Testing Oxygen Depletion as a Mechanism for the FLASH Effect Using Ultra High Dose Rate Electron Beam Irradiation

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Abstract

FLASH radiotherapy is a technique of delivering radiation at an ultra high dose rate. Studies of animal models irradiated at ultra high dose rates show that the technique suppresses tumor growth while reducing negative side effects of radiation as compared to radiation delivered at a conventional dose rate, a response called the FLASH effect. While this effect has been shown in many in vivo studies, in vitro cell survival assays, and in veterinary studies, the mechanism by which it works is unknown. Possible mechanisms include the inter-track hypothesis and the transient oxygen depletion hypothesis, which attribute reduced DNA damage with FLASH radiotherapy to radical recombination or hypoxic radioresistance. This report discusses preliminary tests for two experiments exploring these hypotheses through the lens of varying oxygen conditions in irradiated water and cell models. These tests establish biological and physical considerations for future experiments.

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NEVIS LABORATORIES
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Contents

1 Introduction

Radiotherapy is a key component of many cancer treatment regimens. It can be delivered internally with an internal beam or radioactive material, or it can be delivered externally, via external beam radiation therapy [1]. External beam radiation therapy uses different modes of radiation beams produced by a linear accelerator to attack cancer cells by causing DNA and cellular damage faster than the cancer cell's impaired ability to repair itself. Current methods focus on using 3D imaging to optimize beam placement and modulating beam intensity, dose distribution, and angle to reduce radiation impacts on normal tissue.

Radiation can be delivered using a variety of sources, including proton, x-ray, gamma ray, and electron beams. Each type of beam has a different Bragg curve, which illustrates the energy deposited by the particle as it travels through a material. As a result, the dose and depth of the beam varies depending on the type of irradiation [1]. Beam penetration is lower for photon beams, including x-rays and gamma rays, as well as for electrons. Proton irradiation can reach greater tissue depths, and its Bragg peak is at a greater depth compared to other modes of radiation, so more energy is deposited deeper in the irradiated tissue with proton beams [2]. In this report, electron beam irradiation will be relevant because the linear accelerator in use at the Radiological Research Accelerator Facility (RARAF) at Columbia University's Nevis Laboratories uses electron beams to deliver radiation.

1.1 Clinac

Linear accelerators for medical purposes are small scale accelerators. They generate and use microwave power to accelerate electrons along the length of the accelerator and direct them towards a target [3]. A radiofrequency (RF) driver generates microwaves, which are amplified by a vacuum tube called a klystron. The klystron is powered by a pulse forming network, which is composed of connected inductive and capacitive elements that store energy and discharge it. Energy is discharged when a component called a thyratron, which is a gas-filled high power switch, conducts current.The amplified signal from the klystron is transmitted to the accelerator cavity to generate an oscillating electric field.

In the accelerator, there is an electron gun with a filament that becomes ionized when heated, producing electrons. As they move through the cavity, the oscillating electric field causes the electrons to form bunches to create a beam with uniform energy. The beam is focused and directed using magnetic steering coils along the accelerator cavity. At the end of the waveguide, magnetic coils are used to bend the beam 270 degrees and direct it toward the target. At the head of the beam, there is an ionization chamber to measure the intensity of the beam. There can also be an x-ray target for electrons to scatter via Bremsstrahlung, producing x-ray photons. The beam finally reaches the target, or the patient on the bed if the accelerator is in clinical use.

1.2 FLASH Radiotherapy

The goal of radiotherapy is to kill cancer cells. This can occur by causing irreparable DNA damage to cells. Radiation can ionize DNA directly, or radiation can ionize water, causing water radiolysis [4]. The products of water radiolysis include radicals, which can react with dissolved oxygen in cells to produce reactive oxygen species. These chemicals are dangerous because they can oxidize DNA to compromise its structure and produce double stranded breaks. Since cancer cells can have damaged DNA repair pathways, this kind of DNA damage can prevent cancer cells from surviving or dividing. Even though normal cells have better DNA repair pathways, they can still be killed from irradiation.

