Doctor's Thesis

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A study on fundamental photodynamic hyperthermal therapy with indocyanine green and its clinical application

インドシアニングリーンを用いた光線温熱療法の基礎的検討と臨床応用

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Publications

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Chapter Ⅰ:

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Chapter Ⅱ:

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Abstract

In cancer treatment, the radiation can make a more concentrated attack on cancer cells, and molecular target drugs, which can snipe at cancer cells at a molecular level appeared as anticancer drugs, and the surgery is tried to be less aggressive and keep the function of living in the human medicine. However, on the other hand, the establishment of these standard treatments caused “cancer refugees”, which are patients for whom there is no treatment anymore when a standard treatment becomes less effective. In order to overcome such situation, various new treatments including immunotherapy, gene therapy and hyperthermia are establishing.

In the small animal medicine represented by dogs and cats, which almost imitates the human medicine, it seems that the treatment of animals depends heavily on their owner’s intention, and all animals therefore cannot undergo standard treatments. In addition, because of geographical or economic factor, almost all owners do not seem to receive the benefit of standard treatments. It is real that there are a lot of animals and their owners like “cancer refugees”. Under this situation, there is no doubt that the small animal medicine also needs to fundamentally improve methods of cancer treatment as well as the human medicine and this improvement will have a significant positive impact on human society including animal owners.

We have sought a development of new cancer treatment and an establishment of it. In that process, we focused on hyperthermia with few side effects and photodynamic therapy as reported before. However, hyperthermia was not a popular

treatment in veterinary field, because it was a combination treatment with standard treatments rather than single, it needed expensive equipment, and we could not apply basic data for it though it had a history in human medicine. Furthermore, photodynamic therapy also did not be used widely because of less supply and high cost of sensitizers. Then, we focused on Indocyanine Green (ICG) used basically and clinically also in human medicine, which was safe and low in cost. ICG reacts to light and has a hyperthermic effect and photodynamic effect. We devised and aimed at a clinical application of Photodynamic Hyperthermia (PHT) using ICG as sensitizer and near infrared light (600-800nm). In addition, based on that an anticancer effect was enhanced by hyperthermia, we revised Photodynamic Hyperthermal Chemotherapy (PHCT) combined PHT and anticancer drugs.

In the chapter Ⅰ, on the basis of antitumor effect in PHT reported *in vitro* , we used a tumor-bearing mouse model and made it obvious that antitumor effect was recognized *in vivo*.

In the chapter Ⅱ, we applied PHCT made by adding local anticancer drugs to PHT in 16 cases of dogs and cats with malignant soft tissue sarcoma. It had been considered that malignant soft tissue sarcoma recurred locally very often and radiation was the only way to prevent recurrence of it. However these results suggest that PHCT decreases the risk of recurrence.

From these studies, it is considered that new treatment options for superficial solid cancers will increase in the small animal medicine.

General Introduction

Recently, the life span of companion animals becomes longer because of the vaccination against various infectious diseases, the improvement of eating and environment, etc, and in addition, the development of veterinary medicine. As a result, various illnesses related to aging are increasing. In particular, cancer is one of serious problems.

These days, it is said that cancer became a curable disease with an advance in cancer treatment. The outcome from treatment is certainly improving in human medicine field. However, the number of deaths due to cancer is going on increasing and eight hundred thousand people a year get cancer in Japan. One of out three people, about four hundred thousand people a year, dies from cancer in Japan, where one of out five people died from cancer twenty years ago. On the contrary, the rate of animals dying from cancer is getting higher in the small animal medicine field than that in the human medicine field.

In the case of dogs, about 40% of dogs aged 10 or older die from cancer. Due to the changings of their diet and rearing environment and the stress associated with such changings, diseases are getting diversified. As a result, most diseases such as malignant tumor, heart disease and diabetes, which occur in humans started to develop in rearing small animals. Especially cancer is the leading cause of death.

In the small animal medicine field represented by dogs and cats, which almost imitates the human medicine field, cancer treatment is also standard treatment including surgery, chemotherapy and radiotherapy. However, it is hard that we treat all

cases by these treatments. The treatment of animals depends heavily on their owner's intention, and all animals therefore cannot undergo standard treatments. In addition, because of geographical or economic factor, almost all owners do not seem to receive the benefit of standard treatments. It is real that there are a lot of animals and their owners like "cancer refugees". Under this situation, there is no doubt that the small animal medicine field also needs to fundamentally improve methods of cancer treatment as well as the human medicine and this improvement will have a significant positive impact on human society including animal owners. Therefore it is necessary to develop a new treatment that is safe, simple and applicable.

We noticed that indocyanine green (ICG) generates heat in response to the light near 800 nm (hyperthermia) (Chen *et al*., 1995a; Chen *et al*., 1995b; Chen *et al*., 1996; Liu *et al*., 2002). ICG was developed as a drug to promote liver and bile duct function, and it has been used medically since 1956 (Cherrick *et al*., 1960). This drug is safe, and the principle advantage of ICG is low toxicity (Hope-Ross *et al*., 1994). In particular, ICG is widely used in ophthalmology for hyperthermia therapy with an 808-nm diode laser to treat chorioretinopathy (Dzurinko *et al*., 2004). ICG is also used in sentinel biopsies (Hirche *et al*., 2010; Hojo *et al*., 2010).

Hyperthermia is used as a combined treatment with radiotherapy and chemotherapy because cell-killing effect is recognized at a temperature adaptable to a human body, tumor tissues are easier to be heated than normal tissues, and temperature-sensitive enhances when intracellular pH decreases (Dewey *et al*., 1977; Jähde & Rajewsky, 1982; Storm *et al*., 1979).

Hyperthermia is not a common treatment in the veterinary field because the

device is expensive and little fundamental data are available. On the other hand, there are reports of PDT being used to treat canine and feline spontaneous tumors, and the efficiency of this technique has been recognized (Dougherty *et al*., 1981; Okamoto *et al*., 2005; Osaki *et al*., 2009; Peaston *et al*., 1993; Reeds *et al*., 2004; Roberts *et al*., 1991; Tanabe *et al*., 2004). However, the photosensitizer for PDT is very expensive and a special diode laser is required. Therefore, PDT is rarely used in the veterinary field.

Some reports have suggested that ICG induces the formation of oxygen radicals (Abels *et al*., 1998; Bäumler *et al*., 1999; Bozkulak *et al*., 2009; Diven *et al*., 1996; Mamoon *et al*., 2009). Most of these reports are from in vitro studies of different human cell lines. Bozkulak *et al*. reported that ICG with near-infrared light (809 nm, 60 mW/cm^2, 24 J/cm^2) is very effective in treating human breast cancer cells (Bozkulak *et al*., 2009). That study controlled the temperature to ensure it remained at 37˚C. The authors concluded that ICG is a new photosensitizer. Hirano *et al*. was the first to demonstrate that ICG induces the formation of oxygen radicals in response to irradiation with light of a wavelength of 600-800 nm (Hirano *et al*., 2006).

On the basis of these, we developed a new cancer treatment by using ICG and the broadband light source apparatus. This is a combined treatment with photodynamic therapy using ICG and hyperthermia. We named this treatment to Photodynamic Hyperthemal Therapy (PHT).

Previously, we reported that PHT induced morphological cell death and inhibition of cell proliferation of murine melanoma cell, B16F10 (Radzi *et al*., 2012a). Furthermore, it was found that PHT induced apoptosis and cell cycle arrest *in vitro*

(Radzi *et al*., 2012b). However, there was no *in vivo* experimental data of PHT related with tumor growth and histological change.

In the chapter Ⅰ, on the basis of antitumor effect in PHT reported *in vitro*, we used a tumor-bearing mouse model and made it obvious that antitumor effect was recognized *in vivo*.

Anti-tumor drug effects are enhanced by heat (Hahn *et al*., 1975; Hahn, 1979; Marmor, 1979; Newell & Tannock, 1989). Furthermore, tumor tissues have a tendency to be acidic (Wike-Hooley *et al*., 1985). Therefore, we selected bleomycin as an anti-tumor drug because it is effective in acidic environments (Hahn *et al*., 1975). Platinum drugs such as cisplatin and carboplatin are also useful anti-tumor drugs.

In the chapter Ⅱ, we applied PHCT made by adding local anticancer drugs to PHT in 16 cases of dogs and cats with malignant soft tissue sarcoma. It had been considered that malignant soft tissue sarcoma recurred locally very often and radiation was the only way to prevent recurrence of it. However these results suggest that PHCT decreases the risk of recurrence.

From these studies, it is considered that new treatment options for superficial solid cancers will increase in the small animal medicine.

References

Abels C, Karrer S, Bäumler W, Goetz AE, Landthaler M and Szeimies RM: Indocyanine green and laser light for the treatment of AIDS-associated cutaneous Kaposi's sarcoma. Br J Cancer 77: 1021-1024,1998.

Bäumler W, Abels C, Karrer S, Weiss T, Messmann H, Landthaler M and Szeimies RM: Photo-oxidative killing of human colonic cancer cells using indocyanine green and infrared light. Br J Cancer 80: 360-363,1999.

Bozkulak O, Yamaci RF, Tabakoglu O and Gulsoy M: Photo-toxic effects of 809-nm diode laser and indocyanine green on MDA-MB231 breast cancer cells. Photodiagnosis Photodyn Ther 6: 117-121,2009.

