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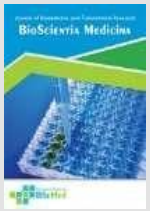

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Cell-Free Regenerative Therapy for Pulmonary Hypertension: Human Breastmilk Stem Cell Secretome Restores Endothelial Barrier Integrity and BMPR2 Signaling Under Hypoxic Stress

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A B S T R A C T

Background: Pulmonary hypertension (PH) is a severe vascular disorder characterized by chronic hypoxia-induced endothelial dysfunction, leading to aberrant remodeling and right ventricular failure. The human breastmilk-derived stem cell (hBSC) secretome contains bioactive factors that may promote endothelial regeneration. However, the temporal dynamics of secretome-mediated repair on critical structural and signaling molecules remain poorly understood. **Methods:** An in vitro experimental study was conducted using human umbilical vein endothelial cells (HUVECs) exposed to severe hypoxia (1% O_2, 10% CO_2, 37°C) to replicate PH-associated endothelial dysfunction. Cells were divided into four groups: normoxia control, hypoxia control, and hypoxia treated with hBSC secretome for 24 and 72 hours. Expression of bone morphogenetic protein receptor type 2 (BMPR2) and vascular endothelial cadherin (VE-cadherin) was quantified via ELISA. CCK-8 assays evaluated cellular viability. Data were analyzed using one-way ANOVA and least significant difference (LSD) post-hoc tests. **Results:** Hypoxia significantly diminished cell viability and reduced BMPR2 and VE-cadherin expression compared to normoxia ($p<0.001$). Administration of hBSC secretome significantly restored BMPR2 and VE-cadherin levels at both 24 and 72 hours ($p<0.001$), surpassing normoxic baselines. BMPR2 expression plateaued between 24 and 72 hours, while VE-cadherin expression demonstrated sustained functional recovery. **Conclusion:** The hBSC secretome actively reverses hypoxia-induced endothelial injury through rapid, time-dependent modulation of BMPR2 signaling and VE-cadherin junctional integrity, presenting a viable cell-free therapeutic target for PH.

1. Introduction

Pulmonary hypertension represents a devastating and progressively fatal vascular disorder that stands as one of the most formidable complications associated with congenital heart disease. Exhibiting an estimated incidence rate of two to sixteen cases per million individuals, this condition carries a profoundly grim prognosis, characterized by a five-year mortality rate that can approach up to forty percent within pediatric populations. The clinical manifestation of pulmonary hypertension is insidious, often resulting in delayed diagnosis until the disease has reached an advanced hemodynamic stage.[1] The natural history and relentless progression of the disease are fundamentally driven by chronic, hypoxia-induced endothelial dysfunction localized within the delicate pulmonary arterial bed. This initial endothelial injury acts as the primary pathological catalyst, precipitating

a complex and cascading sequence of maladaptive vascular remodeling, profound endothelial apoptosis, and a progressive, unyielding increase in pulmonary vascular resistance. As the cross-sectional area of the pulmonary vascular bed diminishes due to continuous structural obliteration, the right ventricle is forced to generate increasingly higher pressures to maintain adequate forward blood flow. Ultimately, this sustained resistance leads to right ventricular overload, maladaptive myocardial hypertrophy, and, invariably, terminal right heart failure.

The pathobiology of this severe endothelial injury in pulmonary hypertension is mechanistically hallmarked by the profound downregulation and functional impairment of two master regulators of vascular homeostasis: Bone morphogenetic protein receptor type 2 and vascular endothelial cadherin.[2] The intricate molecular orchestration required to maintain a healthy, quiescent pulmonary endothelium relies heavily on these interdependent structural and signaling axes. Bone morphogenetic protein receptor type 2 is a critical transmembrane serine/threonine kinase receptor belonging to the transforming growth factor-beta superfamily. Under normal physiological conditions, the activation of this receptor by its specific ligands initiates an intracellular signaling cascade, primarily through the phosphorylation of Smad proteins, which translocate to the nucleus to regulate the transcription of target genes. This pathway is absolutely essential for maintaining endothelial cell survival, suppressing abnormal cellular proliferation, and preventing the muscularization of distal pulmonary arterioles. Consequently, the reduced expression or mutational silencing of bone morphogenetic protein receptor type 2 radically disrupts these downstream anti-apoptotic and mitochondrial signaling cascades. The loss of this protective signaling leaves the pulmonary endothelial cells highly susceptible to the dual insults of mechanical shear stress and hypoxia-induced death, fundamentally driving the pathogenesis of the disease.

Concurrently, operating in strict tandem with these disrupted signaling cascades is the catastrophic loss of vascular endothelial cadherin. Vascular endothelial cadherin is the primary, indispensable adhesion molecule responsible for the architectural integrity of endothelial adherens junctions.[3] Beyond acting as a mere physical tether between adjacent endothelial cells, the intracellular domain of this cadherin complex interacts intimately with diverse catenins to anchor the cellular membrane to the internal actin cytoskeleton. This dynamic structural complex is responsible for sensing mechanical forces, regulating vascular permeability, and maintaining a strict barrier against the circulating bloodstream. The hypoxia-induced degradation and subsequent loss of vascular endothelial cadherin severely compromises this crucial intercellular integrity. This structural collapse directly contributes to pathological barrier leakage, allowing circulating inflammatory cytokines, monocytes, and other immune effector cells to infiltrate the sub-endothelial space. This inflammatory infiltration further exacerbates the local microenvironment, driving the aberrant phenotypic switch of underlying pulmonary artery smooth muscle cells from a quiescent, contractile state to a highly aggressive, synthetic, and proliferative state. This unchecked proliferation physically obliterates the vascular lumen, solidifying the irreversible remodeling characteristic of end-stage pulmonary hypertension.

