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Cytotoxic effects of BPA and DEHP on Human Epithelial Cells

Noah Stickrod

An Analysis of the Cytotoxic effect of BPA and DEHP on Human Epithelial Cells

Noah Stickrod

University of Nebraska at Omaha

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Kristen Johnson, Ph.D.

Faculty Mentor

College of Arts and Sciences

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Abstract

Endocrine disrupting chemicals (EDCs) are substances in the environment, food sources, personal care products, and manufactured products that interfere with the normal function of the body's endocrine system. Most humans are exposed to some amount of these chemicals from many different sources, such as the air we breathe, food we eat, and the water we drink. EDCs can also permeate through the skin. The endocrine system is important because it works with other systems in the body to ensure healthy development and function throughout life. EDCs interfere with the way our body's hormones work. Some EDCs can mimic natural hormones and trick our body, while others can block natural hormones from doing their job. Also, EDCs can upregulate or downregulate levels of hormones in our bodies and can change how sensitive we are to different hormones. For most EDCs, the mechanism in which they function is by binding to steroid hormone receptors such as progesterone receptor and androgen receptor. Disrupting the actions of endogenous hormones may induce abnormal reproduction, stimulation of cancer growth, and dysfunction of the neuronal and immune system.

Research was conducted on the EDCs Bisphenol-A (BPA) and Di(2-ethylhexyl) phthalate (DHEP) to investigate their potential impact on cell stress response, as well as their cytotoxicity levels. Cytotoxicity concentration levels will be investigated through Trypan Blue and Alamar Blue Assay. To examine cell stress and toxic response at the molecular level, RT-qPCR will be used to explore various gene sets in response to EDC treatment below toxic levels. Research on EDCs is still relatively new, there are still questions that have yet to be asked and answers that have yet to be given.

Introduction

Endocrine disrupting chemicals (EDCs) are substances in the environment, food sources, personal care products, and manufactured products that interfere with the normal function of the body's endocrine system. Most humans are exposed to some amount of these chemicals from many different sources, such as the air we breathe, food we eat, and the water we drink. EDCs can also permeate through the skin. The endocrine system is important because it works with other systems in the body to ensure healthy development and function throughout life. EDCs interfere with the way our body's hormones work. Some EDCs can mimic natural hormones and trick our body, while others can block natural hormones from doing their job. Also, EDCs can upregulate or downregulate levels of hormones in our bodies and can change how sensitive we are to different hormones when secreted. For most EDCs, the mechanism in which they function is by binding to steroid hormone receptors such as progesterone receptor and androgen receptor. Disrupting the actions of endogenous hormones may induce abnormal reproduction, stimulation of cancer growth, and dysfunction of the neuronal and immune system.

Research on endocrine disrupting chemicals has gained more attention over the last decade as more data has come out about infertility and declining testosterone levels in men. Let me say that this is all correlation, no causation has been proven unanimously yet, but as more research is conducted it seems that it is leaning towards that way. Research conducted on the cytotoxicity of BPA and DEHP was built on previous studies of BPA and DEHP on differing cell lines. Further interest was developed around the implications of these Endocrine Disrupting Chemicals on the human body in the future. There are many other EDCs in materials used today, as the CDC found 13 phthalate metabolites in the urine of 2,600 people aged 6 years or older in a study conducted in 2004 (CDC, 2023).

Cytotoxicity of BPA

Bisphenol-A (BPA) was first synthesized in the 1890s as a synthetic estrogen, and is currently used as a polymer for polycarbonate and epoxy resins (Son et al., 2018). In the 1950s, scientists discovered that when BPA and carbonyl chloride are combined in a reaction, a clear hard resin, known as polycarbonate. This became widely used in packaging for food and drinks, safety and medical devices, and dental materials (Cimmino et al., 2020). Although the use of plastics in the food industry has allowed us to increase the shelf life of products, there has been a downside to it. Many EDCs like BPA are not bound completely to the plastic, so there is a potential for the chemicals to leach into the food or liquid in the container. In 1936, two biochemists, Edward Dodds and William Lawson, were interested in endocrinology and discovered that BPA has an ability to act like an estrogen agonist in estrogen deficient rats (Dodds & Lawson, 1938). Since there was an inexperience in working with such chemicals and working in a lab on mice, it was decided to shift the direction of the project into the cytotoxic effect of BPA on U2OS cells for this project.

When researchers state that BPA mimics estrogen, it should bring attention to the reader because estrogen (estradiol) plays a key role in many different physiological processes, which include growth, development, and homeostasis. BPA has shown in both *in-vitro* and *in-vivo* data that it can bind estrogen receptors ERα and ERβ. Since BPA acts like estradiol, it can stimulate different cell responses that were not otherwise supposed to happen. Let it be stated that BPAs affinity for the estrogen receptor is lower and its activity is approximately 10,000 to 100,000 times weaker compared to natural hormone 17 beta estradiol (Cimmino et al., 2020). However, that does not mean that BPA cannot have an effect on binding to the estrogen receptor. Binding to estrogen receptors plays a crucial role in the development of tumors. In the BPA-Estrogen

Receptor interactions, there is an increase in proliferation and migration of several ovarian cancer cell lines through a pathway involving Stat3 and ERK1/2 (Ptak & Gregoraszczuk, 2012).

BPA has been shown *in silico* studies, computer simulation studies, to be able to compete with 5α-dihydrotestosterone (DHT) for binding to androgen receptors (Cimmino et al., 2020). The BPA-Androgen receptor pathway has been associated with adverse effects on spermatogenesis, steroidogenesis, and atrophy of the testes. As well as alterations of adult sperm parameters, such as changes in sperm count, motility, and density in experimental animals and humans (Wang et al., 2017). This evidence supports claims the BPA induces several defects in the embryo, during postnatal and pubertal periods, and during adulthood. Researchers believe that this compound affects the hypothalamic-pituitary-testicular function by changing androgen and estrogen synthesis, as well as expression and activity of the respective receptors (Cimmino et al., 2020). The pathway for the production and release of testosterone is a very complicated process. In summary, the hypothalamus will release gonadotropin-releasing hormone (GnRH), which triggers the pituitary gland to release luteinizing hormone (LH). LH will then travel to the tests and stimulate the production and release of testosterone. As one can see, if one step in the developmental pathway of male or females is disrupted, it can cause a cascade effect and disrupt development of gonads and male/female sex characteristics.

Another research group conducted an occupational cohort study to evaluate whether exposure to high levels of BPA affected male sexual functioning (Li et al., 2010). From 2004 to 2008, an occupational cohort study was conducted among workers of manufacturers of BPA and epoxy resin. Epoxy resin was chosen because manufacturers use BPA as one of their raw materials (Li et al., 2010). The study was then given to all the factories participating, which included a personal interview with questions asked regarding demographic characteristics, work

history, medical history, personal behaviors, and sexual activities. Both exposed and unexposed workers performed this task to study health effects of general occupational hazards. The workers were not aware of the specific hypothesis the researchers proposed in relation to BPA. They then conducted spot air sampling for each position in the manufacturing process to determine workplaces with similar exposure (Li et al., 2010). Researchers found that BPA-exposed workers had a significantly higher risk of sexual dysfunction in all of the indices measured. Indices were sexual desire, erectile dysfunction, orgasmic function and overall satisfaction with sex life (Li et al., 2010). Although there are a number of factors that could very well account for these findings, such as stress, age, obesity, it does provide more evidence for a link between exposure to BPA and a reduction in sperm count in the overall population of men.

Cytotoxicity of DEHP

Di(2-ethylhexyl) phthalate (DEHP) is a ester of phthalic acid. It is the most commonly used plasticizer that makes plastic more flexible and elastic (Ito et al., 2019). It is used in various polyvinyl chloride (PVC) products such as plastic sheets, wire coverings, pastes, coating materials, medical products, and adhesive agents. Since it is contained in plastic materials while being chemically bonded, it can easily diffuse into the environment under high temperatures or during contact with hydrophobic materials (Ito et al., 2019). There is a high likelihood that the general population will be exposed to DEHP at some point in their life, given that it can leach easily into the environment and almost everyone is exposed to some sort of plastics on a daily basis. As far as harm the DEHP can cause, it has been shown in animals studies that DEHP exposure induces testicular morphological changes, reproductive tract developmental anomalies, sperm damage, disruption of endocrine hormones, changes in birth sizes of offspring, and anogenital distance (Ito et al., 2019). In Taiwan, child-only or both maternal and child DEHP

metabolite levels were associated with decreased levels of free Testosterone in males (Ito et al., 2019). This is important because free testosterone is what is responsible for creation of secondary sexual characteristics in men, such as facial hair and a deeper voice. Also, it helps male libido, mood, muscle growth, metabolism, and energy regulation. Many endocrinologists believe now that free testosterone can be a better marker for male functioning. Most of the testosterone is bound in albumin and sex hormone binding globulin (SHBG). Only about 1% of testosterone is bioavailable, so if DEHP is affecting free testosterone levels, that is an issue (Shea et al., 2014).

Phthalates such as DEHP have also been shown to possibly have an effect on increasing childhood overweight and obesity. A survey was conducted in China in 2010 where more than 30.43 million children and adolescents aged 7-18 years were either obese or overweight in China. Also, prevalence of obesity or being overweight increased by 7.3 fold in boys and 9.6 fold in girls since 1981 (Xia et al., 2018). It was well established that early childhood and puberty are important periods for the making of adipose (fat) tissue mass and metabolic homeostasis. Exposure to phthalates during this period could have detrimental effects on adipose function and metabolism, which could lead to childhood overweight/obesity (Choi et al., 2014). A unique cohort in the United States reported that exposure to phthalates at 6-8 years of age was positively correlated with increased BMI and weight circumference at 7-13 years of age in girls (Deierlein et al., 2016). The same research group that conducted the study on the 30 million children in China found that increased exposure to phthalate metabolites was correlated with increasing BMI z-score and fat distribution in boys greater than 10 years old (Zhang et al., 2014). Moreover, the researchers conducted another study on 500 children grade 1 to 12, where they analyzed phthalate metabolites in their urine. Results concluded that exposure to phthalates was

significantly associated with childhood overweight/obesity, and may disturb the arginine and proline metabolic pathways (Xia et al., 2018). Furthermore, this study suggested that environmental pollutants like phthalates might also be associated with disruption of carbohydrates and fatty acid metabolism in puberty children (Xia et a., 2018). One limitation with this study was that the sample size was not large enough to confirm their results.

