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# Do Elevated Levels of Glutathione Decrease the Toxicity of Plasticizers in Mouse Liver Cells?

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# DO ELEVATED LEVELS OF GLUTATHIONE DECREASE THE TOXICITY OF PLASTICIZERS IN MOUSE LIVER CELLS?

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by

Khatoon Albahrani

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Graduate Studies

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Dean of Graduate Studies

ABSTRACT

DO ELEVATED LEVELS OF GLUTATHIONE
DECREASE THE TOXICITY OF PLASTICIZERS
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Phthalates are esters of phthalic acid that play a crucial role in the manufacturing industries for enhancing the properties of plastic materials. Di-(2-ethylhexyl) phthalate (DEHP) is one of the most common phthalates used in a variety of products including food packaging and medical equipment. Their use has caused public health concerns because of their toxic effects on reproductive and developmental processes. This study aims to investigate the response of two mouse hepatoma cell lines to the effects of DEHP and its bioactive metabolite mono (2-ethylhexyl) phthalate (MEHP). Cells were exposed to four concentrations (10, 50, 100, and 300 μM) of DEHP and MEHP, which are consistent with levels encountered in clinical and environmental exposures, for two incubation periods (24 and 48-hour). Several assays were conducted to determine the toxicity through observing cell viability, ATP production, mitochondrial membrane potential, and reactive oxygen species (ROS) production. Results indicate significant toxic effects of DEHP and MEHP from cell viability and ATP assessments and show that the two cell lines respond differently to the different concentrations of DEHP and MEHP. However, the mitochondrial membrane potential and reactive oxygen species results show that DEHP and MEHP hepatotoxicity does not apparently involve oxidative stress.

# ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my respected committee members, Dr. Carin Thomas, Dr. Blaise Dondji, and Dr. Todd Kroll, for their patience, support, and guidance in carrying out this research. My thanks also extend to all of those whose research and work assisted me to finalize this thesis. Special thanks to my family and my friends for their love and encouragement during all phases of this research.

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# CHAPTER I

# INTRODUCTION

## Poly(vinyl chloride) and Plasticizers

Poly(vinyl chloride) (PVC) is a synthetic plastic which is produced by the polymerization of vinyl chloride monomers. Pure PVC polymer is brittle and hard to process, but this rigidity can be softened by adding additives called plasticizers. Plasticizers are generally defined as substances that, when mixed with other materials, help to enhance the flexibility of those materials. An example of a plasticizer could be water when it is added to clay to make the clay more controllable. However, the International Union of Pure and Applied Chemistry (IUPAC) have defined a plasticizer as "a substance or material incorporated in [another] material (usually a plastic or an elastomer) to increase its flexibility, workability, or distensibility." [1]Plasticizers are classified into distinct categories and each category gives different characteristics when added to PVC polymers. The most commonly used plasticizers in polyvinyl chloride polymers (PVC) are phthalates.[1]

## Phthalates

Phthalates are esters of phthalic acid (Figure 1) and the most common plasticizers used in the manufacturing industries for enhancing the properties of plastic materials. Because of their unique properties, phthalates have a wide range of applications including food packaging, personal care products, medical equipment, and building materials. With all these various uses of phthalates, human and animal exposure to phthalates has become unavoidable, which emphasizes the importance of scrutinizing and understanding their toxicity. The main reason behind the exposure to phthalates is attributed to the way

phthalates bind to PVC. Phthalates are not covalently bound to PVC but rather interact by non-covalent intermolecular interactions which are weak electrical forces such as van der Waals interactions and dipole moments.[2] One of these interactions results from the dipole-dipole interaction of the polar carbonyl in phthalates with the polar carbon-chloride bonds in PVC. However, the non-covalent bonding is mainly caused by another type of interaction known as van der Waals forces which result from the interaction of the nonpolar parts of phthalates with PVC. Also, these weak forces are easy to disrupt, triggering the phthalates to be released into the environment. Various environmental factors induce the dissociation of phthalates from PVC including variations in temperature, humidity, and oxygen content. In addition, exposure to liquids, UV radiation, and electric fields may play role in the dissociation of phthalates from PVC.[2]

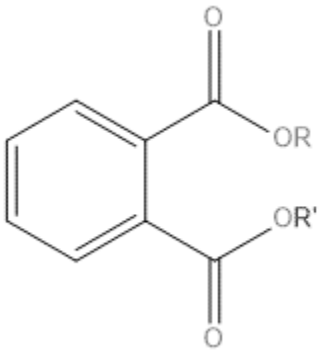



Figure 1: General chemical structure of phthalates

## Bis(2-ethylhexyl) phthalate (DEHP) and Their Potential Health Risks

Using phthalates as plasticizers provides plastic materials with desirable properties for multiple uses such as high flexibility, durability, and oxidation resistance. Bis(2-ethylhexyl) phthalate (DEHP) is the dominant plasticizer used especially in medical applications.[3] The annual production of DEHP has been estimated to be 1.4

million tons.[4] DEHP leaches out from plastic surfaces into the environment because it is not covalently or permanently bound to plastic polymers, as mentioned previously, which has resulted in extensive DEHP contamination of the environment.[3] The various ways that humans and animals are exposed to DEHP include ingestion, inhalation, dermal (skin contact), as well as iatrogenic exposure such as from blood bags, injection syringes, intravenous cannulas and catheters. It has been found that medical devices contain 20-40% DEHP by weight.[5] Phthalates are xenobiotics. These are chemicals that do not occur naturally in the system of living organisms. Thus, these xenobiotics are considered as toxicants to the biological systems and must undergo precise metabolic reactions to eliminate their toxic effects. Several studies have shown that phthalates act as endocrine disruptors and cause reproductive toxicity and developmental toxicity. Recent studies have linked exposure to DEHP to the progression of several types of cancers and to an increased incidence of type II diabetes. [6, 7] Another study has stated that DEHP is associated with allergic symptoms in children. [8]

## Metabolic Pathway of DEHP

The major metabolic pathways of phthalates in mammals involve two phases which are hydrolysis followed by conjugation. However, in the case of DEHP its metabolism is more complicated due to the branched chain which leads to several metabolites.[9] In mammals, the first step of DEHP metabolism is the biotransformation of DEHP into its mono (2-ethylhexyl) phthalate (MEHP) metabolite through a rapid hydrolysis by esterases and lipases in the liver and small intestine.[10] Several studies have suggested that MEHP is more toxic than the parent compound DEHP and it promotes higher levels of reactive oxygen species production and caspase activation which can

damage DNA.[7] In humans and rodents, MEHP is further metabolized into many other secondary metabolites by drug-metabolizing enzymes such as cytochrome P450 and uridine diphosphate glycosyltransferase enzymes (UGT).[10] The detoxified metabolite, the MEHP- glucuronide, is easily excreted into the urine. It has been reported that in the metabolism pathways of DEHP in the human body approximately 7.3 % of DEHP is metabolized and excreted into the urine as MEHP while 25.8% of DEHP accumulates in body tissues. The remaining 66.9 % of DEHP metabolites are excreted into the urine as oxidized monoester metabolites (Figure 2).[11] However, different animal species have been shown to have various pathways of urinary excretion of the MEHP glucuronide conjugates. One study showed that the glucuronidation activities of MEHP (1000 μM) in rat and mouse liver microsomes were similar. It also concluded that the enzymatic function and tissue distribution of uridine diphosphate glycosyltransferase isoforms involved in MEHP glucuronidation is different among different species.[12] Thus, the previous study stated that the toxicity of DEHP would show differences in toxic results among different species.

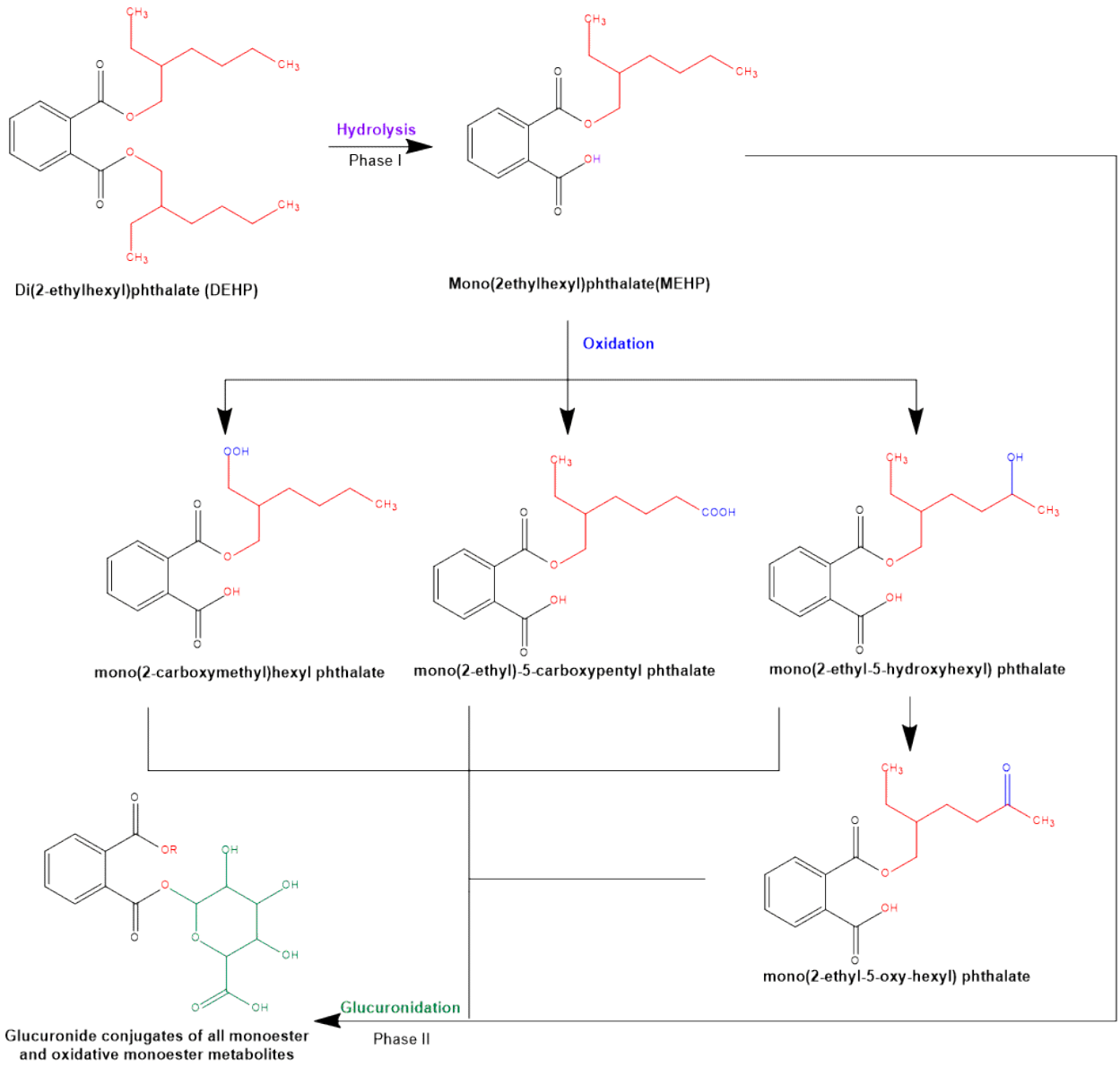



Figure 2: DEHP metabolic pathway which indicates the five metabolites excreted in human urine. Adapted from [3].

