

Sildenafil suppresses the proliferation and enhances the apoptosis of hemangioma endothelial cells

XIAOPO HE^{1,2}, YIRAN LIU³, KAI LI^{1,4}, AIJUN YANG⁵, RUI WANG^{1,4} and SHAOHUA LIU⁴

¹School of Stomatology, Shandong University, Jinan, Shandong 250012;

²Department of Oral and Maxillofacial Surgery, Weifang People's Hospital, Weifang, Shandong 261041;

³School of Medicine, Shandong University; ⁴Department of Oral and Maxillofacial Surgery, Qilu Hospital, Shandong University; ⁵Library of Shandong University, Jinan, Shandong 250012, P.R. China

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Abstract. Treatment of infantile hemangiomas (IH) with propranolol was first reported in 2008. Regressions of lymphatic malformations combined with pulmonary hypertension was first reported in 2012 after three children received treatment with oral sildenafil, which serves as an antagonist of phosphodiesterase isoform-5 (PDE-5). A marked expression of endothelial cells in the cytoplasm of IH tissues was obtained in our previous study. Therefore, the present study hypothesized that the antagonist of PDE-5, sildenafil, may lead to the regression of hemangiomas. To assess this hypothesis, the proliferation and apoptosis of specimen-derived hemangioma endothelial cells (HemECs) was determined *in vitro* by an MTT assay and flow cytometry, respectively, following treatment with sildenafil. The potential mechanisms underlying the mRNA and protein expression levels of inhibitor of differentiation 1 (Id-1) were determined by reverse transcription-quantitative polymerase chain reaction and western blotting. The results demonstrated that 5 μ M sildenafil suppressed the proliferation of HemECs and significantly enhanced the rate of apoptosis after 24 h. Additionally, the mRNA and protein expression levels of Id-1 were downregulated following treatment with sildenafil. Therefore, the present study concluded that PDE-5 may be a potential therapeutic target for hemangiomas and Id-1 may serve a vital role in the associated signaling transduction pathways.

Introduction

Infantile hemangioma (IH), pathologically diagnosed as immature vascular endothelial cell proliferation (1), is the

most common benign neoplasm of infancy and occurs in ~4% of children (2). IH is well known for its rapid growth within the first month after birth, followed by a spontaneous and slow involution. Early intervention is recommended for IH due to the potential disfigurement, dysfunction and psychosocial impairment that may occur (3,4). Currently, oral propranolol is the first-line therapy for IH, which acts by inducing rapid and substantial regression of the lesions with minimal adverse effects (5,6), and results in the occasional successful treatment of IH (7).

In 2012, Swetman *et al* (8) reported an occasional regression of lymphatic malformation (LM) combined with pulmonary hypertension in three children following treatment with oral sildenafil, which served as an antagonist of phosphodiesterase isoform 5 (PDE-5) (8). Inhibition of PDE-5 decreased the contractility of vascular smooth muscle followed by cystic decompression, which potentially explained the therapeutic effects of sildenafil in LM (8). Based on this previous finding, in the current study, tissue sections were selected from patients with LM and IH, and subjected to immunohistochemistry (IHC) testing to confirm the expression of PDE-5 in LM. The results from IHC analysis revealed that PDE-5 was expressed in the cytoplasm of endothelial cells in IH, but not expressed in the endothelia of patients with LM.

According to these results of the current study, it was hypothesized that as an antagonist of PDE-5, sildenafil, may facilitate the regression of hemangiomas, as it is similar to propranolol. To assess this hypothesis, the proliferation and apoptosis of specimen-derived hemangioma endothelial cells (HemECs), following treatment with sildenafil *in vitro* and its potentially associated mechanisms, were investigated via MTT assay and flow cytometry. The potential mechanisms underlying the mRNA and protein expression levels of inhibitor of differentiation 1 (Id-1) were determined by reverse transcription-quantitative polymerase chain reaction and western blotting.

