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Ayşe Demirci Şahin, Emel Güneş, Göktuğ Ömercioğlu, Ayşe Hande Yozgat, Deniz Billur & Metin Baştuğ

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PLATELETS IN ANGIOGENESIS AND PULMONARY PROGENITOR CELL HOMING UNDER CHRONIC INTERMITTENT HYPOXIA

Ayşe Demirci Şahin[1] (ORCID: 0000-0003-0667-8886)

Emel Güneş[1] (ORCID: 0000-0003-3599-5151)

Göktuğ Ömercioğlu[1] (ORCID: 0000-0002-4387-4598)

Ayşe Hande Yozgat[2] (ORCID: 0009-0002-9580-7685)

Deniz Billur[2] (ORCID: 0000-0001-8541-8251)

Metin Baştuğ[1] (ORCID: 0000-0002-8008-6515)

[1] Department of Physiology, Ankara University Faculty of Medicine, Ankara, Türkiye

[2] Department of Histology and Embryology, Ankara University Faculty of Medicine, Ankara, Türkiye

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Address of Corresponding Author:

Ayşe Demirci Şahin, MD

Department of Physiology, Ankara University Faculty of Medicine, Ankara, Türkiye,

E-mail: aysedemirc@yahoo.com

Phone: 00905052215635

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ABSTRACT:

Platelets play a critical role in tissue repair and contain various molecules that regulate angiogenesis, such as stromal cell-derived factor-1 (SDF-1) which stimulates endothelial progenitor cells (EPCs). Chronic intermittent hypoxia (CIH) alters platelet function, but its specific impact on platelet-derived angiogenic factors and endothelial progenitor cell (EPC) recruitment in lung injury remains unclear. This study investigated the effects of CIH on the profile of pro- and anti-angiogenic molecules in platelets and their role in EPC homing during acute lung injury (ALI). Ten-week-old 35 rats were divided into control, ALI, CIH and CIH+ALI groups. CIH was applied for 6 hours per day over 42 days, simulating an altitude of 3,000m., approximately 14% O_2. Blood and lung samples were taken three days after ALI. Vascular endothelial growth factor (VEGF), SDF-1, platelet-derived growth factor (PDGF), endostatin, thrombospondin-1, and plasminogen activator inhibitor-1 (PAI-1) were measured in platelet specimens. EPC homing to the lung was assessed via immunofluorescence for VEGFR2 and CD133. The CIH+ALI group exhibited a significant increase in platelet count. However, concentrations of SDF-1, VEGF, PDGF, PAI-1, endostatin, and thrombospondin-1 *per platelet* were significantly lower in this group. While EPC homing was prominent in the ALI group, it was absent in both CIH and CIH+ALI groups. These findings demonstrate that CIH reprograms the angiogenic cargo of platelets, characterized by a specific reduction in SDF-1 and a general dilution of other factors. This altered platelet profile, coupled with the absence of EPC recruitment, suggests a novel mechanism by which CIH may impair vascular repair in the injured lung via decreased platelet-derived SDF-1.

Keywords: platelet, intermittent hypoxia, angiogenesis, endothelial progenitor cells, SDF-1, acute lung injury

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INTRODUCTION

Platelets play a critical role in tissue repair and contain various molecules that regulate angiogenesis, including stromal cell-derived factor-1 (SDF-1/CXCL12), which aids in neo-alveolarization by promoting progenitor cell homing to damaged lungs [1, 2]. The exact mechanisms of how platelets regulate angiogenesis remain unclear [3, 4]. However, the synchronized action of several signaling molecules, including stem cell activation, angiogenic factors such as vascular endothelial growth factor-A (VEGF-A), and fibroblast growth factors (FGFs) is required for alveolar regeneration [1, 5].

SDF-1 promotes the differentiation of stem cells into endothelial progenitor cells (EPCs) and enhance angiogenesis [6]. Platelet-derived SDF-1 facilitates the binding of CD34+ stem cells from bone marrow to injured endothelium, transforming them into EPCs [7, 8]. Platelets also produce angiogenic molecules like vascular endothelial growth factor (VEGF), FGF, epidermal growth factor (EGF), platelet derived growth factor (PDGF), and matrix metalloproteinases (MMPs), and as well as, angiogenesis inhibitors including endostatin, platelet factor-4, thrombospondin-1, alpha 2-macroglobulin, PAI-1 [3].

Intermittent hypoxia is gaining attention for its protective effects against severe hypoxemia and ischemia [9]. A crucial role in hypoxia-induced angiogenesis is performed by bone marrow-derived mesenchymal stem cells. Platelets may facilitate the communication between oxygen-deprived tissues and bone marrow, acting as reservoirs for growth factors and cytokines [5].

Studies suggest that CIH may enhance their ability to promote angiogenesis and improve microcirculation by regulating platelet activity [10]. This process is particularly significant in conditions such as acute lung injury (ALI), where hypoxia and impaired vascular function are major challenges.

This study aims to examine the effect of CIH on platelet-mediated pro-angiogenic, anti-angiogenic molecules and determine whether changes in platelet-derived SDF-1 levels effect homing of EPCs into damaged lung tissue.

METHODS

Thirty-five 10-week-old male Wistar Albino rats (Rattus norvegicus), purpose-bred in the Experimental Animal Research Laboratory of Ankara University Medical Faculty, were used in this study. The sample size was estimated using the previously described research equation approach [11]. The number of subjects per group was

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determined as n = 6, based on the assumption that this sample size would allow for statistical evaluation and the achievement of normal distribution. To account for anticipated losses due to chronic intermittent hypoxia and lung injury, three reserve animals per group were allocated. Each group initially included six rats, with the study proceeding using the minimum number unless losses were observed. Mortality was observed exclusively following LPS-induced acute lung injury. In the ALI group, two animals died within the first 24 hours. In the CIH+ALI group, three animals died between 12 and 36 hours post-LPS administration. No mortality occurred in the control or CIH-only groups during the 42-day protocol. The study was completed with 7 rats in the control, ALI and CIH groups, 9 rats in the CIH+ALI group. Survival analysis was performed using the Kaplan-Meier method, and comparisons between groups were assessed with the log-rank test. Survival curves for all groups are presented in Supplementary Figure. The log-rank test comparing the survival distributions across all groups did not reveal a statistically significant difference ($p = 0.29$).

