Evaluation of vascular effect of photodynamic therapy in chorioallantoic membrane using different photosensitizers
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**A R T I C L E I N F O**

Article history:
Received 10 January 2014
Received in revised form 2 April 2014
Accepted 12 April 2014
Available online 9 May 2014

Keywords:
Photodynamic Therapy
Vascular effect
Chorioallantoic membrane
CAM
PDT

**A B S T R A C T**

Photodynamic Therapy (PDT) is a local treatment that requires a photosensitizing agent, light and molecular oxygen. With appropriate illumination, the photosensitizer is excited and produces singlet oxygen that is highly reactive and cytotoxic. Tumor vascular network is essential for the tumor growth and the understanding of vascular response mechanisms enables an improvement in the PDT protocol for cancer treatment. Compounds of porphyrin (Photogem®) and chlorin (Photodithazine®) were the photosensitizers tested. The incubation times varied from 20 to 80 min and the concentration ranged between 0.1 and 100 μg/cm². Different light doses were used between 4.8 and 40 J/cm² with irradiance varying between 80 and 100 mW/cm². The light dose of 30 J/cm² was used in the intravenous photosensitizer application. The membrane images were made from 0 to 300 min after treatment. The vascular response was evaluated by the average vessel area. Different responses were observed depending on the photosensitizer concentration and administration form. Intravenous application has been more efficient to produce vessel constriction and the most pronounced effect was observed for the chlorin.

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1. Introduction

Angiogenesis is the phenomenon that involves a new blood vessel formation and is intrinsically linked to several diseases, such as cancer, rheumatoid arthritis and psoriasis [1,2]. In 1971, Folkman described the relation between this phenomenon and cancer. He showed that angiogenesis is essential for both tumor growth and metastasis. This new vascular network development happened to feed the tumor [3]. Therefore, the knowledge about the tumor growth involves the elucidation of its biological properties, including new vessels generation from a pre-existing vascular network [4–7].

A well established model to study angiogenesis uses chicken eggs [8,9]. In the chicken eggs there are porous and rigid eggshell and inner and outer membranes that are permeable to oxygen, carbonic gas and water vapor [10,11]. Due to the need of oxygen for the embryo development, there is the formation of chorioallantoic membrane that is a fusion of the allantoic (responsible for respiration) and chorio (membrane that involves embryo and its structures). This membrane is beneath the porous shell; it presents a lot of vessels and enables enhanced gas exchange [12–14]. The chorioallantoic membrane of chicken eggs is known as CAM and is probably the most used in vivo model to study angiogenesis and compounds activities in vascular endothelium. With a direct access to blood vessels and embryo, this model is simple, cheap and of easy implementation in laboratory environment [15–17].

The scientific community has searched for alternative techniques for oncologic treatments when traditional treatments are inefficient or present limited responses [18,19]. Photodynamic Therapy (PDT) is a treatment modality which involves light (at specific wavelength), a photosensitizing agent and molecular oxygen. The photosensitizer (PS) in tumor cells is activated by light and interacts with cell oxygen, resulting mainly in the production of singlet oxygen, a highly reactive species that induces damage to biomolecules [20–22].

Several groups that work with PDT have invested in the development of improved photosensitizers. Ideal characteristics for the photosensitizer are low dark toxicity, high efficiency for singlet oxygen generation, high penetration by cell membranes and fast post-treatment clearance [23–29]. Other relevant characteristics are needed to a molecule become a clinical photosensitizer, such as a long life-time of the excited triplet state and high molar absorptivity in the electromagnetic “therapeutic window” between 600 nm and 1000 nm, where the light show a measurable penetration into the biological tissues [30–33].

Actually, compounds of porphyrin, chlorin, bacteriochlorin, phthalocyanine and others have been applied with success in PDT, but their individual mechanisms and the resulted differences on the photodynamic response are still not complete understood.
The chlorin compounds are classified as photosensitizers of second-generation and have higher molar absorbance at the red spectrum that results in higher PDT response with the use of lower energy doses. Chlorins are replacing the porphyrin derivatives that are classified as first-generation compound [34].

Among hematoporphyrin compounds, the most related one in literature is Photofrin® (Photofrin, USA) while for chlorin compounds, there is Foscan® (Foscann, Ireland). With these photosensitizers there are several protocols with illumination, ranging from 33 mW/cm² to 150 mw/cm², and doses of 5, 10, 50 and 100 J in CAM model [25].

