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Impact of Microgravity on the Accumulation of DNA damage in Human Embryonic Kidney Cells

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Abstract

In outer space, astronauts undergo direct exposure to oxidative stress and UV radiation due to a low-oxygen environment and the absence of an ozone layer. Although prolonged oxidative stress and UV radiation damages genomic DNA and requires repair mechanisms, the long-term effects of microgravity on these cellular repair mechanisms are not fully understood. The purpose of this study is to determine how microgravity impacts the proliferation and DNA repair pathways of Human Embryonic Kidney (HEK293) cells. We measured the effects of hydrogen peroxide, bleomycin, and camptothecin on HEK293 cells under normal gravitational conditions and microgravity simulation. Hydrogen peroxide (oxidative stress) elicits damage that mimics the conditions of outer space, while bleomycin and camptothecin caused double-stranded and single-stranded breaks, respectively. Treatment occurred at timed increments while the cell viability, physical morphology, and nucleic DNA damage were monitored using a variety of biochemical assays. We found that all reagents induced concentration-dependent DNA damage in normal gravitational conditions while damage was variable under microgravity simulation. Furthermore, DNA damage increased under microgravity simulation alone. We conclude the extent of mutagenic damage observed is specific to HEK293 cells and plan to replicate these studies in cardiomyocytes, dermal fibroblasts, and retinal cells. The experimental outcomes seek to increase the safety of space travel by understanding how microgravitational environments could impact DNA repair mechanisms. This project aligns with NASA's objective by analyzing the microscopic influences of microgravity on DNA repair and how that relates to human health.

Keywords: DNA Damage, Microgravity, Rotary Cell Culture System

1. Introduction

For over three decades, the National Aeronautics and Space Administration (NASA) has been collecting data on the radiation levels of NASA astronauts as a way to monitor changes in biological function.[1] Deoxyribonucleic acid (DNA) damage occurs frequently in outer space as a result of radiation exposure, among other environmental factors. When astronauts are on mission, they undergo direct, constant exposure to mutagens. Environmental mutagens are concerning because unlike other environmental stimuli that may cause physical changes to the cell's composition (such as changes in cell signaling), mutagens directly alter the DNA sequence of a cell.[2]

Research has shown that prolonged ultraviolet (UV) radiation induces cyclobutane pyrimidine dimers and (6,4) pyrimidine-pyrimidone dimers; types of DNA damage that lead to genomic instability if not repaired.[3] Additionally, the increased aerobic activity conducted by astronauts promotes excessive oxidative stress.[4] However, the long-term effects of microgravity on cellular repair mechanisms are not fully understood. This study will determine if a microgravitational environment impacts human cell growth and how microgravity influences DNA repair. Hypothetically, if human cells are grown in microgravity, they will exhibit higher levels of single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) damage, because the change in environmental stimuli increases the cells susceptibility to other external stresses.

In this project, hydrogen peroxide, bleomycin, and camptothecin were three chemicals used to simulate cellular stress. These reagents were chosen because they are known to cause specific types of DNA damage. Previous research has shown hydrogen peroxide creates oxidative stress in human cells.[5] Oxidative stress occurs when oxygen free radicals are in excess and there is a deficiency of antioxidants. When reactive oxygen species interact with DNA, they have the potential to create either ssDNA or dsDNA breaks. Commonly used in cancer treatment, bleomycin and camptothecin were also selected as experimental reagents. Bleomycin induces dsDNA breaks, while camptothecin causes ssDNA breaks.[6] Figure 1 visually depicts the two types of DNA damage monitored throughout the course of this project.

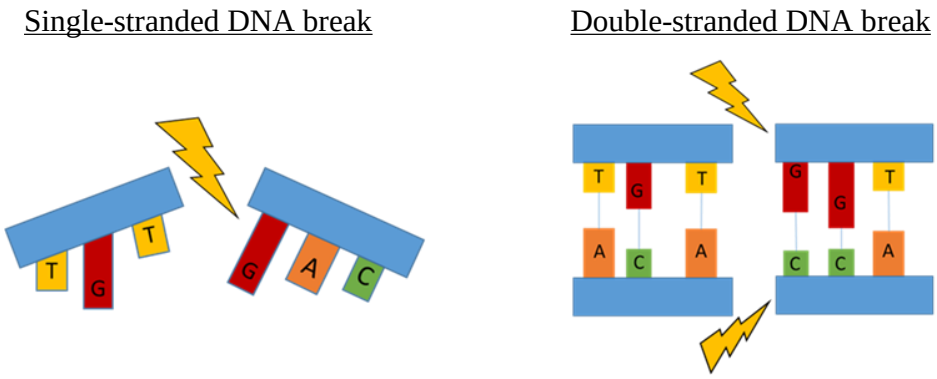

Figure 1. Graphic comparing a ssDNA break to a dsDNA break. Both types of damage occur naturally and can be induced in human cells using chemical mutagens.

