Tumor cells can't stand the heat

Boosting the effectiveness of hyperthermia in cervical carcinoma

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Citation for published version (APA):
Oei, A. L. (2017). Tumor cells can’t stand the heat: Boosting the effectiveness of hyperthermia in cervical carcinoma.
Chapter 6

A short time interval between radiotherapy and hyperthermia reduces in-field recurrence and mortality in women with advanced cervical carcinoma

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Under review
Abstract

Purpose: To determine the effect of the time interval between external beam radiotherapy (EBRT) and same-day hyperthermia on in-field recurrence rate, overall survival and late toxicity in women with cervical cancer.

Materials and methods: Patients with advanced stage cervical cancer who underwent a full-course of curative daily EBRT and (4-5) weekly hyperthermia sessions between 1999 and 2014 were included for retrospective analysis. The mean time interval between EBRT fractions and same-day hyperthermia was calculated for each patient; the cohort was subsequently divided in a ‘short’ and ‘long’ time-interval group. Kaplan-Meier analysis and stepwise Cox regression were used to compare the in-field recurrence and overall survival. Finally, high-grade (≥3) late toxicity was compared across time-interval groups. DNA repair suppression is an important hyperthermia mechanism, DNA damage repair kinetics were therefore studied in patient biopsies to support clinical findings.

Results: Included were 58 patients; the median time interval between EBRT and hyperthermia was 79.2 min. The 3-year in field recurrence rate was 18% and 53% in the short and long time-interval group, respectively (p=0.021); the 5-year overall survival was 52% and 17% respectively (p=0.015). Differences between time-interval groups remained significant for both in-field recurrence (HR=7.7, p=0.007) and overall survival (HR=2.3, p=0.012) in multivariable Cox regression. No difference in toxicity was observed (p=1.00), with only 6 and 5 events in the short and long group, respectively. The majority of DNA damage was repaired within 2 h, potentially explaining a reduced effectiveness of hyperthermia for long time intervals.

Conclusions: A short time interval between EBRT and hyperthermia is associated with a lower risk of in-field recurrence and a better overall survival. There was no evidence for difference in late toxicity.

Novelty and impact

In clinical practice, time interval between radiotherapy and hyperthermia varies from 0.5 - 4 h. This retrospective study shows that time intervals of less than 80 min. resulted in significantly less in-field recurrences and better overall survival in women with advanced cervical cancer, while there was no evidence for an effect on late toxicity. Limiting the time interval to ~1 h is recommended.
Introduction

Cervical cancer is the fourth most common cancer in women worldwide, with 528,000 new cases and 266,000 deaths in 2012 (Ferlay et al, 2015). Standard treatment for locally advanced cervical cancer is cisplatin based chemoradiotherapy (CRT) (2008). Thermoradiotherapy, i.e. a combination of radiotherapy and hyperthermia, is a well-established alternative for patients with a contraindication for chemotherapy and provides similar overall survival (Eifel et al, 2004; Lutgens et al, 2010; Lutgens et al, 2016).

Clinical thermoradiotherapy generally consists of fractionated daily external beam radiotherapy (EBRT) and, during the same period, weekly locoregional hyperthermia. In hyperthermia, the tumor is heated to a temperature of 40-43°C for one hour. The rationale for adding hyperthermia to radiotherapy is that hyperthermia suppresses DNA double strand break (DSB) repair, the most lethal type of DNA damage caused by radiation treatment (Krawczyk et al, 2011; Oei et al, 2015). Additionally, hyperthermia also sensitizes radioresistant (hypoxic) tumors by increasing oxygen delivery (Sun et al, 2010; Vujaskovic & Song, 2004). This radiosensitizing effect increases the efficacy of the radiation treatment (Horsman & Overgaard, 2007). An EBRT fraction and hyperthermia session are usually given sequentially rather than simultaneously, since preclinical studies suggest that this results in the best therapeutic ratio (Horsman & Overgaard, 2007; Overgaard, 1980).

