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ORIGINAL ARTICLE

Effect of 410 nm photodynamic therapy with hemoporfin on the expression of vascular endothelial growth factor (VEGF) in cultured human vascular endothelial cells

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Abstract

Photodynamic therapy (PDT) is considered an effective alternative for the treatment of port-wine stains (PWS) using hemoporfin (hematoporphyrin monomethyl ether, HMME), a novel photosensitizer with better efficacy and lower recurrence. Vascular endothelial growth factor (VEGF) plays an important role in the development of PWS. Therefore, we conducted this study to investigate the effect of HMME-PDT on VEGF expression. Human vascular endothelial cells (HUVECs) were treated with different doses of HMME and irradiated with 410-nm light emitting-diode (LED) light. To assess cell viability, CCK-8 assays were performed. At 48 h after PDT, the expression of VEGF/VEGF receptor (VEGFR) mRNA was detected by reverse transcription-polymerase chain reaction (RT-PCR). Measurement of VEGF protein was carried out using western blotting assays. Cell viability was significantly inhibited after HMME-PDT and was dose-dependent within a certain range. HMME-PDT decreased secretion of VEGF 48 h after irradiation in HUVECs as compared to controls. The downregulation of VEGF and VEGFR mRNA as well as VEGF protein expression was more significant in the high HMME concentration group (4 μg/mL) than in the lower concentration group (2 μg/mL). Our outcomes provide evidence, that HMME-PDT can downregulate VEGF expression in cultured HUVECs and may explain the efficacy of hemoporfin PDT for PWS treatment.

Keywords Port-wine stain · Vascular endothelial growth factor · Photodynamic therapy · Hemoporfin

Introduction

Port-wine stains (PWS) are relatively common congenital low-flow vascular malformations of dermal capillaries and post-capillary venules, found in 0.3–0.5% of newborns [1]. They usually present as flat pinkish-to-purple patches at birth and may progress into darker, hypertrophic or even nodular lesions over time [2, 3]. Studies have shown that the presence of a facial PWS has a significant negative impact on quality of life. In particular, patients with hypertrophy had more emotional and symptomatic impairment such as glaucoma, epilepsy, and limb hypertrophy [4]. Based on the efficacy of selective photothermolysis, pulsed dye laser (PDL) represents the gold standard of treatment for PWS [5]. Most patients can achieve 50–80% improvement after 8–10 sessions of treatment. However, the rate of complete lesion removal has remained less than 20%, and a considerable number of patients with PWS may become resistant to PDL after several treatments [6].

In the 1990s, photodynamic therapy was first applied in treating PWS in China by Gu Ying, et al. [7]. An exogenous photosensitizer was injected intravenously and then activated by laser or other light sources, leading to photochemical-biological reactions that generated singlet oxygen molecules and other reactive oxygen species. These substances damaged the endothelial cells, caused thrombosis, and ultimately blocked the treated vessels. Hematoporphyrin monomethyl ether (HMME), a novel photosensitizer, was recently introduced in PDT on PWS. As a new monomer of porphyrin, HMME has the advantage of strong photo-activity, high photodynamic efficiency, low toxicity, and a fast clearance rate [8].

However, the mechanism of HMME-PDT in the treatment of port wine stain is still not completely clear. Clinical observations have shown that after the treatment of PWS with HMME-PDT, patients usually achieve persistent improvement with few recurrences [9]. When the same method was

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applied to the blood vessels of the rooster combs, regression was achieved but re-vascularization occurred. These positive results suggest that PDT contributes to persistent and long-term inhibition of PWS vascular proliferation. In addition to the immediate damage of endothelial cells by free radicals, other mechanisms may be involved in the effects of PDT against PWS, which induce the continuous degradation and apoptosis of vascular endothelial cells. Vascular endothelial growth factor (VEGF) has been proved to be one of the most important regulators of angiogenesis, which binds to the tyrosine kinase receptors VEGFR-1 and VEGFR-2, thus initiating a downstream signaling cascade that promotes angiogenesis [10]. Therefore, the aim of our study was to determine the expression of VEGF after HMME-PDT toward elucidating the mechanism of PDT in PWS.

