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Original Article

Shehata Marker: A Promising Tool for Assessing Graft Endothelial Dysfunction and Hypoxic Injury

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ABSTRACT

Background: The integrity of the graft vasculature is essential for maintaining homeostasis in transplantation, regulating perfusion, selective permeability, preventing thrombosis, and facilitating immune surveillance. However, despite extensive studies on graft rejection and ischemia-reperfusion injury, the molecular responses of vascular effectors to hypoxic stress during transplantation remain largely uncharacterized.

Objective: This study addresses a critical knowledge gap by evaluating the collective response of established vascular effector molecules to hypoxic injury, a condition commonly encountered in transplantation. The objective is to assess the potential of these molecules, when analyzed as a panel, to serve as early, non-invasive biomarkers for graft function and viability.

Methods: In this study, hypoxia-inducible factor-1 alpha (HIF-1α) was chemically induced using cobalt chloride ($CoCl_2$), a well-established hypoxia mimetic. Unlike conventional methods such as ELISA, which are typically used for detecting subtle changes at the picogram level, immunoblotting was employed in this hypothesis-generating, proof-of-concept pilot investigation to capture more prominent alterations in vascular effectors at the preclinical stage. The study included three independent biological replicates, in which varying concentrations of $CoCl_2$ were compared to untreated controls under normoxic conditions.

Results: Hypoxic stimulation led to significant upregulation of certain key vascular effectors, including eNOS, VEGF-A, sVEGFR1, and endothelin-1. Most notably, a novel and unexpected finding regarding nostrin, a regulator of nitric oxide signaling, is reported: its expression and secretion were differentially modulated by hypoxia in a cell-type-specific manner. While nostrin expression decreased in HuH7 and HEK293T cells, it accumulated in HUVECs—a divergence not documented in prior literature. Furthermore, nostrin secretion exhibited an inverse pattern: a trend toward increased levels in tissue cells, but a significant decrease in endothelial cells under hypoxic conditions—a phenomenon not previously reported.

Conclusion: This study provides the first evidence of differential nostrin regulation across cell types in response to hypoxia, unveiling a novel layer of complexity in vascular biology within the transplant setting. By integrating a novel methodological approach with the discovery of uncharted hypoxia-induced vascular signatures, the findings open new avenues for developing non-invasive biomarkers for graft monitoring, particularly in the early post-transplant phase and during *ex vivo* perfusion.

KEYWORDS: Angiogenesis; Endothelial dysfunction; Hypoxia; Solid organ transplant; Vascular surgery; *Ex vivo* organ perfusion

INTRODUCTION

Solid organ transplantation is a critical therapeutic intervention for various end-stage organ failures, including those affecting the liver, kidney, pancreas, lung, heart, and other vital organs. Despite its life-saving potential, a major challenge in transplantation is the occurrence of both acute and chronic graft rejection, which compromises long-term graft function and survival [1]. The graft vasculature plays a pivotal role in maintaining homeostasis by regulating essential functions, including tissue perfusion, selective permeability, thrombosis prevention, and immune surveillance. Vascular cells in the transplanted organ play a central role in the inflammatory response triggered by ischemia-reperfusion

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injury and allograft rejection, as they interact with mediators from both innate and adaptive immune pathways [2].

Transplant rejection manifests in various forms, each of which primarily targets the graft vasculature. Acute rejection can present as thrombosis, neutrophilic hyperacute rejection, endothelialitis, intimal arteritis, and fibrinoid necrosis, often driven by either cell-mediated or antibody-mediated mechanisms. Chronic rejection, on the other hand, is characterized by diffuse luminal stenosis of the graft vasculature [2]. The fundamental role of vascular integrity is to ensure adequate delivery of oxygen and nutrients to the tissues. Thus, any disruption in the vascular supply, whether through occlusion or injury, leads to ischemic damage, marked by compromised blood flow and resultant tissue hypoxia [3].

Nitric-oxide synthase trafficking inducer (Nostrin) serves as a pleiotropic regulator of endothelial cell function and signaling. It can sequester endothelial nitric oxide synthase 3 (eNOS), thereby reducing nitric oxide (NO) production, and concurrently modulates the functional transcriptome of endothelial cells, down-regulating key genes involved in processes such as cell invasion and angiogenesis [4].

In addition to its role in eNOS regulation, Nostrin influences endothelial cell function through independent mechanisms. For example, Nostrin has been shown to inhibit endothelial cell proliferation by approximately 17%, regardless of its effect on eNOS, suggesting that it may serve as a modulator of endothelial angiogenic potential. Moreover, overexpression of Nostrin significantly reduces the expression of genes associated with cellular invasion, further highlighting its impact on endothelial cell functionality [4].

Nostrin also exerts effects on the endothelial cell adhesion profile, down-regulating various adhesion molecules, including ITGβ3, ITGα5, and FN1. This down-regulation is associated with a reduction in the secretion of pro-inflammatory cytokines and chemokines. One of the key mechanisms by which Nostrin mediates these effects is through the inhibition of the NFκB signaling pathway. Nostrin achieves this by forming a complex with TNF receptor-associated factor 6 (TRAF6), which is thought to impair pro-angiogenic signaling in endothelial cells [5]. Furthermore, ectopic overexpression of Nostrin leads to a reduction in the secretion of pro-inflammatory cytokines, including IL-6, CCL2, and CCL5, while simultaneously upregulating the production of endothelin-1 [4].

