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Cold, heat and hypoxia as a medical tool: The use in a healthy and diseased population

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Editorial: Cold, heat and hypoxia as a medical tool: the use in a healthy and diseased population

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Editorial on the Research Topic
Cold, heat and hypoxia as a medical tool: the use in a healthy and diseased population

1 Introduction

In the complex interplay between humanity and the environment, the profound effects of environmental stressors on human health unfold as an evolving narrative. In recent years, scientific exploration has increasingly focused on the potential advantages of intentional exposures to environmental stressors, such as cold, heat, and hypoxia (Brunt and Minson, 2021; Allan et al., 2022; Burtscher et al., 2023). The recognition of these stressors as effective tools for enhancing health and wellbeing might mark the onset of a new era in therapeutic possibilities.

The appeal lies in the concept of hormesis, where controlled exposure to mild stressors triggers adaptive responses within the body, leading to increased resilience and improved physiological function. Cold therapies, encompassing practices like ice baths and cryotherapy, hold the promise of anti-inflammatory effects and expedited physical recovery (Hohenauer et al., 2015; Garcia et al., 2021). Heat therapy, manifested through saunas and hot-water immersion, is acclaimed for its cardiovascular benefits and potential cognitive enhancements (Von Schulze et al., 2020; Brunt and Minson, 2021). Hypoxic training, mimicking conditions at high altitudes, has emerged as a strategy to bolster endurance and optimize oxygen utilization (Huang et al., 2023).

However, as we navigate the promising terrain of environmental stressors as therapeutic tools, we must also grapple with the intricacies of their physiological consequences. Exposure to extreme temperatures and diminished oxygen levels requires a nuanced understanding how to induce beneficial, stress-induced adaptations rather than potential harms.

This scientific article Research Topic aims to explore the two-sided aspects of environmental stressors, investigating their potentials to positively impact human health and the physiological complexities that determine the balance between

intended therapeutic effects and unintended outcomes. This Research Topic presents current scientific findings, describing the effects of these stressors on various aspects of human physiology. In this endeavor, the current Research Topic provides evidence-based knowledge that accurately exploits the potential benefits of environmental stressors and recognizes the complex interplay between these stressors on the physiological system of our body.

2 Research Topic contributions

This compilation of research articles provides insights into diverse physiological phenomena, spanning from the impact of hypoxic exposure on asthmatics to the effects of cold-water immersion on cardiovascular health. Through investigations into high-altitude adaptability, glucose regulation during exercise, recovery strategies, and more, these studies contribute significantly to our understanding of human physiology across diverse conditions.

Saxer et al. explored acute and subacute effects of hypobaric hypoxic exposures on individuals with mild asthma, undergoing a 3-week rehabilitation program at 3,100 m. Pulmonary artery pressure increased with acute altitude exposure, accompanied by elevated pulmonary vascular resistance, heart rate, and decreased oxygen saturation. Extravascular lung water increased acutely but returned to baseline after 3 weeks. Tee et al. studied glucose regulation in overweight adults during low-intensity exercise under normoxia, moderate hypoxia, and high hypoxia. Post-exercise glucose and insulin responses were lower in moderate hypoxia, suggesting it as an effective stimulus for glucose regulation without excessive stress. Heart rate increased in high hypoxia, indicating physiological strain. Liu et al. identified urine biomarkers for high-altitude adaptability and stamina. High-stamina individuals showed elevated white blood cell counts and specific urine protein expressions. The study introduced potential biomarkers for screening individuals adapting to high altitudes with sustained endurance. Kagelman et al. investigated peripheral skin cooling as a countermeasure during hyper-gravity exposure. Peripheral skin cooling showed no significant impact on hemodynamics, suggesting potential limitations or insufficient cooling. Honorato et al. explored the impact of hypobaric hypoxia during flight on cardiac autonomic function. Hypoxia decreased heart rate variability, indicating sensitivity to hypoxic conditions. Wang and Hurr studied an analgesic cream's effects during temperate-water immersion for exercise-induced hyperthermia. The cream enhanced cooling effects, demonstrated by increased cutaneous vascular conductance and core body heat loss, providing a cost-effective means for improved cooling during exercise-induced hyperthermia. Geng et al. investigated oxygen uptake and deoxyhemoglobin changes in athletes during incremental exhaustive exercise under various conditions. Hypoxia and high temperature decreased exercise capacity, suggesting potential factors contributing to peripheral fatigue under different environmental conditions. Jackman et al. explored acute physiological responses to hot-water immersion following resistance exercise. Hot-water immersion increased intramuscular temperature, suggesting its viability for heat therapy, but did not significantly impact muscle function or soreness. Treigyte et al. examined the effects of cold-water immersions on muscle force and contractility during and after electrically induced contractions. Intermittent/prolonged immersions induced a less pronounced contractile transition, potentially due to reduced vasoconstriction response and enhanced blood perfusion during immersion. Versteeg et al. investigated the effects of a 3-week repeated cold-water immersion on leukocyte counts and cardiovascular factors in healthy men. While leukocyte counts decreased in both cold-water immersion and the control group, cardiovascular factors showed reductions only in the cold-water group.

3 Perspective

In summary, these studies provide insights into the physiological responses under various conditions, offering valuable information for optimizing interventions and countermeasures in situations such as high-altitude exposure, exercise-induced hyperthermia, and hypoxic environments, leading to promising paths for future research.

Based on the findings from Saxer et al. further investigations into hypobaric hypoxic exposures into sustained adaptability in individuals with mild asthma are needed. Tee et al.'s findings on glucose regulation under varying hypoxic conditions invite exploration into molecular mechanisms, particularly the benefits of moderate hypoxia for metabolic health. Liu et al.'s identification of urine biomarkers introduces avenues for personalized screening in high-altitude adaptation. In the study from Kagelmann et al., the exploration of peripheral skin cooling during hyper-gravity exposure highlights the need for optimal cooling strategies. Honorato et al. suggests monitoring heart rate variability in future research for enhanced hypoxia detection during flights. Wang and Hurr's insights into an analgesic cream's efficacy in exercise-induced hyperthermia present cost-effective cooling options. The need for future investigations regarding the molecular mechanisms that underlie the counteractive vasodilatory effect of analgesic creams was highlighted in this study. The study results from Geng et al. regarding oxygen uptake calls for further exploration into peripheral fatigue factors under diverse environmental conditions. Jackman et al.'s exploration of hot-water immersion for heat therapy prompts considerations of its long-term impact on muscle function. Treigyte et al.'s study on cold-water immersions hints at the potential role of sex differences that should be considered in future research. In the study from Versteeg et al., future studies should incorporate larger sample sizes to investigate the true effects of repeated cold-water immersions on immune and cardiovascular factors.

In summary, the included studies in this Research Topic significantly advance our understanding of the effects of cold, heat, and hypoxic exposures. The investigations provide valuable insights into various aspects of the interventions, demonstrating the potential benefits and mechanisms. In conclusion, while these studies have greatly enhanced our physiological knowledge, they also emphasize the ongoing need for additional research in this area to unravel the physiological impact of these environmental stressors to reach definite outcomes.

Author contributions

EH: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. SR: Writing–original draft, Writing–review and editing. RC: Writing–original draft, Writing–review and editing.

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Could cardiac autonomic modulation be an objective method to identify hypobaric hypoxia symptoms at 25.000ft among Brazilian military airmen?

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Hypobaric hypoxia during a flight can cause accidents, resulting in deaths. Heart rate variability may be more sensitive than self-reported hypoxia symptoms to the effects of HH. The level of physical fitness can contribute to efficient cardiac autonomic modulation. However, no studies have examined the association between fitness, heart rate variability, and the time of onset of hypobaric hypoxia symptoms. To analyze the influence of hypobaric hypoxia on cardiac autonomic function at the time of onset of the first symptoms and its association with physical fitness. Male airmen trained and belonging to the staff of the Brazilian Air Force (n = 23; 30 ± 6.7 years) participated in a flight simulation in a 25.000 ft hypobaric chamber. Heart rate variability was recorded with a Polar® cardiac monitor. Data were analyzed in the time-domain method using Kubios software. We evaluated pulse oximetry with the Mindray PM-60 oximeter. Physical fitness assessment test results were collected from the archive. At moments rest vs. hypoxia revealed a decrease in heart rate variability indices iRR and RMSSD (p < 0.001). The individual analysis of hypoxia-rest variation showed that 100% of the airmen had a negative delta for both iRR and RMSSD indices. The time of onset of hypoxia symptoms was not associated with body composition, physical fitness, oxygen saturation, and HRV indices. Also, we suggest that cardiac autonomic modulation seems to be more sensitive to the effects of hypobaric hypoxia at 25.000 ft than the self-reported subjective perception of symptoms. Further devices that alert to a hypoxic condition during a flight should consider heart rate variability allowing more time and security to reestablish control of the flight.

KEYWORDS

hypoxia, altitude, aerospace medicine, physical exercise, heart rate variability, flight safety

1 Introduction

The environmental characteristics of flight (e.g., low partial pressure of oxygen due to high altitude) pose numerous challenges to physiological homeostasis. Bouak et al. (2018). The occurrence of hypobaric hypoxia (HH) is one of the most serious risks during a flight, as its effects can affect the cognitive and psychomotor capacity of the airmen, directly compromising piloting (Rice et al., 2019) and flight performance (Steinman et al., 2017), and increasing the risk of incidents and accidents that can result in death (Files et al., 2005).

The human being submitted to the condition of HH is subject to a reduction in the oxygen (O_2) offered to corporal tissues by the arterial blood due to the reduction in the partial pressure of O_2 in the atmospheric air (de Carvalho et al., 2018; Russomano and Castro, 2020). In an attempt to reestablish physiological homeostasis, the autonomic nervous system acts through biofeedback, promoting the physiological adjustments necessary to provide O_2 to the tissues, such as an increase in heart rate and blood flow (Hawley et al., 2014; Russomano and Castro, 2020).

In this sense, several studies have evaluated the regulation/action of the cardiac autonomic system during HH aiming to guide the best physical preparation and training of airmen so that they can (if necessary) go through this physiological stress without compromising their physical integrity, and ensuring flight safety (Barak et al., 1999; Vigo et al., 2010).

Flight training in a hypobaric hypoxia simulation chamber aims to provide airmen with the perception of the physiological symptoms induced by HH and instructions on risk management for performing the necessary life support procedures, such as: supplementing with O_2 through the mask, reestablishing pressurization of the aircraft, and/or reducing the altitude of the aircraft (Brazil, 2017). In this sense, methods that objectively report the physiological symptoms can serve as confirmation of these symptoms and alert to the occurrence of HH. A non-invasive instrument, providing simple measurement and handling, easy access, and low cost, which could be an important tool in this process, is heart rate variability (HRV).

Studies have used HRV as a tool to assess cardiac autonomic modulation (Freeman and Chapleau, 2013; Taralov et al., 2015), in addition to being efficient to quantitatively report the adjustments in the cardiac autonomic system induced by HH (Barak et al., 1999; Vigo et al., 2010; Zhang et al., 2014; Zhang et al., 2015). It is important to emphasize that the manifestation of these physiological adjustments varies according to the individual and, mainly, with the severity of the hypoxia, that is, exposure time and elevation of altitude (de Carvalho, 2015; Russomano and Castro, 2020).

Furthermore, in hypoxia flight simulations in the hypobaric chamber, the time of useful consciousness (TUC) was studied, that is, the time lapse between the loss of O_2 and the maintenance of the user awareness of the airmen inserted in this environment (Izraeli et al., 1988; Yoneda et al., 2000), commonly assessed by cognitive methods (Rice et al., 2019). Nonetheless, considering the risks to airmen's health and physical integrity and the fact that this training aims to experience hypobaric hypoxia symptoms, TUC was replaced. Nowadays, to guarantee the physical integrity of the airmen, the time of onset of symptoms (TAS) is currently used in training in HH, that is, the time lapse between the loss of the O_2 supply and the moment when the airman notices the first symptoms of the physiological change induced by HH, and reports them to the instruction team (Brazil, 2017). Among the self-reported perceptions are vision changes, slow thinking, limb tingling, headache, and euphoria (Brazil, 2017).

Thus, considering that the TAS time-lapse is shorter than the TUC (commonly used in studies), that HH induces changes in cardiac autonomic function, and that, to date, studies have verified cardiac autonomic changes only in TUC (Barak et al., 1999; Vigo et al., 2010), it is plausible to infer that HRV could be an instrument for confirming the physiological effects of HH during TAS. This would enable the perception of the first symptoms of HH to be confirmed and quantified through HRV indices, optimizing the reaction time in the resumption of flight safety before the proximity of the stage of loss of useful consciousness.

Another important aspect of the training of airmen in the military environment is physical fitness. Therefore, airmen are encouraged to practice physical exercises and are regularly submitted to the Physical Conditioning Assessment Test (TACF), for which a minimum level of physical fitness is required (Brazil, 2011). Furthermore, it has been shown that good physical fitness is related to good balance in cardiac autonomic activity (De Meersman, 1993). This fact is possibly justified due to the consensus of the scientific literature on the cardioprotective effect and positive influence on cardiac autonomic function conferred by physical exercise (Kwon et al., 2014; Carter and Ray, 2015). The numerous benefits conferred by physical exercise to its practitioners, in general, are unquestionable (Hawley et al., 2014; de Sousa et al., 2017; Simoes et al., 2017; Deus et al., 2019; Correa et al., 2021) and those which can help airmen in the exercise of flight safety, by improving tissue resistance to hypoxia are also highlighted (Brown and Moore, 2007; Lalonde et al., 2015; Quindry, 2017). It is reasonable to infer that physical exercise practitioners will have better physiological conditions of response to stressor events such HH.

Finally, it is conjectured that airmen with better physical fitness and better autonomic balance, quantified by HRV, will withstand the effects of HH for a longer time. However, as far as we know, no studies have verified the relationship between the physical fitness of airmen and the time of resistance to the effects of HH, marked by the appearance of the first symptoms (TAS). Thus, it is extremely important to assess whether the minimum physical fitness required of airmen in the Brazilian Air Force can help them to mitigate the effects of HH on 25.000 ft.

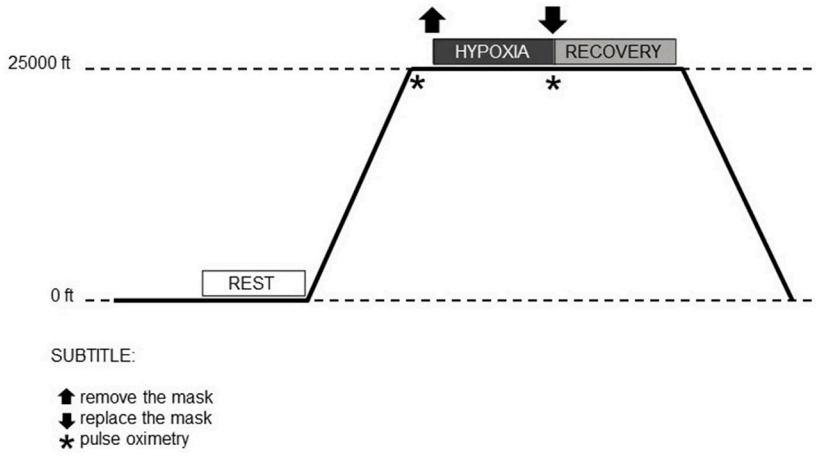

FIGURE 1
Moments of the flight simulation in the hypobaric chamber.

Therefore, the present study aimed to analyze the influence of HH on cardiac autonomic function during TAS, and its association with physical fitness in Brazilian Air Force airmen.

2 Methods

The methods and procedures were approved by the Institute of Aerospace Medicine (IMAE), COMAER protocol nº 67442.003040/2019-01 and by the local Research Ethics Committee under CAAE protocol nº 23003519.80000.0029. The methods and procedures were performed according to the guidelines of the Brazilian Air Force Command in the Physiological Adaptation Stage (EAF) (Brazil, 2017).

All procedures were clearly explained to the airmen, and those who met the requirements of the Institute of Aerospace Medicine and who agreed to participate in the study, signed the Consent Term.

2.1 Sample

Airmen (n = 26) were evaluated for inclusion and exclusion criteria. The inclusion criteria were: 1) members of the Brazilian Air Force who participated in the flight simulation in the hypobaric chamber of the quadrennium corresponding to October and November 2019; 2) airlift pilot; 3) have the health card issued by the Special Health Board valid and without restrictions for air activity; 4) receive a favorable opinion from the physician before starting the flight simulation in the hypobaric chamber; 5) not present joint pain, pneumothorax, bronchitis, convulsive crisis, anemia, cold, infection, joint trauma; 6) not have done any diving in the 48 h before the flight simulation and/or dermatological and/or dental treatment; 5) not be a smoker or be using any type of medication. Exclusions criteria were having otalgia, aerocolia, or decompression illness during the analysis of risk factors for middle ear barotrauma. Among the airmen, three were excluded according to the exclusion criteria and the remainder were included in the final analysis (n = 23).

2.2 Hypobaric flight chamber

Before the flight simulation, participants ate a single standard meal. The flight simulation protocol adopted in the hypobaric chamber was similar to the standard proposed by the Civil Aerospace Medicinal Institute (CAMI) of the Federal Aviation Administration. A hypobaric chamber measuring 7.3 m× 2.7 m× 2.4 m, with a capacity of 16 individuals was used, from the Institute of Aerospace Medicine (IMAE), located in Rio de Janeiro.

The flight simulation was divided into three stages: 1) denitrogenation; 2) ascent to 25,000 ft (7620 m) to demonstrate acute hypoxia; and 3) return to sea level, as illustrated in Figure 1.

The airmen remained seated inside the hypobaric chamber, equipped with an aviation helmet (model HGU-55 CE—Gentex Corporation®, Inc., PA, United States), naval aviation O_2 face mask (standard model MBU-12, Gentex Corporation®, Inc., PA, United States), heart monitor, and pulse oximeter, together with the medical team. Initially, at sea level, the airmen breathed 100% O_2 for approximately 30 min. This procedure aims to minimize the risk of decompression sickness and barotrauma due to body nitrogen concentrations.

Subsequently, the hypobaric chamber simulated ascent to an altitude of 25,000 ft (7620 m), at which point the airmen removed the O_2 face aviation mask and kept it off until the first symptoms of hypoxia were identified (e.g., vision impairment, mental fatigue, shortness of breath, headaches). At the time of exposure to hypobaric hypoxia, the symptoms and their identification were individual. After individual and self-reported recognition of early symptoms (TAS), the airmen resumed O_2 supplementation (100%) by putting the O_2 face mask back on and activating the emergency breathing device.

2.3 Assessment of cardiac autonomic function-HRV

To acquire the RR intervals (iRR), the Polar Team 2 (Polar Heart Rate Monitor®) was used. Before starting the flight simulation, the heart rate monitor belt (Model H10- Polar Electro, Inc, NY, United States) was moistened and positioned on the chest, according to the manufacturer's instructions. The airmen then remained comfortably seated inside the hypobaric chamber with their eyes open and breathing spontaneously. The iRRs were recorded during the hypobaric chamber flight simulation.

For the analysis of cardiac autonomic behavior, the following moments were considered: Rest: at sea level, in the final 2 min and 30 s of the 30 min during which the airmen breathed 100% O_2. Hypoxia: at 25,000 ft, the time lapse between the O_2 mask removal and its replacement after the first individually identified symptoms of hypoxia, with variable time. Recovery: at 25,000 ft, the first 2 min and 30 s from the O_2 mask replacement. The interval time (2m30s) was the maximum time reached during hypoxia, so, in order to match the analysis times between the moments, this time was chosen for all moments.

The data were instantly transmitted to the computer *via* the Polar Team 2® software. HRV analysis was performed using Kubios software (version 2.2 Biosignal Analysis and Medical Imaging Group, Kuopio, Finland). To correct the artifacts, the evaluators used the Kubios automatic filter (medium error correction). In addition, visual inspection of the graphs was carried out by two experienced researchers.

HRV was analyzed in the time domain, including the following indices: R-R (ms)—Average of R-R intervals in milliseconds. SDNN (ms)—Standard deviation of RR intervals in milliseconds. HR (bpm)—Mean heart rate in beats per minute. RMSSD (ms) square root of successive differences between R-R intervals in milliseconds. Also, in the non-linear domain, including the indices: SD1—The standard deviation of the Poincaré plot perpendicular to the identification line. SD2—The standard deviation of the Poincaré plot along the identification line.

2.4 Pulse oximetry analysis

During the entire hypobaric flight simulation, the airmen remained connected to the Mindray PM-60 O_2 saturation sensors (Mindray Bio-Medical Electronics Co, Shenzhen, China) positioned on the second distal phalanx of the non-dominant hand to verify the saturation percentage. (%S O_2). O_2 saturation (S O_2) measurements were evaluated at rest and at the end of the hypoxic period.

2.5 Anthropometric and body composition data

Bodyweight (Filizola®, São Paulo, Brazil) and height (Sanny® Stadiometer) were evaluated following previously published methods (Quetelet, 1994; Keys et al., 2014), before the flight simulation in a hypobaric chamber. The body mass index (BMI) was calculated as kg/m^2 (Quetelet, 1994; Keys et al., 2014). Body fat was estimated using the three skinfold protocol proposed by Jackson and Pollok (Jackson and Pollock, 1978), performed in duplicate using a skinfold caliper (Lange®, Cambridge Scientific Instruments, Maryland, United States). Body density was calculated and converted to body fat percentage using the equation reported by Siri (Siri, 1993).

2.6 Physical Conditioning Assessment Tests

To verify the physical performance of the airmen, twice a year they are obligatorily submitted to physical conditioning evaluation tests. As the cardiorespiratory fitness assessment and neuromuscular tests (push-ups on the ground and trunk flexion on the thighs) were performed 1 month before (September 2019) the flight simulation, a documentary analysis of the results archieved by the airmen was carried out.

2.6.1 VO_2 submaximal

The values of $VO_{2submaximal}$ ($ml.kg^{-1}.min^{-1}$) were obtained following the recommendations of the Cooper test (Cooper, 1968). Airmen were required to run/walk for 12 min on an official athletics track (400 m), covering the greatest possible distance. The distance traveled in meters was then applied to the equation: $VO_{2submaximal}$ = distance traveled in meters—504.9/44.73 (Cooper, 1968).

2.6.2 Push-ups on the ground

In the ventral decubitus position, with hands supported in front on the floor and the shoulders slightly apart about the projection, keeping the body fully extended. The upper limbs are then flexed so that the back exceeds the line of the elbows,

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TABLE 1 Sample characterization and physical assessment results.

Variables	Mean (n = 23)	SD
Anthropometry and body composition		
Age (years)	30.70	±6.70
Body Mass (kg)	83.41	±7.34
Height (m)	1.76	±0.05
Body mass index—BMI (kg/m^2)	26.91	±2.05
Waist circumference (cm)	87.10	±4.35
Waist-to-height ratio—RCE	0.49	±0.02
Body fat (%)	16.91	±4.55
Fat free mass (%)	83.08	±4.55
Fat mass (kg)	14.18	±4.27
Fat free mass (kg)	69.24	±6.38
Physical fitness tests		
Ground push-up (reps)	38.61	±8.14
Trunk flexion (reps)	49.13	±9.21
Distance covered—Cooper 12' (m)	2390.52	±244.28
$VO_{2\ submaximal}$	41.92	±5.43
O_2 saturation		
Initial O_2 saturation (%)	99.61	±0.65
Final O_2 saturation (%)	80.96	±9.45
O_2 saturation delta	18.65	±9.44
TAS	1m40s	±28 ms

and the elbows are projected out approximately 45° to the trunk. Upon reaching this position, the upper limbs are extended, returning to the starting position. The maximum number of repetitions performed without a Pollock adapted time limit was counted (Michael L. Pollock and Wilmore, 1993; Riebe et al., 2018).

2.6.3 Flexion of the trunk on the thighs

Lying in dorsal decubitus, hands crossed at the chest at shoulder height, knees at a 90° angle, feet aligned and fixed with the help of the evaluator. The trunk is then flexed until the elbows touch the distal third of the thighs, returning to the starting position. The maximum number of repetitions performed in 1 min was counted. (Michael L. Pollock and Wilmore, 1993; Riebe et al., 2018).

2.7 Statistical analysis

A posteriori sample size of 23 participants provided a statistical power of 93% (1-β = 0.93), for a significance level of 5% (α = 0.05) and large effect size (f = 0.80). Data normality was assessed using the Shapiro-Wilk test. The significance level adopted was 5% ($p < 0.05$). The variables related to HRV indices showed non-parametric distribution. To compare the values of heart rate variability indices at rest, during hypoxia and recovery, the Kruskal-Wallis test was applied, followed by Dunn's post-test. Data are expressed as mean and standard deviation. For the TAS variables, final O_2 saturation, O_2 saturation delta, weight (kg), height (m), BMI (kg/m2), waist-to-height ratio (RCE), fat percentage, lean mass percentage, fat in kg, fat-free mass in kg, distance covered, and $VO_{2submaximal}$, a descriptive analysis was performed, and data are expressed as mean and standard deviation. Finally, the association between the variables was performed using the Wilcoxon paired difference test. Statistical analyses were performed using G*Power (v3.1), SPSS 21 (IBM, SPSS Statistics® Inc., Illinois, United States), and GraphPad Prism (v6.0).

3 Results

Anthropometric characteristics, body composition, results of physical fitness tests, and O_2 saturation of the airmen are described in Table 1.

Among the first symptoms self-reported by the airmen, there was a frequency of 4% euphoria, 4% headache, 9% tingling in the limbs, 26% slow thinking, and 57% vision alteration.

Comparison of mean values of HRV indices during rest, hypoxia, and recovery are illustrated in Figure 2.

In the comparison between hypoxia vs resting, the values of iRR, RMSSD, NN50 and SD1 indices decreased ($p < 0.0001$). The SDNN and SD2 indices did not change. In the comparisons between recovery vs resting, the value of the iRR decreased ($p < 0.0001$). The SDNN indices increased ($p < 0.0001$). The RMSSD and SD1 indices did not change in the comparisons between recovery vs hypoxia, the iRR, SDNN, RMSSD, SD1 and SD2 indices increased ($p < 0.0001$), and only the HR decreased ($p < 0.0001$).

Visual and individual analysis of hypoxia-rest variation showed that 100% of the airmen had a negative delta for the mean HR, iRR and RMSSD indices. An example of the graphical behavior of iRR is illustrated in Supplementary Figure S1—SF1. From the visual inspection, it was possible to perceive a decrease in the length and amplitude of the RR intervals and the loss of linearity during hypoxia.

We stratified the airmen into "high-HRV" and "low-HRV" considering the rest HRV values in ascending order and compared the values of HRV indices between these groups at rest, hypoxia, and recovery times. See Table 2.

At rest and recovery times, the cardiac autonomic behavior of airmen classified as "high-HRV" differed from those who presented "low-HRV" ($p < 0.000$), except for the SD-HR index. During hypoxia, the iRR ($p = 0.118$), SDNN ($p = 0.151$), Mean HR ($p = 0.134$), and SD2 ($p = 0.151$) indices did not differ between airmen classified as high-HRV when compared to airmen with low-HRV. However, for the representative indices of the parasympathetic activity, RMSSD

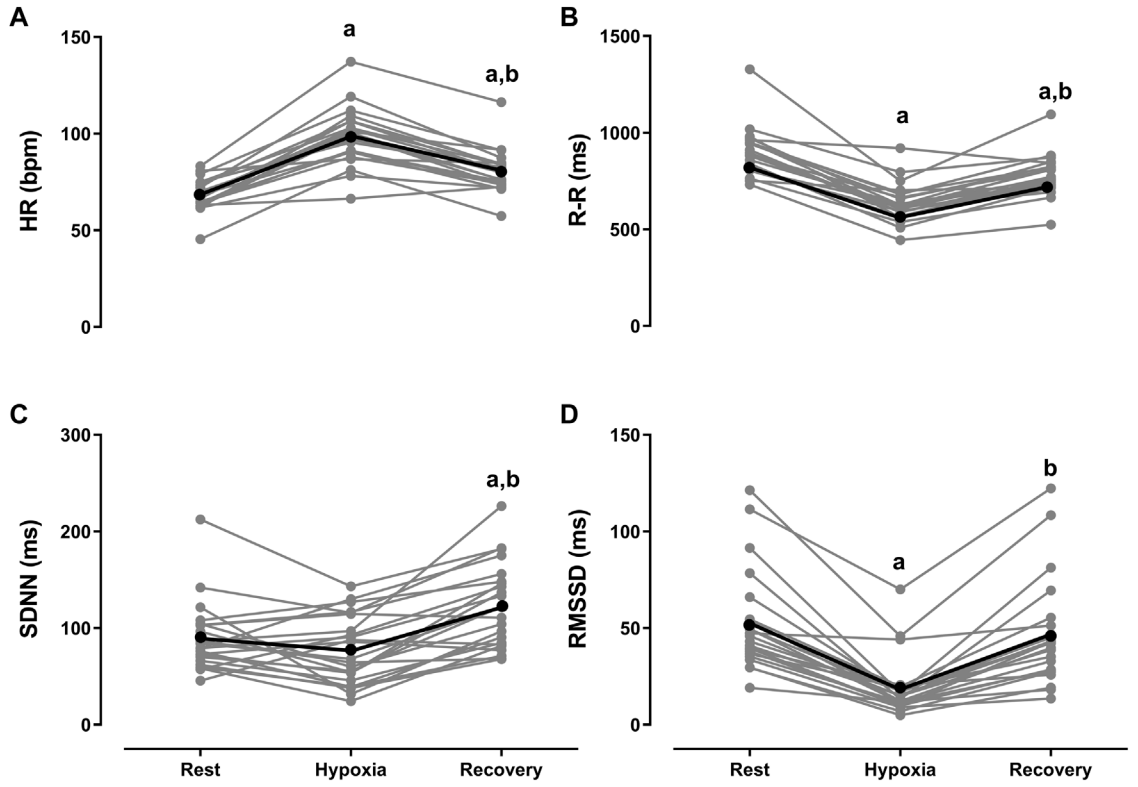

FIGURE 2
Kruskal-Wallis test followed by Dunn's post-test for multiple comparisons. Data expressed as mean ± standard deviation. Ms, milliseconds. **(A)** HR (bpm)—mean heart rate in beats per minute. **(B)** R-R (ms)—mean of R-R intervals in milliseconds. **(C)** SDNN (ms)—standard deviation of RR intervals in milliseconds. **(D)** RMSSD (ms) square root of successive differences between R-R intervals in milliseconds. [a]$p < 0.0001$ vs rest. [b]$p < 0.0001$ vs hypoxia.

($p = 0.027$), NN50 ($p = 0.044$), and SD1 ($p = 0.032$) the airmen classified as "high-HRV" presented higher values when compared to the airmen classified as "low-HRV".

The results of the Spearman correlation between HRV indices at different moments of flight (i.e., rest, hypoxia, and recovery) and the results of physical fitness tests are described in Table 3.

Regarding the estimated VO_2: 1) at rest there was no association; 2) during hypoxia there was an association with the RMSSD and SD1 indices (parasympathetic markers); and 3) in recovery there was an association with the SDNN and SD2 indices (sympathetic markers). Regarding the trunk flexion test: 1) the moment of rest was associated with the Mean RR, SDNN, Mean HR, RMSSD, SD1, and SD2 indices; 2) during hypoxia there was an association with the Mean HR, RMSSD, NN50, and SD1 indices; and 3) in recovery there was an association with the Mean RR, SDNN, Mean HR, RMSSD, and SD1 indices. There was no association between HRV indices and the results of the push-ups on the ground. Therefore, airmen who showed parasympathetic predominance suffered less interference from HH and, therefore, seemed to deal better with this physiological stress.

The Spearman's correlation between TAS (seconds) and body composition variables, and the results of physical fitness tests, O_2 saturation, and HRV indices at rest showed no association between the variables.

4 Discussion

To the best of our knowledge, this is the first study to analyze the influence of HH on cardiac autonomic function during TAS and its association with indices of physical fitness in airmen. Our main findings were: 1) HH induced a decrease in the values of the HRV indices of the airmen during the TAS; 2) the rMSSD index was the most sensitive to changes induced by HH; 3) airmen with high-HRV showed parasympathetic dominance during hypoxia; 4) the results of the physical fitness tests were associated with the HRV indices during the moments of the flight; 5) the HRV indices and the

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TABLE 2 Heart rate variability during baseline, hypoxia, and recovery.

HRV indices	High			Low			Mann-whitney		
	Mean	SD	Median	Mean	SD	Median	U	Z	*P*
RR (ms)									
Baseline	981.75	117.32	944.18	824.81	55.91	834.84	3.000	−3.877	0.000
Hypoxia	678.06	108.02	658.34	593.26	69.95	611.35	40.000	−1.600	0.118
Recovery	841.77	95.87	824.02	722.82	75.89	728.79	14.000	−3.200	0.001
SDNN (ms)									
Baseline	107.94	43.82	100.78	69.34	15.99	64.84	19.000	−2.893	0.003
Hypoxia	89.80	41.25	96.79	65.55	25.33	59.87	42.000	−1.477	0.151
Recovery	144.23	44.27	146.05	101.76	31.97	87.49	28.000	−2.339	0.019
HR (bpm)									
Baseline	62.66	6.07	64.32	73.61	5.17	72.23	3.000	−3.877	0.000
Hypoxia	92.12	13.19	91.26	103.88	13.68	100.19	41.000	−1.539	0.134
Recovery	74.49	7.37	73.63	86.02	10.84	83.35	18.000	−2.954	0.002
RMSSD (ms)									
Baseline	67.42	29.82	52.25	35.99	8.55	36.05	9.000	−3.508	0.000
Hypoxia	25.69	19.08	17.34	12.63	4.65	12.00	30.000	−2.216	0.027
Recovery	62.98	29.69	51.25	30.38	9.93	30.73	10.000	−3.447	0.000
SD1 (ms)									
Baseline	47.83	21.17	37.04	25.51	6.06	25.55	9.000	−3.508	0.000
Hypoxia	18.30	13.51	12.43	9.01	3.29	8.59	31.000	−2.154	0.032
Recovery	44.67	21.07	36.33	21.55	7.04	21.80	10.000	−3.447	0.000
SD2 (ms)									
Baseline	144.01	59.94	136.68	94.42	22.69	87.19	23.000	−2.646	0.007
Hypoxia	124.38	57.83	135.30	91.48	36.01	84.06	42.000	−1.477	0.151
Recovery	197.64	60.84	201.47	141.38	45.41	120.02	28.000	−2.339	0.019

R-R (ms)—average of R-R intervals in milliseconds, SDNN (ms)—standard deviation of RR, intervals in milliseconds, HR (bpm)—mean heart rate in beats per minute, RMSSD (ms) square root of successive differences between R-R intervals in milliseconds, SD1—The standard deviation of the Poincaré plot perpendicular to the identification line, SD2—The standard deviation of the Poincaré plot along the identification line.

TABLE 3 Spearman correlations between HRV indices at different moments and VO_2 estimated, trunk flexion (reps), and push-ups on the ground (reps).

HRV indexes	VO_2 estimated			Trunk flexion (reps)			Push-up on the ground (reps)		
	Baseline	Hypoxia	Recovery	Baseline	Hypoxia	Recovery	Baseline	Hypoxia	Recovery
RR (ms)	0.239	0.326	0.312	0.486	0.414	0.434	0.168	0.050	0.122
SDNN (ms)	0.318	0.229	0.441	0.466	0.359	0.417	0.315	0.380	0.377
HR (bpm)	−0.223	−0.305	−0.256	−0.466	−0.358	−0.402	−0.162	0.006	−0.064
RMSSD (ms)	0.300	0.424	0.371	0.492	0.443	0.463	0.371	0.246	0.289
SD1 (ms)	0.300	0.433	0.371	0.492	0.435	0.463	0.371	0.246	0.289
SD2 (ms)	0.274	0.216	0.449	0.431	0.355	0.394	0.266	0.375	0.377

R-R (ms)—Average of R-R intervals in milliseconds. SDNN (ms)—standard deviation of RR, intervals in milliseconds. HR (bpm)—mean heart rate in beats per minute. RMSSD (ms) square root of successive differences between R-R intervals in milliseconds. SD1—The standard deviation of the Poincaré plot perpendicular to the identification line. SD2—The standard deviation of the Poincaré plot along the identification line.

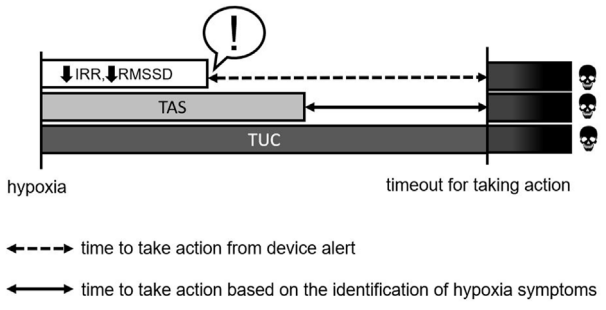

FIGURE 3
R-R (ms)—average of R-R intervals in milliseconds; RMSSD (ms) square root of successive differences between R-R intervals in milliseconds; TAS-time of onset of symptoms; TUC-useful time of consciousness. !—Alert.

results of the physical fitness tests (TACF) did not present a linear correlation with the TAS.

The results of the present study demonstrated a decrease in iRR during TAS, even though its time-lapse was shorter than that of TUC. That is, cardiac autonomic modulation presented similar behavior in both situations. In previous studies, airmen were exposed to HH, on average, for 3 min and 32 s (Barak et al., 1999; Yoneda et al., 2000; Guadagno et al., 2011). In the present study, airmen were exposed to HH for an average of 1 min and 47 s. However, considering only the HRV, the iRR decrease occurred quickly, even before the self-report of the physiological symptoms of HH (TAS), thus minimizing the risks to the physical and mental integrity of the airmen, as well as the risks to flight safety.

In the visual inspection of the airmen's iRR, a decrease in the length and amplitude of the RR intervals and loss of linearity during hypoxia were observed (Supplementary figure). The scientific literature presents a graphic illustration reporting iRR behavior similar to what we saw in the present study. However, the studies differ in the flight simulation protocol regarding altitude (25,000 vs 27,000 ft) and HH exposure time (TAS vs TUC), but together this evidence indicates a reduction in HRV(Barak et al., 1999; Vigo et al., 2010).

Within the first few minutes of exposure to HH at 25,000 ft, the decrease in HRV indices is noticeable. Therefore, there is no need to expose airmen to HH for a long time to identify the physiological symptoms of HH. The analysis of the change in HRV can contribute to the creation of warning devices for airmen (Mellor et al., 2018; Rice et al., 2019). Considering that the time factor in an emergency situation such as HH can be decisive for the occurrence or not of a fatality, the use of a device like this will help to guarantee the maintenance of the physical integrity of the airman and, therefore, the safety of the flight, through an "early" alert. See Figure 3.

TABLE 4 Spearman correlation between TAS (seconds) and body composition variables, physical fitness test results, oxygen saturation, and resting HRV indices.

Variables	R^2	r	p
Body composition			
Weight (kg)	0.0324	−0.18	0.42
waist-to-height ratio	0.0016	−0.04	0.85
Body fat (%)	0.0169	−0.13	0.57
Fat free mass (%)	0.0169	0.13	0.57
Physical fitness indices			
Ground push-up (reps)	0.0324	0.18	0.41
Trunk flexion (reps)	0.0576	0.24	0.26
$VO_{2\ submaximal}$	0.0256	0.16	0.47
Final O_2 saturation	0.0144	−0.12	0.6
O_2 saturation delta	0.0225	0.15	0.5
Resting heart rate variability indices			
RR (ms)	0.0081	0.09	0.70
SDNN (ms)	0.0016	0.04	0.84
HR (bpm)	0.0081	−0.09	0.69
RMSSD (ms)	0.0225	−0.15	0.502
SD1 (ms)	0.0225	−0.15	0.502
SD2 (ms)	0.0036	0.06	0.78

R-R (ms)—average of R-R intervals in milliseconds. SDNN (ms)—standard deviation of RR, intervals in milliseconds. HR (bpm)—mean heart rate in beats per minute. RMSSD (ms) square root of successive differences between R-R intervals in milliseconds. SD1—The standard deviation of the Poincaré plot perpendicular to the identification line. SD2—The standard deviation of the Poincaré plot along the identification line.

It is important to note that among the indices evaluated, the two most sensitive for quantifying cardiac autonomic modulation in HH, paired by exposure time, and were iRR and RMSSD, since in the recovery-hypoxia variation all airmen had a negative delta. Thus, it is suggested that the iRR and RMSSD indices be used as a reference for the creation of a mathematical equation that alerts to the occurrence of HH as soon as possible. However, knowing the complexity of the interaction of physiological systems, multiple factors need to be considered to create this equation, such as: altitude and barometric pressure. In addition, considering the need to evaluate its validity, accuracy, and reproducibility, more accurate analyses and the evaluation of direct measurements and re-tests are necessary.

Another important physiological measure for this equation is peripheral arterial O_2 saturation, which is a useful marker to identify physiological changes related to high altitude, as its values decrease with a drop in atmospheric pressure (Barak et al., 1999; Saito et al., 2005; Wolf and Garner, 2009; Vigo et al., 2010). In the present study, even though the exposure time to HH was shorter, we identified a drop in O_2 saturation, corroborating the

TABLE 5 Relationship between altitude and average time of exposure to HH and SO_2.

Authors	Sample	Altitude	Exposure time average	ΔSO_2
Present study	23	25,000 ft	1 min and 47 s	−19
Guadagno et al. (2011)	4	25,000 ft	2–5 min	−37.4
Yoneda et al. (2000)	369(26.8 years)	25,000 ft	3 min and 57 s	−41.6
	160(45.1 years)	25,000 ft	3 min and 21 s	−35.5
Barak et al. (1999)	21	25,000 ft	4 min and 40 s	−53
Vigo et al. (2010)	12	27,000 ft	1 min and 10 s	−33.5
Hoffman et al. (1946)	25	28,000 ft	2 min and 21 s	−54
	25	30,000 ft	1 min and 38 s	−54
	25	35,000 ft	1 min and 12 s	−58
	25	38,000 ft	47 s	−57

findings of previous studies (Hoffman et al., 1946; Barak et al., 1999; Yoneda et al., 2000; Vigo et al., 2010; Guadagno et al., 2011). However, the O_2 saturation delta of the airmen in the present study was lower than the studies presented in Table 4 and Table 5. In this sense, the evidence shows that the effects of HH depend on the severity of the hypoxia relative to 1) the exposure time (Barak et al., 1999; Guadagno et al., 2011), 2) the magnitude of the altitude (Hoffman et al., 1946; Vigo et al., 2010); and 3) the individual (Temporal, 2005; Russomano, 2012; de Carvalho, 2015; Russomano and Castro, 2020).

Thus, the increase in heart rate, the sharp drop in O_2 saturation, and the appearance of the first subjective symptoms reinforce the idea that the observed changes were caused by hypoxemia and give indications of the manifestation and evolution of the first stages of hypoxia at 25,000 ft (Yoneda et al., 2000; Temporal, 2005; Russomano and Castro, 2020) although the time of exposure to HH was shorter (TAS vs TUC). See Figure 3.

Initially, we hypothesized that the physical fitness and HRV indices of the airmen would be associated with the TAS, so that individuals with a better level of physical fitness and, therefore, higher values of the HRV indices, would have longer TAS, which may resemble the time-lapse of the TUC, without, however, approaching loss of consciousness. Although this association was also considered by other researchers (Barak et al., 1999), to date it has not yet been researched. However, contrary to the initial hypothesis, physical fitness and HRV indices were not associated with TAS.

In this perspective, it is important to point out that, over the years, different methods have been used to define the criterion that delimits the "endpoint" of the time of exposure to hypoxia (Izraeli et al., 1988; Yoneda et al., 2000). This fact makes comparisons between studies difficult. Furthermore, to the best of our knowledge, this is the first study to use the TAS instead of the TUC.

Another important aspect lies in the TAS/TUC evaluation criteria, based on self-reported subjective perception, which in turn, can suffer various interferences, as airmen are placed in an environment that induces changes in cognitive function (Izraeli et al., 1988; Yoneda et al., 2000). Thus, the perception of the first symptoms could be wrong. As an example, it has been suggested that the indication of TUC may be influenced by the tendency of novice airmen not to report the symptoms due to a possible feeling of competitiveness and/or the veterans' experience in recognizing HH symptoms more quickly (Yoneda et al., 2000). Despite the aforementioned limitations, it is possible that in the present study this measure does not reflect the actual moment of onset of the first symptoms.

In this sense, a study showed that smokers, that is, with increased levels of carboxyhemoglobin (responsible for inducing anemic hypoxia) had self-reported subjective symptoms and TUC similar to non-smokers (Yoneda and Watanabe, 1997). Furthermore, it has been suggested that genetic polymorphism (mitochondrial-DNA—haplogroup D) may influence hypoxia tolerance time (Motoi et al., 2016). In addition, the predominance of muscle fiber type seems to influence hypoxia tolerance. Type I muscle fibers are more resistant to the effects of hypobaric hypoxia (Chaudhary et al., 2012).

Sympathetic hyperreactivity and/or maintenance of the fight-or-flight state (sympathetic predominance) during rest cause damage to tissue and, consequently, to health (Rakhshan et al., 2015; Sharma, 2018). Several cardiovascular diseases are associated with hyperreactivity (Fisher and Paton, 2012; Karia et al., 2012; Carter and Ray, 2015). In this sense, a recent study carried out with an animal model reported that intermittent hypoxia generates systemic and cardiac sympathoactivation and is an important risk factor for sudden cardiac death after cardiac ischemia (Morand et al., 2018). On the other hand, physical exercise contributes to a reduction in sympathetic activation and an increase in cardiac vagal modulation, promoting an antiarrhythmic effect (Soares-Miranda et al., 2009; Carter and Ray, 2015). Additionally, the level of physical fitness seems to influence sympathetic reactivity (Ifuku et al., 2007) and positively

regulate the autonomic balance (Kwon et al., 2014; Carter and Ray, 2015; Fisher et al., 2015), and physical training is the best agent to improve physical fitness, by improving cardiac efficiency and O_2 extraction by peripheral tissues (Saltin, 1968). Therefore, effective/efficient cardiac autonomic balance is essential for maintaining health in hypoxic situations. The aforementioned reports corroborate the results of the present study in which the physically trained airmen showed an adequate autonomic response to HH. Furthermore, those classified with high-HRV had higher values of the representative indices of the parasympathetic branch (RMSSD, NN50, and SD1) when compared to airmen classified as "low-HRV" during hypoxia and recovery.

Furthermore, after a stressful event, such as HH, it is of paramount importance that airmen can quickly reestablish physiological homeostasis and resume cognitive, psychomotor, piloting, and flight performance, and flight safety (Files et al., 2005; Steinman et al., 2017; Rice et al., 2019). Nevertheless, cardiac autonomic recovery has been considered an important mortality rate and can be influenced by the level of physical fitness and physical training (de Oliveira and de Lima, 2012).

Finally, for better understanding, we emphasize that the physiological complexity and its adaptations to the hypobaric hypoxic environment require more sophisticated and accurate methods. However, this methodological robustness and its reproduction are too expensive and access to the hypobaric chamber for simulating flights at high altitudes for hypoxia simulation is extremely restricted.

Despite planning and efforts to prevent limitations, we recognize that there are opportunities for improvement in future investigations. First, it was not possible to strictly control the airman's power supply. However, the only meal before the flight simulation in HH was standard for all participants. Second, the lack of individuals with different levels of physical fitness and a control group as access to the flight simulation in HH is extremely restricted and too expensive, preventing testing with different people. Despite this, the present study provides evidence of HRV assessed by a cardiac monitor as a promising tool for assessing cardiac autonomic modulation in HH at 25.000 ft.

In summary, we concluded that HH induced cardiac autonomic changes which could be identified through the cardiac monitor *Polar Team*® even before airmen self-reported the first subjective symptoms. This demonstrates that cardiac autonomic modulation appears to be more sensitive to the effects of HH at 25.000 ft than the self-reported subjective perception of symptoms. Therefore, portable devices capable of providing an early warning of the occurrence of hypobaric hypoxia could be created from the HRV, buying the airman more time to take action to re-establish control and flight safety.

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 Comitê de Ética em Pesquisa da Universidade Católica de Brasília. The patients/participants provided their written informed consent to participate in this study.

Author contributions

FH, LD, and TR conception or design of the work; acquisition, analysis, or interpretation of data for the work; drafting of the work or revising it critically for important intellectual content; agree 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 conceived and designed this study. AR, RN, HC, AM, DH, RM, JP, and CF acquisition, analysis, or interpretation of data for the work; drafting of the work or revising it critically for important intellectual content. All authors have given their approval of the final draft for submission to this Journal.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.1005016/full#supplementary-material

SUPPLEMENTARY FIGURE S1
An example of the graphical behavior of R-R. ms, milliseconds.

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Moderate muscle cooling induced by single and intermittent/prolonged cold-water immersions differently affects muscle contractile function in young males

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Background: We investigated the impact of moderate muscle cooling induced by single and intermittent/prolonged cold-water immersions (CWI) on muscle force and contractility in unfatigued state and during the development of fatigue resulting from electrically induced contractions.

Methods: Twelve young males participated in this study consisting of two phases [single phase (SP) followed by intermittent/prolonged phase (IPP)], with both phases including two conditions (i.e., four trials in total) performed randomly: control passive sitting (CON) and cold-water immersions (10°C). SP-CWI included one 45 min-bath (from 15 to 60 min). IPP-CWI included three baths (45 min-bath from 15 to 60 min, and 15 min-baths from 165 to 180 min and from 255 to 270 min), with participants sitting at room temperature the rest of the time until 300 min. Blood pressure and intramuscular (Tmu) temperature were assessed, and neuromuscular testing was performed at baseline and 60 min after baseline during SP, and at baseline, 60, 90, 150 and 300 min after baseline during IPP. A fatiguing protocol (100 electrical stimulations) was performed after the last neuromuscular testing of each trial.

Results: In unfatigued state, SP-CWI and IPP-CWI reduced electrically induced torque at 100 Hz (P100) but not at 20 Hz (P20), and increased P20/P100 ratio. The changes from baseline for P100 and P20/P100 ratio were lower in IPP-CWI than SP-CWI. Both cold-water immersion conditions slowed down muscle contraction and relaxation, and reduced maximal isokinetic contraction torque, but the changes from baseline were lower after IPP-CWI than SP-CWI. cold-water immersions did not impair maximal voluntary isometric contraction. During the fatiguing protocol, torque fatigue index and the changes in muscle contractile properties were larger after IPP-CWI than SP-CWI, but were in the same range as after CON conditions. The differences of muscle contractile function between SP-CWI and IPP-CWI were accompanied by a lower reduction of superficial Tmu and a smaller increase in systolic blood pressure after IPP-CWI than SP-CWI.

Conclusion: IPP-CWI induces a less pronounced fast-to-slow contractile transition compared to SP-CWI, and this may result from the reduced

vasoconstriction response and enhanced blood perfusion of the superficial muscle vessels, which could ultimately limit the reduction of superficial Tmu.

KEYWORDS

cold exposure, cold-water immersion, muscle contractility, muscle fatigue, cold habituation, muscle force, temperature

1 Introduction

Application of cooling is commonly used in public health to reduce heat strain and improve thermal comfort in hot environment (Lan et al., 2018; Tokizawa et al., 2020), as well as to improve wellbeing and to potentially reduce inflammation in patients (Tipton et al., 2017). Cooling is also applied before and during exercise in hot conditions combined or not with high humidity to attenuate exertional heat strain and optimize physical performance (Coelho et al., 2021; Morito et al., 2022). Moreover, post-exercise cooling such as cold-water immersion (CWI) has become very popular in athletes based on the assumption that it could enhance physical recovery and reduce muscle soreness (Yeargin et al., 2006; Brophy-Williams et al., 2011; Machado et al., 2017). Cold exposure also occurs in numerous sports (e.g., winter sports, open-water swimming, etc.) and occupational activities (e.g., military expedition, emergency rescue, etc.). Severe and/or prolonged cooling can have deleterious effects, such as hypothermia, cold-injuries, and impaired physical performance (Cahill et al., 2011; Fudge et al., 2015).

Exposure to CWI induces acute physiological adjustments, including increased whole-body metabolic heat production (Castellani et al., 1998), reduced muscle metabolic activity (Ihsan et al., 2013), reduced femoral artery blood flow and increased cutaneous vasoconstriction (Mawhinney et al., 2017), reduced muscle and core temperatures (Brazaitis et al., 2014b), and decreased nerve conduction velocity (Algafly and George, 2007). These adjustments are influenced by the duration of exposure, water temperature, and the type of immersion (e.g., single, or intermittent) (Brazaitis et al., 2011; Brazaitis et al., 2014b; Castellani and Young, 2016).

Cooling could also influence skeletal muscle function (Bennett, 1984; Oksa, 2002; Drinkwater, 2008). Numerous human studies have demonstrated that CWI-induced severe muscle cooling (i.e., reduced temperature of 10°C–20°C in the deep portion of the muscle) impairs electrically evoked and maximal voluntary isometric contraction (MVIC) forces (Davies et al., 1982; Giesbrecht et al., 1995; de Ruiter et al., 1999). Severe muscle cooling also elicits a shift towards a slower muscle contractile profile (Davies et al., 1982; de Ruiter et al., 1999), while it could limit force decline during repeated contractions induced by electrical stimulation (Davies et al., 1982). In contrast, moderate muscle cooling, that can be defined as a reduced temperature up to 5°C in the deep portion of the muscle, does not (Petrofsky and Phillips, 1986) or only slightly (Asmussen et al., 1976; Bergh and Ekblom, 1979; Brazaitis et al., 2011) reduces MVIC force. Some evidence indicates that it reduces force during dynamic voluntary contractions, at least of the knee extensors (Bergh and Ekblom, 1979), and force response to 50 Hz electrical stimulations (Brazaitis et al., 2011). However, it may not affect the drop in force (i.e., fatigue resistance) and half relaxation time (HRT) during repeated electrical stimulations induced contractions (Brazaitis et al., 2011). To date, the impact of moderate cooling on muscle force production (isometric and dynamic contractions, and electrically evoked contractions at low and high frequencies) and muscle contractile properties (including rate of force development, rate of force relaxation, contraction time and HRT) in unfatigued state is not well documented. In addition, the effect of moderate cooling on muscle force and contractile properties during the development of fatigue resulting from electrical stimulations requires further investigation.

Human studies evaluating the impact of local cooling on muscle force and contractile properties generally include one single CWI where the immersion time is either not documented [but adjusted so that skeletal muscle reaches a certain temperature (Asmussen et al., 1976; Bergh and Ekblom, 1979)] or limited to a duration <45 min (Davies et al., 1982; de Ruiter et al., 1999; Brazaitis et al., 2011; Brazaitis et al., 2012). To investigate physiological changes in response to prolonged muscle cooling (i.e., several hours), intermittent water immersions can be used to limit cold sensation (Castellani et al., 1998). Single exposure to CWI markedly increases heat production related to shivering and non-shivering thermogenesis and limits heat loss through cutaneous vasoconstriction, while intermittent CWI immersions possibly result in thermoregulatory adjustments and habituation, including reduced shivering and/or cutaneous vasoconstriction response (Castellani and Young, 2016). This latter physiological response may increase the supply of warm blood from the core to the skin and superficial layers of the muscles, while maintaining a reduced temperature in the deep portion of the muscle. In that case, it could be hypothesized that intermittent and prolonged CWI would lead (in comparison to shorter single CWI) to a faster muscle contractile profile in unfatigued state and during the development of fatigue resulting from electrically induced contractions. Intermittent and prolonged CWI may also attenuate the force reduction during dynamic voluntary contractions in unfatigued state due to the faster contractile properties of the muscle (and thus its ability to attain peak force more rapidly), while MVIC would be unaffected. Furthermore, voluntary activation in unfatigued state would most likely not be affected by short and prolonged CWI, as evidenced in recent studies (Brazaitis et al., 2014b; Spillane and Bampouras, 2021). In this study, our aim was to investigate the impact of moderate muscle cooling induced by single and intermittent/prolonged CWI on muscle force production and muscle contractility in unfatigued state and during the development of fatigue resulting from electrically induced contractions in humans.

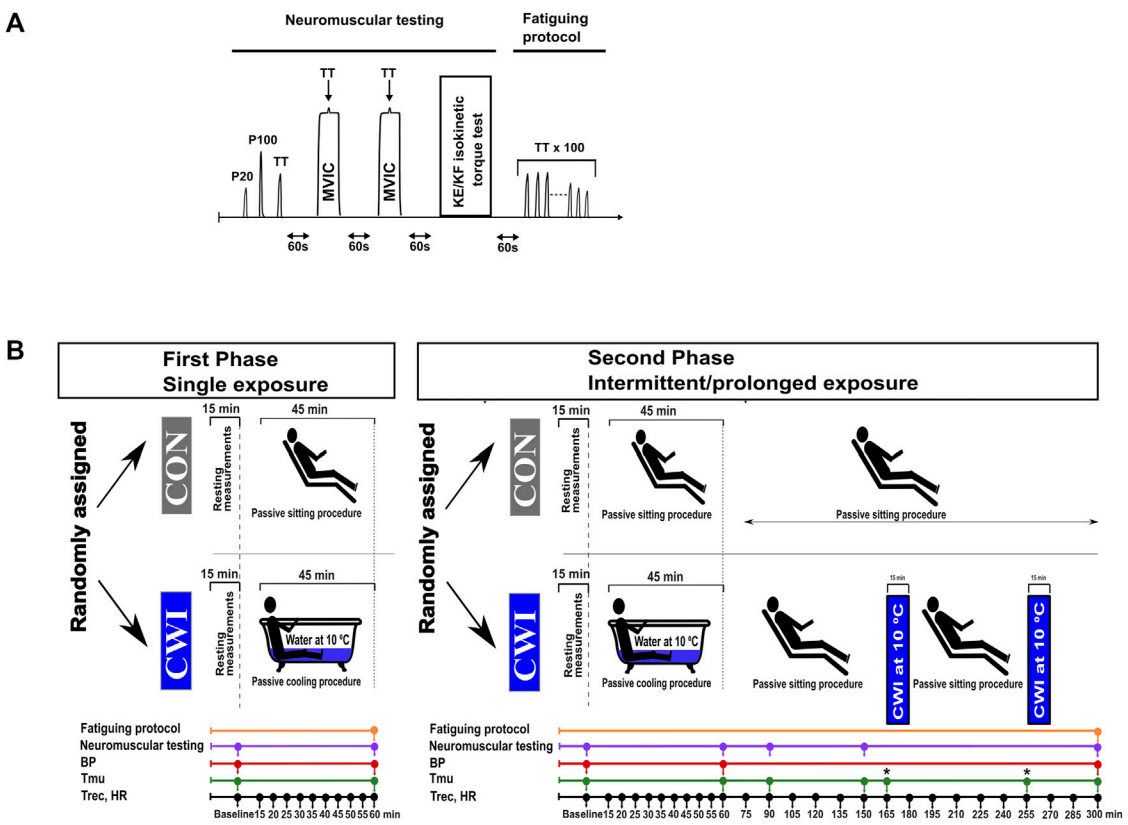

FIGURE 1
Neuromuscular testing and fatiguing protocol **(A)**, and experimental design **(B)**. P20, electrical stimulation at 20 Hz; P100, electrical stimulation at 100 Hz; TT, 250-ms test train stimulation at 100 Hz; MVIC, maximal voluntary isometric contraction; KE, knee extension; KF, knee flexion; BP, blood pressure; Tmu, intramuscular temperature; Trec, rectal temperature; HR, heart rate; CON, control condition; CWI, cold-water immersion condition. Fatiguing protocol consisting of 100 trains of electrical stimulation of the knee extensors (100 x 250-ms TT) was only performed after the last neuromuscular testing (i.e., second neuromuscular testing during the single phase and 5th neuromuscular testing during the intermittent/prolonged phase). *: at the time points 165 and 255 min, Tmu was only measured in the CWI condition.

2 Material and methods

2.1 Participants

A randomized cross-over design was used in this study, in which twelve recreationally active men participated. The inclusion criteria used were: aged between 18 and 45 years, not participating in any other experiments, being healthy, physically active (at least 2–3 times per week) and without medication, and having a body mass index (BMI) < 30 kg.m-2. The exclusion criteria were: asthma, neurological pathology, cardiovascular disease, or conditions that could be worsened by exposure to cold, and suffering from any kind of disease or having physical limitations that would compromise the ability to perform the neuromuscular testing. The age, height, body mass, percentage body fat and BMI assessed at baseline at the beginning of the study were 27.2 ± 6.6 years, 186.7 ± 7.6 cm, 86.5 ± 12.1 kg, 16.9% ± 3.1% and 24.9 ± 2.2 kg/m2, respectively. All experiments were performed at the Lithuanian Sports University (Kaunas, Lithuania). The study protocol was approved by Kaunas Regional Biomedical Research Ethics committee (no. P1-BE-2–14/2022) and was in agreement with the latest revision of the Declaration of Helsinki. The participants were informed of the experimental procedures and gave their written informed consent prior to participation.