IC_{50} of BPA and DEHP

An IC_{50} is a value that represents the concentration at which a substance exerts half of its maximal inhibitory effect, or in this case, the concentration at which half of the cells are killed. Depending upon the time of the treatment of BPA and DEHP, and the cell lines tested, the IC_{50} can have a wide variation. Some research groups will use smaller concentrations over long periods of time, >48 hours, while others will use higher concentrations over a smaller period of time, <24 hours. In this research project, one of the goals was to try and identify an IC_{50} for BPA and DEHP on human epithelial cell line (U2OS) at an exposure time of 24 hours. Concentrations for treatment were based on previous studies of the drugs with other cell lines. The concentrations for BPA were determined from a previous study done by George & Rupasinghe (2018) where they looked at Bisphenol A and Bisphenol S in human bronchial epithelial cells. The researchers suggest that DNA damage at the cellular level can be induced by a reactive oxygen species (ROS) generation due to increased oxidative stress (George & Rupasinghe, 2018).

Concentrations for Di-ethylhexyl phthalate (DEHP) was determined using a number of different studies on various cell lines. Many of the studies, including Ma et al. (2018) and Giovani et al. (2022) exposed the cells to 48-hour treatment times with concentrations in the low

millimolar range (1-5mM). Since the treatment times for the cells we used for exposure were 24 hours, the concentrations were increased to see if an effect was observed.

Material and Methods

Trypan Blue Assay

One of the fundamental tools in cell biology is the establishment of cellular viability under experimental conditions. For this research project, a Trypan Blue Protocol was performed to evaluate the cytotoxicity of BPA and DEHP on U2OS cells, a human bone osteosarcoma epithelial cell line. A six well plate of U2OS cells of >50% confluency was used for each assay. For stock concentration preparation, 100mg of Bisphenol-A was purchased from Research Products International (Mt. Prospect, IL, USA) at a purity of >99%. The total 100mg of BPA was dissolved in 20 ml of 100% ethanol to make a stock concentration of 2 mM. Working solutions were then prepared to the concentrations of 200 µM, 150 µM, 100 µM, and 50 µM. For stock concentration of DEHP, DEHP was purchased from TCI America (Portland, OR, USA) at a purity of >99%. Four milliliters of DEHP was diluted in 10 ml of DMSO and 6 ml of D10 media to make a final stock concentration volume of 20 ml with a stock concentration of 0.507 M. Working solutions were then prepared to the concentrations of 40 mM, 25 mM, 12.5 mM, and 2.5 mM.

For the 6-well plate treatment, the confluency was examined prior to treatment under an inverted microscope. Then, the 2 ml of D10 media was removed from each well, and treatments were then given to the specified well, with a negative and positive control in addition. The treatments were left in the wells for 24 hours to be incubated at 37ºC. Once incubation was complete, treatments were taken out using a transfer pipet and the cells were washed with 1 ml of

1xPBS (Phosphate Buffer Saline), which was then removed. A 1.5 ml aliquot of 0.4% Trypan blue Dye was centrifuged at maximum speed for 20 seconds to pellet unwanted precipitate. Then, 500 µl of 1xPBS and 100 µl Trypan Blue dye was added to each well and mixed by gently rocking the plate. The dye was allowed to sit for 5 minutes and then viewed under the inverted microscope. At least 100 cells were counted to approximate the percentage viability after treatment. Treatments for both BPA and DEHP were performed in triplicate for more consistent results.

qPCR

For the qPCR protocol, a stock concentration of 1M DEHP was made by diluting 8 ml of DEHP into 12 ml of ethanol. A dilution was then made to a final concentration of 50 mM to treat a T25 flask of U2OS cells for 24 hours. Another flask was used with no treatment for the qPCR later. After 24 hours, the treatment was taken out of the flask and the cells were resuspended with 3 ml of trypsin/media, and then pelted in a 15 ml falcon. The media was discarded from the tube and 350 µl of Buffer RLT Plus was added to the cell pellet and centrifuged for 3 min at maximum speed. The supernatant was removed and transferred to homogenized lysate to a gDNA Eliminator spin column placed in a 2 ml collection tube. This was centrifuged at 10,000 rpm for 30 seconds. The column was discarded and the flow-through was saved. Then, 350 µl of 70% ethanol was added to the flow-through and mixed well by pipetting. Next, 700 µl of the sample was transferred to a RNeasy spin column placed in a 2 ml collection tube. The tube was centrifuged at 10,000 rpm for 15 seconds, with the flow through discarded at the end.

One then added 700 µl of Buffer RW1 to the RNeasy mini spin column in a 2 ml collection tube. The tube was then centrifuged at 10,000 rpm for 15 seconds and the flow through was discarded. Then, 500 µl of Buffer RPE was added to the RNeasy spin column and it

was centrifuged at 10,000 rpm for 15 seconds, with the flow through discarded at the end. The same volume of Buffer RPE was added again but it was centrifuged at 10,000 rpm for 2 minutes. The RNeasy spin column was then placed in a 1.5 ml collection tube, with 30 µl of RNase-free water added directly to the spin column membrane. The collection tube was then centrifuged for 1 minute at 10,000 rpm to elute the RNA. The T25 flask with no treatment followed the same protocol as described above.

Reverse Transcriptase Protocol

All reagents were thawed for the Reverse Transcriptase cDNA protocol except for the Reverse Transcriptase Enzyme. Three RNase-free 0.2 ml PCR tubes were prepared, one with the treated mRNA, untreated mRNA, and a control. For the treated tube, 11 µl of the treated mRNA was added, along with 1 µl oligo dT to total 12 µl. For the untreated tube, 8 µl of untreated mRNA was added along with 1 µl oligo dT and 3 µl of RNase free sterile water to total 12 µl. For the negative control tube, 11 µl of RNase free water was added along with 1 µl oligo dT to total 12 µl. All three tubes were placed in the PCR machine and heated at 65ºC for 5 minutes to remove secondary structures from the RNA. The machine was paused at 4ºC and the tubes were opened up and 4 µl 5x RT Buffer, 1 µl RiboLock RNase Inhibitor, 2 µl 10mM dNTP mix, and 1 µl Mt Revert Aid M-MuLV Reverse Transcriptase was added to the tubes. The Reverse Transcriptase was not added to the negative control reaction. The tubes were closed and run through the following program on the PCR machine, 10 minutes at 25ºC, 30 minutes at 45ºC, 5 minutes at 85ºC, with a hold at 4ºC. The tubes were then stored at -20ºC for future processing.

qPCR Protocol

For the qPCR protocol, materials that were gathered were gloves, pipettes, 1.5 ml centrifuge tubes, tubes for qPCR, cDNA templates, SYBR Green Mix, prepared primers, and PCR grade water. A sample plan was developed to get an understanding of what was going to occur. For diluting the cDNA synthesis reactions, one took 20 µl of cDNA and diluted into 80 µl of PCR grade water to make a 1:5 dilution. 10 µl were taken from this tube and diluted into 90 µl of PCR grade water to make a 1:50 dilution. Subsequent steps such as the previous were taken to make a 1:500 dilution and 1:5,000 dilution. Altogether there were 25 reactions made. To prepare the qPCR reactions, one added 7 µl PCR grade water, 2 µl Template, 1.75 µl Forward Primer Solution, 1.75 µl Reverse Primer Solution, and 12.5 µl SYBR Green Mix. This would total a final volume of 25 µl. The reference gene that was used for qPCR was GAPDH. The other genes used were p53, EGFR, and Casp3. There was a no treatment control tube with 2 µl of PCR grade water added in place of the primer set. There was a no Reverse Transcriptase where one added control cDNA in place of PCR grade water and primers. The qPCR program was performed, with 95ºC for 15 minutes, 95ºC for 15 seconds, 60ºC for 30 seconds, 72ºC for 30 seconds. Steps 2 through for 40 cycles afterwards.

Results

Trypan Blue

From performing the Trypan Blue Assay, one was able to get an idea of the impact that the drug treatments had on the U2OS cells. From looking between Figures 1 and 2, one can see that there is a definite difference in cell morphology between treated and untreated, especially in well 2, which had the highest concentration of DEHP. Also, one can observe that there is a definite dose dependent ratio when looking at the cytotoxicity graph of DEHP (Fig. 8). The

images under the microscope were not as profound in the second and third trial (Fig. 4 & 5). However, that does not rule out the impact the DEHP can have on human cells.

To understand if the solvent that DEHP was dissolved in, DMSO, had any effect on the cells, an experiment was performed with DMSO alone at the concentrations it would be at if DEHP was present (Fig. 3). DMSO was not found to have an effect on the cells at the varied concentrations since the concentrations were below 5%. An exposure of 24 hours at 5% will cause cell death, but concentrations below this will cause minimal harm over 24 hours (De Abreu Costa et al., 2017). As for the BPA treatment, one can see that there is a definite morphological change in the cells, but the Trypan Blue Dye is not as clear for unknown reasons that will be explored later. However, when looking at the cytotoxic graph, one can see that there is a definite dose dependent relationship between BPA and its effect on cells. Moreover, the concentrations used in this experiment had similar results to those used in George & Rupasinghe, 2018, where researchers looked at apoptotic potential of Bisphenol A and Bisphenol S in human bronchial epithelial cells.

