The Impact of Microgravity on the Accumulation of DNA Damage in Human Cells

Submitted by

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Abstract

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 (National Aeronautics and Space Administration, 2008). In outer space, astronauts undergo direct exposure to oxidative stress and ultra-violet (UV) radiation due to a low-oxygen environment and the absence of an ozone layer. Although prolonged oxidative stress and UV radiation damages genomic deoxyribonucleic acid (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 cells. We measured the effects of hydrogen peroxide, bleomycin, and camptothecin on human 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. The experimental outcomes seek to increase the safety of space travel by understanding how microgravitational environments could impact DNA repair mechanisms.

Introduction

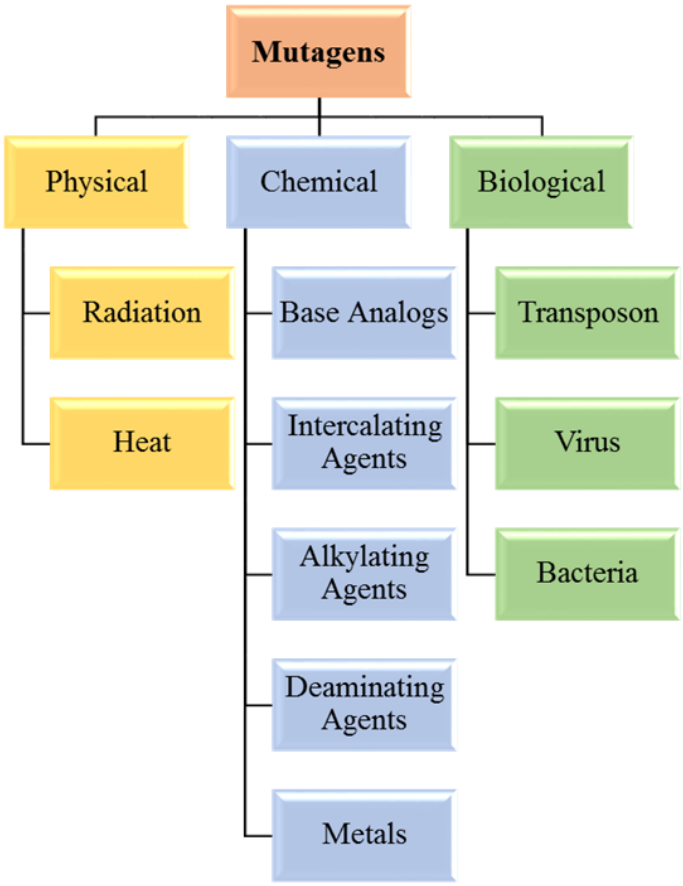
In order to understand the relevance of this research, it is important to first grasp the prevalence of deoxyribonucleic acid (DNA) damage that occurs in outer space. When astronauts are on a mission, they undergo direct, constant exposure to mutagens. The Merriam-Webster dictionary defines a mutagen as, "an agent (such as a chemical or various radiations) that tends to increase the frequency or extent of mutation" (Merriam-Webster, n.d.). Environmental mutagens are concerning because unlike other environmental stimuli that may cause physical changes to the cells composition (such as changes in cell signaling), mutagens directly alter the DNA sequence of a cell. According to a 2008 Nature Education article, the size and structure of many mutagens is advantageous, enabling them to pass through both the cellular and nuclear membranes to interact with DNA directly, resulting in DNA damage (Ralston, A., 2008). There are three main classifications of mutagens: physical, chemical, and biological. Figure 1 outlines subcategories for each main type of mutagen, which includes radiation.

Due to the absence of an ozone layer in outer space, astronauts are exposed to higher levels of ultra-violet (UV) radiation. Research has shown that prolonged UV radiation induces cyclobutane pyrimidine dimers and (6,4) pyrimidine-pyrimidone dimers; types of DNA damage that lead to genomic instability if not repaired (Pelloux et al., 2012). Additionally, the increased aerobic activity conducted by astronauts promotes excessive oxidative stress (Leeuwenburgh, C., & Heinecke, J. W., 2001). However, the long-term effects of microgravity on cellular repair mechanisms are not fully understood. The purpose of this study is to determine how a microgravitational environment impacts human cell growth and to understand how microgravity influences DNA repair. 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. To test this hypothesis, project objectives were created to answer the central questions surrounding this theory. Both the objectives and central questions are outlined in Table 1.

Figure 1

Categorization of mutagens



Note. The diagram above depicts how the umbrella term 'mutagen' can be caused by a number of sources broken into three distinct categories. Physical, chemical, and biological mutagens can be further broken into specifically identified stimuli, such as radiation, that damages DNA. The accumulation of damage within the cell can lead to cell death. Figure 1 was based on a graphic created by biologyboom.com.

Table 1

Steps to answering the hypothesis

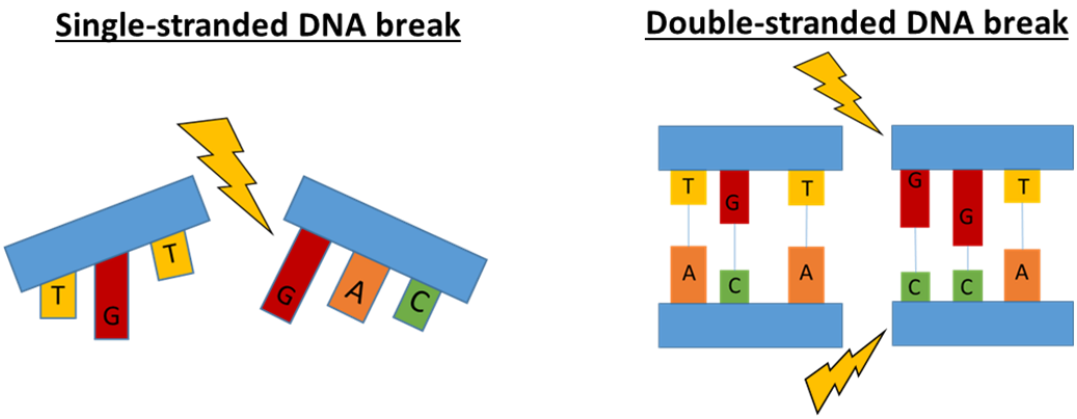
Central Questions
What is the expected trend of DNA damage in cells treated under normal growth conditions?
Are repair mechanisms influenced by microgravity? If so, to what extent?
Does microgravity bias the formation of one type of DNA damage over another (if at all)?
Objectives
Establish baseline ssDNA and dsDNA breaks in standard growth conditions
Introduce mutagens and monitor potential changes in repair mechanisms using biochemical assays
Replicate baseline experiments with cells under microgravity simulation

Note. The central questions and objectives influenced the project methodology, providing the basis for the protocols that were written.

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 (Coyle, C., & Kader, K., 2007). 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 (Povirk, 2012). Figure 2 visually depicts the two types of DNA damage monitored throughout the course of this project.

Figure 2

Types of DNA damage

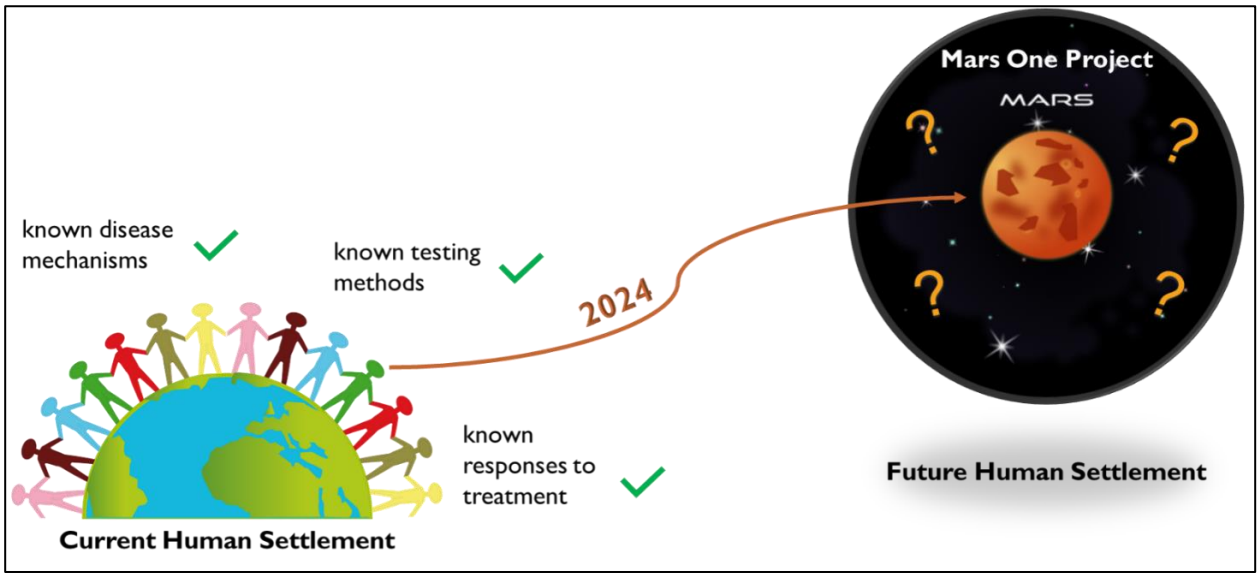


Note. This graphic compares a ssDNA break to a dsDNA break. Both types of damage occur naturally and can be induced in human cells using chemical mutagens.

