Acute effect of a variable pulse width Nd:YAG laser combined with hematoporphyrin monomethyl ether-mediated photodynamic therapy on a cockscomb model of nevus flammeus

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Conflict of interest

None declared

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Abstract

Background. Nevus flammeus (NF) is a congenital vascular malformation.

Objectives. To investigate the acute effect of a variable pulse width Nd:YAG laser combined with hematoporphyrin monomethyl ether (HMME)-mediated photodynamic therapy (PDT) on a cockscomb model of NF.

Material and methods. Forty-two leghorn roosters were randomly divided into the following 7 groups: group A1 (treated with HMME-mediated PDT; energy density of 75 J/cm²), group A2 (treated with HMME-mediated PDT; 125 J/cm²), group A3 (treated with HMME-mediated PDT; 150 J/cm²), group A4 (treated with HMME-mediated PDT; 175 J/cm²), group B (treated with a variable pulse width Nd:YAG laser), group C (treated with a variable pulse width Nd:YAG laser and HMME-mediated PDT), and group D (the control group). Changes in the cockscomb tissues were observed visually and microscopically on days 1, 3, 7, and 14 after treatment. The capillary reduction and the ratio of collagen type I to type III were examined.

Results. The response rate was higher in groups A3 and A4 than in group B. In group A, a higher energy density resulted in a higher response rate and a greater capillary reduction ($p < 0.05$ for all). However, we concluded that PDT at an energy density of 175 J/cm² is not suitable for treating NF, as severe tissue damage, markedly lower capillary numbers, and markedly higher collagen type I:III ratios were observed in the cockscombs treated at this energy density; instead, 150 J/cm² may be a more appropriate energy density. Moreover, HMME-mediated PDT at 150 J/cm² combined with a variable pulse width Nd:YAG laser achieved better treatment outcomes than PDT or a variable pulse width Nd:YAG laser alone ($p < 0.05$ for both).

Conclusions. Compared to PDT or a variable pulse width Nd:YAG laser alone, the combination of the 2 therapies achieved a better acute effect in treating a cockscomb model of NF, and 150 J/cm² may be a suitable energy density for PDT.

Key words: photodynamic therapy, laser, nevus flammeus, hematoporphyrin monomethyl ether

Introduction

Nevus flammeus (NF), also known as port-wine stain, is a congenital vascular malformation characterized by ectasia of capillaries and venules; it often occurs in dermal papillary and reticular layers.^{1,2} The disease does not resolve without treatment, and the incidence rate is about 0.4% in children.^{3,4} Treatment options for NF include laser therapy, cryotherapy and surgery.^{5–7} A pulsed dye laser, which is the most widely used therapy for NF, can achieve good results but may cause irreversible vessel injury, thereby increasing the risk of purpura. Moreover, there may be a recurrence after pulsed dye laser therapy, and it is ineffective in some NF cases.^{7,8} Therefore, it is essential to find a more effective method for the treatment of NF.

In recent years, photodynamic therapy (PDT) has been considered a good method for treating NF.⁹ In this therapy, photosensitizers can accumulate selectively in the vascular endothelial cells, and the recurrence rate is low. The greatest advantage of PDT is that it has high selectivity in the target tissue. Some studies have demonstrated that hematoporphyrin monomethyl ether (HMME) displays characteristic absorption peaks in multiple wavelengths, and a 532-nm laser is often used in PDT for treating NF.^{10,11} Therefore, a 532-nm continuous laser was chosen for our experiment. During PDT, the capillary malformation is targeted by substances generated from the reaction between the laser and photosensitizers. This therapy can be applied in large-area skin damage and offers a low recurrence rate.¹² However, most of the studies to date investigated only the effect of PDT on patients in clinical practice and no effective animal experiments were carried out; moreover, the energy densities applied in those studies were empirical doses. The effectiveness of PDT in the treatment of NF has been demonstrated in many studies.^{5,9,11,13} Different energy densities (75 J/cm², 100 J/cm² and 150 J/cm²) were investigated in some of these studies, but the effects of different energy densities on the treatment outcomes were not thoroughly compared. Therefore, in order to find a suitable energy density for treating NF, we compared 4 energy densities in our experiments. It has been revealed that a variable pulse width Nd:YAG laser can achieve good efficacy in treating some skin diseases.¹⁴ In this therapy, a 532-nm wavelength is often used to treat NF, as a laser at this wavelength can deeply penetrate the skin, cause a large area of thermal damage and achieve good coagulation; the effect of a variable pulse width Nd:YAG laser at a wavelength of 532 nm is especially noteworthy in the management of deep vascular malformation.^{15,16} However, no other study has been performed on the effect of combined variable pulse width Nd:YAG laser and PDT. Therefore, in this study, we investigated the effect of the combined use of the 2 methods and compared the efficacy of such use to the efficacy of a variable pulse width Nd:YAG laser or PDT alone.