One factor limiting the efficacy and maximum dose of radiotherapy is damage to healthy tissues surrounding a tumor [2]. FLASH radiotherapy is a method of delivering radiation at an ultra high dose rate (UHDR), which has been tested for its ability to reduce healthy tissue damage compared to conventional (CONV) dose rate irradiation. The healthy cell sparing observed with FLASH irradiation is called the FLASH effect. FLASH dose rates can be greater than 30 Gy/s all the way to the order of 100 Gy/ms, while conventional dose rates are on the order of Gy/min. Radiation at an ultra high dose rate can be delivered with any type of beam. Gamma ray and electron irradiation of healthy mice with FLASH dose rates resulted in less lung fibrosis compared to mice irradiated with CONV dose rates [5]. The same study found that FLASH irradiation exhibited the same suppression of growth as CONV irradiation in human breast tumor xenografts and injected lung tumors in mice. The FLASH effect has been observed in neurocognitive function, as well. In a series of cognitive tests for memory, anxious behavior, and depression-like behavior, mice irradiated with FLASH dose rates at doses below 14 Gy performed better than those irradiated with CONV dose rates [6]. FLASH radiotherapy has been used to cure one patient with cutaneous T-cell lymphoma and is currently undergoing a clinical trial to treat basal and squamous cell carcinomas that will be completed in 2026 [7, 8].

1.3 Mechanisms of the FLASH Effect

While several mechanisms for the effect are hypothesized, the exact method is not well understood [9]. Proposed mechanisms seek to explain how FLASH irradiations can cause reduced DNA damage in cells. One possible explanation is the transient oxygen depletion hypothesis, where FLASH irradiation results in increased interactions between radicals produced from water radiolysis and dissolved oxygen that deplete oxygen in cells. This creates a transient hypoxic environment, and hypoxic environments are known to confer radioresistance to cancer cells. Tumors can become hypoxic environments, since the requirement for oxygen consumption and access to blood supply changes as cancer cells proliferate [4]. Cancer cells get more distant from blood vessels, receiving less oxygen and forcing them to undergo lactic acid fermentation and reduce the pH of the environment. Cancer cells can also prompt the growth of new, weak vasculature, which can produce an acute hypoxic environment even in regions close to the new blood vessels. As a result of hypoxia, cancer cells can experience less damage in response to radiation compared to cells in normoxic environments. In normoxic environments, when DNA is damaged, oxygen can bind to DNA to stabilize the damaged molecule and prevent it from being repaired [10]. In hypoxic environments, there is not enough available oxygen to stabilize the damaged DNA. Hypoxia can also cause cells to induce DNA repair pathways and produce molecules that inhibit immune cells to contribute to their radioresistance. With FLASH irradiation, normal cells experience local hypoxia, contributing to cell sparing, but cancer cells have an even more dramatic decrease in oxygen, which can cause more damage.

Both in vitro and in vivo experiments testing FLASH irradiation with different oxygen environments rates shed light on how dose rate could affect cellular damage by way of modulating the oxygen environment of cells. In one in vitro study, prostate cancer cells were incubated in hypoxic and normoxic conditions and irradiated with FLASH and CONV dose rates [11]. Cells under hypoxia experienced a sparing effect compared to those incubated in normal oxygen conditions, and this effect was amplified with decreasing concentrations of oxygen in the environment. The study also found that cells irradiated with FLASH dose rates had increased survival compared to those irradiated with CONV dose rates when the dose delivered was greater than 18 Gy. Montay-Gruel et al 2019 describes observations of increased oxygen exposure limiting neuroprotective effects of FLASH irradiation, showing a correlation between oxygen environment and the FLASH effect in vivo [6]. In the same study, FLASH irradiation of water samples equilibrated in a hypoxic environment resulted in

lower hydrogen peroxide production compared to CONV dose rate irradiated samples.

Another possible mechanism is inter-track interactions of radical species [9]. Since FLASH irradiation involves quick repeated pulses that produce multiple tracks in tissue at once, it causes a high local concentration of radicals that can interact with each other, becoming neutral molecules before they are able to oxidize important biological molecules. This hypothesis explains the effect of FLASH irradiation on hydrogen peroxide production in water samples. This report will describe a few preliminary tests in experiments exploring these hypotheses through water radiolysis and cell irradiation.

2 Methods

2.1 Modified Clinac

Irradiations were performed using the UHDR irradiator in RARAF, which is based on a decommissioned Varian Clinac 2100C that was previously in clinical use [12]. The Clinac can deliver electron beams ranging in energy from 6-20 MeV at a pulse repetition of 180 Hz. Each energy has different beam penetrations, with higher energy beams depositing radiation deeper in a sample. Since the irradiator is not in active clinical use, modifications like removing the bed, disabling gantry rotation so the beam is perpetually directed upward, and creating a scaffold for sample placement in the beam path could be made.

