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Original Article

Biological response of human dermal micro-vascular endothelial cells to hypoxia

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Abstract: Venous ulceration is a common ailment that is associated with tissue hypoxia through a complex pathogenic process, but there have been relatively few studies into the effects of hypoxia on human dermal micro-vascular endothelial cells (HDMECs). In this study, we created a hypoxic HDMEC model by culturing cells with $CoCl_2$. We then investigated the migratory capacity of the hypoxic cells using a wound healing assay, and the cellular apoptosis rate using flow cytometry. Additionally, we investigated gene expression using reverse transcription-polymerase chain reaction assays, and protein expression using Western blotting. We found that hypoxia significantly enhanced cellular migration and apoptosis, and significantly delayed cell cycle progression ($p<0.05$). Furthermore, hypoxia caused statistically significant changes in the gene expression of *HIF-1α*, *HIF-1β*, *VEGF*, and *iNOS* ($p<0.05$), with *HIF-1α*, *VEGF*, and *iNOS* expression progressively increasing after 12 h and 24 h of hypoxia when compared with untreated controls ($p<0.05$). Similarly, expression of the apoptosis-related genes *caspase-3* and *Bax* was increased after 12 h and 24 h of hypoxia ($p<0.05$), although there was no further increase in expression between 12 h and 24 h. The expression of the cellular proliferation marker gene *PCNA* gradually decreased after 12 h and 24 h of hypoxia ($p<0.05$). In summary, hypoxia may enhance cell migration via the increased expression of HIF-1α, VEGF, and iNOS. Moreover, hypoxia increases the rate of apoptosis, and slows down cell cycle progression, in HDMECs.

Keywords: Venous ulceration, hypoxia, HDMECs, HIF-1α

Introduction

Many patients with varicose veins go on to develop skin dystrophy, and, eventually, venous ulcers. Some clinical studies indicate that such venous ulceration is correlated with dermal tissue hypoxia [1]; indeed, hypoxia is known to affect gene expression in human dermal micro-vascular endothelial cells (HDMECs), resulting in microenvironmental deficiency and skin ulceration [2]. Furthermore, expression of hypoxia-inducible factor-1α (HIF-1α), a protein known to affect venous endothelial cell function in hypoxic conditions, was incraeased in skin tissues derived from patients with venous ulcers [3]. HIF-1α, which is important for adaptive cellular responses to hypoxia, is known to interact with over 100 genes [4]. The cellular hypoxia response pathway is upregulated in response to HIF-1α stabilization, resulting in changes to gene expression programs involved in cellular proliferation, differentiation, apoptosis, angiogenesis, and energy metabolism [5].

To date, few studies have examined the effect of hypoxia on HDMECs, although we previously reported the use of $CoCl_2$ to model hypoxic conditions in these cells [6]. In the present study, we aimed to investigate the effect of hypoxia on angiogenesis, apoptosis, and cellular proliferation using HDMECs. Additionally, we examined the *in vitro* expression of hypoxia-related proteins, including vascular endothelial growth factor (VEGF), inducible nitric oxide synthase (iNOS), caspase-3, bcl-2-associated X protein (Bax), and proliferating cell nuclear antigen (PCNA), in order to elucidate the molecular mechanisms underlying the observed biological effects.

Materials and methods

Reagents and specialist equipment

$CoCl_2$ (Cobalt (II) chloride hexahydrate; Cat No. C8661) was purchased from Sigma-Aldrich (Saint Louis, USA). TRIzol reagent, Taq DNA polymerase, AMV reverse transcriptase, and

PCR primers were purchased from TaKaRa (Shiga, Japan). Anti-HIF-1α antibody (Cat No. 20960-1-AP), anti-VEGF antibody (Cat No. 19003-1-AP), anti-iNOS antibody (Cat No. 18985-1-AP), anti-caspase-3 antibody (Cat No. 19677-1-AP), anti-Bax antibody (Cat No. 505992-1-AP), and anti-PCNA antibody (Cat No. 10205-2-AP) were purchased from Proteintech (Chicago IL, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L; Cat No. BS13278) was purchased from Bioworld (Minneapolis, USA). All reagents and chemicals, including standard laboratory chemicals, were of analytical grade.

Specialist equipment used in this study included a forma series II water-jacketed CO_2 incubator (Thermo Fisher, USA), an Eclipse TE-2000-U inverted microscope (Nikon, Japan), Z233 MK-2 high-speed refrigerated centrifuges (HEMNLE, Germany), and both a thermocycler (T100 Thermal Cycler)and a ChemiDoc XRS + imaging system from Bio-Rad (Hercules, CA, USA).

Cell culture

HDMECs were purchased from Beijing Dingsheng Corporation (China), were cultured in RPMI 1640 medium (Gibco, USA) containing 10% neonatal calf serum using standard culture protocols, and were maintained at 37°C in a humidified atmosphere containing 5% CO_2. $CoCl_2$ was added to RPMI 1640 medium before use, where appropriate.

