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Determination of Threshold Dose of Photodynamic Therapy to Measure Superficial Necrosis

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Abstract

Background Data: Photodynamic therapy (PDT) involves the photoinduction of cytotoxicity using a photosensitizer agent, a light source of the proper wavelength, and the presence of molecular oxygen. A model for tissue response to PDT based on the photodynamic threshold dose (D_{th}) has been widely used. In this model cells exposed to doses below D_{th} survive while at doses above the D_{th} necrosis takes place. ***Objective:*** This study evaluated the light D_{th} values by using two different methods of determination. One model concerns the depth of necrosis and the other the width of superficial necrosis. ***Materials and Methods:*** Using normal rat liver we investigated the depth and width of necrosis induced by PDT when a laser with a gaussian intensity profile is used. Different light doses, photosensitizers (Photogem, Photofrin, Photosan, Foscan, Photodithazine, and Radachlorin), and concentrations were employed. Each experiment was performed on five animals and the average and standard deviations were calculated. ***Results:*** A simple depth and width of necrosis model analysis allows us to determine the threshold dose by measuring both depth and surface data. Comparison shows that both measurements provide the same value within the degree of experimental error. ***Conclusion:*** This work demonstrates that by knowing the extent of the superficial necrotic area of a target tissue irradiated by a gaussian light beam, it is possible to estimate the threshold dose. This technique may find application where the determination of D_{th} must be done without cutting the tissue.

Introduction

PHOTODYNAMIC THERAPY (PDT) is a treatment for cancer and other lesions involving the application of a photosensitizer that has the ability to localize in tumors.[1–3] The therapeutic action occurs during the absorption of light by the photosensitizer, which leads to the subsequent conversion of chemical reaction products that act upon the cells, inducing necrosis and apoptosis.[4]

The effects of PDT depend on the simultaneous presence of the drug, the excitation light source, and oxygen. Oxygen is essential to promote the production of singlet oxygen, a highly reactive oxygen species that destroys cellular substrates, leading to cell death.[4,5] This is useful information to incorporate when trying to optimize PDT. Recently, we have used the threshold dose to compare the photodynamic efficacy of different photosensitizers.[6]

A model for the tissue response to PDT, based on PDT's threshold dose (D_{th}) has been widely used.[7–12] Cells exposed to doses below D_{th} survive, while at doses above D_{th} necrosis take place.

The concept of the threshold dose is important for the establishment of light dosimetry in PDT. The development of new challenging applications and improvements in dosimetry for PDT depend on a clear understanding of this concept. In a paper by Grossweiner,[13] the idea of threshold dose was investigated using a model for light–tissue interaction which will be used here. We shall also evaluate the effect on the threshold dose of the photosensitizer concentration.

Evaluation of threshold doses is a problem because it requires knowledge of how light penetrates into the tissue, its depth, and correlations with the effects on healthy and necrotic tissues. Because it is easy to determine the irradiance at the surface during PDT, a method based on this value to determine D_{th} would be convenient and simple to apply.

This effect has as its basis the fact that the mechanism behind the generation of toxic products is the production of singlet oxygen, and the amount of these molecules produced

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will depend on the local light fluence and photosensitizer concentration, as well as the local availability of oxygen.[14] It has been established by Patterson et al.[8] that in PDT necrosis will occur if the number of photons absorbed by the photosensitizer per unit of volume exceeds a certain critical value.

Cowled and Forbes[15] found that, assuming no oxygen limitations, clinical and experimental data suggest that tumor destruction is a function of the product of porphyrin concentration and light dose.

Other authors have also investigated the dependence of the extent of necrosis on one or more of the parameters involved in PDT.[16] Bown et al.[17] reported that a logarithmic relationship exists between the extent of necrosis incurred and the total energy delivered. In the last decade, several authors have addressed topics related to the photodynamic threshold dose for many different photosensitizers. Both Lilge et al.[10] and Farrell et al.[11] measured the photodynamic threshold dose for different photosensitizers using the measured light fluence, photosensitizer concentration in tissue, and histological studies, using a rabbit VX2-tumor model.

