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Hypoxia and exercise: Tissue specific and systemic adaptive responses

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Published in
Frontiers in Physiology
Frontiers in Medicine

ISSN 1664-8714
ISBN 978-2-83250-998-2
DOI 10.3389/978-2-83250-998-2

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Hypoxia and exercise: Tissue specific and systemic adaptive responses

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Citation

Yan, X., Girard, O., Duan, R., Suzuki, K., eds. (2022). *Hypoxia and exercise: Tissue specific and systemic adaptive responses*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-83250-998-2

Table of contents

frontiers | Frontiers in Physiology

TYPE Editorial
PUBLISHED 24 November 2022
DOI 10.3389/fphys.2022.1095416

OPEN ACCESS

EDITED AND REVIEWED BY
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SPECIALTY SECTION
This article was submitted to Exercise Physiology, a section of the journal Frontiers in Physiology

RECEIVED 11 November 2022
ACCEPTED 15 November 2022
PUBLISHED 24 November 2022

CITATION
Girard O, Duan R, Suzuki K and Yan X (2022), Editorial: Hypoxia and exercise: Tissue specific and systemic adaptive responses.
Front. Physiol. 13:1095416.
doi: 10.3389/fphys.2022.1095416

Editorial: Hypoxia and exercise: Tissue specific and systemic adaptive responses

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KEYWORDS

exercice, hypoxia, systemic adaptations, tissue-specific adaptation, simulated altitude

Editorial on the Research Topic
Hypoxia and exercise: Tissue specific and systemic adaptive responses

Introduction

Hypoxia exposure leads to lower oxygen availability. Animals and humans dispose of acute and long-term coping mechanisms to protect themselves from hypoxia. In fact, if individual adaptive capacities are insufficient or the environmental stimulus is too severe, hypoxia exposure may become detrimental for many organ systems notably exercising skeletal muscles and the brain. Conversely, positive physiological adaptations not only acutely enhance tolerance to hypoxia but can also induce sustained performance and health benefits. Our intention for this Research Topic was to invite submissions discussing the tissue specific and multi-systemic adaptations to hypoxia, and the combination of hypoxia and exercise. This Research Topic contains a series of eleven articles (i.e., two systematic reviews, two reviews, and seven original articles). This collection of articles provides new knowledge, and most importantly, an integrative view of some of the systemic and molecular mechanisms likely driving any hypoxia-induced adaptation or maladaptation.

Acute and chronic exposure to terrestrial altitude

Acute hypoxia refers to a short exposure when rapid physiological responses occur in order to counterbalance the decrease in oxygen pressure and delivery at the different stages of the oxygen cascade (from alveolar to mitochondria). Zhang and Wang first

demonstrated that pharmacokinetic changes of sildenafil were mainly caused by the decrease in protein expression of CYP3A4 enzyme in rats acutely exposed to 4,300 m, but not 2,300 m, above sea level. Authors argued that a multi-factor regulation mechanism likely dictates changes of the substrate sildenafil pharmacokinetic process, which seems closely related to the adjustments in blood gas, biochemical indicators and metabolic enzymes. Spectral-domain optical coherence tomography was then used by Yin et al. to quantify changes in the retinal structure in 109 healthy individuals after ascent to 3,700 m above sea level. Results showed that the ratios of mean thickness, inferior area, and nasal area were correlated positively with high-altitude headache. This provides new insights into the pathophysiology of high-altitude retinopathy (i.e., papilledema). Unlike acute exposure, chronic continuous hypoxia relies on prolonged exposure or permanent life in altitude with longer-term physiological adaptations. In their cross-sectional study including 475 children and adolescents living at 1,000 or 2,600 m above sea level, Mancero-Soto et al. concluded that the associated effects of endurance training on haemoglobin mass and blood volume were only observed after the onset of puberty. Additionally, authors stated that the large differences in haemoglobin mass and blood volume in adulthood between elite athletes and untrained individuals likely have genetic origins (yet of unknown origin). Overall, the outcome of hypoxia exposure and efficiency of adaptations are largely determined by individual predispositions and vulnerabilities as well as by the "hypoxic dose" (i.e., severity, duration, and frequency of the stimuli).

Intermittent hypoxia exposure

Intermittent hypoxic exposure corresponds to the repetition of hypoxic/normoxic cycles, while its effects range from deleterious (e.g., sleep-disordered breathing) to beneficial (e.g., hypoxia conditioning). The usefulness of intermittent hypoxia exposure (inspired oxygen fraction or FiO_2 = 14.5%) to prevent intense exercise training-induced reductions in haemoglobin concentration was assessed in animal and human studies. Firstly, Weng et al. exposed six-week-old male Sprague-Dawley rats to progressive intense treadmill exercise training over 3 weeks followed by 3 weeks of training with intermittent hypoxia exposure (either for 1, 2, or 1 h + 1 h separated by a 3-h interval after the exercise sessions). Authors concluded that all these intermittent hypoxia exposure strategies (i.e., no difference between treatments) could be used to increase renal erythropoietin and alleviate intense exercise training-induced reductions in haemoglobin concentration. Secondly, Weng et al. reported that 1 h of normobaric hypoxia exposure five times a week over 4 weeks was sufficient to partially restore the low haemoglobin concentration in trained swimmers, but also blunt the decrease in red blood cells and haematocrit. Overall, humans might be more sensitive to the intermittent hypoxia exposure intervention than rats.

Responses to hypoxia and exercise stressors when combined

Ambient hypoxia exposure and exercise, or a combination of both stressors, likely influence the cerebrovascular and muscle regulation interplay. To illustrate, pacing strategy during a 250-kJ cycling time-trial was impaired more after 24 h of hypobaric (3,450 m above sea level) than normobaric (FiO_2 = 13.6%) hypoxia, which may relate to altered cerebrovascular responses (Rupp et al.). By aiming to maintain an equivalent oxygen delivery to the brain, individuals at terrestrial altitude likely adopted a more "protective" strategy, in conjunction with greater impairments in cerebral blood flow and prefrontal motor cortex oxygenation, leading to lower overall cycling performance compared to simulated altitude. Oxygen deprived conditions can also influence exercise-related cardio-vascular system adjustments. A brief exercise bout of mild intensity (30% of maximal aerobic power) in acute normobaric hypoxia (FiO_2 = 13.5%) did not impair systolic or diastolic functions during the ensuing recovery period as evaluated from echocardiographic, Doppler, and tissue Doppler measures (Magnani et al.). Rather, stroke volume was well preserved and systolic and early diastolic functions were actually enhanced by exercise in hypoxia. Finally, the review by Lemieux and Birot on angio-adaptative responses to hypoxia summarizes the remarkable yet complex molecular plasticity of the capillary microvasculature (i.e., capillary-to-myofiber interface).

Therapeutic use of hypoxia

In recent years, the possibility of using hypoxic exposure as a novel therapeutic strategy (i.e., hypoxia conditioning) to improve health outcomes has gained popularity. In their review of the impact of high-altitude hypoxia on bone defect repair, Chen et al. discussed the possible mechanisms related to ion channels, reactive oxygen species production, mitochondrial function, autophagy, and epigenetics. While there is currently no clear optimal treatment plan for bone defects at high altitudes, this review also provides a foundation for future targeted, personalized, and precise bone regeneration therapies. Another systematic review with meta-analysis including 19 studies (a total of 444 participants) showed that the effects of exercise training in hypoxia and normoxia on fat loss in overweight and obese adults are not different (Chen et al.). Subgroup analysis of different age of participants, hypoxia

dose, exercise frequency, and duration failed to demonstrate hypoxia-related effects on body composition, glycometabolism, and lipometabolism. Although hypoxia conditioning has potential in enabling a healthier lifestyle and reduction of related risk factors in cardio-metabolic diseases, more work needs to be done to identify the most effective strategies that are also safe and well tolerated.

Moving forward

A vast number of potential health- and performance-promoting hypoxia applications exist. The strong sense one gets from reading the Hohenauer et al. systematic review is that a positive and small tendency can be seen over the past 40 years for the increase in the methodological quality of clinical trials examining hypoxia-related physiological responses. To accelerate this trend, authors recommended that future studies should incorporate adequate blinding procedures (if possible), concealed allocation, and baseline comparability. By adhering to these principles, well-calibrated hypoxic interventions could be developed and refined to maximize health and performance outcomes.

Author contributions

All authors listed have made substantial, direct, and intellectual contributions to this work. In addition, all authors have approved this work for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

frontiers in Physiology

ORIGINAL RESEARCH
published: 19 February 2021
doi: 10.3389/fphys.2021.627708

Intermittent Hypoxia Exposure Can Prevent Reductions in Hemoglobin Concentration After Intense Exercise Training in Rats

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Intense exercise training can induce low concentrations of hemoglobin, which may be followed by maladaptation. Therefore, it is important for athletes to prevent low concentrations of hemoglobin during intense exercise training. In this study, we explored whether different protocols of intermittent hypoxic exposure (IHE, normobaric hypoxia, 14.5% O_2) could prevent the exercise training-induced reduction in hemoglobin concentration in rats. Six-week-old male Sprague-Dawley rats were subjected to progressive intense treadmill exercise training over three weeks followed by three weeks of training with IHE after exercise. IHE lasted either 1 h, 2 h, or 1 h + 1 h (separated by a 3-h interval) after the exercise sessions. Hematological parameters, including hemoglobin concentration [(Hb)], red blood cells (RBCs), and hematocrit (Hct), and both renal and serum erythropoietin (EPO) were examined. We found that intense exercise training significantly reduced [Hb], RBCs, Hct, food intake and body weight ($P < 0.01$). Analysis of reticulocyte hemoglobin content (CHr) and reticulocyte counts in the serum of the rats suggested that this reduction was not due to iron deficiency or other cofounding factors. The addition of IHE after the intense exercise training sessions significantly alleviated the reduction in [Hb], RBCs, and Hct ($P < 0.05$) without an obvious impact on either food intake or body weight ($P > 0.05$). Increase in reticulocyte count in the rats from the IHE groups ($P < 0.05$ or $P < 0.01$) suggests that IHE promotes erythropoiesis to increase the hemoglobin concentration. Furthermore, the addition of IHE after the intense exercise training sessions also significantly increased the concentration of renal EPO ($P < 0.05$), although the increase of the serum EPO level was statistically insignificant ($P > 0.05$). The different IHE protocols were similarly effective at increasing renal EPO and preventing the training-induced decreases in [Hb], RBCs, and Hct. Collectively, this study suggests that IHE may be used as a new strategy to prevent intense exercise training-induced reductions in [Hb], and deserves future exploration in athletes.

Keywords: hypoxia, IHE, erythropoietin, EPO, hemoglobin

OPEN ACCESS

Edited by:
Gary W. Mack,
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Specialty section:
This article was submitted to Exercise Physiology, a section of the journal Frontiers in Physiology

Received: *10 November 2020*
Accepted: *01 February 2021*
Published: *19 February 2021*

Citation:
Weng X, Chen H, Yu Q, Xu G, Meng Y, Yan X, McConell G and Lin W (2021) Intermittent Hypoxia Exposure Can Prevent Reductions in Hemoglobin Concentration After Intense Exercise Training in Rats. Front. Physiol. 12:627708. doi: 10.3389/fphys.2021.627708

INTRODUCTION

Sport training is a process of carefully applied stress-adaptation (Lorenz and Morrison, 2015; Haugen et al., 2019). Providing stress at the optimal level that an athlete can endure will lead to improvements in physical function and performance (Lorenz and Morrison, 2015; Haugen et al., 2019). In contrast, stress beyond the optimal level will lead to maladaptation and may eventually lead to fatigue or even overtraining (Kellmann, 2010). Therefore, seeking appropriate monitoring, nutritional, and rehabilitation strategies during periods of intense exercise training is critical to help athletes to avoid excessive stress, and to improve physical function and performance.

Blood hemoglobin (Hb) is a routinely used marker for monitoring intense exercise training and physical function (Gleeson, 2002; Halson et al., 2003). Based on previous studies, it has been accepted that a 10% decrease in hemoglobin concentration [(Hb)] can be a practical indicator to predict maladaptation caused by intense exercise training, although this state can result from plasma dilution, which frequently occurs early in prolonged training process (Zhao, 2003; Sheng, 2006). During intense exercise training, the [Hb] of the athletes of low concentration of hemoglobin (at least 10% drop of [Hb)] will keep dropping if they continue to participate the training, which will impair their ability to endure the intense exercise training (Zhao, 2003; Sheng, 2006). Therefore, it is particularly important to avoid low concentrations of hemoglobin by promoting the synthesis of Hb through appropriate strategies.

Currently, altitude training (hypoxic training) is widely used to improve the aerobic capacity of athletes (Millet et al., 2010). Hypoxia can stimulate erythropoietin (EPO) secretion under the regulation of hypoxia inducible factors (HIFs) (Haase, 2013; Ploszczyca et al., 2018; Viscor et al., 2018). HIFs consist of a heterodimer of an oxygen-sensitive α-subunit (HIF1α) and a constitutively expressed β-subunit (HIF1β) (Haase, 2013). Under normoxia, HIFα subunits are rapidly hydroxylated and subjected to either proteosome-mediated degradation or deprivation of transcriptional activity (Haase, 2013). Conversely, during hypoxia, the HIFα subunit is not hydroxylated, and dimerized with HIF1β to activate the transcription of target genes (Haase, 2013). Numerous studies demonstrated that the secretion of EPO induced by altitude training can increase Hb content, oxygen binding, and oxygen transport in athletes (Rodriguez et al., 2000; Ploszczyca et al., 2018; Viscor et al., 2018). For example, intermittent hypoxia exposure (hypobaric hypoxia at a simulated altitude of 4000–5500 m) for 90 min, three times a week for 3 weeks, significantly increased reticulocytes (180%), RBCs (7%), and [Hb] (13%) in athletes (Rodriguez et al., 2000). However, it is noteworthy that the erythropoiesis-promoted effect of altitude training is still under debate since certain studies showed negative results (Julian et al., 2004; Fu et al., 2007). Different outcomes in these studies may result from different experimental design, especially regarding to the dosage and the nature of the hypoxic stimulus.

So far, to our knowledge, hypoxia has not been specifically applied in promoting adaptations to intense exercise training of athletes. In this study, we explored the possibility of such an application in Sprague-Dawley (SD) rats since low concentration of hemoglobin also occurs in rats after intense exercise training (Zhu et al., 2010; Liu et al., 2011) and it seems more challenging to realize a large sample size and genetic homogeneity in athletes. We hypothesized that through intermittent hypoxic exposure (IHE) of a reasonable simulated altitude and a sufficient dosage, SD rats would not display low concentrations of hemoglobin after intense exercise training since hypoxia can stimulate EPO secretion which can subsequently facilitate erythropoiesis to increase Hb content based on human and rodent studies (Rodriguez et al., 2000; Cui et al., 2020). We investigated the effects of hypoxic exposure on the levels of [Hb], RBCs, hematocrit (Hct), and EPO (kidney and serum) in rats undergoing intense exercise training.

MATERIALS AND METHODS

Animals and Diets

Six-week-old male Sprague-Dawley (SD) rats ($n = 50$, mean initial body weights = 158 ± 12g) were purchased from Guangdong Experiment Animal Research Institute (GEARI, Guangzhou, China). The rats were caged (5/cage) in a clean facility with a fixed light-dark cycle (12h/12h), a humidity of 40–60% and a temperature of 24°C, and had free access to fresh water and food purchased from GEARI. The iron content of the rat diet used in this study was 172.6 mg/kg, which should be enough to meet the iron demand of rats based on previous studies (Erikson et al., 2000; Staniek et al., 2020). All animal protocols were approved by the Guangzhou Sport University Animal Ethics Committee.

Study Design

Fifty rats were assigned into 5 groups (10 per group): normoxic sedentary control (SC), normoxic exercise control (EC), 1-h hypoxia exposure after exercise (E + 1h), 2-h hypoxia exposure after exercise (E + 2h), and two separate 1-h hypoxia exposures after exercise [a 3-h interval between two hypoxic exposure, E + (1 + 1)h]. The design of different strategies of IHE aimed to explore the reasonable IHE periods (dosage) for preventing the exercising rats from displaying low concentrations of hemoglobin, while the addition of the E + (1 + 1)h group aimed to increase the number of intermittent hypoxia stimulations (from 1/day to 2/day) that might lead to a different effect from the (E + 2h) group due to the presence of an interval between two hypoxia stimulations. The rats in the EC, E + 1h, E + 2h, and E + (1 + 1)h groups undertook treadmill training sessions 6 days per week for 6 weeks. From the 4th to the 6th week, after the completion of exercise, the rats in Group E + 1h, E + 2h, and E + (1 + 1)h were exposed to normobaric hypoxia (14.5% O_2) in a hypoxic chamber (Hypoxic Tent System, Hypoxico Inc., NY, United States) for their respective durations. The exercise session duration lasted for 10 min on the first day, and increased by 10 min per day thereafter until 1 h was reached and maintained for the remaining training days. In the first week,

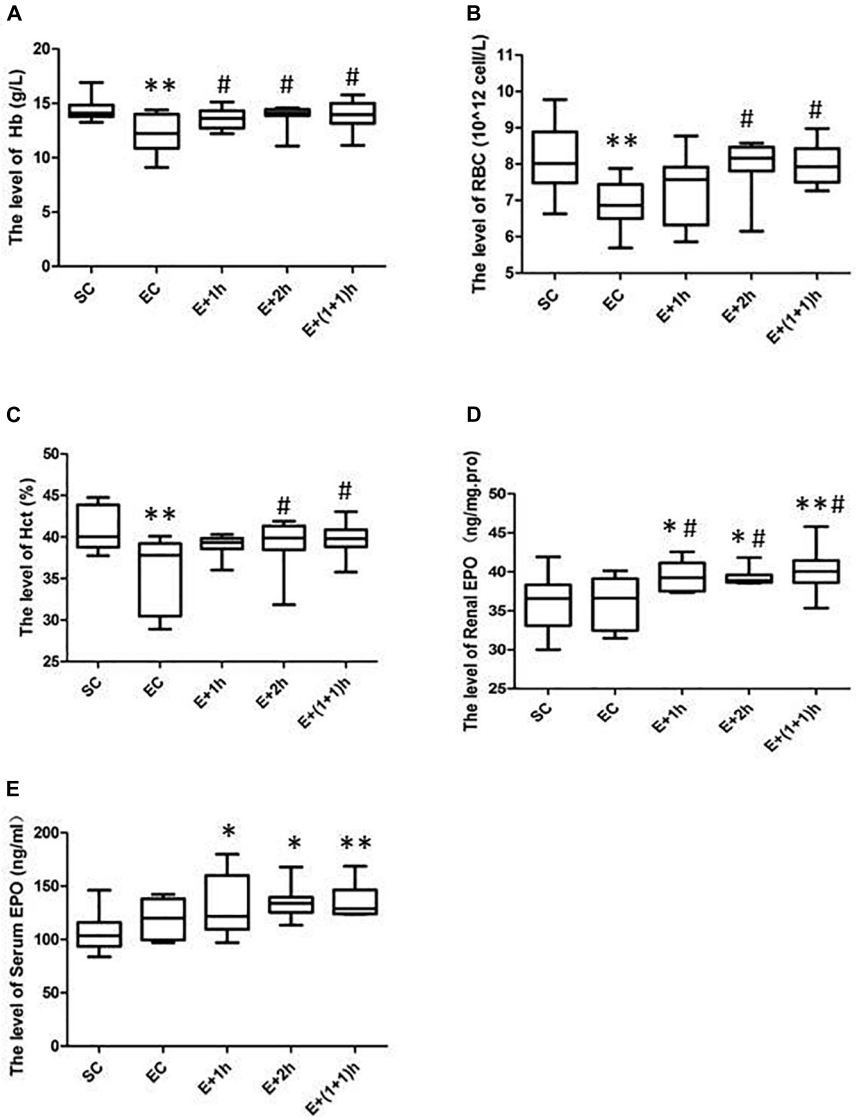

FIGURE 1 | The hematological parameters and EPO levels for various groups. **(A)** Hemoglobin (Hb). **(B)** Red Blood Cells (RBCs). **(C)** Hematocrit (Hct) levels. **(D)** Renal erythropoietin (EPO) levels. **(E)** Serum EPO levels. The groups were normoxia sedentary controls (SC), normoxia exercise control (EC), exercise and 1-h hypoxia exposure (E + 1h), exercise and 2-h hypoxia exposure (E + 2h), and exercise and two one-hour hypoxia exposures [E + (1 + 1)h]. *Significantly different from SC, $P < 0.05$. **Significantly different from SC, $P < 0.01$. #Significantly different from EC, $P < 0.05$.

the exercise velocity commenced at 15m/min, and increased each week by 5m/min, to 40m/min in the sixth week.

Sample Collection and Measurement of Hematological Parameters

Rats were anesthetized by using 50 mg/kg of sodium pentobarbital within 24 h after the end of the experiments. Blood samples were collected from the abdominal aorta into EDTA-containing or EDTA-lacking tubes. Immediately after collection, whole blood collected into EDTA-containing tubes was analyzed to determine [Hb], RBCs count, Hct, reticulocyte hemoglobin content, and reticulocyte count using an automated cell counter (ADVIA120, Bayer AG, Germany). The blood samples collected using EDTA-lacking tubes were centrifuged at 3,000 rpm/min for 20 min at room temperature to collect the serum for EPO measurements (see below). Kidneys were collected and homogenized to measure EPO (see below) as previously described (Garrido et al., 2015; Landau et al., 2018). The rats were then killed by an overdose of the anesthetic.

EPO Measurement

Renal and serum EPO were measured using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's guidelines (E02E0002, Bluegene Biotech CO., LTD, Shanghai, China). This assay applies the technique of quantitative sandwich enzyme immunoassay. A monoclonal antibody specific to rat

EPO was pre-coated onto a microplate. Briefly, 100 μL of conjugate was added to each well in the plate, then 50 μL of standard, control, or sample was added to the plate and incubated for 1 h at 37°C. Each well was aspirated and rinsed with wash buffer for a total of five washes. Substrate (100 μL) was added to each well and incubated for 10–15 min at 37°C in dark. Stop solution (100 μL) was added to each well and the plate was read within 30 min. Plates were read at 450 nm on a VARIOSKAN FLASH (Thermo Fisher Scientific, MA, United States). Each sample was measured in duplicate.

Statistics

The hematological and EPO data were analyzed using a one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test. All statistical calculations were performed using IBM SPSS Statistics for Windows (version 19). *P*-values less than 0.05 were considered statistically significant.

RESULTS

As shown in **Figures 1A–C**, the [Hb], RBC count, and Hct of the EC group all significantly decreased ($P < 0.01$) compared with those of the SC group for whom the [Hb] dropped ∼15%, indicating that the EC group displayed low concentrations of hemoglobin as expected. In addition, from the second week, the average daily food intake of rats in the EC group was significantly lower than that in the SC group ($P < 0.01$) (**Table 1**); from the 3nd week, the body weight of rats in the EC group was significantly lower than that in the SC group ($P < 0.05$, **Table 2**). These data suggested that the food intake and body weight of the rats in the EC group could have been affected by low concentrations of hemoglobin. The low concentrations of hemoglobin in the EC group may be caused by iron deficiency. To explore this possibility, we examined reticulocyte hemoglobin content (CHr or Ret-He), an early marker of iron restricted erythropoiesis (Mast et al., 2002; Roy et al., 2007; Torsvik et al., 2013). We found that the CHr of the EC group was significantly higher than that of the SC group ($P < 0.01$, **Table 3**). This result suggested that the low hemoglobin concentration in the EC group was not due to iron deficiency. To exclude the possibility that low concentrations of hemoglobin in the EC group was caused by cofounding factors such as water intake or manipulation stress, the reticulocyte counts of the rats which can reflects the erythropoietic activity of the bone marrow were examined (Cline and Berlin, 1963; Riley et al., 2001). Reticulocyte count can be reported as absolute reticulocyte count (Retic#) or as a reticulocyte percentage (Retic%) (Riley et al., 2001). After the IHE for three weeks, both the absolute reticulocyte count and reticulocyte percentage of the rats in the EC group were significantly higher than those in the SC group ($P < 0.01$ both, **Table 4**), which revealed that the low concentrations of hemoglobin in the EC group has promoted erythropoiesis. Therefore, it is less likely that the low concentrations of hemoglobin in the EC group were caused by temporary cofounding factors.

As shown also in **Figures 1A–C**, the hematological parameters of all the IHE groups [E + 1h, E + 2h and E + (1 + 1)h] were comparable with those of the SC group ($P > 0.05$), indicating that hypoxic exposure elevates these parameters. Consistently, all the IHE groups displayed statistically significant difference on most of these parameters compared with those of the EC group ($P < 0.05$, **Figures 1A–C**) although their inter-group difference on these parameters were insignificant ($P > 0.05$). The RBC and Hct of the E + 1h group were two exceptions as they didn't display statistically significant difference compared with those of the EC group ($P > 0.05$, **Figures 1A–C**). Furthermore, there was no significant difference between the EC group and the IHE groups in either food intake or body weight (**Tables 1**, **2**). Finally, reticulocyte count and reticulocyte percentage of the rats in the IHE groups were significantly higher than those in the EC group ($P < 0.05$ or $P < 0.01$) (**Table 4**). This result revealed that the amount of reticulocytes was increased significantly after IHE, suggesting that IHE promotes erythropoiesis to increase the hemoglobin concentration. Together, these data indicate that 1-h IHE was sufficient to prevent the decrease of these parameters in the exercised rats and especially the appearance of low concentrations of hemoglobin.

TABLE 1 | The food intake (g) of rats before and during the experiments.

	SC	EC	E + 1h	E + 2h	E + (1 + 1)h
1st week	20.5 ± 2.4	20.5 ± 2.3	19.9 ± 2.0	20.9 ± 2.1	19.7 ± 2.3
2nd week	22.6 ± 1.1	19.0 ± 1.8**	19.5 ± 1.5**	20.3 ± 1.4**	20.2 ± 1.4**
3rd week	22.2 ± 2.0	17.9 ± 1.3**	18.5 ± 1.1**	18.6 ± 1.0**	17.8 ± 1.5**
4th week	23.4 ± 2.1	20.1 ± 2.3**	19.8 ± 2.5**	20.3 ± 2.4**	19.0 ± 2.7**
5th week	25.2 ± 2.8	19.0 ± 4.0**	18.9 ± 4.8**	18.4 ± 4.3**	20.1 ± 3.9**
6th week	26.2 ± 4.0	22.0 ± 5.3**	21.5 ± 5.8**	21.2 ± 6.4**	19.9 ± 7.1**

***Significantly different from SC, P < 0.01.*

As hypoxia can stimulate EPO synthesis that can then stimulate erythropoiesis, renal and serum EPO levels were examined. As revealed in **Figures 1D,E**, renal and serum EPO levels were not altered by exercise ($P > 0.05$, EC vs. SC). However, the renal and serum EPO levels of the IHE groups were significantly increased compared with those of the SC group ($P < 0.05$ or $P < 0.01$). Meanwhile, the renal EPO levels of the IHE groups were also significantly higher than those of the EC group ($P < 0.05$). On the other hand, the differences in serum EPO levels between the IHE groups and the EC group were statistically insignificant ($P > 0.05$). Together, these data suggested that IHE might stimulate the production of EPO to prevent intense exercise-induced low concentrations of hemoglobin.

DISCUSSION

Timely and sufficient recovery of physical function is key for athletes to adapt to intense exercise training (Kellmann, 2010). If the [Hb] drops too much after intense exercise training, it may indicate that the athlete's physical function has not fully recovered and will soon display maladaptation (Zhao, 2003; Sheng, 2006). Currently, one strategy is to supplement athletes with oxygen to compensate their oxygen-carrying capacity that may decline and

TABLE 2 | The body weight (g) of rats before and during the experiments.

	SC	EC	E + 1h	E + 2h	E + (1 + 1)h
Before intervention	188.22 ± 9.65	187.05 ± 10.12	189.38 ± 11.36	185.59 ± 9.98	189.01 ± 10.88
1st week	210.78 ± 12.68	205.40 ± 19.23	205.66 ± 15.98	207.06 ± 17.97	209.65 ± 16.11
2nd week	237.14 ± 26.70	221.10 ± 30.58	219.26 ± 21.55	218.87 ± 26.31	222.18 ± 23.89
3rd week	256.63 ± 31.20	217.35 ± 26.90*	215.36 ± 25.57*	217.01 ± 25.32*	220.10 ± 27.32*
4th week	283.00 ± 32.42	228.25 ± 27.82**	229.37 ± 28.67**	226.86 ± 27.06**	229.63 ± 30.27**
5th week	304.50 ± 37.19	234.25 ± 29.47**	239.56 ± 28.65**	232.28 ± 29.56**	238.51 ± 31.56**
6th week	328.60 ± 36.25	229.37 ± 35.07**	232.78 ± 38.53**	231.37 ± 33.61**	230.79 ± 37.25**

*Significantly different from SC, $P < 0.05$.
**Significantly different from SC, $P < 0.01$.

promote the recovery of their physical function (Sperlich et al., 2011). However, this strategy offers only a temporary solution, since it cannot fully restore exercise-induced low concentrations of hemoglobin in athletes. In contrast, our study tested a strategy that stimulates EPO synthesis and increases RBC and Hb production through moderate exposure of the rats to hypoxia, and that depends on an endogenous machinery in their bodies against the training-induced decreases in [Hb]. The long-term activation of this machinery may not only promote the recovery of physical function and the adaptation to intense exercise training, but also improve physical function and performance.

Hypoxic training was developed in the early 1990s in which the athletes are exposed to systemic and/or local hypoxia at rest (passive) or combined with exercise training (active) (Millet et al., 2010; Ramos-Campo et al., 2020). Currently, there are several strategies of hypoxic training and/or altitude exposure: "live high-train high" (LHTH), "live high-train low" (LHTL), intermittent hypoxic exposure during rest (IHE), and intermittent hypoxic exposure during continuous sessions (IHT) (Millet et al., 2010). Previous studies have revealed that LHTL and IHE (both use passive hypoxic exposures) are the two best strategies to promote Hb generation (Millet et al., 2010). The LHTL strategy was originally designed for athletes to improve their aerobic capacity; however, to promote the production of red blood cells it requires exposure to hypoxia for more than 12 h every day, which might not be feasible for many athletes (Millet et al., 2010). Conversely, methods of IHE (such as those adopted in this study) may be much more feasible. However, to the best of our knowledge, the IHE strategy has not been applied to promote adaptations to periods of the intense exercise training in athletes. This may be partly due to economic reasons such as the need for specialized equipment that can produce hypoxic environments. In the future, increasing the availability of relevant equipment may facilitate the application of the IHE strategy in promoting adaptations to intense exercise training in athletes.

Previous studies have subjected healthy athletes to simulated altitudes in excess of 4000m (Rodriguez et al., 2000; Fu et al., 2007). However, in the current study, we adopted a moderate simulated altitude (3000 m) based on our team's previous research where we found that IHE (12.6% oxygen, simulated altitude of 4000 m) after intense exercise training led the myocardium of rats to partial decompensation, suggesting that irreversible injuries may occur in this protocol (Huang, 2003). However, there was no partial decompensation of myocardium when IHE was conducted at simulated altitude of 3000 m (Huang, 2003). Therefore, we chose 14.5% oxygen to in this study. Indeed, our data show that such a simulated altitude was sufficient to prevent low concentrations of hemoglobin in SD rats. However, laboratory animals, such as the rodents used herein, can be more sensitive to hypoxia than humans (Gonzalez and Kuwahira, 2018). Therefore, future experiments should test the efficacy of the simulated altitude and duration of hypoxic exposure from this study in humans with low concentrations of hemoglobin, but given the hypoxia-EPO-hemoglobin machinery is conserved in both humans and rats, similar findings may be expected (Haase, 2013; Viscor et al., 2018) (unpublished data for another manuscript in preparation). Furthermore, in this study, altitude was not simulated using hypobaric hypoxia whereby hypoxia is caused by reducing the barometric pressure, but normobaric hypoxia whereby hypoxia is caused by decreasing the fraction of inspired O_2. As there may be differences in the physiological responses elicited by hypobaric hypoxia or normobaric hypoxia

TABLE 3 | The CHr of rats after the experiments.

Group	CHr(pg)
SC	17.63 ± 0.16
EC	18.66 ± 0.35*
E + 1h	17.61 ± 0.17##
E + 2h	17.80 ± 0.31##
E + (1 + 1)h	17.52 ± 0.23##

*Significantly different from SC, $P < 0.05$.
##Significantly different from EC, $P < 0.01$.

TABLE 4 | The Retic* and Retic% of rats after the experiments.

Group	Retic# (10^9/L)	Retic% (%)
SC	183.98 ± 13.26	2.22 ± 0.17
EC	226.56 ± 23.93**	2.75 ± 0.19**
E + 1h	301.96 ± 97.31**##	4.17 ± 1.56**##
E + 2h	289.52 ± 121.30**#	3.97 ± 1.70**#
E + (1 + 1)h	321.52 ± 96.17**##	4.59 ± 1.53**##

**Significantly different from SC, $P < 0.01$.
#Significantly different from EC, $P < 0.05$.
##Significantly different from EC, $P < 0.01$.

(Faiss et al., 2013; Saugy et al., 2016), whether hypobaric hypoxia can lead to a better adaptation to intense exercise than normobaric hypoxia deserves future study.

In this study, the differences in serum EPO levels between the IHE groups and the EC group were statistically insignificant ($P > 0.05$). Changes in serum EPO are dynamic, and it is difficult to predict the peak serum EPO during sample collection; therefore, one possibility that cannot be excluded is that the peak serum EPO did not appear at the time when the blood sampling was conducted. In addition, although the hematological parameters of most IHE groups increased significantly ($P < 0.05$ or $P < 0.01$) compared with those of the EC group, the E + 1h group did not display a significant elevation in RBC and Hct compared with the EC group ($P > 0.05$), indicating that 1 h of hypoxia exposure may not be sufficient to prevent the decrease of RBC and Hct in SD rats during intense exercise training. However, the RBC and Hct of the other two IHE groups, E + 2h and E + (1 + 1)h, were significantly higher than those in the EC group. This suggests that increasing the time (dose) of hypoxia exposure can more comprehensively improve the hematological parameters of rats with low concentrations of hemoglobin, although 1 h of hypoxia exposure was sufficient to significantly improve [Hb]. Beside, to increase the number of intermittent hypoxia stimulation, we designed the E + (1 + 1)h group in this study. Unfortunately, the hematological parameters of the E + (1 + 1)h group was not significantly improved compared with those of the E + 2h group. But this design deserves further exploration in future studies.

LIMITATIONS

In this report, we showed that 1 h of normobaric hypoxia exposure (14.5% O_2 per day from the fourth to sixth week) could prevent the rats from low concentrations of hemoglobin after exercise during six-week intense exercise training. However, lack of data regarding blood volume change in rats before and after IHE makes the possibility that increase in [Hb] in the IHE groups might simply reflect some hemoconcentration unable to be excluded completely, although the data of reticulocyte count suggests that IHE promotes erythropoiesis to increase the hemoglobin concentration. In addition, lack of data of the rats before the hypoxic intervention makes that the impact conferred by IHE on preventing from low concentrations of hemoglobin after intense exercise training unable to be evaluated from the intra-group level, although this report provided an evaluation from the inter-group level using the EC group as the control.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Guangzhou Sport University Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

XW and WL conceived and designed the research. XW, HC, GX, and YM conducted the experiments. XW, HC, XY, and GM analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

ACKNOWLEDGMENTS

We thank Dr. Yu Yuan (Guangzhou Sport University, Guangzhou, China) and Dr. Matthew Lee (Victoria University, Melbourne, VIC, Australia) for their kind help in revising the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 23 April 2021
doi: 10.3389/fphys.2021.650696

Systolic and Diastolic Functions After a Brief Acute Bout of Mild Exercise in Normobaric Hypoxia

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Acute hypoxia (AH) is a challenge to the homeostasis of the cardiovascular system, especially during exercise. Research in this area is scarce. We aimed to ascertain whether echocardiographic, Doppler, and tissue Doppler measures were able to detect changes in systolic and diastolic functions during the recovery after mild exercise in AH. Twelve healthy males (age 33.5 ± 4.8 years) completed a cardiopulmonary test on an electromagnetically braked cycle-ergometer to determine their maximum workload (W_{max}). On separate days, participants performed randomly assigned two exercise sessions consisting in 3 min pedalling at 30% of W_{max}: (1) one test was conducted in normoxia (NORMO) and (2) one in normobaric hypoxia with FiO_2 set to 13.5% (HYPO). Hemodynamics were assessed with an echocardiographic system. The main result was that the HYPO session increased parameters related to myocardial contractility such as pre-ejection period and systolic myocardial velocity with respect to the NORMO test. Moreover, the HYPO test enhanced early transmitral filling peak velocities. No effects were detected for left ventricular volumes, as end-diastolic, end-systolic, and stroke volume were similar between the NORMO and the HYPO test. Results of the present investigation support the hypothesis that a brief, mild exercise bout in acute normobaric hypoxia does not impair systolic or diastolic functions. Rather, it appears that stroke volume is well preserved and that systolic and early diastolic functions are enhanced by exercise in hypoxia.

Keywords: blood pressure, cardiac pre-load, myocardial contractility, echocardiography, tissue Doppler

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Exercise Physiology, a section of the journal Frontiers in Physiology

Received: *07 January 2021*
Accepted: *31 March 2021*
Published: *23 April 2021*

Citation:
Magnani S, Mulliri G, Roberto S, Sechi F, Ghiani G, Sainas G, Nughedu G, Vargiu R, Bassareo PP and Crisafulli A (2021) Systolic and Diastolic Functions After a Brief Acute Bout of Mild Exercise in Normobaric Hypoxia. Front. Physiol. 12:650696. doi: 10.3389/fphys.2021.650696

INTRODUCTION

Acute hypoxia (AH) presents some challenges to the human homeostasis, especially during exercise and recovery. During AH, the human circulation experiences rapid changes in the main hemodynamic modulators (i.e., pre-load, after-load, contractility, and chronotropism), which potentially impact on cardiovascular function and regulation (Yan et al., 2007; Dedobbeleer et al., 2013; Goebel et al., 2013; Naeije and Dedobbeleer, 2013). Our group has recently shown that, among cardiovascular parameters, ventricular filling rate was one of the most sensitive to exercise in AH, as it was reduced after brief bouts of acute cycling in normobaric hypoxia (Mulliri et al., 2019, 2020). This suggested that venous return was reduced and led us to

speculate that a reduction in cardiac preload took place in this setting, thereby impairing the Frank-Starling mechanism. Furthermore, this effect was not counterbalanced by any enhancement in cardiac performance.

However, one potential limit of our previous research was that we did not assess echocardiographic parameters. Instead, hemodynamics were assessed by means of impedance cardiography, which does not allow gathering data about cardiac volumes; moreover, it suffers from some limits in the analysis of systolic and diastolic functions.

In this specific area, the research is scarce. Some studies using echocardiography have provided evidence that an increase in left ventricular twist takes place in the acclimatisation at altitude. This response seems to be compensatory to maximise stroke volume (SV) when ventricular filling is impaired. Given that sub-endocardial function was maintained, the elevation in sympathetic activity was proposed as the most likely explanation for this phenomenon (Stembridge and Levine, 2019). In support to this hypothesis, findings have been recently provided that the increase in cardiac twist can be attenuated by the administration of specific β1-adrenergic antagonist, which appears to support the hypothesis that this is an appropriate response to sympathetic activation (Williams et al., 2019). Collectively, these results appear to suggest that left ventricular function is maintained or even enhanced in chronic hypoxia (Stembridge et al., 2016). Moreover, some studies conducted at rest reported an increase in ventricular twist and deformation probably due to sympathetic stimulation and/or peripheral vasodilation (Dedobbeleer et al., 2013; Goebel et al., 2013).

However, little is known about the effect of AH during exercise and recovery. Concerning diastolic functions, evidence has been provided that the diastolic trans-mitral peak early velocity gathered with Doppler is higher during exercise in hypoxia than in normoxia, so suggesting that hypoxic exercise increase ventricular diastolic function (Yan et al., 2007).

Starting from these considerations, the aim of the present study was to investigate the effect of acute dynamic exercise during normobaric hypoxia on echocardiographic parameters related left ventricular volumes, systolic, and diastolic functions. Specifically, the present investigation was devised to verify whether classical echocardiographic measures of ventricular volume confirm or reject our previous hypothesis of a reduced pre-load during the recovery from mild exercise in AH. Moreover, we aimed to verify whether Doppler and tissue Doppler measures confirmed or rejected the hypothesis that systolic and diastolic functions were affected by mild exercise conducted in acute normobaric hypoxia.

MATERIALS AND METHODS

Participants

Twelve healthy Caucasian males aged 24–42 years [mean ± standard deviation (SD) of age 33.5 ± 4.8 years] agreed to participate in the study. All participants were physically active and were regularly involved in leisure-time sports activities such as amateur cycling and running at least 3 times/week. Their average ± SD of body mass and height were 72.5 ± 10.1 kg and 176.5 ± 3.9 cm, respectively. All were non-smokers and none of them suffered from any known diseases or were on medication at the time of the experiment. They were asked for abstaining from drinking alcohol or coffee for at least 24 h before scheduled tests. All experiments were conducted in a room at controlled temperature and humidity (22°C, relative humidity 50%).

To calculate the required sample size, we used a calculator free available on the web.[1] The calculation was conducted using a power of 85%, an overall type 1 error of 0.05, a SD of 10%, and a 15% difference due to conditions in the studied variables. Eight subjects were needed to obtain adequate statistical power.

The study was carried out with approval from the University's Institutional Review Board and in accordance with the Declaration of Helsinki. All the participants signed written informed consent before the beginning of the study.

Experimental Protocol

The experimental protocol consisted in a preliminary screening test and in two experimental sessions in normoxia (test NORMO) and AH (test HYPO). Test NORMO and HYPO were randomly assigned. Randomisation was obtained using an online random sequence generator.[2]

Preliminary Test

All participants underwent a preliminary medical examination to assess their health status. After the medical examination, each participant underwent a cardiopulmonary exercise stress test (CPET) on an electromagnetically braked cycle-ergometer (CUSTO Med, Ottobrunn, Germany). During the CPET, oxygen uptake (VO_2), carbon dioxide production (VCO_2), and V_E were assessed with a gas analyser (Ultima CPX, MedGraphics St. Paul, MN, United States) calibrated immediately before each test accordingly to the manufacturer. Respiratory exchange ratio (RER) was also calculated as VCO_2/VO_2. The exercise consisted of a linear increase of workload (30 W·min^{-1}), starting at 30 W, keeping a pedalling frequency of 60 rpm until exhaustion, which was considered as the point at which the subject was unable to maintain a pedalling rate of at least 50 rpm. Maximum workload (W_{max}), maximum oxygen uptake (VO_{2max}), and maximum heart rate (HR_{max}) were collected. Moreover, anaerobic threshold (AT) was calculated using the *V*-slope method, which detects AT using a regression analysis of the slope of VCO_2 plotted as a function of VO_2 (Beaver et al., 1986). During this preliminary visit, participants familiarised with equipment and the staff of the laboratory, so allowing habituation to the environment and the ergometer that was employed in the successive experimental sessions.

[1]https://clincalc.com/stats/samplesize.aspx
[2]https://www.random.org/sequences/

Sessions to Study Hemodynamics During Normoxia and Hypoxia

After the preliminary test (interval 3–7 days), volunteers performed randomly assigned the NORMO and the HYPO sessions pedalling on the same cycle-ergometer utilised for the CPET. NORMO and HYPO tests were separated by at least 3 days (interval 3–7 days). During both sessions, participants breathed through a mask connected to a hypoxic gas generator (Everest Summit II Generator, Hypoxico, New York, United States). This device utilises a molecular sieve system with zeolites to separate nitrogen from oxygen and allows having a gas mixture with a reduced oxygen content that can be regulated by an operator. A gas mixture with a FiO_2 of 21% and of 13.5% (corresponding to sea level and to an altitude of about 3,500 m) was delivered during the NORMO and the HYPO, respectively. The gas mixture was constantly checked by an operator by means of oxygen analyser provided with the device (Maxtec, Handi+, Salt Lake City, UT, United States). Participants were blinded about the actual content of oxygen they were breathing. During NORMO and HYPO tests, the same cycle-ergometer used for the CPET was utilised. After wearing the mask connected to the hypoxic gas generator, participants sat on the cycle-ergometer for 3 min to collect data at rest. Then, they started pedalling for 3 min against a mild workload corresponding to the 30% of the W_{max} reached during the CPET. A recovery of 6 min was allowed after the exercise. A similar experimental approach was recently used to assess hemodynamics during metaboreflex stimulation during normobaric hypoxia (Mulliri et al., 2020).

Assessment of O_2 Saturation

Peripheral blood O_2 saturation (SO_2) was continuously measured through finger pulse oximetry (Nonin, SenSmart X-100, Plymouth, MN, United States) to confirm that the hypoxic stimulus was effective.

Hemodynamic Measurement

An echocardiographic system (Vivid iq, GE Healthcare, Fairefield, CT, United States) equipped with a hand-held 3.5-MHz adult ultrasound probe was employed to assess cardiovascular functions. Heart rate (HR) was assessed as the reciprocal of the electrocardiogram R-R interval provided by the echocardiograph. Two dimensional and pulsed Doppler recording were acquired with participants in the sitting position. Measures were obtained from the apical four-chamber view. End-systolic volume (ESV) and end-diastolic volume (EDV) were calculated automatically by software using a conventional formula: $8A^2/3\pi L$, where A was the left ventricular area and L was the ventricular longest length (Christie et al., 1987). The ventricular area was determined by tracing along the inner edge of the endocardial targets, and the length was obtained by measuring the distance from the left ventricular apex to the midpoint of the mitral annulus. Echocardiography images were taken at rest and during the recovery from strain (i.e., at the third minute of recovery) by the same operator throughout sessions. When images were considered of good quality, a 6 s frame was recorded and then analysed offline always by the same skilled operator. For each analysis at least three beats were taken into consideration (range 3–6 beats) and data are reported as the average of the measures. Left ventricular ejection fraction (EF) was considered as: (EDV − ESV/EDV)100, and SV as: EDV − ESV.

In the same beats utilised for left ventricular volumes assessment, early and atrial transmitral filling peak velocities (Evel and Avel, respectively) and their ratio (E/A) were assessed using pulse wave Doppler (PWD) with a 5-mm PWD sample volume (3 mm) placed distal to the mitral anulus, between the mitral leaflets. The interrogation beam was aligned with mitral flow (Gardin et al., 1986; Cohen et al., 1996).

Mitral valve motion velocity during early (Em) and late (Am) diastole was determined by Doppler tissue imaging with the pulsed-wave sample volume placed at the lateral mitral anulus from the apical four chamber view. Septal early diastolic mitral anular velocities have been documented to detect impaired left ventricular diastolic functions independent of ventricular loading conditions (Nagueh et al., 1997). This technique has been already employed to analyse the effect of hypoxia on diastolic functions (Allemann et al., 2004). Moreover, systolic myocardial velocity (Sm) was determined to have a measure of longitudinal systolic function. This parameter has been found to correlate with measures of left ventricular EF and peak *dP/dt* (Correale et al., 2012). The ratio Evel/Em was used to estimate left ventricular filling pressure considering that an Evel/Em >10 is correlated with an elevated left ventricular diastolic pressure, whereas a value <8 indicates a normal pressure (Correale et al., 2012; Choudhury et al., 2017).

Aortic Doppler was also conducted from the four-chamber window to assess the pre-ejection period (PEP), which was measured as the time from the beginning of the QRS complex of the electrocardiogram and the opening of the aortic valve, and the ventricular ejection time (VET), which was assessed as the total duration of ejection period in the Doppler trace. Diastolic time (DT) was calculated by subtracting the sum of PEP and VET from the total period of the cardiac cycle (Sainas et al., 2016). We used PEP variations to have an estimate of sympathetic activity towards the left ventricle. Actually, when there is a more rapid development of intraventricular pressure, PEP shortens. Furthermore, the influence of parasympathetic activity on PEP is negligible as ventricles are not innervated by the parasympathetic nervous system. Yet, PEP is not substantially altered by changes in HR (Michael et al., 2017).

All echocardiographic calculations were done by the same expert physician, with a 5-year experience in the field. When comparing pre- and post-experiment echocardiographic measurements, and taking together all the calculation the observer did, the coefficient of variation varied from 8% (very good) to 12% (good).

A manual sphygmomanometer (Heine Gamma GP, Gilching, Germany) was placed in the non-dominant arm and systolic (SBP) and diastolic (DBP) blood pressure were measured by the same physician throughout all protocol sessions. Mean arterial blood pressure (MAP) was calculated using a formula, which takes into consideration changes in PEP, VET, and DT due to tachycardia (Sainas et al., 2016).

Data Analysis

Data are presented as mean ± SD. The Kolmogorov-Smirnov test was employed to assess distribution normality for each variable. Since all variables were normally distributed, parametric tests were used. Paired *t* test was employed to find out differences between the NORMO and the HYPO test at rest and at recovery. Statistical analysis was performed using commercially available software (GraphPad Prism). A $p < 0.05$ was considered to determine statistical significance. For each variable, effect size (ES) was determined using Cohen's statistic, where 0.2, 0.6, and 1.2 were interpreted as small, medium, and large effect, respectively.

RESULTS

Results of the CPET are reported in **Table 1**, while **Table 2** shows the values of variables collected during the third minute of rest preceding the NORMO and the HYPO test. Statistics found out that the HYPO test induced a significant increase in Evel and in E/A ratio, whereas Avel was reduced. Moreover, DT was significantly longer during the HYPO test.

Figure 1 exhibits values of variables collected during recovery from the HYPO and the NORMO test. Panel A shows that SO_2 was significantly reduced during the HYPO test (97.81 ± 1.20 vs. 91.97 ± 2.23% for the NORMO and the HYPO test, respectively, $p < 0.001$, ES = 0.85), while HR (99.08 ± 17.58 vs. 98.00 ± 12.01 bpm, $p = 0.8628$, ES = 0.01) and MAP (90.25 ± 4.41 vs. 89.75 ± 4.49 mmHg, $p = 0.5412$, ES = 0.02) were unaffected by conditions (panels B and C, respectively). Panel D demonstrates that PEP was shorter during the HYPO test than during the NORMO test (124.96 ± 15.30 vs. 112.21 ± 11.00 ms for the NORMO and the HYPO test, respectively, $p = 0.0178$, ES = 0.24), whereas VET (218.14 ± 18.41 vs. 214.60 ± 14.33 ms, $p = 0.6040$, ES = 0.05) and DT (266.40 ± 74.97 vs. 282.59 ± 51.08 ms, $p = 0.4321$, ES = 0.06) were not influenced by condition (panels E and F).

Figure 2 illustrates that ESV, EDV, EF, and SV were not significantly different between conditions (panels A, B, C, and D, respectively). In detail, ESV was 37.88 ± 17.73 vs. 38.56 ± 12.04 ml for the NORMO and the HYPO test, respectively ($p = 0.7993$, ES = 0.01), EDV was 119.77 ± 24.81 vs. 123.30 ± 17.88 ml ($p = 0.3943$, ES = 0.04), EF was 69.70 ± 9.93 vs. 68.99 ± 7.94% ($p = 0.6219$, ES = 0.01), and SV was 81.88 ± 117.79 vs. 84.73 ± 13.40 ml ($p = 0.2312$, ES = 0.05).

HYPO test increased Evel with respect to the NORMO test (75.41 ± 14.01 vs. 67.41 ± 10.69 cm·s^{-1}, $p = 0.0478$, ES = 0.16. **Figure 3**, panel A). Similarly, E/A was also increased by the HYPO with respect to the NORMO test (1.17 ± 0.30 vs. 0.93 ± 0.26, $p = 0.0315$, ES = 0.20, **Figure 3**, panel C), while Avel and Em (panels B and D, respectively) were not different between conditions (75.27 ± 15.60 vs. 67.13 ± 12.95 cm·s^{-1}, $p = 0.1603$, ES = 0.14, and 9.55 ± 1.94 vs. 10.44 ± 3.15 cm·s^{-1}, $p = 0.3343$, ES = 0.08 for Avel and Em during the NORMO and the HYPO test, respectively).

TABLE 1 | Mean values ± SD of metabolic data at the anaerobic threshold (AT) and at maximum workload (W_{max}) collected during the cardiopulmonary test. $\dot{V}O_2$, oxygen uptake expressed indexed for body mass (second line) as well as in absolute values (third line); $\dot{V}CO_2$, carbon dioxide production; RER, respiratory exchange ratio; VE, pulmonary ventilation; HR, heart rate. *N* = 12.

	AT	W_{max}
Workload (W)	165.80 ± 31.68	244.27 ± 38.40
$\dot{V}O_2$ (ml·kg^{-1}·min^{-1})	25.61 ± 3.18	38.45 ± 4.10
$\dot{V}O_2$ (ml·min^{-1})	2,008 ± 350	2,780 ± 528
$\dot{V}CO_2$ (ml·kg^{-1}·min^{-1})	1,771 ± 440	3,920 ± 688
RER	1.08 ± 0.10	1.41 ± 0.05
VE (l·min^{-1})	42.75 ± 10.91	98.34 ± 18.06
HR (bpm)	146.15 ± 8.65	182.50 ± 10.50

TABLE 2 | Hemodynamic values during the third minute of rest preceding the test in normoxia (NORMO) and in hypoxia with FiO_2 at 13.5% (HYPO). *N* = 12.

	NORMO	HYPO	*p*
SO_2 (%)	97.33 ± 1.36	98.04 ± 1.25	0.1840
HR (bpm)	88.92 ± 14.77	80.58 ± 9.98	0.0855
MAP (mmHg)	89.75 ± 4.55	85.83 ± 4.91	0.2106
PEP (ms)	137.90 ± 19.27	135.50 ± 24.16	0.7176
VET (ms)	238.02 ± 25.17	237.85 ± 38.04	0.9876
DT (ms)	261.53 ± 55.57	347.99 ± 82.60	0.0148
ESV (ml)	37.66 ± 12.83	39.27 ± 15.88	0.9828
EDV (ml)	120.81 ± 17.89	120.19 ± 21.82	0.9135
EF (%)	68.87 ± 9.14	67.85 ± 9.06	0.5927
SV (ml)	83.13 ± 15.08	80.91 ± 13.85	0.5244
Evel (cm·s^{-1})	54.47 ± 10.69	62.81 ± 14.54	0.0325
Avel (cm·s^{-1})	62.05 ± 13.41	50.53 ± 7.87	0.0036
E/A	0.91 ± 0.23	1.28 ± 0.38	0.0014
Em (cm·s^{-1})	8.44 ± 2.36	9.14 ± 3.25	0.5695
Am (cm·s^{-1})	8.22 ± 2.33	7.06 ± 1.46	0.1959
Em/Am	1.09 ± 0.40	1.37 ± 0.54	0.0767
Sm (cm·s^{-1})	10.19 ± 2.07	9.86 ± 1.52	0.6189
Evel/Em	6.82 ± 1.60	7.48 ± 2.15	0.3926

Finally, **Figure 4** shows that Sm (panel C) was higher during the HYPO than during the NORMO test (14.30 ± 1.49 vs. 12.72 ± 2.445 cm·s^{-1}, $p = 0.0431$, ES = 0.20), whereas Am (panel A), Em/Am (panel B), and Evel/Em were not different between conditions (9.55 ± 2.75 vs. 9.30 ± 2.05 cm·s^{-1}, $p = 0.7387$, ES = 0.02; 1.10 ± 0.40 vs. 1.19 ± 0.41, $p = 0.4623$, ES = 0.05; and 7.29 ± 1.23 vs. 7.71 ± 1.77, $p = 0.4875$, ES = 0.07 for Am, Em/Am, and Evel/Em during the NORMO and the HYPO test, respectively).

DISCUSSION

In the present study, a group of healthy physically active male subjects performed a brief, mild exercise bout during acute hypoxia to study the cardiovascular response during the following recovery. The fact that our experimental approach was capable of effectively inducing hypoxemia was testified by SO_2, which substantially dropped during the HYPO session, as shown by **Figure 1** (panel A).

The main purpose of the present investigation was to verify whether classical echocardiographic measures of ventricular

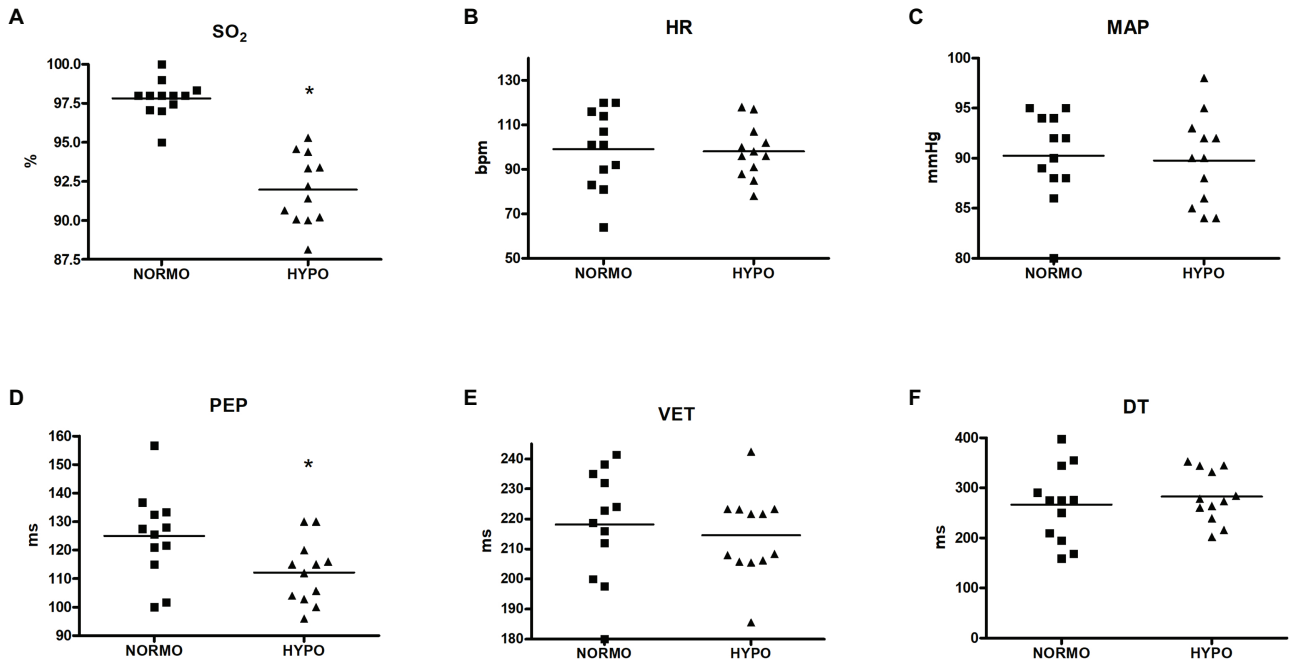

FIGURE 1 | Scatter plot graphs of levels of blood O_2 saturation (SO_2, panel **A**), heart rate (HR, panel **B**), mean arterial pressure (MAP, panel **C**), pre-ejection period (PEP, panel **D**), ventricular ejection time (VET, panel **E**), and diastolic time (DT, panel **F**) during the recovery from sessions of exercise in normoxia (NORMO) and in normobaric hypoxia with a FiO_2 of 13.5% (HYPO). $N = 12$. $^*p < 0.05$ vs. NORMO test.

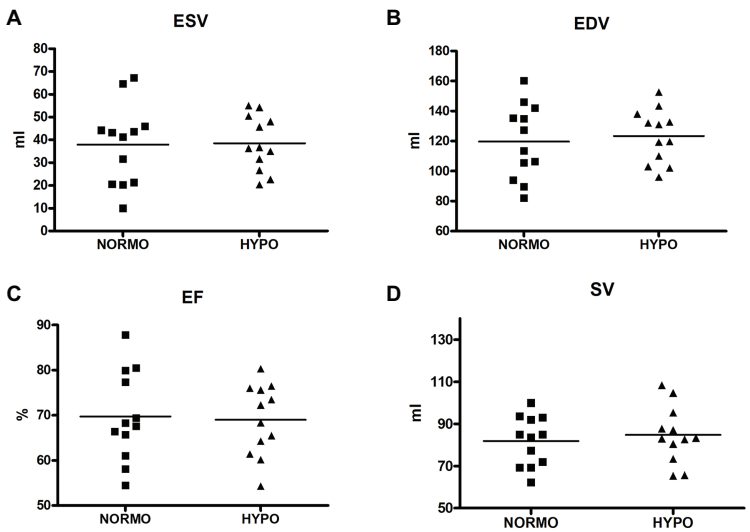

FIGURE 2 | Scatter plot graphs of levels of end-systolic volume (ESV, panel **A**), end-diastolic volume (EDV, panel **B**), ejection fraction (EF, panel **C**), and stroke volume (SV, panel **D**) during the recovery from sessions of exercise in normoxia (NORMO) and in normobaric hypoxia with a FiO_2 of 13.5% (HYPO). $N = 12$.

volume confirmed or rejected our previous hypothesis of a reduced pre-load during the recovery from mild efforts conducted in AH. Another aim was to verify whether Doppler and tissue Doppler measures confirmed or rejected the hypothesis that, during the recovery from exercise in AH, systolic and diastolic functions increased due to sympathetic activation.

Based on results, we must reject the first hypothesis. Indeed, EDV was similar between conditions, thereby indicating that there were not reductions in cardiac pre-load in participants. Moreover, ESV, EF, and SV were not different between the NORMO and the HYPO tests. These findings suggest that a brief bout of mild exercise in AH could not cause any modification in cardiac variables related to heart volumes.

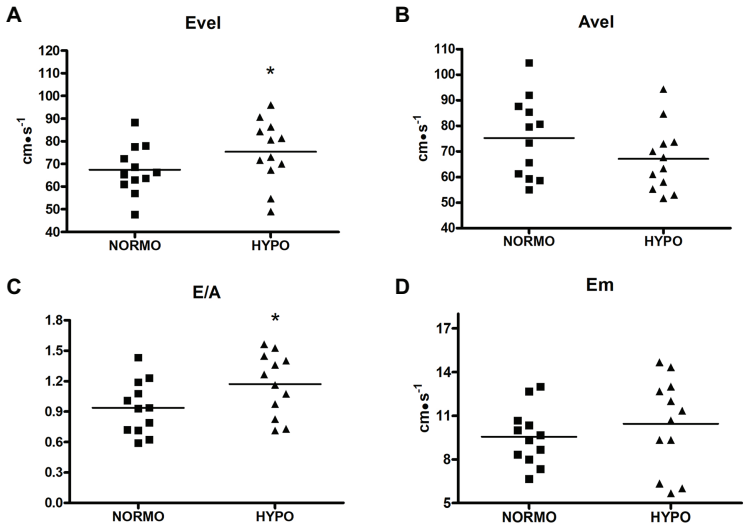

FIGURE 3 | Scatter plot graphs of levels of early transmitral filling peak velocity (Evel, panel **A**), late transmitral filling peak velocity (Avel, panel **B**), their ratio (E/A, panel **C**), and early diastolic mitral valve motion velocity (Em, panel **D**) during the recovery from sessions of exercise in normoxia (NORMO) and in normobaric hypoxia with a FiO_2 of 13.5% (HYPO). $N = 12$. $^*p < 0.05$ vs. NORMO test.

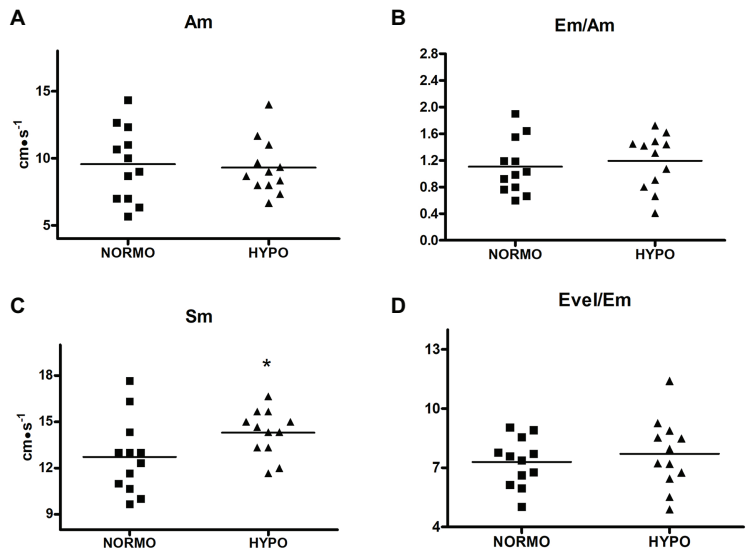

FIGURE 4 | Scatter plot graphs of levels of late diastolic mitral valve motion velocity (Am, panel **A**), ratio between early and late diastolic mitral valve motion velocities (Em/Am, panel **B**), systolic myocardial velocity (Sm, panel **C**), and ratio between early transmitral filling peak and early mitral valve diastolic velocities (Evel/Em, panel **D**) during the recovery from sessions of exercise in normoxia (NORMO) and in normobaric hypoxia with a FiO_2 of 13.5% (HYPO). $N = 12$. $^*p < 0.05$ vs. NORMO test.

This finding appears to contradict our recent investigation, where, in healthy humans, a reduction in the capacity to increase SV due to impairment in ventricular filling rate was found in response to the muscle metaboreflex activation after exercise in AH (Mulliri et al., 2020). In this paper, it was hypothesised that the reduced ventricular filling rate was the consequence of an increase in the production of metabolite-mediated venodilation, such as NO, adenosine, and prostaglandin derived factors, which exerted vasodilatory activity in the venous bed (Marshall, 2015; Dinenno, 2016) and prevented the recruitment of the Frank-Starling mechanism. It should however be considered that, in the quoted study, hemodynamics were studied during the metaboreflex stimulation, which causes a substantial sympathetic activation. Thus, the present and the former studies are quite different in the experimental approach, and their results can be only partially comparable. Moreover, in our previous investigation, we could not conduct any echocardiography assessment.

Thus, out hypothesis was speculative. Further study using echocardiography, during the metaboreflex, should be conducted in order to verify whether metaboreflex-induced sympathetic activation after exercise in AH reveals any impairment in EDV and in the capacity to vasoconstrict the venous bed in healthy humans.

Another result of the present research was that, during the recovery of the HYPO test, PEP significantly shortened in comparison with the NORMO test (**Figure 1**, panel D). This indicated that, in this setting, there was an increase in myocardial contractility, as this parameter is inversely related to the development of intraventricular pressure. Concerning the influence of autonomic activity, it is to be highlighted that PEP responds only to sympathetic stimulation, since the influence of parasympathetic tone is negligible on ventricles. Moreover, PEP does not depend on changes in HR (Michael et al., 2017). Thus, the PEP shortening could be the consequence of an increase in sympathetic tone, although other phenomena may have taken part in the myocardial contractility enhancement (see the following part of Discussion).

The fact that after the exercise bout in AH an increase in contractility took place is also confirmed by the Sm velocity gathered by tissue Doppler, which was faster during the HYPO as compared to the NORMO test (**Figure 4**, panel C). Sm velocity at the lateral mitral anulus is correlated with ventricular peak *dP/dt* and it can be considered an index of inotropism (Correale et al., 2012). Actually, ventricular systole pulls down the atrio-ventricular plane, and it seems reasonable to assume that the displacement of this plane is an expression of the myocardial contractility (Höglund et al., 1988). In support to the notion that Sm is related to myocardial performance there are findings that mitral anulus systolic excursion is reduced in patients with ventricular dysfunction (Grue et al., 2018; Berg et al., 2020).

It is to be noticed that, during the rest period of the HYPO session, neither PEP nor Sm was affected by the administration of the hypoxic gas mixture (see **Table 2**). This result suggests that, rather than hypoxia *per se*, the exercise bout in AH was the real responsible for changes in both parameters related to myocardial contractility. It is also possible that sympathetic activation was not the only responsible for the enhanced myocardial contractility. The concept that several substances produced during exercise in AH can enhance inotropism independently from sympathetic activity has been the subject of active research in the last years. Specifically, apart from sympathetic tone, during AH some metabolic products, such as apelin, may exert positive inotropic effect (Calbet et al., 2009), thus explaining why we noticed an increase in Sm only after exercise in AH and not at rest. For instance, recent findings demonstrated that left ventricular twist mechanic is not impaired by acute hypoxia and that endocardial dysfunction did not occur during AH (Williams et al., 2019). Moreover, it should be mentioned that during exercise in ischemic conditions several metabolites are produced, and these metabolites can trigger the phenomenon termed *ischemic preconditioning*, which confers cardioprotection and favourable hemodynamics effects (i.e., increase in myocardial performance and vasodilation) within few minutes (Marongiu and Crisafulli, 2014). It is then conceivable to hypothesise that exercise in AH leads to a similar metabolites production as during ischemia. To the best of our knowledge, this possibility has never investigated before and it may represent an intriguing field of research in a physiological and clinical perspective.

Whatever the cause responsible for the increased myocardial performance, results of the present investigation confirm that contractile function is preserved after mild exercise bouts in AH and that SV is well preserved by mechanisms, which are only partially known. While a decrease in SV, during acclimatisation at high altitude has been several times reported, this phenomenon is usually not observed during AH (Stembridge et al., 2016).

Another result of the present investigation was that diastolic function was significantly modified by AH both at rest and after the exercise bout. Regarding results at rest, it appears that the hypoxic gas administration shifted ventricular filling from the late to the early phase. This can be at least partially explained by the longer DT during the HYPO in comparison with the NORMO test. This was the result of the slight reduction in HR occurring during the rest period of the HYPO test, which, although insignificant with respect to the NORMO test, nonetheless led to a longer cardiac cycle with respect to the NORMO test. Considering that PEP and VET were quite similar between conditions, then it followed that DT was longer in the HYPO test. We cannot however rule out that the hypoxic condition could improve early diastolic function by any unknown mechanism able to enhance ventricular relaxation. To the best of our knowledge, there are no studies focusing on the potential effect of AH on the myocardial early diastolic proprieties, and further research is warranted in this area. It should however be acknowledged that the presence of any hypoxic-mediated mechanism was unlikely in our setting at rest as the hypoxic stimulus did not significantly reduce SO_2, so indicating that the hypoxic stress was mild.

