Effects of acute and repeated heat therapy on prostate cancer cell survival and viability

by

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Abstract

Background: Despite advances in treatment options, prostate cancer remains the second-most diagnosed and second leading cause of cancer-related death in men in the United States. The efficacy of conventional anti-cancer treatments is dependent, in part, on tumor blood flow and oxygenation. Tumor vasculature is abnormal relative to healthy tissue, leading to reduced blood flow and oxygen delivery, and thus, treatment resistance. An emerging adjuvant to conventional treatment to overcome resistance to radiation is heat therapy. While heat therapy may sensitize tumors to radiation via increases in tumor blood flow and thus, oxygenation, we hypothesized that heat-induced radiosensitization occurs independent, in part, of tumor blood flow or oxygenation. **Methods:** Clonogenic cell survival and cell viability were assessed using human prostate cancer (PC-3) cells *in vitro.* Individual tissue culture flasks of PC-3 cells were randomized into 6 groups, normothermic non-radiated (NT-NR, n=8), normothermic radiated (NT-R, n=8), acute hyperthermic non-radiated (HTA-NR, n=8), acute hyperthermic radiated (HTA-R, n=8), chronic (repeated) hyperthermic non-radiated (HTC-NR, n=8), and chronic (repeated) hyperthermic radiated (HTC-R, n=8) for both clonogenic cell survival and cell viability assays. For assessment of both clonogenic cell survival and cell viability, NT-NR and NT-R flasks were maintained in an incubator at 37° C for the duration of the experiment. HTA-NR and HTA-R flasks were maintained in an incubator at 37° C and heated in a separate incubator to 41° C for 60 minutes prior to radiation. HTC-NR and HTC-R flasks were maintained at 37º C and heated to 41º C for 60 minutes every 48 hours for 3 heat treatments. Non-radiated flasks were subjected to 0 Gy radiation, while radiated flasks were subjected to 2 Gy radiation. For clonogenic cell survival, cells were then plated in 60 mm tissue culture dishes at a density of 500 cells/plate and 1000 cells/plate in 5 replicates each per flask and allowed to

grow for 8 days in an incubator at 37° C. Cell survival was assessed via counting the number of fixed and stained colonies >50 cells at the completion of 8 days of incubation. For cell viability, cells were plated into 96-well plates and incubated for 24, 48, and 72 hours before addition of MTT reagent for quantification of absorbance. Data are presented as mean ± SEM. **Results:** Clonogenic cell survival was significantly reduced between NT-NR vs. NT-R, HTA-NR, HTA-R, and HTC-R (100 % ± 9.7% vs. 59.1 % ± 5.9 %, 72.4% ± 8.5%, 40.3% ± 3.1%, and 43.3% ± 3.4%, respectively; $p < 0.05$). There were no differences between NT-R and HTA-NR or HTC-NR. **Conclusions:** This investigation indicates that 60 minutes of mild-temperature hyperthermia before radiation treatment does not enhance the efficacy of radiation treatment, but that heat therapy alone may be as effective as low-dose radiation *in vitro*. Further, given that 2 Gy radiation alone (representative of one radiation fraction given clinically) was not more effective at reducing cancer cell survival, heat therapy may be an effective strategy to limit cancer progression before radiation therapy begins.

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Chapter 1 - Introduction

Despite advances in treatment options, prostate cancer remains the second-most diagnosed and second leading cause of cancer-related death in men in the United States (30). A significant clinical issue in the progression and severity of the disease is that many tumors are often insensitive to the effects of radiation therapy—termed radioresistance. As such, one of the primary goals in current anti-cancer research is overcoming radioresistance through the addition of adjuvant therapies that reduce tumor hypoxia and/or sensitize tumor cells to radiation.

One emerging adjuvant to conventional treatment aimed at overcoming radioresistance is the use of hyperthermia—or heat therapy. Heat therapy is not a new concept for the treatment of cancer as it was first utilized as early as 5000 years ago (15). More widespread use of heat therapy in pre-clinical and clinical research began in the 1970s and has since been studied in various cancers with varying outcomes. Although heat therapy has primarily been found to result in better tumor control and improved patient outcome(s) (for review, see (5, 8, 9, 15, 37)), techniques and findings vary greatly. As tumors are often characterized by hypoxia and abnormal vasculature (14, 31, 36), pre-clinical research has primarily investigated changes in tissue blood flow and tumor oxygenation in order to enhance the radiosensitivity of cancer cells, while most clinical research has investigated focused heat therapy (microwave antenna heating, radiofrequency heating, interstitial, etc.) (38) and its overall effects on patient survival. Pre-clinical research has found that the use of hyperthermia (41-45º C) before, during, or after radiation sensitizes a variety of tumor types to radiation, with shorter intervals between heat and radiation and higher temperatures leading to the greatest enhancements (for review, see (7, 15)). However, the majority of pre-clinical studies evaluating changes in blood flow and hypoxia in response to heat therapy utilize an ectopic cancer model (15, 33, 34). Ectopic tumors are grown

subcutaneously, typically within the adipose tissue of a hind limb, not tumor host tissue, and these models do not represent tumor host tissue hemodynamics (13). Therefore, interpretation of research regarding tumor blood flow and hypoxia in those studies is severely limited. In addition to pre-clinical research, several clinical investigations indicate that local, regional, interstitial, and whole-body hyperthermia (primarily in breast, cervical, and head and neck cancers), can result in improvements in initial response to radiation and improved disease-free survival compared to radiation alone (for review, see (9, 15)). The use of hyperthermia in conjunction with radiation shows clear potential for significant clinical applications and improved patient outcomes, but the mechanisms by which hyperthermia improves radiosensitivity of tumors are not fully understood.

