Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Zitong Su April 12, 2022

Evaluating a Time Course of Recovery of DNA Damage in HBEC-3KT Cells Following X Ray and Neutron Irradiation

by

Zitong Su

William S. Dynan, PhD
Adviser

Chemistry

William S. Dynan, PhD

Adviser

David G. Lynn, PhD

Committee Member

R. Brian Dyer, PhD

Committee Member

2022

Evaluating a Time Course of Recovery of DNA Damage in HBEC-3KT Cells Following X Ray and Neutron Irradiation

By

Zitong Su

William S. Dynan, PhD

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Chemistry

2022

Abstract

Evaluating a Time Course of Recovery of DNA Damage in HBEC-3KT Cells Following X Ray and Neutron Irradiation

By Zitong Su

Humans are increasingly becoming space-faring creatures. But with that travel comes exposure to galactic cosmic radiation (GCR) and its associated dangers. Especially concerning is GCR's ability to cause double stranded breaks (DSB) in DNA. Therefore, it is critical to study the effects of GCR on DNA damage in order to develop therapeutics. To do this, a local radiation source where high-throughput screening can be conducted is needed. This study evaluates the ability of a deuterium-deuterium (DD) neutron generator at the Georgia Institute of Technology to serve as such a resource. It is hypothesized the neutron irradiation will not only cause a biological effect, but the nature of which will be markedly different than that of a reference X ray irradiation. Human bronchial epithelial cells (HBEC-3KT) were irradiated by the DD generator at 1, 2, and 3 Gy and allowed to recover for 0.5 and 6 h. The DNA damage was compared to a reference X ray source, which irradiated cells at 1 Gy with 0.5, 2, 6, and 24 h recovery times. Compared to X ray radiation, neutron radiation generated a lower number of DSBs, but damage was more clustered and complex. This was evidenced by the slower recovery time and visual differences of neutron-caused damage compared to that of X ray. Overall, the DD neutron generator was indeed able to cause a biological effect, and its pattern of irradiation was distinct from a reference X ray radiation.

Evaluating a Time Course of Recovery of DNA Damage in HBEC-3KT Cells Following X Ray and Neutron Irradiation

By

Zitong Su

William S. Dynan, PhD

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Chemistry

2022

Acknowledgements

I'd like to thank the team at the Georgia Institute of Technology, Dr. Nolan Hertel, Sharmistha Mukhopadhyay, and Victoria Irvin for their help in the neutron irradiation. Thank you for doing all the calculations and optimizations needed to generate a dose and for running the generator during the irradiation. Without your tireless work this project wouldn't have been possible. Thank you also for hosting me and allowing me to use your space to conduct my experimentation.

I'd like to thank Jeff Switchenko from Emory University for his help in conducting the statistical tests for my data. Without your help, interpreting my results would've been much harder. Thank you also to Alexis Chan from my own lab for her consistent assistance throughout the entire project. Alexis ran the X ray generator, tagged along on every trip to the Georgia Institute of Technology for irradiation to assist, created the two dot plots of repair foci, and helped with cell culture.

I'd like to thank my committee members, Dr. David Lynn and Dr. Brian Dyer for not only agreeing to serve on my committee but also for being great professors in classes that I've thoroughly enjoyed during my undergraduate experience.

I'd like to thank my advisor Dr. Willian Dynan, without whom this project wouldn't have been possible. Dr. Dynan was a fantastic mentor, keeping me on track and motivating me to execute the project even when things went awry. He has been an invaluable resource, helping me troubleshoot and guide me through every turn and bump in the road. I can't thank him enough.

Lastly, I'd like to thank my friends and family for their support behind the scenes and in all aspects of my life. Without their presence, I would not have the success I have enjoyed in this project and in the rest of my life.

Thank you all.

Table of Contents

Introduction

A. Galactic Cosmic Radiation

As humanity becomes a multiplanetary species, space travel will become increasingly common. With that travel comes exposure to galactic cosmic radiation (GCR), which originates from outside our solar system, and solar cosmic radiation (SCR), which comes from the Sun. Without the protection of our atmosphere, humans in deep space will be exposed to a much greater degree of this radiation (Rodman et al., 2017). Galactic cosmic radiation can range from high energy photons (i.e., X rays and gamma rays) to protons, neutrons, and high charge and energy (HZE) atomic nuclei such as ^{28}Si and ^{56}Fe. Although a small fraction of GCR overall, these HZE particles are of great concern given that they deposit more concentrated tracks of ionization than other types of radiation when traversing living tissue (Kennedy et al., 2018). It is critical to understand the effects of chronic low dose exposure to galactic cosmic radiation on living organisms during long-haul space travel.

One of the main concerns about the dangers of GCR is their ability to break bonds and cause chemical changes, which can then lead to biological effects. This can affect the functioning of proteins as well as cause damage in DNA in the form of single and double stranded breaks – the latter of which are particularly deleterious because they are hard to repair (Maliszewska-Olejniczak et al., 2021). Since DNA dictates almost everything about how an organism functions and is also the hereditary material that is passed down through the generations, it is imperative to investigate exactly what kinds of DNA damage are caused by different sources of radiation and evaluate the rate of recovery and repair. This study will focus on two types of radiation: X rays and neutrons.

B. Deposition of Radiant Energy

Energy from ionizing radiation is not deposited uniformly in the absorbing medium but is rather found along tracks created by the individual particles. The pattern of these tracks depends on the type of radiation and linear energy transfer (LET), which is the average amount of energy transferred per unit length of the track, typically expressed in units of keV/micron (Rossi, 1960). Low LET radiation deposits energy in a dispersed manner, causing ionization events throughout the absorbing medium, not just along the ionization tracks. On the other hand, high LET radiation deposits energy in a concentrated manner, causing ionization events more strictly along the ionization tracks (Maliszewska-Olejniczak et al., 2021).

Quantity of radiation is expressed in terms of absorbed dose with the unit gray (Gy), which is a measure of the amount of energy absorbed per unit mass of tissue (Gy = J/kg). However, equal doses of different radiation types do not lead to equal biological effects due to the pattern of energy deposition (i.e., 1 Gy of neutrons produces a larger biological effect than does 1 Gy of X rays) (Rossi, 1960).

C. X Ray and Neutron Radiation

X rays are highly energetic, massless photons that are indirectly ionizing: while the photons cannot produce any biological or chemical effect themselves, they can transfer that energy to another particle that is able to. This happens when the electrons of an atom absorb the X ray radiation via the photoelectric effect and eject an energetic electron (Little, 1968). These electrons can then go on to directly interact with DNA, or can first interact with another atom/molecule (often water) to produce free radicals that then go on to interact with DNA (indirect) (Little, 1968). X rays have low LET and are sparsely ionizing, depositing energy in a dispersed manner in tissue (Kennedy et al., 2018).

Neutrons are neutral, subatomic particles that, along with protons, make up the nucleus of atoms. Whereas X rays interact exclusively with the electrons of an atom, neutrons undergo a greater variety of nuclear reactions in biological targets (Maliszewska-Olejniczak et al., 2021). Neutron radiation can generate recoil protons – which are nearly 2,000 times more massive than electrons – and high energy secondary electrons that can extend laterally for several microns as they traverse tissue (Kennedy et al., 2018). At the energies used in this study, neutrons have high LET values and are densely ionizing, leaving behind concentrated tracks of radiation (Little, 1968).

