Study of destruction effect of blood vessels after photodynamic therapy in a model of chorioallantoic membrane

G. Arthuzo, V. S. Bagnato, H. H. Buzza

G. Arthuzo, V. S. Bagnato, H. H. Buzza, "Study of destruction effect of blood vessels after photodynamic therapy in a model of chorioallantoic membrane," Proc. SPIE 11070, 17th International Photodynamic Association World Congress, 11070B6 (7 August 2019); doi: 10.1117/12.2526155

SPIE.

Event: 17th International Photodynamic Association World Congress, 2019, Cambridge, Massachusetts, United States

Study of destruction effect of blood vessels after photodynamic therapy in a model of chorioallantoic membrane

Arthuzo, G.[1], Bagnato, V. S.[1], Buzza, H. H.[1]*

1 Sao Carlos Institute of Physics. University of Sao Paulo.
*Correspondent author – e-mail: hilde.buzza@usp.br

ABSTRACT

Photodynamic therapy, a technique used for several diseases, when carried out in blood vessels, leads to their destruction. However, vessel recovery is observed some time later, which can be an angiogenic process (formation of new blood vessels) induced by the therapy itself or blood reperfusion. For the investigation of this vascular process after photodynamic therapy, the chorioallantoic membrane (CAM) model of chicken eggs was used. Photodynamic therapy was performed on membrane vessels with the Photogem® photosensitizer, at a concentration of 10 μg/mL, and light subdoses to avoid leading the embryo to death. Light doses of 6 and 15 J/cm^2 were established for the experiments and a decrease in vessel density 3 hours after photodynamic therapy was observed, with an increase 24 hours later. For quantification of these effects, an equation was determined and a routine of MATLAB® was designed to determine the percentage of area occupied by blood vessels in the images, which were performed before, every 30 minutes for the first 3 hours after treatment and 24 hours later. Furthermore, for an analysis of the distribution of large and small vessels, the length and diameter of each vessel in the images were measured with the ImageJ® software, which enabled to verify that the smaller vessels are most affected 3 hours after the therapy, with an increase in the number of these vessels after 24 hours.

Keywords: Photodynamic Therapy; Vascular; Chorioallantoic membrane

1.0 INTRODUCTION

There are several diseases that affect blood vessels, such as chronic venous insufficiency (varicose veins), venous thrombosis, diabetic foot, peripheral obstructive arterial disease and abdominal aortic aneurysm. Approximately 20 to 33% of women and 10 to 20% of men will present some degree of chronic venous insufficiency during their lifetime. (1) There are also vascular malformations and proliferative vascular lesions (tumors) that are congenitally malformed capillaries, lymphatic, venous, and arterial vessels. (2-3) Vascular tumors include hemangioma, which are benign and arise from the proliferation of endothelial cells. (2) The infant hemangioma proliferates in the first weeks of life and then regresses spontaneously, leaving scars and facial deformities. (4-5) The blood vessels are also related to other diseases, such as cancer. The formation of new vessels, called angiogenesis, is essential for the growth of solid tumors and for metastasis. (6) In this way, the study of new therapies that aim at the elimination of vessels is fundamental for the development of more effective treatments.

Photodynamic therapy (PDT) is an alternative to conventional treatments, since it has a more localized effect and less adverse reactions. (7) PDT is a technique involving the use of a photosensitizing substance, adequate wavelength light, and oxygen to produce a cytotoxic effect. (8) The photosensitizer

17th International Photodynamic Association World Congress, edited by Tayyaba Hasan, Proc. of SPIE
Vol. 11070, 11070B6 · © 2019 SPIE · CCC code: 0277-786X/19/$21 · doi: 10.1117/12.2526155

absorbs energy from light and transfers it to molecular oxygen to create singlet oxygen, which is the cytotoxic agent that leads to cell death and destruction of the cellular target, such as tumors or vessles. (9) There are different mechanisms for target destruction with the use of PDT. In the case of cancer, tumor cells can be targeted directly or one can reach the blood vessels around the tumor, which are responsible for their nutrition. (9)

The dose of drug, dose of light and time interval between application of the drug and illumination are important parameters of the photosensitizers. The same photosensitizer acts completely differently when these variables are changed. (10)

