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ORIGINAL ARTICLE

# Efficacy of 5-aminolevulinic acid-based photodynamic therapy in different subtypes of canine mammary gland cancer cells

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## Abstract

Canine mammary gland tumors (CMGTs) are heterogeneous disease and subclassified [sarcomas (S), carcinomas (C), and carcinosarcomas (CS)] according to histopathological differentiation. Photodynamic therapy (PDT) is a promising treatment strategy based on the use of a photosensitizer (PS) activated by light. However, the therapeutic potential of PDT in the treatment of CMGTs has not been investigated, yet. Therefore, the aim of this study was to determine the in vitro protocol of 5-ALA-based-PDT for the treatment of three subtypes of CMGTs, for the first time. The intracellular PpIX florescence intensity was measured for 5-ALA (0.5 and 1 mM). After irradiation with different light doses (6, 9, 12, 18, and 24 J/cm$^2$) for two different modes [continuous wave (CW) and pulse radiation (PR)], the cytotoxic effects of 5-ALA (0.5 and 1 mM) on the subtypes (C, S, and CS) of CMGTs were analyzed by WST-1. Finally, the optimal PDT treatment protocol was validated through Annexin V and AO/EtBr staining. Our results showed that 1 mM 5-ALA for 4-h incubation was the best treatment condition in all subtypes of CMGTs due to higher intracellular PpIX level. After irradiation with different light doses, PR mode was more effective in S primary cells at 9 J/cm$^2$. However, a significant decrease in the viability of C and CS cells was detected at 12 /cm$^2$ in CW mode ($p < 0.05$). Additionally, 1 mM 5-ALA induced apoptotic cell death in each subtype of CMGTs. Our preliminary findings suggest that (i) each subtype of CMGTs differentially responds to PDT and (ii) the light dose and mode could play an important role in the effective PDT treatment. However, further studies are needed to investigate the role of the different light sources and PDT-based apoptotic cell death in CMGTs cells.

**Keywords** Photodynamic therapy · 5-Aminolevulinic acid · Canine mammary gland tumor · Apoptosis

## Introduction

Mammary gland tumors (MGT) consist of 50% of tumor cases detected in female dogs [1]. The main treatment options used in canine mammary gland tumors (CMGTs) can be listed as surgery, chemotherapy, radiotherapy, and immunotherapy [2]. Although the most commonly used treatment method in the treatment of CMGTs today is the surgical approach [3], this method is considered an extremely invasive method and causes some complications (posterior limb edema; hematoma; dehiscence and contamination of the surgical site; subcutaneous emphysema;

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infection; bleeding; seroma; hyperesthesia; allodynia; pain; changes in the blood profile of the patient due to long-term anesthesia, post-operative care, invasive intervention and stress) in the patient [4, 5]. Therefore, new treatment modalities are required due to the increased the number of CMGTs cases in humans and dogs and inadequate results from adjuvant therapies.

MGT are heterogeneous disease and subclassified according to histopathological borders and differentiation. Therefore, MGT differentially respond to treatment options [6]. MGT are mostly originated from epithelial cells and called as carcinomas (C) [2]. On the other hand, sarcomas (S) originating in the mammary gland's mesenchymal tissue are a rare type of tumor in dogs, and little is known about their biology [7, 8]. Another histopathological type of MGT is malignant mixed tumors, also called carcinosarcomas (CS). They contain both malignant epithelial cells and malignant connective tissue elements [9].

Photodynamic therapy (PDT) is based on the use of a light-sensitive agent (photosensitizer, PS) to produce singlet oxygen in cells and selectively stimulated into the tumor tissue through a light source at the appropriate wavelength [10–12]. The types of the molecular mechanism of PDT are based on cellular events (signaling pathways leading to both apoptosis and necrosis), vascular events (microvascular destruction), and stimulation of the systemic immunological response [12, 13]. The general feature of apoptosis caused by PDT is the rapid release of mitochondrial cytochrome c into the cytosol leading to apoptosome formation and the activation of procaspase 3. Additionally, the photosensitization process can cause a rapid loss of plasma membrane integrity and necrotic cell death due to the rapid depletion of intracellular ATP. However, apoptotic signaling pathways can be photochemically inactivated due to an inappropriate high light intensity [14]. Furthermore, the most important factors in PDT-mediated cell death are the localization of the PS within the cell and the dose of the light for irradiation [15]. As a result of the administration of high doses of PDT (high PS concentration or high light intensity or both), necrosis leading to undesirable cytotoxicity is observed in the cells [16]. Therefore, the optimal treatment condition should be determined to better response to PDT for especially heterogeneous tumor types.

5-Aminolevulinic acid (5-ALA) is a light-sensitive PS that is commonly used in PDT. It is a natural biochemical precursor of hemoglobin that increases the synthesis and accumulation of fluorescent protoporphyrin IX (PpIX) in different tumor tissues [17, 18]. In veterinary medicine, a limited number of studies have been evaluated the therapeutic potential of 5-ALA-mediated PDT in the treatment of different types of canine tumors, in vitro. In the study of Osaki et al. [17], a total of 15 canine tumor primary cell lines (basal cell carcinoma, prostate cancer, squamous cell carcinoma, lung carcinoma, MGT) originated from S and C are treated with different concentrations (0.2, 0.4, 0.6, 0.8, and 1 mM) of 5-ALA and then irradiated with LED at 630 nm light (20 mW/cm2, 10 J/cm2). They state that the response of these primary cell lines to PDT is different in terms of tumor origin and type in dogs and cats [17]. In another study, Ridgway et al. [18] have determined that the effects of different incubation time (4, 8, 12, 16, 20, and 24 h) and concentrations of 5-ALA (1, 2, 3, 4, 5, and 6 mM) on K9TCC canine transitional cell carcinoma cells after irradiation with a different power density (5, 10, 15, and 20 mW/cm$^2$) of the diode laser. Therefore, further studies are required to determine the best in vitro protocol for canine tumors in veterinary medicine.

