The Effect of Temperature on the Bystander Effect as Examined in Human Prostate Carcinoma Cells with Alpha Particle Irradiation

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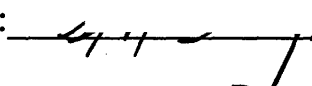IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
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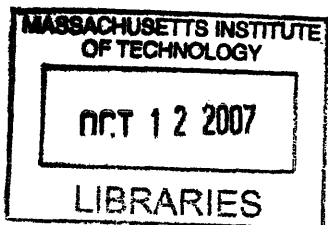
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Abstract

The bystander effect is seen when irradiated cells release a factor that can produce damage or death in neighboring "bystander" cells that are not actually hit by any radiation. One proposed mechanism involves the irradiated cells releasing a soluble factor into the medium that can cause damage to the non-irradiated cells. Previous studies in the Coderre lab showed that the soluble factor released by DU-145 human prostate carcinoma cells was a short-lived, free radical species (Wang and Coderre, Rad. Res., 164, 711-722, 2005). This thesis examined the effect of temperature on the bystander effect. A co-culture system was used to create irradiated and bystander DU-145 cells in the same medium. This thesis showed that a decrease in temperature lessens or prevents the bystander effect. Researching the bystander effect will allow a better understanding of a process that may already be occurring during alpha-particle based therapies such as boron neutron capture therapy (BNCT) and tumor radioimmunotherapy and could provide a means to improve these therapies.

Introduction

Prostate Cancer

Prostate cancer is the most common form of cancer among males in Western countries. In 2006 in the United States, 235,000 new cases are estimated to be diagnosed and 30,000 men will die from prostate cancer. See Jemal *et al* [1] for other cancer statistics.

The prostate is a walnut-sized gland between the bladder and the penis, in front of the rectum, that produces seminal fluid. Small, primary (original) tumors within the prostate are formed in the early stages of prostate cancer. Secondary tumors are formed when the original cancer metastasizes or spreads to other parts of the body. External beam radiotherapy, surgery (radical prostatectomy), brachytherapy and hormone therapy (androgen deprivation therapy) are common treatments used in the early stages of prostate cancer with a 90% cure rate, discussed in [2-4]. Unfortunately, there are no or few symptoms of cancer at this early stage. The new use of prostate-specific antigen (PSA) testing allows physicians to detect tumors in the prostate before they metastasize [5, 6].

About 30,000-40,000 prostate cancer patients per year have metastatic prostate cancer. Once the cancer metastasizes, treatment options are less effective and a significant decrease is seen in cure rates. In about 80% of these cases, the cancer metastasizes to bone. Hormone therapy is initially effective, but the majority of cases develop a resistance. Chemotherapy generally does not work due to systemic toxicity [7]. For

palliative relief of pain, bone-seeking radiopharmaceuticals are used. Radioimmunotherapy, the attachment of radioisotopes to tumor specific monoclonal antibodies, is an experimental treatment for leukemia and metastatic solid tumors, including prostate [8, 9].

Prostate cancer is a good candidate for radioimmunotherapy for multiple reasons. Prostate specific antigens allow a selective targeting of monoclonal antibodies. This is more effective, because each tumor may or may not express the same antigen. When prostate cancer metastasizes, it usually travels to the bone marrow or the lymph nodes. Metastases in these locations are more accessible to the circulating radiolabeled monoclonal antibodies. Often these new tumors are small which allows the antibodies to completely penetrate the metastases and effectively deliver the radiation to all tumor cells. Finally, PSA testing allows physicians to assess the effectiveness of the treatment. [10]

The Bystander Effect

The bystander effect is seen when irradiated cells release a factor that can produce damage or death in neighboring "bystander" cells that are not actually hit by any radiation. Researching the bystander effect will allow a better understanding of a process that may already be occurring during tumor radioimmunotherapy.

The bystander effect was first identified by Nagasawa and Little in 1992 [11]. According to their research, when 1% of Chinese Hamster Ovary (CHO cells were traversed by an alpha particle (0.31 mGy), over 30% of cells showed DNA damage in the form of a sister

chromatid exchange. When the same experiment was repeated with x-rays, a dose of 2 Gy was needed to produce the same effect. Confirmation of the bystander effect occurred 4 years later, when Deshpande *et al* [12] saw the effect using normal human lung fibroblasts and the same sister chromatid exchange endpoint. Micronucleus formation, a severe form of DNA damage due to chromosomal fragmentation, and apoptosis, a type of programmed cell death, are used as endpoints to assess the bystander effect with alpha particle irradiation [13-16]. Other researchers [17-19] demonstrate that the bystander effect can differ considerably depending primarily on cell type and the endpoint examined. Two mechanisms have been suggested for the bystander effect. One mechanism suggests that gap junctions are utilized for intercellular communication, requiring cell-to-cell contact, as discussed in [17-21]. The other mechanism, discussed in [16, 22-25] involves the irradiated cells releasing a soluble factor into the medium that can cause damage to the non-irradiated cells, assuming they are in the same medium. These studies [10-24] used normal cells or tissues; however, a few studies have been conducted using tumor cells. One such study [26] showed that human salivary gland tumor cells released nitric oxide into the medium after heavy ion irradiation, and the medium could inhibit cell growth and increase micronucleus formation in un-irradiated tumor cells. Wang and Coderre [27] performed a study using a co-culture system, showing that the bystander cells need to be present in the medium during the irradiation of the target cells and proposing that a short-lived radical species is involved.

Understanding the bystander effect may contribute to improving the effectiveness of radiation therapy treatments for metastatic prostate cancer. Currently, alpha particles are

used in boron neutron capture therapy (BNCT) and radioimunnotherapy. In BNCT, a high concentration of ^{10}B (boron-10) is delivered to a tumor relative to the surrounding healthy tissue. The tissue is then irradiated with thermal or epithermal neutrons. These neutrons become thermalized in the tissue. ^{10}B will capture a neutron and then the new ^{11}B particle will decay, releasing high linear energy transfer (LET) radiation according to the equation: ^{10}B + ^{1}n → ^{11}B → ^{7}Li + ^{4}He +2.79 MeV. (An alpha particle is a doubly charged helium ion.) These particles have a short range in tissue (5-9 um). Due to uneven ^{10}B concentrations and the short-range radiation, the tumor is usually subject to uneven irradiation. Radioimmunotherapy is the process in which radionuclides are delivered to the tumor site via tumor-specific monoclonal antibodies. Radioimmunotherapy is most effective against blood-borne tumors because the tumor antibody is not obstructed from the tumor [28]. Radioimmunotherapy may be very useful in micrometastatic disease because it is a systemic treatment, capable, in theory, of seeking out and destroying tumors too small to be detected by existing imaging technologies. Radioimmunotherapy does not work as well in solid tumors, because of non-uniform binding of the monoclonal antibody to the tumor [29]. Solid tumors may also be too big for complete penetration by the antibody [30, 31]. In BNCT and radioimmunotherapy, alpha particles may not hit all the tumor cells, thus creating bystander cells. Prostate cancer specifically does not respond well to traditional chemotherapy, so the effectiveness of radioimmunotherapy against prostate cancer is being assessed [7-9, 32-35]. The bystander effect, if it exists, is already present in these alpha-particle therapies, so understanding the bystander effect could allow it to be manipulated to increase the kill rate among tumor cells.

