

Reduced iron bioavailability drives acute high-altitude lung injury through HIF1 α activation and mitophagy

YUMEI GENG^{1-5*}, YU HU^{6*}, HUIJIE WANG¹⁻⁴, FANG ZHANG^{5,7} and RI-LI GE¹⁻⁴

¹Research Center for High Altitude Medicine, Qinghai University, Xining, Qinghai 810001, P.R. China; ²Key Laboratory of High Altitude Medicine (Ministry of Education), Qinghai University, Xining, Qinghai 810001, P.R. China; ³Key Laboratory of Application and Foundation for High Altitude Medicine Research in Qinghai Province (Qinghai-Utah Joint Research Key Lab for High Altitude Medicine), Qinghai University, Xining, Qinghai 810001, P.R. China; ⁴Laboratory for High Altitude Medicine of Qinghai Province, Qinghai University, Xining, Qinghai 810001, P.R. China; ⁵Department of Respiratory and Critical Care Medicine, Qinghai Provincial People's Hospital, Xining, Qinghai 810007, P.R. China; ⁶Department of Pharmacy, Qinghai Provincial Traffic Hospital, Xining, Qinghai 810008, P.R. China; ⁷Department of Internal Medicine I, Guide County People's Hospital, Hainan Tibetan Autonomous Prefecture 811700, P.R. China

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Abstract. High-altitude pulmonary injury, characterized by pulmonary edema and pulmonary hypertension, is mechanistically driven by dysregulated mitophagy, as evidenced by impaired mitochondrial quality control in endothelial cells under hypobaric hypoxia. Iron supplementation for individuals who have ascended rapidly to high altitudes can effectively mitigate the phenomenon of hypoxic pulmonary vasoconstriction; however, the precise role and detailed mechanisms remain to be determined. The present study aimed to explore the role and mechanism of iron in acute hypoxia-induced lung injury. Sprague-Dawley rats were initially placed in a hypobaric hypoxia chamber for various durations to determine the optimal time for acute hypoxia-induced lung injury. The rats were exposed to a hypobaric hypoxia chamber for 3 days, during which they were treated with an iron chelator or iron sucrose. Mean pulmonary artery pressure (mPAP) was measured to assess hypoxic pulmonary vascular response. Furthermore, the degree of lung injury was assessed by

calculating the pulmonary wet/dry weight ratio, and via morphological evaluation of lung tissues and the pulmonary vasculature. Immunofluorescence and western blot analysis were performed to assess hypoxia-inducible factor 1 α (HIF1 α) expression and mitophagy levels. Edu and Cell Counting Kit 8 assays were conducted to evaluate cell proliferation under acute hypoxia. In addition, immunofluorescence and western blot analysis were performed to evaluate the expression levels of proteins associated with cell apoptosis and mitophagy. The results indicated that mitophagy (LC3B-II/LC3B-I expression), pulmonary edema (lung wet/dry weight ratio) and lung injury score were most significant after 3 days of hypoxia. However, mitophagy (LC3B-II/LC3B-I ratio) and lung injury scores peaked after 4 weeks of hypoxic conditions. Furthermore, an iron chelator was observed to promote pulmonary edema, elevate mPAP and cause lung injury. Conversely, iron sucrose was shown to attenuate lung injury in acute hypoxia. The mechanistic findings indicated that acute hypoxia induced HIF1 α activation and increased mitophagy, which promoted a reduction in proliferation and an increase in the apoptosis of pulmonary artery endothelial cells. Furthermore, the iron chelator promoted, whereas iron sucrose ameliorated, the abnormal alterations in pulmonary artery endothelial cells under acute hypoxia. In conclusion, the present study demonstrated that a reduction in iron bioavailability in acute hypoxia may promote HIF1 α activation and increased mitophagy, which in turn has been linked to the development of pulmonary edema, elevated mPAP and lung injury. The administration of iron supplementation may be considered an effective method for the alleviation of the aforementioned abnormalities resulting from acute hypoxia.

Correspondence to: Professor Fang Zhang, Department of Respiratory and Critical Care Medicine, Qinghai Provincial People's Hospital, 2 Gonghe Road, Chengdong, Xining, Qinghai 810007, P.R. China

E-mail: qhsrmzyzf@hotmail.com

Professor Ri-Li Ge, Research Center for High Altitude Medicine, Qinghai University, 16 Kunlun Road, Chengxi, Xining, Qinghai 810001, P.R. China

E-mail: geriligaol@hotmail.com

*Contributed equally

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Introduction

Of the global population ~7% is estimated to reside at altitudes >1,500 meters, whereas ~140 million individuals inhabit regions >2,500 meters above sea level (1) and >40 million

tourists visit high-altitude destinations annually (2). The most notable environmental characteristic of plateaus is the presence of low pressure and low oxygen; this hypobaric hypoxia prompts a cascade of physiological responses. For individuals who ascend rapidly to high altitudes, alveolar tissue hypoxia ensues upon reaching the plateau. This hypoxia results in the physiological phenomenon of hypoxic pulmonary vasoconstriction (HPV), which facilitates the redistribution of pulmonary blood flow to optimize ventilation/perfusion matching; however, when hypoxia persists without resolution, severe HPV induces acute pulmonary hypertension (PH) and may even precipitate high-altitude pulmonary edema. This phenomenon has been observed in individuals who are not acclimatized to high-altitude environments, and can manifest at any point between 1 and 5 days after ascending to an altitude of 2,500 meters above sea level (3). However, the current pharmacological and non-pharmacological preventive measures lack sufficient specificity and targeting (4). Therefore, it is imperative to identify novel strategies to enhance the prevention and management of high altitude-induced lung injury.

Mitophagy, defined as the selective autophagic degradation of damaged mitochondria, represents a crucial process for maintaining mitochondrial quality control. Notably, there is growing evidence to suggest that the process of mitophagy is implicated in the pathogenesis of numerous lung diseases (5). As evidenced by previous studies, autophagy in pulmonary artery smooth muscle cells has been demonstrated to facilitate pulmonary vascular remodeling (6-8). However, the mechanism of mitophagy in HPV remains unclear. Prior research has demonstrated that diminished iron availability enhances HPV (9). While reduced serum iron is attributed to elevated erythropoiesis, it has been hypothesized that pulmonary edema and PH at high altitude may be associated with low serum iron (10,11). Notably, crosstalk exists between iron homeostatic imbalance and mitophagy, and iron chelators have been demonstrated to act as mitophagy inducers (12,13). Therefore, it could be noteworthy to determine whether reduced iron bioavailability under acute hypoxia promotes pulmonary edema and PH by altering mitophagy.

Hypoxia-inducible factor 1 α (HIF1 α) is a principal transcription factor that mediates the acute hypoxic response and is expressed in nearly all cells of diverse animal organs (14). It has been demonstrated that HIF1 α -mediated mitophagy serves a role in the development of ischemic diseases affecting the heart and kidney (15,16). Nevertheless, it remains unclear as to whether a reduction in iron bioavailability under acute hypoxia leads to an increase in HIF1 α expression, thereby triggering mitophagy, and contributing to the development of pulmonary edema and acute PH. The current study aimed to explore the role and mechanism of iron in acute hypoxia-induced lung injury.

Materials and methods

Animal experiments. The experimental protocols and animal care procedures employed in the present study were approved by the Medical Ethics Committee of Qinghai Provincial People's Hospital (approval no. 2023-95; Xinning, China). Male Sprague-Dawley rats (weight, 200-250 g; age, 7-8 weeks) were

procured from Jiangsu Huachuang Xinnuo Pharmaceutical Science and Technology Co., Ltd.