Relative to low LET radiation, high LET radiation creates a tightly clustered and complex mixture of DNA damage that is more challenging to repair (Kennedy et al., 2018). Specifically, high LET radiation can create multiple double stranded breaks (DSB) in close proximity, as well as DSBs accompanied by single stranded breaks (SSB) and oxidative base damage. While low LET radiation can also create DSBs, the damage is less extensive and less complex overall. That is why the biological effects of DNA damage increase with the LET value of radiation (Maliszewska-Olejniczak et al., 2021).

D. DNA Repair Foci

Radiation damages DNA by causing single and double stranded breaks. SSBs are of little consequence as they can be repaired easily using the complementary strand as a template. DSBs, on the other hand, can cleave chromatin in two, and are extremely dangerous and difficult to repair lesions (Asaithamby & Chen, 2011). Enough unrepaired DSBs can eventually lead to cell death and carcinogenesis. Breaks in the phosphodiester backbone of DNA are single molecule events, something too small to visualize directly under a microscope. But with DNA damage comes cellular repair mechanisms, which can be visualized with immunofluorescence. p53-binding protein 1 (53BP1) is one of the most commonly assayed proteins for repair foci formation. 53BP1 (~220 kDa) is a crucial component of DNA DSB signaling and repair in mammalian cells. Specifically, it is recruited to damaged chromatin where it promotes nonhomologous

end-joining (NHEJ) mediated DSB repair while preventing homologous recombination (HR) (Panier & Boulton, 2014). Because each 53BP1 repair foci has a corresponding DSB, quantifying 53BP1 foci also quantifies the number of DSBs. This is done by binding anti-53BP1 primary antibodies to 53BP1 and then attaching a fluorescent tag to those antibodies via secondary antibodies.

E. Experimental Objectives

Currently, any irradiation done to simulate GCR at the Dynan lab is conducted at the NASA space radiation laboratory in Brookhaven, New York. However, only a few hours of irradiation can be done given its high cost and restrictive scheduling. Therefore, it would be a worthwhile endeavor to have a more locally based irradiator where access is not only easier but there is also greater flexibility in scheduling and a greater variety of experiments can be conducted. Potentially, a facility for high throughput countermeasure screening might be constructed around the irradiator (something that is impossible at the NASA lab).

This study aims to test whether a local laboratory-based deuterium-deuterium (DD) neutron generator at the Georgia Institute of Technology would be able to serve as such a resource. This DD generator was designed, and is used, to irradiate metal foils. Never has it been used on biological material. The neutron radiation will be compared to a reference X ray radiation by modeling the time course of recovery of DNA damage. Theoretical modeling shows very similar patterns of LET between a similar deuterium-tritium (DT) generator and the radiation exposure seen on the surface of the moon (Figure 1). Thus, it is hypothesized that the DD generator will not only produce significant biological effects, but these effects will also be markedly different than that of X rays.

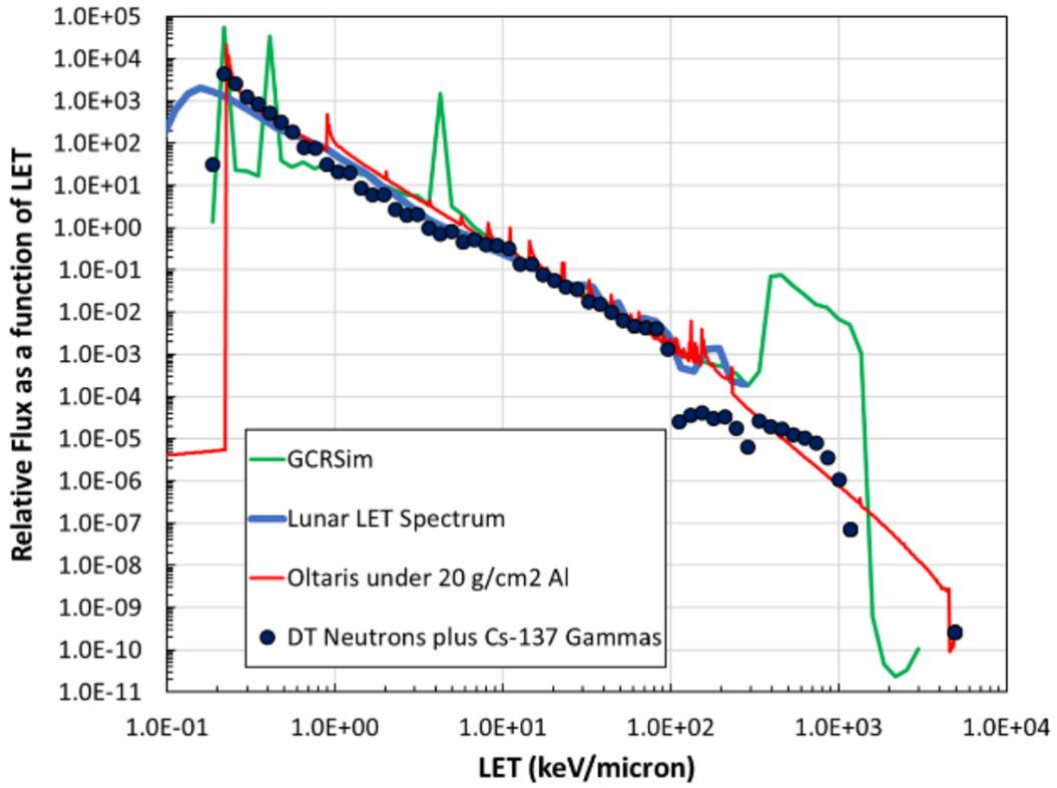

Figure 1. LET spectra of the lunar surface and DT neutrons (Hertel et al., 2022).

Materials and Methods

A. Cell Culture

Immortalized human bronchial epithelial cells (HBEC-3KT) were used for study. Being immortalized, HBEC-3KT can divide indefinitely in vitro and are not subject to normal cellular senescence (Sato et al., 2006). Bronchial epithelial cells were chosen because in space they are one of the cell types potentially at risk of giving rise to lung cancer from galactic cosmic radiation (Kennedy et al., 2018). The media used to grow these cells was 1X Keratinocyte-SFM (ThermoFisher Gibco) with added supplements of 50 µg/mL Bovine Pituitary Extract and 5 ng/mL Epidermal Growth Factor. Aliquots withdrawn for experimentation also had 5 µg/mL Gentamycin (Quality Biological) added to prevent bacterial growth. Media was stored at +4°C.

HBEC-3KT cells were initially kept in liquid nitrogen for long-term storage. To recover the cells, the vial was first rapidly thawed in a 37°C water bath, with extra care taken to prevent temperatures from rising above 4°C due to the damaging nature of the freezing media. The contents of the vial were added to 5 mL of warm prepared media (1X Keratinocyte-SFM with added supplements and gentamycin). The suspension was centrifuged at 180 g for 5 minutes at room temperature and the supernatant was aspirated. The cell pellet was then resuspended in 3 mL media by gently pipetting 3-4 times. The suspension was then plated on a T-75 (Corning) flask with a filter top and diluted to 14 mL of complete media. Cells were incubated at 37°C, 5% CO_2, and 100% humidity.