Tumor vascular network is responsible for delivering the nutrients, oxygen and the photosensitizer to the necrotic cells. Both tumor survival and the PDT response are inherently dependent on vascularization characteristics and any changes in the vascular network can affect the further PDT response. With the CAM model, we can study individually the vascular effect of PDT, helping to analyze the tissue damage. It is possible to vary several parameters associated with this therapy, as drug type and concentration, photosensitizer via, drug-light interval, light dose, fluence and irradiance. The PDT vascular response can be evaluated according to the vessel diameter and extension, post-PDT time interval and embryo age. The understanding of vascular response mechanisms enables an improvement in the PDT protocol for cancer treatment. [23,35,36].

The PDT injury at the blood vessels is particularly useful in the treatment of malignancy since cancer lesions recruit new small immature vessels for the supply of nutrients and, although this phenomenon has not been extensively studied, PDT has shown to cause thrombosis of smaller vessels. However, it is necessary to observe the major blood vessels that are in close proximity to tumor that are extremely important to the life of the patient. For example, in cases of head and neck cancer there is the carotid artery that needs to be preserved. Fatal cases of hemorrhage caused by PDT had been related, showing the relevance of the knowledge of different response to use of different photosensitizers. The evaluation of the PDT vascular response in an animal model is complex, since the tumor overall response is a combination of the damage in cells, extracellular matrix, and vessels. The determination of the induced vascular response for different photosensitizers may improve the safety of the present PDT clinical protocols [37–40].

In this context, the aim of this study was to evaluate the vascular effect of PDT on the CAM model when two different types of photosensitizers are used under different protocols.

2. Materials and methods

2.1. CAM model

Chicken eggs obtained from a local producer (GLOBOAVES, São Carlos/SP, Brazil) were used for the experiments. On the first day of incubation, embryo eggs were wiped with 70% alcohol tissue before being placed in an incubator at 37.7 °C. During the first and second days of development, the eggs were kept under constant slow rotation motion – half cycle each 30 min. On the third day, a small hole was produced with a hand driller in the shell for the removal of 3–4 mL of albumin with a syringe and the rotation was interrupted. A window of 2 cm² was opened on the fourth day and sealed with adhesive tape until 11th day. The survival rate of the CAM model obtained was of 60%. For each PDT protocol, 3–5 eggs were used to verify the response.

2.2. Photosensitizers

Two photosensitizers (PS) were used: a porphyrin compound (Photogem®, Photogem, Russia) and a chlorin compound (Photodithazine®, Veta Grand, Russia). Their structures are showed in Fig. 1a [41] and Fig. 1b [42], respectively.

The PS concentration ranged from 0.1 to 100 μg/cm². A stock solution was prepared using 5 μg of Photogem diluted in 1 mL of distilled water and the stock solution of Photodithazine® also was 5 mg/mL. Both were diluted in distilled water to obtain the desired final concentration.

2.3. Topical administration

Both PS were topically administered to the vascular network of the eggs. A 15 mm Teflon® ring was used to delimit the target site (1.76 cm²) in the vascular network. 200 μL of the PS solution was gently placed inside the ring using a pipette. The times of incubation investigated were of 20, 40, 60 and 80 min to determine the best interval for each PS. After the incubation time, the photosensitizer solution was removed by a syringe and the area inside the ring was washed with a 0.9% NaCl solution.

2.4. Intravenous administration

Photogem® and Photodithazine® were intravenously administered by an insulin syringe with a 29G gauge needle. The egg was a little rotated until the central blood vessel had become slightly stretched. The inject was performed with the gauge angled at approximately 45° and the Teflon® ring placed to delimit the area of illumination. The tested concentrations of PS were 0.2, 0.5 and 5 μL/cm² and the injected volume was 500 μL.

2.5. Photodynamic Therapy

Two diode lasers (EagleEaron®, Quantum Tech, Brazil) were used as light sources, one emitting at 630 nm, for Photogem®, and the other at 660 nm for Photodithazine®. The light was delivered via an optical fiber whose lenses were coupled to the tip so that a uniform irradiation profile could be obtained. The illumination fiber was assembled on a support to fix the distance between fiber tip and egg membrane to obtain an illumination area of the size of the ring area.

The induced vascular changes were first evaluated under different conditions for the establishment of safe parameters to the embryonic annexes (egg white, yolk and embryo). No changes were observed with illumination from 4.8 to 40 J/cm² and irradiance at 80, 100 and 120 mW/cm², which resulted in illumination time between 80 and 300 s. After this analysis, the irradiance was set at 100 mW/cm² and fluence of 30 J/cm².

Based on the results obtained with topical Photogem®, an irradiance of 100 mW/cm² was set for topical chlorin and the tests were conducted with 300 and 600 s, resulting in fluences of 30 and 60 J/cm², respectively.