Cell viability assay

HDMECs were seeded at a density of 5×10^3 cells/well in 96-well culture plates, then pre-incubated at 37°C and 5% CO_2 for 24 h. The culture medium was then replaced with fresh RPMI 1640 medium containing between 0 and 600 μmol/L $CoCl_2$. Control wells containing either untreated cells or no cells ('Blank') were included. After 12 h, cell viability was assessed using Cell Counting Kit-8 reagent (Sigma, USA), according to the manufacturer's instructions. Briefly, cells were washed three times with phosphate-buffered saline (PBS), then 10 μl of CCK-8 reagent was added per well. Cells were incubated for 1 h, then the absorbance at 450 nm was measured for each well using a microplate reader (Bio-Rad, USA). The resulting optical density (O.D.) values were plotted.

Wound healing assay

A wound healing assay was performed to measure HDMEC migration in both hypoxic and normal conditions. Firstly, HDMECs were seeded at a density of 2×10^5 cells/well in a gridded 6-well culture plate, then cultured in complete medium for 48 h under the conditions described above. Once the cells had adhered to the plate, a vertical line was scored across each well, scraping off cells, using a 10 μl aseptic suction head and the existing gridlines. Next, the culture medium was removed, the cells were washed 3 times in PBS, and 3 ml RPMI 1640 without 10% neonatal calf serum was added per well. Two-hundred μmol/L $CoCl_2$ was added to the hypoxia group to induce hypoxia, and cells were cultured at 37°C with 5% CO_2. Images were captured at 200 × magnification using an inverted microscope at 0 h, 12 h, and 24 h after infliction of the wound. The relative wound width under the various conditions and at the measured time points was calculated using ImageJ software.

Cell cycle and apoptosis assays

HDMECs were cultured in 25 cm^2 bottles in the presence or absence of 200 μmol/L of hypoxia-inducing $CoCl_2$. Cells were harvested after 12 h or 24 h, medium was removed, and cells were washed three times in PBS. Next, cells were dissociated from the culture bottle by incubating with 1 ml 0.5% trypsin for 1 min at 37°C and 5% CO_2, then 1 ml of complete medium was added to inactivate the trypsin. Cells were then pelleted by centrifuging for 5 min at 800 rpm, and either resuspended in 1 ml 75% ethanol and stored at -20°C for cell cycle analysis, or resuspended in 1 ml PBS for apoptosis analysis. All analysis was performed by Flow Cytometry Center of Chongqing Medical University.

Reverse transcription polymerase chain reaction (RT-PCR)

HDMECs in the logarithmic growth phase were cultured in 25 cm^2 bottles for 24 h under the conditions described above, and were divided into three treatment groups: group 1 were the untreated, non-hypoxic control group; group 2 were treated with 200 μmol/L $CoCl_2$ for 12 h in order to induce hypoxia; group 3 were treated with 200 μmol/L $CoCl_2$ for 24 h in order to

Table 1. Primer sequences for RT-PCR

Gene	Sequence (5'-3')	Size
HIF-1α-F	CTGCACAGGCCACATTCACG	20
HIF-1α-R	GGTTCACAAATCAGCACCAAGC	22
HIF-1β-F	TCTGGAAACTCTGGACCTGGAA	22
HIF-1β-R	GGCAAACCGCTCCTTATCGT	20
VEGF-F	GAGGGCAGAATCATCACGAAGT	22
VEGF-R	GCACACAGGATGGCTTGAAGA	20
iNOS-F	AATTGAATGAGGAGCAGGTCGA	22
iNOS-R	CCTGTCCTTCTTCGCCTCGTA	20

induce hypoxia. Following treatment, total RNA was extracted from cells using TRIzol reagent according to the manufacturer's instructions and verified the quality by OD ratio, and cDNA was synthesized using AMV reverse transcriptase according to the manufacturer's protocol. To perform PCR reactions, 0.8 μl cDNA template, 0.4 μl each of appropriate forward and reverse primers (listed in **Table 1**), 5 μl SYBR Premix Ex Taq master mix, and 3.4 μl nuclease-free water were mixed, giving a total volume of 10 μl in 96-well PCR plates. Each gene/sample combination was examined in triplicate wells. RT-PCR was then performed using an RT-PCR instrument (BS97My Cycler, Bio-Rad, USA) using the following cycling conditions: 95°C for 30 s; 39 cycles of 95°C for 5 s, 60°C for 30 s; 95°C for 15 s; 60°C for 30 s; 95°C for 15 s; end reaction. All data was analyzed by Bio-Rad Manager software(Bio-Rad, USA).

Western blotting analysis

HDMECs in the logarithmic growth phase were cultured in 25 cm^2 bottles for 48 h under the conditions described above, and were divided into three groups, as described for the RT-PCR experiments. Following treatment, culture medium was removed and cells were washed twice with PBS before being lysed with 50 μl RIPA lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA). Protein quantification was performed using bicinchoninic acid (BCA) protein assay reagent according to the manufacturer's protocol, then equal amounts of protein per sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. For western blotting, membranes were blocked with blocking buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% BSA, 0.1% (v/v) Tween-20) at room temperature for at least 1 h. Membranes were then washed three times for 5 min with TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween-20), before being incubated overnight at 4°C with either anti-HIF-α antibody (1:500 dilution), anti-VEGF antibody (1:1000 dilution), anti-iNOS antibody (1:500 dilution), anti-caspase-3 antibody (1:1000 dilution), anti-Bax antibody (1:2000 dilution), or anti-PCNA antibody (1:2000 dilution). After primary antibody staining, membranes were washed intensively four times for 5 min with TBST, then incubated with secondary antibody (1:5000 dilution) for 1.5 h. Membranes were intensively washed a further four times for 5 mins with TBST, before proteins were detected by chemiluminescence (Beyotime Biotechnology, China).