In order to make the machine suitable for UHDR irradiation, changes to the timer interface card were made to allow for more control of the synchronization of the klystron with the electron gun to control beam pulses better. The conversion target was removed, preventing Bremsstrahlung production of photons from the electron beam. Scattering reduces the efficiency of the 15 MV x-ray beam since many electrons need to hit the target in order to produce enough photons for the beam. With the target removed, the 15 MV setting produces a 15 MeV electron beam with many more electrons compared to the electron beam modes of the machine. FLASH irradiations were performed using this 15 MeV electron beam at a dose rate of 100-1000 Gy/s. CONV dose rate irradiations were performed using the 9 MeV electron beam at a dose rate of 1-2 Gy/min.

For CONV dose rate irradiations, the automatic frequency control (AFC) setting was on to reduce the phase difference between the resonance frequency of the RF driver and the accelerator cavity [13]. For FLASH irradiations, radiation was delivered using the 15 MeV electron beam. An external voltage of 500 mV was applied using a Digilent Analog Discovery 2 digital oscilloscope as a function generator for cell FLASH irradiations. AFC is not available for the 15 MeV beam, so the external voltage contributes to the AFC's role of stabilizing the beam power. No external voltage was applied for water FLASH irradiations.

2.2 Dosimetry

For CONV dose rate irradiation, dosimetry was performed using an Advanced Markus Ion Chamber and UNIDOS E electrometer. The dose reading by the ion chamber is adjusted according to the temperature and pressure of the accelerator hall as well as the beam energy setting. For the 9 MeV and 15 MeV beams, the difference in dose readings is $< 2\%$. It has been previously determined that the ion chamber produces an accurate reading for dose rates up to 0.5 Gy/pulse, so Gafchromic EBT3 and EBT-XD films are used for dosimetry at higher dose rates. EBT3 films are optimized for doses between 0.1 cGy and 10 Gy while EBT-XD films are optimized for doses between 0.4 cGy and 40 Gy and were used during water irradiations for doses up to 100 Gy.

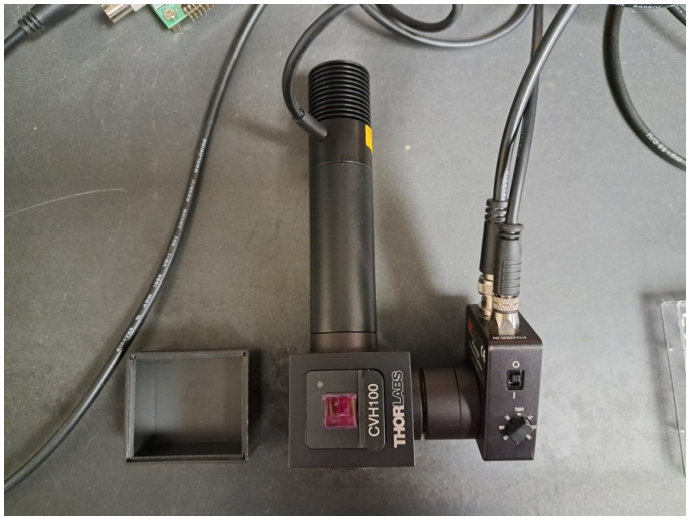

Figure 1: *Fluorescence intensity measurement setup. An LED at the peak excitation wavelength is attached to a cuvette holder with the sample. Light shines through a filter and is received by the detector on the right.*

Films were placed below samples and irradiated during experiments to make dose measurements. Irradiation causes the films to change color, where the optical density of the films post-irradiation is correlated to the dose delivered to the film based on an initial calibration with the ion chamber. Films were scanned using an Epson Perfection V850 Pro scanner. Optical densities from the scanned films were translated to doses to create dose maps using a MATLAB program written by Dr. Guy Garty.

