**Evaluation of radiation response to different dose rates,
with/without mild hyperthermia, in human breast carcinoma cell lines
of different radiosensitivities**

by

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Faculty of Graduate Studies and Research
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for the degree of

Doctor of Philosophy

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ABSTRACT

Computer-controlled radiotherapy technology has motivated the investigation of pulsed dose rate as a replacement for conventional low dose rate treatment in brachytherapy. To determine whether a pulsed dose rate (PDR) treatment of 1.5 Gy given every 3 hours was equivalent to a 0.5 Gy/h low dose rate (LDR) treatment, human breast carcinoma cells of different radiosensitivities (MCF7 and C716), with the resistant C716 overexpressing DNA polymerase β , were irradiated *in vitro* using a variety of biological endpoints. The treatments were also combined with mild hyperthermia to determine if radiosensitization due to hyperthermia occurred. The survival of the cells was assessed using the clonogenic assay, the amount of residual double stranded breaks was determined by the clamped homogeneous electric field and comet assays, and gene expression of polymerases α , β , δ , and ϵ was investigated using RT-PCR. The data showed that for both cell lines there were no significant differences between the pulsed dose rate and low dose rate treatments in terms of all the assays used. From RT-PCR results, it was concluded that possibly the polymerase ϵ may be involved in repair of LDR irradiation damage, and that another pathway, possibly involving PCNA (proliferating cell nuclear antigen) may be involved. For the pulsed dose rate and low dose rate experiments with mild hyperthermia, it was determined that hyperthermia provided significant radiosensitization in both cell lines investigated, and the combination treatments were significantly different from the PDR and LDR treatments alone in terms of the clonogenic and comet assays. In terms of RT-PCR, there were no significant differences between the hyperthermia combination treatments versus the uncombined treatments

except for the α polymerase immediately following PDR irradiation in the C716 cell line. From these experiments it was also concluded that the polymerase ϵ may be involved in repair of radiation damage, however, other DNA polymerases should also be investigated. Although LDR and PDR irradiations were found to be equivalent, it was suggested that caution should be used in the clinic since literature suggests that the equivalence may be cell line dependent.

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LIST OF ABBREVIATIONS

BER	Base Excision Repair
CHEF	Clamped homogeneous electric field
CP	Crossing point (inflection)
DSB	Double-stranded break
DNA	Deoxyribonucleic acid
FAR	Fraction of activity released
Gy	Gray, absorbed dose per unit mass [1 Gy = 1 J/kg]
HDR	High dose rate
ICL	Interstrand Cross-Links
LDR	Low dose rate
LQ	Linear-quadratic (model)
M	Molar
MMR	Mismatch repair
NER	Nucleotide Excision Repair
PCNA	Proliferating cell nuclear antigen
PDR	Pulsed dose rate
PE	Plating efficiency
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SEM	Standard error of the mean
SHM	Somatic hypermutation
SLD	Sublethal damage
SF2	Surviving fraction after 2 Gy
SSB	Single-stranded break
TLS	Translesion synthesis

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1.0 INTRODUCTION

There is strong evidence to indicate that DNA is the principal target for the biological effects of radiation. When cells are irradiated, many breaks of single and some of both strands may occur. In intact DNA single-strand breaks are of little biological consequence as far as cell killing is concerned because they are readily repaired using the opposite strand as a template. In contrast, a double-strand break caused by one ionizing event, may result in cell killing, mutation, or carcinogenesis. If both strands of the DNA are broken, and the breaks are well separated (by more than 20 bases), repair again occurs readily since the two breaks are handled separately. However, if the breaks in the two strands caused by two separated ionizing events are opposite one another, separated by only a few base pairs, this may lead to a double-strand break. The difference between a double-strand break caused by one ionizing event and that caused by two separate ionizing events is that in the latter case the first break (called sublethal damage) can be repaired under certain conditions before the second break occurs (1). These conditions include low dose rate irradiation and fractionating, or pulsing, the dose (pulsed dose rate irradiation). Hence, even though the same total dose may be given for two different exposure conditions, the one where the exposure is spread out in time reduces the effect (2). On the other hand, there are also certain agents that may prevent the repair of the first break before the second occurs under low dose rate or pulsed dose rate irradiation conditions. Such agents are hyperthermia and chemotherapy drugs, both of which inhibit the repair of sublethal damage, so the interaction between radiation and these agents results in double-strand breaks. A double-strand break is believed to be the most important lesion produced in the DNA by radiation, as the interaction of two double-

strand breaks may result in cell killing, mutation, or carcinogenesis (1). This study investigates low dose rate and pulsed dose rate irradiations, as well as the capacity for inhibition of sublethal damage repair by combining these treatments with 41°C hyperthermia.

1.1 Low Dose Rate Irradiation

Although a wide range of doses have been used in radiation biology and radiation therapy (from a few cGy per day to hundreds of Gy in a fraction of a second), the dose rate of importance in radiotherapy extends from about 0.1 Gy/h to several Gy/min (3). Clinical radiotherapy is mostly concerned with either high dose rate (HDR) for external beam therapy or low dose rate (LDR), which is the dose rate commonly used in brachytherapy (although HDR is also used). Brachytherapy (brachy is Greek for “short range”) is a type of treatment in which radioactive sources are placed either in a body cavity in close proximity to the tumour (intracavitary) or directly into the tumour volume (interstitial) (1). The advantage of LDR brachytherapy over external beam irradiation is that the malignant tissue receives a very high dose while the dose to normal tissues is minimized, allowing their sparing. A typical prescribed dose is 30 Gy delivered over a couple of days (4). The total dose is usually adjusted for dose rate, since clinical studies show that both tumour control and effects on the normal tissues vary with the dose rate for a given dose (1). Conventional brachytherapy treatment protocols typically use a 0.4 to 2 Gy/h dose rate, 0.5 Gy/h being most common, with normal tissue tolerance being the limiting factor. Another factor limiting the maximum dose due to normal tissue tolerance is the difference in the rate of repair of sublethal damage (SLD) between malignant and

normal tissues (2). As previously mentioned, SLD can be repaired under normal conditions unless additional SLD is added from a second dose of radiation with which it can interact to produce lethal damage (1,5,6). Sublethal damage, SLD repair and consequently the resulting dose-rate effect can be best observed on the cell survival curve, which describes the relationship between the radiation dose and the proportion of irradiated cells that survive by retaining their reproductive integrity. Figure 1.1 shows two sample survival curves that illustrate the dose-rate effect. The lower curve is a high dose rate (HDR) irradiation response with a significant shoulder, which reflects accumulation of SLD. When the dose rate is progressively reduced, the slope of the survival curve begins to lose its shoulder as a greater proportion of SLD is repaired (3,7,8) until a limiting linear slope is reached, reflecting complete repair of SLD (the top curve in Figure 1.1). The dose-rate effect caused by SLD repair is most dramatic in the 0.01 to 1 Gy/min range, and its magnitude varies greatly between different types of cells (1).

Figure 1.1. Sample LDR and HDR survival curves

1.2 Pulsed Dose Rate Irradiation

The motivation for the development of pulsed dose rate (PDR) brachytherapy was to take advantage of new technology in the form of computer-controlled remote afterloaders (9). An irradiator based on this principle consists of a single high-activity source (usually ^{192}Ir) that is stepped, under computer control, through the catheters of an implant, with dwell times in each position adjusted to obtain the required dose distribution. The incentive to initiate PDR brachytherapy, and to test its feasibility as a replacement for conventional LDR brachytherapy, is the desire to combine the mainly radiobiological advantages of LDR brachytherapy with the mainly practical advantages of the available technology. The advantages of PDR brachytherapy are as follows (9,10):

1. The radiobiological advantages of LDR brachytherapy such as a high level of local control in combination with optimum sparing of normal tissues are retained, as well as there is safety convenience for nursing and for the patient in terms of visiting and mobility during the interval between doses. The interval between doses also offers the possibility of equipment sharing by several patients.
2. The effective dose rate of the treatment can be chosen, independent of the implant volume and the strength of available sources. A stock of sources for manual LDR brachytherapy has to be maintained, and limitations in the available stock can result in undesirable variations in the effective dose rate depending on the catheter separations and the volume of the actual implant. In contrast, a stepping source afterloader enables brachytherapy applications with a constant effective dose rate.

3. The potential for computer-optimized dose distributions, achieved by varying the dwell times of the stepping source. This can be also achieved in the course of the treatment, as well as during the planning stage.
4. PDR treatments offer the possibility to combine brachytherapy with another treatment modality (such as hyperthermia or chemotherapy drugs), applied during the intervals of the PDR treatment.

In a pulsed dose rate treatment, low dose rate is simulated by a series of short high dose rate pulses separated by equally spaced time intervals, such that the overall treatment time and average dose rate is the same as that of continuous low dose rate irradiation (10). Hence, this would offer the radiobiological advantages of low dose rate irradiation (dose-rate effect and normal tissue sparing) if a combination of radiation pulse widths and time intervals that would result in equivalence to LDR irradiation could be found. However, there is need for caution because late responding tissues, such as those in which radiation damage might not be evident until at least six months after treatment, are more sensitive to changes in fractionation patterns (11). Also, although studies are directed at finding equivalence of PDR to LDR irradiation, ultimately, the goal of the treatment is to deliver as high a dose to the tumour as possible while minimizing damage to surrounding normal tissues.

Originally, the theoretical basis for comparison of low dose rate to pulsed dose rate brachytherapy was proposed by Brenner and Hall in 1991 (4). In their paper they analyzed data from 36 cell lines of human origin, as well as late-responding normal tissues in vivo, to which they applied the linear-quadratic formalism (see Materials and Methods). Their results suggest that a pulsed regimen consisting of 0.5 Gy delivered in

10 min (using a dose rate of 3 Gy/h) and repeated every hour would be functionally equivalent to continuous low dose rate irradiation of 0.5 Gy/h. A study by Fowler and Mount that also used the linear-quadratic formalism to predict optimum PDR schedules suggested that pulses of 0.5 Gy given in 10 min to half an hour, as often as once an hour, would also maintain the equivalence between PDR and LDR irradiations (12). This study implied that conditions for equivalence would be met for pulse repetition frequencies up to 2 hours and dose-rates of up to 3 Gy/h. A study by Visser, using the linear-quadratic model as well, agrees with these predictions, and it also suggests that longer pulse intervals of up to 3 hours, keeping the overall average dose rate at 0.5 Gy/h, could be used to obtain PDR-LDR equivalence (10). A longer time interval between pulses would be more convenient in the clinic in terms of equipment sharing, allowing treatment of several patients during this time interval.

There have been many publications of experimental data, both in vitro and in vivo (including in the clinic), on the PDR-LDR equivalence since these theoretical predictions that were based on experimental findings (11,13-21). Although most studies show agreement with the theoretical findings, not all agree, showing that the equivalence between the two treatments has a strong dependence on cell line or tissue. What follows is a summary of published experimental data on the equivalence between pulsed dose rate and low dose rate irradiation.

The earlier studies on experimental PDR-LDR irradiation equivalence were performed using rodent cell lines. A study using 9L gliosarcoma rat cells by Armour et al showed equivalence between PDR and LDR irradiation using an overall average dose rate of 0.5 Gy/h and several fractionation schemes with a dose rate of 66 Gy/h per pulse.

Cell sensitivity using 0.25, 1.00, and 3.00 Gy every 0.5, 2, and 6 hours was identical to that for low dose rate. However, increased cytotoxicity to the tumour cells was observed when the fraction size was increased to 6 Gy delivered every 12 hours (13). This early study showed encouraging results for clinical application, especially from a practical point of view, since a time interval of up to 6 hours between fractions would allow the treatment of many patients. In addition, this study also used a higher dose rate per pulse than was originally determined theoretically, and was still able to maintain the equivalence.

Another study with a rodent cell line (V79) by Fritz et al compared the responses of cells grown as a monolayer, as well as spheroids, which would better simulate an actual tumour. An overall dose rate of 0.94 Gy/h was used in this study, with 0.94 Gy hourly pulses, and with 3.76 Gy delivered every 4 hours. The spheroid model showed that the hourly fractionation was indistinguishable from the low dose rate irradiation, however, increased cytotoxicity was observed when using the 3.76 Gy pulse every 4 hours. The monolayer system, on the other hand, showed increased radiosensitivity to both PDR protocols. The difference between the two responses of the two cell models may be due to the fact that cells grown as spheroids are less radiosensitive than cells grown as a monolayer (14).

A study by Erickson and Shadley investigated the effect of changing the length of a particular PDR pulse. They irradiated rodent cells (mouse SCC VII) at a higher overall dose rate of 1.12 Gy/h. The PDR schemes investigated consisted of hourly 1.12 Gy pulses, but delivered over 5, 10, and 20 min, as well as a 2.24 Gy irradiation pulse delivered over 20 min every 2 hours. This study concluded that all PDR regimens showed

increased cell kill relative to continuous LDR irradiation; however, the increases were not statistically significant (15). An important conclusion from this study, as well as from that of Fritz et al, is that doubling the theoretically proposed dose rate (to 1 Gy/h) appears to have no significant effect on the PDR-LDR equivalence.

Chen et al were among the first to investigate the effect of PDR as compared to LDR brachytherapy on human cells (cervical carcinoma HTB35, and breast carcinoma MCF7 and T47D). This study used an overall average dose rate of 0.6 Gy/h with PDR regimens of 0.6 Gy/1h, 1.2 Gy/2h, 1.8 Gy/3h, 2.4 Gy/4h, 3.6 Gy/6h, and 7.2 Gy/12h (i.e. 0.6 Gy given every hour, 1.2 Gy every 2 hours, etc.), and tested for equivalence in exponentially growing cells. This study showed that there is variability in response among cell lines, with the HTB35 cells showing PDR-LDR equivalence only for the 0.6 Gy/1h scheme, and the MCF7 and T47D cells showing equivalence for the 0.6 Gy/1h and 1.8 Gy/3h fractionation schemes. In addition, the MCF7 showed equivalence for the 3.6 Gy/6h pulse while the T47D cells showed equivalence for the 2.4 Gy/4h pulse (16).

One study with human tumour cells investigated the effect of using two different dose rates during the pulse, 4.25 Gy/h and 63 Gy/h, and changing the time interval between pulses, while keeping the overall treatment time and average dose rate (0.8 Gy/h) constant. This study by Pomp et al using neuroblastoma HX142 and bladder carcinoma RT112 cell lines used fractionation schemes of 0.74 Gy/1h, 1.92 Gy/3h, and 3.2 Gy/6h. For the hourly pulses no significant difference was found in survival between the PDR using the lower dose rate per pulse (4.25 Gy/h) and continuous LDR irradiation in both cell lines. However, survival decreased with increasing dose per pulse, and also even further with increased dose rate per pulse (17). These results show that the PDR

irradiation using the higher dose rate is biologically more effective than continuous LDR irradiation, something that should be taken into account in the clinic.

Another study used a model of tumour cell lines of different radiosensitivities and a normal cell line to investigate how the PDR and LDR treatments compared. The cell lines used were radioresistant and radiosensitive carcinoma cell lines of similar genetic background (A2780cp and A2780s, respectively) and normal fibroblasts (AG1522). They were treated with 0.53 Gy/h LDR irradiation and PDR pulses of 0.53 Gy/1h, 1.1 Gy/2h, 1.6 Gy/3h, 2.1 Gy/4h, and 3.2 Gy/6h. The radioresistant carcinoma cell line, A2780cp, showed PDR-LDR equivalence for all but the largest two pulses used, while the normal cell line showed no equivalence at all, with the PDR treatment being more effective than the LDR. The most interesting result, however, was that for the radiosensitive A2780s parental cell line, which showed adaptive response for all the PDR pulses administered, the cells were becoming resistant to radiation between each pulse (18). The results from this study, therefore, suggest caution when applying PDR irradiation in the clinic, since some normal cells may be more radiosensitive to PDR than LDR irradiation, as well as adaptive response may be induced in some tumours through PDR irradiation. Another study undertaken at the same laboratory with a glioma cell line, U87MG, using the same irradiation protocols showed that the PDR treatments were less effective than LDR irradiation in terms of cell killing, concluding that adaptive response is the most likely explanation for this effect (19,20). Adaptive response of cells to pulsed dose rate irradiation was also found in a Dutch study (21). Human squamous lung carcinoma cell line SW-1573 was irradiated with 0.1 Gy every 6 min, resulting in a mean dose rate of 1 Gy/h. This study observed that following treatment the activities of enzymes

deoxycytidine kinase and thymidine kinase 1 and 2 were increased 1.5-2 fold 6 hours after treatment (compared to untreated controls), and then returned to control levels 24 hours later. It was suggested that this increase of enzyme activities was part of an adaptive response to irradiation. The findings from these studies showed some serious implications for the use of PDR brachytherapy in the clinic, with greater biological effectiveness of the PDR treatment over that of LDR in normal cells, and with adaptive response with some tumour cell lines.

