# Combining hyperthermia and Ionising Radiation: The cell killing effect on mouse leukaemia cells

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by

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## Abstract

Basic *in vitro* cell experiments were conducted on the P388 mouse leukaemia cell line to determine whether a supra-additive cell killing effect from combining hyperthermia with ionising radiation exists in the case of leukaemia. Methods were established to measure the cell kill, using a Coulter counter, from hyperthermia alone, radiation alone and several combined regimes. The cell kill from hyperthermia, in the range of 38-50 degrees for 30 minutes, 1 hour, 2 hours and 3 hours, and radiation, for 1, 3, 5, 9, 11 and 15 Gy was investigated. The approach used had various limitations, such as the underestimation of cell kill. Consistent trends, however, were found for the hyperthermia and radiation data, in accordance with the literature, which killed cells in a predictable manner. Subsequently, after other preliminary combined experiments were completed, the cell kill from both 5 and 11 Gy combined with hyperthermia at 43, 45 and 47 degrees for 2 hours were investigated. 5 Gy in combination with all levels of hyperthermia resulted in a direct additive cell killing effect. This, however, was not observed for 11 Gy in which a diminished effect was found. The overall level of cell kill from 5 Gy combined with hyperthermia was found to be equal, in the case of 43 degrees, or higher, as for 45 and 47 degrees, to that of those combined with 11 Gy. A supra-additive effect was not observed.

## Acknowledgements

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- Lastly, thanks to my husband James for keeping my spirits up and bringing me those late night dinners!

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# List of Abbreviations

**BMT:** Bone marrow transplant

**TBI:** Total body irradiation

**Gy:** gray

**WBH:** Whole-body hyperthermia

**MAF:** Ministry of Agriculture and Fisheries

**MU:** Monitor units

**TLD:** Thermoluminscent dosimeter

**P-value:** Probability value

# Chapter 1

# Research Motivation & Background

## 1.1 INTRODUCTION

Leukaemia is a cancer of the blood that, especially in the case of acute leukaemia, requires a bone marrow transplant (BMT) to fully cure the disease [1-3]. The 'gold standard' conditioning regime used to prepare leukaemia patients for a BMT consists of a course of chemotherapy followed by fractionated total body irradiation (TBI) [4-8]. The chemotherapeutic agent, usually cyclophosphamide, is immunosuppressive and administered daily for approximately seven days. This ensures that there is no immune response when the patient receives the donor marrow [1,3]. Following chemotherapy, the patient is subjected to a fractionated course of TBI where the total dose received by the patient can range from ten to fifteen gray (Gy), depending on the patient and the institution at which it is provided [4]. These doses are delivered either by cobalt-60 treatment units or by using photon beams of 4-6 MV maximal energy from linear accelerators [9]. TBI is administered primarily to kill the leukaemic cells throughout the body and consequently to create new spaces in the bone marrow that the new graft will fill [1,3]. Fractionation allows for a higher biologically effective dose that results in fewer relapses and thus a longer overall survival [10].

Since TBI targets the bone marrow, many organs lie within the constructed radiation fields and receive high radiation doses. Improvements in the delivery of TBI, such as the design of a translational or moving treatment couch as by Papiez *et al.* [11] and Sarfaraz *et al.* [12], are aimed at developing a more homogenous dose

distribution in the patient. The side effects from TBI that can present in a patient are very distinct from those by chemotherapy and are well documented in the literature [4,13]. Partial lung-shielding blocks are common practice at most institutions and serve to reduce the total lung dose. This, consequently, reduces the risk of patients developing interstitial pneumonia, a common side effect from which patients tend to succumb [14]. Recently, Nakagawa *et al.* published two different papers that separately describe the shielding of the kidneys [15] and the ovaries [16]. Renal shielding has become standardised at their institution, where the overall dose to the kidneys has been reduced from 12 to 10 Gy. All patients undergoing standard TBI procedures become sterile and thus Nakagawa *et al.* studied the effects of ovarian shielding in three women in whom they reduced the total dose to the ovaries from 12 to 3.125 Gy. Two out of three women seemingly retained their normal ovarian function and it was indicated that a longer follow up is required to determine whether both can successfully reproduce [16].

Other side effects from TBI include the development of cataracts, endocrine dysfunction and long-term developmental effects in children [9,14,17]. It quickly becomes obvious that there is a distinct *need* to improve the 'gold standard' conditioning regime that is currently in place. An alternative regime that combines TBI and an adjunct therapy to reduce the overall TBI dose would be ideal. Such an adjunct therapy must in itself have minimal side effects and allow for the same therapeutic efficacy as TBI alone currently provides.

Mild hyperthermia as a therapy is known to satisfy the first of these requirements as whole-body hyperthermia (WBH) up to 41.8ºC generally has no significant side effects if its applied using the 'radiant heat device' as described by Robins *et al.* [18]. It is yet to be determined whether the same therapeutic efficacy can be achieved using a regime of mild whole-body hyperthermia and TBI.

## 1.2. BACKGROUND

Hyperthermia has been recognised to be effective in the treatment of various types of cancer for over a century [19,20]. Scientific research using this modality peaked in the 1960s and 1970s. During these time periods, the cell-killing properties of hyperthermia were investigated. Multiple studies have shown that cells most sensitive

to hyperthermia are either hypoxic or in the late S-phase of the cell-cycle [21-26]. This greatly contrasts cell sensitivity to radiation as cells are most radio-resistive in both these situations [20]. It is therefore reasonable to suggest that a synergism ought to exist when combining ionising radiation with hyperthermia. As with most therapies, sequencing for such a combined approach is vital. Deciding whether hyperthermia is to be applied prior to or after the administration of ionising radiation greatly depends on understanding the underlying cell-killing mechanisms by both these modalities.

Firstly, hyperthermia kills cells both directly and indirectly [27]. The 'direct' cell-killing mechanism acts by denaturing proteins, inactivating vital enzymes, disrupting transport mechanisms and ceasing mitochondrial function within the cell [28]. This mode of action is reasonably well understood and most profound if higher temperatures are used. For example, Nikfarjam *et al*., reported that direct cell-kill is observed by the inactivation of vital enzymes in the range of 42-45ºC for periods of 30 to 60 minutes, yet between 60 and 140ºC protein denaturation is observed [29]. The 'indirect' cell-killing mechanism from hyperthermia is not as well understood and is regarded as a progression in cellular injury. This progression is thought to take place due to altered apoptosis mechanisms, cytokine release, vascular injury and a mechanism known as Kupffer cell activation [28,29].

The exposure time to hyperthermia, or the duration of a heat-treatment, is also an issue that needs to be considered. After all, the same cell-killing effect can possibly be achieved by using a lower temperature applied for a longer period compared to a higher temperature for a short period [30]. How this concept was applied in this thesis will be further discussed in the next chapter.

Overall, hyperthermia targets the cytoplasm of the cell and everything within it apart from the nucleus of the cell. This, again, contrasts the effects that x-ray radiation has on a cell, since approximately one third of the radiation will target the DNA in the nucleus directly, and the other two-thirds will create free radicals within the cytoplasm [20]. This, in turn tries to destroy the chromosomes in the nucleus of the cells. The main mode of cell death from radiation is believed to result from the interaction of two DNA double strand breaks within the nucleus of the cell [31]. Single strand breaks can also lead to cell death but have a higher chance of being repaired. Studies have shown that the greatest synergistic effect between ionising radiation and hyperthermia, at lower temperatures, occurs when hyperthermia is administered *after* the radiation, with the best effect if administered directly after [19,23,32-34]. The principal

reason for this is that hyperthermia inhibits cellular repair mechanisms in cells that have only suffered sublethal damage from the radiation, or single strand breaks. There have been suggestions that this combined effect is additive if not supra-additive, yet the disputes between researchers regarding this issue seem to reflect the different cell-lines and/or methodologies used [34-36].

It is interesting to note that fractionating hyperthermia is not beneficial due to the development of thermotolerance [36]. Thermotolerance is the change in cellular sensitivity to succeeding heat treatments produced by the initial thermal dose [24]. This resistance is mainly developed by heat shock proteins [37,38]. Studies have suggested that in a fractionated radiation scheme only one heat treatment, or a maximum of two, is to be incorporated into the overall treatment scheme of patients [36,39]. This then avoids the problems from thermotolerance and ensures the patient receives the benefits from this combined approach. In this study, the development of thermotolerance is of no concern since both the radiation dose and the heat-treatment are administered to the cells in a single fraction.

At present, most hyperthermia treatments are administered to treat solid tumours such as head and neck, liver, renal, bone, prostate, lung, breast and adrenal cancers through the use of percutaneous ablation techniques [30,40,41]. Of all percutaneous ablation techniques available, radiofrequency ablation is the most commonly used ablative therapy [42]. Hyperthermia can also be administered as a regional therapy, in which deep-seated tumours of the pelvis and abdomen are treated. Regional hyperthermia has also been combined with chemotherapy to manage patients with soft tissue sarcomas [43]. Regional therapies can be administered using the Sigma-60 applicator as described by Wust *et al.* [44].

The applications of whole body hyperthermia in the treatment of cancer have been investigated as early as the 1970s. Various methods of applying whole body hyperthermia, such as heating by the use of encompassing patients with hot wax (early technique) and heating by using the radiant heat device (current technique), revealed the various limitations of applying whole body hyperthermia [45]. For instance, it is crucial to ensure that throughout the procedure, the patients fluid intake is continuously replenished to prevent severe dehydration [46]. Toxic effects, such as damage to the liver from the elevation of liver enzymes, were found to occur if the whole body temperature exceeds 41.8ºC [47]. Other reported side-effects below 41.8ºC, such as diarrhea, leg and arm edema, and fatigue are generally observed but are

usually resolved within 48 hours following treatment [48]. More interestingly, whole body hyperthermia has been shown to suppress the immune system and hence might enhance the effects from chemotherapy in the treatment of leukaemia [49]. In this thesis, however, only the combined effects from radiation and hyperthermia in the treatment of leukaemia, on an *in vitro* scale, will be considered.

## 1.3 AIM OF RESEARCH

The aim of this research was to carry out basic *in vitro* experiments using a mouse leukaemia cell line in order to gain a fundamental understanding a supra-additive effect occurs when combining ionising radiation, as used in TBI procedures, with mild hyperthermia. Several experiments were carried out to investigate the cellular response to radiation, mild hyperthermia and a combination of both these regimes. It was hoped that from these experiments a model could be constructed that incorporates the radiation dose plus the 'heating dose' in order to predict the cell survival of leukaemia cells. An extensive literature review did not reveal any published papers that construct such a model for leukaemia cell lines.

In order to achieve this aim, a framework for the experimental set-up at the University of Canterbury for the undertaking of radiation biology experiments needed to be established. Never before have such experiments been performed at the University of Canterbury and hence there not all facilities are in place to carry them out. For radiation biology experiments, one typically requires a biohazard safe room for the growing, maintaining and measurements taken of the cell line and a linear accelerator (linac) for the administration of radiation doses. Preferably, both the linac and the biohazard safe room are found on the same premises for various reasons such as the concerns that the Ministry of Agriculture and Fisheries (MAF) have about the transport of bio-hazardous material. Such facilities however, are not currently available at the University of Canterbury and collaboration was sought with both the Department of Chemistry and Christchurch Hospital to enable this study to be carried out.

The Department of Chemistry at the University of Canterbury has a biohazard safe room used for experiments utilising a mouse leukaemia cell line and were willing to make these available for this project. To handle this cell line, however, one must be

biohazard level 3 qualified as per MAF regulations, resulting in the necessary full cooperation of a highly competent and qualified technician. Approval for this project by both the head of the Department of Chemistry and the Chemistry technician were eventually obtained.

After some investigation, it was found that the Research Department at Christchurch Hospital had access to human leukaemia cell lines. Unfortunately, after some inquiries it was found that these cell lines would be unavailable throughout the duration of this project. Hence, the mouse-leukaemia cell line was the only cell line investigated throughout this study.

The Department of Oncology at Christchurch Hospital was approached with the request for the utilisation of one of their linear accelerators in order to deliver radiation doses to the cells throughout the project. The principal medical physicist approved this request but of course laid restrictions as to 'when' the linac was to be used since patients will always come first. This resulted in 6 a.m. visits to the technician in the Department of Chemistry to receive the cells ready for transportation to the Hospital, to arrive there around 7 a.m. for the administration of radiation doses under the supervision of a medical physicist. Hence, a high level of commitment to the project was required from both the technician and the medical physicist concerned. Logistical problems, throughout this study, were incurred due to the need for MAF approval to transport the cells to and from the hospital, and the lack of several required resources to carry out these experiments.

In this thesis, the experimental set-ups used are discussed in Chapter 2: Materials and Methods; the results and data are analysed in Chapter 3: Results and Analysis; finally, the findings are summarized in Chapter 4: Discussion and Conclusion.

# Chapter 2

# Materials & Methods

## 2.1 THE LEUKAEMIA CELL-LINE

The cell-line used throughout this investigation was the P388 D1 (Murine Leukaemia cells) American Type Culture Collection (ATCC), Manassas, USA, CCL 46. This cell-line was originally obtained from ATCC on the 28th of March 2002 for different experiments under the Ministry of Agriculture and Forestry (MAF) permit: 2002014613. The intrinsic properties of this cell-line, such as the cell doubling time are shown below in table 2.1. It is interesting to note that this type of cell-line cannot be plated. This means that it is not possible to perform a clonogenic assay, as is most commonly done to determine the surviving fraction of cells after irradiation that enables the production of a cell survival curve [20]. In a clonogenic assay, one is able to count the surviving colonies that exist after exposure to radiation. Such an assay therefore, really is a measure of cell viability as the assay assesses the reproductive integrity of the cells.

| | |
|---|---|
| Cell doubling time | Approximately 10-12 hours |
| Natural 'lifetime' of the cells | Approximately 7-10 days |
| Type of culture | Stationary suspension culture |
| Plating efficiency | *Cannot* be plated |

*Table 2.1: The intrinsic properties of the P388 D1 Murine Leukaemia cell-line.* [50].

Since a clonogenic assay could not be performed, it was decided to carry out a cell proliferation assay. A cell proliferation assay determines the inhibition of cell growth, or direct cell-death, rather than looking at long-term reproductive integrity. For this assay, a WST-1 Rapid Cell Proliferation Kit was bought from Merck, Palmerston North, New Zealand. WST-1 is a reagent (dye) used for the colorimetric quantification of cell viability and proliferation. It measures the metabolic activity of mitochondria within the cells to determine whether the cell is still viable. This dye however, must be used within a 96-well plate set-up, as shown in figure 2.1, in order to measure the cell viability. The counter used for this does not accept any other plates.

To be able to interpret any results obtained using this cell-line, it is vital to understand the environment in which the cells live; or in other words, how they are grown, maintained and seeded in experiments. The cells are maintained in large, plastic, tissue culture flasks, also known as stock flasks, and are grown in a growth medium that consists of the following:

| | |
|---|---|
| minimal essential media | |
| 10% foetal calf serum | (protein source) |
| 266 units of penicillin per mL | (antibiotic) |
| 132 μg streptomycin per mL | (antibiotic) |
| 2 mM L-glutamine | (amino-acid source) |
| 2.2 g/L sodium bicarbonate ($NaHCO_3$) | |
| 7.4 mM hepes. | |

Furthermore, the cells are normally grown in a 5% carbon dioxide ($CO_2$) environment that, along with the sodium bicarbonate and the hepes, stabilise the pH of the medium to about 7.5. The cells are kept in a continuous growth phase by the method of 'splitting' on a weekly basis. 'Splitting' is the tipping out of roughly 80% of cells in media and adding 80% new growth medium. This ensures that the stock of cells in culture is always actively growing, as they never seem to run out of growth medium. In all experiments, cells were initially seeded from an actively growing stock flask into a 5% $CO_2$ environment and were incubated overnight with the exposure to hyperthermia, radiation or both occurring on the following day.

The radiation doses to the cells were given using a linear accelerator at Christchurch Hospital. Because of this, travelling to the hospital from the University of Canterbury did not allow the cells to be continuously exposed to $CO_2$. As this condition therefore could not be applied in all experiments at all times, it was decided to eliminate any use of $CO_2$ after the initial incubation time of the cells. A test-run revealed that without this controlled exposure to 5% $CO_2$, the pH of the cells increase. This increase in pH creates an alkaline environment causing the cells to die. It thus became very important to be able to *trap* the carbon dioxide that the cells were initially exposed to into the cell environment, done simply by closing the system from any airflow. Such entrapment was found to create a stable pH environment for the cells, allowing them to survive. The 96-well plate set-up, initially considered for the experiments, could not be sufficiently closed off to prevent air exchange from occurring and it was for this reason that the experimental set-up changed to the use of small culture flasks, as shown in figure 2.1. Due to this change, it was not possible to use the WST-1 dye, since the dye requires the 96-well plate set-up. Hence, determining the cell viability by assessing the mitochondrial changes within a cell was not achievable.

*Figure 2.1: Left: A 96-well plate sealed with a rubber seal that still allowed for air exchange. Right: An empty culture flask that was used to replace the plates as the flask caps screw tightly shut to hold the carbon dioxide inside the flask.*

## 2.2 DETERMINING CELL VIABILITY

Since the logistics of this project prevented the use of WST-1 dye, or any other dye for that matter, a simpler method was employed to determine cell viability.

The Coulter counter Model ZF (Coulter Electronic Limited, Luton, England) [51] can determine whether a cell is dead or alive by measuring the spherical diameter, or volumetric size, of the cell. In general, a dead cell shrinks considerably in comparison to their living counterparts. It becomes obvious that by measuring the size of the cell to determine its viability we are ignoring other vital information that could also indicate the cell is dead. For example, when using the WST-1 dye, the metabolic activity of a cell would show that the cell is no longer viable. Yet when the same cell is passed through the Coulter counter, it could be indicated as alive, as the cell has not shrunk enough to be distinguished from its truly alive counterparts. Hence, the Coulter counter only takes a 'snapshot' of the cells to determine its viability and does not allow for the delayed cell death or loss of reproductive integrity to be measured. Because of this, the cell-kill by hyperthermia, radiation or both as measured by the Coulter counter is most likely underestimated.

### 2.2.1 THE COULTER COUNTER

An aliquot of 1 mL is initially taken from the culture flask and diluted into 10 mL of Isoton II, an electrolyte solution. Once completely mixed, half a milliLitre of this new solution is passed through the aperture of the Coulter counter, which has a tube orifice of 100 microns. Electrodes on either side of this aperture induce a current path between them. Hence, whenever a cell passes through the aperture, the electrolyte is displaced, resulting in a change in the resistance between the two electrodes. This in turn produces a voltage pulse whose magnitude is directly proportional to the volumetric size of the cell producing that change. The voltage pulse is consequently fed into a threshold circuit that discriminates between dead or alive cells by passing the count pulses only for the cells that exceed the threshold level [51]. This threshold level was determined by comparing the cell count known from a haemocytometer, another instrument used for the counting of cells in a solution, to that of the Coulter counter. The threshold is set by changing the 'aperture' and 'threshold' settings on the machine, (see schematic diagram in Appendix A), and were set to 32 and 43 respectively. This was done on a trial and error basis, resulting in these values coinciding with the measurements as obtained from the haemocytometer in previous experiments performed in the Chemistry Department. The level of sensitivity was set to one, the highest value available. It should be noted

that the calibration of the Coulter counter was done years before this thesis commenced, and could not be redone since it was also simultaneously used for other experiments in the Chemistry Department. In addition, the settings on the Coulter counter are very arbitrary, as the values indicated above do not have any units associated with them. The uncertainty in the Coulter counter was found to be negligible when it was first calibrated and has since been ignored [52].

The total pulse count measured by the Coulter counter represents one tenth of the number of viable cells per half a millilitre. To obtain the number of viable cells per millilitre the total pulse count is simply multiplied by twenty. It should be noted that living cells are counted, resulting in an indirect measure of cell-kill. In fact, the experiments arguably determine the growth inhibition of the cells rather than direct cell-death. Throughout this thesis, however, it will be referred to as cell-death.