Statistical analysis

All statistical analyses were performed using SPSS version 18.0 software (IBM, USA) for Windows. Differences in wound healing ability under the various conditions were assessed using an unpaired Student's t-test. All others differences were assessed using one-way ANOVA. Statistical significance was defined as $p<0.05$, and all values are expressed as the mean ± standard deviation of three independent experiments.

Results

Determination of the optimal $CoCl_2$ concentration

$CoCl_2$, as well as promoting hypoxia, is toxic to cells. It is therefore critical that the $CoCl_2$ concentration is carefully controlled to maximize cell viability during the induction of hypoxia. Thus, we examined HDMEC viability at a range of $CoCl_2$ concentrations by performing CCK-8 cell viability assays, as described in Materials and Methods. As expected, cell viability decreased in a non-linear dose-dependent manner with increasing $CoCl_2$ concentration (**Figure 1**). Cell survival at 200 μmol/L $CoCl_2$ was 65.10%, which was deemed an acceptable level of loss, and all subsequent

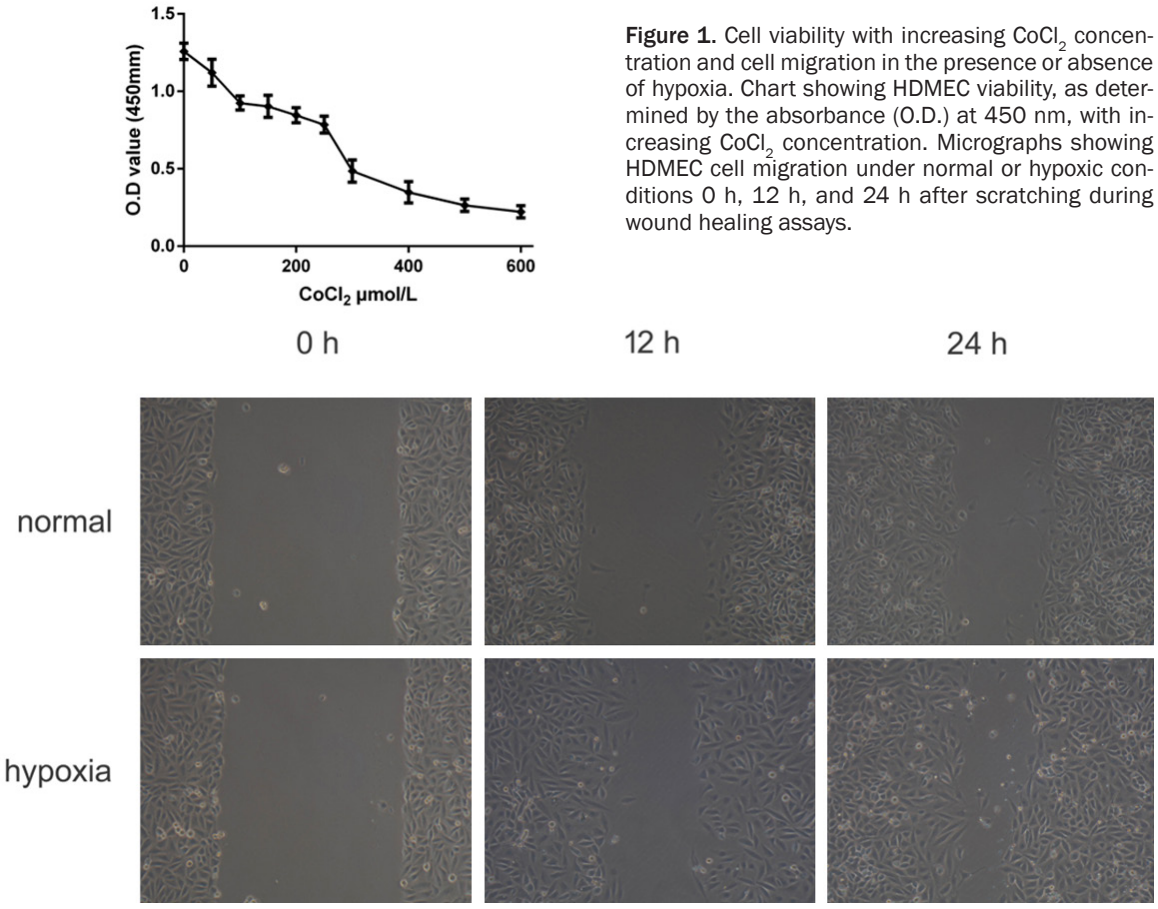

Figure 1. Cell viability with increasing $CoCl_2$ concentration and cell migration in the presence or absence of hypoxia. Chart showing HDMEC viability, as determined by the absorbance (O.D.) at 450 nm, with increasing $CoCl_2$ concentration. Micrographs showing HDMEC cell migration under normal or hypoxic conditions 0 h, 12 h, and 24 h after scratching during wound healing assays.

experiments were performed with this concentration of $CoCl_2$.