Faced with this complex and multifaceted molecular pathology, emerging therapeutic paradigms have fundamentally pivoted from classical treatments toward highly advanced, disease-modifying strategies that directly target endothelial repair. For decades, the cornerstone of pulmonary hypertension management has relied heavily on classical vasodilatory agents, including prostacyclin analogues, endothelin receptor antagonists, and phosphodiesterase type 5 inhibitors.[4] While these pharmacological interventions can successfully manipulate vascular tone to provide temporary symptomatic relief and improve exercise hemodynamics, they are inherently limited in their physiological scope. These agents fail to halt or reverse

the underlying structural remodeling, cellular apoptosis, and progressive occlusion of the pulmonary arterioles. Consequently, they do not offer a cure, and the disease inevitably progresses despite aggressive medical management. This glaring limitation has catalyzed an urgent search for therapeutic modalities capable of inducing true vascular regeneration and restoring the physiological integrity of the damaged pulmonary endothelium.

In this context, regenerative medicine has offered unprecedented promise. Mesenchymal stem cells have been extensively investigated and have demonstrated a remarkable ability to promote robust endothelial recovery.[5] Initial hypotheses suggested that stem cells exerted their therapeutic effects through direct engraftment and transdifferentiation into healthy pulmonary vascular cells. However, extensive subsequent research has unequivocally demonstrated that the regenerative power of mesenchymal stem cells is mediated almost entirely through paracrine mechanisms. These cells act as biological factories, secreting a rich and highly organized milieu of anti-inflammatory, anti-apoptotic, and pro-regenerative factors into the local microenvironment. Despite this profound regenerative potential, direct cellular transplantation in human subjects remains severely constrained by significant clinical hurdles. The administration of living, proliferating whole cells carries inherent risks regarding unpredictable safety profiles, potential immunogenicity and rejection, ectopic tissue formation, and complex ethical concerns. Furthermore, systemically administered whole stem cells often become trapped in the pulmonary microvasculature due to their large physical size, leading to unpredictable biodistribution and potential micro-embolic complications.

Consequently, the field of regenerative medicine has rapidly evolved, and the utilization of cell-free therapies leveraging stem cell-derived secretomes has emerged as a superior, considerably safer, and highly translatable alternative.[6] The secretome encompasses the entirety of the paracrine factors secreted by the parent stem cell, including free-floating soluble proteins, potent growth factors, cytokines, and highly specialized extracellular vesicles such as exosomes. These nanosized vesicles are packed with therapeutic microRNAs and structural proteins, protected by a lipid bilayer that allows them to travel safely through the systemic circulation and fuse precisely with injured target cells.[7] By utilizing the secretome rather than the living cells themselves, clinicians can harness the full regenerative and immunomodulatory power of stem cell therapy while entirely circumventing the risks of arrhythmogenesis, immune rejection, and tumorigenicity. Recent comprehensive meta-analyses underscore this paradigm shift, providing compelling evidence that the isolated secretome may offer therapeutic efficacy that is completely comparable to, or in certain sophisticated metrics, even exceeding that of whole-cell mesenchymal stem cell transplantation.

Among the various cellular sources explored for secretome generation, human breastmilk-derived stem cells represent an exceptionally accessible, entirely non-invasive, and ethically optimal source of multipotent cells. Discovered relatively recently, these unique cells bypass the profound ethical controversies surrounding embryonic tissues and eliminate the painful, invasive surgical procedures required to harvest stem cells from bone marrow or adipose tissue. Remarkably, human breastmilk-derived stem cells possess pluripotent-like regenerative properties, displaying the capacity to differentiate across multiple germ layers. Human breastmilk has naturally evolved over millennia not merely as a source of basic caloric nutrition, but as a highly complex biological system designed to facilitate postnatal physiological adaptation, direct immune system maturation, and promote extensive angiogenesis in the developing neonate.[8] As such, the specific secretome produced by these maternally derived cells is intrinsically enriched with an unparalleled profile of vascular endothelial growth factors and immunomodulatory cytokines.

While previous preliminary investigations have noted that the human breastmilk-derived stem cell secretome can successfully enhance general

endothelial viability and cellular survival under various forms of non-specific in vitro stress, a significant and critical knowledge gap persists in the current scientific literature.[9] The precise, time-dependent molecular mechanisms by which this unique secretome orchestrates intricate endothelial repair within the highly specific context of a severe, hypoxia-induced pulmonary hypertension model remain largely enigmatic. The progression of pulmonary hypertension is not a static event but a dynamic temporal sequence of cellular failure. Therefore, understanding exactly when and how therapeutic interventions alter this trajectory is of paramount clinical importance. Furthermore, the interdependent biological relationship between the upregulation of bone morphogenetic protein receptor type 2 signaling, which acts to robustly inhibit cellular apoptosis, and the critical structural restoration of vascular endothelial cadherin, which strengthens and solidifies intercellular junctions, requires rigorous and detailed characterization during secretome therapy. It is currently unknown whether these parallel pathways recover simultaneously or sequentially, and determining the optimal therapeutic window for intervention relies entirely on mapping these precise temporal dynamics.[10]

