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CITOTOXICITY OF DI-ETHYLHEXYL PHTHALATE AND MONO ETHYLHEXYL PHTHALATE ON HUMAN (TK6) LYMPHOBLAST CELLS

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by:
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DEDICATORY

Para mí, es admirable aquel que lucha contra sus propios prejuicios en su intento de ayudar a otros, Gracias Papá.

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ABSTRACT

Phthalates are ubiquitous compounds in the manufacturing industry. Some are known estrogen disrupters; others induce reproductive toxicity and DNA damage. Studies on apoptosis induction and mitochondrial damage on the immune system are limited. This study aims to determine cell viability inhibition and apoptosis induction of diethylhexyl phthalate (DEHP) and monoethylhexyl phthalate (MEHP) on human TK6 lymphoblasts. Mitochondrial membrane permeability, reactive oxygen species (ROS) and activation of caspase were measured. TK-6 cells were cultured on 25cm^2 flasks with modified RPMI culture media with 10% FBS, and incubated at 37°C with 5% CO_2. Concentration that inhibits viability of 50% (IC_{50}) of cells was determined at 48 and 72 hours with a dose range of 10µM to 500µM. Viability was assessed by an automated cell counter. For each assay an ANOVA with a Tukey's HSD Post Hoc Test was also performed. The experimental IC_{50} at 48 hours resulted in 234µM and 196µM for DEHP and MEHP respectively. Both compounds affected mitochondrial membrane potential, promoted ROS generation and activated caspases 3 and 7 with significant difference over the negative control. These findings provide additional information on the adverse effects of phthalates on lymphoblast cells at concentrations found in the environment, specifically their capacity to induce changes on the mitochondrial membrane potential, generation of ROS and activation of caspases, which are hallmark events in the induction of apoptosis.

RESUMEN

Algunos son conocidos como perturbadores endocrinos otros inducen toxicidad en el sistema reproductor y daño al ADN entre otros efectos. Los estudios sobre la inducción de apoptosis y la capacidad de dañar el mitocondria en el sistema inmune de los ftalatos son limitados. Este estudio busca determinar la inhibición de la viabilidad celular y la inducción de apoptosis de di etilhexil ftalato (DEHP) y monoetilhexil ftalato (MEHP) en la línea de células de limfoblastos humanas (TK6). Se evaluaron eventos claves de la apoptosis como la permeabilización de la membrana mitocondrial, generación de especies reactivas de oxigeno y la activación de las caspasas 3 y 7. La concentración que inhibe el 50% de la población celular fue determinada para dos periodos de tiempo 48 y 72 horas exponiendo las células a concentración entre10µM y 500µM. La viabilidad se pudo determinar a través del método de exclusión por Trypan Blue. Los cambios en la permeabilidad de la membrana mitocondrial, la generación de ROS y la activación de las caspasas 3 y 7 se auscultaron para determinar el mecanismo de muerte celular. Para cada uno de los ensayos se trabajó una ANOVA con efectos fijos; en caso de encontrar resultados significativos se realizó un Tukey HSD Post Hoc Test El IC_{50} de 48 horas fue 234µM y 196µM para DEHP y MEHP respectivamente. Ambos compuestos afectaron el potencial de la membrana mitocondrial, promovieron la generación de ROS y la activación de las caspasas 3 y 7. MEHP es mas tóxico, promovió mayor activación de caspasas y producción de ROS. DEHP promueve una leve pero mayor permeabilización de la membrana mitocondrial. Los hallazgos de este estudio sugieren que DEHP y MEHP tienen la capacidad de inducir apoptosis en células del sistema inmune a concentraciones encontradas en el medio ambiente.

