every 2 s. Baseline SmO_2 (SmO_{2base}) and baseline [THb] were computed as a 30 s average while subjects performed the standard 3 min period of unloaded baseline pedaling (8 W) at their preferred cadence before the beginning of each test. Minimum SmO_2 (SmO_{2min}) was the lowest 6 s average reached during each test. Maximum SmO_2 (SmO_{2max}) and maximum [THb] were the highest 6 s average reached during each test with recovery phase included. Average SmO_2 from 30 to 120 s after the end of each test was used to assess recovery SmO_2 ($SmO_{2recovery}$). For each test, baseline SmO_{2base} and SmO_{2min} are expressed as % of SmO_{2max} (relative-SmO_{2base} and relative-SmO_{2min}, respectively). Change in SmO_2 (ΔSmO_2) and change in [THb] ([ΔTHb]) were calculated as the difference between relative-SmO_{2min} and relative-SmO_{2base} and, the difference between maximal and baseline [THb].

Statistical Analysis

A total of 10 participants per group were considered for a type I error of 5%, a power of 80%, with statistical significance, and an average to be detected with probability population effect size of 0.5 (as it indicates a moderate to large difference; G*Power software version 3.1.9.2). *A priori* type of power analysis was used considering the student's *t*-test as the statistical test performed. Shapiro–Wilk test confirmed the data normality and Levene's test the variance homogeneity. Data are presented as mean ± SD. A two-way (group × time) ANOVA test was used to test main and interaction effects for the studied variables in performance (*post-hoc* comparisons when appropriate: Bonferroni test). Student's *t*-tests were used to test: (i) performance differences at moderate altitude when high-intensity exercise training sessions were performed at moderate altitude, compared to the condition where they were performed at normoxia (HNO + HPL vs. CON) and (ii) performance differences at moderate altitude when chronic dietary NO_3^- supplementation was used together with high-intensity exercise training sessions performed at hypoxia, compared to the condition where placebo condition was used (HNO vs. HPL). Repeated measures *T*-Tests were used to test both pre- vs. post-performance differences in-between each intervention group. Pearson's correlation coefficient (*r*) was employed to analyze the bivariate correlations between the different variables. Magnitude of the changes using effect sizes (ES) = (post-test mean − pretest mean)/(pretest standard deviation) was obtained for each test, and threshold values considered were "trivial" (<0.2), "small" (0.2–0.6), "moderate" (0.6–1.2), "large" (1.2–2.0), and "very large" (>2.0). Magnitudes of standardized effects (Cohen's *d* calculated as the difference in means divided by the pooled SD) were determined against the following criteria: small, 0.2–0.5; moderate, 0.5–0.8, and large, >0.8. All statistical procedures were conducted with SPSS 24.0 and the significance level was set at 5%.