The purpose of this work is to demonstrate that knowing the extent of the surface necrotic area of a target tissue irradiated by a gaussian-shaped light beam, it is possible to determine the threshold dose.[18] The amounts of width and depth of necrosis obtained via other methods are compared to verify the validity of the method proposed here. The use of a gaussian beam has also recently been applied in the context of CO_2 laser ablation to characterize tissue properties such as absorption coefficient, thermal relaxation time, and ablation threshold, in a matter similar to that described here.[19]

Materials and Methods

Photosensitizers

The photosensitizers employed in this study consist of three commercial hematoporphyrin derivatives (HpDs): Photogem® (PG) from the Moscow Institute of High Chemical Technology, Photofrin® (PF) from Axcan Pharma of Canada, and Photosan® (PS) from Seehof Laboratories of Germany, and three chlorine type photosensitizers: Foscan® (FOS) from Biolitec Pharma in the United Kingdom, Photodithazine® (PDZ) from Veta Grand of Russia, and Radachlorin® (RADA) from Rada Pharma of Russia.

Laser light source

The light source employed for HpD illumination was a commercial CeramOptec Ceralas 630 diode laser unit (CeramOptec GmbH, Bonn, Germany) operating at 630 nm, and the light source used to illuminate the chlorine-type agents was a commercial Quantum Tech® Eagle Heron 660 diode laser unit (São Carlos, Brazil) operating at 660 nm. Both units have controlled power, and were coupled to an optical fiber that allowed variations in spot size and intensity. These variations were achieved by modifying the distance between the fiber tip and the tissue surface. The light spot was a nearly gaussian beam, that was fully characterized before starting the experiments and expressed by a gaussian function.[20] The fitting provided the central intensity and the half width of the gaussian profile. We worked with a distance between the fiber tip and the tissue that provided a central intensity of 250 mW/cm^2 and a spot size of 10 mm.

Animals

Male Wistar rats weighing 200–250 g were used in this study, and they were maintained in accordance with the Guidelines of the Committee on the Care and Use of Laboratory Animals of the National Research Council, and the Commission for Ethics in Research of the Hospital of Ribeirão Preto, School of Medicine, at the University of Sao Paulo (Ribeirão Preto, Brazil).

Experimental design

A total of 147 animals were divided into groups of three animals each that were used for each photosensitizer concentration and light dose. These parameters were chosen based on our previous experience and examples in the literature.[6,21,22] The time between injection and liver treatment was determined for all photosensitizers (PG, 30 min; PF, 2 h; PS, 12 h; FOS, 9 h; PDZ, 1 h and 30 min; and RADA, 7 h) based on previous studies carried out in our laboratory.[23,24]

Prior to any manipulation, the animals were anesthetized using 5% ketamine at a dose of 0.08 mL/100 g body weight, and xylazine at a dose of 0.04 mL/100 g of body weight.[14]

The photosensitizers were diluted in 0.9% NaCl and were injected via the vena cava.[21] The HpD concentrations used were 1.0 and 2.0 mg/kg of body weight. After the predetermined time had elapsed, each animal was submitted to an open surgery in which the liver was illuminated by a gaussian beam with central intensity of 250 mW/cm^2 (fixed intensity) and a spot size of 10 mm. The total surface light dose was controlled by the exposure time, to 10 J/cm^2 (40 sec), 50 J/cm^2 (200 sec), 100 J/cm^2 (400 sec), or 200 J/cm^2 (800 sec). For the chlorine-type agents the concentration and light doses used for each group were: FOS 0.1, 0.2, and 0.3 mg/kg body weight and illuminated with 127 mW/ cm^2, delivered at 10 (79 sec),

