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Hematological and biochemical responses to extreme hypoxia exposure after hypoxia preconditioning in Sprague–Dawley rats

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ABSTRACT

Hypoxia preconditioning (HP) is postulated to induce adaptive changes in the body for endurance and hypoxic acclimatization. Its dosage (severity, intermittence, duration) determines its effectiveness. Male SD rats were subjected to HP by exposing them to intervals of hypoxia for different durations in a normobaric hypoxia chamber at 12 % FiO_2 for 4h consecutively for 1, 2, 3, 4 and 5 days. To assess the acclimating effect of HP, the animals were further subjected to severe hypoxic exposure to 8 % FiO_2 for 6h. Physiological variables (peripheral oxygen saturation-SpO_2, heart rate-HR, and respiratory rate-RR), protein expression parameters (HIF-1α, EPO, VEGF, and uNOS), biochemical metabolites and hematology and blood gas variables were studied during the course of the hypoxia preconditioning schedule. All the statistical comparisons were performed using one-way ANOVA following Tukey's correction. It was found Day 3-HP was associated with a greater SpO_2 level ($p < 0.05$) compared with those of other hypoxia preconditioned groups, the percentage of NRBC was lowest in day 3-HP. PCO_2 was lower during days 2, 3 and 4-HP. Circulatory metabolites (nitrate + nitrite-NO, L-arginine, citrulline, succinate, blood urea nitrogen, and L-lactate) changed significantly with different durations of hypoxia preconditioning. HIF-1α showed peak expression on HP-3 day, whereas EPO was highest during HP-2 day, and VEGF was significantly lower at $p < 0.001$ as compared to extreme hypoxia without HP. Reduced oxidative stress (ROS) and inflammation (histopathology) were observed during HP-3 day. Hypoxia preconditioning at 12 % FiO_2 for 3 days can be postulated to be a potent non-pharmacological modality for inducing physiological and molecular responses that can influence hypoxic acclimatization during exposure to extremely hypoxic conditions.

1. Introduction

The effects of high altitude on humans are mostly the consequences of the reduced partial pressure of oxygen in the atmosphere. As the altitude increases, the atmospheric pressure decreases, resulting in low oxygen saturation levels in the blood of people ascending to high altitudes. The oxygen saturation of hemoglobin determines the content of oxygen in the blood. Studies have revealed that, as an individual reaches an altitude of approximately 2100 m above sea level, the oxygen saturation of hemoglobin begins to decline rapidly (Young and Reeves, 2003). The greater the altitude increase is, the greater the risk of altitude-related problems (Cymerman and Rock, 1994), resulting to the mild signs of acute mountain sickness(AMS) to the fatal signs of high altitude pulmonary edema(HAPE) and high altitude cerebral edema (HACE). When exposed to high-altitude hypoxic environments, the body makes several adjustments over time for hypoxic acclimatization. These acclimatization responses can take many forms. It can be induced by continuously residing at high altitudes for a long period, resulting in adaptation to a high-altitude atmosphere. Another alternative to altitude acclimatization is gradual advancements while ascending to no more than 305 m (1000 ft) in the day (Bezruchka, 2005) or sojourning at intermediate altitudes (Muza, 2007). Another alternative to altitude acclimatization involves hypoxia preconditioning via intermittent hypobaric hypoxia (Coimbra-Costa et al., 2021) or normobaric hypoxia exposure (Wang et al., 2024). Furthermore, hypoxia preconditioning has been suggested for military applications meant for high-altitude operations (Muza, 2007). Hypoxia Conditioning consists of 3 elements: 1. Inspired partial pressure of oxygen; 2. Hypoxia session duration; and 3. Total number of hypoxia preconditioning sessions. The schedule of hypoxia preconditioning (simulated altitude or FiO_2 %, exposure

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duration and number of sessions) can be standardized as per the required operation, such as targeted altitude ascent and risk of developing high-altitude problems.

Hypoxia preconditioning is known to elicit physiological responses in our system, ranging from alterations in the ventilatory pattern to the modulation of molecular and cellular functions (Bouhamida et al., 2022; Coimbra-Costa et al., 2021). The transcription factor hypoxia-inducible factor-1 (HIF-1) is critical for mediating oxygen homeostasis via the activation of a signaling molecular cascade after hypoxia exposure (Semenza, 2011) and is responsible for the expression of various physiologically important proteins involved in hypoxic acclimatization: nitric oxide synthase (NOS), vascular endothelial growth factor (VEGF), erythropoietin (EPO), etc. Hypoxia preconditioning promotes the upregulation of HIF-1α (Viscor et al., 2018). The activation of HIF-1α results in a metabolic shift from aerobic to anaerobic glycolysis (McNamara and El- Khuffash, 2017). It is thus speculated that increased glucose metabolism, with its glycolytic intermediates succinate, pyruvate, lactate and blood urea nitrogen, is a hallmark of high altitude hypoxic acclimatization (Murray et al., 2018). Nitric oxide (NO), a signaling molecule produced through metabolism by nitric oxide synthase (NOS), plays a pivotal role in acclimatization to high-altitude hypoxia (Levett et al., 2011). Hypoxia leads to an increase in the expression of different NOS isoforms via up-regulation of HIF-1α (Brüne and Zhou, 2007). Considering these factors, NO can act as a potential marker for hypoxic acclimatization. Therefore, considering these previous findings, we hypothesize that hypoxia preconditioning through repeated hypoxia exposure can result in the priming of several late-onset cellular and molecular mechanisms and synergistically contributes to systemic responses, resulting in early onset of acclimatization to high-altitude exposure. Henceforth, the present study aimed to determine the optimal HP period and its efficacy as a non-pharmacological therapeutic modality for hypoxic acclimatization, as well as to outline the determinants of hypoxic acclimatization.

2. Materials and methods

2.1. Animal grouping

Male Sprague Dawley rats weighing 180 ± 10 g were used as an animal model for the present study. A total of 35 animals were divided into seven groups (n = 5; N = 35). The present study was performed on male Sprague Dawley rats because evidence indicates that the physiological responses of these rats are similar to those of humans while acclimatizing to high altitude (Dempsey and Forster, 1982), and males are more susceptible to high-altitude maladies than females are (Schoene, 2008). All animal procedures and experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC Number: DIPAS/IAEC/2019/05) and followed the standards outlined by the statutory body, Committee for Control and Supervision of Experiments on Animals (CCSEA) under Ministry of Fisheries, Animal Husbandry and Dairying, Government of India and Guide for the Care and Use of Laboratory Animals. National Academy Press, Washington, D.C., 1996. The animals were grouped as follows: **Group A**: Normoxia, 21 % FiO_2. **Group B**: Hypoxia preconditioning; Group B1: HP 4 h/1day. Group B2: HP 4 h/2 Days. Group B3: HP 4 h/3day Group B4: HP 4 h/4 days. Group B5: HP 4 h/5 days. Furthermore, after 24 h of each HP exposure schedule, the animals were exposed to 8 % FiO_2 for 6 h to assess their hypoxia tolerance and validation. **Group C**: This group of animals were directly exposed to 8 % FiO_2 for 6 h. A schematic representation of the same process is depicted in Fig. 1. After each stipulated time period of the hypoxia preconditioning schedule, the animals were returned to normoxic conditions by slowly increasing the FiO_2 % of the chamber from 8 % (equivalent altitude, 25,000 ft) to 21 % (sea level). The inlet and outlet valves of the normobaric chamber are set such that the FiO_2 % increases or decreases slowly and gradually. This process takes approximately 25 min (re-pressurization rule of thumb @1000 ft/min). Furthermore, blood and tissue samples were collected half an hour after the animals were removed from the normobaric chamber to avoid reoxygenation injury. This whole procedure takes approximately 1 h from normalization of the chamber to sample collection and analysis.

2.2. Hypoxic preconditioning

The animals were exposed to hypoxia under normobaric hypoxic conditions via a hypoxicator (Hypoxicator-Jarvis 50 LN, Biostag Technologies) (Fig. 2), which is based on the principle of pressure swing absorption technology (PSA). Briefly, a hypoxicator has an air compressor that compresses ambient air and passes through cooling air to remove moisture from compressed air. Afterward, the samples were transferred to a PSA-based desiccant dryer to make dry air. Dry air was passed through a nitrogen purifier membrane to obtain hypoxic gas. The temperature and humidity in the chamber were maintained at optimum values of 25 ± 1 °C and 50–60 %, respectively. The animals were allowed access to food and water *ad libitum*. These parameters were kept

