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ORIGINAL ARTICLE

Extracellular heat shock protein 70 levels in tumour-bearing dogs and cats treated with radiation therapy and hyperthermia

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Abstract

Hyperthermia is a form of a cancer treatment which is frequently applied in combination with radiotherapy (RT) to improve therapy responses and radiosensitivity. The mode of action of hyperthermia is multifactorial; the one hand by altering the amount of the blood circulation in the treated tissue, on the other hand by modulating molecular pathways involved in cell survival processes and immunogenic interactions. One of the most dominant proteins induced by hyperthermia is the major stress-inducible heat shock protein 70 (Hsp70). Hsp70 can be found in the blood either as a free-protein (free HSP70) derived from necrotic cells, or lipid-bound (liposomal Hsp70) when it is actively released in extracellular vesicles (EVs) by living cells. The aim of the study was to evaluate the levels of free and liposomal Hsp70 before and after treatment with RT alone or hyperthermia combined with radiotherapy (HTRT) in dogs and cats to evaluate therapy responses. Peripheral blood was collected from feline and canine patients before and at 2, 4, 6 and 24 h after treatment with RT or HTRT. Hsp70 enzyme-linked immunosorbent assays (ELISAs) were performed to determine the free and liposomal Hsp70 concentrations in the serum. The levels were analysed after the first fraction of radiation to study immediate effects and after all applied fractions to study cumulative effects. The levels of free and liposomal Hsp70 levels in the circulation were not affected by the first singular treatment and cumulative effects of RT in cats however, after finalizing all treatment cycles with HTRT free and liposomal Hsp70 levels significantly increased. In dogs, HTRT, but not treatment with RT alone, significantly affected liposomal Hsp70 levels during the first fraction. Free Hsp70 levels were significantly increased after RT, but not HTRT, during the first fraction in dogs. In dogs, on the other hand, RT alone resulted in a significant increase in liposomal Hsp70, but HTRT did not significantly affect the liposomal Hsp70 when cumulative effects were analysed. Free Hsp70 was significantly induced in dogs after both, RT and HTRT when cumulative effects were analysed. RT and HTRT

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treatments differentially affect the levels of free and liposomal Hsp70 in dogs and cats. Both forms of Hsp70 could potentially be further investigated as potential liquid biopsy markers to study responses to RT and HTRT treatment in companion animals.

KEYWORDS
cancer, canine, feline, liposomal Hsp70, radiotherapy, thermoradiotherapy

1 | INTRODUCTION

Solid, inoperable or recurrent tumours in dogs and cats represent a challenge in anticancer therapy. While radiotherapy (RT) remains an option for treating locally advanced disease, adequate tumour control often cannot be achieved due to intrinsic radioresistance of solid tumours. Combining RT with the treatment modality of mild or moderate (loco-regional) hyperthermia (HT), where tumour tissue is radiosensitized by heating to temperatures between 40 and 44°C, has been shown to increase the therapeutic potential in various tumours of humans and companion animals.[1–5] The mechanism of this sensitization is multifold, ranging from the cellular level by impairing DNA-double strand break repair, modification of cytoskeleton, membranes, cell metabolism etc. to the tissue and systemic effects such as modifications of the tumour microenvironment by impacting vascular support, or immunological parameters.[6,7] HT not only impairs DNA repair in the nucleus but also exerts its damaging activity to cytosolic proteins. It is mechanistically different and complementary to the manner by which ionizing radiation leads to cellular damage, thereby resulting in an additive tumoricidal effect. The radiosensitizing effect of HT on canine cancer cell lines strongly depends on the tumour cell type and hyperthermia settings.[8]

Among a multitude of other stress factors, hyperthermia induces the synthesis of heat shock proteins (HSPs) and their increased levels coincide with a temporary induced thermotolerance. This thermotolerance leads to an unwanted transient resistance to subsequent heating and is—at least in part—mediated by anti-apoptotic HSPs, such as Hsp70, the major stress-inducible member of the Hsp70 family.[6] In various cancers of humans and dogs baseline Hsp70 levels are upregulated, possibly conveying resistance to radiation as well as chemotherapeutic agents even in the absence of stress such as HT. Therefore, elevated levels of Hsp70 could be relevant in a predictive and prognostic setting[9–16]: In a tumour-bearing mouse model, Hsp70 plasma levels correlated positively with increasing tumour volume and the levels decreased significantly after a radiation-induced tumour regression.[17] Similarly, in human patients with hepatocellular and squamous cell carcinoma, non-small-cell lung and pancreatic cancer, serum levels of Hsp70 were elevated and in part reflected tumour burden, rendering serum Hsp70 as a potential biomarker for tumour size, disease stage and predictor of therapy response.[12,13,18]

Other types of cellular stress, either of physiological or pathophysiological nature can also upregulate Hsp70 in the cytosol of normal and tumour cells. In contrast to normal cells and due to a tumour-specific lipid composition which enables membrane anchorage, tumour cells also present Hsp70 on their cell surface as a tumour-specific biomarker.[19] Moreover, membrane Hsp70 positive tumour cells actively release Hsp70 in extracellular vesicles with biophysical characteristics of exosomes.[20] The amount of Hsp70 in the cytosol and on the cell surface increases upon anticancer treatment such as RT, HT or chemotherapy.[15] Serum levels of Hsp70 increased in patients with prostate cancer during and after fractionated radiation therapy.[21] Hence, transiently increased levels of Hsp70 on cellular surfaces and in the blood of tumour patients might coincide with the occurrence of resistance-promoting thermotolerance after HT predominantly by lipid-bound Hsp70 on the one hand, but also might be able to enhance immunostimulatory anti-cancer effects of HT on the other hand.[15,22] An optimal scheduling of a combined thermoradiotherapy could help to stimulate the anti-cancer immune effects of extracellular Hsp70 whilst avoiding thermotolerance. The major proportion of circulating Hsp70 originates from lipid-bound Hsp70 which is released predominantly by living tumour cells, whereas a smaller proportion of free Hsp70 is derived from dying tumour cells.[20,23] The lipid-associated conformation of Hsp70 impedes the binding of many commercially available Hsp70-specific ELISA kits and demands for specialized Hsp70 antibodies. The antibody cmHsp70.1 and cmHsp70.2 are capable to detect Hsp70 in the lipid conformation on the plasma membrane of metabolically active, viable tumour cells.[24,25] A recently developed Hsp70 ELISA reliably detects both, the free as well as lipid-bound Hsp70 in the blood of humans and this method could be extended to other species.[24,26,27]