After growing to a 90% confluency, the cells needed to be split to prevent overgrowth and crowding. The cell flask was first opened and spent media was aspirated with a sterile plastic pipette. The cells were washed using 5 mL of 1X phosphate-buffered saline (PBS) (Gibco). A stock 0.25% trypsin (Gibco) needed to first be diluted into 0.05% trypsin (1:5 in PBS) before 2 mL of the 0.05% trypsin was pipetted

onto the washed cells. The flask was incubated for 10 minutes to allow cell detachment from the flask (trypsin digests extracellular proteins anchoring the cell to the flask bottom). The trypsin buffer was then neutralized to prevent over digestion by adding 2 mL of soybean trypsin inhibitor (1 mg/mL in PBS). The suspension was transferred into a sterile 15 mL centrifuge tube and centrifuged at 180 g for 7 minutes at room temperature. The supernatant was aspirated, and the cell pellet was washed in 5 mL complete Keratinocyte-SFM (gently pipetting 3-4 times) and again centrifuged at 180 g for 7 minutes. The supernatant was aspirated, and the pellet was resuspended in 4 mL media. Cell density was determined using an automated cell counter with a 0.4% trypan blue vital stain. For passaging, 10^6 cells were plated in a new T-75 flask with 15 mL of fresh media. As a backup, 15 mL of media was also added to the old flask. The cells could alternatively be split 1:4. The flasks were then returned to the incubator. On average, the HBEC-3KT cells had a doubling time of about 48 hours. For experimentation – in addition to plating a new T-75 flask – 250,000 to 500,000 cells (depending on the total cell count available and the number of experimental groups needed) were plated in 3 mL of fresh media in slide flasks. These cells were plated 3-4 days before experimentation (plated on Monday/Tuesday for Friday irradiation) to allow for adequate cell growth and coverage of the flask.

B. X Ray Irradiation

HBEC-3KT cells were irradiated in a Thermo Scientific SlideFlask at 1 Gy X ray radiation and allowed to recover for 0.5, 2, 6, and 24 h. There were 5 flasks prepared: one sham (0 Gy; 0.5 h recovery), and four experimental (1 Gy; 0.5, 2, 6, and 24 h recovery) (Table 1). The flasks were irradiated in the Emory University Whitehead Biomedical Research Building using the RadSource RS-2000-Pro-225 Biological System. Radiation was emitted along the z-axis and struck the flask normal to the slide, which was placed flat, allowing tracks to be formed orthogonal to the plane of the cell monolayer. The cells were then returned to the incubator after irradiation to recover. Two independent replicate experiments were performed on different dates using different batches of cells.

Sample #	Dose (Gy)	Recovery (h)
1	0	0.5
2	1	0.5
3	1	2
4	1	6
5	1	24

Table 1. Experimental set-up for X ray irradiation.

The general mechanism for the production of X ray radiation in a laboratory setting involves the heating of a metal filament (cathode) to emit electrons by thermionic emission. The electrons are then accelerated by a high voltage field. Upon striking a metal target (anode), the high-speed electrons cause the emission of high energy photons with frequencies in the range of X rays (Seibert, 2004).

C. Neutron Irradiation

HBEC-3KT cells were irradiated in Flaskette Chamber Slide Culture Chambers at 1, 2 ,and 3 Gy neutron radiation and allowed to recover for 0.5 and 6 h. There were 7 flasks prepared: one sham (0 Gy; 0.5 h recovery), and six experimental (1, 2, and 3 Gy; 0.5 and 6 h recovery) (Table 2). The flasks were irradiated at the Georgia Institute of Technology Gilbert Hillhouse Boggs Building in the Radiation Safety and Engineering laboratory using the nGen800b Deuterium-Deuterium Neutron Generator.

Because the cells needed to be irradiated at the Georgia Institute of Technology, the flasks were sealed shut with Parafilm and transported in a foam box with a heat pack from Emory University on the day of irradiation. While there, the sealed slide flasks cells were stored in an incubator at 35°C to 37°C.

Radiation was emitted along a horizontal axis and struck the flask normal to the slide, which was placed on its side, allowing tracks to be formed orthogonal to the plane of the cell monolayer. To allow this, the flask was filled full of media so that even while on its side, all cells were submerged under media. The

cells were then returned to the incubator after irradiation to recover. Two independent replicate experiments were performed on different dates using different batches of cells.

Note that the doses for the neutron irradiation were estimates and the result of calculations. Metal foil activation experiments gave an estimated dose rate of 11 Gy/hour using a quality factor of 10, meaning doses of 1, 2, and 3 Gy translated to 5.5, 11, and 16.5 minutes of radiation exposure, respectively. The neutron beam also didn't turn on instantaneously – it required about 30-45 seconds to ramp up to 100%. The time of irradiation only began after this ramp up period was complete.

Sample #	Dose (Gy)	Recovery (h)	Time (min)
1	0	0.5	–
2	1	0.5	5.5
3	1	6	5.5
4	2	0.5	11
5	2	6	11
6	3	0.5	16.5
7	3	6	16.5

Table 2. Experimental set-up for neutron irradiation.

The general mechanism for the production of neutron radiation in a laboratory setting using a deuterium-deuterium generator involves the acceleration of deuterium into a metal hydride target that also contains deuterium. The fusion of two deuterium atoms generates a ^{3}He (helium-3) ion and a neutron with a kinetic energy of approximately 2.5 MeV (Reijonen, 2005).

D. Staining for Imaging

Following recovery, the cells were washed 3 times with 2 mL of non-sterile Dulbecco's phosphate-buffered saline (DBPS) (Gibco), incubated at room temperature for 15 minutes with 1 mL of 4% paraformaldehyde (PFA) (Electron Microscopy Sciences) in PBS to fix the cells, and washed again 3 times with 2 mL of DPBS. Following fixation with PFA, the cells became preserved and any biochemical reactions (i.e., DNA repair) were terminated (Celikkan et al., 2020). At this point, the flasks could be

stored for an extended period of time at 4°C, if needed. The flasks were then incubated at room temperature for 15 minutes with 1 mL of 0.5% Triton X-100 (Fisher Chemical) in PBS. Triton X-100 is a non-ionic surfactant and emulsifier that solubilizes proteins and increases membrane permeability, which in this context allows antibody access to the nucleus (Koley & Bard, 2010). Cells were washed 3 times with 2 mL of DPBS and incubated at room temperature for 10 minutes with 1 mL of 0.1% (w/v) sodium borohydride ($NaBH_4$) (Sigma Aldrich) to reduce any background autofluorescence due to PFA fixation when imaging (Clancy & Cauller, 1998). Cells were washed again 3 times with 2 mL DPBS. The slides containing the cells were then separated from the rest of the flask. A 2 cm^2 area was marked off with a liquid-repellent slide marker for staining to conserve reagent. The area was incubated with 50 µL of 10% normal goat serum (NGS) in DPBS for 1 hour to block any non-specific epitopes followed by incubation with 50 µL of primary antibodies for 1-2 hours at room temperature (1:500 dilutions in 10% NGS of rabbit anti-53BP1 (Novus NB 100-904)). The cells were then washed 4 times with 50 µL of DPBS, with each wash staying on for 3 minutes. The area was then incubated with 50 µL of fluorescently-labeled secondary antibody for 1 hour at room temperature (1:500 dilutions in 10% NGS of Invitrogen Alexa Fluor 488 goat anti-rabbit, which binds to anti-53BP1 and fluoresces green). Cells were again washed 3 times with 50 µL DPBS for 3 minutes each. VectaShield mounting media with DAPI (which stains the nucleus blue) was added and a cover slip was placed on top, which was sealed with nail polish. The nail polish was left to dry overnight at room temperature and the slides were covered with aluminum foil to prevent light exposure. The slides were then stored long-term at 4°C.

