

## Original Article

# Effects of cobalt chloride on phenotypes of normal human saphenous vein smooth muscle cells

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Received October 3, 2014; Accepted December 8, 2014; Epub December 15, 2014; Published December 30, 2014

**Abstract:** To explore the cellular adaptations and responses to hypoxia in normal human saphenous vein smooth muscle cells (SMCs) and presume what roles phenotypic modulation of normal human saphenous vein SMCs would play in varicose vein of lower extremity, we used cobalt chloride ( $\text{CoCl}_2$ ), a hypoxia mimetic, to treat normal human saphenous vein SMCs in vitro. The proliferating ability of cells exposed to serial dilutions of  $\text{CoCl}_2$  (0, 200, 300, 400 and 500  $\mu\text{M}$ ) at 24 h, 48 h and 72 h respectively was detected by MTT assay. Wound healing assay was used to observe the migrating ability of cells under  $\text{CoCl}_2$  (200  $\mu\text{M}$ ) treatment for 8 days continuously. Hoechst 33258 stain was used to determine whether hypoxia induced by  $\text{CoCl}_2$  could cause apoptosis of normal human saphenous vein SMCs. We found that  $\text{CoCl}_2$  enhanced the proliferation and inhibited the migration of normal human saphenous vein SMCs. The apparent morphous of normal human saphenous vein SMCs under chronic  $\text{CoCl}_2$  treatment was significantly changed compared to no  $\text{CoCl}_2$  treated control, but this process did not relate to cell apoptosis. To conclude, our results support the concept that the phenotypes of normal human saphenous vein SMCs could be influenced by hypoxia stimulus. Cellular structural and functional changes under chronic hypoxia in normal human saphenous vein SMCs might play important roles in the development of varicose veins of lower extremity.

**Keywords:** Varicose veins, vascular smooth muscle cells, hypoxia, cobalt chloride, phenotype

## Introduction

The development of primary varicose veins (VVs) is the most common disease of peripheral veins and affects 10% to 40% of individuals between the ages of 30 and 70 years [1]. The mechanisms underlying the pathogenesis of VVs remain unclear [2]. Vein wall changes as the primary events of VVs were supported by duplex ultrasonography [3]. The dilatation of vein wall is controlled by vascular smooth muscle cells (VSMCs), collagen and elastin. Defects of these components could lead to loss of tone of vein wall [4]. VSMCs as the important component of blood vessels, function to contract or relax vessel, to regulate blood pressure and distribute blood flow. VSMCs display striking plasticity and can undergo phenotype switch, dedifferentiating from a quiescent contractile state to a highly migratory synthetic state, in response to vascular injury or various disease states [5, 6]. The underlying hypothesis is that the formation of VVs is secondary to defects in cellular and extracellular matrix (ECM) components, causing weakness and altered venous tone.

Several stresses including hypoxia, mechanical stretch and low shear stress, have been postulated to contribute to vein wall changes [7, 8]. Hypoxia is critical in many pathological conditions. Cells respond to hypoxia by activation of the hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ), a transcription factor that modulates the expression of genes involved in angiogenesis, survival, metabolism and cell migration. In the normoxic state the HIF-1 $\alpha$  protein is hydroxylated, ubiquitinated and degraded in the proteasome. In the hypoxic state the activity of specific hydroxylase is quenched and HIF-1 $\alpha$  is stabilized [9]. Ferrous iron is an important cofactor in the enzyme activity of hydroxylase. It is well known that cobalt chloride ( $\text{CoCl}_2$ ) can mimic hypoxia through replacing iron in hydroxylase and promote the stability of HIF-1 $\alpha$  by inhibiting its hydroxylation [10].

Venous hypoxia as an etiological factor of VVs is poorly studied [11]. Although Chung et al [12] have demonstrated that an increased activation of HIF pathway in VVs compared with non-VVs using organ culture, the effects of hypoxia

on the phenotypes of VSMCs from normal human saphenous veins at cellular level have not been reported. We hypothesized that hypoxia would change the structure and function of VSMCs from normal human saphenous veins and promote the pathogenesis of VVs. We designed to culture normal human saphenous vein SMCs in vitro, use  $\text{CoCl}_2$  treatment to cause chemical hypoxia environment and observe the phenotypic changes of normal human saphenous vein SMCs in this condition. We wanted to give a cue for the etiology of VVs from the point of view of hypoxia induction at cellular level.