Hypoxia enhances HDMEC migration

To study the effect of hypoxia on cellular migration, a wound healing assay on treated or untreated cells was performed as described in Materials and Methods. As seen in **Figure 1**, treatment with 200 μmol/L $CoCl_2$ significantly increased the distance travelled by HDMECs, by an average of 172% and 124% after 12 h and 24 h, respectively. No significant differences between the groups were seen at 0 h. Such migration is involved in the initial steps of angiogenesis, suggesting that the HDMEC movement that is activated in hypoxic conditions may be involved in the formation of new blood vessels.

Hypoxia induces apoptosis and impairs cell cycle progression in HDMECs

The effect of hypoxia on both apoptosis and cell cycle progression in HDMECs was assessed after 12 h and 24 h using flow cytometric analysis, as described in Materials and Methods. Apoptosis, shown as the proportion of the cell population that were apoptotic, was significantly increased as hypoxia progressed, with the baseline level at 0 h of 7.64±2.12% increasing to 21.43±2.3% and 19.98±3.45% after 12 h and 24 h of hypoxia, respectively (**Figure 2**). There was statistcally significant between 0 h and 12 h or 24 h, interestingly, there was no significant difference in the proportion of apoptotic cells between 12 h and 24 h, suggesting that this effect had reached a plateau after 12 h.

Considering cell cycle progression, the S phase fraction of the total population was 39.52 +2.12% in the baseline controls at 0 h, but reduced to 36.78+2.01% and 29.86±1.93% after 12 h and 24 h of hypoxia, respectively (**Figure 3**). The differences between 0 h and both 12 h and 24 h were statistically significant, as was the difference between the 12 h

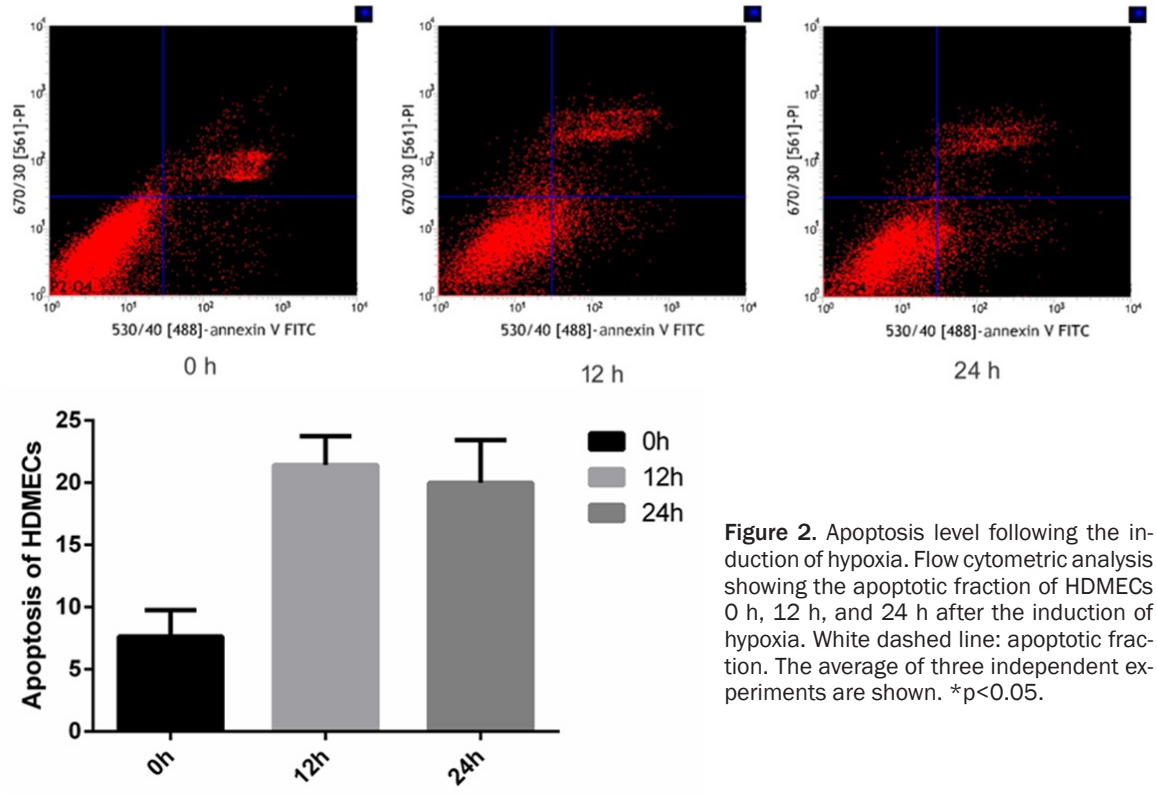

Figure 2. Apoptosis level following the induction of hypoxia. Flow cytometric analysis showing the apoptotic fraction of HDMECs 0 h, 12 h, and 24 h after the induction of hypoxia. White dashed line: apoptotic fraction. The average of three independent experiments are shown. *p<0.05.