E. Visualizing DNA Repair Foci

Slides were imaged using the DeltaVision Elite microscope under the 60X magnifying objective (immersion oil needed between the lens and slide) and 1024 X 1024 image size. The excitation/emission filters used include DAPI (blue nucleus) and FITC (green 53BP1) at an exposure of 0.0050 seconds and

100% transmittance. Because the cell was thicker than the depth of field the microscope can image in focus, a series of around 15 planes along the z-axis was imaged instead, with each plane having a section of the cell in focus. These z-sections were then deconvolved to remove background noise (i.e., out of focus areas from the plane above and below) so that each image was perfectly in focus. The sections were finally projected on top of one another, combining all the z-sections into one, allowing the entire cell to be in focus in one single image.

F. Quantifying DNA Repair Foci

The number of repair foci per cell (visualized as individual, distinct dots in the nucleus) was counted to quantify the magnitude of DNA damage and the rate of repair. Every complete cell visible in each field (not partially cut off by the boundaries of the field) was included in the count. There were typically about 5 to 10 cells per field and 10 fields were taken of every experimental group (for example, for X ray irradiation, 10 fields were taken each of the sham, 0.5, 2, 6, and 24 h groups, for 50 fields total).

G. Statistical Tests

Replicate data was pooled for testing. X ray and neutron groups were compared using the Kruskal–Wallis test, which is a nonparametric analysis that tests whether or not samples originated from the same distribution (Kruskal & Wallis, 1952). It can be used to compare two or more independent samples of equal or different sample sizes. Pairwise assessments (testing whether two experimental groups, such as 1 Gy X ray 0.5 h recovery and 1 Gy X ray 2 h recovery, are statistically significantly different) were handled using Dunn's test with a Bonferroni correction. Dunn's test is the nonparametric pairwise multiple comparison test that is used when a Kruskal–Wallis test is rejected (Dinno, 2015). The Bonferroni correction is a multiple-comparison correction used when several independent or dependent statistical tests are being performed simultaneously (because although a particular alpha value may be suitable for a particular comparison, it is not suitable for every comparison) (Napierala, 2012).

Results

A. X Ray Irradiation

Non-irradiated cells presented with sparse foci, the presence of which was due to normal cellular processes. Irradiated cells exhibited a far greater number of foci, with the most abundant being cells that had the shortest recovery time of 0.5 h, followed by 2 h, 6 h, and 24 h (Figure 2). This indicates the ability of cells to repair DNA damage caused by X ray irradiation. Overall, foci were consistent in size, shape, and brightness across all cells irradiated by X rays.

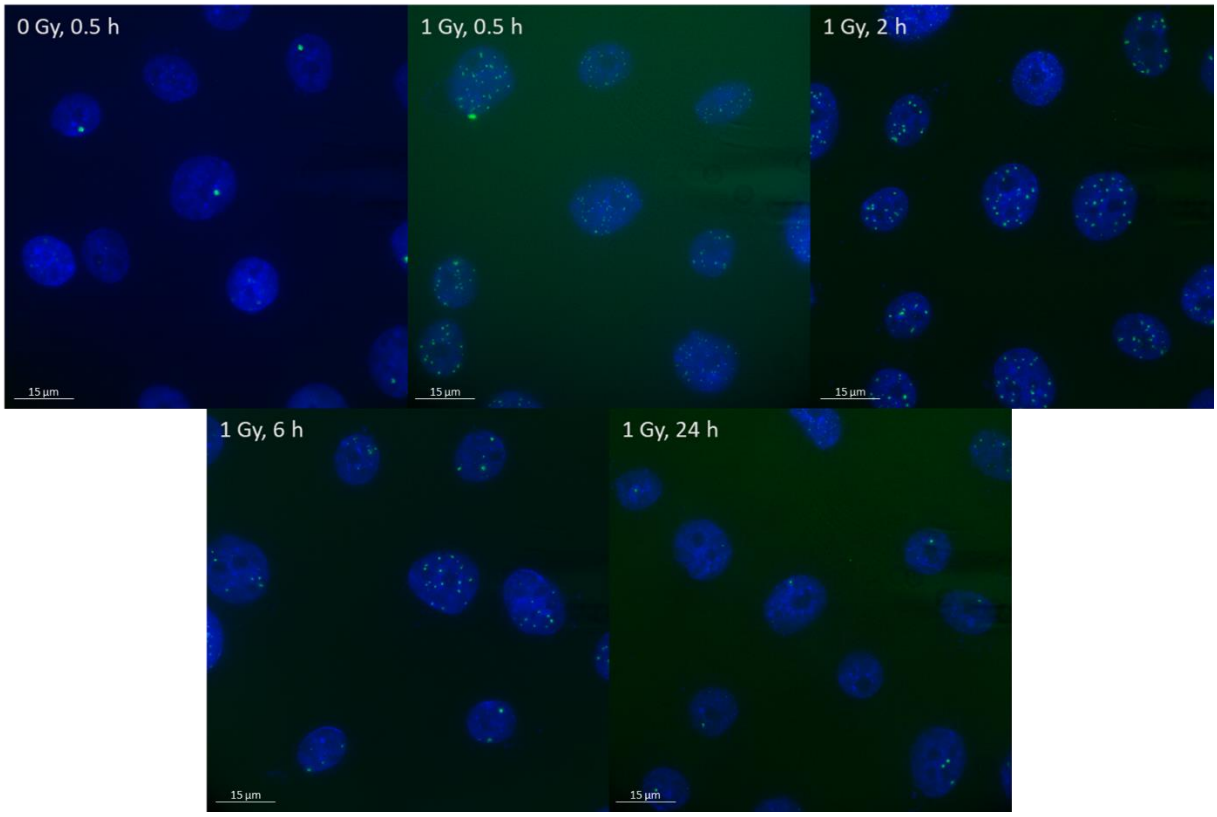

Figure 2. Representative images of HBEC-3KT cells at different times of recovery following X ray irradiation. The nuclei are blue and 53BP1 repair foci are green.

Foci counts were highest immediately following irradiation, with counts decreasing over time (Figure 3). The foci counts of each experimental group were significantly different from every other group (p value

< 0.001) with the exception of sham and 24 h recovery (p value = 1.0000). This means that after 24 hours, cellular mechanisms were able to repair virtually all DNA damage caused by X ray irradiation. This also means that each time point had significant amounts of recovery relative to the time points prior. The results suggest that DNA damage caused by X ray irradiation was not very complex and was essentially all repaired within 24 hours.

