

# One Possible Mechanism of Pulsed Dye Laser Treatment on Infantile Hemangioma: Induction of Endothelial Apoptosis and Serum vascular endothelial growth factor (VEGF) Level Changes

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## Abstract:

**Introduction:** Pulsed dye laser (PDL) is an important treatment for superficial infantile hemangioma, but few studies report on its cellular mechanism. The aim of this study was to evaluate alterations of serum vascular endothelial growth factor (VEGF) level in infantile hemangioma (IH) patients after laser treatment and effects of PDL irradiation on human umbilical vein endothelial cells (HUVECs) in vitro, as well as to explore the biomolecular mechanisms and ultrastructure changes of the PDL effect.

**Methods:** 74 children with infant hemangioma including 45 patients in proliferating phase, 18 patients in involuting phase, 11 patients in involuted phase and 10 healthy children were engaged in this study. The plasma VEGF levels of children were measured with the enzyme-linked immunosorbent assay (ELISA). 24 hours after, HUVECs cultured in vitro were irradiated with PDL, cell apoptosis, mRNA levels of VEGF, and changes of ultrastructure were evaluated using flow cytometry, real-time reverse transcriptase polymerase chain reaction (RT-PCR), and transmission electron microscopy, respectively.

**Results:** The serum VEGF concentrations in children with proliferating hemangiomas were significantly higher than in patients with involuting / involved hemangiomas and healthy patients. After receiving 3 laser treatments, the plasma VEGF levels of IH patients in proliferating hemangiomas decreased significantly. PDL irradiation could down-regulate VEGF mRNA expression of HUVECs, and increase cell apoptosis rate.

**Conclusion:** The present study demonstrates that PDL irradiation imparts apoptosis induction effects on HUVECs in vitro. Furthermore, our results suggest that vascular endothelial growth factor may be of particular importance in pathophysiology and PDL treatment of hemangiomas, also serum VEGF levels may be used as an aid in the follow up of IH. This provides valuable evidence of the PDL effect on infantile hemangioma.

**Keywords:** infantile hemangioma; pulsed dye lasers; apoptosis; vascular endothelial growth factor

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## Introduction

Infantile hemangiomas (IH), localized tumors of blood vessels, is the most common tumor of infancy. It grows

faster than the rate of the child during the first year of age (proliferating phase); After 9 to 12 months of age, the tumor begins to shrink (involuting phase). Recent studies show that after 3.5 years of age, most infantile

hemangiomas do not improve significantly<sup>1</sup>. However, IH often does not completely resolve following involution. Residual evidence with scar formation, fibrofatty masses, atrophic wrinkling, yellowish discoloration, and telangiectasias is usually seen after complete involution<sup>2</sup>. Significant cosmetic disfigurement and psychological problems related to appearance cannot be neglected. In order to reduce the incidence of these sequelae, we think that early interventions are necessary for some IH patients.

Pulsed dye laser (PDL) treatment is an important treatment for superficial infantile hemangiomas, which could lighten the lesions, accelerate regression, or stop growth. The up-regulation of capillary endothelial cell apoptosis may be responsible for reducing the size of the hemangiomas, but not so much is known about the real mechanism triggering hemangioma regression induced by the laser. Basic lasers-tissue interactions remains unclear. In this study, we aimed to clarify the mechanism(s) underlying the PDL treatment on IH.

It has been demonstrated that vascular endothelial growth factor (VEGF) is the most potent stimulator of hemangioma derived endothelial cell (HemEC) proliferation and differentiation<sup>3</sup>. Many reports have confirmed that excessive VEGF expression in IH tissue parallels the proliferating phase of IH tissue growth. Conversely, during the involuting phase, VEGF expression rapidly decreases, and many inhibitors of angiogenesis are instead expressed<sup>4-6</sup>. However, published reports on the observation of serum VEGF levels of IH are limited. This report present the relationship of serum VEGF levels and IH progress receiving PDL treatment, trying to look for an objective basis and even clinical marker for assessing of IH progress .

## Methods

**1. Laser treatment.** A tunable pulsed dye laser (Nlite PDL, PHOTONICS Corp, England) was used. The parameters were as follows: a wavelength of 585nm, pulse duration of 10-20 milliseconds, spot size of 5 mm and energy fluency of 4-8.5J/cm<sup>2</sup>. The energy adjusted to produce purpura was believed to be suitable. The energy density was adjusted according to the age of the patient, color, site, history of the lesion, and the patient response to the previous treatment. Photographs before and after treatment have been taken with an effort to use the same magnification, lighting and exposure. The treatments were repeated at intervals of 4 - 6 weeks until the lesion was cleared, stopped proliferating or

responding, or the parents discontinued the treatment. Additionally, icepacks were applied to the treated area as analgesia and to minimize adverse epidermal effects. All patients were instructed on post-treatment sun protection and protection from trauma until the purpura faded.