Material and methods

Study subjects

Forty-two leghorn roosters (age: 8–9 months; weight: approx. 3 kg) were chosen as subjects. The cockscombs (thickness: 7–9 mm) were ruddy and without damage, necrosis or ulcers. The subjects were divided into the following 7 groups: group A1 (treated with HMME-mediated PDT; an energy density of 75 J/cm²), group A2 (treated with HMME-mediated PDT; 125 J/cm²), group A3 (treated with HMME-mediated PDT; 150 J/cm²), group A4 (treated with HMME-mediated PDT; 175 J/cm²), group B (treated with variable pulse width Nd:YAG laser), group C (treated with variable pulse width Nd:YAG laser and HMME-mediated PDT), and group D (the control group). The study was approved by the Animal Ethics Committee of our hospital.

The cockscombs of the leghorn roosters met the criteria for the disease model of NF.¹⁷

Treatment methods

Photodynamic therapy

The HMME is the most commonly used photosensitizer in PDT. For the treatment in our study, HMME (Fudan-Zhangjiang Bio-Pharmaceutical, Shanghai, China) was diluted with normal saline solution (10 mg/mL/kg). The subjects received chloral hydrate (2 mL/kg) orally for anesthesia, and the experimental area on the left side of the comb was marked in a circle with a diameter of 1.5 cm. The HMME was then injected intravenously into the root of the chicken wings in group A, and laser radiation with a power density of 150 mW/cm² was applied to the experimental areas. During the radiation, both the experimental and non-experimental areas were covered. The energy densities in groups A1, A2, A3, and A4 were 75 J/cm², 125 J/cm², 150 J/cm², and 175 J/cm², respectively.

Variable pulse width Nd:YAG laser

Before applying the variable-pulse frequency-doubled Nd:YAG (532 nm) laser, the subjects received chloral hydrate (2 mL/kg) orally for anesthesia, and the experimental areas on the combs were marked in the same way as that in the PDT groups. The energy density was 20 J/cm², the pulse-width was 10–50 ms and the spot diameter was 8 cm.

Combination therapy

After comparing the effects in the 4 PDT groups, we chose 150 J/cm² as the most suitable energy density and applied it with the variable pulse width Nd:YAG laser for the combination therapy. The PDT was first conducted, followed by variable pulse width Nd:YAG laser treatment 10 min later. The 2 procedures were carried out in the same way as described above.

Outcome measures

Main outcome measures

On days 1, 3, 7, and 14 after treatment, samples from the experimental areas of the combs were collected in each group for visual and microscopic inspection, and the non-experimental area was used as a control. The blood capillaries in the combs were observed under a microscope, and the percentage decrease of the capillary number was calculated with the following formula: percentage decrease of the capillary number = (the number of capillaries before treatment – the number of capillaries after treatment)/the number of capillaries before treatment × 100%.