While heat therapy may elicit changes in tumor blood flow or hypoxia, it is likely that radiosensitizing effects of heat on cancer cells are due in part to blood flow- and hypoxia-independent pathways. For example, it has been suggested that heat therapy may disrupt tumor cell DNA damage repair (17, 18, 28), enhance anti-tumor immune response via cell surface expression of heat shock proteins (HSPs) (18, 20, 29, 35), and alter cell cycle progression (18).

Before investigating changes in orthotopic tumor blood flow and hypoxia in response to heat therapy, this study was designed to investigate the effects of heat therapy on human prostate cancer cell characteristics, independent of blood flow or hypoxia. Of the studies performed *in vitro* that have utilized heat as an adjuvant to radiation, those performed on cancer cells have typically consisted of a single bout of heat therapy of varying durations and have been aimed at determining the optimal radiation dose and timing of heat therapy (before, during, or after radiation) (7, 15, 22). Much like radiation therapy, a single, high dose of heat could damage healthy cells while also targeting cancer cells. Moreover, directly killing cancer cells with high

temperature heat therapy may not be the most efficacious method of radiosensitization. As such, we hypothesized that independent of increased blood flow or decreased hypoxia, both acute and repeated mild temperature hyperthermia (39-42º C) will enhance the radiosensitivity of human prostate cancer cells *in vitro*.

Chapter 2 - Methods

Cell Culture

Human-derived prostate adenocarcinoma cancer cells (PC-3) were used in this study (ATCC). All cells were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum and 2 μM l-glutamine. Cells used were cultured according to manufacturer guidelines from passages 12-16 and were maintained in a humidified incubator at 37º C and 5% CO_2.

Heat Therapy

All heat therapy was performed before radiation. For administration of heat therapy, cells were placed in a separate humidified incubator calibrated to maintain cell temperature at 41º C ± .5º C and 5% CO_2. Before administration of acute heat therapy, cells were allowed to attach to tissue culture flasks overnight. After the initial increase in temperature to 41º C, (as measured by monitoring the temperature directly inside an identical tissue culture flask containing equal volumes of media as flasks containing cells), cells were maintained at 41º C for 60 minutes. Cells were then randomly assigned to radiated or non-radiated conditions. Administration of repeated heat therapy occurred in 3 sessions over the course of 6 days, with all bouts separated by 48 hours and lasting 60 minutes. As with acute heat therapy, the time for flasks to reach 41º C was monitored via the use of a flask containing equivalent amounts of media. Between each heat exposure, cells were maintained in the standard 37º C incubator. Cells were then randomly assigned to radiated or non-radiated conditions.

Radiation

All cells were seeded in 25 cm^2 flasks for radiation, with 8 flasks plated per experimental condition. After administration of heat therapy, radiated and non-radiated cells were subjected to 2 Gray and 0 Gray (Gy) radiation, respectively, with a linear accelerator (Clinac 2100 CD, Varian Medical Systems, Palo Alto, CA). Low-dose radiation was selected as 2 Gy is the dosage of a single fraction that would be administered clinically. All radiation was carried out at room temperature. Cells subjected to one bout of heat therapy were returned to a 37º C incubator and then radiated within 30 minutes after the completion of heat therapy. Cells subjected to repeated heat therapy were radiated 24 hours after the last bout of heat therapy in order to avoid any acute effects of heat exposure.

Clonogenic Survival

Cells were harvested within 2 hours after the completion of radiation for determination of clonogenic survival according to the methods of Franken, et al., (11) and Feoktistova, et al., (10). Cells were isolated via trypsinization and re-suspended in media, followed by seeding in 60mm tissue culture dishes at densities of 500 and 1000 cells per plate. Five replicates per plating density were performed, for a total of 10 plates per flask. Plates were placed in a humidified incubator maintained at 37º C and 5% CO_2 for 8 days for development of cell colonies. After 8 days, cells were fixed and stained with 20% methanol and 0.5% crystal violet in water (Figure 1). Using a microscope at 10x magnification, individual colonies containing >50 cells from each plate were counted as a single colony (Figure 2). Plating efficiency was determined from the fraction of control cells initially plated that formed colonies. Survival fraction for each group was determined by dividing the number of colonies formed by the number of cells seeded

multiplied by the plating efficiency determined from the control condition. The survival fraction was expressed as an average of the two plating densities for each condition.