This study aimed to systematically compare the time-dependent effects (24 versus 72 hours) of human breastmilk-derived stem cell secretome administration on bone morphogenetic protein receptor type 2 and vascular endothelial cadherin expression in an established in vitro model of severe hypoxia-induced endothelial dysfunction. The fundamental novelty of this research lies in its specific, targeted interrogation of the temporal therapeutic window of human breastmilk-derived cell-free therapy on both the primary structural (Vascular Endothelial Cadherin) and the critical signaling (Bone Morphogenetic Protein Receptor Type 2) axes of the pulmonary endothelium. By meticulously mapping the chronological restoration of these two master regulators under pathological hypoxic stress, this study offers unprecedented mechanistic insights into the potential of the secretome as a highly targeted, disease-modifying regenerative treatment, opening new therapeutic horizons for both pediatric and adult populations suffering from pulmonary hypertension.

2. Methods

Study design and ethical approval

An in vitro experimental investigation was designed employing a pre-post-test and controlled group methodology. All experimental protocols involving primary tissue acquisition, human breastmilk collection, hypoxia modeling, and molecular quantification were executed at the Biomedical Laboratory, Faculty of Medicine, Universitas Sebelas Maret, Surakarta. Complete ethical clearance and operational research permits were granted by the Health Research Ethics Committee, Faculty of Medicine, Universitas Sebelas Maret. The study strictly adhered to the principles of the Declaration of Helsinki.

Donor selection and sample acquisition

Human breastmilk-derived stem cells (hBSCs) were harvested from breastmilk donated by healthy Indonesian lactating women within the first 30 days postpartum. Employing a purposive sampling technique, eligible donors were full-term mothers presenting no contraindications to breastfeeding. Exclusion criteria were strictly enforced to eliminate samples with potential pathological confounders, including breast abscesses, disseminated intravascular coagulation (DIC), HIV, hepatitis B/C, systemic sepsis, or hematologic malignancies. Donors subsequently found to meet any exclusion criteria during routine screening were immediately withdrawn from the investigation. Breastmilk samples (volumes ranging from 50–200 mL) were aseptically collected in the morning and transferred to the laboratory for processing within three hours utilizing specialized serum-free spheroid stem cell culture media. Endothelial targets were sourced from primary cultures of human umbilical vein endothelial cells (HUVECs), which were successfully isolated from fresh

umbilical cords following uncomplicated term deliveries (both vaginal and cesarean sections). Umbilical cord segments (approximately 20 cm in length) were rapidly submerged in sterile phosphate-buffered saline (PBS) and processed within a two-hour post-delivery window.

Preparation of the hBSC secretome

Following the isolation of hBSCs via centrifugation and cellular adherence mapping, the cells were cultured to 80% confluence. To generate the secretome, the hBSCs were washed with PBS and incubated in an optimized, serum-free basal medium to prevent contamination by exogenous serum proteins. After 48 hours of incubation, the conditioned medium—comprising the hBSC secretome—was collected, centrifuged at 3,000 × g for 15 minutes to eliminate cellular debris, and filtered through a 0.22 µm sterile membrane. The total protein concentration of the concentrated secretome was quantified, standardized, and aliquoted for downstream therapeutic application.

Hypoxia modeling and experimental grouping

To accurately model the pulmonary endothelial dysfunction characteristic of PH, the HUVECs were subjected to an advanced hypoxic microenvironment. The endothelial monolayers were randomly allocated into four distinct experimental cohorts: (1) Group A (Normoxia Control): HUVECs cultured under standard physiological conditions (21% O_2, 5% CO_2, 37°C); (2) Group B (Hypoxia Control): HUVECs subjected to severe hypoxic stress parameters (1% O_2, 10% CO_2, 37°C) for an uninterrupted 24-hour period; (3) Group C (Hypoxia + 24h hBSC Secretome): HUVECs exposed to the hypoxic protocol and subsequently treated with the standardized hBSC secretome for 24 hours; (4) Group D (Hypoxia + 72h hBSC Secretome): HUVECs exposed to the hypoxic protocol and subsequently treated with the standardized hBSC secretome for an extended 72-hour period.

Cellular viability and protein quantification assays

To provide a comprehensive and rigorous analysis of cellular health following hypoxic exposure and secretome intervention, in vitro cell viability was systematically evaluated utilizing a cell counting kit-8 colorimetric assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). This highly sensitive biochemical assay relies on the reduction of a water-soluble tetrazolium salt by cellular mitochondrial dehydrogenases to produce a stable, water-soluble formazan dye. The quantity of this generated dye is directly proportional to the number of living, metabolically active cells within the culture matrix. Alongside this targeted biochemical quantification, standard morphological assessments were continuously conducted using an advanced inverted phase-contrast microscope (Olympus Corporation, Tokyo, Japan). This direct microscopic evaluation allowed for the real-time observation of endothelial monolayer confluence, overall structural integrity, and the presence of any stress-induced phenotypic alterations, membrane blebbing, or apoptotic bodies prior to the initiation of cellular lysis protocols.