**2. Sample Collection.** Infantile hemangiomas with at least one lesion >1 cm in diameter and involving the skin were included in the study. 74 children with infant hemangioma including 45 patients in proliferating phase, 18 patients in involuting phase and 11 patients in involuted phase were engaged in this study, 10 non-hemangioma children were selected as control. All patients received PDL treatments or not, without other treatments. Peripheral blood samples (1mL) were drawn from each patient according to a protocol approved by the Local Ethical Committee at Provincial Hospital affiliated to Shandong University, at the time points: before treatment and after 3 treatments, and were collected to identical tubes, also serum was separated from the samples at the same time within 1-2 h and frozen at -20°C pending analysis. Subsequently all samples were thawed to room temperature and analyzed for VEGF levels using an ELISA kit according to the manufacturer's instructions (BioSource International, Camarillo, CA, USA).

**3. Observation of ultrastructure of HUVECs by transmission electron microscopy.** The HUVECs were trypsinized and collected into an Eppendorf tube after washing. They were washed by PBS, fixed by 3% glutaraldehyde at 0°C-4°C, then dehydrated by dimethylketone. After embedment in Epon-812, the samples were cut into ultrathin sections (70nm). The ultrathin sections were dyed with uranium acetate and plumbum citrate. They were examined with JEM-100sX electron microscopy.

**4. Assessment of cell apoptosis by flow cytometry.** The HUVECs were treated as indicated above. At the end of the incubation period, cells were harvested by trypsinization and washed twice with cold PBS. Briefly, cells were re-suspended in 500µl binding buffer, 5µl Annexin V-FITC and 5µl Propidium Iodide were added and mixed gently. After incubation for 5 min, the cells were analyzed by flow cytometry (Beckman Coulter Epics XL-4, Brea, CA).

**5. Assays of mRNA levels of VEGF by quantitative real-time polymerase chain reaction (RT-PCR).** In accordance with the manufacturer's instructions (TaKaRa Corp, Shiga, Japan), total RNA was harvested with RNAisoTM Plus (TaKaRa Corp). For the reverse transcriptase polymerase chain reaction (RT-PCR), 2 mg of RNA was reverse-transcribed into complementary

DNA (cDNA) by incubating with 1 ml of reverse transcriptase (TaKaRa Corp) in 20 µl of reaction buffer containing 0.1 nM of random 6 mers, 0.05 pM of Oligo dT primer, and 5×Prime Script™ buffer at 37°C for 15 min and 85°C for 15 sec. The PCR reaction system (20µl) contains 0.4µl of cDNA, 10 µl of SYBR Premix Ex Taq™ (2×), 0.2µl of Rox Reference Dye (2×), 0.4µl of forward primer (10µ M) and reverse primer (10µM) of VEGF or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and double-distilled water. The PCR was performed in accordance with the two-step procedure of ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems Corp, Foster

City, CA), which contains stage 1 of predegeneration, 95°C for 30 sec, and stage 2 of PCR reaction, 40 cycles of 95°C for 15 sec and 60°C for 1 min. The PCR products were scanned with an ultraviolet gel imaging system, and fluorescence intensity was analyzed by 7500 System SDS software. Compared with GAPDH product, the expression level of detected mRNA transcription was determined. The primers were as follows:

VEGF: forward: 5'-TGCAGTTATGCGGATCAAACC-3';

reverse: 5'- TGCATTCACATTTGTTGTGCTGTA-3'.

GAPDH: forward: 5'- TCCACCTCCAGCAGATGTGG-3';

reverse: 5'- GCATTTGCGGTGGACGAT-3'.

**6. Statistical analysis.** Data are shown as the mean±standard deviation (SD). Statistical analyses were performed with Statistical Package for Social Sciences

(SPSS) software, version 16.0, One-way analysis of variance (ANOVA) was used to determine the statistical significance of differences. The level of statistical significance was set at 0.05.