The rat model of hypoxia was created by exposing the animals to a hypobaric hypoxia chamber (cat. no. DYC-300; Guizhou Fenglei Oxygen Chamber Co., Ltd.). Previous studies have shown that acute mountain sickness usually occurs within a few hours to 24 h upon entering high-altitude environments, with symptoms peaking within 24-72 h and then gradually subsiding (17-20). Thus, the present study centered on the first 3 days of acute hypoxia. Meanwhile, for an improved comparison of the physiological changes under acute hypoxia, it additionally included the observation results of hypoxic treatments at 7 days and 4 weeks. The initial animal models were exposed to hypoxia or normoxia for 0, 1, 2, 3 and 7 days and for 4 weeks ($n=60$; 5/group; each group comprised different animals). The iron intervention animal models were exposed to hypoxia for 3 days ($n=40$; 10/group). The air pressure and oxygen content corresponded to an altitude of 6,000 meters above sea level. The pressure was 52.9 kPa, the oxygen concentration was 10% (hypoxia), the temperature was 22.0°C, the relative humidity was 50% and there was a 12-h light/dark cycle (21-23).

The pharmacological intervention rat model was generated as follows: Male Sprague-Dawley rats were administered deferrioxamine (DFO; 200 mg/kg; cat. no. HY-B0988; MedChemExpress) in the hypoxia + DFO (HD) group or iron sucrose (20 mg/kg; cat. no. HY-B2068; MedChemExpress) in the hypoxia + iron sucrose (HI) group via daily intraperitoneal injection (24). The rats were then placed in a hypoxic (10%) environment for 3 days, as previously described (19-21). The control groups received an equivalent volume of solvent via intraperitoneal injection under either hypoxic (10% O₂; HC group) or normoxic (21% O₂; NC group) conditions.

Invasive hemodynamic monitoring. The rats ($n=3$ /group) were anesthetized with isoflurane gas (induction, 2%; maintenance, 1.5-2.0%; inhalation) and the mean pulmonary artery pressure (mPAP) was determined using a right heart catheter. The catheter was positioned in the right external jugular vein and advanced into the pulmonary artery via the right ventricle. The position of the insertion was determined by analyzing the waveforms produced by a biosignal acquisition system (MP150; Biopac Systems, Inc.). After right cardiac catheterization, the rats were sacrificed via cervical dislocation under isoflurane anesthesia, and their death was confirmed by observing the arrest of cardiac and respiratory functions and the disappearance of the pain response. The lung tissue was then excised and its wet weight was precisely measured. Subsequently, the excised lung tissue underwent a 72-h drying process at 65°C; after the completion of this period, the tissue was re-weighed to obtain the dry weight. The lung wet/dry weight ratio was calculated to reflect the water content of the lung tissue.

Detection of hematological parameters and serum iron. Blood samples (1 ml) ($n=3$ /group in the hypoxia study at different time points; $n=5$ /group in the pharmacological intervention rat model) were collected from rats via cardiac puncture under 1.5% isoflurane inhalation anesthesia, followed by euthanasia through cervical dislocation while maintaining anesthesia. Aliquots were transferred to EDTA-coated anticoagulant

tubes for complete blood count analysis following gentle inversion (5–10 cycles), or were transferred to clot activator tubes for centrifugation (4°C, 1,006 x g, 10 min) to obtain serum. Complete blood count was performed using a YAN-305A hematology analyzer (Shanghai Yuyan Scientific Instrument Co., Ltd.) with species-specific calibration. Serum iron levels were quantified using a commercial kit (cat. no. A039-1-1; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's guidelines. Raw data were processed for statistical analysis.

Transmission electron microscopy. Following anesthesia and euthanasia of the rats, the heart and lungs were simultaneously removed and placed in 1X PBS. The main pulmonary artery and its branches were meticulously isolated from the right ventricle. Subsequently, the artery was stabilized with 3% glutaraldehyde for 2 h at 4°C, followed by post-fixation with 1% osmium tetroxide for 1 h at 4°C. A graded series of acetone treatments was employed for dehydration, followed by infiltration with Epon 812 and embedding. The semithin sections (50 nm) were subjected to methylene blue staining at room temperature for 2 min, whereas the ultrathin slides were meticulously sliced using a single diamond knife and were dual-stained with uranyl acetate at room temperature for 30 min and lead citrate at room temperature for 15 min. Subsequently, the slides were subjected to observation under a JEM-1400-FLASH transmission electron microscope (JEOL Ltd.).

Morphological analysis of the lung vessels. The lung tissue and main pulmonary artery from 3 rats/group were fixed in 4% paraformaldehyde at room temperature for 12 h, dehydrated in a graded series of ethanol, cleared with xylene (2x10 min) and embedded in paraffin via triple wax immersion (56°C, 1 h/cycle). Sections (5 µm) were then stained with hematoxylin at room temperature for 5 min and eosin at room temperature for 3 min following standard hematoxylin and eosin (H&E) staining protocols. An image was acquired using a BA400 digital light microscope with a digital interface (Motic Incorporation, Ltd.). The rat lung tissue was evaluated for evidence of lung injury in accordance with the established criteria for pathological assessment of lung injury, as outlined by the American Thoracic Society (25). The scoring indexes were based on the following criteria: Neutrophil infiltration of lung tissue, hyaline membrane formation, fibrin deposition in alveoli and thickening of alveolar septa. Each index was scored on a 5-point scale, with the severity of injury corresponding to the following categories: No injury, 0 points; mild injury, 1 point; moderate injury, 2 points; severe injury, 3 points; and very severe injury, 4 points (22,23). A total of five randomly selected fields of H&E-stained lung tissue from each animal were scored and two experts scored the sections in a double-blind manner.

Immunohistochemical staining. The protocols for fixation, embedding and sectioning of the main pulmonary artery and alveolar tissue were the same as those performed for H&E staining. Sections were deparaffinized in xylene (2x10 min), rehydrated through a graded ethanol series (5 min/step), and subjected to microwave-mediated antigen retrieval in citrate

buffer (pH 6.0) at sub-boiling temperature for 10 min. After cooling, endogenous peroxidase was blocked with 3% H₂O₂ for 10 min at room temperature, followed by blocking with 5% BSA (cat. no. SW3015; Beijing Solarbio Science & Technology Co., Ltd.) for 30 min at room temperature. Primary anti-HIF1α antibody (1:100; cat. no. ab179483; Abcam) was applied overnight at 4°C, followed by an incubation with a HRP-conjugated goat anti-rabbit secondary antibody (1:100; cat. no. GB23303; Wuhan Servicebio Technology Co., Ltd.) for 1 h at room temperature; Subsequently, DAB was employed to develop the color (cat. no. GB1212; Wuhan Servicebio Technology Co., Ltd.), hematoxylin was used to counterstain the nuclei at room temperature for 10 min and neutral balsam was used to seal the mount. Observations were conducted using a BA400 digital light microscope (Motic Incorporation, Ltd.).

Cell culture. Human-derived primary pulmonary artery endothelial cells (cat. no. 337714) were obtained from BeNa Culture Collection; Beijing Beina Chunglian Institute of Biotechnology. The Research Ethics Committee of Qinghai Provincial People's Hospital confirmed that commercially procured human primary cells fall outside the scope of required ethics approval. At the time of purchase, the cells were in the first passage. The cells were cultured in endothelial cell medium (cat. no. ECM 1001; ScienCell Research Laboratories, Inc.) containing 1% vascular endothelial growth factor at 37°C with 5% CO₂. The cells were passaged, and those between passages 3 and 5 were used in the present study. The cells in the hypoxia group were cultured at 37°C under hypoxia (1% O₂) for 2 h.

Cellular pharmacological interventions. Human-derived pulmonary artery endothelial cells were treated with DFO (100 µmol/l; cat. no. HY-B0988; MedChemExpress) in the HD group or with iron sucrose (50 µmol/l; cat. no. HY-B2068; MedChemExpress) in the HI group as previously described (26). The control groups received an equivalent volume of solvent under either hypoxic (1% O₂, HC group) or normoxic (21% O₂, NC group) conditions.