Viability Assay

Viable cell number was quantified using CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT) (Promega, Madison, WI). PC-3 cells (5×10^3) were seeded in 96-well tissue culture dishes immediately after radiation for 24, 48, and 72 hours. At the completion of each incubation time, dye solution was added to each well and plates were re-incubated for 4 hours before the addition of solubilization/stop solution. Absorbance was measured using an accuSkan GO UV/Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA) at 570 nm. Viable cell number was expressed as a percentage of cell number in the NT-NR group and calculated from two replicates for each time period.

Data Analysis

Prism (Version 8, GraphPad Software, San Diego, CA) data analysis software was used to perform all statistical analyses. All comparisons were made with two-way analysis of variance (ANOVA) and followed by Holm-Sidak post hoc tests when appropriate to assess statistical differences between groups for all measures. Data are expressed as a percentage of control within each measure and presented as mean ± SEM. The level of significance was set as $p \leq 0.05$ for all measures.

Chapter 3 - Results

Clonogenic Survival

Quantification of surviving fraction of cells was used to assess the colony-forming ability of cells exposed to different combinations of treatments. The survival fraction of cells was significantly reduced in the NT-R (59.1% ± 5.9%), HTA-NR (72.4% ± 8.5%), HTA-R (40.3% ± 3.1%), and HTC-R (43.4% ± 3.4%) groups compared to the NT-NR group (100% ± 9.7%). There was also a significant reduction in the survival fraction of cells from HTA-R compared to HTA-NR and HTC-R compared to HTC-NR (78.6% ± 7.6%) (all $p \leq 0.05$). There were no differences found for HTA-NR, HTA-R, HTC-NR, or HTC-R compared to NT-R, or HTC-NR compared to NT-NR ($p > 0.05$) (Figure 3).

Cell Viability

Measurement of absorbance was used to determine differences in viable cell number between cells exposed to different combinations of treatments. No differences in viability were detected at 24 hours of incubation. After 48 hours of incubation, viable cell number was significantly reduced in HTC-NR compared to HTA-NR (76.5 % ± 6.5% vs. 114.7% ± 10.2%; $p \leq 0.05$). No other significant differences in viability were detected at 48 hours. After 72 hours of incubation, viability was significantly increased in HTC-NR compared to NT-NR (135.3 % ± 4.8% vs. 100% ± 8.9%; $p \leq 0.05$) (Figure 4). No other differences in viability were found at any time point.

Figure 1. *Representative Images of Clonogenic Plates*

Representative plates from each experimental condition; dark spots represent individual cell colonies. **1x magnification**.