A different diastolic behaviour between tests was present also during the recovery phase. Indeed, Evel and E/A were significantly higher after AH. An increase in Evel, during exercise in AH, has been already reported in the scientific literature (Yan et al., 2007), but the phenomenon has been never replied by other groups to date. Authors of the quoted paper suggested that acute hypoxic exercise increased diastolic function, although no explanation for the phenomenon was provided. Our results seem to confirm these previous findings. Moreover, our results suggest that the increased contractility could be at least in part responsible for it. Both PEP and Sm indicated that, during the HYPO test, myocardial contractility was more elevated with respect to the NORMO test. It was observed that the energy generated during systole is stored in the extracellular collagen matrix and then released during diastole, thereby supporting ventricular filling (Notomi et al., 2008). Furthermore, it was also proposed that the atrio-ventricular plane acts like a piston driven by ventricular contraction and that its movement

during systole pulls blood from the venous tree to the atria (Arutunyan, 2015). In short, when the ventricles contract, the A-V plane descends towards the apex, while the pulmonary veins remain fixed in the mediastinum. The descent of the A-V plane aspirates blood from the pulmonary circulation and generates one of the forces able to fill the atria (Chung et al., 2015). In humans, it has been demonstrated that up to 70% of atrial filling occurs during ventricular emptying and is driven by ventricular longitudinal contraction (Steding-Ehrenborg et al., 2013).

Then, the increased myocardial performance during the HYPO test may have enhanced early diastolic filling with at least two different phenomena: (a) an increase in the energy generated during systole and recoiled during diastole and (b) a more efficient A-V displacement, which allowed a more effective atrial filling.

A third phenomenon that could theoretically affect diastolic filling could be the increase in left ventricular filling pressure due to hypoxia-induced pulmonary vasoconstriction (Naeije and Dedobbeleer, 2013; Stembridge et al., 2016). We employed Evel/Em to estimate the left ventricular filling pressure, but we did not find out any significant difference between the HYPO and the NORMO test. It can be then concluded that a brief bout of exercise in AH cannot significantly affect left ventricular filling pressure.

Limitations of the Study

Some limitations of the present investigation should be honestly acknowledged.

In detail, echocardiographic measures were conducted only during recovery and not during exercise. This is because, in a pilot study, we could not collect good images during cycling mainly because of chest movements due to respiration. Probably, the best position in this kind of research is the recumbent one. However, this position is not very natural as normally individuals exercise standing or sitting, as in the present investigation. Moreover, the recumbent position increases venous return, thereby affecting EDV and diastolic functions.

It should be pointed out that diastolic measures obtained with tissue Doppler yielded different results with respect to trans-mitral Doppler. Specifically, while Evel was significantly increased by the HYPO test, Em was not affected by this condition. One explanation for this different outcome could be that tissue Doppler measures are highly dependent on the angle between scan beam and the vector of ventricular motion, which should be parallel (Bassareo et al., 2010). It is then possible that chest movements due respiration after effort may have rendered problematic tissue Doppler measures in our experimental setting, thus affecting assessment precision.

Another limit could be that we did not directly assess myocardial inotropism. Instead, indirect measures were used, i.e., PEP and Sm. However, the direct assessment of myocardial inotropism is problematic in humans as it requires the use of invasive technologies, which are not advisable in study such the present one.

Finally, the present study was conducted in healthy male individuals, thus its results cannot be applicable for elderly people, for females, or for patients suffering from any disease. Further research in different groups of individuals is needed to have a clearer picture of the hemodynamic consequences of hypoxia during exercise in these sub-groups.

CONCLUSION

Overall, the results of the present investigation support the hypothesis that a brief exercise bout of mild intensity in acute normobaric hypoxia does not impair systolic or diastolic functions. Rather, it appears that SV is well preserved, thanks to an improvement in inotropism and in early diastolic function. It remains to be ascertained whether the described improvement in diastolic function is a direct consequence of the enhanced systolic activity or it is due to an unknown metabolic process triggered by exercise in hypoxia. Taking into consideration that exercise in hypoxia has been proposed as a useful tool for training as well as for therapeutic purposes, its effects should be further investigated to better understand its hemodynamics and its capacity to product regulating metabolites.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AC, SM, GM, and PB conceived the protocol, conducted experiments, analysed data, ran statistics, interpreted results, and wrote the manuscript. SR, FS, GS, GG, GN, and RV conducted experiments, analysed data, ran statistics, and interpreted results. All authors contributed substantially to the article and approved its final form.

FUNDING

This study was supported by the University of Cagliari and by the Regione Autonoma della Sardegna (Legge 7/2007 – annualità 2017. Risorse FSC 2014–2020 – Patto per lo Sviluppo della Regione Sardegna).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 06 September 2021
doi: 10.3389/fphys.2021.735557

Altitude, Exercise, and Skeletal Muscle Angio-Adaptive Responses to Hypoxia: A Complex Story

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Hypoxia, defined as a reduced oxygen availability, can be observed in many tissues in response to various physiological and pathological conditions. As a hallmark of the altitude environment, ambient hypoxia results from a drop in the oxygen pressure in the atmosphere with elevation. A hypoxic stress can also occur at the cellular level when the oxygen supply through the local microcirculation cannot match the cells' metabolic needs. This has been suggested in contracting skeletal myofibers during physical exercise. Regardless of its origin, ambient or exercise-induced, muscle hypoxia triggers complex angio-adaptive responses in the skeletal muscle tissue. These can result in the expression of a plethora of angio-adaptive molecules, ultimately leading to the growth, stabilization, or regression of muscle capillaries. This remarkable plasticity of the capillary network is referred to as angio-adaptation. It can alter the capillary-to-myofiber interface, which represent an important determinant of skeletal muscle function. These angio-adaptive molecules can also be released in the circulation as myokines to act on distant tissues. This review addresses the respective and combined potency of ambient hypoxia and exercise to generate a cellular hypoxic stress in skeletal muscle. The major skeletal muscle angio-adaptive responses to hypoxia so far described in this context will be discussed, including existing controversies in the field. Finally, this review will highlight the molecular complexity of the skeletal muscle angio-adaptive response to hypoxia and identify current gaps of knowledges in this field of exercise and environmental physiology.

Keywords: skeletal muscle, capillary, angiogenesis, VEGF-A, thrombospondin, hypoxia, altitude, exercise

OPEN ACCESS

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Specialty section:
This article was submitted to Exercise Physiology, a section of the journal Frontiers in Physiology

Received: *02 July 2021*
Accepted: *16 August 2021*
Published: *06 September 2021*

Citation:
Lemieux P and Birot O (2021) Altitude, Exercise, and Skeletal Muscle Angio-Adaptive Responses to Hypoxia: A Complex Story. Front. Physiol. 12:735557. doi: 10.3389/fphys.2021.735557

INTRODUCTION

Our review aims to revisit the complexity of the skeletal muscle angio-adaptive response to hypoxia, particularly when combining exposure to ambient hypoxia and exercise-induced tissue hypoxia. Indeed, in the context of high-altitude expeditions, mountaineers usually engage into prolonged periods of intense physical activity over several weeks or months (West, 2006, 2012). In the context of sport performance at sea level, hypoxia training has become a complex and very specialized area of research (Millet et al., 2010; Lundby et al., 2012; Girard and Chalabi, 2013; Girard and Pluim, 2013; Girard et al., 2013). Finally, the use of exercise training under hypoxia has recently emerged as a new and promising therapeutic avenue to improve some metabolic and cardiovascular conditions

(obesity, type-2 diabetes, hypertension) as well as for the training of elderly subjects (Verges et al., 2015; Millet et al., 2016; **Figure 1**).

Skeletal muscles represent one of our largest tissues, accounting for about 40% of human body weight. Skeletal muscles adapt to environmental, physiological, and pathological conditions with a remarkable plasticity. This can include changes in muscle mass, in the size of myofibers and their metabolic and contractile phenotype, as well as changes in muscle capillarization (Booth and Thomason, 1991; Hudlicka et al., 1992; Hudlicka, 2011).

Since August Krogh's pioneering work about a century ago (Krogh, 1919a,b,c), our understanding of the regulation of muscle blood flow and oxygen delivery to muscle cells has considerably evolved and was recently revisited in great review articles (Angleys and Østergaard, 2020; Poole et al., 2020, 2021; Kissane et al., 2021). The oxygen cascade from skeletal muscle arterioles to capillaries, interstitial tissue, sarcolemma, and mitochondria can be influenced at several levels: The vasomotricity of upstream arterioles and the subsequent regulation of capillary blood flow; the content and velocity of red blood cells; the hemoglobin and myoglobin concentrations; the tortuosity and number of capillaries; and the surface area of myofibers.

The capillary-to-myofiber interface plays a crucial role for muscle function. Indeed, it represents the site of exchange for oxygen, nutrients, metabolic heat and waste between the blood and the myofibers. The density of capillaries within a given area of muscle tissue will greatly contribute to matching the delivery of oxygen and nutrients with the myofibers' metabolic needs, particularly during contractile activity (Hudlicka et al., 1987; Hoppeler and Kayar, 1988; Mathieu-Costello, 1994; Hudlicka, 2011). The capillary network can therefore be considered as a key determinant of skeletal muscle function and several studies have reported strong correlations between the level of muscle capillarization and mitochondria volume density, muscle oxidative capacity, and oxygen consumption (Hoppeler et al., 1987; Hudlicka et al., 1987; Hoppeler and Kayar, 1988; Poole and Mathieu-Costello, 1996; Howlett et al., 2003). For instance, Howlett et al. (2003) reported a strong correlation between skeletal muscle capillary density and muscle oxygen conductance in rats selectively bred for running endurance (Howlett et al., 2003).

SKELETAL MUSCLE CAPILLARIZATION AND THE CONCEPT OF MUSCLE ANGIO-ADAPTATION

The capillary density in a muscle section can vary in response to various environmental, physiological, or pathological conditions. An increase in muscle capillary density is usually observed in human subjects and animal models in response to prolonged endurance training or high-altitude sojourn (Hudlicka et al., 1992; Breen et al., 2008; Hudlicka, 2011). Conversely, skeletal muscle capillary rarefaction has been described in response to physical deconditioning as well as in the context of some pathologies such as chronic obstructive pulmonary disease, chronic heart failure, or diabetes (Roudier et al., 2010; Gouzi et al., 2013; Olfert et al., 2015; Aiken et al., 2019).

Skeletal muscle angio-adaptation refers to the complex and dynamic processes of capillary formation, stabilization, or regression in response to acute and chronic physiological or pathological conditions. These processes are regulated at the molecular level by a plethora of pro- and anti-angiogenic molecules (Hoppeler, 1999; Breen et al., 2008; Egginton, 2009; Olfert and Birot, 2011; Egginton and Birot, 2014; Olfert et al., 2015). At the cellular level, endothelial cells proliferate, migrate, and assemble to form new capillaries in the context of angiogenesis, or conversely, undergo apoptosis during capillary regression. Myofibers can represent an important source of production and release of angio-regulatory molecules such as the well-described pro-angiogenic Vascular Endothelial Growth Factor-A (VEGF-A).

Importantly, changes in capillary density can also be a direct consequence of alterations in the size of myofibers (hypertrophy or atrophy) without any true capillary loss or formation. Also, no change in capillary density is not necessarily synonymous of an absence of angio-adaptive activity. For example, during myofiber hypertrophy, a formation of new capillaries might occur simply to prevent a decrease in the capillary density that would result from the increase in the myofibers surface area. It is also important to note that some angio-adaptive molecules, such as the pro-angiogenic VEGF-A, are not only required for the growth of capillaries but also to maintain existing ones. Evidence suggests an autocrine expression of VEGF-A being important for endothelial cell survival and vascular homeostasis (Lee et al., 2007; Domigan et al., 2015). Finally, the skeletal muscle could also be seen as an endocrine organ releasing "angio-adaptive myokines" in circulation, potentially affecting distant tissues.

SKELETAL MUSCLE HYPOXIA: IMPACT OF ALTITUDE AND EXERCISE

Hypoxia, defined as a lack of oxygen supply to a given tissue, is a powerful pro-angiogenic stimulus for various cell types and tissues including skeletal muscle (Hoppeler and Vogt, 2001; Semenza, 2001; Breen et al., 2008; Fraisl et al., 2009; Favier et al., 2015). Ambient hypoxia is a hallmark of the altitude environment and results from a drop in the oxygen pressure in the atmosphere with elevation. However, the impact of ambient hypoxia *per se* on skeletal muscle angio-adaptive responses and capillarization still remains a source of scientific debate. A hypoxic stress can also be generated locally at the muscle tissue level in response to intense exercise if oxygen delivery cannot match the metabolic needs of contracting myofibers (**Figure 1**).

Several studies have aimed at determining the oxygen partial pressure (PO_2) cascade in human and animal models using different techniques such as proton magnetic resonance spectroscopy, surface electrodes, microcatheter, and more recently phosphorescence quenching. The intramuscular PO_2 at rest is estimated around 27 mmHg with some variations (10-34 mmHg) among studies (different species, muscles, and techniques), below the microvascular PO_2 but well above the

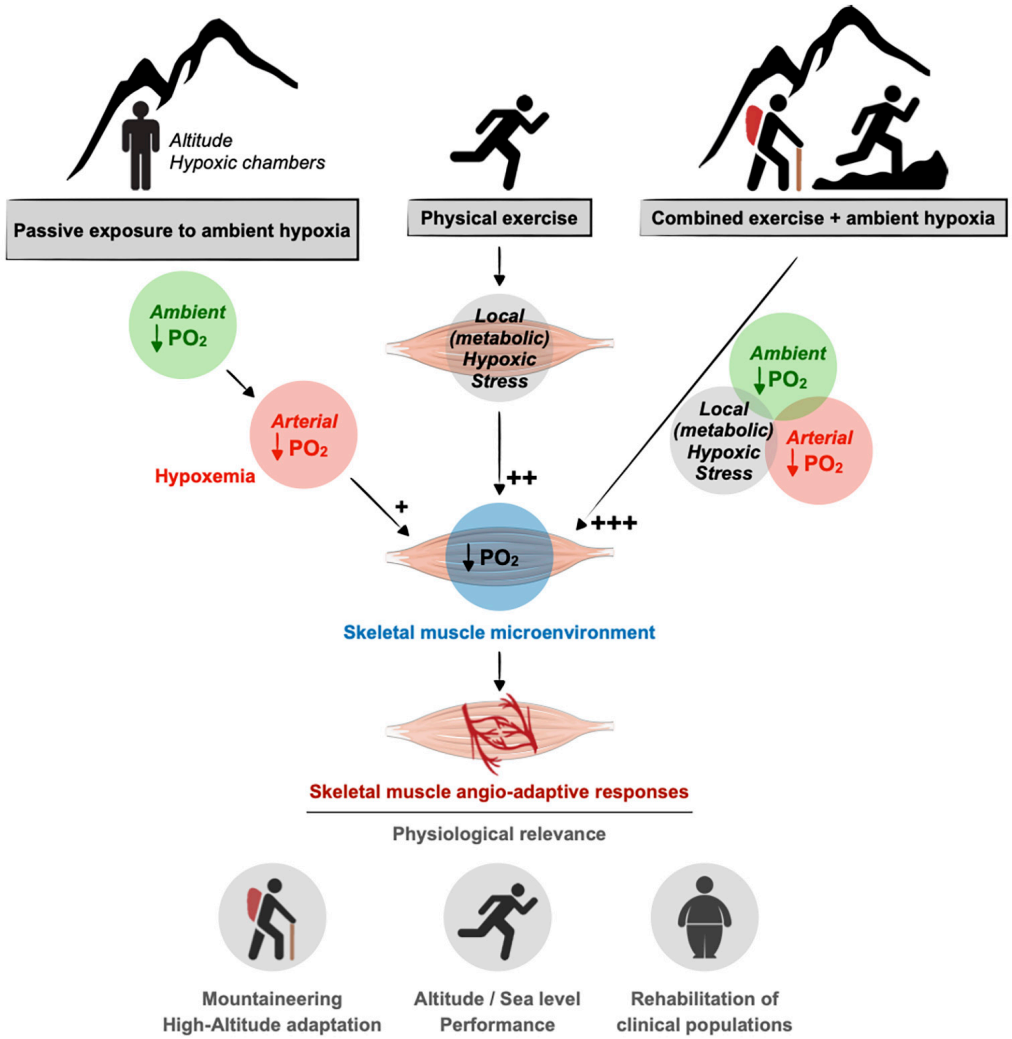

FIGURE 1 | Contribution of ambient hypoxia and exercise to induce a hypoxic stress in skeletal muscle. A drop in the partial pressure of oxygen (PO_2) in the muscle tissue can result from a decrease in ambient PO_2 (e.g., altitude) as well as an unbalanced between blood supply to the muscle cells and their increase metabolic needs during exercise. In response to muscle hypoxia angio-adaptive responses will take place to maintain an optimal oxygen supply to myofibers and to preserve muscle function. This is of particular relevance in the context of mountaineering activity as well as exercise training strategies using ambient hypoxia for athletes or clinical populations. As discussed in the text, the impact of ambient hypoxia on skeletal muscle PO_2 seems rather modest (+) compared to the impact of exercise in generating a local hypoxic stress (++). The combination of ambient hypoxia exposure and exercise might exacerbate this hypoxic stress (+++).

intra-myocyte PO_2 (1-3 mmHg) (Prewitt and Johnson, 1976; Boegehold and Johnson, 1988; Richardson et al., 1995, 2006; Richmond et al., 1997, 1999; Hutter et al., 1999; Molé et al., 1999; Lombard et al., 2000; Richardson, 2000; Kindig et al., 2003; Johnson et al., 2005; Liu et al., 2012; Hirai et al., 2018, 2019; Colburn et al., 2020; Poole et al., 2020, 2021).

A few of these studies have investigated the impact of ambient hypoxia on skeletal muscle PO_2. A modest reduction in PO_2 (from 34 mmHg under normoxia to 23 mmHg under hypoxia) was observed in human quadriceps muscle following an exposure to an inspired O_2 fraction of 10% (F_iO_2 0.10), equivalent to an altitude of about 5,800 m (Richardson et al., 2006). In another study, the interstitial PO_2 in rat cremaster muscles was reduced from 26 to about 10 mmHg following one minute exposure to an inspired O_2 fraction of 7% (F_iO_2 0.07), equivalent to an altitude of about 8,300 m (Johnson et al., 2005). However, the physiological relevance of this conditioning (1 min of exposure at F_iO_2 0.07) can be questioned. These few studies suggest that ambient hypoxia exposure might not alter muscle PO_2 to a large extent. Conversely, the impact of physical exercise on the muscle PO_2 seems much more important. One bout of exercise, even at moderate intensity (50% maximal leg O_2 uptake) was shown to decrease muscle PO_2 to values around 3-5 mmHg (Richardson et al., 1995, 2006; Molé et al., 1999; Angleys and Østergaard, 2020). This exercise-induced hypoxic stress could be further exacerbated (muscle PO_2 down to 2 mmHg) when exercise was performed in a hypoxic environment (Richardson et al., 2006).

At a molecular level, the Hypoxia Inducible Factor-1 (HIF-1) is a transcription factor widely recognized as a hallmark of the hypoxia signaling pathway (Semenza and Wang, 1992; Semenza, 2001). HIF-1 is a heterodimeric complex formed of two subunits alpha and beta. Whereas the expression of the beta subunit remains stable under both normoxic and hypoxic conditions, the alpha subunit (HIF-1α) confers on HIF-1 most of its regulation. Under normoxia, the HIF-1α protein is constantly synthesized in the cytoplasm, and rapidly undergoes proteosomal degradation within a few minutes. This involves HIF-1α hydroxylation on certain proline residues by HIF prolyl hydroxylases (PHD1-3) (Ke and Costa, 2006; Lindholm et al., 2014). If the PO_2 drops enough to generate a hypoxic stress at the cellular level, the function of PHD1-3 is inhibited, HIF-1α is stabilized and can translocate into the nucleus to dimerize with the beta subunit. HIF-1 binds to hypoxia responsive elements (HRE) in the promoter regions of target genes to regulate their transcription. Both the erythropoietin (EPO) and pro-angiogenic VEGF-A genes possess HRE sites in their promoters (Semenza, 2001).

Whether ambient hypoxia exposure results in an increased expression of HIF-1α protein in skeletal muscle remains largely understudied. Stroka et al. (2001) have observed a strong HIF-1α protein expression level in mouse skeletal muscle even under normoxic conditions (Stroka et al., 2001). In the same study, 1 h exposure to extreme normobaric hypoxia (F_iO_2 0.06, equivalent to 9,100 m) did not seem to increase the expression level much further. As noted previously, the physiological relevance of conditionings combining very short exposures (1 min to 1 h) and extreme hypoxia levels (F_iO_2 0.06-0.07) are questionable (Stroka et al., 2001; Johnson et al., 2005).

Intense physical exercise can be at the origin of a local hypoxic stress in the muscle tissue. In an elegant study, Ameln et al. (2005) quantified HIF-1α protein expression in response to one single bout of moderate intensity exercise in human vastus lateralis muscle biopsies (Ameln et al., 2005). HIF-1α protein levels were increased immediately after exercise and remained elevated for up to 6 h post-exercise. An increased nuclear staining for HIF-1α was also observed as well as an increased DNA binding to HRE binding sites. mRNA levels for HIF-1 target genes EPO and VEGF-A were also higher post-exercise. These results suggest that HIF-1 expression and activity were both increased post-exercise in human skeletal muscle.

An increase in HIF-1α protein expression could result from the inhibition of HIF-1α protein degradation but also from an increased expression of HIF-1α mRNA and protein translation. Vogt et al. (2001) have measured HIF-1α mRNA in human vastus lateralis muscle biopsies before and after an endurance training program conducted at low or high intensity either under normoxia or normobaric hypoxia (simulated altitude of 3,850 m) (Vogt et al., 2001). HIF-1α mRNA expression was increased after hypoxia training regardless of the training intensity whereas training under normoxia had no effect. Interestingly, VEGF-A mRNA levels were also measured and found to be increased only in the high-intensity hypoxia training group but not under low exercise training conditions. This could reveal a synergetic effect of combining ambient hypoxia and exercise-induced local hypoxia. Here, biopsies were performed at least 24 h post-exercise to assess the effect of prolonged training on HIF-1α basal levels. In line with this study, Lundby et al. (2006) have evaluated the impact of prolonged exercise training on HIF-1α acute response to one single bout of exercise (Lundby et al., 2006). HIF-1α mRNA levels were increased in vastus lateralis biopsies at 6 h post-exercise only in the untrained group. Whether exercise training could blunt the acute HIF-1α response to one single bout of exercise was then questioned by Lindholm et al. (2014), Lindholm and Rundqvist (2016) who showed that several inhibitors of HIF-1α expression and HIF-1 activity were increased in trained muscles. The analysis of trained muscle biopsies indeed revealed higher expression levels of prolyl hydroxylases (PHD1-3), Factor Inhibiting HIF-1 (FIH) and Sirtuin-6 (SIRT6) (Lindholm et al., 2014). By catalyzing hydroxylation on specific prolyl residues, PHD1-3 target HIF-1α for proteasomal degradation. FIH, a HIF-1α-specific asparagine hydroxylase, can inhibit HIF-1 transactivation. SIRT6, a histone deacetylase, can act as an epigenetic co-repressor of HIF-1. Another explanation to the results from Lundby et al. (2006) could be an increased level of muscle capillarization post-training. Angiogenesis is a well-described tissular adaptation of the skeletal muscle tissue to endurance training. This would result in a better capillary-to-myofiber interface and oxygen delivery to contracting myofibers, and as such, a reduced exercise-induced hypoxic stress in trained muscles.

In addition to inducing a local cellular hypoxic stress, exercise combines other pro-angiogenic stimuli such as increased shear stress on endothelial cells due to enhanced muscle blood flow, mechanical tissue stretch, and oxidative stress (Hudlicka et al., 1992; Milkiewicz et al., 2001; Breen et al., 2008; Egginton, 2009; Hoier et al., 2012; Egginton and Birot, 2014; Hellsten and Hoier, 2014; Haas and Nwadozi, 2015; Olfert et al., 2015). The increase in muscle blood flow results from active vasodilation, and the resultant increase in shear stress represents a well-described pro-angiogenic stimulus for skeletal muscle endothelial cells, mediating the production of angio-adaptive molecules such as nitric oxide, metalloproteinases, and VEGF-A (Milkiewicz et al., 2001, 2011; Egginton, 2009, 2011; Hudlicka and Brown, 2009; Hellsten and Hoier, 2014; Haas and Nwadozi, 2015). If local cellular hypoxia and shear stress are often presented side by side as exercise-induced pro-angiogenic stimuli, passive exposure to ambient hypoxia also stimulates vasodilation and increases blood flow in skeletal muscle (Casey and Joyner, 2011, 2012; Joyner and Casey, 2014; Dinenno, 2016). Therefore, ambient hypoxia could then represent an upstream stimulus of muscle shear stress. The degree of vasodilation observed seems linked to the degree of ambient hypoxia, at least for acute exposures. Interestingly, the combination of ambient hypoxia exposure and exercise seems to synergistically induce greater vasodilation and muscle blood flow than what could be expected by simply adding their respective contributions (Casey and Joyner, 2011, 2012; Joyner and Casey, 2014). Finally, to add more complexity, HIF-1α expression can also be stimulated under normoxic conditions in skeletal muscle in response to increased shear stress or mechanical tissue stretch (Milkiewicz et al., 2007).

ALTITUDE, EXERCISE, AND SKELETAL MUSCLE HYPOXIA: KEY POINTS

- Exercise is a more powerful stimulus than ambient hypoxia to decrease muscle PO_2.
- Combining ambient hypoxia and exercise might further decrease muscle PO_2.
- Hypoxia Inducible Factor-1 is a well-established hallmark of hypoxia signaling.
- Skeletal muscle angio-adaptive activity can locally change the level of muscle capillarization and can also release angio-adaptive myokines in the circulation for distant effects.

INCREASED MUSCLE CAPILLARIZATION IN RESPONSE TO EXERCISE TRAINING AND AMBIENT HYPOXIA

We previously discussed how increasing the capillary-to-myofiber interface could be beneficial for maintaining or improving muscle function when oxygen delivery becomes a challenge. The capillary density (CD), which represents the number of capillaries per surface unit of tissue, is often used to assess the level of muscle capillarization (Andersen, 1975; Hudlicka et al., 1992). The CD can however be influenced both by changes in the number of capillaries and alterations in the size of myofibers. Changes in CD might therefore not always be representative of angiogenesis or capillary regression. Conversely, the capillary-to-fiber ratio (C/F) represents one of the best histological parameters to truly appreciate capillaries formation or rarefaction.

Exercise training is a powerful and well-established pro-angiogenic stimulus for the skeletal muscle tissue (Andersen, 1975; Andersen and Henriksson, 1977; Hudlicka et al., 1992; Egginton, 2009). Human and animal studies have shown that one single bout of exercise leads to the production and release of several angio-adaptive molecules both in the muscle microenvironment, for example to stimulate skeletal muscle endothelial cells (Roudier et al., 2012; Aiken et al., 2016), and in the circulation as myokines (Hudlicka et al., 1992; Vogt et al., 2001; Egginton, 2009; Olfert and Birot, 2011; Hoier et al., 2012; Egginton and Birot, 2014). During prolonged exercise training, the chronic repetition of these exercise-induced angiogenic responses can ultimately lead to the formation of new capillaries. This increase in muscle capillarization will usually be reflected by higher C/F and CD in trained muscles (Andersen, 1975; Andersen and Henriksson, 1977; Hudlicka et al., 1992; Egginton, 2009). As previously mentioned, the exercise stimulus combines several stressors such as local tissue hypoxia, increased shear stress, tissue stretch, and oxidative stress. The exact contribution of hypoxia *per se* during exercise remains difficult to study and still unclear. Conversely to regular exercise, muscle hypokinesia or deconditioning can lead to capillary regression (Fujino et al., 2005; Egginton, 2009; Malek et al., 2010; Roudier et al., 2010; Olfert and Birot, 2011).

The effect of passive exposure to ambient hypoxia on skeletal muscle capillarization is less clear than the impact of exercise training. A well-established consensus of the literature is that prolonged exposure to field or simulated high altitude hypoxia results in improved skeletal muscle capillarization and increased capillary density. This alteration in capillary density seems however mainly due to the atrophy of myofibers rather than a true angiogenic response. Several studies have indeed reported an increase in CD concomitantly to a decrease in the myofiber surface area, with no change in the C/F ratio (Oelz et al., 1986; Green et al., 1989; Hoppeler et al., 1990a,b; MacDougall et al., 1991; Kayser et al., 1996).

This should be taken with a certain caution since there are in fact almost as many original studies reporting an increase in muscle CD as studies showing no change (**Figure 2A**; Banchero et al., 1976; Sillau and Banchero, 1977; Oelz et al., 1986; Poole and Mathieu-Costello, 1989; Hoppeler et al., 1990a,b; Bigard et al., 1991; Green et al., 1992; Kayser et al., 1996; Olfert et al., 2001; Lundby, 2004; Mizuno et al., 2008; Levett et al., 2012). We searched the PubMed database for original research studies that analyzed skeletal muscle CD and C/F in animals or human subjects passively exposed to normobaric or hypobaric hypoxia. Studies combining ambient hypoxia and exercise interventions were included only if they had all the required experimental groups to assess the impact of passive hypoxia exposure independently of the exercise training stimulus. We identified a total of 22 original research studies (**Figure 2**). Interestingly, 55% reported a significant increase or a trend for an increase in CD by at least 10% whereas 45% showed no change (**Figure 2A**).

The lack of change in CD could be attributed to an absence of muscle atrophy, and some authors have in fact observed no reduction in myofiber size at high altitude (Green et al., 1992; Lundby, 2004; Levett et al., 2012; D'Hulst et al., 2016). It was then suggested that both the exposure duration and the altitude level could determine whether myofibers would atrophy or not, leading to the notion of hypoxic dose (D'Hulst and Deldicque, 2017; Millet et al., 2017). Some conditionings were indeed performed at moderate altitude (3,000-4,000 m) over 2-3 weeks only, as opposed to longer exposures (8-10 weeks) at higher altitudes (>5,200 m) (Oelz et al., 1986; Green et al., 1989, 1992; Hoppeler et al., 1990a,b; MacDougall et al., 1991; Lundby, 2004; Mizuno et al., 2008; Levett et al., 2012).

It is difficult to identify the source of discrepancy between these studies regarding myofiber size and CD. In rodent studies for example, the age of the animals can be a confounding factor if they are still growing (Banchero, 1985; Snyder et al., 1992). Calorie intake can also influence body and muscle weights. Prolonged mountaineering expeditions at high-altitude can involve logistical constraints for proper nutrition and gastroenteritis disorders are often described (West, 2012; Swenson and Bärtsch, 2013). Hypophagia has been well observed in rodents exposed to prolonged hypoxia, usually requiring the use of pair-fed control animals (Daneshrad et al., 2001, 2003).

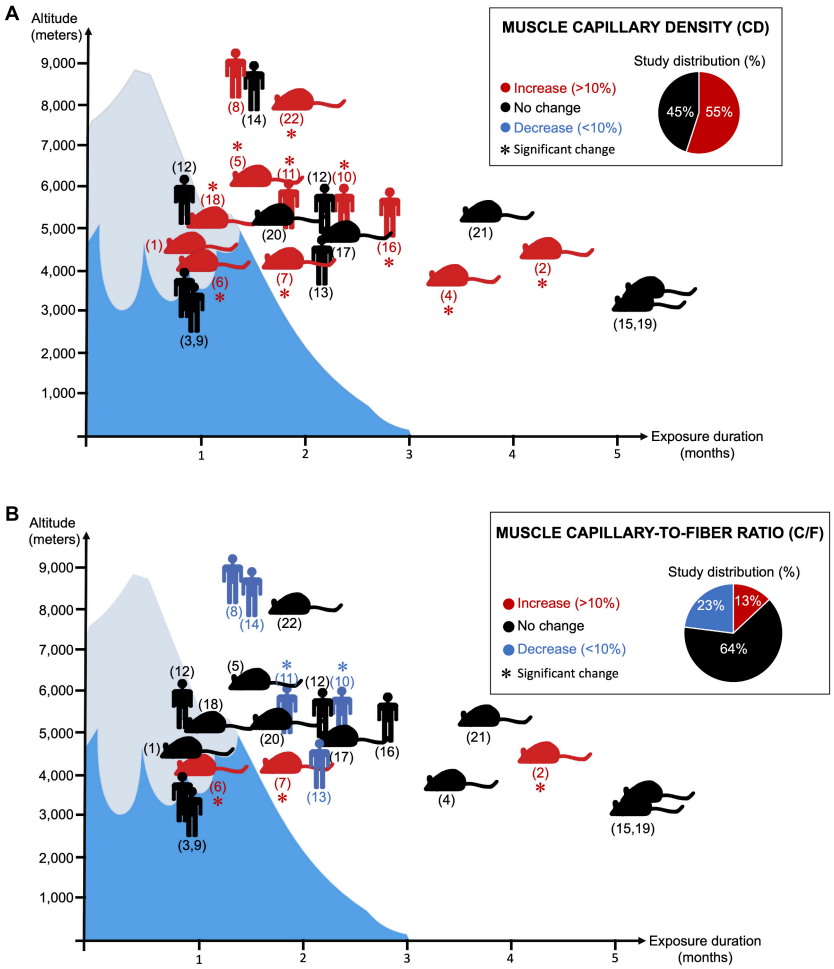

FIGURE 2 | Twenty-two research articles analyzing changes in skeletal muscle capillary density (CD) (Panel **A**) or capillary-to-fiber ratio (C/F) (Panel **B**) in response to prolonged exposure to ambient hypoxia were surveyed. (🧍) indicates human studies. (🐀) indicates the use of animal models (not restricted to rodents). (*) indicates significant finding. Red coloration indicates an increase in magnitude by 10% or greater. Blue coloration indicates a decrease in magnitude by 10% or greater. Black coloration indicates no change. *Y*- and *X*-axis, respectively, reflect the level of altitude (real or simulated) and the duration exposure. Corresponding references: **(1)** Banchero et al. (1976); **(2)** Banchero et al. (1985); **(3)** Basset et al. (2006); **(4)** Bigard et al. (1991); **(5)** Cassin et al. (1971); **(6)** Deveci et al. (2001); **(7)** Deveci et al. (2002); **(8)** Green et al. (1989); **(9)** Green et al. (1992); **(10)** Hoppeler et al. (1990a); **(11)** Hoppeler et al. (1990b); **(12)** Levett et al. (2012); **(13)** Lundby (2004); **(14)** MacDougall et al. (1991); **(15)** Mathieu-Costello and Agey (1997); **(16)** Mizuno et al. (2008); **(17)** Olfert et al. (2001); **(18)** Panisello et al. (2008); **(19)** Poole and Mathieu-Costello (1989); **(20)** Sillau and Banchero (1977); **(21)** Sillau et al. (1980a); **(22)** van Ekeren et al. (1992).

Muscle atrophy could also result from reduced physical activity and muscle deconditioning, particularly during prolonged and passive exposure in hypoxic chambers. For example, when maintaining an isocaloric state and matching physical activity levels, Green et al. (1992) did not observe any atrophy in their study. There is however still a lack of consensus around the contribution of physical activity or the notion of a hypoxic dose on muscle atrophy. This can nicely be illustrated by two studies from Mizuno et al. (2008), Levett et al. (2012). Both compared very active versus less active subjects during similar conditions of prolonged exposure (66-75 days) to high altitude (>5,000 m). Whereas the less active participants remained moderately active at the Everest Base Camp (5,250-5,300 m), active subjects were repeating climbing sessions at higher altitudes. In Levett's study, climbers reached Camp 2 (6,400 m) and some even summited the Mount Everest (8,848 m) (Levett et al., 2012). Mizuno et al. (2008) observed a significant increase in muscle CD (+14%) associated with a reduction in muscle circumferences and myofiber size. Conversely, Levett et al. (2012) did not observe any significant myofiber atrophy or change in CD.

Some authors have also concluded that ambient hypoxia alone might not be sufficient to stimulate skeletal muscle angiogenesis and that a combination of cold and hypoxia stressors might be required (Banchero, 1985; Jackson et al., 1987; Snyder et al., 1992; Hoppeler, 1999). Interestingly, cold *per se* was recently identified as a pro-angiogenic stimulus in rodent skeletal muscle (Sillau et al., 1980b; Egginton, 2002; Deveci and Egginton, 2003).

An increase in muscle CD can also result from the formation of new capillaries via the process of angiogenesis.

The capillary-to-fiber ratio (C/F) is often used to assess capillary formation. Some earlier studies in species adapted to high altitude, including deer mice, gooses, finches or pigeons have reported increased C/F values compared to sea level animals (Mathieu-Costello and Agey, 1997; Hepple et al., 1998; Hepple, 2000; Lui et al., 2015; Scott et al., 2015). Yet, as previously mentioned, a general interpretation is that the increase in CD in response prolonged exposure to hypoxia results from tissue remodeling and myofiber atrophy without any angiogenesis. Methodological artifacts in tissue preparation for histology procedures, such as variation in the sarcomere length, were pointed out (Sillau and Banchero, 1977; Sillau et al., 1980b; Banchero, 1985; Banchero et al., 1985; Hoppeler and Kayar, 1988; Poole and Mathieu-Costello, 1989; Hoppeler et al., 1990a,b; Hudlicka et al., 1992; Mathieu-Costello, 1994). The impact of prolonged hypoxia on muscle C/F was however, revisited by Deveci et al. (2001, 2002) in rats passively exposed to 3 or 6 weeks of hypoxia (F_iO_2 0.12, equivalent to 4,400 m). The effect of hypoxia on C/F could vary accordingly to muscle and fiber types as well as exposure duration. After 3 weeks of conditioning, CD and C/F were increased in the diaphragm and soleus muscles without any significant myofiber atrophy, suggesting a true angiogenic response to ambient hypoxia. No indication of angiogenesis was observed in the tibialis anterior and extensor digitorum longus muscles (Deveci et al., 2001). At 6 weeks, an increase in C/F was observed all muscles (Deveci et al., 2002). Interestingly, the analysis of different areas from each muscle also suggested that angiogenesis might occurs predominantly around larger and glycolytic myofibers (Deveci et al., 2001, 2002).

When re-analyzing our 22 original research studies that measured the C/F ratio in animal and human skeletal muscles in response to prolonged exposure to hypoxia, we found that about 64% mentioned no change and only 13% reported a significant increase or a trend for it (**Figure 2B**). Interestingly, 23% indicated a significant or a trend for a decrease in C/F (Green et al., 1989; Hoppeler et al., 1990a,b; MacDougall et al., 1991; Lundby, 2004). For example, in the study from Hoppeler et al., 1990a,b, the significant decrease in C/F (-10%) is of the same magnitude as the increase in CD (+11%).

Based on the current literature, the understanding of the effect of prolonged exposure to hypoxia on skeletal muscle capillarization is still unclear and difficult to generalize. Responses can vary between species, muscles, and fiber types, and can be different when combining different stressors such as cold or physical activity. The idea of CD increasing because of myofiber atrophy can be seen as an interesting and economic way to improve the capillary-to-myofiber interface without the need of angiogenesis. However, having smaller myofibers might represent a disadvantage when it comes to muscle performance.

Divergent from passive exposure to ambient hypoxia, the utilization of hypoxia in conjunction with exercise has gained popularity as a possible training avenue for improving sea-level exercise performance (Levine, 2002; Levine and Stray-Gundersen, 2006; Millet et al., 2010; Lundby et al., 2012; Chapman, 2013; Girard and Chalabi, 2013; Girard et al., 2013, 2020; Brocherie et al., 2018). As discussed earlier, exercise can generate a local hypoxic stress in the skeletal muscle tissue. It is therefore appealing to consider how the combination of ambient hypoxia and exercise training would affect muscle capillarization.

Several rodent and human studies suggest that skeletal muscle capillarization might improve to a larger extent when exercise training is performed under ambient hypoxia compared to sea level conditions (Terrados et al., 1988; Bigard et al., 1991; van Ekeren et al., 1992; Desplanches et al., 1993, 1996; Olfert et al., 2001; Vogt et al., 2001). To assess the impact of ambient hypoxia on the angiogenic effect of training, some authors trained their animals or subjects at the same relative intensity, for example at a similar percentage of VO_2 max determined under hypoxia and normoxia, thus expecting similar endurance times. Conversely, some authors utilized training protocols with the same absolute intensity, which then can represent a higher stimulus in hypoxic conditions.

Bigard et al. (1991) observed a greater increase in C/F ratio in the plantaris, extensor digitorum longus and soleus muscles of rats housed and trained in a hypobaric chamber at an altitude equivalent to 4,000 m compared to animals trained under normoxic conditions (Bigard et al., 1991). Yet, in the same study C/F values were not different when comparing sedentary normoxic and hypoxic animals, suggesting that hypoxia alone is insufficient to promote muscle angiogenesis. Similarly, Olfert et al. (2001) trained rats for 8 weeks under ambient hypoxia (FiO_2 0.12) or normoxia. The C/F ratio was only increased in muscles from animals trained under hypoxia (Olfert et al., 2001).

Interestingly, Vogt et al. (2001) have also evaluated the effect of exercise intensity during hypoxia training in human subjects. They reported a significant increase in capillarization only in the vastus lateralis muscles of subjects trained at high intensity for 12 weeks under hypoxia (simulated altitude of 3,850 m). Training under normoxia, even at high intensity, did not improve significantly muscle capillarization.

Overall, these laboratory and well-controlled studies indicate a greater angio-adaptive response of the skeletal muscle tissue when exercise training is performed in ambient hypoxia. This reflects into a higher level of muscle capillarization. Yet, it remains unknow whether muscle angio-adaptation occurs faster.

Field studies in humans for prolonged sojourn to altitude focusing on exercise and muscle angiogenic activity have provided mixed results. Mizuno et al. (1990) reported a significant increase in the triceps C/F of competitive cross-country skiers after 2 weeks of training at an altitude of 2,700 m (Mizuno et al., 1990). However, because of the absence of proper control groups (sedentary and trained subjects in normoxia and hypoxia) it cannot really be discerned that these results were simply a response to exercise training. No change in C/F ratio was described in the rectus femoris or biceps brachii of climbers regularly engaged into intense climbing, walking, carrying activities at altitudes above 5,250 m (Mizuno et al., 2008; Levett et al., 2012).

Finally, the use of exercise training in hypoxia for improving certain health outcomes (biomechanical limitations, obesity, hypertension, aging) is also gaining a high interest (Burtscher et al., 2004; Wiesner et al., 2010; Verges et al., 2015; Millet et al., 2016; Kong et al., 2017; Pramsohler et al., 2017; Camacho-Cardenosa et al., 2018, 2019, 2020; Ramos-Campo et al., 2019;

Jung et al., 2021). In regard to skeletal muscle blood flow, capillarization and angio-adaptation, we and others have shown in rodent models and human patients that exercise interventions could represent a powerful therapeutic avenue to prevent, delay or improve alterations in chronic conditions such as peripheral limb ischemia, diabetes, obesity, chronic obstructive pulmonary diseases (Armstrong et al., 1986; Hudlicka et al., 1994; Gardner and Poehlman, 1995; McDermott et al., 2009; Roudier et al., 2009; Gouzi et al., 2013; Amouzou et al., 2016; Aiken et al., 2019). Yet, to the best of our knowledge the impact of hypoxia training as a therapeutic or preventive approach specifically for conditions affecting muscle capillarization and angio-adaptive activity remains to be investigated.

MUSCLE CAPILLARIZATION, EXERCISE TRAINING, AND AMBIENT HYPOXIA: KEY POINTS

- Prolonged exposure to hypoxia can improve skeletal muscle capillary density likely because of myofiber atrophy. Yet the implication of a true angiogenic response cannot be ruled out.
- Combining training and ambient hypoxia can result in a higher muscle angiogenic response than training alone. Yet whether it could also result in an earlier response remains unknown.
- The existing literature obviously shows non-negligible discrepancy imputable sometimes to a lack of proper controls in early studies and to uncontrolled confounding factors such as activity level and training status, cold exposure, restriction in calory intake, animal growth.

SKELETAL MUSCLE CAPILLARIZATION IN HIGH-ALTITUDE NATIVE POPULATIONS

As mentioned previously, a classical idea is that prolonged exposure of lowlanders to high altitude could result in increased skeletal muscle CD often attributed to myofiber atrophy. Could this represent a phenotypic transition towards highlanders' muscles (Gilbert-Kawai et al., 2014)?

Interestingly, CD measured in highlanders' muscles do not seem to differ much from lowlanders. Kayser et al. (1991) have compared the CD from Tibetan Sherpas' muscles with average CD values from sedentary lowlanders or active climbers before and after a high altitude expedition (Kayser et al., 1991). The average CD was significantly higher (+20%) in Sherpas' muscles than in sedentary lowlanders' muscles. However, the training status of the subjects was not taken into consideration. In fact, there was no difference between Sherpas and active climbers before expedition, and a trend for a lower CD (-13%) was even observed in Sherpas' muscles when compared with post-expedition lowlander climbers' muscles (Kayser et al., 1991). It is also important to note that the CD values measured in Sherpas' muscles in this study (Kayser et al., 1991) were compared with CD obtained from different studies (Hoppeler et al., 1990a,b; Oelz et al., 1986). In another study, Kayser et al. (1996) have compared muscles from second-generation Tibetans living at low altitude with Nepalese controls: No difference was observed for muscle CD despite smaller fibers in Tibetans (Kayser et al., 1996).

In an interesting study, Lundby (2004) have compared muscle CD from Aymara subjects, who live permanently around 3,800-4,100 m altitude in Bolivia, with CD values from lowlanders' muscles before and an 8-weeks sojourn at 4,100 m (Lundby, 2004). Aymara muscle CD was 12% (trend) and 15% (significant) lower than lowlanders' CD, respectively, measured before and after the altitude sojourn. This apparent lower CD is intriguing given that high-altitude natives had 25-30% smaller myofibers. The C/F ratio was in fact significantly 40% lower in Aymara's muscles compared to lowlanders.

Altogether, these results suggest that skeletal muscles from high-altitude residents, although presenting smaller fibers, do not have a higher CD than lowlanders' muscles. The observation of a much lower C/F ratio in high-altitude natives reminds the few studies reporting a tendency for a decreased C/F in lowlanders following a prolonged exposure to hypoxia (Green et al., 1989; Hoppeler et al., 1990a,b; MacDougall et al., 1991; Lundby, 2004). It is then tempting to conclude this section by questioning whether a long-term angio-adaptation of the skeletal muscle to hypoxia could in fact result in some capillary regression. Angio-adaptation ensures an optimal match between the muscle capillarization and the metabolic needs of myofibers. If prolonged hypoxia results in an increased CD and smaller myofibers with decreased mitochondria volume density (Horscroft and Murray, 2014; Favier et al., 2015; Murray and Horscroft, 2016; Horscroft et al., 2017), some existing capillaries might at some point become unnecessary.

MOLECULAR ASPECT OF SKELETAL MUSCLE ANGIO-ADAPTIVE RESPONSES TO HYPOXIA

The skeletal muscle capillary network possesses a remarkable plasticity and whether capillaries regress, stabilize or grow in response to a stimulus is largely determined by an intricate balance of pro- and anti-angiogenic molecules (Hoppeler, 1999; Breen et al., 2008; Olfert and Birot, 2011; Egginton and Birot, 2014; Olfert et al., 2015). Among the plethora of angio-adaptive molecules described in the literature, the use of transgenic animal models identified two of them as key regulators of skeletal muscle angio-adaptation: The pro-angiogenic Vascular Endothelial Growth Factor-A (VEGF-A) (Tang et al., 2004; Olfert et al., 2009, 2010; Baum et al., 2017); and the anti-angiogenic thrombospondin-1 (THBS-1) (Malek and Olfert, 2009; Slopack et al., 2014). Different methodological approaches have been used, some targeting VEGF-A in the whole muscle tissue, some targeting it specifically in myofibers (Tang et al., 2004; Breen et al., 2008; Malek and Olfert, 2009; Olfert et al., 2010; Baum et al., 2017). VEGF-A deletion results in: (1) decreased basal level of muscle capillarization, (2) blunted exercise-induced angiogenic response, (3) severely reduced exercise capacity.

Targeting the anti-angiogenic THBS-1 has somehow opposite consequences: Better vascularized muscles and greater exercise capacity (Breen et al., 2008; Malek and Olfert, 2009; Olfert et al., 2009, 2015). In the context of the present review on skeletal muscle angio-adaptive responses to hypoxia, it is therefore important to recapitulate the response of VEGF-A and THBS-1 to exercise and ambient altitude (**Figure 3**).

Most of studies in exercise and altitude physiology have essentially focused on measuring VEGF-A mRNA and protein expression levels. However, how hypoxia can influence VEGF-A expression and activity is in fact very complex (Semenza, 2001; Breen et al., 2008). The *VEGFA* gene can be considered as a hypoxia-sensitive gene since it possesses several Hypoxia Responsive Elements (HRE) recognized by the transcription factor HIF-1. Under hypoxic conditions, the stabilization of HIF-1α and HIF-1 enhanced transcriptional activity lead to increased VEGF-A mRNA levels (Hoppeler et al., 2003). VEGF-A mRNA can then be stabilized via interaction between its 3' untranslated region and the Human antigen R (HuR protein) (Amadio et al., 2008; Morfoisse et al., 2015; Osera et al., 2015). Interestingly, Tang et al. (2002) have described such interaction between HuR and VEGF-A mRNA in rat ischemic gastrocnemius muscles (Tang et al., 2002). VEGF-A mRNA translation into proteins can also be affected by hypoxia. Indeed, whereas the

FIGURE 3 | Skeletal muscle angio-adaptation is a complex and dynamic process in which much earlier molecular and cellular events lead to eventual changes observed at the tissular level through modifications in the skeletal muscle's capillarization. In response to a given stimulus, whether it be the growth, regression, or stabilization of skeletal muscle capillaries, the process is dictated by an intricate balance of pro- and anti-angiogenic factors. Among the plethora of angio-adaptive factors two have been identified as central in the regulation of skeletal muscle capillarization: the pro-angiogenic VEGF-A; and the anti-angiogenic THBS1. Majority of current literature concerning hypoxic muscle angio-adaptation has focused on the regulation of VEGF-A and as such there are current gaps in knowledge concerning the hypoxic regulation of THBS1. Additionally, there is a lack of consensus regarding the impact of ambient hypoxia eliciting changes at the tissular level concerning CD and C/F.

classical mechanism of mRNA cap-dependent translation can be inhibited under hypoxia, some proteins such as VEGF-A can still be translated by using an alternative translational mechanism where cap-independent translation is initiated via internal ribosome entry sites (IRESs) (Huez et al., 1998; Miller et al., 1998; Stein et al., 1998). The endoplasmic reticulum chaperone Oxygen Regulated Protein-150 (ORP-150) can finally facilitate VEGF-A protein secretion, and an increase in ORP-150 has been reported in rat plantaris muscles following an acute bout of running exercise (Ozawa et al., 2001a,b; Birot et al., 2003). Finally, hypoxia can also stimulate the expression of VEGF-A receptors (Gerber et al., 1997).

A stimulatory effect of exercise on VEGF-A expression has been well described in human and rodent studies. Both mRNA and protein levels are transiently increased after one bout of exercise (Breen et al., 1996, 2008; Gustafsson and Kraus, 2001; Hoppeler and Vogt, 2001; Gustafsson et al., 2002, 2005; Birot et al., 2003; Ameln et al., 2005; Croley et al., 2005; Gavin et al., 2006, 2007; Roudier et al., 2012; Aiken et al., 2016). A study from Breen et al. (1996) has shown that increases in mRNA in rat skeletal muscle were higher and lasted longer when exercise was performed at a higher intensity (Breen et al., 1996). An attenuation of VEGF-A mRNA responsiveness to exercise stimulus has be observed after short-term (few days) training (Gavin and Wagner, 2001; Olfert et al., 2001; Vogt et al., 2001; Kraus et al., 2004).

The effect of acute or chronic exposure to ambient hypoxia itself on VEGF-A expression are less conclusive. Most of the studies that have assessed VEGF-A expression in the muscle tissue have measured mRNA levels only, whereas the protein was essentially quantified in the plasma or serum by ELISA (Asano et al., 1998; Gunga et al., 1999, 2003; Schobersberger et al., 2000; Hanaoka et al., 2003; Oltmanns et al., 2006; Bahtiyar et al., 2007; Dorward et al., 2007; Ding et al., 2012; Morici et al., 2013; Brinkmann et al., 2017; Boos et al., 2018; Kasai et al., 2019; Kasperska and Zembron-Lacny, 2020). Results from the literature remain largely inconsistent, reporting some increase in mRNA or protein (Breen et al., 1996; Gavin et al., 2006), some attenuation (Olfert et al., 2001; Oltmanns et al., 2006; Morici et al., 2013; Boos et al., 2018), or no change (Gunga et al., 2003; Lundby, 2004; Bahtiyar et al., 2007). Additionally, circulating VEGF-A levels measured by ELISA do not necessarily reflect skeletal muscle VEGF-A production.

Finally, some studies have analyzed VEGF-A responsiveness when combining exercise and ambient hypoxia (Breen et al., 1996; Asano et al., 1998; Olfert et al., 2001; Vogt et al., 2001; Gunga et al., 2003; Zoll et al., 2006; Nagahisa et al., 2016; Brocherie et al., 2018; Kasperska and Zembron-Lacny, 2020). Breen et al. (1996) have shown that VEGF-A mRNA expression in rat skeletal muscle was higher and last longer when exercise was performed in ambient hypoxia compared to a normoxic environment (Breen et al., 1996). Brocherie et al. (2018) have compared the effect of different modalities of exercise training combined with hypoxia exposure (Brocherie et al., 2018). VEGF-A mRNA levels were only increased in human skeletal muscle in their model of Live High-Train Low and High (LHTLH) that combined passive exposure to ambient hypoxia and exercise training session at maximal intensity under hypoxia, and no change in VEGF-A was observed in the other two models (Live High-Train Low, LHTL, and Live Low-Train Low, LLTL).This study is in line with previous results reported by Vogt et al. (2001) who analyzed VEGF-A mRNA expression in response to a training program realized either at high or low intensity and either in normoxia or hypoxia (6 weeks at an equivalent of 3,850 m) (Vogt et al., 2001). Whereas training at high intensity in normoxia and training at low intensity in hypoxia only led to a trend for an increase in VEGF-A mRNA (respectively, +13 and +17%), only the combination of training at high intensity in hypoxia resulted in significant increase (+72%). These studies support the idea that combining exercise and ambient hypoxia could exacerbate the angio-adaptive response of the skeletal muscle tissue. If the duration, nature and intensity of the training program are key parameters, all these studies also point out the importance of the duration and intensity of hypoxia exposure. This has recently led to the notion of hypoxic dose (Lundby et al., 2009, 2012; D'Hulst and Deldicque, 2017; Millet et al., 2017; Girard et al., 2020).

Thrombospondin-1 has been identified as a potent anti-angiogenic factor in the skeletal muscle tissue (Malek and Olfert, 2009; Hellsten and Hoier, 2014). Similar to VEGF-A, one bout of exercise stimulates the expression of THBS1mRNA and protein in skeletal muscle (Olfert et al., 2006; Malek and Olfert, 2009; Slopack et al., 2014) whereas short-term training is accompanied by a progressive loss of responsiveness of THBS1 to exercise stimulus (Olfert et al., 2006; Slopack et al., 2014; Hoier et al., 2020; **Figure 3**). This responsiveness, however, appears to be restored with long-term training (Olfert et al., 2006; Hoier et al., 2012; Slopack et al., 2014). The decreased responsiveness of THBS1 during training could in fact contribute to shifting the skeletal muscle angio-adaptive balance towards its pro-angiogenic side, reflected at the tissue level by a pro-angiogenic microenvironment prone to capillary formation. This has led to the hypothesis that exercise-induced angiogenesis might in fact be more controlled by a decrease in anti-angiogenic factors rather than an increase in pro-angiogenic ones (Olfert and Birot, 2011; Hellsten and Hoier, 2014; Olfert et al., 2015; Olfert, 2016). This could apply to capillary regression, as during detraining or muscle hypokinesia, with an increase expression of anti-angiogenic factors shifting the angio-adaptive balance the opposite way (Roudier et al., 2009, 2010; Kishlyansky et al., 2010; Olfert and Birot, 2011; Olenich et al., 2014).

Long-term training does not seem to alter the basal expression level of THBS1 in rodent and human healthy skeletal muscles (Olfert et al., 2006; Hoier et al., 2012; Gliemann et al., 2015) although Yoshioka et al. (2003) have reported higher THBS1 gene expression in skeletal muscles from endurance athletes compared to sedentary subjects (Yoshioka et al., 2003). Interestingly, Gouzi et al. (2013) have shown that muscle THBS1 protein levels could be reduced in response to prolonged training in patients with chronic obstructive pulmonary disease (Gouzi et al., 2013). This reinforces the idea of using exercise training as a therapeutic avenue for clinical conditions with skeletal muscle microvascular alterations. THBS1 expression was indeed found to be increased in rodent skeletal muscles in the context of diabetes, pre-diabetes

and hindlimb ischemia (Kivelä et al., 2006, 2008; Roudier et al., 2013; Dunford et al., 2017; Aiken et al., 2019).

The effect of hypoxia alone on THBS1 expression has provided mixed results essentially from *in vitro* experiments. Phelan et al. (1998) have described an increased expression of THBS1 mRNA and protein in endothelial cells exposed to severe hypoxia (1%O_2) (Phelan et al., 1998). Conversely, Yadav et al. (2014) have observed a decrease in THBS1 expression in differentiated murine C2C12 myotubes in response to hypoxia (Yadav et al., 2014). Finally, THBS1 expression was found to be increased both in rodent and human ischemic skeletal muscles (Roudier et al., 2013).

To our knowledge, there is very limited data regarding THBS1 expression in skeletal muscle when combining exercise stimulus and ambient hypoxia. Olfert et al. (2006) have analyzed THBS1 mRNA expression in skeletal muscles from rats kept sedentary or enrolled into a 8-weeks endurance running program (Olfert et al., 2006). At the end of the training program, animals were performing one bout of intense running exercise. Data suggests that endurance training did not alter the basal expression level of THBS1. However, chronic hypoxia exposure resulted in lower basal expression level (–44%) as well as THBS-1 expression in response to one bout of exercise (–48%).

As a conclusion to this section, it is important to keep in mind that VEGF-A and THBS1 are only two members of the large family of angio-adaptive molecules susceptible to influence the skeletal muscle angio-adaptive responses to exercise and ambient hypoxia. The expression levels of some other angio-adaptive molecules have been measured in skeletal muscle tissue in the context of exposure to ambient hypoxia, such as basic Fibroblast Growth Factor (bFGF), Transforming Growth Factor-β (TGF-β), VEGF receptors, leptin (Breen et al., 1996; Olfert et al., 2001; Patitucci et al., 2009; Morici et al., 2013). However, these measurements remain very anectodical, and more research in this area is needed.

Here, we have also essentially reviewed VEGF-A and THBS1 expression levels, which do not reflect the functionality of these molecules, their interaction between each other's and their receptors.

As illustrated in **Figure 3**, the angio-adaptive response to a given stimulus is a complex and dynamic process that involves molecular, cellular, and tissular responses. There is for example a lack of knowledge regarding cross-talks between muscle cells. For instance, how angio-adaptive signals originating from a contractile myofiber will stimulate neighboring endothelial cells to proliferate and migrate to form new capillaries.

Ideally, evaluating the muscle angio-adaptive response to hypoxia should be integrative. For example, an absence of significant change in capillarization does not rule out any angio-adaptive responses at a cellular or molecular level. Finally, the interpretation of results from the literature is very complex with a certain discrepancy between studies. Such divergence can obviously have several origins: Animal versus human studies; healthy versus pathological conditions; training status; exercise protocols; hypoxia level and duration; confounding environmental stressors (cold, air pollution); time of sample collection; methodology (northern blotting, qPCR, western blotting, ELISA, histochemistry).

THE SKELETAL MUSCLE AS AN ENDOCRINE ORGAN AND THE ROLE OF ANGIO-ADAPTIVE MYOKINES

As previously mentioned, many studies studying angiogenic responses to hypoxia and exercise have quantified circulating VEGF-A protein levels by ELISA. Several studies have aimed to link changes in circulating VEGF-A levels with the susceptibility to develop acute or chronic mountain sickness (Tissot van Patot et al., 2005; Dorward et al., 2007; Nilles et al., 2009; Schommer et al., 2011; Espinoza et al., 2014). Regarding THBS1, this anti-angiogenic molecule seems to be involved in the pathophysiology of hypoxia-induced pulmonary hypertension and right ventricular hypertrophy (Ochoa et al., 2010; Bauer et al., 2012; Rogers et al., 2017). Kaiser et al. (2016) have reported elevated serum THBS1 levels and strong correlations of serum THBS1 to mean pulmonary artery pressure and pulmonary vascular resistance in patients suffering from pulmonary hypertension (Kaiser et al., 2016).

Interestingly, many circulating angio-adaptive molecules, either pro- or anti-angiogenic, could therefore represent valuable biomarkers to evaluate for example the response of athletes to a specific training program in hypoxia or the impact of a therapeutic exercise intervention in a group of patients. More research in identifying the patho-physiological relevance of circulating angio-adaptive biomarkers would be exciting and essential.

The quantification of circulating angio-adaptive molecules also points out the role of the skeletal muscle as an endocrine organ secreting myokines to act on distant organs such as bones, brain, fat, and liver (Fabel et al., 2003; Schnyder and Handschin, 2015; Delezie and Handschin, 2018; Kim et al., 2019; Gomarasca et al., 2020). Fabel et al. (2003) have demonstrated that peripherally produced VEGF-A seems necessary for running-induced improvements in hippocampal neurogenesis (Fabel et al., 2003). Rich et al. (2017) confirmed that VEGF-A produced by skeletal myofibers plays an important role in hippocampal neurogenesis (Rich et al., 2017). Interestingly, it was also suggested that VEGF-A meditated neurogenesis could provide a neuroprotective effect and could be essential for attenuating decrements to cognitive function experienced with ambient hypoxia during high altitude exposure (Koester-Hegmann et al., 2019).

MOLECULAR ASPECT OF SKELETAL MUSCLE ANGIO-ADAPTIVE RESPONSES TO HYPOXIA: KEY POINTS

- Skeletal muscle angio-adaptation is a complex and dynamic process combining molecular and cellular responses that will ultimately alter muscle capillarization.

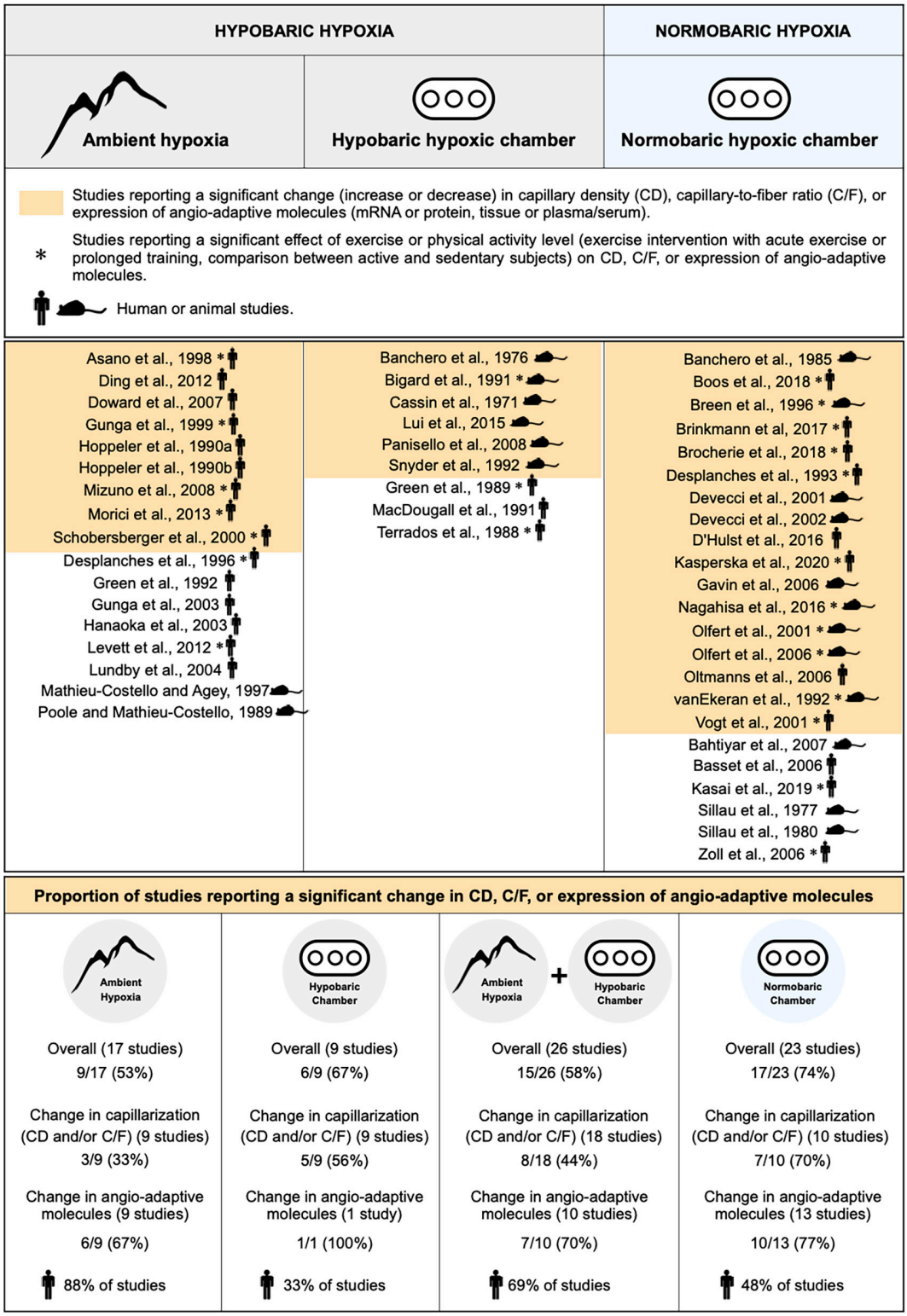

FIGURE 4 | Characterization of the impact of hypobaric and normobaric hypoxia on skeletal muscle angio-adaptive responses. Original research studies cited in the review and analyzing capillary density (CD), capillary-to-fiber ratio (C/F), and expression levels of angio-adaptive molecules in skeletal muscle tissue in response to ambient hypoxia exposure were characterized based on the nature of their hypoxic environment: Hypobaric or normobaric. Studies reporting significant changes in capillarization (CD or C/F), in molecular expression, or in both ("Overall") are highlighted in color. Studies involving a physical activity component are identified by an asterisk. The mouse and human silhouette symbols distinguish studies conducted in animal models or human subjects.

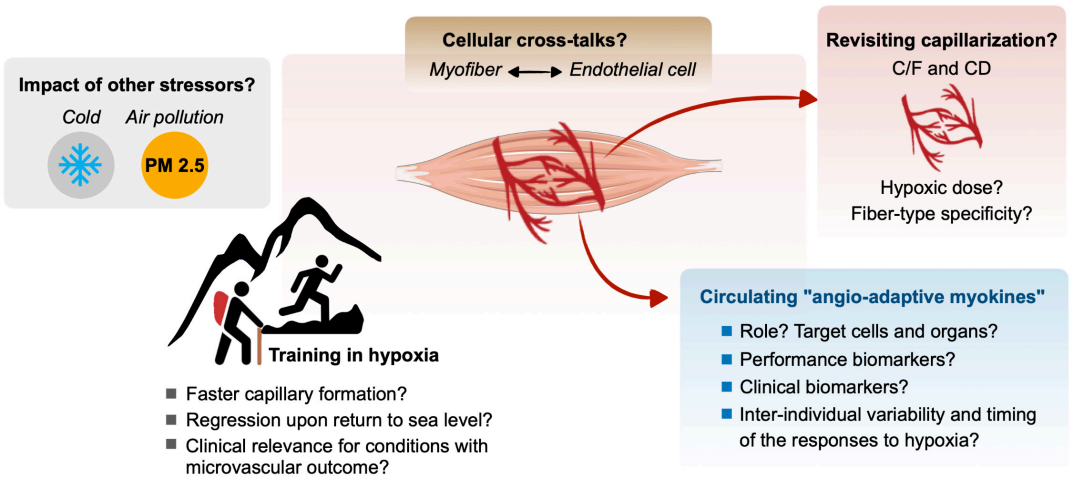

FIGURE 5 | Gap of knowledge and future research directions for a better understanding of the molecular, cellular and tissular angio-adaptive responses of the skeletal muscle tissue to hypoxia, particularly in the context of ambient hypoxia and exercise-induced local hypoxia. Refer to the different text sections for details.

- Several angio-adaptive molecules have been described in the skeletal muscle tissue in the context of exercise-induced angio-adaptation. Yet, their characterization in the context of exposure to ambient hypoxia remains largely unknown.
- There is a current lack of consensus in the literature largely due to confounding experimental variables regarding the expression of muscle VEGF-A and circulating VEGF-A in response to ambient hypoxia alone and to training in hypoxia.
- Anti-angiogenic molecules, such as THBS1, could in fact impact skeletal muscle capillarization to a greater extent than VEGF-A. Their contribution in skeletal muscle angio-adaptive responses to exercise and hypoxia is however, understudied.
- The patho-physiological relevance of circulating angio-adaptive molecules as biomarkers remains poorly documented.
- The role of the skeletal muscle tissue as an endocrine organ secreting angio-adaptive myokines to act on distant organs in the context of exercise and hypoxia is an exciting research avenue.

SKELETAL MUSCLE ANGIO-ADAPTIVE RESPONSES TO HYPOXIA: DO NORMOBARIC AND HYPOBARIC CONDITIONS DIFFER?