Collectively, Nostrin modulates the expression of proteins critical to both angiogenesis and inflammation. One of its key actions involves the down-regulation of pro-angiogenic factors, which interact with their respective receptor tyrosine kinases (RTKs) on the cell surface. Notably, Nostrin reduces the expression of several RTKs, including FLT-1/VEGFR1 and KDR/VEGFR2 [6]. It also significantly down-regulates FLT-1 and placental growth factor, which may play a role in fine-tuning the VEGF signaling pathway [4].

Endothelial nitric oxide synthase (eNOS) is a critical enzyme that protects the vascular system by producing nitric oxide (NO), a key signaling molecule [7]. eNOS is activated through the VEGFR-2/PI3K/Akt-PKB pathway in endothelial cells, leading to NO production [7]. NO serves both autocrine and paracrine roles in various physiological processes, including the regulation of blood flow, thrombosis, inflammation, immune responses, neural activity, cell migration, and angiogenesis. Additionally, NO functions as an antioxidant, mitigating the effects of free radicals that contribute to immune activation and inflammation. This protective role is partially mediated by the upregulation of heme oxygenase-1 and ferritin expression, which help to reduce the concentration of harmful superoxide anions in the vasculature [7].

The vascular endothelial growth factor (VEGF) family comprises several members, including VEGF-A, VEGF-B, VEGF-C, VEGF-D, placental growth factor (PlGF), non-human genome encoded VEGF-E, and

snake venom-derived VEGF (svVEGF). Among these, VEGF-A is crucial for angiogenesis and the maintenance of endothelial function, playing a vital role in tumor growth, proliferation, invasion, and metastasis, and contributing to tumor resistance to therapeutic agents. VEGF-B is involved in promoting neuronal survival and stimulating cardiovascular growth, particularly in organs such as the heart. VEGF-C and VEGF-D primarily mediate VEGFR-3-dependent lymphangiogenesis and lymphatic metastasis [8]. For further details on the diverse biological roles of the VEGF family and the signaling mechanisms of its receptors (VEGFR1, VEGFR2, and VEGFR3), readers are referred to other comprehensive reviews [9]. Fig. 1 provides a concise overview of the primary functions of the VEGF family and its receptors.

Soluble vascular endothelial growth factor receptor 1 (sVEGFR1), also known as sFlt-1, is a 110 kDa truncated splice variant of the 180 kDa membrane-bound VEGFR1. It is primarily produced through alternative splicing and, to a lesser extent, by proteolytic cleavage of the full-length receptor [10]. Soluble VEGFR1 is thought to act as an anti-angiogenic factor through several mechanisms [11, 12], including:

- Sequestering VEGF ligands: Soluble VEGFR1 can bind VEGF ligands in monomeric, dimeric, or even multimeric forms, reducing their ability to activate pro-angiogenic receptors.

- Heterodimerization with full-length VEGFR: Soluble VEGFR1 can heterodimerize with full-length VEGFR monomers, rendering the receptor dimer inactive.

Evidence suggests that soluble VEGFR1 exists as a family of at least four alternatively spliced variants [11]. Its expression is notably upregulated in the placenta under hypoxic conditions, a mechanism that may contribute to the pathogenesis of preeclampsia [12]. The overall effects of soluble VEGFR1 in neutralizing VEGF signaling include:

i. Anti-angiogenesis: By reducing VEGF-VEGFR2 signaling, soluble VEGFR1 inhibits angiogenesis.

ii. Anti-oedema: It interferes with VEGFR1- or VEGFR2-mediated vascular permeability in response to VEGF, thereby reducing edema.

iii. Anti-inflammation: By attenuating VEGF-VEGFR1-dependent activation and migration of monocytes and macrophages, it exerts anti-inflammatory effects.

The endothelin system comprises three main isoforms: ET-1, ET-2, and ET-3, which exert their effects through two distinct endothelin receptors, A (ETRA) and B (ETRB). ETRA exhibits a higher affinity for ET-1 and ET-2 compared to ET-3, whereas ETRB binds all three isoforms with equal affinity. The ET-1 isoform is predominantly expressed in the vascular endothelium and smooth muscle cells, where it plays a crucial role in the process of angiogenesis. ET-1 directly stimulates the growth and proliferation of endothelial and peri-vascular cells via the MAPK signaling pathway [13], thereby contributing to tumor angiogenesis. Moreover, ET-1 enhances VEGF expression and promotes angiogenesis through multiple signaling pathways, including ETAR, integrin-linked kinase (ILK), Akt, and HIF-1α [13].