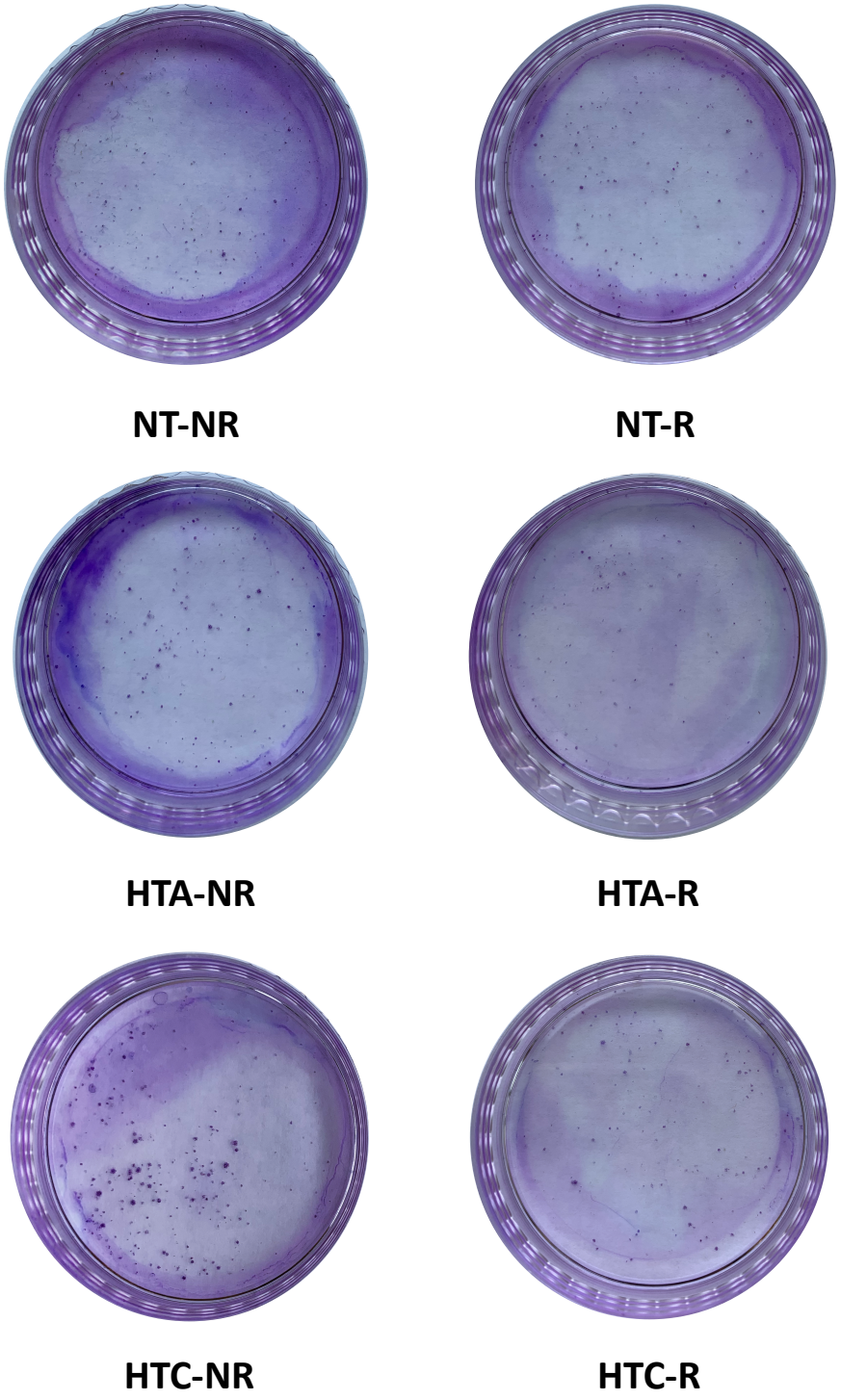


Figure 2. *Representative Image of Individual Clonogenic Colony*

Example of single colony >50 cells counted for determination of survival fraction. **10x magnification.**

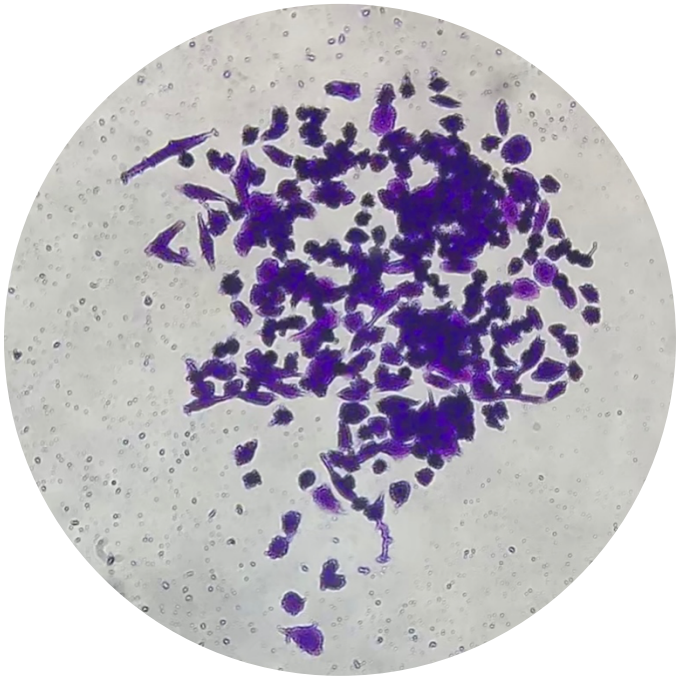


Figure 3. *Clonogenic Survival*

Effect of radiation, acute heat, and repeated heat on clonogenic cell survival. There were no significant differences in survival fraction of HTA-R or HTC-R vs NT-R. There was, however, a significant decrease in survival fraction in NT-R, HTA-NR, HTA-R, and HTC-NR vs. NT-NR (*$p \leq 0.05$). There was also a significant decrease in survival fraction in HTA-R vs. HTA-NR (#$p \leq 0.05$) and HTC-R vs. HTC-NR (†$p \leq 0.05$).