Chen WR, Adams RL, Heaton S, Dickey DT, Bartels KE and Nordquist RE: Chromophore-enhanced laser-tumor tissue photothermal interaction using an 808-nm diode laser. Cancer Lett 88: 15-19, 1995a.

Chen WR, Adams RL, Bartels KE and Nordquist RE: Chromophore-enhanced in vivo tumor cell destruction using an 808-nm diode laser. Cancer Lett 94: 125-131, 1995b.

Chen WR, Adams RL, Higgins AK, Bartels KE and Nordquist RE: Photothermal effects on murine mammary tumors using indocyanine green and an 808-nm diode laser: an in vivo efficacy study. Cancer Lett 98: 169-173, 1996.

Cherrick GR, Stein SW, Leevy CM and Davidson CS: Indocyanine green: observations on its physical properties, plasma decay, and hepatic extraction. J Clin Invest 39: 592-600,1960.

Dewey WC, Hopwood LE, Sapareto SA and Gerweck LE: Cellular responses to combinations of hyperthermia and radiation. Radiology 123: 463-477,1977.

Diven DG, Pohl J and Motamedi M: Dye-enhanced diode laser photothermal ablation of skin. J Am Acad Dermatol 35: 211-215,1996.

Dougherty TJ, Thoma RE, Boyle DG and Weishaupt KR: Interstitial photoradiation therapy for primary solid tumors in pet cats and dogs. Cancer Res 41: 401-404,1981.

Dzurinko VL, Gurwood AS and Price JR: Intravenous and indocyanine green angiography. Optometry 75: 743-755,2004.

Hahn GM, Braun J and Har-Kedar I: Thermochemotherapy: synergism between hyperthermia (42-43 degrees) and adriamycin (of bleomycin) in mammalian cell inactivation. Proc Natl Acad Sci USA 72: 937–940,1975.

Hahn GM: Potential for therapy of drugs and hyperthermia. Cancer Res 39: 2264-2268,1979.

Hirano T, Kohno E, Gohto Y and Obana A: Singlet oxygen generation due to ICG irradiation. Photomed Photobiol 28: 15-16,2006.

Hirche C, Murawa D, Mohr Z, Kneif S and Hünerbein M: ICG fluorescence-guided

sentinel node biopsy for axillary nodal staging in breast cancer. Breast Cancer Res Treat 121: 373-378,2010.

Hojo T, Nagao T, Kikuyama M, Akashi S and Kinoshita T: Evaluation of sentinel node biopsy by combined fluorescent and dye method and lymph flow for breast cancer. Breast 19: 210-213,2010.

Hope-Ross M, Yannuzzi LA, Gragoudas ES, Guyer DR, Slakter JS, Sorenson JA, Krupsky S, Orlock DA and Puliafito CA: Adverse reactions due to indocyanine green. Ophthalmology 101: 529-533, 1994.

Jähde E and Rajewsky MF: Tumor-selective modification of cellular microenvionment in vivo: effect of glucose infusion on the pH in normal and malignant rat tissues. Cncer Res 42: 1505-1512,1982.

Liu VG, Cowan TM, Jeong SW, Jacques SL, Lemley EC and Chen WR: Selective Photothermal Interaction Using an 805-nm Diode Laser and Indocyanine Green in Gel Phantom and Chicken Breast Tissue. Laser Med. Sci 17**:** 272-279,2002.

Mamoon AM, Gamal-Eldeen AM, Ruppel ME, Smith RJ, Tsang T and Miller LM: In vitro efficiency and mechanistic role of indocyanine green as photodynamic therapy agent for human melanoma. Photodiagnosis Photodyn Ther 6: 105-116,2009.

Marmor JB: Interactions of hyperthermia and chemotherapy in animals. Cancer Res 39: 2269-2276,1979.

Newell KJ and Tannock IF: Reduction of intracellular pH as a possible mechanism for killing cells in acidic regions of solid tumors: effects of carbonylcyanide-3 chlorophenylhydrazone. Cancer Res 49: 4477-4482,1989.

Okamoto Y, Ogura K, Okamura Y, Ishii H, Sakata I, Hakamada K, Miyaki S, Nakajima S and Minami S: Canine hemangiopericytoma treated by combination of surgical resection and photodynamic therapy with novel photosensitizer, PAD-S31. Jpn J Vet Anesth Surg 36: 69-73,2005.

Osaki T, Takagi S, Hoshino Y, Okumura M, Kadosawa T and Fujinaga T: 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: 125-132,2009.

Peaston AE, Leach MW and Higgins RJ: Photodynamic therapy for nasal and aural squamous cell carcinoma in cats. JAVMA 202: 1261-1265,1993.

Radzi R, Osaki T, Tsuka T, Imagawa T, Minami S and Okamoto Y: Morphological Study in B16F10 Murine Melanoma Cells after Photodynamic Hyperthermal Therapy with Indocyanine Green (ICG). J Vet Med Sci 74: 465-472,2012a.

Radzi R, Osaki T, Tsuka T, Imagawa T, Minami S, Nakayama Y and Okamoto Y: Photodynamic Hyperthermal Therapy with Indocyanine Green (ICG) induces Apoptosis and Cell Cycle Arrest in B16F10 Murine Melanoma Cells. J Vet Med Sci 74: 545-551,2012b.

Reeds KB, Ridgway TD, Higbee RG and Lucroy MD: Non-coherent light for photodynamic therapy of superficial tumours in animals. Vet Comp Oncol 2:157-163,2004.

Roberts WG, Klein MK, Loomis M, Weldy S and Berns MW: Photodynamic therapy of spontaneous cancers in felines, canines, and snakes with chloro-aluminum sulfonated phthalocyanine. J Natl Cancer Inst 83:18-23,1991.

Storm FK, Harrison WH, Elliott RS and Morton DL: Normal tissue and solid tumor effects of hyperthermia in animal models and clinical trials. Cncer Res 39: 2245-2251,1979.

Tanabe S, Yamamaguchi M, Iijima M, Nakajima S, Sakata I, Miyaki S, Takemura T, Furuoka H, Kobayashi Y, Matsui T, Uzuka Y and Sarashina T: Fluorescence detection of a new photosensitizer, PAD-S31, in tumour tissues and its use as a photodynamic treatment for skin tumours in dogs and a cat: a preliminary report. Vet J 167: 286-293,2004.

Wike-Hooley JL, van den Berg AP, van der Zee J and Reinhold HS: Human tumour pH and its variation. Eur J Cancer Clin Oncol 21: 785-791,1985.

Chapter I

Effects of photodynamic hyperthermal therapy with indocyanine green on tumor growth in a colon 26 tumor-bearing mouse model

Abstract

The present study used indocyanine green (ICG) and a broadband light source apparatus [photodynamic hyper-thermal therapy (PHT) group] in order to treat a colon 26 tumor-bearing mouse model. The other groups were administered either ICG alone (ICG group), light alone (light group) or no treatment (control group). Following the treatment, tumor growth was measured. Nine days after the treatment, the tumors were resected and histological and immunohistological examinations were performed. In the PHT group, the growth rates of the tumor tissues were significantly decreased compared with those observed in the other groups ($P<0.05$). The proportion of necrotic areas in the PHT and light groups were increased significantly compared with those observed in the ICG and control groups. However, there were no significant differences between the PHT and light groups. The proportion of Ki-67 in the PHT and light groups was less than that observed in the ICG and control groups. The number of terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling-positive cells in the PHT group was significantly increased compared with that observed in the other groups. These data indicate that PHT is effective *in vivo* and *in vitro.*

1. Introduction

The life span of animals has grown longer due to vaccinations for various infectious diseases, improvements in food and environment and the development of veterinary medicine. As a result, the incidence of various illnesses that are associated with aging have been increasing in pet populations. In particular, cancer is a significant problem. As in human medicine, there are three major treatments for cancer in veterinary medicine, surgery, chemotherapy and radiation. However, it is difficult to treat all cases with these therapies. Therefore, it is necessary to develop new treatments.

Indocyanine green (ICG) generates heat in response to light near a wavelength of 800 nm (hyperthermia) (Chen *et al.*, 1995a; Chen *et al.*, 1995b; Chen *et al.*, 1996; Liu *et al.*, 2002). Furthermore, ICG generates active oxygen in response to light at 600-800 nm (photodynamic effect) (Abels *et al.*, 1998; Bäumler *et al.*, 1999; Bozkulak *et al.*, 2009; Hirano *et al.*, 2006). The principle advantage of ICG is low toxicity (Hope-Ross *et al.*, 1994). ICG has become widely used during sentinel biopsies (Hirche *et al.*, 2010; Hojo *et al.*, 2010; Ito *et al.*, 2004). Based on this, we developed a new cancer therapy, called photodynamic hyperthermal therapy (PHT), using ICG and a broadband light source apparatus. We previously reported that PHT induced morphological cell death and inhibited the proliferation of the murine B16F10 melanoma cell line (Radzi *et al.*, 2012a). Furthermore, it has been demonstrated that PHT induces apoptosis and cell cycle arrest *in vitro* (Radzi *et al.*, 2012b). However, there are no *in vivo* experimental data with regard to PHT-related tumor growth and histological changes.

The present study aimed to investigate the effects of PHT on tumor growth and histological changes using colon 26 tumor-bearing mice *in vivo*.