2.2 Experimental design

The study included two phases: the phase “single exposure”, called single phase (SP), followed by the phase “intermittent/prolonged exposure”, called intermittent/prolonged phase (IPP), with both phases consisting of two experimental conditions performed in a random order: control sitting (CON) and cold-water immersion (CWI). Each experimental trial (four in total) was separated by at least 1 week. A summary of the experimental design is presented in Figure 1. The week before the first experiment, the participants were familiarized with the protocols, equipment, the neuromuscular testing, and fatiguing protocol (see description below). Air temperature in the laboratory was controlled and maintained at 23.0°C ± 0.5 °C, and relative humidity was 35% ± 3%.

The participants arrived at the laboratory and started the experiments in the morning (9.00 a.m.) after overnight fasting. Body mass, body composition, heart rate (HR), blood pressure (BP), rectal temperature (Trec) and intramuscular temperature

TABLE 1 Changes in the physiological measurements after the single and intermittent/prolonged phases.

	SP-CON	IPP-CON	P	*d*	SP-CWI	IPP-CWI	P	*d*
Δ HR (bpm)	−2.50 ± 8.42	−0.67 ± 5.1	0.60	−0.15	−4.67 ± 6.07	−6.33 ± 7.05	0.52	0.19
Δ Diastolic BP (mmHG)	−0.17 ± 7.31	0.83 ± 7.35	0.74	−0.10	2.17 ± 9.92	2.92 ± 5.82	0.80	−0.07
Δ Systolic BP (mmHG)	−0.08 ± 8.56	2.67 ± 11.42	0.54	−0.18	7.08 ± 10.34	0.17 ± 5.46	0.04	0.65
Δ Trec (°C)	−0.01 ± 0.17	−0.18 ± 0.34	0.12	0.48	−0.01 ± 0.17	−0.78 ± 0.37	<0.001	1.80
Δ Tmu (°C) at 3 cm	−0.05 ± 0.42	−0.66 ± 0.63	0.02	0.88	−3.78 ± 1.89	−4.29 ± 1.17	0.34	0.30
Δ Tmu (°C) at 2 cm	−0.38 ± 0.65	−0.79 ± 1.29	0.29	0.34	−6.09 ± 1.72	−4.57 ± 1.25	0.02	−0.81
Δ Tmu (°C) at 1 cm	−0.50 ± 0.73	−0.65 ± 0.81	0.62	0.15	−9.73 ± 2.66	−4.83 ± 1.30	<0.001	−1.96

d, Cohen's *d*, HR, heart rate; BP, blood pressure; Trec, rectal temperature; Tmu, intramuscular temperature. SP, single phase; IPP, intermittent/prolonged phase.
Data are shown as mean ± SD. Δ change was calculated as: end value—baseline value.
N = 12 for all parameters except for Tmu (N = 11).

(Tmu) were assessed at baseline (after sitting for 15 min). Then, they performed the first neuromuscular testing, which was followed by either 45 min control passive sitting in the laboratory (CON condition) or by 45 min sitting in a cold-water bath (CWI condition, immersion up to the waist in an acrylic bathtub, water temperature of 10.0°C ± 0.2°C). Ice slush was added into the bath to maintain the required water temperature. HR and Trec were recorded every 5 min during this period. Then, HR, BP, Trec and Tmu were measured, and the second neuromuscular testing (endpoint during the single phase and at 60 min during the intermittent/prolonged phase) followed. During the single phase, the second neuromuscular testing was directly followed by a fatiguing protocol consisting of 100 trains of electrical stimulation of the knee extensors (Table 1; see below for details). During IPP-CWI, two additional bouts of cold-water immersion (15 min each, immersion up to the waist, 10.0°C ± 0.2°C) were added between 165 and 180 min, and between 255 and 270 min. During this experimental trial, participants were sitting at room temperature the rest of the time: between 60 and 165 min (duration of 105 min), between 180 and 255 min (duration of 75 min), and between 270 and 300 min (duration of 30 min). During IPP-CON, participants were sitting at room temperature for 300 min. During the intermittent/prolonged phase, HR and Trec were recorded every 15 min between 60 min and the end of the protocol (i.e., 300 min), and BP was measured at the end. Tmu was measured at 90 min, 150 min and at the end of CON condition (300 min), and two additional Tmu measurements were added after 165 min (before the second bath) and 255 min (before the third bath) in the IPP-CWI condition to monitor possible changes in Tmu due to passive sitting between baths. The number of Tmu measurements was limited to limit discomfort and tissue damage. Additional neuromuscular testing was performed at 90 min, 150 min, and at the end of the intermittent/prolonged phase. After the last neuromuscular testing, a fatiguing protocol consisting of 100 trains of electrical stimulations of the knee extensors was directly performed.

During the intermittent/prolonged phase, a strawberry breakfast cereal bar (87 kcal; Fitness, South Africa) was provided at 120 min and 225 min, while the participants remained fasted during the single phase. Participants were told to take similar meals on the day before experiments. During the experiments, participants did not drink water, but were allowed to rinse their mouth with cool water. Participants wore only swimming shorts during the baths, and quickly wiped off at the end of each bath before starting the neuromuscular testing. Between the baths (only for intermittent/prolonged cooling) and in the CON conditions, they wore shorts and a tee-shirt.

2.3 Physiological measurements

2.3.1 Anthropometric measurements

Body mass and percentage body fat were assessed with a body composition analyzer (Tanita, TBF-300, IL, United States). The height of the participants was measured with a height gauge, and BMI was calculated.

2.3.2 Blood pressure and heart rate measurements

Diastolic and systolic BP were measured on the left arm (one measurement each time) with an automatic BP monitor (Gentle+, Microlife, FL, United States). HR was recorded with a HR monitor (S-625X, Polar, Kempele, Finland). The time points of measurements are presented in the section "experimental design" and in Figure 1.

2.3.3 Body temperature measurements

Trec and Tmu were similarly assessed as in some studies from our research group (Brazaitis et al., 2011; Brazaitis et al., 2014b) and the time points are presented in the section "experimental design" and in Figure 1. More precisely, Trec was measured using a thermocouple (Rectal Probe; Ellab, Hvidovre, Denmark; accuracy, ±0.01°C) inserted to a depth of 12 cm past the anal sphincter. Tmu was measured using a needle microprobe (Intramuscular Probe MKA, thermometer model DM-852, Ellab) inserted into the vastus lateralis muscle of the right leg at mid-thigh and slightly lateral to the femur at three different depths (1, 2 and 3 cm beneath the skin surface).

2.4 Rationale of the CWI protocols

In this study, we developed our CWI protocols with the objective of inducing a moderate reduction of Tmu (<5°C) in the deep portion of the vastus lateralis muscle. We defined deep Tmu at a depth of

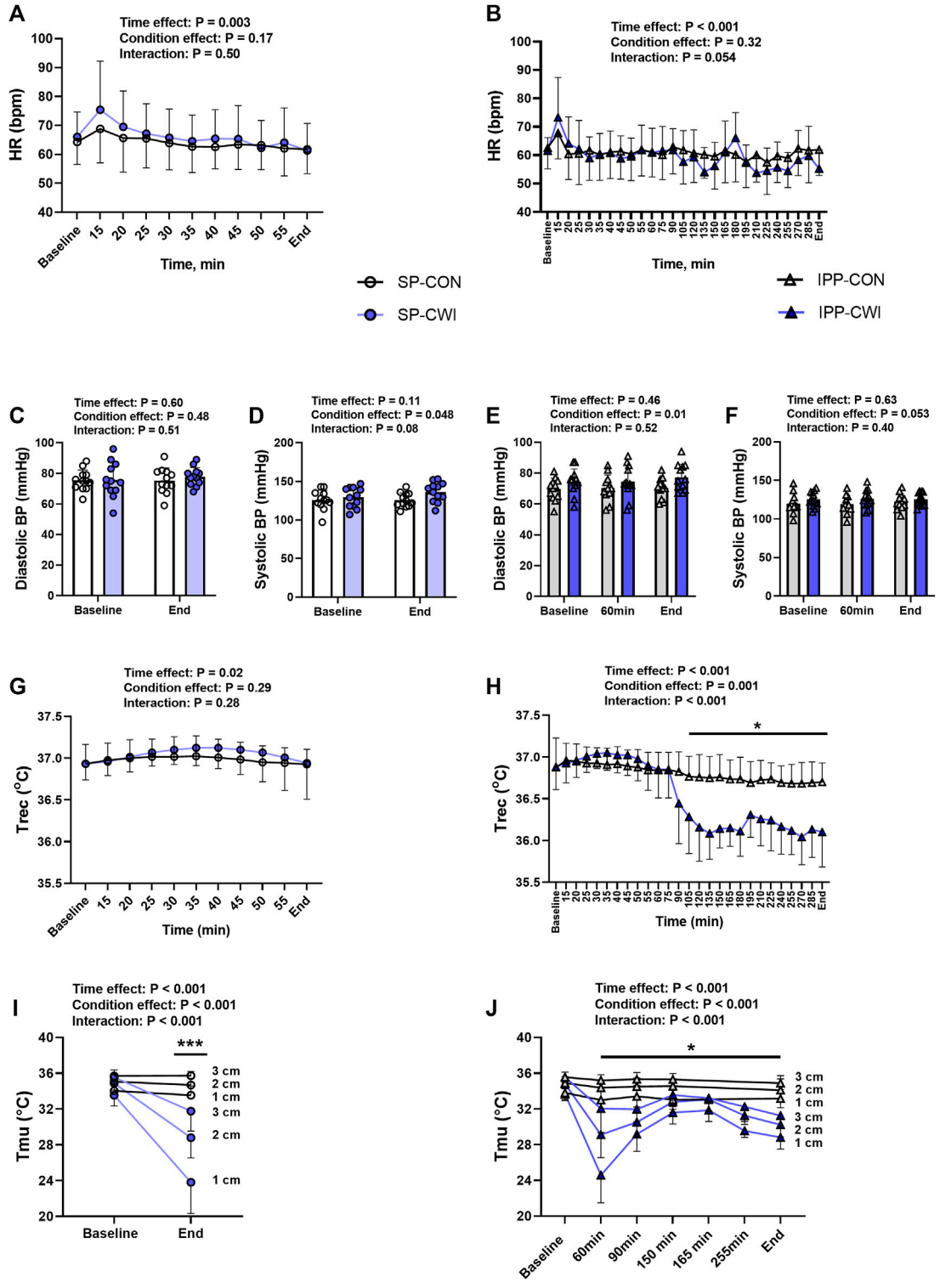

FIGURE 2
Physiological measurements during the single and intermittent/prolonged phases. Heart rate (HR) **(A,B)**, diastolic **(C,E)** and systolic **(D,F)** blood pressures (BP), rectal temperature (Trec) **(G,H)** and intramuscular temperature (Tmu) at three depths (1, 2, and 3 cm) **(I,J)** during the single and intermittent/prolonged phases, respectively. Data are shown as mean ± SD and individual values are presented in panels C–F. SP, single phase; IPP, intermittent/prolonged phase. *, $p < 0.05$; ***, $p < 0.001$: Significant differences CON vs. CWI. In I and J, significant differences are between CON and CWI are at the three depths. In J, Tmu was not assessed in CON at the time points 165 and 255 min. N = 12 for all parameters except for Tmu (N = 11) **(I,J)**.

3 cm in the vastus lateralis muscle, which is commonly used in studies investigating force in the knee extensors in response to CWI (Asmussen et al., 1976; Bergh and Ekblom, 1979; Beelen and Sargeant, 1991; Brazaitis et al., 2011; Brazaitis et al., 2012). Previous studies and data from our laboratory have shown that after a relatively short exposure to CWI (10 min), deep Tmu continues to decrease for at least 30–40 min post-immersion (Mawhinney et al., 2013; Mawhinney et al., 2017; Eimonte et al., 2021). To maintain a relatively stable deep Tmu after the first immersion and during the second neuromuscular testing (and during the fatiguing protocol of SP), the first bath was set for 45 min. Based on pilot experiments and as illustrated in Figure 2J, we found that deep Tmu remained relatively stable for approximately 100 min following the first 45 min-CWI. For this reason, two additional 15 min-CWI were included during IPP: one at 165-min (i.e., 105 min after the first 45 min-bath and directly after the fourth neuromuscular testing) and one at 255 min. The duration of these two baths was only of 15 min to limit participants' discomfort and to maintain Trec higher than 35.5°C, a set point previously used in studies from our group (Brazaitis et al., 2014a; Brazaitis et al., 2014b; Brazaitis et al., 2015).

2.5 Neuromuscular testing, fatiguing protocol and data analysis

Neuromuscular testing, consisting of electrically evoked torque of the knee extensors, maximal voluntary isometric contraction (MVIC) of the knee extensors, and maximal isokinetic concentric contraction of the knee extensors (KE-isoK) and knee flexors (KF-isoK) is illustrated in Figure 1A. Involuntary contractions and voluntary contractions were previously performed in studies from our research group (Skurvydas et al., 2011; Brazaitis et al., 2014b; Eimantas et al., 2022). More precisely, the participants sat upright in an isokinetic dynamometer (System 4; Biodex Medical Systems, Shirley, NY, United States) calibrated according to the manufacturer's recommendations, with a correction for gravity performed using the Biodex Advantage program (Version 4. X). Shank, trunk and shoulders were stabilized with belts. The dynamometer was set with the knee joint positioned at an angle of 90° (180° = full extension) during MVIC and electrical stimulations, and at a joint angle between 85° and 176° during KE-isoK and between 176° and 85° during KF-isoK.

Electrical stimulations were applied using three carbonized rubber surface electrodes (MARP Electronic), lubricated with electrode gel (ECG-EEG Gel, medigel, Modi'in, Israel). Two electrodes (12 × 8 cm) were positioned vertically and transversely across the width of the proximal portion of quadriceps muscle, and the third electrode (12 × 8 cm) covered the distal portion of the quadriceps muscle above the patella. Caution was made to keep a similar position of the electrodes during the four experimental trials. An electrical stimulator (Digitimer DS7A, Digitimer, Hertfordshire, United Kingdom) was connected to the electrodes and delivered 0.5-ms square wave pulses at a constant current set at 100 mA and constant voltage limit set at 200 V. This selected current ensures full contraction and activation of the muscle (Eimantas et al., 2022).

Neuromuscular testing began with three electrical stimulations separated with 3 s of rest: 1-s stimulation at 20 Hz (P20), 1-s stimulation at 100 Hz (P100) and 250-ms test train stimulation at 100 Hz (TT). Peak torques were determined during these three electrical stimulations, and P20/P100 was calculated to estimate the contractile profile. The contractile properties in the unfatigued state were determined during TT by calculating contraction time/peak torque, half-relaxation time (HRT), peak rate of torque development (RTD) and peak rate of torque relaxation (RTR). Contraction time was defined as the time taken to reach peak torque. Contraction time progressively decreased in our fatiguing protocol (see the description below) as a result of the progressive reduction of torque production and thus the fact that it takes less time to reach a lower torque (data not shown). Consequently, we chose to normalize contraction time to peak torque in both protocols (i.e., unfatigued state and fatiguing protocol). This ratio represents the average time required to increase the torque by 1 Nm. HRT was calculated as the time taken for the torque to decline from the peak value to 50% of the peak value. RTD and RTR were calculated by using Excel software and raw data exported from Biodex system (100Hz sampling rate) and were defined as the peak slope of torque per 10 ms (Δtorque/Δ10 ms) (Eimantas et al., 2022).

Following these three electrical stimulations, two MVIC of the knee extensor muscle were performed and separated by 1-min rest period. The participants were verbally encouraged to exert and maintain maximal effort for ~5 s, and a 250-ms test train stimulation at 100 Hz was superimposed on voluntary contraction 3–4 s into the MVIC. Central activation ratio (CAR), a measure of voluntary activation level, was calculated as (MVIC torque/total peak torque generated with the superimposed 250-ms test train stimulation) x 100. The highest MVIC torque was selected for analysis. One minute after the second MVIC, three continuous repetitions of KE-isoK (90°/s) and KF-isoK (90°/s) were performed with 1 min rest between KE and KF contractions. The highest torque during these maximal isokinetic contractions was selected for analysis.

Directly after the last neuromuscular testing (i.e., second during the single phase and 5th during the intermittent/prolonged phase), a fatiguing protocol consisting of 100 trains of electrical stimulation of the knee extensor muscle [250-ms test train stimulation at 100 Hz (TT) interspaced with 1s break] was included. TT torque and the contractile properties (contraction time/peak torque, HRT, RTD and RTR) were determined during the fatiguing protocol and are presented as the average of the first three contractions, 4th to 20th contraction, 21st to 40th contraction, 41st to 60th contraction, 61st to 80th contraction, and 81st to 100th contraction. The torque fatigue index and the changes in contractile properties during fatiguing protocol were determined from the first three and last three contractions (Table 2).

2.6 Statistical analysis

Data are presented as mean ± standard deviation (SD) and data presented in figures also include individual values (except in some panels of Figures 2, 6). Statistical analyses were performed using GraphPad Prism (Graphpad Prism 9.0.2, San Diego, CA, United States) and SPSS Statistics (version 28; for analysis of

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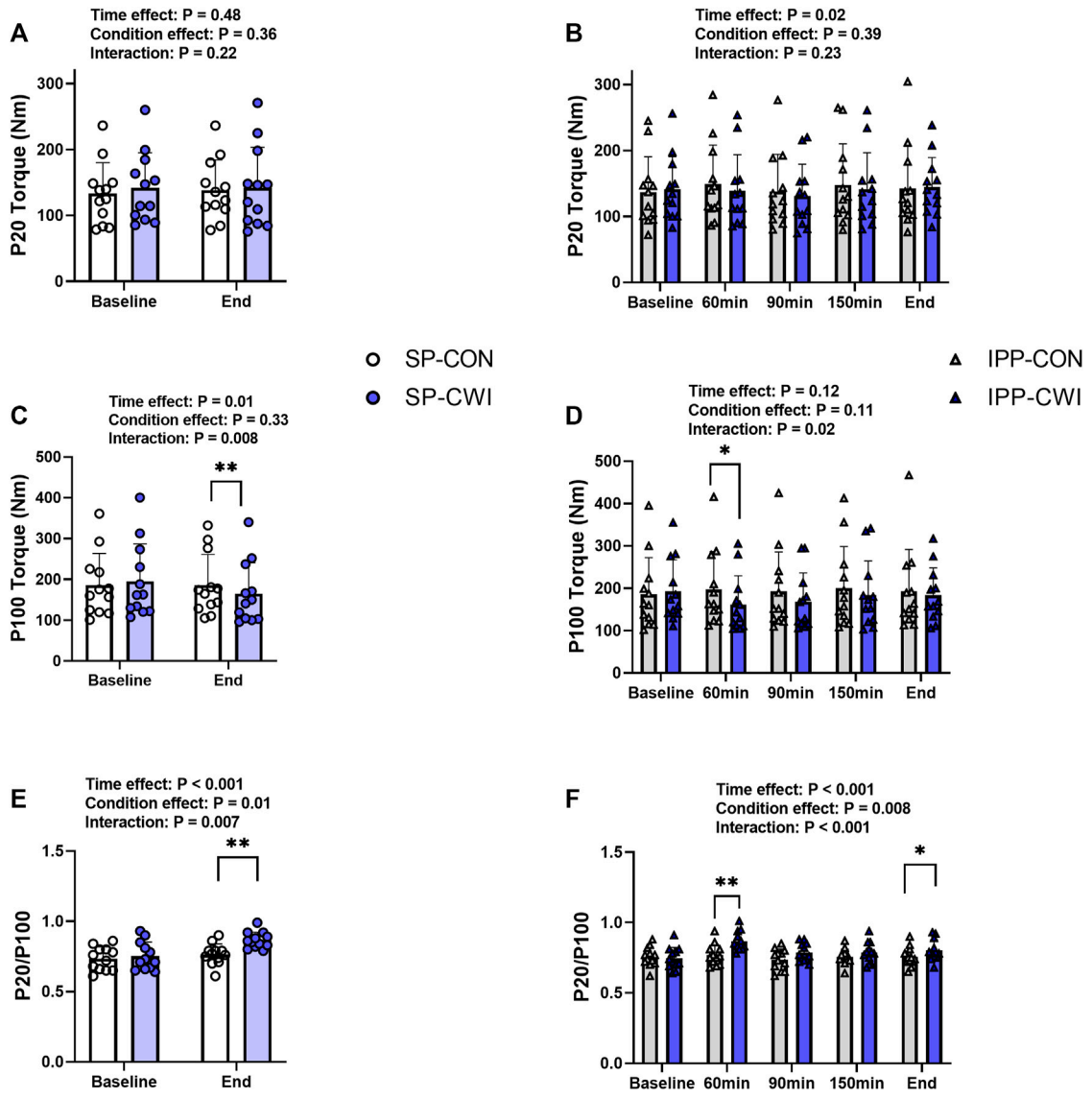

FIGURE 3
Electrically induced isometric torques in the unfatigued state. Peak torques at 20 Hz (P20) **(A,B)** and 100 Hz (P100) **(C,D)**, and P20/P100 **(E,F)** during the single and intermittent/prolonged phases, respectively. Data are shown as mean ± SD and all panels include individual values. SP, single phase; IPP, intermittent/prolonged phase. *, $p < 0.05$; **, $p < 0.01$: Significant differences CON vs. CWI. N = 12.

effect size only). Data were tested for normality using the Shapiro–Wilk test before conducting parametric statistical analyses, and all data were found to be normally distributed. For the physiological parameters (HR, BP, Trec and Tmu; Figure 2), and torques and contractile properties in unfatigued state (Figure 3; Figure 4; Figure 5), two-way repeated-measures analyses of variance (ANOVA) were performed to assess the effects of condition (CON vs. CWI), time and the condition × time interaction during both the single and intermittent/prolonged phases. When an interaction was observed, Sidak's multiple comparisons test was used to compare the two conditions (CON vs. CWI). For the physiological parameters (Table 1) and torques and contractile properties in unfatigued state (Table 3; Table 4), the Δ change (i.e., end value—baseline value) and % change (i.e [(end value—baseline value)/baseline value] x 100) between SP-CON and IPP-CON, and between SP-CWI and IPP-CWI were assessed using paired t-tests. For TT torque and the contractile properties during the fatiguing protocol (Figure 6), two-way repeated-measures ANOVA were performed to assess the effects of condition (CON vs. CWI), contraction number (1–3, 4–20, 21–40, 41–60, 61–80, 81–100) and the condition x contraction number interaction during both phases. When an interaction was observed, Sidak's multiple comparisons test was used to compare the two conditions (CON vs. CWI). In Supplementary Table S1, two-way repeated-measures ANOVA were also performed to assess the effects of the phase (single vs. intermittent/prolonged), contraction number (1–3, 4–20, 21–40, 41–60, 61–80, 81–100) and the phase x contraction number interaction. When an interaction was observed, Sidak's multiple comparisons test was used to compare the two phases for both CON (SP-CON vs. IPP-CON) and CWI (SP-CWI vs. IPP CWI) conditions. All two-way repeated-measures ANOVA included

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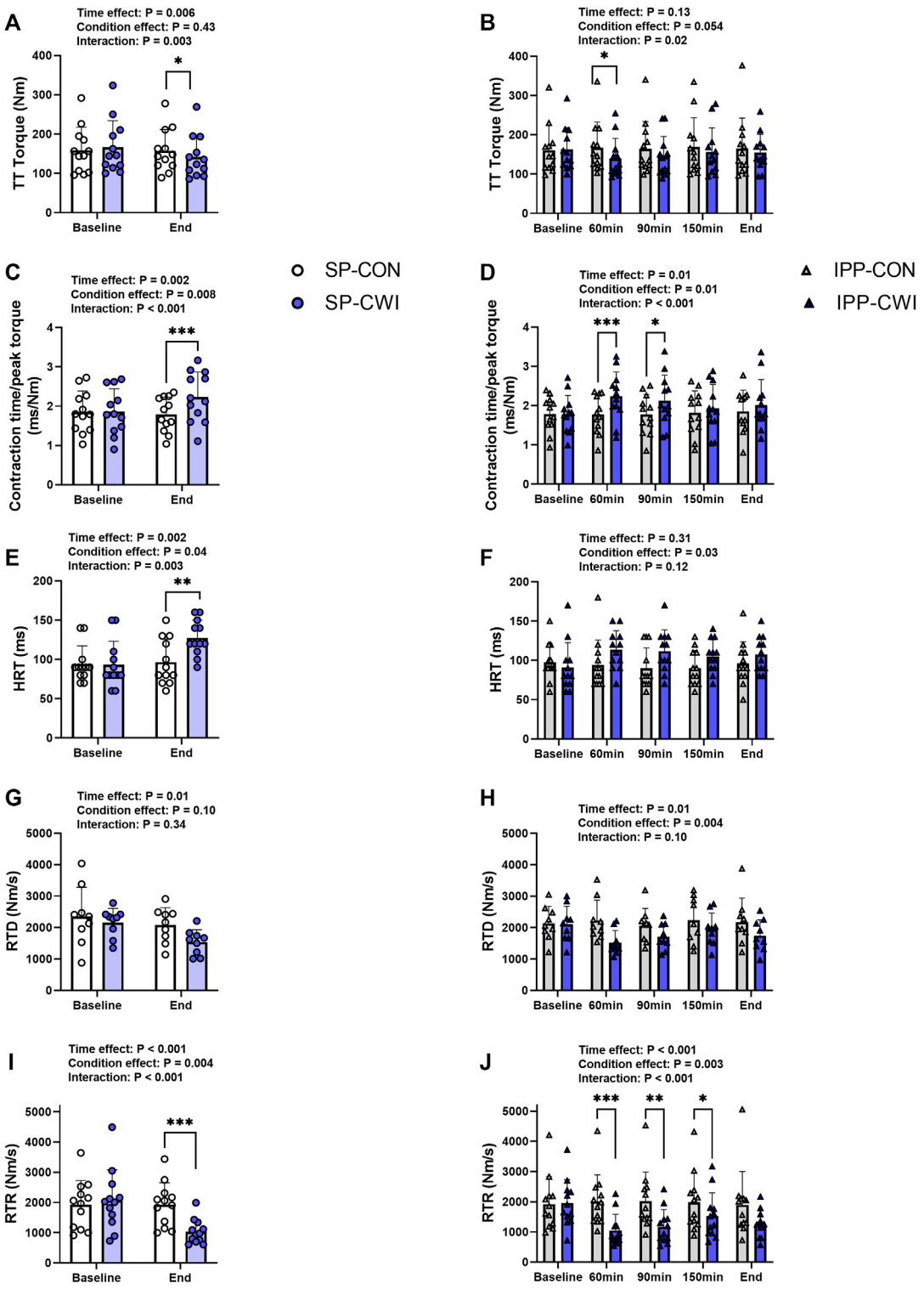

FIGURE 4
Peak torques and contractile properties derived from a 250-ms test train stimulation at 100 Hz (TT) in the unfatigued state. TT torques **(A,B)**, contraction time/peak torque **(C,D)**, half-relaxation time (HRT) **(E,F)**, peak rate of torque development (RTD) **(G,H)** and peak rate of torque relaxation (RTR) **(I,J)** during the single and intermittent/prolonged phases, respectively. Data are shown as mean ± SD and all panels include individual values. SP, single phase; IPP, intermittent/prolonged phase. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$: Significant differences CON vs. CWI. N = 12 for all parameters except for RTD (N = 9) **(G,H)**.

Geisser-Greenhouse corrections to correct for violation of the sphericity assumption. To compare the torque fatigue index and the changes in contractile properties during fatiguing protocol between SP-CON and IPP-CON, and between SP-CWI and IPP-CWI (Table 2), paired t-tests were used. Three participants were excluded from the analysis of RTD in the unfatigued state, and two

TABLE 2 Torque fatigue index and changes in contractile properties during the fatiguing protocol.

	SP-CON	IPP-CON	P	*d*	SP-CWI	IPP-CWI	P	*d*
Torque fatigue index (%)	54.4 ± 13.4	56.4 ± 12.4	0.16	−0.44	49.8 ± 13.0	55.5 ± 11.7	0.01	−0.86
Contraction time/peak torque (% change)	126.4 ± 81.2	129.5 ± 90.7	0.79	−0.08	92.0 ± 76.3	121.8 ± 77.2	0.02	−0.82
HRT (% change)	125.1 ± 81.0	121.4 ± 60.2	0.78	0.08	66.7 ± 60.1	108.1 ± 73.2	0.01	−0.88
RTD (% change)	−58.8 ± 17.3	−57.8 ± 15.8	0.77	−0.10	−53.5 ± 16.5	−58.2 ± 15.2	0.08	0.62
RTR (% change)	−75.3 ± 11.0	−74.9 ± 9.4	0.86	−0.06	−60.5 ± 12.9	−66.2 ± 20.8	0.19	0.45

d, Cohen's *d*, HRT, half-relaxation time; RTD, peak rate of torque development; RTR, peak rate of torque relaxation; SP, single phase; IPP, intermittent/prolonged phase.
Data are shown as mean ± SD., Torque fatigue index was calculated as: [(average of the first 3 contractions—average of the last 3 contractions)/average of the first 3 contractions] x 100.% change was calculated as: [(average of the last 3 contractions—average of the first 3 contractions)/average of the first 3 contractions] x 100.
N = 12 for all parameters except for RTD, and RTR (N = 10).

TABLE 3 Changes in torques and contractile properties in the unfatigued state.

	SP-CON	IPP-CON	P	*d*	SP-CWI	IPP-CWI	P	*d*
Δ P20 torque (Nm)	5.13 ± 15.87	5.70 ± 20.50	0.95	−0.02	0.03 ± 11.92	3.46 ± 17.17	0.54	−0.18
Δ P100 torque (Nm)	−0.02 ± 27.99	6.84 ± 27.09	0.65	−0.13	−30.29 ± 19.71	−9.34 ± 25.87	0.02	−0.79
P20/P100 (% change)	4.24 ± 7.01	0.28 ± 4.81	0.14	0.46	16.00 ± 10.96	7.95 ± 5.55	0.041	0.67
Δ TT torque (Nm)	−1.01 ± 14.39	4.68 ± 18.63	0.49	−0.20	−24.12 ± 18.50	−8.37 ± 20.80	0.047	−0.64
Δ Contraction time/peak torque (ms/Nm)	−0.06 ± 0.23	0.07 ± 0.15	0.16	−0.43	0.37 ± 0.19	0.24 ± 0.28	0.17	0.42
Δ HRT (ms)	2.50 ± 20.50	−1.67 ± 29.18	0.66	0.13	34.17 ± 21.51	16.67 ± 18.26	0.01	0.89
Δ RTD (Nm/s)	−270.0 ± 945.3	−36.7 ± 449.2	0.36	−0.32	−625.6 ± 158.1	−361.1 ± 225.9	0.007	−1.19
Δ RTR (Nm/s)	2.5 ± 287.3	−25.8 ± 385.9	0.88	0.05	−1041.7 ± 724.8	−691.7 ± 408.4	0.03	−0.74

d, Cohen's *d*, P20, electrically induced isometric torque at 20 Hz; P100, electrically induced isometric torque at 100 Hz; TT, 250-ms test train stimulation at 100 Hz; HRT, half-relaxation time; RTD, peak rate of torque development; RTR, peak rate of torque relaxation; SP, single phase; IPP, intermittent/prolonged phase.
Contractile properties (i.e., contraction time/peak torque, HRT, RTD, and RTR) are derived from the TT, stimulation. Data are shown as mean ± SD. Δ change was calculated as: end value—baseline value. % change was calculated as: [(end value—baseline value)/baseline value] x 100.
N = 12 for all parameters except for RTD (N = 9).

TABLE 4 Changes in maximal voluntary contraction torques and central activation ratio in the unfatigued state.

	SP-CON	IPP-CON	P	*d*	SP-CWI	IPP-CWI	P	*d*
Δ MVIC torque (Nm)	−16.96 ± 15.84	−21.38 ± 32.93	0.66	0.13	−26.58 ± 28.64	−16.55 ± 16.90	0.26	−0.35
CAR (% change)	−0.12 ± 2.14	0.49 ± 4.16	0.67	−0.13	−0.14 ± 1.17	0.94 ± 4.15	0.58	−0.16
Δ KE isokinetic torque (Nm)	−15.03 ± 15.23	−5.55 ± 15.20	0.14	−0.45	−33.85 ± 13.13	−24.69 ± 9.99	0.01	−0.90
Δ KF isokinetic torque (Nm)	1.37 ± 11.88	−7.10 ± 10.37	0.08	0.56	−29.76 ± 10.69	−16.58 ± 15.60	0.008	−0.93

d, Cohen's *d*, MVIC, maximal voluntary isometric contraction; CAR, central activation ratio; KE, knee extension; KF, knee flexion; SP, single phase; IPP, intermittent/prolonged phase.
Data are shown as mean ± SD. Δ change was calculated as: end value—baseline value. % change was calculated as: [(end value—baseline value)/baseline value] x 100.
N = 12.

participants were excluded from the analysis of RTD and RTR during the fatiguing protocol due to technical issues. One participant was also excluded from the analysis of Tmu due to incorrect measurements. The α-level of significance was set at $p < 0.05$. Partial eta squared (η_p^2) was determined to estimate the effect size for the two-way repeated-measures ANOVA. Cohen's d was calculated to interpret the magnitude of the mean difference between two conditions, and effect sizes were classified as small ($|d|$ from 0.2 to 0.5), moderate ($|d|$ from 0.5 to 0.8) and large ($|d|$ above 0.8).

3 Results

3.1 Physiological measurements

HR slightly decreased during the single and intermittent/prolonged phases (time effect during SP: $p = 0.003$, $\eta_p^2 = 0.31$; time effect during IPP: $p < 0.001$, $\eta_p^2 = 0.36$; Figures 2A,B) but was not affected by cooling. Diastolic BP was higher in CWI than CON during the intermittent/prolonged phase (condition effect:

p = 0.01, η_p^2 = 0.44; Figure 2E), but not during the single phase (Figure 2C). In addition, a significant condition effect was found for systolic BP during the single phase (p = 0.048, η_p^2 = 0.31; Figure 2D), while it almost reached the level of significance during the intermittent/prolonged phase (p = 0.053, η_p^2 = 0.30; Figure 2F). Trec remained roughly constant in both the CON and CWI conditions during the single phase and during the first 60 min of the intermittent/prolonged phase (Figures 2G,H). During the latter phase, Trec decreased in the CWI condition from 105 min and remained lower compared with the CON condition until the end (p < 0.05, d: 1.21 to −2.74). Tmu (at the three depths) were similarly reduced in CWI compared with CON at the end of the single phase and at 60 min during the intermittent/prolonged phase (p < 0.05, d: −1.18 to −2.89; Figures 2I,J). Tmu (at the three depths) remained lower in CWI than in CON until the end of the intermittent/prolonged phase (p < 0.05, d: 1.18 to −2.73). However, Tmu at 1 and 2 cm depth varied in CWI from 60 min to the end of the intermittent/prolonged phase. The Δ changes from baseline (i.e., end values—baseline values) were not different between the single and intermittent/prolonged phases in the CON condition for any of these physiological parameters, except for Tmu at 3 cm depth (p = 0.02, d = 0.88) (Table 1). The Δ changes for systolic BP, Trec, and Tmu (1 and 2 cm depth) were lower in IPP-CWI than in SP-CWI (p < 0.05, d reported in Table 1), while the Δ changes between these two conditions were not significantly different for HR, diastolic BP, and Tmu at 3 cm depth.

3.2 Electrically induced torques and contractile properties in the unfatigued state

Representative 20 Hz (P20) and 100 Hz (P100) torques are illustrated in Supplementary Figure S1A and results are presented in Figure 3. P20 torques remained constant during the single phase and slightly increased during the intermittent/prolonged phase (time effect, p = 0.02, η_p^2 = 0.25), but they were not affected by the cooling condition (Figures 3A,B). P100 torques were lower in CWI than in CON at the end of the single phase (p < 0.01, d = −1.13; Figure 3C) and only at 60 min during the intermittent/prolonged phase (p < 0.05, d = −0.95; Figure 3D). As a result, P20/P100 ratio increased in CWI compared with CON at the end of the single phase (p < 0.01, d = 1.28) and at 60 min during the intermittent/prolonged phase (p < 0.01, d = 1.57; Figures 3E,F). P20/P100 ratio was not different between CON and CWI at both 90 and 150 min during the intermittent/prolonged phase, while it became higher again in CWI than in CON at the end of this phase (p < 0.05, d = 0.98). The Δ changes from baseline (for P20 and P100 torques) and the % changes from baseline (for P20/P100 ratio) were not different between SP-CON and IPP-CON (Table 3). The Δ changes for P20 torques were not different between SP-CWI and IPP-CWI. However, the Δ changes for P100 torques and the % changes for P20/P100 ratio were larger in SP-CWI than in IPP-CWI (P100 torque: p = 0.02, d = −0.79; P20/P100 ratio: p = 0.041, d = 0.67).

A 250-ms test train stimulation (TT) was performed after P100 to assess the contractile properties in the unfatigued state. Representative TT torques are illustrated in Supplementary Figure S1 and results of TT and contractile properties are presented in Figure 4. Similar to P100 torques, TT torques were reduced in CWI compared with CON at the end of the single phase (p < 0.05, d = −0.96; Figure 4A) and only at 60 min during the intermittent/prolonged phase (p < 0.05, d = −1.11; Figure 4B). Contraction time/peak torque ratio was higher in CWI than in CON at the end of the single phase (p < 0.001, d = 1.51; Figure 4C) and after 60 min (p < 0.001, d = 1.63) and 90 min (p < 0.05, d = 1.05) during the intermittent/prolonged phase (Figure 4D). HRT was also greater in CWI compared with CON at the end of the single phase (p < 0.01, d = 1.25; Figure 4E). A significant condition effect was found for HRT during the intermittent/prolonged phase (p = 0.03, η_p^2 = 0.36; Figure 4F) without any significant condition × time interaction (p = 0.12, η_p^2 = 0.17). A significant condition effect was also found for RTD during the intermittent/prolonged phase (p = 0.004, η_p^2 = 0.67; Figure 4H) while only a tendency was observed during the single phase (p = 0.10, η_p^2 = 0.31; Figure 4G), without any significant condition × time interaction in both phases. RTR highly decreased in CWI compared with CON at the end of the single phase (p < 0.001, d = −1.59; Figure 4I) and at 60, 90, and 150min during the intermittent/prolonged phase (p < 0.05, d: 0.91 to −1.60; Figure 4J). The Δ changes from baseline were not different between SP-CON and IPP-CON conditions for TT torque, contraction time/peak torque ratio, HRT, RTD, and RTR (Table 3). Except for contraction time/peak torque ratio, the Δ changes for the other parameters of contractile properties were lower in IPP-CWI than in SP-CWI conditions (p < 0.05, d reported in Table 3).

3.3 Maximal voluntary contraction torques in the unfatigued state

Voluntary torque production was assessed from maximal voluntary isometric contraction (MVIC) and maximal isokinetic contraction of the knee extensors (KE-isoK) and knee flexors (KF-isoK). MVIC torques slightly decreased during the single (time effect, p = 0.001, η_p^2 = 0.62; Figure 5A) and intermittent/prolonged phases (time effect, p = 0.006, η_p^2 = 0.36; Figure 5B) but were not affected by cooling. CAR remained constant in both the CON and CWI conditions during the intermittent/prolonged phase (Figures 5C,D). KE-isoK torques were lower in CWI than in CON at the end of the single phase (p < 0.001, d = −1.50; Figure 5E) and only at 60min during the intermittent/prolonged phase (p < 0.01, d = −1.17; Figure 5F). KF-isoK torques decreased in CWI compared with CON at the end of the single phase (p < 0.001, d = −2.12; Figure 5G) and a similar result was found at 60 min (p < 0.01, d = −1.44) and 90 min during the intermittent/prolonged phase (p < 0.01, d = −1.21; Figure 5H). The Δ changes from baseline (for MVIC, KE-isoK torques, and KF-isoK torques) and the % changes from baseline (for CAR) were not different between SP-CON and IPP-CON conditions (Table 4). The Δ changes for MVIC torques and the % changes for CAR were not different between SP-CWI and IPP-CWI conditions, while the Δ changes for KE-isoK and KF-isoK torques were larger in SP-CWI than in IPP-CWI (KE-isoK torque: p = 0.01, d = −0.90; KF-isoK torque: p = 0.008, d = −0.93) (Table 4).

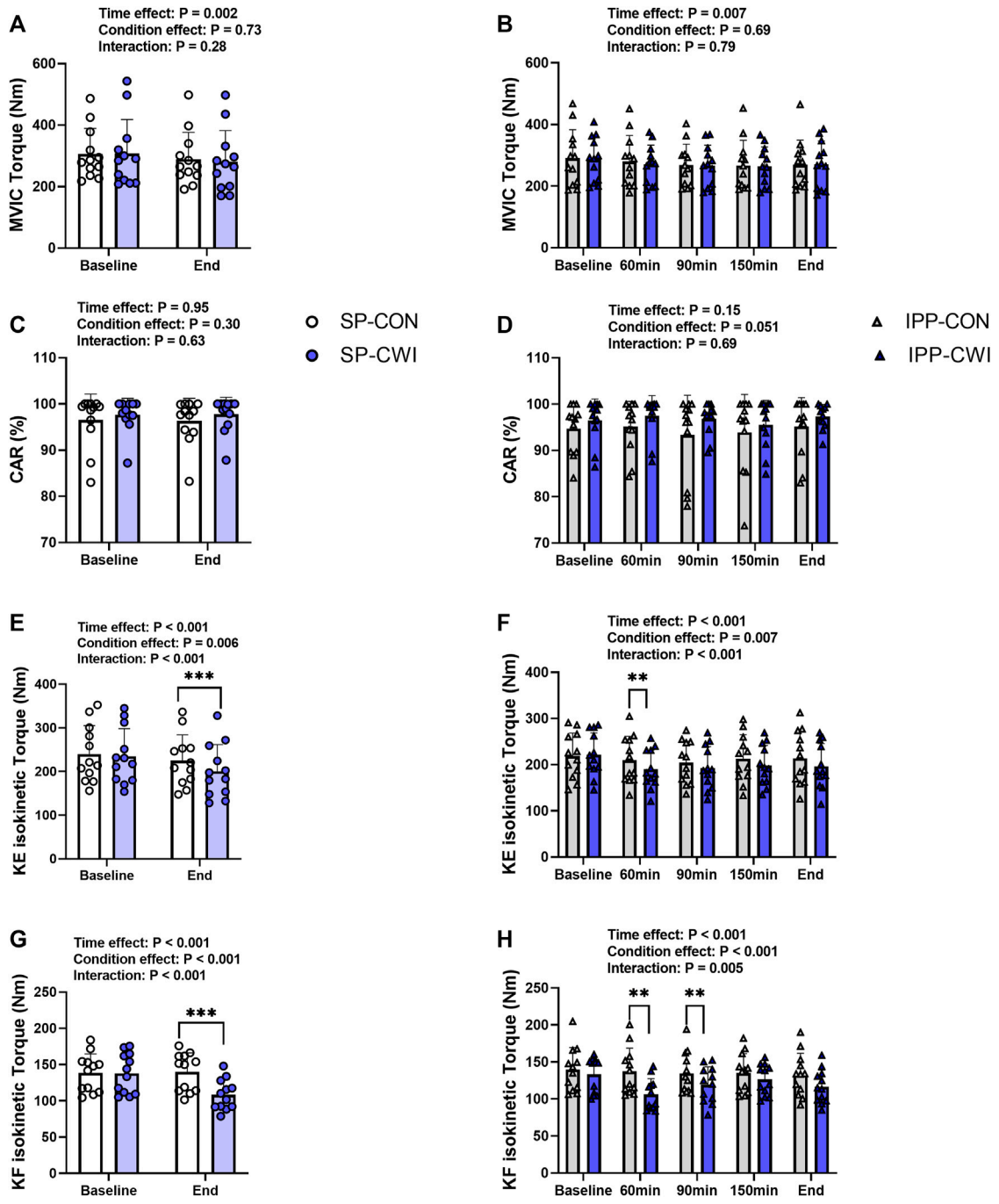

FIGURE 5
Maximal voluntary contraction torques and central activation ratio (CAR) in the unfatigued state. Maximal voluntary isometric contraction (MVIC) torques **(A,B)**, CAR **(C,D)**, knee extension (KE) maximal isokinetic torques **(E,F)**, and knee flexion (KF) maximal isokinetic torques **(G,H)** during the single and intermittent/prolonged phases, respectively. Data are shown as mean ± SD and all panels include individual values. SP, single phase; IPP, intermittent/prolonged phase. **, $p < 0.01$; ***, $p < 0.001$: Significant differences CON vs. CWI. N = 12.

3.4 Electrically induced torques and contractile properties during fatiguing protocol

A fatiguing protocol, consisting of 100 electrical stimulations of the knee extensor muscle [250-ms test train stimulation at 100 Hz (TT) interspaced with 1-s break], was performed at the end of the single (i.e., at 60 min) and intermittent/prolonged (i.e., 300 min) phases. TT torques and the contractile properties were determined, and results are presented in Figure 6, Supplementary Table S1 and in Table 2. Representative torques are illustrated in Supplementary Figure S1B. Decreases in TT torques, RTD and RTR, and increases in contraction time/peak torque ratio and HRT were found over the fatiguing protocol in CON and CWI conditions in both phases, as

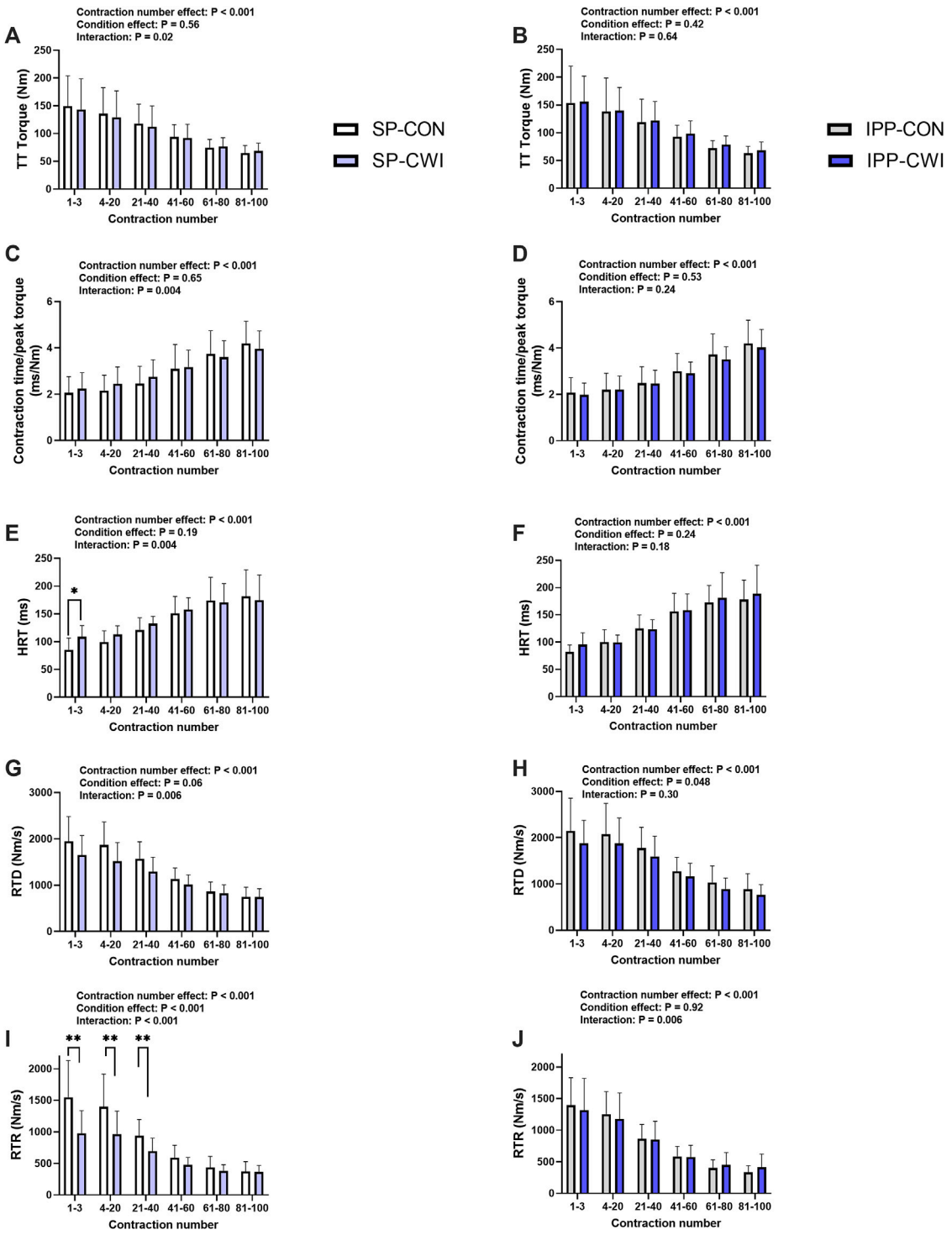

FIGURE 6
Torques and contractile properties during the fatiguing protocol. Torques during 250-ms test train stimulations at 100 Hz (TT torques) **(A,B)**, contraction time/peak torque **(C,D)**, half-relaxation time (HRT) **(E,F)**, peak rate of torque development (RTD) **(G,H)**, and peak rate of torque relaxation (RTR) **(I,J)** during the single and intermittent/prolonged phases, respectively. Data are shown as mean ± SD. SP, single phase; IPP, intermittent/prolonged phase. *, $p < 0.05$; **, $p < 0.01$: Significant differences CON vs. CWI. N = 12 for all parameters except for RTD (N = 10) **(G,H)** and RTR (N = 10) **(I,J)**.

illustrated in Figure 6 (contraction number effect, $p < 0.001$, η_p^2: 0.72–0.84) and Supplementary Table S1, and in Table 2. Significant condition x contraction number were found for all these parameters during the single phase ($p < 0.02$, η_p^2: 0.35–0.76), but not during the intermittent/prolonged phase (except for RTR, $p = 0.006$, $\eta_p^2 = 0.40$) (Figure 6). In addition, two-way repeated-measures ANOVA revealed significant phase x contraction number interactions for all these parameters in the CWI condition ($p < 0.02$), but not in the

CON condition (Supplementary Table S1). During the single phase, *post hoc* analyses detected higher HRT during the first 3 contractions in CWI compared with CON ($p < 0.05$, $d = 1.08$; Figure 6E), and lower RTR during the first 40 contractions in CWI compared with CON ($p < 0.01$, d: −1.74 to −1.76; Figure 6I). Although similar tendencies were observed for the other parameters during the single phase (i.e., higher contraction time/peak torque ratio, and lower TT torques and RTD specifically during the first 40 contractions in CWI vs. CON conditions), comparisons between the two conditions did not reach the level of significance (Figures 6A, C, G). Similarly, tendencies to higher TT torques, RTD and RTR, and tendencies to lower contraction time/peak torque ratio and HRT were noticeable in IPP-CWI compared with SP-CWI during the first 40 contractions, but Sidak's multiple comparisons tests did not reveal any significant differences (Supplementary Table S1). Torque fatigue index and changes in contractile properties during the fatiguing protocol were not different between SP-CON and IPP-CON (Table 2). In contrast, torque fatigue index, and the increases in contraction time/peak torque ratio and in HRT were lower in SP-CWI than in IPP-CWI ($p < 0.02$, d: −0.82 to −0.88), while they were in the same range after IPP-CWI and after CON conditions. The decreases in RTD and RTR during the fatiguing protocol were not significantly different between SP-CWI and IPP-CWI.

4 Discussion

To our knowledge, this is the first study investigating the impact of moderate muscle cooling induced by single and intermittent/prolonged CWI exposures on muscle force production and muscle contractility in unfatigued state and during repeated electrically induced contractions in humans. In the unfatigued state, both SP-CWI and IPP-CWI reduced P100 and TT torques, and increased P20/P100 ratio, but the changes from baseline were less pronounced in IPP-CWI than in SP-CWI. Overall, moderate muscle cooling slowed down muscle contraction and relaxation to a lesser extent after IPP-CWI than SP-CWI. Muscle cooling neither affected MVIC torque nor CAR, while the reductions of KE and KF isokinetic torques by cooling were less marked after IPP-CWI than SP-CWI. During the early phase of the fatiguing protocol, SP-CWI impaired muscle contractile properties (especially HRT and RTR) compared with SP-CON, while IPP-CWI did not affect these parameters. During the fatiguing protocol, torque fatigue index and the changes in muscle contractile properties were larger (with $p < 0.05$ only found for contraction time/peak torque ratio and HRT) after IPP-CWI than SP-CWI, but were in the same range as after CON conditions. These differences between the two cooling modalities were accompanied by a lower reduction of superficial Tmu and a smaller increase in systolic BP directly after intermittent/prolonged than single CWI, suggesting a reduced vasoconstriction response after intermittent/prolonged cold water immersions.

4.1 Impact of moderate muscle cooling on MVIC torque and CAR in unfatigued state

Our results showed that moderate muscle cooling, as evidenced by a ~4°C decline in Tmu at 3 cm depth, did not impair MVIC. This result is in contrast with previous studies including voluntary isometric contractions of the KE muscles, in which moderate muscle cooling reduced MVIC torque by ~6% (Bergh and Ekblom, 1979) and ~20% (Brazaitis et al., 2011) compared with the control condition. In Brazaitis et al. study (Brazaitis et al., 2011), the torques developed by the male participants in the control condition were ~20% higher than those reported in the current study, and some methodological aspects were different compared to this work (e.g., KE performed with a different knee joint angle, different dynamometers used, different CWI protocols, etc.). Although it remains unclear, these differences might explain the discrepancy between the two studies. Previous works indicated that MVIC force declined by 1%–2% for every 1°C of decreasing Tmu (Asmussen et al., 1976; Bergh and Ekblom, 1979), while others proposed that maximal isometric force production is relatively stable within the muscle temperature range from 27°C to 40°C (Clarke et al., 1958; Petrofsky and Phillips, 1986). Altogether, the moderate reduction of Tmu observed in the current study was not sufficient to impair MVIC torque production. Furthermore, and in line with previous studies (Brazaitis et al., 2014b; Spillane and Bampouras, 2021), we observed that CWI did not affect voluntary activation (assessed from CAR) during MVIC, providing additional evidence that muscle cooling does not impact motor drive when the muscle is unfatigued.

4.2 Impact of moderate muscle cooling on maximal isokinetic torque and muscle contractile properties in unfatigued state

In our study, moderate muscle cooling impaired maximal isokinetic torque, a result previously observed in the KE muscles (Bergh and Ekblom, 1979), and in other types of dynamic exercises (Asmussen et al., 1976). To our knowledge, this is the first time that it is reported during isokinetic contractions of the KF muscles. Interestingly, the torque decline was more pronounced for KF-isoK contraction than for KE-isoK contraction (−23% and 11% at the end of SP-CWI, respectively). This may result from the faster contraction profile of the hamstrings muscle compared to the quadriceps muscle (Garrett et al., 1984), which may make the hamstring muscle more sensitive to thermal changes (Bennett, 1984). Muscle cooling particularly impairs maximal concentric strength because peak torque is only reached at a specific joint angle during the movement, and thus the time available to attain peak torque is much shorter than during MVIC (Bergh and Ekblom, 1979). Muscle cooling may extend the time required to fully activate the motor units due to lower nerve conduction velocity (Algafly and George, 2007). In addition, muscle cooling could reduce the speed of chemical reactions, leading to a slower excitation of the sarcolemma (Brazaitis et al., 2016), a slower rate of Ca^{2+} release (and uptake) from the sarcoplasmic reticulum (Kössler et al., 1987), and a delay in the formation (and dissociation) of the cross-bridges (Faulkner et al., 1990). These changes would consequently reduce the rate of force development and ability to quickly reach peak torque during dynamic movements. Furthermore, some studies have demonstrated that severe muscle cooling impairs muscle contractile properties and induces a left shift of the stimulation frequency-isometric force relationship (Davies et al., 1982; de Ruiter

et al., 1999). In the current study, we provided clear evidence that moderate muscle cooling also elicits a shift towards a slower muscle contractile profile, as shown by an increase in P20/P100 ratio and by altered muscle contractile properties (increased contraction time/peak torque ratio and HRT, decreased RTR). These changes are certainly the result of the peripheral muscle effects mentioned above and are most likely independent of changes in Trec (Giesbrecht et al., 1995). The fact that P20 torque was not affected by moderate cooling, while P100 torque was reduced, suggests that actomyosin sensitivity to Ca^{2+} was probably not impaired by cooling in our study (de Ruiter et al., 1999).

4.3 Comparison of the changes in muscle contractile function between SP-CWI and IPP-CWI in unfatigued state and during the development of fatigue

In accordance with our hypothesis, we showed that the changes in muscle contractile function observed in response to moderate muscle cooling were less pronounced after IPP-CWI than SP-CWI. This was observed in unfatigued state for KE- and KF-isoK torques, P100 and TT torques, P20/P100 ratio, and muscle contractile properties (HRT, RTD, and RTR), but not for MVIC torque. Compared with the CON condition, SP-CWI impaired muscle contractile properties (especially HRT and RTR) during the early phase of the fatiguing protocol, while IPP-CWI did not substantially affect these parameters. Upon cold exposure, early physiological responses include cutaneous vasoconstriction and reduced skin blood flow, leading to decreased convective heat transfer between the body's core and shell (i.e., skin, subcutaneous adipose tissue, and skeletal muscle), which results in an effective maintenance of core temperature (Castellani and Young, 2016). In our study, the reduction of Tmu observed superficially (at 1 cm depth) was less pronounced after IPP-CWI than SP-CWI (−4.83°C vs. −9.73°C, respectively), and the changes in systolic BP were lower after IPP-CWI than SP-CWI. Although skin temperature and cardiac output were not assessed in this study, our findings suggest that peripheral resistance and cutaneous vasoconstriction response were likely lower after intermittent/prolonged compared with single cooling. These physiological adjustments may have led to an increased supply of warm blood from the core to the cutaneous and superficial muscle vessels, which consequently limited the reduction of Tmu (superficially) and decreased body insulation and core temperature during IPP-CWI (Tansey and Johnson, 2015). Several factors could explain this physiological response to intermittent cooling and referred as cold habituation (Castellani and Young, 2016). For instance, a reduced sensitivity of the thermoreceptors [transient receptor potential (TRP) channels], and a decreased discharge frequency of cold-sensitive thermoreceptors may result in a reduced neural flux to the neural system for thermoregulatory response (Hensel and Zotterman, 1951; Vybíral et al., 2000; Brazaitis et al., 2014a). Fatigue of smooth muscle has been previously reported (Yamanishi et al., 1994), suggesting that this could also occur in vascular smooth cells during intermittent/prolonged cooling, which may have compromised the vasoconstriction response (Castellani et al., 1998). Furthermore, it is unlikely that sitting at thermoneutral room temperature has evoked superficial vasodilatation because TRPM8 cold channels are normally activated at temperature below 27°C (Fujita et al., 2013). It is however possible that muscle contractions and dynamic movements from the bath to the chair (and *vice versa*) and on the dynamometer induced small changes in muscle blood perfusion, but they were insufficient to affect Tmu, as evidenced by the absence of differences in Tmu between 150 and 165 min (i.e., directly before and after the fourth neuromuscular testing) in CWI condition (see Figure 2J).

As shown in Table 2, torque fatigue index and the changes in contractile properties (contraction time/peak torque ratio and HRT) during repeated contractions induced by high frequency electrical stimulation were more elevated after intermittent/prolonged-CWI than after single-CWI but were in the same range as after CON conditions. This result indicates that a moderate and homogenous reduction of Tmu (4°C–5°C at the three depths) after intermittent/prolonged CWI has no major effect on muscle fatigability and contractility during repeated contractions, while these Tmu changes led to a mild impairment of muscle contractile function in unfatigued state. The homogenous (4°C–5°C) and heterogeneous (4°C–10 °C) decline in Tmu after intermittent/prolonged and single cooling, respectively, most likely explain the differences in functional outcomes observed in this study. Differences in muscle blood perfusion between the two cooling modalities, especially at the more superficial layers, might be another explanatory factor. Indeed, recent findings indicate that changes in muscle perfusion in response to CWI at low temperature (8 °C for 10 min), although modest, are not uniform across the quadriceps muscle (Mawhinney et al., 2020).