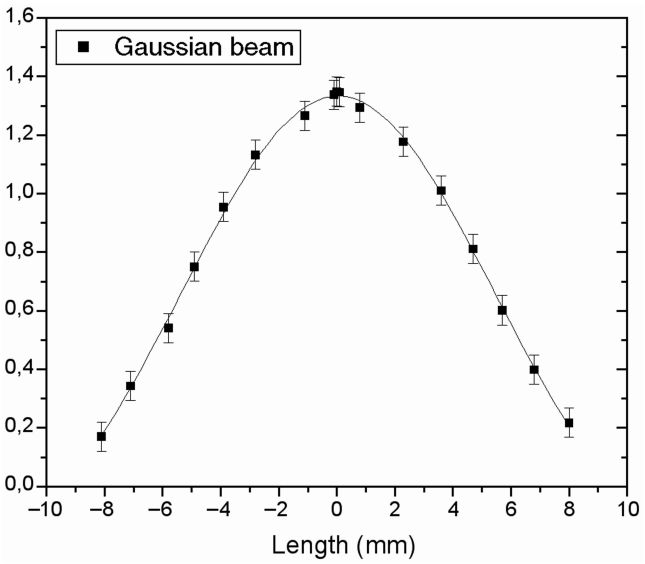

FIG. 1. Measured gaussian-shaped light beam.

20 (158 sec), and 30 (237 sec) J/cm^2, and the PDZ and RADA concentrations were 0.5 and 1.0 mg/kg body weight and illumination with 200 mW/cm^2, to deliver 20 (100 sec), 50 (250 sec), 100 (500 sec), and 200 (1000 sec) J/cm^2. The doses of the sensitizers were chosen according to examples in the literature.[5,7–9,11–14,18,20] We used the sizes of necrosis as the points of data analysis. After illumination, the animals were closed and allowed to recover from surgery. Then the animals were killed by an anesthetic overdose 30 h after illumination and the livers were removed.[24]

Macroscopic and microscopic analysis

The removed livers were macroscopically inspected to assess the extent of necrosis and their overall aspect.

Microscopic analysis was carried out through histological sections from the rat livers exposed to photodynamic therapy. Serial sections of liver tissues were cut with a Leica RM 2125RT microtome (Leica, Houston, TX). The 4-μm paraffin sections were mounted on glass slides and stained with hematoxylin and eosin. Morphometric evaluation allowed us to determine the depth of necrotic liver tissue, as well as the superficial width of necrosis.[24]

This study protocol was approved by the Ethics Committee of Animal Experimentation of the School of Medicine, University the of São Paulo (Ribeirão Preto, Brazil).

Model for the depth of necrosis

The concept of the threshold dose in PDT corresponds to the existence of a minimum dose beneath which no necrosis is induced.[8,11,14,25] Below this threshold dose any cell damage is reversible, and leads to recovery of the original tissue. There is a simple model to use to study the depth of necrosis, as well as the determination of the threshold dose, and was proposed by Ferreira et al.[14] Based on the assumption of validity of Lambert's law of light penetration given by equation 1 below, the model begins with the suppositions that light intensity decreases exponentially as it penetrates into the tissue,[23] and that this penetration depends on the tissue's photosensitizer concentration (c).

$$I = I_0 e^{-ad} \tag{1}$$

where α is the effective absorption coefficient, which is dependent on the photosensitizer concentration ($\alpha = \alpha$[c]); I_0 is light intensity at the tissue surface; d is the distance of penetration; and I is the light intensity at a distance d from the surface.

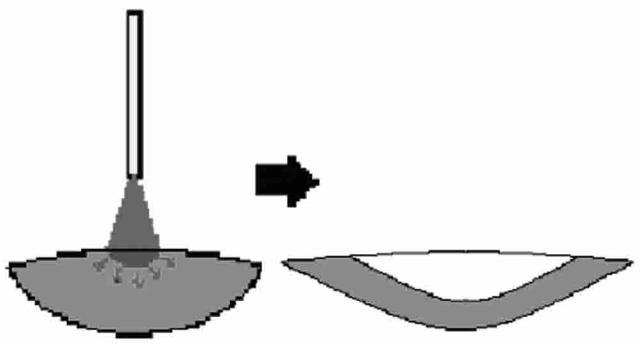

FIG. 2. Schematic representation of the illumination geometry and clinical parameters for assessing the necrotic tissue volume.