Immunofluorescence assay. For immunofluorescence experiments conducted *in vivo* (n=3/group), lung vascular tissue samples underwent fixation, dehydration, embedding and sectioning following the aforementioned standard H&E protocols. Subsequently, the sections were dewaxed, rehydrated and subjected to microwave-assisted antigen retrieval in citrate buffer (pH 6.0), as per the immunohistochemistry methods. After blocking with immunostaining buffer (cat. no. P0102; Beyotime Institute of Biotechnology) for 1 h at room temperature, the following primary antibodies were used to incubate the sections overnight at 4°C: PTEN-induced putative kinase 1 (PINK1 1:100; cat. no. GB114934-100; Wuhan Servicebio Technology Co., Ltd.), smooth muscle actin (SMA; 1:100; cat. no. A2547; MilliporeSigma) and HIF1α (1:100; cat. no. ab179483; Abcam). Samples were washed three times with 1X PBS (10 min each), followed by incubation with a fluorescent secondary antibody [Goat Anti-Rabbit Alexa Fluor® 488 for HIF1α and Caspase 3 (cat. no. ab150077), Alexa Fluor 647 for PINK1 (cat. no. ab150079) or Goat Anti-Mouse Alexa Fluor 647 for SMA (cat. no. ab150115); 1:1,000; Abcam] for 1 h

at room temperature. Nuclei were counterstained with DAPI, and images were acquired using a confocal laser microscope (FV1000; Olympus Corporation).

Pulmonary arterial endothelial cells were routinely cultured to an appropriate density and were treated with hypoxia and DFO or iron sucrose for 2 h. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100 for 10 min at room temperature and blocked with immunostaining buffer (cat. no. P0102; Beyotime Institute of Biotechnology) for 1 h at room temperature. Subsequently, the sections were incubated with the primary antibodies against Caspase 3 (1:100; cat. no. 19677-1-AP; Proteintech Group, Inc.) and HIF1 α (1:100; cat. no. ab179483; Abcam) overnight at 4°C. The secondary antibody incubation, nuclear counterstaining with DAPI and confocal imaging (FV1000; Olympus Corporation) were performed according to the aforementioned protocols.

To assess mitochondrial membrane potential (MMP) and apoptosis of pulmonary arterial endothelial cells, mitochondrial superoxide and Annexin V-FITC fluorescence assays were conducted in accordance with the instructions provided in the MitoSOX and Annexin V-FITC kits (cat. nos. S0061M and C1071M; Beyotime Institute of Biotechnology). The images were examined using a fluorescence microscope (THUNDER Imager; Leica Microsystems, Inc.) and the immunofluorescence intensity of protein signals was subsequently semi-quantified using ImageJ software (version 1.54f; National Institutes of Health).

Western blot analysis. Proteins were extracted from lung tissues and pulmonary arterial endothelial cells using RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) and were quantified using a BCA kit (Thermo Fisher Scientific, Inc.). A 20- μ g protein sample from each group was then subjected to SDS-PAGE on a 10% gel, followed by transfer to a polyvinylidene fluoride membrane (PVDF; 0.22 μ m; Merck KGaA). Following the transfer of the proteins, 5% skimmed milk (BD Biosciences) was used to block the PVDF membranes for 1 h at room temperature. The membranes were then incubated overnight at 4°C with the following primary antibodies: LC3 (1:1,000; cat. no. 14600-1-AP) and β -actin (1:5,000; cat. no. 66009-1-Ig) (both from Proteintech Group, Inc.). Subsequently, the membranes were incubated with HRP-conjugated secondary antibodies (1:5,000; cat. nos. E-AB-1003 and E-AB-1001; Wuhan Elabscience Biotechnology Co., Ltd.) for 1 h at room temperature and chemiluminescence signals were detected using an ECL kit (Abbkine Scientific Co., Ltd.). Finally, ImageJ software (version 1.54f) was employed to semi-quantify the intensity of the bands (27–29).

Cell proliferation assay. The Cell Counting Kit 8 (CCK8) assay (cat. no. E-CK-A362; Wuhan Elabscience Biotechnology Co., Ltd.) was used to investigate cell proliferation in accordance with the manufacturer's instructions. The absorbance at 450 nm was measured using a microplate reader (Biotek; Agilent Technologies, Inc.). In addition, cell proliferation was detected using the Edu Cell Proliferation Kit (cat. no. C0071L; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Images were captured using a

fluorescence microscope (ECHO/RVL-100; Discover Echo, Inc.) and ImageJ software (version 1.54f) was employed for data analysis.

Statistical analysis. All data were processed using the statistical software package SPSS 28.0 (IBM Corp.) and the graphical software package GraphPad 10.0 (Dotmatics). Data with a normal distribution are presented as the mean $\pm$ standard deviation. Statistical comparisons of parameters (including lung wet/dry weight ratio, serum iron and erythroid parameters) across different durations of hypoxic treatment were performed using two-way ANOVA, followed by Tukey's honestly significant difference post hoc test for multiple comparisons. One-way ANOVA followed by the Tukey's post hoc test was performed for multiple group comparisons when the data met the assumptions of normality and homogeneity of variance. Non-normally distributed ordinal data (lung injury scores) are presented as the median (IQR), and were analyzed using Kruskal-Wallis test with Dunn's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Hypoxic lung injury is most severe on day 3 of acute hypoxia in rats. The lung dry/wet weight ratio was significantly increased on days 2 and 3 of hypoxia compared with day 0 (Fig. 1C), indicating the presence of pulmonary edema. Lung morphology results indicated that there were no abnormal changes in lung tissue morphology during days 1–7 under normoxia. However, under hypoxia, erythrocyte leakage from alveolar spaces accompanied by inflammatory cell infiltration was observed. This phenomenon was most pronounced on day 3 of acute hypoxia and then gradually resolved, with a marked infiltration of inflammatory cells reappearing after 4 weeks of hypoxia (Fig. 1A). Semi-quantitative analysis further confirmed that hypoxia-induced lung tissue injury was most pronounced on day 3 and peaked at 4 weeks compared with on day 0 under hypoxia (Fig. 1B).

Reduced serum iron concentration and compensatory erythropoiesis occur in a rat model under acute hypoxia. To elucidate the involvement of iron in acute mountain sickness, serum iron and erythropoiesis levels were quantified under normoxia/hypoxia at multiple timepoints. The results demonstrated that, under normoxia, there were no statistically significant differences in serum iron, red blood cell (RBC), hemoglobin and hematocrit (HCT) levels among the groups. By contrast, under hypoxia, as the duration of hypoxia increased, serum iron concentration exhibited a gradually decreasing trend, the serum iron concentration in the 4-week hypoxia group exhibited statistically significant differences compared with that in the day 0 group. By contrast, RBC, HCT and hemoglobin levels were increased with prolonged hypoxia exposure. RBC parameters in hypoxia groups at day 3 and 7 and week 4 were significantly different compared with those in the day 0 group under hypoxia, whereas hemoglobin and HCT levels in hypoxia groups day 1, 3 and 7, and week 4 exhibited significant changes relative to the day 0 control under hypoxia (Fig. 1D and E). These findings indicated that the oxygen content in the rats was diminished and compensatory