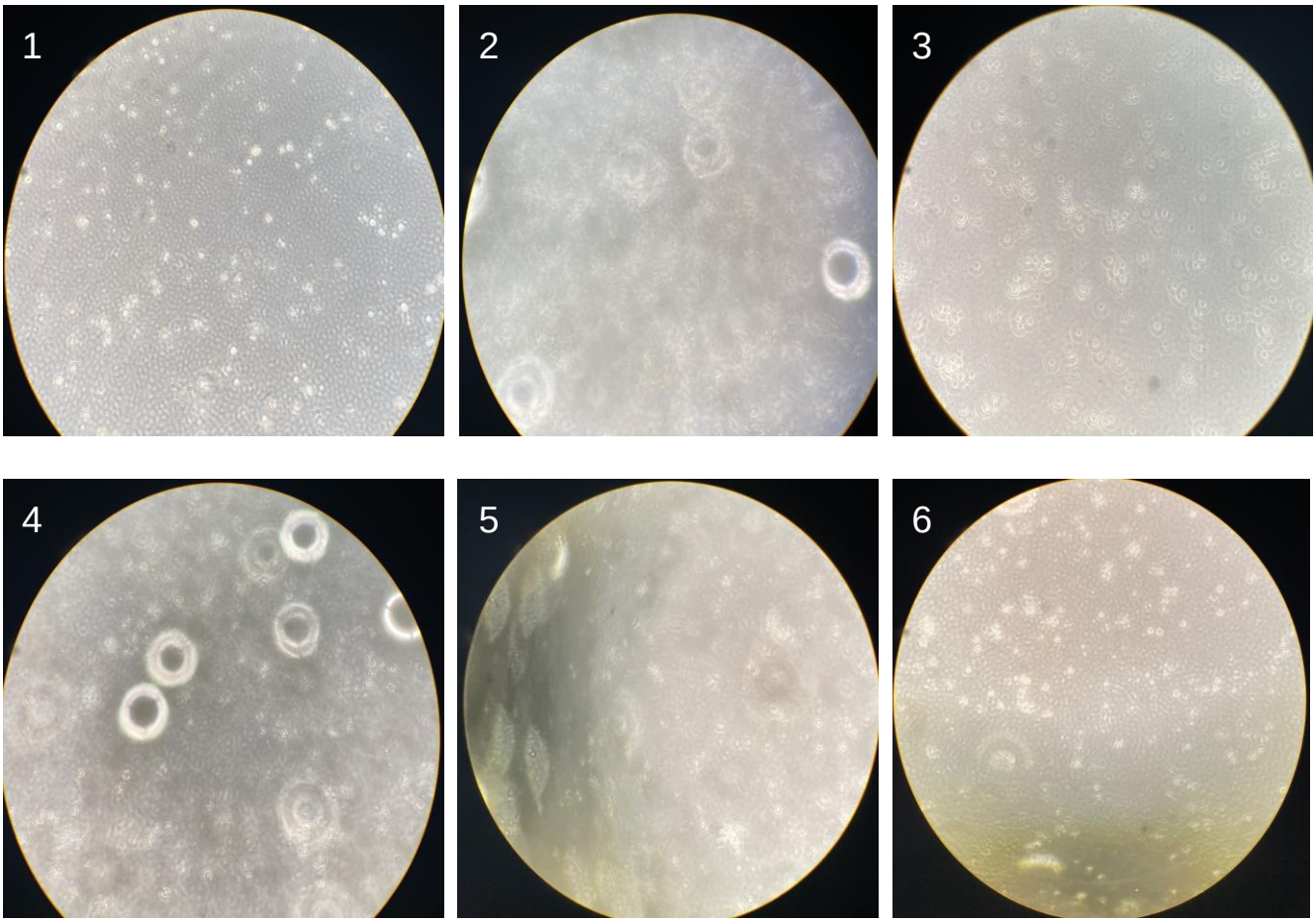


Figure 1: From the top (Left to Right) Wells 1-6 of U2OS cells prior to treatment with DEHP for 24 hours.

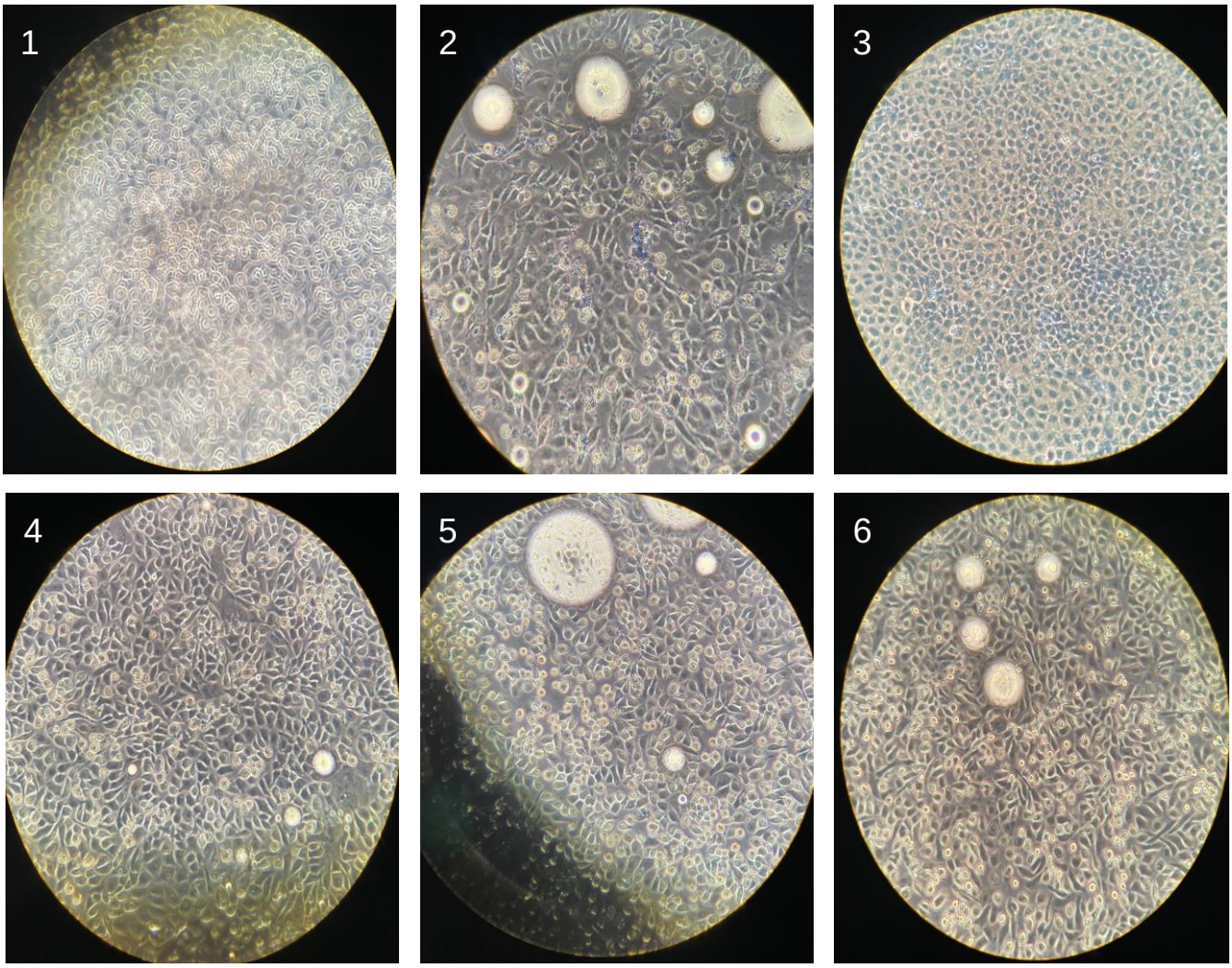


Figure 2: Trypan Blue Assay following 24 h exposure to varying concentration of DEHP. From the top (Left to Right) 1-Negative Control, 2-40 mM DEHP, 3-Positive Control (70% ethanol), 4-25 mM DEHP, 5-12.5 mM DEHP, 6-2.5 mM DEHP.

