Research Article

Intermittent short-duration reoxygenation protects against simulated high altitude-induced pulmonary hypertension in rats

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Abstract
High-altitude pulmonary hypertension (HAPH) is a severe and progressive disease caused by chronic hypoxia and subsequent pulmonary vascular remodeling. No cure is currently available owing to an incomplete understanding about vascular remodeling. It is believed that hypoxia-induced diseases can be prevented by treating hypoxia. Thus, this study aimed to determine whether daily short-duration reoxygenation at sea level attenuates pulmonary hypertension under high-altitude hypoxia. To this end, a simulated 5000-m hypoxia rat model and hypoxic cultured human pulmonary artery smooth muscle cells were used to evaluate the effect of short-duration reoxygenation. Results show that intermittent, not continuous, short-duration reoxygenation effectively attenuates hypoxia-induced pulmonary hypertension. The mechanisms underlying the protective effects involved that intermittent, short-duration reoxygenation prevented functional and structural remodeling of pulmonary arteries and proliferation, migration, and phenotypic conversion of pulmonary artery smooth muscle cells under hypoxia. The specific genes or potential molecular pathways responsible for mediating the protective effects were also characterised by RNA sequencing. Further, the frequency and the total time of intermittent reoxygenation affected its preventive effect of HAPH, which was likely attributable to augmented oxidative stress. Hence, daily intermittent, not continuous, short-duration reoxygenation partially prevented pulmonary hypertension induced by 5000-m hypoxia in rats. This study is novel in revealing a new potential method in preventing HAPH. It gives insights into the selection and optimisation of oxygen supply schemes in high-altitude areas.

Abbreviations: ACh, acetylcholine; ASMCs, artery smooth muscle cells; CCK-8, cell counting Kit-8; DHE, dihydroethidium; GSH-Px, glutathione peroxidase; HAPH, high-altitude pulmonary hypertension; HPASMCs, human pulmonary artery smooth muscle cells; MDA, malondialdehyde; OPN, osteopontin; PE, phenylephrine; PFA, paraformaldehyde; ROS, reactive oxygen species; RVSP, right ventricular systolic pressure; SNP, sodium nitroprusside; VEGF, vascular endothelial growth factor; α-SMA, α-smooth muscle actin.
Qiang Lyu and Yungang Bai contributed equally to this work.

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1 | INTRODUCTION

In recent years, the number of lowlanders who need to work and stay in high-altitude areas for several months or years has increased. Compared with permanent residents, sojourners living at high altitudes experience more challenges.\(^1,2\) One of the most serious problems that individuals chronically exposed to hypoxia may suffer from is high-altitude pulmonary hypertension (HAPH).\(^3\) HAPH is a progressive disease that results in right heart dysfunction and is associated with poor prognosis. The hallmarks of HAPH include impaired pulmonary vasoreactivity and increased muscularisation of distal pulmonary vessels.\(^3,4\) Therefore, a clear understanding about the mechanisms of vascular remodeling may lead to the development of novel therapeutic approaches for the prevention and/or treatment of HAPH.

Current treatments for HAPH involve lowering the altitude to increase oxygen supply and administering pharmacological drugs, including prostanoids, endothelin receptor blockers, and phosphodiesterase-5 inhibitors.\(^5-7\) These pharmacological treatments mainly focus on reversing the abnormal arterial remodeling. However, these medications are not sufficient to totally regress vascular remodeling and prevent right heart dysfunction due to the multiple mechanisms involved in arterial remodeling after hypoxia. Therefore, the development of more effective prevention and treatment options against HAPH remains the primary focus.

The best way to prevent hypoxia-induced diseases is to relieve individuals from hypoxia.\(^4,8\) In aviation physiology, oxygen supply based on physiological equivalent altitude is one of the main ways to prevent the effects of hypoxia. According to this principle, when the inspired oxygen concentration is 42% at 5000 m, the physiological equivalent altitude is about sea level. A previous work in our laboratory confirmed that rats staying at 5000 m in a hypobaric chamber for 2 weeks did not experience growth inhibition or cardiovascular dysfunction as long as their oxygenation levels were equivalent to the one at sea level. However, maintaining the inspired oxygen concentration at approximately 42% at 5000 m all day for individuals staying in high-altitude areas is impractical and uneconomical.

Our previous work suggested another possible and effective method to prevent HPAH, which consisted of short-duration reoxygenation. Specifically, we used the hindlimb unweighting rat model to explore the effect of simulated weightlessness on the cardiovascular system. In the study, we observed that 1 hour of intermittent artificial gravity (1 hour of restoring to normal posture) daily prevented the remodeling of some arteries caused by 23 hours of simulated microgravity.\(^9,10\) Inspired by that possible “arterial memory effect” in above work, we speculate whether intermittent recovery of normal oxygen supply can also block or alleviate pulmonary arterial remodeling caused by chronic hypoxia. Furthermore, previous studies reported that frequent oxygen inhalation alleviated the symptoms in residents living at high altitudes.\(^11,12\) However, these studies focused more on relieving the symptoms of acute or chronic mountain sickness rather than paying attention to the development of pulmonary arterial remodeling disease. Moreover, research focusing on the relationship between protective effect and reoxygenation level, frequency, or oxygen saturation is lacking.

In this study, we used a hypobaric chamber to simulate 5000-m altitude hypoxia. By changing the gas composition in the chamber based on the physiological equivalent altitude, we observed whether daily short-duration reoxygenation at sea level could prevent pulmonary hypertension caused by 5000-m altitude exposure for 2 weeks in rats.