The research studies discussed in our review were conducted in three types of environments: Field experiments with real altitude exposure versus hypoxic chambers simulating altitude. Chambers where the barometric pressure and partial oxygen pressure are decreased represent a hypobaric environment, closer to the physics of real altitude, whereas normobaric chambers generate a hypoxic environment by decreasing the fraction of oxygen in the inspired air. Whether normobaric and hypobaric hypoxia are equivalent and then interchangeable has been an intense source of scientific debate, particularly with regards to their impact on exercise performance and various physiological parameters. Systematic reviews and "points-counterpoints" discussions have however, not enable researchers to reach any consensus (Girard et al., 2012; Millet et al., 2012a,b, 2013; Mounier and Brugniaux, 2012a,b; Faiss et al., 2013; Debevec and Millet, 2014; Richard et al., 2014; Coppel et al., 2015; DiPasquale et al., 2016; Richalet, 2020a,b). Discrepancy between studies can be attributable to many cofounding factors: Different degrees of hypoxia, themselves determined either by barometric pressure, inspired PO_2, oxygen fraction; seasonal and geographical differences in barometric pressure; air temperature and humidity; additional environmental stressors such as cold exposure; duration of exposure; presence or not of exercise interventions, and if so different exercise protocols; characteristics of subjects; animal versus human studies.

To the best of our knowledge, whether normobaric and hypobaric hypoxia could differently affect the angio-adaptive responses of the skeletal muscle has never been investigated. We have therefore revisited the original research studies cited in our review that analyzed CD, C/F, or the expression levels of angio-adaptive molecules (mRNA or protein levels, tissue or circulating levels) in response to ambient hypoxia exposure. This represents 49 original studies (**Figure 4**). We separated them accordingly to the hypoxic environment used: Field studies (hypobaric), hypobaric chambers, combined field and hypobaric chambers (both hypobaric environments), and normobaric chambers. Studies involving physical activity (comparing active versus less active subjects, including one bout of exercise or prolonged training) were considered only if they possessed all required

control groups enabling the evaluation of the impact of ambient hypoxia *per se*. We also distinguished studies conducted in animal models or human subjects. Finally, we determined in each category the percentage of studies reporting significant changes (increase or decrease) in capillarization (CD and/or C/F) and expression of angio-adaptive molecules.

Unfortunately, the information presented in **Figure 4** does not really help in answering the question on whether normobaric and hypobaric hypoxia could differently affect the angio-adaptive responses of the skeletal muscle. Indeed, when considering only the studies reporting significant changes in capillarization, a distinction could be made between those conducted in normobaric chambers (70% presenting significant changes) versus studies run in a hypobaric environment (33% for field studies, 56% for hypobaric chambers, and 44% for combined hypobaric environments). However, no such distinction could be made when considering only the studies reporting significant changes in angio-adaptive molecules (67% for field studies, 70% for combined hypobaric environments, and 77% for studies conducted in normobaric chambers). Finally, when looking at "overall" changes (capillarization and angio-adaptive molecules), it seems that a larger proportion of studies conducted in hypoxic chambers, whether normobaric or hypobaric, report significant changes (respectively, 74% and 67%) compared to field studies (53%). As mentioned earlier, a possible explanation could be that field studies often present cofounding factors. Another interesting observation from **Figure 4** is that these conditionings in hypoxic chambers were mainly performed in animal models whereas field studies were essentially involving human subjects (respectively, 33% and 48% of human studies involving hypobaric and normobaric chambers versus 88% for field studies). Similar durations of exposure to hypoxia will obviously not represent the same proportion of a lifespan between rodents and humans.

Based on this analysis, we do not believe that any strong consensus can be established regarding the impact of hypobaric versus normobaric hypoxia on skeletal muscle angio-adaptive responses.

CONCLUSION

Hypoxia, defined as a reduction of oxygen availability can occur in the skeletal muscle tissue of an individual exposed to ambient hypoxia as well as during physical exercise if the oxygen supply to contracting myofibers cannot match their increased metabolic needs. The superimposition of these two stressors, ambient hypoxia exposure and exercise-induced local hypoxia, can lead to an exacerbation of the hypoxic stress experienced by the skeletal muscle. The capillary-to-myofiber interface serves as the site for the exchange of oxygen, nutrients, metabolic heat, and waste between the blood and myofibers. As such, the capillary microvasculature is tightly related to the functional capacity of the skeletal muscle. The capillary microvasculature is a highly adaptive tissue with remarkably plasticity that can grow or regress to various physiological, pathological, and environmental stressors, a process named angio-adaptation. Skeletal muscle angio-adaptation involves complex and dynamic molecular and cellular responses. Given the relevance of skeletal muscle angio-adaptation in response to hypoxia to mountaineers, athletes, and clinical populations, this review aimed to delineate the existing literature and identify current gaps in the knowledge of this field of environmental and exercise physiology (**Figure 5**).

AUTHOR CONTRIBUTIONS

Both authors PL and OB have equally contributed to the design and writing of the manuscript and its figures.

FUNDING

This research was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants Program, grant RGPIN-2017-05675 to OB. PL was supported by the Alexander Graham Bell Canada Graduate Scholarship-Master (CGS-M) from the Natural Sciences and Engineering Research Council of Canada (NSERC).

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frontiers in Medicine

ORIGINAL RESEARCH
published: 26 November 2021
doi: 10.3389/fmed.2021.759273

Elevated Serum Tenascin-C Predicts Mortality in Critically Ill Patients With Multiple Organ Dysfunction

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Background: Multiple organ dysfunction is a complex and lethal clinical feature with heterogeneous causes and is usually characterized by tissue injury of multiple organs. Tenascin-C (TNC) is a matricellular protein that is rarely expressed in most of the adult tissues, but re-induced following injury. This study aimed to evaluate serum TNC in predicting mortality in critically ill patients with multiple organ dysfunction.

Methods: Adult critically ill patients with at least two organs dysfunction and an increase of Sequential Organ Failure Assess (SOFA) score ≥ 2 points within 7 days were prospectively enrolled into two independent cohorts. The emergency (derivation) cohort was a consecutive series and the patients were from Emergency Department. The inpatient (validation) cohort was a convenience series and the patients were from medical wards. Their serum samples at the first 24 h after enrollment were collected and subjected to TNC measurement using ELISA. The association between serum TNC level and 28-day all-cause mortality was investigated, and then the predictive value of serum TNC was analyzed.

Results: A total of 110 patients with a median age of 64 years (53, 73) were enrolled in the emergency cohort. Compared to the survivors, serum TNC in the non-survivors was significantly higher (467.7 vs. 197.5 ng/ml, $p < 0.001$). Multivariate logistic regression analysis revealed that the association between serum TNC and 28-day mortality was independent of sepsis or critical illness scores such as SOFA, Acute Physiology and Chronic Health Evaluation (APACHE II), and Simplified Acute Physiology Score (SAPS II), respectively ($p < 0.001$ for each). The area under receiver operating characteristic curve of serum TNC for predicting mortality was 0.803 (0.717–0.888) ($p < 0.001$), similar with SOFA 0.808 (0.725–0.891), APACHE II 0.762 (0.667–0.857), and SAPS II 0.779 (0.685–0.872). The optimal cut-off value of serum TNC was 298.2 ng/ml. Kaplan–Meier analysis showed that the survival of patients with serum TNC ≥ 300 ng/ml was significantly worse than that of patients with serum TNC < 300 ng/ml. This result was validated in the inpatient cohort. The sensitivity and specificity of serum TNC ≥ 300 ng/ml for predicting mortality were 74.3 and 74.7% in the emergency cohort, and 63.0 and 70.1% in the inpatient cohort, respectively.

OPEN ACCESS

Edited by:
Anna Maria Piccinini, University of Nottingham, United Kingdom

Reviewed by:
Kyoko Imanaka-Yoshida, Mie University, Japan
Izabela Duda, Medical University of Silesia, Poland

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *16 August 2021*
Accepted: *21 October 2021*
Published: *26 November 2021*

Citation:
Xu Y, Li N, Gao J, Shang D, Zhang M, Mao X, Chen R, Zheng J, Shan Y, Chen M, Xie Q and Hao C-M (2021) Elevated Serum Tenascin-C Predicts Mortality in Critically Ill Patients With Multiple Organ Dysfunction. Front. Med. 8:759273. doi: 10.3389/fmed.2021.759273

Conclusion: Serum TNC was associated with mortality in critically ill patients with multiple organ dysfunction, and would be used as a prognostic tool for predicting mortality in this population.

Keywords: tenascin-C, critically ill patients, multiple organ dysfunction (MODS), mortality, biomarker

INTRODUCTION

Multiple organ dysfunction, defined as more than one organ system deranged, is a complex and lethal clinical feature with highly heterogeneous causes and clinical manifestations. Different diseases, such as sepsis, malignant tumor, cardiovascular/cerebrovascular diseases, and surgery or trauma can lead to multiple organ dysfunction. The most common organs affected are kidneys, lungs, heart, hematologic system, liver, and central nervous system (1). Prognoses of critically ill patients with multiple organ dysfunction are usually poor. Therefore, accurate prediction of outcomes in these patients can guide physicians in their communication and decision making. However, the outcome of these patients at risk has many relative effects including age, gender, the severity of illness, comorbidities, diagnosis, and response to therapy, which makes the prediction of prognosis difficult and inaccurate. During the last three decades, several physiology-based ICU prognostic models have emerged. The main prognostic models for assessing the overall severity of illness in critically ill adults are Acute Physiology and Chronic Health Evaluation (APACHE), Simplified Acute Physiology Score (SAPS), and Mortality Prediction Model (MPM) (2). These models, as well as Sequential Organ Failure Assess (SOFA), which was primarily designed to describe the degree of organ dysfunction in critically ill patients (3), have been found to predict mortality effectively in different clinical conditions (4–6). However, limitations for these scoring systems do exist since they are all obtained by calculating a lot of components, which makes their clinical practice complicated. So, new effective outcome examination and guidance in critically ill patients with multiple organ dysfunction are strongly demanded.

Serum tenascin-C (TNC) has been reported to be significantly increased in critically ill patients and associated with the severity of diseases (7). TNC is a matricellular protein that is widely expressed during embryonic development and absent in most of the adult tissues, but re-induced following injury (8). TNC contains multiple functional domains that primarily regulate the interaction of cells with other extracellular matrix components and growth factors, thereby modulating cellular processes such as cell adhesion, proliferation, survival, migration, and differentiation (8, 9). Previous studies showed that TNC expression can be induced in different organs without disease specificity, such as heart tissue with myocarditis (10, 11), acute myocardial infarction (12), and dilated cardiopathy (10, 13), liver tissue with hepatitis (14), lung tissue with fibrosis (15, 16), and kidney tissue with glomerulonephritis and fibrosis (17, 18). The increased TNC in injured tissues can be released into a circulating system that results in serum TNC elevation. There were several studies, which revealed that serum TNC was associated with the mortality of different critical illnesses, such as sepsis, acute aortic dissection (AD), and myocarditis (7, 19, 20). However, the clinical significance of serum TNC levels in critically ill patients with multiple organ dysfunction remains uncertain.

Based on the previous studies, we hypothesized that serum TNC, representative of the quantity and severity of organ damage, was associated with mortality of critically ill patients with multiple organ dysfunction. In this prospective study, we detected serum TNC in critically ill patients with multiple organ dysfunction from two independent cohorts (emergency cohort and inpatient cohort) and aimed to evaluate the serum TNC. First, we investigated the association between serum TNC and mortality, and then examined the predictive performance of serum TNC for 28-day mortality in the emergency cohort and validated it in the inpatient cohort. We determined the cut-off value using the Youden index and evaluated the dichotomized predictive ability, and further combined the TNC value with critical scores to improve the predictive values. This work provided a strong basis for the adoption of serum TNC as an effective prognostic biomarker in critically ill patients with multiple organ dysfunction.

MATERIALS AND METHODS

Study Design and Participants

The study was a prospective observational cohort with prespecified outcome and procurement of biological specimens. Other than blood draws, there were no study-related interventions, and all clinical care was at the discretion of the clinical teams caring for the study subjects. The study subjects were recruited in two separated cohorts, although they were from a single hospital—Huashan Hospital, Fudan University, which is a large size tertiary comprehensive hospital with more than 1,200 beds in the city of Shanghai, China. The emergency cohort, which was used as a derivation set, was a consecutive series and the subjects were recruited in Emergency Department from January 2018 to December 2019. Different from the emergency cohort, the inpatient cohort was a convenience series and the subjects were recruited in Divisions of Internal Department (medical wards) from January 2015 to December 2016. This cohort was used as a validation set. The study design was presented in **Supplementary Material 1**.

All the study subjects were adults and met the criteria of at least two organs dysfunction and acute organ injury with an increase of SOFA $\geq$ 2 points within 7 days caused by risk factors including infection, malignancy, rheumatic diseases, trauma, cardiovascular events, and others such as diabetic ketoacidosis and pancreatitis. Organ dysfunction was defined by reference

to SOFA, which is composed of scores from six organ systems. Patients with (1) age < 18 years; (2) died within 48 h after acute onset (e.g., trauma, acute myocardial infarction, and stroke); and (3) no information on follow-up outcome were excluded. Sepsis was defined as an acute change in total SOFA score ≥ 2 points consequent to the infection (21). These patients were first to our hospital or transferred from others and followed up prospectively from enrollment to death or 28 days. The primary endpoint was all-cause mortality. This study was approved by the ethics review board at the Huashan Hospital, Fudan University. The informed consent was signed by the patients or their authorized representatives.

Blood Sample and TNC Measurement

Serum specimens in both inpatient and emergency cohorts were obtained within 24 h after study enrollment and stored at −80°C for analysis. TNC in serum was measured in 1:50 dilution using quantitative ELISA kits (IBL, Lot. 27767), according to the instructions of the manufacturer. TNC detected by this ELISA kit is the large molecular weight variant.

Clinical Data

At enrollment, clinical characteristics of the patients including age, gender, comorbidities, and vital signs were recorded by the researchers. Laboratory measurements including blood routine, blood biochemistry, arterial blood gas analysis, coagulation markers, and D-dimer, lactic dehydrogenase (LDH) and C-reactive protein (CRP) were carried out within 24 h after enrollment. Major causes of acute organ dysfunction were determined by two independent physicians. The disease severity was assessed by critical illness scoring systems, including SOFA, APACHE II, and SAPS II. As mentioned above, SOFA was calculated by scores from six organ systems which were graded from 0 to 4 by the degree of dysfunction (3). APACHE II was calculated based on the worst values of 12 physiologic criteria [body temperature, mean arterial blood pressure, heart rate, respiratory rate, oxygenation, arterial PH, hematocrit, white blood cell count, serum levels of sodium, potassium, creatinine, and Glasgow Coma Scale (GCS)] during the first 24 h after enrollment, as well as age and previous health status (22). SAPS II was calculated by 17 variables including 12 physiologic factors (body temperature, mean arterial blood pressure, heart rate, urine output, oxygenation index, white blood cell count, arterial bicarbonate, serum levels of sodium, potassium, urea nitrogen, bilirubin, and GCS), as well as age, type of admission, and three variables regarding underlying diseases (23).

Statistical Analysis

Continuous variables were presented as mean (SD) for normal distribution and median (IQR) for non-normal distribution. Categorical variables were presented as frequencies and percentages. Comparisons between two groups were performed using independent *t*-test or Mann–Whitney *U*-test for continuous variables and Pearson's Chi-square test or Fisher's exact test for categorical variables. The data were analyzed in the following steps. First, the association between serum TNC and 28-day all-cause mortality were analyzed and multivariable logistic regression was used to adjust the potential confounders including age, gender, and severity of the disease. The covariates sepsis, SOFA, APACHE II, or SAPS II, which were clinically associated with the survival were included in the multivariable logistic regression, respectively. Second, the predictive performance of serum TNC for 28-day mortality was evaluated by the area under the receiver operating characteristic (ROC) curve (AUC) in both cohorts. Third, the optimal cut-off value was determined by the Youden index. According to the optimal cut-off value of serum TNC in the emergency (derivation) cohort, the study population was divided into TNC ≥ 300 ng/ml and TNC < 300 ng/ml groups. Then the association between serum TNC and disease severity, as well as all-cause mortality were analyzed. Kaplan–Meier survival curves were drawn to evaluate the difference of mortality between serum TNC ≥ 300 ng/ml and <300 ng/ml groups, and the log-rank test was used for comparison. Finally, according to different serum TNC levels, the sensitivity and specificity were also calculated. These results were obtained from the emergency (derivation) cohort and validated in the inpatient (validation) cohort. All statistical analyses were performed with SPSS 24.0 and Graph-Pad Prism 7.0 with a statistical significance of p-value < 0.05.

RESULTS

Cohort Characteristics

Baseline patient characteristics for the emergency and inpatient cohorts are shown in **Table 1**. A total of 110 critically ill patients with median (IQR) age of 64 years (53, 73) and 67% men were enrolled in the emergency cohort. The organ dysfunction defined as a SOFA score ≥ 1 included 60.0% in coagulation disorder, 50.9% in liver, 46.4% in respiration system, 43.6% in the neurological system, 38.2% in kidney, and 11.8% in cardiovascular systems. Among them, 58 (52.7%) had a history of chronic organ dysfunction. In the patients with chronic organ dysfunction, the predominant causes of acute organ injury were also acute exacerbation of chronic disease (63.8%) and infection (89.7%). In the patients without chronic organ dysfunction, the predominant causes of acute organ injury were also infection (86.5%) and subsequent metabolic diseases (19.2%), malignancy (17.3%), and activity of connective tissue disease (13.5%). Of the 110 patients, 36.4% had acute kidney injury, 55.5% had sepsis, and 16.4% were ventilation dependent at the enrollment, and 31.8% died during the follow-up.

A total of 115 critically ill patients with median (IQR) age of 56 years (38, 66) and 65.2% men were enrolled in the inpatient cohort. Seventy percent were from the Division of Nephrology or had nephrology consultation. The organ dysfunction, with the same inclusion criteria, included 68.7% in kidney, 45.2% in coagulation, 35.7% in respiration, 28.7% in liver, 21.7% in the neurological system, and 13.0% in cardiovascular systems. Among them, 78 (67.8%) had a history of chronic organ dysfunction. In the patients with chronic organ dysfunction, the predominant causes of acute organ injury were acute exacerbation of chronic disease (79.5%) and infection (46.2%). In the patients without chronic organ dysfunction, the predominant causes of acute organ injury were infection (94.6%),

TABLE 1 | Clinical characteristics of patients with multiple organ dysfunction.

	Emergency (derivation) cohort				Inpatient (validation) cohort			
	Total	**Survivor**	**Non-survivor**	***P***	**Total**	**Survivor**	**Non-survivor**	***P***
	***n* = 110**	***n* = 75**	***n* = 35**		***n* = 115**	***n* = 88**	***n* = 27**	
Age (years)	64 (53, 73)	63 (52, 72)	65 (54, 73)	0.672	56 (38, 66)	53 (36, 65)	62 (50, 73)	0.054
Male, *n* (%)	74 (67.3%)	51 (68.0%)	23 (65.7%)	0.813	75 (65.2%)	59 (67.0%)	16 (59.3%)	0.459
Laboratory								
Creatinine (mg/dL)	1.0 (0.6, 1.9)	78.5 (55.5, 143.5)	109.0 (57.0, 272.0)	0.249	3.0 (0.9, 6.2)	271.5 (79.3, 672.5)	231.0 (80.0, 383.0)	0.229
LDH (U/L)	297.0 (222.0, 595.0)	286.0 (209.8, 594.0)	419.0 (245.0, 626.0)	0.283	244.0 (196.0, 363.0)	237.0 (186.0, 360.0)	327.5 (221.3, 1,091.5)	0.080
CRP (mg/L)	68.7 (17.3, 185.8)	78.2 (13.8, 190.3)	39.5 (23.5, 178.3)	0.642	16.8 (5.5, 78.7)	13.5 (3.3, 73.6)	53.0 (16.8, 92.8)	0.063
Platelet ($\times 10^9$/L)	116.0 (54.3, 214.0)	139.0 (78.0, 220.0)	70.0 (46.0, 173.0)	0.019	115.0 (72.5, 227.0)	171.0 (104.0, 239.0)	62.0 (32.0, 155.5)	<0.001
INR	1.2 (1.1, 1.6)	1.2 (1.1, 1.4)	1.4 (1.1, 1.9)	0.013	1.1 (1.0, 1.4)	1.1 (1.0, 1.2)	1.4 (1.2, 1.9)	<0.001
D-dimer (μg/mL)	3.8 (1.9, 7.8)	3.4 (1.5, 6.5)	4.3 (2.3, 9.8)	0.116	2.3 (1.0, 6.7)	1.8 (0.5, 4.0)	5.3 (2.5, 13.0)	<0.001
Total Bilirubin (mg/dL)	1.2 (0.6, 3.5)	20.3 (8.6, 59.3)	26.5 (11.1, 75.4)	0.194	0.6 (0.3, 1.4)	8.1 (4.6, 16.3)	20.9 (11.0, 65.0)	<0.001
Involved organs								
Cardiovascular dysfunction	13 (11.8%)	9 (12.0%)	4 (11.4%)	0.931	15 (13.0%)	9 (10.2%)	6 (22.2%)	0.107
Respiratory dysfunction	51 (46.4%)	30 (40.0%)	21 (60.0%)	0.051	41 (35.7%)	27 (30.7%)	14 (51.9%)	0.045
Renal dysfunction	42 (38.2%)	25 (33.3%)	17 (48.6%)	0.127	79 (68.7%)	60 (68.2%)	19 (70.4%)	0.831
Hepatic dysfunction	56 (50.9%)	37 (49.3%)	19 (54.3%)	0.630	33 (28.7%)	19 (21.6%)	14 (51.9%)	0.002
Neurological dysfunction	48 (43.6%)	28 (37.3%)	20 (57.1%)	0.052	25 (21.7%)	13 (14.8%)	12 (44.4%)	0.001
Coagulation disorder	66 (60.0%)	41 (54.7%)	25 (71.4%)	0.096	52 (45.2%)	33 (37.5%)	19 (70.4%)	0.003
Chronic organ dysfunction	58 (52.7%)	37 (49.3%)	21 (60.0%)	0.227	78 (67.8%)	58 (65.9%)	20 (74.1%)	0.429
Causes of acute organ injury in patients with chronic organ dysfunction								
Acute exacerbation of chronic disease	37 (63.8%)	23 (30.7%)	14 (40.0%)	0.337	62 (79.5%)	48 (54.5%)	14 (51.9%)	0.807
Infection	52 (89.7%)	31 (41.3%)	21 (60.0%)	0.069	36 (46.2%)	21 (23.9%)	15 (55.6%)	0.002
Other	19 (32.8%)	12 (16.0%)	7 (20.0%)	0.607	33 (42.3%)	29 (33.0%)	4 (14.8%)	0.070
Causes of acute organ injury in patients without chronic organ dysfunction								
Infection	45 (86.5%)	32 (42.7%)	13 (37.1%)	0.585	35 (94.6%)	25 (28.5%)	10 (37.0%)	0.996
Malignancy	9 (17.3%)	6 (8.0%)	3 (8.6%)	0.919	6 (16.2%)	2 (2.3%)	4 (14.8%)	0.011
Activity of connective tissue disease	7 (13.5%)	5 (6.7%)	2 (5.7%)	0.850	5 (13.5%)	5 (5.7%)	0	0.207
Metabolic disease (e.g., DKA)	10 (19.2%)	8 (10.7%)	2 (5.7%)	0.402	3 (8.1%)	2 (2.3%)	1 (3.7%)	0.685
Surgery/Trauma	5 (9.6%)	5 (6.7%)	0	0.120	2 (5.4%)	2 (2.3%)	0	0.431
Cardiovascular/Cerebrovascular events	4 (7.7%)	2 (2.7%)	2 (5.7%)	0.429	8 (21.6%)	6 (6.8%)	2 (7.4%)	0.917
Other	15 (28.8%)	10 (13.3%)	5 (14.3%)	0.893	9 (24.3%)	9 (10.2%)	0	0.085
Sepsis, *n* (%)	61 (55.5%)	35 (46.7%)	26 (74.3%)	0.007	31 (27.0%)	18 (20.5%)	13 (48.1%)	0.005
Critical illness score								
SOFA	6.0 (3.0, 8.0)	4.0 (3.0, 7.0)	8.0 (7.0, 10.0)	<0.001	6.0 (4.0, 9.0)	5.0 (3.0, 8.0)	8.5 (6.0, 12.0)	<0.001
APACHE II	16.0 (11.0, 21.0)	14.0 (10.0, 18.0)	20.0 (15.0, 25.0)	<0.001	14.0 (10.0, 20.0)	13.5 (8.3, 17.0)	19.0 (14.0, 23.0)	<0.001
SAPS II	43.0 (35.0, 54.0)	39.0 (32.0, 47.0)	55.0 (43.0, 65.0)	<0.001	37.0 (30.0, 43.0)	33.5 (26.3, 40.5)	44.0 (41.0, 59.0)	<0.001
TNC	229.4 (141.6, 472.5)	197.5 (97.7, 343.8)	467.0 (267.4, 786.3)	<0.001	210.2 (96.8, 469.6)	202.6 (91.4, 324.9)	584.4 (164.4, 902.6)	0.002

TNC, tenascin-C; LDH, lactate dehydrogenase; CRP, C-reactive protein; INR, International Normalized Ratio; DKA, diabetic ketoacidosis; SOFA, Sequential Organ Failure Assessment; APACHE II, Acute Physiology and Chronic Health Evaluation II; SAPS II, Simplified Acute Physiology Score II.

and subsequent cardiovascular/cerebrovascular events (21.6%), malignancy (16.2%), and the activity of connective tissue disease (13.5%). Of the 115 patients, 45.2% had acute kidney injury, 31% had sepsis and 7% were ventilation dependent at the enrollment, and 23.5% died during the follow-up.

Serum TNC Was Significantly Higher in the Patients Who Died Within 28 Days

In the emergency cohort, serum TNC in the non-survivors was 467.7 ng/ml, significantly higher than that of 197.5 ng/ml in the survivors ($p < 0.001$) (**Table 1**; **Supplementary Material 2**). The severity was even more in non-survivors with significantly higher critical illness scores, including SOFA (8.0 vs. 4.0), APACHE II (20.0 vs. 14.0), and SAPS II (55.0 vs. 39.0), and had a higher rate of sepsis (74.3 vs. 46.7%, $p = 0.007$) than the survivors ($p < 0.001$ for all). They also had significantly lower blood platelet levels and higher internal normalized ratio (INR). For age, gender, involved organs, and causes of acute organ injury, there was no significant difference between the survivors and non-survivors (**Table 1**). Multivariate logistic regression analysis revealed that the association between serum TNC [adjusted OR (95% CI), 1.656 (1.288, 2.130)] and 28-day mortality was independent of sepsis [adjusted OR (95% CI), 1.255 (0.433, 3.635)] or critical illness scores such as SOFA [adjusted OR (95% CI), 1.423 (1.181, 1.715)], APACHE II [adjusted OR (95% CI), 1.142 (1.053, 1.239)], and SAPS II [adjusted OR(95% CI), 1.076 (1.031, 1.123)], respectively ($p < 0.001$ for all) (**Table 2**).

In the inpatient cohort, serum TNC had a similar direction and magnitude (584.4 ng/ml in the non-survivors vs. 202.6 ng/ml in the survivors, $p = 0.002$) with that in the emergency cohort (**Table 1**; **Supplementary Material 2**). The severity was more in non-survivors and had more sepsis, higher INR, and lower blood platelet levels (**Table 1**). However, for involved organs, the non-survivors had more lung, liver, brain, and coagulation system involved than the survivors, and the cause of acute organ injury was more infection and malignant diseases (**Table 1**). Multivariate logistic regression analysis revealed that the association between serum TNC [adjusted OR (95% CI), 1.261 (1.084, 1.467)] and 28-day mortality was independent of sepsis [adjusted OR (95% CI), 1.718 (0.576, 5.121), $p = 0.004$] or critical illness scores such as SOFA [adjusted OR (95% CI), 1.275 (1.088, 1.495), $p = 0.016$] and APACHE II [adjusted OR (95% CI), 1.138 (1.034, 1.253), $p = 0.030$] respectively, but not SAPS II [adjusted OR (95% CI), 1.092 (1.032, 1.157), $p = 0.057$] (**Table 2**).

The Performance of Serum TNC for Predicting Mortality in Critically Ill Patients

In the emergency cohort, the AUCs of serum TNC, SOFA, APACHE II, and SAPS II for predicting 28-day mortality were 0.803 (0.717–0.888), 0.808 (0.725–0.891), 0.762 (0.667–0.857), and 0.779 (0.685–0.872), respectively ($p < 0.001$ for all). There was no statistically significant difference in AUCs between serum TNC and the three critical illness scores (**Supplementary Material 3**; **Figure 1**). The optimal cut-off value of serum TNC calculated by the Youden index was 298.2 ng/ml. In the inpatient cohort, the AUCs of serum TNC, SOFA, APACHE II, and SAPS II for predicting 28-day mortality were 0.745 (0.624–0.865), 0.844 (0.776–0.912), 0.846 (0.780–0.912), and 0.872 (0.808–0.936), respectively ($p < 0.001$ for all). The ROC of serum TNC was lower than SAPS II ($p = 0.032$), whereas there was no significant difference between serum TNC and SOFA or APACHE II (**Supplementary Material 3**; **Figure 1**).

According to the optimal cut-off value, the study population was divided into TNC $\geq$ 300 ng/ml and TNC $<$ 300 ng/ml groups. Compared to the patients with lower TNC, patients with higher TNC were older and more severe with significantly higher critical illness scores including SOFA, APACHE II, and SAPS II ($p < 0.01$ for all), and had a significantly higher 28-day mortality (57.8 vs. 13.8%, $p < 0.001$). This result was validated in the inpatient cohort, which showed that the mortality was 38.6% in the non-survivors and 14.1% in the survivors ($p = 0.003$) (**Table 3**). Kaplan–Meier analysis showed that the survival of patients with serum TNC $\geq$ 300 ng/ml was significantly worse than that of patients with serum TNC $<$300 ng/ml in both cohorts (log-rank test, $p < 0.001$ in the emergency cohort and $p = 0.002$ in the inpatient cohort) (**Figure 2**). As a single biomarker, the sensitivity and specificity of serum TNC $\geq$ 300 ng/ml for predicting mortality was 74.3 and 74.7% in the emergency cohort, while they were 63.0 and 70.1% in the inpatient cohort, respectively. If TNC $\geq$ 450 ng/ml, the specificity was 85.3% in the emergency cohort and 79.3% in the inpatient cohort (**Table 4**).

DISCUSSION

In this prospective study, we found that serum TNC was significantly higher in the critically ill patients with multiple organ dysfunction who died within 28 days in two independent cohorts. We revealed that the AUC of serum TNC for predicting mortality was similar to SOFA, APACHE II, and SAPS II. Kaplan–Meier analysis showed that the survival of patients with higher serum TNC was significantly worse than that of patients with lower serum TNC in both cohorts. These findings suggested that serum TNC was a useful prognostic tool for predicting 28-day mortality in critically ill patients with multiple organ dysfunction.

Previous studies had developed several models to predict the prognosis of critically ill patients. Among them, the APACHE II score and SAPS II, which were initially developed to predict hospital mortality in general ICU patients, have good prognostic performance (22, 23). However, they are calculated using the worst values from data collected in the first 24 h after ICU admission and are therefore not immediately available at the time of admission. They use 14 or 17 variables, also making their clinical practice not convenient. SOFA, another confirmed useful predictive model of critical illness, which is much simpler than APACHE II score and SAPS II, also measures six variables (4–6). Even so, the discrimination of these models remains unsatisfactory with AUCs varied from 0.7 to 0.9 in previous studies (24). It has been also reported that C-reactive protein (CRP), procalcitonin (PCT), lactate, and the markers of platelet function

TABLE 2 | Multivariate analysis for risk factors of all-cause mortality in patients with multiple organ dysfunction.

Variables	Emergency (derivation) cohort		Inpatient (validation) cohort	
	Adjusted OR (95% CI)	*p*-value	Adjusted OR (95% CI)	*p*-value
Model 1				
Age	0.980 (0.950, 1.010)	0.191	1.012 (0.985, 1.040)	0.377
Gender	1.341 (0.474, 3.794)	0.581	0.604 (0.219, 1.668)	0.331
Sepsis	1.255 (0.433, 3.635)	0.676	1.718 (0.576, 5.121)	0.332
TNC (per 100 ng/ml increase)	1.656 (1.288, 2.130)	<0.001	1.261 (1.084, 1.467)	**0.004**
Model 2				
Age	0.979 (0.946, 1.013)	0.215	1.014 (0.986, 1.042)	0.341
Gender	1.475 (0.469, 4.634)	0.505	0.651 (0.214, 1.978)	0.449
SOFA	1.423 (1.181, 1.715)	<0.001	1.275 (1.088, 1.495)	0.003
TNC (per 100 ng/ml increase)	1.678 (1.276, 2.206)	<0.001	1.137 (1.024, 1.262)	**0.016**
Model 3				
Age	0.976 (0.945, 1.009)	0.150	1.007 (0.980, 1.035)	0.629
Gender	1.516 (0.496, 4.638)	0.466	0.761 (0.262, 2.210)	0.616
APACHE II	1.142 (1.053, 1.239)	0.001	1.138 (1.034, 1.253)	0.008
TNC (per 100 ng/ml increase)	1.630 (1.276, 2.083)	<0.001	1.118 (1.011, 1.237)	0.030
Model 4				
Age	0.969 (0.936, 1.002)	0.558	0.993 (0.962, 1.025)	0.663
Gender	1.429 (0.468, 4.363)	0.556	0.653 (0.217, 1.964)	0.449
SAPS II	1.076 (1.031, 1.123)	0.001	1.092 (1.032, 1.157)	0.002
TNC (per 100 ng/ml increase)	1.524 (1.203, 1.930)	<0.001	1.109 (0.997, 1.234)	0.057

TNC, tenascin-C; SOFA, Sequential Organ Failure Assessment; APACHE II, Acute Physiology and Chronic Health Evaluation II; SAPS II, Simplified Acute Physiology Score II.

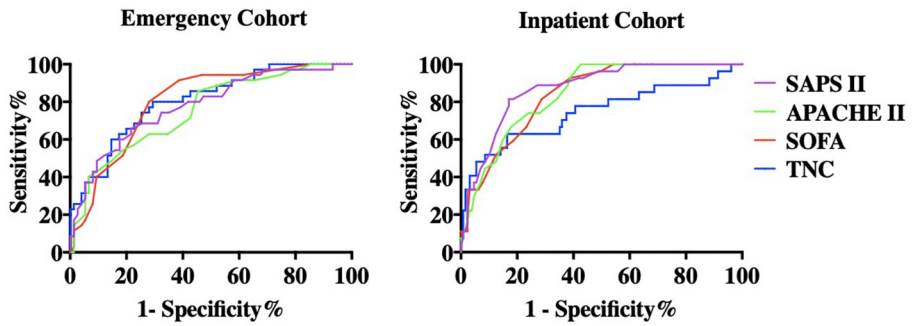

FIGURE 1 | Receiver Operating Characteristics (ROC) curve in both cohorts. The areas under ROC (AUC) of serum TNC, SOFA, APACHE II, and SAPS II for predicting all-cause mortality were 0.803, 0.808, 0.762, and 0.779 ($p < 0.001$ for all) in the emergency (derivation) cohort and 0.745, 0.844, 0.846, and 0.872 ($p < 0.001$) in the inpatient (validation) cohort. In the emergency cohort, there was no significant difference between serum TNC and the three critical illness scores. In the inpatient cohort, the AUC of serum TNC was significantly lower than SAPS II ($p = 0.032$) while there was no significant difference between serum TNC and SOFA or APACHE II.

(such as thrombocytopenia and impaired platelet aggregation) were associated with the severity and mortality of multiple organ dysfunction (25–27). However, these biomarkers were more relevant to inflammation and infection and only showed limited value in predicting mortality in sepsis. Differently, serum TNC was upregulated in response to tissue injury regardless of causes and could be used to predict mortality in multiple organ dysfunction patients with the AUC as high as SOFA. As a single biomarker, it was convenient in clinical application.

The elevation of serum TNC may be explained by the upregulation of TNC in the injured tissues. It was postulated that persistent TNC expression was caused by prolonged inflammation and tissue injury. For example, intense TNC expression was observed at the site of infarction or active inflammation of the heart during the active phase but not in scar tissue during the healing phase (10, 11, 13, 28). Increased TNC expression was also found in lungs with progressive idiopathic pulmonary fibrosis (16), and in synovium with arthritis such as rheumatoid arthritis (29). Basic studies revealed that TNC could

TABLE 3 | Comparisons between patients with serum TNC ≥ 300 ng/ml and serum TNC < 300 ng/ml.

	Emergency (derivation) cohort			Inpatient (validation) cohort		
	TNC < 300 ng/ml *n* = 65	TNC ≥ 300 ng/ml *n* = 45	*P*	TNC < 300 ng/ml *n* = 71	TNC ≥ 300 ng/ml *n* = 44	*P*
Age (years)	61 (48, 71)	67 (60, 78)	0.002	51 (35, 62)	64 (49, 74)	0.001
Male, *n* (%)	46 (70.8%)	28 (62.2%)	0.350	42 (59.2%)	33 (75.0%)	0.084
Creatinine (mg/dL)	0.8 (0.6, 1.5)	1.3 (0.7, 3.2)	0.043	1.9 (0.7, 5.0)	4.5 (2.4, 7.8)	0.001
CRP (mg/L)	42 (7, 169)	135 (32, 200)	0.023	11 (3, 23)	81 (10, 135)	0.002
D-dimer (μg/mL)	3.4 (1.4, 4.6)	5.6 (2.6, 11.1)	0.002	1.5 (0.5, 2.8)	4.6 (2.3, 12.6)	<0.001
Sepsis, *n* (%)	25 (38.5%)	36 (80.0%)	<0.001	11 (15.5%)	20 (45.5%)	<0.001
SOFA	4.0 (3.0, 7.5)	7.0 (6.0, 8.5)	0.003	5.0 (3.0, 8.0)	7.0 (5.0, 9.0)	0.001
APACHE II	14.0 (10.0, 18.0)	18.0 (14.0, 22.5)	0.003	11.0 (7.0, 16.0)	17.0 (15.0, 22.0)	<0.001
SAPS II	38.0 (31.0, 49.8)	47.0 (41.5, 62.0)	<0.001	33.0 (26.0, 41.0)	41.5 (35.5, 53.0)	<0.001
Mortality, *n* (%)	9 (13.8%)	26 (57.8%)	<0.001	10 (14.1%)	17 (38.6%)	0.003

TNC, tenascin-C; CRP, C-reactive protein; SOFA, Sequential Organ Failure Assessment; APACHE II, Acute Physiology and Chronic Health Evaluation II; SAPS II, Simplified Acute Physiology Score II.

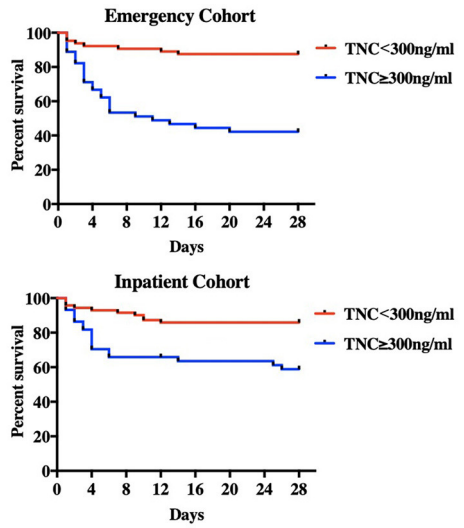

FIGURE 2 | Kaplan-Meier survival curve. By reference to the optimal cut-off value, the patients were divided into serum TNC ≥ 300 ng/ml and TNC < 300 ng/ml groups. Compared with lower TNC group, the higher TNC group had a significantly lower survival rate in both emergency (derivation) cohort (log rank test, $p < 0.0001$) and inpatient (validation) cohort (log rank test, $p = 0.002$).

be induced by inflammatory and growth factors, oxidative stress, and hypoxia (30). Therefore, the increased TNC expression may reflect disease activity and progression of various diseases without organ specificity. This was the rationale we included patients with acute organ injury by an increase of SOFA ≥ 2 points within 7 days.

Consistent with TNC in tissues, serum TNC was also associated with the activity of various diseases (31–33). Page et al. reported that serum TNC, in rheumatoid arthritis, was associated with ultrasound-determined erosion scores and was decreased after treatment with infliximab and methotrexate (31). Zavada et al. found that serum TNC was positively associated with the disease activity score (SLEDAI) in SLE patients (34). Serum TNC was also associated with the severity and prognosis of various diseases (35–38). For example, serum TNC was correlated with the total occlusion and inflammation in myocardial infarction (39), and levels on day 5 after admission was an independent predictor for cardiac events during the follow-up period (24 ± 13 months) (40). In patients with heart failure, serum TNC was also positively correlated with the severity of left ventricular dysfunction and was an independent predictor for 12-month major adverse cardiac events (37). In patients with acute AD, serum TNC was a valuable biomarker for predicting in-hospital deaths (38). Acute, active, and systemic injury is a common feature of critically ill patients with multiple organ dysfunction, and serum TNC is probably significantly increased. But now, only a few studies focus on the predictive value of serum TNC for mortality in these patients. Meijer et al. examined plasma TNC during sepsis and non-septic critical illness and found that plasma TNC was reflective of disease severity more than an independent predictor of mortality (7). However, serum TNC, in our study, was not only positively associated with the critical illness scores such as SOFA, APACHE II, and SAPS II, but also independently associated with mortality after adjusting for these scores. This result was also supported by another study that focused on patients with sepsis and showed that serum TNC was positively correlated with SOFA scores and associated with 30-day mortality (41).

This study has several limitations. First, serum TNC in different stages may show different clinical significance. For example, higher serum TNC on admission predicted more hospitalization deaths in patients with acute AD (19), whereas a higher serum TNC on hospital day 7 predicted a lower risk of enlargement of the aortic lesion during the chronic stage (42). However, our study measured serum TNC one time in the acute stage. So, further studies with dynamic changes

TABLE 4 | The diagnostic sensitivity and specificity of serum TNC and critical illness scores in emergency (derivation) and inpatient (validation) cohorts.

	Emergency (derivation) cohort			Inpatient (validation) cohort		
	N	Sensitivity	Specificity	*N*	Sensitivity	Specificity
TNC≥200 ng/ml	67	85.7%	50.7%	63	66.7%	48.2%
TNC≥300 ng/ml	45	74.3%	74.7%	63	66.7%	48.2%
TNC≥450 ng/ml	29	51.4%	85.3%	31	51.9%	79.3%

of serum TNC or in the chronic stage are required in the future. Second, the clinical utility of serum TNC may lie in the low negative predictive value, especially in patients with acute cardiovascular events. For example, patients with acute myocardial infarction or acute cerebral hemorrhage will probably die very soon after the events, but their basic serum TNC will not be as high as the patients with multiple organs injury, because the injured tissues may be limited to heart or brain in the early stage. By contrast, serum TNC is relatively useful for the assessment of illness severity in patients with multiple organ dysfunction. Third, the inpatient cohort enrolled the patients from different departments but not consecutive patients in ICUs. Selective bias included that more patients with kidney injury were enrolled than with other organs involved. The imbalance of patient enrollment influenced the interpreter of the results. However, the emergency cohort enrolling patients from the emergency department did not have this limitation. Fourth, there was no specific definition for critical illness, which also led to the various types of mortalities in different studies on this population. The inclusion criteria of our study were the patients with at least two organ dysfunction, which might be different from the traditional critically ill patients in the ICUs. Fifthly, TNC has multiple protein isoforms and which isoform has the strongest relationship with the outcome remains unknown. The ELISA kit used in this study measured the large TNC variant which was characteristic for some tumors. Finally, the low sample size also influenced the power of this study. So, larger, independent validation studies with consecutive patients in ICUs are needed to further support the utility of serum TNC in critically ill patients.

In summary, serum TNC, representative of tissue injury, is a novel, promising predictive marker for mortality in critically ill patients with multiple organ dysfunction. Incorporation of serum TNC into clinical practice and future investigation may bring better understanding and management in critically ill patients.

CONCLUSIONS

Serum TNC was positively associated with the severity of illness and mortality, and could be used as a prognostic tool for predicting mortality in critically ill patients with multiple organ dysfunction.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Review Board at Huashan Hospital, Fudan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YX collected, analyzed, interpreted data, and drafted the manuscript. NL and JG collected and analyzed the data. DS and YS collected the data. QX and MC conceived the study, participated in its design and coordination, analyzed and interpreted data, drafted the manuscript, had full access to all the study data, and assume responsibility for the integrity of the data and the accuracy of the analysis. C-MH conceived the study, participated in its design, and helped to draft the manuscript. All authors approved the final manuscript.

FUNDING

The study was supported by the National Natural Science Foundation of China Grants 81520108006, 81930120, and 81700673.

ACKNOWLEDGMENTS

We thank Wanghong Xu (Department of Epidemiology, School of Public Health, Fudan University) and Hui Cai (Vanderbilt University Medical Center) for helping with the data analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.759273/full#supplementary-material

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frontiers in Physiology

ORIGINAL RESEARCH
published: 29 November 2021
doi: 10.3389/fphys.2021.736108

Intermittent Hypoxia Exposure Helps to Restore the Reduced Hemoglobin Concentration During Intense Exercise Training in Trained Swimmers

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In prolonged intense exercise training, the training load of athletes may be reduced once their hemoglobin concentrations ([Hb]s) are decreased dramatically. We previously reported that intermittent hypoxia exposure (IHE) could be used to alleviate the decrease of [Hb] and help to maintain the training load in rats. To further explore the feasibility of applying IHE intervention to athletes during prolonged intense exercise training, 6 trained swimmers were recruited to conduct a 4-week IHE intervention at the intervals after their [Hb] dropped for 10% or more during their training season. IHE intervention lasted 1 h and took place once a day and five times a week. Hematological and hormonal parameters, including [Hb], red blood cells (RBC), hematocrit (Hct), reticulocytes, serum erythropoietin (EPO), testosterone (T) and cortisol (C) were examined. After the IHE intervention was launched, [Hb], RBC and Hct of the subjects were increased progressively with their maximum levels ($P < 0.01$) showing at the third or fourth week, respectively. An increase in reticulocyte count ($P < 0.01$) suggests that IHE intervention promotes erythropoiesis to increase [Hb]. Besides, serum level of EPO, the hormone known to stimulate erythropoiesis, was overall higher than that before the IHE intervention, although it was statistically insignificant. Furthermore, the serum level of T, another hormone known to stimulate erythropoiesis, was increased progressively with the maximum level showing at the fourth week. Collectively, this study further confirms that IHE intervention may be used as a new strategy to prevent intense exercise training-induced reductions in [Hb].

Keywords: hypoxia, IHE, erythropoietin, EPO, testosterone, hemoglobin

OPEN ACCESS

Edited by:
Hassane Zouhal,
Rennes 2 University, France

Reviewed by:
José Ramón Alvero Cruz,
University of Malaga, Spain
Filipe Dinato De Lima,
University Center of Brasilia, Brazil

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Specialty section:
This article was submitted to Exercise Physiology, a section of the journal Frontiers in Physiology

Received: *04 July 2021*
Accepted: *25 October 2021*
Published: *29 November 2021*

Citation:
Weng X, Lin J, Yuan Y, Lin B, Huang W, Tin HT, Li J, Yan X, Lin W and Chen H (2021) Intermittent Hypoxia Exposure Helps to Restore the Reduced Hemoglobin Concentration During Intense Exercise Training in Trained Swimmers. Front. Physiol. 12:736108. doi: 10.3389/fphys.2021.736108

INTRODUCTION

Blood hemoglobin (Hb) serves as a routinely used marker for monitoring intense exercise training and physical function (Gleeson, 2002; Halson et al., 2003). There is literature supporting that a decrease in hemoglobin concentration ([Hb]) can be caused by acute or prolonged plasma volume (PV) expansion which can aid the athletes adapt to exercise training (Kargotich et al., 1998).

However, higher [Hb] usually leads to better performance (Calbet et al., 2006; Cai et al., 2019), as long as the hematocrit (Hct) is at the optimum range (Reinhart, 2016; Sitina et al., 2021). Actually, based on our long-term observation and other studies (Hasibeder et al., 1987; Zhao, 2003), the training load of athletes will usually be reduced once their [Hb] were 10% lower than their baselines. Seeking appropriate strategies to restore the decreased [Hb] could be able to prevent the reduction in training load during prolonged intense training periods (Weng et al., 2021).

Altitude training (hypoxic training) has been widely adopted by athletes to improve their aerobic capacity (Millet et al., 2010). Hypoxia can induce expression of genes regulated by hypoxia inducible factors (HIFs) and then stimulate erythropoietin (EPO) production, which ultimately promotes the synthesis of Hb (Haase, 2013). When laboratory rats were subjected to intense exercise training for 6 weeks, one hour of hypoxic exposure (simulated altitude: 3,000 m) at the interval of exercise from the fourth to the sixth week could prevent the 10% decrease in [Hb] (Weng et al., 2021). These results suggest that hypoxia exposure can be potentially used to slow or prevent intense exercise training-induced decrease of [Hb] in athletes. However, in the rat study, the physiological and biochemical parameters of the rats were not dynamically monitored. To further explore the feasibility of applying hypoxia exposure to athletes during intense exercise training period, 6 trained swimmers were recruited to conduct hypoxic exposure at the intervals during their intense exercise training period. The effects of hypoxic exposure on the levels of [Hb], RBC, hematocrit (Hct), reticulocytes, serum EPO, testosterone (T) and cortisol (C) in athletes undergoing intense exercise training were investigated.

MATERIALS AND METHODS

Subjects

Male trained swimmers who were members of the Guangzhou Sport University Team with at least ten-year training experience participated in this study. This study was conducted during their training season. Since this study was based on the training monitoring of the Guangzhou Sport University Swimming Team during its training season lasting about half a year prior to the Guangdong Provincial University Games, the swimmers were not subjected to various training loads to induce low hemoglobin concentration in certain groups, as it may produce negative outcomes to their training. All swimmers were trained by their coaches, and conducted the same training program with the same relative intensity. Their [Hb]s were examined every Monday. If the instant [Hb] of a swimmer was 10% lower than his baseline [Hb], the athlete was subjected to the IHE intervention from the same day on. In total, 6 swimmers were subjected to the IHE intervention (mean ± SD: age, 20.85 ± 0.634 yr; body weight, 68.71 ± 2.22 kg; height, 182 ± 2.49 cm; training duration, 11.85 ± 0.96 yr). None of the subjects have hematologic diseases, liver diseases, kidney diseases, or endocrine disorders. None of the subjects smoked, drank alcohol, or were taking medication known to alter the hormonal response. The study was conducted according to the Declaration of Helsinki, and the study was approved by the Guangzhou Sport University Ethics Committee (ID number: 2018LCLL-11). All the subjects provided written informed consent to participate in this study.

Training Routine

The swimmers performed 10 training sessions per week (usually two sessions on Monday, Tuesday, Thursday, and Friday, respectively, 1 session on Wednesday and Saturday, respectively, and a break on Sunday), ∼150 min per session. Each session included two parts: swimming training and strength training. In the swimming training, the swimmers were subjected to interval training, continuous training or sprint training, and they completed a mean value of 40.0 km swimming per week. About 50% of the training volume aimed for the aerobic power of the swimmers, 20% aimed for the anaerobic power, and the remaining 30% aimed for both aerobic and anaerobic power. The strength training part included dry-land training and in-water training. Dry-land training, which was conducted 2–3 times per week with 1 h each time, included squat, running, bench press, pull-up, etc. There were various forms of in-water training regimes, which were conducted twice per week with half an hour each time, such as tethered training with elastic bands.

Intermittent Hypoxia Exposure Design

The IHE intervention sessions were conducted in the evening after daily training. The subjects seated comfortably and conducted IHE intervention by breathing through a face mask (hypoxia generator hyp-100, hypoxia company, New York, United States). A simulated altitude of 3,000 m was chosen based on our previous rat study (Weng et al., 2021). Each IHE intervention session lasted for one hour and the study lasted for 4 weeks (5 days/week). No special load or schedule changes were made specifically for the subjects before, during and after the study.

Sample Collection and Measurement of Hematological Parameters

The baseline values for [Hb], red blood cell count (RBC) and hematocrit (Hct) were collected one week before the training season. On every Monday during the IHE intervention period, the venous blood samples of the subjects were collected with EDTA-containing or EDTA-lacking tubes. Immediately after collection, 1 ml whole blood was analyzed to measure hemotological parameters: [Hb], RBC, Hct and reticulocyte count using an automated cell counter (ADVIA120, Bayer AG, Germany). Another 4 ml blood samples was collected using EDTA-lacking tubes and centrifuged at 3,000 rpm/min for 10 min at room temperature to collect the serum for the measurement of EPO, testosterone (T) and cortisol (c) (see below).

Measurement of Erythropoietin, Testosterone, and Cortisol

Serum EPO, T and C were measured using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's guidelines (Catalog #: E01E0002, E11T0007 and E11C0008, respectively, Bluegene Biotech CO., LTD, Shanghai, China).

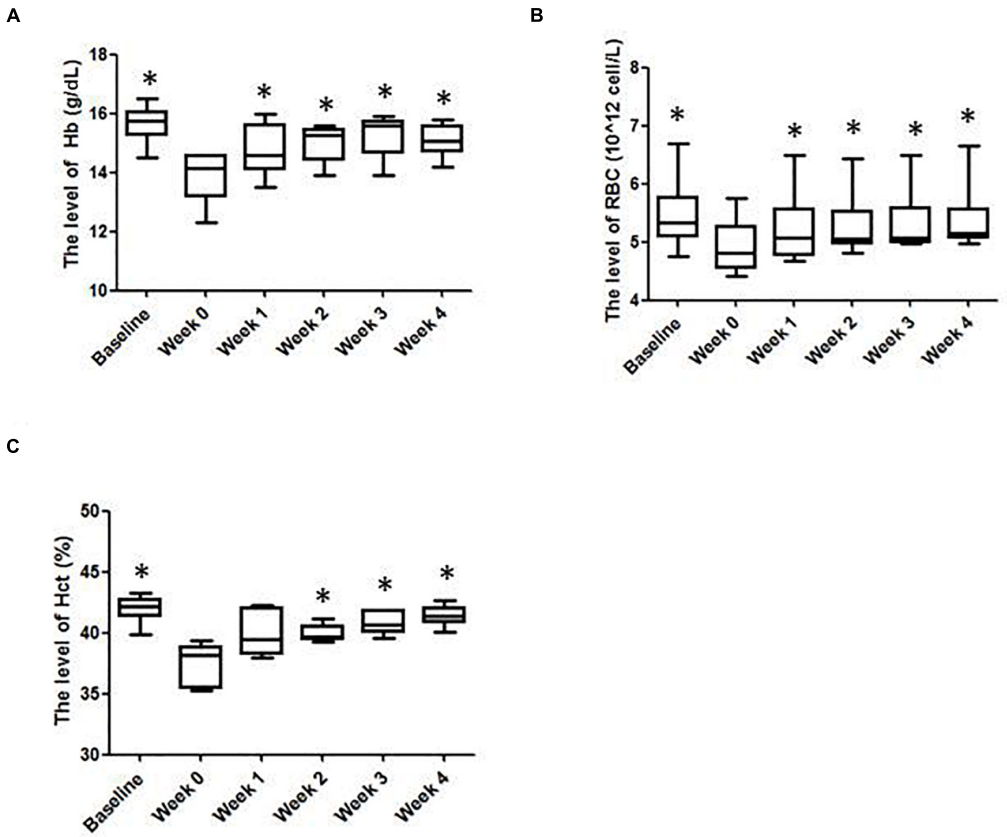

FIGURE 1 | The hematological parameters of the subjects in various weeks. **(A)** Hemoglobin (Hb); **(B)** red blood cells (RBCs); **(C)** hematocrit (Hct) levels. *Significantly different from week 0, $P < 0.05$.

TABLE 1 | Some hematological parameters of subjects during IHE intervention.

Group	MCV (fL)	RDW (%)	MCHC (g/dL)	HDW (g/dL)	CH (pg)	MCH (pg)
Week 0	76.98 ± 7.83	12.78 ± 0.79	38.10 ± 1.49	2.66 ± 0.16	29.30 ± 3.85	28.53 ± 3.65
Week 1	77.10 ± 7.35	12.78 ± 0.67	37.98 ± 2.13	2.59 ± 0.09	29.28 ± 4.17	28.63 ± 3.91
Week 2	77.20 ± 7.94	12.85 ± 0.75	38.38 ± 1.38	2.65 ± 0.13	29.60 ± 3.91	28.85 ± 3.63
Week 3	78.13 ± 8.06	12.81 ± 0.73	38.08 ± 1.45	2.64 ± 0.15	29.68 ± 3.96	28.73 ± 3.72
Week 4	77.81 ± 8.01	12.78 ± 0.66	38.11 ± 1.45	2.63 ± 0.14	29.65 ± 3.93	28.65 ± 3.67

Briefly, 100 μl of conjugate was added to each well in the plate, then 50 μl of standards, control, or sample were added to the plate and incubated for 1 h at 37°C. Each well was aspirated and rinsed with wash buffer for a total of five washes. Substrate (100 μl) was added to each well and incubated for 10–15 min at 37°C in dark. Stop solution (100 μl) was added to each well and the plate was read within 30 min. Plates were read at 450 nm on a VARIOSKAN FLASH (Thermo Fisher Scientific, MA, United States). Each sample was measured in duplicate.

Statistics

Non-parametric analyses of the data were analyzed using the procedure of Friedman test to locate overall significant differences among various weeks. Significant differences between various weeks were then determined with the Wilcoxon test. All statistical calculations were performed using IBM SPSS Statistics for Windows (version 22). Data were expressed as mean ± standard deviation (SD). *P*-values less than 0.05 were considered statistically significant.

RESULTS

The Hematological Parameters of the Subjects Were Increased During Intermittent Hypoxia Exposure Intervention

As shown in **Figure 1A**, after prolonged intense exercise training, the [Hb] of the subjects were about 11% lower than their baseline

TABLE 2 | The Retic# and Retic% of subjects during IHE intervention.

Group	Retic#(10^9/L)	Retic% (%)
Week 0	50.23 ± 6.84	0.99 ± 0.15
Week 1	60.75 ± 15.09	1.20 ± 0.29
Week 2	66.13 ± 12.96*	1.28 ± 0.31
Week 3	63.53 ± 13.23*	1.25 ± 0.28
Week 4	65.81 ± 12.26*	1.27 ± 0.26

**Significantly different from week 0, $P < 0.05$.*

values. After the IHE intervention was launched, the [Hb] of the subjects increased progressively with the maximum level achieved at the third week. Similar outcomes also occurred for RBC and Hct. The RBC and Hct of the subjects increased progressively with both of their maximum levels achieved at the fourth week (**Figures 1B,C**).

Besides, the results of mean corpuscular volume (MCV), red cell distribution width (RDW), mean corpuscular hemoglobin concentration (MCHC), hemoglobin distribution width (HDW), mature red blood cell hemoglobin content (CH) and mean corpuscular hemoglobin (MCH) were slightly different from week to week and displayed no statistical significance (**Table 1**). Reticulocyte count can reflect the erythropoietic activity of the bone marrow (Cline and Berlin, 1963). Reticulocyte count can be reported as absolute reticulocyte count (Retic#) or as a reticulocyte percentage (i.e., reticulocytes per total RBCs examined, Retic%) (Riley et al., 2001). As shown in **Table 2**, after the IHE intervention was launched, the Retic# and Retic% of the subjects increased progressively. The Friedman test indicated that there was a statistically significant difference after the IHE intervention was launched for Retic# ($P = 0.006$), but not for Retic% ($P = 0.054$). As Retic# is an absolute value and more reliable, these results indicate that IHE intervention promoted erythropoiesis in the subjects.

Erythropoietin and Testosterone of the Subjects Were Increased During Intermittent Hypoxia Exposure Intervention

As shown in **Figure 2A**, after IHE intervention was launched, the serum EPO level was overall higher than that before the IHE intervention, especially at the second week and the fourth week, although the Friedman test indicated that there was not a statistically significant difference for the EPO level. These results suggest that the IHE intervention might promote the production of serum EPO, which could subsequently stimulate erythropoiesis.

Besides EPO, testosterone (T) can also enhance erythropoiesis and [Hb] (Bachman et al., 2014; Cervi and Balitsky, 2017). As shown in **Figure 2B**, after IHE intervention was launched, the serum T of the subjects were increased progressively with the maximum level showing at the fourth week. T is known as an anabolic hormone and cortisol (C) is a catabolic hormone (Urhausen et al., 1995). On most occasions, their levels inside human bodies are well coordinated. Moreover, The T/C ratio can serve as an indicator of both the anabolic and catabolic balance and the fatigue state (Urhausen et al., 1995). The decrease of this ratio marks a potential physical function decrease (Urhausen et al., 1995). In this study, the level of the serum C remained overall stable before and during IHE intervention (**Figure 2C**). Therefore, the T/C ratio displayed the same shift as that of T (**Figure 2D**). These results suggest an increase of anabolic metabolism for the subjects during the HE intervention.

DISCUSSION

In our previous report, we adopted a moderate simulated altitude (3,000 m) and our data showed that it was sufficient to prevent low [Hb] in rats subjected to intense exercise training (Weng et al., 2021). This current study further confirmed the feasibility of this simulated altitude in athletes. In addition, we have previously found that one-hour IHE intervention was sufficient to prevent the appearance of low [Hb] in rats subjected to intense exercise training, although it may not be sufficient to prevent the decrease of RBC and Hct (Weng et al., 2021). However, in this study, one hour of normobaric hypoxia exposure can not only prevent the subjects from decreasing [Hb] during intense exercise training, but also blunt the decrease in RBC and Hct. These results suggest that humans might be more sensitive to the IHE intervention than rats.

There is literature in which roughtly 14 h per day are needed to stimulate erythropoiesis in healthy subjects in hypoxic interventions (Rusko et al., 2003; Brugniaux et al., 2006). However, in the current study, only one hour per day was needed. This design was justified as follows. Firstly, this study was conducted based on our research performed in laboratory rats previously published (Weng et al., 2021). In that study, rats undergoing intense exercise training were subjected to the IHE intervention lasting for three weeks for 1 h/day, 2 h/day and 3 h/day (the simulated height was 3,000 m), respectively. We found that IHE with different hours displayed comparable improvement in [Hb]. Therefore, we chose one-hour intervention in this study. Secondly, there is literature in which IHE intervention of 1–3 h is sufficient for enhanced erythropoiesis (Rodriguez et al., 1999, 2000; Casas et al., 2000). IHE intervention of different durations in various studies may be attributed to divergent physical characteristics of subjects and divergent types and/or intensities of exercise training involved in these studies (Millet et al., 2010; Viscor et al., 2018).

After IHE intervention was launched, the serum EPO level in the subjects reached the peak in the second week. However, the levels of RBC and Hb reached the peak in the fourth or third week, respectively, which indicated that the level shift of EPO was not strictly correlated with those of RBC and Hb. Moreover, the Friedman test failed to detect a statistically significant difference for the EPO level after IHE intervention was launched. These results showed that the increase in the serum EPO level upon hypoxic stimulation could be transient. These results are consistent with our previous study in rats, in which the serum EPO level did not display a significant increase compared with the exercise control group ($P > 0.05$) after the 3 weeks of IHE

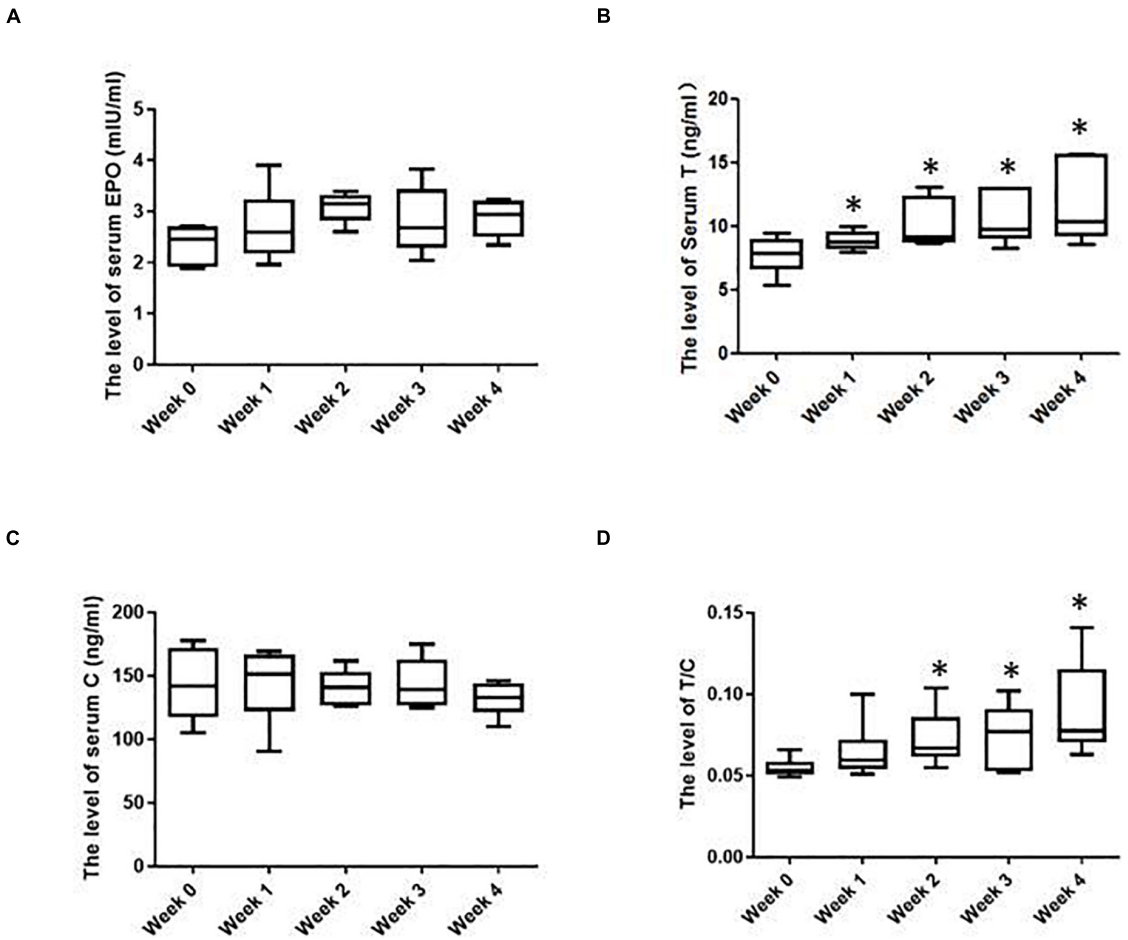

FIGURE 2 | The hormonal parameters of the subjects in various weeks. **(A)** Serum erythropoietin (EPO) levels; **(B)** serum T levels; **(C)** serum C levels; **(D)** serum T/C levels. *Significantly different from week 0, $P < 0.05$.

intervention, although [Hb] did increase (Weng et al., 2021). This can be attributed to the fact that it takes time from EPO secretion to the production of RBC and Hb. Therefore, the level shift of RBC, Hb and Hct occurred after that of EPO. The reason beyond this may be that the level increase of RBC and Hb can enhance the blood oxygen level, which imposes a negative feedback on the production of EPO (Maxwell et al., 1999; Ivan et al., 2001).

During the IHE intervention in this study, the level of serum T progressively increased with the maximum level showing at the fourth week. In previous studies, the level shifts of serum T during or upon hypoxia exposure displayed divergent results. For instance, the levels of serum T of 52 male soldiers decreased significantly after a 5,380 m altitude exposure for half a year, while increased significantly after another half a year (He et al., 2015). However, Ding et al., found that although 113 male subjects showed a significant increase in the serum T level one day after a 3,700 altitude exposure, their serum T levels fell below their baselines seven days later (Ding et al., 2018). These divergent results may be attributed to divergent physical characteristics of subjects and different duration of hypoxia exposure. It would be interesting to explore whether IHE intervention can keep increasing or maintaining the level of serum T after periods longer than 4 weeks in future studies.

This study has shown that the IHE intervention could partially restore the low [Hb] caused by intense exercise training in the subjects. We noticed that there is literature supporting that the [Hb] decrease during exercise training can be due to PV expansion (Kargotich et al., 1998) and that hypoxic expsure can reduce PV (Siebenmann et al., 2017). Thus, one potential interpretation of our results is that the IHE intervention may simply decrease PV and reverse the possible PV expansion induced by intense exercise training. However, there are studies reporting that the PV remains unchanged after IHE interventions. For instance, Rodriguez et al. (2000) reported an increase of [Hb] and Hct without signs of decreased PV (hemoconcentration). To support our statement, moreover, the "live high-train low" strategy has shown an increase of the hemoglobin mass without a decrease in PV (Wehrlin et al., 2006; Wehrlin and Marti, 2006; Saugy et al., 2014). On the other hand, we think that the recovery of [Hb] in the subjects benefited substantially from the stimulated erythropoiesis induced by the IHE intervention based on the following evidences. Firstly,

the reticulocyte counts which can reflect the erythropoietic activity of the bone marrow (Cline and Berlin, 1963) of the subjects were increased significantly during the IHE intervention (**Table 2**); Secondly, the serum level of EPO, the hormone known to be induced by IHE to stimulate erythropoiesis, was overall higher than that before the IHE intervention, although it was statistically insignificant probably due to a relatively transient increase (**Figure 2A**); Finally, the serum level of T, another hormone known to be induced by IHE to stimulate erythropoiesis, was increased progressively during the IHE intervention (**Figure 2B**). Moreover, the T/C ratio, serving as an indicator of anabolic and catabolic balance and is of the fatigue state (Urhausen et al., 1995) displayed the same shift as that of T (**Figure 2D**), which may suggest, although indirectly, that the interaction between training-induced PV expansion and IHE-induced plasma loss can be overall beneficial for the subjects. Future investigation, including exercise tests, blood flow and blood volume measurements are warranted for clarification of outcomes of this interaction.