![Fig. 1. Structures of photosensitizers used. (a) Photogem®. (b) Photodithazine®.](image-url)
Two types of energy delivery were tested for the topical PDT: continuous and fractionated (1/1 min with light/dark cycles) for both PS. For the intravenous PS application, the illumination was performed during, immediately after, one minute and five minutes after injection.

2.6. Control groups

Treatment control groups were performed to evaluate the vascular effect of the light and the PS. The dark toxicity of both photosensitizers was evaluated by monitoring the vascular network after 40 min of incubation time at 1 μg/cm². The same procedure of the PDT group was repeated without the illumination. The illumination effect was analyzed for both excitation wavelengths, under 100 mW/cm² and 30 J/cm². The influence of the injection procedure was evaluated after the injection of a saline solution at the same total volume. In all tests, the vascular network was monitored until 300 min after treatment.

2.7. Evaluation of the photodynamic vascular response

Images were captured every 30 min after illumination until 300 min with a USB Digital Microscope® (AVANTGARDE, China). They were processed by Photoshop® software (ADOBE PHOTOSHOP CS4, version 11, USA). The pixels corresponding to vessels were defined as black and the remaining structures as white pixels (embryo, yolk and egg white). To quantify the overall vascular response, the decrease or increase in the total area of blood vessels was calculated using a percentage of black pixels in the region of interest as a function of time. This analysis was performed using the ImageJ® software (National Institutes of Health, USA, public domain).

A maximum increase and minimum decrease in the total amount of blood vessels were calculated from the maximum and minimum percentage values of black pixels normalized for the initial value (t = 0). The maximum diameter of the damage vessels was determined for each PDT condition.

The vessel diameters were defined by calibrating the pixels with the fixed measurement of ring width. Using this analysis, a correlation between the vessel diameter and the final changes after the PDT could be established for each protocol.

3. Results and discussion

The analysis of vascular damage using PDT in CAM presented in the literature is usually based on a score method. This kind of method depends of the vessel diameter and if there is a total occlusion, partial occlusion or no occlusion. This score ranges from zero to five or in relative arbitrary units. The analysis shows the response at specific vessels, giving a local response, but lacking studies presenting a vasculature overall effect.

The blood vessel quantification in CAM model is a complex problem and several methods can be used aiming a best analysis. Variations involve image acquisition and its processing, with several computational or manual methods. In the literature, it is possible to highlight the restriction of visual field and manual counting of blood vessels, analysis of individual parameters, methods using fractal analysis, among others methods.

The quantification of small changes in the vascular network is the main difficulty of the proposed methods, since this counting is manual. Furthermore, some alternatives of image acquisition are done using microscopes however, this analysis requires the CAM removal and prevents the monitoring of the vascular damage over time [43]. The use of binary image is already known using several methods, including the fractal dimensional method [44–46].

Our analysis was based on the overall vascular response, quantifying the vessel pixels with image acquisition before and after the treatment, in the total illuminated area. This procedure involved the area inside the Teflon® ring and the number of pixel is directly related to the area of blood vessels. A qualitative analysis was also performed to determine the relation of the individual vessel response, depending on its diameter and the PDT protocol.

3.1. Topical administration

For all incubation times tested, the results showed that 40 min was an appropriate drug light interval, because shorter times did not induce any alteration in the vascular network and longer times provided similar results.

The analysis of the control treatment groups, where the toxicity of only light or PS was investigated, revealed no major changes in the vascular network and the illumination and PS parameters, when applied individually, induce no vascular damage.

3.1.1. Porphyrin

Setting the incubation time at 40 min and fluence at 30 J/cm², the initial concentration tested was of 1 μg/cm². The images of the vascular network after PDT (Fig. 2a) were processed for a quantitative analysis. The ratio of black/white pixels was calculated for each image, resulting in the total blood vessel area as a function of time (Fig. 2b).

Fig. 2a shows vascular damage at 150 min and a more pronounced effect at 300 min after PDT, indicating that the parameters were effective to induce vessels destruction. A decrease in the CAM overall vascularization due to vessel shutdown or a reduction in its diameter could be observed.

From the graphs of Fig. 2b, the average reduction was calculated for the three eggs treated using the same PDT parameters, and the result is a decrease of 66% ± 17%. Each egg shows an initial different network and therefore an individual analysis was performed. The average value shows a significant and general vessels reduction.

The correlation between the vessel diameter and the PDT response was established for diameters between 3 and 360 μm. In wider vessels (over 151 μm) a vascular constriction was observed, with a diameter reduction between 30% and 70%. Thinner vessels (below 144 μm) exhibited completed destruction.