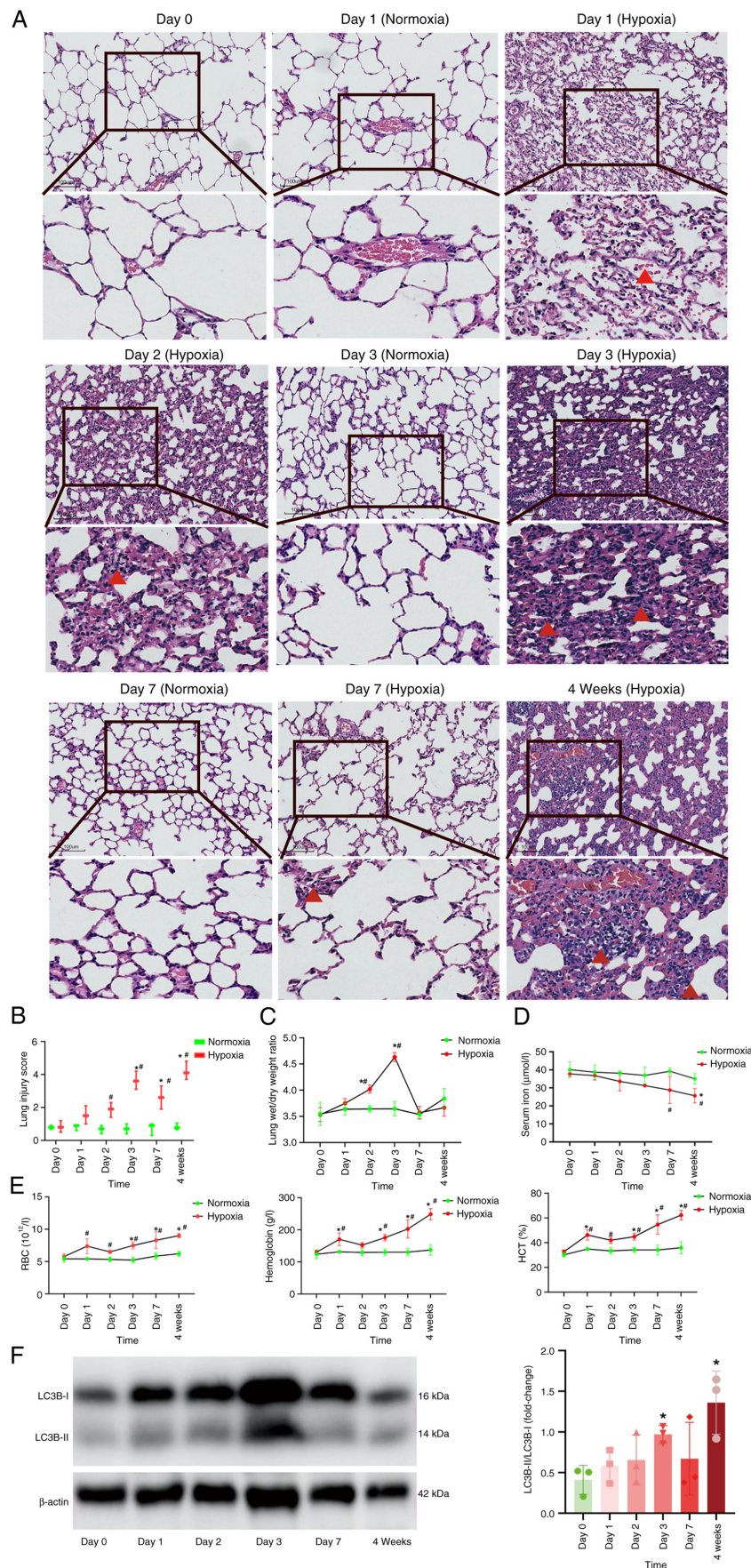


Figure 1. Alterations in rats under hypoxia at different time intervals. (A) Overview and magnified views of hematoxylin and eosin staining micrographs of rat lung tissues (magnification, x100). Red triangles indicate erythrocytes in alveolar spaces on day 1 under hypoxia and inflammatory cells on days 2, 3 and 7 and week 4 under hypoxia. (B) Analysis of the injury score of rat lung tissues, n=3. (C) Analysis of the wet/dry weight ratio of rat lung tissues, n=3. (D) Serum iron concentration in rats, n=3. (E) RBC, hemoglobin and HCT levels in rats, n=3. (F) Representative western blotting images and analysis in rat lung tissues, n=3. *P<0.05 vs. Day 0 group under hypoxia, *P<0.05 vs. the normoxia group at the same time point. RBC, red blood cell; HCT, hematocrit.

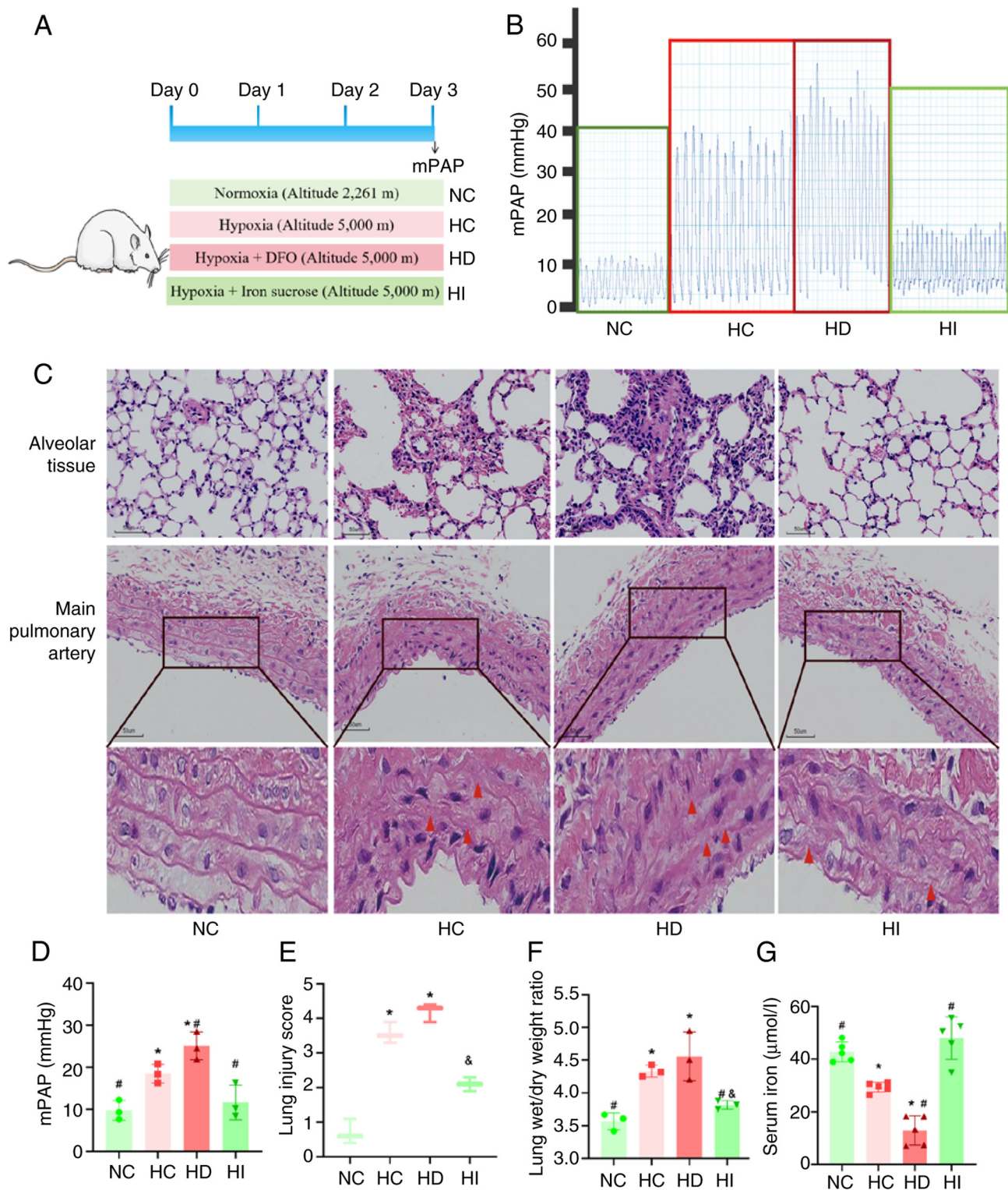


Figure 2. Effects of iron on the pulmonary vasculature and lung tissue under acute hypoxia. (A) Schematic diagram of animal modeling. (B) Representative images of mPAP waves. (C) Representative images of hematoxylin and eosin staining of lung tissues and the main pulmonary artery in rats (magnification, $\times 200$). Red triangles indicate regions exhibiting elastic fiber layer disruption and structural disorganization in the pulmonary aorta. (D) Analysis of mPAP, $n=3$. (E) Analysis of the injury score of lung tissues in rats, $n=3$. (F) Analysis of the wet/dry weight ratio of lung tissues in rats, $n=3$. (G) Serum iron concentration in rats, $n=5$. * $P<0.05$ vs. NC group; # $P<0.05$ vs. HC group; & $P<0.05$ vs. HD group. NC, normoxia control; HC, hypoxia control; HD, hypoxia + deferoxamine; HI, hypoxia + iron sucrose; mPAP, mean pulmonary artery pressure.