### Materials and methods

#### *Specimen*

Normal human saphenous veins were obtained from patients undergoing coronary bypass surgery or limb amputation. All patients were under pre-operative evaluation of the absence of varicose abnormalities or retrograde flow by echo-Doppler studies. All patients provided informed consents prior to the procedure and the study was approved by the ethics committee of the 89 hospital of PLA. The investigation conformed to the principles outlined in the Declaration of Helsinki.

#### *Cell culture*

Explants from the medial layer of saphenous veins were prepared as previously described [13]. After cutting the vessel into 2×2 mm explants, we placed the explants with the luminal side down in a T 25 flask. Cells were grown in  $\alpha$ -MEM (Gibco®, Life technologies™, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco®, Life technologies™, NY, USA), 105 U/L penicillin and 100  $\mu\text{g}/\text{mL}$  streptomycin (Solarbio Science & Technology Co., Beijing, China). Incubation conditions were: 37°C, 5%  $\text{CO}_2$ . The culture medium was changed every 2 days. Cell growth began within 7 to 14 days. After attaining confluence, cells were trypsinized and subcultured. Cells from passage 4 to 5 were used for experiments.

#### *Immunohistochemical analysis*

All procedures were under room temperature. Every operation was followed by phosphate-buffered saline (PBS) rinsing for 3 times. Monolayer cells grown in normoxic culture were

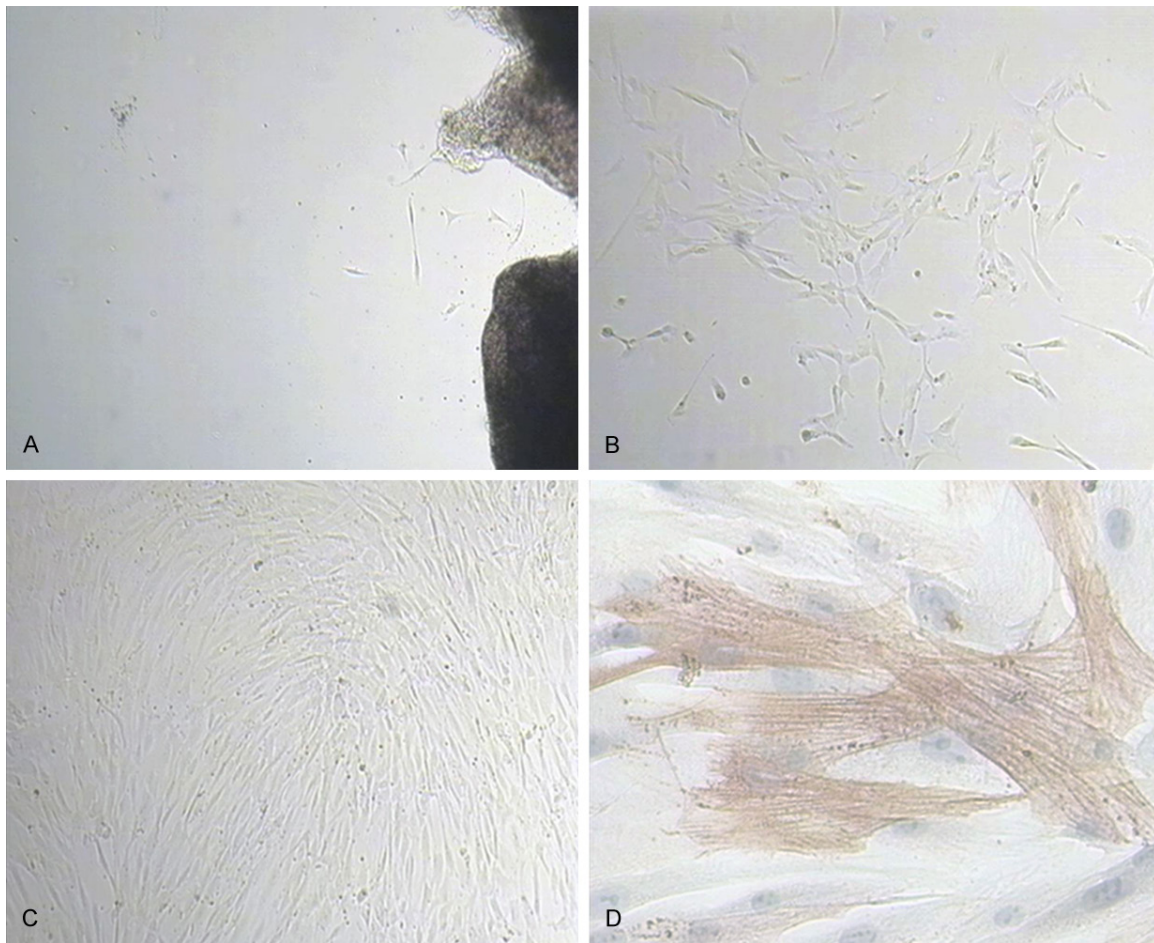
fixed with 4% paraformaldehyde (Solarbio Science & Technology Co., Beijing, China) for 30 min. 0.3% Triton X-100 (MaiXin Biology Co., Fuzhou, China) was added to fixed cells for 10 min to enhance the permeability. 3%  $\text{H}_2\text{O}_2$  was added to block endogenous peroxidase for 10 min. After incubating with mouse to human  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) monoclonal antibody (MaiXin Biology Co., Fuzhou, China) for 1 h, goat to mouse peroxidase-labeled secondary antibody work solution (MaiXin Biology Co., Fuzhou, China) was loaded for 15 min. Then freshly prepared DAB chromogenic agent (MaiXin Biology Co., Fuzhou, China) was added for 5 min to reveal peroxidase activity. Finally, sections were counterstained with hematoxylin. The negative control was treated similarly as above except for PBS instead of  $\alpha$ -SMA antibody.