2. Materials and methods

2.1 Preparation of the tumor-bearing mouse model

A total of 23 female five-week-old BALB/c mice were purchased from CLEA Japan, Inc. (Osaka, Japan). The animals were maintained under conventional conditions. The use of these animals and the procedures they underwent were approved by the Animal Research Committee of Tottori University. Colon 26 tissue, which is of murine colon cancer origin, was transplanted subcutaneously into the dorsal regions of the mice.

The mice were bred for nine days with free access to food and water, following which, the experiments were performed. The mice whose tumors grew to 5 mm in size were used in this study.

2.2 Study design

The mice (n=23) were divided into four groups that were subjected to light + ICG (PHT group; n=8), ICG alone (ICG group; n=5), light alone (light group; n=5) or were untreated (control group; n=5).

All the treatments were performed at day 0 under general anesthesia with inhalation of 5% isoflurane. In the PHT group, 25 mg ICG (Diagnogreen; Daiichi Sankyo, Tokyo, Japan) was dissolved in 10 ml saline and adjusted to pH 5.0. A total of 0.5 ml ICG solution was injected into the tumor tissue, following which, irradiation was

performed using a near-infrared light source (Super Lizer™, Hyper 5000; maximum output, 5.0 W; 600–1600 nm of output wavelength bands; Tokyo Iken Co., Ltd., Tokyo, Japan). Irradiation was performed for 10 min using 20% output so that the distance from the tumor to the light source was 3-5 cm. The temperature of the tumor tissue and on the tumor surface during irradiation was measured using a digital temperature indicator (Anritsu Meter Co., Ltd., Tokyo, Japan) and maintained in a range of 42.5-45.0˚C in the tumor and <45.0˚C on the tumor surface. In the ICG group, the ICG solution was injected without the administration of irradiation. In the light group, irradiation was performed under similar conditions to those that were used in the PHT group.

Following nine days of treatment (day 9), all the mice were sacrificed by inhalation of 5% isoflurane followed by cervical dislocation. On days 0 and 9, the volume of tumor tissue was calculated by measuring the mediastinum and the transverse length, and the depth of the tumor. Based on volumes of the tumor on days 0 and 9, the tumor growth rate (mm^3/day) was calculated as follows: (tumor volume on day 9 - tumor volume on day 0)/9. The tumor tissue was removed and fixed in 10% buffered formalin.

2.3 Histological examination

The fixed samples were embedded in paraffin and sectioned in a routine manner. The sections were stained with hematoxylin and eosin (HE staining) and examined immunohistologically for Ki-67 and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining.

For the Ki-67 staining, 3-μm tissue sections were placed on glass slides and deparaffinized, then washed with ethanol and water and soaked in phosphate-buffered saline (PBS). The sections were autoclaved using 0.01 M citrate buffer (pH 6.0) for 15 min at 121˚C, washed with PBS and incubated with rabbit polyclonal anti-Ki-67 antibodies (1:50; code no. E0468; Dako, Glostrup, Denmark) for 30 min at room temperature. Subsequent to being washed with PBS, the sections were incubated with rat anti-immunoglobulin G antibodies (1:100; sc-372; Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min at room temperature. The slides were washed with PBS and avidin/biotin complex methods were performed (PK-4000; Vector Laboratories, Inc.) for 30 min. The tissue sections were counterstained with histogreen and then stained with nuclear fast red.

For the TUNEL staining, 3-*μ*m tissue sections were placed on glass slides and deparaffinized, then washed with ethanol and water and soaked in diluted water. The TUNEL staining was performed using an *In situ* Apoptosis Detection kit (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. The tissue sections were counterstained with histogreen and then stained with nuclear fast red. A total of 10 random high‑power fields were selected and the number of positive cells was counted.

2.4 Image analysis of HE and Ki‑67 staining

An analysis of the necrotic regions was performed using the bio‑imaging analysis system (Lumina Vision; Mitani Corporation, Tokyo, Japan). The necrotic regions were assessed based on the inhibition of cytoplasm, denaturation and nuclear

fragmentation. In brief, the images of 10 randomly chosen high-power fields (magnification, x200) in each cross section were captured using a digital camera attached to an Olympus microscope system (Olympus Corporation, Tokyo, Japan). The proportion of the necrotic areas among the total area was calculated. All the tumor tissues were analyzed. The mean proportion of the necrotic areas was calculated.

With regard to the Ki-67 staining, a quantitative digital morphometric analysis of the Ki-67-positive areas was performed. In brief, the images of 10 randomly chosen high-power fields (magnification, x200) in each cross section were captured using a digital camera attached to an Olympus microscope system (Olympus Corporation). The color wavelengths of the copied image were transformed into digital readings using the Lumina Vision software program (Mitani Corporation), allowing for the quantification of the various color wavelengths, with pixels as the unit of measurement. The proportion of the positive areas in the tumor tissues was calculated by dividing the total pixel area of the positive areas by the total pixel area that corresponded with the total tumor tissue in the field of view. The tumor tissues of three mice in each group were analyzed. The mean proportion of the positive areas in 30 fields was calculated in each group.

2.5 Statistical analysis

The data are expressed as the mean ± SE. The statistical analyses were performed using one-way ANOVA, followed by Tukey-Kramer's test. $P<0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1 Effects of PHT on tumor growth

The tumor growth rates are shown in Fig. 1. In the PHT group (93.6±5.7 mm^3/day), the growth rates of the tumor tissues were significantly decreased compared with those observed in the ICG (175.4±16.5 mm^3/day), light (142.0±6.3 mm^3/day) and control (184.8±13.0 mm^3/day) groups ($P<0.05$).

3.2 Histological observations

In the PHT group, uniform, large necrotic regions were observed at the tumor margin on the skin side. By contrast, numerous foci of necrosis were observed in the light group, primarily at the tumor margin on the skin side. Among the necrotic areas, no immunocytes, including lymphocytes or neutrophils, were observed in any of the groups.

The proportions of the necrotic areas are shown in Fig. 2. The values in the PHT (40.0±9.1%) and light (37.0±11.4%) groups were increased significantly compared with those observed in the ICG (5.0±2.3%) and control (5.0±1.8%) groups. However, there were no significant differences between the PHT and light groups.

3.3 Immunohistological analysis

The results of the Ki-67 immunohistochemistry are shown in Fig. 3. The proportions of the Ki-67-positive areas in the PHT (29.0±1.6%/field) and light (20.1±6.1%/field) groups were less than those observed in the ICG (51.6±1.7%/field) and control (35.4±8.9%/field) groups. In the light group, the proportion of the

Ki-67-positive areas was significantly decreased compared with that observed in the ICG group ($P<0.05$).

The results of the TUNEL immunohistochemistry are shown in Fig. 4. The number of TUNEL-positive cells (85.5±16.9 cells/field) in the PHT group was increased significantly compared with that observed in the other groups. In the ICG and control groups, almost no TUNEL-positive cells were observed in the tumor tissues. In the light group, TUNEL-positive cells were observed in the tumor tissues (3.0±2.3 cells/field).

4. Discussion

In the present study, PHT was observed to be effective *in vivo* and *in vitro*. The tumor growth rate in the PHT group was decreased significantly compared with that observed in the other groups. It has been reported that the combination of ICG and a 805 nm diode laser exhibits anticancer efficacy *in vivo* and *in vitro* (Chen *et al*., 1995a; Chen *et al*., 1995b; Chen *et al*., 1996; Ito *et al*., 2004). In these studies, it was speculated that ICG generated heat in response to light near the 800 nm wavelength, which indicated hyperthermia. In the present study, a broadband light source was used instead of a diode laser in the PHT group, as ICG generates active oxygen in response to light at 600-800 nm (photodynamic effect) in addition to hyperthermia (Abels *et al.*, 1998; Bäumler *et al.*, 1999; Bozkulak *et al.*, 2009; Hirano *et al.*, 2006). The present results revealed that PHT is more effective in suppressing tumor growth compared with hyperthermia alone.

Histologically, the proportion of the necrotic areas was similar between the PHT

and light groups. This indicates that hyperthermia alone induces tumor necrosis. With regard to the tumor growth rates, the rate that was observed in the PHT group was significantly decreased compared with the rate that was measured in the light group, indicating that the tumor cells in the areas without necrosis in the PHT group proliferated slowly compared with those in the light group.

The slow proliferation of tumor cells implies that numerous tumor cells do not proliferate due to causes such as cell cycle arrest and apoptosis. The present study investigated cell cycle arrest and apoptosis using immunohistochemical methods. TUNEL staining is one method that is used to detect apoptosis (Allen *et al.*, 1997; Otsuki *et al.*, 2003). The present results revealed that the number of TUNEL-positive cells was greater in the PHT group than in the light group. This result indicates that PHT strongly induces apoptosis compared with hyperthermia. Ki-67 is a cell population marker that is detected during all the active phases of the cell cycle, but is absent in resting cells (Brown & Gatter, 2002). With regard to Ki-67 staining, the proportions of the Ki-67-positive areas in the PHT and light groups were less than those observed in the ICG and control groups, which indicates that PHT and hyperthermia induce cell cycle arrest. However, there were no significant differences between the PHT and light groups. Our previous data has shown that PHT induces cell cycle arrest at an early time following the treatment (Radzi *et al.*, 2012b). The present results do not support our previous data. One reason for this discrepancy may be differences in the sampling time following the treatment. In the present study, the samples were obtained at nine days post-treatment. Periodical sampling (days 1, 3, 5 and 7) following treatment is therefore required in future studies.