## *In Vitro* Liver Cell Models for DEHP Hepatotoxicity

DEHP belongs to a chemical family known as peroxisome proliferators. Peroxisome proliferators have been reported as compounds that induce liver tumors through "peroxisome proliferation, induction of hepatic DNA synthesis and the suppression of apoptosis". [13] Various hypotheses were initiated to understand the

mechanism that links peroxisome proliferation and liver tumors. One hypothesis states that elevated levels of peroxisome proliferators lead to oxidative stress which is triggered by increased formation of reactive oxygen species such as hydrogen peroxide and hydroxyl free radical. [14] Hepatocytes are the parenchymal cells of the liver and were chosen for this study because of their high responsiveness to exposure to peroxisome proliferators.[15] Large-scale studies have reported that using hepatocytes is the optimal available way to compare the responses of rodents and humans to peroxisome proliferators. [15]

## Glutathione and Its Detoxification Role

Glutathione (GSH), the major intracellular thiol compound, is a tripeptide composed of three amino acids: glutamate, glycine, and cysteine with a thiol group. The highest level of GSH is found in the liver and it is found in different intracellular organelles such as endoplasmic reticulum, nucleus, and mitochondria. GSH plays a crucial role in protecting cells from oxidative stress that results from the production of reactive oxygen species (ROS) such as free radicals, peroxides, and lipid peroxides. Oxidative stress is a serious indicator of cellular damage which leads to diseased tissues and potentially cancer. Mitochondria have sites that are capable of producing ROS, and they have a unique ROS defense system. The mitochondrial glutathione (mGSH) has a significant role in maintaining mitochondrial function and cell survival. Superoxide anion is the primary ROS produced by the electron transport chain (ETC) in the inner mitochondrial membrane through transferring a small fraction of electrons from the electron transport chain, and its production can lead to producing other types of ROS. Superoxide anion is converted into hydrogen peroxide ($H_2O_2$) by dismutation. $H_2O_2$ then

is reduced into water by glutathione peroxidase and its substrate GSH. As a result, GSH becomes oxidized glutathione (GSSG). Then the enzyme glutathione reductase recycles GSSG back to GSH with the concurrent oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) as shown in figure 3. [17] When cells undergo oxidative stress, this results in accumulating GSSG which leads to a decrease in the ratio of GSH to GSSG.

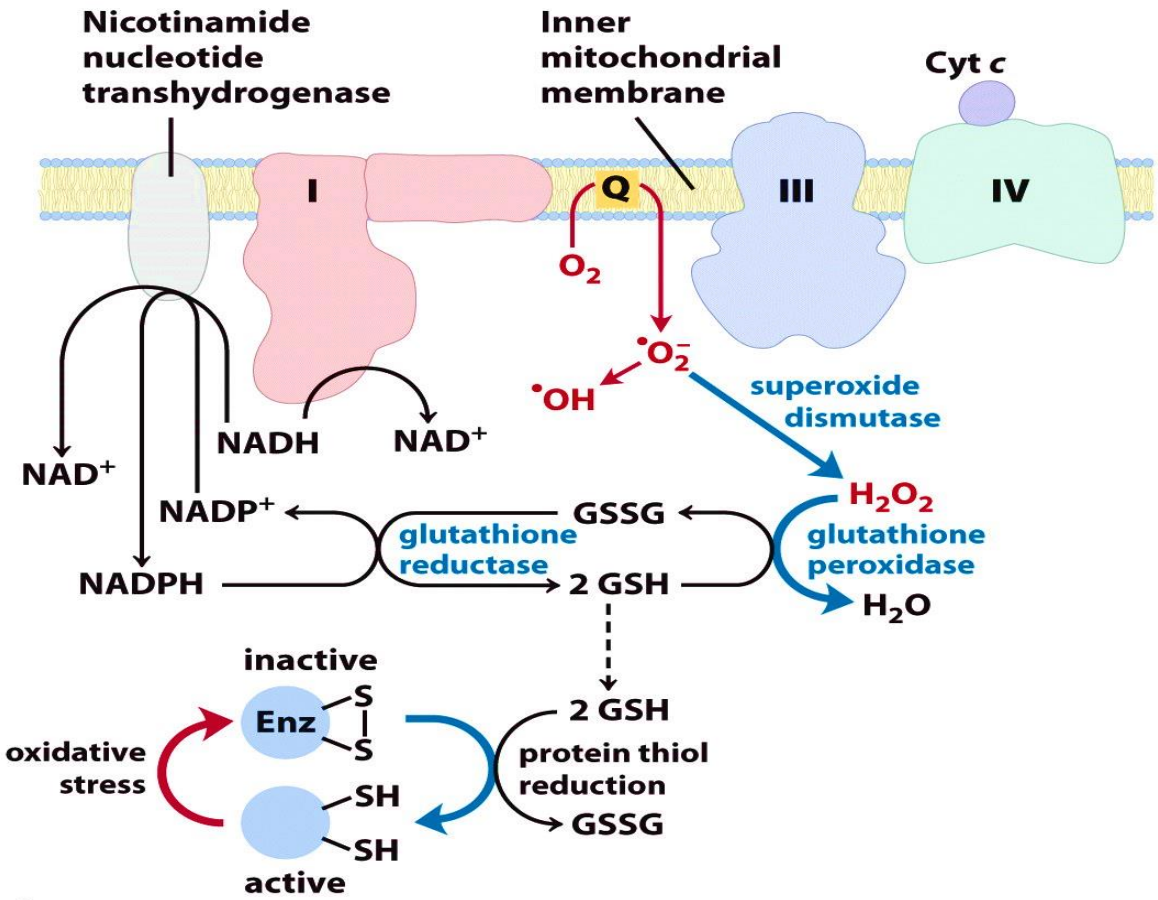



Figure 3: An example of mitochondrial reactive oxygen species (ROS) generation and glutathione defense role.[18]

In this study, we examine the response of two mouse liver hepatoma (Hepa-1c1c7) cell lines to the toxic effects of DEHP and MEHP. One of these cell lines has higher levels of glutathione due to its overexpressing of both subunits of the rate-limiting

enzyme in glutathione synthesis. [16] The key hypothesis addressed in this study is whether DEHP and MEHP hepatoxicity involves oxidative stress. Thus, four experimental approaches were used in this study to answer our hypothesis. These experimental approaches are measurements of cell viability, bioenergetics by Adenosine Triphosphate (ATP) content, mitochondrial membrane function, and reactive oxygen species production.

# CHAPTER II

# METHODS AND MATERIALS

Two mouse liver cell lines were used in this study. One is clone transfected with the plasmid vector alone (Hepa-V) and the other is clone transfected with both subunits of the rate-limiting enzyme in GSH synthesis (CR-17) resulting in elevated levels of glutathione which is known for preventing cell damage by removing toxic compounds and reactive oxygen species. Both cell lines were acquired from our collaborator, Dr. Terry Kavanagh's laboratory, at the University of Washington. The toxicities of DEHP and MEHP were tested in this research by conducting the four previously mentioned experimental approaches.

## Cell Culture and Seeding Cells in Microplate Wells

The two mouse liver cell lines were obtained and maintained in our laboratory. The following details were applied for both cell lines exactly. Cell culture media was prepared by adding 50 mL of Nu Serum and 5 mL of 100 U/mL penicillin-streptomycin into 500 mL of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12(DMEM/F-12). Cells were grown in 25 $cm^2$ cell culture flasks and incubated at 37$^oC$ in a humidified atmosphere of 95% air and 5% carbon dioxide. The media was used to feed cells every two or three days by replacing the old media with fresh media warmed to 37$^oC$. Cells were passed when they were 70-80 % confluent. The passage process starts by aspirating the old media of the adhered cells. Then, the cells were washed with 5 mL of warmed phosphate buffered saline (PBS) at pH 7.4. After that the PBS was aspirated from the flask and 1 mL of 0.05 % of trypsin dissolved in PBS was added. The purpose of adding trypsin is to allow the cells to detach from the wall of the flask. The flask was incubated

for two minutes at 37ºC in a humidified atmosphere of 95% air and 5% carbon dioxide. After the cells were detached from the wall, 4 mL of media was added in order to deactivate trypsin. Then, the content of the flask was transferred into a 15-mL sterile conical tube and centrifuged for 6 minutes at 800 rpm. After the centrifuging, the trypsin and media were aspirated off the cell pellet and 5 mL fresh media was added into the conical tube. The media and cells were pipetted up and down to breakdown the pellets and resuspended all the cells. When no large clumps were visible, 0.5 of the re-suspended cells was transferred into new sterile flask. Then 4.5 mL of fresh media was added into the new flask and this flask was incubated at 37ºC in a humidified atmosphere of 95% air and 5% carbon dioxide. The rest of the re-suspended cells in the conical tube, 100 µL was transferred into a microtube. And the 100 µL was diluted to 300 µL using deionized water. This microtube was used to count cells with a hemocytometer.

The counting process begins with transferring 10 µL of cell solution from the microtube to each side of the hemocytometer microscope slide after a coverslip was placed. The hemocytometer has nine boxes and each box consists of 16 squares. The number of cells were counted under an inverted microscope only in the highlighted five boxes as shown in Figure 4. After that the average was calculated by dividing the sum of cells by 5. In order to obtain the number of cells in each mL of media the following equation was followed: Number of cells / mL= Average number of cell counted × Dilution factor $\times 10^4$. After calculating the number of cells per mL, cells were diluted with the needed volume of media to obtain 5000 cells per 100 µL for 96 microplate wells and $10^5$cells per 500 µL for 24 well plate.

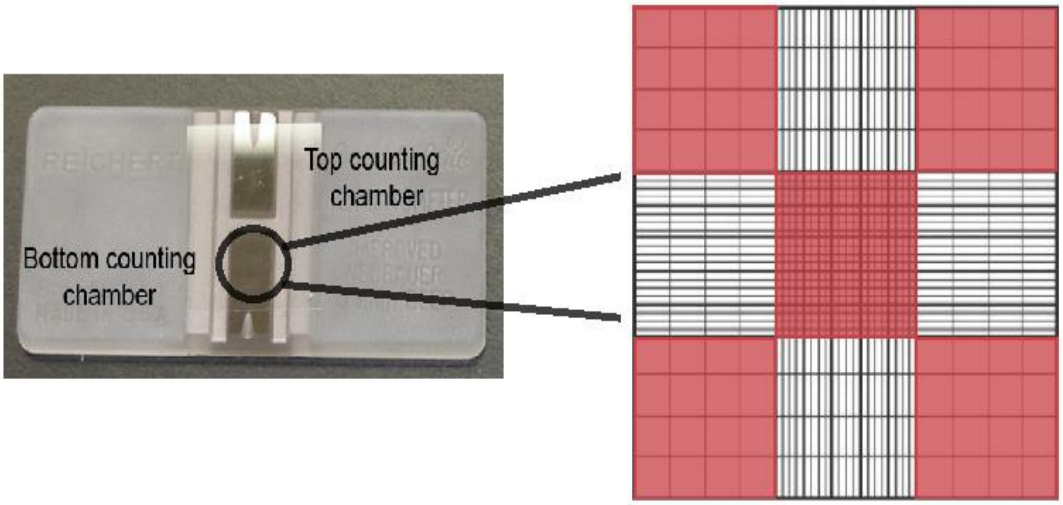



Figure 4: Schematic illustration of a hemocytometer microscope slide (on left), and a diagram of chambers used for counting cells as seen under the microscope (on right).

## Treating Cells with DEHP and MEHP

Four concentrations of DEHP and MEHP (10, 50, 100, and 300 µM), which were consistent with levels encountered in clinical and environmental exposures [10], were prepared. The stock solution of 10 mM of each DEHP and MEHP in dimethyl sulfoxide (DMSO) were diluted to the final concentration using media; the final concentration of DMSO was less than 5% (v/v) as shown in Table 1.