The reality of intergalactic human settlements will almost certainly occur within the next 50 years. The Mars One Project aims to establish a permanent human settlement on Mars by the year 2024.[7] As humanity expands into alternative settlements, it is essential to determine how DNA integrity could be impacted by new environmental stimuli. Scientists have identified the pathogenesis of countless diseases and have established accurate testing methods as well as effective forms of treatment on earth. Acute Lymphocytic Leukemia (ALL), for example, is a type of cancer of the blood and bone marrow that has an established pathogenesis, particularly in children. On earth, children with ALL have a high recovery rate following an efficient diagnosis and appropriate treatment. In space, however, blood flow is altered due to the alteration of the gravitation pull. The subsequent effects of a microgravity environment of the effective diagnosis and treatment of this disease, as well as many others, is currently not known. This highlights the need for further research on the effects of microgravity on human cells.

2. Materials and Methodology

2.1 Cell Growth and Maintenance

The cell line initially used at the start of this project were HeLa cells. This cell type was originally derived from a cervical cancer biopsy and is well-known within the scientific community as the first immortal cell line. Initial experiments were performed on HeLa cells to establish researcher competency of the cell culture protocols before advancing to more complex growth procedures. During this project, data was collected using Human Embryonic Kidney (HEK293) cells.

The cell stocks were frozen at a concentration of $1x10^6$ cells/mL to ensure the cells were seeded at a concentration of approximately 1 x 105 cells/mL. They were seeded on a T-25 flask at a 1:5 dilution and grown in an incubator set to 37ºC with 5.0% carbon dioxide and high humidity. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS). Additionally, 5.0% penicillin and streptavidin (Pen/Strep) antibiotics were added to reduce the risk of bacterial contamination.

The growth media was changed every other day. After 48 hours, when the cells had reached 80-90% confluency, the cells were split and seeded in either a new T-25 culture flask, a 60 mm culture plate, or a 10 mL rotary cell culture vessel. While the type of culture apparatus varied based on the experiment performed, the overall cell concentration

and cell generation remained constant for each experiment by using the same dilution and passage number, respectively.

2.2 Cell Treatment

Before introducing microgravity, a baseline of DNA damage in normal growth conditions had to be established. To accomplish this, a concentration-dependent treatment plan was designed with the three reagents: hydrogen peroxide, bleomycin, and camptothecin. The criteria for selecting the four concentrations was based on a visible increase in cell death that correlated to increased treatment concentration. The concentrations (for example 350 µM, 250 µM, 150 µM, and 0 µM of H2O2) had to vary enough from each other so that a noticeable cytopathic effect could be seen between conditions. The observed change in cell growth patterns was confirmed using biochemical assays to assess DNA damage.

Once the cells were treated, changes in morphology were monitored using a Nikon Eclipse Ts2 microscope. A high-resolution, live-imaging Motic microscopy camera attached to the microscope made it possible to track changes in morphology each day by capturing images directly from the live view of the cells. Additionally, the nucleic DNA damage was analyzed using Comet Assay. A set of control cells were made alongside each set of treated cells. The untreated cells were handled in the same manner as the treated cells, and the data was processed to control for endogenous levels of DNA damage.

2.2.1 hydrogen peroxide, bleomycin, and camptothecin

The chosen set of concentrations for hydrogen peroxide (H_2O_2) treatment were 150 µM, 250 µM, and 350 µM. For each experiment, 50 mL of 100 mM solution was prepared from a 13.5 M stock solution of H_2O_2, using MQ water as the diluent. Then, the range of concentrations was made by adding the appropriate amount of 100 mM H_2O_2 to DMEM. A total volume of 5mL was used for T-25 flasks, while a 10 mL total volume was needed for both the 60 mm culture plates and the 10 mL rotary cell culture vessels.

Bleomycin was purchased as a 10 mg dehydrated powder. The maximum solubility of bleomycin in phosphate-buffered saline (PBS) buffer is 10 mg/mL.[8] To prevent precipitation of the drug, a conservative stock concentration of 2 mg/mL was made by dissolving the entire stock powder in 5 mL of PBS buffer. The appropriate set of experimental concentrations for bleomycin treatment was determined to be 1 µg/mL, 10 µg/mL, and 100 µg/mL based on previous research in which similar ranges were used.[9]

Camptothecin was purchased as a 250 mg dehydrated powder, and the maximum solubility of camptothecin in dimethyl sulfoxide (DMSO) was found to be 3 mg/mL.[10] A 1.5 mg/mL stock concentration was made by dissolving 15 mg of stock powder into 10 mL of DMSO. The suitable set of concentrations for camptothecin treatment was determined to be 1 µg/mL, 10 µg/mL, and 100 µg/mL based on previous research.[11]

2.3 Rotary Cell Culture System (RCCS)

Once the baseline data for DNA damage had been established in regular growth conditions, the studies were replicated in a microgravity environment. To simulate the low-gravity environment, a Rotary Cell Culture System (RCCS) was used, depicted in Figure 2. The RCCS was developed and recommended by NASA as an effective way to simulate microgravity. The system contains four spinning vessels which made it possible to culture four different conditions simultaneously, under low-gravity simulation.[12] The cells were spun in the horizontal plane and experienced a state of constant free-fall. Due to the nature of the rotation, the cells did not adhere to the culture vessel and remained free-floating in DMEM.

