Published in final edited form as:

Int J Hyperthermia. 2012 ; 28(5): 431–440. doi:10.3109/02656736.2012.689087.

Thermal Dose Fractionation Affects Tumor Physiologic Response

Donald E Thrall¹, Paolo Maccarini², Paul Stauffer², James MacFall², Marlene Hauck¹, Stacey Snyder¹, Beth Case¹, Keith Linder¹, Lan Lan², Linda McCall², and Mark W. Dewhirst²

¹College of Veterinary Medicine, North Carolina State University, Raleigh NC

²Duke University Medical Center, Durham NC

Abstract

Purpose—It is unknown whether a thermal dose should be administered using a few large fractions with higher temperatures or a larger number of fractions with lower temperatures. To evaluate this, we assessed the effect of administering the same total thermal dose, approximately 30 CEM43T₉₀, in 1 versus 3–4 fractions per week, over 5 weeks.

Materials and Methods—Canine sarcomas were randomized to receive one of the hyperthermia fractionation schemes along with fractionated radiotherapy. Tumor response was based on changes in tumor volume, oxygenation, water diffusion quantified using MRI, and a panel of histologic and immunohistochemical endpoints.

Results—There was a greater reduction in tumor volume and water diffusion at the end of therapy in tumors receiving 1 hyperthermia fraction per week. There was a weak but significant association between improved tumor oxygenation 24 hours after the first hyperthermia treatment and extent of volume reduction at the end of therapy. Finally, the direction of change of HIF 1 α and CA IX immunoreactivity after the first hyperthermia fraction was similar and there was an inverse relationship between temperature and the direction of change of CA IX. There were no significant changes in interstitial fluid pressure, VEGF, wVf, apoptosis or necrosis as a function of treatment group or temperature.

Conclusions—We did not identify an advantage to a 3–4/week hyperthermia prescription and response data pointed to a 1/week prescription being superior.

Introduction

After decades of investigation into the biology and physical principles of hyperthermia, randomized clinical trials have shown that hyperthermia led to improved local control of the following irradiated human tumors compared to radiation alone: melanoma (1), glioblastoma multiforme (2), breast cancer (3), head and neck tumors (4), pelvic tumors (5) and superficial tumors (6). Despite these successes, the application of radiation and hyperthermia for treatment of solid tumors has not been adopted widely. One reason for this is the lack of well-defined thermal dosimetry goals. Even today, many clinical hyperthermia treatments are undertaken without *a priori* establishment of a hyperthermia dose prescription. Hyperthermia may be the only cancer treatment modality where that philosophy is accepted. We know that the total cumulative thermal dose is related to duration of tumor control (6, 7) and it is also very likely that there are differences in tumor response depending on how the

Declarations of Interests

Supported by Grant P01 CA42745 from the National Institutes of Health

total cumulative thermal dose is fractionated. Until the fractionation effect is understood and thermal dose is defined and administered prospectively, clinical trials will be suboptimal and the cancer community's confidence in hyperthermia treatments will remain tentative.

Current day thermal dose prescriptions may be ill-defined because of the need for invasive thermometry to characterize the temperature distribution adequately. Wider application of noninvasive magnetic resonance (MR) thermometry would solve this problem. However, establishing a noninvasive MR thermometry program is time, labor and cost intensive. In the meantime, many hyperthermia treatments will continue to be assessed using invasive thermometry. This does not mean that thermal prescriptions should not be defined, as administering a prescribed thermal dose accurately based on limited thermometry is possible (7, 8), and, as already noted, thermal dose-response relationships based on invasive thermometry have been characterized in canine (7) and human (6) tumors. Nevertheless, thermal dose relationships will be even more evident when noninvasive thermometry is more widespread.

Even though thermal dose can be delivered as prescribed, the optimal time-dose prescription is not known. Both cytotoxicity and alteration of tumor oxygenation, likely the major mechanisms by which hyperthermia exerts its antitumor effect, are a function of the time-temperature relationship. For example, higher temperatures are more likely to lead to cytotoxicity (9, 10) but these could also have a deleterious effect on tumor vasculature (11), leading to hypoxia, radioresistance and phenotypic aggressiveness. On the other hand, lower temperatures would be less cytotoxic but might result in improved perfusion and oxygenation (11–13) with better drug delivery and a reduction in tumor hypoxia that leads to improved radiation response. It remains to be determined which of these strategies is optimal in the clinic. For example, should higher temperatures be achieved over a few fractions with the aim being to kill tumor cells, or should the temperature be reduced intentionally and a larger number of fractions administered to take advantage of improved tumor oxygenation? This is a critical question and the answer could have a profound impact on how hyperthermia should be administered in human treatments. Our goal was to assess this by characterizing the physiologic effect of two different fractionation schemes in spontaneous canine sarcomas where the same total cumulative thermal dose was administered.

Materials and Methods

This study was conducted in spontaneous canine soft tissue sarcomas, which include undifferentiated sarcoma, fibrosarcoma, myxosarcoma, hemangiopericytoma, liposarcoma and neurofibrosarcoma. Extremity tumors are most common but canine soft tissue sarcomas also arise on the head, oral cavity, and trunk. They are locally invasive into soft tissue, but rarely invade bone, except in the oral cavity. Canine soft tissue sarcomas have metastatic potential but metastasis does not occur with such a high frequency or sufficiently early to interfere with evaluation of the primary tumor. Tumors were measureable and had a volume between 10 cm³ and 400 cm³ and there was no evidence of local bone invasion in any dog.

Before treatment, dogs underwent quantification of tumor volume (using calipers), tumor oxygenation and interstitial fluid pressure, followed by tumor biopsy. Pretreatment diffusion-weighted MRI was performed in some dogs after the project had begun.