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 (Mars One, n.d.). As humanity expands into alternative settlements, it is essential to determine how DNA integrity could be impacted by new environmental stimuli (Figure 3). The goal of the Human Research Project (HRP) developed by the National Aeronautics and Space Administration (NASA) is to, "provide human health and performance countermeasures…to enable safe, reliable, and productive human space exploration…by [developing] technologies that serve to reduce medical and environmental risks…" (Hanson, D. & Febus, D., n.d.). This project aligns with HRP's objective by analyzing the microscopic influences microgravity may have on DNA repair to predict the potential changes of human health in space.

Figure 3

Reality of Settlements in Space



Note. The cartoon above depicts the current dilemma facing interplanetary human settlements. 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. It is recognized by the identification of immature white blood cells (WBC) in a peripheral blood smear and can be confirmed using established molecular testing. 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 various human cells.

Materials and Methods

Replicates

Each baseline experiment was replicated at least twice, to ensure the data obtained was precise and reproducible. Since every microgravity trial was based on the initial baseline data for each cell type, the microgravity experiments were only performed once.

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. Other cell lines grown during the course of this project include Human Embryonic Kidney (HEK293) cells, Retinal (RPE-1) cells, Cardiomyocytes (HCM), and Breast Cancer (MCF-7) cells.

The human cell stocks were frozen at a concentration of 1×10^6 cells/mL to ensure the cells were seeded at a concentration of approximately 1×10^5 cells/mL. Each cell line was seeded on a T-25 flask at a 1:5 dilution and growth in a cell culture incubator set to 37ºC with 5.0% carbon dioxide and high humidity. All cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS). Hela, HEK293 and RPE-1 cells were grown with the addition of 5.0% penicillin and streptavidin (Pen/Strep) antibiotics to reduce the risk of bacterial contamination, while MCF-7 cells were grown in DMEM enhanced with 10% insulin. The HCM cells were not grown with 5% Pen/Strep because of the fragility of their growth pattern; HCM cells would grow very slowly, if at all, when antibiotics were added.

The growth media was changed every other day. After 48 or 72 hours (depending on the growth speed of the cell line), when the cells had reached 80-90% confluency, the confluent 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.

Experimental reagents

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 chosen reagents: hydrogen peroxide, bleomycin, and camptothecin. The selected concentrations of hydrogen peroxide treatment were first established in HeLa cells, while the concentrations of bleomycin and camptothecin were determined first in HEK293 cells. 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 H_2O_2) 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. Following preliminary experiments, the concentrations of each reagent remained the same across each cell type. 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.

Hydrogen Peroxide

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 were 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

Bleomycin was purchased as a 10 mg dehydrated powder. The maximum solubility of bleomycin in phosphate-buffered saline (PBS) buffer is 10 mg/mL (Cayman Chemical Company, 2018). 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 (Fiorentzis et al, 2019).

Camptothecin

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 (Cayman Chemical Company, 2016). 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 in which similar ranges were used (Lee et al., 2003).

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, which is depicted in Figure 4. 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 (Mitteregger et al., 1999). 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 (Figure 5), a different approach was taken. After the cells had been sheared off the original plate and resuspended in media, they were concentrated using a centrifuge set at 1000 g's for 3 minutes. 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.

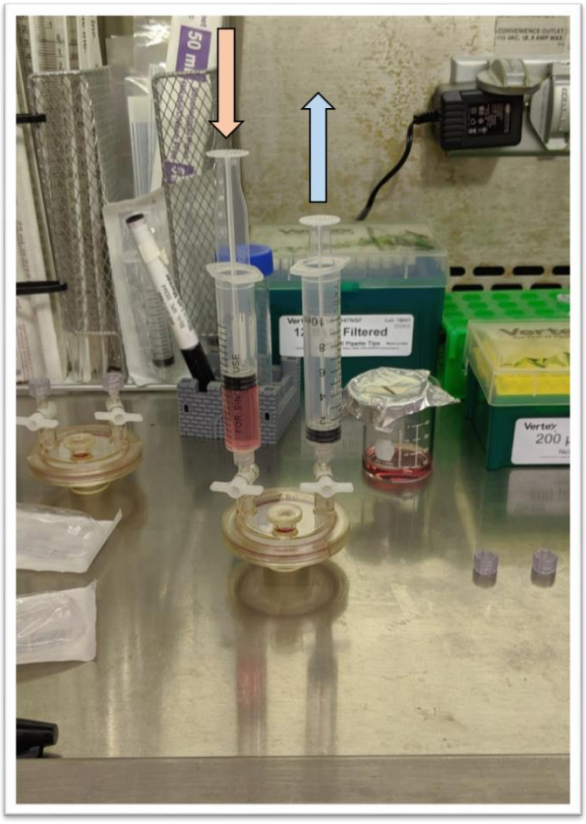
Figure 4

Synthecon© RCCS-4D Bioreactor and vessels

Note. The RCCS simulates microgravitational conditions.

Figure 5

Seeding a 10 mL Rotary Cell Culture Vessel



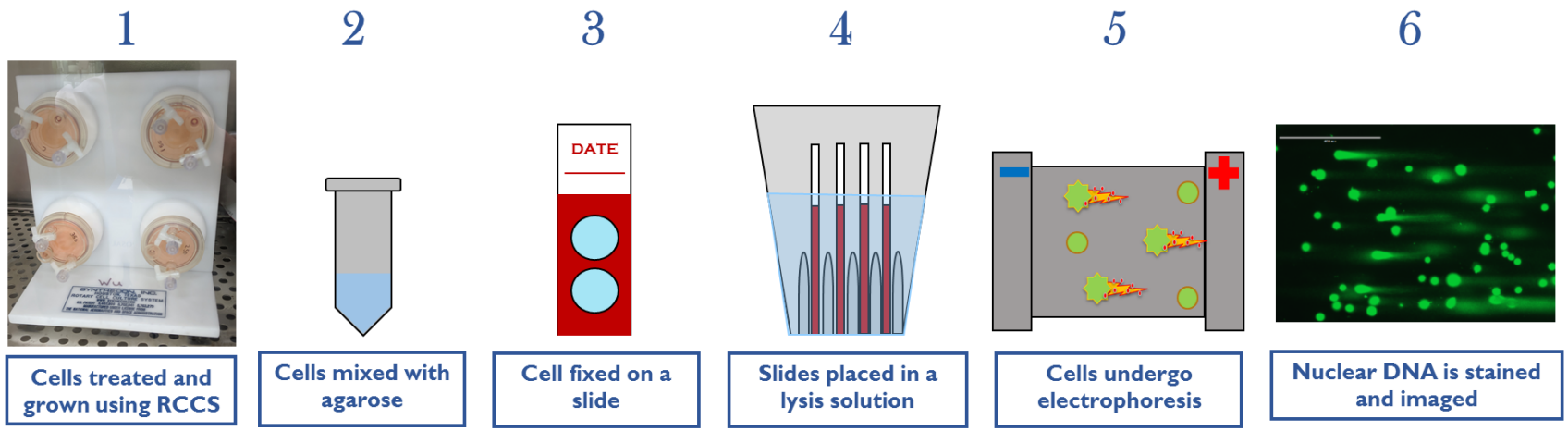
Note. The image above shows how human cells were loaded into the rotary cell culture vessel. A complex valve system ensured there were minimal air bubbles inside the vessel. In this picture, both ports are locked in closed position. When the valves are both opened, air is suctioned out of the vessel (blue arrow) while cells in DMEM are simultaneously flushed into the vessel (orange arrow). Loading cells into the vessel is a time-sensitive process, which is why the protocol was modified to minimize exchanging media and increase efficiency.