The Photogem® concentration was increased to 100 μg/cm² to evaluate if the vessel destruction could be improved. With higher PS concentration, there is more drug availability for cells.

Fig. 2c shows that after PDT the peripheral vessels became more pronounced, due to a vessel dilatation in this region. A qualitative analysis suggests an increased vascular network resulted from the appearance of thin vessels, not evident immediately after treatment (zero time). No angiogenesis occurred due to the short investigation time when the event was observed. These thin vessels were already present in the CAM, but with no active function, since no blood was inside them. After PDT, which occurred in the thicker vessels, that retained higher concentrations of the photosensitizer, the damage was induced in the vessel wall resulting in the constriction, and finally the blood flow was directed to other vessels, that were not previously required in the vascular network.

Through image processing, the graph of Fig. 2d shows this behavior quantitatively. The graphs shows maximum values of reduction of (10% ± 3%) and increase of (20% ± 10%), calculating from the average of three eggs.

The measurements of vessel diameter revealed that vessels whose diameter above 143 μm suffered a decrease of approximately 30%. Vessels measuring above 447 μm did not undergo any change. In deeper layers, no changes were observed in larger vessels, however, narrow vessels measuring less than 52 μm showed dilatation.
3.1.2. Chlorin

The same parameters applied to porphyrin were used with a chlorin, starting with concentration of $1 \mu g/cm^2$. A sequence of images is provided in Fig. 3a, with pixel transformation image and plot of blood vessel area as a function of time (Fig. 3b).

A qualitative analysis of the vascular effect induced by PDT using chlorin at $1 \mu g/cm^2$ showed a very similar behavior to porphyrin when used at $100 \mu g/cm^2$.

This behavior, suggests a faster photodynamic response of the chlorin compound in the larger vessel in comparison to the porphyrin compound. If a vascular damage had occurred more slowly, there would be enough time for a vessel activation and dilation in the peripheral environment.

The calculated average reduction was of $(15\% \pm 5\%)$ and increase of $(15\% \pm 2\%)$, showing no significant overall vascular change in the network area, similar to the one observed with Photogem at $100 \mu g/cm^2$. In the analysis of the vessel diameter, vessels smaller than $230 \mu m$ were completely destroyed in some regions over the investigated time post therapy. Vessels between $230 \mu m$ and $402 \mu m$ suffered a diameter reduction of approximately $30\%$ and $70\%$. Vessels whose diameter was larger than $402 \mu m$ or in deeper layers showed a reduction of $30\%$ or less. The dilatation happened with vessels smaller than $49 \mu m$.

From these results, a lower concentration of chlorin at $0.1 \mu g/cm^2$ was tested, reducing the availability of this PS. The images in Figs. 3c clearly show a reduction in the vascular network diameter after PDT. This result is similar to the one obtained when Photogem was used at $1 \mu g/cm^2$ (Figs. 2a and b). From the image processing for the quantitative analysis the graph in Fig. 3d was plotted, confirming this similar behavior. The average of 3 eggs testing the same parameters resulted in a reduction of $43\% \pm 9\%$ and increase of $10\% \pm 4\%$, in vessel diameter.

For chlorin at $0.1 \mu g/cm^2$, vessels thinner than $156 \mu m$ were completely destroyed while wider vessels suffered only a reduction of $30\%–60\%$. All results are summarized in Table 1.

When both photosensitizers topically applied were compared, two main observations could be pointed: equivalent effect of porphyrin and chlorin at different concentrations and distinct vascular for the same compound, depending on its concentration. The similar behaviors of porphyrin and chlorin at different concentrations were qualitatively and quantitatively observed. Considering the overall vascular effect, chlorin has been the most efficient compound, since a faster effect occurs with lower concentration when compared to porphyrin. This probably occurs because Photodithazine has smaller molecules than Photogem that is a mixture of oligomers of several sizes, increasing the time of diffusion between the CAM layers. Therefore, chlorin molecules penetrates in the CAM faster and more deeply and a higher amount of the PS will be available to both the endothelial cells and the blood. If there is more PS in the cell and in the blood flow, and the photons are delivered there with the illumination, then a higher PDT effect will be present.

3.2. Intravenous administration

For intravenous injection, the illumination performed at immediately after, 1 min and 5 min after injection, no or only small changes were induced in the vascular network. This occurs because the PS is taken by the blood flow and is not absorbed by the
endothelial cells within those drug-light intervals. Under such conditions, no photodynamic effect occurs. The vascular effect was observed only when the illumination was performed during the intravenous injection of the PS. It is important to highlight that when the needle is removed from the vessel, the resulting bleeding was intense and prolonged, no images could be acquired. Therefore only eggs with small bleeding were considered for the image processing analysis.

