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**The radiobiological equivalence of low dose rate irradiation
and pulsed dose rate irradiation, as it relates to
brachytherapy, using the U-87MG glioblastoma cell line**

by

Debbie Michelle Smith

A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfillment of the requirements
for the degree of

Master of Science

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**The radiobiological equivalence of low dose rate irradiation and pulsed
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submitted by

Debbie Michelle Smith, B.Sc.

in partial fulfillment of the requirements
for the degree of Master of Science

Thesis Supervisor

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ABSTRACT

Recently, pulsed dose rate brachytherapy has emerged as a possible alternative to the conventional low dose rate brachytherapy used clinically to treat malignancies. Studies have shown that an equivalence between pulsed dose rate and low dose rate can be achieved if certain conditions are met, which include restrictions on the duration of the pulse and on the period between consecutive pulses.

This thesis investigates the effects of replacing a low dose rate irradiation protocol with a variety of pulsed dose rate irradiations using U-87MG glioma cells *in vitro*. The pulses were delivered at 0.5 Gy/1 h, 1 Gy/2 h, 1.5 Gy/3 h, 2Gy/4 h, or 3 Gy/6 h, in order to maintain the same average dose rate (0.5 Gy/h) as the low dose rate irradiation. The clonogenic assay results show an increase in radiation resistance when comparing the pulsed irradiation data to the low dose rate data. A change in radiosensitivity parameters indicates that an adaptive response may be occurring, possibly due to an increase in DNA double strand break repair. The DNA double strand break assay (CHEF) however showed no significant differences at the strand break level between pulsed and low dose rate. This could be due to limitations in the sensitivity of the assay, or to a higher level of misrepair in the low dose rate case. Overall, none of the pulses investigated in this study showed equivalent survival to the low dose rate data, and the greatest amount of kill was observed with the low dose rate treatment.

ACKNOWLEDGEMENTS

In the completion of this thesis, I have had the assistance and encouragement of many individuals, and I would like to thank them all. First, many thanks to my thesis supervisor Peter Raaphorst, for his ongoing encouragement, his support, both emotional and financial, and for taking the time and effort to share his scientific knowledge and experience so that I may benefit from it. I would also like to thank Cheng Ng for his help, both in the lab and with the writing of this thesis.

I am also grateful to my fellow researchers at the Ottawa Regional Cancer Center. In particular, to the graduate students and technicians in the physics lab, who made the work atmosphere pleasant and enjoyable. In particular, I would like to thank the lab manager Sheryl Cybulski, first and foremost for sharing her expertise in tissue culture, but also for her friendship and support. I would also like to thank Gosia Neidbala for her help and encouragement, especially during our 24 hour experiments. I am also grateful to Daron Owen, for his help with the CHEF and low dose irradiator, and for always having a joke at hand to make me laugh.

Finally, I would like to thank those closest to me. I am very grateful to my parents and my two brothers, for their support, encouragement, and their understanding over the last few years. I would also like to thank my boyfriend Robert Wright, who believed in me, and gave me love and strength to see this thesis through to completion. I am grateful for his patience and understanding, especially during the hectic hours I spent at the lab.

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LIST OF ABBREVIATIONS

CHEF	Clamped homogeneous electric field
CPM	Counts per minute
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
FAR	Fraction of activity released
HDR	High dose rate
LDR	Low dose rate
LQ	Linear quadratic (model)
MG	Malignant glioblastoma
PBS	Phosphate buffered saline
PDR	Pulsed dose rate
PE	Plating efficiency
SEM	Standard error of the mean
SF	Surviving fraction
SLD	Sublethal damage
SSD	Source to surface distance
$T_{1/2}$	Repair half-time
TAE	Tris/Acetic acid EDTA (buffer)
TBE	Tris/Boric acid EDTA (buffer)
TLD	Thermoluminescent dosimetry

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1.0 INTRODUCTION

The use of radiation to treat cancer has been a common clinical practice for many years, but with technological advances, the methods used for delivering such radiation treatments have changed greatly. The most widely used methods today are external beam teletherapy and brachytherapy. More recent developments in radiotherapy have supported two different branches of brachytherapy, the continuous low dose rate (LDR) approach and the high dose rate (HDR) approach. However, a third technique for brachytherapy, called pulsed dose rate (PDR), is slowly gaining popularity. This study investigates the radiobiological equivalence between LDR and PDR brachytherapy, using human glioma cells *in vitro*.

1.1 Radiation Therapy Modalities

In clinical radiotherapy, the most commonly used system to deliver a radiation treatment is the linear accelerator. It provides an external beam therapy and is used to treat various types of cancers, which include sites such as the prostate, breast, and lungs. External beam therapy is very often combined with other modalities, including brachytherapy and chemotherapy. When brachytherapy is chosen as a treatment option, which is the case for the 5-10% of patients with accessible tumors [1], the oncologist must decide if the radiation dose will be administered at a low dose rate (LDR) or at a high dose rate (HDR). The choice is often based on the type and stage of the disease in question. A new brachytherapy treatment given with a pulsed dose rate hopes to

maintain the practical advantages of HDR and the radiobiological advantages of continuous LDR treatment, while eliminating the late normal tissue complications of HDR and the practical disadvantages of LDR treatments.

1.1.1 Low Dose Rate (LDR) brachytherapy

The origins of low dose rate brachytherapy date back many years to when radium was first discovered by the Curies in the early 20th century [2]. Its use in cancer treatment predates external beam teletherapy simply due to the technologies available at the time of inception. It was not until the second world war that teletherapy was widely used, and since the principles behind brachytherapy were simple and required no sophisticated equipment, it was used in earlier times. The word brachytherapy takes its origins from the Greek, meaning *short range*, which describes the proximity of the radiation source to the disease. Brachytherapy is defined as the insertion of a radioactive source directly into or very near a tumor. This is done by loading radioactive seeds or wires into catheters which are implanted in the tumor. Brachytherapy provides a significant advantage over external beam therapy in that the majority of the dose is delivered directly to the malignant tissue, thus sparing healthy normal tissues (such as the skin). Another advantage is the short overall treatment time, which is important to counter tumor repopulation [3]. Brachytherapy can be divided into two main applications: intracavitary brachytherapy, and interstitial brachytherapy.

In an intracavitary treatment, the source is implanted in a body cavity in close proximity to the tumor. This type of treatment is temporary, with the removal of sources

usually occurring after 1 to 5 days. The most common location for this type of brachytherapy is the uterine cervix, although it can also be used for prostate, lung, and lower GI (gastrointestinal) type malignancies. Historically, the radionuclide of choice for intracavitary brachytherapy was radium, although cesium was sometimes used. Today, the most commonly used isotope is Iridium-192. Iridium has a shorter half life, and lower energy gamma rays, making radiation protection easier to handle [2].

For an interstitial treatment, radioactive seeds are implanted directly into the tumor volume. This method can be either permanent or temporary. Originally, radium implants were used, but now the radioisotope which is most commonly used for temporary implants is also Iridium-192. A common isotope for permanent implantation is Iodine-125. There are some advantages to using a permanent implant method in that no operation is required to remove the implant, and the patient hospital time is reduced, since he/she can return home with the implant. One of the disadvantages for the clinics is the expense of purchasing new sources [2].

Clinical brachytherapy has historically been given with low dose rates, over one continuous fraction. A typical dose rate for this type of treatment is 0.5 Gy/h, with a typical treatment consisting of a 30 Gy dose delivered over 60 hours [1]. One of the advantages of a LDR approach is that a long treatment time allows the normal cells to repair sublethal damage. Sublethal damage (SLD) is defined as damage which is not lethal to cells, and under normal conditions can be repaired, unless subsequent damage is sustained which in combination produces lethal, irreparable damage. Differences in SLD

repair between normal and malignant cells is what helps determine the maximum dose which can be delivered to a tumor while still maintaining acceptable normal tissue damage [4]. SLD repair occurs during the continuous low dose rate irradiation, as well as in between fractions for the pulsed dose rate irradiation.

As a consequence of cells being able to repair sublethal damage, a concept known as the dose rate effect becomes important, in which cells exhibit their potential for damage repair and show differences in survival for different rates of irradiation. Cell survival is expressed using a survival curve, which plots, on a logarithmic scale, the surviving fraction of cells after irradiation against, on a linear scale, the dose of irradiation given to those cells (Figure 1.0).

Figure 1.0: Example of a survival curve, showing the surviving fraction of cells as a function of dose

This curve has distinct characteristics and can therefore be described in relatively simple terms. At low doses, the survival of the cells is an exponential function of the dose.

Hence the curve is straight with a finite initial slope, and tends to maintain this shape between zero and small doses (up to a few Gy). As the dose gets larger, the curve tends to bend and then straighten out once again for very large doses. The bending region is typically known as the shoulder of the survival curve [2]. Generally, for high dose rate irradiation, a large shoulder is associated with a greater dose rate effect, whereas a smaller shoulder would indicate a more modest dose rate effect. Therefore the accumulation of sublethal damage is reflected by the shoulder of the curve, and the dose rate effect is representative of the capacity of the cells for sublethal damage repair [4]. As the dose rate is continually reduced, the cells are able to repair more sublethal damage, resulting in a smaller shoulder and a shallower slope in the survival curve [2], [4], and [5]. This dose rate effect is usually most pronounced between dose rates of 0.01 Gy/min and 1 Gy/min [2].

In vitro studies have shown that Chinese Hamster Cells (CHL-F line) have a large capacity for repair, and hence show much shallower survival curves with decreasing dose rates (from 1.07 Gy/min down to 0.0086 Gy/min) [6]. Another study which looked at HeLa cells, also grown in vitro, showed that a change in dose rate is much less significant when compared to the Chinese Hamster Cells, even though the dose rate range was larger, from 7.3 Gy/min down to 0.0054 Gy/min [2]. The shoulders of the survival curves for the HeLa cells do not change significantly, and hence the dose-rate effect is much more modest. This would also lead to the conclusion that the intrinsic capacity for SLD repair in HeLa cells is not very large in comparison to that shown for the Chinese Hamster Cells, and that not all cells have the same intrinsic SLD repair capacity. Since

many normal tissues are late responding, and hence have a greater capacity for repair of SLD, a reduced dose rate would allow for this repair and consequently spare late effects in normal tissues. These normal tissue complications are a very troublesome problem in radiotherapy, often being the dose limiting factor. Since many tumor cells are early responding tissues, the dose rate would affect the malignant cells less than the normal tissues. One should note, however, that if the dose rate is too low, the malignant cells will proliferate, thereby defining a biological limit to how low a dose rate can be in order to remain effective for local tumor control.

Although continuous low dose rate brachytherapy offers many biological advantages in terms of damage repair and sparing of normal tissue, it does have some practical disadvantages. When a LDR brachytherapy treatment begins, many sources are placed into the patient via catheters or needles inserted at the diseased site. These sources remain in the patient for the duration of the treatment. Traditionally, the insertion of these sources into the patient is done manually, resulting in radiation exposure to the immediate medical staff while the sources are being implanted. Furthermore, since the sources are fixed in the patient for the entire treatment time, any hospital, nursing, or medical staff, or family members wishing to interact with this patient during the course of the treatment would also receive small doses of radiation. With continuous LDR, a large inventory of sources is necessary, and an added expense is incurred in storing and preparing the sources. This results in an added radiation protection inconvenience due to the large inventory.

Due to advances in brachytherapy, some centers now choose to use a remote afterloader. With small sources like Cesium-137 or Iridium-192, which can be encapsulated into very small seeds or as thin wires, a remote afterloading device is able to load the sources by computer control, thereby eliminating some of the radiation protection issues present with a manual afterloading technique. The medical, nursing, and hospital personnel can also enter a room during a treatment with very little risk of radiation exposure. When this situation occurs, the afterloader automatically removes the sources from the patient, storing them in a shielded lead safe within the afterloader. However, this results in the interruption of the treatment, and thus the total treatment time must be adjusted and increased accordingly. Also, because of the large number of sources, the afterloading equipment required to administer LDR brachytherapy is often expensive and complex [1]. Another important disadvantage is in terms of the equipment limitations. The equipment and sources are confined to a single patient for the entire duration of the treatment, which can be as long as 4 or 5 days. It would therefore be beneficial to find a treatment method which would eliminate many of the disadvantages of a continuous low dose rate approach, while maintaining the radiobiological advantages mentioned above.

1.1.2 Pulsed Dose Rate (PDR) brachytherapy

This method of treatment is aimed at maintaining the radiobiological advantages of LDR and eliminating many of the aforementioned disadvantages. Pulsed dose rate is directly comparable to low dose rate, since the overall dose rate is the same. In other words, the overall treatment time is the same, and the given dose is the same. The

difference lies in the fact that PDR uses short, intermittent, high dose rate pulses to deliver the dose in various fractions spaced one to several hours apart instead of being continuous. For example, a typical LDR protocol which delivers a 30 Gy dose continuously over 60 hours has a dose rate of 0.5 Gy/h. An equivalent PDR treatment could be given in hourly pulses, say for ten minutes, at a dose rate of 3 Gy/h, thereby giving 0.5 Gy every hour. The instantaneous dose rate during the pulse is higher than the continuous 0.5 Gy/h for LDR. However, since the overall treatment time and the overall dose are the same, the average dose rate is equivalent to that used in the LDR case.

The advantages of PDR brachytherapy are numerous. First, it would require a single high activity source (~ 1 Ci), and would provide optimized dose distributions [7]. This is mainly due to the use of the afterloader and its computer controlled dwell times, where the position of the source is adjusted as it travels within the catheter in the patient. The source can be positioned at any location, for any length of time, thereby making the treatment volume and dose distributions much more precise and easier to define. This method also allows for corrections due to a decaying source as the computer simply increases the dwell time of the source at any particular position [7]-[9]. A single source also reduces factors such as maintenance costs, time, and certain radiation protection issues as previously mentioned [1], [8], and [9]. Also, by giving a fractionated treatment, there is no radiation exposure risk to nurses and hospital staff. They may enter the treatment room in between the pulses, since the source is shielded in a lead safe. Another beneficial feature of PDR is that it would allow, in a well organized clinic, the source to

be used for different patients in between pulses [9]. This would allow more patients to be treated in shorter time periods, and would eliminate some of the treatment waiting lists.

One of the important questions which remains is whether a pulsed treatment is radiobiologically equivalent to continuous LDR brachytherapy. This equivalence can be further divided into two categories: the acute effects and the late effects. Ideally, acute effects such as local tumor control would remain the same, without an increase in such late effects as normal tissue complications. This equivalence obviously depends on certain parameters such as the duration of the pulse, the dose per pulse, and the time interval between pulses. Many studies have been done, both *in vitro* and *in vivo*, for early and late effects, to determine the parameters which make a pulsed dose rate treatment equivalent to the continuous low dose rate approach.