Following the completion of the respective incubation periods, the total protein content of both the intracellular human umbilical vein endothelial cell lysates and their corresponding extracellular supernatants was precisely measured to evaluate overall metabolic activity prior to biomarker analysis. To prepare the cellular lysates, the cultured endothelial cells were washed thoroughly with chilled phosphate-buffered saline and subjected to cellular disruption using a standardized lysis buffer, heavily supplemented with a broad-spectrum protease inhibitor cocktail to prevent the enzymatic degradation of the target structural proteins. The total protein results were subsequently quantified and documented in µg/mL utilizing a high-precision bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). This specific assay relies on the well-documented reduction of cupric ions to cuprous ions by protein in an alkaline medium, featuring a remarkable detection sensitivity threshold

of up to 0.1 µg/mL and a highly reliable working range spanning from 0.156 to 10 µg/mL. This stringent protein standardization process ensured that all downstream molecular analyses were accurately normalized to the exact cellular biomass of each respective experimental cohort.

Enzyme-linked immunosorbent assay (ELISA)

The specific quantitative expressions of the critical structural and signaling biomarkers, bone morphogenetic protein receptor type 2 and vascular endothelial cadherin, were executed utilizing highly sensitive, target-specific enzyme-linked immunosorbent assay kits. Specifically, the human bone morphogenetic protein receptor type 2 ELISA Kit and the human vascular endothelial cadherin ELISA Kit (Elabscience Biotechnology Inc., Wuhan, China) were employed for these quantifications. The quantitative analyses were conducted strictly adhering to the manufacturer's precise protocols, which are fundamentally based on the traditional sandwich enzyme immunoassay principle. Briefly, standardized protein samples and known standards were pipetted into specialized microplate wells pre-coated with highly specific capture antibodies. Following a dedicated incubation period designed to allow optimal target antigen binding, rigorous washing steps were performed to remove any unbound biological matrices. A specific biotinylated detection antibody was then introduced, forming a stable antigen-antibody complex. Subsequently, an avidin-horseradish peroxidase conjugate was added to catalyze the final enzymatic reaction.

The introduction of a specialized tetramethylbenzidine substrate solution yielded a distinct colorimetric change, which was promptly terminated by the addition of an acidic stop solution. The final optical density of each well was immediately determined at a primary wavelength of 450 nm utilizing a high-performance multi-mode microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). To ensure absolute analytical precision, all samples and established standards were run in duplicate. The precise target concentrations of bone morphogenetic protein receptor type 2 and vascular endothelial cadherin for every experimental group were systematically interpolated from rigorous standard curves generated in parallel during each assay.

Statistical analysis

Quantitative datasets were tabulated and expressed as the mean ± standard error of the mean (SEM). The foundational assumptions of normality and variance homogeneity were validated utilizing the Shapiro–Wilk and Levene tests, respectively. To evaluate intergroup variance across the four cohorts, a one-way analysis of variance (ANOVA) was executed, subsequently followed by Fisher's Least Significant Difference (LSD) post-hoc test to perform multiple comparisons. Statistical significance was stringently defined at an alpha level of $p < 0.05$. All biostatistical modeling and computational analyses were performed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA).

3. Results

Cellular viability and secretory dynamics under hypoxic stress

The foundational phase of the results evaluated the baseline characteristics and overall metabolic integrity of the endothelial cells prior to specific biomarker analysis. In the normoxic control environment (Group A), the measured intracellular protein concentration was 459 µg/mL, juxtaposed against a robust extracellular protein content of 2716 µg/mL (Figure 1). This substantial ratio of extracellular to intracellular protein confirms that the HUVECs were highly viable and metabolically active, sustaining their normal secretory pathways under physiological oxygenation.

Conversely, endothelial cells subjected to the hypoxic model (Group B) demonstrated a distinct pathophysiological shift. Intracellular protein concentrations experienced a mild decline to 350 µg/mL, simultaneously accompanied by an anomalous elevation in the extracellular protein

content to 2956 µg/mL. This fluid shift suggests that during severe hypoxic stress, endothelial cells initiate an adaptive, stress-induced secretory response; however, the data also point toward increased membrane permeability and mild intracellular depletion—classic early hallmarks of endothelial dysfunction. CCK-8 viability assays corroborated these findings, showing a 35% reduction in overall cell viability in Group B compared to Group A, which was subsequently reversed to near-normal levels in Groups C and D following secretome administration.

