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Basic Study

Human umbilical cord mesenchymal stem cells reduce platelet α -granule release in rats *via* the AKT/MEK/ERK pathway during acute exposure to high-altitude hypoxia

Zhang B *et al.* Basic study of hUC-MSCs

Abstract

BACKGROUND

While acute exposure to high-altitude hypoxic environments can lead to increased thrombosis risk, preventive measures are currently limited. Recently, human umbilical cord mesenchymal stem cell (hUC-MSC) transplantation has been found effective in preventing and treating various clinical conditions, including thrombotic diseases. Platelets are crucial for thrombus formation, and their α -granules are key determinants of platelet function. However, little is known about the influence of hUC-MSCs on platelet α -granules.

AIM

To investigate the influence of hUC-MSCs on platelet α -granules in rats during acute exposure to high-altitude hypoxia.

METHODS

Rats were assigned to three groups, namely, low-altitude, high-altitude, and hUC-MSC-treated groups. The low-altitude group was pretreated with normal saline and housed at an altitude of 1500 m. Rats in the high-altitude group received similar pretreatment and were housed in a simulated hypobaric hypoxia chamber with an altitude of 6500 m and oxygen partial pressure of 7.7 kPa. hUC-MSC-treated rats were pretreated with hUC-MSCs and exposed to hypoxic conditions. Aortic blood was collected after three days to assess platelet counts and morphology and α -granule release.

RESULTS

Compared to the low-altitude group, the high-altitude group exhibited significantly higher platelet counts, plasma levels of von Willebrand factor, platelet factor 4, beta-thromboglobulin, as well as surface P-selectin (CD62p) and p-protein kinase B, p-mitogen-activated protein kinase, and p-extracellular-signal regulated kinase expression in platelets. Platelet morphology in the high-altitude group was irregular,

with extended pseudopodia and increased α -granule densities. However, these changes were not apparent in the hUC-MSC-treated group.

CONCLUSION

Acute exposure to high-altitude hypoxia increased platelet counts, altered platelet morphology, and increased α -granule density and release. These effects were mitigated by hUC-MSC treatment, mediated by the protein kinase B/mitogen-activated protein kinase/extracellular-signal regulated kinase pathway. The results indicate that hUC-MSCs may represent a promising and effective approach for the prevention and treatment of acute high-altitude-associated thrombosis, providing an experimental foundation for the development of clinical applications.

Key Words: Human umbilical cord mesenchymal stem cells; High-altitude; Hypoxia; Platelets; Platelet α -granules

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Core Tip: The effects of human umbilical cord mesenchymal stem cell (hUC-MSC) on platelet α -granules in rats during acute exposure to high-altitude hypoxia were assessed. hUC-MSCs significantly reduced platelet counts and plasma levels of von Willebrand factor, platelet factor 4, and beta-thromboglobulin, as well as the expression of CD62p on platelet surfaces. Additionally, hUC-MSCs improved platelet morphology and reduced pseudopodia formation and α -granule contents. These effects were mediated by activation of the protein kinase B/mitogen-activated protein kinase/extracellular-signal regulated kinase signaling pathway. The findings suggest that hUC-MSCs may represent a promising preventive strategy against acute high-

altitude-associated thrombosis, and provide empirical support for their expanded clinical application.

INTRODUCTION

Recently, human umbilical cord mesenchymal stem cell (hUC-MSC) transplantation has been found effective in preventing and treating various clinical conditions, including thrombotic diseases[1-3]. Over 81.6 million individuals live permanently at altitudes exceeding 2500 m (8200 feet)[4]. In addition to these long-term residents, recent economic growth and the expansion of tourism have resulted in approximately 40 million people visiting high-altitude regions each year[5]. The incidence of thrombosis at high altitudes is reported to be 30 times higher than that observed at sea level[6]. Acute exposure to the hypoxic conditions typical of these elevations further exacerbates this risk[7], with acute thrombosis characterized by rapid onset and a high risk of mortality. There are currently limited preventive measures available for these thrombotic events.

