Quantifying the Effects of Common *in vitro* Cell Culture Techniques on Glutathione Depletion and Cellular Viability via Breast Cancer Cells and Reactive Oxygen Species (ROS)

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by

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MASTER OF SCIENCE

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PREFACE:

My plan coming out of high school was to go into medicine and become a physician. Quickly, I learned that I also had a passion for research. As an undergraduate at UM-Flint, I was very fond of my research experiences even though 75 % of the time I felt like nothing worked. My professors saw early on in my career that I would make a promising scientist one day. To that end, my professors pushed for me to go to graduate school and obtain my Ph.D. For the past five years, I would go to an American Chemical Society (ACS) national meeting with my professors and they would have the "life conversion" with me, always steering me towards a Ph.D. career. I was stern on becoming a medical doctor; however, I did listen to them…kind of! While a 3rd year undergraduate in the Honors Biochemistry program, I decided to complete another bachelor's degree in Molecular Biology and Biotechnology. Then in my 4th year, I decided on taking my professors' advice and went to graduate school at UM-Flint. I applied to the 2 year BS/MS Biochemistry program at UM-Flint still as an undergraduate completing a 2nd bachelors degree. At the time, the program was only accepting "in house" students and because of the passion for science I showed, I was accepted without a problem into the program. There were a lot of hurdles to overcome such as actually graduating from the program. Since its beginning, a handful of students have tried to complete the program but were unsuccessful. To date, I will be the first and only student to graduate from the program with a Masters in Biochemistry degree. Sadly, during my last year in the program I was told that the program would be stopped. So (for now) I will be the first and last

Biochemistry student to graduate from the department. Completing my master's degree is not an end to my research career even though I will attend medical school in the fall. In fact, I know that my passions for research will continue from a clinical perspective in the future.

ABSTRACT:

Glutathione (GSH), an important antioxidant, is essential for proper mammalian biochemical function. Its mechanism as an antioxidant involves the neutralization of reactive oxygen species (ROS) to less harmful compounds like water. ROS are neutralized using oxidation/reduction reactions, where glutathione reacts with a ROS forming oxidized glutathione (GSSG) and a neutral species, like water, in the process. GSSG is then reduced to GSH by glutathione reductase. Therefore, a proper *in vivo* concentration of GSH may determine a cell's ability to survive stressful conditions, such as the presence of drug compounds: benserazide, hydrogen peroxide, or buthionine sulfoximine (BSO). The research presented in this thesis shows that common cell culture techniques, such as media replacement and cell washing, causes cells to be more susceptible to these drugs. To analyze the effects of the drugs, IC_{50} curves were constructed with results from cell viability assays under three cell treatment conditions: no media replacement, media replacement, and two times wash with PBS followed by media replacement. These experiments were performed using two triple-negative breast cancer cell lines, MDA-MB-231 and MDA-MB-468. One suspected cause of increased susceptibility is loss of total GSH inside of the cells and to measure total GSH, a luminescent assay was used. However, we concluded from experimentation that an increase in cell susceptibility to drugs is not due to loss of GSH. Future research is needed to determine the cause. Finally, these results suggest that procedures used in *in vitro* cell culture studies can cause changes to the cell viability and that these results should urge

researchers to develop a more universal cell culture protocol that is comparable to *in vivo* conditions.

ACKNOWLEDGMENTS:

me through life; even now you continue to be. Her final wishes were to see me complete graduate school and walk down the aisle.

Dedication:

I dedicate this thesis to my future family.

That their relationships will be as rewarding for them as these have been for me.

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CHAPTER 1

Introduction:

Scientist Joseph de Rey-Pailhade may have first described glutathione (GSH) in the late 1880's, but it was not until 1921 that the term "glutathione" was coined and the polypeptide rediscovered by British, Nobel Prize winner Sir Frederick Gowland Hopkins. [1-3] Hopkins spent many years characterizing this protein, which he described as a dipeptide, in the newly established and built biochemistry building at Cambridge [2-4]. He determined that two forms of the proteins existed, the thiol-reduced form and a disulfide-oxidized form known as GSSG, which at the time was not characterized. Although Hopkin's is credited with this milestone discovery, his error in structure has not been forgotten. Another group of scientists characterized and published the protein as a tripeptide [5], where in turn Hopkins reiterated in another publication that the protein was indeed a dipeptide. [6] It was not until a novel crystallization technique of glutathione was developed and used by Hunter and Eagle that Hopkins realized he was wrong. [7] He published a final article in 1929 that detailed his mistake and suggested that glutathione is a tripeptide made up of glutamic acid, glycine, and cysteine; later glutathione was shown to have exactly that structure. [7]

Later, in the early 1950's, Robert B. Johnston and Konrad Bloch were the first to describe the biosynthesis of glutathione with the help of enzymes,

cofactors, and ATP hydrolysis. [1, 8-9] Since that time, advancement in science and technology have allowed for a more in-depth characterization of the biosynthesis mechanism. The first reaction of the biosynthesis involves the amino acids, glutamate and cysteine, and the heterodimeric enzyme, γ-glutamylcysteine ligase (GCL). This rate-limiting step depends on ATP as an energy source and at least one of the subunits of GCL, catalytic subunit (GCLC), to catalyze the reaction to produce γ-glutamylcysteine. [10] It was established that the catalytic subunit's activity was inhibited by GSH, and in fact, the two subunits could reversibly disassociate when too much GSH was present. This feedback inhibition allows for regulation of the biosynthesis of GSH. [11] Furthermore, it has been found that oxidative stress, specifically reactive oxygen species (ROS), improves the re-formation of GCL. [12] The last step is catalyzed by GSH synthase, a non-GSH-inhibited homodimeric enzyme, by adding glycine to the C terminus of γ-glutamylcysteine. [13] The resulting GSH structure is special because of its stability inside of the cell. The stability of the protein is due to the glutamate and cysteine residues not linking through the normal α-carboxyl group but through γ-carboxyl group. This type of bond is hydrolyzed only by γ-glutamyltranspeptidase (GGT), which is normally found on the external surface of most cells. [14] Unsurprisingly, more then 90% of GSH is found within the cell either in the cytosol or mitochondria. [10]

As previously mentioned, regulation is vitally important in the biosynthesis pathway, especially for the GCL and GSH synthase enzymes. Regulation of these enzymes is induced through many biological molecules such as hormones