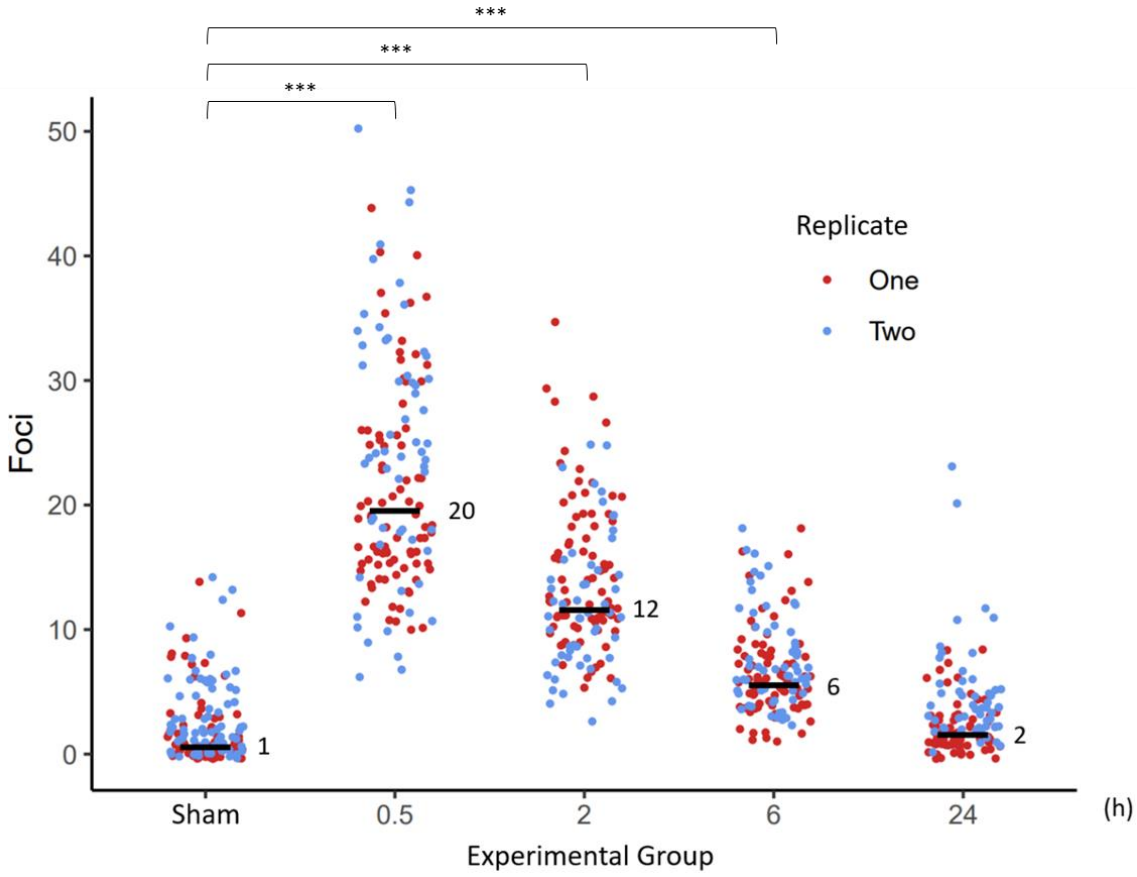

Figure 3. DNA repair foci counts following X ray irradiation (sham and 1 Gy with 0.5, 2, 6, and 24 h recovery). Each dot represents a cell, and the bar marks the median (because a nonparametric test is being used).

Foci counts were fairly evenly distributed, albeit with a slight right skew. The majority were concentrated loosely around the median and there were not many extreme or isolated outliers. The

median was highlighted rather than the mean to mitigate the effects of any outliers. Replicates were identical, validating the methodology and reproducibility of the experiment.

B. Neutron Irradiation

Neutron-irradiated cells presented with more foci than non-irradiated control cells but had less foci on average compared with cells that received the same dose of X ray irradiation (Figure 4). This may be because neutrons transfer much more energy per particle to the cells than do X rays (high LET) and so 1 Gy of neutrons is comprised of much fewer particles than 1 Gy of X rays. Thus, fewer tracks were formed following neutron irradiation, but each track was more densely ionizing and caused more severe and difficult to repair DNA damage. This is reflected in that fact that neutron foci appeared much larger in size than X ray foci, and foci were less uniform in shape (tended to be more jagged whereas X ray foci were typically circular) (Figure 5).

Both sham and irradiated groups had cells with abnormally high numbers of foci. These cells numbered on average one to two per image (out of around 10 cells total per image). While also present in X ray images, cells used for neutron irradiation exhibited this phenomenon more often. This may have been due to non-irradiation related effects that occurred during the transport of cells to and from the Georgia Institute of Technology as well as the conditions of their storage (poor temperature control of the incubator and flasks were full of media) (Figure 6). These foci appeared markedly different (smaller and fainter) than other foci present in neutron irradiated cells.

The dose-dependent effects of neutron irradiation based on the images alone appeared to be quite weak. There was very little increase visually in the number of foci between the 1, 2, and 3 Gy doses (Figure 4). And for all dose rates, the number of foci appeared to be quite similar between 0.5 h and 6 h of recovery. This could indicate the presence of more complicated and harder to repair DNA damage.