Secondary outcome measures

On day 14 after treatment, 5 fields in the experiment area of each comb were randomly picked and drilled for immunohistochemistry staining to examine the levels of collagen types I and III. The samples were fixed with 10% formaldehyde, sectioned after paraffin embedment, and then dewaxed and hydrated. After antigen retrieval, the samples were blocked with serum and incubated with primary antibodies to collagen types I and III. Next, the samples were incubated with the secondary antibodies after rewarming. Then, they were stained with DAB followed by counterstaining with hematoxylin. The sections were then sealed for observation, and the ratio of collagen type I to type III were calculated.

The response rate in each group was observed and classified into 5 levels as listed in Table 1.¹⁸ The total response rate = the total number of samples with response rate in levels 1, 2 and 3/the total number of samples × 100%.

Adverse reactions in each group such as pigmentation, blistering and scar formation were recorded 14 days after treatment.

Statistical analysis

SPSS v. 19.0 software (IBM Corp., Armonk, USA) was used for the statistical analysis. The measurement data is expressed as means ± standard deviation (SD). Comparison between 2 groups was conducted using a t-test for independent samples. Measurement data at different time points between the 2 groups were compared with repeated-measures analysis of variance (ANOVA) and a Bonferroni post hoc test. A p-value <0.05 was considered to indicate a statistically significant difference.

Results

Results of visual inspection

After treatment, the color and morphology remained the same in the combs of group D and on the right side of the combs (opposite to the experimental side) in other groups. In contrast, changes were observed in the experimental areas of combs in groups A, B and C. In groups A1–4, the higher energy density resulted in a better clearance of NF. However, the energy density in group A4 was not optimal, as scars were formed on the combs in this group 14 days after treatment. Therefore, we chose the energy density from group A3 for PDT in the combination therapy. The results showed that the outcome in group C was much better than that of the other groups. In group C, 14 days after treatment, marked blanching was observed in some areas and no noticeable scars had formed (Table 2).

Results of microscopic observation

On days 1, 3, 7, and 14 after treatment, the combs in each group were observed under a microscope. Prior to the treatment, the structure of the stratum corneum and the dermis was intact in each group; however, changes were observed after treatment in the blood vessels in the experimental areas of groups A, B and C, whereas the combs in group D and the right side of the combs in other groups remained unchanged. As with the results of the visual inspection, good clearance of NF was achieved in group A4, but proliferative collagen fibers, which could lead to scars, were observed in this group. Thus, the energy density from group A3 was chosen for the combination therapy. Fourteen days after treatment in group C, the capillaries had almost disappeared, the epidermis had thickened, no blisters had formed, and the results of the treatment were much better than that of other groups (Table 3).

Capillary reduction in each group

Compared to group D at each time point, the other groups had a higher percentage of decrease in capillary number ($p < 0.05$ for all), suggesting that the treatment in these groups may have damaged the blood vessels. In groups A1–4, we found that a higher energy density resulted in a greater percentage of decrease in capillary number. Moreover, compared to groups B and A3, group C had a greater percentage decrease in capillary number ($p < 0.05$ for both; Table 4).

Table 1. Response rate level

Variable	Level 1: Excellent response	Level 2: Good response	Level 3: Fair response	Level 4: Poor response	Level 5: No response
Percentage of NF clearance	80–100%	60–79%	40–59%	20–39%	0–19%

NF – nevus flammeus.