### 2.2.2 CONTROL GROUPS AND UNCERTAINTIES

Every culture flask used throughout this study contained an initial 15 mL of cells in media, allowing approximately 10 readings to be taken from each flask, as 1 mL is needed per reading with the extra 5 mL ensuring that the cells have enough media when the last reading is taken for all experiments performed in this thesis. These readings were taken over a period of a week, and for each week of experiments, data from at least one control group were obtained. This was done to ensure that each control group was obtained from the same actively growing stock flask as the other cells in separate culture flasks receiving hyperthermia, radiation or combined treatments. The control groups were continuously maintained at 37.0 ± 0.1 ºC and had measurements of cell-death taken at the same time as those cells in media undergoing hyperthermia, radiation or both. See Section 2.4 for the specifications of incubators used.

The reproducibility of the initial culture flask set-up, in which cells are dispensed into small culture flasks from the stock culture flask, was tested by dispensing 15 mL of cells in media from the same stock flask into 10 separate culture flasks and taking a measurement from each at the same time. The mean Coulter counter reading from this was found to be 370916 cells/mL with a standard deviation of 8% (±25673). This uncertainty can be ignored since the analysis of the data obtained throughout these experiments only concerns itself with the relative growth

response to the control groups, rather than the response to every first measurement taken. Hence, a comprehensive analysis of the control groups is required and has been completed in Section 3.1.

The reproducibility of the initial set-up as explained above was also found to vary by approximately 10% when compared on a weekly basis. In simpler terms, each week when cultures where dispensed from the stock flask it was found that a difference of 10% existed between the readings taken in separate weeks. The method of splitting creates this particular uncertainty in the measurements. A way to eliminate this uncertainty was to normalise all experiments in one week using the control group used in that particular week before comparing the results obtained in all weeks.

The reproducibility of a single cell count measurement from one culture flask was also tested by dispensing 15 mL into a single culture flask and taking 10 measurements from it at the exact same point in time. This variation resulted in a standard error of 10%. This uncertainty possibly arises from the cells still clinging to one another even after being vigorously shaken, thus disallowing an equal distribution of viable cells in media to be measured. This uncertainty could not be eliminated and was accounted for in the analysis.

## 2.3 TRANSPORTATION PROTOCOL AND MAF APPROVAL

The original MAF approval for the cell-line when it was first obtained from the American Tissue Culture Collection indicates that the cell-line is classed as a Category A Infectious Substance, affecting animals only. Because of this, the cell-line was to be kept within the biohazard laboratory in the Chemistry Department at the University of Canterbury at all times. For this project however, the cells needed to be transported from the University of Canterbury to Christchurch Hospital and back. This was necessary so that a linear accelerator, in the Department of Oncology, could be used to administer the radiation doses. The logistics of this project therefore required approval from MAF. This approval was obtained through a formal proposal, which contained a transportation protocol that was approved by both a health and safety officer at the University of Canterbury and MAF as included in Appendix B.

The transportation protocol required the cells in media to be housed in two leak-proof containers that were carried in a well-labelled chilly bin. Figure 2.2 shows

both the chilly bin and the containers. The flasks needed to be kept upright within the chilly bin at all times to prevent contamination of the flask-caps. Cotton wool was used to reduce movement of the flasks within the container and to serve as an absorbent medium if spillage were to occur. Seven culture flasks fit into the inner leak-proof container and, when housed by the outer container, allowed four such packages to be transported in the chilly bin (an insulated picnic cooler). One of these packages, however, contained a single separately packaged culture flask instead of seven as it was used as the control group. This control group therefore underwent the exact same travelling conditions as the groups receiving radiation.

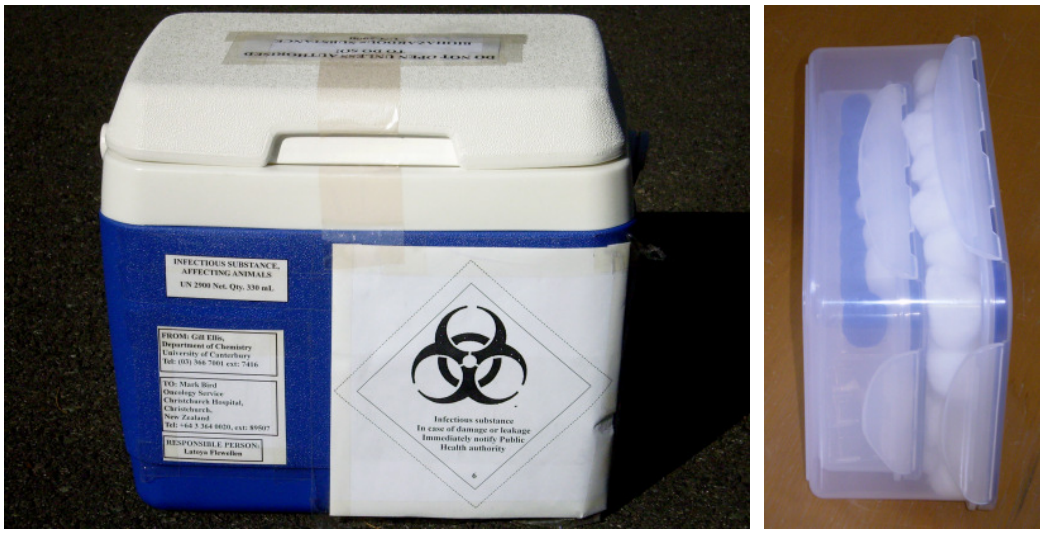



*Figure 2.2: The chilly bin required some basic labels including the biohazard symbol and contact details of people involved in the case of accident or severe spillage. The containers on the right show how seven culture flasks fit into the inner container housed by the outer container.*

## 2.4 SET-UP PROCEDURE FOR HYPERTHERMIA EXPERIMENTS

The following set-up was employed to determine the cell-kill from hyperthermia alone. The cells dispensed in their culture flasks after overnight incubation, as described in Section 2.1, were exposed to various regimes and different levels of hyperthermia in several incubators. All the incubators used (models: CAT 7050-7150 and CAT 1050-1400 manufactured by Contherm, Lower Hutt, New Zealand) regulate temperature to within 0.1ºC and allows for heat settings in the range of the ambient room temperature plus 5ºC up to 100ºC [53].

In the initial experiments conducted the cells were exposed to a temperature range of 37–43ºC, in intervals of 1ºC, with one batch exposed to 50ºC. The lower temperatures provide data on the mild hyperthermia range whereas the 50ºC investigates hyperthermia at a more extreme level. The exposure times used for each temperature were 30 minutes and 1 hour to mimic a practical clinical setting, along with 2- and 3-hour long exposures used to determine whether the effects of hyperthermia were more pronounced by increasing the exposure time. For logistical reasons, the heat treatments were carried out approximately 22 hours after the seeding of the cells. Measurements of cell survival were taken as indicated in table 2.2, resulting in 10 readings taken over the span of a week. Due to logistical reasons, measurements could not be obtained at night or on weekends. It should be noted that the exact time of each measurement was recorded and was corrected for in the results. After these results were analysed, it was also decided to expose the cells to hyperthermia regimes of 44, 45, 46, 47, 48 and 49 degrees Celsius due to various reasons as outlined in Chapter 3.

| **Measuring Cell-Survival** | | |
|---|---|---|
| **Day** | **Time of measurement** | **Notes** |
| Monday | Morning | Original number of cells seeded |
| Tuesday | Morning | Number of cells present after incubation time |
| Tuesday | Directly after heat treatment and late afternoon | Measuring cell-survival |
| Wednesday | Morning and late afternoon | Measuring cell-survival |
| Thursday | Morning and late afternoon | Measuring cell-survival |
| Friday | Morning | Afternoon measurement was not possible |
| Saturday & Sunday | None | Could not measure on these days |
| Monday | Morning | Last measurement of cell-survival |

*Table 2.2: Measurements of cell survival were taken on a regular basis.*

## 2.5 IRRADIATION SET-UP

The linear accelerator in Treatment room 3, Oncology, at Christchurch Hospital was used to provide the radiation doses to the cells contained as outlined in Section 2.3 above. A standard dosimetry check was initially performed to determine the output of the linac (Section 2.5.1). Solid water with a thickness of 10 cm was placed on the treatment table with the containers holding the flasks placed on top in an upright position. The solid water and the containers were centred on the treatment table by both the light field produced for a 25 cm x25 cm field size and by using the optical lasers. This ensured that the reproducibility of the set-up method for all containers to be irradiated was both consistent and accurate. The gantry of the linac was placed at 180º with the collimator rotated to 90º, resulting in a source to skin distance (SSD) of 100 cm. Irradiation thus took place from underneath the treatment table as can be seen in figure 2.3 below. The carbon fibre treatment couch has an absorption factor of less than 1%. This factor was ignored throughout the rest of the calibration procedures as it was found to be negligible [54].

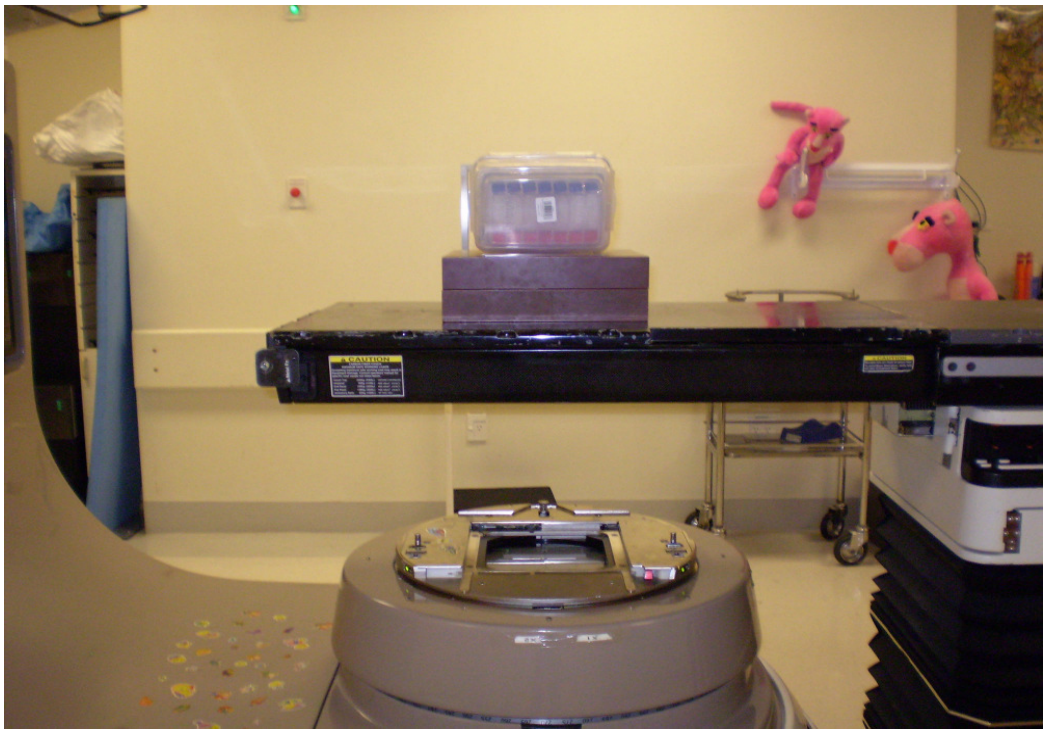


*Figure 2.3: Irradiation set-up showing the cells in their pinkish media. Note how seven flasks were irradiated at once.*

The above set-up was calibrated using thermoluminescent dosimeters (TLDs) that were attached to the bottom of each flask as outlined further in Section 2.5.2 below.

### 2.5.1 DETERMINING THE OUTPUT OF THE LINAC

The nominal photon energy as used by this linear accelerator was 6 MV. Before performing the calibration of TLDs, a standard dosimetry check was carried out. For this dosimetry check, the field size of the linac was set to 10 cm x10 cm along with a source to skin distance of 90 cm, a measurement depth of 10 cm and with 10 cm of solid water as backscatter material. The Baldwin-Farmer dosimeter, consisting out of a Farmer chamber and an electrometer, was used to determine the output of the linac. The Farmer chamber provides a stable and reliable standard for x-rays of all energies in the therapeutic range [31]. This chamber was connected to an electrometer that measures the ionisation charge. This reading was subsequently corrected for both the pressure and temperature of the room [7]. The output of the linac was determined by the following formula:

$$Linac\ output\ \ (cGy/MU) = \frac{R_{av} \cdot F_{cal}}{Number\ of\ \ Monitor\ Units\ \ (MUs)\ \ delivered} \qquad (1)$$

Where $R_{av}$ is the average of three readings taken from the electrometer and $F_{cal}$ is the chamber calibration factor. 200 monitor units were delivered to check the output of the linac. This output was determined to be 0.997 cGy/MU, which is very close to 1.0 cGy/MU as expected and has been carried through all subsequent dose calculations.

### 2.5.2 TLD CALIBRATION AND MEASUREMENTS OF DOSE

TLDs were chosen for the calibration of the irradiation set-up, as they were readily available at Christchurch Hospital and inexpensive. The phenomenon of thermoluminescence is observed when a crystalline material, such as lithium fluoride (LiF), releases both absorbed energy and energy trapped from irradiation as visible photons through heating the material [6]. The heating of the material, also known as the

heating cycle, occurs in a planchet and the visible light emitted is measured by a photomultiplier tube (PMT). The PMT converts the light into an electrical current that is subsequently amplified and then measured by an electrometer. The heating of the material occurs over a range of temperatures resulting in the formation of a glow curve that can be correlated to the dose given through proper calibration methods. The most common crystalline material used for clinical dosimetry is LiF that requires a trace amount of impurities to induce the thermoluminescent phenomenon. In this study, lithium fluoride with both magnesium and titanium impurities (LiF:Mg,Ti) were used. These TLDs, also known as the Harshaw TLD-100, have a size of 3.2 mm x 3.2 mm x 0.9 mm and display a main peak in the glow curve in the temperature range of 180º to 260 ºC.

The average calibration factor for the TLDs was determined by placing three calibration TLDs in a standardised set-up, with a field size of 10 cm x10 cm, and exposing them to 200 MUs. The calibration TLD readings, multiplied by each individual TLD's sensitivity factor, were used to determine the calibration factors as given by the following formula:

$$Calibration\ \ Factor = \frac{Linac\ \ output \cdot MUs\ \ delivered}{TLD\ \ reading \cdot individual\ \ sensitivity\ \ correction\ \ factor} \qquad (2)$$

From this, the average calibration factor was determined to be 0.015 cGy. TLDs were then placed underneath each of the seven culture flasks, as it was not possible to place them accurately inside due to the bottleneck shape of the flasks. Underneath was thus the closest place to where the cells would actually be. All of the culture flasks were filled with 15 mL of water to mimic the cells in media. These flasks were put into the leak-proof containers with cotton wool and set-up on the treatment table as seen in figure 2.3 above. The whole set-up was exposed to 200 MUs. Each TLD reading was corrected by the average calibration factor to obtain the actual dose to which each had been exposed. This procedure was repeated twice to observe the variability in dose with reproduction of the set-up. The depth dose variation was also determined, with 15 mL of cells in media mimicking a distance of 2 cm in water.

The average dose given to the first batch of TLDs was found to be 163 cGy with a standard deviation of 1.9 cGy (1.2%). The second batch received an average

dose of 150 cGy with a standard deviation of 3 cGy (2%). It is known that the intrinsic uncertainty in LiF TLDs lies in the range of 3-5% and this variation is therefore within the expected range [55]. Other factors that contribute to this difference of dose measurements in both batches are the uncertainties found in the spatial variation of the dose given to the TLDs and the reproducibility of the set-up. To determine the overall uncertainty, the TLDs of both the first and second batches in the same position were compared and averaged. Over both batches of TLDs in the same positions, it was found that the average dose given was 157 cGy with a standard deviation of 7.8 cGy (5%). The depth dose variation for the 2 cm of cells in media was found to be less than 0.3% and deemed negligible. Hence, the total uncertainty in the dose as determined by TLDs was found to be 9%, of which 4% was taken as the intrinsic uncertainty of the TLDs and the other 5% results from both the spatial variation of the radiation and the reproducibility of the set-up used.

The overall factor used to determine the number of MUs to be given to cells in media in subsequent experiments was 157 cGy/200 MUs, or in other words, 127 MUs for every one Gray administered. This factor can be regarded as rather low, but since backscatter material was not used to increase the overall dose administered, it can be considered sensible. Backscatter material in the form of rice bags was originally considered for this project but was not used since they complicated both the reproducibility and the stability of the set-up. It was later realised that perhaps a tank of water surrounding the set-up would have been ideal as backscatter material. The obvious problems with a tank of water of course are the possibilities of leaks in the plastic containers and the floating of these containers. Such problems could have been overcome if more time was available for the completion of the project. At the stage of realisation, however, over half of the experiments had already been completed and consistency was considered as more important.

## 2.6 COMBINING RADIATION WITH HYPERTHERMIA

The radiation set-up, as outlined in Section 2.5, allowed seven flasks to be irradiated at the same time with the same number of MUs resulting in the same dose with an uncertainty of 9% as determined in Section 2.5.2. From a logistical point of view, it made sense to use one of these flasks to observe the effects from radiation

alone and subsequently expose the other six to various regimes of hyperthermia, thus resulting in combined regimes. The levels of radiation that the cells received were 1, 3, 5, 9, 11 and 15 Gy. The various regimes of hyperthermia were administered to the six flasks, back at the University of Canterbury, after the radiation had been applied using the same methods as described in Section 2.4.

The temperatures used for the hyperthermia regimes were chosen to be 41 and 42 degrees after a literature review revealed that whole-body hyperthermia in humans can only be safely administered up to a maximum temperature of 41.8ºC as described in Chapter 1. These temperatures were applied for 30 minutes, 1 hour and 3 hours. The 30 minute and 1 hour exposure time was used to mimic a treatment time that would be practical in a hospital setting. The exposure time of 3 hours was merely chosen to observe how the increase of exposure time changes the results of the combined treatment. It should be noted that the time between the radiation treatment and subsequent hyperthermia treatment was approximately one hour on average. According to the literature review as discussed earlier, this time should be as short as possible to observe the greatest synergistic effect between hyperthermia and radiation. Both the transportation logistics and MAF requirements in this project prevented any reduction in this time between treatments and resulted in an inconsistent travel time between the hospital and the university that ranged between 20-30 minutes.

Measurements of the control group, which underwent the same travelling conditions, and the cells exposed to both radiation alone and the combined regimes were made in the same way as for hyperthermia alone. The radiation treatment, however, could only be applied on Wednesday mornings and therefore delayed the experiments by a day. Table 2.3 indicates when measurements of cell-survival were taken of the cells that had undergone either radiation alone or a combined treatment of radiation and hyperthermia. Again, the exact time of each measurement was recorded and was corrected for in the results. It should be noted, however, that the ‘directly after’ measurement for radiation exposures and those combined with hyperthermia were taken approximately one to four hours after the exposures were completed, due to logistical reasons.

| Measuring Cell-Survival | | |
|---|---|---|
| **Day** | **Time of measurement** | **Notes** |
| Tuesday | Morning | Original number of cells seeded |
| Wednesday | Morning, directly after radiation and/or heat treatment and late afternoon | The morning measurement gives the number of cells present after the incubation time |
| Thursday | Morning and late afternoon | Measuring cell-survival |
| Friday | Morning and late afternoon | Measuring cell-survival |
| Saturday & Sunday | None | Could not measure on these days |
| Monday | Morning | Last measurement of cell-survival |

*Table 2.3: Measurements of cell survival were taken on a regular and reproducible basis for all experiments conducted.*

## 2.7 STATISTICAL METHODS

The two statistical methods used throughout this thesis were the unpaired student t-test and the analysis of variance (ANOVA). Both statistical tests were performed using Statview 4.0 for the Macintosh computer by Abacus Concepts, Berkeley, USA. It is important to understand how these tests work so that the results from them can be interpreted correctly. Section 2.7.1 deals with the unpaired student t-test and Section 2.7.2 with ANOVA.