In the current study, we aimed to determine the in vitro protocol for 5-ALA-based PDT in the treatment of CMGTs, for the first time. Firstly, primary cell culture was performed from three different subtypes (C, S, and CS) of CMGTs. The intracellular PpIX fluorescence intensity was measured for the different concentration of 5-ALA (0.5 and 1 mM) in these cells before irradiation. Then, the cytotoxic effects of different concentration of 5-ALA (0.5 and 1 mM) on these cells after irradiation with different light doses (6, 9, 12, 18, and 24 J/cm2) in two different [continuous wave (CW) mode and pulse radiation (PR)] modes were analyzed by WST-1 analysis. Finally, the best optimal PDT treatment protocol for each subtype (C, S, CS) was validated through Annexin V and acridine orange/ethidium bromide staining.

## Material and methods

### Ethical approval

The study was approved by the Local Animal Ethical Committee (08.03.2019, no. 2019/15). Dog owners were informed about the study and signed an agreement to allow participation of their dogs in the study.

### Case description and tissue sampling

Tissue samples were taken from the surgically removed masses from bitches admitted to the clinic (Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Istanbul University-Cerrahpaşa) with palpable masses in mammary glands. The tissues submitted for histopathological examination were fixed in 10% buffered formalin. Then, a part of the tissue was transferred to RPMI medium for primary cell culture. After histologic processing and staining with Hematoxylin&Eosin, sections were examined under the light microscope and evaluated based on the criteria defined by Goldschmidt et al. [19]. Then, tissues obtained from three dogs were diagnosed with carcinoma (C), sarcoma (S), and

carcinosarcoma (CS) according to histopathological evaluation and included in the study.

Accordingly, case 1 was an 8-year-old intact Terrier bitch, weighing 8 kg with a 5-year history of mammary mass and diagnosed as grade I tubular carcinoma (C). Case 2 was a 12-year-old intact golden retriever bitch, weighing 28 kg with an 8-month history of mammary mass and diagnosed as liposarcoma (S). Case 3 was an 8-year-old intact mix-breed bitch, weighing 35 kg with a 3-month history of mammary mass, and diagnosed as carcinosarcoma (CS). After careful clinicopathological examinations, the cases were classified and staged according to the TNM classification system and staging proposed by Owen [20]. The cases of this study were classified and staged as T2N1M0/stage IV; T3N1M0/stage IV; and T2N0M0/stage II for C, S and CS tumors, respectively.

## Primary cell isolation and cell culture

The CMGT tissues were minced into 3 to 4 mm of fragments by a scalpel. The fragments were transferred into a 15-mL centrifuge tube, washed with PBS, and then incubated with collagenase type IV (1 mg/mL) (Sigma, USA) at 37 °C for 1 h. Following, the tissue sample was further digested with trypsin-EDTA (0.25%) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) for 15 min. Then, the cells were centrifuged at 1500×*g* for 10 min. After centrifugation, the pellets were washed with phosphate buffer saline (PBS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Finally, the primary mixed cell cultures were seeded in T-25 culture flasks with medium. The primary CMGT cells were grown in Dulbecco's Modified Eagle Media (DMEM, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 1% penicillin/streptomycin (Gibco,Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C in a humidified atmosphere with 5% $CO_2$. After seven passages, the initial cell culture experiments were performed. The cells were not irradiated with laser and not incubated with 5-ALA served as control.

## 5-ALA incubation and cell photosensitization

Stock solution of 5-ALA (St Louis, MO, USA, A3785) was prepared in sterile distilled water. The cells were seeded in the 96-well plate at $2\times10^4$ density and treated with two concentration of 5-ALA (0.5 and 1 mM) in DMEM medium without FBS for 4 h at 37 °C. Then, the medium was immediately changed and replaced with fresh medium including FBS. Afterwards, the cells were irradiated with different light doses for two different modes (CW and PR).

## Photodynamic treatment in vitro

In experiments, the diode laser was used and the laser light source's wavelength was 635 nm with ±3 FWHM (full width half maximum). In the experiment, continuous wave (CW) mode and pulse radiation mode (PR) were used for laser irradiation at 6, 9, 12, 18, and 24 J/cm$^2$. The optical power and wavelength spectrum validation were performed through a power meter and spectrometer (PM100 and C series spectrometer, Thorlabs, Germany) at 30 mW/cm$^2$ fluence rate. Control groups are incubated in the same medium without irradiation (Table 1).

## Measurement of protoporphyrin IX (PpIX) accumulation

To measure intracellular protoporphyrin IX (PpIX) level, the cells were seeded in the 96-well plate at $2\times10^4$ density and cultured for 24 h. Then, the cells were subsequently incubated with 0.5 and 1 mM 5-ALA for 4 h. After incubation with 5-ALA, the cells were replaced with fresh medium with FBS, and the intracellular PpIX level was immediately analyzed. Images were obtained with the EVOS FL Cell Imaging System (Thermo Fisher Scientific, USA). Additionally, the corrected total cell fluorescence (CTCF) was analyzed by the Image J program.

## Cell viability assay

To determine the cytotoxicity of 5-ALA-based PDT, the cells were seeded in the 96-well plates at $2\times10^4$ density. After incubation for 24 h, the cells were treated with different concentrations (0.5 mM and 1 mM) of 5-ALA for 4 h. Following incubation, the media was changed, and the cells were irradiated with different light doses (6, 9, 12, 18, and 24 J/cm$^2$) in both CW and PR modes. After 24-h incubation, 10 µL of WST-1 reagent was added to each well and incubated for 45 min at

**Table 1** The radiation parameters of the 5-ALA-based PDT experiments

| Radiation parameters | | |
|---|---|---|
| Wavelength (nm) | 635 ± 3 nm | 635 ± 3 nm |
| Mode | CW | PR |
| Power output | 30 mW | 30 mW |
| Exposure time (s) | 200 s, 300 s, 400 s, 600 s, 800 s | 400 s, 600 s, 800 s, 1200 s, 1600 s |
| Energy density (J/cm$^2$) | 6, 9, 12, 18, 24 (J/cm$^2$) | 6, 9, 12, 18, 24 (J/cm$^2$) |

37 °C in the dark. The cell viability analysis was performed through the microplate reader (Allsheng, China) at the absorbance values at 450 nm. According to WST-1 results, the cells treated with the most effective 5-ALA concentration for further experiments.