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(insulin, cortisone, etc.) as well as xenobiotics such as ROS. [10] GCL's heterodimeric structure is regulated at both catalytic (GCLC) and modifier (GCLM) subunits. Many researchers have shown that both subunits are transcriptionally regulated, specifically upregulated, through a series of unrelated pathways induced by both hormones and xenobiotics, such as several PI3K signaling pathways (i.e. PI3K/Akt/p70S6K), MEK, and p38 MAPK causing the upregulation of GCLC promoter activity. [15-17] In addition, posttranslational regulation of GCLC occurs when it is phosphorylated by protein kinase A, protein kinase C, or calcium calmodulin kinase II, causing a down regulation in GSH synthesis [18]. Xenobiotic-induced regulation of GCLM also occurs both at the transcriptional and translational level. One example involves β-naphthoflavone (β –NF) binding Nrf2 transcription factor allowing for a higher affinity binding to the GCLM promoter sequence, thereby causing up regulation of the gene. [19] Unfortunately when it comes to GSH synthase, little is known about how it is regulated. [10] However, researchers have shown that GSH synthase is not regulated by GSH and that both GCLC and GSH synthase are needed for efficient production of GSH. [20] Finally, no posttranslational regulation of GSH synthase has been published to date.

The biochemical functions of GSH are critical because both forms of glutathione (GSH and GSSG) function in many human biochemical processes, including cell proliferation, apoptosis, and redox cell signaling to maintain human homeostasis. Furthermore, very low and very high levels of GSH result in pathological symptoms resulting in human disease. In a 1995 study, researchers

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showed that upon decrease of GSH through buthionine sulfoximine (BSO) treatment, cells would not continue through the cell cycle. [21] Furthermore, it was found that GSH is directly involved in transition from S phase to G_2. [21] The researcher attributed this finding with DNA synthesis and its coupling with GSH and ribonucleotide reductase. Although the connection may not be clear, GSH is used to maintain thioredoxin, which is a coenzyme of ribonucleotide reductase needed to maintain proper function. [22] In fact, when GSH leaves the cell, apoptotic proteins are triggered due to the decrease in GSH levels. [23] Furthermore, as previously mentioned, both forms of GSH function in redox reactions. The GSH redox cycle involves oxidized GSSG being reduced to GSH while NADPH molecules from metabolic pathways such as pentose phosphate pathway are also oxidized for energy and protons. The enzyme that reduces GSSG back to 2 GSH molecules is glutathione reductase (GR). [23-24] From there GSH can be oxidized again for many other roles or be used in signaling where GSH binds to a thiol residue of a protein through a glutathionylation reaction. [25] Furthermore, because of the glutathionylation reaction, many signaling molecules containing cysteine residues are protected from oxidative damage. Upon deglutathionylation, a protein may continue its normal function. The role of GSH in protection from oxidative damage is the central theme of this thesis.

One of glutathione's most important functions involves using sulfur chemistry to eliminate oxidative stress, specifically ROS, through its oxidation-reduction cycle. The body produces many types of molecules that induce oxidative stress such as superoxide and hydrogen peroxide. [26] These molecules

are byproducts of many metabolic processes such as fatty acid oxidation and the breakdown of superoxide by superoxide dismutase, creating hydrogen peroxide. [27] The breakdown of hydrogen peroxide is then catalyzed by several enzymes such as catalase. However, one of the most important systems is that of glutathione and glutathione peroxidases (GPx), 8 of which (GPx1-8) have been discovered thus far. [28] Furthermore, these selenoproteins, abbreviated SecGPx, (selenocysteine, Sec, in the catalytic center), specifically the selenolate (-SeH) group, reacts with hydrogen peroxide to produce water and selenic acid (-SeOH), which is reduced back to –SeH by 2 GSH molecules, resulting in GSSG as shown in the reactions below. [28]

$$GPx\text{-}Se^- + H_2O_2 \quad \rightarrow \quad GPx\text{-}SeOH + OH^-$$

$$GPx\text{-}SeO^- + H^+ + GSH \quad \rightarrow \quad GPx\text{-}Se\text{-}SG + H_2O$$

$$GPx\text{-}Se\text{-}SG + GSH \quad \rightarrow \quad GPx\text{-}Se^- + H^+ + GSSG$$

The human body is constantly trying to balance equilibrium levels of hydrogen peroxide so that levels are not high enough to damage the cells. High levels cause cancer in some cases because the oxidative agents oxidize DNA, RNA, and other biological molecules needed for living.

Because of GSH's importance in the body (*in vivo*), especially in cell viability and cell death, the question becomes how do we further test its role *in vitro*? Furthermore, is it proper scientific technique to use what we obtain *in vitro* to represent *in vivo* processes? How is GSH affected in common plating

techniques such as media replacement and washing of cells? Reiners showed that 2 of their cell lines showed a loss (40-90%) of GSH while passaging, but returned back to normal after 24 hours. [29] In another study, researchers showed that 50% of the GSH was depleted in cell isolation procedures. [30] Lee (2009) concluded that H9c2 cardiac myocytes experienced a decrease in GSH when cells had their media replaced, ultimately leading to increased cell death by increased susceptibility to oxidative stress such as ROS. [31] There has been very little research done on other cell lines to further solidify the need for a change in common global cell culture and cell plating techniques. More research is needed to push biotechnology companies to develop assays and systems that do not require the removal or wash of cells to better mimic *in vivo* conditions. This thesis quantifies the effects of common cell plating techniques on cell viability via GSH levels using two breast cancer cell lines, MDA-MB-231 and MDA-MB-468. The conclusions made in this thesis were the result of a 2-year research project.

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CHAPTER 2

Experimental Procedures:

Initial Growth and Maintenance of MDA-MB-231 and MDA-MB-468. Most cell culture techniques were done in a LabGard ES Class II, Type B2 Biological Safety Cabinet (NuAire: Plymouth, MN). All equipment and chemicals entering and exiting the cabinet were sterilized using 70% ethanol. All chemicals and equipment used in cell culture were either sterile or sterilized and kept sterile. All chemicals that were used in cell culture were either at room temperature or heated to 37°C in an Isotemp water bath (Fisher Scientific: Pittsburg, PA). Both breast cell lines were purchased from ATCC (Manassas, VA). The original stock cell lines were thawed, pelleted via centrifugation at 250 g, and the supernatant was then removed. The cells were resuspended with 1 mL of respective media and added separately to BioLite 75 cm^3 vented cell culture flasks by Thermo Scientific (Waltham, MA) containing 30 mL of respective media and 1 mL of Heat Inactivated Fetal Bovine Serum (HI FBS), certified Performance Plus (Gibco: Carlsbad, CA). The MDA-MB-231 cell line was maintained using Gibco 1X minimum essential media + GlutaMAX-I (MEM) with Earle's salts supplemented with 1.0 % Gibco 100X antimycotic-antibiotic, 1.0 % Gibco 100X (100 mM) sodium pyruvate, and 10 % HI FBS. The MDA-MB-468 cell line was maintained using Gibco 1X Dulbecco's Modified Eagle Medium (DMEM) + GlutaMAX-I (DMEM) with 4.5 g/L D-Glucose supplemented with 1.0 % Gibco 100X