### 2.7.1 UNPAIRED T-TEST

The t-distribution is a probability distribution used when the mean of a normally distributed population is estimated for a small sample size [56]. This distribution is the basis for performing an unpaired t-test in which the statistical significance between two sample means is found [56,57]. In simple terms, an unpaired t-test is used when two groups undergoing different conditions are compared. One example of this, as applied in this project, is the comparison between cells that have

undergone hyperthermia at 40ºC and those that underwent hyperthermia at 41ºC. Essentially two different populations exist that are being compared in terms of their means. The null-hypothesis tested is that the two population means are the same, and hence that there is no difference between the two groups. This test produces a probability value, known as a p-value that indicates whether the two population means are significantly different. Throughout this thesis, significance will be assumed to be for p-values less than 0.05, meaning that there is less than a 5% chance that the two population means are the same.

### 2.7.2 ANALYSIS OF VARIANCE

The analysis of variance tests for the heterogeneity of the mean differences between several populations, based on samples taken from each population [58,59]. It is very similar to the unpaired t-test described above where two population means are compared. In the analysis of variance, however, multiple population means can be compared with the null-hypothesis being that all population means are the same. Again, p-values are produced in this test to indicate how different each group is from one another. In the hyperthermia experiments, for example, one of the hypotheses to be tested is whether there is indeed a statistically significant effect from applying heat to the cells in comparison with the control groups. This requires an analysis of variance since the means of the control groups will be compared to the means of groups that underwent different parameters, such as groups exposed to 38 degrees and those exposed to 50 degrees.

# Chapter 3

# Results & Analysis

## 3.1 Initial Basic Analysis and Control Groups

Over the span of several weeks of data collection, eleven 37ºC control groups were obtained of which four underwent the travelling conditions to and from Christchurch Hospital. These four control groups were used to find the cell death relative to 37ºC of cells undergoing radiation or combinations of radiation and hyperthermia. The other seven control groups are indicators of how the cells normally proliferate in a stable environment and were used to find the cell death relative to 37ºC of cells undergoing hyperthermia only.

For every dataset obtained throughout this project a second order polynomial was fitted using the Matlab 7 software package. Normally, cell growth is considered to be exponential and hence, an exponential fit is often more appropriate. An exponential fit however is only warranted if the cells are allowed to grow with a sufficient amount of media to fuel such an exponential growth. In our case, such growth was restricted by the amount of media present and hence, the cells eventually ran out of nutrition and died. The logistic function, in the form of an S-shape, models this situation accurately as seen in figure 3.1. The initial exponential growth is observed followed by a slowing down as the cells run out of media. In our experiments, it was found that the initial number of cells seeded, on average being $4x10^5$ cells/ml, was so large that the initial exponential growth on the logistic curve is not seen. The second part of the graph after the exponential growth can be modelled either by a log function or by a second order polynomial.

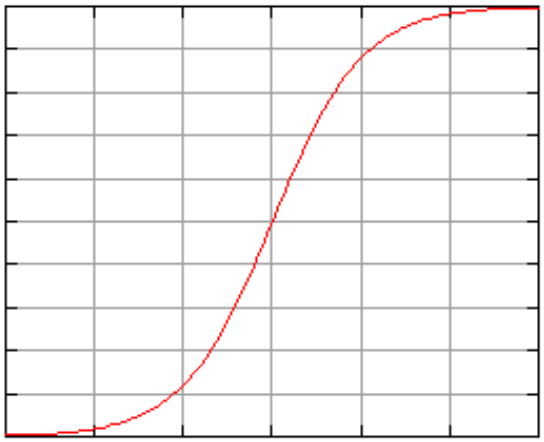


*Figure 3.1: The logistic function.*

Due to restrictions on our data acquisition of only being able to record two data points per day, as explained in Chapter 2, it was found that a second order polynomial fits the data better than a log function. Recall that all the data obtained contains an uncertainty of 10% due to the method of splitting as discussed and determined in section 2.2.2. An example of how well a second order polynomial fit one control group of the 37-degree data is shown in figure 3.2, with the 10% uncertainty shown by the uncertainty bars.

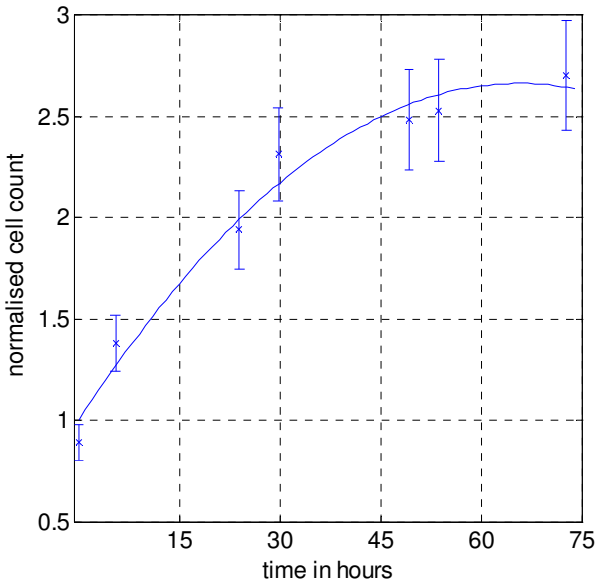



*Figure 3.2: The second order polynomial fit to one of the 37-degree control groups models all the data accurately and lies within the uncertainty.*

The second order polynomial fit, applied using Matlab's polyfit command, was used throughout this thesis as a means of presenting the data. These plots were generated for all datasets and it was found that a second order polynomial models the data accurately.

### 3.1.1 ANALYSING THE CONTROL GROUPS

As indicated earlier, there are two different sets of control group data to consider; namely, the control groups that travelled to and from Christchurch Hospital and those that remained in their stable 37-degree environments at the University of Canterbury. The seven control groups that remained, consisted of data taken over five different weeks. In this group of seven, three lots of control group data were acquired in a single week whilst the other four groups were all acquired in individual weeks. The data of those control groups that 'travelled' however were all taken in individual weeks. Figure 3.3 shows the compilation of both sets of control group data side by side with their averages calculated.

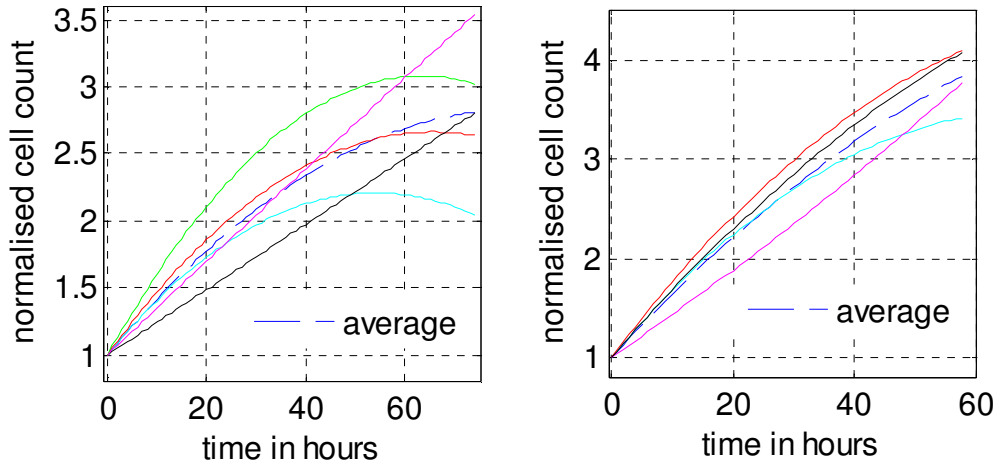



*Figure 3.3: The normalised cell counts of the normal 37-degree control groups (left) are more spread out than those of the groups that travelled (right).*

It should be noted that it seemed reasonable to average the three sets of data acquired in a single week of the normal control groups, as they were close together, and hence only five curves, instead of seven are drawn. The uncertainty bars and data points on these plots have been removed for reasons of clarity.

The real question to be answered, however, is how do the two different types of control groups compare? Is there a statistical significant difference between them

or does the travel to and from the hospital have little or no effect? Figure 3.4 was generated to get some insight into the answers to these questions.

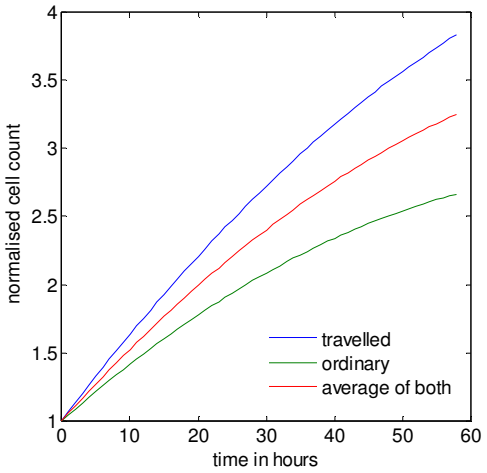



*Figure 3.4: Comparison of the travelling and ordinary control groups.*

It should be noted that figure 3.4 only shows the cell growth up to 58 hours since the control groups that were exposed to travelling conditions only had measurements taken over a period of three instead of four days when compared with the ordinary groups. This is due to the logistical restrictions that were applied when obtaining data for cells that underwent radiation exposures.

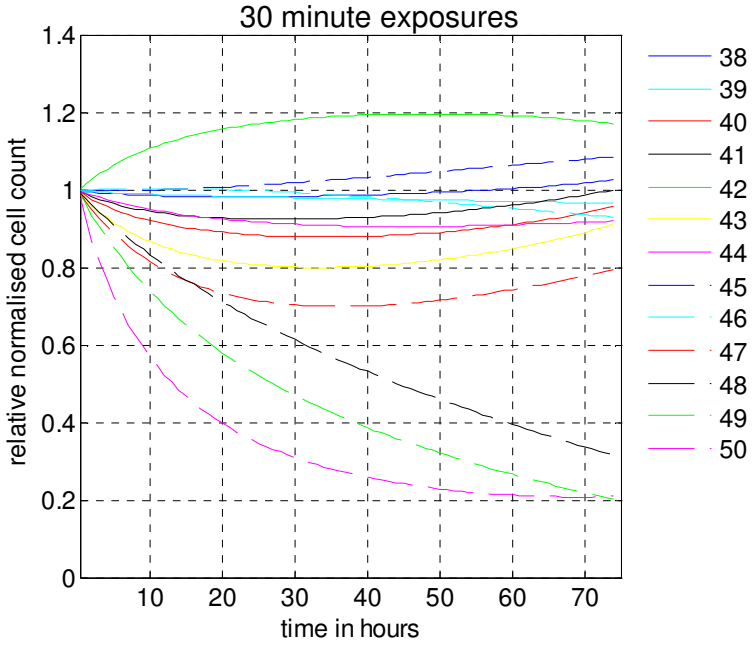
Figure 3.4 seems to indicate an obvious difference between the travelled and the ordinary control groups. A statistical unpaired t-test, however, was performed to test whether this difference was statistically significant. Recall that the null hypothesis used in an unpaired t-test assumes that the means of the two datasets are the same. A p-value of 0.07, however close to 0.05, was found confirming the null hypothesis and thus there is no statistical significant difference between the two control groups. It should be noted the cells were only out of their incubators for approximately 2 to 3 hours. Hence, it is not surprising to find that the travelling conditions made no statistically significant difference to the growth rate of the cells. Also, the visual difference that is seen might be an indication that by the vibration of the car, gases are vibrationally dispensed into the medium resulting in a slightly better growth rate.

Before analysing the data obtained from the cells exposed to hyperthermia, radiation or a combination of both, one must consider whether it is reasonable to average all the control group data. One could argue as to whether this average can be used to generate the relative response of the cells to radiation, hyperthermia or a

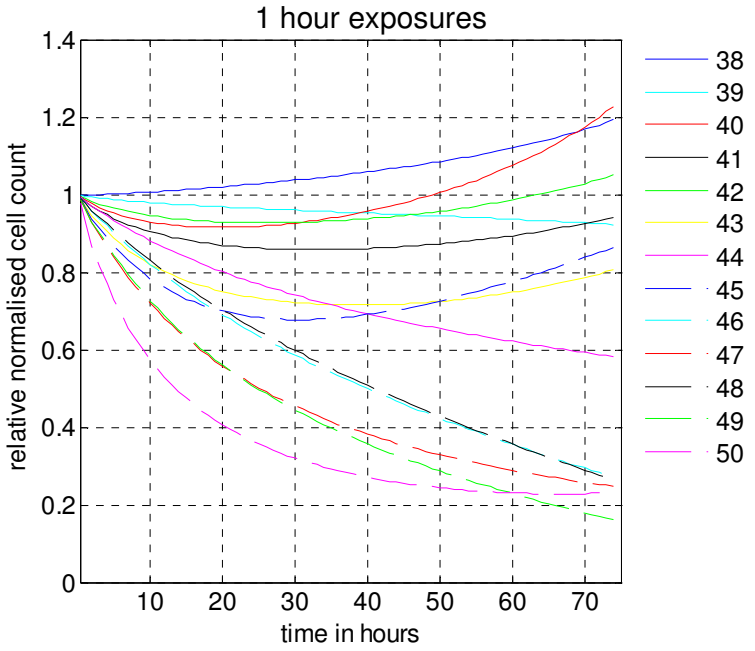
combination of both, or if the week-to-week individual datasets are to be used for this. The argument in favour of averaging is, that because there is no statistical significant difference between the groups and the variation in each individual dataset is small (10% standard deviation) it is reasonable to average all the individual control groups together. By averaging, the overall noise found within the control groups is also reduced. The argument against, however, debates that the individual control groups are representative of all the data taken in one week and model the natural growth rate occurring in each week more accurately. It can also be said that uncontrollable outside influence parameters change on a weekly basis, such as air humidity, and we do not know what this effect has on those cells that underwent hyperthermia and/or radiation exposures. In addition, when looking at figure 3.4, the difference between the two groups is reasonably large, even though not statistically significant. Hence, it is argued that all the data should be corrected for on a week-to-week basis rather than on an 'averaged' basis. Since both arguments are very reasonable it was decided to compare all the data obtained throughout this thesis, visually through a graphical analysis, in terms of both arguments. This comparison showed that the trends were the same using either method. Hence, after careful consideration the convention of correcting on a weekly basis was adopted, so that uncontrollable parameters can be accounted for.

## 3.2 HYPERTHERMIA EXPERIMENTS

The initial hyperthermia experiments consisted of the temperature range of 38-43 and 50 degrees, for exposure times of 30 minutes, 1, 2 and 3 hours. Soon after these datasets were acquired, it was also decided to perform the same experiments in the temperature range of 44-49 degrees. This was done to obtain a more complete set of data but mainly because the lower temperatures showed little to no cell kill, as can be seen in the analysis of them. Figure 3.5 contains four plots of the normalised cell count relative to 37ºC for all levels of hyperthermia per exposure time.

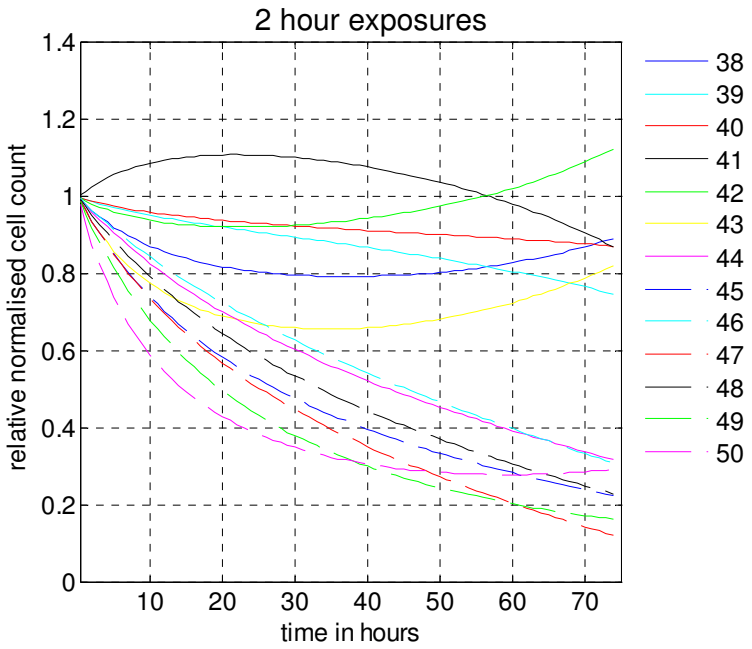



***a)***

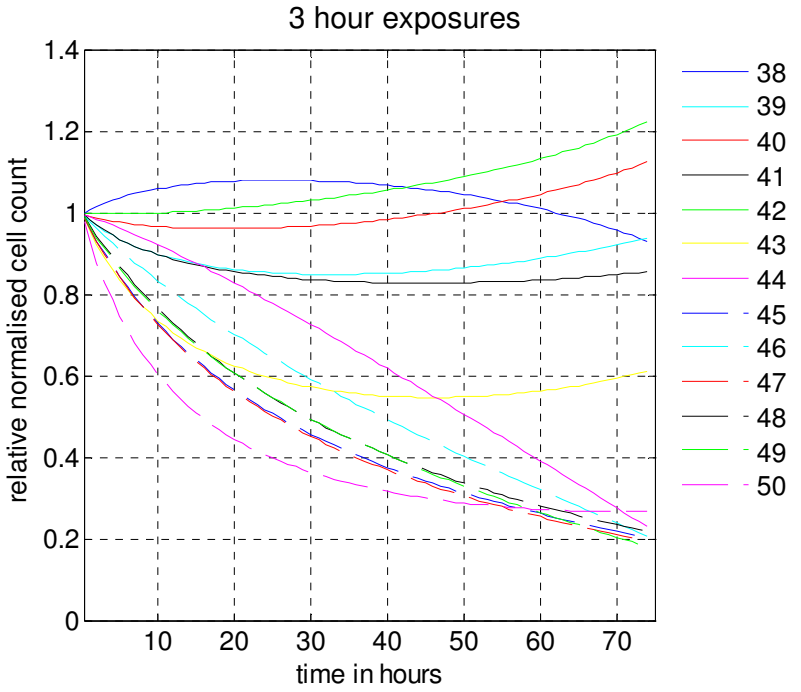



***b)***

*Figure 3.5: The above plots show the effects of various levels of hyperthermia for* ***a)*** *30 minutes and* ***b)*** *1 hour.*




***c)***




***d)***

*Figure 3.5 continued: The above plots show the effects of various levels of hyperthermia for* ***c)*** *2 hour and* ***d)*** *3 hour exposure times.*

On these plots 37ºC, even though not plotted, can be visualized as a horizontal line through the y-value of one. For reasons of clarity, the individual data points and

uncertainty bars are not shown. It is obvious from the graphs that lower temperatures in the range of 38-43 degrees have little or no effect, which is why data for the range of 44-49 degrees was also acquired. Only 43 degrees appears to be breaking away after a 3-hour exposure from the low temperatures that cluster around the y-value of 1, or in other words cluster around the 37 degree control group. The higher temperatures, ranging from 44-50 degrees, seem to show the same effect for all exposure times with 44 degrees joining this group more clearly after one hour. The extreme level of hyperthermia applied at 50 degrees shows virtually no change in the level of cell kill over the various exposure times. Considering 50 degrees, it was found that the lowest cell fraction after about 72 hours, was 0.2, meaning that approximately 80% of the cells were killed.

