Life Sciences 233 (2019) 116685

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Effects of laser irradiation on growth factors and cell apoptosis of *in vitro* cultured infant hemangioma endothelial cells

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ARTICLE INFO

Keywords:
Laser
Infant hemangioma
Growth factors
Cell apoptosis
In vitro

ABSTRACT

Aims: The present study aimed to investigate the effects of laser irradiation on the growth factors and cell apoptosis of *in vitro* cultured infant hemangioma endothelial cells.

Main methods: Endothelial cells of infant hemangioma were cultured *in vitro* and irradiated using a variable pulse width 1064 nm Nd:YAG laser and intense pulsed light (IPL), the expression of VEGF, VEGFR-2, bFGF and their mRNAs before and after irradiation were measured by ELISA, western blot, RT-PCR and flow cytometry, and changes in the apoptotic rate of endothelial cells in hemangioma were monitored.

Key findings: The mRNA and protein expressions of VEGF, VEGFR-2, bFGF in hemangioma endothelial cells were inhibited by both Nd:YAG laser and ILP compared to the control cells. The apoptotic rates of hemangioma endothelial cells were also decreased after both laser irradiation treatments in comparison to the blank group. The differences were statistically significant.

Significance: Laser irradiation treats hemangioma not only through a selective photothermal mechanism, but also through cytokine signaling pathways.

1. Introduction

Some present studies have concluded that the mechanism of laser treatment of hemangioma is the “principle of selective photothermal interaction”. However, in recent years, with the deepening of basic research on hemangioma, the role of endothelium-related growth factor in the development and apoptosis of infant hemangioma has attracted increasing attention [1–10]. The previous studies conducted by the investigators revealed that propranolol could inhibit the growth activity of cytokines, and the growth factor and apoptosis of *in vitro* cultured hemangioma endothelial cells, and that this had a certain correlation with action duration and drug concentration [11,12]. Therefore, it was speculated that in the laser treatment of infant hemangioma, in addition to the “selective photothermal interaction”, the laser treatment can also inhibit the growth of hemangioma endothelial cells through other mechanisms, thereby playing a role in treatment. In the present study, we compared the effects of intense pulsed light (IPL) and a long wavelength Nd:YAG laser on the regulation of cytokine signaling in infant hemangioma endothelial cells. The levels of vascular endothelial growth factor (VEGF) and its receptor were measured at different time points before and after irradiation. We also detected the expression of an angiogenic factor, basic fibroblast growth factor (bFGF), at designated time points. Moreover, the apoptotic rate was measured before and after irradiation to further investigate the mechanism of laser treatment in hemangioma, laying a foundation for exploring better treatment for hemangioma in the future.

2. Experimental materials

2.1. Experimental cells

The infant hemangioma endothelial cells originated from primarily cultured infant hemangioma endothelial cells in previous experiments (Cite the reference: YALIN ZHU, AERZIGULI TUERXUN, YAN HUI and PARIDE ABLIZ. Effects of propranolol and isoproterenol on infantile hemangioma endothelial cells *in vitro*. Experimental and Theraputic Medicine, 2014, 8(2):647–651). The present study was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University. The *in vitro* cultured infant hemangioma endothelial cells were identified by immunohistochemistry, and the purity was determined as 75%. The cell culture conditions are as follows: EGM-2 medium, 37 °C, 5% CO^2, and saturated humidity.

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https://doi.org/10.1016/j.lfs.2019.116685
Received 30 May 2019; Received in revised form 15 July 2019; Accepted 22 July 2019
Available online 23 July 2019

Table 1
ELISA data analysis of infant hemangioma endothelial cells at different time points after different laser irradiations ($\bar{x} \pm s$, n = 9).

	VEGF(pg/ml)			bFGF(pg/ml)		
	1d	3d	7d	1d	3d	7d
Control	339.458 ± 4.396	344.003 ± 4.127	334.506 ± 13.084	8.661 ± 0.590	9.241 ± 0.136	9.165 ± 0.232
Intense pulsed light	267.307 ± 23.148	195.629 ± 14.504	128.858 ± 6.063	6.533 ± 0.378	4.684 ± 0.254	2.723 ± 0.471
l064 nm Nd: YAG laser	250.204 ± 3.273	159.641 ± 13.458	69.389 ± 24.179	5.915 ± 0.315	4.369 ± 0.445	2.386 ± 0.151

2.2. Major reagents

Fetal bovine serum (FBS) was purchased from Life Technologies Inc. (USA). EGM-2 (CC-4176, LONZA), 0.25% trypsin (Trypsin-EDTA), and phosphate buffer powder for phosphate buffered saline (PBS) were purchased from Beijing ZSGB-BIO (China). The penicillin-streptomycin double antibody (10,000 U) was purchased from GIBCO (USA). The annexin V-FITC apoptosis detection kit was purchased from BD (USA).