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antimycotic-antibiotic, 1.0 % Gibco 100X (100 mM) sodium pyruvate, and 10 % HI FBS. All media were then subjected to filtration via Nalgene Rapid-Flow 75mm Filter Unit (Thermo Scientific: Waltham, MA). All cells were grown in a Thermo Scientific Forma Steri-Cycle CO_2 incubator at 37°C and 5.0 % CO_2. After 24 hours, the media was removed and replaced with 30 mL of respective media. The cells were then incubated for another 48 hours for confluent growth.

Long Term Maintenance and Freezer Stocks. Using the initial growth, the media was removed using the vacuum pipette system. Then 10 mL of Thermo Scientific HyClone 1X DPBS/Modified supplemented with calcium and magnesium was added to the flask. After 30-60 seconds the DPBS was removed using an electronic pipette and 3-5 mL of Gibco 0.25 % (1X) trypsin-EDTA was added. After 30 seconds all of the trypsin was removed except for 1 mL. For the MDA-MB-231 cells, the trypsinization process was done at room temperature, while the MDA-MB-468 cells were incubated at 37°C for 5-7 min. Once the cells had detached, the cells were split with their respective media into new, labeled flasks. Each split constitutes a new passage (p). A split ratio was recorded based on the following: 6 mL of appropriate media was added to the flask containing 1 mL trypsin, then 1 mL was taken out and added to the new flask thereby recording a 1:7 split. The cells were split every 3rd then 4th day (Monday and Thursday). Freezer stocks were made following a couple of growth passages. After media was added following trypsinization, the entire sample was added to a Falcon tube and counted using a Bright-Line hemocytometer with a volume of 0.1

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mm^3 (Hausser Scientific: Horsham, PA). The hemocytometer was viewed using a EVOS XL Cell Imaging Microscope System (Life Technologies: Carlsbad, CA). The stock was then diluted to about 1-2 million cells/mL followed by the addition of 5 % sterile DMSO (Sigma Aldrich: St. Louis, MO). Then 1 mL of cells were aliquoted into Fischer Scientific cryogenic vials and frozen at -80°C. A freezer stock of cells was used after the cells grew for 25 passages. The frozen freezer stocks were melted and maintained as described in initial growth section above.

Cell Treatment and CellTiter-Glo Luminescent Cell Viability Assay. During the cell treatment process, cells were resuspended using the same appropriate media but without sodium pyruvate. Furthermore, before the cells were split for a new passage, the cells were counted using the hemocytometer. The appropriate dilutions were made with media without sodium pyruvate for plating using a bio-one CELLSTAR 96 well, cell culture plate (Greiner: Kremsmünster, Austria) with 70 μL of liquid at cell concentrations ranging from 1000-10,000 cells/well. After overnight incubation, the cells in triplicate underwent three cell culture treatments using a Thermo Scientific 12 channel electronic pipette: no media replacement, media replacement, and wash twice with 70 μL of DPBS then media replacement. One experiment involved determining the best technique to remove the media without removing cells by using either electronic pipette, vacuum pipette, or inverting the plate. Following these treatments, cells were treated with one type of drug: either benserazide, hydrogen peroxide, or buthionine sulfoximine (BSO) ranging in concentration from 244 nM – 250 μM,– 17 nM –

10,000 µM, and 56 µM – 2,000 µM, respectively. The serial dilutions were made with the appropriate diluent, which maintains a constant concentration of the vehicle, when needed using a 12 well trough and 2 or 3 fold serial dilutions per well. Finally, 10 µL of varying drug concentrations were added in each row of the plate using a 12 channel electronic pipette, with each column of the plate having the same concentration and the last column having a 0 mM concentration (media only) of drug added. After the plates had incubated for 72 hours, they were removed and 80 µL of CellTiter-Glo reagent (Promega: Madison, WI) was added to each well. The plate was shaken using a Thermo Scientific Titer Plate Shaker for 5 min on a speed setting of 4 then centrifuged at 250 g for 60 seconds using a Thermo Scientific Sorvall ST 8. The plate was then incubated at room temperature. After 15 min. incubation, 80 µL from each well was transferred to a white-walled 96 well plate. The results of the luminescent assay were obtained using a Promega GloMax Multi Detection System. The preprogrammed CellTiter-Glo assay parameters were used. Finally, IC_{50} curves were constructed using variable dose response curve parameters in GraphPad Software (La Jolla, CA). All curves were normalized by taking the CellTiter-Glo values and dividing it by the top IC_{50} values calculated by the program.

GSH/CellTiter-Glo Assays. To begin these assays, the same plating procedure as described in the Cell Treatment section above was conducted. However, when plating the cells, only 3 rows with 8 columns were plated. After 24 hours, the same plating treatments (i.e. no media replacement, etc.) were conducted as

described in the Cell Treatment section above, preparing the cells for GSH/CellTiter-Glo assays. The GSH/CellTiter-Glo assays use GSH-Glo assay and Cell-Titer-Glo assay on the same plate but not the same wells for normalization of GSH levels against viable cells. The GSH-Glo assay was conducted per Promega protocol. To begin, all media was removed from 4 columns and 3 rows and discarded. Then 50 µL of Total Glutathione Lysis (TGL) Reagent was added to the appropriate wells containing cells and 4 (2 each) other wells containing no cells as a background control. The TGL reagent was made with the following per well composition: 1.0 µL Luciferin-NT, 10.0 µL Passive Lysis Buffer, 5X, and 39.0 µL Nuclease-Free Water. After 5 min. on the plate shaker, 50 µL of Luciferin Generation Reagent was added to the wells containing TGL or OGL Reagent with the following composition per well: 1.25 µL 100 mM DTT, 3.0 µL Glutathione-S-Transferase, and 45.75 µL Glutathione Reaction Buffer. Following a brief shake on the plate shaker and 30 min. incubation at room temperature, the contents of each well were transferred to a white-walled plate. Then 100 µL of Luciferin Detection Reagent was added per well and incubated for 15 minutes. The results of luminescent assay were obtained using a Promega GloMax Multi Detection System. The CellTiter-Glo cell viability assay was conducted on the other 3 rows and 4 columns worth of cells as described in the CellTiter-Glo Luminescent Cell Viability Assay section above. The preprogrammed CellTiter-Glo assay parameters were used. Finally, bar graphs were constructed by utilizing GraphPad Software containing two bars, total glutathione as a percent (red) and total glutathione normalized to cell number