### Annexin V assay

For apoptotic analysis, the cells were seeded in 6-well plate ($1 \times 10^5$) and incubated for 24 h. After incubation, the cells were treated with 1 mM 5-ALA for 4 h. After incubation, the media was changed, and the cells were irradiated with the different light doses in CW and PR modes for each group. Following 24-h incubation, the cells were collected with trypsin-EDTA (0.25%) and washed in PBS. The cells were stained with Muse™ Annexin V and Dead Cell reagent (Millipore, Germany) and incubated for 30 min in the dark at room temperature. The analysis was performed using Muse Cell Analyzer (Millipore, Germany) (Annexin at excitation/emission = 485/535 nm; 7-AAD at excitation/emission = 555/655 nm).

### Acridine orange/ethidium bromide staining

To observe the morphological changes in the cells after irradiation, the cells were seeded in 6-well plate ($5 \times 10^5$) and treated with 1 mM 5-ALA for 4 h. After incubation, the cells were irradiated with the different light doses in CW and PR modes for each group. Following 24-h incubation, the cells were fixed with 4% paraformaldehyde and washed with PBS. After washing, the cells were stained with AO/EtBr (1 µg/mL) for 30 min in the dark. Finally, the images were analyzed by EVOS FL Cell Imaging System (Thermo Fisher Scientific, USA) (excitation 502 nm (dsDNA), 460 nm (ssDNA and RNA) and emission 525 nm (dsDNA), 650 nm (ssDNA and RNA).

### Statistical analysis

Statistical analysis was performed by SPSS 21.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA with the post-hoc Tukey test was used for multiple comparisons. Additionally, a two-way ANOVA analysis was performed to compare groups. All experiments were repeated at least 3 times and the obtained data presented as the mean ± standard deviation. $p < 0.05$ was considered significant.

## Results

### Accumulation of PpIX-fluorescence in 5-ALA-treated CMGTs primary cells

To evaluate the PpIX accumulation levels in 5-ALA-treated subtypes of CMGTs, the CTCF intensity was measured by ImageJ software (Fig. 1). For this purpose, CMGTs were treated with two different concentrations of 5-ALA (0.5 and 1 mM) for 4 h. As shown in Fig. 1, the intracellular level of PpIX increased in a dose-dependent manner in all CMGTs compared with control cells (Fig. 1a). Additionally, the mean PpIX fluorescence intensity was significantly higher in C and CS cells than S cells after 4-h incubation with 1 mM 5-ALA (Fig. 1b). Therefore, 1 mM 5-ALA for 4 h was the optimal treatment before irradiation for each CMGTs primary cells.

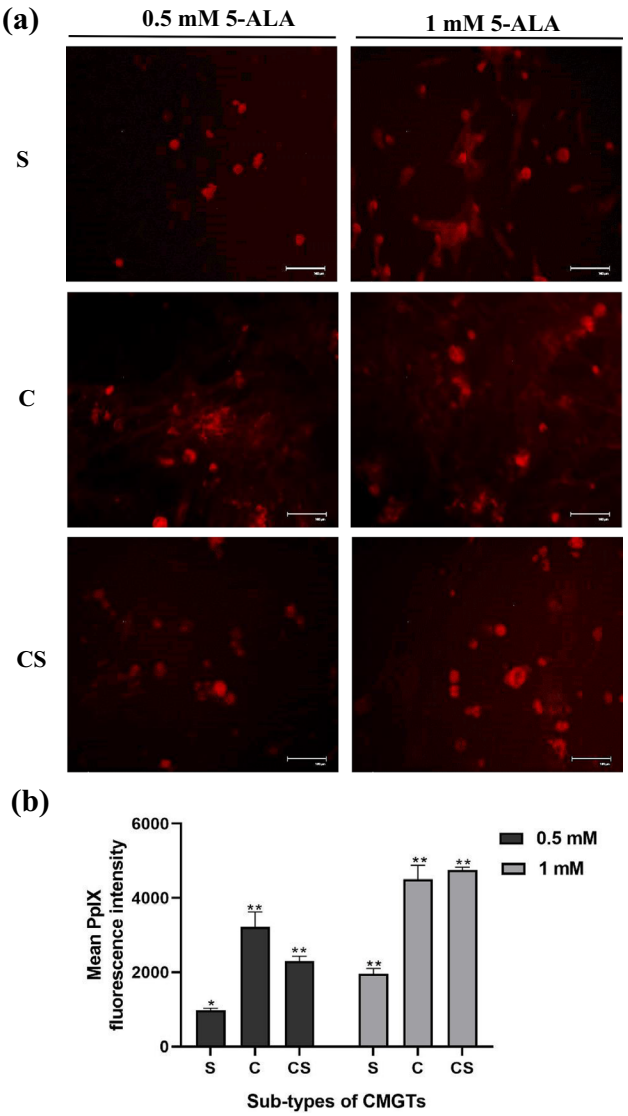


**Fig. 1** Intracellular PpIX accumulation in the subtypes of CMGTs after treatment with different concentrations (0.5 and 1 mM) of 5-ALA. **a** Cell images of sarcoma (S), carcinoma (C), and carcinosarcoma (CS) cells were obtained at 628 ± 40 nm red filter and (**b**) statistical comparison of the mean PpIX fluorescence in the subtypes of CMGTs compared with control cells ($p < 0.05$*, $p < 0.01$**)

## Evaluation of the viability of 5-ALA-treated CMGTs primary cells after irradiation

To evaluate the inhibitory effects of 5-ALA on the viability of three subtypes of CMGTs after irradiation, we performed WST-1 analysis (Fig. 2). For this purpose, three subtypes of CMGTs were treated with two different concentrations (0.5 and 1 mM) of 5-ALA and then irradiated with 6, 9, 12, 18, and 24 J/cm$^2$ laser energy in two different laser mode (CW and PR) (Fig. 2). Briefly, 1 mM 5-ALA treatment after irradiation with 6, 9, 12, 18, and 24 J/cm$^2$ light doses in two different modes was more considerably decreased the viability of each subtypes of CMGTs than 0.5 mM dose of 5-ALA treatment compared with control cells ($p < 0.05$). The obtained results were consistent with the level of intracellular PpIX level. However, the efficacy of 1 mM 5-ALA in each subtype was different in light doses and mode.