CHAPTER I

INTRODUCTION

Problem background

Phthalates are a family of multifunctional organic chemicals (Hauser, Meeker, Park, Silva, & Calafat, 2004) used in the manufacturing industry, as well as the fabrication of personal care products including: nail polishes, hair spray, perfumes, deodorants, and cosmetics, (Koo & Lee, 2004). This family of compounds has also been used in the manufacturing of shampoo and electrical cables, clothing, wall coverings (Wahl, Hong, Hildenbrand, Risler, Luft, & Liebich, 2004), medical devices, (Wahl et al., 2004; Hauseret al., 2004) toys, and in polyvinyl chloride (PVC) materials (Wahl et al., 2004). These compounds are easily released into the environment; because of this, phthalate residues can be found indoors (Nakamiya, Takagi, Nakayama, Ito, Tsuruga, Edmonds, & Morita, 2005), in urine samples (Silva, Samandar, Preau Jr, Reidy, Needham, & Calafat, 2007), human plasma (Cobellis, Latini, De Felice, Razzi, Paris, Ruggieri, Mazzeo, & Petraglia, 2003) and foods (Jen & Liu, 2006).

In Puerto Rico, several phthalates have been detected in soil and underground water systems including the superfund site at Guayama, (U.S. Environmental Protection Agency EPA, 2002) and can possibly be present in numerous sites around the whole island such as landfills, which invariably contain discarded materials made with several types of phthalates that can be washed out and released into the environment. As reported in several studies, Di-ethylhexyl phthalate (DEHP) has been detected in superficial water samples at concentrations ranging between 0.19 to 0.88μg/L (Garrido,

Barco, López, Martínez, & Romero-González, 2009) in sewages and leachates at levels up to 122µg/L studied by Marttinen, Kettunen, and Rintala (2003).

Most of the studies conducted with phthalates have reported on their physical and chemical characteristics, their environmental release, sources and rate of exposure, and absorption in humans and animals. Several studies have focused on the health effects including damage to the reproductive and endocrine system or their capacity to induce developmental abnormalities, or even cancer (Kicheva, Richter, & Popova, 2004) with infants as the most vulnerable group (Sathyanarayana, Karr, Lozano, Brown, Calafat, Liu, and Swan, 2008).

Study problem

The selected phthalates for this study are Di-ethylhexyl phthalate (DEHP), one of the most abundant, and most commonly used plasticizers (Cobellis et al., 2003), and its main metabolite Mono ethylhexyl phthalate (MEHP) (Cobellis et al., 2003; Nakamiya, Takagi, Nakayama, Ito, Tsuruga, Edmonds, & Morita, 2005) (Figure 1). Both compounds have caused concern among scientific groups and governmental agencies because there is limited confirmative information available regarding the possible health impact on humans (Chou & Wright, 2006) or the environment.

Study justification

The presence of phthalates on personal or health care products and their release into the environment and eventual absorption of DEHP and MEHP in humans has been documented. Since DEHP is a ubiquitous environmental contaminant (Cobellis et al., 2003), humans live constantly exposed to phthalates during normal activities. Human exposure to these compounds, their possible health risks and the mechanism of action of DEHP and MEHP are a cause of concern in the scientific community (Fabja, Hulzebos, Mennes, & Piersma, 2006).

Both DEHP and MEHP have been found in human tissue samples such as blood and the umbilical cord. Some researchers have indicated a possible relationship between exposure to phthalates and some diseases, conditions and potentially adverse effects to human health such as infertility based on animal models (Cobellis et al., 2003). Research on molecular effects of DEHP has reported up and down the regulation of genes as well as an androgen antagonist (Hokanson, Hanneman, Hennessey, Donnelly, McDonald, Chowdhary, & Busbee, 2006).

Limited information, however, has been reported on the effect of phthalates on the immune system. Studying the exposure of human lymphoblast cells to DEHP and MEHP can bring new aspects to the analysis of the toxicity of these compounds. Cells from the immune system such as lymphoblasts provide the body with a vital defense mechanism.

This research will contribute to better understand the effects of DEHP and MEHP on the immune system and their potential impact on human health.

Hypothesis

- The viability of TK6 human lymphoblast cells can be affected when exposed to Di-ethylhexyl phthalate (DEHP).
- The viability of TK6 human lymphoblast cells can be affected when exposed to MEHP, a DEHP metabolite..