Comet Assay

An overview of the Comet Assay protocol is depicted in Figure 6 on the following page.

Figure 6

Comet Assay Overview



Note. Step 1 depicts cells grown in the RCCS located in the Wu Lab incubator. After being cultured, the cells were harvested and combined with low melting agarose shown in Step 2. Cells were then evenly spread onto a two-well slide depicted in Step 3 before being washed with a Lysis shown in Step 4. After the slides were fully processed so that only the nucleus of each cell remained, they were subjected to an electrophoresis system shown on Step 5, and finally, stained to quantify the comets formed, which is shown in Step 6.

Buffers and Reagents

Table 2

Materials Used in Comet Assay

Material Name	Content/Trevigen® Catalog number	Purpose
Comet LM Agarose	# 4250-050-02	"LM" refers to the low melting point of the agarose, which is advantageous to use with live cells because the melting temp. near 37°C prevents heat damage. LM Agarose fixed the cells on the slides.
Lysis Buffer	# 4250-050-01	The 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	1. Tris Base 2. Boric Acid 3. EDTA 4. MQ H_2O 5. 10X stock was diluted to 1X working strength	TBE was the Neutral Assay electrophoresis running buffer first used in this project. It was discontinued due to poor comet image quality.
TSA Neutral Electrophoresis Buffer	1. Tris Base (MW 121.14) 2. Sodium Acetate (MW 82) 3. MQ H_2O 4. glacial acetic acid (to pH buffer to 9.0) 5. 10X stock was diluted to 1X working strength	TSA has become the preferred Neutral Assay electrophoresis running buffer because recent images have shown this system produces a comet with better definition.
DNA Precipitation Buffer	1. 7.5 M NH4Ac (6.7mL) 2. 95% EtOH (43.3mL)	This buffer was used for the Neutral Comet Assay following electrophoresis to precipitate out double-stranded damage.
Alkaline Unwinding Solution	1. NaOH Pellets (0.4 g) 2. 200 mM EDTA (250 µL) 3. MQ H_2O (49.75 mL)	This buffer was used for the Alkaline Comet Assay to unwind the DNA to be analyzed as single strands during electrophoresis

Material Name	Content/Trevigen® Catalog number	Purpose
Alkaline Electrophoresis Buffer	1. NaOH Pellets (8.0 g) 2. 500mM EDTA (2mL) 3. MQ H_2O	This buffer was used as the running buffer for the Alkaline Electrophoresis Comet Assay to analyze ssDNA brakes.
SYBR Green Dye	1. 10,000X SYBR® Green in DMSO (1µL) 2. TE Buffer, pH 7.5 (10mL)	The dye was used to stain the cell nuclei following electrophoresis. Any damaged DNA would appear as a green comet.

Note. The table above outlines the complete list of buffers and reagents used for Comet Assay runs, including the name of the material, the chemical components as to how it was made (or the catalog number if purchased), and the purpose it served for the experiment.

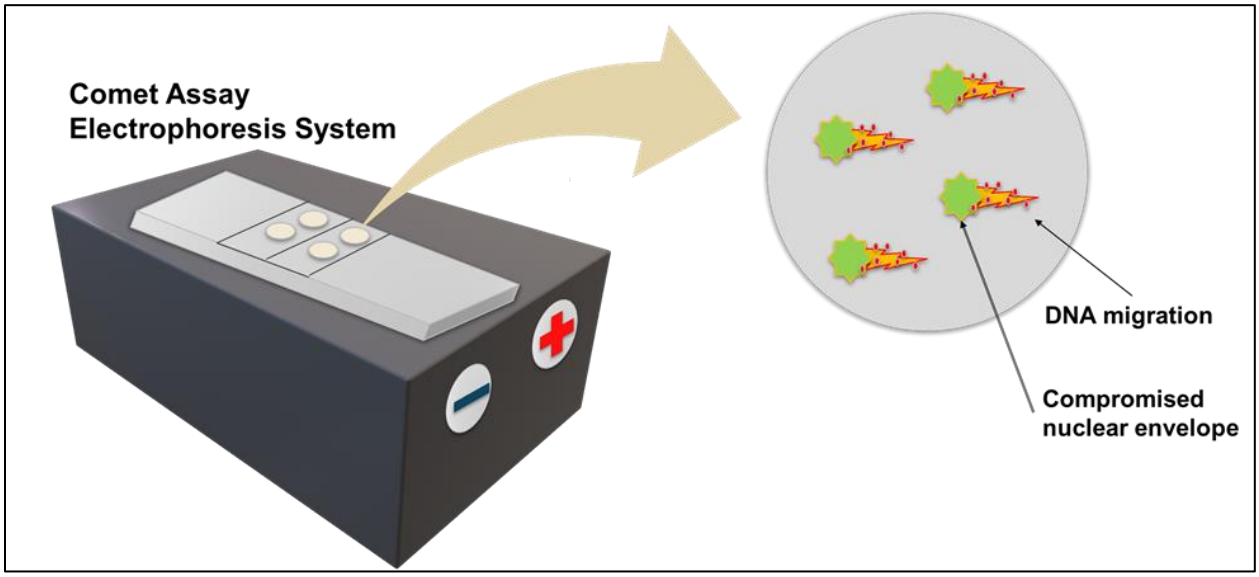
Electrophoresis System and Cell Preparation

After the cells were grown and treated, the cells 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 spun in a centrifuge set at 1000 g's for 3 minutes. 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 analyze dsDNA breaks or ssDNA breaks, respectively. The details of these two applications can be found under *DNA-damage specific applications,* pg. 19.

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" (Trevigen Reagent Kit, Catalog # 4250-050-K). Gel electrophoresis is an electrolytic cell event, which essentially means the electrodes are reversed; 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. The 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 causes 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 7 represents a simplified form of the electrophoresis system.

Figure 7

Comet Assay Electrophoresis Cartoon



Note. The cartoon above depicts 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. DNA damage can be quantified by measuring the length of the comet tail that forms from the migration of the damaged DNA.

DNA-damage specific applications

Trevigen Comet Assay® Electrophoresis System has the ability to specifically analyze either ssDNA breaks or dsDNA breaks based on the buffer system used. The Neutral Comet Assay was used to detect dsDNA breaks, whereas the Alkaline Comet Assay, which is a more sensitive assay, was used to detect ssDNA breaks.

Staining and Imaging

DNA damage (or lack thereof) was seen on each sample by staining the wells of 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 passed, 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), the slides 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.