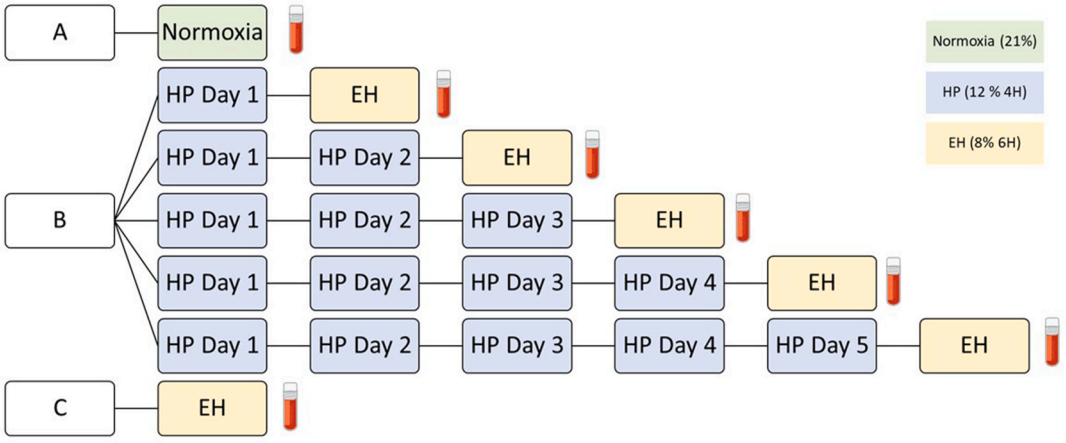


Fig. 1. Schematic representation of Animal grouping and blood collection **schedule.** The animals were grouped as follows: **Group A**: Normoxia, 21 % FiO_2. **Group B**: Hypoxia preconditioning; Group B1: HP 4 h/1day. Group B2: HP 4 h/2 Days. Group B3: HP 4 h/3day Group B4: HP 4 h/4 days. Group B5: HP 4 h/5 days. Furthermore, after 24 h of each HP exposure schedule, the animals were exposed to 8 % FiO_2 for 6 h to assess their hypoxia tolerance and validation. **Group C**: This group of animals were directly exposed to 8 % FiO_2 for 6 h. A schematic representation of the same process is depicted in Fig. 1. After each stipulated time period of the hypoxia preconditioning schedule, the animals were returned to normoxic conditions by slowly increasing the FiO_2 % of the chamber from 8 % (equivalent altitude, 25,000 ft) to 21 % (sea level).

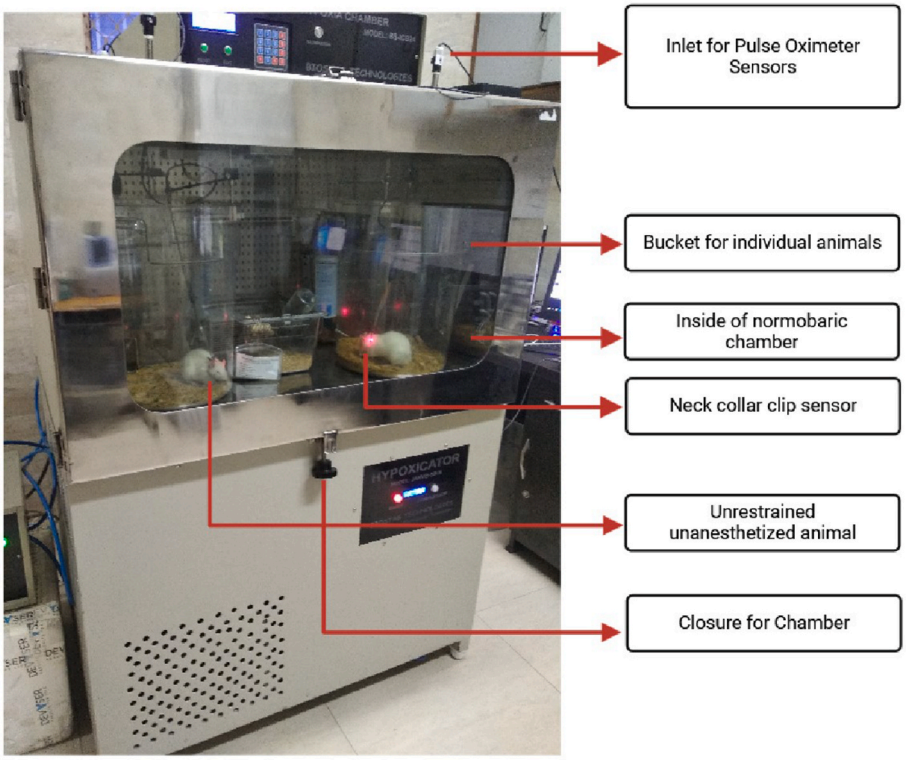


Fig. 2. The integrated hypoxia chamber and pulse oximet**er system.** The normobaric hypoxia chamber was customized to be integrated with a multichannel pulse oximeter system. Physiological parameters viz., peripheral capillary oxygen saturation (SpO_2), heart rate (HR) and respiratory rate (RR) were observed constantly throughout the exposures using MouseOx Plus Pulse Oximeter System (Starr Life Science, Oakmont, PA, USA) in unanaesthetised animals. A multi-channel data recording unit (Powerlab, Ad Instruments) integrated the physiological records in real-time. Labchart software (Ad Instruments) allowed storage and analysis of the recorded data.

constant throughout the experiment.

2.3. Physiological parameter recording

Peripheral capillary oxygen saturation (SpO_2), heart rate (HR), and respiratory rate (RR) were monitored while the conscious and unrestrained animals were exposed to HP and extreme hypoxia via a pulse oximeter system (MouseOx plus, STARR Life Sciences, Oakmont, PA, USA). A multichannel data recording unit using LabChart software (Powerlab, ADInstruments, New Zealand) was used to record and analyze the data. Briefly, the rats were first habituated to the collar clip sensors for one week before the actual experiments began. The hair around the neck of each rat was shaved, and a dummy collar clip sensor was placed on the back of the neck daily.

2.4. Blood collection for analysis of hematological, blood gas and biochemical metabolites

Blood samples were collected from the retro-orbital plexus (venous plexus located in the retrobulbar space) under general anesthesia (ketamine @ 100 mg/kg body wt combined with xylazine @10 mg/kg body wt), followed by a drop of opthalmic anesthesia (proxacaine hydrochloride). Heparinized capillary tubes were used to collect blood from the retro-orbital plexus, collected in EDTA-coated vacutainer tubes (Becton Dickinson-BD, US) and immediately used for analysis of hematological variables, blood-gas analysis and biochemical metabolites.

2.5. Hematological variables

Freshly collected blood from all the groups was used for whole blood routine complete blood count (CBC) and reticulocyte tests through a flow cytometry-based 5-part differential veterinary hematology analyzer (Spincell vet5 Compact, Spinreact, Spain).

2.6. Blood gas analysis

Freshly collected blood samples were analyzed immediately through the i-STAT analyzer (Abbott, East Windsor, N. J. USA). Blood pH, blood gas composition (pCO2- partial pressure of carbon dioxide, pO2- partial pressure of oxygen in the venous blood), and blood electrolytes (BE-Base Excess, HCO3−–bicarbonate) were measured.

2.7. Biochemical metabolites

Nitrate and nitrite estimation was performed through a colorimetric assay kit, which works on the principle of the Griess reagent, which converts nitrite into a colored product for quantification. (Cayman chemical cat no. 780001). NO is a highly unstable molecule with a half-life of approximately 0.005–1 s in the circulation (Beckman and Koppenol, 1996). It rapidly reacts with oxygen and water to produce more stable metabolic byproducts, nitrites (half-life of 110 s; Kelm, 1999) and nitrates (half-life of 5–8 h; Tannenbaum, 1979). Hence, in present study, by products of nitric oxide, nitrate (NO_3^-) and nitrite (NO_2^-) were assayed along with L-arginine, citrulline, succinate, lactate and blood urea nitrogen (BUN).

L-arginine, a substrate for the generation of NO by NOS, was estimated via an enzyme-based assay (Abcam L-arginine assay kit, cat no. ab241028). Citrulline, a byproduct of NO synthesis, was estimated via a colorimetric assay (Cell Biolabs Inc., Homocitrulline/citrulline Assay Kit cat no. MET-5027). Succinate estimation was performed following the EnzyChrom™ Succinate Assay Kit (Bioassay systems, ESNT-100). The glycolytic intermediate lactate was assayed via an L-lactate assay kit

(ElabSciences, E-BC-K044-M). Blood urea nitrogen (BUN) was assayed via a colorimetric kit (ElabSciences, E-BC-K183-S).

2.8. Lung tissue collection and processing

After the stipulated time period of hypoxic exposure, the experimental animals were removed from the normobaric hypoxia chamber. Furthermore, the animals were anesthetized with a combination of ketamine @ 100 mg/kg body weight and xylazine @ 10 mg/kg body weight for the collection of blood and lung tissue samples. The lungs were perfused with ice-cold PBS and collected and processed immediately to quantify reactive oxygen species (ROS). The remaining samples were snap-frozen in liquid nitrogen and stored at −80 °C until further use for estimation of protein expression.