3. Experimental methods

3.1. Experimental grouping

According to the experimental design, these cells were divided into three groups: blank group, intense pulsed light (IPL) irradiation group and laser irradiation group. The laser energy was determined by a previous pre-experiment. For each group, four repeated wells were set:

Blank control group:

IPL irradiation group: the irradiation intensity was 23 j/cm2.

Laser irradiation (Nd:YAG) group (l064 nm Nd: YAG laser): the irradiation intensity was 90 j/cm^2.

3.2. Laser intervention

Cells in the logarithmic growth phase were obtained, and cultured at 37 °C with 5% CO^2 and saturated humidity for 24 h. When cell adherence and fusion reached 80% in the culture flask (25 cm^2), the supernatant was discarded, adherent cells were washed with PBS twice, 5 ml of complete medium was added, and cells were irradiated using a laser, according to the above experimental groups. Each group had four repeated wells. The detection was carried out on post-irradiation day 1, 3 and 7, and the detection time points were determined by previous pre-experiments. Cells were obtained at the corresponding time points, the supernatant was discarded, the adherent cells were washed with 3 ml of PBS twice per flask, PBS was discarded, 1 ml of Trizol was added to lyse cells for 10–15 min, cells were transferred to 1.5-ml EP tubes, and the serial numbers were labeled for detection.

3.3. Detection method

The expression of VEGF and bFGF was detected by enzyme-linked immunosorbent assay (ELISA). The mRNA expression of VEGF, vascular endothelial growth factor receptor-2 (VEGFR-2) and bFGF-1 were detected by polymerase chain reaction (PCR). The expression of VEGFR-2 was detected by western blot. The cell apoptosis rate was detected by flow cytometry. The specific detection methods could be found in the kit.

4. Statistics analysis

All data were expressed as mean ± standard deviation (x ± SD). SPSS 19.0 software was used for the statistical analysis of the levels of the biochemical indexes in ELISA and in each group. If data was normally distributed, these were compared using univariate analysis of variance. If the variance was homogeneous, the least significant difference (LSD) method was used for multiple comparisons. If the variance was not homogeneous, the Tamhane method was used for multiple comparisons. $P < 0.05$ was considered statistically significant. If the data was not normally distributed, the data was first logarithmically transformed to make the data normally distributed. Then, the data was statistically analyzed according to the above univariate analysis of variance. GraphPad Prism 5.0 was used to assist in the plotting.

5. Experimental results

5.1. Changes in the expression of VEGF and bFGF at different time points after laser irradiation

ELISA was performed to detect the expression of VEGF and bFGF at different time points before and after irradiation with IPL and a 1064-nm Nd:YAG laser. The results revealed that after IPL and Nd:YAG laser irradiation, the levels of VEGF and bFGF significantly differed from those in the blank group ($P < 0.05$). On post-irradiation day one, the expression of VEGF in the IPL irradiation group had no significant change, when compared to the blank group. However, on day 3 and 7, the difference in levels of VEGF and bFGF between these two groups were statistically significant after irradiation using the two laser techniques ($P < 0.05$). Furthermore, the reduction in the expression of VEGF and bFGF were significantly lower in the Nd:YAG group, when compared to the IPL irradiation group, and the differences were statistically significant ($P < 0.05$, Table 1). In addition, over time, the reduction in VEGF and bFGF was more obvious, while the levels of VEGF and bFGF did not change in the blank group at the same time point ($P > 0.05$). Hence, it can be speculated that after IPL and 1064-nm Nd:YAG laser irradiation on *in vitro* cultured hemangioma endothelial cells, the expression of VEGF and bFGF were both inhibited, and for a certain period of time, the inhibition effect became more obvious over time (Figs. 1-2).