(blue bar), over all three cell treatment conditions. Total glutathione as a percent was obtained from the GSH-Glo raw data. After correcting for background, an average of the no media replacement row was calculated then all numbers were divided by that number. Total glutathione normalized to cell number was calculated using normalized numbers from GSH-Glo and normalizing CellTiter-Glo numbers the same way as described above. Then the normalized GSH-Glo numbers were divided by average normalized CellTiter-Glo numbers. Two one-way ANOVA statistical analyses via SPSS were conducted on the red and blue bars to determine if changes among the cell treatments were statistically significant. If ANOVA received a p value less than 0.05, then Tukey's statistical test was conducted to determine where the difference is.

CHAPTER 3

Results and Discussion:

On November 14th, 2014 the first experiments were conducted with MDA-MB-231 and eseroline at a cell count of 1,000 cells/well to become familiar with the experimental techniques of cell culture and drug treatment (results not known). Eseroline was initially chosen as a drug to induce cell death through ROS; however, after further experiments in the lab, a better ROS drug inducer was chosen. [1] The drug functions as a toxic metabolite of physostigmine, an anti-cholinesterase drug. [2] Its mechanism of action causes cell death by inducing lactic acid dehydrogenase (LDH) leakage, ultimately causing ATP loss. [2] Furthermore, it became clear from those experiments that using a pipette connected to a vacuum was not the best way to remove the media because there was cell loss based on the IC_{50} curve (or lack thereof). Data for IC_{50} curves were obtained by using the CellTiter-Glo assay system. This system measures the number of living cells indirectly by measuring ATP, an essential energy molecule for living cells, using a luminescent signaling though the use of luciferase. [4] An optimization experiment was developed, which included plating cells at the same cell count and in triplicate removing the media using a pipette connected to a vacuum, using a 12 channel electronic pipette, and finally just inverting the plate on paper towel. All of these were done at the side of the well to minimize any cell loss. Researchers have implemented the last technique and although it makes

sense, the technique lends itself to contamination issues, media retention inside the wells, lack of analytical method, and a large mess. The results (not shown) indicated that the best way to remove the media was through a 12 channel electronic pipette, because it showed the least amount of cell loss.

A better ROS inducer, benserazide, was selected the following semester, and to see a more profound effect in cell death, a cell count of 10,000 cells/well was used for plating. Benserazide, a drug used in Parkinson treatment, was shown by another colleague in the lab to induce more hydrogen peroxide, H_2O_2, in media than eseroline, especially media without sodium pyruvate. [1] The structural components of benserazide, specifically the hydroxyl groups on the phenyl ring, permits oxidation reactions creating ROS in medium. [3] Results of Benserazide triplicates from one experiment are shown in Figure 1. Results indicate a drop in IC_{50} values from both plating treatments compared to the control as hypothesized. The plating treatments included: no media replacement, media replacement, and 2 time wash with PBS following by media replacement resulting in 13.78 µM, 10.40 µM, and 8.45 µM IC_{50} values, respectively. The IC_{50} is defined as the concentration of the inhibitor, in this case drug, which reduces the response by half, or in this case reduces the viable cells. These results were in line with the hypothesis that common cell maintenance procedures cause loss of essential proteins and compounds necessary for viable cell growth. We hypothesized that the most important loss was that of GSH, thereby causing a decrease in viability due to the inability to fight ROS. To corroborate these findings, we tried to quantify the amount of total GSH (GSH and GSSG) inside

the cells and the media itself after treatment. Unfortunately, the results (not shown) were not usable because of the cysteine found in the MEM media, which interfered with measuring the total GSH content in the media. Moreover, the results from the IC_{50} curves were not as profound as were hypothesized. Along with the PI, it was decided to forgo ROS induction and use low concentrations of H_2O_2 to induce cell death. Experiments with hydrogen peroxide began on March 6th, 2015 with a cell count of 10,000 cells/well. These results (not shown) were unreliable and there was not enough time to do more experiments before the safety cabinet was shut down for almost 5 months. Before the shutdown, one simple experiment was conducted using a drug called BSO. BSO was found to deplete cells of GSH. [5] So to see a more intense effect on cell death, we decided to use BSO. However, at any concentration, BSO would cause cell death after 24 hours, as shown in Figure 2. It was not possible to use BSO, because we wanted to see a more profound effect of the drug over a three-day period. After 96 hours, the cells were not viable at 25 µM BSO, and by adding another drug such as peroxide, the results would only show zero viable cells.

Following the shutdown, experimental results were not obtained again until October 2015. During the shutdown, there was a break in the cryogenic freezers, leading to thawing of freezer stocks. All new cell lines were ordered. In September 2015, MDA-MB-231 and MDA-MB-468 were thawed and new freezer stocks were made. It was during this time that the PI thought it was best to see this hypothesized effect over two different cell lines. Cell viability experiments began with MDA-MB-468 at 1,000 cells/well; however, the results were not

credible (not shown). It was determined to run another optimization experiment on the cell line to decide the best cell plating count. The results (not shown) concluded that cell plating should be done at 5,000 cells/well for optimal cell viability results. From that point on, all experiments in both cell lines were conducted at that cell concentration. In 2 months, 4 cell viability experiments done in triplicate were conducted on MDA-MB-231 with hydrogen peroxide and 5 cell viability experiments done in triplicate were conducted on MDA-MB-468 with hydrogen peroxide. The results were plotted on one IC_{50} graph for each cell line. In Figure 3, the results for MDA-MB-231 are shown for plating treatments: no media replacement, media replacement, and 2 time wash with PBS following by media replacement resulting in IC_{50} values of 47.33 µM, 32.59 µM, and 26.92 µM, respectively. In Figure 4, the same results are shown but for MDA-MB-468 cell line for plating treatments: no media replacement, media replacement, and 2 time wash with PBS followed by media replacement resulting in IC_{50} values of 71.55 µM, 33.90 µM, and 29.97 µM, respectively. When looking at the results separately we can conclude that common cell culture maintenance techniques lead to a decrease in cell viability after drug treatment. When comparing the two cell lines, it can be deduced that MDA-MB-468 is more resistant to drug ROS treatment throughout the control, no media replacement, treatment because of the increase in IC_{50} values from 47.33 µM to 71.55 µM. However, upon plating treatments, such as media replacement and wash, the cell lines result in an almost identical drop in IC_{50} values. This may further solidify the finding that common cell culture maintenance techniques are detrimental to cell viability.