For each experiment, four 60 mm culture plates and four 10 mL rotary cell culture vessels were seeded from the primary culture flask. Traditionally, the media of a newly seeded cell culture is changed within the first 10 hours to remove excess Trypsin, a proteolytic enzyme that breaks down proteins. Trypsin is used to lift the cells off of the culture flask, but excess Trypsin is toxic to cell growth over time. Due to the complexity of the valve lock system on the rotary cell culture vessels and the potential to lose free-floating cells while changing growth media, a different approach was taken. After the cells had been sheared off the original plate and resuspended in media, they were concentrated using centrifugation. The media containing trypsin was aspirated off and fresh media was used to resuspend the cells. A 1:5 dilution of the cells was made and administered to both the 60 mm culture plates and the 10 mL rotary cell culture vessels; 24 hours after the cells were seeded, treatment occurred.

Figure 2. Synthecon© RCCS-4D Bioreactor and vessels. The RCCS simulates microgravitational conditions.

2.4 Comet Assay

The Comet Assay was used to quantify specific types of DNA damage using two distinct buffer systems. The components of each buffer as well as the reagents used in the cell preparation process are listed in Table 1.

Table 1. The complete list of buffers and reagents used for running Comet Assays.

Material Name	**Content/Trevigen® Catalog number**	**Purpose**
Comet LM Agarose	# 4250-050-02	"LM" refers to low melting point agarose; the melting temp. near 37°C prevents heat damage. LM Agarose fixed the cells to the slides.
Lysis Buffer	# 4250-050-01	Detergent in the lysis buffer disrupts the cell membrane and breaks open the cell, leaving only the nucleus and nuclear DNA.
TBE Neutral Electrophoresis Buffer	Tris Base, Boric Acid, EDTA and MQ H_2O	TBE was the Neutral Assay running buffer first used in this project. It was discontinued due to poor image quality.
TSA Neutral Electrophoresis Buffer	Tris Base, Sodium Acetate, MQ H_2O, and glacial acetic acid (pH buffer to 9.0)	TSA was the preferred Neutral Assay running buffer; images depicted comets with better definition.
DNA Precipitation Buffer	7.5 M NH4Ac (6.7mL) and 95% EtOH (43.3mL)	Precipitates out double-stranded damage following the Neutral Comet Assay.
Alkaline Unwinding Solution	NaOH Pellets (0.4 g), 200 mM EDTA (250 µL), and MQ H_2O (49.75 mL)	Unwinds DNA to be analyzed as single strands during Alkaline Comet Assay electrophoresis.
Alkaline Electrophoresis Buffer	NaOH Pellets (8.0 g), 500mM EDTA (2mL) and MQ H_2O	Running buffer for the Alkaline Electrophoresis Comet Assay.
SYBR Green Dye	10,000X SYBR® Green in DMSO (1µL) and TE Buffer, pH 7.5 (10mL)	Stains the cell nuclei following electrophoresis. DNA would be appear as green.

2.4.1 electrophoresis system and cell preparation

After the cells were grown and treated, they were harvested for Comet Assay electrophoresis. First, the cells were lifted off each culture plate or flask using Accutase®, transferred to a 15 mL centrifuge tube, and concentrated using centrifugation. The DMEM (in addition to the treatment added) was aspirated, and the cells were washed in Phosphate-

Buffered Saline without calcium or magnesium (PBS -Ca^{2+} -Mg^{2+}). The cells were then resuspended in PBS -Ca^{2+} -Mg^{2+} at a 1:50 dilution and mixed with Comet LM Agarose®. While the agarose-cell suspension remained liquid, each sample was pipetted onto Comet Slides that were pre-treated to promote adherence. The slides were set to solidify at 4°C for 10 minutes before being transferred to Lysis Buffer for 30 minutes, also at 4°C. Following the lysis step, either a neutral buffer system or an alkaline buffer system was prepared to specifically analyze dsDNA breaks or ssDNA breaks, respectively.

Trevigen Comet Assay® Electrophoresis System is a single-cell gel electrophoresis assay that, according to Trevigen Inc., "provides a simple, effective method for evaluating DNA damage…based upon the ability of denatured, cleaved DNA fragments to migrate out of the nucleoid under the influence of an electric field."[13] Gel electrophoresis is an electrolytic cell event; the electrodes are reversed so that the anode is positively charged, and the cathode is negatively charged. The broken, negatively charged DNA strands are electrically attracted to the anode and will therefore migrate away from the nucleus towards the positive end of the charged buffer system. DNA inside the nucleus will also migrate towards the anode, but the larger size of the undamaged DNA molecule, as well as the decreased permeability of the intact nuclear membrane contributes to a much slower migration. Any damaged fragments of DNA migrate out of the compromised nuclear envelope and create a tail that mimics the celestial comets in space, hence the name Comet Assay. Figure 3 represents a simplified form of the electrophoresis system.