RESULTS

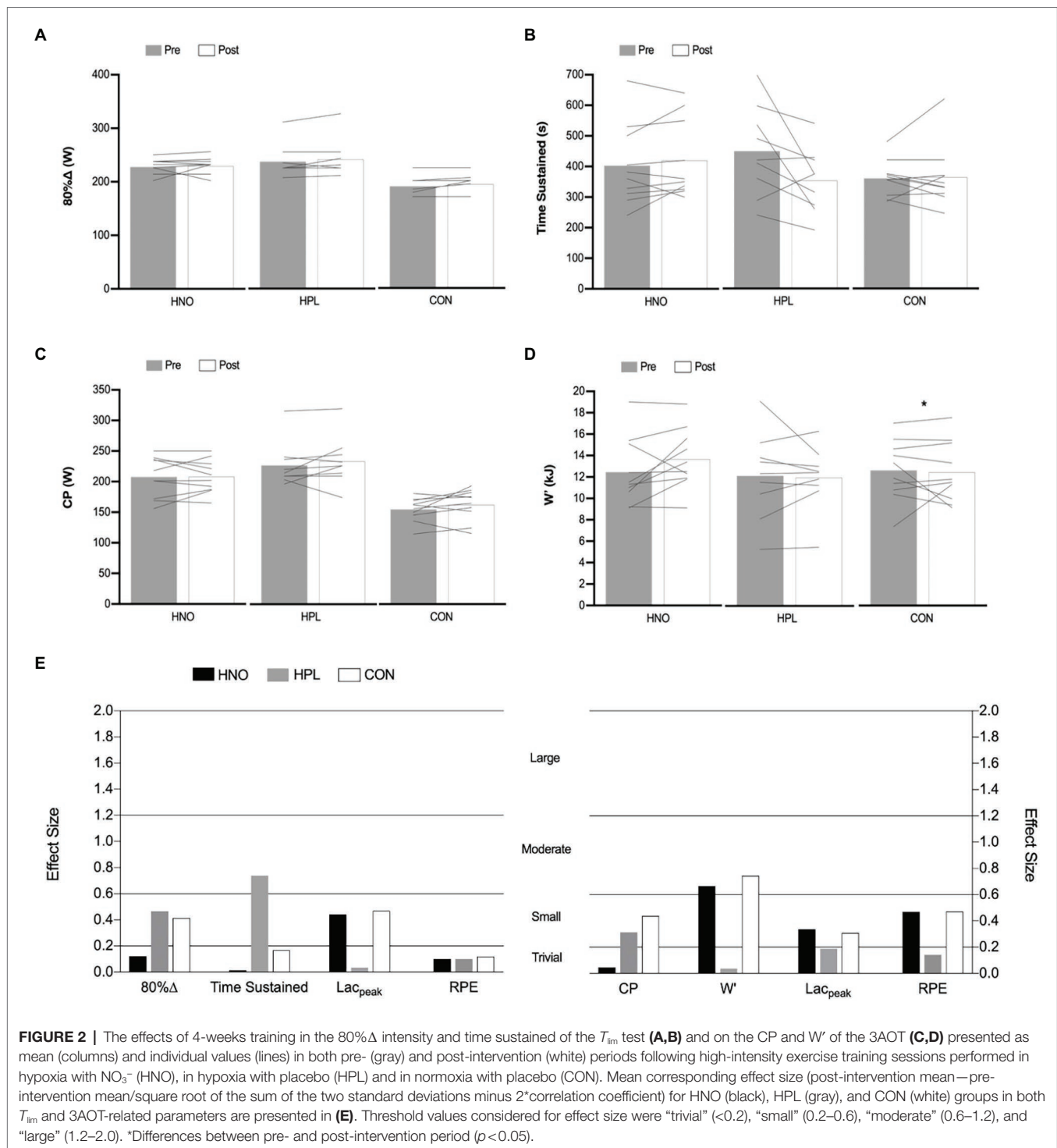### Incremental Continuous Protocol Until Exhaustion Test

Figure 1 shows the effects of 4 weeks of training in the INC test' related parameters for all experimental groups. Except for VT_1-W (time effect: *p* = 0.04) which was higher in hypoxia compared to normoxia (*p* = 0.03, *d* = 0.62), no other differences were found, inclusively between chronic dietary NO_3^- supplementation vs. placebo condition. From all the INC test-related parameters, only VT_1-W, $\dot{V}O_2$max (ES: 1.14 and 0.65, respectively, in HNO group), VT_2-W (ES: 0.72 in HPL group), and $\dot{V}O_2$max (0.77 in CON group) percentage differences had a moderate effect, with the remaining one being small (0.2–0.6) and trivial (<0.2). From pre- to post-intervention period, VT_1-W was significantly higher (*p* = 0.006, *d* = 0.48) and $[Lac]_{max}$ significantly lower (*p* = 0.009, *d* = 0.46) in HNO group, and $\dot{V}O_2$max significantly higher (*p* = 0.037, *d* = 0.94, *r* = 0.7) in CON group. No significant differences were found for HPL group.
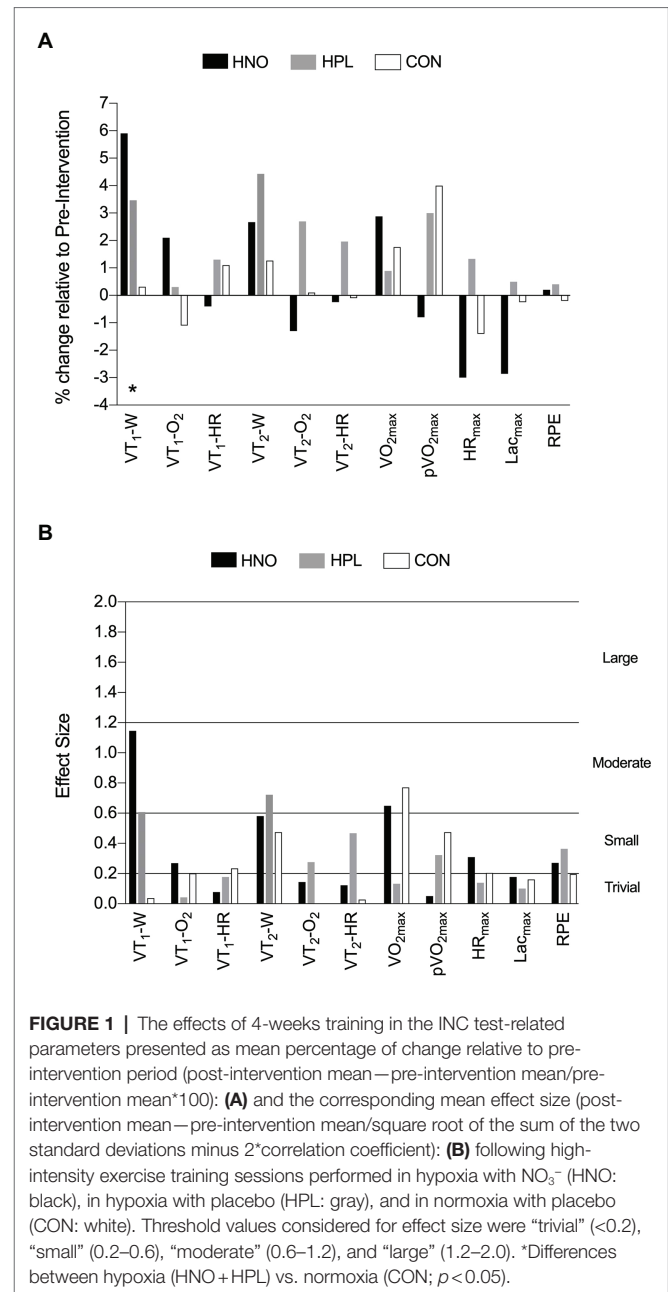
FIGURE 1 | The effects of 4-weeks training in the INC test-related parameters presented as mean percentage of change relative to pre-intervention period (post-intervention mean − pre-intervention mean/pre-intervention mean*100): **(A)** and the corresponding mean effect size (post-intervention mean − pre-intervention mean/square root of the sum of the two standard deviations minus 2*correlation coefficient): **(B)** following high-intensity exercise training sessions performed in hypoxia with NO_3^- (HNO: black), in hypoxia with placebo (HPL: gray), and in normoxia with placebo (CON: white). Threshold values considered for effect size were "trivial" (<0.2), "small" (0.2–0.6), "moderate" (0.6–1.2), and "large" (1.2–2.0). *Differences between hypoxia (HNO + HPL) vs. normoxia (CON; *p* < 0.05).