In clinical practice, the time interval between EBRT and hyperthermia treatment typically varies from 0.5-4 h for various reasons (Lutgens et al, 2016), such as availability of the treatment machines. Pre-clinical data suggest that longer time intervals between radiotherapy and hyperthermia reduce the efficacy of thermoradiotherapy (Li & Kal, 1977; Overgaard, 1980), but clinical evidence is scarce. Only two studies investigated the effect of time interval, and only for a small and heterogeneous series of superficial tumors (Arcangeli et al, 1983; Lindholm et al, 1987).

Aim of this retrospective study is to determine the effect of the time interval between EBRT and hyperthermia treatments on in-field recurrence, overall survival and late toxicity in cervical cancer patients. Furthermore, by examining the effect of time interval between EBRT and hyperthermia on the prevalence of DSBs in patient biopsies we demonstrate a potential mechanism supporting the clinically observed relationship.
### Table 1. Characteristics of the included patients, stratified by the mean time interval between RT and HT.

|                          | Short group (n = 30) | Long group (n = 28) | Statistical test | p-value |
|--------------------------|----------------------|---------------------|------------------|---------|
| **t_{int,mean} [min]**   | 65.8 (33.8-79.2)     | 91.7 (80.0-125.2)   | Mann-Whitney U test | <0.001 |
| **Age [y]**              | 67.5 (33-90)         | 65.0 (29-85)        | Mann-Whitney U test | 0.45   |
| **T_{90,mean} [°C]**     | 40.0 (38.6-41.9)     | 40.3 (38.2-41.1)    | T-test           | 0.71   |
| **HT duration\_mean [min]** | 60.0 (52.6-63.8)     | 60.8 (34.5-64.6)    | Mann-Whitney U test | 0.16   |
| **Warm-up time [min]**   | 5.2 (0.75-17.8)      | 8.5 (2.75-26.6)     | Mann-Whitney U test | 0.001 |

| Histology                | N        | %   | N        | %   |
|--------------------------|----------|-----|----------|-----|
| Squamous cell carcinoma  | 27       | 90  | 26       | 93  |
| Adenocarcinoma           | 3        | 10  | 2        | 7   |
| Figo stage | Chi-square test | 0.40 |
|------------|----------------|------|
| IB         | 3 10 4 14      |      |
| IIA        | 1 3 0 0        |      |
| IIB        | 6 20 8 29      |      |
| IIIA       | 2 7 5 18       |      |
| IIIB       | 14 47 10 36    |      |
| IVA        | 4 13 1 4       |      |

| Lymph node status | Fisher’s exact test | 1.00 |
|-------------------|---------------------|------|
| Negative          | 15 50 14 50         |      |
| Positive          | 15 50 14 50         |      |

| Number of hyperthermia treatments | Fisher’s exact test | 0.22 |
|-----------------------------------|---------------------|------|
| 4                                 | 5 17 9 32           |      |
| 5                                 | 25 83 19 68         |      |

| Smoking | Fisher’s exact test | 1.00 |
|---------|---------------------|------|
| Yes     | 7 23 7 25           |      |
| No      | 23 77 21 75         |      |
Results

Fifty-eight patients were included. The median time interval was 79.2 min, defining the short and long time-interval groups as $t_{\text{int,mean}} \leq 79.2$ min and $t_{\text{int,mean}} > 79.2$ min respectively. Out of all clinical and treatment characteristics, only warm-up time and the time interval itself were significantly different across time-interval groups (Table 1). Median follow-up for censored patients was 18 months (range, 2-130 months) for in-field recurrence and 37 months (range, 3-195 months) for overall survival.

The in-field recurrence rate and overall survival were significantly better in the short time interval group (Figure 1). The 3-year in-field recurrence rate was 18% (0-35%) in the short time-interval group and 53% (18-82%) in the long time-interval group. The 5-year overall survival was 52% (35-77%) in the short time-interval group and 17% (7-41%) in the long time-interval group; median overall survival was 61 months (38-83 months) and 19 months (13-26 months) respectively.

The last iteration of the stepwise Cox regression for in-field recurrence included three significant factors for a favorable outcome: a short time interval, advanced age and long warm-up time (Table 2). In the overall survival analysis,
the last iteration included three prognostically favorable factors: a short time interval (significant), high $T_{90,\text{mean}}$ (significant) and negative lymph node status (trend, not significant).