5.2. Western blot results

Western blot was used to detect the expression of VEGFR-2 after IPL and Nd:YAG laser irradiation. The results revealed that both laser irradiation methods could downregulate the expression of VEGFR-2 (Fig. 3), and the difference was statistically significant. However, the difference in the inhibition of these two laser irradiation methods on VEGFR-2 was not statistically significant. Although the expression of VEGFR-2 did not significantly change on day 3, the expression of

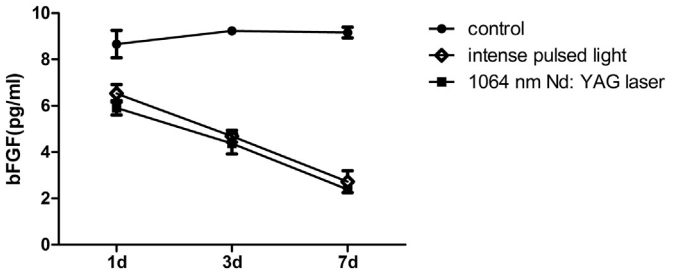

Fig. 1. Chart of the bFGF level.

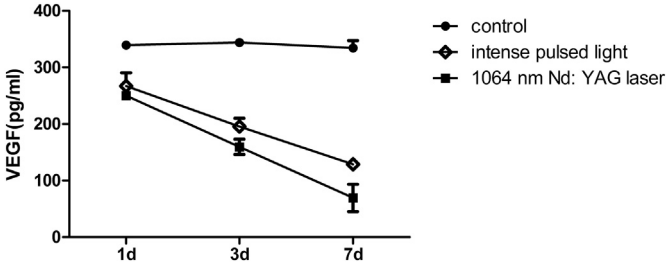

Fig. 2. Chart of the VEGF level.

VEGFR-2 was downregulated on day seven (Fig. 4).

5.3. Reverse-transcription polymerase chain reaction (RT-PCR) experimental results

The expression of VEGF mRNA, and the mRNAs of its receptor VEGFR-2 and bFGF-1 were detected by RT-PCR. The result revealed that after IPL and Nd:YAG laser irradiation, the mRNA expression of all these genes decreased, when compared to those in the blank group. Furthermore, on post-irradiation day 3 and 7, the inhibition of the Nd:YAG laser on the mRNAs of all these genes were more significant, and the differences were statistically significant (Table 2).

This result revealed that in the blank group, the expression of all these genes did not significantly change on day 1, 3 and 7. However, after IPL and Nd:YAG laser irradiation, the results of the measurements revealed that after IPL irradiation, the expression was lower to a certain extent on day three than on day one, and lower to a certain extent on day seven than on day three, and the differences were statistically significant. A similar change occurred after Nd:YAG irradiation. This suggests that both IPL and Nd:YAG can decrease the mRNA expression of VEGF, VEGFR-2 and bFGF, and for a certain period of time, this inhibitory effect persists with the prolongation of treatment time (Fig. 5).

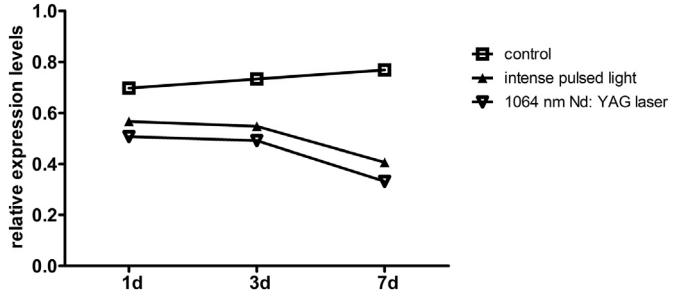

Fig. 4. Line chart of the relative expression level of VEGFR-2 protein in infant hemangioma endothelial cells after different laser irradiations. Bands of three repetitions from the same group were shown in each representative image.

5.4. Experimental results for the apoptosis of cells

The apoptotic rate of hemangioma endothelial cells was detected by flow cytometry after two laser irradiations. The result revealed that on post-irradiation day 3 and 7, the apoptotic rates increased in the Nd:YAG group and IPL irradiation group, and the difference between these two groups and the blank group was statistically significant. Within a period of time after irradiation, the upregulation effects of laser on hemangioma endothelial cells between these two groups differed, but the general trend was the increase in apoptotic rate for hemangioma endothelial cells. The apoptotic rate in the blank group did not change among these time points (Figs. 6-7).

