Photodynamic therapy for cutaneous hemangiosarcoma in dogs

Martha S.T. Rocha[a], Carolina M. Lucci[a], Jairo A.M. dos Santos[b], João Paulo F. Longo[c],
Luis Alexandre Muehlmann[d], Ricardo B. Azevedo[c,*]

[a] Department of Physiological Sciences, Institute of Biological Sciences, University of Brasilia, Brasilia, DF, Brazil
[b] Autonomous Veterinarian, Brasilia, DF, Brazil
[c] Department of Genetics and Morphology, Institute of Biological Sciences, University of Brasilia, Brasilia, DF, Brazil
[d] Faculty of Ceilandia, University of Brasilia, Brasilia, DF, Brazil

ARTICLE INFO

ABSTRACT

Cutaneous hemangiosarcoma is a malignant neoplasia that frequently occurs in dogs. The most effective treatment requires wide surgical excision of the tumor. To avoid mutilating surgeries, photodynamic therapy (PDT) could serve as an alternative treatment. This study aimed to treat cutaneous hemangiosarcomas in dogs using PDT with aluminium-chloride-phthalocyanine nanoemulsion (AlClPc-nano) as photosensitizer. Eight dogs with histopathological diagnosis of naturally occurring cutaneous hemangiosarcoma were treated. Animals were given intra and peritumoral injections of AlClPc-nano (13.3 μM). After 15 min, the masses were LED irradiated at a wavelength of 658–662 nm (80 mW potency) for 25 min (120 J/cm^2 fluency). The number of sessions was based on lesion observations, with PDT sessions repeated every 7 days until the mass was no longer macroscopically visible. On that occasion, an excisional biopsy of the area was taken for histopathology analysis. Blood was collected from each animal before each PDT session and excisional biopsy for hematological analysis (blood counts; liver and kidney function). The number of PDT sessions varied from 2 to 4, depending on the size of the initial mass. Seven of the eight cases demonstrated complete remission of neoplasia. Microscopic analysis of the excisional biopsies showed necrosis and hemorrhage only, with no cancer cells, except in one case. During the treatment, inflammation and necrosis were macroscopically observed in the treated areas. The dogs did not show any alteration in blood parameters that could be related to the PDT. In conclusion, PDT with AlClPc-nano is a safe and effective treatment for cutaneous hemangiosarcoma in dogs.

1. Introduction

Photodynamic therapy (PDT) is a treatment modality for different types of tumors. PDT uses photoactivated molecules (called photosensitizers), light and molecular oxygen. Following light activation of the photosensitizers, a photochemical reaction occurs and promotes the formation of reactive oxygen species (ROS), mainly singlet oxygen, that can kill cells directly and/or cause blood vessel shutdown. In some cases, this reaction can induce an immune response against cancer cells [1,2].

Photosensitizers are classified as first, second or third generation depending on their time of development and specific characteristics. Hematoporphyrins and their derivatives belong to the first-generation group of photosensitizers. However, they exhibit relatively poor tissue selectivity, low light absorption, and poor tissue penetration of the light used to promote excitation [3]. The second-generation of photosensitizers include porphyrins, chlorins and phthalocyanines. The main advantages of using these molecules include better tumor selectivity, faster clearance from normal cells, and preferential absorption of light at longer wavelengths so they can be used to treat tumors in deeper tissues [4]. However, some of the second-generation photosensitizers, such as the phthalocyanines, are hydrophobic molecules and lose their photodynamic capability in aqueous environments. The use of carriers to deliver photosensitizers not only solves this problem, but can also increase its accumulation in the tumor site. Third generation photosensitizers consist of the association of photosensitizers with carriers at the nanoscale (nanocarriers) [4,5].

One of the first studies into the effects of PDT application in veterinary medicine was conducted in the 1980s, involving the treatment of primary solid malignant lesions in pet cats and dogs with a hematoporphyrin derivative [6]. The authors reported that this first-generation photosensitizer produced side effects, especially local erythema and late photosensitization. Other photodynamic agents, such as aminolevulinic acid (ALA), have since been used in the topical treatment of

---

squamous cell carcinoma in cats with reduced side effects [7,8]. However, although PDT represents a recognized treatment option for different diseases in human medicine, it is not yet an established treatment option in veterinary medicine [9].