Materials and methods

Cell lines and cell culture

The human vascular endothelial cells (HUVECs) were purchased from ALLCELLS Inc. (CA, USA). HUVECs were maintained in complete RPMI-1640 medium supplemented with 10%(*v*/*v*) heat-inactivated fetal bovine serum and 1% streptomycin-penicillin (ALLCELLS. CA, USA) at 37 °C with 5% CO2 and 95% air in a humidified atmosphere.

Photosensitizer and light source

A stock solution of HMME (Shanghai Fudan-zhangjiang Bio-Pharmaceutical Co. China) was prepared by adding 50 μl of dimethyl sulfoxide, DMSO (Sigma Aldrich Inc., St. Louis MO, USA) to 1 mg of HMME to obtain 20 mg/mL stock solution, which was stable in solution at 0 ± 4 °C in the dark. A working concentration of 20 μg/mL was prepared by further dilution with RPMI-1640 medium (ALLCELLS). Working drug solutions (0–8 μg/ml) were prepared by diluting the stock solution with RPMI-1640 medium (ALLCELLS. CA, USA). A light-emitting diode (LED) at a wavelength of 410 nm (GSD company, Shenzhen China) was used as the light source.

In vitro PDT

HUVECs were seeded into cell culture plate. After 24 h, the cells were incubated with different concentrations of HMME for 3 h in the dark. After removing the drug and washing with phosphate-buffered saline (PBS), the treated cells were irradiated using the LED at 100 mW/cm^2 for 1 min. All irradiations were performed at room temperature (25 °C). Controls for each experiment were cells exposed to PDT light without HMME and cells incubated with different concentrations of HMME without being exposed to PDT light.

Observation of cell morphology

The HUVECs were seeded at a density of 1.5×10^5 cells per well in six-well plates (Corning, Carlsbad, CA, USA). HUVECs treated in a similar manner but without light irradiation were used as controls. After PDT, the changes of cell morphology were observed at 24 h and 48 h under an inverted microscope (Olympus, TAKACHIHO SEISAKUSHO, Japan).

Cell viability assays

CCK-8 assays were conducted to determine the photocytotoxic effects of HMME-PDT on HUVECs. The cells were divided into two groups: the PDT and non-PDT groups. Approximately 1.0×10^4 cells were seeded onto 96-well plates. After overnight growth, the cells were treated with different concentrations of HMME (0, 2, 4, 6, 8, 16 μg/mL) for 3 h. The culture medium was removed and replaced with fresh medium after washing with PBS three times. The PDT group was then exposed to the 410-nm LED light at a dose of 10 mW. The non-PDT group was not treated with light irradiation. Subsequently, both groups were incubated for an additional 24 h in the dark. Then, 10 μL of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)- 5-(2,4-disulfophenyl)-2H-tetrazolium, Dojindo Molecular Technologies, Inc., Japan) was added to each well, and the cells were incubated for another 3 h. The absorbance at 450 nm was measured using a microplate reader. The cell viability of the treated cells was compared with that of the vehicle-only control cells (100%) to calculate the half-maximal inhibitory concentration (IC_{50}) value.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The HUVECs were seeded at a density of 3.0×10^5 cells per well onto six-well plates and cultured for 24 h before PDT. Forty-eight hours after treatment, the cells were then washed twice with ice-cold PBS. Total RNA was extracted from the PDT-treated cells using an RNAiso Plus Kit (TaKaRa,Tokyo, Japan) following the manufacturer's instructions. Reverse transcription of the extracted RNA was performed using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa). Two-step qRT-PCRs were performed using SYBR® Premix Ex Taq™ II (TaKaRa) on a Real-Time qPCR System (QuantStudio™ 3 Real-Time PCR Systems, Thermo Fisher Scientific Inc. Carlsbad, CA, USA) using the primers listed in Table 1. The relative fold change in the mRNA expression level of the target gene was quantified using the $2^{-\Delta\Delta CT}$ method and the QuantStudio™ Design and Analysis Software. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was

Table 1 Primers used for quantitative real-time PCR

Template	Forward primer (5′-3′)	Reverse primer (5′-3′)
VEGFA	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
VEGFR2	CAGCATCACCAGTAGCCAGA	GATGCTCCAAGGTCAGGAAG
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

used as a housekeeping gene. A vehicle-only control was used as a calibrator for quantification.