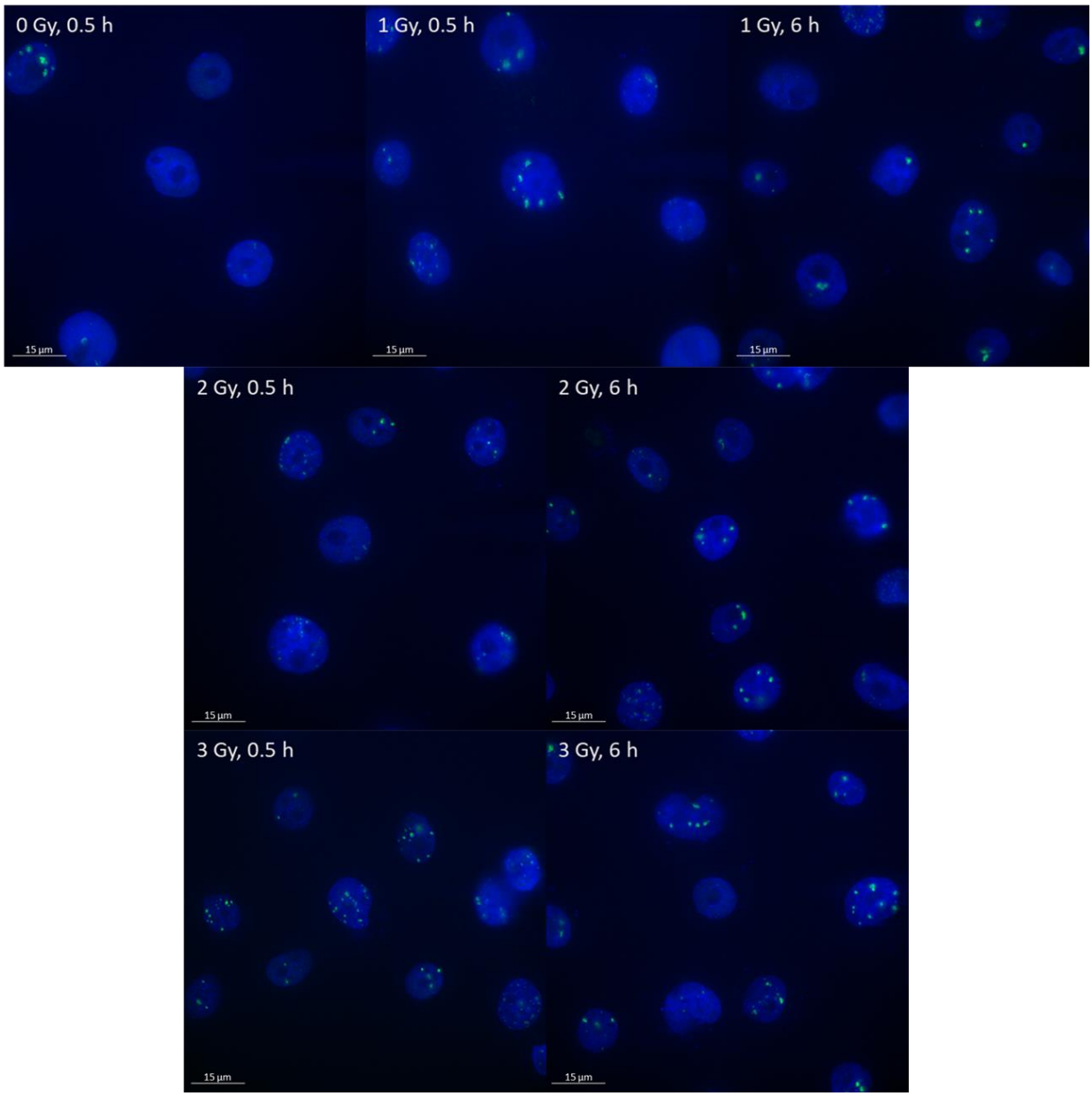

Figure 4. Representative images of HBEC-3KT cells at different times of recovery following neutron irradiation. The nuclei are blue and 53BP1 repair foci are green.

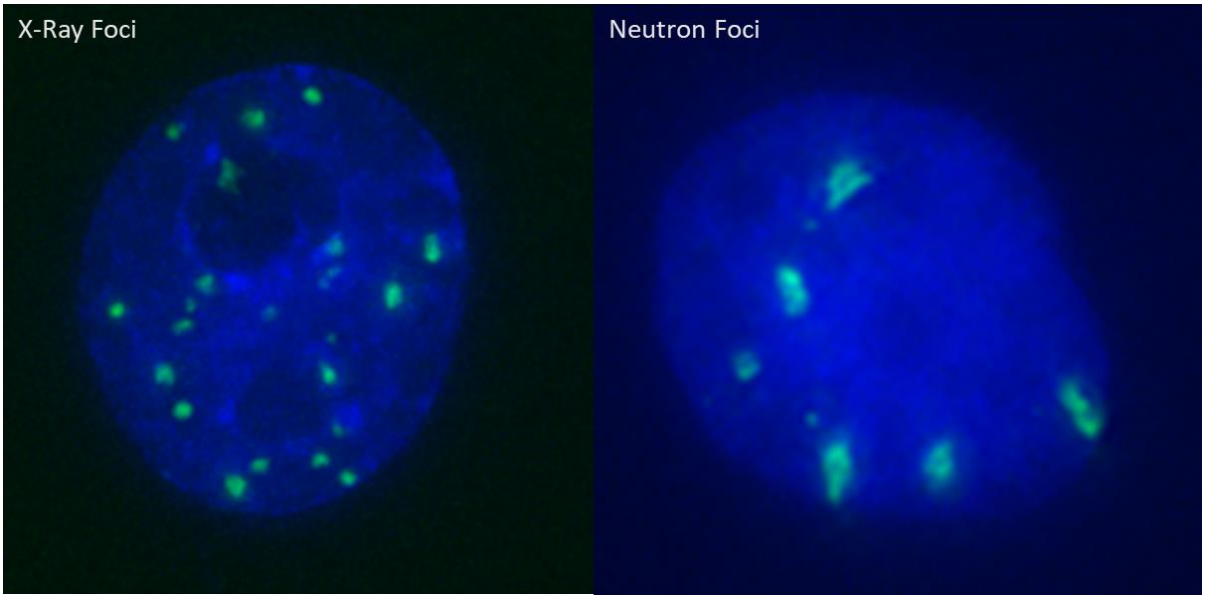

Figure 5. X ray and neutron foci close-up.

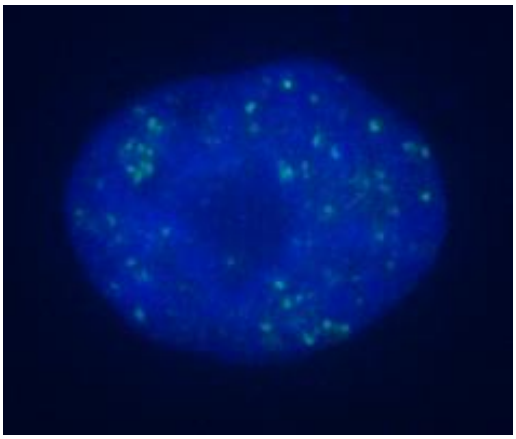

Figure 6. Non-irradiation related foci present in neutron experiment cells.

The foci count of the sham group (0 Gy, 0.5 h recovery) was significantly different from that of the 1, 2, and 3 Gy, 0.5 h recovery groups (p value < 0.001) (Figure 7). The DD neutron generator indeed caused a significant biological effect in the damage of DNA. Furthermore, the foci count of the sham group wasn't significantly different from the 1 Gy, 6 h recovery group (p value = 0.1683) but was significantly different from the 2 and 3 Gy, 6 h recovery group (p value < 0.001). While cells were able to repair neutron related damage at the lower 1 Gy dose, it wasn't able to at the higher 2 and 3 Gy doses. The foci counts

between the 0.5 and 6 h recovery times for 1 and 2 Gy doses weren't significantly different (p value = 0.1036 and 0.1213, respectively) but were significantly different for the 3 Gy dose (p value = 0.0030). Unlike the cells following X ray irradiation, there wasn't significant DNA repair at low doses of 1 and 2 Gy but there was at 3 Gy. Moreover, compared to X rays, which had a more than threefold decrease in foci between 0.5 and 6 h at 1 Gy (from 20 down to 6), neutrons exhibited a much smaller decrease (from 4 down to 3). The slower rate of recovery indicates more complex damage. Finally, at both 0.5 h and 6 h recovery, there weren't significant differences in the foci counts between 1, 2, and 3 Gy. Thus, there seems to be little dose-dependent effects of neutron irradiation.