Cutaneous hemangiosarcoma (HSA) is a malignant neoplasia that arises from endothelial cells. It manifests as a reddish tumor mass located on the skin with histological features of pleomorphic endothelial cells, with increased nuclear size, the presence of mitotic figures, which line blood-filled vascular spaces of variable sizes infiltrating into surrounding tissues, together with severe bleeding [10–12]. Ulceration may be present, and the owners of affected animals often believe such areas to be bruises resulting from trauma [10,11]. This type of tumor occurs in dogs and can appear anywhere on the skin, but is most commonly present on the ventral abdomen, prepuce, and pelvic limbs, or other areas of skin that are sparsely haired and light in color [13]. The most effective treatment requires a wide surgical excision of the tumor, together with some of the normal surrounding tissue, and, depending on the size and localization of the cutaneous HSA, the surgery can lead to animal mutilation [13,14]. In addition, Ward et al. (1994) observed that complete surgical margins excision was only achieved in 7 out of 9 cases (78%) for superficial cutaneous HSA in dogs. For cats, complete surgical margins were only achieved in 5 out of 10 cases (50%) [11]. In these cases, PDT could serve as an excellent alternative treatment or co-treatment for surgery.

The aim of this study was to evaluate the effectiveness of PDT for cutaneous HSA in dogs, using a third-generation photosensitizer consisting of a nanoemulsion of aluminum-chloride-phthalocyanine (AlClPc-nano).

2. Materials and methods

2.1. Animals

Eight adult dogs with histopathologically confirmed cutaneous HSA of natural occurrence were included in the study. Breed, sex, age, body weight and tumor data of the dogs used are presented in Table 1. All animals were patients of the Oncologic Service for Companion Animals at the Antonio Clemenceau Veterinary Hospital assisted by veterinarian Martha Rocha (Register # 1639 CRMV-DF). All pet owners signed a written Informed Consent form and the procedures were preapproved by the Animal Ethics Committee of the University of Brasilia (protocol#36972/2008).

2.2. Animal preparation for treatment

Prior to treatment, blood samples were collected from all animals for hematological and biochemical evaluation (blood cell counts, creatinine, urea, ALT and alkaline phosphatase). Only when blood parameters were normal were the dogs subjected to the treatment.

Animals received PDT under anesthesia. The animals were fasted for 8 h prior to the procedure. Before anaesthetic induction, each animal received fentanil (5 μg/kg i.v.). Anesthesia was induced with propofol (5 mg/Kg i.v.) and maintained by ventilation with isoflurane in pure oxygen. Hemoglobin oxygen saturation and heart rate were monitored continuously. Ringer's lactate solution was administered i.v. at 4 mL/kg/h. Animals were medicated with dipirona (25 mg/kg i.v.) immediately after the procedure for analgesia.

2.3. PDT

The nanoemulsion containing 40 μM AlClPc (AlClPc-nano) was produced by a self-nanoemulsification protocol, previously published by our group (Muehlmann et al., 2015). The light source used was a LED device with 658–662 wave length and potency of 80 mW ($0.08\,W/cm^2$). Irradiation was maintained for 25 min (1500 s) resulting in a light energy dose of $120\,J/cm^2$ ($0.08\,W/cm^2$.1500sec).

The AlClPc-nano was diluted with 0.9% saline solution (1:3, with a final concentration of 13.3 μM) and the dogs given intra- and peritumoral injections in a volume sufficient to cause tumescence of the tumor area. After 15 min, non-contact (4 cm distance) surface illumination of the entire tumor area was implemented (Fig. 1). PDT sessions were repeated every 7 days until the tumor was no longer macroscopically visible. An excisional biopsy of the area was subsequently taken, following the same previously described anesthetic procedure. The excised tissue was then submitted to histopathological analysis by a professional veterinary histopathology laboratory (Histopato, Brasilia-DF, Brazil). Blood was collected before each PDT session and immediately prior to excisional biopsy for hematological and biochemical evaluations.