erythrocyte hyperplasia was exhibited. Given the pivotal role of iron chelators in the initiation of mitophagy, western blot analysis was performed to assess the expression levels of the mitophagy protein LC3B. The results demonstrated that

LC3B-II/LC3B-I expression was significantly upregulated in response to acute hypoxia, particularly on day 3 of hypoxia exposure compared to day 0 under hypoxia, and peaked after 4 weeks of hypoxic treatment (Fig. 1F).

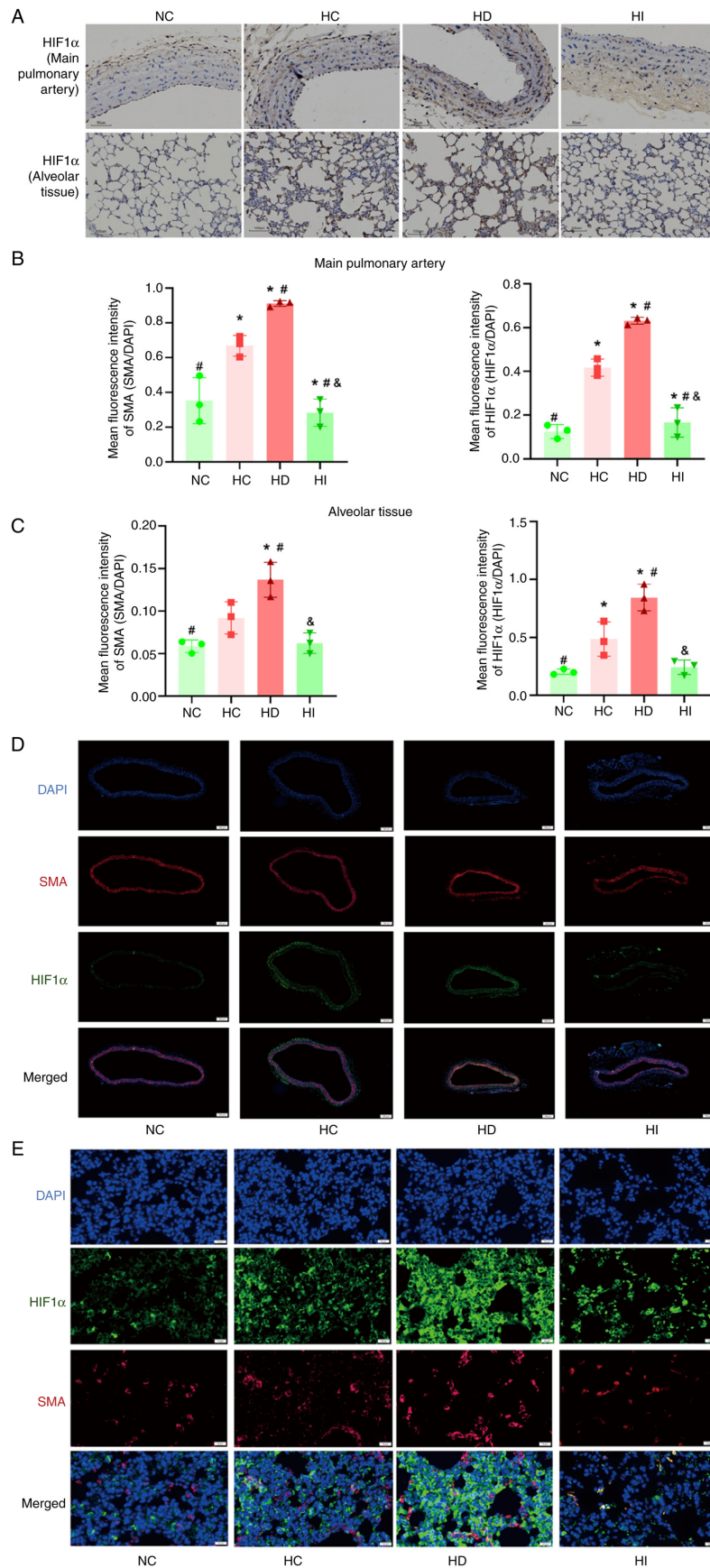


Figure 3. Role of iron in the regulation of HIF1α. (A) Representative HIF1α immunohistochemical staining of the main pulmonary artery (magnification, x200) and alveolar tissue (magnification, x100); brown regions indicate HIF1α-positive areas detected by immunohistochemical staining. Analysis of immunofluorescence staining of the (B) pulmonary vasculature and (C) pulmonary tissue, n=3. Representative images of immunofluorescence staining of the (D) pulmonary vasculature (magnification, x500) and (E) pulmonary tissue (magnification, x400). *P<0.05 vs. NC group; #P<0.05 vs. HC group; &P<0.05 vs. HD group. NC, normoxia control; HC, hypoxia control; HD, hypoxia + deferoxamine; HI, hypoxia + iron sucrose; HIF1α, hypoxia-inducible factor 1α; SMA, smooth muscle actin.