The chorioallantoic membrane (CAM) is formed by fusion of the corium and the allantoid into chicken eggs. In this membrane, a vascular network develops and acts as the respiratory organ of the embryo until the moment of leaving the egg. (11) Because of its high vascularity and easy access to blood vessels, CAM is a suitable model for studying the vascular effects of PDT and other therapies. Experiments with Avastin®, for example, have shown that this drug strongly inhibits angiogenesis in CAM when applied topically on the 7th and 8th day of embryo development. (12) The model allows the delivery of the substance under study in a topical or intravenous administration. (13)

Another great advantage of this model is that the use of anesthetics is not necessary because the experiments are performed before the embryo develops the perception of pain and, therefore, this model is considered in many countries an alternative method to the use of animals. (14)

Research shows that PDT, when performed in blood vessels, induces reversible or permanent damage. However, after some time, vessel recovery is observed, which may be caused by an angiogenic process induced by PDT itself. The formation of new blood vessels is an undesirable effect in the treatment of tumors and vascular diseases themselves, and therefore understanding of the mechanisms of PDT-induced angiogenesis may help to enhance its vascular targeting and its combination with inhibitors of angiogenesis. (12-13,15-16)

2.0 MATERIAL AND METHODS

2.1 Chorioallantoic Membrane Model

Fertilized eggs were provided by Ad'oro SA Company (São Carlos, SP, Brazil). They arrived to the lab on the first embryo development day (EDD1). To obtain the model, the procedures described in the literature was followed, with the window opened on the EDD3. (7) To avoid any type of contamination, all procedures were performed inside of laminar flow, with disinfected tools. The eggs were kept in the incubator at 37ºC and were taken from it only the time needed to the experiment, to avoid temperature changes. The experiments with antiangiogenic formulations or PDT were performed until EDD14, because during this period there is no development of pain receptors in the embryo. Therefore, it is not necessary the use of analgesic and anesthetic during the procedures. (14)

2.2 Bevacizumab

The experiments with bevacizumab, commercially called Avastin® (Genentech, South San Francisco, USA) started from EDD7, to be used as a positive inhibitor of angiogenesis. Avastin® was diluted in saline and the concentrations tested were 1 mg/mL and 10 mg/mL to evaluate the anti-angiogenic effect of the substance. The number of applications was varied between the EDD7 and EDD11 and the volume of the Avastin® solution in each application was varied between 20 and 100 μL

2.3 Photodynamic Therapy

The photosensitizer chosen was Photogem® (Photogem LLC Company, Moscow, Russia). The concentration of the solution was 10 μg/mL diluted in saline solution with topical application and incubation time of 40 minutes. After incubation, the membrane was washed with physiological saline to remove the excess photosensitizer, which remained on the membrane. Immediately after washing, the membrane was

illuminated with the Lince equipment (MMOptics, São Carlos, Brazil), which has a set of LEDs with emission at 635 nm.

The PDT parameters were varied in relation to the volume of the Photogem® solution applied (200 and 400 μL) and the irradiance used (20 and 50 mW/cm^2), and the lighting time was fixed in 5 minutes, totalizing doses of 6 and 15 J/cm^2, respectively. The control groups tested were: photosensitizer only (200 and 400 μL), light only (6 and 15 J/cm^2) and only saline solution (400 μL).

2.3 Aquisition and analysis of images

CAM images were obtained with a 400x magnification camera (Digital Microscope, China) positioned above the egg and, therefore, they could be made without removal of the membrane and with the live embryo, following the development of vessels both as necessary. In the PDT experiments, the membrane images were taken before the procedure, immediately thereafter, every 30 minutes for the first 3 hours and the following day (24 hours after PDT). In the Avastin® experiments, the CAM was photographed daily after the first application of the drug. The acquisition of each image was always done in order to obtain the same CAM region.

The quantitative analysis was done with a MATLAB® routine (MATrix LABoratory, MathWorks, USA) that calculates the percentage of area occupied by the vessels from the amount of red, green and blue present in each image. As the blood absorbs in the spectral region of 500-600 nm, the green channel of the images was used to allow automatic detection of the vessels. For this, an 8-bit green channel array convolution was performed with a disk mask with 30 radius pixels. This process basically generated an image where the value of each pixel was the average of all pixels within a disk in a given given radius. Therefore, the ratio provided the percentage of area that the blood vessels occupied. Since each egg has a distinct vascular network, all data from the same egg were normalized to the value corresponding to the first image of each CAM, made before the experiment. (17)

3.0 RESULTS AND DISCUSSION

To evaluate the angiogenic effect after PDT, the parameters were chosen so as to guarantee the survival of the embryo 24 hours after the experiment, with the use of light subdoses. The doses of light that showed vascular effect, but allowed the survival of the embryo, were: 6 and 15 J/cm^2.