Six high-grade late toxicities were observed in the short time-interval group, compared to five high-grade toxicities in the long time-interval group (Table 3).

The γ-H2AX staining of patient biopsies showed a substantial increase in DSBs for the six samples fixated 15 min after irradiation, compared to control (Figure 2A). For the six samples fixated at two hours, the number of DSBs was similar to that of untreated samples (Figure 2B). Patients included in this retrospective study had time intervals between EBRT and hyperthermia ranging from 30 min to two hours. Thus, patients with short time intervals received hyperthermia when substantial DNA damage was still present, while patients with long time intervals received hyperthermia when the majority of DNA damage was already repaired.

**Discussion**

This is the first clinical study to demonstrate the effect of time interval on treatment outcome in patients with advanced stage cervical cancer. The results of both univariable and multivariable analyses indicate that a short time interval between EBRT and hyperthermia treatments results in a lower
in-field recurrence rate and better overall survival. Although the confidence intervals were substantial due to the limited size of the patient group, the estimated effect size was large enough to yield a significant result. At the same time, the results provide no evidence for an effect of time interval on high-grade late toxicity; a possible relationship may however have been obscured by the low number of toxicity events.

In addition to time-interval group, age is identified as a significant factor in the multivariable analysis, with older patients doing better (Table 1). This effect may be explained by our patient policy, whereby younger

| CTC-score | Short group | Long group |
|-----------|-------------|------------|
| <3        | Unspecified (16) | Unspecified (17) |
| 3         | Radiation cystitis (1), Pelvic fracture (1), rectovaginal fistula (1), local radiation ulcer (1) | Local radiation ulcer (1) |
| 4         | Secondary in-field malignancy (1), local radiation ulcer (1) | Radiation enteritis (1), complex/multiple (1), vesicovaginal fistula (1) |
| 5         | - | Gastrointestinal perforation (1) |
| Insufficient follow-up | 8 | 6 |

Table 3. Number of patients experiencing late effects, stratified by time-interval group. The γ-H2AX staining of patient biopsies showed a substantial increase in DSBs for the six samples fixated 15 min after irradiation, compared to control (Figure 2A). For the six samples fixated at two hours, the number of DSBs was similar to that of untreated samples (Figure 2B). Patients included in this retrospective study had time intervals between EBRT and hyperthermia ranging from 30 min to two hours. Thus, patients with short time intervals received hyperthermia when substantial DNA damage was still present, while patients with long time intervals received hyperthermia when the majority of DNA damage was already repaired. p=1.00
Figure 2. γ-H2AX foci staining of patient biopsies treated ex vivo. Presenting unrepaired DNA double strand breaks fixated at different time points after 4 Gy irradiation in patient biopsies.
patients usually received thermoradiotherapy instead of chemoradiotherapy because of hydronephrosis from large local tumors and/or extensive lymph node metastasis, whereas elderly women were often denied chemotherapy due to their generally frail condition. Warm-up time was also identified as a significant factor, where patients with longer warm-up time did better. Since, after warm-up, a fixed duration of steady state (60 min) is aimed for, patients with longer warm-up time will have a longer total heating time, and have therefore received a higher thermal dose. The observed significance of warm-up time can therefore be understood in view of the well-established correlation between thermal dose and clinical outcome (Franckena et al., 2009; Myerson et al., 1990; Oleson et al., 1993; Overgaard et al., 1996; Sherar et al., 1997; Wust et al., 1998). For overall survival, a high $T_{90,\text{mean}}$ was identified as a significantly favorable factor, also supporting a thermal dose effect relationship.