The irradiation of S cells with 6, 9, 12, 18, and 24 J/cm$^2$ energy in CW mode, the viability was $62.7 \pm 2.5\%$, $61.0 \pm 1.2\%$, $61.0 \pm 2.4\%$, $62.9 \pm 2.6\%$, and $65.8 \pm 2.5\%$, respectively ($p < 0.05$). However, a significant decrease ($42.3 \pm 3.7\%$) was observed following irradiation with 9 J/cm$^2$ energy in PR mode ($p < 0.01$). The viability of S cells was higher in all light doses in PR mode than CW mode. Therefore, the best optimal PDT treatment condition for S cells was the irradiation with 9 J/cm$^2$ energy for both CW and PR mode. The irradiation of C cells with 6, 9, 12, 18, and 24 J/cm$^2$ light in CW mode, the viability was $74.0 \pm 2.1\%$, $75.2 \pm 1.8\%$, $59.9 \pm 2.3\%$, $79.2 \pm 1.9\%$, and

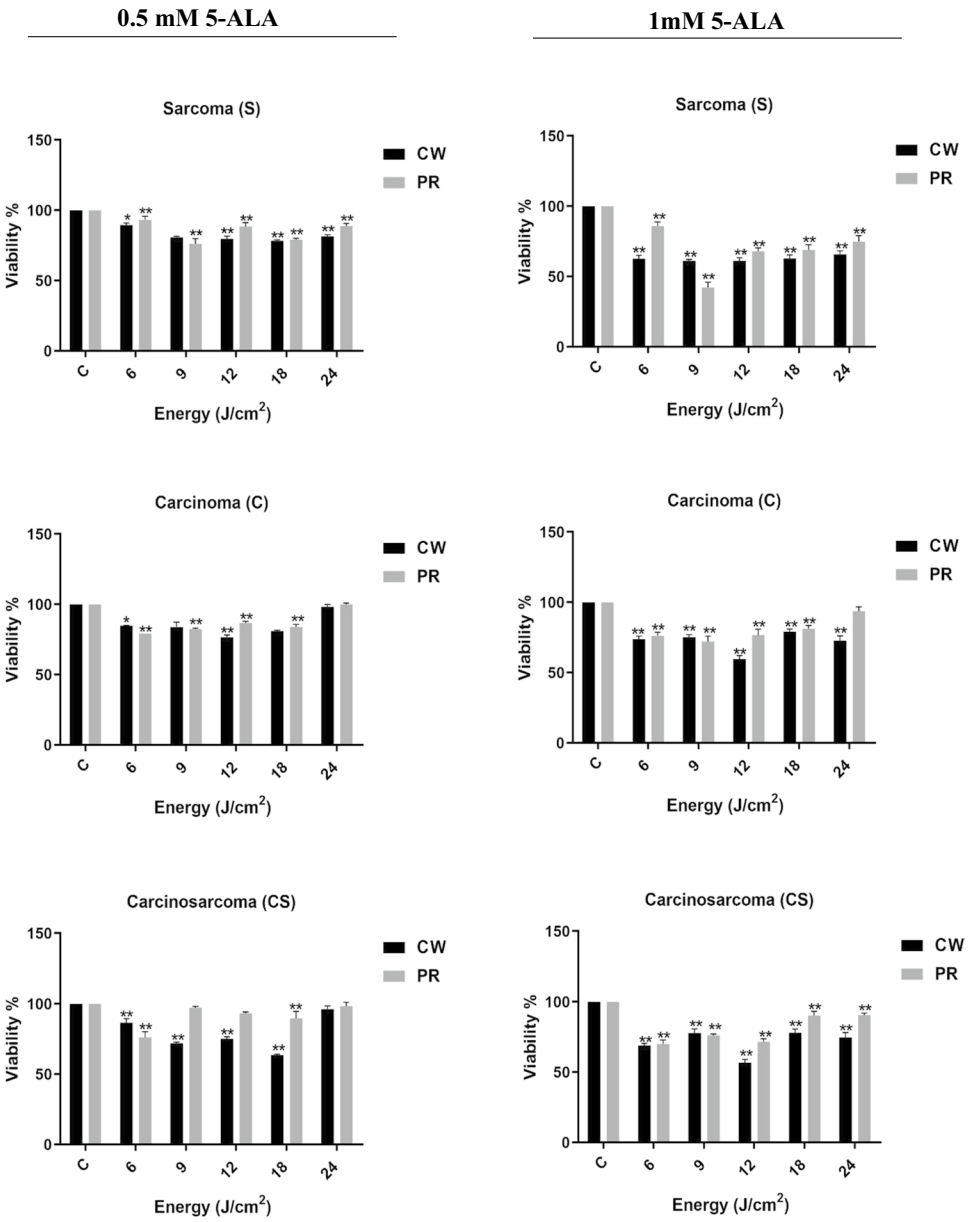


**Fig. 2** The cytotoxic effects of different concentration of 5-ALA on the subtypes of CMGTs. After irradiation with different light doses in both CW and PR modes, the viability of sarcoma (S), carcinoma (C), and carcinosarcoma (CS) cells was analyzed by WST-1 analysis for 24 h (CW: continuous mode, PR: pulse mode, J/cm$^2$: Joule/cm$^2$, $p < 0.05$*, $p < 0.01$**)