Table 2. Results of visual inspection in each group

Group	Before treatment	1 day after treatment	3 days after treatment	7 days after treatment	14 days after treatment
Group A1	The comb was ruddy and intact	No noticeable edema was observed. The skin color in the experimental area was darker compared with the surrounding area	The skin was slightly yellowish-white. No skin damage was observed.	The blanching became more noticeable.	The blanching decreased, and the color in part of the experimental area was restored to normal.
Group A2		Mild edema was observed. Part of the skin was slightly purplish-red.	Edema was gone. Evident blanching was observed in part of the experimental area.	The purplish-red color faded in some areas. The blanching became more evident.	The blanching decreased, and the skin color became darker compared with the surrounding area and was not restored to normal.
Group A3		Moderate edema occurred. Evident purplish-red was observed in part of the experimental area.	The edema reduced partially, and the purplish-red color faded slightly.	The edema decreased significantly. The blanching was noticeable in the experimental area.	The edema was gone. The blanching decreased slightly. Thin crust was formed on the skin. No blister or scar was formed.
Group A4		Severe edema occurred. Part of the skin was purplish-black. Blister was formed	The edema was noticeable. Part of the skin was deep to moderate purplish-red.	The edema reduced. The blanching was evident in some areas. The skin was mild purplish-red. Crust was formed on the skin.	The edema was gone. The blanching was evident in some areas. The purplish-red color faded. The scar was formed.
Group B		Mild edema occurred. Blanching was observed in part of the experimental area.	Edema was gone. The blanching became more evident.	The blanched area turned darker.	The blanching reduced significantly but the skin color still looked a little different from the normal skin color.
Group C		Moderate to severe edema occurred. The skin was deep purplish-red. No blister was formed.	Both the edema and the purplish-red color were in a moderate level.	The edema and the purplish-red color became mild. Blanching was evident in some areas.	The edema and the purplish-red color was gone. Blanching was evident in some areas. No scar was formed.
Group D	No noticeable changes were observed.				

Collagen type I:III ratio in each group

The collagen type I:III ratios in groups A1, A2, A3, B, and C were similar to that in group D, while group A4 had a higher ratio than group D ($p < 0.05$). This result indicates that the energy density in group A4 was too high and that it may cause a risk of fibrosis in the combs (Fig. 1).

Response rate and adverse reactions in each group

The results from groups A1–4 demonstrated that the response rate increased with the increase of energy density. In fact, the energy density of 175 J/cm² used in group A4 could easily cause blisters and scarring. Considering that NF usually occurs on the facial and neck area, any therapy that may lead to blisters and scarring in these areas is not suitable for clinical treatment. Thus, we chose an energy density of 150 J/cm² for further experiments. The results showed that group C achieved a better response rate than groups A3 and B ($p < 0.05$ for both), and had a similar incidence rate of adverse reactions in comparison to groups A3 and B (29.2% vs 29.2%, 29.2% vs 25.0%; $p > 0.05$ for both; Tables 5, 6).

Fig. 1. Collagen fiber type I:III ratios in the experimental area of each group ([#] $p < 0.05$ vs group D)

Discussion

Methods for treating NF include surgical incision, cryo-therapy with liquid nitrogen and skin grafting if the wound is large.^{19,20} However, these methods cannot achieve excellent results and are likely to cause scarring or pigmentation.