#### *Cell proliferation*

$10^4$  cells per well were plated in 96-well plates. After cultured for 24 h, cells were subjected to exposure to serial dilutions of  $\text{CoCl}_2$  (0, 200, 300, 400 and 500  $\mu\text{M}$ ).  $\text{CoCl}_2$  was solved in ultrapure water as 40 mM stock concentration and stored at 4°C after autoclaving.  $\text{CoCl}_2$  work solution was diluted with serum free  $\alpha$ -MEM. The viabilities of cells at 24 h, 48 h and 72 h were evaluated using methyl thiazolyl tetrazolium (MTT) quantitative colorimetric assay (Solarbio Science & Technology Co., Beijing, China). Cells absent of  $\text{CoCl}_2$  were used as controls. The absorbance of the converted purple formazan crystals from the yellow solution was measured at 492 nm. Cell survival ratio was calculated by the equation: relative survival ratio =  $A_{\text{test}}/A_{\text{control}}$ . The experiments were performed 3 times independently.

#### *Wound healing assay*

$2 \times 10^5$  cells per well were plated in 12-well plates and maintained in  $\alpha$ -MEM containing 10% FBS, 105 U/L penicillin and 100  $\mu\text{g}/\text{mL}$  streptomycin until confluence. The monolayers were wounded with a sterile plastic pipette tip to create an acellular line within the well. Cells were washed in PBS and fresh serum free  $\alpha$ -MEM with or without  $\text{CoCl}_2$  (200  $\mu\text{M}$ ) were added. Cells were continuously cultured for 8 days to monitor cells migration. Media was changed every 2 days. Images of wound at the start moment and after the treatment were taken with DMIL Leica inverted microscope.



**Figure 1.** Characteristics of normal human saphenous vein smooth muscle cells. (A) Cell culture was established by an explants technique. At the 5th day, spindle-like morphous cells were migrated from explants. (B) At the 7th day, cells started to form colony. (C) After cultured for 14 days, cell confluence was reached and presented typical “peak and valley” morphous. (D) Immunohistochemical analysis with  $\alpha$ -SMA monoclonal antibody showed that abundant yellowish-brown cytoskeleton fibers, that is smooth muscle actin in the isolated saphenous vascular smooth muscle cells. Magnification in (A-C) is  $\times 40$ . Magnification in (D) is  $\times 200$ .

## Fluorescence staining

$2 \times 10^5$  cells per well were plated in 12-well plates. After cultured for 24 h, cells were exposed to serum free  $\alpha$ -MEM with or without  $\text{CoCl}_2$  (200  $\mu\text{M}$ ). Media was changed every 2 days. After treated for 8 days, cells morphology was observed with DMIL Leica inverted microscope. Pyknosis was evaluated through Hoechst 33258 stain. All procedures were under room temperature. Cells, after washing with PBS, were fixed with 4% paraformaldehyde for 30 min. Stain with Hoechst 33258 (Solarbio Science & Technology Co., Beijing, China) was performed for 5 min. Then cells were washed with PBS and fluorescence was detected with the DMIL Leica fluorescence inverted microscope.