Despite the critical role of vascular integrity in transplant rejection, the molecular mechanisms underlying endothelial dysfunction in graft vasculature, particularly in response to hypoxia, remain poorly understood. It is hypothesized that the proteins mentioned above play key roles in the tissue's reaction to hypoxia and can impact the graft vasculature; therefore, they may serve as markers to reflect the tissue's hypoxic status. Accordingly, this study aims to serve as a hypothesis-generating, proof-of-concept pilot investigation examining the alterations in these key vascular effectors in response to hypoxia. To minimize variability arising from different forms of hypoxia, which predominantly activate hypoxia-inducible factor-1α (HIF-1α), chemical hypoxia induction with cobalt chloride ($CoCl_2$)

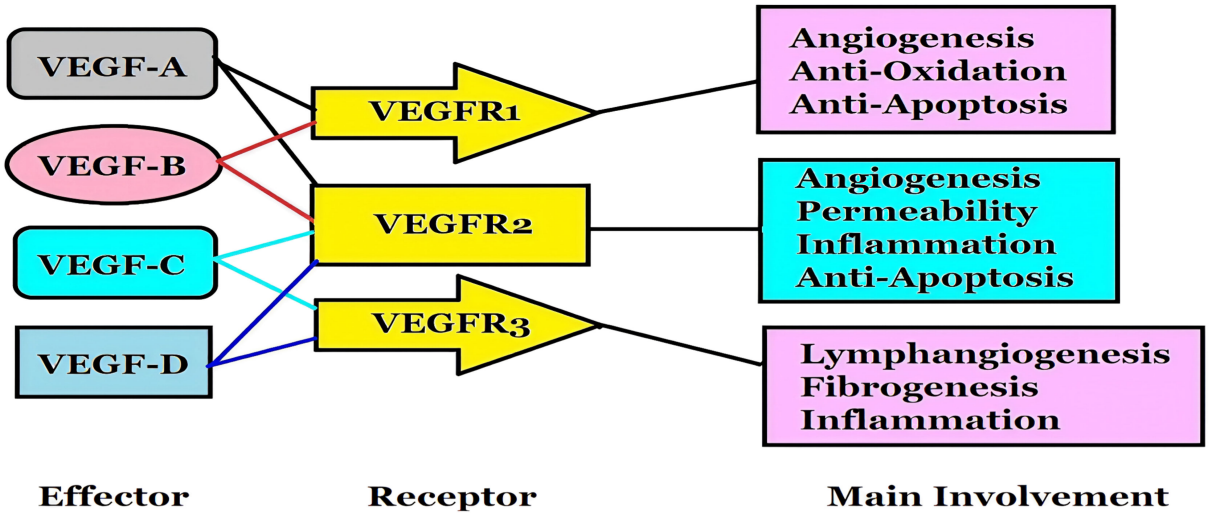


Figure 1: Diagrammatic representation of the major involvements of the VEGF family.

was employed in this study. While changes in target effectors are often subtle and typically measured at the picogram level, making techniques like ELISA ideal for detecting such small alterations, immunoblotting was used to assess more prominent changes at the preclinical level.

MATERIALS AND METHODS

Commercially available human cell lines were used, obtained from collaborating laboratories. Cell line identity was verified by the original supplier; however, no additional in-house authentication (e.g., STR profiling) or mycoplasma testing was performed. Cells were cultured under standard conditions and utilized at passages 3–7 to minimize phenotypic drift.

All experiments were conducted in three independent biological replicates. Cells (2×10^5 per well) were seeded in standard 6-well plates and incubated overnight at 37°C in 2 mL of Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS; Gibco), 1× sodium pyruvate, 1× penicillin–streptomycin (Gibco), 1× GlutaMAX (Gibco), and 25 mM HEPES.

To model hypoxic-like stress, cells were treated with cobalt chloride ($CoCl_2$; Sigma-Aldrich), a chemical hypoxia mimetic that stabilizes hypoxia-inducible factor (HIF) signaling. Treatment conditions included:

- Untreated control (0 μM $CoCl_2$)
- 200 μM $CoCl_2$
- 300 μM $CoCl_2$
- 400 μM $CoCl_2$
- 500 μM $CoCl_2$ (HUVECs only)

Following 72 hours of incubation at 37°C, cells were washed with phosphate-buffered saline (PBS) and lysed in RIPA buffer containing protease and phosphatase inhibitors (Roche). Conditioned media (supernatants) were also collected to evaluate secreted protein levels.

Protein concentration was quantified using the Bradford assay, and equal amounts of total protein were loaded per lane. Internal loading controls were validated and used for normalization of target protein expression levels. To enable valid comparison of secreted protein levels across different treatment conditions, cells were initially seeded at equal densities, and equal amounts of total secreted protein

$CoCl_2$ Concentration	HIF-1α	Nostrin (Intracell.)	eNOS	VEGF-A	sFlt-1	ET-1
200 μM	0.73	↑ 0.01	0.06	0.03	0.04	0.01
300 μM	0.02	↓ 0.01	0.05	0.02	0.04	0.00
400 μM	0.00	↓ 0.00	0.02	0.09	0.27	0.23