2.9. The oxidative stress marker reactive oxygen species (ROS)

Briefly, the lung tissues from all the groups were rinsed with cold saline and homogenized in 10 % 0.154 M KCl (pH 7.4) buffer. The generation of oxidative stress markers (ROS) in hypoxia exposed lung tissue homogenates were determined via the use of the cell permeable non-fluorescent dye H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate). In the presence of ROS, predominantly H_2O_2, H_2DCF is rapidly oxidized to 2′,7′-dichlorofluorescein (DCF), which is highly fluorescent, with excitation at 498 nm and emission at 522 nm. Fluorescence was measured with an **Agilent Cary Eclipse fluorescence spectrophotometer (**McLennan and Degli Esposti, 2000**) and expressed as nmol/min/mg protein.**

2.10. Protein expression studies: HIF-1α and its regulated proteins

HIF-1α and its downstream proteins were studied in lung tissue samples from all control and hypoxia-preconditioned rats through western blotting. Hypoxia inducible factor 1α (HIF1α; MA1-16511, Thermo Fisher Scientific, Inc., Waltham, MA, USA) was measured in the nuclear extracts (extraction kit; NE-PER™ Thermo Scientific™, 78833) of the isolated snap-frozen lung tissues. Vascular endothelial growth factor (VEGF; Sc57496, Santa Cruz Biotechnology, Inc.), erythropoietin (EPO; Sc5290, Santa Cruz Biotechnology, Inc.) and nitric oxide synthase (uNOS; NOS–3F7–B11 B5, Invitrogen) were measured in whole-tissue homogenates. The protein bands were visualized with an Alliance UVitec chemiluminescence imaging system (Cleaver Scientific Ltd., England, United Kingdom). Densitometric analysis of the differential expression of proteins was carried out by using ImageJ software (ImageJ 1.52a). Furthermore, the housekeeping protein α-tubulin (MA5-38221**;** Thermo Fisher Scientific, Inc., Waltham, MA, USA) was assessed in the same samples as an internal standard.

2.11. Histology for assessing pathological damage

The rats were anesthetized and perfused transcardially with ice-cold PBS, followed by ice-cold 4 % paraformaldehyde. Lungs were extracted and stored in 10 % formalin for 24 h at room temperature and then processed further for paraffin embedding. Approximately 5-micron-thick lung tissue sections were cut with a microtome and then stained with hematoxylin and eosin. The slides were observed under a light microscope (Olympus BX51TF, Olympus Corporation, Centre Valley, PA, USA), and images were captured with a digital camera (Nikon, Tokyo, Japan) attached to the microscope. The data were analyzed by comparing three lung tissue sections obtained from each group for histopathological changes.

2.12. Statistical analysis

The results are expressed as the means ± SDs. Differences were considered statistically significant at $p < 0.05$. Intergroup comparisons of the physiological variables, hematological variables, blood gas variables, ROS and biochemical metabolites were performed via one-way ANOVA with Tukey's correction. All the statistical tests were performed with GraphPad Prism 8.4.3. The relative densities of the HIF-1α bands and related proteins were determined via semiquantitative densitometric analysis with ImageJ software (V 1.8.0; http://imagej.nih.gov/ij). The extent of pathological changes in the histology of the lung tissue sections from all the groups of animals were graded quantitatively as follows: , no change; +, mild; ++, moderate; +++, high and ++++, severe change.

3. Results

3.1. Changes in physiological variables

The baseline physiological variables of the rats recorded under normoxic conditions were 98.33 ± 2.03 % (SpO_2), 334.93 ± 41.11 bpm (HR), and 94.63 ± 15.02 brpm (RR). Different durations of HP had variable effects on SpO_2, HR and RR. The results revealed variations in the mean SpO_2 level across the groups. Compared with the extreme hypoxia (EH) group without hypoxia preconditioning, the HP-2 day, HP-3 day and HP-4 day hypoxia preconditioning groups presented significantly greater levels of SPO_2 at $p < 0.0001$ (HP-3 day) and $p < 0.05$ (HP-2 day and HP-4 day), respectively, by the 6th hour of extreme hypoxia exposure (Fig. 3a). The rest of the groups differed non-significantly. Compared with that in the EH group, the heart rate increased, which was not significantly different (Fig. 3b). The respiratory rate increased in all the hypoxia-preconditioned and extreme hypoxia-exposed groups compared with the normoxia groups (94.63 ± 15.02 brpm to 147.03 ± 15.68 brpm). Compared with EH group, RR was significantly different ($p < 0.05$ through HP-2 day, $p < 0.05$ through HP-3 day, $p < 0.001$ through HP-4 day and $p < 0.05$ through HP-5 day) (Fig. 3c).

3.2. Hematological changes

Upon exposure of the rats to extreme hypoxia after the stipulated time period of hypoxia preconditioning, hematological variables, white blood cells (WBCs), red blood cells (RBCs), hemoglobin (HGB), hematocrit (HCTs), platelets (PLTs) and nucleated red blood cells (NRBCs), were found to increase subsequently from normoxic values. Statistical comparison of the hypoxia preconditioned groups with that of EH group via one-way ANOVA revealed significant differences (Table 1).

3.3. Blood gas analysis

The blood gas parameters changed with decreasing FiO_2 %. Blood pH increased significantly ($p < 0.01$) during the 3-day HP, whereas other groups showed non-significant difference (Fig. 4a). pCO_2 was significantly lower ($p < 0.05$) during the 2-day HP, 3-day HP and the 4-day HP, whereas 1-day HP, 5-day HP and EH were found to be significantly greater ($p < 0.01$) (Fig. 4b). PO_2 was found to be significantly higher ($p < 0.01$) during the 3-day HP treatment group as compared with the EH group, while other HP groups showed a non-significant difference (Fig. 4c). Changes in HCO_3^- were found to be non-significant in all the HP groups as compared to EH group (Fig. 4d). The base excess (BE) were significantly higher in all the hypoxia preconditioned groups as compared to EH (Fig. 4e).

3.4. Biochemical metabolite assay

NO (nitrate + nitrite) increased in all the hypoxia-preconditioned groups and EH groups, showing a statistically non-significant difference (Fig. 5a). An elevated plasma level of the precursor molecule L-arginine was observed in the peripheral blood of the hypoxia-preconditioned groups (day 1 to day 5) and the EH group compared with that of the normoxia group (Fig. 5b). Citrulline was found to

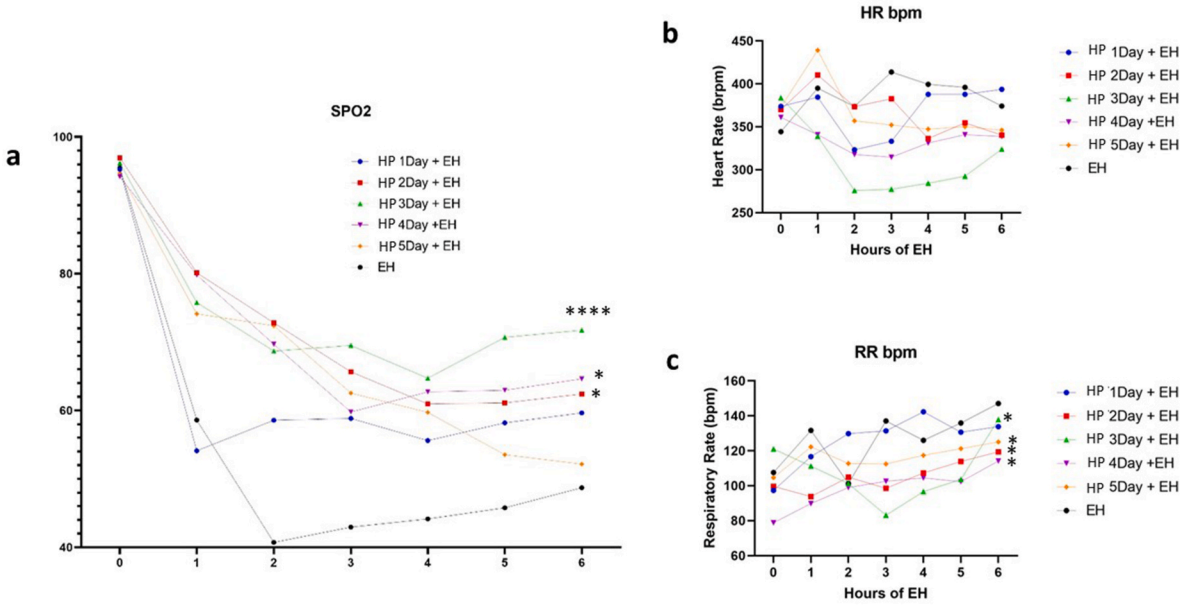


Fig. 3. Changes in Physiological Variables during Extreme Hypoxia Exposure at **8 % FiO_2. A)** Oxygen saturation (SpO_2 %) **B)** Respiratory Rate (bpm) **C)** Heart Rate (brpm) **(N)** Normoxia, **(HP1Day + EH; blue line with circle data point)** Hypoxia preconditioning for 4 h/1 Day followed by exposure to extreme hypoxia (8 % FiO_2, 6 h) **(HP 2Day + EH; red line with square data point)** Hypoxia preconditioning for 4 h/2 Day followed by exposure to extreme hypoxia (8 % FiO_2, 6 h). **(HP 3Day + EH; green line with triangle data point)** Hypoxia preconditioning for 4 h/3 Day followed by exposure to extreme hypoxia (8 % FiO_2, 6 h). **(HP 4Day + EH; purple line with inverted triangle data point)** Hypoxia preconditioning for 4 h/4 Day followed by exposure to extreme hypoxia (8 % FiO_2, 6 h). **(HP5Day + EH; orange line with square data point)** Hypoxia preconditioning for 4 h/5 Days followed by exposure to extreme hypoxia (8 % FiO_2, 6 h). **(EH; black line with inverted circle data point)** Direct exposure to extreme hypoxia (8 % FiO_2, 6 h). Data points are mean ± SD (n = 5). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Changes in Hematological variables through hypoxia preconditioning.