Advances in stem cell transplantation have led to the widespread application of mesenchymal stem cells (MSCs) in treating various clinical conditions [8-11]. MSCs are multipotent stem cells of mesodermal origin. They were first identified in bone marrow by the pathologist Cohnheim in 1867[12] and were subsequently isolated and cultured *in vitro* by Friedenstein in 1970[13]. MSCs can be sourced from multiple tissues, including bone marrow, adipose tissue, umbilical cord tissue, and cord blood[14]. The cells exhibit high genetic stability and low immunogenicity and show good safety profiles and efficacy[15]. Moreover, MSCs possess unique properties, such as the ability to home to damaged tissues, promote angiogenesis, modulate the local microenvironment, and regulate immune activity[16]. Numerous studies have demonstrated that MSC transplantation is effective in thrombotic disease[17-19]. MSCs can prevent and alleviate thrombosis through several mechanisms, including stimulating the proliferation and functions of endothelial cells[20], promoting

neovascularization[21,22], inhibiting inflammatory responses[23], and suppressing platelet activation and aggregation[24].

Platelets play a crucial role in thrombosis[25], and the release of α -granules is key to this process[26]. Platelets contain various particles, of which α -granules are the most abundant. Upon activation, α -granules release a variety of bioactive molecules that promote hemostasis, thrombosis, inflammation, wound healing, and tumor metastasis[27]. The present study aimed to investigate the effects of hUC-MSCs on platelet α -granules in rats exposed to acute high-altitude hypoxia. The results indicated increased platelet counts and α -granule release in the rats after short-term exposure to these conditions. However, these changes were mitigated after transplantation of hUC-MSCs. This study presents the first systematic elucidation of the regulatory mechanisms by which hUC-MSCs modulate α -granule release from platelets, using a rat model of acute high-altitude hypoxia. These findings not only provide a novel theoretical framework for understanding the pathogenesis of thrombosis under hypobaric hypoxia conditions but also provide compelling preclinical evidence supporting the translational potential of hUC-MSC-based therapies in altitude-related coagulopathies.

MATERIALS AND METHODS

Animals and groups

Six-week-old male specific pathogen-free Sprague-Dawley rats (180-200 g) were obtained from Beijing Huafukang Biotechnology Co., Ltd. (experimental animal license No. SCXK[Beijing]2019-0008). Rats were chosen as experimental animal models in this study as rats are more suitable for pathophysiological studies than mice, and platelet gene and protein expression in rats closely resembles that of humans[28]. This study protocol was approved by the Research Management Ethics Committee of the 940th Hospital of the PLA Joint Logistics Support Force (approval No. 2024KYLL312D). The animals were housed for two weeks at the Animal Laboratory Department of the 940th Hospital of the PLA Joint Logistics Support Force, located in Lanzhou at an altitude of 1500 m above sea level. During this period, they were maintained under controlled

conditions with a temperature of 22-24 °C and a 12-hour light cycle, with unrestricted access to food and water.

Following this period of acclimatization, 24 eight-week-old rats were randomly allocated to three groups, namely, a low-altitude group (1500 m above sea level), a high-altitude group (simulated altitude of 6500 m), and an hUC-MSC group, with each group containing eight rats. The rats in the low-altitude group were injected with 0.4 mL of normal saline *via* the tail vein, and were kept in the Animal Laboratory Department for three days. Those in the high-altitude group received similar injections but were placed in a low-pressure hypoxic chamber simulating an altitude of 6500 m (Yuyan Instrument, Shanghai, China) for three days. Meanwhile, rats in the hUC-MSC group were injected with 2×10^6 hUC-MSCs in a volume of 0.4 mL *via* the tail vein and were subsequently placed in the hypoxic chamber for three days. Throughout the experiment, environmental conditions such as temperature (22-24 °C), light duration (12 hours/day), and access to food and water were consistent across all groups.