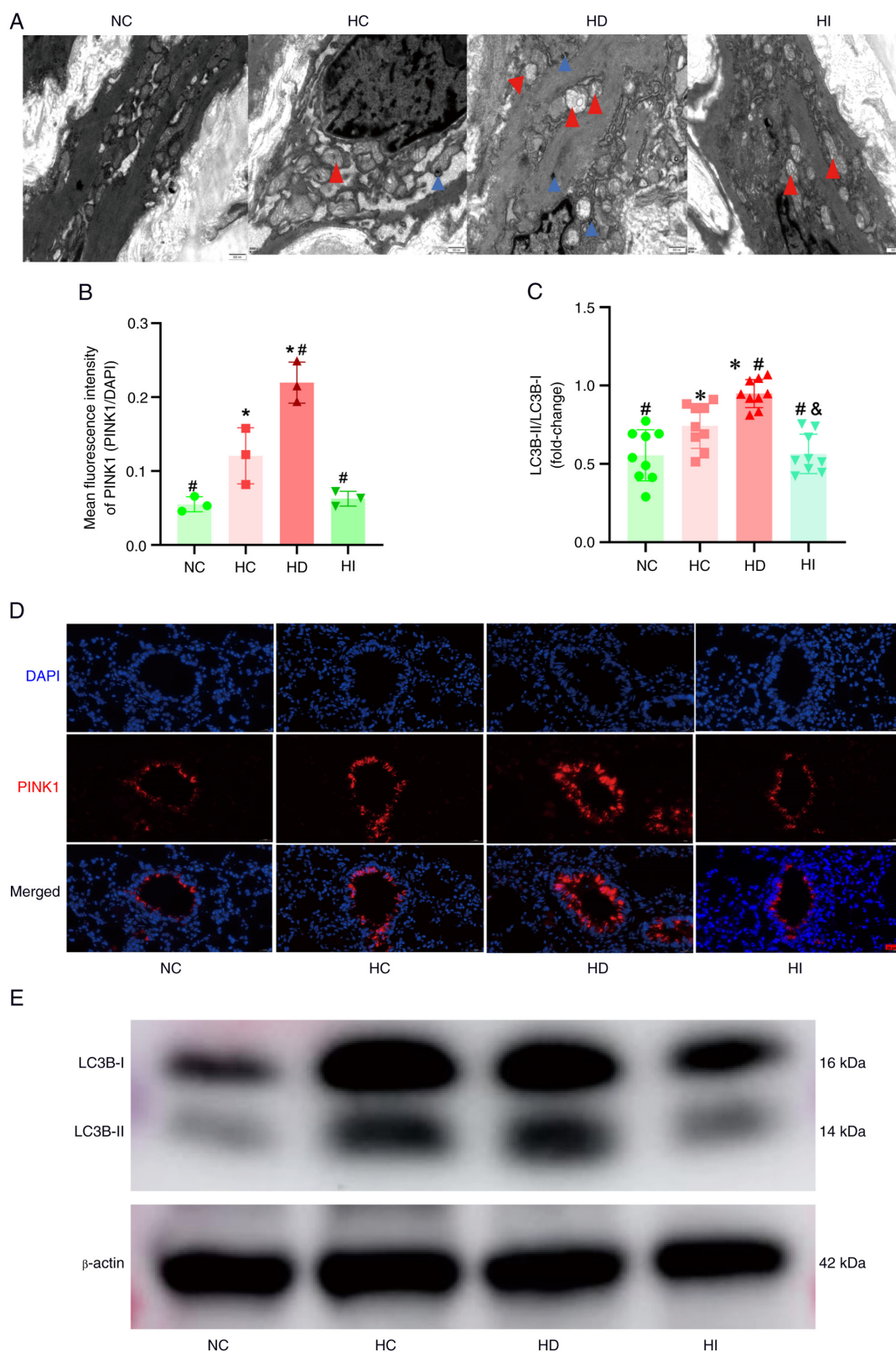


Figure 4. Role of iron in mitophagy in the pulmonary vasculature. (A) Representative images of transmission electron microscopy of the main pulmonary artery (magnification, x20,000). Red triangles represent swollen mitochondria, blue triangles represent mitophagy. (B) Semi-quantitative analysis of PINK1 immunofluorescence, n=3. (C) Semi-quantitative analysis of LC3B-II band intensity, n=9. (D) Representative images of PINK1 immunofluorescence in the pulmonary vasculature (magnification, x400). (E) Representative images of western blot analysis. *P<0.05 vs. NC group; #P<0.05 vs. HC group; &P<0.05 vs. HD group. PINK1, putative kinase protein 1; NC, normoxia control; HC, hypoxia control; HD, hypoxia + deferoxamine; HI, hypoxia combined + iron sucrose.

Iron chelator treatment exacerbates lung tissue injury and elevated mPAP under acute hypoxia, whereas iron sucrose treatment attenuates lung tissue injury and elevated mPAP

induced by acute hypoxia. Given the reduced serum iron concentration observed in rats during acute hypoxia, the present study aimed to intervene in iron metabolism in a rat model

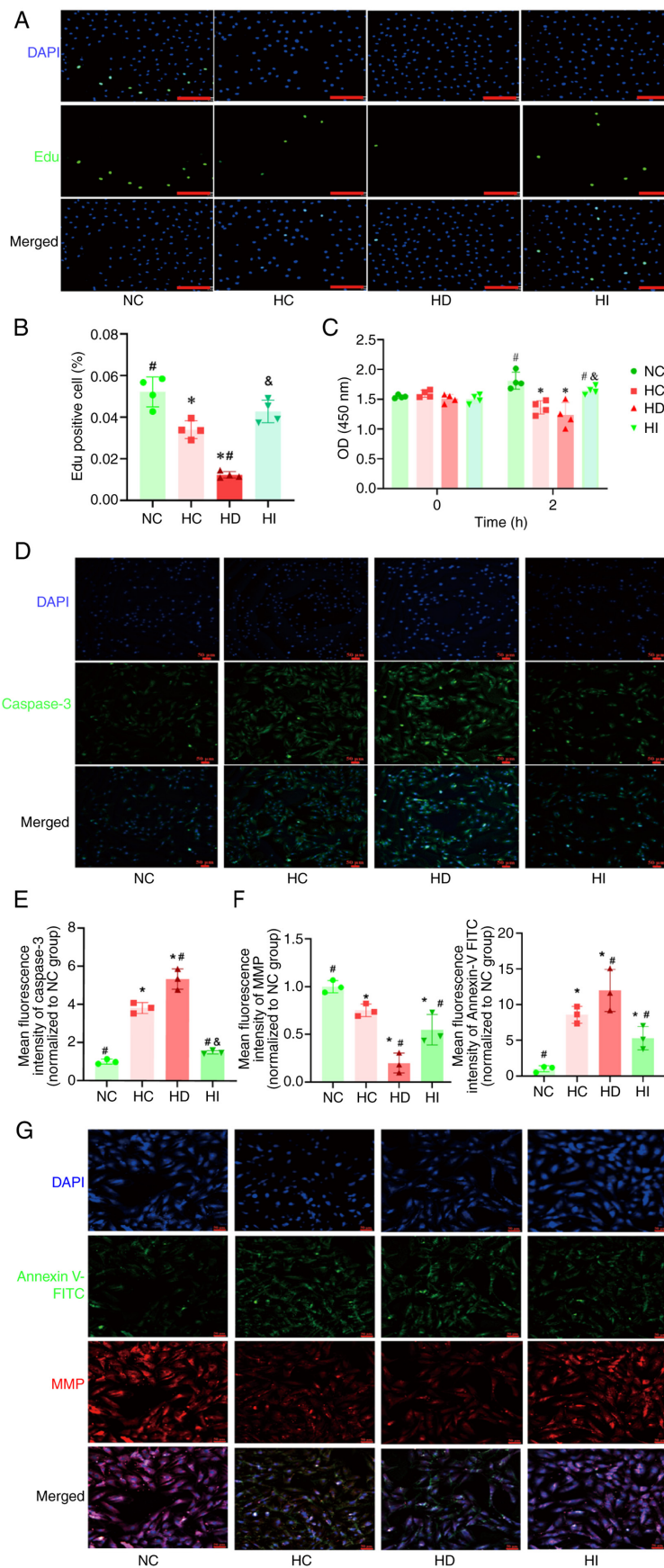


Figure 5. Role of iron in the proliferation and apoptosis of pulmonary artery endothelial cells under acute hypoxia. (A) Representative images of Edu staining (scale bar, 170 μ m). (B) Quantitative analysis of the Edu assay, n=4. (C) Quantitative analysis of the Cell Counting Kit 8 assay, n=4. (D) Representative images of Caspase 3 immunofluorescence staining (magnification, x200). (E) Semi-quantitative analysis of Caspase 3 immunofluorescence, n=3. (F) Semi-quantitative analysis of MMP and Annexin V-FITC, n=3. (G) Representative images of MMP and Annexin V-FITC (magnification, x100). *P<0.05 vs. NC group, #P<0.05 vs. HC group; &P<0.05 vs. HD group. NC, normoxia control; HC, hypoxia control; HD, hypoxia + deferoxamine; HI, hypoxia + iron sucrose; MMP, mitochondrial membrane potential.

using an iron chelator and iron sucrose to elucidate the role of iron in acute high-altitude sickness (Fig. 2A). The results of right heart catheterization for mPAP measurements indicated that the iron chelator DFO was associated with an elevation in mPAP under acute hypoxia. By contrast, iron sucrose was associated with a reduction in mPAP elevation under acute hypoxia (Fig. 2B and D). Morphological analysis indicated the presence of alveolar tissue destruction and inflammatory cell infiltration of the alveolar tissue under acute hypoxia, which was accompanied by fracturing and disorganization of elastic fiber septa in the main pulmonary artery (Fig. 2C). Quantitative data corroborated the findings of H&E staining (Fig. 2E). Furthermore, the wet/dry weight ratio of the lungs was increased under acute hypoxia (Fig. 2F). The administration of an iron chelator resulted in more pronounced acute hypoxia-induced lung injury, whereas iron sucrose demonstrated a protective effect against this injury. The serum iron concentration analysis provided additional evidence supporting the efficacy of the iron chelator and iron sucrose modeling approaches (Fig. 2G).

