Tumor cells can’t stand the heat
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Chapter 5

Radiosensitization by hyperthermia: the effects of temperature, sequence and time interval

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Under Review
Abstract

Background: Hyperthermia (HT) is a clinically proven radiosensitizer. However, there is no consensus about the sequence of HT and radiotherapy (RT) and the optimal time interval between these modalities, since there are different explanations for how HT sensitizes RT. One mechanism is that HT inactivates DNA repair for several hours resulting in the persistence of more fatal radiation-induced DNA breaks. The sequence and interval yielding optimal radiosensitization thus depend on the balance between the duration of inhibition of DNA repair and the contributions of the fast and slow repair of radiation induced DNA damage. This study investigates the contributions of DNA repair and the hyperthermic inhibition of DNA repair by establishing the impact of different sequence, time interval and temperatures on hyperthermic radiosensitization on in vitro models.

Methods: Clonogenic cell survival, DNA damage, cell cycle distribution and apoptosis were studied in SiHa, HeLa and C33A cells after varying sequence, time interval (0 up to 4 h) and temperature (37 to 42°C).

Results: The sequence, HT either before or after RT, yields similar levels of DNA damage and similar effects on cell survival. The time interval is an important factor: shorter time intervals result in more unrepaired DNA damages, thus more cell kill. Even for 4 h intervals the additional cell kill is considerable, suggesting relatively long DNA repair times. Treatment at a higher temperature was more effective for all time intervals.

Conclusions: Our results suggest that HT is more effective on the slow than on the fast DNA repair mechanisms.

Novelty and impact

Optimizing radiosensitization of hyperthermia
Introduction

The combination of radiotherapy (RT) and cisplatin-based chemotherapy is presently standard treatment for cervical cancer. The survival after daily RT plus weekly hyperthermia (HT) is comparable to that of chemoradiotherapy in cervical cancer (van der Zee et al., 2000; Vernon et al., 1996), but since HT can only be given in a limited number of centers, patients sometimes have to travel several hours. RT plus HT therefore is a good second choice for women with a contraindication for chemotherapy, usually because of limited renal function or frailty. Mild HT (i.e. heating the tumor to 40-42.5°C for 1 h) has been used in the clinic to sensitize RT since the 1980’s, with excellent results for various tumor types (Cihoric et al., 2015; Datta et al., 2015) including cervical cancer (Datta et al., 2016; Lutgens et al., 2010). HT has the ability to change the microenvironment, in hypoxic areas of the tumor, characterized by poor vascularization and low pH, enabling cells to become more sensitive to therapy (Valdagni & Amichetti, 1994). Another major advantage of mild HT is that it induces no side-effects and hardly increases radiation induced side-effects (van der Zee et al., 2000). Since simultaneous HT and RT is technically challenging, common clinical practice is to deliver HT and RT sequentially. However, there is no consensus on the optimal sequence and time interval in applying RT and HT. Another practical question is whether it is safe to give the patient the daily fractionated RT in a facility nearby and have her travel once a week to a remote HT facility, usually several hours from home, or that both modalities should be given with the shortest interval possible in one (remote) center.

Multiple mechanisms are responsible for the radiosensitization effect by HT, each operating at different temperatures and each with different optimal sequence and time interval (Crezee et al., 2016; Dewhirst et al., 2016; Horsman & Overgaard, 2007; Issels et al., 2016). First, pre-heating the tumor, prior to RT causes induction of oxygen radicals and as a consequence ionizing radiation can in turn induce more DNA damage (Song, 1984; van Vulpen et al., 2002). Second, HT interferes with the homologous recombination (HR), preventing the radiation-induced DNA breaks from being repaired (Kampinga & Dikomey, 2001; Krawczyk et al., 2011). The HR requires a sister chromatid and is thus mainly active during the S-phase and G2-phase of the cell cycle and is considered error-free (Fleck & Nielsen, 2004), but also considered a slow DNA repair pathway. An accumulation of DNA damage will result in eradicating more cancer cells. Simultaneous application of RT and HT will cause the highest amount of DNA damage, not only in the tumor but also in the normal tissue (Overgaard, 1980). Third, HT leads to direct cell kill, particularly of hypoxic tumor cells and at higher temperatures (Sharif-Khatibi et al., 2007). Sequence and time interval are not relevant for this mechanism, but the temperature level is.