Western blot analysis

The cells were grown on a six-well plate (3.0×10^5 cells per well) before PDT. The HUVECs were treated with HMME (2 μg/mL and 4 μg/mL, respectively) in combination with VEGF-A (25 ng/ml) for 3 h. Forty-eight hours after treatment, the media were aspirated and the cells were washed twice with ice-cold PBS. The proteins were harvested from the cells using cell lysis buffer containing 1 mM polymethanesulfonyl fluoride (Beyotime, Shanghai, China), and the protein concentrations were measured using the BCA Protein Assay Kit (Beyotime, Shanghai, China).

The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore, MA, USA). The membranes were blocked with 6% non-fat milk at 37 °C for 1 h and then incubated overnight with the following primary antibodies at 4 °C: anti-VEGFA (ab1316, Abcam, USA) and anti-GAPDH (Beyotime, Shanghai, China). The blots were then incubated with peroxidase-conjugated AffiniPure goat anti-mouse IgG (1:10,000,

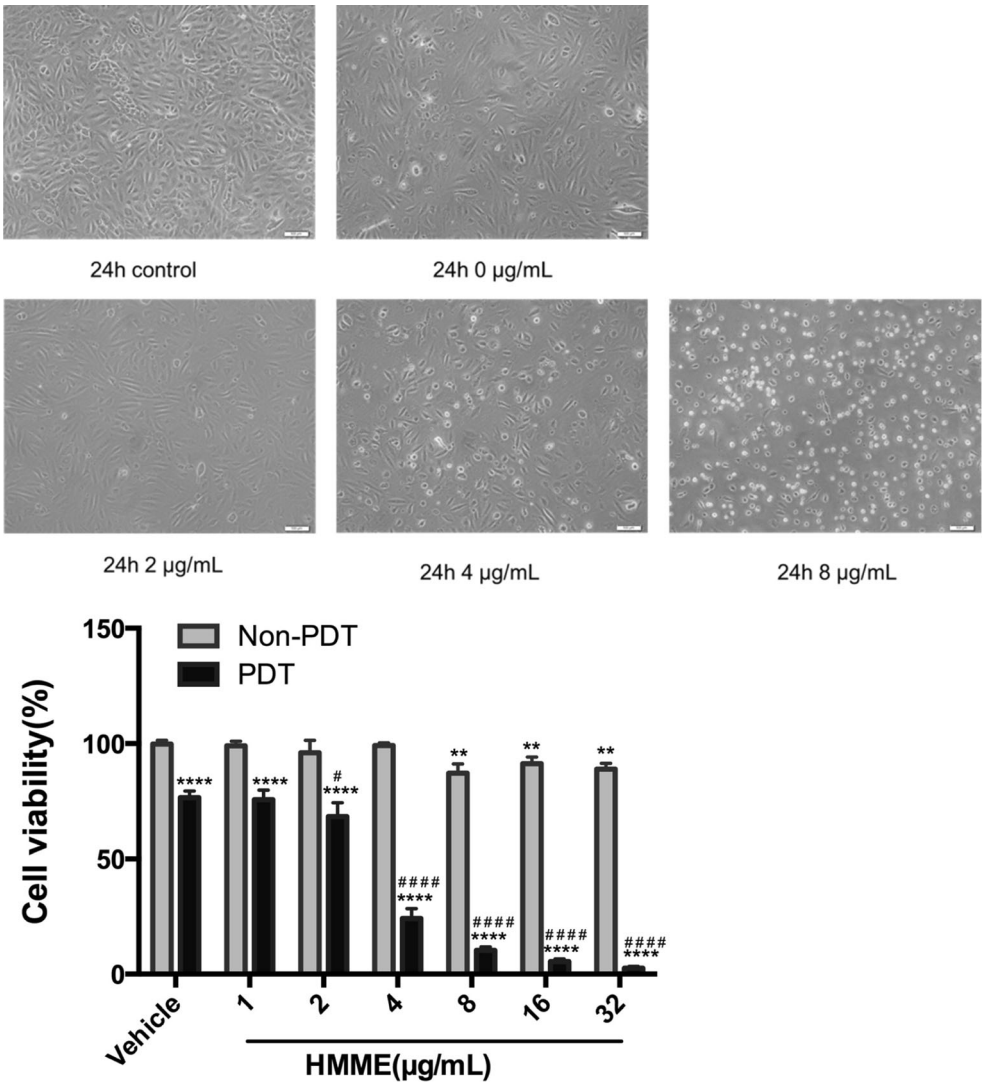

Fig. 1 Effects of HMME-PDT on the growth of HUVECs at 24 h after PDT. Cells were treated with increasing concentrations of HMME (2–32 μg/mL), and cell viability was measured 24 h after PDT. Data are presented as means ± SDs ($n = 4$). $^{**}P < 0.01$, $^{****}P < 0.0001$, compared with the vehicle control; $\#P < 0.05$, $\#\#\#\#P < 0.0001$, compared with cells exposed to PDT without HMME. The cell viability decreased in PDT group compared to vehicle controls. Cell viability had a significant decrease in 4 μg/mL PDT group