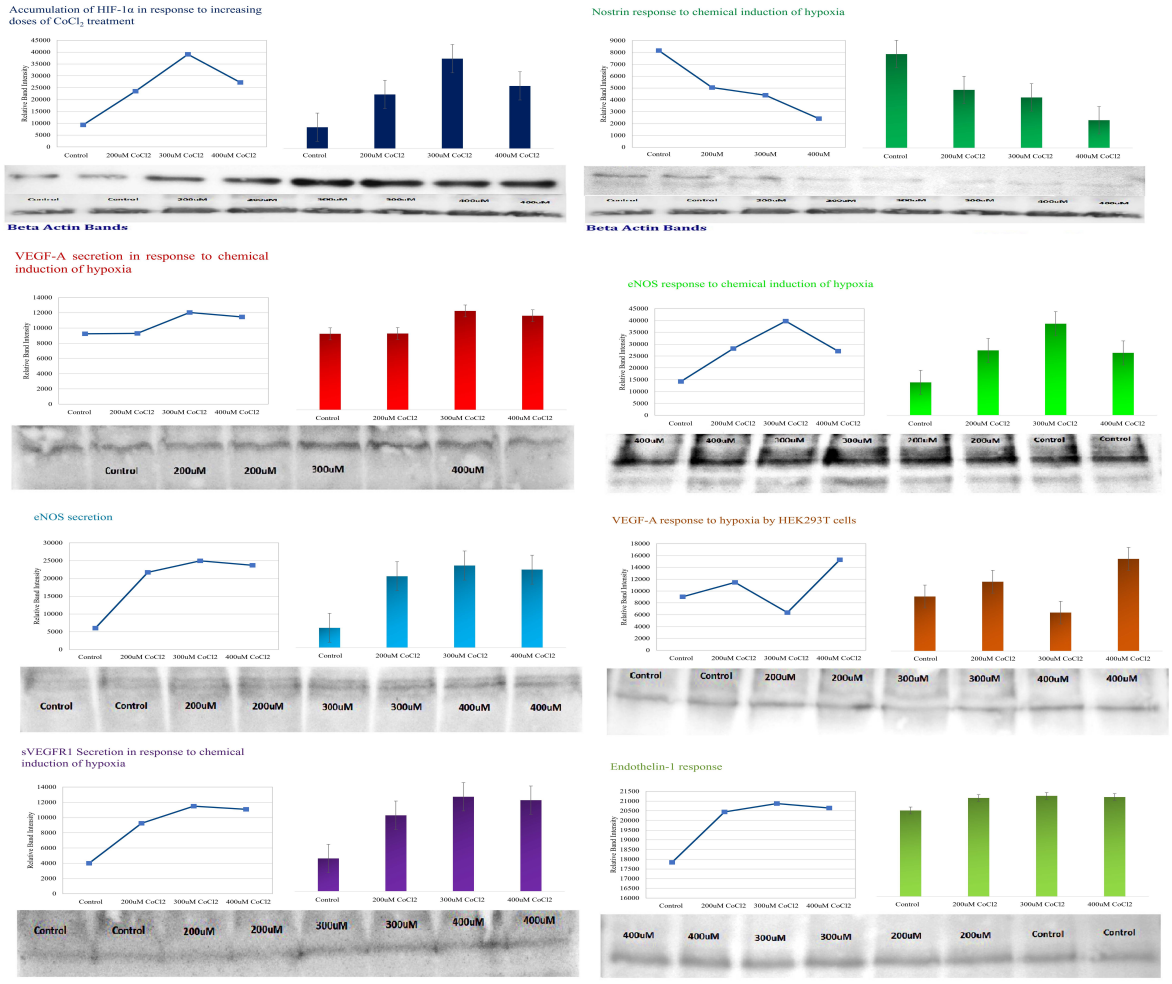
P-values for proteins secreted by HuH7 cells and changes in intracellular HIF-1α & nostrin levels. Positive (↑) or negative (↓) changes in nostrin where applicable. P-values less than 0.05 are considered significant.

Figure 2: Chemical induction of hypoxia in HuH7 liver cells using $CoCl_2$ leads to multiple cellular and secretory changes. Treatment with 300 μM and 400 μM $CoCl_2$ significantly increases HIF-1α expression. This hypoxic response is accompanied by a significant decrease in intracellular nostrin levels, alongside increased secretion of nostrin (though not reaching statistical significance using this technique), eNOS (P= 0.02 at 400 μM), VEGF-A (P= 0.02 at 300 μM), and endothelin-1 (P< 0.001 at 300 μM). Additionally, a corresponding increase in soluble VEGFR1 (sVEGFR1) was detected in the culture medium (P= 0.04 at 200 μM and 300 μM). P-values less than 0.05 were considered statistically significant.

were loaded for analysis. This approach controls for variations in overall protein secretion, allowing for a relative assessment of treatment-induced changes in the secreted proteins of interest.

Immunoblotting (Western blot) was employed to analyze both intracellular and secreted protein expression. Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. After blocking, membranes were incubated with primary antibodies against eNOS (Cat# 27120-1-AP), VEGF-A (Cat# 19003-1-AP), HIF-1α (Cat# 66730-1-IG), sVEGFR1 (Cat# 13687-1-AP), ET-1 (Cat# 12191-1-AP), and nostrin (Cat# 20116-1-AP), all purchased from Proteintech and used at a 1:1000 dilution according to the manufacturer's instructions. This was followed by incubation with the corresponding HRP-conjugated secondary antibodies (Thermo Fisher Scientific, Germany) according to the manufacturer's instructions. Signal detection was performed using enhanced chemiluminescence (ECL; Bio-Rad). Band intensities were quantified by densitometry using ImageJ software and normalized to internal controls.