The aim of the study was to analyse the time course of serum levels of both, free and lipid-bound Hsp70 protein after RT alone or combined thermoradiotherapy (HTRT) in tumour-bearing cats and dogs as a potential predictive marker for therapy response. A better understanding of the dynamics of these serum levels would help to avoid thermo-resistance, elucidate how representative either measurement (i.e. free and lipid-bound) is for the total amount of Hsp70 and provide insights into the therapy response in general.

2 | MATERIALS AND METHODS

The current study involved sampling and analysing of peripheral blood from companion animals. Blood was collected from feline and canine patients before and after treatment with (thermo)radiotherapy. Repeated sampling was performed to evaluate changes in Hsp70 expression in response to (thermo)radiotherapy over time. ELISAs were performed to determine extracellular Hsp70 concentrations in serum.

2.1 | Patients and treatment

Dogs and cats presented between December 2014 and March 2017 for treatment of malignant tumours. Each patient had a clinical work up (tumour staging) as is appropriate to the type of presenting disease. Written owner's consent was obtained for invasive sampling in this study.

Radiation was delivered with a 6 MV linear accelerator (Clinac iX, Varian, Palo Alto, USA) using either photons or electrons, depending on tumour size and location. Treatment planning was performed on the basis of CT for photon plans or by hand calculation for electron plans and was done by a board-certified radiation oncologist (CRB). The recommendations for specifying dose and volumes as proposed for veterinary medicine were adhered to as proposed in the corresponding literature.[28,29] The radiation protocols used (radiation only group) correspond to commonly used protocols for treatment in animals. Cats and dogs treated with HTRT underwent a 5 × 6 Gray (Gy) (total dose of 30 Gy) radiation therapy protocol, applied twice per week for sarcomas.[30] For oral melanoma, a total of 32 Gy was prescribed, delivered in 4 × 8 Gy, weekly.[31,32]

Hyperthermia was performed in three sessions once a week, in each case immediately prior to radiation therapy. Duration of hyperthermia was 45 min from the time that at least either one intra-tumoral temperature sensor achieved ≥41°C or 15 min of heating-up time had elapsed. The steady tumour temperature aimed for was at least 41°C and should not exceed 44°C throughout treatment. Superficial skin temperatures were supposed to remain below ≤42°C. Details about heating technique, treatment planning and the computation of the CEM43 thermal dose for these animals are described elsewhere.[33]

2.2 | Collection and preparation of serum samples

Blood was collected by a peripheral venous catheter used for introducing anaesthesia for (thermo)radiotherapy-treated animals. Repeated sampling was performed before, 2, 4, 6 and 24 h after treatment at different fractions. These time points were chosen within the anticipated duration of DNA repair processes initiated by radiotherapy and sample the period during which thermotolerance following heating is expected.[22,34] In particular, the last measurement (24 h after treatment) was obtained to check whether the Hsp70 levels would remain elevated for at least 24 h. For each withdrawal approximately 1–2 mL (depending on species and body weight) of blood were collected in a serum tube (serum clotting activator, Sarstedt, Nümbrecht, Germany) and kept at room temperature for 30 min until centrifugation at 1000 RPM for 15 min at 4°C. The serum was aliquoted into at least 2 Eppendorf tubes and stored at ≤ −20°C.

2.3 | Western blot analysis

The specificity of the Hsp70 antibodies cmHsp70.1 and cmHsp70.2 for cat cells was demonstrated by Western blot analysis using a lysate of cat colon tissue (FT-311, Zyagen, San Diego, CA, USA). Tissue lysates ready for immediate use in Western blotting were derived from an adult domestic colon cat tissue of a single donor. As an internal control, tumour cell lysates of two human glioblastoma cell lines (U87, LN18) and Hsp70 negative human fibroblasts were subjected to the SDS/PAGE. As shown on the original Western blot (Figure S1), lysates of human tumour cells and normal cells were subjected into four sequential lanes, the cat colon tissue lysate was subjected only into one lane. Briefly, the protein content of the lysate was determined by BCA protein assay kit (Pierce). After subjection of 80 µg protein of the feline colon lysate and 40 µg protein of the tumour cell lines in lysis (RIPA) buffer to an SDS-PAGE (10%), proteins were transferred on PVDF membranes (Biorad, Hercules, CA, USA). Blots were blocked in 5% skim milk for 1 h at room temperature and incubated with the cmHsp70.1 or cmHsp70.2 antibodies (multimmune GmbH, Munich, Germany). An antibody against ß-actin (Sigma-Aldrich) was used as a control. After three washing steps in 1× TBST a horseradish-peroxidase (HRP)-conjugated rabbit anti-mouse antibody (Promega, Fitchburg, WI, USA; dilution 1:10000) was used as a secondary antibody (1 h at room temperature) and immune complexes were detected by using the ECL detection system (GE Healthcare, Chicago, IL, USA) and imaged digitally (ChemiDoc MP/ChemiDOC XRS Touch Imaging System, Biorad). The molecular weight was determined by using a molecular weight marker.