A few limitations of the current study have to be acknowledged. First of all, the sample size of 6 was not large and there was a lack of a control group. Due to the availability of swimmers with similar competition level and training history, we were not able to recruit more than 6 participants, nor a control group. Yet because of the strict selection criteria, we observed very similar physiological parameters among our participants, and similar response of the participants to the IHE intervention. Beside, this study was based on the training monitoring of the Guangzhou Sport University Swimming Team. Since this study was conducted during the training season before the Guangdong Provincial University Games, the subjects needed a uniform intervention for a fair competition, which made us unable to set a control group. Regardless, further studies with a big sample size and a control group are warranted. Secondly, the benefits of IHE need to be interpreted with caution. It is known that sport anemia is partially due to the increase of PV, which is associated improved microcirculation and better distribution of blood flow to exercising muscle (Fellmann, 1992). On the other hand, hypoxia is known to induce erythropoiesis, which can increase the number red blood cells and hematocrit (Haase, 2013), yet this increase could be transient and return to pre-hypoxia level in less than 2 weeks (Berglund, 1992). What we have demonstrated in the current study was that [Hb], red blood cell counts and hematocrit were decreased after intense training, while IHE was able to restore the reduced parameters partially or in full, but not above the pre-training baseline (**Figure 1**). Therefore, we could conclude that the IHE is not likely to increase the blood viscosity in these athletes. Moreover, we did not notice a decrease of training load or intensity for the swimmers, which could demonstrate the benefits of the IHE intervention. Finally, we did not monitor the [Hb]s of the subjects beyond the 4-week intervention period, nor study how long the restored [Hb] can maintain. Although the subjects can rejoin the IHE intervention whenever their [Hb]s drop again, future studies are warranted to explore the "shelf life" upon IHE intervention against the training-induced [Hb] decrease.

CONCLUSION

One hour of normobaric hypoxia exposure (14.5% O2) each day during training intervals was sufficient to partially restore the low [Hb] in trained swimmers during prolonged exercise training. However, limitations, including a small sample size, lacking of a control, exercise tests and blood flow and blood volume measurements, are noticeable for this study. Future studies of more comprehensive design are warranted.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Guangzhou Sport University Ethics Committee. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XW and HC conceived and designed the research. XW, JeL, YY, BL, WH, WL, and HC conducted the experiments. XW, HTT, JaL, XY, WL, and HC analyzed the data. XW, XY, and HC prepared the original manuscript. XW, JeL, YY, HTT, JaL, XY, WL, and HC revised the manuscript. All authors read and approved the manuscript.

ACKNOWLEDGMENTS

We thank Guoqin Xu and Yan Meng for their kind help in revising the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.736108/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 27 January 2022
doi: 10.3389/fphys.2022.755769

Changes in CYP3A4 Enzyme Expression and Biochemical Markers Under Acute Hypoxia Affect the Pharmacokinetics of Sildenafil

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To investigate the effects of pathological, physiological, biochemical and metabolic enzymes CYP3A4 on the pharmacokinetics of sildenafil under acute hypoxia, rats were randomly divided into the plain group (50 m above sea level), acute plateau group 1 (2300 m above sea level), and acute plateau group 2 (4300 m above sea level), and blood samples and liver tissues were collected. Our results showed that the blood gas, physiological and biochemical indexes of rats changed under acute hypoxia, and the protein expression of CYP3A4 enzyme decreased. The process of absorption, distribution, metabolism and excretion of sildenafil in rats has changed. Compared with the P group, the area under the drug-time curve and the average resident in the H2 group increased to 213.32 and 72.34%, respectively. The half-life and peak concentration increased by 44.27 and 133.67%, respectively. The clearance rate and apparent distribution volume decreased to 69.13 and 46.75%, respectively. There were no statistical differences in the pharmacokinetic parameters between the P group and the H1 group. In conclusion, the pharmacokinetic changes of sildenafil have a multi-factor regulation mechanism, and changes in blood gas, pathology, and biochemical indicators and metabolic enzymes affect the absorption, distribution, excretion, and metabolism of sildenafil, respectively. This study provides experimental evidence and new ideas for the rational use of sildenafil under acute hypoxic conditions.

Keywords: acute hypoxia, CYP3A4, sildenafil, pharmacokinetics, blood gas, pathological and biochemical indicators

OPEN ACCESS

Edited by:
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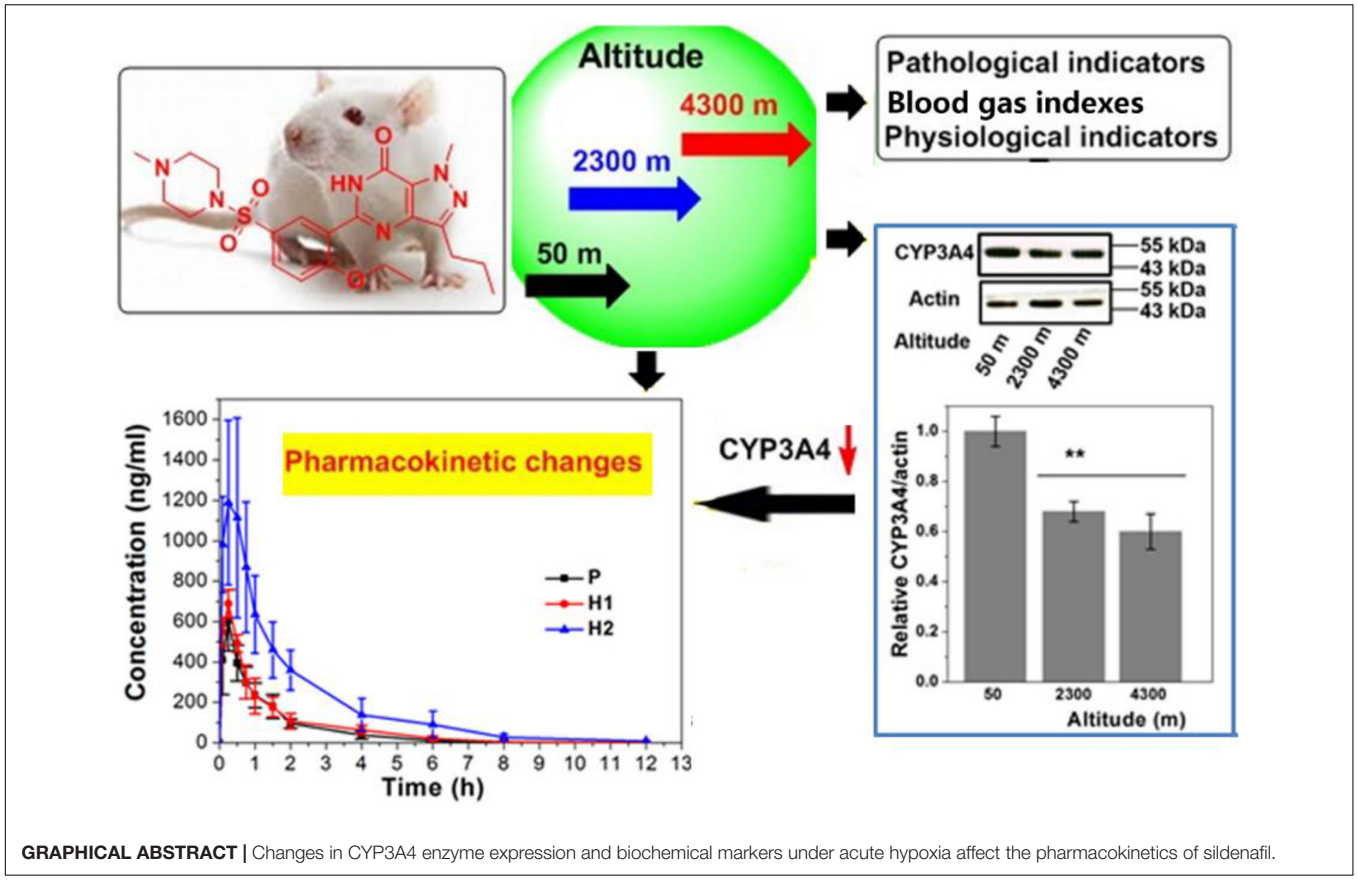
Specialty section:
This article was submitted to Environmental, Aviation and Space Physiology, a section of the journal Frontiers in Physiology

Received: *09 August 2021*
Accepted: *03 January 2022*
Published: *27 January 2022*

Citation:
Zhang J and Wang R (2022) Changes in CYP3A4 Enzyme Expression and Biochemical Markers Under Acute Hypoxia Affect the Pharmacokinetics of Sildenafil. Front. Physiol. 13:755769. doi: 10.3389/fphys.2022.755769

HIGHLIGHTS

- We first demonstrated that the pharmacokinetic changes of sildenafil have a multi-factor regulation mechanism under acute hypoxia.
- We demonstrated that these changes were mainly caused by the decrease in protein expression of CYP3A4 enzyme under acute hypoxic conditions.
- Our investigation provides experimental evidence and new ideas for the rational use of sildenafil under acute hypoxic conditions.



GRAPHICAL ABSTRACT | Changes in CYP3A4 enzyme expression and biochemical markers under acute hypoxia affect the pharmacokinetics of sildenafil.

INTRODUCTION

At present, under high altitude hypoxia conditions, the research on the factors affecting the changes of pharmacokinetic parameters mainly focuses on drug-metabolizing enzymes. The changes in pharmacokinetic characteristics are closely related to the changes in the expression and activity of drug-metabolizing enzymes. The expression and activity of P450 enzymes are affected by hypoxia, which in turn affects the metabolism of related substrates. Previous studies have shown that compared with rats in the plain group, the activity and protein expression of CYP2C9 in the middle-altitude acute hypoxia group and the high-altitude acute hypoxia group have no significant changes, while acute altitude hypoxia may reduce the activity of CYP3A4, which in turn affects the metabolism of its substrate *in vivo* (Jurgens et al., 2002; Duan et al., 2020; Yuan et al., 2020). However, for some enzymes, the degree of influence is inconsistent or controversial, and further experiments are required according to specific conditions. Specifically, under acute hypoxic conditions, the activity of CYP450 enzymes *in vivo* will be affected to varying degrees, further affecting the pharmacokinetic characteristics of the relevant substrates (Ku et al., 2008; Zeng et al., 2017; Sychev et al., 2018), thus studying the expression of CYP450 enzymes and changes in activity are important for the clarification of the metabolic characteristics of the relevant substrate under acute hypoxic conditions. It is a direction worthy of further exploration in the research of rational use of drugs on the plateau.

Sildenafil has been found to have anti-hypoxia effects and is a widely used oral drug (Ku et al., 2008; Bates et al., 2011). It is rapidly absorbed by the gastrointestinal tract after oral administration, and the absolute bioavailability is about 40%. It is mainly cleared by the liver microsomal enzyme CYP3A4, and its second clearance pathway is CYP2C9 (Hyland et al., 2001; Tang et al., 2020). The main N-demethyl metabolite is about half of the parent compound *in vitro*, so about 20% of its pharmacological action may come from N-demethyl metabolites (Hyland et al., 2001; Ku et al., 2008). Changes in drug metabolism and pharmacokinetic properties are important for the pharmacological activity, toxicology, and safety of sildenafil (Francis and Corbin, 2005; Tang et al., 2020; Lee et al., 2021). There are many studies on the pharmacokinetics of sildenafil, but there are no pharmacokinetic data under acute hypoxia (Mekjaruskul and Sripanidkulchai, 2015; Hakamata et al., 2016; Ghoneim and Mansour, 2020; De Bie et al., 2021; Salerno et al., 2021). The change in the metabolic enzymes CYP2C9 and CYP3A4 under acute hypoxic conditions plays an important role in the metabolism of sildenafil, which has aroused great interest in our research. The expression changes and activity changes of CYP2C9 and CYP3A4, which are the main metabolic enzymes of sildenafil (Tang et al., 2020), have a highly important effect on their metabolism under acute hypoxic conditions.

Previous work showed that the expression and activity of the metabolic enzyme CYP2C9 under acute hypoxia were not affected, and the expression of CYP3A4 may be altered (Wang et al., 2017). As part of our continued exploration of high altitude hypoxia-mediated drug metabolism (Zhang et al., 2018, 2019, 2020), the change of CYP3A4 with increasing altitude and the effect of this alteration on the pharmacokinetics of its substrate sildenafil are issues of our concern. Therefore, we report here for the first time that the pharmacokinetic changes of sildenafil under acute hypoxic conditions have a multifactorial regulatory mechanism, and prove that these changes are mainly caused by the decrease of CYP3A4 protein expression under acute hypoxic conditions. Our research provides experimental evidence and new ideas for the rational use of sildenafil under acute hypoxic conditions.

MATERIALS AND METHODS

Chemicals and Reagents

The sildenafil citrate tablets (lot no.1283007) were purchased from Pfizer Pharmaceuticals Ltd (New York NY, United States). The standard substance of sildenafil citrate (lot no. 100783-200401) was obtained from the China Drug and Biologic Product Standardization Station (Beijing, China). HPLC-grade acetonitrile and ammonium formate were purchased from Merck KGaA (Darmstadt, Germany). Other reagents are of analytical grade.

Apparatus

The Ultra-Fast LC (UFLC, Agilent Technologies, United States), API 3200 MS system (Applied Biosystems, United States), an automatic blood gas system (ABL80, Denmark) and automatic biochemistry analyzer (LX20, United States) were used.

Animal Groups

Twenty-four Wistar rats (6–8 weeks, weighing 180–220 g, certificate: 2007000524909) were divided into three groups in random as the low altitude group of 50 m in Shanghai (P), the short-term exposure to high altitude group of 2300 m in Xining of Qinghai province (H1) and the short-term exposure to high altitude group of 4300 m in the Huashixia town of Qinghai province (H2). Rats in group H1 were airlifted from Shanghai to Xining by plane and bus 7 h later, involving a distance of about 2250 kilometers. Rats in the H2 group were traveled from Shanghai to Huashixia Town by plane and bus in 15 h, a journey of about 2900 kilometers. Rats in the H1 and H2 groups started relevant experiments 24 h after reaching the destination. The study of the P group was performed at the second military medical university. All experiments were executed according to the guidelines and regulations of the Chinese Association for Laboratory Animal Sciences.

Measurements of Blood Gas Parameters

The rats were anesthetized by intraperitoneal injection of 10% chloral hydrate, and blood samples were collected from the abdominal aorta for blood gas analysis. The blood gas indicators included blood pH, standard bicarbonate (SBC), buffer base (BB), base access (BE), carbon dioxide partial pressure ($PaCO_2$), arterial oxygen partial pressure (PaO_2), arterial oxygen saturation (SaO_2), Hemoglobin (Hb), Lactic acid (Lac), sodium ion concentration (cNa^+), potassium ion concentration (cK^+) and calcium ion concentration (cCa^{2+}) (Wang et al., 2016).

Pharmacokinetic Study

The treatment methods of the three groups were the same, *i.e.,* after the rats have fasted overnight, the animal dose was converted according to the normal dose taken by the human body, and each rat was given 4.8 mg/kg (aqueous solution) sildenafil by gavage. A series of blood samples (0.2 mL) were collected from the orbital vein at 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, and 24 h after administration and placed in a heparinized centrifuge tube. The blood sample was then centrifuged at 3000 × *g* for 5 min to obtain plasma, which was stored at −20°C until quantitative analysis.

For analysis, aspirate 30 μL of plasma, add 75 μL of acetonitrile, vortex for 1 min, and then centrifuge at 13,000 × *g* for 5 min. Aspirate the supernatant for liquid chromatography-mass spectrometry (LC/MS/MS) analysis.

Measurements of Plasma Biochemical Parameters

Blood was collected from the rat orbital venous plexus and centrifuged at 3000 $r{\cdot}min^{-1}$ for 10 min for biochemical analysis. The indexes included alkaline phosphatase (ALP), total protein (TP), albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TBIL), direct bilirubin (DBIL), C-reaction protein (CRP), total cholesterol (TCHO), blood urea nitrogen (BUN), creatinine (Cr) and uric acid (UA) (Li et al., 2015; Wang et al., 2016).

Observation of Liver Histomorphology and Measure of CYP3A4 by Western Blotting

After the rats were anesthetized, the liver was taken out and washed with 0.9% NaCl saline, and the part was then fixed with 10% formaldehyde. The other part uses western blotting to analyze the expression of CYP3A4.

Data Analysis

Software DAS 2.0 was used to analyze the pharmacokinetic parameters of sildenafil. All data were expressed as mean ± SD. Analysis of statistical significance was performed, and $P < 0.05$ was considered to indicate a statistically significant result. The analysis was carried out using SPSS software, version 13.0.

RESULTS

Method Validation

The mass spectrum scan of sildenafil is shown in **Figure 1**, and the detection ion pair is *m/z* 475.0→99.9. Ion spray

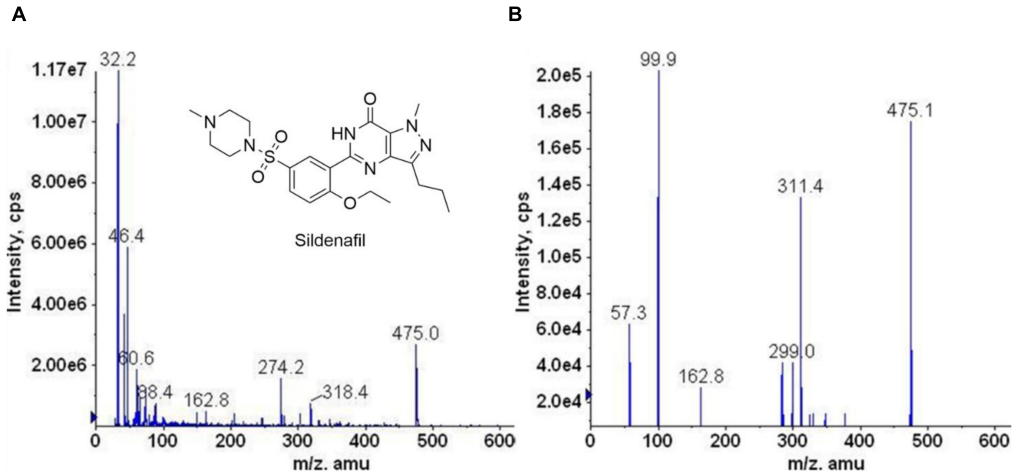


Figure 1 | The chemical structure of sildenafil and its MS/MS spectrum. **(A)** Mass-spectrogram of the parent ion. The illustration shows the chemical structure of sildenafil. **(B)** Mass-spectrogram of fragmentation.

voltage (IS): 5500 V, ion source temperature (TEM): 250°C, collision energy (CE): 38 eV, dissociation voltage (DP): 50 V. The shim-pack XR-ODS column (3.0 mm × 75 mm, 2.0 μm) chromatographic column is performed. The mobile phase is a mixture of acetonitrile: 2 mM ammonium formate (85:15, v/v).

The chromatogram of sildenafil is shown in **Figure 2**. The retention time is 1.33 min, and there is no matrix interference nearby, which is suitable for plasma sample analysis. The standard curve equation is Y = 187X-25.4, and the correlation coefficient is 0.9993. The linear relationship is good in the range of 2.5∼4000 ng·mL^{-1}. Plasma samples of sildenafil were placed at room temperature for 24 h, three times of repeated freezing and thawing, and stored at 4°C for 1 month, and then taken out for determination following the law. The RSD% are 1.09, 0.98, 3.80%, respectively, indicating that the sample is stable under the above conditions. The method is sensitive, accurate, and simple, and can be used to study the pharmacokinetics of sildenafil in rats.

Analysis of Blood Gas Indexes

Changes in physiologic parameters induced by acute hypoxia are shown in **Table 1**. Compared with the low altitude group, the SBC and SaO_2 of the acute exposure group (H1) were significantly decreased by 19.04 and 16.72%, respectively, while the Lac was significantly increased by 54.74% ($P < 0.05$). The SBC, $PaCO_2$, PaO_2, and SaO_2 values of the acute exposure group (H2) were significantly decreased by 3.10, 29.63, and 32.50%, respectively, while the pCO_2 and pO_2 were remarkably increased by 15.09 and 17.81%, respectively ($P < 0.05$).

Pharmacokinetics

This study found significant alterations in the pharmacokinetics of sildenafil under the special environment of high altitude hypoxia. The mean pharmacokinetic parameters of sildenafil are listed in **Table 2**. The concentration-time profiles in plasma obtained from the three groups have similar shapes. **Figure 3** shows mean plasma concentration-time profiles for sildenafil. There were no statistically significant differences in pharmacokinetic parameters between the group P and group H1. Compared with the P group, the area under the curve-time curve (AUC) and the mean residence time (MRT) in the H2 group increased significantly and increased by 213.32 and 72.34% in the 0–24 h. The half-life ($t_{1/2}$) and peak concentration (C_{max}) increased by 44.27 and 133.67%, respectively. Plasma clearance (CL) and apparent volume (V) decreased by 69.13 and 46.75%, respectively. Compared with the H1 group, the AUC and MRT of the H2 group increased significantly and increased by 160.02 and 30.12% within 0–24 h. The $t_{1/2}$ and C_{max} increased by 47.69 and 105.54%, respectively. The clearance and volume distribution values decreased by 61.85 and 44.55%, respectively. It can be seen from the parameter changes that the process of absorption, distribution, metabolism and excretion of sildenafil in rats is changed after acute hypoxia.

Effect of Hypoxia on the Biochemical Indicators

The results of the biochemical analysis are listed in **Table 3**. Compared with the low altitude group, it shows that the AST values of the acute exposure group (H1) were significantly increased. The TP, ALB, AST, ALT, CRP, BUN, and UA levels of the high altitude group (H2) were significantly increased by 13.72 ± 1.61, 12.71 ± 1.29, 28.18 ± 5.15, 54.81 ± 5.15, 18.53 ± 0.83, 13.11 ± 4.09, and 16.45 ± 0.80%, respectively, compared with those in the plain group ($p < 0.05$), but the Cr levels was severely reduced by 30.97 ± 8.18% ($p < 0.05$). The TP, ALB, AST, ALT, CRP, BUN, and UA levels of the H2 group increased by 10.31 ± 1.34, 14.86 ± 0.55, 15.78 ± 8.25, 59.64 ± 7.75, 18.34 ± 3.94, 27.19 ± 1.18, and 23.81 ± 5.23%,

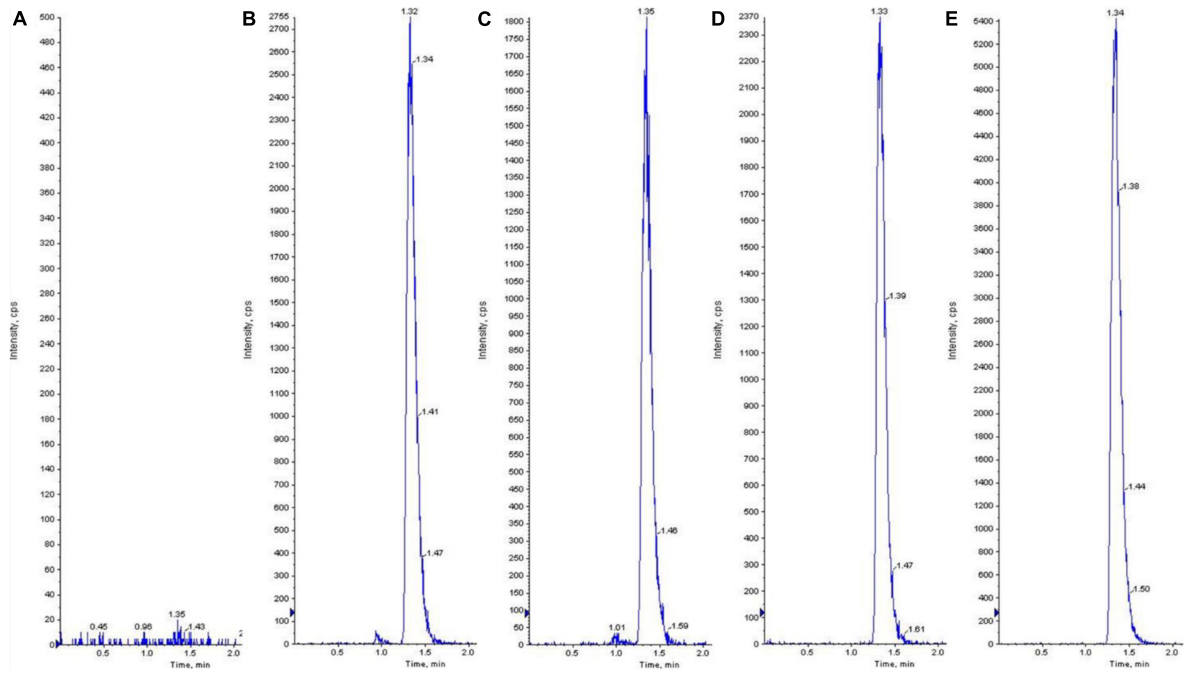


Figure 2 | Determination of the typical chromatograms of sildenafil in rat plasma. **(A)** The samples of blank plasma. **(B)** The blank plasma was spiked with standard sildenafil of 100 ng·mL^{-1}. **(C)** The rat plasma 2 h after intragastric administration of sildenafil at low altitude. **(D)** The rat plasma 2 h after intragastric administration of sildenafil at the high altitude of 2300 m. **(E)** The rat plasma 2 h after intragastric administration of sildenafil at the high altitude of 4300 m.

respectively, compared with the H1 group, whereas the Cr levels significantly decreased.

Comparison of Pathological Changes

The results show significantly pathological changes in the liver among the groups. The liver lobules of group P were integrity and clarity, the liver cells were arranged neatly (**Figure 4A**). Compared with group P, the rats in group H1 and group H2 revealed the liver injury and inflammation, had much inflammatory cell infiltration and edema (**Figures 4B,C**).

TABLE 1 | Results of blood gas analysis in rats at different groups (mean ± SD, $n = 8$).

Blood gas indicators	Low altitude (P)	Acute exposure (H1)	Acute exposure (H2)
pH	7.43 ± 0.03	7.37 ± 0.04	7.45 ± 0.02
SBC (mmol/L)	25.63 ± 0.82	21.53 ± 0.25*	22.17 ± 0.51*
BB (mmol/L)	1.58 ± 0.57	−1.85 ± 0.29*	−2.86 ± 1.19*
BE (mmol/L)	1.42 ± 0.73	−2.2 ± 0.52*	−5.30 ± 1.15*
$PaCO_2$ (mmHg)	41.83 ± 2.39	40.50 ± 3.67	26.90 ± 1.40*#
PaO_2 (mmHg)	102.13 ± 2.62	87.5 ± 4.59*	55.91 ± 4.77*#
SaO_2 (%)	98.30 ± 0.54	96.33 ± 0.85	90.24 ± 2.08*#
Hb (g/dL)	12.99 ± 0.35	13.32 ± 0.87	15.87 ± 1.00*
Lac (mmol/L)	1.37 ± 0.28	2.12 ± 1.04*	2.90 ± 0.95*#
cNa^+(mmol/L)	140.59 ± 1.29	140.86 ± 1.06	145.73 ± 1.74*#
cK^+(mmol/L)	4.19 ± 0.37	4.35 ± 0.53	4.30 ± 0.21
cCa^{2+}(mmol/L)	1.19 ± 0.05	1.32 ± 0.05*	1.30 ± 0.04*

*$P < 0.05$ versus the low altitude group.
#$P < 0.05$ versus the H1 group.

Effects of High Altitude on CYP3A4 Protein Expression

Protein expression levels of CYP3A4 in the liver were studied using western blot (**Figure 5**). The results showed a remarkable decrease in the level of CYP3A4 protein in 2300 and 4300 m groups. High altitude could significantly down-regulate the protein expression of CYP3A4 compared to the plain group. The values for group 4300 m were significantly decreased by 40% compared with group 50 m ($p < 0.05$).

DISCUSSION

First, the blood gas index of each group of rats was measured, because its changes can prove the hypoxia of the body and affect the pharmacokinetic characteristics of the drug (Wang et al., 2016). Previous studies have shown that hypoxia increases hemoglobin levels in rats and cells (Grek et al., 2011; Ivy et al., 2021). This study also found that acute hypoxia reduced SaO_2, $PaCO_2$, PaO_2, and SBC, and increased Lac and Hb. It indicates that the body has symptoms of compensatory respiratory alkalosis and hypoxemia (Palmer, 2012; Brinkman and Sharma, 2021), and it is proved that the rats are in an anoxic state. Blood gas analysis also showed that changes in Na^+ concentration in the H2 group may affect the absorption function of the gastrointestinal tract, which may

TABLE 2 | The main pharmacokinetic parameters of sildenafil in rats at low altitude and after acute exposure to high altitude (Mean ± SD, n = 8).

Parameters	Low altitude (P)	Acute exposure (H1)	Acute exposure (H2)
AUC_{0-24}/μg·L^{-1}·h^{-1}	747.96 ± 78.34	901.26 ± 65.78	2343.43 ± 131.20*#
AUC_{0--8}/μg·L^{-1}·h^{-1}	751.81 ± 76.90	906.58 ± 66.31	2356.57 ± 131.29*#
MRT_{0-24}/h	1.41 ± 0.18	1.66 ± 0.32	2.16 ± 0.74*#
MRT_{0--8}/h	1.47 ± 0.21	1.72 ± 0.34	2.25 ± 0.77*#
$t_{1/2}$/h	1.07 ± 0.22	1.30 ± 0.39	1.92 ± 0.73*#
T_{max}/h	0.25 ± 0.00	0.25 ± 0.00	0.28 ± 0.19
CL/L·h^{-1}	83.52 ± 11.86	67.57 ± 10.07	25.78 ± 4.22*#
Vd/L	133.07 ± 27.19	127.80 ± 24.37	70.86 ± 18.13*#
C_{max}/μg·L^{-1}	605.83 ± 41.85	688.75 ± 57.31	1415.67 ± 84.40*#

*$P < 0.05$ versus the low altitude group.
#$P < 0.05$ versus the H1 group.

further affect drug absorption. Subsequent studies examined the pharmacokinetic characteristics of sildenafil. Before the evaluation of pharmacokinetics, an LC-MS/MS method for the determination of sildenafil was established, which is superior to previous analytical methods in terms of analysis time and accuracy. Pharmacokinetic experiments show that acute high altitude hypoxia does affect the pharmacokinetic characteristics of sildenafil: including absorption, distribution, metabolism, and excretion, and the degree of influence is related to the altitude. The change of Na^+ concentration in the blood gas index affects the AUC and Cmax of the drug, which mainly affects the absorption process.

Secondly, we analyzed the biochemical indicators of rats because it is a method to assess changes in blood sugar, proteins, enzymes, and metabolites in the body. At the same time, it is an important indicator to evaluate liver and kidney function and drug clearance rate (Li et al., 2015). The results of this experiment showed significant changes in biochemical indicators, especially the decrease in renal clearance. This is one of the important reasons for the decreased clearance of sildenafil in rats after acute hypoxia, *i.e.*, changes in biochemical indicators mainly affect the distribution, clearance, and excretion of sildenafil. Several studies have shown that high-altitude environments have varying degrees of impact on human physiological functions and their circadian rhythms, brain functions, and behavioral functions (Ma, 1988; Rostrup et al., 2005; Xie et al., 2020; Wei et al., 2021). Therefore, it is expected that similar results may be observed in humans in this study. The pharmacokinetic study of sildenafil in high-altitude populations is currently underway. In addition, it should be pointed out that this study alone cannot fully recommend clinical dosages. Thus, health care providers can consider recommending that patients with acute hypoxia should pay attention to medication and seek medical attention if they have adverse reactions.

Finally, this study analyzed the pathological changes of rat liver tissue and the expression of CYP3A4. After acute hypoxia, rat liver tissue has many inflammatory cell infiltration and edema, which may affect the metabolism of the drug (Wang et al., 2017). The analysis of CYP3A4 protein expression further indicated that acute hypoxia significantly down-regulated the protein expression of CYP3A4. The half-life of the substrate sildenafil is prolonged, and the average residence time in the

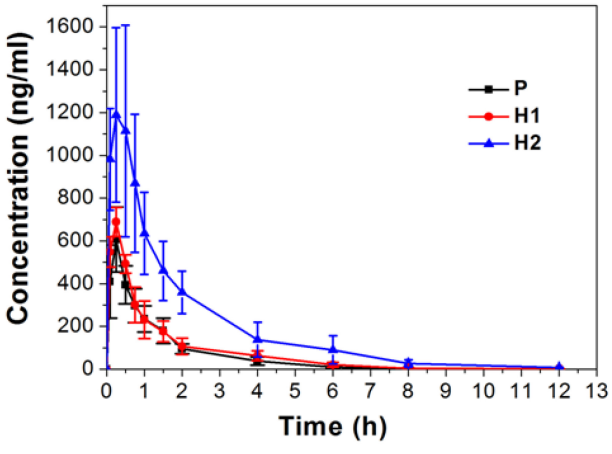


Figure 3 | Plasma concentration-time profiles of sildenafil in rats at low altitude and after acute exposure to high altitude.

TABLE 3 | The comparison of main biochemical parameters between the three groups (mean ± SD, n = 8).

Biochemical parameters	Low altitude (P)	Acute exposure (H1)	Acute exposure (H2)
ALP (IU/L)	106.75 ± 13.29	101.50 ± 23.34	110.38 ± 26.22
TP (g/L)	52.13 ± 1.66	53.74 ± 1.78	59.28 ± 2.50*#
ALB (g/L)	22.43 ± 0.75	22.01 ± 0.92	25.28 ± 1.04*#
AST (IU/L)	79.38 ± 5.26	87.88 ± 16.68*	101.75 ± 9.35*#
ALT (IU/L)	33.75 ± 3.24	32.73 ± 5.97	52.25 ± 31.32*#
TBIL (μmol/L)	7.84 ± 0.70	8.20 ± 0.63	8.30 ± 0.19
DBIL (μmol/L)	−0.68 ± 0.12	−0.79 ± 0.31	−1.25 ± 0.73*#
CRP (mg/mL)	12.41 ± 1.05	12.43 ± 1.53	14.71 ± 2.02*#
TCHO (mmol/L)	1.19 ± 0.05	1.18 ± 0.15	1.39 ± 0.31
BUN (mmol/L)	4.88 ± 0.39	4.34 ± 0.71	5.52 ± 1.22*#
Cr (μmol/L)	20.30 ± 3.89	21.38 ± 3.44	15.50 ± 2.23*#
UA (μmol/L)	154.19 ± 8.83	145.03 ± 11.7	179.56 ± 21.10*#

*$P < 0.05$ versus the low altitude group.
#$P < 0.05$ versus the H1 group.

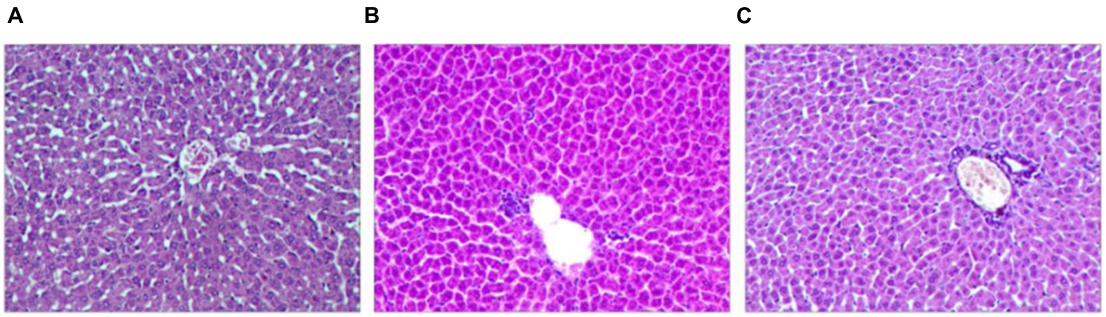


Figure 4 | Hematoxylin and eosin staining of rat liver tissue (×200). **(A)** The plain group(P). **(B)** The high altitude group (H1). **(C)** The high altitude group (H2).

body is prolonged, indicating that its metabolism is slowed down. Thus, down-regulation of CYP3A4 protein expression is closely related to the slowing down of sildenafil metabolism (De Denus et al., 2018).

Sildenafil has antioxidant and anti-fatigue properties (Uthayathas et al., 2007; Maziero Alves et al., 2021), it thus may be suitable for people who exercise at altitude. Due to the increasing popularity of sildenafil at high altitude areas, it is important to understand the effects of location altitude on the body and the drug pharmacokinetics. This study demonstrates that most of the pharmacokinetic parameters of sildenafil have changed under acute hypoxic conditions, and there are many influencing factors, and there is a certain correlation between these factors. For example, physiological and pathological changes in the body and changes in metabolic enzymes are important factors influencing the changes in the pharmacokinetic parameters of sildenafil. The concentration of the drug in the body must be maintained within the therapeutic window to play its due role. Although there are reports that food reduces the rate and extent of systemic exposure to sildenafil, these reductions are not clinically meaningful (Nichols et al., 2002). In addition, compared with humans, the apparent volume of distribution of sildenafil in rats has no significant change, but the clearance rate is increased and the elimination half-life is shortened (Nichols et al., 2002). Thus, our current results are necessary for reference when conducting subsequent human trials. In aggregate, this study provides a valuable reference and new ideas for the study of drug metabolism under acute hypoxic conditions and guides clinical rational drug use and personalized drug use in high altitude areas.

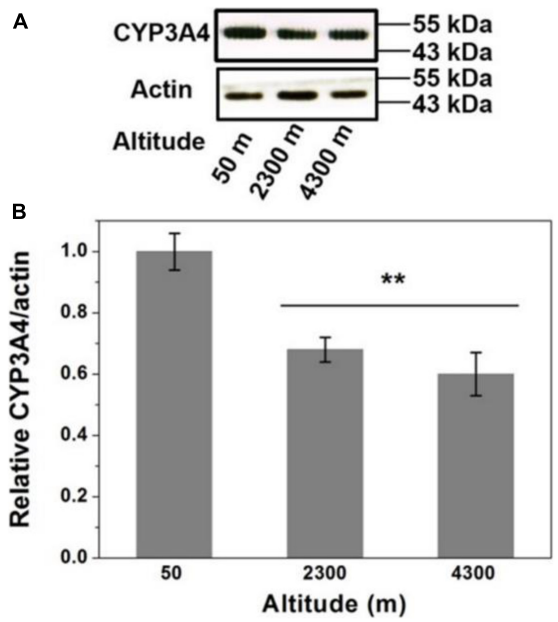


Figure 5 | Western blots analysis of protein expression of CYP3A4 in the liver. **(A)** Downregulation of protein expression of CYP3A4 in high altitude. Actin was used as a loading control. **(B)** The quantification of the blots. The data are expressed as the means ± SE of three experiments. $^{**}P < 0.01$ versus the low altitude group (50 m).

CONCLUSION

In conclusion, we demonstrated that the acute hypoxia down-regulates the protein expression of the metabolic enzyme CYP3A4, and at the same time demonstrates that there is a multi-factor regulation mechanism in the changes of the substrate sildenafil pharmacokinetic process, which is closely related to the changes of blood gas, biochemical indicators and metabolic enzymes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by 940th Hospital of Joint Logistics Support Force of CPLA.

AUTHOR CONTRIBUTIONS

JZ and RW designed the research, analyzed the results, and performed most laboratory experiments. JZ drafted and revised the manuscript, analyzed the results, drafted the figure, performed part laboratory experiments and assisted all laboratory experiments. JZ and RW. Both authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (82001995 and 81673508), Natural Science Foundation of Gansu Province (20JR10RA010), Macao Young Scholars Program (AM2020018), Northwest Normal University Ph.D. Research Startup Fund (202003101602), and Northwest Normal University Young Teachers' Research Ability Improvement Program (NWNU-LKQN2021-06).

ACKNOWLEDGMENTS

We want to express their sincere thanks to the Second Military Medical University, the Fourth People's Hospital of Xining and Army Service Station of Huashixia of Qinghai province for their support, transportation, and housing at the investigation site to facilitate this study. We are also grateful to Junyi Ma and Kun Sun for their help in preparing and revising our manuscript.

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ORIGINAL RESEARCH
published: 04 May 2022
doi: 10.3389/fphys.2022.864222

Thickened Retinal Nerve Fiber Layers Associated With High-Altitude Headache

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OPEN ACCESS

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Specialty section:
This article was submitted to Exercise Physiology, a section of the journal Frontiers in Physiology

Received: 28 January 2022
Accepted: 01 April 2022
Published: 04 May 2022

Citation:
Yin X, Li Y, Ma Y, Xie Y, Wang K, Sun D, Liu X, Hao M, Liang M, Zhang S, Guo Y, Jin L, Wang N and Wang J (2022) Thickened Retinal Nerve Fiber Layers Associated With High-Altitude Headache. Front. Physiol. 13:864222. doi: 10.3389/fphys.2022.864222

Purpose: This study aimed to quantify the different quadrants of the optic nerve head (ONH) and macular parameters and their changes during exposure to high altitude, and to assess their correlation with high-altitude headache (HAH).

Methods: Spectral-domain optical coherence tomography (OCT) was used to quantify changes in the retinal structure in 109 healthy subjects during acute exposure to high altitude (3,700 m). Self-reported symptoms of HAH and acute mountain sickness AMS were assessed using Lake Louise Score (LLS), alongside measurements of physiological parameters (oxygen saturation [SpO_2], heart rate [HR], hemoglobin level [Hb], and red blood cell [RBC] count). Measurements were taken before and after exposure to the high-altitude environment. The correlations of these parameters and changes at ONH were examined.

Results: With the exposure to high altitude, the incidence of AMS was 44.0% and the frequency of HAH was 67.0% (54.1% mild, 12.9% moderate-severe). As for systemic parameters measured at high altitude, the participants exhibited significantly lower SpO_2, higher resting HR, higher Hb, and a higher RBC (all $p < 0.05$). Key stereometric parameters used to describe ONH [superior, inferior, nasal, temporal, and mean retinal nerve fiber layer (RNFL) thickness] and macula (macular thickness) increased at high altitude compared with baseline. Most parameters of ONH changed, especially superior, inferior, and mean RNFL thickness ($p < 0.05$). There was a significant correlation between the ratios of RNFL at ONH and HAH [mean thickness ($r = 0.246$, $p = 0.01$); inferior ($r = 0.216$, $p = 0.02$); nasal ($r = 0.193$, $p = 0.04$)]. No associations between parameters of ONH and AMS or LLS were observed.

Conclusion: The high-altitude environment can increase RNFL thickness at ONH. Furthermore, we found that the ratios of mean thickness, inferior area, and nasal area correlated positively with HAH, which provides new insights for understanding of the underlying pathological mechanisms of high-altitude retinopathy (HAR).

Keywords: headache, high-altitude headache, HAH, retinal nerve fiber layer, RNFL, OCT

INTRODUCTION

High-altitude headache (HAH) is the most frequent, most unpleasant, and sometimes only symptom experienced when rapidly ascending from sea level to high altitude (Serrano-Dueñas, 2005; 2007; Burtscher et al., 2011; Carod-Artal, 2014; Wang et al., 2018). According to the widely accepted Lake Louise Consensus scoring system, which was revised in 2018, HAH is also the core symptom of acute mountain sickness (AMS) (Roach et al., 2018). HAH, as defined by the International Headache Society, occurs within 24 h after rapidly ascending to high altitude and is resolved within 8 h after descending; it can be so severe that it can induce life-threatening, high-altitude cerebral edema (HACE) or high-altitude pulmonary edema (HAPE) (Headache Classification Subcommittee of the International Headache Society, 2004; Headache Classification Committee of the International Headache Society, 2013; Lopez et al., 2013; Guo et al., 2017). Given that the incidence of HAH is approximately 80% (Headache Classification Subcommittee of the International Headache Society, 2004; Wilson et al., 2009; Lopez et al., 2013; Marmura and Hernandez, 2015) in subjects who rapidly ascend to high altitude, HAH has become a public health problem that requires urgent resolution (Queiroz and Rapoport, 2007).

The underlying pathological mechanism of HAH is not clear, which is deemed to be an ongoing pathophysiological process of AMS and HACE (Basnyat, 2005). Direct or indirect evidence has shown that increased intracranial pressure (ICP) is the main cause of high-altitude illness, including HAH, AMS, HACE, and high-altitude retinopathy (HAR) (Wilson et al., 2014). Noninvasive methods for direct measuring of ICP do not yet exist. However, parameters of the retina may be ideal candidates for noninvasive indirect assessment of ICP for anatomical (closely adjacent to intracranial tissues) and technical (highly amenable to acquisition) reasons (Clarke et al., 2019). Papilledema (optic disc edema, ODE) refers to the swelling of the optic disc, which clinically serves as a biomarker of increased ICP (Chen and Bhatti, 2019; Eikenberry et al., 2020). ODE was first reported in 1969 as a clinical manifestation of HAR and has been linked with HACE (Singh et al., 1969).

The present study was based on a hypothesis that some retinal parameters may be related to HAH. Accordingly, we explored the association between quantitative measurements of the retina nerve fiber layer (RNFL) and HAH. After collecting demographic information, we performed a repeated measurement of retinal fundus changes and collected physical parameters before and after high-altitude ascent. LLS scores were obtained from 109 healthy young Han Chinese males within 24 h after high-altitude ascent.

MATERIALS AND METHODS

Participants

A total of 109 subjects (all male; mean age: 19.6 years, SD: 1.7 years) lived at 50 m and traveled to Tibet by plane were recruited to our observational cohort study. To participate in the study, subjects had to be healthy Han Chinese men between the age of 18 and 35 years, and no high-altitude exposure in recent 2 years. All of the subjects completed a self-reported questionnaire (structured case report forms, CRFs) to report their disease status and medical history. We did not include participants who had suffered from cardio-cerebrovascular, respiratory, ophthalmic, or migraine diseases, or had had a headache or cold, or were taking any medications during ascent to high altitude. To motivate and recruit subjects, the purpose of the study was explained in detail to all of the subjects who volunteered for participation, and all of the participants signed an informed consent before the examinations. The Human Ethics Committee of Fudan University approved the protocol.

Study Procedures and Measurements

All of the participants underwent baseline examination 1 week before departure and within 24 h after their arrival at 3,700 m. Demographic (age, body mass index (BMI), smoking and drinking history), physiological [heart rate at rest (HR, beats/min), oxygen saturation (SpO_2, %)], and hematological data [hemoglobin level (Hb, g/L) and red blood cell count (RBC, $*10^9$)] were obtained. To measure the severity of HAH and AMS, the Lake Louise Scoring (LLS) system for acclimatization grading was used. Using the self-reported questionnaire, Lake Louise points were assigned on a 0 to 3 scale for headache, gastrointestinal symptoms, fatigue, and dizziness. All subjects with a headache and LLS ≥3 were considered to have AMS (Roach et al., 2018).

All optical coherence tomography (OCT) scans were acquired by the same operator using the Cirrus OCT device (Cirrus 5,000, Carl Zeiss Meditec Inc. Dublin, California, United States; software version 6.5.0772). The Cirrus HD-OCT 5,000 has an A-scan velocity of 27,000 scans/second with a 5 μm axial resolution and a scanning depth of 2 mm. The instrument uses light of 840 nm wavelength, and images of the optic disc and macula. The optic disc scan was centered on the optic disc (optic disc tube 200 × 200 protocol), while macular scan was centered on the fovea (macular cube 512 × 128 protocol). To eliminate binocular confounding factors, we only included the left eye. Only high-quality images (signal strength ≥6) of the left eye in each participant were taken. The scans were used to measure RNFL thickness and macular thickness. RNFL thickness was automatically calculated by the fast RNFL procedure. The software allows the mapping of thickness data on a quadrant-by-quadrant basis. We considered the average values of three different measurements per quadrant (superior, inferior, nasal, and temporal), and the overall data obtained in all of the quadrants were used to determine the average RNFL thickness (**Figure 1**). OCT software calculates macular retinal thickness as the distance between the first signal from the inner limiting membrane and the signal from the anterior boundary of the retinal pigment epithelium. The map of the early treatment diabetic retinopathy study (ETDRS) grid is composed of nine sectorial thickness measurements in three concentric circles with diameters of 1 mm, 3 mm, and 6 mm. The area bounded by the outer (6 mm) and middle (3 mm) circles forms the outer ring, and the area bounded by the middle (3 mm) and inner circles (1 mm) forms the inner ring. The central 1-mm circular region represents the foveal area (**Figure 2**).

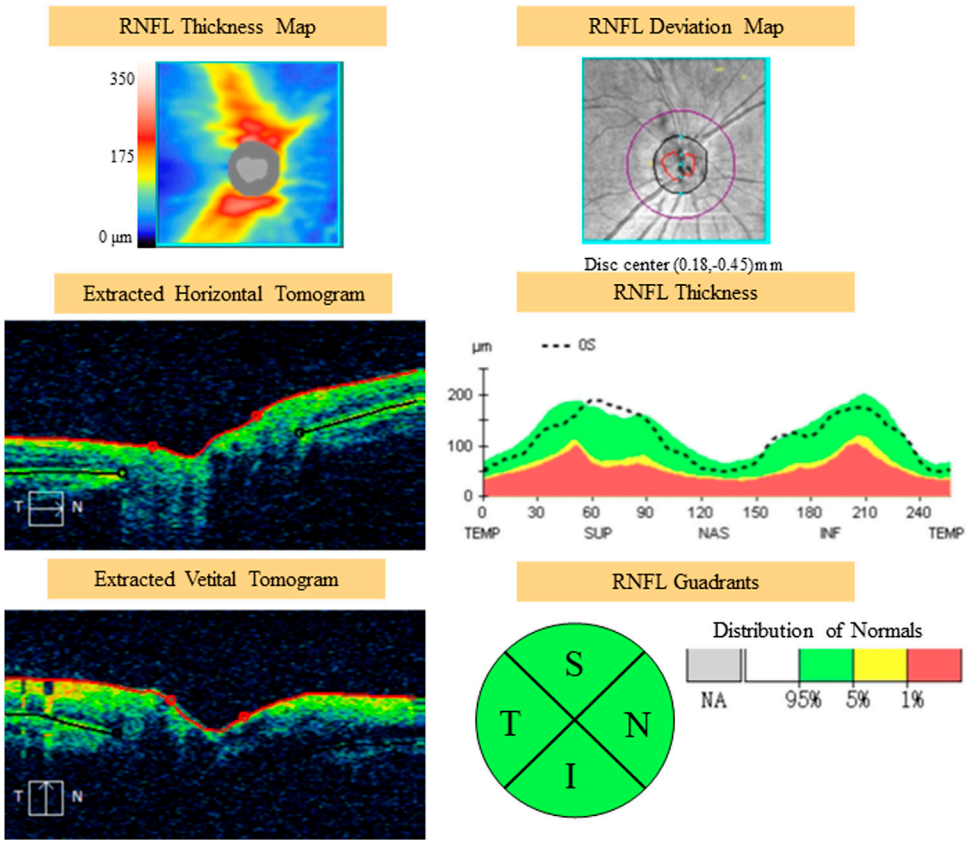

FIGURE 1 | Example of scanning and measurements of RNFL with OCT. RNFL, retinal nerve fiber layer; OCT, optical coherence tomography; S, superior area; I, inferior area; N, nasal area.

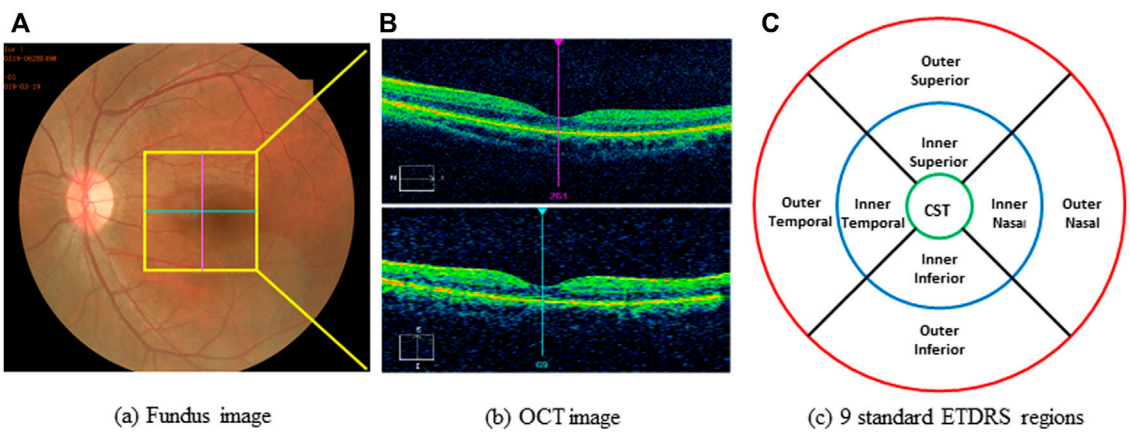

FIGURE 2 | Example of scanning and measurements with OCT. **(A)** funds photograph of subject; yellow box indicates the area of macula. **(B)** OCT image of macula. **(C)** Standard of ETDRS map, divided into 9 regions with 3 concentric rings measuring 1 mm (green ring), 3 mm (blue ring), 6 mm (red ring). ETDRS, early treatment diabetic retinopathy study; OCT, optical coherence tomography; CST, center subfield thickness.

Statistical Analyses

Continuous data were presented as the means ± standard deviations (SDs), and their normality was assessed by the Shapiro–Wilk test. Categorical variables were presented as percentages. Differences between baseline and high altitude were analyzed using a paired-sample Student's *t* test for Gaussian distribution. When Gaussian distribution was not satisfied for continuous variables, the nonparametric Mann–Whitney *U* test was used to compare the

TABLE 1 | Demographic characteristics and systemic parameters of the study participants (*N* = 109).

Characteristics		Sea Level	High Altitude	*p*
Age, years	—	19.6 (1.7)	—	—
BMI, kg/m^2	—	23.4 (2.4)	—	—
Smoking, yes (%)	—	70 (64.2%)	—	—
Drinking, yes (%)	—	48 (44.0%)	—	—
LLS	—	0	2.4 (2.0)	—
AMS	—	0	48 (44.0%)	—
Headache	None	0	36 (33.0%)	—
	Mild	0	59 (54.1%)	—
	Moderate	0	11 (10.1%)	—
	Severe	0	3 (2.8%)	—
HR (beats/min)	—	69.0 (8.8)	86.1 (10.5)	<0.001**
SpO_2 (%)	—	98.3 (1.6)	83.0 (15.1)	<0.001**
Hemoglobin (Hb) [g/L]	—	152.6 (9.4)	159.9 (21.2)	<0.001**
RBC (*10^9)	—	5.03 (0.28)	5.34 (0.62)	<0.001**

Continuous variables are presented as mean ± standard deviation and categorical variables are presented as percentages, compared using a paired-sample t *test. Non-normally distributed variables were compared using the Mann–Whitney* U *test.*
p *value <0.05;* ***p** *value <0.001.*

high altitude. In terms of the hematological measurements, Hb and RBC showed a significant increase ($p < 0.001$; **Table 1**).

Changes in RNFL and Macular Thickness in the Peripapillary Sectors and ETDRS Grid, Respectively

In this study, we investigated the retina of the subjects before and after ascent to high altitude. As shown in **Table 2**, there were evident changes in the thickness of the RNFL and macular thickness in the participants after their ascent to high altitude. In the optic disc, there was also a significant increase in the thickness of the RNFL in the superior and inferior quadrants and mean RNFL ($p < 0.05$), with an insignificant increase in RNFL thickness in the nasal and temporal quadrants ($p > 0.05$). There was a significant increase in macular thickness in the outer superior and outer nasal quadrants ($p < 0.05$). However, the central subfield thickness exhibited no significant decrease ($p < 0.05$). Changes in thickness of the RNFL in the peripapillary sectors were evident following ascent to high altitude. No

TABLE 2 | Retinal nerve fiber layer of the optic disc and macular thickness at baseline and after the ascent to high altitude (x ± s, μm).

Characteristics	Parameters	Baseline	High Altitude	*p*
Optic disc	Superior area	131.45 (15.09)	136.34 (16.52)	0.024*
	Inferior area	126.51 (30.65)	131.02 (19.51)	0.028*
	Nasal area	63.83 (10.03)	66.25 (10.41)	0.061
	Temporal area	74.99 (12.14)	76.16 (11.86)	0.437
	Mean thickness	99.35 (8.84)	101.84 (9.08)	0.030*
Macular thickness	Central subfield thickness	243.77 (19.37)	242.95 (19.69)	0.925
	Inner superior	327.32 (30.98)	327.93 (13.16)	0.119
	Inner inferior	319.57 (15.69)	323.78 (13.81)	0.054
	Inner nasal	311.25 (33.24)	312.98 (11.87)	0.106
	Inner temporal	324.76 (14.67)	328.29 (13.48)	0.089
	Outer superior	281.85 (13.46)	287.1 (13.47)	0.005**
	Outer inferior	270.22 (14.14)	273.51 (14.21)	0.089
	Outer nasal	266.14 (12.73)	269.27 (11.83)	0.045*
	Outer temporal	301.84 (18.28)	306.74 (15.09)	0.053
	Mean thickness	99.45 (11.07)	103.26 (8.96)	0.017*

baseline and high-altitude measurements. To evaluate possible correlations between changes at ONH, HAH, or AMS parameters, Spearman correlations were used. The significance level (two-tailed) was set at 0.05.

RESULTS

Subjects' Characteristics

All of the 109 participants completed the self-reported questionnaire, and systemic and hematological measurements at sea level and at high altitude were obtained. The incidence rates of AMS and HAH following acute exposure to high altitude were 44.0 and 67.0%, respectively. AMS scores within 24 h after arriving at high altitude ranged from Lake Louise scores of 0–10, with an average of 2.4. As for the physiological measurements, SpO_2 decreased from 98.3 to 83.0% ($p < 0.001$), while HR significantly increased from sea level to

significant differences were observed between HAH and non-HAH subjects (**Figure 3**).

Relationship Between the Changes of the Optic Disc Parameters and HAH

Spearman correlation analyses were used to explore the relationships between the optic disc measurements and HAH severity. The correlation analysis revealed a significant correlation between the optic disc measurements and HAH severity. HAH severity significantly correlated with the ratio of mean thickness ($r = 0.246$, $p = 0.01$ in **Figure 4A**), inferior thickness ($r = 0.216$, $p = 0.02$ in **Figure 4C**), and nasal thickness ($r = 0.193$, $p = 0.04$ in **Figure 4D**). The ratios of superior and temporal RNFL thickness did not display a significant correlation with HAH severity ($p > 0.05$ in **Figures 4B,E**). However, the ratios of mean RNFL thickness did not significantly correlate with AMS (**Figure 4F**), LLS scores

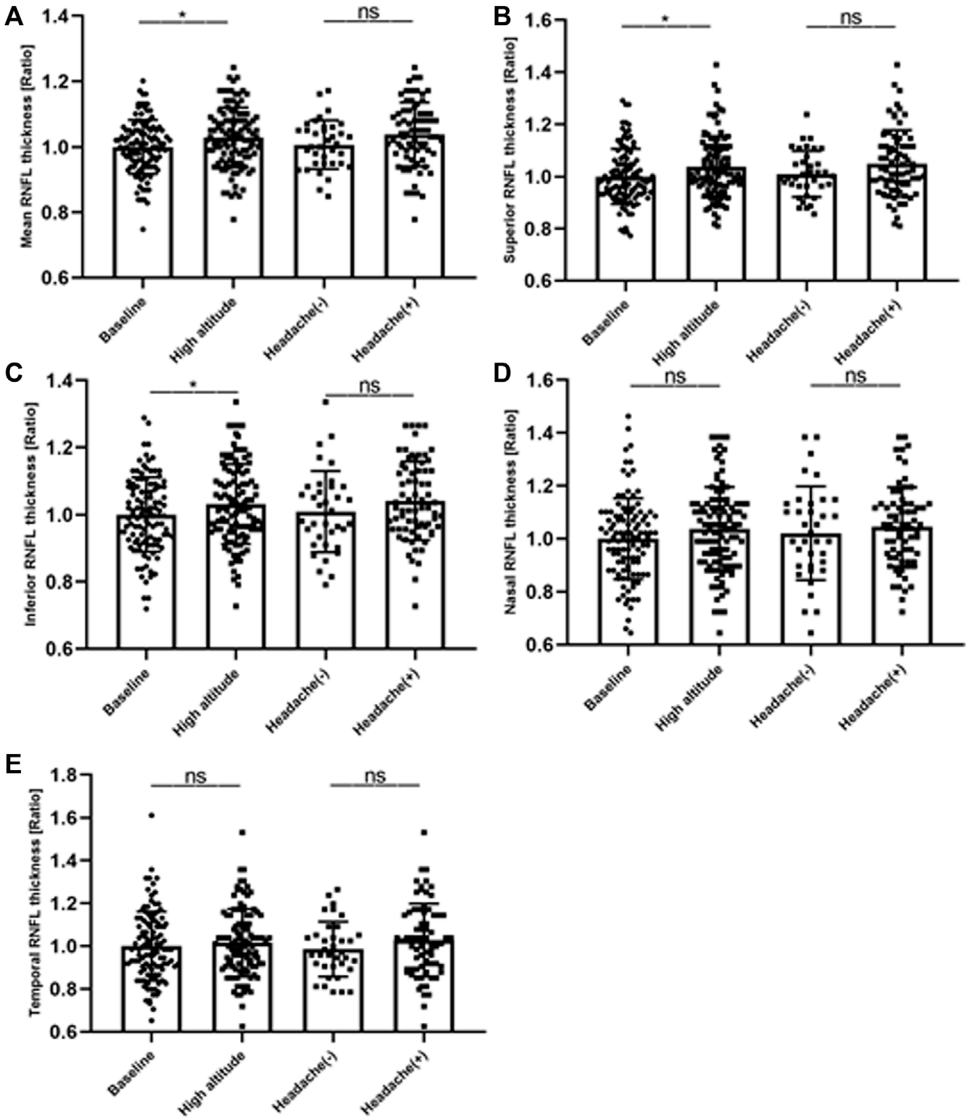

FIGURE 3 | Changes in thickness of RNFL in peripapillary sectors. Intraindividual changes (expressed as ratios) at high altitude. **(A)** mean RFNL thickness, **(B)** superior RNFL thickness **(C)** inferior RNFL thickness, **(D)** nasal RNFL thickness and **(E)** temporal RNFL thickness. RNFL, retinal nerve fiber layer; HAH (+), high altitude headache (HAH); (HAH) (–), none-HAH; *p value indicates $p < 0.05$; **$p < 0.01$.

(**Figure 4G**), gastrointestinal symptoms, fatigue, dizziness, hemoglobin, or RBC (**Supplementary Figure S1**).

DISCUSSION

Alterations in RNFL of the Optic Disc and Macular Parameters

This study was undertaken to objectively quantify structural changes in the optic disc and macula during acute, high-altitude exposure to low atmospheric oxygen, low humidity, and strong ultraviolet radiation. In this study, OCT was used to determine alterations in the retinal morphology, including macular thickness and the RNFL. Evaluating changes in the optic disc that are highly related to the structure of the brain can provide a better understanding of the pathophysiology of HAH.

According to the findings of the RNFL parameters of the optic disc during exposure to high altitude, young Chinese males enrolled in this study had a thicker RNFL in the superior and inferior areas and a thinner RNFL in the nasal and temporal areas, which is consistent with histological changes of the RNFL reported in previous studies (Hsu and Tsai, 2008; Ascaso et al., 2012). A week before the subjects entered Tibet, the RNFL thickness in each quadrant of the optic disc exhibited different degrees of thickening after rapid ascent to high altitude, especially in the superior and inferior quadrants, which is in agreement with Tian et al. (2018). However, we did not assess the effects of long-term exposure to high altitude and subsequent return to low altitude using

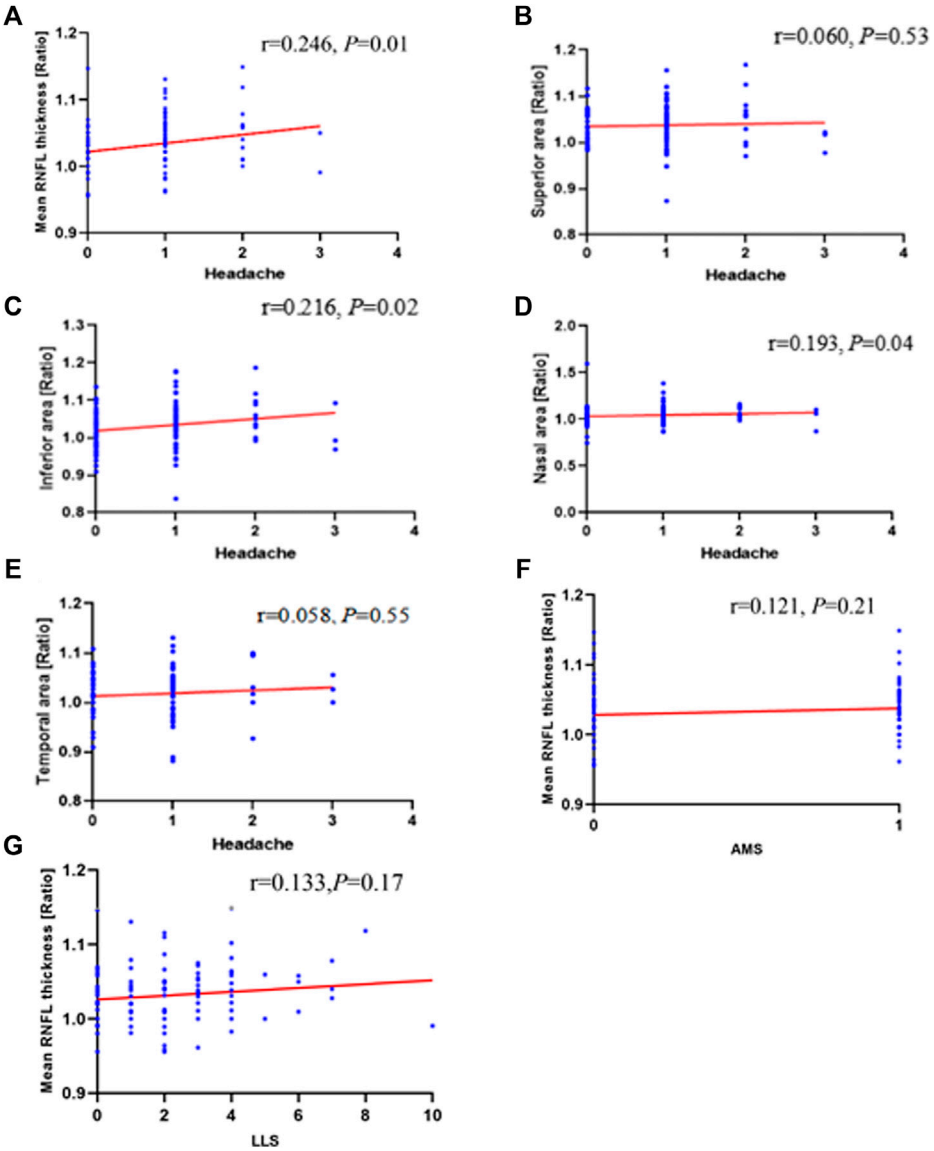

FIGURE 4 | Correlation of changes in ONH with HAH and AMS parameters during high-altitude exposure. Correlation analyses between **(A)** mean retinal nerve fiber layer (RNFL) thickness ratios ($r = 0.246$; $p = 0.01$), **(B)** superior area thickness ratios ($r = 0.060$; $p = 0.53$), **(C)** inferior area thickness ratios ($r = 0.216$; $p = 0.02$), **(D)** nasal area thickness ratios ($r = 0.193$; $p = 0.04$), **(E)** Temporal area thickness ratios ($r = 0.058$; $p = 0.55$) and headache. Mean retinal nerve fiber layer (RNFL) thickness ratios does not correlate with **(F)** AMS ($r = 0.121$; $p = 0.21$) or **(G)** LLS ($r = 0.133$; $p = 0.17$). ONH, optic nerve head; HAH, high-altitude headache; AMS, acute mountain sickness; LLS, Lake Louise score.

OCT. In the studies by Tian (Tian et al., 2018) and Willmann (Willmann et al., 2011), the optic nerve did not show permanent damage after short-term exposure, which may indicate that HAR is a benign high-altitude illness.

Our results indicated that the thickness of the macula notably increased, and the outer superior and outer nasal zones were much thicker than at baseline. A study on macular thickness conducted by Fischer et al. quantified macular structure in 14 healthy subjects before and after ascent to high altitude; the authors found a minor increase in total retinal thickness (Fischer et al., 2012). Tian et al. adopted OCT to scan the retinal structure of 91 healthy subjects after 1-month exposure to high altitude (4,600 m above the sea level), and their results indicated a significant increase in RNFL thickness in the superior and inferior zones (Tian et al., 2018). However, previous OCT studies on retinal changes associated with high-altitude exposure and AMS showed no significant alteration in any of the ETDRS subfields (Ascaso et al., 2012; Fischer et al., 2012). Our present findings indicated that acute exposure to high altitude did not result in macular edema, but it did increase the perimacular thickness of ETDRS. This indicates that the macular region has a superior self-regulation potential to meet the demands of oxygenation upon acute exposure to high altitude in healthy subjects.

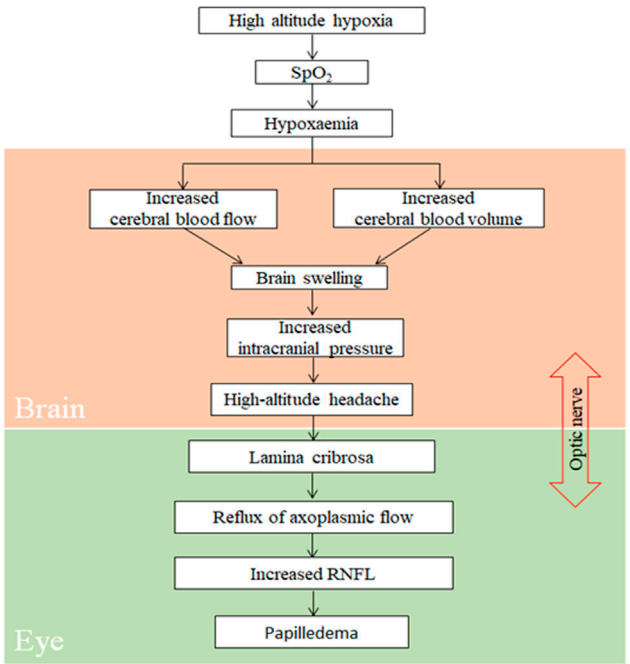

FIGURE 5 | Proposed pathophysiology of retinal nerve fiber layer during exposure to high altitude. Spo2, oxygen saturation; RNFL, retinal nerve fiber layer.