Numerous studies have been done to predict the theoretical parameters which would result in the equivalence of LDR and PDR. One of the first studies to investigate this theoretical equivalence was done by Brenner and Hall [1]. They looked at determining optimal pulse times and periods which would generate equivalence between PDR and LDR. The investigators analyzed data from 36 human cell lines to model the acute effects (cell kill) and looked at the late effects using three *in vivo* models, two animal and one human. The two animals studies were done using the mouse lung, the endpoint being late damage (pneumonitis), and the rat spinal cord, with the endpoint being paralysis. The human data originated from a patient's skin, with the endpoint being telangiectasia. The authors concluded that in practice, a period of 1 hour between 0.5 Gy pulses of ten

minutes in duration would be indistinguishable from LDR irradiation at 0.5 Gy/h, in terms of both early and late effects.

Another study done by Fowler *et al* [10] also looked at the conditions which would result in no significant loss of therapeutic ratio. They found that a good equivalence could be had if the pulses were given as often as once an hour, and if the dose rate during the pulse was kept below 3 Gy/h. This would deliver a 0.5 Gy pulse in minimum of 10 minutes, and agrees with the results of Brenner and Hall [1]. They also suggested that increasing the pulse period to 2 hours would also maintain this equivalence, although not as closely as with an hourly pulse schedule. Furthermore, a study done by Visser and his colleagues [11] suggested that PDR schedules equivalent to LDR could be designed with time intervals as long 3h in between pulses. These longer time intervals would allow for a greater patient load to be treated, and would be beneficial in a clinical setting.

Another study was done to look at the acute response from a pulsed system and its equivalency to the continuous LDR treatment [8]. The overall dose rate for all treatments was kept at 0.6 Gy/h and the instantaneous pulsed dose rate was 9.48 Gy/h, while the pulse width (duration of the pulse) and period between pulses was varied. This study was done using exponentially growing human carcinoma cells *in vitro*, and concluded that there was no significant difference in cell survival between PDR and LDR if the period between pulses was kept at less than 1 hour. This study, however, also found significant differences in early response if the period was lengthened, especially for periods of 6 or 12 hours, with PDR being the more effective treatment showing decreased cell survival.

Furthermore, variability in cell survival among the various cell lines was observed, indicating that perhaps the equivalence between PDR and LDR is dependent on cell type and/or cell line. Another *in vitro* study recently compared the biological effectiveness of PDR to LDR by using two different dose rates during the pulses, namely 4.25 Gy/h and 63 Gy/h, while keeping the overall treatment time constant but changing the period between pulses and the dose per pulse [12]. The results showed that PDR was indistinguishable from LDR for the 4.25 Gy/h pulses, but increasing the dose per pulse (fewer, large pulses) showed a decrease in cell survival. This decrease was even more pronounced when the dose rate during the pulses was increased to 63 Gy/h. This result is significant in that during a pulsed treatment, the source moves through the catheters, delivering the pulses at a high dose rate locally, to the tumor tissue.

Mason et al [9] also looked at testing these predictions using an *in vivo* acutely responding normal tissue. The acute response was measured using the intestinal mucosa of mice, and tested two LDR and two PDR schemes. The two PDR regimens consisted of an hourly 10 minute pulse, at 4.2 Gy/h, and an hourly 1 minute pulse, at 42 Gy/h, giving an overall dose rate of 0.7 Gy/h. Their results showed a LDR/PDR equivalence for both (hourly) pulsed regimens. Reducing the pulse time would also be beneficial by allowing more patients to be treated.

An early study was done to look at the response of rat cells to the various PDR schedules [13]. The equivalence to LDR was shown for an overall dose rate of 0.5 Gy/h repeated as often as every half hour, up to every 6 hours. The instantaneous dose rate

during the pulses was 66 Gy/h, which is somewhat higher than the dose rate used by other investigators. This group did observe some cytotoxic effects when the pulses were delivered every 12 hours in fractions of 6 Gy. This study did provide some indication that a PDR schedule with longer inter-pulse times would be feasible, making the clinical application of PDR more attractive and more effective.

Brenner and Hall also studied the late effects of PDR using an *in vivo* rat study [7]. The chosen parameter for the measurement of late effects was the induction of cataracts, since this is a highly quantifiable system. They exposed the rats' lenses to a total dose of 15 Gy. Some were exposed continuously for 24 hours, others with one of three pulsed regimens: an hourly 10 minute pulse, a 10 minute pulse every four hours, and a 100s hourly pulse. The overall dose rate was 0.6 Gy/h, while the instantaneous dose rate for each pulse varied from 3.6 Gy/h to 21.6 Gy/h. Their results showed that there were no significant increases in late effects when compared to the LDR. Another group also looked at an *in vivo* rat model, but investigated the damage to the spinal cord of the rat [14]. Their experiments differed somewhat, in that they looked at a daytime only schedule, which consisted of a 0.69 Gy pulse every hour (9 pulses per day), and a 2 Gy pulse every 3 hours (4 pulses per day). The dose rate during the day was 0.89 Gy/h and 0.78 Gy/h respectively for each pulse treatment. The LDR treatment was given at 0.5-1 Gy/h. The overall treatment time of the PDR schedule was not matched with the LDR treatment time, with the former being longer in duration (6-8 days, compared with 3 days for the LDR treatment). The investigators concluded that the PDR schedules were more

effective than continuous LDR irradiation, with a dose modifying factor of at least 1.11-1.17.

Recently, PDR has been introduced in certain clinics in Europe and in the United States. One study reported the results of a clinical experience using pulsed dose rate brachytherapy to treat squamous cell carcinoma of the tonsillar fossa and soft palate [15]. The pulses used were less than or equal to 2 Gy, and were delivered 4-8 times a day (4 times in 12 h, or 8 times in 24 h). The results shown were encouraging, and comparable to the best results in the literature, which was particularly significant since 30% of the patients had a high grade tumor (T3/4). The patients who were treated with the pulses also showed superior local control of the tumor compared with patients who received only external radiation therapy. Swift *et al* [16] reported their clinical experience treating pelvic malignancies using hourly pulses of 0.4-0.85 Gy, delivered in 10 to 25 minutes each, around the clock without any planned interruptions. They observed no significant increase in acute toxicity compared with the standard LDR approach, and excellent local tumor control, but suggest that a longer follow-up (greater than the 16.1 month median used in this study) would be needed to fully assess local control and late toxicity. In a more recent clinical application, PDR brachytherapy was used to treat uterine cervix carcinoma [17]. The patients were treated using hourly pulses of 0.4-0.7 Gy. This group also concluded that PDR was a safe and efficient method of treatment for this particular malignancy, and that the outcome (disease free survival rates) and complication rates were comparable to a continuous low dose rate protocol. Jensen and his colleagues [18] studied the use of hourly 0.6 Gy PDR irradiation as salvage treatment for locally

advanced or recurrent gynecologic cancer, and found that it was an effective treatment when combined with external radiation therapy. However, they also observed substantial toxicity in patients with large treatment volumes and recurrent disease.

Two separate groups investigated the application of PDR to the clinical treatment of anal carcinomas [19], [20]. Their PDR schedules consisted of 0.5 Gy or 0.6 Gy pulses delivered every hour, and both groups found that the pulsed approach was an effective and feasible alternative to continuous LDR brachytherapy, although one group did also find substantial toxicities. They concluded that improvements might be possible with a lower total dose, and/or a reduction in the implanted volume [19]. A German group recently implemented a clinical PDR protocol to treat head and neck malignancies [21]. The pulses were 0.5 Gy or 0.7 Gy, given as hourly pulses, 24 hours a day. Their results showed that PDR irradiation provided permanent locoregional tumor control in 93% of the patients (37/40), and that the PDR approach was a safe one, comparable to continuous low dose rate brachytherapy. A retrospective study done by De Pree *et al* [22] analyzed the feasibility and tolerance of pulsed dose rate brachytherapy as used on pelvic, head and neck, and breast malignancies. The studies they reported on also delivered hourly pulses of 0.4-0.5 Gy. They concluded that the clinical use of PDR was feasible, and that the acute and late toxicities were acceptable.

1.2 Linear Quadratic (LQ) Model

The response of tissues and cells to various doses of radiation has been described using various empirical and mechanistic models. In the past, the nominal standard dose

(NSD) model by Ellis was most commonly used to compare different fractionation regimens [2]. More recently, models which are more mechanistic in nature have replaced such empirical models as the NSD. These newer models, which include the Repair-Misrepair model [23], the Saturable Repair model [24], the Lethal, Potentially Lethal model, and the Incomplete Repair model [25], have described the mechanisms for the time-dose effects of radiation. Interestingly, many of these models result in the same overall predictions, even though they each suggest somewhat different mechanisms for the repair of DNA (deoxyribonucleic acid) damage [26]. However, the most commonly used model today is the Linear Quadratic model (LQ) [2], [26]. It is this model, and its variations, which will be used in the data analysis of the experiments done in this study.

The Linear Quadratic (LQ) model was originally proposed by Chadwick and Leenhouts [27]. They wanted to suggest a formulation to explain the effects of radiation on cell survival. Their theory was a molecular based model, which made some basic assumptions in describing the interaction of radiation with DNA. They suggested that DNA is the critical molecule, or target, within a cell, and that the critical damage to this target is a double strand break to the DNA helix. Furthermore, the damage to the DNA can be divided into two different components, the first of which is proportional to dose (linear component α), and the second, which is a function of the square of the dose (quadratic component β). These two components represent the two modes of “radiation action”. The linear component represents the double strand breaks which are caused by a single ionizing event, while the quadratic component represents two independent and individual ionizing events, separated by both time and distance. In the latter case, each

individual event is thought to cause a single strand break, and as such is deemed sublethal. However, if these two events are close enough together, both spatially and temporally, the result is a lethal double strand break. This type of damage has the possibility of being repaired, and thus remaining sublethal, if there is a sufficient time period between the two events, or if they are located far enough apart in the DNA helix. The quadratic (β) component is therefore representative of the amount of sublethal damage (SLD) which can be repaired if the dose rate is reduced. If the dose rate is sufficiently low, the quadratic component becomes essentially zero, indicating that the cells are repairing most of the SLD sustained from the radiation.

The mathematical representation of the LQ model is as follows:

$$S.F. = \exp(-\alpha D - \beta D^2) \quad (1)$$

where S.F. is the surviving fraction of cells after a dose D. The linear component dominates at low doses, as previously stated, with the quadratic component contributing to the shoulder of the survival curve at higher doses. This model is widely used and is valid for a wide range of doses.

An important concept when dealing with sublethal damage repair is the quantification of the cellular repair rate. The most commonly used parameter is the repair half-time, $T_{1/2}$, defined as the time required to repair half of the total attainable repair. Another common way to represent the repair capacity of a cell is to define a related parameter, μ , the repair rate. It is defined with respect to the repair half-time as follows:

$$\mu = \frac{\ln 2}{T_{1/2}} \quad (2)$$

The repair rate is an important concept when dealing with radiation damage, especially with dose/time protracted or fractionated treatments. This repair rate is assumed to be monoexponential in this particular study [25], [28], and [29] although some rodent cell systems have shown bi-phasic recovery kinetics [28]. One particular study also suggested that this repair rate is dependant on dose rate, indicating that for certain tissues, there can be many possible repair rates depending on the rate at which the radiation damage is inflicted [30].

1.2.1 Modified Linear Quadratic (LQ) Model

A new LQ formalism, taking into account the cellular repair rate, was proposed by Lea and Catcheside [5] and involves a time factor G . This new factor reduces the contribution of the quadratic term β from equation (1) by taking into account the repair of sublethal damage which occurs during time protracted irradiation. This reduction in cell killing can occur during low dose rate irradiation or in between treatment fractions. In other words, if one sublethal ionizing event occurs with a sufficient time lapse before a second similar event occurs, there is a probability that the first event will be repaired before there is any interaction with the second event. The generalized Lea-Catcheside G factor is applied to the linear quadratic model (only the quadratic term) as follows:

$$S.F. = \exp(-\alpha D - G\beta D^2) \quad (3)$$

For the simple case of a continuous irradiation at a constant dose rate, the G factor is defined as:

$$G = 2 \left(\frac{1}{\mu T} \right)^2 \left[\mu T - 1 + e^{-\mu T} \right] \quad (4)$$

where T is the overall treatment time, and μ is the repair rate as defined in equation (2).

This modified LQ model can be used to analyze low dose rate survival [5], [26].

The G function has also been derived for a general fractionation scheme of N equal fractions of duration c and period b between fractions. This mathematical derivation was done by Dale [30] and others, and defines G as follows:

$$G = \frac{2}{(Nc)^2} \left[\frac{Nc}{\mu} + \frac{N}{\mu^2} \left(1 - \frac{1}{x} \right) + \frac{1}{\mu^2} \left(x + \frac{1}{x} - 2 \right) \left(\frac{N}{1-y} - \frac{y(1-y^N)}{(1-y)^2} \right) \right] \quad (5)$$

where $x = \exp(-\mu c)$ and $y = \exp(-\mu b)$. This adaptation of the LQ formalism can be used to fit fractionated, or pulsed data. The underlying assumptions of this model are that there is no change in the radiosensitivity of the cells in between the fractions, and that no cell cycle redistribution effects are occurring [30], [31].

1.3 Gel Electrophoresis

The technique of gel electrophoresis is used to separate DNA fragments based on molecular size. More specifically, gel electrophoresis can be used to quantify the amount of radiation damage to DNA by separating the large intact molecules from the smaller, broken fragments. The technique is based on the fact that DNA is a charged molecule, and hence will migrate in the presence of an electric field. The DNA samples are traditionally placed in a solid agarose gel matrix, and then an electric field is placed across the gel. The DNA will begin to migrate, with many factors influencing its mobility through the gel. Generally, smaller fragments will move more easily through the matrix than larger molecules [32]. Other factors which become important for DNA mobility include the agarose concentration in the gel matrix, the type and concentration of buffer used during the electrophoresis, the temperature of this buffer, and the magnitude of the electric field being applied across the gel.

During a continuous field electrophoresis, large molecules of DNA (larger than 30-50 kilobase pairs) migrate at approximately the same rate, regardless of size. This is due to the mode of DNA migration termed reptation [32], [33], where the head of the DNA selects the path through the gel, with the rest of the molecule following behind, independent of size. However, if the orientation of the electric field is changed, separation of larger fragments is possible. This concept resulted in the development of pulsed field electrophoresis by Schwartz and Cantor in 1984, referenced by Blocher *et al* [34].

