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Microvascular effects of Photofrin®-induced photodynamic therapy

Cheng-Jen Chang MD, PhD[a,*], Sally M.H. Cheng[b], J. Stuart Nelson[c]

[a] *School of Medicine, Chang Gung University, and Department of Plastic Surgery, Chang Gung Memorial Hospital, 199, Tung Hwa North Road, Taipei, Taiwan*
[b] *School of Medicine, University of Melbourne, Melbourne, Australia*
[c] *Beckman Laser Institute and Medical Clinic, University of California, Irvine, CA, USA*

KEYWORDS
Photodynamic therapy;
Photofrin®;
Chick chorioallantoic membrane

Summary
Background and objective: The object of our study is to evaluate the feasibility of photodynamic therapy (PDT) for complicated hemangiomas. The photosensitizing activities of Photofrin® have been used *in vivo* models for our goal of evaluation.
Study design/materials and methods: The *in vivo* biological activities of Photofrin® exposed to the total laser energy density of 100 J/cm^2 with the power density of 100 or 120 mW/cm^2 at 630 nm wavelength was studied. The amount of vascular damage produced in the chick chorioallantoic membrane (CAM) was evaluated. At 630 nm wavelength, those individual vessels with a diameter of 40 μm or less and those with a diameter between 40 and 100 μm were treated with Photofrin® at a concentration of about 2.5 mg/mL, and injected intraperitoneally at 2.5 mg/kg, illuminated at 100 and 120 mW/cm^2, respectively. Both exhibited coagulation.
Results: There were no statistically significant differences between the two groups (100 and 120 mW/cm^2) on vessel damage grade 1. With vessel damage grades 2 and 3, the differences were statistically significant between two groups. Vessel damages between arterioles and venules also demonstrated differences in the 100 mW/cm^2 treated group but not in the 120 mW/cm^2 group. Statistically significant differences were also shown in arteriole and venules damage between 100 and 120 mW/cm^2 treated groups. The severity of vessel damage between grades 1 and 2, 1 and 3, and 2 and 3, were compared. The differences were statistically significant in 100 mW/cm^2 treated group. There was no statistically significant difference in 120 mW/cm^2 treated group.
Conclusion: Photofrin® has the capabilities for destruction of microvascular vessels of CAM. Extension of this study to the second-generation photosensitizers is underway. The most important treatment variables seem to be the power density.

Introduction

Hemangioma of infancy (strawberry hemangioma) is a common birthmark that occurs on many anatomical sites [1]. This condition is self-limiting in that the majority will

* Corresponding author. Tel.: +886 2 27135211x3502; fax: +886 2 25140600.
E-mail address: chengjen@adm.cgmh.org.twl (C.-J. Chang).

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spontaneously involute by age 3—7 years [2,3]. When obstruction of body orifice(s), ulceration, or bleeding occurs, these otherwise benign lesions cause considerable morbidity. Occasionally, ulceration is accompanied by pain. Wound contamination can lead to secondary infection. Scarring is an expected sequela of ulceration [1,2]. This scarring consists of a depressed white area that is clearly distinguishable from the adjacent normal skin [3]. When secondary infection is present, there may be permanent loss of tissue [1].

Photodynamic therapy (PDT), using a photosensitizing drug specifically activated by a specific wavelength of light to cause photoreaction in biological systems, dates back to the beginning of this century [4]. Since that time, numerous examples of the ''photodynamic effect'' have been reported for a wide range of photosensitizers both *in vitro* and *in vivo*. The basic concept of PDT is that certain molecules can function as photosensitizers. The presence of these photosensitizers in biological tissues makes it vulnerable to light at the specific wavelengths which are absorbed by the chromophore. The photosensitizer absorbs photons of the appropriate wavelength and is elevated to an excited state. The excited photosensitizer subsequently reacts with a substrate, such as oxygen, to produce highly reactive singlet molecular oxygen that causes irreversible oxidative damage to biologically important molecules [5,6]. Previous basic science studies have attempted to achieve an understanding of the mechanism of tumor destruction following PDT [7—9]. Histopathology of PDT treated tumors shows that apparent internal hemorrhage and red cell extravasations are common findings after PDT, not only in experimental animal tumors but in tumors of patients as well. The rationale for use to treat hypervascular cutaneous anomalies is based on the fact that it will allow the destruction of the target blood vessels buried deep within the skin without the production of heat. Therefore, risks inherent in conventional photothermal laser therapy, such as hypertrophic scarring, changes in the normal skin pigmentation, atrophy, or induration, would not occur. The purpose of our study is to evaluate the feasibility of photodynamic therapy in vessels of the chick chorioallantoic membrane (CAM) using the Food Drug Administration (FDA) approved photosensitizer, Photofrin®.