4.4 Limitations

One aspect that remains unclear is whether cold habituation (i.e., lower decline in peripheral Tmu and potential reduction of vasoconstriction response) observed in this study is the result of intermittent cooling, prolonged body cold exposure, or the combination of both. As explained in the method section, intermittent CWI was used to induce a moderate and relatively stable reduction of Tmu in the deep portion of the vastus lateralis muscle, while avoiding hypothermia and too much discomfort for the participants. Cold habituation has been observed during repeated CWI (3 times 2 h with 2 h break; whole-body CWI at 20°C) where Trec was fully recovered before each cold bath (Castellani et al., 1998). Although the exact cause of cold habituation remains unclear, our study suggests that cold habituation could also occur when core temperature is reduced. As shown in this study, the reduction of superficial Tmu was larger than that of deep Tmu after SP-CWI, while the reduction of Tmu was homogeneous across the 3 depths after IPP-CWI. To be able to clearly answer whether the duration of muscle cooling affects muscle contractile function, an adequate protocol in which the reduction of both superficial and deep Tmu remain stable over several hours would be required. In addition, additional physiological parameters, including skin temperature, cardiac output, and cutaneous and muscle blood flow could be examined to further investigate vasoconstriction response. Furthermore, although Trec is almost exclusively used in the literature to estimate core temperature in

response to CWI, it may underestimate the cooling rates (Miller et al., 2017). Finally, core temperature (estimated from Trec) seems to decrease faster in females than males in response to CWI following hyperthermia, which may be due to several factors, including sex-differences in body surface area, lean body mass, and body surface area to lean body mass ratios (Boehm and Miller, 2019). Therefore, it is possible that changes in Tmu in response to cooling are different between females and males, which may influence the outcomes of muscle contractile function assessed in this study.

4.5 Conclusion

We provide evidence that moderate muscle cooling impairs force production during electrically stimulated contractions at high frequency and during voluntary isokinetic contractions, but not during MVIC. In addition, it slows down muscle contractile properties in unfatigued state and during the early phase of the fatiguing protocol. Our results indicate that in comparison with single CWI, intermittent/prolonged CWI has a less severe impact on muscle function in unfatigued state. In contrast, during repeated contractions, muscle fatigability and the changes in muscle contractile properties are larger after IPP-CWI than after SP-CWI but are in the same range as after CON conditions. We believe that intermittent/prolonged CWI induces a less pronounced fast-to-slow contractile transition compared to single CWI, and this physiological response may result from the reduced vasoconstriction response and enhanced blood perfusion of the superficial layers of the muscle, which could ultimately limit the reduction of superficial Tmu.

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 authors.

Ethics statement

The study protocol was approved by Kaunas Regional Biomedical Research Ethics committee (no. P1-BE-2–14/2022). The patients/participants provided their written informed consent to participate in this study.

Author contributions

VT, TV, MB, and TC conceived and designed the research study. VT and NE performed experiments. VT, NE, and TC analyzed data. VT, TV, MB, and TC interpreted results of experiments. VT, NE, and TC prepared figures. VT and TC drafted the manuscript. VT, NE, TV, MB, and TC edited and revised the manuscript. VT, NE, TV, MB, and TC approved the final version of 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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1172817/full#supplementary-material

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Combined effects of exercise and different levels of acute hypoxic severity: A randomized crossover study on glucose regulation in adults with overweight

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Purpose: The aim of this study was to investigate the influence of manipulating hypoxic severity with low-intensity exercise on glucose regulation in healthy overweight adults.

Methods: In a randomized crossover design, 14 males with overweight (age: 27 ± 5 years; body mass index (BMI) 27.1 ± 1.8 kg·m^2) completed three exercise trials involving 60 min aerobic exercise cycling at 90% lactate threshold in normoxia (NM, FiO_2 = 20.9%), moderate hypoxia (MH, FiO_2 = 16.5%) and high hypoxia (HH, FiO_2 = 14.8%). A post-exercise oral glucose tolerance test (OGTT) was performed. Venous blood samples were analyzed for incremental area under the curve (iAUC), plasma glucose and insulin, as well as exerkine concentrations (plasma apelin and fibroblast growth factor 21 [FGF-21]) pre- and post-exercise. A 24-h continuous glucose monitoring (CGM) was used to determine interstitial glucose concentrations. Heart rate, oxygen saturation (SpO_2) and perceptual measures were recorded during exercise.

Results: Post-exercise OGTT iAUC for plasma glucose and insulin concentrations were lower in MH vs. control (p = 0.02). Post-exercise interstitial glucose iAUC, plasma apelin and FGF-21 were not different between conditions. Heart rate was higher in HH vs. NM and MH, and MH vs. NM ($p < 0.001$), while SpO_2 was lower in HH vs. NM and MH, and MH vs. NM ($p < 0.001$). Overall perceived discomfort and leg discomfort were higher in HH vs. NM and MH ($p < 0.05$), while perceived breathing difficulty was higher in HH vs. NM only (p = 0.003).

Conclusion: Compared to higher hypoxic conditions, performing acute aerobic-based exercise under moderate hypoxia provided a more effective stimulus for improving post-exercise glucose regulation while concomitantly preventing excessive physiological and perceptual stress in healthy overweight adults.

KEYWORDS

hypoxia, low-intensity exercise, exerkines, apelin, FGF-21

Introduction

The current obesity epidemic is a global health issue evidenced through a tripling of obesity incidence since 1975 and has emerged as the leading cause of non-communicable diseases (WHO, 2021). Individuals with overweight or obesity, defined as body mass index (BMI) ≥25 and ≥30 kg·m^2, respectively, are at higher risk of impaired metabolic homeostasis, reduced insulin sensitivity (Krogh-Madsen et al., 2010) and postprandial lipid metabolism (Booth et al., 2012), which can be contributed by physical inactivity (WHO, 2021). Regular physical activity or exercise exerts numerous health benefits such as improved cardiovascular fitness, anabolic (e.g., increased muscle mass) (Callahan et al., 2021) and metabolic (e.g., enhanced mitochondrial biogenesis and substrate metabolism) adaptations (Coffey and Hawley, 2007; Gibala and McGee, 2008; MacInnis and Gibala, 2017), and reduced levels of circulating pro-inflammatory markers that collectively reduce all-cause mortality and improve quality of life (Chakravarty et al., 2008; Hawley et al., 2014).

Hypoxic exposure (i.e., reduction of oxygen supply to tissues) combined with exercise can synergistically increase adaptations associated with exercise (Tee et al., 2023). For instance, elite athletes regularly engage in hypoxic training to improve muscular adaptations (i.e., citrate synthase, mitochondria density) (Hoppeler et al., 2008), physiological regulatory systems (i.e., haemoglobin mass) and ultimately, physical performance (Wilber, 2007; Saunders et al., 2009). In clinical settings, acute (i.e., single bout) (Mackenzie et al., 2011; Mackenzie et al., 2012b) and chronic (i.e., 3 times/week for 4–6 weeks) (Haufe et al., 2008; Wiesner et al., 2010; De Groote et al., 2018) exercise in combination with hypoxia (~2,700–3,100 m) can lead to improved metabolic health markers (e.g., plasma glucose and insulin), body compositional changes and cardiorespiratory health in people with overweight or obesity (Fernandez Menendez et al., 2018). These beneficial effects are of greater relevance to people with overweight or obesity considering the addition of hypoxia to exercise training can induce similar or greater physiological adaptations despite a lower absolute exercise intensity (Fernandez Menendez et al., 2018). Moreover, greater metabolic stress induced at a lower exercise intensity under hypoxic conditions can also reduce muscle/joint load and mechanical strain, which is important considering such extraneous strain is an established source of injury and exercise reluctance due to increased body mass in cohorts with overweight and obesity (Millet et al., 2016; Pramsohler et al., 2017; Jung et al., 2021; Tee et al., 2022).

While the underlying mechanisms of blood glucose regulation with combined exercise and hypoxia remain largely unknown, exercise-secreted factors (i.e., "exerkines") may play a role in orchestrating this process (Chow et al., 2022). Exerkines such as apelin and fibroblast growth factor 21 (FGF-21) have been implicated in mediating improvements in insulin sensitivity and substrate metabolism with exercise, respectively (Carson, 2017; Garneau and Aguer, 2019; Laurens et al., 2020). Studies have reported that acute aerobic exercise under normoxia can regulate the secretion of apelin (Besse-Patin et al., 2014; Son et al., 2019) and FGF-21 (Kim et al., 2013; Taniguchi et al., 2016; Tanimura et al., 2016) in humans. While the effects of exercise under hypoxia on circulating apelin and FGF-21 remain unknown, hypoxia stimulation in cultured adipocytes has been shown to induce the secretion of apelin (Glassford et al., 2007; Geiger et al., 2011) and FGF-21 (Wu et al., 2020). Such data suggest that the secretion of exerkines such as apelin and FGF-21 could be modulated by combined exercise and hypoxia. To the best of our knowledge, no studies have examined the plasma concentrations of apelin and FGF-21 in response to combined exercise and hypoxia. Therefore, it is important to examine the changes in these exerkines to understand the potential mechanisms of blood glucose regulation in response to combined exercise and hypoxia.

Accumulating research demonstrates numerous health benefits from performing acute or chronic exercise under low to moderate hypoxic conditions. However, some evidence suggests that exercising at severe levels of hypoxia (>3,000 m) may lead to detrimental physiological responses such as increased oxidative stress and inflammation (Ristow and Schmeisser, 2014; Burtscher et al., 2022). While combined exercise and hypoxia can improve blood glucose regulation (Mackenzie et al., 2011; De Groote et al., 2018), there is no current consensus in the literature regarding the effective level of hypoxia to induce the greatest improvements. Previous studies have shown either decreased (Lei et al., 2022) or increased blood glucose levels (Kon et al., 2015) following sprint interval exercise in healthy active participants, regardless of different hypoxic levels. However, no study to date has compared different levels of hypoxic stimuli combined with acute low-intensity exercise on glucose tolerance, or incorporated continuous blood glucose monitoring, to provide a more comprehensive evaluation of whether potential improvements in blood glucose responses can be optimized with different levels of hypoxic severity in overweight adults (Tee et al., 2023). Therefore, the primary aim of this study was to investigate the acute effect of normoxia, moderate hypoxia, or high hypoxia combined with low-intensity aerobic cycle exercise, on blood glucose regulation. A secondary aim of our study was to determine whether different levels of hypoxic stimuli differentially regulate the expression of apelin and FGF-21. Compared to normoxia and high hypoxia, we hypothesized that the addition of a moderate hypoxic stimulus to low-intensity exercise would improve glucose regulation and exerkines in adults with overweight.

Methods

Participants

Fourteen overweight, physically inactive males (mean ± SD, age: 27 ± 5 years; height 175 ± 0 cm; body mass (BM) 83.0 ± 7.0 kg; body mass index (BMI) 27.1 ± 1.8 kg·m^2; body fat percentage (BF) 28.4% ± 2.5%; lactate threshold (LT) 90 ± 25 W), participated in this study after meeting the eligibility criteria. Eligible participants were those with a BMI between 25.0–29.9 kg·m^2, normotensive (90–120 and 60–80 mmHg systolic and diastolic blood pressure, respectively), no known cardiovascular, metabolic, or physiological disease, physically inactive (<150 min/week of physical activity), and no exposure to altitude (≥1,000 m) within 3 months prior to participation. Participants were screened for eligibility and their physical activity levels were measured using the Adult Pre-exercise Screening System (APSS) (Exercise & Sports Science Australia,

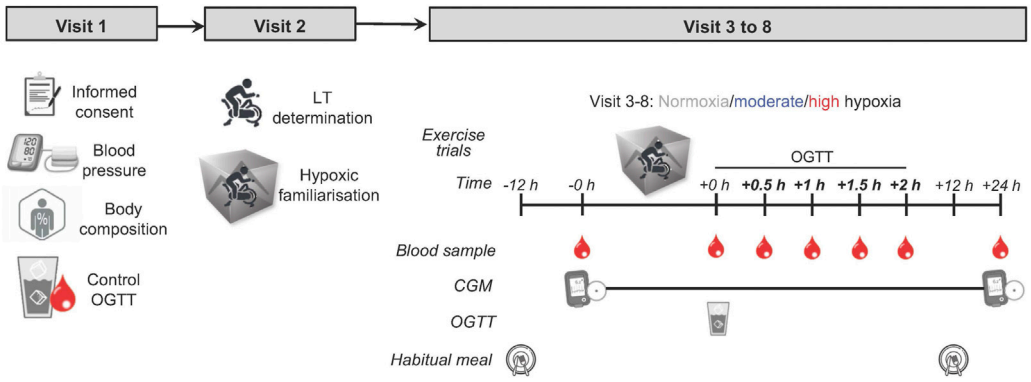

FIGURE 1
Study protocol: an overview of the entire study. Eligible healthy, overweight participants (n = 14) completed three exercise trials in three altitude conditions in a randomized order separated by ≥ 7 days 75 g of glucose dissolved in water was provided immediately following each exercise trial for the measurement of OGTT. Blood samples were collected pre, immediately post, every 0.5 h for 2 h post and post-24 h each exercise trial. OGTT, oral glucose tolerance test; LT, lactate threshold test; CGM, continuous glucose monitor.

2019). This study was approved by the Human Research Ethics Committee of the National Sports Institute of Malaysia (ISNRE/A/008/2020–003/2020), Swinburne University of Technology (Australia) (20225950-9155) and registered on ClinicalTrials.gov (trial identifier: NCT05577429). The study was undertaken in accordance with the *Declaration of Helsinki* and written informed consent was obtained from each participant.

Experimental protocol

An overview of the experimental protocol is shown in Figure 1. Participants attended the laboratory for a total of eight visits (two baseline visits, three exercise trials, and three post-24 h follow-up visits). Throughout the experimental period, participants were instructed to maintain their habitual diet and activities of daily living. For each visit, participants were instructed to attend the laboratory following an overnight fast for at least 10 h. During the first visit, blood pressure and body composition were measured and a control (CTL) 2 h oral glucose tolerance test (OGTT) was conducted (described subsequently). Body composition parameters including body mass, height, and body fat percentage (BF) were measured using bioimpedance analysis (Inbody 770, Cerritos, CA, United States) in light clothing.

During the second visit, participants were asked to perform a lactate threshold (LT) test. Participants cycled on a cycle ergometer (Velotron Racermate, Seattle, United States) with an initial load of 50 W. The load was increased progressively in 15 W increments every 4 min, with participants maintaining a constant pedal frequency (cadence ~90 rpm) until reaching LT, which is defined as the power output preceding a sudden and sustained increase in lactate (≥1.0 mmol/L greater than baseline), as described previously (Farrell et al., 1979). Heart rate (HR) was recorded throughout the test using a heart rate monitor (Polar H10, Polar Electro OY, Kempele, Finland). Ratings of perceived exertion (RPE) were recorded at the end of every 4 min stage using Borg 1–10 scale. The first and second visits were conducted at least a week prior to the third visit (exercise trial).

For visits 3 to 8, three exercise trials were carried out in a single-blinded randomized crossover design. The order of conditions was determined by random assignment and implemented by an investigator who was not involved with the exercise trials. Each trial was separated by at least 7 days. All exercise trials were conducted at 7:30 a.m. The exercise trials consisted of a 60-min cycling bout at 90% of LT under three simulated hypoxic conditions: i) normoxia near sea level (NM; FiO_2 = 20.9%), ii) normobaric moderate hypoxia (MH; FiO_2 = 16.5% corresponding to a simulated altitude of ~2000 m), and iii) normobaric high hypoxia (HH; FiO_2 = 14.8%, ~3,000 m). Considering excessive hypoxia (severe, >3,000 m) can cause adverse health effects such as high-altitude sickness, the levels of hypoxia selected in our study were chosen based on safety and practical reasons while also ensuring adequate metabolic stress was induced (Millet et al., 2016; Burtscher et al., 2022). All exercise trials were conducted in an environmental chamber (Welltech Instruments, Hong Kong) with temperature and relative humidity maintained at 20 °C and 40%, respectively.

Measurements on exercise trial days

A continuous glucose monitor sensor (CGM; FreeStyle Libre™, Abbott Diabetes Care, Witney, UK) was placed on the back of the upper arm, according to the manufacturer's instructions. Participants were instructed to scan the sensor with a CGM reader every 8 h to minimize missing data.

Venous blood samples (EDTA) were collected from antecubital vein via venipuncture at pre-, immediately post- (within 5 min after the cessation of exercise), and 24 h post-exercise. During the cycling exercise, HR and oxygen saturation (SpO_2) were recorded. After the exercise, participants were asked to reflect on their subjective perceptions, including their ratings of overall perceived discomfort, perceived breathing difficulty, and leg discomfort, using modified Borg CR10 scales (Tee et al., 2022). Symptoms of acute mountain sickness were assessed using the Lake Louise Questionnaire (Roach et al., 2018) at the end of the exercise.

Participants displayed no symptoms of acute mountain sickness during hypoxic exposures.

Oral glucose tolerance test (OGTT)

A 2 h oral glucose tolerance test was performed immediately after exercise. A 20-gauge cannula was inserted into the dorsal hand vein for the collection of arterialized-venous blood. Participants consumed 75 g of glucose (Glucolin glucose powder) dissolved in 250 ml of water. Venous blood samples (EDTA and sodium fluoride) were drawn immediately post glucose consumption and at 30-min intervals up to 120 min (30, 60, 90, and 120 min).

Biochemical analysis

Upon collection, blood samples were centrifuged at 2,000 x *g* for 10 min at 4 °C. Plasma glucose was measured using a biochemistry analyser (YSI 2900D Biochemistry Analyzer, Yellow Springs, OH, United States) with a coefficient of variation (CV) of <2.0%. Commercially available enzyme-linked immunoassay kits were used to analyse plasma insulin (Insulin ELISA, DE2935, Demeditec, Germany), plasma apelin (Apelin-12 EIA, EK-057–23, Phoenix Pharmaceuticals, Inc., CA, United States; $n = 8$), and plasma FGF-21 (Quantikine Human FGF-21 ELISA, DF2100, R&D Systems, MN, United States; $n = 8$) concentrations. The intra-assay CV was <2.6%, <10.0% and <3.9% for insulin, apelin and FGF-21, respectively, whereas the inter-assay variability was <6.0%, <15.0%, and <10.9%. All assays were performed according to the manufacturer's instructions and were measured in duplicate.

Statistical analysis

Sample size estimation was determined from *a priori* power analysis, using software G*Power (v3.1.9.7) to detect differences (effect size = 0.58, power of 0.80, alpha of 0.05) based on previous work comparing the effects of exercise cycling under normoxic and normobaric hypoxic (FiO_2 = 14.6%) conditions on the glucose area under the curve in response to an intravenous glucose tolerance test (Mackenzie et al., 2011). It was determined that 12 participants were required, with 14 recruited allowing for a 20% attrition rate. Incremental area under the curve (iAUC) for 2 h OGTT venous plasma glucose and insulin concentrations were calculated using the trapezoid method (Potteiger et al., 2002). Total AUC (AUC_{total}) for 24 h interstitial glucose derived from CGM was calculated using the trapezoid method. All data were analyzed using one-way repeated-measures analysis of variance (ANOVA) to compare between hypoxic conditions. Physiological and perceptual measures between hypoxic conditions were analyzed using one-way repeated-measures ANOVA. Differences over time (pre, post, and 24 h post) and conditions (NM, MH and HH) of plasma apelin and FGF-21 concentrations were evaluated by two-way repeated measures of ANOVA. Bonferroni-adjusted *p* values were performed if the main effect was observed. Effect sizes were described in terms of partial eta-squared (η^2, with $\eta^2 \geq 0.06$ representing a moderate effect and $\eta^2 \geq 0.14$ a large effect) (Cohen, 2013). Due to our sample size (n < 20), Hedge's *g* effect sizes were assessed to determine meaningful differences (with g = 0.38–0.75 representing a moderate effect and $g \geq 0.76$ representing large effect sizes) (Brydges, 2019). All data are expressed as mean ± SD. All statistical tests were carried out using GraphPad Prism version 9.2.0 (GraphPad Software, San Diego, CA). Statistical significance was set at the level of $p < 0.05$.

TABLE 1 Participant characteristics.

Variables	Males (n = 14)
Fasting concentration	
Fasting glucose (mmol/L)	4.9 ± 0.4
Fasting insulin (μU/mL)	7.2 ± 1.9
HOMA-IR	1.6 ± 0.5
Systolic blood pressure (mm Hg)	116 ± 7
Diastolic blood pressure (mm Hg)	76 ± 5
Physical activity	
Time (min/week)	95 ± 59
Cycling lactate threshold power output W)	90 ± 25

Values are presented as mean ± SD.
HOMA-IR, homeostatic model assessment for insulin resistance.

Results

Participant characteristics and energy intake

Baseline characteristics of participants are presented in Table 1. Participants complied with recording 24-h dietary intake on exercise trial days, with no differences observed between conditions for macronutrients and energy intake ($p > 0.05$; Figure A and B, Supplementaary Figure S1).

Biochemical analyses

There was a significant main effect of hypoxic condition on plasma glucose iAUC ($p = 0.02$; $\eta^2 = 0.28$; Figure 2A). Plasma glucose iAUC was significantly lower in MH compared to CTL (−35% ± 4%; $p = 0.046$; $g = 0.84$; Figure 2B). No significant difference was observed in either NM or HH (−20% ± 18%; −12% ± 17%; $p > 0.05$, respectively) compared to CTL as well as between NM and MH or HH, or MH and HH (−19% ± 11%; +10 ± 30%; +35 ± 20%; $p > 0.05$, respectively). There was no difference between conditions for interstitial glucose iAUC during 2-h OGTT (Figure 2C), despite it was lower in HH compared to MH and NM (−8 ± 25%; −6 ± 6%; $p > 0.05$, respectively; Figure 2D). A main effect of hypoxic condition was detected for plasma insulin iAUC ($p = 0.02$; $\eta^2 = 0.24$; Figure 2E). Plasma insulin iAUC was significantly lower in MH compared to CTL (−22% ± 20%; $p = 0.03$; $g = 0.51$; Figure 2F). No significant differences were observed between NM or HH and CTL

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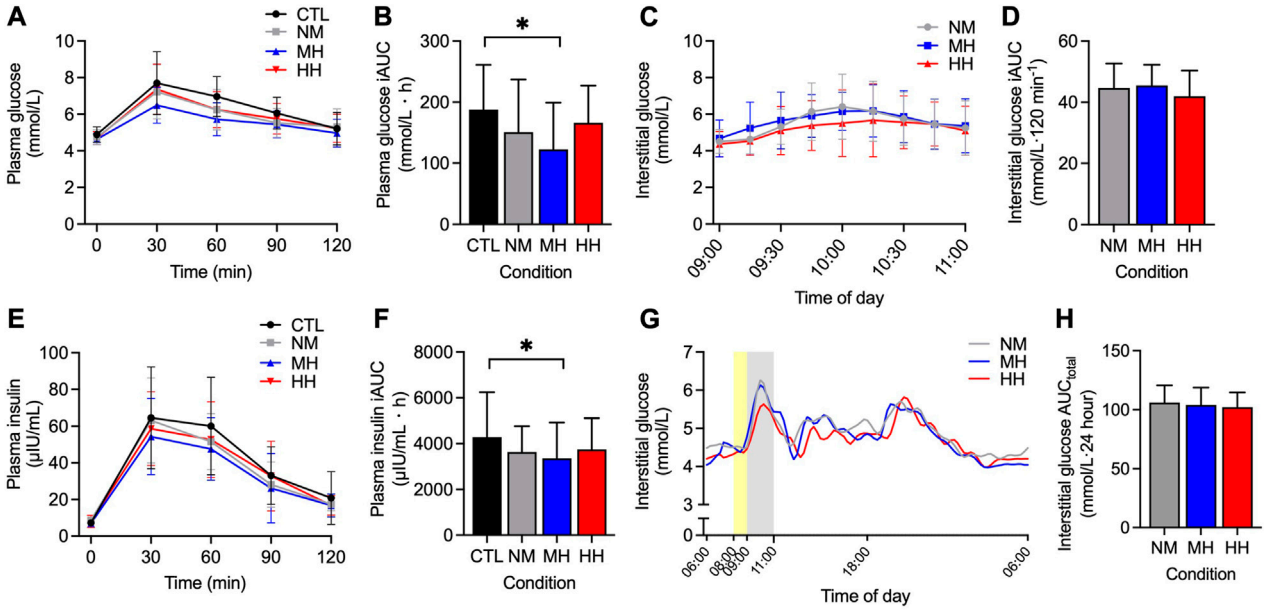

FIGURE 2
Two-hour responses following an OGTT after each of three exercise altitude conditions for venous plasma glucose **(A)**, interstitial (CGM) glucose **(C)**, plasma insulin **(E)** concentrations and the subsequent 2 h incremental AUC for plasma glucose **(B)**, plasma insulin **(F)** and interstitial glucose **(D)** concentration. 24 h interstitial (CGM) glucose concentration **(G)** and CGM AUC **(H)** from 06:00 until 06:00 the following morning. Values are mean ± SD. *$p < 0.05$ denotes a statistically significant difference between conditions. Area shaded with yellow represents 1 h exercise and grey represents OGTT. AUC, area under the curve; CGM, continuous glucose monitor; CTL, control; HH, high hypoxia; MH, moderate hypoxia; NM, normoxia; OGTT, oral glucose tolerance test.

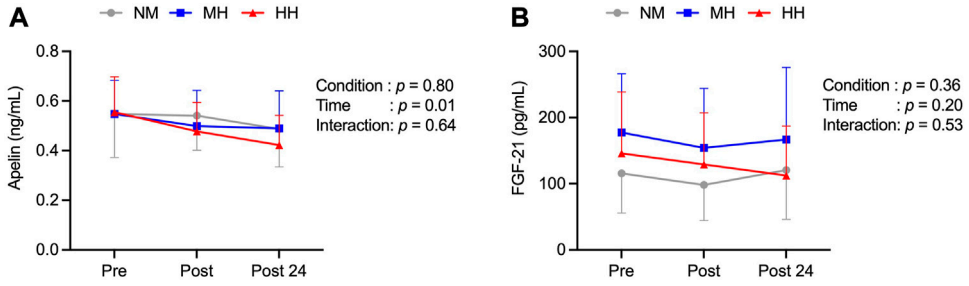

FIGURE 3
Pre, immediately post and 24 h post-exercise concentration of plasma apelin **(A)** and plasma FGF-21 **(B)** at three exercise altitude conditions ($n = 8$). Values are mean ± SD.

(−15% ± 43%; −13% ± 30%; $p > 0.05$, respectively), as well as NM compared to either MH or HH, or between MH vs. HH (−8 ± 39%; +3 ± 22%; +11 ± 12%; $p > 0.05$, respectively). For interstitial glucose 24 h AUC$_{total}$, no differences between conditions were detected ($p > 0.05$; Figures 2G, H).

There was a main effect of time on plasma apelin ($p = 0.01$; Figure 3A), however, *post hoc* analysis did not reveal any significant changes between time points. No differences in hypoxic condition as well as time × hypoxic condition interaction effects were observed for plasma apelin ($p > 0.05$). Similarly, there were no differences in time, hypoxic condition as well as interaction effects for plasma FGF-21 ($p > 0.05$; Figure 3B).

Physiological measures

There were significant differences between hypoxic conditions for HR ($p < 0.001$; $\eta^2 = 0.61$; Figure 4A), with higher HR responses in HH compared to both NM (8% ± 5%; $p < 0.001$; $g = 0.78$) and MH (3% ± 6%; $p = 0.03$; $g = 0.29$), as well as between MH and NM ($p = 0.01$; $g = 0.47$). There were also significant differences between hypoxic conditions for SpO_2 ($p < 0.001$; $\eta^2 = 0.87$; Figure 4B). SpO_2 was significantly lower in HH (88.1% ± 3.3%) compared to both NM (97.1% ± 0.7%, $p < 0.001$; $g = 3.89$) and MH (92.1% ± 1.9%; $p < 0.001$; $g = 1.41$), as well as between MH and NM ($p < 0.001$; $g = 3.74$).

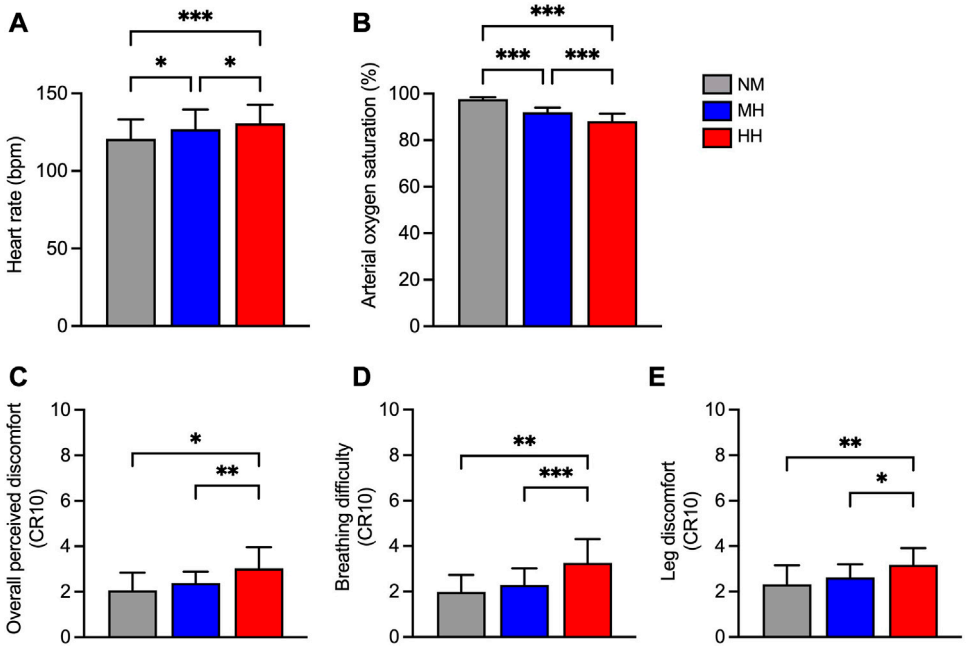

FIGURE 4
Heart rate **(A)**, arterial oxygen saturation (SpO_2; **(B)**, overall perceived discomfort **(C)**, difficulty breathing **(D)** and leg discomfort **(E)** at three exercise altitude conditions. Values are mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ denotes a statistically significant difference between condition.

Perceptual measures

There were significant differences between hypoxic conditions for overall perceived discomfort rating ($p < 0.01$; $\eta^2 = 0.41$; Figure 4C). Overall perceived discomfort rating was greater in HH compared to both NM and MH (+32 ± 17%; $p = 0.02$; $g = 1.09$; +21 ± 45%; $p = 0.005$; $g = 0.68$, respectively). No significant differences were observed in overall perceived discomfort between MH and NM (+13 ± 51%; $p > 0.05$). Significant differences were found between hypoxic conditions for perceived breathing difficulty ($p < 0.001$; $\eta^2 = 0.55$; Figure 4D) and perceived leg discomfort rating ($p < 0.01$; $\eta^2 = 0.42$; Figure 4E). Perceived breathing difficulty and leg discomfort rating were greater in HH compared to both NM and MH (+39 ± 29%; $p = 0.003$; $g = 1.37$; +30 ± 30%; $p < 0.001$; $g = 1.05$; +27 ± 13%; $p = 0.008$; $g = 1.07$; +18 ± 20%; $p = 0.03$; $g = 0.82$, respectively). No significant difference was observed in either perceived breathing difficulty or leg discomfort rating in MH compared to NM (+17 ± 45%; +12 ± 42%; $p > 0.05$, respectively).

Discussion

Our study has demonstrated that low-intensity cycle exercise performed under moderate hypoxia (2000 m) improved post-exercise OGTT plasma glucose and insulin iAUC compared to near sea-level and high hypoxia (3,000 m). Furthermore, HR and perceptual responses (i.e., overall perceived discomfort, perceived breathing difficulty and leg discomfort) were lower and SpO_2 was higher with moderate hypoxia as compared to high hypoxia. Collectively, low-intensity exercise in combination with moderate hypoxia may provide effective conditions for enhancing acute blood glucose responses without inducing superfluous levels of respiratory and lower extremity discomfort in physically inactive and overweight adults.

Fasting glucose and insulin levels as well as insulin sensitivity have been previously demonstrated to be improved with low-intensity cycle exercise performed under high hypoxia (~3,000 m) in individuals with type 2 diabetes (T2D) and overweight (Mackenzie et al., 2011; Mackenzie et al., 2012a). Our study is the first to compare different levels of hypoxic exposure as an adjuvant to exercise and we provide new information to indicate that significant improvements in acute blood glucose regulation can be attained with low-intensity cycle exercise without exposure to high or severe levels of hypoxia (i.e., >3,000 m). Such knowledge can be practically relevant for individuals with overweight or obesity. For instance, previous research has indicated that such population cohorts lose their enjoyment of exercise when exercise intensity is 10% greater than a self-selected speed (Ekkekakis and Lind, 2006). As adequate cardiorespiratory stimulation to induce metabolic adaptations can be achieved with lower exercise intensities when performed in combination with hypoxic loading (Hobbins et al., 2021; Tee et al., 2022), individuals with overweight or obesity may have better exercise adherence under such conditions compared to exercise with greater intensity undertaken in normoxia. Furthermore, in adults with overweight and T2D, acute hypoxic exercise was more effective than normoxic exercise at improving glucose tolerance (Mackenzie et al., 2011). While we acknowledge the current study is acute in nature, the findings provide an initial basis to show that overweight adults performing low-intensity cycle exercise at moderate hypoxia, as compared to normoxia or high

hypoxia, can also promote significant improvements in post-exercise 2 h OGTT plasma glucose and insulin iAUC responses. Incorporating exercise and moderate hypoxia into habitual training program of adults with overweight and/or pre-diabetes may thus provide an effective approach to prevent potential later incidences of hyperglycemia and reduce their risk of developing diabetes.

In contrast to the observed venous plasma glucose responses, no differences between hypoxic conditions were apparent in the post-exercise interstitial glucose response to the OGTT using CGM. Disparities between venous and interstitial blood glucose measures have been reported previously (Cengiz and Tamborlane, 2009). Such differences may relate to the interstitial glucose reading from the CGM having a delayed effect due to glucose in the blood taking time to appear in the interstitial fluid (Rebrin et al., 2010), as well as the intervals of readings (15 min for CGM vs. 30 min for venous blood). When assessing 24 h CGM data, although not statistically significant, the AUC_{total} showed a trend to decrease after exercising in both hypoxic conditions as compared to normoxia. Of note, the 24-h macronutrient and energy intake during the exercise day were not significantly different between conditions, eliminating the confounding effects of dietary intake on blood glucose regulation. Together, our data suggest that adding hypoxia to exercise may help improve blood glucose regulation. To the best of our knowledge, this is the first investigation of 24 h blood glucose patterns in response to a combined exercise and hypoxia stimulus incorporating CGM measures. Whether further beneficial effects in daily blood glucose responses manifest with repeated exercise sessions performed under moderate hypoxia remains an area of future investigation.

Our current study also observed changes in physiological (e.g., heart rate and arterial oxygen saturation) and perceptual responses that have important implications for exercise adherence. As expected, HR significantly increased during low-intensity exercise under acute exposure to moderate and high hypoxia. Similarly, SpO_2 values were progressively lower as hypoxic severity increased. Perceptual responses (i.e., ratings of overall perceived discomfort, perceived breathing difficulty and leg discomfort) were greater as hypoxic severity increased. These results are in line with previous observations examining physiological and perceptual responses to interval walking in adults with overweight and obesity at high hypoxia (~3,750 m) (Hobbins et al., 2021). However, a notable distinction in the current study is that the overall perceived discomfort rating, perceived breathing difficulty and leg discomfort rating were all greater in high hypoxia compared to moderate hypoxia. Such findings are important when considering moderate hypoxia was able to significantly improve glucose regulation without inducing greater physiological and psychological distress. Furthermore, a significant decrease in self-reported pleasure that led to reduced exercise adherence was reported in people with overweight, despite exercising at greater intensity similar to normal-weight counterparts (Ekkekakis and Lind, 2006). Thus, from a perceptual standpoint, our results suggest that low intensity exercise combined with moderate hypoxia may be suitable for overweight adults to acquire metabolic health improvements without diminishing enjoyment and adherence due to greater perceptual discomfort. Further investigation regarding the manipulation of exercise intensity and duration of aerobic exercise under hypoxia on enjoyment and adherence in similar cohorts is required.

To elucidate possible mechanisms of how exercise and hypoxia can promote metabolic adaptations, we investigated the expression of the "exerkines," apelin and FGF-21 in plasma. Apelin and FGF-21 are released with exercise-induced muscle contraction and are involved in metabolic regulation by controlling glucose and fat metabolism. As such, these exerkines are implicated in improving insulin sensitivity and preventing obesity and diabetes mellitus with exercise (Son et al., 2018; Khalafi et al., 2021). However, secretion of these exerkines after hypoxic exposure in combination with exercise remains poorly investigated. We found no significant changes in plasma apelin or FGF-21 post-exercise or between different conditions despite post-exercise glucose and insulin iAUC being significantly lower in moderate hypoxia. Previous studies have reported inconsistent findings in plasma apelin concentration following aerobic-based exercise under normoxic conditions in either healthy (Waller et al., 2019) or individuals with overweight or obesity (Son et al., 2019). Similarly, acute exercise under normoxic conditions has been shown to selectively increase plasma FGF-21 in lean individuals (Tanimura et al., 2016), but this response was attenuated in individuals with obesity (Slusher et al., 2015). Disparities in both plasma apelin and FGF-21 responses to acute exercise between studies may relate to differences in participant characteristics (i.e., age, physical activity levels and health status), exercise intensity and/or the timing of post-exercise sample collection and measurement (Slusher et al., 2015; Tanimura et al., 2016). Moreover, it cannot be ruled out that any significant changes in plasma apelin may have occurred outside our pre-, immediately post-, and 24 h post-exercise sampling window (Brame et al., 2015). Future studies with increasing post-exercise sampling frequency, as well as standardization and consistency in sample process time are needed to better clarify the patterns of circulating FGF-21 and apelin in response to exercise and hypoxia.

Several limitations of the present study are acknowledged. Firstly, our study recruited only overweight males with normal glycemia levels, therefore the results may not be able to be generalized to female and clinical cohorts. Furthermore, our study only included one type of exercise (aerobic cycle exercise) and one exercise intensity (low intensity). In this regard, it is plausible that alternative exercise modes, such as high-intensity interval training, or contractile types (i.e., walking, running), may induce different effects on blood glucose regulation to that currently observed with low-intensity aerobic exercise (Tee et al., 2023). Another limitation in the current study is that glucose and insulin responses were determined using OGTT rather than using the gold standard glucose tolerance assessment (hyperinsulinemic-euglycemic clamp technique) (DeFronzo et al., 1979), which was not possible for practical reasons. Lastly, previous studies have reported a positive effect of acute aerobic exercise on glucose tolerance in individuals with T2D and/or overweight (Mackenzie et al., 2011; Mackenzie et al., 2012a), and robust changes in blood glucose and exerkine regulation with exercise may be more apparent with repeated hypoxic exposure over the course of several weeks to months. While our study aimed to examine the acute effects of hypoxic stimuli combined with exercise, we acknowledge that repeated exercise sessions under

hypoxic conditions may be required to observe more substantial changes in blood glucose responses.

Conclusion

In conclusion, our results show that an acute bout of low-intensity exercise in combination with moderate hypoxia was most effective for improving post-exercise blood glucose regulation in healthy overweight adults. These results suggest that increasing hypoxic severity to 3,000 m and beyond may not be necessary to improve glucose regulation in such cohorts while also minimizing undue physiological stress and discomfort on participants. Thus, our findings provide an initial basis for determining safe and optimal hypoxic stimuli that can promote metabolic adaptations while reducing unnecessary physiological and psychological stress.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Human Research Ethics Committee of the National Sports Institute of Malaysia (ISNRE/A/008/2020-003/2020) and Swinburne University of Technology Human Research Ethics Committee (20225950-9155). The patients/participants provided their written informed consent to participate in this study.

Author contributions

WK and CT conceived the project. WK, DC, MC, and CT designed the experiments. CT and NR performed data collection. CT, MC, and NR performed and analyzed the experiments. CT, MC, and MR analyzed the data. CT, EP, MC, MC, WK, and DC prepared the figures and wrote the manuscript. All authors commented on and approved 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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1174926/full#supplementary-material

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Identification of novel urine proteomic biomarkers for high stamina in high-altitude adaptation

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Introduction: We aimed to identify urine biomarkers for screening individuals with adaptability to high-altitude hypoxia with high stamina levels. Although most non-high-altitude natives experience rapid decline in physical ability when ascending to high altitudes, some individuals with high-altitude adaptability continue to maintain high endurance levels.

Methods: We divided the study population into two groups: the LC group (low change in endurance from low to high altitude) and HC group (high change in endurance from low to high altitude). We performed blood biochemistry testing for individuals at high altitudes and sea level. We used urine peptidome profiling to compare the HH (high-altitude with high stamina) and HL (high-altitude with low stamina) groups and the LC and HC groups to identify urine biomarkers.

Results: Routine blood tests revealed that the concentration of white blood cells, lymphocytes and platelets were significantly higher in the HH group than in the HL group. Urine peptidome profiling showed that the proteins ITIH1, PDCD1LG2, NME1-NME2, and CSPG4 were significantly differentially expressed between the HH and HL groups, which was tested using ELISA. Urine proteomic analysis showed that LRG1, NID1, VASN, GPX3, ACP2, and PRSS8 were urine proteomic biomarkers of high stamina during high-altitude adaptation.

Conclusion: This study provides a novel approach for identifying potential biomarkers for screening individuals who can adapt to high altitudes with high stamina.

KEYWORDS

urine biomarkers, proteomic, high stamina, high-altitude adaptation, blood routine tests

Introduction

The high-altitude regions of plateaus are characterized by environmental factors such as hypoxia, cold temperatures, low pressure, and high radiation (Jeong et al., 2016). Each year, millions of people travel to high altitudes for various reasons including altitude training for athletes (Khodaee et al., 2016). Altitude training is often used to improve physical fitness and competitive performance in endurance sports such as middle-distance running, cross-country skiing, and cycling (Bailey et al., 1997). However, the results of altitude training

are often uncertain, and not all athletes achieve the desired outcomes (Hamlin et al., 2018). Therefore, the question of why some individuals tolerate high altitudes and adapt well to high-altitude hypoxia training, whereas others do not remains unanswered. Additionally, individual athletes differ from each other. Every year during military screening, several individuals show high stamina at sea level, whereas at high altitudes, only some maintain high stamina, and others show rapid decline in stamina. This may be related to the characteristics specific to individuals. Therefore, the ability to screen for individuals who adapt well to high altitudes would be of great significance, particularly for military purposes.

To date, research on high-altitude adaptability has mainly focused on the genetic or biochemical differences between high-altitude residents such as Tibetans and sea-level residents, the physiological or other differences between individuals at sea level and high altitude, or the effect of altitude training on performance improvement (Simonson et al., 2010; Eichstaedt et al., 2020; Chiou et al., 2022). However, few studies have explored the mechanism of maintaining high performance during high-altitude adaptation. Recent advances in proteomics have enabled the identification of protein expression profiles in high stamina and low stamina individuals at high altitudes, providing a better understanding of the mechanisms underlying the functional adaptations of individuals in high-altitude hypoxic environments (Gracey et al., 2007).

This study aimed to screen for novel urine proteomic biomarkers for high-stamina in high-altitude adaptation. The study population was divided into two groups: the LC group (low change in endurance from low altitude to high altitude) and HC group (high change in endurance from low altitude to high altitude). We aimed to identify the differentially expressed proteins (DEPs) as potential biomarkers by comparing the generated urine peptidome profiles. In addition to military purposes, this research could be highly useful for screening individuals with good high-altitude adaptability in the sports field. Thus, identifying individuals with high physical fitness would be useful for choosing competitive personnel that can fulfil combat needs in plateau environments in a targeted manner.

Materials and methods

Study participants and sample collection

This study included 200 healthy Chinese men aged 18–25 years. The exclusion criteria included any health problems; known liver, lung, or cardiovascular disease; history of migraine or head injury; and smoking. None from the study population had prior experience at high altitudes. The study began with the participants undergoing 3,000 m training at sea level. Urine and blood samples were collected from these participants at sea level. They were then transported to high altitudes in Ali, Tibet (average elevation, >3,000 m). After 7 days of acclimatisation to high altitude (4,000 m), the participants underwent another 3,000 m training., and urine and blood samples were collected again. The participants were divided into HH (high-altitude with high stamina, n = 50), HL (high-altitude with low stamina, n = 50), LH (low-altitude with high stamina, n = 50), and LL (low-altitude with low stamina, n = 50) groups. Notably, collecting results for 3,000 m training for 200 participants at high altitudes (after only 7 days of acclimatisation) is quite difficult. Ethical approval for the study was obtained from the Chinese PLA General Hospital ethical committee (approval identifier S2019-035-01), and all protocols followed the established national and institutional ethical guidelines. All participants provided signed written informed consent.

Routine blood tests

All blood samples collected from the HH and HL group participants at high altitude and LH and LL group participants at sea level were subjected to routine testing for haemoglobin (HGB), red blood cell (RBC) count, white blood cell (WBC) count, lymphocyte (Lym) count, neutrophil (Neu) count, monocyte (Mon) count, eosinophil (Eos) count, basophil (Bas) count, lymphocyte percentage (Lym%), monocyte percentage (Mon%), eosinophil percentage (Eos%), basophil percentage (Bas%), haematocrit (HCT), mean corpuscular volume (MCV), red blood cell distribution width coefficient of variation (RDW-CV), red blood cell distribution width standard deviation (RDW-SD), platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), and platelet volume (PCT). All routine blood test results were statistically analysed using SPSS software, and only the representative changes are shown in this paper.

Urine proteome profiling and analysis

The urine samples were centrifuged at 12,000 × g for 30 min at 4°C, and the pellets were removed. The protein concentration in each sample was measured using the Bradford protein assay. The proteins were then digested with trypsin (Promega, United States), and 100 μg of the protein sample was loaded onto a 10 kDa filter unit (Pall, United States). The protein solution was reduced with 4.5 mM DTT for 4 min at 95°C. The proteins were digested with trypsin (enzyme-to-protein ratio of 1:50) overnight at 37°C. Formic acid (10%) was added to obtain a final formic acid concentration of 1%. The supernatant was centrifuged and the pH was adjusted to 10 with ammonia water. The digested samples were desalted, and different gradient eluents were configured to manually divide the samples into six components. They were rotary evaporated to dryness at 60°C for 90 min, reconstituted with formic acid, and placed in the machine for analysis.

LC–MS/MS setup for DDA

An Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific, Germany) coupled with an Ultimata 3000-HPLC system (Thermo Scientific, Germany) was used for analysis. Each peptide sample was dissolved in 0.1% formic acid, and 1 μg of peptide was loaded onto a reversed-phase trap column (75 μm × 2 cm, 3 μm, C18, 100 Å, Thermo Scientific). The eluent was then transferred to a reversed-phase analytical column (50 μm × 500 mm, 2 μm, C18,

TABLE 1 Baseline characteristics of the enrolled subjects.

	LH	LL	HH	HL	*p*-Value
Sex					
Male Female	50 (100%) 0 (0%)	50 (100%) 0 (0%)	50 (100%) 0 (0%)	50 (100%) 0 (0%)	NA
Age(years)					
Mean (SD) Median [min,max]	20 (1.41) 20 [18,23]	20 (1.66) 20 [18,25]	20 (1.65) 20 [18,25]	20 (1.58) 20 [18,25]	$P_{12} = 0.99$; $P_{34} = 0.99$; $P_{13} = 0.91$; $P_{24} = 0.95$
Height(cm)					
Mean (SD) Median [min,max]	175.17 (6.58) 175 [162,190]	174.31 (5.58) 173.25 [161,188]	174.18 (6.21) 174.5 [161,188]	173.25 (5.21) 172 [160,188]	$P_{12} = 0.89$; $P_{34} = 0.86$; $P_{13} = 0.84$; $P_{24} = 0.82$
Weight(kg)					
Mean (SD) Median [min,max]	67.75 (7.23) 67.3 [52,82.3]	73.17 (10.06) 71.2 [58.1,104.5]	62.8 (6.94) 62.1 [51.4,77.4]	67.20 (8.58) 65.25 [54.3,89.9]	$P_{12} = 0.009$; $P_{34} = 0.06$; $P_{13} = 0.02$; $P_{24} = 0.003$
3000m(s)					
Mean (SD) Median [min,max]	12′11” (22″) 12′16” [11′09″,12′39”]	14′22” (39″) 14′14’ [13′40″,16′40”]	13′04” (18″) 13′02” [12′20″,13′30”]	14′58” (46″) 14′40” [14′20″,18′29”]	$P_{12} < 0.0001$; $P_{34} < 0.0001$; $P_{13} < 0.0001$; $P_{24} < 0.0001$

TABLE 2 Results of routine blood tests in LH/LL groups at low altitude and HH/HL groups at high altitude.

	LH	LL	HH	HL	*p*-Value
WBC(10^9/L)	5.43 ± 0.88	5.92 ± 1.09	5.72 ± 0.99	6.27 ± 1.25	$P_{12} = 0.09$; $P_{34} = 0.05$; $P_{13} = 0.69$; $P_{24} = 0.57$
Lym (10^9/L)	1.75 ± 0.39	1.89 ± 0.45	2.18 ± 0.52	2.59 ± 0.54	$P_{12} = 0.09$; $P_{34} = 0.01$ $P_{13} = 0.001$; $P_{24} < 0.0001$
Lym%	32.56 ± 6.40	32.34 ± 7.10	38.34 ± 7.68	41.55 ± 8.68	$P_{12} = 0.83$; $P_{34} = 0.10$ $P_{13} = 0.007$; $P_{24} < 0.0001$
RBC(10^{12}/L)	5.32 ± 0.26	5.43 ± 0.43	5.79 ± 0.48	5.76 ± 0.48	$P_{12} = 0.25$; $P_{34} = 0.28$ $P_{13} < 0.0001$; $P_{24} = 0.0002$
HGB (g/L)	161.6 ± 7.94	160.7 ± 8.57	187.53 ± 13.94	185.8 ± 16.11	$P_{12} = 0.55$; $P_{34} = 0.48$ $P_{13} < 0.0001$; $P_{24} < 0.0001$
HCT (%)	47.3 ± 2.36	47.5 ± 2.29	53.79 ± 6.39	54.59 ± 5.83	$P_{12} = 0.74$; $P_{34} = 0.64$ $P_{13} < 0.0001$; $P_{24} < 0.0001$
PLT (10^9/L)	241.38 ± 56.19	253.63 ± 38.32	228.65 ± 43.26	250.8 ± 38.42	$P_{12} = 0.27$, $P_{34} = 0.05$ $P_{13} = 0.69$; $P_{24} = 0.99$
MPV(fL)	10.48 ± 0.97	10.27 ± 0.77	10.46 ± 1.07	10.09 ± 0.88	$P_{12} = 0.25$; $P_{34} = 0.09$ $P_{13} = 0.99$; $P_{24} = 0.86$

100 Å). A gradient elution of 5%–30% buffer B (0.1% formic acid in 80% acetonitrile; flow rate 0.6 μL/min) was used for 98 min. The MS data were acquired in data-dependent acquisition mode. Survey MS scans were acquired in the Orbitrap using a mass-to-charge ratio range of 300–1,500 with a resolution set to 70,000. The most intense ions per survey scan (top speed mode) were selected for collision-induced dissociation fragmentation, and the resulting fragments were analysed in the Orbitrap with a resolution of 30,000. Dynamic exclusion was employed with a 20 s window to prevent repetitive selection of the same peptide. The normalized collision energy for the HCD-MS2 experiments was set to 32%.

LC–MS/MS data analysis

Raw MS data files were processed using Proteome Discoverer 2.1-Sequest software (Thermo Scientific). Features with only one charge or more than five charges were excluded from analysis. Only peptides with a Mascot score >30 and a $p < 0.01$ for the identified proteins were included for further quantitation. Proteins identified using at least one peptide were retained. MS/MS spectra were exported and processed using Mascot software (version 2.5.1, Matrix Science, London, United Kingdom) against the NCBI database (human reference sequence 2017-11-01) with the following search parameters: 10 ppm precursor mass tolerance, 0.05 Da fragment mass tolerance, and up to two missed cleavage sites allowed in trypsin digestion. Only highly confident peptide identifications (FDR ≤0.01) were imported into Progenesis software for further analysis. DEPs were identified using statistical criteria of a *t*-test *p*-value <0.05, a minimum of two peptides matched to a protein, and a fold change >1.5. Principal component analysis (PCA) was performed on the DEPs without missing values. A heatmap of the DEPs was generated using OmicShare software.

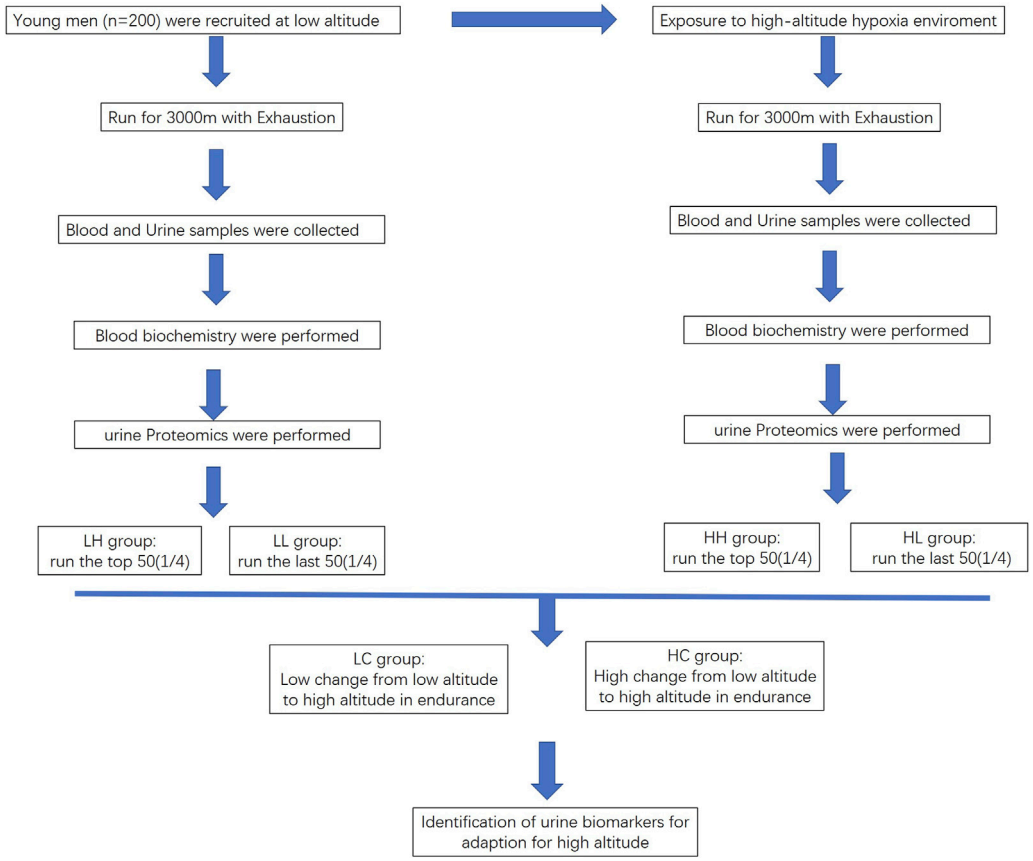

FIGURE 1
Flowchart outlining the process for identifying biomarkers for high endurance in high-altitude adaptation.

Enzyme-linked immunosorbent assay

All urine samples were analysed in a blinded fashion with standards and the samples analysed in triplicate. The concentrations of inter-α-trypsin inhibitor heavy chain H1 fragment (ITIH1), recombinant programmed cell death protein 1 ligand 2 (PDCD1LG2), human NME1-NME2 complex (NME1-NME2), and chondroitin sulphate proteoglycan 4 (CSPG4) were quantified using a Human ITIH1 ELISA Kit (No. MM61243H1), Human PDCD1LG2 ELISA Kit (No. MM61263H1), Human NME1-NME2 ELISA Kit (No. MM61229H1), and Human CSPG4 ELISA Kit (No. MM6125H1) respectively. A standard curve was generated and used to determine the concentrations of ITIH1, PDCD1LG2, NME1-NME2, and CSPG4 in the samples.

Statistical analyses

The IBM Statistical Package for the SPSS software was used for statistical analysis. Data were tested for normality and parametric tests were used for normally distributed data and nonparametric tests for data not normally distributed. Independent sample t-tests or Mann–Whitney U tests were used to determine the differences between groups with the appropriate test being chosen based on the normality of the data. All data are presented as the mean ± standard deviation (SD). $p < 0.05$ was considered statistically significant. For DEP analysis, we used an adjusted p-value based on multitest. Comparisons between LL, LH, HL, and HH groups were performed using non-parametric t-tests for routine blood test analyses and ELISA. Comparisons between the LC and HC groups were performed using parametric t-tests.

Results

Baseline characteristics of participants

The baseline characteristics of all the participants are listed in Table 1. No significant differences were observed in sex, age, or height between the individuals. The weight of the HH and HL groups did not show significant differences, but some differences were noted at low altitude. The scores on the 3,000 m run were significantly higher for the individuals in the LH group than for those in the LL group (12′11” ± 22″ vs. 13′04” ± 18″, $p < 0.0001$) and for those in the HH group than those in the HL group (14′22” ± 39″ vs. 14′58” ± 46″, $p < 0.0001$) (Table 1; Figure 1).

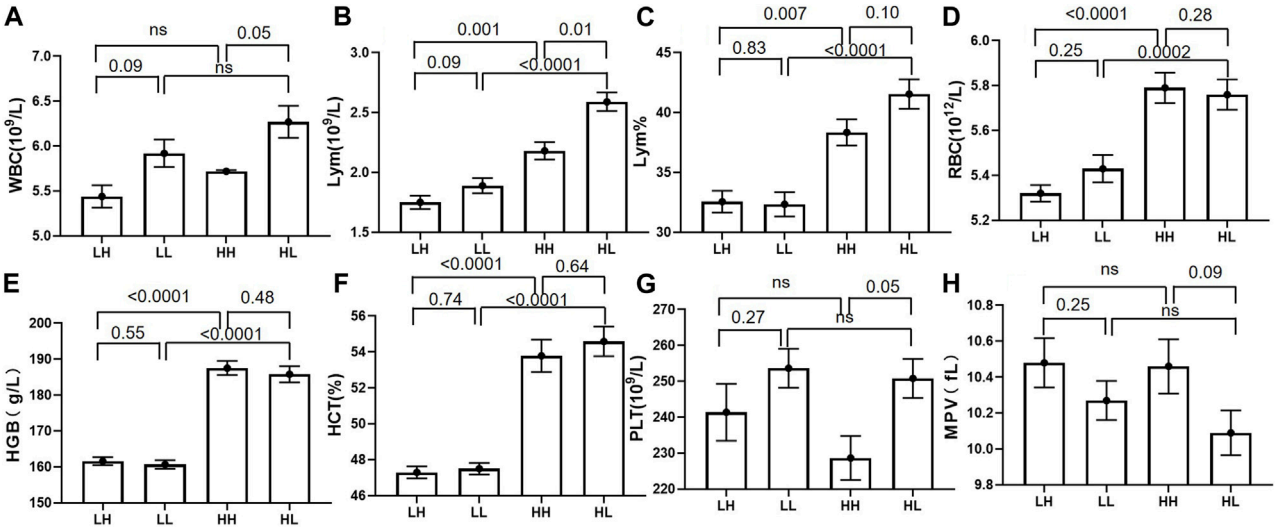

FIGURE 2
The routine blood test results for low-altitude with high stamina (LH) and low-altitude with low stamina (LL) groups at low altitude and high-altitude with high stamina (HH) and high-altitude with high stamina (HL) groups at high altitude. **(A, B, G, H)** White blood cell (WBC), lymphocyte (LYM), and platelet (PLT) counts in the HH group were significantly higher than those in the HL group. Mean platelet volume (MPV) was higher in the HH group than that in the HL group, however the difference was not significant. **(B–F)** The corresponding indexes including lymphocyte percentage (LYM%), haematocrit (HCT)%, and LYM, red blood cell (RBC), and haemoglobin (HGB) counts in HH and HL groups were significantly higher than those in the LH and LL groups.