### 3.2.1 ARRHENIUS PLOT

An Arrhenius plot can be used to ensure that no systematic error is present within the hyperthermia datasets. In an Arrhenius plot, one graphs the inverse of temperature on the abscissa against the natural log of the reaction rate, with a straight line confirming that there is indeed no systematic error. In our case, this reaction rate is simply the cell count at a single point in time. The point in time chosen for this plot was 72 hours as this is where all curves, in figure 3.5 **a**–**d**, are most divergent

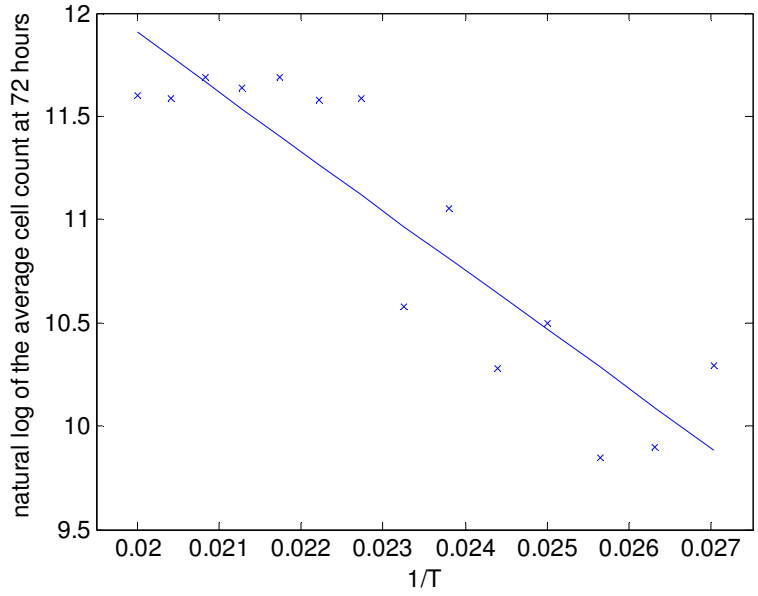



*Figure 3.6: The Arrhenius plot for the average of all data points of each temperature taken at 72 hours after the exposure to hyperthermia.*

All data for each temperature, regardless of exposure time, at 72 hours was averaged so that one single data point exists for each temperature level, as seen on figure 3.6. The Arrhenius plot does indeed follow a straight line and thus indicates that there was no systematic error in our data. Please note, that the values for 37 – 43 degrees all cluster around the same level confirming that there is indeed no difference between them and that hyperthermia has little or no effect at these levels.

### 3.2.2 STATISTICAL ANALYSIS OF HYPERTHERMIA DATA

An analysis of variance was performed, in which the means of all datasets tested are assumed to be the same, to determine whether there is a statistically significant difference in the data due to the exposure time. Only in the case of 30-minute exposures was a statistically significant difference found, p-value less than 0.05. The 30-minute data however, was found to be exceedingly noisy and this difference is hence attributed to the level of noise present. Hence, the exposure times that we tested, have no effect on the level of cell kill from hyperthermia. An analysis of variance also revealed that the level of cell kill was indeed dependent on temperature, as the means between the control groups and the data exposed to different temperatures were statistically significantly different with a p-value less than 0.01.

An unpaired t-test was also conducted which compared every dataset with respect to the temperature they had been exposed to. From this, it was confirmed that there is no statistically significant difference between 38-42 degrees and 37 degrees. It was found that 43 degrees is statistically significantly different from 37 degrees with a p-value less than 0.05 as suspected from figure 3.5. All other temperatures were statistically significantly different with p-values less than 0.0001. Appendix C includes all the p-values found from the unpaired t-test.

## 3.3 RADIATION EXPERIMENTS

The cell killing effect of 1, 3, 5, 9, 11 and 15 Gy were observed. Initially only a single dataset for each of these radiation levels was obtained. After the initial set of combined experiments, it was decided to acquire more data of the 5 and 11 Gy radiation levels; reasons for this are given in section 3.4. Three extra datasets of both 5 and 11 Gy were obtained resulting in four datasets for each of these two levels, with the other levels consisting of only a single dataset each. Figure 3.7, shows how almost all four individual datasets of both the 5 and 11 Gy groups follow similar trends and hence their averages were found. Only the 5 Gy dataset that was acquired in a different week seems to slightly deviate from the main trend. The cell kill found from radiation alone normalised with respect to the control group is shown in figure 3.7 where 5 and 11 Gy are the averages as found in figure 3.6. Note that the data points and uncertainty bars were omitted on both figures 3.7 and 3.8 for reasons of clarity.

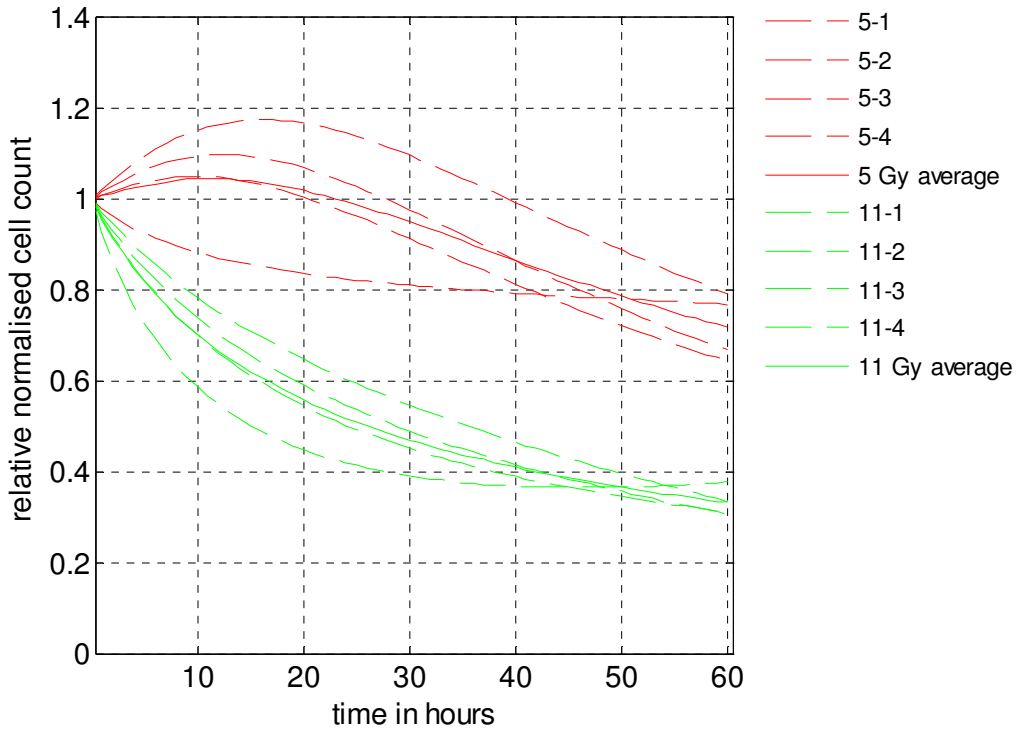



*Figure 3.7: Finding the averages of the 5 and 11 Gy datasets.*

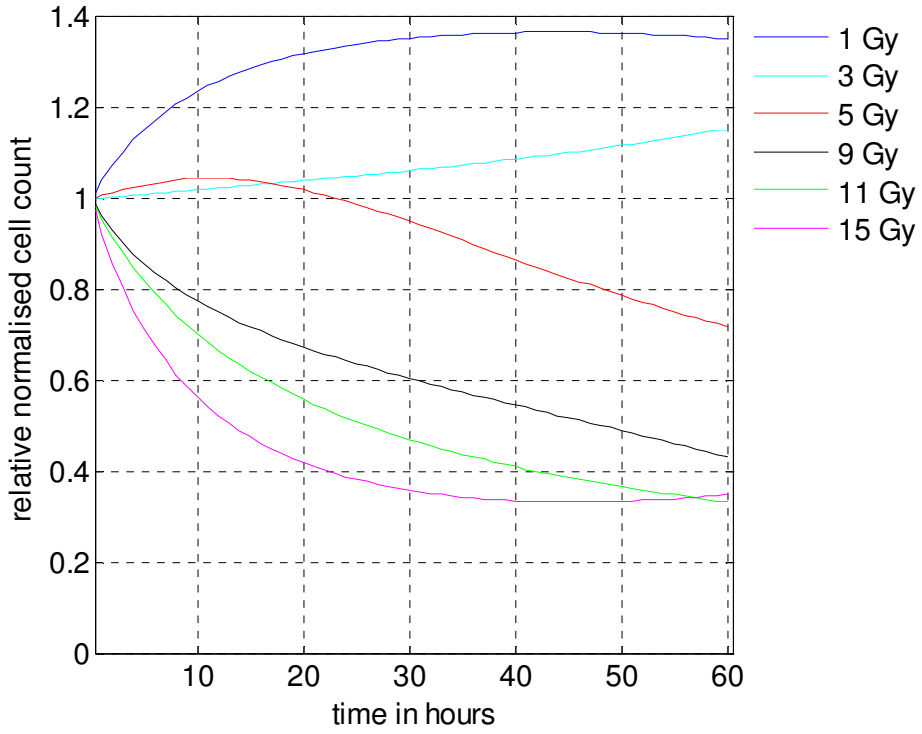



*Figure 3.8: Cell kill from various levels of radiation.*

These graphs indicate that the cell kill from radiation follows the familiar and well-known pattern that higher levels of radiation constitute increases in the level of cell kill. The level of cell kill from all these radiation levels, however, is a lot lower, even though consistent, than what other cell experiments have shown [20]. It is believed that this is due to the limitations of our set-up and due to the Coulter counter, which underestimates the level of cell kill. It is interesting, however, to observe that the plots indicate an increase in the growth of the cells, relative to the control group, for both 1 and 3 Gy. This is a possible indication that the hormesis effect, in which cell growth is encouraged by small amounts of radiation [60,61], might apply for this particular cell line. It should be noted that these experiments were not designed to test this effect and that since only one dataset for both 1 and 3 Gy is available conclusions in regards to this cannot be drawn.

### 3.3.1 STATISTICAL ANALYSIS OF RADIATION DATA

An analysis of variance found that there is a statistical significant difference between the radiation levels and the control groups, with a p-value less than 0.01, as expected. An unpaired t-test, which compared the cell kill effects of each radiation level to one another, found that there was no statistically significant difference between 0 Gy and 1, 3 and 9 Gy of radiation (p-values all above 0.05). This is not surprising as for all three radiation levels only a single dataset was obtained. All other levels were found to be statistically significantly different with p-values less than 0.04. All p-values from the unpaired t-test can be found in Appendix C. These statistics cannot verify if a hormesis effect is seen for 1 and 3 Gy, as much more data is required to confirm such a hypothesis.

## 3.4 INITIAL COMBINED EXPERIMENTS

It was initially decided to combine the levels of radiation as investigated in section 3.3 with hyperthermia at 41 and 42 degrees for exposure times of 30 minutes, 1 hour and 3 hours. When these experiments were carried out, all the data for the hyperthermia experiments had not yet been obtained. Namely, the range of 44-49 degrees had not yet been tested and hence, even though in retrospect it is known that different exposure times have no effect, as determined in Section 3.2.2, they were still investigated here. It was thought that even though hyperthermia at the low levels of 41 and 42 degrees does not show any significant cell killing effect as shown in section 3.2, that a combined regime could still show an overall supra-additive effect. It was hoped that the radiation exposure would allow the hyperthermia to inhibit critical cell repair mechanisms resulting in greater cell death even though hyperthermia by itself at these levels does not kill the cell. Graphs were generated for each set of parameters in which the normalised relative cell count, or relative response to the control groups, is shown.

Figure 3.9 considers the effect of combining various levels of radiation for the different exposure times for both 41 and 42 degrees, thus resulting in six separate plots.

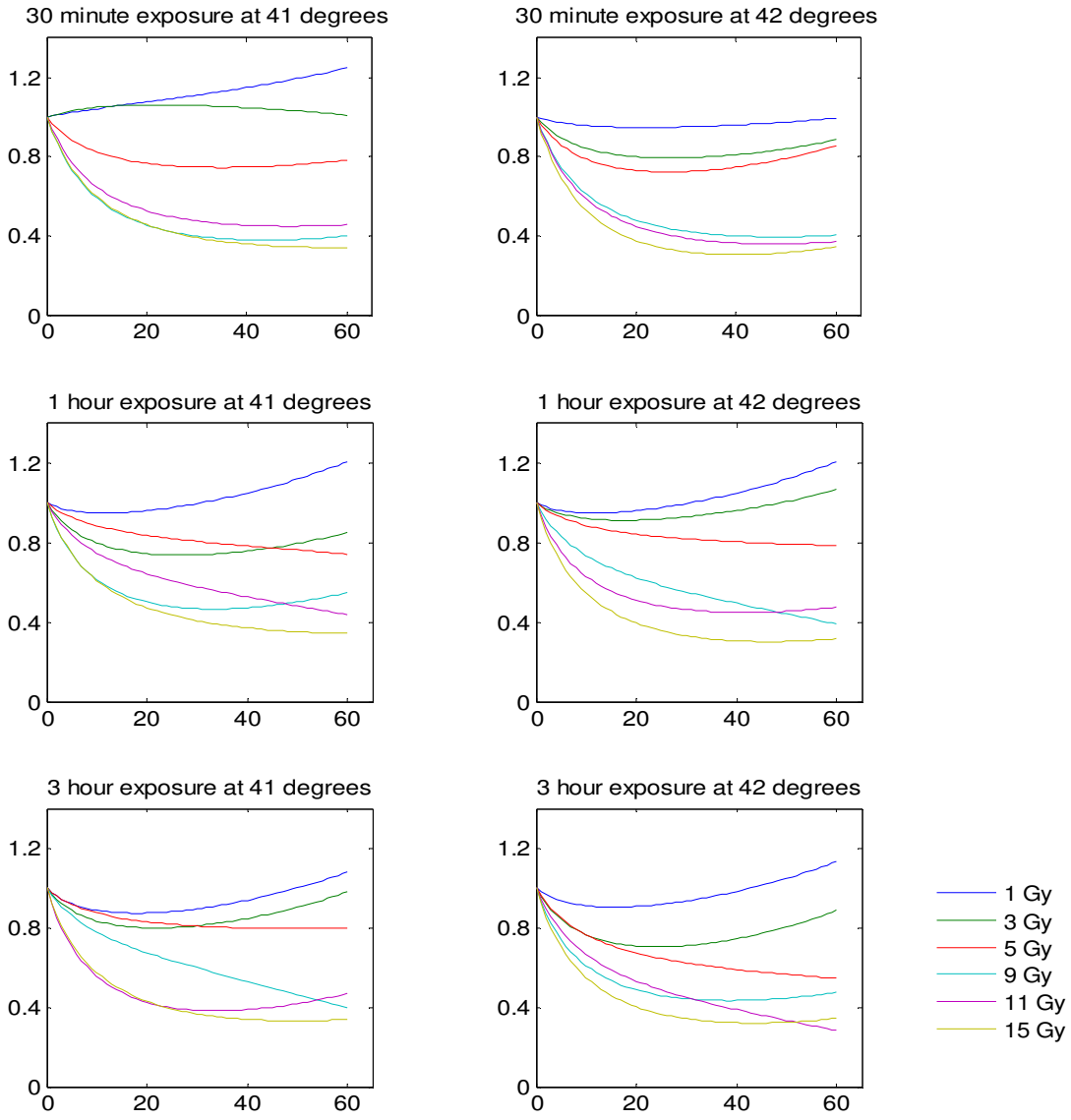



*Figure 3.9: Combinations of various levels of radiation with hyperthermia at both 41 and 42 degrees for exposure times of 30 minutes, 1 and 3 hours.*

At first glance, no real obvious differences exist between these graphs and it is difficult to analyse if there is a beneficial effect from combining these levels of radiation with the hyperthermia regimes. For these reasons, the level of cell kill from the hyperthermia alone and the radiation alone were plotted on the same plots, resulting in 13 curves on the same graph. As it is difficult to present this data clearly, these graphs were split up to show only seven curves on each plot. They have also been divided into three groups with respect to the length of hyperthermia exposures

applied, denoted as figures 3.10, 3.11 and 3.12. Please note that the legends correspond to each row of plots they are beside, 'H' stands for hyperthermia.

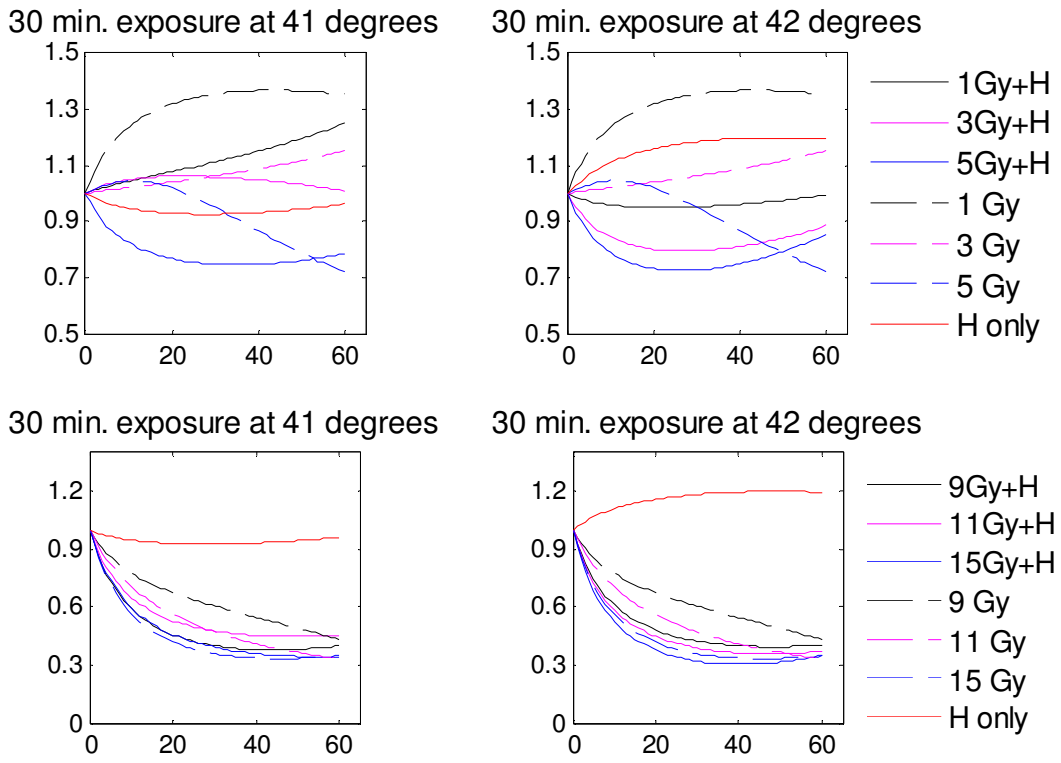



*Figure 3.10: 30-minute exposures to hyperthermia at 41 and 42 degrees combined with various levels of radiation. Each legend corresponds to each row of plots.*

The above plots indicate that there is an overall effect from combining levels of radiation with 30-minute exposures to hyperthermia at both 41 and 42 degrees. In almost all cases, there is some form of additive effect as the solid lines all fall well below the dashed ones. More interestingly, this effect does not seem to be observed as strongly when hyperthermia is applied for 1 hour as can be seen in figure 3.11. The 1-hour exposures seems to indicate that there might be some small enhancement of cell kill from combining radiation with hyperthermia levels as for the lower radiation levels. However, the higher radiation levels (9, 11 and 15 Gy) do not display a clear distinction between radiation alone and the combined regimes. This may indicate that some form of saturation effect exists, in which the radiation at these higher levels dominates the level of cell kill that can be achieved. The level of hyperthermia applied might not be strong enough to overcome such an effect causing saturation. It

should be noted that this is of course is only speculation as testing this hypothesis was not in the scope of this project.