3.1 Image processing

The percentage of area occupied by blood vessels was given by the ratio of the number of pixels per vessel obtained with our image processing routine to the number of total pixels. To avoid comparison between distinct vascular networks, the quantification of each egg was obtained by the ratio between the percentage of vessel area of each image over time and the percentage of vessel area of the initial image of the same egg.

In each study group, the average of the 5 egg group ratios and the error bar were calculated, which allowed to observe the behavior of the vessels over time. This type of analysis was done for all groups described in the next subsections.

3.2 Anti-angiogenic response of Bevacizumab (Avastin®)

Avastin® is a substance that has an anti-angiogenic effect already known, (14) including on the CAM model. (36) Thus, this drug was used as a positive control of inhibition and established the protocol with the highest inhibition of blood vessel growth. Some tests were performed and a qualitative and quantitative analysis of CAM images showed the effect of Avastin®.

In these tests, the substances were applied topically, since this way of administration has the advantage of do not causes any vascular damage. Different parameters were tested to establish a protocol that showed inhibition of vessel growth with the application of Avastin®. After the tests, the concentration chosen was 1 mg/mL, with the solution applied in EDD7, 8, 9 and 10 in a volume of 100 μL per day. The

other parameters tested showed no high effect on blood vessels. For comparison, the control group received 100 µL of saline on those same days. The application was topical, directly in the region of blood vessels, and the CAM images were made daily from EDD7 to EDD11.

Figure 1 shows CAM images obtained with the camera positioned above the egg, showing an example of the last day of the group that received Avastin® and the group that receive saline.

The images made in EDD7 show CAM without the application of substances. It is noted that each egg has a different initial vascularization. Over time, more vessels arise and the displacement of some vessels occurs due to the movement of the embryo. In EDD11 it is possible to notice regions with absence of blood vessels, different from what is seen on the same day of the control group.

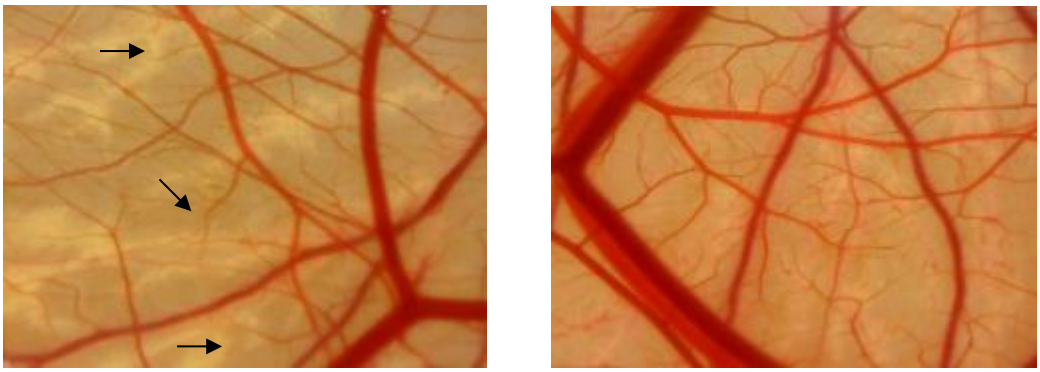


Figure 1 - CAM images obtained in EDD11. (a) CAM with daily application of Avastin®, where the arrows indicate the regions without the presence of visible vessels. (b) CAM with daily application of serum, presenting a higher density of small vessels.

In the quantitative analysis performed with the MATLAB® routine, 5 eggs per group were used. Figure 2 shows a graph with the ratio of the percentage of area related to the vessels in each day and the percentage of area in the initial day (EDD7), showing the growth of the vascular network along the days of embryonic development for each of the groups. The values shown in the graph are the average and error bar of the 5 eggs.