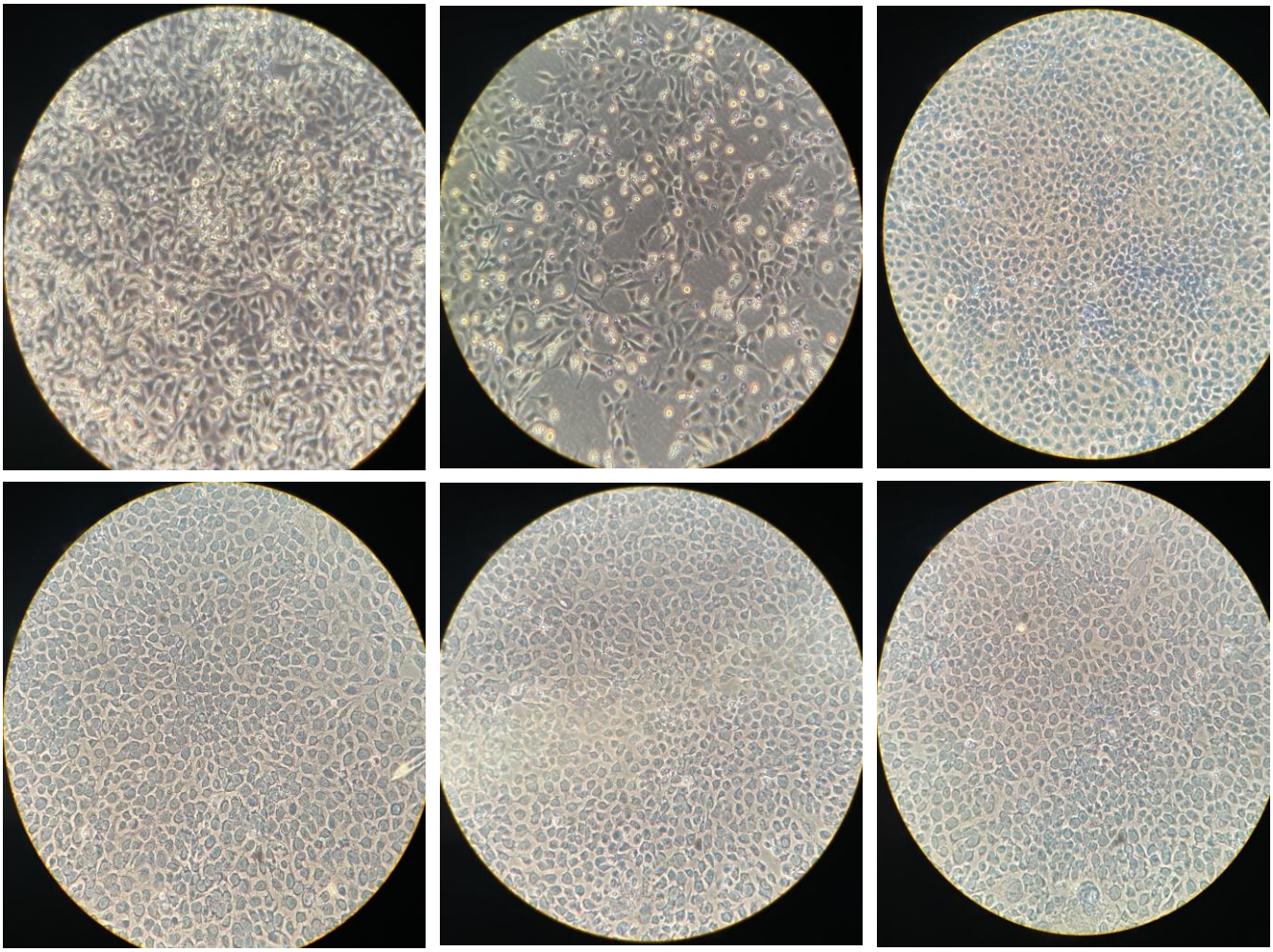


Figure 3: Trypan Blue Assay following 24 h exposure to varying concentration of DMSO without DEHP. (Left to Right) Negative Control, 2.5% DMSO, Positive Control (70% ethanol), 1.25% DMSO, 0.625% DMSO, 0.125% DMSO.

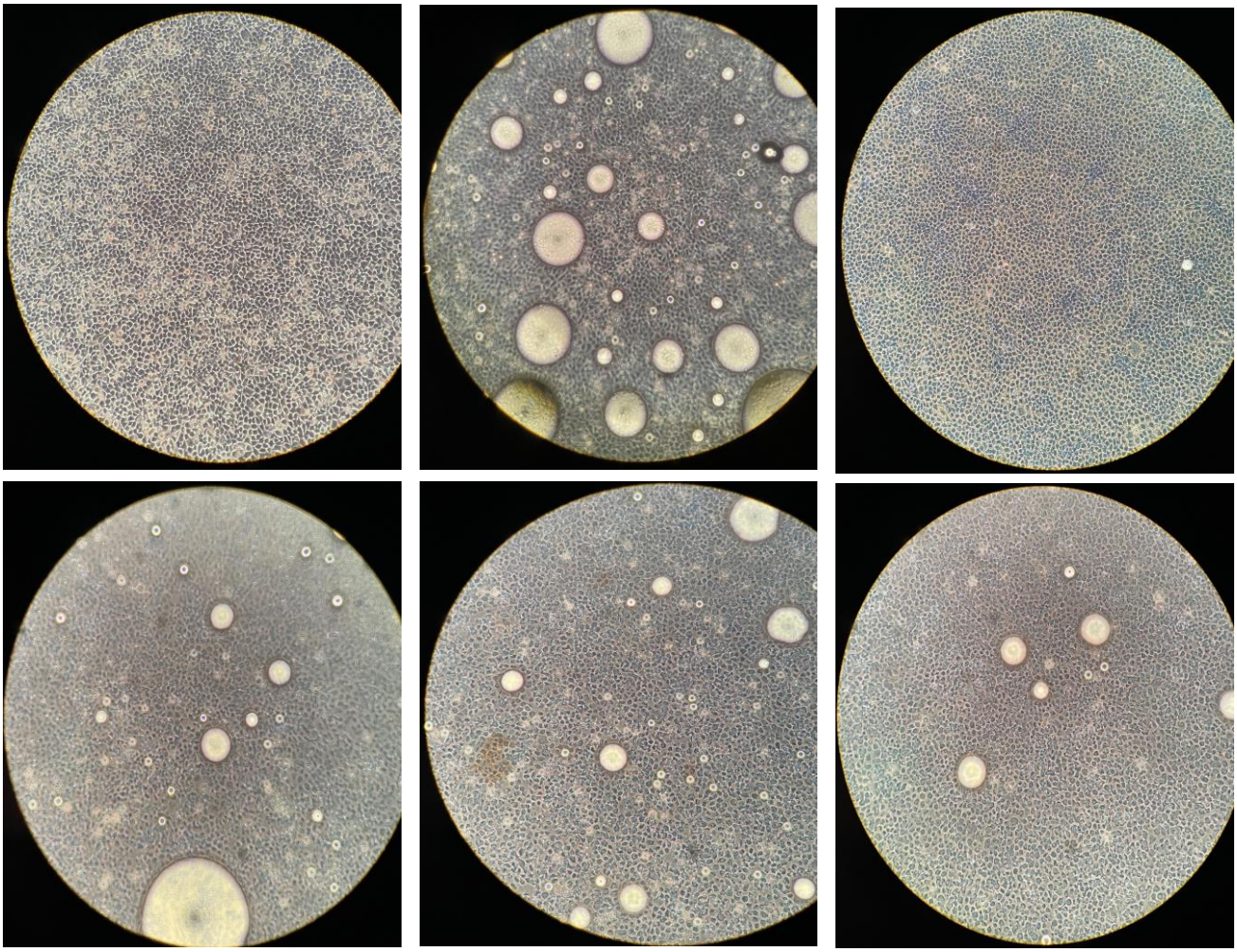


Figure 4: Trypan Blue Assay following 24 h exposure to varying concentration of DEHP. (Left to Right) Negative Control, 40 mM DEHP, Positive Control (70% ethanol), 25 mM DEHP, 12.5 mM DEHP, 2.5 mM DEHP. (2nd trial)

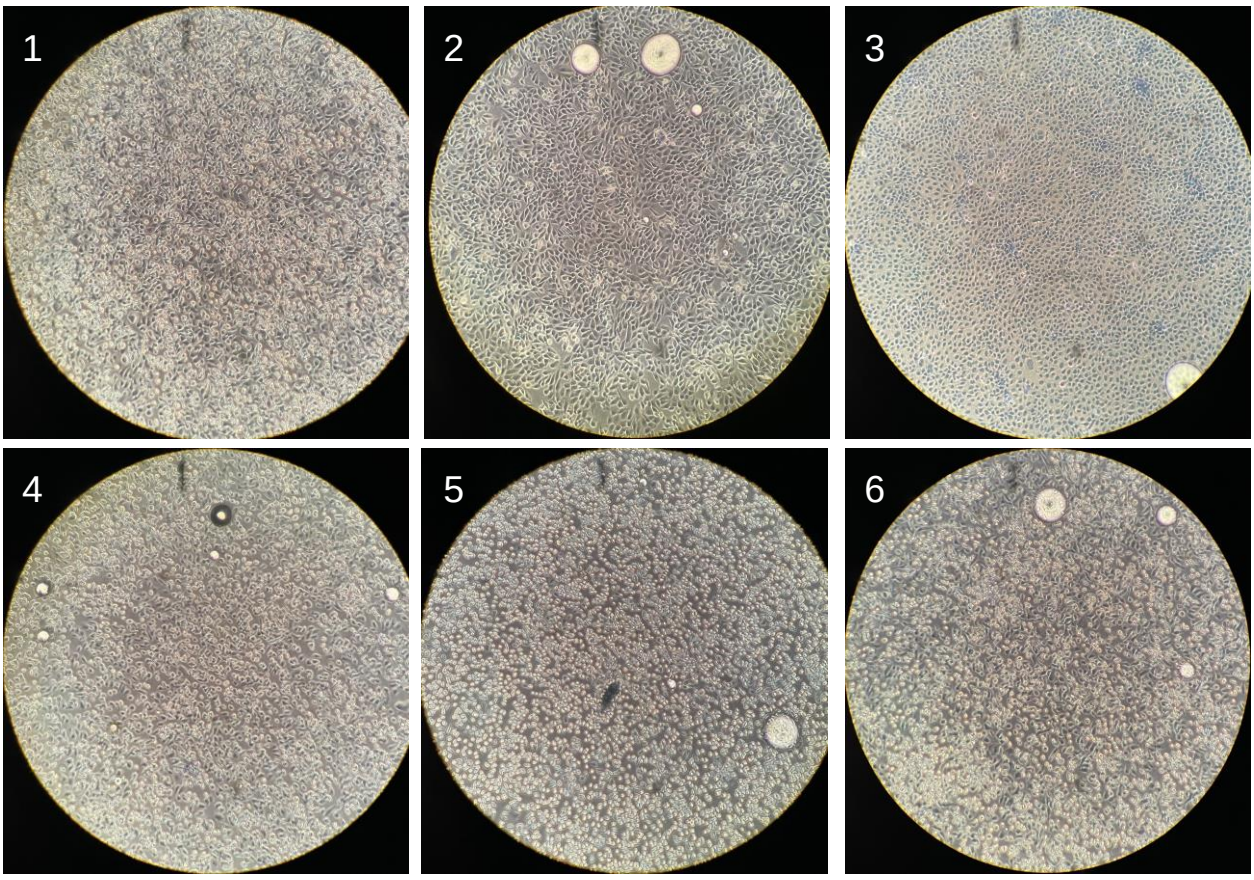


Figure 5: Trypan Blue Assay following 24 h exposure to varying concentration of DEHP. (Left to Right) Negative Control, 40 mM DEHP, Positive Control (70% ethanol), 25 mM DEHP, 12.5 mM DEHP, 2.5 mM DEHP. (3rd trial)