2 | MATERIALS AND METHODS

2.1 | Animals

Male Sprague–Dawley (SD) rats (body weight, 220-250 g) were supplied by the Laboratory Animal Centre of the Fourth Military Medical University. All animals were placed in a room with a 12:12-h light-dark cycle at 23 ± 1°C with standard lab chow and water available ad libitum.

2.2 | Ethics statement

All experimental treatments and procedures employed in this study were approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University. Furthermore, all animal experiments complied with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

2.3 | Hypoxia rat model

In order to investigate whether intermittent short-duration reoxygenation at sea level can prevent HAPH, rats were
randomly assigned into five groups and subjected to different oxygenation treatments each day (Figure 1, protocol 1). For the normoxia (N) group, rats were exposed to 400 m (laboratory altitude, gas composition: 21% O₂-79% N₂) for 24 hours each day. For the hypobaric normoxia (HN) group, rats were exposed to a hypobaric chamber with a pressure of improved text.
405 mm Hg to simulate hypoxia at about 5000 m as well as to 42% O2-58% N2 to ensure that the physiological equivalent altitude in the chamber was continuously 0 m for 24 hours each day. For the hypoxia (H) group, rats were exposed to a hypobaric chamber with a pressure of 405 mmHg to simulate hypoxia at about 5000 m for 24 hours each day. For the hypoxia + continuous 3-h reoxygenation (C3) group, rats were exposed to a hypobaric chamber similar to group H as well as to 42% O2-58% N2 continuously for 3 hours each day. For the hypoxia + intermittent 3-h reoxygenation (I3) group, rats were treated similarly as group H, with 42% O2-58% N2 intermittently for 3 hours each day. After a 2-week treatment, pulmonary arterial remodeling experiments were performed.

To explore the protective effect of different reoxygenation frequencies on pulmonary artery remodeling after hypoxia, we fixed the reoxygenation level to 0 m and the duration of each time to 1 hours and changed the frequency to two, three, and six times each day. Correspondingly, we also observed the effects of continuous reoxygenation (2, 3, and 6 hours) on pulmonary artery remodeling after hypoxia (Figure 1, protocol 3).

The hypobaric chamber was continuously flushed with room air or 42% O2-58% N2 to wash out CO2, H2O, and NH3. Sodium lime and discoloured silica gel were used to absorb excess carbon dioxide and water in each chamber. Each chamber was opened for 20-25 min every 2 days in order to weight the rats, clean the cages, and replenish food and water.

2.4 | Haemodynamic measurement and tissue preparation

To measure right ventricular (RV) haemodynamics, rats were first intraperitoneally (ip) anaesthetised with 10% chloral hydrate (0.35 g/kg). A 1.2-F pressure catheter was then passed into the right ventricle via the jugular vein to measure the right ventricular systolic pressure (RVSP). Data were analysed using a PowerLab data acquisition system (AD Instruments, Bella Vista, NSW, Australia). In brief, pulmonary artery rings were mounted onto two gracile steel hooks and suspended in a four-chamber organ bath system (Radelco, Covina, CA, USA), with one hook attached to the holder in the chamber and the other to the transducer connected to PowerLab (AD Instruments, Bella Vista NSW, Australia) for vasoreactivity recording. The chambers were filled with Krebs solution, kept at 37°C, and continuously bubbled with 95% O2 and 5% CO2. After a 30 min equilibration, artery segments were stimulated with 60 mM KCl and thoroughly washed to re-establish the initial resting force; this was repeatedly performed until the optimal resting tension was achieved. The left pulmonary artery was used to detect the vasoconstriction function, and concentration response curves for exposure to 10⁻⁹-10⁻⁴ M phenylephrine (PE), 5-100 mM KCl, or 10⁻¹¹-10⁻⁶ M U-46619 were obtained. The right pulmonary artery was used to detect the vasodilation function; the rings were pre-contracted with 10⁻⁶ M PE and concentration response curves were obtained with 10⁻⁹-10⁻⁴ M acetylcholine (ACh), 10⁻⁹-10⁻⁴ M sodium nitroprusside (SNP) or 10⁻⁷-10⁻⁴ M NS1619. The overall vasoconstriction or vasodilation capacity was expressed as the area under the curve.

2.5 | Isometric force measurement

The arteries were carefully dissected under a microscope and cut into 3-mm lengths. Isometric force measurement was performed according to the methods described by Wang et al. In brief, pulmonary artery rings were mounted onto two gracile steel hooks and suspended in a four-chamber organ bath system (Radelco, Covina, CA, USA), with one hook attached to the holder in the chamber and the other to the transducer connected to PowerLab (AD Instruments, Bella Vista NSW, Australia) for vasoreactivity recording. The chambers were filled with Krebs solution, kept at 37°C, and continuously bubbled with 95% O2 and 5% CO2. After a 30 min equilibration, artery segments were stimulated with 60 mM KCl and thoroughly washed to re-establish the initial resting force; this was repeatedly performed until the optimal resting tension was achieved. The left pulmonary artery was used to detect the vasoconstriction function, and concentration response curves for exposure to 10⁻⁹-10⁻⁴ M phenylephrine (PE), 5-100 mM KCl, or 10⁻¹¹-10⁻⁶ M U-46619 were obtained. The right pulmonary artery was used to detect the vasodilation function; the rings were pre-contracted with 10⁻⁶ M PE and concentration response curves were obtained with 10⁻⁹-10⁻⁴ M acetylcholine (ACh), 10⁻⁹-10⁻⁴ M sodium nitroprusside (SNP) or 10⁻⁷-10⁻⁴ M NS1619. The overall vasoconstriction or vasodilation capacity was expressed as the area under the curve.