2.3 Water Radiolysis

2.3.1 Dihydrorhodamine 123 Staining

Hydrogen peroxide concentrations of irradiated water samples were measured to compare the production of reactive oxygen species between samples irradiated with CONV and FLASH dose rates. In order to quantify hydrogen peroxide levels in the irradiated samples, hydrogen peroxide standards were created with different concentrations of H2O2 and dihydrorhodamine 123 (DHR 123) dye diluted with water. DHR 123 was chosen as a dye because it is oxidized by hydrogen peroxide to produce rhodamine, which is fluorescent, allowing the relative concentration of hydrogen peroxide in the original solution to be determined [14]. The peak excitation and emission wavelengths for rhodamine are 507 nm and 529 nm, respectively.

Preliminary tests were performed using different concentrations of hydrogen peroxide and DHR 123 to determine an optimal range of concentrations for hydrogen peroxide standards as well as an optimal concentration of dye for testing hydrogen peroxide concentration following water radiolysis. The range of concentrations tested for hydrogen peroxide was 3 uM to 15 M, and the range of DHR 123 concentrations tested was 10 uM to 1.28 mM. The DHR 123 concentration chosen for adding to water samples prior to irradiation was 30 uM.

Fluorescence of the samples was measured by shining a 470 nm LED through 1.5 mL of each sample through a cuvette (Fig. 1). The emitted light passed through a 540 +/- 10 nm filter and was collected by a THORLabs PDA100A Si Amplified Detector, which employs a photodiode to produce an output voltage proportional to the light it collects. fluorescence intensity data was recorded from the output voltage of the detector using a digital oscilloscope. The fluorescence intensity is

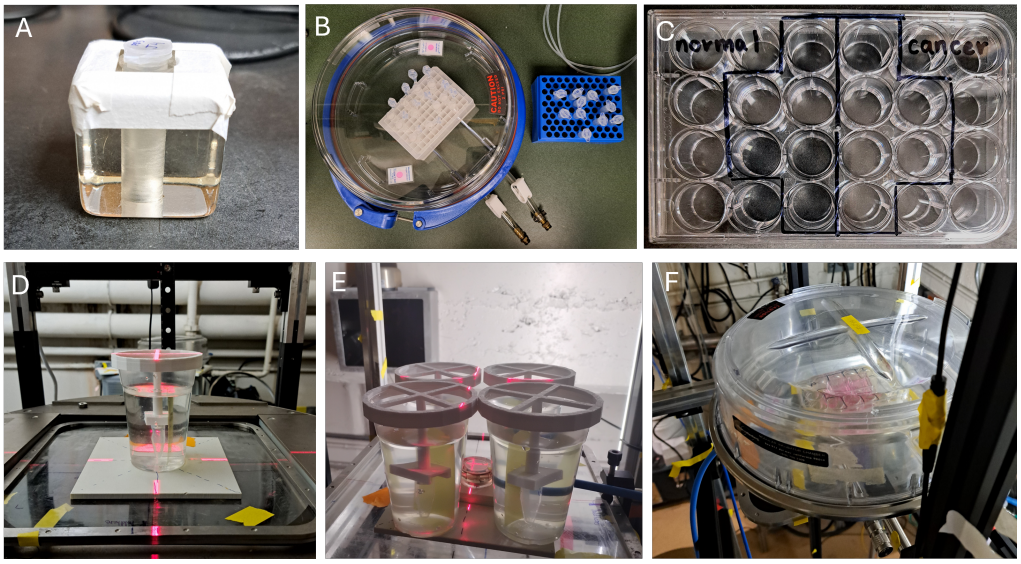

Figure 2: *Experimental setups and sample placements for water and cell irradiations. For the DHR123 assay, water samples were irradiated in solid water blocks wrapped in foil (A). For the Amplex Red assay, water was equilibrated in hypoxic and normoxic conditions prior to irradiations (B). Cells were irradiated in 24 well plates using only the center 12 wells to ensure uniform beam exposure (C). Water samples were irradiated in cups of water one at a time at SSD=20 for FLASH (D) and four at once at SSD=170 for CONV (E) with films attached vertically. All cell irradiations were done in incubator chambers at SSD=170 (F).*

proportional to the concentration of hydrogen peroxide in the sample.

Water samples with DHR 123 at a concentration of 30 uM were irradiated in solid water blocks using a 9 MeV electron beam at a dose rate of about 5 Gy/s (Fig. 2A). Doses ranged from 2.7 Gy to 105.8 Gy. fluorescence intensity values were recorded as the difference between fluorescence intensity measured before irradiation and 15 minutes post-irradiation.