Exercise Transition From Rest to Severe Intensity Until Exhaustion and 3 Min All-Out Tests

Figure 2 shows the effects of 4 weeks of training in the T_{lim} and 3AOT-related parameters for all experimental groups. No differences were found between hypoxia vs. normoxia intervention and between chronic dietary NO_3^- supplementation vs. placebo condition in any of the tests. Only time sustained (ES: 0.74, in HPL) and W′ (ES: 0.67 and 0.74 in HNO and CON groups, respectively) differences had a moderate effect, with the remaining one being small (0.2–0.6) and trivial (<0.2). From pre- to post-intervention period, W′ was significantly lower (*p* = 0.04,

FIGURE 2 | The effects of 4-weeks training in the 80%Δ intensity and time sustained of the T_{lim} test **(A,B)** and on the CP and W' of the 3AOT **(C,D)** presented as mean (columns) and individual values (lines) in both pre- (gray) and post-intervention (white) periods following high-intensity exercise training sessions performed in hypoxia with NO_3^- (HNO), in hypoxia with placebo (HPL) and in normoxia with placebo (CON). Mean corresponding effect size (post-intervention mean—pre-intervention mean/square root of the sum of the two standard deviations minus 2*correlation coefficient) for HNO (black), HPL (gray), and CON (white) groups in both T_{lim} and 3AOT-related parameters are presented in **(E)**. Threshold values considered for effect size were "trivial" (<0.2), "small" (0.2–0.6), "moderate" (0.6–1.2), and "large" (1.2–2.0). *Differences between pre- and post-intervention period ($p < 0.05$).

$d = 0.74$, $r = 0.86$) in CON group, and no differences were found for both HNO and HPL groups.

Table 1 shows the mean values for the $\dot{V}O_2$ kinetics' parameters obtained during the T_{lim} test. In the pre-intervention period, $\dot{V}O_2$ kinetics' analysis showed that from the 30 participants, only nine individual responses (30%) were better

modeled using a double exponential approach ($A_2 = 13.03 \pm 3.4$ ml·kg^{-1}·min^{-1}: mean ± SD) with an overall $R^2 = 0.85 \pm 0.06$. In the post-intervention period, only eight individual responses (~27%) were better modeled using a double exponential approach ($A_2 = 12.66 \pm 4.1$ ml·kg^{-1}·min^{-1}: mean ± SD) with an overall $R^2 = 0.85 \pm 0.09$. For comparison

TABLE 1 | Mean ± SD values for the $\dot{V}O_2$ kinetics' parameters obtained in pre- and post-intervention periods during T_{lim} for high-intensity exercise training sessions performed in hypoxia with NO_3^- (HNO), in hypoxia with placebo (HPL), and in normoxia with placebo (CON).