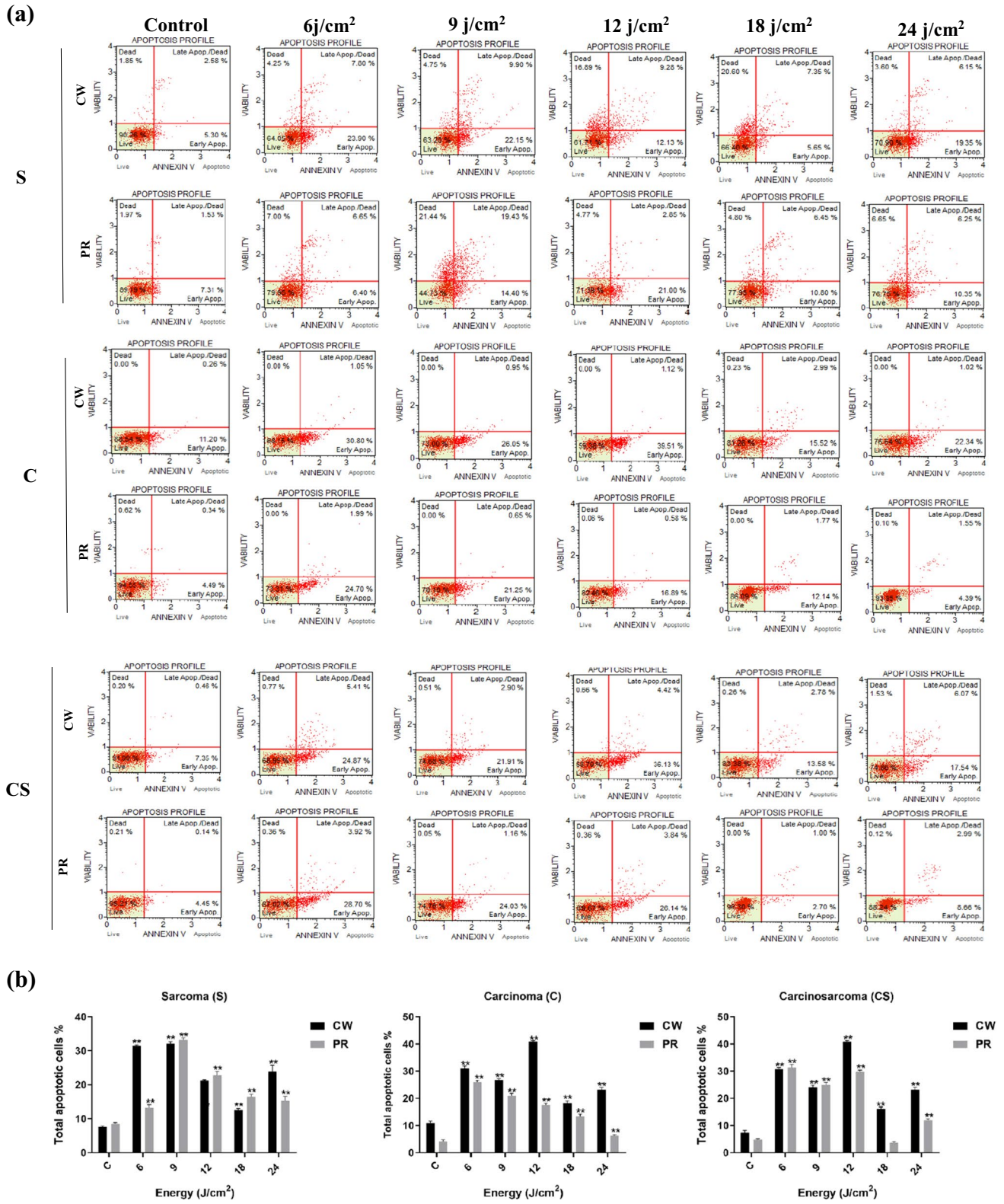


**Fig. 3** The apoptotic effects of 5-ALA (1 nM) on the subtypes of CMGTs. After irradiation with different light doses in two modes by using laser. **a** The apoptotic cell death of sarcoma (S), carcinoma (C), and carcinosarcoma (CS) cells was analyzed by Annexin V analysis, and (**b**) statistical comparison of the percentage of total apoptotic cell death in the subtypes of CMGTs compared with a control cells (CW: continuous mode, PR: pulse mode, J/cm$^2$: Joule/cm$^2$, $p < 0.05$*, $p < 0.01$**)

72.9 ± 3.4%, respectively ($p < 0.05$). The irradiation of CS cells with 6, 9, 12, 18, and 24 J/cm$^2$ energy in CW mode, the viability of cells reduced to 69.0 ± 1.6%, 78.0 ± 2.8%, 56.8 ± 2.2%, 78.1 ± 2.7%, and 74.7 ± 3.5%, at 1 mM dose of 5-ALA, respectively. Additionally, after irradiation with different light doses, the viability of C and CS cells was more decreased in CW mode than PR mode. Therefore, CW mode was more effective for C and CS cells than PR mode unlike S cells.

## Evaluation of 5-ALA-treated CMGTs primary apoptotic cells after irradiation

To further evaluate the apoptotic effects of 5-ALA on the subtypes of CMGTs after irradiation, we perform Annexin V analysis (Fig. 3). The apoptotic cell death was significantly increased in subtypes of CMGTs following irradiation with 6, 9, 12, 18, and 24 J/cm$^2$ in two different laser mode compared with control cells ($p < 0.05$).