ZSGB-BIO, Beijing, China) as the secondary antibodies for 1 h at 37 °C. The blots were detected with the Immobilon Western Chemiluminescent HRP Substrate (Millipore). GAPDH was used as an internal control.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance. $P < 0.05$ was regarded as a statistically significant difference.

Results

Morphology of HUVECs after PDT

To investigate whether HMME-mediated PDT-induced cell death, HUVECs were observed under an inverted microscope at 24 h and 48 h after treatment. As shown in Fig. 2, the control cells showed adherent growth with clear contours. However, PDT-treated cells had altered morphology, showing a condensed cytoplasm or a floating growth pattern within 24–48 h.

Cell viability

The effects of different concentrations of HMME with PDT on HUVEC viability were determined by CCK-8 assays. As shown in Fig. 1, the viability of HUVECs decreased from 75.7 to 2.61% following irradiation in cells treated with HMME concentrations ranging from 1 to 32 μg/mL ($P < 0.05$ versus the vehicle control). At 48 h after PDT, the PDT-treated cells' viability was between 11.1 and 57.2%, which showed a significant decrease compared to controls. At 48 h after PDT, inhibition of cell viability was stronger than that at 24 h (Fig. 2). After 48 h of incubation, the IC50 for HMME was between 0 and 2 μg/mL. In the non-PDT group, cell viability was not inhibited in HUVECs incubated with 2 or 4 μg/mL HMME ($P > 0.05$ versus the vehicle control), but was significantly inhibited when the HMME concentration was 8 μg/mL or more ($P < 0.01$ versus the vehicle