No complementary assays such as ELISA, qPCR, or functional analyses (e.g., migration, apoptosis, or permeability assays) were included. Therefore, detection sensitivity and quantification remain limited to the semi-quantitative resolution of immunoblotting. The absence of orthogonal validation restricts the interpretability of subtle or low-abundance changes.



$CoCl_2$ Concentration	HIF-1α	Nostrin (Intracell.)	eNOS	eNOS (Secretion)	VEGF-A	sFlt-1
200 μM	0.03	↓ 0.19	↑ 0.03	0.01	0.91	0.04
300 μM	0.00	↓ 0.12	↑ 0.01	0.00	0.01	0.00
400 μM	0.00	↓ 0.02	↑ 0.03	0.01	0.01	0.02

P-values for proteins secreted by HEK293T cells and changes in intracellular HIF-1α & nostrin levels. Positive (↑) or negative (↓) changes in nostrin where applicable. P-values less than 0.05 are considered significant.

Figure 3: Chemical induction of hypoxia in HEK293T cells resulted in multiple cellular and secretory changes.

HIF-1α expression was significantly increased across all treatment conditions, with the highest levels observed at 300 μM $CoCl_2$ (P= 0.001). This was accompanied by a decrease in intracellular nostrin levels, an increase in intracellular eNOS, and enhanced secretion of eNOS. VEGF-A expression and secretion were both elevated, along with increased levels of soluble VEGFR1 (sVEGFR1) in the culture medium. Expression of endothelin-1 also increased, although this change did not reach statistical significance using the current method (western blot). P-values less than 0.05 were considered statistically significant.

Statistical Analysis

Statistical analysis was performed using GraphPad QuickCalcs (GraphPad Software, San Diego, CA). Group comparisons were initially conducted using unpaired, two-tailed Student's t-tests. Each treatment group was compared individually to the control group, representing independent, hypothesis-driven comparisons rather than a global group analysis. Consequently, multiple t-tests were applied without correction for multiple comparisons, as the comparisons were predefined and non-overlapping in interpretation.

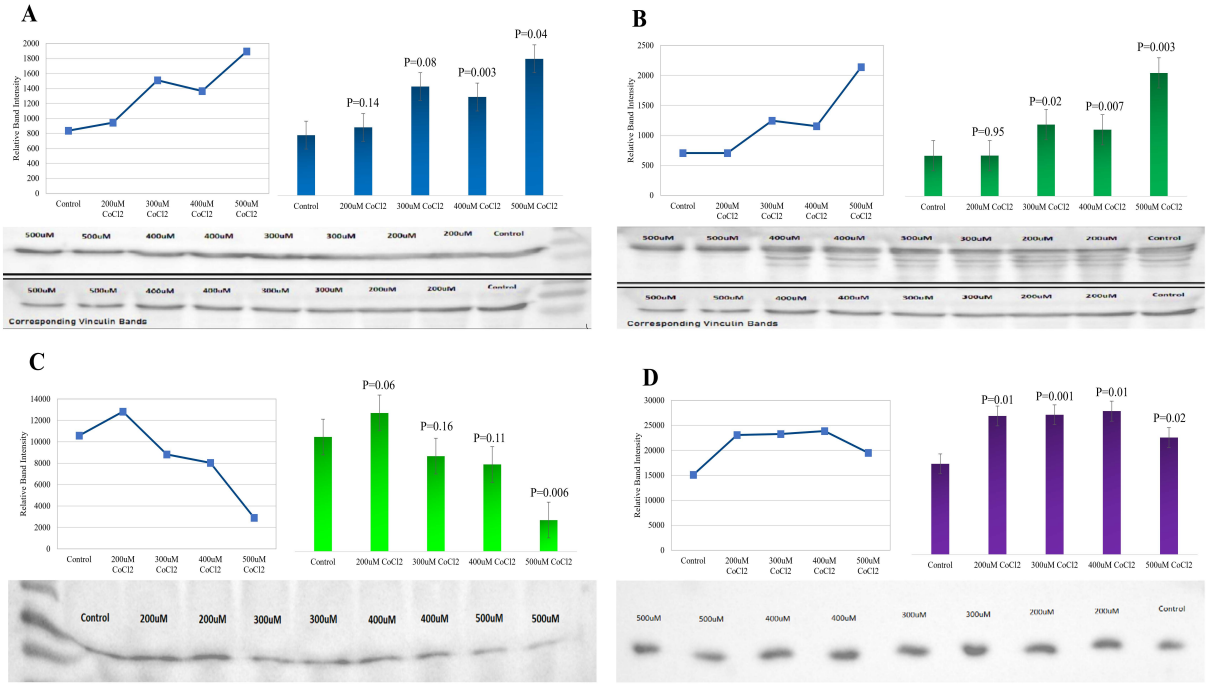


Figure 4: Chemical induction of hypoxia in HUVECs led to intracellular and secretory alterations. HIF-1α expression was significantly increased at 400 μM and 500 μM $CoCl_2$ (**A**). This hypoxic response was associated with an accumulation of intracellular nostrin (**B**) and a corresponding decrease in its secretion (**C**). Additionally, secretion of endothelin-1 was significantly elevated at all treatment doses (**D**). P-values less than 0.05 were considered statistically significant.