Only male rats were used in this study to eliminate potential confounding effects of the estrous cycle and sex hormones on platelet reactivity, angiogenic factor levels, and the response to hypoxia, thereby reducing biological variability within the relatively small sample groups.[12]

The study was approved by the Institutional Animal Experiments Ethics Committee of Ankara University Medical Faculty (reference number 2021-11-91). All animal handling adhered to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals, as published by the United States National Institutes of Health [13]. The results of the study are reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

Experimental Interventions:

Prior to study interventions, the animals were kept in an environment with a 12-hour light/dark cycle, a temperature range of 22-24°C, and a humidity level of $50 \pm 5\%$. Food and water were provided ad libitum. After one week of adaptation, animals were randomly divided into four groups: control, ALI, CIH, and CIH+ALI.

The hypoxia groups (CIH and CIH+ALI) were placed in a hypobaric hypoxic chamber for 42 days, with each session lasting 6 hours per day. The chamber was set to an atmospheric pressure of 69.3 kPa, equivalent to an altitude of 3,000 meters, with an oxygen concentration of about 14%. This CIH protocol, including the duration, pressure, and session length, was adapted from the study by Akat et al, which

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demonstrated its efficacy in inducing cardiovascular and angiogenic adaptations [14].

Lipopolysaccharide (LPS) was administered intratracheally to provoke lung injury and stimulate epithelial cell regeneration.[15] The animals in the lung injury groups (ALI and CIH+ALI) were administered intratracheal LPS (from Escherichia coli O55:B5) at a concentration of 100 µg/100 g, 24 hours after the final session of the 42-day CIH protocol for the CIH+ALI group or at an equivalent time point for the ALI group. The administration was performed under anesthesia (90 mg/kg ketamine and 10 mg/kg xylazine). The procedure was carried out under sterile conditions, tracheostomies were closed with absorbable sutures before the animals recovered from anesthesia.

Since previous research indicates that the peak level of SDF-1 occurs on the third day following lung injury, when endothelial progenitor cells start to migrate and settle in the lung, stem cells were evaluated on the third day after lung injury [16]. Animals were euthanized via exsanguination under anesthesia (intramuscular administration of 90 mg/kg ketamine and 10 mg/kg xylazine) 3 days after LPS administration, and 4 days after the final hypoxia session for groups without LPS application (Figure 1).

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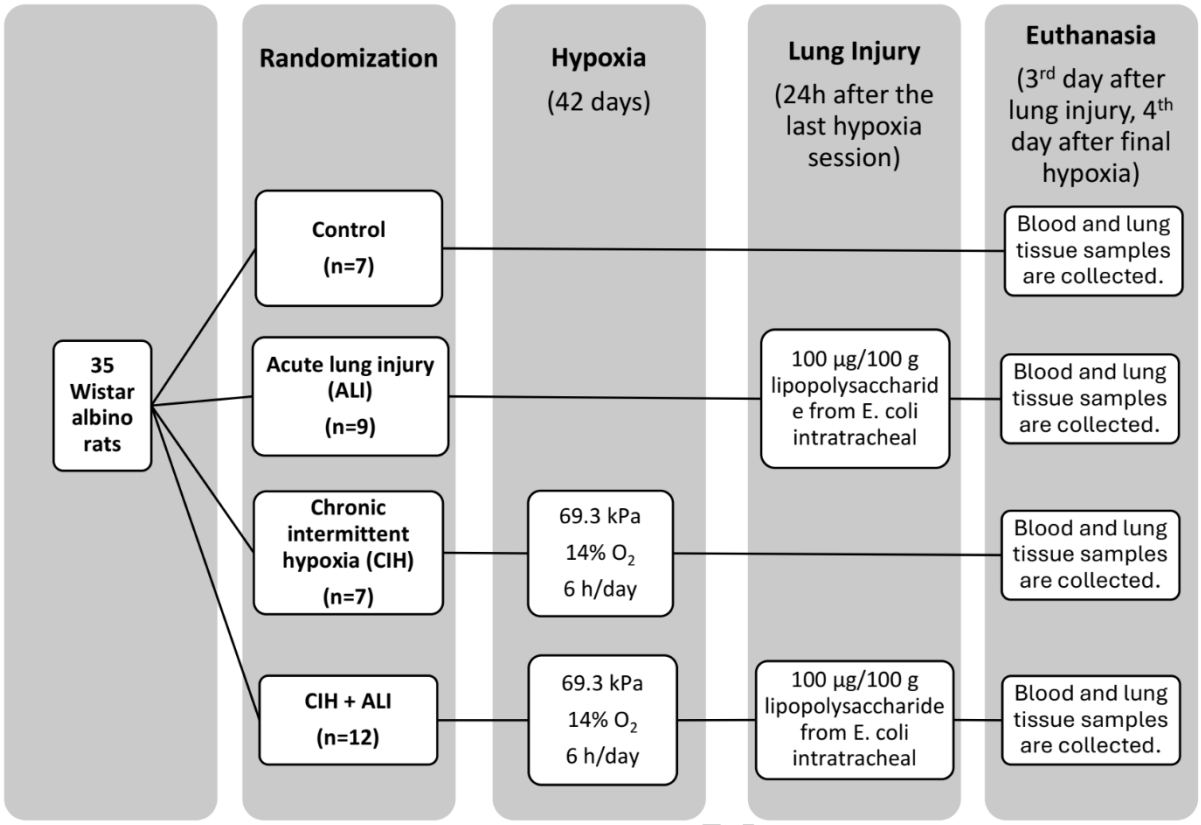


Figure 1. *Timeline of the experimental protocol detailing the sequence and timing of interventions, including hypoxia exposure, lipopolysaccharide administration, sample collection, and endpoint assessments.*

Blood Sample and Tissue Processing:

Blood samples were taken directly from the heart into tubes containing citrate and the animals were euthanized by exsanguination. Lung tissue was extracted after blood collection. Each rat's lungs were carefully removed and divided into two portions for subsequent analyses. Consistent with established fixation protocols, tissue samples designated for light microscopic examination were immediately fixed in buffered 10% formalin to preserve histological architecture. The remaining portions intended for immunofluorescence staining were fixed in 4% paraformaldehyde (PFA) solution to maintain cellular antigenicity.[17]

Platelet rich plasma (PRP) was produced through a three-step process of preparing a washed platelet suspension as previously described [18]. Blood was collected from the abdominal aorta using a 25-gauge needle into tubes with anticoagulant citrate dextrose solution (ACD-A) at a ratio of 6 parts blood to 1 part ACD-A. Blood

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samples were centrifuged at 2300 g for 10 minutes to obtain PRP. Using a sterile pipette, PRP was transferred to separate tubes.