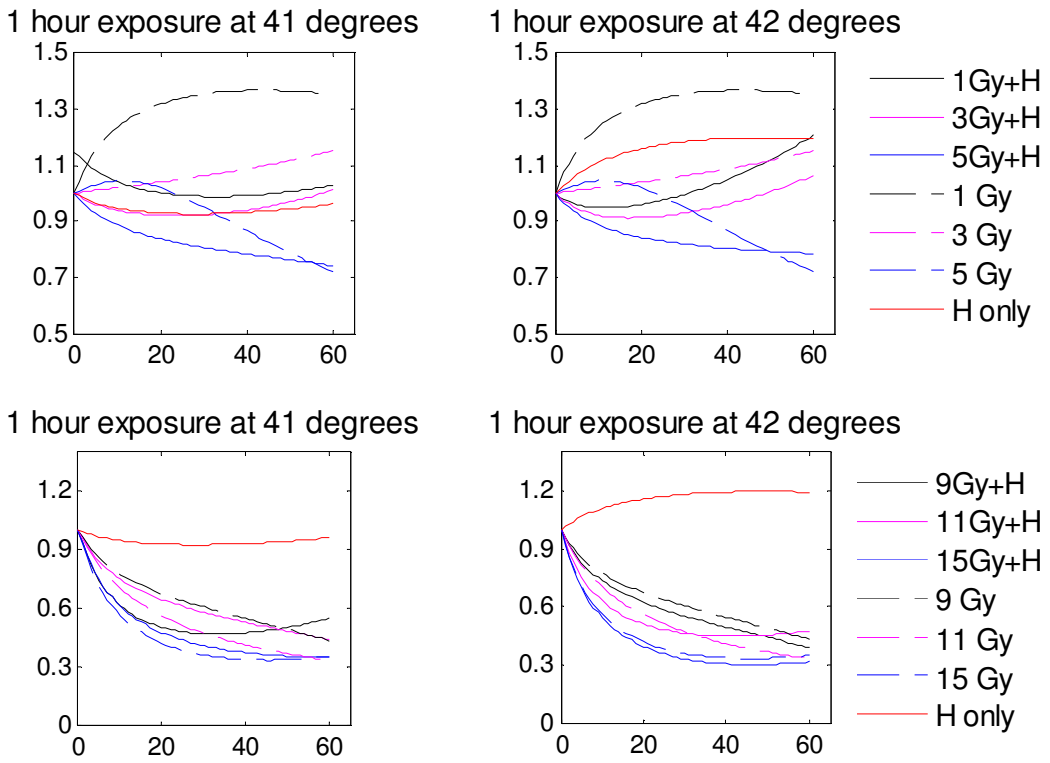



*Figure 3.11: 1-hour exposures to hyperthermia at 41 and 42 degrees combined with various levels of hyperthermia. Each legend corresponds to each row of plots.*

Similarly, in figure 3.12, the 3 hour exposures to both hyperthermia and radiation levels seems to indicate that indeed the lower levels of radiation benefit from a combined regime whereas the higher levels do not. Solely from this set of data, we cannot determine any of these factors with a high degree of certainty since the number of variables exceed the number of datasets significantly. Hence, it was not possible to run a statistical analysis of this data to either confirm or deny any possible trends that might appear to present themselves on the graphs. Section 3.5 presents multiple datasets for the same parameters thus enabling statistics to answer important questions such as if there is an overall benefit from combining radiation with levels of hyperthermia.

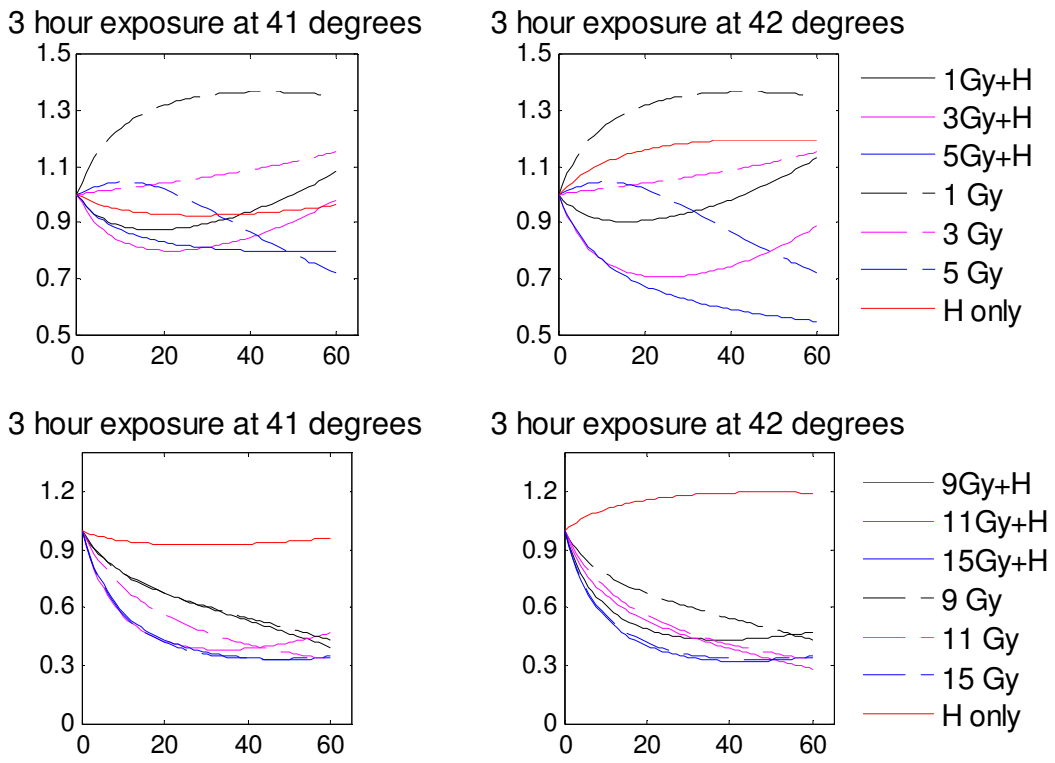



*Figure 3.12: 3-hour exposures to hyperthermia at 41 and 42 degrees combined with various levels of radiation. Each legend corresponds to each row of plots.*

## 3.5 COMBINED EXPERIMENTS – MULTIPLE DATASETS

Since the results from the first initial combined experiments can be regarded as inconclusive as to whether combining hyperthermia with radiation is beneficial, it was decided to acquire several other datasets. Due to logistical reasons imposing time constraints on the acquisition of these new sets, selective decisions were made as to which combinations of hyperthermia and radiation should be tested. Since radiation kills cells in a predictable manner, only two levels of radiation were selected alongside with three new levels of hyperthermia. Out of the radiation levels used previously, 5 and 11 Gy were chosen for these final experiments. This was done in order to observe the effects of a lower level of radiation that still showed cell kill, as seen in figure 3.7, along with a high level of radiation that seemed relatively unaffected by the addition of heat as observed in the initial combined experiments. The temperatures chosen for the addition of the hyperthermia regimes to radiation were 43, 45 and 47 degrees, for durations of 2 hours. This particular exposure time

was chosen since the previous data from 30 minutes was very noisy along with the data from 1 hour, whereas there is no difference in the effects from 2 and 3 hours as indicated in the statistics done for hyperthermia alone.

### 3.5.1 COMBINING 5 GY WITH HYPERTHERMIA REGIMES

In this section, only the data from the combined experiments containing 5 Gy of radiation will be considered. For each individual temperature, 43, 45 and 47 degrees, combined with 5 Gy of radiation, 6 datasets were acquired. All datasets were averaged in order to reduce the overall noise in each individual dataset (figure 3.13).

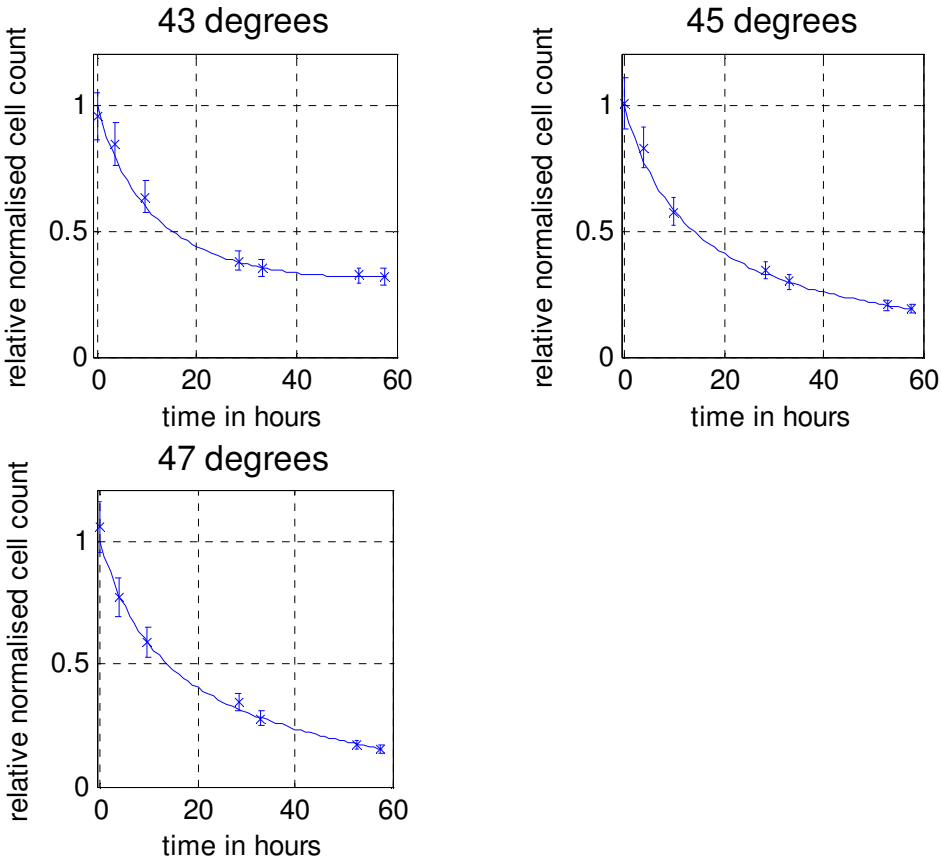



*Figure 3.13: Cell response to 5 Gy of radiation combined with 2 hour exposures to three levels of hyperthermia. All the data presented are the averages of six datasets.*

Figures 3.14 to 3.16 show the cell killing effects from hyperthermia alone, radiation alone and the combined regimes. Furthermore, one can add a curve that represents the theoretical levels of cell kill expected from the combined regimes,

based on the direct multiplication of the level of cell kill from hyperthermia alone with that from radiation alone. The data points and uncertainty bars for the experimental combined level of cell kill are included. This thus allows direct conclusions to be drawn as to whether there is a supra-additive effect from combining radiation with hyperthermia, simply an additive effect or whether one regime reduces the effect of the other.

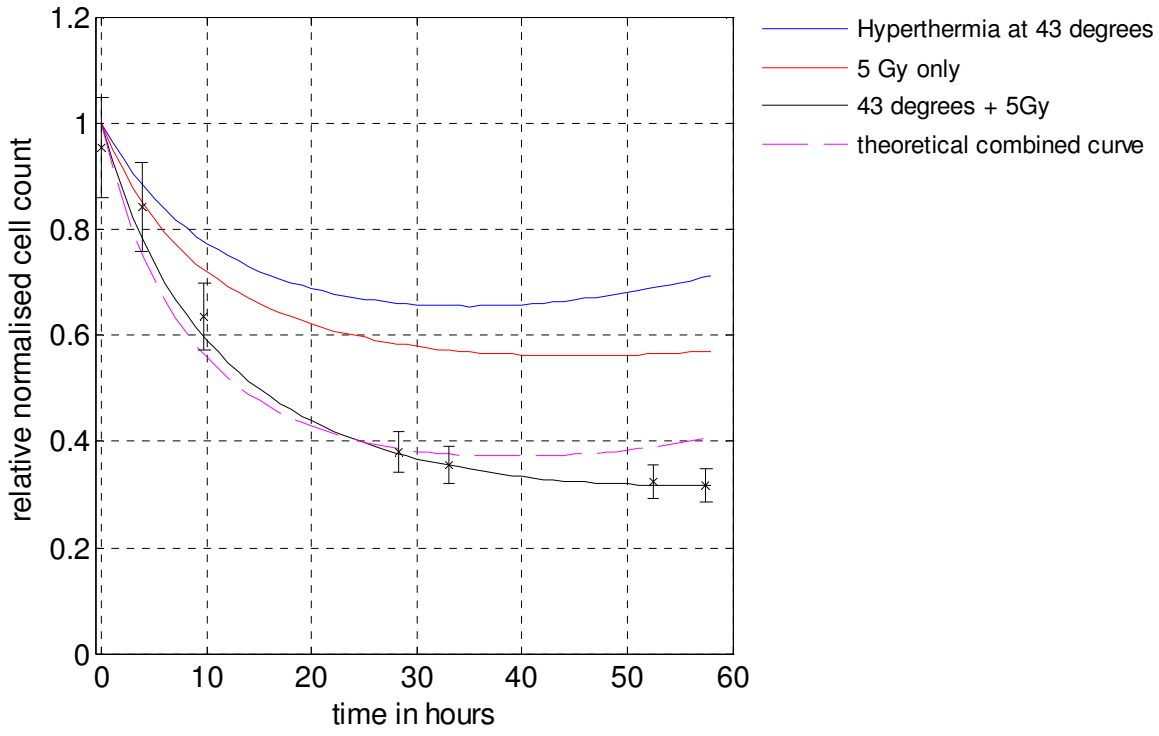



*Figure 3.14: Combining 43 degrees for 2 hours with 5 Gy of radiation. Comparison of the experimental and theoretical curves.*

Figures 3.14 to 3.16 all indicate that 43, 45 and 47 degrees, when applied for 2 hours, dominate over 5 Gy alone. The experimental combination of 43 degrees with 5 Gy of radiation agrees, within uncertainty, with the theoretical combined curve for almost all data points. This would be expected for a directly additive effect from combining radiation with hyperthermia. For both 45 and 47 degrees, it can be seen that the experimental and theoretical curves agree within experimental uncertainty in most instances apart from the data points around 30 hours. It should be said, however, that this agreement is often only marginal and can only be interpreted as suggestive evidence that a direct additive effect is seen.

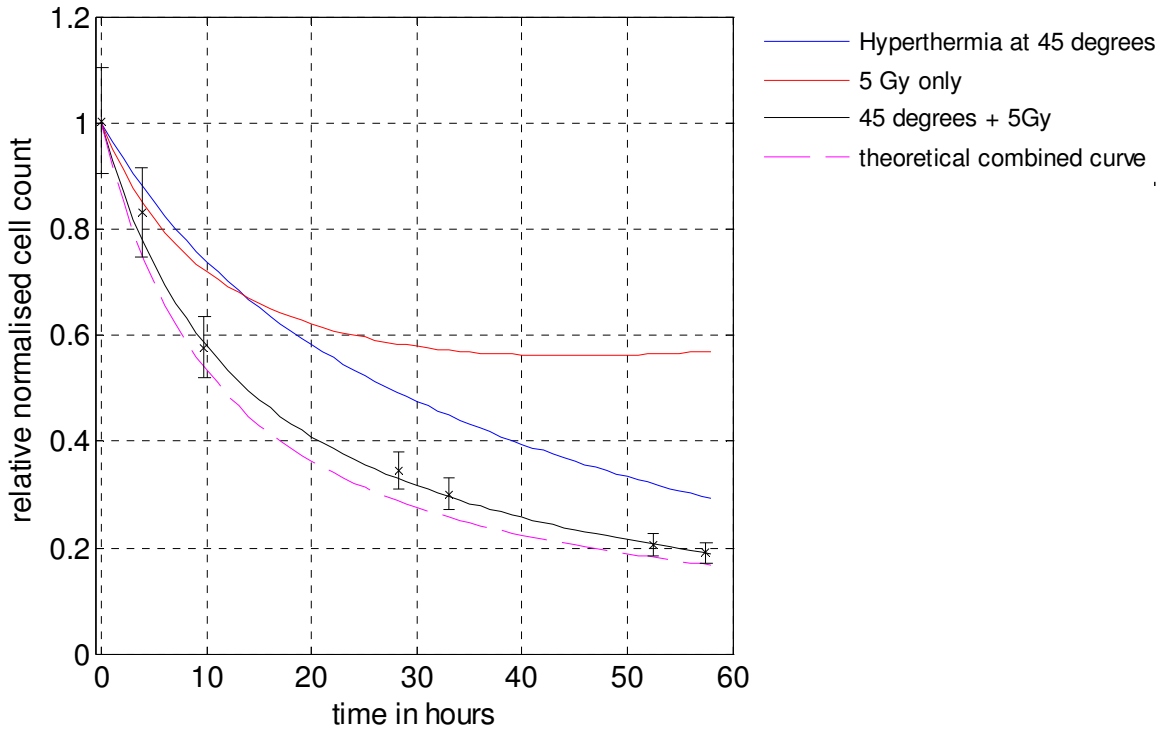



*Figure 3.15: Combining 45 degrees for 2 hours with 5 Gy of radiation. Comparison of the experimental and theoretical curves*

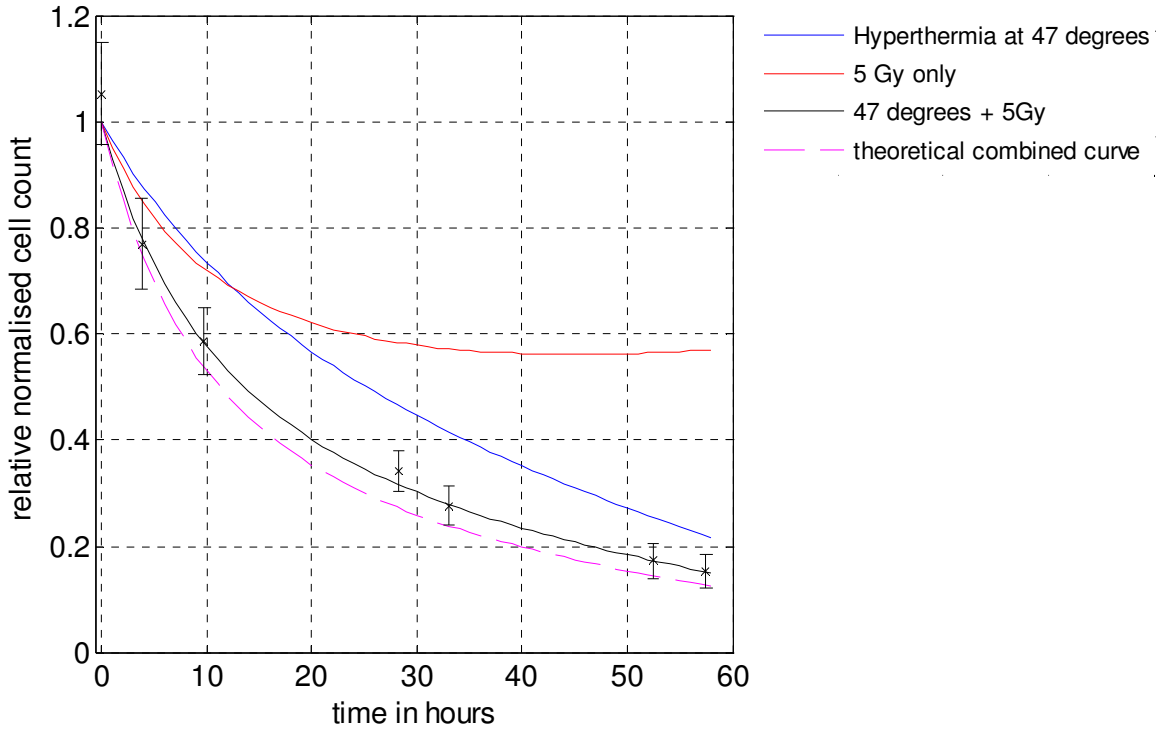



*Figure 3.16: Combining 47 degrees for 2 hours with 5 Gy of radiation. Comparison of the experimental and theoretical curves.*

To test this statistically, an analysis of variance on all of these datasets was conducted. The null hypothesis tests that all sample means of all datasets is the same. From this it was found, that the cell killing effects of hyperthermia alone when compared with those of radiation alone are statistically significantly different with a p-value less than 0.001. This means that the cell kill induced by radiation is not dependent on that induced by hyperthermia. Since the difference between both the theoretical curves and the actual experimental data is very small it can therefore be said that hyperthermia when added to radiation presents a straightforward additive effect rather than a supra additive or a diminished effect.