Table 3. Results of microscopic observation in each group

Group	Before treatment	1 day after treatment	3 days after treatment	7 days after treatment	14 days after treatment
Group A1	The epidermal layer was intact. Many dilated capillaries were observed in the dermis. Plenty of red blood cells were present in the vessels.	Mild edema occurred in the epidermal layer. Mild exudation was observed in the dermis. The number of capillaries slightly decreased.	The edema in the epidermal layer slightly reduced. Exudation in the dermis still existed. The capillary number and vessel diameter decreased.	Edema disappeared in the epidermal layer. Exudation in the dermis reduced.	There was no noticeable edema or exudation. The capillary number and the vessel diameter reduced slightly.
Group A2		Mild to moderate edema and exudation occurred. A small number of inflammatory cells were observed. The number of superficial capillaries in dermis reduced. The vessel diameter decreased.	Edema and exudation in dermis were aggravated a bit. The number of inflammatory cells increased a little.	Edema and exudation reduced in the epidermal and dermal layers. The number of inflammatory cells reduced.	No evident exudation or edema was observed. The number of inflammatory cells reduced. The capillary number decreased significantly. The vessel diameter reduced.
Group A3		Moderate to severe edema and exudation occurred. Many inflammatory cells were observed. The capillary number and the vessel diameter reduced markedly.	Edema and exudation were aggravated. The number of inflammatory cells increased. The capillary number and the vessel diameter reduced further.	Edema and exudation decreased. The number of inflammatory cells began to decline. The capillary number and the vessel diameter reduced further.	Edema and exudation reduced markedly. The number of inflammatory cells reduced markedly. The epidermal layer was thickened. No blister was formed. The capillary number and the vessel diameter reduced significantly.
Group A4		Severe edema and exudation occurred. Blisters were present in dermis. Infiltration of many inflammatory cells was observed. The capillary number and the vessel diameter reduced markedly.	Edema and exudation were aggravated. The capillary number and the vessel diameter reduced further.	Moderate to severe edema and exudation occurred. The number of inflammatory cells did not decrease significantly. The capillaries were almost gone.	Edema and exudation began to decrease. Inflammatory cells reduced. More capillaries disappeared. Proliferative collagen fibers were formed.
Group B		Mild to moderate edema and exudation occurred. Few inflammatory cells were present. The number of superficial capillaries in dermis reduced. The vessel diameter decreased.	Edema and exudation in dermis were aggravated a little. The number of inflammatory cells increased.	Edema and exudation in epidermal and dermal layer reduced markedly. The number of inflammatory cells reduced markedly.	No evident exudation or edema was observed. The inflammatory cells almost disappeared. The capillary number and the vessel diameter reduced.
Group C		Moderate to severe exudation occurred. Many inflammatory cells were observed. The capillary number and the vessel diameter reduced markedly.	Exudation and edema were aggravated. The number of inflammatory cells increased. Capillary number and the vessel diameter reduced further.	Edema and exudation decreased. The number of inflammatory cells decreased. Capillary number and the vessel diameter reduced further.	Edema and exudation reduced markedly. The number of inflammatory cells reduced markedly. The epidermal layer was thickened. No blister was formed. Capillaries almost disappeared.
Group D	No noticeable changes were observed.				

Table 4. Decrease in the number of capillaries (%; $\pm$ SD)

Group	1 day after treatment	3 days after treatment	7 days after treatment	14 days after treatment
Group A1	15.43 $\pm$ 5.21 [#]	18.54 $\pm$ 4.22 [#]	24.21 $\pm$ 5.92 ^{##}	27.64 $\pm$ 4.11 ^{###}
Group A2	25.67 $\pm$ 6.31 ^{##}	31.88 $\pm$ 4.23 ^{###}	37.23 $\pm$ 5.43 ^{###}	40.21 $\pm$ 4.37 ^{###}
Group A3	43.65 $\pm$ 5.26 ^{####}	59.35 $\pm$ 4.77 ^{####}	74.68 $\pm$ 5.43 ^{####}	86.55 $\pm$ 5.29 ^{####}
Group A4	55.42 $\pm$ 6.91 ^{###}	70.67 $\pm$ 4.16 ^{###}	87.37 $\pm$ 6.15 ^{###}	93.23 $\pm$ 5.54 ^{###}
Group B	26.44 $\pm$ 4.25 ^{####}	32.17 $\pm$ 4.16 ^{####}	40.51 $\pm$ 5.24 ^{####}	45.33 $\pm$ 4.65 ^{####}
Group C	52.87 $\pm$ 4.69 ^{###}	68.53 $\pm$ 5.21 ^{###}	86.33 $\pm$ 6.89 ^{###}	94.25 $\pm$ 4.11 ^{###}
Group D	1.22 $\pm$ 3.54	1.34 $\pm$ 3.61	2.11 $\pm$ 2.24	1.18 $\pm$ 3.85

Compared with Group D: [#]p < 0.05, ^{##}p < 0.01, ^{###}p < 0.001; compared with Group C: ^{*}p < 0.05, ^{**}p < 0.01, ^{***}p < 0.001.

Table 5. Efficacy in each group

Group	Excellent response	Good response	Fair response	Poor response	No response	Total response rate [%]
Group A1	0	0	2	3	1	33.3
Group A2	0	2	1	2	1	50.0
Group A3	3	2	0	1	0	83.3 ^{**}
Group A4	5	1	0	0	0	100.0 ^{**}
Group B	0	2	2	1	1	66.7 ^{##}
Group C	6	0	0	0	0	100

Compared with Group C: [#]p < 0.05, ^{##}p < 0.01; compared with Group B: ^{*}p < 0.05, ^{**}p < 0.01.