Parameters	Intervention	Groups		
		HNO	HPL	CON
A_0 (ml·kg^{-1}·min^{-1})	Pre	16.1 ± 0.9	15.4 ± 1.0	17.2 ± 1.0
	Post	16.4 ± 1.3	15.1 ± 0.9	17.0 ± 0.9
A_1 (ml·kg^{-1}·min^{-1})	Pre	25.0 ± 1.3	24.8 ± 1.0	23.4 ± 1.3
	Post	24.6 ± 1.9	25.8 ± 2.2	23.4 ± 1.7
τ_1 (s)	Pre	39.6 ± 1.7	39.1 ± 1.6	42.1 ± 1.9
	Post	39.6 ± 1.9	39.6 ± 1.8	42.5 ± 1.4
Gain	Pre	11.1 ± 0.6	10.5 ± 0.6	12.3 ± 0.8
(ml·kg^{-1}·min^{-1}/W)	Post	10.9 ± 1.0	10.8 ± 0.9	12.1 ± 0.9

A_0 is the VO_2 at rest (ml·kg^{-1}·min^{-1}), A_1 (ml·kg^{-1}·min^{-1}), and τ_1 (s) are the amplitudes and time constants of the fast VO_2 components, respectively. A_1 was used to determine the gain (G=A_1/power) of the primary component.

TABLE 2 | Mean ± SD values for the oxygen saturation' parameters obtained during the T_{lim} for high-intensity exercise training sessions performed in hypoxia with NO_3^- (HNO), in hypoxia with placebo (HPL), and in normoxia with placebo (CON).

Parameters	Intervention	Groups		
		HNO	HPL	CON
SmO$_{2base}$ (%)	Pre	59.2 ± 8.8	58.6 ± 12.3	50.1 ± 7.3[#]
	Post	58.8 ± 9.3	59.0 ± 11.7	54.9 ± 9.9
SmO$_{2min}$ (%)	Pre	2.8 ± 2.3[#]	3.1 ± 1.9	3.5 ± 1.2
	Post	6.33 ± 4.8	2.1 ± 0.4	5.25 ± 2.6
SmO$_{2max}$ (%)	Pre	71.2 ± 7.5	66.1 ± 10.9	64.1 ± 10.1
	Post	67.8 ± 13.4	64.0 ± 13.2	61.8 ± 6.9
SmO$_{2recovery}$	Pre	57.3 ± 13.6	51.1 ± 18.7	39.6 ± 14.7
(%)*	Post	56.6 ± 11.1	54.9 ± 13.4	27.1 ± 15.5
Relative-	Pre	83.4 ± 11.7	88.2 ± 6.8	79.1 ± 11.5
SmO$_{2base}$ (%	Post	87.8 ± 10.8	92.7 ± 5.6	88.7 ± 12.1
of SmO$_{2max}$)				
Relative-	Pre	3.80 ± 1.7[#]	3.4 ± 1.6	3.7 ± 1.5
SmO$_{2min}$ (% of	Post	10.1 ± 8.9	6.2 ± 4.1	6.6 ± 3.9
SmO$_{2max}$)				
ΔSmO$_2$ (%)	Pre	−79.7 ± 13.05	−68.8 ± 35.2	−70.2 ± 11.7
	Post	−77.7 ± 12.6	−70.2 ± 36.2	−74.3 ± 12.7
[ΔTHb] (AU)	Pre	0.11 ± 0.01	0.14 ± 0.01	0.13 ± 0.09
	Post	0.11 ± 0.05	0.14 ± 0.03	0.10 ± 0.05

SmO_{2base}, SmO_{2min}, SmO_{2max}, and $SmO_{2recovery}$: Baseline, minimum, maximum, and recovery SmO_{2base}, respectively; Relative-SmO_{2base} and Relative-SmO_{2min}: SmO_{2base} and SmO_{2min} expressed as % of SmO_{2max}, respectively; ΔSmO_2 and [ΔTHb]: change in SmO_2 and [THb], respectively. *Differences between hypoxia (HNO+HPL) vs. normoxia (CON). [#]Differences between pre- and post-intervention period ($p < 0.05$).

purposes, only common $\dot{V}O_2$ kinetics' parameters were presented in **Table 1**.