Biological studies in the late 70’s already suggested that the time interval between irradiation and hyperthermia affects outcome (Li & Kal, 1977; Overgaard, 1980). These in vitro and in vivo experiments have shown that the radiosensitizing effect of hyperthermia in tumor tissue decays substantially in the first 2h, in particular when EBRT is given before hyperthermia (Figure 2 in (Li & Kal, 1977) and Figure 5 in (Overgaard, 1980). These data support the difference in local control observed in this study. Additionally, Overgaard’s data suggest that the radiosensitizing effect decays even more rapidly in normal tissue, regardless of the order in which radiotherapy and hyperthermia are applied. If a substantial part of the radiosensitizing effect in normal tissue has already disappeared for a time interval of one hour, this could explain why no difference in late toxicity was observed between the short time-interval group (median 65.8 min) and the long time-interval group (median 91.7 min).

Two previous clinical studies investigated the effect of time interval in superficial recurrent and metastatic tumors of mixed primary origin. Lindholm et al compared time intervals of 0.5-1.5 h to 3-4 h and saw no significant difference between both groups in terms of either tumor response or skin toxicity (Lindholm et al., 1987). However, the short time-interval group in this study only included 15 tumors and time-interval groups were not comparable with respect to the numbers and mode of hyperthermia treatment. Arcangeli et al describe three trials involving hyperthermia (Arcangeli et al., 1983). The second trial compared hyperthermia immediately after radiotherapy, with delayed hyperthermia (4 h between treatments) and radiation alone. Local tumor control at 6 months was 5/7, 4/7 and 3/9 respectively. While these differences were not significant, this trend is in agreement with our finding that a short time interval improves local tumor control. Moist desquamation was observed in 64%, 46% and 36% respectively, suggesting that very short time intervals are best avoided. We did not observe differences in normal tissue toxicity in our cohort, but this
may be because hyperthermia was delivered immediately after radiotherapy in the short arm of Arcangeli’s trial, whereas in our cohort time intervals were around 1 h in the short arm.

In a more recent study, a more homogenous group of superficial tumors (all recurrent breast cancers) was studied (Linthorst et al., 2013). One factor that was investigated was whether patients received EBRT and hyperthermia in the same or in different institutes. For patients treated within a single institute, local control was worse (not significant) and late toxicity was increased (significant). However, although the factor ‘institute’ likely correlated with the time interval between treatments, it also reflects potential differences in patient selection and treatment. Any relation between this factor and treatment outcome thus cannot be exclusively attributed to an effect of time interval.

Multiple mechanisms have been suggested for the radiosensitizing effect of hyperthermia (Crezee et al., 2016). A mechanism particularly important for hyperthermia after irradiation, is the ability of heat to interfere with DNA damage (Krawczyk et al., 2011; Oei et al., 2015). This mechanism can only be effective if unrepaired DNA damage is still present. Radiobiological studies on clinical data have shown that the exponential repair time constant for radiation damage is roughly 1.5h (Fowler et al., 2001; Joiner, 2008; Kal & Van Gellekom, 2003; Roberts et al., 2004). A similar repair time is suggested by the results of our experiments on patient biopsies. While a substantial amount of DNA DSBs was observed 15 min after irradiation (Figure 2A), almost all DNA damage was repaired after two hours (Figure 2B). As time intervals in the long time-interval group ranged up to 2h, our biopsy data could explain why hyperthermia is less effective in this group: since much of the radiation damage has already been repaired when hyperthermia is given, the efficacy of the repair-blocking mechanism is substantially reduced. In contrast, a substantial amount of unrepaired damage is still present at the time of hyperthermia treatment in the short time-interval group, thus the repair-blocking mechanism is effective.

The Dutch Deep Hyperthermia Trial (DDHT), which compared radiotherapy to thermoradiotherapy in advanced stage cervical cancer, reported a 3-year overall survival of 27% for the radiotherapy group and 51% for the thermoradiotherapy group (van der Zee et al., 2000). In our study, the 3-year overall survival for all 58 patients was 48% (36-64%), almost similar as in the DDHT. However, 3-year overall survival was considerably lower in the long time-interval group at 34% (20-59%) compared to 62% (46-84%) in the short time-interval group. Comparison of the two series could lead to two conclusions. First, the overall survival in the long time-interval group was close to that of the radiotherapy-alone arm in the DDHT trial, suggesting that long time-interval patients had very little
benefit from the hyperthermia treatment. Second, if a short time interval can be ensured for all patients, an additional improvement of approximately 10% in overall survival of the results in the DDHT may be attained.