This study investigates in an in vitro model which sequence and time interval
(0 up to 4 h) between applying RT and HT, causes most damage to the tumor cells at different temperatures (37 to 42 °C). Effects on cell cycle, DNA damage, and cell survival are assessed. Finally, different mechanisms explaining these results will be discussed.

Results

A short time interval between RT and HT results in a lower cell survival

To evaluate the effects from the sequence of and the time interval between RT and HT on cell survival, clonogenic assays were performed after HT followed by RT and after the opposite order. Furthermore, different time intervals between RT and HT (0, 2 and 4 h) were tested and the effect of different temperatures was investigated for SiHa, HeLa and C33A. The heat-maps presented in Figure 1, demonstrate the survival fraction after each treatment, a darker color is related to a lower cell survival, SiHa presented in red, HeLa in green and C33A in blue. From top to bottom, the dose of radiation increases (2, 4, 6 and 8 Gy) and per dose of radiation elevated levels of HT are applied (39, 41 and 42 °C). From left to right the time between RT and HT is shown, e.g. -4 h means that HT is applied before RT with a time interval of four hours. First, these heatmaps demonstrate that a higher dose of radiation is correlated with an increase in cell death. Second, cells treated with a higher temperature show a lower fraction of cell survival. Third, from the outsides towards the middle columns, the color gets darker, indicating a lower survival fraction after treatments with a shorter time interval between two therapies. In conclusion, there is no significant difference noticeable between different sequence. However, there is a significantly lower cell survival after a short (0 h) than after a long (4 h) time interval between the two treatment modalities. In all cell lines therapy with the shortest time interval (0 h) resulted in the lower cell survival. The heat-maps of all three cell lines, as shown in Figure 1, illustrate that a higher temperature and a higher dose of ionizing radiation resulted in a lower survival fraction compared to a lower temperature or a lower dose of ionizing radiation.

In supplementary Figure 1, bar graph of at least three independently performed experiments show means with standard deviation of clonogenic cell survival of SiHa (red), HeLa (green) and C33A (blue). For each cell lines, the top lane shows the results of HT prior to RT, the bottom lane the results of HT after RT, and for each sequence also, from left to right, the results after different HT temperatures (39, 41 and 42 °C) and different radiation doses (0, 2, 4, 6 and 8 Gy). As confirmed by the previously described statistical analysis, the bar graphs show for most cases that a longer time interval between the two therapies resulted in a higher survival fraction compared to a shorter time interval.
Figure 1. A shorter time interval between ionizing radiation and hyperthermia decreases cell survival. To study the effect of different time intervals (0, 2 or 4 h) between ionizing radiation and hyperthermia, clonogenic assays were performed for SiHa, HeLa and C33A cells. (A) Schematic overview of treatments. (B) Survival fraction is demonstrated by a colour gradient; a darker colour indicates lower cell survival. From top to bottom, different doses of ionizing radiation are presented (0, 2, 4, 6 and 8 Gy). Within one dose of radiation, cells are also treated with different temperatures of hyperthermia (39, 41, 42 °C). From left to right, within one heatmap, cell survival at different time intervals between ionizing radiation and hyperthermia (-4, -2, -0, 0, 2 and 4 h) is shown, e.g. -4 h time intervals means that hyperthermia is applied before ionizing radiation with 4 hours between the two therapies. Means of at least 3 experiments are presented.
A higher G₂ arrest after a short time interval between HT and RT