Table 1: Calculations for the preparation of four concentrations of DEHP and MEHP

| DEHP or MEHP Stock Solutions | V1=C2xV2/C1 | | | Treatment Conditions |
|---|---|---|---|---|
| Initial Concentration | Initial Volume | Final Concentration | Final Volume | % DMSO |
| C1=10000 µM | V1 (µL) | C2 (µM) | V2 (µL) | (vehicle) |
| 10000 | 30 | 300 | 1000 | 3 |
| 10000 | 10 | 100 | 1000 | 1 |
| 10000 | 5 | 50 | 1000 | 0.5 |
| 10000 | 1 | 10 | 1000 | 0.1 |

For the first two assays, cells were seeded in the appropriate well plate for 48 hours. 70-80 % confluent cultures then were treated with the four concentrations of DEHP and MEHP and incubated for two different times of exposure, 24 hours and 48 hours, at 37$^{o}$C in a humidified atmosphere of 95% air and 5% carbon dioxide. For the last two assays, cells were treated with only one concentration of DEHP and MEHP.

## Cell Viability Assay

In this assay, WST-8, which is a highly water-soluble tetrazolium salt [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2*H*-tetrazolium, monosodium salt], was used to monitor the mitochondrial dehydrogenase activity to determine cell viability. The mechanism of the metabolic cell death in this essay as shown in Figure 5 includes the reduction of WST-8 by mitochondrial dehydrogenase activities in cells which will give a yellow color formazan dye which is soluble in the cell culture media. Only active and healthy cells have the capability to produce this dehydrogenase activity. The amount of the generated formazan dye is directly proportional to the number of living cells. On the day of assay 30 minutes before the 24 hours or 48 hours incubation period ends, 10 % v/v of DMSO was added for the negative control. After 24 hours or 48 hours of incubation, 10 µl of WST-8 was added to each well and then the 96 well plate was incubated for 2 hours at 37$^{o}$C in a humidified atmosphere of 95% air and 5% carbon dioxide. After incubation, the fluids were moved from all wells with cells into wells without cells then the absorbance values were obtained by using a Synergy II plate reader at 450 nm, an instrument available in the shared instrument room of the Chemistry and Biology Departments at CWU. [19]

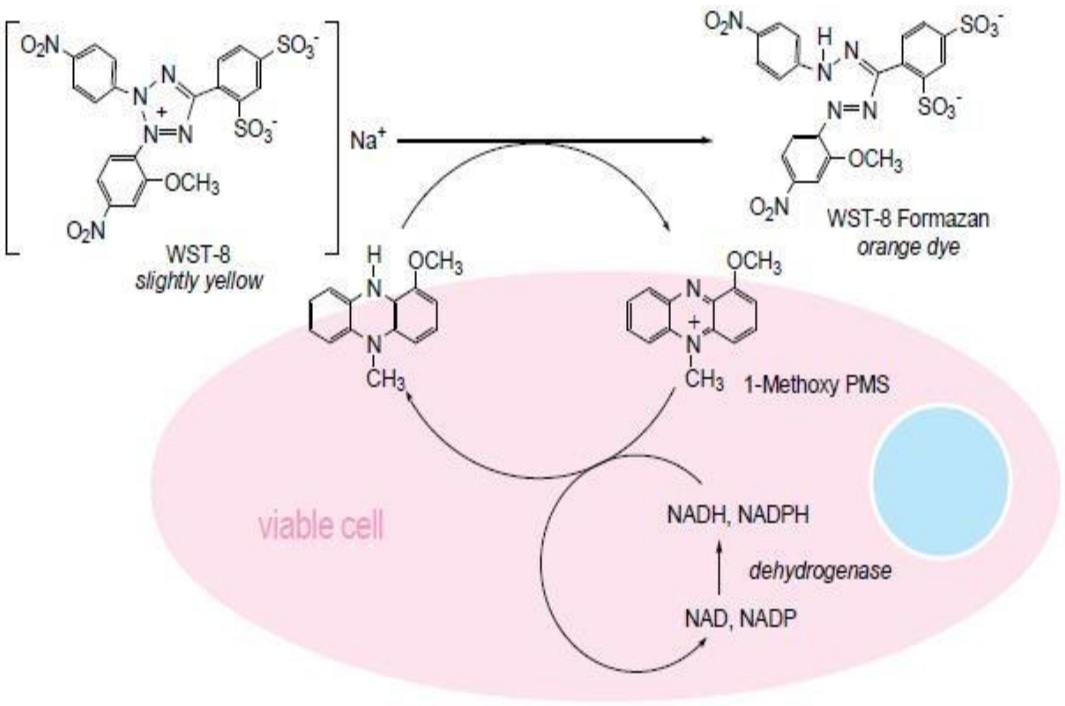



Figure 5: Cell viability detection mechanism with WST-8. [19]

## Adenosine Triphosphate (ATP) Luminescence Assay

Adenosine triphosphate (ATP) is the cellular energy that is required for cell growth and metabolism. The ATP level produced by cells is affected when cells are treated with xenobiotics such as phthalates. Thus, a quantitative measurement of ATP can be used to signal cell cytotoxicity. The main goal of this assay is to measure the amount of ATP produced in cells after they were treated with DEHP and MEHP compared to untreated control. Since it is a luminescence assay, 96 well white luminescence plates were used. After 24 hours and 48 hours exposures to DEHP and MEHP, cells were washed by PBS buffer and treated with 35 µL of 1.5% triton X-100 at room temperature for 20 minutes to solubilize the membrane to release the intracellular ATP. For the negative control, producing lower concentration of ATP, 10 µM of antimycin A, which is known as a mitochondrial electron transport chain inhibitor of complex III, was added to the control wells and incubated for 6 hours. Then 100 µL of the ATP luciferin luciferase

buffer solution (250 mM glycylglycine buffer, pH of 7.3, 2 mM EGTA, 2 mM $MgCl_2 \cdot 6H_2O$, 7.5 mM dithiothreitol, 15 µM luciferin and 10 µg/mL luciferase) was added to each well in the plate then the luminescence intensity values were measured using a Synergy II luminescent plate reader with plugging the excitation filter wheel. The reagents added in this assay were prepared as given in a previous study. [20] ATP amounts produced by cells were measured by comparison to an ATP standard curve of different concentrations (0.1, 0.2, 0.5, 1, 5, 10, 20, 40, 60, 80, and 100 nM). The mechanism of this assay relies on the luciferin-luciferase reaction which depends on ATP to emit light. Hence, ATP levels and light emission in this reaction are correlated as shown in Figure 6.[21]

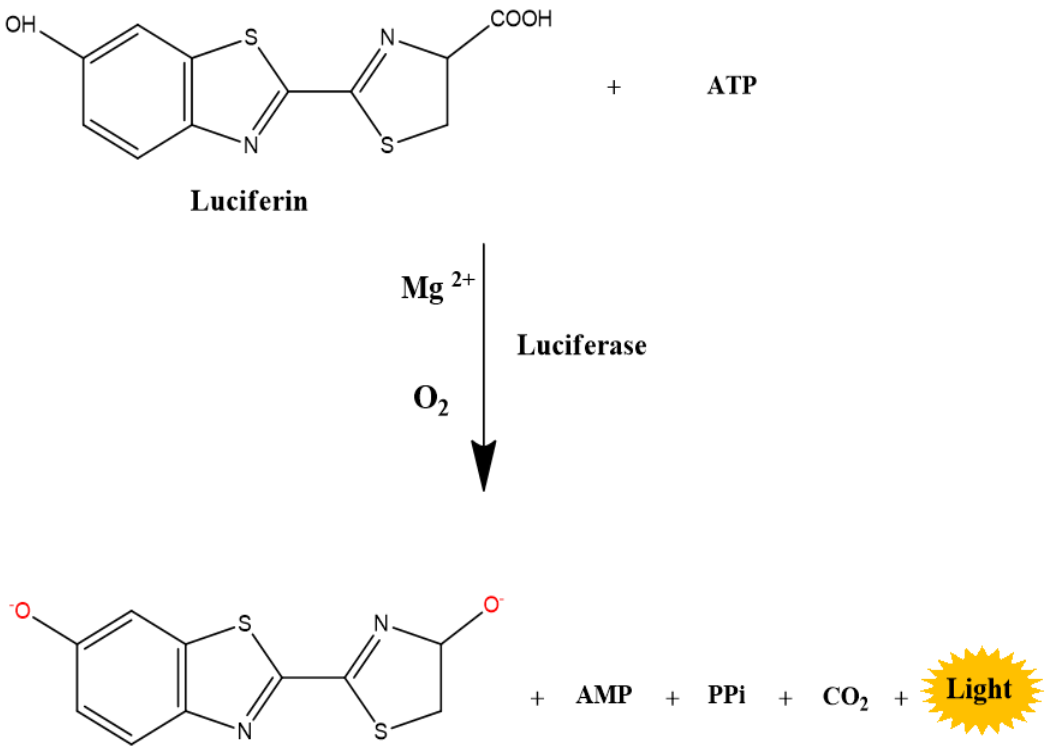



Figure 6: Reaction of Luciferin and ATP in the presence of Luciferase. Adapted from [(21)].

## Flow Cytometry

In the following two assessments, the mitochondrial membrane potential assay and the reactive oxygen species assay, samples were analyzed using the Bio-RAD S3e cell sorter at the Keck-Murdock Flow Cytometry Facility at CWU. Flow cytometry is used to analyze various physical and chemical properties of individual particles. In the flow cytometer, a stream of a single line of cells is formed which then flows through a laser beam. As the laser beam hits each individual cell, light will be scattered in different directions. Scattering of light will result in diffracted scatter (forward scatter) and refracted and reflected scatter (side scatter). Each scatter is proportional to different properties of cells. The size of cells is detected via the forward scatter while the internal complexity and shape of cells are detected via the side scatter. When the laser beam hits cells, a fluorochrome inside the cell will fluoresce a certain color or spectrum of light. Optical filters are used to direct a spectrum of light to the appropriate photomultiplier detector. In this study, we used only two filters, filter 1 and filter 2. The filter 1 channel collects green light emission (525±30 nm) while filter 2 channel collects orange light emission (586±25 nm). The information collected by the photomultiplier tube is converted into digital data, which appears in an attached computer. Figure 7 shows a visual illustration of the concept by which flow cytometry works. [22]

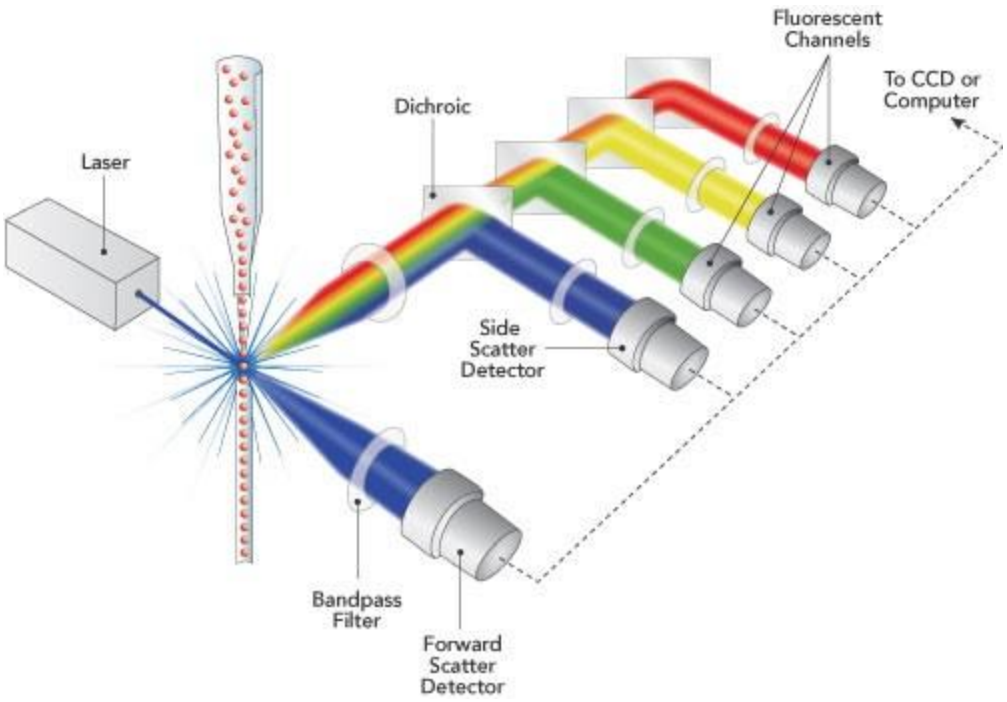



Figure 7: Illustration of the concept by which flow cytometry works. [23]

## Flow Cytometry Data Analysis

After collecting the desired number of single cells which are called events by flow cytometry, the acquired data are analyzed using a software program called FlowJo (V.10.1). In FlowJo, we separated the cell population of interest from all the collected events by using a tool called Gating. Another gating was then applied which included only the singlet events and to avoid any debris or double events (two cells stuck together) that may have interfered with the analysis. After that a histogram was obtained from the singlet events from which the median fluorescence intensities were calculated.