No differences were found between hypoxia vs. normoxia intervention and between chronic dietary NO_3^- supplementation vs. placebo condition. $\dot{V}O_2$ kinetics' parameters differences were all trivial (ES < 0.2) for every experimental group. From pre- to post-intervention period, no differences were found for any experimental group.

Table 2 shows the mean values for the oxygen saturation' parameters obtained during the T_{lim} test in both pre- and post-intervention periods. Except for SmO$_{2recovery}$ (group main effect: $p = 0.001$, $d = 0.4$), which was significantly higher in hypoxia compared with normoxia ($p = 0.04$, $d = 0.82$), no other differences were found, inclusively between chronic dietary NO_3^- supplementation vs. placebo condition. The 4 weeks of training induced an increase in both SmO$_{2min}$ ($p = 0.04$) and Relative-SmO$_{2min}$ ($p = 0.05$) in HNO group (ES = 0.72 and 0.67, respectively). Moreover, CON group presented significantly higher SmO$_{2base}$ mean values in the post-intervention period ($p = 0.03$, ES = 1.05). No differences were found for the HPL group.

Table 3 shows the mean values for the oxygen saturation' parameters obtained during the 3AOT in both pre- and post-intervention periods. Except for SmO$_{2recovery}$ (group main effect: $p = 0.008$, $d = 0.2$; interaction effect: $p = 0.02$, $d = 0.2$) which was significantly higher in hypoxia compared with normoxia ($p = 0.03$, $d = 1.43$), no other differences were found, inclusively between chronic dietary NO_3^- supplementation vs. placebo condition. In HPL group, the 4 weeks of training induced an increase in SmO$_{2base}$ ($p = 0.001$, ES = 1.5), Relative-SmO$_{2base}$ ($p = 0.05$, ES = 1.14), and ΔSmO$_2$ ($p = 0.013$, ES = 1.32). Moreover, CON group presented significantly higher SmO$_{2base}$ ($p = 0.04$, ES = 0.79) and Relative-SmO$_{2base}$ ($p = 0.016$, ES = 1.11) mean values in the post-intervention period compared to baseline. No differences were found for the HNO group.

DISCUSSION

The present study is the first investigation to analyze the influence of an extended dietary NO_3^- supplementation period combined with high-intensity training sessions at simulated altitude on the aerobic and anaerobic performance at simulated altitude. Contradicting our initial hypothesis, we did not observe that performance at simulated altitude is improved to a larger extent when training sessions are performed in hypoxia, compared to the same training in normoxia, as no differences in many of the variables studied were found. Moreover, our second hypothesis was also not confirmed as we did not find evidence that chronic dietary NO_3^- supplementation potentiates performance at simulated altitude.

Training in Hypoxia

Although some improvements in anaerobic performance have been reported (Daniels and Oldridge, 1970; Gore et al., 1998; Friedmann et al., 2005; studies "uncontrolled"), it is extensively reported that no additional benefit on the aerobic performance of endurance athletes occurs when conducting interval training in a hypoxic environment, compared with the same training performed in normoxia (see Faiss et al., 2013a for a review). To overcome these limitations, it was recently proposed a new hypoxic training method: RST in hypoxia (RSH; Faiss et al., 2013b). As the training stimulus is maximal, allowing one to maintain high fast twitch fibers recruitment, additional positive results on performance could be expected when hypoxic stimulus is added. The consensus is that RSH leads

TABLE 3 | Mean $\pm$ SD values for the muscle oxygenation parameters obtained during the 3AOT for high-intensity exercise training sessions performed in hypoxia with NO_3^- (HNO), in hypoxia with placebo (HPL), and in normoxia with placebo (CON).

