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Evaluation of vascular effect of Photodynamic Therapy in chorioallantoic membrane using different photosensitizers

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ABSTRACT

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/cm^2. Different light doses were used between 4.8 and 40 J/cm^2 with irradiance varying between 80 and 100 mW/cm^2. The light dose of 30 J/cm^2 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 was 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.

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 absorbance at 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.

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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® (Foscan, Ireland). With these photosensitizers there are several protocols with illumination, ranging from 33 mW/cm^2 to 150 mw/cm^2, 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 neoplastic 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, photosensitization 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 fecundation, 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 cm^2 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/cm^2. A stock solution was prepared using 5 mg of Photogem diluted in 1 mL of distilled water and the stock solution of Photoditazine® 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 cm^2) 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 administrated 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/cm^2 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/cm^2 and irradiance at 80, 100 and 120 mW/cm^2, which resulted in illumination time between 80 and 300 s. After this analysis, the irradiance was set at 100 mW/cm^2 and fluence of 30 J/cm^2.

Based on the results obtained with topical Photogem®, an irradiance of 100 mW/cm^2 was set for topical chlorin and the tests were conducted with 300 and 600 s, resulting in fluences of 30 and 60 J/cm^2, respectively.

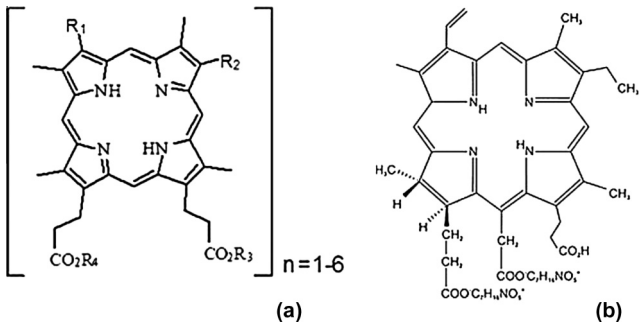

Fig. 1. Structures of photosensitizers used. (a) Photogem®. (b) Photodithazine®.