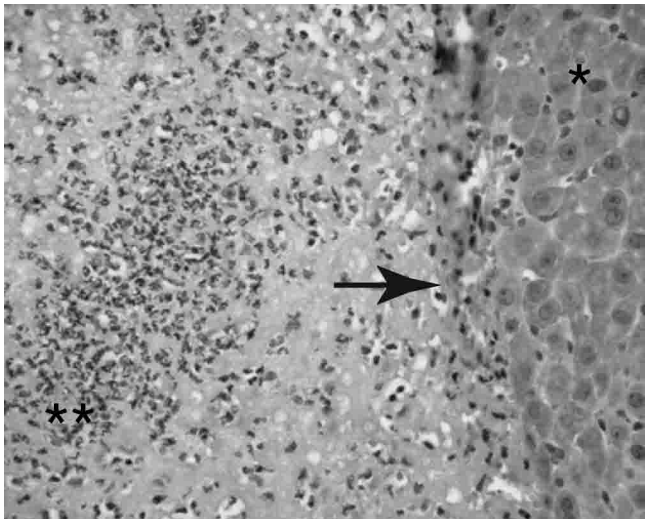

FIG. 3. Typical histological image showing the transition (➔) between necrotic (**) and normal (*) tissue (100× magnification).

These considerations and the fact that the light dose at any point is the product of the local light intensity and the time of illumination, allows one to define the superficial dose D_0, as

$$D_0 = I_0 t \tag{2}$$

The necrosis will take place up to the depth at which $D = D_0 e^{-\alpha d}$ corresponds to the threshold dose (D_{th}). This leads to a relationship between the surface light dose (D_0), the depth of necrosis (d_{nec}), and the threshold dose (D_{th}) given by

$$d_{nec} = 1/\alpha \ln (D_o/D_{th}) \tag{3}$$

where $1/\alpha = \delta$ is the effective penetration depth of the light.[6,11,26] d_{nec} is the depth of necrosis at which $D = D_{th}$. δ can be directly measured as described previously,[23] or it can be used as a fitting parameter in equation (3), when d_{nec} is measured as a function of D_0, which is the procedure we adopted in this work.

Equation (3) can be linearized and fitted to a linear equation ($y = A + Bx$), being ($x = \ln D_0$) and ($y = d_{nec}$), and A and B are both defined in equations 4 and 5. The factor α and D_{th} can be obtained by fitting the parameters A and B. The depth of necrosis (d_{nec}) is measured as a function of D_0, D_{th}, and α, and is obtained by mathematical fitting procedures.

$$\begin{cases} B = 1/\alpha & (4) \\ A = 1/\alpha \ln D_{th} & (5) \end{cases}$$

Model for the width of necrosis at the surface

A light beam emitted by a bare fiber is represented by a gaussian distribution,[20] as measured in this work and presented in Fig. 1, and expressed by the mathematical relation

$$I = I_0 e^{-r^2/2\sigma^2} \tag{6}$$

where I_0 is the intensity of light at the beam's center, r is the radius of the illuminated region, I is the intensity at r from the center, and σ^2 is related to the width of the gaussian beam. This connection with the gaussian beam can also be characterized by its full width at half maximum (FWHM), denoted below by w.

Lets assume a light beam with width w, centered at $r = 0$. The width w is related to σ by

$$w = FWHM = 2\sqrt{2 \ln 2\sigma} \quad (7)$$

Assuming that when $D = D_{th}$, $r = r_{nec}$, leads to a relation between the light dose at the surface (D_0), the square of the necrotic area (r^2_{nec}), and the threshold dose (D_{th}) given by

$$r^2_{nec} = 2\sigma^2 \ln D_0/D_{th} \quad (8)$$

Since there is light side-scatter, some light goes beyond the illuminated area. So, we define

$$w_{nec} = w\,\beta \quad (9)$$

Table 1. Measures of Depth (D_{NEC}) and Width ($2R_{NEC}$) of Necrosis for Each Dose Used for Each Photosensitizer