2.6 | Histological analyses

The fixed lungs and hearts were embedded in paraffin, and cross sections (5 μm) were prepared. The medial cross sections of the heart were stained with haematoxylin and eosin. The lung sections were stained with Masson or used for immunostaining. To determine the location of ki67 and vascular endothelial growth factor (VEGF) proteins in the lungs and blood vessels, lung sections were double-stained with antibodies against α-smooth muscle actin (α-SMA) (1:500, ab7817, Abcam) and either ki67 (1:200, ab15580, Abcam) or VEGF (1:100, ab46154, Abcam). Images were acquired using a fluorescence confocal laser scanning microscope (Nikon Eclipse TI-SP; Nikon Corporation, Tokyo, Japan). The degree of muscularisation was determined according to the methods described by Omura et al. This analysis was performed on vessels with an external diameter of...
25-75 μm, which were not associated with bronchi from lung sections, and were immunostained with α-SMA. Specifically, a vessel was considered partially or fully muscularised if it exhibited double-elastic visible lamina throughout at least half or the complete diameter of the vessel cross-section, respectively. Pulmonary vascular wall thickness was detected by measuring the medial thickness of distal pulmonary vessels with a diameter of 50-200 μm from lung sections immunostained with α-SMA. The percent wall thickness was expressed as the medial wall divided by the area of the vessel. The proliferation of artery smooth muscle cells (ASMCs) in pulmonary arteries was evaluated by Ki67 staining. The ratios of Ki67-positive arteries were detected in vessels with an external diameter of 50-200 μm from lung sections co-immunostained with α-SMA and Ki67. For the above three analyses, two lung sections were performed for each rat, and more than eight vessels were analysed using a computer-assisted imaging system (CaseViewer; 3DHISTECH, Hungary).

2.7 | Cell culture

Human pulmonary artery smooth muscle cells (HPASMCs) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). Cells were routinely cultured in a medium containing 2% foetal bovine serum, 1% smooth muscle cell growth supplement, and 1% penicillin/streptomycin (all obtained from ScienCell Research Laboratories) and were subsequently incubated in a humidified incubator at 37°C with 5% CO₂. The cells of passages 3-6 were used in this study.

2.8 | Hypoxia-induced cell culture system

To investigate the effect of intermittent short-duration re-oxygenation on HPASMCs after chronic hypoxic exposure, different groups of HPASMCs were exposed to normoxia, chronic hypoxia, or chronic hypoxia with reoxygenation for the indicated time spans (Figure 1, protocol 2). Hypoxic culture conditions, defined as 3% O₂, were established using an oxygen-regulated cell culture incubator (Thermo Fisher Scientific, Waltham, MA, USA) and were subsequently incubated in a humidified incubator at 37°C with 5% CO₂. The cells of passages 3-6 were used in this study.

2.9 | Proliferation assay

Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Kumamoto, Japan) and EDU labelling/immunofluorescence assays (RiboBio, Guangzhou, China) were used to measure HPASMC proliferation in different groups.

For the CCK-8 assay, cells were counted and seeded into 96-well culture plates (4 × 10³ cells/well). As per the relevant protocol, HPASMCs were exposed to different groups for 55 hours and were subsequently treated with 10 μL CCK-8 solution for 2 hours. The absorbance of the samples was detected using a microplate reader at 450 nm. For EDU labelling and immunofluorescence analysis, HPASMCs growing in the logarithmic phase were digested and incubated in a 96-well plate (4 × 10³ cells/well). As per the relevant protocol, HPASMCs in different groups were subsequently incubated with 50 μM EDU medium for 2 hours. EDU medium was subsequently discarded, and the cells were washed with PBS and fixed with 4% PFA for 30 min. Afterwards, the cells were decolourised in 2 mg/mL of glycine, permeabilised with 0.5% Triton X-100, and subsequently stained with buffer Apollo and Hoechst. Cells were observed under a fluorescence microscope (Nikon Eclipse Ti-SR; Nikon Corporation, Tokyo, Japan), and the percentage of positive cells was calculated.

2.10 | Migration

The wound healing test was used to measure the migration of HPASMCs. Cells cultured in a six-well plate until confluency were pre-treated with 1 μg/mL of mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) for 1 hour and washed with culture medium. Subsequently, the cells were scraped in a straight line to create a scratch using a 200-μL pipette tip. The scratched cells were washed with culture medium to remove debris and ensure that the edges of the scratch were smooth. The plates were then incubated at 37°C for 55 hours. The migration was quantified by the percent recovered area in the wound area.