Goal

The goal of this study is to determine the concentrations of DEHP and MEHP that affect the viability of TK6 lymphoblast cells and determine if key steps on apoptosis (programmed cell death pathway) are activated after exposure to DEHP and MEHP.

Objectives

1. To determine the IC_{50} (viability inhibition of 50 % of the treated cells) of DEHP and MEHP on human lymphoblast cells (TK6) when exposed for 48 and 72 hours.

2. To determine if the exposure of the TK6 human lymphoblast cells to DEHP and MEHP at the IC_{50} concentrations for 48 hours can induce mitochondrial membrane permeability.

3. To determine if the exposure of the TK6 human lymphoblast cells to DEHP and MEHP at the IC_{50} concentrations for 48 hours can induce an increase in the generation of reactive oxygen species (ROS).

4. To determine if the exposure of the TK6 human lymphoblast cells to DEHP and MEHP at the IC_{50} concentrations for 48 hours can induce the activation of the caspases 3 and 7.

CHAPTER II

LITERATURE REVIEW

Background

The manufacturing industry has experienced a great transformation through the last few decades. During the twentieth century, one of the greatest changes of the manufacturing industry was the employment of lightweight materials like plastics, which have caused an increase in the use of compounds such as the phthalates. Phthalates are multifunctional plasticizers with great economical importance for their high industrial utility (Wichert, Andrade, Talsness, Grote, & Chahoud, 2006). The first reported industrial use of phthalates dates from the 1930's (Chou & Wright, 2006) and their use in the manufacturing industry has increased dramatically during the course of the last century. Since the 1970's, studies related to phthalates have been developed with the goal of understanding and clarifying aspects regarding the adverse effects on human health and the environment. Several latent repercussions due to phthalate exposure have been determined partially through research with animals, tissue culture and through some public health research on humans. Some of the effects observed are hepatocarcinogenesis, tumors, DNA mutations, embryo development alterations, reproductive toxicity, and male fertility diminution. Human exposure to phthalates is constant through oral, epidermal and respiratory routes. The excretion of these compounds or their metabolites in inferior vertebrate organisms and humans has been documented with di-ethylhexyl phthalate (DEHP) and mono-ethylhexyl phthalate (MEHP). Human exposure to some of the phthalate compounds, specifically DEHP, has

been related with certain health conditions such as asthma, allergies, rhinitis and eczema in children (Chou & Wright, 2006), fertility reduction, shortened gestation age (Chou & Wright, 2006) and the alteration of gene expression in human cells (Hokanson et al., 2006).

Theoretical approach

Phthalates are organic compounds that impart flexibility and durability to poly vinyl chloride (PVC) acting as intermolecular lubricants (Chou & Wright, 2006). The basic structure of these compounds is phthalic acid, also known as 1,2-benzenedicarboxylic acid, derived from the phthalates dialkyl or alkyl aryl esters (Kato, Silva, Needham, & Calafat, 2005). Although there are many phthalate compounds that exist, the focus of this study will be on Di-ethylhexyl phthalate (DEHP), the most used phthalate compound in the manufacturing industry and one of its principal metabolites, Mono ethylhexyl phthalate (MEHP).

A. Phthalates:

DEHP is a stable, hydrophobic low volatility liquid, with a molecular formula of $C_{24}H_{38}O_4$. Its metabolism has been under study for some years and several studies have indicated that DEHP is hydrolyzed inside the mitochondria and MEHP becomes the principal metabolite and 2-ethylhexanol (Carter, Roll, & Petersen, 1974) (Figure 1). Phthalate 2-ethylhexanol undergoes a series of oxidations as a result of the body's capacity to bio transform the compounds into a more hydrophilic molecule.

Mono ethylhexyl phthalate (MEHP), also known as 1,2-Benzenedicarboxylic acid with molecular formula $C_{16}H_{22}O_4$, is a stable colorless to cloudy viscous liquid. The

material safety data sheet (MSDS) of MEHP lists its potential health effects as irritation to the eyes, skin, mucous membrane and upper respiratory system.