The number of platelets per milliliter of plasma was determined using an Invitrogen Countess automated cell counter. The platelet count was determined by multiplying the number of platelets per milliliter by the volume of the sample in milliliters.

PRP was then subjected to another round of centrifugation at 2200 g for 4-10 minutes, depending on the volume obtained. After centrifugation, platelets were isolated from the bottom of the tube, and the upper layer, designated as platelet-free plasma (TFP), was carefully aspirated

Platelets were resuspended by adding 5 mL of a standard solution containing albumin (0.35% w/v), heparin (10 U/mL), and prostaglandin I2 (PGI2, 0.5 µM). After a 10-minute incubation at 37°C, the sample was centrifuged at 1900 g for 5 minutes. The supernatant was removed, and platelets were resuspended in 5 ml of the solution containing albumin and PGI2. After a 10-minute incubation at 37°C, PGI2 (0.5 µM) was added, and the sample was centrifuged at 1900 g for 4 minutes. After centrifugation, 0.5 mL of liquid was left on the platelet pellet, and the remaining part was removed.[19] Platelet pellets were stored at -80°C.

Enzyme-linked Immunosorbent Assay (ELISA) Protocols:

The concentrations of SDF-1α, VEGF, PDGF, PAI-1, endostatin and thrombospondin-1 in platelet supernatants were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturers' instructions. The following kits from Bioassay Technology Laboratory (BT Lab, Shanghai, China) were used: rat stromal cell-derived factor 1a (SDF-1a) ELISA kit (catalog # E0327Ra; detection range: 0.3 - 90 ng/L; sensitivity: 0.152 ng/L; sample volume: 50 µL), rat vascular endothelial cell growth factor (VEGF) ELISA kit (catalog # E0659Ra; detection range: 10 - 3000 ng/L; sensitivity: 5.01 ng/L; sample volume: 50-100 µL), rat platelet-derived growth factor (PDGF) ELISA kit (catalog # E0691Ra; detection range: 0.05 - 15 ng/mL; sensitivity: 0.023 ng/mL; sample volume: 50 µL), rat plasminogen activator inhibitor 1 (PAI-1) ELISA kit (catalog # E0636Ra; detection range: 0.1 - 40 ng/mL; sensitivity: 0.49 ng/mL; sample volume: 50 µL), rat endostatin (ES) ELISA kit (catalog # E0467Ra; detection range: 3 - 900 ng/mL; sensitivity: 1.52 ng/mL; sample volume: 50 µL), rat thrombospondin 1 (TSP-1) ELISA kit (catalog # E0687Ra; detection range: 0.5 - 150 ng/mL; sensitivity: 0.25 ng/mL; sample volume: 50 µL). All kits were sandwich ELISA formats. The absorbance of the samples was measured at a wavelength of 450 nm using a spectrophotometer

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(BioTek® µQuant Microplate Reader, Bio-Tek Instruments, Inc., VT, USA). The concentrations of the analytes were determined by interpolating from the standard curve generated for each assay. The measured concentrations were then normalized by the platelet count in each sample and are expressed as concentrations per platelet.[20, 21]

Light Microscopic Examination:

Lung specimens spared for light microscopic examination were fixed in buffered 10% formalin for 72 hours at room temperature, then washed in tap water. Samples were dehydrated in 70%, 80%, 90%, and 100% ethanol, respectively, rinsed in xylene for 5-10 minutes, and embedded in paraffin wax. Serial sections of 5 µm in thickness were obtained with a rotary microtome and stained with hematoxylin and eosin (H&E). The stained sections were evaluated and photographed under a light microscope.[22] For each slide, 3 randomly selected microscopic fields were evaluated under ×400 magnification.

Immunofluorescent Staining Procedure:

Lung specimens spared for immunofluorescence were fixed in 4% PFA solution at +4°C for one night, then kept in 1.2 M sucrose + 0.1% fixative PFA mixture overnight. After drying, tissues were embedded in cryomatrix and stored at -18°C until sectioning. Subsequently, 10 µm-thick serial sections were prepared and mounted on Poly-L-Lysine-coated slides.[22] After washing and blocking, the sections were incubated with rabbit anti-VEGFR2 polyclonal antibody (catalog # bs-10412R; Bioss Antibodies, Woburn, MA, USA) diluted 1:50 at 4°C overnight. The next day, sections were washed, incubated with Cy3-conjugated goat anti-rabbit IgG (H+L) secondary antibody (catalog # 111-165-003; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:800 at 37°C for 2 hours, and then washed and blocked again. The second staining was performed using mouse monoclonal anti-CD133 antibody (E-11 clone; sc-365537; Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:50 at 4°C overnight. The next day, sections were washed, incubated with Cy5-conjugated goat anti-mouse IgG (H+L) secondary antibody (catalog # 115-175-146; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:400 for 2 hours at 37°C, washed again, covered with Hoechst, and examined with Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). For each specimen, confocal images were obtained from 3 randomly selected fields. The images presented in both light microscopic (Figure 5) and immunofluorescence (Figure 6) examinations are illustrative of the observed

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morphological changes and protein localization patterns. The study did not include pixel-based quantification.

Statistical Analysis:

Normal distribution of variables was analyzed using visual (histograms and probability plots) and analytical methods (Shapiro-Wilk test). Descriptive statistics provided mean ± standard deviation for normally distributed data and median and minimum/maximum values for non-normally distributed data. ANOVA and Bonferroni post-hoc tests were used for normally distributed data. Kruskal-Wallis test and Mann-Whitney U test with Bonferroni correction were used for non-normally distributed data. Statistical significance was accepted at $p < 0.05$. Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 25.0 (released 2017, Armonk, NY: IBM Corp). Data was visualized using GraphPad Prism 8.4.3 for Windows (www.graphpad.com).