Multiple pretreatment tumor biopsies were acquired with a Tru-Cut biopsy needle and archived. The Oxford-Optronix Oxylite system was used for oxygen measurement¹. The

¹<http://www.oxford-optronix.com/ptissuemonitoring.htm>

flexible probes, 250–500 μ in diameter, were inserted through a pre-placed catheter and measurements were recorded in 5mm increments, or less in small tumors, as the probe was withdrawn. A minimum of 20 oxygen determinations was sampled from 4 tracks, as recommended by others (14). Tumor oxygenation was expressed as Mean pO₂, Median pO₂, % measured points $\geq$ 2.5 mmHg, % measured points $\geq$ 5 mmHg and % measured points $\geq$ 10 mmHg.

Interstitial fluid pressure was measured with a wick and needle system (15); four sites were measured at each time point. Interstitial fluid pressure was expressed as mean and median values.

Water diffusion was quantified using diffusion weighted imaging (DWI) with a 1.5 T magnet (Siemens Symphony, Siemens Medical Systems). Dogs were under general anesthesia, breathing isoflurane in 100% oxygen, for MR imaging. DWI was performed using a half Fourier acquisition, diffusion-weighted, single shot turbo spin echo (HASTE) sequence with b values of 0 and 500 sec/mm² (TR 3,000 msec, TE 132 msec, echo train length 256, 128 $\times$ 128 matrix, NEX=2 for b= 0 sec/mm² and NEX=6 for b=500 sec/mm², 4.0 mm slice thickness, 0.5 mm gap). Diffusion-weighted images were used to compute the apparent diffusion coefficient (ADC) using the magnet's proprietary software. T2-weighted spin echo images were also acquired and used for anatomic registration of the region of interest (ROI) used for quantification of the ADC. The ADC was quantified in every pixel in the tumor by drawing a ROI around the tumor in every T2-weighted image and then transferring the ROI to the corresponding ADC image. ADC was quantified using ImageJ (16)(<http://rsbweb.nih.gov/ij/>).

After pretreatment measurements, dogs received local hyperthermia on a subsequent day, under general anesthesia, using a stationary or scanning spiral 433 MHz superficial microwave applicator coupled to the skin with deionized water. We have used these applicators to heat canine tumors for many years. The maximum tumor volume in this study was 400 cm³, according to protocol design. We have heated tumors of this volume satisfactorily in prior studies with the equipment used herein. Additional details on the hyperthermia technique can be found elsewhere (7). Thermometry probes were translated automatically through preplaced intratumoral catheters by computer control to record temperatures at 0.5–1.0 cm increments across the tumor at 3–5 min intervals. Generally, temperature was monitored at approximately 20 discrete points from 2–4 catheters. Thermal dose descriptors were calculated according to standard thermal isoeffect dose relationships (10, 17) using software designed at Duke University. The target cumulative thermal dose was between 20–50 CEM43T₉₀, with the aim of being as close to 50 CEM43T₉₀ as possible at the end of the treatment course. The dose of 20–50 CEM43T₉₀ was the target because it was the dose associated with significant improvement in local tumor control compared to a dose of 2–5 CEM43T₉₀ in a prior canine soft tissue sarcoma study (7).

Heating a tumor with an external power source results in temperature heterogeneity that fluctuates with time, between treatments and between tumors. This heterogeneity creates problems in quantifying thermal dose for accurate reproduction of dose from treatment to treatment. When combined with radiation, the beneficial effects of hyperthermia appear related to the lower temperatures in the heterogeneous temperature distribution (18, 19). To quantify thermal dose from a heterogeneous temperature distribution, a unit descriptor, CEM43T₉₀ (Cumulative Equivalent Minutes that the T₉₀ temperature was equal to 43°C), was developed (20) that takes into account both treatment time and the low end of the temperature distribution since T₉₀ is the temperature reached or exceeded by 90% of measured temperature points during a HT fraction. In this way, CEM43T₉₀ represents a

volumetric thermal dose descriptor that relates the tissue temperature distribution and time of heating.

The target T_{90} required for study entry was 40.1°C. This is the same definition of tumor heatability that we used in previous canine trials (7). To evaluate whether this target T_{90} could be achieved, temperature descriptors were assessed in real time during the first hyperthermia fraction beginning immediately following power application. Once an instantaneous T_{90} of 40.1°C was observed, the tumor was randomized to receive either 1 hyperthermia fraction per week for 5 weeks or 3–4 hyperthermia fractions per week, also for 5 weeks (16–18 total). Monitoring tumor heatability and performing randomization during the first hyperthermia fraction eliminated the possibility of entering a non-heatable tumor (there were none) and then allowed for tailoring of the remainder of the first hyperthermia fraction according to whether 1 fraction/week or 3–4 fractions/week were desired.

The hypothesis was that the lower fraction-specific temperatures associated with administration of the total thermal dose in 16–18 fractions vs. 5, would lead to a favorable increase in tumor oxygenation in the 16–18 fraction group compared to 5 fractions and that the increased oxygenation would lead to decreased tumor volume and increased apoptosis and necrosis in irradiated tumors. Randomization was stratified by tumor volume (1–60 cm³ vs. 60–400 cm³) and pretreatment tumor oxygenation (median pO_2 <15mmHg vs 15mmHg) to balance these outcome variables between groups. The cutpoint for tumor volume of 60 cm³ and for median pO_2 of 15mmHg was chosen as these were the median values for these variables in a prior canine trial (7).