6. Discussion

Hemoglobin has absorption peaks at 417, 542 and 577 nm. After laser irradiation on the blood vessels, hemoglobin selectively absorbed the energy of the laser and heated the blood to 70 °C, thereby achieving the purpose of destroying the function of blood vessels. This is the principle of selective photothermal interaction. At present, the laser wavelengths for vascular dermatosis are 585 and 532 nm. Oxyhemoglobin has another absorption peak at 800–1100 nm.

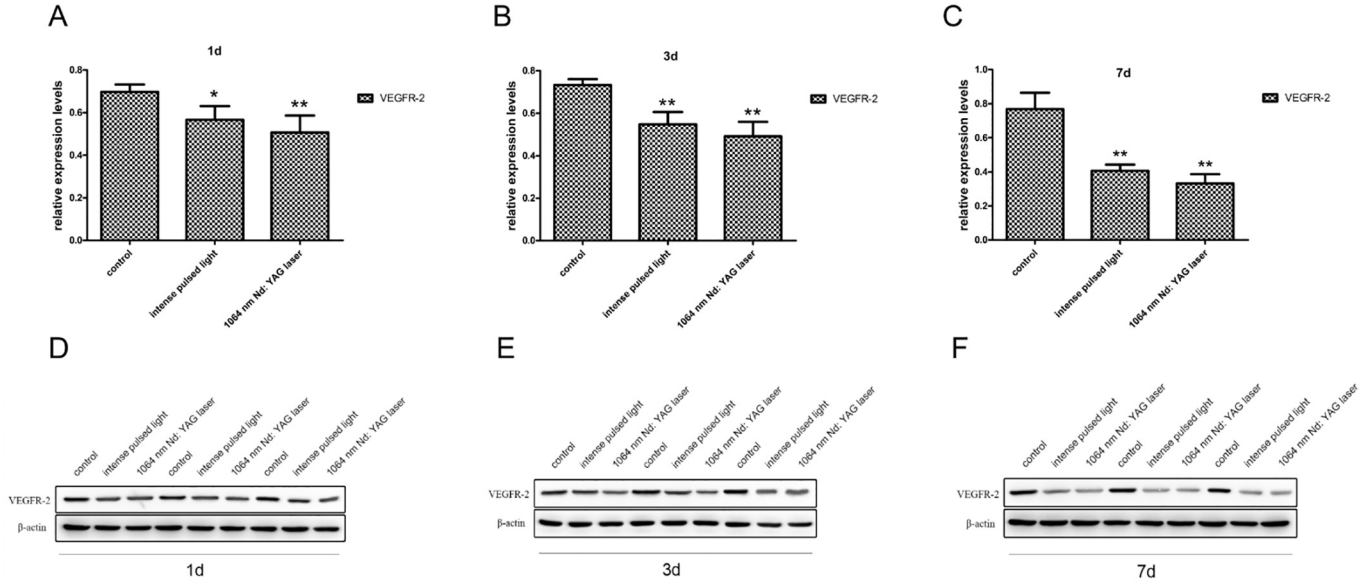

Fig. 3. Histogram and band image of the relative expression level of VEGFR-2 protein.
Notes: *Compared with the blank group, $P < 0.05$, $P < 0.01$; **Compared with the blank group, $P < 0.01$. (A) Histogram of the relative expression level of VEGFR-2 protein on post-irradiation day one. (B) Histogram of the relative expression level of VEGFR-2 protein on post-irradiation day three. (C) Histogram of the relative expression level of VEGFR-2 protein on post-irradiation day seven. (D) Band image of the relative expression level of VEGFR-2 protein on post-irradiation day one. (E) Band image of the relative expression level of VEGFR-2 protein on post-irradiation day three. (F) Band image of the relative expression level of VEGFR-2 protein on post-irradiation day seven.

Table 2
Data analysis of the relative mRNA expression levels of genes in infant hemangioma endothelial cells at different time points after different laser irradiations ($\bar{x} \pm s$, n = 9).