2.11 | Immunofluorescence

HPASMCs were seeded on cell culture slides (Solarbio, Beijing, China) and fixed using 4% PFA in PBS for 20 min at room temperature. The cells were subsequently blocked and permeabilised with PBS containing 1% BSA and 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. After washing with PBS, the cells were incubated overnight at 4°C with the following primary antibodies: anti-α-SMA (1:500, ab7817, Abcam), anti-SM22 (1:200, 10493-1-AP, Proteintech, Rosemont, IL, USA), and anti-osteopontin (anti-OPN) (1:100, ab8448, Abcam). The cells were washed again with PBS and incubated with a secondary fluorescent antibody (1:500, Thermo Fisher Scientific, Waltham, MA, USA) for 1 hour at 37°C in the dark. The cells were rinsed and stained with DAPI. Images were acquired using a fluorescence microscope (Nikon Eclipse Ti-SR; Nikon Corporation, Tokyo, Japan).
2.12  |  Western blot

Western blot was conducted, as previously described. Briefly, pulmonary tissues or cells were lysed by resuspending them with a protein extraction reagent (Thermo Fisher Scientific, Waltham, MA, USA) containing protease inhibitor (Thermo Fisher Scientific, Waltham, MA, USA) on ice. The protein concentrations of lysates were detected using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples containing 20-30 μg protein were subsequently electrophoresed in 4%-12% Bis-Tris PAGE gels within the NuPAGE Bis-Tris Precast Gel System (Thermo Fisher Scientific, Waltham, MA, USA) and were then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Unspecific antibody binding was blocked by incubating the membranes for 4 hours with 5% BSA in TBS containing 0.1% Tween 20 (TBS-T) at room temperature. Subsequently, the membranes were incubated overnight at 4°C with primary antibodies against the following proteins: VEGF (1:1000, ab46154, Abcam), OPN (1:1000, ab84484, Abcam), α-SMA (1:1000, ab7817, Abcam), SM22 (1:1000, 10493-1-AP, Proteintech, Rosemont, IL, USA), HIF-1α (1:1000, ab1, Abcam), HIF-2α(1:500, ab199, Abcam) and GAPDH (1:1000, # 2118, Cell Signaling Technology, Boston, MA, USA). The membranes were then washed and incubated with secondary antibodies (1:10 000, ZSGB-BIO, Beijing, China) conjugated to horseradish peroxidase for 90 min. Bound antibodies were detected using enhanced chemiluminescence detection reagents (Millipore, Billerica, MA, USA) in a Gel Image Analysing System (Tanon-4200; Tanon Science & Technology, Shanghai, China). Densitometry analysis of bands was conducted using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.13  |  RNA-seq

Whole lung RNA-seq was detected in the N, H, C3, and I3 groups (n = 3 for each group). The specific steps were performed as follows. First, RNA was extracted from the total lung tissue samples, electrophoresed in agarose, and purified, followed by assessment of their quality and quantity using a Nanodrop; then, RNA was enriched with oligo(dT) magnetic beads. Next, the RNA sequencing library was prepared. Specifically, RNA was fragmented, random primer was inverted into the first-chain DNA, and dUTP was added to synthesise the second DNA chain. Third, the end of the double-stranded DNA was repaired and connected with the Illumina matching junction. The final library was obtained by PCR amplification and quantified using the qPCR method. After quality control of the raw sequencing data produced by Illumina HiSeq 4000, we conducted quantitative analysis of gene and transcript expression, including analyses based on gene expression levels (PCA, correlation analysis), and screened for differentially expressed genes. Moreover, GO functional and pathway enrichment analyses were performed. The sequencing procedure was primarily completed by Shanghai Kangcheng Biological Co. Ltd.

2.14  |  Oxidative stress

To assess the production of reactive oxygen species (ROS), the frozen lung sections were incubated in PBS containing 10 μM of dihydroethidium (DHE) (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37°C in a dark room. DHE-positive cells were observed under a fluorescence microscope (Nikon Eclipse Ti-SR; Nikon Corporation, Tokyo, Japan), and the fluorescence intensity was analysed using ImageJ software. Superoxide dismutase, catalase, and glutathione peroxidase (GSH-Px) activity and malondialdehyde (MDA) levels were determined using assay kits purchased from Nan Jing Jian Cheng Company (Jiangsu, China). All procedures were performed according to the manufacturers' protocols.

2.15  |  Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis was performed using SPSS 16.0 (SPSS Inc, Chicago, IL, USA) and Prism 7.0 (GraphPad Inc, La Jolla, CA, USA). Body weight changes, food consumption and isometric force measurements were analysed by two-way ANOVA with post hoc Tukey’s HSD analysis. In the other experiments, values were compared using t-tests for two groups or one-way ANOVA with post hoc Tukey’s HSD analysis for multiple groups. A P value <.05 was considered statistically significant.

3  |  RESULTS

3.1  |  Intermittent short-duration reoxygenation effectively attenuates the increased RVSP and RV hypertrophy

To investigate the effect of intermittent short-duration reoxygenation in the presence of chronic hypoxia, we first detected body weight changes in different groups. Compared with the N and HN groups, all rats exposed to hypoxia showed a rapid and significant body weight loss during the first 2 days of hypoxia. A slight recovery was subsequently observed on the 4th and 6th days of hypoxia. The lost body weight was regained on about the 8th day of hypoxia and body weight then continued to slowly increase (Figure 2A, Table S1). Next, the ratio of average food consumption of each animal to its body weight for 24 hours was evaluated at different time points. Compared with the N group, the food consumption
of all rats exposed to hypoxia was significantly decreased on the 2nd day (Figure S1A). However, there was no difference among the groups on the 10th and 12th days (Figure S1A). Simple hypobaric exposure did not restrict animal growth (Figure 2A) or reduce food consumption (Figure S1B). We then observed the increase in haematocrits in the H, C3, and I3 groups. However, haematocrit values were lower in the I3 group than in the H group (Figure 2B). Most importantly, in all hypoxia groups, rats displayed significant elevations in RVSP (Figure 2C,E) and RV hypertrophy (Figure 2D,F); however, rats in the I3 group experienced lower levels of RVSP and RV hypertrophy than those in the H and C3 groups (Figure 2C–F).