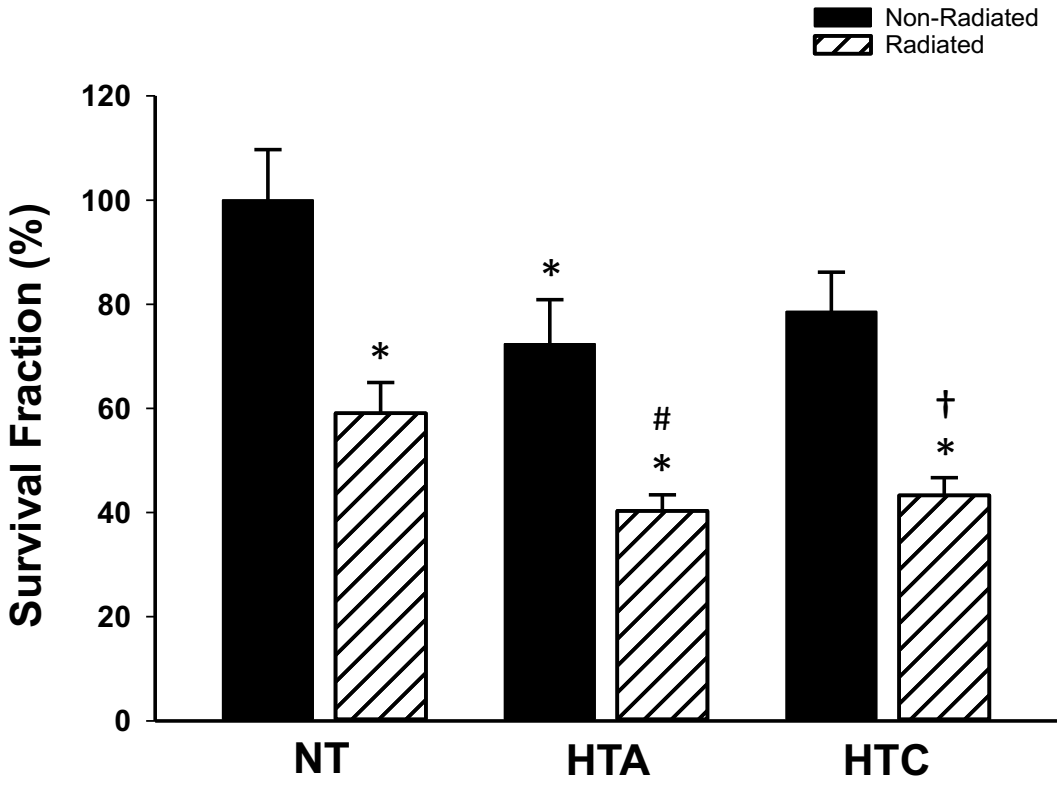
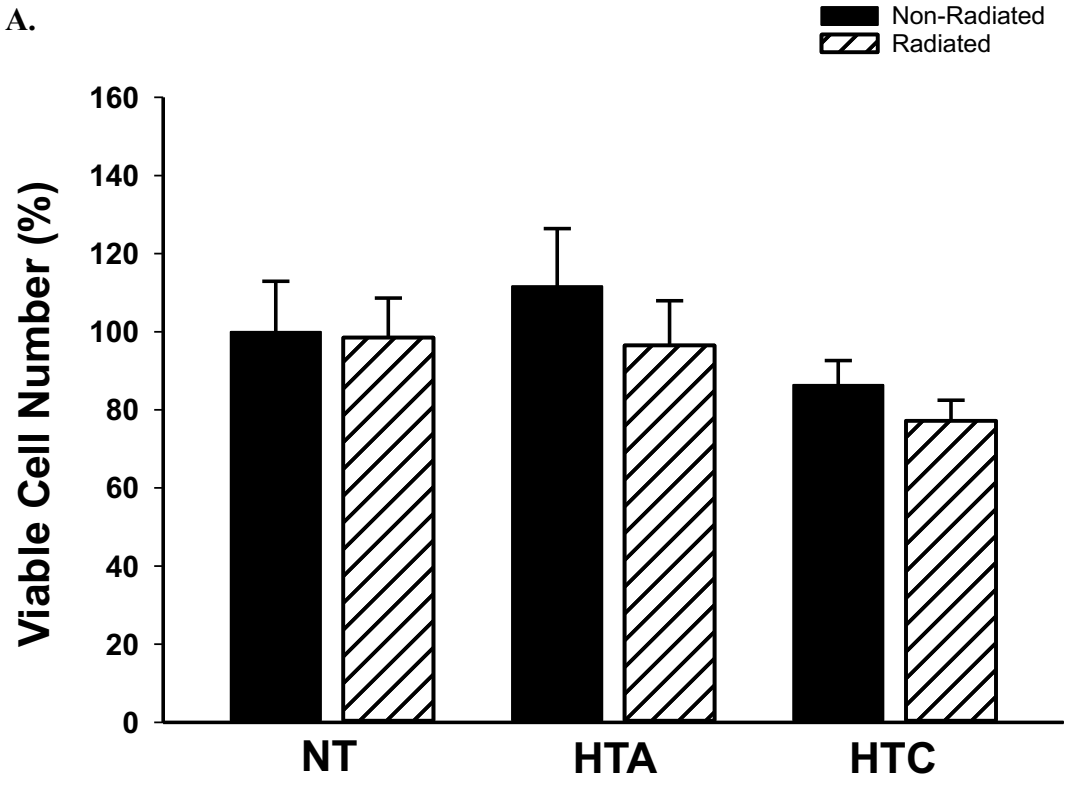
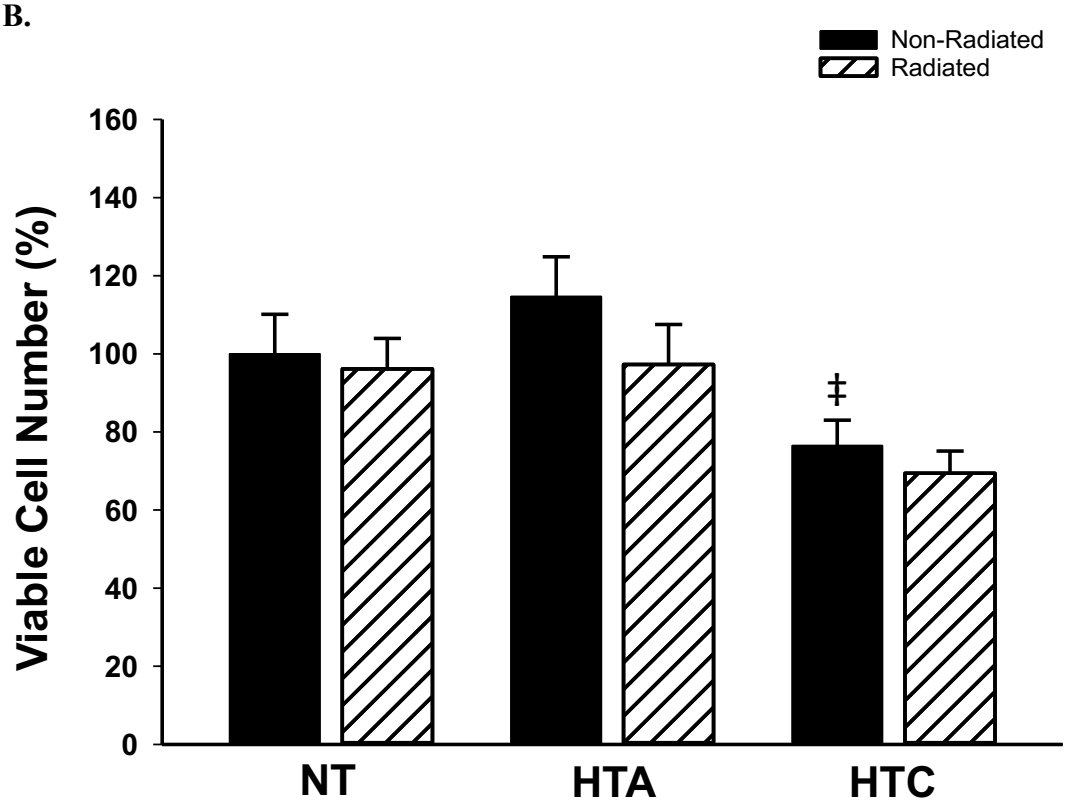