Routine blood tests of HH and HL groups

The mean values for RBC, HGB, HCT, LYM, and LYM% were significantly higher at high altitude in the HH group (mean value 5.79 ± 0.48; 187.53 ± 13.94; 53.79 ± 6.39; 2.18 ± 0.52; and 38.34 ± 7.68) than that at low altitude in the LH group (mean value 5.32 ± 0.26; 161.6 ± 7.94; 47.3 ± 2.36; 1.75 ± 0.39; 32.56 ± 6.40) (p < 0.005). Similarly, the mean values for RBC, HGB, HCT, LYM, and LYM% were significantly higher at high altitude in the HL group (mean value 5.76 ± 0.48; 185.8 ± 16.11; 54.59 ± 5.83; 2.59 ± 0.54; 41.55 ± 8.68) than at low altitude in the LL group (mean value 5.43 ± 0.43; 160.7 ± 8.57; 47.5 ± 2.29; 1.89 ± 0.45; 32.34 ± 7.10) (p < 0.005). Furthermore, the concentrations of WBC, LYM, and PLT were significantly lower in the HH group (mean value 5.72 ± 0.99; 2.18 ± 0.52; 228.65 ± 43.26) than in the HL group (mean value 6.27 ± 1.25; 2.59 ± 0.54; 250.8 ± 38.42) (p < 0.05). Although the WBC, LYM, and PLT concentrations were lower in the LH group than in the LL group, the difference was not significant (Table 2; Figure 2).

Urine peptidome profiles of HH and Hl groups

The reproducibility and stability of the mass spectra results were evaluated using an HPLC-Orbitrap MS-based proteome platform, and the samples in the same group showed closely reproducible peaks (Figures 3A,B). The Heatmap of the HH and HL groups showed that most of the higher-expression proteins were concentrated in the HH group (Figure 3C). PCA demonstrated that the sample distribution pattern of the HH group was distinct from that of the HL group (Figure 3D).

Identification of HH urea biomarkers

A total of 144 different proteins were identified between the HH and HL groups with 33 of them showing fold changes >2 (p < 0.05). The four most significantly differential proteins are listed in Table 3 (p < 0.005, fold change >2), and their receiver operating characteristic (ROC) curves are shown in Figure 4. The area under the curve (AUC) values for these four proteins were 0.84, 0.71, 0.70, and 0.70 (Figure 4). All four proteins were upregulated in the HH group (Table 3; Figure 4).

Validation of protein expression in HH and HL groups

To validate the expression levels of PDCD1LG2, ITIH1, NME1-NME2, and CSPG4 obtained from the LC–MS/MS analysis, the urine concentrations of these proteins were further examined in 96 samples from HH and HL groups using ELISA. Urinary concentrations of the four proteins in the two groups are shown in Table 4 and Figure 5. The mean concentration of ITIH1 was 144.21 ± 12.64 in the HH group and 55.10 ± 40.11 ng/mL in the HL group (p = 0.001), indicating that ITIH1 was expressed at significantly higher levels in the HH group. Similarly, the mean concentration of PDCD1LG2 was 32.35 ± 22.40 ng/mL in the HH group and 20.14 ± 14.39 ng/mL in the HL group (p = 0.02), indicating that PDCD1LG2 was also expressed at significantly higher levels in the HH group. The urine concentrations of NME1-NME2 and CSPG4 were also higher in the HH group with mean concentrations of 232.99 ± 134.63 ng/mL and 443.06 ± 251.56 ng/mL, respectively, compared with that of the HL group with mean concentrations of 203.73 ± 123.12 ng/mL and

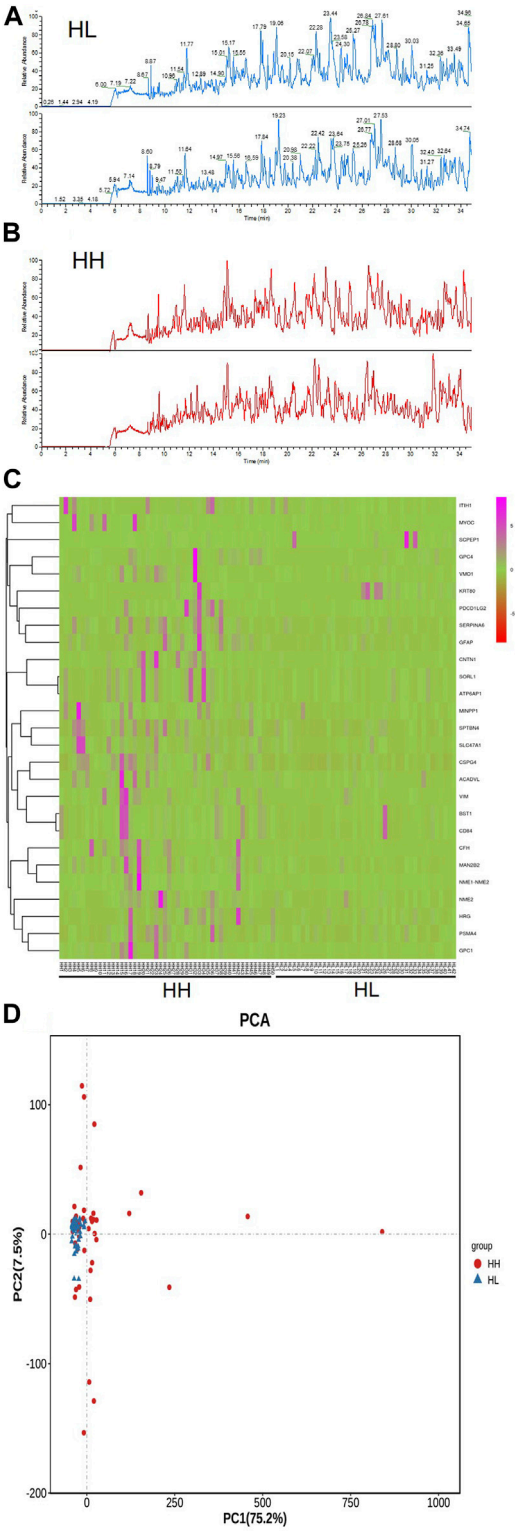

FIGURE 3
Reproducibility of mass spectra generated in individuals from the HH and HL groups, and comparative analysis of urine peptidome profiling between the HH and HL group samples collected at high altitude. **(A)** Representative mass spectra of an individual from the HL group (blue) and **(B)** representative mass spectra of an individual from the HH group (red), indicating low variability between replicates of this sample. **(C)** A heat-map representing the expression levels of proteins in the HH and HL groups. **(D)** Principal component analysis (*Continued*)

FIGURE 3 (Continued)
(PCA) of gene expression variations in HH and HL groups. A bivariate plot comparing the peptidome profiles of individuals from the HH (red) and HL (blue) groups.

356.98 ± 278.80 ng/mL, respectively. This indicated that NME1-NME2 and CSPG4 were also expressed at higher levels in the HH group but the difference was not statistically significant ($p > 0.05$) (Table 4; Figure 5). These results were consistent with the findings of the UPLC-MS/MS analysis.

Urine biomarkers filtrates of LC and HC groups

The urine of individuals in the LC and HC groups were screened for biomarkers associated with changes in endurance. Results showed that six proteins were significantly upregulated in high altitude compared to low altitude. The six proteins were leucine rich alpha-2-glycoprotein 1 (LRG1), glutathione peroxidase 3 (GPX3), and decreases in protein expression of nidogen 1 (NID1), vasorin (VASN), acid phosphatase 2 (ACP2), and serine protease 8 (PRSS8). The ROC curve and AUC values were used to assess the combined diagnostic value of the six proteins for high stamina in high-altitude adaptation. The ROC curve is shown in Figure 6, and the combined AUC value for the six proteins was 0.748 (Figure 6).

Validation of inflammation factor in LC and HC groups

As the level of inflammation is an important factor in high-altitude adaptation, we validated the expression levels of tumour necrosis factor a (TNFα) in different groups using ELISA. The mean concentration of TNFα was 2.40 ± 0.76 in the LH group, 5.30 ± 1.06 ng/mL in the LL group ($p = 0.03$), 18.09 ± 3.47 in the HH group, and 34.47 ± 7.89 ng/mL in the HL group, indicating that TNFα was expressed at significantly higher levels in the HL group compared to the other groups. Increased expression of TNFα was also observed in low altitude to high altitude adaptation and LC to HC groups (Figure 7).

Discussion

The study of high-altitude adaptation is a well-established area in the field of biological anthropology with a focus on the physiological effects of hypobaric hypoxia on high-altitude populations (Hartman-Ksycińska et al., 2016). However, few studies have explored the physical fitness of athletes during high-altitude adaptation. To the best of our knowledge, this study is the first to analyse urine proteomics in the HL/HH and LC/HC groups to investigate the adaptability of lowland athletes to high-altitude hypoxia with high performance. We have demonstrated the feasibility and safety of using proteomics to identify urine

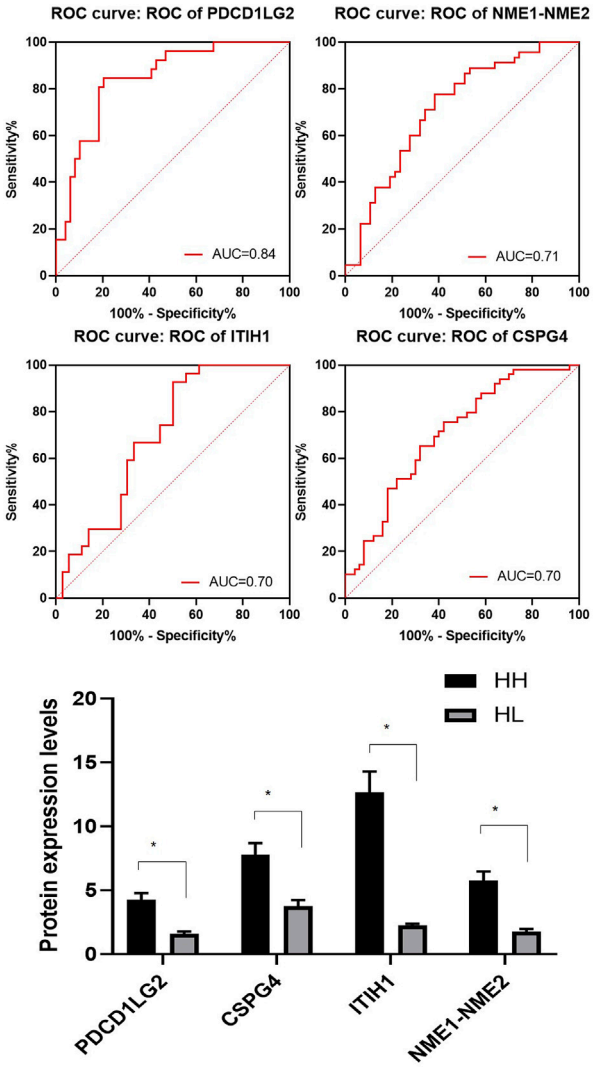

FIGURE 4
Representative spectra of four potential biomarkers in the HH group at high altitude. Receiver operating characteristic (ROC) curves for the four selected proteins with their area under the curve (AUC) values. The average expression levels of the four selected proteins in the HH (black) and HL samples (grey) and their *p* values expressed as mean ± SD.

TABLE 3 Mean levels of four differentially expressed proteins in HH and HL samples collected at high altitude.

Protein	HH	HL	Fold	*p*-value
PDCD1LG2	4.24 ± 3.78	1.60 ± 1.25	2.65	0.005
ITIH1	12.66 ± 11.50	2.25 ± 0.86	5.62	0.004
NME1-NME2	5.73 ± 5.08	1.75 ± 1.55	3.27	0.0005
CSPG4	6.65 ± 5.68	3.20 ± 3.16	2.07	0.0003

TABLE 4 Mean levels of four differentially expressed proteins verified by ELISA in HH and HL samples.

	HH	HL	*p*-value
ITIH1 (range) ng/mL	16.71-469.58	3.34-231.82	0.001
Mean ± Std	144.21 ± 126.64	55.10 ± 40.11	
PDCD1LG2 (range) ng/mL	2.08-83.13	2.08-53.66	0.02
Mean ± Std	32.35 ± 22.40	20.14 ± 14.39	
NME1-NME2 (range) ng/mL	46.87-524.21	32.18-465.46	>0.05
Mean ± Std	232.99 ± 134.63	203.73 ± 123.12	
CSPG4 (range) ng/mL	80.59-942.07	43.13-848.43	>0.05
Mean ± Std	443.06 ± 251.56	356.98 ± 278.80	

biomarkers. Four potential urinary protein biomarkers were identified in the HL and HH groups, and six combined urine protein biomarkers were screened from the LC and HC groups, which may play a role in maintaining high stamina during high-altitude adaption.

Routine blood tests revealed that the concentrations of HGB, RBC, and HCT levels in the high-altitude samples were significantly higher than those in low-altitude samples regardless of stamina levels. This is consistent with the response of lowland mammals to chronic hypoxia, which typically involves increased erythropoietic activity, leading to an increase in HGB, RBC, and HCT concentrations (Wagner et al., 2015). The moderate increase in HGB concentration observed in the high-stamina group may have contributed to the increased blood oxygen-carrying capacity and improved tissue oxygenation, helping these individuals to maintain high stamina during high-altitude adaptation (Yang et al., 2020). Additionally, the concentrations of WBC, LYM, PLT, and LYM% were lower in high-stamina samples than in low-stamina samples, with a greater difference observed between HH and HL groups than between LH and LL groups. These results show that the difference in protein expression between different stamina groups is more significant at high-altitudes than at low-altitudes. The lower expression of WBC, LYM, and LYM% in the HL group compared with that of the HH group suggests that soldiers with higher altitude running speeds have lower levels of inflammatory stress and better antioxidant capacity (Xiong et al., 2020). Kostrzewa et al. confirmed that the increase in WBC count after exercise is related to the immune response and is not solely caused by dehydration (Nowak et al., 2020). WBC counts often decline after prolonged training, and high altitudes are associated with an inflammatory response (Galun et al., 1987). In comparison with individuals with poor stamina, those with high stamina maintain a low inflammatory level to adapt to high altitudes. Additionally, we found that PLT was highly expressed in the HL samples. PLT plays a role in blood coagulation (Nader et al., 2021). In the past decade, many epidemiological studies have shown that high PLT expression is a major risk factor for cardiovascular disease (Picker et al., 2013). Additionally, blood coagulation glycoproteins are highly expressed in patients with high-altitude hypoxia (Yang et al., 2013). The present study suggests that the high expression levels of PLT in HL samples may lead to enhanced blood coagulation, resulting in low stamina and difficulty in adapting to high-altitude hypoxic environments.

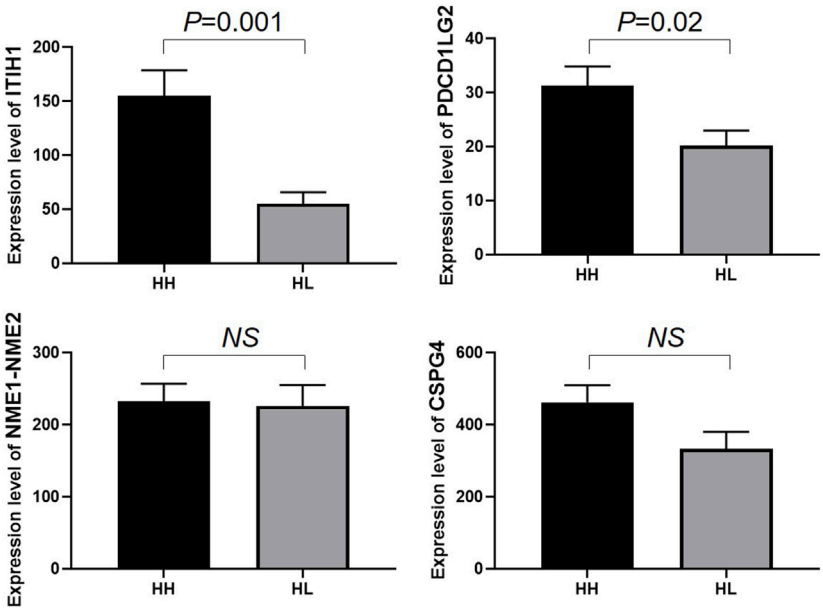

FIGURE 5
Enzyme-linked immunosorbent assay (ELISA) analysis of ITIH1, PDCD1LG2, NME1-NME2, and CSPG4 expression in the HH and HL groups. The y-axis represents protein expression levels (ng/mL) in different groups. Statistical analysis was performed using Prism 8.0 software. All data with *p* values are represented as the mean ± SD.

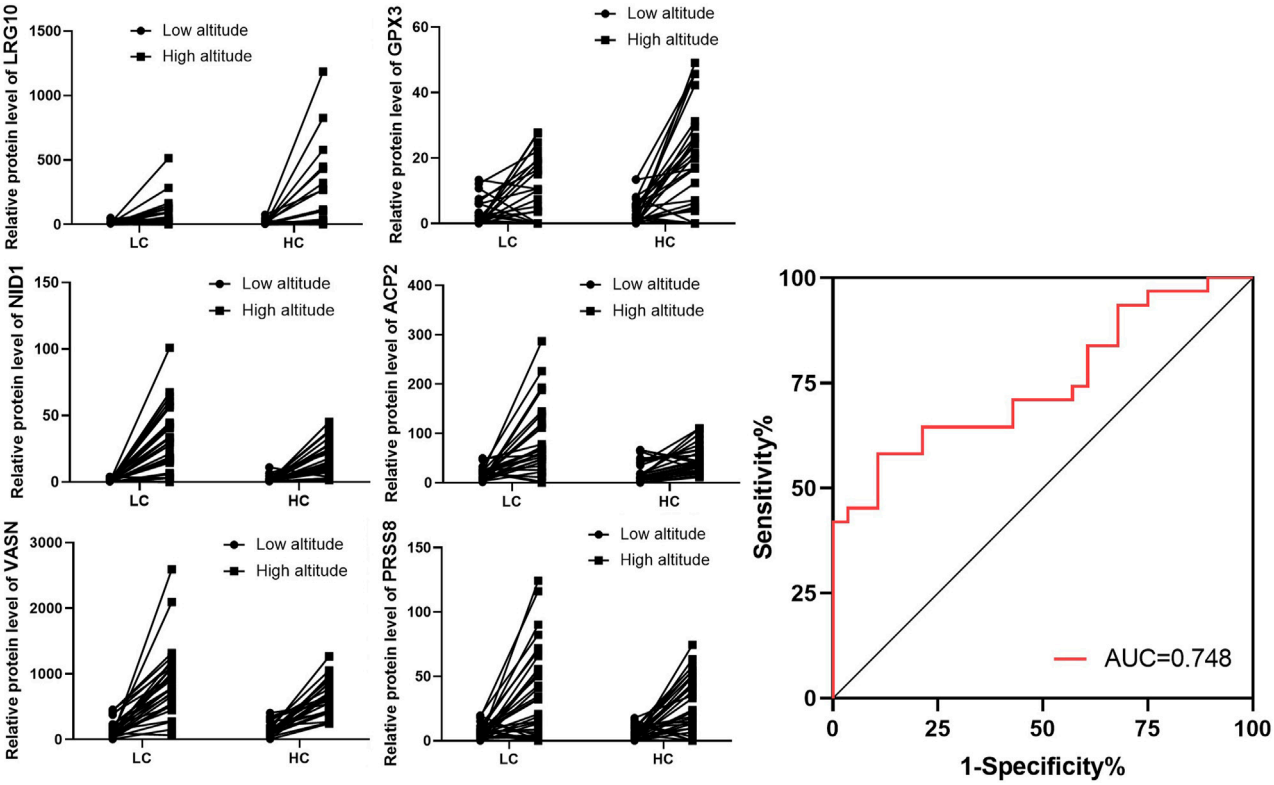

FIGURE 6
Expression of individual screened proteins for LC and HC groups at low and high altitudes. Expression of the six selected proteins in the LC and HC groups from low to high altitude. Combined Receiver operating characteristic (ROC) curve for all six selected proteins with their area under the curve (AUC) values.

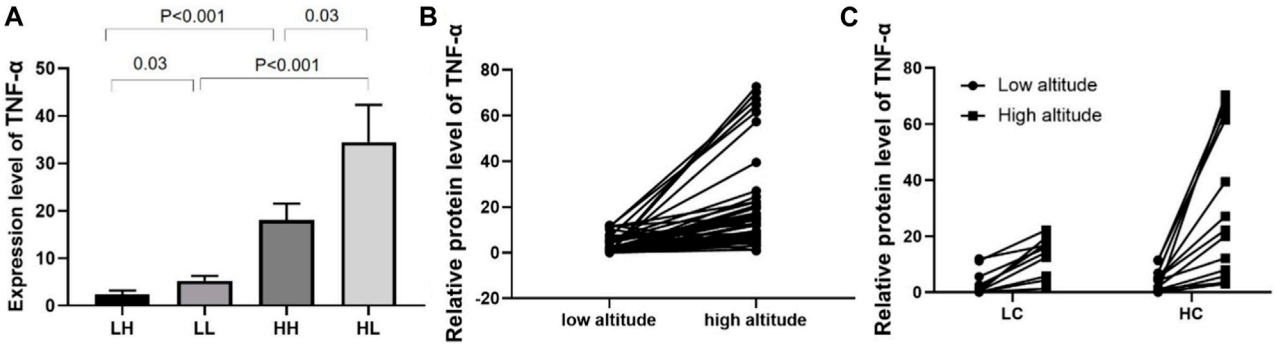

FIGURE 7
ELISA analysis of TNFα in different groups. **(A)** The expression of TNFα in HH/HL was significantly higher than in LH/LL group. **(B)** The expression of TNFα was significantly increased from low altitude to high altitude. **(C)** Individual expression of TNFα in the LC and HC groups from low to high altitude.

In our urine peptidome study, we identified four DEPs that varied in levels between the HH and HL groups. All four proteins were upregulated in the HH group compared to the HL group, and their mean recognition capacity had an ROC value of ≥70%. The four potential urine biomarkers identified for HH were ITIH1, PDCD1LG2, NME1-NME2 and CSPG4. The expression levels of these four proteins were verified using ELISA in the HH and HL groups. ELISA results confirmed the upregulation of ITIH1 and PDCD1LG2 in the HH group. ITIH1 is a heavy chain of a serine protease inhibitor that may act as a carrier of hyaluronan or as a binding protein between hyaluronan and other matrix proteins, playing a role in inflammation and carcinogenesis (Hamm et al., 2008). ITIH1 contains a putative binding site for hyaluronic acid, a ubiquitous component of the extracellular matrix (ECM) (Scott et al., 2009). Therefore, ITIH1 could be involved in ECM stabilization (Huang et al., 1993). Thus, they accumulate in the vascular endothelium and may play a role in the stabilization of endothelial cells and ECM damaged by high-altitude hypoxia. Low expression of ITIH1 in HL urine samples may be a vital clue for the inability to adapt to high-altitude hypoxic environments as the conditions of vascular endothelial cells have been affected. PDCD1LG2 is involved in costimulatory signalling that is essential for T-cell proliferation and IFNG production in a PDCD1-independent manner. Interaction with PDCD1 inhibits T-cell proliferation by blocking cell cycle progression and cytokine production (Huang et al., 2020). PDCD1LG2 is highly expressed in the heart, placenta, pancreas, lung, and liver and weakly expressed in the spleen, lymph nodes, and thymus (Takamochi et al., 2022). It is upregulated by IFNG/IFN-gamma stimulation in monocytes and induced in dendritic cells grown from peripheral blood mononuclear cells with CSF2 and interleukin-4 (Yang et al., 2022). High expression of PDCD1LG2 in HH urine samples may signify its role in adaptation to high-altitude hypoxic environments with high endurance by inhibiting T-cell proliferation and maintaining low inflammation.

Furthermore, we identified six DEPs that varied between the LC and HC groups. All six proteins were upregulated in high altitude compared to low altitude with increase in the protein expression of LRG1 and GPX3 and decreases in the protein expression of NID1, VASN, ACP2, and PRSS8 in the LC group compared with that of the HC groups. LRG1 is a Rho-GTPase-activating protein that acts as a GTPase activator and has metal ion-binding activity (Lin M et al., 2022).Several studies have investigated the biological effects of LRG1 as a factor in inflammation, vascular growth regulation, cell adhesion, and cell viability (Liu TT et al., 2021). Therefore, lower LRG1 expression in the LC group compared with that in the HC group suggests that individuals with little change in running speed with change in altitude have lower levels of inflammatory stress and better antioxidant capacity. GPX3 is a component of the glutathione peroxidase-like protective system against oxidative damage (Reddy AT et al., 2018). Lower GPX3 expression in the LC group than in the HC group suggests that individuals with little change in altitude running speed have lower levels of ROS stress and better antioxidant capacity. NID1 is a sulphated glycoprotein that is widely distributed in basement membranes and tightly associated with laminin, which plays a role in cell–ECM interactions (Ferraro DA et al., 2019). The relatively low expression of NID1 in HC urine samples may indicate the inability to adapt to high-altitude hypoxic environments as the cell–ECM interaction conditions have been affected. VASN acts as an inhibitor of TGF-β signalling, which has been shown to exert anti-apoptotic effects, and hypoxia has been reported to stimulate a robust elevation in VASN expression (Choi JA et al., 2022). We observed an increase in VASN expression at high altitudes. The accumulation of VASN in the LC group is indicative of its role in adaptation to high altitudes through anti-apoptotic and anti-TGF-β activities. ACP2 belongs to the histidine acid phosphatase family and is a carrier of the growing fatty acid chain in fatty acid biosynthesis (Yi Z et al., 2019). The relatively low expression of ACP2 in HC urine samples may indicate its significant role in the individual's inability to adapt to high-altitude hypoxic environments owing to alteration of fatty acid metabolism. PRSS8 is a potential regulator of epithelial sodium channel (ENaC) function, causing increased blood pressure (Shipway A et al., 2004). Reduced prostasin expression in the IBD mucosa is linked to the deterioration of local anti-inflammatory activity (Sugitani Y et al., 2020). The lower expression of PRSS8 in the HC group might be related to the deterioration of the local anti-inflammatory activity.

In conclusion, this study aimed to identify urine protein biomarkers for people with high stamina levels and good adaptability to high altitudes. Our findings indicate that WBC, lym, PLT, and lym% were significantly higher in HH individuals

than in HL individuals. Additionally, urine proteomics analysis revealed several differentially expressed proteins between HH and HL samples, with ITIH1 and PDCD1LG2 being identified as potential biomarkers for HH. Furthermore, our analysis identified six proteins (LRG1, NID1, VASN, GPX3, ACP2, and PRSS8) as urine proteomic biomarkers for high stamina in high-altitude adaptation, and a combination of these proteins could potentially be used in the screening of individuals with high-altitude adaptability. The use of proteomics in this study offers a new approach to identifying biomarkers for screening individuals who can adapt to high altitudes with high stamina levels. We hope to expand this study by verifying these findings using larger sample sizes.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: http://proteomecentral.proteomexchange.org; PXD039791.

Ethics statement

The studies involving human participants were reviewed and approved by the All study participants provided informed consent, and the study design was approved by from the Chinese PLA General Hospital ethical committee with the approval identifier S2019-035-01. The patients/participants provided their written informed consent to participate in this study.

Author contributions

CL, GG, XL, YS, and YC analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft. HL, XX, and JH analyzed the data. KH conceived and designed the experiments, approved the final draft. XL and CL confirm the authenticity of all the raw data.

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Acknowledgments

The Clinical research was reviewed, approved and granted by the Ethical Committee of the Chinese PLA General Hospital (Reference number: S2019-035-01). Each participant had signed informed consent.

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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Glossary

HAPH	high-altitude pulmonary hypertension
LC group	low change in endurance from low altitude to high altitude
HC group	high change in endurance from low altitude to high altitude
HH group	high-altitude with high stamina
HL group	high-altitude with low stamina
HGB	haemoglobin
RBC	red blood cell count
WBC	white blood cell count
Lym	lymphocyte count
Neu	neutrophil number
Mon	monocyte number
Eos	eosinophil number
Bas	basophil number
Lym%	lymphocyte percentage
Mon%	monocyte percentage
Eos%	eosinophil percentage
Bas%	basophil percentage
HCT	haematocrit
MCV	mean corpuscular volume
RDW-CV	red blood cell distribution width coefficient of variation
RDW-SD	red blood cell distribution width standard deviation
PLT	platelet count
MPV	mean platelet volume
PDW	platelet distribution width
PCT	platelet volume
DEPs	Differentially expressed proteins
PCA	Principal component analysis
ITIH1	inter-α-trypsin inhibitor heavy chain H1 fragment
PDCD1LG2	Recombinant Programmed Cell Death Protein 1 ligand 2
NME1-NME2	Human NME1-NME2 complex
CSPG4	chondroitin sulphate proteoglycan 4
LRG1	leucine rich alpha-2-glycoprotein 1
GPX3	glutathione peroxidase 3
NID1	nidogen 1
VASN	Vasorin
ACP2	acid phosphatase 2
PRSS8	serine protease 8
ROC	receiver operating characteristic curve
AUC	area under the curve
FOT	fraction of total
TNF-a	tumour necrosis factor

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Effects of cutaneous administration of an over-the-counter menthol cream during temperate-water immersion for exercise-induced hyperthermia in men

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Introduction: Hyperthermia impairs various physiological functions and physical performance. We examined the effects of cutaneous administration with an over-the-counter (OTC) analgesic cream containing 20% methyl salicylate and 6% L-menthol during temperate-water immersion (TWI) for exercise-induced hyperthermia.

Methods: In a randomized crossover design, twelve healthy males participated in both of two experiments. Firstly, participants underwent a 15-min TWI at 20°C with (CREAM) or without (CON) cutaneous application of an analgesic cream. Cutaneous vascular conductance (CVC) was measured using laser doppler flowmetry during TWI. In a subsequent experiment, same participants performed a 30-min strenuous interval exercise in a heated (35°C) environment to induce hyperthermia (39°C), which was followed by 15 min of TWI.

Results: Core body temperature, as measured by an ingestible telemetry sensor, and mean arterial pressure (MAP) were measured. CVC and %CVC (% baseline) were higher during TWI in CREAM than in CON (Condition effect: $p = 0.0053$ and $p = 0.0010$). An additional experiment revealed that core body heat loss during TWI was greater in CREAM than in CON (Cooling rate: CON 0.070 ± 0.020 vs. CREAM 0.084°C ± 0.026°C/min, $p = 0.0039$). A more attenuated MAP response was observed during TWI in CREAM than in CON (Condition effect: $p = 0.0007$).

Conclusion: An OTC analgesic cream containing L-menthol and MS augmented cooling effects when cutaneously applied during TWI in exercise-induced hyperthermia. This was, at least in part, due to the counteractive vasodilatory effect of the analgesic cream. The cutaneous application of OTC analgesic cream may therefore provide a safe, accessible, and affordable means of enhancing the cooling effects of TWI.

KEYWORDS

hyperthermia, skin cooling, topical analgesics, core temperature, cutaneous vasodilation, menthol

1 Introduction

Hyperthermia, a condition characterized by elevated body temperature, impairs various physiological functions and physical performance (Periard et al., 2021). Numerous studies have verified the effectiveness of a variety of cooling methods, including cold water immersion (CWI) (Proulx et al., 2003; Luhring et al., 2016), tarp-assisted cooling with oscillations (TACO) (Luhring et al., 2016; Hosokawa et al., 2017), application of a cooling garment (Abdallah et al., 2015; Xu et al., 2021), ice-sheet cooling (Butts et al., 2017), etc. Among these, CWI is currently regarded by numerous professional organizations as the gold standard treatment for hyperthermia (VanScoy et al., 2016; Belval et al., 2018).

External cold stimulation leads to vasoconstriction in the cutaneous microvasculature (Alba et al., 2019). This vasoconstricting response helps maintain a stable core temperature in the body as cutaneous blood flow is significantly dampened in response to external cold exposure, thereby decreasing the amount of heat transferred from core to skin (Charkoudian, 2010; Tansey and Johnson, 2015). In the case of hyperthermia, however, the cold-induced vasoconstriction in the skin could be a restriction factor, as the effect of external cooling can be partly limited to the cooled regions via conduction while heat exchanged between skin and core via the circulatory system is reduced (Taylor et al., 2008). Regarding this, the cooling effect of temperate-water immersion (TWI) at 26°C has been shown to be comparable to that of CWI at 14°C because of the maintenance of a higher peripheral blood flow during TWI (Taylor et al., 2008). Proulx et al. have also reported no differences in the cooling rate between the immersions at 8, 14, and 20°C for treating exercise-induced hyperthermia (Proulx et al., 2003). These studies suggest that TWI could be a safe, comfortable, and effective cooling modality for treating hyperthermic individuals. Also, there is potential that the cooling effects of TWI would be further augmented by overcoming the cold-induced cutaneous vasoconstriction associated with the immersion.

For decades, topical analgesic products have been utilized by sportspersons to relieve pain in local muscles and joints. The two main ingredients of over-the-counter (OTC) analgesics are L-menthol (1%–10%) and methyl salicylate (MS) (12%–30%) (Martin et al., 2004; Higashi et al., 2010). These two ingredients have a vasodilatory effect in the skin (Green and Flammer, 1989; Dolen et al., 2015; Craighead and Alexander, 2016; Craighead et al., 2017). Moreover, they have a synergistic effect on skin absorption, such that MS is more efficiently absorbed into the cutaneous vasculature in the presence of L-menthol (Yano et al., 1991). Our laboratory recently showed that the cutaneous application of analgesic cream can attenuate cold-induced vasoconstriction in the skin and potentially promote heat loss during hyperthermia (Wang et al., 2022). However, the effectiveness of OTC analgesic application administered with TWI as part of a hyperthermia treatment remains unknown.

In this paper, we determined whether an OTC analgesic cream containing L-menthol and MS augmented cooling effects when applied in TWI for treatment exercise-induced hyperthermia. We first tested whether the application of analgesic cream attenuated cold-induced vasoconstriction (*i.e.*, counteractive vasodilation) during TWI and compared this result to a control TWI. We then tested whether analgesic cream accelerated a reduction in core body temperature during TWI for treatment of exercise-induced hyperthermia.

2 Materials and methods

2.1 Ethical approval and participants

The study was conducted in accordance with the Declaration of Helsinki (2013) and all study procedures used in the current experiment were approved by the Institutional Review Board (IRB) at Jeonbuk National University (IRB #: JBNU 2022-01-004-002). All participants were verbally informed of the risks and discomforts associated with experimental trials, and written informed consent was obtained from all participants prior to their participation.

Twelve healthy males (age: 26.8 ± 2.6 years, height: 177.9 ± 5.4 cm, body weight: 75.7 ± 7.9 kg, BMI: 23.4 ± 1.6 kg · m^{-2}, body fat: 12.3% ± 3.9%) participated in this study. Participants with a history of genetic, cardiovascular, metabolic, or respiratory disease, allergic reactions to cold stimulation or topical analgesic cream, or a history of smoking, were excluded from participation. No participants reported taking medicines or supplements.

2.2 Overall experimental procedures

A schematic experimental protocol is shown in Figure 1. During their first visit, participants were familiarized with the experimental protocol and instrumentation after providing written and verbal consent. Body mass (kg) and body fat (%) were measured (InBody 720, InBody Co., Seoul, South Korea) during the first visit. Each participant subsequently visited the laboratory a total of four times in the morning (7–10 a.m.) with at least 72-h separation between visits, two visits for Study 1 and two visits for Study 2. The order of each of the two visits within Study 1 and 2 was randomly assigned (*i.e.*, a randomized crossover design). Thus, all participants completed both Study 1 and 2. Participants were instructed to refrain from strenuous exercise, alcohol, and caffeine consumption during the 24 h prior to all visits and were asked to fast overnight for the ~12 h preceding the visit. Participants were also asked to wear the same clothes (short-sleeved T-shirt and tight swim shorts) during all four visits.

2.2.1 (Study 1) experiment procedures and measurements

All procedures in Study 1 were conducted in a temperature-controlled laboratory (~23°C and 50% humidity). On the experimental day, participants were instructed to rest in a patient bed in a semi-supine position for 10 min (baseline). During the baseline period, two laser doppler probes (VP7 A/T with VMS-LDF2; Moor Instruments, Wilmington, DE, United States of America), wrapped with transparent plastic bags as

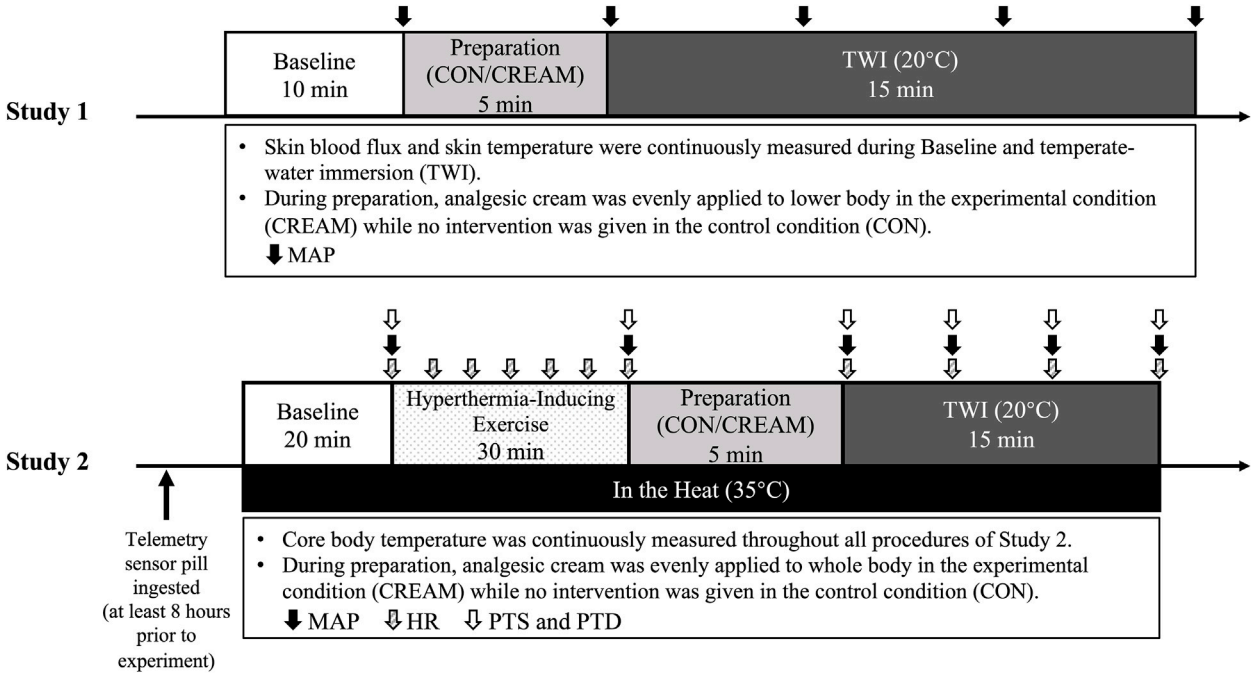

FIGURE 1
Experimental Protocols. Experimental procedures of two studies (Study 1 and 2). CON, a control condition; CREAM, an experimental condition that received cutaneous application of analgesic cream; TWI, temperate-water immersion at 20°C; MAP, mean arterial pressure; HR, heart rate; PTD, perceived thermal sensation; PTD, perceived thermal discomfort. Study 1 was performed in a thermoneutral environment (~23°C, 50% humidity) while all procedures in Study 2 were conducted under hyperthermic conditions (35°C, 50% humidity).

waterproofing, were placed on the anterior thigh (15 cm apart to minimize inter-probe interaction). A line was drawn from the anterior superior iliac spine to the superior border of the patella. Two laser doppler flowmetry (LDF) probes were then placed in two spots from the center of the line of both thighs. Using LDF, skin blood flux and skin temperature (T_{sk}) were recorded and averaged during the last minute of the baseline period. This was followed by three measurements of intermittent blood pressure via automated brachial auscultation (GE S/5 Light Patient Monitor; Datex-Ohmeda, Madison, WI, United States of America). Mean arterial pressure (MAP) was determined as one-third pulse pressure plus diastolic blood pressure. Cutaneous vascular conductance (CVC) was calculated as the ratio of skin blood flux to MAP.

Following the baseline measurement, the LDF probes were removed and analgesic cream was evenly applied to the entirety of the skin surface of both legs (CREAM), which was followed by re-attachment of the probes. For the control condition (CON), probes were removed and re-attached without administration of analgesic cream. In our pilot study, SkBF readings were neither affected by the re-attachment of the laser doppler probes nor the transparent plastic bag for waterproofing (data not shown). Participants were then immersed in a bathtub (498L) in which the water temperature was maintained at 20°C (temperate-water immersion, TWI) to the iliac crest for 15 min (Mawhinney et al., 2017). During the 15-min TWI period, skin blood flux and T_{sk} were continuously measured, and intermittent blood pressure was measured three times every 5 min. It should be noted that, rather than whole body immersion, TWI was performed only for the lower body in Study 1 as a result of technical difficulties associated with laser doppler measurement in the water (*i.e.*, waterproofing issue).

2.2.1.1 Cutaneous application of topical analgesic cream

The OTC analgesic cream that was used in our study consisted of 6% L-menthol, 20% methyl salicylate (MS), and other ingredients, including lanolin and mineral oil, sorbitanmonostearate (surfactant), polysorbate 60 (surfactant), trolamine, and purified water (Antiphlamine-S, Yuhan, South Korea). In a previous study, we confirmed that dose of 0.64 μ L/cm^2 placed on the skin surface exhibited a potent vasodilatory effect (increasing blood flux by 80%–115% relative to baseline) over the course of 1 h (Wang et al., 2022).

2.2.2 (Study 2) experimental procedures and measurements

Based on findings in Study 1, we performed Study 2 to assess whole body heat loss. Along with overnight fasting, participants were asked to ingest a telemetry sensor pill (CorTemp Ingestible Core Body Temperature Sensor, HQ, Palmetto, FL, United States) to measure core body temperature (T_{CORE}) at least 8 h before the trial to avoid temperature deviation due to stomach contents (Domitrovich et al., 2010). Upon arrival, a core temperature recorder (CorTemp Data Recorder, HQ, Palmetto, FL, United States) was wirelessly connected with the sensor pill and placed on the back of participants to allow for continuous recording of their core temperature. Participants drank 200 mL of thermoneutral water (37°C) to minimize interference during the core temperature reading and no additional water was allowed

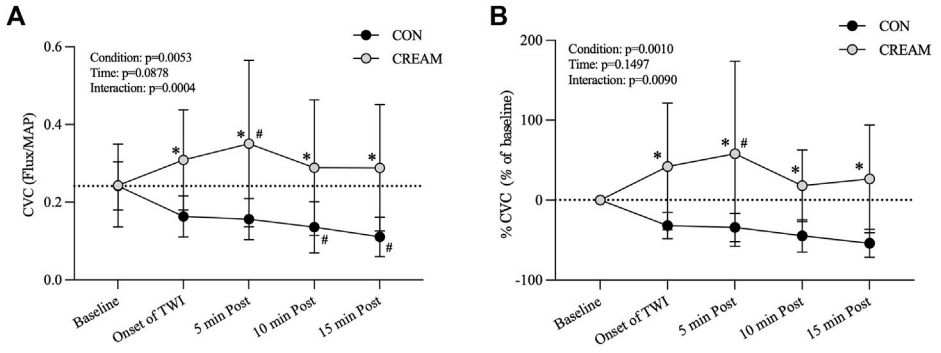

FIGURE 2
Cutaneous Vascular Conductance (CVC) and % CVC. Changes in CVC **(A)** and % CVC **(B)** during Baseline and 15-min TWI. CON, a control condition; CREAM, an experimental condition that received cutaneous application of analgesic cream. Two-way repeated measures ANOVA with a Šidák's *post hoc* test for all. Data are presented as the mean ± SD (n = 12). *p < 0.05 between the conditions. #p < 0.05 vs. baseline of each condition.

afterwards. Subjects entered the temperature-controlled room which was maintained at 35°C (~50% humidity). All procedures conducted in Study 2 were completed in a hot environment (Figure 1).

Following a 20-min baseline period in the sitting position (baseline), participants performed a 30-min regimen of strenuous interval exercise that consisted of 30 s each of jumping jacks, high knees, squat, lunges, and cycling with 5 s breaks given between each activity. During this exercise protocol, T_{CORE} increased by 2.02°C ± 0.18°C from the Baseline (Baseline 37.02 ± 0.14 vs. Post Exercise 39.04°C ± 0.15°C for the two conditions combined). Based on the age-predicted exercise intensity (heart rate reserve or HRR) (Lui et al., 2011), the exercise intensity in the current study was between 75% and 85%.

Once hyperthermia was induced, participants removed all clothes except for the tight swim shorts and toweled off their sweat. They then were immersed in a recumbent position in a 20°C water bathtub (498L) without (CON) or with (CREAM) the analgesic cream administered over the entire surface of the legs and arms as well as torso (0.64 μ L/cm^2 of skin surface), which took approximately less than 1 min. During the 15 min of TWI, participants completely submerged their bodies up to the sternum. The water temperature was measured every 3 min and was maintained by adding an appropriate amount of ice-water and then stirring slightly.

Perceived thermal sensation (Brazaitis et al., 2014), perceived thermal discomfort (Mekjavic et al., 2021), and blood pressure (BP) were measured at the end of the baseline and exercise, at the onset and throughout TWI (every 5 min), and post TWI. The thermal sensation scale ranged from 1 (very cold) to 9 (very hot), with 5 being neutral. The scale of perceived thermal discomfort ranged from 1 (neutral) to 5 (extreme discomfort).

2.3 Statistical analysis

Data are expressed as mean ± SD. Statistical significance was set as p < 0.05. The assumption of normality was verified using the Shapiro-Wilks W-Test. Variables were analyzed using two-way repeated-measures ANOVA, which was followed by Šidák's multiple comparisons; the factors were intervention conditions (CON/CREAM) and time. A two-tailed paired t-test was used to determine a statistical difference in cooling rates between the conditions. Cohen's d effect size (ES) was reported (ES > 0.2, small; >0.5, moderate; >0.8, large) when statistical differences were found between conditions. Statistical analyses were performed using GraphPad Prism 9.2.0 software (GraphPad, La Jolla, CA, United States).

TABLE 1 Changes in Skin Temperature. Skin temperature (°C) during baseline and 15-min TWI. CON, a control condition; CREAM, an experimental condition that received cutaneous application of analgesic cream. Two-way repeated measures ANOVA with a Šidák's *post hoc* test for all. Data are presented as the mean ± SD (n = 12). No significant differences were found between the conditions.

	Baseline	TWI (min)			
		Onset	5	10	15
CON	30.75 ± 0.92	23.14 ± 0.99	21.59 ± 0.52	21.51 ± 0.69	21.67 ± 0.77
CREAM	30.44 ± 1.06	23.17 ± 1.12	21.92 ± 0.94	21.83 ± 0.88	21.94 ± 0.82

3 Results

3.1 Study 1

3.1.1 Cutaneous vascular conductance (CVC), % CVC, and skin temperature (T_{sk})

During the 15-min TWI, CVC in CREAM remained elevated at levels higher than those seen in CON (Condition effect: p = 0.0053) (Figure 2A). Also, a significant interaction (condition × time) was seen (p = 0.0004). From the *post hoc* analyses, CVC was elevated from the onset of TWI to the end of TWI (Onset of TWI: p = 0.0001, ES = 1.67; 5 min Post: p < 0.0001, ES = 1.52; 10 min Post: p < 0.0001, ES = 1.33; 15 min Post: p < 0.0001, ES = 1.74) (Figure 2A). CVC was lower in CON relative to the baseline during the TWI (10 min Post:

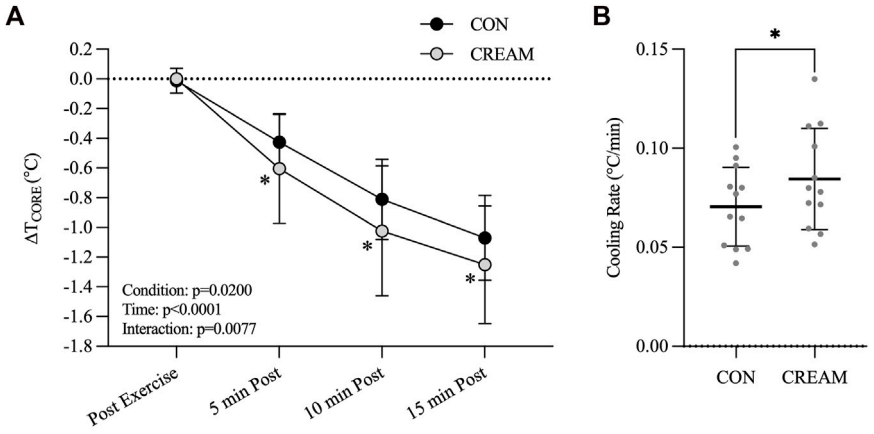

FIGURE 3
Core Temperature and Cooling Rate. Changes in T_{CORE} (ΔT_{CORE}) **(A)** and cooling rate **(B)** during 15-min TWI. CON, a control condition; CREAM, an experimental condition that received cutaneous application of analgesic cream. Two-way repeated measures ANOVA with a Šidák's *post hoc* test and a paired *t*-test was used for **(A and B)**, respectively. Data are presented as the mean ± SD (n = 12). *p < 0.05 between the conditions.

p = 0.0112, ES = 1.73; 15 min Post: p = 0.0009, ES = 2.43) while an increased CVC was observed in CREAM (5 min Post: p = 0.0095, ES = 0.70).

Similarly, % CVC was higher in CREAM relative to CON (Condition effect: p = 0.0010) (Figure 2B). A significant interaction was found (p = 0.0090). % CVC was greater in CREAM from the onset of TWI to the end of TWI (Onset of TWI: p = 0.0012, ES = 1.61; 5 min Post: p < 0.0001, ES = 1.45; 10 min Post: p = 0.0071, ES = 2.02; 15 min Post: p = 0.0004, ES = 1.98) (Figure 2B). % CVC was increased in CREAM relative to the baseline during the TWI (5 min Post: p = 0.0287, ES = 1.05) but no significant differences were observed in CON (p > 0.05 for all).

T_{sk} decreased similarly in both conditions during TWI (Time and Condition effect: p < 0.0001 and 0.6455, respectively). No differences in T_{sk} were found between conditions during TWI (Onset of TWI: p > 0.9999; 5 min Post: p = 0.7019; 10 min Post: p = 0.7129; 15 min Post: p = 0.8322) (Table 1).

3.2 Study 2

3.2.1 Core temperature (T_{CORE}) and cooling rate

The baseline T_{CORE} was similar between conditions (CON: 37.02 ± 0.15 vs. CREAM: 36.95°C ± 0.14°C, p = 0.8763) (Figure 3A). During the 30-min exercise, T_{CORE} increased similarly in both conditions (Baseline: 37.02 ± 0.15 vs. Post Exercise: 39.04°C ± 0.15°C, Time effect: p < 0.0001, Condition effect: p = 0.5066) with no differences observed between conditions by the end of exercise (Post Exercise) (CON: 39.03 ± 0.16 vs. CREAM: 39.04°C ± 0.15°C, p = 0.598).

During 15-min TWI, a decline in T_{CORE} (ΔT_{CORE}) was observed in both conditions (Time effect: p < 0.0001), though an even a greater reduction in T_{CORE} was detected in CREAM (Condition effect: p = 0.0200; 5 min Post: CON − 0.425 ± 0.186 vs. CREAM − 0.604°C ± 0.369°C, p = 0.0027, ES = 0.67; 10 min Post: CON − 0.811 ± 0.270 vs. CREAM − 1.024°C ± 0.438°C, p = 0.0003, ES = 0.63; 15 min Post: CON − 1.070 ± 0.286 vs. CREAM − 1.252°C ± 0.396°C, p = 0.0022, ES = 0.56) (Figure 3A). A significant interaction was found (Condition × Time) (p = 0.0077).We also analyzed the cooling rate during the 15-min TWI; this was higher in CREAM relative to CON (CON 0.070 ± 0.020 vs. CREAM 0.084°C ± 0.026°C/min, p = 0.0039, ES = 0.65) (Figure 3B).

3.2.2 Blood pressure and heart rate (HR)

During the TWI, a reduced systolic blood pressure (SBP) was observed in CREAM relative to CON (Condition effect: p = 0.0205, Onset of TWI: CON 136.58 ± 10.05 vs. CREAM 128.58 ± 7.65 mmHg, p = 0.015, ES = 0.94; 5 min Post: CON 132.67 ± 9.19 vs. CREAM 126.83 ± 6.78 mmHg, p = 0.1397; 10 min Post: CON 132.17 ± 10.85 vs. CREAM 122.92 ± 10.09 mmHg, p = 0.0034, ES = 0.92; 15 min Post: CON 131.08 ± 9.77 vs. CREAM 121.58 ± 10.07 mmHg, p = 0.0025, ES = 1.00) (Figure 4A). Also, significant time effect and interaction (Condition × Time) were found (Time effect: p < 0.0001; Interaction: p = 0.0058).

We also noted a trend towards lowered diastolic blood pressure (DBP) during TWI (Condition effect: p = 0.0044, Onset of TWI: CON 80.67 ± 8.51 vs. CREAM 73.75 ± 8.30 mmHg, p = 0.0186, ES = 0.86; 5 min Post: CON 82.75 ± 8.44 vs. CREAM 76.08 ± 6.91 mmHg, p = 0.0254, ES = 0.91; 10 min Post: CON 80.25 ± 7.93 vs. CREAM 74.58 ± 7.06 mmHg, p = 0.0822; 15 min Post: CON 78.75 ± 6.62 vs. CREAM 75.08 ± 6.29 mmHg, p = 0.4925) (Figure 4B). A significant time effect (Time effect: p < 0.0001) was detected while no significant interaction (Condition × Time) was found (p = 0.0538).

In both conditions, MAP significantly increased during TWI (Time effect: p < 0.0001). Interestingly, MAP was lower in CREAM than in CON during TWI (Condition effect: p = 0.0007) (Onset of TWI: CON 99.31 ± 7.36 vs. CREAM 92.03 ± 6.44 mmHg, p < 0.0001, ES = 1.10; 5 min Post: 98.86 ± 7.76 vs. CREAM 93.00 ± 6.05 mmHg, p = 0.0028, ES = 0.89; 10 min Post: CON 97.83 ± 8.24 vs. CREAM 90.81 ± 6.80 mmHg, p = 0.0051, ES = 0.98; 15 min Post: CWI 96.19 ± 7.25 vs. 90.58 ± 6.58 mmHg, p = 0.0495, ES = 0.85) (Figure 4C). No significant interaction (Condition × Time) was found (p = 0.0515).

No differences in HR were seen during baseline between conditions (Condition effect: p = 0.9906). Both conditions

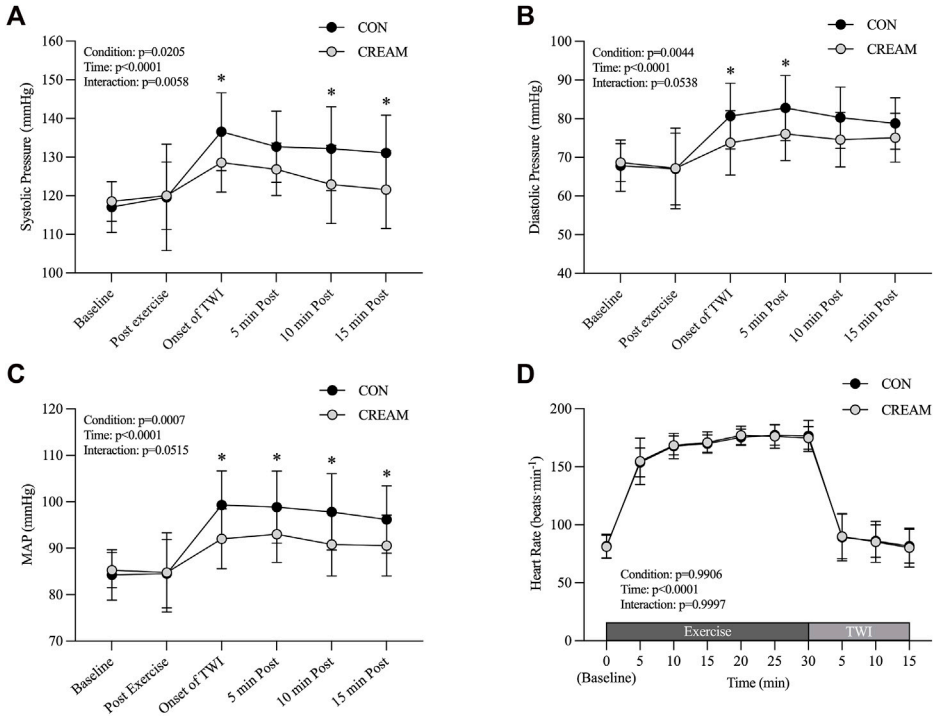

FIGURE 4
Blood Pressure. Changes in systolic blood pressure (SBP) **(A)**, diastolic blood pressure (DBP) **(B)**, mean arterial pressure (MAP) **(C)**, and heart rate (HR) **(D)** during baseline, post-exercise, and 15-min TWI. CON, a control condition; CREAM, an experimental condition that received cutaneous application of analgesic cream. Two-way repeated measures ANOVA with a Šidák's *post hoc* test for all. Data are presented as the mean ± SD (n = 12). *p < 0.05 between the conditions.

TABLE 2 Perceived Thermal Sensation (PTS) and Perceived Thermal Discomfort (PTD). PTS (score range: 1–9) and PTD (score range: 1–5) during baseline, post-exercise, and 15-min TWI is shown. CON, a control condition; CREAM, an experimental condition with cutaneous application of analgesic cream. Two-way repeated measures ANOVA with a Šidák's *post hoc* test for all. Data are presented as the mean ± SD (n = 12). No significant differences were found between the conditions.

		Baseline	Post exercise	TWI (min)			
				Onset	5	10	15
PTS	CON	6.17 ± 0.39	8.83 ± 0.39	1.50 ± 0.79	1.75 ± 0.87	1.75 ± 1.06	1.83 ± 1.03
	CREAM	6.33 ± 0.49	8.67 ± 0.49	1.25 ± 0.62	1.33 ± 0.49	1.67 ± 0.65	1.67 ± 0.65
PTD	CON	1.50 ± 0.52	3.75 ± 0.45	3.75 ± 0.45	3.42 ± 0.67	3.42 ± 0.67	3.33 ± 0.49
	CREAM	1.75 ± 0.45	3.75 ± 0.45	3.83 ± 0.39	3.42 ± 0.52	3.50 ± 0.52	3.17 ± 0.39

showed similar increases in HR during the 30-min exercise, and ultimately no discrepancy was seen by the end (p > 0.9999 for all time points during exercise). HR also declined significantly in both conditions with no noticeable differences between them (p > 0.9999 for all time points during TWI (Figure 4D).

3.2.3 Perceived thermal sensation (PTS) and perceived thermal discomfort (PTD)

PTS was similar between conditions during baseline (p = 0.9872), post exercise (p = 0.9872), and TWI (Onset of TWI: p = 0.9106; 5 min Post: p = 0.4964; 10 min Post: p = 0.9997; 15 min Post: p = 0.9872) (Table 2). Similarly, no differences in PTD were observed between conditions during baseline (p = 0.6234), post exercise (p > 0.9999), and TWI (Onset of TWI: p = 0.9974; 5 min Post: p > 0.9999; 10 min Post: p = 0.9974; 15 min Post: p = 0.9135) (Table 2).

4 Discussion

We investigated the effects of an OTC analgesic cream containing 20% methyl salicylate (MS) and 6% L-menthol administered cutaneously during TWI at 20°C as a treatment for exercise-induced hyperthermia. In Study 1, a significant vasoconstriction in the skin occurred during a control TWI (a decrease by 32%–59% from baseline); and a counteractive

vasodilatory effect was observed during the TWI after the OTC analgesic cream was applied to the skin, such that cutaneous vascular conductance (CVC) was higher than the baseline counterpart (by 19%–44%) (Figure 2A). Skin temperature (T_{sk}) dropped in both conditions during the TWI with no difference seen between conditions (Table 1).

Relying on our findings in Study 1, we performed a subsequent experiment (Study 2) and found that cutaneous administration of analgesic cream promoted core body heat loss during TWI administered in response to exercise-induced hyperthermia (Figures 3A, B). Together, the analgesic cream improved the cooling effect of TWI potentially via vasodilation that counteracted the cold-induced vasoconstriction. Interestingly, we also observed that the analgesic cream lowered the blood pressure during the TWI (Figure 4).