Table 6. Adverse reactions in each group

Variable	Blister	Scar	Pigmentation	Discoloration
Group A1	0	0	0	1
Group A2	0	0	1	2
Group A3	0	0	3	4
Group A4	5	6	6	6
Group B	0	0	4	2
Group C	1	0	3	3
p-value	<0.001	<0.001	<0.001	<0.001
χ^2	23.546	25.264	28.153	21.823

χ^2 – chi-squared test.

Therefore, it is essential to find a safer and more effective method for treating NF. In recent years, PDT and variable pulse width Nd:YAG lasers have often been employed in clinical practice. The PDT can achieve good results for treating superficial vascular lesions, but not for deep vascular malformations, due to its weak laser penetration. In contrast, variable pulse width Nd:YAG lasers, with stronger laser penetration, can treat the capillary malformations in deep tissues.^{15,21} Therefore, in order to effectively treat both superficial and deep vascular malformations in NF, we investigated the effect of a combination therapy of PDT and a variable pulse width Nd:YAG laser.

We found that in the PDT groups, the clearance improved with increased energy density, and that PDT at an energy density of 175 J/cm² achieved the highest clearance rate. However, the group with this energy density had the most adverse reactions, including severe blistering and scarring, and had markedly higher collagen type I:III ratios, suggesting the potential risk, or even the presence, of fibrosis in the combs.²² Therefore, an energy density of 175 J/cm² cannot be employed clinically; instead, 150 J/cm² may be considered a suitable energy density. The PDT at 150 J/cm² in group A3 was found to achieve a good clearance rate and a similar incidence rate of adverse reactions as found in groups B and C; likewise, PDT at this energy density did not cause a noticeable elevation in the collagen type I:III ratio, indicating a low risk of fibrosis.

In this study, we found that the combination of PDT and a variable pulse width Nd:YAG laser was able to achieve

a better outcome than PDT or variable pulse width Nd:YAG laser alone. The combination therapy achieved a similar response rate with a lower incidence of adverse reactions in comparison with the PDT therapy at 175 J/cm². The combined use of PDT and a variable pulse width Nd:YAG laser can treat both deep and superficial capillary malformations. So far, many scholars have explored the combination of 2 or more therapies for treating NF, including cryotherapy combined with laser therapy, surgery combined with laser therapy, microneedle therapy combined with PDT, and pulsed dye laser therapy combined with long-pulse 1064-nm Nd:YAG laser therapy. However, many of these methods can easily cause adverse reactions, including scarring and pigmentation. The combination of microneedle therapy and PDT, even though it can achieve good outcomes, requires a more complicated operation than the combination of PDT and a variable pulse width Nd:YAG laser. Therefore, the combined use of PDT and variable pulse width Nd:YAG laser therapy can be recommended for further clinical trials.

Due to time constraints, there were some limitations in our study. Firstly, there are still some differences between cockscomb skin and human skin, even though the cockscomb can be used as a disease model of NF. Secondly, since human facial skin is more delicate, whether PDT at 150 J/cm² can be used to treat faces needs to be investigated further. In this study, we found that the combination of 2 therapies, even though it did not increase the incidence of adverse reactions, could still lead to blisters and scars on the subjects.

Therefore, we do not recommend its clinical application on facial areas to avoid scarring or other adverse reactions. Lastly, the study only demonstrated the short-term effect within 14 days of treatment, and more studies need to be carried out in the future to investigate the adverse reactions and recurrence rate after a longer course of treatment.

Conclusions

In conclusion, 150 J/cm² can serve as a suitable energy density in PDT for the treatment of NF, and the combination of HMME-mediated PDT and variable pulse width Nd:YAG laser therapy can achieve a better outcome than PDT or such a laser alone, so it can be recommended for clinical application.

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