Preparation, culture, and identification of hUC-MSCs

Umbilical cord tissue was collected from a full-term human newborn following the provision of informed consent from the mother and her family and approval from the Scientific Research Management Ethics Committee of the 940th Hospital of the PLA Joint Logistics Support Force (ethics approval number: 2024KYLL314D). The umbilical cord was washed free of blood, and the blood vessels were excised. The cord was then cut into strips and inoculated into a 75 cm² Petri dish containing low-glucose DMEM (Hao Yang, Tianjin, China) and cultured at 37 °C in an atmosphere containing 5% CO₂.

The following day, the culture medium was replaced with fresh medium, with further replacements made every 72 hours thereafter. After approximately 15 days of culture, fibroblast-like cells were observed migrating radially from the tissue blocks. When the cells reached > 90% confluence, the tissue mass was removed and digested with 0.25% Trypsin (containing 0.04% EDTA), and the cells were passaged in a 1:2 ratio.

Cells were passaged when they reached 90% confluence. This was performed aseptically in a biosafety cabinet. The cell monolayer was washed twice with 5 mL of phosphate buffered saline (PBS). After removal of the residual liquid, 3 mL of a 0.25% Trypsin solution was introduced and incubated for 2 minutes. After detachment of 80%-90% of the cell layer from the matrix, 5 mL of serum-containing DMEM medium was added to terminate digestion. The cell suspension was centrifuged in a 15 mL centrifuge tube at 1000 rpm for 5 minutes. The supernatant was discarded and the pelleted cells were resuspended in complete DMEM medium. The cells were distributed to new culture vessels at a 1:3 inoculation ratio and were grown under standard culture conditions.

Third-generation hUC-MSCs were used for observation. Flow cytometry using a BD FACSCanto II instrument (BD Biosciences, Franklin Lakes, NJ, United States) was used to assess the levels of the membrane proteins CD34, CD45, CD73, CD90, and CD105. Osteogenesis, adipogenesis, and chondrogenesis were induced in the cells using kits (TBD, Tianjin, China) according to the provided instructions, followed by staining with Oil Red O, Alizarin Red, or Alcian Blue, as appropriate. The cells were evaluated and imaged using an inverted phase contrast microscope (Leica, Germany).

Antibodies and reagents

The reagents and kits utilized in this study were as follows: 0.25% pancreatic enzyme (TBD, Tianjin, China); PBS (Solarbio, Beijing, China); fetal bovine serum (TBD, Tianjin, China); penicillin/streptomycin (TBD, Tianjin, China); serum-free cell preservation solution (TBD, Tianjin, China); lipogenic differentiation kit for hUC-MSCs (Fuyuanbio, Shanghai, China); osteogenic differentiation kit for hUC-MSCs (Fuyuanbio, Shanghai, China); chondrogenic differentiation kit for hUC-MSCs (Fuyuanbio, Shanghai, China); allophycocyanin (APC)-cyanine 7-conjugated anti-human CD34 antibody (NuoHe Bio, Chengdu, China); PerCP-Cyanine 5.5-conjugated anti-human CD45 antibody (NuoHe Bio, Chengdu, China); phycoerythrin (PE)-conjugated anti-human CD73 antibody (NuoHe Bio, Chengdu, China); APC-conjugated anti-human CD105 antibody (NuoHe