1.3.1 Clamped Homogeneous Electric Field electrophoresis

Clamped homogeneous electric field (CHEF) electrophoresis is an application of the pulsed field technique to gel electrophoresis. By applying an initial electric field, the DNA is elongated in the direction of the field. When the electric field is reoriented at a different angle, the DNA will also begin to reorient itself towards this new field. It must do so before it can begin to migrate forward in the gel. The time of reorientation is dependent on the size of the DNA as larger molecules take more time to realign than small fragments [35]. This results in the smaller fragments being able to migrate sooner than large fragments, thus providing a physical separation between the different sized DNA molecules [32], [34], and [35]. One particular application of this technique is the field inversion gel electrophoresis (FIGE), where the electric field is simply reoriented at an angle of 180° with respect to the initial field [36], [37]. In the CHEF technique, the two homogeneous fields are oriented 120° apart, at $+60^\circ$ and -60° , which results in a net DNA migration along the resulting vector in the forward direction at 0° . By adjusting the various electrophoresis parameters, such as the voltage gradient, the time between the 120° reorientation of the field, and the total overall electrophoresis time, a good separation can be had between large fragments (up to approximately 10 megabase pairs) and smaller fragments [34], [38].

This principle of DNA size separation implemented with a pulsed field electrophoresis technique can be a useful tool for assessing the double strand breaks caused by various radiation treatments in mammalian cells [34]-[36], [39], and [40]. Measuring these

double strand breaks becomes important since it is thought that they are the main lesions that lead to cell lethality [41]. The CHEF technique can also provide quantitative information pertaining to cellular repair mechanisms, since these mechanisms directly affect the amount of double strand breaks present in the DNA [34], [42]. The CHEF assay has also been shown to be sensitive for doses as low as 2-3 Gy [34], [35]. The useful statistic which is obtained from an electrophoresis technique is a measure of the amount of DNA which has migrated out of well (small, damaged fragments), compared to the amount of intact DNA which remains in the well (large fragments). This is expressed as a ratio, namely the fraction of activity released. The technique relies on radiolabelling of the cellular DNA, after which the gel is counted in a liquid scintillation counter.

1.4 Hypothesis and Specific Aims

The hypothesis of this thesis is that pulsed dose rate irradiation can be equivalent to low dose rate irradiation, *in vitro* using a human gliomas cell line, if certain conditions are met with respect to the dose per pulse and the time period between subsequent pulses.

Specific aims are:

- Determine the intrinsic radiosensitivity of the cells using both high dose rate and low dose rate irradiation in combination with the clonogenic cell survival assay; fit the results using the linear quadratic model, and estimate the related radiosensitivity parameters α , β
- Establish the response of the cells to a protocol of pulsed dose rate irradiation, by giving pulses of different widths and varying the time interval between these pulses;

fit these responses using the linear quadratic model, and compare the results to those obtained for low dose rate irradiation; determine which combination of pulse width and frequency provide an equivalence between pulsed dose rate and low dose rate. and qualify the equivalence using a statistical test

- Compare predicted values of the repair rate and repair half-time from the linear quadratic model with those obtained from the data
- Obtain a measure of the double strand breaks present in the cell following high, low, and pulsed dose rate irradiation using the CHEF electrophoresis; determine if these results follow the trends observed with the clonogenic cell survival data; determine if the equivalence between pulsed and low dose rate still holds with respect to the induction of double strand breaks

2.0 MATERIALS AND METHODS

2.1 Glioblastoma Cell Line

The cell line which was used for all the experiments is the U-87 MG cell line, derived from a malignant glioblastoma and obtained from the human tumor cell bank at the American Type Culture Collection. These cells are of human origin, and were originally established from a 44 year old female caucasian with a stage III astrocytoma, which is a specific classification for high grade brain cancer. The cells are epithelial in nature and are known to be quite radioresistant *in vivo* when compared to other solid tumors [43]. Glioblastomas are also the most common type of primary brain tumor, and usually offer patients a very poor prognosis. Furthermore, recent surgical or radiotherapy advances have not significantly improved the median survival time of approximately 8 months. However, most glioblastoma cell lines *in vitro* show a certain degree of radiosensitivity [43].

2.2 Cell Culture Methods

For most experiments, the cells were grown in polystyrene flasks or dishes, each with a surface of 25 cm² available for cell growth and proliferation. All experiments were done with the cells grown to confluence, having reached the plateau phase of their growth curve. In order to achieve this confluence, the cells were seeded (on day 0) at 10⁵ cells per 25 cm² flask. The cells were fed on days 4 and 7, with the experiment done on day 10.

The cells were incubated at 37°C in 97.5% air and 2.5% CO₂, with the humidity level kept at 100%. The cells were subcultured and fed with a medium consisting of 1:1 Dulbecco's Modified Eagle's Medium : Ham's F12 (DMEM : F12) with L-glutamine (Gibco), 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Wisent), 10 mM sodium bicarbonate (Fisher Scientific), and 1 mM MEM non-essential amino acids (Gibco). This medium was further supplemented with 10% Fetal Bovine Serum (Wisent).

To release the cell monolayer from the plastic surface of the culture flasks, the depleted medium was aspirated and the cells rinsed with a citrate saline solution (134 mM KCl, 17.6 mM Citric Acid, Sigma) to remove any residual traces of serum or other substances which might inhibit the action of trypsin [44]. The cells were subsequently treated with 0.5 mL of trypsin (0.2% w/v in citrate saline, Gibco), which was left on the monolayer. They were then incubated at 37°C for 6 minutes, to allow the trypsin to break any intercell adhesions, as well as the adhesions between the cells and the growing surface. The cells, detached from the flask, were then resuspended in 4 mL of fresh medium to provide a single cell suspension. A 300 µL sample was then taken and counted on an electronic cell counter (Particle Data Elzone 80, calibrated with a haemocytometer) to determine the number of cells per milliliter. The cells were then seeded in flasks/plates at the concentration mentioned above.

For the LDR experiments however, the cells were grown in glass vials with a growing surface of 1.4 cm². The reason for using these glass vials is simply that the holder for the LDR irradiator was designed specifically for using vials, with flasks being too large and

impractical for the current irradiator setup. To detach the cells from the vials, they were first rinsed with 300 μL of citrate saline and then treated with only 200 μL of trypsin. After the incubation, they were resuspended in 500 μL of medium (with serum), and counted as above.

2.3 Irradiation Procedures

2.3.1 Calibration of X-ray unit

The irradiation for most of the experiments were carried out with a Pantak Bipolar HF320 Industrial X-ray unit with a tube voltage of 250 kV. A new tube was recently installed since the previous unit was taken out of service and replaced. Upon receipt and installation, the new unit required calibration to determine the quality and quantity of its output. This would allow and facilitate the precise calculation of the dose rate produced by the tube, necessary to determine the irradiation time to deliver a given dose to the cells.

The main assessment of beam quality is for flatness across the field of interest. The cells were to be irradiated at a distance of 55.6 cm from the x-ray tube, and therefore the flatness measurements were also made at that distance. X-ray film (Kodak X-Omat V) was placed in the center of the field and irradiated for 10 seconds. The films were then developed and read by a film density scanner (Lumisys Laser Film Scanner) to produce dose profiles in both the horizontal (cathode to anode) direction and in the vertical direction (perpendicular to the cathode-anode). To produce a flat field, a flattening filter was designed, with an aluminum base of 1.87 mm. Then, the flatness was further

improved by placing various layers of aluminum, cut into smaller shapes, at different areas on the base filter. This was done until an acceptable flatness was obtained ($\pm 2\%$)

The protocol used for the dose calibration originated from the Institution of Physics and Engineering in Medicine and Biology [45] and was followed for medium energy X-rays, which are defined as those generated at tube voltages of 160 kV up to 300 kV. The main reference measurement is made in order to calculate the absorbed dose to water, in a known amount of time, therefore providing a dose rate calibration. This measurement, made at a depth of 2 cm in a full scatter water phantom, was done using a graphite ionization chamber (NE model 2571) and a Farmer Dosimeter (model 2570). The measurement was done with a source to surface distance (SSD) of 55.4 cm. The measurement was then corrected to standard temperature and pressure (20°C, 101.3 kPa) using the following correction factor:

$$k_{T,P} = \frac{(273.15 + T)}{293.15} \times \frac{101.3}{P} \quad (6)$$

where $k_{T,P}$ is the correction factor, T is the temperature in degrees Celsius, and P is the pressure in kPa. The chamber calibration factor is also required, which is taken from the National Research Council Calibration report for the NE2571 ionization chamber. The dose to water at a depth of 2 cm is then calculated using the following equation:

$$D_{w,z=2} = M N_k k_{ch} \left[\left(\frac{\bar{\mu}_{en}}{\rho} \right)_{w,air} \right]_{z=2,\phi} \quad (7)$$

where $D_{w, z=2}$ is the dose to water at a depth of 2 cm when the chamber is replaced by water, M is the electrometer reading corrected to standard temperature and pressure using $k_{T,P}$ defined above in equation (6), N_k is the chamber calibration factor in grays per scale reading, $[(\mu_{en} / \rho)_{w,air}]_{z=2,\phi}$ is the mass energy absorption coefficient ratio of water to air, averaged over the photon spectrum at 2 cm depth and with a field diameter of ϕ , and k_{ch} is a factor which accounts for the change in the response of the ionization chamber between calibration in air and measurement in a phantom. The values for the mass energy absorption coefficient $[(\mu_{en} / \rho)_{w,air}]_{z=2,\phi}$ and the k_{ch} factor were taken from the protocol.

Since the cells to be irradiated are at a depth of only 2 mm from the surface of the medium, the calculated dose to water at 2 cm then needed to be corrected to obtain a dose to water at a 2 mm depth. A parallel plate chamber (NACP) was used in a plexiglass phantom, and measurements were done with 2 cm of solid water above the chamber, and then repeated with only 2 mm of solid water. The SSD was kept at 55.4 cm for both measurements. The ratio of these 2 measurements was then used to correct the dose to water at 2 cm to a dose to water at 2 mm. Since all the measurements were done for a one minute irradiation, the final result was easily converted to a dose rate at the level where the cells would be irradiated.

2.3.2 HDR irradiation

The high dose rate (HDR) irradiation was done on day 10 of the experiment with the aforementioned 250 kV X-ray unit (12.5 mA), at a dose rate of 168 cGy/min (101 Gy/h).

The cells were irradiated in 25 cm² flasks on ice to inhibit repair during the irradiation time [32]. The cells which required shorter irradiation times were also placed on ice after the irradiation so that all cells at all dose points were on ice for the same amount of time. The cells were then trypsinized and prepared for the appropriate assay (either the clonogenic cell survival assay or the CHEF assay)

2.3.3 LDR irradiation

The continuous low dose rate (LDR) irradiation was done with a circular array of ²²⁶Ra sources (total activity of 800 mCi) contained in a temperature controlled incubator. The cells were irradiated in 1.4 cm² glass vials, at the center of the array of sources. The dose rate of 0.88 cGy/min (0.53 Gy/h) was previously determined using TLD dosimetry relative to a ⁶⁰Co reference protocol [46]. The incubator was kept at 37°C to allow the cells to repair during the irradiation.

2.3.4 PDR irradiation

The pulsed dose rate experiments were done using the 250 kV X-ray (12.5 mA) unit with an instantaneous dose rate of 168 cGy/min (101 Gy/h). The cells were irradiated at room temperature, and immediately placed in a 37°C water bath during the interpulse time. The cells were irradiated in 25 cm² flasks with one of 5 different pulse sequences: 0.5 Gy every hour, 1 Gy every 2 hours, 1.5 Gy every 3 hours, 2 Gy every 4 hours or 3 Gy every 6 hours, to produce an overall dose rate of 0.5 Gy/h in each case.

2.3.5 Split dose experiments

For the split dose experiments, the cells were grown to confluence and irradiated in 25 cm² flasks. The irradiation was done using the 250 kV (12.5 mA) X-ray unit with a dose rate of 168 cGy/min (101 Gy/h). All the cells were given a total dose of 6 Gy but this dose was delivered in two separate 3 Gy fractions. These two fractions were separated by various amounts of time, ranging from 1 to 8 hours. During this time, the cells were kept in a temperature controlled incubator at 37°C. After the second fraction was delivered, the cells were trypsinized and plated immediately.

2.4 The Clonogenic Cell Survival Assay

The clonogenic cell survival assay was used to determine the level of survival of cells after various radiation doses [2], [44], and [47]. This assay, done *in vitro*, defines cell survival as the retention of reproductive integrity. Conversely, non survival does not necessarily imply cell death, but only requires that the cell loses its ability to proliferate. To quantify the cell survival level, a known number of cells are plated in a petri dish, and incubated for a given amount of time. Each individual viable cell will then proliferate and give rise to a clonogenic population in the form of a colony. The colonies are scored and counted, and the survival level is then determined. To normalize the data, a plating efficiency must be calculated, which reflects the survival of the cells that have not received any treatment. This plating efficiency (PE) is defined as follows:

$$PE = \left(\frac{\text{colonies counted}}{\text{cells plated}} \right)_{Dose = 0} \quad (8)$$

Once the PE is obtained, the survival data for a given radiation dose or treatment is then normalized according to the following equation:

$$SF = \frac{\text{colonies counted}}{\text{cells plated} * PE} \quad (9)$$

where SF represents the surviving fraction at that particular dose point.

For this assay, the cells were grown to confluence as previously described, with the experiment done on day ten. The cells were then irradiated and removed from the growth surface with trypsin. After counting the cells, a specific number of them were seeded into petri dishes in order to have approximately fifty surviving colonies. When less than 10^5 cells were plated, 60 mm dishes were used with 4 mL of medium in each plate. However, when a greater number of cells was required, 100 mm plates were used along with 10 mL of medium per plate. In most cases, each dose point, including the zero dose point to determine the PE, was plated three times in separate plates. The plates were then incubated at 37° C for fourteen days to allow the colonies to form and become large enough to be seen with the naked eye. They were then stained with methylene blue (0.2% w/v in 70% ethanol, BDH) and the colonies counted. Any colony which consisted of more than fifty individual cells was scored. Each experiment was repeated three times unless otherwise indicated. The mean of these experiments and the standard error of the mean (SEM) were calculated and plotted. The resulting graphs show the surviving fraction (on a logarithmic scale) as a function of dose.

2.4.1 Curve fitting

The survival curves obtained from the clonogenic cell survival assay were fitted using the linear quadratic model [27]. The fitting, plotting, and estimation of various parameters was done with graphing/statistical software packages, namely Grapher (version 2.0, Golden Software) and Sigma Plot (version 4.0), which use a non-linear least squares fitting method.