To verify the hypothesis that the results from Figure 3 and 4 are not just from cell loss but from loss of GSH, two assays were conducted, CellTiter-Glo and GSH-Glo. The GSH-Glo assay system couples the reaction of GSH and glutathione-S-transferase (GST) with a derivative of luciferin, luciferin-NT, to create a luminescent product only in the presence of GSH, thereby measuring the total amount of GSH in the cell. [6] To that end, we performed both assays on the same plate in order to see the total amount of GSH (%) normalized to the amount of cells present. The findings are found in Figures 5 and 6 (MDA-MB-231 and MDA-MB-468, respectively). The red columns in Figure 5, MDA-MB-231, show only normalized total GSH percentage values. To determine if the difference between each treatment is significant, a one-way ANOVA revealed that there is a difference ($p = 0.02$). To determine where the difference lies, Tukey's statistical test was conducted and revealed that the difference is between No Media Replacement & PBS Wash and Media Replacement ($p = 0.02$). This means that we do see a significant decrease in GSH values between those two treatments. However, further testing, as shown by the blue bars, shows that the GSH decrease is due to cell loss because one-way ANOVA reveals that there is no significant change ($p > 0.05$) across treatments when normalized to cell number.

In Figure 6, MDA-MB-468, the red columns show only normalized total GSH percentage values. A one-way ANOVA revealed that there is a significant difference between treatments ($p < 0.001$). Tukey's test reveals that there is difference between No Media Replacement & Media Replacement ($p < 0.001$),

and No Media Replacement & PBS Wash and Media Replacement ($p < 0.001$) but there is no difference between Media Replacement & PBS Wash and Media Replacement ($p > 0.05$). This means that we do see a significant decrease in GSH values between those two treatments. This difference may only be due to a loss of cells. To account for this loss, another one-way ANOVA ($p = 0.05$) was conducted on the blue bars showing total GSH percentage normalized to cell number. Finally, to determine where this difference occurs, Tukey's test shows one difference between No Media Replacement & PBS Wash and Media Replacement ($p = 0.04$). Therefore, the loss of GSH is not due to cell loss and that we do see a statistical significant decrease in total GSH when we wash the cells. To conclude for certain that this loss is significant in this cell lines, we still believe that further testing needs to be done. The statistical values are too close to threshold, where the difference may still be due to chance. It is certain though that each cell line exhibits different GSH loss when exposed to common cell plating techniques. Finally, although we see very little evidence for loss of GSH causing a decrease in cell viability, more testing is needed to replicate these results and draw the same conclusions.

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Effects of Benserazide on MDA-MB-231

No Media Replacement

$IC_{50} = 13.78\ \mu M$

Media Replacement

$IC_{50} = 10.40\ \mu M$

PBS Wash & Media Replacement

$IC_{50} = 8.45\ \mu M$

Y-axis: % Viable Cells

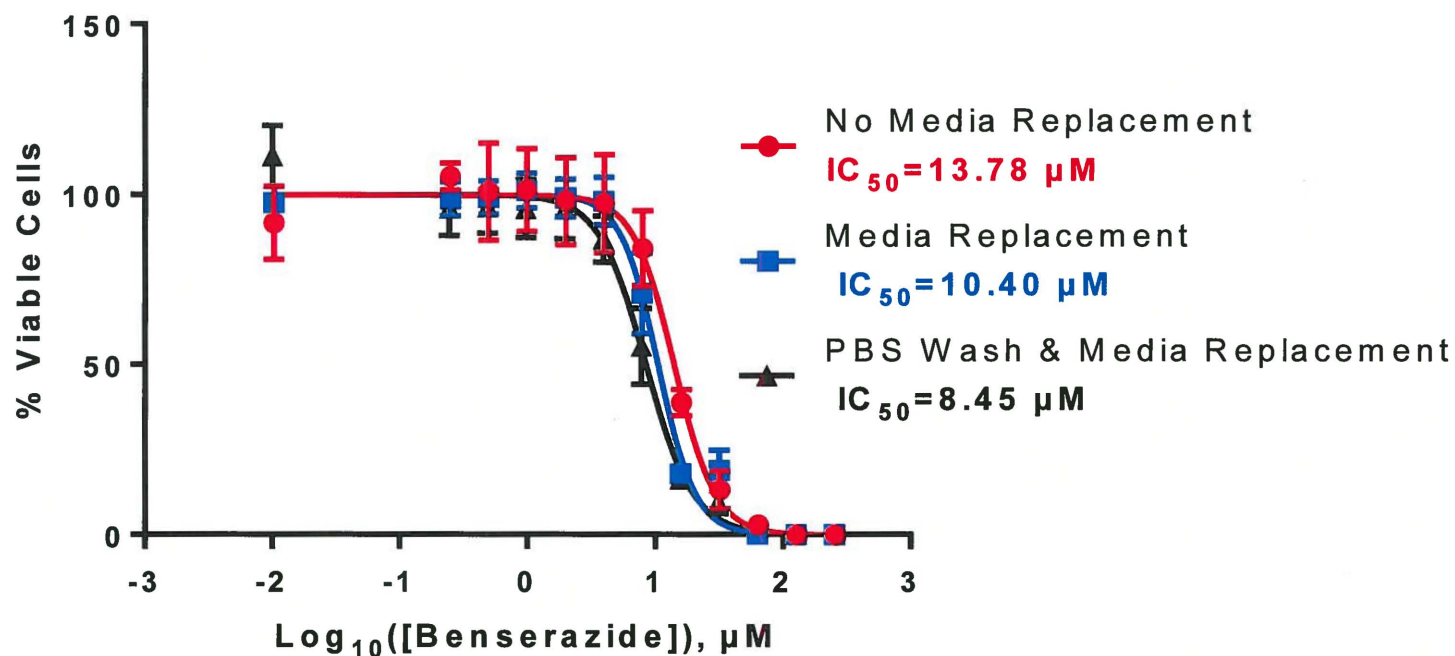X-axis: $Log_{10}([Benserazide]),\ \mu M$

Figure 1: Data were plotted using triplicates of MDA-MB-231 at a seeding density of 10,000 cells/well from one experiment. The relative light units (RLU) were normalized using the top IC_{50} values (results not shown) from each plating treatment resulting in a plateau at 100 % Viable Cells. The x-axis is a log scale of Benserazide concentrations used in drug treatment ranging from 250 μM to 0 μM in 11 2-fold serial dilutions. Three resulting IC_{50} values were calculated for each plating treatment: No Media Replacement – 13.78 μM (Red), Media Replacement – 10.40 μM (Blue), and PBS Wash (2X) then Media Replacement (Black) – 8.45 μM.