Parameters/groups	Normoxia	HP1Day + EH	HP2Day + EH	HP3Day + EH	HP4Day + EH	HP5Day + EH	EH
WBC (10^6/μl)	9.15 ± 1.36	10.27 ± 1.97**	10.62 ± 0.68*	12.22 ± 1.82	11.88 ± 1.14	13.27 ± 1.15	14.23 ± 2.16
RBC (10^6/μl)	5.88 ± 0.51	7.18 ± 0.45	7.82 ± 0.30	7.40 ± 0.13	7.87 ± 0.66	7.77 ± 0.18	7.96 ± 0.37
HGB (g/dl)	13.78 ± 0.67	15.60 ± 0.71**	17.62 ± 0.34	17.80 ± 1.70	18.76 ± 1.76	17.26 ± 0.32	18.58 ± 0.56
HCT (%)	32.74 ± 2.1	38.20 ± 1.08***	42.82 ± 0.58	41.82 ± 0.42	45.66 ± 4.16	42.52 ± 0.97	46.62 ± 3.42
PLT (10^3/μl)	480.2 ± 51.72	672.2 ± 17.82	635.4 ± 27.01	635.6 ± 137.9	619.4 ± 127.2	670.4 ± 43.96	761.0 ± 47.82
NRBC (%/μl)	2.32 ± 1.16	55.16 ± 17.13***	59.61 ± 8.58**	43.28 ± 1.27****	57.39 ± 6.72***	77.45 ± 7.32	82.71 ± 3.75

White blood cells (WBC) in 10^6/μl; Red blood cells (RBC) in 10^6/μl; Hemoglobin (HGB) in g/dl; Hematocrit (HCT) in %; Platelet (PLT) in 10^3/μl; Nucleated RBC (NRBC) in %/μl. Values are mean ± SD (n = 5). NS, Non-significant; *p < 0.05, compared to EH; **p < 0.01, compared to EH; ***p < 0.001, compared to EH.

decrease while exposure to hypoxia (both HP and EH groups). The hypoxia preconditioned groups (HP-2 day to HP-5 day) was found to be statistically higher as compared to EH at $p < 0.05$ (Fig. 5c). Succinate was found to increased while exposure to hypoxia showing a non-significant difference between the hypoxia preconditioned groups and EH (Fig. 5d). BUN in the HP groups showed a non-statistical difference as compared to EH (Fig. 5e). L-lactate was found to be statistically lower in the HP-2 day and HP-3 day at $p < 0.05$ as compared to EH (Fig. 5f).

3.5. Reactive oxygen species (ROS) generation due to the hypoxia preconditioning effect

There was increased generation of ROS in all the hypoxia-exposed groups. Statistically significant differences were observed during HP-2day, HP-3day, HP-4day and HP-5day as compared to EH at $p < 0.05$ (Fig. 6).

3.6. Changes in the expression of hypoxia-inducible protein-1 alpha (HIF-1α) and its regulated proteins through hypoxia preconditioning

HIF-1α and its regulatory protein were studied in lung tissue samples via western blotting (Fig. 7A) and semi-quantified via densitometric analysis (Fig. 7B). HP-3 day was statistically significant different as compared to EH at $p < 0.05$. Rest of the hypoxia preconditioned groups showed a non-significant difference as compared to EH (Fig. 7B–i). EPO was non-significantly difference between all the HP groups as compared to EH (Fig. 7B–ii). VEGF was significantly lower in the HP-1 day ($p < 0.01$), HP-3 day ($p < 0.001$) and HP-4day ($p < 0.01$) as compared to EH (Fig. 7B–iii). The nitric oxide synthase (uNOS) level was significantly lower during the HP-2 day ($p < 0.05$) and HP-3 day ($p < 0.01$) as compared to EH (Fig. 7B–iv). The standard housekeeping protein α-tubulin was expressed at equal levels in all the groups of animals (Fig. 7A–e).

3.7. Histopathological changes due to hypoxia preconditioning in lung tissue sections

The lung tissue sections from all the groups of animals exhibited marked morphological changes because of HP and extreme hypoxia exposure (Fig. 8a–g). Lung tissue sections from normoxic rats demonstrated no pathological changes, as normal lung parenchyma was observed (Fig. 8a). Animals subjected to HP sessions (Fig. 8 b, c, d, e, f) and extreme hypoxia exposure without HP (Fig. 8 g) presented perivascular edema (PVE) along the blood vessels and interstitial edema as

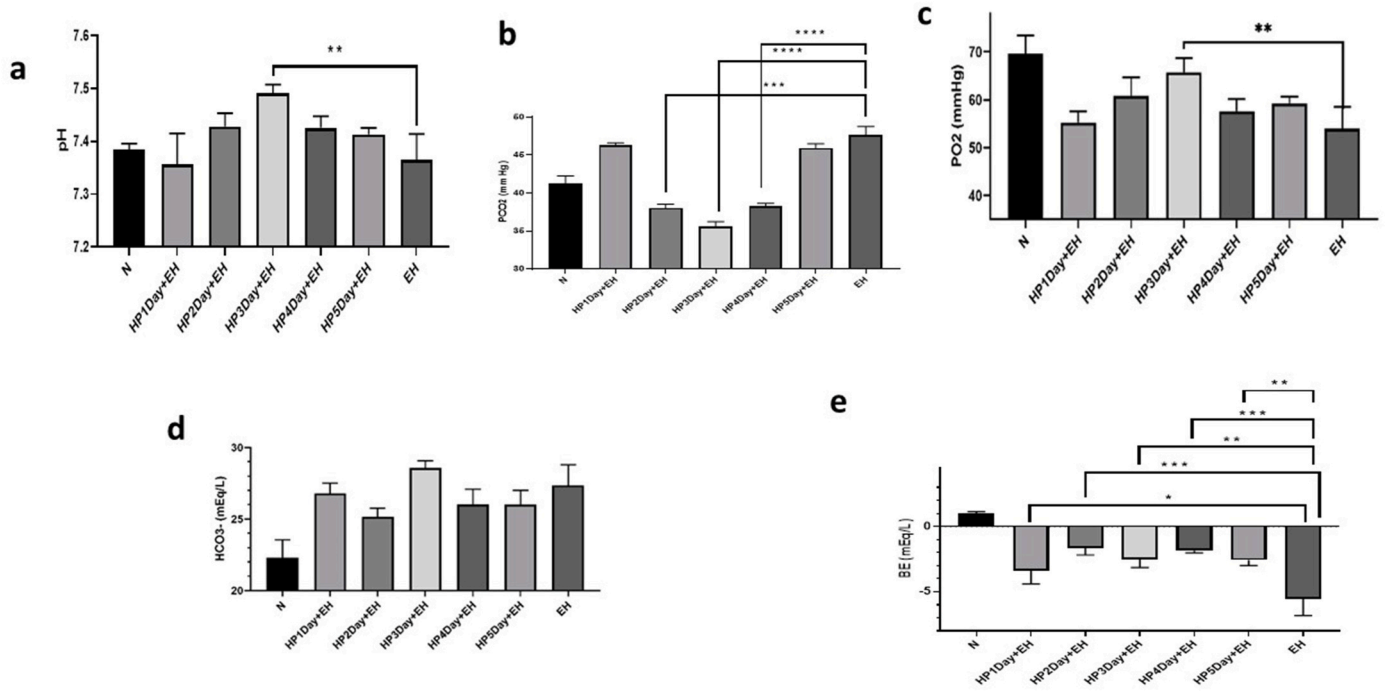


Fig. 4. Changes in blood gas variables due to hypoxia pre-conditioning. PCO_2 mm Hg (Partial Pressure of Carbon Dioxide), PO_2 mm Hg (Partial Pressure of Oxygen), HCO_3^- mmol/L (Bicarbonate), BE mmol/L (Base Excess). **(N)** Normoxia, **(HP1Day + EH);** Hypoxia preconditioning for 4 h/1 Day followed by exposure to extreme hypoxia, **(HP 2Day + EH);** Hypoxia preconditioning for 4 h/2 Day followed by exposure to extreme hypoxia, **(HP 3Day + EH);** Hypoxia preconditioning for 4 h/3 Day followed by exposure to extreme hypoxia, **(HP 4Day + EH)** Hypoxia preconditioning for 4 h/4 Day followed by exposure to extreme hypoxia, **(HP5Day + EH)** Hypoxia preconditioning for 4 h/5 Days followed by exposure to extreme hypoxia, **(EH)** Direct exposure to extreme hypoxia. Data points are mean ± SD (n = 5).*p < 0.05, compared to EH; **p < 0.01, compared to EH; ***p < 0.001, compared to EH.