2.5 CHEF Methods

2.5.1 Cell preparations

The CHEF electrophoresis technique used here required the cells to be radioactively labelled, and in this case, the isotope of choice was ^{14}C . The cells were grown to confluence as previously described, with the only difference being the addition of 0.02 $\mu\text{Ci/mL}$ ^{14}C -thymidine (Mandel NEN Products) to the growth medium. The cells were thus able to incorporate the radioactive label into their DNA. On the day of the experiment, after the radiation dose was given, the depleted medium was aspirated, the cells washed with citrate saline, and then treated with 0.5 mL of trypsin. The cells were incubated for 6 minutes at 37°C and resuspended in PBS (Phosphate Buffered Saline, Sigma). The cells were then counted on an electronic cell counter to determine the number of cells per milliliter. The remaining cells were centrifuged at 1200 rpm, at a temperature of 4°C, for 5 minutes. Then, the supernatant was carefully aspirated, leaving only the cell pellet at the bottom of the tube. These cells were then resuspended at a concentration of 10^6 cells/mL, first using one half the final volume of PBS at 37°C and

then adding an equal volume of 2% low melting point agarose (Gibco BRL) also at 37°C, resulting in a final agarose concentration of 1%. Using a glass Pasteur pipette, the agarose-cell suspension was placed into disposable plug molds (BIO-RAD) and allowed to solidify on ice for approximately 5 minutes. The plugs were extruded from the molds (using the attachment provided for that purpose). They were immediately placed in lysis buffer (0.5 mM EDTA (ethylene diamine tetra acetic acid), 1.0% w/v N-lauryl-sarcosine, Sigma) supplemented with 50 µg/mL Proteinase K (Sigma). The samples were placed in a dry bath incubator (Fisher Scientific) at 50°C for 24 hours. Following the lysis, the agarose plugs were washed with TE buffer (10mM Tris base, Gibco BRL, 1mM EDTA) for 1 hour at 4°C. This washing was repeated a total of three times, and the plugs stored in TE buffer at 4°C until the CHEF electrophoresis was run.

2.5.2 Electrophoresis setup and parameters

To run the CHEF electrophoresis, a CHEF DR II system was used, complete with Model 1000 mini-chiller (BIO-RAD). The buffer, 0.5 x TBE (Tris/Boric acid EDTA) (BIO-RAD) or 1 x TAE (Tris/Acetic acid EDTA) (BIO-RAD), was placed into the electrophoresis cell, circulated and cooled to 14°C. The gel was made using 0.8 g Pulse Field Certified Agarose (BIO-RAD) which was dissolved in 100 mL of 0.5 x TBE or 1x TAE buffer by heating in a microwave. After it had cooled to below 60°C, the gel was cast into a 13 x 14 cm casting stand (BIO-RAD) complete with combs, and allowed to solidify at room temperature for approximately 15 minutes, and then cooled at 4°C for 10 minutes. The plugs were cut into 3 x 2 mm samples, and the combs removed from the

solidified gel. A single plug was loaded into each well. DNA standards were also placed in random wells in order to provide controls for the electrophoresis. The standards consisted of the yeast chromosomes (DNA) *Schizosaccharomyces pombe* (S. pombe) and *Saccharomyces cerevisiae* (S. cerevisiae) (BIO-RAD). Finally, the wells were covered with 0.8% low melting point agarose to obtain a homogenous environment for the DNA to migrate.

The gel was placed into the CHEF electrophoresis cell, and properly levelled. The parameters were programmed into the Control Module as listed in Table 2.0 and the gel was run.

Table 2.0: Run parameters for the CHEF DR II electrophoresis system for the various experiments. The switch time is the time before the reorientation of the electric fields.

	Buffer	Total Run Time (h)	Switch Time (min)	Volts/cm
HDR experiments	0.5 x TBE	18	75	1.5
all other experiments	1 x TAE	50	300	1.5

2.5.3 Liquid scintillation analysis

When the electrophoresis was complete, the gel was removed from the electrophoresis cell and placed into a staining container with 1L of distilled water and 100 μ L of 10 mg/mL ethidium bromide (EtBr, Gibco BRL). The container was then gently agitated on a rotating plate for approximately 1h. EtBr was used because it attaches to the DNA

which is present in the gel, but it also seeps into the entire gel volume. Therefore, after the staining, the EtBr solution was removed and the gel destained for 20 minutes in distilled water, again with gentle agitation. The gel was then placed on a 312 nm transilluminator (Fisher Biotech) and viewed. The ultraviolet light causes the EtBr to fluoresce, thus allowing the DNA bands to be observed and photographed.

The gel was then carefully cut to prepare for the liquid scintillation. For each dose point, the well and the lane were cut and put into separate glass scintillation vials. A volume of 50 μ L of HCl was added to each vial, and the vial heated slowly to allow the gel to melt. The HCl was added to prevent the solidification of the gel after it was melted. The scintillation cocktail (Ecolite, ICN Biochemicals) was then added, 10 mL to each vial. The samples were placed into the liquid scintillation counter (TR 1900, Packard), along with a ^{14}C standard used to calibrate the unit. The samples were individually read, for a period of four minutes each, and the resulting counts per minute (CPM) data was output to a local printer. The data was then entered into a spreadsheet software program (Microsoft Excel) and the Fraction of Activity Released (FAR) was calculated based on the CPMs in the wells and in the lanes:

$$FAR = \frac{(CPM)_{Lane}}{(CPM)_{Well} + (CPM)_{Lane}} \quad (10)$$

3.0 RESULTS

3.1 Calibration of the X-ray unit

The X-ray tube which was used to deliver the radiation for most of the experiments in this study was calibrated in order to characterize the radiation dose delivered to the cells. The calibration was done with the X-ray unit operating at a tube voltage of 250 kV and at 15 mA. The objective of the calibration was to first ensure that the radiation field was flat, so that cells being irradiated at various locations in the field would receive the same dose of radiation regardless of their position. The first films irradiated by the new X-ray tube were done using only the 1.87 mm aluminum base filter. Profiles were obtained from the digitization of these films, in both the horizontal direction (cathode to anode) and in the vertical direction (perpendicular to the cathode-anode). These first profiles, seen in Figures 3.0 and 3.1, showed the radiation field as very uneven across the region of interest, and also falling off rather rapidly at the edges.

To alter this field, various layers and shapes of aluminum were added to the base filter. After each change, a new film was taken and digitized for verification. When the field was determined to be appropriately flat, the final films were taken and digitized. The final horizontal profile, seen in Figure 3.2, shows a much smoother distribution across the field than the original horizontal profile. The region defined from -12 cm to 12 cm (24 cm total) was chosen to be the limit for the horizontal field since it represents a relatively flat area where the dose would be constant anywhere in that region. The final vertical profile, seen in Figure 3.3, is also much flatter than the original vertical profile.

These characteristics are maintained in the -12 cm to 12 cm range. The final radiation field was therefore chosen to be 24 cm x 24 cm.

To quantify the radiation field, equations (6) and (7) were used as stated in section 2.3.1. The measurements were done and the final dose rate was calculated to be 168 cGy/min (101 Gy/h). This dose rate was used for most of the experiments, including the high dose rate, pulsed dose rate, repair, and adaptive response experiments.

Figure 3.0: Original X-ray beam profile in the horizontal (anode to cathode) direction, using only a 1.87 mm aluminum filter.

Figure 3.1: Original X-ray beam profile in the vertical (perpendicular to the anode/cathode) direction, using only a 1.87 mm aluminum filter.

Figure 3.2: Final X-ray beam profile in the horizontal (anode to cathode) direction, using a 1.87 mm aluminum base filter and a custom-made aluminum flattening filter.

Figure 3.3: Final X-ray beam profile in the vertical (perpendicular to the anode/cathode) direction, using a 1.87 mm aluminum base filter and a custom-made aluminum flattening filter.

3.2 Clonogenic Assay Results

3.2.1 HDR irradiation

The experiments done using the clonogenic cell survival assay technique were designed to provide information on the intrinsic radiosensitivity of the cells, through survival curves and their associated radiosensitivity parameters [48]. These experiments were done *in vitro*, and quantify the relationship between cell survival and varying doses of radiation. Several studies have demonstrated the link between survival curve parameters and the radioresponsiveness of cells in a clinical situation [49]-[52].

The HDR irradiation experiments were done at a dose rate of 168 cGy/min. Using equations (8) and (9), the surviving fraction was obtained from the cell survival assay and plotted as a function of dose. This survival curve is shown in Figure 3.4. This HDR survival curve was fitted using the linear quadratic model as described in equation (1) resulting in an estimate of the radiosensitivity parameters α and β . These parameters can be seen in Table 3.0. The survival curve has an initial slope which is defined by the α parameter, and the shoulder which is described by the β component. The steepness of the curve, described by these parameters, shows that it is of average radioresistance, not being extremely radioresistant but not considered to be radiosensitive either. These parameters are consistent with previous findings with this cell line [28], [46], and [53].

Table 3.0: Comparison of radiosensitivity parameters α and β , obtained using the linear quadratic model, for high dose rate (HDR) and low dose rate (LDR) experiments.

	α (Gy ⁻¹)	β (Gy ⁻²)
HDR	0.31 ± 0.04	0.046 ± 0.018
LDR	0.17 ± 0.03	0.016 ± 0.009

3.2.2 LDR irradiation

For the low dose rate experiments, the cells were irradiated at a continuous dose rate of 0.88 cGy/min, a decrease of almost 200 fold compared with the high dose rate experiments. The survival curve for the low dose rate is plotted, along with the high dose rate survival curve, in Figure 3.5. As expected for LDR, the cells show a dose rate sparing effect when compared with the HDR curve. This is due to the fact that the cells are irradiated over a much longer period of time for the low dose rate, giving them a sufficient amount of time to repair any sublethal damage which is occurring during the continuous irradiation. Table 3.1 shows a comparison of the duration of irradiation for HDR and LDR.

The LDR survival curve was also fit and the linear quadratic parameters obtained are found in Table 3.0. As expected, the β component is reduced when compared with the HDR value, and is closer to a value of zero, indicating that most of the sublethal damage is being repaired. One thing of importance to note however is the change in the parameter α , when comparing the HDR value to the LDR value. This parameter represents the damage which is termed 'unrepairable', such as a double strand break caused by a single ionizing event. The significance of this change in α will be addressed in further sections. Using the modified linear quadratic model in equation (3), the value of G was calculated. From equation (4), an estimate of the repair rate was obtained, and is shown in Table 3.2.

Table 3.1: Irradiation times used for HDR and LDR experiments, showing the dose and the time to deliver each dose.

Dose (Gy)	Time (min:s) HDR	Time (h:min) LDR
1	0:39	1:53
2	1:14	3:46
4	2:26	7:32
6	3:37	11:18
8	4:38	15:04

Table 3.2: Radiosensitivity parameters obtained from the low dose rate experimental data.

	LDR experimental data
G-value	0.35 ± 0.11
Repair rate $\mu \text{ (h}^{-1}\text{)}$	0.14 ± 0.06
Repair half-time $T_{1/2} \text{ (h)}$	4.9 ± 2.2

Figure 3.4: Survival curve showing the response of the U-87MG cells after high dose rate irradiation on ice. Each point represents the average of at least 3 experiments, with the error bars representing the standard error of the mean between experiments. The line through the points represents the linear quadratic fit of the data.

Figure 3.5: Survival curve showing the response of the U-87MG cells after low dose rate irradiation at 37°C. Each point represents the average of at least 3 experiments, with the error bars representing the standard error of the mean between experiments. The high dose rate curve is shown as a reference. The lines through the points represent the linear quadratic fit of the data.

3.2.3 Split dose experiments

The split dose experiments were done to provide information on the repair capabilities of the cells. By giving the doses in two separate fractions, one can assess how much sublethal damage repair is occurring in the period separating the fractions. This repair is quantified by calculating the recovery ratio. It is defined as the ratio of the cell survival when cells are given time to repair in between fractions to the cell survival when this time period is zero. This ratio is greater than one, and is expected to saturate with time, after which all the possible sublethal damage repair has occurred. Figure 3.6 shows the results of the split dose experiments in a plot of the recovery ratio as a function of the time of incubation. As expected, the recovery ratio reaches a plateau after about 6 or 7 hours. From this graph, the repair half-time can be calculated, since the plateau region represents full repair. This repair half-time, along with the corresponding repair rate constant, are shown in Table 3.3. Using this value for the repair rate, a value of G was calculated to fit the LDR data. A comparison between the parameters obtained with LDR and the split dose experiments can also be seen in Table 3.3.

Table 3.3: A comparison of radiosensitivity parameters obtained from the split dose experimental data and the low dose rate experimental data.

	Split dose experimental data	Low dose rate experimental data
G-value	0.10 ± 0.01	0.35 ± 0.11
Repair rate $\mu \text{ (h}^{-1}\text{)}$	0.58 ± 0.05	0.14 ± 0.06
Repair half-time $T_{1.2} \text{ (h)}$	1.2 ± 0.1	4.9 ± 2.2

Figure 3.6: Curve showing the recovery ratio of the U-87MG cells after split dose repair experiments. The cells were given two fractions of 3 Gy each for a total dose of 6 Gy. These fractions were separated by various amounts of time, where the cells were placed in a 37°C incubator. Each point represents the average of at least 3 experiments, with the error bars representing the standard error of the mean between experiments.

3.2.4 PDR irradiation

To simulate a pulsed irradiation protocol, the cells were irradiated at various time intervals with different doses. In all, five different pulsed schemes were used. The pulses were scheduled to closely resemble the dose rate used in the low dose rate experiments (0.53 Gy/h) in order to make valid comparisons between the results. The parameters for the five different pulse schemes can be seen in Table 3.4. The overall resulting dose rate for each pulse sequence is 0.5 Gy/h, with most experiments delivering 12 Gy in a total time of 24 h. The cells were irradiated at room temperature and placed in a 37°C waterbath during the interpulse time so that all possible repair could occur.

Table 3.4: Irradiation parameters used for the pulsed dose rate experiments, including the dose per pulse, the period between pulses, and the length of each pulse. All doses were given with the X-ray tube set at 12.5 mA unless otherwise indicated.

Pulse dose (Gy)	Period between pulses (h)	Pulse length (min:s)
0.5	1	0:49 (at 5 mA)
1	2	0:39
1.5	3	0:57
2	4	1:14
3	6	1:50

The survival curve for the first pulse sequence, 0.5 Gy delivered every hour, can be seen in Figure 3.7. The HDR and LDR curves are included for comparison. The PDR curve has a smaller shoulder and the cells show an overall higher level of survival when compared to the low dose rate protocol. Figure 3.8 shows the survival curve for the 1 Gy

pulse delivered every 2 hours. This survival curve, similar in shape to the 0.5 Gy/1 h curve, also shows higher radiation resistance when compared with the low dose rate curve. In Figure 3.9, which shows the survival curve for the 1.5 Gy delivered every 3 hours, the curve for the pulsed scheme also lies above the low dose rate curve. In Figures 3.10 and 3.11, the survival curves for 2 Gy delivered every 4 hours, and 3 Gy delivered every 6 hours are shown, respectively. In these figures the survival curves seem to approach the low dose rate survival curve, at times even overlapping it. Linear quadratic fits were done on the pulsed curves, and these fits are shown as solid lines in Figures 3.7-3.11. The fits for all the pulse schemes are shown together in Figure 3.12. The fits were done by limiting the values of α and β to the positive domain, since this is an assumption of the linear quadratic model. The values of α and β , along with their uncertainties, are shown in Table 3.5. If the data is fit using the linear quadratic model without any restrictions on α and β , some of the parameters take on negative values. The unrestricted fit parameters are shown in Table 3.6.