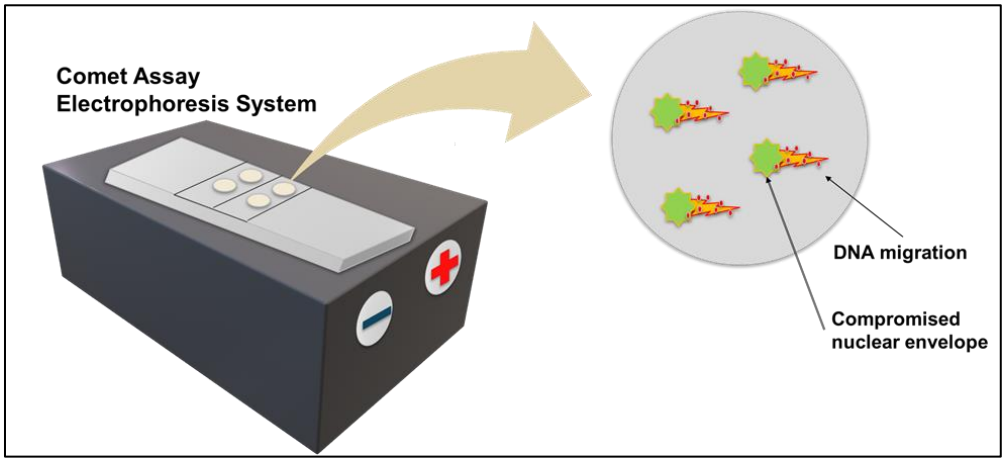

Figure 3. The cartoon depicts the cell nuclei that have been compromised due to the introduction of a mutagenic reagent. The increased permeability in the damaged cell membrane allows fragmented DNA to migrate out of the nuclear envelop, toward the anode of the electrolytic system.

2.4.2 staining, imaging, and software analysis

DNA damage (or lack thereof) was seen on each sample by staining each slide with 50µL of SYBR Green dye. The dye was placed on each well for 5 minutes, during which time the slides were placed in a dark box to prevent ambient light from quenching the dye's fluorescence. After 5 minutes, excess dye was removed from the edge of each well and the slides were dried in an incubator set at 37ºC that was likewise impervious to ambient light. Once the slides were fully dried (approximately 15 minutes), they were kept in a dark box and imaged within 24 hours of staining.

The DNA slides were originally imaged using the EVOS XL Core Cell Imaging System located in the Villa-Diaz laboratory within the biology department of Oakland University. Each well was imaged manually, with a minimum of 10 images per well. Recently, a Cytation 1 automated microscope (BioTek) was purchased to improve the accuracy of comet image acquisition and the speed of data collection. Once the images were obtained, each comet could be individually assessed. DNA damage was quantified by measuring the length of the comet tail that forms from the migration of the damaged DNA.

Trevigen Comet Assay® Software was used to process the images from each experiment. Data sets were created from each condition. The software system was programmed to recognize comets from an Alkaline Comet Assay or a Neutral Comet Assay, depending on the selected protocol. During the scanning step, comets were selected by the program based on the expected parameters, including height, width, and intensity. Once images were scanned, they were manually examined during the review step. This step was used to rate the quality of the comets found, to

distinguish a true comet from debris, and to determine if the selected box size was appropriate for the length and width of the comets scanned. The review step proved to be key to the accurate analysis of DNA damage for each sample.

3. Results

3.1 Oxidative Stress in Standard Growth Conditions

The HEK293 cells indicated a concentration-dependent increase in DNA damage when treated with H_2O_2. The images taken before and after treatment revealed increased apoptosis and decreased viability, which can be seen in Figure 4. Apoptosis is referred to as programmed cell death and is a normal part of the cell cycle. It serves to eliminate old cells that have decreased function and are near the end of their lifespan. Apoptosis also removes cells that have accumulated irreversible DNA damage that could lead to cancer if the cell is not eliminated.

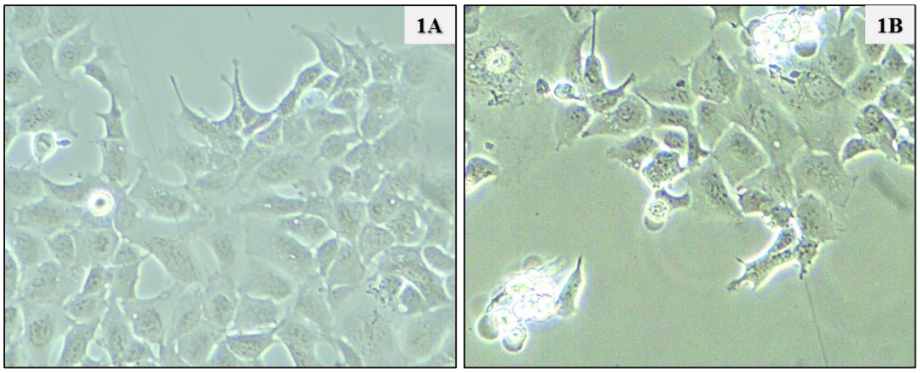

Figure 4. HEK293 cells untreated (panel 1A) vs. treated with H_2O_2 (panel 1B).