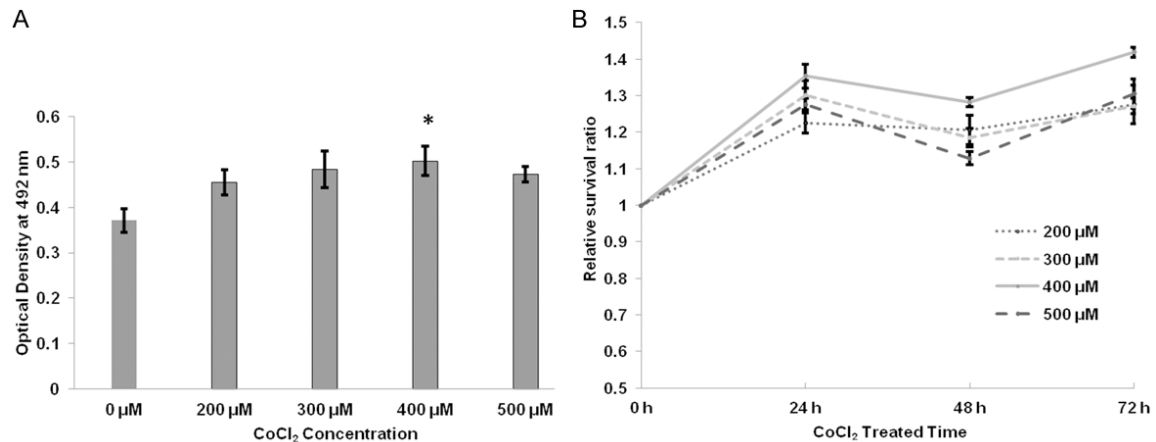
## Statistics

All experiments were repeated three independent times. All statistical analyses were done and graphs were plotted using Excel 2007 software. A two-tailed student t-test was used and  $P < 0.05$  was considered statistically significant.

## Results

### *Characteristics of vascular smooth muscle cells isolated from normal human saphenous veins*

Cell culture was established by an explants technique. At the 5th day, some spindle-like cells migrated from the explants observed by



**Figure 2.** Cobalt chloride enhanced the proliferation of normal human saphenous vein smooth muscle cells in dose- and time-dependent manner detected by MTT assay. A. Cells were exposed to serial dilutions of CoCl<sub>2</sub> (0, 200, 300, 400 and 500 μM). After 24 h, the optical densities at 492 nm of CoCl<sub>2</sub> treated cells were significantly higher than that of no treated cells, especially at 400 μM concentration. B. As CoCl<sub>2</sub> treated time extended, relative survival ratios of CoCl<sub>2</sub> treated cells kept increasing in time-dependent manner. Each column indicated the mean and vertical lines indicated standard deviation (S.D.). \*indicated significant difference compared with control (0 μM) ( $P = 0.017$ ).

light microscopy (**Figure 1A**, ×40 magnification). After removing the explants at the 7th day, cell colonies were observed at the sites of removal explants (**Figure 1B**, ×40 magnifications). When reaching to 80% confluence, cells appeared typical “peak and valley” growth morphous (**Figure 1C**, ×40 magnification). To confirm cell identity as VSMCs, we used α-SMA monoclonal antibody to detect the smooth muscle actin in saphenous vein SMCs by immunohistochemistry analysis. Results showed that there were abundant yellowish-brown cytoskeleton fibers in isolated cells (**Figure 1D**, ×200 magnifications). Therefore, we could use these saphenous vein SMCs in the following experiments.

#### *Cobalt chloride enhanced the proliferation of normal human saphenous vein smooth muscle cells*

We used MTT assay to detect the effect of CoCl<sub>2</sub> on the proliferating ability of normal human saphenous vein SMCs. To eliminate impact of FBS, we used serum free medium as solvent of CoCl<sub>2</sub>. Saphenous vein SMCs were exposed to serial dilutions of CoCl<sub>2</sub> (0, 200, 300, 400 and 500 μM). After treated for 24 h, cells treated by CoCl<sub>2</sub> appeared markedly proliferating state compared to no treated cells under light microscopy (result not shown). Results of MTT assay showed that optical densities at 492 nm of CoCl<sub>2</sub> treated cells were significantly higher than that of no treated cells,

especially at 400 μM concentration (**Figure 2A**). As CoCl<sub>2</sub> treated time extended, relative survival ratios of CoCl<sub>2</sub> treated cells maintained increase in time-dependent manner (**Figure 2B**).