FS (mg/kg)	*Dose (J/cm²)*	*d_{nec} (mm)*	*$2r_{nec}$ (mm)*
PG 1.0 mg/kg	20	0.3 ± 02	4.0 ± 0.6
	50	1.6 ± 0.3	6.5 ± 0.4
	100	2.1 ± 0.2	7.0 ± 0.3
	200	2.8 ± 0.3	9.0 ± 0.6
PG 2.0 mg/kg	20	1.4 ± 0.3	7.7 ± 0.6
	50	1.8 ± 0.2	10.0 ± 0.3
	100	2.6 ± 0.3	11.1 ± 0.5
	200	3.0 ± 0.2	14.1 ± 0.6
PF 1.0 mg/kg	20	0.0 ± 0.0	0.0 ± 0.0
	50	0.0 ± 0.0	0.0 ± 0.0
	100	0.8 ± 0.2	9.7 ± 0.3
	200	1.0 ± 0.3	11.8 ± 0.5
PF 2.0 mg/kg	20	0.0 ± 0.0	0.0 ± 0.0
	50	0.5 ± 0.3	4.8 ± 0.5
	100	1.0 ± 0.4	10.4 ± 0.6
	200	2.0 ± 0.3	14.2 ± 0.5
PS 1.0 mg/kg	20	0.0 ± 0.0	0.0 ± 0.0
	50	0.5 ± 0.3	2.6 ± 0.6
	100	1.5 ± 0.1	6.9 ± 0.5
	200	2.0 ± 0.3	8.5 ± 0.3
PS 2.0 mg/kg	20	0.0 ± 0.0	0.0 ± 0.0
	50	1.0 ± 0.3	4.3 ± 0.5
	100	1.3 ± 0.3	7.8 ± 0.6
	200	2.0 ± 0.4	10.5 ± 0.3
PDZ 0.5 mg/kg	20	1.8 ± 0.2	7.9 ± 0.4
	50	2.7 ± 0.1	8.5 ± 0.3
	100	2.8 ± 0.1	11.0 ± 0.4
	200	2.9 ± 0.1	12.0 ± 0.5
PDZ 1.0 mg/kg	20	2.8 ± 0.2	8.0 ± 0.5
	50	3.9 ± 0.1	9.7 ± 0.3
	100	4.0 ± 0.3	11.6 ± 0.4
	200	4.4 ± 0.3	15.2 ± 0.5
RADA 0.5 mg/kg	20	0.0 ± 0.0	0.0 ± 0.0
	50	0.0 ± 0.0	0.0 ± 0.0
	100	0.0 ± 0.0	0.0 ± 0.0
	200	2.8 ± 0.2	10.0 ± 0.3
RADA 1.0 mg/kg	20	0.0 ± 0.0	0.0 ± 0.0
	50	0.0 ± 0.0	0.0 ± 0.0
	100	2.3 ± 0.3	10.2 ± 0.4
	200	3.5 ± 0.4	13.2 ± 0.3
FOS 0.1 mg/kg	10	0.0 ± 0.0	0.0 ± 0.0
	20	1.3 ± 0.2	5.0 ± 0.4
	30	3.0 ± 0.3	7.0 ± 0.3
FOS 0.2 mg/kg	10	0.0 ± 0.0	0.0 ± 0.0
	20	2.2 ± 0.2	7.2 ± 0.3
	30	3.2 ± 0.3	10.0 ± 0.4
FOS 0.3 mg/kg	10	1.3 ± 0.3	6.2 ± 0.5
	20	2.7 ± 0.2	10.5 ± 0.4
	30	3.4 ± 0.4	12.2 ± 0.5

where β is a coefficient that depends on the light scatter, and increases the influence of light on w, depending on the medium. The factor β (dependent on the photosensitizer concentration, $\alpha = \alpha[c]$) is related to the light scattered in the tissue by the photosensitizer.

Combining equations (7), (8), and (9), one gets the following relation:

$$r^2_{nec} = w^2\,\beta^2/4 \ln 2 \ln D_0/D_{th} \quad (10)$$

Again, we can linearize this equation, and fit it to the linear equations ($y = A' + B'x$), ($x = \ln D_0$), ($y = r^2_{nec}$), and ($A' = w^2\,\beta^2/4 \ln 2 \ln D_{th}$), where A' and B' are related to β and D_{th}, and their values can be obtained by fitting the linear equations with the data obtained for r_{nec} for many different values of D_0. Normally A– will be a negative value, due to the inversion of the logarithmic term ($\ln 1/Dth = -\ln Dth$). Explicitly, we have:

$$B' = w^2\,\beta^2/4 \ln 2 \quad (11)$$

$$D_{th} = e^{A'/B'} \quad (12)$$

and from the values of A– and B–, D_{th} is determined.

Results

Fig. 2 shows a schematic illumination of the liver and the macroscopic necrotic tissue volume typically obtained.