Cell cycle distribution was studied by incorporation of BrdU. In all cell lines, untreated samples have approximately 55% of cells in G₁-phase, around 35% in S-phase and only 10% in G₂-phase. As shown in Figure 2, cells treated with a short time interval have the highest increase of cells in G₂-phase compared to the untreated samples (ctrl), indicating that the cells treated with 0 h time interval are not capable of cell division, whereas more cells treated with a four hours time interval have progressed to cell division S-phase (Figure 2). No significant difference was found in cell cycle distribution between HT before or after ionizing radiation. However, there seems to be a slight trend in the C33A cell-line toward higher levels of cells in G₂-phase, when HT was given before

![Cell cycle distribution graphs](image)

*Figure 2. More pronounced G₂ arrest after a shorter time interval between ionizing radiation and hyperthermia.* Cell cycle distribution using BrdU incorporation after different time intervals and sequence of ionizing radiation and hyperthermia is performed on three cervical cell lines, from left to right: SiHa, Hela and C33A cells. Untreated samples are marked as control (ctrl). In the sample treated with a short time interval (0 h) between the two therapies, an increase in G₂-phase is observed, while after a longer time interval (4 h) between the two therapies, the S-phase is increased. Means of at least three replicates are presented.
than after irradiation: however this difference is not significant, and not consistent in all cell lines.

Apoptotic levels are the highest after a short time interval between ionizing radiation and HT
Apoptosis after different time intervals and for the different sequences of ionizing radiation and HT was measured by the Nicoletti assay. A short time interval between ionizing radiation and HT resulted in the highest apoptotic levels measured by flow cytometry (Figure 3). SiHa, HeLa and C33A show that ~30% of cells are apoptotic after 0 h time interval, compared to about ~20% after a 4 h time interval between ionizing radiation and HT. A shorter time interval between the two treatment modalities induces more cell kill. However, the sequence of applying ionizing radiation or HT did not result in a significant difference in apoptosis levels, not for any of the cell lines.

γ-H2AX foci levels are increased at higher temperatures and at shorter time intervals between RT and HT
DNA damages, specifically DNA double strand breaks, were measured by nuclear γ-H2AX staining, which may be very important to understand the earlier described differences in cell survival. The bar graphs in Figure 4 give the amount of DNA DSBs expressed as the fold-change of γ-H2AX foci after treatment for each of the three cell lines, and for three different temperatures and four different time intervals. In these graphs, the fold induction in γ-H2AX is presented, compared to the untreated sample. Although the fold change were different per cell line, the trend is equal in SiHa, HeLa and C33A cells. Levels of γ-H2AX are higher after HT at 42°C compared to 40 and 41°C. Furthermore, a shorter time interval between RT and HT resulted in higher γ-H2AX levels. Cells
were fixated and stained 24 h after treatment, indicating that there is more remaining DNA damage after a short time interval between the two therapies. In Figure 4B, SiHa cells demonstrate a higher number of γ-H2AX foci after 42°C than after 40 and 41°C, but there was no difference if HT preceded or followed irradiation.

Discussion and conclusion

The results of the present in vitro do not support that the sequence of RT and HT makes a major difference in terms of radiosensitization, since both sequences induce similar amounts of cell kill, similar changes in cell cycle distributions, similar induction of DNA damages and similar changes in apoptotic levels. However, the clinically relevant reduction in time interval from four to zero hours between HT and radiation clearly increased tumor