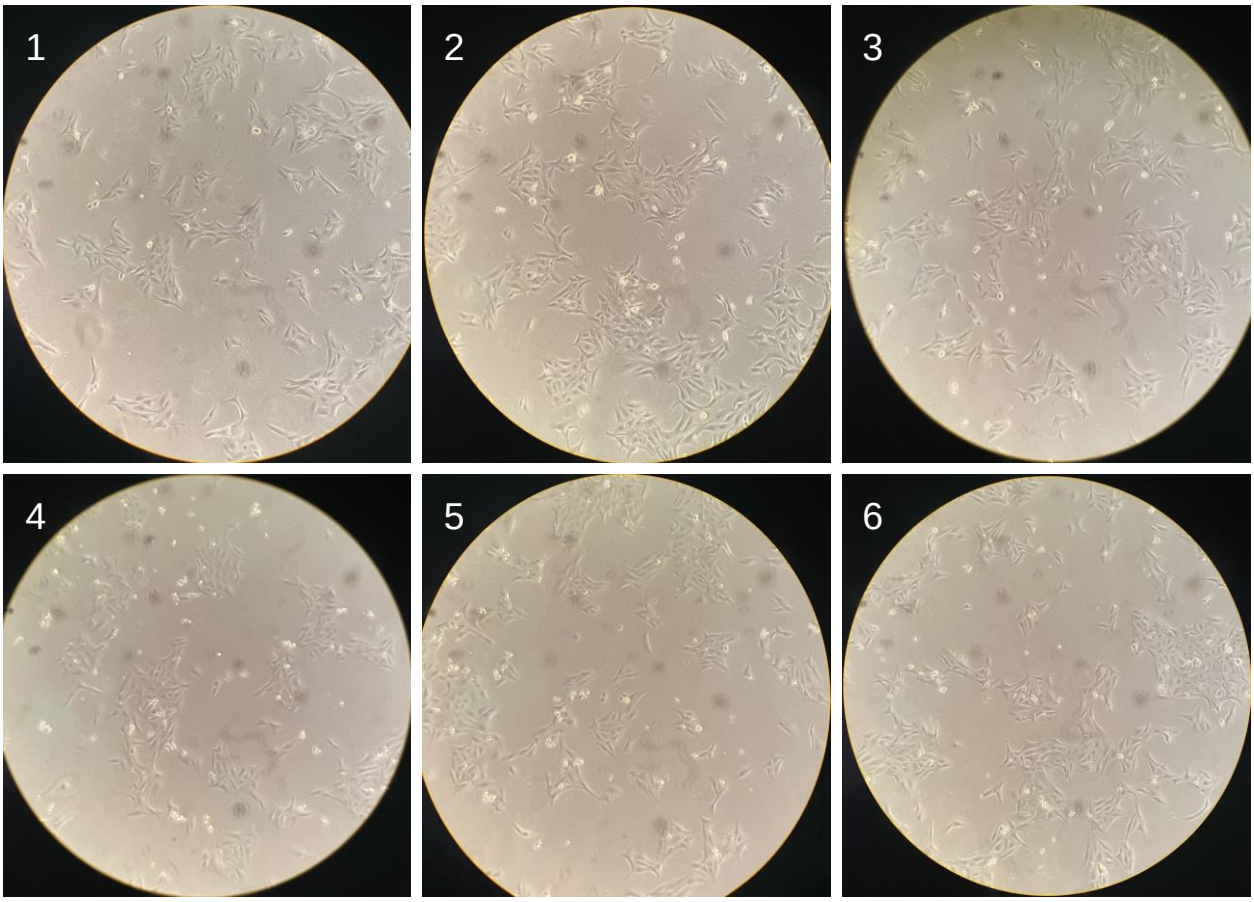


Figure 6: Well 1-6 of U2OS cells before BPA treatment.

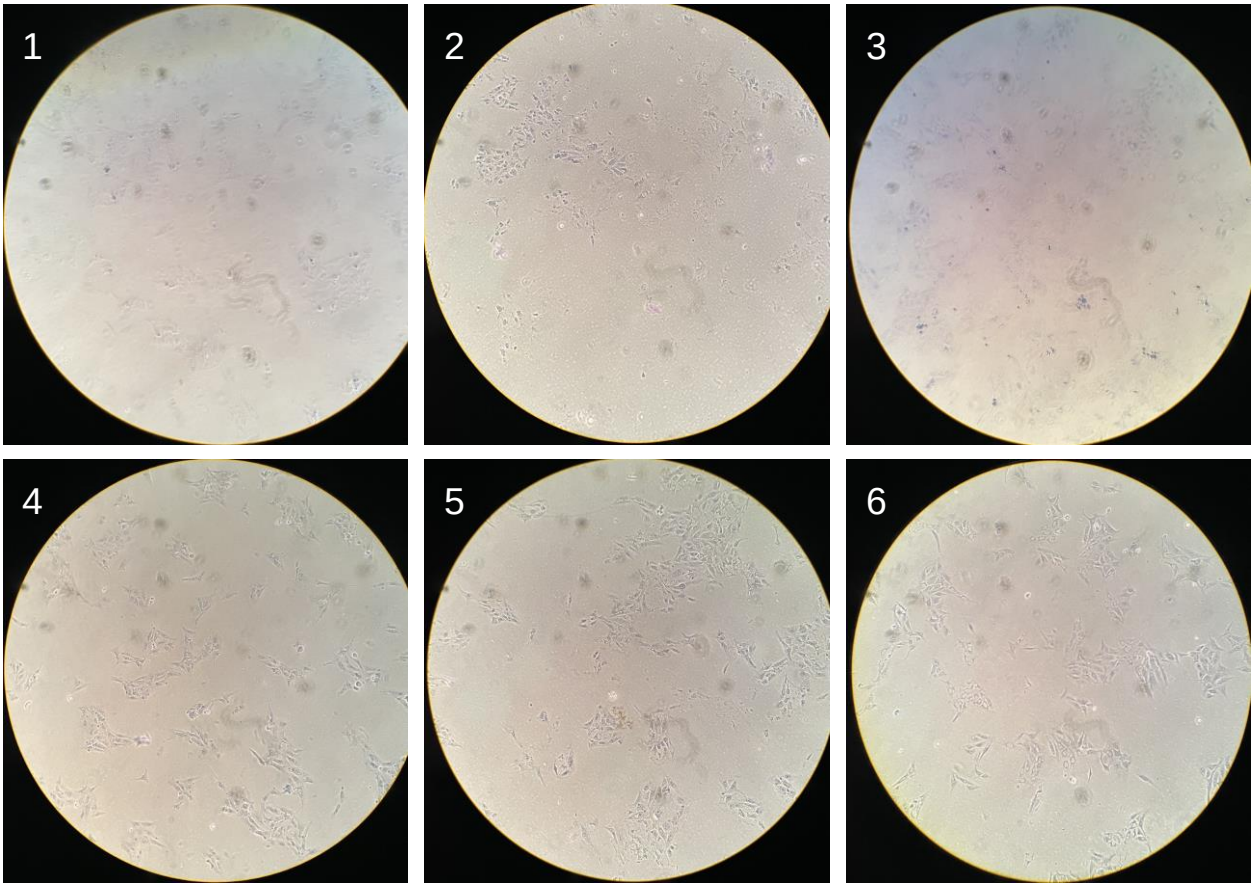


Figure 7: USOS cells treated with varying BPA concentrations for 24 hr. Well 1-Negative Control, Well 2-200 µM, Well 3-Positive Control (70% Ethanol), Well 4-150 µM, Well 5-100 µM, Well 6-50 µM.

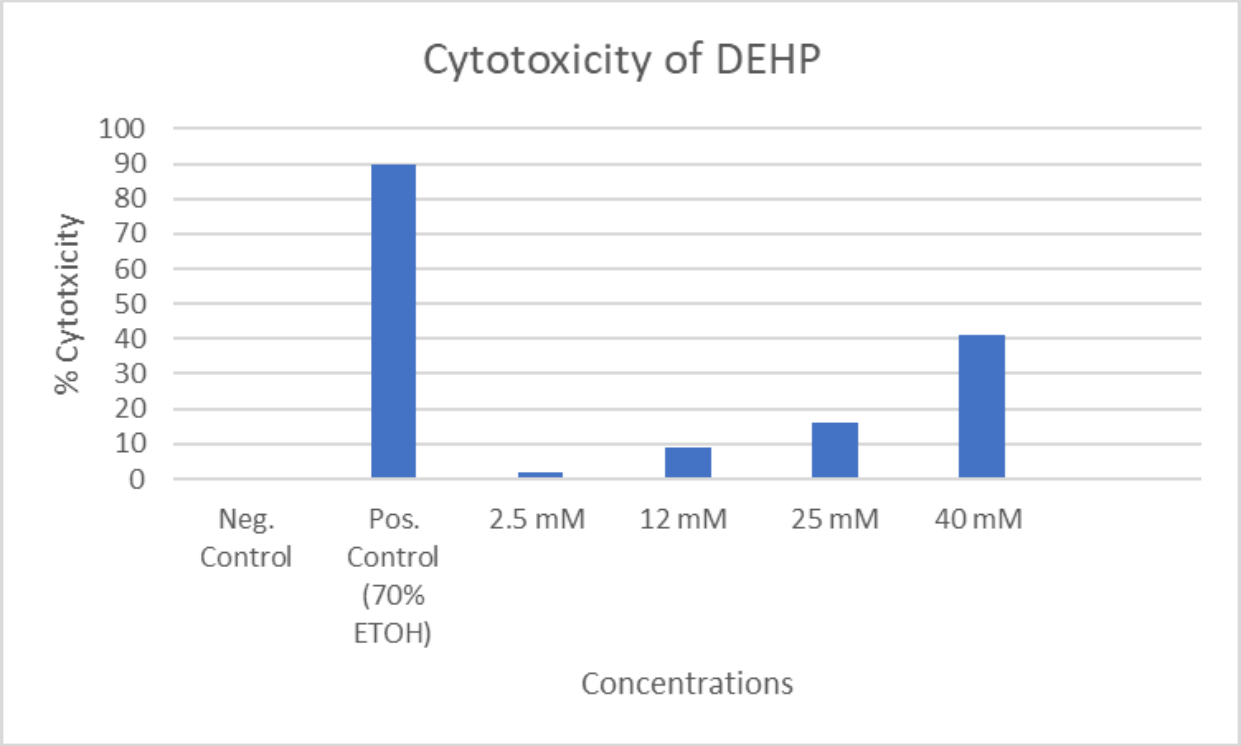


Figure 8: Cytotoxicity graph of DEHP from Trypan Blue Assay.