Parameters	Intervention	Groups		
		HNO	HPL	CON
SmO_{2base} (%)	Pre	61.9 ± 10.5	57.2 ± 10.8#	50.1 ± 8.9#
	Post	63.1 ± 6.5	61.7 ± 11.3	59.8 ± 11.2
SmO_{2min} (%)	Pre	2.7 ± 2.1	4.5 ± 3.2	4.9 ± 3.1
	Post	5.4 ± 2.3	3.1 ± 1.8	6.9 ± 3.5
SmO_{2max} (%)	Pre	69.1 ± 6.8	67.8 ± 9.4	59.3 ± 12.9
	Post	69.1 ± 11.8	63.8 ± 10.9	66.4 ± 7.5
$SmO_{2recovery}$ (%)*	Pre	60.7 ± 8.9	56.7 ± 10.9	36.7 ± 15.6#
	Post	56.9 ± 16.3	54.1 ± 12.9	53.9 ± 6.7
Relative-SmO_{2base} (% of SmO_{2max})	Pre	89.4 ± 9.6	84.5 ± 11.8#	85.7 ± 12.0
	Post	92.6 ± 10.8	96.8 ± 8.3	92.1 ± 8.8
Relative-SmO_{2min} (% of SmO_{2max})	Pre	4.06 ± 3.2	6.5 ± 3.1	8.4 ± 3.4
	Post	8.11 ± 3.1	4.9 ± 2.4	10.2 ± 4.2
ΔSmO_2 (%)	Pre	-85.4 ± 12.3	-65.1 ± 35.9#	-77.3 ± 17.6
	Post	-84.48 ± 14.3	-76.83 ± 41.1	-80.2 ± 11.8
$[\Delta THb]$ (AU)	Pre	0.12 ± 0.1	0.19 ± 0.1	0.13 ± 0.1
	Post	0.09 ± 0.06	0.07 ± 0.05	0.1 ± 0.09

SmO_{2base}, SmO_{2min}, SmO_{2max}, and $SmO_{2recovery}$: Baseline, minimum, maximum, and recovery SmO_{2base}, respectively; Relative-SmO_{2base} and Relative-SmO_{2min}: SmO_{2base} and SmO_{2min} expressed as % of SmO_{2max}, respectively; ΔSmO_2 and $[\Delta THb]$: change in SmO_2 and $[THb]$, respectively.*Differences between hypoxia (HNO+HPL) vs. normoxia (CON). #Differences between pre- and post-intervention period ($p < 0.05$).

to superior (1–5%) repeated-sprint ability in normoxic conditions, while larger increase in $\dot{V}O_2$max was not observed (Brocherie et al., 2017).

In a protocol similar to ours (eight RSH sessions during a 4-week period: 3*10 s all-out with 20 s and 5 min recovery between repetition and sets, respectively), Faiss et al. (2013b) showed that RSH (~at 3,000 m) delayed fatigue during a repeated-sprint test to exhaustion. However, endurance performance (during a 3AOT) was not increased from pre- to post-intervention period in either group, neither hypoxic stimulus evidenced larger improvements compared to the same training performed in normoxia (for both 3AOT and Wingate test). Also, a 5-week intervention of RSH (30 s sprints interspersed by 4.5 min recovery intervals, 3 weekly sessions, 4–6 sprints per session), performed at ~2,750 m altitude, did not alter endurance ($\dot{V}O_2$max test and 30-min simulated time trial) or sprinting (30 s sprint test) performance to a higher extent compared to the same training stimulus performed in normoxia (De Smet et al., 2016). Even when exposure time was extended to 7 weeks (two sessions of high-intensity at 100 or 90% of p$\dot{V}O_2$max), Roels et al. (2005) did not report a greater increase in performance (submaximal cycling test, 10 min cycle time trial and incremental test to exhaustion).

In line with most of the previous studies, we did not find differences in performance between hypoxic and control groups, except for VT_1-W in INC test ($p = 0.09$, $d = 0.62$). This was significantly higher when subjects were exposed to interval training in hypoxia compared to normoxia, achieving a moderate

effect (ES = 1.14). In fact, only HNO group evidenced significantly higher VT_1-W ($d = 0.48$) and lower $[Lac]_{max}$ mean values ($d = 0.46$) from pre- to post-intervention period. Moreover, in both T_{lim} and 3AOT, local O_2 saturation in the recovery period ($SmO_{2recovery}$) was significantly higher in hypoxia compared with the control group ($d = 0.82$ and 1.43, respectively). Therefore, the participants who were exposed to the hypoxic stimulus during the 4-weeks intervention were able to recover O_2 saturation faster after T_{lim} and 3AOT ended. Of interest, the addition of a single RSH session per week did not bring additional benefits and collectively, the combination of high-intensity sessions in hypoxia used in the present study (two HIT sessions and one RSH session) did not bring an additional aerobic and anaerobic performance enhancement compared to the same program performed in normoxia. However, an innovative and unexpected output of the present study is that this combination was also non effective for preparing athletes for simulated altitude performance.