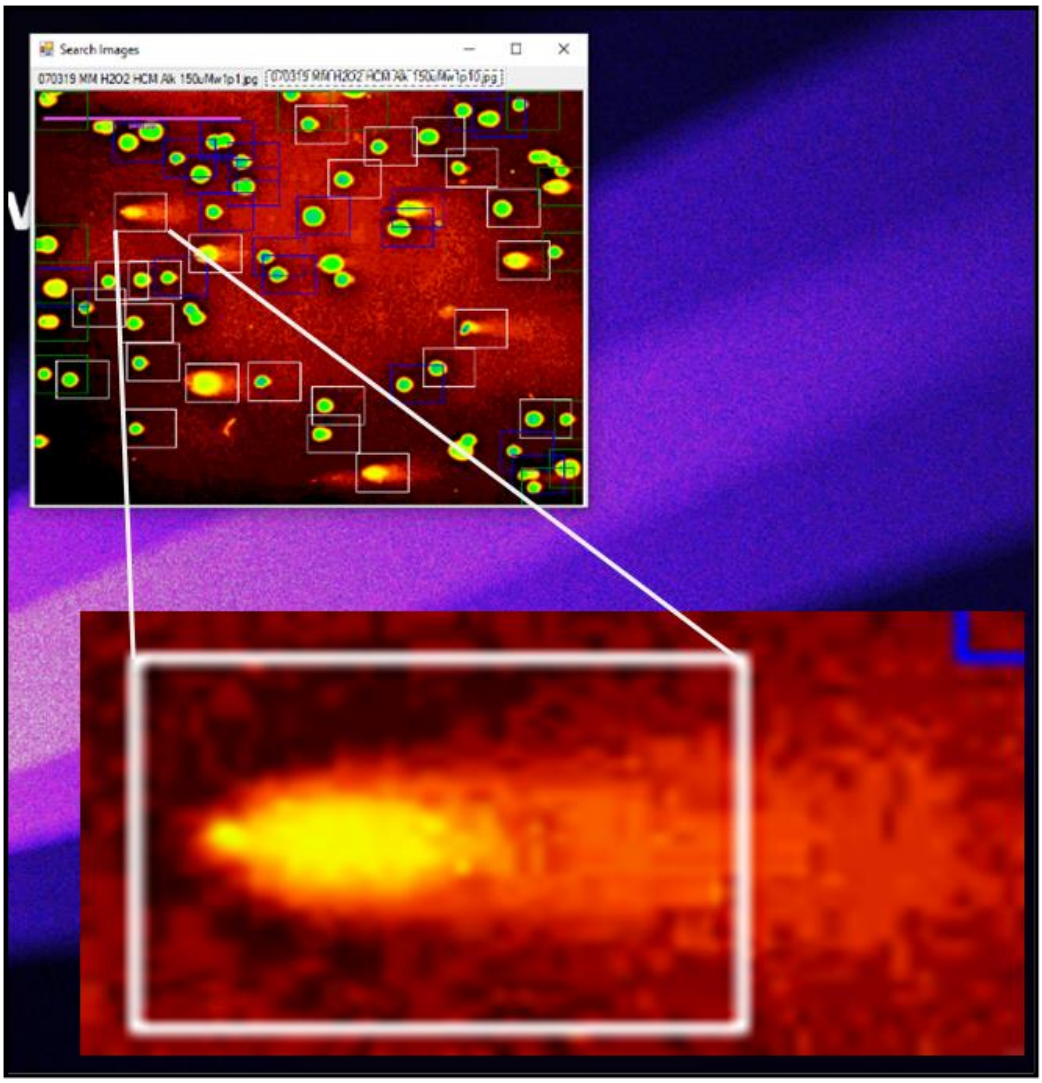
Software Analysis

Trevigen Comet Assay® Software was used to process the images from each experiment. Data sets were created for each condition. For example, *HEK293 Micro Alk. Assay Control* represents a data set of kidney cells that were untreated, grown in microgravity simulation, and analyzed using a Comet Assay with an alkaline buffer system. The images from each well were uploaded and scanned for possible comets. 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. Figure 8 represents a snapshot of this process.

After the well 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. Examples of pseudo-comets that showed high levels of DNA damage but were repeatedly excluded from computational analysis are shown in Figure 9.

Figure 8

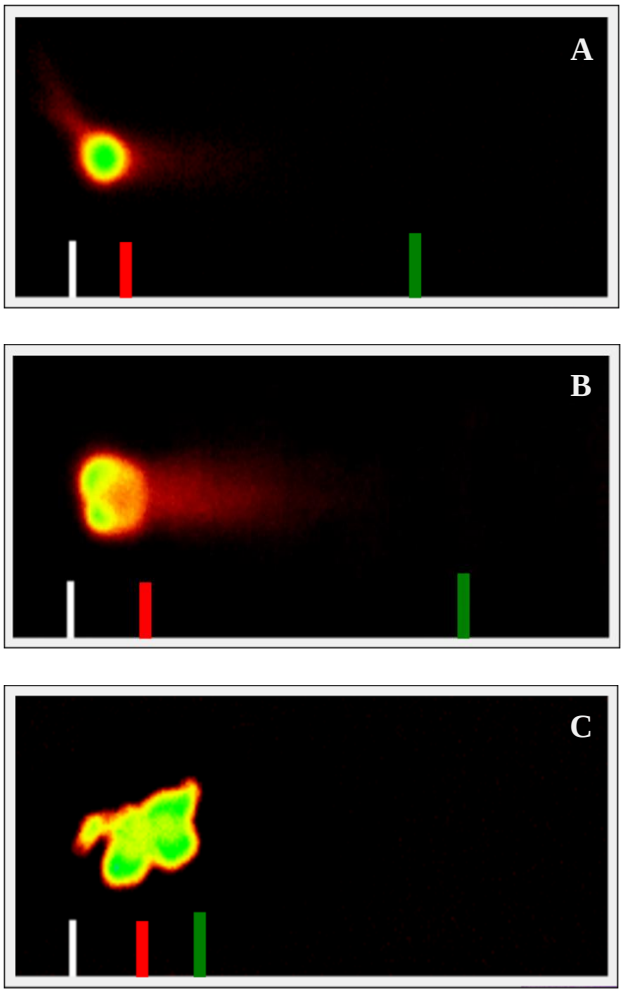
Comet Scanning Step



*Note***.** The figure above describes the scanning step of the Comet Assay. White boxes are placed around comets that meet the programmed criteria for acceptance, while blue boxes are placed around potential comets that are not selected.

Figure 9

Sample Comets Excluded from Analysis



Note. In the figure above, panel A represents a comet that has DNA fragmented in multiple directions. The "multi-tail" comets were excluded because the tail-length could not be measured in multiple directions simultaneously. Similarly, comets that were bi-nucleated had to be excluded, as seen in panel B. It was impossible to tell if two individual nuclei had fused together, or if one nucleus had split in half, so all comets exhibiting a bi-nucleated appearance were excluded. Lastly, panel C represents debris that absorbed the SYBR Green dye and met the length/width standard of a comet but was clearly not a cell nucleus. They were likewise excluded.

Results

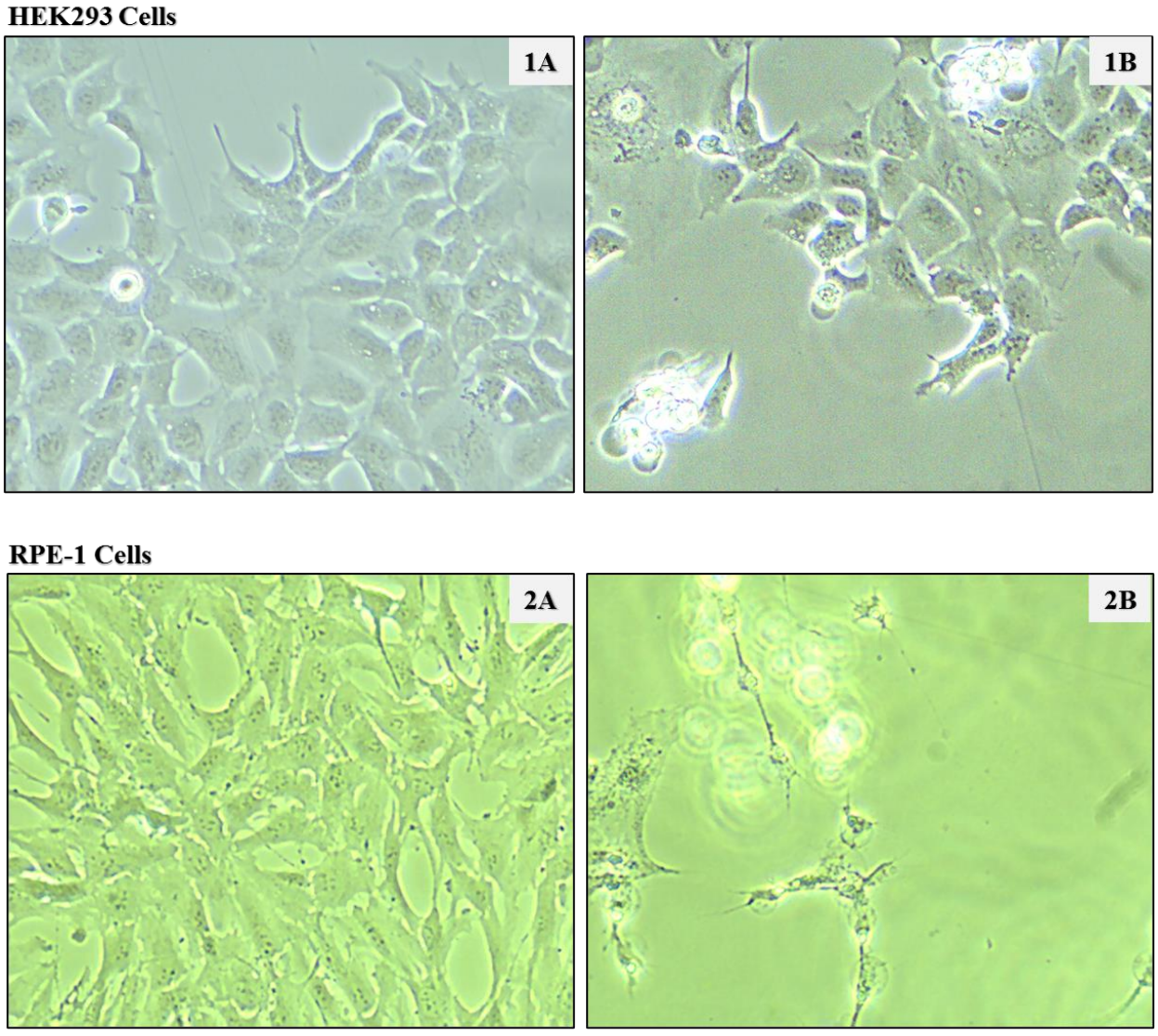
Oxidative Stress in Standard Growth Conditions