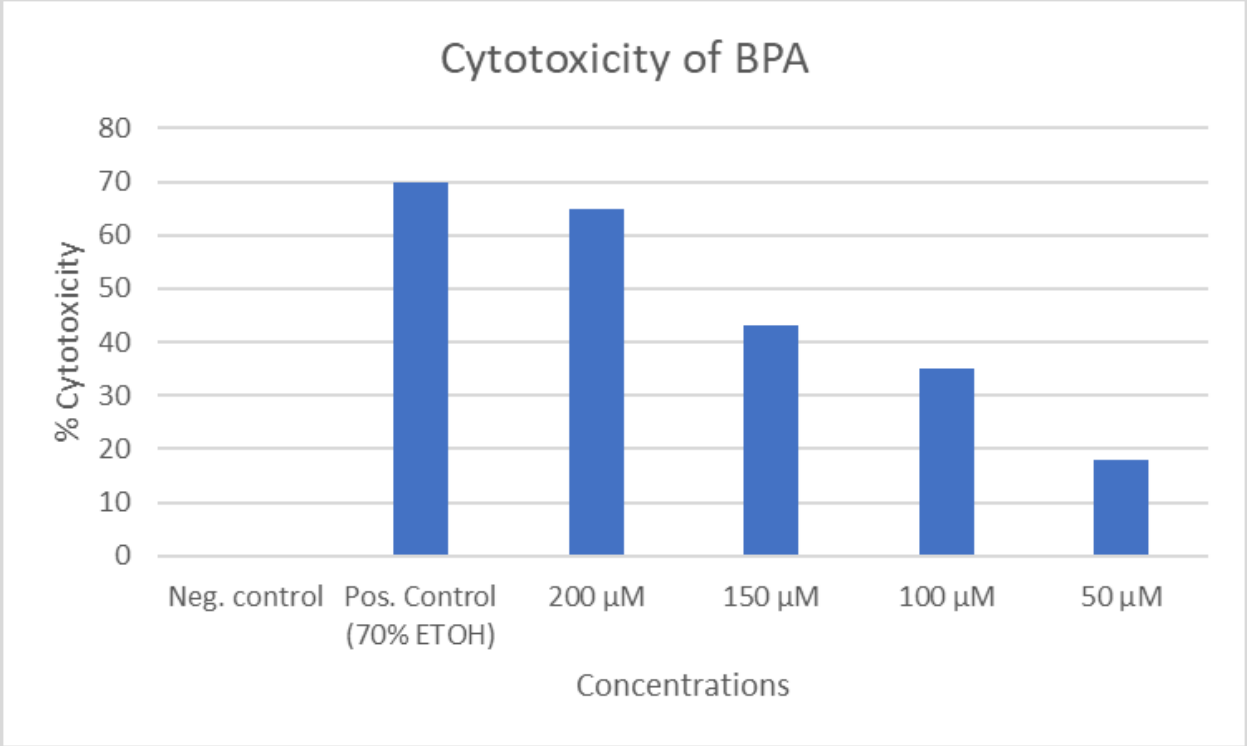


Figure 9: Cytotoxicity graph of BPA from Trypan Blue Assay.

qPCR

Three genes were chosen based on their apoptotic effects on cellular activity if expression is altered. The three genes that were chosen were EGFR, Casp-3, and p53. The Rotor-Gene program took all 25 samples that were made during the lab, and spun them while recording gene expression for all three genes chosen in addition to one "housekeeping" gene (GAPDH) that was used as a marker. QUANT and MELT data were provided through the use of the Rotor-Gene, and each figure can be seen below. There were no C_t values produced for any of the genes that were investigated, except for GAPDH. Therefore, we were not able to determine the effect that DEHP had on gene expression.

Figure qPCR

Table 1: RNA yield from RNeasy Kit

Sample	Nucleic Acid (ng/µl)	260/280
Treated	41.6	2.07
Untreated	139.6	2.17

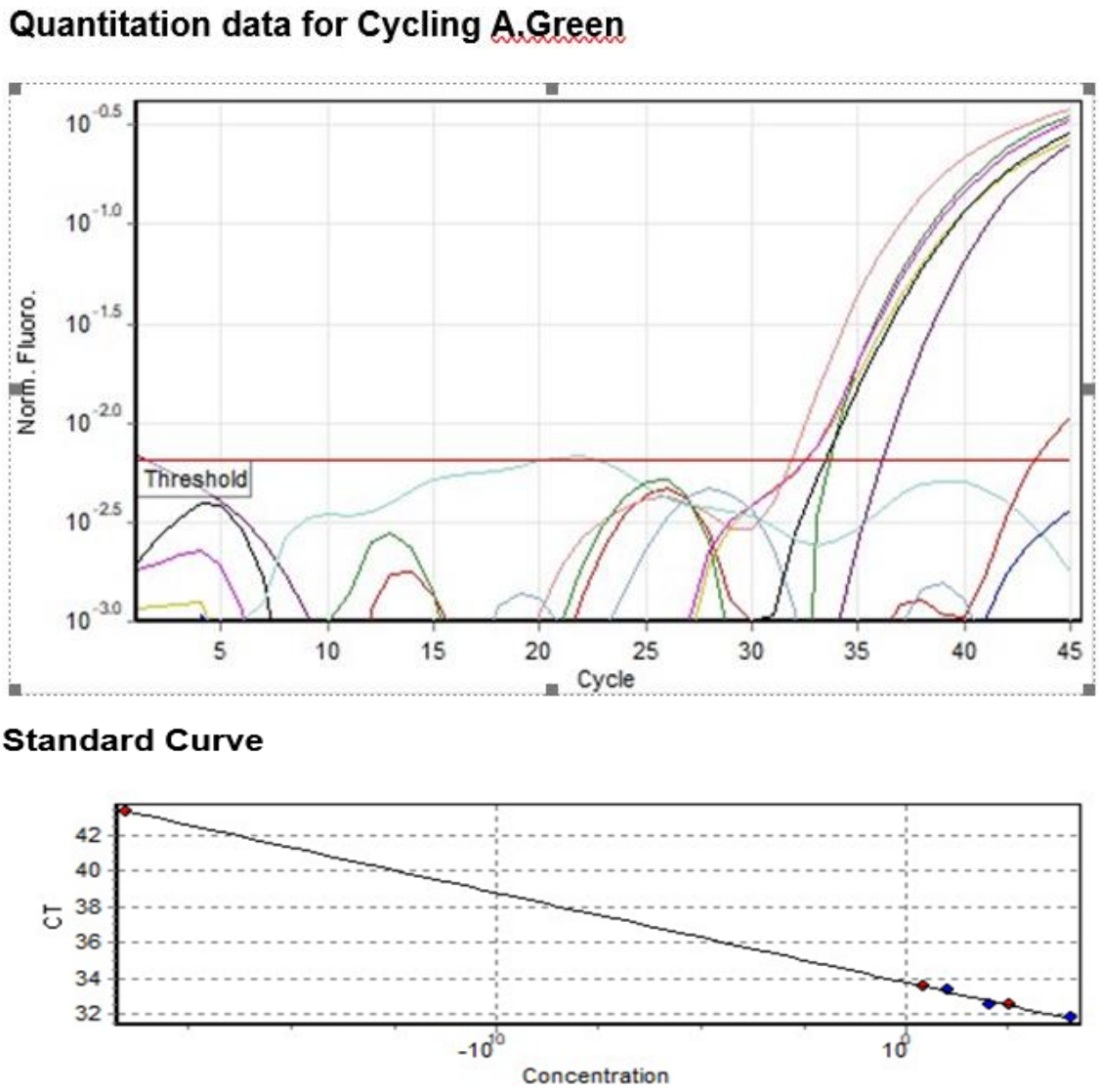


Figure 10: Quantitation data for Cycling Green (GAPDH). The reported efficiency value (E) was 98.04293, while the reported correlation (R) value was 0.97354.