Figure 4. *Cell Viability*

Effect of radiation, acute heat, and repeated heat on cell viability at 24 (A), 48 (B), and 72 (C) hours. There were no significant differences in viable cell number between any groups at 24 hours ($p > 0.05$). At 48 hours, viable cell number was significantly reduced in HTC-NR vs. HTA-NR (‡$p \leq 0.05$). At 72 hours, viable cell number was significantly increased in HTC-NR vs. NT-NR (*$p \leq 0.05$).



B.


Non-Radiated
Radiated
Viable Cell Number (%)
160
140
120
100
80
60
40
20
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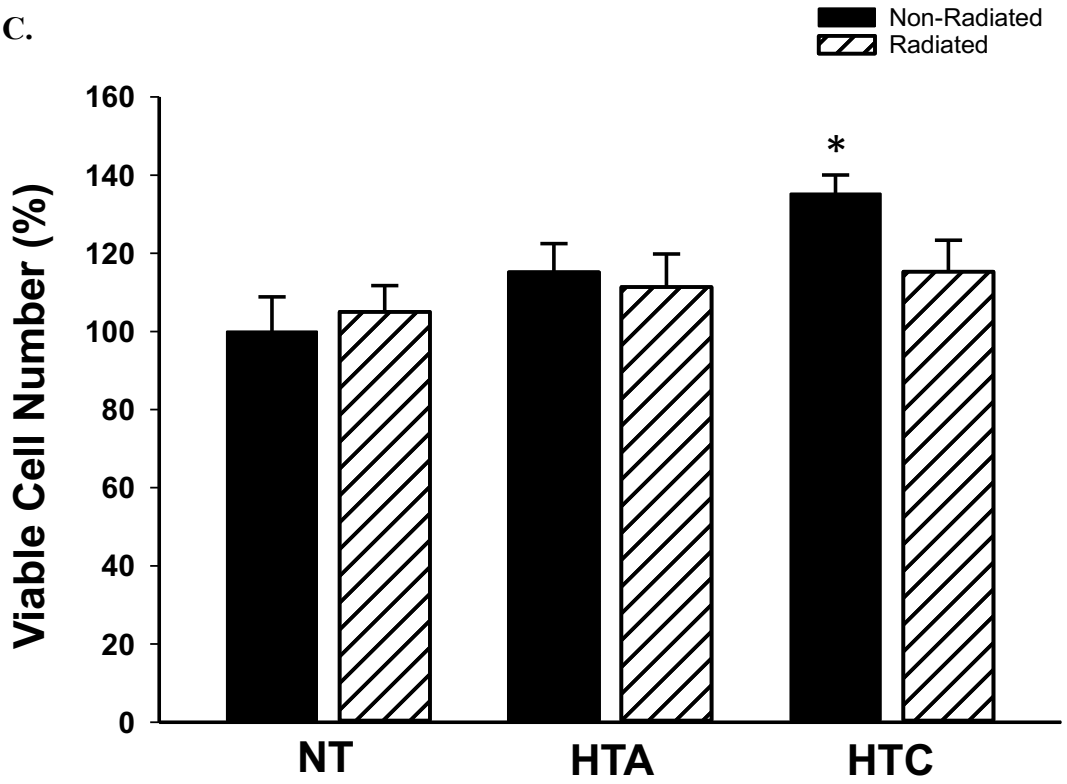
‡
NT
HTA
HTC

C.