2.4. Animal accompaniment post-treatment

Post-treatment, each animal was given anti-inflammatory and analgesic drugs if necessary. All animals returned 7 days after each PDT session for re-evaluation of the tumor, blood sample collection and another PDT session, if necessary. All animals were clinically accompanied for at least 1 year and up to 3 years after the end of the treatment.

3. Results

PDT with AlClPc-nano was performed on 8 dogs (10 lesions) presenting a cutaneous HSA of natural occurrence. No vomiting, diarrhea, fever or other systemic side effects were observed at any time following the therapy. Furthermore, PDT treatment did not alter the hematological or biochemical parameters of the dogs.

The number of PDT sessions varied from 1 to 4 in these dogs. In most dogs (five out of eight), 2 PDT sessions were sufficient for the
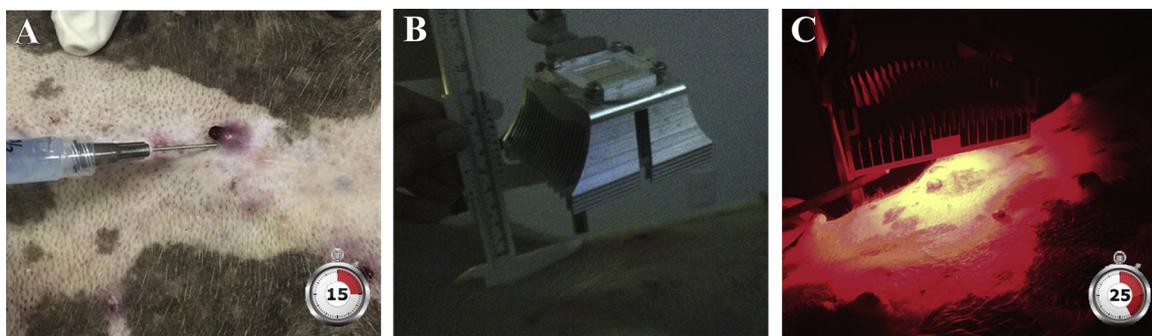
Table 1
Breed, sex, age, body weight (BW) and tumor data of the 8 dogs treated with PDT.

Case Number	Breed	Age (Years)	Sex	BW (kg)	Site of tumor	Single (S) or Multiple (M) tumors	Tumor Size (cm)
1	Pitbull	10	M	30.0	Groin	S	0.5 × 0.6
2	Pitbull	9	F	37.0	Abdomen	M	1.1 × 1.3
							2.0 × 1.1
							0.7 × 0.8
3	Mixed breed	unknown	M	22.7	Prepuce	S	3.1 × 3.4
4	Boxer	11	M	38.2	Groin	S	1.0 × 2.4
5	Pinscher	12	M	2.4	Prepuce	M	0.4 × 0.3
6	Pitbull	10	F	21.5	Abdomen	S	0.6 × 0.7
7	Pitbull	9	F	33.6	Abdomen	S	0.9 × 0.5
8	Mixed breed	9	M	9.0	Prepuce [a]	S	1.2 × 0.7

[a] This patient had a penectomy 2 years prior to this treatment due to another HSA. The new lesion treated in this work was localized in the area which was previously the prepuce. It is probable that this lesion was a relapse from the first tumor.

Fig. 1. Illustration of PDT procedure. A) intra- and peritumoral injection of aluminum-chloride-phthalocyanine nanoemultion (AlClPc-nano) followed by a 15-minute waiting period; B) positioning of the LED device 4 cm distant from the surface of the lesion; C) illumination of the entire tumor area with 658–662 wave length LED light for 25 min.

complete clinical disappearance of the tumor. One patient needed 3 sessions and another needed 4 sessions. In one animal, a single PDT session was sufficient for the tumor to be no longer macroscopically visible.

Swelling and hyperemia were noted at the treatment site 7 days after the first PDT session and tumors showed marked necrosis in all animals. Moreover, a wound crust was usually present at the irradiation site 7 days after the initial PDT session. This crust was mechanically removed prior to the second PDT session. At the end-point excisional biopsy, the macroscopic tumor lesion was completely absent in general with only a scar visible. Fig. 2 shows the tumor region of patient #2 before, during, and after PDT treatment.