Dead cells appear under the microscope as pinpoint, floating circles. On panel 1B of Figure 4, a cluster of dead cells can be seen floating in the lower left corner. By using microscopic imaging alone, it is impossible to determine the number of cells that were removed solely because they accumulated mutagenic changes to their genomic DNA. Thus, the addition of the Comet Assay data was key in determining the quantity of cells that were programmed to die due to irreversible damage and not simply old age. The difference between an undamaged nucleus, a nucleus with single-stranded DNA damage and a nucleus with double-stranded DNA damage is shown in Figure 5.

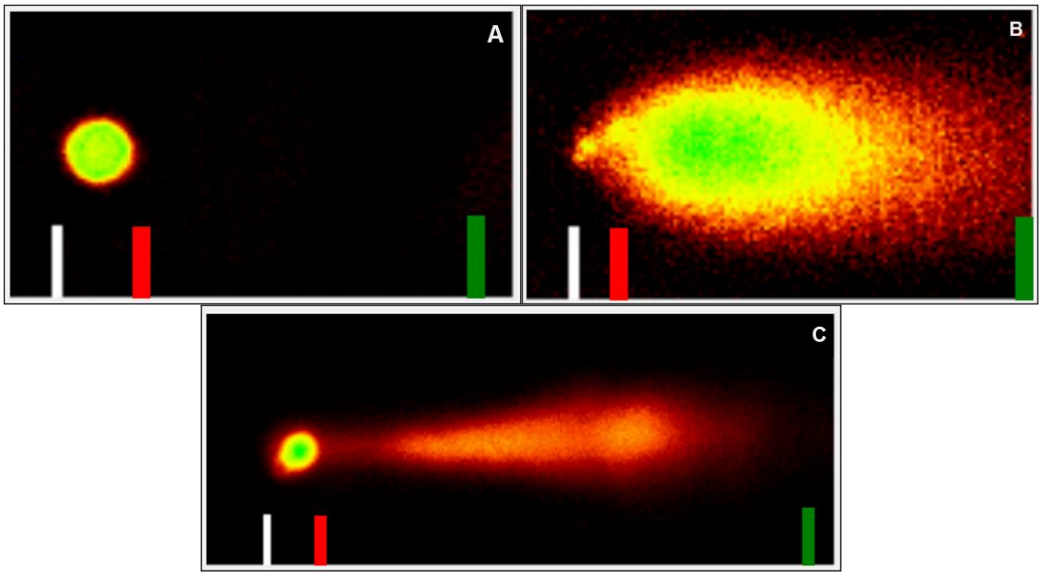

Figure 5. Panel A is an untreated nucleus with no DNA damage. Panel B represents a treated nucleus depicting a shortened, broad-tail comet characteristic of ssDNA breaks. Panel C is a damaged nucleus with a long narrow comet representing dsDNA breaks.

3.2 Microgravity Simulation

Microgravity studies performed on HEK293 cells were successful. The extent of the data obtained from the experiment is shown in Table 2. It is clear that in kidney cells, microgravity has a synergistic effect on DNA damage. When coupled with any of the three treatments, the microgravity environment increased the occurrence of both single-stranded and double-stranded DNA breaks. Additionally, in untreated cells (where the only variable was microgravity) endogenous DNA damage spiked. A graphical comparison of the effects of microgravity on DNA damage accumulation for each treatment condition is detailed in Figures 6, 7, and 8.

Table 2: Comparison of Normal Growth to Microgravity Simulation in Embryonic Kidney Cells

Normal Growth Conditions			
Alkaline Assay			
Treatment	% DNA inTail (Mean)	StDev	No. of Comets
Untreated	2.47	3.04	288
350 µM H_2O_2	40.42	24.06	167
100 µg/mL Camp.	25.56	13.29	189
Nuetral Assay			
Treatment	% DNA inTail (Mean)	StDev	No. of Comets
Untreated	4.49	6.17	112
350 µM H_2O_2	27.18	9.4	331
100 µg/mL Bleo.	33.65	10.14	109

Microgravity Conditions			
Alkaline Assay			
Treatment	% DNA inTail (Mean)	StDev	No. of Comets
Untreated	14.63	11.72	277
350 µM H_2O_2	72.71	19.68	169
100 µg/mL Camp.	43.15	12.37	258
Nuetral Assay			
Treatment	% DNA inTail (Mean)	StDev	No. of Comets
Untreated	25.14	12.85	138
350 µM H_2O_2	32.23	13.63	86
100 µg/mL Bleo.	66.47	19.71	86