DEHP and MEHP metabolism and toxicity to humans remain incompletely characterized and controversial (Shea, 2003), especially as possible contributors to respiratory conditions, stimulants of premature breast development or as anti estrogenic agents (Gustaf, Sundell, Weschler, Sigsgnard, Lundgren, Hasselgren, & Hägerhed-Engman, 2004; Colón, Caro, Bourdony, & Rosario, 2000). More research on their cellular damage and human health effects are needed and there are several research approaches that can be applied. The *in vitro* cell culture approach is one alternative used to accomplish a more complete understanding of their toxicity through cells in culture exposed to DEHP and MEHP. *In vitro* cytotoxicity studies with lymphoblasts can provide additional information on the mechanism of action of this family of compounds. Understanding if these compounds can be harmful to the human immune system at the cellular level is of outmost importance.

B. Immune System:

When foreign substances known as antigens make their way inside an organism, a group of tissues and cells is responsible for the protection of the organism against any threat or exogenous substance. This group of tissues and cells is part of the lymphatic and immune system; it is a defense system that can perform several kinds of responses based on the nature of the foreign substance or organism (antigen). The immune system is divided into two general types; the acquired or adaptive immune system and the innate immune system. The adaptive is the arm of the immune system that specifically responds to an antigen with a dynamic antigen pathogen recognition system. However, the innate

is not specific in nature and uses either passive barriers or receptors that recognize conserved microbial molecules (Lippolis, 2008) providing the first line of defense against pathogens.

Lymphocytes are blood cells employed by the immune system. The blood cells are divided into two groups: erythrocytes or red cells and leukocytes. The leukocytes or white blood cells are the main functional cells of the lymphatic or immune system (Martini, 2006; Ross, Kaye, & Pawlina, 2003). The white blood cells are classified or separated into two major groups: the granulocytes and the agranulocytes, based on the content or absence of specific granules. Granulocytes are categorized into three types of cells: the neutrophils, the eosinophils and the basophils. The agranulocytes lack specific granules and are composed of monocytes and lymphocytes.

Granulocytes are involved in the innate immunity response also known as the nonspecific defense. From the granulocyte population, neutrophils are among the first cells to enter the area of damaged tissue. They recognize, attach and phagocyte antigens and secrete interleukin-1 (IL-1), a fever inducing agent (pyrogen). The eosinophils moderate the potentially deleterious effects of inflammatory vasoactive agents in sites of allergic reactions and in phagocytosis of antigen-antibody complexes. The basophils are a small group of white blood cells involved with severe vascular disturbances associated with the hypersensitivity and anaphylaxis (Rosset et al., 2003).

The monocytes are agranulocyte cells that differentiate into various phagocytes playing a role in both the innate and adaptive immune response. Meanwhile the lymphocytes are a very important factor of the specific immune response. The types of lymphocytes are Bone Marrow derived cells (B cells or B lymphocytes), Natural Killer

cells (NK cells, NK lymphocytes or Large Granular Lymphocytes), and the Thymus-dependent cells (T cells or T lymphocytes). The lymphocyte types are differentiated by their function and by proteins in their plasma membrane surface.

B-lymphocytes are involved in the production and secretion of some circulating antibodies known as immunoglobulins (immune proteins associated with the humoral immunity or antibody-mediated immunity). These B cells express in their membrane the immunoglobulins IgD (binds antigens in the extracellular fluid), IgM (is secreted after an antigen arrives and is recognized) and the Major Histocompatibility Complex II (MHC II), integral membrane component proteins (glycoproteins), also known as human leukocyte antigens (HLAs) that are present in the membrane of antigen-presenting cells (APCs), cells responsible for activating T cell defenses, such as those found in lymphocytes. The B cells have present in their plasma membrane surface specific clusters of differentiation (CD) markers CD9, CD19, CD24 and CD20 (Ross et al., 2003).