The therapeutic response of cutaneous HSA to PDT with AlClPc-nano was complete in 90% of the treated lesions (9/10) and partial in 10% of the treated lesions (1/10). Histopathological analysis showed no tumor cells in all animals but one (patient #5). Patient #5 did not show a macroscopically visible lesion on the skin at the time of end-point excisional biopsy. Analysis of the biopsy revealed remaining HSA tumor cells. However, the incision revealed that this patient had a very deep tumor that probably could not be reached by the light irradiation during PDT. Common histopathological findings were: inflammatory infiltrate, necrosis and ulcer areas, and initial scarring lesions. In all, seven dogs had a complete response to PDT, with no gross or microscopic signs of the tumor after treatment. Dogs were followed for at least 18 months after the treatment, and no tumor recurrence was observed in any of them.

As the volume of AlClPc-nano was not predetermined (the AlClPc nanoemulsion was injected intra- and peritumorally in a volume sufficient to cause tumescence of the tumor area), the drug dose varied among dogs (Table 2). Irrespective of this, even the lowest dose (0.76 mL/cm^2 – patient #3) was sufficient to elicit a total response to PDT treatment of cutaneous HSA in 7 out of 8 dogs, with the number of sessions ranging from 1 to 4, at 7-day intervals.

4. Discussion

The purpose of the study was to investigate the efficacy of PDT using a third-generation photosensitizer - an AlClPc-nano - in dogs with naturally occurring neoplasia. Overall, our results presented an excellent complete remission for all of the dogs treated with one exception. This shows that, not only was AlClPc-nano effective as a photosensitizer, but also that the nanoemulsion carrier used was capable of protecting it from degradation and agglomeration. The average number of sessions used was two, with a minimum of one (one case) and a maximum of 4 (one case). Several factors could account for this variation observed in the number of applications, including tumor size and depth, and/or differences in photosensitizer uptake by the tumor tissue that can differ between lesions and/or patients [2,15]. In addition, the number of biological molecules present in the tumor tissue and animal

skin that are capable of absorbing and/or reflecting light is also a critical parameter with regards to the efficacy of PDT [15].

In the present study, one of the treated lesions, despite presenting clinical absence of lesion, presented tumor cells in deep skin layers on histopathological analysis. As PDT is dependent on light penetrating tissue, the general efficacy of PDT decreases when it is applied for deep tumor treatments, even for second generation photosensitizers [5,16,17]. Another common cause of PDT failure is hypoxia [18,19]. However, it is certainly not a probable cause for HSA.

Systemic side effects such as hyperemia, edema, cyanosis and pruritus have already been observed after PDT in pets, however they are rarely seen when using second generation photosensitizers [9], and it is in resonance with our results. Common local adverse effects following PDT are the occurrence of inflammation and pain, which in our work was successfully managed with medication. Another major problem in PDT relates to photosensitivity. Systemically administered photosensitizers, especially of the first generation, can induce skin photosensitivity and patients must be protected from light for several days [2,4,17]. In our work, this was avoided by using a second-generation photosensitizer, which is more readily cleared from normal cells than first generation photosensitizers. Furthermore, intra and peritumoral injections were given, instead of systemic administration. Besides avoiding nonspecific photosensitization, the rational for using this specific protocol (direct injection of photosensitizer in the tumor site and immediate light application - after 15 min) was also chosen to induce preferential PDT intravascular damage, since HSA has a rich network of blood vessels [20,21]. The biological target of PDT depends upon the interval between drug injection and light irradiation. PDT with a short drug-light interval targets tumor vasculature and acts as anti-vascular therapy [20,21]. Anti-vascular PDT can cause endothelial cell death and expose subendothelium leading to platelet aggregation, thrombus formation and vessel occlusion resulting in tissue hypoxia, nutrient deprivation and tumor destruction [20,21]. In the present study, after AlClPc-nano based PDT, tumor necrosis was only observed in the treated area, attesting the selectivity of PDT. As a highly vascularized type of tumor, HSA offer a large supply of oxygen for fueling the type II photoreactions during the application of PDT. These photoreactions yield singlet oxygen, which is a reactive oxygen species [5,16]. Thus, the efficacy of PDT against HSA reported in this work may be at least partially due to the high availability of oxygen in the tumor tissue. It is worth noting that healing of the treated area occurred rapidly, which is another valuable advantage of PDT.