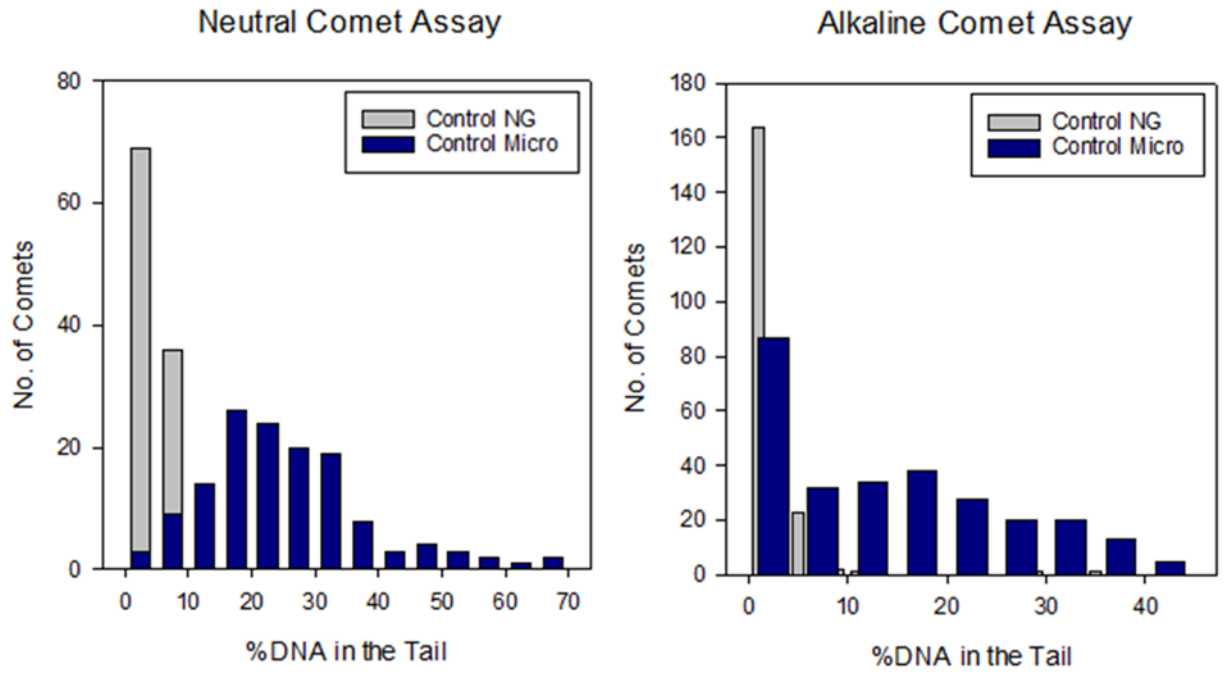

Figure 6. Graphs demonstrate the increased in ds- and ss-damage in untreated cells, represented by Neutral Comet Assay and Alkaline Comet Assay, respectively. “Control NG” refers to kidney cells that were grown in standard growth conditions without treatment. “Control Micro” refers to kidney cells that were grown in the RCCS microgravity simulator (without treatment).