	VEGF-mRNA			VEGFR-2-mRNA			bFGF-mRNA		
	1d	3d	7d	1d	3d	7d	1d	3d	7d
Control	1.000 ± 0.021	1.000 ± 0.015	1.000 ± 0.023	1.000 ± 0.017	1.000 ± 0.017	1.001 ± 0.031	1.000 ± 0.008	1.000 ± 0.023	1.000 ± 0.004
Intense pulsed light	0.829 ± 0.020	0.630 ± 0.007	0.436 ± 0.041	0.797 ± 0.052	0.686 ± 0.062	0.493 ± 0.037	0.743 ± 0.043	0.628 ± 0.042	0.490 ± 0.044
l064 nm Nd: YAG laser	0.803 ± 0.090	0.560 ± 0.046	0.363 ± 0.021	0.808 ± 0.046	0.655 ± 0.051	0.483 ± 0.017	0.739 ± 0.008	0.548 ± 0.022	0.402 ± 0.040

Although its absorptivity is lower than those at 585 and 532 nm, its penetration is stronger. Therefore, both the 1064-nm Nd:YAG laser and IPL (560 nm) can play a role in the treatment of infant hemangioma. However, in addition to the principle of selective photothermal interaction, it remains to be determined whether it has any other mechanism. The high expression of angiogenic factors, such as VEGF and bFGF, in the body and tissues of infants are the main pathogenesis of proliferative hemangioma [13–17]. In particular, VEGF is the most potent [18–20]. Changes in VEGF and bFGF play an important role in the occurrence and development of hemangioma. The previous studies conducted by the investigators also revealed that propranolol can inhibit the expression of β receptors and cytokines, such as VEGF and bFGF, on the surface of *in vitro* cultured hemangioma endothelial cells [11,12]. Laser irradiation has a significant effect on the growth curve of VEGF and bFGF. The growth curve of *in vitro* cultured hemangioma endothelial cells significantly changed after the application of these two laser irradiation methods. Therefore, it was speculated that the mechanism of the laser treatment of hemangioma is not only the "principle of selective photothermal interaction", laser also the important role of affecting cytokines that affect the growth of hemangioma endothelial cells.

In the present study, a 1064-nm Nd:YAG laser and IPL were used for the irradiation of *in vitro* cultured hemangioma endothelial cells. The expression of VEGF, VEGFR-2, bFGF, VEGF mRNA, VEGFR-2 mRNA and bFGF mRNA were detected at different time points before and after irradiation. Then, the apoptosis of hemangioma endothelial cells was detected to further investigate the molecular biological mechanism of the laser treatment for hemangioma.

1. The present study revealed that after irradiation with a 1064-nm Nd:YAG and IPL laser, both methods could reduce the expression of VEGF, VEGFR-2 and bFGF, and both were correlated to time to a certain extent. After the application of these two laser irradiation methods, the expression of VEGF and bFGF did not significantly change on post-irradiation day one. Furthermore, on post-irradiation day 3 and 7, the expression continued to decrease, and the difference in the effects of laser irradiation on the expression between these two groups were not statistically significant. The expression of VEGFR-2 changed on post-irradiation day seven, but this expression was inhibited. These reveal that the Nd:YAG laser and IPL not only plays a role in the treatment of hemangioma through the absorption of hemoglobin, but also plays a role in the treatment of hemangioma by inhibiting the expression of VEGF, VEGFR-2 and bFGF, and the inhibition persisted with time. However, further studies are needed to determine how long this specifically lasts.

2. The present study revealed that after irradiation with the 1064 nm Nd:YAG and IPL laser, both methods could affect the mRNA expression of upstream VEGF, VEGFR-2 and bFGF. After the irradiation of these two laser methods, the expression of the above factors were downregulated, when compared with the blank group. On post-irradiation day one, the difference in the effect of the laser on mRNA expression between the two groups was not statistically significant. On post-irradiation day 3 and 7, the inhibition on the mRNA expression was stronger in the Nd:YAG group, when compared to the IPL group. These reveal that laser irradiation can inhibit VEGF, VEGFR-2 and bFGF by inhibiting their upstream mRNA.

3. In the present study, the apoptotic rates of hemangioma endothelial cells were compared before and after irradiation, and the results revealed that both laser irradiation methods could increase the apoptotic rate of hemangioma endothelial cells. However, the difference between these two laser irradiation groups was not statistically significant.