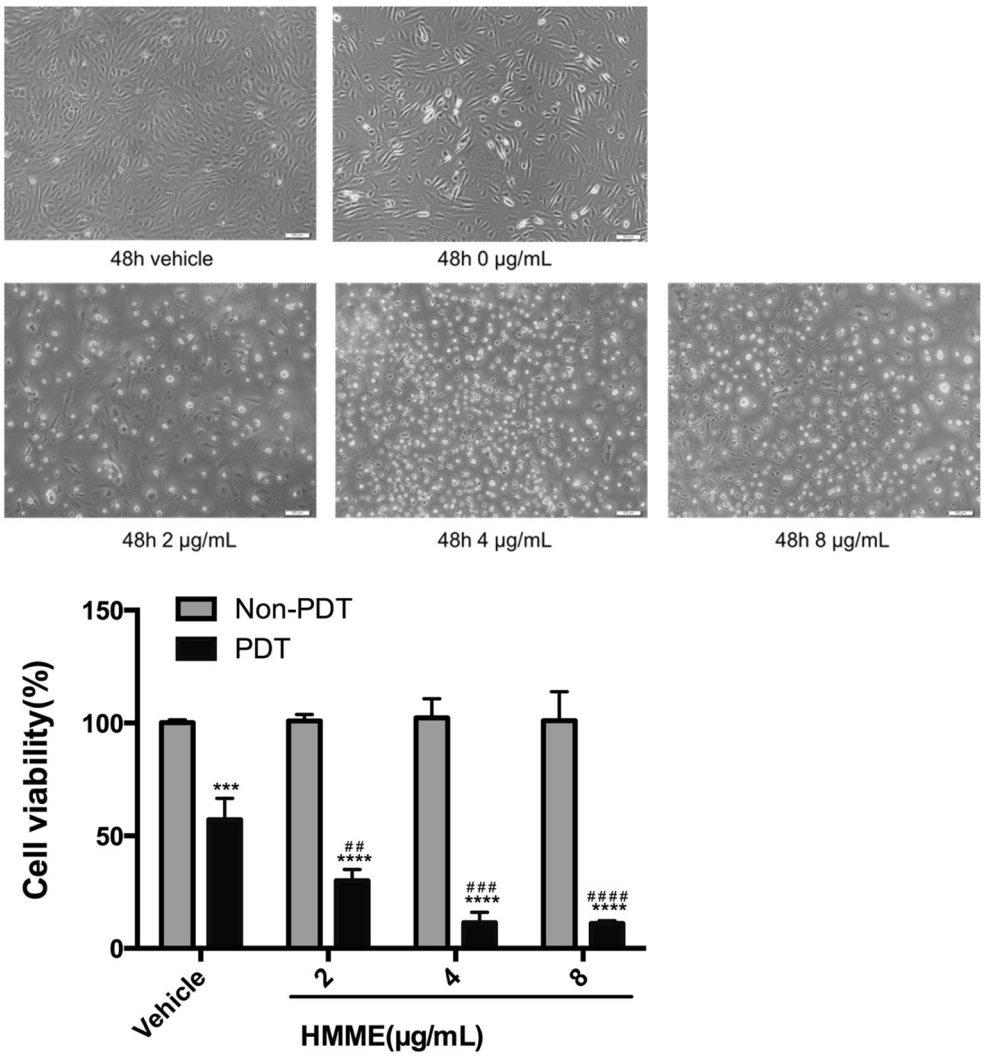

Fig. 2 Effects of HMME-PDT on the growth of HUVECs at 48 h after PDT. Cells were treated with increasing concentrations of HMME (2–8 μg/mL), and cell viability was measured 48 h after PDT. Data are presented as means ± SDs ($n = 4$). ***$P < 0.001$, ****$P < 0.0001$, compared with the vehicle control; ##$P < 0.01$, ###$P < 0.001$; ####$P < 0.0001$, compared with cells exposed to PDT without HMME. The cell viability decreased in PDT group compared to controls. Cell viability had a significant decrease in 4 μg/mL PDT group. The cell viability in 8 μg/mL group showed no significant difference compared with 4 μg/mL group at 48 h after PDT

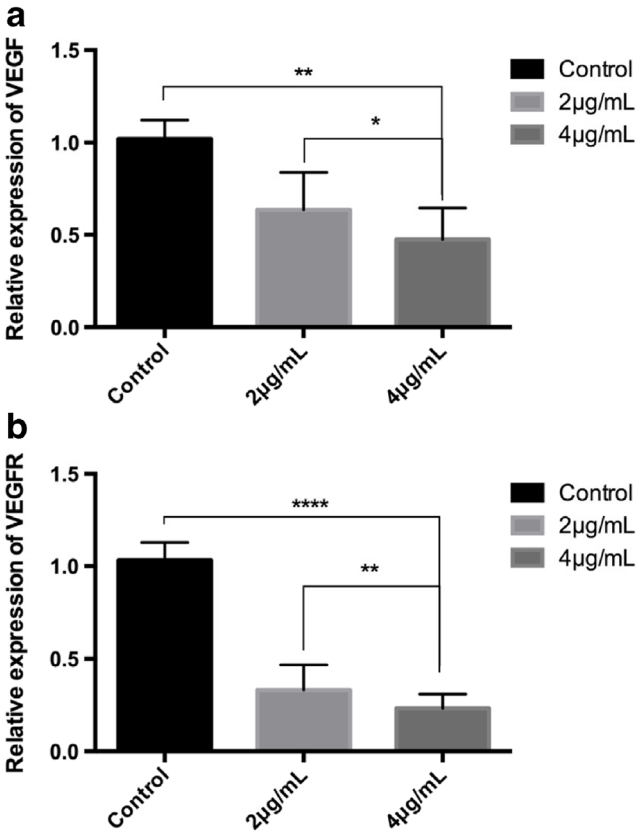

Fig. 3 Expression of *VEGF* mRNA in HUVECs after HMME-PDT. Two-step qRT-PCR was performed to analyze the mRNA expression levels of *VEGF* (**a**) and *VEGFR* (**b**). Data are presented as means ± SDs ($n = 3$). *$P < 0.05$, **$P < 0.01$. The mRNA level of *VEGF* and *VEGFR* showed a significant decrease at 48 h after PDT in HMME-PDT group ($P < 0.05$ vs. vehicle control)