Iron modulates the HIF1 α activity of the pulmonary vasculature and lung tissue under acute hypoxia. Given the pivotal role of iron as a cofactor for HIF1 α activation, the present study assessed HIF1 α expression. Immunohistochemistry and immunofluorescence staining of the main pulmonary artery and alveolar tissues in rats demonstrated that acute hypoxia resulted in an increase in HIF1 α expression. In addition, the iron chelator promoted an increase in HIF1 α expression, whereas iron sucrose inhibited the increase in HIF1 α expression under acute hypoxic conditions (Fig. 3A-E). Furthermore, alterations in the expression of SMA in the pulmonary vasculature and lung tissue in response to acute hypoxia were in accordance with the expression of HIF1 α (Fig. 3B-E).

Iron modulates mitophagy in the pulmonary vasculature under acute hypoxia. Given the pivotal role of iron chelators and hypoxia in triggering mitophagy, the current study conducted a detailed examination of this process. The results of transmission electron microscopy of the main pulmonary artery demonstrated that acute hypoxia resulted in mitochondrial swelling, accompanied by increased mitophagy (Fig. 4A). The results of immunofluorescence analysis of the pulmonary vasculature demonstrated that acute hypoxia resulted in elevated expression of PINK1, a pivotal protein for mitophagy (Fig. 4B and D); this was accompanied by an increase in the expression of the mitophagy protein LC3B-II. Furthermore, the results indicated that DFO promoted hypoxia-driven PINK1 and LC3B-II upregulation, whereas iron sucrose inhibited this response (Fig. 4B-E).

Iron regulates the proliferation and apoptosis of pulmonary artery endothelial cells under acute hypoxia. To further validate the role of iron in acute HPV, *in vitro* experiments were conducted to examine the effects of iron on the proliferation of human pulmonary artery endothelial cells. The Edu and CCK8 assay results demonstrated that the proliferation of human pulmonary artery endothelial cells was diminished under acute hypoxia (Fig. 5A-C). Furthermore, the administration of an iron chelator led to a further reduction in endothelial cell proliferation under hypoxic conditions, whereas the use of iron sucrose inhibited this reduction (Fig. 5A-C).

The present study also employed immunofluorescence to elucidate the effect of iron on the apoptosis of pulmonary artery endothelial cells under acute hypoxia. The findings demonstrated that acute hypoxia resulted in the upregulation of the apoptotic protein Caspase 3 and Annexin V-FITC (Fig. 5D-G), accompanied by a reduction in MMP (Fig. 5G). The administration of an iron chelator led to a further exacerbation of these hypoxia-induced abnormalities, whereas iron sucrose was observed to exert a protective effect, ameliorating the aforementioned acute hypoxia-induced abnormalities in pulmonary artery endothelial cells (Fig. 5D-G).

Iron modulates HIF1 α expression and mitophagy in pulmonary artery endothelial cells under acute hypoxia. The expression of HIF1 α was observed to be elevated in pulmonary artery endothelial cells subjected to acute hypoxia (Fig. 6A and B). The administration of an iron chelator was revealed to facilitate HIF1 α expression, whereas the introduction of iron sucrose was demonstrated to impede HIF1 α expression in the context of acute hypoxia. The results of western blot analysis of endothelial cells demonstrated that acute hypoxia induced an increase in LC3B-II expression, DFO promoted LC3B-II expression under acute hypoxia, whereas iron sucrose suppressed its upregulation (Fig. 6C and D).

Discussion

A considerable number of individuals climb for recreational, occupational and competitive sporting purposes. The typical pulmonary manifestation of acute hypobaric hypoxia is HPV, which, if left undiagnosed, can result in excessive HPV, leading to PH and pulmonary edema. The onset of these disorders can occur at any point between a few hours and 5 days after the individual has ascended to a given altitude (4). The present study employed a hypoxia administration protocol to investigate the effects of prolonged hypoxia on pulmonary edema and lung tissue injury in rats. The results demonstrated that the degree of pulmonary edema and lung tissue injury was most significant on day 3 of hypoxia. Additionally, it was shown that serum iron exhibited a sustained decline, RBC levels demonstrated an increase and the expression of mitophagy markers in lung tissues was markedly elevated on day 3 of hypoxia. These findings indicated that day 3 of plateau entry may be a period of high incidence of acute altitude sickness. Furthermore, it may be observed that after day 3, with the prolongation of the duration of hypoxia, the performance of the acute plateau response improves; this may be related to the physiological adaption of the body to hypoxia (30).

Iron is an essential element for almost all living organisms, as it is an important component of hemoglobin and iron-sulfur proteins. Furthermore, it is essential for a number of physiological processes, including immune surveillance, oxygen transport and cell proliferation (31). Notably, long-term residents at high altitudes respond to the diminished partial pressure of inhaled oxygen by stimulating erythropoiesis. The increase in hemoglobin levels necessitates a substantial intake of dietary iron supplementation, which can result in an iron deficiency if not replenished in a timely manner (32). The present study demonstrated that rats subjected to acute hypoxia exhibited a reduction in serum iron levels and an increase in hemoglobin concentration. In light of the continuing debate