7. Conclusions

In summary, (1) after the IPL and 1064-nm Nd:YAG laser irradiation on *in vitro* cultured hemangioma endothelial cells, growth factors, such as VEGF, VEGFR-2, bFGF and their upstream mRNA, were inhibited. This reveals that the therapeutic effect of the laser on hemangioma is not only through "the principle of selective photothermal interaction", but also the exertion of its therapeutic effects through influencing cytokine signaling pathways and the apoptotic rate, and these inhibitory effects lasts for a certain period of time. The present study revealed that the difference in effects on related factors of vascular endothelial cells between these two laser methods was not statistically significant. Therefore, during treatment, these two can be combined according to the need of treatment, in order to take advantages of the depth penetration of the long wavelength and the good selectivity of the short wavelength, with the hope of achieving a better effect in the treatment

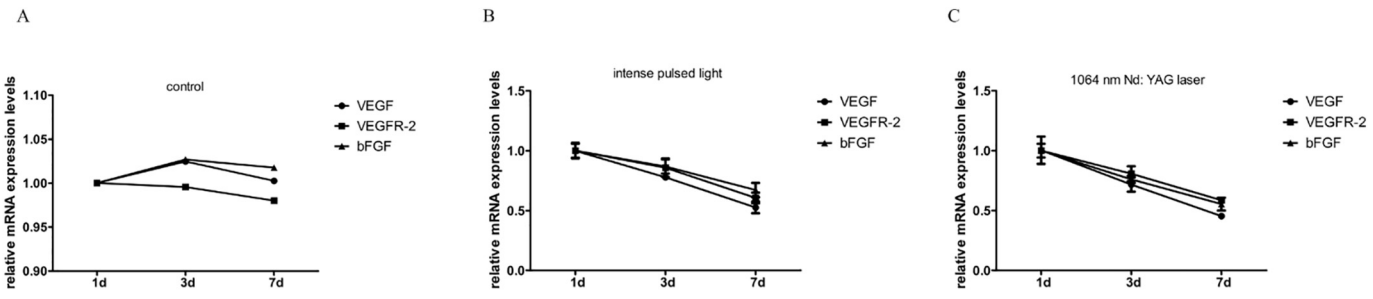

Fig. 5. Charts of the relative mRNA expression level of genes in hemangioma endothelial cells in the different groups on post-irradiation day 1, 3 and 7. (A) Blank group; (B) Intense pulsed light (IPL) irradiation group; (C) laser irradiation (Nd:YAG) group.