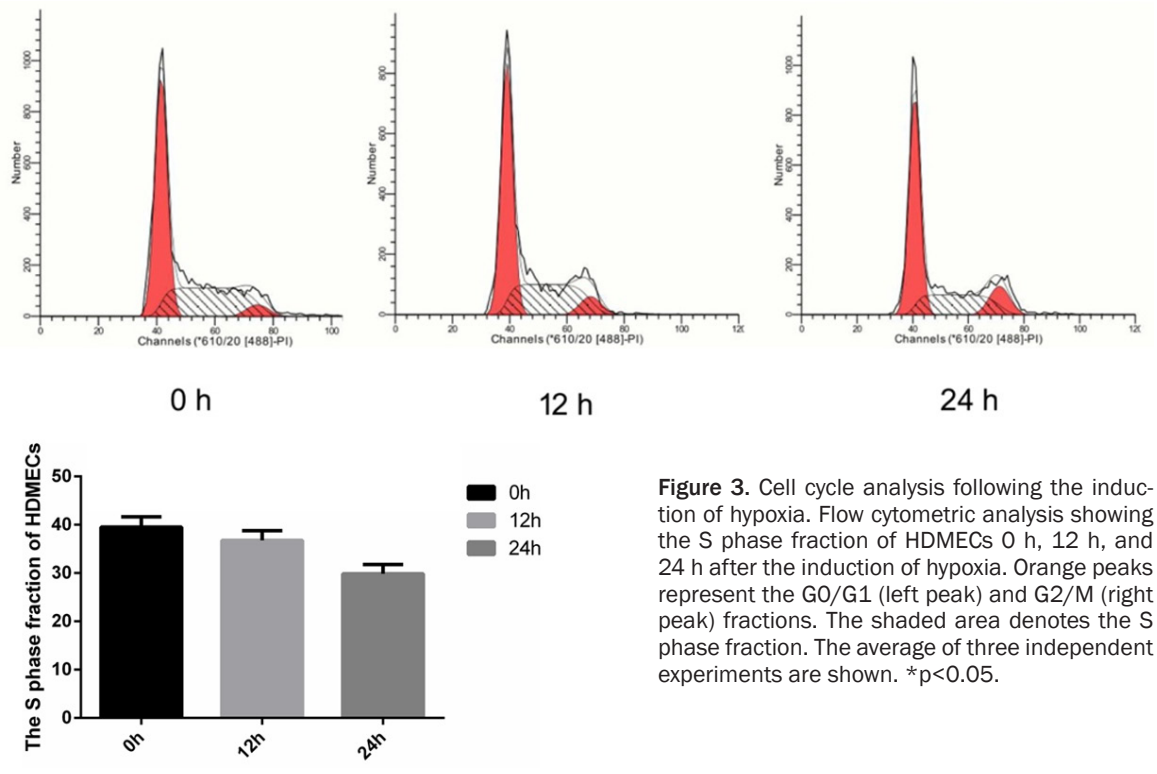

Figure 3. Cell cycle analysis following the induction of hypoxia. Flow cytometric analysis showing the S phase fraction of HDMECs 0 h, 12 h, and 24 h after the induction of hypoxia. Orange peaks represent the G0/G1 (left peak) and G2/M (right peak) fractions. The shaded area denotes the S phase fraction. The average of three independent experiments are shown. *p<0.05.

and 24 h samples. This finding suggests that hypoxia significantly impairs cell cycle progression, which would be expected to lead to a concomitant reduction in cellular proliferation.

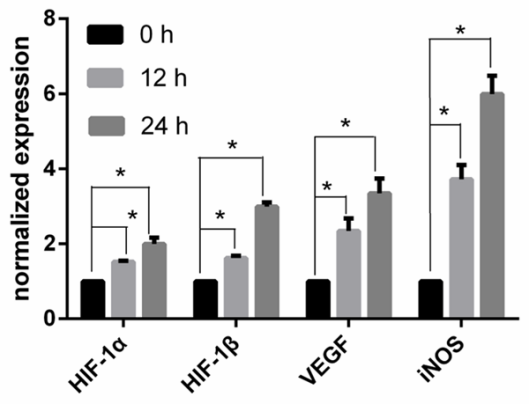

Figure 4. Gene expression analysis following the induction of hypoxia. Chart showing the relative gene expression of *HIF-1α*, *HIF-1β*, *VEGF*, and *iNOS*, normalized to β-action, 0 h, 12 h, and 24 h after the induction of hypoxia. *p<0.05.