2.4 | ELISA assays

Serum Hsp70 levels of companion animals were measured using the exoHsp70 ELISA (multimmune GmbH, Munich, Germany) for detecting free and liposomal Hsp70 and as a control, the commercial DuoSet IC Human/Mouse/Rat Total Hsp70 ELISA (R&D Systems, Minneapolis, MN, USA) for detecting free Hsp70. The exoHsp70 ELISA was conducted as follows: 96-well MaxiSorp Nunc-Immuno plates (Thermo, Rochester, NY, USA) were coated overnight with cmHsp70.2 monoclonal antibody (3.67 µg/plate; multimmune GmbH), diluted in sodium carbonate buffer (0.1 M sodium carbonate, 0.1 M sodium hydrogen carbonate, pH 9.6). After washing three times with phosphate buffered saline (PBS, Life Technologies, Carlsbad, CA, USA) with 0.05% Tween-20 (Calbiochem, Merck, Darmstadt, Germany) the wells were blocked with PBS/2% milk powder (Carl Roth, Karlsruhe, Germany) for 1.5 h at 27°C. Following another washing step, samples were diluted 1:5 for dog sera and 1:15 for cat sera in CrossDown Buffer (Applichem, Chicago, IL, USA) were added to the wells for 2 h at 27°C. After, another washing step the biotinylated mouse monoclonal antibody cmHsp70.1 (multimmune, Munich, Germany) diluted in PBS/2% milk powder was added for 2 h at 27°C. Finally, after another washing step, 0.2 µg/mL horseradish peroxidase-conjugated streptavidin (Pierce, Thermo, Rockford, IL, USA) in 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) was added for 1 h at 27°C. Binding was quantified by adding substrate reagent (R&D Systems, Minneapolis, MN, USA) for 30 min at 27°C and absorbance was read at 450 nm, corrected absorbance at 570 nm, using a Microplate Reader (BioTek, Winooski, VT, USA). An eight-point concentration standard curve of Hsp70 protein diluted in CrossDown Buffer (0–50 ng/mL) was included into each ELISA test. The blank was determined by measuring the absorbance of PBS diluted in CrossDown Buffer 1:5 and 1:15, respectively.

The exoHsp70 ELISA detects free and liposomal Hsp70 whereas the R&D Hsp70 ELISA only detects free Hsp70. By subtracting the amount of

TABLE 1 Characteristics of cats (upper panel) and dogs (lower panel) sorted by main tumour categories.[a]

Classification of tumours[a]	Breed	Sex	Tumour type Location	Treatment	Dose per fraction (Gray)	Maximum cumulative dose (Gray)	Average CEM43 per fraction (±SD, min)
1. Mesenchymal	European Shorthair	Fs	Fibrosarcoma Right scapula	HTRT	6	30	56.02 (±24.83)
	European Shorthair	Fs	Fibrosarcoma Right lip	HTRT	6	18	[b]
	European Shorthair	Mc	Extraskeletal osteosarcoma Right abdominal wall	HTRT	6	18	55.49 (±71.93)
	European Shorthair	Mc	Hemangiosarcoma Nasal planum and dorsum	RT	4.8	4.8	
	European Shorthair	Mc	Fibrosarcoma Right scapula	HTRT	6	18	22.40 (±5.99)
2. Epithelial	European Shorthair	Fs	Adenocarcinoma Left nasal cavity	RT	4.2	4.2	
	European Shorthair	Mc	Adenocarcinoma Right nasal cavity	RT	4.2	4.2	
	European Shorthair	Fs	Adenocarcinoma Right nasal cavity	RT	4.2	4.2	
	European Shorthair	Fs	Squamous cell carcinoma Sublingual	RT	10	10	
	European Shorthair	Fs	Squamous cell carcinoma Nasal planum	RT	4.8	4.8	
3. Discrete round cell	Siamese	Fs	Lymphoma Left nasal cavity	RT	3.8	34.2	
	European Shorthair	Fs	Lymphoma Nasal cavities	RT	4.2	4.2	
1. Mesenchymal	Belgian Shepherd (Malinois)	F	Soft tissue sarcoma Left elbow	RT	6	30	
	Mixed	Mc	Soft tissue sarcoma Left elbow	RT	3	48	
	Irish Red and White Setter[c]	F	Soft tissue sarcoma Right elbow	HTRT	3	33	26.7 (±13.86)
	English Bulldog	M	Hemangiosarcoma Left inguinal	HTRT	6	30	55.93 (±6.47)
	German Boxer	Fs	Soft tissue sarcoma Right carpus	HTRT	5	15	9.57 (±3.79)
	Labrador Retriever	Mc	Soft tissue sarcoma Right popliteal	HTRT	6	30	5.59 (±3.16)
	Irish Red and White Setter[c]	Fs	Soft tissue sarcoma Right elbow	HTRT	6	30	103.22 (±34.93)
	Mixed	Mc	Oral fibrosarcoma Left mandible	RT	6	24	
2. Epithelial	Pug	Fs	Adenocarcinoma Nasal cavities	RT	4.2	4.2	
	Golden Retriever	Fs	Adenocarcinoma Left nasal cavity	RT	4.2	25.2	
	Labrador Retriever	F	Squamous cell carcinoma Rostral maxilla	RT	3	24	
	Labrador Retriever	M	Carcinoma Thyroid gland	RT	3	24	