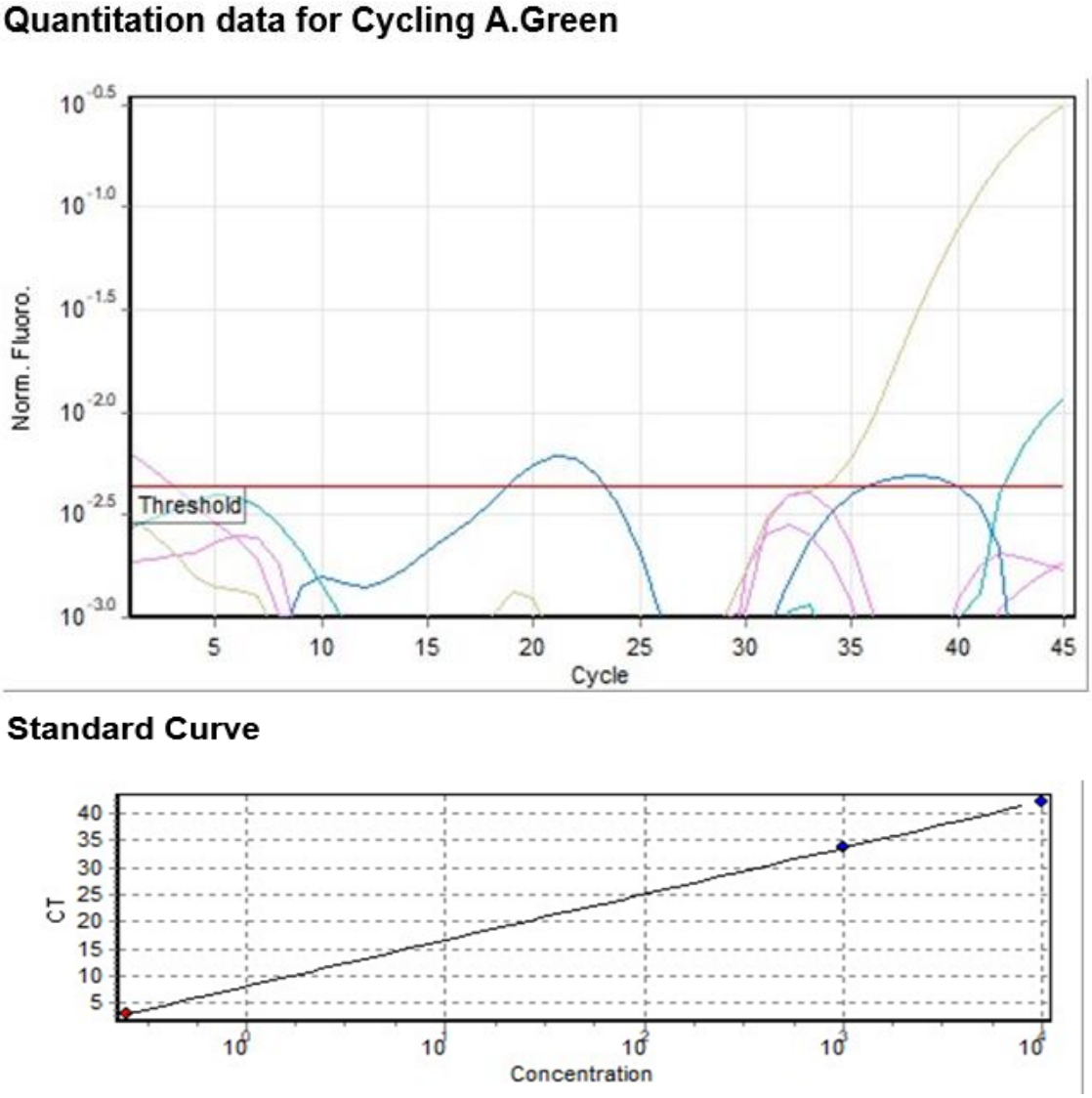


Figure 11: Quantitation data for Cycling Green (p53). The reported efficiency value (E) was -0.23724, while the reported correlation (R) value was 1.000.

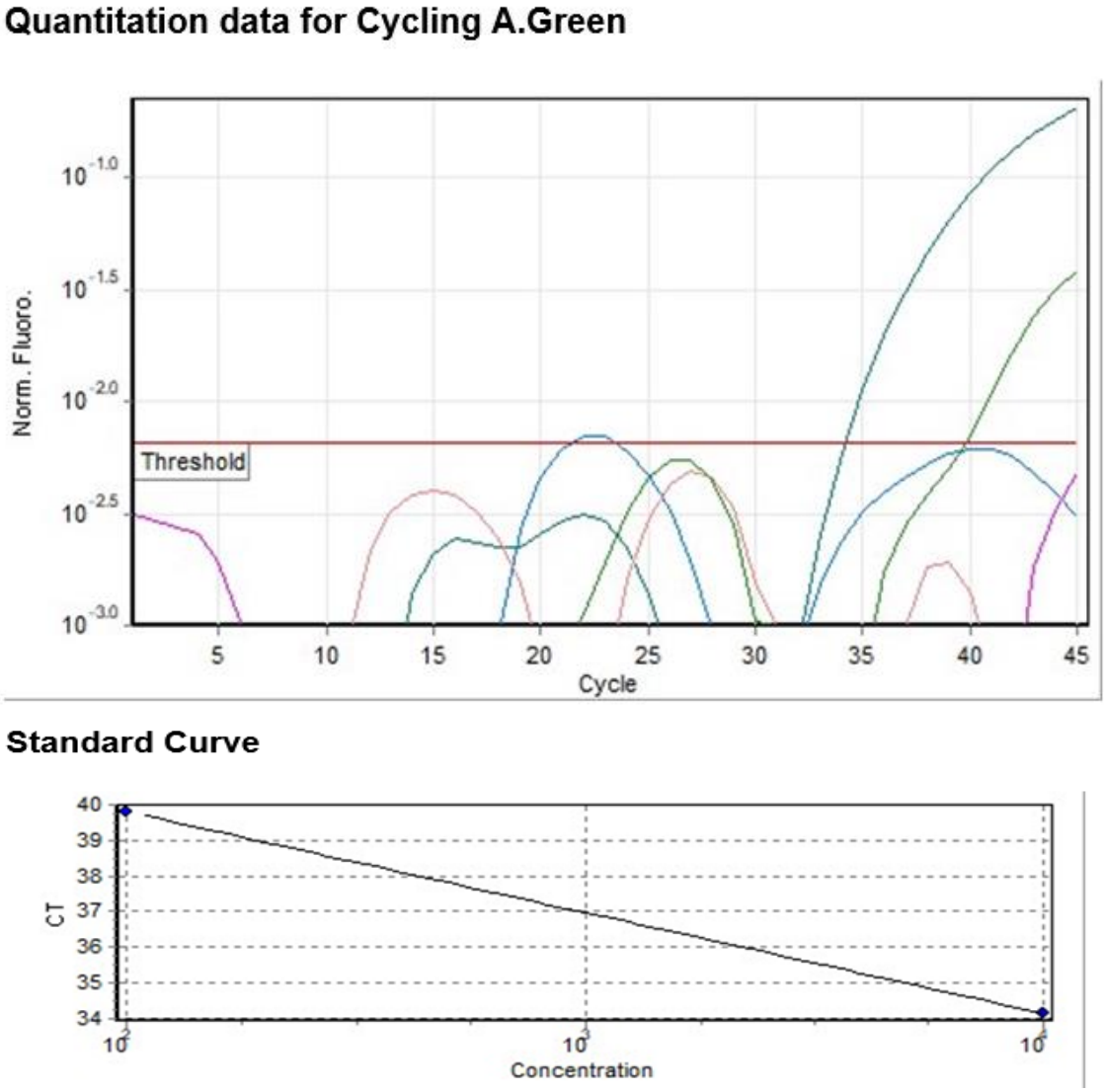
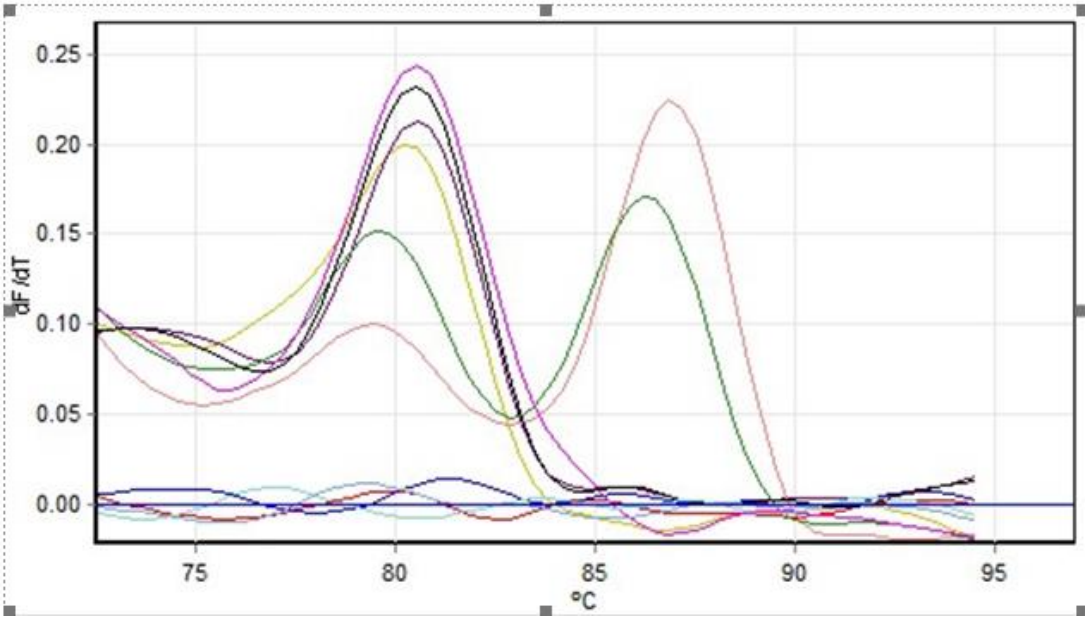
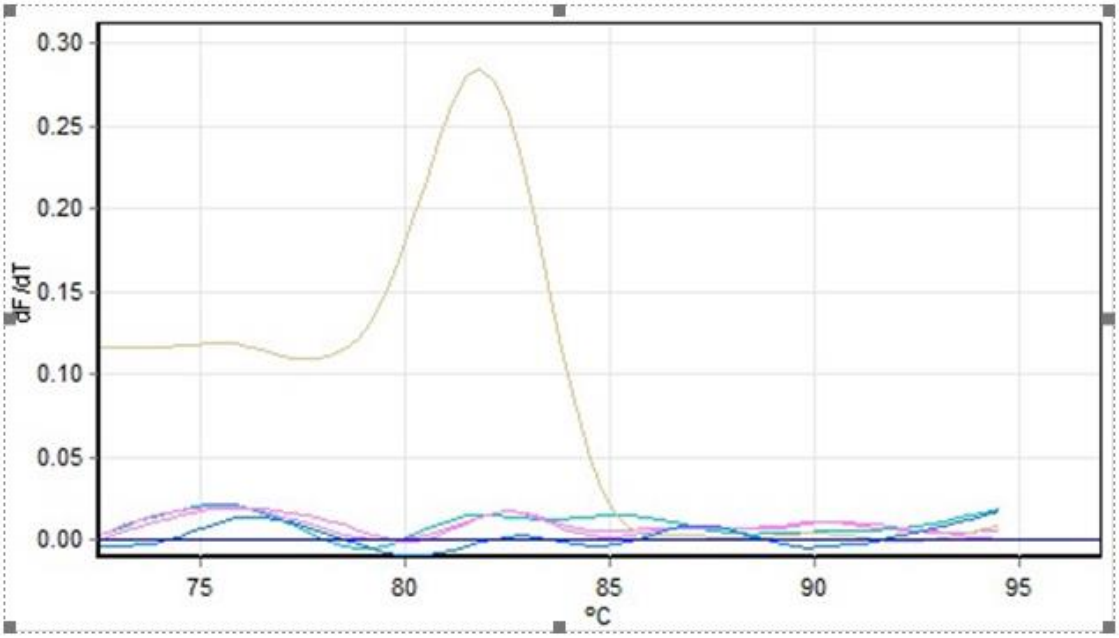