For the 5 hyperthermia fraction group, the target T_{90} was 40.5°C, with the duration of each fraction adjusted such that 4–10 CEM43 T_{90} were given per fraction to meet the total thermal dose goal of 20–50 CEM43 T_{90} . For the 16–18 hyperthermia fraction group, the target T_{90} remained 40.1°C, with the duration of each fraction adjusted such that ~1.5–2 CEM43 T_{90} were given per fraction to meet the total thermal dose goal of 20–50 CEM43 T_{90} . Dogs received concurrent daily fractionated radiation therapy of 25 fractions of 2.25Gy using 6MV photons for a total dose of 56.25Gy in five weeks. Hyperthermia was always administered prior to irradiation; the inter-treatment interval was approximately 1 hour.

Measurements of tumor volume, tumor oxygenation, and diffusion-weighted MRI were repeated 24 hours after the first hyperthermia fraction. Tumor biopsies were also repeated 24 hours after the 1st hyperthermia fraction and during the 2nd and 3rd weeks of treatment. Tumor oxygenation was measured 10–12 additional times during treatment and at the end of treatment. Interstitial pressure was measured midway through treatment and at the end of treatment. Diffusion-weighted MRI and tumor biopsies were repeated at the end of treatment.

Tumor biopsies were assessed quantitatively for apoptosis, necrosis, microvascular density (von Willebrand factor, wVf), vascular endothelial growth factor (VEGF), hypoxia inducible factor 1 α (HIF1 α and carbonic anhydrase IX (CA IX). The initial tumor biopsy was also assessed for tumor grade using the number of mitotic figures per high field as the measure.

For all immunohistochemistry, tru-cut biopsy samples were paraffin embedded and cut into 5 μ m sections that were deparaffinized and rehydrated using Citri-Solve and graded ethanol. Antigen retrieval was performed on all samples using 10 mM sodium citrate buffer at pH 6.0 heated to 95°C for 20 minutes. All samples were blocked against secondary antibody background with 10% normal donkey serum in PBS for 1 hour and all sections were incubated overnight at 4°C with primary antibody. Endogenous peroxidase was quenched with 3% H₂O₂ for 30 minutes at room temperature following primary antibody incubation. Following secondary antibody incubation all slides had avadin-biotin complex (Vector Labs

ABC Elite avidin-biotin linker kit, # PK-6100) applied for 30 minutes at room temperature. Lastly, DAB substrate (Vector Labs, # SK-4100) was applied and sections were incubated for 5 minutes. Slides were counterstained with hematoxylin for 30 seconds, dehydrated and mounted.

VEGF: sections were incubated overnight at 4°C with R & D systems ab # MAB 1603, at a concentration of 25 ug/mL (1:20 dilution). The secondary antibody, biotinylated donkey anti-mouse (Jackson Immuno #715-065-150) at a concentration of 1:2000 was applied and incubated for 1 hour at room temperature.

CA IX: sections were incubated overnight at 4°C with Abcam # ab15086, at a concentration of 2 ug/mL (1:500 dilution). The secondary antibody, biotinylated donkey anti-rabbit (Jackson Immuno #715-065-152) at a concentration of 1:2000 was applied and incubated for 1 hour at room temperature.

vWF: sections were incubated overnight at 4°C with Diapharma # SACWF-IG, at a concentration of 2.5 ug/mL (1:2000 dilution). The secondary antibody, biotinylated donkey anti-sheep (Jackson Immuno #715-065-003) at a concentration of 1:2000 was applied and incubated for 1 hour at room temperature.

HIF1 α : sections were incubated overnight at 4°C with Affinity bioreagents #PA1-16601 at a concentration of 2.5 ug/mL (1:100 dilution). The secondary antibody, biotinylated donkey anti-rabbit (Jackson Immuno #715-065-152) at a concentration of 1:2000 was applied and incubated for 1 hour at room temperature.

Apoptosis, based on Activated Caspase 3: sections were incubated overnight at 4°C with R&D systems #AF835, at a concentration of 0.5 ug/mL (1:1000 dilution). The secondary antibody, biotinylated donkey anti-rabbit (Jackson Immuno #715-065-152) at a concentration of 1:2000 was applied and incubated for 1 hour at room temperature.

All sections were anonymized and randomized using a random number generator available at www.random.org. Values were recorded and then slides were un-anonymized.

Assessment Criteria for HIF1 α , CAIX, and VEGF

To be considered assessable by immunohistochemistry, tumor sections needed to contain viable tissue of at least one 40X field of view. Any necrotic areas were not assessed with this parameter. The tumor biopsies that contained multiple pieces of tumor had each piece scored individually and those scores averaged. The percentage of area stained and the intensity of the staining were both scored at 10X. Intensity was classified as a 0, 1, 2, or 3. Percentage area was classified as none (0), 1–25% (1), 26–50% (2), 51–75% (3), or 76% (4) or greater. A final immunohistochemistry score was determined by multiplying the intensity by the percent area staining.

Assessment Criteria for vWF (Microvessel Density)

Tumor sections were assessed with three 40X fields. Any necrotic areas were not assessed with this parameter. Cells that stained positive were counted and the total number of cells contained in three hotspots was recorded.

Assessment Criteria for Caspase 3 (Apoptosis)

Tumor sections were assessed with five 100X fields. Any necrotic areas were not assessed with this parameter. Cells that stained positive were counted and the total number of cells contained in the five hotspots was recorded.

The primary response endpoints were necrosis and apoptosis in the tumor as assessed histologically in the tumor biopsies, and % tumor volume change at the end of treatment. The effect of temperature and fractionation on water diffusion was also assessed.