Correspondence to: Professor Shaohua Liu, Department of Oral and Maxillofacial Surgery, Qilu Hospital, Shandong University, 107 Wenhuxi Road, Jinan, Shandong 250012, P.R. China
E-mail: lshabccba@126.com

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Materials and methods

Specimens of vascular anomalies. Formalin-fixed and paraffin-embedded specimens of vascular anomalies for IHC

were obtained randomly from the Department of Pathology of Qilu Hospital (Shandong University, Jinan, China) between January 2000 and December 2013. Specimens were from 20 patients; 8 males and 12 females, aged 4 months-39 years (mean, 5 years). Patients were included if they had undergone no other treatments, such as drug therapy, laser therapy, and cryotherapy, in the past but were excluded if they presented with infection, systemic diseases, had undergone sclerotherapy or had a history of drug use. Specimens included lymphatic malformations (n=10), proliferating hemangiomas (n=8), and involuting hemangiomas (n=2). The diagnosis of these anomalies was confirmed by patient medical histories, physical examinations, magnetic resonance imaging (MRI) and final pathological examinations. The present study was approved by Qilu Hospital Ethics Committee of Shandong University and all the patients provided their written informed consent.

Expression of PDE-5 in vascular anomalies. Paraffin-embedded 4- μ m serial tissue sections were obtained and IHC was performed to assess the expression of PDE-5 in different types of vascular anomalies using an ABC kit (Gene Tech Co., Ltd., Shanghai, China). Lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), cluster of differentiation 34 (CD34) and glucose transporter-1 (GLUT-1) were used as markers for lymphatic endothelial cells, vascular endothelial cells and hemangioma tissues, respectively, to confirm the type of vascular anomalies. Subsequently, the expression of PDE-5 was assessed in the tissues of all the vascular anomalies.

Tissue sections were deparaffinized in xylene (Guangcheng Chemical Reagent Co., Ltd., Tianjin, China) and rehydrated in graded alcohol, followed by three rinses (each for 5 min) in phosphate-buffered saline (PBS). Endogenous peroxides were quenched by incubating the tissue sections with 10% (v/v) methanol and 3% (v/v) hydrogen peroxide for 15 min at room temperature. A citrate buffer solution (10 mM; pH 6.0; Beijing Solarbio Life Sciences Co., Ltd., Beijing, China) was used to unmask antigens at 100°C for 15 min and was slowly cooled down to room temperature. Following blocking with 10% normal goat serum (Beijing Zhongshan Jinqiao Biological Technology Co., Ltd., Beijing, China) for 1 h at 37°C, the slices were incubated with goat anti-rabbit LYVE-1 (ab36993; 1:100; Beijing Zhongshan Jinqiao Biological Technology Co., Ltd.), anti-rabbit CD34 polyclonal IgG (sc-9095; 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-human GLUT-1 polyclonal antibody (GR-004G1; 1:200; Sun Bio Technology Co., Ltd., Shanghai, China); and anti-rabbit PDE-5 polyclonal IgG (sc-32884; 1:100; Santa Cruz Biotechnology, Inc.), which were diluted in PBS overnight at 4°C. Following several rinses with PBS, tissue sections were incubated for 1 h at room temperature with anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary antibodies (GK500705; undiluted; Gene Tech Co., Ltd., Shanghai, China). Following three rinses in PBS, tissue sections were developed by incubation with 3,3'-diaminobenzidine tetrahydrochloride (Gene Tech Co., Ltd.) for 10 min at room temperature. Following counterstaining with hematoxylin (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) to stain

the nuclei, the tissue sections were dry mounted and cover slips were treated with neutral balsam (Yi Yang Instrument Co., Ltd., Shanghai, China).

Isolation and cultivation of HemECs. A specimen of proliferating hemangioma was obtained from a 3-month-old female by surgical resection and was confirmed by pathological examinations in the Department of Oral & Maxillofacial Surgery of Qilu Hospital, Shandong University. Tissue samples were rinsed with PBS and cut into pieces 1cmx1cmx1 cm large. The majority of the pieces were used for culturing HemECs and the residue was maintained to estimate the expression of GLUT-1, which served as the molecular marker for hemangioma.