From the photomicrographic images, we confirmed the observations reported by other authors,[14,24] that the transition between the necrotic and normal tissue is a seen as well-defined line, as shown in Fig. 3.

The observed depth of necrosis (d_{nec}), as well as the superficial width of necrosis ($2r_{nec}$) for all doses are shown in Table 1. Using the data shown and the laser beam characteristics (I_0,w), the expressions for the presented model (D_{th}, β, and α) were determined via mathematical fitting.

Tables 2 and 3 present D_{th}, α, and β for all experimental conditions. α Values were obtained by using the fitting of equation 3, and β values were determined using the fitting of equation 10.

TABLE 2. D_{TH} AND EFFECTIVE LIGHT ATTENUATION COEFFICIENT α VALUES OBTAINED BY FITTING EQUATION 3 AFTER LIVER PDT USING DIFFERENT CONCENTRATIONS OF HEMATOPORPHYRIN DERIVATIVE AND CHLORINE TYPE PHOTOSENSITIZERS

Photosensitizer	*mg/kg*	D_{th}	α
PG	1.0	15.0 ± 2.0	0.88 ± 0.10
	2.0	11.0 ± 2.0	0.98 ± 0.10
PF	1.0	55.0 ± 2.0	1.38 ± 0.10
	2.0	28.0 ± 2.0	1.19 ± 0.10
PS	1.0	26.0 ± 4.0	1.08 ± 0.10
	2.0	21.0 ± 4.0	1.24 ± 0.10
PDZ	0.5	1.5 ± 0.5	1.68 ± 0.10
	1.0	1.1 ± 0.5	1.21 ± 0.10
FOS	0.1	11.7 ± 2.0	0.31 ± 0.10
	0.2	9.7 ± 2.0	0.36 ± 0.10
	0.3	5.3 ± 2.0	0.49 ± 0.10
RADA	0.5	150 ± 2.0	0.29 ± 0.10
	1.0	52.6 ± 2.0	0.33 ± 0.10

TABLE 3. D_{TH} AND EFFECTIVE LIGHT SCATTERING β COEFFICIENT VALUES OBTAINED BY FITTING EQUATION 10 AFTER LIVER PDT USING DIFFERENT CONCENTRATIONS OF HEMATOPORPHYRIN DERIVATIVE AND CHLORINE TYPE PHOTOSENSITIZERS

Photosensitizer	*mg/Kg*	D_{th}	β
PG	1.0	12.5 ± 2.0	0.83 ± 0.10
	2.0	10.8 ± 2.0	1.24 ± 0.10
PF	1.0	52.8 ± 2.0	1.30 ± 0.10
	2.0	26.2 ± 2.0	1.89 ± 0.10
PS	1.0	25.5 ± 4.0	1.04 ± 0.10
	2.0	19.5 ± 4.0	1.16 ± 0.10
PDZ	0.5	2.8 ± 0.5	1.03 ± 0.10
	1.0	1.9 ± 0.5	1.41 ± 0.10
FOS	0.1	11.05 ± 2.0	1.10 ± 0.10
	0.2	9.4 ± 2.0	1.58 ± 0.10
	0.3	5.8 ± 2.0	1.67 ± 0.10
RADA	0.5	154.0 ± 2.0	1.44 ± 0.10
	1.0	55.2 ± 2.0	1.87 ± 0.10

Fig. 4 shows the D_{th} values obtained using the procedure described here involving the depth and width of necrosis for the HpD and chlorine type photosensitizers.

Discussion

Based on the presented results, there are many features that are common for all the photosensitizers tested, independently of the technique used to obtain the results (depth or width). One common factor is that necrotic and normal regions are always separated by a well-defined boundary (Fig. 3), independent of the type or concentration of photosensitizer used. During our histological observations, findings that appeared to be specific to the porphyrin or chlorine type photosensitizers were observed, but they will not be explored in this discussion. Rather, we shall focus our attention on our main goal, the determination of D_{th}.

The first and most important observation is that the values obtained for D_{th} by measuring the depth and width of necrosis agree quite well within the observed experimental error, here taken as the standard deviations of the data (Fig. 4). This certainly helps to validate the use of superficial necrosis as a way to determine D_{th}, once the parameters of the light beam are known.