TABLE 1 (Continued)

Classification of tumours[a]	Breed	Sex	Tumour type Location	Treatment	Dose per fraction (Gray)	Maximum cumulative dose (Gray)	Average CEM43 per fraction (±SD, min)
3. Discrete round cell	German Boxer	M	Mast cell tumour Nasal dorsum	RT	3	51	
	Magyar Vizsla	M	Mast cell tumour Right nasal cavity	RT	6	30	
	Rottweiler	F	Oral malignant melanoma Left mandible	RT (fraction 1) HTRT (fraction 2, 3, 4)	8	24	63.54 (±44.65)
4. Brain tumours	Bullterrier	M	Glioma Left frontal lobe	RT	4	24	
	Golden Retriever	Mc	Meningioma Brain stem	RT	2.5	2.5	
	Staffordshire Bull Terrier	Mc	Peripheral nerve sheath tumour Right trigeminal nerve	RT	2.5	2.5	
	Mixed	Fs	Meningioma Right caudal fossa	RT	4	4	
	American Staffordshire Terrier	Mc	Choroid plexus tumour Left lateral ventricle	RT	4	24	
	Maltese	F	Meningioma Brain stem	RT	4	24	
	French Bulldog	Mc	Macroadenoma Pituitary gland	RT	4	24	

Abbreviations: CEM43: thermal dose expressed in cumulative equivalent minutes at 43°C; F, female; Fs, female spayed; HTRT, combined thermoradiotherapy; M, male; Mc, male castrated; RT, radiotherapy.
[a]Classification of tumours as: (1) mesenchymal neoplasm: tumours derived from mesenchymal and connective tissues (e.g., spindle-shaped and stellate cells, vascular endothelium, smooth muscle and stroma, striated muscle, bone). (2) Epithelial neoplasm: tumours derived from epithelial tissue; include tumours of epithelial surfaces (e.g., skin, respiratory, gastrointestinal, and urogenital tract, as well as tumours of glands and organs). (3) Discrete round cell neoplasm: tumours derived from haematopoietic origin, including neoplasms of mast cells, plasma cells, lymphocytes, and histiocytes. In malignant melanoma, the cells can adopt the appearance of epithelial, mesenchymal, or discrete round cell tumours. (4) Brain tumours: divided into primary and secondary. Primary brain tumours originating from brain parenchyma (e.g., glial cells and neurons, cells that line the interior and exterior brain surfaces [such as meningeal and ependymal] or cells from vascular structures (such as choroid plexus). Secondary brain tumours include metastatic neoplasia (e.g., mammary, pulmonary) and tumours that affect the brain by local extension (e.g., pituitary tumours, nerve sheath tumours, nasal and frontal sinus carcinoma). Definitive histopathological diagnosis was not available in these cases; radiological diagnosis was made based on MRI findings.
[b]No intratumoral temperature measurement available.
[c]Patient number 86 experienced a relapse and was treated again as 201.

free Hsp70 determined by the R&D Hsp70 ELISA form the values obtained by the exoHsp70 ELISA the amount of liposomal Hsp70 can be estimated.

2.5 | Statistical analysis

Statistical analysis was performed using the environment for statistical computing and graphics R (version 4.0.2, R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was assessed as follows: First, the fold change was calculated for the samples acquired 2, 4, 6 and 24 h after treatment, using the Hsp70 sample from before the treatment as a baseline. Employing fold change in this manner addresses the variability present in the baseline values. Any treatment effect yields a fold change different from 1, and the fold change is expected to be 1 in the absence of a treatment effect. For a given point in time, the samples were therefore tested for an expected value different from 1 using one-sample *t* tests. A *p* value below .05 was considered statistically significant and denoted with a star (*). Two stars were used for p-values below 0.01 (**) and three stars for be values below 0.001 (***) and four stars for values below 0.0001 (****). In case no Hsp70 measurement could be acquired in the baseline case, the baseline for the fold change calculation was missing and in consequence, the fold change for these cases is missing. Note that although the fold-change to a baseline of 0 ng/mL would mathematically be infinite, this potential issue did not arise since all (non-missing) measurements reported Hsp70 levels greater than 0 ng/mL. GraphPad Prism 8 (San Diego, CA, USA) was used to draw the figures.

3 | RESULTS

3.1 | Post-treatment Hsp70 serum levels

Sixteen dogs and eight cats were treated with RT alone and six dogs and four cats were treated with HTRT. Characteristics of the animals