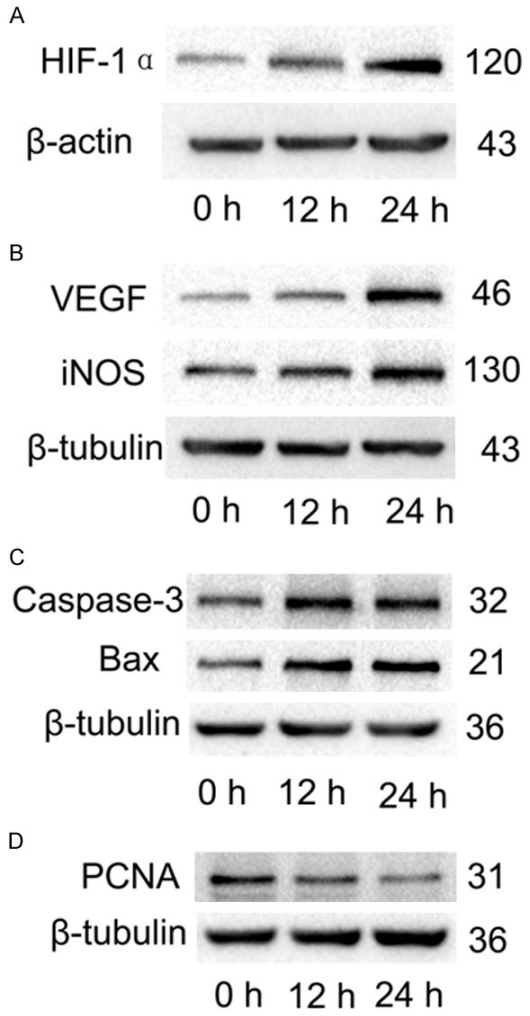

Figure 5. Western blot data showing protein expression after the induction of hypoxia. Representative western blot images showing expression of HIF-1α (A), VEGF and iNOS (B), caspase-3 and Bax (C), and PCNA (D) in either untreated HDMECs (0 h) or HDMECs undergoing hypoxia for either 12 h or 24 h. β-actin and β-tubulin control expression is also shown. Numbers to the right of the image denote the molecular weight of the proteins in kDa.

Hypoxia increases mRNA expression of angiogenesis-related genes

To further examine the effect of hypoxia on angiogenesis, RT-PCR analysis was performed to examine changes in the gene expression of *HIF-1α*, *HIF-1β*, *VEGF* and *iNOS* in HDMECs that were either untreated (0 h) or treated with 200 μmol/L $CoCl_2$ for 12 h or 24 h. The relative expression of each gene, normalized to that of the *β-action*, are presented in **Figure 4**. The expression of all of the genes under consideration progressively increased in a time-dependent manner following the induction of hypoxia. In all cases, the increases in expression between 0 h and 12 h, and between 12 h and 24 h, were statistically significant.

The effect of hypoxia on protein expression

The effect of hypoxia on angiogenesis, apoptosis, and cellular proliferation in HDMECs was investigated further by measuring protein expression using Western blot analysis. The proteins investigated were the angiogenesis-related factors HIF-1α, VEGF, and iNOS, the apoptosis-related factors caspase-3 and Bax, and the cellular proliferation marker PCNA. As above, HDMECs were either untreated, or treated with 200 μmol/L $CoCl_2$ for 12 h or 24 h, as indicated. Representative Western blots and expression data, shown relative to β-actin or β-tubulin control expression, are shown in **Figures 5** and **6**, respectively. The expression of the angiogenesis-related proteins HIF-1α, VEGF, and iNOS increased progressively over time, with significant increases in expression observed between 0 h and 12 h, and between 0 h and 24 h (**Figure 6A-C**). Similarly, protein expression of the apoptosis-related proteins caspase-3 and Bax was significantly higher after 12 h or 24 h of hypoxia when compared to the untreated control (0 h), although there was no significant difference between 12 h and 24 h, indicating that expression had plateaued by the earlier time point (**Figure 6D**, **6E**). Interestingly, protein expression of the proliferation marker PCNA decreased steadily after