RESULTS

Platelet Counts:

Platelet counts varied significantly between groups ($p<0.01$). The CIH+ALI group had significantly higher platelet concentration (mean 103.7 ±11.6 x 10^5/mL) compared to control (25.6 ±3.8 x 10^5/mL), ALI (mean 31.0 ±5.4 x 10^5/mL) and CIH (mean 32.5 ±9.5 x 10^5/mL) groups ($p<0.01$). The CIH+ALI group had also significantly higher total platelet counts (mean 455.5 ±47.1 x 10^5) compared to control (mean 105.6 ±16.0 x 10^5/mL), ALI (mean 112.8 ±16.6 x 10^5/mL) and CIH (mean 146.6 ± 40.8 x 10^5/mL) groups ($p<0.01$) (Figure 2).

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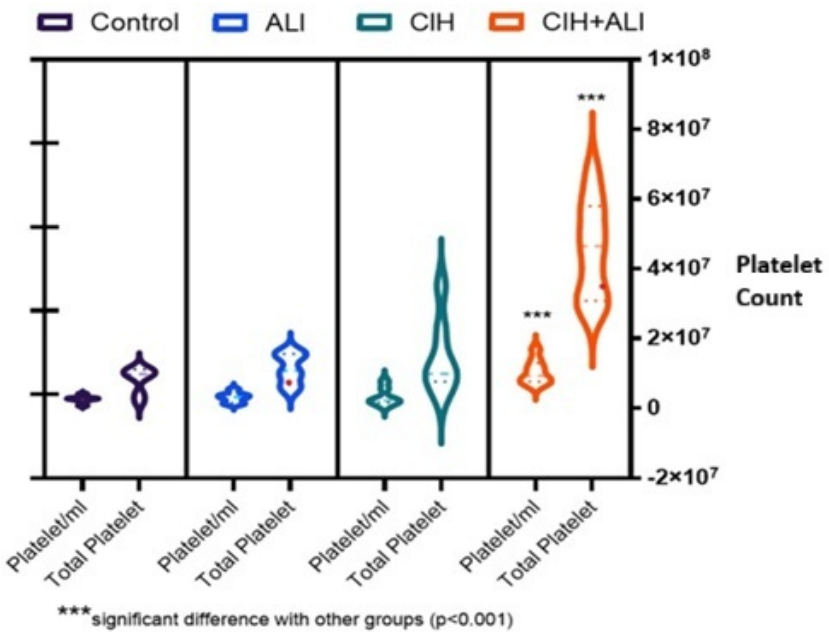


Figure 2. *Distribution and comparison of mean total platelet yields and corresponding mean platelet concentrations per unit volume among study groups*

Platelet-Derived Pro-angiogenic and Anti-angiogenic Factors:

SDF-1 levels differed significantly between groups (p=0.04). In post-hoc analyses, CIH (median 1.04 ng/L; range 0.56-2.19) had significantly lower levels when compared to control (median 2.47 ng/L; range 1.40-2.55) and ALI (median 2.06 ng/L; range 2.05-4.21) groups (p=0.001 and p = 0.038, respectively); CIH+ALI (median 1.33 ng/L; range 0.88-3.26) had similar levels when compared to control, CIH and ALI (Figure 3A). SDF-1/platelet concentrations varied significantly between groups (p=0.001). In post-hoc analyses, CIH+ALI (median 1.0 x 10^{-8} ng/L; range <5.0) had significantly lower levels than control (median 10.0 x 10^{-8} ng/L; range 6.0-27.0) and ALI (median 10.0 x 10^{-8} ng/L; range 3.0-18.0) (p<0.01 and p=0.001, respectively); CIH (median 4.0 x 10^{-8} ng/L; range <14.0) had similar levels when compared to control, ALI and CIH+ALI (Figure 3B).

The median VEGF concentrations were 243.6 ng/L (range 160.2-258.2), 180.0 ng/L (range 162.9-290,2), 167.5 ng/L (range 134.5-237.2) and 179.3 (range 146.4-269,8) in control, ALI, CIH and CIH+ALI, respectively. No significant difference was observed among groups (p=0.29) (Figure 3C). However, VEGF/platelet concentrations varied significantly (p<0.01). In post-hoc analyses, CIH+ALI had significantly lower VEGF/platelet concentrations (median 1.9 x 10^{-6} ng/L; range 1.3-4.5) compared to control (median 10.9 x 10^{-6} ng/L; range 4.7-31.0), ALI (median 9.1

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x 10^{-6} ng/L; range 5.2-17.3) and CIH (median 8.7 x 10^{-6} ng/L; range 2.2-23.5) ($p<0.01$, $p<0.01$, $p=0.002$, respectively) (Figure 3D).

The mean PDGF concentrations were 2.09 ng/mL [standard deviation (SD) ±0.13], 1.86 ng/mL (SD ±0.13), 2.06 ng/mL (SD ±0.07) and 2.12 ng/mL (SD ±0.06) in control, ALI, CIH and CIH+ALI, respectively. There was no significant difference between groups ($p=0.26$) (Figure 3E). However, PDGF/platelet concentrations varied significantly ($p=0.001$). In post-hoc analysis, CIH+ALI had significantly lower PDGF/platelet concentrations (median 2.2 x 10^{-8} ng/mL; range 1.5-3.8) compared to control (median 9.7 x 10^{-8} ng/mL; range 4.0-28.0), ALI (median 10.0 x 10^{-8} ng/mL; range 4.0-14.0), CIH (median 10.1 x 10^{-8} ng/mL; range 2.0-27.0) groups ($p<0.01$, $p<0.01$, $p=0.002$, respectively) (Figure 3F).

The mean PAI-1 concentrations were 4.82 ng/mL (SD ±0.37), 5.11 ng/mL (SD ±0.47), 6.85 ng/mL (SD ±0.42) and 5.64 ng/mL (SD ±0.46) in control, ALI, CIH and CIH+ALI, respectively. A significant difference in PAI-1 concentrations was observed between groups ($p=0.02$). In post-hoc analysis, there was a significant difference between control and CIH ($p<0.05$) (Figure 4A). PAI-1/platelet concentrations also varied significantly ($p=0.001$). In post-hoc analysis, CIH+ALI had significantly lower PAI-1/platelet concentrations (median 5.0 x 10^{-8} ng/mL; range 2.0-13.0) compared to control (median 2.5 x 10^{-7} ng/mL; range 1.2-6.2), ALI (median 2.4 x 10^{-7} ng/mL; range 1.3-4.5), CIH (median 27.0 x 10^{-8} ng/mL; range 9.0-127.0) groups ($p<0.01$, $p<0.01$, $p=0.001$, respectively) (Figure 4B).