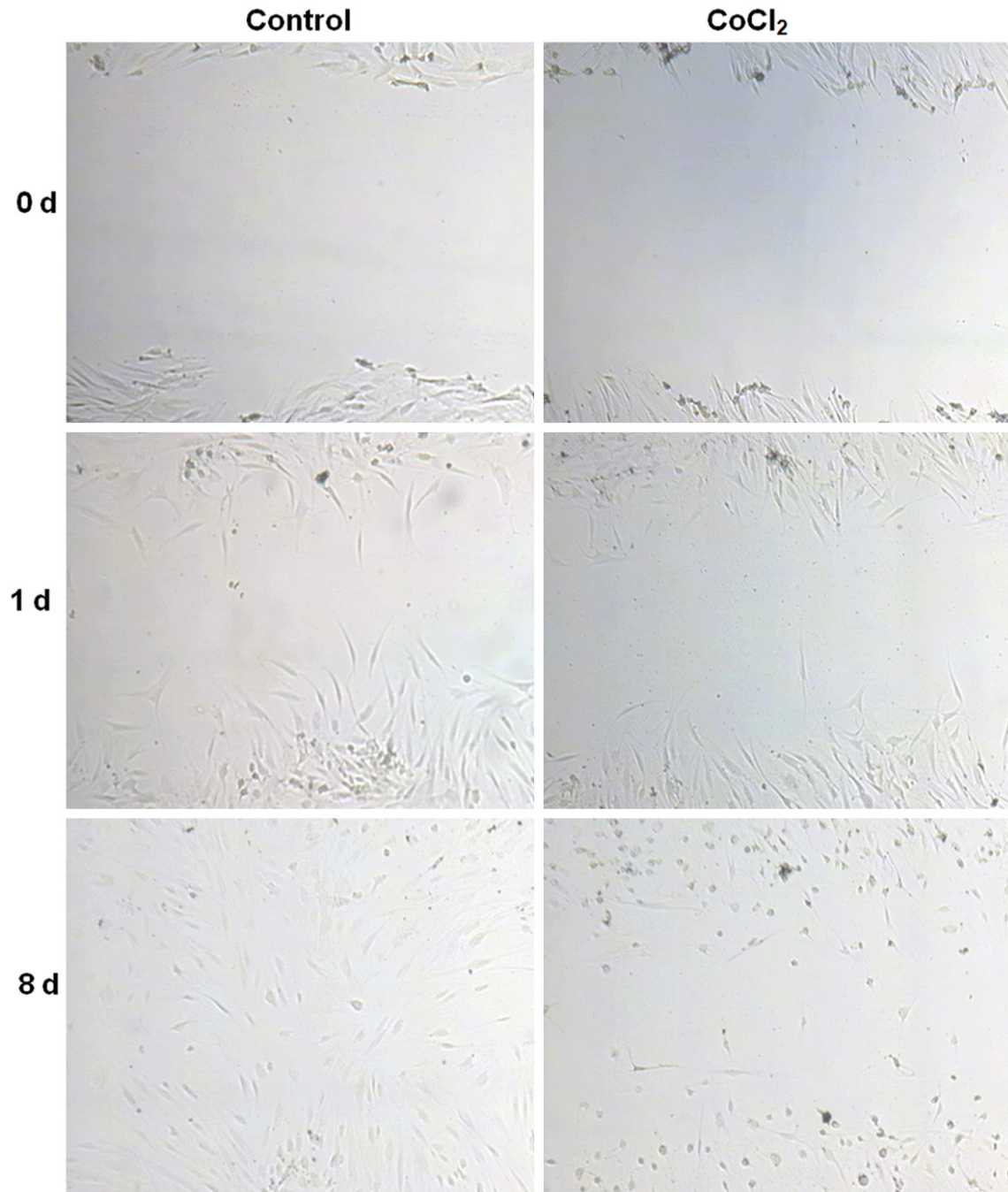
#### *Cobalt chloride inhibited migration of normal human saphenous vein smooth muscle cells*

In wound healing assay, we found that the migration of normal human saphenous vein SMCs treated by CoCl<sub>2</sub> (200 μM) was slower than that of no treated cells. Cells with or without CoCl<sub>2</sub> (control) started to migrate toward acellular area after cultured for 1 d. Cells in the control gradually covered the acellular area as time extended, while cells treated by CoCl<sub>2</sub> migrated more slowly than control. At the 8th day, initial spindle-like cells had covered the acellular area in the control, while fewer cells with depauperate morphous in the acellular area of CoCl<sub>2</sub> treated group (**Figure 3**).