All four cell types 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 Figures 10 and 11. It is important to note the images for both Figure 10 and Figure 11 were not enhanced in any way. The change in color was due to the position of the condenser on the microscope, which changed the amount of light directed towards the objective to improve focus.

Apoptosis is referred to as programmed cell death, and it is a normal part of the cell cycle. It serves as a means 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. Dead cells appear under the microscope as pinpoint, floating circles. On panel 1B of Figure 10, for instance, a cluster of dead cells can be seen floating in the lower left corner. By using microscopic imaging alone, it is not possible to determine the number of cells that were removed 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 12. Figure 13 is a graph depicting the percent of single-stranded breaks that occurred in each cell type as a result of oxidative stress. Similarly, Figure 14 is a graphical representation of the percent of double-stranded breaks that occurred in the same cell types under the same conditions.

Figure 10

Nikon Eclipse Ts2 microscope images of kidney and retinal cells

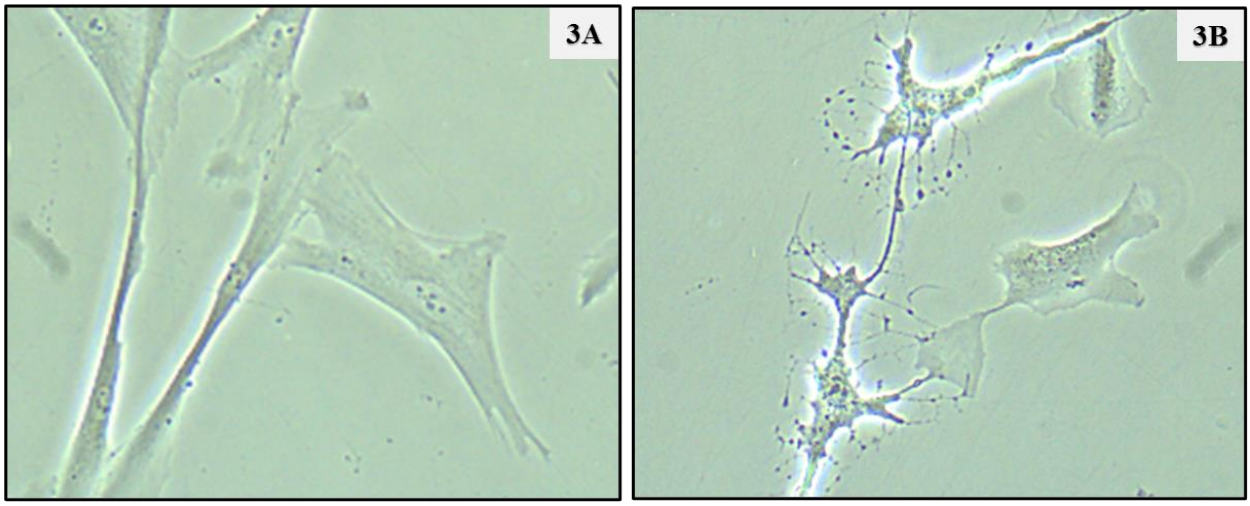


Note. HEK293 refers to the type of kidney cells cultured for this experiment, while RPE-1 refers to the type of retinal cells studied in the same manner. Image 1A represents untreated kidney cells while image 1B represents kidney cells treated with 350 µM H_2O_2. Likewise, image 2A represents untreated retinal cells, while image 2B represents retinal cells treated with 350 µM H_2O_2. Cell death (floating cells) and increased cellular stress are visible under treatment.