Statistical Methods

Descriptive statistics were used to summarize the baseline tumor information and thermal parameters, tumor oxygenation, interstitial fluid pressure and immunohistochemistry scores. The Wilcoxon rank-sum test or two sample test was used to study the treatment difference in continuous variables. The chi-square test was applied to examine differences of categorical endpoints between two treatment groups. Fisher's exact test was conducted to study if there was a nonrandom association between the categorical endpoints and the treatment group. Pearson's product moment correlation coefficients and p values to test the significance of correlation were calculated to assess the correlation between two variables

Results

Thirty-seven dogs were entered; 21 were randomized to one hyperthermia fraction per week and 16 to 3–4 hyperthermia fractions per week. Twenty-nine of the 37 dogs had diffusion-weighted MRI.

Most tumors in each treatment group were extremity tumors (Table 1). There were no significant differences in any of the following pretreatment variables between treatment groups: tumor volume (Table 1), tumor oxygenation, interstitial fluid pressure, VEGF, CAIX, HIF, wVf, apoptosis and necrosis. There were more high grade tumors in the 3–4 fraction per week group (Table 1).

Individual hyperthermia treatments were longer in the 3–4 fraction per week group and intratumoral temperatures at each fraction were lower, compared with the 1 fraction per week group (Table 2). This was intentional and occurred because of adjustment of the T_{90} and treatment time to give between 4–10 CEM43 T_{90} per fraction in the 1 fraction per week group, and between 1.5–2 CEM43 T_{90} per fraction in the 3–4 fraction per week group so that the total prescribed dose of 20–50 CEM43 T_{90} would be met in both groups despite the large difference in the number of hyperthermia fractions. The total cumulative thermal dose, quantified as CEM43 T_{90} was statistically different between treatment groups but was within the target range (20–50 CEM43 T_{90}). Although statistically different, it is doubtful that the small difference in total CEM43 T_{90} values between groups was clinically significant.

The main finding of this fractionation comparison was a statistically greater reduction in tumor volume at the end of treatment in the 1 fraction per week group compared to the 3–4 fraction per week group ($p = 0.0022$) (Figure 1). Additionally, but without respect to treatment group, tumor oxygenation tended to decrease 24 hours after the first hyperthermia fraction (Table 3). However, there was a weak (correlation coefficients ~ 0.4) association between an improvement in oxygenation at 24 hours and reduction in tumor volume at the end of treatment, whether tumor oxygenation was based on median pO_2 ($p=0.0146$), % measured O_2 points ≥ 2.5 mmHg ($p=0.0138$) (Figure 2) or % measured O_2 points ≥ 5 mmHg ($p=0.0286$). A similar relationship was also found for changes in oxygenation between 7 and 15 days relative to the pretreatment value (data not shown as relationship is similar to that for 24 hours after treatment).

At the end of treatment, the lower end of the range of ADC values decreased in the 1 fraction per week group in comparison to a slight increase in the lower end values in the 3–4 fraction per week group (Figure 3). In other words, if one looks at the change in water diffusion in an individual tumor in the 1 fraction per week group, regions where water

diffusion was more restricted before treatment were characterized by greater decreases in water diffusion following treatment than regions where water diffusion was less restricted before treatment (Figure 4).

The direction of change of HIF1 α and CA IX immunoreactivity 24 hours after the first hyperthermia fraction were related directly. Of 19 dogs with a reduction in CA IX immunoreactivity at 24 hours, 17 (89%) also had reduction in HIF1 α immunoreactivity. Also, of 19 other dogs with an increase in CA IX immunoreactivity at 24 hours, 14 (74%) also had an increase in HIF1 α immunoreactivity ($p < 0.0001$, Fisher's exact test). The direction of change of CA IX was also related to thermal dose administered during the first hyperthermia fraction. Median CEM43T₉₀ for the first hyperthermia fraction in dogs with a decrease in CA IX at 24 hours was 4.6 min vs. 1.9 min in dogs with an increase in CA IX at 24 hours.

There were no treatment or temperature effects on changes in interstitial fluid pressure, VEGF, wVf, apoptosis or necrosis (data not shown).

Discussion

Based on percent tumor volume change at the end of treatment, administering a prescribed hyperthermia dose using one fraction per week was more effective than when administering it in 3–4 fractions per week (Figure 1). As the temperatures were higher at each fraction in the one fraction per week group, this greater volume reduction may have been due to greater cytotoxicity at each fraction. Alternatively, thermotolerance developing in the 3–4 fraction per week group may have diminished the efficacy of that prescription, and/or temperatures might not have been high enough to cause clinically significant cytotoxicity.

Percent change in tumor volume is not the most robust endpoint to use to assess treatment efficacy in a solid tumor. The time to an event, such as local recurrence, would have been superior but survival analyses are difficult to implement in a canine tumor model due to the large number of subjects required to detect a clinically significant difference between treatment groups, unless the difference is very large. Although we previously completed a time-to-local-control analysis in a canine sarcoma model, the on-study time was approximately 7 years (7); this is too long to complete exploratory trials. Additionally, using only radiation and hyperthermia to treat canine soft tissue sarcomas is no longer acceptable clinically due to the proven value of surgery for this tumor type. Nevertheless, the use of tumor volume change as a surrogate for local control has been documented as a valid endpoint (21, 22). Importantly, a volume reduction of >50% for human sarcomas is highly predictive of a good pathologic response (22). Approximately 75% of the canine sarcomas in this trial treated with one hyperthermia fraction per week had a volume reduction of at least 50% compared to very few sarcomas receiving 3–4 hyperthermia fractions per week, as seen in Figure 1. Thus, applying the guidelines of Roberge (22), most sarcomas receiving 1 fraction per week would be expected to have a good pathologic response compared to few to no sarcomas treated with 3–4 fractions per week. This greater effect of the one fraction per week on volume reduction is an important finding and one that should be considered when future clinical trials are designed.