3.2.1. Porphyrin

500 μL of solution with concentration of 0.2 mg/mL were slowly injected, and during this processes, a fluence 30 J/cm² was delivered. The injection lasted approximately 2 min and the total illumination was performed in 5 min. An evident acute vascular shutdown was observed in Fig. 4a. With the graph in Fig. 4b, the reduction of the vascular network area was calculated in 73% ± 20%.

Table 1

<table>
<thead>
<tr>
<th>Administration</th>
<th>PS</th>
<th>Concentration</th>
<th>Total decrease (em %)</th>
<th>Total increase (em %)</th>
<th>Behavior of vessel after PDT</th>
<th>Vessel diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Topical</td>
<td>Porphyrin</td>
<td>1 μg/cm²</td>
<td>66 ± 17</td>
<td>9 ± 6</td>
<td>Total constriction</td>
<td>&lt;151 μm</td>
</tr>
<tr>
<td></td>
<td>Chlorine</td>
<td>1 μg/cm²</td>
<td>15 ± 5</td>
<td>15 ± 2</td>
<td>Total constriction</td>
<td>&gt;153 μm</td>
</tr>
<tr>
<td></td>
<td>Porphyrin</td>
<td>100 μg/cm²</td>
<td>10 ± 3</td>
<td>20 ± 10</td>
<td>Total constriction</td>
<td>&gt;230 μm</td>
</tr>
<tr>
<td></td>
<td>Chlorine</td>
<td>0.1 μg/cm²</td>
<td>43 ± 9</td>
<td>10 ± 4</td>
<td>Total constriction</td>
<td>&gt;333 μm</td>
</tr>
<tr>
<td>Intravenous</td>
<td>Porphyrin</td>
<td>0.2 mg/mL</td>
<td>59 ± 13</td>
<td>3 ± 2</td>
<td>Total constriction</td>
<td>&lt;156 μm</td>
</tr>
<tr>
<td></td>
<td>Chlorine</td>
<td>0.2 mg/mL</td>
<td>73 ± 20</td>
<td>10 ± 10</td>
<td>Total constriction</td>
<td>&gt;186 μm</td>
</tr>
</tbody>
</table>

* There was no destruction because it was possible to observe regions of total occlusion, but not the complete vessel occlusion.

Fig. 3. PDT response with chlorin and topical application. (a) 1 μg/cm² – images of CAM as a function of post-treatment time and corresponding processed images. (b) 1 μg/cm² – graph of the ratio of black/white pixels. (c) 0.1 μg/cm² – images of CAM as a function of post-treatment time and corresponding processed images. (d) 0.1 μg/cm² – graphs of the ratio of black/white pixels.
3.2.2. Chlorin

The same PDT parameters for porphyrin were used for Photodithazine. There was an evident reduction in the vascular network (Fig. 5a). The peripheral blood vessels disappeared and only the vessel of larger diameter in the deeper layers of the CAM was still present. The mean reduction among the three eggs was 59% ± 13%, calculating with graphs of Fig. 5b. This value was higher than calculated for chlorin in topical application and they are provided in Table 1.

The analysis of the vascular response depending on the vessel diameter, for both photosensitizers, could not be performed due to the impossibility of image analysis as a function of time.

The results show the intravenous injection was more effective than the topical application. The direct injection in the vessel showed no diffusion barrier confronted by the PS molecules in the topical photosensitization.

In intravenous administration a more pronounced destruction of vessels was observed when compared to topical application, including vessels in deeper layers of the membrane. Even in vessels that are not destroyed, a reduction in their diameters was observed and, therefore it is considered the decreased vascular network as overall effect.

4. Conclusions

The PDT vascular response in the CAM model depends on the parameters used for porphyrin and chlorin. For the same photosensitizer, the vascular response may be distinct, depending on the photosensitization procedure (topical or intravenous), the drug-light interval, and the PS concentration. Comparing the results, the intravenous injection is the most efficient photosensitization method for the vascular PDT response and chlorin showed a higher and faster effect.

Acknowledgements

The authors acknowledge the financial support provided by FAPESP (CEPOF-CEPID Program) and CAPES (HH Buzzá scholarship).

References


Fig. 4. PDT response with Photogem and intravenous application. (a) 0.2 mg/mL – images of CAM as a function of post-treatment time and corresponding processed images. (b) 0.2 mg/mL – graph of the ratio of black/white pixels.

Fig. 5. PDT response with chlorin and intravenous application. (a) 0.2 mg/mL – images of CAM as a function of post-treatment time and corresponding processed images. (b) 0.2 mg/mL – graph of the ratio of black/white pixels.