The percentage of total apoptotic S cells considerably increased from 7.5 ± 1.7% to 31.5 ± 0.3%, 32.1 ± 0.6%, 21.3 ± 0.8%, 12.5 ± 0.6%, and 23.9 ± 1.8% at 6, 9, 12, 18, and 24 J/cm$^2$, respectively, in CW mode ($p < 0.01$). Additionally, a significant increase (33.2 ± 0.5%) in the total percentage of apoptotic cells was detected at 9 J/cm$^2$ in PR mode ($p < 0.01$, Fig. 3b). For C cells, the percentage of total apoptotic cells was detected as 31.1 ± 0.7%, 26.8 ± 0.5%, 40.9 ± 0.3%, 18.2 ± 0.9%, and 23.2 ± 0.9%, at 6, 9, 12, 18, and 24 J/cm$^2$, respectively, in CW mode, whereas a remarkably increase (30.8 ± 0.5%, 24.1 ± 0.9%, 40.9 ± 0.4%, 16.2 ± 0.7%, and 23.3 ± 1.3%, respectively) was observed in total apoptotic CS cells after irradiation ($p < 0.01$; Fig. 3b). Therefore, 5-ALA induces apoptotic cell death in each subtype of CMGTs. Additionally, the total apoptotic percentage of C and CS cells was more profound in CW mode than PR mode following irradiation with different light doses. The most effective PDT protocol for C and CS primary cells was the irradiation with 12 J/cm$^2$ in CW mode. However, CW and PR modes at 9 J/cm$^2$ were the most effective PDT protocol for S primary cells unlike C and CS primary cells. These findings were consistent with WST-1 results.

## Morphological changes in 5-ALA-treated CMGTs primary cells after irradiation

To observe the morphological effects of 5-ALA on subtypes of CMGTs after irradiation, AO/EtBr staining was performed (Fig. 4). We observed chromatin condensation, DNA fragmentation, membrane blebbing, and some vacuolization in subtypes of CMGTs following irradiation with different light doses compared with control cells. Additionally, some necrotic cells were also observed after irradiations in CW and PR modes. In S primary cells, the most obvious changes (cell shrinkage, chromatin condensation, and membrane blebbing) in the cell morphology were observed at 9 J/cm$^2$ in both CW and PR modes. Additionally, the irradiation

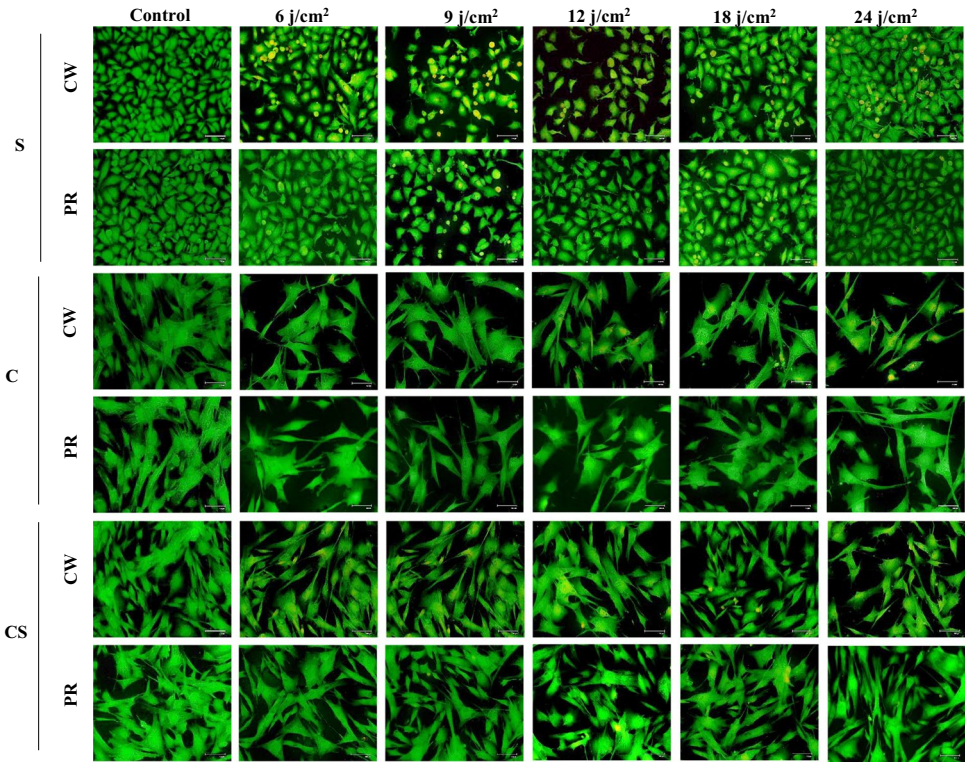


**Fig. 4** The effects of 5-ALA (1 nM) on the cell morphology in the subtypes of CMGTs. After irradiation with different light doses in CW and PR modes, the apoptotic death of sarcoma (S), carcinoma (C), and carcinosarcoma (CS) cells was analyzed by AO/EtBr staining. Cell images were obtained at 510 ± 42 nm green and 628 ± 40 nm red filters (CW: continuous mode, PR: pulse mode, J/cm$^2$: Joule/cm$^2$)

with 12 J/cm$^2$ in CW mode resulted in greater DNA damage in C and CS primary cells. Consequently, S primary cells were more sensitive to 5-ALA-mediated PDT than C and CS primary cells due to subtypes' different characteristic features.

## Discussion

In the present study, we for the first time proposed the optimum PDT protocol for three different subtypes of CMGTs. According to our findings, 1 mM 5-ALA for 4-h incubation was the best treatment condition for PDT in all subtypes of CMGTs. However, each subtype could differentially respond to PDT due to their molecular properties. PR mode was more effective in S primary cells at 9 J/cm$^2$ than CW mode. On the other hand, the viability of C and CS primary cells was more decreased in CW mode than PR mode at all light doses. A significant decrease in the viability of C and CS cells was detected at 12 J/cm$^2$ in CW mode. However, S cells were significantly more responded to 5-ALA-mediated PDT than C and CS cells.