Materials and methods

In vivo chicken chorioallantoic membrane

The chicken chorioallantoic membrane is an elegant and inexpensive biological model for studying. Because the structure of the CAM contains a transparent matrix, it is possible to view directly the microvasculture. For the direct viewing, the upper of the chick egg shell is removed by opening a 2 cm diameter round window. Effects of each photosensitizer and PDT can be quantitatively assessed and compared.

Preparation of chicken chorioallantoic membrane

The protocol for CAM preparation was a modification of a previously described technique [10]. Fertilized eggs (Hyline W36 white leghorn) were washed with 70% alcohol, incubated at 37 °C in 60% humidity, and rolled over hourly. On days 3—4 of embryonic development, a hole was drilled in the apex and 2—3 mL albumin was aspirated from each egg to create a false air sac. On the following day, part of the CAM was exposed by opening a round window of 20 mm diameter in the shell that was covered with a Petri dish. The eggs were placed in a stationary incubator until the CAM was fully developed and ready for experimentation. On days 10—12, sterile Teflon O-rings (6.2 mm inner diameter, 9 mm outer diameter, and 1.4 mm height) were placed on the surface of the CAM, each demarcating a location where individual blood vessels and capillaries were clearly visible and to which the laser beam was directed. A drop of normal saline was added within the ring area to reduce spurious light reflection and to prevent desiccation of the CAM during the experiment [11]. Outside of the incubator, eggs were kept at 35 °C in a heating block filled with glass beads. At the time of irradiation, the CAM was illuminated with a cold white-light fiber optic source (Volpi, Intralux, model 100HL) and placed under a stereomicroscope (Olympus, model SZH), equipped with a video camera (Panasonic, model AC-2510), giving a total magnification of 70× on a color monitor (Sony, model KV-1393R).

Vessel selection

It was convenient to subdivide the extensive microvascular network of the CAM according to the following branching pattern [12]. About 100 capillaries were estimated to be in the field of view. The capillaries served as a reference and were designated vessels of ''order 0.'' The smallest precapillary vessels (arterioles, a) as well as the smallest post-capillary vessels (venules, v) were assigned ''order 1.'' The convergence of two order 1 vessels was assigned as an ''order 2'' vessel and similarly two order 2 vessels formed as an ''order 3'' vessel.

Photosensitizers

Photofrin®obtained from Quadra Logic Technologics, Inc. (Vancouver, BC, Canada) was stored in the dark at 4 °C. Photofrin® was prepared with 5% dextrose to a final concentration of 2.5 μg/mL [10]. Photofrin® powder was prepared with 5% dextrose to a final concentration of 2.5 mg/mL. Intraperitoneal (IP) embryo injection of Photofrin® with the concentration of 2.5 mg/kg. No side effects were found. Three hours post-intraperitoneal embryo injection of Photofrin®, the CAM were treated with the laser (λ = 630nm).

Laser light delivery system

Laser irradiation was performed with a Coherent (Palo Alto, CA, USA) Innova 20 Argon ion laser stimulating a Coherent 599-01 dye laser. The dye laser was tuned to emit radiation at 630nm, for each experiment. The wavelengths were verified using a Jobin Yvon #5/354 UV monochromator (Lonjuneau, France). Irradiation was coupled into a 400 μm fused silica fiber optic using a Spectra-Physics (Mountain View, CA, USA) model 316 fiber optic coupler. The output end of the fiber terminated with a microlens that focused the

Figure 1 Arrow heads indicate empty vessel (damage 2 in ''order 3'' vein).

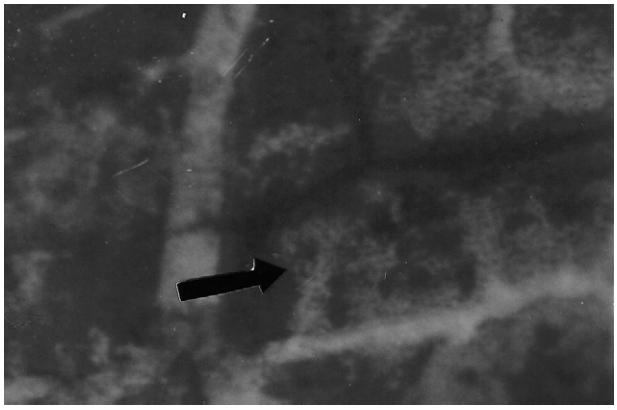

Figure 2 After PDT. Arrows denote hemorrhage (damage 3 in ''order 1'' arteriole).

laser radiation into a circular field of uniform light intensity. Laser irradiation emitting from the fiber was monitored with a Coherent Model 210 power meter before and after treatment.