3.2 Intermittent short-duration reoxygenation prevents functional remodeling of pulmonary arteries

To explore the effect of intermittent short-duration reoxygenation after chronic hypoxia on the functional remodeling of pulmonary arteries, concentration-dependent vasoconstriction and vasodilation were examined on pulmonary arterial segments. Relative to the N group, vasoconstrictions in the arteries of the H, C3, and I3 groups, which were dependent on KCL, PE, or U-46619 concentrations, were significantly decreased; however, vasoconstrictions were higher in the I3 group than those in the H group (Figure 3A–C, Figure

FIGURE 2 Intermittent short-duration reoxygenation attenuates pulmonary hypertension induced by chronic hypoxia. (A) Body weight changes and growth curves in different groups. (B) Haematocrit levels. (C) Representative traces of RVSP variations; scale bar, 100 ms (D) Representative haematoxylin-eosin staining of the heart; scale bar, 1 mm. (E) RVSP values in different groups. (F) Relative RV weight determined as the ratio of RV weight to LV and septum weights (RV/LV + S). Results are expressed as mean ± SEM. n ≥ 6, *P < .05, **P < .01 vs N; †P < .05, ††P < .01 vs H
Similarly, compared to the N group, the vasodilatory responses elicited by ACh, SNP, and NS1619 were impaired in the H, C3, and I3 groups. Nevertheless, the arteries in the I3 group showed enhanced vasodilatory responses compared to those in the H group (Figure S2D–F). Moreover, no significant difference in vasoreactivity was observed between the N and HN groups (Figure S3A–D).

### 3.3 Intermittent short-duration reoxygenation prevents structural remodeling of pulmonary arteries

Vascular structure remodeling, characterised by an increase in the degree of muscularisation and wall thickness of pulmonary arterioles, was observed in all groups that were subjected to hypoxia, and it was significantly attenuated in the I3 group compared to the H group (Figure 4A–C). In accordance with these findings, the ratio of Ki67-positive pulmonary arteries was increased in the H group. However, it was partly restored in the I3 group (Figure 4D; Figure S4). The expression of VEGF in the lung tissue, which was evaluated by western blot and immunostaining, was also enhanced after hypoxia and was partly reversed by intermittent reoxygenation (Figure 4E,F; Figure S4). Not remarkable difference in the above characters between the N and HN groups was observed (Figure S3E–H).

### 3.4 Intermittent short-duration reoxygenation prevents proliferation and phenotype conversion of HPASMCs

To investigate the effect of intermittent short-duration reoxygenation on HPASMCs after exposure to chronic hypoxia,
HPASMCs were distributed into four groups and exposed to normoxia, chronic hypoxia, or chronic hypoxia with reoxygenation for the indicated time spans (Figure 1, protocol 2). Exposure to hypoxia (3% O₂) for 55 hours resulted in higher proliferation of HPASMCs (Figure 5A) and an increase in the proportion of EDU-positive cells in this cell population (Figure 5B,C), whereas intermittent short-duration reoxygenation markedly alleviated the hypoxia-induced proliferation. Furthermore, HPASMCs exposed to hypoxia (3% O₂) for 55 hours exhibited significantly larger areas of wound recovery compared to cells exposed to normoxia. However, hypoxia-induced migration was attenuated by intermittent short-duration reoxygenation (Figure 5D,E). We observed a marked downregulation of contractile phenotype markers (SM22 and α-SMA) and an upregulation of synthetic phenotype marker (OPN) levels when HPASMCs were exposed to hypoxia. Similarly, these changes were attenuated by intermittent short-duration reoxygenation (Figure 5F,G).
FIGURE 5 Intermittent short-duration reoxygenation prevents proliferation, migration, and phenotype conversion of HPASMCs under hypoxia. (A) Relative cell numbers under normoxia or hypoxia, denoted by relative A450 nm values, were detected by the CCK-8 test, n ≥ 5. (B) Cell proliferation was determined using EDU labelling; scale bar, 100 μm. (C) The percentage of EDU-positive PASMCs was analysed, n ≥ 5. (D) Wound healing test was performed to assess the migratory capacities of HPASMCs; scale bar, 200 μm. (E) The percentage of recovered areas was analysed, n ≥ 4. (F) Expression of α-SMA, SM22, and OPN proteins was determined using western blotting and immunofluorescence; scale bar, 100 μm. (G) Statistical analysis of α-SMA, SM22, and OPN expression, n ≥ 5. Results are expressed as mean ± SEM. *P < .05, **P < .01 vs H

3.5 | Intermittent short-duration reoxygenation changes the expression of HAPH genes and disrupts HPASMCs HIF-1α and HIF-2α switch

To investigate whether intermittent 3 hours/day reoxygenation affected HAPH at the molecular level, we analysed differences in mRNA expression between the groups by RNA-seq. An overlap among differentially expressed genes of H vs N (≥1.2 times), differentially expressed genes of C3 vs N (≥1.2 times) and pulmonary hypertension-related genes (from PubMed, up to February 2018) was first sorted out. Among the genes included in that intersection, those which were not differentially expressed in I3 vs N or differentially expressed in I3 vs N but the difference is different from that of H vs N or C3 vs N were selected as targeted genes (Figure 6A). Cluster and gene enrichment pathway analyses of all the selected genes were performed (Figure 6B). Genes selected according to Figure 6B were involved in several pathways, including the PI3K-Akt signalling pathway, cancer pathways, and ECM-receptor interactions etc (Figure 6C). In addition, we used cell models to detect the effects of intermittent reoxygenation on HIF-1α and HIF-2α protein expression. It was found that compared with H group, the protein expression of HIF-1α was increased while the protein expression of HIF-2α was decreased in I3 group (Figure 6D).