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**Figure 3.** Higher levels of apoptosis are detected after a shorter time interval between ionizing radiation and hyperthermia. Using the Nicoletti assay, apoptotic levels were measured. (A) Representative flow charts of apoptosis levels. (B) Higher levels of apoptosis were observed after a short time interval between ionizing radiation and hyperthermia compared to a longer time interval. Applying hyperthermia before or after ionizing radiation did not result in any significant differences on SiHa, HeLa or C33A cells. Untreated samples are marked as control (ctrl). Means with standard deviation of at least three replicates are presented.
Figure 4. Higher levels of DNA damage were observed after treatment with a shorter time interval between ionizing radiation and hyperthermia. (A) γ-H2AX levels are higher after a shorter time interval between ionizing radiation and hyperthermia. Also, hyperthermia treatment with a higher temperature increased the amount of γ-H2AX. (B) SiHa cells demonstrating the DNA damages using γ-H2AX foci.
cell kill which may be pivotal in tumor control and patient cure. This bigger effect of shorter intervals can be explained by an increase of cells with DNA damages, resulting in more G2-arrest and more apoptosis. Cell cycle checkpoints prevent cells from being replicated or divided if DNA is damaged, by prolonging cell cycle phases. Therefore, changes in cell cycle can indicate the presence of DNA damage. Treatments with a short time interval led to a higher percentage of cells in G2-phase, indicating that cells cannot pass the last check before cell division due to compromised DNA integrity. The percentage of cells in S-phase increased after longer time intervals (~4 h) between the two treatment modalities, suggesting cells are halted and damage is too severe to continue to the G2-phase. Moreover, for long time intervals, the fraction of cells in G1-phase decreases when time intervals get longer, perhaps cells that were halted in G1 recover after a certain time and are then released to the next phase, the S-phase.

All the in vitro data converge to the conclusion that the shortest time interval (0 h) between ionizing radiation and HT results in the lowest cancer cell survival. In three different cell lines, experiments were conducted independently and treated all at least three times.

HT has been described to downregulate BRCA2 (Krawczyk et al., 2011), a protein of the HR and as the HR requires a sister chromatid (Fleck & Nielsen, 2004), this pathway is not immediately activated once DNA damage occurs. The downregulation of BRCA2 lasts a few hours. Since blocking of HR by HT, when HT is applied before RT, did not result in different cell survival compared to the opposite order of treatment, it may indicate that mild HT with temperatures ≤ 42°C has minimal effects of the non-homologous end joining (NHEJ). The NHEJ is considered as an error-prone pathway, which is active during all phases of the cell cycle, and is furthermore also known as the fast DNA repair pathway (Fleck & Nielsen, 2004). The long duration of the radiosensitization found in our results supports this conclusion. Therefore, our results may suggest that mild HT mainly affects the slow DNA repair pathway, the HR.

Decades ago, similar experiments were performed on mammalian cell lines, in which Chinese hamster ovary (HA-1) and mouse mammary sarcoma (EMT-6) cells were investigated regarding the interaction of HT and ionizing radiation (Li & Kal, 1977). Li and Kal concluded that in HA-1 cells the radiosensitizing effect of HT was bigger when applied before RT; but in EMT-6 cells the opposite sequence had a larger effect. We did not find a preference for a sequence to achieve the most cell kill. In the studies with HA-1 and EMT-6 cells were treated for 1 h at 43°C, which already can result in direct cell kill, and received 600 rad (6 Gy); the highest radiation dose we gave was 4 Gy and to best simulate clinical practise, we gave not 1 h at 42°C or lower. Another explanation for the differences in sensitivity to ionizing radiation and HT between the previous study and our present study, may results from the
biological difference in rodent sarcoma-like tumor cell lines HA-1 and EMT-6 and the human carcinoma cell lines that we used.

A limitation of the present study is that we only studied effects in tumor cell lines, whereby we cannot exclude potential thermal enhancement in normal cells after radiation. Therefore, the next step is find the optimal balance between damaging the tumor and sparing of the normal tissue. This will require in vivo models. These in vivo models will not only provide additional information of the normal tissue toxicity, but they will also give the opportunity to establish the role of re-oxygenation within the tumor. In an in vivo model for mammary cancer heated to 42.5°C, Overgaard found a more pronounced enhancement in radiosensitization for HT treatment given before than after RT, which could be attributed to re-oxygenation of the tumor. He also found more pronounced enhancement in radiosensitization for simultaneous treatment (time interval 0 h) compared to - 0.5 h and 0.5 h which could also indicate that a faster DNA repair pathway was involved at that temperature (Overgaard, 1980).