We hypothesized that administration of a defined thermal dose in 3–4 fractions per week would lead to improved tumor oxygenation but there was no treatment group effect on changes in tumor oxygenation, based on invasive probe measurements. Invasive oxygen measurements have been considered to be the gold standard in many clinical trials where tumor oxygenation was being quantified and results have led to the identification of important clinical principles. Regardless, invasive measurements are subject to sampling

error and may also perturb perfusion due to the invasiveness of the procedure, especially when conducted longitudinally over 5 weeks, as herein. Also, in human trials, serial invasive measures are not likely to be feasible due to patient comfort and compliance issues. To solve these problems, noninvasive functional imaging of oxygenation using PET, with radiopharmaceuticals such as ^{18}F -EF5 (23) or ^{62}Cu -ATSM (24) would be better tolerated and will also allow quantification of tumor oxygenation volumetrically, such that it can be compared on a 3D voxel basis to other parameters measured volumetrically, such as perfusion and temperature.

Although we did not find a significant effect of treatment group on the change in tumor oxygenation, we did find a significant association between improvement in tumor oxygenation at 24 hours after the first hyperthermia fraction, and also improvement in oxygenation between 7 and 15 days (data not shown), and percent reduction in tumor volume at the end of treatment (Figure 2). This is logical in that tumors with improved oxygenation would be expected to be more responsive to radiation. A significant treatment group effect may not have been found due to the variation in oxygenation changes within each group and the association only became apparent when looking specifically at individual oxygen parameters. This result suggests that quantification of the change in tumor oxygenation 24 hours after the first hyperthermia fraction may be a reliable endpoint to use to predict the success of a fractionated hyperthermia prescription when combined with radiation, regardless of the specific hyperthermia fractionation details.

Developed originally as a way to characterize acute thrombotic brain infarction, diffusion weighted imaging is also receiving attention as a predictor of tumor response (25). As water diffusion in tumors is restricted by cellular membranes and macromolecular structures, cytotoxic treatment can lead to loss of cell membrane integrity, which can be detected as an increase in water diffusion values for the tumor. This will be reflected as an increase in the apparent diffusion coefficient (ADC) of water in diffusion weighted imaging (25). The assessment of the ADC is best done at the percentile level rather than simply looking at median or mean values because changes in the distribution pattern can occur without a change in the mean value (26). This is similar in concept to assessing tumor temperature percentiles, i.e. the T90, rather than the median or mean. We found that the lower percentiles of the ADC values were decreased in the 1 fraction per week group at the end of treatment (~5 weeks) in comparison to a slight increase in the lower end percentiles of the ADC values in the 3–4 fraction per week group (Figure 3). In other words, in tumors treated with one fraction per week (higher temperatures per fraction), regions where water diffusion was more restricted before treatment were characterized by greater decreases in water diffusion following treatment than regions where water diffusion was less restricted before treatment (Figure 4). This is an interesting observation, but based on a greater tumor volume decrease in dogs receiving 1 hyperthermia fraction per week, the finding of reduced water diffusion goes against the conventional interpretation of the predictive value of the ADC where favorable tumor responses are usually associated with increases in the ADC. What specifically caused the greater reduction in water diffusion in tumors treated with fewer fractions of higher temperatures is unknown. With treatments such as radiation therapy or chemotherapy, an increase in the ADC is generally associated with greater cytotoxicity as a result of cell killing leading to less diffusion restriction. We observed the opposite; a reduction in the ADC at the end of therapy in tumors treated with more effective hyperthermia fractionation (1/week) in terms of observed volume reduction. However, there is very little known about the relationship between the direction of change or temporal kinetics of change of the ADC and tumor response in hyperthermia trials, or the effect of tissue geometry on the value of the ADC. In uterine fibroids treated with radiofrequency ablation, the response of the ADC parameters was variable, increasing in some tumors and decreasing in others, with no predictive power (27). In normal canine prostate treated with

radiofrequency ablation, the ADC value decreased immediately after treatment but increased subsequently as prostate recovered (28). Our finding of greater restriction in water diffusion at the end of treatment, in tumors with reduced volume, may be related to a condensation of stroma, producing a water restriction barrier, rather than to increased cellularity as might be concluded from application of conventional thinking regarding the change in the ADC value vs. cellularity. It is important to re-emphasize that, in addition to pretreatment, we applied diffusion weighted MRI to quantify ADC at 24 hours after the first hyperthermia fraction and at the end of therapy, approximately 5 weeks later. These times are probably too soon and too late, respectively, to quantify water diffusion as a reflection of effectiveness of tumor therapy. Further work is needed with diffusion weighted imaging being performed more often and closer to the beginning of therapy but longer than 24 hours after the first fraction, before significant volume change from the radiation/hyperthermia becomes widespread.

Unfortunately, there were more high grade tumors in the 3–4 fraction per week group, which may have contributed to the smaller treatment effect, in terms of tumor volume change, in this group (Figure 1). We did not stratify randomization by tumor grade as there is a limit to the number of stratification variables that can be employed in studies where the overall number of subjects is relatively small. We thought it more important to balance the primary study endpoint variables, i.e. tumor volume and tumor oxygenation. Regardless, there was no association between tumor volume change at the end of therapy and tumor grade. Intermediate/low grade tumors underwent 52.4% volume reduction compared to 37.8% volume reduction in high grade tumors ($p=0.11$, ANOVA), suggesting that tumor grade was not a highly influential variable. Also, one might expect a more dramatic volume reduction in high grade tumors due to the higher mitotic index, leading to greater responsiveness, as has been observed in chemotherapy trials (29). In that scenario, having more high grade tumors in a group might actually inflate the treatment effect. However, it is just not possible to know how the disparity of tumor grade affected the results. Regardless, the available results supported 1 hyperthermia fraction per week being more effective in terms of tumor volume reduction compared to 3–4 hyperthermia fractions per week, when a constant total thermal dose was given. In future trials, balancing tumor grade between groups should be considered.