Immunocytochemistry. HemECs were isolated from adherent tissue and the expression levels of CD34, von Willebrand factor (vWF) and PDE-5 were determined by immunocytochemistry. Cells (5.0×10^3 cells/well) were seeded in gelatin-coated 24-well plates in endothelial basal medium (EBM-2; Lonza Inc., Allendale, NJ, USA), supplemented with 20% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA), SingleQuot (Lonza Inc.), penicillin (100 U/ml) and streptomycin (100 μ g/ml; both Gibco; Thermo Fisher Scientific Inc.). Isolated cells were cultivated in a humidified and sterilized incubator, in an atmosphere containing 5% CO₂ at 37°C. Fresh medium was added following removal and washing of the initial medium was performed every 2-3 days. All experiments were performed using endothelial cells between generations three and five.

Viability of HemECs after sildenafil treatment. An MTT assay was used to assess the effects of sildenafil on the viability of HemECs. Sildenafil (Selleck Chemicals; Houston, TX, USA) stock solution at 100 μ M was prepared by dissolving sildenafil in dimethyl sulfoxide (DMSO) and was stored at 4°C. HemECs were seeded in 96-well plates (5.0×10^3 cells/well) and were cultivated in EBM-2, containing all the supplements mentioned, for 24 h with five replicates per concentration. The medium was subsequently changed to serum-free EBM-2 for another 24 h. The medium was removed and replaced with 200 μ l fresh medium, containing increasing concentrations of sildenafil (0, 1, 3, 5, 10 and 15 μ M) and cultured for different durations (24, 48 and 72 h). Cells in the control groups (0 μ M sildenafil) were cultivated in the medium with 0.1% DMSO (sildenafil-free medium). Following treatment, HemECs were incubated with MTT (20 μ l/well) for 4 h at 37°C, and the optical density at 490 nm was measured after the cell contents were dissolved in DMSO. Absorbance was measured using an automatic microplate reader (BioTek Instruments, Inc., Winooski, VT, USA), and a minimal effective concentration and duration was calculated for late apoptosis and the expression of Id-1.

Apoptosis of HemECs by sildenafil. Following treatment with 0 and 5 μ M sildenafil for 24 h, HemECs (1×10^5 clones) were trypsinized, rinsed with cold PBS and blended in binding buffer using the Annexin V-fluorescent isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection kit

(Merck Millipore, Darmstadt, Germany). Annexin V-FITC and PI were added to the fixed cells for 20 min at room temperature in the dark. Subsequently, Annexin V-FITC binding buffer was added to the mixture prior to the fluorescence being measured on a FACSort flow cytometer (BD Biosciences, San Jose, CA, USA). Cellular apoptosis was analyzed using FlowJo 7.6.1 software (Tree Star, Ashland, OR, USA).

Total RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). HemECs were exposed to 0 and 5 μM sildenafil for 24 h. Total RNA in HemECs was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific Inc.) and the Id-1 RNA was reverse transcribed into cDNA using a PrimeScript RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China). cDNA was amplified by PCR using specific primers for Id-1 (GeneChem Co., Ltd. -Shanghai, China): Forward, 5'-CATTCTGTTTCA GCCAGTCG-3' and reverse, 5'-AGCTCCTTGAGGCGT GAGTA-3'; (120-bp fragment). Amplification conditions were as follows: 35 cycles at 95°C for 5 min, 95°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec, and 72°C for 5 min, using SYBR-Green I dye real-time fluorescent quantitative detection (Takara Biotechnology Co., Ltd) on a Mastercycler ep realplex assay system (Eppendorf; Hamburg, Germany). A primer pair for β -actin (Genechem) was used as an internal control: Forward, 5'-GTGGGGCGCCCCAGGCACCA-3' and reverse, 5'-CTCCTTAATGTCACGCACGATTTTC-3'. Differences in the threshold cycles between the target genes and the reference gene (β -actin) were calculated. The value of the relative mRNA quantity for the control group was arbitrarily set to one for normalization and the data were analyzed using the comparative Cq method ($2^{-\Delta\Delta Cq}$) (9).