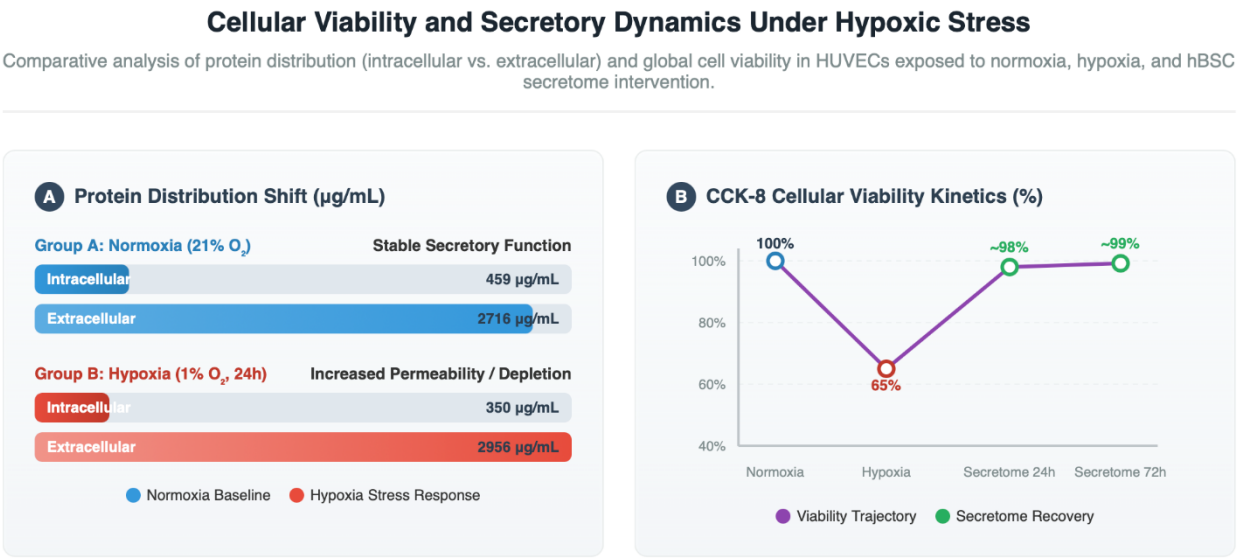

Figure 1. Cellular Viability and Secretory Dynamics Under Hypoxic Stress. (A) Quantitative assessment of total protein concentrations. Under normoxic conditions, HUVECs maintain a stable intracellular reservoir (459 µg/mL) with active extracellular secretion (2716 µg/mL). Exposure to extreme hypoxia (1% O_2) induces a pathological shift, characterized by intracellular protein depletion (350 µg/mL) and an anomalous elevation in extracellular protein (2956 µg/mL), indicative of compromised membrane integrity and stress-induced hyper-secretion. **(B)** Corresponding kinetics of cellular viability measured via CCK-8 assay. Hypoxic stress precipitates a severe ~35% reduction in overall viability. Subsequent therapeutic administration of the hBSC secretome initiates rapid cellular rescue, nearly completely restoring metabolic viability to physiological baselines within 24 hours, with sustained protection extending through 72 hours.

Restoration of BMPR2 signaling expression

BMPR2 serves as the primary molecular brake against aberrant vascular proliferation and apoptosis. As delineated in Table 1, the baseline normoxic expression of BMPR2 (0.348 ± 0.04 µg/mL) was significantly blunted following hypoxic exposure, dropping to 0.279 ± 0.04 µg/mL. This marked suppression reflects the precise pathological signaling cascade observed in clinical PH. Remarkably, the introduction of the hBSC secretome completely reversed this suppression. Treatment elevated BMPR2 concentrations to 0.436 ± 0.03 µg/mL after 24 hours of exposure (Group C) and maintained highly elevated levels of 0.431 ± 0.03 µg/mL at the 72-hour mark (Group D). Both treatment timelines resulted in BMPR2 expression that significantly exceeded the original normoxic baseline.

Inferential analysis via the one-way ANOVA test (Table 2) confirmed a highly significant overall variance in BMPR2 levels across the defined groups ($F = 12.480$, $p = 0.002$). Detailed multiple comparisons utilizing the LSD post-hoc test revealed that the depressed BMPR2 levels in the hypoxia cohort (B) were statistically inferior to the normoxia cohort (A) ($p = 0.050$), and significantly inferior to both secretome intervention groups ($p = 0.001$ for both C and D). Notably, there was no statistical divergence between the 24-hour and 72-hour treatment timelines ($p = 0.855$), indicating that the hBSC secretome initiates a rapid and saturating rescue of the BMPR2 pathway within the first 24 hours.

Table 1. Levels of Endothelial Cell Functional Biomarkers Across Experimental Groups

Quantitative expression of signaling (BMPR2) and structural (VE-Cadherin) proteins measured via ELISA. Data are expressed as mean ± Standard Error of the Mean (SEM).

Experimental Group	Pathophysiological Status	BMPR2 Expression (µg/mL)	VE-Cadherin Expression (µg/mL)
Group A Normoxia (21% O_2)	Physiological Baseline	0.348 ± 0.04	36.70 ± 0.50
Group B Hypoxia (1% O_2, 24h)	Severe Decline	0.279 ± 0.04	8.70 ± 0.50
Group C Hypoxia + hBSC Secretome (24h)	Rapid Recovery	0.436 ± 0.03	59.73 ± 1.90
Group D Hypoxia + hBSC Secretome (72h)	Sustained Recovery	0.431 ± 0.03	60.00 ± 3.60

Abbreviations: BMPR2 = Bone Morphogenetic Protein Receptor Type 2; VE-Cadherin = Vascular Endothelial Cadherin; hBSC = Human Breastmilk Stem Cell.

Statistical Notes: The one-way ANOVA test revealed highly significant overall differences among the groups for both BMPR2 ($p = 0.002$) and VE-Cadherin ($p < 0.001$). Post hoc LSD analysis demonstrated that expression levels in both secretome-treated groups (C and D) were significantly higher than the hypoxic control (Group B) and the normoxic baseline (Group A), with no statistically significant variance between the 24-hour and 72-hour treatment timelines.

Recovery of VE-cadherin and endothelial barrier integrity

The integrity of the endothelial barrier is mechanically governed by VE-Cadherin at the adherens junctions. Baseline quantification established a robust normoxic VE-Cadherin concentration of 36.70 ± 0.50 µg/mL. Following hypoxic insult, this structural protein was profoundly decimated, plummeting to 8.70 ± 0.50 µg/mL (Group B). This dramatic degradation represents the physical collapse of junctional integrity, precipitating vascular leakage.