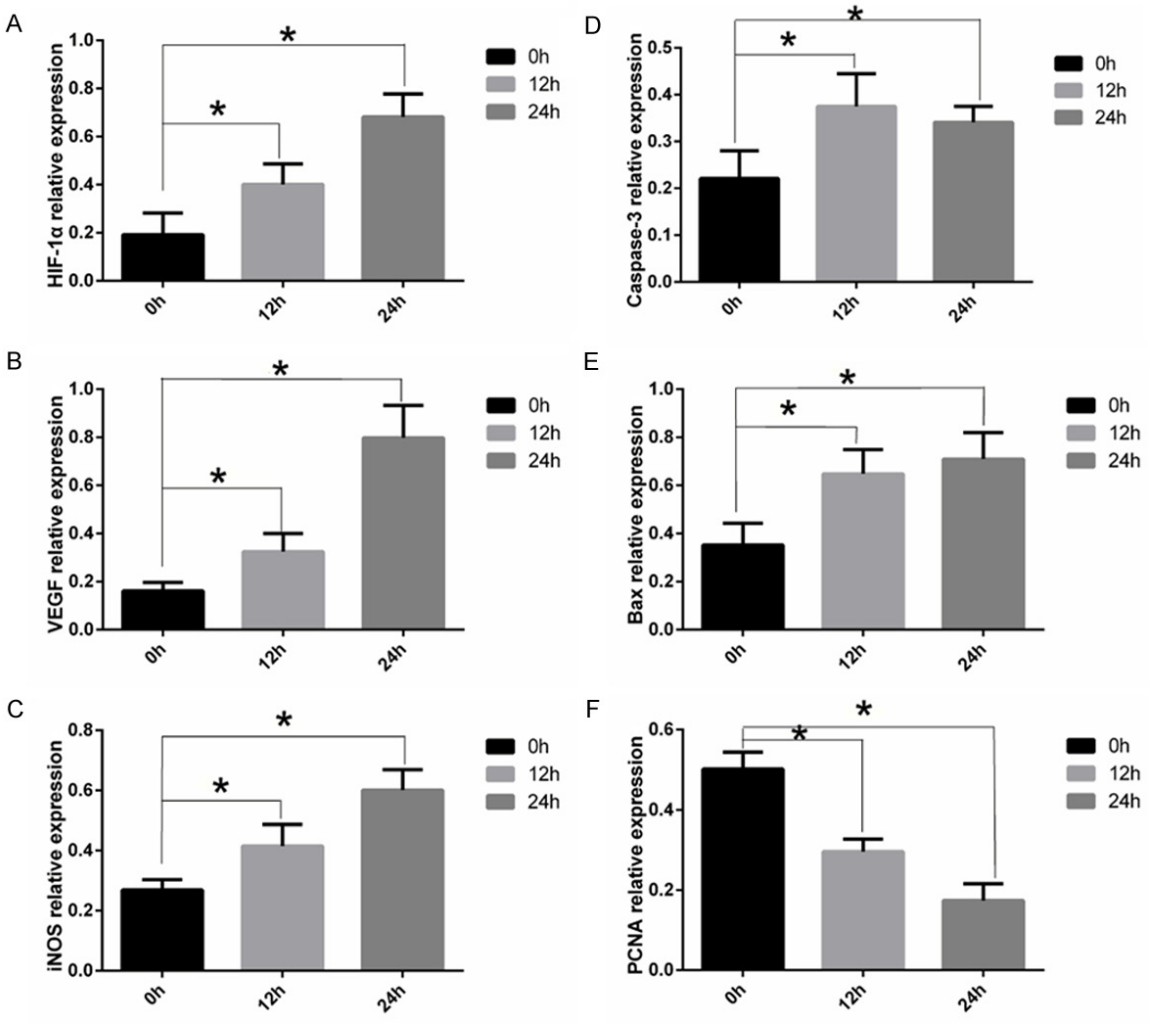

Figure 6. Relative protein expression following the induction of hypoxia. Summary data showing the protein expression of HIF-1α (A), VEGF (B), iNOS (C), caspase-3 (D), Bax (E), and PCNA (F), relative to that of either β-actin or β-tubulin control, as noted in **Figure 6**. HDMECs were either untreated (0 h) or underwent hypoxia for 12 h or 24 h, as indicated. The average of three independent experiments are shown. *p<0.05.

the induction of hypoxia, with expression after both 12 h and 24 h being significantly lower than at 0 h (**Figure 6F**). These results are consistent with those seen in the wound healing assay and with flow cytometry.

Discussion

Presently, venous ulceration is a very common condition that is often difficult to treat. Venous ulcers are chronic wounds that are associated with ambulatory venous hypertension of the lower extremities; they occur either as a consequence of vein reflux or in conjunction with venous obstruction, and are often a manifestation of severe chronic venous insufficiency (CVI). In the USA, 10-35% of adults suffer from some form of CVI, and venous ulcers affect 4% of people over 65 years of age [7]. The exact mechanisms underlying the pathophysiological transition from chronic venous disease to venous ulcers are currently poorly understood, and the factors contributing to this disease have not been absolutely defined [8].

Several cellular mechanisms link dermal tissue hypoxia with the progression of ulceration. Tissue hypoxia is known to activate leukocytes, which then adhere to the venous endothelium and migrate into and through the vein wall. Such interactions alter the shear stress on the endothelial cells, causing them to release vaso-

active agents, inflammatory mediators, chemokines, adhesion molecules, and prothrombotic precursors [9]. Furthermore, clinical studies have indicated that dermal tissue hypoxia may cause venous ulceration [10, 11], and hypoxia reportedly injured skin tissue during oxidative stress and inflammation [12]. Hypoxia has also been shown to induce wide-spread alterations in gene expression, with signals relayed via venous endothelial cell membrane oxygen receptors triggering a complex array of gene up-regulation and down-regulation [13]. Successful wound healing depends upon the efficient restoration of the blood supply, and increased oxygen consumption within a wound, leading to a hypoxic microenvironment, is believed to be the primary promoter of angiogenesis in this context [14].

In the present study, we investigated the effect of hypoxia on angiogenesis in HDMECs using an *in vitro* $CoCl_2$-induced severe hypoxia model. Currently, there are two principal methods of building a hypoxia model in common usage: a physical method involving the use of a CO_2-N_2 cell incubator, and the chemical method used here. The disadvantage of the physical method is that cells must be removed from this environment during medium changes and cellular passage, meaning that the hypoxic state cannot be maintained continuously. HIF-1α is unstable in a normoxic environment and will degrade within 5 minutes, meaning any expression could easily be lost within the time taken to passage the cells. By contrast, $CoCl_2$ reproduces the hypoxic condition through Co^{2+} replacing the Fe^{2+} in hemoglobin molecules, leading to disruption of cellular oxygen receptors [15], and *in vitro* $CoCl_2$-induced hypoxia is the method most commonly used. We therefore chose to use the $CoCl_2$ method to build our hypoxia model, and performed experiments to determine the appropriate dosage. We found that there was no straight-forward linear relationship between $CoCl_2$ dose and HDMEC survival, and while 200 μmol/L gave an acceptable survival rate of 65.10%, this decreased sharply at concentrations above 250 μmol/L.