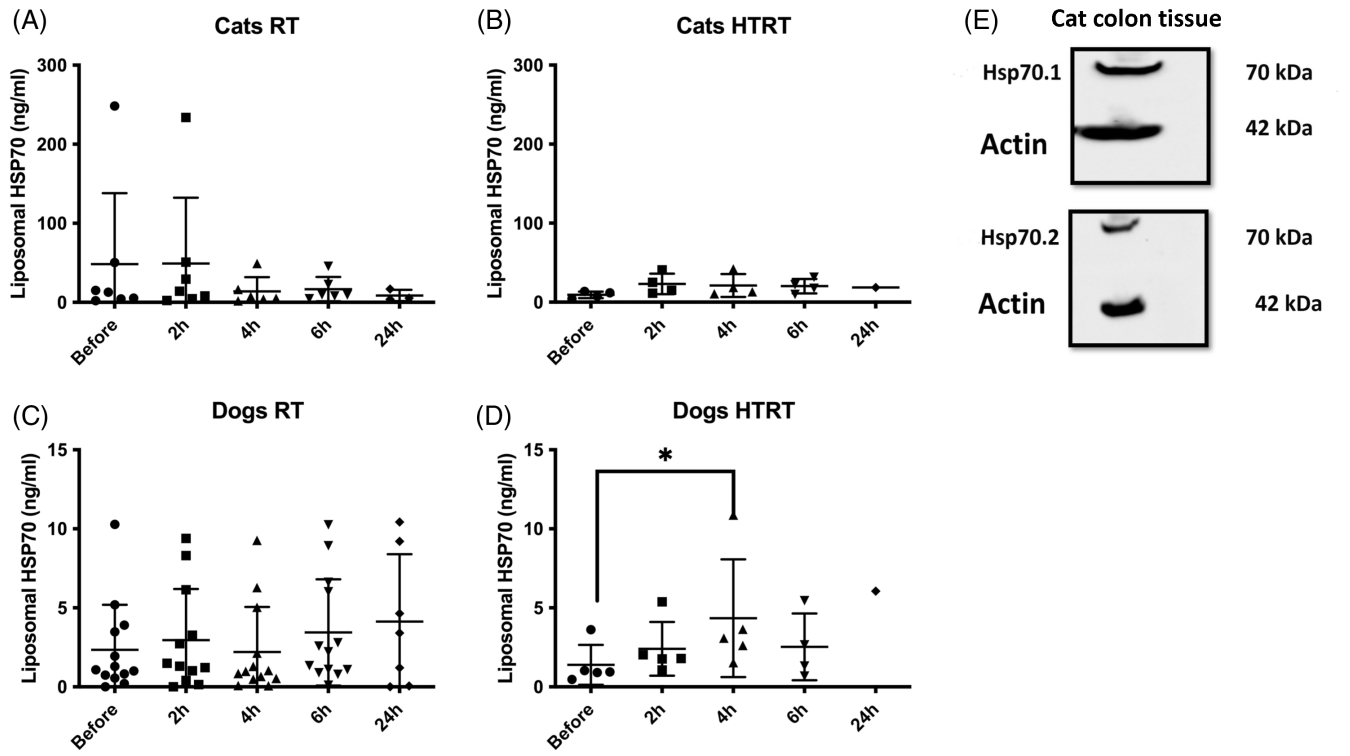

FIGURE 1 Levels of liposomal Hsp70 during first fraction of treatment (immediate effect). Levels of liposomal Hsp70 before and after treatment with radiation alone, radiotherapy (RT) (A) or with thermoradiotherapy, hyperthermia combined with radiotherapy (HTRT) (B) in cats. Levels of liposomal Hsp70 before and after treatment with radiation alone, RT (C) or with thermoradiotherapy, HTRT (D) in dogs. The specificity of the cmHsp70.1 and cmHsp70.2 antibodies for cat cells was shown by Western blot analysis using the lysate of a cat colon tissue (E). Upper graph, staining of the blot with cmHsp70.1 antibody (Hsp70.1, 70 kDa), lower graph staining of the blot with cmHsp70.2 antibody (Hsp70.2, 70 kDa), β-actin (50 kDa) was used as a loading control. The molecular weights (70, 50 kDa) on the right side of the Western blots are based on the bands of a molecular weight marker.

and their diseases are summarized in Table 1 along with the radiation dose per fraction and the thermal dose per fraction (CEM43). Applied fraction sizes varied between 2.5 and 10 Gy, while the total radiation doses ranged from 2.5 to 51 Gy depending on the animals' treatment protocol and on the number of fractions which were applied. In order to differentiate between immediate and cumulative effects of the different treatment schedules on the circulating Hsp70 levels, we analysed the levels of liposomal Hsp70 and free Hsp70 during the first fraction of treatment only (immediate effects) and the combined levels of all the fraction applied to every animal (cumulative effects). We compared the levels of Hsp70 2, 4, 6 and 24 h after treatment to the Hsp70 levels before treatment for every animal.

3.2 | Liposomal Hsp70 levels in cats and dogs during first fraction of treatment

The levels of liposomal Hsp70 were neither significantly affected by the treatment with RT alone nor by HTRT during the first fraction in cats (Figure 1A,B). In dogs, a significant increase of liposomal Hsp70 was observed 4 h after treatment with HTRT (Figure 1D), treatment with RT did not affect the levels of liposomal Hsp70 in dogs (Figure 1C).

The specificity of the Hsp70 antibodies cmHsp70.1 and cmHsp70.2 for cat cells which was used in the exoHsp70 ELISA was demonstrated by Western blotting. As shown in Figure 1E, a 72 kDa protein band was visible in the lysate of a cat colon tissue with both antibodies.

3.3 | Free Hsp70 levels in cats and dogs during first fraction of treatment

The levels of free Hsp70 were not affected by the treatment with RT and HTRT during the first radiation dose in cats (Figure 2A,B). In dogs, free Hsp70 levels were significantly increased at 2, 4 and 6 h after treatment with RT (Figure 2C). HTRT did not significantly affect free Hsp70 levels during first fraction in dogs (Figure 2D).