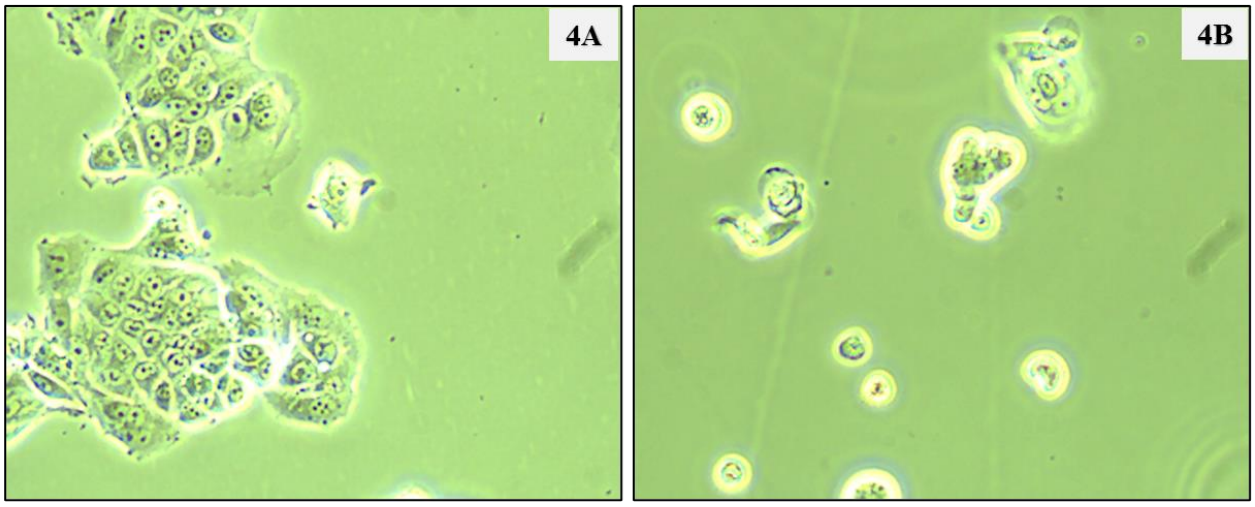
Figure 11

Nikon Eclipse Ts2 microscope images of heart and breast cancer cells

HCM Cells



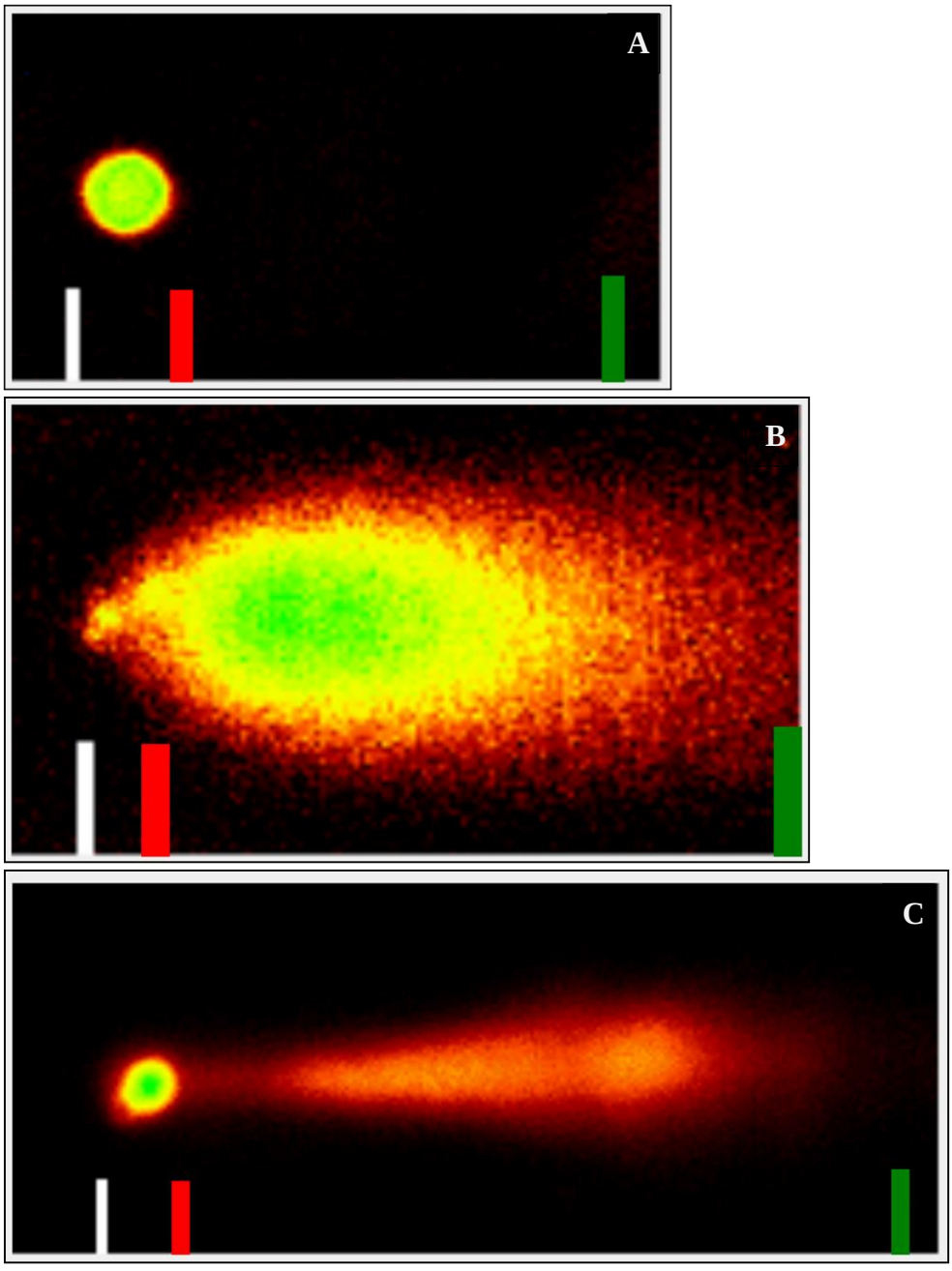
MCF-7 Cells



Note. HCM refers to the type of heart cells cultured for this experiment, while MCF-7 refers to the type of breast cancer cells studied in the same manner. Image 3A represents untreated heart cells while image 3B represents the same cells treated with 350 µM H_2O_2. Likewise, image 4A represents untreated breast cancer cells, while image 4B represents the same cells treated with 350 µM H_2O_2. Cell death (floating cells) and increased cellular stress are visible under treatment.

Figure 12

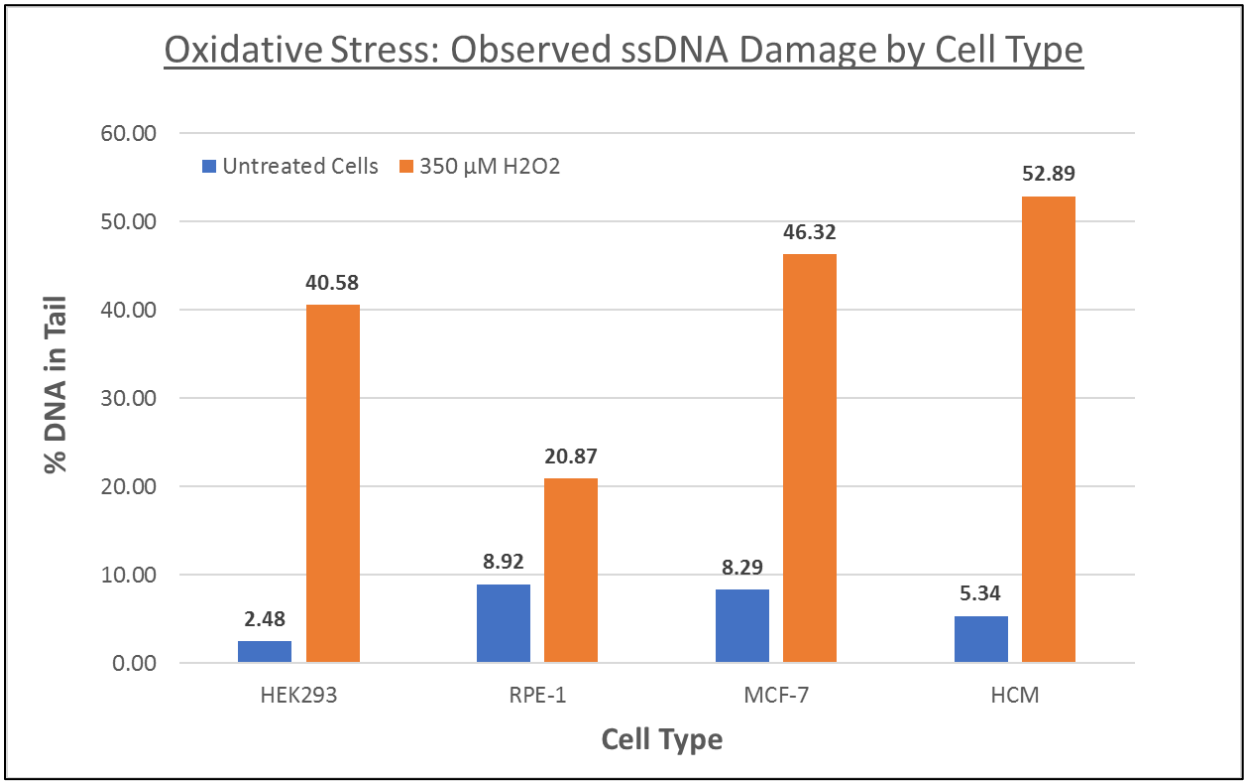
Nuclei Results from Comet Assay



Note. In the figure above, 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.