To validate the robustness of statistical findings, a one-way ANOVA followed by Tukey's post-hoc test was also performed. This analysis reproduced the same patterns of statistical significance relative to the control as observed with the t-tests. However, inter-group comparisons between $CoCl_2$ doses were not considered biologically meaningful within the study design, as $CoCl_2$ was employed solely as a hypoxia mimetic, not to model graded hypoxic conditions.

A P-value< 0.05 was considered statistically significant. Data are presented in the figures as Mean ± Standard Error (SE), unless otherwise indicated. Given the limited sample size (n= 3 biological replicates), these findings are preliminary and warrant further validation *in vivo*.

RESULTS

Chemical induction of hypoxia was successfully achieved in HuH7 liver cells, where HIF-1α accumulated and significantly increased, reaching its peak in the 400 μM treatment sample (ANOVA P<0.0001, highly significant). The treatment at 300 μM and 400 μM significantly increases HIF-1α levels compared to the control. The 200 μM group does not differ significantly from the control, but it does differ significantly from the higher doses. The dynamic response of target proteins to various doses of $CoCl_2$ treatment is shown in Fig. 2.

To confirm that these changes were not specific to the cell line origin, similar experiments were conducted on HEK293T cells (non-tumorigenic human embryonic kidney cells). Chemical induction of HIF-1α occurred in a dose-dependent manner. The dynamic

changes in response to various doses of $CoCl_2$ treatment are presented in Fig. 3.

To assess the response of the vascular endothelium, human umbilical vein endothelial cells (HUVECs) were treated with increasing doses of $CoCl_2$. Successful chemical induction of HIF-1α was confirmed by Western blot, accompanied by a dose-dependent reduction in cellular confluence observed under the microscope. Control untreated cells showed full confluence after 72 hours of incubation, while cells treated with 200 μM, 300 μM, 400 μM, and 500 μM $CoCl_2$ displayed approximately 90%, 80%, 70%, and 60% confluency, respectively. The endothelial changes in response to hypoxia are presented in Fig. 4, where HIF-1α induction reached statistical significance at the 400 μM and 500 μM $CoCl_2$ treatment doses, leading to a corresponding increase in intracellular nostrin and a decrease in nostrin secretion. Notably, the secretion of ET-1 was significantly increased at all treatment doses ($P<0.05$).

DISCUSSION

As a step towards improving the clinical outcomes of solid organ transplantation, where the availability of suitable donor organs and the incidence of post-transplant graft dysfunction or rejection are major limitations [1], certain target effectors were selected for investigation. These target proteins are well-established and thoroughly studied effectors that play crucial roles in both physiology and pathology. They were chosen for their known ability to reflect the hypoxic status of tissues and their involvement in angiogenesis. While each of these proteins has been studied individually, to the best of my knowledge, this work is the first to combine them under the same experimental conditions.

To avoid potential variations associated with studies using physical hypoxia, such as differences in the type of hypoxia (sustained vs. intermittent), its duration (short-term vs. long-term), or its extent, this work employed the previously accepted chemical induction of hypoxia using $CoCl_2$. $CoCl_2$ has been reported to induce the accumulation of HIF-1α and HIF-2α under normoxic conditions [14].

The current results confirmed that liver cells exposed to hypoxia show decreased intracellular nostrin and increased levels of intracellular eNOS, VEGF-A, and VEGFR1. Hypoxia is also associated with an increase in the secretion of nostrin, eNOS, VEGF-A, sVEGFR1, and ET-1.

Notably, the secretion of ET-1 and VEGF-A increased significantly at the lowest $CoCl_2$ treatment dose, even though HIF-1α accumulation did not reach statistical significance at this dose. This observation may suggest that both effectors are more sensitive to hypoxia and potentially play leading roles in the tissue's response to hypoxia, particularly at the paracrine or remote level.

Since the liver cells used in this study have a tumorigenic origin, it was important to replicate the findings using benign human renal cells (HEK293T), which responded similarly (Fig. 3). These results confirmed that the observed reactions to hypoxia, such as the increased production of eNOS, VEGF-A, sVEGFR1, and ET-1, are consistent responses across both liver and renal cells.

When HUVECs were treated with increasing doses of $CoCl_2$, a similar increase in ET-1 secretion was observed. However, the changes in nostrin differed, mirroring the opposite pattern seen in tissue cells. Intracellular nostrin levels accumulated (or, more accurately, there was more nostrin degradation in the control and 200 μM treatment samples, which tended to decrease as the $CoCl_2$ concentration increased), while nostrin secretion decreased in response to hypoxia (Fig. 4). This suggests that VEGF-A, VEGFR1, and its soluble form, sVEGFR1, as well as ET-1, play significant roles in both the local and remote tissue responses to hypoxia, particularly in angiogenesis. In contrast, nostrin appears to have differential roles in tissue and endothelial cells, warranting further investigation.