Wang and Coderre developed a co-culture system to test the presence of a soluble factor in medium creating a bystander effect [27]. This co-culture system allowed the target cells and bystander cells to be grown separately. The bystander cells were grown on an insert that was placed in the same media as the target cells before irradiation. Micronucleus formation was used as an endpoint to assess damage. Wang and Coderre showed the bystander effect is present in DU-145 cells after alpha particle irradiation. Medium irradiation and medium transfer experiments were conducted as well. Medium was irradiated and then added to the cells in the insert and incubated. No bystander effect was seen. Next, the medium was irradiated with the target cells present, the media was then removed, filtered, and added to the cells on the insert and incubated. Again, no bystander effect was observed. These two experiments indicate that the bystander effect, as seen under these conditions, is not purely a chemical effect. Experiments were also conducted with radical scavengers DMSO and PTIO to help identify the soluble factor responsible for the bystander effect. The results from these studies indicated that the soluble factor is a free radical other than nitric oxide. [27]

The Effect of Temperature on Enzyme Activity

Enzymes are catalysts, usually proteins, that bind to the reactant and speed up the reaction by lowering the activation energy or energy needed for the reaction to go forward). There is an ideal pH and temperature for each enzyme to work. Most enzymes work best at 37 °C or body temperature. As temperature increases, the kinetic energy of the molecules increases, thus more effective collisions occur and fewer molecules are needed for the same reaction rate. Simply, as temperature increases to a certain limit, enzyme activity increases. Each enzyme will have optimum activity in a certain

temperature range. Outside this range, the difference in temperature is enough to alter the structure of the enzyme. The temperature change provides enough energy to break the intramolecular attractions, such as hydrogen bonding or dipole-dipole interactions, or hydrophobic forces between non-polar groups in the protein. These changes cause a change in the secondary structure, 3D conformation of protein chains, and tertiary structure, interactions between the side chains of a protein. Often this conformational change can alter the active site, so it can no longer catalyze the substrate molecules it was supposed to catalyze. [36]

Specific Goals of this Research

This thesis was aimed at determining whether the short-lived radical species proposed by Wang and Coderre [27] is the result of a chemical or biological effect. Wang and Coderre's medium irradiation experiments indicated that this effect is not solely a chemical effect. This research studied the alpha-particle bystander effect on non-irradiated prostate tumor cells by examining the effect of temperature during irradiation and incubation to further determine whether the effect is chemical or biological. If the effect is biological, or based on an enzyme, there would be more of an effect at 37 °C and little or no effect at 4 °C. If the alpha bystander effect is due uniquely to a chemical effect, i.e. Ca^{+2} releases from damaged mitochondria, there should be no change in damage as temperature is varied. Chromosomal damage in the bystander cells was assessed at these different endpoints and compared to determine if temperature affected the bystander effect, and whether this effect is chemical or biological.

Materials and Methods

Wang and Coderre [27] developed a system, shown in figure 1, to examine the bystander effect due to a soluble factor using alpha particle irradiation. This system allows cells to be irradiated with alpha particles while maintaining bystander cells in close proximity (4 mm) in the same media, but beyond the range of the alpha particles. The cells to be irradiated are seeded on 1.4 um Mylar film in a custom cell dish and the bystander cells on a Snapwell insert. After 24 hours, the media is changed and the insert is placed upside down in the media of the Mylar dish. The cells on the Mylar are irradiated from below with different doses of alpha particles at either room temperature or 4 $^{\circ}$C using an Americium-241 source. After the irradiation, the Mylar dish with the insert is incubated at 4 or 37 $^{\circ}$C for 2 hours. Irradiation at room temperature and incubation at 37 $^{\circ}$C were the control conditions. The insert was then removed from the Mylar dish. Bystander cells were then harvested and a micronucleus assay was conducted. Chromosomal damage was assessed by comparing the percentage of binucleated cells containing micronuclei, counted under 400x magnification on a fluorescent microscope, in the non-irradiated and experimental samples.
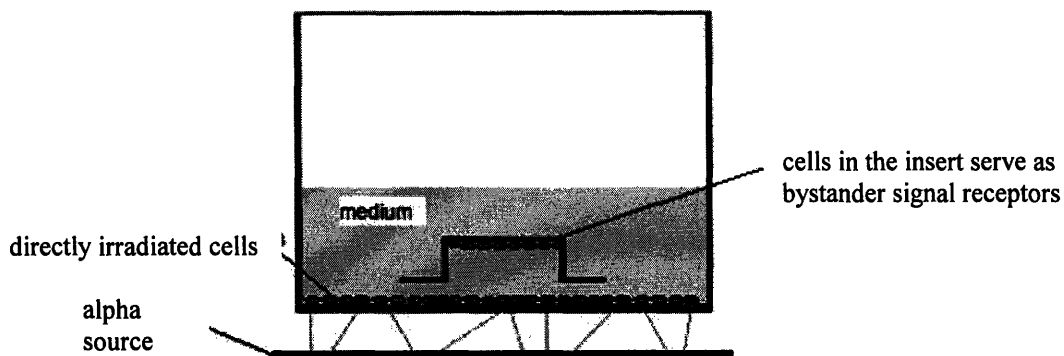
Figure 1. Schematic view of the co-culture system for the bystander effect study. The bystander cells were 4 mm above the directly irradiated cells. Source: Wang.

Cell Culture

The DU-145 human prostate carcinoma cell line was ordered from American Type Culture Collection (passage number 57). After their arrival, the cells were cultured for three passages then frozen in cryovials (~ 10^7 cells per vial) and stored in liquid nitrogen by Wang [27]. These cells were defrosted and used for 11 passages in the experiments described in this paper. Cells used in experiments were between 2 and 11 passage numbers. Cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 with Eagle's Minimum Essential Medium containing Earle's Balance Salt Solution. The nutrients were added to the media in the following concentrations: 2mM L-glutamine (MEM/EBSS; HyClone), 1.0 mM sodium pyruvate (SH30239.01, HyClone), 0.1 mM nonessential amino acids (SH30238.01, HyClone), 1.5 g/L sodium bicarbonate (SH30033.01, HyClone) and 14% fetal bovine serum (FBS; Sigma). The cell doubling time was about 34 hours. The media was changed every 3-4 days and cells were passaged every week, when cells grew to confluence. Before passaging, the trypsin and the media

were warmed to 37 °C in a water bath. To passage, cells were first washed with 2 ml of

trypsin and then 1 ml of trypsin was added to cover the cells. The cells were incubated for

3 minutes at 37 °C and 5% CO_2. Fresh media was added to make a total volume of 10 ml.

The solution was pipetted up and down to make a single cell suspension. Approximately

10^5 cells were seeded into a new T75 flask with 10 ml fresh media and placed in the

incubator.

Mylar Dishes

Stainless steel cell culture dishes were machined similar to the design in [37]. The dishes

used were the same ones used by Wang and Coderre [27]. Wang acquired the thinnest

Mylar film commercially available (Steinerfilm, Inc., Williamstown, MA), which was 1.4

um, to use for the bottoms. The Mylar dish was 4.05 cm tall and had a growth area of

11.4 cm^2 (3.81 cm inner diameter). Stainless steel dishes with a 1.4 µm Mylar bottom for

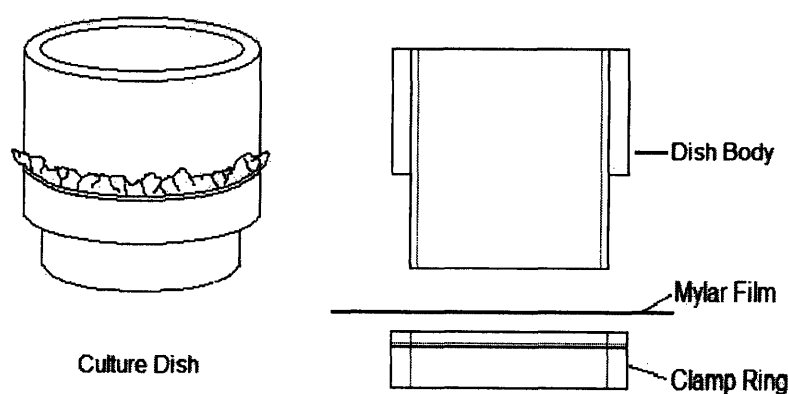the cell growth were assembled as shown in Figure 2.