Total protein extraction and western blotting. HemECs were exposed to 0 and 5 μM sildenafil for 24 h, and were collected for protein extraction using radio immunoprecipitation assay lysis buffer/phenylmethanesulfonyl fluoride (100:1; Beijing Solarbio, Beijing, China). Protein concentration was determined using the Micro BCA Protein Assay kit (Thermo Fisher Scientific, Inc). Protein samples were separated using an SDS-PAGE gel kit (15% separating gel and 5% stacking gel; CoWin Biotech, Beijing, China) and were electrophoretically transferred onto a nitrocellulose membrane (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China). Following blocking with 5% non-fat milk for 1 h at room temperature, the membrane was incubated with the following antibodies: Anti-rabbit Id1 polyclonal IgG (sc-488; 1:400; Santa Cruz Biotechnology, Inc.) and anti-mouse β -actin polyclonal antibody (TA-09; 1:1,000; Beijing Zhongshan Jinqiao Biological Technology Co., Ltd., Beijing, China) in Tris-buffered saline containing Tween-20 [TBST; 10 mM Tris (pH 7.4), 138 mM NaCl and 0.05% Tween-20] at 4°C overnight. Subsequently, the membrane was washed with TBST three times and incubated with the appropriate secondary antibody: Goat anti-rabbit IgG conjugated to horseradish peroxidase (ZB-2301; 1:3,000; Beijing Zhongshan Jinqiao Biological Technology Co., Ltd) or goat anti-mouse IgG (GAM0041; 1:4,000; Lianke Biotech Co., Ltd., Hangzhou, China), for 1 h at room temperature. Immunopositive bands were examined

using an enhanced chemiluminescence (ECL) detection system FluorChem Q (Alpha Innotech Corp., San Leandro, CA, USA) and an ECL kit (EMD Millipore, Billerica, MD, USA). Images were captured on X-ray film, according to the instructions of FluorChem Q. A human TCA 8113 tongue squamous cell carcinoma cell line (American Type Culture collection, Manassas, VA, USA) was used as a positive control for the protein expression of Id-1 (10).

Statistical analysis. The SPSS 20.0 software package (IBM SPSS, Armonk, NY, USA) was used to analyze the data and all the figures were generated by GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, California, USA). Paired data from the two groups were analyzed using a paired Student's *t* test. Repeated measurement data with two variances, two-way analysis of variance was used and an intergroup *t*-test was applied. $P < 0.05$ was considered to indicate a statistically significant difference and data are expressed as the mean \pm standard error of the mean.

Results

PDE-5 is expressed in the cytoplasm of endothelial cells of hemangiomas. CD34, LYVE-I and GLUT-1 were considered as positive markers for vascular endothelial cells, lymphatic malformation endothelial cells and hemangiomas endothelial cells, respectively (11-13). Expression levels of PDE5 demonstrated that the expression of PDE5 was present in the cytoplasm of endothelial cells of hemangiomas, but not in the lymphatic malformation endothelia (Fig. 1).

Sildenafil suppresses the viability of HemECs. Specimen-derived HemECs were identified using vWF and CD34 markers, and positive expression of PDE-5 was also determined (Fig. 2). Viability of HemECs was determined via MTT assay following treatment with sildenafil (0, 1, 3, 5, 10 or 15 μM) for different durations (24, 48 and 72 h). As the concentration increased, the suppressive effects gradually increased in a dose-dependent manner (Fig. 3A). It was demonstrated that 5 μM sildenafil exerted significant suppressive effects on the viability of HemECs following incubation for 24 h, compared with the control group (0 μM sildenafil; $t=3.220$, $**P < 0.01$; Fig. 3B). However, insignificant suppression of viability was observed in HemECs treated with 10-15 μM sildenafil ($P > 0.05$). Therefore, the present study considered 5 μM and 24 h to be the minimal effective concentration and duration of sildenafil treatment, respectively, for the optimal suppression of HemECs.

Sildenafil enhances the apoptosis of HemECs. Following treatment with 5 μM sildenafil for 24 h, the morphology of HemECs changed, indicated by the destruction of intercellular junctions and increasing particles in the cytoplasm observed under light microscopy (Fig. 3C). Results of the FITC-PI labeled flow cytometric analysis demonstrated a higher apoptosis rate of 10.5% compared with 2.53% in the control group (Fig. 3D).