In summary, in vitro data shows that the equivalence between PDR and LDR irradiation is cell line-dependent, even at the smaller fraction sizes. In addition, adaptive response may present a serious problem in the clinical application of PDR brachytherapy.

There have also been a number of in vivo studies published using animal systems to investigate the equivalence between pulsed dose rate and low dose rate irradiation, using both acute and late responding tissues. Mason et al used jejunal stem cells (acute responding normal tissues) to show that for an average dose rate of 0.7 Gy/h, the dose given over a 10 min (4.2 Gy/h) hourly pulse resulted in biological equivalence of pulsed to continuous treatment, while the same dose delivered in 1 min (42 Gy/h) resulted in a modest 3-4% decrease in survival. This study therefore validates the prediction between pulsed and continuous brachytherapy at a clinically relevant average dose rate (22). Another in vivo animal study (Brenner et al) compared the two treatments using a late responding system, namely the induction of cataracts in the rat lens. Using an overall average dose rate of 0.6 Gy/h, it was found that 10 min pulses each hour, 10 min every 4 hours, and 100 second pulses every hour showed no significant differences from the LDR protocol (11). These two in vivo studies, therefore, validate the notion that practical PDR

treatments can be designed that are biologically equivalent to conventional LDR, not only in terms of early responses such as tumour control, but also in terms of late effects on normal tissues, which are often more serious.

Recently, the biological effectiveness of PDR has been investigated in clinics across Europe and the United States. One of the earliest studies reported the results of using pulsed dose rate brachytherapy in the treatment of anal carcinoma. This study used hourly 0.6 Gy pulses for a total of 42 pulses (25.2 Gy total dose), and found the treatment to be highly effective, however since there was substantial toxicity, it was recommended to decrease the pulses to 0.5 Gy and to reduce the total dose to 20 Gy (23). This conservative regimen in the treatment of anal cancer was followed by a recent French study. It was found that using 10-min pulses with 0.5 Gy delivered every hour (10-25 Gy total dose) was comparable to LDR brachytherapy results found in the literature for this type of malignancy (24). Another study of pelvic malignancies also used the conservative approach of hourly 0.40-0.85 Gy delivered in 10-20 min with no significant increase in acute toxicity compared with the low dose rate protocol. They concluded that even though there was excellent local tumour control, caution should be used when attempting to increase the dose per pulse, as well as the time interval beyond 4 hours, or decreasing the pulse length to less than 10 min (25).

In another clinical application, pulsed dose rate was employed as salvage treatment of locally advanced or recurrent gynecologic cancer. Jensen and colleagues used hourly 0.6 Gy pulses and found this to be effective, except for increased toxicity in patients with large treatment volumes and recurrent disease. They recommend the treatment, but suggest informing patients about the increased toxicity (26). Rogers et al

also applied pulsed dose rate brachytherapy to gynecological malignancies with favourable results. A median dose rate of 0.55 Gy delivered every hour was used to treat cervical carcinoma, with a range of 0.40 to 0.70 Gy per pulse (27).

There have also been studies of pulsed dose rate brachytherapy with head and neck malignancies. A clinical trial performed by a Dutch group used pulsed dose rate brachytherapy with 8 fractions per day of 1.5 Gy delivered every 3 hours (1.5 Gy/3h) to treat squamous cell carcinoma of tonsillar fossa and soft palate. Their results were found to be comparable to those of LDR treatments in the literature and therefore support the replacement of LDR by PDR brachytherapy (28). A Swiss study found that a regimen of hourly 0.4-1 Gy pulses (median pulse 0.5 Gy) supports the PDR-LDR brachytherapy equivalence with head and neck malignancies, as well as pelvic and breast cancers (29). A German study also found support for the equivalence in the treatment of head and neck malignancies using 0.5 Gy or 0.7 Gy pulses every hour (30), as well as a French group using 0.5 Gy hourly pulses (31).

In the treatment of breast cancer, in addition to the favourable Swiss study already mentioned (29), 0.5-1 Gy/pulse/h brachytherapy was found to be effective with a high local control rate and acceptable toxicity by a recent German study (32,33).

In summary, clinical data shows that there is pulsed dose rate to low dose rate brachytherapy equivalence with the conservative 0.5 Gy hourly-pulsed regimen originally proposed by Brenner and Hall (4). However, there is tissue-dependent response to changing the size of the pulses and the interfraction interval, which was also observed in in vitro findings. The question therefore remains, as to why some cell lines show PDR and LDR irradiation equivalence and some do not. Furthermore, some studies using cells

from the same genetic background found that one cell line shows PDR-LDR equivalence while the other cell line does not. To address that, mechanisms of radiation damage repair should be studied, to investigate whether there are different mechanisms of repair of PDR and LDR irradiation and how these mechanisms of repair differ in isogenic cell lines with different radiosensitivities. In addition, the mechanisms of adaptive response should also be investigated, since there would be a detrimental impact on using PDR brachytherapy in the clinic if tumour cells developed radiation resistance and normal cells did not.

1.3 Hyperthermia

Hyperthermia is achieved by heating the body or localized parts of the body to temperatures above the normal 37°C. Regarding cancer therapy, based on a substantial body of data, it is generally agreed that local hyperthermia alone has little role in the curative treatment of tumours (1). However, data from animal studies and clinical trials have shown that hyperthermia is primarily efficacious when applied in combination with radiotherapy (34-36). Research on the response to combined treatments of hyperthermia and radiation of mammalian cells cultured in vitro supports the clinical findings and further experimentation is leading to characterization and understanding of the role of many factors (such as pH, oxygen, cell cycle distribution, etc.) in thermal and radiosensitivity (37,38). One of the mechanisms of radiosensitization is believed to be through the inhibition of sublethal damage repair (39-43). In low dose rate brachytherapy, where extensive repair of SLD occurs, the effectiveness of the treatment would be enhanced by the simultaneous application of heat. In fact, some in vitro studies done primarily with rodent cells show that hyperthermia is a more efficient radiosensitizer

when combined with low dose rate than with high dose rate irradiation due to inhibition of SLD repair, which occurs during LDR, but to a lesser extent during HDR (36,44,45). Studies with animal tumours also show the effectiveness of hyperthermia as a radiosensitizer in combination with brachytherapy, with hyperthermia usually administered before or after irradiation, although one study showed that hyperthermia at the midpoint of the brachytherapy treatment was most effective, indicating the possibility of simultaneous treatments (36).

Although many studies have demonstrated the effectiveness of hyperthermia when given at temperatures above 42-43°C (34-36,46), difficulties arise when heating many types of tumours above these temperatures and maintaining uniformity throughout the tumour. However, recently, it has been shown that 39-41°C mild hyperthermia can completely inhibit the sparing effect observed with low dose rate irradiation. Mild hyperthermia and brachytherapy administered simultaneously was shown to be very effective in several in vitro studies (36,45,47). One study with human cells suggests that the degree of thermo-radiosensitization may be inversely related to radiosensitivity by showing that the most radioresistant cell line became the most radiosensitive when subjected to the combined treatment of mild hyperthermia and low dose rate irradiation, while the most radiosensitive cell line was the most unaffected by the combined treatments (36). To support the in vitro data, there have been many phase I and phase II studies showing the efficacy of hyperthermia when combined with radiation, and recently, positive phase III trials of LDR brachytherapy and hyperthermia have been reported in Europe and the U.S. (2,39,48-51).

Since in these studies hyperthermia has proved to be effective in combination with low dose rate brachytherapy, its effectiveness should also be investigated in combination with pulsed dose rate irradiation with a possible application to clinical brachytherapy. If PDR demonstrates similar radiobiological advantages to LDR irradiation, especially in the dose rate sparing effect and repair of sublethal damage, then theoretically mild hyperthermia combined with pulsed dose rate irradiation would also show the inhibition of sublethal damage repair. In the literature, there are only two such studies to date, one using a rodent cell line (13), while the other used human cell lines (52). The rodent cell study shows equivalence between LDR and PDR irradiations using hourly 0.5 Gy pulses, and also shows that 41°C hyperthermia combined with LDR and PDR treatments maintains this equivalence, but at much lower survival levels. From this study there is evidence that mild hyperthermia may sensitize cells to pulsed dose rate irradiation in a similar way to that when combined with low dose rate irradiation. The only human cell study published to date investigated the responses of normal cells, and radiosensitive and radioresistant isogenic ovarian carcinoma cells. This study used 0.53 Gy and 1.6 Gy pulses delivered every one and three hours, respectively, combined with 40°C mild hyperthermia. The results showed that 0.53 Gy/1h did not show radiosensitization with added hyperthermia in any of the cell lines, while the 1.6 Gy/3h combined with hyperthermia radiosensitized the tumour cell lines but not the normal cells. Therefore these results showed a potential clinical advantage when using the larger pulse and interfraction interval in combination with 40°C mild hyperthermia. However, this is only one study to date using human cells, therefore more studies should be performed using other human cell lines to investigate PDR irradiation combined with mild hyperthermia.

In addition, a higher temperature of 41°C combined with PDR irradiation should also be investigated, since it should prove to be a more efficient radiosensitizer.

1.4 DNA double-stranded breaks and radiosensitivity

The primary target for cell inactivation by ionizing radiation is the DNA, and cell death induced by radiation is most likely the result of lack of repair or misrepair of complex lesions in the DNA (53). Ionizing radiation is believed to produce about 1000 single-strand breaks (SSBs) and 25-40 double-strand breaks (DSBs) per diploid cell per Gray regardless of cell type (with a few exceptions). However, the response to these lesions can differ widely for different cell types, and this variation in the intrinsic radiosensitivity is often considered to be a result of differences in the ability of cells to repair DNA damage accurately. The cellular complement and efficiency of repair enzymes are critical for DNA repair. In addition, other less obvious factors to consider that can affect the efficiency of DNA repair include (1) higher-order chromatin structure, influencing lesion distribution, recognition and accessibility to repair enzymes; (2) gene induction, leading to cell cycle arrest and apoptosis; and (3) the cellular environment.

There has been an extensive effort in correlating DNA DSBs with cell radiosensitivity. DNA DSBs were originally measured in 1968 by neutral velocity sedimentation after high radiation doses (54). Since then, other assays have been developed including the modified neutral velocity sedimentation procedure (55), the neutral filter elution assay (56), asymmetric field inversion gel electrophoresis (AFIGE) (57), clamped homogenous electric field gel electrophoresis (CHEF) (58), and most recently, the comet assay (59).

Although these assays have been used to gain insights into the induction and repair of DSBs and how they relate to the radiation response of the cells to radiation, no consistent results have been found to date. In some studies both initial damage and repair of DNA DSBs have been correlated with sensitivity (60-62), while others have shown that only residual damage after a given repair time is related to radiosensitivity, either after high dose rate (63) or low dose rate irradiation (64).

Most recently, some of these assays have been used to determine whether a radiation treatment would result in fewer residual DSBs than this radiation treatment combined with a radiosensitizer such as hyperthermia or a chemotherapeutic agent, and whether these findings were correlated to the corresponding survival curves.

One study investigated the ability of camptothecin to potentiate cell killing by low dose rate irradiation and whether this potentiation was associated with an increase in the level of residual DNA DSBs using the comet assay (65). This study found strong correlations between the levels of residual DNA DSBs and surviving fraction after concurrent treatment with 25 μ M camptothecin and low dose rate irradiation.

A study by Raaphorst et al found a correlation between survival and DSB for analysis at the same dose levels. It investigated the response of human melanoma cells (SK mel-3) to combinations of high dose rate irradiation and hyperthermia given 1 h immediately after irradiation, using a range of temperatures from 42 to 45°C (66). The same research group conducted another study where CHEF was used to determine whether the amount of residual DSBs reflected the clonogenic assay findings following low dose rate and pulsed dose rate irradiation in human glioma cells, finding the two treatments equivalent,

although the findings did not correlate with the clonogenic assay results, where for most fractionation schemes survival was higher for PDR than for LDR irradiation (20).

Another study using two human ovarian carcinoma cell lines (A2780s and A2780cp, parental and radioresistant strain, respectively) found no correlation between clonogenic assay and induction of DSBs measured immediately after irradiation and allowing 6 h repair before assessment (67).

In the present study, the CHEF and the comet assays (described in the materials section) were used to determine whether the amount of residual DSBs following a combination treatment of pulsed dose rate or low dose rate irradiation with 41°C hyperthermia was greater than either treatment without hyperthermia. The CHEF assay was also used to determine whether there was a correlation between cell line sensitivity and the amount of initial DSBs following a high dose rate treatment for two cell lines of different radiosensitivity. The rationale for using the CHEF assay is that PDR and LDR irradiation combined with 41°C hyperthermia should result in residual DSB levels similar to HDR irradiation levels if repair of radiation damage is inhibited. These levels are detectable by CHEF. The comet assay, on the other hand, is more sensitive at lower doses therefore results would be obtained in a shorter period of time compared to CHEF. In addition, while CHEF electrophoresis uses bulk DNA, comet assay uses DNA from single cells, which allows elimination of apoptotic and mitotic cells, increasing the sensitivity of the assay. Another advantage of the comet assay over the CHEF assay is that it does not require radiolabeling DNA.

1.5 DNA polymerases and their role in repair of radiation damage

An enzyme that can synthesize a new DNA strand on a template strand is called a DNA polymerase (68). A major function of DNA polymerases is to accurately replicate the six billion nucleotides that constitute the human genome. This function is complicated by an assortment of DNA-damaging agents, both endogenous and exogenous. Damage to the DNA results in the loss or modification of bases, single- and double-strand breaks in the DNA, and intra- and interstrand cross-links. Such damage presents a threat to genomic stability by creating obstacles for DNA replication and transcription complexes and by altering base coding potential, which leads to replication and transcription errors (69). However, cells possess specialized repair pathways that remove various types of damage, and several DNA polymerases are known to be the key components of these repair systems. In addition, DNA polymerases can act as sensors in cell cycle checkpoint pathways that prevent entry into mitosis until damaged DNA is repaired and replication is completed.

Over the past few years, several new DNA polymerases have been discovered. They are listed in Table 1.1, along with their proposed functions (69). The next subsections are based on a review of the functions of DNA polymerases (69), and they will describe the different repair pathways and, in each case, which DNA polymerases come into play in the repair process, with special emphasis on the four polymerases investigated in this study, alpha, beta, delta, and epsilon.

1.5.1 Nucleotide Excision Repair (NER)

A variety of helix-distorting lesions, including radiation-induced damage (direct action of radiation), are removed by nucleotide excision repair (NER). NER removes DNA lesions by excising an oligonucleotide containing the lesion, leaving a single-stranded DNA gap of ~30 nucleotides. There is substantial evidence that these gaps are filled by one or both polymerases δ or ϵ .

Table 1. Known DNA polymerases and their proposed functions.

Polymerase	Proposed Function
α (alpha)	Chromosomal replication, S-phase checkpoint, DSB repair
β (beta)	BER, SSB repair
γ (gamma)	Mitochondrial replication, mitochondrial BER
δ (delta)	Chromosomal replication, NER, BER, MMR, DSB repair
ϵ (epsilon)	Chrom. repl., NER, BER, MMR, DSB repair, S-phase checkpoint
ζ (zeta)	TLS, DSB repair, ICL repair?, SHM
η (eta)	TLS, SHM
θ (theta)	ICL repair?
ι (iota)	TLS?, BER?, SHM
κ (kappa)	TLS
λ (lambda)	DSB repair, BER?
μ (mu)	DSB repair
σ (sigma)	Sister chromatid cohesion
REV1	TLS

1.5.2 Base Excision Repair (BER)

Damaged bases, such as those produced by reactive oxygen species (which may have been produced by the indirect action of radiation) are removed primarily by base excision repair (BER). During BER, damaged bases are first removed by a DNA glycosylase, a repair enzyme which recognizes and removes nitrogenous bases in the DNA molecule that have lost an amino group, generating an abasic (AP) site (apurinic or apyrimidinic site resulting from the loss of a purine or pyrimidine residue from the DNA). The subsequent action of an AP endonuclease creates a nick with 3'-OH and 5'-dRP termini. This can be further processed by more than one BER pathway involving different DNA polymerases. In the major mammalian BER pathway, polymerase β inserts a single nucleotide and removes the 5'-dRP. This generates a nick that is sealed by DNA ligase, with the entire reaction accomplishing the replacement of a single damaged nucleotide (single-nucleotide BER). In some cases, a DNA polymerase may instead perform limited displacement synthesis to incorporate 2 to 13 nucleotides (long patch BER). Polymerases β , δ , and ϵ can perform DNA resynthesis during long patch BER in vitro. The identity of the polymerase involved in the long patch BER in vivo is not certain, but it has been reported that polymerase β always initiates DNA synthesis.

It has been observed that two of the newly discovered DNA polymerases, ι and λ , have dRP lyase activity similar to that of the β polymerase, therefore, it is possible that they also participate in BER.

DNA polymerase γ is the only DNA polymerase found in mitochondria; therefore, it is responsible for all DNA synthesis transactions in this organelle, including DNA replication and repair of DNA damage.