The cell kill induced in our in vitro experiments represents two mechanisms: inhibition of DNA damage repair and direct cell kill. These data do not shed light on the effect of HT on reduction of the hypoxic fraction by direct cell kill, since studies on cells under hypoxic conditions were not included. Also the effect of re-oxygenation could not be evaluated as that requires the induction of a physiological response to heat and can therefore only be determined from in vivo experiments (Crezee et al., 2016).

In summary, HT is an effective tumor sensitizer for ionizing radiation. However, optimizing therapies is important to find the best clinical outcome. Our in vitro results demonstrated that the time interval between HT and radiation is an important factor that determines the effectiveness of this combinational therapy.

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Competing interests
The authors report no conflict of interest.
Materials and Methods

Cell lines and cell culture. The cervical carcinoma cell, SiHa, HeLa and C33A, were obtained from the American Type Culture Collection (ATCC). These cell lines were grown in EMEM, containing 25 mM Hepes (Gibco-BRL life technologies, Breda, The Netherlands) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM glutamine. Cells were maintained in a 37°C incubator with humidified air supplemented with 5% CO₂. The cell division time of these cells was approximately 24 h.

In vitro treatments. Cells were treated with either of the two sequences and 0, 1, 2, 3 or 4 h intervals between ionizing radiation and HT. Varying dose of ionizing radiation and temperatures of HT were used. HT was performed in a thermostatically controlled water bath (Lauda aqualine AL12, Beun de Ronde, Abcoude, The Netherlands) by partially submerging the culture dishes for approximately 1 h at 39, 41 or 42°C. In order to check the temperature, thermocouples were placed in parallel culture dishes and the desired temperature (±0.1°C) was reached in approximately 5 min. HT on all cells were performed in a 5% CO₂/95% air atmosphere, both with an air inflow of 2 L/min.

Cell survival assay. Clonogenic cell survival on SiHa, HeLa and C33A cells was studied to investigate the effect of different sequence and different time interval of applying ionizing radiation and HT. Cells plated four hours prior to treatment with different doses (0, 2, 4, 6, and 8 Gy) of ionizing radiation and several time intervals (0, 1, 2, 3 and 4 h) between these two therapies were investigated. Furthermore, these set ups were tested on varying temperature of HT (37, 40, 41 and 42°C). Surviving fractions were calculated by dividing the plating efficiency of treated cells by that of control cells ± SEM (van Bree et al., 2001). Statistical analysis was performed using a t-test.

Detection of DNA DSBs via γ-H2AX foci and γ-H2AX FACS. To study DNA damage in cells, γ-H2AX foci were performed. Cells were seeded, at a density of 300.000 cells per coverslip, 24 h before treatment with ionizing radiation (2 Gy) and HT (37, 40, 41 and 42°C) on sterile coverslips place in culture dishes. One day after treatment, cells were fixed for 15 min with PBS containing 2% paraformaldehyde for immunocytochemistry staining. After three times washing with PBS, cells were permeabilized during a 30-min incubation with TNBS (PBS containing 0.1% Triton X-100 and 1% FCS). Then cells were stained with a primary mouse monoclonal antibody anti-γ-H2AX (Millipore, dilution 1:100 in TNBS for 90 min at room temperature). Cells were washed once with PBS and two times with TNBS before staining with the secondary goat anti-mouse-Cy3 (Jackson-Immunoresearch, dilution 1:100 in TNBS for 30 min at room temperature, in the dark). Finally, vectashield-containing DAPI (Life technologies, USA) was dropped at the slide before placing the upside down on the slide. DSBs were scored under the fluorescence microscope.