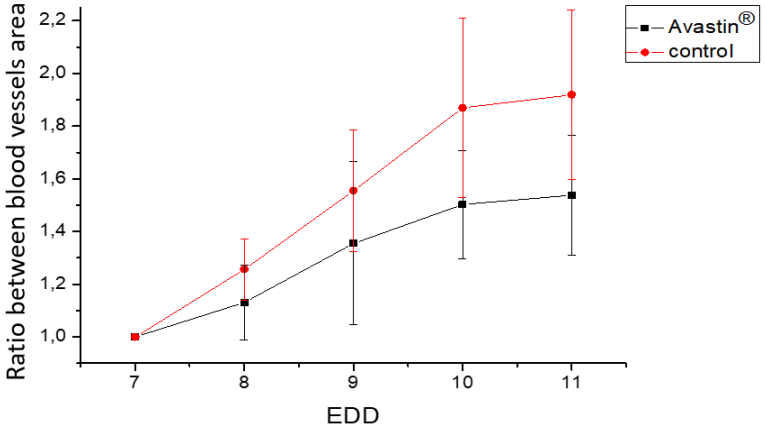


Figure 2 - Graph of the growth of the relative area of blood vessels along the days of embryonic development of the group that received Avastin® solution and the control group.

In EDD8 there is already a difference between the groups, with the control group showing a growth of 26% and the Avastin group a growth of 13% in relation to the previous day. In addition to the difference between them increasing over the days, the chart shows a much lower growth rate for the Avastin® group

compared to the control. In EDD11, the control increased by 92% compared to EDD7, while the Avastin group increased by 54%. Despite the error bars, there is a trend of behavior that shows that Avastin® inhibited the growth of blood vessels.

3.3 Evaluation of vascular damage of PDT

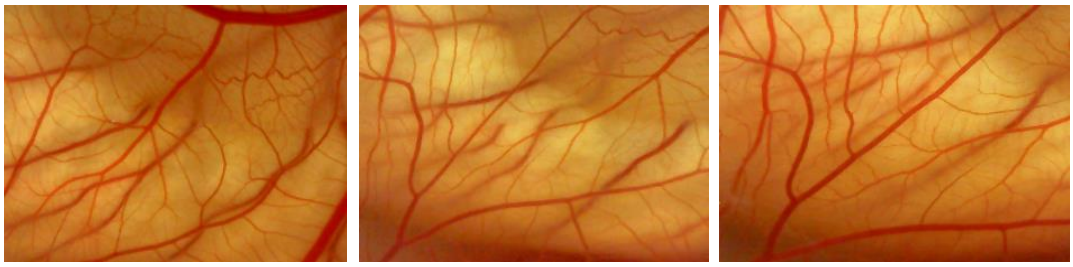
Some tests were performed to verify vascular damage after PDT. In one group, 200 μL of Photogem® was used with 20 mW/cm^2 illumination for 5 minutes (group A), totaling a dose of 6 J/cm^2. In another group (group B), the volume of Photogem® was increased to 400 μL, maintaining the same lighting parameters, to verify if there was a greater damage in the vessels without bringing the embryo to death. In addition, another group was performed and 400 μL of Photogem® was maintained, increasing the irradiance to 50 mW/cm^2 for 5 minutes (Group C), totaling a dose of 15 J/cm^2, in order to verify if the damage would be even greater. A summary of the differences between groups A, B and C is shown in Table 1.

Table 1 – Photogem® solution volume and total light dose in groups A, B and C.

Group	Photogem® volume (μL)	Total dose of light (J/cm^2)
A	200	6
B	400	6
C	400	15

The first CAM image was obtained before the photosensitizer was applied and the following images were obtained immediately after the PDT, every 30 minutes for the first 3 hours and 24 hours after PDT.

Figure 3 shows an example of CAM with decreased vessel density during the 3 hours after PDT, especially in regions of smaller vessels, and their recovery the next day (an example of group C egg). A quantitative analysis with the MATLAB® routine was performed as in the previous subsection. Figure 4 shows the behavior of blood vessels over time in the groups where PDT was performed, with the average and error bar for 5 eggs per group.



Before 3 hours 24 hours

Figure 3 – CAM images obtained before PDT, 3 and 24 hours later, with application of 400 μL of Photogem® solution and irradiance of 50 mW/cm^2.