3.6 | Increasing intermittent reoxygenation’s frequency and total time does not further attenuate HAPH

To investigate whether increasing the frequency and total time of intermittent reoxygenation further attenuated hypoxia-induced HAPH, rats were randomly distributed into groups exposed to normoxia, chronic hypoxia, or chronic hypoxia with reoxygenation for the indicated time spans (Figure 1, protocol 3). Two weeks of chronic hypoxic exposure resulted in HAPH development, as indicated by an increase in RVSP (Figure 7A), right heart hypertrophy (Figure 7B), degree of muscularisation (Figure 7C), and wall thickness of small pulmonary vessels (Figure 7D), as well as ASMCs proliferation (Figure 7E). Intermittent 2 hours/day (I2) or 3 hours/day (I3) reoxygenation attenuated HAPH development, as contrary to intermittent 6 hours/day reoxygenation (I6) (Figure 7A–E).

Furthermore, I3 further repressed the degree of muscularisation and wall thickness of small pulmonary vessels, compared to I2 (Figure 7C,D). Continuous 2 hours/day (C2), 3 hours/day (C3), and 6 hours/day (C6) reoxygenation did not significantly protect against HAPH development during 2 weeks of hypoxia (Figure S5A–E).

3.7 | Increasing the frequency and total time of reoxygenation augments oxidative stress in the lungs

To investigate the reason behind no further attenuation of hypoxia-induced pulmonary hypertension after increasing the frequency and total time of intermittent reoxygenation, we detected oxidative stress markers in the lungs of I3 and I6 groups. DHE staining revealed markedly increased ROS levels in the I6 group (Figure 8A,B). The MDA level was also significantly increased in the I6 group (Figure 8C). However, the activities of the antioxidant enzymes catalase and GSH-Px decreased in the I6 group (Figure 8D,E). No obvious difference in superoxide dismutase activity was noted (Figure 8F).

4 | DISCUSSION

In this study, we determined whether daily intermittent short-duration reoxygenation at sea level attenuated pulmonary hypertension under hypoxia. The primary findings of the study were as follows: Intermittent, not continuous, short-duration reoxygenation effectively attenuates hypoxia-induced pulmonary hypertension. The mechanisms underlying the protective effects involved that intermittent short-duration reoxygenation prevented functional and structural remodeling of pulmonary arteries and proliferation, migration, and phenotypic conversion of pulmonary artery smooth muscle cells under hypoxia. The specific genes or potential molecular pathways responsible for mediating the protective effects were also characterised by RNA sequencing. Furthermore, increasing the frequency and the total time of intermittent reoxygenation did not further attenuate HAPH, which was likely attributable to augmented oxidative stress.

HAPH is a chronic and progressive disease characterised by pulmonary arterial function and structure remodeling,
FIGURE 6  Intermittent short-duration reoxygenation changes the expression of HAPH genes. (A) Schematic diagram of target gene isolation. (B) Cluster analysis of the target genes. (C) Gene enrichment pathways corresponding to target genes, n = 3. (D) Representative western blotting bands and statistical analysis of HIF-1α and HIF-2α expression. Results are expressed as mean ± SEM, n = 3. †P < .05 vs H
The pathogenesis of HAPH is different from other types of pulmonary hypertension. Before the onset of HAPH, that is, before entering a high-altitude hypoxic environment, HAPH patients have no diseases that could lead to pulmonary hypertension. HAPH is caused only by a decrease in partial oxygen pressure in the high-altitude environment. The initiating factors for pulmonary vascular remodeling are primarily a decrease in alveolar partial oxygen pressure and changes in pulmonary haemodynamics, which provided the premise for our hypothesis of “intermittent short-duration restoration to normoxia.”

Previous studies reported that the increase in pulmonary arterial pressure and the remodeling of pulmonary arterioles in SD rats were stabilised after simulation of 5000-m hypoxia for 2 weeks. Longer duration of hypoxia did not further augment the pulmonary arterial pressure nor the degree of pulmonary arterial muscularisation. Consequently, we investigated the preventive effect of intermittent short-duration reoxygenation on HAPH by simulating 5000-m hypoxia for 2 weeks in SD rats. After the successful establishment of the animal model, we selected a series of indicators to comprehensively evaluate the effectiveness of intermittent short-duration reoxygenation. Firstly, we found that intermittent short-duration reoxygenation did not improve body weight loss induced by hypoxia obviously. Body weight change is one of the most direct and sensitive indicators to reflect the physiological health of animals. Intermittent short-duration reoxygenation may not be powerful enough to eliminate the effects of hypoxia on rats totally. In general, it is believed that increased in haematocrit and haemoglobin concentrations after hypoxia are compensatory responses to the hypoxic environment; however, they negatively influenced pulmonary arterial pressures and resistance.
the reduced levels of haematocrit caused by 3 hours intermittent reoxygenation is of great value. Enhanced RVSP and RV hypertrophy are the most important indicators of pulmonary hypertension severity. In the current study, we found that intermittent short-duration reoxygenation effectively reduced RVSP and improved RV hypertrophy. These findings suggested that HAPH could partially be prevented by intermittent short-duration recovery of alveolar oxygen partial pressure (3 times/day, 1 hour/time, reoxygenation at sea level), which indicated that intermittent oxygen supply was an effective method for preventing HAPH.