Prior to illumination, the window of CAM was covered by a metal shield containing a 1.5 cm diameter circular hole exposed the illuminated area. The total laser energy density is 100 J/cm^2 at power densities of 100 or 120 mW/cm^2. Photofrin® and laser illumination was only in control groups, respectively. The vascular changes in CAM were recorded immediately and 1, 7, and 14 after PDT.

Damage assessment and statistic analysis

The PDT-induced vascular damage, recorded on videotape, was evaluated in a double-blind fashion and graded as follows: 0, no observable damage; 1, slight damage, vasodilatation/constriction, temporary occlusion; 2, moderate damage, permanent occlusion; 3, severe damage, capillary extravasation, hemorrhage (Figs. 1 and 2). Effects of the delivery method on the relative importance of various treatment variables in Photofrin®-based photodynamic therapy were examined in the vascular coagulation of the CAM system by means of the analysis of variance (ANOVA) test [18]. The average of damaged vessels in each group was then used for statistical analysis. Statistical significance was taken as $P < 0.05$ for vessel type (arteriole versus venule), vessel order (1 versus 2 and 3; 1 and 2 versus 3), and level of power densities.

Results

Information regarding the mean number of blood vessels and the types of damage after Photofrin®-PDT is presented in Table 1. Based on the analysis of variance (ANOVA), there were no statistically significant differences between the two groups (100 and 120 mW/cm^2) on vessel damage grade 1 ($P > 0.05$). Based on vessel damage grades 2 and 3, the difference was statistically significant between the two groups ($P < 0.05$). Assessment of the vessel damage between arterioles and venules demonstrated that the difference was statistically significant in 100 mW/cm^2 treated group ($P < 0.05$). There were no statistically significant differences between the vessel damage of arterioles and venules in 120 mW/cm^2 treated group ($P > 0.05$). Assessment of the arteriole damage demonstrated that the difference was statistically significant between 100 and 120 mW/cm^2 treated groups ($P < 0.05$). There were also statistically significant dif-

Table 1 Mean arterial and venous damage of CAM in different power densities

Vessel type	Order	Mean number of vessels/cm^2 before PDT	Damage 0	Damage 1, mW/cm^2 (%)		Damage 2, mW/cm^2 (%)		Damage 3, mW/cm^2 (%)	
			Control group[a]	100	120	100	120	100	120
Artery	3	18	18	13 (72.2)	4 (22.2)	15 (83.3)	10 (55.6)	17 (94.4)	11 (61.1)
	2	61	61	48 (78.7)	33 (54.1)	57 (93.4)	43 (70.5)	58 (95.1)	57 (93.4)
	1	170	170	161 (94.7)	163 (95.9)	166 (97.6)	164 (96.5)	166 (97.6)	164 (96.5)
Capillary	0								
Vein	1	109	109	51 (46.8)	11 (10.1)	65(59.6)	53 (48.6)	71 (65.1)	55 (50.5)
	2	39	39	17 (43.6)	6 (15.4)	23(59)	12 (30.8)	25 (64.1)	16 (41)
	3	17	17	3 (17.6)	2 (11.8)	6 (35.3)	4 (23.5)	8 (47.1)	6 (35.3)

0: no damage; 1: coagulation; 2: angiostasis; 3: hemorrhage.
[a] 100 or 120 mW/cm^2 illumination, with Photofrin® respectively.

ferences of venule damage between 100 and 120 mW/cm^2 treated group ($P > 0.05$). The severity of vessel damage between grade 1 and 2, 1 and 3, and 2 and 3, were compared. The differences were statistically significant ($P < 0.05$) in 100 mW/cm^2 treated group. There was no statistically significant difference in 120 mW/cm^2 treated group ($P > 0.05$).