1.5.3 Repair of Interstrand Cross-Links (ICLs)

Interstrand cross-links present a special challenge to DNA repair systems since both strands are damaged and thus neither strand retains the correct genetic information. Little is known about the mechanism by which ICLs are repaired in mammalian cells. From studies of model organisms polymerases ζ and θ have been identified as participating in this type of damage repair. It remains to be determined whether these are also involved in repair of ICLs in human cells.

1.5.4 Double Strand Break (DSB) Repair

Double strand breaks can be repaired by recombination with a homologous DNA sequence (homologous recombination or HR) or by nonhomologous DNA end joining (NHEJ) in vertebrate cells. Homologous recombination (HR) represents the major mechanism for repair of DSBs in lower organisms, while it shows a low frequency of activity in mammalian cells. It requires an extensive region of homology between the strand under repair and an undamaged homologous strand, and since any sequence information lost at the site of the break is repaired using information from the undamaged homolog, it represents a very efficient, high-fidelity repair mechanism (70). Repair of DSBs by HR requires DNA synthesis that is initiated by invasion of the 3' end of the broken DNA into a donor sequence. In a yeast cell line, all three DNA polymerases required for S-phase replication of chromosomes (α , δ , ϵ) were found to be necessary for DNA synthesis associated with DSB-induced recombination. Replicative DNA polymerases are characterized by high fidelity, which is consistent with the notion that recombinational repair of DNA damage does not frequently generate mutations (i.e.

“error-free” repair). Polymerase ζ has also been found to be involved in this type of repair pathway.

In contrast, nonhomologous DNA end joining (NHEJ) plays the dominant role in rejoining DSBs in mammalian cells. This type of DSB repair involves a complex of enzymes, which bind to the double-strand DNA ends and facilitate the rejoining of the break. Because little or no homology is required, NHEJ is more error prone than homologous repair (70). At least two DNA polymerases are involved in the NHEJ pathway in eukaryotic cells. Several studies have implied that polymerases λ and μ are possibly involved in this repair pathway.

1.5.5 Cell cycle checkpoints

The dependence of cell cycle progression on completion of DNA replication and repair of DNA damage is controlled by regulatory mechanisms called checkpoints. Studies, mostly with yeast cell lines, have shown that a defect in the DNA damage checkpoint manifests itself by a lack of inhibition of cell cycle progression. These studies have shown that polymerases α and ϵ are involved in S-phase checkpoints.

To advance our understanding of the mechanisms of radiation damage repair, this study investigated the levels of expression of four DNA polymerases believed to be involved in repair (α , β , δ , and ϵ) following low dose rate and pulsed dose rate irradiation, with and without mild hyperthermia, as well as hyperthermia alone. In addition, the cell lines chosen for this study were selected to further this goal. Although the two cell lines studied are isogenic, they have different radiosensitivities, and one of

the cell lines overexpresses polymerase β , which as mentioned, is believed to be involved in radiation damage repair, specifically base excision repair (BER). Therefore differences in radiation response in the two cell lines theoretically should be reflected in differences in gene expression in the cells, in particular in polymerase β .

1.6 Hypothesis and Specific Aims

The hypothesis of this thesis is that pulsed dose rate irradiation using a fraction of 1.5 Gy delivered every 3 hours can be equivalent to a conventional low dose rate treatment of 0.5 Gy/h, in combination with or without mild hyperthermia, as determined from cell survival curves using clonogenic assay, as well as from double strand break assays using clamped homogenous electric field and the comet assay, and that the two radiation treatments have similar repair mechanisms as determined from DNA polymerase expression.

Specific aims are:

- Determine the intrinsic radiosensitivities of the two cell lines using high dose rate and low dose rate irradiation, and determine the radiosensitivity parameters and the sublethal damage component from fitting the survival curves with the linear quadratic model.
- Establish the pulsed dose rate irradiation response of the cell lines, and compare the results to those from the low dose rate irradiation by using the Student's t-test.
- Combine the two radiation treatments with mild hyperthermia and assess the degree of thermosensitization, and whether it would present a therapeutic advantage from a clinical perspective.

- Determine whether the clonogenic assay results correlate with results from the double strand break assays.
- Investigate the role of four DNA polymerases in repair of damage resulting from the two radiation treatments, with and without hyperthermia, especially in their possible roles in sublethal damage repair.

This thesis is comprised of five chapters and two Appendices. The first chapter is the introduction, followed by materials and methods, then results, discussion, and conclusions. Appendix A describes the design and setup of the low dose rate irradiator used in the experiments in this study. Appendix B describes preliminary results using another set of cell lines, which proved to be less suitable for this study.

2.0 MATERIALS AND METHODS

2.1 The cell lines

Two cell lines were used in this study; both are breast carcinoma cell lines, one being the parental cell line, MCF7, and the other its transfected clone, C716, which was transfected with a plasmid containing polymerase β (71). The C716 was considered to be radioresistant compared to the parental MCF7 cell line. These cells were kindly donated by Dr R. Sobol and Dr S. Wilson. These particular cell lines were selected for this study because they have different radiosensitivities yet are isogenic. Most importantly, one of the cell lines overexpresses a gene believed to be involved in repair of radiation damage, hence the differences in radiation response between the two cell lines should result in differences in gene or genes expression.

The cells were grown in Wisent DMEM/F12 medium supplemented with 10% Fetal Bovine Serum (Wisent). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

The cells were plated at 5×10^5 cells per 25cm² flask or 2×10^5 cells per 35 mm petri dish. The medium was changed on day 7, and the experiments were performed on day 10. All experiments were performed at plateau phase to eliminate variations arising from cycling cells, especially from cells in the S-phase, which are more resistant. Growth curves were performed to ensure that cells remained at plateau phase during the entire length of the experiments. Briefly, 5×10^5 cells were plated per petri dish on day 0, and each day (at about the same time of the day) one petri dish was scored for total number of cells for 14 days (medium was changed on day 7). This allowed identification of the length of the exponential growth and plateau phase of the cells.

2.2 LDR, HDR, and PDR procedures

For continuous low dose rate (LDR) irradiation, cells were irradiated in petri dishes by a low dose rate Cesium-137 irradiator that was designed for this project (see Appendix A). The dose rate used was 0.47 Gy/h, and irradiations were carried out at 37°C to allow repair to occur during irradiation, as well as at 41°C for the hyperthermia experiments by adjusting the thermocontroller settings (Versa Therm Proportional Electronic Temperature Controller, Model 2156, Cole-Parmer). Hyperthermia alone control flasks were maintained at 41°C in a temperature-controlled water bath.

For high dose rate (HDR) and pulsed dose rate (PDR) irradiations, cells were irradiated in 25 cm² flasks with a 250 kV_p x-ray unit (Pentak) at a dose rate of 103.8 Gy/h. The HDR irradiations were carried out at room temperature due to the short irradiating times that would not be sufficiently long to allow repair. The exception is the CHEF experiments for which very large doses were used and therefore the cells were irradiated on ice (~ 4°C) in order to minimize repair during irradiation (72). For PDR irradiations, cells were kept in a temperature-controlled water bath during the entire experiment, using a setup described in the next section. The dose to the cells was measured in the initial calibration of the x-ray unit by an ion chamber (73), and subsequent quality assurance measurements were taken regularly to ensure dose rates and exposure times were within accepted limits. This was done by averaging 3 separate measurements taken using an ion chamber placed inside a plexiglass slab. A correction factor was applied for temperature and pressure, and the value was compared to the accepted value. If the measured value was not within 4% of the accepted value, the time settings to obtain total doses were adjusted accordingly.

2.2.1 PDR apparatus setup

Pulsed dose rate irradiations required long irradiating times during which a 1.5 Gy pulse is given every 3h. Some experiments, such as those for CHEF, Comet Assay, and RT-PCR, can run for several days, and it would be very difficult for an experimenter to complete them with no sleeping periods. To circumvent this, a setup was designed to allow irradiations to take place automatically. A schematic diagram of the apparatus set up is shown in Figure 2.1.

Figure 2.1. PDR irradiation set up.

A timer was connected to the x-ray control panel and it was programmed to activate the unit anytime daily during one week for up to 14 times. The irradiation time

for a given dose can be set on the control panel and it gets reset every time, so the timer switches the machine on just for the length of time that is set on the control panel. Following irradiation, the timer on the control panel switched the x-rays off, and the programmable timer switched itself off as well until the next irradiation. During the irradiations, the cells were maintained at either 37°C or 41°C, and this was achieved by placing the cell flasks in a water-filled holder with the bottom of the holder lined with heating cable, which is connected to a thermoregulator (VWR or Cole-Parmer). The flask holder and thermoregulator with its power source were placed inside the irradiator such that the distance from the x-ray tube to the top of the flasks was the same as during regular irradiating procedures. This allowed for PDR experiments to be performed in a temperature-controlled environment for as long as necessary.

2.3 Clonogenic assay

Following treatment, cell survival was determined by the colony-forming assay, an *in vitro* method originally described by Puck et al (1,74,75). This method is a measure of the reproductive integrity of cells since cells that are unable to reproduce are functionally dead, while cells that have retained their reproductive integrity are able to proliferate and produce colonies.

After treatment, the depleted medium was aspirated, and the cell monolayer was rinsed with either citrate saline (134 mM KCl (BDH), 17.6 mM citric acid (Sigma)) or PBS (phosphate buffered saline, 2.6mM KCl (Sigma), 1.5mM KH₂PO₄ (Sigma), 0.1M NaCl (EM Science), 8mM Na₂HPO₄ (EM Science)) to neutralize the trypsin inhibiting effect of the medium. After aspirating the citrate saline or PBS, trypsin was then rinsed

over the cells to detach them from the surface, and fresh medium was then used to resuspend the cells into single cell suspension. The suspended cells were counted using an electronic cell counter (Particle Data Elzone 80, calibrated by hemacytometer). The cells were diluted to the desired concentration and plated such that about 50 colonies per plate would be achieved. For low concentrations of cells 60 mm petri dishes were used with 4 ml of medium, while for concentrations greater than 10^5 cells per plate 100 mm plates were used with 10 ml of medium. Following an incubation period of 14 days, the cells were fixed with 75% ethanol for 15 min and then stained with crystal violet (0.2% w/v in 70% ethanol) for at least 20 min. The plates were then examined under a microscope and colonies of at least 50 cells were scored.

The average of the number of colonies of three plates per dose was taken. In addition, the average of three control plates, 0 Gy, was used to calculate the plating efficiency (PE) as follows:

$$P.E. = \frac{\text{number of colonies (untreated)}}{\text{number of cells plated (untreated)}} \quad (2.1)$$

Therefore the surviving fraction was calculated according to the formula:

$$S.F. = \frac{\text{number of colonies}}{P.E. (\text{number of cells plated})} \quad (2.2)$$

Each experiment was repeated at least twice and the mean and the standard error of the mean were calculated for each set of experiments. The surviving fraction data points were plotted on a semi-logarithmic scale as a function of dose. The curves were fitted with Chadwick and Leenhouts's Linear Quadratic Model (76), expressed as follows:

$$S.F. = \exp(-\alpha D - \beta D^2) \quad (2.3)$$

This model is based on the assumptions that a double strand break in the DNA is the critical target for radiation damage, and that there are two components of DNA damage by radiation, one proportional to dose, α , the other proportional to the square of the dose, β (1). The linear component α accounts for double strand breaks from a single ionizing event, and is considered difficult to repair, while the quadratic component β accounts for double strand breaks from two separate ionizing events, and it is considered repairable if the dose is split into two fractions with sufficient elapsed time between them for the first break to repair before the second break occurs (1). A large β therefore implies accumulation of sublethal damage and hence potential for repair when fractionating the dose. In low dose rate irradiation survival curves this component is usually very small or insignificant since the ionizing events are sufficiently spaced for repair of single strand breaks to occur before the second break takes place. This model has shown to describe adequately high dose rate and low dose rate irradiation survival curves, and in some cases pulsed dose rate irradiation survival curves (77).

2.4 Double-stranded break assays

Two different double-stranded break assays were used to determine whether there is a correlation between cell line radiosensitivity (as determined by the clonogenic assay) and the amount of double-stranded breaks in the DNA incurred by the cells. Also, these assays were used to determine whether a concurrent treatment of radiation and hyperthermia would increase the number of residual DNA double-stranded breaks when compared to radiation treatment alone. The two assays work on the principle that DNA is negatively charged and will migrate in a porous matrix when an electric field is applied to

it (electrophoresis). Therefore in both assays an electric field is applied to the DNA of lysed cells that has been encased in agarose (a polysaccharide isolated from seaweed), which is a porous gel that allows DNA to migrate (78). The main difference between the two assays is that CHEF uses bulk DNA lysed from a large number of cells, while comet assay examines DNA from individual cells.

2.4.1 CHEF

The Clamped Homogeneous Electric Field or CHEF assay (CHEF-DR II (Bio Rad)) consists of an array of 24 electrodes arranged in a hexagon as shown in Figure 2.2 (79). This allows the electric field to pulse, changing direction, as indicated on the figure, by 120° . This system uses homogenous electric fields with a voltage gradient that is constant across the entire gel, and this allows the DNA to travel in a straight line. Changing the direction of the field allows the DNA to reorient itself, resulting in fragment size separation, since it takes longer for very large DNA to reorient itself (77). This allows separation of DNA molecules up to 10 Mbp, and this size includes DNA fragments from irradiated cells. The fraction of DNA fragments is correlated with the number of radiation induced double stranded breaks.

Figure 2.2. CHEF-DR II setup (Bio-Rad).

Agarose gels are electrophoresed horizontally, submerged under a cooled recirculating buffer (14°C to avoid degradation of samples due to heat generated by the electric field). A 14 x 13 cm² gel is cast separately on a platform and then placed in the center of the hexagon (80).

Cells were pre-treated with medium containing 0.2 µl of C-14 thymidine per 1 ml of medium during setup and feeding of cells before experiments.

Cell preparation following treatment involved trypsinization as per clonogenic assay, and resuspension of cells. A concentration of 10⁶ cells/ml was used as the optimum concentration (79). The cells were then centrifuged, the medium was aspirated, and cells were re-suspended in 500 µl of PBS. A volume of 500 µl of 2% low melting point (LMP) agarose (2g of agarose (Gibco BRL) in 100 ml of PBS) was added to the cells and mixed well with a pipetter. The cell/agarose mixture was pipetted into well molds and left on ice for 10 min. The solidified gel plugs were dropped into tubes with lysis buffer and proteinase K (500 mM EDTA, 1% w/v sarkosyl, 1.0 mg/ml Proteinase K) maintained at 50°C on a hot plate and the cellular material was digested during the 18-24 hour incubation period. The lysis buffer was aspirated after incubation and 1 ml of TE buffer (1M Tris-HCl, 0.5 EDTA) per tube was added and left for 30 min. This procedure was repeated 3 more times for a total of 4 washes per sample. The gel was prepared (100 ml 0.5xTBE buffer with 0.8 g GIBCO pulsed field certified agarose (79), dissolved in the microwave and cooled) and cast in a mold (Bio-Rad). Well combs were placed on the gel box and the gel was allowed to solidify for 30 min at room temperature, and then in the fridge for an additional 10 min. While the gel was cooling, the CHEF gel chamber was filled with 2L of 0.5xTBE buffer (Bio-Rad) and the cooling module was set to 14°C. The

combs were removed from solidified gel and plugs cut to size (3mmx5mm) were placed in the wells. Plugs were sealed in the wells with 1% LMP agarose, and once the molding tray was removed, the gel was placed in the CHEF chamber. For gel runs exceeding 24 hours, the 0.5xTBE running buffer was changed halfway through. The gel was run at 1.5 V/cm with 75 min switch times (total 18 hour run) for HDR (79) and with 1.5 h switch times (total of 50 hours run) for PDR (67,81). The difference in parameters reflects the fact that DNA following PDR irradiation is in much larger fragments than following HDR irradiation, therefore it requires longer switch times.

The gels were stained with SYBR Gold nucleic acid gel stain (Molecular Probes, 20 µl per 200 ml of 0.5xTBE buffer) for 20-30 min. Then the gels were cut separating wells from lanes under a UV-transilluminator. The separated wells and lanes were placed into respective labeled scintillation counter glass vials. Vials were placed on a hot plate at the low setting until plugs were at least mostly melted. An amount of 50 or 100 µl of 10M HCl was added (depending whether lanes were shorter or longer, respectively) to the plugs and then they were put back on the hot plate until melted completely. A volume of 10 ml of scintillation fluid was poured into each vial with the melted sample and then the samples were placed on a scintillation rack. The counter was operated at a protocol for C-14, and from the readout, fraction of activity released (FAR), was calculated using:

$$FAR = \frac{\text{activity in lane}}{\text{activity in lane} + \text{activity in well}} \quad (2.4)$$

Average FAR from three experiments was then plotted as a function of dose.