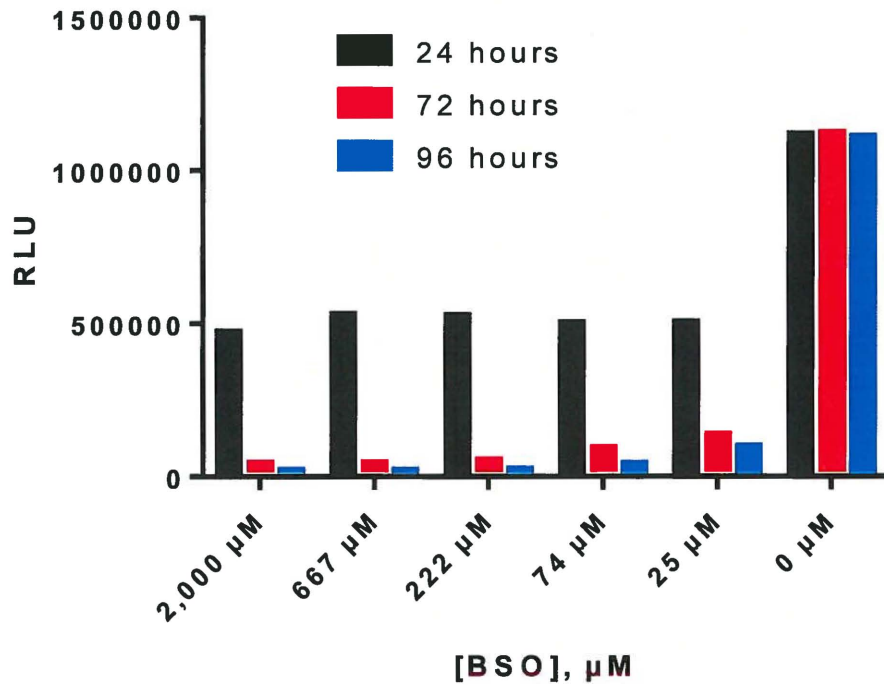## Effects of BSO on MDA-MB-231 Cell Viability

Figure 2: Data were plotted using triplicates of MDA-MB-231 at a seeding density of 10,000 cells/well from one experiment. The relative light units (RLU) obtained from the experiment were plotted against Buthionine Sulphoximine (BSO) concentrations used in drug treatment ranging from 2000 µM to 0 µM in 5 3-fold serial dilutions. Luminescent cell viability results were obtained after 24 (Black), 72 (Red), and 96 (Blue) hours via the CellTiter-Glo Assay.

Effects of Hydrogen Peroxide on MDA-MB-231

Legend:
No Media Replacement — $IC_{50} = 47.33\ \mu M$ (Red)
Media Replacement — $IC_{50} = 32.59\ \mu M$ (Blue)
PBS Wash & Media Replacement — $IC_{50} = 26.92\ \mu M$ (Black)

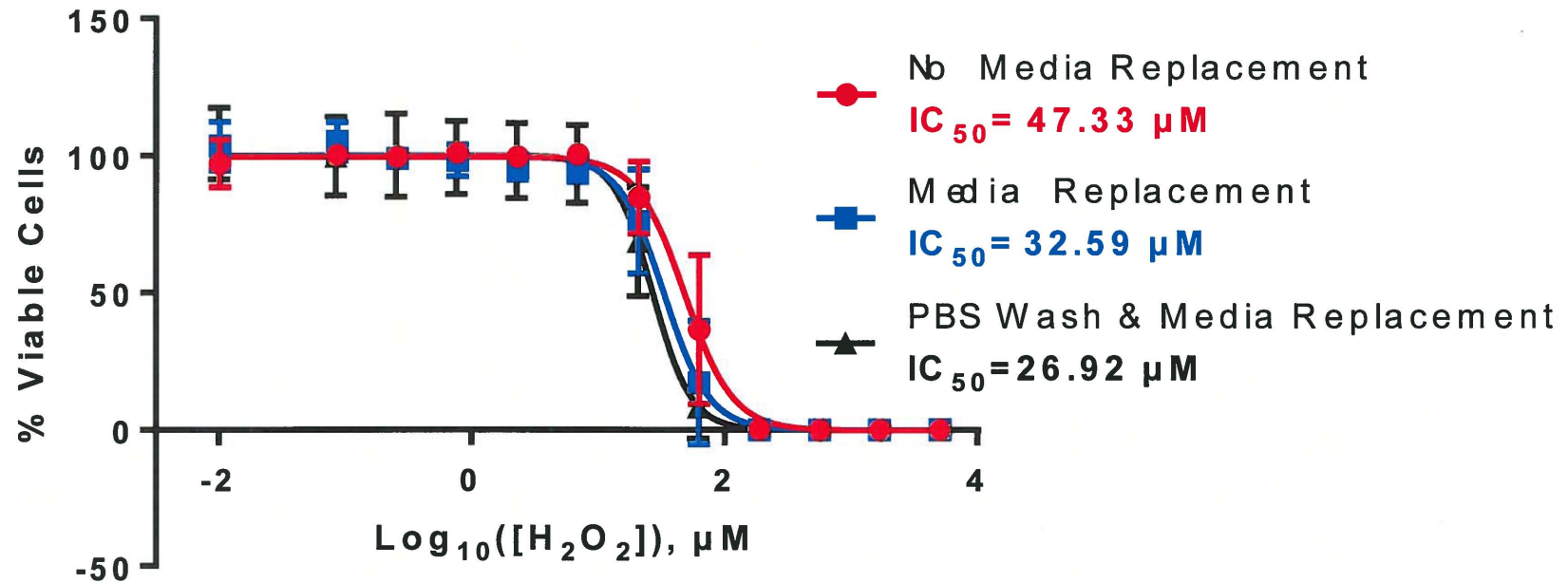X-axis: $Log_{10}([H_2O_2])$, μM
Y-axis: % Viable Cells