To measure γ-H2AX on FACS stainings, cells were seeded one day before treatment at a density of 250.000 cells per petri dish. Cells were again treated with ionizing radiation (4 Gy) and HT (37, 40, 41 and 42°C). Fixation was performed at 24 h after treatment, in order to study the remaining γ-H2AX in cells after therapy. Cells were washed with PSB, trypsinised and afterwards centrifuged (1200 rpm/10 min), prior to fixation with
2 ml PBS and 100% 6 ml ethanol. Next day, ethanol was washed out with PBS. And cells were incubated on ice for 10 min with PBS containing 0.1% triton and 4% BSA. After centrifugation, cells were incubated for 60 min at room temperature with γ-H2AX-FITC (4 µg/ml, Merck Millipore, USA). Then cells were washed once with PBS, centrifuged and resuspended in PBS for FACS measurements. Samples were analyzed using the flow cytometry (FACS Canto, BD Biosciences, USA).

**Cell cycle analysis.** Experiments on of cell cycle distribution were performed using the thymidine analogue 5-Bromo-2'-deoxy-uridine (BrdU, Sigma Aldrich, USA). At 16 h after treatment cells incubated for 1 h with BrdU at 37°C, before washing with PBS, trypsinizing cells and transferring them to 15 ml tubes. After a centrifugation cells at 1200 rpm for 10 min were fixed in 2 ml PBS and 6 ml of 100% ethanol. The next day, cells were washed with PBS and centrifugated and the pellet was resuspended in pepsin-HCl (0.4 mg/ml, 0.1 N HCl) for 30 min at room temperature. Then, cells were washed using PBT (0.5% Tween-20, Sigma Aldrich USA in 0.5 l PBS) and after centrifugation, the pellet was resuspended in HCl (2 N, Merck) and cells were incubated 30 min at 37°C. After one washing with PBTg, the pellet is incubated with the primary antibody rat-anti-BrdU (Abcam, UK) diluted 1:100 in PBTb (1% bovine serum albumin, Sigma, in PBT) for 60 min at 37°C. After washing two times with PBT and once with PBTg, cells are incubated for 60 min at 37°C with a secondary antibody IgG goat-anti-rat FITC (Abcam, UK) diluted 1:100 in PBTg (1% normal goat serum, Dako, USA, in PBT). Eventually, propidium iodide (Sigma-Aldrich, USA) was added and cell suspensions were vortexed before analyzing samples with the flow cytometry (FACS Canto, BD Biosciences, USA).

**Apoptosis assay.** To study apoptosis after all treatment options, the Nicoletti assay (Riccardi & Nicoletti, 2006) was performed. Directly after treatment, cells were stored in a 37°C incubator with the desired percentage of CO₂. After 48 h, cells were collected and pellets were resuspended in Nicoletti buffer (0.1% w/v Sodium citrate, 0.1% v/v Triton X-100 in demi water, pH 7.4). Analyses were performed using flow cytometry (FACS Canto, BD Biosciences, USA). Differences were tested for using a t-test.
Supplementary Figures

**HT prior to RT**

**HT after RT**

![Graphs showing survival fraction vs dose for different temperatures and times of heat treatment](image)

- **HT39°C**
- **HT41°C**
- **HT42°C**

Legend:
- RT
- 0h
- 2h
- 4h
Chapter 5 | Temperature, sequence and time interval between RT and HT - in vitro

**HT prior to RT**

**HT after RT**

- HT39°C
- HT41°C
- HT42°C

Surviving fraction vs. Dose (Gy)

- RT
- 0h
- 2h
- 4h

Surviving fraction

Dose (Gy)

10^-3

10^-4

10^-5

10^-6

10^-7

10^-8

10^-9

10^-10

10^-11

10^-12

10^-13

10^-14

10^-15
Supplementary Figure 1. Clonogenic assays demonstrating that a shorter time interval increases cell death. Lower cell survival after a shorter time interval between ionizing radiation and hyperthermia is observed after performing clonogenic assays. A higher temperature of hyperthermia treatment resulted in lower cell survival. Three cervical cell lines are depicted in different colors: SiHa (red), HeLa (green), C33A (blue). A darker color indicated a longer time interval. Means with standard deviation are shown of at least three replicates. * p<0.05, ** p<0.01, *** p<0.001
References


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