Tumor cells are more sensitive to heat under acidic conditions (Gerweck, 1977). Therefore, saline that was adjusted to pH 5.0 using acetic acid was used as a solvent for ICG. In a preliminary experiment, saline that was adjusted to a pH of 4.0 was observed to induce inflammation in the tissue.

In the present study, single treatments were performed, following which, tumor growth was observed. Consequently, it was identified that the single treatments were not adequate to induce complete tumor remission. In order to achieve complete remission, several rounds of treatment are necessary. Further studies are therefore required.

In conclusion, the growth rates of the tumor tissues were significantly decreased in the PHT group. Necrosis and apoptosis were induced by the PHT treatment. The Ki-67-positive areas were significantly decreased by the PHT treatment. The data indicate the PHT has the potential to be a novel cancer treatment. Further studies using clinical patients are required.

References

Abels C, Karrer S, Bäumler W, Goetz A E, Landthaler M and Szeimies RM: Indocyanine green and laser light for the treatment of AIDS‐associated cutaneous Kaposi's sarcoma. Br J Cancer 77: 1021‐1024, 1998.

Allen RT, Hunter WJ 3rd and Agrawal DK: Morphological and biochemical characterization and analysis of apoptosis. J Pharmacol Toxicol Methods 37: 215‐228, 1997.

Bäumler W, Abels C, Karrer S, Weiss T, Messmann H, Landthaler M and Szeimies RM: Photo-oxidative killing of human colonic cancer cells using indocyanine green and infrared light. Br J Cancer 80: 360-363, 1999.

Bozkulak O, Yamaci RF, Tabakoglu O and Gulsoy M: Photo-toxic effects of 809-nm diode laser and indocyanine green on MDA-MB231 breast cancer cells. Photodiagnosis Photodyn Ther 6: 117-121, 2009.

Brown DC and Gatter KC: Ki67 protein: the immaculate deception? Histopathology 40: 2-11, 2002.

Chen WR, Adams RL, Heaton S, Dickey DT, Bartels KE and Nordquist RE: Chromophore-enhanced laser-tumor tissue photothermal interaction using an 808-nm diode laser. Cancer Lett 88: 15-19, 1995a.

Chen WR, Adams RL, Bartels KE and Nordquist RE: Chromophore-enhanced in vivo tumor cell destruction using an 808-nm diode laser. Cancer Lett 94: 125-131, 1995b.

Chen WR, Adams RL, Higgins AK, Bartels KE and Nordquist RE: Photothermal effects on murine mammary tumors using indocyanine green and an 808-nm diode laser: an in vivo efficacy study. Cancer Lett 98: 169-173, 1996.

Gerweck LE: Modification of cell lethality at elevated temperatures. The pH effect. Radiat Res 70: 224‐235, 1977.

Hirano T, Kohno E, Gohto Y and Obama A: Singlet oxygen generation due to ICG irradiation. Photomed Photobiol 28: 15‐16, 2006.

Hirche C, Murawa D, Mohr Z, Kneif S and Hünerbein M: ICG fluorescence‐guided sentinel node biopsy for axillary nodal staging in breast cancer. Breast Cancer Res Treat 121: 373‐378, 2010.

Hojo T, Nagao T, Kikuyama M, Akashi S and Kinoshita T: Evaluation of sentinel node biopsy by combined fluorescent and dye method and lymph flow for breast cancer. Breast 19: 210‐213, 2010.

Hope‐Ross M, Yannuzzi LA, Gragoudas ES, Guyer DR, Slakter JS, Sorenson JA, Krupsky S, Orlock DA and Puliafito CA: Adverse reactions due to indocyanine green. Ophthalmology 101: 529‐533, 1994.

Ito N, Fukuta M, Tokushima T, Nakai K and Ohgi S: Sentinel node navigation surgery using indocyanine green in patients with lung cancer. Surg Today 34: 581‐585, 2004.

Liu VG, Cowan TM, Jeong SW, Jacques SL, Lemley EC and Chen WR: Selective photothermal interaction using an 805‐nm diode laser and indocyanine green in gel phantom and chicken breast tissue. Lasers Med Sci 17: 272‐279, 2002.

Otsuki Y, Li Z and Shibata MA: Apoptotic detection methods ‐ from morphology to gene. Prog Histochem Cytochem 38: 275‐339, 2003.

Radzi R, Osaki T, Tsuka T, Imagawa T, Minami S and Okamoto Y: Morphological study in B16F10 murine melanoma cells after photodynamic hyperthermal therapy with indocyanine green (ICG). J Vet Med Sci 74: 465‐472, 2012a.

Radzi R, Osaki T, Tsuka T, Imagawa T, Minami S, Nakayama Y and Okamoto Y: Photodynamic hyperthermal therapy with indocyanine green (ICG) induces apoptosis and cell cycle arrest in B16F10 murine melanoma cells. J Vet Med Sci 74: 545‐551, 2012b.

Figures

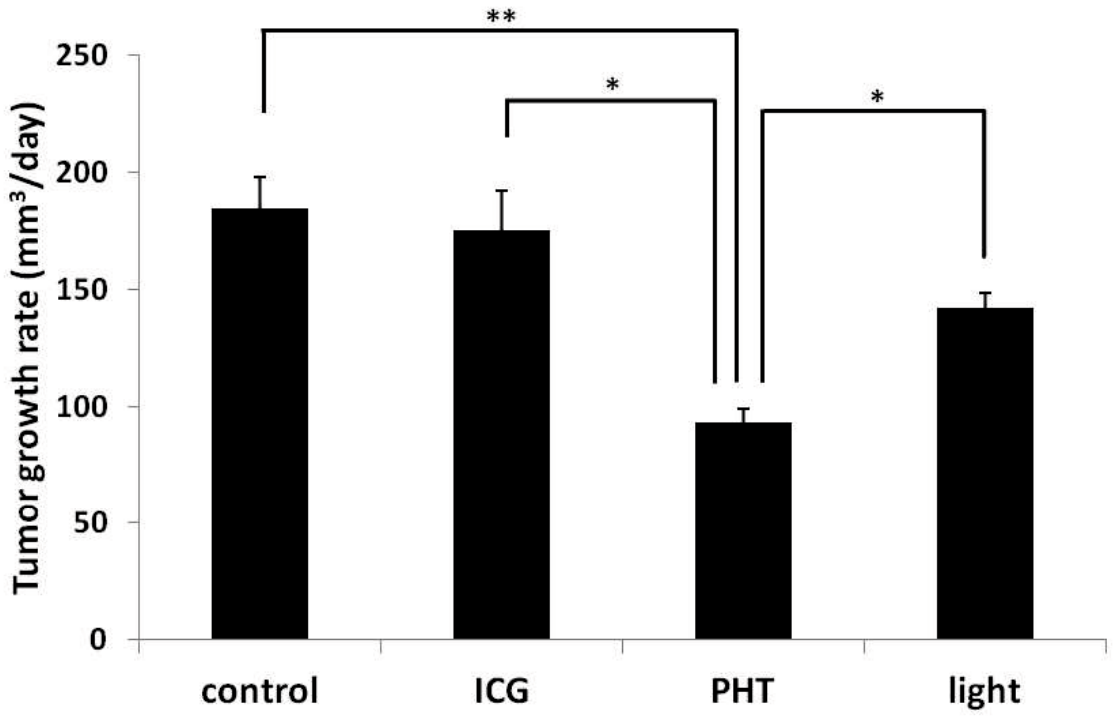

Figure 1. Effects of photodynamic hyperthermal therapy (PHT) on tumor growth. The tumor volume was measured on days 0 and 9. The tumor growth rates (mm^3/day) were calculated according to the tumor volumes. The data are presented as the mean ± SE of each group. Statistical significance was determined according to the Tukey‐Kramer test. **P<0.01 and *P<0.05. ICG, indocyanine green.

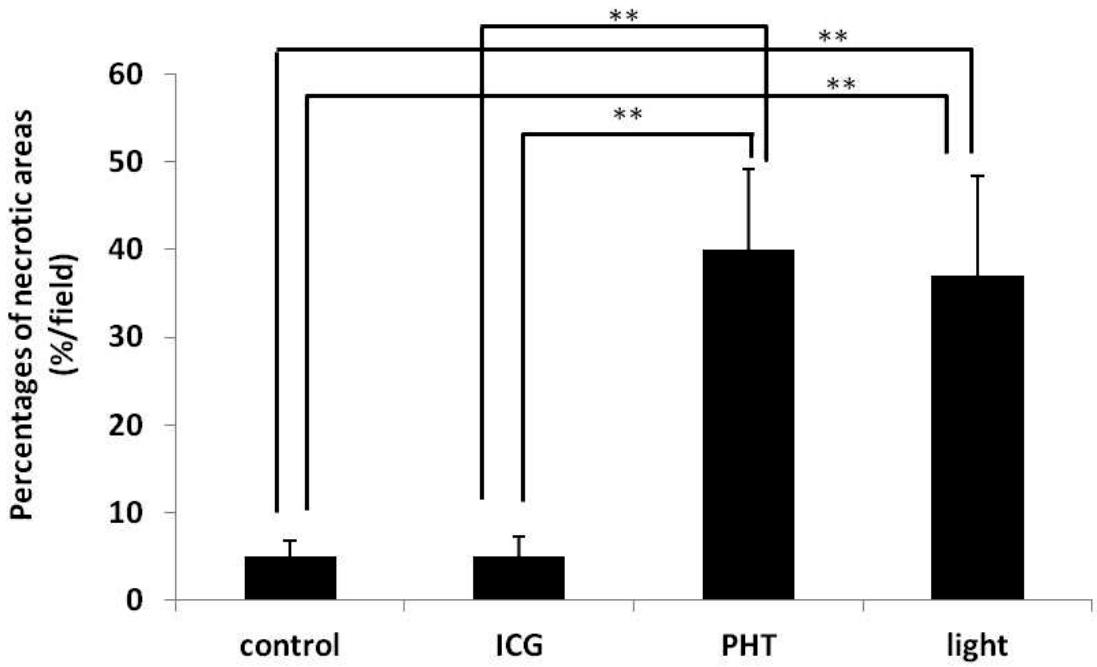

Figure 2. Effects of photodynamic hyperthermal therapy (PHT) on the proportion of necrotic areas in the tumor tissue. The proportions of necrotic areas were calculated. The data are presented as the mean ± SE of each group. Statistical significance was determined according to the Tukey‐Kramer test. **P<0.01. ICG, indocyanine green.