2.4.2 Comet Assay

In 1984, Ostling and Johanson presented the novel idea of performing gel electrophoresis on individual cells instead of on bulk DNA (82). This provides a method to measure initial levels of DNA damage in individual cells. There are many advantages of using the comet assay (named so for the appearance of the cells following electrophoresis) over electrophoresis methods that use bulk DNA: (a) requires fewer cells, (b) looking at individual cells allows identification of apoptotic cells as well as cells undergoing mitosis, (c) sensitive detection of DNA damage is possible without radiolabeling DNA (82, 83).

Cells were trypsinized as per clonogenic assay and a dilution of $3\text{-}4 \times 10^5$ cells/ml was obtained. A volume of 50 μl of the cell mixture was mixed with 450 μl of 1% LMP agarose (2g of agarose (Gibco BRL) in 100 ml of PBS), which has been melted and maintained at 40°C. A volume of 120 μl of the cell/agarose mixture for each dose point was then poured into one of the two wells of a Supercell Lab-Tek II chamber slide (Nalge Nunc) that has been attached to the hydrophilic side of a strip of Gelbond film (Mandel) that has been cut to size. Once the cell/agarose mixture has solidified, the Supercell chambers were removed from the Gelbond strips, and the strips were placed in 50ml of lysis buffer (2.5M NaCl, 100mM Tetrasodium EDTA, 10mM Tris base, and 1% Sodium Lauryl Sarcosine, pH 10.0), which had a concentration of 1% of Triton-X 100 added to it on the day of the experiment. The strips were lysed overnight at 4°C, after which they were rinsed in ddH₂O three times. They were then incubated in 50 ml 1xTBE buffer for 20 min at room temperature, and finally electrophoresed in 220 ml of 1xTBE buffer at 1 V/cm (13V DC) in a mini horizontal submarine gel electrophoresis chamber (Hoefer) for

20 min. The optimum duration of electrophoresis was determined experimentally. Following electrophoresis, the gels were rinsed in ddH₂O and placed in absolute ethanol for 2 hours before air-drying overnight.

Before analysis, the gels were stained in SYBR Gold (Molecular Probes, 1:1000 dilution in ddH₂O) for 10-15 min, and then rinsed in ddH₂O. Each strip of gel was placed on a microscope slide and covered with a cover slip, and viewed under oil using the 40x objective lens with narrow blue-cube filter. The microscope used to view the samples (Olympus BX60) had a KP-D 581 digital camera (Hitachi) attached to it, and the samples were illuminated with 495 nm light excitation from a 100W mercury burner, with the digitized image displayed into a pixel matrix on a computer screen. The images were analyzed using the ALKOMET v3.1 image analysis system (Richard Brancker Research Ltd., Ottawa, Ontario). Figure 2.3 shows treated and untreated comets. For each comet, the head and tail regions were defined, and the DNA damage parameters (total intensity, tail length, comet length, and percentage of DNA in head and tail regions) were determined. A total of at least 50 cells were analyzed per dose point.

Figure 2.3. Untreated (left) and HDR treated (right) comets.

The tail moment, which is a popular and sensitive endpoint (84), was calculated using the following equations:

$$T.M. = \phi_{tail} | cm_{head} - cm_{tail} | \quad (2.5)$$

where ϕ_{tail} is the length of the tail multiplied by the distance in microns from the centre of mass of the head to the centre of mass of the tail. Tail moments were calculated for at least 50 cells for a given dose point, and then averaged. The tail moment was then normalized as follows:

$$T.M. = \left(\frac{T.M.(D)}{T.M.(0)} \right) \times 100 \quad (2.6)$$

where T.M.(D) is the dose point to be normalized by T.M.(0), which is the unirradiated control. An average of three experiments was plotted as a function of dose.

2.5 RT-PCR

Polymerase Chain Reaction (PCR) amplifies short (approximately 100-500bp) segments of a longer DNA molecule (85). A typical amplification reaction includes the sample of target DNA, a thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), reaction buffer, magnesium salt and optional additives. The components of the reaction are mixed and the reaction is placed in a thermal cycler, which takes the reaction through a series of different temperatures for varying amounts of time. This series of temperature and time adjustments is referred to as one cycle of amplification. The reaction is taken through a number of cycles of amplification, typically 25-35, resulting in a theoretical 2^{25} - 2^{35} amplification, and the reaction products can be electrophoresed on an agarose gel, which can be stained with

nucleic acid stain and viewed and analyzed under a UV transilluminator with a computer imaging program.

The thermostable polymerases used in the PCR process require a DNA template so the technique is limited to the analysis of DNA samples. However, numerous instances exist in which the amplification of mRNA would be preferred, for example, in analysis involving the differential expression of genes in tissues during development or cloning of cDNAs from rare messages (86). Therefore in order to be able to use RNA samples for PCR, the RNA samples must first be reverse transcribed to cDNA to provide the necessary DNA template for the thermostable polymerase, a process known as reverse transcription (RT-PCR). In reverse transcription, a retroviral enzyme (reverse transcriptase M-MuLV) is used to produce a DNA copy of the RNA template, using (in this case) an oligo(dT) primer. The primer anneals to the RNA, and the cDNA is extended toward the 5' end of the mRNA through the RNA-dependant DNA polymerase activity of the reverse transcriptase. Other additives such as dNTPs, RNase inhibitors, and reaction buffer are also present in the reaction.

Prior to the RT-PCR protocol, following irradiation (6 Gy, with different lengths of time following irradiation to allow time for expression), treated cells, as well as control cells, were trypsinized as per clonogenic assay and a total volume containing 1.5×10^6 cells was obtained. The cells were centrifuged and the RNA was extracted from the cells as per RNeasy kit instructions (87). Following extraction, the concentration of RNA obtained was calculated using a spectrometer (Eppendorf). The RNA could be frozen at this point at -80°C until further use. Reverse transcription (RT) was performed in a PTC-200 Peltier Thermal Cycler (MJ Research) using a kit (First Strand cDNA Synthesis Kit,

MBI Fermentas), which allowed 1 µg of RNA to be reverse transcribed into cDNA. Samples could be frozen at this point at -20°C until required for PCR. Polymerase Chain Reaction (PCR) was performed on the samples to look at expression of four different genes (in this case DNA polymerases), all believed to be involved in DNA repair (α , β , δ , ϵ), and one reference (β_2 -microglobulin). The sequences of the genes were found in the literature and a computer program (Vnti40) was used to select appropriate regions in the genes to be used as primers. The forward and reverse primer sequences, along with their size and melting temperatures (determined from entering the sequences in the Applied Biosystems technical tools website) are shown in Table 2.1.

Table 2.1. DNA polymerase primers

Gene	Primer sequence	Size (bp)	T _m
β_2 micro globulin	5'-GACTGGTCTTTCTATCTCTTGTACTACAC-3'	503	57.38
	3'-GAAGGTAAATGTATGAGACGAATCTTA-5'		57.73
α	5'-ATATGTACAGTGAATCTTCTCAACTGTTATA-3'	683	57.24
	3'-TTCGAGTGTCGCTTGTCATACA-5'		57.62
β	5'-GGTGAGACAAAGTTCATGGGT-3'	205	57.38
	3'-GTGTTAGTTACTCATGTGGTAGG-5'		57.76
δ 1 st try	5'-TTCTCTGTCTGCTGCCACAT-3'	600	57.96
	3'-AGTAGGTCTAGACGAGCGA-5'		57.99
δ 2 nd try	5'-CGACCGCGTCCCCTACGTG-3'	318	66.6
	3'-GTGCCGAGGGACCAACGAC-5'		66.7
ϵ	5'-CCAACATCATCCTGACCAAC-3'	320	57.17
	3'-TCTTCTAGGTGTAGTGGTTCC-5'		57.47

The PCR reaction ingredients are listed in Table 2.2. The reaction took place in the LightCycler (Roche). During the PCR reaction the samples were heated to 95°C for 20 seconds to allow initial cDNA denaturation. Then the samples underwent between 35-45 cycles of denaturing at 95°C for less than a second, annealing at 56°C (65°C for the second set of δ primers) for 9 seconds, and DNA synthesis or extension at 72°C for a length of time estimated as the length of the gene divided by 25 (for example, β had an extension duration of 205/25 ~ 8 seconds). Finally, during the melting step, the samples were brought to 96°C for less than a second, heated to 62°C for 30 seconds, and then gradually brought to a temperature of 99°C.

Table 2.2. PCR reaction

SOLUTION	β, δ, β_2-microglobulin (μ l)	α, ϵ (μ l)
RT product	2 (1:5 dilution)*	2 (1:5 dilution)
H ₂ O	11.2	12.0
10X buffer	2	2
MgCl ₂	1.2	2
DNTPs	1.6	1.6
Primer (x2)	0.4 (0.8)	0.4 (0.8)
BSA	0.4	0.4
SYBR Green	0.4	0.4
Taq	0.4	0.4
Total	20	20

*Note: since the C716 cell line overexpresses polymerase β , its samples were diluted to 1:50.

The samples were analyzed directly during the procedure by using fluorescence emitted by SYBR I green (Molecular Probes, 1:1000 dilution), which bound itself to the amplified DNA. To view the products to ensure that the desired region of the DNA was being amplified, a 2% agarose gel (2 g in 100 ml 1xTBE buffer, Gibco) was cast, and after solidifying, it was submerged in 1xTBE buffer in a mini horizontal submarine gel electrophoresis chamber (Hoefer). For each sample, 5 μ l was mixed with 1 μ l of loading buffer and this mixture was loaded into each well. The samples were then electrophoresed at 100V for about 1-1½ hours. The gel was then stained with SYBR Gold (Molecular Probes, 1:1000 dilution in dH₂O) for 20-25 minutes, and then the products could be viewed under an UV Transilluminator.

To be able to analyze samples, standard curves for each target gene and the reference gene had to be established. To accomplish this, an untreated sample of cDNA (in this case untreated MCF7 cells were used for all 4 genes) was amplified in duplicates in the Lightcycler at various concentrations (undiluted and dilutions 1:2, 1:4, 1:8, 1:16, 1:32). Each of these dilutions was assigned an arbitrary concentration (proportional to the dilution, so the undiluted sample was assigned a concentration of 400, 1:2 was 200, etc.). The standard curves were then plotted with the sample concentrations as functions of the crossing points, which are inflection points on the exponential part of the curves (where the second derivative of the slope is equal to zero). When experimental samples were analyzed, the standard curves were used to eliminate samples which crossing point (CP) did not fall on the range of the standard curve. A program called RelQuant 1.01 was then used to calculate coefficients from each standard curve that were later used on experimental samples for relative quantification.

Each PCR amplification also included a calibrator sample. The purpose of the calibrator was to account for variations during the RT step (the Lightcycler is very stable while the Thermocycler used for RT reactions is more variable). The calibrator came from the same RNA as the samples used for standard curves. After amplification, Rel Quant 1.01 was used to set the ratio of the calibrator for the target gene to that for the reference gene as 1.0, and then used to determine the experimental normalized crossing point ratios for target to reference genes. The normalized crossing point (CP) ratios were then plotted as bar graphs as functions of the time allowed for gene expression.

2.7 Curve Fitting and Statistical Analysis

Survival curves were fitted using the Linear Quadratic Model described in section 2.3. The model was used because it has shown to describe adequately high dose rate and low dose rate irradiation survival curves, and in some cases pulsed dose rate irradiation survival curves. It was also used to fit survival curves of radiation combined with hyperthermia, and although the linear quadratic parameters cannot be interpreted as described by the model, the model still provides an excellent fit. Hence the survival curves were fit to the Linear Quadratic Model with the graphing software Sigmaplot for Windows Version 4.0. This software uses the Levenberg-Marquardt algorithm to determine the parameters of the independent variables using weighted non-linear least squares fit method (chi-square minimization) that give the best fit of the model to the data. This software was used to display all the graphs in this study.

The equivalence between the low dose rate and pulsed dose rate responses for each assay was determined by using p values from the Student's t-test at the 0.05 significance level, using matched data points.

The quadratic component of the pulsed dose rate and low dose rate survival curves was tested for significance, to determine the extent of sublethal damage repair. This was performed using the F-statistic, which compares two population variances, by testing the full LQ model (with both linear and quadratic terms) against a reduced model with only the linear component (77). To accomplish this, the sum of squares for both the full LQ model and the reduced LQ model was calculated as follows:

$$SSE = \sum_{i=1}^n (S_i - f(D_i))^2 \quad (2.7)$$

Where S_i is the transformed surviving fraction, and $f(D_i)$ is the negative natural logarithm of the linear quadratic model.

The mean square was calculated by using:

$$MSE = \frac{SSE}{n-2} \quad (2.8)$$

where (n-2) is the number of degrees of freedom (total number of data points minus the two parameters, alpha and beta).

The test was then calculated using:

$$F^* = \frac{SSE_r - SSE_f}{2 \cdot MSE_f} \quad (2.9)$$

where the subscripts r and f mean reduced (linear term only) and full model (linear and quadratic terms), respectively.

If $F^* < F(1-\alpha; 1, n-2)$, then the quadratic component is not statistically different from zero, implying complete sublethal damage repair (alpha here is not the LQ model, but denotes confidence intervals). This test was estimated at the 95% level of significance.

3.0 RESULTS

3.1 HDR and LDR Irradiations

High dose rate and low dose rate survival curves were performed in order to establish the relative radiosensitivities of the two cell lines. Studies have shown that differences in radiosensitivities in cell lines can be measured in vitro using the clonogenic assay, and that these represent the intrinsic sensitivities of the cells (88-95).

Figure 3.1 shows the survival curves after HDR and LDR treatments for both cell lines, the MCF7 and C716. These curves were fit with the Linear Quadratic model and the estimated parameters α and β are shown in Table 3.1 along with their estimated standard errors. The radiosensitivity parameter SF2, which is the surviving fraction at 2 Gy (a typical dose per fraction used in clinical therapy), is also shown with its standard error. As expected, Figure 3.1 shows that the parental cell line MCF7 has a lower survival than its transfected clone C716, for both HDR and LDR irradiations. In Table 3.1, from the radiosensitivity parameter, the SF2, it can be observed that since the MCF7 is more radiosensitive than the C716, it has lower SF2 values for both types of irradiation. In addition, predictably, the SF2 values for HDR irradiation are lower than those for LDR irradiation for both cell lines. The other radiosensitivity indicators, the α parameters, are similar, within errors, between the two radiation treatments for both cell lines.

From Figure 3.1, it can be observed that both cell lines have considerable shoulders on the HDR curves, while the LDR curves shows virtually linear fits, with very small shoulders. The shoulder on the survival curve can be measured with the β parameter. The HDR β value for the parental MCF7 is 0.1001, which is larger nearly by a

Figure 3.1. Survival curves for the MCF7 and C716 cell lines after high dose rate (HDR) and low dose rate (LDR) irradiation. Each point represents the average of three experiments and the error bars represent the SEM between experiments.

factor of 2 than the value of 0.0627 for the C716 cell line. The LDR β values were 0.0023 for the MCF7 cell line and 0.0080 for the C716 cell line. Both cell lines show a dose rate sparing effect caused by repair of sublethal damage when changing dose rate from HDR to LDR. The significance of the β parameter in the LDR fits is tested in the next section, along with the PDR β parameters.

Table 3.1 LQ model parameters for the MCF and C716 cell lines for HDR and LDR irradiations.

Parameter	HDR		LDR	
	MCF7	C716	MCF7	C716
SF2	0.421 ± 0.035	0.604 ± 0.031	0.557 ± 0.033	0.718 ± 0.045
α (Gy⁻¹)	0.240 ± 0.031	0.134 ± 0.014	0.369 ± 0.031	0.149 ± 0.021
β (Gy⁻²)	0.1001 ± 0.0092	0.0627 ± 0.0031	0.0023 ± 0.0015	0.0080 ± 0.0028

3.2 PDR and LDR Irradiations

A pulse of 1.5 Gy given every 3 hours was chosen for all PDR irradiations because most studies have shown that this is the largest pulse of radiation that still maintains PDR-LDR equivalence, and larger intervals between fractions are more clinically feasible, allowing more patients to be treated at once (**10, 16, 18**). The low dose rate used was 0.5 Gy/h.

3.2.1 Clonogenic assay

Figure 3.2. Survival curves for the MCF7 cell line after pulsed dose rate (PDR), low dose rate (LDR), and high dose rate (HDR) irradiation. Each point represents the average of three experiments and the error bars represent the SEM between experiments.

Figure 3.2 shows the survival curves for the 1.5 Gy given every 3 hours pulsed dose rate and 0.5 Gy/h low dose rate treatments for the parental MCF7 cell line. The high dose rate curve is shown as well for comparison. When evaluating these curves, it can be observed that the pulsed dose rate treatment data has similar survival to the low dose rate treatment. Results from the LQ model are shown in Table 3.2a. To determine the statistical significance of the β parameter for both types of treatment, the LQ model is tested against a reduced model with only the linear component, and the significance was measured using the F^* statistic. From the table it can be seen that the α parameters for the two treatments are similar, within their errors. The β parameters are very small, and not statistically significant for either treatment, implying complete repair of sublethal damage (SLD).