Our findings may have important consequences for treatment policy of women with inoperable cervical cancer. Although underpowered, results from a trial comparing standard chemoradiotherapy with thermoradiotherapy in women with inoperable cervical cancer suggest that chemoradiotherapy and thermoradiotherapy are equally effective, with approximately 60% long term event free survival in both arms (Lutgens et al., 2016). However, time interval between EBRT and hyperthermia in this trial ranged from 1-4 h, which may have resulted in sub-optimal results for the thermoradiotherapy group. Thus, thermoradiotherapy could potentially be even more effective than standard chemoradiotherapy, provided a short time interval between EBRT and hyperthermia is ensured. This hypothesis would need confirmation through a clinical trial, and would require EBRT and hyperthermia to be delivered with a short time interval.

The importance of a short time interval has implications for clinical practice. In recent years an increasing number of patients received hyperthermia at our institute, but radiation treatment elsewhere (5 out of 11 patients in 2014). This is more convenient for patients who live close to a radiotherapy center, but far from a hyperthermia facility, since these patients then only have to travel to the hyperthermia center once a week. However, considering the substantial reduction in efficacy of the hyperthermia, delivering both treatments in separate institutes should be strongly discouraged. A solution would be for patients to receive EBRT on the day of hyperthermia within the same institute, while all other EBRT treatments are delivered in a center closer to the patients residence. However, this requires radiotherapy treatment plans to be designed for both institutes and will yield additional workload. Even when both treatments are given within a single institute, long time intervals should be avoided. While the optimal time interval cannot be established based on the current data and an increase in normal toxicity may be expected for very short time intervals based on pre-clinical data, a time interval of 1 h appears to be a reasonable tradeoff between feasibility, efficacy and safety (Li & Kal, 1977; Overgaard, 1980).

In conclusion, a short time interval between EBRT and hyperthermia is associated with a lower risk of in-field recurrence and a better overall survival. Efficacy is reduced for longer time intervals, likely because of the reduced amount of unrepaired DNA damage present at the time of hyperthermia treatment. There was no evidence for a difference in long-term toxicity, however, the low number of events in both arms means that statistical power is limited. Limiting the time interval between EBRT and hyperthermia to approximately one hour is recommended.
Materials and Methods

Patient population. Included were patients treated at the Academic Medical Center for cervical cancer (ICD-9: 180, ICD-10: C53) with curative thermoradiotherapy, between January 1999 and January 2014. Excluded were patients who received concurrent chemotherapy and patients who received less than four out of the intended five hyperthermia sessions. Patients who received EBRT at other institutes were also excluded, because the variation between institutes (e.g. different treatment guidelines, radiation schedules and techniques) would have introduced too many potential confounding factors. All patients had a histologically confirmed cervical carcinoma, and were staged by FIGO clinical staging, including investigation under general anesthesia with cystoscopy, and lymph node staging by imaging (CT, MRI and/or PET). Patients with bulky lymph nodes (>2cm) received a lymph node debulking first. Patients were referred for primary radiotherapy with hyperthermia for locally inoperable tumors (large FIGO IIB tumors, IIIA, IIB and IVA) and for lymph node positive patients with FIGO IB and IIA. Since 2005, chemoradiotherapy became the standard treatment, and thermoradiotherapy was reserved for patients with a medical contraindication for cisplatin-based chemotherapy (i.e. hydrenephrosis, renal insufficiency, poor performance, old age). In-field recurrence, overall survival and late toxicity data were extracted from patient files. Subsequently, overall survival for patients who were Dutch citizens was updated using the Dutch civil registry. Late toxicities, occurring or persisting at least 6 months after completion of thermoradiotherapy, were scored according to CTCAE v4.0. Only high-grade (≥3) toxicities were analyzed, since retrospective analysis of low-grade toxicity is less reliable.