Figure 2. Schematic view of the Mylar dish. The Mylar dish was composed of three parts:
the dish body, 1.4 µm-thick Mylar film and a clamp ring to tighten the Mylar film as dish
bottom. Source: Wang.

First, the stainless steel cell culture dishes were fitted with 1.4 um Mylar bottoms. The dishes were autoclaved to create a tight seal of the Mylar dish. The high pressure and temperature of the autoclave also sterilized the Mylar dish so the cells would not be contaminated for the experiment.

Irradiation system

The irradiation system was designed by Wang [27]. There was a 5 mm air gap between the Americium-241 alpha particle source and the Mylar dish bottom, as seen in Figure 3.
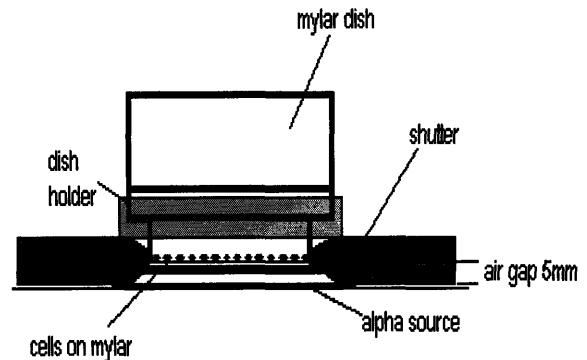

Figure 3. Schematic view of the alpha-particle irradiation system. There was a 5 mm air gap between the Mylar membrane that the cells were grown on and the alpha particle source. Source: Wang (2005).

Experimental Procedure

Cells were seeded onto Mylar dishes or Snapwell inserts. The cells were irradiated, and then co-incubated at different temperatures. Next the cells were seeded onto 2-well chamber slides. Finally, a micronucleus assay was conducted.

Seeding the Cells on the Mylar Dishes and Snapwell Inserts

Previous to the start of the experiment, DU-145 cells were grown to confluence according to the technique described above (see "Cell Culture"). On Day 1 of the experiment, the cells were seeded on the Mylar dishes and the Snapwell inserts (Corning Inc, available through Fischer Scientific Education at www.fishersci.com, number 3407). The polycarbonate Snapwell inserts have a growth area of 1 square centimeter. The membranes were porous to allow diffusion of particles smaller than 0.4 um. The cells did not adhere well to the Mylar, so the stainless steel Mylar culture dishes were treated with 0.5 ml of FNC coating mix from AthenaES (Athena Environmental Sciences, Inc. Baltimore, MD) for 30 seconds. Immediately after FNC treatment of the Mylar, 3 ml of media was added to the dish. Cells were trypsinized as described above (see "Cell Culture") and a single cell suspension was made. The concentration of the cells in this solution was evaluated by using a Z2 Coulter Counter from Beckman Coulter. This machine counts the cells and displays the concentration of the solution. This procedure was performed three times and the mean of the three concentrations was taken as the final concentration of the solution. This concentration was used to seed 4-5 x 10^5 DU-145 cells on the Mylar dishes and 4-5 x 10^4 cells on the Snapwell inserts. The dishes and the inserts were placed in the incubator at 37 $^\circ$C for 24 hours to allow the cells to attach before irradiation.

Irradiation

The next day, dishes and inserts were removed from the incubator and the media in the Mylar dishes was changed. Fresh media, either pre-warmed to 37 $^\circ$C or cooled to 4 $^\circ$C, was added to the Mylar dishes. Using a pair of sterile tweezers, also sterilized in the autoclave, the plastic support rings were removed from the Snapwell inserts. The inserts

were then placed upside down in the media in the Mylar dishes, taking care to not create any air bubbles. This system was co-incubated for 10 minutes in a VWR cold box (4 °C) or an incubator (37 °C, 5% CO_2). The Mylar dish was placed on the alpha particle source and the target cells were irradiated for a given dose.

Co-Incubation

The cells on the insert were co-incubated in the medium in the Mylar dish at either 4 °C or 37 °C for two hours.

Seeding the Cells Onto the 2-Well Chamber Slide

After two hours, the Snapwell inserts were removed from the Mylar dishes, placed in T-25 cell culture dishes and 1 ml of fresh media was added to each Snapwell insert and inserts were returned to the incubator (37 °C, 5% CO_2). Cells were trypsinized and the solution was pipetted to create an evenly distributed single cell suspension. These cells were seeded onto a two well chamber slide (Lab-Tek II, Nalge Nunc Int., Rochester, NY), each with 2 ml fresh media. Slides were incubated at 37 °C, 5% CO_2 for 3-5 hours to allow the cells to attach.

Micronucleus Assay

A cytokinesis-block technique was used to assess micronuclei (MN) [38]. A chemical called cytochalasin-B was used for the micronucleus assay. Cytochalasin-B blocks the cell cycle at the point where the binucleated cell separates into two daughter cells (cytokinesis). The cells were incubated with this chemical for one and a half doubling times to allow bi-nucleated cells (BN) to accumulate. A micronucleus was formed when chromosomal fragmentation occurred. This was a sign of cellular damage. This process is

shown in Figure 4. Figure 5 shows a picture under different magnifications using an
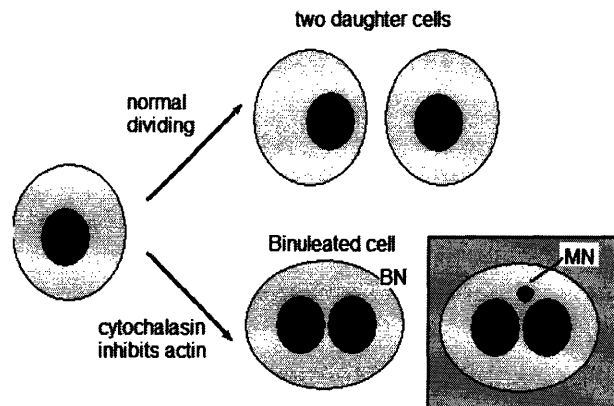
Olympus BX51 epi-fluorescence microscope.

Figure 4. The formation of a micronucleus and binucleated cell. In the process seen on the top, normal division of the cell will produce two distinct daughter cells, each with a single nucleus. When incubated with cytochalasin-B, an actin inhibitor, a binucleated cell results, as shown by the process on the bottom. When chromosomal fragmentation occurs, it will produce one or more micronuclei (a piece of chromosome) within the binucleated cell. Source: Wang.
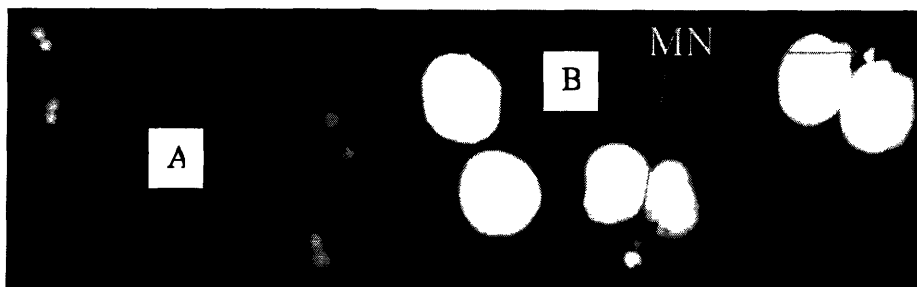

Figure 5. Photograph of binucleated cells (BN) with and without micronucleus (MN). The cells were stained with DAPI and recorded by an Olympus BX51 epi-fluorescence microscope. A) 100 x magnification. B) 400× magnification. Source: Wang.