2.3.2 Amplex Red Staining

Hydrogen peroxide concentrations were compared between water samples irradiated with CONV and FLASH dose rates in normoxia and hypoxia conditions. LCMS grade water in microcentrifuge tubes was equilibrated in normoxic or hypoxic conditions for 20 hours prior to irradiation. The hypoxia condition was created by purging a Billups-Rothenberg modular incubator chamber, flushing it continuously with nitrogen gas for 75 minutes with open microcentrifuge tubes inside the chamber (Fig. 2B). Samples under normoxia were kept in closed microcentrifuge tubes.

Water samples were irradiated in the range of 0 to 100 Gy using both FLASH and CONV dose rates. Radiation was delivered at dose rates of 0.9 to 1.3 Gy/ms for the FLASH irradiations and a dose rate of about 2 Gy/min for the CONV dose rate irradiations. During irradiation, samples were held in place by a custom holder that was designed and 3D printed using PLA (Fig. 2D,E). The holder was placed in a plastic cup such that the base of the microcentrifuge tube was suspended less than 1 cm above the bottom of the cup, and the cup was filled with water to act as a water tank. EBT-XD films were attached vertically to the holder to measure dose lengthwise across the microcentrifuge tube. The 9 MeV beam for CONV irradiations has a penetration depth of about 4.3 cm, and the 15 MeV beam for FLASH irradiations has a penetration depth of about 7 cm. Since the tubes are 4 cm in height, electrons from both beams were able to penetrate the entirety of the

sample.

Due to variability in the 15 MeV beam power over the course of FLASH irradiations, the actual doses the samples were irradiated with were not consistent with the intended doses, so the delivered doses were between 0 and 80 Gy. For CONV dose rate irradiations, four setups were irradiated at once, with two samples inserted or removed at a time according to the final dose delivered to each sample (Fig. 2E). As a result, 100 Gy and 60 Gy samples were irradiated discontinuously.

The ThermoFisher Amplex Red Assay kit was used to measure H2O2 concentration in irradiated water samples. In the presence of peroxidase, the Amplex Red reagent reacts with hydrogen peroxide to form resorufin, which has excitation and emission peaks at 571 and 581, respectively. Hydrogen peroxide standards were created with hydrogen peroxide concentrations ranging from 0 to 10 uM in sodium phosphate buffer. Amplex Red and horseradish peroxidase was added to the water samples at 45 minutes post irradiation. The solutions had a concentration of 16.67 uM Amplex Red, previously determined to be optimal by Montay-Gruel et al 2019 [6]. Samples were incubated protected from light and fluorescence intensity was measured 120 minutes post-irradiation. fluorescence intensity was measured using the setup described above, with a 530 nm LED and 590 +/- 40 nm filter (Fig. 1).

2.4 Cell Preparation and Irradiation

Frozen IMR90 normal lung fibroblast cells and A549 cancer cells were thawed and incubated at 37 C for 24 hours. Cells were then trypsinized and passaged onto 24 well plates according to the setup shown in Fig. 2C.

In order to study the impact of oxygen levels on the FLASH effect, cells were incubated in normoxia and hypoxia conditions and irradiated using FLASH and CONV dose rates. The hypoxia condition was created with the chamber described above, with the chamber being purged for 15 minutes before closing off its tubes. For each oxygen condition, there was a 24 well plate that was irradiated at a FLASH dose rate, CONV dose rate, and an unirradiated control plate. All cell irradiations, including both FLASH and CONV irradiations were performed at an SSD of 170 cm (Fig. 2F). Both normoxia and hypoxia plates were irradiated to approximately 11 Gy in an incubator chamber. For FLASH irradiations, plates were irradiated at a rate of 100 Gy/s, and for the CONV dose rate irradiations, plates were irradiated at a rate of 1 Gy/min.

2.5 Clonogenic Assay

Following irradiations, cells were collected with trypsin and [] and counted. High and low concentrations of cells were chosen and plated on petri dishes for colony counting. All plates were incubated for 7 days and then fixed and stained with Crystal Violet for counting to assess cell survival. Colonies with over 50 cells were counted, and cell survival was determined by dividing the number of colonies by the number of plated cells scaled by the plating efficiency.

3 Results

In order to compare the production of reactive oxygen species between water samples irradiated with FLASH and CONV dose rates, water samples were irradiated with different final doses and fluorescence intensity was measured using two different dyes.