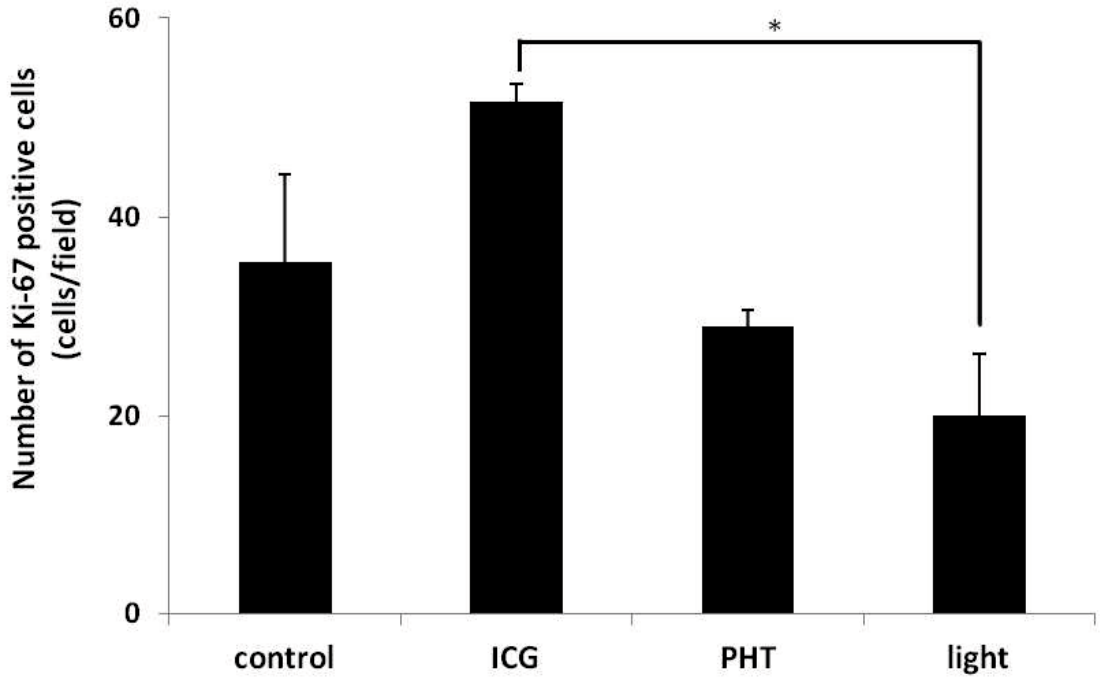

Figure 3. Effects of photodynamic hyperthermal therapy (PHT) on the proportion of Ki-67-positive areas in the tumor tissue. The proportions of Ki-67-positive areas were calculated. The data are presented as the mean ± SE of each group. Statistical significance was determined according to the Tukey-Kramer test. *P<0.05. ICG, indocyanine green.

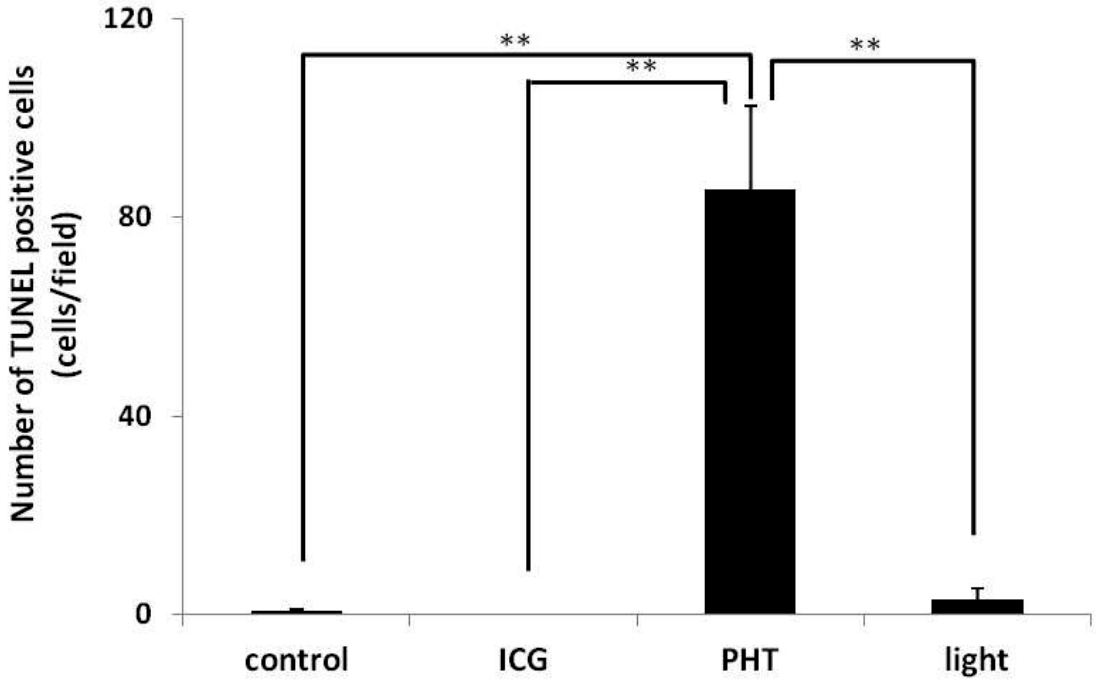

Figure 4. Effects of photodynamic hyperthermal therapy (PHT) on the number of terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL)-positive cells in the tumor tissue. The numbers of TUNEL-positive cells were calculated. The data are presented as the mean ± SE of each group. Statistical significance was determined according to the Tukey-Kramer test. **P<0.01 ICG, indocyanine green.

Chapter II

Photodynamic hyperthermal chemotherapy with indocyanine green: a novel cancer therapy for 16 cases of malignant soft tissue sarcoma

Abstract

Sixteen cases of malignant soft tissue sarcoma (STS; 10 canines and six felines) were treated with a novel triple therapy that combined photodynamic therapy, hyperthermia using indocyanine green with a broadband light source, and local chemotherapy after surgical tumor resection. This triple therapy was called photodynamic hyperthermal chemotherapy (PHCT). In all cases, the surgical margin was insufficient. In one feline case, PHCT was performed without surgical resection. PHCT was performed over an interval of 1 to 2 weeks and was repeated three to 21 times. No severe side effects, including severe skin burns, necrosis, or skin suture rupture, were observed in any of the animals. No disease recurrence was observed in seven out of 10 (70.0%) dogs and three out of six (50.0%) cats over the follow-up periods ranging from 238 to 1901 days. These results suggest that PHCT decreases the risk of STS recurrence. PHCT should therefore be considered an adjuvant therapy for treating companion animals with STS in veterinary medicine.

1. Introduction

Soft tissue sarcoma (STS) develops in a variety of mesenchymal tissues (Kuntz

et al., 1997). In general, local recurrence is common following conservative excision. Therefore, other therapies including radiation therapy and chemotherapy are used to prevent recurrence after performing a wide first excision (Ettinger, 2003; Liptak & Forrest, 2012; McChesney *et al.*, 1986; Ogilvie *et al.*, 1989; Ogilvie *et al.*, 1991). Radiation therapy plays a particularly important role in the management of STS (Dernell, *et al*., 1998). However, this type of therapy can only be performed in restricted facilities. Some patients cannot undergo radiation therapy due to financial difficulties or because the number of available facilities is limited. Therefore, it is necessary to develop novel and effective techniques to treat STS.

Indocyanine green (ICG) induces heat generation in response to light at a wavelength of 808 nm (Chen *et al*., 1995a; Chen *et al*., 1995b; Chen *et al*., 1996) and oxygen radicals upon exposure to light at wavelengths of 600−800 nm (Hirano *et al*., 2006). Based on these evidences, we developed a novel cancer therapy using the properties of ICG and a broadband light source instead of a diode laser to establish a combination of photodynamic therapy and hyperthermia. This method is known as photodynamic hyperthermia (PHT) and can be combined with local chemotherapy to create a triple therapy strategy (photodynamic therapy, hyperthermia therapy, and chemotherapy) called photodynamic hyperthermal chemotherapy (PHCT). To date, no studies on the application of PHT and PHCT for the treatment of STS have been published. This is the first report of the use of PHCT to treat STS.