Figure 12: Quantitation data for Cycling Green (Casp-3). The reported efficiency value (E) was 1.25312, while the reported correlation (R) value was 1.000.



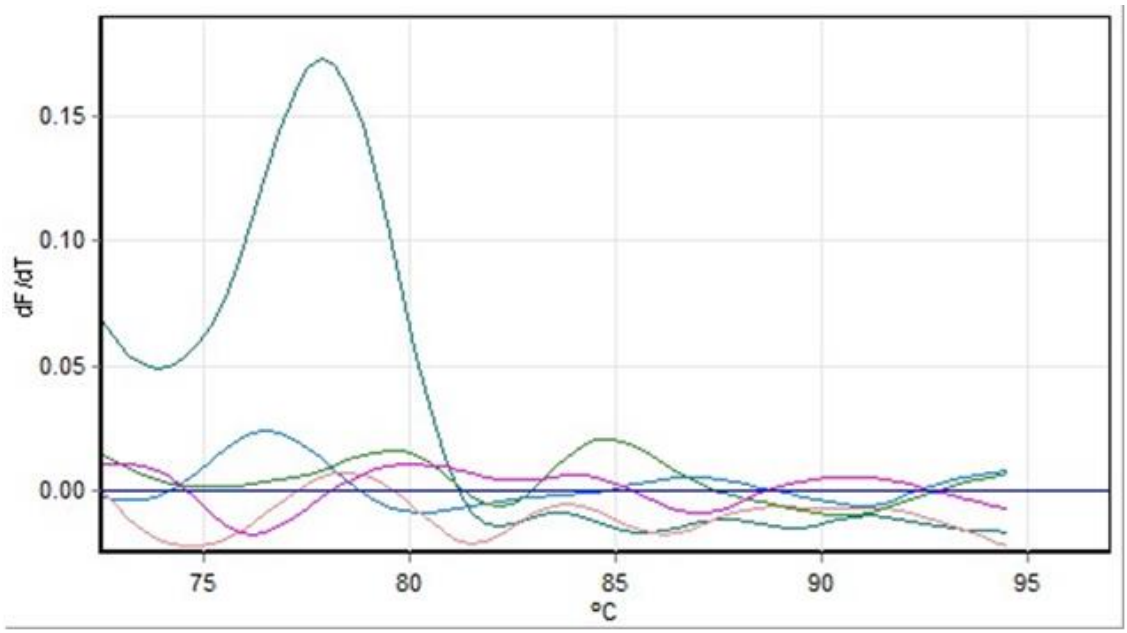
No.	Color	Name	Genotype	Peak 1	Peak 2	Peak 3	Peak 4
1		T-5-GADPH		80.0	84.8	93.2	
2		T-50-GADPH		80.3			
3		T-500-GADPH		74.3	81.3	85.7	89.3
4		T-5000-GADPH		73.5	80.5	85.5	90.3
8		NT-5-GADPH		79.5	86.8		
9		NT-50-GADPH		79.5	86.3		
10		NT-500-GADPH		80.5			
11		NT-5000-GADPH		73.3	80.5	85.7	89.5
47		X		77.0	83.7	87.3	92.0
48		RT		79.2	88.2	91.5	

Figure 13: GAPDH MELT data provided by qPCR.



No.	Color	Name	Genotype	Peak 1	Peak 2	Peak 3	Peak 4
5		T-5-p53		75.7	82.5	87.0	90.2
12		NT-5-p53		75.5	81.8	85.2	
13		NT-50-p53		75.5	81.8	86.5	90.0
37		NT-500-p53		75.5	82.5	87.2	90.5
38		NT-5000-p53		76.3	82.8	87.2	

Figure 14: p53 MELT data provided by qPCR.



No.	Color	Name	Genotype	Peak 1	Peak 2	Peak 3
6		T-5-Casp3		76.5	87.0	
39		NT-5-Casp3		77.8		
40		NT-50-Casp3		78.5		
41		NT-500-Casp3		79.5	84.8	
42		NT-5000-Casp3		80.0	84.0	90.8

Figure 15: Casp3 MELT data provided by qPCR.

Table 2: C_t and ratios

Values	GAPDH	EGFR	Casp-3	p53
Untreated C_T	31.83	-	34.13	42.16
Treated C_T	43.30	-	-	-
Untreated Normalized Expression		-	0.203	.000777
Treated Normalized Expression		-	-	-

Fold Diff.		-	-	-

Discussion

The purpose of this research was to gain a better understanding of potential effects of endocrine disrupting chemicals DEHP and BPA on the human body. Through researching many of the previous studies that have been performed with these chemicals, whether that be *in-vitro* or *in-vivo*, one can see that these chemicals may play some role in the reduction of testosterone in men and reduction in the anogenital distance (Swan et al., 2005). The anogenital distance (AGD) between the center of the anus to the anterior base of the penis. Masculinization of external male genitalia, represented by a longer AGD, is controlled by dihydrotestosterone (Swan et al., 2005). Another phthalate study found that this metabolite of testosterone is decreased by prenatal administration of monobutyl phthalate (MBP), suggesting that it could act as an antiandrogen (Ema and Miyawaki, 2001).

The Trypan Blue Experiment did allow us to gain an understanding of the effects that BPA and DEHP may be having on the cellular level. Under the microscope, we were able to observe a noticeable difference in the morphology of the cells. Although the DEHP produced clumps in Trials 2 and 3, we were still able to observe some changes in the cells. DEHP may have clumped because it is a viscous liquid, similar to vegetable oil, so polarities may have been a factor. Although it is possible to see these changes in the cells anyway since they are being exposed to a higher concentration in a smaller area. However, when looking at the cytotoxicity of BPA in the 6-well plate, one can see that similar results were found in George and Rupasinghe, 2018. It would have been more convincing if we were able to do three trials with BPA, but since there was a limit on time and funding we were only able to do one experiment. Also, we were not able to perform an Alamar Blue assay because of outside factors, i.e. getting

sick and missing the week that it was supposed to be performed. This would have allowed us to potentially get a more accurate IC_{50} of BPA and DEHP.

For the qPCR experiment, outcomes were not as anticipated. Particularly there were no C_t values for the treated cells with the genes that were selected. Thus, this does not allow us to see if there is any real change in the gene expression for the selected genes. This could possibly be because DEHP could possibly inhibit mRNA translation. It was found in a study done by Ling et al., 2016 that DEHP does inhibit mRNA translation *in vitro.* However, it is interesting to point out that the GAPDH in the treated cells did produce a C_t, so there could be some underlying mechanism where GAPDH is not as affected as p53, EGFR, and Casp-3 are. Also, there could have been errors in pipetting since the volumes were below 5 µl. Another reason why we did not see any C_t values for the treated cells could be due to too small of an amount of mRNA to make cDNA in the reverse transcriptase step. To get good results we wanted the mRNA to be around 60 ng/µl at least, but ours was 41.6 ng/µl. Due to lack of remaining time in the schedule for the experiment and dwindling resources, we were not able to reproduce the qPCR.

Overall, this research project broadened my knowledge of the potential effects of Endocrine Disrupting Chemicals (EDCs) on the human body. We were able to get an idea of the cytotoxic effects that these chemicals can have on U2OS cells, and they may have effects on other cell lines, such as germ cell tumors. It would have been interesting to see how the three genes, EGFR, p53, and Casp-3 would have been affected by treatment with DEHP. However, based off of other research conducted we would have hypothesized to see a downregulation in p53 (Dairkee et al., 2013), an upregulation in in EGFR (Sauer et al., 2017), and an upregulation in Casp-3 (Li et al., 2009). If there was more time for this research project, we would have performed an Alamar Blue Assay to gain a better understanding of the IC_{50} of BPA and DEHP.

Also, we would like to perform the qPCR experiment again and try BPA since DEHP has been shown to suppress mRNA translation. I gained a plethora of knowledge into researching techniques and the amount of time and planning that studies take to be performed. However, there are still a lot of unanswered questions relating to phthalates in the environment.

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