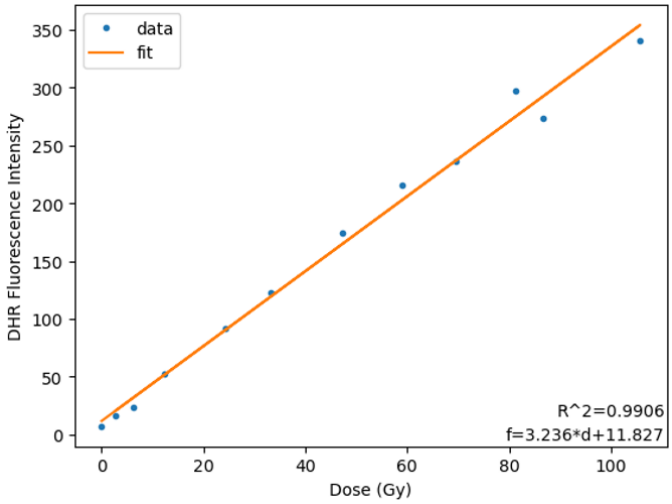

Figure 3: *Fluorescence intensity for irradiated water samples with DHR 123. Measured fluorescence intensity for water samples irradiated with DHR 123 is shown as blue dots. A linear fit is shown in orange, and the equation describing the fit is shown.*

3.1 DHR123 Hydrogen Peroxide Assay

DHR 123 was added to water samples prior to irradiation, and fluorescence measurements were taken immediately after irradiation. Fluorescence intensity and dose have an almost linear relationship, as indicated by the R^2 value for the linear fit (Fig. 3). When the data is fit to a line, we get an equation for the relationship between DHR fluorescence intensity and dose with the specifications of this setup. This indicates that DHR123 does react with hydrogen peroxide post-irradiation in a predictable manner, reflecting the dose that was delivered to samples. However, when creating hydrogen peroxide standards to compare fluorescence intensity to, there was difficulty in producing standards whose fluorescence was predictably related to hydrogen peroxide concentration. Water samples without hydrogen peroxide had as strong of a signal as samples with hydrogen peroxide, indicating that the purity of the water samples, exposure to light, or another chemical reaction was interfering with clean standard measurements.

3.2 Amplex Red Hydrogen Peroxide Assay

Amplex Red reagent was added to irradiated water samples 45 minutes after the beginning of irradiation. Measurements of fluorescence intensity were taken 2 hours after cells were incubated with the reagent. Fluorescence intensity is a relative measurement of peroxide concentration because hydrogen peroxide reacts with the Amplex Red reagent to create a fluorescent product. Interestingly, water samples incubated in normoxic conditions had less fluorescence relative to samples incubated in hypoxic conditions (Fig. 4). It was expected that under normoxic conditions, there would be more dissolved oxygen with which radicals in the water could interact and form hydrogen peroxide. It was also observed that samples irradiated with CONV dose rates had slightly less fluorescence compared to those irradiated with FLASH dose rates. This did not support results from other experiments that show that FLASH dose rate irradiation produces lower concentrations of hydrogen peroxide compared to CONV dose rate irradiation.

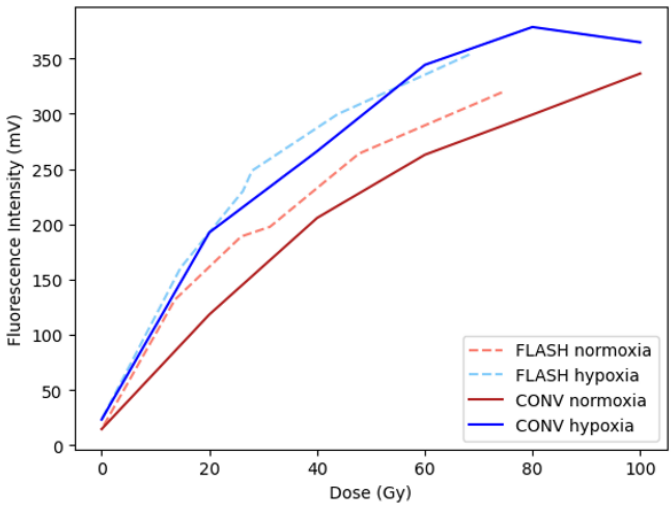

Figure 4: *Fluorescence intensity of irradiated water samples with Amplex Red reagent. Each curve contains 6 data points corresponding to samples irradiated at 5 different doses along with an unirradiated control. CONV data are shown with solid lines and FLASH data are shown with dashed lines. Normoxia sample data are shown in red and hypoxia sample data are shown in blue.*