2. Materials and Methods

2.1 Animals

Table 1 presents a summary of the 10 canine and six feline cases. These animals were treated at Veterinary Teaching Hospital of Tottori University (six dogs and four cats; Tottori, Japan), Takayama Pet Clinic (two dogs; Osaka, Japan), Aino Animal Hospital (one dog and two cats; Shizuoka, Japan) and Tokyo Animal Medical Center (one dog; Tokyo, Japan), respectively. The animals were diagnosed with STS based on preoperative biopsies. The ages of the animals ranged from 4 to 15 years. The dogs included two Labrador retrievers, two Golden retrievers, one Cocker spaniel, one Welsh corgi, one Miniature schnauzer, one French bulldog, and two Mongrels. The cats included five Domestic shorthair (DSH) cats and one American shorthair. We measured the original tumor size by caliper before surgery. All cases were classified according to TNM (T, size of the primary tumor; N, condition of the regional lymph nodes; M, absence/presence of distant metastasis) stage (Liptak & Forrest, 2012). Tumors in five dogs and six cats were classified as T1 (≤ 5 cm in diameter at the greatest dimension), and T2 (> 5 cm in diameter at greatest dimension) in five dogs.

Among the dogs, the tumor types included three cases of malignant schwannoma, three cases of hemangiopericytoma, one case of liposarcoma, one case of fibrosarcoma, and two cases of undifferentiated soft tissue tumors. In the cats, the tumor types included three cases of fibrosarcoma, one case of malignant schwannoma, one case of rhabdomyosarcoma, and one case of an undifferentiated soft tissue tumor. The tumor sites included the trunk in four cases (one in the dorsal region, two in the axilla, and one in the perineum) and the limbs in 12 cases. No lung metastasis was observed by radiography in any case.

We explained the risk of recurrence and treatment options, including surgery,

radiation, and chemotherapy, to the animal owners. When tumors had developed in the limbs, we proposed amputation as the first choice of treatment to the owners. However, the owners did not desire amputation and radiation. We then proposed to all owners other treatments including the combination of PHCT and surgery. We explained that PHCT is an experimental therapy, and all owners of the pets enrolled in this clinical trial provided informed consent.

2.2 Surgical treatment

For tumors that had developed in the limbs, we maintained sufficient skin for skin closure with skin sutures after the tumors were removed because the owners did not want us to perform reconstructive surgery such as skin grafting. As a result, we could not obtain sufficient surgical margins. We removed the underlying fascia of the tumors. In some cases, we could not perform perfect skin closure due to the presence of skin ulcers (Fig. 1A). In some cases (cases C06, C09, and F04), we removed the tumors with an ultrasonic aspiration device (Qucer; M & M, Japan). For case F02, only PHCT was performed because the owner did not wish the animal to undergo surgery.

2.3 PHCT

ICG (25 mg/vial, Giagnogreen; Daiich Sankyo, Japan) was dissolved in 9 ml of saline with an adjusted pH of 5.0. As an anti-tumor drug, 1 ml of bleomycin (1 mg/ml, Buleo; Nippon Kayaku, Japan) or carboplatin (10 mg/ml; Nippon Kayaku) was added to the ICG solution. For some cases, a small volume (0.1 or 0.2 ml) of paclitaxel (Bristol-Myers Squibb, USA.) was added to the ICG solution. For one case (case C04),

no anti-tumor drugs were administered in accordance with the owner's request. A broadband light source (Super Lizer 5000; Tokyo Iken, Japan) emitting a wavelength spectrum from 600 to 1,600 nm with a 5,000 mW maximum output power was used because ICG responses to light at wavelength of 600−800 nm.

For each case, the tumors were resected and ICG solution was injected into the resected area 3-dimensionally, including the skin surgical margin (3 cm). One ml of the ICG solution was administered per cm^2 of the wound bed. Irradiation was administered at a distance of 10 cm from the resected area (irradiation area: 113 cm^2, 40 mW/cm^2) for 20 min per 113 cm^2 (48 J/cm^2; Fig. 1B) immediately after the ICG solution was injected. The temperature at the surface of the resected area was kept under 45℃ by moving light source near and away from the skin surface and monitored with a thermometer. The first round of PHCT was performed immediately after skin suturing following surgery. The treatment interval between the second and fourth round of PHCT was generally 1 week, and then the treatment was performed at intervals of 2 to 4 weeks. At a minimum, treatment was continued for 3 months after surgery. At that point, we continued the treatment if the owner desired. For the second and subsequent rounds of PHCT, the treatments were performed with all animals under sedation. In some cases, local anesthesia was induced by Lidocaine of 15～50 mg/head (Xylocaine; AstraZeneca, Japan).

2.4 Follow-up after PHCT

In all cases, follow-up examinations for STS recurrence and metastasis were performed at intervals of several months for 1 year after the first round of PHCT.

Thereafter, follow-up examinations were performed once a year for 5 years.

3. Results

Table 2 presents a summary of the study results. PHCT was performed three to 21 times. The treatment frequency depended on the wishes of the owner. No severe side effects, including severe skin burns, necrosis, or rupture of skin sutures, were observed in any of the animals although skin redness and minor skin burns occurred. The overall canine survival time (ST) except for 1 case of amputation (case C06) ranged from 225 to 1,901 days (median survival time: 767 days). In seven out of 10 dogs (70.0%), no recurrence was observed during the follow-up periods ranging from 238 to 1,901 days. In five of these seven dogs, no recurrence was observed after more than 2 years. The remaining three dogs (two with undifferentiated soft tissue sarcomas and one with liposarcoma) experienced recurrence or metastasis over intervals ranging from 72 to 162 days after surgery. One case with liposarcoma (case C06) experienced local recurrence occurring within 1 month after the first surgery. In this animal, local recurrence was also observed 72 days after the first round of PHCT following the second surgery. The disease-free time (DFT) was prolonged by the use of PHCT. However, amputation was performed in accordance with the owner's desire, and the dog is currently alive. One dog (case C08) of the remaining three dogs died due to tumor progression. In case 08, metastasis to the sublumbar lymph nodes was observed. In that animal, no local recurrence was found. In case C09, local recurrence was observed 162 days after the first round of PHCT. PHCT was repeated for this animal five times at intervals of 1 to 2 weeks. Thereafter, the dog was only

followed up. Sixty-seven days after the fifth round of PHCT, local recurrence was observed. The dog eventually died of cardiac failure 225 days after the first round of PHCT.

The overall feline ST, excluding two cases of amputation (cases F04 and F06), ranged from 383 to 1,521 days (median survival time: 1,344 days). In three out of the six cats (50.0%), no recurrence was observed over the follow-up periods ranging from 1,173 to 1,521 days. The remaining three cats (one case of fibrosarcoma, one case of rhabdomyosarcoma, and one case of undifferentiated STS) experienced recurrence over intervals ranging from 20 to 175 days after surgery. In two (cases F04 and F06) out of the remaining three cases, amputation was performed on one cat (case F04) that is currently alive and one cat (case F06) that died due to unknown cause. The remaining cat died due to tumor progression. In case F02 for which no surgery was performed, we periodically assessed tumor status using fine needle aspiration (FNA). No living tumor cells were observed by FNA during the eighth round of PHCT. We diagnosed the tumor as degenerative following PHCT, and the degenerative tumor tissue was removed using an ultrasonic aspiration device (Sono Cure; Tokyo Iken, Japan). Thereafter, PHCT was performed for 3 months. This cat had no recurrence for about 5 years.

4. Discussion

Malignant STSs rarely metastasize; however, they are locally invasive (Bostock & Dye, 1980). If the surgical margin is insufficient, the rate of recurrence is 10 times of that when the surgical margin is sufficient (Kuntz *et al.*, 1997). Therefore, complete

resection with a sufficient surgical margin is necessary. In general, adjuvant therapies including radiation therapy and chemotherapy are administered to cases of insufficient surgical margins (Ettinger, 2003; Liptak & Forrest, 2012; McChesney *et al*., 1986; Ogilvie *et al.*, 1991). In particular, radiation therapy plays an important role in the management of STS. In the present study, the surgical margins were insufficient in all cases. However, 70.0% (7/10) of the dogs and 50.0% (3/6) of the cats did not experience recurrence over follow-up periods ranging from 238 to 1,901 days. These results suggest that PHCT decreases the risk of recurrence.

As an alternative to radiation therapy, we developed a new therapy, PHT, by combining Photodynamic therapy (PDT) and hyperthermia with ICG using a broadband light source. Furthermore, we administered PHT in combination with local chemotherapy and designated this technique “PHCT”. No previous studies have demonstrated the efficacy of the combination of ICG and use of a broadband light source in cancer therapy.

It has been suggested that fibrosarcomas, malignant schwannomas, and hemangiopericytomas are sensitive to PHCT considering the relationship between tumor type and PHCT efficacy. The local control rate of fibrosarcoma, malignant schwannoma, and hemangiopericytoma was 100% in the present study. However, PHCT is not effective for other tumor types including liposarcoma, rhabdomyosarcoma, and undifferentiated STSs occurring in the limbs. In these cases, the tumors recurred ranging from 20 to 175 days after the first PHCT. These results suggest that the effects of PHCT might differ depending on tumor type. Further investigation into this possibility is necessary. In the present study, no relationship

between treatment frequency and recurrence was observed. In one case (case C09), local recurrence was observed 63 days after the final round of PHCT. This phenomenon suggests that it is important to periodically continue treatment with PHCT. Additionally, no relationship was observed between the use of anti-cancer drugs and outcomes.