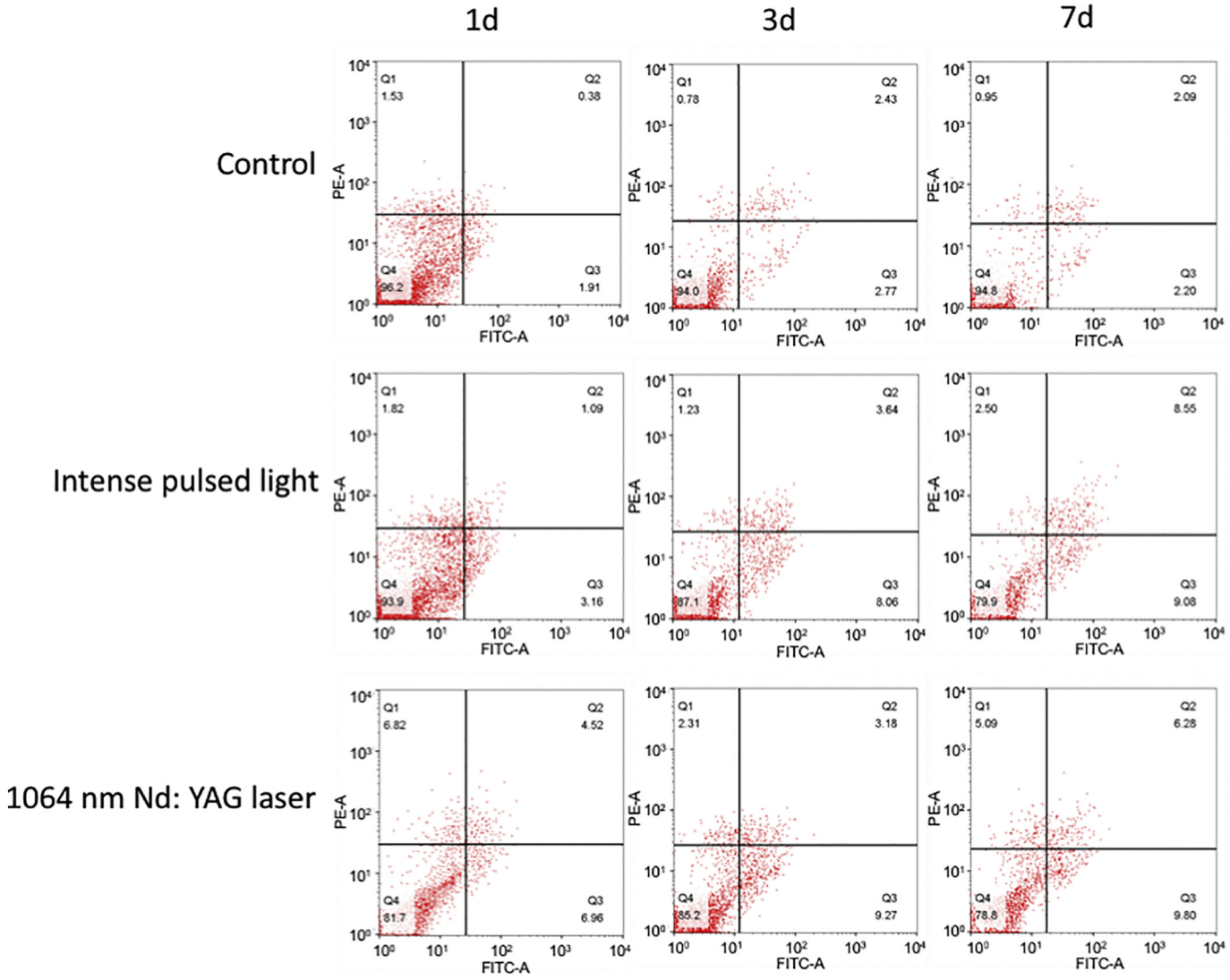

Fig. 6. The florescence-activated cell sorting plots of infant hemangioma endothelial cells at different time points after performing the different irradiation methods.

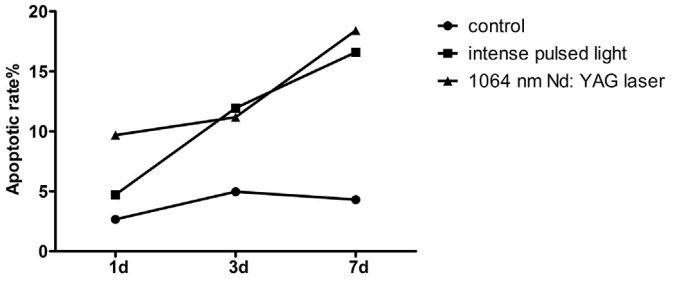

Fig. 7. Line chart of the apoptotic rate of infant hemangioma endothelial cells at different time points after performing the different irradiation methods.

of vascular dermatosis. However, it remains to be further studied whether the combination of these two approaches in the treatment of hemangioma can induce a synergistic inhibition on cytokines. After laser irradiation, the inhibition on hemangioma endothelial cell factors lasted for some time. This result suggests that there is no need to rush for repetitive treatment after laser treatment, because the inhibition persists after treatment. This is consistent with the time interval required for current laser therapy. However, further studies are needed to determine how long the inhibition of the laser specifically lasts. (2) It was revealed in the present study that laser and drugs can treat hemangioma through the influence of growth factors, receptors and upstream mRNAs related to hemangioma. Therefore, the result suggests that the mechanism of occurrence and development of hemangioma is correlated to cytokine signaling pathways. As a result, it can be explained that although hemangiomas are presently classified as benign tumors derived from blood vessels, and these have no trend of metastasis, proliferation and invasion, and the logarithmic growth trend is similar to that of malignant tumors. Furthermore, these can also cause serious and even fatal effects on important organs around the tumor and their functions. Therefore, the result suggests that it is valuable to find new targets for the treatment of hemangioma, such as cytokine antagonists and the corresponding receptor antagonists, in order to curb its growth and development. The present study elucidates the effects of a 1064-nm Nd:YAG laser and IPL on cytokines and their upstream mRNAs to some extent. However, the effects of specific signaling pathways needs to be further investigated.

Funding

Joint Project of Natural Science Foundation of Xinjiang Province Uygur Autonomous Region, China (NO. 2016D01C257).

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