Finally, we observed a temperature dependent decrease in CA IX immunoreactivity, and a direct association between changes in CA IX and HIF1 α immunoreactivity. The expected association between hyperthermia and the directional change in HIF1 α immunoreactivity has not been characterized completely. An inverse association between temperature and HIF1 α immunoreactivity was documented in macrophages (30), but a direct association between temperature and HIF1 α immunoreactivity observed in tumor cells (31, 32) and murine tumors (33). As such, we would have expected a direct association between temperature and either HIF1 α immunoreactivity or one of its downstream products, but we observed a decrease. The reason for this unexpected result is not known. This may be a temperature dependent effect, which has not been characterized completely, especially in the situation of highly heterogeneous temperatures, as occurred in these canine tumors. Finally, it might have been expected to see increased VEGF immunoreactivity as a consequence of the upregulation of HIF 1 α . We did not observe this and this may be due to sampling error, which undoubtedly affected the quantification of all histologic parameters, again pointing to the value of noninvasive volumetric determination of tumor parameters in future studies where tumor physiology is being assessed.

We found no significant change in interstitial fluid pressure, VEGF, wVf, apoptosis or necrosis. This should not be taken as evidence that these endpoints are not important in

terms of tumor response to hyperthermia. Perhaps the sensitivity of our sampling and quantification methods was not adequate to detect the changes that occurred.

Conclusion

In conclusion, based on changes in tumor volume, our evaluation of hyperthermia fractionation in canine sarcomas suggests that administration of a large number of relatively low-temperature hyperthermia fractions on a multiple fraction per week schedule is not associated with improved tumor response. We found greater tumor volume reduction in tumors treated with one fraction per week. Importantly, we are not implying that one fraction per week is optimal, only that 3–4 fractions of relatively low-temperature administration does not seem to confer a benefit. The value of administering a large number of hyperthermia fractions has been debated for years, with the suggestion that the lower temperatures would lead to greater increases in tumor oxygenation. Although this study was undoubtedly influenced by the invasive methods used to characterize the tumor, and possibly by the difference in tumor grade between groups, we found no advantage of a highly fractionated prescription. In fact, there was evidence that a coarsely fractionated prescription of one fraction per week was superior. This needs to be tested in a spontaneous tumor where the physiologic endpoints are quantified volumetrically and not invasively as herein, and all influential variables are balanced between groups by means of stratification, or counteracted by a large sample size. Only through further prospective investigation where thermal dose is defined a priori and administered strictly according to protocol will optimum hyperthermia fractionation be defined. Defining optimal hyperthermia fractionation will likely be paramount in re-establishing the value of hyperthermia for cancer treatment in the United States.

References

1. Overgaard J, Gonzalez Gonzalez D, Hulshof MC, Arcangeli G, Dahl O, Mella O, et al. Randomised trial of hyperthermia as adjuvant to radiotherapy for recurrent or metastatic malignant melanoma. *European Society for Hyperthermic Oncology Lancet*. 1995; 345(8949):540–3.
2. Sneed PK, Stauffer PR, McDermott MW, Diederich CJ, Lamborn KR, Prados MD, et al. Survival benefit of hyperthermia in a prospective randomized trial of brachytherapy boost +/- hyperthermia for glioblastoma multiforme. *Int J Radiat Oncol Biol Phys*. 1998; 40(2):287–95. [PubMed: 9457811]
3. Vernon CC, Hand JW, Field SB, Machin D, Whaley JB, van der Zee J, et al. Radiotherapy with or without hyperthermia in the treatment of superficial localized breast cancer: results from five randomized controlled trials. *International Collaborative Hyperthermia Group. Int J Radiat Oncol Biol Phys*. 1996; 35(4):731–44. [PubMed: 8690639]
4. Valdagni R, Amichetti M. Report of long-term follow-up in a randomized trial comparing radiation therapy and radiation therapy plus hyperthermia to metastatic lymph nodes in stage IV head and neck patients. *Int J Radiat Oncol Biol Phys*. 1994; 28(1):163–9. [PubMed: 8270437]
5. van der Zee J, Gonzalez Gonzalez D, van Rhoon GC, van Dijk JD, van Putten WL, Hart AA. Comparison of radiotherapy alone with radiotherapy plus hyperthermia in locally advanced pelvic tumours: a prospective, randomised, multicentre trial. *Dutch Deep Hyperthermia Group. Lancet*. 2000; 355(9210):1119–25. [PubMed: 10791373]
6. Jones EL, Oleson JR, Prosnitz LR, Samulski TV, Vujaskovic Z, Yu D, et al. Randomized trial of hyperthermia and radiation for superficial tumors. *J Clin Oncol*. 2005; 23(13):3079–85. [PubMed: 15860867]
7. Thrall DE, LaRue SM, Yu D, Samulski T, Sanders L, Case B, et al. Thermal dose is related to duration of local control in canine sarcomas treated with thermoradiotherapy. *Clin Cancer Res*. 2005; 11(14):5206–14. [PubMed: 16033838]