The intracellular response of nostrin to hypoxia has been previously reported, where the accumulation of HIF-1α leads to a reduction in nostrin, even in endothelial cells [4,15]. To the best of my knowledge, this work is the first to explore nostrin secretion in response to hypoxia and the first to report an increase in intracellular nostrin, rather than a decrease, in endothelial cells. The independent findings in this study suggest a complementary response, where nostrin decreases in liver and renal cells, accompanied by increased secretion, while in endothelial cells, intracellular nostrin increases, along with a decrease in secretion.

Nostrin is an F-BAR protein involved in several important cellular processes, including endocytosis, phagocytosis, exocytosis, angiogenesis, and migration [16]. It has been reported that eNOS is translocated from the plasma membrane to intracellular vesicular structures through caveolar endocytosis, a key mechanism for VE-cadherin internalization [17]. However, nostrin has also been shown to down-regulate certain genes critical for invasion and angiogenesis, independent of eNOS activity. Ectopic overexpression of nostrin has been reported to functionally restrict endothelial cell proliferation, invasion, adhesion, and VEGF-induced capillary tube formation [4].

Therefore, the changes observed in nostrin may be part of an orchestrated sequence of events or a physiological response to the extent of hypoxic injury, particularly since caveolar VE-cadherin and eNOS activity are crucial for endothelial sprout and tube formation [18,19]. In other words, the differential changes in nostrin between tissue and endothelial cells in response to hypoxia could reflect different cell migration responses across various functional cell types.

A decrease in eNOS activity and NO production has been reported in HUVECs in response to hypoxia [20]. This may, in part, be explained by the enhanced intracellular accumulation of nostrin at high doses of $CoCl_2$ treatment, as nostrin overexpression is associated with reduced eNOS activity, and consequently, decreased NO production [20]. Conversely, the findings in tissue cells, where nostrin was decreased and eNOS increased in response to hypoxia, align with this concept. Indeed, the roles of eNOS, NO, and VEGF in angiogenesis are well-documented. Most clinically approved anti-angiogenic therapies target this signaling axis [21]. VEGF induces eNOS and promotes NO production in vascular endothelial cells, and reduced NO production, in turn, diminishes angiogenesis and vascular permeability induced by VEGF [22].

The variations in the experimental findings and discrepancies among studies may be due to differences in the type, duration, or extent of hypoxia, as well as the physiological sequence of events that occur during the angiogenesis process.

Hypoxia-induced VEGF also stimulates the production of its truncated soluble form, sVEGFR1, through VEGFR-2-MEK-PKC signaling, which serves as a regulatory mechanism to control excessive VEGF activity [23]. As hypoxia triggers angiogenesis, a process that involves a balance between pro-angiogenic and anti-angiogenic factors, sVEGFR1 functions as one of the endogenous anti-angiogenics to prevent excessive angiogenesis, or more precisely, to regulate VEGF signaling [11, 12].

These concepts align well with the results of the present work, where sVEGFR1 was increased in response to the chemical induction of hypoxia in both liver and kidney cells. However, contradictory findings have been reported by other researchers, who observed a reduction in sVEGFR1 expression under hypoxic conditions. Their experiments, however, were conducted on human microvascular endothelial cells, primarily isolated from neonatal dermis [24].

Soluble VEGFR-1 can play an active role in angiogenesis by binding to its isoforms through GM3 ganglioside, which affects the dynamics of the actin cytoskeleton. This destabilizes pericyte-endothelial interactions and modifies adhesion contacts with the basement mem-

brane, thus facilitating vessel sprouting [25]. The presence of sVEGFR-1 has also been shown to shift α5β1 integrin signaling from a classic adhesion pathway to a more dynamic one, and even enhance its expression [26, 27]. Therefore, sVEGFR-1 in the endothelial cell microenvironment during vessel sprouting is influential [28]. Taken together, these findings confirm the importance of sVEGFR-1 in vessel sprouting and angiogenesis through mechanisms beyond VEGF binding [29], which aligns with the findings of the present work.

Another major finding in this study is the increased secretion of ET-1 in response to hypoxia. Similar results have been reported in both preclinical and clinical *in vivo* studies, where intermittent hypoxia increased the expression of ET-1 in animals, and chronic intermittent hypoxia, as seen in patients with obstructive sleep apnea, led to elevated circulating ET-1 levels. Increased ET-1 has been confirmed to be associated with vascular complications and endothelial dysfunction in both non-transplant and transplant patients [30, 31].

A recent study aimed to investigate the effects of sustained and intermittent hypoxia (SH and IH, respectively) on HIF-1α, VEGF, and ET-1 in HepG2 cells (hepatocellular carcinoma cell line). The study reported overexpression of HIF-1α and VEGF in response to intermittent hypoxia (IH), but not ET-1 [32]. While this may seem contradictory to the findings of the present study, several important points should be considered when interpreting their results:

i. Hypoxia Induction Method: In their study, hypoxia was induced physically through exposure to a low-oxygen gas mixture, whereas the present study employed chemical induction of hypoxia with $CoCl_2$.

ii. Cell Type: The HepG2 cells used were of cancerous origin, which may already exhibit certain proangiogenic characteristics, making the induction of ET-1 less detectable. Cancerous cells, due to their rapid growth rates, may naturally experience a certain degree of hypoxia in culture, which could complicate the detection of additional ET-1 induction. In my HuH7 experiments, ET-1 secretion was increased at the 200 μM treatment dose and tended to decrease at the 400 μM treatment dose, where HIF-1α levels peaked (Fig. 2).