Acceptable cooling rates for treating hyperthermic condition are >0.08°C/min with an ideal rate of cooling being >0.16°C/min (McDermott et al., 2009). Thus, the proposed cooling method in our study (CON: 0.070°C ± 0.020°C/min and CREAM: 0.084°C ± 0.026°C/min) would not be the best options for individuals with heat-related illnesses such as exertional heat stroke (EHS) although the proposed TWI (*i.e.*, CREAM) did meet 'minimal' guideline for EHS treatment (>0.08°C/min). In athletic competition, however, athletes may not have adequate time to cool between breaks in competition, which would be ~10 min at most between halves or quarters. In our study, cooling rates during the first 5-min of TWI were 0.121°C ± 0.061°C/min in CREAM while a control TWI showed 0.085°C ± 0.036°C/min. Therefore, an OTC analgesic cream administered during TWI would be applicable for those who have limited time for cooling.

Cold-induced vasoconstriction is mediated by a variety of intrinsic mechanisms including norepinephrine (NE) synthesis and release, adrenergic receptors, nitric oxide (NO), as well as activation of Rho-associated kinase (ROCK) signaling mechanisms (Lang et al., 2009; Alba et al., 2019). Our data revealed that cutaneous vasoconstriction during TWI was clearly lower in CREAM from Study 1, such that skin blood flux or CVC were 2-3 times higher in CREAM than in CON during the TWI. Considering the main ingredients in our analgesic cream (and in the majority of OTC analgesic products), we speculate that the pharmacological actions of MS and L-menthol may play an important role in decreasing cold-induced vasoconstriction during TWI.

MS, a common ingredient of topical analgesics, is a non-steroidal anti-inflammatory drug (NSAID) made from natural plant extracts. As an agonist for transient receptor potential vanilloid subtype 1 (TRPV1), MS is involved in the nociceptive signaling of sensory nerves (Ohta et al., 2009). A recent study showed that TRPV1 mediates thermoregulation, such that inhibition of TRPV1 activates sympathetic nervous activity (SNA) and associated heat gaining responses such as cutaneous vasoconstriction (Alawi et al., 2015). Cold stimulation is known to suppress TRPV1 activation (Chung and Wang, 2011), which may partly explain the cold-induced thermogenic responses observed in the human body. Thus, MS that binds to TRPV1 may blunt sympathetic-induced vasoconstriction despite the fact that cold stimulation concomitantly suppresses TRPV1. The opposite actions for TRPV1 might explain the blunted cutaneous vasoconstriction we observed in the CREAM during TWI. In addition, the TRPV1 expressed in endothelial cells lead to increased endothelial NO synthase (eNOS) phosphorylation and NO production (Wang et al., 2017), which may also explain the counteractive vasodilation in the skin of the CREAM as we observed during the TWI in our study. However, at this point these proposed mechanisms, as an explanation for this phenomenon, are merely speculative and future studies are needed to verify the mechanisms.

L-menthol is a known vasodilator that operates through eNOS, an L-type voltage-gated calcium blockade, and endothelium-derived hyperpolarizing factor (EDHF) (see review paper by Silva (Silva, 2020)). L-menthol also suppresses the reactive oxygen species (ROS)-induced RhoA/ROCK pathway via transient receptor potential melastatin 8 (TRPM8) channels (Fernandez-Tenorio et al., 2011; Xiong et al., 2017), a signaling pathway associated with cold-induced vasoconstriction. Along with its vasodilatory effects, L-menthol may blunt the increased activation of the RhoA/ROCK signaling induced by cold stimulation, and prompt the translocation of the α_{2C}-adrenergic receptor (Chotani et al., 2000; Bailey et al., 2004). Two recent studies showed that L-menthol application triggers a sympathetic-mediated vasoconstriction that delays the cooling effects associated with CWI (*i.e.*, heat-conservation responses) (Kounalakis et al., 2010; Botonis et al., 2018). However, the analgesic cream used in our study consisted of a large amount of MS (20%), which complicates the comparison between their findings and ours. MS-induced TVPV1 activation may blunt sympathetic-mediated responses (Alawi et al., 2015) and inhibit sensory nerves and perception (Ohta et al., 2009). Although previous studies have reported a decrease in muscle and skin temperature in response to menthol application, possibly indicating menthol-induced vasoconstriction (Lasanen et al., 2016; Hunter et al., 2018; Gillis et al., 2021), the idea that the ethanol contained in a menthol product induces evaporative heat loss cannot be ruled out (Hunter et al., 2018). Nevertheless, it is obvious that the OTC analgesic product used in this current study resulted in an increase in cutaneous blood perfusion by 80%–115% from baseline, a potent vasodilating effect (Wang et al., 2022). Further data is needed to explain this discrepancy in menthol's effects on vascular function during cooling.

An elevation in blood pressure is normally seen during skin cooling due to SNA-mediated peripheral vasoconstriction (Koehn et al., 2020), which indicates a potential risk to the cardiovascular system (Koehn et al., 2012; Tipton et al., 2017). It was also reported that medical professionals are sometimes hesitant to utilize whole-body CWI due to the fear of inducing shock (Hosokawa et al., 2019). Nevertheless, the majority of cooling studies have not reported such changes in blood pressure during cooling. In the current study, MAP increased in response to the 15-min TWI by 12–15 mmHg with systolic and diastolic pressure elevated by 12–17 mmHg and 12–14 mmHg, respectively. Intriguingly, participants cooled with analgesic cream experienced a lower blood pressure (Figures 4A–C). The effect size ranged from 0.85 to 1.10, so the blood pressure lowering effect of the analgesic cream seems clinically significant. It is well known that MS exerts an analgetic effect by desensitizing TRPV1 on sensory nerves (Ohta et al., 2009). Recent data has also revealed that TRPV1 plays a key role in the regulation of blood pressure via SNA modulation (Zhong et al., 2019). We accordingly speculate that cold-induced activation of sensory afferent nerves might have been blunted by MS-induced desensitization of TRPV1, which consequently attenuated reflex vasoconstriction and blood pressure responses during TWI. In addition to MS, L-menthol may have entered the systemic circulation and suppressed the RhoA/

ROCK pathway, which has previously been observed in humans (Martin et al., 2004). Other studies have also shown that menthol supplementation can decrease blood pressure in hypertensive animals (Sun et al., 2014; Xiong et al., 2017).

With reference to the perceived thermal discomfort (PTD) data in our study, perceived discomfort during TWI was comparable to that measured post exercise (Table 2); TWI at 20°C was quite unpleasant. The water temperature of TWI was set between 20°C–26°C in previous investigations (Proulx et al., 2003; Taylor et al., 2008). It may be worth investigating the effects of TWI when a higher water temperature is used in combination with additional interventions that can improve the cooling effects of TWI. We speculate that this may result in a greater counteractive vasodilatory effect triggered by the analgesic cream, as warmer water would lead to a decrease in cold-induced SNA response and consequent peripheral vasoconstriction.

4.1 Limitations

There are some limitations in this study. Firstly, we were not able to measure CVC or %CVC during TWI in Study 2 because of technical difficulties associated with waterproofing LDF probes. To measure CVC during the whole-body water immersion, LDF probes should have been waterproofed for longer length (>50 cm). Thus, we chose to perform a separate experiment (lower body TWI of Study 1) that could only provide an indirect explanation of the greater cooling effect of TWI observed with the application of the analgesic cream in Study 2.

Although we observed a potent counteractive vasodilatory effect from the analgesic cream during TWI in Study 1, its cooling effect on a hyperthermic body did not seem to be clinically meaningful (Figure 3). This may be because Study 2 involved whole-body TWI, and therefore elicited greater SNA activation and vasoconstriction (*i.e.*, greater reflex vasoconstriction), while Study 1, which involved only the lower body, led to less cold-induced reflex responses. Supporting this theory, we observed no increases in blood pressure during the lower body TWI in Study 1 relative to blood pressure in the baseline period (data not shown).

Sweat loss is closely associated with thermoregulatory function (Periard et al., 2021). There is a possibility that sudomotor function might have been affected by the analgesic. However, in our recent study, there was no discrepancy in sweat loss between conditions (CON vs. CREAM) (Wang et al., 2022) and we did not measure sweat loss in the current study. Also, female participants were not included in the current study as a subcutaneous fat layer largely affects cooling efficiency (Boehm and Miller, 2019). Data interpretation of our study, therefore, should be limited to males and future studies with both sexes included are warranted.

We speculate that the TRPV1 and RhoA/ROCK signaling pathways are involved in mediating the beneficial effects in application of analgesic cream containing 20% MS and 6% L-menthol. However, an analgesic product that does not contain active ingredients (*i.e.*, a product without MS and L-menthol) may better serve as a control intervention. Future studies investigating the molecular mechanisms that underlay the counteractive vasodilatory effect of analgesic cream applied in response to external cold stimulation are also needed.

5 Perspectives

We determined that an OTC analgesic cream containing L-menthol and MS augments TWI's cooling effects when applied in response to exercise-induced hyperthermia. The improved heat loss may be, at least in part, due to the counteractive vasodilatory effect of the analgesic cream. This tested cooling method also blunted the increased blood pressure normally observed during external cooling. A cutaneous application of OTC analgesic cream therefore suggests itself as a safe, accessible, and affordable means of improving the cooling effects of TWI.

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 studies involving human participants were reviewed and approved by The Institutional Review Board (IRB) at Jeonbuk National University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

GW and CH designed the studies; GW and CH performed and analyzed the experiments; GW and CH wrote the paper; GW and CH participated in important discussions of the data and edited the paper. All authors approved the final version of 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.

Publisher's note

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Peripheral skin cooling during hyper-gravity: hemodynamic reactions

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Introduction: Orthostatic dysregulation occurs during exposure to an increased gravitational vector and is especially common upon re-entering standard Earth gravity (1 g) after an extended period in microgravity (0 g). External peripheral skin cooling (PSC) has recently been described as a potent countermeasure against orthostatic dysregulation during heat stress and in lower body negative pressure (LBNP) studies. We therefore hypothesized that PSC may also be an effective countermeasure during hyper-gravity exposure (+Gz).

Methods: To investigate this, we designed a randomized short-arm human centrifuge (SAHC) experiment ("Coolspin") to investigate whether PSC could act as a stabilizing factor in cardiovascular function during +Gz. Artificial gravity between +1 g and +4 g was generated by a SAHC. 18 healthy male volunteers completed two runs in the SAHC. PSC was applied during one of the two runs and the other run was conducted without cooling. Each run consisted of a 10-min baseline trial followed by a +Gz step protocol marked by increasing g-forces, with each step being 3 min long. The following parameters were measured: blood pressure (BP), heart rate (HR), stroke volume (SV), total peripheral resistance (TPR), cardiac output (CO). Furthermore, a cumulative stress index for each subject was calculated.

Results: +Gz led to significant changes in primary as well as in secondary outcome parameters such as HR, SV, TPR, CO, and BP. However, none of the primary outcome parameters (HR, cumulative stress-index, BP) nor secondary outcome parameters (SV, TPR, CO) showed any significant differences—whether the subject was cooled or not cooled. Systolic BP did, however, tend to be higher amongst the PSC group.

Conclusion: In conclusion, PSC during +Gz did not confer any significant impact on hemodynamic activity or orthostatic stability during +Gz. This may be due to lower PSC

Abbreviations: +Gz, hyper-gravitational stress; CP, cooling protocol; CVP, central venous pressure; LBNP, lower body negative pressure; NCP, non-cooling protocol; PSC, peripheral skin cooling; SAHC, short-arm human centrifuge; VO_2max, maximal oxygen consumption.

responsiveness of the test subjects, or an insufficient level of body surface area used for cooling. Further investigations are warranted in order to comprehensively pinpoint the exact degree of PSC needed to serve as a useful countermeasure system during +Gz.

KEYWORDS

hyper-gravity, hyper-gravity centrifuge model tests, spaceflight countermeasures, G-induced loss of consciousness, short-arm human centrifuge, orthostatic instability, cardiovascular stability, peripheral external cooling

1 Introduction

Astronauts experience a multitude of physiological adaptations during space travel including cardiovascular deconditioning, loss of bone density, reduced aerobic capacity, and decreased plasma volume, which all contribute to the occurrence of orthostatic intolerance (Komorowski et al., 2016). This factor is particularly problematic for astronauts when returning to Earth after long-term space flight after having been conditioned to microgravity (Pavy-Le Traon et al., 2007; Lee et al., 2015). For astronauts returning from space, the resumption of standard gravity is considered hyper-gravitational stress (+Gz). Exposure to +Gz can lead to g-induced loss of consciousness where the redistribution of blood induced by high g-forces leads to a cranial to caudal blood volume shift, thereby creating a state of relative hypovolemia, hypoxia, and thus, if not countered, a loss of consciousness (Ryoo et al., 2004; Iwasaki et al., 2012; Habazettl et al., 2016; Ogawa et al., 2016).

High-g training and occupational exposure to +Gz are also experienced by military/fighter jet and test pilots. Orthostatic failure can lead to dizziness, reduced responsiveness, and—in the worst case—to (fatal) accidents and thus addressing this hazard is of utmost importance for human health in the aerospace environment. Different countermeasures have been tested in the past to i) counteract the acute effects of +Gz and ii) to attenuate complications after long-term deconditioning in microgravity when re-exposed to terrestrial standard gravity.

One apparatus that has already been developed is the so-called “anti-g suit” or “g-suit”. The underlying principle behind the design was laid out by physiologist Frank Cotton (which led to the design of the “Cotton Aerodynamic Anti-G Suit”) (Brook, 1990). The first suit (“Franks Flying Suit”) was then designed in the 1940s by Wilbur Franks and included inflatable trousers governed by g-sensitive faucets. However, this is no doubt effective we speculated that cooling might also be a valid and effective way of stabilizing the cardiovascular system in hyper-gravity.

It is well-known that cooling (also known as skin surface cooling) can play a protective role in attenuating orthostatic symptoms during an orthostatic challenge (Raven et al., 1980; Raven et al., 1981; Durand et al., 2004; Cui et al., 2005). Most of these studies were assessed using lower body negative pressure (LBNP). Among others, the lab of Crandall et al. investigated this topic extensively (Durand et al., 2004; Cui et al., 2005). Several factors are known to improve orthostatic stability via skin cooling. For example, skin surface cooling leads to an activation of the sympathetic nervous system accompanied by an increase in catecholamines. This leads to the stimulation of α1-receptors on the surface of blood vessels, leading to vasoconstriction of small vessels in the skin (Durand et al., 2004; Cui et al., 2005; Bender et al., 2020). Consequently, the blood distribution moves from the periphery to the body core, which increases the central venous pressure (CVP) and the mean arterial pressure (MAP) (Cui et al., 2005; Wilson et al., 2007).

Moreover, Opatz et al. have shown that higher baseline limb skin temperatures can predict presyncopal episodes during a head-up tilt test combined with LBNP (Opatz et al., 2018). It has been shown that skin surface cooling can be a countermeasure in heat stress subjects in a LBNP device (Durand et al., 2004). However, there is a lack of evidence that shows whether external cooling is feasible and provides +Gz tolerance time in a short-arm human centrifuge (SAHC). A SAHC can generate +Gz along the head-feet axis and is a useful terrestrial-based analogue for +Gz countermeasure system development (Watenpaugh et al., 2004).

To date, cooling has not been tested in a +Gz environment. Our study, therefore, deployed peripheral skin cooling (PSC) in a max +Gz graded run via SAHC. Based on the expected response to cooling, we hypothesized that:

1. PSC will lead to higher orthostatic tolerance which will be noticeable in a higher blood pressure (BP), lower heart rate (HR), and a higher cumulative stress index.
2. Cardiovascular parameter changes, such as higher stroke volume (SV), higher total peripheral resistance (TPR), equal cardiac output (CO), will be observed in the cooling protocol (CP) subjects compared to the non-cooling protocol (NCP) subjects.

2 Materials and methods

2.1 Subjects

Eighteen civilian, healthy, male volunteers participated in the SAHC study. Written informed consent was obtained prior to beginning the study. The experiment was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Medical Council North Rhine. Each volunteer passed a medical examination and spiroergometry test that was overseen by an independent aerospace physician. Only subjects without cardiovascular or metabolic diseases (e.g., orthostatic dysregulation, arrhythmia, diabetes mellitus, etc.), a body mass index (BMI) between 18–26 kg/m^2, a height between 170–210 cm and a maximal oxygen consumption (VO_2max) of at least 35 mL/kg/min were included. In the end, eight SAHC experienced and ten SAHC naive subjects took part in this study. All experiments were performed at the DLR (German Aerospace Center, Institute of Aerospace Medicine, Cologne, Germany) and all tests and experiments were supervised by a DLR aerospace physician.

2.2 SAHC

The DLR's SAHC g-force range runs up to +6G at a subject's feet due to a maximum rotation velocity of 38 rpm. The SAHC has a maximum radius of 3.80 m and a range of translation of 2.20 m (Supplementary Figure S1). The maximal acceleration amounts to 0.325 g/s. The volunteers were positioned in a nacelle with the head pointing to the gantry in the center. The feet of all subjects were the same distance from the gantry. The subjects were secured with four belts while lying in this supine position. The subjects' left arm was placed in an arm sling with the hand placed on the breast to optimize the recording of continuous blood pressure measurements via finger plethysmography. The subjects´ right hand was placed right to the body in supine position. The force vector generated by the centrifugation was pointed from head to feet. Ambient temperature was maintained between 23°C–25°C. To ensure the highest safety standards were met during the run, continuous visual and verbal communication between subjects and the aerospace physician were maintained. Three cameras monitored alterations in the face, body, and legs. Subjects were asked about symptoms of pre-syncope and freezing three times during each phase (*Uncomfortably cold? Are you okay?*). Before the run subjects were briefed to answer only with yes or no. Questions were phrased as clearly and simply as possible to assess any occurrence of mental confusion signifying presyncope. Criteria for termination were pre-syncope/syncope (dizziness, vision changes, mental confusion), cardiac arrhythmia, narrowing pulse pressure, or termination at the discretion of the subjects themselves. Narrowing pulse pressure was defined as a drop to at least 25% of systolic blood pressure. The subjects were able to stop the centrifuge by pushing an emergency stop button at any time throughout the runs.

2.3 Cooling: the Arctic Sun 5000™ and the cooling protocol

Cooling was applied using the Artic Sun 5000™ (C.R. Bard, Inc., United States), an apparatus normally used for temperature management in patients after cardiac arrest. It consisted of a main cooling apparatus and two cooling pads. The cooling apparatus of the Artic Sun 5000™ was fixed on the center of the centrifuge and connected to the pads via two isolated tubes each. The water running through the pads had an internal temperature of 8°C and this was maintained during each run. Skin temperature was indirectly controlled by two sensors measuring the temperature of inflowing and outflowing water from the cooling pads. The aim of PSC was to elicit a maximum effect on skin-related sympathetic activation and vasoconstriction. This was done by securing cooling pads around the thighs (Supplementary Figure S2). The pads were also applied in the NCP to avoid the compression of the thighs instigating bias. In the CP, cooling was initiated at the beginning of the 10-min rest. The cooling pads were kept in place during baseline and the step protocol until the end of the run.

2.4 Measurements

Before starting the experiment, the weight and height of each subject were determined by the study physician and the subjects were informed of the details of the centrifuge run. To verify the maintenance of body core temperature during skin cooling, transtympanal body core temperature was determined before and after the run in a subgroup of participants (N = 11 in the CP group; N = 8 in the NCP group).

A cumulative stress index mirrored the orthostatic tolerance of the subjects. This was calculated by adding up the products of each g-level in relation to the duration in seconds (e.g., 1 g × 180 s + 2 g × 180 s + 3 g × 180 s + 4 g × 50 s) (Zhang et al., 1997; Durand et al., 2004).

ECG was recorded at 2000 Hz (IntelliVue X2, Philips, Netherlands).

Continuous arterial blood pressure at the finger (BP_F) was measured by plethysmography (Finometer Midi model 2, Finapres Medical Systems BV, Netherlands) and recorded by the AcqKnowledge Software™ (v4.4.5, MP150, BIOPAC Systems Inc., United States). Analysis of the cardiovascular raw data was conducted using the LabChart Pro™ Software (v8.1.16 12.12.2019, ADInstruments, New Zealand). HR was derived beat-by-beat from the ECG. SV, CO, and TPR were calculated from the pulse wave using Windkessel Three-Element Model (Wesseling et al., 1993).

Discontinuous blood pressure at the brachial site (BP_B) was measured 90-s after beginning of each g-level deriving mean arterial blood pressure (MAP), systolic blood pressure (SYS) and diastolic blood pressure (DIA). Brachial and finger blood pressures may differ for physiological and technical reasons. Therefore, before extracting beat-by-beat blood pressure values we calibrated BP_F using SYS and DIA values measured with the arm-cuff device 90-s after the start of the 1-g centrifugal acceleration. For this aim, we considered the 3-s segment of the BP_F recording synchronous with the arm-cuff measure and calculated the maximum (MAX (BP_F)) and minimum (MIN(BP_F)) values of BP_F within the 3-s window. The calibrated finger blood pressure (BP_{FC}) was calculated as:

$$BP_{FC} = \frac{(SYS - DIA)(BP_F - MIN(BP_F))}{MAX(BP_F) - MIN(BP_F)} + DIA$$

To increase data interpretability, we used a 60-s rolling average for analyzing all continuous BP data. This was necessary as a reaction to the decreasing quality of pulse wave recordings under increasing g-forces.

2.5 Experimental protocol

For the study, a randomized cross-over design was used. The subjects were divided into two groups (A and B). Group A performed its first run without cooling and underwent the CP on the second day of the trial (and *vice versa* for group B). The wash-out phase between both study days was at least 3 days. The protocol consisted of a 10-min baseline run at +1Gz, followed by a 10 min recovery phase and a protocol of +1Gz stepwise acceleration. The reported g-levels referred to subjects' feet levels. The +Gz step protocol ranged from +1Gz to +4Gz, whereby each phase was 3 min long. The period of acceleration to the next higher g-level lasted 6 s. The centrifuge experiment was split into 8 phases (Figure 1): Phase 1 (P1) was the acceleration from 0 to 1 g, Phase 2 (P2) the steady-state condition in the last 60 s of 1 g,

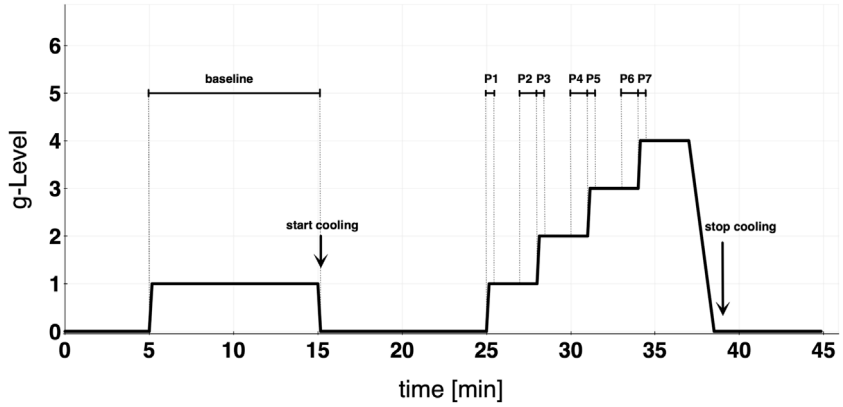

FIGURE 1
Study protocol detailing baseline and graded step protocol of increasing +Gz. Cooling was started at the end of baseline. Each acceleration to the next higher g-level lasted approximately 6 s. Phase 1 (P1) = 0–1 g; Phase 2 (P2) = last 60 s of 1 g; Phase 3 (P3) = 1–2 g; Phase 4 (P4) = last 60 s of 2 g; Phase 5 (P5) = 2–3 g; Phase 6 (P6) = last 60 s of 3 g; Phase 7 (P7) = 3–4 g.

Phase 3 (P3) was the acceleration from 1 to 2 g, Phase 4 (P4) was the last 60 s of 2 g, Phase 5 (P5) was the acceleration from 2 to 3 g, Phase 6 (P6) was the last 60 s of 3 g, Phase 7 (P7) was the acceleration from 3 to 4 g, and Phase 8 (P8) was the last 60 s of 4 g. Only one subject was able to complete P8 successfully in both protocols. Because of that, P8 was neglected in the analysis and illustrations. These periods of time were chosen to investigate how the cardiovascular system reacts to acute +Gz increase and after approximately 2min at the same +Gz level. A single brachial blood pressure measure was determined 90 s after the beginning of each g-level (1, 2, and 3 g). Therefore, blood pressure analyses differed from the analyses of the other cardiovascular variables which instead were performed on the P1-P7 phases.

2.6 Statistics

IBM SPSS Statistics™ (v.25, IBM, United States) was used for the statistical analysis. Values are indicated as mean ± standard deviation (SD). In order to establish the normality of the cardiovascular parameters, graphical and statistical analyses (Shapiro-Wilk-Test, with a significance level of $\alpha = 0.05$) were conducted. A *t*-test for normally distributed parameters and a Wilcoxon test for non-normally distributed data were used and subsequently adjusted via Bonferroni-Holm correction (Holm, 1979). In order to reduce multiple testing, which would greatly increase the α-error, linear mixed models were created for cardiovascular parameters with repeated measurements (HR, SV, CO, TPR). Fixed estimates for cardiovascular parameters regarding i) different phases of the +Gz-protocol, ii) different protocol groups (CP and NCP), and iii) the interaction between phase and protocol groups were calculated for each subject. The primary outcome was orthostatic tolerance, and this was tested with a significance level of 5%. To evaluate the orthostatic tolerance cumulative stress index, HR and BP were considered. The secondary endpoints SV, CO, TPR were also tested with a significance level outcome of 5%. We conducted a repeated-measures ANOVA with Bonferroni-Holm corrected *post hoc* test to assess differences in BP between baseline and +Gz for all data and separately for CP and NCP.

3 Results

3.1 Subjects

The mean age of the subjects was 28 years old (range: 22–50 years old). Weight and height were determined, with the average being 80 ±7 kg and 180 ±7 cm, respectively. Every subject completed the 2–3 g phase, except for one in the NCP group. The last 60 s of 3 g were completed by 11 subjects and the 3–4 g phase was completed by 9 subjects in each protocol group. Body core temperature showed only small changes after the run in both groups (CP pre: 36.5 ± 0.5°C post: 35.9 ± 0.6°C; NCP pre: 36.7 ± 0.4°C post: 36.2 ± 0.4°C).

Not all subjects managed to reach the same phases in both protocols. Depending on the subject, the run was aborted earlier or later in the one protocol than compared to the other. This problem has been considered in the statistical analysis. No analysis of a specific phase between CP and NCP was performed if a subject did not complete this specific phase in both protocols. Due to the insufficient signal quality of the pulse wave, two datasets (CP and NCP) from two subjects were omitted from the analysis regarding SV, CO, and TPR.

3.2 Brachial blood pressure

The calibration of the continuous finger BP waveform allows estimating the profile of the brachial BP during the whole experimental sessions as in the example of Figure 2 which shows beat-by-beat MAP data in the same participant during the cooling and non-cooling protocol.

Figure 3 shows the increments in brachial BP measures (SYS, MAP, DIA) from baseline under increasing +Gz steps (see Table 1 for the absolute values). The increasing trend was highly significant ($p < 0.001$).

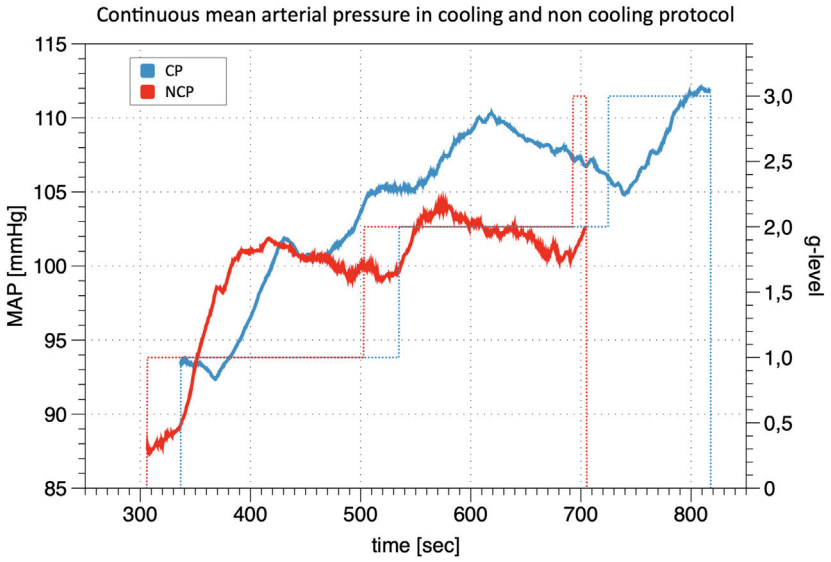

FIGURE 2
Line Plot of continuous mean arterial pressure (MAP) during +Gz: Depiction of continuous mean arterial pressure profiles for one individual subject in cooling protocol (CP) and non-cooling protocol (NCP). The run was quit at 3 g after 817 s in CP and at 3 g after 704 s in NCP. _ indicates MAP; . . . indicates g-level.

SYS increased from baseline to 1 g and from baseline to 2 g during CP while during NCP SYS increased under 2 g only. Subjects in CP experienced a slightly higher increase in SYS from baseline to 2 g (+16.9 mmHg) than in NCP (+10.6 mmHg). The subsequent drop in SYS under 3 g could be observed in CP (−7.0 mmHg) as well as in NCP (−11.3 mmHg). For all hyper-gravity states, SYS in CP visually showed greater values than in NCP, although the difference did not reach statistical significance. Considering MAP and DIA, there was also a lack of interprotocol significant differences.

3.3 Stress index

The cumulative stress index reflects the quantitative resilience to the orthostatic SAHC challenge, where 1800 g*s was the maximum that could be achieved by completing the whole protocol (Figure 4). Comparisons between CP and NCP revealed no differences in the cumulative stress index (CP: 1,214 g*s ± 310; NCP: 1,152 g*s ± 327; $p > 0.05$).

3.4 Heart rate

Table 2 shows results from the linear mixed model regarding HR, SV, TPR, and CO. As to HR, the CP as well as the NCP revealed no elevation in HR in Phase 2. In both protocols, the course of the HR showed a steady increase, as illustrated in Figure 5, with significant changes compared to baseline (original values are reported in the Supplementary Table S1). Estimations from linear mixed model revealed no significant differences in HR between CP and NCP during the +Gz step protocol (see further details in the Supplementary Table S2). Regarding individual values, 13 of the 17 subjects showed tendencies to lower values in the CP (Phase 3: −4.49 ± 7.84 beats/min; Phase 4: −6.67 ± 5.42 beats/min; Phase 5: −6.35 ± 7.78 beats/min; Phase 6: −9.89 ± 9.99 beats/min; Phase 7: −7.74 ± 4.54 beats/min). Four volunteers showed tendencies to have a higher HR in the CP (Phase 3: +0.83 ± 3.13 beats/min; Phase 4: 3.09 ± 1.24 beats/min; Phase 5: 2.16 ± 3.1 beats/min; Phase 6: 1.5 ± 8.62 beats/min).

3.5 Stroke volume, cardiac output, and total peripheral resistance

Significant decreases in SV were observed from Phase 3 onwards in CP and NCP in comparison to the baseline. However, no inter-protocol differences were found (Figure 6). CO continuously decreased during the +Gz challenge until Phase 6 where it shortly plateaued (Figure 7). Changes were only significant from Phase 4 until Phase 7. Furthermore, Table 2 indicates a congruent CO in both protocol groups.

From Phase 3 onwards both graphs outline a significantly increasing TPR when compared to baseline, with almost identical values and without differences between CP and NCP (Figure 8). See original SV, CO, and TPR values in the Supplementary Table S1, and further details of the linear mixed-model analysis in the Supplementary Tables S3–S5.

4 Discussion

4.1 Key findings

Hyper-gravity led to significant changes in primary as well as secondary outcome parameters such as HR, SV, TPR, CO, BP. However, neither primary outcome parameters (HR, cumulative stress index, BP) nor secondary outcome parameters (SV, TPR, CO) with PSC showed significant differences when compared to NCP.

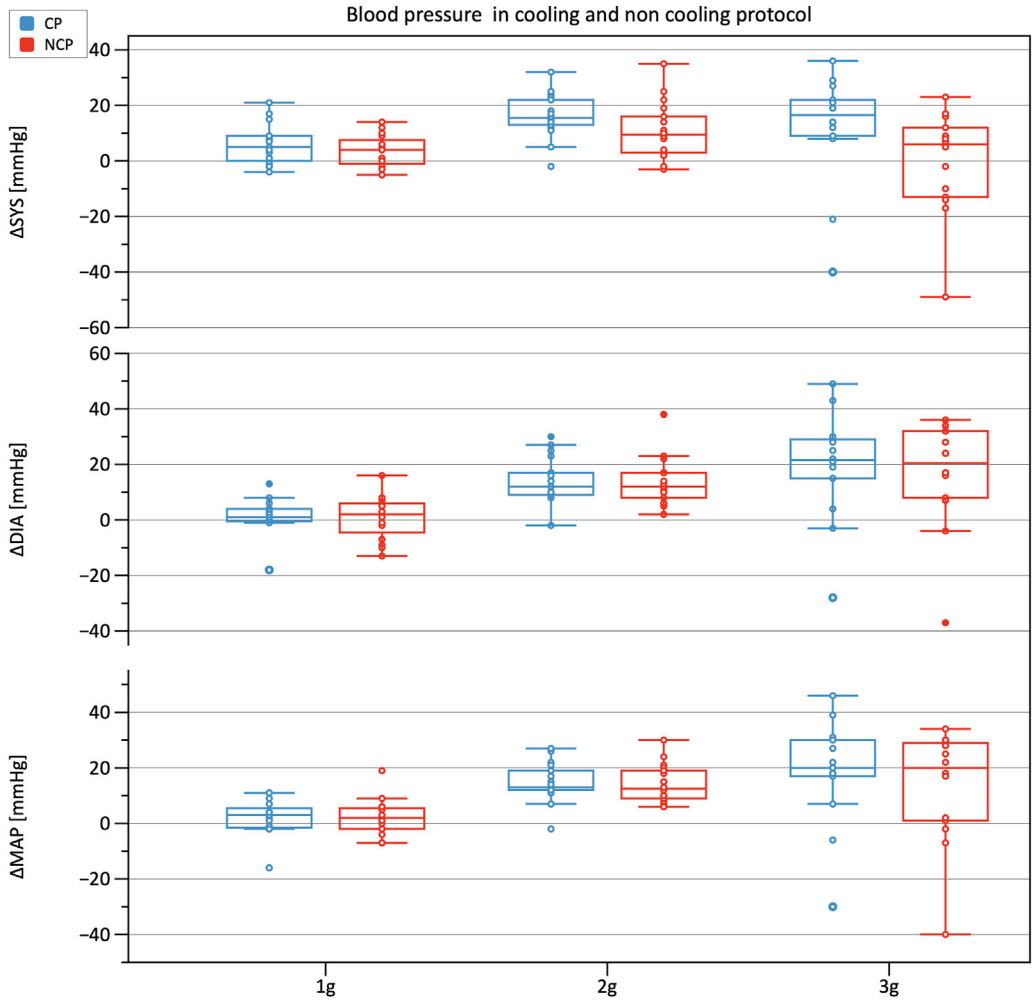

FIGURE 3
Boxplots of relative changes in systolic (SYS), diastolic (DIA), and mean arterial blood pressure (MAP) with respect to baseline values in the cooling protocol (CP) versus the non-cooling protocol (NCP). Blood pressure was recorded at 90 s after the start of each g-level.

TABLE 1 Brachial blood pressure values. Mean ± SD of systolic (SYS), diastolic (DIA) and mean arterial blood pressure (MAP) values for the different phases per each group (CP: cooling protocol NCP: non-cooling protocol). Brachial blood pressure was taken 90 s after the start of each g-level.

Phases	SYS [mmHg]	MAP [mmHg]	DIA [mmHg]
Baseline	**CP**: 134 ± 10 **NCP**: 136 ± 11	**CP**: 94 ± 9 **NCP**: 93 ± 8	**CP**: 82 ± 9 **NCP**: 82 ± 8
1 g	**CP**: 142 ± 11 **NCP**: 140 ± 13	**CP**: 98 ± 7 **NCP**: 97 ± 10	**CP**: 85 ± 7 **NCP**: 84 ± 9
2 g	**CP**: 150 ± 12 **NCP**: 146 ± 17	**CP**: 108 ± 9 **NCP**: 107 ± 11	**CP**: 96 ± 9 **NCP**: 96 ± 11
3 g	**CP**: 145 ± 21 **NCP**: 135 ± 20	**CP**: 111 ± 18 **NCP**: 105 ± 18	**CP**: 100 ± 18 **NCP**: 97 ± 18

4.2 Cardiovascular response

4.2.1 Stress-index

There were no differences observed between the two protocols regarding cumulative stress index. This contradicts prior studies that showed improved orthostatic tolerance when applying skin surface cooling (Durand et al., 2004; Keller et al., 2011). Nevertheless, there are some possible explanations for these differences. Contrary to these studies where LBNP was used, we utilized the SAHC to generate orthostatic stress. Unlike LBNP, a SAHC produces increased vascular pressure from the head to the feet. The SAHC additionally activates the vestibular system which leads to vestibulo-sympathtic reflex resulting in decreased tibial muscle sympathetic nerve activity (Cui et al., 2001). This mechanism could be an additional orthostatic stressor and indicates a higher load on the cardiovascular system, especially due to larger amount of blood

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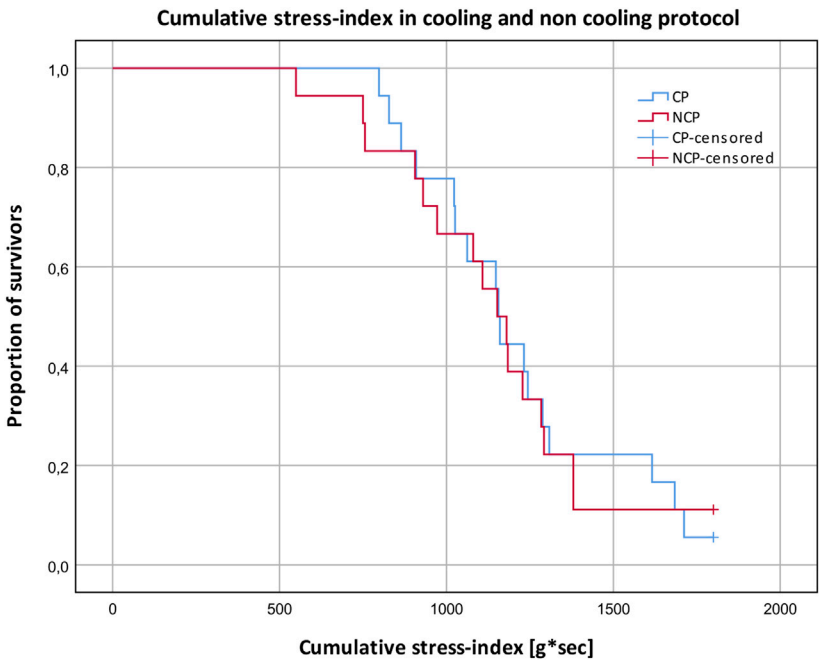

FIGURE 4
Cumulative stress-index and proportion of subjects surviving different g-levels in the cooling protocol (CP) versus the non-cooling protocol (NCP). 180 g*s ≙ survived 1 g; 540 g*s ≙ survived 2 g; 1,080 g*s ≙ survived 3 g; 1800 g*s ≙ survived 4 g.

TABLE 2 Results from the linear mixed model. Estimates and significances from the linear mixed model of heart rate (HR), stroke volume (SV), cardiac output (CO), and total peripheral resistance (TPR) adjusted to the protocol relating to different phases of the +Gz-protocol. Cooling (CP)/non-cooling (NCP) protocol groups regarded as fixed effects and individual subjects as random effects. Estimates are given as differences to baseline. The term "phase" relates to differences to baseline in NCP and express if SAHC forces have an effect to illustrated parameters regardless of cooling. The terms CP*phase indicate the differences in shown parameters in the CP compared to NCP during different phases. CP*phase reveal if changes in HR, SV, CO, TPR are only based on effects of SAHC or even on cooling effects. If cooling effects were present, this would be reflected in significant values in CP*phase. *indicates $p < 0.05$, **indicates $p < 0.01$.

Parameter	HR [bpm]	SV [ml]	CO [L/min]	TPR [mmHg*min/L]
Phase = P1	8*	14.66**	1.49	−0.36**
phase = P2	4	0.73	0.26	−0.07
phase = P3	17**	−16.36**	−0.42	0.27*
phase = P4	31**	−29.36**	−0.89**	0.53**
phase = P5	49**	−41.78**	−1.48**	0.97**
phase = P6	72**	−46.92**	−1.45**	1.18**
phase = P7	83**			
CP * P1	4	−0.35	0.25	−0.02
CP * P2	−1	1.97	0.08	0.01
CP * P3	−3	2.75	0.15	−0.01
CP * P4	−4	2.59	0.15	−0.05
CP * P5	−4	1.21	−0.08	0.13
CP * P6	−7	5.90	0.56	−0.26
CP * P7	−5			

volume pooling in the lower limbs. The protocol in the mentioned study was not conducted on a centrifuge but also involved linear acceleration, so the influence on the vestibular system is comparable. Hence, comparisons between SAHC and LBNP study results must be considered with caution. We applied PSC only around the thighs, unlike in experiments by Keller et al. and Durand et al., where the

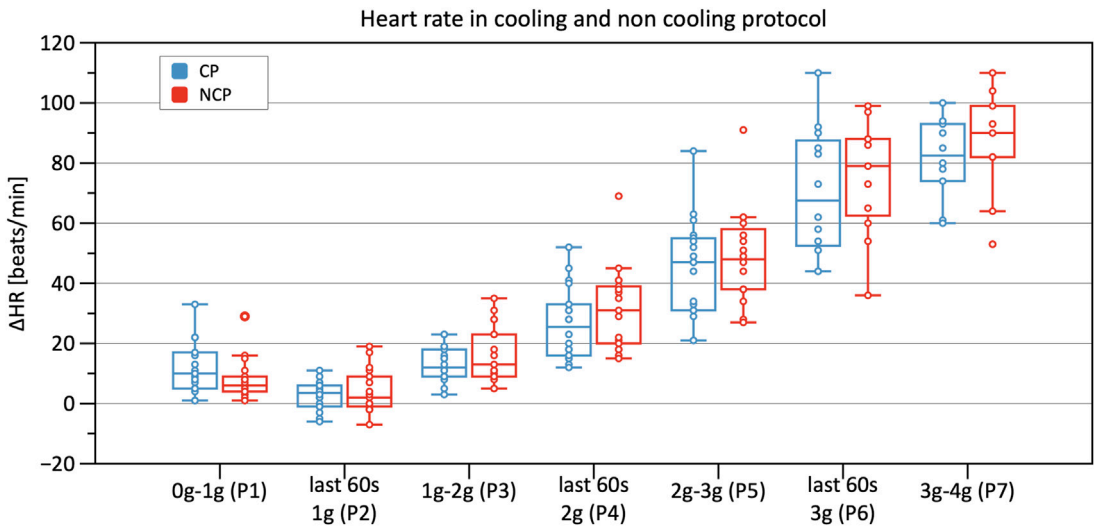

FIGURE 5
Boxplots of relative changes in heart rate (HR) with respect to baseline HR values in the cooling protocol (CP) versus the non-cooling protocol (NCP) during the first 30 s at the beginning of acceleration to the next higher g level (0–1 g, 1–2 g, 2–3 g, and 3–4 g) and during the last 60 s of 1, 2, and 3 g.

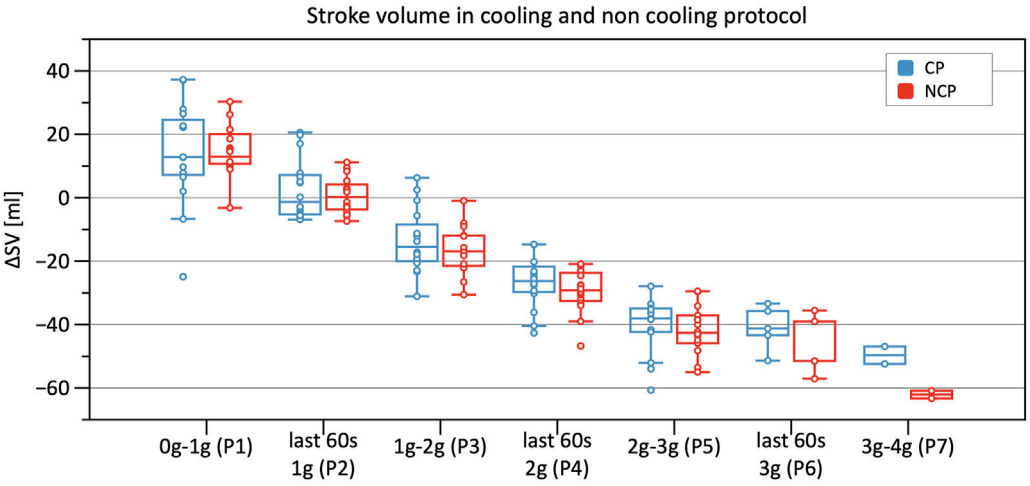

FIGURE 6
Boxplots of relative changes in stroke volume (SV) with respect to baseline SV values in the cooling protocol (CP) versus the non-cooling protocol (NCP) during the first 30 s from the beginning of acceleration to the next higher g level (0–1 g, 1–2 g, 2–3 g, and 3–4 g) and during the last 60 s of 1, 2, and 3 g.

subjects wore water-perfused suits covering the entire body surface except for the hands, feet, and head (Durand et al., 2004; Keller et al., 2011). As such, the magnitude of blood distribution from the periphery to the body core was presumably lower in this study. Another possible factor that may have led to our results being different is that we were forced to rotate the aerospace physicians (due to illness). This resulted in different physicians providing their evaluations on different days. Despite clear instructions, they differed in their interpretations of the critical point of pre-syncope. Hence, on one study day, the run was, for example, aborted earlier compared to another testing day, indicating a bias in the assessment of the cumulative stress-index. Despite this, data from the survivability index (see Figure 4) did indicate that PSC tended to increase cumulative stress either early in the protocol (between 500–1,000 g*sec), and at 1,500 g*sec. Applying PSC during mild to moderate +Gz levels (up to +2 g) may provide some level of cumulative stress tolerance amongst certain cohorts, however, more investigations are needed.

4.2.2 Heart rate

Statistically, HR did not differ during the CP runs when compared to NCP runs. Despite this, when investigating single values, 13 of the

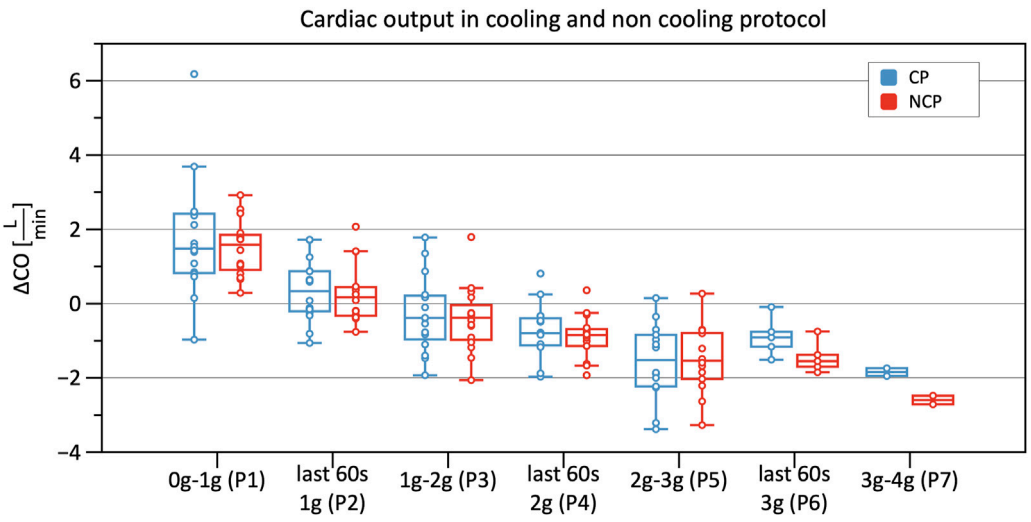

FIGURE 7
Boxplots of relative changes in cardiac output (CO) with respect to baseline CO values in the cooling protocol (CP) versus the non-cooling protocol (NCP) during the first 30 s from the beginning of acceleration to the next higher g-level (0–1 g, 1–2 g, 2–3 g, and 3–4 g) and during the last 60 s of 1, 2, and 3 g.

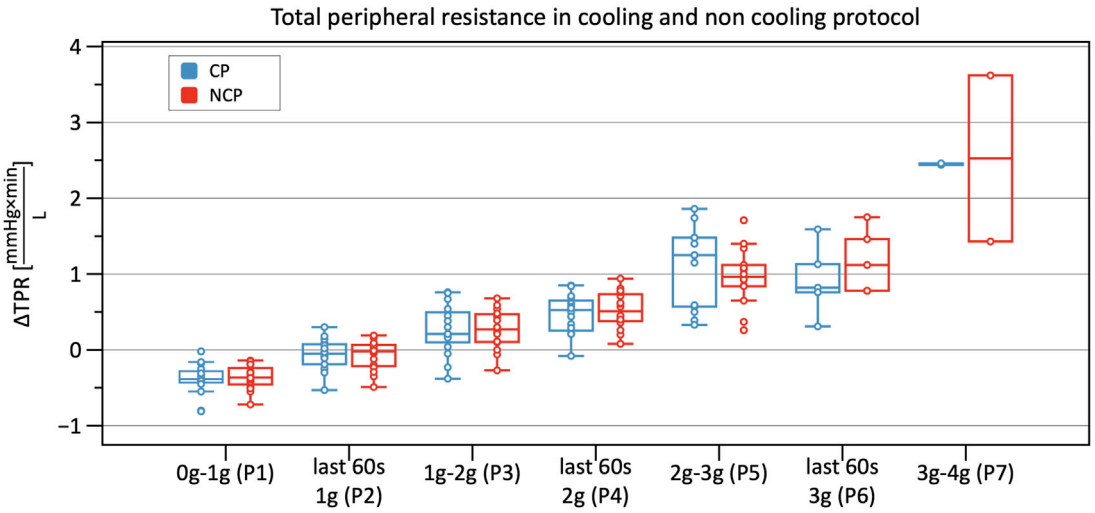

FIGURE 8
Boxplots of relative changes in total peripheral resistance (TPR) with respect to baseline TPR values in the cooling protocol (CP) versus the non-cooling protocol (NCP) during the first 30 s from the beginning of acceleration to the next higher g level (0–1 g, 1–2 g, 2–3 g, and 3–4 g) and during the last 60 s of 1, 2, and 3 g.

17 subjects showed tendencies to lower values in the CP. A minority of only 4 subjects tended to have higher values. Even though these results are not supported by statistical significance, they nevertheless showed a trend that suggests a cooling effect. We, therefore, suspect that there are subjects who are immune to the benefits of cooling. This may be caused, among other things, by a higher individual threshold regarding cooling. To achieve a higher magnitude of cooling, a greater body surface area should be exposed to surface cooling.

Moreover, according to Sawasaki et al., there are high inter-subject differences regarding the magnitude of the skin sympathetic nerve activity which induces cutaneous vasoconstriction (Sawasaki et al., 2001). Their findings outline a positive correlation between increases in sympathetic nerve activity and tympanal-measured body core temperature that could mirror a lower cutaneous heat loss and a greater blood distribution out of the splanchnic area.

Hachija et al. wrote that their "high tolerance group" in an LBNP experiment showed delayed responses in vasoconstriction, presumably caused by higher reserve blood volume from the splanchnic area, which preserves circulation (Arbeille et al., 2005; Hachiya et al., 2010). Investigations with a somatostatin analog

during a tilt table test contribute to the hypothesis that splanchnic blood volume is crucial for orthostatic tolerance (Jarvis et al., 2012). To induce splanchnic vasoconstriction the following aspects are particularly important: i) activation of atrial volume receptors; ii) increased arterial baroreceptor activity; iii) vasoconstriction response of splanchnic resistant vessels (Karim and Hainsworth, 1976; Greenway et al., 1994). Therefore, a lack of adequate response in one of these three domains could lead to lower orthostatic tolerance. This failure or attenuated response time to acute hypovolemia results in higher HR. Moreover, inadequate mobilization of splanchnic blood also likely contributes to the absence of cumulative stress-index differences, leading to the assumption that this study randomly included a subject population with several "low tolerance" subjects (Hachiya et al., 2010).

4.2.3 Blood pressure

The analysis of BP showed an increase of SYS, MAP, and DIA up until +2 g with a subsequent reduction in BP. These results add to the inhomogeneous literature on BP reaction to hyper-gravity in SAHC (Watenpaugh et al., 2004; Verma et al., 2018; Kourtidou-Papadeli et al., 2021). Increases in BP up to 2 g were descriptively larger in the CP than in the NCP, possibly indicating a cardiovascular stabilization by the CP. Furthermore, BP decreased from 2 to 3 g in the CP, as well as corresponding to increased cardiovascular stability. Also, PSC applied to a greater body surface area may elicit a greater impact on BP during +Gz.

The recording of continuous BP data allowed the visual interpretation of individual subjects' BP profiles in CP and NCP. Oscillometric measurement is the gold standard to determine BP non-invasively. Therefore, we relied on the oscillometrically measured BP to conduct our quantitative analyses. However, we successfully applied the continuous BP measurement paradigm to improve visual interpretation of BP dynamics. We recommend its adoption for statistical analyses in studies with more subjects, averaging out the issues of lower measurement certainty and decreased signal quality during higher g-levels.

4.2.4 Stroke volume, cardiac output, and total peripheral resistance

Analyzing SV, CO, and TPR data revealed that pulse wave quality decreased with increasing g-forces, leading to limitations when calculating these parameters. This problem occurred in Phase 5 and even more so in Phases 6 and 7. Hence, only 5 subjects in Phase 6 and only 2 subjects in Phase 7 were included in the analysis. Therefore, Phase 7 was excluded from statistical analysis. A particularly remarkable finding was that poor signal quality (i.e., high oscillations) during the >+2 g phases was observed in the NCP, suggesting a more stable cardiovascular state in the CP group. However, interpretation of Phase 6 should be treated with caution, due to the low number of subjects included in the analysis. Utilizing a perfusion index probe on the finger may reveal subtle changes in peripheral perfusion for future studies of this nature.

Moreover, assessing SV, CO, and TPR revealed no differences between the two protocols. Excluding SV, these results are in line with prior studies (Durand et al., 2004; Cui et al., 2005; Wilson et al., 2007). After applying cooling, it was expected that SV would increase during Phase 2 (same conditions as baseline). This was not supported in our study, although increases in left ventricular filling pressure have been described in prior studies (shown by increases in CVP and pulmonary capillary wedge pressure) (Wilson et al., 2007). The explanation put forth by Wilson et al., postulates that cooling leads to a right shift of the operating point of the Frank-Starling mechanism, with that of a flatter curve (Wilson et al., 2009). Furthermore, these latter findings show reduced decreases in CVP and SV under cooling conditions during an orthostatic challenge in contrast to our SV results (Durand et al., 2004; Cui et al., 2005; Wilson et al., 2009).

Our results therefore contradict our initial hypothesis and previous studies, demonstrating that SV was not different under CP versus NCP conditions during gravitational stress (Durand et al., 2004; Cui et al., 2005; Wilson et al., 2009). This divergent finding could be explained either due to the PSC system used in this study (water based), or that PSC needs to be applied on a greater surface area to achieve a greater impact on SV. This assumption can only be conclusively clarified by further studies utilizing PSC in a similar +Gz run, albeit covering a larger body surface area.

4.3 Limitations

One confounding factor was the rotation of physicians throughout the experiment which influenced the definition or threshold at which pre-syncope was established. The physicians were given instructions and concrete abort criteria but could still be affected by bias and personal interpretation. Consequently, the assessment of the cumulative stress-index is not only dependent on the subject's own g-tolerance or the cooling effect, but also on the supervising physician.

Furthermore, in order to avoid shivering during +Gz runs, PSC was applied only around the thighs. This most likely led to inadequate hemodynamic stimulation amongst the CP cohort.

Another limitation is the difficulty concerning blood pressure measurements. There were inconsistencies between baseline as well as +Gz reference arm cuff values and Finapres™ values. This issue may have occurred due to cold induced vasoconstriction of the extremities or the tightness of the finger cuffs (Langewouters et al., 1998).

Using the Arctic Sun 5000™ to produce a significant cooling stimulus does work in a clinical scenario where patients are cooled after cardiac arrest, as well as the absence of a gravitational stress. Through its feedback slope, it moreover provides a clinically tested system to prevent overcooling. However, the system is not optimally designed for a peripheral cooling protocol during SAHC runs and the efficiency of the cooling water flow during +Gz phases may have been reduced. Moreover, it is not clear how much body surface is needed to cool, and which anatomical areas needed to cool in order to provide a sufficient cooling stimulus during gravitational stress.

Finally, this study only encompassed male subjects and took place on a SAHC as such, generalizing the results may not be appropriate when considering females and real-life +Gz scenarios, such as high speed aircraft. Additional research involving female subjects in a similar setting is, therefore necessary in order to perform a gender based hemodynamic comparison, as females do show different responses to +Gz compared to males (Masatli et al.,

2018). Furthermore, PSC would need to be tested during parabolic flight, as well as trained +Gz subjects such as jet fighter pilots.

5 Conclusion

Although PSC did not demonstrate significant impacts upon +Gz tolerance time and cardiovascular function compared to a control group, this study was the first ever to deploy a novel PSC system during +Gz runs in a SAHC. However, the cardiovascular reactions during progressively increasing +Gz did show a trend for PSC suggesting a positive impact upon vital signs. It is conceivable that cooling a greater surface area and different pad placement (i.e., whole body + neck) could increase the physiological impact of cooling, thereby more effectively increasing orthostatic tolerance. Nevertheless, it seems that some subjects were more responsive to cooling than others, though the exact reason for this remains unknown. This matter warrants further investigation so that individualized cooling countermeasures can be optimized for astronauts to counteract orthostatic symptoms during launch or directly after return to earth. Furthermore, the analysis of microcirculatory, neuroendocrine, and hematological factors, as well as cardiopulmonary testing, which were all investigated during this study, are expected to be published in the coming year, which will further explore the physiological impacts of PSC during +Gz.

Future implications for PSC could be the integration into a wearable device or garment. While the cooling countermeasure deployed in this study may not increase a person's individual g-threshold, it could still provide additional comfort and reduce adverse effects, such as heat-stress during orbital re-entry.

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 Ethics committee of the Medical Council North Rhine. The patients/participants provided their written informed consent to participate in this study.

Author contributions

OO, MG, and MN designed the study and secured funding. NK and AN collected data. NK, AR, and PC processed the analyzed data. NK and TLB performed the statistical analysis. NK prepared figures and drafted the manuscript. H-CG, MAM, OO, HH, and DJ revised and edited the manuscript. OO and H-CG supervised the experiment. All authors approved the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1173171/full#supplementary-material

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Effect of hot water immersion on acute physiological responses following resistance exercise

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Purpose: Hot water immersion (HWI) is a strategy theorised to enhance exercise recovery. However, the acute physiological responses to HWI following resistance exercise are yet to be determined.

Methods: The effect of HWI on intramuscular temperature (IMT), muscle function, muscle soreness and blood markers of muscle cell disruption and inflammatory processes after resistance exercise was assessed. Sixteen resistance trained males performed resistance exercise, followed by either 10 min HWI at 40°C or 10 min passive recovery (PAS).

Results: Post-intervention, the increase in IMT at all depths was greater for HWI compared to PAS, however this difference had disappeared by 1 h post at depths of 1 and 2 cm, and by 2 h post at a depth of 3 cm. There were no differences between groups for muscle function, muscle soreness or any blood markers.

Conclusion: These results suggest that HWI is a viable means of heat therapy to support a greater IMT following resistance exercise. Recovery of muscle function and muscle soreness is independent of acute changes in IMT associated with HWI.

KEYWORDS

heat therapy, strength training, temperature, recovery, muscle damage, inflammation

Introduction

Post-exercise hydrotherapy is common practice among athletic individuals, with the goal of enhancing acute recovery following training and competition (Vaile et al., 2010). While cold water immersion has received growing attention in the literature, there is a paucity of research investigating the effectiveness of hot water immersion (HWI) on exercise recovery in humans, with equivocal findings to date (McGorm et al., 2018). The efficacy of such

Abbreviations: CK, Creatine kinase; DXA, Dual-energy x-ray absorptiometry; ELISA, Enzyme-linked immunosorbent assay; hsCRP, High-sensitivity C-reactive protein; HWI, Hot water immersion; IL-6, Interleukin-6; MMP-9, Matrix metalloproteinase-9; MVIC, Maximal voluntary isometric contraction; μM, Micromolar; N, Newtons; PAS, Passive recovery; RFD, Rate of force development; RM, Repetition maximum; VAS, Visual analogue scale.

strategies is likely related to the specific recovery needs of the individual (Minett and Costello, 2015), therefore a greater understanding of the impact of HWI on acute physiological responses will aid in the application of this strategy in exercise recovery.