Subsequently, we explored the protective effects of intermittent 3 hours reoxygenation on the impaired pulmonary vasoreactivity and increased muscularisation of distal pulmonary vessels induced by hypoxia. HAPH is associated with impaired responsiveness of the pulmonary arterial circulation to both vasoconstrictor and vasodilator stimuli. PE, α-adrenergic agonist, is coupled to activation of GTP-binding protein and induces contraction through both Ca²⁺-dependent and -independent (Ca²⁺ sensitisations) mechanisms. The contraction induced by high concentration of KCl, a receptor independent response, mainly involves stimulation of Ca²⁺ entry through voltage gated channels. U-46619 is a thromboxane prostanoid receptor activator. Thromboxane prostanoid receptor stimulation causes sustained contraction of vascular smooth muscle with modest increase in intracellular Ca²⁺ concentration, indicating that U-46619-induced vasoconstriction has a robust effect on Ca²⁺ sensitisation of the contractile apparatus. ACh-induced vasodilation is endothelium dependent and requires endogenous NO production. SNP is an exogenous NO donor that directly increases cGMP levels in ASMC and induces endothelium-independent vasodilation. NS1619, activates endothelial and vascular smooth muscle BKCa channels, and causes both endothelial-dependent and -independent vasodilation. In the current study, we demonstrated that 5000-m hypoxia reduced responsiveness of the pulmonary arterial circulation to various vasoconstrictor and vasodilator stimuli. However, intermittent reoxygenation partially reversed or prevented it. Studies have reported that the pulmonary arterial endothelial cell dysfunction and smooth muscle cell phenotype switch play central roles in the arterial remodeling. The reason behind the decrease in contraction in the pulmonary artery induced by PE, KCl, and U-46619, and its impairment by ACh, SNP and NS1619 is probably related to the excessive vascular remodeling and ASMCs phenotypic switch and proliferation. The proliferative or synthetic ASMCs make the artery lose its original vasoconstrictive or vasodilative function. Intermittent short-duration reoxygenation may prevent the above process and thus inhibit the artery function remodeling.

In the study of structural remodeling of pulmonary arteries, we evidenced that 2 weeks of hypoxia significantly augmented the thickness of pulmonary arterial medial layers with a diameter of 50-200 μm and the degree of muscularisation of pulmonary small vessels (25-75 μm in diameter). However, intermittent 3 hours reoxygenation had protective effects on these changes. Human and animal experimental
studies demonstrated that VEGF is abundant in the lungs and is a lung structure maintenance factor. Abnormal VEGF in plasma or lung tissue is involved in the occurrence of pulmonary hypertension. Our study indicated that the proportion of pulmonary small vessels positive for Ki-67, an antigen closely related to cell proliferation, and the expression of VEGF in lung tissue were significantly increased after 2 weeks under hypoxia, reduced after intermittent short-duration reoxygenation, and not affected by to continuous 3 hours reoxygenation. Thus, these results demonstrated that hypoxia was associated with enhanced proliferation of ASMCs and muscularisation of distal pulmonary arteries. Intermittent short-duration reoxygenation prevented the above processes, and thus maintained the normal function of the vasculature. The results of the structural changes in rats were also verified in the cell experiments. It has been reported that enhanced proliferation, migration, and conversion of ASMCs from a contractile to synthetic phenotype increased the muscularisation of distal pulmonary vessels, as also observed in the current study, when HPASMCs were exposed to hypoxia (3% O₂) for 55 hours. However, exposure to hypoxia for 7 hours followed by normoxia for 1 hours effectively reversed or alleviated the above changes, but continuous 3 hours reoxygenation did not induce any change. In summary, intermittent short-duration reoxygenation (3 hours/day, 1 hours/time; reoxygenation at sea level) effectively prevented HAPH through inhibition of pulmonary arterial function and structure remodeling after hypoxia.

In order to determine the specific genes or potential molecular pathways that mediate the protective effects of intermittent reoxygenation, lung tissue RNA-seq was performed. We evidenced that intermittent reoxygenation altered the expression of Kcnk3, Nos3, Smad1, and other genes that are involved in pulmonary arterial remodeling, thereby preventing pulmonary hypertension after hypoxia. According to pathway analysis, those genes were involved in the PI3K-Akt signaling pathway, cancer pathways, and transcriptional dysregulation in cancer, among others. These pathways have been widely investigated and associated with the pathogenesis of pulmonary hypertension. However, the results of RNA-seq suggest that multiple mechanisms may be involved in the improvement of pulmonary arterial remodeling after intermittent short-duration reoxygenation and no single mechanism can fully explain the protective effects. We will look for clues in the results of sequencing studies to lay the foundation for future studies on mechanisms. In addition, HIF pathway is an unavoidable topic in the study of hypoxic diseases. The most important transcription factors driving cell responses to hypoxia are the hypoxia-inducible factors 1 and 2 (HIF-1 and HIF-2). HIF-1 drives the initial response to hypoxia, but during chronic hypoxia exposure it is HIF-2 that plays the major role in driving the hypoxic responses. It’s reported that when the HPASMCs is exposed to hypoxia, HIF-1α will rapidly accumulate and reach maximal levels from 2 to 6 hours of hypoxia. Afterwards, HIF-1α abundance was reduced and the HIF-2α reaches maximal levels at about 8 hours of hypoxia and remains elevated for more than 48 hours. This HIF “switch” results in HIF-1 and HIF-2 playing divergent but complementary roles during the hypoxic response of tissues under pathophysiological conditions. In our study, we used cell models to detect the effects of intermittent reoxygenation on HIF-1α and HIF-2α protein expression in HPASMCs. We found that intermittent short-duration reoxygenation disrupted the “switch” of HIF-1α and HIF-2α, which was probably one of the reasons why intermittent short-duration reoxygenation had a protective role in pulmonary arterial remodeling induced by hypoxia.