Discussion

The photosensitizer absorbs photons of the appropriate wavelength and is elevated to an excited state. The excited photosensitizer subsequently reacts with a substrate, such as oxygen, to produce highly reactive singlet molecular oxygen that causes irreversible oxidative damage to biologically important molecules [14,15]. The intersystem transfer from an excited singlet photosensitizer to a triplet state is essential for the production of singlet oxygen. The phototoxic reaction is a local phenomenon that takes place within the same cell on a time scale of microseconds. Irradiation at the appropriate wavelength absorbed by the photosensitizer provides the energy to drive photodynamic reactions without the generation of heat, provided the incident power density is kept below 100 mW/cm^2 [16]. The optimal maximum temperature would be below 40 °C to prevent any biotissue degeneration. Efforts to define the mechanisms of PDT action have led to a controversy that attributes cytotoxicity to vascular-mediate events (i.e., indirect cell kill) or to cellular targets (i.e., direct cell kill) of photochemically produced $^1O_2$ or other oxygen radicals [17,18]. One reasoned that if systemic Photofrin® treatment could lead to effective vascular photosensitization, it would likely result from cellular events of endothelial cells [8,19,20]. Hence, experiments were performed to ascertain the time course and dose relationship of Photofrin® in CAM [15]. Photofrin® is a derivative of hematoporphyrin. This photosensitizer located throughout cytoplasm was highly susceptible to functional inhibition by PDT [21,22]. We proposed that PDT damage to mitochondrial function and liposomes could be the major factor responsible for the effectiveness of PDT. Those results *in vivo* demonstrated marked PDT-induced destruction of the chicken comb in our previous studies [13].

In the evaluation of the arterial and venous response, one of the phenomena observed in our study was the higher vulnerability for PDT injury of arterioles as compared to venules. This occurred for the three vessel calibers. A possible explanation of these findings might be based on considerations of vascular anatomy. The arteriolar walls consist of three concentric layers: an endothelial tube, an intermediate layer of smooth muscle cells, and an outer coat of fibrous elements. The thickness of the arteriolar wall varies with vessel caliber and function; the walls of the venules are always thinner than those of arterioles of equal caliber. In the case of PDT, we might anticipate less damage to the thicker, more resilient arteriolar wall. However, another point of difference is the platelet aggregation initiated by the chain of biochemical reactions triggered by PDT, which is different in arterioles and venules. This seems to be consistent with reports of PDT-induced vasoconstriction, where it was shown that 90% of the arterioles were affected by photochemical injury versus 70% of the venules [23]. Finally, it should be noted that coagulated blood emboli, consisting of agglutinated damaged RBCs [17], can be transported downstream in venules, but not in arterioles because blockage occurs when they reach capillaries. This difference in permanent clotting is a component in our interpretation for the lower threshold for PDT-induced vascular damage in arterioles then venules.

In our study, assessment of the vessel damage between arterioles and venules demonstrated that the difference was statistically significant in 100 mW/cm^2 treated group ($P < 0.05$). There were no statistically significant differences between the vessel damage of arterioles and venules in 120 mW/cm^2 treated group ($P > 0.05$). This was possibly because at 120 mW/cm^2 level, all vessels were destroyed. These results support our theories, and also demonstrated that the power density was one of the factors that contribute to the differences of vessel damage between arterioles and venules. Based on vessel damage grade, the vessel damage in grades 2 and 3 was more significant in 120 mW/cm^2 than in 100 mW/cm^2 treated group ($P < 0.05$). There were no statistically significant differences between the two groups (100 and 120 mW/cm^2) on vessel damage grade 1 ($P > 0.05$). This result was possibly owing to the power densities of 120 mW/cm^2 that had better PDT-induced vessel damage. These results were also demonstrated in arteriole and venule damage with significant difference between 100 and 120 mW/cm^2 treated groups, respectively. The severity of vessel damage between grades 1 and 2, 1 and 3, and 2 and 3, were compared. The differences were statistically significant ($P < 0.05$) in 100 mW/cm^2 treated group. There was no statistically significant difference in 120 mW/cm^2 treated group ($P > 0.05$). This result was also possibly owing to the power densities of 120 mW/cm^2 that had better PDT-induced vessel damage.

In conclusion, our preliminary study demonstrated that Photofrin® has the ability to destroy blood vessels *in vivo* of CAM models. This can be effective in treating tumors as hypervascularity are commonly found in tumors. Photofrin® can target the vessels within the tumor and causes destruction thereby resulting in the shrinkage in size. However, continued improvement in treatment results for patients with hypervascular cutaneous anomalies such as hemangiomas will depend on the ability to cause selective destruction of only the targeted blood vessels, buried deep within the skin, without the production of heat by non-thermal mechanisms. In addition, the greater tissue penetration of the longer wavelengths used in PDT should make it ideal for treatment of deeper, larger hemangiomas, thus substantially expanding the population of patients expected to benefit from laser treatment.

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