#### *Persistent treatments of cobalt chloride promoted morphous changes of normal human saphenous vein smooth muscle cells*

After treatment with CoCl<sub>2</sub> (200 μM) for 8 days, the macroscopical morphous of normal human saphenous vein SMCs changed markedly. Initial spindle-like cells became more roundish and diacaustic under light microscope compared with cells absent of CoCl<sub>2</sub> (**Figure 4**, ×40 magnification). To confirm whether cell apoptosis led

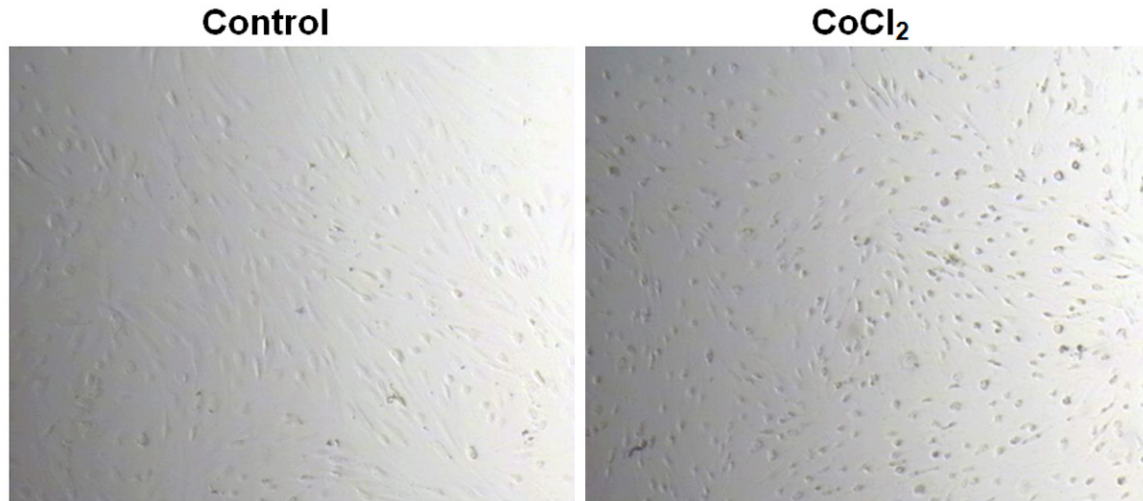




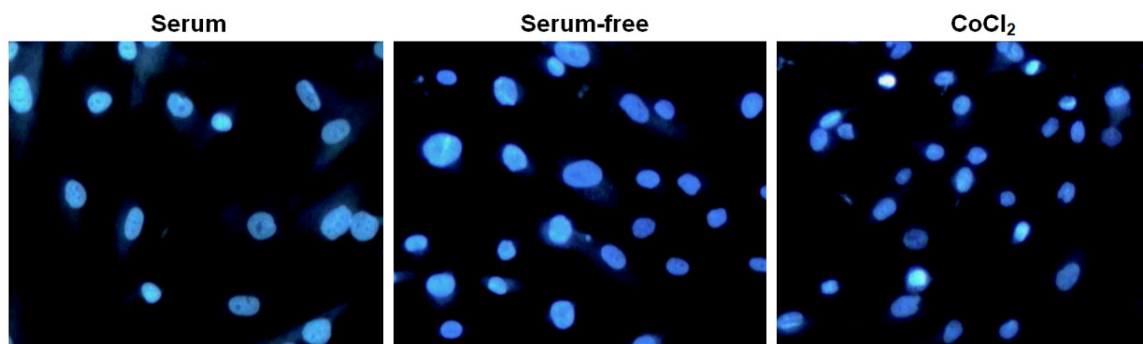
**Figure 3.** Cobalt chloride inhibited the migration of normal human saphenous vein smooth muscle cells determined by wound healing assay. An acellular area was prepared in confluent monolayer normal human saphenous vein SMCs at first. Cells with CoCl<sub>2</sub> (200  $\mu$ M) or without CoCl<sub>2</sub> (control) started to migrate toward acellular area after cultured for 1 d. After cultured for 8 days, initial spindle-like cells had covered the acellular area in the control, while there were fewer cells with depauperate morphous in the acellular area of CoCl<sub>2</sub> treated group (magnification is  $\times 40$ ).

to this phenotype, we used Hoechst 33258 to stain nuclei of cells with or without CoCl<sub>2</sub> (200  $\mu$ M). Fluorescence stain results showed that there was no significant difference between

cells with FBS and cells (serum free) with or without CoCl<sub>2</sub> (**Figure 5**,  $\times 100$  magnifications). There were a few pyknotic nuclei in CoCl<sub>2</sub> treated cells, but quantity results (result not shown)



**Figure 4.** Cobalt chloride led to morphous changes of normal human saphenous vein smooth muscle cells under light microscopy (magnification is  $\times 40$ ). After cultured for 8 days, cells in control (serum free medium) almost remained initial spindle-like morphous, while cells treated by  $\text{CoCl}_2$  (200  $\mu\text{M}$ ) became more roundish and diacaustic.