There were also no differences in $\dot{V}O_2$ kinetics' parameters assessed in T_{lim}. It was previously reported that τ_1 and A_2 are highly plastic and decrease substantially with endurance exercise training in previously untrained individuals and that these adaptations are remarkably rapid (7–14 days; Womack et al., 1995). The authors proposed that the initial speeding (faster $\dot{V}O_2$ kinetics) might be related to faster muscle blood flow kinetics or increased capillary-to-fiber ratio. In our study, at least for the HNO group, SmO_{2min} was significantly lower in T_{lim} at post-compared to pre-intervention period. Although acute exposure to simulated altitude induces a slower $\dot{V}O_2$ kinetics (Sousa et al., 2021), there are no studies that analyzed the chronic impact of hypoxic exposure on altitude' $\dot{V}O_2$ kinetics performance.

The lack of performance differences found in our study may be due to several factors. It has been purported that intermittent exposure and/or training under hypoxic conditions may enhance exercise sea-level performance through increasing circulating erythropoietin levels and hemoglobin mass, upregulation of hypoxia-inducible factor 1α (causing structural changes within the muscle fiber), and ventilatory adaptation (Chapman et al., 2013). However, intermittent training in hypoxia is quite likely to have a minimal effect on erythropoiesis since a large "hypoxic dose" is required for significantly "stimulating the erythropoietic pathway" to the point that it enhances post-altitude sea-level endurance performance [Levine and Stray-Gundersen, 2006; e.g., $\geq$3,000 m for at least 3 h/day for 1–3 weeks (Katayama et al., 2003)]. Intermittent training in hypoxia has more potential to induce muscle skeletal and ventilatory adaptations (Faiss et al., 2013a), being suggested that an exposure altitude $\geq$4,000 m for at least 1.5 h/day repeated for 5–6 days is required to stimulate ventilatory acclimatization and concomitant improvements in performance (Muza, 2007). Moreover, greater buffering capacity, lactic acid tolerance, and/or O_2 extraction in the working muscle can be expected as well (Brocherie et al., 2017).

Collectively, it seems that the simulated altitude used in our study (~3,000 m), as well as the exposure time (~3 h per week for 4 weeks), may have been insufficient to promote the total ergogenic effect of training in hypoxia, either through hematological changes (hemoglobin mass) or *via* ventilatory

acclimatization. It is also possible that the high-intensity exercise stimulus, which necessarily induces a higher acidosis, has blunted the erythropoiesis, making HIIT less effective, as previously suggested (Millet et al., 2010).

Training in Hypoxia Combined With Dietary NO_3^- Supplementation

It is well documented that the one-electron reduction of NO_2^- to NO is enhanced in conditions of hypoxia (Kelly et al., 2014). Therefore, it would be expected that lowering skeletal muscle oxygenation would augment the potential for dietary NO_3^- supplementation to improve physiological responses during exercise and performance at simulated altitude. However, we did not find evidence that chronic dietary NO_3^- supplementation potentiates simulated altitude performance' responses when high-intensity training sessions were held at moderate altitude, as no differences were found between HNO and HPL groups in any of the variables studied. This latter corroborates previous reports where acute NO_3^- supplementation has shown to be ineffective in enhancing cycling and running' performance in hypoxia (Arnold et al., 2015; Bourdillon et al., 2015). However, significant results were also shown for single-leg knee extension high-intensity (Vanhatalo et al., 2011) and cycling exercise (Masschelein et al., 2012; Kelly et al., 2014). Some factors may potentially contribute to the lack of agreement in these data, namely, the supplementation period (Wylie et al., 2016), dosage used (Wylie et al., 2013), and training status of the participants (Wilkerson et al., 2012).

With a protocol of 6 weeks in duration (five training sessions/week, 5×30 min/week at 4–6 mmol/L blood lactate), Puype et al. (2015) concluded that dietary NO_3^- supplementation did not enhance the effects of intermittent hypoxic training on endurance exercise performance at sea level. However, the authors pointed out that higher daily doses of NO_3^- (in contrast with the 0.07 mmol NO_3^-/kg body weight used) and administered as a bolus 2–3 h (and not 2.5–2 h) prior to the training sessions could result in significant training adaptations. With a protocol similar to ours (5 weeks: 4–6*30-s sprints interspersed by 4.5 min recovery intervals; three sessions/week), and a NO_3^- dosage of 6.45 mmol administered 3 h before each session, De Smet et al. (2016) showed that performance at sea level increased in all experimental groups (with and without NO_3^- supplementation) to a similar extent. The only exception was that NO_3^- supplementation combined with training sessions performed in hypoxia increased the proportion of type IIa fibers in muscle. Taken together, it would appear that even when chronic NO_3^- supplementation is combined with HIIT training sessions performed in hypoxia, no effect on performance at sea level in healthy and/or moderately trained participant is observed. Our results extend these findings by suggesting that dietary NO_3^- does not have the potential to improve training adaptation even at simulated altitude conditions. Therefore, given the lack of consistent outcomes, at least for well-trained endurance athletes, we suggest that chronic dietary NO_3^- supplementation cannot be currently recommended to improve performance or training adaptation in hypoxic conditions, even when combined with HIIT training sessions, as previously suggested (Mujika et al., 2019).