Non-Radiated
Radiated
Viable Cell Number (%)
160
140
120
100
80
60
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NT
HTA
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Chapter 4 - Discussion

The purpose of this study was to determine the effects of both acute and repeated heat exposure on prostate cancer cell characteristics and radiosensitivity. Contrary to our hypothesis, no significant differences were found that indicate mild hyperthermia sensitizes PC-3 cells to radiation. However, given a lack of statistical differences between HTA-NR and HTC-NR compared to NT-R, our results suggest that one or 3 bouts of heat therapy in the absence of radiation are as effective as radiation alone on cancer cell colony formation *in vitro*. Our results also suggest that one bout of heat therapy without radiation significantly reduces the surviving fraction of PC-3 cells compared to untreated cells, but that the addition of radiation kills a significantly larger percentage of cells compared to heat alone. No differences in cell viability were found, except for a decrease in viability of non-radiated cells exposed to repeated heat compared to non-radiated cells exposed to one bout of heat at 48 hours. Conversely, at 72 hours, there was a significant increase in the viability of non-radiated cells exposed to repeated heat compared to non-radiated cells exposed to one bout of heat.

Mechanisms of heat-induced changes in cancer cell characteristics

While the exact mechanisms by which heat affects cancer cell characteristics are not fully understood, several mechanisms appear to play a significant role. One mechanism that has been a major focus in hyperthermia research is the induction of DNA damage and disruption of DNA damage repair. Much research over the last 30 years has led to the proposition that heat both directly and indirectly damages DNA. Specifically, in malignant and non-malignant mammalian cell lines heat exposure can directly elicit DNA synthesis-associated protein damage, apoptotic cell death, single stranded breaks, double stranded breaks, or changes in chromosomal structure

(16, 18, 23, 26, 38). However, it is suggested that heat-induced DNA damage is not consistently measurable with hyperthermia alone, but requires 1) a combination of hyperthermia with other modalities designed to damage DNA (e.g., radiation) and/or 2) hyperthermia at higher temperatures (>46° C) (23, 26). In addition to directly damaging DNA, heat causes unfolding of proteins, including those associated with regulating various aspects of DNA replication (e.g. proteins that facilitate and stabilize DNA unwinding), which stalls DNA replication and may be a significant contributor to double stranded breaks (26). While the mechanisms are not well understood, it is likely that heat also diminishes the potential for various methods of DNA damage repair (6, 23, 24, 26, 28) and, as such, may sensitize cells to other DNA damaging agents. Additionally, hyperthermia may result in G1/S cell cycle transition arrest, or alter the distribution of cells in each phase, resulting in an increased number of cells in phases more sensitive to the effects of other agents that are, in part, dependent on cell cycle phase (24, 25).

Another potential mechanism mediating the effects of heat on cancer cell characteristics is an upregulation of heat shock proteins (HSPs). Certain HSPs are constitutively active in many types of cells, but others are upregulated in cancer cells and in response to various stressors including, but not limited, to heat (21, 29). Many of these HSPs have been implicated as negative regulators of apoptosis and studied as potential targets in anti-cancer treatments (e.g., the stress-inducible HSP72) (12). However, the pathways associated with HSPs are complex and they may serve dual roles. Recent research suggests that upregulation of HSPs on the surface of cancer cells (extracellular expression as opposed to intracellular expression) may actually serve as a signal to the immune system to recognize foreign cells (18, 20, 29) and, thus, could provide a beneficial role in eliciting a greater local immune response and mitigate tumor progression. If HSPs do in fact act as an immune-signaling cell in various cancer cells, further upregulation by

heat therapy may be a potential means of improving patient prognosis, but proper investigation into any immune-mediated changes requires the use of *in vivo* experiments.