The incidence of MGTs in canine is three times higher than in humans, and treatment options continue to be a major problem due to aggressive behavior of CMGTs [21]. Therefore, new treatment options including PDT, photodynamic detection (PDD), and photodynamic hyperthermia (PHT) have drawn attention for the treatment of canine tumors in veterinary medicine. In preclinical studies, different PS (5-ALA, verteporfin, G-Ce6, hematoporphyrin monomethyl ether (HMME), ICG, and HPPH) have been used for the treatment of various canine tumors including canine mammary carcinoma, canine oral squamous cell carcinoma, fibrosarcoma, liposarcoma, malignant shwannoma, and canine osseous tumors [22–28]. In clinical studies, PDT alone as well as the combination of PDT with PDD or PHT using different PS (HPPH, 5-ALA, ICG) have promising results in the treatment of canine tumors [17, 22–25]. However, there is limited evidence about the optimum PDT protocol and the underlying molecular mechanism of PDT in CMGTs, in vitro. The studies of Liu et al. [26] and Li et al. [27] state that HMME-based PDT induces apoptotic cell death through the mitochondrial apoptosis pathway including increased the level of caspase-9, caspase-3, and cytochrome c and mRNA and protein levels of Bax in canine mammary tumor cells after irradiation with He–Ne laser equipped with a 632.8 nm light source at a fluence of 2.8 J/cm$^2$. However, the knowledge of pathology and subtype of these cells is not presented in these studies. Another study shows that G-Ce6-mediated PDT inhibits the viability of in SNP canine mammary carcinoma cells irradiated with four light doses (0, 1, 5, or 15 J/cm$^2$) at 650 nm by a semiconductor laser, in vitro and in vivo [28]. Furthermore, only one study has investigated the clinical efficacy of 5-ALA-based PDT (10 dogs and 4 cats) and PDD (109 dogs and 15 cats) with different tumors using a laser diode and LED [17]. Additionally, a total of 15 canine tumor primary cell lines are produced, and these cells are incubated with different concentrations (0, 0.03, 0.1, 0.3, and 1 mM) of 5-ALA after irradiation at 630 nm light (20 mW/cm$^2$, 10 J/cm$^2$) using LED lights. Cell Counting Kit-8 in this study evaluates the viability of these primary cell lines. Among them, melanoma and nasal cavity adenocarcinoma are more sensitive to 5-ALA-PDT than other types. In contrast, some canine tumor cell lines including lung adenocarcinoma, liposarcoma, and mammary gland tumor (malignant pleural effusion) are resistant to PDT in this study [17]. However, the efficacy of laser diode and different light doses has not been investigated, in vitro [17]. Therefore, further studies have required the determination of PDT protocol for different types of canine tumors. Herein, we determined the efficacy of 5-ALA-mediated PDT in different subtypes of CMGTs using a laser diode. To assess the light doses effect, the cells were incubated with 0.5 and 1 mM 5-ALA and irradiated with 6, 9, 12, 18, and 24 J/cm$^2$ in CW and PR modes. Our findings demonstrated that 1 mM 5-ALA was more efficient in S, C, and CS cells than 0.5 mM 5-ALA due to higher intracellular PpIX level. In the study of Osaki et al.'s [28], the cytotoxicity of 1 mM 5-ALA is significantly correlated with the intracellular PpIX concentration in carcinomas unlike sarcomas [17]. However, our results showed that the cytotoxicity of 5-ALA was associated with a higher PpIX level for all CMGTs cells. These differences could result from the light source, light doses, and canine tumor cell types and molecular features. 5-ALA is a hydrophilic PS [29], and its uptake into tumor cell is affected by the aquosity of the tumor cells [30]. Intracellular pH is another factor that affects PS uptake into the cell [31]. Therefore, we investigated the different paramaters including light doses and modes and different concentrations of 5-ALA to determine the best PDT protocol for the treatment of canine mammary tumors.

Furthermore, we found that the response of subtype of CMGTs was different to 5-ALA-based PDT. In S cells, both CW and PR modes effectively inhibited the cell viability at especially 9 J/cm$^2$. However, the viability of C and CS cells was significantly reduced at particularly 12 J/cm$^2$ in CW mode. Therefore, the identification of specific PDT protocol has great importance for effective treatment response in CMGTs. Additionally, we assessed the apoptotic effects of 5-ALA through Annexin V analysis and AO/EtBr staining on each subtype of CMGTs cells irradiated with five light doses in CW and PR modes. Our preliminary findings indicated that the apoptotic effect of 5-ALA was correlated with the cytotoxicity of 5-ALA. However,

further investigations should be performed to elucidate the underlying molecular mechanisms of PDT-based apoptotic cell death in CMGTs cells.

## Conclusion

Herein, we determined 5-ALA-based PDT protocol in three subtypes of CMGTs cells after irradiation with five light doses in CW and PR modes using a laser diode. Our preliminary findings showed that each subtype of CMGTs differentially responded to PDT. S primary cells were more sensitive to 5-ALA-mediated PDT than C and CS cells. Additionally, the determination of light dose and mode could play a crucial role in the effective PDT treatment. However, further studies are needed to investigate the role of different light sources for the treatment of CMGTs. Additionally, the number of primary CMGTs cells for each subtype should be increased for further studies.

**Authors' contributions** All authors contributed to the study conception and design. Experimental analyses were performed by Özge Turna, Gamze Guney Eskiler, Asuman Deveci Ozkan, Elif Sözen Kucukkara, Ozge Ozten, Ali Furkan Kamanlı, and Aslihan Baykal. The data analysis was performed by Salih Zeki Yildiz, Hyun Soo Lim, Cemil Bilir, Suleyman Kaleli, Melih Ucmak, and Guven Kasıkcı. The first draft of the manuscript was written by Ozge Turna and Gamze Guney Eskiler, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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## Declarations