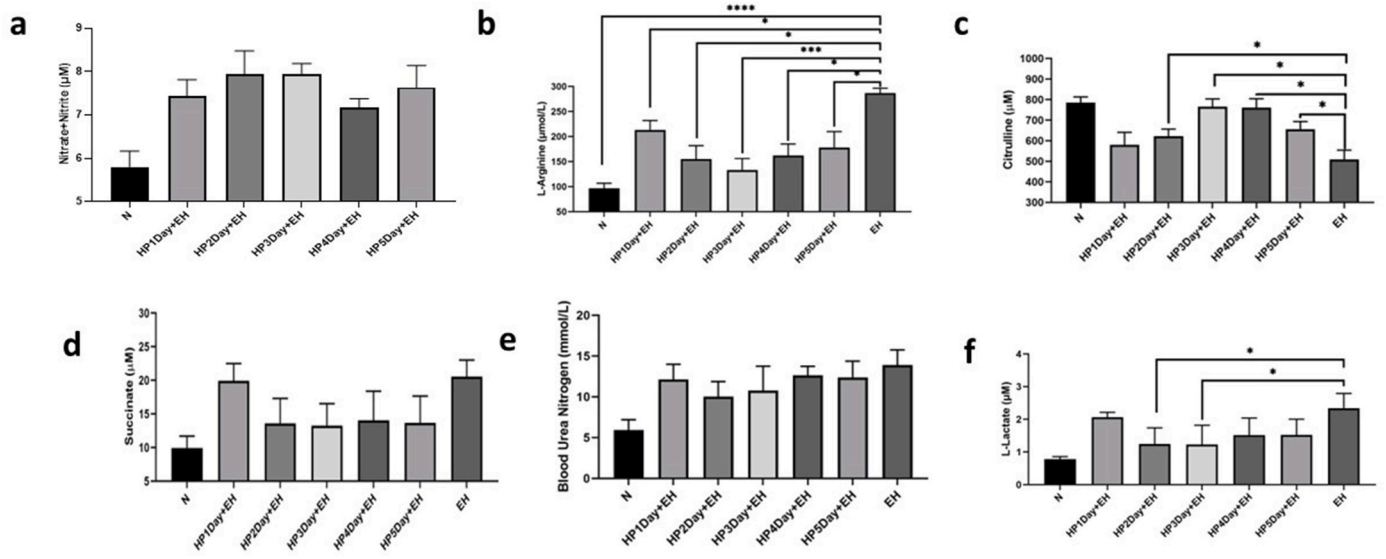


Fig. 5. Changes in Biochemical and metabolic variables due to hypoxia pre-conditioning. **(N)** Normoxia, **(HP1Day + EH);** Hypoxia preconditioning for 4 h/1 Day followed by exposure to extreme hypoxia (8 % FiO_2, 6 h), **(HP 2Day + EH);** Hypoxia preconditioning for 4 h/2 Day followed by exposure to extreme hypoxia (8 % FiO_2, 6 h), **(HP 3Day + EH);** Hypoxia preconditioning for 4 h/3 Day followed by exposure to extreme hypoxia (8 % FiO_2, 6 h), **(HP 4Day + EH)** Hypoxia preconditioning for 4 h/4 Day followed by exposure to extreme hypoxia (8 % FiO_2, 6 h), **(HP5Day + EH)** Hypoxia preconditioning for 4 h/5 Days followed by exposure to extreme hypoxia (8 % FiO_2, 6 h), **(EH)** Direct exposure to extreme hypoxia (8 % FiO_2, 6 h). Data points are mean ± SD (n = 5).*p < 0.05, compared to EH; **p < 0.01, compared to EH; ***p < 0.001, compared to EH.

well as peri-bronchial and intraparenchymal inflammatory cell infiltration in all hypoxia preconditioned groups as well as the extreme hypoxia group without hypoxia preconditioning. However, the intensity of occurrence differed (depicted in the summary table of histopathological changes). Epithelial proliferation was observed in the extreme hypoxia group without HP (Fig. 8g). Emphysematous lesions were observed in the HP-1 day, HP-5 day and EH groups.

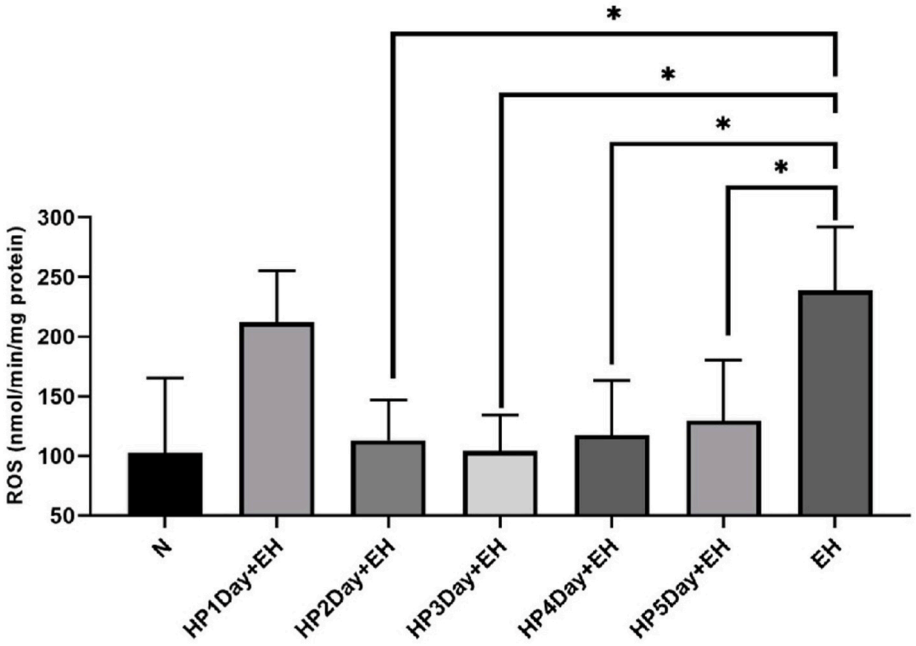


Fig. 6. Effect of hypoxia pre-conditioning on Reactive Oxygen species (ROS).**(N)** Normoxia, **(HP1Day + EH);** Hypoxia preconditioning for 4 h/1 Day followed by exposure to extreme hypoxia (8 % FiO_2, 6 h), **(HP 2Day + EH);** Hypoxia preconditioning for 4 h/2 Day followed by exposure to extreme hypoxia (8 % FiO_2, 6 h), **(HP 3Day + EH);** Hypoxia preconditioning for 4 h/3 Day followed by exposure to extreme hypoxia (8 % FiO_2, 6 h), **(HP 4Day + EH)** Hypoxia preconditioning for 4 h/4 Day followed by exposure to extreme hypoxia (8 % FiO_2, 6 h), **(HP5Day + EH)** Hypoxia preconditioning for 4 h/5 Days followed by exposure to extreme hypoxia (8 % FiO_2, 6 h), **(EH)** Direct exposure to extreme hypoxia (8 % FiO_2, 6 h). Data points are mean ± SD (n = 5).*p < 0.05, compared to EH; **p < 0.01, compared to EH; ***p < 0.001, compared to EH.

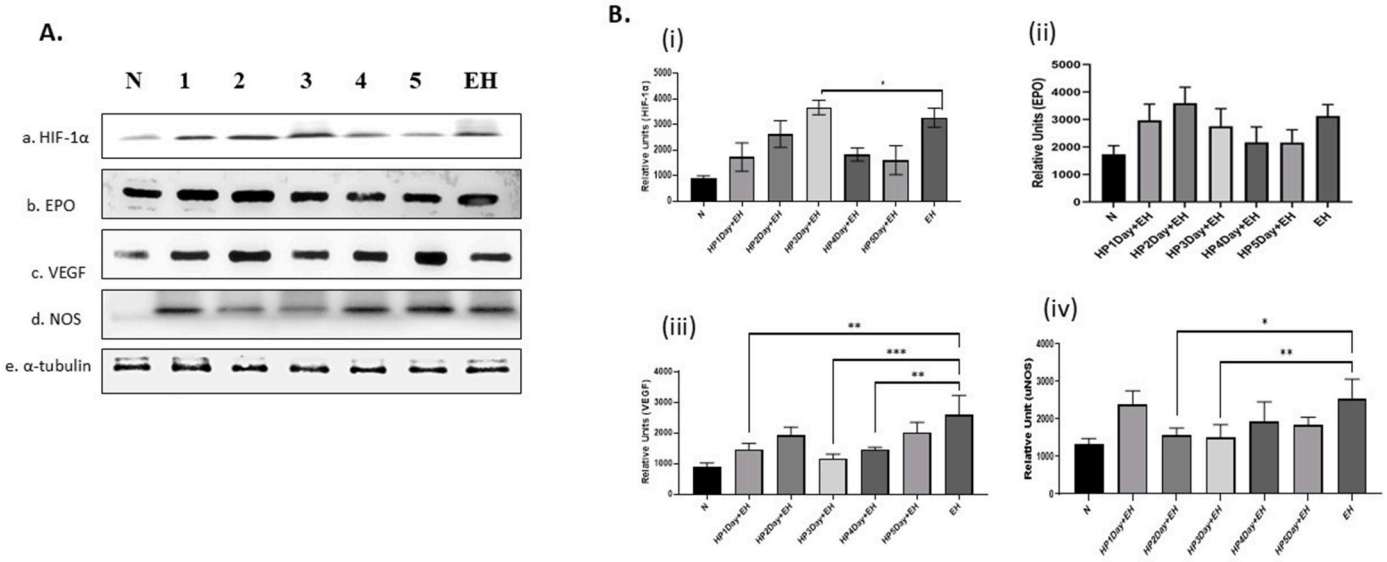
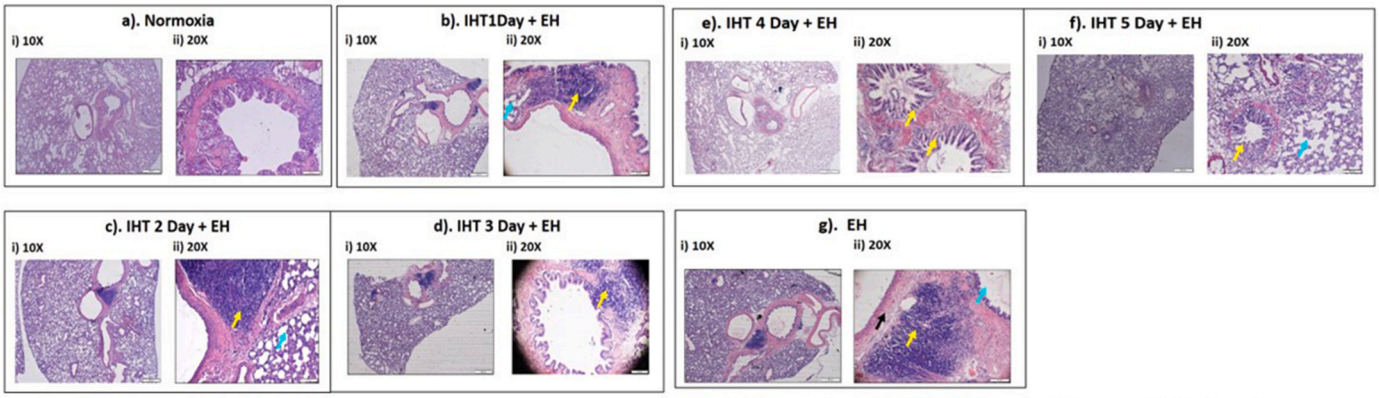