The Underlying Mechanism of Increasing RNFL Induced by HAH

Consistent with the results of other studies (Bian et al., 2013; Shin, 2014), the incidence of HAH, a core symptom of AMS, was 67.0% in our participants. To objectively and quantitatively analyze correlation between parameters of RNFL at ONH and HAH, AMS, or LLS, we monitored changes in the optic disc that were acquired within 24 h of high-altitude exposure, as LLS scores attain their peak values at the same time. According to the proposed model (**Figure 5**), exposure to high altitude can significantly reduce blood oxygen saturation, thereby inducing hypoxemia (Abdolrahimzadeh et al., 2017). By further stimulating hypoxia through the activation of the corresponding signaling pathway, hypoxemia can result in a significant increase in cerebral blood flow and blood volume, thereby inducing brain swelling and increased ICP (Hackett and Roach, 2001; Rosenblum, 2007; Schoonman et al., 2008; Bailey et al., 2009). The increase in ICP can lead to HAH and elevate the trans-lamina pressure difference (Jonas et al., 2013; Hou et al., 2016). This would directly obstruct axoplasmic flow at ONH (Tso and Hayreh, 1977), and an obstruction to axoplasmic transport could increase RNFL and cause papilledema.

In a large sample, we confirmed that the ratio in the mean nerve fiber layer thickness did not significantly correlate with AMS or LLS scores, which is consistent with the results of previous studies (Willmann et al., 2011; Fischer et al., 2012). Some other studies showed significant positive correlations between AMS and optic disc swelling (Bosch et al., 2008), optic nerve sheath diameter (Bosch et al., 2010), corneal thickness (Bosch et al., 2009), and retinal capillary blood flow (Sutherland et al., 2008). Our study was the first to explore the relationship between changes in RNFL thickness of the peripapillary sectors and HAH; specifically, it illustrated a consistent and robust association between them.

Limitations

Several limitations to our study should be mentioned. First, the association between alterations of RNFL and HAH was described, but no causal relationship could be determined because of the observational nature of the study. Therefore, longitudinal studies should be conducted to validate our findings in the future. Second, the participants in our study were all young male individuals, which decreases the generalizability of the results. More subjects distributed in different age groups will be recruited in a future study. Third, a longitudinal follow-up study to observe the dynamic changes of the retina at multiple time points and altitudes should be conducted in the future. More parameters of the eyes should be considered, including intraocular pressure (IOP), optic nerve sheath diameter (ONSD), optical coherence tomography angiography (OCTA), and fundus images. Finally, the onset of headache in participants was subjective and not precise. Studies on biomarkers with high sensitivity and specificity for HAH may be desirable in the future investigations.

CONCLUSION

In conclusion, our study detected alterations in physiological and retinal parameters after rapid exposure to high altitude (3,700 m above the sea level). We observed a correlation between changes in the RNFL in the optic disc and HAH before and after ascent to 3,700 m, which may offer further insights into the elaborate pathophysiology of papilledema. However, we did not find any correlation between AMS or LLS scores and changes in the optic disc.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Human Ethics Committee of Fudan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XY contributed to study supervision, and were responsible for data collection, drafting and revision of the manuscript. YL

and YM collected the questionnaires data analysis, and revision of the manuscript. YX and KW contributed to the revise of the manuscript. DS, XY, MH, ML, and XY contributed to the data collection and data analysis. YG contributed to collect the questionnaires. JW, NW and LJ were responsible for study supervision and contributed to the study concept and design, data collection, data analysis, drafting and revision of the manuscript. All authors were involved in final approval of the submitted and published version.

FUNDING

This work was supported by the Shanghai Municipal Science and Technology Major Project (2017SHZDZX01), CAMS Innovation Fund for Medical Sciences (2019-I2M-5-066), the National Natural Science Foundation of China (31330038,31871436), and Science and Technology Committee of Shanghai Municipality (16JC1400500).

ACKNOWLEDGMENTS

The authors thank Wei Chen, Qian Chen, MD, and Jun Zhang (Carl Zeiss Shanghai Co. Ltd., China) for their invaluable support during data collection.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.864222/full#supplementary-material

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REVIEW
published: 10 May 2022
doi: 10.3389/fmed.2022.842800

Impact of High-Altitude Hypoxia on Bone Defect Repair: A Review of Molecular Mechanisms and Therapeutic Implications

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Reaching areas at altitudes over 2,500–3,000 m above sea level has become increasingly common due to commerce, military deployment, tourism, and entertainment. The high-altitude environment exerts systemic effects on humans that represent a series of compensatory reactions and affects the activity of bone cells. Cellular structures closely related to oxygen-sensing produce corresponding functional changes, resulting in decreased tissue vascularization, declined repair ability of bone defects, and longer healing time. This review focuses on the impact of high-altitude hypoxia on bone defect repair and discusses the possible mechanisms related to ion channels, reactive oxygen species production, mitochondrial function, autophagy, and epigenetics. Based on the key pathogenic mechanisms, potential therapeutic strategies have also been suggested. This review contributes novel insights into the mechanisms of abnormal bone defect repair in hypoxic environments, along with therapeutic applications. We aim to provide a foundation for future targeted, personalized, and precise bone regeneration therapies according to the adaptation of patients to high altitudes.

Keywords: hypoxia, plateau, bone defect, bone regeneration, high altitude

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *24 December 2021*
Accepted: *15 April 2022*
Published: *10 May 2022*

Citation:
Chen P, Liu Y, Liu W, Wang Y, Liu Z and Rong M (2022) Impact of High-Altitude Hypoxia on Bone Defect Repair: A Review of Molecular Mechanisms and Therapeutic Implications. Front. Med. 9:842800. doi: 10.3389/fmed.2022.842800

INTRODUCTION

Due to commercial activities, military deployment, tourism, and entertainment, high-altitude areas (altitude $\geq$2,500–3,000 m) are among the most important residential and business spaces for modern humans. There are three major plateaus in the world, including the Tibetan Plateau, the Andes Mountains, and the Ethiopian Plateau. The total plateau area is estimated at more than 11,000,000 km^2, with a population of ~107 million. The main characteristics of the high-altitude environment include (1) low pressure, oxygen deficiency, and thin air; (2) cold, dry, and strong winds; and (3) long sunshine time and intense ultraviolet radiation. Specifically, the main factors affecting the body are thin air, low atmospheric pressure, and reduced oxygen partial pressure in a high-altitude environment.

There is a relationship between altitude and the decreased partial pressure of oxygen (PO_2; i.e., the tension produced by oxygen dissolved in the blood). At an altitude of ~3,000 m, although arterial PO_2 (PaO_2) is reduced, oxygen saturation can be well maintained (1). The high-altitude environment affects humans mostly because reduced PaO_2 in the blood leads to hypoxia (2, 3). At sea level plains (henceforth referred to simply as "plains"), the PaO_2 is ~21.15 kPa. However, as the altitude increases by 100 m, atmospheric pressure decreases by 5.9 mmHg, and PaO_2 decreases by

1.2 mmHg (4). When the pressure in the air inhaled by humans is lower than 16 kPa (2,500–3,000 m), the symptoms of hypoxia start to appear, including increased breathing and heart rate, headache, loss of appetite, poor sleep, decreased exercise ability, and even mountain sickness (5).

The major metabolic phases of bone defect healing overlap with biological stages, including several stages: haematoma, unmineralized cartilage (soft callus), fibrous tissue, sencodary bone (hard callus), remodeled bone (6). Bone defect repair is affected in high-altitude environments. Indeed, the high-altitude environment has a systemic effect on humans, negatively impacting bone mass, microstructure, and biomechanics of normal bone (7), resulting in declined repair ability of bone defects as follows. First, the healing time of bone defects is significantly prolonged. The incidence of non-union at plateaus is 20–30%, which is significantly higher than that of plains (0.4%) (8). Bone repair efficiency is also significantly reduced (9), and fracture healing time is significantly longer, especially at extreme altitudes (5,400–6,700 m), than that found in coastal areas (10). Furthermore, X-ray findings reveal no periosteal hyperplasia and bony callus generation at the fracture end, while osseointegration is also poor after implantation (9, 11). Second, the different physiological components of bone defect healing are compromised. The capacity to regenerate bone is weaker based on the structural, geometrical, and material properties in a high-altitude environment (12). In addition, callus reconstruction is difficult, and the bone defect is filled with less callus tissue, with mostly new bone and cartilage (13). Moreover, the number of osteoblasts is reduced, and bone defect healing mainly involves endochondral bone (14). Third, different challenges are faced as part of bone defect treatment. For instance, in high-altitude areas, due to the special natural geographical environment of plateaus, the treatment of bone defects caused by war trauma differs from therapies applied in low altitude areas (15). After autogenous bone transplantation, hyperbaric oxygen therapy (HBOT) is often needed to achieve a relatively high success rate (16).

Herein, we review the physiological factors influencing bone repair, the cellular mechanisms of abnormal bone repair, and therapeutic research progress made in addressing the impact of a high-altitude environment on bone defect repair.

FACTORS INFLUENCING BONE DEFECT REPAIR IN HIGH-ALTITUDE ENVIRONMENTS

The high-altitude environment can exert systemic effects on humans that presents as a series of compensatory reactions. Although these compensations are conducive to adaptation to low pressure and oxygen at high altitudes, they may affect bone defect repair (**Figure 1**).

Blood Compensation

The blood system is among the first responses to the high-altitude environment, leading to a series of compensatory reactions. When staying at a high altitude for a short time, the pulmonary arterioles contract, pulmonary blood flow resistance increases, pulmonary arterial pressure increases, and volume vessels contract (peripheral veins), resulting in a release of reserved blood, thus ensuring blood supply to important organs (heart and brain). After living on a plateau for a longer time, acclimatization to the environment occurs by increasing oxygen transport, erythropoietin (EPO) concentration, the number of red blood cells, and hemoglobin concentration (17). However, when the physiological capacity to adapt is exceeded, maladaptation occurs *via* increased blood viscosity, reduced oxygen transport and oxygen release to vital tissues, and further aggravation of tissue hypoxia. Due to genetic differences, the Andes and Han populations living on the elevated altitudes of Tibet are more susceptible to this type of maladaptation, while the Tibetan residents have higher resting and hypoxic ventilation responses (HVRs), lower arterial oxygen saturation, and reduced hemoglobin concentration at the same altitude (18).

Blood compensation may affect the healing of bone defects through increased EPO production in response to the low pressure and oxygen environment at high altitudes. This in turn affects mobilization and differentiation (osteoblasts, osteoclasts, and mature blood cells) in mesenchymal stem cells (MSCs) and hematopoietic stem and progenitor cells (HSPCs). It is known that low EPO concentration is conducive to bone formation and bone defect healing (19, 20), while high EPO levels lead to the stimulation of osteoclast precursors and induces bone loss, preventing healing (21).

Blood compensation may lead to insufficient blood supply through increased blood viscosity, thus affecting the healing of bone defects. During bone defect healing, the blood vessels and interstitial tissue around the bone need to continuously grow into the center of the defect; hence, the abundance of blood supply affects the healing process. At high altitudes, blood compensation leads to an increase in blood cells. When blood viscosity exceeds a certain limit, it leads to local microcirculation disturbance (22). Platelets in stagnant blood promote the release of thrombin and damage endothelial cells, resulting in increased 5-hydroxytryptamine and histamine content (23), basement membrane exposure, the release of coagulation factors, and even thrombosis (24–26). Therefore, poor blood supply induced by blood viscosity may be one of the factors influencing poor bone defect healing.

Sympathetic Nervous Excitement

In the high-altitude environment, low oxygen stimulates the sympathetic nervous system, prompts catecholamines secretion, accelerates the heartbeat, enhances myocardial contractility, increases cardiac output, and elevates arterial blood pressure to a certain extent. On the skeleton, the sympathetic nerve fibers run along the major artery and nourish the bones through nutrient pores. Both the periosteum and bone marrow receive nutrients from noradrenergic fibers (often associated with the vascular system), vesicular acetylcholine transporter (VAChT), and vasoactive intestinal polypeptide immunoreactive fibers (often associated with the parenchyma). Sympathetic fibers on the periosteum branch overlaying the bone marrow and dense mineralized bone region receive the greatest mechanical stress and load, while also having the highest metabolic rate.

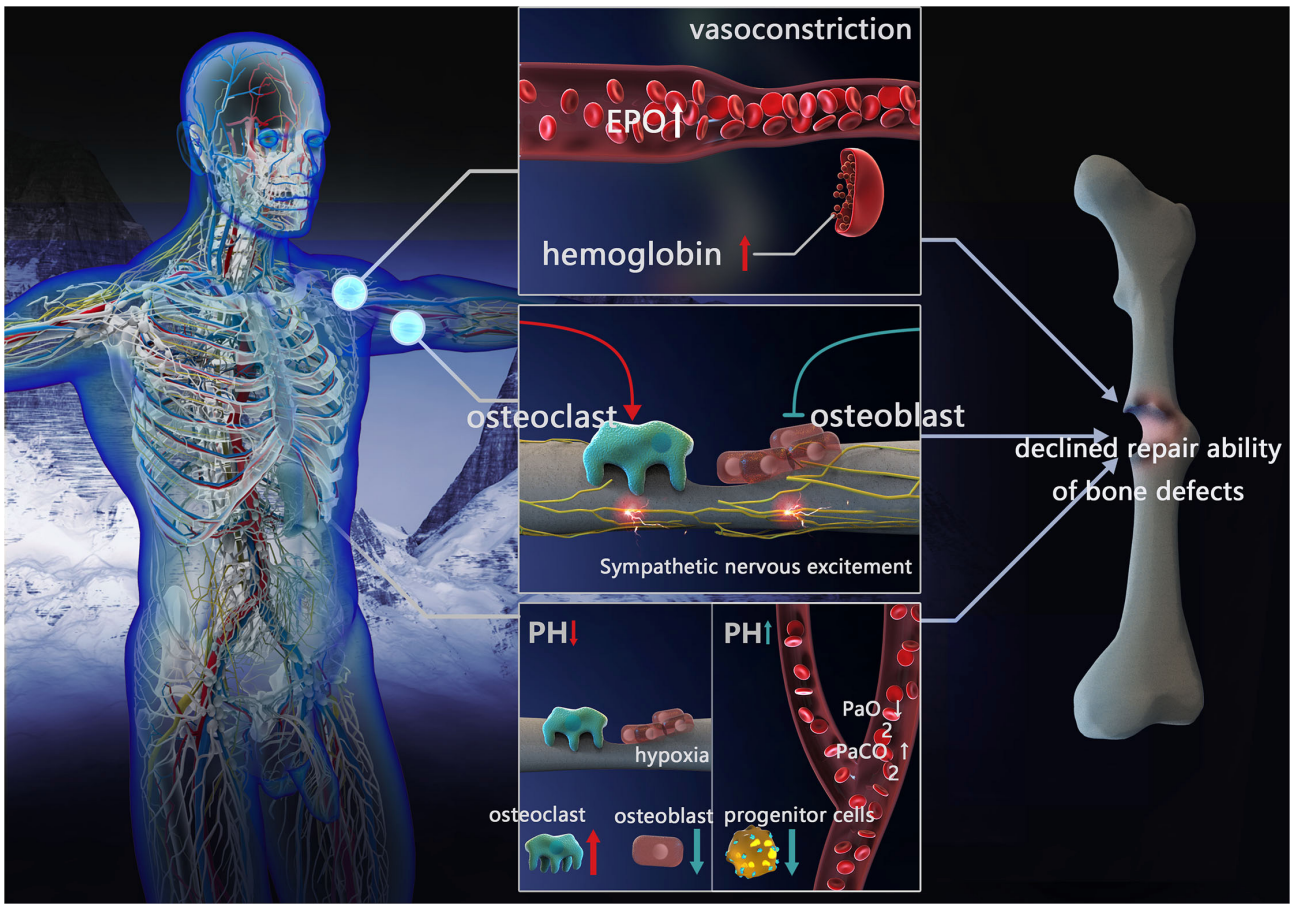

FIGURE 1 | Factors influencing repair of bone defects in high-altitude environment. Blood compensation: vessels constrict, EPO concentration and hemoglobin increased. Sympathetic nervous excitement: inhibit osteoblast activity and promote osteoclast formation. Acid-base compensation: In local acid-base compensation, decreased pH impeding osteoblasts differentiation and enhanced osteoclast activation. In systemic acid-base compensation, with the decreasing of PaO_2 and increasing of $PaCO_2$, increased pH inhibits bone marrow progenitor cell proliferation (EPO, erythropoietin; $PaCO_2$, partial pressure of arterial CO_2; PaO_2, partial pressure of oxygen).

Furthermore, the periosteum is the site with the most abundant blood vessels and has the highest density of sympathetic and sensory fibers.

Sympathetic nervous excitement may affect bone defect repair through vasoconstriction, leading to an inadequate blood supply. In high-altitude environments, sympathetic nerves are excited, form a large number of sympathetic active substances, and act on the vascular smooth muscle α receptor, leading to vasoconstriction, enhanced resistance of surrounding blood vessels, and reduced perfusion flow of the tissue (27). Furthermore, it results in decreased blood supply and hypoxia in the soft tissue at and around the bone defects, affecting hematoma organization and callus formation, and interrupting the healing of the bone fracture. The effect of vasoconstriction on the reduction of local blood flow in bone defects is more pronounced with increasing altitude (28).

Sympathetic nervous excitement regulates bone homeostasis and promotes bone resorption. Sympathetic nervous excitement at high altitude may result in noradrenaline nerve endings releasing noradrenaline and stimulating β2-adrenergic receptor (β2AR) near osteoblasts and osteocytes, bone formation inhibition, increased receptor activator of nuclear factor-κB ligand (RANKL) expression, promotion of osteoclast formation, and increased bone resorption. Altogether, these effects impede bone formation. Lastly, the secretion of adrenergic agonists (catecholamines) also stimulates bone resorption, inducing bone loss (29).

Acid-Base Compensation

High-altitude hypoxia induces hyperventilation and increases pH, due to the decreased oxygen availability and subsequent lower PaO_2. The HVR occurs when the body attempts to maintain PaO_2 to adapt to the high altitude. Although the PaO_2 level during HVR is corrected to a certain degree, partial pressure of arterial CO_2 ($PaCO_2$) is simultaneously reduced, further leading to decreased plasma H_2CO_3 concentration and increased

pH (30). Minor changes in pH have negligible physiological effects, however, when the pH keeps increasing, a compensatory acid retention mechanism and increased excretion of HCO_3 in urine is triggered, causing diuresis of sodium bicarbonate and potassium bicarbonate (31), in turn leading to decreased pH in arterial blood (pHa) compared to the normal level (pHa $\approx$ 7.4) (32, 33). Insufficient renal compensation may cause a continuous increase of blood pH, weaken HVR, and reduce oxygen saturation leading to the onset of acute mountain disease.

Nevertheless, local pH change in bone defects differs from that of systemic responses. In a high-altitude environment, due to decreased vascular perfusion, tissue hypoxia generates an acidic environment in the bone defect area. Moreover, when a bone defect occurs, disruption of the blood supply can have negative consequences for the bone *via* the direct actions of hypoxia and acidosis on bone cells (34). In severe hypoxia, glycometabolism at the injury site is incomplete, anaerobic glycolysis is enhanced, and local acidic products are increased (35).

Healing of the bone defect is affected by systemic and local acid-base compensation. A continuous increase of physiological pH caused by the high-altitude environment affects oxygen-carrying capacity, as well as the transportation and release of hemoglobin and blood. In addition, it increases the affinity of hemoglobin to oxygen (Bohr effect) and allows tissues to absorb more oxygen (33). Therefore, in terms of oxygen delivery to the bone tissue, the acid-base compensatory response under the high-altitude environment is advantageous for increasing the local oxygen content in bone defects. However, the pH increase resulting from this compensation does not promote bone marrow progenitor cell proliferation and may affect bone defect healing. Studies have reported that promyelocytic KG-1a cells (hematopoietic stem cells) cultured under high pH have significantly decreased proliferation and enhanced apoptosis (35). The bone contains a large number of alkaline minerals (hydroxyapatite), and bone cells are extremely sensitive to the direct effects of pH. When the acid-base balance cannot be maintained within a narrow range, these alkaline minerals can eventually be used to neutralize pH, in turn reducing alkaline mineral deposition by osteoblasts in the bone (36), and impeding osteoblasts differentiation (34, 37). In contrast, the osteoclastic differentiation and activation are enhanced (37, 38) resulting in detrimental bone defect repair.

MECHANISM OF ABNORMAL BONE DEFECT REPAIR IN HIGH-ALTITUDE

High-altitude areas have thin air and reduced PaO_2. Therefore, cellular structures closely related to oxygen sensing, including the mitochondria and cell membrane ion channels, produce corresponding functional changes causing oxidative stress response, ion permeability changes, signaling pathway activity changes, autophagy, apoptosis, etc. In addition, the high-altitude environment alters the epigenetic modification-related effects on genes, thus affecting protein function that may have consequences for bone defect repair (**Figure 2**).

Changes in Ion Permeability of the Cell Membrane

Ion channels participate in a variety of high-altitude adaptive compensations such as HVR and vascular tension changes. It is generally accepted that high-altitude environments inhibit K^+ channels and activate Ca^{2+} channels (39, 40). Altered K^+ permeability affects osteoblast differentiation and proliferation (41). Meanwhile, altered Ca^{2+} permeability affects the mineralization of extracellular inorganic Ca^{2+}, which acts as a second messenger, impacting the expression of downstream osteogenesis-related signaling pathways (42), thereby influencing bone defect repair.

High-altitude-related low oxygen inhibits the activity of K^+ channels, leading to membrane depolarization. Acute hypoxia depolarizes the membrane potential by 15–20 mV (43). It has been confirmed that K^+ channels affected by oxygen in bone cells include voltage-gated potassium channels (Kv), inward rectifying K^+ channels (Kir), Ca^{2+}-activated K^+ channels (K_{Ca}), ATP-sensitive K^+ channels (K_{ATP}), and two-pore K^+ channels (KT) (44). However, the mechanism by which these K^+ channels sense oxygen changes is not fully elucidated. Studies have found that K^+ channels retain their hypoxic reactivity after recombination (45, 46), indicating that oxygen not only directly regulates K+ channels but also affects ion permeability through pore-forming subunits or regulatory β-subunits. Six transmembrane subunits and one pore-forming subunit (Kv1.2, Kv1.5, Kv2.1, Kv3.1, Kv3.3, Kv4.2, and Kv9.3) of the Kv channels are reversibly blocked by hypoxia (47). Furthermore, four transmembrane and two pore-forming subunits of the double-pore potassium ion channel KT are also involved in oxygen sensing peripheral chemoreceptors (48). However, whether other subunits play a role in the low-oxygen environment at high altitudes remains unclear.

High-altitude hypoxia increases intracellular Ca^{2+} levels and causes calcium overload, which results from Ca^{2+} channel activation. The main Ca^{2+} channels affected by oxygen in bone cells include voltage-sensitive Ca^{2+} channels (VSCCs) and transient receptor potential channels (TRPs). In these Ca^{2+} channels, TRPs are the most predominant oxygen sensors (49). TRP expression differs on the surface of different bone cells; e.g., TRP vanilloid-5 (TRPV5) is missing in osteoblasts (50). However, only the hypoxic responses of TRPV1 and TRPV4 have been examined. Nevertheless, TRPV5 and TRPV6 are also closely associated with bone defect healing (51). Different from the other TRPV channels, these are highly selective for Ca^{2+} and their changes under hypoxia need to be confirmed. In addition, although VSCC expression is greatly reduced on osteoblasts compared with excitable cells, reduced PaO_2 can lead to rapid activation of L-type VSCCs (52). However, the change in T-type VSCC under hypoxic conditions has not been reported. This may be a result of the T-type VSCC only being involved at the early stage of cell differentiation, after which its activity significantly reduces (53).

In addition to oxygen-sensitive ion channels, only a few studies have examined changes in other ion channels that may be associated with high-altitude environments. For example, compensatory responses to high-altitude have been reported

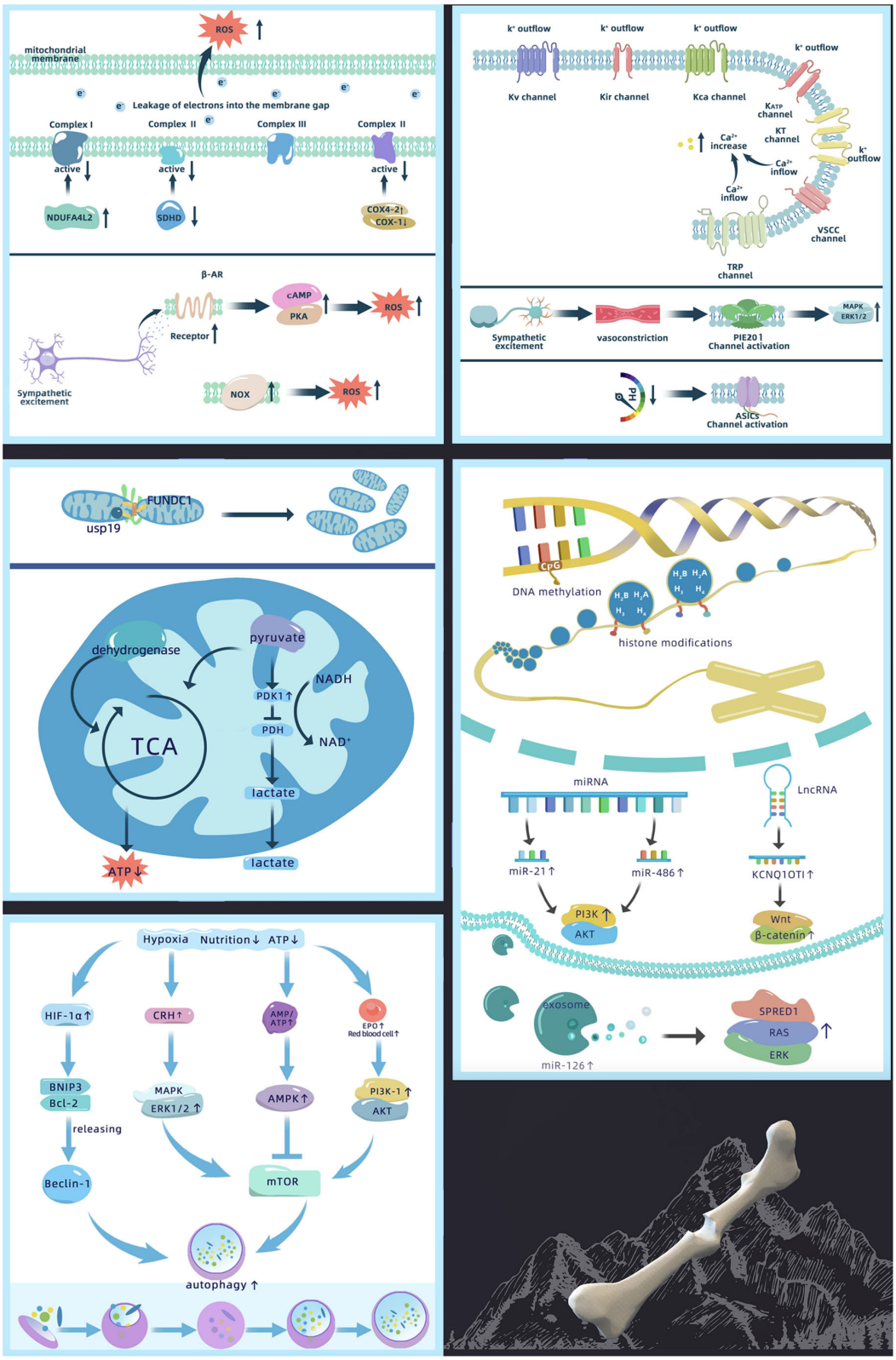

FIGURE 2 | Mechanism of abnormal bone defect repair in high-altitude, including changes in ion permeability of the cell membrane, ROS production, changes in mitochondrial function, activation of autophagy, and epigenetics regulation [Akt, protein kinase B; AMPK, adenosine 5′-monophosphate-activated protein kinase; ASIC, acid sensitive ion channel; ATP, adenosine triphosphate; Bcl-2, B-cell lymphoma 2; BNIP3, Bcl-2 19 kDa interacting protein 3; COX, cytochrome C; CRH, *(Continued)*

FIGURE 2 | corticotropin releasing hormone; EPO, erythropoietin; ERK1/2, extracellular regulated protein kinases 1/2; FUNDC1, FUN14 domain-containing 1; HIF-1α, hypoxia inducible factor-1α; KATP, ATP-sensitive K^+ channels; KCa, Ca^{2+} activated K^+ channel; KCNQ1OT1, potassium voltage-gated channel subfamily Q member 1 opposite strand 1; Kir, inward rectifying K^+ channel; KT, double-pore K^+ channels; Kv, voltage-gated potassium channel; lncRNA, long non-coding RNA; MAPK, mitogen-activated protein kinase; miRNA, micro-RNA; mTOR, mammalian target of rapamycin; NADH, nicotinamide adenine dinucleotide; NDUFA4L2, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4-like 2; NOX, NADPH oxidase; PDH, pyruvate dehydrogenase; PDK1, pyruvate dehydrogenase kinase 1; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; ROS, reactive oxygen species; SDHD, succinate dehydrogenase complex subunit D; SPRED1, sprouty-related EVH1 domain containing 1; TCA, tricarboxylic acid cycle; TRP, transient receptor potential channel; USP19, ubiquitin specific protease 19; VSCC, voltage sensitive Ca^{2+} channel].

whereby sympathetic excitation can lead to local vasoconstriction with increased expression of the mechanically-gated channel Piezo1 during vasoconstriction (54), activating the downstream MAPK/ERK1/2 signaling pathways. In addition, the local extracellular environment of bone defects is acidified under hypoxia, which can activate acid-sensitive ion channels (34).

It may be speculated from existing studies that oxygen-sensitive ion channels play the main role in high-altitude bone defects. Most literature assessing the effects of high altitude on ion channels focused on pulmonary edema. However, whether other subtypes of TRPs (such as TRPC, TRPA, TRPM) also present similar changes in bone cells as pulmonary vascular cells have not been reported and should be considered for future research. In addition, there are few studies on Na^+ channels. The latest study showed that Na^+ acts as a second messenger to regulate the permeability of the inner mitochondrial membrane in an acute hypoxic environment (55). Therefore, attention should be paid to the changes of Na^+ channels in high-altitude environments as well as the related effects on bone defect repair.

ROS Production

Both acute and long-term exposures to high-altitude environments induce the production of reactive oxygen species (ROS), which results in oxidative stress response (56, 57). The ROS level increase in the bone can affect bone defect healing by decreasing osteoblast activity and accelerating bone resorption by osteoclasts (58). In addition, ROS can also induce osteoblast and osteocyte apoptosis (59), affecting the quality of bone defect healing.

Hypoxia is the main factor increasing ROS in high-altitude environments. At a plateau, PaO_2 gradually decreases with altitude elevation. The mitochondria, a main source of ROS, are affected by the changes in intracellular and extracellular PaO_2. Multiple studies have suggested that mitochondrial complex III (ubiquinol-cytochrome c oxidoreductase) and complex I (NADH-ubiquinone oxidoreductase) are major sites of ROS production (60–62). Hypoxia reduces complex I activity by upregulating NDUFA4L2 (63). However, studies have found that complex IV (cytochrome C oxidase) and complex II (succinate dehydrogenase) in the mitochondria are also sensitive to hypoxia. This is because complex IV has a binding site for oxygen, and hypoxia can reduce its activity by upregulating COX4-2, an isoform of cytochrome C oxidase (COX), and downregulating COX-1 (64). It also affects the activity of complex II by decreasing SDHD expression (62). In addition, insufficient oxygen supply can also obstruct mitochondrial electron transport by partially leaking single electrons from complexes I–III directly to oxygen leading to the production of large amounts of ROS (56).

In addition to the effects of hypoxia, sympathetic nervous system excitement at high altitude may be one of the factors that contribute to increased ROS production. It was found that adrenaline receptors mediate the production of cellular ROS induced by sympathetic system over-excitation. β-AR promotes the mitochondrial tricarboxylic acid cycle, enhances oxidative respiration, and increases oxygen consumption through the classical cAMP/PKA pathway, which leads to enhanced electron leakage into the mitochondrial inner and outer membrane spaces, thereby increasing ROS production (65). In addition to the mitochondria, NOX (NADPH oxidase), distributed on the plasma membrane and multiple organelle membranes, also produces ROS upon sympathetic system excitation (66) and promotes vasoconstriction through a RhoA kinase-dependent pathway (67). However, studies examining sympathetic nervous excitement with ROS are currently limited to cardiomyocytes and vascular smooth muscle cells. Thus, whether a similar mechanism also exists in bone cells needs confirmation.

The results of *in vitro* mechanistic studies evaluating high-altitude-related ROS production as well as the actual *in vivo* situation in high-altitude populations may differ due to the genetic polymorphisms of ROS production in high-altitude environments. Studies have found that the mtDNA 10609T in Han people living on the plateau promotes the increase of intracellular ROS in hypoxia, while the mtDNA T8414 does not (68). In addition, the high-altitude environment increases ROS, which has a negative effect on bone defects. On the other hand, increased ROS can stabilize the activity of hypoxia-inducible factor 1-alpha (HIF-1α), which is helpful for the body to cope with the high-altitude environment. Therefore, balancing the advantages and disadvantages ROS still needs quantitative investigation.

Changes in Mitochondrial Function

Mitochondrial dysfunction can affect osteoblast, osteocyte, and vascular endothelial cell functions (69, 70) as well as inflammatory reactions in chondrocytes. In turn, this leads to metabolic disorders in chondrocytes, affecting endochondral osteogenesis (71). Moreover, osteogenic differentiation of human marrow MSCs can be impeded by mitochondrial dysfunction (72), which is not advantageous for bone defect repair.

Mitochondria are the main consumers of oxygen in cells and need to adapt collectively to the decrease in available oxygen at high altitudes. Low oxygen environments can lead to an increased number of mitochondria and imbalanced

proteostasis. A possible mechanism is that hypoxia induces mitochondrial fission *via* mitochondrial outer-membrane protein FUNDC1 signaling. Under hypoxia, the deubiquitinase USP19 accumulates at the ER-mitochondria contact sites with FUNDC1. USP19 then interacts with and removes ubiquitin chains from FUNDC1 at the ER-mitochondria contact sites. After USP19 stabilizes FUNDC1 and subsequently promotes Drp1 oligomerization (73, 74), hypoxia-induced mitochondrial division occurs thus increasing the number of mitochondria. In addition, recent studies have found that hypoxia suppresses mTORC1 signaling and mediates homeostasis remodeling of mitochondrial proteins by regulating substrate-related mitochondrial metabolism through the mTORC1-LIPIN1-YME1L signaling axis (75).

In addition, high-altitude environments may also cause changes in mitochondrial respiration and aerobic capacity. However, how this dysfunction relates to the degree of hypoxia is unknown. Mitochondrial respiratory function is not affected in mild or early hypoxia. It was found that the effect of 15 days of mild normobaric hypoxia on mitochondrial function is negligible as mitochondria adapt to the environment by increasing LON protease content, optimizing respiratory chain function (76). However, severe hypoxia leads to mitochondrial dysfunction. When the PaO_2 in the mitochondria drops to the critical point of 0.1 kPa (<1 mmHg), dehydrogenase activity decreases, reducing the respiratory function of the mitochondria and decreasing ATP production (77). Moreover, mitochondrial metabolic pathways switch from aerobic to anaerobic metabolism under hypoxic conditions. The possible explanation is that hypoxia upregulates pyruvate dehydrogenase kinase 1 (PDK1), inactivates pyruvate dehydrogenase (PDH), transforms pyruvate to acetyl-CoA, and reduces the availability of substrates for oxidative metabolism, thereby promoting the conversion of pyruvate to lactate (78).

However, changes in mitochondrial function under high-altitude environments are correlated with mitochondrial genes. Studies have found that mitochondrial haplogroups B and M7 may be related to inadaptability to hypoxia, while haplogroups G and M9a1a1c1b are related to hypoxia adaptation. Specifically the T3394C and G7697A mutations in haplogroup M9a1a1c1b may be the main factor improving the ability to adapt to the environment in Tibetans living on the plateau for generations (79). In addition, the mitochondrial genes *MT-ND1* and *MT-ND2*, encoding two subunits of mitochondrial NADH dehydrogenase, play an important role in the oxidative phosphorylation electron transport chain and contribute significantly to high-altitude hypoxia adaptation of the mitochondria (80).

In addition to hypoxia, high-altitude factors such as cold (81) and strong ultraviolet radiation (82, 83) may also impact mitochondrial function. Nonetheless, no relevant studies have assessed their role on mitochondria in bone tissue, which may be an important future direction.

Autophagy

Both acute and chronic high-altitude exposures activate autophagy and increase cell death (84). Overall, autophagy exerts a protective effect under short-term moderate stimulation induced by the high-altitude environment (85, 86), which can increase the expression of vascular endothelial growth factor (VEGF) by stabilizing HIF-1α, thereby benefiting angiogenesis (87). Meanwhile, as a carrier of osteoblasts to secrete hydroxyapatite crystals, autophagosomes participate in bone formation (86, 88). However, a sustained high level of the oxidative stress response can overstimulate autophagy, leading to premature cell death (89).

Studies have found that autophagy acts in a HIF-1α-dependent manner under hypoxia (90). HIF-1α activates downstream BNIF3, then competitively binds Bcl-2 with Beclin-1 for subsequent activation of autophagy. However, the regulatory mechanism involved in autophagy has not been studied in bone cells. Findings in other cell types reveal that hypoxia affects autophagy *via* the HIF-1α/Beclin-1 pathway in SH-SY5Y cells (neuroblastoma cells), dendritic cells (DCs) (91), and vascular endothelial cells (87). However, this autophagy activating pathway is not present in all cell lines. For example, autophagy of NP cells (nucleus pulposus cells) in the hypoxic environment is independent of the HIF-1α pathway. Therefore, further investigation is needed to determine whether the same mechanism exists in skeletal cells.

In addition, a high-altitude environment can also regulate autophagy through the mTOR-related signaling pathway as mTOR kinase can be inhibited under conditions such as malnutrition, decreased ATP levels, and hypoxia. Thus, reduced metabolic activity induces autophagy (90). Cellular energy deficiency resulting from high altitude leads to increased AMP/ATP ratio (92), whereas hypoxia induces the phosphorylation of AMPK (93), all of which regulate the AMPK/mTOR signaling pathway. Moreover, the increased number of red blood cells and EPO caused by high-altitude compensation can activate the PI3K-1/Akt/mTOR signaling pathway (94). The low-oxygen environment at high altitude also upregulates corticotropin-releasing hormone (CRH) and regulates mTOR in autophagy through the MAPK/ERK1/2 pathway (95, 96).

However, the basal level of autophagy is not the same in different populations; hence, the effects of autophagy on bone cells may also differ between individuals in high-altitude environments. Studies have shown significant differences in the levels of autophagy markers, including LC3 and BNIP3, between Tibetans living at an altitude of 3,000 m and Han nationals living below 500 m (97). Long-term living in high-altitude environments could increase the level of basal autophagy—possibly by resisting the negative effects of oxidative stress through a higher level of autophagy.

Besides, bone marrow is in a hypoxic environment under physiological conditions. Thus, whether *in vitro* effects of hypoxia on autophagy signaling pathways could be translated *in vivo* need to be confirmed. However, through literature review, we found that altitude affects autophagy by various mechanisms, emphasizing the complexity of the autophagy signaling pathways. Future research should investigate whether autophagy is affected by the degree and duration of bone defects at high altitudes.

Epigenetics

Populations living at high altitudes for generations all have a genetic basis for adaptation to high-altitude environments. However, genetics alone cannot fully explain the mechanism of impaired bone defect repair at high altitudes. An increasing number of studies have confirmed that epigenetic alterations integrate genetic and environmental stimuli to participate in hypoxia adaptation of tissues and cells, thereby affecting bone defect repair by regulating related downstream genes. Some of the most prominent epigenetic mechanisms include DNA methylation, histone modifications, and non-coding RNAs.

The most intensively studied epigenetic mechanism to date is DNA methylation. DNA sequencing in high-altitude and plain populations reveals that genes with differential methylation are mainly involved in HIF-related signaling pathways (98, 99). The methylation levels of HIF-1α and HIF-2α promoter regions are significantly lower in plateau animals (99, 100). HIF-dependent hypoxia response element (HRE)-related genes are sensitive to methylation, and a large number of CpG dinucleotides are present at the binding site of the HRE sequence of HIF (101). When methylation changes occur at the above sites, binding to various transcription factors is inhibited. Studies have shown that chronic hypoxia induces CpG for VHL promoter methylation, reduces VHL expression, and increases EPO production by elevating HIF-2α expression in the bone marrow, leading to erythrocytosis (102). Meanwhile, HIF-1α cannot be degraded by ubiquitination and further accumulates in cells (103). Evidence suggests that DNA hypomethylation in high-altitude environments favors the expression of *HIF* genes, in turn triggering the hypoxia adaptation response of the tissue and cells by regulating the activity of downstream genes.

It is known that high-altitude hypoxia also leads to changes in histone modifications. The modification sites are generally located on four common histones, including H2A, H2B, H3, and H4—with H3 and H4 being the most common. Studies have shown that high-altitude environments increase the levels of H3K14ac, H4R3me2, H3K4me2, H3K4me3, H3K79me2, H3K9/27me2, H3K9me2, H3K27me3, and H3K4mel in mice (104). In addition, the Jumonji domain (JMJD) protein family comprises enzymes catalyzing the demethylation of arginine and lysine residues in histones. Both JMJD1A and JMJD2B retain their activities, bind to specific recognition sites of HIF-1α, and induce its expression under high-altitude hypoxia (105). The chemical modification of these histones ultimately alters the expression of the genes by changing the affinities of the promoter regions of genes associated with hypoxia response.

Non-coding RNAs are expressed differently in plateau and plain environments. Examining the expression profiles of miRNAs between these environments revealed a total of 26 differentially expressed miRNAs (106). The hypoxic environment upregulates the expression of miRNA-21 (107) and miR-486 (108), while increasing osteogenic differentiation of bone MSCs (BMSCs) through the PI3K/Akt pathway. Studies have shown that lncRNAs play an important role in the direct or indirect regulation of HIF-1α expression and related pathways, and interrupt angiogenesis and bone formation in hypoxia by negatively regulating HIF-1α at the mRNA level. The lncRNA *KCNQ1OT1* exerts the primary effects in delayed fracture healing, inducing cell proliferation and inhibiting cell apoptosis by activating the Wnt/β-catenin signaling pathway (109). In addition, hypoxia promotes the production of miR-126 production in exosomes to enhance bone defect healing *via* the SPRED1/Ras/ERK signaling pathway (110).

It remains controversial whether epigenetic factors are beneficial to bone defect repair at high altitudes. Most studies investigating the impact of epigenetic factors started from the aspect of hypoxia in the high-altitude environment. However, it is important to identify other key factors involved in the healing of bone defects to determine how they are regulated under a high-altitude environment. Moreover, the histone modification process is extremely complex and has not been comprehensively studied. For example, the histone protease LSD1 is a key factor regulating endochondral ossification during bone regeneration (111). Yet, how it changes under high-altitude environments has not been elucidated, which deserves further attention.

IMPLICATIONS FOR TREATMENT

Oxygen Therapy

Oxygen therapy is the primary treatment for acute and chronic altitude sickness. It primarily serves to improve PaO_2 and arterial oxygen saturation, increase the content of arterial oxygen, and correct various hypoxia levels caused by the high altitude. Furthermore, oxygen administration can be divided into systemic and local types.

The most common systemically administered oxygen therapy is HBOT (112). Hyperbaric oxygen can reduce the cardiac workload, improve cardiac function, block the vicious cycle of hypoxia leading to excessive erythrocyte proliferation, reduce the respiratory rate, and correct the acid-base balance. Hyperbaric oxygen can play a role in promoting the repair of both fresh and old bone defects. It helps reduce tissue edema, restore venous return, improve microcirculation (113, 114), and stimulate angiogenesis (115), Furthermore, it also increases PaO_2 in the fractured area (especially in the callus and medullary cavity), enhances the activities of osteoclasts and osteoblasts, and accelerates bone callus formation (116). In addition, hyperbaric oxygen also enhances the anti-infective ability of local tissues, especially against anaerobic bacteria (117). Clinically, hyperbaric oxygen chambers have been widely used in patients with bone defects to shorten the recovery time. In response to peri-implant tissue stimulation by titanium (Ti) particle exposure, ROS production (118), pro-inflammatory cytokines, infiltration of inflammatory response cells, and activation of the osteoclast activity (119), HBOT can be used in combination with bone grafts (120). Lastly, HBOT also improves the implantation of osseointegration (121).

Topical oxygen therapy (TOT) has been used in diabetic skin ulcers, post-operative infections, and gangrenous lesions. The most common method uses the micro-oxygen wound therapy instrument, which delivers pure oxygen to the wound for 24 h *via* an oxygen administration tube yet is not readily applied in bone defect repair. Studies have also found that local oxygen administration improves post-operative local PaO_2 and oxygen

saturation at sternal defects and reduces the risk of infection (122). In addition, reports have adopted innovative local oxygen delivery methods. For instance, perfluoro-octane-loaded hollow particles can be used as a local oxygen source, increasing cell viability, and maintaining the osteogenic differentiation potential of human periosteum-derived cells under hypoxic conditions (123, 124). However, only local oxygen-releasing dressings, including OxygeneSys, Oxyzyme, and Oxyband, have been marketed to date, and oxygen released from these products can only act on the wound surface and cannot improve PaO_2 to address deep bone defects.

In general, HBOT has been widely used in clinical practice with a definite curative effect, but measures should be taken according to local conditions. Specifically, the treatment pressure should not be too high, and the speeds of pressure increase and decrease rates should be reduced, with the times of pressure increase and decrease extended appropriately. In addition, the high-pressure oxygen chamber has various shortcomings, including inconvenient mobility, which cannot meet the criteria of emergency treatment to combat altitude sickness. Thus, scientists have also developed a vehicle-mounted mobile hyperbaric oxygen chamber. However, the effect of local oxygen therapy in the treatment of bone defects remains unclear and deserves further investigation.

Systemic Administration

Some drugs can promote the healing of bone defects by improving the physiological ability to adapt to hypoxic environments, reducing ROS, and improving ion permeability of the cell membrane.

As far as traditional Chinese medicine (TCM) is concerned, the anti-altitude sickness drug rhodiola has the obvious effect of inhibiting bone resorption. Studies have found that rhodiola reduces ROS production, significantly decreases the expression and activity of MMPs, and upregulates the TIMP protein (125). Salidroside, the major bioactive compound of rhodiola, has various pharmacological effects and acts through HIF-1α-VEGF (126) and BMP (127) pathways, simultaneously promoting angiogenesis and osteogenesis, thereby accelerating bone defect healing. Rosavin, a rhodiola component, can block the NF-κB and MAPK pathways, inhibit RANKL-induced osteoclast formation *in vitro* and *in vivo*, decrease the expression of genes related to osteoclast differentiation, and promote osteogenesis in BMSCs (128). In addition, Jiuerjiegusan and Jieguling capsules are used in the treatment of high-altitude traumatic fractures, also significantly accelerating fracture healing.

Supplementation of antioxidants can reduce the negative effects of oxidative stress on bone defect repair by reducing ROS amounts (129). Common oral exogenous antioxidants include vitamin C, vitamin E, and trace elements. Polyphenols in fruits and vegetables also act as natural antioxidants. The effects of oral antioxidants are currently controversial. Studies have found that antioxidant cocktail therapy has no effects on bone resorption or formation (130). Epidemiologic studies showed that although systemic administration of vitamin C improves the soft tissue healing of tooth extraction wounds (131, 132), there is no significant difference in the percentage of X-ray density of new bone formation (133). The main polyphenolic catechins in green tea increase the survival, proliferation, increasesdifferentiation, and mineralization of osteoblasts by promoting osteogenic differentiation of MSCs (134). Conjugated linoleic acid (CLA) is an important component of the Tibetan diet, with a strong antioxidant effect. CLA the quality and mechanical strength of bone callus fracture healing in rats (135), and significantly reduces alveolar bone loss in rats with periodontitis and diabetes (136).

Calcium ion antagonists, which dilate blood vessels and relax bronchial smooth muscles, are commonly used to treat high-altitude reactions, improving the anti-hypoxia ability to various degrees. Ronacaleret, a novel calcium-sensing receptor antagonist, stimulates the release of parathyroid hormone (PTH) and increases the expression of bone formation markers. A phase I and II clinical study found that it acts as a potent oral anabolic agent to promote bone fracture healing (137). However, it has not been reported whether other calcium antagonists can be used for the treatment of high-altitude-related bone defects by systemic administration.

Most of the above reports are basic research, and large-scale, placebo-controlled, long-term randomized trials with optimal timing of protocol interventions are still needed to determine the efficacy of drugs on bone defect repair. In addition, non-targeted mitochondrial antioxidants cannot accumulate in large quantities in key steps of mitochondrial ROS production, and may eventually interfere with subsequent physiological signal transduction (138). Therefore, the safety of these proof–of–concept drugs remains to be confirmed in further experiments. Under the guidance of the TCM theory, TCM has its advantages in fundamentally preventing and treating altitude sickness. Its combined application with Western medicine is expected to become the future therapeutic standard for treating plateau bone defects.

Local Drug Delivery

Drugs directly acting on bone defects are administered by local drug delivery. With the development of biomaterials and bone tissue engineering technology, local drugs are sometimes combined with biomaterials to enhance angiogenesis and osteogenesis abilities, which effectively repair bone defects and promote bone regeneration to a certain extent.

Bioactive materials have been widely reported in the treatment of bone defects, but whether they have the same efficacy in high-altitude environments remains to be confirmed. In view of the potential mechanism of poor bone defect repair at high altitudes, existing studies can serve as a certain reference. Amorphous silicon nitride ($Si(On)_x$) can be used as nano-coating for titanium plates, which have a strong attachment to the surface of scaffolds and induces the sustainable release of Si (+4-valent) for a prolonged time (139), enhancing the expression of superoxide dismutase. Thus, its therapeutic use is also expected to promote bone tissue repair and angiogenesis by reducing the influence of ROS in the high-altitude environment (140). Bioactive borosilicate glass (BG) scaffolds and tricalcium phosphate (TCP) are commonly used materials in bone repair. DMOG, a small

molecule angiogenic drug that can adjust the stability of HIF-1α (141), can be added to BG and TCP to promote new bone formation and neovascularization in bone defects (142).

HIF is a key factor in response to hypoxic stress in high-altitude environments; direct application of HIF or the use of HIF-related drugs may promote bone defect repair at high altitudes. For instance, DBBM-C (deproteinized bovine bone +10% collagen) combined with HIF-1α promotes the formation of new bone (143). HIF-1α mediates DNA delivery *via* the protein transduction domain (PTD), and local administration of HIF-1α *via* PTD promotes bone growth (142). Under severe hypoxia, mechanical growth factor E inhibits the expression of HIF-1α and its transfer to the nucleus, thus regulating the proliferation and osteogenic differentiation of BMSCs (144). In addition, local application of HIF-related drugs may also be helpful for cartilage repair. Studies have shown that HIF-1α combined with collagen scaffold can repair osteochondral defects of the condyle of the temporomandibular joint in rabbits (145). Local injection of icariin inhibits the NF-κB/HIF-2α signaling pathway, thereby enhancing chondrocyte viability (146).

As discussed earlier, autophagy plays an important role in poor bone defect repair at high altitudes. Therefore, applying drugs regulating autophagy may be a potential therapeutic approach. Metformin, which is commonly used to combat hyperglycemia, has also been shown to affect bone regeneration. Metformin increases autophagy in BMSCs under hypoxic conditions and upregulates the osteogenic markers Runx2, osteocalcin, and alkaline phosphatase to significantly accelerate the formation of new bone (147, 148). Simvastatin, a serum cholesterol-lowering drug, has been shown to promote bone regeneration. Topical administration of simvastatin enhances autophagy and reduces the activity of osteoclasts (149), as well as induces homing of endothelial progenitor cells and promotes angiogenesis (150). It is as effective in repairing long tubular and flat bone defects as autografts (151). Other studies have found that under hypoxic conditions, resveratrol and angiopoietin 2 improve the survival and differentiation of BMSCs through autophagy (152, 153). Furthermore, intraperitoneal administration of the autophagy inducer rapamycin in fractured rats improves the autophagy level, increases bone callus formation, and accelerates fracture healing (154). Nevertheless, rapamycin has many adverse reactions. Therefore, identifying autophagy modulators with reduced toxicity and good efficacy is a direction of future research.

Local drug therapy can act directly on the bone defect site to accelerate fracture healing and enhance bone graft stability. However, the condition of bone defects faced by clinicians in high-altitude environments is complex and often occurs in combination with hypothermia, hypoxemia, and infection. Therefore, it is possible to combine drugs that promote osteogenesis with those conferring anti-infection and rehydration to establish a suitable rescue treatment approach for bone defects in high-altitude areas.

Stem Cells

The regeneration ability of bone tissue cells is compromised in high-altitude environments. Fortunately, stem cell therapy by itself or in combination with drugs, surgery, biomaterials, etc., can play a role in accelerating wound repair by improving tissue differentiation ability.

In high altitude environment, EPO was increased. Erythropoietin receptor (EPOR) was expressed in bone marrow stromal cells (BMSC), and it was verified that increased EPO results in reduced bone by regulating BMSCs (155). Although the local transplantation of BMSCs accelerated wound repair of high-altitude femoral defects, the healing rate of the high-altitude group remained lower than that of the plain group (156). Therefore, improving the survival rate and osteogenic ability of transplanted BMSCs in hypoxic regions is crucial. Studies have shown that BMSC transplantation combined with FG4592 administration can further accelerate fracture healing by increasing the proliferation and migration of BMSCs (157). BMSCs are reprogrammed into induced pluripotent stem cells (iPSCs), called iPSC-MSCs, whose morphology, immunophenotype, *in vitro* differentiation potential, and DNA methylation pattern are similar to those of BMSCs. Nevertheless, iPSC-MSCs have a higher proliferative capacity and promote bone repair and angiogenesis more pronouncedly (158). A 3D cell oxygen permeation culture device "Oxy Chip" was developed to generate and supply oxygen to cell spheroids to prevent hypoxia (159). It could promote osteoblastic differentiation of MSCs.

Adipose-derived stem cells (ADSCs) could differentiate into chondroblasts after 2 days of *in vitro* culture without the addition of growth factors at low oxygen partial pressure (<1% PO_2) (160). However, for osteogenic differentiation to be achieved, different growth factors or biomaterials should be combined to provide an osteogenic induction environment for ADSCs. The combination of ADSCs and autologous platelet-rich plasma (PRP) was not statistically significant compared with autologous bone grafts (161). This may be because the growth factor mixture in PRP has a short half-life *in vivo*, which cannot guarantee osteogenic induction of ADSCs during the whole process of bone repair. Nonetheless, tissue engineering scaffolds have advantages in providing a continuous environment for osteogenic induction. Some scholars have developed epigallocatechin gallate (EGCG)-coated synthetic fibers encapsulating ADSCs to fabricate stem cell spheroids for bone tissue regeneration. These EGCG fibers effectively delivered osteo-inductive and ROS scavenging signals to ADSCs in spheroids, upregulating the osteogenic markers RUNX2 and OPN (162). In addition, a novel 3D BG scaffold (BG-XLS/GelMA-DFO) combined with ADSCs could also promote bone regeneration under simulated hypoxia conditions (163).

The cell viability and survival time of ordinary 2D-cultured MSCs after transplantation is not ideal. It is the current trend to aggregate cells into 3D spheres or to bind, extend, and grow them on porous 3D scaffolds. Since blood supply plays a crucial role in bone defect repair, whether the 3D culture of stem cells can be vascularized has become an important hotspot for research. Organoids are stem cell-derived 3D culture systems. It is now possible to re-create the architecture and physiology of human organs in remarkable detail. A preliminary breakthrough has shown that organoids can form a vascular network through the Organ Bud technology (164, 165), which is expected to become a

future research direction for developing treatment tools for bone defects at high altitudes by stem cell transplantation.

Gene Modifications

The rapid development of modern molecular biology theory and technology has given rise to innovative solutions to address bone defect repair. For instance, using gene therapy, a target gene combined with a carrier can be injected directly into the target tissue. Alternatively, gene-modified stem cells can be used to promote new bone formation and repair bone defects. The most common methods of gene modifications are viral and non-viral vectors, including retroviruses, lentiviruses, adenoviruses, liposomes, and cationic polymers.

Osteogenesis-related genes can be introduced to target cells by vectors. This enables achieve long-term stable expression of the genes of interest within the bone defect, which could promote bone defect repair. Studies have shown that adenovirus transduction of human *BMP2* promotes osteogenic differentiation of adipose tissue fragments (166). Introducing the *BMP2* gene into BMSCs can induce bone differentiation and accelerate the healing process (167–169). Furthermore, using gene modification, the *OPG* gene was introduced into BMSCs seeded on a hydroxyapatite (HA) scaffold to form a novel OPG-BMSC-HA complex, which could promote the osteogenic effect of BMSCs and facilitate bone defect reconstruction therapy (170). *Runx2* gene-modified MSC-derived 3D spheroids have also been used to effectively promote bone regeneration (171). VEGF transfected with recombinant adenovirus vector preserves the oxygen sensitivity of HIF-1/HRE and promotes vascularization (172).

As small non-coding RNAs that regulate gene expression, miRNAs have become new targets for poor bone healing. Studies have shown that ADSCs transfected with miR-26a (173), as well as BMSCs transfected with miR-218 (174) and miR-29b-3p (175), can improve bone regeneration capacity. In addition, miR-378 can stimulate both osteogenesis and angiogenesis (176). Therefore, it can be used as a reference target for the treatment of bone defects at high altitudes (177). Regarding the mechanism underlying the effect of high altitude on bone repair, the hypoxic environment can upregulate miR-21 (107) and miR-486 (108). Therefore, these two miRNAs are also potential therapeutic targets. It was found that miR-21 activates the PI3K/Akt signaling pathway (178, 179) and significantly increases the volume of new bone formation and mineralization at the bone defect site. miR-486-3p targeting catenin β-interacting protein 1 can activate the Wnt/β-catenin signaling pathway and promote the bone formation of BMSCs (180).

Exosomes exhibit biological characteristics similar to those of the parent cells and can be used as carriers to deliver genes to cells (181). In addition, direct application of exosomes can also reduce the risk of immunogenicity, avoiding the ethical and technical issues linked to cell therapy (182). BMSC-derived exosomes carrying miR-335 promote fracture recovery through activation of the Wnt/β-catenin signaling pathway (183) and enhance bone regeneration by inhibiting hypoxia-induced osteocyte apoptosis (184)—thus, significantly preventing bone loss and increasing the blood vessel volume of the femoral head (185). Ther exosomal release is induced upon hypoxia (186) and this approach may be used in the treatment of bone defects in high-altitude-related hypoxic environments. In addition, umbilical cord MSC-derived exosomes increase the expression of VEGF and HIF-1α, which may accelerate fracture healing by promoting angiogenesis (181). The combination of exosomes with biomaterials is also one of the potential therapeutic strategies for repairing cartilage (187) and bone defects (188). Lastly, a novel "NANOBIOME" approach based on the biobanking of exosomes secreted by MSCs has shown promise as an innovative "cell-free" regenerative medicine (182).

Genetic regulation in organisms is a highly sophisticated dynamic process. The safety of gene-modified therapies in humans still needs consideration as their improper use may lead to cell dysfunction and other side effects. For example, miRNAs can be involved in multi-target regulation and may cause adverse reactions in tissues other than the bone when applied *in vivo*. In addition, human manipulation of epigenetic regulation requires higher legal requirements. Therefore, convenient, rapid, efficient, and safe epigenetic modification methods are required to achieve accurate treatment of bone defects at high altitudes.

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

In most *in vitro* experiments, researchers applied oxygen concentrations of 1–5% as hypoxic conditions and used oxygen concentrations of ∼21% at standard atmospheric pressure for comparison. However, the latter is indeed much higher than the oxygen concentration in the physiological state of the tissue microenvironment, whereas the former is close to normal physiological conditions in humans [e.g., the oxygen concentration in the bone marrow is 4–7% (189)]. Furthermore, studies examining high-altitude environments are often limited to reduced oxygen content which can be misleading. First, actual oxygen concentration at the plateau is not low as all oxygen levels are ∼21% between sea level to an altitude of 100,000 m. Altitude sickness is actually caused by a drop in PaO_2. At this elevation, hypoxia occurs when PaO_2 in the air inhaled by the human body falls below 16 kPa (2,500–3,000 m). Since the key molecule in oxygen sensing and adaptation is HIF-1, it may be a more accurate method to simulate the effect of high altitude on cells by regulating the expression of intracellular HIF-1. For example, prolyl hydroxylase inhibitors can be used to effectively increase HIF-1α protein stability (190).

At present, there is no clear optimal treatment plan for bone defects at high altitudes. The existing clinical work in high-altitude areas is mainly based on systemic oxygen therapy and bone transplantation. This is because many treatment methods involving signaling pathways and gene modifications are still in the basic research stage. Therefore, drugs targeting mitochondrial dysfunction and novel bone graft materials with oxygen-carrying capacity are urgently needed. Elabel (ELA), a peptide hormone, has been shown to promote the growth, survival, and pluripotency of human embryonic

stem cells (191, 192). However, to date, ELA has only been studied for the treatment of cardiovascular diseases (193, 194), and its therapeutic effect on bone defects at high altitudes requires investigation.

As biomaterial and bone tissue engineering technology is advancing, developing biomaterials for plateau environments could be a future research hotspot. A new type of bone graft with an oxygen-carrying function can provide both local oxygen supply and bone substitute. Wang et al. (195) developed a novel oxygen sustained-release biomaterial composed of CaO_2/gelatin microspheres and a 3D printed polycaprolactone/nano-HA composite porous scaffold. Their results showed that CaO_2/gelatin microspheres continuously released oxygen for 19 days, improving the survival rate of transplanted BMSCs in the rabbit model by reducing local apoptosis. However, further experiments evaluating high-altitude animal models are needed to confirm the repair effect of this material on bone defects.

With the discovery of mitochondrial dysfunction, autophagy, signaling pathways, and epigenetic mechanisms of high-altitude bone defects, the development of better, targeted, personalized, and precise bone defect repair methods adjustable according to the high-altitude adaptation of patients should be expected.

AUTHOR CONTRIBUTIONS

MR and ZL conceptualized this manuscript. YW and PC retrieved and interpreted the information. PC and YL wrote the manuscript. WL revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the Natural Science Foundation of Tibet Autonomous Region (Grant No. XZ2017ZR-ZY37); Guangdong Province Science and Technology Innovation Strategy Special Fund Project (Grant No. 2018KJY2014); Scientific Research Project of Southern Medical University Stomatological Hospital (Grant No. PY2018014), and the Medical Scientific Research Foundation of Guangdong Province of China (Grant No. A2020435).

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frontiers | Frontiers in Physiology

ORIGINAL RESEARCH
published: 03 June 2022
doi: 10.3389/fphys.2022.892247

Hemoglobin Mass, Blood Volume and VO_2max of Trained and Untrained Children and Adolescents Living at Different Altitudes

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Exercise Physiology, a section of the journal Frontiers in Physiology

Received: 08 March 2022
Accepted: 18 April 2022
Published: 03 June 2022

Citation:
Mancera-Soto EM, Ramos-Caballero DM, Rojas J. JA, Duque L, Chaves-Gomez S, Cristancho-Mejía E and Schmidt WF-J (2022) Hemoglobin Mass, Blood Volume and VO_2max of Trained and Untrained Children and Adolescents Living at Different Altitudes. Front. Physiol. 13:892247. doi: 10.3389/fphys.2022.892247

Introduction: To a considerable extent, the magnitude of blood volume (BV) and hemoglobin mass (Hbmass) contribute to the maximum O_2-uptake (VO_2max), especially in endurance-trained athletes. However, the development of Hbmass and BV and their relationships with VO_2max during childhood are unknown. The aim of the present cross-sectional study was to investigate Hbmass and BV and their relationships with VO_2max in children and adolescents. In addition, the possible influence of endurance training and chronic hypoxia was evaluated.

Methods: A total of 475 differently trained children and adolescents (girls $n = 217$, boys $n = 258$; untrained $n = 171$, endurance trained $n = 304$) living at two different altitudes (~1,000 m, $n = 204$, ~2,600 m, $n = 271$) and 9–18 years old participated in the study. The stage of puberty was determined according to Tanner; Hbmass and BV were determined by CO rebreathing; and VO_2max was determined by cycle ergometry and for runners on the treadmill.

Results: Before puberty, there was no association between training status and Hbmass or BV. During and after puberty, we found 7–10% higher values in the trained groups. Living at a moderate altitude had a uniformly positive effect of ~7% on Hbmass in all groups and no effect on BV. The VO_2max before, during and after puberty was strongly associated with training (pre/early puberty: boys +27%, girls +26%; mid puberty: +42% and +45%; late puberty: +43% and +47%) but not with altitude. The associated effects of training in the pre/early pubertal groups were independent of Hbmass and BV, while in the mid- and late pubertal groups, 25% of the training effect could be attributed to the elevated Hbmass.

Conclusions: The associated effects of training on Hbmass and BV, resulting in increased VO_2max, can only be observed after the onset of puberty.

Keywords: puberty, tanner stage, erythropoiesis, hypoxia, lean body mass

INTRODUCTION

The maximum oxygen uptake (VO_2max) of an adult athlete depends, among other factors, such as muscle fiber composition and cardiac compliance (Levine, 2008), to a large extent on the level of blood volume (BV) and hemoglobin since the BV determines the maximum cardiac output (CO), and the hemoglobin concentration ([Hb]) influences the maximum arterial-mixed venous oxygen difference ($a - \bar{v}O_2$ diff) (Lundby et al., 2017; Schierbauer et al., 2021). Therefore, the hemoglobin mass (Hbmass), as an integrative parameter of BV and [Hb], is closely associated with VO_2max, and a change in Hbmass of 1 g is related to a change in VO_2max of ~4 ml min^{-1} (Schmidt and Prommer, 2010). Training in adulthood only causes a relatively small adjustment of the Hbmass by 3–6.5%, (Schmidt and Prommer, 2008; Garvican et al., 2010), so that a genetic predisposition or an early training adjustment in childhood and adolescence has been discussed to explain the high values in elite athletes exceeding those of untrained subjects by more than 40% (Heinicke et al., 2001). Findings from Martino et al., (2002) showed that completely untrained people with a VO_2max of ~65 ml kg^{-1} min^{-1} possess a significantly increased BV and a higher Hbmass, suggesting a genetic determination.

This work is part of a larger cross sectional study involving nearly 500 Colombian children living at two different altitudes: at 1,000 m and above 2,600 m. The main goals were to study the general development of hemoglobin and BV throughout puberty in boys and girls and the hormonal regulation of erythropoiesis by testosterone and erythropoietin. Further central points were the determination of the influences of training status and altitude on these hematological parameters as well as on VO_2max over the entire pubertal development period and the possible dependencies between these variables. Finally, possible differences in terms of hematological and performance parameters between children and adolescents in different sports disciplines at different altitudes should be pointed out. In a first, recently published step, we showed the normal course of the increase in Hbmass during puberty in male and female adolescents and the influence of the increasing testosterone concentration in boys (Mancera-Soto et al., 2021). The work presented here represents the main part of the study and, in addition to the normal development of Hbmass, BV and VO_2max in children and adolescents, describes the influence of training status and different altitudes on the above parameters and their interactions.

According to our first paper, Hbmass increased by 33% in girls during puberty, while an increase of 95% was observed in boys—a percentage closely associated with the occurrence of testosterone and its direct and indirect effects via lean body mass (LBM). Studies of the adaptation of Hbmass in children and adolescents through long-term training processes are difficult to perform using a longitudinal design since it is barely possible to follow a statistically sufficient number of children over several years from an untrained to a highly trained state. The few existing longitudinal studies have shown only small training effects (~7%) over a period of 3 years [initial age 8–11 years old (Prommer et al., 2018)] and no effects over 1 year [11–15 years old (Eastwood et al., 2009)], over 18 months [15–17 years old, (Ulrich et al., 2011)] and over 3 years [12–15 years old (Landgraff and Hallén, 2020) and 16–19 years old (Steiner et al., 2019)]. In trained male adolescents, an increase in Hbmass up to the age of 21 was observed (Steiner and Wehrlin, 2011). It is undisputed that Hbmass is primarily determined by LBM, even in childhood and adolescence. However, elite adult endurance athletes also show greater Hbmass when normalized to LBM, suggesting additional, possibly training-specific influences, as is also known from the development of cardiac structures in adolescence (Bjerring et al., 2020).

Since it is hardly possible to quantify training-related effects on Hbmass and BV during all stages of puberty in a single longitudinal study with a statistically sufficient number of test subjects, we performed the present study using a cross-sectional format with a large number of endurance-trained and untrained children and adolescents in the range between 9 and 18 years old.