8. Thrall DE, Rosner GL, Azuma C, Larue SM, Case BC, Samulski T, et al. Using units of CEM 43 degrees C T90, local hyperthermia thermal dose can be delivered as prescribed. *Int J Hyperthermia*. 2000; 16(5):415–28. [PubMed: 11001575]
9. Dewey WC, Hopwood LE, Sapareto SA, Gerweck LE. Cellular responses to combinations of hyperthermia and radiation. *Radiology*. 1977; 123(2):463–74. [PubMed: 322205]
10. Dewey WC. Arrhenius relationships from the molecule and cell to the clinic. *Int J Hyperthermia*. 2009; 25(1):3–20. [PubMed: 19219695]
11. Vujaskovic Z, Poulson JM, Gaskin AA, Thrall DE, Page RL, Charles HC, et al. Temperature-dependent changes in physiologic parameters of spontaneous canine soft tissue sarcomas after combined radiotherapy and hyperthermia treatment. *Int J Radiat Oncol Biol Phys*. 2000; 46(1): 179–85. [PubMed: 10656391]
12. Teicher BA. Acute and chronic in vivo therapeutic resistance. *Biochem Pharmacol*. 2009; 77(11): 1665–73. [PubMed: 19428320]
13. Song CW, Shakil A, Osborn JL, Iwata K. Tumour oxygenation is increased by hyperthermia at mild temperatures. *Int J Hyperthermia*. 1996; 12(3):367–73. [PubMed: 9044906]
14. Doll CM, Milosevic M, Pintilie M, Hill RP, Fyles AW. Estimating hypoxic status in human tumors: a simulation using Eppendorf oxygen probe data in cervical cancer patients. *Int J Radiat Oncol Biol Phys*. 2003; 55(5):1239–46. [PubMed: 12654433]
15. Milosevic MF, Fyles AW, Wong R, Pintilie M, Kavanagh MC, Levin W, et al. Interstitial fluid pressure in cervical carcinoma: within tumor heterogeneity, and relation to oxygen tension. *Cancer*. 1998; 82(12):2418–26. [PubMed: 9635535]
16. Rasband, WS. ImageJ. Bethesda, MD: U.S. National Institutes of Health; 2009. <http://www.rsb.info.nih.gov/ij/>
17. Sapareto SA, Dewey WC. Thermal dose determination in cancer therapy. *Int J Radiat Oncol Biol Phys*. 1984; 10(6):787–800. [PubMed: 6547421]
18. Dewhirst MW, Sim DA, Sapareto S, Connor WG. Importance of minimum tumor temperature in determining early and long-term responses of spontaneous canine and feline tumors to heat and radiation. *Cancer Res*. 1984; 44(1):43–50. [PubMed: 6690058]
19. Oleson JR, Sim DA, Manning MR. Analysis of prognostic variables in hyperthermia treatment of 161 patients. *Int J Radiat Oncol Biol Phys*. 1984; 10(12):2231–9. [PubMed: 6511520]
20. Oleson JR, Samulski TV, Leopold KA, Clegg ST, Dewhirst MW, Dodge RK, et al. Sensitivity of hyperthermia trial outcomes to temperature and time: implications for thermal goals of treatment. *Int J Radiat Oncol Biol Phys*. 1993; 25(2):289–97. [PubMed: 8420877]
21. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst*. 2000; 92(3):205–16. [PubMed: 10655437]
22. Roberge D, Skamene T, Nahal A, Turcotte RE, Powell T, Freeman C. Radiological and pathological response following pre-operative radiotherapy for soft-tissue sarcoma. *Radiation Oncol*. 2010; 97(3):404–7. [PubMed: 21040989]
23. Komar G, Seppanen M, Eskola O, Lindholm P, Gronroos TJ, Forsback S, et al. 18F-EF5: a new PET tracer for imaging hypoxia in head and neck cancer. *J Nucl Med*. 2008; 49(12):1944–51. [PubMed: 18997048]
24. Wong TZ, Lacy JL, Petry NA, Hawk TC, Sporn TA, Dewhirst MW, et al. PET of hypoxia and perfusion with 62Cu-ATSM and 62Cu-PTSM using a 62Zn/62Cu generator. *AJR Am J Roentgenol*. 2008; 190(2):427–32. [PubMed: 18212229]
25. Thoeny HC, Ross BD. Predicting and monitoring cancer treatment response with diffusion-weighted MRI. *J Magn Reson Imaging*. 2010; 32(1):2–16. [PubMed: 20575076]
26. Pope WB, Lai A, Mehta R, Kim HJ, Qiao J, Young JR, et al. Apparent diffusion coefficient histogram analysis stratifies progression-free survival in newly diagnosed bevacizumab-treated glioblastoma. *AJNR Am J Neuroradiol*. 2011; 32(5):882–9. [PubMed: 21330401]
27. Pilatou MC, Stewart EA, Maier SE, Fennessy FM, Hynynen K, Tempny CM, et al. MRI-based thermal dosimetry and diffusion-weighted imaging of MRI-guided focused ultrasound thermal ablation of uterine fibroids. *J Magn Reson Imaging*. 2009; 29(2):404–11. [PubMed: 19161196]

28. Chen J, Daniel BL, Diederich CJ, Bouley DM, van den Bosch MA, Kinsey AM, et al. Monitoring prostate thermal therapy with diffusion-weighted MRI. *Magn Reson Med*. 2008; 59(6):1365–72. [PubMed: 18506801]
29. Italiano A, Delva F, Mathoulin-Pelissier S, Le Cesne A, Bonvalot S, Terrier P, et al. Effect of adjuvant chemotherapy on survival in FNCLCC grade 3 soft tissue sarcomas: a multivariate analysis of the French Sarcoma Group Database. *Ann Oncol*. 2010; 21(12):2436–41. [PubMed: 20439343]
30. Jackson IL, Batinic-Haberle I, Sonveaux P, Dewhirst MW, Vujaskovic Z. ROS production and angiogenic regulation by macrophages in response to heat therapy. *Int J Hyperthermia*. 2006; 22(4):263–73. [PubMed: 16754348]
31. Katschinski DM, Le L, Heinrich D, Wagner KF, Hofer T, Schindler SG, et al. Heat induction of the unphosphorylated form of hypoxia-inducible factor-1alpha is dependent on heat shock protein-90 activity. *J Biol Chem*. 2002; 277(11):9262–7. [PubMed: 11779866]
32. Wartenberg M, Gronczynska S, Bekhite MM, Saric T, Niedermeier W, Hescheler J, et al. Regulation of the multidrug resistance transporter P-glycoprotein in multicellular prostate tumor spheroids by hyperthermia and reactive oxygen species. *Int J Cancer*. 2005; 113(2):229–40. [PubMed: 15389514]
33. Moon EJ, Sonveaux P, Porporato PE, Danhier P, Gallez B, Batinic-Haberle I, et al. NADPH oxidase-mediated reactive oxygen species production activates hypoxia-inducible factor-1 (HIF-1) via the ERK pathway after hyperthermia treatment. *Proc Natl Acad Sci U S A*. 2010; 107(47):20477–82. [PubMed: 21059928]