HIF-1 is a heterodimer comprising an oxygen-dependent α-subunit and an oxygen-independent β-subunit. There are three HIF-α isoforms, HIF-1α, HIF-2α, and HIF-3α [16], and two isoforms of HIF-β (also known as aryl hydrocarbon receptor nuclear translocator or ARNT), namely HIF-1β and HIF-2β [17]. Among these HIFs, the most important is HIF-1α, which is responsible for the hypoxia-mediated activation of transcription. Additionally, HIF-1α is important in venous endothelial cell biology and angiogenesis; loss of HIF-1α prevents angiogenesis-related behaviors, including migration, wound healing, and chemotaxis, in venous endothelial cells [18]. During both pathophysiological and physiological angiogenesis, the HIF-1α pathway is a principal regulator of vascular formation, triggering the up-regulation of proangiogenic factors such as VEGF and iNOS in response to hypoxia [19, 20]. Once up-regulated, the primary function of these factors is to respond to the hypoxic conditions by increasing vasopermeability and angiogenesis. Consistent with these reports, we found that hypoxia induced the overexpression of HIF-1α in HDMECs, with corresponding increases in VEGF and iNOS expression at both the mRNA and protein levels. This result also serves to verify our hypoxia-inducing model.

In addition to increasing vasopermeability, VEGF, a 35 kDa heparin-binding glycoprotein and critical mediator, acts to regulate angiogenesis by enhancing cellular migration. The biological effects of VEGF on endothelial cells are exerted through the binding of VEGF-1 and VEGF-2 to tyrosine kinase receptors, which are expressed predominantly on endothelial cells. Through these receptors, VEGF then activates the mitogen-activated protein kinase pathway, whose principal components include ERK, p38, JNK, and BMK1 [21]. Previous studies have indicated that the microenvironment within a skin wound was hypoxic because of an increased tissue demand for oxygen, and that this greatly increased angiogenic activity [14, 22]. In the present study, we confirmed that VEGF expression was increased in hypoxic conditions at both the mRNA and protein level, and that cellular migration was increased.

Endothelial cell dysfunction affects angiogenesis through many different mechanisms; oxidative stress, inflammation, and autophagy all affect endothelial cell activation, apoptosis, and migration. Oxidants and oxygen radical formation, critical factors in the cellular response to post-hypoxic injury, are reportedly involved in

these processes [23]. One of the most important free radical signaling molecules, nitric oxide (NO), plays diverse physiological and pathological roles. Endogenous NO, synthesized and secreted by endothelial cells, is a vasoactive substance which can relax vascular smooth muscle, expand vessels, and improve microcirculation of the blood supply. Furthermore, endogenous NO promotes angiogenesis and blood vessel regeneration by stimulating leucocytes to secrete vascular active substances, which regulate vascular endothelial growth. However, NO is extremely unstable *in vivo* and degrades easily [24]. iNOS, which is maintained at a constant level *in vivo*, is the principal NO synthase during hypoxia and ischemia, and can thus induce the stable release of NO in these scenarios. Previously, iNOS expression was shown to be up-regulated in hypoxic rat microglial cells via the phosphoinositol 3-kinase pathway [25]. We found that HDMEC migration was increased under hypoxic conditions, with corresponding increases in the gene and protein expression of iNOS. This suggests that both VEGF and iNOS may be critical in the regulation of cellular responses to hypoxic injury.

The effects of hypoxia on HDMECs are complex. We found that the cellular apoptosis rate was increased during hypoxia, and, consistent with this, the expression of the apoptosis-related proteins caspase-3 and Bax also increased following treatment. Caspase-3, as we known, played a siginificant role in cell apoptosis process. Inapproproate activation of the executioner caspase-3 is prevented by their production as inactive procaspase dimers that must be cleaved by initiator caspases. This cleavage between the large and small subunits allows a comformational change that brings the two active sites of yje executioner caspases dimer together and creates a funtional mature protease. Once activated, a single executioner caspase can cleave and activate other executioner caspases, leading to an accelerated feedback loop of caspase actvation. It means the whole quanitity of caspase-3 increased although a part of caspase-3 cleaved [26]. According to previous reports, activation of the JNK signaling cascade and expression of Bax and caspase-3 leads to apoptosis during hypoxia [27]. Results of our study suggested that hypoxia increased apoptosis of HDMECs through the high expression of Bax and caspase-3.

Furthermore, we found that the cell cycle is prolonged under hypoxic conditions, and the expression of the cellular proliferation marker PCNA was decreased accordingly. PCNA is a kind of intranuclear protein, which is an assistant protein of DNase. It has no specificity of species, genus and tissue, expressing in phages G1 and S, so it has been widely used to mark cells of S phage. In this point PCNA is a perfect marker to evaluate cell proliferation [28]. And it has been used as a routing method to test proliferation cells at present. It was also demonstrated that the change of PCNA expression increased DNA duplication and cell proliferation. Our results suggested that hypoxia decreased proliferation of HDMECs.

From these results, we infer that these biological changes may augment the effects of hypoxia in the immediate microenvironment, especially in areas of extensive tissue damage.

In summary, the response of HDMECs to hypoxia is complex, with hypoxic conditions conferring some advantages, such as enhanced migration ability, and some disadvantages, such as increased apoptosis and a prolonged cell cycle. Such biological responses would be expected to make the immediate microenvironment less hospitable. However, whether HIF-1α, or indeed hypoxia *perse*, directly influence these biological responses in HDMECs remains to be confirmed. This will be investigated further in future experiments involving the silencing of HIF-1α expression using RNA interference methods. The pathogeny of venous ulceration is correlated with peripheral varicose veins, hypostasis, alogotrophy, and inflammation. Further study is required to elucidate which factor is most important in ulceration, and the specific mechanisms by which this occurs.

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Disclosure of conflict of interest

None.

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