In addition to possible training effects, other factors influencing Hbmass must be considered. The excellent performance of endurance athletes, e.g., from Kenya and Colombia, has often been explained by their childhoods spent at moderate altitudes. While elevated Hbmass levels were not found in Kenyan runners (Prommer et al., 2010), elevated levels in Colombian cyclists have indeed been proved (Schmidt et al., 2002). Because [Hb] is much greater in higher altitude populations from the Andes than in those from East Africa (Beall et al., 2002), it seems possible that their erythropoietic system is more sensitive to hypoxia, and early training of an Andean population could have a pronounced influence on Hbmass. The first aim of this part of the project was, therefore, using a cross-sectional design with a large number of participants, to show the possible relationships of Hbmass and BV with endurance training activity and the place of residence. As in the first part of this project, a small, general effect of altitude and training on Hbmass was shown (Mancera-Soto et al., 2021); in this part, we increased the number of participants and focused on the training and altitude effects occurring selectively during pre/early puberty, mid-puberty and late puberty.

While the relationship between Hbmass level and VO_2max has been well documented in adults, there are only a few data on such a dependency in children and adolescents. According to Wagner (Mancera-Soto et al., 2021), VO_2max in untrained subjects is primarily limited to O_2 consumption in muscle cells. In endurance-trained athletes, however, muscular aerobic processes are optimized, and the amount of O_2 supplied by the blood circulation becomes increasingly a limiting factor. To date, it is not known whether Hbmass and BV might already have a performance-limiting influence in children or whether an increase in VO_2max is based solely on muscular adaptation. The second aim of this study was therefore to investigate the extent to which a possible training-related change in Hbmass and BV could contribute to an increase in VO_2max.

During acute stays at moderate altitude, VO_2max decreases by ~0.65% per 100 m (Clark et al., 2007). During stays of several weeks at moderate altitudes, the body adapts, among other ways, by increasing the Hbmass (Wachsmuth et al., 2013) but without achieving the VO_2max at low altitudes (Schuler et al., 2007). It is known, however, that cyclists living permanently at moderate altitudes and performing on a national level achieve a similar VO_2max at their accustomed altitudes to that of comparable athletes living at sea level or low altitudes (Schmidt et al., 2002). A third aim of this study was therefore to determine whether children and adolescents growing up at moderate altitudes might adapt to chronic hypoxia so that the altitude-related decrease in VO_2max observed in sea-level residents can be completely compensated for.

MATERIALS AND METHODS

Ethical Approval

Ethical approval was granted by the ethics committee of the National University of Colombia at Bogotá (reference: ID 06/2015). The study conformed to the standards set by the Declaration of Helsinki. Written informed consent was obtained from all of the children and their parents. The subjects volunteered to participate in the study and were free to withdraw at any time without any need to provide a reason.

Subjects

In total, 475 healthy children and adolescents (girls n = 217, boys n = 258; untrained group n = 171, endurance trained group n = 304) living at two different altitudes (~1,000 m, n = 204, ~2,600 m, n = 271) and aged from 9 to 18 years old participated in the study. Data on Hbmass and BV in relation to serum testosterone and erythropoietin concentrations were already determined from 313 participants in a recent publication (Mancera-Soto et al., 2021). Hematological data were recorded for all 475 children, and maximum oxygen uptake (VO_2max) was determined in 404 participants. For anthropometrical data and the exact distribution of the test subjects to the individual subgroups, see **Tables 1**, **2**.

All of the trained children and adolescents practiced endurance sports, i.e., medium- and long-distance running, cycling, speed skating, race walking and triathlon. The precondition for participation in the trained group was a training history of at least 1 year, a training volume of at least 6 h per week and a training frequency of at least 3 times per week (for detailed information, see **Table 2**). The participants in the untrained group did not practice any other sport apart from school sports. The subjects were recruited in the Colombian regions around Bogotá (Central East Andean region, ~2,600 m above sea level) and Tuluá/Cali (West Andean region, ~1,000 m). Ethnicity of the participants corresponded to the regional distribution, which differed only slightly between both regions [Central East/West Andes: African origin 5.1%/14.0%, European origin 58.9%/55.4%, Native American origin: 36.0%/30.5% (Ossa et al., 2016)]. With the exception of 17 participants from the Central-East group who had lived in Bogotá for at least 5 years, they had lived their entire lives at their respective altitudes and had not changed their altitude for more than a week over the past year.

None of the female participants indicated the use of hormonal contraceptives, and none used iron, folic acid or any supplements that could affect the parameters determined in this study.

Study Design

This cross sectional study with an independent groups design was conducted in Bogotá and in Tuluá on two consecutive days. On the first day, a medical examination was performed, followed by an anthropometric evaluation. Hemoglobin mass was determined using the CO-rebreathing method according to Schmidt and Prommer, (2005) and Prommer and Schmidt, (2007) and a cubital venous blood sample; i.e., 4 ml of heparinized blood were obtained for the determination of basic hematological parameters. On the second day, an incremental step test on a cycle ergometer or treadmill was performed to assess VO_2max. The cyclists, speed skaters, race walkers and triathletes, as well as the untrained children, were tested on a cycle ergometer, and the runners were tested on a treadmill.

Medical Examination and Anthropometrical Evaluation

Before the study, the children and their parents were advised not to practice exhaustive sports before both test days, to sleep at least 8 h, and to eat carbohydrate-rich meals. Health status was checked by medical examination, including electrocardiography, under resting conditions. The stage of biological maturation was evaluated using the method of coevaluation by a physician and self-report, according to Tanner, (1962), and the children were classified in stages I to V according to their external primary and secondary sexual characteristics.

For the anthropometrical evaluation, skinfold caliper measurements were performed by an identical scientist using the triceps, subscapular, supra-iliac, abdominal, thigh and medial calf (Stewart and Marfell-Jones, 2011). Percentage body fat and absolute lean body mass were estimated using a correction for the age and gender of the participants (Slaughter et al., 1988).

Sample Transport and Storage

The whole procedure, including blood sampling, sample transport, sample storage and sample analyses, was performed under standardized conditions oriented to current WADA guidelines (WADA, 2019). Four milliliters of blood were drawn from a cubital vein using EDTA vacutainers after the subject was left in a sitting position for

TABLE 1 | Number of participants in the respective subgroups.

Sex	Training status	Altitude (m)	Pre/early puberty	Mid puberty	Late puberty
Boys	Untrained	1,000	22	8	12
		2,600	22	7	19
	Trained	1,000	10	17	46
		2,600	22	19	54
Girls	Untrained	1,000	17	9	9
		2,600	18	10	18
	Trained	1,000	20	20	14
		2,600	30	33	19

TABLE 2 | Anthropometric data and training history.

		Pre/early puberty	Mid puberty	Late puberty	ANOVA $p \leq$ (Tanner, Sex, Interaction)
Number of boys/girls		76/85	51/72	131/60	
Age (years)	Boys	10.7 ± 1.3	13.8 ± 1.8^{+++}	15.8 ± 1.5^{+++}	T 0.001
	Girls	10.4 ± 1.4	13.8 ± 1.8^{+++}	15.4 ± 1.7^{+++}	S n.s.
					I n.s.
Body mass (kg)	Boys	36.3 ± 8.2	48.2 ± 8.4^{+++}	57.3 ± 7.0^{+++}	T 0.001
	Girls	34.3 ± 8.1	47.7 ± 6.9^{+++}	52.2 ± 7.3^{++} ***	S 0.001
					I 0.05
Height (cm)	Boys	141.9 ± 9.4	159.0 ± 8.8^{+++}	168.3 ± 6.8^{+++}	T 0.001
	Girls	140.1 ± 9.1	155.7 ± 6.3^{+++} *	159.4 ± 6.9^{+} ***	S 0.001
					I 0.001
BMI	Boys	18.0 ± 2.5	18.9 ± 2.0^{+}	20.2 ± 2.0^{+++}	T 0.001
	Girls	17.3 ± 2.5	19.6 ± 2.1^{+++}	20.5 ± 2.5	S n.s.
					I 0.05
Body fat (%)	Boys	16.8 ± 6.7	14.2 ± 5.3	12.8 ± 4.6	T n.s.
	Girls	18.0 ± 5.0	20.0 ± 5.6 ***	22.0 ± 6.5 ***	S 0.001
					I 0.001
LBM (kg	Boys	29.9 ± 5.9	41.2 ± 6.9^{+++}	50.0 ± 6.0^{+++}	T 0.001
	Girls	27.9 ± 5.6 *	38.0 ± 4.9^{+++} **	40.5 ± 4.7^{+} ***	S 0.001
					I 0.001
Number of boys/girls		32/50	36/53	100/33	
Training volume (h week^{-1})	Boys	10.6 ± 3.9	14.1 ± 5.0^{+}	16.8 ± 6.5	T 0.001
	Girls	11.8 ± 3.6	14.4 ± 5.5^{+}	15.8 ± 6.9	S n.s.
					I n.s.
Training history (years)	Boys	2.4 ± 1.9	3.2 ± 2.0	4.0 ± 2.7	T 0.001
	Girls	3.0 ± 2.1	4.0 ± 2.4^{+}	4.9 ± 3.0	S 0.05
					I n.s.

*Values are means ±SD. The right column presents the results of the two-way ANOVA (step 1 in the statistics section). Significance of differences between boys and girls in the same stage of maturation (t-test): * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Significance of differences from the previous stage of maturation (Bonferroni test): $^{+}$ = $p < 0.05$, $^{+++}$ = $p < 0.001$.*

at least 15 min. The samples were stored in a +4°C refrigerator until they were transported within 24 h under controlled, cool conditions to the WADA-related Institute for Doping Analysis in Bogotá, Colombia.

Blood Analytical Procedures

The hematological measurements were obtained at the Institute of Doping Analysis in Bogotá. A Sysmex XT 2000i hematological analyzer (Sysmex, Norderstedt, Germany) was used for the

determination of hemoglobin concentrations ([Hb]) and hematocrit (Hct).

Hemoglobin Mass Determination

Hemoglobin mass (Hbmass) was determined using the CO-rebreathing method as described by Schmidt and Prommer, (2005) and modified by Prommer and Schmidt, (2007). Briefly, a bolus of 99.97% carbon monoxide (boys and girls < 14 years old—untrained 0.7 ml/kg body mass, trained 0.8 ml/kg; boys > 14 years old—untrained 1.0 ml/kg, trained 1.2 ml/kg; girls > 14 years old—untrained 0.8 ml/kg, trained 1.0 ml/kg) was administered to subjects and rebreathed along with 2–3 L of 100% O_2 for 2 min. The COHb concentration was measured in sextuplicate before and 7 min after starting the CO-rebreathing in capillarized blood from an earlobe using the CO-oximeter OSM3 (Radiometer, Copenhagen, Denmark). The total Hbmass was then calculated from the difference in the COHb concentration before and after the rebreathing maneuver. A more detailed description was previously provided by Mancera-Soto et al., (2021). During the whole study, identical equipment was used by the same staff. The typical error (TE) of this method obtained in our laboratory was 2.2% and was in accordance with the TE published by Gore et al., (2005). BV, red cell volume (RCV) and plasma volume (PV) were calculated using Hb mass, venous [Hb] and venous hematocrit values (obtained from the Sysmex XT 2000i system) as described by Schmidt and Prommer, (2005).

VO_2max Tests

VO_2max was determined by an incremental step test until subjective exhaustion. Except for the runners, all of the trained and untrained children were tested on the identical cycle ergometer (Monarc Ergomedic 839, Monark Exercise AB, Vansbro, Sweden). The reason why the untrained children were tested on the bicycle ergometer was that they could be graded much more precisely than on the treadmill. Among the trained children, cyclists, triathletes and speed skaters were sufficiently familiar with bicycles to reach their discipline-specific maxima, while runners were tested on a treadmill (HP Cosmos Quasar, h/p/cosmos sports and medical GmbH, Nussdorf—Traunstein, Germany) to obtain their discipline-specific maximum values. Since in untrained subjects and in moderately trained endurance athletes, the VO_2max on the treadmill is 5–10% higher than on the bicycle ergometer because of the greater muscle mass involved (Millet et al., 2009), the values of the runners were corrected by 7% (Hermansen and Saltin, 1969). Before starting the testing, all of the children were familiarized with the ergometer or treadmill. In all of the tests, a 3-min warm-up was provided with the initial setting, and the load was increased differently in the individual groups according to the scheme presented in the supplemental material. The test was ended when the participant could not maintain the number of revolutions (cycle ergometer) or speed (treadmill). Ninety-seven percent reached at least two of the following criteria: 1) occurrence of a plateau of VO_2 despite an increase in exercise intensity; 2) heart rate greater than 180 beats/min; and 3) respiratory exchange ratio (RER) > 1.1. Measurement of VO_2max was always performed using the identical portable spirometry system (COSMED Model Quark CPET, COSMED, Rome, Italy). For VO_2max, values were averaged over a period of 30 s before exhaustion.

Statistics

For statistical analysis, IBM SPSS software, version 28, was used. Data refer to the stage of biological maturation and are presented as the means and standard deviations for the respective stage of puberty. To increase the statistical power, we pooled the data on Tanner stages I (pre puberty) and II (early puberty) and named them pre/early puberty, as well as stages IV and V (late puberty), and compared them with Tanner stage III (mid puberty). These pooled data are presented in this paper, and the data divided into the five Tanner subgroups are shown in the **supplemental material**.

The following methods were used for the statistical analysis.

1) In the first step, changes in the hematological variables and VO_2max over the course of pubertal development were examined. For the hematological parameters, a two-way ANOVA with puberty and sex as the independent variables was used. For VO_2max, training status was added as an additional independent variable, so a three-way ANOVA was used here.
2) Additionally, a one-way ANOVA with puberty as a single independent factor was performed to show the development during puberty separately in boys and girls, as well as for VO_2max separately in the trained and untrained groups. The significance of mean differences was tested using the Bonferroni test when comparing more than two groups (comparison of the three pubertal stages) and using the unpaired t-test when comparing two groups (e.g., sex differences or differences between the trained and untrained groups within one pubertal stage). To exclude the possible influences of different group sizes, effect sizes (Cohen's d) were calculated with pooled standard deviations to judge the differences between the individual puberty, sex and training groups (Sullivan and Feinn, 2012), where d = 0.2 presents small, d = 0.5 presents medium, and d = 0.8 presents large effects (Lakens, 2013).
3) Multifactorial ANOVA followed by multiple regression analyses were conducted with the whole group to determine and quantify the possible influences of puberty, sex, altitude and training status on the dependent variables of Hbmass, BV and PV. The same procedure was performed to determine the effects of puberty, sex, altitude, training status, and Hbmass on VO_2max.
4) The same procedure used for the whole group was applied separately to the pre/early-, mid-, and late puberty groups, and the main effects were calculated for each independent variable.
5) Bivariate linear regression analyses were performed with Hbmass as the dependent variable and LBM as the independent variable and with VO_2max as the dependent variable and Hbmass as the independent variable.

TABLE 3 | Hematological data and blood volumes in boys and girls in different stages of sexual maturation.

		Pre/early puberty	Mid puberty	Late puberty	ANOVA $p \leq$ (Tanner, Sex, Interaction)
number of boys/girls		76/85	51/72	131/60	
[Hb] (g dl^{-1})	Boys	14.6 ± 1.0	15.1 ± 1.2^{+++}; *0.5*	15.8 ± 1.2^{+++}; 0.6	T 0.001
	Girls	14.3 ± 0.9	14.2 ± 1.0; 0.1	14.2 ± 1.1; 0.0	S 0.001
	d_{cohen}	0.3	***; 0.8	***; 1.5	I 0.001
Hct (%)	Boys	42.0 ± 2.3	43.9 ± 2.9^{+++}; 0.7	46.5 ± 3.0^{+++}; 0.9	T 0.001
	Girls	41.8 ± 2.3	42.2 ± 2.6; 0.2	42.3 ± 3.1; 0.0	S 0.001
	d_{cohen}	0.1	***; 0.6	***; 1.4	I 0.001
Hbmass (g)	Boys	408 ± 88	636 ± 152^{+++}; 1.9	788 ± 133^{+++}; 1.1	T 0.001
	Girls	367 ± 86	503 ± 104^{+++}; 1.4	531 ± 107; 0.3	S 0.001
	d_{cohen}	**; 0.5	***; 1.1	***; 2.2	I 0.001
Hbmass (g kg^{-1})	Boys	11.4 ± 1.5	13.1 ± 1.8^{+++}; 1.0	13.8 ± 1.5^{+}; 0.4	T 0.001
	Girls	10.8 ± 1.4	10.6 ± 1.7; 0.1	10.2 ± 1.3; 0.3	S 0.001
	d_{cohen}	*; 0.4	***; 1.5	***; 2.5	I 0.001
Hbmass (g $kgLBM^{-1}$)	Boys	13.7 ± 1.5	15.3 ± 1.7^{+++}; 1.0	15.8 ± 1.4; 0.3	T 0.001
	Girls	13.2 ± 1.6	13.2 ± 1.9; 0.0	13.0 ± 1.5; 0.1	S 0.001
	d_{cohen}	*, 0.3	***; 1.5	***; 2.0	I 0.001
RCV (ml)	Boys	1,182 ± 260	1849 ± 452^{+++}; 1.9	2,320 ± 399^{+++}; 1.1	T 0.001
	Girls	1,082 ± 259	1,490 ± 307^{+++}; 1.4	1,581 ± 326; 0.3	S 0.001
	d_{cohen}	*; 0.4	***; 1.0	***; 2.0	I 0.001
RCV (ml kg^{-1})	Boys	32.7 ± 4.6	38.2 ± 5.2^{+++}; 1.1	40.5 ± 4.5^{+}; 0.5	T 0.001
	Girls	31.7 ± 4.1	31.3 ± 5.0; 0.1	30.3 ± 4.3; 0.2	S 0.001
	d_{cohen}	0.2	***; 1.4	***; 2.3	I 0.001
RCV (ml $kgLBM^{-1}$)	Boys	39.4 ± 4.6	44.5 ± 5.1^{+++}; 1.1	46.3 ± 4.2; 0.4	T 0.001
	Girls	38.7 ± 4.8	39.1 ± 5.6; 0.1	38.9 ± 4.8; 0.0	S 0.001
	d_{cohen}	0.1	***; 1.0	***; 1.7	I 0.001
BV (ml)	Boys	3,092 ± 661	4,622 ± 1050^{+++}; 1.8	5,495 ± 931^{+++}; 0.9	T 0.001
	Girls	2,836 ± 657	3,883 ± 756^{+++}; 1.5	4,112 ± 770; 0.3	S 0.001
	d_{cohen}	*; 0.4	***; 0,8	***; 1.6	I 0.001
BV (ml kg^{-1})	Boys	85.4 ± 10.4	95.6 ± 11.4^{+++}; 0.9	95.9 ± 10.1; 0.0	T 0.01
	Girls	83.5 ± 11.5	81.6 ± 11.9; 0.2	78.8 ± 10.2; 0.3	S 0.001
	d_{cohen}	0.2	***; 1.2	***; 1.7	I 0.001
BV (ml $kgLBM^{-1}$)	Boys	102.9 ± 10.8	111.6 ± 12.4^{+++}; 0.8	109.6 ± 9.4; 0.2	T 0.01
	Girls	101.9 ± 13.0	102.0 ± 13.3; 0.2	101.3 ± 12.2; 0.1	S 0.001
	d_{cohen}	0.1	***; 0.7	***; 0.8	I 0.01
PV (ml)	Boys	1909 ± 414	2,772 ± 629^{+++}; 1.7	3,175 ± 581^{+++}; 0.7	T 0.001
	Girls	1756 ± 409	2,393 ± 473^{+++}; 1.4	2,531 ± 479; 0.3	S 0.001
	d_{cohen}	*; 0.4	***; 0.7	***; 1.42	I 0.001
PV (ml kg^{-1})	Boys	52.7 ± 6.5	57.4 ± 7.3^{+++}; 0.7	55.4 ± 7.0; 0.3	T n.s.
	Girls	51.8 ± 7.9	50.3 ± 7.5; 0.2	48.6 ± 6.9; 0.2	S 0.001
	d_{cohen}	0.1	***; 1.0	***; 1.0	I 0.001
PV (ml $kgLBM^{-1}$)	Boys	63.6 ± 7.0	67.1 ± 8.7^{+}; 0.5	63.4 ± 7.0^{+}; 0.5	T n.s.
	Girls	63.2 ± 8.9	62.8 ± 8.6; 0.0	62.4 ± 8.6; 0.0	S 0.05
	d_{cohen}	0.1	**; 0.5	0.1	I n.s.

*The data are presented as absolute values and as values normalized to body mass and lean body mass (LBM). Hbmass, hemoglobin mass; RCV, red cell volume; BV, blood volume; PV, plasma volume. The right column presents the results of the two-way ANOVA (step 1 in the statistics section). Significance of differences between boys and girls in the same stage of maturation (t-test): *p < 0.05, **p < 0.01, ***p < 0.001, Significance of differences from the previous stage of maturation (Bonferroni test): +p < 0.05, ++p < 0.01, +++p < 0.001. The effect size for the comparison between boys and girls, as well as for the comparison of different stages of maturation, is presented in italics as d_{cohen} next to the symbols for significance. Hbmass, hemoglobin mass; RCV, red cell volume; BV.blood volume; PV, plasma volume.*

TABLE 4 | Multiple analyses of variance with hematological parameters **(A)** and VO_2max **(B)** as dependent variables followed by a multiple regression analysis.

A	**Whole group *n* = 475**					
	Main effect				**Interaction between**	
	Puberty Pre-, mid-, end-puberty	**Sex Boys/girls**	**Training Trained/untrained**	**Altitude 2600m/1000 m**	**Sex and puberty**	**Training and puberty**
[Hb] (g dl^{-1})	0.3 ± 0.1***	2.3 ± 0.1***	n.s.	1.0 ± 0.1***	***	
Hct (%)	1.3 ± 0.1***	2.3 ± 0.2***	0.8 ± 0.2**	2.3 ± 0.2***	***	
Hbmass (g)	138 ± 7***	143 ± 11***	65 ± 11***	35 ± 11***	***	*
Hbmass (g kg^{-1})	0.41 ± 0.1***	2.2 ± 0.1***	1.4 ± 0.1***	0.7 ± 0.1***	***	*
Hbmass (g $kgLBM^{-1}$)	0.44 ± 0.1***	1.8 ± 0.1***	1.0 ± 0.1***	0.8 ± 0.1***	***	*
BV (ml)	924 ± 46***	764 ± 78***	438 ± 81***	n.s.	***	*
BV (ml kg^{-1})	n.s.	10.6 ± 1.0***	9.3 ± 1.0***	n.s.	***	*
BV (ml $kgLBM^{-1}$)	n.s.	5.7 ± 1.1***	6.5 ± 1.1***	n.s.	***	*
PV (ml)	508 ± 28***	365 ± 48***	231 ± 49***	-95 ±47*	***	
PV (ml kg^{-1})	n.s.	4.6 ± 0.6***	5.0 ± 0.7***	-2.1 ± 0.6**		
PV (ml $kgLBM^{-1}$)	n.s.	n.s.	3.2 ± 0.8***	-2.7 ± 0.7***		

B	**Whole group n = 404**					
	Puberty Pre-, mid-, end-puberty	**Sex Boys/girls**	**Training Trained/untrained**	**Altitude 2600m/1000 m**	**Hbmass g or g/kg or g/kgLBM**	**Interaction between training and Hbmass**
VO_2max (ml min^{-1})	91 ± 25***	201 ± 36***	531 ± 33***	n.s.	2.8 ± 0.1***	***
VO_2max (ml min^{-1} kg^{-1})	-1.2 ± 0.4**	5.1 ± 0.8***	12.4 ± 0.8***	1.7 ± 0.7***	2.0 ± 0.2***	*
VO_2max (ml · min^{-1} $kgLBM^{-1}$)	-1.2 ± 0.5**	5.9 ± 0.9***	14.5 ± 0.8***	2.4 ± 0.8***	1.2 ± 0.2***	**

*Presented are significant main effects with standard errors and interactions. The effects were calculated as described in step 3 in the statistics section by multiple regression analysis. [Hb], hemoglobin concentration; Hct, hematocrit; Hbmass, hemoglobin mass; BV, blood volume; PV, plasma volume; LBM, lean body mass. Significance of effects: *p < 0.05, **p < 0.01, ***p < 0.001, n.s., not significant.*

[Hb], hemoglobin concentration; Hct, hematocrit; Hbmass, hemoglobin mass; BV, blood volume; PV, plasma volume; LBM, lean body mass; n.s., not significant.

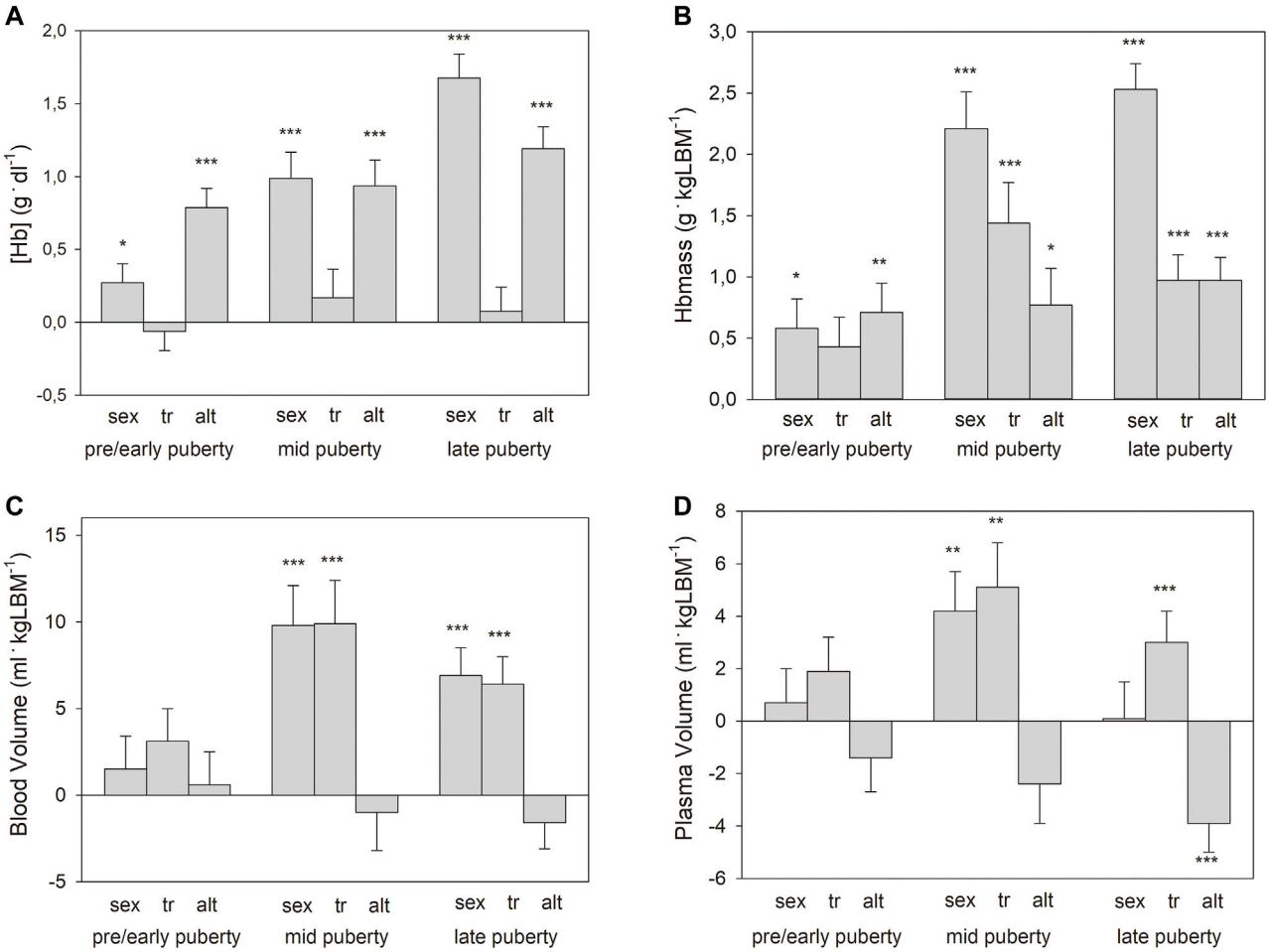

FIGURE 1 | Main effects with standard errors calculated by multiple regression analysis separately for the pooled puberty stages for sex (boys vs. girls), training status (tr, trained vs. untrained), and altitude (alt, 2,600 m vs. 1,000 m) on: **(A)** hemoglobin concentrations ([Hb]); **(B)**. Hbmass; **(C)**. blood volume; and **(D)**. plasma volume. Data for Hbmass, BV, and PV are normalized to lean body mass (LBM). Significance of effects: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

RESULTS

The anthropometric characteristics show the well-known time course during development, with higher fat mass in girls than in boys in the mid and late puberty and a larger LBM in the boys than in the girls during these stages of maturation (**Table 2**).

Hemoglobin and Blood Volumes

In both sexes, Hbmass was elevated in the later puberty stages compared to pre/early puberty; this was, however, much more pronounced in the boys (from 408 ± 88 g to 788 ± 133 g) than in the girls (from 367 ± 86 g to 531 ± 107 g; **Table 3**). Significantly higher absolute Hbmass and blood volumes (RCV, BV, PV) were observed in girls during mid puberty compared to pre/early puberty, and in boys during late puberty compared to pre/early and mid puberty. When Hbmass, RCV, BV and PV were normalized to LBM, no difference or only small differences were detected between girls and boys at pre/early puberty, and no significant difference existed between the female puberty groups. In boys, however, considerably higher values for Hbmass, RCV and BV were detected in the mid puberty and late puberty groups compared to pre/early puberty (**Table 3**). The PV did not differ between the sex and puberty groups with the exception of higher values in mid-puberty boys (**Table 3**).

Hemoglobin concentrations ([Hb]) in the boys was higher at late puberty (15.8 ± 1.2 g · dl^{-1}) than at pre/early puberty (14.6 ± 1.0 g · dl^{-1}) while in the girls, no difference was observed among the different puberty groups (**Table 3**). At pre/early puberty, no sex-related differences in [Hb] and Hct were detected, while at mid puberty and late puberty, all of the hematological values were considerably higher in the boys (**Table 3**).

Multifactorial ANOVA (see #3 in the section "statistics") with absolute Hbmass and with normalized Hbmass (g kg^{-1} and g $kgLBM^{-1}$) as the dependent variables yielded significant main effects of phase of puberty, sex, training status and place of residence, as well as of the interaction of phase of puberty with sex and training status (**Table 4**). No

TABLE 5 | VO_2max in trained and untrained boys and girls in different stages of sexual maturation.

			Pre/early puberty	Mid puberty	Late puberty	ANOVA $p \leq$ (Tanner, Sex, Training, Interaction)
Number of boys/girls		Untrained	43/32	13/17	28/19	
		Trained	29/40	32/37	87/27	
VO_2 max ($ml \cdot min^{-1}$)	Untrained	Boys	1,561 ±427	2,141 ± 395^{+++}; *1.4*	2,408 ± 447; *0.6*	T 0.001
		Girls	1,624 ± 350^{+}; *0.7*	1,365 ±355	1,704 ± 299; *0.2*	S 0.001
		d_{cohen}	**; 0.6*	****; 1.4*	****; 1.8*	Tr 0.001
	Trained	Boys	1,981 ± 483	2,900 ± 521^{+++}; *1.8*	3,449 ± 463^{+++}; *1.1*	T × S 0.001
		Girls	1,660 ±328	2,296 ± 290^{+++}; *2.0*	2,337 ± 383; *0.1*	T × Tr 0.001
		d_{cohen}	***; 0.8*	****; 1.5*	****; 2.5*	S × Tr 0.05
VO_2 max ($ml\ min^{-1}\ kg^{-1}$)	Untrained	Boys	43.7 ± 11.2	42.9 ± 4.7; *0.1*	42.3 ± 6.4; *0.1*	T n.s.
		Girls	39.5 ± 6.5	33.7 ± 5.6^{++}; *0.9*	31.8 ± 3.7; *0.4*	S 0.001
		d_{cohen}	**; 0.4*	****; 1.8*	****; 1.9*	Tr 0.001
	Trained	Boys	55.8 ± 7.2	61.0 ± 8.1^{++}; *0.7*	60.5 ± 6.2; *0.1*	T × S 0.001
		Girls	49.6 ± 8.4	48.9 ± 5.1; *0.1*	46.9 ± 5.9; *0.4*	T × Tr 0.001
		d_{cohen}	***; 0.8*	****; 1.8*	****; 2.2*	S × Tr n.s.
VO_2 max ($ml \cdot min^{-1} \cdot kgLBM^{-1}$)	Untrained	Boys	53.3 ± 11.8	52.5 ± 7.2; *0.1*	49.8 ± 6.7; *0.4*	T n.s.
		Girls	48.9 ± 7.9	43.4 ± 7.1^{+}; *0.7*	42.5 ± 3.8; *0.2*	S 0.001
		d_{cohen}	*0.4*	***; 1.9*	****; 1.3*	Tr 0.001
	Trained	Boys	66.0 ± 8.7	69.9 ± 7.7; *0.5*	68.8 ± 6.3; *0.2*	T × S 0.05
		Girls	59.9 ± 8.7	60.2 ± 5.8; *0.0*	58.1 ± 6.8; *0.3*	T × Tr 0.01
		d_{cohen}	***; 0.7*	****; 1.4*	****; 1.7*	S × Tr n.s.

*The data are presented as absolute values and as values normalized to body mass and lean body mass (LBM). Significance of differences between boys and girls in the same stage of maturation: * = p < 0.05, ** = p < 0.01, *** = p < 0.001. The right column presents the results of the two-way ANOVA (step 1 in the statistics section). Significance of difference from the previous stage of maturation (Bonferroni test): + = p < 0.05, ++ = p < 0.01, +++ = p < 0.001. Significance of differences between trained and untrained groups in the same stage of maturation (t-test): p < 0.001 in all cases. The effect size for the comparison between boys and girls, as well as for the comparison of different stages of maturation, is presented in italics as dcohen next to the symbols for significance. The effect size for the comparison between the trained and untrained groups in all cases: dcohen > 0.8.*

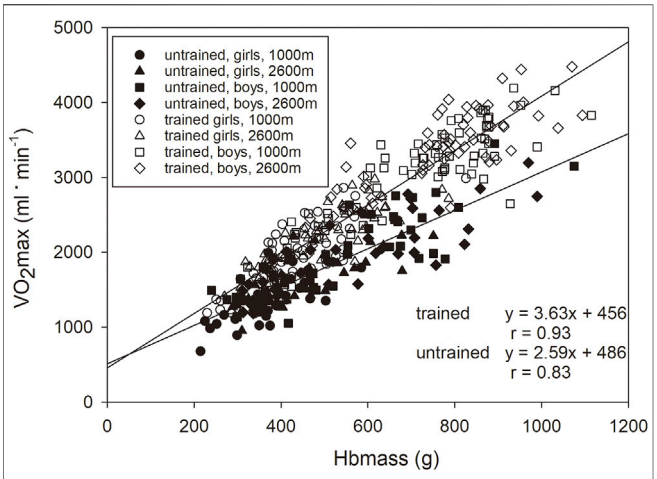

FIGURE 2 | Relationship between Hbmass and VO_2max normalized to LBM presented for the trained (y = 2.3x + 31.0, r = 0.54, $p < 0.001$) and untrained groups (y = 1.4x + 30.4, r = 0.31, $p < 0.01$).

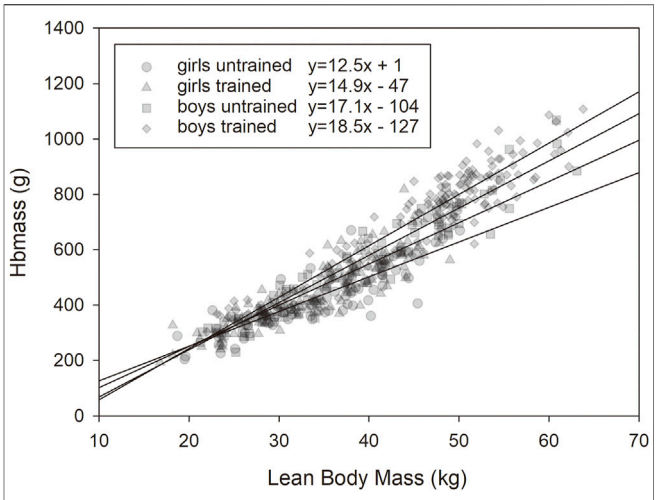

FIGURE 4 | Relationship between lean body mass (LBM) and Hbmass in the trained and untrained male and female groups.

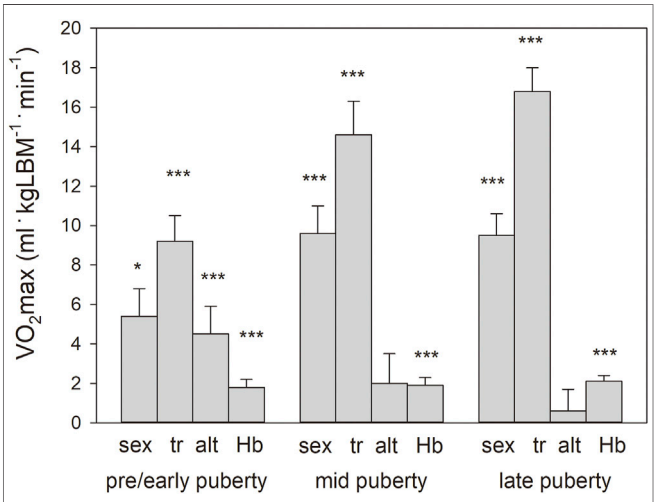

FIGURE 3 | Main effects with standard errors calculated by multiple regression analysis separately for the pooled puberty stages for sex (boys vs girls), training status (tr, trained vs. untrained), altitude (alt, 2,600 m vs. 1,000 m), and Hbmass (g $kgLBM^{-1}$) on VO_2max normalized to lean body mass. Because of the interaction in the mid-and late puberty groups, the main effects for sex and Hbmass were calculated independently. Significance of effects: * = $p < 0.05$, *** = $p < 0.001$.

interaction was found for the phase of puberty with place of residence.

When the whole group was divided and the pre/early-, mid- and late pubertal phases were separately analyzed (see #4 in the section "statistics"), only a slight, significant effect of sex and no effect of training status were observed in the pre/early pubertal group for absolute and normalized Hbmass, while both effects were highly significant in the later stages. (**Figure 1B** shows Hbmass normalized to kg LBM). Moderate altitudes had significant, positive effects in all puberty stages (**Figure 1B**).

Multifactorial ANOVA (see #3 in the section "statistics") for BV as a dependent variable showed significant, positive main effects of sex and training status, as well as interactions for sex and training status, with the phase of puberty but no effect of altitude (**Table 4**). The main effects of sex and training status (see #4 in the section "statistics") were highly pronounced in the mid- and late pubertal stages but did not exist pre/early puberty (**Figure 1C**). The effects of sex and training status on PV were similar to those on BV, while moderate altitude exerted a negative effect on PV (**Table 4**; **Figure 1D**).

Maximum Oxygen Uptake (VO_2max)

The absolute VO_2max showed a well-known sex- and training-specific increase with increasing sexual maturity (**Table 5**). When VO_2max was normalized to LBM, there was still a clear, positive influence of sex and training status but not of the puberty stage.

The linear regression analysis between Hbmass and VO_2max showed a clear difference between the trained and untrained groups, with a steeper slope of the regression line of the trained group (3.63 vs. 2.59. The relationship between the two variables became weaker when the values related to LBM were used (trained, absolute vs. normalized: r = 0.93 vs r = 0.54, untrained: r = 0.83 vs r = 0.30), but the difference between the groups persisted (**Figure 2**).

Multifactorial ANOVA (see #3 in the section "statistics") with the absolute VO_2max of the entire group as the dependent variable showed significant main effects for puberty status, sex, training status, and Hbmass and interactions between training status and Hbmass (**Table 4**). For normalized VO_2max as the dependent variable, also a small positive effect was found for altitude.

In all stages of puberty, there were significant main effects (see #4 in the section "statistics") of the confounding factors of sex, training, and Hbmass. Moderate altitude also tended to have a

positive effect on VO_2max. For the magnitude of the main effects, see **Figure 3**.

DISCUSSION

In our recent study conducted with ~65% of the participants of this study, we monitored the normal development of Hbmass and BV during childhood and adolescence and the role that testosterone plays during male puberty. In this part of the project, we demonstrated that an association of Hbmass and BV with training status does not exist until the onset of puberty, but close associations are found thereafter. Living at a considerable altitude, in contrast, stimulates Hbmass age independently.

An important result is that the training status determines VO_2max much more via other factors than via Hbmass and that VO_2max is influenced by an endurance training-associated increase in Hbmass only after the onset of puberty. Living at a moderate altitude completely compensates for the hypoxic conditions and does not negatively affect VO_2max.

Hemoglobin and Blood Volumes During Sexual Maturation

The development of Hbmass, BV and PV during maturation corresponds to the data we recently published (Mancera-Soto et al., 2021). In girls, there was an equal percentage increase in RCV or Hbmass and PV by approximately 45% for both parameters from pre/early to late puberty, reflected in unchanged [Hb] and Hct levels. However, in boys, Hbmass increased by 95%, while PV increased by only 65%, resulting in an increase in [Hb] from 14.6 g dl^{-1}–15.8 g dl^{-1}. These data clearly show that [Hb] is not a good indicator of the absolute amount of hemoglobin in the body and does not represent the quantitative development of hemoglobin in childhood and adolescence.

Hbmass and Training

The influence of training has only been investigated in a few studies with a small number of test subjects. Prommer et al., (2018) described a small training effect of 25 g (7%) over the course of 2.5 years in children aged 8–11 years old at the start of the study, whereas other studies did not find any specific training effects in 12–15-year-old boys and girls (Landgraff and Hallén, 2020) or in 15–17-year-old boys (Ulrich et al., 2011).

As demonstrated in **Figure 1**, at pre/early puberty, there were no associations between training status and Hbmass or blood volumes. In the mid- and late pubertal states, however, there were significant effects ranging between 10 and 12% occurring independently on LBM in both sexes (**Figures 1**, **4**). Possible reasons for these delayed training adaptations could be the training status of the different age groups, although the youngest participants already had a training history of at least 1 year (mean 2.7 years) and a training volume of at least 6 h/week (mean 11 h/week) completed. In contrast, the mean training history and the weekly training volume in the end-pubertal group were up to twice as high (see **Table 2**).

Conversely, the relatively small increase in Hbmass at later developmental ages, which is only slightly greater than in adults who begin intensive endurance training (Schmidt and Prommer, 2008), demonstrates only slight erythropoietic stimulation from exercise. Since there was no interaction between training status and sex, the increase in Hbmass in the mid- and late pubertal training groups was not achieved through training-related testosterone stimulation. This outcome is in line with our recently published data, which showed no association between training status and serum testosterone levels during puberty (Wagner, 2000).

BV shows a very similar association with training status as Hbmass, which could indicate that the increase in Hbmass occurs less via primary erythropoietic stimulation but rather as a consequence of the training-related increase in BV with subsequent normalization of [Hb] and Hct *via* a so-called critmeter (Montero and Lundby, 2018). This outcome would mean that the undoubtedly higher and pronounced Hbmass values in elite endurance athletes are mainly genetically determined and can only be modulated to a relatively small extent by training. However, data on the genetic mechanisms are scarce. Studies with endurance athletes have identified only NFIA-AS2 polymorphisms as a mediator of erythropoiesis, with carriers of the variant genotype having higher hemoglobin mass, BV, and PV (Malczewska-Lenczowska et al., 2020; Kim et al., 2022). The view of the involvement of the kidney as a critmeter is also supported by the behavior of the PV, which was significantly increased in the mid- and late puberty training groups and thus, together with the larger erythrocyte volume, contributes to the increase in BV.

Hbmass and Altitude

The influence of moderate altitude on the [Hb] is approximately 1.0 g/dl in all stages of puberty and does not show sex-specific characteristics, corresponding to data from Böning et al., (2004); Schmidt et al., (2002) in adults from the same regions, as well as data from Gassmann et al., (2019), who showed significantly greater [Hb] in inhabitants of the Andes compared to inhabitants of similar high altitudes from Tibet and East Africa. The influence of altitude on Hbmass and BV has not yet been investigated in children. For the first time, we showed here that an equal erythropoietic adaptation as in adults also occurs in childhood and adolescence. There are data available from adults from the same regions where this study was conducted showing almost the same absolute Hbmass values and altitude-related differences in women as we do here in Bogotá for late pubertal girls (Böning et al., 2004). In men, however, trained and untrained subjects from Bogotá show significantly higher values than adolescents during late puberty (Schmidt et al., 2002). This outcome demonstrates that, in contrast to the girls, the development of Hbmass and the associated BV is not yet complete at the end of puberty in boys, which is in good agreement with the data of Steiner and Wehrlin, (2011), who observed an increase in Hbmass up to the age of 21 years old.

In our recently published study, we did not find any altitude-related effects on serum erythropoietin and testosterone concentrations in children and adolescents (Wagner, 2000) and can therefore exclude a simple increase in these hormones as the cause of the higher Hbmass at higher altitudes. However, it seems possible that, at higher altitudes, there occurs a decrease in the soluble EPO receptor, improving the binding of EPO to its membrane receptor (Villafuerte et al., 2014) and thereby augmenting the erythropoietic effectiveness at identical serum EPO concentrations.

The whole BV did not differ between the groups from low and moderate altitudes since the higher erythrocyte volume at moderate altitudes is compensated for by a lower PV (**Table 3**; **Figure 1**), which can be attributed to a hypoxia-related change in the concentration of the volume-regulating hormones aldosterone and ANP (Schmidt et al., 1999). In adults, this behavior of the PV has not only been described for short-term stays at different altitudes (Beidleman et al., 2017) but also for chronic stays at the identical moderate altitudes to those in this study (Böning et al., 2004).

In this study, we did not detect any interactions between the training effects and the altitude effects on Hbmass but only the main effects, which are demonstrated in **Figure 1**. This outcome suggests independent regulatory mechanisms, which at moderate altitudes are likely to be hypoxic renal stimulation and, with regard to the training effects, compensation for the expansion of the PV (Schmidt et al., 1988).

VO_2max and Training

The increase in the absolute VO_2max with age, as well as the sex differences, correspond to the model developed by Armstrong and Welsman, (2019). Since LBM is the main influencing factor on VO_2max (Armstrong and Welsman, 2019), the normalized VO_2max should be constant during the maturation and growth process of children and adolescents. Landgraff et al., (2021), however, demonstrated a slight decrease over the course from 12 to 15 years of age in endurance-trained boys. Our data do not show any significant differences in the normalized values during the growth and maturation process but a clear trend toward decreasing values with increasing age in the untrained children and increasing values in the trained children. Because in our study, the normalized data differed greatly between the trained and untrained children, factors other than LBM also influenced VO_2max and even made variation in the normalized values during maturation likely.

The improvements through training shown by Bar-Or for children and adolescents (Bar-Or and Rowland, 2004) were less than 5% in pre/early pubescent children and more than 15% in mid- and late pubertal adolescents. This different behavior in the age groups could in principle also be observed in this study. However, the differences between the trained and untrained groups in all stages of development were greater than those in Bar-Or's review and amount to ~20% in the pre/early pubertal stage and ~40% in the mid- and late pubertal stages for both sexes (**Table 5**). While Bar-Or does not provide any definitive causes for better trainability at older ages, the finding in our study could also be due to a longer training history and possibly more intensive training in the older children.

To date, VO_2max has been examined several times in children and adolescents, but the importance of both Hbmass and BV on VO_2max has rarely been quantified. In statistical analysis, Hbmass and BV have almost the same effect on VO_2max. Both parameters are associated with the maximum stroke volume (Schierbauer et al., 2021), and a high Hbmass guarantees an advantageous [Hb] and thus O_2 transport capacity. In children, both maximum SV and $a - \bar{v}O_2$ diff are mainly determined by the magnitude of LBM (Armstrong and Welsman, 2020). The effects of endurance training on VO_2max, in contrast, are—like in adults (Lundby et al., 2017)—mainly brought about by increasing the SV and less so by increasing the $a - \bar{v}O_2$ diff (Obert et al., 2003). As a reason for this change, cardiac remodeling through the development of cardiac morphology and function in youth athletes has been reported (Nottin et al., 2002). Recently, Bjerring et al., (2020) demonstrated distinct phases in the development of the young athletic heart with a tendency toward concentric remodeling in endurance athletes at the age of 12, with increased wall thickness and cardiac mass compared to sedentary peers. In later ages, this development changed to eccentric remodeling. Cardiac changes during adolescence appear to be associated with the development of BV and Hbmass, as Perkins et al., (2022) showed a simultaneous increase in left ventricular mass and expansion of Hbmass and BV in trained children in the later stages of puberty.

In this study, the effect of the training status on VO_2max, already present in the pre/early pubescent stage but even more pronounced in the mid- and late pubertal stages, was remarkable and indicates—because LBM was similar in the trained and untrained groups—LBM-independent training adjustments of the O_2 supply, likely via an increased stroke volume, as mentioned above.

In the pre/early pubertal stage, there is merely a tendency toward an expansion of the BV, and the hypothesized higher SV should be obtained by functional improvements within the circulation. In the mid- and late pubertal stages, however, the increase in BV of ~400–600 ml in both sexes should lead to an increase in the maximum SV of ~12 ml (Schierbauer et al., 2021). This increase, in turn, should lead to a higher maximum cardiac output (Q_{max}) of ~2.3 L min^{-1} and thus a higher VO_2max of ~200 ml min^{-1} (Schierbauer et al., 2021). Conversely, although this adjustment indicates an important improvement, it can only explain part of the difference in VO_2max between the trained and untrained groups. In the later stages of development, both the functional improvements as in the pre/early pubertal stage and the BV increase-related optimizations should be mainly responsible for the improved VO_2max.

A similar conclusion emerges when one examines the relationship between Hbmass and VO_2max, as demonstrated in **Figure 2**. With the same Hbmass of 13.1 g kg LBM^{-1} (average value of boys in the mid pubertal stage), untrained children had a VO_2max of 49.1 ml $min^{-1.}$ kg LBM^{-1}, but trained children had a VO_2max of 61.3 ml/

min/kg LBM^{-1}, indicating 25% more effectiveness in O_2 transport and/or O_2 metabolism. Since the trained groups of both sexes in the mid- and late pubertal stages also possess ~1.5 g kg LBM^{-1} more Hbmass (see **Figure 1B**), their VO_2max increases by an additional 3.5 ml min^{-1}· kg LBM^{-1}, i.e., by 7% more. The adaptation processes via increased erythropoiesis thus constitute ~22% of the total training adaptations.

VO_2max and Altitude

Immediately after ascent to a moderate altitude, VO_2max decreases by ~0.65% per 100 m, i.e., by 17% from sea level to 2,600 m (Clark et al., 2007). During longer stays in altitude training camps at ~2,300 m, the VO_2max improves, but after 3 weeks, it is still ~5% less than at sea level (Schuler et al., 2007), and the performance, measured as competition pace, is still decreased by ~3% (Chapman and Levine, 2007). In the case of chronically altitude-adapted cyclists at the national level from Bogotá, however, comparable values (69 ml kg^{-1}· min^{-1}) were measured at moderate altitudes to those of European cyclists with the same performance level at sea level (68 ml kg^{-1}· min^{-1}) (Schmidt et al., 2002). To date, however, there are no data available comparing adolescent athletes born and living at different altitudes.

In the present study, we did not detect any negative, but even a tendency to a positive effect of altitude on VO_2max (**Figure 3**). This result cannot be attributed to methodological causes since, when comparing the cycle ergometer and treadmill tests, the discipline-specific performance determined as power and speed at 2,600 m did not differ from that at 1,000 m. Rather, the following mechanisms could contribute to the complete compensation for the hypoxic surroundings at 2,600 m. 1) There is an increase in Hbmass, which is ~7% in all stages of development; 2) The Hb-O_2 binding properties are adapted to the altitude in the form of a steeper Hb-O_2 binding curve, resulting in a left shift in the upper part and a right shift in the lower part (Schmidt et al., 1990). This process enables a higher arterial O_2 saturation in the lungs and a more effective O_2 release in the muscle tissue. In that study, we were able to show that, with a capillary pO_2 of 15 mm Hg, this mechanism delivers ~4% more O_2 to the tissue. 3) Adequate ventilation and improved diffusion properties in the lungs (Wagner et al., 2002) lead to higher arterial O_2 saturation at rest (Böning et al., 2004), especially during exercise, which is much more pronounced than in altitude-adapted lowlanders (Favier et al., 1995).

Practical Importance

Based on these considerations, the importance of a high Hbmass for VO_2max can be emphasized. Since the effects of the training-related increase in Hbmass are almost nonexistent at pre/early pubescent ages and relatively small at mid- and late pubertal ages, genetic predispositions for a high Hbmass and thus high VO_2max in adulthood are absolute prerequisites. Screening Hbmass as one biological marker at a young age was therefore recommended by Wehrlin and Steiner, (2021) to characterize possible talent for endurance sports. In this study, for example, after correction for the training and altitude effects, 9% of all of the values of the children and adolescents from the untrained groups were greater than the 90th percentile, and 3% were greater than the 95th percentile of the sex- and puberty stage-specific Hbmass values, while the numbers for the trained children were 12 and 5%, respectively. This outcome indicates that a large number of children who have not yet practiced any sports might still have the potential to become successful endurance athletes. These subjects could be those individuals who respond to endurance training with particularly large increases in VO_2max, as described by Bouchard et al., (1999) in their genetic studies.

Living at a moderate altitude from childhood fully compensates for the decreased ambient oxygen pressure so that VO_2max is not restricted. Athletes from moderate altitudes, therefore, have a clear advantage in competitions at altitude over athletes from sea level even if the latter have adapted to high-altitude training camps. This advantage should also exist at lower altitudes and could be one reason for the success of elite Colombian cyclists, who mainly grew up in the area around Bogotá that we examined in this study. However, it is still unclear to what extent and in what time frame this advantage over athletes from sea level will be reduced over time when competing at low altitudes.

Limitations

The study was conducted in Colombia, whose residents are of different ethnic origins. Although the ethnic groups in the two regions from which the children studied here derived are fairly homogeneous, it must be noted that the majority of residents of these regions are mestizos with more or less European or Native American origin. In order to be able to assess possible ethnic influences on the parameters determined here, it would therefore be desirable to carry out a genetic typing, which, however, could not be done in this study.

In the present cross-sectional study, relationships between Hbmass or BV and important confounding factors were derived by means of regression analysis. Highly significant correlations with Hbmass and other influencing factors were also established for VO_2max. However, the correlations calculated here do not prove cause and effect but only associations between the respective variables. Cause and effect can only be estimated through longitudinal studies over several years. However, since it is extremely difficult to observe training effects from the untrained state to the competitive athlete over the course of child and adolescent development on a statistically sufficiently large number of test subjects, cross-sectional studies such as these could be valuable. Therefore, it cannot be excluded that children with a genetically predetermined high Hbmass and BV facilitating a higher VO_2max are more likely to be found in the trained group and thus influence the conclusions about the trainability of Hbmass and BV.

In boys, the parameters related to body mass and LBM agree very well. In the case of girls, however, the data diverge notably over the course of puberty due to the increasing body fat percentage. Therefore, the hematological variables are presented as normalized to LBM to better compare boys

and girls. The LBM was not measured directly here but was estimated by skin fold measurements, and based on these measurements, the body fat percentage was determined. Although this method correlates sufficiently with the gold standard methods [dual-energy X-ray absorptiometry (Hofsteenge et al., 2015), three-component method (Aguirre et al., 2015)], the results should be interpreted with caution.

The VO_2max of the runners was corrected by 7% because it was determined on the treadmill and not on the bicycle ergometer, as was the case with the other participants. This adjustment was necessary because very well-trained cyclists show almost identical values on both devices, but runners are significantly less efficient on the ergometer. Since there are interindividual differences when comparing the VO_2max on the treadmill and on the bicycle ergometer, inaccuracies due to the 7% correction cannot be excluded. However, since the same number of runners were tested at both altitudes, this possible inaccuracy should not affect the validity of this work.

CONCLUSIONS

The present cross-sectional study showed, on the basis of data from 475 children and adolescents, that increases in Hbmass and BV through endurance training do not yet occur before puberty and that they are relatively moderate during and after puberty, being ~10–12%. The large differences in Hbmass and BV in adulthood between elite athletes and untrained subjects are therefore likely to be due to genetic causes, although the underlying mechanisms are still largely unclear. Growing up at a moderate altitude, however, leads to moderate stimulation of Hbmass, being ~7% in all stages of development.

The VO_2max does not differ in children and adolescents growing up at different altitudes when tested in their respective places of residence; the hypoxic circumstances at moderate altitudes are completely compensated for. The training effects on VO_2max are already clearly present before puberty and are reinforced by the onset of puberty, partly due to hematological adaptations.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the data are still needed for more extensive analysis. Requests to access the datasets should be directed to WS, walter.schmidt@uni-bayreuth.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics committee of the National University of Colombia at Bogotá (reference: ID 06/2015). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

All authors contributed to the study. EM, WS, and EC were involved in the conception and design of the study, the acquisition of data, the analysis and interpretation of the data and the drafting of the manuscript. DR, JR, LD, and SC were involved in the acquisition of data, analysis, and interpretation of data for the work as well as in the critical revision of the manuscript. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

FUNDING

This study was financially supported by the funds from the National Program of Projects for the strengthening of research, creation and innovation in postgraduate courses of the National University of Colombia in the editions of 2013-2015 and 2016-2018. We also received funding from the Central Unit of the Valley (UCEVA, Colombia) for conducting the research in Tuluá. Publishing was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation, 491183248) and by the Open Access Publishing Fund of the University of Bayreuth, Germany.

ACKNOWLEDGMENTS

The authors would like to thank all the study participants, parents and coaches for their support. They also thank William C. Byrnes for his critical revision of the manuscript and his valuable comments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.892247/full#supplementary-material

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Conflict of Interest: WS is a managing partner of the company Blood tec GmbH, but he is unaware of any direct or indirect conflicts of interest regarding the content of this paper. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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SYSTEMATIC REVIEW
published: 16 June 2022
doi: 10.3389/fphys.2022.919359

The Methodological Quality of Studies Investigating the Acute Effects of Exercise During Hypoxia Over the Past 40 years: A Systematic Review

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OPEN ACCESS

Edited by:
Olivier Girard, University of Western Australia, Australia

Reviewed by:
Tadej Debevec, University of Ljubljana, Slovenia
Alba Camacho-Cardenosa, University of Extremadura, Spain

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Specialty section:
This article was submitted to Exercise Physiology, a section of the journal Frontiers in Physiology

Received: *13 April 2022*
Accepted: *09 May 2022*
Published: *16 June 2022*

Citation:
Hohenauer E, Freitag L, Herten M, Siallagan J, Pollock E, Taube W and Clijsen R (2022) The Methodological Quality of Studies Investigating the Acute Effects of Exercise During Hypoxia Over the Past 40 years: A Systematic Review. Front. Physiol. 13:919359. doi: 10.3389/fphys.2022.919359

Exercise under hypoxia and the physiological impact compared to normoxia or hypoxia has gained attention in the last decades. However, methodological quality assessment of articles in this area is lacking in the literature. Therefore, this article aimed to evaluate the methodologic quality of trials studying exercise under hypoxia. An electronic search was conducted until December 2021. The search was conducted in PubMed, CENTRAL, and PEDro using the PICO model. (P) Participants had to be healthy, (I) exercise under normobaric or hypobaric hypoxia had to be (C) compared to exercise in normoxia or hypoxia on (O) any physiological outcome. The 11-item PEDro scale was used to assess the methodological quality (internal validity) of the studies. A linear regression model was used to evaluate the evolution of trials in this area, using the total PEDro score of the rated trials. A total of $n = 81$ studies met the inclusion criteria and were processed in this study. With a mean score of 5.1 ± 0.9 between the years 1982 and 2021, the mean methodological quality can be described as "fair." Only one study reached the highest score of 8/10, and n = 2 studies reached the lowest observed value of 3/10. The linear regression showed an increase of the PEDro score of 0.1 points per decade. A positive and small tendency toward increased methodologic quality was observed. The current results demonstrate that a positive and small tendency can be seen for the increase in the methodological quality in the field of exercise science under hypoxia. A "good" methodological quality, reaching a PEDro score of 6 points can be expected in the year 2063, using a linear regression model analysis. To accelerate this process, future research should ensure that methodological quality criteria are already included during the planning phase of a study.

Keywords: hypoxia, exercise, review, methodological quality, PEDro

INTRODUCTION

Increased interest in altitude training as well as a popular trend toward reaching higher altitudes for sporting activities or traveling justifies the importance of understanding physiologic changes at higher altitudes, particularly during exercise. Several altitude classifications exist (Mazzeo, 2008; Dietz and Hackett, 2019). The most common of these is by Bärtsch and Saltin (2008): sea level is considered to lie between 0 and 500 m, low altitudes range from 500 to 2000 m, moderate altitude from 2000 to 3,000 m, high altitude above 3,000 m, and extreme above 5,000 m (Bärtsch and Saltin, 2008). Most mountain and ski resorts are located at a moderate altitude (Dietz and Hackett, 2019). Rapid ascent, representing acute hypoxic conditions, can lead to symptoms of altitude illness, which is the result of impaired acclimatization (Dietz and Hackett, 2019).

The main factor, associated with (acute) exposure to altitude is hypoxia, which is defined as tissue oxygen supply below the needed levels, to maintain normal physiological function (Loiacono and Shapiro, 2010). There are four main types of hypoxia, which can be classified into hypoxic hypoxia, anemic hypoxia, circulatory (stagnant or ischemic) hypoxia, and histotoxic hypoxia (Pittman, 2016; Cheung and Ainslie, 2022). The most common form of hypoxia is hypoxic hypoxia, which is the result of reduced arterial oxygen tension (Cheung and Ainslie, 2022). The physiologic response of the human body to hypoxia depends on the duration of exposure (short and long term), the magnitude of reduced ambient pressures, reduction in ambient oxygen pressure, the rate of occurrence, and severity of the exposure (Dietz and Hackett, 2019; Cheung and Ainslie, 2022). These physiological responses have been highlighted and explained in the literature (Michiels, 2004; Millet et al., 2012; Mounier and Brugniaux, 2012).

Hypoxic hypoxia can be the result of reduced barometric pressures at altitude, leading to a reduced partial pressure of inspired oxygen (Cheung and Ainslie, 2022). Barometric pressure decreases with increased terrestrial altitude, resulting in proportionally lower atmospheric oxygen partial pressures, while the oxygen percentage stays constant (20.9%) (Brown and Grocott, 2013). The direct consequences of lowered atmospheric oxygen partial pressure are a decrease in the partial pressure of oxygen in the body and blood tissues, a decrease in the arterial O_2 partial pressure, and a reduction of the oxygen tension in the alveoli (Rodway et al., 2003; Sharp and Bernaudin, 2004). In the setting of hypobaric hypoxia and normobaric hypoxia, the respiratory ventilation is increased to compensate the reduced partial pressure of inspired oxygen.

The effects of altitude on human physiology were described as early as 1644. Evangelista Torricelli (1,608–1,647), a student of the great Galileo, was the first person to clearly state that the atmosphere exerts pressure. Over the proceeding years, many experiments with hypobaric and hyperbaric chambers and those at effective altitudes (e.g., balloon and mountain) were performed (West, 2016). Over the last few centuries, a large amount of scientific knowledge about altitude exposure was gained. Rapid exposure to high altitudes can result in acute hypoxia that affects many physiological systems, including the respiratory, cardiovascular, and neurologic systems (Cheung and Ainslie, 2022; Hohenauer, 2022). One of the most important responses of the body to hypoxia is to increase ventilation. As a result of lower partial pressure of oxygen, the increase in minute ventilation is triggered by oxygen-sensing cells in the carotid body (Dietz and Hackett, 2019). Further physiologic adaptions to altitude include an increased resting and sub-maximal heart rate, increased blood pressure, and decreased maximal oxygen consumption (Wyatt, 2014).

A considerable number of review studies about physical activity during hypoxia exposure have been performed over the past few decades (Ando et al., 2020; Pojskić et al., 2021), investigating its physiologic consequences (Coppel et al., 2015; Fernandez-Lazaro et al., 2019; Griffiths et al., 2019). Exercise training under hypoxia, as part of elite sports training, was established in the late 1960s, as it is advantageous compared to sea-level training to increase oxygen delivery capacity and aerobic exercise capacity (Park et al., 2016). Exercise under hypoxia is currently not only integrated into elite sports but also used in the field of health and rehabilitation (Nishiwaki et al., 2011; Kong et al., 2014; Schreuder et al., 2014). However, evidence from the literature shows that several methods and variables have to be taken into account during hypoxic training which determine its effectiveness (Millet et al., 2010). In general, exercise under hypoxia is associated with a compensatory increase in blood flow toward active muscles, resulting in pronounced shear stress and nitric oxide release (González-Alonso et al., 2006; Casey and Joyner, 2012). Exercise under hypoxia, therefore, seems to stimulate arterial remodeling/function and angiogenesis (Geiser et al., 2001; Ridnour et al., 2005; Tinken et al., 2010; Hellsten and Hoier, 2014). However, this is controversially discussed, with reports indicating superior (Geiser et al., 2001; Nishiwaki et al., 2011; Kon et al., 2015) or similar (Desplanches et al., 2014; Kong et al., 2014; Schreuder et al., 2014) vascular adaptations following hypoxic versus normoxic exercise. Debates on the difference between normobaric and hypobaric hypoxia highlight the increased interest and developments in this area (Millet and Debevec, 2020; Richalet, 2020).

Internal validity and external validity are the most relevant components when critically appraising randomized controlled trials although there is no gold standard method available (Jung et al., 2022). The internal validity of a study reflects the systematic error or bias in a clinical trial (Higgins and Green, 2011; Boutron et al., 2019), expressing the methodological robustness of a study (Jung et al., 2022). External validity is known by several definitions, and the terms generalizability, external validity, applicability, or transferability are used interchangeably in the literature (Weise et al., 2020). In an internally valid trial, external validity refers to the ability of the results to be generalized to the "real world" population (Akobeng, 2008). Consequently, a lack of internal validity adversely influences the quality of the evidence that can be derived from a trial. Without internal validity, an experiment cannot demonstrate a causal link between two variables. The main errors that could negatively affect the internal validity are bias (systematic error) and random error (chance error or statistical error) (Keirse and Hanssens, 2000; Stephenson and Babiker, 2000; Akobeng, 2008). Therefore, it is an

TABLE 1 | Screened databases, keywords, and identified studies.

Database	Keywords	Total Studies
PEDro	Hypoxia	72
PubMed	((((“hypoxia” [MeSH Terms] OR “hypoxia” [All Fields]) OR “hypoxia s” [All Fields]) OR “hypoxias” [All Fields]) AND (“hypobaric” [All Fields] OR “hypobarism” [All Fields])) OR “normobaric” [All Fields])	5,492
Cochrane trials	hypoxia AND normobaric OR hypobaric	879

important step to assess the methodological quality of trials, build an evidence base that informs clinical practice, and identify areas of healthcare that require further research (de Morton, 2009). In general, there are three types of tools for establishing internal validity: scales, checklists, and items (Jüni et al., 2001; Zeng et al., 2015).

Although there are many scales available that assess the methodological quality of clinical trials (Ma et al., 2020), the PEDro scale is commonly employed to assess the internal validity (Maher et al., 2003) and was already used in the field of hypoxia (Camacho-Cardenosa et al., 2019). The PEDro scale considers two aspects of trial quality, namely, the “believability” (or “internal validity”) of the trial and whether the trial contains sufficient statistical information to make it interpretable. It does not rate the “meaningfulness” (or “generalizability” or “external validity”) of the trial or assess the size of its treatment effect.

To the authors’ knowledge, no systematic review has evaluated the methodological quality of studies that investigated the effects of exercises in the setting of acute hypoxia on physiological parameters over the past 40 years. The present systematic review uses the PEDro scale to evaluate the methodological quality of studies that examined the effects of exercise under acute hypoxic conditions (normobaric or hypobaric) vs. normoxic conditions or acute hypoxic conditions under different barometric pressure on physiologic parameters and to assess the evolution of the methodological quality of these trials over the last 40 years.

MATERIALS AND METHODS

Literature Search Strategies and Data Sources

A literature search was conducted using the PICO model from the PRISMA guidelines (Page et al., 2021): 1) Population: healthy, female and male study participants could be of any training status; 2) Intervention: exercise under hypobaric or normobaric hypoxia; 3) Comparator: exercise under normoxia or hypoxia; and 4) Outcomes: physiological parameters including, but not limited to heart rate, oxygen saturation (of the blood or muscle), blood flow, core temperature, blood markers, and respiration characteristics.

A systematic search was performed electronically until December 2021 in the following databases: MEDLINE (PubMed), Cochrane Central Register of Controlled Trials (CENTRAL), and Physiotherapy Evidence Database (PEDro), according to the PRISMA statement. The keywords and their combinations that were used in this work are shown in **Table 1**.

Selection Criteria

Eligibility criteria were based on the PICO approach. The following selection criteria were used: 1) all participants were healthy humans, 2) healthy participants had to perform any exercise under acute (<24 h) normobaric or hypobaric hypoxic conditions, 3) physiologic values were measured during and/or after exercise, and 4) only experimental studies were included. Studies were excluded in cases of 1) participants were exposed to hypoxia for longer than 24 h, 2) supplement (caffeine, vitamins, saline, etc.) or medication intake (e.g., formoterol, beta-blocker, sildenafil), 3) studies were published in a language other than English, or 4) no physical exercises were performed.

Assessment of Methodological Quality Using the PEDro Scale

The German version of the PEDro scale was used to assess the methodological quality of the included trials. The German version of the PEDro scale demonstrated good inter-reliability for individual items and the total PEDro score (Costa et al., 2015). The PEDro scale, which is based on the Delphi list, is a valid (convergent and construct validity) and reliable tool for assessing trial methodologic quality (Maher et al., 2003; Macedo et al., 2010). The use of the PEDro scale, outside the classical field of physiotherapy, is growing (Elkins et al., 2013). This shows that its use is not limited to currently practiced methods in physiotherapy, nor that the trials have to be conducted by a physiotherapist (PEDro, 2017). The PEDro scale was used to assess the internal validity and has already been used in studies dealing with healthy participants (Fradkin et al., 2006; Ganio et al., 2009), patients (Pinto et al., 2012; Kouloutbani et al., 2019) and also in the field of hypoxia (Camacho-Cardenosa et al., 2019). The scale includes 11 items, but item 1 (eligibility criteria were specified) is not included in the calculation of the total score. A maximum of 10 points is therefore possible. A brief description of each PEDro item (English version) can be seen in **Table 2**. A PEDro score of 9–10 is considered to reflect an “excellent,” 6 to 8 a “good,” 4 to 5 a “fair,” and <4 a “poor” methodological quality (Cashin and McAuley, 2020).