3.4 | Effect of RT/HTRT treatment during all the fractions applied (cumulative) on liposomal Hsp70 levels

When we analysed the time course of liposomal Hsp70 levels during all treatment fractions we observed a significant increase

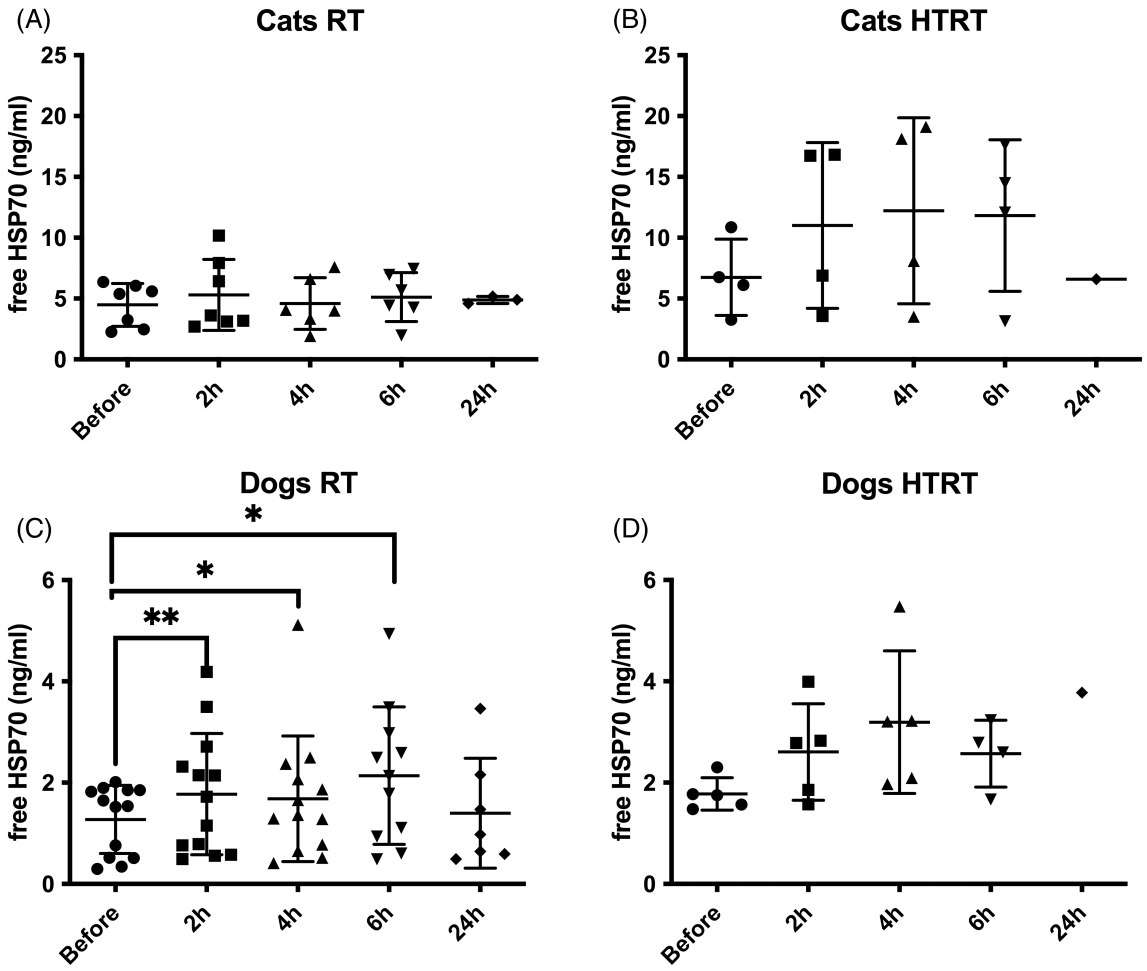

FIGURE 2 Levels of free Hsp70 during first fraction of treatment (immediate effect). Levels of free Hsp70 before and after treatment with radiation alone, radiotherapy (RT) (A) or with thermoradiotherapy, hyperthermia combined with radiotherapy (HTRT) (B) in cats. Levels of free Hsp70 before and after treatment with radiation alone, RT (C) or with thermoradiotherapy, HTRT (D) in dogs.

at the 4 h time point in response to HTRT in cats (Figure 3B), but no significant differences were observed in liposomal Hsp70 levels in response to RT alone (Figure 3A). In dogs, on the other hand, RT alone resulted in a significant increase in Hsp70 at 2 and 6 h after treatment (Figure 3C), but HTRT did not significantly affected the liposomal Hsp70 concentrations in dogs (Figure 3D).

3.5 | Effect of RT/HTRT treatment during all the fractions applied (cumulative) on free Hsp70 levels

Similarly to liposomal Hsp70, free Hsp70 levels were not affected by RT treatment in cats (Figure 4A). However, HTRT treatment significantly increased free Hsp70 levels at 4 and 6 h after treatment in cats (Figure 4B). In dogs, the RT treatment resulted in a significant induction of free Hsp70 at every time point (Figure 4C) and also HTRT resulted in a significant increase in free Hsp70 levels at 2, 4 and 6 h after treatment (Figure 4D).

4 | DISCUSSION AND CONCLUSION

Prior work has shown that the exoHsp70 ELISA is able to detect free as well as the lipid-bound Hsp70 in the blood (serum, plasma) of patients with cancer.[24] Due to a sequence homology of the Hsp70 antibody epitopes in different animals, serum Hsp70 values can also be determined in tumour-bearing companion animals.[26] In this study we investigated the Hsp70 levels in the circulation of dogs and cats undergoing anticancer treatments in the course of therapy to evaluate the kinetics of their up-regulation.