Figure 3: Data were plotted using triplicates of MDA-MB-231 at a seeding density of 5,000 cells/well from 4 experiments. The relative light units (RLU) were normalized using the top IC_{50} values (results not shown) from each plating treatment resulting in a plateau at 100 % Viable Cells. The x-axis is a log scale of Hydrogen Peroxide concentrations used in drug treatment ranging from 5,000 µM to 0 µM in 11 3-fold serial dilutions. Three resulting IC_{50} values were calculated for each plating treatment: No Media Replacement – 47.33 µM (Red), Media Replacement – 32.59 µM (Blue), and PBS Wash (2X) then Media Replacement (Black) – 26.92 µM.
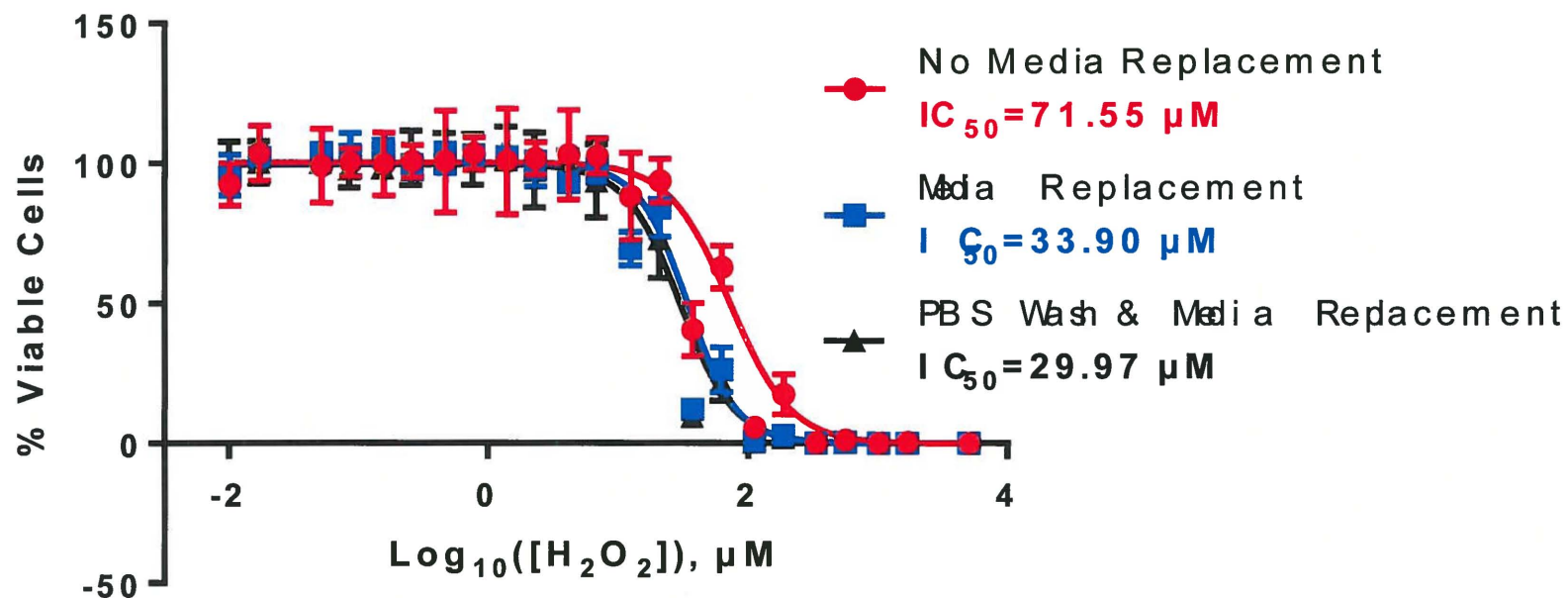
Figure 4: Data were plotted using triplicates of MDA-MB-468 at a seeding density of 5,000 cells/well from 5 experiments. The relative light units (RLU) were normalized using the top IC_{50} values (results not shown) from each plating treatment resulting in a plateau at 100 % Viable Cells. The x-axis is a log scale of Hydrogen Peroxide concentrations used in drug treatment ranging from 5,000 µM to 0 µM in 11 3-fold serial dilutions and 1,000 µM to 0 µM in 11 3-fold serial dilutions. Three resulting IC_{50} values were calculated for each plating treatment: No Media Replacement – 71.55 µM (Red), Media Replacement – 33.90 µM (Blue), and PBS Wash (2X) then Media Replacement (Black) – 29.97 µM.
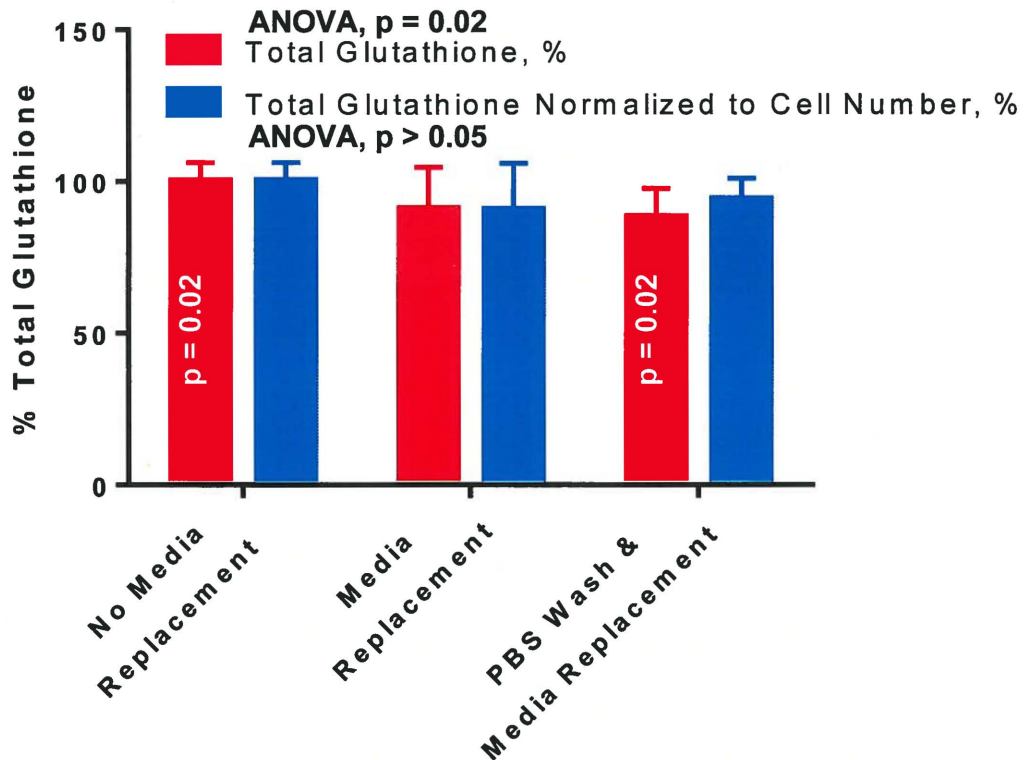
Figure 5: To investigate the finding from Figure 3, data were plotted using quadruplicates of MDA-MB-231 at a seeding density of 5,000 cells/well from 3 experiments. The total cellular glutathione percentages (Red Bars) were measured using GSH-Glo Assay and plotted against three plating treatments. Luminescent cell viability results were also obtained using the CellTiter-Glo Assay. The percentage total glutathione was then normalized using cell viability percentages (Blue Bars). A one-way ANOVA across the red data shows that there is a statistical significant difference ($p < 0.05$). Tukey's test shows that the difference occurs when comparing No Media Replacement to PBS Wash and Media Replacement. However, no statistical significant difference is shown in the blue bars ($p > 0.05$).
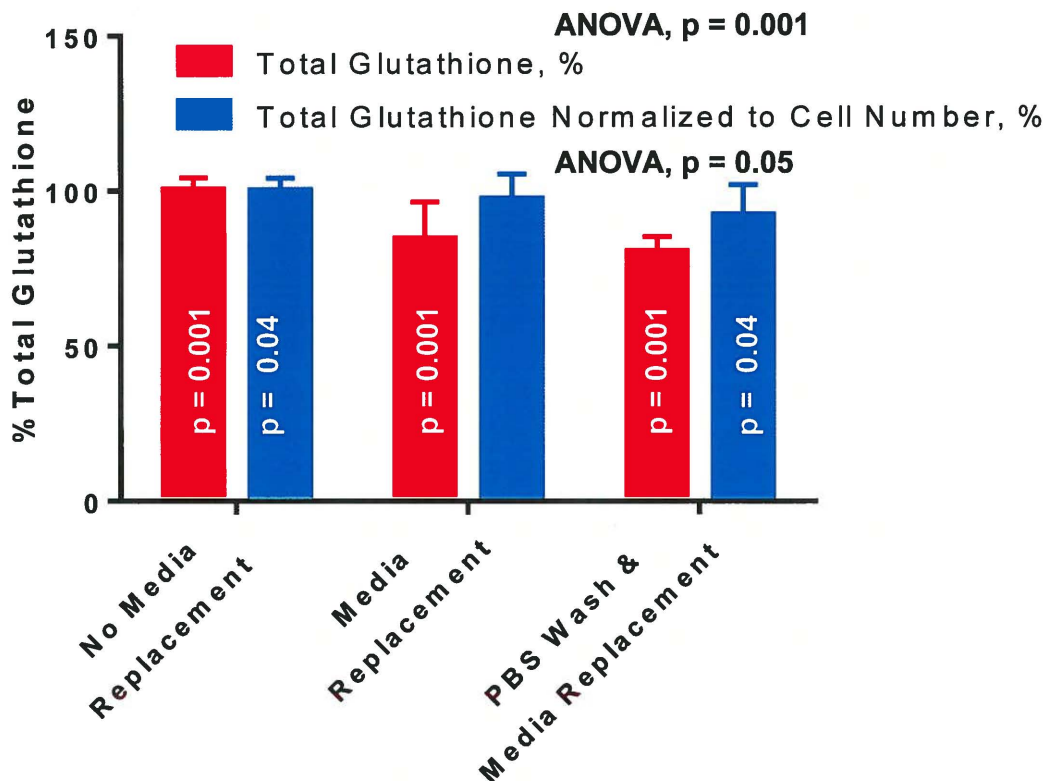
GSH/CTG Assays for MDA-MB-468