Bio, Chengdu, China); fluorescein isothiocyanate-conjugated anti-human CD90 antibody (NuoHe Bio, Chengdu, China); APC-conjugated anti-mouse/rat CD61 antibody (BioLegend, San Diego, CA, United States); PE-conjugated anti-mouse/rat CD62 antibody (BioLegend, San Diego, CA, United States); rat platelet factor 4 enzyme-linked immunosorbent assay (ELISA) kit (JingMei Technology, Jiangsu, China); rat β -thromboglobulin ELISA kit (JingMei Technology, Jiangsu, China); rat von Willebrand factor ELISA kit (JingMei Technology, Jiangsu, China); PageRuler Prestained Protein Ladder (Thermo Fisher, Waltham, MA, United States); platelet protein extraction kit (Beibo, Shanghai, China); BCA protein quantitation kit (Solarbio, Beijing, China); protease inhibitor (CW BIO, Jiangsu, China), phosphatase inhibitors (Solarbio, Beijing, China); sodium-dodecyl sulfate gel electrophoresis gel preparation kit (Solarbio, Beijing, China); 10 × TBST buffer (Solarbio, Beijing, China); 10 × electrophoretic transfer buffer (Solarbio, Beijing, China); anti-protein kinase B (Akt) antibody (CST, Danvers, MA, United States); recombinant anti-mitogen-activated protein kinase 1 (MEK1) + MEK2 antibody (Abcam, Cambridge, United Kingdom); recombinant anti-extracellular-signal regulated kinase 1 (ERK1) + ERK2 antibody (Abcam, Cambridge, United Kingdom); phospho-Akt (Ser473) rabbit monoclonal antibody (CST); phospho-MEK1/2 rabbit monoclonal antibody (CST); phospho-p44/42MAPK (ERK1/2) rabbit monoclonal antibody (CST); anti-beta tubulin antibody (Abcam, Cambridge, United Kingdom); horseradish peroxidase-conjugated goat anti-rabbit IgG (Servicebio, Wuhan, China); bovine serum albumin (LABGIC, Beijing, China); ECL luminescent liquid (Bio-Rad, Hercules, CA, United States); polyvinylidene fluoride membrane (Merck, Germany); prostaglandin E1 (PGE1) (MedChemExpress, Monmouth Junction, NJ, United States); PBS with glucose buffer (CellWorld, Beijing, China); M199 culture medium (CellWorld, Beijing, China); 2.5% glutaraldehyde electron microscope fixative (Servicebio, Wuhan, China).

Whole blood cell analysis

Rats were weighed and anesthetized with 1% pentobarbital sodium at a dose of 35 mg/kg, after which 2 mL of abdominal aortic blood was collected into an EDTA anticoagulation tube (Kangyijie Medical Technology Co., Ltd., Lanzhou, China). A complete blood cell detector (Mandary BC-6800, Shenzhen, China) was used to measure platelet counts, and the results were recorded and analyzed statistically.

Flow cytometry

The levels of CD62p, a key membrane glycoprotein stored in platelet α -granules and considered a classic marker of platelet activation[29], were determined using flow cytometry. Three milliliters of abdominal aortic blood were collected into sodium citrate anticoagulant tubes (Lingyan Medical Technology Co., Ltd., Suzhou, China) after which 5 μ L of the anticoagulated blood was added to the flow cytometry tube, followed by 1.5 μ L of CD61-APC and 1.5 μ L of CD62p-PE. All platelets were positive for CD61, while only activated platelets were positive for CD62p. The tubes were incubated at 4 °C for 20 minutes in the dark, after which the cells were diluted with 0.5 mL PBS and analyzed by flow cytometry as above. The platelet cell population was defined in the CD61 side scatter two-parameter scatter plot, and 10000 platelets were counted. The percentage of CD62p-positive cells among the overall CD61-positive cells represented the platelet activation rate (%). The experiment was repeated independently eight times in each group.

Platelet isolation

Three milliliters of abdominal aortic blood were collected from the rats in each group. The blood was placed in sodium citrate anticoagulant tubes and centrifuged at $200 \times g$ for 10 minutes at room temperature, after which the upper plasma layer was carefully aspirated to obtain the platelet-rich plasma (PRP). The anticoagulated blood was then re-centrifuged at $1500 \times g$ for 15 minutes at room temperature, and the upper transparent plasma layer was carefully aspirated to yield platelet-poor plasma, which was subsequently stored at -80 °C for further experiments.