To quantify the differences between the low dose rate survival curve and the pulsed dose rate survival curves, an unpaired student t-test was performed on the matching dose points between the LDR and PDR data. A p-value of less than 0.05 indicates that the results are statistically significant. In this case, the null hypothesis was that there was no difference between the pulsed schedule and the low dose rate irradiation. This hypothesis was rejected for most points, as seen in Table 3.7, since most of the p-values were less than 0.05.

Table 3.5: Comparison of radiosensitivity parameters α and β , obtained using the linear quadratic model, for different irradiation procedures. The fits were obtained by limiting α and β to positive values.

	α (Gy ⁻¹)	β (Gy ⁻²)
HDR	0.31 ± 0.04	0.046 ± 0.018
LDR	0.17 ± 0.03	0.016 ± 0.009
0.5 Gy/1 h	0.03 ± 0.02	0.017 ± 0.003
1 Gy/2 h	0.00 ± 0.02	0.022 ± 0.004
1.5 Gy/3 h	0.00 ± 0.03	0.024 ± 0.006
2 Gy/4 h	0.19 ± 0.02	0.010 ± 0.003
3 Gy/6 h	0.03 ± 0.01	0.026 ± 0.002

Table 3.6: Comparison of radiosensitivity parameters α and β , obtained using the linear quadratic model, for different irradiation procedures. The fits were obtained by not placing any restrictions on either α or β .

	α (Gy ⁻¹)	β (Gy ⁻²)
HDR	0.31 ± 0.04	0.046 ± 0.018
LDR	0.17 ± 0.03	0.016 ± 0.009
0.5 Gy/1 h	0.03 ± 0.02	0.017 ± 0.003
1 Gy/2 h	-0.02 ± 0.02	0.025 ± 0.004
1.5 Gy/3 h	-0.06 ± 0.03	0.036 ± 0.005
2 Gy/4 h	0.19 ± 0.02	0.010 ± 0.003
3 Gy/6 h	0.03 ± 0.01	0.026 ± 0.002

Table 3.7: Results of unpaired student t-tests, showing the p-value when each dose point for the pulse schedule was compared to the corresponding dose point for low dose rate. A p-value of less than 0.05 is considered to be statistically significant. N/A- Not Applicable, please refer to text for explanation.

	0.5 Gy/1 h	1 Gy/2 h	1.5 Gy/3 h	2 Gy/4 h	3 Gy/6 h
2 Gy	0.0168	0.0414	N/A	0.0247	N/A
4 Gy	0.0005	0.0021	N/A	0.0248	N/A
6 Gy	0.0109	0.0135	0.0105	0.348	0.045
8 Gy	0.0102	0.0176	N/A	0.0448	N/A
12 Gy	N/A	0.0473	0.0206	0.131	0.0615

Since the pulsed sequences were given in discrete amounts, not all dose points matched up with the doses given in the LDR experiment. The comparisons in Table 3.7 were done only for corresponding experimental data points. Therefore, some dose points found in the LDR sequence are not matched up with points in the PDR sequence. For example, a comparison at the 2 Gy level is not possible for the 3 Gy/6 h scheme since the first pulse, and hence the first experimental data point, is at 3 Gy. In other cases, such as the 1.5 Gy/3 h, a comparison at the 2 Gy level is not possible since the pulses are only in multiples of 1.5 Gy. Such instances are labeled N/A in Table 3.7.

The experimental results from the PDR experiments were used with equations (3) and (5) to estimate the value of G and to calculate the repair rate and repair half-time values.

These parameters can be seen in Table 3.8. Most of the PDR schemes show large values for the repair half-time, in the order of hours, and often larger than the period between the fractions. Many of the repair rate values are in agreement with the calculated value using the LDR experimental data, with the exception of the 2 Gy/4 h result. However, when comparing these repair rates to the value obtained from the split dose experiment (Table 3.3), only the 2 Gy/4 h fraction is in agreement, while all other fraction schedules demonstrate a significantly lower value of repair rate (and higher value for the repair half-time).

Table 3.8: Radiosensitivity parameters obtained from the pulsed dose rate experimental data.

	0.5 Gy/1 h	1 Gy/2 h	1.5 Gy/3 h	2 Gy/4 h	3 Gy/6 h
G-value	0.37 ± 0.06	0.48 ± 0.07	0.52 ± 0.10	0.22 ± 0.04	0.56 ± 0.04
Repair rate $\mu \text{ (h}^{-1}\text{)}$	0.21 ± 0.05	0.12 ± 0.03	0.10 ± 0.04	0.45 ± 0.20	0.10 ± 0.01
Repair half-time $T_{1/2} \text{ (h)}$	3.4 ± 0.8	5.9 ± 1.3	6.8 ± 2.2	1.5 ± 0.6	7.3 ± 1.0

Since the repair half-times from the PDR experiments were so significantly different from those of the split dose experiments, the latter was used as a constant in the following calculations. The split dose value for the repair half-time was obtained from experiments which were designed to measure repair parameters, and therefore it is more likely to

represent an accurate and true measure of the intrinsic repair capacity of the cells than the fit data from the PDR experiments. This repair rate (0.58 h^{-1}) was used with equation (5) to estimate the G-value, and to predict the theoretical value of the quadratic coefficient $G\beta$ from equation (3) (The value of β was taken from the high dose rate parameters) These values are seen in Table 3.9. Also included in this table are the experimental values of the coefficient, obtained directly from the linear quadratic fits of the PDR data.

Table 3.9: Radiosensitivity parameters obtained from the split dose experimental data, using the repair rate as a constant, and comparing the calculated value of the quadratic coefficient against the experimental value from fitting the pulsed scheme data.

	0.5 Gy/1 h	1 Gy/2 h	1.5 Gy/3 h	2 Gy/4 h	3 Gy/6 h
Repair rate $\mu (\text{h}^{-1})$	0.58 ± 0.05	0.58 ± 0.05	0.58 ± 0.05	0.58 ± 0.05	0.58 ± 0.05
G-value	0.16 ± 0.01	0.15 ± 0.01	0.17 ± 0.01	0.20 ± 0.02	0.26 ± 0.01
Predicted $G\beta$	0.008 ± 0.001	0.007 ± 0.001	0.008 ± 0.001	0.009 ± 0.001	0.012 ± 0.001
Experimental $G\beta$	0.017 ± 0.033	0.022 ± 0.004	0.024 ± 0.006	0.010 ± 0.003	0.026 ± 0.002

There is a significant difference, up to a three fold increase in some cases, when comparing the predicted value of the quadratic coefficient (obtained using the split dose repair rate) to the coefficient obtained from the fit of the experimental PDR data. The predictions resulted in smaller values of the coefficient, indicating a more complete repair of damage than what is shown with the data.

Figure 3.7: Survival curve showing the response of the U-87MG cells after pulsed dose rate delivered at 0.5 Gy every hour, low dose rate, and high dose rate irradiation. Each point represents the average of at least 3 experiments, with the error bars representing the standard error of the mean between experiments.

Figure 3.8: Survival curve showing the response of the U-87MG cells after pulsed dose rate delivered at 1 Gy every 2 hours, low dose rate, and high dose rate irradiation. Each point represents the average of at least 3 experiments, with the error bars representing the standard error of the mean between experiments.

Figure 3.9: Survival curve showing the response of the U-87MG cells after pulsed dose rate delivered at 1.5 Gy every 3 hours, low dose rate, and high dose rate irradiation. Each point represents the average of at least 3 experiments, with the error bars representing the standard error of the mean between experiments.

Figure 3.10: Survival curve showing the response of the U-87MG cells after pulsed dose rate delivered at 2 Gy every 4 hours, low dose rate, and high dose rate irradiation. Each point represents the average of 3 experiments, with the error bars representing the standard error on the mean between experiments.

Figure 3.11: Survival curve showing the response of the U-87MG cells after pulsed dose rate delivered at 3 Gy every 6 hours, low dose rate, and high dose rate irradiation. Each point represents the average of 3 experiments, with the error bars representing the standard error of the mean between experiments.

Figure 3.12: Survival curves showing the linear quadratic fits obtained from each pulsed dose rate data set (limiting the α and β parameters to positive values), as well as the linear quadratic fit of the low dose rate data.

3.2.5 Adaptive response experiments

To further investigate the increase in radiation resistance seen when comparing the PDR to LDR, a different set of experiments was designed. These were done to determine if the pulses of irradiation were causing a change in the behavior of the cells, perhaps inducing an adaptive response, which would alter the ability of the cells to repair radiation damage.

The pulse sequence which was chosen for these experiments was the 1 Gy/2 h, as the PDR response for this sequence showed a significant difference from the LDR response. The cells were initially irradiated with pulses, delivering 6 Gy in a 12 h period. The total number of pulses given was 6, and the total dose was 6 Gy, which represents one half of the total dose used in the PDR alone experiments. The total dose was chosen to be less since the majority of the radiation resistance was observed below 6 Gy. After the pulses were delivered, the cells were seeded in 25 cm² flasks at an appropriate concentration and allowed to proliferate for four days. They were then trypsinized and seeded at the concentration required for a HDR experiment (section 2.2). Ten days later, a high dose rate experiment was performed on these cells to determine if they would show a different HDR survival level after being primed with pulses given every two hours. The survival curve for this adaptive response experiment is seen in Figure 3.13, and also included on this graph is the original HDR survival curve from Figure 3.4. The two curves seem to differ in the low dose regions, with the adapted cells (those having received the pulses) showing a slightly greater radiation resistance. However, at higher doses, the effect is almost reversed, with the adapted cells showing a lower survival curve and a steeper

slope. The value of the linear quadratic parameters are shown in Table 3.10. The first fit for this curve was done by limiting the values of α and β to the positive domain, since this is an assumption of the linear quadratic model. These parameters are listed under the HDR-adapted, restricted model section. If this same data is fit using the linear quadratic model without any restrictions on α and β , some of the parameters take on negative values. The unrestricted fit parameters are also shown in Table 3.10.

To quantify the differences seen in the survival curves, an unpaired student t-test was done between pairs of corresponding dose points. Again, a p-value of less than 0.05 indicates that the two results are statistically significant. The results of the t-test, showing the p-values, are shown in Table 3.11.

Table 3.10: Comparison of radiosensitivity parameters α and β , obtained using the linear quadratic model, for different irradiation procedures. The results for the adapted cells are shown with both the unrestricted fit, and the fit restricting α and β to positive values. The values of the HDR and LDR experiments are included as references.

	α (Gy ⁻¹)	β (Gy ⁻²)
HDR- adapted restricted model	0.00 ± 0.09	0.11 ± 0.04
HDR- adapted unrestricted model	-0.13 ± 0.07	0.17 ± 0.04
HDR	0.31 ± 0.04	0.046 ± 0.018
LDR	0.17 ± 0.03	0.016 ± 0.009

Table 3.11: Results of unpaired t-tests, showing the p-value when each dose point for the HDR survival was compared to the corresponding dose point for the adapted HDR survival. A p-value of less than 0.05 is considered to be statistically significant.

Dose	p-value
1 Gy	0.0053
2 Gy	0.0904
4 Gy	0.2165
6 Gy	0.4102

From the p-values in this table, only the low dose point (1 Gy) shows statistical significance, while the largest dose points (4 and 6 Gy) show the least amount of statistical significance.

Figure 3.13: Survival curve showing the response of the U-87MG cells after high dose rate irradiation on ice. These cells had previously received a 6 Gy dose, delivered in pulses of 1 Gy every 2 hours, and are labeled as adapted cells. The original HDR curve is shown as a reference. Each point represents the average of 3 experiments, with the error bars representing the standard error of the mean between experiments

3.3 CHEF Results

3.3.1 HDR irradiation

The clonogenic cell survival assay showed that there were significant differences in the radiation resistance of the cells if the LDR protocol was replaced with various PDR schemes. The CHEF assay was used to determine if these same differences are present at the molecular or strand level, since this assay provides information on the double strand breaks present in the cells. It is thought that double strand breaks are the most important lesions causing cell lethality.

The measure of double strand breaks was done with the CHEF assay by determining the amount of DNA which had migrated out of the well (small, broken fragments) in relation to the amount of intact DNA which remained in the well. It is expected that a larger dose will result in a greater number of double strand breaks, seen as an increase in the fraction of activity released (FAR) with an increase in dose. The CHEF assay was run according to the HDR conditions specified in Table 2.0 of the Materials and Methods section. From the results of the liquid scintillation, the FAR was calculated according to equation (10) and plotted against the dose delivered to the cells. The relationship between FAR and dose was found to be linear, as expected, and can be seen in Figure 3.14. The greater the dose, the more broken fragments are induced, and the greater the number of fragments which run into the gel.

The data was normalized so that a dose of zero represents a FAR of zero, since no radiation induced strand breaks should be present with zero dose of radiation. This

eliminates any background DNA strand breaks which may have been naturally present prior to the experiment, or which were induced with cell manipulation. This background level of FAR was typically in the range of 2% to 12% for untreated cells. This normalization was done for all the FAR data, including the LDR and PDR experiments.

3.3.2 LDR irradiation

The CHEF assay was also done on the cells after low dose rate irradiation. In order to have a large enough FAR signal during the liquid scintillation analysis, a large concentration of cells must be used in the preparation of the agarose plugs (10^6 /mL). For the LDR experiments, glass vials must be used because of the irradiator setup, which limits the number of cells available due to the smaller growth surface area of the vials. Another constraint with the LDR irradiator is that the setup limits the number of vials to 14 at any one time in the irradiator. For these reasons, there were only enough cells to allow for 4 dose points: 0, 2, 6 and 12 Gy. The CHEF assay was run according to the conditions in Table 2.0 of the Materials and Methods section, with the parameters listed under the “other experiments” heading. Figure 3.15 shows the FAR plotted against the dose. As expected, the curve is nearly flat, since the cells are repairing much of the sublethal damage during the LDR irradiation. This results in a smaller number of double strand breaks, seen here as a smaller FAR. Since the CHEF parameters were different for HDR and LDR, the two results were not plotted on the same graph and no quantitative comparison was done. However, some qualitative conclusions can be drawn since the LDR electrophoresis was run for a longer time period and yet still showed less FAR than the HDR.

3.3.3 PDR irradiation

Since differences were seen in the radiation survival of cells when the LDR was replaced with a PDR schedule, the CHEF analysis was also done to cells that had undergone pulsed irradiation to determine if these differences were perceivable at the double strand break level. The assay was run according to the parameters in Table 2.0 of the Materials and Methods section, which are identical to the parameters used for the LDR experiments. Figures 3.16-3.20 show the FAR versus dose curves for the 0.5 Gy/1 h, 1 Gy/2 h, 1.5 Gy/3 h, 2 Gy/4 h, and 3 Gy/6 h respectively. In these figures, the LDR curve is included for comparison, as well as the linear fits of both the PDR and LDR data. The curves for the PDR closely resemble the LDR data, although in most cases, the PDR shows a lower level of FAR than the LDR, which is in agreement with the clonogenic assay results. The shallower slopes of the pulsed schemes (when compared to LDR) indicate a smaller number of double strand breaks, which in turn implies more sublethal damage repair and a higher level survival as seen in the clonogenic assay. The linear fits for all five of the pulsed schedules are shown together in Figure 3.21, which also includes the LDR fit for comparison.