**Ethical approval** The study was approved by the Local Animal Ethical Committee (08.03.2019, no. 2019/15).

**Conflict of interest** The authors declare no competing interests.

## References

1. O'Kefee DA (1995) Tumors of the genital system and mammary glands. In: Ettinger SJ, Feldman EC (eds) Textbook of veterinary internal medicine, 4th edn. W.B.Saunders Company, Philadelphia, pp 1702–1704
2. Sorenmo KU, Worley DR, Goldschmidth MH (2013) Tumours of the mammary gland. In: Withdrow SJ, Vail DM, Page RL (eds) Withrow & MacEwen's small animal clinical oncology, 5th edn. Saunders/Elsevier Inc., St. Louis, pp 538–556
3. Aiello SE (1998) The Merck Veterinary Manual. Merck&CO., INC. 1043–1045
4. Al-Asadi RN, Al-Kaledarm NAR, Al-Kadi KK (2010) An evaluation of mastectomy for removal of mammary glands tumors in bitches. Bas J Vet Res 10:141–152
5. Horta RS, Figueiredo MS, Lavalle GE, Costa MP, Cunha RMC, Araujo RB (2015) Surgical stress and postoperative complications related to regional and radical mastectomy in dogs. Acta Vet Scand 57(34):1–10
6. Misdorp W, Cotchin E, Hampe JF, Jabara AG, von Sandersleben J (1971) Canine malignant mammary tumours. I. Sarcomas. Vet Pathol 9:99–117
7. Goldschmidt M, Peria L, Rasotto R, Zapulli V (2011) Classification and grading of canine mammary tumors. Vet Pathol 9:117–131
8. Goldschmidt MH, Shofer FS, Smelstoys JA (2001) Neoplastic lesions of the mammary gland. In: Mohr U (ed) Pathobiology of the aging dog. Iowa State University Press, Ames, pp 168–178
9. Weigelt B, Geyer FC, Reis-Filho JS (2010) Histological types of breast cancer: how special are they? Mol Oncol 4(3):192–208
10. Juarranz Á, Jaén P, Sanz-Rodríguez F (2008) Photodyanamic theraphy of cancer. Basic principles and applications. Clin Transl Oncol 10:148
11. Allison RR, Moghissi K (2013) Photodynamic therapy (PDT): PDT mechanism. Clin Endosc 46:24–29
12. Buytaert E, Dewaele M, Agostinis P (2007) Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. Biochim Biophys Acta 1776:86–107
13. Nagata S, Obana A, Gohto Y, Nakajima S (2003) Necrotic and apoptotic cell death of human malignant melanoma cells following photodynamic therapy using an amphiphilic photosensitizer ATX-S10(Na). Lasers Surg Med 33:64–70
14. Wachowska M, Muchowicz A, Firczuk M, Gabrysiak M, Winiarska M, Wańczyk M, Bojarczuk K, Golab J (2011) Aminolevulinic acid (ALA) as a prodrug in photodynamic therapy of cancer. Molecules 16:4
15. Kessel D, Poretz RD (2000) Sites of photodamage induced by photodynamic therapy with a chlorin e6 triacetoxymethyl ester (CAME). Photochem Photobiol 71:94–96
16. Stummer W, Tonn JC, Mehdorn HM et al (2011) Counterbalancing risks and gains from extended resections in malignant glioma surgery: a supplemental analysis from the randomized 5-aminolevulinic acid glioma resection study. Clin Article J Neurosurg 114:613–623
17. Osaki T, Yokoe I, Sunden Y, Ota U, Ichikawa T, Imazato H, Li L (2019) Efficacy of 5-aminolevulinic acid in photodynamic detection and photodynamic therapy in veterinary medicine. Cancers 11(4):495
18. Ridgway TD, Lucroy MD (2003) Phototoxic effects of 635-nm light on canine transitional cell carcinoma cells incubated with 5-aminolevulinic acid. Am J Vet Res 64(2):131–136
19. Owen LN (1980) TNM classification of tumors in domestic animals, 1st edn. World Health Organization, Geneva, pp 1–53
20. Goldschmidt M, Pena L, Rasotto R, Zappulli V (2011) Classification and grading of canine mammary tumors. Vet Pathol 48:117–131
21. Li HT, Song XY, Yang C, Li Q, Tang D, Tian WR, Liu Y (2013) Effect of hematoporphyrin monomethyl ether-mediated PDT on the mitochondria of canine breast cancer cells. Photodiagn Photodyn Ther 10(4):414–421
22. McCaw DL, Pope ER, Payne JT, West MK, Tompson RV, Tate D (2000) Treatment of canine oral squamous cell carcinomas with photodynamic therapy. Br J Cancer 82(7):1297–1299
23. Onoyama M, Tsuka T, Imagawa T, Osaki T, Sugiyama A, Azuma K, Okamoto Y (2015) Photodynamic hyperthermal chemotherapy with indocyanine green in feline vaccine-associated sarcoma. Oncol Lett 10(4):2118–2122
24. Osaki T, Yokoe I, Ogura S, Takahashi K, Murakami K, Inoue K, Ishizuka M, Tanaka T, Li L, Sugiyama A, Azuma K, Murahata Y, Tsuka T, Ito N, Imagawa T, Okamoto Y (2017) Photodynamic detection of canine mammary gland tumours after

oral administration of 5-aminolevulinic acid. Vet Comp Oncol 15(3):731–739
25. Burch S, London C, Seguin B, Rodriguez C, Wilson BC, Bisland SK (2009) Treatment of canine osseous tumors with photodynamic therapy: a pilot study. Clin Orthop Relat Res 467(4):1028
26. Liu Y, Ma XQ, Jin P, Li HT, Zhang RR, Ren XL, Tian WR (2011) Apoptosis induced by hematoporphyrin monomethyl ether combined with he–ne laser irradiation in vitro on canine breast cancer cells. Vet J 188(3):325–330
27. Li H, Tong J, Bao J, Tang D, Tian W, Liu Y (2016) Hematoporphyrin monomethyl ether combined with he–ne laser irradiation-induced apoptosis in canine breast cancer cells through the mitochondrial pathway. J Vet Sci 17(2):235–242
28. Osaki T, Hibino S, Yokoe I, Yamaguchi H, Nomoto A, Yano S, Okamoto Y (2019) A basic study of photodynamic therapy with glucose-conjugated chlorin e6 using mammary carcinoma xenografts. Cancers 11(5):636
29. Peng Q, Berg K, Moan J, Kongshaug M, Nesland JM (1997) 5-Aminolevulinic acid-based photodynamic therapy: principles and experimental research. Photochem Photobiol 6:235–251
30. Derycke AS, de Witte PA (2004) Liposomes for photodynamic therapy. Adv Drug Deliv Rev 56(1):17–30
31. Mehraban N, Freeman HS (2015) Developments in PDT sensitizers for increased selectivity and singlet oxygen production. Materials (Basel) 8(7):4421–4456