Radiation and Heat Therapy

Radiation induces DNA damage and the presence of free radicals formed in the presence of oxygen is critical for preventing DNA repair after the radiation-induced DNA damage (19). Studies demonstrate that tumor hypoxia is associated with poor patient outcomes and leads to more aggressive cancer phenotypes (3, 4, 14), which may be due, in part, to radioresistance caused by tumor hypoxia. *In vivo*, improved radiation responses with heat therapy are typically attributed to enhanced oxygen delivery to the tumor via increases in tumor blood flow. However, as the tumor vasculature is aberrant, both structurally and functionally (31, 36), the results of many studies are highly variable and improved oxygen delivery with heat therapy would likely impact only adequately perfused areas of the tumor. Further, whole body heat therapy may reduce peripheral vascular resistance in compliant regions (e.g., skin), and shunt blood away from the tumor and transiently enhance tumor hypoxia. As a complete abrogation of tumor hypoxia and radioresistance is unlikely due solely to changes in blood flow (particularly in anatomically deep-seeded tumors where blood flow is less likely to increase in response to heat due to thermoregulatory redistribution of blood flow away from the core), the interaction between hyperthermia and radiation is dependent, in part, upon other molecular mechanisms, such as inhibition of DNA damage repair, as mentioned previously. As radiation causes DNA damage that may be reversible without the presence of an adequate supply of oxygen, heat likely prevents repair of radiation-induced DNA damage or alters cell cycle, via a combination of several mechanisms not yet fully understood. In our study, however, we did not observe an

improvement in radiation response with the addition of heat *in vitro*. The effects of hyperthermia on cancer cell characteristics are largely dependent on the temperature, duration, and sequence of heat therapy, along with the thermo-sensitivity of individual cell lines. For example, *in vivo,* it has been found that short periods of heat exposure, ranging from 4 hours pre- to post-radiation all result in some degree of radiosensitization, but that the greatest effect is seen when heat exposure and radiation occur simultaneously (7, 15). Duration of heating is also an important consideration, with highly variable findings found for exposures ranging from 30 minutes to 24 hours (27, 33). Finally, temperature is an important consideration. Most studies investigating the radiosensitizing effects of heat utilize temperatures in the range of 42-45° C. We chose 41° C for our studies, as some studies have still shown radiosensitizing effects from acute exposure at 41° C (27), and it represents a much more clinically relevant temperature than those typically used for *in vitro* studies. Although targeted heating methods (microwave, radiofrequency, etc.) have become much more refined, utilizing any non-local method of heating above 41° C in both animal subjects and human patients has the potential for much greater side effects and radiosensitization of surrounding normal tissues.

Clinical Relevance

Although several clinical trials have implemented hyperthermia as an adjuvant to radiation and seen promising results (reviewed in (9, 15)), hyperthermia in combination with radiation is not widely used in routine practice. There is little substantial evidence for any specific combination of temperature, time, duration, and method of heating that are most effective for individual cancer types. However, the research clearly indicates that hyperthermia may be an effective strategy to overcome radioresistance in certain types of cancers, or as our

study suggests, may independently be an effective technique to reduce disease progression. While it is unlikely that heat alone would ever lead to complete tumor control or prevention of cancer progression, it could serve as an effective strategy to limit cancer progression before radiation can begin. In general, the use of hyperthermia in patients would be more practical and carry less side effects compared to other potential means of enhancing radiation sensitivity or tumor control (e.g., experimental medications).

Limitations

There are several limitations to address in this study. First, the presence of few differences in cell viability could be due to several limitations associated with the MTT assay. The assay reagent itself has the potential to cause cytotoxicity if incubated for an extended period of time (32). To prevent this, the recommended incubation time is 1-4 hours. Our plates were incubated with the reagent for 4 hours, so it is unlikely that the reagent caused any cytotoxicity in our study. Additionally, the reaction that occurs between the cells and the reagent is dependent on the presence of NADH for the reduction of tetrazolium and production of a colored solution, which may negatively affect the health of cells, as NADH is essential for the survival of cells (32). As such, if a significant amount of NADH was diverted away from cells in order for the chemical conversion to occur, the number of viable cells could be significantly altered. Over time as cells continue to grow, the pH of the cell media can change significantly, which can impact the formation of formazan and increase background absorbance. Finally, although typically reflective of cell number, the MTT reaction is technically dependent on metabolic activity and rate of NADH production (1, 2), rather than an absolute number of viable cells. Thus, cell viability interpreted from absorbance readings may be more reflective of the

overall metabolic activity of cells and not of the absolute number of viable cells. In addition to limitations with interpretation of cell viability, the dosage of radiation used in our study could be a limitation. While 2 Gy is representative of a single dose of fractionated radiation, only one fraction was administered. *In vivo,* multiple fractions of radiation may lead to slightly different effects of hyperthermia on cancer cell characteristics.

Conclusions

In conclusion, our study did not demonstrate a radiosensitizing effect of mild temperature hyperthermia on PC-3 cells. However, both acute and repeated heat exposure resulted in significant reductions in cell survival that were comparable to the effects of radiation alone. While heat therapy would likely not be efficacious enough for long-term treatment, based on our findings, it could serve as a relatively safe and effective way to control or potentially limit cancer cell progression before radiation treatment is begun. Importantly, our findings are not necessarily reflective of *in vivo* responses to heat therapy, as the tumor microenvironment is an important factor in cancer cell radiosensitivity and tumor progression. As such, pre-clinical studies utilizing orthotopic cancer models should be performed to fully elucidate the response of cancer cells to the effects of hyperthermia in combination with radiation. Additionally, dose response experiments could indicate different combinations of duration, sequence, and temperature that are most efficacious for enhancing radiosensitivity of prostate cancer cells.

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