Nevertheless, the main findings of the present study focus on ET-1 secretion, which may not directly correlate with changes in mRNA or protein expression levels. In cancer-related angiogenesis, where local relative hypoxia is often present within the tumor microenvironment, multiple reports document elevated circulating ET-1 [33, 34]. Therefore, the findings of the present study appear to align more closely with the clinical observations.

Additionally, it's essential to note that the presented results are from a preclinical pilot study conducted using commercially available cell lines. While other techniques, such as ELISA and mRNA expression analyses, might have provided more precise measurements of the changes, immunoblotting was chosen for its ability to detect significant changes despite the limited financial resources available for these experiments. Further studies are planned to explore this topic in more detail.

As the intervention in the present study was the CoCl2 treatment (chemical induction of HIF-1α), the observed changes can be attributed to the effects of HIF-1α. While the potential dependency of one effector on the upregulation or downregulation of another may be somewhat diminished by the observed variations in response at different treatment doses, it cannot be entirely ruled out. The suggestion that nostrin is responsible for the increase in ET-1 secretion is further weakened by the finding that ET-1 secretion increased in liver and kidney cells despite a decrease in intracellular nostrin. In contrast, in endothelial cells, ET-1 secretion increased alongside an increase in intracellular nostrin. This complexity suggests that the relationship between nostrin and ET-1 secretion may not be as straightforward as initially hypothesized.

In addition to their essential roles in vascular

and endothelial survival, the target effectors also play significant roles in immune reactions and inflammation. As mentioned earlier, nostrin has been shown to inhibit NFκB signaling and the production of proinflammatory cytokines [4]. Similarly, VEGF exhibits immunosuppressive activities [35], modulating both innate and adaptive immunity through its interactions with endothelial cells and various immune cells, thereby influencing vascular permeability and protein expression. Key actions of VEGF include:

Macrophage Polarization: VEGF promotes the polarization of macrophages into the M2 phenotype, which is associated with immunosuppressive actions [36, 37].

Dendritic Cell Maturation: VEGF can reduce the maturation of dendritic cells (DCs) [36].

Expression of Checkpoint Inhibitors: VEGF increases the expression of checkpoint inhibitors on dendritic cells and CD8+ T cells [36].

Natural Killer (NK) Cell Cytotoxicity: VEGF decreases the cytotoxicity of NK cells through VEGF-C/VEGFR-3 signaling, inhibiting the proliferation, cytotoxicity, and recruitment of CD3+ T cells [36].

These findings highlight the multifaceted roles of VEGF in immune regulation, contributing to its involvement in various immune-mediated processes, including inflammation and tissue remodeling.

In contrast, ET-1 functions as a chemokine that enhances vascular permeability and chemotaxis. It also stimulates the production of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6, by monocytes. These effectors play crucial roles in inflammation and significantly influence the clinical outcomes of transplantation [38].

Furthermore, recent accumulating evidence suggests that angiogenesis is not just an accompanying process important for increased vascular permeability, where VEGF plays a predominant role, or for immune cell migration to the site of inflammation, but is also an integral part of inflammation itself. In fact, angiogenesis has been identified as a preceding process involved in the initiation of inflammation [39, 40]. The target proteins investigated in the present study are deeply involved in endothelial function and its response to various insults. While their detailed contributions to cell migration, angiogenesis, and inflammation can be further studied or reviewed, they are already recognized as key players in many processes related to graft vasculature dysfunction and rejection reactions, based on current knowledge. Thus, the combined five effectors can serve as a surrogate marker for hypoxia, i.e, for angiogenesis. This could be particularly valuable for the immediate follow-up of post-operative transplant patients or for assessing the efficacy of pre-transplant interventions, such as *ex vivo* perfusion. In this context, the persistence of a hypoxic profile post-operation would indicate a perfusion issue, while the reversal and subsiding of the hypoxic profile would be a positive sign. Conversely, a truncated or inadequate response to hypoxia before transplantation (without perfusion) could suggest a poor proangiogenic profile, potentially predicting a poor prognosis. Further clinical studies are needed to assess the reliability of this proposed panel marker, explore its correlations with pro-inflammatory mediators, and determine potential cut-off levels for clinical use.

In conclusion, tissue hypoxia leads to significant enhancement of eNOS, VEGF-A, sVEGFR1, and ET-1 production. Nostrin levels remain, however, to be confirmed *in vivo* in preclinical and clinical studies. As the circulating levels of these effectors can indicate hypoxia and affect endothelial function and vasculature contributions to inflammation, they may be collectively used as a surrogate marker to reflect the graft condition during *ex vivo* perfusion and/or the first hours or days post-transplantation. Further preclinical and clinical studies are required to rigorously validate the concepts discussed above.

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CONFLICTS OF INTEREST: None declared.

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