**Results**

1. Downregulation of serum levels of VEGF after PDL treatment in IH children and VEGF mRNA expression in HUVECs

The serum levels of VEGF in the different patient groups were compared (Table 1). The VEGF levels in proliferative hemangiomas group were significantly higher than those in the involuting and involuted hemangiomas (p<0.05) and in the control group (p<0.05). As shown in Table 2, Figure 1 and 2, after 3 laser treatment, accompany with the improvement of local lesions, such as regression or cessation of growth, shrinkage or flattening of the lesion, and lightening of the surface color, serum levels of VEGF decreased significantly (p<0.05), compared with those before laser treatment.

**Table 2.** The changes of plasma levels of VEGF in IH children ( $\bar{x}\pm S$ , n=23)

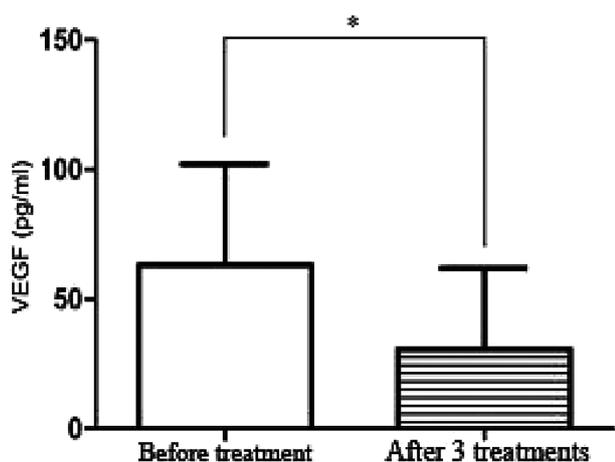
	Before treatment	After 3 treatments
Age (months)	3.79±1.86	6.31±1.76
Male/Female	8/15	8/15
Weight (Kg)	7.67±2.39	9.13±2.39
Size (cm <sup>2</sup> )	9.46±10.99	9.88±8.53
VEGF (pg/mL)	63.22±38.84	30.74±31.22

**Table 1.** The plasma levels of VEGF in IH children ( $\bar{x}\pm S$ )

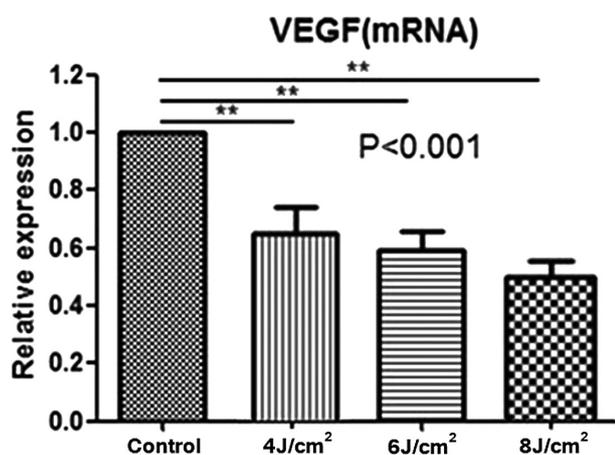
	Proliferating phase (n=45)	Involuting phase (n=18)	Involuted phase (n=11)	Control group (n=10)
Age (months)	3.88±2.16	9.17±2.68	19.18±5.80	7.83±6.32
Male/Female	16/29	7/11	7/4	5/5
Weight (Kg)	7.05±1.54	9.92±2.87	11.32±1.66	8.05±2.05
Size (cm <sup>2</sup> )	6.67±6.94	7.44±11.52	0	0
VEGF (pg/mL)	268.64±256.16	42.89±37.90	25.55±21.76	26.50±23.50



**Figure 1.** 2-month-old girl before, during, and after treatment with PDL therapy. A. Segmental hemangioma on the inner left thigh at the age of 2 month, before treatment. B. Significant improvement in the IH could be seen after 3 times of PDL treatment. C. With fading of surface color and shrinkage of lesion, near-complete clearance of superficial IH achieved after receiving 9 PDL sessions.



**Figure 2.** Serum levels of VEGF in the same patients before and after laser treatment. After 3 laser treatment, serum levels of VEGF decreased significantly ( $P < 0.05$ ), compared with those before laser treatment.



**Figure 3.** Effect of PDL irradiation on mRNA expression of VEGF. HUVECs were irradiated using PDL as described in the Methods section. Twenty-four hours after irradiation, HUVECs were harvested for RNA isolation and RT-PCR. PDL irradiation energy-dependently decreased the mRNA level of VEGF, statistical significance was obvious compared with the control group ( $p < 0.05$ ).