Figure 6: To investigate the finding from Figure 4, data were plotted using quadruplicates of MDA-MB-231 at a seeding density of 5,000 cells/well from 3 experiments. The total cellular glutathione percentages (Red Bars) were measured using GSH-Glo Assay and plotted against three plating treatments. Luminescent cell viability results were also obtained using the CellTiter-Glo Assay. The percentage total glutathione was then normalized using cell viability percentages (Blue Bars). A one-way ANOVA across the red data shows that there is a statistical significant difference between the red bars ($p < 0.05$). Tukey's test shows that the difference is among all treatments when comparing to the control. Furthermore, a one-way ANOVA across the blue data shows that there is a statistical significant difference between the blue bars ($p = 0.05$). Tukey's test shows that the difference occurs when comparing No Media Replacement to PBS Wash and Media Replacement.

CHAPTER 4

Conclusion and Future Work:

The research done for this thesis does in fact show strong evidence that common cell culture and plating techniques do decrease cell viability; however, the mechanism is not through GSH loss and is still unknown. This evidence is shown by thedecrease in IC_{50} in both cell lines as presented in Figures 3 and 4. In addition, Figures 5 and 6 show that the loss of GSH is due to cell loss and therefore, a decrease in cell viability is not due to a loss of GSH. Further research is needed to speculate on the cause of this decrease in cell viability.

The two year long thesis research project had many difficulties, the biggest being time. To make this project better in the future, more time in the lab is necessary. In addition, when we began, the cell lines were not growing well, it is advised that fresh cell lines be purchased along with high quality serum, which we did almost 1.5 years in. Furthermore, knowledge and optimization are key. Before starting the project, having a broad and somewhat in-depth understanding of the experiments is helpful. Many of the optimizations we conducted would have saved a lot of time had we done them in the beginning. This way equipment and chemicals are not wasted.

Future projects with this research are endless. For example, extending to more plating treatments among 10 different cell lines is a possibility. It is not unheard of to have 5 undergraduate students running three cell lines or having 3

undergraduates run 3 cell lines each. Moreover, the drugs can be changed; expanding into clinically tested ROS drugs or even reactive nitrogen species. In addition, expanding to other assays or experimental techniques that measure GSH may also be an option. An understanding of GSH from this perspective may lead to other projects such as continued research discovery on human GSH transporters. A grant has been developed and written for this research topic, if the PI would like to explore this route.

The possibilities are endless for GSH research; however, with this research evidence, we urge the scientific community to develop better *in vitro* techniques that are more consistent with *in vivo* conditions.