As previously stated the most effective treatment for cutaneous HSA is surgery which, depending on the size and localization of the lesion, can lead to animal mutilation. In fact, one of the animals included in our study had previously been submitted to a penectomy due to a HSA. In some cases, this is a collateral damage that PDT can overcome. Here, another patient presented a lesion on the prepuce which PDT was sufficient to treat without the requirement of a radical recession
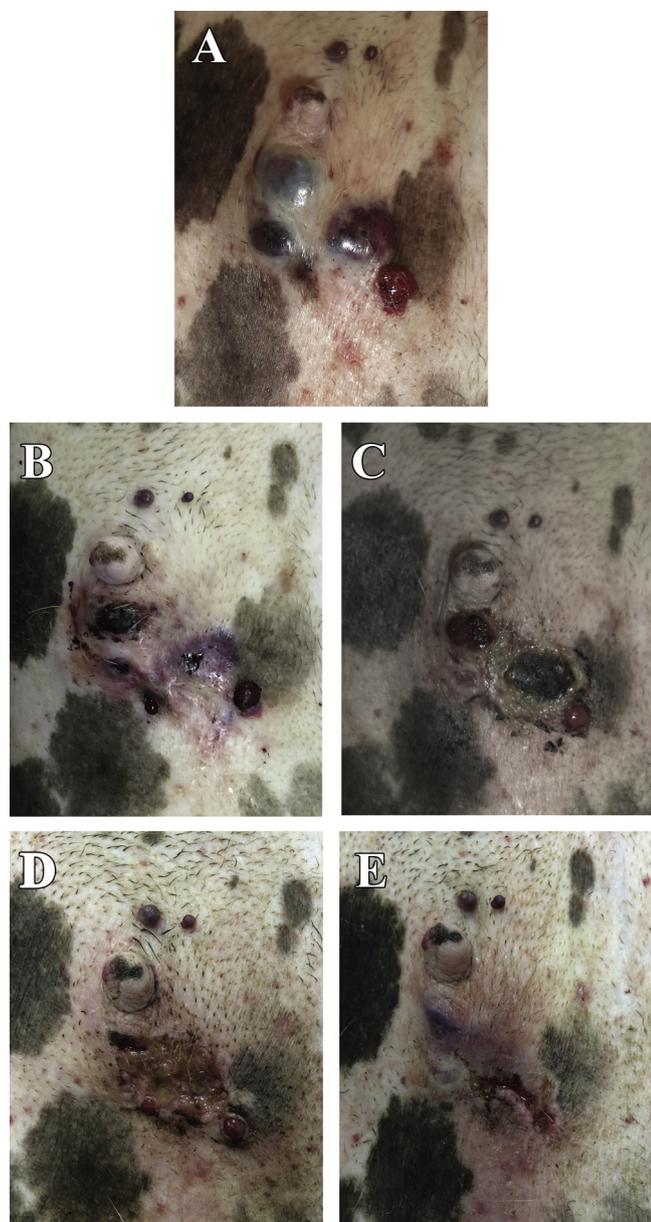
Fig. 2. Photographs of patient #2 tumor area. A) Before the first PDT session. Note the characteristic purple nodes of HSA lesions. B) Before the second PDT session. Swelling and hyperemia of the area and focal necrosis. C) Before the third PDT session. Wound crust on the tumor site. D) Before the fourth PDT session. The tumor lesion is barely visible, only necrosis. E) Before the excisional biopsy. Tumor lesion is absent; only a scar is visible.

demonstrating that PDT was not only effective, but also minimally invasive. On the other hand, PDT may be inefficient for very deep tumors, since light penetration into tissue is limited. Moreover, treatment of lesion on regions of dark skin may require an adjustment of the light dose, since the amount of energy effectively delivered to the tissue is reduced by the concentration of pigment [22].