Treatment. Treatment consisted of daily EBRT (23x2Gy or 28x1.8Gy), five (occasionally four) weekly hyperthermia treatments, followed by a pulsed dose rate brachytherapy boost (24Gy). Initially, EBRT was delivered using 3D conformal techniques, with a transition to IMRT in 2011. Hyperthermia was delivered during the period of EBRT treatment, approximately 1 h after the corresponding EBRT fraction. Hyperthermia was delivered by the AMC-4 phased array system, a 70MHz radiofrequency heating system designed for deep-seated tumors (van Dijk et al, 1990).16 Temperature was monitored during treatment using intracavitary multisensor thermocouple probes. A steady state duration of 60 min was aimed for, where the start of the steady state period is defined as the moment when (after an initial warm-up period) one of the temperature sensors in the target region reached 41°C. If a temperature of 41°C was not reached within the tumor after a 30 min, start of the steady state was defined as 30 min after start of the warm-up period.

Statistical analysis. Since multiple hyperthermia treatments are delivered, treatment of a patient is not characterized by a single time interval between EBRT and hyperthermia. Thus, for each patient, the mean time interval (tint,mean) between their hyperthermia treatments and corresponding EBRT fractions was calculated. The median of tint,mean was then used to split the population into a ‘short’ and a ‘long’ time-interval group. The following patient and treatment characteristics were described for each time-interval group. Pre-treatment variables: age, histology, FIGO stage, lymph node status, smoking status. Hyperthermia parameters: tumor temperature (T90,mean), the steady state duration (HT duration mean), warm-up time, and number of hyperthermia...
treatments. $T_{90,\text{mean}}$ represents the temperature reached in at least 90% of the tumor volume, averaged over each patient’s hyperthermia treatment series. Warm-up time was defined as the time between the start of power on to the start of the steady state. Differences between both time-interval groups in terms of these patient and treatment characteristics were tested for using Fisher’s exact test, the Chi-square test, the independent samples t-test and the Mann-Whitney U test depending on the type of data. In-field recurrence rate and overall survival were calculated by the Kaplan-Meier method, and groups were compared by the log-rank test. Time to event (in-field recurrence or death) and censoring were calculated from the date of diagnosis. Multivariable analysis of in-field recurrence and overall survival was done by (backwards) stepwise Cox regression, including time-interval group, age, $T_{90,\text{mean}}$, HT duration$_{\text{mean}}$, histology, FIGO stage, lymph node status, number of hyperthermia treatments and smoking status as factors. A Fisher’s exact test was used to test for differences in the incidence of high-grade toxicity between time-interval groups. All analyses were performed using SPSS version 23, all tests were two-sided and p<0.05 was considered significant. Accuracy of statistical estimates is reported using 95% confidence intervals.

**Patient biopsies.** An effect of time interval on clinical outcome could be related to the amount of unrepaired DNA DSBs present at the time the hyperthermia is given, since DSB repair suppression is an important mechanism for the radiosensitizing effect of hyperthermia. To investigate this, experiments were performed on 12 cervical carcinoma biopsies (AMC/MEC 03/137). After informed consent was given, biopsies were obtained from patients diagnosed in 2015 with advanced stage cervical cancer and eligible for thermoradiotherapy. Biopsies were divided in two parts: one half was treated ex vivo with RT (4Gy), the other was left untreated (control). Six samples were fixated at approximately 15 min and six samples at approximately 2h after RT. After treatment, biopsies were submerged in paraformaldehyde, to be used for paraffin coupes. Before antigen retrieval, they were deparaffinized and rehydrated. Afterwards a heat-induced antigen retrieval at pH 9.0 for 20 min was performed, followed by a 30-min cooling period. Next, a 15-min PO block including H2O2 was performed. Then coupes were incubated overnight at 4°C with γ-H2AX mAb (Millipore, Merck). Next, tissue was embedded in Alexa Fluor 488 (Invitrogen Life Technologies), after washing with PBS. DAPI was used to stain the nuclei blue before covering tissue with a drop of ProLong Gold anti-fade reagent (Invitrogen Life Technologies) and a coverslip.

**Competing interests**
Authors declare that they have no competing interests.

**Acknowledgements**
This work was supported by the Dutch Cancer Society (UVA 2012-5540).
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