HWI is thought to exert a physiological impact primarily through an increase in cutaneous and subcutaneous tissue temperature which induces peripheral vasodilation and a subsequent increase in blood flow (Wilcock et al., 2006). The ensuing increase in permeability of cellular, lymphatic and capillary vessels may drive an increased rate of metabolism, nutrient delivery and clearance of waste products (Coté et al., 1988; Baker et al., 2001), which could aid exercise recovery. The research behind these proposed mechanisms has typically occurred in the field of physiotherapy or with techniques of heat application including ultrasound and heat packs (Wyper and McNiven, 1976; Knight and Londeree, 1980; Bonde-Petersen et al., 1992). Whether similar effects would be seen with HWI and following an exercise session which stimulates acute physiological responses typical of a 'real world' training session requires further investigation.

Despite the limited insights into the mechanisms that would rationalise enhanced recovery in an exercise setting, research from human studies using post-exercise heating has produced some promising evidence (Vaile et al., 2010; McGorm et al., 2018). Cheng et al. (2017) reported that acute recovery following exhaustive intermittent arm cycling is influenced by intramuscular temperature, with mean power output better preserved after the upper limbs were heated to ~38°C compared to being cooled to ~15°C for 2 h post-exercise. Based upon replication of these findings in an animal model, the authors attributed the enhanced recovery to better rates of glycogen resynthesis with heating versus cooling, due to the increased tissue temperature promoting rates of enzymatic processes (Cheng et al., 2017). Others have also reported HWI to improve the recovery of strength (Clarke, 1963; Vaile et al., 2008) and power (Viitasalo et al., 1995) following fatiguing isometric exercise, a leg press protocol designed to elicit delayed onset-muscle soreness and throughout a strength/power training week for track and field athletes, respectively. Enhancing recovery in this way would be of benefit to those performing resistance exercise as part of a progressive programme, however the effect of HWI on these measures following an ecologically valid exercise session are yet to be determined.

The effect of post-exercise heating on other acute physiological responses is inconclusive. Following high-intensity intermittent exercise (Pournot et al., 2011) and an intense strength/power training week (Viitasalo et al., 1995), HWI did not influence the appearance of intramuscular enzymes in the blood. Whereas after a single bout of eccentric exercise, Vaile et al. (2008) reported a reduction in plasma creatine kinase with HWI. Heat therapy may reduce pain via an analgesic effect on nerves (Baker et al., 2001) and has been shown to reduce soreness following lumbar extension exercise (Mayer et al., 2006). Conversely, other reports have demonstrated post-exercise heating to exert no impact upon muscle soreness (Viitasalo et al., 1995; Kuligowski et al., 1998; Vaile et al., 2008; Pournot et al., 2011). Differences in exercise modality, HWI protocol and timing of recovery measures make conclusions problematic, lending to the assertion that further research utilising ecologically valid protocols are required in this area (Vaile et al., 2010; McGorm et al., 2018).

TABLE 1 Participant characteristics.

	Intervention group	
	PAS (n = 8)	HWI (n = 8)
Age (yrs)	24 ± 4	25 ± 4
Height (m)	1.77 ± 0.05	1.80 ± 0.07
Body mass (kg)	88 ± 17	89 ± 14
Surface area:body mass	32 ± 3	32 ± 4
1 RM (kg)	158 ± 30	158 ± 31

PAS, passive recovery group; HWI, hot water immersion group; RM, repetition maximum.

A disparity currently exists between the hypothesised benefits of post-exercise heating and the evidence for HWI to improve acute exercise recovery (Vaile et al., 2010; McGorm et al., 2018). Given the growing use of thermotherapy in recreationally active and athletic populations, further insights are required to provide evidence-based rationale supporting or refuting the application of HWI as a recovery aid. Therefore, the aim of the present study was to investigate the effect of HWI on a range of acute physiological responses following resistance exercise.

Methods

Participants

Sixteen strength trained males volunteered to take part in the study (Table 1). Participants were considered to be resistance-trained if they had performed ≥3 resistance sessions per week for ≥2 years with a minimum of one session per week including exercises that targeted the lower limbs (Buckner et al., 2017). Prior to any experimental procedures, written informed consent was obtained from all individual participants and the study conformed to the latest revision of the Declaration of Helsinki (World Medical Association, 2013). All participants completed a health screening questionnaire and were excluded from the study if the investigator deemed they were contraindicated to the study procedures. The study was granted ethical approval (application number: 1,686) by the London Sports Institute Ethics Sub-Committee at Middlesex University.

Experimental design

Using a between-subject design, participants were pair matched for baseline strength (1 repetition maximum [RM] back squat), and body composition (body surface area to body mass ratio) in line with previous research (Roberts et al., 2015) and assigned to HWI (n = 8) or passive recovery (PAS) (n = 8) groups. Previous research has shown the relationship between body surface area relative to body mass to be an important influencer on thermal and physiological responses to hydrotherapy (Stephens et al., 2014). Participants attended the laboratory on four occasions; during the first visit,

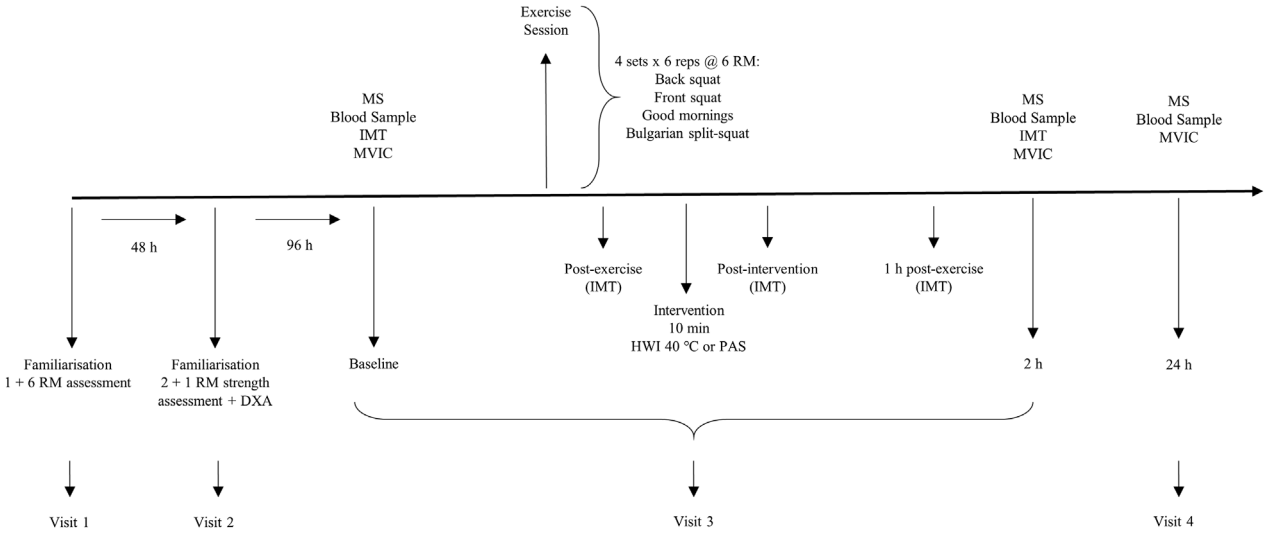

FIGURE 1
Schematic overview of study timeline. The order of assessments is depicted for the Baseline session. MS, muscle soreness; IMT, intramuscular temperature; MVIC, maximal voluntary isometric contraction; RM, repetition maximum; DXA, dual-energy x-ray absorptiometry; HWI, hot water immersion; PAS, passive recovery.

anthropometric data was collected (height and body mass), before participants were familiarised with experimental procedures. Additionally, participants performed a strength assessment to determine a 6 RM for the exercise techniques used in the exercise session (back squat, front squat, good morning, Bulgarian split-squat). The second visit involved a body composition assessment prior to a further familiarisation with experimental procedures. Participants then performed a strength assessment to determine a 1 RM for the back squat.

Visit 3 formed the start of the experimental period and required participants to perform baseline assessments in the following order: muscle soreness, blood sample, intramuscular temperature and maximal voluntary isometric contraction (MVIC). Following baseline assessments, participants performed a resistance exercise session and then completed either the HWI or PAS interventions. Intramuscular temperature assessments were repeated: post-exercise, post-intervention, 1 h and 2 h post-exercise. Muscle soreness, blood sample and MVIC were repeated at 2 h post-exercise. During visit 4, participants returned to the laboratory to perform 24 h post-exercise assessments for muscle soreness, blood sample and MVIC (Figure 1).

Prior to visit 3, participants were provided with a standardised meal (27 g porridge oats, 180 mL semi-skimmed milk, 200 g high protein yoghurt) to consume 2 h before arrival at the laboratory. Participants also consumed a ready to drink protein milk (30 g protein) following completion of the exercise session and after the 2 h post-exercise assessments, between which they were required to be fasted (Roberts et al., 2015). Additionally, participants were asked to refrain from food consumption in the 2 hours prior to any testing procedures. Aside from the control measures, participants were instructed to maintain their habitual dietary intake throughout the study. Participants were required to avoid the following throughout the study: exercise external to the protocol, any therapeutic interventions or nutritional supplements, alcohol and non-steroidal anti-inflammatory drugs.

TABLE 2 Standardised warm up.

Exercise	Distance/Repetitions
Forward/backward shuttle run	10 m x 3 reps
Side to side shuttle	10 m x 3 reps
Forward/backwards lunge	10 m x 1 rep
Inch worms into spiderman	5 reps
Single leg RDL with high knee pull	5 reps each side
Bodyweight squat	5 reps
Glute bridge into heel walkouts	5 reps
Single leg glute bridge	5 reps each side
Jump squats	5 reps

RDL, Romanian deadlift.

6 RM strength assessment

Strength assessments were performed using a 6 RM testing protocol in accordance with recognised guidelines (Haff and Triplett, 2015). Following a standardised warm-up (Table 2), participants performed three sets of six repetitions of back squat with a progressively increasing load that corresponded to 50, 75% and 90% of their perceived 6 RM. Participants then performed sets of six repetitions with an increasing load for the determination of 6 RM with 2 min rest afforded between attempts. All 6 RM determinations were made within four attempts which were deemed successful by an investigator if a participant had reached a position in which the thigh was at least parallel to the floor. Participants repeated the above procedure for the determination of

6 RM on the front squat, good mornings and Bulgarian split-squat exercises with successful attempts determined by an investigator against standardised techniques. During the Bulgarian split-squat, participants placed the top of the toes of the trail leg on a 12 inch platform (McCurdy et al., 2010). The lead leg was placed approximately 39–45 inches from the front edge of the platform supporting the trail leg. Participants were then required to squat to a depth where the thigh of the lead leg was parallel to the ground before returning to the start position. These exercises were chosen to target a range of lower limb musculature and are commonly included in strength and conditioning programmes (Haff and Triplett, 2015). All resistance exercises were performed using free weights and a standard 20 kg bar (ELEIKO SPORT, Illinois, United States).

1 RM strength assessment

For the purposes of matching groups for baseline strength, maximal lower-body strength was assessed by 1 RM testing in the back squat consistent with recognized guidelines established by the National Strength and Conditioning Association (Baechle and Earle, 2008).

Resistance exercise session

Following the standardised warm up (Table 2), participants performed three sets of six repetitions of back squat with a load corresponding to 50, 75% and 90% 6 RM. Participants then performed four sets of six repetitions with a load corresponding to 6 RM for the following exercises: back squat, front squat, good mornings and Bulgarian split-squat. The intensity (100% 6 RM or ~85% 1 RM) and volume (12 sets targeting the quadriceps muscle group) of the session were selected based upon recommendations that loads of 80%–95% 1 RM elicit maximal gains in strength (Peterson et al., 2005), and hypertrophy (Fry, 2004). The performance of at least 8–10 weekly sets per muscle group has also been suggested to be required to maximise increases in muscle strength (Peterson et al., 2005) and size (Schoenfeld et al., 2016) in trained individuals. Participants were instructed to perform the eccentric phase of the exercises in a controlled fashion lasting approximately 2 s, whilst the concentric phase was to be performed with maximal acceleration. This method of lifting was chosen given the suggestion that it is the intended rather than the actual velocity that determines the velocity-specific training response (Behm and Sale, 1993). Two minutes rest was afforded between sets and exercises, which has been recommended as a minimum for maximising gains in muscle size (Schoenfeld et al., 2015).

Interventions

The HWI or PAS interventions were performed within 10 min post-completion of the resistance exercise session. Participants in the HWI group sat in an inflatable bath (iSprint, iCoolsport, Miami, Australia) and were required to submerge their legs in the water up to their waist in a seated position (hip angle of ~90°), with their legs outstretched and relaxed. Water temperature was maintained at 40°C using a circulatory heating unit (iCool dual temperature LITE, iCoolsport, Miami, Australia) and participants were required to remain immersed for 10 min. Participants in the PAS group sat on a physiotherapy bed and were required to remain still in a seated position (hip angle of ~90°), with their legs outstretched and relaxed for 10 min. To avoid any external confounding influences on temperature, all participants were required to remain in the laboratory until after all the assessments were complete on visit 3.

Body composition

In order to match groups for body surface area to body mass ratio, body composition was assessed by dual-energy x-ray absorptiometry (DXA; GE Lunar Prodigy, GE Healthcare, Bucks, United Kingdom) in accordance with previous research (Bell et al., 2016).

Intramuscular temperature

Intramuscular temperature was measured using a re-useable sterile needle thermistor (MKA08050-A, Ellab A/S, Rodovre, Denmark) with data read via a thermocouple system (E-Val Flex, Ellab A/S, Rodovre, Denmark). Previous studies have reported precision of 0.1°C for this system (Mohr et al., 2004). The needle thermistors were sterilised using an autoclave prior to each use according to manufacturer guidelines. The site of insertion was identified as the mid-point of the vastus lateralis muscle of the right limb, between the superior border of the patella and the inguinal fold and was marked using a pen for consistency between assessments with the site of insertion sterilised using a topical antiseptic (Betadine, Purdue Products LP, CT, United States). To ensure consistency in the depth of insertion, skin thickness and adipose tissue was measured using a caliper and a piece of medical tape was placed from the end of the needle at a distance which corresponded to 3 cm plus half of the skinfold thickness. The needle was then inserted into the vastus lateralis muscle until the tape contacted the skin surface, ensuring an intramuscular temperature of 3 cm. Once the reading had stabilised (approximately 2 s), the temperature was recorded. The needle was then manually removed to a depth of 2 cm and the temperature recorded once the reading had stabilised. This process was repeated for a depth of 1 cm prior to the needle being removed. Previous research has used this method to determine the effect of water immersion on intramuscular temperature following resistance exercise (Mawhinney et al., 2017).

Maximal voluntary isometric contraction

Participants were seated on the dynamometer chair (Biodex 3, Biodex Medical Systems, NY, United States) with a hip joint angle of 90° and a knee joint angle of 70° (Eddens et al., 2017), set by the investigator using a goniometer. A knee joint angle of 70° has been shown to be sensitive to detect reduced muscle function following eccentric exercise, with no difference between this angle and the torque produced at 90° (McHugh and Tetro, 2003). Participants completed a standardised warm-up consisting of efforts at 50, 75% and 90% of perceived maximal force. Participants then performed

TABLE 3 List of blood markers and associated time points of collection.

Marker	Baseline	2 h	24 h
CK	✓		✓
hs-CRP	✓		✓
IL-6	✓	✓	✓
IL-10	✓	✓	✓
MMP-9	✓	✓	✓

CK, creatine kinase; hs-CRP, high-sensitivity c-reactive protein; IL-6, interleukin-6; IL-10, interleukin-10; MMP-9, matrix metalloproteinase-9.

three maximal voluntary isometric contractions (MVIC) of the right limb, each lasting 3 s, with standardised verbal instruction and encouragement provided throughout. Sixty seconds rest was afforded between attempts with peak force (N) recorded and the best attempt used for subsequent analysis.

Active muscle soreness

Active muscle soreness was determined using a 200 mm visual analogue scale (VAS) with "no pain" indicated at one end and "pain/soreness as bad as it could be" at the other (Bell et al., 2014). Participants were instructed to stand with hands on hips and feet shoulder width apart prior to performing a squat to a depth whereby the thigh was parallel to the floor. Upon completion, participants indicated the pain felt in the lower limbs by drawing a line on the VAS.

Blood sample collection and analysis

Venous blood samples were collected using the venepuncture technique from a vein in the ante-cubital fossa region by a trained phlebotomist. Blood was collected into two 5 mL serum separator tubes and left to clot for 30–60 min prior to being centrifuged at 3,000 *g*, 23°C for 8 min. The serum was then removed and immediately stored in aliquots at −80°C for later analysis. Blood samples were analysed for markers of: muscle cell disruption (creatine kinase [CK]) and inflammatory processes (interleukin-6 [IL-6], high-sensitivity c-reactive protein [hsCRP], matrix metalloproteinase-9 [MMP-9]). The time of collection for all blood markers was based upon likely known time-course responses, and peak changes post-exercise (Table 3).

Serum CK and hs-CRP were determined by electrochemiluminescence using an automated analyser (Roche c702 chemistry module, Roche Diagnostics Ltd., United Kingdom). Serum IL-6 (Invitrogen Corporation, California, United States, MAN0006706) and MMP-9 (Thermo Scientific, Maryland, United States, BMS 2016-2 and BMS 2016-2TEN) were determined by an enzyme-linked immunosorbent assay (ELISA) using commercially available kits.

Statistical analysis

Raw data is reported as mean ± SD. All data (except soreness) were checked for normality using Shapiro-Wilk, which is recommended for sample sizes less than 30. Data identified as non-normal was log-transformed and all further data analysis was conducted on the transformed data. A mixed model ANOVA was used to analyse each dependent variable with a between-subjects factor of group (HWI and PAS) and a within-subject factor of time (baseline and post resistance exercise timepoints). Mauchley's test of sphericity was used to assess the homogeneity of variance and, where necessary, Greenhouse-Geisser corrections were applied. Significant main and interaction effects were analysed using Bonferonni *post hoc* pairwise comparisons. Partial eta squared (ηp^2) was used to indicate the effect sizes for main and interaction effects with ≥ 0.01, ≥ 0.059, ≥ 0.138 indicating small, moderate and large effects, respectively (Cohen, 1988). SPSS (IBM Corp., IBM SPSS Statistics for Windows, Version 28, Armonk, NY) was used for statistical analysis with a significance level of $p < 0.05$.

The mean ± 95% confidence interval (CI) for the differences between groups for changes between baseline and post measurement points were calculated (HWI minus PAS group). 'Unclear' was used to describe 95% CI's that crossed 0. Cohen's *d* effect sizes were used to determine the magnitude of the difference between the groups at each time point with < 0.20 indicating a trivial effect, 0.20—0.49 a small effect, 0.50—0.79 a medium effect, 0.80—1.19 a large effect, and ≥ 1.2 a very large effect (Cohen, 1988).

Results

The average (mean ± SD) values for each dependent variable for both groups at all time points are shown in Table 4. The same volume load was performed in the exercise session by both groups, with no significant differences seen between groups for any of the exercises. Post hoc analyses using the group means for post-intervention IMT at 1 cm depth, determined an effect size of 0.92 with an alpha level of 0.05, and yielded a statistical power of 1.00.

MVIC

There was a significant main effect of time ($F_{1.348,\ 18.870} = 13.005$, $P = <0.000$, $\eta p^2 = 0.482$), and *post hoc* pairwise comparisons determined significant decreases in MVIC between baseline-2 h, and −24 h post resistance exercise. There was no significant interaction effect ($F_{1.348,\ 18.870} = 0.041$, $p = 0.904$, $\eta p^2 = 0.003$) or main effect of group ($F_{1,14} = 0.347$, $p = 0.565$, $\eta p^2 = 0.024$) (Figure 2A). Comparisons for the group difference in the change

TABLE 4 Mean and SD for all dependent variables at each time point in both HWI and control.

Variable	Time point	HWI	PAS	Variable	Time point	HWI	PAS
MVIC (N)	Baseline	258 ± 99	226 ± 39	IMT at 1 cm (°C)*$	Baseline	33.52 ± 0.76	33.17 ± 1.18
	2 h*	214 ± 76	196 ± 49		P-ex	34.60 ± 1.51	33.95 ± 1.02
	24 h*	235 ± 79	212 ± 45		P-int	35.00 ± 0.81	33.15 ± 0.54
Soreness (mm)	Baseline	3 ± 3	11 ± 9		1 h	33.97 ± 1.06	33.16 ± 0.56
	2 h*	38 ± 41	50 ± 49		2 h$	32.89 ± 0.97	32.28 ± 0.72
	24 h*	52 ± 53	93 ± 40	IMT at 2 cm (°C)*$	Baseline	35.06 ± 0.73	34.81 ± 1.11
MMP-9 ($ng.mL^{-1}$)	Baseline	644 ± 242	802 ± 378		P-ex*	36.66 ± 0.96	36.28 ± 0.52
	2 h*	1,031 ± 368	1,278 ± 349		P-int$	36.60 ± 0.56	34.98 ± 0.42
	24 h	536 ± 134	626 ± 307		1 h$	35.42 ± 0.59	34.39 ± 0.69
hs-CK ($U.L^{-1}$)	Baseline	379 ± 324	352 ± 277		2 h$	34.22 ± 0.88	33.79 ± 0.82
	24 h*	1,167 ± 1,285	823 ± 668	IMT at 3 cm (°C)*$	Baseline	35.95 ± 0.67	35.85 ± 0.76
CRP ($mg.L^{-1}$)	Baseline	1.2 ± 1.0	1.2 ± 1.1		P-ex*	37.73 ± 0.38	37.45 ± 0.38
	24 h	0.9 ± 0.6	1.7 ± 1.7		P-int*$	37.20 ± 0.31**	36.12 ± 0.26
IL-6 ($pg.mL^{-1}$)	Baseline	0.9 ± 0.5	1.3 ± 0.8		1 h$	36.30 ± 0.42**	35.55 ± 0.41
	2 h*	1.4 ± 0.5	2.5 ± 1.5		2 h$	35.30 ± 0.70	35.16 ± 0.48
	24 h	1.4 ± 1.1	2.2 ± 2.2				

HWI, hot water immersion; PAS, passive recovery group; MVIC, maximum voluntary contraction; MMP-9, matrix metalloproteinase-9; CK, creatine kinase; hs-CRP, high sensitivity C-reactive protein; IL-6, interleukin-6; IMT, intramuscular temperature.
*significantly ($p < 0.05$) different from baseline (main effect of time); $ significantly ($p < 0.05$) different from post-exercise (main effect of time); ** significantly ($p < 0.05$) different from PAS; *$ significant ($p < 0.05$) difference between PAS, and HWI (main effect of group).

from baseline to 2 h post (−14 N, 95%CI [-51, 22], $d = 0.4$) and baseline to 24 h (−9 N, 95% CI [-32, 14], $d = 0.4$) were unclear.

Active muscle soreness

There was a significant main effect of time ($F_{2,28} = 17.632$, $P = <0.001$, $\eta p^2 = 0.557$) with *post hoc* comparisons showing significant increases in soreness from baseline-2 h, and −24 h after resistance exercise. No significant interaction ($F_{2,28} = 1.287$, $p = 0.292$, $\eta p^2 = 0.084$) or group effect ($F_{1,14} = 2.064$, $p = 0.173$, $\eta p^2 = 0.128$) was determined (Figure 2B). Comparisons for the group difference in the change from baseline to 2 h post (−3 mm, 95% CI [-48, 42], $d = 0.07$) and baseline to 24 h (−32 mm, 95% CI [-84, 20], $d = 0.7$) were unclear.

MMP-9

Changes in MMP-9 were significant over time ($F_{2,28} = 33.777$, $P = <0.001$, $\eta p^2 = 0.707$) with *post hoc* comparisons identifying significant increases between baseline and 2 h post resistance exercise. There was no significant interaction ($F_{2,28} = 0.582$, $p = 0.565$, $\eta p^2 = 0.040$) or main ($F_{1,14} = 1.620$, $p = 0.224$, $\eta p^2 = 0.104$) effect of group. Comparisons for the group difference in the change from baseline to 2 h post (−98 ng mL^{-1}, 95% CI [-410, 215], $d = 0.3$) and baseline to 24 h (81 ng mL^{-1}, 95% CI [-209, 370], $d = 0.3$) were unclear.

CK

Due to high baseline values in two participants (one per group) data were analysed for seven participants per group. There were significant increases between baseline and 24 h in CK ($F_{1,12} = 56.402$, $p = <0.001$, $\eta p^2 = 0.825$). There was no significant interaction effect ($F_{1,12} = 0.189$, $p = 0.672$, $\eta p^2 = 0.015$) or main effect of group ($F_{1,12} = 0.039$, $p = 0.848$, $\eta p^2 = 0.003$). There were no clear differences for group comparisons between baseline and 24 h (316 $U.L^{-1}$, 95% CI [-2,967, 3,599], $d = 0.1$).

Hs-CRP

Due to a high baseline value in one participant in the PAS group, data were analysed as seven (PAS) versus eight (HWI). There were no significant main effects of time ($F_{1,13} = 1.339$, $p = 0.268$, $\eta p^2 = 0.093$) or group ($F_{1,13} = 0.260$, $p = 0.618$, $\eta p^2 = 0.020$) and no significant interaction effect ($F_{1,13} = 2.312$, $p = 0.152$, $\eta p^2 = 0.151$). Group comparisons between baseline and 24 h were unclear (−0.8 $mg.L^{-1}$, 95% CI [-2.0, 0.4], $d = 0.8$).

IL-6

Due to high baseline values in two participants (one per group) data were analysed for seven participants per group. There was a significant main effect of time ($F_{2,24} = 4.783$, $p = 0.018$, $\eta p^2 = 0.285$)

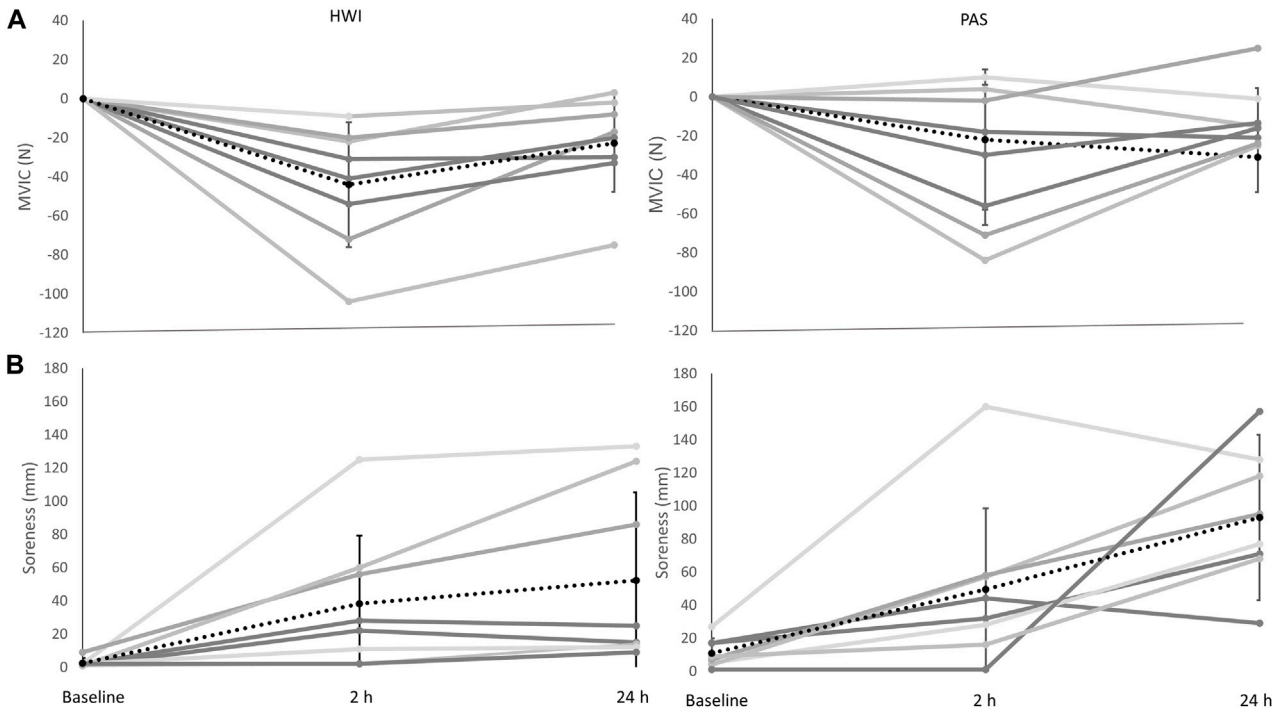

FIGURE 2
(A) Change in MVIC in the passive recovery (PAS) and hot water immersion (HWI) group (from left to right). **(B)** Change in muscle soreness in PAS and HWI (from left to right). Black dashed line represents the corresponding group average across time and standard deviation; grey lines represent individual responses across time. Significant differences ($p < 0.05$) between baseline, and 2 h and 24 h were evident for both MVIC and muscle soreness (main effect of time). Note: for MVIC the value shown at 2 h post is the change from baseline to 2 h post, and the value shown at 24 h is the change from baseline to 24 h.

with *post hoc* tests determining a significant increase between baseline and 2 h post. There was no significant interaction effect ($F_{2,24} = 0.093$, $p = 0.911$, $\eta p^2 = 0.008$) or main effect of group ($F_{1,12} = 1.286$, $p = 0.279$, $\eta p^2 = 0.097$). Effects were unclear for group comparisons between baseline and 2 h post (−0.7 pg mL^{-1}, 95%CI [-1.8, 0.4, $d = -0.8$), and baseline and 24 h (−0.3 pg mL^{-1}, 95%CI [-2.1, 1.5), $d = -0.2$).

Intramuscular temperature

There were significant main effects of time for intramuscular temperature (IMT) at 1 cm ($F_{2.232,\ 31.250} = 9.144$, $p = <0.001$, $\eta p^2 = 0.395$), 2 cm ($F_{1.866,\ 26.124} = 29.305$, $p = <0.001$, $\eta p^2 = 0.677$) and 3 cm ($F_{4,56} = 60.753$, $p = <0.001$, $\eta p^2 = 0.813$). Post-exercise resulted in the highest IMT at all depths, with significant increases at this point from baseline at 2 cm and 3 cm, and significant decreases from post-exercise to post-intervention (2 and 3 cm), 1 h post (2 and 3 cm) and 2 h post (all depths). Significant increases from baseline to intervention were only observed at 3 cm.

There were main effects of group at all IMT depths (1 cm: $F_{1,14} = 9.758$, $p = .007$, $\eta p^2 = 0.411$; 2 cm: $F_{1,14} = 11.866$, $p = 0.004$, $\eta p^2 = 0.459$; 3 cm: $F_{1,14} = 10.321$, $p = 0.006$, $\eta p^2 = 0.424$) with HWI eliciting higher intramuscular temperatures versus PAS.

There were no significant interaction effects at 1 cm ($F_{2.232,\ 31.250} = 1.745$, $p = 0.188$, ηp^2 square = 0.111) or 2 cm ($F_{1.866,\ 26.124} = 2.735$, $p = 0.087$, $\eta p^2 = 0.163$). However, at 3 cm there was a significant interaction effect ($F_{4,56} = 3.419$, $p = 0.014$, $\eta p^2 = 0.196$). Post-hoc tests showed that at both post-intervention and 1 h post intramuscular temperature in HWI was significantly greater than PAS, furthermore between baseline and post-intervention there was only a significant increase in HWI.

Comparisons for the group differences are shown in Table 4. All effects were unclear except between baseline and post-intervention HWI resulted in a greater increase in intramuscular temperature compared to PAS at all depths.

Discussion

The purpose of this study was to investigate the effect of HWI on acute physiological responses to resistance exercise. The results of this study indicate that HWI is a viable method of heat therapy that can maintain the rise in intramuscular temperature following resistance exercise. Despite this, there were no effects of HWI on measures of muscle function, muscle soreness or blood markers of muscle cell disruption or inflammatory processes. These results represent the first investigation into the acute physiological responses of a "real-world" HWI protocol following resistance exercise, alongside the use of a trained cohort, applied exercise session, and utilising good nutritional practice.

This is the first study to investigate the effects of HWI on intramuscular temperature during recovery from resistance exercise. After the post-exercise elevation in intramuscular temperature for both groups, the PAS group saw a decline following the intervention, whilst the HWI group showed a second increase post-intervention. Immediately following the intervention, HWI had the greatest effect on intramuscular temperature. HWI was able to maintain a higher intramuscular temperature compared to PAS up until 1 h post exercise at a depth of 3 cm.

As was previously hypothesised (Wilcock et al., 2006), the HWI-induced increase in tissue temperature would be expected to induce peripheral vasodilation and a subsequent increase in muscle blood flow. In the acute post-exercise period, a reduction in muscle blood flow, associated with cold water immersion, has typically been viewed as beneficial for exercise recovery due to reductions in inflammation, oedema and pain (Lee et al., 2005). This viewpoint has been challenged by recent research showing cold water immersion to exert no influence on muscle inflammatory or cellular stress responses (Peake et al., 2016).

It has previously been suggested that increases in muscle blood flow facilitate increased permeability of cellular, lymphatic and capillary vessels (Wilcock et al., 2006), leading to greater clearance rates of inflammatory markers in the blood. Given this, it is surprising that none of the blood markers in this study displayed enhanced rates of clearance following HWI. Only two previous studies investigating HWI have collected inflammatory markers from the blood after exercise, and in line with the current study, neither reported any effect of HWI (Vaile et al., 2008; Pournot et al., 2011). A mixture of findings related to post-exercise HWI and the appearance of intramuscular proteins in the blood currently exists with evidence of: a reduction (Vaile et al., 2008), a rise (Viitasalo et al., 1995) and no effect (Pournot et al., 2011). The only study to find an enhanced clearance utilised multiple immersions for each day up to 72 h post-exercise (Vaile et al., 2008). It is therefore reasonable to suggest that like others, the single bout of HWI in this study did not enhance the clearance of markers in the blood.

Research has shown both passive heat therapy (Uehara et al., 2004; Kobayashi et al., 2005; Yoshihara et al., 2013), and heat therapy in addition to a mechanical stimulus (Goto et al., 2003; Kakigi et al., 2011) to upregulate key anabolic signalling pathways and protein expression. The increased intramuscular temperature *per se* may therefore exert direct effects on cell signalling, impacting both inflammatory and anabolic pathways, although further research is required to confirm this.

Despite the manipulation of intramuscular temperature that could potentially aid the recovery of muscle function, we found no effect of HWI on MVIC. These results may be partially attributed to a reduction in acute physiological responses seen here in comparison to those reported by Jackman et al. (2019) which would have reduced the likelihood for an effect of the intervention. For example, at 24 h post-exercise the reduction in MVIC was 13% in the study by Jackman et al. (2019) and 7.2% in the present study. A possible cause could be differences in participant cohort, whereby those in the study by Jackman et al. (2019) had a 1 RM of 1.4 x BM, whilst the participants in the present study demonstrated 1.8 x BM. However, strength is not the primary determinant of training status (Buckner et al., 2017) and participants were recruited from an identical inclusion/exclusion criteria. Another possible explanation is the addition in the present study of added nutritional control which included the provision of protein supplements in the acute post-exercise period. Previous research has demonstrated that consuming protein (in the form of branched chain amino acids) can attenuate reductions in muscle function in the post-exercise period in resistance trained individuals (Howatson et al., 2012). This may therefore explain the reduced response seen between Jackman et al. (2019) and the present study. However, best practice recommendations (Jäger et al., 2017) suggest that individuals consume protein in the post-exercise period and therefore, in line with the applied nature of this study, the inclusion of the nutritional control enhanced ecological validity.

Previous research has typically produced mixed results related to the effect of post-exercise heat therapy on the recovery of muscle function, with those in support showing beneficial effects on all-out exercise performance (Cheng et al., 2017), strength (Vaile et al., 2008) and power (Viitasalo et al., 1995), although the mechanisms are still undefined. Cheng et al. (2017) reported that mean power output was better maintained following endurance exercise when the upper limbs were heated (~38°C) compared to control (~33°C) or being cooled (~15°C). The authors attributed this to higher rates of glycogen resynthesis with heating. It is unlikely that MVIC measured in the present study were limited by muscle glycogen stores and highlights the need to match post-exercise strategies to specific recovery demands (Minett and Costello, 2015). Participants in the study by Cheng et al. (2017) were heated to an intramuscular temperature of ~38°C at a depth of 1.5 cm, which is greater than the post-intervention intramuscular temperature of 35.0°C and 36.6°C reported in the present study for 1 and 2 cm, respectively. Despite differences in exercise modality, those that have reported beneficial effects of HWI, have used water temperatures of 37°C–38°C and durations of 14–20 min (Viitasalo et al., 1995; Vaile et al., 2008). It is unlikely that these protocols would have elevated intramuscular temperatures to greater levels than the present study, therefore we speculate that the intramuscular temperatures we report would have been comparable to previous studies that have reported beneficial effects. This lends to the assertion that in this instance, recovery of muscle function during maximal strength tasks following resistance exercise is independent of short-term changes in intramuscular temperature associated with HWI. Although isometric muscle function testing is considered the gold standard for absolute strength testing, it cannot be used as a proxy for dynamic muscle actions. As such, the results presented here may not accurately reflect the impact of HWI on more sport specific or ecologically valid sporting actions, and further research is warranted to investigate the utility of acute HWI for recovery in performance settings.

Our finding that HWI exerted no positive effect on ratings of muscle soreness is consistent with several reports (Kuligowski et al., 1998; Vaile et al., 2008; Pournot et al., 2011). Studies investigating heat therapy that have demonstrated beneficial effects have been limited to those that included underwater massage alongside HWI (Viitasalo et al., 1995) or continuous (8 h) low-level heat wrap therapy (Mayer et al., 2006) and would therefore be expected to elicit a markedly different response to HWI in the present study. Heat therapy has been suggested to reduce pain via an analgesic effect on nerves (Baker et al., 2001), although this theory has been proposed within the field of physiotherapy. Together, these results suggest that for those interested in applying HWI following

resistance exercise, it is unlikely to produce reductions in muscle soreness.

A number of points are worth considering when interpreting the results of the present study. Firstly, a passive recovery protocol was employed as the comparator to HWI. Although it is unlikely that athletic individuals will employ a sedentary post-exercise strategy, to understand the effect of HWI on acute physiological responses, it is recommended to compare with no recovery method (White and Caterini, 2017). Secondly, it is important to understand the implications of our findings in the context of the study. We recruited trained individuals, employed an applied exercise modality, delivered a realistic HWI protocol and utilised good nutritional practice around the session to enhance the ecological validity of our findings. We do not rule out the possibility that the influence of heat therapy on physiological responses may differ in other situations. For example, passive heat acclimation has been shown to improve muscle contractile properties which may have clinical relevance for individuals unable to exercise (Racinais et al., 2016). Lastly, the authors acknowledge that the relatively small sample size could have influenced the findings, particularly given the large interindividual variation in certain dependent variables. Therefore, further research is warranted to verify the results presented herein.

In summary, this is the first study to demonstrate HWI as a viable means of heat therapy that can increase intramuscular temperature. We also offer insights into the effect of HWI on acute physiological responses in a real-world environment. In the context of the present study it appears that HWI is no more effective than passive recovery for the recovery of muscle function, soreness and markers of muscle cell disruption and inflammation following resistance exercise. Further research is required to investigate the application of HWI in other contexts including: following other exercise modalities, using different durations/temperatures of immersion, and when used chronically as part of a long-term training programme.

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 London Sport Institute Ethics Sub-Committee, Middlesex University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JJ, PB, KV, and EC conceived and designed the study. JJ collected the data. JJ, MG, and FH analysed the blood samples. JJ, PB, and EC assembled and analysed the data. JJ, PB, KV, and EC interpreted the data. JJ, PB, LW, and EC drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

JJ and PB were empolyed by Art Health Solutions.

The remaining 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.

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Echocardiography and extravascular lung water during 3 weeks of exposure to high altitude in otherwise healthy asthmatics

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Background: Asthma rehabilitation at high altitude is common. Little is known about the acute and subacute cardiopulmonary acclimatization to high altitude in middle-aged asthmatics without other comorbidities.

Methods: In this prospective study in lowlander subjects with mostly mild asthma who revealed an asthma control questionnaire score >0.75 and participated in a three-week rehabilitation program, we assessed systolic pulmonary artery pressure (sPAP), cardiac function, and extravascular lung water (EVLW) at 760 m (baseline) by Doppler-echocardiography and on the second (acute) and last day (subacute) at a high altitude clinic in Kyrgyzstan (3100 m).

Results: The study included 22 patients (eight male) with a mean age of 44.3 ± 12.4 years, body mass index of 25.8 ± 4.7 kg/m^2, a forced expiratory volume in 1 s of 92% ± 19% predicted (post-bronchodilator), and partially uncontrolled asthma. sPAP increased from 21.8 mmHg by mean difference by 7.5 [95% confidence interval 3.9 to 10.5] mmHg ($p < 0.001$) during acute exposure and by 4.8 [1.0 to 8.6] mmHg ($p = 0.014$) during subacute exposure. The right-ventricular-to-pulmonary-artery coupling expressed by TAPSE/sPAP decreased from 1.1 by −0.2 [−0.3 to −0.1] mm/mmHg ($p < 0.001$) during acute exposure and by −0.2 [−0.3 to −0.1] mm/mmHg ($p = 0.002$) during subacute exposure, accordingly. EVLW significantly increased from baseline (1.3 ± 1.8) to acute hypoxia (5.5 ± 3.5, $p < 0.001$) but showed no difference after 3 weeks (2.0 ± 1.8).

Conclusion: In otherwise healthy asthmatics, acute exposure to hypoxia at high altitude increases pulmonary artery pressure (PAP) and EVLW. During subacute

Abbreviations: ANOVA, analysis of variance, AT, acceleration time; BSA, body surface area; CI, cardiac index; CO, cardiac output; EF, ejection fraction; FEV1, forced expiratory volume in 1 s; HR, heart rate; LVOT, left ventricular outflow tract; RA, right atrial area; RAP, right atrial pressure; RV-EDA, right ventricular end-diastolic area; RV-ESA, right ventricular end-systolic area; RV-FAC, right ventricular fractional area change; RVIDd/LVIDd, right to left ventricular internal diastolic diameter ratio; sPAP, systolic pulmonary artery pressure; SV, stroke volume; SVI, stroke volume index; TAPSE, tricuspid annular plane systolic excursion; TASV, tricuspid annular systolic velocity; TRV, tricuspid regurgitation jet velocity; TRPG, tricuspid regurgitation pressure gradient; O_2, oxygen.

exposure, PAP remains increased, but EVLW returns to baseline values, suggesting compensatory mechanisms that contribute to EVLW homeostasis during acclimatization.

KEYWORDS

hypoxia, altitude, echocardiography, asthma, acclimatization

Introduction

Worldwide, an estimated 300 million people are affected by asthma, with a varying prevalence of 1%–25% (Gourgoulianis et al., 2001; Droma et al., 2007; Global initiative for Asthma, 2023). Very little is known about pulmonary vascular and cardiac acclimatization in asthmatic but otherwise healthy middle-aged lowlanders traveling to and staying at high altitude (Cogo and Fiorenzano, 2009). Due to the potentially favorable effects of the altitude climate, many asthmatics are sent to high altitude for asthma rehabilitation in order to improve symptoms and lung function (Fieten et al., 2022). The fall in barometric pressure and decreased partial pressure of oxygen (PaO_2) at high altitude cause compensatory increased minute ventilation and thus lead to hypocapnia as well as hypoxic pulmonary vasoconstriction (HPV). The resulting elevated pulmonary artery pressure (PAP) while traveling to high altitude has been associated with right-sided cardiac dysfunction and may predispose the patient to high-altitude pulmonary edema (HAPE), which is a life-threatening condition associated with increasing extravascular lung water (EVLW, B-lines) (Allemann et al., 2000; Maggiorini et al., 2001; Mounier et al., 2011; Swenson and Bartsch, 2012). When the severity of asthma is increased and during exacerbations, patients may develop hyperinflation that may challenge the cardiopulmonary system. Thus, the known increase in PAP while traveling to high altitude might increase the risk of right heart decompensation for these patients (Eniseeva and Sizykh, 1995; Bobrov et al., 2003).

Previous studies on the PAP and right heart function at high altitude have focused mainly either on acute short-term exposure in healthy younger travelers or highlanders permanently living at high altitude (Soria et al., 2016; Soria et al., 2019). However, studies investigating the subacute acclimatization of the right heart function are rare but are of particular interest for people traveling to high altitudes during vacations and for asthma patients undergoing high-altitude climate therapy (Fieten et al., 2022). Rehabilitation is an add-on of a non-pharmacological intervention in patients with asthma (Global Initiative for Asthma, 2023).

Therefore, the purpose of the current study was to describe the cardiac function and hemodynamics and perform sonographic assessments of EVLW in otherwise healthy asthmatic lowlanders staying for 3 weeks at a high altitude (3100 m) while participating in a comprehensive rehabilitation program.

Materials and methods

Design

This prospective study was embedded in a randomized controlled trial investigating the effect of 3 weeks of asthma rehabilitation at a high altitude (Saxer et al., 2019). We conducted three sequential measurements of PAP and cardiac function by echocardiography and lung sonography in patients with asthma. The first measurement was made at a low altitude baseline in Bishkek (Kyrgyzstan, altitude 760 m), and the second was made the day after arrival at the Too Ashu high-altitude clinic (Kyrgyzstan, altitude 3,100 m) by minibus (acute exposure), and the last was made after 17 nights at 3100 m (subacute hypoxia).

The patients participated in a rehabilitation program during the high-altitude stay, including patient education, endurance training, muscle strength training, breathing exercises, and guided walks. A detailed description of the intervention can be found in the previous publication (Saxer et al., 2019).

The study was performed between June and July 2016 and was approved by the Ethics Committee of the National Center of Cardiology and Internal Medicine, Bishkek, Kyrgyzstan (No. 01-8/151). All patients provided written informed consent.

Patients

Patients with mild to moderate, atopic, or non-atopic asthma that was not optimally controlled were included in this current trial. All patients had lived in or near Bishkek below 1,000 m for the last 3 months. Patients with an underlying cardiac pathology, assessed by taking a history and echocardiography, were excluded; for details, see Saxer et al. (2019).

Measurements and procedures

Resting transthoracic echocardiography was conducted by three experienced cardiac sonographers (P.B., M.L., and S.U.). The images were obtained with a CX 50 Ultrasound System (Philips®, Bothell, United States of America), using a 5 to 1 MHz sector array transducer, and analyzed after the examination on the same system by the same physicians. A standard echocardiographic investigation was performed according to guidelines (Quiñones et al., 2002; Rudski et al., 2010; Lang et al., 2015).

The tricuspid regurgitation pressure gradient (TRPG) was derived from the maximal tricuspid regurgitation jet velocity (TRVmax) using the modified Bernoulli equation. The systolic PAP (sPAP) was calculated by adding the maximal TRPG to the right atrial pressure (RAP), which was rated as 3, 8, or 15 mmHg, depending on the diameter and collapsibility of the inferior vena cava (Rudski et al., 2010; Lichtblau et al., 2019). The mean PAP (mPAP) was estimated by the equation mPAP = sPAP * 0.61 + 2 (Chemla et al., 2004).

The left ventricular systolic function was assessed by the ejection fraction (EF) according to the modified Simpson's rule.

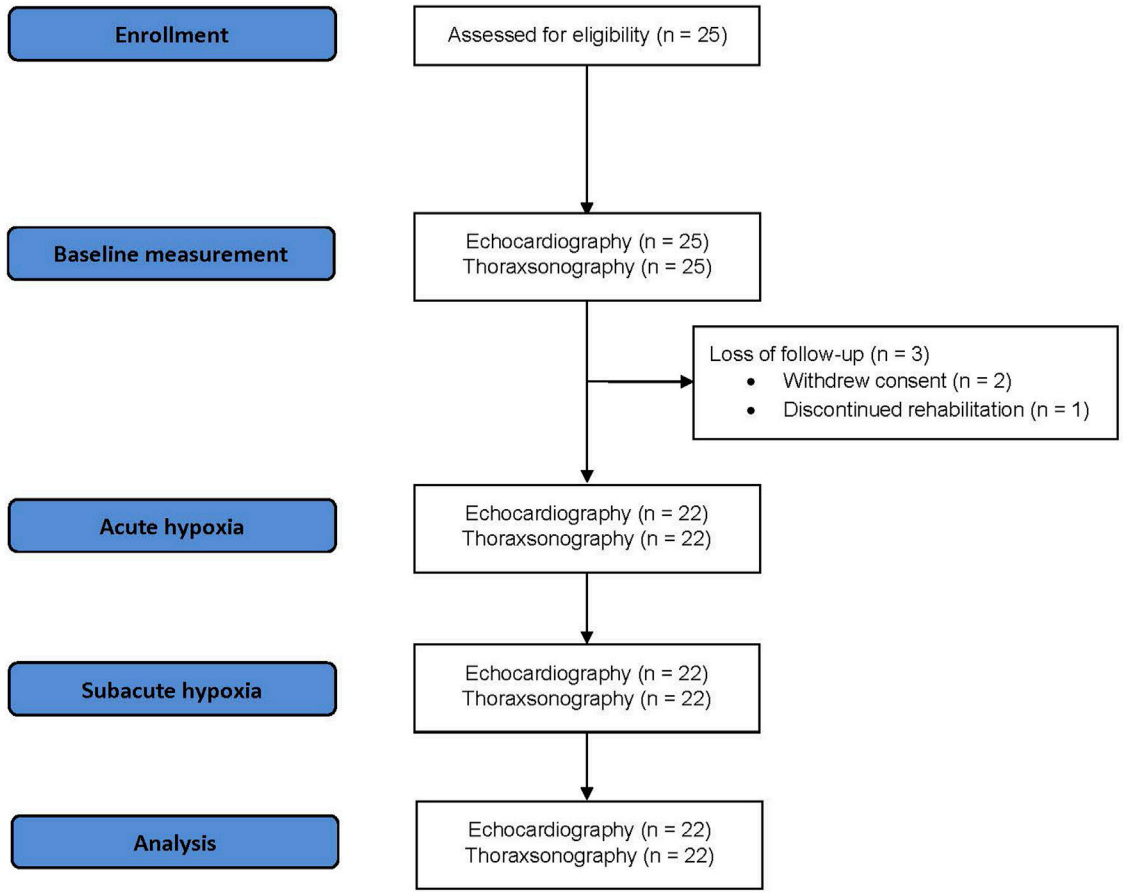

FIGURE 1
Patient flow.

Stroke volume (SV) was calculated using the velocity time integral and the diameter of the left ventricular outflow tract (LVOT). Cardiac output (CO) was derived by multiplying the SV by the heart rate (HR). Stroke volume index (SVI) and cardiac index (CI) were obtained by dividing SV and CO by the body surface area (BSA). The assessment of the right ventricular function included the following parameters: tricuspid annular plane systolic excursion (TAPSE), tricuspid annular systolic velocity (TASV), RV end-diastolic area (RV-EDA), RV end-systolic area (RV-ESA), right ventricular fractional area change (RV-FAC), diastolic right and left ventricular internal diameters and their ratios (RVIDd/LVIDd) from an apical four-chamber view, and right atrial end-systolic area (RA-ESA). The total pulmonary resistance was calculated with mPAP/CO, the right ventricular arterial coupling was calculated with TAPSE/sPAP (Tello et al., 2019), the pulmonary arterial wedge pressure (PAWP) was calculated with the formula (PAWP = 1.24 × (E/e') + 1.9) (Nagueh et al., 2016), and the pulmonary vascular resistance (PVR) was calculated as with PVR = (mPAP − PAWP)/CO.

Sonographic assessment of the lung

After the echocardiographic examination, all patients were assessed for EVLW by looking for the number of B-lines in a supine position with the same ultrasound machine and transducer described previously (Lichtblau et al., 2021a). A B-line is defined as an echogenic linear signal with a narrow origin from the pleural line that crosses the image parallel to the sector arrays. These B-lines, observed at 28 different intercostal sites (on the left hemithorax from the second to the fourth and on the right from the second to the fifth intercostal spaces in the parasternal, midclavicular, anterior axillary, and midaxillary lines), were totaled (Picano et al., 2006).

Other measurements

In addition, systemic blood pressure (BP), heart rate (HR) and pulse oximetry (SpO_2) spirometry, and a 6-min walk test (6 MWT) were obtained in the same time frame (Saxer et al., 2019).

TABLE 1 Baseline characteristics at low altitude.

Subjects, n	22
Male	8 (36)
Female	14 (64)
Age, years	44.3 ± 12.4
Body mass index, kg/m^2	25.8 ± 4.7
Body surface area, m^2	1.78 ± 0.20
Height, m	1.66 ± 0.1
Spirometry pre-bronchodilator	
FEV1/FVC, %	63 ± 11
FEV1% predicted, %	80 ± 18
Spirometry post-bronchodilator	
FEV1% predicted, %	92 ± 19
Asthma severity by spirometry	
Mild (FEV1 predicted > 80%), n	20 (91)
Moderate (FEV1 predicted > 60%), n	2 (9)
Asthma Control Questionnaire score	2.33 ± 0.87
6-min walk distance, m	538 ± 58
Smokers, n	0 (0)
Medication	
Inhaled corticosteroids, n	22 (100)
Beta-adrenergics, n	17 (77)
Anticholinergics, n	1 (5)
Antihistamines, n	1 (5)
Leukotriene receptor antagonists, n	2 (9)

Data are presented as mean ± standard deviation or as number (%). SpO_2, pulse oximetry; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity.

Intervention

Patients performed a comprehensive 3-week, in-patient rehabilitation program at a high altitude that was described previously (Saxer et al., 2019).

Statistical analysis

The analyses included all available measurements at different time points. The analysis was performed per protocol. Missing data were not imputed.

Data are presented as mean and ± SD. EVLW (B-lines) and echocardiographic and physiological parameters were analyzed in linear mixed models with different time points (baseline, acute, and subacute hypoxia as fixed effects and subjects as random intercept). The average marginal effects (mean difference) induced by time were extracted from these regression models and expressed as mean change with 95% confidence intervals. Model assumptions were tested by visual inspection of the homogeneity and normality of the residuals and the random effects (Kentucky–Ascombe and Q–Q plots). A p-value threshold of <0.05 or a confidence interval not including zero was considered to be statistically significant. All statistical analyses were performed using R Studio (version 1.0.153, R Studio Inc., San Francisco, United States).

Results

Of 25 patients in the high altitude asthma rehabilitation study, 22 were included in this per-protocol study. Three patients were not available due to personal reasons (one discontinued the rehabilitation program, and two denied undergoing echocardiographic measures); see Figure 1.

Characteristics of the 22 patients (14 women), aged between 24 and 66 years, all non-smokers with no relevant cardiac comorbidity, are displayed in Table 1.

Table 2 displays the number of B-lines, oxygen saturation, and blood pressure. Almost all patients increased the number of B-lines in the acute exposure with a mean difference of 4.2 (2.7–5.7, $p < 0.001$) with a subsequent reduction over the course of the high-altitude stay and did not show a significant difference to the baseline (mean difference 0.7 [−0.8 to 2.2], $p = 0.341$). Oxygen saturation significantly decreased at altitude in the acute phase and only slightly recovered in the subacute phase (baseline: 95.5% ± 2.5% with a mean difference of −6.5 [−8.3 to −4.7]% during acute exposure and −4.1 [−5.9 to −2.3]% in the subacute exposure; all $p < 0.001$).

There was no significant change in systolic and diastolic blood pressure (systolic: baseline 120 ± 24 to acute −3.2 [−10.6 to 4.2] mmHg to subacute −3.1 [−10.5 to 4.1] mmHg and diastolic: baseline 76 ± 13 to acute 1.0 [−5.0 to 6.0] mmHg to subacute 3.3 [−1.7 to 8.3] mmHg, all $p > 0.05$).

Figure 2 displays the association of the EVLW and the SpO_2 at the different measurement times −0.28 (−0.42 to −0.13, $p < 0.001$).

The echocardiographic results are shown in Table 3 and Figure 3. Traveling to a high altitude revealed a significantly increased PAP and pulmonary vascular resistance as well as a reduced right-ventricular-to-pulmonary-artery coupling in acute hypoxia that was mainly maintained during subacute hypoxia.

sPAP increased from 21.8 mmHg by 7.5 [3.9 to 10.5] mmHg in the acute exposure and by 4.8 [1.0 to 8.6] mmHg in the subacute exposure compared to baseline. Pulmonary vascular resistance increased from 1.5 WU at baseline by 1.5 [0.9 to 2.1] WU in the acute exposure and 0.8 [0.1 to 1.5] WU in the subacute exposure, and the right ventricular coupling expressed by TAPSE/sPAP decreased from 1.1 at baseline by −0.2 [−0.3 to −0.1] mm/mmHg in the acute exposure and −0.2 [−0.3 to −0.1] mm/mmHg in the subacute exposure; all values are statistically significant.

The FAC of the right ventricle was significantly reduced in the subacute phase, and the size of the right ventricle was significantly larger than the baseline.

Most patients tolerated the high altitude very well; only one patient suffered from asthma exacerbation, defined as a decline in PEF of >12%, and one patient, who suffered from AMS during the high-altitude stay, was treated with acetazolamide (Saxer et al., 2019).

TABLE 2 Vital signs and extravascular lung water analyzed with a linear mixed model.

	Baseline, 760 m	Acute hypoxia, day 2 at 3,100 m	*p*-value	Subacute hypoxia at day 17 at 3,100 m	*p*-value
Heart rate, bpm	72.1 ± 10.9	74.5 ± 9.0	0.261	79.0 ± 9.1	0.001
Systolic blood pressure, mmHg	120 ± 24	117 ± 13	0.838	117 ± 11	0.410
Diastolic blood pressure, mmHg	76 ± 13	77 ± 12	0.708	80 ± 10	0.197
Oxygen saturation, %	95.5 ± 2.5	89.0 ± 5.4	<0.001	91.3 ± 2.7	<0.001
Weight, kg	71.0 ± 14.6	73.1 ± 13.2	0.070	73.0 ± 12.5	0.096
Lung comets, B-lines	1.3 ± 1.8	5.5 ± 3.5	<0.001	2.0 ± 1.8	0.341

Values are displayed as mean ± standard deviation. *p*-value linear mixed model compared to baseline.

Discussion

In this study, we showed for the first time the acute and subacute effects of exposure to the hypobaric hypoxic environment on PAP, heart function, and EVLW in patients with mostly mild but partly uncontrolled asthma who participated in a three-week asthma rehabilitation program at high altitude (3100 m). We could show that in accordance with healthy subjects, sPAP increased with acute exposure to high altitude, albeit remaining below a level that would define pulmonary hypertension (PH), along with an increased PVR and a slightly lower TAPSE/sPAP ratio at high compared to low altitude but still within normal range. These changes went along with a decrease in SpO_2 and an increase in HR with altitude and were maintained after 17 nights at high altitude. All changes, with the exception of the PVR, remained below a level considered pathological at low altitude. EVLW was assessed as B-lines increased upon acute exposure to the high altitude but returned to low altitude baseline values after 17 days, suggesting compensatory mechanisms that contribute to EVLW homeostasis during acclimatization.

Oxygenation and hemodynamic changes

As expected, SpO_2 decreased from 95% to 89% upon acute exposure to the high altitude and slightly rose during subacute exposure but remained decreased compared to the low altitude. Mean systemic blood pressure did not change with acute or subacute exposure to the high altitude. Previous studies have shown that blood pressure tends to be elevated only in the first 10–24 h and then normalizes over the next days at a moderate altitude (~2500 m), but several weeks of acclimatization are needed to normalize the blood pressure at higher altitudes (Calbet et al., 2003; Parati et al., 2014; Sagoo et al., 2017).

In contrast to most reports, resting HR was only slightly but not significantly increased during acute exposure to the high altitude. This may be due to increased HR at the low altitude due to sympathetic activity caused by inhaled sympathomimetic bronchodilators with no further changes at the high altitude under maintained therapy (Huez et al., 2009; Gaur et al., 2021). As in other studies of acclimatization to high altitudes, the HR further increased after 17 nights at high altitude (Huez et al., 2009; Gaur et al., 2021). The acute effect of high altitudes on PAP and PVR due to hypoxic pulmonary vasoconstriction is well known (Grünig et al., 2000; Maggiorini and Leon-Velarde, 2003; Grunig et al., 2009) and is usually reversible after descent (Hilty et al., 2016). As previously described in healthy subjects and patients with lung diseases, PAP rose upon acute exposure to the high altitude in our asthma patients (Lichtblau et al., 2019; Lichtblau et al., 2021b; Schneider et al., 2022). However, sPAP remained below what would be considered pathological. At the end of the 3-week rehabilitation program, the PAP was still higher than at the low altitude, which is consistent with other reports (Lichtblau et al., 2021a; Gaur et al., 2021).