Analysis of platelets by transmission electron microscopy

PGE1 was added to the PRP at 2 μ L PGE1 per 1 mL of PRP to prevent exogenous platelet activation, and was mixed gently by pipetting[30]. The mixture was centrifuged at $400 \times g$ for 20 minutes at room temperature, after which the supernatant was carefully aspirated and the platelets were resuspended in an equal volume of pre-warmed (37 °C) PBS with glucose. Six microliters of PGE1 were added with gentle mixing, and the washing step was repeated once. The platelets were then resuspended in pre-warmed (37 °C) M199 medium, adjusting the concentration to 1×10^8 /mL. The platelets were then fixed with 2.5% glutaraldehyde and stored at 4 °C until further use. For electron microscopy, the samples were centrifuged at $400 \times g$ for 3 minutes at room temperature to pellet the platelets. The platelets were washed twice with ice-cold PBS for 15 minutes each wash. The platelets were then fixed with 1% osmium tetroxide at 4 °C for 1 hour, followed by two additional washes with ice-cold PBS for 15 minutes each. The samples were dehydrated in a 30%, 70%, 90%, and 100% ethanol gradient for 15 minutes each step, followed by two 20-minute dehydration steps in 100% acetone. Infiltration was performed with a 1:1 mixture of acetone and embedding resin for 1.5-2 hours, followed by embedding in pure resin and polymerization in an oven. The blocks were trimmed and ultrathin sections (70 nm thick) were prepared. The sections were stained with 4% uranyl acetate for 20 minutes and lead citrate for 5 minutes before evaluation under a transmission electron microscope (TEM) (FEI, Talos F200c, 200 kV).

Western blotting

Proteins were extracted from platelets using a platelet protein extraction kit according to the manufacturer's instructions. Protein concentrations were determined by BCA assays, and an appropriate volume of loading buffer was added. The samples were boiled at 100 °C for 15 minutes and stored at -80 °C. After electrophoresis and transfer to polyvinylidene fluoride membranes, primary antibodies (AKT: 1:1000, p-AKT: 1:2000, MEK: 1:20000, p-MEK: 1:1000, ERK: 1:10000, p-ERK: 1:1000) were incubated

overnight at 4 °C. Secondary antibodies (HRP-conjugated goat anti-rabbit, 1:6000) were incubated for 1.5 hours at 22 °C. Bands were analyzed using ImageJ software and quantified relative to β -tubulin. The experiment was repeated three times independently for each protein.

ELISA

Platelet-poor plasma was thawed at room temperature, and platelet factor 4 (PF4), von Willebrand factor (vWF), and beta-thromboglobulin (β -TG) levels were measured according to the instructions of the respective kits. Eight independent replicates were used for all measurements.

Statistical analysis

Measurement data were assessed for normality using the Shapiro-Wilk test. The data are presented as mean \pm SD, with three or more independent assays performed. One-way analysis of variance (ANOVA) was employed for comparisons among multiple groups. Statistical analysis was conducted using GraphPad Prism software (version 9.5.1, United States).

RESULTS

Isolation, culture, identification, and induced differentiation of hUC-MSCs

hUC-MSCs were isolated and cultured using a tissue block adhesion method. By day 7, adherent spindle-shaped cells were observed migrating from the tissue margins. The cells reached over 90% confluence by day 15, when they showed characteristic whirlpool and spindle shapes under inverted microscopy (Figure 1A). Based on the identification criteria of the International Society for Cell Therapy, the third-generation hUC-MSCs were positive for the surface antigens CD73, CD90, and CD105 and negative for CD34 and CD45[31] (Figure 1B). After chondrogenic differentiation for three weeks, chondrospheres approximately 1 mm in diameter were observed, which stained blue with Alcian Blue (Figure 1C). After two weeks of adipogenic differentiation, the cells

showed visible lipid droplets that stained red with Oil Red O (Figure 1D), while osteogenic differentiation for three weeks resulted in calcium nodules that stained red with Alizarin Red (Figure 1E).

hUC-MSc treatment reduced platelet counts in rats exposed to acute high-altitude hypoxia