## 2. Downregulation of VEGF mRNA expression in HUVECs 24h after PDL irradiation

The VEGF mRNA expression of HUVECs decreased apparently after PDL irradiation in comparison with the control group ( $p < 0.05$ ). As the energy increase, the expression of VEGF mRNA decreased more significantly, in an energy-dependent manner.

## 3. Apoptosis of HUVECs exposed to laser treatments

Considering PDL treatments could inhibit the proliferation of IH, we investigated its role in apoptosis induction of HUVECs. The apoptosis rates were detected with flow cytometry after Annexin V/PI staining. The apoptosis rate for the 4 J/cm<sup>2</sup> group was 0.56%, and the apoptosis rates were increased to 1.71 and 3.55% at the 6, 8 J/cm<sup>2</sup> group respectively (Figure 4). A significant

difference was observed compared to the control cells ( $p < 0.05$ ).

## 4. Ultrastructure changes of HUVECs after PDL irradiation

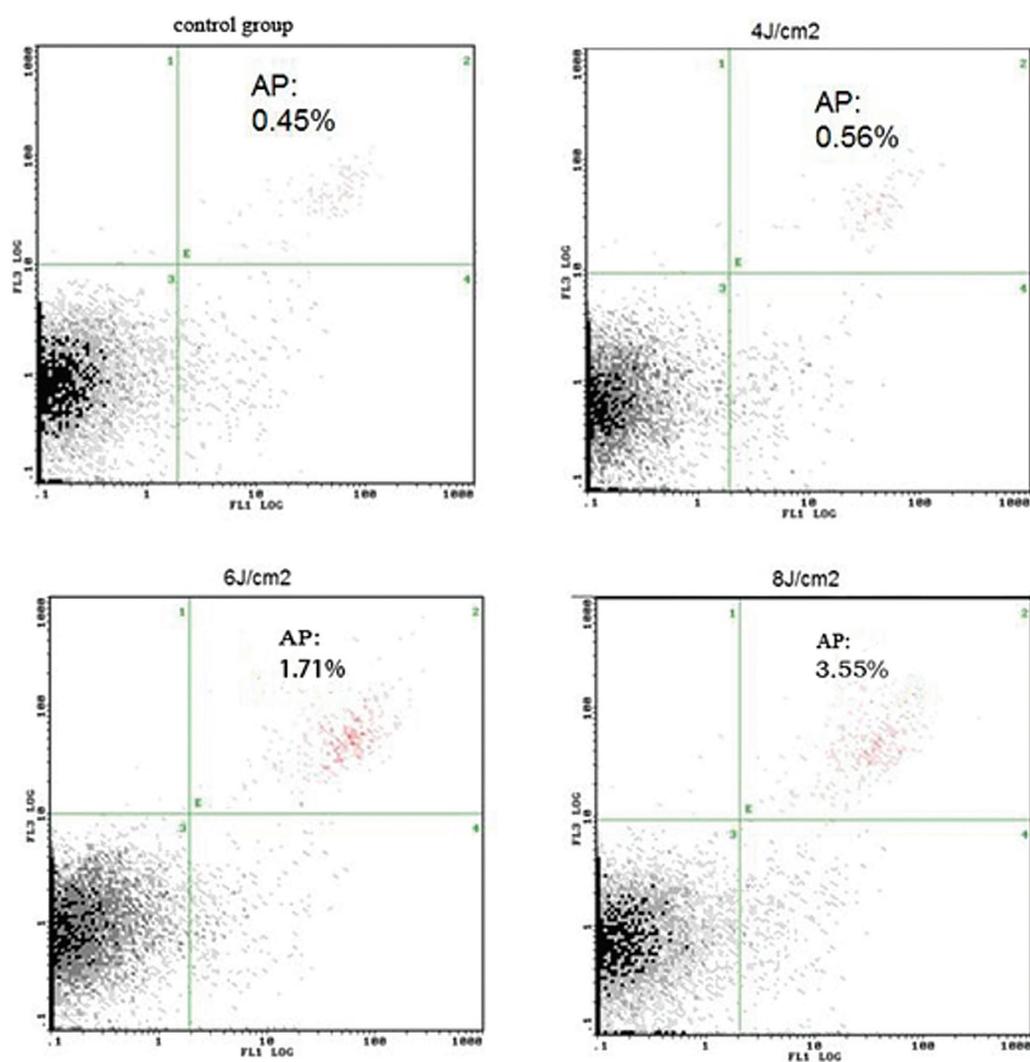
The transmission electron microscope study (Figure 5) indicated that the cells showed more apoptosis characteristics after the PDL irradiation. Serrated nucleus, chromatin condensation, swollen mitochondria and more bubbles in the cytoplasm were obviously seen under TEM, compared with the control group.