While waiting for the cells to attach to the chamber slides, a 3 µg/ml solution of

cytochalasin-B (Sigma) in media was made and placed in the incubator. After 3-5 hours,

the media in the chamber slides was changed and 2 ml of the cytochalasin-B media was added to each chamber. The slides were incubated for 42 hrs at 37 °C, 5% CO_2. After 42 hrs the slides were removed from the incubator and washed twice with 1x phosphate buffered saline (PBS). Cells were fixed to the slides with a 4 °C 3:1 solution of methanol: acetic acid in a 4 °C environment for 10 minutes. The process was repeated and the slides were washed with PBS twice. After the second washing, the slides were allowed to dry for 5-10 minutes. The slides were stained with 1 ml of 10 µg/ml DAPI (Sigma-Aldrich) for 10 minutes, and washed with PBS. The chambers were removed and slides were allowed to dry completely. A drop of anti-fade reagent (Fluoro Guard, Bio-Rad Laboratories) was placed on each half and a cover slip was placed on the slides with a little bit of pressure to evenly distribute the anti-fade reagent and get rid of any air bubbles. The slides were sealed with nail polish. The slides were examined under 400X magnification on an Olympus BX51 epi-fluorescence microscope. The ratio of micronuclei to binucleated cells was counted for 1000-2000 cells. The slides were able to be stored at 4 °C for 2-3 months.

Data Analysis

Experiments were repeated 4-6 times. Each experiment produced two samples, n=2; one for each side of the 2-well chamber slide. The ratios of MN/BN were determined from each experiment and compiled into a Microsoft Excel spreadsheet. The mean, standard deviation, confidence interval, T and a student's T-test were calculated using Microsoft Excel. A 95% confidence interval was used to assess the reproducibility of the experimental results.

Results

The first step in this experiment was to reproduce the bystander effect as seen in the control set up by Wang and Coderre [27]. The control set up was an irradiation at room temperature with a co-incubation at 37 °C, 5 % CO_2. Next, variations of the experiment were conducted by changing the irradiation temperature and the incubation temperature. A micronucleus assay was conducted to assess damage through comparing the ratio of MN to BN.

For multiple experiments with control conditions, n=12, the mean damage for a 0 Gy dose was 5.24%. The 95% confidence interval, as calculated by Microsoft Excel, was 0.00135. When the same system was given a dose of 1.2 Gy, the mean damage, as assessed by the MN to BN ratio, was 9.29%. The 95% confidence interval, as calculated by Microsoft Excel, was 0.00520. These values are shown in Figure 6. The student's t-test between these two values, 1.41×10^{-9}, showed that these values were statistically different (p<0.05).

Frequency of Micronuclei in Binucleated Cells for Room Temperature Irradiation and 37 °C Incubation (Error Bars Confidence Interval Excel)
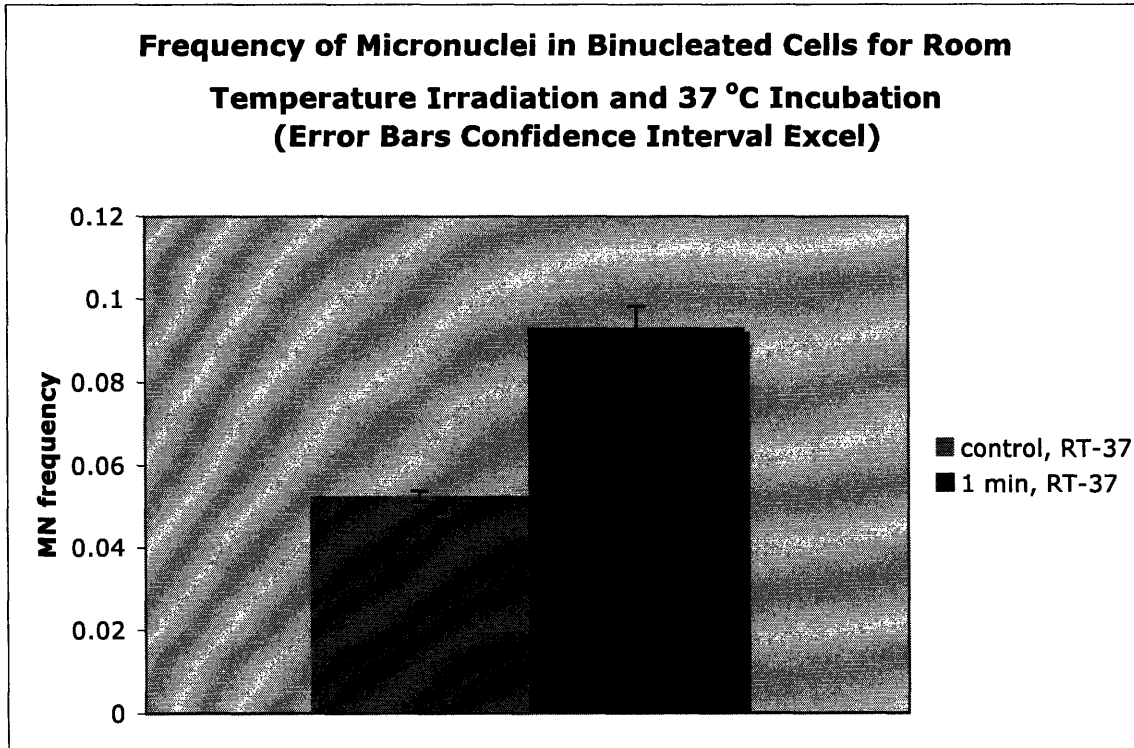
Figure 6. Mean frequency of micronuclei in binucleated cells for room temperature irradiation and 37 °C incubation. Error bars shown are for a 95% confidence interval. The mean was taken from 12 samples.