**Figure 5.** Fluorescence stain with Hoechst 33258. There was no significant apoptosis existing in human saphenous vein SMCs treated by different media including with 10% FBS (Serum), with  $\text{CoCl}_2$  (200  $\mu\text{M}$ ) and without  $\text{CoCl}_2$  (Serum free) (magnification is  $\times 200$ ).

from at least 10 fields showed no significant difference compared with no treated cells.

### Discussion

Varicose veins (VVs) load significant burdens on patients, but the mechanism of VVs formation remains unclear. Vein wall changes are now thought to be the primary events of VVs formation [2, 14]. Smooth muscles in the tunica media of vessels are responsible for wall tone. VSMCs as the important component of tunica media play important roles in contraction and relaxation of vessels. Understanding the upstream regulation of vein wall changes at cellular level may help to identify new therapeutic targets for VVs.

Venous hypoxia has long been postulated as a potential cause of varicosity formation. There are two potential mechanisms of vein wall hypoxia in VVs. Stagnation of venous blood flow results in reduced oxygen replenishment and first affects the endothelial and inner layers of the vein wall termed as endoluminal hypoxia. Distension of the vein by hydrostatic pressure secondary to blood stasis cause compression of vasa vasorum and affect the media and outer layers of the vein wall termed as medial hypoxia [11]. Venous endothelial responses to hypoxia have often been studied with cell culture and ex vivo explants. However, direct effects of hypoxia on the phenotypes of normal human saphenous vein SMCs have not been reported as far as we know.

In this study, we isolated normal human saphenous vein SMCs from tunica media and cultured in vitro. We used  $\text{CoCl}_2$  as a chemical hypoxia agent to mimic hypoxia environment due to the advantages of simple operation and stable hypoxic effects [15], and observed the phenotypic changes of normal human saphenous vein SMCs responding to hypoxia in vitro. Our results showed that treatment with  $\text{CoCl}_2$  enhanced the proliferation of normal human saphenous vein SMCs. This effect was markedly obvious especially at 24 h. As the treated time extended, the relative survival ratio of  $\text{CoCl}_2$  treated cells continue to increase in time-dependent manner. This result was consistent with previous reports about responses of vascular cells to hypoxia. For example, Li et al [16] observed  $\text{CoCl}_2$  increased proliferation of human pulmonary arterial smooth muscle cells in dose- and time-dependent manner. Ahmad et al [17] demonstrated exposure of human pulmonary artery endothelial cells to hypoxia (10%  $\text{O}_2$  or 5%  $\text{O}_2$ ) increased proliferation over 48 h when compared to cells in normoxia (21%  $\text{O}_2$ ), while human pulmonary artery smooth muscle cells pre-exposed to chronic hypoxia (96 h) showed increased proliferation, both at 10%  $\text{O}_2$  and 3%  $\text{O}_2$ . HIF-1 $\alpha$  and HIF-2 $\alpha$  played different roles in both vascular cells. Moreover, research also showed that SMCs derived from varicose veins increased proliferation 2 folds than that from normal veins [18]. These phenomena could be explained by the principle of ischemic/hypoxic preconditioning. When faced to endogenous or exogenous hypoxia stimulus, cells would change their phenotypes and functions to adapt to varied environment and increase the hypoxia tolerance [19, 20].

We used wound healing assay to evaluate hypoxia induced by  $\text{CoCl}_2$  how to influence the migrating ability of normal human saphenous vein SMCs. Cells with or without  $\text{CoCl}_2$  (control) started to migrate toward acellular area after cultured for 24 h. During the treated time of 8 days, cells in the control gradually covered the acellular area, while cells treated by  $\text{CoCl}_2$  migrated very slowly. Moreover, the appearance of cells treated by  $\text{CoCl}_2$  became significantly different from that of control as the time extended. Light microscopy data of the 8th day showed that cells treated by  $\text{CoCl}_2$  became more roundish and diacoustic compared with spindle-like cells absent of  $\text{CoCl}_2$ . Using Hoechst