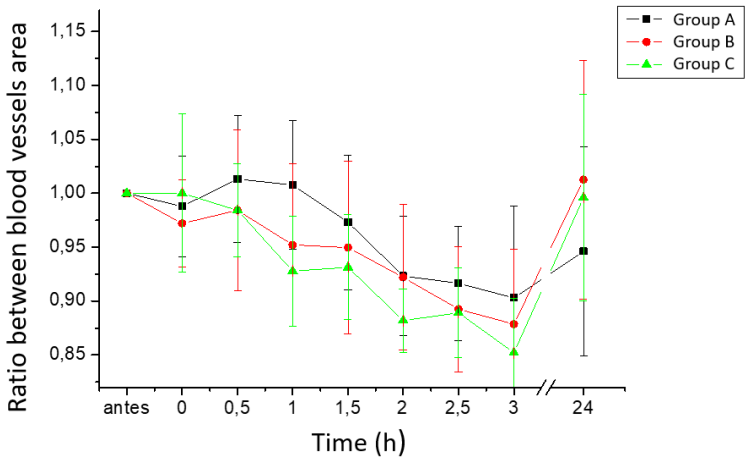


Figure 4 – Graph of post-PDT vascular damage using different parameters.

For group A, vascular damage begins 1 hour after PDT. By increasing the amount of Photogem® applied (groups B and C), it can be seen from the graph that the damage begins immediately after PDT. In addition, in group C, in which the total light dose is the largest, there is a more pronounced drop in vessel area in the image. Group B showed a greater reduction in blood vessels compared to group A, that is, the application of double volume of Photogem® solution did not show double the vascular effect, but it was possible to increase vessel damage without bringing the embryo to the death. At the end of the first 3 hours of follow-up, group A had a 10% reduction of vessels compared to the amount before PDT and group B had a 12% reduction in the same period. The group that presented the greatest reduction in blood vessels in the first 3 hours after PDT was group C, with a reduction of 15%.

The next day, we can see that there was recovery of the vessels in all groups.

Therefore, even with the variation of light and photosensitizer dose parameters, the reduction of the vessels happened between 10 and 15% at 3 hours post-PDT, and 24 hours later, there was complete recovery of vessels.

From the values obtained with the graph, another quantitative analysis can be done to better distinguish the reduction presented by the three groups. If we consider that the area occupied by the vessels varies in time with a growth factor, and a damage factor given by the PDT itself, an equation for vascular damage can be written as:

$$\frac{dA_V}{dt} = L - \alpha A_V$$

where A_V is the area of vessels, L represents the growth and α is the rate of damage in the vessels. The equation can be integrated and obtained the equation:

$$A_V = \frac{L}{\alpha} - \frac{(L - \alpha A_0)}{\alpha} e^{-\alpha t}$$

The equation shows that, immediately after PDT, the vessel area decreases. After a certain time, the vessel area reaches a constant value of L/α. For αt small (away from saturation), it is possible to make the approximation:

$$A_V = A_0 + (L - A_0\alpha)t$$

Analysis of a time interval of 3 hours does not show a significant growth of the blood vessels, therefore it can be considered L = 0. Thus, when considering the time interval of 3 hours after the PDT, it is possible to approximate the behavior of the graph by a line of equation:

$$A_V = A_0 - A_0\alpha t$$

From the graph of Figure 4, the best fit line for each group has linear coefficient A_0 and angular coefficient $-A_0\alpha$. Therefore, as higher α value as greater is the vessel damage, which corresponds to the greater effect of PDT. The ratio between the coefficient and the linear coefficient is equal to the value of -α, which enables the calculation of the damage rate in the vessels of the groups, in which the PDT was performed. The α value of groups A, B and C is shown in Table 2.

Table 2 – Value of α of the groups in which the PDT was made.

Grupo	α
A	0,037
B	0,036
C	0,049

In this analysis, with the values of α, group C also showed a greater efficiency in reducing the area occupied by blood vessels, followed by group A and group B. Although group B received a greater amount of photosensitiser compared to the group A, the α values of these groups do not differ significantly. However, the value of α in group C shows that, with a higher dose of light in PDT, there is a higher rate of vessel damage. Therefore, the rate of damage did not increase with the increase in the amount of photosensitizer, but rather with the increase in the dose of light.

3.4 Control Groups

The groups only light (50 mW/cm^2 during 5 minutes), only PS (volume of 400 μL and incubation time of 40 minutes) and only saline (volume of 400 μL) did not show significate changes, totaling the maximum reduction of 6%. This result showed a similar behavior for all control groups, confirming the vascular effect of photodynamic therapy.