Fig. 7. Changes in protein expression due to hypoxia preconditioning. (a) HIF-1α, (b) EPO, (c) VEGF, (d) NOS, and (e) α – tubulin A) Representative western blotting results from 3 independent experiments and B) Relative densitometric analysis expressed as relative units, Values are mean ± SD (n = 3).).*p < 0.05, compared to EH; **p < 0.01, compared to EH; ***p < 0.001, compared to EH.

4. Discussion

4.1. Changes in physiological variables

Reports have suggested that the association of SpO_2 with AMS is proportional to altitude increase, as reported by Chen et al. (2012) and Karinen et al. (2010). They reported that an increase in altitude between 3500 m and 5300 m corresponds to an increased risk of developing AMS with a reduced SpO_2 level. We observed that hypoxia preconditioning at 12 % FiO_2 for as little as 1 day led to improved SpO_2 levels compared with those of the group exposed to hypoxia without preconditioning. Hypoxia preconditioning for 3 days was the most efficient group for reducing the extent of oxygen desaturation, reflecting a gradual decrease and maintaining a constant and relatively high SPO_2 level. Our study is therefore consistent with previous studies conducted under high-altitude hypoxic conditions (Nagel et al., 2020; Taralov et al., 2018) as well as normobaric hypoxic conditions (Nimje et al., 2020), showing that preacclimatization with HP leads to an improved SpO_2 %



Interstitial edema as well as peribranchial and intraparenchymal inflammatory cell infiltrations (yellow arrows). Emphysematous lesions (blue arrows). Epithelial proliferation (black arrows)

Summary of Histopathological changes

Groups	Perivascular Edema	Alv. Septal Thickening	Inflamm. Cells in Alv. spaces	Emphysematous lesions
Normoxia	-	-	-	-
HP 1 Day + EH	+++	+	++	++
HP 2 Day + EH	++	+	++	+
HP 3 Day + EH	+	-	+	-
HP 4 Day + EH	+	-	+	-
HP 5 Day + EH	+	-	+	+
EH	+++	++	++++	++

Fig. 8. Histological analysis of lung tissue sections as observed with hematoxylin-eosin **staining.** (i) 10X. (ii) 20X a) Normoxia (N). Lung from normoxia rats showing a normal lung parenchyma b) HP1Day + EH. c) HP2Day + EH. d) HP3Day + EH. e) HP4Day + EH. f) HP5Day + EH. g) EH. Yellow arrows- Interstitial edema and peribronchial and intraparenchymal inflammatory cell infiltrations; Blue arrows-Emphysematous lesions; Black arrows- Epithelial proliferation. Table showing summary of histopathological changes through hypoxia preconditioning. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

compared with that of the innate group without hypoxia preconditioning. The elevated levels of SpO_2 maintained in the hypoxia preconditioned groups may be attributed to the augmented sensory response of the peripheral chemoreceptors to acute hypoxia, leading to an increased hypoxic ventilatory response. Ainslie et al. (2007) and Peng et al. (2001) reported an increase in SpO_2 % following hypoxia preconditioning.

4.2. Hematological changes

Hypoxia leads to an increase in the WBC count (Kasperska and Zembron-Lacny, 2020; Wang et al., 2018). WBCs are thought to be biomarkers of inflammation (Wirth et al., 2018). WBC, RBC, HGB, HCT and PLT increased with hypoxia preconditioning and extreme hypoxia exposure. Also a significant increase in the number of nucleated red blood cells (NRBCs) was observed in all the hypoxia-exposed groups, with a significantly lower percentage increase observed at 3 HP than in the other groups. Nucleated red blood cells are immature cells that spiked due to hypoxia. The size and nucleus of NRBCs are similar to those of lymphocytes. As a result, NRBCs are *reported in % (per* 100 WBC*)*. The presence of a significant number of NRBCs in peripheral blood may interfere with the WBC count, so a corrected WBC count was calculated and reported. Up to 1 nRBC/100 WBCs or absolute counts of $<$100 nRBCs/μL are most often considered normal. Nucleated RBCs of more than 5/100 WBCs or $>$400 nRBCs/μL are considered indicative of severe normo-blastosis (Moretti et al., 1999). Increased erythropoiesis might have induced the early release of NRBCs into the circulation, as observed in the present study, with elevated EPO. The EPO expression pattern was consistent with the NRBC% changes with each HP duration. The significantly lowest percentage of NRBC in the day 3 HP in comparison to EH and other HP groups, indicates neo-cytolysis representing the down-regulation of red blood cells, thereby decreasing the excess elevation in erythrocytes and hemoglobin. This indicates a physiological response to hypoxic acclimatization. Alfrey et al. (1997) reported that neo-cytolysis represents hypoxic acclimatization, indicating that young RBCs and red cell mass are no longer needed, thereby decreasing blood viscosity under hypoxic conditions. Furthermore, the increase in hematological variables, namely, RBC, HGB, and HCT, might be due to hemoconcentration owing to enhanced diuresis and dehydration as a result of the high-altitude physiological response, as reported earlier by Bartsch and Gibbs (2007) on acute physiological adjustments and early acclimatization to high altitude. The latter needs to be investigated.

4.3. Changes in blood gas parameters in response to hypoxia preconditioning

Changes in blood gas parameters in response to hypoxia exposure are influenced by high-altitude acclimatization (Samaja, 1997). Venous blood sampling was used in this experiment because it is an alternative method to arterial blood sampling during blood gas analysis for estimating systemic carbon dioxide and pH (Schütz et al., 2019). The significantly reduced PCO_2 and higher PO_2 compared with those under EH group on HP-3 day and HP-4 day are the result of a hyperventilatory acclimatization response leading to metabolic alkalosis, as observed with significantly higher base excess in the HP-3 day and HP-4 day as compared to EH, which further increases the pH in these groups of animals, thereby accelerating acclimatization.

4.4. Changes in biochemical, metabolic and HIF-1α and downstream protein expression in response to hypoxia preconditioning