In two out of three canine and two out of three feline recurrence cases, the tumor cells had infiltrated deeply. We speculate that the light used for PHCT did not reach the tumor cells located in deep tissues. In a preliminary experiment, we found that the temperature at a depth of 2 cm from the skin surface did not reach 40℃ (data not shown), indicating that PHCT is not effective for treating tissues beyond this depth. Additionally, the ICG solution was not equally distributed. These drawbacks will be investigated in the future.

Hyperthermia is not a common treatment in veterinary medicine because the required device is expensive and few fundamental data related to the efficacy of hyperthermia are available. On the other hand, there are reports of PDT being used to treat spontaneous tumors in canines and felines, and the efficiency of this technique has been recognized (Dougherty *et al*., 1981; Okamoto *et al*., 2005; Osaki *et al*., 2009; Peaston *et al*., 1993; Reeds *et al*., 2004; Roberts *et al*., 1991; Tanabe *et al*., 2004). However, the photosensitizer for PDT is very expensive and a special diode laser is needed. Therefore, PDT is also rarely administered by veterinarians. ICG was developed as a drug to promote liver and bile duct function, and it has been used medically since 1956. This drug is safe for both human and animals (Cherrick *et al.*, 1960). In particular, ICG is widely used in ophthalmology for hyperthermia therapy

with an 808-nm diode laser to treat chorioretinopathy (Dzurinko *et al.*, 2004). ICG is also used for sentinel biopsies (Hirche *et al*., 2010; Hojo *et al*., 2010).

Some reports have suggested that ICG induces the formation of oxygen radicals (Abels *et al*., 1998; Bäumler *et al*., 1999; Bozkulak *et al*., 2009; Diven *et al*., 1996; Mamoon *et al*., 2009). Most of these findings are from in vitro studies of different human cell lines. Bozkulak et al. (Bozkulak *et al*., 2009) reported that ICG with near-infrared light (809 nm, 60 mW/cm^2, 24 J/cm^2) is very effective for eliminating human breast cancer cells. In this study, the temperature of the medium was controlled to ensure that it remained at 37℃. The authors concluded that ICG acts as a new photosensitizer. Hirano et al. (Hirano *et al.*, 2006) was the first to demonstrate that ICG induces the formation of oxygen radicals in response to irradiation with light at a wavelength of 600−800 nm. The concentration of oxygen radicals produced by ICG dissolved in ethanol is similar to that generated by other photosensitizers. Oxygen radicals are formed even if ICG is dissolved in water although the concentration is lower. The total volume of oxygen radicals produced is thought to result from the accumulation of oxygen radicals produced with each wavelength. Therefore, a broadband light emitting light over a range of 600−800 nm would induce the formation of more oxygen radicals than a diode laser. Additionally, a broadband light that has an emission range of 600−800 nm would stimulate ICG to simultaneously induce heat and oxygen radical production in tumor tissues.

We previously investigated the effects of PHT on B16F10 murine melanoma cells in vitro (Radzi *et al*., 2012a; Radzi *et al*., 2012b). Our results demonstrated that PHT induces early morphological changes in tumor cells that promote more cell death

than hyperthermia alone. Furthermore, PHT induces early apoptosis and cell arrest. These in vitro data support the present in vivo results.

Tumor cells are more sensitive to heat under acidic conditions (Gerweck, 1977). Therefore, saline with a pH adjusted to 5.0 with acetic acid was used as a solvent for ICG. In a preliminary experiment, we found that saline with a pH of 4.0 induced tissue inflammation (data not shown).

The effects of anti-tumor drugs are enhanced by heat (Hahn *et al*., 1975; Hahn, 1979; Marmor, 1979; Newell & Tannock, 1989). Furthermore, tumor tissues have a tendency to be acidic (Wike-Hooley *et al*., 1985). Therefore, we selected bleomycin as an anti-tumor drug because it is effective in acidic environments (Hahn *et al*., 1975). Platinum-based drugs such as cisplatin and carboplatin are also useful anti-tumor reagents. In the present study, we were concerned that PHCT would affect the stability of the anti-tumor drug. However, our preliminary data showed that the anti-tumor effects of PHCT were enhanced by the addition of an anti-tumor drug (data not shown). This means that PHCT does not only affect the stability of the anti-tumor drug but also enhances its effects.

In conclusion, PHCT is a simple procedure, is not associated with any severe side effects, and requires no special facilities. However, further investigation is necessary to establish PHCT as a therapeutic technique due to some associated problems involving treatment times and intervals, and selection of anti-cancer drugs. Nevertheless, this modality is expected to become a useful alternative to radiation therapy for treating superficial tumors such as STS in companion animals.

References

Abels C, Karrer S, Bäumler W, Goetz AE, Landthaler M and Szeimies RM: Indocyanine green and laser light for the treatment of AIDS-associated cutaneous Kaposi's sarcoma. Br J Cancer 77: 1021-1024,1998.

Bäumler W, Abels C, Karrer S, Weiβ T, Messmann H, Landthaler M and Szeimies RM: Photo-oxidative killing of human colonic cancer cells using indocyanine green and infrared light. Br J Cancer 80: 360-363,1999.

Bostock DE and Dye MT: Prognosis after surgical excision of canine fibrous connective tissue sarcomas. Vet Pathol 17: 581-588,1980.

Bozkulak O, Yamaci RF, Tabakoglu O and Gulsoy M: Photo-toxic effects of 809-nm diode laser and indocyanine green on MDA-MB231 breast cancer cells. Photodiagnosis Photodyn Ther 6: 117-121,2009.

Chen WR, Adams RL, Heaton S, Dickey DT, Bartels KE and Nordquist RE: Chromophore-enhanced laser-tumor tissue photothermal interaction using an 808-nm diode laser. Cancer Lett 88: 15-19,1995a.

Chen WR, Adams RL, Bartels KE and Nordquist RE: Chromophore-enhanced in vivo tumor cell destruction using an 808-nm diode laser. Cancer Lett 94: 125-131,1995b.

Chen WR, Adams RL, Higgins AK, Bartels KE and Nordquist RE: Photothermal effects on murine mammary tumors using indocyanine green and an 808-nm diode laser: an in vivo efficacy study. Cancer Lett 98: 169-173,1996.

Cherrick GR, Stein SW, Leevy CM and Davidson CS: Indocyanine green: observations on its physical properties, plasma decay, and hepatic extraction. J Clin Invest 39: 592-600,1960.

Dernell WS, Withrow SJ, Kuntz CA and Powers BE: Principles of treatment for soft tissue sarcoma. Clin Tech Small Anim Pract 13: 59-64,1998.

Diven DG, Pohl J and Motamedi M: Dye-enhanced diode laser photothermal ablation of skin. J Am Acad Dermatol 35: 211-215,1996.

Dougherty TJ, Thoma RE, Boyle DG and Weishaupt KR: Interstitial photoradiation therapy for primary solid tumors in pet cats and dogs. Cancer Res 41: 401-404;1981.

Dzurinko VL, Gurwood AS and Price JR: Intravenous and indocyanine green angiography. Optometry 75: 743-755,2004.

Ettinger SN: Principles of treatment for soft-tissue sarcomas in the dog. Clin Tech Small Anim Pract 18: 118-122,2003.

Gerweck LE: Modification of cell lethality at elevated temperatures: the pH effect. Radiat Res 70: 224-235,1977.

Hahn GM, Braun J and Har-Kedar I: Thermochemotherapy: synergism between hyperthermia (42-43 degrees) and adriamycin (of bleomycin) in mammalian cell inactivation. Proc Natl Acad Sci U S A 72: 937-940,1975.

Hahn GM: Potential for therapy of drugs and hyperthermia. Cancer Res 39: 2264-2268,1979

Hirano T, Kohno E, Gohto Y and Obana A: Singlet oxygen generation due to ICG irradiation. Photomed Photobiol 28: 15-16,2006.

Hirche C, Murawa D, Mohr Z, Kneif S and Hünerbein M: ICG fluorescence-guided sentinel node biopsy for axillary nodal staging in breast cancer. Breast Cancer Res Treat. 121: 373-378,2010.

Hojo T, Nagao T, Kikuyama M, Akashi S and Kinoshita T: Evaluation of sentinel node biopsy by combined fluorescent and dye method and lymph flow for breast cancer. Breast 19: 210-213,2010.

Kuntz CA, Dernell WS, Powers BE, Devitt C, Straw RC and Withrow SJ: Prognostic factors for surgical treatment of soft-tissue sarcomas in dogs: 75

cases (1986-1996). J Am Vet Med Assoc 211: 1147-1151,1997.

Liptak JM and Forrest LJ: Soft tissue sarcoma. In: Withrow SJ, Vail DM, Page RL (eds.). Small Animal Clinical Oncology. 5th ed. pp. 356-380, Elsevier Saunders, St. Louis, 2012.

Mamoon AM, Gamal-Eldeen AM, Ruppel ME, Smith RJ, Tsang T and Miller LM: In vitro efficiency and mechanistic role of indocyanine green as photodynamic therapy agent for human melanoma. Photodiagnosis Photodyn Ther 6: 105-116,2009.

Marmor JB: Interactions of hyperthermia and chemotherapy in animals. Cancer Res 39: 2269-2276,1979.