4 °C Irradiation – 4 °C Incubation

The first experiment involved an alpha particle irradiation at 4 °C and incubation at 4 °C. Figure 7 shows the frequency of MN per BN cells for this system. For 12 samples, the mean damage for a dose of 0 Gy was 2.19%. For a dose of 1.2 Gy with the same number of samples, the mean damage was 3.33%. The confidence intervals for these samples were 0.00636 and 0.00690, respectively. The student's t-test between the 0 Gy and 1.2 Gy doses was 0.0136, showing that the damage values are statistically different ($p < 0.05$).
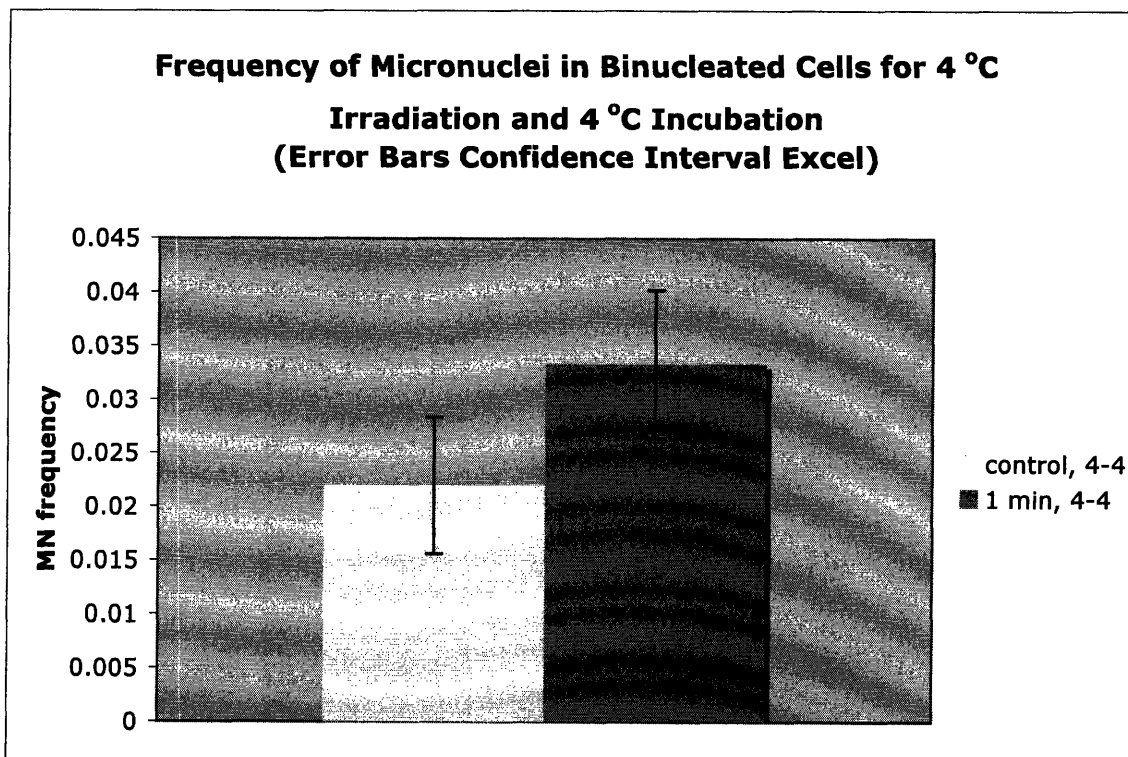
Figure 7. Mean frequency of micronuclei in binucleated cells for 4 °C irradiation and 4 °C incubation. Error bars shown are for a 95% confidence interval. The mean was taken from 12 samples.

4 °C Irradiation – 37 °C Incubation

The second experiment involved an alpha particle irradiation at 4 °C and incubation at 37 °C. There were 10 samples taken for this case. The MN to BN ratios for the 0 Gy and 1.2 Gy were very similar, 3.21% and 3.78%, with 95% confidence intervals of 0.00804 and 0.00964, respectively. The student's t-test showed the values for damage not to be statistically different (p>0.05). Figure 8 shows the frequency of MN to BN for this experiment.
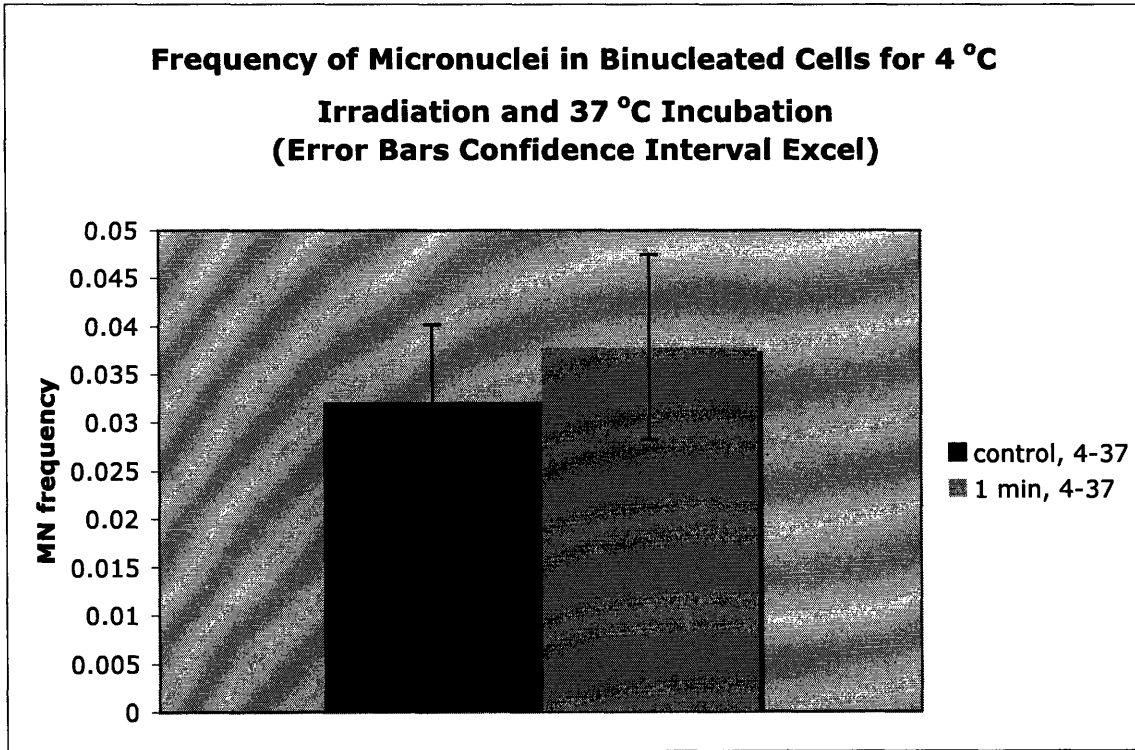
Figure 8. Mean frequency of micronuclei in binucleated cells for 4 °C irradiation and 37 °C incubation. Error bars shown are for a 95% confidence interval. The mean was taken from 10 samples.

Room Temperature °C Irradiation – 4 °C Incubation

In the third and final experimental case, eight samples were irradiated at room temperature and incubated at 4 °C. The frequency of MN to BN was shown to be statistically different for a dose of 0 Gy, 3.40%, and 1.2 Gy, 5.70%, by the student's t-test value of 1.12×10^{-7} (p<0.05). The 95% confidence intervals were 1.89×10^{-3} for 0 Gy and 3.46×10^{-3} for 1.2 Gy. Figure 9 shows the frequency of MN to BN for this experimental case.
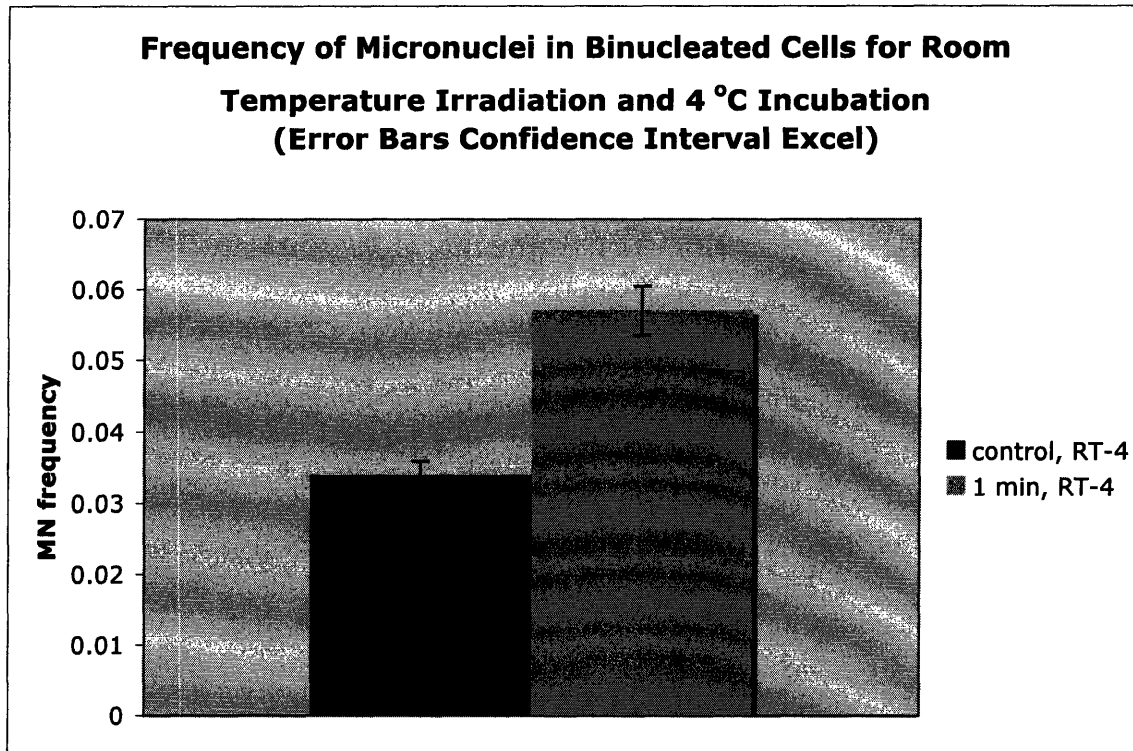
Figure 9. Mean frequency of micronuclei in binucleated cells for room temperature irradiation and 37 °C incubation. Error bars shown are for a 95% confidence interval. The mean was taken from 8 samples.