## Mitochondrial Membrane Potential Assay

This assay is used to directly measure a cell's mitochondrial function and indirectly its capacity to produce ATP after they have been treated with DEHP and MEHP. To investigate the effect of DEHP and MEHP on mitochondrial membrane

potential, cells were treated with a cationic lipophilic dye JC-10 which produces two different colors to indicate low or high mitochondrial membrane potential. Normal cells gave emission profile at 570 nm due to JC-10 aggregation in mitochondria which results in red fluorescence. However, the affected cells shifted the emission profile to green emission at 520 nm due to the production of JC-10 monomers which indicates a decrease in the membrane potential function as shown in Figure 8.

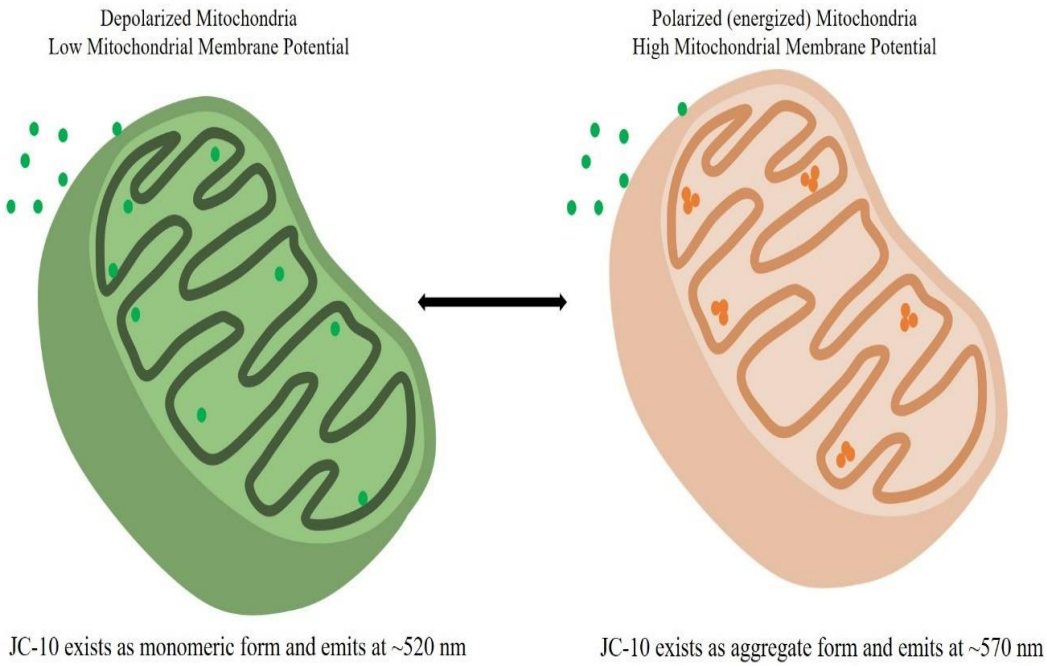



Figure 8: Mechanism of action for JC-10 Adapted from [24].

In the mitochondrial membrane potential assay 24 well plates were used and cell concentration was $1\times10^5$ cells /mL. Cells were treated with only one concentration of DEHP and MEHP which was 100 µM. The JC- 10 dye was dissolved in DMSO to 1:1 ratio with HHBS buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer in Hank's Balanced Salt Solution (HBSS) with final pH of 7.3 with 0.02% pluronic F-127). On the day of experiment, 200 µL of PBS was used to wash the

cells after DEHP and MEHP treatments were aspirated. After that, all cells were treated with 493.3 μL of fresh media and 6.7 μL of 1.5 mM JC- 10 making the final concentration of dye to be 20 μM in each well. The cells with JC-10 dye were incubated for 30 minutes at 37$^{o}$C in a humidified atmosphere of 95% air and 5% carbon dioxide. After the incubation, cells were washed with 200 μL of PBS and then trypsinized with 0.05% trypsin to detach cells from the culture flasks and then 500 μL of media was added to stop the activity of trypsin. Cells were transferred to a 2-mL microfuge tube and centrifuged for 6 minutes at 800 rpm. Then the media and trypsin mix were aspirated, and cells were re-suspended in 500 μL HHBS buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer in Hank's Balanced Salt Solution (HBSS) with final pH of 7.3 with 0.02% pluronic F-127). Florescence intensities were measured using the Bio-RAD S3e cell sorter flow cytometer from channels FL1 and FL2 and the data were analyzed using FlowJo (V.10.1) software. Both the flow cytometer instrument and the analysis software are available in the Biology Department at CWU.

## Reactive Oxygen Species Production Assay

Oxidative stress results when cells are unable to scavenge or remove excess, damaging reactive oxygen species (ROS). ROS production is considered an important indicator for abnormal metabolic activities in live cells such as the metabolism of xenobiotics. After exposing cells to DEHP and MEHP, DEHP and MEHP treatments were aspirated and cells were washed with 7.5% PBS. Then, all cells received 499 μL of media except the positive control cells which received 400 μL of media plus 99 μL of 2.032 mM tert-butyl hydroperoxide dissolved in media. The positive controls were incubated for 45 minutes at 37$^{o}$ C in a humidified atmosphere of 95% air and 5% carbon

dioxide. Tert-butyl hydroperoxide is used as a positive control in this assay because it is known to induce the formation of reactive oxygen species. The reactive oxygen species were measured by treating cells with a fluorescent dye called CellROX green which is a DNA dye that binds to DNA when cells undergo oxidation. Reactive oxygen species are known to oxidize CellROX green. According to the manual provided by the producer, this dye is weakly fluorescent when it is in a reduced state, but it exhibits a bright green fluorescence upon oxidation by reactive oxygen species (ROS) which is followed by binding to DNA, with absorption/emission maxima of approximately 485/520 nm. 1 μL of CellROX green was added to all wells to give a final concentration of 5 μM and incubated for 45 minutes at 37$^{o}$ C in a humidified atmosphere of 95% air and 5% carbon dioxide. After that, cells were trypsinized and centrifuged for 6 minutes at 800 rpm in 500 μL of FACS buffer (1% bovine serum albumin in PBS). Cells were re-suspended in fresh 500 μL of FACS buffer and the green fluorescence intensities were monitored by the Bio-RAD S3e cell sorter flow cytometer from channel FL1 and then data were analyzed using FlowJo (V.10.1) software. [20]

## Statistical Analysis

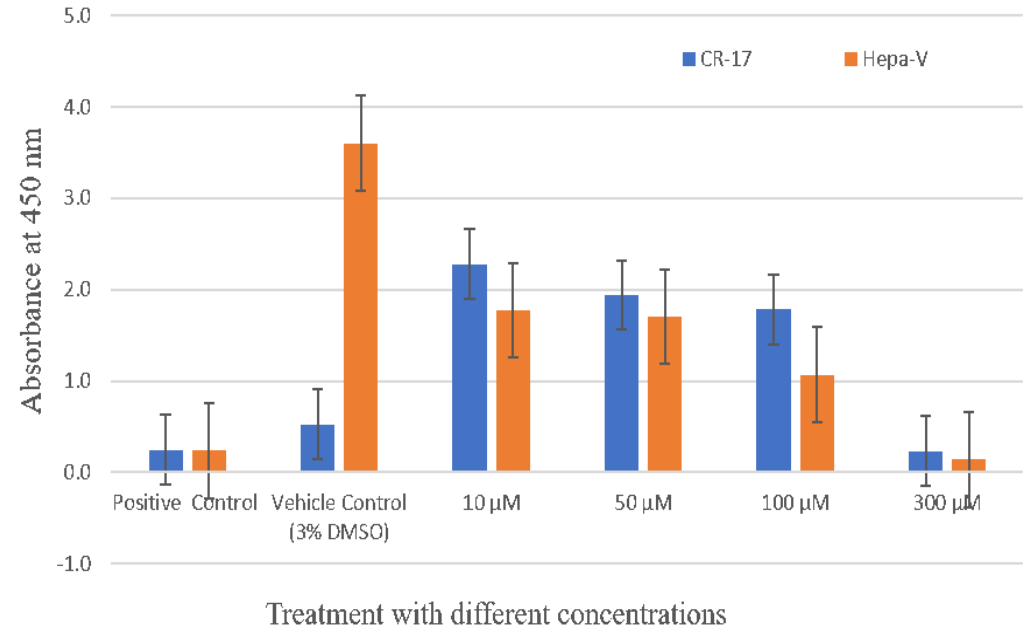
All experiments were repeated a minimum of three times. Calculations were carried out using Microsoft Excel. Data were expressed as means ± SD. The mean values were compared using one-way ANOVA and/or two-way ANOVA. The one-way ANOVA was used to test for significant differences within one cell line in terms of dose of phthalate while the two-way ANOVA was used to test for significant differences between the two cell lines in response to phthalate exposure. A difference for which $P < 0.05$ is considered statistically significant.