To further quantify the differences between the low dose rate and pulsed dose rate responses, the slopes of the graphs were analyzed and a student's t-test was done comparing the LDR slope to each PDR slope. These results can be seen in Table 3.12.

Table 3.12: Comparison of the slopes from the CHEF data for the various irradiation protocols, the coefficient of determination R^2 , and p-values from unpaired student t-tests. A p-value of less than 0.05 is considered to be statistically significant.

	Slope	R^2	p-value
LDR	0.0061 ± 0.0006	0.980	N/A
0.5 Gy/1 h	0.0040 ± 0.0011	0.704	0.098
1 Gy/2 h	0.0028 ± 0.0015	0.551	0.108
1.5 Gy/3 h	0.0014 ± 0.0014	0.385	0.059
2 Gy/4 h	0.0072 ± 0.0019	0.744	0.668
3 Gy/6 h	0.0049 ± 0.0012	0.851	0.216

A p-value of less than 0.05 indicates that the two results are statistically significant.

From these results, none of the PDR schemes show small p-values, indicating no significant difference between the pulsed data and the LDR data.

Figure 3.14: Graph showing the Fraction of Activity Released (FAR) of the U-87MG cells after high dose rate irradiation on ice. Each point represents the average of 3 experiments, with the error bars representing the standard error of the mean between experiments.

Figure 3.15: Graph showing the Fraction of Activity Released (FAR) of the U-87MG cells after low dose rate irradiation at 37°C. Each point represents the average of 3 experiments, with the error bars representing the standard error of the mean between experiments.

Figure 3.16: Graph showing the Fraction of Activity Released (FAR) of the U-87MG cells after pulsed dose rate irradiation at 0.5 Gy/1 h. The LDR curve is included for comparison. Each point represents the average of 3 experiments, with the error bars representing the standard error of the mean between experiments

Figure 3.17: Graph showing the Fraction of Activity Released (FAR) of the U-87MG cells after pulsed dose rate irradiation at 1 Gy/2 h. The LDR curve is included for comparison. Each point represents the average of 3 experiments, with the error bars representing the standard error of the mean between experiments

Figure 3.18: Graph showing the Fraction of Activity Released (FAR) of the U-87MG cells after pulsed dose rate irradiation at 1.5 Gy/3 h. The LDR curve is included for comparison. Each point represents the average of 3 experiments, with the error bars representing the standard error of the mean between experiments

Figure 3.19: Graph showing the Fraction of Activity Released (FAR) of the U-87MG cells after pulsed dose rate irradiation at 2 Gy/4 h. The LDR curve is included for comparison. Each point represents the average of 3 experiments, with the error bars representing the standard error of the mean between experiments

Figure 3.20: Graph showing the Fraction of Activity Released (FAR) of the U-87MG cells after pulsed dose rate irradiation at 3 Gy/6 h. The LDR curve is included for comparison. Each point represents the average of 3 experiments, with the error bars representing the standard error of the mean between experiments

Figure 3.21: Graph showing the linear fits for all the pulsed dose rate CHEF data. The LDR curve is included for comparison. Each point represents the average of 3 experiments, with the error bars representing the standard error of the mean between experiments

4.0 DISCUSSION

4.1 Clonogenic Assay

For this study, the U-87MG glioblastoma cell line was chosen to assess the cellular response to various radiation treatment protocols, including high dose rate, low dose rate, and pulsed dose rate irradiation. Measurement of the intrinsic radiosensitivity of these malignant cells could aid in the prediction of the *in vivo* clinical response in a patient, which makes such studies important when implementing new treatment modalities. The clonogenic assay provides information on the survival of cells after such radiation treatments.

4.1.1 HDR irradiation

The cells were first irradiated with a high dose rate irradiator, delivering the dose at a rate of 168 cGy/min. The survival curve showed a relatively large shoulder with a steep curve. From the linear quadratic fit of the survival curve, an estimate of the parameters of this model were obtained. The coefficient of the linear term, α , describes the initial slope of the survival curve. It has been shown that the steepness of the curve, given by α , has a positive correlation to the clinical response of human malignant cells [48], [52]. In this study, the value of α was found to be 0.31 Gy^{-1} , which is in the range of $0.2\text{-}0.6 \text{ Gy}^{-1}$ found in the literature [31], [52] for acute irradiation of human tumor cells, but it is slightly below the average of 0.35 Gy^{-1} . This indicates that the cell line is somewhat radioresistant. This result agrees with published data that claims that *in vitro* gliomas do not show the extreme radioresistance that is seen *in vivo* [43].

The coefficient of the quadratic term, β , is representative of the shoulder of the survival curve, and was found to be 0.046 Gy^{-2} in this study. The range of β values from other human *in vitro* cell lines following an acute radiation treatment is $0.02\text{-}0.06 \text{ Gy}^{-2}$, with an average value of 0.032 Gy^{-2} [31], [52]. The β value found in this study is slightly above this average, but still within the range of the published values. The relatively large value of β indicates that there is a significant amount of sublethal damage accumulation which is leading to cell lethality, and that a lower dose rate would benefit these cells by allowing for some damage repair and cellular recovery.

The α parameter from this study is greater than the values found in the literature for the U-87MG cell line [28], [46], and [53]. The β value for these cells is in agreement with these previous studies, although the estimate found in this study was slightly lower. The observed differences in the parameters can be explained by changes in the radiosensitivity of the cells due to time and repeated subculturing of the cell line, factors which may alter certain cellular mechanisms. The growth conditions for the cells (media, etc) are not identical in all studies, and this could also contribute to the variability in the radiation response of the cells.

4.1.2 LDR irradiation

The low dose rate irradiation was delivered at a rate of 0.53 Gy/h . This represents a significant decrease in the dose rate compared with the HDR treatments, the dose therefore being delivered over a much longer period of time. The survival curve for these

LDR experiments showed a dose rate sparing effect, as the cells are capable of sublethal damage (SLD) repair. As expected, the β component was reduced to a value of 0.016 Gy^{-2} , which is much lower than the HDR value of 0.046 Gy^{-2} . This measured value is in agreement with previous studies with gliomas [28], [46], although it is slightly lower than the average value of $0.068 \pm 0.039 \text{ Gy}^{-2}$ taken from a compilation of 36 human *in vitro* data sets [1]. The reduction in the quadratic term is due to the repair of SLD occurring during the irradiation when two single strand breaks are separated by sufficient amounts of time. This time allows them to be individually repaired before being able to combine to form a lethal lesion.

The α component, by definition, is thought to be independent of dose rate and of repair, and thus should not change from HDR to LDR [54]. However, in this study, the value of the linear term does show a reduction, which cannot be accounted for by the range of the standard deviations of the data. The LDR value of α is 0.17 Gy^{-1} , compared with 0.31 Gy^{-1} from the HDR experiments. The value is still within the range of previously published data [1], although this change indicates that the intrinsic radiosensitivity of the cells has changed. This change in α with change in dose rate has also been seen in other studies on various cell lines [31], [55]. The same trend is seen with the PDR data, and will be addressed in section 4.1.4.

From the LDR data, the value of G was calculated, which represents the reduction of the quadratic term due to SLD repair, when going from the HDR value to the LDR value. A small value of G indicates that most of the SLD has been repaired and that only a small

amount of unrepaired SLD damage remains. The G-value of 0.35 indicates that the quadratic term is reduced to 35 % of the HDR value, which means that not all the SLD is being repaired. From this G-value, the repair rate was estimated to be 0.14, with a repair half-time of 4.95 hours. This value is considered large, and does not take into consideration any double strand break repair which could be occurring and affecting the parameter α . Other measured values of the repair half-time for SLD repair in U-87MG cells include 7.9 ± 1.3 h, 4.0 ± 1.0 h, 2.0 ± 0.4 h [28], [46] and 2.7 h. The value of the repair half-time found with the LDR data is much larger than the average of 0.54 ± 0.91 h taken from a compilation of 36 human *in vitro* data sets [1]. It is, however, within the range of some of the glioma data. A long repair half-time, such as that seen here, would be therapeutically disadvantageous if a PDR scheme were used instead of the conventional LDR, since it has been shown that late responding normal tissues with shorter half-times than early responding tumor cells would be relatively more damaged in such a case [10], [54], and [56].

4.1.3 Split dose experiments

Another method of obtaining an estimate of the repair rate and the repair half-time is to do a split dose experiment. With this method, all the cells receive the same dose of radiation, but it is delivered in two separate fractions. In this study, the cells were given a total dose of 6 Gy divided into two 3 Gy fractions. To obtain information on the repair capabilities of the cells, the two fractions are separated by various amounts of time during which the cells are incubated at 37°C. This experiment is a more direct measure of the

SLD repair which takes place during the inter-fraction period, when there is no radiation at all. The recovery ratio was plotted against the time of incubation, and it was observed that the ratio increases and then reaches a plateau, showing that the cells have reached their maximum achievable repair.

From this data, the repair half-time was determined to be 1.2 h, which is significantly less than the 4.9 h found with the LDR data. Several studies have shown a variation in the repair half-time based on the total dose (6 Gy in this study) and also on the dose rate [24], [30]. Therefore it is not difficult to accept that there is no single repair rate or repair half-time for a particular cell line, but that many repair half-times exist, and are dependant on the experiments done to measure them. A smaller value for the repair half-time such as 1.2 h would make PDR a more feasible option. Also, this repair half-time is measured using a fractionated method, (with only two fractions) and hence may be more representative of the clinical situation with a PDR schedule in place.

4.1.4 PDR irradiation

If a LDR protocol is to be replaced by an equivalent PDR scheme, certain parameters must be considered. First, the period between pulses must be long enough to allow for SLD repair. Most studies used a low dose rate in the range of 0.5-0.7 Gy/h, and used this same dose rate when simulating the pulsed treatments [1], [7]-[10], [12]-[14], and [16]-[22]. All five pulse sequences for this study were chosen accordingly, with an overall dose rate of 0.5 Gy/h, but with varying pulse lengths and pulse periods (seen in Table 3.4 of the Results section).

When looking at the survival curves for all five pulses, all seem to show a greater level of survival and radiation resistance when compared with the LDR. Furthermore, these differences are statistically significant for most dose points, as seen by the p-values shown in Table 3.7 in the Results section. The behavior of the cells is characteristic of an adaptive response, a process by which a small dose of radiation affects the response of cells to subsequent doses of radiation. Many studies have demonstrated an adaptive response among a wide range of mammalian cells [57-63]. Traditionally, an adaptive response has been seen when a small single priming dose was given, followed by a large challenge dose. However, it is possible that an adaptive response is affecting the cellular response in this study if a PDR pulse is considered as a priming dose capable of inducing an adaptive response. A similar response has been observed after pulsed irradiation with an ovarian carcinoma cell line [31], [63].

The first pulse sequence delivered 0.5 Gy once every hour. The results of the linear quadratic fit show that the α component, the linear coefficient of the linear quadratic model, has decreased significantly to a value of 0.03 Gy^{-1} . This value is lower than both the HDR and LDR values. Again, this indicates that the radiosensitivity of the cells is somehow changing with the pulsed treatment, perhaps the result of an adaptive response. The lower value of α also points towards cells which are more radioresistant, with the initial slope of the survival curve being much more shallow. The value of α also approaches the value of zero, the minimum value dictated by the linear quadratic model.

The same trend is seen in the α value for the 1 Gy given every 2 hours and for the 1.5 Gy delivered every 3 hours. The linear quadratic fit of the data was done with restrictions imposed on the two coefficients α and β . The linear quadratic model intends for these values to be greater than or equal to zero, and therefore the variables were restricted to positive values. It is seen that the parameter α has a value of zero for both these pulse sequences, which indicates that the initial slope of the survival curve is zero. This implies that the double strand breaks caused by one ionizing event have all been repaired, and that the effect of low doses of radiation is very minimal.

To better understand the data, the linear quadratic fits were also done without any restrictions, which showed α values to be -0.03 Gy^{-1} and -0.02 Gy^{-1} for the 1 Gy/ 2h and 1.5 Gy/ 3h pulses respectively. A negative α value has no meaning in the context of the linear quadratic model, as it indicates that the cells are showing a greater survival with small doses of radiation than without any dose at all. The linear quadratic model is not designed to account for such results, and perhaps some modifications to this model are required. That, however, is beyond the scope of this study. Some of these changes could be due to an adaptive response in the cells, making them more radiation resistant, and better able to deal with subsequent pulses of radiation. Although the linear quadratic model with its restrictions does not accurately describe the data, further analysis was done with the restricted values of the α parameter since it is under those restrictions that the model was intended to be used.

The pulse sequence of 2 Gy delivered every 4 hours was the only pulsed scheme to show an α value in agreement with the LDR value, although it was still significantly different from the HDR value. However, the 3 Gy delivered every 6 hours did show a decrease in α similar to that seen in the other pulses, especially with the 0.5 Gy /1h. Once again, the overall trend towards small and varying values for α indicates a change in the radiosensitivity of the cells. One possible explanation is that the α parameter is in fact dose rate dependant, and that it does not simply reflect the non-repairable damage [55]. This dose rate dependence was speculated to be the result of an adaptive response [55]. It could also represent the repair of double strand breaks, those that originated from a single event.

In the linear quadratic model, the α component represents the damage due to a single track of radiation, and resulting in a double strand break. The assumption of the linear quadratic model is that double strand break events are unrepairable and lethal to the cells. However, it is possible that the α value is decreasing (initial slope becoming shallower) because some of the DNA double strand breaks are being repaired [55], either through non-homologous end-joining, or through homologous recombination [64-67]. These two processes are the main pathways for DNA double strand break repair in mammalian cells, and could be happening or be triggered during the low dose rate irradiation or during the pulsed treatments. Another possible explanation for the change in radiosensitivity is the cell cycle re-distribution of the cells during the experiment, during which certain cells

could partially cycle to a radioresistant stage of the cell cycle. The cells in this study were grown to a plateau phase for all experiments, where the cells do not proliferate or progress through the cell cycle. A previous analysis of the U-87MG cells [46] found that less than 5% of cells were in the S-phase, which is the most radioresistant, and that the cells were indeed at a true plateau, with no cell growth or cell cycling throughout the experiments.