Further Analysis

The control was an irradiation at room temperature and incubation at 37 °C, 5 % CO_2. For the control set up, the student's t-test showed that the damage between the 0 Gy and 1.2 Gy samples were statistically different (p<0.05). This result proves that there is a bystander effect occurring.

In two of the three experimental cases a bystander effect was produced. A student's t-test showed that the ratios of MN to BN, for the dose of 0 Gy compared with 1.2 Gy, were statistically different (p<0.05) for the room temperature irradiation with 4 °C incubation and 4 °C irradiation with 37 °C incubation. For the experimental case of 4 °C irradiation and 4 °C incubation, the MN to BN ratios, for a dose of 0 Gy and a dose of 1.2 Gy, were

shown to be statistically insignificant (p>0.05) by a student's t-test. This indicates that for the condition of 4 °C irradiation and incubation there is no bystander effect. These results show that temperature does affect the bystander effect.

Comparing the damage, as assessed by the frequency of MN to BN, shows temperature affects the bystander effect. The damage ratio for a 1.2 Gy irradiation at 4 °C and an incubation at 4 °C is 3.33% compared to 3.78% for the same irradiation conditions with an incubation at 37 °C. Figure 10 shows these frequencies. The student's t-test shows these ratios for irradiations at the same temperature of 4 °C but varying incubation temperatures, 4 and 37 °C, to be statistically similar. These results indicated that for low irradiation temperature, the irradiation temperature is the crucial step and the incubation temperature is less significant, as supported by the media transfer experiments by Wang and Coderre, [27], as discussed in the introduction.
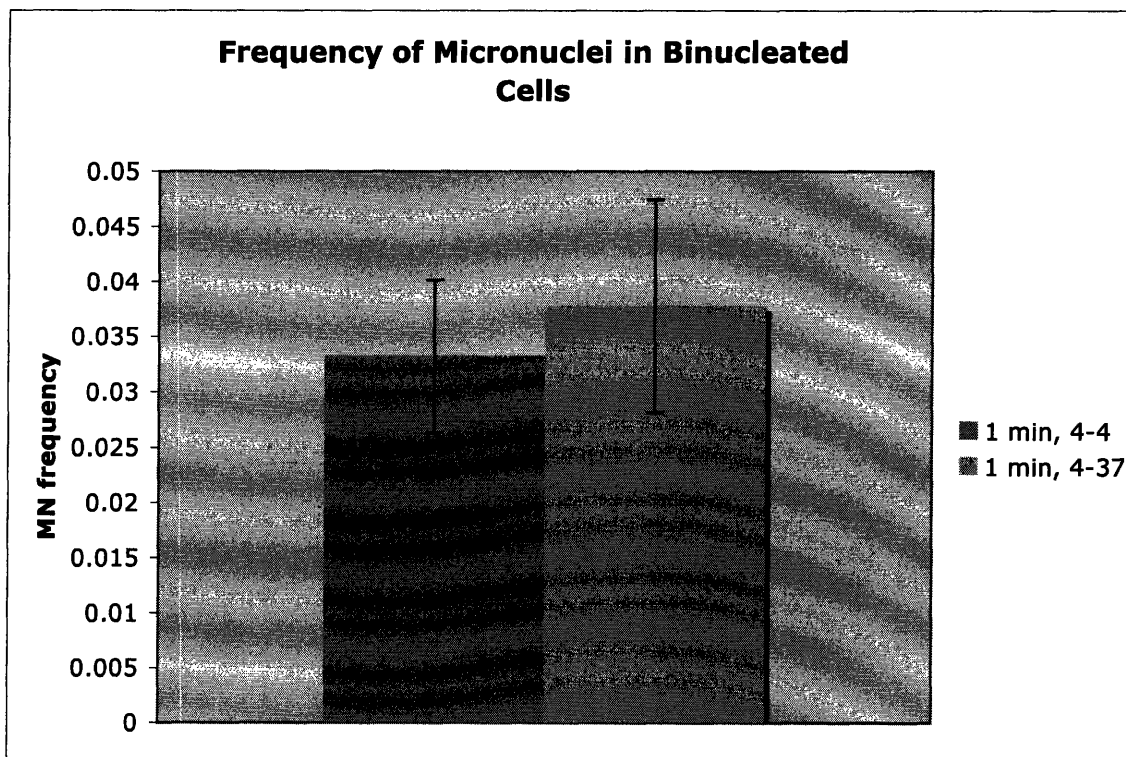
Figure 10. Mean frequency of micronuclei in binucleated cells for 1.2 Gy dose 4 °C irradiation and varying incubation temperature. Error bars shown are for a 95% confidence interval.

As described above, for irradiations at 4 °C, the incubation temperature did not make a significant difference in damage. This is not the case for 1.2 Gy irradiations at room temperature. For a case of 1.2 Gy room temperature irradiation and incubation at 4 °C, 5.70% MN frequency, or 37 °C, 9.29% MN frequency, not only are the damage ratios statistically different as calculated by a student's t-test (value 9.49×10^{-10}, $p < 0.05$) but the frequency of MN increases by more than a third when the incubation temperature is increased from 4 °C to 37 °C. The frequency of MN to BN for this case is compared in Figure 11. Also, a student's t-test shows that the frequencies of MN to BN for a 0 Gy room temperature irradiation with a 37 °C, 5.24%, and 1.2 irradiation at room temperature and incubation at 4 °C, 5.70%, are barely statistically different (0.0192, $p < 0.05$). These values, shown in Figure 12, show that the decrease in incubation

temperature decreases the damage to almost the same level as the control values observed

for the irradiations at room temperature.