The mean endostatin concentrations were 149.9 ng/mL (SD ±3.6), 187.1 ng/mL (SD ±9.0), 163.3 ng/mL (SD ±7.0) and 150.6 ng/mL (SD ±6.5) in control, ALI, CIH and CIH+ALI, respectively. A significant difference in endostatin levels was present between the groups ($p=0.002$), with higher levels in ALI compared to control and CIH+ALI ($p<0.01$ and $p<0.01$, respectively) (Figure 4C). Endostatin/platelet concentrations also varied significantly ($p=0.002$). The CIH+ALI group had significantly lower endostatin/platelet concentrations (median 1.64 x 10^{-6} ng/mL; range 1.11-3.10) compared to control (median 7.44 x 10^{-6} ng/mL; range 3.69-31.0), ALI (median 8.44 x 10^{-6} ng/mL; range 5.76-15.7) and CIH (median 8.56 x 10^{-6} ng/mL; range 2.24-21.1) ($p<0.01$, $p<0.01$, $p = 0.03$, respectively) (Figure 4D).

Thrombospondin concentrations differed significantly between groups ($p=0.002$). Thrombospondin concentration was significantly higher in CIH (median 43.4 ng/mL; range 39.1-56.3) when compared to control (median 29.1 ng/mL; range 22.7-38.6), ALI (median 28.3 ng/mL; range 22.1-32.3) and CIH+ALI (median 29.2 ng/mL; range

19.5-48.8) (p=0.001, p=0.001 and p=0.003, respectively) (Figure 4E). Thrombospondin/platelet concentrations varied significantly (p<0.01). In post-hoc analyses, CIH+ALI had significantly lower levels (median 3.3 x 10^{-7} ng/mL; range 1.6-7.5) when compared to control (median 14.7 x 10^{-7} ng/mL; range 6.1-39.5), ALI (median 13.4 x 10^{-7} ng/mL; range 9.0-21.2) and CIH (median 21.9 x 10^{-7} ng/mL; range 6.0-57.4) (p<0.01, p<0.01, p<0.01, respectively) (Figure 4F).

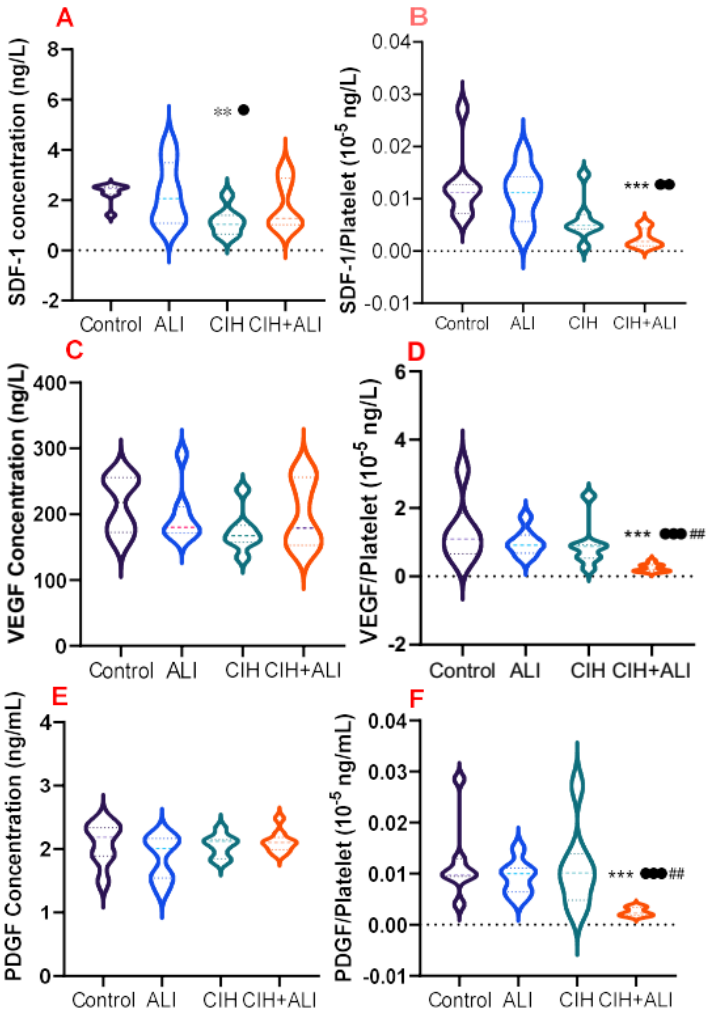


Figure 3. *Platelet-derived proangiogenic factors. (**A:** Total SDF-1 concentration in the supernatant, **B:** SDF-1 concentration per platelet, **C:** Total VEGF concentration in the supernatant, **D:** VEGF concentration per platelet, **E:** Total PDGF concentration in the supernatant, **F:** PDGF concentration per platelet*