Sildenafil downregulates the mRNA and protein expression levels of Id-1. Following treatment with 5 μM sildenafil for

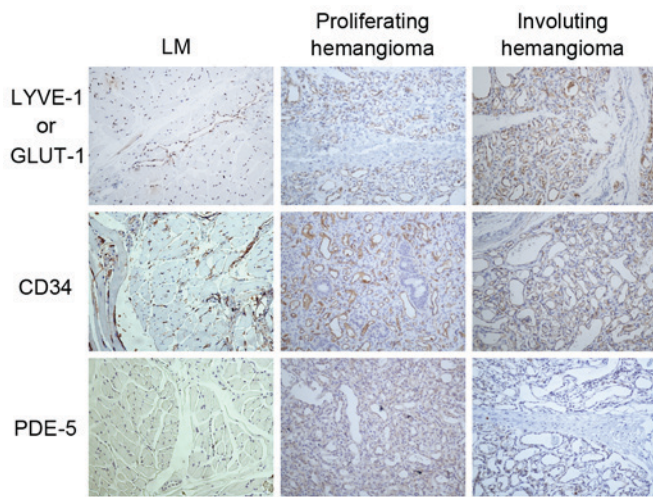


Figure 1. Expression levels of LYVE-1, CD34, GLUT-1 and PDE-5 in various tissue types. Expression levels of LYVE-1, CD34, GLUT-1 and PDE-5 were determined in tissues of LM, proliferating hemangioma and involuting hemangioma by immunohistochemical staining (magnification, x200). LYVE-1 was positively expressed in LM, and CD34, GLUT-1 and PDE-5 were positively expressed in proliferating hemangioma and involuting hemangioma. LYVE-1, lymphatic vessel endothelial hyaluronan receptor-1; CD34, cluster of differentiation 34; GLUT-1, glucose transporter-1; PDE-5, phosphodiesterase isoform-5; LM, lymphatic malformation.

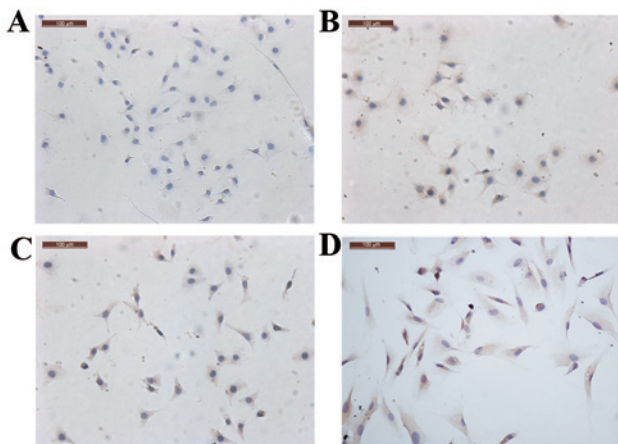


Figure 2. Immunocytochemical staining to identify the specimen-derived HemECs. Specimen-derived HemECs were identified by the markers, vWF and CD34, and PDE-5 positive expression was observed in these positive tissues compared with the control group by immunocytochemical staining (magnification, x200). (A) Control group, (B) CD34 group, (C) vWF group and, (D) PDE-5 group. HemECs, hemangioma endothelial cells; vWF, von Willebrand factor; CD34, cluster of differentiation 34; PDE-5, phosphodiesterase isoform-5.

24 h, the relative mRNA expression levels of Id-1 in the HemECs markedly decreased by 44.2%, according to the $2^{-\Delta\Delta Cq}$ method ($t=9.749$; $df=4$; $P=0.0006$; Fig. 4A). Additionally, the protein expression levels of Id-1 markedly decreased following treatment with sildenafil, according to the grey scale of obtained immunoblotting bands (Fig. 4B). These results suggest that, the Id-1 gene was downregulated at the mRNA and protein expression levels following treatment with 5 μ M sildenafil. Therefore, it was suggested that sildenafil may inhibit the proliferation of HemECs by downregulating the gene expression of Id-1 in HemECs.

Discussion

Infantile hemangioma is a common benign vascular tumor that is distinctive for its perinatal presentation, rapid growth during the first year of life and subsequent involution (14); although, the pathogenesis remains to be elucidated, the predominant theory is that abnormal EC (HemECs) proliferation triggers dysregulation of angiogenesis with high expression of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor during the proliferative phase (15). Gradual changes in the proliferation and apoptosis of HemECs occur between the proliferating and involuting phases (16). Determining the precise cellular mechanisms that drive hemangioma growth and apoptosis will provide a basis for targeted therapies to inhibit the proliferating phase or accelerate the involuting phase, in order to cure hemangiomas.