Figure 13

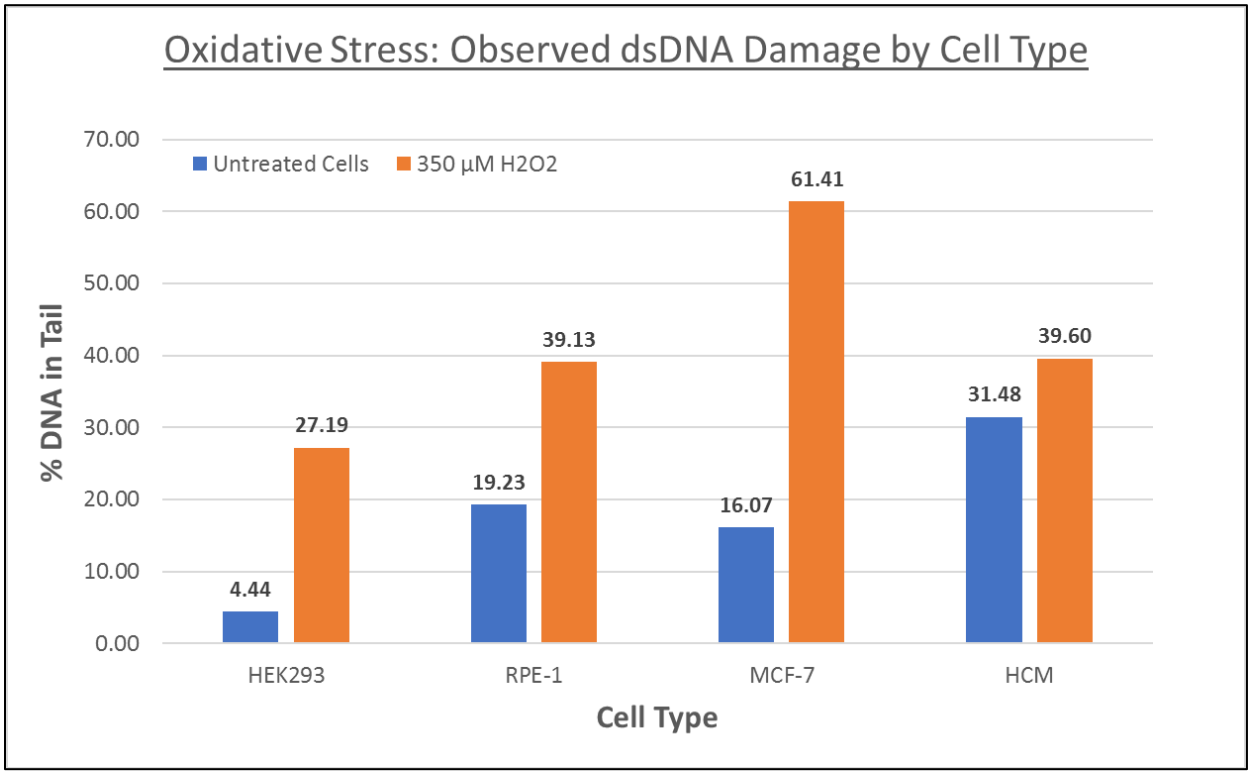
Single-stranded DNA Damage per Cell Type



Note. The graph above shows the amount of single-stranded DNA damage that accumulated when cells were subjected to oxidative stress. The four cell types; kidney cells (HEK293), retinal cells (RPE-1), breast cancer cells (MCF-7), and human cardiomyocytes (HCM) were treated with hydrogen peroxide and analyzed using an Alkaline Comet Assay. From this data, we were able to analyze the change in ss-DNA damage between treated and untreated cells. Additionally, we determined in which cell type oxidative stress created the highest percent of single-strand damage overall.

Figure 14

Double-stranded DNA Damage per Cell Type



Note. The graph above shows the amount of double-stranded DNA damage that accumulated when cells were subjected to oxidative stress. The four cell types; kidney cells (HEK293), retinal cells (RPE-1), breast cancer cells (MCF-7), and human cardiomyocytes (HCM) were treated with hydrogen peroxide and analyzed using a Neutral Comet Assay. From this data, we were able to analyze the change in ds-DNA damage between treated and untreated cells. Additionally, we were able to determine in which cell type oxidative stress had the greatest overall impact.

Microgravity Simulation

Microgravity studies were performed on both kidney cells and cardiomyocytes. Growing cells in the RCCS proved to be challenging as the unique culture system was a difficult adjustment for certain cell lines. Unfortunately, no feasible data was obtained from the initial cardiomyocyte experiment due to a drastic drop in cell count (the Comet Assay recovered less than 10 nuclei per condition). However, the microgravity trials on kidney cells were successful. The extent of the data obtained from the experiment is shown in Table 3. 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 15, 16, and 17.

Table 3

Microgravity Simulation of Kidney Cells

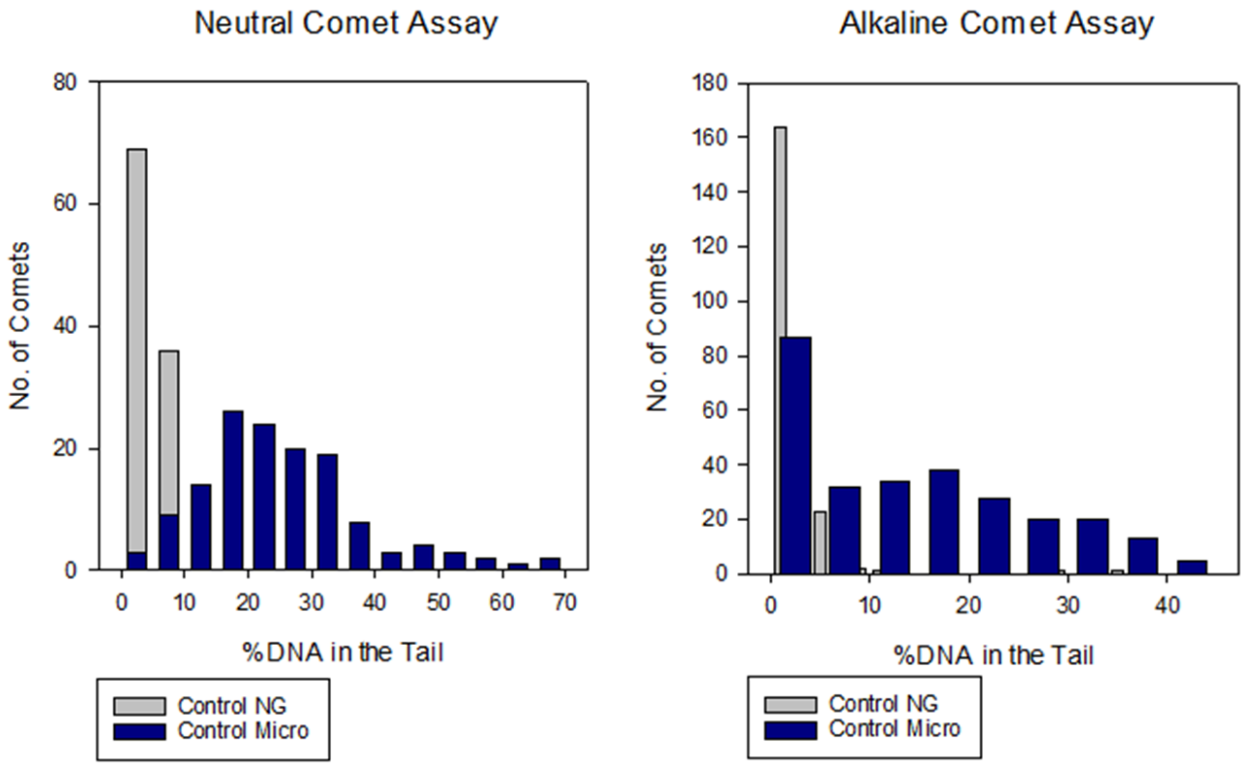
Normal Growth Conditions			
Alkaline Assay			
Treatment Type	% DNA in Tail Mean	Standard Deviation	Comets Counted
Untreated	2.47	3.04	288
350 µM Hydrogen Peroxide	40.42	24.06	167
100 µg/mL Camptothecin	25.56	13.29	189
Neutral Assay			
Treatment Type	% DNA in Tail Mean	Standard Deviation	Comets Counted
Untreated	4.49	6.17	112
350 µM Hydrogen Peroxide	27.18	9.40	331
100 µg/mL Bleomycin	33.65	10.14	109

Microgravity Conditions			
Alkaline Assay			
Treatment Type	% DNA in Tail Mean	Standard Deviation	Comets Counted
Untreated	14.63	11.72	277
350 µM Hydrogen Peroxide	72.71	19.68	169
100 µg/mL Camptothecin	43.15	12.37	258
Neutral Assay			
Treatment Type	% DNA in Tail Mean	Standard Deviation	Comets Counted
Untreated	25.24	12.85	138
350 µM Hydrogen Peroxide	32.23	13.63	86
100 µg/mL Bleomycin	66.47	19.71	86

Note. Microgravity increased the occurrence of both single-stranded and double-stranded DNA breaks in kidney cells. Even endogenous DNA damage increased for cells grown in the RCCS microgravity simulator.