The stroke volume index was reduced in the acute and subacute phases at high compared to the low altitude, whereas the cardiac index remained stable due to the increase in the heart rate during the subacute phase.

In contrast to our study, Huez et al. (2009) showed a higher cardiac output in lowlanders during acute exposure and after acclimatization. They showed adaptive changes in the diastolic function of both ventricles; however, pulmonary vascular resistance was not directly calculated but was stated as being only moderately increased.

Gaur et al. (2021) studied young, healthy male Kyrgyz and Indian residents during a comparable length of stay at a higher altitude (4100 m). They showed comparable changes for HR, stroke volume, PAP, and PVR as shown in the investigated asthma cohort and thus may indicate that the presently investigated patients with mostly mild asthma could stay at a high altitude without exaggerated changes in pulmonary or systemic hemodynamics. The acute effect of high altitudes on PAP and PVR due to hypoxic pulmonary vasoconstriction is well known (Grünig et al., 2000; Maggiorini and Leon-Velarde, 2003; Grunig et al., 2009) and is reversible after descent (Hilty et al., 2016). In contrast to our study, the cardiac index increased at high altitude, probably due to a higher increase in heart rate. An increase in cardiac output during acute exposure was also found in patients with other obstructive lung diseases such as chronic obstructive pulmonary disease (Lichtblau et al., 2023).

In non-acclimatized Chinese Han lowlanders, Liu et al. (2022) showed that subacute exposure led to an enlargement of the right ventricle and a decrease of the diastolic right ventricular function, whereas the systolic function only decreased after long-term exposure to high altitudes (working at high altitudes for more than two decades). In our study, only the right ventricular end-systolic area was significantly enlarged at acute and subacute measurements at high altitude. Right ventricular systolic

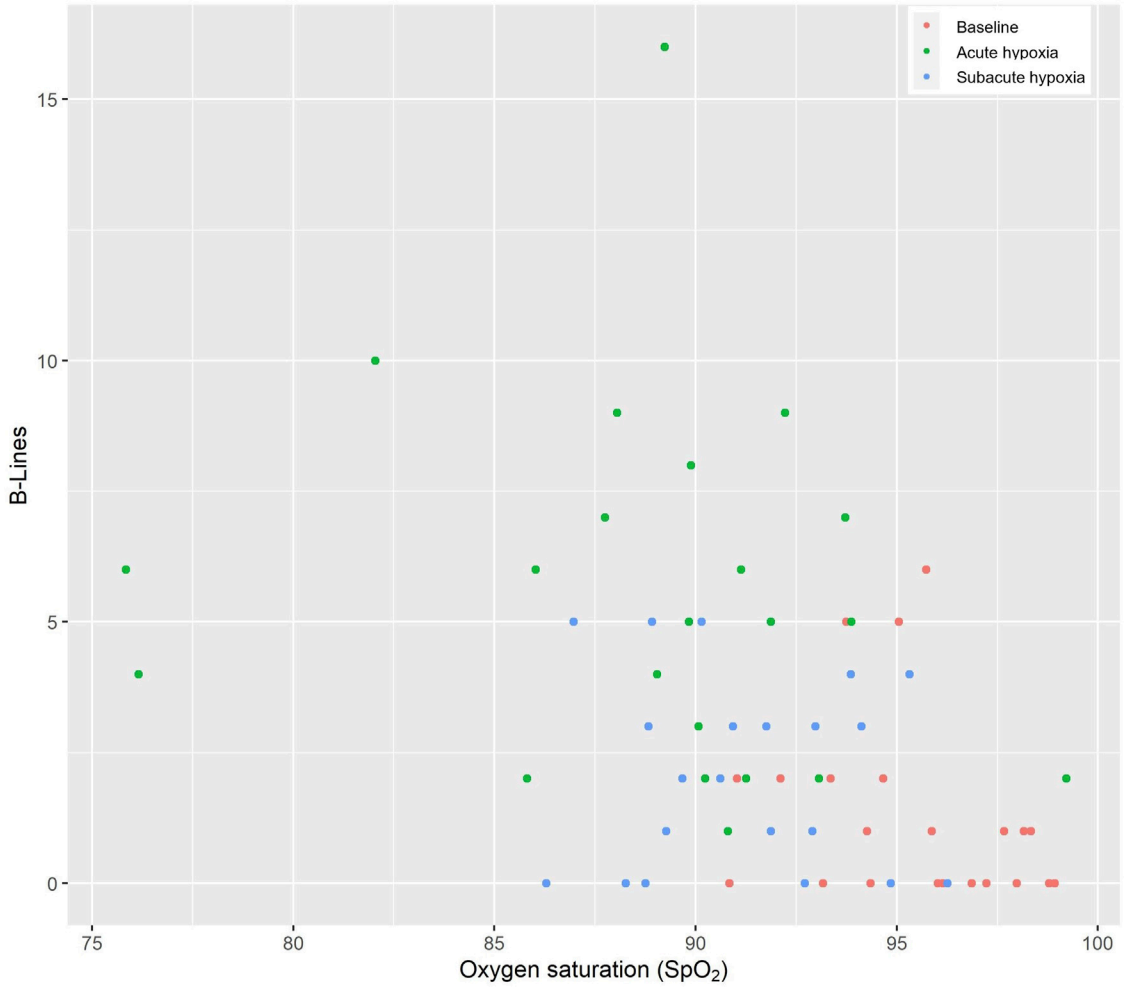

FIGURE 2
Thoraxsonography of the extravascular lung water (B-lines) and the oxygen saturation. The red circles indicate the baseline values at low altitude, the green circles indicate acute hypoxia, and the blue circles indicate subacute hypoxia.

function was preserved; TAPSE did not significantly change during the high-altitude stay; FAC decreased slightly and was significantly lower in the subacute phase. We do not have a certain explanation for these changes; however, baseline examinations were performed during the summer at Bishkek with temperatures ranging around 45°C, and participants might have been dehydrated during the assessment leading to reduced intravascular and intracardiac fluid volumes, whereas the temperatures were significantly colder at the higher altitude. However, all values measured at high altitude were still within the normal range, and changes were slight and in accordance with those expected in healthy subjects (Gaur et al., 2021).

A study from Turkey focused on the long-term effect of migrating to higher altitudes in lowlanders. That study performed echocardiography within 48 h and 6 months after arrival at a moderate altitude (1890 m) but did not take baseline measurements at the low altitude. In contrast to the Chinese study, the Turkish study showed that RV diastolic function is altered upon long-term exposure to moderate altitudes, whereas systolic function was preserved (Arisoy et al., 2016).

Extravascular lung water

B-lines can be used to measure extravascular lung water. High-altitude exposure can lead to acute mountain sickness and HAPE (Fagenholz et al., 2007). The increase in EVLW might be explained initially by elevated hydrostatic pressure that causes capillary leakage followed by extravasation of large-molecular-weight proteins and erythrocytes in the interstitial space, creating an increased osmotic gradient. This can then lead to pulmonary edema (Bartsch et al., 2005). The participants of this study did not show any clinical symptoms or signs of HAPE. Studies have shown that HAPE usually develops after 2–5 days upon exposure to high altitudes (Maggiorini, 2006). Similar to the study of Lichtblau et al. (2021a) that assessed healthy subjects at a low (720 m) and a high altitude (5050 m) in Chile, we have shown in this study that upon acute exposure to a high altitude, the number of B-lines initially increased and then decreased upon acclimatization. Our study showed acclimatization in terms of B-lines and also a moderate recovery of SpO_2. This is consistent with compensatory mechanisms that assure adequate homeostasis of lung water during prolonged exposure to hypobaric hypoxia.

TABLE 3 Echocardiographic parameters analyzed by a linear mixed model.

	Baseline, 760 m	Acute hypoxia, day 2 at 3,100 m	*p*-value	Subacute hypoxia at day 17 at 3,100 m	*p*-value
TRPG, mmHg	18.7 ± 3.7	25.1 ± 6.8	<0.001	22.4 ± 4.6	0.043
sPAP, mmHg	21.8 ± 3.9	29.2 ± 7.8	<0.001	27.1 ± 5.5	0.014
mPAP, mmHg	15.3 ± 2.4	19.8 ± 4.8	<0.001	18.5 ± 3.4	0.014
RAP, mmHg	3.2 ± 1.1	4.1 ± 2.1	0.081	4.1 ± 2.2	0.081
RA area, cm^2	13.7 ± 2.1	13.3 ± 2.2	0.571	12.9 ± 2.6	0.210
RV FAC, %	42.1 ± 6.2	38.3 ± 8.2	0.071	35.6 ± 6.1	0.003
RV ESA, cm^2	10.1 ± 2.6	11.8 ± 2.2	0.006	12.8 ± 2.8	<0.001
RV EDA, cm^2	17.5 ± 4.5	19.3 ± 3.6	0.081	19.8 ± 4.0	0.028
TAPSE, cm	2.3 ± 0.3	2.4 ± 0.3	0.487	2.2 ± 0.2	0.101
TASV, cm/s	12.9 ± 1.9	12.8 ± 1.7	0.690	12.9 ± 1.7	0.947
SVI, mL/m^2	38.5 ± 7.4	34.2 ± 7.1	0.009	34.6 ± 5.6	0.017
CI, $L/min/m^2$	2.7 ± 0.5	2.5 ± 0.5	0.102	2.7 ± 0.4	0.806
mPAP/CO, mmHg/L/min	3.2 ± 0.8	4.6 ± 1.7	<0.001	4.1 ± 1.0	0.058
TAPSE/sPAP, mm/mmHg	1.1 ± 0.2	0.9 ± 0.3	<0.001	0.9 ± 0.2	0.002
TAPSE/TRPG, mm/mmHg	1.3 ± 0.2	1.0 ± 0.3	<0.001	1.1 ± 0.3	0.005
PAWP, mmHg	8.4 ± 1.4	7.4 ± 1.3	0.003	7.9 ± 1.5	0.155
PVR, WU	1.5 ± 0.6	3.0 ± 1.4	<0.001	2.3 ± 0.9	0.027
LFEF biplan, %	59.9 ± 4.8	61.0 ± 5.8	0.390	59.2 ± 4.4	0.582
LVID end-systolic, cm	3.0 ± 0.4	3.1 ± 0.5	0.138	3.0 ± 0.3	0.966
LVID end-diastolic, cm	4.5 ± 0.4	4.4 ± 0.5	0.182	4.3 ± 0.5	0.044
MV E/A	1.3 ± 0.5	1.0 ± 0.3	0.003	1.1 ± 0.3	0.014
Eccentricity index end-systolic	1.0 ± 0.1	1.0 ± 0.1	0.979	1.1 ± 0.1	0.033
Eccentricity index end-diastolic	1.1 ± 0.1	1.0 ± 0.1	0.297	1.1 ± 0.1	0.520
RV/LV, %	79 ± 15	78 ± 9	0.766	75 ± 9	0.206

Data are presented as mean ± SD. *p*-value linear mixed model compared to baseline. TRPG, tricuspid regurgitation pressure gradient; sPAP, systolic pulmonary artery pressure; mPAP, mean pulmonary artery pressure; RAP, right atrial pressure; RA, right atrium; RV, right ventricle; EDA, end-diastolic area; ESA, end-systolic area; FAC, fractional area change; TAPSE, tricuspid annular plane systolic excursion; TASV: tricuspid annular systolic velocity; SVI, stroke volume index; CI, cardiac index; mPAP/CO, total pulmonary resistance; PAWP, pulmonary arterial wedge pressure; PVR, pulmonary vascular resistance; LV, left ventricle; ID, internal dimension; MV, mitral valve; E/A, ratio of transmitral early diastolic to late diastolic velocity.

Limitations

Because all patients underwent an asthma rehabilitation program during the high-altitude stay, a distinction between training and altitude effects cannot be made. However, it seems unlikely that asthma rehabilitation would have had a significant effect on PAP and heart function. Furthermore, with the background knowledge of similar changes found in acclimatization studies of healthy lowlanders without rehabilitation programs, it is well conceivable that changes seen here are due to the high-altitude exposure and subsequent acclimatization and not due to the asthma rehabilitation program, albeit the 3 weeks of asthma rehabilitation were associated with a significant increase in exercise capacity measured by the 6-minute walk distance and the 1 min sit-to-stand test and better asthma control. In addition to forced expiratory volume in 1 s, exhaled nitric oxide and hemoglobin were also significantly increased after the rehabilitation at the high altitude compared to the rehabilitation at the low altitude (Saxer et al., 2019).

All but two patients in this study had mild asthma but initially revealed an asthma control questionnaire score >0.75, which improved with the rehabilitation program, and all were otherwise healthy (Saxer et al., 2019). High-altitude-induced changes found in this cohort were in the range expected from studies of healthy subjects (Gaur et al., 2021; Liu et al., 2022); however, we cannot know whether they would apply to patients with more severe and less controlled asthma who wish to undertake rehabilitation programs at a high altitude.

There were many missing values of the TRPG at the last assessment in subacute hypoxia; whereas TRPG was assessable in 82% of subjects at

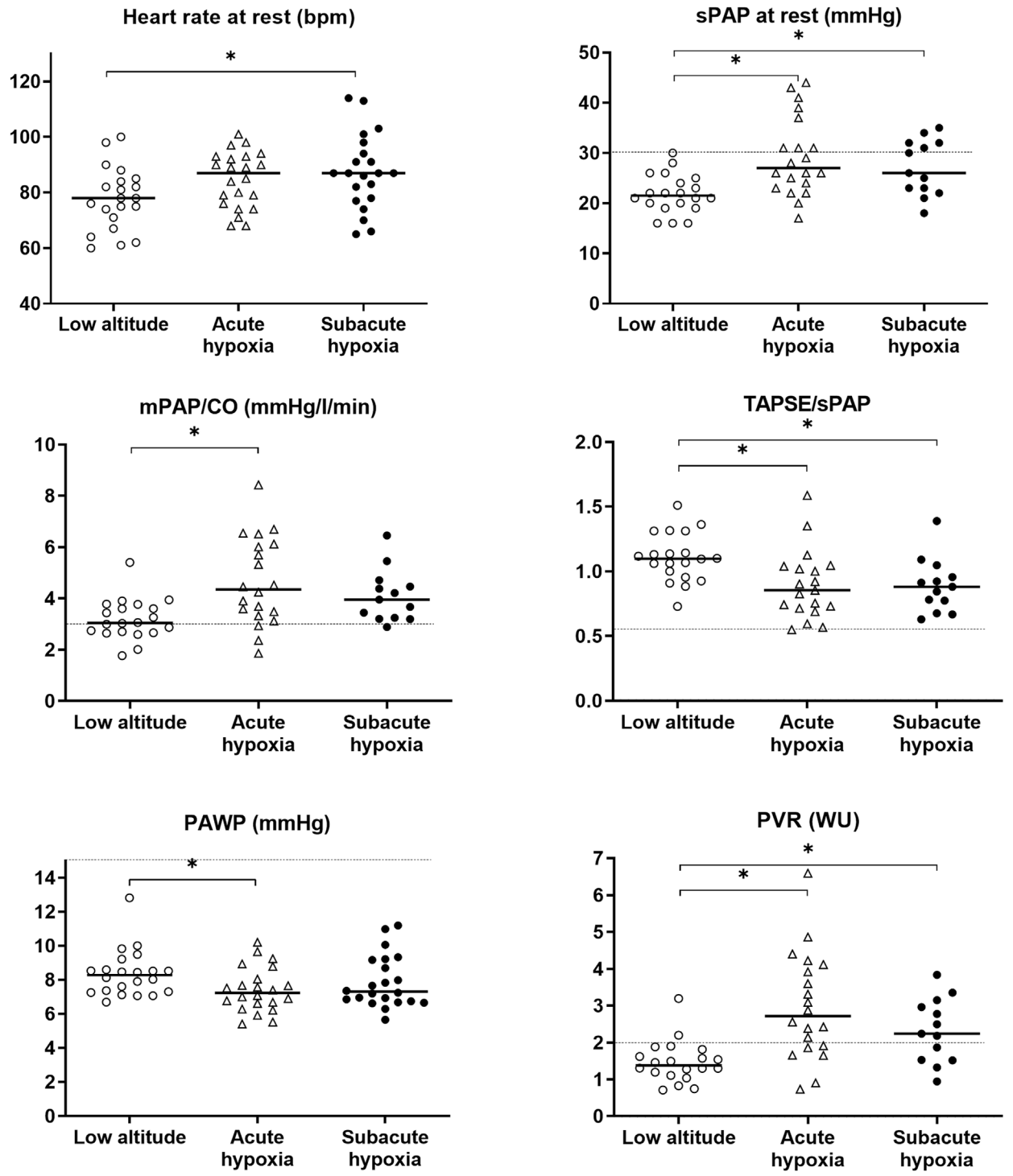

FIGURE 3
Grape plot of the mean pulmonary artery pressure (mPAP), the systolic PAP (sPAP), the total pulmonary resistance as the relationship of the cardiac output (mPAP/CO), the surrogate marker of right ventricular coupling (TAPSE/sPAP), the pulmonary arterial wedge pressure (PAWP), and the pulmonary vascular resistance (PVR). Empty circles display the individual values at baseline (low altitude), empty triangles indicate the values at high altitude 3,100 m (acute hypoxia), and full circles indicate the values at altitude with subacute exposure after 3 weeks. The dotted line shows the reference or cut-off values for healthy subjects under normobaric conditions (Humbert et al., 2022); the asterisks (*) mark the statistically significant differences.

baseline and even in 86% of subjects at acute high altitude measures, it was only present in 59% of subjects after 17 nights at the high altitude. The reason for the failing presence of the TRPG during subacute exposure to the high altitude is unclear but may be explained by hitherto unexplained acclimatization mechanisms. Echocardiography is associated with a potentially relevant intra- and interobserver

variability, which was reduced by using the same echocardiographers throughout the study and double-checking the measurements. Because no blood gas analysis was carried out, we were not able to determine arterial oxygen content and oxygen delivery.

Conclusion

In this first comprehensive assessment of the PAP, heart function, and EVLW upon acute and subacute exposure to a high altitude during asthma rehabilitation, we showed that in patients with mostly mild asthma, in accordance with findings in healthy subjects, PAP increases with altitude and remains elevated for up to 3 weeks, albeit with all changes being below pathological values proposed at a low altitude.

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 Ethics Committee of the National Center of Cardiology and Internal Medicine, Bishkek, Kyrgyzstan. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SS, PB, TS, KB, SU, and ML contributed to the conception and design of the study. SS, PB, PA, and SRS organized the database. SS, PB, and JM performed the statistical analysis. SS and PB wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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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.

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Effects of 3-week repeated cold water immersion on leukocyte counts and cardiovascular factors: an exploratory study

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Aim: This exploratory study aimed to investigate the effects of a 3-week repeated cold water immersion (CWI) intervention on leukocyte counts and cardiovascular factors (mean arterial pressure [MAP], heart rate [HR]) in healthy men.

Methods: A total of n = 12, non-cold-adapted men (age: 25.2 ± 4.0 years; height: 177.8 ± 5.6 cm; weight: 73.8 ± 6.5 kg) were randomly allocated to the CWI or control (CON) group. The CWI group underwent a 3-week repeated CWI intervention (12min at 7°C, 4x/week). The CON group did not receive any cold exposure or therapy. Total leukocyte numbers and proportions (neutrophils, basophils, eosinophils, monocytes, lymphocytes) and cardiovascular factors (MAP, HR) were assessed at baseline and after the 3-week intervention period.

Results: Total leukocyte count decreased in CWI (p = 0.027, 95% CI −2.35 to −0.20 × 10^3/μL) and CON (p = 0.043, 95% CI −2.75 to −0.50 × 10^3/μL). CWI showed a decrease in neutrophil number (p = 0.028, 95% CI −1.55 to −0.25 × 10^3/μL) and proportion (p = 0.046, 95% CI −6.42 to 0.56%). In contrast, CON showed no significant change (p > 0.05). No differences were found for other leukocyte subtypes in CWI or CON (all p > 0.05). MAP (p = 0.028, 95% CI −17 to −8 mmHg) and HR (p = 0.027, 95% CI −7 to −2 bpm) were reduced in CWI, whereas CON showed no change (p > 0.05).

Conclusion: The results suggest no relevant effects of 3-week repeated CWI on leukocyte counts in healthy men. Due to methodological limitations, the effects on the investigated cardiovascular factors remain unclear. Further studies with larger sample sizes are needed to examine the effects on immune function and cardiovascular health.

KEYWORDS

cold water immersion, cryotherapy, immune system, blood count, leukocytes, cardiovascular factors

1 Introduction

Cold water immersion (CWI) in the form of cold/ice bathing and cold water swimming has become increasingly popular due to its potential health benefits (Tipton et al., 2017; Manolis et al., 2019; Knechtle et al., 2020; Espeland et al., 2022). In non-cold-adapted humans, initial whole-body CWI at water temperatures <15°C has a stressful physiological impact, potentially leading to a so-called 'cold shock response'. This acute thermoregulatory reaction to cold exposure is manifested by hyperventilation, cutaneous vasoconstriction, increased muscle shivering, metabolic heat production, and the release of catecholamine and glucocorticoid hormones (Tipton, 1989; Castellani and Young, 2016; Eimonte et al., 2021a). Repeated CWI leads to adaptive processes that attenuate the initial physiological responses (Keatinge and Evans, 1961; Golden and Tipton, 1988; Castellani and Young, 2016). It has been proposed that short-term stress activates the cardiovascular, musculoskeletal, and neuroendocrine systems for fight-or-flight and, under certain circumstances, prepares the immune system for challenges (Dhabhar, 2009; Dhabhar, 2014). Building on this idea, frequent activation of short-term stress through repeated CWI may induce adaptive psychophysiological mechanisms that enhance immune protection, improve cardiovascular health and, in turn, prevent the occurrence of a wide variety of diseases or attenuate the adverse effects of surgical stress (Harper, 2012; Tipton et al., 2017; Manolis et al., 2019; Knechtle et al., 2020; Espeland et al., 2022).

An essential component of the immune system is the rapid recruitment of immune cells to specific sites of infection, wound, surgery or vaccination. The absolute numbers of circulating leukocytes and leukocyte proportions (i.e., percentage of each leukocyte subtype) provide information about the state of leukocyte distribution in the body and activation state of the immune system. Previous investigations indicate that acute short-term stress stimulates the leukocytes to exit the spleen, lung and marginated pool, as well as other organs, to enter the bloodstream (Dhabhar et al., 1995; Benschop et al., 1996; Dhabhar and McEwen, 1997; Dhabhar, 2009; Dhabhar et al., 2012). It has been consistently shown that short-term stress reactions triggered by a single CWI can rapidly alter the numbers and proportions of leukocytes (Janský et al., 1996a; Brazaitis et al., 2014; Eimonte et al., 2021a; Eimonte et al., 2021b). This modification seems to be dose-dependent (Eimonte et al., 2021a): While more extended CWI protocols (60min at 14°C (Janský et al., 1996a) or intermittent cooling with 120min maximum total immersion time at 13–14°C (Brazaitis et al., 2014; Eimonte et al., 2021b)) tended to increase the total leukocyte count, a shorter (10min at 14°C) CWI protocol produced no change (Eimonte et al., 2021a). In addition, studies reported an increase in the proportion of neutrophils (Brazaitis et al., 2014; Eimonte et al., 2021a; Eimonte et al., 2021b), a decrease in the proportion of lymphocytes (Brazaitis et al., 2014; Eimonte et al., 2021a; Eimonte et al., 2021b) and a reduced proportion of monocytes (Brazaitis et al., 2014; Eimonte et al., 2021b). Taken that the leukocyte counts have been demonstrated to fully recover to pre-exposure levels within 6–12 h after CWI, the observed changes are likely to reflect a re-distribution of leukocytes within different body compartments rather than a formation or destruction of these cells (Cox and Ford, 1982; Eimonte et al., 2021a; Eimonte et al., 2021b).

This raises the question of whether frequent CWI, repeated over several weeks, modifies the mobilisation and recruitment of specific leukocytes to enhance immune protection. To our knowledge, only one study has examined the effects of repeated CWI (60min at 14°C, 6-week intervention, 3x/week) on leukocyte counts. This study found a small increase in the monocyte numbers and the proportion of certain lymphocyte subpopulations, but no change in the total leukocyte count (Janský et al., 1996a). As this initial evidence is based on a within-subject design without a control group, the effects of repeated CWI on leukocyte counts are inconclusive.

Habitual winter-swimmers have been shown to have increased numbers of monocytes compared to inexperienced individuals. Since the winter-swimmers often practise ice-cold water swimming in combination with systemic heat (i.e., hot sauna), the observed differences in leukocyte levels suggest adaptive mechanisms in response to thermal stress in general (Dugué and Leppänen, 2000). Therefore, the effects of cold exposure through repeated CWI remain to be fully elucidated. In addition, several studies have pointed out the difficulty of separating the effects of swimming and/or exercise before, during or after CWI. Acute exercise and cold exposure are both complex physiological conditions that stimulate a stress response, and their combined effects may exceed the individual effects of either stimulus (Tipton et al., 2017; Eimonte et al., 2021a; Espeland et al., 2022). Accordingly, it is essential to explore the effects of a static repeated CWI intervention alone to avoid the confounding effect of exercise.

Among other cardiovascular parameters, elevated resting blood pressure and heart rate (HR) are recognised risk factors for the development of cardiovascular disease (Böhm et al., 2015; Liu et al., 2021). In particular, mean arterial pressure (MAP) predicts cardiovascular health and disease in young men (Sesso et al., 2000). Physiological responses during CWI, including blood pressure and HR, are well documented (Tipton, 1989; Janský et al., 1996b). In addition, cold adaptation through repeated CWI has been shown to alter blood pressure responses during CWI (Muza et al., 1988; Janský et al., 1996b). However, the overall effects of repeated CWI and cold adaptation on cardiovascular health are not clarified. Previous research indicates a beneficial effect of cold adaptation on lipoprotein parameters and antioxidative markers (Kralova Lesna et al., 2015). Nonetheless, investigations focusing on the effects of repeated CWI on resting MAP and HR remain to be completed.

Since research has mainly focused on physiological responses to initial CWI, further investigation is needed to examine the effects of repeated CWI on leukocyte counts and cardiovascular factors. Therefore, the aim of this study was to explore the effects of a 3-week repeated CWI intervention on total leukocyte numbers and proportions (neutrophils, basophils, eosinophils, monocytes, lymphocytes) and cardiovascular factors (MAP, HR) in healthy men. Based on previous studies (Janský et al., 1996a; Dugué and Leppänen, 2000), it was hypothesized that a 3-week repeated CWI intervention may increase the number of monocytes. Furthermore, it was hypothesized that a 3-week repeated CWI intervention could potentially reduce resting MAP and HR.

2 Methods

2.1 Participants

A total of n = 12 men (age: 25.2 ± 4.0 years; height: 177.8 ± 5.6 cm; weight: 73.8 ± 6.5 kg, estimated lower body fat percentage: 16.4 ± 3.5%) volunteered for this study. Participants were included if they were between the age of 18 and 60 years and engaged in recreational activity for 30–60 min, 2–3x/week. Furthermore, they were included if they were non-habituated to CWI (no prior experience with regular systemic or local cold exposure was reported). Participants were excluded if they were smokers, had a cold allergy, had a history of cardiovascular or respiratory disease, had any pain symptoms, or were taking medication. All participants were fully informed of the experimental protocol, the aims, risks and discomforts related to this study, before signing an informed consent form. The study was approved by the Swiss Ethical Committee of Zurich (Req-2021-00989) in accordance with the Declaration of Helsinki (ICH-GCP). The study was conducted during the winter season in the laboratory of the University of Applied Sciences and Arts of Southern Switzerland (RESlab, Landquart, Switzerland).

2.2 Study design

The study was based on a randomised controlled design. The participants were randomly assigned by drawing lots to the CWI group ($n = 6$) or the CON ($n = 6$) group. Participant's characteristics for each intervention group are displayed in Supplementary Table S1. Age, height, mass and estimated lower body fat percentage did not differ significantly between the intervention groups at baseline (BL) (all $p > 0.05$).

2.3 Experimental protocol

To our knowledge, there is currently no standardised repeated static CWI intervention protocol specifically designed for healthy individuals to induce meaningful physiological cold adaptation effects. In the CWI literature, cold water is generally defined as a temperature <15°C (Tipton et al., 1991). Post-exercise CWI protocols have demonstrated significant metabolic effects in non-cold habituated individuals when using temperatures between 5-13°C for durations of 10–24 min (Leeder et al., 2012; Hohenauer et al., 2015). Considering these findings, the water temperature and CWI duration were selected within this range. The CWI group underwent a 3-week repeated CWI intervention with CWI sessions 4x/week, on consecutive days. During the CWI, participants sat in a pool (168 cm × 168 cm) filled with cold water (12min at 7 ± 0.5°C) and submerged to the level of the sternum with their arms on the outside. Participants wore swimming trunks. The water temperature was constantly monitored with a multimeter device (Voltacraft MT52, Wollerau, Switzerland). Crushed ice was added if needed. After the immersion, the participants were patted dry and laid in a supine position. The CON group was not allowed to perform any cold exposure or therapy. All participants were instructed to maintain their regular daily habits during the 3-week intervention period, including their usual food intake and caffeine consumption. BL measurements were performed on the first measurement day of the 3-week intervention period, and follow-up (F/U) measurements were taken 2–3 days post-intervention. BL and F/U measurements were performed in the morning. Anthropometric characteristics at BL included height (GPM Stadiometer, Zurich, Switzerland), body mass, and lower body fat percentage estimation. Body mass and lower body fat percentage estimation were measured using a TANITA-TBF 611 scale (Tokyo, Japan). Estimate of lower body fat percentage was chosen because the participants were submerged without arms to sternum level, which mainly affects the lower body. Blood samples were collected, and cardiovascular factors were analysed at BL and F/U.

2.4 Leukocyte counts

Venous blood samples were collected from a cubital vein (K2E EDTA, 4000 µL, BD Vacutainer, Plymouth, UK). Samples were swirled, stored at room temperature and analysed for differential blood cell count using flowcytometry (Sysmex, Norderstedt, Germany) within 12 h after collection by the laboratory Dr. Risch (Labormedizinische Analytik FAMH, Buchs, Switzerland). The proportions of each leukocyte subtype (neutrophils, basophils, eosinophils, monocytes, lymphocytes) were calculated as a percentage of the total leukocyte count. The reference values for the hematologic parameters used were taken from a meta-analysis based on practice of a group of large hospitals and a literature review (Herklotz et al., 2006), which are used by the laboratory Dr. Risch (total leukocyte count: 3.9-10.2 10^3/µL; neutrophils: 1.5-7.7 10^3/µL, basophils: 0.0-0.2 10^3/µL; eosinophils: 0.02-0.5 10^3/µL; monocytes: 0.1-0.9 10^3/µL, lymphocytes: 1.1-4.5 10^3/µL, neutrophil proportion: 42–77%, eosinophil proportion: 2–4%, basophil proportion: <2.0%, monocyte proportion: 2.0–9.5%, lymphocyte proportion: 20–44%).

2.5 Cardiovascular factors

Resting MAP was assessed after 10min rest in the supine position using an automatic sphygmomanometer monitor (Beurer, BM77, Beurer GmbH, Ulm, Germany) from the left brachial artery. MAP was calculated according to the following formula: MAP = DBP+([SBP-DBP]/3), where SBP is systolic blood pressure and DBP is diastolic blood pressure (Grillo et al., 2021). HR was measured using a Polar watch (Polar, V800, Kempele, Finland) and a Bluetooth chest belt (Polar, H10, Kempele, Finland) at a time interval of 10min and averaged. The Polar V800 is a valid instrument for measuring RR intervals at rest (Giles et al., 2016). MAP and HR were assessed by the same investigator who was not blinded to assignment to the intervention groups. Reference values for resting MAP (70–100 mmHg) were approximated from the normal blood pressure (SBP: <120 mmHg SBP, and DBP: <80 mmHg) as recognized by the American Heart Association (Whelton et al., 2018). A mean reduction of minimally 5–10 mmHg for SBP, or 3–5 mmHg for DBP can be considered a clinically meaningful reduction in clinical trials (Kandzari et al., 2022). Accordingly, this study's potential reductions of MAP of this magnitude were considered meaningful. Resting HR was considered normal between 50–80 bpm, as approximated from reports of resting HR in healthy men (Ostchega et al., 2011; Quer et al., 2020).

TABLE 1 Leukocyte counts at baseline (BL) and after the intervention period (follow-up, F/U). Wilcoxon matched-pairs signed rank test results indicate the before-after differences for each intervention group.

	Unit	Group	Median		Median difference (F/U-BL)	BCa 95%CI		Z-value	*p*-value	Effect size
			BL	F/U		Lower	Upper			
Total leukocytes	count [10^3/µL]	CWI	6.15	4.75	−1.10	−2.35	−0.20	−2.207	0.027*	0.637
		CON	6.10	5.50	−0.80	−2.75	−0.50	−2.023	0.043*	0.584
Neutrophils	count [10^3/µL]	CWI	3.20	2.55	−0.65	−1.55	−0.25	−2.201	0.028*	0.635
		CON	3.30	2.65	−0.65	−2.90	0.05	−1.782	0.075	0.514
	proportion [%]	CWI	58.75	53.32	−2.77	−6.42	0.56	−1.992	0.046*	0.575
		CON	51.70	50.35	−3.67	−12.43	3.50	−1.153	0.249	0.332
Basophils	count [10^3/µL]	CWI	0.05	0.10	0.00	-	-	−1.000	0.317	0.289
		CON	0.00	0.00	0.00	-	-	0.000	1.000	0.000
	proportion [%]	CWI	0.50	1.45	0.34	0.00	1.38	−1.826	0.068	0.527
		CON	0.00	0.00	0.00	-	-	−1.000	0.317	0.289
Eosinophils	count [10^3/µL]	CWI	0.10	0.10	0.00	-	-	−0.447	0.655	0.129
		CON	0.15	0.10	−0.05	−0.10	0.00	−1.000	0.317	0.289
	proportion [%]	CWI	1.97	2.44	0.18	−0.71	3.04	−1.153	0.249	0.333
		CON	2.70	2.18	−0.41	−1.40	0.68	−0.524	0.600	0.151
Lymphocytes	count [10^3/µL]	CWI	2.20	1.70	−0.20	−0.70	0.10	−1.378	0.168	0.398
		CON	2.05	2.05	−0.10	−0.25	0.00	−0.921	0.357	0.266
	proportion [%]	CWI	30.58	33.58	1.38	−1.06	4.65	−1.153	0.249	0.334
		CON	33.79	37.38	3.31	0.08	5.87	−1.363	0.173	0.393
Monocytes	count [10^3/µL]	CWI	0.55	0.40	−0.10	-	-	−1.667	0.096	0.481
		CON	0.60	0.60	−0.05	−0.10	0.00	−1.134	0.257	0.327
	proportion [%]	CWI	8.63	9.24	0.03	−2.19	2.03	−0.314	0.753	0.09
		CON	10.65	10.14	1.17	0.14	1.82	−1.153	0.249	0.333

BL = baseline, F/U = follow-up, BCa 95% CI = Bias-corrected and accelerated 95% confidence interval, CWI = cold water immersion, CON = control, *$p < .05$, **$p < .01$, ***$p < .001$ (asymp. sign. 2-tailed).

2.6 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (29, IBM Corp). Demographic data were reported descriptively (mean ± SD). Independent t-tests were used to observe between-group differences at BL. Given the small sample size (n = 12), a non-parametric Wilcoxon matched-pairs signed rank test was used to compare the before-and-after differences for each variable. Effect sizes (Cohen's d) were calculated using the formula $r = \frac{|Z|}{\sqrt{n}}$ (Rosenthal, 1984) and defined as follows: small: ≤0.2; medium: ≤5; large: ≤0.6 (Cohen, 1998; Fritz et al., 2011). In all analyses, statistical significance was set at $p < 0.05$. Figures were created using Prism (9, Graphpad, Software Inc.). Post hoc power analysis for Wilcoxon signed-rank test (matched pairs, 2-tailed, normal parent distribution, $\alpha = 0.05$, $n = 12$) was performed for each outcome using G*Power (version 3.1.9.6, Franz Faul, Germany).

3 Results

3.1 Leukocyte counts

Total leukocyte count decreased significantly in CWI ($p = 0.027$, r = 0.637,1-ß = 0.49) with a median difference of −1.10 × 10^3/µL (95%CI −2.35 to −0.20), and CON ($p = 0.043$, r = 0.584, 1-ß = 0.43) with a median difference of −0.8 × 10^3/µL (95%CI −2.75 to −0.50). Leukocyte differential count revealed a significant decrease in the number of neutrophils in CWI ($p = 0.028$, r = 0.635, 1-ß = 0.50) with a median difference of −0.65 × 10^3/µL (95%CI −1.55 to −0.25). At the same time, no change was observed in CON ($p = 0.075$, r = 0.514, 1-ß = 0.35). The neutrophil proportion was reduced significantly in CWI ($p = 0.046$, r = 0.575, 1-ß = 0.42) with a median difference of −2.77% (95%CI −6.42 to 0.56). In contrast, CON did not show a significant change ($p = 0.249$, r = 0.332, 1-ß = 0.18). There were no significant differences in the numbers or proportions of basophils, eosinophils, monocytes or lymphocytes in CWI or CON (all $p >$

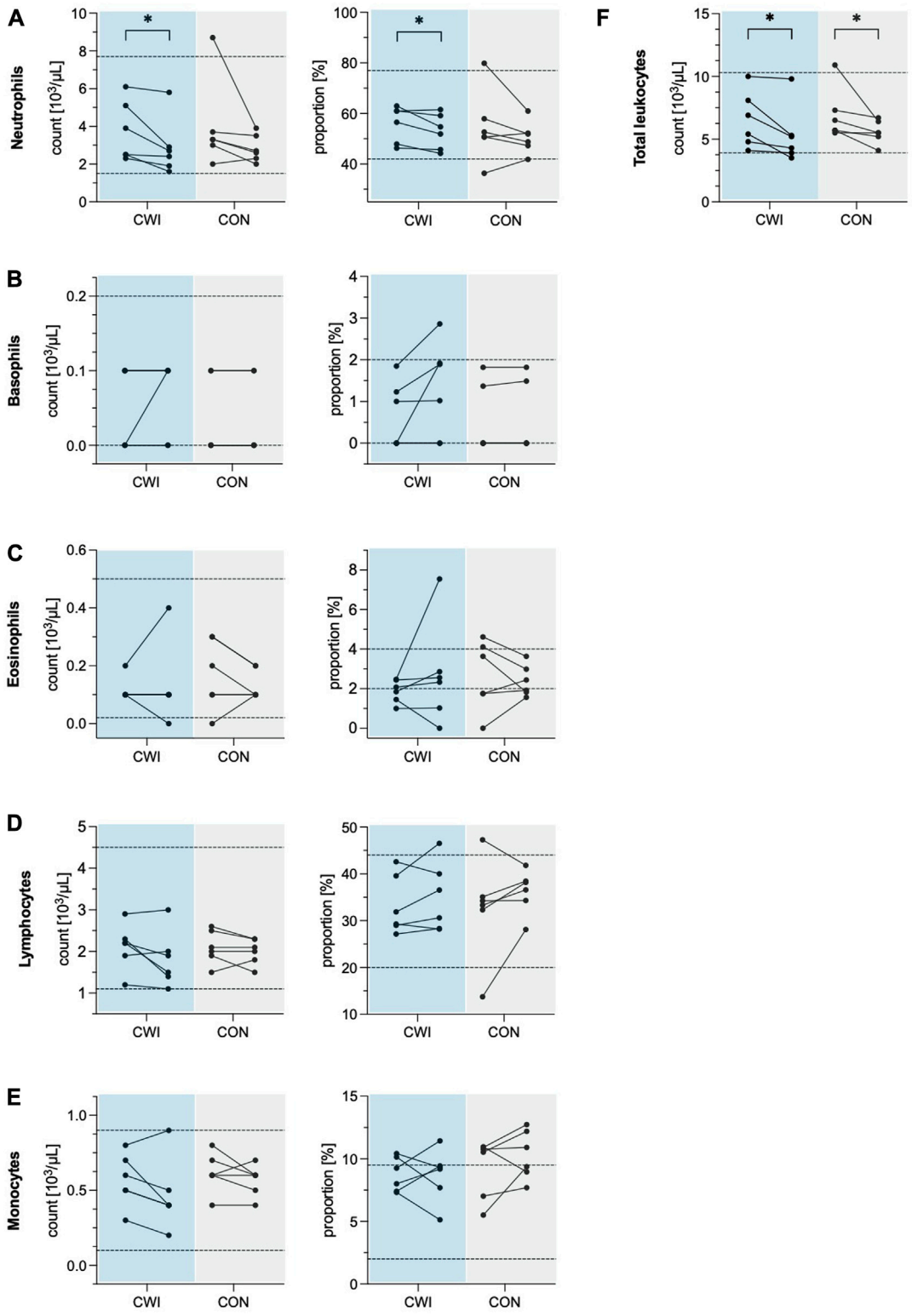

FIGURE 1
Leukocyte numbers and proportions of the cold water immersion (CWI) and control group (CON), at baseline (BL, left) and after the intervention (follow-up, F/U, right). Solid lines connect individual participants' values. Dashed lines represent the upper and lower limits of the reference values for adults (18–65 years). **(A)** Neutrophils. **(B)** Basophils. **(C)** Eosinophils. **(D)** Lymphocytes. **(E)** Monocytes. **(F)** Total leukocytes. *$p < .05$, **$p < .01$, ***$p < .001$. N = 12.

TABLE 2 Cardiovascular factors at baseline (BL) and after the intervention period (follow-up, F/U). Wilcoxon matched-pairs signed rank test results indicate the before-after differences for each intervention group.

	Group	Median		Median difference (F/U-BL)	BCa 95%CI		Z-value	*p*-value	Effect size
		BL	F/U		Lower	Upper			
MAP [mmHg]	CWI	109	98	−12	−17	−8	−2.201	0.028*	0.635
	CON	101	96	−5	−9	9	−0.211	0.833	0.061
HR [bpm]	CWI	68	66	−4	−7	−2	−2.207	0.027*	0.637
	CON	62	63	1	−16	10	−0.314	0.753	0.091

BL = baseline, F/U = follow-up, BCa 95% CI = Bias-corrected and accelerated 95% confidence interval, CWI = cold water immersion, CON = control, *$p < .05$, **$p < .01$, ***$p < .001$ (asymp. sign. 2-tailed).

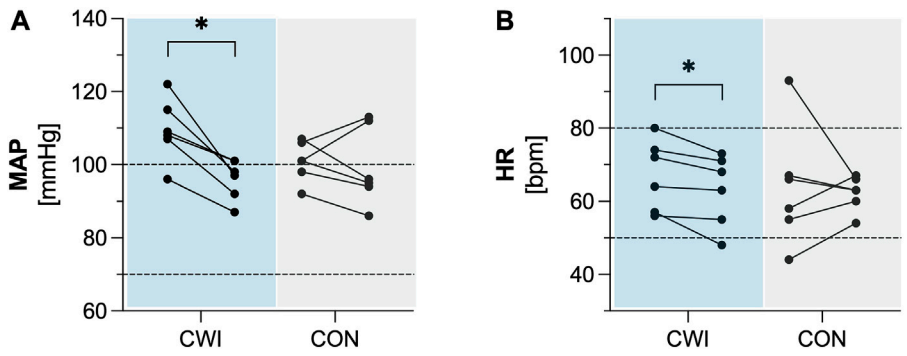

FIGURE 2
Cardiovascular factors **(A)** Mean arterial pressure (MAP). **(B)** Heart rate (HR) of the cold water immersion (CWI) and control group (CON) at baseline (BL, left) and after the intervention (follow-up, F/U, right). Solid lines connect individual participants' values. Dashed lines represent the upper and lower limits of reference values for adults. *$p < .05$, **$p < .01$, ***$p < .001$. N = 12.

0.05). Complete results of the leukocyte counts are displayed in Table 1. Participants' values are shown in Figure 1.

3.2 Cardiovascular factors

CWI showed a significant decrease in MAP ($p = 0.028$, r = 0.635, 1-ß = 0.50) with a median difference of −12 mmHg (95%CI −17 to −8), as well as in HR ($p = 0.027$, r = 0.637, 1-ß = 0.50) with a median difference of −4 bpm (95%CI −7 to −2). In contrast, CON did not show a significant change in MAP ($p = 0.833$, r = 0.061, 1-ß = 0.06) or HR ($p = 0.753$, r = 0.091, 1-ß = 0.06)). Complete results of the cardiovascular factors are available in Table 2. Participants' values are shown in Figure 2.

4 Discussion

The aim of this study was to investigate the effects of repeated CWI on leukocyte counts and cardiovascular factors. Blood samples from healthy men were assessed, and cardiovascular factors were assessed at BL and after a 3-week repeated CWI intervention compared to a CON group. The main findings of this exploratory study suggest that a 3-week repeated CWI intervention has no relevant effects on leukocyte counts.

The current study revealed a minimal reduction in total leukocyte counts in both the CWI and CON groups, suggesting that repeated CWI did not result in relevant changes in the total number of circulating leukocytes. This finding is consistent with previous research by Janský et al. who also showed no changes in the total leukocyte count after a 6-week repeated CWI intervention (Janský et al., 1996a). In the same study, Janský et al. reported a slight increase in monocyte numbers (Janský et al., 1996a) and Dugué et al. found that winter-swimmers exposed to thermal stress had higher numbers of monocytes compared to non-habituated individuals (Dugué and Leppänen, 2000). Contrary to these observations, a 3-week repeated CWI intervention in this present study had no significant effect on monocyte numbers but only resulted in a minimal decrease in neutrophil numbers (median difference: $-0.65 \times 10^3/\mu L$) and proportion (median difference: −2.77%). Considering that a mean peripheral neutrophil number of 4.4 (4.3–4.9) $10^3/\mu L$ is considered normal for healthy adults (Zini et al., 2011) and taken that neutrophil counts show dynamic fluctuations (Scheiermann et al., 2015), the observed reductions in neutrophils in CWI do not seem relevant.

Research has predominantly focused on the immediate physiological responses to a single CWI (Brazaitis et al., 2014; Eimonte et al., 2021a; Eimonte et al., 2021b). It has been consistently shown that a single CWI triggers short-term

adaptive physiological mechanisms involving the re-distribution of immune cells rather than the formation or destruction of these cells (Cox and Ford, 1982; Eimonte et al., 2021a; Eimonte et al., 2021b). According to Dhabhar et al. (2014), short-term stress is defined as lasting minutes to hours, and chronic stress is persistent for hours each day for weeks or months (Dhabhar and McEwen, 1997; Dhabhar, 2014). Short-term stress can stimulate adaptive physiological responses that enhance immune protection by modulating the innate/primary and adaptive/secondary immune responses, including changes in immune cell trafficking, maturation and function. However, long-term or chronic stress is considered harmful because it suppresses or dysregulates the innate and adaptive immune responses (Dhabhar, 2014). Based on this concept, it has been proposed that regular activation of the short-term stress response by repeated CWI could mediate adaptive physiological mechanisms that enhance immune protection (Harper, 2012; Tipton et al., 2017; Manolis et al., 2019; Knechtle et al., 2020; Espeland et al., 2022). In the present study, it was assumed that short-term re-distribution of immune cells occurred 6–12 h after each CWI, as it was shown previously (Eimonte et al., 2021a; Eimonte et al., 2021b). However, since there were no relevant changes following the 3-week intervention period, repeated CWI does not appear to alter the numbers or proportions of leukocytes.

A critical issue to consider is whether the repeated CWI protocol used in this study was adequate to stimulate potential changes in leukocyte counts. In the study of Janský et al., it was concluded that the cold stimulus (60min at 14°C, 6-week intervention, 3x/week) may not have been strong enough to induce significant changes (Janský et al., 1996a). However, there is conflicting evidence to support this interpretation. It is reasonable to assume that the 3-week repeated CWI protocol used in the present study (12min at 7°C, 4x/week) was appropriate for two reasons: 1) Given that the participants were non-cold adapted, the frequency and the intensity of the cold stimulus were relatively high; 2) The participants experienced several minutes of sustained shivering after CWI. Therefore, it could even be speculated whether repeated CWI protocols could potentially induce chronic stress as defined above (Dhabhar and McEwen, 1997; Dhabhar, 2014). Nonetheless, responses to CWI vary widely between individuals, and an optimal dose-response relationship for non-cold-adapted humans has not yet been defined (Tipton et al., 2017). Although changes in mature leukocyte mobilisation may occur within a few days (Scheiermann et al., 2015), meaningful adaptations in leukocyte mobilisation and recruitment associated with repeated CWI may take longer than 3 weeks.

To our knowledge, this is the first study to explore the effects of repeated CWI on resting MAP and HR. The results of this study showed a significant reduction in MAP and HR for CWI but not for CON. Even though the reduction of MAP (median difference: −12 mmHg) found in CWI might be considered clinically meaningful (Kandzari et al., 2022), this finding must be interpreted with caution given the following considerations. Resting MAP was generally elevated in all participants, with the BL measurements being exceptionally high in the CWI group (median: 109 mmHg). Participants in the CWI group were measured on the first day of the intervention period, presumably contributing to the elevated blood pressure due to nervousness associated with their first CWI session. As the CWI group became more familiar and accustomed to the experimental protocol, their blood pressure may have decreased. Therefore, this reduction cannot be directly related to the repeated CWI. Moreover, in this study blood pressure was only measured once for each time point. According to blood pressure measurement guidelines for adults, resting blood pressure levels should be estimated based on the mean of ≥2 assessments on ≥2 occasions (Whelton et al., 2018). Consequently, due to the methodological limitations in this exploratory study, the effects of repeated CWI on resting MAP and HR remain inconclusive and require verification using adjusted measurement protocols.

The observations in this study suggest no relevant effect of repeated CWI on leukocyte counts. Due to the preliminary and exploratory nature of this study, the conclusions are provided considering several limitations. 1) A *post hoc* power analysis revealed that the study was underpowered to reliably detect the effects of interest. It is therefore essential to validate the findings with a larger sample size. 2) Since this study focused solely on males, the absence of females in this sample may restrict the generalisability of the findings. Future research should examine the effects in both males and females to provide a more comprehensive understanding of the physiological responses to repeated CWI. 3) Participants in the CON group were free-living, making it impossible to separate the physiological effects of the hydrostatic pressure exerted from the water itself (Wilcock et al., 2006). Therefore, future research could consider using a thermoneutral bath to test the specific contribution of exposure to cold water. 4) Even though leukocyte counts provide a valuable insight into immune system function, their clinical importance in the context of CWI is not apparent (Tipton et al., 2017; Knechtle et al., 2020). The numbers and proportions of circulating leukocytes vary and reflect the current need for specific leukocyte subtypes at a given time point (Scheiermann et al., 2015). Consequently, leukocyte counts do not provide a complete picture of the immune function and should not be used as a sole marker in future studies.

To better understand cold adaptation responses and potential immune stimulation by repeated CWI over time, it is recommended that future investigations include measurements of acute physiological responses after each CWI session. This would provide insight into the dynamic changes in leukocyte counts (Scheiermann et al., 2015). There is evidence that cold-adapted humans may be more resistant to certain illnesses and infections (Kormanovski et al., 2010; Collier et al., 2021). Although this information is based on participants' self-reports of illness, infection or general wellbeing, such additional information could be included in similar studies.

Various CWI protocols have been used over the past decades. The magnitude of physiological responses to CWI depends on the protocol (i.e., water temperature, duration, time points of measurements) and participant characteristics (i.e., age, body fat percentage, degree of cold acclimation, training status, health style, social interaction, mindset) (Tipton et al., 2017; Espeland et al., 2022). In order to investigate physiological responses to CWI more reliably, it is necessary to establish standardised CWI protocols and

to account for confounding variables. Finally, this study used a static CWI protocol to avoid the confounding effects of exercise. Nevertheless, investigating the synergistic effects of CWI and exercise, particularly in the context of cold water swimming, remains an important research area (Harper, 2012; Tipton et al., 2017; Manolis et al., 2019; Knechtle et al., 2020; Espeland et al., 2022).

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 studies involving humans were approved by the Swiss Ethical Committee of Zurich. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

EH designed and coordinated the study, performed the experiment, discussed the data and reviewed the manuscript. NV performed data analysis, discussed the data and wrote the manuscript. RC performed the experiment and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1197585/full#supplementary-material

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Differential impact of heat and hypoxia on dynamic oxygen uptake and deoxyhemoglobin parameters during incremental exhaustive exercise

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Purpose: This study aims to explore the relationship between the dynamic changes in oxygen uptake ($\dot{V}O_2$) and deoxyhemoglobin (HHb) and peripheral fatigue in athletes during incremental exhaustive exercise under different environmental conditions, including high temperature and humidity environment, hypoxic environment, and normal conditions.

Methods: 12 male modern pentathlon athletes were recruited and performed incremental exhaustive exercise in three different environments: normal condition (23°C, 45%RH, FiO_2 = 21.0%, CON), high temperature and humidity environment (35°C, 70%RH, FiO_2 = 21.0%, HOT), and hypoxic environment (23°C, 45%RH, FiO_2 = 15.6%, HYP). Gas metabolism data of the athletes were collected, and muscle oxygen saturation (SmO_2) and total hemoglobin content in the vastus lateralis muscles (VL) were measured to calculate the deoxyhemoglobin content. Linear and nonlinear function models were used to fit the characteristic parameters of $\dot{V}O_2$ and HHb changes.

Results: The results showed that compared to the CON, $\dot{V}O_2$, $\dot{V}CO_2$, and exercise time were decreased in the HOT and HYP ($p < 0.05$). $\Delta E_{\dot{V}O2}$ and OUES were reduced in the HOT and HYP compared to the CON ($p < 0.05$). The Gas exchange threshold in the CON corresponded to higher $\dot{V}O_2$ than in the HYP and HOT ($p < 0.05$). $\Delta E_{\dot{V}O2\text{-}1}$ was reduced in the HOT compared to the HYP ($p < 0.05$). ΔE_{HHb} was higher in the HOT compared to the CON ($p < 0.05$). $\Delta E_{HHb\text{-}1}$ was increased in the HYP compared to the CON ($p < 0.05$). There was a negative correlation between ΔE_{HHb} and corresponding $\dot{V}O_{2\,max}$ in the HOT ($r = -0.655$, $p < 0.05$), and a negative correlation between $\Delta E_{HHb\text{-}1}$ and corresponding $\dot{V}O_{2\,max}$ in the HYP ($r = -0.606$, $p < 0.05$).

Conclusion: Incremental exhaustive exercise in hypoxic environment and high temperature and humidity environments inhibits gas exchange and oxygen supply to skeletal muscle tissue in athletes. For athletes, the accelerated deoxygenation response of skeletal muscles during incremental exhaustive exercise in high temperature and humidity environments, as well as the excessive

deoxygenation response before BP of deoxyhemoglobin in hypoxic environment, may be contributing factors to peripheral fatigue under different environmental conditions.

KEYWORDS

high temperature and humidity environment, hypoxic environment, incremental exhaustive exercise, deoxyhemoglobin dynamics, oxygen uptake dynamics

1 Introduction

Competitive sports athletes frequently encounter various challenging training and competition environments, such as high temperature, high humidity, and hypoxic conditions. However, when the human body operates in these environments, it often experiences a decline in physical performance and an accelerated onset of exercise fatigue, among other negative effects (Osawa et al., 2017; Jung et al., 2021).

Hypoxia exposure, in comparison to normal environmental conditions, can restrict respiratory function and affect gas exchange in the human body. It can lead to a decrease in arterial oxygen saturation (SpO_2), capillary oxygen partial pressure, and ultimately limit oxygen supply to peripheral tissues (Twomey et al., 2017). Numerous studies have consistently demonstrated that hypoxia exposure leads to a decrease in exercise time, maximal oxygen uptake ($\dot{V}O_{2\,max}$), and peak output power among athletes during incremental exhaustive exercise (Lawler et al., 1988; González-Alonso and Calbet, 2003; Subudhi et al., 2007). Furthermore, it induces a leftward shift in the Gas Exchange Threshold (GET) and Respiratory Compensation Point (RCP) (Zerbini et al., 2013; Bowen et al., 2016). Furthermore, research focusing on the impact of hypoxia exposure have demonstrated that during incremental exhaustive exercise, break point (BP) of active muscle deoxy-hemoglobin (HHb) undergoes a leftward shift under hypoxic conditions (Azevedo et al., 2020a). Additionally, when athletes engage in physical activities in high-temperature and humid environments, heat exposure can contribute to the acceleration of peripheral fatigue by influencing gas exchange and oxygen transport in skeletal muscle. The challenging conditions in such environments often lead to disruptions in heat dissipation, elevated core temperature (Tc), excessive dehydration, and impaired functional regulation of the cardiovascular, central nervous, and musculoskeletal systems (Périard et al., 2013; Yamaguchi et al., 2021). Consequently, these factors can restrict the capacity for oxygen transport and utilization in skeletal muscle tissue. As a result, athletes experience a leftward shift in the GET, along with a decrease in $\dot{V}O_{2\,max}$ and exercise time, when performing incremental exhaustive exercise under high-temperature and humid conditions (Tatterson et al., 2000; Sawka et al., 2011).

The dynamic characteristics of Oxygen Uptake ($\dot{V}O_2$) and HHb during incremental exercise, particularly in special environments such as high temperature and humidity, and hypoxic environments, have not been extensively studied. Previous research has shown that $\dot{V}O_2$ exhibits a linear increase with exercise intensity, while HHb in skeletal muscle demonstrates a bilinear increase with exercise load (Vieth, 1989; Spencer et al., 2012). However, the specific differences in dynamic parameters between $\dot{V}O_2$ and HHb during incremental exhaustive exercise under these special environments remain unclear. Exploring the dynamic changes of HHb in body gas exchange and skeletal muscle microcirculation during exercise can provide valuable insights into the mechanisms underlying premature peripheral fatigue in these environments.

Therefore, it is crucial to investigate the characteristics of oxygen supply in peripheral tissues and body gas exchange among athletes in high temperature, high humidity, and hypoxic environments. In our study, we utilized Near-Infrared Spectroscopy (NIRS) as a non-invasive method to assess oxygen levels in micro vessels. By comparing the characteristic parameters of oxygen uptake kinetics and deoxyhemoglobin kinetics in athletes during incremental exhaustive exercise under high temperature and high humidity, hypoxic, and normal environments, we aim to explore the influence of these different special environments on the dynamic changes of $\dot{V}O_2$ and Muscle Oxygen Saturation (SmO_2) during exercise.

2 Materials and methods

2.1 Subject

Twelve male modern pentathletes (age = 17.91 ± 2.94 years; height = 1.81 ± 0.06 m; body mass = 70.95 ± 8.38 kg; body mass index = 21.69 ± 1.83 kg/m^2; training years = 5.33 ± 2.92 years) participated in the study, with no dropouts recorded. Prior to the study, the participants were provided with detailed information about the experimental procedures and the purpose of the study. They were also informed about the potential risks and benefits associated with their participation. Informed consent forms were provided to the participants, and they were given sufficient time to review and understand the information before signing the consent forms. For athletes under the age of 18, approval was sought from the athlete's legal guardian or close relative. The study specifically involved athletes from the modern pentathlon team of Shanghai Chongming Sports Training Base, who voluntarily agreed to participate in the research. Confidentiality and anonymity of the participants' personal information were ensured throughout the study.

2.2 Experimental design

2.2.1 Environmental parameters

This study was conducted in the Special Environment Laboratory at the Shanghai Institute of Sports Science. Three different exercise environments were set up: high temperature and humidity with normoxia (HOT), normal temperature and humidity with hypoxia (HYP), and normal temperature and humidity with normoxia (CON). The environmental parameters were as follows: High temperature and humidity with normoxia:

35°C, 70% relative humidity (RH), and fractional inspired oxygen concentration (FiO_2) = 21.0%. Normal temperature and humidity with hypoxia: 23°C, 45% RH, and FiO_2 = 15.3%. Normal temperature and humidity with normoxia: 23°C, 45% RH, and FiO_2 = 21.0%.

2.2.2 Experimental procedure

The athletes performed incremental exercise tests in the three different environments. The CPET test was administered by researchers following a standardized procedure tailored to the characteristics of modern pentathlon athletes, conducted on a treadmill. Prior to the experiment, athletes engaged in a 10-min standardized warm-up and stretching routine, donning necessary equipment such as breathing masks and heart rate monitors before commencing the formal test.