PDE-5 is a subtype of the PDE family, which preferentially hydrolyzes cyclic guanosine monophosphate (cGMP) and is abundant in vascular smooth muscle cells (17). Previously, PDE-5 was implicated in the proliferation and apoptosis of pulmonary ECs and cancer cells (18-20). Sildenafil, as an antagonist of PDE-5, has been widely applied to treat pulmonary hypertension and erectile disorders (21-23). To the best of our knowledge, the present study revealed for the first time that PDE-5 was expressed in HemECs. In addition, it was demonstrated that sildenafil exerted a significant proliferative suppression effect on HemECs *in vitro*. These findings are consistent with results published by Erdogan *et al* (24), in which sildenafil significantly suppressed the proliferation of human umbilical vein endothelial cells.

Distinct from that of necrosis, apoptosis is the process of programmed cell death that has been regarded as a recommended and privileged strategy for clearing tumor cells. Apoptosis of HemECs increased by 5-fold in the involuting phase compared with that in the proliferating phase in a previous study (16). Therefore, a strategy that triggers apoptosis in HemECs may be an effective treatment for hemangiomas. PDE-5 inhibitors have been demonstrated to induce apoptosis in human breast tumor cells by increasing cGMP levels (25). The present study revealed that treatment with 5 μ M sildenafil for 24 h markedly increased the ratio of apoptotic HemECs labeled with Annexin V-FITC/PI, as determined by flow cytometry. This suggested that PDE-5 may contribute to revealing the pathogenesis of hemangiomas, and its antagonists may be considered as novel drug candidates for the treatment of hemangioma.

Id-1 is an important subfamily member of the helix-loop-helix proteins. Increased expression levels of Id-1 have been implicated in regulating the growth, proliferation, migration and differentiation of cells (10,26). Additionally, Id-1 promoted the proliferation and survival of endothelial progenitor cells (27). Knockdown of Id-1 with RNA interference abolished proliferation, activation, and angiogenic processes of human umbilical vein endothelial cells (HUVECs) induced by VEGF (28). In the present study, following treatment with 5 μ M sildenafil for 24 h, the mRNA and protein expression levels of Id-1 in HemECs markedly decreased. These findings indicated proliferative suppression by sildenafil was exhibited in HemECs via the regulation of