# CHAPTER III

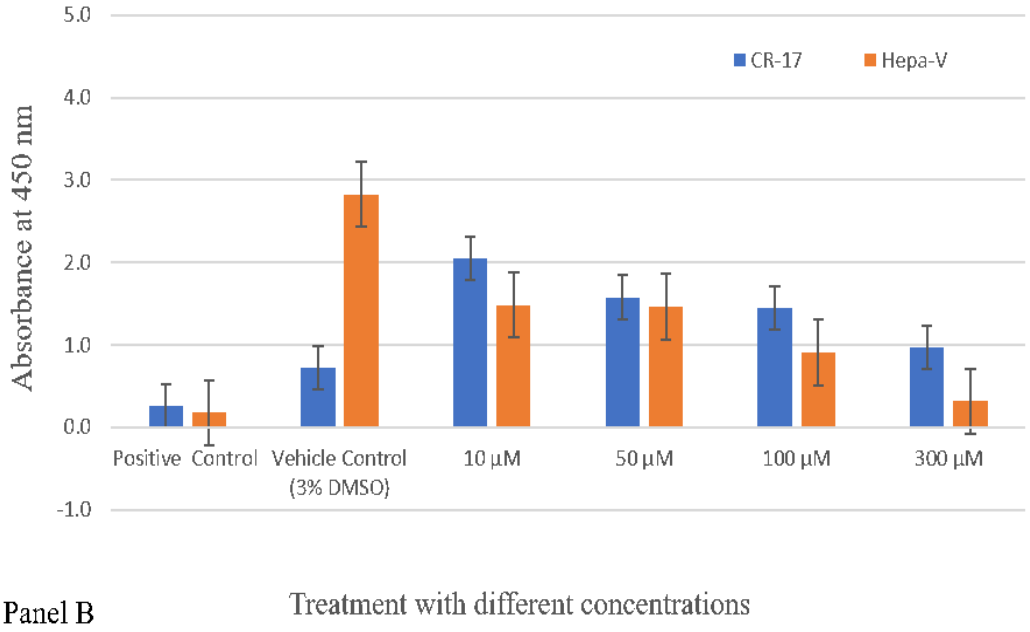
# RESULTS

## Cell Viability

Cell viability was assessed in this study by using the WST-8 assay. The determination of cell viability was acquired by measuring the activity of mitochondrial dehydrogenases produced by healthy cells. DEHP and MEHP induced dose-dependent cytotoxic effects on both cell lines used in this experiment. For the positive control, cells were treated with 10 % dimethyl sulfoxide (DMSO) and incubated for 30 minutes. For the vehicle control, cells were treated with 3 % of DMSO which is the percent of DMSO in the highest concentrations of DEHP and MEHP (300 µM). All results of this assay are shown in Figures 9 and 10. According to the trends shown in the figures, both cell lines showed decreasing cell viability with increasing phthalate concentrations. At 300 µM DEHP or MEHP, cell viability was decreased the most. When comparing the effects of DEHP and MEHP on metabolic cell death, we found that MEHP was more toxic than DEHP at both times of exposure. No significant differences in the pattern of phthalate-disrupted cell viability were observed between Hepa-V and CR-17 cells. The two cell lines responded similarly to the phthalates. However, at 50 µM DEHP or MEHP, the time variable affected the responses of these two cell lines. After 48 h exposure, CR-17 shows more resistance to the toxic effects of DEHP and MEHP which was not clear at 24 h exposure.

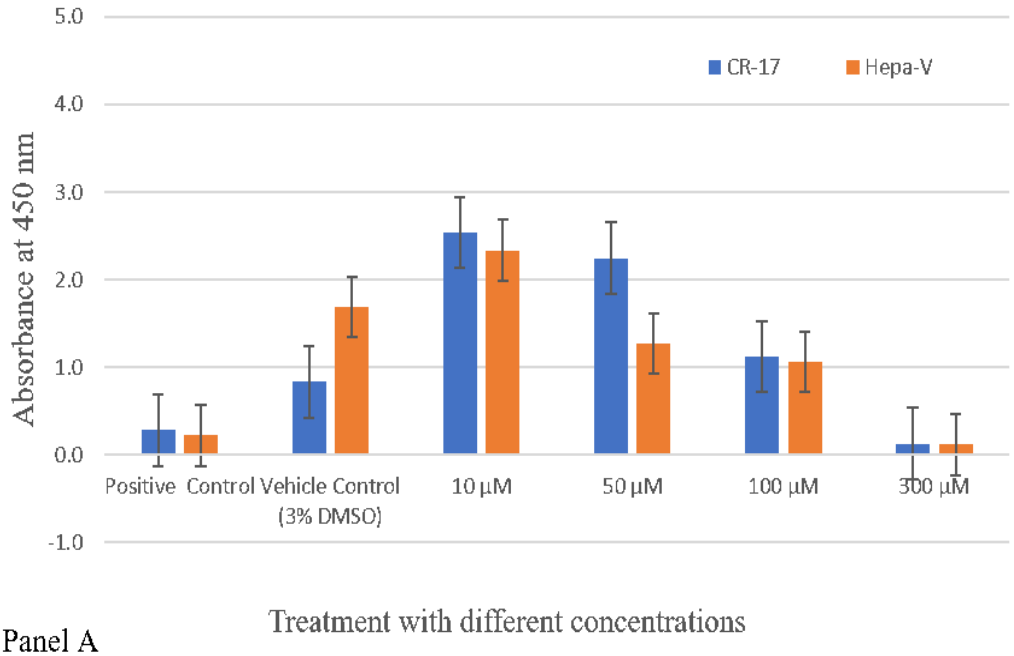



Panel A

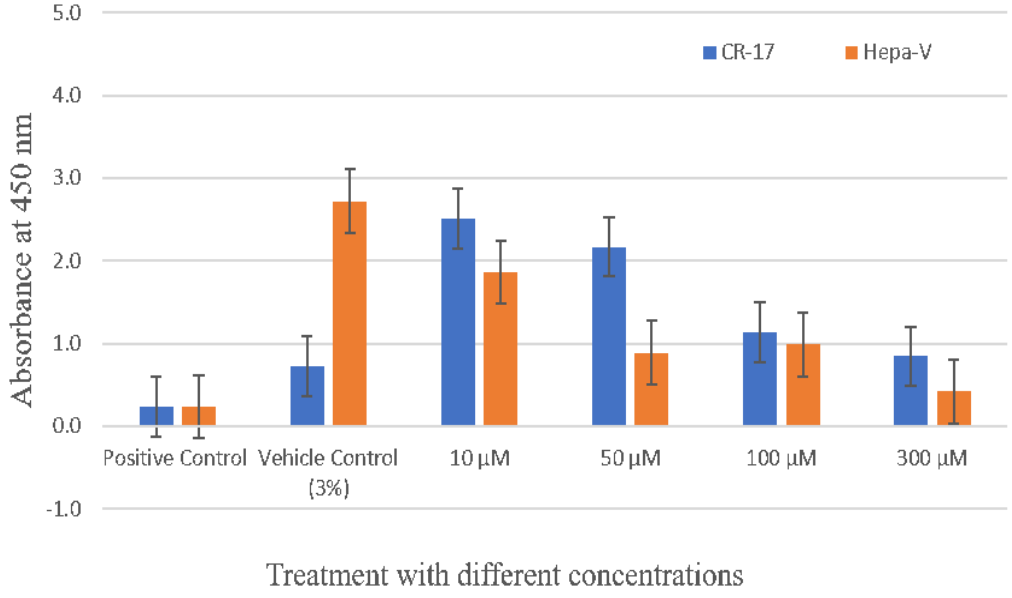



Panel B

Figure 9: Cell viability after 24-hour exposure. Panel A shows the results of MEHP exposure while panel B shows the results of DEHP exposure. Liver cell mitochondrial dehydrogenases activity measured by (WST-8). For the positive control, cells were treated with 10 % dimethyl sulfoxide (DMSO) and incubated for 30 minutes. For the vehicle control, cells were treated with 3 % of DMSO. Analysis by Two-Way ANOVA ($p<0.05$: P-value for data in panel A is 0.68 while for panel B is 0.96) indicates no significant differences in the response of the two cell lines to the toxic effects of MEHP and DEHP in terms of metabolic cell death. However, analysis by One-Way ANOVA for the effects of each phthalate on each cell line indicates significant effects of MEHP and DEHP on metabolic cell death in CR-17 and Hepa-V cells. P-values from One-Way ANOVA analysis ($p<0.05$, $n=3$) for each these data are shown in Table 2.




Panel A




Panel B

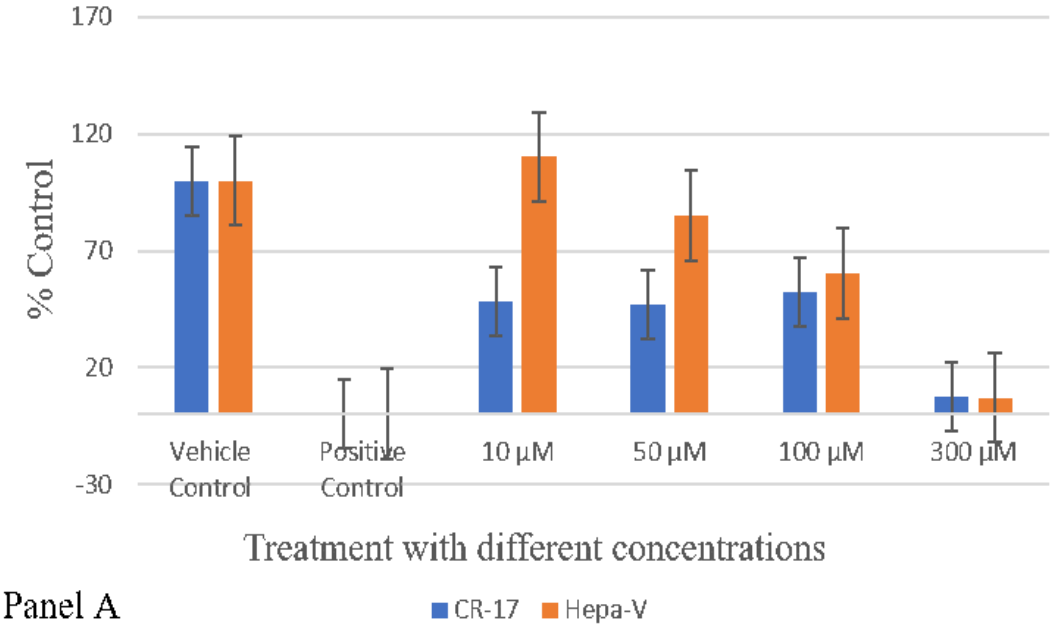
Figure 10: Cell viability after 48-hour exposure. Panel A shows the results of MEHP exposure while panel B shows the results of DEHP exposure. Liver cell mitochondrial dehydrogenases activity measured by (WST-8). For the positive control, cells were treated with 10 % dimethyl sulfoxide (DMSO) and incubated for 30 minutes. For the vehicle control, cells were treated with 3 % of DMSO. Analysis by Two-Way ANOVA ($p<0.05$: P-value for data in panel A is 0.76 while for panel B is 0.86) indicates no significant differences in the response of the two cell lines to the toxic effects of MEHP and DEHP in terms of metabolic cell death. However, analysis by One-Way ANOVA for the effects of each phthalate on each cell line indicates significant effects of MEHP and DEHP on metabolic cell death in CR-17 and Hepa-V cells. P-values from One-Way ANOVA analysis ($p<0.05$, $n=3$) for these data are shown in Table 2.

Table 2: P-values from One-Way ANOVA analysis for WST-8 data

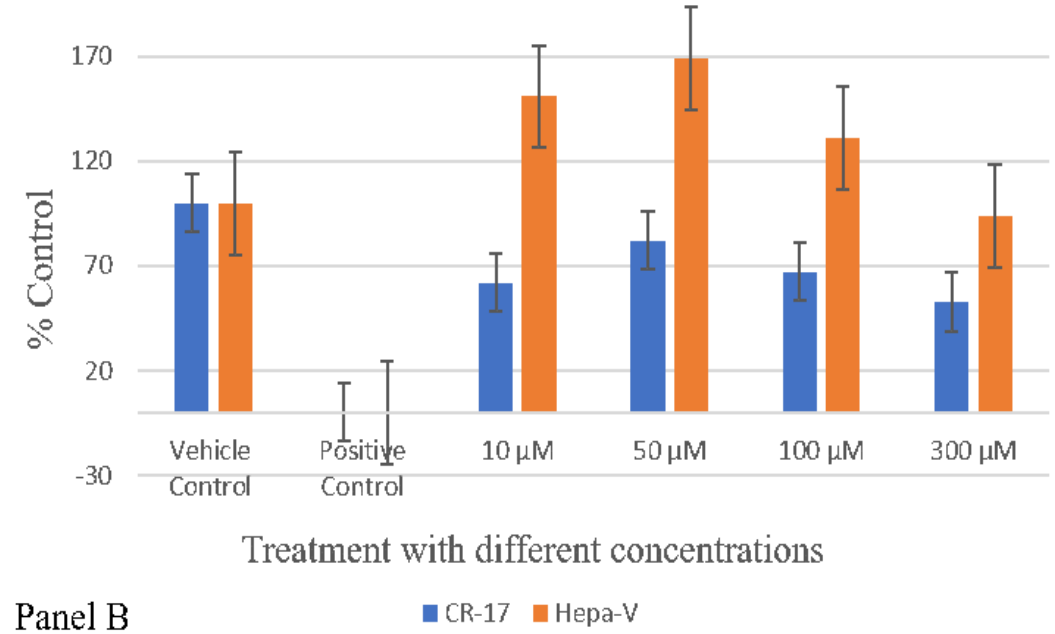
| Cell Line, Phthalate, Exposure Time | P-Value |
|---|---|
| CR-17, MEHP,24 h | 1.61E-05 |
| Hepa-V, MEHP,24 h | 8.30E-10 |
| CR-17, DEHP,24 h | 2.45E-05 |
| Hepa-V, DEHP,24 h | 7.19E-08 |
| CR-17, MEHP, 48 h | 5.95E-11 |
| Hepa-V, MEHP,48 h | 5.39E-04 |
| CR-17, DEHP,48 h | 4.01E-05 |
| Hepa-V, DEHP,48 h | 1.32E-04 |