A team internal briefing took place, during which each item of the PEDro score was discussed for reliability reasons. It was set forth, that a point for an item could only be awarded if the criterion was fulfilled. For Item 1, a list of inclusion criteria and exclusion criteria were required to fulfill the criterion. Furthermore, it had to be clearly described how the subjects were recruited. For Item 4, the trial must have performed a baseline measure of the severity of the condition being treated and at least one key outcome. In addition, it had to be shown that

TABLE 2 | PEDro score.

Nr	Item	No	Yes	Where
1	Eligibility criteria were specified			
2	Subjects were randomly allocated to groups (in a crossover study, subjects were randomly allocated an order in which treatments were received)			
3	Allocation was concealed			
4	The groups were similar at baseline regarding the most important prognostic indicators			
5	There was blinding of all subjects			
6	There was blinding of all therapists who administered the therapy			
7	There was blinding of all assessors who measured at least one key outcome			
8	Measures of at least one key outcome were obtained from more than 85% of the subjects initially allocated to groups			
9	All subjects for whom outcome measures were available received the treatment or control condition as allocated or, where this was not the case, and data for at least one key outcome was analyzed by "intention to treat"			
10	The results of between-group statistical comparisons are reported for at least one key outcome			
11	The study provides both point measures and measures of variability for at least one key outcome			

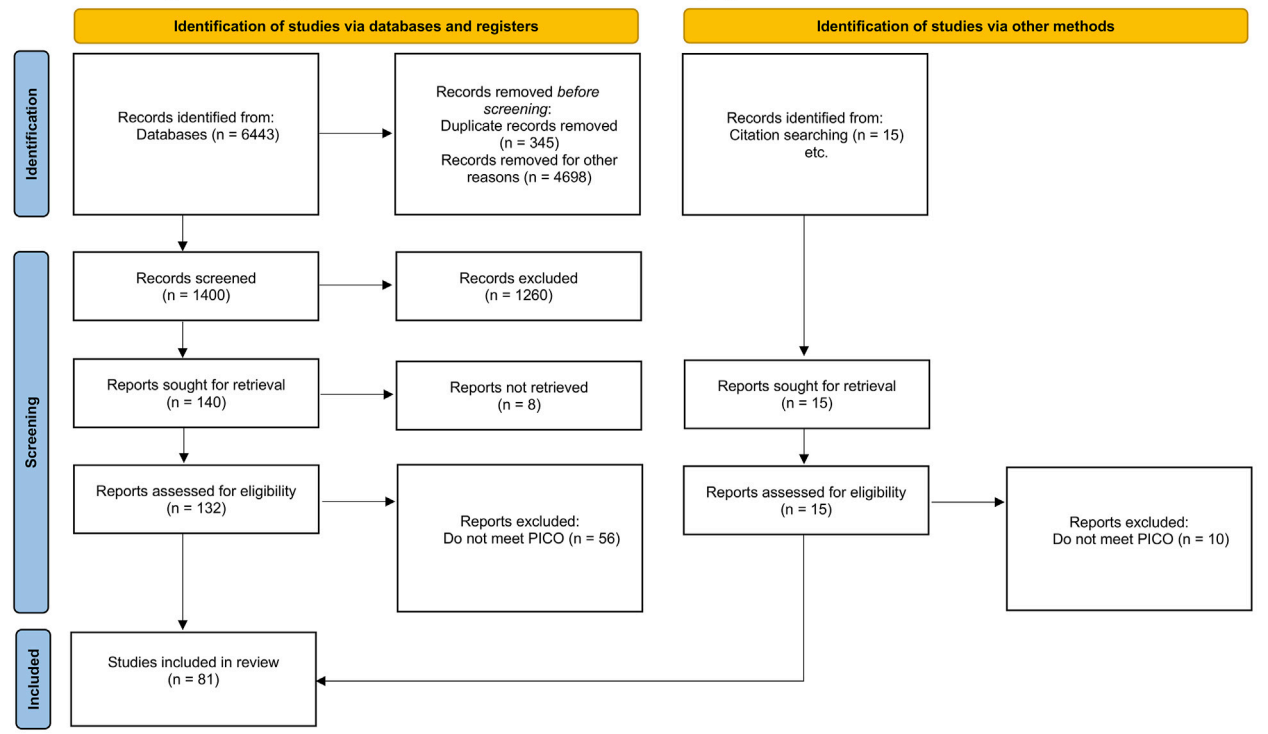

FIGURE 1 | Flowchart describing the systematic selection procedure.

these parameters did not differ significantly between the different groups (e.g., through a *p*-value). Finally, for Item 11, both point measures and measures of variability were required.

Data Extraction

A total of n = 6,443 studies were identified from the main search strategy (**Table 1**) in databases and registers. After removing duplicates (n = 345) and for other reasons (n = 4,698), a total of n = 1,400 studies were used for the screening process. During this process, a total of n = 1,316 were excluded because they did not meet the PICO scheme or due to other reasons, and n = 8 reports were not retrieved. This resulted in the inclusion of n = 76 studies from databases and registers. A total of n = 15 studies were retrieved from citation searching. From these n = 15 studies, a total of n = 10 studies were extracted because they did not meet the defined PICO scheme, resulting in an inclusion of n = 5 studies from other methods. **Figure 1** depicts the systematic search strategy and selection process.

Included articles were downloaded and saved in alphabetical order in a pdf format. The following variables were extracted: author names, article title, and publication year. Four researchers (EP, JS, LF, and MH) independently scored all trials (n = 81) for methodological study quality with the PEDro score (each item and the total score of the PEDro scale). Two researchers each rated the same article. In case of disagreement between the researchers, a third researcher rated the questionable item, and agreement was sought by consensus.

Data Analysis

All data were analyzed using the Statistical Package for the Social Sciences (SPSS version 27.0, IBM, Armonk, United States). A bubble plot was created (DataGraph 4.7.2beta, Visual Data Tools Inc. Chapel Hill, United States) based on the total score of the PEDro scale (dependent variable) and the publication year (independent variable) of each study. The size of the bubble was dependent on the number of studies with the same total PEDro score for each year, to assess the relationship between the total PEDro score and time. The different colors of the bubbles represent the different PEDro scores: green represents a high PEDro score (greater than or equal tosix) and red represents a lower score (lower than six). A linear regression model was used to evaluate the development of methodological quality over time.

RESULTS

Distribution of Scientific Literature

The included clinical trial dates ranged from 1982 to 2021. A total of n = 81 studies were included in the final analysis to evaluate the methodological quality over the past 40 years. Notably, no study was already listed in the PEDro database.

A total of n = 2 studies were retrieved between the years 1982 and 1989 (Squires and Buskirk, 1982; Wagner et al., 1986), n = 5 between 1990 and 1999 (Fulco et al., 1994; Koistinen et al., 1995; Naughton et al., 1995; Fulco et al., 1996; Taylor and Bronks, 1996), n = 18 between 2000 and 2009 (Casas et al., 2001; Takase et al., 2002; Bocqueraz et al., 2004; Shave et al., 2004; Choukèr et al., 2005; Friedmann et al., 2005; Heubert et al., 2005; Saito et al., 2005; Sandiford et al., 2005; Schiffer et al., 2005; Wehrlin and Hallen, 2006; Mackenzie et al., 2008; Richardson et al., 2008; Subudhi et al., 2008; Zhou et al., 2008; Richardson et al., 2009a; b; Wang and Chiu, 2009), n = 46 between 2010 and 2019 (Fukuda et al., 2010; Miyagawa et al., 2011; Basualto-Alarcón et al., 2012; Degache et al., 2012; Kroepfl et al., 2012; Maire r et al., 2012; Schommer et al., 2012; Faiss et al., 2013; Fan et al., 2013; Mairer et al., 2013; Sandfeld et al., 2013; Feriche et al., 2014; Ho et al., 2014; Julia-Sanchez et al., 2014; Kröpfl et al., 2014; Slivka et al., 2014; Trapp et al., 2014; DiPasquale et al., 2015; Seo et al., 2015; Brocherie et al., 2016; Girard et al., 2016; Shrestha and Singh, 2016; Filopoulos et al., 2017; Girard et al., 2017; Klenze et al., 2017; Lira et al., 2017; Lühker et al., 2017; Machado et al., 2017; Matu et al., 2017; Sweeting et al., 2017; Tymko et al., 2017; Wadley et al., 2017; Willis et al., 2017; Alhammoud et al., 2018; Cooke et al., 2018; Lee and Thake, 2018; Angeli et al., 2019; Charkoudian et al., 2019; da Mota et al., 2019; Gronwald et al., 2019; Lei et al., 2019; Morawetz et al., 2019a; Morawetz et al., 2019b; Mulliri et al., 2019; Sharma et al., 2019; Valenzuela et al., 2019), and n = 10 between 2020 and 2021 (Faulhaber et al., 2020; Jung et al., 2020; Limmer et al., 2020; Nell et al., 2020; Willis et al., 2020; De Groote et al., 2021; Kong et al., 2021; Magnani et al., 2021; Vasquez-Bonilla et al., 2021; Yamaguchi et al., 2021).

The mean PEDro score of the included studies over time was 5.1 ± 0.9. In the period from 1982 until 1989, only two studies were published in peer-reviewed journals, with a mean PEDro score of 4.5 ± 0.7. In the period from 1990 to 1999, five studies achieved a mean PEDro score of 5.0 ± 0.8. From 2000 to 2009, a total of n = 18 studies achieved a mean PEDro score of 4.6 ± 0.7, from 2010 to 2014 a total of n = 17 studies reached a score of 4.8 ± 0.9, and from 2015 to 2020 a mean PEDro score of 5.4 ± 1.0 was reached by n = 35 included studies. Four studies reached a mean PEDro score of 5.7 ± 0.5 out of 10 in the year 2021.

Methodological Quality

The bubble plot in **Figure 2** depicts the evolution of the PEDro scores of the included manuscripts from 1982 to 2021. Green bubbles represent a PEDro score of 6 and higher and red bubbles represent studies with a PEDro score of 5 or lower. The linear regression analyses demonstrated that 2.6% of the variance of the y-variable can be explained, and the linear regression line was calculated using the following equation: y = (0.0188 x year) + (−32.794). The slope of the linear regression line suggests that the mean PEDro score increases by 0.1 points each decade.

The methodological quality, measured with the PEDro score, ranged from 3/10 to 8/10, between the years 1982 and 2021. None of the included studies reached the maximum PEDro score of 10.

The highest achieved PEDro score in the assessed studies was a score of eight, which was awarded to an article published by one research group in the year 2016 (Brocherie et al., 2016). The second highest PEDro score was 7/10, which was achieved by four studies (Wehrlin and Hallen, 2006; Faiss et al., 2013; DiPasquale et al., 2015; Nell et al., 2020). A total of 23 studies achieved a PEDro score of 6/10. Total PEDro scores of six to eight, which are considered reflective of "good" methodological quality, were achieved in 34.6% (n = 28 studies) of all included studies (Cashin and McAuley, 2020).

"Fair" methodological quality (PEDro score of four to five was achieved by 51 studies, reflecting 63.0% of the 81 included studies. Only 2.4% (2/81) of works are rated to have "poor" methodologic quality, which is considered a PEDro score of <4. A detailed overview of the number of studies and their PEDro scores is shown in **Figure 3**.

Analysis of PEDro score single items can be seen in **Figure 4**. All included trials (100%) fulfilled the item for point estimates and variability, 97.5% included "between-group comparisons", and 95.0% performed "adequate follow-up." Lower relative frequency scores were observed for the items regarding "intention-to-treat analysis" (83.9%) and "random allocation" (74.0%). The items "eligibility criteria" (excluded from the total PEDro score ratings) and "blind subjects" each reached 39.5%. "Baseline comparability" (11.1%), "blind therapists and blind assessors" (each 4.9%), and "concealed allocation" (0%) had the lowest relative frequency scores.

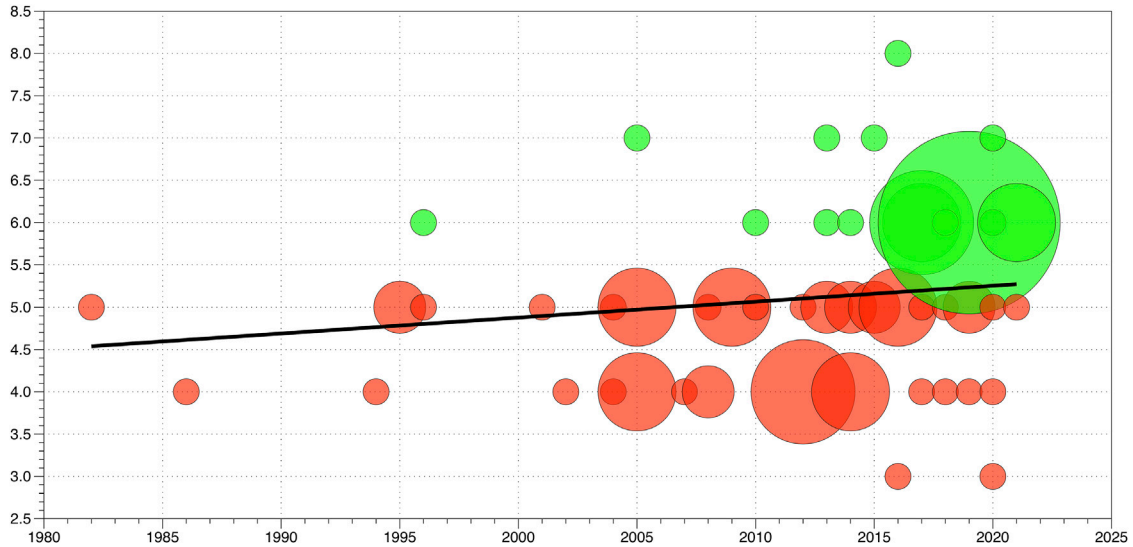

FIGURE 2 | Bubble plot of the $n = 81$ included studies. Green dots represent studies with "good" methodological quality and a PEDro score ≥6 points. Red bubbles represent studies with lower PEDro scores of <6 points, indicating "fair" (4–5 points) or "poor" (<4 points) methodological quality. The size of the bubbles is related to the proportion of studies having the same PEDro score at a specific time point. The black line represents the linear regression line as a function of time.

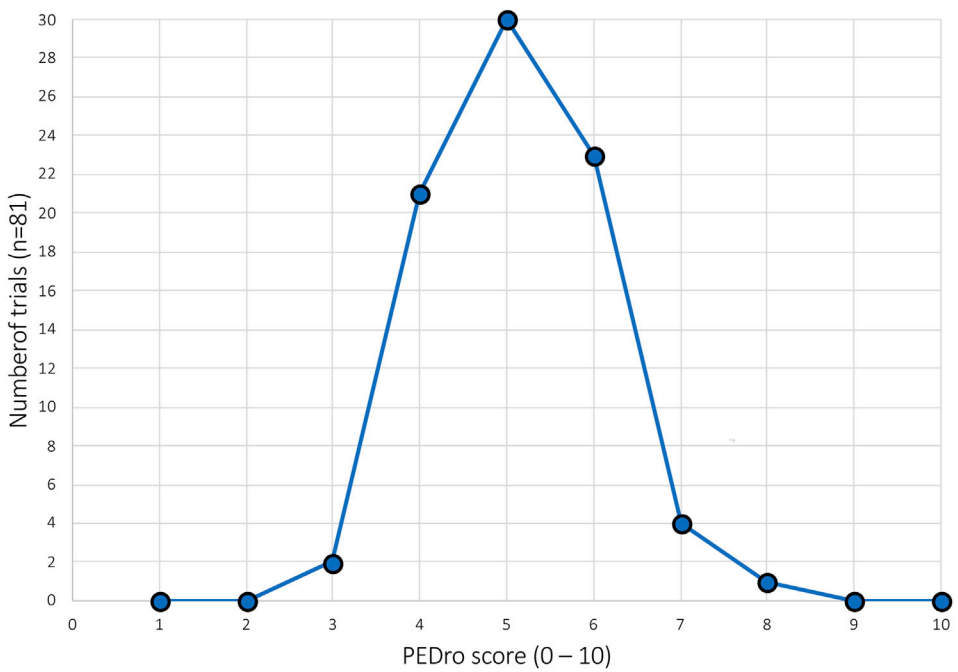

FIGURE 3 | Absolute frequency distribution of the total PEDro scores of all included studies.

DISCUSSION

This systematic review aimed to assess the methodological quality of clinical trials examining the physiological response to exercise under hypoxic conditions by considering PEDro scores. We also aimed to assess changes in the mean methodological quality over time.

The calculated mean PEDro score of the $n = 81$ included studies was 5.1 ± 0.9. Research groups that were able to blind the subjects and assessors (Brocherie et al., 2016) or subjects and therapists (Nell et al., 2020) had higher PEDro scores. Single- or double-blinding procedures are important to avoid bias. It has been demonstrated that trials without double-blinding yielded larger estimates of treatment effects than trials using double-

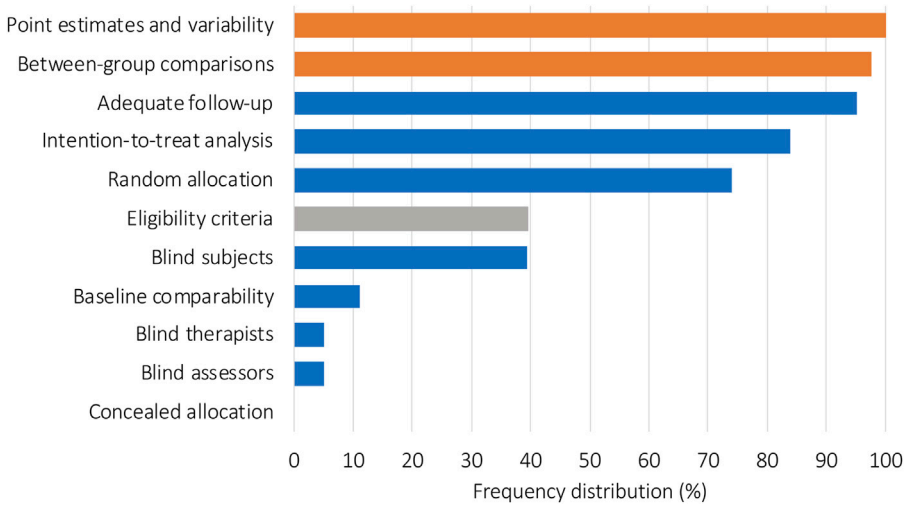

FIGURE 4 | Relative frequency distribution of all included studies for each PEDro item. Orange bars represent items 10 and 11 of the PEDro score, which represent the interpretability of the data. Blue bars represent items 2–9, which are used to evaluate internal validity. The gray bar represents item 1, which evaluated external validity and was not used to calculate the total PEDro score.

blinding procedures (Schulz et al., 1995). From a theoretical perspective, studies that compared the effects of different partial pressures of inspired oxygen and barometric pressure could reach a maximum score on the PEDro scale (10/10). In particular, studies using closed chamber systems, where barometric pressure and the fraction of inspired oxygen can be controlled, are particularly predisposed to reaching the maximum methodological score on the PEDro score. It is nearly impossible to blind participants, therapists, or assessors, in case a study evaluates the difference between terrestrial altitude and a laboratory condition at the sea level. The use of mask systems or oxygen tents might help contribute to the blinding procedure, but barometric pressure can only be simulated in a closed chamber or at a real altitude. Studies that investigated the difference between terrestrial altitude (or did not have an environmental chamber system) and a laboratory setting at the sea level could only have reached a maximum PEDro score of 7/10 since the blinding of participants, therapists, and assessors could be a problematic issue in those setups. In the articles analyzed in the present study, only five works, representing around 6% of the included 81 studies, reached a PEDro score of seven or higher (Wehrlin and Hallen, 2006; Faiss et al., 2013; DiPasquale et al., 2015; Brocherie et al., 2016; Nell et al., 2020). Following the PEDro database, only these 6% could be considered to have good methodologic quality (PEDro, 1999; Cashin and McAuley, 2020). The modal PEDro score was 5 and the mean score was 5.1, suggesting that the overall methodological quality of these studies can be rated as "fair" (Cashin and McAuley, 2020).

Interestingly, none of the included studies concealed their sample allocation or failed to describe it based on their PEDro score. Random allocation is known to be an important method to ensure that the groups being compared are on an equivalent basis at the study start (Schulz, 2001). This point could be easily achieved because a point is awarded for this category even if it is not stated in the work that allocation was concealed. In this case, the study must state that allocation was via sealed opaque envelopes or that allocation involved contacting the holder of the allocation schedule who was "off-site" (PEDro, 1999). Only 11% of the included studies received a point for comparing groups that were similar at baseline for the most important prognostic indicators. Here, the ratings could be theoretically higher as at a minimum the report must describe at least one measure of the severity of the condition being treated and at least one (different) key outcome measure at baseline. The rater must be satisfied that the groups' outcomes would not be expected to differ based on baseline differences in prognostic variables alone by a clinically significant amount (PEDro, 1999). However, on the other site, the meaningfulness of statistical testing for baseline differences has been questioned (Harvey, 2018).

Random allocation was satisfactory in 74% of the included studies. Although this number is relatively high, it should be considered with caution from a methodologic quality perspective. An article receives a point for this item just by mentioning that the allocation was random without further explanation. Good methods of generating a random allocation sequence include using a random-number table or a computer software program (Dettori, 2010). Less recommended methods to achieve random allocation are tossing a coin, drawing lots, or throwing a dice (Dettori, 2010). Quasi-randomization allocation procedures such as allocation by hospital record number, birth date, or alternation do not satisfy this criterion (PEDro, 1999).

The PEDro scale is used to assess the methodological quality of trials and to specifically identify those trials with good internal validity (PEDro items 2–9) that report enough data to make their results interpretable (PEDro items 10–11) (Moseley et al., 2020). Looking into the results section we assume that the results of the included studies can be rated as interpretable, as item 10

(between-group statistical comparison) and item 11 (point estimates and variability) achieved nearly perfect fulfillments. However, the specific items that define good internal validity demonstrate a wide range of scores among the included studies. Using the linear regression formula, it can be expected that "good" mean methodological quality as defined by a PEDro score of 6 (Cashin and McAuley, 2020) will be reached in the year 2063. However, this process could be accelerated, if upcoming studies in this area, especially consider items two to nine of the PEDro score. The number of published articles in the field of exercise under hypoxic conditions is increasing.

Our article not only demonstrates that the mean methodologic quality of studies is "fair" and increasing over time but also demonstrates that clear guidelines are needed to further increase the methodologic quality in the field. To increase internal validity, researchers should ensure careful study planning and implementation strategies. The results of our analysis demonstrate that adequate blinding procedures should be incorporated into studies whenever possible. However, as already mentioned, blinding procedures are now always easy to implement in studies investigating the effects of hypoxia, especially when no closed chamber systems are available or in the case the study is performed during terrestrial altitude. The results of the current analysis further show that future studies in this area are advised to realize concealed allocation and ensure baseline comparability, to increase their internal validity. It is important to mention that internal validity, the extent to which the design and conduct of a trial eliminate the possibility of bias, is a prerequisite for external validity (Moher et al., 2010). The effective use of external validity has the potential to speed up the implementation of worthwhile innovations and avoid unworthwhile efforts (Dyrvig et al., 2014).

It should be acknowledged that the PEDro score is only one tool for evaluating the methodological quality of clinical trials that is typically used (but not limited to) in current practice methods in physiotherapy. The use of the summary score from the PEDro scale has also been critically questioned, as it showed poor construct validity in addition to other limitations (Albanese et al., 2020). Other methodological quality assessment tools are available in the literature, such as the Cochrane risk of bias 2.0 tool, the EPOC risk of bias tool, and the CASP checklist, which can be found elsewhere (Ma et al., 2020). Researchers should therefore carefully choose and report the methodological quality assessment tool that they chose to use and try to achieve the highest score for their internal validity. A limitation of the current study might be that only studies written in English were used, and the inclusion of studies was limited to the aforementioned search strategies. Therefore, the current findings might over- or underestimate the true methodological quality of the entire current literature in this field.

CONCLUSION

The mean PEDro score of trials investigating the difference between different hypoxic and normoxic conditions during exercise over the last 40 years is 5.1 ± 0.9, indicating "fair" methodological quality. This work's linear regression showed a small positive trend toward higher scores in the future, with an increase of 0.1 point each decade. "Good" mean methodological quality in this research field can be expected in the year 2063 at the earliest given current trends. Although the results of the studies are interpretable, future studies in this field should incorporate adequate blinding procedures (if possible), concealed allocation, and baseline comparability. Future studies should consider including the relevant criteria during the planning of the study to achieve the highest possible methodological quality score.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Conceived and designed the study: EH and RC. Analyzed the data: EH, LF, MH, JS, and EP. Wrote the manuscript: EH, LF, WT, and RC.

ACKNOWLEDGMENTS

We thank Thim van der Laan AG for the financial support.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Physiology

TYPE Systematic Review
PUBLISHED 23 August 2022
DOI 10.3389/fphys.2022.940749

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Exercise Physiology,
a section of the journal
Frontiers in Physiology

RECEIVED 10 May 2022
ACCEPTED 22 July 2022
PUBLISHED 23 August 2022

CITATION
Chen S, Su H, Liu X, Li Q, Yao Y, Cai J, Gao Y, Ma Q and Shi Y (2022), Effects of exercise training in hypoxia versus normoxia on fat-reducing in overweight and/or obese adults: A systematic review and meta-analysis of randomized clinical trials.
Front. Physiol. 13:940749.
doi: 10.3389/fphys.2022.940749

Effects of exercise training in hypoxia versus normoxia on fat-reducing in overweight and/or obese adults: A systematic review and meta-analysis of randomized clinical trials

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Objective: Fat loss theory under various oxygen conditions has been disputed, and relevant systematic review studies are limited. This study is a systematic review and meta-analysis to assess whether hypoxic exercise training (HET) leads to superior fat-reducing compared with normoxic exercise training (NET).

Methods: We searched PubMed, Web of Science, CNKI, ProQuest, Google Scholar, Cochrane Library, and EBSCOhost from inception to June 2022 for articles comparing the effects of hypoxic and normoxic exercise on body composition indicators, glycometabolism, and lipometabolism indicators in obese and overweight adults. Only randomized controlled trials (RCTs) were included. The effect sizes were expressed as standardized mean difference (SMD) and 95% confidence intervals (CI). Between-study heterogeneity was examined using the I^2 test and evaluated publication bias via Egger's regression test. The risk of bias assessment was performed for each included trial using Cochrane Evaluation Tool second generation. The meta-analysis was performed by using R 4.1.3 and RevMan 5.3 analytic tools.

Results: A total of 19 RCTs with 444 subjects were analyzed according to the inclusion and exclusion criteria. Among them, there were 14 English literature and five Chinese literature. No significant difference in body composition (SMD -0.10, 95% CI -0.20 to -0.01), glycometabolism and lipid metabolism (SMD -0.01, 95% CI -0.13 to -0.10) has been observed when comparing the HET and NET groups. We only found low heterogeneity among trials assessing glycometabolism and lipometabolism (I^2 = 20%, p = 0.09), and no publication bias was detected.

Conclusion: The effects of HET and NET on fat loss in overweight or obese people are the same. The application and promotion of HET for fat reduction need further exploration.

KEYWORDS

hypoxic exercise, normoxic exercise, obesity, body composition, glycometabolism, lipometabolism, meta-analysis

Introduction

Obesity has gradually become a global public health issue and a leading cause of death in most countries' populations. According to the World Health Organization (WHO, 2020), the global prevalence of obesity had nearly tripled since 1975. Obesity is normally accompanied by the development of metabolic disorders such as insulin resistance, type II diabetes, and cardiovascular disease, which all have severe health effects for humans (Engin, 2017). In 2016, 39% of adults were overweight, and 13% were obese (WHO, 2020). As of 2017, nearly four million people die yearly due to obesity or overweight (WHO, 2020).

Obese people are more likely to have dyslipidemia (Gardner and Poehlman, 1995; Lu et al., 2008; Meyer et al., 2012). The type of dyslipidemia arising from the concerted action of obesity has been identified as "related metabolic dyslipidemia," which is a clinical sign of elevated total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and reduced high-density lipoprotein cholesterol (HDL-C) in human blood (Su et al., 2021). Lipid metabolism reflects the metabolism of body fat and, to a certain extent, the risk of associated diseases (Lacour, 2001). Correction of dyslipidemia promotes body fat consumption and reduces cardiovascular risk in overweight and obese people (Kopin and Lowenstein, 2017; Vekic et al., 2019). Therefore, we need to focus on encouraging obese individuals to lose weight and correcting abnormalities in lipid metabolism.

Dietary intervention, exercise intervention, and medication intervention, among other things, are effective fat removal strategies (Hao et al., 2021). In terms of long-term fat reduction, exercise intervention outperforms diet and pharmaceutical interventions (Maillard et al., 2018). Traditional fat reduction workouts performed under normoxia efficiently lower extra body fat in obese patients. For example, moderate-intensity continuous training effectively boosts fat-burning capacity, improves body glycolipid levels, and enhances aerobic fitness (Rugbeer et al., 2021). High-intensity interval training can enhance aerobic capacity and insulin sensitivity, decrease blood glucose, promote adipose tissue breakdown, and improve vascular endothelial function (Batacan et al., 2017; Ryan et al., 2020). Sustained physical exercise decreases TC, TG, and LDL-C levels while increasing HDL-C levels (Burgomaster et al., 2008; Moholdt et al., 2012).

Hypoxia is a decrease in oxygen delivery to the body's tissues caused by a fall in arterial blood oxygen saturation (Heinonen et al., 2016). Hypoxia and exercise synergistically affect lipid metabolism (Vogt et al., 2001). Hypoxia activates the peroxisome proliferator-activated receptor γ (PPARγ) and peroxisome proliferator-activated receptor -γ co-activator 1α (PGC1) (Zoll et al., 2003), which play an essential role in mediating the adaptive regulation of muscle fatty acid oxidation (Gilde and Van Bilsen, 2003). Similarly, active or passive hypoxia prevents obesity by activating the hypoxia-inducible factor (HIF) to maintain body mass and glucose homeostasis (Gaspar and Velloso, 2018). Reportedly, tissue hypoxia stimulates the production of HIF-1 (Vogt et al., 2001), improves glucose absorption and transport, enhances glycolysis, and accelerates lactate production, thereby increasing the efficiency of oxygen transport (Wenger, 2002) and ATP synthesis (Zoll et al., 2006). In addition, hypoxia increases blood serotonin levels in humans and rats (Urdampilleta et al., 2012). Serotonin regulates appetite, and rats show an anorexic response to serotonin administration (Urdampilleta et al., 2012). Long-term residents of high altitudes have appetite suppression and reduced body mass (Hill et al., 2011). Thus, a hypoxic environment may lead to a reduction in appetite in humans, which in turn may improve body mass loss.

Hypoxic exercise training (HET) is a method of exercise and fitness in a naturally occurring or artificially simulated plateau where the body is below normal oxygen conditions (Weng et al., 2006). An artificial hypoxic environment mainly includes a "hypoxic chamber" and a "hypoxic generator (Gürpinar Ö et al., 2018)." The hypoxic generator produces an absolute low partial pressure of oxygen by wearing a respiratory mask for the body to inhale hypoxia, resulting in a moderate hypoxic environment in the body (Hamlin et al., 2018). This hypoxic environment causes a series of anti-hypoxic physiological and biochemical reactions in the human body, affecting several physical signs (Wheelock et al., 2020). Studies have shown that exercising in hypoxic conditions helps alleviate a variety of cardiovascular, metabolic, and pulmonary illnesses, including obesity (Camacho-Cardenosa et al., 2018; Camacho-Cardenosa et al., 2019). Some studies claim that overweight or obese adults who perform HET experience more significant weight loss and greater improvements in body composition than those who perform normoxic exercise training (NET) (Kong et al., 2014; Park et al., 2019), whereas others found the opposite (Gatterer et al., 2015; Gonzalez-Muniesa et al., 2015). In another study (Netzer et al., 2008), the heart rate during exercise was same in the HET and NET groups (150 ± 5.3 beats/min vs.150 ± 4.6 beats/min), but the exercise load was significantly lower in the HET group than in the NET group (1.39 ± 0.20 w/kg vs.1.67 ± 0.15 w/kg, $p < 0.001$). At the same metabolic level, it is obvious that the subjects had to perform the exercise at a significantly lower intensity in hypoxic conditions than in normoxic conditions (Girard et al., 2017). In brief, hypoxia allows obese patients to achieve higher metabolic demands. At the same time, lower exercise intensity may also have a protective effect on the

muscles/joints of obesity with orthopedic co-morbidities (Wiesner et al., 2010).

Previous literature reviews have critically examined the research potential of HET as an intervention for reducing body fat and cardiovascular health (Quintero et al., 2010; Kayser and Verges, 2013; Montero and Lundby, 2016; Girard et al., 2017; Hobbins et al., 2017; Camacho-Cardenosa et al., 2019; Dünnwald et al., 2019; Ramos-Campo et al., 2019; Kayser and Verges, 2021). Only five of them were systematic reviews. The research population for the systematic review by Montero and Lundby (2016) focused on the effects of exercise under hypoxic conditions on vascular health and was not overweight or obese. The systematic review by Hobbins et al. (2017) included both animal and human trials, and data from the included studies were not integrated and statistically analyzed to determine the intervention effects of hypoxic exercise on outcome indicators. The systematic review of (Camacho-Cardenosa et al. (2019) included only five studies and the conclusions obtained may be highly heterogeneous. The systematic review of Ramos-Campo et al. (2019) did not restrict the age of the subjects included in the study, and the age range may also induce bias into the study results. The systematic review by Dünnwald et al. (2019) studied the regular exercise population, but our review was conducted on a sedentary population. Accordingly, the purpose of this study was to systematically review and meta-analyze the differences in the effects of HET and NET on fat loss in overweight and obese individuals in order to determine whether exercise under hypoxic conditions can further contribute to fat loss and improved lipid metabolism levels.

Methods

The review is reported according to the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines (Page et al., 2021).

Search strategy

We performed a literature search using online databases such as PubMed, Web of Science, CNKI, ProQuest, Google Scholar, Cochrane Library, and EBSCOhost for relevant publications up to 9 June 2022. We used the standard Boolean operators (AND, OR) to concatenate search terms. The following combination of terms was used: “hypoxia” or “altitude” or “oxygen deficiency” or hypoxic exercise training” or “normoxia” or “normoxic exercise training.” The Boolean operator “AND” was used to combine these descriptors with “obesity” or “overweight,” “fat loss,” or “sedentary adults.” We also searched the aforementioned databases for systematic reviews and manually verified reference lists to identify studies that could have been omitted.

Studies selection

The retrieved literature was imported into the reference management software EndNote. Duplicate publications were removed. The first round of preselection was based on the title and abstract. Those with incompatible topics were removed. The full text was downloaded and examined for the second preselection round to determine whether it met the inclusion criteria. Two investigators (SC and XL) independently conducted the database searches to find relevant publications. When conflicts occurred, a group discussion was held with a third investigator (QL).

Inclusion criteria

Inclusion criteria followed the PICOS principles (i.e., population, intervention, comparison, outcome, and study design): 1) Participants were overweight or obese adults (18 years old), with criteria for diagnosing overweight (Body Mass Index >25 kg/m^2) or/and obesity (all obesity categories; Body Mass Index >30 kg/m^2) based on World Health Organization (WHO, 2020), and no physical restrictions or health conditions that would preclude evaluation and exercise intervention; 2) Studies that used a randomized controlled trial (RCT) design; 3) The original study article had to perform the exercise intervention under (normoxic or hypobaric) hypoxic condition; 4) The control group performed the same exercise as the intervention group under normoxic conditions ($FiO_2 = 0.21$); 5) Outcome indicators were body mass (BM), body mass index (BMI), body fat percentage (BFR), fat body mass (FBM), waist circumference (WC), hip circumference (HC), waist-to-hip ratio (WHR), TC, TG, HDL-C, LDL-C, Glucose, and Insulin.

Exclusion criteria

Studies that met these criteria were excluded: 1) Conference abstracts, dissertations, case reports, and animal experiments; 2) Participants who exercised frequently before the intervention; 3) Participants who were generally unable to exercise due to various diseases; 4) Inadequate data, with only a sub-set of data available before and after the intervention; 5) Repeatedly published studies; 6) Not English or Chinese studies.

Data extraction

The extracted content primarily included the first author of the literature, the year of publication, the sample size, the gender and age of the subjects, the training details (intervention period, frequency, length, and intensity), the hypoxic condition, the type of hypoxia, and outcome indicators (Table 1). Information that

TABLE 1 Main characteristics of studies included in the meta-analysis.

Study	Participants characteristics					Intervention						
	Participants (M/F)		Age		BMI/ BFR	Duration	Frequency	Type/ modality	Exercise intensity	Hypoxic condition	Type of hypoxia	Outcome
	HET	NET	HET	NET								
Camacho-Cardenosa, A,2018	15 (0/15)	18 (0/18)	37.40 ± 10.25	40.05 ± 8.66	>25 kg/m^2	12 weeks	3 days/wk	Repeated-sprint training; cycling	Exercise: (130% Wmax; 30 s); Active recovery: (55–65% Wmax; 3 min)	FiO_2 = 17.2%	Normobaric	WC, HC, WHR, BFR,TG, TC, Glucose
Camacho-Cardenosa, A,2018[a]	13 (0/13)	15 (0/15)	44.43 ± 7.18	43.14 ± 7.67	>25 kg/m^2	12 weeks	3 days/wk	IT, cycling	Exercise: (90% Wmax; 3 min); Active recovery: (55–65% Wmax; 3 min)	FiO_2 = 17.2%	Normobaric	WC, HC, WHR, BFR,TG, TC, Glucose
Chacaroun, S,2020	12 (11/1)	11 (8/3)	52 ± 12	56 ± 11	> 27 kg/m^2	8 weeks	3 days/wk	Endurance; cycling	75% (±2%) HRmax; 45 min	FiO_2 = 13%	Normobaric	FBM, BMI, WC, HC, Glucose, Insulin
Fernandez, M,2018	12 (2/10)	11 (2/9)	34.8 ± 4.7	32.2 ± 8.4	>30 kg/m^2	3 weeks	—	Endurance; walking	six different speeds; 60 min	FiO_2 = 14.5%	Normobaric	BM, BMI,FBM,TC,TG,HDL-C,LDL-C, Glucose, Insulin
Gatterer, H,2015	16 (4/12)	16 (6/10)	50.3 ± 10.3	52.4 ± 7.9	>30 kg/m^2	8 m	2 days/wk	Endurance; cycling	65–70% HRmax; 90 min	Exercise: FiO_2 = 14.0 ± 0.2%; Rest: FiO_2 = 12.2 ± 0.3%	Normobaric	BM, BMI, WC, HC, WHR, BFR,TG, TC, HDL-C, Glucose
Gonzalez-Muniesa, P,2015	14 (14/0)	12 (12/0)	25–50	25–50	>30 kg/m^2	13 weeks	2 days/wk	Endurance/ Strength; cycling	No mention; 1 h	FiO_2 = 16.7%–13.7%	Normobaric	BM, BMI, WC, HC, WHR, BFR,TG, TC, LDL-C, HDL-C
Hobbins, L,2021	8 (4/4)	8 (5/3)	32.1 ± 10.2	41.1 ± 13.0	27–35 kg/ m^2	2 weeks	4 days/wk	IT; walking	self-paced,60min	FiO_2 = 13.0%	Normobaric	BM, BMI
Jung, K,2020	12 (0/12)	10 (0/10)	47.2 ± 6.4	43.8 ± 8.6	>25 kg/m^2	12 weeks	4~5 days/wk	Endurance; Pilates	75% HRmax; 50 min	FiO_2 = 14.5%	Normobaric	BM, BMI, BFR, TG, TC, LDL-C,HDL-C, Glucose, Insulin
Klug, L,2018	12 (12/0)	11 (11/0)	57.6 ± 2.2	55.0 ± 2.1	>305 kg/m^2	6 weeks	3 days/wk	Endurance; running	50–60% HRmax; 60 min	FiO_2 = 15%	Normobaric	BM, BMI, WC,HC,WHR,BFR,TG,HDL-C,LDL-C, Glucose, Insulin
Kong, Z,2014	10 (5/5)	8 (5/3)	19.8 ± 2.2	22.3 ± 1.7	>27.5 kg/m^2	4 weeks	3~4 days/wk	Endurance/ Strength; cycling, running, stepping	60–70% HRmax; 1 h.40–50% maximal strength, three sets of 15–20 RM with 2–3 min of rest between sets	FiO_2 = 16.4%–14.5%	Normobaric	BM, FBM, BMI, WHR
Kong, Z,2017	11	13	18–30	18–30	25.8 ± 2.3 kg/m^2	5 weeks	4 days/wk	IT; cycling	8-s maximum; 60 repetitions	FiO_2 = 15%	Normobaric	BM, BMI, FBM,BFR,TC,TG,HDL-C,LDL-C
Morishima, T,2014	9 (9/0)	11 (11/0)	30 ± 2	32 ± 3	>25 kg/m^2	4 weeks	3 days/wk	Endurance; cycling	55%VO_{2max}; 60 min	FiO_2 = 15%	Normobaric	BM, FBM, BMI, BFR, TG, TC, LDL-C,HDL-C, Glucose, Insulin
Nishiwaki, M,2016	7 (0/7)	7 (0/7)	56 ± 1	56 ± 1	>24 kg/m^2	8 weeks	4 days/wk	Endurance; aquatics	50% VO_{2max}; 0.5 h	Simulation of 2000 m altitude (6001–6038 mmHg)	Normobaric	BM, BMI,BFR,Glucose

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TABLE 1 (*Continued*) Main characteristics of studies included in the meta-analysis.

Study	Participants characteristics					Intervention						
	Participants (M/F)		Age		BMI/ BFR	Duration	Frequency	Type/ modality	Exercise intensity	Hypoxic condition	Type of hypoxia	Outcome
	HET	NET	HET	NET								
Park, H,2019	12 (12/0)	12 (12/0)	66.50 ± 0.90	66.50 ± 0.67	>25 kg/m^2	12 weeks	3 days/wk	Endurance/ Strength; cycling, running	60–70% HRmax; 90 min–100 min	FiO_2 = 14.5%	Normobaric	BM, BFR, TG, TC, LDL-C,HDL-C
Wang, N,2012	11 (6/5)	7 (4/3)	19.5 ± 1.64	22.4 ± 2.07	H:34.62 ± 5.05 kg/m^2 N:35.19 ± 5.07 kg/m^2	4 weeks	6 days/wk	Endurance/ Strength; cycling, running	M:100W, F:75 W; 4 h	FiO_2 = 15.4%–14.8%	Normobaric	BM, FBM,BFR,WC,BMI,TC,TG,HDL-C,LDL-C
Wiesner, S,2010	24 (10/14)	21 (8/13)	42.2 ± 1.2	42.1 ± 1.7	H:33.1 ± 0.3 kg/m^2 N: 32.5 ± 0.8 kg/m^2	4 weeks	3 days/wk	Endurance, running	65% VO_{2max},60 min	FiO_2 = 15%	Normobaric	LDL-C, BFR, WC, Insulin
Yang, X,2014	10 (10/0)	8 (8/0)	22.50 ± 1.27	22.13 ± 2.17	>25 kg/m^2	4 weeks	5 days/wk	Endurance; cycling, running	HR:140–150 beats/ min; 1 h	FiO_2 = 15.4%	Normobaric	BM, FBM, BMI, WHR, BFR, TG, TC, LDL-C,HDL-C
Zhang, N,2019	20 (20/0)	20 (20/0)	22.34 ± 2.15	21.87 ± 2.31	>25 kg/m^2	1 m	5 days/wk	Endurance; cycling	65% $V0_{2max}$; 60 min	FiO_2 = 15%	Normobaric	BM, FBM, BMI, BFR, TG, TC, LDL-C, HDL-C
Zhao, S,2016	9	9	18.08 ± 1.79	18.24 ± 2.23	>30 kg/m^2	8 weeks	5 days/wk	Endurance; cycling	65–75%$V0_{2max}$	FiO_2 = 14.7%	Normobaric	BM, BFR
Zheng, B,2020	10 (0/10)	10 (0/10)	19.60 ± 1.26	19.38 ± 0.74	>28%	6 weeks	3 days/wk	IT; cycling	Exercise: (64–76% HRmax; 10 min); Intervals: 3 min	FiO_2 = 14.0 ± 0.2%	Normobaric	BM, FBM, BMI, BFR

Data are presented as mean or range. An article presented separate study groups distinguished by the presence or absence of a.

M, male; F, female; wk, weeks; d, day; FiO_2, the fraction of inspired oxygen; IT, interval training; HR, heart rate; RM, repetition maximum; VO_{2max}, maximal oxygen consumption; BM, body mass; BMI, body mass index; BFR, body fat rate; FBM, fat body mass; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

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was not accessible in the full text had partial data or was ambiguously contacted via email or other means to the authors, and if no answer was received, it was not included in the literature. If the standard deviation was not expressly stated in the text, it was calculated using the following formula (Higgins JPT et al., 2022):

$$\sqrt{\mathrm{SDpre}^2 + \mathrm{SDpost}^2 - 2 \times \mathrm{Corr}(\mathrm{pre}, \mathrm{post}) \times \mathrm{SDpre} \times \mathrm{SDpost}}$$

SDpre is the pre-intervention SD, SDpost is the post-intervention SD, and Corr (pre, post) is the within-participant correlation coefficient, with the within-participant correlation set to 0.5 if no correlation is reported.

Quality assessment

The included studies were assessed separately using the Revised Cochrane risk-of-bias tool for randomized trials (RoB 2) (Higgins JPT et al., 2022) in terms of the risk of bias from the randomization process, the impact of intervention assignment, the risk of bias due to missing outcome data, the risk of bias in outcome measures, the five major components, and the overall evaluation of the articles.

Data synthesis and statistical analysis

1) The collected trials were meta-analyzed using R (RStudio V4.13, Boston, MA, United States) and RevMan (RevMan 5.3, Cochrane Collaboration, Oxford, United Kingdom) analytic tools, and all experimental data were continuous variables. The units of measurement for the outcome indicators were different, so standardized mean difference (SMD) effect scales and 95% confidence interval (CI) were utilized for statistical purposes.
2) I^2 was used to assess the heterogeneity of a study. When $I^2 = 0$, there was no heterogeneity among the included trials, and a fixed-effects model was used for analysis. When $I^2 > 40\%$, there was a significant probability of heterogeneity, and a random-effects model was used for analysis, along with a sensitivity analysis to eliminate the test for high heterogeneity. When the heterogeneity was too tremendous and determining the cause was problematic, descriptive analysis was performed.
3) Assessment of potential modifiers (the age of participants, the duration of the exercise intervention, the frequency of the intervention, and the level of hypoxia) of body composition indicators, glycometabolism, and lipometabolism indicators through subgroup analysis. Sensitivity analysis was done to investigate the sources of heterogeneity and assess the results' stability by removing each test individually.
4) We evaluated publication bias using Egger's regression test (Egger et al., 1997). When the sample size of the included literature was less than 15, publication bias testing was not necessary because of the small sample size and low test power (Cumpston et al., 2019).
5) If $p < 0.05$, it is considered a significant difference.

Results

Study selection and characteristics

The original literature search yielded 2,104 studies, of which 2082 were written in English and 22 were written in Chinese. After deleting duplicates with Endnote software and reviewing the titles and abstracts of the literature for initial screening, 486 studies were collected, of which 23 were discarded due to the lack of full text. Four hundred forty-four were excluded after reading the full text of the remaining 463 studies, and 19 RCTs were finally included, including 14 English and five Chinese studies (Wiesner et al., 2010; Wang et al., 2012; Kong et al., 2014; Morishima et al., 2014; Yang et al., 2014; Gatterer et al., 2015; Gonzalez-Muniesa et al., 2015; Nishiwaki et al., 2016; Zhao and Shi, 2016; Kong et al., 2017; Camacho-Cardenosa et al., 2018; Fernández Menéndez et al., 2018; Klug et al., 2018; Park et al., 2019; Zhang, 2019; Chacaroun et al., 2020; Jung et al., 2020; Zheng et al., 2020; Hobbins et al., 2021). Figure 1 depicts the procedure for incorporating the literature.

Table 1 contains the essential information on 19 studies, one of which incorporated two types of HET (Camacho-Cardenosa et al., 2018); hence the study was included twice. A total of 444 subjects were included in the study, all of whom were overweight or obese adults (aged 18–66 years) without metabolic diseases such as heart disease or type II diabetes. Six studies contained only male subjects, four studies contained only female subjects, seven studies contained both male and female subjects, and two studies did not specify the gender of the subjects in the text. The exercise mode was identical between the HET and NET groups, with 11 studies involving continuous aerobic exercise, primarily jogging, cycling, and aerobic exercise, with an average duration of more than 20 min per exercise session; four studies involving intermittent exercise, with three studies involving high-intensity interval exercise, and one study involving moderate-intensity interval exercise. The four studies used a combination of aerobic and strength training. The FiO_2 of the HET group ranged from 13% to 17.2%, whereas the NET group was exposed to normal atmospheric conditions (FiO_2 = 21%).

Risk-of-bias assessment

Among the 19 included studies, 18 were considered bias-free overall, while one was determined to be high-risk overall because

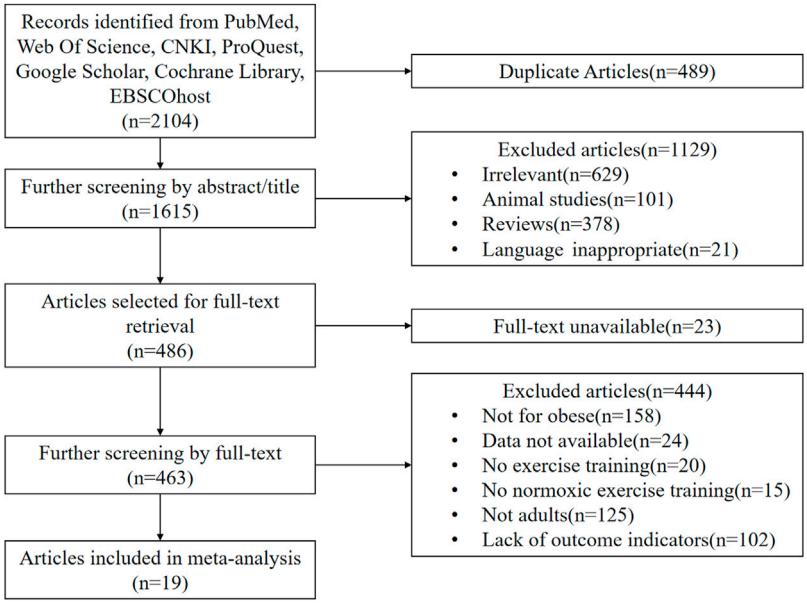

FIGURE 1
Flow diagram of the process of article selection.

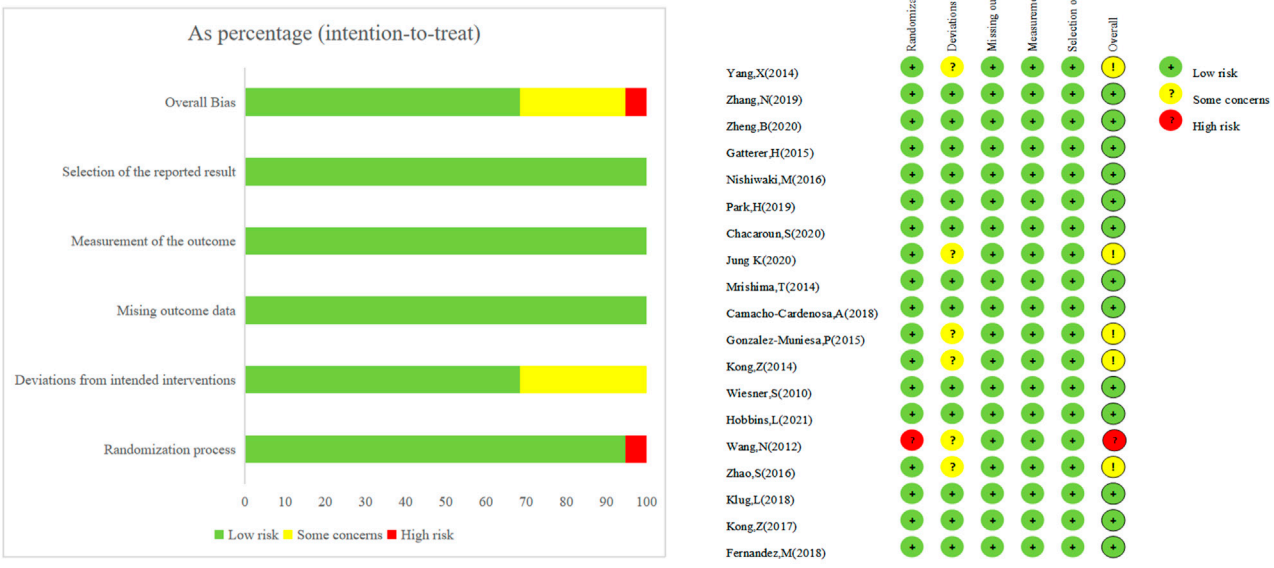

FIGURE 2
The risk assessment of bias.

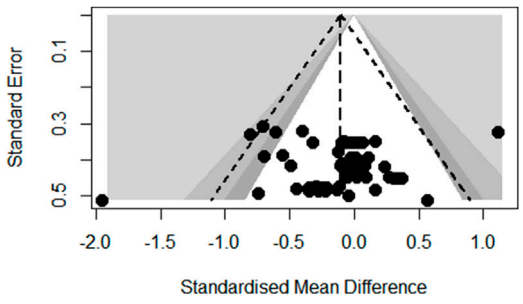

FIGURE 3
Funnel plot of body composition outcomes.

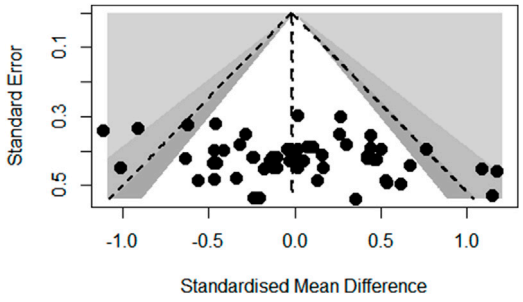

FIGURE 4
Funnel plot of glycometabolism and lipometabolism outcomes.

randomization grouping was not specified in the text. Figure 2 provides further details. Regarding blinded assessment, one study utilized the double-blinded approach, six used the single-blinded method, while the remaining 12 did not accomplish experimental implementer and participant blinded evaluation. The remaining 12 research was not blinded to participants or personal information, and the outcome indicators of six of these studies may have been impacted due to the absence of blinding, putting these six studies at risk. In contrast, the remaining 13 studies were at low risk.

Publication bias

Funnel plot analysis of body composition outcomes showed slight asymmetry but Egger's regression test did not reach statistical significance (p = 0.6672; see Figure 3). Also, the funnel plot of glycometabolism and lipometabolism outcomes showed reasonable symmetry and Egger's test did not reach statistical significance (p = 0.9647; see Figure 4).

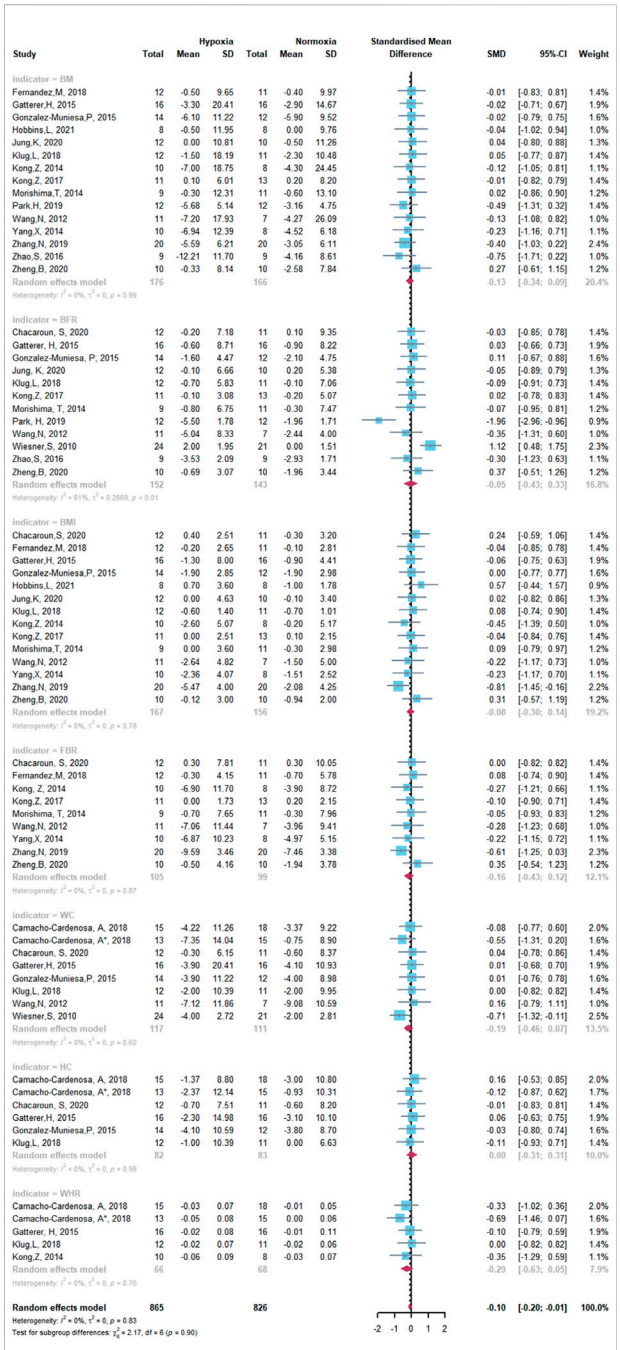

FIGURE 5
Forest Plot of the Meta-analysis on body composition.

Meta-analysis

The results of the meta-analysis of all studies are shown as forest plots in Figure 5 and Figure 6. A random effects model was considered the main method for meta-analysis to account for the heterogeneity between studies.

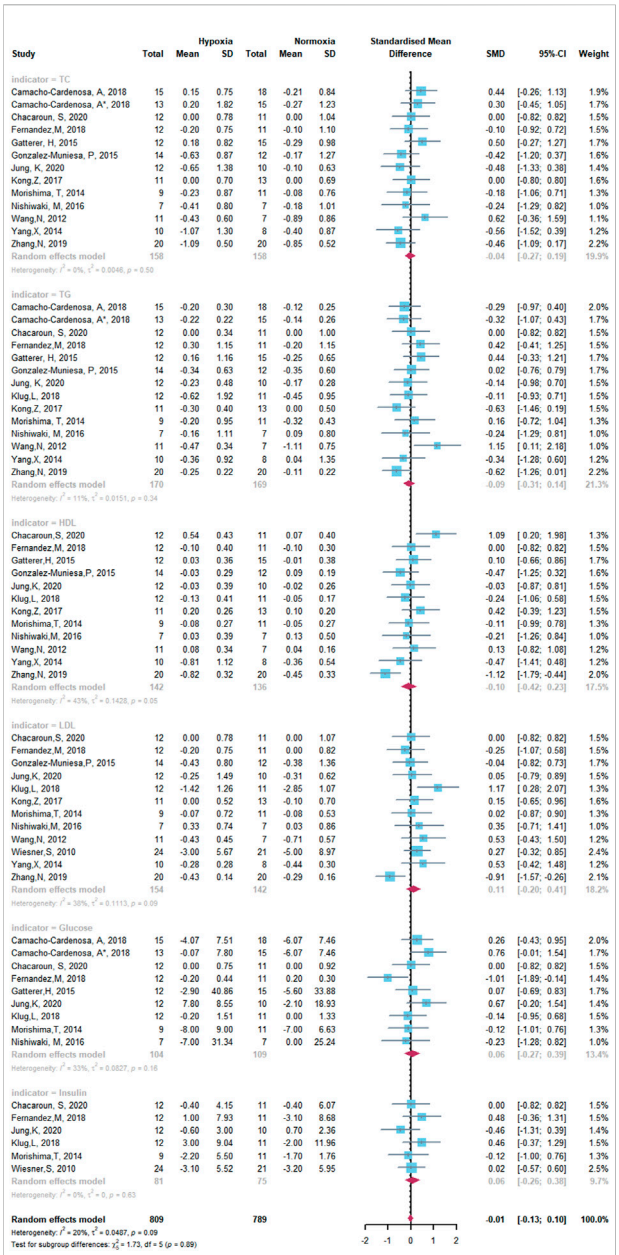

FIGURE 6
Forest Plot of the Meta-analysis on glycometabolism and lipometabolism.

The overall meta-analysis of the effects of HET and NET on body composition comprised 69 SMDs from 19 trials (Figure 5). There was no significant difference in the improvement of body composition with HET and NET and no heterogeneity among the studies (SMD -0.10, 95% CI -0.20 to -0.01; $p = 0.90$, $I^2 = 0\%$). Similarly, for studies conducted on glycometabolism and lipometabolism, our meta-analysis reveals no difference between HET and NET and low heterogeneity among the studies (SMD -0.01, 95% CI -0.13 to -0.10; $p = 0.89$, $I^2 = 20\%$) (Figure 6).

Subgroup analyses

The effects of HET and NET on the body composition, glycometabolism, and lipometabolism of overweight and obese adults may be influenced by the age of participants, intervention duration, frequency, and level of hypoxia. Subgroup analysis demonstrated that the age of participants, intervention duration, frequency, and level of hypoxia did not change the results of the meta-analysis (Table 2).

Discussion

This review aimed to examine the effect of hypoxia on fat loss compared to equivalent training in normoxia in overweight or obese adults. The key finding of this meta-analysis was that training in hypoxia elicited comparable responses in body composition, glycometabolism, and lipometabolism in individuals. In addition, we found that the subgroup analysis of different training duration, training frequency, hypoxia levels, and the age of participants not significantly improved body composition, glycometabolism, and lipometabolism values. These findings may have important implications for obese patients and multidisciplinary teams involved in obesity management.

Hypoxic exercise training and normoxic exercise training effects on body composition in obese and overweight people

BM, BMI, FBM, and BFR were reliable and valid for identifying adults at increased risk for death and morbidity related to overweight and obesity (Aronne, 2002). This review showed no significant positive effect on body composition for both hypoxic and normoxic conditioning. This is an important finding because it has previously been reported that active hypoxia may reduce BM in animals and humans (Hobbins et al., 2017). Early studies have found that hypoxia exposure at high altitudes can cause weight loss and changes in other body composition (Ge et al., 2010). A meta-analysis of studies by Dünnwald et al. (2019) showed that greatest reductions in BM and FBM were observed in moderate altitudes (1,500–3,500 m) when exposure was active and the duration of hypoxia exposure was more than 42 days (6 weeks). Active exposure primarily consisted of hiking, trekking, and sometimes swimming (e.g., activity during moderate altitude vacations) (Dünnwald et al., 2019). The studies included in this review simulated oxygen concentration conditions at moderate altitudes (FiO_2: 13.5%–17.4%), and the active exposure consisted of running and cycling in the hypoxic chamber. It follows that subjects in the studies included in this review were exposed to hypoxic environments

TABLE 2 Subgroup analyses of the effects of exercise training performed in hypoxia versus normoxia on reducing the fat.

Subgroup	Frequency (d/wk)						Duration (wk)						FiO_2 (%)						age (yr)					
		n	SMD [95%CI]	*p* values of SMD	I^2	*p* values of I^2		n	SMD [95%CI]	*p* values of SMD	I^2	*p* values of I^2		n	SMD [95%CI]	*p* values of SMD	I^2	*p* values of I^2		n	SMD [95%CI]	*p* values of SMD	I^2	*p* values of I^2
BM	<4	6	0.03 [−0.31, 0.36]	0.88	0%	0.99	<8	9	−0.03 [−0.32, 0.26]	0.84	0%	1	<15	6	−0.06 [−0.40, 0.29]	0.74	0%	0.77	<45	10	−0.14 [−0.41, 0.13]	0.33	0%	0.96
	≥4	7	−0.19 [−0.56, 0.18]	0.31	0%	0.84	≥8	5	−0.13 [−0.53, 0.27]	0.52	0%	0.59	≥15	5	0.02 [−0.46, 0.50]	0.94	0%	0.99	≥45	3	−0.14 [−0.58, 0.30]	0.54	0%	0.06
BMI	<4	7	0.03 [−0.28, 0.34]	0.83	0%	0.94	<8	9	−0.09 [−0.40, 0.23]	0.58	0%	0.71	<15	5	0.13 [−0.21, 0.47]	0.44	0%	0.92	<45	9	−0.10 [−0.38, 0.18]	0.48	0%	0.71
	≥4	6	−0.09 [−0.49, 0.32]	0.67	0%	0.44	≥8	4	0.08 [−0.27, 0.44]	0.65	0%	0.96	≥15	5	−0.12 [−0.55, 0.31]	0.58	0%	0.52	≥45	3	0.07 [−0.38, 0.51]	0.77	0%	0.86
FBM	<4	4	−0.10 [−0.44, 0.23]	0.55	0%	0.87	<8	3	−0.10 [−0.44, 0.23]	0.55	0%	0.87	<15	2	0.13 [−0.35, 0.62]	0.60	0%	0.84	<45	8	-	-	-	-
	≥4	5	−0.31 [−0.93, 0.31]	0.33	0%	0.44	≥8	5	−0.01 [−0.40, 0.38]	0.97	0%	0.87	≥15	5	−0.22 [−0.73, 0.29]	0.39	0%	0.67	≥45	1	-	-	-	-
BFR	<4	5	0.29 [−0.12, 0.70]	0.16	41%	0.13	<8	4	0.22 [−0.26, 0.70]	0.37	51%	0.07	<15	4	0.02 [−0.39, 0.43]	0.91	0%	0.78	<45	6	0.19 [−0.32, 0.69]	0.46	54%	0.05
	≥4	3	−0.22 [−0.74, 0.30]	0.41	0%	0.88	≥8	4	−0.03 [−0.42, 0.37]	0.90	0%	0.92	≥15	3	0.38 [−0.00, 0.76]	0.05	63%	0.04	≥45	5	−0.46 [−1.29, 0.36]	0.27	75%	0.007
TC	<4	6	0.14 [−0.18, 0.46]	0.39	0%	0.51	<8	3	0.05 [-0.38, 0.48]	0.82	0%	0.64	<15	3	0.00 [−0.40, 0.41]	0.98	0%	0.41	<45	8	−0.01 [−0.28, 0.27]	0.97	0%	0.40
	≥4	5	−0.14 [−0.55, 0.27]	0.43	0%	0.50	≥8	7	0.01 [−0.28, 0.30]	0.95	8%	0.37	≥15	5	0.18 [−0.20, 0.57]	0.36	0%	0.69	≥45	3	0.15 [−0.34, 0.65]	0.54	0%	0.49
TG	<4	5	−0.03 [−0.33, 0.26]	0.84	0%	0.83	<8	3	0.07 [−0.40, 0.54]	0.76	41%	0.13	<15	4	0.19 [−0.22, 0.60]	0.36	0%	0.68	<45	8	−0.13 [−0.50, 0.25]	0.51	42%	0.10
	≥4	5	−0.08 [−0.65, 0.49]	0.78	46%	0.11	≥8	7	−0.08 [−0.38, 0.22]	0.62	0%	0.85	≥15	5	−0.33 [−0.71, 0.05]	0.09	0%	0.85	≥45	4	0.06 [−0.36, 0.49]	0.76	0%	0.70
HDL	<4	3	0.04 [−0.33, 0.41]	0.83	48%	0.11	<8	3	−0.04 [−0.43, 0.36]	0.86	0%	0.66	<15	4	0.37 [−0.30, 1.03]	0.28	49%	0.14	<45	6	−0.32 [−0.69, 0.05]	0.09	59%	0.05
	≥4	5	0.01 [−0.40, 0.41]	0.98	0%	0.70	≥8	5	0.08 [−0.30, 0.46]	0.73	45%	0.12	≥15	3	0.03 [−0.45, 0.52]	0.89	0%	0.50	≥45	4	0.19 [−0.24, 0.62]	0.39	41%	0.13
LDL	<4	3	0.18 [−0.23, 0.60]	0.39	30%	0.22	<8	4	0.28 [−0.12, 0.68]	0.18	30%	0.21	<15	5	−0.07 [−0.54, 0.41]	0.78	0%	0.87	<45	7	−0.08 [−0.52, 0.37]	0.73	48%	0.09
	≥4	5	0.34 [−0.13, 0.82]	0.15	0%	0.86	≥8	4	0.06 [-0.37, 0.48]	0.26	0%	0.94	≥15	4	0.54 [−0.61, 1.68]	0.36	78%	0.03	≥45	4	0.50 [−0.22, 1.22]	0.17	46%	0.16
Glucose	<4	6	0.16 [−0.16, 0.48]	0.32	0%	0.62	<8	3	−0.41 [−0.91, 0.08]	0.10	25%	0.26	<15	4	−0.05 [−0.46, 0.36]	0.81	59%	0.06	<45	4	0.06 [−0.34, 0.45]	0.78	68%	0.02
	≥4	2	0.31 [−0.36, 0.98]	0.37	40%	0.20	≥8	6	0.29 [−0.04, 0.61]	0.09	0%	0.56	≥15	5	0.17 [−0.20, 0.54]	0.37	0%	0.43	≥45	4	−0.05 [−0.47, 0.37]	0.81	0%	0.97
Insulin	<4	4	-	-	-	-	<8	4	0.18 [−0.20, 0.55]	0.36	0%	0.64	<15	3	0.01 [−0.47, 0.49]	0.96	15%	0.31	<45	3	0.10 [−0.32, 0.52]	0.63	0%	0.57
	≥4	1	-	-	-	-	≥8	2	−0.22 [−0.81, 0.37]	0.47	0%	0.45	≥15	3	0.10 [−0.32, 0.52]	0.65	0%	0.60	≥45	2	0.23 [−0.36, 0.81]	0.45	0%	0.44

n, number of studies. -, absence of studies for a given subgroup. BM, body mass; BMI, body mass index; BFR, body fat rate; FBM, fat body mass; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

10.3389/fphys.2022.940749

for less hours per day than those included in the studies by Dünnwald (Dünnwald et al., 2019). Thus, the insufficient daily hypoxic exposure time may have contributed to the insignificant difference between HET and NET in terms of fat loss.