In view of our results, it can be concluded that PDT as used in the present study, i.e. using a third-generation photosensitizer, is effective for the treatment of cutaneous HSA in dogs. Our study demonstrated that in addition to the excellent percentage of complete remission of HSA with no recurrence to date; this treatment also benefits from a good cosmetic outcome over a short recuperation period. These are important parameters not only for company animals, but also for translational studies in humans.

Table 2
Dose calculation of AlClPc-nano used in each patient in relation to the superficial area of the initial lesion.

Case Number	Initial Tumor Size (cm)	Superficial Tumor Area (cm^2)	Volume of AlClPc-nano administered (mL)	Dose [a] (mL/cm^2)
1	0.5 × 0.6	0.30	1	3.33
2	1.1 × 1.3	4.19	10	2.39
	2.0 × 1.1			
	0.7 × 0.8			
3	3.1 × 3.4	10.54	8	0.76
4	1.0 × 2.4	2.40	5	2.08
5	0.4 × 0.3	0.12	1.6	13.33
6	0.6 × 0.7	0.42	1.6	3.81
7	0.9 × 0.5	0.45	3	6.67
8	1.2 × 0.7	0.84	3.5	4.17

[a] Dose was calculated as volume of AlClPc-nano (mL) injected by total tumor area (cm^2).

Conflict of interest

The authors report no conflict of interest.

Acknowledgements

References

[1] D.E. Dolmans, D. Fukumura, R.K. Jain, Photodynamic therapy for cancer, Nat. Rev. Cancer 3 (2003) 380–387, https://doi.org/10.1038/nrc1071.

[2] D.V. Straten, V. Mashayekhi, H.S. Bruijn, S. Oliveira, D.J. Robinson, Oncologic photodynamic therapy: basic principles, current clinical status and future directions, Cancers 9 (19) (2017) E19, https://doi.org/10.3390/cancers9020019.

[3] I.O. Bacellar, T.M. Tsubone, C. Pavani, M.S. Baptista, Photodynamic efficiency: from molecular photochemistry to cell death, Int. J. Mol. Sci. 16 (2015) 20523–20559, https://doi.org/10.3390/ijms160920523.

[4] I. Yoon, J.Z. Li, Y.K. Shim, Advance in photosensitizers and light delivery for photodynamic therapy, Clin. Endosc. 46 (2013) 7–23, https://doi.org/10.5946/ce.2013.46.1.7.

[5] L.A. Muehlmann, G.A. Joanitti, J.R. Silva, J.P.F. Longo, R.B. Azevedo, Liposomal photosensitizers: potential platforms for anticancer photodynamic therapy, Braz. J. Med. Biol. Res. 44 (2011) 729–737, https://doi.org/10.1590/S0100-879X2011007500091.

[6] T.J. Dougherty, R.E. Thoma, D.G. Boyle, K.R. Weishaupt, Interstitial photoradiation therapy for primary solid tumors in pet cats and dogs, Cancer Res. 41 (1981) 401–404.

[7] A.J. Stell, J.M. Dobson, K. Lagamark, Photodynamic therapy of feline superficial squamous cell carcinoma using topical 5-aminolaevulinic acid, J. Small Anim. Pract. 42 (2001) 164–169, https://doi.org/10.1111/j.1748-5827.2001.tb01796.x.

[8] M.D. Lucroy, M.H. Bowles, R.G. Higbee, M.A. Blaik, J.W. Ritchey, T.D. Ridgway, Photodynamic therapy for prostatic carcinoma in dog, J. Vet. Intern. Med. 17 (2003) 235–237, https://doi.org/10.1111/j.1939-1676.2003.tb02440.x.

[9] J. Buchholz, H. Walt, Veterinary photodynamic therapy: a review, Photodiagn. Photodyn. Ther. 10 (2013) 342–347, https://doi.org/10.1016/j.pdpdt.2013.05.009.

[10] H. Ward, L.E. Fox, M.B. Calderwood-Mays, A.S. Hammer, C.G. Couto, Cutaneous hemangiosarcoma in 25 dogs; a retrospective study, J. Vet. Intern. Med. 4 (1994) 345–348, https://doi.org/10.1111/j.1939-1676.1994.tb03248.x.