## Cellular ATP Levels

ATP levels produced by cells work as indicators for cell cytotoxicity. ATP in the presence of luciferase enzyme and luciferin substrate generates luminescent signals which were quantified using a Synergy II plate reader. The vehicle control in this experiment consisted of 3% DMSO. For the positive control in this experiment, cells were treated with 10 µM Antimycin A for 24 h When comparing the two phthalates in terms of times of exposure (24 and 48 h) as shown in Figures 11 and 12, the two cell lines exhibited different responses. After 24-hour exposure of DEHP and MEHP, there is a dose dependent decrease in ATP levels in Hepa-V cells. This dose dependent trend was not obvious in CR-17 cells. CR-17 cells also had lower ATP levels than Hepa-V at most MEHP and DEHP doses as shown in Figure 11. However, after 48 h as shown in Figure 12, CR-17 cells exposed to MEHP had higher levels of ATP than Hepa-V cells. Cells treated with DEHP varied in their response but at most DEHP concentrations (10, 50, 300 µM) ATP levels were higher for CR-17 cells than Hepa-V cells. In both times of exposure, Hepa-V and CR-17 cells treated with 300 µM of MEHP had the lowest ATP levels, an indication of the significant effect of this dose.




Panel A




Panel B

Figure 11: ATP production in liver cells after 24-hour exposure. Panel A shows the results of MEHP exposure while panel B shows the results of DEHP exposure. ATP production of liver cells was determined by the luciferase/luciferin luminescence assay. Vehicle control consists of DMSO (3%). Positive control cells were treated with 10 µM Antimycin A for 24 h. Analysis by Two-Way ANOVA ($p<0.05$: P-value for data in panel A is 0.004, while for data in panel B is 0.010) indicates significant differences in the response of the two cell lines to the toxic effects of MEHP and DEHP on ATP production.

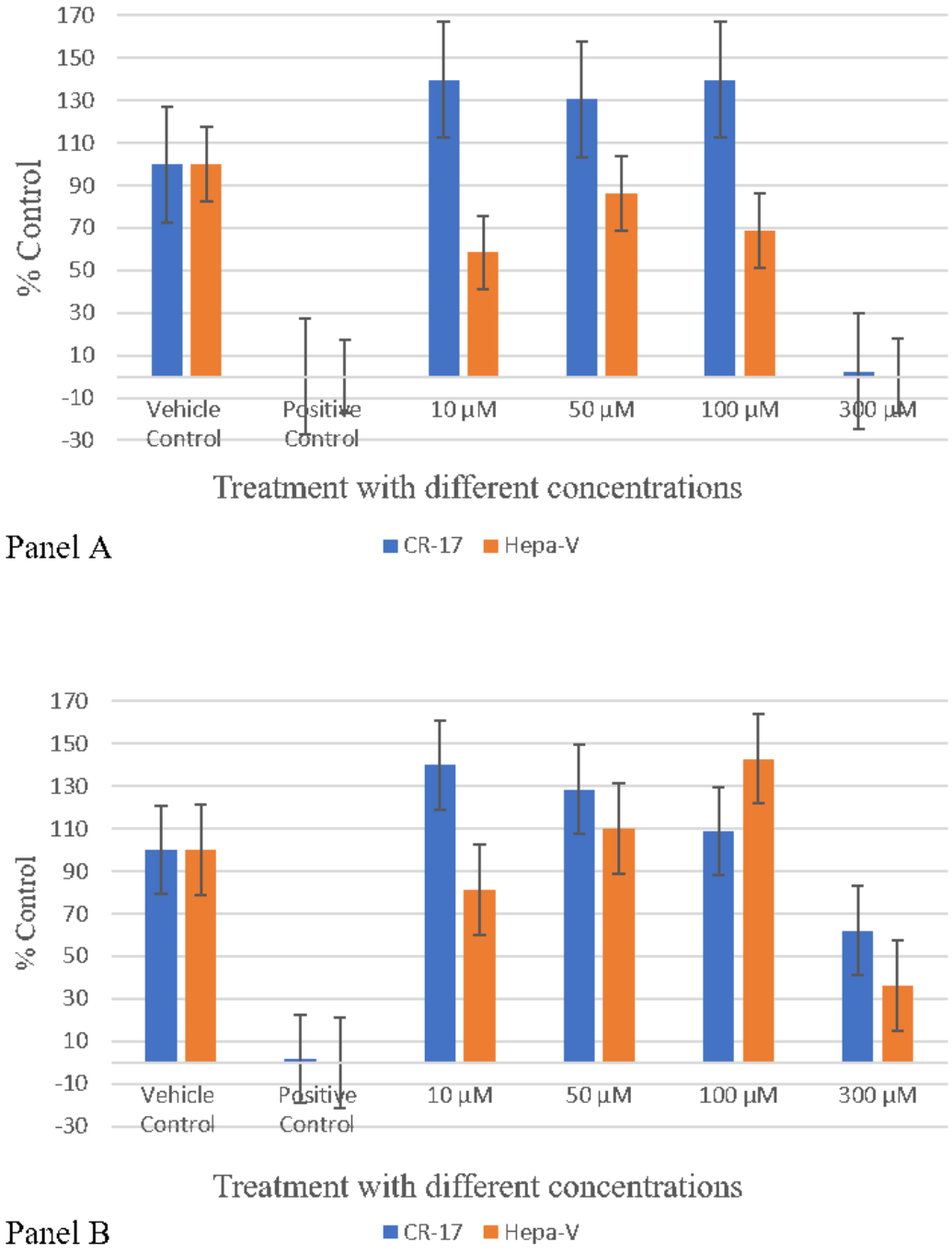



Figure 12: ATP production in liver cells after 48-hour exposure. Panel A shows the results of MEHP exposure while panel B shows the results of DEHP exposure. ATP production of liver cells was determined by the luciferase/luciferin luminescence assay. Vehicle control consists of DMSO (3%). Positive control cells were treated with 10 µM Antimycin A for 24 h. Analysis by Two-Way ANOVA ($p<0.05$: P-value for data in panel A is 0.036, while for data in panel B is 0.007) indicates significant differences in the response of the two cell lines to the toxic effects of MEHP and DEHP on ATP production.

## Mitochondrial Membrane Potential

Mitochondrial membrane potential is a main indicator for mitochondrial function and its ability to produce ATP. Based on the results collected from the previous two assays, only one concentration was chosen to be tested in this assay and the ROS assay. This concentration was 100 µM due to its moderate toxic effects on both cell lines. Moderate toxicity permits us to study the cell death mechanism which is the main purpose of the mitochondrial membrane potential assay and ROS assay. A decrease in mitochondrial membrane potential could be used to signal cell apoptosis or programmed cell death. JC-10 dye was used in this research to measure the effects of DEHP and MEHP on mitochondrial membrane potential. After 24 h exposure of MEHP and DEHP, Hepa-V cells had lower mitochondrial membrane potential than CR-17 cells although the values were not different from vehicle control, where the cells were treated with 1% of DMSO. The 48-hour data show a similar lower trend of mitochondrial membrane potential in Hepa-V cells. However, One-Way ANOVA analysis showed that CR-17 cells treated with MEHP and DEHP for 48 h had mitochondrial membrane potential values that were significantly increased as compared to the vehicle control as shown in Figure 13 and Table 3. Figure 14 shows a representative data figure for how data were analyzed with FlowJo.

Table 3: P-values from One-Way ANOVA analysis for mitochondrial membrane potential assay compared to vehicle control.

| Cell Line, Exposure Time | P-Value |
|---|---|
| CR-17, 24 h | 9.51E-01 |
| Hepa-V, 24 h | 1.04E-01 |
| CR-17, 48 h | 6.98E-05 |
| Hepa-V, 48 h | 1.40E-01 |

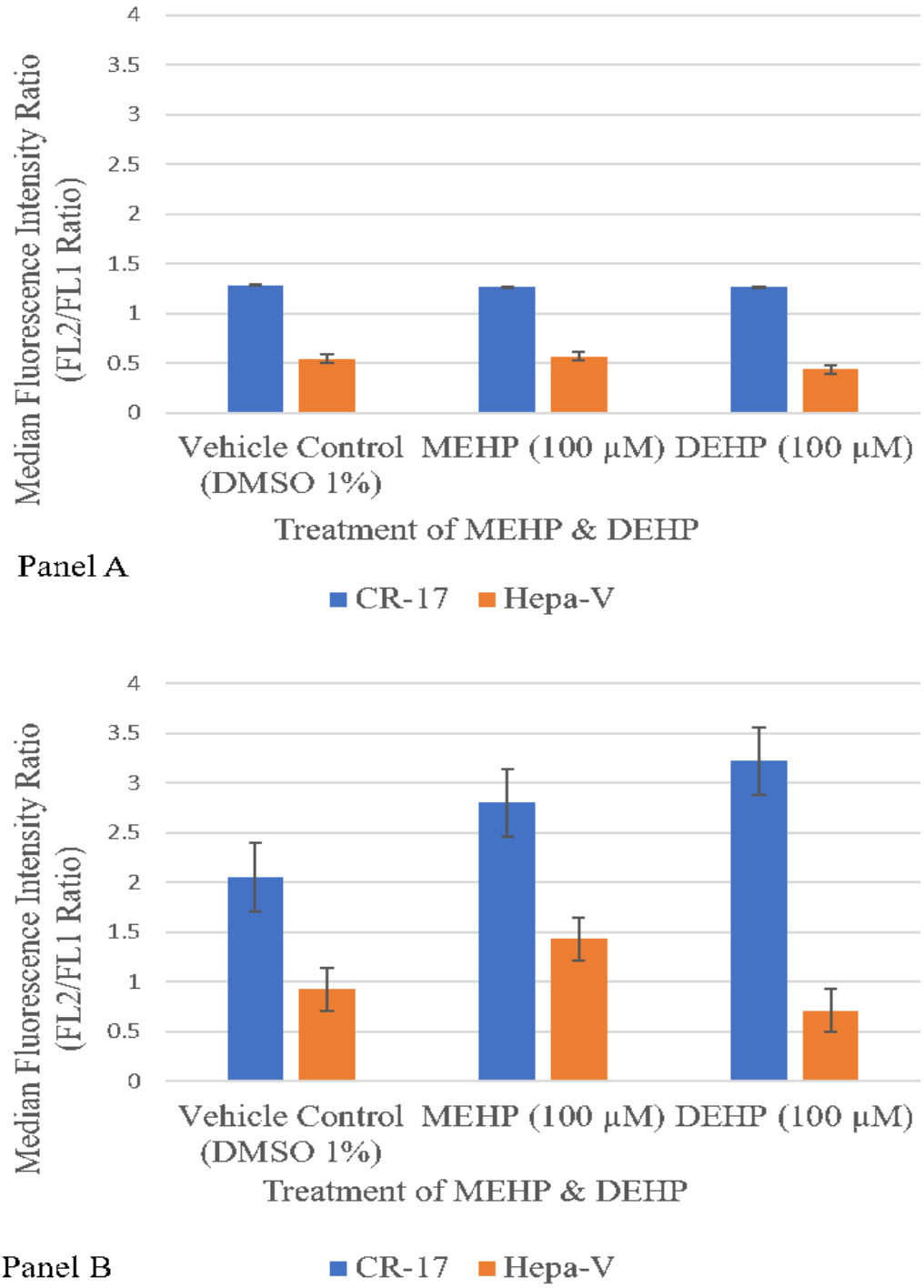



Figure 13: Effects of DEHP and MEHP on mitochondrial membrane potential. Panel A shows results after 24-hour exposure while panel B shows results after 48-hour exposure. Two-Way ANOVA analysis indicates no significant difference of cell lines responses to the effect of DEHP and MEHP on mitochondrial membrane (P=0.495 for 24 hr. exposure, P=0.835 for 48 hr. exposure). Median fluorescence intensities (FL2/FL1) represents the ratio of JC-10 aggregate form which was measured at Ex/Em=488/590 nanometer over JC-10 the monomeric form which was measured at Ex/Em= 488/525 nanometer.