According to the literature, the exoHsp70 ELISA yields higher basal Hsp70 levels in healthy humans compared to the R&D ELISA.[24] The levels of free Hsp70 measured by the R&D ELISA ranged between 1 and maximal 20 ng/mL whereas the levels of liposomal and free Hsp70 ranged between 10 and 400 ng/mL. This reflects that most Hsp70 in the circulation is of liposomal origin. Recent study shows similar results in healthy dogs and cats[26,35] as determined with the compHsp70 ELISA which is based on the same Hsp70 antibodies and reagents like the exoHsp70 ELISA. These findings are in line with

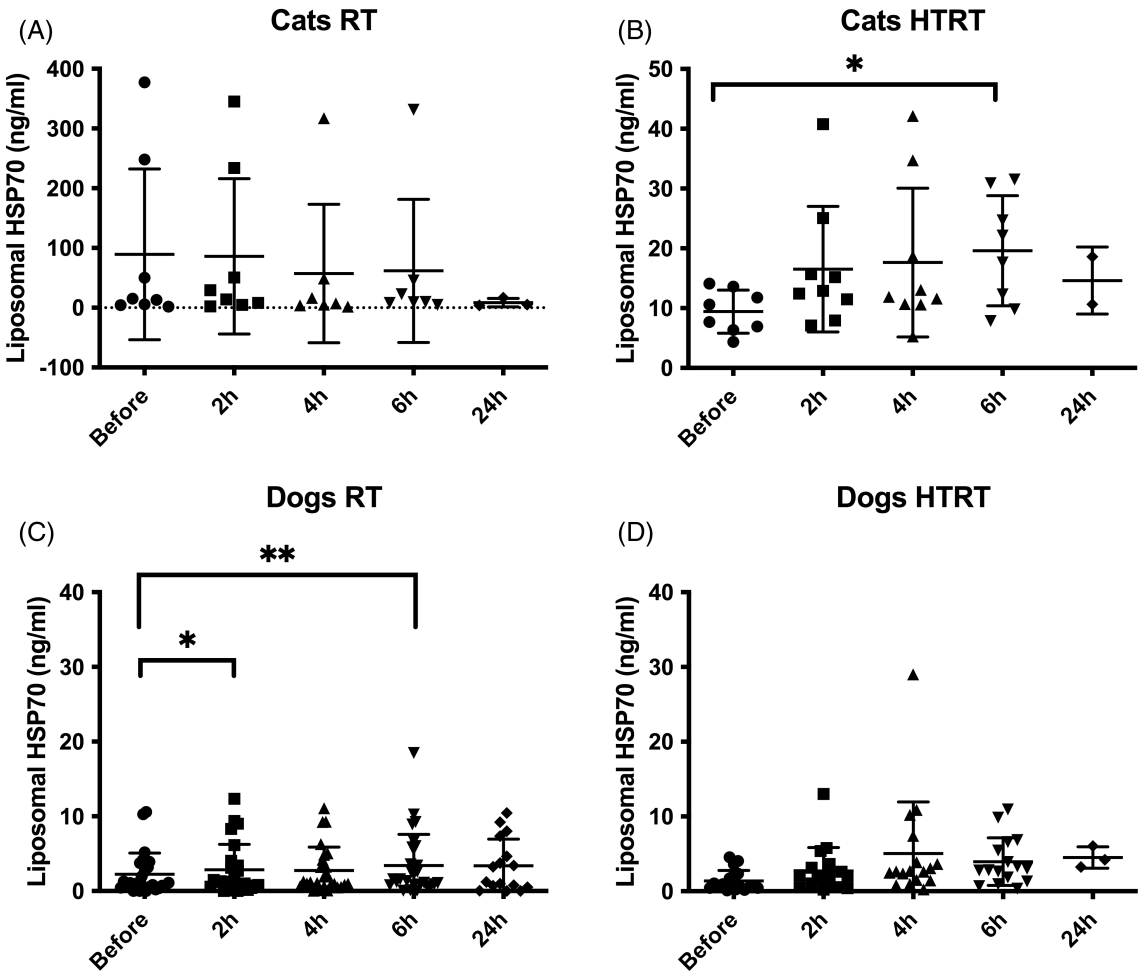

FIGURE 3 Levels of liposomal Hsp70 during all treatment fractions (cumulative effect). Levels of liposomal Hsp70 before and after treatment with radiation alone, radiotherapy (RT) (A) or with thermoradiotherapy, hyperthermia combined with radiotherapy (HTRT) (B) in cats. Levels of liposomal Hsp70 before and after treatment with radiation alone, RT (C) or with thermoradiotherapy, HTRT (D) in dogs.

data reported in the current study regarding tumour-bearing animals. The herein reported basal levels of serum Hsp70 indicate an inter-species difference with canine levels being lowest, and feline levels being higher than that of humans. The differences between species can possibly be explained with the different-sized compartments of neutrophil granulocytes in the blood of cats and dogs. Boyko et al. found an increased expression of stress-inducible proteins including HSPs in neutrophil granulocytes in humans.[36] In all species, the total blood neutrophil pool (TBNP) is divided into the circulating neutrophil pool (CNP) and the marginal neutrophil pool (MNP). While the CNP represents the quantitated neutrophil count derived in the routine WBC (white blood count), the MNP is a hidden population, which is associated to the vascular endothelium. The latter is known to be larger in cats (21.0×10^8/kg) than in dogs (4.8×10^8/kg). Stress situations lead to a massive shift of neutrophils into the CNP of cats, resulting in a pronounced leukocytosis.[37]

Elevated tissue and intracellular Hsp70 levels have been reported for various tumours in dogs.[38–41] Significantly elevated serum concentrations of Hsp70 were specifically identified in dogs with round cell tumours.[26] RT for localized prostate cancer in humans can increase circulating serum levels up to 3.5-fold.[21] Our observations, however, were somewhat inconsistent between the assays and between species: We observed an increase of free Hsp70 but not liposomal Hsp70 in response to RT in dogs (during first fraction, Figures 1, 2), but not in cats. This effect was even stronger when we analysed the levels of free Hsp70 during all fractions applied (Figures 3, 4), though this may also result from increased statistical power due to a larger sample size. Liposomal Hsp70 levels were also significantly increased after RT treatment in dogs, but not in cats when all fractions were analysed. We hence observed RT to influence the free Hsp70 levels in dogs with currently unknown implications. While such an increase can convey temporary therapy resistance on the one hand, it could also not only serve as a recognition structure for targeted therapies, but also play a role in eliciting anti-tumour immune responses on the other hand.[42] Moreover, Hsp70 might interact with number of other molecules, especially involved in immunological response and in that manner the combined effects of Hsp70 increase and