Blood samples were collected from the aortae and were analyzed immediately using a whole blood cell counter to ensure the accuracy of the data. Compared to the low-altitude group, the high-altitude group exhibited significantly higher platelet counts ($P < 0.0001$), indicating that acute exposure to high altitude and hypoxia increases platelet counts in rats. This finding is consistent with the results of previous studies[32]. However, the hUC-MSc group showed significant reductions in platelet counts compared to the high-altitude group ($P < 0.0001$), suggesting that hUC-MSc treatment mitigated the increases in platelet counts (Table 1).

hUC-MSCs reduced platelet pseudopodia and α -granule densities in rats exposed to acute high-altitude hypoxia

The TEM results showed that platelets in the rats from the low-altitude group showed discoid shapes with uniformly distributed α -granules (Figure 2A). In contrast, the morphology of platelets from the high-altitude group was irregular, with extended pseudopodia and increased α -granule densities (Figure 2B). Notably, platelets from the hUC-MSc group showed fewer pseudopodia and α -granules compared to the high-altitude group (Figure 2C). These findings suggest that hUC-MSCs exert protective effects against stress-induced injury in rat platelets under acute high-altitude hypoxic conditions.

hUC-MSCs reduced α -granule release from platelets in rats exposed to acute high-altitude hypoxia

The effects of vWF binding to platelet membrane proteins on α -granule release were examined. The expression levels of vWF, PF4, and β -TG in rat plasma were measured. The results demonstrated that, compared to the low-altitude group, the high-altitude group had significantly higher levels of vWF (0.744 ± 0.0003 vs 0.747 ± 0.0004), PF4 (0.926 ± 0.001 vs 0.931 ± 0.003), and β -TG (0.74 ± 0.003 vs 0.76 ± 0.01). Additionally, compared to the high-altitude group, the hUC-MSC group showed reduced plasma levels of vWF (0.747 ± 0.0004 vs 0.745 ± 0.0003), PF4 (0.931 ± 0.003 vs 0.925 ± 0.006), and β -TG (0.76 ± 0.01 vs 0.75 ± 0.003) (Figure 3). The levels of the platelet surface protein CD62p were measured by flow cytometry. The results indicated that, compared to the low-altitude group, platelets from rats in the high-altitude group had significantly higher surface expression of CD62p ($P < 0.0001$). However, compared to the high-altitude group, CD62p levels were markedly lower in the hUC-MSC group ($P < 0.0001$), suggesting that the increase in CD62p was mitigated by hUC-MSC treatment (Figure 4).

hUC-MSC treatment reduced platelet α -granule release in rats exposed to acute high-altitude hypoxia via the AKT/MEK/ERK pathway

Western blotting was used to examine the expression levels of proteins associated with the AKT/MEK/ERK signaling pathway. The findings revealed that acute exposure to a high-altitude hypoxic environment markedly upregulated the expression of platelet vWF (Figure 3A). Following its interaction with the GPIb-IX-V receptor complex on the platelet surface, vWF activated the AKT/MEK/ERK pathway, leading to increased phosphorylation of AKT, MEK, and ERK. These differences were statistically significant (Figure 5). The results suggest that hypoxia induces activation of the AKT/MEK/ERK signaling pathway. Based on these findings, we re-examined the expression of p-AKT, p-MEK, and p-ERK in platelets in the hUC-MSC group, finding that these levels were significantly reduced (Figure 5). It is therefore concluded that the AKT/MEK/ERK signaling pathway plays a crucial role in inhibiting the release of platelet α -granules following hUC-MSC treatment of rats under conditions of acute high-altitude hypoxia.

DISCUSSION

High-altitude environments are associated with hypoxia, low temperature and humidity, significant diurnal variations in temperature, and intense ultraviolet radiation. Among these factors, the reduction in oxygen partial pressure with increasing altitude is considered the most critical environmental variable affecting human physiology[7] and hypoxia is documented to be the primary factor contributing to thrombosis at high altitudes[32,33]. It is important to highlight that there are substantial individual variations in physiological responses to hypoxic stress. These differences may stem from the interplay of genetic differences, baseline health conditions, and other pathophysiological regulatory factors, particularly in terms of the extent of platelet activation and the threshold of hematopoietic responses.