With exposure to high-altitude hypoxia, significant transformation of tissue metabolism occurs, which alters circulating metabolic levels (Chicco et al., 2018; O'Brien et al., 2019). Nitric oxide (NO) formed by the oxidation of nitrogen is a metabolite of nitric oxide synthase (NOS), which plays a significant role as an antioxidant (Wink et al., 2001), regulator of intermediary metabolism (Jobgen et al., 2006) and cellular energy production by mitochondria (Taylor and Moncada, 2010). Increased NO production acts as an integral part of the human physiological response to hypoxia (Levett et al., 2011) and is produced by the enzymatic conversion (nitric oxide synthase) of its precursor molecule L-arginine into its substrate L-citruline (Luiking et al., 2010). Furthermore, NO is acutely oxidized to nitrite and nitrate. Under hypoxic conditions, the oxidized product of NO, which is nitrite, can be reduced to form NO (Shiva, 2013). Our finding of significantly higher NO (nitrate + nitrite) and lower L-arginine in the hypoxia-preconditioned group in comparison to the EH group might be an adaptive physiological response to hypoxia preconditioning associated with angiogenesis and vascular remodeling. Concomitantly, exposure to hypoxia resulted in a significantly lower level of circulating citrulline, a nonessential amino acid and a precursor of L-arginine. However, the hypoxia preconditioning effect resulted in a higher level of citrulline than that in the group exposed directly to extreme hypoxia without preconditioning. Previous studies on human subjects revealed a positive correlation between low plasma citrulline levels and acute respiratory distress syndrome in patients with severe sepsis (Ware et al., 2013). Furthermore, a recent study on human subjects exposed to 4559 m above sea level revealed positive correlations of citrulline with alveolar PO_2 and SpO_2 (Hannemann et al., 2023). Thus, the results of the present study, which revealed relatively higher citrulline levels in the hypoxia-preconditioned groups in comparison to EH, indicate hypoxic acclimatization. Furthermore, the expression of NOS, a group of enzymes that catalyzes the production of NO from L-arginine, was assessed via the use of a uNOS antibody (Thermo Fisher), which can detect all 3 isoforms of NOS: nNOS, iNOS and eNOS. In the present study, there was a lower NOS in the lung tissue samples from HP-2 day and HP-3 day sessions than in those from the other groups. The lower level of NOS in these groups might be attributed to reduced inflammatory and oxidative stress responses through HP-2 day and HP-3 day exposure. Studies have revealed that under various pathological conditions, neuronal and inducible NO synthases (nNOS and iNOS) become uncoupled and generate the harmful free radical superoxide (O^{2-}) in addition to synthesizing NO, which potentiates oxidative stress (Karbach et al., 2014). Moreover, succinate and lactate, which are intermediates of tricarboxylic acid, are very important metabolites. Succinate is often known as an inflammatory signal and a marker of tissue hypoxia (Lukyanova et al., 2018) and is also involved in the formation and elimination of reactive oxygen species. There are reports of succinate induced oxidative stress and neuronal injury in hypoxia-exposed rats (Zhang et al., 2022). In the present study, rats subjected to hypoxia preconditioning (HP-2 day to HP-5 day) presented non-significant decreases in succinate and lactate concentrations as compared to EH. These findings indicate that the hypoxia preconditioning effect significantly reduces oxidative stress and the inflammatory response, as evidenced by reduced ROS generation and inflammatory cell infiltration in the lung tissues of rats subjected to hypoxia preconditioning (HP-2 day to HP-5 day). Hypoxia preconditioning for 1 day duration was not sufficient enough to reduce the extreme hypoxia induced oxidative stress and the inflammatory response. Similar to our study, earlier clinical trials in human subjects with hypoxic preconditioning reported reduced oxidative stress and inflammatory responses caused by hypoxia (Baillieul et al., 2017; Coimbra-Costa et al., 2021). Furthermore, the histopathological results revealed that direct exposure of the rats to extremely hypoxic conditions resulted in histopathological damage to the lung tissue sections, with interstitial edema, peribronchial and intraparenchymal inflammatory cell infiltration, and emphysematous lesions. Similarly, protein expression studies revealed a similar pattern of changes with respect to changes in VEGF. Reports have shown that increased expression of VEGF is associated with lung tissue damage (Kaner and Crystal, 2001), as observed with elevated VEGF in the epithelial lining of the alveoli, which induces pulmonary edema.

5. Conclusion

Taken together, moderate preconditioning to hypoxia at 12 % FiO_2 for 4 h/day for 3 consecutive days was postulated as a potent approach for non-pharmacological therapeutic modalities that triggers effective physiological and molecular changes to induce hypoxic acclimatization. Our results revealed that the pro-acclimatization responses of the hypoxia preconditioned group to extremely hypoxic conditions are associated with an increase in potential hematological markers along with a resultant up-regulation of erythropoietic processes and alterations in various molecules (HIF-1α, VEGF, EPO, and uNOS) and biochemical pathways (NO and related metabolites). All these changes further help achieve tissue oxygenation under extremely hypoxic conditions via ventilatory and hematopoietic acclimatization. The optimized Hypoxia preconditioned schedule can further be extrapolated and implemented in designing the strategies for faster acclimatization to high altitude hypoxia and improved endurance under extreme high-altitude condition. Further studies can be conducted to test the duration of the effectiveness of hypoxic acclimatization through the optimized hypoxia preconditioning duration. Additionally, hypoxia preconditioning can be standardized to maintain its effectiveness for long-term durations.

6. Limitations of the study

The pulse oximeter system used in the present study is a noninvasive method of monitoring and measuring the physiological parameters of conscious and unanaesthetised animals. However, this system is limited with respect to the movement of the animal. Accurate oxygen saturation cannot be measured while the animal is moving excessively. The working principle of the pulse oximetry system allows calculations of oxygen saturation to be obtained from light absorption measurements. The prerequisite for calculations without errors is that the distance between the sensor pads does not change. Moreover, the sensors must not be obstructed, such that the emitted light is not absorbed by any other material except peripheral blood. However, when a rat agitates or flexes muscles under the skin without noticeable surface movement, the measurement of oxygen saturation (SpO_2) is violated and does not provide accurate readings. Thus, an animal that is conscious and unrestrained must also remain quiet while the physiological parameter measurement is being recorded. The same might be responsible for the large amount of standard deviation observed in the physiological data. Another limitation of the present study is that, on the basis of the literature, it was assumed that 12 % FiO_2 was the most suitable percentage of hypoxia for inducing adaptive responses. The standardization of hypoxia preconditioning was thus performed for the duration of the exposures and not for FiO_2 %. The present study provides evidence of a suitable combination of intermittence and duration of hypoxia preconditioning for 12 % FiO_2 but does not provide any suggestions on the most accurate combination of FiO_2, duration, and intermittence.

CRediT authorship contribution statement

Megha A. Nimje: Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Himadri Patir:** Project administration, Methodology, Investigation, Data curation, Conceptualization. **Rajesh Kumar Tirpude:** Writing – review & editing, Supervision, Resources. **Prasanna K. Reddy:** Supervision, Resources. **Bhuvnesh Kumar:** Writing – review &

editing, Supervision, Resources.

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Data availability statement

The data used in this study are available in the manuscript and associated data files. Any data not readily available for this study will be provided from the corresponding author upon reasonable request.

Ethical compliance

All animal procedures and experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of the institute (IAEC Number: DIPAS/IAEC/2019/05) and followed the standards outlined in the Committee for the Control and Supervision of Experiments on Animals (CCSEA), Animal Welfare Board, Ministry of Agriculture, Government of India.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Ainslie, P.N., Barach, A., Cummings, K.J., Murrell, C., Hamlin, M., Hellemans, J., 2007. Cardiorespiratory and cerebrovascular responses to acute poikilocapnic hypoxia following intermittent and continuous exposure to hypoxia in humans. J. Appl. Physiol. 102, 1953–1961. https://doi.org/10.1152/japplphysiol.01338.2006.

Alfrey, C.P., Rice, L., Udden, M.M., Driscoll, T.B., 1997. Neocytolysis: physiological down- regulator of red-cell mass. Lancet 349, 1389–1390. https://doi.org/10.1016/S0140-6736(96)09208-2.

Bärtsch, P., Gibbs, J.S., 2007. Effect of altitude on the heart and the lungs. Circulation 116, 2191–2202. https://doi.org/10.1161/CIRCULATIONAHA.106.650796.

Beckman, J.S., Koppenol, W.H., 1996. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am. J. Physiol. 271, C1424–C1437. https://doi.org/10.1152/ajpcell.1996.271.5.C1424.

Bezruchka, S., 2005. Altitude Illness: Prevention & Treatment. The Mountaineers Books.

Bouhamida, E., Morciano, G., Perrone, M., Kahsay, A.E., Della Sala, M., Wieckowski, M. R., Fiorica, F., Pinton, P., Giorgi, C., Patergnani, S., 2022. The interplay of hypoxia signaling on mitochondrial dysfunction and inflammation in cardiovascular diseases and cancer: from molecular mechanisms to therapeutic approaches. Biology 11, 300. https://doi.org/10.3390/biology11020300.

Brüne, B., Zhou, J., 2007. Nitric oxide and superoxide: interference with hypoxic signaling. Cardiovasc. Res. 75, 275–282. https://doi.org/10.1016/j.cardiores.2007.03.005.

Chen, H.C., Lin, W.L., Wu, J.Y., Wang, S.H., Chiu, T.F., Weng, Y.M., Hsu, T.Y., Wu, M.H., 2012. Change in oxygen saturation does not predict acute mountain sickness on Jade Mountain. Wilderness Environ. Med. 23, 122–127. https://doi.org/10.1016/j.wem.2012.03.014.

Chicco, A.J., Le, C.H., Gnaiger, E., Dreyer, H.C., Muyskens, J.B., D'Alessandro, A., Nemkov, T., Hocker, A.D., Prenni, J.E., Wolfe, L.M., Sindt, N.M., Lovering, A.T., Subudhi, A.W., Roach, R.C., 2018. Adaptive remodeling of skeletal muscle energy metabolism in high- altitude hypoxia: lessons from AltitudeOmics. J. Biol. Chem. 293, 6659–6671. https://doi.org/10.1074/jbc.RA117.000470.

Coimbra-Costa, D., Garzón, F., Alva, N., Pinto, T.C.C., Aguado, F., Torrella, J.R., Carbonell, T., Rama, R., 2021. Intermittent hypobaric hypoxic preconditioning provides neuroprotection by increasing antioxidant activity, erythropoietin expression and preventing apoptosis and astrogliosis in the brain of adult rats exposed to acute severe hypoxia. Int. J. Mol. Sci. 22, 5272. https://doi.org/10.3390/ijms22105272.

Cymerman, A., Rock, P.B., 1994. Medical Problems in High Mountain Environments: A Handbook for Medical Officers. US Army Research Institute of Environmental Medicine, Natick, Mass. T94-2.