Besides the agreement in values, we also observed that among the agents in the porphyrin group, Photogem was the photosensitizer with the lowest D_{th}. Similarly, among the chlorine type agents, Photodithazine was the one with the lowest D_{th}. Such comparisons are important to show that there are no generalizations to be made from the D_{th} values we found.

The values of D_{th}, as defined here, are dependent on the concentration present on in the tissue as is evident from the data presented in Tables 2 and 3. As general rule, D_{th} decreased with increasing photosensitizer concentration.

Mathematical fitting was used to provide the values of α and β for each test condition. With regard to α (the attenuation coefficient), we observed that it was dependent on the photosensitizer concentration, but its value increased with increasing concentrations. This is the expected result, be-

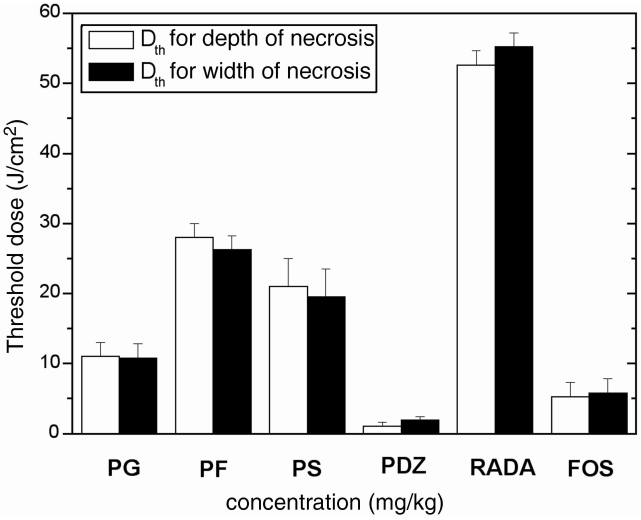

FIG. 4. Threshold dose obtained using the model for depth and superficial width of necrosis as a function of the photosensitizer used, for the highest light dose (J/cm^2) and photosensitizer concentration (mg/kg) tested for each group.

cause it corresponds to increasing absorption (α is higher) as higher concentrations of photosensitizer are present. We also observed that α was strongly dependent on the photosensitizer used.

As for β, its value also increases with concentration, but it seems to be more independent from the type of photosensitizer used. The reason for this is derived from the fact that the scattered portion of β is independent from the photosensitizer used, and is more closely dependent on the tissue's intrinsic properties.

Finally we point out that the use of surface necrosis in the determination of D_{th} has the clear advantage of being an easier method compared to the traditional method using the depth of necrosis. When using the surface method only a single measurement is necessary. By using a gaussian light beam, the surface is automatically exposed to many different doses, and therefore single measurements allow the determination of D_{th}. Besides, the method using the width of necrosis does not depend on α or heterogeneities in the tissue along the optical path of the light energy. We should also emphasize that the values of D_{th} found here are in agreement with the findings of others, when such comparisons are possible.[14,20,21]

For potential clinical application, we have to consider lack of data available about D_{th} in humans due to the difficulty in precisely evaluating the depth of necrosis. Certainly observation of the width of necrosis is much simpler, and may finally allow more accurate calculations of D_{th} in human tissues. In clinical situations we cannot remove the treated tissue to analyze the depth of necrosis; on the other hand, the superficial necrotic region can always be measured. This is a clear advantage of the method presented here compared to more traditional ones.

Conclusions

We have extended the depth-of-necrosis model proposed by Ferreira et al.[14] to determine threshold PDT doses using a model that employs the superficial width of necrosis caused by a gaussian laser beam. When determining D_{th}, we need explicit knowledge of the optical properties of the tissues involved. In the present study this information was not necessary since the penetrating properties of light are not relevant to the superficial width of necrosis. The side-scatter coefficient β could also eventually be used as an alternative to determine the most effective attenuation coefficient α in the bulk of the tissue. However, further investigation is needed and this issue will be the subject of future studies. The method presented here adds to the literature an alternative method of determining D_{th}, which is an important parameter for effective PDT. In addition, in clinical application the method presented here may be the simplest alternative to use to obtain the proper D_{th} for PDT.

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Disclosure Statement

No competing financial interests exist.

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