Figure 3.17 combines all of the data above on a single plot along with the cell killing effect from both hyperthermia and radiation alone. The 'theoretical' cell kill of each has been omitted from this graph for reasons of clarity.

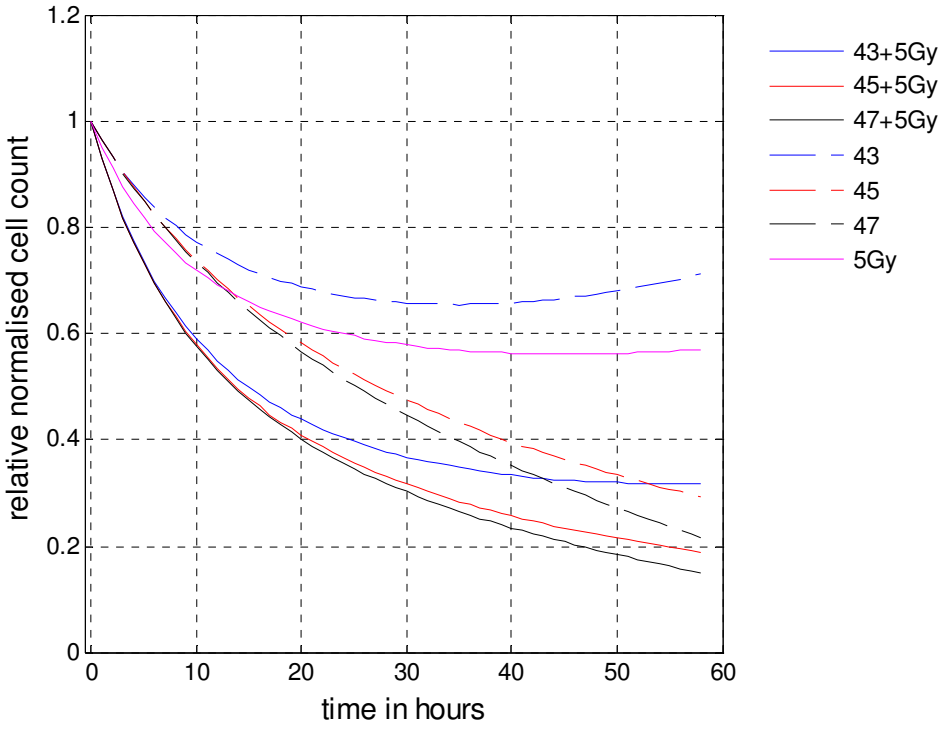



*Figure 3.17: Comparing the various levels of hyperthermia applied with the overall combined cell killing effects of 5 Gy plus 43, 45 and 47 degrees.*

Figure 3.17 allows for a direct comparison between the levels of hyperthermia applied, or in other words shows whether the different temperatures achieve different levels of cell kill. This graph is consistent with the convention that higher levels of

hyperthermia produce higher levels of cell kill than the lower levels. Combining radiation with hyperthermia does not change this convention as can be expected.

### 3.5.2 COMBINING 11 GY WITH HYPERTHERMIA REGIMES

The same procedure was followed to analyse the results from all three hyperthermia levels combined with 11 Gy of radiation. It was initially hoped to again acquire 6 datasets for each individual temperature combined with 5 Gy, but unfortunately, due to unforeseeable circumstances, both one dataset and the 10 hour data points in all sets, had to be discarded, resulting in only 5 datasets analysed with 6 data points on each. The averages of these datasets are shown in figure 3.18.

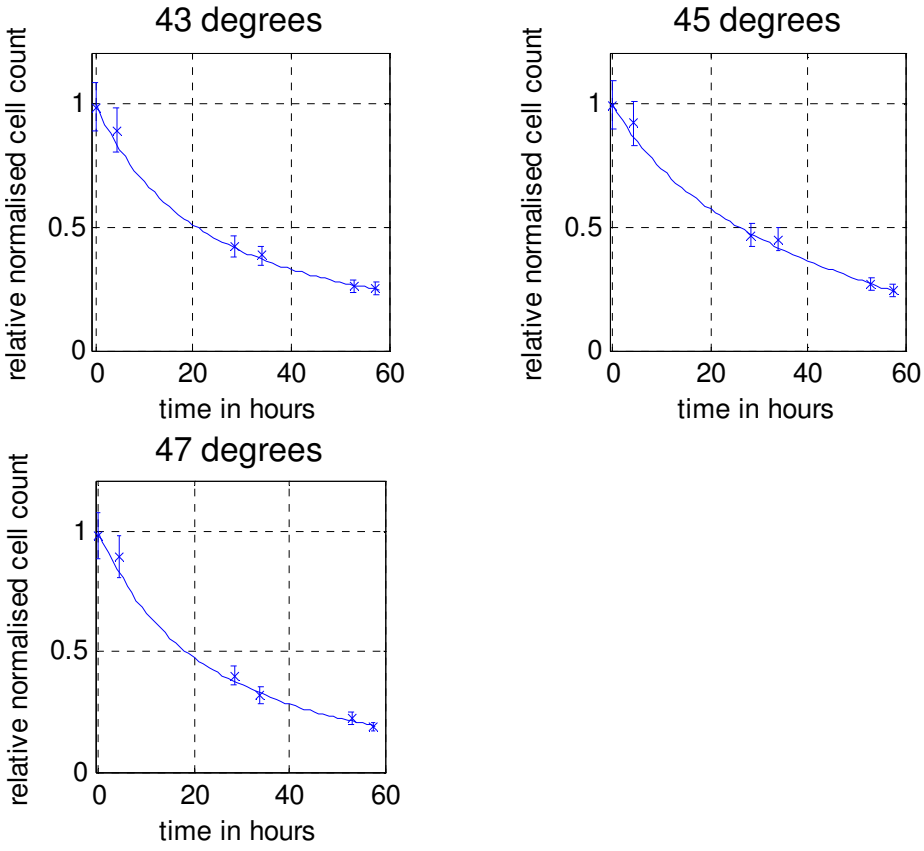



*Figure 3.18: Cell response to 5 Gy of radiation combined with 2 hour exposures to three levels of hyperthermia. All the data presented are the averages of five datasets.*

Redrawing these graphs to include the cell killing effects of radiation and hyperthermia alone, along with the theoretical estimate of direct addition of these two components resulted in figures 3.19 through to 3.21.

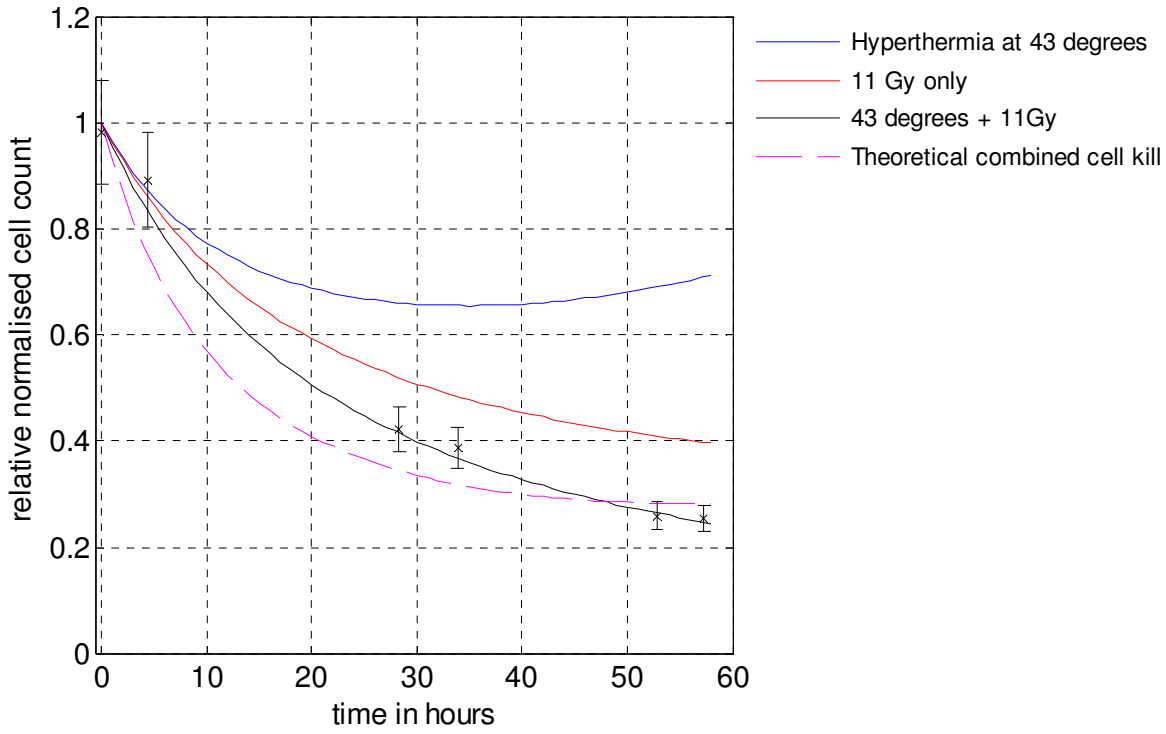



*Figure 3.19: Combining 43 degrees for 2 hours with 11 Gy of radiation and comparing the experimental and theoretical curves.*

It is interesting to see that the cell killing effect from hyperthermia at 43 degrees does not dominate over the cell killing effect of 11 Gy of radiation. At the higher levels of 45 and 47 degrees, however, as seen in figures 3.20 and 3.21, this is not the case, as hyperthermia strongly dominates the level of cell kill over 11 Gy of radiation. Furthermore, figure 3.19 shows that there might be an additive effect for combining hyperthermia with radiation as both the experimental and theoretical curves are reasonably close together even though both curves do not fall within the uncertainty bars most of the time. Figures 3.20 and 3.21, however, clearly do not present this phenomenon, as the curves do not agree at all. In fact, these curves tend to show that the higher levels of hyperthermia are in agreement with the experimental combined curves. Thus, suggesting that there is no added effect from combining hyperthermia with radiation. Another analysis of variance conducted on all of these datasets found that the combination of these levels of hyperthermia with radiation at 11 Gy is still statistically significant with a p-value less than 0.001.

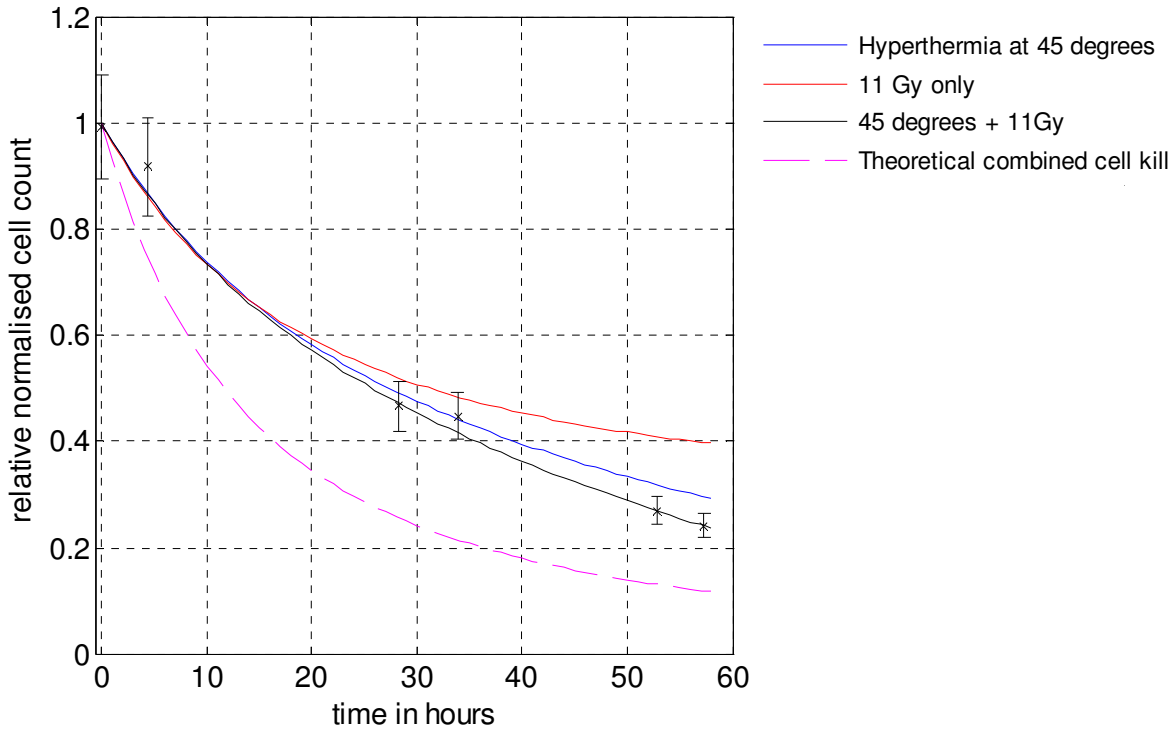



*Figure 3.20: Combining 45 degrees for 2 hours with 11 Gy of radiation and comparing the experimental and theoretical curves.*

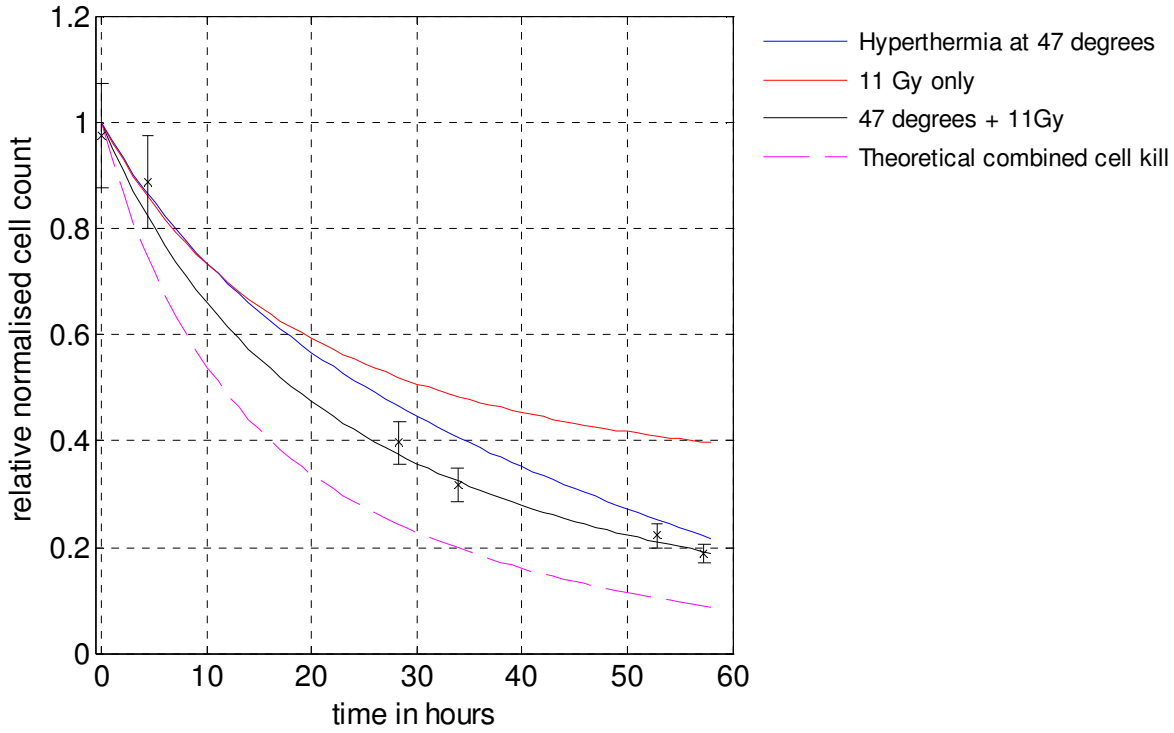



*Figure 3.21: Combining 47 degrees for 2 hours with 11 Gy of radiation and comparing the experimental and theoretical curves.*

This means that the means of the cell killing effects from hyperthermia alone when compared to those of radiation alone are statistically different. This confirms what was found for these levels of hyperthermia combined with 5 Gy as explained in Section 3.5.1. A statistically significant difference is to be expected since the experimental combined curve falls clearly below the hyperthermia alone and radiation alone curves. However, as noted earlier there is no agreement between the experimental and theoretical curves for 11 Gy and hence no direct additive effect is seen. It is unsure why this effect is seen for 5 Gy but not for 11 Gy. It could be possible that higher levels of radiation, when combined with high hyperthermia levels results in some form of saturation effect, in which the cell kill from the most dominant treatment takes over, in this case the hyperthermia treatment.

When all this data is combined, as seen in figure 3.22, with the theoretical curves eliminated, then the combined levels of hyperthermia and radiation follow a chronological convention in which higher levels of hyperthermia result in higher levels of cell kill, as expected.

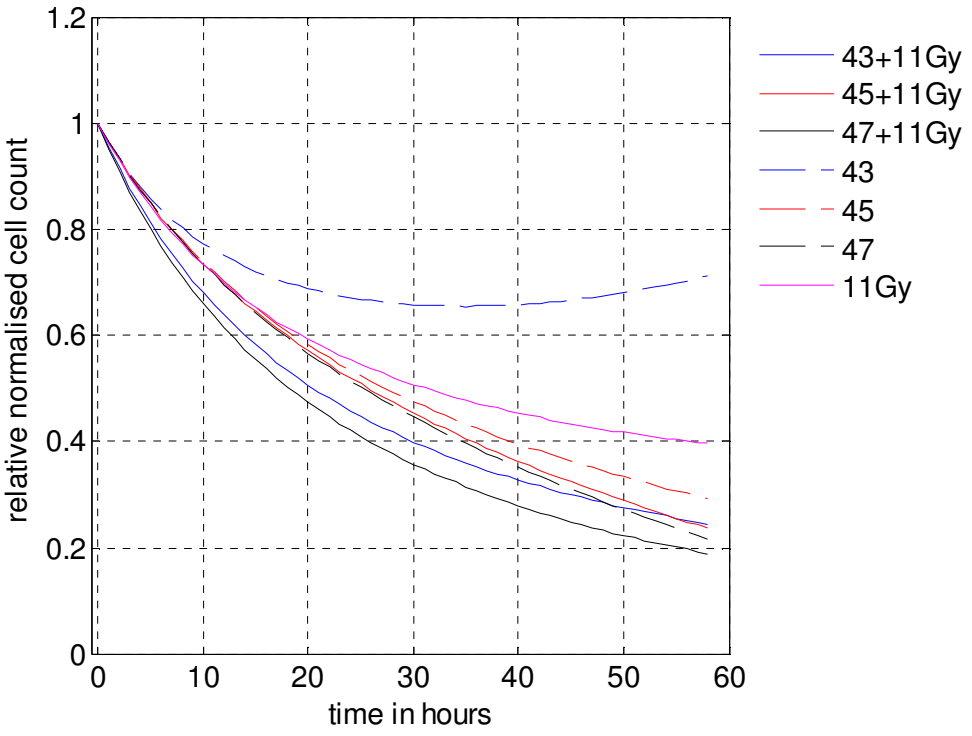



*Figure 3.22: Comparing the cell killing effects of 43, 45 and 47 degrees, and 11 Gy of radiation with their corresponding combined regimes.*

### 3.5.3 EXPLORING THE DIFFERENCES BETWEEN 5 AND 11 GY

Finally, after the combined regimes for 5 and 11 Gy were analysed separately, it is now possible to compare the radiation levels to one another to see if there is also a consistent chronological effect for radiation when combined with hyperthermia. This comparison results in three different graphs, figures 3.23 to 3.25, for each hyperthermia level and includes the levels of radiation by itself and their combined effects. Note that the data points and uncertainty bars have been added for the combined curves in order to determine whether there is a real difference between them. Recall, that the 11 Gy datasets are all missing the data point at 10 hours as seen for the 5 Gy datasets.