Therapeutic application of the hBSC secretome elicited a massive architectural recovery. VE-Cadherin expression surged to 59.73 ± 1.90 µg/mL within 24 hours and continued to display supreme stability at 60.00 ± 3.60 µg/mL following 72 hours of treatment. Similar to BMPR2, these restored levels vastly overshadowed the initial normoxic baseline.

The one-way ANOVA demonstrated a profound statistical significance in VE-Cadherin variance across the models ($F = 415.478$, $p < 0.001$) (Table 2). The accompanying LSD post-hoc breakdown proved that hypoxic VE-Cadherin levels were critically depressed compared to all other parameters ($p < 0.001$). Furthermore, the comparison between the 24-hour and 72-hour secretome groups yielded no statistical difference ($p = 0.878$), establishing that structural adherens junction regeneration is rapidly mobilized and durably sustained by the stem cell milieu.

4. Discussion

This investigation conclusively demonstrates that the human breastmilk stem cell-derived secretome functions as a highly potent, cell-free biological modifier capable of dramatically reversing severe hypoxia-induced endothelial dysfunction. Within the clinical and molecular landscape of pulmonary vascular diseases, chronic hypoxia acts as the

absolute dominant pathogenic driver in the evolution of pulmonary hypertension. The hypoxic microenvironment does not merely stress the endothelial cells; it systematically and mechanically dismantles the highly organized endothelial monolayer. It achieves this cellular destruction by simultaneously inhibiting essential survival signals, most notably the bone morphogenetic protein receptor type 2, and enzymatically cleaving the critical structural anchor points defined by vascular endothelial cadherin.[11]

Table 2. One-Way ANOVA of Biomarker Expression Among Groups

Analysis of variance detailing the robust statistical differences in signaling and structural protein levels across the normoxic, hypoxic, and secretome-treated cohorts.

Biomarker	Source of Variation	Sum of Squares	df	Mean Square	F	Sig. (p-value)
BMPR2	Between Groups	0.050	3	0.017	12.480	0.002
	Within Groups	0.011	8	0.001		
	Total	**0.061**	**11**			
VE-Cadherin	Between Groups	5320.190	3	1773.397	415.478	< 0.001
	Within Groups	34.147	8	4.268		
	Total	**5354.337**	**11**			

Notes:
- **df:** Degrees of Freedom; **F:** F-ratio determining the variance between sample means; **Sig:** Significance level indicating the probability of the null hypothesis.
- The analysis confirms highly significant variance across the four experimental groups for both Bone Morphogenetic Protein Receptor Type 2 ($p = 0.002$) and Vascular Endothelial Cadherin ($p < 0.001$), validating the profound impact of the hBSC secretome intervention.

At the core of this pathological transformation is the stabilization of hypoxia-inducible factor 1-alpha. Under normal physiological conditions, this transcription factor is rapidly degraded.[12] However, under the severe oxygen deprivation modeled in this study, hypoxia-inducible factor 1-alpha avoids degradation, accumulates within the cytoplasm, and translocates to the nucleus to fundamentally reprogram the cellular metabolism and transcriptional profile. Under pathological chronicity, this stabilization initiates widespread, unrelenting endothelial apoptosis. Concurrently, the breakdown of the endothelial barrier removes the inhibitory signals that normally keep the underlying pulmonary artery smooth muscle cells in a quiescent state. The resulting unchecked proliferation, hyper-muscularization, and phenotypic switching of these smooth muscle tissues represent the definitive histological signature and primary cause of right ventricular failure in pulmonary hypertension.[13]