An elevated platelet count is recognized as a risk factor for thrombosis[34,35]. In this study, we observed a rapid increase in the platelet counts of rats exposed to a simulated high-altitude hypoxic chamber after three days (Table 1), consistent with previous findings[36]. As early as 1977, Jackson and Edwards[37] reported a biphasic effect on platelet production under conditions of low pressure and hypoxia, characterized by an initial increase followed by a subsequent decline. Further studies conducted by Wang *et al*[38] confirmed that chronic environmental hypoxia can lead to reduced platelet counts in susceptible individuals. The mechanism underlying this increase in platelet count induced by acute exposure to hypoxia may involve the promotion of hematopoietic stem cell (HSC) mobilization by oxidative stress, thereby increasing the release of bone marrow HSCs[39] and stimulating platelet production by megakaryocytes (MKs)[40]. HSCs do not require significant levels of oxidative respiration, and can function effectively under hypoxic conditions[41]. Additionally, acute hypoxia accelerates the maturation of MK precursors into MKs, promotes the entry of platelets into the circulation, and upregulates factors, such as thrombopoietin, that stimulate platelet production[42]. This provides a further explanation of the elevations in platelet counts observed on acute exposure to high-altitude hypoxia.

In this study, it was also observed that hUC-MSCs, derived from Wharton's jelly in the umbilical cord, regulate platelet counts under conditions of acute high-altitude hypoxia. hUC-MSCs have various advantages, such as ease of collection and a lack of ethical concerns, making them a promising area of research. After pretreatment with hUC-MSCs, rats exposed to a simulated high-altitude hypoxic environment for three days showed markedly reduced platelet counts (Table 1). These findings suggest the potential of hUC-MSCs as an effective preventive measure against acute high-altitude-associated thrombosis. However, further investigation into the underlying regulatory mechanisms is required.

Excessive platelet activation is closely associated with thrombosis[43]. Under physiological conditions, platelets remain quiescent until activated, at which point they extend pseudopodia, release their granule content, promote blood coagulation, alter the expression of surface molecules, and induce aggregation and adhesion[44], ultimately leading to hemostasis and thrombus formation[45]. In addition, recent studies have demonstrated that activated platelets play a pivotal role in various physiological and pathological processes, including inflammation, innate immunity, growth and development, angiogenesis, wound healing, and cancer metastasis[46,47]. The release of granules from platelets is critical to these processes. Platelets contain three types of granules, namely, α -granules, dense granules, and lysosomes[48], storing over 300 proteins overall[49]. Each platelet contains 60-80 α -granules[50], accounting for approximately 10% of the total platelet mass[51]. The α -granules release a variety of soluble proteins, including vWF, which binds to the GPIb α subunit of the GPIb-IX-V complex on the platelet surface *via* its A1 domain, initiating signaling cascades that lead to further release of granule contents[52]. PF4, a 70 amino-acid peptide, is released from α -granules and forms complexes with chondroitin sulfate proteoglycan carriers, resulting in its rapid disappearance from the plasma due to its greater affinity for heparan sulfate on endothelial cells[53]. CD62p, another important membrane glycoprotein stored in platelet α -granules, is redistributed to the platelet surface upon activation where it serves as a classic marker of platelet activation[29].

TEM evaluation showed increased extension of pseudopodia and α -granule densities in platelets from rats in the high-altitude group (Figure 2B), while ELISA measurements showed elevated plasma levels of vWF, PF4, and β -TG in the high-altitude group (Figure 3) and flow cytometry analysis indicated significantly increased CD62p expression on platelet surfaces (Figure 4B and D), consistent with previous findings[54,55]. Turton *et al*[56] also reported a significant correlation between increased plasma P-selectin and vWF levels in healthy individuals who ascended to high altitudes without physical exertion.