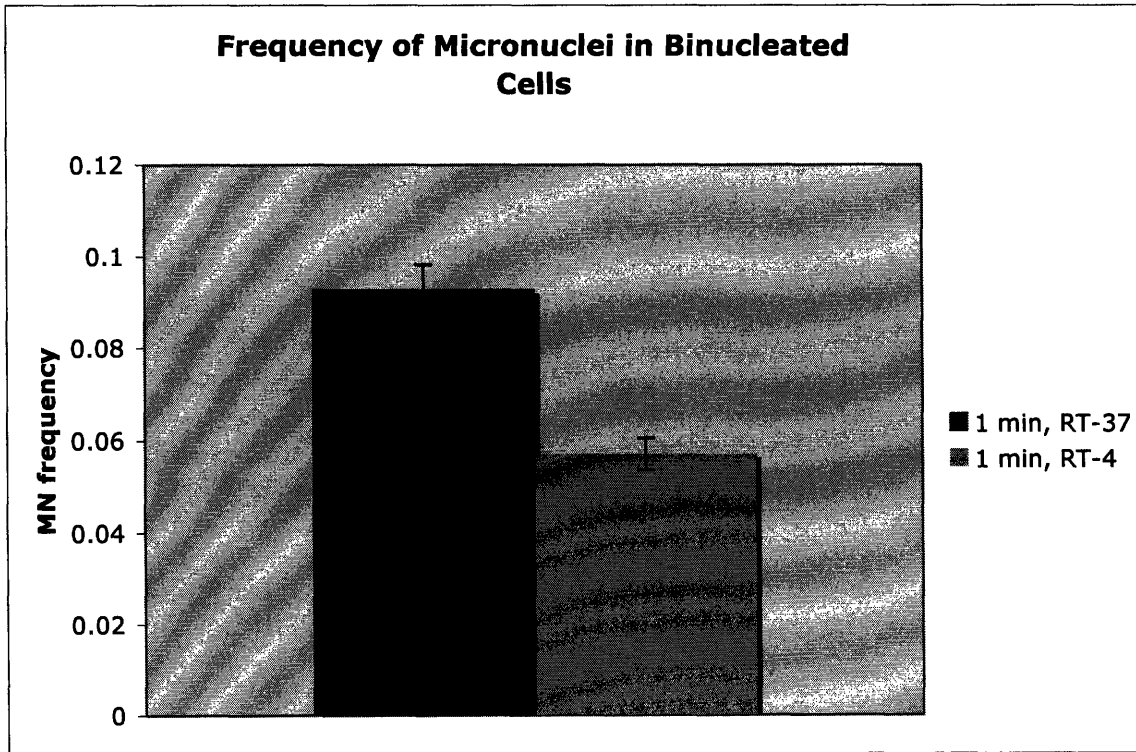

Frequency of Micronuclei in Binucleated Cells

Figure 11. Mean frequency of micronuclei in binucleated cells for 1.2 Gy dose room temperature irradiation and varying incubation temperature. Error bars shown are for a 95% confidence interval.

Frequency of Micronuclei in Binucleated Cells

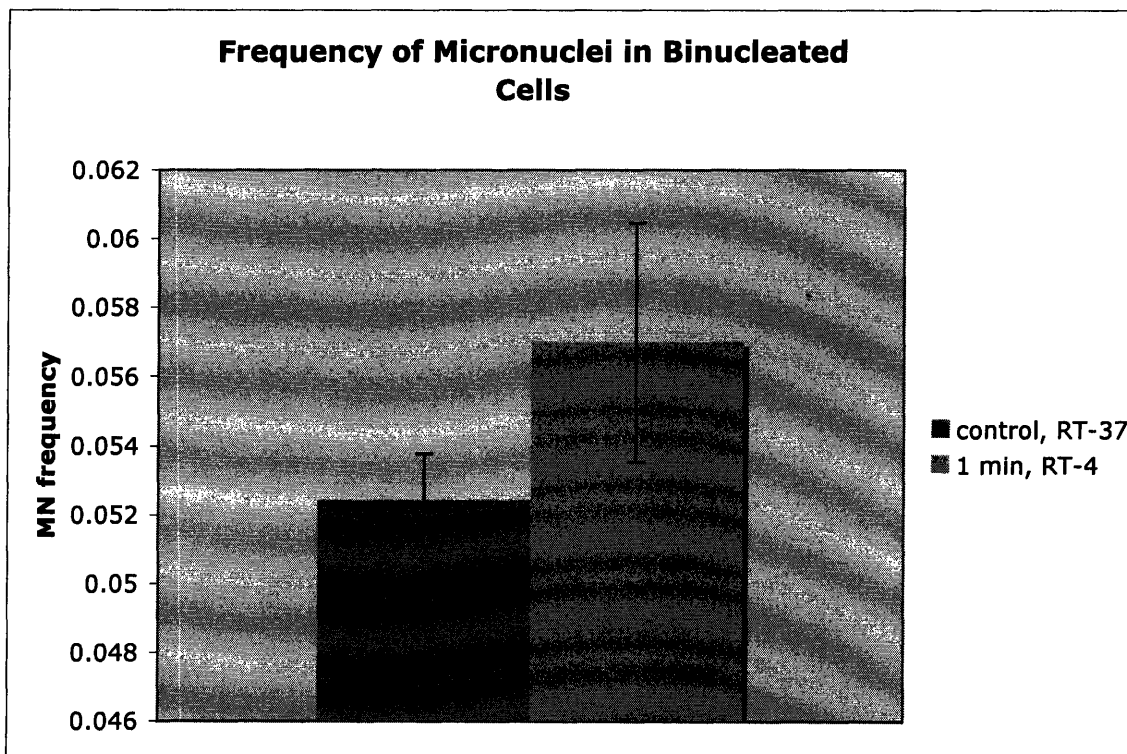Legend: ■ control, RT-37; ■ 1 min, RT-4

Figure 12. Mean frequency of micronuclei in binucleated cells for varying dose room temperature irradiation and varying incubation temperature. Error bars shown are for a 95% confidence interval.

The next case to compare is different irradiation temperatures with the same incubation temperature. For a 1.2 Gy dose, the mean damage seen for a room temperature irradiation and 37 °C incubation is 9.29% compared with 3.78% for a 4 °C irradiation and 37 °C incubation. These two values are shown in Figure 13. A student's t-test showed these values to be statistically different (5.51×10^{-8}, $p < 0.05$). These results show that there is less damage, and therefore less of a bystander effect, when the irradiation temperature is decreased and the incubation temperature is held constant.

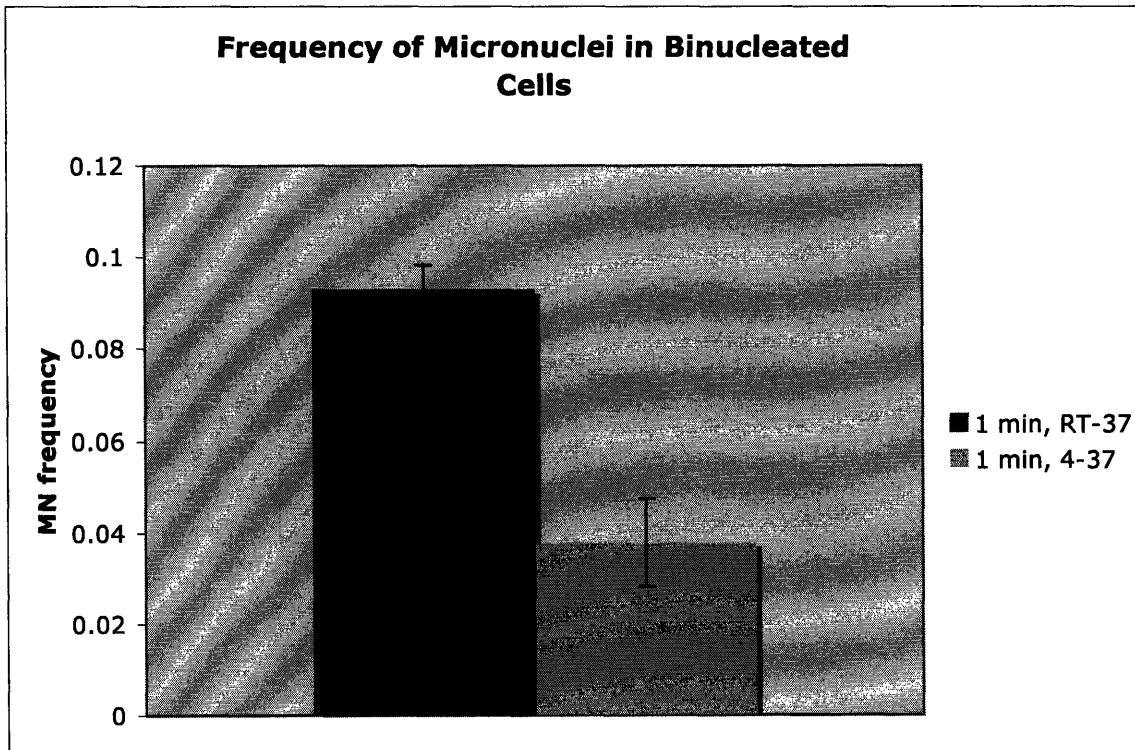**Frequency of Micronuclei in Binucleated Cells**