4.0 CONCLUSION

The CAM model was used to study the vascular effects of PDT with the use of Photogem® at a concentration of 10 μg/mL and light sub-doses. With light doses of 6 and 15 J/cm^2, there was a decrease in vessel density 3 hours after PDT, but an increase in this density was observed 24 hours later. To quantify the observed effects, a routine was developed in MATLAB® to calculate the percentage of area occupied by the blood vessels in the images. The routine enabled to construct a graph that shows the area of vessels related to the time after the therapy and, from the line of best fit considering the first 3 hours after the PDT, the damage rate in the vessels of these groups was calculated. As group C (PDT with 400 μL of Photogem® solution and light dose of 15 J / cm2) showed a greater reduction in vessel area with these analyzes.

References

1 SOCIEDADE BRASILEIRA DE ANGIOLOGIA E DE CIRURGIA VASCULAR REGIONAL SÃO PAULO. *Doenças Vasculares*. Disponível em: <https://sbacvsp.com.br/doencas-vasculares/>. Acesso em: 29 out. 2018.

2 WASSEF, M. et al. Vascular anomalies classification: recommendations from the International Society for the Study of Vascular Anomalies. *Pediatrics*, v. 136, n. 1, p. 1–12, 2015.

3 KOLLIPARA, R. et al. Vascular anomalies in pediatric patients: updated classification, imaging, and therapy. *Radiologic Clinics of North America*, v. 51, n. 4, p. 659–672, 2013.

4 HAGGSTROM, A. N. et al. Prospective study of infantile hemangiomas: clinical characteristics predicting complications and treatment. *Pediatrics*, v. 118, n. 3, p. 882–887, 2006.

5 LEE, K. C.; BERCOVITCH, L. Update on infantile hemangiomas. *Seminars in Perinatology*, v. 37, n. 1, p. 49–58, 2013.

6 SHIH, S.-C. et al. Molecular profiling of angiogenesis markers. *American Journal of Pathology*, v. 161, n. 1, p. 35–41, 2002.

7 HH Buzzá, et al. Fluorescence analysis of a tumor model in the chorioallantoic membrane used for the evaluation of different photosensitizers for photodynamic therapy. *Photodiagnosis and photodynamic therapy*, v. 19, p. 78-83, 2017.

8 KHURANA, M. et al. Quantitative in vitro demonstration of two-photon photodynamic therapy using Photofrin® and Visudyne®. *Photochemistry and Photobiology*, v. 83, n. 6, p. 1441–1448, 2007.

9 BROWN, S. B.; BROWN, E. A.; WALKER, I. The present and future role of photodynamic therapy in cancer treatment. *Lancet Oncology*, v. 5, n. 8, p. 497–508, 2004.

10 ALLISON, R. R.; MOGHISSI, K. Oncologic photodynamic therapy: clinical strategies that modulate mechanisms of action. *Photodiagnosis and Photodynamic Therapy*, v. 10, n. 4, p. 331–341, 2013.

11 BLACHER, S. et al. Quantification of angiogenesis in the chicken chorioallantoic membrane (CAM). *Image Analysis & Stereology*, v. 24, n. 3, p. 169–180, 2005.

12 NOWAK-SLIWINSKA, P. et al. Processing of fluorescence angiograms for the quantification of vascular effects induced by anti-angiogenic agents in the CAM model. *Microvascular Research*, v. 79, n. 1, p. 21–28, 2010.

13 NOWAK-SLIWINSKA, P. et al. Vascular regrowth following photodynamic therapy in the chicken embryo chorioallantoic membrane. *Angiogenesis*, v. 13, n. 4, p. 281–292, 2010.

14 RIBATTI, D. The chick embryo chorioallantoic membrane (CAM). A multifaceted experimental model. *Mechanisms of Development*, v. 141, n. 3, p. 70–77, 2016.

15 NOWAK-SLIWINSKA, P. et al. Angiostatic kinase inhibitors to sustain photodynamic angio-occlusion. *Journal of Cellular and Molecular Medicine*, v. 16, n. 7, p. 1553–1562, 2012.

16 DEBEFVE, E. et al. Combination therapy using verteporfin and ranibizumab; optimizing the timing in the CAM model. *Photochemistry and Photobiology*, v. 85, n. 6, p. 1400–1408, 2009.

17 BUZZA, H. H., FREITAS, L. C. F., MORIYAMA, L. T., et al. Vascular Effects of Photodynamic Therapy with Curcumin in a Chorioallantoic Membrane Model. *Int J Mol. Sci*, v. 20, n.5, p. 1-12, 2019.