control). Thus, these findings suggested that high concentrations of HMME may lead to cell damage.

Expression of *VEGFA* and *VEGFR2* mRNA after HMME-PDT

Next, we investigated the mRNA expression levels of *VEGFA* and *VEGFR2* to determine the anti-angiogenic effects of PDT. The expression levels of *VEGFA* and *VEGFR2* were reduced by PDT at 48 h after HMME-PDT, and significant ($P < 0.05$) decreases were observed in HUVECs treated with 2 or 4 μg/mL HMME-PDT compared with those detected in the vehicle control (Fig. 3). No significant differences were found in the expression levels of *VEGFA* or *VEGFR2* mRNAs in HUVECs treated with 2 or 4 μg/mL HMME-PDT.

Expression of VEGF protein after HMME-PDT

To further examine the anti-angiogenic effects of PDT, we detected the protein expression of VEGF-A using western blotting. The HUVECs were treated with HMME for 3 h, subjected to PDT, and then lysed for analysis of protein expression. As shown in Fig. 4, the expression of VEGF-A was reduced by PDT plus 4 μg/mL HMME after 48 h. Notably, VEGF expression downregulation was greater in the 4 μg/mL group than in the 2 μg/mL group at 48 h.

Discussion

HMME, a porphyrin derivative, is absorbed by light at a wide range of wavelengths, with five absorption peaks between 400 and 700 nm, in which the highest is 418 nm. Therefore, in vitro, we choose 410-nm LED as the light source in hope of achieving high PDT effect, in which singlet oxygen species play an important role [11].

PDT induces cell death via the combined effects of light, a photosensitizer, and molecular oxygen. The specific effects of PDT depend on the selective distribution of photosensitizer in the target tissue and the absorption of light energy by photosensitizers. Therefore, PDT is highly targeted. In PDT of PWS, HMME was highly distributed in the endothelial cells of capillaries and well activated by 410-nm LED light, close to the absorption peak of HMME. Thus, PDT selectively destroys the PWS lesions with minimal injury of the normal surrounding tissue [12].

VEGF-A (also known as VEGF) is the most potent member of VEGFs family that induces proliferation, sprouting, and tube formation of endothelial cells. VEGF binds to a variety of

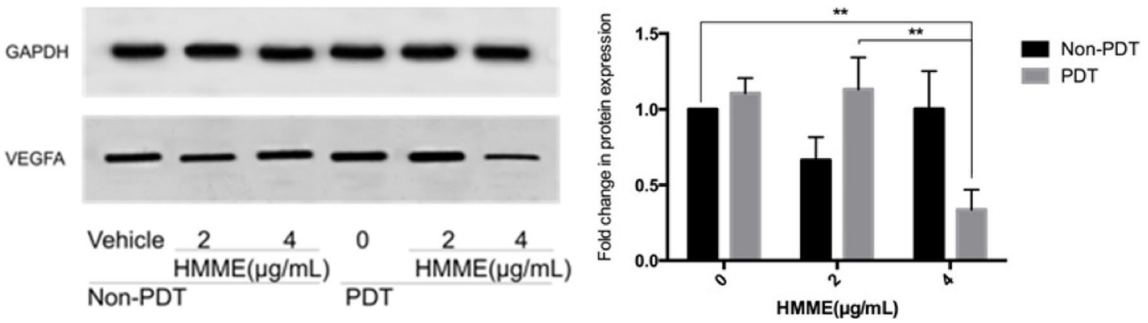

Fig. 4 Expression of VEGF in HUVECs after HMME-PDT. The VEGF protein level was reduced by PDT both in the 2 μg/mL and 4 μg/mL group after 48 h compared to that of the control group. The reduction of VEGF expression was more significant in 4 μg/mL than 2 μg/mL group

transmembrane tyrosine kinase receptors (VEGF- R1, VEGF-R2, and VEGF-R3), and VEGF-R2 has been reported to be the main receptor for proangiogenic effects of VEGF [13]. VEGF and VEGF-R may contribute to the pathogenesis of PWS by inducing vessel proliferation and vasodilatation. It also induces endothelial nitric oxide synthase to stimulate vasodilatation [14].