Table 3.2a. Linear quadratic parameters from the fits of the low dose rate and pulsed dose rate experiments for the MCF7 cell line, with estimated standard errors. The significance of the beta parameter is also shown for both treatments.

Parameter	LDR		PDR		β significance (F^*)	
	Full	Reduced	Full	Reduced	LDR	PDR
α (Gy^{-1})	0.369 ± 0.031	0.394 ± 0.020	0.327 ± 0.049	0.366 ± 0.016	-	-
β (Gy^{-2})	0.0023 ± 0.0015	0	0.0037 ± 0.0019	0	2.48 (<4.30)	0.48 (<4.30)

*denotes statistically significant

Figure 3.3 shows the pulsed dose rate and low dose rate results for the C716 cell line, along with the high dose rate curve. As with the parental cell line, the pulse dose rate data has similar survival to the low dose rate treatment. Table 3.2b shows the results of

Figure 3.3. Survival curves for the C716 cell line after pulsed dose rate (PDR), low dose rate (LDR), and high dose rate (HDR) irradiation. Each point represents the average of three experiments and the error bars represent the SEM between experiments.

the LQ model fit, as well as the statistical significance of the β parameter for both types of treatments. These results show that the α parameters for the two treatments are similar, within their errors, and that the β parameter is statistically significant for the LDR treatment (implying not all SLD is being repaired) and not for the PDR treatment.

Table 3.2b. Linear quadratic parameters from the fits of the low dose rate and pulsed dose rate experiments for the C716 cell line, with estimated standard errors. The significance of the beta parameter is also shown for both treatments.

Parameter	LDR		PDR		β significance (F*)	
	Full	Reduced	Full	Reduced	LDR	PDR
α (Gy ⁻¹)	0.149 ± 0.021	0.209 ± 0.008	0.167 ± 0.021	0.248 ± 0.008	-	-
β (Gy ⁻²)	0.0080 ± 0.0028	0	0.0071 ± 0.0029	0	6.24* (>4.17)	2.37 (<4.30)

* denotes statistically significant

Table 3.3. p-values calculated using the Student's t-test to determine equivalence between LDR doses and PDR fractionation cumulative doses for the MCF7 and C716 cell lines.

Dose (Gy)	MCF7	C716
1.5	0.980	0.905
3	0.871	0.959
4.5	0.852	0.448
6	0.601	0.464
7.5	0.429	0.376
9	0.499	0.654
10.5	0.711	0.914

The equivalence between pulsed dose rate and low dose rate irradiations was tested for both cell lines using the student's t-test, and the results are displayed in Table 3.3. A p-value of less than 0.05 denotes statistical significance. It can be observed from this table that the two treatments are equivalent for both cell lines.

3.2.2 Double-stranded break assays

3.2.2.1 CHEF gel electrophoresis

Figure 3.4 shows the fraction of activity released (FAR) after a treatment of high dose rate irradiation for the parental MCF7 cell line and its variant C716 cell line. The treatment was administered at room temperature as well as on ice, and samples were taken immediately following treatment. The treatment at room temperature shows that there are more initial DSBs for the MCF7 cell line than for the C716, however, the treatment delivered on ice, which is the customary procedure, shows that there is no difference in the amount of initial DSBs between the two cell lines.

Figure 3.5 shows FAR for the MCF7 cell line after a treatment of PDR irradiation, with HDR irradiation for comparison. Samples were taken immediately after treatment, however, since there was repair occurring during the treatments, residual DSBs were measured. It can be observed from the figure that PDR irradiation resulted in a very slight increase in the amount of residual DSBs from background levels (dashed line), even at higher doses.

Figure 3.6 shows that for the C716 cell line there is a more substantial increase in the amount of residual DSBs from background levels (dashed line) after PDR irradiation at higher doses.

Figure 3.4. Fraction of activity released following high dose rate irradiation for the MCF7 and C716 cell lines. Each point represents the average of three experiments and the error bars represent the SEM between experiments.

Figure 3.5. Fraction of activity released following PDR and HDR irradiation for the MCF7 cell line. Each point represents the average of three experiments and the error bars represent the SEM between experiments.

Figure 3.6. Fraction of activity released following PDR and HDR irradiation for the C716 cell line. Each point represents the average of three experiments and the error bars represent the SEM between experiments.

3.2.2.2 Comet Assay

Comet assay experiments were performed since data showed that the CHEF gel electrophoresis might not be sensitive enough to detect residual DSBs after PDR and LDR irradiations.

Figure 3.7 shows the tail moments for the MCF7 after PDR and LDR irradiations. In contrast to the CHEF gel electrophoresis results, this assay shows a more definite increase in the amount of residual DSBs at lower doses. It can be observed from the figure that the PDR and LDR treatments are similar for the MCF7 cell line. The equivalence is confirmed in Table 3.4 using the student's t-test on matched data points.

Table 3.4. p-values calculated using the Student's t-test to determine equivalence between LDR doses and PDR fractionation cumulative doses for the MCF7 and C716 cell lines.

Dose (Gy)	MCF7	C716
6	0.938	0.178
9	0.464	0.114
10.5	0.934	0.143

*denotes statistically significant (not equivalent to LDR)

Figure 3.8 shows the tail moments for the C716 after PDR and LDR treatments. It can be observed from the figure that, as with the parental cell line, the two treatments are similar for the C716 cell line. The results of the student's t-test on matched data points are also displayed in Table 3.4, and they support the graphical data.

Figure 3.7. Normalized tail moments following LDR and PDR irradiation for the MCF7 cell line. Each point represents the average of three experiments and the error bars represent the SEM between experiments.

Figure 3.8. Normalized tail moments following LDR and PDR irradiation for the C716 cell line. Each point represents the average of three experiments and the error bars represent the SEM between experiments, except for LDR, which was only two experiments.

3.2.3 RT-PCR

Figures 3.9-3.11 show the expression levels of DNA polymerases α , β , and ϵ , respectively, for the MCF7 cell line after LDR and PDR treatments. Cells were treated with 6 Gy and levels of expression were measured immediately after treatment, as well as 6 hours and 12 hours after treatment. Only the crossing point values within the range of the standard curves are displayed. Data were normalized to the 0 Gy control. Note that results from polymerase δ were not measurable and are not shown. This is likely due to very low levels of expression of this polymerase in these cell lines. A positive control was used with the same set of primers, cDNA from colorectal cancer cells, which showed proper quantification (data not shown).

From Figure 3.9 it can be observed that the alpha polymerase expression levels are the same after 0h and 12 hours after the 6 Gy dose as for untreated cells for both LDR and PDR. The gene is downregulated after 6h following the dose for both LDR and PDR treatments. Table 3.5a shows the results of Student's t-test on the normalized crossing point (CP) ratios, between LDR and PDR data points, as well as the 6Gy dose with different times for expression versus the untreated control. The table confirms what's seen on the figure, except that for LDR the downregulation after 6h is not significant.

Figure 3.10 shows that the β polymerase is downregulated after PDR and LDR irradiation at 0h and 6h expression times compared to the control, then recover back to untreated control levels. Results in Table 3.5a show that the PDR and LDR treatments are equivalent, and that the changes in expression levels in this polymerase are not significantly different from untreated levels.

Table 3.5a. p-values calculated using the Student's t-test to determine equivalence between LDR and PDR irradiation in terms of gene expression for the MCF7 cell line.

Treatment	LDR vs. PDR			LDR 0 Gy vs. treatment			PDR 0 Gy vs. treatment		
	α	β	ϵ	α	β	ϵ	α	β	ϵ
6Gy 0h	0.945	0.210	0.906	0.860	0.266	0.094	0.703	0.213	0.177
6Gy 6h	0.739	0.085	0.111	0.305	0.311	0.150	0.008*	0.182	0.227
6Gy 12h	0.991	0.966	0.390	0.857	0.801	0.347	0.826	0.478	0.890

*denotes statistically significant (the two treatments are different from each other)

Figure 3.11 shows that the ϵ polymerase is upregulated immediately after irradiation, then for PDR ϵ is downregulated while for LDR it is upregulated after 6h, and after 12h the expression levels are similar to untreated levels for both types of treatment. Results from Table 3.5a show that the LDR and PDR treatments are equivalent, and that the changes in expression levels in this gene are not significantly different from untreated control levels.

It should be noted that the PCR assay is difficult to work with and there is a lot of variability between experiments due to the doubling of the products during each cycle. Thus while the relative changes within each experiment were very reproducible there were differences amongst experiments which resulted in large error bars when combining experimental data.

Figures 3.12-3.14 show the expression levels of the three DNA polymerases for the C716 cell line after LDR and PDR treatments using the same protocol as for the parental cell line

Figure 3.9. Normalized CP ratios for the α polymerase following LDR and PDR irradiation for the MCF7 cell line. Each point represents the average of at least two experiments and the error bars represent the SEM between experiments.

Figure 3.10. Normalized CP ratios for the β polymerase following LDR and PDR irradiation for the MCF7 cell line. Each point represents the average of at least two experiments and the error bars represent the SEM between experiments.

Figure 3.11. Normalized CP ratios for the ϵ polymerase following LDR and PDR irradiation for the MCF7 cell line. Each point represents the average of at least two experiments and the error bars represent the SEM between experiments.

. From Figure 3.12 it can be observed that compared to the untreated levels, the alpha polymerase is downregulated after 0h, further downregulated after 12 hours, and then it recovers almost to the levels immediately after irradiation for the PDR treatment. For LDR, the CP values for the treatment immediately after irradiation did not fall within the standard curve range, hence they were rejected. This was done because points outside of the range of the standard curve may not have a linear relationship (which exist in the standard curve range), therefore the CP values obtained by interpolation might be inaccurate. After 6h following irradiation, the gene is downregulated, then further downregulated after 12h following irradiation. Table 3.5b shows the results of Student's t-test on the normalized CP ratios, between LDR and PDR data points, as well as the 6Gy dose with different times for expression versus the untreated control. According to the results in the table, the LDR and PDR treatments are equivalent in terms of polymerase α expression, and that any changes in expression for either treatment are not statistically different from untreated control levels.

Table 3.5b. p-values calculated using the Student's t-test to determine equivalence between LDR and PDR irradiation in terms of gene expression for the C716 cell line.

Treatment	LDR vs. PDR			LDR 0 Gy vs. treatment			PDR 0 Gy vs. treatment		
	α	β	ϵ	α	β	ϵ	α	β	ϵ
6Gy 0h	-	0.876	0.670	-	0.399	0.844	0.181	0.551	0.482
6Gy 6h	0.114	0.484	0.060	0.374	0.128	0.021*	0.074	0.217	0.256
6Gy 12h	0.653	0.995	0.054	0.263	0.908	0.997	0.110	0.952	0.444

*denotes statistically significant (the two treatments are different from each other)

Figure 3.13 shows the expression levels of DNA polymerase β . It can be observed that the polymerase is slightly downregulated immediately after treatment and further after 6h following treatment, but it recovers to background levels at 12h following treatment. This is observed for both PDR and LDR treatments. These changes in expression are very small and Table 3.5b shows that they are not significantly different from background levels, and that the PDR and LDR treatments are equivalent.

The expression levels of the DNA polymerase ϵ are shown on Figure 3.14. There is little change in expression for PDR irradiation at the times measured following treatment compared to background levels. For LDR irradiation, the levels of expression are similar to background when measured immediately after irradiation and 12h following treatment. However, there is a dramatic increase in polymerase expression at 6h following the LDR treatment. This increase was also observed with the parental cell line, also at 6h following LDR treatment. In this case, however, the change from background levels of expression is statistically significant, as determined by the Student's t-test shown in Table 3.5b.

Figure 3.12. Normalized CP ratios for the α polymerase following LDR and PDR irradiation for the C716 cell line. Each point represents the average of at least two experiments and the error bars represent the SEM between experiments.

Figure 3.13. Normalized CP ratios for the β polymerase following LDR and PDR irradiation for the C716 cell line. Each point represents the average of at least two experiments and the error bars represent the SEM between experiments.

Figure 3.14. Normalized CP ratios for the ϵ polymerase following LDR and PDR irradiation for the C716 cell line. Each point represents the average of at least two experiments and the error bars represent the SEM between experiments.

3.3 PDR and LDR with 41°C hyperthermia

A temperature of 41°C was delivered continuously during the PDR irradiation period, which was a pulse of 1.5 Gy given every 3 hours for all PDR irradiations, as well as during the LDR irradiation. A temperature of 41°C was chosen because two published studies on hyperthermia and pulsed dose rate show that 40°C does not cause a lot of sensitization, while 41°C has a larger effect (13, 52). Hyperthermia alone experiments were also performed and used to normalize the hyperthermia and radiation combination treatments (the hyperthermia plus radiation combination treatment result was divided by the hyperthermia alone result).

3.3.1 Clonogenic assay

Figure 3.15 shows the response of the MCF7 cell line to PDR and LDR with/without 41°C hyperthermia, along with a HDR curve for comparison. From these curves, it can be observed that the pulsed dose rate plus hyperthermia response has lower survival compared to that of the low dose rate plus hyperthermia treatment at higher doses (although from Table 3.6b the difference is not significant). Results from the LQ model are shown in Table 3.6a. From the table it can be seen that the α parameters for the PDR and LDR plus hyperthermia treatments are similar, within their errors. In addition, the α parameters for the treatment with and without hyperthermia are also similar to each other within their errors. The β parameters are large, although not as large as that for high dose rate irradiation. This supports the theory that hyperthermia, when added to a radiation treatment, inhibits sublethal damage repair. In this case, some sublethal damage

repair is being inhibited when combining LDR or PDR with hyperthermia, but this inhibition is not as much as for HDR irradiation.

Table 3.6a. Linear quadratic parameters from the fits of the low dose rate and pulsed dose rate in combination with 41°C hyperthermia experiments for the MCF7 cell line, with estimated standard errors. The parameter values for treatments without hyperthermia are also shown for comparison.

Parameter	LDR	LDR + 41°C	PDR	PDR + 41°C
α (Gy ⁻¹)	0.369 ± 0.031	0.336 ± 0.042	0.327 ± 0.049	0.329 ± 0.046
β (Gy ⁻²)	0.0023 ± 0.0015	0.0302 ± 0.0083	0.0037 ± 0.0019	0.0425 ± 0.0096

The equivalence between the combination treatments of pulsed dose rate and low dose rate irradiations with 41°C hyperthermia was tested, as well as the equivalence between PDR with/without hyperthermia (PDRH and PDR, respectively), and LDR with/without hyperthermia (LDRH and LDR, respectively) to establish the effectiveness of concurrent hyperthermia as a radiosensitizer. The equivalence was determined using the student's t-test, and the results are displayed in Table 3.6b. A p-value of less than 0.05 denotes statistical significance. It can be observed from this table that for the MCF7 cell line the two treatments of LDR and PDR are equivalent. In addition, the p-values show that concurrent hyperthermia is an effective radiosensitizer at higher doses.

Figure 3.15. Survival curves for the MCF7 cell line after pulsed dose rate (PDR) with/without hyperthermia, low dose rate (LDR) with/without hyperthermia, and high dose rate (HDR) irradiation. Each point represents the average of three experiments and the error bars represent the SEM between experiments.

Table 3.6b. p-values calculated using the Student's t-test to determine equivalence between LDR doses and PDR plus hyperthermia fractionation cumulative doses for the MCF7 cell line.

Dose (Gy)	PDRH vs. LDRH	PDR vs. PDRH	LDR vs. LDRH
1.5	0.666	0.135	0.077
3	0.917	0.183	0.065
4.5	0.392	0.149	0.313
6	0.124	0.055	0.023*
7.5	0.089	0.029*	0.003*
9	0.051	0.017*	0.004*

*denotes statistically significant (the two treatments are not equivalent)

Figure 3.16 shows the survival curves for the C716 cell line after PDR and LDR with/without 41°C hyperthermia, along with a HDR curve for comparison. This cell line shows similar results to that of the parental cell line. PDR irradiation plus hyperthermia (PDRH) shows lower levels of survival at higher doses than LDR plus hyperthermia (LDRH). Results from the LQ model are shown in Table 3.7a, and it can be observed that, as with the parental cell line, the α parameters for PDR and LDR plus hyperthermia treatments are similar, within their errors. As with the parental cell line, the α parameters for the treatment with and without hyperthermia are also similar to each other within their errors. The β parameters are large, although not as large as for HDR irradiation, implying that there is some SLD repair inhibition, however, not as much as for HDR irradiation.

Table 3.7a. Linear quadratic parameters from the fits of the low dose rate and pulsed dose rate in combination with 41°C hyperthermia experiments for the C716 cell line, with estimated standard errors. The parameter values for treatments without hyperthermia are also shown for comparison.