The β values from the fits of the PDR experimental data show varying trends. For the small fraction size, the 0.5 Gy every hour, the value of β does not change when compared to the corresponding LDR value. This indicates that the cells are not repairing any additional SLD with the pulsed treatment. The G-value which was calculated is almost identical to that from the LDR data, since both β values were similar. However, the repair half-time was calculated to be 3.4 h, which is between the value found from the LDR data (4.9 h) and the value from the split dose experiments (1.2 h). With the split dose repair rate as a constant, a predicted value of 0.008 Gy^{-2} was obtained for the quadratic component, which is about half the value found experimentally (0.016 Gy^{-2}). The model predicts that more SLD repair is occurring, if the repair rate from the split dose experiment is used in the calculations. The experimental data does not agree with the predicted values from the model.

For the next two pulse sequences, 1 Gy/ 2h and 1.5 Gy/ 3h, the β components are showing increases when compared to their corresponding LDR values. This indicates that there is less sublethal damage repair, and that more of it is accumulating to become

lethal. The values of β are not, however, larger than the HDR value. The values of G from Table 3.8 show that there is a reduction of the β value when compared to the HDR result. A value of 1 for G would indicate that no additional repair is occurring compared with the high dose rate case. The G values for these two pulses are also greater than the LDR value. An interesting observation is that it is these two pulse sequences which showed large reductions in α . This could be an indication that the cells are changing their repair from sublethal repair of single strand breaks to the repair of double strand breaks. The repair half-time calculated from the G values for the 1 Gy every 2 hours was 5.9 h, which is much larger than the values found from the split dose data (1.2 h) and from the LDR data (4.9 h). The same trend is observed with the 1.5 Gy/3h pulse, which has a calculated value of 6.8 h for the half-time of repair.

A similar trend was also seen with the 3 Gy delivered every 6 hours. The quadratic parameter and the G value were also greater than the LDR value. The repair half-time calculated from this G -value is the largest of all the pulses, with a value of 7.3 h. Looking at the repair half-times for all the PDR experimental data (Table 3.8, Results section), there seems to be an increase in the repair half-times with increasing pulse size, with the exception of the 2 Gy/ 4h value of 1.5 h. Such changes in the repair half-times (and also in the repair rates) are feasible if these repair rates are indeed dose rate dependant [28], [55]. None of these pulse sequences (1 Gy/2h, 1.5 Gy/3h, 3 Gy/6h) showed agreement with the predictions of the model when the repair rate from the split dose experiment was used in the equations. The predictions for the quadratic coefficient were all significantly lower than the actual values obtained from the experimental data.

The main reason for the lack of agreement with the model is most likely that an adaptive response is being exhibited by the cells, which is not accounted for in the theoretical models.

The only pulse sequence which seemed to show some agreement in terms of the LDR β parameter was the 2 Gy delivered every 4 hours. The β value of $0.010 \pm 0.003 \text{ Gy}^{-2}$ value for this data set was slightly lower than the $0.016 \pm 0.009 \text{ Gy}^{-2}$, although the two values agree when the errors are taken into consideration. The β value for this pulse sequence is also much smaller than the corresponding HDR value, and is the only pulse sequence to show a β value which is smaller than the HDR result. The G value for this data set is also slightly lower than the LDR value. For this pulse sequence, the model predictions for the quadratic coefficient are in agreement with the experimental data when the split dose repair rate value was used in the calculations. This agreement for the 2 Gy/ 4h seems to be the exception, since all other PDR experimental data showed significant differences with the predicted values of the linear quadratic model. The survival curve for the 2 Gy/ 4h does, however, show significant radiation resistance at the 2 and 4 Gy level ($p < 0.025$) when compared with the LDR data.

For the U-87MG cell line, the clonogenic cell survival data shows that there does not seem to be a pulse sequence which provides equivalence to the low dose rate data. In most cases, the cells show a greater radiation resistance, and a high repair-half time, which make low dose rate irradiation the more desirable choice.

4.1.5 Adaptive response experiments

Since the cells are showing an adaptive response when the LDR irradiation is replaced by a PDR schedule, some adaptive response experiments were done to further investigate this finding. The cells were given some pulses at 1 Gy/2 h, with a total dose of 6 Gy delivered in 12 hours. These pulses were collectively given as a priming dose, with the intention of giving larger challenge doses at a later time. After these priming doses, the cells were allowed to proliferate, and were consequently set up for a high dose rate experiment ten days later. The results of this experiment shows a very dramatic change in the radiosensitivity parameters α and β . The linear parameter α has a value of zero with the restricted model, and this changes to -0.13 Gy^{-1} when the data is fit without any restrictions. It is obvious that the cells are demonstrating a behavior which is not intended to be represented by the linear quadratic model, and that such an upward slope at low doses shows an adaptive response and a change in radiosensitivity of the cells. The β value of both the restricted and unrestricted fits are also much larger than any values seen thus far in this study, including those from the original HDR curve. This would indicate that the sublethal damage is accumulating and is not being repaired.

These results show that there is a definite change in the response of the cells after being given a priming dose in the form of pulses at 1 Gy/2 h. To qualify these differences, t-tests were done, which showed significant differences between the original HDR curve and the adapted HDR curve. The difference was most pronounced at the low dose level, specifically at the 1 Gy level. The cells are showing an adaptive response which is most evident after small challenging doses, and they seem to show a

radiosensitization effect at higher doses. This could have implications in the treatment of glioblastomas in a clinic, especially if there is a combination of treatments such as teletherapy and brachytherapy.

4.2 CHEF Data

4.2.1 HDR irradiation

The double strand break analysis done with the CHEF electrophoresis was done to gain information on the repair capacities of the cells, specifically when the low dose rate treatments were replaced by pulsed irradiation. The first experiments were done to establish a response to high dose rate irradiation. The fraction of activity released (FAR), which is a measure of the double strand breaks in the cells, was plotted against the radiation dose to yield a linear relation. This shows that a higher dose produces more double strand breaks, and consequently, more fragments travel out of the well and into the lanes of the gel.

This particular graph was not quantitatively analyzed since the results would not be comparable to the LDR or PDR graphs. This is because the CHEF parameters were different for the HDR, since the experiment produces many more strand breaks, which run relatively easily and quickly into the gel. Therefore the electrophoresis run time required to move the fragments into the gel is much less than with either LDR or PDR.

4.2.2 LDR irradiation

When using the CHEF after a low dose experiment, only four different dose points were possible. This is due to the fact that the vials used to grow the cells have a small surface area, making it difficult to have large numbers of cells. A large number of cells is required in order to get an adequate signal, and the optimal cell concentration was shown to be 10^6 cells/mL [39]. The FAR resulting from low dose rate irradiation is much lower than the HDR FAR, especially considering the LDR gel was run for more than double the amount of time used with the HDR gel. Also, the linear response is nearly flat, and the resulting slope was very close to zero. This was an expected result, as many of the strand breaks are being repaired during the low dose rate treatments [32]. However, one important limitation of this gel technique is that it does not measure the correctness of the repair, and hence does not account for misrepair which would lead to lethality. The relation between double strand breaks and cell survival is therefore still controversial. Some studies have correlated double strand breaks with survival [40], [68], while others have shown no correlation [67], [69].

4.2.3 PDR irradiation

The clonogenic results for the pulsed data showed that overall, the pulsed schemes resulted in a greater radiation resistance than the LDR treatment. The gel electrophoresis was done to determine if these trends are seen at the double strand break level. All pulse sequences showed a relatively flat linear response, indicating that repair was occurring and therefore reducing the FAR, which is representative of the double strand breaks. Based on the clonogenic assay results and the possible correlation between double strand

breaks and survival, it was expected that the PDR schemes would show a flatter slope than the LDR, since it is speculated that it is the increased repair of double strand breaks that is causing an increase in survival. However, the slopes of the PDR graphs were all very close to zero, and after performing t-tests on the data, none of the slopes were significantly different from the LDR curve.

This result can be explained in several ways. The first is that the CHEF technique, as it was used in this study, did not have a high enough resolution to detect the small differences that may exist between the pulsed FAR response and the LDR FAR response. This is highly likely, as the FAR signal is low to begin with since many of the strand breaks are repaired. Another possible explanation is that the amount of double strand breaks detected in the CHEF assay are not correlated to the survival seen in the clonogenic assay, as some studies have shown [67], [69]. It is possible that the repair mechanisms present or triggered during low dose rate irradiation are more error prone than those present during PDR irradiation, causing more strand breaks to be repaired incorrectly (misrepaired) in the LDR case. This would result in a low FAR response (few strand breaks) when in fact the survival would be lower than expected due to the lethal misrepair. The higher level of survival and radiation resistance seen in the clonogenic assay for the PDR sequences could be due to increased levels of correct double strand break repair. If this is the case, it would explain why the LDR and PDR show similar FAR values, with differences seen only at the level of the clonogenic cell survival assay. This change in the fidelity of repair (increased fidelity) for the PDR irradiation could be the result of the adaptive response seen in the clonogenic PDR curves. It has also been

suggested that the formation of cross-links between strands of DNA may give misleading information that there are fewer fragments present in the gel. If the repair mechanisms in the LDR case are more error prone than those for the PDR sequences (due to an adaptive response) then it is also feasible that radiation induced DNA cross-linking is not repaired in the low dose rate case, which would result in the appearance of fewer strand breaks and lower FAR in the gel analysis.

5.0 CONCLUSIONS

It has been suggested that LDR brachytherapy can be replaced by an equivalent PDR schedule, provided certain conditions are met with respect to the duration of the pulses and the time between the pulses. In this study, the PDR sequences were chosen to have the same overall dose rate as the LDR irradiation, with each pulse sequence having differing amounts of time between sequential pulses. To determine if in fact there was an equivalent PDR scheme, the clonogenic assay was used to measure cell survival and the CHEF assay was used to measure the double strand breaks resulting from each protocol.

The clonogenic cell survival data showed that overall, all pulse sequences had a greater level of radiation resistance than the LDR case. Furthermore, t-tests show that these differences are statistically significant for most dose points, for most pulse sequences. An analysis of the linear quadratic parameters revealed that there was a change in radiosensitivity occurring with the cells, and that perhaps this was due to an adaptive response. Many of the α parameters showed a significant decrease, some even leaning towards negative values, which are not possible under the assumptions of the linear quadratic model. This model and its variations do not account for such differences in the α and β parameters, and thus may not be adequate to represent the data. The repair half-times found with the PDR data were very long, and were similar to the value obtained from the LDR data. However, the half-time found from the split dose recovery was much smaller than those from the LDR and PDR data. Further calculations with this smaller value of repair half-time predicted values of the quadratic coefficient which did

not agree with the data, suggesting that perhaps this method of measuring the repair half-time was not appropriate when modeling the PDR data, or that the model was simply not adequate to describe the data. Also, the adaptive response experiments showed similar trends with changes in the radiosensitivity parameters, and increased radiation resistance at low doses. This leads to the conclusion that the pulses are inducing an adaptive response in the cells, and that this response could be responsible for the change in radiosensitivity observed in the cells.

The CHEF data did not measure differences in the amount of double strand breaks present after LDR and PDR and therefore did not show the same trend as the clonogenic data. This result was somewhat unexpected, and is most likely due to a combination of factors, including the low level limitations of the CHEF electrophoresis, and a possible difference in the fidelity of repair caused by an adaptive response.

Based on these findings, it would seem that none of the pulses are equivalent to the LDR result, although the sequences with larger pulses (2 Gy/ 4h, and 3 Gy/ 6h) show the least amount of increased resistance. This has serious implications for the possible applications of a pulsed protocol in a clinical situation. The glioma cell line used in these experiments would show a greater level of kill with the traditional low dose rate brachytherapy than with the pulse sequences investigated in this study. However, great caution must be taken when translating *in vitro* results to a clinical *in vivo* situation. This is especially true in light of the favorable results found with PDR in studies of various other cell lines.

5.1 Future Work

It is obvious that the equivalence of LDR to PDR is influenced by a great number of factors, such as the cell line under investigation [8]. Many of the positive results from PDR were with very different cell lines, but this should not imply that all cell lines will show positive results, as seen in this study. Therefore more work is needed in the investigation of the varying responses among different cell lines. This could also lead to more information regarding the clinical differences seen in cancer patients, and why some malignancies respond well to treatment, while others have high failure rates. It would also be useful to investigate variations on the PDR schemes used in this study, such that the overall dose and overall treatment time are not identical to the low dose rate case. This would have important clinical applications, especially with the possibility of a daytime only schedule for the pulsed treatment. In certain countries, regulations require that a medical physicist be present at all times during a pulsed brachytherapy treatment, therefore the implementation of a daytime only schedule would be beneficial and could even encourage the use of this treatment (in cases where it would be advantageous).

Another current area of interest in radiobiology is the combination of hyperthermia with radiation in the hopes of making the malignant cells more sensitive to radiation. It would be interesting to examine the behavior of this glioma cell line with a pulsed treatment in combination with hyperthermia. Also, more work should be done to investigate the adaptive response seen in this study, as there are not many studies which have used priming doses in the form of pulses. Most studies, for traditional adaptive

response experiments, have used a single small priming dose of radiation, followed by large challenge doses.

REFERENCES

- [1] Brenner, D. J., Hall, E. J., Conditions for the equivalence of continuous to pulsed dose rate brachytherapy, *Int. J. Radiat. Oncol. Biol. Phys.*, **20**:181-190, 1991.
- [2] Hall, E. J., Radiobiology for the Radiologist, 4th edition, J. B. Lippincott Company, Philadelphia, 1994.
- [3] Brenner, D. J., Radiation biology in brachytherapy, *J. Surg. Oncol.*, **65**:66-70, 1997.
- [4] Hall, E. J., Brenner, D. J. The dose-rate effect revisited: radiobiological considerations of importance in radiotherapy, *Int. J. Radiat. Oncol. Biol. Phys.* **21**:1403-14, 1991.
- [5] Lea, D. E., Catcheside, D. G., The mechanism of the induction by radiation of chromosome aberrations in *Tradescantia*, *J. Genet.*, **44**:216-245, 1942.
- [6] Bedford, J. S., Mitchell, J. B., Dose rate effects in synchronous mammalian cells in culture, *Radiat. Res.*, **54**:316-327, 1973.
- [7] Brenner, D. J., Hall, E. J., Randers-Pherson, G., Huang, Y., Johnson, G. W., Miller, R. W., Wu, B., Vazquez, M., Medvedovsky, C., Worgul, B. V., Quantitative comparisons of continuous and pulsed low dose rate regimens in a model late effect system, *Int. J. Radiat. Oncol. Biol. Phys.*, **34**:905-910, 1996.
- [8] Chen, C.-Z., Huang, Y., Hall, E. J., Brenner, D. J., Pulsed brachytherapy as a substitute for continuous low dose rate: an *in vitro* study with human carcinoma cells, *Int. J. Radiat. Oncol. Biol. Phys.*, **37**:137-143, 1997.
- [9] Mason, K. A., Thames, H. D., Ochran, T.G., Ruifrok, A. C. C., Janjan, N., Comparison of continuous and pulsed low dose rate brachytherapy: biological equivalence in vivo, *Int. J. Radiat. Oncol. Biol. Phys.*, **28**:667-671, 1994.
- [10] Fowler, J., Mount, M., Pulsed brachytherapy: the conditions for no significant loss of therapeutic ratio compared with traditional low dose rate brachytherapy, *Int. J. Radiat. Oncol. Biol. Phys.*, **23**: 661-669, 1992.
- [11] Visser, A. G., Van Den Aardweg, G. J. M. J., Levendag, P. C., Pulsed dose rate and fractionated high dose rate brachytherapy: Choice of brachytherapy schedules to replace low dose rate treatments, *Int. J. Radiat. Oncol. Biol. Phys.*, **34**:497-505, 1996.
- [12] Pomp, J., Woudstra, E. C., Kampinga, H. H., Pulsed-dose-rate and low-dose-rate brachytherapy: Comparison of sparing effects in cells of a radiosensitive and a radioresistant cell line, *Radiat. Res.*, **151**:449-453, 1999.