Dempsey, J.A., Forster, H.V., 1982. Mediation of ventilatory adaptations. Physiol. Rev. https://doi.org/10.1152/physrev.1982.62.1.262.

Hannemann, J., Freytag, J., Schiefer, L.M., Macholz, F., Sareban, M., Schmidt-Hutten, L., Stang, H., Schwedhelm, E., Swenson, E.R., Böger, R., Berger, M.M., 2023. Asymmetric and symmetric dimethylarginine in high altitude pulmonary hypertension (HAPH) and high altitude pulmonary edema (HAPE). Front. Physiol. 14, 1297636. https://doi.org/10.3389/fphys.2023.1297636. PMID: 38093907; PMCID: PMC10716225.

Jobgen, W.S., Fried, S.K., Fu, W.J., Meininger, C.J., Wu, G., 2006. Regulatory role for the arginine-nitric oxide pathway in metabolism of energy substrates. J. Nutr. Biochem. 17, 571–588. https://doi.org/10.1016/j.jnutbio.2005.12.001.

Kaner, R.J., Crystal, R.G., 2001. Compartmentalization of vascular endothelial growth factor to the epithelial surface of the human lung. Mol. Med. 7, 240–246.

Karbach, S., Wenzel, P., Waisman, A., Munzel, T., Daiber, A., 2014. eNOS uncoupling in cardiovascular diseases–the role of oxidative stress and inflammation. Curr. Pharm. Des. 20, 3579–3594. https://doi.org/10.2174/13816128113196660748.

Karinen, H.M., Peltonen, J.E., Kähönen, M., Tikkanen, H.O., 2010. Prediction of acute mountain sickness by monitoring arterial oxygen saturation during ascent. High Alt. Med. Biol. 11, 325–332. https://doi.org/10.1089/ham.2009.1060.

Kasperska, A., Zembron-Lacny, A., 2020. The effect of intermittent hypoxic exposure on erythropoietic response and hematological variables in elite athletes. Physiol. Res. 69, 283–290. https://doi.org/10.33549/physiolres.934316.

Kelm, M., 1999. Nitric oxide metabolism and breakdown. Biochim. Biophys. Acta Bioenerg. 1411, 273–289. https://doi.org/10.1016/S0005-2728(99)00020-1.

Levett, D.Z., Fernandez, B.O., Riley, H.L., Martin, D.S., Mitchell, K., Leckstrom, C.A., Ince, C., Whipp, B.J., Mythen, M.G., Montgomery, H.E., Grocott, M.P., Feelisch, M., 2011. The role of nitrogen oxides in human adaptation to hypoxia. Sci. Rep. 1, 109. https://doi.org/10.1038/srep00109.

Lukyanova, L.D., Kirova, YuI., Germanova, E.L., 2018. The role of succinate in regulation of immediate HIF-1α expression in hypoxia. Bull. Exp. Biol. Med. 164, 298–303. https://doi.org/10.1007/s10517-018-3976-2.

Luiking, Y.C., Engelen, M.P., Deutz, N.E., 2010. Regulation of nitric oxide production in health and disease. Curr. Opin. Clin. Nutr. Metab. Care 13, 97–104.

McNamara, P.J., El-Khuffash, A., 2017. 71-Oxygen transport and delivery. In: Fetal and Neonatal Physiology. Elsevier Inc, pp. 724–737.

Murray, A.J., Montgomery, H.E., Feelisch, M., Grocott, M.P.W., Martin, D.S., 2018. Metabolic adjustment to high-altitude hypoxia: from genetic signals to physiological implications. Biochem. Soc. Trans. 46, 599–607. https://doi.org/10.1042/BST20170502.

Muza, S.R., 2007. Military applications of hypoxic training for high-altitude operations. In: Medicine and Science in Sports and Exercise, pp. 1625–1631. https://doi.org/10.1249/mss.0b013e3180de49fe.

Nagel, M.J., Jarrard, C.P., Lalande, S., 2020. Effect of a single session of intermittent hypoxia on erythropoietin and oxygen-carrying capacity. Int. J. Environ. Res. Publ. Health 17, 1–10. https://doi.org/10.3390/ijerph17197257.

Nimje, M.A., Patir, H., Tirpude, R.K., Reddy, P.K., Kumar, B., 2020. Physiological and oxidative stress responses to intermittent hypoxia training in Sprague Dawley rats. Exp. Lung Res. 46, 376–392. https://doi.org/10.1080/01902148.2020.1821263.

O'Brien, K.A., Horscroft, J.A., Devaux, J., Lindsay, R.T., Steel, A.S., Clark, A.D., Philp, A., Harridge, S.D.R., Murray, A.J., 2019. PPARα-independent effects of nitrate supplementation on skeletal muscle metabolism in hypoxia. Biochim. Biophys. Acta Mol. Basis Dis. 1865, 844–853. https://doi.org/10.1016/j.bbadis.2018.07.027.

Peng, Y., Kline, D.D., Dick, T.E., Prabhakar, N.R., 2001. Chronic intermittent hypoxia enhances carotid body chemoreceptor response to low oxygen. Adv. Exp. Med. Biol. 499, 33–38. https://doi.org/10.1007/978-1-4615-1375-9_5.

Samaja, M., 1997. Blood gas transport at high altitude. Respiration 64, 422–428. https://doi.org/10.1159/000196718.

Schoene, R.B., 2008. Illnesses at high altitude. Chest 134, 402–416. https://doi.org/10.1378/chest.07-0561.

Schütz, N., Roth, D., Schwameis, M., Röggla, M., Domanovits, H., 2019. Can venous blood gas Be used as an alternative to arterial blood gas in intubated patients at admission to the emergency department? A retrospective study. Open Access Emerg. Med. 11, 305–312. https://doi.org/10.2147/OAEM.S228420. PMID: 31920407; PMCID: PMC6934126.

Semenza, G.L., 2011. Regulation of metabolism by hypoxia-inducible factor 1. Cold Spring Harbor Symp. Quant. Biol. 76, 347–353. https://doi.org/10.1101/sqb.2011.76.010678.

Shiva, S., 2013. Nitrite: a physiological store of nitric oxide and modulator of mitochondrial function. Redox Biol. 1, 40–44. https://doi.org/10.1016/j.redox.2012.11.005.

Tannenbaum, S.R., 1979. Nitrate and nitrite: origin in humans. Science 205, 1332–1335.

Taralov, Z.Z., Terziyski, K.V., Dimov, P.K., Marinov, B.I., Kostianev, S.S., 2018. Assessment of the impact of 10-day intermittent hypoxia on the autonomic control measured by heart rate variability. Physiol. Int. 105. https://doi.org/10.1556/2060.105.2018.4.31.

Taylor, C.T., Moncada, S., 2010. Nitric oxide, cytochrome C oxidase, and the cellular response to hypoxia. Arterioscler. Thromb. Vasc. Biol. 30 (4), 643–647. https://doi.org/10.1161/ATVBAHA.108.181628. Epub 2009 Aug 27. PMID: 19713530.

Viscor, G., Torrella, J.R., Pages, T., Corral, L., Ricart, A., Javierre, C., Ventura, J., 2018. Physiological and biological responses to short-term intermittent hypobaric hypoxia exposure : from sports and mountain medicine to New biomedical applications. Front. Physiol. 9, 1–20. https://doi.org/10.3389/fphys.2018.00814.

Wang, K., Zhang, M., Li, Y., Pu, W., Ma, Y., Wang, Y., Liu, X., Kang, L., Wang, X., Wang, J., Qiao, B., Jin, L., 2018. Physiological , Hematological and Biochemical Factors Associated with High- Altitude Headache in Young Chinese Males Following Acute Exposure at 3700 M.

Wang, Y., Zhang, Q., Ma, Q., Wang, Q., Huang, D., Ji, X., 2024. Intermittent hypoxia preconditioning can attenuate acute hypoxic injury after a sustained normobaric hypoxic exposure: a randomized clinical trial. CNS Neurosci. Ther. 30 (3), e14662. https://doi.org/10.1111/cns.14662. PMID: 38477221; PMCID: PMC10934266.

Ware, L.B., Magarik, J.A., Wickersham, N., et al., 2013. Low plasma citrulline levels are associated with acute respiratory distress syndrome in patients with severe sepsis. Crit. Care 17, R10. https://doi.org/10.1186/cc11934.

Wink, D.A., Miranda, K.M., Espey, M.G., Pluta, R.M., Hewett, S.J., Colton, C., Vitek, M., Feelisch, M., Grisham, M.B., 2001. Mechanisms of the antioxidant effects of nitric oxide. Antioxidants Redox Signal. 3, 203–213. https://doi.org/10.1089/152308601300185179.

Wirth, M.D., Sevoyan, M., Hofseth, L., Shivappa, N., Hurley, T.G., Hébert, J.R., 2018. The dietary inflammatory index is associated with elevated white blood cell counts in the national health and nutrition examination survey. Brain Behav. Immun. 69, 296–303. https://doi.org/10.1016/j.bbi.2017.12.003.

Young, A., Reeves, J., 2003. Human Adaptation to High Terrestrial Altitude.

Zhang, M., Cheng, Y., Zhai, Y., et al., 2022. Attenuated succinate accumulation relieves neuronal injury induced by hypoxia in neonatal mice. Cell Death Discov. 8, 138. https://doi.org/10.1038/s41420-022-00940-7.