Many studies have shown that VEGF expression decreases in tumor tissues after PDT [15]. Moreover, PDT also decreases VEGF expression in the aqueous humor in patients with macular degeneration [16]. However, the lesion of PWS is mainly composed of vascular endothelial cells. Thus, studying the changes of VEGF expression in vascular endothelial cells after photodynamic therapy could better explain the biological influence of PDT on PWS.

Previous studies have shown that using the clinical parameters of HMME-PDT in animal models can lead to short-term vascular regression but that revascularization occurs soon thereafter [17]. VEGF and VEGFR expression have been reported to be increased in PWS lesions, suggesting that VEGF and VEGFR play an important role in the pathogenesis of PWS by inducing vessel proliferation, vasodilatation, or both [18]. Our results showed that VEGF and VEGFR levels were significantly decreased at 48 h after 410-nm LED-HMME-PDT, indicating that HMME-PDT is capable of downregulating VEGF expression. This may explain why PWS rarely relapses after PDT. In the clinical setting, after treatment with HMME-PDT, there is often a delay in the resolution of PWS, probably because it takes some time for PDT to downregulate VEGF expression.

In this study, we found that VEGF and VEGFR mRNA and protein levels decreased most significantly in the 4 μg/mL group at 48 h after HMME-PDT, suggesting a seemingly dose-dependent inhibitory effect of PDT on endothelial cells. These findings were also consistent with the results of CCK-8 assays which indicated that HUVEC viability was significantly inhibited 48 h after PDT especially in 4 μg/mL HMME group. It is likely that 410-nm-LED-HMME-PDT downregulated VEGF expression in vascular endothelial cells, thereby leading to the inhibited viability of these cells. This could in turn result in the apoptosis of endothelial cells and occlusion of dilated capillaries in PWS lesions. Such inhibitory effect of PDT is likely to be prolonged, and this may explain the longer duration of PDT efficacy in the treatment of PWS.

The downregulation of VEGF and VEGFR may play an important role in the inhibition of cell viability after PDT according to our study. Inhibition of cell viability after PDT is concentration-dependent within a certain range. Through analysis of HUVEC viability after HMME-PDT, we found that greater concentrations of photosensitizer (within a certain range) resulted in stronger inhibitory effects on cell proliferation. Our result suggested that higher dose of photosensitizer is likely to have better clinical response. This also suggested that PDT may have a longer duration of efficacy in the treatment of PWS. Thus, optimization of the photosensitizer dosage in the clinical setting is necessary to reduce toxicity to normal cells while achieving the desired efficacy. Increase the energy of light source may also increase the efficacy of PDT and further study is required to prove this.

There are several limitations in the present study. First, our study focused on the effect of HMME-PDT in vitro. In clinical practice, the efficacy of PDT in PWS can be affected by many factors, such as blood vessel depth, blood vessel diameter, and the distribution of lesions. It may be better to test the expression of VEGF in human PWS lesions after HMME-PDT. However, due to ethical considerations and difficulty in obtaining specimens, we did not do the biopsy in patients. Second, this experiment mainly studied the expression changes of VEGF and VEGFR after PDT treatment, whereas its upstream regulation mechanism remains to be elucidated. Therefore, further experiments are needed to explore it.

Conclusion

In conclusion, our study demonstrated that 410-nm LED-HMME-PDT can downregulate the expression of VEGF and VEGFR. This may partially explain the mechanism of PDT of PWS. Further in vivo study will be necessary to elucidate the exact mechanism.

Role of funding source The research was sponsored by the Natural Science Foundation of Shanghai.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the Ethical Committee of Shanghai Huashan Hospital, Fudan University.

Informed consent Informed consent was obtained from each participant.

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