McChesney SL, Gillette EL, Dewhirst MW and Withrow SJ: Influence of WR 2721 on radiation response of canine soft tissue sarcomas. Int J Radiat Oncol Biol Phys 12: 1957-1963,1986.

Newell KJ and Tannock IF: Reduction of intracellular pH as a possible mechanism for killing cells in acidic regions of solid tumors: effects of carbonylcyanide-3 chlorophenylhydrazone. Cancer Res 49: 4477-4482,1989.

Ogilvie GK, Reynolds HA, Richardson RC, Withrow SJ, Norris AM, Henderson

RA, Klausner JS, Fowler JD and McCaw D: Phase II evaluation of doxorubicin for treatment of various canine neoplasms. J Am Vet Med Assoc 195: 1580-1583,1989.

Ogilvie GK, Obradovich JE, Elmslie RE, Vail DM, Moore AS, Straw RC, Dickinson K, Cooper MF and Withrow SJ: Efficacy of mitoxantrone against various neoplasms in dogs. J Am Vet Med Assoc 198: 1618-1621,1991.

Okamoto Y, Ogura K, Okamura Y, Ishii H, Sakata I, Hakamada K, Miyaki S, Nakajima S and Minami S: Canine hemangiopericytoma treated by combination of surgical resection and photodynamic therapy with novel photosensitizer, PAD-S31. Jap J Vet Anesth Surg 36: 69-73,2005.

Osaki T, Takagi S, Hoshino Y, Okumura M, Kadosawa T and Fujinaga T: 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: 125-132,2009.

Peaston AE, Leach MW and Higgins RJ: Photodynamic therapy for nasal and aural squamous cell carcinoma in cats. J Am Vet Med Assoc 202: 1261-1265,1993.

Radzi R, Osaki T, Tsuka T, Imagawa T, Minami S and Okamoto Y: Morphological

study in B16F10 murine melanoma cells after photodynamic hyperthermal therapy with indocyanine green (ICG). J Vet Med Sci 74: 465-472,2012a.

Radzi R, Osaki T, Tsuka T, Imagawa T, Minami S, Nakayama Y and Okamoto Y: Photodynamic hyperthermal therapy with indocyanine green (ICG) induces apoptosis and cell cycle arrest in B16F10 murine melanoma cells. J Vet Med Sci 74: 545-551,2012b.

Reeds KB, Ridgway TD, Higbee RG and Lucroy MD: Non-coherent light for photodynamic therapy of superficial tumours in animals. Vet Comp Oncol 2: 157-163,2004.

Roberts WG, Klein MK, Loomis M, Weldy S and Berns MW: Photodynamic therapy of spontaneous cancers in felines, canines, and snakes with chloro-aluminum sulfonated phthalocyanine. J Natl Cancer Inst 83: 18-23,1991.

Tanabe S, Yamamaguchi M, Iijima M, Nakajima S, Sakata I, Miyaki S, Takemura T, Furuoka H, Kobayashi Y, Matsui T, Uzuka Y and Sarashina T: Fluorescence detection of a new photosensitizer, PAD-S31, in tumour tissues and its use as a photodynamic treatment for skin tumours in dogs and a cat: a preliminary report. Vet J 167: 286-293,2004.

Wike-Hooley JL, van den Berg AP, van der Zee J and Reinhold HS: Human

tumour pH and its variation. Eur J Cancer Clin Oncol 21: 785-791,1985.

Figures and Tables

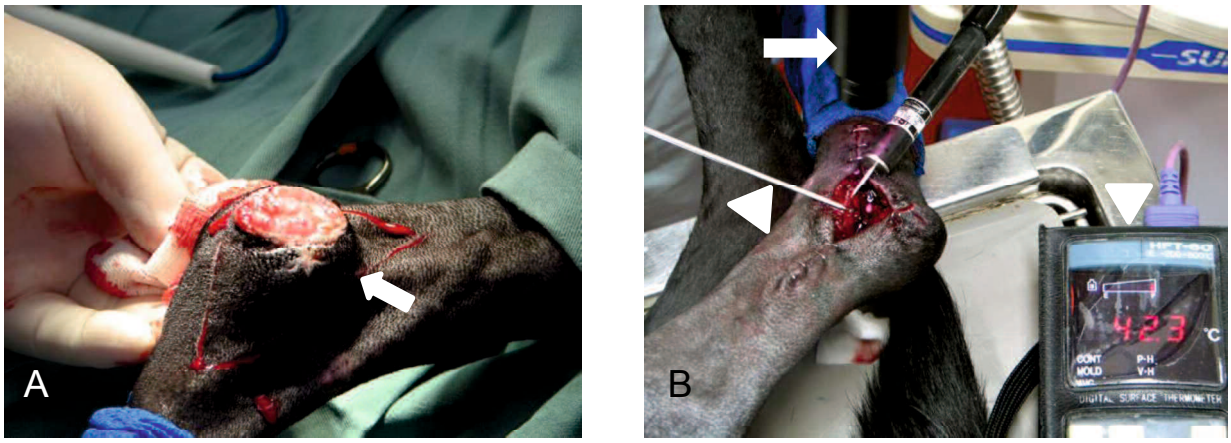

Fig. 1. (A) Skin incision (arrow) in case C07. (B) PHCT was performed after surgery. The arrow indicates the broadband light source while the arrowheads indicate the thermometer and thermometer sensor.

Table 1. Data for the clinical cases

Species	Case number	Breed	Age	Sex	BW (kg)	Type of tumor	Site	Size (cm)	TNM stage
Dogs	C01	Mongrel	10	♀	10.0	Malignant schwannoma	L. forlimb	3.0 × 3.0	T1N0M0
	C02	L. retriever	9	♀	29.0	Malignant schwannoma	R. hindlimb	1.9 × 3.1	T1N0M0
	C03	Miniature schunauzer	13	♂	7.9	Malignant schwannoma	L. axilla	6.0 × 5.0	T2N0M0
	C04	G. retriever	13	♀	22.7	Hemangiopericytoma	L. forlimb	5.0 × 8.0	T2N0M0 (recurrent case)
	C05	Welsh corgie	15	♂	11.0	Hemangiopericytoma	L. hindlimb	8.0 × 8.0	T2N0M0
	C06	French bulldog	11	♀	3.0	Liposarcoma	L. hindlimb	3.0 × 3.0	T1N0M0 (recurrent case)
	C07	L. retriever	7	♂	31.4	Fibrosarcoma	R. hindlimb	3.0 × 3.0	T1N0M0
	C08	G. retriever	10	♂	33.0	Undifferential soft tissue tumors	Perineum	3.0 × 3.0	T1N0M0
	C09	Cocker spaniel	10	♂	10.0	Undifferential soft tissue tumors	R. forlimb	8.0 × 8.0	T2N0M0
	C10	Mongrel	8	♂	10.0	Hemangiopericytoma	L. forlimb	5.0 × 8.0	T2N0M0
Cats	F01	DSH	11	♂	6.7	Malignant schwannoma	R. forlimb	2.0 × 2.0	T1N0M0
	F02	DSH	13	♀	3.5	Fibrosarcoma	Dorsal region	2.7 × 3.1	T1N0M0
	F03	DSH	12	♀	3.2	Fibrosarcoma	R. hindlimb	3.0 × 4.0	T1N0M0
	F04	American short-hair	4	♀	3.2	Fibrosarcoma	L. axilla	0.8 × 0.8	T1N0M0
	F05	DSH	11	♀	3.5	Undifferential soft tissue tumors	R. forlimb	1.5 × 1.0	T1N0M0
	F06	DSH	7	♂	5.0	Rhabdomyosarcoma	R. forlimb	2.0 × 3.0	T1N0M0

BW: body weight, TNM: T, size of the primary tumor; N, condition of the regional lymph nodes; M, absence/presence of distant metastasis.

Table 2. Summary of treatment outcomes

Species	Case number	Treatment time	Anti-cancer drug	DFT	ST	Update	Comments
Dogs	C01	7	CA	1041	1041	Alive	
	C02	3	CA	1185	1185	Alive	
	C03	12	BL	238	238	Alive	
	C04	8	No use	1774	1774	Alive	
	C05	8	CA, PA	767	767	Alive	
	C06	8	CA, PA	72	453	Alive	Tumor removal using ultrasonic device. Underwent amputation
	C07	4	CA	1901	1901	Alive	
	C08	11	CA	151	269	Deceased	Died due to tumor progression.
	C09	5	BL	162	225	Deceased	Tumor removal using ultrasonic device. Died due to cardiac failure.
	C10	9	CA	286	286	Alive	
Cats	F01	5	CA, PA	1173	1173	Alive	
	F02	17	BL, CA	1521	1521	Alive	No surgery
	F03	13	BL	1515	1515	Alive	
	F04	21	CA	82	1254	Alive	Tumor removal using ultrasonic device. Underwent amputation
	F05	15	CA, PA	175	383	Deceased	Died due to tumor progression.
	F06	6	BL, CA	20	1879	Deceased	Underwent amputation. Died due to unknown cause

DFT: disease-free time, ST: survival time, CA: carbopulatin, BL: bleomycin, PA: paclitaxel.

Conclusions

- PHT has an anticancer efficacy in a tumor-bearing mouse model .

- PHCT is expected to become a useful alternative to radiation therapy for superficial tumors such as STS in companion animals.

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