The initial load was set at 8 km/h with 0% incline. Subsequently, the speed was increased by 1 km/h every 1 min while maintaining the incline. When the treadmill speed reached 18 km/h, the speed was no longer increased, but instead, the incline was increased by 1% every 1 min. There were no breaks between the levels. Gas metabolism, heart rate, and other relevant data were continuously collected without intervals between levels. The test termination criteria included various indicators such as dyspnea, cyanosis, dizziness, tinnitus, nausea, chest pain, extreme fatigue, painful expression, pale face, and body shaking. Additionally, the test could be terminated if the participant's heart rate reaches the expected maximum heart rate, if the subject requests to stop the test, if the Borg Rating of Perceived Exertion (RPE) reaches or exceeds 17, or if the participant is unable to maintain the required speed. In any of these situations, the test was immediately stopped to ensure the safety and wellbeing of the participants.

We implemented a randomized crossover design, conducting participant tests in diverse environments at the same time of day on days 1, 3, and 5, with a 48-h interval between each session. Before each test, the athletes' physiological parameters were checked to ensure their good health and normal physical function.

2.2.3 Cardiopulmonary responses

Gas metabolism data during the incremental exercise tests were collected using the COSMED gas analyzer (COSMED Quark PFT ergo, OMNIA CPET, Italy). The following parameters were obtained: $\dot{V}O_2$, carbon dioxide production ($\dot{V}CO_2$), respiratory exchange ratio (RER), respiratory rate (RR), tidal volume ($\dot{V}T$), and minute ventilation ($\dot{V}E$). Heart rate (HR) changes during exercise were recorded using a heart rate monitor (Polar RS800CX, Polar Electro, Kempele, Finland), and Tc was recorded using a core temperature capsule (e-Celsius®, BMedical Pty LTb, BodyCap, Australia). Immediately after exercise, SpO_2 was measured using a finger pulse oximeter (YX302, Yuwell Medical, China), and fingertip blood samples were collected to assess the maximal blood lactate (Bla) concentration.

2.2.4 Tissue oxygenation

The NIRS signal in human tissues predominantly originates from the absorption of light by hemoglobin (Hb) in arterioles, capillaries, and venules. In muscle tissue, myoglobin (Mb) contributes approximately 10% to the NIRS light absorption signal (Seiyama et al., 1988; Chance et al., 1992). However, due to the overlap of Mb and Hb absorption spectra, they are indistinguishable in NIR spectra. Near-infrared spectral signals primarily indicate the availability of oxygen in tissue microcirculation (Boushel et al., 2001). Furthermore, serving as a monitoring instrument, the MOXY (Moxy Muscle Oxygen Sensor, Hutchinson, Minnesota, United States) has demonstrated reliability in monitoring SmO2 and THb (Crum et al., 2017). Muscle oxygen saturation data were collected using the MOXY near-infrared spectroscopy (NIRS) device. The device utilizes NIRS to measure the concentrations of oxyhemoglobin (O_2Hb), deoxyhemoglobin (HHb), and total hemoglobin (THb) in the muscle tissue during incremental exercise tests and recovery after exercise. The data were sampled at a frequency of 1 Hz. The NIRS probe was placed on the skin surface above the vastus lateralis muscles (VL) belly of the dominant lower limb and securely covered to prevent light interference (Yamaguchi et al., 2021). SmO_2 was calculated based on a modified form of the Beer-Lambert law (Saitoh et al., 2010).

2.3 Data analyses

2.3.1 Oxygen uptake kinetics

In order to analyze the data in our study, we applied a smoothing technique to $\dot{V}O_2$, VE, RF and VT for a 10-s interval during the incremental exhaustive exercise. Given the variations in athletes' exercise duration across the three environments, we calculated the mean value of $\dot{V}O_2$ during the last 30 s of each stage load within the initial 10 min of the exercise test for further analysis. Additionally, average values of VT, RF, and VE were calculated every 30 s during exercise to evaluate the influence of environmental factors before reaching GET. Recognizing variations in individual GET, we specifically analyzed data collected before 270s. To further analyze the data, we employed Origin software (OriginLab, 2018 Pro, United States) to fit the entire motion process data using two formulas. The first Formula (1) represents a linear fit between $\dot{V}O_2$ and time during the exercise, and the second Formula (2) represents a logarithmic function fit between $\dot{V}O_2$ and VE during the exercise. This fitting process allows us to examine the relationship between $\dot{V}O_2$ and time, as well as $\dot{V}O_2$ and VE throughout the exercise protocol.

To determine the GET during the exercise, we utilized the V-slope method (Beaver et al., 1986). The inflection point of the VE/ $\dot{V}CO_2$ relationship was employed to determine RCP (Whipp et al., 1989). The GET served as a pivotal point in the exercise protocol, dividing the incremental exhaustive exercise into two stages for bilinear fitting. The bilinear fitting was performed using Formula (1) to analyze the relationship between $\dot{V}O_2$ and time. Model parameter estimation was carried out using linear least squares regression analysis. The slopes of the calculated linear fitting equations were designated as $\triangle E_{\dot{V}O2\text{-}1}$ and $\triangle E_{\dot{V}O2\text{-}2}$, representing the two stages of the exercise from the start to GET and from GET to exhaustion, respectively.

$$\dot{V}O_{2(t)} = \dot{V}O_{2baseline} + \triangle E_{VO2} \cdot t \quad (1)$$

$$\dot{V}O_{2(VE)} = \dot{V}O_{2baseline} + OUES \cdot log\,10(VE) \quad (2)$$

Where $\dot{V}O_{2(t)}$ is the oxygen uptake at time t, $\dot{V}O_{2baseline}$ is the oxygen uptake at baseline, $\triangle E_{\dot{V}O2}$ is the linear fitting slope, $\dot{V}O_{2(VE)}$

is the oxygen uptake corresponding to VE. OUES is the Oxygen Uptake efficiency Slope, that is the rate of change of VE/$\dot{V}O_2$.

2.3.2 Deoxyhemoglobin kinetics

In the incremental exhaustive exercise, the NIRS measurements of SmO_2, [HHb], and [THb] were smoothed using a 10-s interval. To account for variations in athletes' exercise time in different environments, the mean values of SmO_2, [HHb], and [THb] were calculated for the last 30 s of each stage load during the initial 10 min of the exercise test.

The software Origin was utilized for analyzing the dynamic changes of [HHb] measured by NIRS during the exercise test and to calculate the dynamic parameters of [HHb] over the course of exercise. Formula (3) was employed to perform a linear fit between [HHb] and time during exercise. Additionally, the exercise duration was divided into two sections based on the BP, namely, from the start of exercise to BP and from BP to the point of exhaustion. Bilinear fitting using Formula (3) was applied to these two sections. The model parameters were estimated using linear least squares regression analysis, and the slopes of the linear fitting equations were represented as $\triangle E_{HHb\text{-}1}$ and $\triangle E_{HHb\text{-}2}$, respectively (Spencer et al., 2012).

To assess the dynamic relationship between oxygen uptake and oxygen utilization during exercise, $\triangle$[HHb]/$\triangle\dot{V}O_2$ was normalized for both time and amplitude. The baseline value was assigned 0%, while the steady-state value was assigned 100%. The standardized $\dot{V}O_2$ was shifted left by 20 s to align with the [HHb] (Murias et al., 2014), after which the ratio of the two was calculated to obtain the $\triangle$[HHb]/$\triangle\dot{V}O_2$ curve (Boone et al., 2015).

$$[HHb]_{(t)} = [HHb]_{baseline} + \triangle E_{HHb} \cdot t \quad (3)$$

Where $[HHb]_{(t)}$ is the deoxyhemoglobin value at time t, $[HHb]_{baseline}$ is the baseline deoxy hemoglobin value, and $\triangle E_{HHb}$ is the linear fitting slope.

2.4 Statistical analysis

The statistical analysis of the experimental data was conducted using SPSS 21.0 software (IBM SPSS Statistics 21, IBM Cooperation, Chicago, IL). The data were presented as mean ± standard deviation (Mean ± SD). For normally distributed data with homogeneous variance, the parameter test was chosen. ANOVA with Repeated Measures was used to analyze the experimental data, and the Bonferroni method was employed for *post hoc* comparisons between groups to identify any significant differences. If the data did not meet the assumptions of normal distribution or homogeneity of variance, non-parametric tests were used instead. The goodness of fit of the regression model coefficients was evaluated using regression analysis and the Coefficient of Determination (R^2). Additionally, the Pearson correlation coefficient (r) was utilized to analyze the correlation between $\triangle E_{HHb\text{-}1}$, $\triangle E_{HHb}$, and $\dot{V}O_{2\,max}$ during exercise under various environmental conditions. The confidence interval was set at 95%, and the significance level was $\alpha = 0.05$.

3 Results

3.1 Gas metabolism

The results of the study showed that compared to the CON, athletes in the HOT and HYP exhibited reductions in $\dot{V}O_2$ at exhaustion ($F(2,22) = 6.832$, $p = 0.005$, $\eta_p^2 = 0.383$, CON vs. HOT $p = 0.012$, 95%CI [149.652, 397.937]; CON vs. HYP $p = 0.038$, 95% CI [-47.983, 2001.856]), as well as relative $\dot{V}O_2$ ($F(2, 22) = 17.161$, $p < 0.001$, $\eta_p^2 = 0.609$, CON vs. HOT $p = 0.012$, 95%CI [1.231, 20.459]; CON vs. HYP $p = 0.002$ [95%CI 4.282, 23.423]; HOT vs. HYP $p = 0.012$ 95%CI [0.358, 5.657]). They also showed decreases in $\dot{V}CO_2$ ($F(2, 22) = 6.288$, $p = 0.007$, $\eta_p^2 = 0.364$, CON vs. HOT $p = 0.058$, 95%CI [-160.213, 2024.822]; CON vs. HYP $p = 0.010$ 95%CI [-291.809, 2021.921]) and relative $\dot{V}CO_2$ ($F(2, 22) = 13.938$, $p = 0.003$, $\eta_p^2 = 0.559$, CON vs. HOT $p = 0.003$, 95%CI [3.540, 23.322]; CON vs. HYP $p = 0.013$, 95%CI [1.345, 24.095]), as well as a decrease in exercise time ($F(2, 22) = 10.158$, $p = 0.001$, $\eta_p^2 = 0.480$, CON vs. HOT $p = 0.055$, 95%CI [-0.253, 3.421]; CON vs. HYP $p = 0.011$, 95%CI [95%CI 0.279, 3.985]). Compared to the HOT, the HYP exhibited a increase in RER ($F(1.899, 20.892) = 5.396$, $p = 0.014$, $\eta_p^2 = 0.329$, HOT vs. HYP $p = 0.017$, 95%CI [0.004, 0.144]) and an elevated Bla ($F(1.583, 17.416) = 7.383$, $p = 0.007$, $\eta_p^2 = 0.402$, HOT vs. HYP $p = 0.025$, 95%CI [0.015, 9.402]). There was not a reduction in Tc in Hyp and CON, but rather an increase in Tc in HOT conditions compared to the others ($F(1.842, 20.259) = 14.663$, $p < 0.001$, $\eta_p^2 = 0.571$, CON vs. HOT $p < 0.001$, 95%CI [0.230, 0.838]; HOT vs. HYP $p = 0.012$, 95%CI [0.500, 0.813]). Additionally, we would expect a lower SpO_2 in HYP compared to CON and HOT conditions ($F(2, 22) = 49.023$, $p < 0.001$, $\eta_p^2 = 0.817$, HYP vs. HOT $p < 0.001$, 95%CI [6.062, 17.104]; CON vs. HYP $p < 0.001$, 95%CI [6.880, 15.453]), are shown in Table 1.

After linear regression analysis of $\dot{V}O_2$ and exercise time in each group, it was found that the HOT had $R^2 = 0.84 \pm 0.15$ ($p < 0.01$), the HYP had $R^2 = 0.83 \pm 0.06$ ($p < 0.01$), and the CON had $R^2 = 0.91 \pm 0.07$ ($p < 0.01$) as shown in Figure 1A. Compared to the CON, both the HOT and HYP showed reduced $\triangle E_{\dot{V}O2}$ ($F(1.747, 19.217) = 4.837$, $p = 0.023$, $\eta_p^2 = 0.305$, CON vs. HOT $p = 0.029$, 95%CI [0.880, 13.264]; CON vs. HYP $p = 0.034$, 95%CI [0.556, 12.019]). Additionally, by comparing the nonlinear logarithmic regression analysis of $\dot{V}O_2$ and VE during exercise, it was found that the HOT had $R^2 = 0.91 \pm 0.07$ ($p < 0.01$), the HYP had $R^2 = 0.93 \pm 0.07$ ($p < 0.01$), and the CON had $R^2 = 0.96 \pm 0.04$ ($p < 0.01$), as shown in Figure 1B. Compared to the CON, both the HYP and HOT showed reduced OUES ($F(2, 22) = 8.333$, $p = 0.002$, $\eta_p^2 = 0.431$, CON vs. HOT $p = 0.024$, 95%CI [162.155, 1873.418]; CON vs. HYP $p = 0.008$, [95%CI 385.768, 1996.751]). Furthermore, it was found in this study that the exercise time corresponding to GET was shorter in the HYP compared to the CON($F(1.666, 18.330) = 7.491$, $p = 0.003$, $\eta_p^2 = 0.405$, CON vs. HYP $p = 0.015$, 95%CI [0.303, 2.863]). The $\dot{V}O_2$ at GET was higher in the CON compared to the HOT ($F(1.766, 19.421) = 7.285$, $p = 0.004$, $\eta_p^2 = 0.398$, CON vs. HOT $p = 0.004$, 95%CI [165.044, 799.057]), and the relative $\dot{V}O_2$ at GET was higher in the CON compared to the HYP and HOT ($F(1.743, 19.170) = 9.805$, $p = 0.002$, $\eta_p^2 = 0.471$, CON vs. HOT $p = 0.001$, 95%CI [3.484, 11.090]; CON vs. HYP $p = 0.049$, 95%CI [0.027, 10.807]).

TABLE 1 Gas metabolism parameters at exhaustion during incremental exhaustive exercise in different environments.

Variables	HOT	HYP	CON	*p*
RF (cpm)	57.88 ± 7.00	59.00 ± 12.09	58.79 ± 11.21	0.916
VT (L)	2.59 ± 0.55	2.63 ± 0.61	2.7 ± 0.76	0.918
VE (L/min)	148.81 ± 29.17	151.49 ± 26.69	153.38 ± 24.4	0.916
$\dot{V}O_2$ (mL/min)	3791.91 ± 613.39$^{\#}$	3581.09 ± 574.27$^{\#}$	4543.11 ± 807.96	0.005*
$\dot{V}O_2$ (ml/min·kg)	53.53 ± 5.04$^{\#}$	50.52 ± 4.54$^{\#}$	64.37 ± 7.76	<0.001*
$\dot{V}CO_2$ (mL/min)	3943.74 ± 536.64$^{\#}$	4010.99 ± 707.4$^{\#}$	4876.05 ± 792.23	0.007*
$\dot{V}CO_2$ (ml/min·kg)	55.79 ± 4.59$^{\#}$	56.5 ± 5.83$^{\#}$	69.23 ± 8.22	0.003*
HR (bpm)	193.45 ± 9.3	191 ± 8.67	190.91 ± 9.48	0.763
RER	1.04 ± 0.05$^{\&}$	1.12 ± 0.05	1.08 ± 0.06	0.014*
Bla (mmol/L)	11.42 ± 2.56$^{\&}$	16.13 ± 4.75	13.43 ± 2.93	0.007*
Tc (°C)	39.06 ± 0.21$^{\&\#}$	38.52 ± 0.31	38.63 ± 0.36	<0.001*
SpO_2 (%)	97.83 ± 1.27$^{\&}$	86.25 ± 5.26$^{\#}$	97.42 ± 1.78	<0.001*
Exercise time (min)	13.14 ± 1.42$^{\#}$	12.59 ± 1.59$^{\#}$	14.73 ± 1.17	0.001*

*Indicates statistical of intergroup differences ($p < 0.05$), # indicates difference compared to the CON ($p < 0.05$), and and indicates difference compared to the HYP ($p < 0.05$).

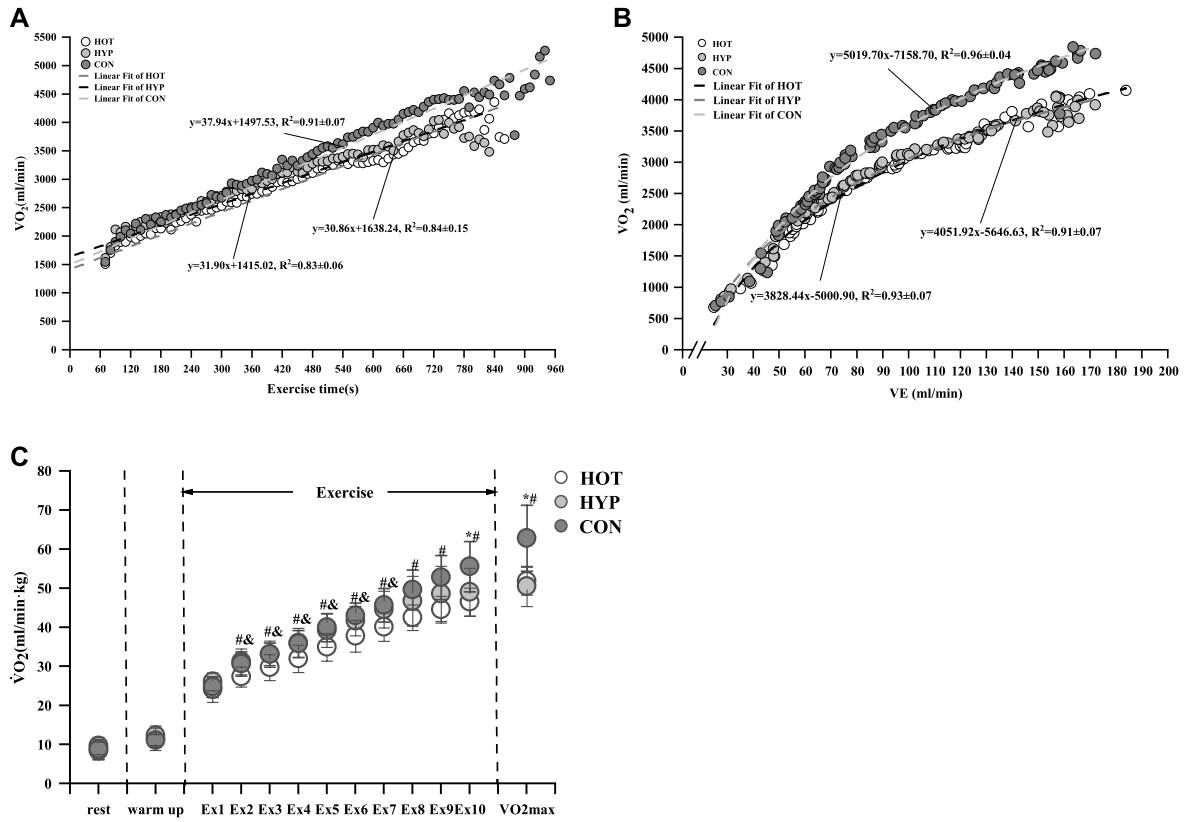

FIGURE 1
Linear fit of $\dot{V}O_2$ **(A)** and logarithmic fit of $\dot{V}O_2$ and VE **(B)** and changes in $\dot{V}O_2$ **(C)** during incremental exhaustive exercise under different conditions. Note: * indicates a difference between the CON and HYP ($p < 0.05$), # indicates a difference between the CON and HOT ($p < 0.05$), and & indicates a difference between the HOT and HYP ($p < 0.05$).

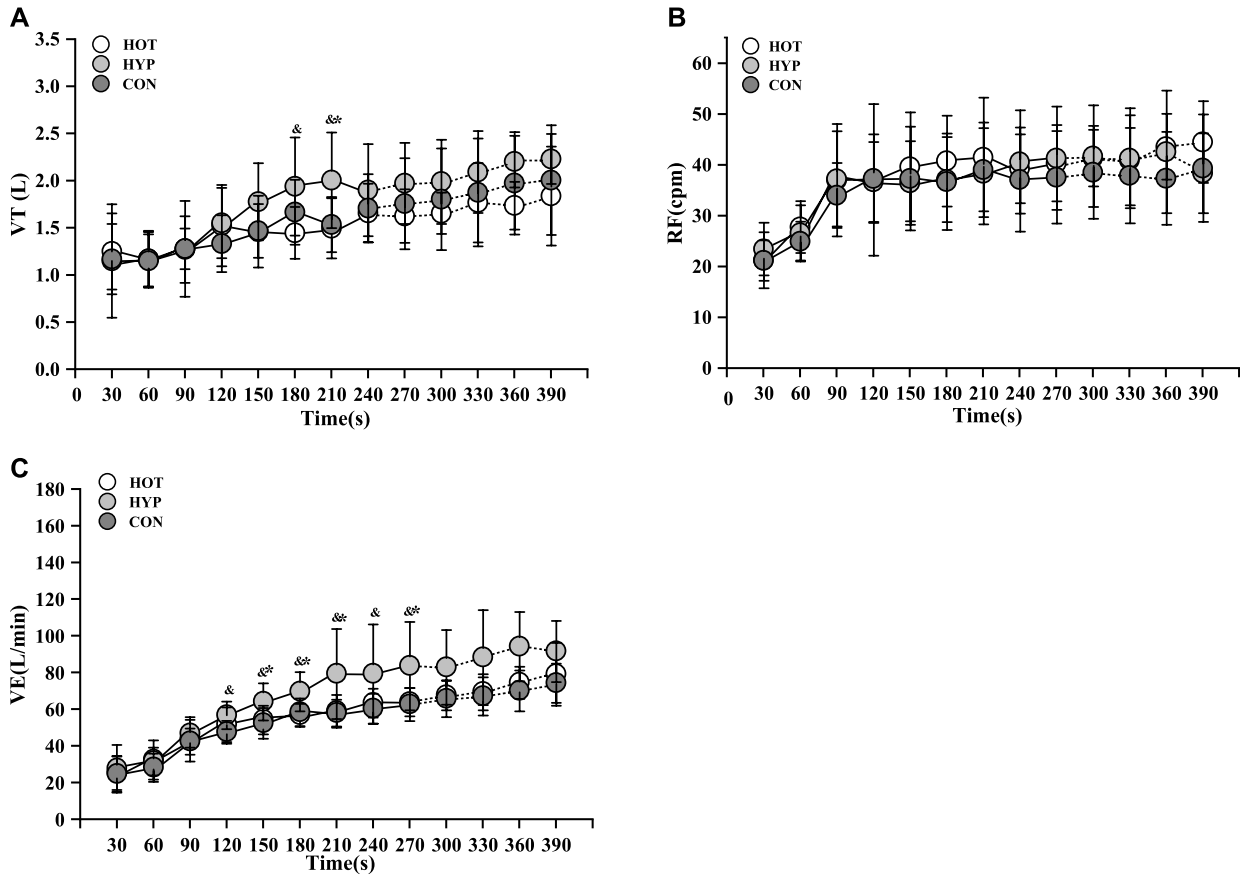

FIGURE 2
Changes of VT **(A)** and RF **(B)** and VE **(C)** in incremental exhaustive exercise in different environments. Note: * indicates a difference between the CON and HYP ($p < 0.05$), # indicates a difference between the CON and HOT ($p < 0.05$), and & indicates a difference between the HOT and HYP ($p < 0.05$).

Using GET as a reference point, linear regression analysis was performed on $\dot{V}O_2$ and exercise time before and after GET in each group. The results showed that the HOT had $R^2_{\dot{V}O2\text{-}1} = 0.84 \pm 0.04$ ($p < 0.01$), $R^2_{\dot{V}O2\text{-}2} = 0.89 \pm 0.08$ ($p < 0.01$). The HYP had R $R^2_{\dot{V}O2\text{-}1}{}^2 = 0.81 \pm 0.08$ ($p < 0.01$), $R^2_{\dot{V}O2\text{-}2} = 0.87 \pm 0.08$ ($p < 0.01$). The CON had $R^2_{\dot{V}O2\text{-}1} = 0.86 \pm 0.03$ ($p < 0.01$), $R^2_{\dot{V}O2\text{-}2} = 0.91 \pm 0.08$ ($p < 0.01$). Compared to the HYP, both the HOT and CON showed reduced $\triangle E_{\dot{V}O2\text{-}1}$ values ($F(2, 22) = 8.181$, $p = 0.002$, $\eta_p{}^2 = 0.427$, HYP vs. HOT $p = 0.002$, 95%CI [10.096, 29.136]; CON vs. HYP $p = 0.030$, 95%CI [2.052, 32.785]) as shown in Table 2.

For the change in $\dot{V}O_2$ during exercise, differences were observed in the main effect at various time points ($F(12, 396) = 323.664$, $p < 0.001$, $\eta_p{}^2 = 0.907$). There were disparities in group main effects ($F(2, 33) = 7.509$, $p = 0.002$, $\eta_p{}^2 = 0.313$), and differences in time and group interaction effects were also identified ($F = 2.790$, $p < 0.001$, $\eta_p{}^2 = 0.145$). Specifically, compared to the HOT, the HYP and CON showed a increase in $\dot{V}O_2$ from the 2nd to the 7th minute ($p < 0.05$). From the 7th to the 9th minute, the HOT exhibited a decrease in $\dot{V}O_2$ compared to the CON ($p < 0.05$). At the 10th minute and $\dot{V}O_{2\,max}$, both the HOT and HYP demonstrated a reduction in $\dot{V}O_2$ compared to the CON ($p < 0.05$) are shown in Figure 1C.

For the change in VT during exercise, differences were observed in the main effect at various time points ($F(9, 297) = 31.103$, $p < 0.001$, $\eta_p{}^2 = 0.485$). There were disparities in group main effects ($F(9, 297) = 31.103$, $p < 0.001$, $\eta_p{}^2 = 0.485$), and differences in time and group interaction effects were also identified ($F = 2.551$, $p = 0.001$, $\eta_p{}^2 = 0.134$). The results indicated that, in comparison to HYP, CON exhibited a lower VT at 210s ($p < 0.05$), and HOT showed a lower VT at 180s and 210s shown in Figure 2A.

For the change in RF during exercise, differences were observed in the main effect at various time points ($F(9, 297) = 35.231$, $p < 0.001$, $\eta_p{}^2 = 0.516$). There was no discernible difference in the group main effect ($F(2, 33) = 1.057$, $p = 0.359$, $\eta_p{}^2 = 0.060$), and no substantial difference in the time and group interaction effect ($F = 1.122$, $p = 0.329$, $\eta_p{}^2 = 0.064$). While there was no increase in RF within the first 270s for HYP, the RF remained relatively high during this period shown in Figure 2B.

For the change in VE during exercise, differences were observed in the main effect at various time points ($F(9, 297) = 150.155$, $p < 0.001$, $\eta_p{}^2 = 0.820$). There were disparities in group main effects ($F(2, 33) = 5.468$, $p = 0.009$, $\eta_p{}^2 = 0.249$), and differences in time and group interaction effects were also identified ($F = 3.901$, $p < 0.001$, $\eta_p{}^2 = 0.191$). Additionally, in the comparison of VE, it was observed that CON had a lower VE than HYP from 120s to 270s ($p < 0.05$), and HOT had a lower VE than HYP at 180s, 210s, and 270s ($p < 0.05$) shown in Figure 2C.

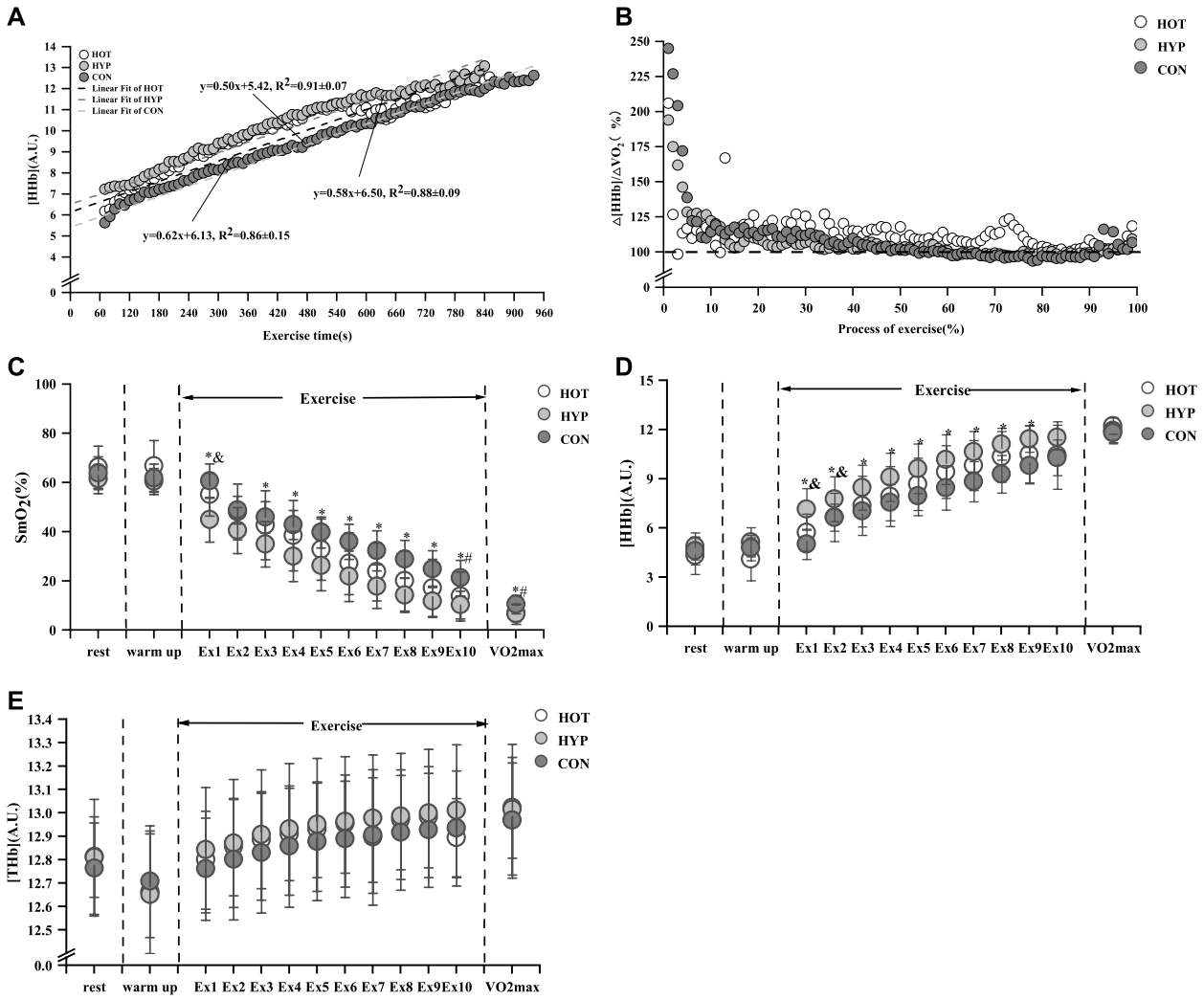

FIGURE 3
Linear fit of HHb **(A)** and characteristics of $\triangle$[HHb]/$\triangle\dot{V}O_2$ **(B)** and changes of SmO_2 **(C)** and HHb **(D)** and THb **(E)** in incremental exhaustive exercise under different environments. Note: * indicates a difference between the CON and HYP ($p < 0.05$), # indicates a difference between the CON and HOT ($p < 0.05$), and & indicates a difference between the HOT and HYP ($p < 0.05$).

3.2 Skeletal muscle hemoglobin

The study results indicate that after linear fitting of [HHb] values with exercise time for each group, the HOT had $R^2 = 0.86 \pm 0.15$ ($p < 0.01$), the HYP had $R^2 = 0.88 \pm 0.09$ ($p < 0.01$), and the CON had $R^2 = 0.91 \pm 0.07$ ($p < 0.01$), as shown in Figure 3A. The results show that compared to the CON, the HOT exhibits a increase in $\triangle E_{HHb}$ (F (1.695, 18.643) = 3.796, $p = 0.047$, $\eta_p^2 = 0.257$, CON vs. HOT $p = 0.044$ 95%CI [0.003, 0.248]). Additionally, the study results reveal that compared to the CON, both the HOT and HYP exhibit a decrease in the exercise time corresponding to BP (F (2, 22) = 4.860, $p = 0.018$, $\eta_p^2 = 0.306$, CON vs. HOT $p = 0.049$ 95%CI [0.004, 2.857]; CON vs. HYP $p = 0.031$, 95%CI [0.144, 2.420]). Furthermore, compared to the CON, the HOT shows a decrease in $\dot{V}O_2$ at the BP (F (2, 22) = 7.488, $p = 0.003$, $\eta_p^2 = 0.405$, CON vs. HOT $p = 0.048$, 95%CI [5.893, 1173.387]), and both the HOT and HYP exhibit a decrease in relative $\dot{V}O_2$ at the BP (F (2, 22) = 8.177, $p = 0.009$, $\eta_p^2 = 0.426$, CON vs. HOT $p = 0.035$ 95%CI [0.517, 15.574]; CON vs. HYP $p = 0.046$, 95%CI [0.088, 10.001]). Subsequently, using BP as a breakpoint, a bilinear fitting was performed on the $\dot{V}O_2$ and exercise time before and after BP for each group. The results show that the HOT had $R_{HHb\text{-}1}^2 = 0.88 \pm 0.08$ ($p < 0.01$) and $R_{HHb\text{-}2}^2 = 0.80 \pm 0.10$ ($p < 0.01$), the HYP had $R_{HHb\text{-}1}^2 = 0.84 \pm 0.10$ ($p < 0.01$) and $R_{HHb\text{-}2}^2 = 0.83 \pm 0.22$ ($p < 0.01$), and the CON had $R_{HHb\text{-}1}^2 = 0.87 \pm 0.05$ ($p < 0.01$) and $R_{HHb\text{-}2}^2 = 0.87 \pm 0.10$ ($p < 0.01$). The results also show that compared to the CON, the HYP exhibits a increase in $\triangle E_{HHb\text{-}1}$ (F (2, 22) = 4.984, $p = 0.016$, $\eta_p^2 = 0.312$, CON vs. HOT $p = 0.005$, 95%CI [0.109, 0.566]; CON vs. HYP $p = 0.029$, 95%CI [0.039, 0.782]), as shown in Table 3 Correlations were found between GET and BP in all groups ($r_{CON} = 0.635$, $P_{CON} = 0.027$; $r_{HYP} = 0.872$, $P_{CON}<0.001$; $r_{HOT} = 0.931$, $P_{HOT}<0.001$).

Furthermore, there were differencesand CON reached in $\triangle$[HHb]/$\triangle\dot{V}O_2$ during exercise under different conditions.

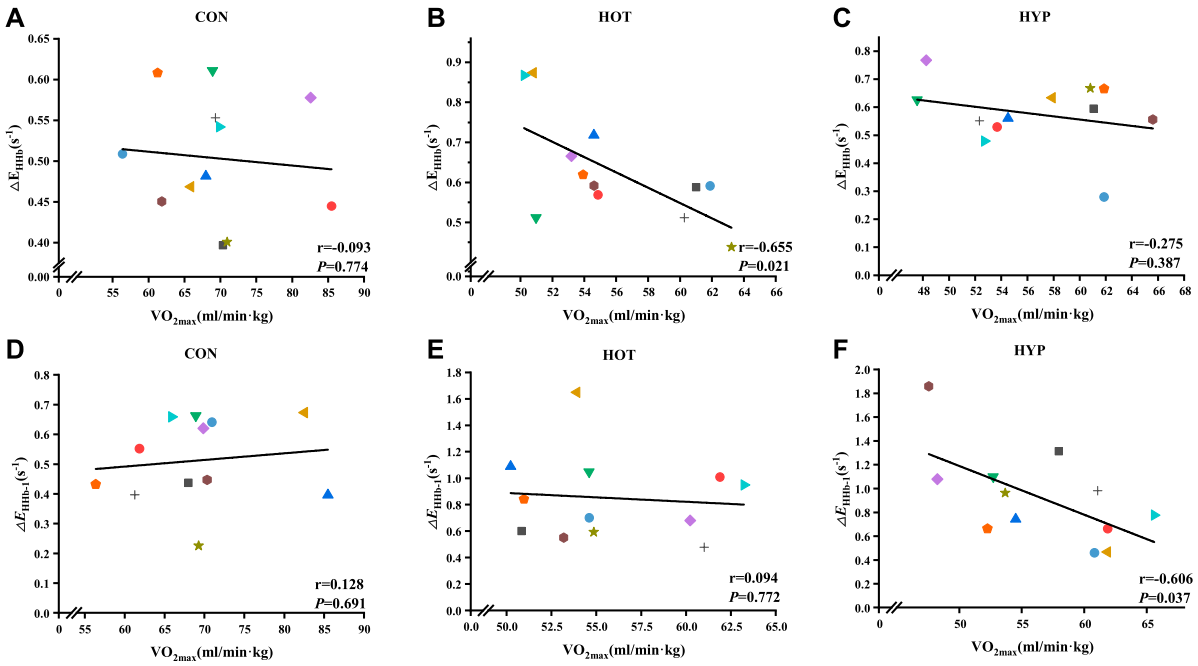

FIGURE 4
Correlation analysis results for $\triangle_{EHHb\text{-}1}$ and $\dot{V}O_{2\,max}$ in CON **(A)**, HOT **(B)**, and HYP **(C)**, as well as correlation analysis results for $\triangle_{EHHb}$ and $\dot{V}O_{2\,max}$ in CON **(D)**, HOT **(E)**, and HYP **(F)** during incremental exhaustive exercise under different environments.

Compared to the HOT, the HYP and CON reached 100% of $\triangle$[HHb]/$\triangle\dot{V}O_2$.as shown in Figure 3B.

After analyzing the data from the first 10min of exercise, For the change in SmO_2 during exercise, differences were observed in the main effect at various time points ($F(12, 396) = 323.664$, $p < 0.001$, $\eta_p^2 = 0.907$). There were disparities in group main effects ($F(2, 33) = 7.509$, $p = 0.002$, $\eta_p^2 = 0.313$), and differences in time and group interaction effects were also identified ($F = 2.790$, $p < 0.001$, $\eta_p^2 = 0.145$). It was found that in the first minute of incremental load testing, the HOT and CON had a increase in SmO_2 compared to the HYP ($p < 0.05$). From the 3rd to the 9th minute, the HYP had a decrease in SmO_2 compared to the CON ($p < 0.05$). At the 10th minute of the incremental load test, the HOT had a decrease in SmO_2 compared to the CON ($p < 0.05$). When reaching $\dot{V}O_{2\,max}$, both the HOT and HYP had a decrease in SmO_2 compared to the CON ($p < 0.05$) are shown in Figure 3C. Analyzing the changes in [HHb] during exercise, For the change in [HHb] during exercise, differences were observed in the main effect at various time points ($F(12, 396) = 257.178$, $p < 0.001$, $\eta_p^2 = 0.886$). There were disparities in group main effects ($F(2, 33) = 6.378$, $p = 0.005$, $\eta_p^2 = 0.279$), and differences in time and group interaction effects were also identified ($F = 2.254$, $p < 0.001$, $\eta_p^2 = 0.120$). It was found that from the 1st to the 2nd min, both the CON and HOT had a decrease in [HHb] compared to the HYP ($p < 0.05$). From the 3rd to the 9th minute, the CON had a decrease in [HHb] compared to the HYP ($p < 0.05$) are shown in Figure 3D.

In addition, For the change in [THb] during exercise, differences were observed in the main effect at various time points ($F(12, 396) = 31.572$, $p < 0.001$, $\eta_p^2 = 0.489$). There was no discernible difference in the group main effect ($F(2, 33) = 0.208$, $p = 0.813$, $\eta_p^2 = 0.012$), and no substantial difference in the time and group interaction effect ($F = 0.893$, $p = 0.612$, $\eta_p^2 = 0.051$). we found that no difference in [THb] during exercise among the three environments ($p > 0.05$), as shown in Figure 3E.

Additionally, the study analyzed the correlation between $\triangle E_{HHb}$ and corresponding $\dot{V}O_{2\,max}$ under different conditions and found a correlation between the two in the HOT ($r_{HOT} = -0.655$, $P_{HOT} = 0.021$), as shown in Figures 4A–C. Moreover, the study analyzed the correlation between $\triangle E_{HHb\text{-}1}$ and the corresponding $\dot{V}O_{2\,max}$ under different conditions, and found a negative correlation bewteen the two in the HYP condition ($r_{HYP} = -0.606$, PHYP = 0.037), as shown in Figures 4D–F.

4 Discussion

The aim of this study was to compare the effects of incremental exhaustive exercise on the dynamic changes of athletes' $\dot{V}O_2$ and HHb under different environments, in order to elucidate the relationship between the dynamic changes of $\dot{V}O_2$ and HHb and peripheral fatigue in different environments. Our results demonstrate that compared to normal condition, both hypoxia exposure and heat exposure can lead to a reduction in exhaustion time and an acceleration of fatigue development, thereby negatively impacting aerobic capacity, such as gas exchange and oxygen supply in skeletal muscle tissue. Additionally, the dynamic changes of HHb in different

TABLE 2 Differences in kinetic parameters of $\dot{V}O_2$ during incremental exhaustive exercise in different environments.

Variables	HOT	HYP	CON	*p*
$\dot{V}O_2$@GET (mL/min)	2643.74 ± 436.69#	2770.26 ± 390.49	3125.79 ± 390.71	0.004*
$\dot{V}O_2$@RCP (mL/min)	3131.59 ± 424.58#	3053.44 ± 539.85#	3642.27 ± 310.47	0.004*
$\dot{V}O_2$@GET (ml/min·kg)	37.4 ± 4.62#	39.26 ± 4.70#	44.64 ± 4.42	0.002*
$\dot{V}O_2$@RCP (ml/min·kg)	44.37 ± 4.47#	43.18 ± 5.70#	52.22 ± 5.02	<0.001*
Time@GET (min)	6.25 ± 1.02	6.13 ± 0.83#	7.67 ± 1.38	0.003*
Time@RCP (min)	8.79 ± 1.43&	7.35 ± 1.08#	9.96 ± 1.33	<0.001*
$\triangle E_{\dot{V}O2}$ (ml/min·s)	30.86 ± 6.10#	31.90 ± 5.46#	37.94 ± 6.51	0.023*
$\triangle E_{\dot{V}O2\text{-}1}$ (ml/min·s)	47.08 ± 6.97&	66.69 ± 18.35	49.27 ± 9.83	0.002*
$\triangle E_{\dot{V}O2\text{-}2}$ (ml/min·s)	34.33 ± 22.00	27.96 ± 17.68	35.19 ± 14.77	0.580
OUES	4051.92 ± 818.58	3828.44 ± 678.62#	5019.70 ± 840.35	0.002*

*Indicates statistically differences between groups ($p < 0.05$), # indicates differences compared to the CON ($p < 0.05$), & indicates differences compared to the HYP ($p < 0.05$).

TABLE 3 The dynamic parameter difference of HHb in the incremental exhaustive exercise under different environments.

Variables	HOT	HYP	CON	*p*
Time@BP (min)	6.58 ± 1.08	6.73 ± 0.69	8.01 ± 1.64	0.018*
$\dot{V}O_2$@BP (mL/min)	2702.14 ± 391.06#	2924.22 ± 385.05	3291.78 ± 606.11	0.003*
$\dot{V}O_2$@BP (ml/min·kg)	38.36 ± 5.07#	41.36 ± 3.47#	46.40 ± 5.38	0.009*
$\triangle E_{HHb}$ (s^{-1})	0.63 ± 0.13#	0.58 ± 0.12	0.50 ± 0.08	0.047*
$\triangle E_{HHb\text{-}1}$ (s^{-1})	0.85 ± 0.33#	0.92 ± 0.39#	0.51 ± 0.14	0.016*
$\triangle E_{HHb\text{-}2}$ (s^{-1})	0.42 ± 0.23	0.37 ± 0.19	0.48 ± 0.20	0.918

*indicates statistically differences between groups ($p < 0.05$), # indicates differences compared to the CON ($p < 0.05$), and and indicates differences compared to the HYP ($p < 0.05$).

modes indicated that hypoxic and heat stress might individually contribute to the reduction of athletes' aerobic capacity.

When comparing the dynamic changes of $\dot{V}O_2$ during the process of incremental exhaustive exercise under different environments, our study revealed a significant reduction in $\Delta E_{\dot{V}O2}$ and OUES in HOT and HYP. These findings indicate that the gas exchange ability of athletes was inhibited in both environments. Specifically, during exercise in a high-temperature and high humidity environment, the $\dot{V}O_2$ between 2 min and 10 min in the HOT was lower compared to the CON, which aligns with the research findings of Sawka (Sawka et al., 1985), González (González-Alonso and Calbet, 2003), and Wingo (Wingo et al., 2020). This can be attributed to the impact of heat stress on the cardiovascular system and oxygen delivery (Cheuvront et al., 2010). Heat stress reduces Cardiac Output (Q) and Mean Arterial Blood Pressure (MAP) by decreasing cardiac output, resulting in reduced skeletal muscle blood flow, oxygen delivery, and $\dot{V}O_2$ during exercise. Heat stress can reduce blood flow in skeletal muscles during exercise by enhancing sympathetic nerve activity (Sawka et al., 1985). Therefore, during exercise under heat stress, athletes may reduce their oxygen consumption, which can result in a decrease in $\dot{V}O_2$ and $\Delta E_{\dot{V}O2}$. Additionally, our study observed a significant decrease in $\Delta E_{\dot{V}O2}$ and OUES of athletes under low-oxygen conditions, which aligns with the research findings of Wagner (Wagner et al., 1986) and Loeppky (Loeppky et al., 2020). However, it is important to note that these two studies did not specifically compare the slope of $\dot{V}O_2$ before and after the GET. Nevertheless, our study found an increase in $\Delta E_{\dot{V}O2\text{-}1}$ in the hypoxic environment. This finding may be attributed to the fact that the initial stage of exercise in a hypoxic environment effectively stimulates peripheral chemoreceptors, leading to increased depth and acceleration of respiration through reflex mechanisms. Furthermore, the utilization of oxygen reserves in the body results in a decrease in SpO_2. We believe that one of the reasons for the increase in $\triangle E_{VO2\text{-}1}$ is the elevation in VE resulting from enhanced and accelerated respiration due to hypoxic exposure. Firstly, the decrease in oxygen reserve in the body during exercise affects oxygen kinetics. The increased VE under hypoxic conditions improves ventilation efficiency in the early stages of exercise, thereby offsetting the reduction in oxygen reserve (Engelen et al., 1996), resulting in an overall increase in VO_2 slope with decreasing blood oxygen saturation. Secondly, the elevated VE under hypoxic exposure leads to increased oxygen consumption of respiratory muscles

and subsequently increased ventilation cost (Coast et al., 1993; Benoit et al., 1997), thereby influencing VO_2 production. Consequently, the $\dot{V}O_2$ and working rate during the initial stage of exercise increase as the body ingests more oxygen to meet its normal work demands. Therefore, the results of $\dot{V}O_2$ in HYP differ from those in HOT, with no significant decrease observed in $\dot{V}O_2$ during the initial stage of exercise in HYP. However, as the duration of exercise increases, athletes may experience a decrease in blood perfusion to the respiratory muscles and limitations in gas diffusion. This can result in a mismatch between ventilation and perfusion, ultimately leading to a decrease in $\dot{V}O_2$. Therefore, the response of VE to exercise under hypoxic environment throughout the entire exercise process contributes to the reduction in $\Delta E_{\dot{V}O2}$ (Amann and Calbet, 2008).

Regarding the oxygen supply to skeletal muscles, our findings are consistent with previous literature reports (Zhang et al., 2010), indicating a positive correlation between BP and GET. In terms of selecting the appropriate linear fitting model for HHb, Spencer (Spencer et al., 2012) have noted that the bilinear model provides a more accurate description of the potential physiological response of HHb in subjects compared to the S-type regression model. Thus, we employed the bilinear model to assess the dynamic changes in HHb in VL during exercise. To evaluate the HHb response throughout the entire exercise process, we also calculated the slope of HHb from the onset of exercise to the point of exhaustion. Interestingly, our observations demonstrate that the ΔE_{HHb} in the HOT is higher than that in the CON, and the significance of SmO_2 is lower in the HOT compared to the CON at the 10-min mark during exercise. Previous research by Dennis (Dennis et al., 2023) has shown that exercise at 35°C and 40°C, compared to a single exercise at 20°C, can enhance the deoxygenation reaction in skeletal muscles. Girard (Girard et al., 2016) have highlighted that high-intensity exercise in a high temperature and humidity environment can affect the efficiency of output power and accelerate peripheral fatigue. Our study indicates that the efficiency of output power of skeletal muscles increases during the initial stage of exercise in the high-temperature and high-humidity environment, offsetting some of the negative effects (Racinais et al., 2017). Consequently, we did not observe a significant decrease in SmO_2 in the VL during the initial stage of exercise. However, as exercise intensity and duration increased, along with an increase in Tc, peripheral fatigue eventually set in. Throughout the entire exercise process, the significant increase in ΔE_{HHb} may be attributed to the rightward shift of the oxygen dissociation curve caused by a decrease in Hb affinity for oxygen as temperature rises, promoting oxygen release (Webb et al., 2022). Simultaneously, in the analysis of $\dot{V}O_2$ and HHb, the $\Delta\dot{V}O_2/\Delta HHb$ value in the HOT also revealed an enhancement in skeletal muscle Hb deoxygenation reaction. In particular, during the initial stage of exercise, we observed an increase in $E_{HHb\text{-}1}$ in the HOT condition compared to the CON condition, while $E_{VO2\text{-}1}$ remained unchanged. This observation aligns with the findings of Nybo et al. (Nybo et al., 2001), who reported that heat stress did not influence the oxygen uptake rate in the early stages of exercise but led to a reduction in oxygen uptake and oxygen pulse. It has been suggested that oxygen uptake kinetics are influenced by both the rise in skin temperature and core temperature (Rowell et al., 1966; José et al., 1997), although they do not increase simultaneously during the early stages of exercise. Despite the acceleration of skeletal muscle deoxygenation in these early stages, compensatory mechanisms appear to address the lack of oxygen delivery. Our investigation revealed an increase in HR in the HOT condition, accompanied by an elevation in cardiac output within a certain heart rate range to compensate for the mismatch between oxygen intake and utilization. As heart rate increased within a specific range, cardiac output(Q) also rose to compensate for the discrepancy between oxygen intake and utilization. Additionally, it has been noted that higher Q, hemoconcentration, and enhanced O_2 extraction contribute to a similar initial rate of rise in $\dot{V}O_2$ (González-Alonso and Calbet, 2003). Therefore, the early-stage mismatch between oxygen uptake and utilization in exercise under high temperature and high humidity may result from various factors. Furthermore, the correlation results showed that ΔE_{HHb} in the HOT was negatively correlated with $\dot{V}O_{2\,max}$, suggesting that the increase in Hb deoxygenation reaction during exercise, resulting from the aforementioned mechanism, may be associated with a decrease in exercise duration. Hence, athletes engaging in physical activity within high-temperature, high-humidity environments conditions may potentially influence the interplay between oxygen uptake in skeletal muscles and lungs.

Hypoxia exposure negatively affects athletes during incremental exhaustive exercise, resulting in decreased SpO_2, SmO_2, and VO_{2max} at the point of exhaustion. This is consistent with findings from previous studies (Osawa et al., 2011; Azevedo et al., 2020a). Osawa (Osawa et al., 2017) and Bowen (Bowen et al., 2016) have also demonstrated that the SmO_2 curve of skeletal muscle decreases while the HHb curve increases during incremental exhaustive exercise in a hypoxic environment. However, these studies did not compare the slope of HHb during exercise. In the initial stage of incremental exhaustive exercise in a hypoxic environment, the metabolism is immediately affected. Hypoxia exposure causes the anaerobic energy supply system to be utilized earlier to maintain ATP demand (Linnarsson et al., 1974), leading to a left shift in BP and GET. Previous studies have shown that the value of ΔE_{HHb} during incremental exhaustive exercise can be influenced by factors such as body position (DiMenna et al., 2010) and metabolic diseases (Gildea et al., 2019). In our study, we observed a significant increase in $\Delta E_{HHb\text{-}1}$ before BP, indicating that as skeletal muscle deoxygenation accelerates, the ability to increase peripheral oxygen delivery and meet the increased oxygen demand decreases. However, in the study by Azevedo (Azevedo et al., 2020b), only the leftward shift of BP and the increase in HHb during exercise under hypoxia were observed, without affecting $\Delta E_{HHb\text{-}1}$. In comparison, our study employed a lower FiO_2 for exercise, which may explain the enhanced skeletal muscle deoxygenation response. Acute hypoxia exposure leads to a significant reduction in oxygen delivery to skeletal muscles during exercise, but this can be compensated by increased oxygen uptake in the body (Calbet

et al., 2009). The increase in $\Delta E_{\dot{V}O2\text{-}1}$ and $\Delta \dot{V}O_2/\Delta HHb$ before GET reflects the relationship between the decrease in oxygen reserve and the increase in oxygen uptake. The earlier deployment of the anaerobic energy supply system causes the slope of HHb to rise rapidly in the initial stage, and impaired hemodynamic response can be considered a potential mechanism for decreased exercise capacity (Gildea et al., 2019). Additionally, the decrease in muscle O_2 flow may potentially limit the possibility of $\dot{V}O_2$ kinetics during exercise (Koga et al., 2007). The observed correlation between $\Delta E_{HHb\text{-}1}$ and $\dot{V}O_{2\,max}$ in the HYP group supports this perspective.

In our study, we also observed that $\Delta E_{HHb\text{-}2}$ in the HHb plateau after BP appeared to be unaffected by the environmental factors, supporting the notion that the increase in HHb near the critical exercise intensity is not limited by the oxygen diffusion capacity (Murias et al., 2013). Iannetta (Iannetta et al., 2018) have indicated that an oxygen reserve can still be observed in the deep layer of the VL during incremental exhaustive exercise, and this is not influenced by gender or training level (Inglis et al., 2019). Therefore, the presence of an HHb plateau does not indicate the upper limit of oxygen extraction. After reaching BP, the diffusion capacity of oxygen from the capillaries to the muscle fibers may have reached its peak, and the subsequent increase in oxygen uptake depends more on increased oxygen delivery. In our study, we did not observe any difference in $\Delta E_{\dot{V}O2\text{-}2}$ among the three environments, which consequently led to no difference in $\Delta E_{HHb\text{-}2}$. This suggests that the oxygen extraction capacity during the HHb plateau phase is not influenced by the environmental conditions.

Some criticisms persist regarding the use of near-infrared spectroscopy in the determination of tissue oxygenation. NIRS signals capture changes in hemoglobin Hb and Mb oxygenation, enabling a robust assessment of muscle oxygenation status throughout all exercise stages with high precision (Lucero et al., 2018). Muscle oxygenation reflects the equilibrium between oxygen delivery and oxygen utilization (Koga et al., 2007). However, the contributions of Hb and Mb to NIRS signals differ during muscle contraction (Spires et al., 2011). Other factors, including hematocrit, blood volume, arterioles, capillaries, and venous distribution, can also influence the interpretation of NIRS dynamics in oxygenation and deoxygenation (Koirala et al., 2021). With increased blood flow during exercise, THb may be affected (Alvares et al., 2020). However, even when skin and muscle blood flow increase simultaneously, changes in NIRS-derived oxygenation signals (SmO_2, HHb) can still accurately reflect alterations in muscle oxygenation (Tew et al., 2010). Some studies have suggested that the oxygenated signal is influenced by increased skin blood flow, while the deoxygenation signal is not sensitive to changes in blood volume (Grassi et al., 2003; Grassi and Quaresima, 2016). Additionally, Koirala et al.'s study (Koirala et al., 2021) found that changes in blood volume have an additional impact on oxygenation Hb-Mb, primarily associated with capillary oxygenation Hb. In contrast, the effect on deoxidation HHb-Mb is less pronounced, given its association with abundant O_2 delivery due to the high O_2 saturation of Hb and Mb. Therefore, based on the aforementioned evidence, we maintain confidence in exploring deoxyhemoglobin dynamics in athletes during increasing load exercises under different environments.

5 Conclusion

When athletes engage in incremental exhaustive exercise in a hypoxic environment or a high temperature and high humidity environment, the gas exchange in the body and the oxygen supply to skeletal muscle tissue can be compromised. This can have implications for athletes, as accelerated deoxygenation of skeletal muscle during increasing load exercise under high temperature and high humidity, and excessive deoxygenation of skeletal muscle before the break point of deoxygenated hemoglobin under hypoxic environments, may contribute to peripheral fatigue in different conditions.

In high temperature and high humidity environments, exercise intensity can negatively impact the skeletal muscle deoxygenation response. On the other hand, low to moderate load training in a hypoxic environment can accelerate the skeletal muscle deoxygenation response. Therefore, coaches should take into account the specific characteristics of peripheral fatigue during training or competition in different environments and design appropriate training or competition programs accordingly. It is important to consider the limitations imposed by these environments and develop strategies to optimize performance and mitigate the negative effects of reduced oxygen availability and increased heat and humidity.

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 humans were approved by the Shanghai Research Institute of Sports Science (Shanghai Anti-Doping Center) Research Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

All authors of this study actively contributed to the design and development of the research. ZG took the lead in writing the manuscript, while JQ reviewed and edited the written content.

All authors contributed to the article and approved the submitted version.

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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.

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Glossary

$\triangle E_{HHb}$	Linear fitting slope of HHb
$\triangle E_{HHb\text{-}1}$	Linear fitting slope of HHb before BP
$\triangle E_{HHb\text{-}2}$	Linear fitting slope of HHb after BP
$\triangle E_{\dot{V}O2}$	Linear fitting slope of $\dot{V}O_2$
$\triangle E_{\dot{V}O2\text{-}1}$	Linear fitting slope of $\dot{V}O_2$ before GET
$\triangle E_{\dot{V}O2\text{-}2}$	Linear fitting slope of $\dot{V}O_2$ after GET
ANOVA	Analysis of variance
Bla	Blood lactate
BP	Break point
CON	Normal temperature and humidity with normoxia
FiO_2	Fractional inspired oxygen concentration
GET	Gas exchange threshold
HHb	Deoxyhemoglobin
HOT	High temperature and humidity with normoxia
HR	Heart rate
HYP	Normal temperature and humidity with hypoxia
Mb	Myoglobin
NIRS	Near-infrared spectroscopy
OUES	Oxygen uptake efficiency slope
RCP	Respiratory compensation point
RER	respiratory exchange ratio
RF	Respiratory frequency
RH	Relative humidity
RPE	Borg rating of perceived exertion
SD	Standard division
SmO_2	Muscle oxygen saturation
SpO_2	Oxygen saturation
Tc	Core temperature
THb	Total hemoglobin
$\dot{V}CO_2$	Carbon dioxide production
VE	Minute ventilation
VL	Vastus lateralis muscles
$\dot{V}O_2$	Oxygen uptake
$\dot{V}O_{2\,max}$	Maximal oxygen uptake
VT	Tidal volume

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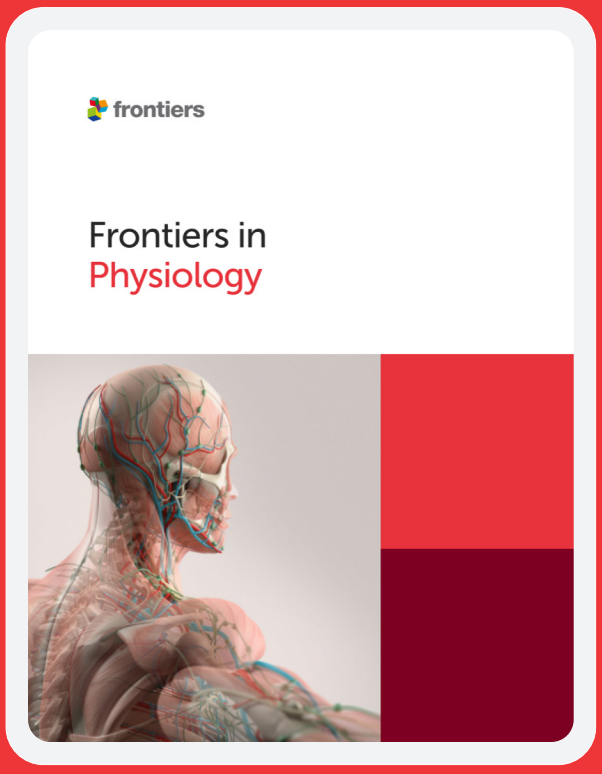

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