Figure 15

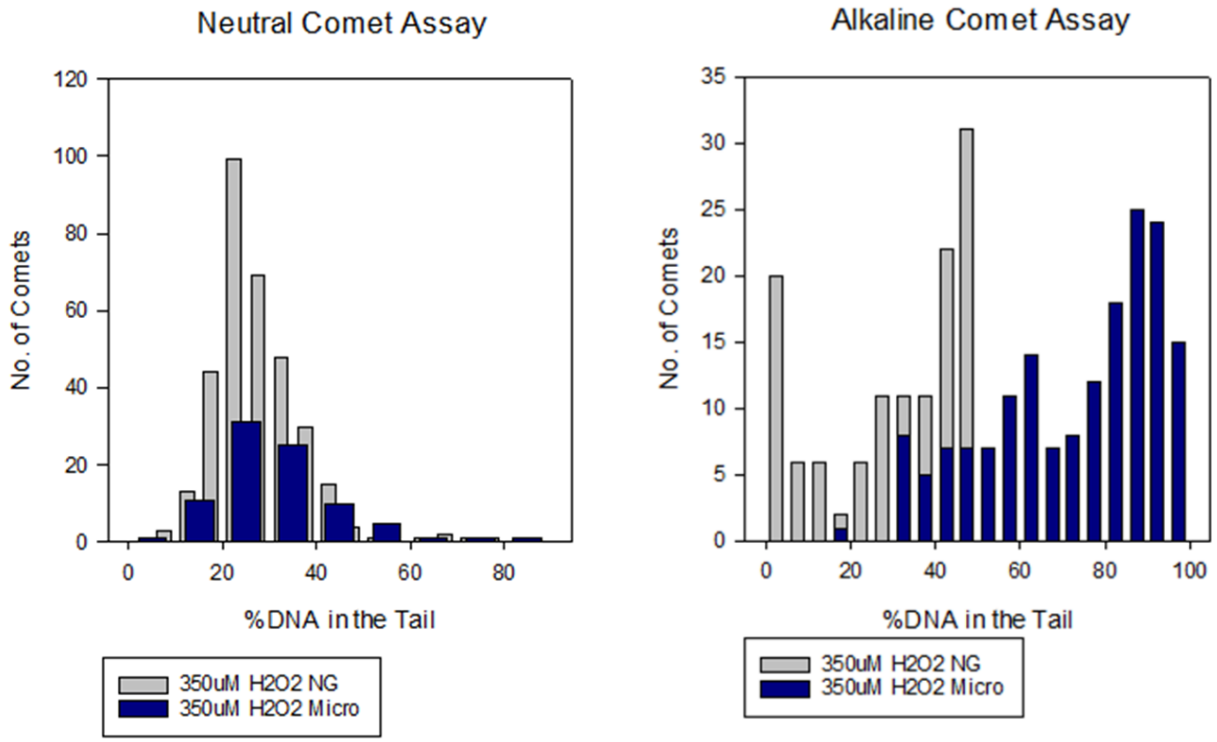
Effects of Microgravity alone



Note. These graphs demonstrate the increased in double-stranded and single-stranded 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 the addition of mutagenic reagents. "Control Micro" refers to kidney cells that were grown in the RCCS microgravity simulator (without mutagens present).

Figure 16

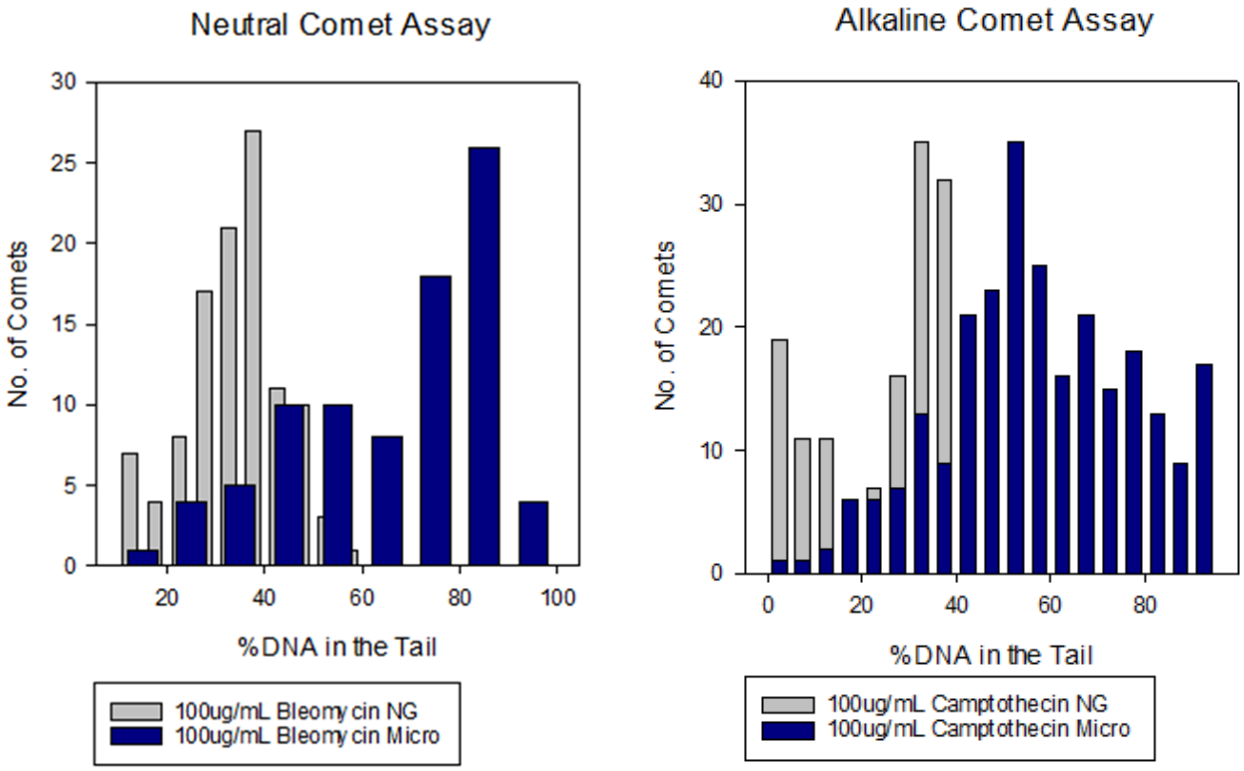
Effects of Microgravity and Oxidative Stress



Note. These graphs demonstrate the change in double-stranded and single-stranded damage in kidney cells treated with H_2O_2. "NG" refers to kidney cells grown in standard growth conditions, while "Micro" refers to cells that were grown in the RCCS microgravity simulator. The Neutral Comet Assay monitors changes in ds-breaks while the Alkaline Comet Assay monitors changes in ss-breaks.

Figure 17

Effects of Microgravity and DNA-specific Mutagens



Note. These graphs demonstrate the change in double-stranded and single-stranded damage in 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 microgravity simulator. The Neutral Comet Assay monitors changes in ds-breaks while the Alkaline Comet Assay monitors changes in ss-breaks.

Discussion

During the course of this project, we succeeded in answering the central questions surrounding the hypothesis. 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. Therefore the data is fit for publication, as 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 we acquired, it is likely that this trial will be repeated to establish reproducibility before being published.

Overall, the data provides new insight into how 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 all four cell lines 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 retinal cells were treated with hydrogen peroxide, they exhibited 39.13% double-stranded DNA breaks compared to 20.87% single-stranded DNA breaks (in reference to Figures 13 and 14). 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 because 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.

After comparing the kidney cells, retinal cells, heart cells and breast cancer cells, it became apparent that certain cells could better withstand the effects of induced oxidative stress. For example, the cardiomyocytes exhibited the highest amount of single-stranded damage, with an average of 52.89% ssDNA in the tail of the comet, while the retinal cells showed the least amount of ssDNA damage (20.87%) due to oxidative stress. 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. In this case, we believe the cardiomyocytes showed higher levels of endogenous damage due to the fragility of the cell line. The cardiomyocytes were particularly challenging to culture as they had a longer growth period and were more sensitive to cell splitting. Nonetheless, the application can be used to accurately compare the other three cell types. The kidney cells showed the most genomic stability in the presence of increased oxidative stress, followed by the retinal cells and the breast cancer cells, respectively. This can be deduced because the kidney cells had the least amount of DSBs, so they were less likely to exhibit genomic mutations that could inhibit proper cellular function or cause premature cell death.

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 3). 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.

Future Plans

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. For one, microgravity simulation performed on kidney cells 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).

This microgravity trial that was performed on kidney cells will be replicated in the retinal cells, cardiomyocytes, and additionally, 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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