Parameter	LDR	LDR + 41°C	PDR	PDR + 41°C
α (Gy ⁻¹)	0.149 ± 0.021	0.185 ± 0.032	0.167 ± 0.021	0.154 ± 0.044
β (Gy ⁻²)	0.0080 ± 0.0028	0.0328 ± 0.0060	0.0071 ± 0.0029	0.0469 ± 0.0088

The equivalence between the combination treatments of pulsed dose rate and low dose rate irradiations with 41°C hyperthermia was tested, as well as the equivalence between PDR with/without hyperthermia, and LDR with/without hyperthermia. The equivalence was determined using the student's t-test, and the results are displayed in Table 3.7b. A p-value of less than 0.05 denotes statistical significance. It can be observed from this table that for the C716 cell line the two treatments are equivalent for all cumulative dose points except for the 9 Gy dose. In addition, the p-values show that as with the MCF7 cell line, concurrent hyperthermia is an effective radiosensitizer at higher doses as tested between PDR with/without hyperthermia, and LDR with/without hyperthermia.

Figure 3.16. Survival curves for the C716 cell line after pulsed dose rate (PDR) with/without hyperthermia, low dose rate (LDR) with/without hyperthermia, and high dose rate (HDR) irradiation. Each point represents the average of three experiments and the error bars represent the SEM between experiments.

Table 3.7b. p-values calculated using the Student's t-test to determine equivalence between LDR doses and PDR plus hyperthermia fractionation cumulative doses for the C716 cell line.

Dose (Gy)	PDRH vs. LDRH	PDR vs. PDRH	LDR vs. LDRH
1.5	0.575	0.521	0.990
3	0.634	0.212	0.118
4.5	0.857	0.041*	0.005*
6	0.116	0.001*	0.004*
7.5	0.089	0.002*	0.004*
9	0.024*	0.004*	0.002*
10.5	0.053	0.029*	0.003*

*denotes statistically significant (the two treatments are not equivalent)

3.3.2 Double strand break assays

3.3.2.1 CHEF gel electrophoresis

Figure 3.17 shows FAR for the MCF7 cell line after a combination treatment of PDR irradiation and concurrent 41°C hyperthermia, with HDR irradiation for comparison. It can be observed from the figure that the unnormalized PDR curve shows a substantial increase in the amount of residual DSBs when compared to PDR alone, approaching HDR levels. However, after normalization was performed (by dividing FAR of the combination treatment by FAR of hyperthermia alone), the PDR plus hyperthermia data falls close to background levels. Although thermodynamically hyperthermia by itself does not cause DSBs, it may induce apoptosis or the breakdown of S phase replication sites, which could result in an increase in DSB.

Figure 3.17. Fraction of activity released following PDR with/without hyperthermia and HDR irradiation for the MCF7 cell line. Each point represents the average of two (three for HDR) experiments and the error bars represent the SEM between experiments.

Figure 3.18. Fraction of activity released following PDR with/without hyperthermia and HDR irradiation for the C716 cell line. Each point represents the average of two (three for HDR) experiments and the error bars represent the SEM between experiments.

Figure 3.18 shows similar results for the C716 cell line after the PDR irradiation plus hyperthermia treatment. As with the parental cell line, the unnormalized PDR curve shows an increase in the amount of DSBs from the PDR alone treatment that approaches HDR levels. However, after normalization was performed, the PDR plus hyperthermia curve for the C716 shows no difference in DSBs from the PDR alone until the last dose point. Again, as with the parental cell line, hyperthermia may have induced apoptosis or S-phase replication sites failure, resulting in increased DSB.

3.3.2.2 Comet Assay

Figure 3.19 shows the tail moments for the MCF7 after PDR and LDR irradiations in combination with/without 41°C mild hyperthermia. Hyperthermia alone was not found to cause any significant increases in DSBs levels. The heating time of 21h was much shorter and better tolerated by the cells compared to the 81h for CHEF. It can be observed from the figure that the PDR plus hyperthermia treatment has slightly more DSBs than the LDR plus hyperthermia treatment for the MCF7 cell line, except for the last dose point, where the values for the two treatments overlap. The equivalence between the two treatments is tested in Table 3.8a where the results of the student's t-test on matched data points show that the two treatments are equivalent. The PDR with/without hyperthermia treatments were also tested and it can be observed from the table that the two treatments are significantly different, except for the last data point (which has a large error in the PDR irradiation alone). Similarly, the LDR with/without hyperthermia treatments were found to be statistically different for the same data points as for PDR, and not for the last data point. In addition, the LDR and PDR treatments were combined

and tested against the LDR and PDR plus hyperthermia, the two also combined. This was done to be able to conclude whether the two treatments with/without hyperthermia were significantly different from each other. It was already concluded that PDR and LDR were equivalent and PDR and LDR with hyperthermia as well. Combining treatments allowed for more degrees of freedom. The results are displayed in the last column of Table 3.8a, and they show that the treatments with no hyperthermia are statistically different from the combination treatments of radiation and hyperthermia.

Table 3.8a. p-values calculated using the Student's t-test to determine equivalence between LDR doses and PDR fractionation cumulative doses with/without hyperthermia for the MCF7 cell line.

Dose (Gy)	PDRH vs. LDRH	PDR vs. PDRH	LDR vs. LDRH	PDR+LDR vs. PDRH + LDRH
6	0.078	0.017*	0.027*	0.001*
9	0.130	0.009*	0.042*	0.001*
10.5	0.946	0.071	0.127	0.004*

*denotes statistically significant (the two treatments are not equivalent)

Table 3.8b. p-values calculated using the Student's t-test to determine equivalence between LDR doses and PDR fractionation cumulative doses with/without hyperthermia for the C716 cell line.

Dose (Gy)	PDRH vs. LDRH	PDR vs. PDRH	LDR vs. LDRH	PDR+LDR vs. PDRH + LDRH
6	0.788	0.129	0.058	0.011*
9	0.305	0.012*	0.704	0.019*
10.5	0.245	0.019*	0.019*	0.001*

*denotes statistically significant (the two treatments are not equivalent)

Figure 3.19. Normalized tail moments following LDR and PDR irradiation with/without hyperthermia for the MCF7 cell line. Each point represents the average of three experiments and the error bars represent the SEM between experiments.

Figure 3.20. Normalized tail moments following LDR and PDR irradiation with/without hyperthermia for the C716 cell line. Each point represents the average of three experiments and the error bars represent the SEM between experiments, except for LDR, which was only two experiments.

Figure 3.20 shows the tail moments for the C716 cell line after the PDR and LDR irradiation plus hyperthermia. It can be observed from the figure that, as with the parental cell line, the two treatments are similar to each other for the C716 cell line, and the results from the t-test confirming the equivalence are shown in Table 3.8b. As with the parental cell line, the equivalence between PDR alone and PDR plus hyperthermia treatments was also tested, and the results show that the two treatments are statistically different from each other. Results from the t-test show that the LDR with/without hyperthermia treatments were not significantly different from each other, except for the last data point. LDR and PDR results for this cell line were also combined and tested against these treatments combined with hyperthermia and are shown in the last column of Table 3.8b. It can be observed that as with the parental cell line, the treatments without hyperthermia are different from the treatments combined with hyperthermia.

3.3.3 RT-PCR

Figures 3.21-3.23 show the expression levels of DNA polymerases α , β , and ϵ , respectively, for the MCF7 cell line after LDR and PDR treatments combined with hyperthermia, as well as hyperthermia alone, using the same protocol as for the treatments without hyperthermia. It should be noted that on the figures, 6Gy is referred to the radiation treatments, while 12h is referred to the duration of the hyperthermia alone treatment (the length of time to deliver 6Gy of radiation using PDR or LDR is 12h). For example, 6Gy6h/12h6h means 6Gy or 12h of hyperthermia measured at 6h following treatment. From Figure 3.21 it can be observed that the alpha polymerase is downregulated for both LDR and PDR combined with hyperthermia, although for LDR,

the downregulation starts at 6h after the treatment. For hyperthermia alone, it can be observed that the polymerase is upregulated, reaching the highest level after 12h following heating. Table 3.9a shows the results of Student's t-test on the normalized CP ratios, between LDR and PDR plus hyperthermia (LDRH and PDRH, respectively), as well each of the radiation treatment plus hyperthermia compared to hyperthermia alone. The table shows that the two radiation plus hyperthermia treatments are equivalent to each other except for the PDRH treatment measured immediately following the last radiation fraction, which is lower than the LDRH treatment. The LDRH and hyperthermia alone treatments are not statistically different from each other (LDRH is averaged from two experiments), and the same is true for the PDRH treatment versus hyperthermia, except for the point taken 12h after treatment, which is statistically different between the two treatments.

Table 3.9a. p-values calculated using the Student's t-test to determine equivalence between LDR and PDR irradiation plus hyperthermia, as well as each radiation combination treatment against hyperthermia alone, in terms of gene expression for the MCF7 cell line.

Treatment	LDRH vs. PDRH			LDRH vs. H			PDRH vs. H		
	α	β	ϵ	α	β	ϵ	α	β	ϵ
6Gy/12h0h	0.004*	0.940	0.988	0.727	0.925	0.947	0.163	0.804	0.962
6Gy/12h6h	0.430	0.376	0.919	0.396	0.558	0.534	0.187	0.577	0.770
6Gy/12h12h	0.877	0.892	0.781	0.094	0.982	0.672	0.042*	0.959	0.769

*denotes statistically significant.

Figure 3.21. Normalized CP ratios for α polymerase expression following LDR and PDR irradiation with hyperthermia, as well as hyperthermia alone, for the MCF7 cell line. Each point represents the average of at least two experiments and the error bars represent the SEM between experiments.

Figure 3.22. Normalized CP ratios for β polymerase expression following LDR and PDR irradiation with hyperthermia, as well as hyperthermia alone, for the MCF7 cell line. Each point represents the average of at least two experiments and the error bars represent the SEM between experiments.

Figure 3.23. Normalized CP ratios for ϵ polymerase expression following LDR and PDR irradiation with hyperthermia, as well as hyperthermia alone, for the MCF7 cell line. Each point represents the average of at least two experiments and the error bars represent the SEM between experiments.

Table 3.9b. p-values calculated using the Student's t-test to determine the significance of the gene expression levels after treatment as compared to untreated controls for the MCF7 cell line.

Treatment	0Gy vs. LDRH			0Gy vs. PDRH.			0h vs. H		
	α	β	ϵ	α	β	ϵ	α	β	ϵ
6Gy/12h0h	0.983	0.441	0.731	0.002*	0.037*	0.641	0.787	0.381	0.566
6Gy/12h6h	0.114	0.705	0.854	0.006*	0.043*	0.934	0.982	0.250	0.761
6Gy/12h12h	0.141	0.366	0.769	0.030*	0.098	0.768	0.300	0.439	0.978

Table 3.9b shows the statistical significance of the different levels of expression of the polymerase α for the three treatments compared to the untreated control levels. From the table, it can be observed that the only treatment that has significant changes in expression levels compared to the untreated levels is PDR plus hyperthermia.

Figure 3.22 shows that the β polymerase is downregulated compared to untreated controls for all three types of treatment investigated. Table 3.9a shows that all three types of treatment are not statistically different from each other. Table 3.9b shows that the downregulation in the gene is significant only for the PDR plus hyperthermia treatment for the measurements taken immediately after treatment, and following 6h after treatment.

In contrast, Figure 3.23 shows that the polymerase ϵ is upregulated immediately after treatment, then returns to control levels after 6h and 12h following treatment for the combination treatments of radiation and hyperthermia. For the hyperthermia alone treatment, the polymerase levels are increased immediately after heating, then they return to control levels after 12h following heating. Table 3.9a shows that, as with polymerase

beta, the three types of treatment are not statistically different from each other, and from Table 3.9b, the differences in levels in gene expression for the three treatments are not significant compared to untreated controls.

Figures 3.24-3.26 show the expression levels of DNA polymerases α , β , and ϵ , respectively, for the C716 cell line after LDR and PDR treatments combined with hyperthermia, as well as hyperthermia alone, using the same protocol as for the treatments without hyperthermia.

Table 3.10a. p-values calculated using the Student's t-test to determine equivalence between LDR and PDR irradiation plus hyperthermia, as well as each radiation combination treatment against hyperthermia alone, in terms of gene expression for the C716 cell line.

Treatment	LDRH vs. PDRH			LDRH vs. H			PDRH vs. H		
	α	β	ϵ	α	β	ϵ	α	β	ϵ
6Gy/12h0h	0.716	0.058	0.701	0.364	0.706	0.776	0.355	0.151	0.025*
6Gy/12h6h	0.233	0.892	0.740	0.988	0.543	0.903	0.164	0.144	0.777
6Gy/12h12h	0.317	0.487	0.500	0.007*	0.161	0.701	0.069	0.250	0.980

* denotes statistically significant

Table 3.10b. p-values calculated using the Student's t-test to determine the significance of the gene expression levels after treatment as compared to untreated controls for the C716 cell line.

Treatment	0Gy vs. LDRH			0Gy vs. PDRH.			0h vs. H		
	α	β	ϵ	α	β	ϵ	α	β	ϵ
6Gy/12h0h	0.094	0.630	0.560	0.203	0.023*	0.030*	0.346	0.763	0.342
6Gy/12h6h	0.958	0.864	0.417	0.282	0.329	0.232	0.943	0.506	0.492
6Gy/12h12h	0.051	0.161	0.603	0.270	0.364	0.179	0.878	0.441	0.596

Figure 3.24. Normalized CP ratios for α polymerase expression following LDR and PDR irradiation with hyperthermia, as well as hyperthermia alone, for the C716 cell line. Each point represents the average of at least two experiments and the error bars represent the SEM between experiments.

Figure 3.25. Normalized CP ratios for β polymerase expression following LDR and PDR irradiation with hyperthermia, as well as hyperthermia alone, for the C716 cell line. Each point represents the average of at least two experiments and the error bars represent the SEM between experiments.

Figure 3.26. Normalized CP ratios for ϵ polymerase expression following LDR and PDR irradiation with hyperthermia, as well as hyperthermia alone, for the C716 cell line. Each point represents the average of at least two experiments and the error bars represent the SEM between experiments.

From Figure 3.24, it can be observed that polymerase α is downregulated immediately after treatment for all three types of treatment. After 6h α for PDRH recovers slightly, while it recovers back to untreated levels for the LDRH and hyperthermia alone treatments. At 12h after treatment the polymerase levels remain unchanged from those at 6h for the PDRH and hyperthermia alone treatments, while they decrease slightly below levels observed immediately after treatment for LDRH. From Table 3.10a, it can be observed that the three treatments are not statistically different from each other except for the levels between LDRH and hyperthermia alone measured 12h after treatment. Table 3.10b shows that the changes in levels of expression are not statistically significant from untreated levels for any of the three types of treatment.

Figure 3.25 shows that the levels of expression for the polymerase β decrease immediately after treatment for the PDRH treatment, but recover back to untreated levels over the next 12h. For the LDRH treatment, the polymerase levels remain virtually unchanged from untreated levels, until 12h after treatment, when there is a slight decrease in expression. For hyperthermia alone, the β polymerase is gradually upregulated starting immediately after heating. Table 3.10a shows that all three types of treatment are not statistically different from each other. Table 3.10b shows that only the downregulation immediately after the PDRH treatment is statistically different from the untreated levels.

Figure 3.26 shows that the levels of expression for the ϵ polymerase are upregulated for the three types of treatment, especially for PDRH immediately after treatment. Table 3.10a shows that the three types of treatment are equivalent to each other, except for PDRH and hyperthermia alone, immediately after treatment. Table

3.10b shows that only the measurement taken immediately after PDRH treatment is statistically different from the untreated measurement.

4.0 DISCUSSION

4.1 HDR and LDR Irradiations

The relative radiosensitivities of the parental and variant cell lines were determined by comparing their high dose rate and low dose rate irradiation survival curves. Since the C716 transfected clone overexpresses polymerase β , and this polymerase is thought to be involved in repair, the survival of this cell line is expected to be greater than for its parental cell line. Both the high dose rate and the low dose rate survival curves show that this is true for the two cell lines. The MCF7 parental cell line had a lower survival response to high dose rate and low dose rate irradiations than the C716 cell line. This can be observed on the survival curve, as well as in the analysis of the radiosensitivity parameters SF2 and α . The SF2 parameter is defined as the surviving fraction after a 2 Gy dose, which is a typical dose per fraction used in clinical radiotherapy, and there is evidence that the radiosensitivity of human tumour cell lines to low radiation doses correlates with clinical experience (91). For HDR irradiation, the SF2 for the MCF7 cell line was 0.421, which is lower than the 0.604 value for the C716 cell line. For LDR irradiation, the SF2 for the parental cell line was again lower with a value of 0.557 than that for its mutant with its 0.718 value. In addition, although there was a slight change in survival for the two types of radiation for each cell line, the difference is not significant, since it is at a very low dose. However, these differences become significant at higher doses.