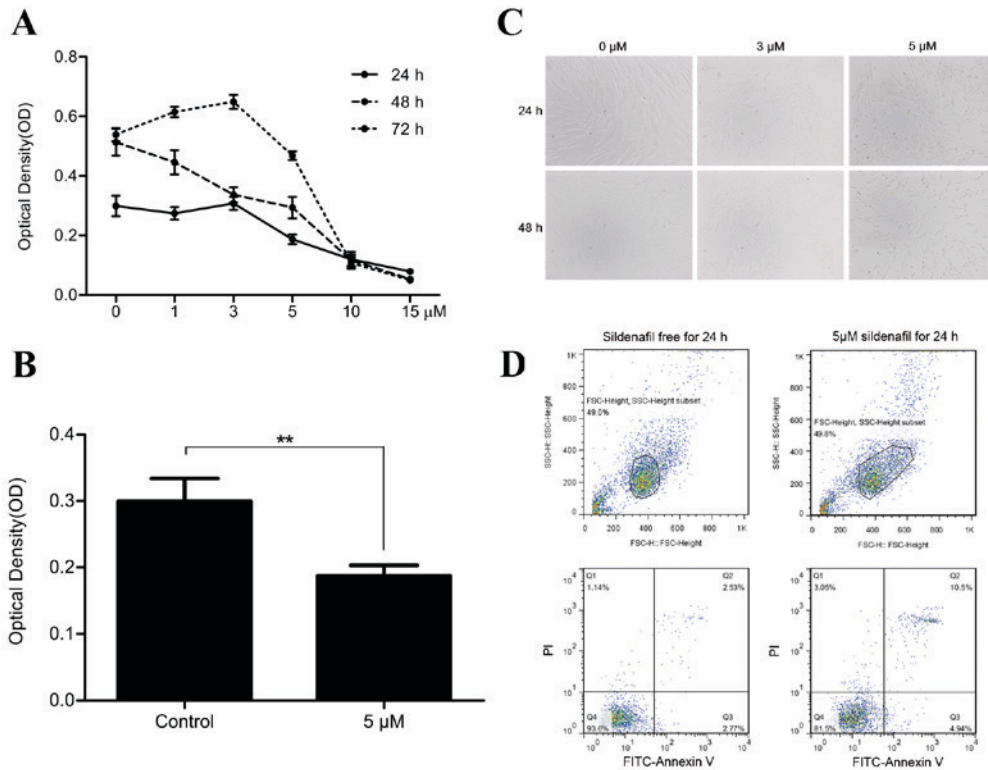


Figure 3. Sildenafil treatment suppresses viability and induces apoptosis in HemECs. (A) Viability of HemECs was determined via MTT assay following treatment with sildenafil (0, 1, 3, 5, 10 or 15 μM) for different durations (24, 48 and 72 h). As the concentration increased, the suppressive effects gradually increased in a dose-dependent manner. (B) Optical density after 24 h of Control and 5 μM groups (**P<0.01 vs. the control group). (C) Following treatment with 5 μM sildenafil for 24 h, the morphology of the HemECs changed, as indicated by the destruction of intercellular junctions and increasing particles identified in the cytoplasm, when compared with 0 and 5 μM sildenafil treatment using a light microscope (magnification, x200). (D) Annexin V-fluorescent isothiocyanate/propidium iodide flow cytometric analysis demonstrated a higher apoptosis rate of 10.5% following treatment with 5 μM sildenafil for 24 h compared with 2.53% in the control group. HemECs, hemangioma endothelial cells.

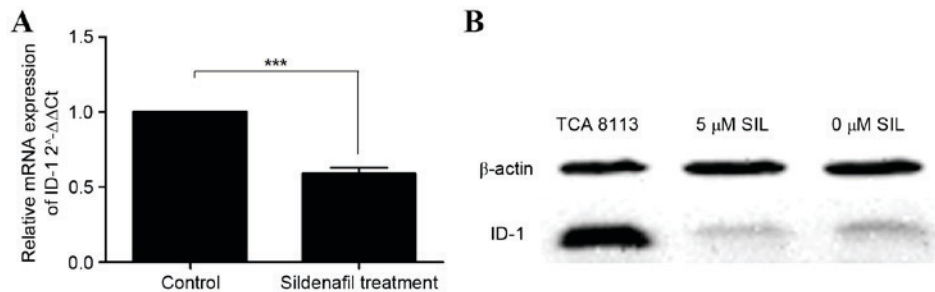


Figure 4. mRNA and protein expression levels of Id-1 following treatment with 5 μM sildenafil for 24 h. (A) Relative mRNA expression levels of Id-1 in the HemECs were significantly decreased by 44.2%, according to the 2^{-ΔΔCt} method (***P=0.0006 vs. control). (B) Western blotting revealed that the protein expression levels of Id-1 markedly decreased following treatment with sildenafil when compared with the negative control. The TCA 8113 cell line was used as a positive control. HemECs, hemangioma endothelial cells; SIL, sildenafil; ID-1, inhibitor of differentiation 1.

Id-1 expression. It has been reported that PDE-5 inhibitors reduced the expression of VEGF in a rat model of cyclosporine-induced nephrotoxicity (29). We therefore considered that sildenafil can reduce the VEGF-induced angiogenic processes in hemangioma by downregulation of Id-1 to lead to hemangioma regression.

In conclusion, the present findings demonstrated that PDE-5 was expressed in hemangioma tissues, and its antagonist, sildenafil, has the ability to suppress the proliferation and induce apoptosis of HemECs *in vitro*. Id-1 is important in this process, although further studies are required to elucidate the

cellular and molecular mechanisms that occur. It is essential that future *in vivo* hemangioma animal model studies are performed to investigate the effectiveness of sildenafil, prior to it being introduced into clinical practices.

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