3.3 Clonogenic Assay

Healthy and cancer lung cells were incubated in normoxic and hypoxic conditions and irradiated at different dose rates. Following irradiation, they were counted and plated to test for cell survival 1 week post irradiation. For healthy cells, the average survival fraction was greatest for cells incubated in the hypoxia chamber and irradiated with FLASH dose rates (Fig. 5). This was expected since the hypoxic environment would compound the sparing effect of FLASH.

Cancer cells had lower average survival compared to healthy cells, showing that CONV and FLASH dose rate irradiations both reduced cancer cell proliferation. Cancer cells incubated in the hypoxia chamber experienced lower survival, which is not consistent with the result from Adrian et al 2020 that prostate cancer cells incubated in a hypoxic environment had higher survival fractions at both dose rates [11]. This could be due to the harsh environment of the hypoxia chamber.

4 Discussion

4.1 Water Irradiations

The Amplex Red assay and DHR assay were preliminary tests for measuring H2O2 concentrations following irradiation. This is an important measurement because hydrogen peroxide is implicated in causing DNA damage, and it is a product of water radiolysis [15]. DHR123 has been tested as a dye for measuring reactive oxygen species in cells, but this experiment tested its efficacy as a dye in water [14]. DHR123 produced a large background signal when added to unirradiated water. The reaction between hydrogen peroxide and DHR123 in the calibration standards took at least 4 hours to produce a signal that could be differentiated for low concentrations of peroxide. One aspect of the Amplex Red assay is that horseradish peroxidase was included to catalyze the reaction between the reagent and hydrogen peroxide. It is possible that peroxidase was also required to improve the rate and progression of the reaction between DHR123 and hydrogen peroxide. Another possibility is that inconsistent fluorescence intensity measurements with varying DHR123 concentrations may be due

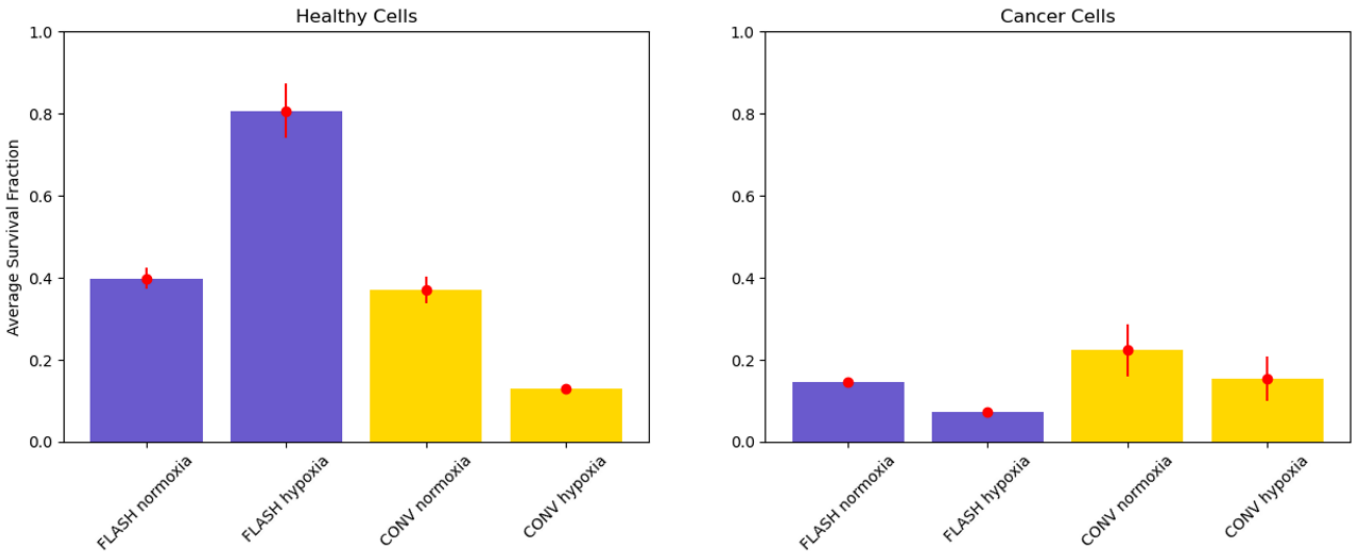

Figure 5: *Average survival fractions 1 week post irradiation for healthy and cancer cells incubated in different oxygen environments and irradiated at FLASH and CONV dose rates. Each value was calculated as the average survival fraction of two plates for each condition, and error bars indicate standard deviation. Cells irradiated with FLASH are shown in blue and cells irradiated with CONV dose rates are shown in yellow.*

to DHR123's role as a quencher of rhodamine, meaning that excess DHR123 decreases sensitivity of rhodamine [16].