Figure 1.

Percent tumor volume change at end of treatment as a function of treatment group. There was a statistically significant greater reduction in tumor volume at the end of treatment in tumors treated with one fraction per week compared to tumors treated with 3–4 fractions per week ($p=0.0022$).

Figure 2.

Median number of measured pO_2 points $<2.5\text{mmHg}$ (24 hours post treatment minus pretreatment) as a function of the % change in tumor volume at the end of treatment. Note the direction of the x-axis; decreasing tumor volume proceeds to the right. The pattern is a greater tumor volume reduction in tumors where the median number of measured points $<2.5\text{mmHg}$ decreases compared to the pretreatment value, i.e. improving oxygenation ($p=0.0138$, test for zero correlation; correlation coefficient 0.41).

Figure 3.

Difference in median percentile values for the apparent diffusion coefficient of water (ADC), post treatment relative to pretreatment, as a function of the individual percentile as a function of treatment group. The greatest decrease occurred in the low end of the diffusion range in dogs receiving 1 fraction per week (0.0023 p 0.4322). 5HT = 1 hyperthermia fraction per week; 20 HT = 3–4 hyperthermia fractions per week..

Figure 4. Percentile distribution of apparent diffusion coefficient (ADC) values in an individual tumor before treatment and at the end of treatment. In this subject, treatment led to a generalized decrease in water diffusion but regions where water diffusion was more restricted prior to treatment (low ADC values) were characterized by greater decreases. Every 1000th point is shown. Fewer points are present after treatment because the tumor was smaller. Pre = before treatment.

Table 1

Distribution of tumor grade, tumor site and tumor volume between treatment groups. Proportionally more high grade tumors were present in tumors treated with 3–4 fractions per week.

Variable	Arm		p-value
	1 per week	3–4 per week	
Tumor Grade			
Low/Intermediate	20 (95.2%)	7 (43.8%)	0.0007 [*]
High	1 (4.8%)	9 (56.2)	
Tumor Site			
Head	1 (4.8%)	2 (12.5.5)	0.23 [*]
Trunk	2 (9.5.5)	4 (25.0%)	
Extremity	18 (85.7%)	10 (62.5%)	
Tumor Volume (cm ³)			
Mean	69.2	89.7	0.87
Median	72.3	60.1	
Min	11.0	10.0	
Max	142.6	360.3	

* Fisher's exact p-value

Table 2

Thermal parameters as a function of treatment group. Tumor temperatures were higher and duration of each hyperthermia treatment shorter in the 1 fraction per week group compared to the 3–4 fraction per week group. The total cumulative thermal dose, quantified as CEM43T₉₀ was slightly higher in the 1 fraction per week group but was within the target range of 20–50 CEM43T₉₀. It is doubtful that the small absolute difference in total CEM43T₉₀ values between groups is clinically significant.

Variable	Arm		p-value
	1 per week (n=21 dogs)	3–4 per week (n=16 dogs)	
T₁₀ (°C) at First Hyperthermia Treatment			
Mean	44.90	43.37	0.0013
Median	45.13	43.38	
T₅₀ (°C) at First Hyperthermia Treatment			
Mean	42.91	41.47	<0.0001
Median	43.10	41.31	
T₉₀ (°C) at First Hyperthermia Treatment			
Mean	41.15	40.08	<0.0001
Median	41.02	40.04	
Total Number of Hyperthermia Treatments			
Mean	4.9	15.4	<0.0001
Median	5.0	16.0	
Duration of First Hyperthermia Treatment (min)			
Mean	55.8	82.6	0.009
Median	45.0	89.5	
Total CEM43T₁₀ (min)			
Mean	1185.7	2109.1	0.044
Median	749.8	1814.0	
Total CEM43T₅₀ (min)			
Mean	251.7	304.0	0.318
Median	200.5	291.1	
Total CEM43T₉₀ (min)			
Mean	29.9	24.9	0.007
Median	32.1	24.2	
Total Duration of Hyperthermia (min)			
Mean	240	1459	<0.0001
Median	206	1506	

Table 3

Median descriptors of tumor oxygenation prior to treatment vs 24 hours after the first hyperthermia treatment. These data are for all tumors, in both fractionation groups combined. The change in median values is in a direction consistent with reduction in oxygenation, without respect to fractionation group.

Variable		Pretreatment	24 hours after first HT treatment	P-Value
Median pO ₂		10.5	6.2	0.06
Median % measured pO ₂ points	2.5 mmHg	22.5	34.2	0.44
Median % measured pO ₂ points	5 mmHg	33.4	50.0	0.03
Median % measured pO ₂ points	10 mmHg	48.9	61.8	0.02

HT = hyperthermia. S.D. = standard deviation. P value is from signed rank test to compare medians.