The other radiosensitivity indicator, the estimated α parameter from the linear quadratic model, describes the initial slope of the survival curve. A positive correlation has been established between the steepness of the initial slope of the survival curve for

human tumour cells and their clinical response (88, 96). The α values found in the literature for acute irradiation lie in the 0.2-0.6 Gy⁻¹ range, with the lower end indicating radioresistance, and the upper indicating radiosensitivity (88, 91). The α value for the parental cell line was found to be 0.240 Gy⁻¹, which is at the radioresistant limit of the cell lines found in the literature. The value for the C716 cell line was determined to be 0.134 Gy⁻¹, which is much lower than the lower radioresistant limit of the range (lower α means more radioresistant), indicating that this cell line is very radioresistant. The α value for the MCF7 parental cell line from the low dose rate irradiation experiments changed from the high dose rate irradiation value. However, rather than indicating a possible change in radiosensitivity (which would be expressed by a lower α in LDR results compared to HDR (77)), this is most likely due to the fact that the α and β parameters are correlated. The α value for the C716 cell line from the low dose rate irradiation experiments changed only slightly from the high dose rate irradiation value. This suggests that the dose-rate effect did not affect the intrinsic radiosensitivity of this cell line.

The shoulder region on the survival curves can be analyzed using the β parameter from the linear quadratic model. The shoulders on the high dose rate survival curves for the two cell lines were observed to be considerable, as quantified by substantial β values (0.1001 Gy⁻² and 0.0627 Gy⁻² for the MCF7 and C716, respectively). This indicates that at higher doses the damage is lethal due to sublethal damage accumulation and fixation and the survival decreases more rapidly, therefore the accumulated sublethal damage could potentially be repaired when using a fractionated regimen. On the other hand, low dose rate irradiation results show that, as expected, the β value for the parental cell line is

not statistically different from zero, which means that sublethal damage is being repaired due to the dose rate sparing effect. The C716 cell line, however, has a significant β parameter, which implies that not all SLD is being repaired. This may indicate that polymerase β , which is overexpressed in this cell line, is not involved in SLD repair.

The response of the two cell lines to HDR irradiation has been previously established, and the results found in this study are consistent with the published data (71).

4.2 PDR and LDR Irradiations

4.2.1 Clonogenic Assay

The survival curves for the parental MCF7 cell line showed that the 1.5 Gy delivered every 3 hours fractionation scheme was similar to the low dose rate treatment. The LQ model parameters were compared between the two types of radiation, and the results showed no apparent change in radiosensitivity between fractions (similar α values), and a negligible β component, indicating complete repair. In addition, the student's t-test determined that the two treatments are equivalent. The C716 cell line showed similar results. There was no apparent change in radiosensitivity between fractions, and the β component was negligible, with results from the t-test showing equivalence between the two treatments. This equivalence between the two treatments is in agreement with a study that used mathematical modeling with this fraction size (10), as well as with several in vitro studies (13,77). However, there are a couple of studies that show no equivalence between the two treatments using different cell lines, showing that perhaps PDR to LDR equivalence is cell line dependent (16,20,77). In vivo data from animal work and clinical trials mainly used conservative fractionation schemes of 0.5 Gy

delivered over 10 min every hour. Only one study used the same fraction size as the present investigation, using 8 fractions per day of 1.5 Gy delivered every 3 hours to treat squamous cell carcinoma of tonsillar fossa and soft palate, with results comparable to those of LDR treatments in the literature (28).

4.2.2 Double-strand break assays

Double-strand break assay results showed that the CHEF system is adequate for high dose rate irradiation experiments, but not for pulsed dose rate irradiation. HDR irradiation results showed that there is no difference in the amounts of initial DSBs between the two cell lines when the irradiations take place on ice. At room temperature the C716 cell line had less DSBs than the MCF7, indicating it may be more efficient at repairing damage while undergoing irradiation (takes about 45 min to deliver 150 Gy) than its parental cell line.

Pulsed dose rate irradiation results showed that the amount of relative DSBs is not significantly different from background levels for the parental cell line. This is most likely due to the fact that it may take only one DSB to kill a cell, while this assay uses bulk DNA so that a few breaks that may result in cell lethality in the clonogenic assay may not be detected in the CHEF assay. In addition, during PDR irradiation cells may undergo misrepair, where two double strands of DNA are joined incorrectly and may result in cell death as measured per clonogenic assay. This, however, would not be reflected in the CHEF assay, where this particular DNA fragment may be too large to be detected as a DSB. Although PDR DSBs were not detectable by this assay for either cell line, CHEF was used in one study to compare the DSBs following PDR (0.5 Gy/1h, 1

Gy/2h, 1.5 Gy/3h, 2 Gy/4h, and 3 Gy/6h) and LDR (0.5 Gy/h) irradiation using human glioma cell lines and found the two to be equivalent for all fraction sizes investigated (20). This did not correlate with the clonogenic assay results, where for most fraction sizes survival was significantly higher than for LDR irradiation. The study concluded that the CHEF assay does not have a high enough resolution to detect the difference between the two treatments.

The comet assay measures DNA damage in individual cells, so it is more sensitive than the CHEF assay. The comet assay shows similar results to the clonogenic assay. For both cell lines the low dose rate treatment is equivalent to the pulsed dose rate treatment, as shown both graphically and statistically. However, it is interesting to note that in this case the comet assay does not provide a correlation between DSBs and radiosensitivity for the two cell lines. The normalized tail moment have similar values for both cell lines at each dose, hence the more radioresistant cell line does not have less DSBs than the more radiosensitive cell line. This does not correlate with the clonogenic assay where the more radioresistant cell line has greater survival. However, this may be explained as the result of the way in which cells identify or define a DSB. Radiosensitive cells could view both opposed and more widely spaced opposing lesions as DSBs while in resistant cells, a DSB could be identified as two closely opposed SSBs (although it would still be viewed as a DSB by the assay). Hence resistant cells could repair this break as independent SSBs, while the sensitive cells may use other, more error-prone repair pathways (53). This would explain the lack of correlation between the cells radiosensitivity and amount of residual DSBs observed in the present study. In the clonogenic assay results, where the more sensitive cell line, with possibly more error-

prone repair pathways (as previously observed already in our laboratory (67)), has lower survival than the more radioresistant one. The comet assay, on the other hand, would not distinguish DSBs as seen by the sensitive cell line from two closely spaced SSBs as seen by the radioresistant cell line, hence resulting in similar response in the two cell lines. In addition, rejoining of DSBs shows both fast and slow components. Rapidly rejoined lesions that are considered to be DSBs by the assay, but which are viewed as SSBs by the cells should be rejoined quickly. “Correct” rejoining has been shown to occur more likely within the first 2 hours, and misrejoining happens later (53). This may be evidenced in the HDR CHEF results. The C716 cell line was shown to undergo repair while being irradiated at room temperature compared to on ice, while the MCF7 had similar amounts of DSBs whether irradiated at room temperature or on ice. This could be due to the C716 cell line may have a fast component of repair of DSBs and the parental cell line may have a slow component of repair, and this was evidenced in the CHEF HDR irradiation results where the C716 cell line had some repair when the cells were irradiated at room temperature. In addition, there may be more misrepair in the MCF7 cell line resulting in lower residual DSB detection (67). However, in the end, both cell lines appear to repair DSBs to the same degree.

4.2.3 RT-PCR

Both cell lines showed practically undetectable δ polymerase activity, and to ensure that this was not due to the primer design, a second set of primers was obtained, showing similar results. A positive control (cDNA from human colorectal cancer cell line) was used with the second set of primers and showed typical quantification. The

consequences of a defect in proofreading involving a point mutation in polymerase δ have recently been studied in mice (69). The study concludes that the results imply that DNA polymerase errors that are not proofread contribute to carcinogenesis. Consequently, the low level of expression in this polymerase in the cell lines in present study may possibly be due to a defective DNA polymerase δ , which may also have resulted in carcinogenesis in these cells.

From Figures 3.9 and 3.12 it can be observed that for both cell lines levels of gene expression of the polymerase α following LDR and PDR are similar. This polymerase is downregulated in both cell lines, although only one point is significantly different from untreated values (6Gy after 6h for MCF7 PDR). This downregulation is consistent with the proposal that this polymerase is mainly involved in replication, hence during repair of radiation damage cell reproduction is decreased (69).

Figures 3.10 and 3.13 show that the levels of expression of the polymerase β change very little in both cell lines following both LDR and PDR treatments. Most studies in the literature propose that this polymerase is involved in ionizing radiation damage repair, particularly in base excision repair (71,97,98). However, one study showed that cells overexpressing polymerase β were more sensitive to irradiation treatments by increasing apoptosis (99), while another showed that two cell lines, a wild type and the other a polymerase β knockout, had identical clonogenic assay responses, as well as same levels of apoptosis (100). This latter study by Miura et al proposes two pathways for base excision repair (BER), one dependent on polymerase β , the other (concluded as the predominant one in the study) on proliferating cell nuclear antigen

(PCNA). It may be that in the present study the PCNA-dependent pathway is the main route in the repair of radiation-induced DNA damage, as well.

Figures 3.11 and 3.14 show that for both cell lines the LDR and PDR treatments are equivalent and unchanged from untreated levels in terms of expression of polymerase ϵ , except for at 6Gy after 6h for LDR, where there is a great increase (significant for the C716) in gene expression. Since this effect happens in both cell lines, it may be speculated that the polymerase ϵ may have a role in repair of sublethal damage after LDR treatment. This may not happen following PDR treatments because 1.5 Gy may be too large a fraction to activate this particular enzyme. The study by Miura et al mentioned previously indicates in their discussion that in vitro studies support efficient long-patch BER by the polymerase δ or polymerase ϵ enzymes (100). These studies support the finding in the present study that the polymerase ϵ may have a role in the repair of radiation damage due to LDR irradiation. As for PDR irradiation, another polymerase may be involved that was not investigated here, such as iota and lambda (which are proposed to be involved in BER (69)).

4.3 PDR and LDR with 41°C hyperthermia

4.3.1 Clonogenic assay

The results in this study confirmed the effectiveness of mild 41°C hyperthermia administered concomitantly with the 1.5 Gy/3h fractions, supporting data from several studies that have demonstrated the effectiveness of mild hyperthermia as a radiosensitizer (13,35,45,47).

Survival data for the MCF7 and C716 cell lines show that both PDR and LDR combined with 41°C hyperthermia have a much lower survival than either treatment alone, and statistical analysis shows that the combination treatments are significantly different from the uncombined treatments. This is especially true for the C716 where the difference between the two treatments starts already at 4.5 Gy compared to 6 and 7.5 Gy for the MCF7. This radiation and hyperthermia combination effectiveness in both cell lines is most likely due to SLD repair inhibition by the added hyperthermia treatment. In addition, since both cell lines are thermally radiosensitized to a similar degree, it indicates that polymerase β , which is overexpressed in the radioresistant cell line, may not play a role in repair of this type of radiation damage (101).

The combination hyperthermia treatment appears on the graphs to be more effective for the PDR treatment than for the LDR for both cell lines, however, the two combination treatments are not statistically different from each other (except for one data point for the C716). Therefore the LDR-PDR equivalence is maintained with or without hyperthermia for this cell line. These results support those in one study that used rat cells treated with PDR (0.25 Gy/0.5h, 1 Gy/2h, 3Gy/6h) with and without 41°C hyperthermia, as well as 0.5 Gy/h LDR irradiation. This study also found that the hyperthermia treatment sensitized the PDR and LDR treatments to the same degree (13). Another study using human cell lines found that the 1.5 Gy/3h treatment combined with hyperthermia was only slightly effective for two tumour cell lines, and not at all for the normal cells, although the hyperthermia used was 40°C, and therefore slightly lower (52).

Currently there are no mathematical models to fit the pulsed dose rate combined with hyperthermia treatment, however, the linear quadratic model was used in this study.

Graphically, the LQ model fits the data well for the low and intermediate doses, but diverges at higher doses, and results from the fits show that in this case, the LQ model adequately describes the differences between the two radiation treatments with hyperthermia and without. In both LDR and PDR irradiation the α parameter changes very little when compared to LDR and PDR plus hyperthermia. This suggests that the intrinsic radiosensitivity of the cell does not change when hyperthermia is administered. The change occurs in the β parameter, which is larger for the hyperthermia combination treatments than the LDR and PDR treatments alone, suggesting SLD repair inhibition. This gives support to the theory that hyperthermia combined with radiation inhibits sublethal damage repair. One study that used the LQ model to fit LDR plus hyperthermia data found that although the LQ model fit the data adequately, it did not describe the data correctly biologically, since the α parameter changed (77).

4.3.2 Double-strand break assays

CHEF data showed for both cell lines that although PDR with hyperthermia caused significant amounts of DSBs (supporting the clonogenic assay results), normalizing PDR to hyperthermia brought the DSBs values to background levels. Hyperthermia alone does not cause DSBs, however, to reach 40 Gy the cells are at 41°C for four days, which is a very long time at this temperature for cells to function normally. The effect observed is most likely due to apoptosis caused by the long exposure to hyperthermia, causing the cells to suicide resulting in fragmented DNA. This apoptosis effect may be greater on DSB formation than the combination of PDR with hyperthermia

hence the low DSB levels after normalizing. Failure in S phase replicating sites may also result in a greater detection of DSBs.

Results from the comet assay show that, for both cell lines, PDR and LDR plus hyperthermia treatments are equivalent to each other. In addition, although PDR/LDR and PDR/LDR plus hyperthermia are statistically different from each other for most of the doses investigated in both cell lines, there are some dose points that do not show this difference. In order to be able to draw definite conclusions about these experiments, the treatments without hyperthermia were combined together as were those combined with hyperthermia. This was done to increase the number of degrees of freedom, and also it was justified since the equivalence between LDR and PDR with/without hyperthermia has already been established. This resulted in significant differences between the treatments with and without hyperthermia. This supported the clonogenic assay data. However, just as with the irradiation treatments alone, the results of the combination irradiation and hyperthermia treatments are not correlated to the clonogenic assay results since the amounts of DSBs are not different between the two cell lines, meaning the more radioresistant cell line does not have less DSBs (in fact they are slightly greater than for the radiosensitive parental cell line). This could be due to the C716 cell line may have a fast component of repair of DSBs and the parental cell line may have a slow component of repair, as well as there may be more misrepair in the MCF7 cell line resulting in lower residual DSB detection (67). There have been no studies to date using the comet assay to compare PDR or LDR to these treatments combined with hyperthermia. There is however one study comparing LDR to LDR combined with camptothecin, which found strong

correlations between the levels of residual DNA DSBs and surviving fraction after concurrent treatment with 25 μ M camptothecin and low dose rate irradiation (65).

4.3.3 RT-PCR

There have been very few studies investigating the effect of irradiation and hyperthermia on DNA polymerases expression. Several studies have been performed using hyperthermia alone, and it was found that for higher hyperthermia temperatures (42.2-45.5°C) polymerase β was more sensitive than polymerase α (102-106). Mild hyperthermia (40-41°C), on the other hand, has shown that polymerase β is more resistant to hyperthermia than polymerase α (107).

In the present study polymerase α expression was not affected significantly by mild hyperthermia in either cell line, although there was an upward trend in the parental cell line, which has been observed in the literature, followed by decreased levels of expression as time for expression progressed (107). Hyperthermia combined with LDR or PDR treatments caused decreased levels of expression of this polymerase in both cell lines, which is consistent with the hypothesis that this is mainly a replicative polymerase, hence during repair of radiation damage cell reproduction is decreased.

Polymerase β expression was not affected in a significant way by hyperthermia alone, which is consistent with the published data that this polymerase is not sensitive to mild hyperthermia (107). Hyperthermia combined with the LDR and PDR treatments did not have a significant effect on polymerase β expression in either cell line, except for the C716 immediately after PDR treatment. This is consistent with the findings from the previous section in that this polymerase does not appear to be involved in radiation

damage repair, and there are some studies in the literature that support these findings (101). As discussed in the previous section, PCNA may be the predominant BER pathway.

Polymerase ϵ expression was slightly upregulated for the treatments studied, however, the upregulation was not significant for the MCF7 and significant for only one point for the C716 (PDR plus hyperthermia immediately after treatment). Therefore this polymerase may have some involvement with repair of these types of treatment. This polymerase has already been proposed to be involved in radiation damage repair in the previous section and other studies (100). However, other polymerases that have not been investigated may be involved instead or as well as this polymerase in the response to mild hyperthermia and in the repair of damage following PDR and LDR treatments combined with mild hyperthermia.

5.0 CONCLUSIONS

The relative radiosensitivities of two cell lines were investigated, and it was found that the parental cell line, MCF7 was more radiosensitive than its β polymerase-overexpressing clone, C716. The MCF7 cell line had a smaller SF2 value and a larger α parameter from the LQ model (larger α meaning more sensitivity to radiation), as well as a larger shoulder on the survival curve, which reflected in a larger β parameter.

The pulsed dose rate (PDR) to low dose rate (LDR) irradiation responses were studied, and both cell lines showed equivalence between the two types of treatment in terms of the clonogenic assay and the comet assay (the other double strand break assay, the CHEF assay, was determined insufficiently sensitive for these types of treatments). Results from RT-PCR also showed the two treatments to be equivalent in terms of gene expression of three polymerases, alpha, beta and epsilon. The two types of treatment, therefore, were found to be equivalent in terms of several biological endpoints. The α polymerase was found to be downregulated, which could be interpreted as a decrease in replication during radiation damage repair. The β polymerase was found to be unresponsive to the treatments, implying that it is not a major pathway in base excision repair and that there may be another pathway, such as the PCNA (proliferating cell nuclear antigen). The ϵ polymerase was found to have a possible role in the repair of radiation damage following LDR irradiation.