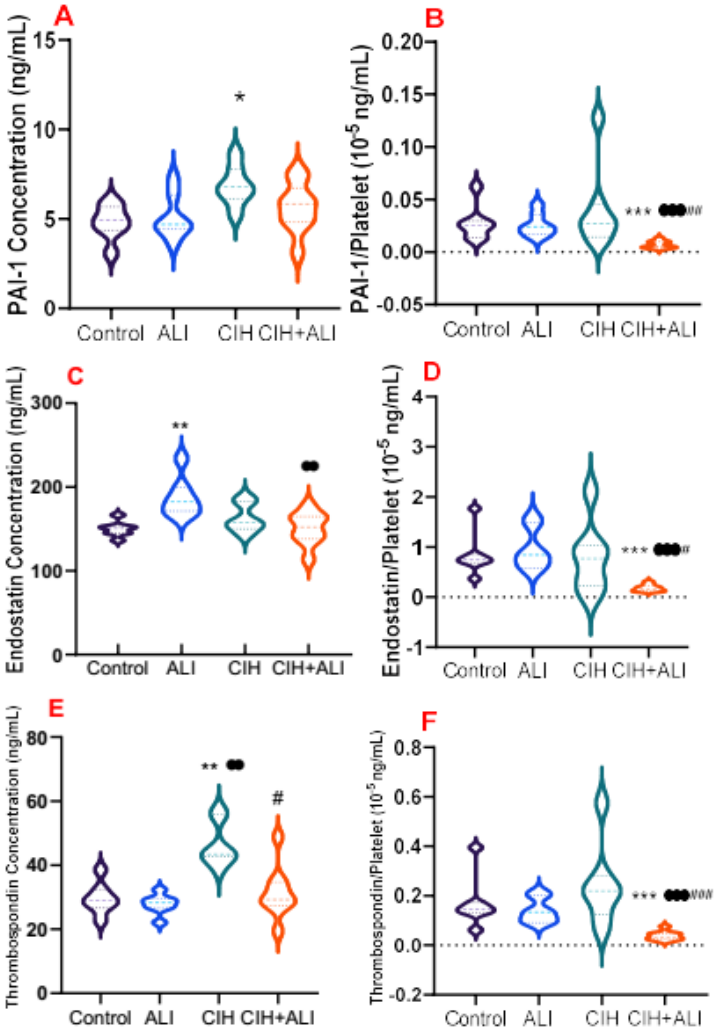


Figure 4. *Platelet-derived antiangiogenic factors. (**A:** Total PAI-1 concentration in the supernatant, **B:** PAI-1 concentration per platelet, **C:** Total endostatin concentration in the supernatant, **D:** Endostatin concentration per platelet, **E:** Total thrombospondin concentration in the supernatant, **F:** Thrombospondin concentration per platelet.)*

Light Microscopic Examination:

Lung samples were stained with H&E. Interstitial mononuclear cell infiltrations and hemorrhage, indicating bleeding, were observed in the ALI, CIH, and CIH+ALI groups. Whereas, mononuclear cells in the airway lumen were present in the ALI and CIH+ALI groups (Figure 5).

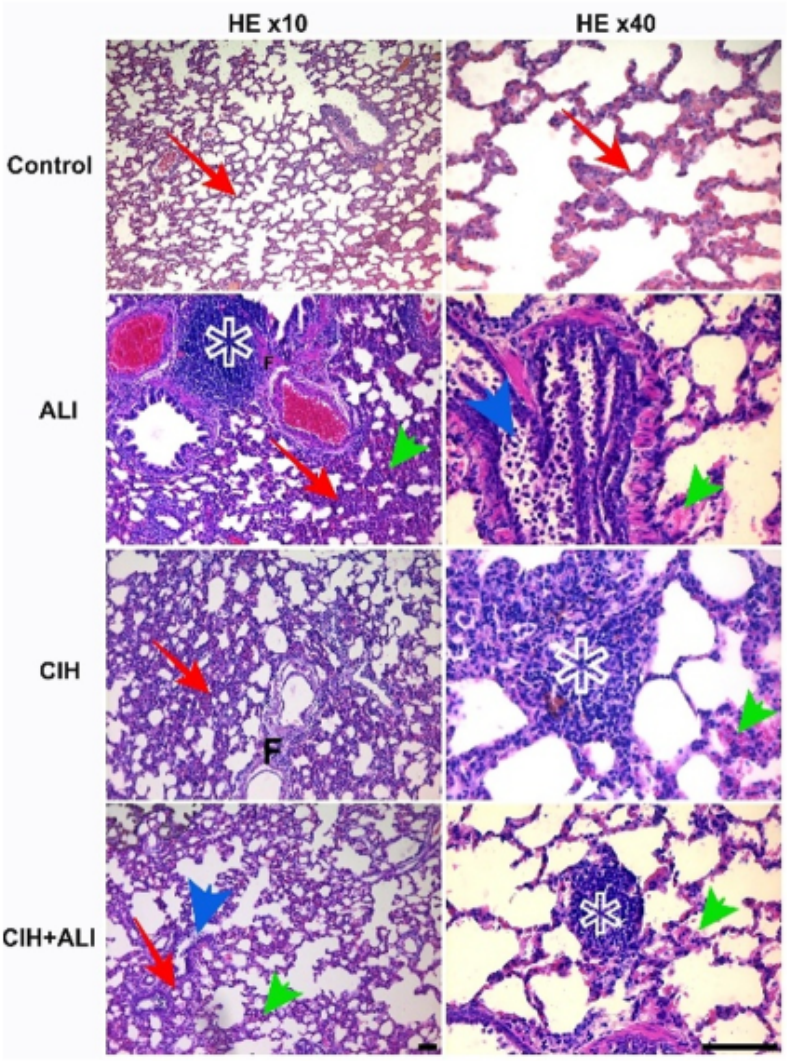


Figure 5. *Light microscopic image of the lung tissue micrographs from experimental groups. (Red arrow: interalveolar septum; asterix: interstitial mononuclear cell infiltration; green arrow: hemorrhage; blue arrow: mononuclear cell in airway lumen. Dyes: Haematoxylin and Eosin. Scale bars: 100 µm)*

Immunofluorescence Staining of EPCs in Lung Tissue:

Since simutaneous expression of VEGFR2 and CD133 has been reported to define endothelial progenitor cells, lung samples were labeled with VEGFR2 (CD309), CD133 [23]. The control group exhibited a marked presence of double-positive VEGFR2+/CD133+ cells. The ALI group had more pronounced co-expression of both markers compared to control. The CIH group exhibited low expression of VEGFR2+ and CD133+ cells, with no co-expression. The CIH+ALI group showed similar VEGFR2 expression as CIH, but more prominent CD133 expression, with no co-expression (Figure 6).