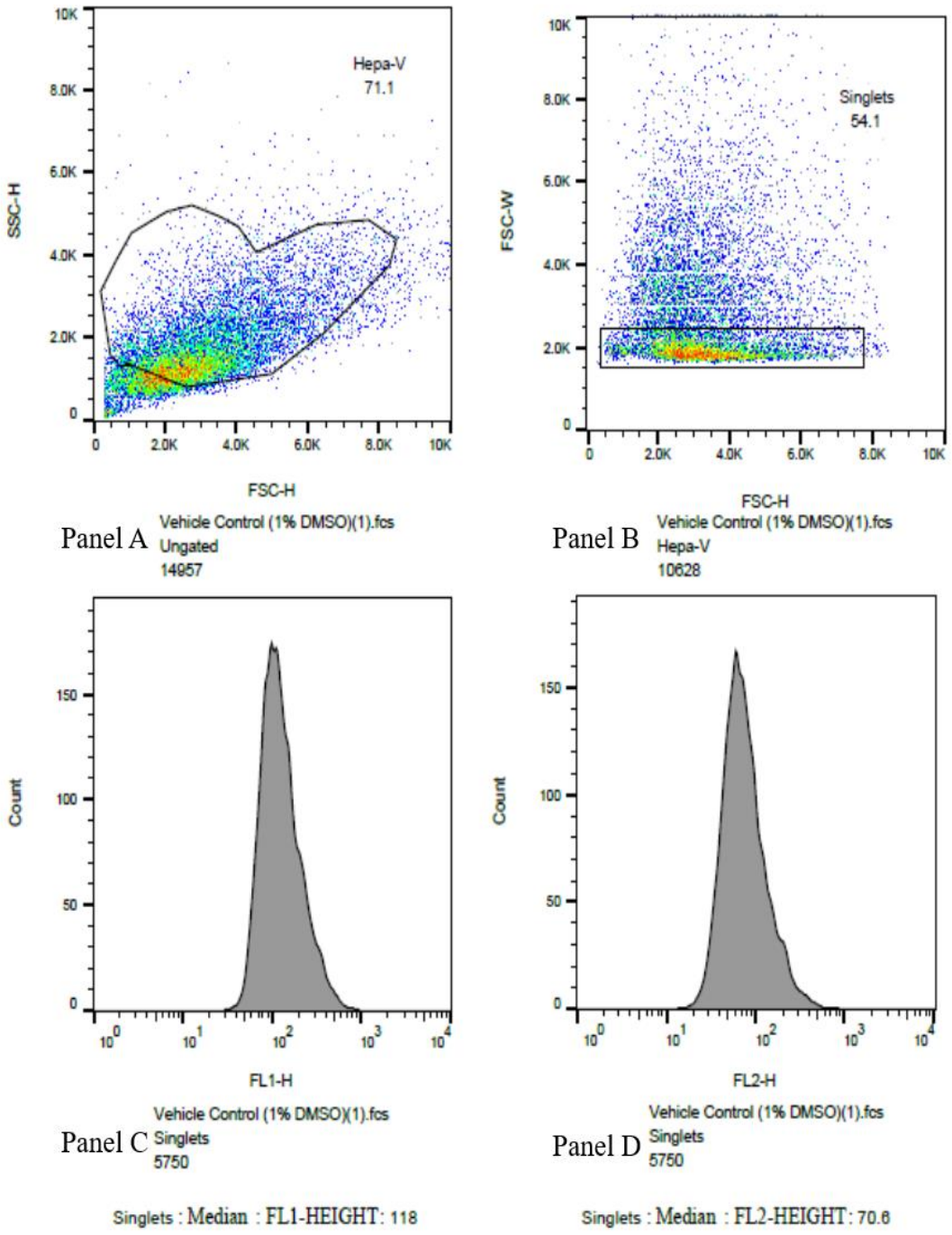


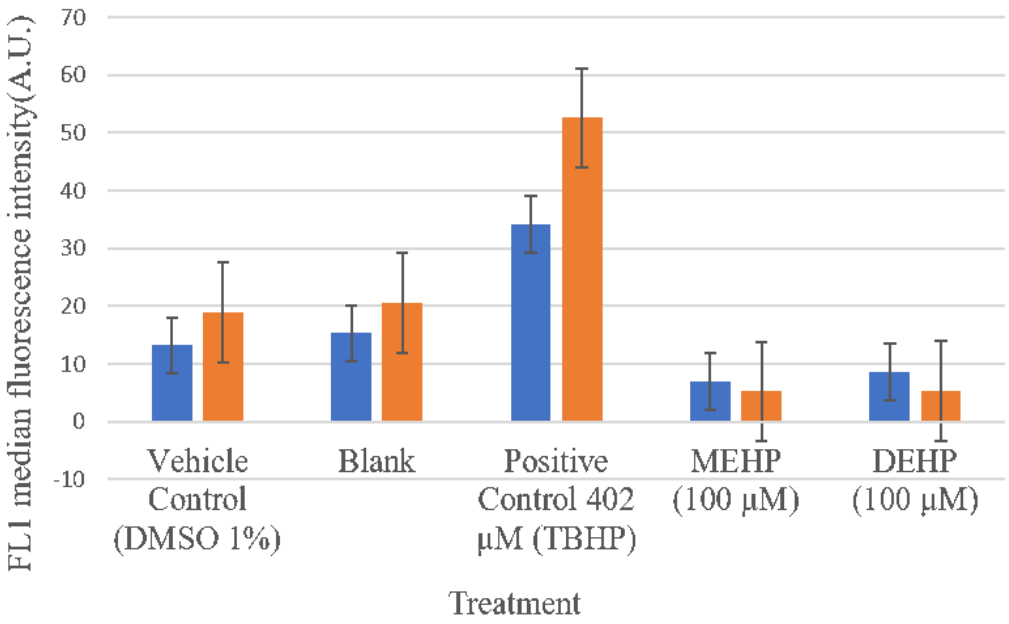

Figure 14: Flow data for mitochondrial membrane potential assay. Panel A shows the first applied gating to separate the population of interest while panel B shows the second applied gating to separate the singlet events from debris and double events. From Panel B, two histograms were obtained, panel C and D, to give us the median fluorescence intensities at FL1 and FL2, respectively. The ratio of the two emissions (FL2/FL1) was used to assess mitochondrial membrane potential.

## Reactive Oxygen Species Generation

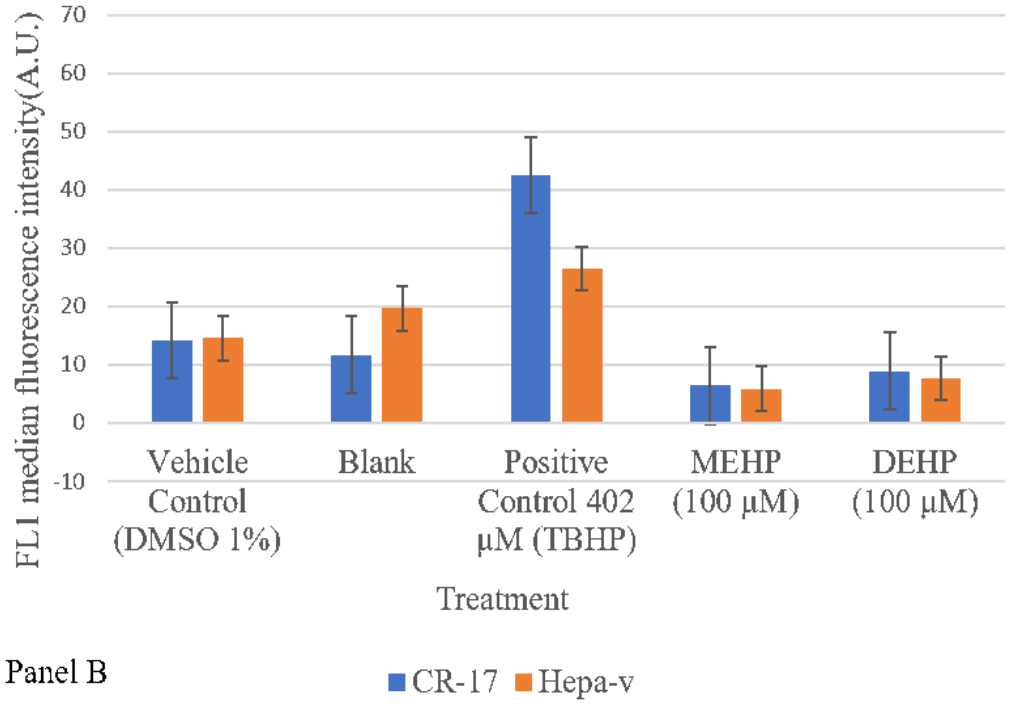
Oxidative stress occurs when cells become unable to scavenge reactive oxygen species (ROS) that are generated because of exposure to xenobiotics such as phthalates. The production of ROS was measured using the CellROX green dye which produces green fluorescence when oxidized by ROS. For the vehicle control, cells were treated with 1% DMSO. For the positive control, cells were treated with tert-butyl hydroperoxide which is known to induce the formation of reactive oxygen species. Results of this assay indicate that DEHP and MEHP did not increase ROS production if data were compared with the control samples (vehicle control and positive control) as shown in Figure 15. However, One-Way ANOVA analysis indicates that there is a significant decrease in ROS production in both cell lines and for the two times of exposure as shown in Table 4. After 24-hour exposure, CR-17 cells show more resistance than Hepa-V cells toward the positive control and with untreated cells. However, in the positive control, the trend was reversed after 48-hour exposure of DEHP and MEHP. Figure 16 shows a representative data figure for how data were analyzed with FlowJo.

Table 4: P-values from One-Way ANOVA analysis for reactive oxygen species assay.

| Cell Line, Exposure Time | P-Value |
|---|---|
| CR-17, 24 h | 1.04E-06 |
| Hepa-V, 24 h | 2.36E-06 |
| CR-17, 48 h | 3.00E-02 |
| Hepa-V, 48 h | 7.68E-05 |




Panel A




Panel B

Figure 15: Effects of DEHP and MEHP on ROS production. Panel A shows the effects on ROS after 24 h while panel B shows the effects on ROS after 48 h. Blank samples are cells which did not receive any treatments, its purpose was to help with data analysis through FlowJo software. No ROS generation was observed after the exposure of DEHP and MEHP for 24 h and 48 h. Median fluorescence intensities were measured at Ex/Em=488/530 nanometer. Vehicle control, cells were exposed to 1% DMSO. Positive control, cells were exposed to 402 µM tert-butyl hydroperoxide to induce the formation of reactive oxygen species.