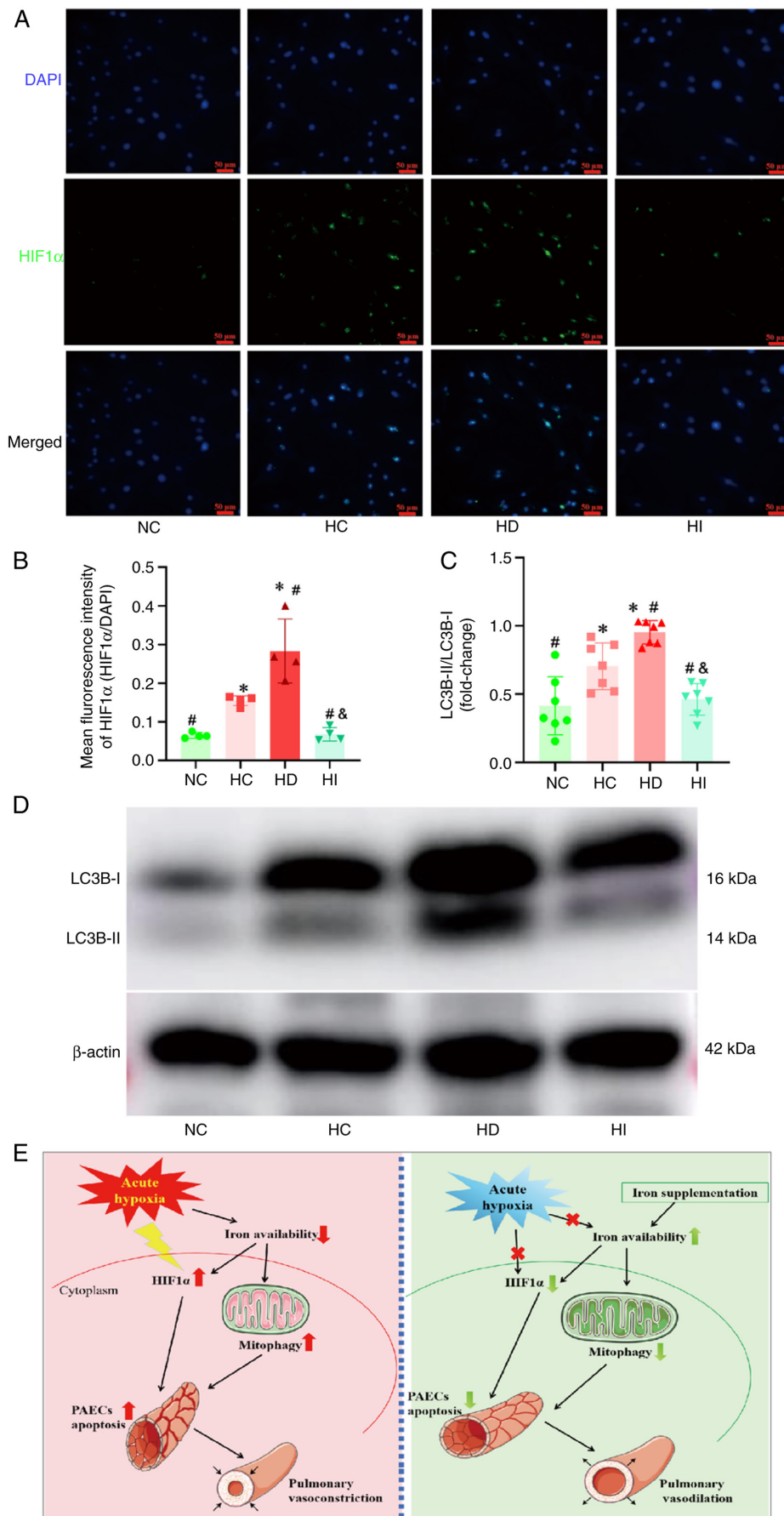


Figure 6. Role of iron in the mitophagy of PAECs under acute hypoxia. (A) Representative images of HIF1α immunofluorescence staining (magnification, x100). (B) Semi-quantitative analysis of HIF1α immunofluorescence staining, n=4. (C) Semi-quantitative analysis of western blotting, n=7. (D) Representative images of western blotting. (E) Graphical abstract. *P<0.05 vs. NC group; #P<0.05 vs. HC group; &P<0.05 vs. HD group. HIF1α, hypoxia-inducible factor 1α; NC, normoxia control; HC, hypoxia control; HD, hypoxia + deferoxamine; HI, hypoxia + iron sucrose; PAECs, pulmonary artery endothelial cells.

surrounding the role of iron status in HPV (9,10,33,34), the current study revealed that low iron levels may intensify HPV, whereas iron supplementation diminished it. This finding aligns with the results of prior clinical investigations (9,34). Nevertheless, the role of iron in high altitude-induced pulmonary edema remains relatively understudied, with current research largely focused on HPV. The present study demonstrated that iron supplementation may be an effective intervention for acute hypoxic pulmonary edema; however, the observed effect may be attributed to its ability to improve acute HPV. Further in-depth studies are required to elucidate the precise relationship between iron and pulmonary edema.

HIF1 α is a principal transcription factor that mediates the acute hypoxic response; its expression is increased following exposure to acute hypoxia (35). In addition, HIF1 α serves a pivotal role in the induction of mitophagy and may be linked to the progression or inhibition of disease states (36). Furthermore, iron, which acts as a crucial cofactor for proline hydroxylase, has been demonstrated to regulate HIF1 α expression under hypoxic conditions (37). Concurrently, iron chelators have also been demonstrated to induce mitophagy (10,11). It could be hypothesized that the combination of HIF1 α and iron chelators may intensify mitophagy during acute hypoxia. The findings of the present study demonstrated that acute hypoxia resulted in an elevation in HIF1 α expression. Notably, an iron chelator was observed to enhance HIF1 α expression and mitophagy under acute hypoxia. By contrast, iron sucrose was shown to mitigate the aforementioned abnormalities. This provides a comprehensive illustration of the relationship between iron, HIF1 α and mitophagy in acute high altitude-induced lung injury.

Beyond mitophagy, excessive pulmonary inflammation and oxidative stress are established contributors to the pathogenesis of acute lung injury (38). The present findings demonstrated that acute hypoxia could induce inflammatory cell infiltration in alveolar tissues, indicating an inflammatory response. This response involves cytokine interactions, with HIF1 α playing a crucial regulatory role. Acute lung injury exhibits a pro-inflammatory positive feedback loop, where inflammatory factors upregulate HIF1 α , which in turn amplifies inflammation and exacerbates injury (39). Notably, ferritin, an iron storage protein, serves as an inflammatory marker (40,41). The reduction of iron in the circulation during acute hypoxia may be due to the increased inflammation in lung tissues and the elevation of ferritin levels; future studies should elucidate the precise mechanisms underlying iron-mediated inflammatory regulation in acute lung injury. Additionally, a previous study has verified that the increase in reactive oxygen species under hypoxia may contribute to the progression of acute lung injury; this occurs through the autoxidative disruption of cellular microstructures and the structural or functional damage to pulmonary vascular endothelial cells, epithelial cells and smooth muscle cells, resulting in dysfunction of the alveolar-vascular endothelial barrier and vasodilatory function (42). The present study demonstrated that acute hypoxia can lead to an imbalance between proliferation and apoptosis in pulmonary vascular endothelial cells, thereby inducing acute lung injury. Notably, excessive iron aggregation has been shown to promote oxidative stress and exacerbate lung injury (43). Therefore,

precisely controlling circulating iron levels may help to mitigate lung injury.

Taken together, the present study identified the most critical time point of acute lung injury during hypobaric hypoxia. This finding offers a scientific foundation for the clinical transportation of patients suffering from acute altitude sickness. Simultaneously, the current study identified the changes in iron levels under acute hypoxia and performed iron-intervention experiments to determine the role and mechanism of serum iron status in high altitude-induced hypoxic lung injury, thereby providing a novel target for intervention in the treatment of acute lung injury at high altitude (Fig. 6E). However, the present study has several limitations. First, human trials were not conducted due to laboratory constraints and ethical restrictions in human genetics. Future research will include human trials, pending improved laboratory conditions and ethics approval, to further elucidate the disease mechanism. Second, the animal study design lacked an intervention treatment group under normoxic conditions. While the hypoxia-based intervention effectively demonstrated its effect on acute hypoxic lung injury, this still represents a shortcoming of the study. Finally, although the present study focused on mitophagy, the pathogenesis of acute hypoxic lung injury is multifactorial, involving mechanisms such as inflammation and oxidative stress. Furthermore, the evaluation of lung injury encompasses diverse indicators including pulmonary function tests. However, the present study primarily concentrated on mitophagy-related mechanisms and employed histopathological analysis along with wet/dry weight ratio as principal evaluation metrics for lung injury, which constitutes one of the limitations in this work. Future studies will employ diverse methodologies to assess the modeling efficacy of lung injury and explore these additional mechanistic pathways, aiming to provide a more comprehensive understanding of high altitude-induced acute hypoxic lung injury.

In conclusion, the present study demonstrated the role of iron-mediated HIF1 α activation and mitophagy in acute lung injury. The onset of acute hypoxia was revealed to result in a reduction in iron bioavailability, which subsequently triggered the activation of HIF1 α and facilitated mitophagy. This ultimately led to the development of HPV and pulmonary edema. In the future, it may be possible to increase iron bioavailability to prevent high altitude-induced acute lung injury, and to inhibit HIF1 α activity and mitophagy in order to treat acute lung injury. Both of these approaches provide new potential therapeutic targets for high altitude-induced acute lung injury.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YG and RG conceived and designed the experiments. YG, YH, HW and FZ performed experiments and analyzed data. YG, YH and RG wrote and revised the manuscript. YG, FZ and RG confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The experimental protocols and animal care procedures employed in the present study were approved by the Medical Ethics Committee of Qinghai Provincial People's Hospital (approval no. 2023-95). The Research Ethics Committee of Qinghai Provincial People's Hospital confirmed that commercially procured human primary cells fall outside the scope of required ethical approval.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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