Figure 13. Mean frequency of micronuclei in binucleated cells for 1.2 Gy dose with varying room temperature irradiation and 37 °C incubation temperature. Error bars shown are for a 95% confidence interval.

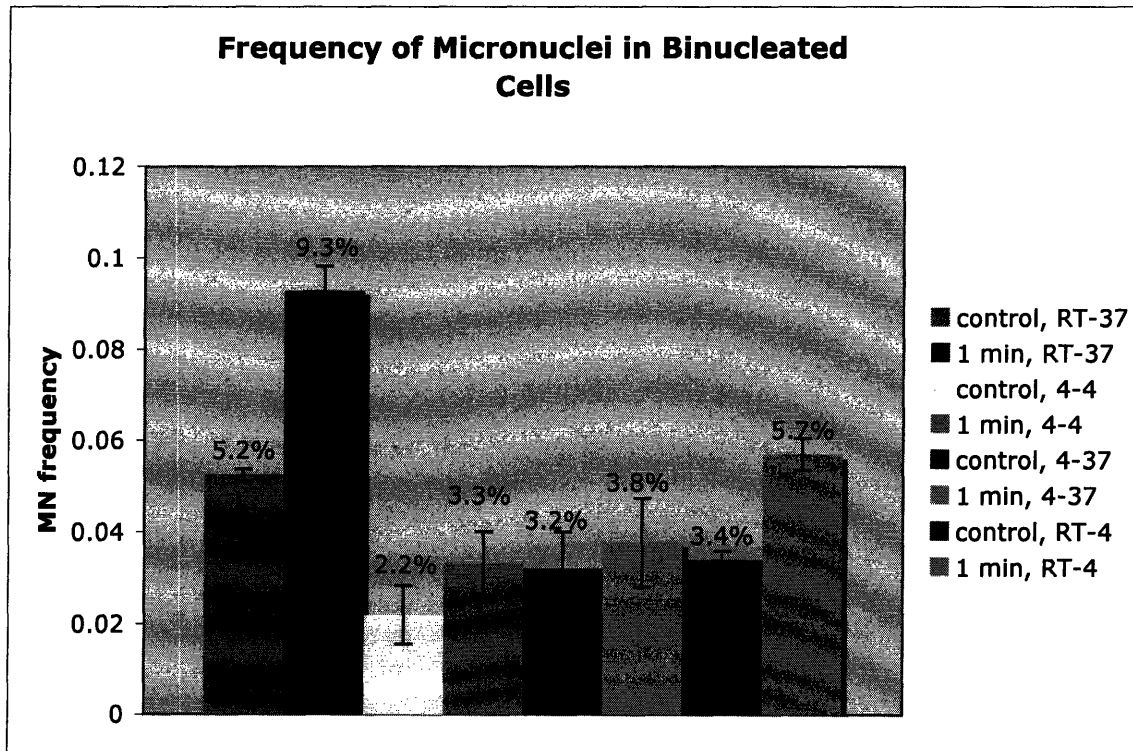Figure 14 summarizes the results from all the experiments.

Figure 14. Mean frequency of micronuclei in binucleated cells for all conditions. Error bars shown are for a 95% confidence interval.

Discussion

The results presented above indicate that temperature affects the bystander effect. For experiments conducted with the irradiation temperature at 4 °C, no bystander effect was seen. Examining the trend, there is less damage when the irradiation is conducted at 4 °C rather than room temperature, suggesting the bystander effect decreases as temperature decreases. Even in the non-irradiated control samples, there is less damage for the conditions at 4 °C than the room temperature or the 37 °C samples. Like the data seen in this experiment, enzyme activity slows as temperature decreases, especially at 4 °C. Moreover, a decrease in temperature, especially in incubation temperature, lessens or inhibits the bystander effect. This correlation indicates that enzyme activity is involved in the bystander effect, and thus the bystander effect is a biological effect. It suggests that an

enzyme is creating a short-lived signal. To examine the hypothesis further, more experiments should be conducted with irradiations at 37 °C. Preliminary experiments (2 samples), with both the irradiation and incubation at 37 °C, show a low level of damage, 3.5% MN frequency, for a dose of 0 Gy and a high level of damage, 11.2% MN frequency, for a 1.2 Gy dose. The MN frequency in the control for the irradiation and incubation at 37 °C is lower than the MN frequency in the control for the irradiation at room temperature and the incubation at 37 °C. These date are not in agreement with the other results in the thesis. A new study should be conducted with these conditions and the results should be compared with the data from this study to more thoroughly conclude that enzyme activity is involved in the bystander effect.

Assuming enzyme involvement, the next goal would be to identify the enzyme. An enzyme is a type of protein and proteins are usually identified by gel electrophoresis. SDS could be used to denature the proteins in two solutions: one a solution of fresh media and the other the media from a just irradiated system (this is assuming that the short-lived particle/signal identified by [27] is related to enzyme activity). The solutions would then be run side by side with a molecular weight marker on a gel by electrophoresis. The two lanes could be compared to distinguish the protein unique to the irradiated solution. This protein could be compared to the molecular weight marker to help identify the molecular weight of the protein. This band with the protein could then be cut out of the gel and purified for further analysis.

If it is not the enzyme itself, but a product produced by the enzyme that triggers a pathway, another method should be used to identify the specific signaling pathway.

Recently, the cyclo-oxygenase 2 (COX-2) gene has been implicated in bystander effect signaling [39]. Upregulation of the COX-2 gene leads to increased production of prostaglandins, which have been associated with a variety of effects. The COX-2 signaling pathway also activates the mitogen-activated protein kinase (MAPK) pathways, such as extracellular signal-related kinase (ERK). Using microarray analysis of bystander cells to show overexpression of the COX-2 gene and NS-398, a COX-2 inhibitor, to suppress COX-2 activity, Zhou et al showed that the COX-2 signaling pathway is a critical signaling event for producing a bystander effect [39]. Use of drugs to inhibit specific signaling pathways, such as COX-2 inhibitors, could be combined with the temperature studies of the alpha-particle bystander effect to see if there is any correlation.

A better understanding of this bystander effect will allow for the development of more effective cancer treatments that utilize alpha particle irradiation. Cancer treatments that utilize alpha particle irradiation already create bystander cells. The ability to harness this bystander effect would allow scientists to develop treatments to kill more of the target (tumor) cells, while being even more sure about not damaging the surrounding healthy body tissue.

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