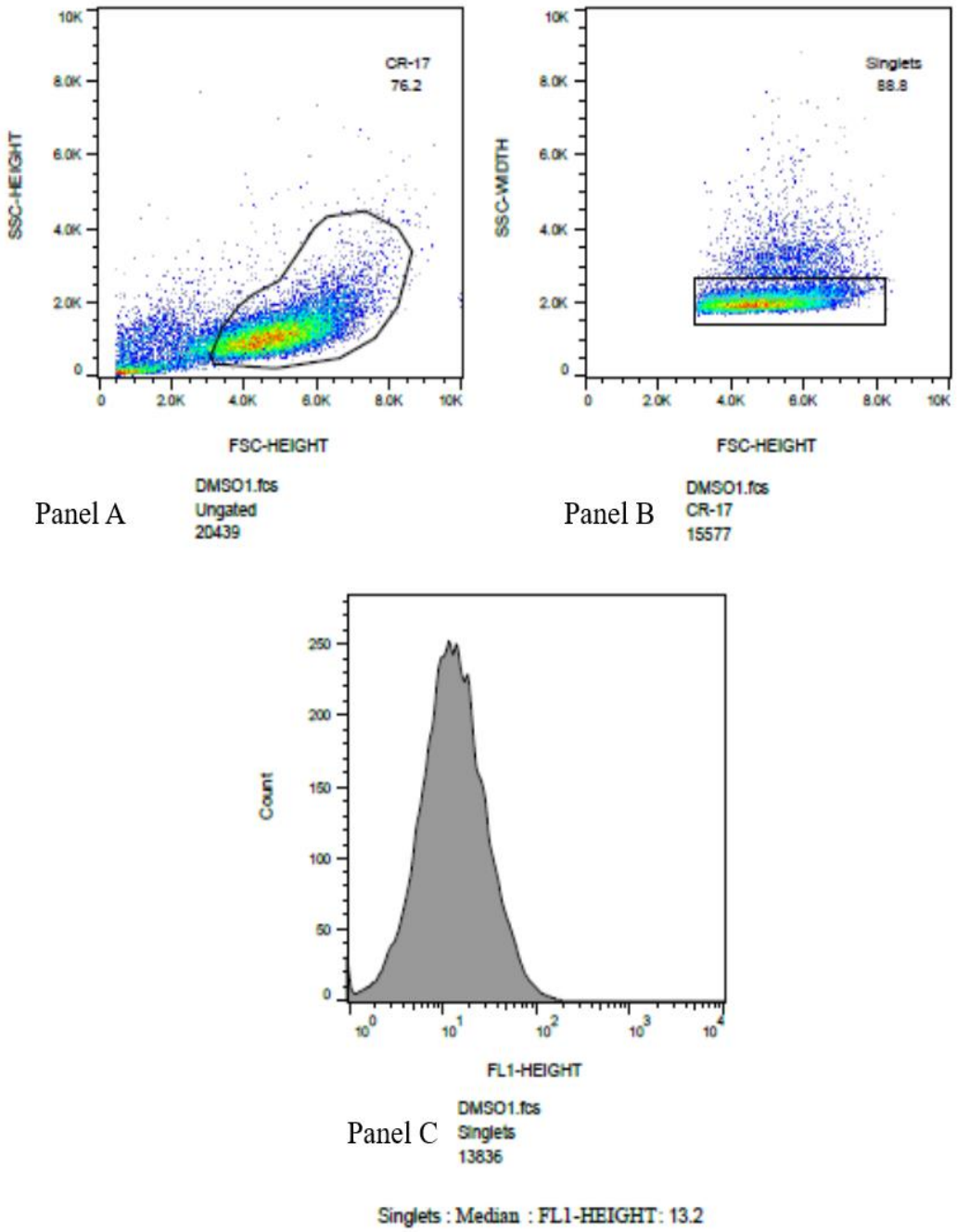



Figure 16: Flow data for the reactive oxygen species assay. Panel A shows the first applied gating to separate the population of interest while panel B shows the second applied gating to separate the singlet events from debris and double events. From Panel B, one histogram, panel C, was obtained to provide the median fluorescence intensity at FL1.

# CHAPTER IV

## DISCUSSION AND CONCLUSION

Because of their wide range of applications, phthalates have been the focus of researchers' interests for several decades. [3] The concerns over phthalate use have been raised because phthalates leach out from plastic surfaces into the environment. High concentrations of several types of phthalates such as DEHP have been found in the water and soil. [4] The applications of DEHP in medical devices and food packing put DEHP on the list of the most questionable phthalates. Studies of DEHP continue to be published because each animal species and each internal organ responds differently to the effects of DEHP.

In this study, the hepatotoxic effects of DEHP and its bioactive metabolite MEHP were investigated using various parameters. These parameters include concentration, times of exposure, and study models. Four concentrations of DEHP and MEHP were prepared, which were similar to the levels found in the environment and in medical applications. Two exposure times were tested to see if the toxic effect would express any time-dependent trend. This study involved two study models of two hepatoma cell lines. One of these cell lines, CR-17, has higher levels of an antioxidant called glutathione.

Our results from the cell viability assay showed a dose-dependent significant hepatoxicity of DEHP and MEHP, with MEHP exhibiting higher cytotoxicity. These results are in agreement with previous reports.[7] It was reported that in mammalian and bacterial cell bioassays, MEHP showed more carcinogenic and mutagenic activities than DEHP. MEHP, and not DEHP, showed dose-dependent DNA-damaging effects to *Hay bacillus* bacteria that was evaluated using a Rec-assay. [25] Similarity in the response of the

two hepatocyte cell lines to the toxic effects of DEHP and MEHP in cell viability assays observed in this thesis work suggests that the mechanism of toxicity does not involve oxidative stress. It was expected that CR-17 hepatocytes would be more resistant to cell death via an oxidative stress mechanism due to elevated levels of the antioxidant glutathione.

The ATP assay also indicated that DEHP and MEHP have effects on ATP production, and these effects are most pronounced at 300 µM concentration. Differences in hepatocyte cell line responses were observed in the ATP assay. After the longer exposure to DEHP and MEHP, CR-17 cells, with higher levels of glutathione, were more resistant to the effects of DEHP and MEHP. An explanation for this could be that upregulating GSH synthesis in CR-17 cells assisted in repair processes that helped the cells to recover from the deleterious effects of the phthalates and this upregulation may require a certain dose or time limit to show its effect. MEHP had a greater effect on ATP production than DEHP in both cell lines. The majority of cellular ATP production occurs in the inner mitochondrial membrane through mitochondrial respiration. Thus, measuring the rate of mitochondrial respiration is another way to indirectly observe ATP levels. Studies of isolated rat liver mitochondria exposed to a dose of 1000 µM of DEHP and MEHP, showed that MEHP caused a total loss of mitochondrial respiration while DEHP had no effect on the respiration rate. [26] Total loss of mitochondrial respiration means that MEHP impaired the capability of mitochondria to generate ATP.

The mitochondrial membrane potential assay demonstrated that DEHP and MEHP toxicities did not involve mitochondrial dysfunction leading to ROS production since no significant decrease in mitochondrial membrane potential was observed. In a

previous study, where the effects of DEHP and MEHP were investigated on isolated rat liver mitochondria, it was demonstrated that at a dose of 1000 µM, DEHP had no significant effect on liver mitochondrial activities while MEHP acted as an uncoupler of mitochondrial energy-linked reactions.[26] At the longer time exposure, CR-17 cells exposed to DEHP and MEHP had higher mitochondrial membrane potential values than Hepa-V cells. This trend was also observed with ATP levels, which suggests that glutathione enhanced the abilities of CR-17 hepatocytes to recover from the toxic effects of DEHP and MEHP.

Results from the ROS assay also suggest that the production of reactive oxygen species resulting in oxidative stress was not the mechanism for cell death. At the 24-hour exposure, the antioxidant effects of glutathione in CR-17 cells on ROS production were observed as lower ROS production in the positive control, vehicle control, and untreated cells as compared to Hepa-V cells. This suggests that using glutathione as an antioxidant could be an effective mechanism for preventing cell damage due to ROS. However, our results indicate that no ROS were generated due to phthalate exposure. Previous studies conducted on human lymphoblast cells also stated that glutathione worked as a protecting agent toward the toxicities of DEHP and MEHP. In the human lymphoblast cells study, higher levels of reactive oxygen species were detected when cells were treated with 234 µM of DEHP and 196 µM of MEHP for 48 hours; however, cells with more glutathione showed significantly lower levels of reactive oxygen species.[27]

The ROS assay results also conflict with the results from another study conducted in rat hepatocytes. [28] In the rat hepatocyte study, cells were treated with 200 µM DEHP for 24 hours and then reactive oxygen species were measured by a dichlorofluorescein

assay. An increase in reactive oxygen species was observed leading to induction of oxidative stress as it was reported. Another study conducted in human prostatic adenocarcinoma reached a similar conclusion that the oxidative stress caused by elevation in reactive oxygen species is the mechanism that underlies DEHP and MEHP toxicities. In the human prostatic adenocarcinoma study, cells were treated with 3 mM DEHP and 3 μM MEHP for 24 hours. [29] Therefore, in this study, either the dose of DEHP and MEHP was not enough to induce reactive oxygen species or the mouse hepatocytes respond differently to DEHP and MEHP exposure.

The general metabolism of xenobiotics consists of two phases. Phase I is the hydroxylation of the xenobiotics which is catalyzed by certain enzymes. Phase II is the conjugation of the compounds produced in phase I, in which compounds are conjugated with glucuronic acid, glutathione, sulfate, acetate, or certain amino acids. [30] However, by looking at the metabolic pathway of DEHP shown in Figure 2, the conjugation occurs with glucuronic acid and glutathione does not appear to play a significant role in the metabolism of DEHP, which may explain the trends of the similar responses of the two cell lines where the elevated level of glutathione in CR-17 did not exhibit a significant difference in the detoxification of DEHP.

It is crucial to remember that each species has various sensitivities toward the toxicity of xenobiotics including DEHP [10] and since here we tested mouse liver cells, the results of this research should not be generalized to other animal species or even to other internal mouse organs. To illustrate the differential sensitivity among different internal organs, a previous study compared the effects of DEHP with two other phthalates on testes and livers obtained from the same rats. The results from this comparison indicated

that the liver was more resistant to DEHP than testis since more apoptosis was observed within testis, which explained the reproductive toxicity of DEHP discussed in several other studies. [31] Furthermore, another study adopted the same principle but aimed to highlight differential sensitivities among different species by conducting *in vitro* analysis of the hepatic and intestinal glucuronidation of MEHP among four species, including humans, mice, dogs, and rats using microsomal fractions. [10] Microsomes are small membrane-bound vesicles formed from endoplasmic reticulum when cells undergo disruption. The results from that analysis indicated that amongst the four species, dogs had the highest values for liver microsomes while mice had the highest values for intestinal microsomes. Thus, understanding these differences is the key point if we aim to address the toxicities of these phthalates.

The mechanism behind phthalate toxicity is still a controversial and challenging debate due to the all variables that must be considered with the occurrence of phthalate exposure. Theses variables include time, dose, and route of exposure. Along with all of these variables, differences in the response of different species and organs to the exposure of phthalates demand extra caution in the interpretation of any toxicological assessment. For future research, alternative mechanisms, rather than the use of antioxidants, should be investigated for the detoxification effectiveness of phthalate hepatoxicity. Moreover, following divergent experimental approaches in measuring phthalate toxicity could lead us to the mechanism by which phthalates induce hepatoxicity.

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