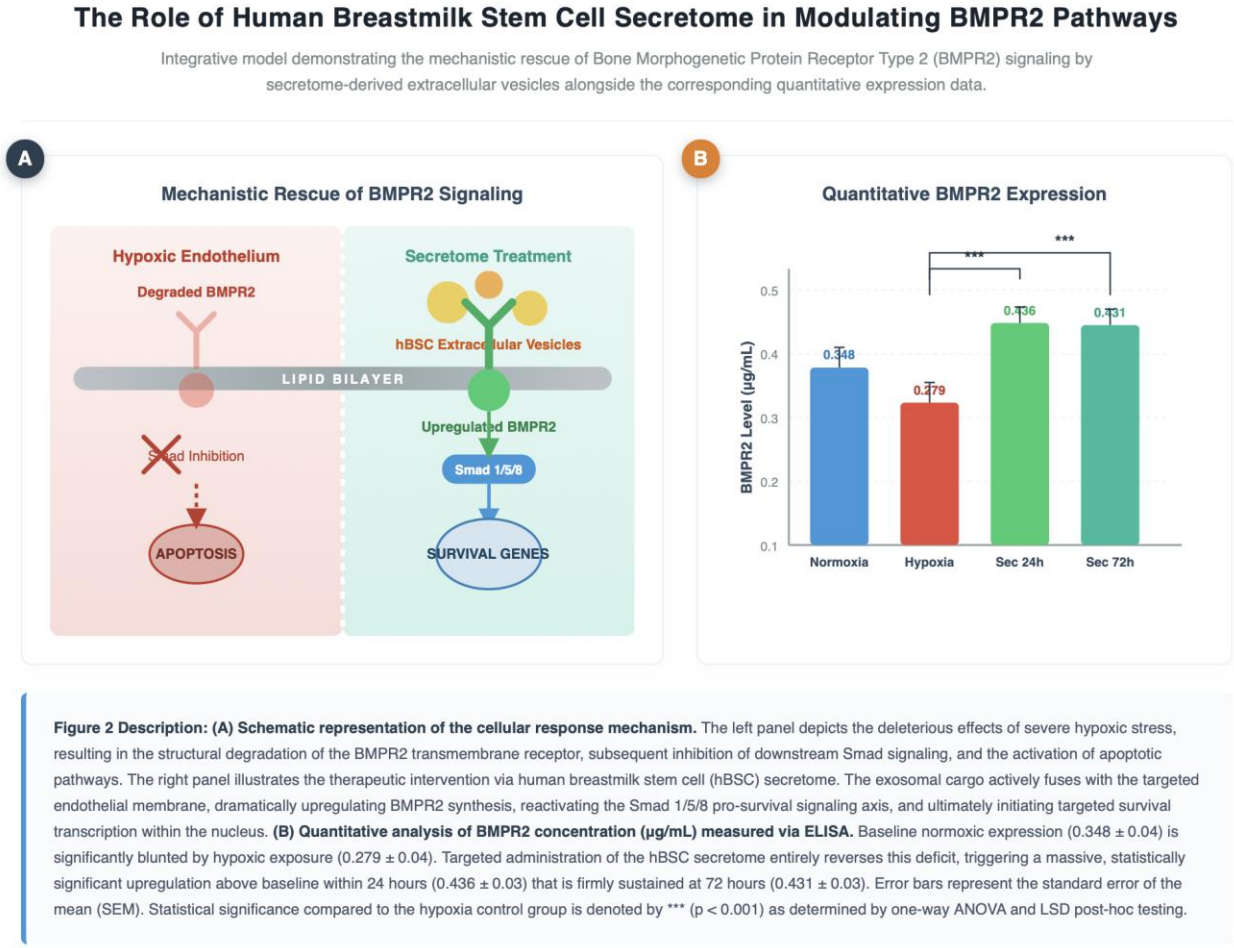

Figure 2 Description: (A) Schematic representation of the cellular response mechanism. The left panel depicts the deleterious effects of severe hypoxic stress, resulting in the structural degradation of the BMPR2 transmembrane receptor, subsequent inhibition of downstream Smad signaling, and the activation of apoptotic pathways. The right panel illustrates the therapeutic intervention via human breastmilk stem cell (hBSC) secretome. The exosomal cargo actively fuses with the targeted endothelial membrane, dramatically upregulating BMPR2 synthesis, reactivating the Smad 1/5/8 pro-survival signaling axis, and ultimately initiating targeted survival transcription within the nucleus. **(B) Quantitative analysis of BMPR2 concentration (µg/mL) measured via ELISA.** Baseline normoxic expression (0.348 ± 0.04) is significantly blunted by hypoxic exposure (0.279 ± 0.04). Targeted administration of the hBSC secretome entirely reverses this deficit, triggering a massive, statistically significant upregulation above baseline within 24 hours (0.436 ± 0.03) that is firmly sustained at 72 hours (0.431 ± 0.03). Error bars represent the standard error of the mean (SEM). Statistical significance compared to the hypoxia control group is denoted by *** ($p < 0.001$) as determined by one-way ANOVA and LSD post-hoc testing.

Figure 2. The role of human breastmilk stem cell secretome in modulating BMPR2 pathways.

Bone morphogenetic protein receptor type 2 deficiency is irrefutably implicated as a cornerstone molecular anomaly in both heritable and idiopathic forms of pulmonary hypertension.[14] Under healthy physiological conditions, the binding of specific bone morphogenetic proteins to this transmembrane serine/threonine kinase receptor activates the highly conserved Smad 1/5/8 signaling axis. Upon phosphorylation, these Smad proteins translocate directly to the cellular nucleus to act as master transcription factors, rigorously regulating a suite of target genes that maintain endothelial quiescence, promote cellular survival, and actively inhibit apoptosis. In our experimental model, severe hypoxic exposure successfully decoupled and dismantled this essential protective mechanism, as evidenced by the highly significant reduction in bone morphogenetic protein receptor type 2 expression observed in the hypoxic control cohort.

Crucially, following the targeted therapeutic administration of the human breastmilk stem cell secretome, bone morphogenetic protein receptor type 2 expression was not merely salvaged from hypoxic degradation but was paradoxically upregulated well beyond the original, healthy normoxic baseline. This extraordinary biological phenomenon heavily aligns with contemporary molecular understanding that stem cell-derived secretomes exert profound, pleiotropic regenerative effects. The secretome is not a single active ingredient; rather, it is a highly complex,

concentrated biological fluid extraordinarily rich in nanosized extracellular vesicles, diverse messenger RNAs, regulatory microRNAs, and a vast array of specialized growth factors.[15]

We theorize that the complex exosomal cargo inherent to the breastmilk secretome directly interfaces and fuses with the damaged human umbilical vein endothelial cells. Once internalized, these targeted microRNAs and regulatory proteins actively suppress destructive oxidative stress pathways while aggressively reactivating pro-survival transcription at the genomic level. This targeted genetic modulation forces the endothelial cells to hyper-express the survival receptors to overcompensate for the hypoxic insult. Furthermore, the quantitative data confirmed that there was absolutely no statistical disparity between the 24-hour and 72-hour treatment timelines regarding receptor expression. This plateau highly implies that the secretome possesses exceptional, rapid bioavailability, successfully triggering and saturating the bone morphogenetic protein receptor type 2 dependent recovery cascades within a highly abbreviated and clinically favorable therapeutic window.