The PDR and LDR treatments combined with mild 41°C hyperthermia were found to be equivalent to each other, but statistically different from their counterpart treatments without hyperthermia in terms of the clonogenic assay and the comet assay. RT-PCR studies showed relatively no change, except for the C716 cell line, which had a

lower level of expression of the polymerase α immediately after a combination treatment of PDR and mild hyperthermia. The response to mild hyperthermia in terms of the expression of the three polymerases was also established in the two cell lines. It was found that the α polymerase was the most thermoresponsive of the polymerases studied, which would translate into a decrease in cell replication during radiation damage repair. The same was observed in the radiation-hyperthermia combination treatments. The β polymerase was found to be only slightly downregulated in the treatments studied, as previously concluded, it may not be a major component in BER, but another pathway may be involved. The ϵ polymerase was slightly (insignificantly) upregulated, therefore it may have a role in radiation damage repair.

From the treatments investigated, the PDR and LDR treatments were found to be equivalent, hence the use of the 1.5 Gy given every 3 hours should be studied in the clinic. In addition, combining the two radiation treatments with hyperthermia was found to be effective, therefore the use of hyperthermia as a radiosensitivity enhancer in the treatment of tumours should be investigated further in the clinic as well.

5.1 Future Research

Although this study showed equivalence between PDR and LDR irradiation, there is a large body of literature that shows that the equivalence may be cell line dependent (16, 18, 77). The reason for this cell line dependence should be studied by examining the response of different cell lines to PDR and LDR irradiation, particularly those of the same genetic background but of different radiosensitivities, using the assays studied here.

This would allow the study of different responses between cell lines in terms of their survival, its correlation to the amount of DSBs, and differences in gene expression.

With tumour cell lines, especially those that are used to derive radioresistant clones, there is a danger of adaptive response (18). However, there are not many studies in the literature that investigate this effect, which could have a crucial impact in using PDR brachytherapy in the clinic (18-21). It is very important, therefore, to investigate adaptive response by expanding on the current findings, such as the role of the enzymes deoxycytidine kinase and thymidine kinase 1 and 2. The effect of PDR irradiation on the activities of these enzymes has been studied following treatment (21), however, it would be useful to track its expression during the treatment to determine what dose or which fraction size would trigger the adaptive response. In addition, cell lines known to exhibit adaptive response following PDR irradiation, such as the human ovarian carcinoma cell line A2780s, should be examined in terms of the gene expression of polymerases and other enzymes (such as the kinases mentioned above) believed to be involved in repair of radiation damage. Other cell lines, including the ones used in present study, should be investigated for possibility of adaptive response by irradiating them with small doses followed by HDR irradiation and compare those results to HDR alone for changes in survival (18).

The RT-PCR experiments should be followed up with the Western Antibodies assay, which examines protein levels. This would be useful because it would determine whether the upregulation or downregulation of the genes studied here, especially polymerase ϵ , is being translated into a respective increase or decrease in protein production. DNA polymerase ϵ should also be further studied in other cell lines following

PDR and LDR treatments to determine whether it is expressed differently following these treatments. In addition, the δ polymerase should be further investigated in other cell lines for its role in radiation response and thermal radiosensitization, as well as in the cell lines presently used to determine why it did not amplify during PCR. The role of PCNA in base excision repair should also be studied, especially in the cell lines used in present study, since PCNA and not polymerase β may be a major pathway of this repair mechanism (100).

This study investigated only four DNA polymerase genes. To date there are 14 DNA polymerases, and some have a yet undetermined role in the repair of radiation damage. Therefore further studies should be undertaken to investigate which DNA polymerases may be involved in the repair of radiation damage. In addition, a host of other repair genes should also be evaluated. It would be constructive to design a DNA micro array chip with all known repair genes in order to assay gene regulation in response to radiation and thermal radiosensitization.

Appendix A

Low Dose Rate Irradiator

There are two low dose rate irradiators belonging to the Cancer Research Group at the Ottawa General Hospital that are used to irradiate cell cultures in glass vials. The high dose rate unit, however, is used to irradiate cells in plastic flasks that have a slight negative charge, which helps cells adhere to the surface of the flask (74). While for many cell lines the difference between plating them in glass vials or plastic flasks is not significant when regarding plating efficiencies, some cell lines may not attach as well to the glass surface as they do to plastic. Also, the surface area of the glass vial is very small (1.3 cm^2) and does not yield the necessary number of cells required for many of the assays used in this study. Therefore to use a uniform system to irradiate cells, a new low dose rate irradiator that is able to irradiate cells in flasks or petri dishes has been designed and built. This irradiator has also been used to irradiate mice by other investigators.

On January 1997 the Cancer Research Group at the Ottawa General Hospital received decommissioned cesium sources from the Manitoba Cancer Treatment and Research Foundation, and from the Selectron remote afterloading unit at the Civic Division. The sources are described in Table A-1 along with their activities and active lengths. Total activity of all the sources combined is 1.358 Ci.

The half life ($T_{1/2}$) of cesium is 30 years with γ ray energy of 0.662 MeV. The half value layer (HVL) is 5.5 mm Pb.

Table A-1. Cesium sources.

Source I.D.	No. of sources	Activity (mCi)	Description
A1	7	16.9	10 mg ²²⁶ Ra equivalent “tube” sources. Active volume 15.6 mm x 1.7 mm; 20.9 mm x 3.0 mm overall. Cs active length 15 mm.
A1	3	17.0	Same as above
A2	2	24.8	15 mg ²²⁶ Ra equivalent “tube” sources. Active volume and length as above.
A3	2	25.3	Same as above
A4	10	33.0	20 mg ²²⁶ Ra equivalent “tube” sources. Active volume and length as above.
M1	14	16.6	Micrad line sources. Active volume 20.0 mm x 0.84 mm; each source at tip of a 31 cm long stainless steel wire.
S1	34	15.48	Selectron point sources; 2.5 mm in diameter.

Initially, the dose rate was desired to be 0.5 Gy/h at a distance of 4 cm from the source plane. The irradiation plane was desired to be at least 15x15 cm² to be able to fit an adequate number of flasks or an animal cage. For smaller areas (not more than 80 cm²) brachytherapy tables such as the Manchester system are usually used (108), but because this was such a large plane, the source distribution on the plane was based on an intuitive estimate. The higher activity sources were placed closer together at the peripheries of the plane (especially on the corners), while in the centre of the plane the sources were more spread out (Figure A1.1).

Figure A1.1 Cesium sources distribution

Once the source configuration was estimated, the sources were entered into the Theraplan in the dosimetry lab at the General Division and the dose rate at 4 cm from the source plane was calculated. The dose rate, however, was not uniform throughout the plane, but it was highest in the centre of the plane and then it fell off at the peripheries. Further spreading of the sources in the centre of the plane caused the appearance of cold spots, or areas of low dose rate, therefore a flattening filter (0.89 mm of lead) was

required that would even the dose. The irradiation plane was to be 20x20 cm² as desired. The cesium sources were then sandwiched between 0.5 cm thick plexiglass plates with grooves carved in them to hold the sources in place. Since the total activity of the source plate is very high, the whole source plate was divided into four sections (as denoted by the vertical dashed lines in Figure A1.1) to make it safer by handling one section at a time when placing the sources in their tray, which was made of aluminum.

To determine the dose rate, sixteen 35 mm petri dishes, each with a TLD inside it, were arranged evenly in a 4x4 array on the irradiating plane and irradiated for half an hour. Table A1.2 shows the total dose at each TLD position (position 1 starting at the left back corner of the plane and following left-to-right and back-to-front order). After discarding two positions due to very low doses (position 1 and 13), an average dose rate of 0.472 ± 0.037 Gy/h was calculated. The irradiation times required to achieve the desired doses were calculated using this average dose rate.

Table A1.2. Dose distributions.

TLD number	Dose after 0.5h (cGy)	TLD number	Dose after 0.5h (cGy)
1	19.035	9	25.039
2	23.448	10	26.039
3	20.121	11	24.675
4	20.654	12	23.034
5	23.017	13	18.190
6	21.817	14	22.162
7	25.076	15	25.791
8	25.273	16	24.172

The irradiating chamber size had to be reasonable since it was also to be used with caged animals, therefore the dimensions of the irradiating chamber were to be 20cm high by 20x20 cm². The shielding required was calculated by restricting the exposure rate at just outside the box to be less than 1 mR/h. Using 0.5 mR/h just outside the shielding:

$$\text{Exposure Rate without shielding} = \frac{X}{t} = \frac{\Gamma A}{d^2} \quad (A-1)$$

where X is the exposure, t is time, Γ is the exposure rate constant which is defined at the exposure rate in R/h at a point 1 m from a 1 Ci source, A is the activity of the source in Ci and d is the distance from the source (108). Using the thickness of the lead box to be about 7 cm and the source plate to be in the middle of the box (10 cm from top and bottom), the exposure rate without shielding was calculated to be 14.55 R/h just outside the irradiator box. Using the exposure rate with shielding (assuming 0.5 mR/h just outside the box), the attenuation factor required was calculated as follows:

$$\text{Attenuation factor required} = \frac{\text{unshielded dose rate}}{\text{shielded dose rate}} \quad (A-2)$$

The attenuation factor required to decrease exposure from 14.55 R/h to 0.5 mR/h just outside the box was calculated to be 2.91×10^4 . The number of tenth value layers (TVL) required was calculated to be 4.464 using the following formula:

$$\text{number of TVLs} = \log_{10}(\text{attenuation factor}) \quad (A-3)$$

The HVL for lead shielding for cesium is 5.5 mm, therefore 8 cm of lead were required. In addition, a storage container for the source tray was also designed using similar calculations but for smaller inner dimensions, giving 9 cm of lead. For additional radiation protection, a gap was made in the back of the irradiator and the open storage container could be physically attached to the irradiator with the gap matching the opening of the storage chamber. This allowed for pushing the source tray into the storage chamber using a rod through a hole in the front of the irradiator to allow for safer opening of the irradiator. For irradiating, the source tray could be pushed into the irradiator chamber using the rod through a hole in the back of the storage chamber. When the irradiator was not in use, the source tray remained in the storage chamber.

Experiments involving animals were performed at room temperature. Cells, on the other hand, need 37°C for proper biological function, or 41°C for the hyperthermia experiments. To achieve this, heating cables were coiled on each of the four exposed outer surfaces of the irradiator with heat-conducting material filled in the gaps between the coils, as well as a plate sandwiching the coils for maximum thermal contact. The cable was then connected to a thermocontroller. To estimate the time it took to heat the irradiator, the following was used:

Volume of irradiator box = outer volume – inner volume

$$V = 0.38^3 \text{ m}^3 - 0.20^3 \text{ m}^3 = 0.0469 \text{ m}^3$$

$$\rho \text{ of lead} = 11360 \text{ kg/m}^3$$

$$M \text{ of lead box} = \rho V = 532.44 \text{ kg}$$

Cable was rated at 5W/ft and 120ft were used, so $5\text{W/ft} \times 120\text{ft} = 600\text{W} = 600\text{J/s}$

Specific heat capacity of lead = $128\text{ J/(kg}^\circ\text{C)}$

Room temperature = 23°C and want temperature to increase to 37°C , so $\Delta T = 14^\circ\text{C}$

Time it takes to bring irradiator from room temperature to 37°C :

$$t = 128\text{ J/(kg}^\circ\text{C)} \times 1/(600\text{J/s}) \times 532.44\text{kg} \times 14^\circ\text{C} = 1590.23\text{s} = 26.5\text{ min}$$

In addition, heat loss has to be assumed, so for 90% heat loss $t = 265\text{ min}$ or 4 hours.

However, the heat loss was overestimated since it took less than 5 hours to heat the irradiator to 45°C .

Appendix B

B1. Introduction

Originally, this project included another set of cell lines, human gliomas U373MG and their radioresistant and radiosensitive variants U373MG-CPA and U373MG-CPP, respectively (109). The idea was to have a cell system with normal, resistant, and sensitive responses to radiation from cells of the same genetic background. It has been shown that another human glioma cell line (the U87MG) has exhibited adaptive response by using the same PDR schedule as that used in this study (19). Therefore it would have been interesting to see if there is an adaptive response from any of the three cell lines of the same genetic background to PDR irradiation. In contrast, another study also using human gliomas (U251) showed that after exposure to continuous ultra low dose rate irradiation (0.03 Gy/h), the cells exhibited a sensitization when the treatment was followed by high dose rate irradiation (110). Both types of responses are clinically important in the treatment of malignant human gliomas, and although the scope of the present study was to include these experiments and the three cell lines, in the results section it can be observed that these cell lines lost their variability in response to radiation. However, there were some interesting preliminary results obtained with the U373MG cell line that helped to make decisions regarding experiments with the other cell lines, the MCF7 and C716.

B2. Materials and Methods

The variants U373MG-CPA and U373MG-CPP were derived from the parental cell line U373MG (obtained from the American Type Culture Collection) by intermittent

exposure to cisplatin, resulting in cross-resistance or sensitivity to both radiation and cisplatin. Cells were grown and irradiated using the methods described in sections 2.1 and 2.2, with the exception of the PDR irradiations, which were performed by the experimenter manually, as the automated irradiation system was not yet available. The cells used in the experiments were plated at 10^5 cells per 25cm^2 flask, then fed on day 4 and 7, and experiments were performed on day 10 (at plateau phase). The procedure for the clonogenic and CHEF experiments performed is described in sections 2.3 and 2.4.1.

B3. Results

Figure B1 shows the high dose rate survival curves for the parental U373MG and the variant CPA cell lines. It can be observed from these curves that the two cell lines have identical responses to high dose rate. A second variant cell line, CPG, was also investigated and its response to HDR irradiation from a single experiment is shown on the same curve. The response of this cell line is identical to that of the other two cell lines as well.

Figure B2 shows the response of the U373MG cell line to PDR irradiation, and it can be observed that there is a significant increase in survival when compared to the HDR irradiation curve.

The response to PDR with 41°C hyperthermia is shown in Figure B3, along with the PDR alone response for the U373MG. The combination treatment shows great radiosensitization to radiation when compared to the PDR treatment alone, the survival approaching HDR levels.

Figure B1. Survival curves for the U373MG parental and U373MG-CP variant cell lines after high dose rate (HDR) irradiation. Each point represents the average of three experiments and the error bars represent the SEM between experiments.

Figure B2. Survival curves for the U373MG parental cell line after pulsed dose rate (PDR) and high dose rate (HDR) irradiation. Each point represents the average of three experiments and the error bars represent the SEM between experiments.

Figure B3. Survival curves for the U373MG parental cell line after pulsed dose rate (PDR) with/without hyperthermia and high dose rate (HDR) irradiation. Each point represents the average of three experiments and the error bars represent the SEM between experiments.

Figure B4. Fraction of activity released following PDR with/without hyperthermia and HDR irradiation for the U373MG cell line. Each point represents the average of three experiments and the error bars represent the SEM between experiments.

The results from the CHEF experiments for the U373MG cell line are shown in Figure B4. It can be observed that for most of the doses the PDR alone and the PDR combined with hyperthermia treatments have similar responses, however, the last two dose points, at 15 and 19.5 Gy, show that the two curves may start to diverge.

B4. Discussion

The three cell lines in this study, U373MG with its variants U373MG-CPA and U373MG-CPP, were chosen because of their different radiosensitivities. However, as shown in Figure B1, the difference in responses is absent, perhaps because the radiosensitivity or radioresistance of these cells was only a temporary effect, not lasting more than a few months from when they were originally derived. This has led to the decision to exclude these cells from the present study. However, there were enough interesting results to warrant their inclusion in this section.

The first result of note was the degree of radiosensitization observed when combining PDR with 41°C hyperthermia is shown in Figure B3. When compared to PDR alone, the combination treatment showed a large degree of radiosensitization that brought survival almost to HDR levels. Another study by the experimenter using 1.6 Gy /3h with 40°C hyperthermia showed very little sensitization (77) therefore this result was important in choosing the appropriate temperature for radiosensitization.

The other noteworthy result was that obtained using CHEF, shown in Figure B4. When PDR with 41°C hyperthermia was compared to PDR alone, at the higher doses, it seemed that there was a trend of increased radiosensitization as shown by the increased number of residual double strand breaks. The difference was very small, but it was

thought that irradiating at even higher doses would make the difference significant. This led to the development of the automated PDR irradiation system, which was crucial in accomplishing most of the experiments in this study.

B5. Conclusion

Although the three cell lines U373MG, U373MG-CPA, and U373MG-CPP did not have different radiation responses as expected, results from experiments performed with the U373MG cell line were applied to the other cells used in this study, the MCF7 and the C716 cell lines.

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