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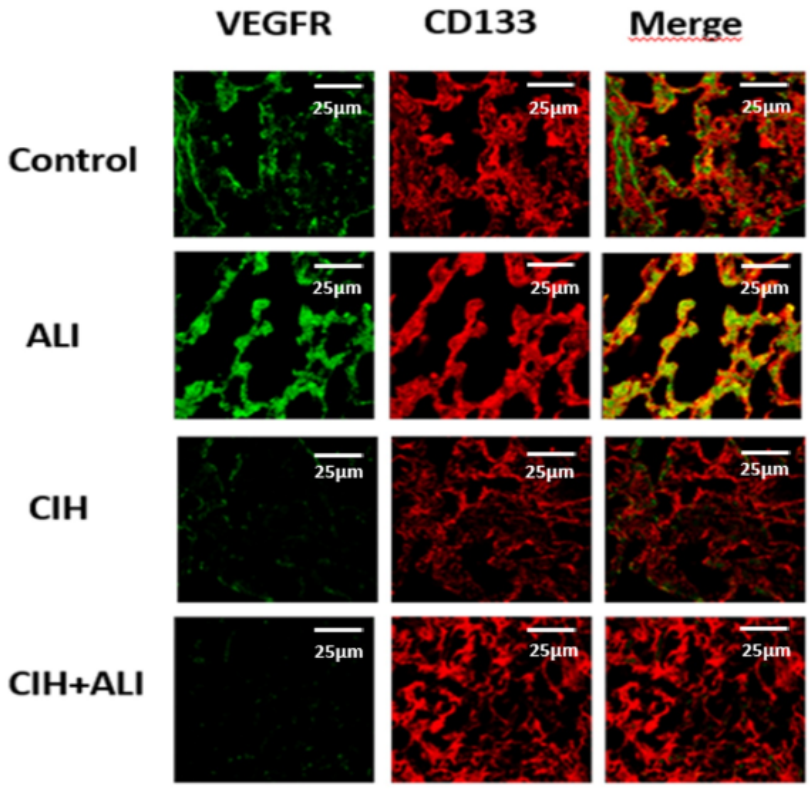


Figure 6. *Immunofluorescently labelled micrographs of the lung tissue from control, ALI, CIH and ALI+CIH groups.*

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DISCUSSION

Our study examined the changes in platelet-derived pro-angiogenic and anti-angiogenic molecules in response to CIH and their effects on EPC homing. The findings underscore the complex role of platelets in modulating the angiogenic milieu, particularly in the context of CIH combined with ALI.

A key observation was the significantly higher platelet count in the CIH+ALI group. Elevated platelet counts in severe inflammatory conditions like pneumonia have been linked to increased mortality and morbidity [24]. However, in our study, this quantitative increase did not correspond to a proportional rise in the concentration of the measured angiogenesis-related factors *per platelet*. In fact, the concentrations of SDF-1, VEGF, PDGF, PAI-1, endostatin, and thrombospondin-1 per platelet were significantly lower in the CIH+ALI group. This discrepancy suggests that platelet function and content may be altered, a phenomenon observed in other contexts where platelet count does not linearly correlate with functional capacity [25]. It is plausible that under CIH, megakaryocyte maturation is shifted towards producing a higher quantity of platelets, potentially at the expense of granular protein synthesis, leading to a population of platelets with an "immature" or altered

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secretory profile [26, 27]. While our study did not perform direct morphological analysis of platelets, the functional data on granular content indirectly supports the concept of CIH-induced alterations in platelet biology, which has been documented in other studies showing that hypoxia can increase platelet size and alter the phospholipid structure of their membranes [28, 29].

The impact of hypoxia on platelet-derived SDF-1 is a central and distinctive finding of our study. While the existing literature frequently documents an increase in SDF-1 expression under acute hypoxia or ischemia—often from endothelial or stromal sources, for instance, in callus tissue after fracture or in neonatal lung tissue [30, 31]—it is crucial to note that the cellular source in these contexts is often endothelial or stromal cells, not platelets. In contrast, our investigation specifically into *platelet-derived* SDF-1 revealed that its levels were lower in the CIH group compared to controls, and its concentration per platelet was markedly reduced in the CIH+ALI group. This divergent profile underscores the critical importance of considering the cellular origin of angiogenic factors. Given that platelet-derived SDF-1 is a key chemoattractant for the homing of bone marrow-derived progenitor cells to injured tissues [1, 7], its reduction provides a plausible explanation for our immunofluorescence findings. We observed prominent co-expression of VEGFR2 and CD133 (markers of EPCs) in the ALI group, indicating active EPC recruitment, whereas this co-expression was absent in both the CIH and CIH+ALI groups. Therefore, we propose that the specific reduction in platelet-derived SDF-1 may be contributing to the impaired homing of EPCs observed in our chronic hypoxia models, an interpretation strongly supported by the established crucial role of the SDF-1/CXCR4 axis in EPC differentiation and survival [5-8]. The observed reduction in platelet-derived SDF-1, alongside the impaired EPC homing observed in our immunofluorescence data, may suggest a potential mechanism for the compromised vascular repair in CIH. Our data on platelet-derived factors provide a foundation for future studies to directly measure functional angiogenesis.

We found that the total levels of VEGF and PDGF were similar across groups. However, when normalized to platelet count, their concentrations per platelet were significantly lower in the CIH+ALI group. This indicates that the relative contribution of each platelet to the pool of these pro-angiogenic factors is diminished under combined stress, which may not be apparent when measuring total plasma or supernatant levels. This finding offers a novel insight into the specific profile of platelet-derived, as opposed to tissue-derived, growth factors in the context of CIH

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and ALI. While some studies report that CIH does not significantly alter VEGF expression, others demonstrate that hypoxic conditions can upregulate VEGF levels. CIH-exposed rats' lung tissue gene microarrays showed increased VEGF-A and PDGF-B gene expression [32]. HIF-1 and VEGF near bone fractures increased with CIH administration but reverted to control by week 8 [30]. CIH also increased VEGF levels in testicular microcirculation and enhanced testicular vascularization [33]. Chronic hypoxia increased VEGF mRNA in rat hippocampus, whereas intermittent hypoxia did not [34]. Results may have varied due to hypoxia severity, duration, and recovery. Similarly, hypoxia has been shown to affect PDGF in some other studies. Normobaric hypoxia doubled PDGF-B mRNA after six hours and normalized after three days [35]. PDGF was highest in 3% hypoxia in rabbit stem cell cultures [36]. Hypoxia raised PDGF-B transcripts within one hour and remained elevated for up to 32 hours [37]. Some studies, however, found no effect of hypoxia on PDGF levels, such as in renal PDGF gene expression after chronic normobaric hypoxia [38]. While the cellular response to PDGF under hypoxia is documented, our data provide new insight into the specific profile of platelet-derived, rather than tissue-derived, PDGF in the context of CIH and ALI.