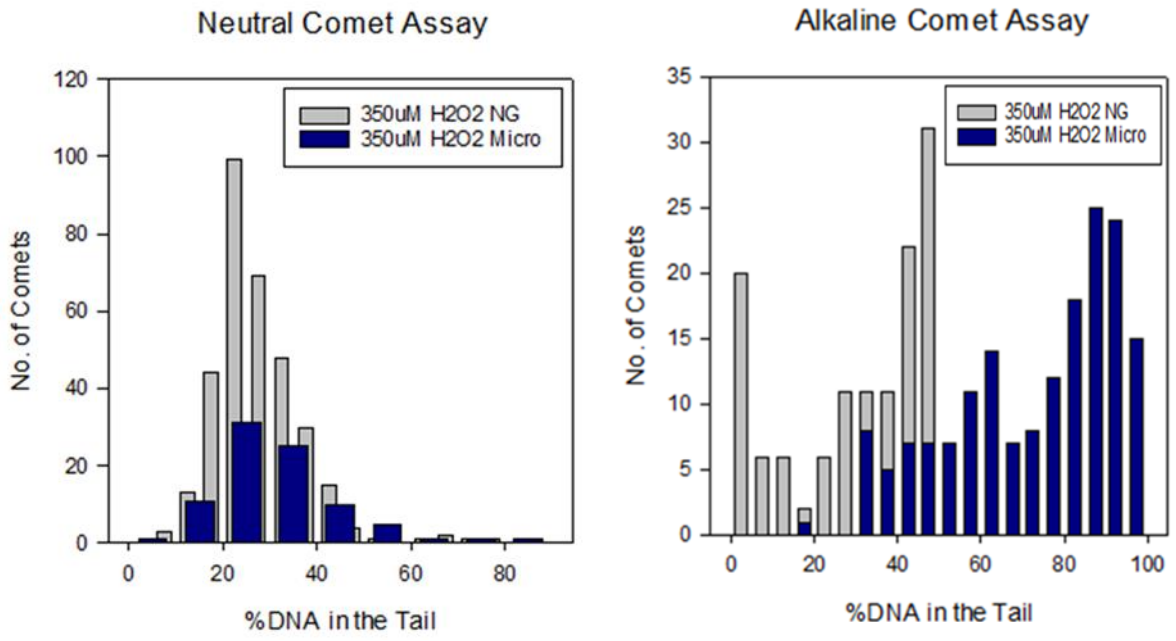

Figure 7. Graphs demonstrate the change in DNA damage for kidney cells treated with H2O2. "NG" refers to kidney cells grown in standard growth conditions, while "Micro" refers to cells that were grown in the RCCS microgravity simulator.

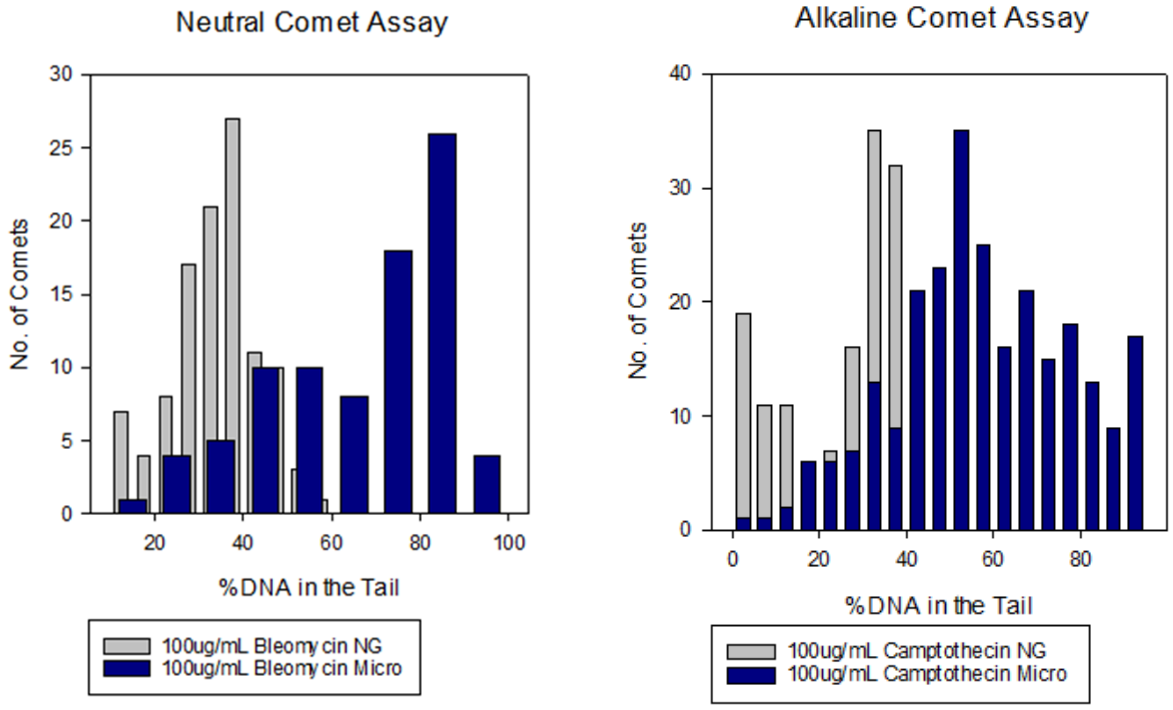

Figure 8. Graphs demonstrate the change in ds- and ss-breaks for kidney cells treated with either bleomycin (to create ds-breaks) or camptothecin (to create ss-breaks). "NG" refers to kidney cells grown in standard growth conditions, while "Micro" refers to cells that were grown in the RCCS.

4. Conclusion and Discussion

The results of the microgravity trial that was performed on kidney cells and analyzed using an Alkaline Comet Assay met the minimum comet count for statistical accuracy; many scientific journals, including *Nature*, publish Comet Assay data with a minimum of 100 comets. Considering the new Comet Assay Neutral Electrophoresis Buffer and the new Cytation 1 microscope imaging software that was acquired, it is likely that this trial will be repeated to establish reproducibility before further data is published.

Overall, the data provides new insight into how kidney cells respond to oxidative stress and microgravity simulation. In the standard growth environment, where the only variable was hydrogen peroxide treatment, it was clear that the cells exhibited a higher accumulation of DNA damage when treated. While oxidative stress has already been proven to cause DNA damage in prior research, this work identified type-specific damage that was not previously known. For example, when the kidney cells were treated with hydrogen peroxide under normal growth conditions, they exhibited 27.18% double-stranded DNA breaks compared to 40.42% single-stranded DNA breaks (Table 2).