- [13] Armour, E., Zhenhua, W., Corry, P., Martinez, A., Equivalence of continuous and pulse simulated low dose rate irradiation in 9L gliosarcoma cells at 37°C and 41°C, *Int. J. Radiat. Oncol. Biol. Phys.*, **22**:109-114, 1991.
- [14] Haustermans, K., Fowler, J., Landuyt, W., Lambin, P., van der Kogel, A., van der Schueren, E., Is pulsed dose rate more damaging to spinal cord of rats than continuous low dose rate?, *Radiother. and Oncol.*, **45**: 39-47, 1997.
- [15] Levendag, P. C., Schmitz, P. I. M., Jansen, P. P., Senan, S., Eijkenboom, W. M. H., Sipkema, D., Meeuwis, C. A., Kolkman-Deurloo, I.-K., Visser, A. G., Fractionated high-dose-rate and pulsed-dose-rate brachytherapy: First clinical experience in squamous cell carcinoma of the tonsillar fossa and soft palate, *Int. J. Radiat. Oncol. Biol. Phys.*, **38**: 497-506, 1997.
- [16] Swift, P. S., Purser, P., Roberts, L. W., Pickett, B., Powell, C. B., Phillips, T. L., Pulsed low dose rate brachytherapy for pelvic malignancies, *Int. J. Radiat. Oncol. Biol. Phys.*, **37**: 811-817, 1997.
- [17] Rogers, C. L., Freel, J. H., Speiser, B. L., Pulsed low dose rate brachytherapy for uterine cervix carcinoma, *Int. J. Radiat. Oncol. Biol. Phys.*, **43**: 95-100, 1999.
- [18] Jensen, P. T., Roed, H., Engelholm, S. A., Rosendal, F., Pulsed dose rate (PDR) brachytherapy as salvage treatment of locally advanced or recurrent gynecologic cancer, *Int. J. Radiat. Oncol. Biol. Phys.*, **42**:1041-1047, 1998.
- [19] Roed, H., Engelholm, S. A., Svendsen, L. B., Resendal, F., Olsen, K. J., Pulsed dose rate (PDR) brachytherapy of anal carcinoma, *Radiother. and Oncol.*, **41**:131-134, 1996.
- [20] Gerard, J.-P., Mauro, F., Thomas, L., Castelain, B., Mazon, J.-J., Ardiet, J.-M., Peiffert, D., Treatment of squamous cell anal carcinoma with pulsed dose rate brachytherapy. Feasibility study of a French cooperative group, *Radiother. and Oncol.*, **51**:129-131, 1999.
- [21] Strnad, V., Lotter, M., Grabenbauer, G., Sauer, R., Early results of pulsed-dose-rate interstitial brachytherapy for head and neck malignancies after limited surgery, *Int. J. Radiat. Oncol. Biol. Phys.*, **46**: 27-30, 2000.
- [22] De Pree, C., Popowski, Y., Weber, D., Nouet, P., Rouzaud, M., Kurtz, J. M., Feasibility and tolerance of pulsed dose rate interstitial brachytherapy, *Int. J. Radiat. Oncol. Biol. Phys.*, **43**: 971-976, 1999.
- [23] Tobias, C. A., The repair-misrepair model in radiobiology: Comparison to other models, *Radiat. Res.*, **106**(Suppl.): S77-S95, 1985.

- [24] Goodhead, D. T., Saturable repair models of radiation action in mammalian cells, *Radiat. Res.*, **104**(Suppl.): S58-S67, 1985.
- [25] Thames, H. D., An "incomplete-repair" model for survival after fractionated and continuous irradiations, *Int. J. Radiat. Biol.*, **47**: 319-339, 1985.
- [26] Brenner, D. J., Hlatky, L. R., Hahnfeldt, P. J., Huang, Y., Sachs, R. K., The linear quadratic and most other common radiobiological models result in similar predictions of time-dose relationships, *Radiat. Res.*, **150**: 83-91, 1998.
- [27] Chadwick, K. H., Leenhouts, H. P., A molecular theory of cell survival, *Phys. Med. Biol.*, **18**: 78-87, 1973.
- [28] Heller, D. P., Raaphorst, G. P., The kinetics of cellular recovery in exponential and plateau growth phase human glioma cells following γ -irradiation, *Int. J. Radiat. Oncol. Biol. Phys.*, **30**: 383-390, 1994.
- [29] Dale, R. G., The application of the linear-quadratic dose-effect equation to fractionated and protracted radiotherapy, *Br. J. Radiol.*, **58**: 515-528, 1985.
- [30] Dale, R. G., Huczkowski, J., Trott, K. R., Possible dose rate dependence of recovery kinetics as deduced from a preliminary analysis of the effects of fractionated irradiations at varying dose rates, *Br. J. Radiol.*, **61**: 153-157, 1988.
- [31] Niedbala, M., A Comparison of Pulsed Dose Rate to Low Dose Rate Irradiations with/without Mild Hyperthermia Using Three Human Cell Lines, M.Sc. thesis, Carleton University, 1999.
- [32] Brown, R. C., The prediction of patient radiosensitivity using the clonogenic assay and asymmetric field inversion gel electrophoresis, Ph.D. thesis, Carleton University, 1996.
- [33] Joppa, B., Li, S., Cole, S., Pulsed field electrophoresis for separation of large DNA, National Agriculture Library, US Department of Agriculture, <http://www.nal.usda.gov/pgdic/Probe/v2n3/puls.html>, March 2000.
- [34] Blocher, D., Einspinner, M., Zajackowski, J., CHEF electrophoresis, a sensitive technique for the determination of DNA double-strand breaks, *Int. J. Radiat. Biol.*, **56**: 437-448, 1989.
- [35] Ager, D. D., Dewey, W. C., Gardiner, K., Harvey, W., Johnson, R. T., Waldren, C. A., Measurement of Radiation-Induced DNA double-strand breaks by pulsed-field gel electrophoresis, *Radiat. Res.*, **122**: 181-187, 1990.

- [36] Stamato, T. D., Guerriero, S., Denko, N., Two methods for assaying DNA double-strand break repair in mammalian cells by asymmetric field inversion gel electrophoresis, *Radiat. Res.*, **133**: 60-66, 1993.
- [37] Stamato, T. D., Denko, N., Asymmetric field inversion gel electrophoresis: a new method for detecting DNA double-strand breaks in mammalian cells, *Radiat. Res.*, **121**: 196-205, 1990.
- [38] Blocher, D., In CHEF electrophoresis a linear induction of dsb corresponds to a nonlinear fraction of extracted DNA with dose, *Int. J. Radiat. Biol.*, **57**: 7-12, 1990.
- [39] Blocher, D., Kunhi, M., DNA double-strand break analysis by CHEF electrophoresis, *Int. J. Radiat. Biol.*, **58**: 23-34, 1990.
- [40] Murray, D., Simpson, R., Rosenberg, E., Carraway, A., Britten, R., Correlation between γ -ray-induced DNA double-strand breakage and cell killing after biologically relevant doses: analysis by pulsed-field gel electrophoresis, *Int. J. Radiat. Biol.*, **65**: 419-426, 1994.
- [41] Ward, J.F., DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation and repairability, *Progress in Nucleic Acids and Molecular Biology*, **35**: 95-125, 1988.
- [42] Contopoulou, C. R., Cook, V. E., Mortimer, R. K., Analysis of DNA double strand breakage and repair using orthogonal field alternation gel electrophoresis, *Yeast*, **3**: 71-76, 1987.
- [43] Kaaijk, P., Troost, D., Sminia, P., Hulshof, M.C.C.M., van der Kracht, A.H.W., Leenstra, S., Bosch, D.A., Hypofractionated Radiation Induces a Decrease in Cell Proliferation but no Histological Damage to Organotypic Multicellular Spheroids of Human Glioblastomas, *Eur. J. Cancer.*, **33**: 645-651, 1997.
- [44] Freshney, R. I., Culture of Animal Cells: A Manual of Basic Technique, Wiley/Liss, New York, 1994.
- [45] Working Party of the IPEMB (Institution of Physics and Engineering in Medicine and Biology), The IPEMB code of practice for the determination of absorbed dose for x-rays below 300 kV generating potential (0.035 mm Al – 4 mm Cu HVL; 10-300 kV generating potential), *Phys. Med. Biol.*, **41**(12): 2605-2625, 1996.
- [46] Heller, D. P., Radiobiological aspects of cellular recovery following high and low dose-rate irradiation with/without mild hyperthermia in a human glioma cell model. Ph.D. thesis, Carleton University, 1993.

- [47] Puck, T. T., Morkoving, D., Marcus, P. I., Cieciura, S. J., Action of X-rays on mammalian cells. II. Survival curves of cells from normal human tissues, *J. Exp. Med.*, **106**:1957.
- [48] Fertil, B., Malaise, E.P., Inherent radiosensitivity as a basic concept for human tumor radiotherapy, *Int. J. Radiat. Oncol. Biol. Phys.*, **7**: 621-629, 1981.
- [49] Fertil, B., Malaise, E.P., Intrinsic radiosensitivity of human cell lines is correlated with radioresponsiveness of human tumors: analysis of 101 published survival curves, *Int. J. Radiat. Oncol. Biol. Phys.*, **9**:1699-1707, 1985.
- [50] Girinsky, T., Lubin, R., Pignon, J.P., Chavaudra, N., Gazeau, J., Dubray, B., Cosset, J.M., Socie, G., Fertil, B., Predictive value of in vitro radiosensitivity parameters in head and neck cancers and cervical carcinomas: preliminary correlations with local control and overall survival, *Int. J. Radiat. Oncol. Biol. Phys.*, **25**: 3-7, 1993.
- [51] Deacon, J., Peckham, M.J., Steel, G.G., The radioresponsiveness of human tumors and the initial slope of the cell survival curve, *Radiother. and Oncol.*, **2**:317-323, 1984.
- [52] Steel, G.G., Peacock, J.H., Why are some human tumors more radiosensitive than others? *Radiother. and Oncol.*, **15**: 63-72, 1989.
- [53] Shahine, B.H., Models of radiation treatment responses after continuous and fractionated irradiation with/without mild hyperthermia, M.Sc. thesis, Carleton University, 1995.
- [54] Fowler, J.F., Why shorter half-times of repair lead to greater damage in pulsed brachytherapy, *Int. J. Radiat. Oncol. Biol. Phys.*, **26**: 353-356, 1993.
- [55] Badie, C., Alsbeih, G., Reydellet, I., Arlett, C., Fertil, B., Malaise, E.P., Dose-rate effects on the survival of irradiated hypersensitive and normal human fibroblasts, *Int. J. Radiat. Biol.*, **70**: 563-570, 1996.
- [56] Brenner, D., Armour, E., Corry, P., Hall, E., Sublethal damage repair times for a late-responding tissue relevant to brachytherapy (and external-beam radiotherapy): implications for new brachytherapy protocols, *Int. J. Radiat. Oncol. Biol. Phys.*, **41**: 135-138, 1998.
- [57] Boyden, S. N., Investigation of adaptive response in human tumor and normal cell lines with varying radiosensitivity, M.Sc. thesis, Carleton University, 1998.
- [58] Raaphorst, G.P., Boyden, S., Adaptive response and its variation in human normal and tumour cells, *Int. J. Radiat. Biol.*, **75**: 865-873, 1999.

- [59] Wiencke, J.K., Afzal, V., Olivieri, G., Wolff, S., Evidence that the [³H]thymidine-induced adaptive response of human lymphocytes to subsequent doses of X-rays involves the induction of a chromosomal repair mechanism, *Mutagenesis*, **1**: 375-380, 1986.
- [60] Wolff, S., Afzal, V., Wiencke, J.K., Olivieri, G., Michael, A., Human lymphocytes exposed to low doses of ionizing radiations become refractory to high doses of radiation as well as to chemical mutagens that induce double-strand breaks in DNA *Int. J. Radiat. Biol.*, **53**: 39-48, 1988.
- [61] Shadley, J.D., Wiencke, J.K., Induction of the adaptive response by X-rays is dependant on radiation intensity, *Int. J. Radiat. Biol.*, **56**:107-118, 1989.
- [62] Joiner, M.C., Induced radioresistance : an overview and historical perspective, *Int. J. Radiat. Biol.*, **65**:79-84, 1994.
- [63] Raaphorst, G.P., Ng., C.E., Smith, D.M., Neidbala, M., Evidence for adaptive response and the implication in pulse simulated low dose rate radiotherapy, Accepted for publication, *Int. J. Radiat. Oncol. Biol. Phys.*, 2000.
- [64] Johnson, R.D., Liu, N., Jasin, M., Mammalian XRCC2 promotes the repair of DNA double-strand breaks by homologous recombination, *Nature*, **410**: 397-399, 1999.
- [65] Hiom, K., Rad52-the means to an end, *Curr Biol*, **9**:R446-448, 1999.
- [66] Takata, M., Sasaki, M.S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A., Takeda, S., Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells *EMBO J.*, **17**: 5497-5508, 1998.
- [67] Britten, R.A., Kuny, S., Perdue, S., Modification of non-conservative double-strand break (DSB) rejoining activity after the induction of cisplatin resistance in human tumour cells, *Br. J. Cancer*, **79**: 843-849, 1999.
- [68] Kiltie, A.E., Orton, C.J., Ryan, A.J., Roberts, S.A., Marples, B., Davidson, S.E., Hunter, R.D., Margison, G.P., West, C.M.L., Hendry, J.H., A correlation between residual DNA double strand breaks and clonogenic measurements of radiosensitivity in fibroblasts from preradiotherapy cervix cancer patients, *Int. J. Radiat. Oncol. Biol. Phys.*, **39**: 1137-1144, 1997.
- [69] Olive, P.L., Banath, J.P., MacPhail, H.S., Lack of a correlation between radiosensitivity and DNA double-strand break induction or rejoining in six human tumor cell lines, *Cancer Res.*, **54**: 3939-3946, 1994.