After confirming the validity of intermittent short-duration reoxygenation, we preliminarily investigated different combinations of level, frequency, and duration of reoxygenation. For these studies, we set the physiological equivalent altitude of reoxygenation at sea level (0 m), reoxygenation to 1 hour/time, and frequencies of intermittent reoxygenation to 2 (I2), 3 (I3), and 6 (I6). We observed that increasing the frequency of reoxygenation to six times per day did not further decrease RV or pulmonary arterial remodeling under hypoxia. Through literature review, we found that dysregulation of oxidative signaling caused by an excess of ROS has had been heavily implicated in the underlying pathophysiology of Pulmonary hypertension. Experimental models of Pulmonary hypertension demonstrated dysregulation of oxidative stress, with elevated ROS, reduced superoxide dismutase (SOD), glutathione peroxidase, and catalase. Moreover, dysregulation of oxidative signaling plays an important role in other well-known phenotypes where hypoxia/reoxygenation induces bursts of reactive oxygen species such as reperfusion injury. Therefore, we examined the indicators of oxidative stress in different groups and found that oxidative stress injury in lung tissues in I6 group was significantly increased. These results indicated that an excessive frequency of intermittent reoxygenation could further aggravate the oxidative stress injury occurring in the lung tissue under hypoxia, which possibly counteracted the protective effect of intermittent reoxygenation itself on pulmonary arterial remodeling. Unlike cardiomyocytes and kidney cells that are sensitive to ischemia/reperfusion injury, pulmonary artery smooth muscle cells will undergo enhanced proliferation and apoptosis resistance under hypoxia. This unique biological characteristic is the basis for the formation of pulmonary hypertension. The restoration of oxygen supply just normalizes the functional characteristics of pulmonary artery smooth muscle cells. Moreover, cardiomyocytes and kidney cells will experience mitochondria swell and increase apoptosis during ischemia or hypoxia. When these cells in a poor state resume their blood or oxygen supply,
they will produce a large amount of oxygen free radicals, which will aggravate the damage. Therefore, the unique biological characteristics of pulmonary artery smooth muscle cells are also the cellular basis for intermittent reoxygenation. But when we excessively increase the frequency of intermittent reoxygenation, the pulmonary artery smooth muscle cells are always in a relatively changing environment. Under this context, the cells are in an abnormal state, which may induce an increase in oxidative stress damage.

Our study has some limitations. First, we used first-branch pulmonary arteries to detect arterial function, which may not totally reflect the pulmonary circulation, particularly the interlobar small arteries, which are thought to be more important in the control of pulmonary vascular resistance. Future studies should examine whether the reduced vasoreactivity in first-branch pulmonary arteries also occurs in distal resistance arteries. Second, in current study, the molecular mechanisms underlying the protective effects of intermittent reoxygenation were not fully discussed. It focused more on a phenomenal observation. The demonstration of molecular mechanisms needs more studies in future. Lastly, we only preliminarily explored the protective effect of different reoxygenation frequencies on pulmonary artery remodeling after hypoxia. However, an optimal combination of different factors, such as intermittent reoxygenation level, frequency, and duration, may exist. Establishing the optimal combination of these factors may allow for the selection of oxygen supply schemes for treating medium-term and long-term high-altitude sojourners. However, the optimal combination of different factors needs further exploration in the future.

In conclusion, daily intermittent short-duration reoxygenation at sea level partially prevented pulmonary arterial remodeling, and thus alleviated pulmonary hypertension in SD rats exposed to 5000-m hypoxia. Investigation of genes and pathways involved in the prevention of the high-altitude pulmonary arterial remodeling effect induced by intermittent short-duration reoxygenation provided potential targets for preventing HAPH. Finally, by increasing the frequency and total time of intermittent reoxygenation, HAPH was not further attenuated, which was likely attributed to augmented oxidative stress. However, an optimal combination of intermittent reoxygenation physiological altitude, frequency, and duration could be determined and needs further exploration.

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**CONFLICTS OF INTEREST**

All authors declare that they have no conflicts of interest.

**AUTHOR CONTRIBUTIONS**

Q. Lyu and Y. Bai performed most experiments and wrote the manuscript. J. Cheng helped in maintaining the hypobaric chamber and raising the animals. H. Liu, S. Li, and J. Yang helped in raising the animals, collected the tissues, and analysed the data. Z. Wang, Y. Ma, and M. Jiang helped in performing the cell culture. D. Dong, Y. Yan, and Q. Shi helped in performing the molecular work. X. Ren directed the study and revised the article. J. Ma designed and directed the study, provided the funding for the research, and took part in manuscript writing. All authors have provided final approval of the manuscript version to be published and 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.

**DATA AVAILABILITY STATEMENT**

The authors declare that all the data supporting the study findings are available within the article, in the Supporting Information Files, and from the authors upon request.

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the Supporting Information section.

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