One important feature of the present study has to do with an O_2 "sparing-effect" of oral NO_3^- supplementation, which was initially demonstrated by Larsen et al. (2007). In addition to liberating bioactive NO, NO_2^- acts as both a potent vasodilator in hypoxia and as an alternative electron acceptor, replacing O_2 in respiration (Bourdillon et al., 2015). Therefore, it would be expected the O_2 "sparing effect" of NO_3^- supplementation to be reflected in muscle tissue oxygenation in hypoxic conditions. In fact, our results show that from pre- to post-intervention period only the HNO group increased SmO_{2min} (from ~3 to 6%), at least in T_{lim} test, with the remaining experimental groups not evidencing this difference in any of the tests performed. These findings were previously observed by Masschelein et al. (2012) during steady-state cycling where an improvement in muscle tissue oxygenation index (and reduced HHb) during hypoxic exercise after 6 days of oral NO_3^- supplementation occurred. In this present study, as performance was not significantly different between HNO and HPL groups, we attribute the greater improvement in SmO_{2min} to a better matching of O_2 delivering to the metabolic requirements of the working muscles in T_{lim} test.

Limitations

In our assay, we did no measure total baseline hemoglobin mass (Hb_{mass}) or both plasma $[NO_2^-]$ and $[NO_3^-]$. Although baseline Hb_{mass} influence on the potential increase of total Hb_{mass} after exposure to a Living High-Training Low design is unclear and remains debated (Millet et al., 2019), this may have influenced the results of the present study. In recent years, several studies have assessed the acute and chronic effect of NO_3^- supplementation and reported increases in plasma $[NO_3^-]$ (~500%) and $[NO_2^-]$ (~40–140%; see Jones et al., 2018 for more details). Participants were instructed to maintain their normal diet, but they chose their own meals and were responsible for keeping their diets consistent. These could have influenced the effects of NO_3^- supplementation on the outcome variables. Moreover, the well-trained status of the participants recruited, and consequently, their higher plasma NO_2^- and NO_3^- concentrations, may have played an influential role (Wilkerson et al., 2012). In support of this, data from Bescos et al. (2012) showed that 7 out of 13 trained endurance athletes were non-responders, with only small elevations in plasma NO_3^- concentration observed following a 3-day supplementation protocol. Moreover, well-trained athletes experience less severe localized hypoxia and acidosis in the muscle compared with untrained populations at normoxia conditions (Wilkerson et al., 2012). However, when exposed to hypoxic environments, decreasing pulse oxygen saturation (SpO_2) is larger in endurance-trained athletes (compared to less trained counterparts) due to the prevalence of exercise-induced hypoxemia caused by a higher cardiac output (Mollard et al., 2007). Collectively, this seems to suggest that the training status of some of our participants may have influenced any NO_3^- supplementation effects or at least, expressed them differently. The fact that only male participants were part of this study reduces the applicability of the results found since our results are relevant to 50% of the population at large. Considering these latter, an important future direction for research is to investigate the moderating effect of training status

and gender on the response to NO_3^- supplementation in hypoxic conditions. Moreover, the inclusion of another experimental group (normoxia + supplementation) could have helped in the interpretation of the results obtained. Other sources of inter-individual variability are likely to be influential. Consequently, the study design would have been improved by better controlling these factors, particularly for the allocation of the participants in the different groups.

CONCLUSION AND IMPLICATIONS

The current experiment did not provide evidence that performance at simulated altitude (~3,000 m) would be improved to a larger extent, with high-intensity training sessions held at ~3,000 m simulated altitude for 4 weeks, compared to the condition where the training design was held in normoxia. In line with most of the previous studies on interval training in hypoxia providing no additional benefits, when compared to interval training in normoxia, the present study also reported that the weekly addition of a single RSH session was not sufficient to promote differences in performance. Also, the present study failed to show that dietary supplementation in NO_3^- would further improve performance in simulated altitude than with a placebo. As many well-trained endurance athletes spend some of their preparation time under hypoxic environments (normobaric or hypobaric hypoxia) to compete at moderate altitude conditions, this study will assist athletes, practitioners, and coaches to consider the best LLTH design (longer exposure periods) and nutritional protocols (NO_3^- supplementation currently not recommended) to improve performance or training adaptation in hypoxic conditions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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