Conversely, our data on anti-angiogenic factors revealed that CIH alone led to a significant increase in the total levels of PAI-1 and thrombospondin-1, consistent with studies showing hypoxia-induced upregulation of these inhibitors via HIF-dependent pathways [39-41]. The CIH+ALI group also exhibited high absolute platelet counts, but the concentration of these anti-angiogenic factors per platelet was lower than in other groups. Despite this per-platelet decrease, the overall burden of these inhibitors in the circulation might still be significant due to the high platelet count, potentially contributing to an anti-angiogenic environment. Previous research has shown that human platelets increased PAI-1 and HIF-2α expression in low oxygen applications [42]. Plasma and platelet PAI-1 concentrations were elevated on the first day of acute myocardial infarction, declined by the fifth day [43]. Obstructive sleep apnea (associated with intermittent hypoxia) patients had elevated platelet-derived microparticles and plasma PAI-1 than controls [39]. Hypoxia upregulated HIF-1, activating PAI-1 expression in chondrocytes and fibroblasts [44, 45]. HIF-1 suppression in pulmonary fibrosis decreased PAI-1 [40]. Our findings support the literature showing PAI-1's role in inhibiting angiogenesis. The inconsistencies seen in some studies may be explained by the complexity of the system, since these proteases act on multiple substrates and exert their effects on many molecules [46]. Further research is needed to explore the role of platelet-

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derived PAI-1 in regulating angiogenesis under CIH. Similarly, studies on thrombospondin-1 regulation indicated that hypoxia-induced increases control angiogenesis potentially via a feedback mechanism in response to pro-angiogenic factors. High expression of of thrombospondin-1 and VEGF suppresses endothelial cell proliferation [41]. Under various hypoxic conditions thrombospondin-1 expression increases and peaks between 24-48 hours and decreases as endothelial cell cultures increase HIF-1, VEGF, and miR-21 after 24 hours of hypoxia [47]. Endostatin, a powerful angiogenesis inhibitor, is crucial for lung growth. Although no studies have examined platelet-derived endostatin, lung damage has been linked to higher tissue endostatin levels and platelet activity. Acute respiratory distress syndrome patients with high endostatin levels had disrupted endothelial barriers, neutrophil migration, and platelet aggregation [48]. Early in ALI, plasma and bronchoalveolar lavage fluid endostatin levels rise [49]. Endostatin levels peak on the first postnatal day in neonates, indicating lower lung maturity and prenatal lung development [50]. Elevated endostatin in cord plasma of preterm infants with bronchopulmonary dysplasia supports this finding [51]. Lung ischemia rats had higher MMP-9 and endostatin levels during reperfusion [52]. Elevated platelet-derived endostatin during lung injury may positively affect pathological processes. Consistently, endostatin levels were elevated in the ALI group compared to both the control and CIH+ALI groups, while endostatin-to-platelet ratios were significantly reduced in the CIH+ALI group, potentially reflecting the deleterious effects of tissue hypoxia.

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Histological examination confirmed tissue injury and inflammation in the ALI, CIH, and CIH+ALI groups, which may indicate the exacerbation of an inflammatory response and tissue damage in the lung parenchyma via ALI and CIH. In addition, immunofluorescence staining for VEGFR2 and CD133 revealed differential expression patterns among the groups. The observed qualitative patterns in VEGFR2 and CD133 expression suggest a potential dysregulation of EPCs in our disease models. The prominent co-expression in the ALI group and its absence in the CIH-exposed groups point to a divergent endothelial response to acute versus chronic injury. This qualitative pattern aligns with the measured reduction in the key homing factor, platelet-derived SDF-1. We acknowledge that the statement on a "compromised angiogenic response" is inferred from the combination of 1) reduced pro-angiogenic signals (SDF-1/platelet), 2) elevated anti-angiogenic signals (PAI-1, thrombospondin) under CIH, and 3) the absence of EPC homing. While direct measures of angiogenesis (e.g., microvessel density) would strengthen this claim,

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the collective data on the mediators and the cellular (EPC) response strongly suggest an impaired reparative angiogenic process. However, it is a key limitation of this study that due to technical constraints, these findings are descriptive and lack the quantitative, statistical analyses required for definitive conclusions. The trends reported here should therefore be considered preliminary. Future work employing rigorous cell-counting or pixel-based co-localization analysis is essential to objectively quantify these differences and validate the potential role of EPC impairment in the pathophysiology of CIH. Furthermore, direct functional assays of angiogenesis would be a valuable addition to corroborate the inferred angiogenic capacity.

In summary, our results demonstrate that CIH alters the profile of key platelet-derived angiogenic factors, notably reducing SDF-1 and elevating PAI-1 and thrombospondin. This, coupled with the observed impairment in EPC homing, suggests a platelet-mediated mechanism that may contribute to inadequate vascular repair in the context of chronic intermittent hypoxia.

CONCLUSION

In conclusion, our study demonstrates that CIH fundamentally alters the angiogenic cargo of platelets, creating a profile that is detrimental to vascular repair. We propose that CIH induces a dysfunction in platelet biology characterized by an "immature" or altered secretory profile. The key findings—a specific reduction in platelet-derived SDF-1 coupled with elevated levels of anti-angiogenic factors like PAI-1 and thrombospondin—provide a plausible platelet-mediated mechanism for the impaired EPC homing and compromised angiogenic response observed in our models. This platelet-mediated mechanism offers a possible explanation for the inadequate angiogenic response in conditions marked by CIH. While the lack of peripheral blood measurements and variations in hypoxia protocols present challenges for direct comparison with other studies, our internally consistent results highlight the critical role of platelet-specific contributions to the angiogenic milieu in lung injury. Future research should focus on elucidating the underlying mechanisms and exploring therapeutic strategies that target these specific platelet functions to restore vascular repair.

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STATEMENTS AND DECLARATIONS:

Ethics Approval and Consent to Participate

This study was approved by the Institutional Animal Experiments Ethics Committee of Ankara University Medical Faculty (reference number 2021-11-91). All animal handling adhered to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals, as published by the United States National Institutes of Health.

Availability of Data and Material

The datasets generated during and/or analysed during the current study are not publicly available due to institutional policies, but are available from the corresponding author on reasonable request.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

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Authors’ Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ayşe Demirci Şahin and Ayşe Hande Yozgat. The first draft of the manuscript was written by Ayşe Demirci Şahin and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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