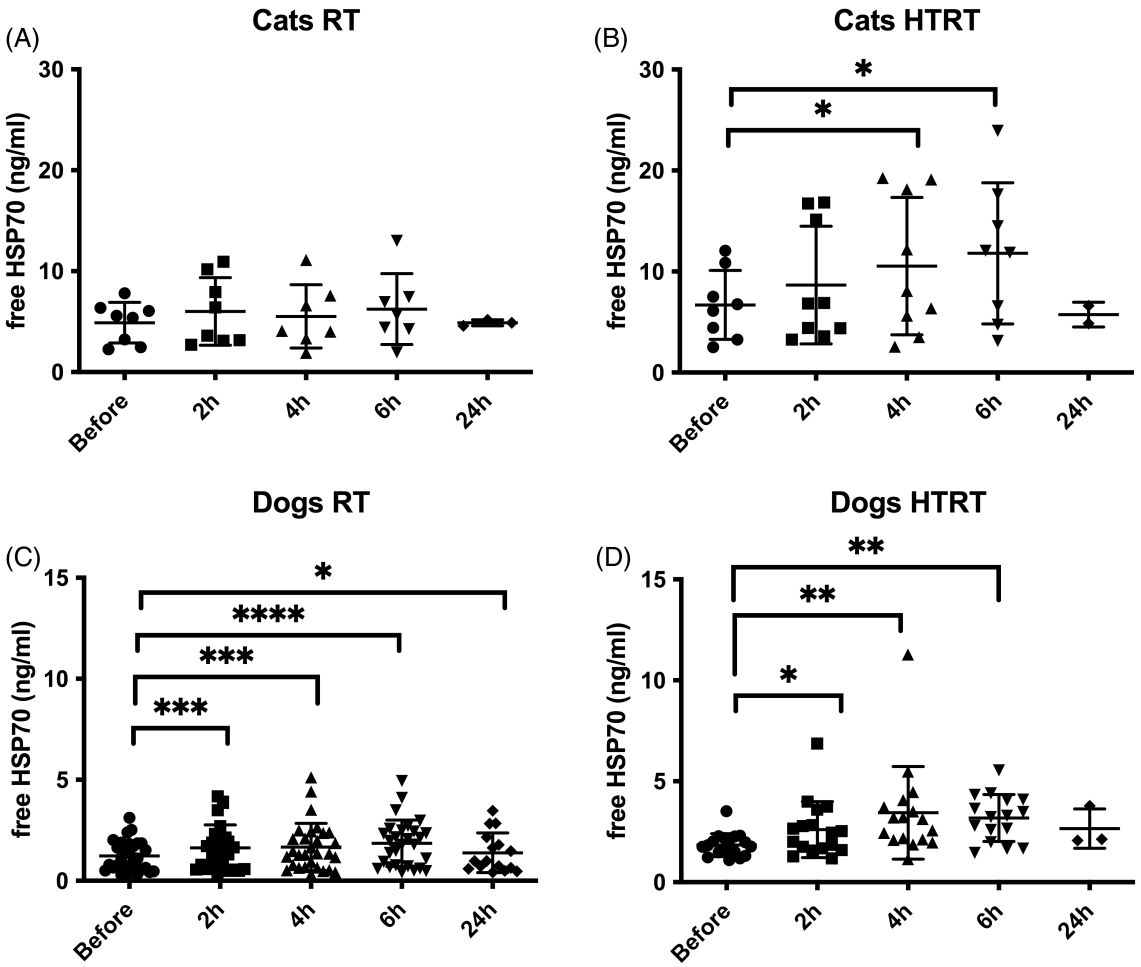

FIGURE 4 Levels of free Hsp70 during all treatment fractions (cumulative effect). Levels of free Hsp70 before and after treatment with radiation alone, radiotherapy (RT) (A) or with thermoradiotherapy, hyperthermia combined with radiotherapy (HTRT) (B) in cats. Levels of free Hsp70 before and after treatment with radiation alone, RT (C) or with thermoradiotherapy, HTRT (D) in dogs.

immunological modulation could affect tumour microenvironment.[15,17] Interestingly, cats seem to respond differently to RT than dogs. Alternatively, it could be that the treatment settings induce strong stress response in cats that masks the effects of RT.

For animals subject to HTRT treatments, we did not observe any correlation between the thermal dose (CEM43) and the peak-level of Hsp70 measured after the respective treatment. Thus, such a relationship may not exist, or may simply be too weak to be detected in this in vivo study, where many parameters cannot be controlled. We acknowledge the limitations imposed by the small number of patients, varies treatment protocols and resulting different fractions sizes, as well as the lack of samples at later time points in some patients treated with RT only. Moreover, different tumour types were included in the study design and therefore this could thus it influence Hsp70 serum levels. Additional study, where comparison between Hsp70 serum levels of patients with tumours of similar origin/histotypes would be more useful to evaluate changes in Hsp70 serum levels in response to RT or HTRT.

In conclusion, we showed that RT and HTRT treatments differentially affect the levels of free and liposomal Hsp70 in dogs and cats. Both forms of Hsp70 could potentially be further investigated as liquid biopsy markers of response to RT and HTRT treatment in companion animals, the R&D and exoHsp70 ELISAs allow for quantification.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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