This new data is useful because it allows us to quantify the percent of double-stranded breaks (DSBs) and single-stranded breaks (SSBs) that will arise from increased oxidative stress in each cell type. DNA damage due to oxidative stress is inevitable since reactive oxygen species are common byproducts of cellular metabolism. By distinguishing the type of DNA damage that was more likely to occur, we identified which cells were more prone to DSBs, and furthermore, we can predict which cells will show higher frequencies of DNA mutations and cell death for future work.

The microgravity simulation studies additionally provided new information about how kidney cells tolerate a low gravity environment. As untreated kidney cells grew in the RCCS, they experienced a 20.75% increase in endogenous double-stranded DNA breaks in comparison to the same passage of cells grown in standard conditions (Table 2). As previously mentioned, the microgravitational environment had a negative impact on cellular repair and increased the effects of oxidative stress, bleomycin, and camptothecin. This discovery is critical as it provides insight that is relevant to the expansion of human civilization into space. As Mars Project One prepares to establish human settlements in lower gravity environments, knowing the impact of microgravity on the accumulation of DNA damage across various human cells will be essential. Simulations done with different cells in an RCCS can be used to predict the likelihood of human survival in space.

5. Preliminary Studies and Future Work

Preliminary studies comparing the effects of oxidative stress between different cell types have begun; the details are shown in Figure 9. After comparing multiple cell types, it became apparent that certain cells could better withstand the effects of induced oxidative stress. For instance, cardiomyocytes exhibited the highest amount of ss-damage, with an average of 52.89% DNA in the tail of the comet, while the retinal cells showed the least amount of ssDNA damage (20.87%). When the cells were analyzed for double-stranded breaks, breast cancer cells had the greatest increase in DNA damage, while cardiomyocytes had only an 8.12% increase in double-stranded breaks in treated cells.

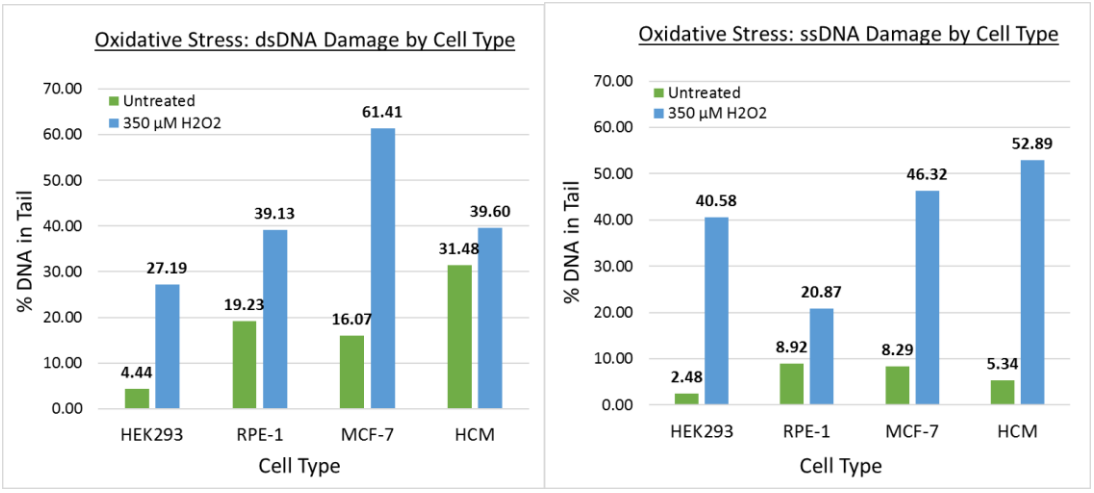

Figure 9. DNA damage seen in kidney (HEK293), retinal (RPE-1), breast cancer (MCF-7), and heart (HCM) cells.

For future experiments, data acquisition and analysis will be much more efficient. The previously mentioned Cytation 1 microscope can automatically image entire sets of slides and stitch together an overall image for each well in a quarter of the time it took to manually image the slides. Moving forward, this project has many potential applications, including microgravity simulation performed on kidney cells that could be expanded into time-dependent studies. The levels of genomic DNA damage may increase, decrease, or remain constant over larger periods of microgravity exposure (greater than 48 hours).

The microgravity trial that was performed on kidney cells will be replicated in retinal cells, cardiomyocytes, and dermal fibroblasts – a type of skin cell. Eventually, this project may expand to include serology studies to determine if microgravity affects the coagulation cascade by altering hemostatic mechanisms, or if microgravity changes the oxygen-carrying capacity of hemoglobin. Finally, cancer-cell-specific trials can be done to compare the repair mechanisms of cancerous breast cells (MCF-7) to cancerous ovarian cells (HeLa) under microgravity simulation.

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