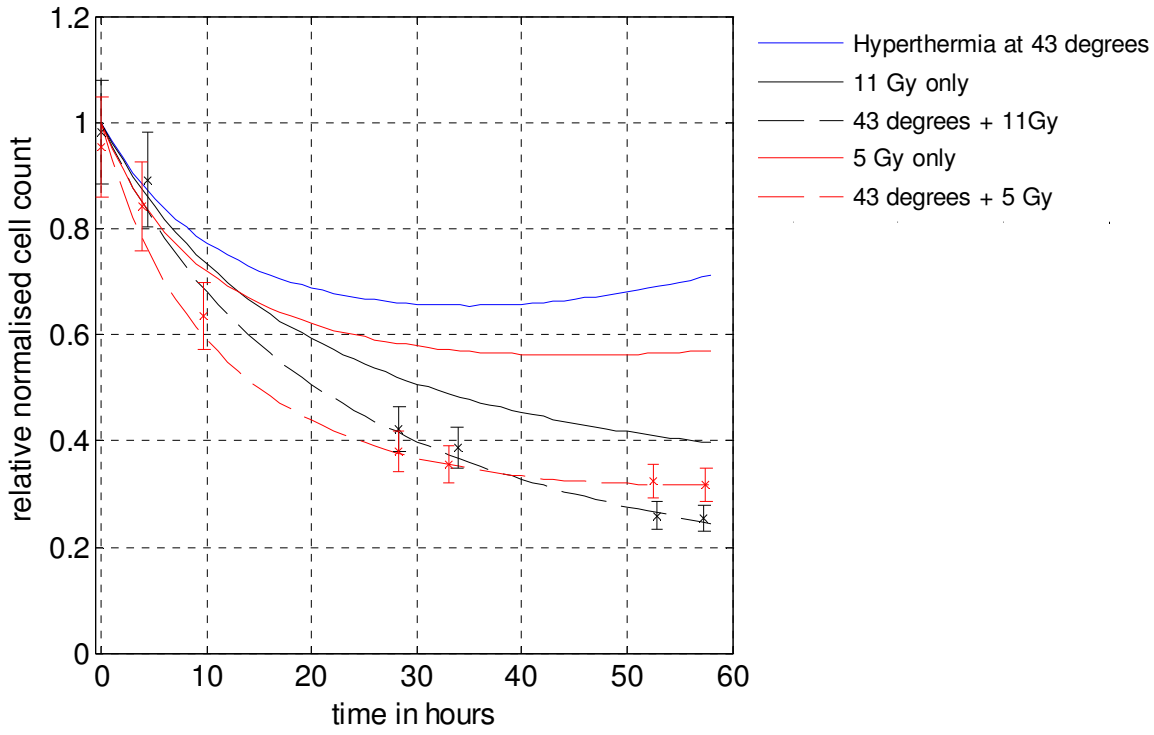



*Figure 3.23: The difference between 5 and 11 Gy when combined with 43 degrees*

After careful reanalysis of the data, it was found that the trends observed are indeed correct, however odd. From figures 3.23 to 3.25 the level of cell kill increases with temperature alone, with radiation alone, with respect to hyperthermia when combined with radiation but not with respect to radiation when combined with hyperthermia. The latter is somewhat unexpected.

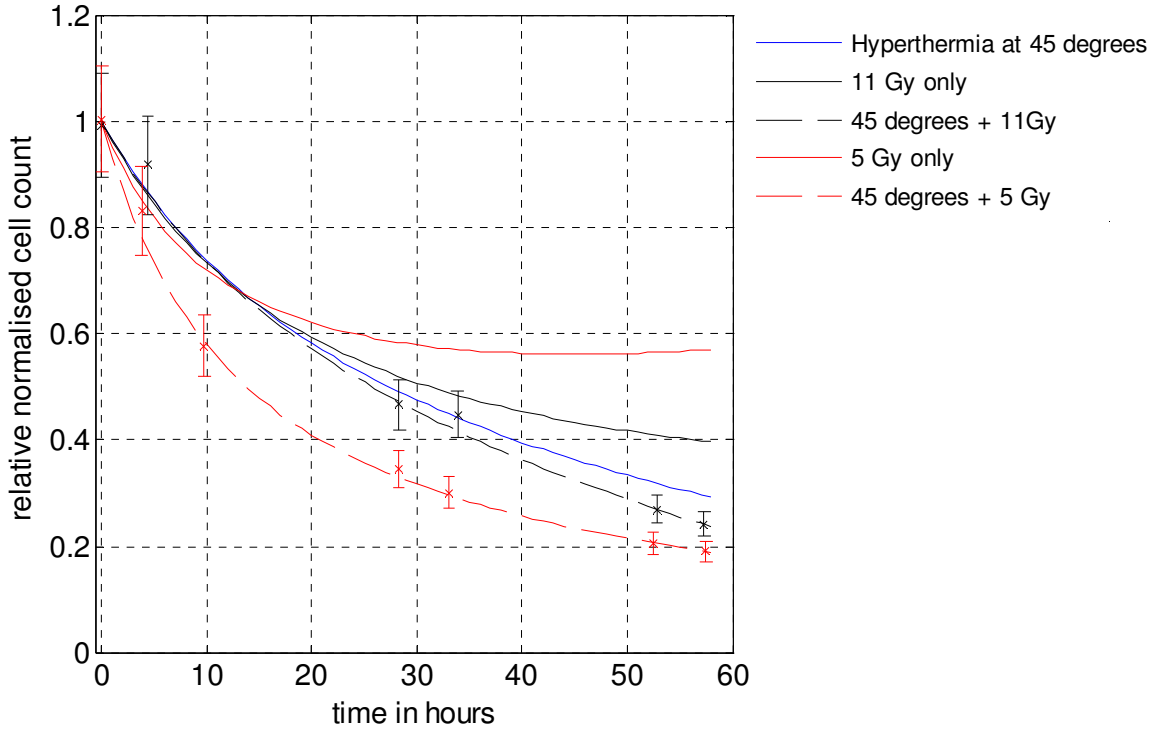



*Figure 3.24: The difference between 5 and 11 Gy when combined with 45 degrees*

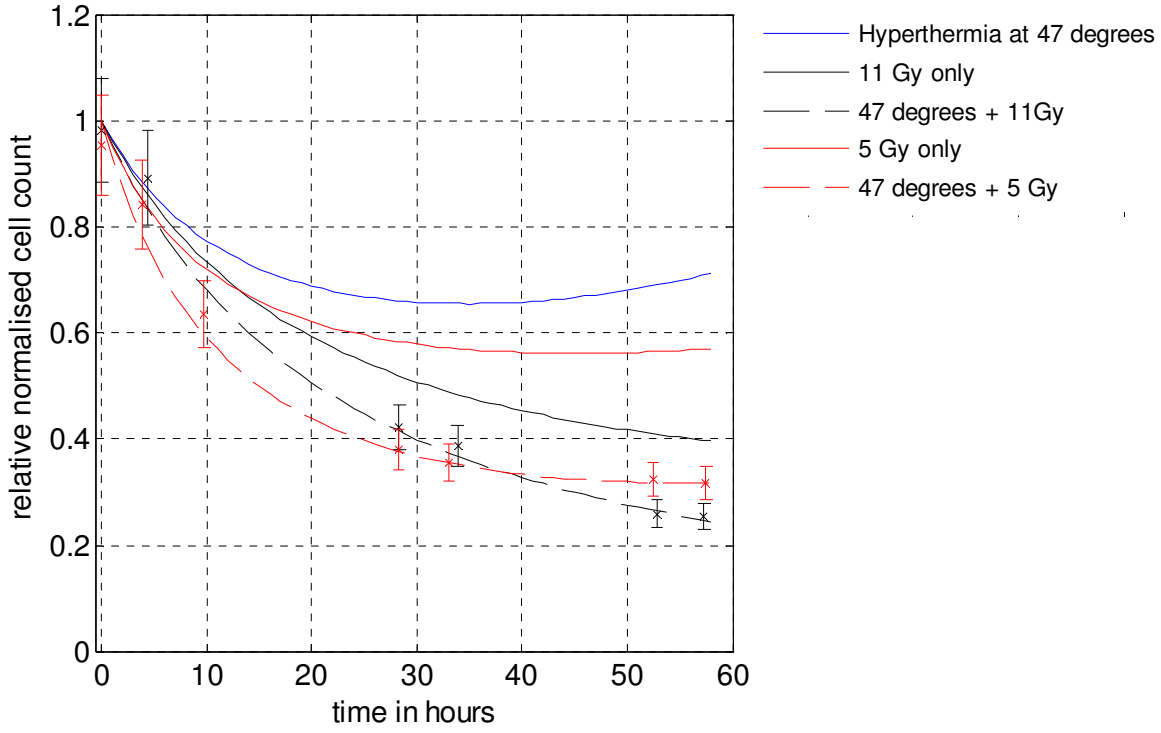



*Figure 3.25: The difference between 5 and 11 Gy when combined with 47 degrees*

Furthermore, 5 Gy when combined with hyperthermia levels either agrees with, within uncertainties, or exceeds the combined levels found for 11 Gy as seen in the case of 45 degrees. Again, this can be a very good indication that some form of saturation effect exists, causing smaller amounts of radiation at certain temperatures to have more cell killing effect than higher levels of radiation. It is interesting to recall that the initial combined experiments showed the opposite effect, as radiation dominated instead of hyperthermia and no combined effect was seen. Reasons for not observing an overall combined effect, such as the possibility of saturation effects, are only speculation and could not be tested within the scope of this project.

In summary, an additive effect from combining radiation with hyperthermia was found for 5 Gy combined with hyperthermia at 43, 45 and 47 degrees for 2 hours. This effect was marginal in the combinations of 45 and 47 degrees. 11 Gy when combined with the same hyperthermia regimes was not found to present an additive effect at all for any of the temperatures applied. Hence, a supra-additive effect was never observed and it was found that a direct additive effect of cell kill does not exist in a consistent fashion when combining hyperthermia with radiation.

# Chapter 4

# Discussion & Conclusion

## 4.1 DISCUSSION

The initial aim of this research was to carry out basic *in vitro* experiments on the P388 mouse leukaemia cell line, in order to gain a fundamental understanding as to whether a supra-additive effect occurs when combining radiation with hyperthermia in the treatment of leukaemia. A framework was established, with MAF approval, at the University of Canterbury to allow these experiments to be carried out. The facilities and equipment available imposed various limitations on the set-ups of the experiments. In summary, the most important limitations of this project were the underestimation of cell kill from the Coulter counter and having to travel to and from Christchurch Hospital to use a linear accelerator to administer the radiation doses. Delays in obtaining the MAF approval and various pieces of equipment laid restrictions on the time available for this project and eventually resulted in fewer experimental datasets obtained than originally was hoped.

Throughout this project, 13 control group datasets were obtained and investigated. Normally, a logistic curve is used to represent population growth data that is restricted by its food supply, in our case the cells are restricted by the amount of media present. It was found that the ratio of number of cells seeded to media available was large, and that because of this, the initial exponential part of the logistic curve was not observed. The data was consequently fitted to a second order polynomial and this fit was found to approximate the data accurately and fall within the uncertainties. Following this, the control groups that travelled to and from

Christchurch Hospital were compared with those that remained at the University of Canterbury. A visual difference between these two types of control groups was seen but a p-value of 0.07, however close to 0.05, indicated that these groups were not statistically significantly different. It was important to determine whether to correct all other datasets to their respective control groups on a weekly basis or to correct all datasets by one single averaged control group. It was decided, after careful consideration, to correct the rest of the data presented throughout this thesis on a weekly basis, in order to account for uncontrollable variations in factors such as the change in humidity of the room.

Hyperthermia experiments were conducted for a temperature range of 38-50 degrees for durations of 30 minutes, 1 hour, 2 hours and 3 hours and used to determine the cell killing effects from hyperthermia alone. This data was plotted on an Arrhenius plot, which followed a straight line, indicating that no systematic error was present throughout the data. The statistical analysis of the hyperthermia data revealed that there was no dependence on exposure time.

The hyperthermia experiments also indicated that strong temperature dependences exist for cell kill by hyperthermia, with a p-value less than 0.05. This general trend, however, did not exist for each temperature when compared with the control groups as seen in an unpaired t-test. The lower temperature range of 38-42 degrees was found to have no effect whereas the range of 43-50 degrees displayed very high statistical significance with p-values less than 0.0001. This study also confirmed the findings by Harisladis *et al.* who showed that there is no cell kill for temperatures below 43 degrees and that for 43 degrees the exposure time must be longer than 30 minutes in order to achieve a detectable amount of cell kill [62].

The cell kill from 1, 3, 5, 9, 11 and 15 Gy were investigated and demonstrated the familiar pattern that an increase in the radiation dose corresponds to an increase in levels of cell kill [20,31]. The statistics indicated that there is indeed a statistically significant difference from applying radiation, as would be expected.

When initially combining hyperthermia with radiation, not all results from hyperthermia alone had been obtained and hence the conclusion that exposure times had no effect, as previously discussed had not yet been drawn. At this time, it was proposed to investigate 41 and 42 degrees Celsius for 30 minutes, 1 hour and 3 hour exposure times combined with 1, 3, 5, 9, 11 and 15 Gy of radiation. Consequently, there were too many variables and not enough datasets to be able to draw concrete

conclusions, about the combination of hyperthermia and radiation. Visually, through graphs no strong additive effects were seen.

The final combined experiments conducted, consisted of 5 and 11 Gy as radiation levels, each combined with 43, 45 and 47 degrees applied for 2 hours. The theoretical level of cell kill was estimated based on a direct additive effect from the cell kill from hyperthermia and radiation alone. The main assumption for a direct additive effect is that the cell kill from one variable is not dependent on the other. In the case of 5 Gy combined with these various levels of hyperthermia, a direct additive effect was observed as the theoretical and experimental curves agreed to a reasonable level of certainty. This however, was not seen for 11 Gy when combined with the various hyperthermia regimes in which the overall experimental cell kill did not agree with the predicted theoretical level of cell kill required for an additive effect. Instead, at 11 Gy the overall effect seemed diminished. When the two levels of radiation were compared, it was also found that 5 Gy when in combination with these levels of hyperthermia, induces higher levels of cell kill than 11 Gy for the same combinations. Reasons for this inconsistency cannot be provided since the mechanisms involved that produce the cell kill for both radiation and hyperthermia alone are known to be highly complex. In addition, the investigation of the combined effects of hyperthermia and radiation, at the microscopic level, which could possibly provide such reasons, was outside the scope of this thesis. It is suggested however, that some form of saturation effect could be present causing these results, but this is sheer speculation. An analysis of variance found that the combined regimes are statistically significantly dependent on both radiation and hyperthermia levels, as p-values of less than 0.05 were found.

Overall, these results do not indicate that when combining hyperthermia with radiation, a straightforward additive effect will be consistently observed. Hence, the hypothesis of combining radiation with hyperthermia in the treatment of leukaemia is not supported by the results found in this thesis, as a consistent additive effect would be necessary. These experiments have thus found what is known as a null-result that does not support any further on going work for this particular cell line. It is difficult to compare these results with findings in the literature, as the differences in the methodologies used are often substantial. Most cell lines investigated, for example, can be plated in order to run clonogenic assays and the cell lines used are often solid tumours rather than lymphoid neoplasms or leukaemias. The studies conducted on local hyperthermia combined with radiation were discussed in Chapter 1: Research

motivation and background, and will not be reiterated here as deemed irrelevant. It is interesting, however, to discuss the findings and trials by Robbins *et al.* who have thus far had successful results from combining radiation with hyperthermia in the treatment of chronic leukaemias and lymphomas [63,64].

In the studies by Robbins *et al.* the AKR murine leukaemia cell line has been investigated both *in vitro* and *in vivo.* These studies have maintained a focus on comparing the cell kill effects from hyperthermia on healthy haemopoietic cells with those on the AKR cell line [65]. They found that hyperthermia at 41.8 degrees, or higher temperatures, selectively kills leukaemia cells both *in vitro* and *in vivo* whereas in this study no effect from 42 degrees was found. No cell experiments were carried out however, to test the combined effect from radiation and hyperthermia *in vitro* and hence our findings cannot be directly compared. The AKR mouse model was used, however, to test the combination of hyperthermia with TBI and found an increase in the survival of the mice [66]. Shen *et al.* investigated the effects on the erythroleukaemia mouse for the combination of whole body hyperthermia with cyclophosphamide, a chemotherapeutic agent. The Cyclophosphamide combined with whole-body hyperthermia at 41.8 degrees also showed a prolonged survival of the mice undergoing this treatment [67]. Robbins *et al.* continued to write a paper on the interaction of whole-body hyperthermia and irradiation in the treatment of AKR mouse leukaemia. They found a supra-additive killing effect from the hyperthermia [35]. This is obviously different to what was found in this thesis, where no supra-additive effect has been observed. This difference cannot, however, be compared since *in vitro* cell lines react very differently to *in vivo* mechanisms.

Ever since, a number of case studies have been performed on patients with Burkitt's lymphoma and uncurable B-cell neoplastic diseases [64,68]. One case study conducted by Wessalowski *et al.* combined whole-body hyperthermia with various chemotherapeutic agents in order to try and save a child with myelomonocytic leukaemia [69]. Proposals and calls for phase II randomised trials to combine whole-body hyperthermia with either radiation or chemotherapy or both have been made [63,70]. On a final note, the clinical studies performed aim at increasing the overall cell kill of the leukaemias or lymphomas present in order to reduce the risk of relapse. They do not seem to aim at reducing the overall radiation or chemotherapy doses, which was why the investigations in this thesis were originally performed.

## 4.2 FUTURE WORK

Even though in this thesis, a null-result was obtained, and hence, no further work is to be done using the P388 mouse leukaemia cell line, investigations into human leukaemia cell lines should be performed. This is because mouse cell lines are only an approximation of human cell lines and it is possible that in the case of combining hyperthermia with radiation, the human cell lines will respond differently. Similarly, to the investigations made by Robbins *et al.*, one should investigate the different response from the human leukaemia cell line to its healthy counterparts. In addition, one must keep in mind that in the case of leukaemia one is discussing the possibilities of combining whole-body hyperthermia with radiation. Hence, the effects of whole-body hyperthermia when combined with radiation ought to be tested in an *in-vivo* mouse model to obtain any real indication as to whether combining hyperthermia with radiation is beneficial for the treatment of leukaemia. Robbins *et al.* only showed that heat selectively kills the cells but no papers were found showing exactly how much additional cell kill is obtained from adding hyperthermia to radiation. Hence, in their studies it was not determined if the possibility for lowering the radiation dose whilst maintaining the therapeutic index exists.

## 4.3 CONCLUSION

In this thesis, basic *in vitro* experiments were carried out on the P388 mouse leukaemia cell line. It was investigated whether combining radiation with hyperthermia, in the case of leukaemia, results in an overall supra-additive cell killing effect or in an additive effect. The cell killing effects from both hyperthermia and radiation alone were initially determined. It was found that the exposure time to hyperthermia has no significant effect, but varying temperature, as was expected, does have statistically significant effects. Radiation levels were found to show the familiar pattern in which an increase in the level of radiation increases the amount of cell kill. Some initial combined experiments were performed but too many parameters were present in the data, resulting in no concrete conclusions. Because of this, multiple datasets were acquired for only two radiation levels, 5 and 11 Gy, and three hyperthermia temperatures, 43, 45 and 47 degrees Celsius. The analysis of these datasets revealed that no supra-additive effect is observed from combining radiation

with hyperthermia. In the case of 5 Gy, a consistent additive effect was found but for 11 Gy a diminished effect in overall cell kill was seen. When comparing the combined effects based on the different radiation levels, it was found that 5 Gy, when combined with hyperthermia, results in the same or a higher level of cell kill than the cell killing effect from 11 Gy combined with hyperthermia. This is rather counter intuitive and inconsistent with the expectations that a higher level of radiation when combined with hyperthermia regimes will result in a higher level of cell kill. Reasons for this inconsistency cannot be given as the mechanisms that induce the cell kill from a combined regime should be investigated on the microscopic level, and such an investigation was outside the scope of this thesis.