## Discussion

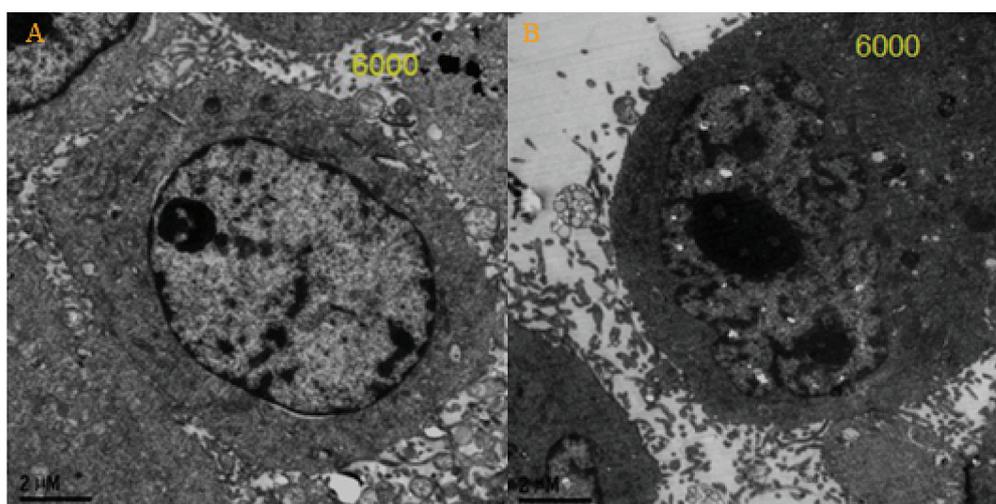
The goal of laser treatment of hemangiomas is to maximize vascular damage while minimizing dermal and epidermal injury. In spite of the expanding role of  $\beta$ -blockers for treatment of infantile hemangiomas, there are still irreplaceable advantages for laser therapy for IH. One advantage of PDL treatment lies in decreasing the proliferative phase of IH and increasing the rate of involution, with the added benefit of no systemic side effects<sup>7</sup>. PDL can be used to treat ulcerated hemangiomas both acutely and early to hasten healing of the troublesome and painful ulceration. As one of efficient measures for precursor lesions and small superficial hemangiomas, PDL treatment should be used at the earliest sign of hemangioma as soon as possible in the proliferative phase. Especially in anatomically or cosmetically sensitive areas such as perineum, periocular areas and the extremities, early laser treatment can safely and effectively diminish proliferative growth of superficial IH. To this day, the most commonly used PDL systems are generally considered the first treatment of choice for hemangiomas and portwine stains in pediatric population<sup>8</sup>. Used for many years as a safe and effective option, it was well known that PDL treatment could limit superficial proliferation and increase complete clearance rates of IH<sup>9,10</sup>, but the reports about the cellular mechanism of PDL are seldom seen.

The tissue reaction to the laser treatment is not only described by microvascular destruction but with a more presumable induction of apoptosis secondary to an inflammation process. Our scanning electron microscopy results showed nuclear condensation and fragmentations were visualized, the presence of swollen mitochondria and more bubbles were revealed in the laser treatment group. The ultrastructural changes showed that the PDL irradiation had a significant apoptosis-inducing effect on HUVECs, and the irradiation had a direct impact on cells.

In addition to subcellular structure changes of PDL



**Figure 4.** Cell apoptosis rates were assessed with flow cytometry 24 h after PDL irradiation. The apoptosis rates were increased to 1.71 and 3.55% in the group of 6, 8 J/cm<sup>2</sup> respectively. A significant difference was observed compared to the control cells ( $p < 0.05$ ).



**Figure 5.** Ultrastructure of HUVECs after PDL irradiation observed by transmission electron microscope. A. The control group. B. The PDL irradiation group; serrated nucleus, swollen mitochondria and more bubbles were obviously seen in the cytoplasm of the cells after PDL irradiation, compared with the control group ( $\times 6000$ ).

irradiation, the present study showed the PDL irradiation could increase the apoptosis rate of HUVECs. In the current study, the elevated apoptosis rates with flow cytometry after PDL irradiation are consistent with ultrastructural changes of HUVECs. The irradiated cells are inclined to initiate apoptosis, also repress the VEGF production of HUVECs. Furthermore, the apoptosis phenomena of HUVECs provide structural bases for the lower expression of VEGF mRNA.