These results seem to indicate that DHR123 can only be successfully used in cells to detect reactive oxygen species. However, DHR123 is currently being tested in clay based dosimeters for its reaction with hydrogen peroxide in irradiated water, so it is possible to use the reaction with DHR123 and hydrogen peroxide to determine dose [16]. In the test reported here, fluorescence intensity changed proportionally with dose, but it was difficult to reduce the background noise in order to produce standards that allow accurate determinations of dose in the irradiated samples. The background signal could be further reduced by increasing water purity in the samples and protecting them better from light. With more accurate standards, DHR123 could be used for dosimetry or for redoing this assay, since the linear relationship between fluorescence and dose indicates that peroxide concentration could be predicted easily if the dose is known.

More replicates of the Amplex Red assay are needed to distinguish the effects of hypoxia and FLASH on hydrogen peroxide production during water radiolysis. Other ways to improve this assay would be to add the Amplex Red reagent closer to the end of irradiation and continue to optimize the stability of the electron beam. This could make the fluorescence intensity measurements more consistent with the results from Montay-Gruel et al 2019, which shows hydrogen peroxide concentration to be directly proportional to dose and a clear difference between hydrogen peroxide concentration with FLASH and CONV irradiations [6]. With these changes, the Amplex Red assay could be used to measure hydrogen peroxide concentrations with more accuracy than the DHR123 assay.

Beyond measuring peroxide concentration, a similar assay could be performed to measure hydroxyl radical concentration immediately after irradiation. Another way of studying the radical-radical recombination hypothesis involves using Monte Carlo simulations to predict interactions between reactive oxygen species to understand how they cause cell damage and how FLASH irradiation may impact cells differently [17]. Simulations can model interactions much faster than measurements can be taken. Instantaneous reactions can be predicted in a way that is not possible with chemical assays,

which are limited by irradiation time and the time needed to incubate samples with a reagent.

4.2 Cell Irradiations

The water and cell irradiation experiments allowed us to test a preliminary setup for the hypoxia incubator chamber. The hypoxia chamber setup could be improved by using a device or assay to measure the oxygen concentration in the chamber. Placing cells in a hypoxic environment also subjected them to stress, which caused a change in the pH of the cell media overnight. This may have contributed to the low plating efficiency of cells in the hypoxia group. Redoing this experiment with the cells incubated in the hypoxia chamber for less time and using a device to measure oxygen concentration may help increase the plating efficiency of cells in the hypoxic condition. This is necessary to ensure that there are enough colonies to count 1 week post-irradiation. Additionally, measuring survival fraction with cells irradiated with different doses would provide more insight to the impact of dose on oxygen level and cell survival since oxygen depletion is dependent on dose and available oxygen [11]. The next step following cell irradiations is an organoid irradiation experiment. While cells provide a 2D model to understand the FLASH effect, an organoid model would be 3D and be derived from tumor and healthy cells from a real patient.

5 Summary and Conclusions

The FLASH effect is well characterized in several animal models and cell cultures, but its mechanism is unclear. The goal of the preliminary tests for water irradiation and cell irradiation described in this report was to test possible mechanisms by varying oxygen conditions. Oxygen conditions are known to have a role in mediating the FLASH effect, with less production of reactive oxygen species and hypoxia increasing the cell sparing effect of FLASH. By testing two dyes for measuring hydrogen peroxide concentration in water, the background signal with water and respective protocols could be compared. The DHR123 assay would require reduction of the background signal to create usable standards, but the Amplex Red assay could be performed again with few changes to streamline the process. Performing a clonogenic assay allowed us to establish a protocol for irradiating cells in a hypoxic environment. These preliminary tests contribute to the development of protocols for future experiments, working toward organoid irradiation.

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