The overall conclusion drawn from this work is that there is no real beneficial effect for combining radiation with hyperthermia for the P388 mouse leukaemia cell line. No further investigations into the combination of hyperthermia and radiation for this cell line are needed. Human leukaemia cell lines and *in vivo* mouse models, however, should in the future be investigated. Human cell lines would benefit from a similar investigation, as it is possible that they will react differently to the combined regimes than the P388 mouse leukaemia cell line has. *In vivo* mouse models will take blood circulation effects throughout the body into account, which is the only way to determine whether there are any beneficial effects from combining radiation with hyperthermia in the treatment of leukaemia.

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# APPENDIX A

## Schematic diagram of the Coulter counter

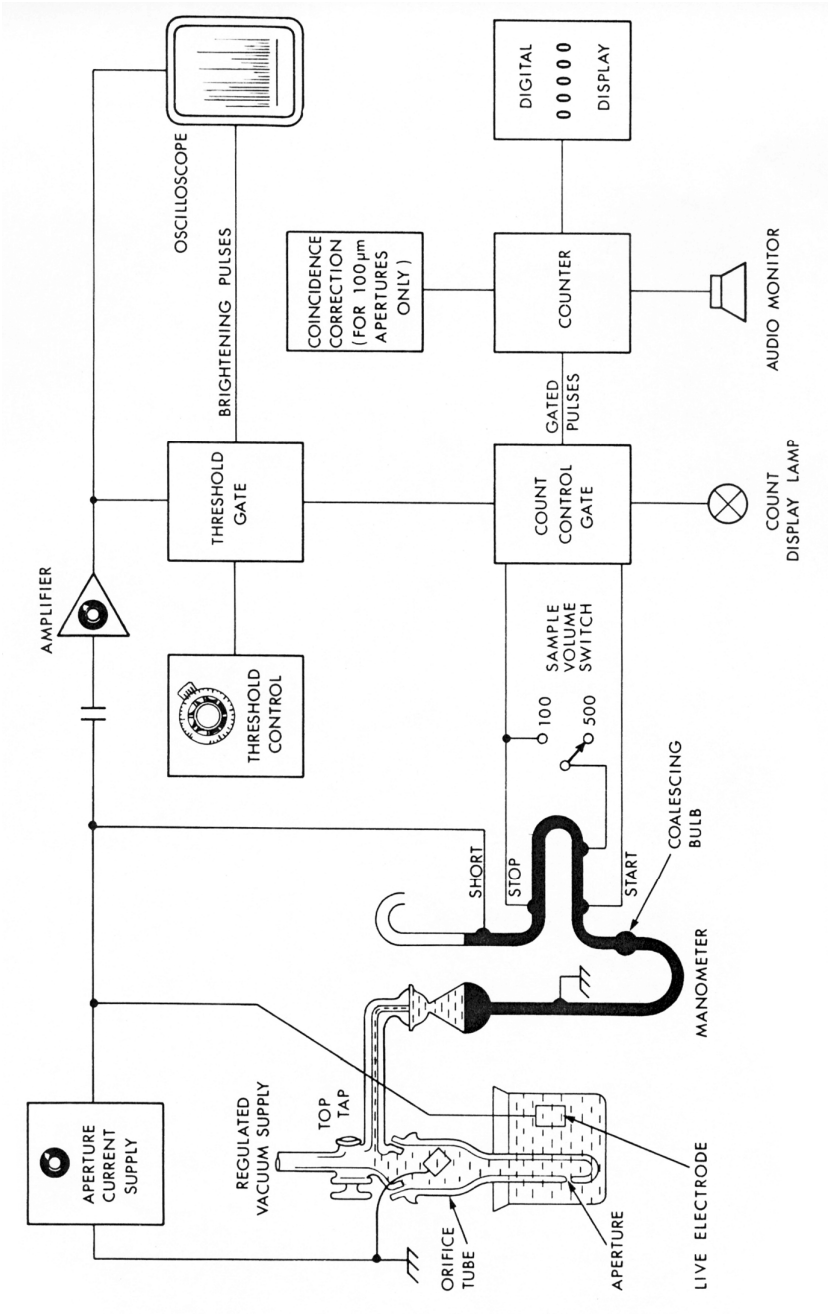


*Figure A.1: Schematic diagram of the Coulter counter taken from the Coulter counter manual*

# APPENDIX B

# Formal proposal & Transportation protocol for MAF approval

*Note: changes in set-up as occurred throughout this thesis have been verbally approved by both the health and safety officer of the University of Canterbury and by MAF*

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## PROPOSAL TO PROF. BILL DAVISON WITH THE REQUEST FOR HEALTH AND SAFETY APPROVAL OF THIS PROJECT AS OUTLINED BELOW

**3RD APRIL 2007**

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## COMBINING MILD HYPERTHERMIA AND IONISING RADIATION; THE CELL-KILLING EFFECT ON RODENT LEUKAEMIA CELLS

### MEDICAL PHYSICS MASTERS THESIS

**Latoya Flewellen**

Under the supervision of: Assoc. Prof. Lou Reinisch and Dr. Juergen Meyer
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The experimental work in Chemistry is conducted by the technician Gill Ellis and the principal physicist Dr. Mark Bird at Christchurch Hospital oversees the radiation experiments.

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**DETAILS OF THE PROJECT:**

It has been proposed to observe the cell-killing effect of P388 mouse leukaemia cells after hyperthermia, ionising radiation and after a combination of both these modalities has been applied. The details of the cell-line are as follows: P388 D1 (Murine Leukaemia cells) ATCC CCL 46 imported from ATCC on 28/03/02 under MAF permit: 2002014613.
The prime reason for observing this effect is to observe whether the hyperthermia will make the cells more sensitive to radiation, if so then

hyperthermia might have important beneficial consequences for future patients undergoing total body irradiation followed by a bone-marrow transplant.

### TEST-RUN AND HYPERTHERMIA EXPERIMENTS

This work will be conducted by the technician Gill Ellis in her biohazard room (room 745 in Chemistry) when it is PC 2 approved. This work includes the following:
The growing of these cells in culture and establishing what concentration of cells will work best for our experiments by doing a test-run. That is the filling of a 96-well-plate with different concentrations of cells and observing their natural cell-kill for a period of days. This cell-kill is read out by a spectrometer in this laboratory.

The hyperthermia experiments are to be conducted by placing the same well plates into an incubator in this room and increasing the temperature in the incubator; from this, the cell-killing effect from different levels of hyperthermia alone can be established.

### IONISING RADIATION EXPERIMENTS

The ionising radiation experiments are to be conducted in Treatment room 3, Lower Ground Level, Oncology Service, Christchurch Hospital, during after hours. The experiments will establish the cell-killing effect of different levels of ionising radiation on the leukaemia cells, and will determine which levels of radiation are best to be used in the combined experiments. The supervisor for the application of the radiation is the medical physicist Dr. Mark Bird.
To do this, the cells will be dispensed into their wells in the biohazard room (room 745) in Chemistry by Gill Ellis. The cells have been classified as Infectious substances Category A, affecting animals only. Hence, the transport regulations UN 2900, set by the International Air Transport Association (IATA) apply. An outline for the design of the packaging including the primary receptacle, the secondary watertight container and the outer packaging can be found at the very end of this proposal. It should be noted that with these regulations in place, any spillage that might occur will be safely contained within the packaging.

The package will be transported to Christchurch Hospital by car and there it will be placed under the Linear Accelerator, which will irradiate the whole package including the outer packaging. The x-rays will pass through the package and irradiate the cells inside. The entire package is then to be taken back to Chemistry immediately and placed back inside the biohazard room where the cells can be analysed. The whole procedure will take less than a few hours. The experiment is to be repeated a number of times with different radiation doses.
*Please Note:* There is no need to take the cells out of the biohazard safe packaging during the experiment. The only place they are removed is in Chemistry laboratory, in the safe hands of Gill Ellis.
There will be a maximum of 50 wells (on a 96 well plate) filled with a concentration of cells in culture. The concentration can only be established during the initial test-run but it is estimated to be around $8.4 \times 10^4$ cells/ml dispensed into 400 µl wells of which about 150 µl will be used.

### COMBINING IONISING RADIATION AND HYPERTHERMIA

This part of the experiment will follow the exact same protocols as outlined in

the separate hyperthermia and ionising sections above. It is proposed to apply hyperthermia to the cells before they travel their biohazard safe packaging to the hospital in the same fashion.

All the above experiments that are to be conducted at Christchurch Hospital will in total comprise 1 trip per week over a total of approximately 10 weeks. In each trip up to 4 containers will be transferred to the hospital and back.

### SPILLAGE AND CONTAINMENT

At all times the cells will either be contained in the Biohazard Room (745) in Chemistry or be contained in the biohazard safe packaging.

If at any time spillage does occur within the packaging, it will not be noted until the secondary packaging is examined in the biohazard room at which point the entire secondary packaging has become biohazard waste and will be disposed off according to MAF PC 2 regulations, which apply within that room.

### WASTE PRODUCTS

The only waste products from this experiment are the dead cells either due to us killing them or due to their own natural death. These waste products will be disposed of within the biohazard room according to MAF regulations as they apply within that room.

### CONTACT DETAILS

If you have any queries please do not hesitate to contact me, my supervisors or the qualified technicians (in their respective areas) who are helping me with this project:

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# DESIGN OF THE BIOHAZARD CONTAINER

*The following outlines the design of the biohazard packaging as required according to IATA packing instructions 602 and the Guidance Document for infectious substances. Please note that the packing instructions follow infectious substances category A, affecting animals only (UN 2900)*

### THE PRIMARY RECEPTACLE:

We initially have a plate with 96 wells of which approximately 20 to 50 wells are filled at a time with cells dispersed in their media. Since the well is open at the top, it needs to be sealed tightly:

The rubber seal shown contains all the liquid within each well. This has been tested by using some blue and red food colouring. Next, we place the original lid of the plate on top and then wrap it all tightly up with glad-wrap. This serves as our primary receptacle.

### THE SECONDARY PACKAGING

The secondary packaging is both water- and air-tight, and can withstand temperatures of at least 100 degrees Celsius and it is estimated that it will at least withstand temperatures below -15 degrees Celsius.

The packaging has been tested to indeed be watertight (it was filled full with water and shaken vigorously upside down and left upside down for over an hour and nothing leaked through).

The primary receptacle is placed inside the secondary packaging and is surrounded with cotton wool. The cotton wool holds the primary receptacle in place, and will absorb any leakage from the primary receptacle if it were to occur.

The secondary packaging locks on all four sides of the container and is made of thick polypropylene. A picture of it is shown below:


A specimen record is to be attached to the secondary packaging that includes an itemized list of contents. In this case, it will read along the following lines:

P388 mouse lymphoid leukaemia cells dispersed in media of concentration *such and such* with *X* amount of wells filled at a total quantity of *X* ml.

**THE OUTER PACKAGING:**

The outer packaging must be rigid and of adequate strength for its weight, capacity and intended use as in accordance with Packing instruction 602 from the IATA. We have bought a container of the same description as the secondary packaging but slightly bigger so that the secondary fits inside. To stop the secondary from moving the container is filled up with more cotton wool and this has been tested to show that it holds it securely in place.

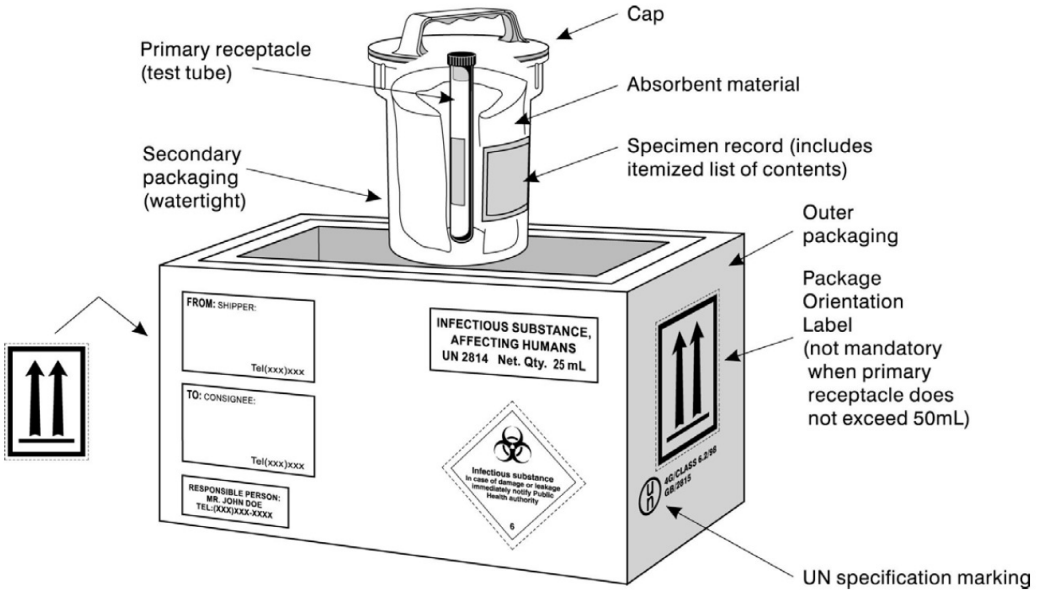
The outer packaging will be labelled in accordance with packing instructions 602 and the IATA Guidance Document for infectious substances resulting in the following labels to be made and attached to the outer packaging.

The picture on the following page shows these labels and comes from Annex 3 in the IATA Guidance Document for infectious substances.

ANNEX 3

Example of Packing and Marking for Category A Infectious Substances

(See Packing Instruction 602 for additional requirements)




*Please Note:* The supplier will be Gill Ellis from the Department of Chemistry at the University of Canterbury and the consignee will be Dr. Mark Bird at Christchurch Hospital. I, or one of my supervisors can/will be the person responsible for the package. Finally, the UN code used will be UN 2900 as the infectious substance only affects animals and thus the above label shown as an example will be changed slightly. Professor Bill Davison will be included as a contact person as the Operator of Transitional Facilities (rm 559) in the School of Biological Sciences.

A '**DO NOT OPEN'** label will also be affixed to the outer packaging.

**TRANSPORT TO THE HOSPITAL**

Finally, the entire package as described above will be placed in a small chilli-bin such that three or four of these packages can be transported at once to the hospital and back. This will allow us to complete the experiments faster and make fewer trips to the hospital. Please note that we would only ever have four such packages as described above at any one time.

# APPENDIX C

## P-values from unpaired t-tests for hyperthermia and radiation levels

This first table provides all the p-values as determined from an unpaired t-test in which data from every radiation level has been compared to one another.

| Radiation levels compared | p-value |
|---|---|
| 0 Gy, 1 Gy | .0859 |
| 0 Gy, 3 Gy | .3961 |
| 0 Gy, 5 Gy | .0340 |
| 0 Gy, 9 Gy | .0741 |
| 0 Gy, 11 Gy | .0360 |
| 0 Gy, 15 Gy | .0283 |
| 1 Gy, 3 Gy | .0187 |
| 1 Gy, 5 Gy | .0186 |
| 1 Gy, 9 Gy | .0273 |
| 1 Gy, 11 Gy | .0209 |
| 1 Gy, 15 Gy | .0185 |
| 3 Gy, 5 Gy | .0260 |
| 3 Gy, 9 Gy | .0598 |
| 3 Gy, 11 Gy | .0313 |
| 3 Gy, 15 Gy | .0254 |
| 5 Gy, 9 Gy | .3692 |
| 5 Gy, 11 Gy | .0453 |
| 5 Gy, 15 Gy | .0277 |
| 9 Gy, 11 Gy | .0245 |
| 9 Gy, 15 Gy | .0167 |
| 11 Gy, 15 Gy | .1098 |

*Table C.1: Statistical comparison of every radiation levels in an unpaired t-test.*

The following table provides all the p-values as found from an unpaired t-test conducted on all hyperthermia data comparing every temperature to one another. It should be noted that exposure times is not taken into account. P-values less than 0.05 indicate that the difference between the two temperatures is statistically significant. As an example: 37,38 compares 37 degrees with 38 degrees and the p-value of .2764 indicates that there is no statistically significant difference between the two.

| Temperatures compared | p-value |
|---|---|
| 37, 38 | .2764 |
| 37, 39 | .9722 |
| 37, 40 | .0643 |
| 37, 41 | .0681 |
| 37, 42 | .0803 |
| 37, 43 | <.0001 |
| 37, 44 | <.0001 |
| 37, 45 | <.0001 |
| 37, 46 | <.0001 |
| 37, 47 | <.0001 |
| 37, 48 | <.0001 |
| 37, 49 | <.0001 |
| 37, 50 | <.0001 |
| 38, 39 | .3453 |
| 38, 40 | .4597 |
| 38, 41 | .5234 |
| 38, 42 | .0018 |
| 38, 43 | <.0001 |
| 38, 44 | .0176 |
| 38, 45 | <.0001 |
| 38, 46 | <.0001 |
| 38, 47 | <.0001 |
| 38, 48 | <.0001 |
| 38, 49 | <.0001 |
| 38, 50 | <.0001 |
| 39, 40 | .0985 |
| 39, 41 | .1067 |
| 39, 42 | .1200 |
| 39, 43 | .0001 |
| 39, 44 | <.0001 |
| 39, 45 | .0026 |
| 39, 46 | <.0001 |
| 39, 47 | <.0001 |
| 39, 48 | <.0001 |
| 39, 49 | <.0001 |
| 39, 50 | <.0001 |
| 40, 41 | .9820 |
| 40, 42 | .9198 |
| 40, 43 | .0134 |
| 40, 44 | <.0001 |
| 40, 45 | .0635 |
| 40, 46 | <.0001 |
| 40, 47 | <.0001 |
| 40, 48 | <.0001 |
| 40, 49 | <.0001 |
| 40, 50 | <.0001 |
| 41, 42 | .9388 |
| 41, 43 | .0141 |
| 41, 44 | <.0001 |
| 41, 45 | .0634 |
| 41, 46 | <.0001 |
| 41, 47 | <.0001 |
| 41, 48 | <.0001 |
| 41, 49 | <.0001 |
| 41, 50 | <.0001 |

*Table C.2: Statistical comparison of all hyperthermia levels in an unpaired t-test.*

| | |
|---|---|
| 42, 43 | .0105 |
| 42, 44 | <.0001 |
| 42, 45 | .0544 |
| 42, 46 | <.0001 |
| 42, 47 | <.0001 |
| 42, 48 | <.0001 |
| 42, 49 | <.0001 |
| 42, 50 | <.0001 |
| 43, 44 | .0107 |
| 43, 45 | .9091 |
| 43, 46 | .0343 |
| 43, 47 | .0010 |
| 43, 48 | <.0001 |
| 43, 49 | <.0001 |
| 43, 50 | <.0001 |
| 44, 45 | .1446 |
| 44, 46 | .9671 |
| 44, 47 | .1774 |
| 44, 48 | .0009 |
| 44, 49 | <.0001 |
| 44, 50 | <.0001 |
| 45, 46 | .1828 |
| 45, 47 | .0308 |
| 45, 48 | .0027 |
| 45, 49 | .0009 |
| 45, 50 | .0005 |
| 46, 47 | .2338 |
| 46, 48 | .0114 |
| 46, 49 | .0019 |
| 46, 50 | .0008 |
| 47, 48 | .2983 |
| 47, 49 | .0975 |
| 47, 50 | .0589 |
| 48, 49 | .1954 |
| 48, 50 | .0570 |
| 49, 50 | .6254 |

*Table C.2 continued: Statistical comparison of all hyperthermia levels in an unpaired t-test.*