33258 to stain nuclei, we observed there was no significant apoptosis appearance among cells treated by different conditions, including medium containing serum, serum free medium with or without  $\text{CoCl}_2$ , except that there were a few pyknotic nuclei in  $\text{CoCl}_2$  treated cells with no significant difference. The fact that chronic hypoxia induced by  $\text{CoCl}_2$  could make normal human saphenous vein SMCs become depauperate morphous and decrease migrating ability implied that hypoxia would impair the contractile function of normal human saphenous vein SMCs and these changes might be associated with cell internal structure. The principal function of mature SMCs is contraction. These cells have a high cytoplasmic volume fraction of myofilaments, a low rate of proliferation, and synthesize only small amounts of matrix proteins [18]. The electron microscopy findings have shown that SMCs in VVs are poorly differentiated and filament bundles in them are reduced, which imply the decreased contractility of SMCs in VVs [21]. Contractility is essential for the maintenance of vessel wall tone in human saphenous vein SMCs. A reduction in contractility of SMCs cultured from VVs also have been demonstrated and such cells exhibit decreased actin cytoskeleton organization and fibronectin matrix deposition compared with those from non-varicose veins [22]. These changes at cellular levels might contribute to the structural abnormalities of VVs [4]. On the other hand, a marked reduction in migrating ability of SMCs may loosen their attachments to the basement membrane and endothelial cells and cause the dilatation of vein wall [23].

A genetic modification induced by hypoxia might exist. It is well established that there is a genetic component to the formation of VVs [7, 24]. Cytoskeleton is a dynamic signaling platform involved in cell growth, differentiation, and other fundamental biological behaviors. Altering expression of cytoskeletal proteins in VSMCs might influence the phenotypes and functions of VSMCs [25, 26]. In our study,  $\text{CoCl}_2$  mimicked hypoxia through stabilizing HIF-1 $\alpha$  which was identified as a transcriptional factor. HIF pathway dysregulation contributing to formation of varicose veins had been suggested. Chronically recurring stresses lead to repeated activation of the HIF pathway in the vascular wall, causing excessive changes that ultimately contribute to pathologic remodeling of the



blood vessels [27]. This hypothesis is partially supported by our results at cellular level. Future studies focusing on the HIF pathway at molecular level will further illuminate these phenotypic changes.

In our study, significant apoptosis induced by chronic  $\text{CoCl}_2$  treatment was not observed in normal human saphenous vein SMCs. Some researchers have observed dysregulated apoptosis and cell cycle dysfunction occur in varicose veins. The overall number of apoptotic cells and activity are reduced in varicose compared with non-varicose veins. Dedifferentiation of SMCs may be due to dysregulated apoptosis [28, 29]. Apoptosis is an important physiological process in normal development, morphogenesis and tissue regulation. Improperly regulated apoptosis may contribute to pathological conditions involving faulty DNA repair mechanisms and cell cycle disruption [30]. The existence of a few pyknotic nuclei in  $\text{CoCl}_2$  treated cells seemed to be the consequence of dysregulation of apoptosis.

This study has a number of limitations that warrant mention. Due to restricted experiment conditions, we only detected the macroscopical appearance. Signaling molecules involved in HIF pathway and cytoskeleton proteins related to contractility of VSMCs should be further identified by expression analysis of nucleic acids and proteins. Newer molecular techniques including assessing the cellular responses and molecular changes to hypoxia in normal human saphenous vein SMCs may be more useful. To our knowledge, such research has not been made to date. Further functional studies are also needed to elucidate the precise molecular mechanisms about the effects of hypoxia on normal human saphenous vein SMCs. Moreover, we did not assess contractility of normal human saphenous vein SMCs. Given the apparent morphous changes reported herein, we presumed that consequential changes in contractility would be apparent. Further studies are warranted to confirm this. A positive control should be set up in apoptosis analysis.

As our data have shown, the proliferating and migrating abilities of normal human saphenous vein SMCs could be influenced by hypoxia induced by  $\text{CoCl}_2$  treatment. These processes might involve in the cell adaptive responses to

hypoxia and the cellular structural and functional changes except for apoptosis. Phenotypic modulation of normal human saphenous vein SMCs responding to hypoxia stimulus might play a key role in varicose vein development. Understanding the internal molecular mechanisms could help to develop useful pharmacologic targets for the therapy of VVs of lower extremity.

### Acknowledgements

We thank Dr Hai Bo Chu and his colleagues for specimen collection.

### Disclosure of conflict of interest

None.

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