[11] K.P. McAbee, L.L. Ludwig, P.J. Bergman, S.J. Newman, Feline cutaneous hemangiosarcoma: a retrospective study of 18 cases (1998-2003), J. Am. Anim. Hosp. Assoc. 41 (2005) 110–116, https://doi.org/10.5326/0410110.

[12] N. Jayasree, A. Nasreen, S.H. Naik, R.V.R. Murthy, Ch. Srilatha, K. Sujatha,

Histopathological features of cutaneous tumours arising from the vascular endothelial cells in dogs, J. Exp. Biol. Agric. Sci. 4 (2016) 78–82, https://doi.org/10.18006/2016.4(Spl-3-ADPCIAD).S78.S82.

[13] G. Hosgood, Canine hemangiosarcoma, Compend. Cont. Educ. Pract. Vet. 13 (1991) 1065–1075.

[14] C.A. Clifford, A.J. Mackin, C.J. Henry, Treatment of canine hemangiosarcoma: 2000 and beyond, J. Vet. Intern. Med. 14 (2000) 479–485, https://doi.org/10.1111/j.1939-1676.2000.tb02262.x.

[15] B.C. Wilson, M.S. Patterson, L. Lilge, Implicit and explicit dosimetry in photodynamic therapy: a new paradigm, Lasers Med. Sci. 12 (1997) 182–199, https://doi.org/10.1007/BF02765099.

[16] L.A. Muehlmann, M.C. Rodrigues, J.P.F. Longo, M.P. Garcia, K.R. Py-Daniel, A.B. Veloso, P.E. de Souza, S.W. da Silva, R.B. Azevedo, Aluminium-phthalocyanine chloride nanoemulsions for anticancer photodynamic therapy: development and in vitro activity against monolayers and spheroids of human mammary adenocarcinoma MCF-7 cells, J. Nanobiotechnol. 13 (2015) 36, https://doi.org/10.1186/s12951-015-0095-3.

[17] J. Zhang, C. Jiang, J.P.F. Longo, R.B. Azevedo, H. Zhang, L.A. Muehlmann, An updated overview on the development of new photosensitizers for anticancer photodynamic therapy, Acta Pharm. Sin. B 8 (2018) 137–146, https://doi.org/10.1016/j.apsb.2017.09.003.

[18] T. Osaki, S. Takagi, Y. Hoshino, M. Okumura, T. Fujinaga, Antitumor effects and blood flow dynamics after photodynamic therapy using benzoporphyrin derivative monoacid ring A in KLN205 and LM8 mouse tumor models, Cancer Lett. 248 (2007) 47–57, https://doi.org/10.1016/j.canlet.2006.05.021.

[19] H.S. Jung, J. Han, H. Shi, S. Koo, H. Singh, H.J. Kim, J.L. Sessler, J.Y. Lee, J.H. Kim, J.S. Kim, Overcoming the limits of hypoxia in photodynamic therapy: a carbonic anhydrase IX-targeted approach, J. Am. Chem. Soc. 139 (2017) 7595–7602, https://doi.org/10.1021/jacs.7b02396.

[20] K. Kurohane, A. Tominaga, K. Sato, J.R. North, Y. Namba, N. Oku, Photodynamic therapy targeted to tumor-induced angiogenic vessels, Cancer Lett. 167 (2001) 49–56, https://doi.org/10.1016/S0304-3835(01)00475-X.

[21] T. Osaki, S. Takagi, Y. Hoshino, M. Okumura, T. Kadosawa, T. Fujinaga, Efficacy of antivascular photodynamic therapy using benzoporphyrin derivative monoacid ring A (BPD-MA) in 14 dogs with oral and nasal tumors, J. Vet. Med. Sci. 71 (2009) 125–132, https://doi.org/10.1292/jvms.71.125.

[22] F.H. Mustafa, M.S. Jaafar, Comparison of wavelength-dependent penetration depths of lasersin different types of skin in photodynamic therapy, Indian J. Phys. 87 (2013) 203–209, https://doi.org/10.1007/s12648-012-0213-0.