Despite multiple biochemical, pathologic, and genetic studies, the mechanisms underlying growth and subsequent involution of infantile hemangiomas remain unclear. Impaired balance between proangiogenic and antiangiogenic factors has been implicated in the development of hemangiomas. VEGF is the one of the most potent angiogenic factor involved in hemangioma growth, which increases vascular permeability, stimulates endothelial cell proliferation and prevents endothelial cell apoptosis. The serum VEGF concentrations in children with proliferating hemangiomas have been confirmed significantly higher than in patients with involuting hemangiomas, vascular malformations and healthy patients<sup>11</sup>. The circulating levels of VEGF have been proposed as reflections of the angiogenic activity and prognosis in hemangiomas patients. In this report, the changes of serum VEGF concentration in 23 patients with proliferating hemangiomas were dynamically observed before and after the treatment, compared with 18 patients with involuting hemangiomas, 11 patients with involuted hemangiomas and 10 negative control subjects. After laser treatment for 3 times, the level of VEGF had fallen 51.4% ( $p < 0.05$ ), although the mean lesion size had not decreased significantly. This indicated that after laser treatment, the VEGF concentration decreased prior to hemangiomas volume reducing. And our results are consistent with the changes of VEGF level after oral propranolol or steroid therapy<sup>12</sup>.

Up to now, VEGF serum level is regarded by many studies as the most sensitive and convenient objective indicator for the treatment of IH<sup>13</sup>. However, Vivian T's reports<sup>14</sup> showed that they did not find VEGF expression changes in eight angioma biopsies before and 7 days after PDL treatment, or angioma compared to normal skin by immunohistochemistry staining. Reasons accounted for this might include that their samples were little, and detection time was 7 days after PDL treatment, inconsistent with other reports. Our results in IH patients confirmed that in the process of laser treatment, along with regression or cessation of hemangioma growth, the VEGF serum level significantly decreased. Additionally,

we found that changes of serum VEGF levels in IH children receiving PDL treatment are consistent with the decreased mRNA expression of this cytokine in HUVECs exposed to PDL irradiation. The serum VEGF levels are partly influenced by the local lesion's cellular VEGF concentration at least. That is to say, serum VEGF levels variations may reflect the developing trend of hemangiomas.

Our results confirm the prognostic role of VEGF in IH, which show if a patient was sensitive to laser treatment, satisfying results would achieve in a short time, accordingly the downregulation of VEGF serum level was obvious. On the contrary, if a patient's VEGF level did not reduce significantly, he might accept more treatments than others. For those patients, combination treatments may be more advisable, such as oral propranolol or steroid therapy, even pingyungmycin local injection. Combination treatments may have potential benefits, including possibilities of greater efficacy, synergistic effects, and lower toxicity. Reddy's retrospective studies showed that facial-segmental IH treated with propranolol and PDL displayed more rapid and complete clearance and required a lower cumulative propranolol dose to achieve near-complete clearance<sup>15</sup>. Although there are various choices for IH treatment, in appropriately selected cases, mono or adjuvant therapy with PDL can lead to more rapid response and may decrease the risk of more serious sequelae.

## Conclusions

At first, the present study demonstrates that PDL irradiation imparts an apoptosis-inducing effect on HUVECs in vitro, and serum VEGF levels of IH children decrease significantly after receiving PDL treatments. PDL irradiation can promote cell apoptosis rate, decrease VEGF mRNA expression. This provides valuable evidence regarding the mechanism of PDL treatment on IH in vivo. Although various previous studies have demonstrated the laser treatment effects on infantile hemangioma by providing clinical and histologic evidence, the exact molecular mechanism underlying IPL irradiation remains to be elucidated. Secondly, we confirm that serum VEGF levels of IH children decrease significantly after receiving PDL treatments and suggest that the serum VEGF level detection may be of particular importance in estimation of laser treatment and even an objective indicator for prognosis. Having advantages of minor injury and convenient operation, serum VEGF measurement of IH children may be a useful tool for

predicting hemangioma course and monitoring the effectiveness of laser treatment.

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The authors declare that there are no conflicts of interest.

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