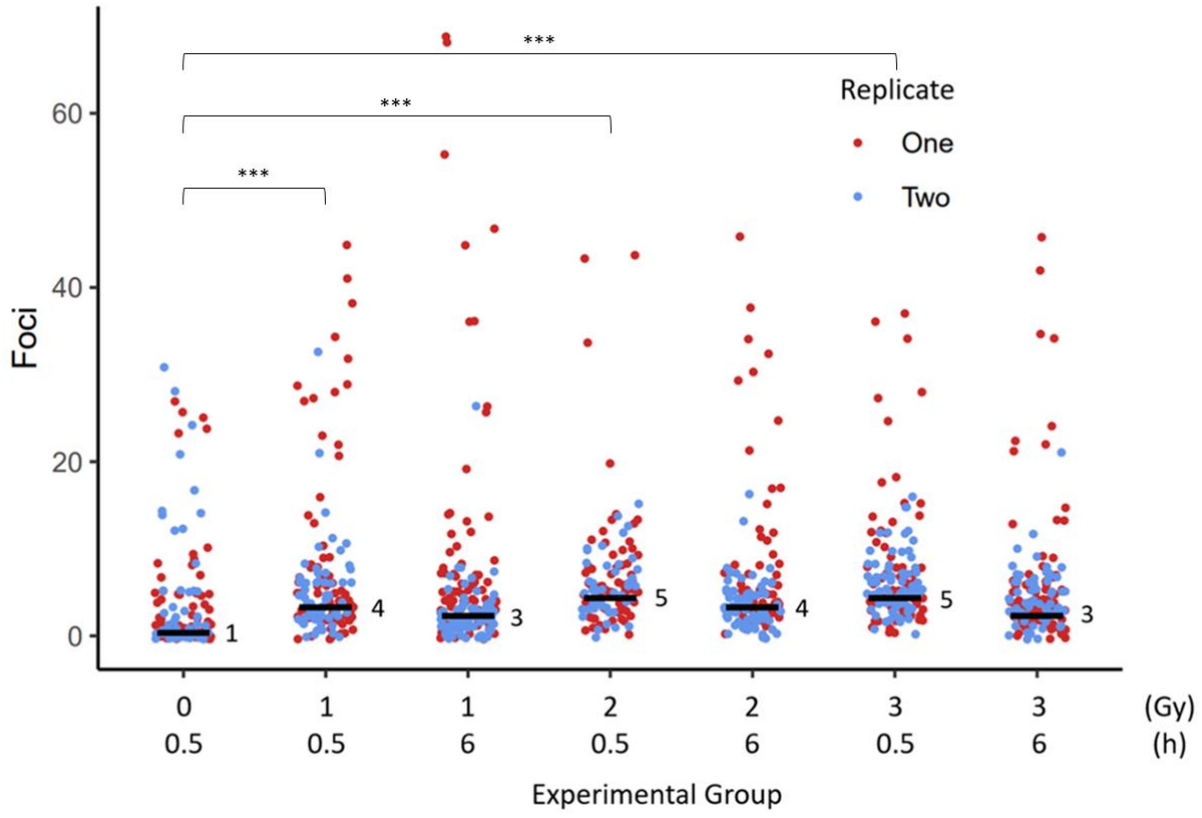

Figure 7. DNA repair foci counts following neutron irradiation (0, 1, 2, and 3 Gy with 0.5 and 6 h recovery). Each dot represents a cell, and the bar marks the median (because a nonparametric test is being used).

The vast majority of foci were tightly concentrated around the median, but there were a few extreme and isolated outliers that gave the distribution an overall right skew. Whereas the means and medians for the X ray groups were quite similar, the means were consistently higher than the medians for the neutron groups because of the greater number of abnormal cells in the neutron experiments (Tables 3 and 4). For example, whereas the median for both the X ray and neutron sham groups was 1, the mean for the neutron was 4.41 and the mean for the X ray was 2.60. Given the presence of these high foci count cells in both irradiated and non-irradiated groups, their cause was likely non-irradiation related effects that originated from the stress of traveling from Emory University to the Georgia Institute of Technology. Thus, the median was highlighted rather than the mean to mitigate the effects of these outliers. Replicates were identical, validating the methodology and reproducibility of the experiment.

X Ray Groups	Median	Mean
Sham	1	2.60
0.5 h	20	22.4
2 h	12	13.5
6 h	6	6.96
24 h	2	3.31

Table 3. Median and mean values for X ray groups.

Neutron Groups	Median	Mean
Sham	1	4.41
1 Gy, 0.5 h	4	7.07
1 Gy, 6 h	3	6.12
2 Gy, 0.5 h	5	6.69
2 Gy, 6 h	4	5.81
3 Gy, 0.5 h	5	6.99
3 Gy, 6 h	3	5.53

Table 4. Median and mean values for neutron groups.

Discussion

Cells irradiated by X rays displayed few foci prior to irradiation and a large amount immediately following, which decreased in number over time as cellular mechanisms repaired DNA. After 24 hours, virtually all DNA damage was repaired. This was expected given the low-LET nature of X ray radiation, which deposits energy in a sparser manner, causing less complex DNA damage. The foci themselves were generally uniform in size, shape, and brightness. The distribution of foci counts was fairly even and lacked many extreme or isolated outliers.

Cells irradiated by neutrons had a more complex story. Due to non-irradiation related effects during the neutron experiment (likely due to the transport and storage of cells), certain cells in every experimental group exhibited abnormally high counts of foci. These foci were different from other observed foci in that they were much smaller and fainter. The presence of these foci caused an extreme right skew in the data. Because there were more of these cells in the neutron groups than in the X ray groups, data collection was more difficult. An alternate explanation for the presence of these foci is that the fluorescence signal was higher in the X ray cells (due to the presence of more foci in the cells) and so any background glow was less of an issue than with the neutron cells, where it was more visible.

The incubator used to store cells while at the Georgia Institute of Technology lacked precise control over temperature and no control over humidity and CO_2 levels. Temperatures varied between 35°C to 37°C (the cells could tolerate lower temperatures more than they could higher ones). Future experimentation should seek to minimize transport and store cells in more equipped incubators.

At the same dose and recovery times, cells irradiated by neutrons exhibited less foci per cell than did cells irradiated by X rays. This was because neutrons deposit more energy per track and thus created fewer tracks than X rays at the same dose (Gy). But because these tracks were more densely ionizing (a

characteristic of neutrons and other high LET radiation), the DNA damage was more complex, which is why neutron foci were much larger and more jagged in shape compared to X ray foci. Furthermore, compared to X ray groups, there was a much smaller difference in the foci counts between the 0.5 and 6 hour recovery at 1 Gy, indicating potentially more complex damage that was more challenging to repair.

The neutron groups exhibited weak dose-dependent effects. At both 0.5 and 6 hour recovery times, there weren't significant differences between the foci counts of the different doses (1, 2, and 3 Gy). Ordinarily, a higher dose is expected to produce a greater number of foci. Such a trend indicates a dose saturation of the system. Radiation experienced by astronauts in space are at fractions of a Gy (mGy). Thus, it would be interesting to see effects at even lower doses.

There were, however, other differences at the different doses. At 1 Gy, cells were able to repair virtually all DNA damage caused by neutron irradiation after 6 hours, whereas they weren't able to at higher doses of 2 and 3 Gy. So perhaps the effects of increasing dose using the DD generator doesn't present itself in the creation of more tracks of radiation (and thus more foci) but rather in the degree of damage caused along those already existing tracks.