While bone morphogenetic protein receptor type 2 signaling is absolutely paramount for ensuring basic cellular survival and resisting apoptosis, the actual physical functionality and fluid dynamic capacity of the pulmonary vasculature rely completely on the strict mechanical integrity of the endothelial barrier. Vascular endothelial cadherin is the fundamental molecular glue and strictly specific adhesion molecule of endothelial adherens junctions.[16] Beyond merely acting as a physical, passive zipper linking adjacent cell membranes, the intracellular domain of vascular endothelial cadherin is highly dynamic. It interacts intimately with the catenin protein family—specifically beta-catenin and p120-catenin—to firmly anchor the junctional complex directly to the internal actin cytoskeleton. This continuous structural bridge is absolutely essential for orchestrating mechanotransduction, sensing shear stress from blood flow, and maintaining strict, impermeable vascular stability.

In advanced clinical pulmonary hypertension, the degradation and breakdown of vascular endothelial cadherin directly precipitate catastrophic paracellular hyper-permeability. This loss of barrier function removes the protective shield of the vessel, actively permitting circulating inflammatory cytokines, activated leukocytes, and circulating fibrocytes to freely infiltrate the deep sub-endothelial space. This aggressive infiltration fuels the severe inflammatory microenvironment that drives maladaptive vascular remodeling. Our strictly controlled hypoxic cohort perfectly mirrored this catastrophic junctional collapse, presenting severely decimated vascular endothelial cadherin levels that indicated a total loss of cellular cohesion.[17]

Remarkably, targeted treatment with the human breastmilk stem cell secretome produced a monumental and rapid structural reversal. The intervention increased vascular endothelial cadherin concentrations nearly seven-fold against the decimated hypoxic baseline, rocketing the expression levels to significantly outperform even the healthy, undisturbed normoxic cells. This sustained hyper-expression, statistically identical at both the 24-hour and 72-hour measurement intervals, solidifies a critical physiological concept. It proves that breastmilk-derived bioactive factors do not simply patch the cell membrane; they fundamentally and genetically reorganize the entire cellular cytoskeleton.[18] By massively upregulating the production of junctional proteins, the secretome reinforces the adhesive strength of the adherens junctions and successfully re-establishes a tight, highly impermeable endothelial monolayer, stubbornly maintaining structural homeostasis despite the ongoing, severe oxygen deprivation (Figure 2).

The synchronous, dual-axis recovery of both internal signaling mechanisms through bone morphogenetic protein receptor type 2 and external structural parameters through vascular endothelial cadherin elevates the human breastmilk stem cell

secretome from a purely theoretical scientific concept to a remarkably high-potential translational therapy.[19] Current medical management of pulmonary hypertension relies heavily on classical pharmacotherapies that predominantly manipulate vasomotor tone via nitric oxide enhancement or prostacyclin receptor activation. While these standard therapies provide symptomatic relief, they completely fail to halt the underlying cellular destruction. In stark contrast, this cell-free secretome specifically, directly targets and aggressively reverses the upstream pathophysiological endothelial injury that causes the disease.

Furthermore, the biological source of this therapy provides a unique evolutionary advantage. Human breastmilk naturally evolved over millions of years precisely to facilitate postnatal cardiopulmonary adaptation, drive the maturation of the newborn immune system, and promote safe, highly regulated angiogenesis in developing tissues. Because it is intrinsically packed with these specific developmental cues, its secretome profile may be singularly suited and uniquely powerful for treating severe pediatric and neonatal vascular pathologies. This includes highly lethal conditions such as persistent pulmonary hypertension of the newborn and prematurity-associated pulmonary vascular disease resulting from chronic lung dysplasia.[20]

While this rigorously controlled in vitro molecular framework is robust and yields highly significant statistical data, the study design is inherently limited by the absence of multi-dimensional, systemic endothelial assessments. Crucial physiological metrics, including in vivo hemodynamic evaluations utilizing right ventricular systolic pressure tracking via cardiac catheterization, direct nitric oxide quantification, and comprehensive functional angiogenic tube-formation assays, were not evaluated within this specific experimental scope. To responsibly advance this therapy toward human application, future research must aggressively pivot toward highly controlled in vivo mammalian models of pulmonary hypertension. These advanced animal studies are absolutely necessary to definitively map the in vivo biodistribution of the extracellular vesicles, establish the pharmacokinetic durability of the protein cargo, and determine the optimal, safe dosing regimens of the purified breastmilk secretome prior to the initiation of any clinical trials.

5. Conclusion

This comprehensive investigation provides compelling, highly significant molecular evidence that the human breastmilk stem cell secretome serves as an aggressive and highly effective therapeutic intervention against severe, hypoxia-induced endothelial dysfunction. By rapidly upregulating the critical bone morphogenetic protein receptor type 2 signaling cascade to actively halt cellular apoptosis, while simultaneously hyper-expressing vascular endothelial cadherin to physically reconstruct and fortify structural adherens junctions, the secretome comprehensively and simultaneously targets the two fundamental pathobiological pillars of pulmonary hypertension. The remarkable discovery that these profound, dual-axis regenerative effects manifest robustly within the first 24 hours of administration and are reliably, statically sustained through 72 hours of continuous hypoxic stress underscores the vast clinical viability of this approach. Ultimately, these findings highly support the development of human breastmilk-derived cell-free therapies as a truly paradigm-shifting, safely translatable, and definitively disease-modifying regenerative treatment for severe pediatric and adult pulmonary vascular diseases.

6. References

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