Another factor contributing to weight loss in hypoxic conditions was decreased caloric intake through meals as a result of diminished appetite (Westerterp-Plantenga et al., 1999). Highlanders' negative energy balance was the primary cause of weight loss (Hamad and Travis, 2006). According to animal studies, 21-day-old rats exposed to a low-pressure chamber at a simulated altitude of 6100 m only had 54.6% of the food intake and 32.7% of the body weight gain of normoxic rats (Bozzini et al., 1987). Energy intake was also reduced in humans exposed to artificial hypoxia (De Glisezinski et al., 1999). Due to the short duration of hypoxia exposure per day in the studies included in this review, subjects may not have been affected by reduced appetite.

More studies have also found non-significant improvements in body composition in the hypoxic condition. For instance, Wiesner et al. (Wiesner et al., 2010) discovered that in a 4-weeks HET trial of normal-weight people, HET was not considerably more successful in lowering BM compared to NET. Netzer et al. (Netzer et al., 2008)reported an insignificant reduction in BM and BMI in the hypoxic group. Similarly, in another study, subjects exercising in a hypoxic (FiO_2 = 15.0%) and normoxic environment (moderate intensity cycling, three times per week for 4 weeks) did not show significant changes in BMI and FBM. However, the normoxic group demonstrated a slight decrease in BM following the intervention compared to the hypoxic group (-1% and -0.5%, respectively) (Morishima et al., 2014). Subgroup analysis of this review showed comparable effects of HET and NET on body composition at less than 8 weeks. Therefore, hypoxia may take time to have a physiological effect on the body. Nevertheless, the longer duration of the intervention (8 weeks–8 months) did not result in significant differences between the HET and NET groups either. This could have led to subjects already having adapted to the hypoxic environment. When people have been exposed to hypoxia for long periods of time, the adaptation of body composition may rapidly plateau and, without periodic adjustment, may not continue the benefits of hypoxia to the organism (Hobbins et al., 2017).

In addition to BM, BMI, FBM, and BFR, the risk of overweight and obesity was strongly associated with excess abdominal fat and lower fitness level. The results of two previous studies (Wiesner et al., 2010; Shin et al., 2018) showed a significant decrease in WC in subjects after HET (aerobic training, 60 min/d, VO_{2max} of 65%, and FiO_2 of 14.5–15%), but no significant changes in NET. Another study (Camacho-Cardenosa et al., 2018) also found a significant reduction in WC and WHR after 12 weeks of HET. These studies suggested that HET positively affected abdominal fat reduction, which may be related to the enhanced oxidation of lipids after exercise (Camacho-Cardenosa et al., 2018). It is noteworthy that reducing abdominal fat requires adequate exercise duration and intensity. According to the American College of Sports Medicine (Wilkins, 2006), people who are overweight or obese should achieve the following recommended program of moderate-intensity exercise for 50–60 min/day and 300 min/week when exercising for weight loss. Only four trials included in this review had exercise protocols that met the exercise regimen recommended by the American College of Sports Medicine (Wang et al., 2012; Yang et al., 2014; Zhao and Shi, 2016; Zhang, 2019). In addition, when people exercised in a hypoxic environment as opposed to a normoxic one, there was a tremendous increase in heart rate, more production of blood lactate, and more significant subjective fatigue (Bouissou et al., 1987). It follows that exercise in a hypoxic environment may be more "challenging", especially for obese individuals. In other words, exercising in an environment with low levels of hypoxia may cause physiological discomfort, leading to reduction in total workload. Hence, more data from randomized trials are required to determine the optimal intervention duration, intervention frequency, and hypoxia level for fat loss in NETs.

Hypoxic exercise training and normoxic exercise training effects on glycometabolism and lipometabolism in obese and overweight people

The current meta-analysis showed that HET and NET did not significantly improve lipid metabolism, which is generally consistent with previous studies (Netzer et al., 2008; Wiesner et al., 2010). Differences in the current findings may be primarily related to the total duration of the training. It has been shown that subjects with NET have significantly increased HDL-C levels (Costa et al., 2019). Netzer (Netzer et al., 2017) found that subjects who completed 8 weeks of exercise under hypoxia or normoxia showed a greater increase in TG, TC, and HDL-C. In other studies of similar exercise intensity, TG, TC, and HDL-C did not change after completing 4 weeks of exercise training in the HET and NET groups (Wiesner et al., 2010; Morishima et al., 2014). Interestingly, all intervention groups of these findings completed the same type of exercise at an 'absolute' intensity, i.e., an intensity regardless of the environmental condition. The proportion of carbohydrate oxidation increase under hypoxia condition compared to the normoxic condition (Jung et al., 2020). However, exercising in a hypoxic environment puts extra stress on the metabolism of lipids, which the body must overcome to oxidize further substances that provide energy in the muscles (Kelly and Basset, 2017; Jung et al., 2020). As mentioned above, the hypoxic environment makes exercising more "harder." Therefore, we suggest that the intensity of exercise achieved by people exercising in a normoxic environment may be what stimulates lipid metabolism.

Previous studies have shown HET increases the relative glucose oxidation rates of subjects (Geiser et al., 2001; Vogt et al., 2001; Hoppeler et al., 2003). This phenomenon was attributed to the trans-activation of HIF-1 (Wiesner et al., 2010). In this review, exercise training under hypoxic and normoxic conditions elicited similar responses in obese patients, suggesting that HET had no additional effect on fasting insulin and glucose metabolism in obese subjects.

It is well-established the age-related variations in glycometabolism and lipometabolism (Tessari, 2000). Nevertheless, subgroup analysis of this review demonstrated that neither youth (18–44 years) nor middle-aged subjects (45–66 years) exhibited changes in lipid metabolism between HET and NET. Only five trials in this review had subjects over 45 years of age (Nishiwaki et al., 2016; Klug et al., 2018; Park et al., 2019; Chacaroun et al., 2020), which may lead to biased results. Furthermore, most trials included in this review (Wiesner et al., 2010; Yang et al., 2014; Gatterer et al., 2015; Nishiwaki et al., 2016; Kong et al., 2017; Camacho-Cardenosa et al., 2018; Fernández Menéndez et al., 2018; Klug et al., 2018; Park et al., 2019; Zhang, 2019; Chacaroun et al., 2020; Jung et al., 2020; Zheng et al., 2020; Hobbins et al., 2021) did not investigate dietary intake and daily activity in the HET and NET groups during the hypoxic exercise intervention. As a result, we cannot rule out the effect of the age of participants, food intake, and daily activities on glycometabolism and lipometabolism.

There were several limitations to this review that should be taken into account. Even though a systematic literature search was conducted, it was possible that some relevant studies were not included in this meta-analysis if our search algorithm did not capture them. In addition, there was unavoidable variation in the design protocols of different literature, such as individual subject differences, compliance, intervention duration, and exercise format. These uncontrollable variables may have contributed to the heterogeneity in this study.

To conclude, this systematic review with meta-analysis showed that HET and NET have similar effects on fat loss in overweight and obese adults. Furthermore, subgroup analysis also revealed that hypoxia had no effect on body composition, glycometabolism, and lipometabolism when potential parameters (i.e., the age of participants, hypoxia dose, exercise frequency, and duration) are altered. Thus, application and promotion of HET in fat reduction require additional exploration. However, due to some limitations, more RCTs with larger sample sizes are needed to further understand exercise's effectiveness on fat loss under different oxygen conditions.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

SC, XL: Conceptualization, Formal analysis, Data Curation, Writing-Original Draft, Visualization. QL, JC, YY: Formal analysis, Data Curation. YG, QM, YS: Writing-Review and Editing. HS: Writing-Review and Editing.

Funding

This work was supported by the National Key Research and Development Program of China (NO.2018YFCC2000600) and Fok Ying Tung Education (NO.161094). The funders had no role in the study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Physiology

TYPE Original Research
PUBLISHED 25 August 2022
DOI 10.3389/fphys.2022.893872

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Exercise Physiology, a section of the journal Frontiers in Physiology

RECEIVED 10 March 2022
ACCEPTED 27 July 2022
PUBLISHED 25 August 2022

CITATION
Rupp T, Saugy JJ, Bourdillon N and Millet GP (2022), Brain-muscle interplay during endurance self-paced exercise in normobaric and hypobaric hypoxia. *Front. Physiol.* 13:893872. doi: 10.3389/fphys.2022.893872

Brain-muscle interplay during endurance self-paced exercise in normobaric and hypobaric hypoxia

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Purpose: Hypoxia is one major environmental factor, supposed to mediate central motor command as well as afferent feedbacks at rest and during exercise. By using a comparison of normobaric (NH) and hypobaric (HH) hypoxia with the same ambient pressure in oxygen, we examined the potential differences on the cerebrovascular and muscular regulation interplay during a self-paced aerobic exercise.

Methods: Sixteen healthy subjects performed three cycling time-trials (250 kJ) in three conditions: HH, NH and normobaric normoxia (NN) after 24 h of exposure. Cerebral and muscular oxygenation were assessed by near-infrared spectroscopy, cerebral blood flow by Doppler ultrasound system. Gas exchanges, peripheral oxygen saturation, power output and associated pacing strategies were also continuously assessed.

Results: The cerebral oxygen delivery was lower in hypoxia than in NN but decreased similarly in both hypoxic conditions. Overall performance and pacing were significantly more down-regulated in HH *versus* NH, in conjunction with more impaired systemic (e.g. saturation and cerebral blood flow) and prefrontal cortex oxygenation during exercise.

Conclusions: The difference in pacing was likely the consequence of a complex interplay between systemic alterations and cerebral oxygenation observed in HH compared to NH, aiming to maintain an equivalent cerebral oxygen delivery despite higher adaptive cost (lower absolute power output for the same relative exercise intensity) in HH compared to NH.

KEYWORDS

altitude, cerebral oxygen delivery, near-infrared spectroscopy, pacing strategies, performance, time-trial exercise

1 Introduction

Hypoxia is one major environmental factor of interest when considering training and performance at altitude since it mediates cerebral oxygen supply and central motor command as well as afferent feedbacks at rest and during exercise (Amann et al., 2007; Subudhi et al., 2009; Subudhi, Panerai and Roach, 2010; Vogiatzis et al., 2011; Verges et al., 2012; Goodall, Twomey and Amann, 2014; Marillier et al., 2021). Only a few studies investigated self-paced exercise when arterial oxygenation is manipulated, suggesting marginal differences (Clark et al., 2007; Beidleman et al., 2014) or similar trends (Amann et al., 2006; Périard and Racinais, 2016) in pacing between normoxia and hypoxia, despite reduced average power output in the latter. A major concern regarding these studies is that subjects were exposed to simulated altitude for only 5–40 min before time-trials. Oxygen delivery to the tissues (e.g. muscle, brain) progressively evolves in the first hours of exposure to hypoxia (e.g. time delay in changes between arterial and cerebral oxygenation (Rupp and Leti, 2013), and from a practical point of view, exposure time preceding training/competing at altitude is usually longer and prone to influence how the practitioner may feel (Luks, 2015). Altogether, pacing strategies adopted after a more prolonged exposure period (e.g. 24-h) are relevant and may strongly differ from what has been observed with short-term acute exposure (Tucker, 2009). Discrepancies in the literature regarding altered pacing in hypoxia may also result from the type of hypoxia (normobaric hypoxia NH *versus* hypobaric hypoxia HH) used in these models. Indeed, numerous disparities have been recently found in subjects resting and exercising at a given pressure of inspired oxygen (P_iO_2), but among NH or HH conditions (Faiss et al., 2013). For instance, a combination of a higher tidal volume and lower respiratory frequency leading to a higher minute ventilation in NH compared to HH (Conkin and Wessel, 2008) has been reported and markers of oxidative stress have recently been shown lower in NH compared to HH (Faiss et al., 2013; Ribon et al., 2016). The mechanisms underlying these slight physiological differences are not so clear yet. Nevertheless, we have just shown that such differences are associated with impaired global performance in HH compared to NH on a 250-kJ cycling time-trial at 3,450 m (Saugy et al., 2016). However, the extent to which underlying physiological responses driving pacing strategies would be differentially affected in HH *versus* NH remains unknown.

We therefore examined if there are differences in cerebrovascular regulation and muscular activation in relation to oxygen consumption, arterial saturation, cerebral and muscle hemodynamics and deoxygenation, subjective discomfort, RPE and how it would influence or be influenced by different pacing strategies during a 250-kJ cycling time-trial conducted after 24 h: 1) in normobaric hypoxia (NH) at a simulated altitude of 3,450 m, 2) in hypobaric hypoxia (HH) at a terrestrial altitude of 3,450 m and, 3) in normobaric normoxia (NN) as a control condition. We hypothesized that there might be subtle differences in the regulation of oxygen delivery to the brain between NH and HH, inducing different pacing strategies and consequently, the supposed better endurance performance in NH, when compared to HH. It was also anticipated that SpO_2 and cerebral hemodynamics would be more affected in HH, compared to NH, leading to suboptimal pace management and a greater performance decrement. By comparing cerebrovascular and brain *versus* muscle deoxygenation time-course during self-paced endurance exercise in NH *versus* HH, we aim to better understand how exercise is regulated in hypoxia and to explore new mechanisms on the interplay between cerebrovascular and muscular regulation in humans.

2 Methods

Experimental design and partial parts of the methods have already been presented in a previous paper (Saugy et al., 2016) focusing on global exercise performance in HH *versus* NH but with no mention to the present interests (*i.e.*, exercise intensity regulation and underlying cerebrovascular and muscular regulation). For the convenience of the reader, key methodological information is redefined in the present paper.

2.1 Subjects

Sixteen healthy, trained male subjects volunteered to participate to this study (mean ± SD; age 34.7 ± 9.5 years, body weight 75.2 ± 7.2 kg, height 180 ± 6 cm, maximal oxygen consumption VO_{2max} 60.2 ± 9.9 ml kg^{-1} min^{-1}). Participants were all experienced (recreational or competitive but not elite) cyclists. Written informed consent was obtained from each participant before participation. Subjects were non-smokers, and neither acclimatized nor recently exposed to altitude. All procedures conformed to the standards set by the *Declaration of Helsinki* and the study was approved by a Medical Ethics Committee (Commission Cantonale Valaisanne d'Ethique Médicale, CCVEM; Agreement 051/09).

2.2 Experimental design

The experimental design consisted in a preliminary visit and three testing sessions. During the first meeting to the laboratory, subjects completed the baseline anthropological measurements and filled the consent form. Participants then performed 1) a maximal incremental exercise test (F_iO_2: 0.21; 60 W + 30 W min^{-1}) to determine VO_{2max} and peak workload on a braked cycle ergometer including a Powertap sensor (Cycleops IC 400 Pro, Madison, Wisconsin, United States);

and 2) a familiarization test with the 250-kJ time-trial on the same ergocycle.

The experimental design was then composed of three different sessions in a randomized order separated by at least 12 days. One session was performed in a hypobaric hypoxia (HH) environment at the Altitude Research Station in Jungfraujoch (3,450 m, F_iO_2 of 20.9%, BP of 481.8 ± 4.7 mmHg, P_iO_2 of 90.9 ± 1.0 mmHg, temperature of 21.3 ± 0.6°C, humidity of 45.1 ± 8.3%). Two sessions were conducted in a hypoxic chamber (ATS Altitude, Sydney, Australia) built in our laboratory (Sion, 485 m, Switzerland). Temperature inside the chamber was maintained constant by an internal air conditioning system. One of the two sessions completed in the chamber was performed in normobaric hypoxia (NH) with a F_iO_2 of 13.6% (BP of 715.8 ± 3.8 mm Hg, P_iO_2 of 91.0 ± 0.6 mmHg, a temperature of 22.7 ± 0.8°C, and a humidity of 41.0 ± 4.8%) corresponding to a simulated altitude of 3,450 m. The other session was performed in normobaric normoxia (NN) with a F_iO_2 of 20.9% (BP: 718.1 ± 3.3 mmHg, P_iO_2 of 140.5 ± 0.6 mmHg, a temperature of 23 ± 1°C, and a humidity of 42.8 ± 4.4%.). These parameters were controlled regularly with an electronic device (GOX 100 oximeter, Greisinger, Regenstauf, Germany). In order to blind subjects to altitude, the system was also running normoxic airflow into the chamber during the NN sessions.

Each session consisted of 26 h of exposure in each condition (NN, NH, HH) in a randomized blinded order. After 24 h of exposure, subjects completed a self-paced 250 kJ time-trial, being free to increase/decrease the resistance to adjust the workload as they became familiar with it in the preliminary session (up/down electronic shifter at the handlebar). All trials were preceded by a rest period of 5 min, followed by 3 min warm-up period at 70 W. Setting (bike dimensions and braking resistance) was individualized but strictly identical between the three conditions for each subject. The participants were instructed to complete the 250 kJ as rapidly as possible. Work progressively completed from 0 to 250 kJ was the only information (visually) provided to subjects during the time-trial. With regard of the participants expertise in cycling and their VO_2max, it was expected that the self-paced exercise would last 15–20 min in normoxia. In order to prevent excessive thermal stress, a fan providing a high wind speed was placed directly in front (~80 cm) of the subjects during the trials.

Particular attention was given to the standardization and the control of the conditions. The participants were asked to maintain their usual training and physical activities during the whole experimental protocol to avoid fitness changes between sessions. Similar standardized meals were provided at the same time under each condition. The bedding was similar among conditions and sleep quality was not different between HH and NH (Saugy et al., 2016). The daily schedule and activities were exactly the same for all the conditions and the time trial was performed at the same time of the day in each condition.

2.3 Measurements

2.3.1 Gas exchanges, heart rate, SpO_2 and perceptual variables

Breath-by-breath pulmonary gas exchange (oxygen uptake, VO_2; carbon dioxide production and end-tidal carbon dioxide partial pressure, $P_{ET}CO_2$), minute ventilation ($\dot{V}$ E) and respiratory frequency (Rf) were measured at rest and throughout time-trial using a portable gas analyzer (MetaMax 3B, Cortex, Leipzig, Germany) with an oronasal mask (Vmask™, 7,500 series; Hans Rudolph Inc., Shawnee, United States). Heart rate (HR) and peripheral oxygen saturation (SpO_2) were continuously recorded (Radical-7®, Masimo Corporation, Irvin, United States) and stored for offline analyzes with commercially available software (Labchart software, AD Instrument, Colorado Springs, United States). The VO_{2max} was not measured in hypoxic conditions but estimated by decrementing the NN VO_{2max} (*i.e.*, during the preliminary visit) by 22.7% following the 7.7% estimated linear VO_{2max} decrement per 1,000 m described by Wehrlin et al. (Wehrlin and Hallén, 2006).

Subjects were regularly asked to qualify their rate of perceived exertion (RPE, Borg Scale, 6–20) during the time-trial. Legs and breathing feelings were also assessed with visual analog scales (VAS, 0–10), ranging from "no difficulty" to "extremely difficult".

2.3.2 Power, workload and cadence

Power output, speed, cadence and total workload were continuously recorded during the time-trial by the cycle ergometer (Cycleops IC 400 Pro, Madison, United States).

2.3.3 Electromyographic recordings

Quadriceps electromyography (EMG) was continuously recorded from the right *vastus lateralis* (VL) using bipolar silver chloride surface electrodes of 10-mm diameter (Kendall Meditrace 100). Electrodes were taped lengthwise on the skin over the muscle belly following SENIAM recommendations, with an interelectrode distance of 20 mm. Positions of the electrodes were marked on the skin to ensure precise replacement in other sessions. Reference electrode was attached on the patella. Low impedance (<10 kΩ) at the skin-electrode was obtained by shaving and abrading the skin with an abrasive sponge and cleaning with alcohol. EMG data were recorded at 2 kHz with Biopac system (MP150, Biopac System, Goleta, United States) and amplified with a bandwidth frequency ranging from 10 to 500 Hz. For data analysis, the integral of the EMG activity was calculated over 10-kJ time-periods throughout time-trial using the formula:

$$iEMG\ (|m(t)|) = \int_0^1 |m(t)|dt$$

where m is the raw EMG signal.

2.3.4 Cerebrovascular variables

Mean middle cerebral artery blood flow velocities (MCAv) were measured bilaterally using a 2-MHz pulsed Doppler ultrasound system (ST3, Spencer technology, Seattle, United States). The Doppler ultrasound probes were positioned over right and left temporal windows and held firmly in place with an adjustable headband (Marc 600 Headframe, Spencer technology). The signals were at depths ranging from 44 to 58 mm. Signal quality was optimized using an M-mode screen shot and insonation depth, probes and headband locations were marked to ensure within-subject repeatability. Bilateral MCAv were averaged to represent an index of global cerebral blood flow at rest and during exercise. Cerebral O_2 delivery (cDO_2) before and during exercise was calculated using the equation: cDO_2 = mean MCAv x CaO_2, where CaO_2 refers to the oxygen content of the arterial blood estimated as follows: CaO_2 = [hemoglobin concentration assessed in each condition after 20 h of exposure x 1.36 x current SpO_2/100], oxygen dissolved in plasma being neglected. cDO_2 was then expressed as a percentage of the resting normoxic (NN) pre-exercise value.

2.3.5 Near-infrared Spectroscopy measurements

Cerebral oxygenation in the left prefrontal (PFC) and motor (MC) cortex was assessed by monitoring changes in oxy- and deoxy-hemoglobin (O_2Hb and HHb, respectively) obtained with spatially resolved, continuous wave near-infrared spectroscopy (NIRS) (Oxymon MkIII, Artinis, Zetten, Netherlands). Theoretical and performance details of NIRS have been previously described (Perrey, 2008). PFC NIRS probes were centered between Fp1 and F3 locations according to the international 10–20 EEG system, with 3.5-cm interoptode distance. MC NIRS data were expressed as the average of a 4-channel square setting (3-cm interoptode distance) fixed with headbands between Cz and C3 locations. Muscle oxygenation was assessed from the right *vastus lateralis (at mid thigh)* using a 4-cm interoptode distance. For PFC and muscle, probe holders were secured to the skin using double-sided adhesive tape to minimize any change in its relative position and all optodes were covered with black sweatbands for them to be shield from ambient light. Total hemoglobin changes (THb = O_2Hb + HHb) were calculated to reflect the changes in tissue blood volume within the illuminated areas and difference in hemoglobin (HbDiff = HbO_2 - HHb) was calculated as a reliable estimator of change in tissue (de-) oxygenation status (Rooks et al., 2010). NIRS data were recorded at 10 Hz, filtered with a 2-s moving Gaussian window smoothing algorithm and expressed as relative changes (Δμmol) from the stable baseline preceding each time-trial.

2.4 Statistics

Data are reported as means and standard deviations with 95% confidence intervals. Data were tested for equality of variance (Fisher-Snedecor *F-test*) and for normality (Shapiro-Wilk test). To investigate the pacing strategies, we divided the time-trial in 25 slices of 10 kJ (increments of 4% of the total work completed). One-way ANOVA were used to determine if systemic (SpO_2, VO_2) and cerebrovascular (MCAv, cDO_2) variables were different between conditions before the time-trial (*i.e.*, at baseline, BL). When a significant main effect was found, Bonferroni *post-hoc* tests were used to localize differences between conditions (NN, NH, HH). Two-way ANOVA (condition x time) with repeated measures were used for each parameter during the time-trial. When significant main or interaction effects were found, Bonferroni *post-hoc* tests were used to localize differences between conditions (NN, NH, HH) and/or time (each 10 kJ slice from 0 to 250 kJ). Null hypothesis was rejected at $p < 0.05$. All analyses were made using Sigmaplot 11.0 software (Systat Software, San Jose, United States).

3 Results

3.1 Time-trial performance and pacing

Time-trial performance data are summarized in Table 1. Compared to NN (*i.e.*, 1,041 ± 151 [955.9–1,126.9] s), the mean time required to complete 250 kJ was 24.1 ± 9.6 [18.7–29.5] % and 33.2 ± 12.4 [26.2–40.2] % higher for NH and HH, respectively (both $p < 0.001$, cf. Supplementary Material). The mean time was 7.5 ± 7.5 [3.2–11.7] % higher in HH than in NH ($p < 0.01$). Compared to NN the whole time-trial pace was reduced for both hypoxic conditions (Figure 1A, $p < 0.001$). The HH power output was significantly reduced compared to NH from 140 to 220 kJ ($p < 0.05$). When expressed as a function of the average power sustained in each respective condition (Figure 1B), an interaction effect was observed ($p < 0.05$) showing inverse trends between NN and both NH and HH, despite a comparable range of variation (~30%, ~21 and ~23%, for NN, NH and HH, respectively). In HH, normalized power output followed a similar pattern to NH. Between 85% and the end of the time-trial (so called "final burst" or "end-spurt"), the relative increase in power output was 9, 18 and 21% in NH, HH and NN, respectively.

3.2 Pulse oxygen saturation

As presented in Figure 2A, SpO_2 was significantly higher at baseline (97.7 ± 1.2 [97.1–98.4] %; $p < 0.001$) and during cycling in NN compared to both hypoxic conditions (0–250 kJ; $p < 0.001$) and SpO_2 was significantly lower in HH compared to NH at baseline

TABLE 1 Baseline physiological measurements before exercise and performance results during the self-paced 250-kj time-trial in normobaric normoxia (NN), normobaric hypoxia (NH) and hypobaric hypoxia (HH). ***$p < 0.001$ for difference between NN and both hypoxic conditions; ##$p < 0.01$ for difference between NH and HH.

	Normobaric normoxia	#auto; normobaric Hypoxia	#auto; Hypobaric Hypoxia
#auto; Baseline Measurements			
#auto; *Sp O 2 (%)*	98 ± 1 ***	92 ± 2	90 ± 2 ##
#auto; *HR (bpm)*	69 ± 11 ***	77 ± 12	81 ± 8
#auto; Time-trial Performance data			
#auto; *Mean duration (s)*	#auto; 1,041 ± 151 ***	#auto; 1,286 ± 167	#auto; 1,379 ± 187 ##
#auto; *Mean Power (W)*	#auto; 245 ± 39 ***	#auto; 197 ± 26	#auto; 184 ± 25
#auto; *Mean cadency (rpm)*	#auto; 95 ± 7	#auto; 93 ± 9	#auto; 88 ± 6 #

(92.2 ± 2.1 [91.1–93.3] vs 89.9 ± 1.9 [88.9–91.1] %; $p < 0.01$) and during the first half of the time-trial (0–140 kJ; $p < 0.05$).

3.3 Cerebrovascular variables

MCAv at baseline was not different between conditions. MCAv was increased in the first quarter of the time-trial in both NN, NH and HH (+31%, +15% and +25%, respectively), the increase being lower in NH compared to HH (from 10 to 150 kJ, $p < 0.05$) and NN (from 10 to 100 kJ, $p < 0.05$) while no difference was found along time-trial between HH and NN (Figure 2B), where MCAv decreased from 40 kJ onward. Baseline cDO_2 values were not different between conditions (Figure 2C). In contrast, during time-trial cDO_2 was lower in both NH and HH (on average, by 17%) compared to NN ($p < 0.05$), with no significant difference between NH and HH. In the latter conditions cDO_2 decreased to near baseline values at ~100 kJ while it remained elevated in NN ($p < 0.05$).

3.4 Cardio-respiratory parameters

VO_2 was higher for NN compared to both hypoxic conditions (from 70 to 250 kJ; $p < 0.001$) and trend to be lower for HH compared to NH before the end-spurt (from 140 to 210 kJ; $p = 0.08$) (Figure 3A). No difference was observed between conditions in VO_2 relative to VO_{2max} (Figure 3B). Heart rate was higher at baseline for both hypoxic conditions (78 ± 12 [71–84] and 81 ± 8 [76–85] bpm for NH and HH, respectively) compared to NN (69 ± 11 [62–75] bpm) but no difference was observed during the time-trial (Figure 3C). $P_{ET}CO_2$ at baseline was lower for NH (26.8 ± 2.9 [25.1–28.4] mmHg) than NN (30.6 ± 3.3 [28.7–32.5] mmHg; $p < 0.05$) and HH (29.9 ± 2.5 [28.5–31.4] mmHg; $p < 0.05$), but no statistical difference was reached during the time-trial (Figure 3D). $\dot{V}$E was higher in NH than NN at baseline (15.8 ± 5.2 [12.8–18.8] and 12.8 ± 4.2 [10.4–15.1] $L.min^{-1}$, respectively, $p < 0.05$) but no difference was found between NH and HH at rest or between conditions during exercise (Figure 3E). No difference was found neither at baseline, nor during exercise for Rf (Figure 3F).

3.5 Cerebral oxygenation

These results are presented in Figures 4A–F. PFC and MC HbDiff were higher for NN than for the two hypoxic sessions throughout the time-trial ($p < 0.001$, Figures 4A,B). Lower values were found in HH compared to NH in PFC HbDiff at the beginning of the time-trial (20–120 kJ, $p < 0.05$, Figure 4A), while higher values were observed in HH compared to NH in MC HbDiff in the second part of the time-trial (160–240 kJ, $p < 0.05$, Figure 4B). Exercise induced an increase in cerebral HHb that was lower for NN than the two hypoxic conditions in both PFC and MC ($p < 0.001$, Figures 4C,D). This increase was particularly limited during the first half of the time-trial in NN. During the second half of the time-trial, both MC HbDiff and HHb tended to "plateau". MC HHb was also slightly lower for HH compared to NH in this last part of exercise ($p < 0.05$). Increase in cerebral THb along exercise was similar in the three conditions in PFC (Figure 4E) but was higher for NN in MC during the second half of the time-trial (from 100 kJ onward, $p < 0.001$, Figure 4F) compared to the increase observed in both hypoxic conditions.

3.6 Muscle oxygenation

Muscle HbDiff was lower for NH compared to NN from 20 to 210 kJ ($p < 0.05$) and lower for NN compared to HH from 190 to 240 kJ ($p < 0.05$) (Figure 5A). HbDiff was also lower in NH

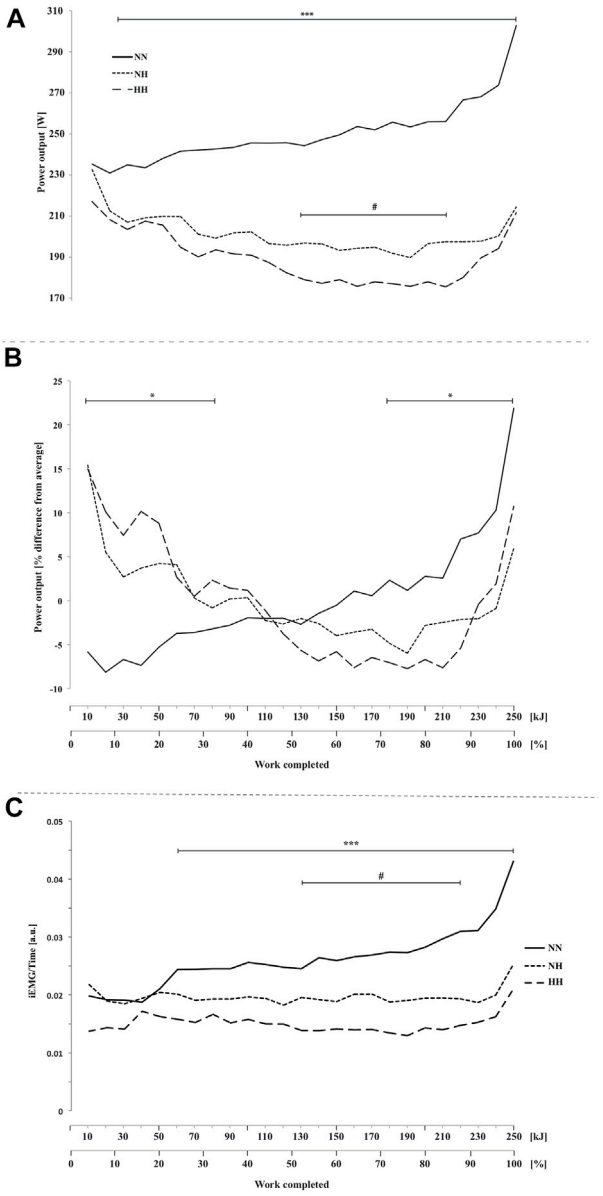

FIGURE 1
Power output **(A)**, normalized power output **(B)** and electromyographic activity (iEMG) measured for the *vastus lateralis* **(C)** during the self-paced 250-kJ time-trial in normobaric normoxia (NN), normobaric hypoxia (NH) and hypobaric hypoxia (HH). *$p < 0.05$, ***$p < 0.001$ for difference between NN and both hypoxic conditions; $^{\#}p < 0.05$ for difference between NH and HH.

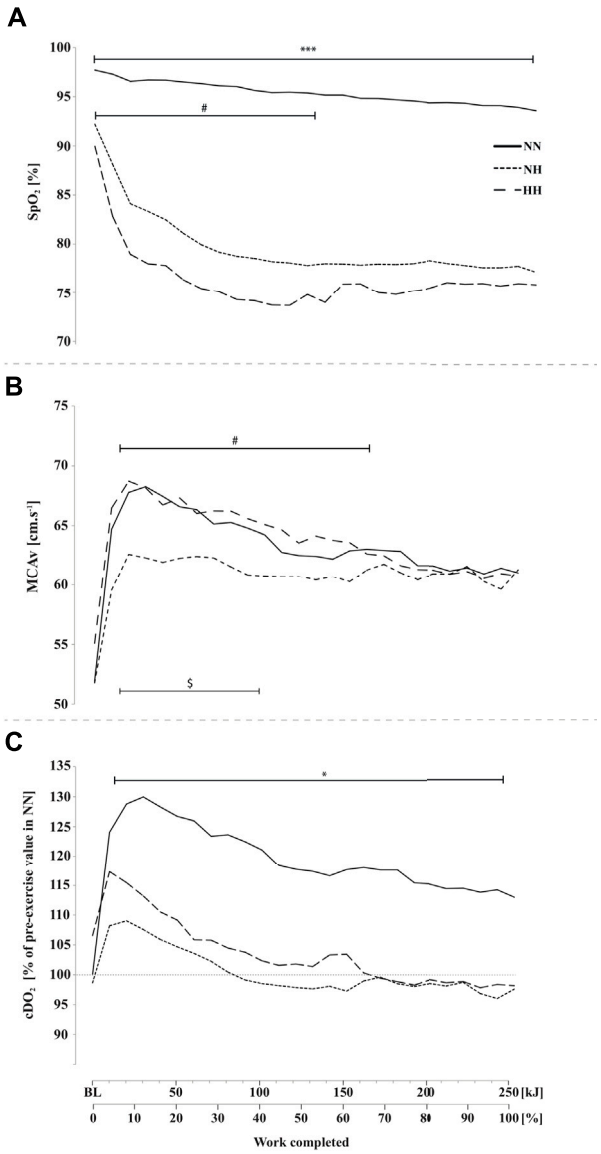

FIGURE 2
Peripheral oxygen saturation (SpO_2, panel **(A)**, middle cerebral artery blood flow mean velocity (MCAv, panel **(B)** and cerebral oxygen delivery cDO_2, panel **(C)** during the self-paced 250-kJ time-trial in normobaric normoxia (NN), normobaric hypoxia (NH) and hypobaric hypoxia (HH). *$p < 0.05$, ***$p < 0.001$ for difference between NN and both hypoxic conditions; $^{\$}p < 0.05$ for difference between NN and NH; $^{\#}p < 0.05$ for difference between NH and HH.

compared to HH from 50 kJ onward ($p < 0.05$). No difference has been found in muscle HHb increase during time-trials between conditions (Figure 5B). Muscle THb was higher in HH compared to both NN and NH conditions from 90 kJ onward ($p < 0.05$) (Figure 5C), with no difference between NN and NH.

3.7 Electromyographic activity

EMG activity was higher for NN compared to both hypoxic conditions from 60 kJ onward (Figure 1C, $p < 0.001$) and it was lower for HH compared to NH from 130 to 210 kJ ($p < 0.05$).

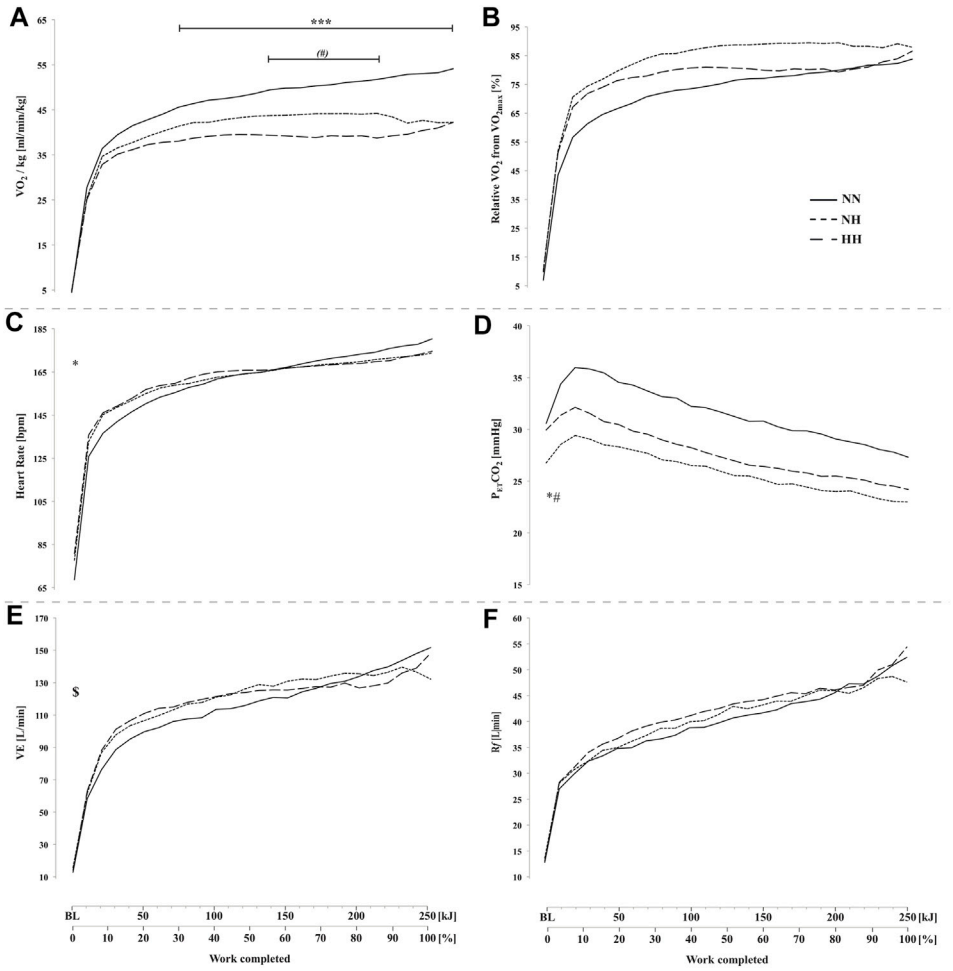

FIGURE 3
Absolute oxygen uptake VO_2, panel **(A)**, oxygen uptake relative to VO_{2max} panel **(B)**, heart rate (panel **(C)**, end-tidal CO_2 pressure ($P_{ET}CO_2$, panel **(D)**, minute ventilation ($\dot{V}$ E, panel **(E)** and respiratory frequency (R*f*, panel **(F)** during the self-paced 250-kj time-trial in normobaric normoxia (NN), normobaric hypoxia (NH) and hypobaric hypoxia (HH). *$p < 0.05$; ***$p < 0.001$ for difference between NN and both hypoxic conditions; $^{\$}p < 0.05$ for difference between NN and NH; $^{(\#)}p = 0.08$; $^{\#}p < 0.05$ for difference between NH and HH.

3.8 Subjective feelings

Perceived exertion (Figure 6A) and legs discomfort (Figure 6B) were not different between conditions along cycling. However, breathing discomfort (Figure 6C) was higher for both hypoxic conditions from the beginning to the end of the time-trial ($p < 0.001$, with no difference between NH and HH. In addition, a biphasic evolution from 0 to 70 kJ (i.e. improvement or plateau) and then from 70 to 250 kJ (i.e. worsening) was observed with a time effect on both legs and breathing discomfort (both $p < 0.001$, Figures 6B,C).

4 Discussion

This study was designed to investigate time-trial performance in conjunction with systemic, muscle and cerebrovascular variables after a 24-h exposure to hypoxia. The major findings were that, for the same pressure in oxygen, there were subtle differences in cerebrovascular regulation during an endurance exercise performed in normobaric *versus* hypobaric hypoxia likely resulting in differences in pacing and total performance. Moreover, there was an inverse and more variable pacing pattern in hypoxia compared to normoxia, in conjunction with depressed cerebrovascular function for a same relative intensity of exercise (*i.e.*, %VO_{2max}, HR, $\dot{V}$ E, VO_2, RPE).

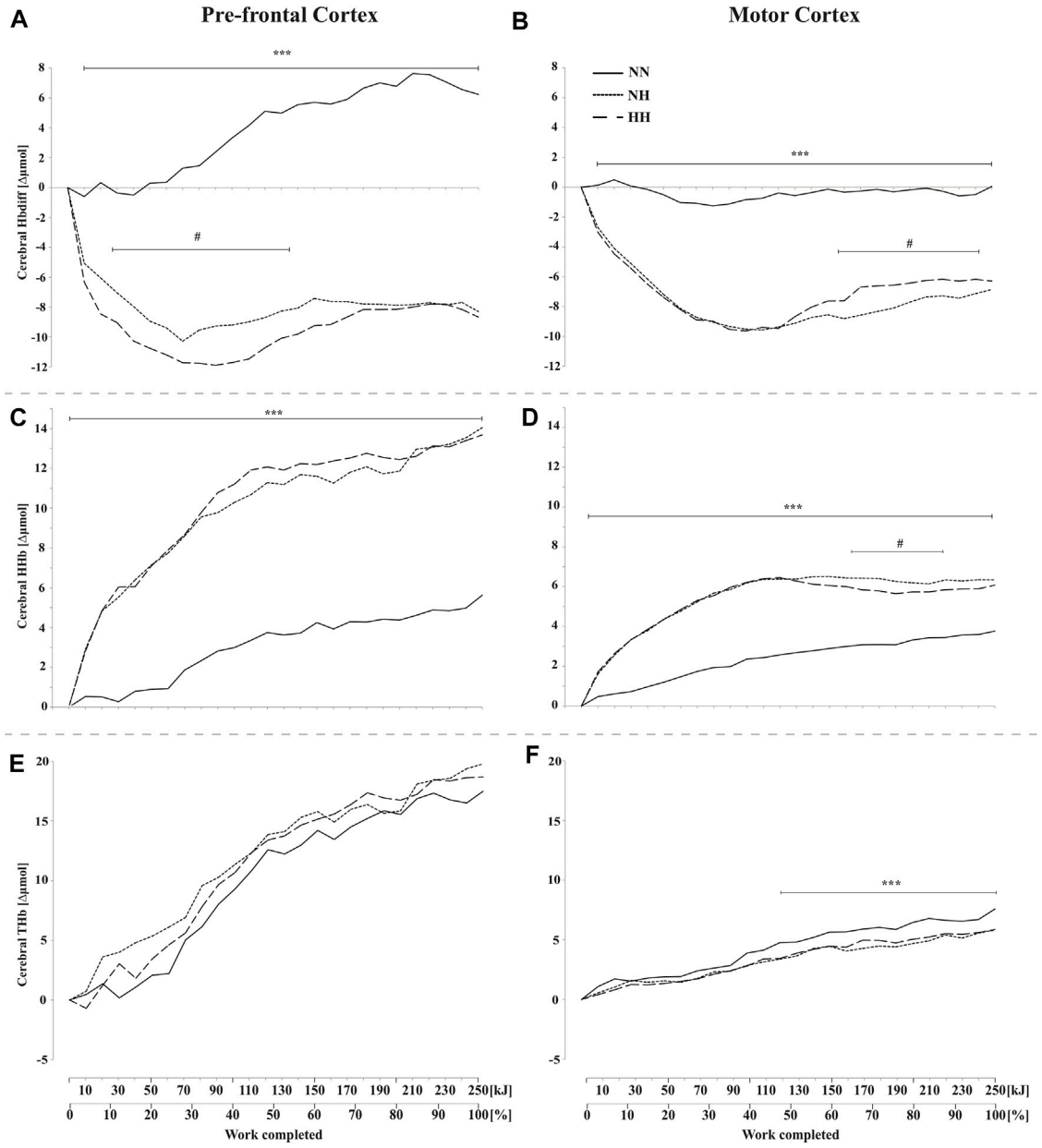

FIGURE 4
Mean changes in cerebral hemoglobin difference (HbDiff = O_2Hb-HHb), deoxy-(HHb), and total-hemoglobin (THb), during the self-paced 250-kJ time-trial in normobaric normoxia (NN), normobaric hypoxia (NH) and hypobaric hypoxia (HH). Data are shown for the prefrontal cortex (PFC, panels **(A,C,E)**) and for the motor cortex (MC, panels **(B,D,F)**). ***$p < 0.001$ for difference between NN and both hypoxic conditions; $^{\#}p < 0.05$ for difference between NH and HH.

Pacing in hypoxia versus normoxia

Hypoxia-induced impairments of aerobic performance have been extensively studied by the past but scarce studies have described how pacing strategy (*i.e.*, self-selected distribution of power output or energetic reserves) may help to understand why we fatigue when oxygen availability becomes challenging.

Due to the experimental design, participants started exercise at the same workload (~226 W) in normoxia and hypoxia but acutely reduced power output until end-spurt in hypoxia whereas a slight positive trend was seen in normoxia over the same period. Variation in power outputs (as a function of mean power during the trial) from the start to 85% of time-trial was twice higher in hypoxia compared to normoxia (~22 and ~11%, respectively).

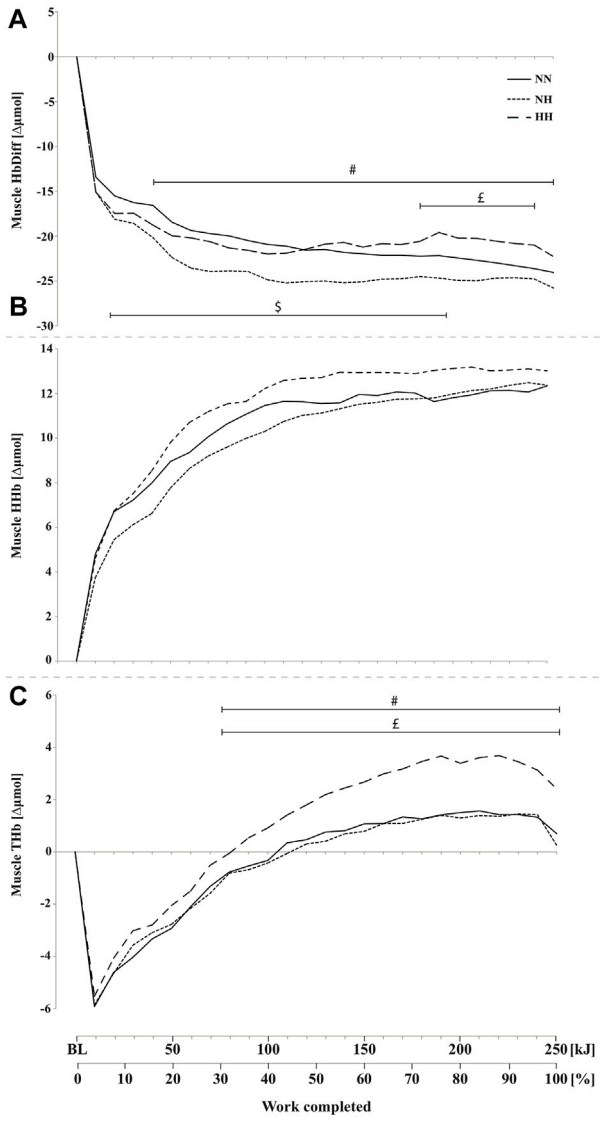

FIGURE 5
Mean changes in muscle hemoglobin difference (HbDiff = O_2Hb-HHb, panel **(A)**, deoxy-(HHb, panel **(B)**, and total-hemoglobin (THb, panel **(C)**, during the self-paced 250-kJ time-trial in normobaric normoxia (NN), normobaric hypoxia (NH) and hypobaric hypoxia (HH) for the *vastus lateralis*. $^{\$}p < 0.05$ for difference between NN and NH; $^{£}p < 0.05$ for difference between NN and HH; $^{\#}p < 0.05$ for difference between NH and HH.

Previous reports examined exclusively time-trials performed after very short exposure to hypoxia, ranging from only 3–40 min (Clark et al., 2007; Fan and Kayser, 2013; Périard and Racinais, 2016) up to 2 h (Beidleman et al., 2014). Sudden and acute hypoxic exposure provides an interesting model that stresses oxygen transport systems but in real-world training/competing (particularly at terrestrial altitude), exposure time preceding exercise is usually longer and is prone to deeply influence how pace would be regulated (Tucker, 2009). We previously showed that the first hours at altitude progressively affect motor cortical excitability (Rupp et al., 2013a) or muscle and cerebral oxygenation (Rupp and Leti, 2013) for instance. In the present experiment, the prooxidant/antioxidant balance became impaired from 10 h in hypoxia (Ribon et al., 2016), hematologic parameters were affected after 20 h in hypoxia (Saugy et al., 2016) and sleep quality was significantly disturbed in hypoxia the night before time-trial (Heinzer et al., 2016). This might, at least partly explain why available evidence suggests that the acute reduction in both maximal aerobic power and endurance performance is maximal within 16–24 h of exposure (Schuler et al., 2007), and resorbs progressively thereafter.

Initial phase. The intensity set at the start of the time-trial in hypoxia led to a rapid decrease in power output over that period (from start to kJ 70), with a 15% decrease in normalized power output compared to a +3% variation only in normoxia (Figure 1A). Interestingly, similar rate of increase in RPE were observed in all conditions in the present study in the first 30% of completed work (0–70 kJ). These RPE were however generated from a very different balance between central drive and afferent feedbacks (physiological adaptations) in normoxia and hypoxia. A wide range of variables can influence pacing but recently, tactical adaptations in the chosen pacing strategies have been incredibly manipulated with the use of central nervous system drugs and selective block of the central projection of ascending sensory pathways (*e.g.*, fentanyl, opioid analgesic) (Amann et al., 2009), so that the understanding of the role of neurophysiological processes has grown.

Cortical representation of muscle involved in cycling is served dominantly by MCA (Jorgensen, Perko and Secher, 1992). Transcranial Doppler can provide quantitative information on cerebral hemodynamic changes at the macrovascular level (*i.e.*, cerebral arteries) but is unable to assess directly the qualitative repercussions of such changes for the tissue at the microvascular level. How much reductions in SpO_2 and cDO_2 translate into changes in cerebral tissue oxygenation when self-paced exercise is performed in hypoxia remains largely unknown. Hence, NIRS is increasingly used to measure the (mis)balance between oxygen supply and utilization directly in tissue micro-vessels (venules, arterioles and capillaries), with a predominant venous contribution (70–80%) (Hamaoka et al., 2007).

We found that the cerebrovascular responses in NN over the first part of exercise appeared appropriate (*e.g.*, cDO_2 maintained at ~125% from baseline value, preserved/increased brain oxygenation), while it is likely that the brain function was threatened in hypoxia over that period from a rapid decrease in SpO_2, cDO_2 (Figures 2A,C) and cerebral oxygenation towards low levels (Figures 3A–D).

Main phase. From 30 to 85% of the time-trial mean power output difference between NN and both hypoxic conditions continued to grow (Figure 1A), still without any difference in

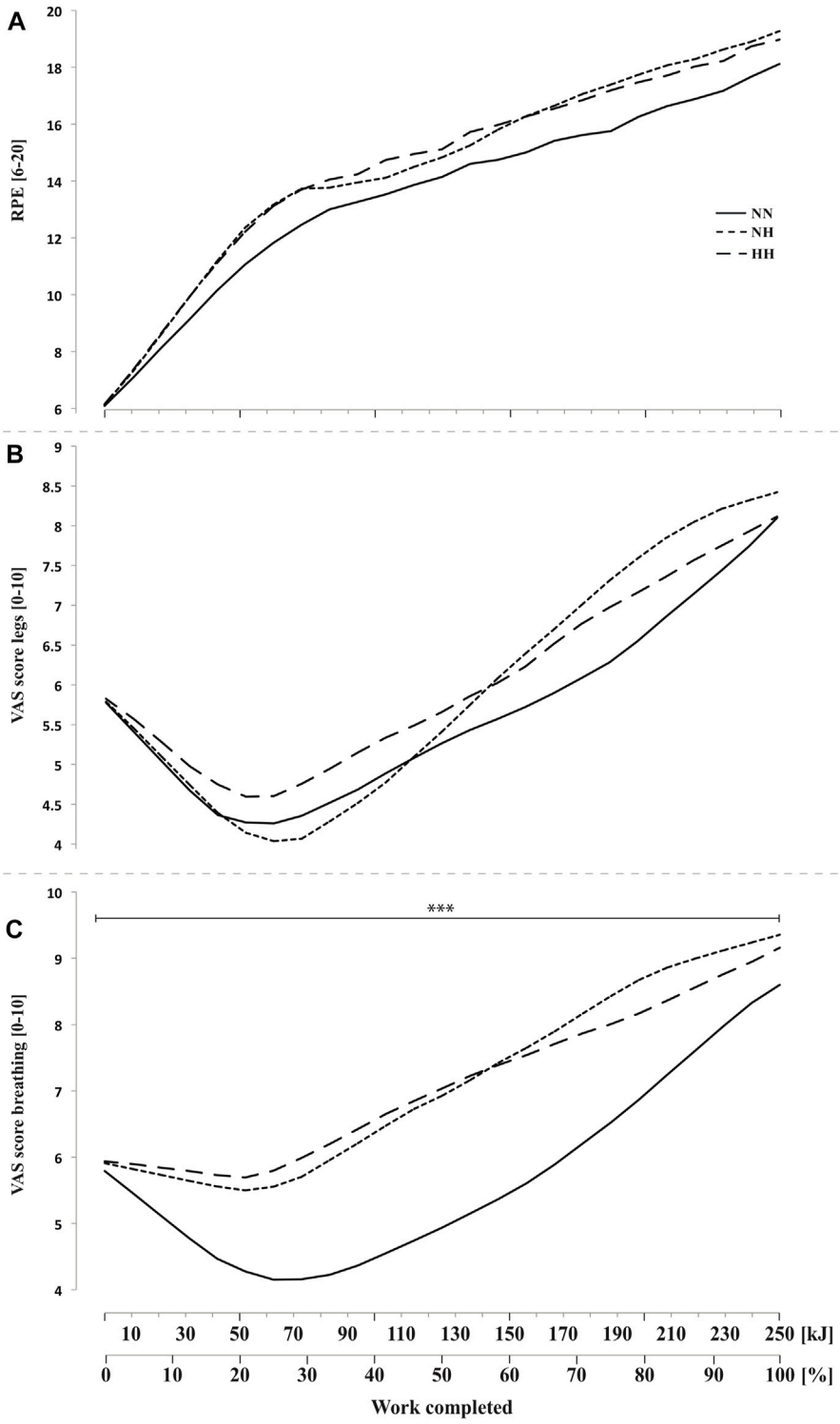

FIGURE 6
Rate of perceived exertion (RPE, panel **(A)** and visual analog scale (VAS) scores for legs feelings (panel **(B)** and breathing discomfort (panel **(C)** during the self-paced 250-kJ time-trial in normobaric normoxia (NN), normobaric hypoxia (NH) and hypobaric hypoxia (HH). ***$p < 0.001$ for difference between NN and both hypoxic conditions.

RPE (Figure 6) and relative exercise intensity as expressed from $\%VO_{2max}$ (Figure 3B) HR (Figure 3C) or ventilation parameters (Figures 3E,F). The lower work-rate in hypoxia was achieved by the alteration in the degree of skeletal muscle recruitment (*i.e.*, lower iEMG) (Figure 1C) in accordance with Peltonen et al. (Peltonen et al., 1997). This is corroborated for the first time by a concomitant significant cerebral hypoperfusion at the micro-vascular level (from THb with NIRS, Figure 4F) over the motor cortex, which directly drives the muscles.

Synthesizing information from a wide range of brain systems and exerting control over cognitive and executive behavior (*e.g.* sensory information integration, decision-making, movement planning, pacing strategies and motivation), PFC associative areas play a central role in the orchestration of thoughts and actions in accordance with internal goals (Ramnani and Owen, 2004) (of particular interest in our study), but the central motor drive is ultimately conducted from the premotor and primary motor areas, which have never been investigated during self-paced exercise before. From the study of Subudhi et al. (Subudhi et al., 2009) it has been often argued that there is a good correlation between prefrontal, premotor and motor cortices oxygenation measurements during exercise. However, this has been shown during a short, maximal incremental exercise, where pacing strategy was minimal as power output was compelled throughout the test. We recently demonstrated that PFC and MC oxygenation profiles can differ during submaximal fatiguing exercise (Rupp et al., 2013b) and to our knowledge the present study is the first to present simultaneous macro-circulation in MCA and both PFC and MC micro-hemodynamics and oxygenation during a self-paced exercise.

Here, PFC and MC oxygenation were both markedly depressed in hypoxia from the start of the time-trial (Figures 4A–D), confirming what has been reported before almost exclusively during progressive maximal exercises in hypoxia and in the PFC (Subudhi et al., 2009; Vogiatzis et al., 2011; Marillier et al., 2021). Presumably, the traditionally-observed exercise-induced increase in cerebral cortex oxygenation reflects progressive increase in oxygen metabolic demand with increased neuronal networks activation. Despite lower central drive and muscle activity during time-trial in hypoxia, it is likely that the challenging environmental conditions (cf., low F_iO_2) blunted the ability of the neurovascular coupling to increase or even maintain cerebral oxygenation to habitual levels aiming at preserving a positive balance between oxygen supply and consumption. An hypothesis might be that power output is rapidly then steadily decreased in hypoxia to prevent the body to be exposed to unacceptable levels of SpO_2, cDO_2 and cerebral oxygenation or, at least, to values that would be considered as incompatible with the remaining expected time before exercise completion. The diminished power output certainly allowed cDO_2 and cerebral hemodynamics not to decrease further (e.g. under baseline values for cDO_2). Of interest is that both PFC and MC HbDiff plateaued in hypoxia during the second half of the time-trial while the rate of HHb increase was lower. On the other hand it is important to stress that PFC and MC oxygenation would be only one of many afferent signals influencing complex regulation of motor drive during self-paced exercise (St Clair Gibson and Noakes, 2004).

In the present study, CBF declined in both NN and HH despite increased *versus* decreased power output, respectively. This adaptation mirrors the $P_{ET}CO_2$ decrease over the same period (Figure 3D) and might thus be triggered by hyperventilation-induced hypocapnia (cf. Figures 3E,F). This assumption is corroborated by previous results who observed a similar decrease in MCAv during a 750-kJ time-trial in normoxia (Périard and Racinais, 2016). Conversely, other results demonstrated a maintain MCAv (in normoxia) or an increased MCAv (and almost maintained cDO_2) in hypoxia during a 15-km time-trial (Fan and Kayser, 2013). To explain these results, authors underlined a higher RPE during the time-trial in hypoxia (5,000 m) and suggested a greater sensorimotor activation compared to normoxia.

End-spurt. In accordance with well-known field observations and as previously described in the literature (Roelands et al., 2009) our results indicated a characteristic end-spurt phenomenon in the last 10–15% of the time-trial. This end-spurt was seen whatever the condition (Figure 1B). Our results confirm that the subjects have the drive and/or motivation to augment power output when approaching the end-point in normoxia but also in hypoxia (what is not the case anymore after administration of a serotonin reuptake inhibitor in normoxia) (Roelands et al., 2009).

Normobaric versus hypobaric hypoxia

When comparing HH and NH, it should be noted that power output at the immediate onset of the time-trial (0–20 kJ, 8%) was similar and also similar to NN, suggesting that the initial selection of work rate was more likely based on previous experience and expectations of exercise duration, rather than on an instantaneous (baseline) afferent input from hypoxemic or disturbed organ/tissue homeostasis (*e.g.*, modified baseline HR, SpO_2, $P_{ET}CO_2$). Indeed, even the knowledge of the condition (no possibility to blind HH) had no influence on the initial power output, and we recall participants had no feedback on their power output throughout the time-trials. However, the tendency towards lower power output in HH in the first part of the time-trial appeared significant in the second part (from ~30% of total work onward). Trends and normalized variation in power output (Figure 1A) were similar in HH and NH throughout the time-trial, but for the first time we were able to identify subtle differences in

underlying physiological adaptations (i.e., cerebrovascular responses) with a potential explanation of the lower performance in HH.

Part of the explanation may arise from distinct baseline status in HH and NH after the 24-h exposure; time-window of particular interest with regards to the early signs of AMS for instance (Subudhi, Panerai and Roach, 2010), which are known to be correlated to the degree of hypoxemia (Fulco et al., 2011). Accordingly, baseline SpO_2 before the time-trial was significantly lower in HH despite similar P_iO_2 (Figure 2A). This might have impacted subjective feelings (e.g. RPE, leg and ventilation discomforts) during exercise so that power output had to be further reduced in HH compared to NH to match an equivalent relative exercise intensity ($\%VO_{2max}$, HR, ...). Besides, underlying mechanisms associated with the same relative exercise intensity showed differences, likely explaining lower power output in HH in the second half (140–220 kJ, corresponding to 55–85%) of the time-trial duration.

Mass oxygen delivery to the brain is dependent on cerebral blood flow (cDO_2 assessed from MCAv in the present study) and on arterial content in oxygen, which is a function of hemoglobin concentration and arterial saturation in oxygen. It seems consistent from the literature to report that the rate of cDO_2 is an important information integrated by the central nervous system to regulate central drive. We demonstrated that cDO_2 was extremely reduced in both HH and NH compared to NN, already in the first part of the time-trial. It is particularly interesting to see how the progressive impairments in power output mirrored the decrease and plateau in cDO_2, conceivably preventing any further decrease under dramatically low values in hypoxia. This was observed in a very similar extent in both HH and NH but we show here for the first time that the comparable cDO_2 levels achieved, resulted from distinct mechanisms in NH and HH (cf. SpO_2-MCAv profiles) and were seen in conjunction with significantly different cerebral oxygenation states in both conditions.

First SpO_2 was significantly lower at rest and during the first half of the time-trial in HH compared to NH, what may explain *1*) the higher hemoconcentration measured before exercise in HH (+6%, (Saugy et al., 2016)) as a compensatory mechanism and, *2*) the higher MCAv values in HH (+7.5% over 10–150 kJ) throughout exercise likely due to a higher hypoxia-induced cerebral vasodilation (Casey and Joyner, 2012). At the same time, exercise-induced decrease in $P_{ET}CO_2$ was approximately the same in NN, NH and HH (-8 mmHg on average over 20–250 kJ with similar $\dot{V}$ E and Rf kinetics, Figure 3D–F), but this decrease started from a significant hypocapnic state only in NH (*e.g.* significantly lower value of $P_{ET}CO_2$ at baseline). Accordingly, MCAv appeared to be much more affected by hypocapnia-induced vasoconstriction in NH compared to HH (Figure 2B). Altogether, cDO_2 was maintained at comparable levels in NH and HH, but likely from significantly lower values of CaO_2 (despite a slight hemoconcentration) and significantly higher values of MCAv in HH.

However, impaired cDO_2 is only part of the equation (*i.e.*, reduction in the ability of CNS to voluntarily activate skeletal muscle, (Goodall, Twomey and Amann, 2014)) and explains mainly the decreased performance in both NH and HH compared to NN. Cerebral oxygenation kinetics described in the present study also help to understand how and why pacing might be more down-regulated in HH compared to NH. From our data, one may speculate that a comparable central drive was produced in HH and NH in the initial part of the time-trial (*e.g.*, same MC deoxygenation, iEMG, power output, muscle deoxygenation, absolute VO_2). However, PFC deoxygenation was significantly higher in HH (Figure 4A) during that period. As cDO_2 was the same in HH and NH along the time-trial, the lower PFC oxygenation may have resulted from higher extraction rate of oxygen (*i.e.*, higher neuronal activity) in this part of the brain. Whatever the explanation (*e.g.*, greater integrative process, higher planning activity), the fact is that lowering power output in HH allowed PFC HbDiff to progressively "restore" to NH levels. Finally, these observations explain similar PFC activity in the second part of the time-trial for lower power output in HH than in NH. Consistently (from 55 to 85% of the total duration, prior end-spurt), the lower power output in HH *vs.* NH was seen in conjunction with a lower degree of muscle recruitment (*i.e.*, lower MC HHb, lower muscle deoxygenation and blood perfusion, lower iEMG).

Methodological considerations

Some limitations inherent to NIRS measurement should be noted. Part of the detected NIR light may be affected by the changes in optical properties of superficial tissue layers between the optode and the investigated tissue (*e.g.* scalp and skull for the brain; skin and fat for the muscle) (Takahashi et al., 2011). We sought to minimize the effects of near-surface blood flow in the observed chromophore concentration changes by controlling room air temperature, by giving attention to ensure NIRS setup to be non-compressive and by using enlarged inter-optode distances to reach the maximal light path providing a sufficient signal-to-noise ratio of the optical density measurements (Rolfe, 2000). Moreover, CBF may be heterogeneously distributed at exercise and under hypoxia (Pagani et al., 2011), we investigated MCA macrocirculation plus multiple sites of interest by NIRS and we acknowledge that observed tissue oxygenation cannot be generalized to whole brain (or to other muscles from NIRS on *vastus lateralis*). Finally, we contend that MCAv is a reliable index of changes in global cerebral blood flow during exercise in normoxia and hypoxia, as the cross-sectional area of the MCA has been shown unchanged within a wide range of changes in $P_{ET}CO_2$ (Valdueza et al., 1997) and in comparable hypoxic situations (<5,000 m) (Poulin and Robbins, 1996).

Conclusion

This study showed that pacing strategy during a cycling time-trial is impaired after 24 h in hypoxia (different trend and higher variability) compared to normoxia and this is likely the result from a compromised ability of the central nervous system to voluntarily activate skeletal muscles, owing to inadequate oxygen delivery to the brain. With simultaneous multi-systemic parameters, cerebrovascular function, muscle activity and subjective feelings, light is shed on tissue-specific adaptations and new insights are provided into the mechanisms underlying the higher pacing down-regulation in hypobaric *versus* normobaric hypoxia. Despite equivalent PiO_2, HH is a more stressful stimulus than NH (*e.g.*, lower SpO_2 and higher PFC deoxygenation for a given power output during the first half) and suggests a higher "adaptive cost" (same RPE, leg and ventilation discomforts for a lower power output during the second half). As a consequence, aiming to maintain an equivalent oxygen delivery to the brain, the system likely adopts a more "protective" strategy, leading to a further impaired performance in HH. In addition, same relative exercise intensity and physiological disturbances were achieved in HH from the production of lower absolute power when compared to NH, emphasizing once more time that exercise at terrestrial and simulated altitude cannot be carelessly interchanged.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Commission Cantonale Valaisanne d'Ethique Medicale, CCVEM; Agreement 051/09. The patients/participants provided their written informed consent to participate in this study.

Author contributions

TR, JS and GM designed the protocol. TR, JS and NB collected the data. TR and JS performed the data analysis with close support from NB. TR, JS and GM prepared the figures and wrote the manuscript. TR, JS, NB and GM reviewed the manuscript, approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

This study was funded by grants from Bundes Amt für Sport (BASPO; Switzerland). The authors have no conflict of interest, source of funding or financial ties to disclose and no current or past relationship with companies or manufacturers who could benefit from the results of the present study.

Acknowledgments

The authors thank Alexandre Lamon and Rémi Favre for their technical support. The authors declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.893872/full#supplementary-material

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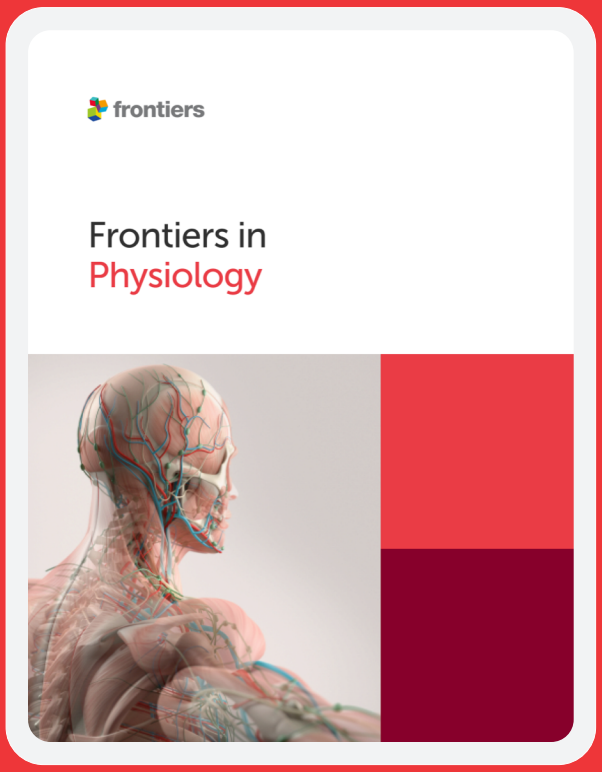

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