With regards to the initial hypothesis, the DD neutron generator at the Georgia Institute of Technology was indeed able to produce significant amounts of DNA damage relative to the sham group. Furthermore, the damage caused by neutrons was markedly different from that of X rays in their appearance, quantity, and rate of recovery.

Further study should analyze the cells at 2 and 24 hours following neutron irradiation and compare them with the X ray cells at those times to generate a more complete time course of recovery. Even longer periods of recovery (i.e., 3 days) can be conducted too since neutron damage has such a slow rate of repair. Long term viability should also be investigated. Since the fixing and imaging procedure

kills the cells, some cells should be left to recover completely (over several days) and not imaged, then compared to non-irradiated cells in their cell counts and doubling times.

Since living organisms in space receive radiation in very low doses over a long period of time, experiments should mimic that. Cells can be irradiated at fractions of a Gy over several days. Note that this type of experiment is exactly the kind that would only be possible with a local resource – there is no way to conduct such a long irradiation at the NASA lab.

Background signals and non-irradiation related foci need to be minimized as much as possible by optimizing the procedure and improving the transport and storage of cells when irradiating at the Georgia Institute of Technology.

Finally, a more robust and precise method of foci quantification should be developed to count foci. Specifically, a higher resolution is needed to distinguish one focus from two that are simply close together and identify foci that are fainter. A more defined set of guidelines also needs to be established to draw the line between when a focus is defined as one or two and when faint foci are included in the count or not. Ideally, foci can be segregated as due to irradiation or non-irradiation related effects.

References

Asaithamby, A., & Chen, D. J. (2011). Mechanism of cluster DNA damage repair in response to high-atomic number and energy particles radiation. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *711*(1), 87-99. https://doi.org/https://doi.org/10.1016/j.mrfmmm.2010.11.002

Celikkan, F. T., Mungan, C., Sucu, M., Uysal, F., Kahveci Hayme, S., Hayme, S., Kuscu, N., Ozkavukcu, S., Celik-Ozenci, C., & Can, A. (2020). PFA is superior to glyoxal in preserving oocyte, embryo, and stem cell proteins evidenced by super-resolution microscopical surveys of epitopes. *Journal of Assisted Reproduction and Genetics*, *37*(2), 369-384. https://doi.org/10.1007/s10815-019-01666-9

Clancy, B., & Cauller, L. J. (1998). Reduction of background autofluorescence in brain sections following immersion in sodium borohydride. *J Neurosci Methods*, *83*(2), 97-102. https://doi.org/10.1016/s0165-0270(98)00066-1

Dinno, A. (2015). Nonparametric Pairwise Multiple Comparisons in Independent Groups using Dunn's Test. *The Stata Journal*, *15*(1), 292-300. https://doi.org/10.1177/1536867x1501500117

Kennedy, E. M., Powell, D. R., Li, Z., Bell, J. S. K., Barwick, B. G., Feng, H., McCrary, M. R., Dwivedi, B., Kowalski, J., Dynan, W. S., Conneely, K. N., & Vertino, P. M. (2018). Galactic Cosmic Radiation Induces Persistent Epigenome Alterations Relevant to Human Lung Cancer. *Sci Rep*, *8*(1), 6709. https://doi.org/10.1038/s41598-018-24755-8

Koley, D., & Bard, A. J. (2010). Triton X-100 concentration effects on membrane permeability of a single HeLa cell by scanning electrochemical microscopy (SECM). *Proceedings of the National Academy of Sciences*, *107*(39), 16783-16787. https://doi.org/doi:10.1073/pnas.1011614107

Kruskal, W. H., & Wallis, W. A. (1952). Use of Ranks in One-Criterion Variance Analysis. *Journal of the American Statistical Association*, *47*(260), 583-621. https://doi.org/10.1080/01621459.1952.10483441

Little, J. B. (1968). Cellular Effects of Ionizing Radiation. *New England Journal of Medicine*, *278*(6), 308-315. https://doi.org/10.1056/nejm196802082780605

Maliszewska-Olejniczak, K., Kaniowski, D., Araszkiewicz, M., Tymińska, K., & Korgul, A. (2021). Molecular Mechanisms of Specific Cellular DNA Damage Response and Repair Induced by the Mixed Radiation Field During Boron Neutron Capture Therapy [Review]. *Frontiers in Oncology*, *11*. https://doi.org/10.3389/fonc.2021.676575

Mladenova, V., Mladenov, E., Stuschke, M., & Iliakis, G. (2022). DNA Damage Clustering after Ionizing Radiation and Consequences in the Processing of Chromatin Breaks. *Molecules*, *27*(5), 1540. https://www.mdpi.com/1420-3049/27/5/1540

Napierala, M. A. (2012). What is the Bonferroni correction? [Article]. *AAOS Now*, 40. https://link.gale.com/apps/doc/A288979427/HRCA?u=anon~da57b2d9&sid=googleScholar&xid=8e7d6e31

Panier, S., & Boulton, S. J. (2014). Double-strand break repair: 53BP1 comes into focus. *Nature Reviews Molecular Cell Biology*, *15*(1), 7-18. https://doi.org/10.1038/nrm3719

Reijonen, J. (2005). *Compact Neutron Generators for Medical Home Land Security andPlanetary Exploration* Conference: Particle Accelerator Conference 2005, Knoxville,Tennessee, 05/16-20/2005, United States. https://www.osti.gov/biblio/881393

https://www.osti.gov/servlets/purl/881393

Rodman, C., Almeida-Porada, G., George, S. K., Moon, J., Soker, S., Pardee, T., Beaty, M., Guida, P., Sajuthi, S. P., Langefeld, C. D., Walker, S. J., Wilson, P. F., & Porada, C. D. (2017). In vitro and in vivo assessment of direct effects of simulated solar and galactic cosmic radiation on human

hematopoietic stem/progenitor cells. *Leukemia*, *31*(6), 1398-1407. https://doi.org/10.1038/leu.2016.344

Rossi, H. H. (1960). Spatial Distribution of Energy Deposition by Ionizing Radiation. *Radiation Research Supplement*, *2*, 290-299. https://doi.org/10.2307/3583601

Sato, M., Vaughan, M. B., Girard, L., Peyton, M., Lee, W., Shames, D. S., Ramirez, R. D., Sunaga, N., Gazdar, A. F., Shay, J. W., & Minna, J. D. (2006). Multiple Oncogenic Changes (K-RASV12, p53 Knockdown, Mutant EGFRs, p16 Bypass, Telomerase) Are Not Sufficient to Confer a Full Malignant Phenotype on Human Bronchial Epithelial Cells. *Cancer Research*, *66*(4), 2116-2128. https://doi.org/10.1158/0008-5472.Can-05-2521

Seibert, J. A. (2004). X-Ray Imaging Physics for Nuclear Medicine Technologists. Part 1: Basic Principles of X-Ray Production. *Journal of Nuclear Medicine Technology*, *32*(3), 139-147. https://tech.snmjournals.org/content/jnmt/32/3/139.full.pdf