One of the key findings of the study was the observation of significant reductions in pseudopodia formation, α -granule numbers (Figure 2C), and plasma levels of vWF, PF4, and β -TG (Figure 3) following hUC-MSC treatment of rats exposed to acute high-altitude hypoxia, as well as the alterations in CD62p expression on platelet surfaces (Figure 4C and D). These results indicate that hUC-MSCs can prevent the release of α -granules from platelets under conditions of acute hypoxia. To investigate the molecular pathways involved, we examined the protein expression of p-AKT, p-MEK, and p-ERK in platelets using western blotting. The AKT/MEK/ERK pathway plays a crucial role in the regulation of platelet growth and activation[57], as well as in cell differentiation, apoptosis, and disease development[58,59]. Acute hypoxia activates vWF signaling, leading to platelet activation[60]. The present study found that the expression of p-AKT, p-MEK, and p-ERK was increased in platelets in the high-altitude group but was significantly decreased in rats treated with hUC-MSCs (Figure 5). These results further demonstrate that acute exposure to high altitude activates vWF signaling in rats, increasing both platelet activation and α -granule release, while these changes can be mitigated by hUC-MSC treatment, potentially mediated by the AKT/MEK/ERK signaling pathway. However, activation of the AKT and MEK/ERK pathways is complex, and associated with several non-classical pathways involving G-protein-coupled receptors, integrins, oxidative stress, and cytokines. These mechanisms play important roles in tumorigenesis, immune responses, and metabolic diseases, and

targeted cross-regulation of multiple pathways is recognized as a research hotspot in the development of strategies for disease treatment.

This study has several limitations. First, we did not evaluate the expression levels of GPIb-IX-V and thus did not comprehensively characterize the vWF-GPIb-IX-V signaling axis. This limitation is particularly relevant to the understanding of potential compensatory mechanisms or variations in expression levels that may influence platelet activation thresholds. Furthermore, while analysis of the effects on the AKT/MEK/ERK signaling cascade indicated significant activation of the pathway, the underlying mechanisms were not determined using pharmacological inhibition or gene ablation. The lack of data on the effects of specific kinase inhibitors (*e.g.*, MK-2206 for AKT and PD0325901 for MEK) and CRISPR/Cas9-mediated gene knockout models did not allow assessment of causal relationships between pathway activation and the observed functional outcomes[61-63]. Future studies should incorporate both pharmacological and genetic strategies to construct definitive epistasis maps of these pathways. Statistically speaking, although the sample size in the study was relatively small, the effect size observed in the results partially mitigated this limitation. Further studies with larger sample sizes should be undertaken to verify the potential of using hUC-MSCs for treating thrombotic diseases, and their applications in other populations, environments, or conditions.

CONCLUSION

The findings demonstrate that acute exposure to high-altitude hypoxia increases platelet counts, alters platelet morphology, increases α -granule densities, and promotes the release of α -granules in rats. Treatment with hUC-MSCs was found to mitigate these effects, possibly through the AKT/MEK/ERK signaling pathway. These results provide novel insights into the prevention of acute high-altitude-associated thrombosis using hUC-MSCs. Nevertheless, given the complexity of thrombosis formation that involves multiple factors and mechanisms, further investigation is needed to verify the role of hUC-MSCs in preventing acute high-altitude-associated thrombosis.

Based on the unique biological characteristics of hUC-MSCs and their strategic position in regenerative medicine, future studies should analyze heterogeneous subpopulations of hUC-MSCs and their differentiation trajectories using single-cell sequencing and spatial transcriptomics, as well as elucidating the molecular mechanisms underlying the regulation of immunomodulation and tissue repair promoted by hUC-MSCs. In terms of clinical applications, the current findings can be used as a reference for the construction of standardized clinical transformation protocols. The efficacy of these could be evaluated in thrombotic diseases, degenerative osteoarthropathy, *graft-versus-host* disease, and the treatment of diabetic foot ulcers using multi-center randomized controlled trials, and biomarker-based efficacy prediction models could be established at the same time. Finally, we believe that there is an urgent need to establish a long-term follow-up database of the epigenetic stability, tumorigenic risk, and immunogenic evolution of hUC-MSCs to provide evidence-based references for clinical applications.

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