The natural killer (NK) cells have the specific markers CD16, CD56, and CD94 (Ross et al., 2003). NK cells can recognize transformed cells and are able to destroy virus-infected cells and tumor cells.

The Thymus-dependent cells (T cells) represent the major population of lymphocytes in the body, the smallest in size, and are involved in cell-mediated immunity also known as the collective response of T cells. T lymphocyte cells are key players of the adaptive cellular immune responses (Andersen, Schrama, Straten, & Becker, 2006). They develop and differentiate (lymphopoiesis) in the thymus, a grainy organ located in the mediastinum, in the bone marrow and the peripheral lymphoid tissues (Martini, 2006). The T cells contain specific membrane markers, the clusters of differentiation

CD2, CD3, CD7 and the T cells receptors (TCR) (Ross et al., 2003). There are three major varieties of T cells (Thymocytes) which are the Cytotoxic T cells, the Helper T cells, and the Suppressor T cells (Martini, 2006; Ross et al., 2003), identifiable on the basis of the presence or absence of the markers CD4 and CD8 (Ross et al., 2003). The ones that express CD4 are known as the Helper $CD4^+$ T lymphocytes and those that express CD8 are the Cytotoxic. The Suppressor T cells are also known as $CD8^+$ T lymphocytes.

The Helper $CD4^+$ T lymphocytes are involved with the induction of the immune response and are crucial for the defense against both intracellular and extracellular pathogens. They recognize extracellular antigens bound to the major histocompatibility complex II (MHC II), which activates the Helper $CD4^+$ T lymphocytes to become effector helper T cells. In this state they stimulate the proliferation and differentiation of more helper $CD4^+$ T lymphocytes that can differentiate into two options, the T_{H1} and T_{H2} cells. These effector helper T cells can be distinguished by the cytokines, a diverse group of soluble proteins or peptides that they secrete influencing the type of adaptive immune response to be built up against the pathogen.

T_{H1} cells defend against intracellular pathogens and secrete the cytokine interferon - γ (INF -γ) which binds to the macrophages with the display of the costimulatory protein CD-40 ligand to activate macrophages. T_{H1} also secretes the tumor necrosis factor - α (TNF - α) and can activate dendritic cells, cytotoxic T cells and B cells, which can also be stimulate to secrete specific subclasses of IgG antibodies.

T_{H2} cells secrete interleukin 4, 5, 10, and 13, and mainly defend against extracellular pathogens, and stimulate B cells to make most classes of antibodies including IgE and some subclasses of IgG.

The Cytotoxic $CD8^+$ T lymphocytes cells recognize antigens bound to the MHC I molecules (glycoproteins present in the membrane of all the nucleated cells that pick up small peptides in the cytoplasm and take them to the cell surface). The Cytotoxic $CD8^+$ T cells are primary effectors in cell-mediated immunity and express at the plasma membrane the TCR's (T cells receptors) proteins which recognize and bind antigens bound to the MHC I molecules and in combination activate the proteins after which $CD8^+$ T cells secrete lymphokines and perforins leading to lysis of the infected cells (Ross et al., 2003). The suppressor (and /or cytotoxic $CD8^+$, $CD45RA^+$,) T lymphocytes diminish or suppress antibody formation by B cells, and also down regulate the ability of T lymphocytes to initiate a cellular immune response (et al., 2003).

The T cell activation through antigenic stimulation is characterized by cell growth (blastogenesis) and proliferation (Frauwirth & Thompson, 2004). When these cells are stimulated there is a moderate increase of oxygen consumption, but the increase in glycolysis is dramatically greater (Frauwirth et al., 2004).

When cells are exposed or treated with stressor substances, several organelles can respond to them on certain ways depending on the intensity resulting on cytotoxic effects. These effects can induce the cellular system to fail in a manner in which different organelle actions could coordinate a programmed cell death mechanism. Any type of cell exposed to any kind of antigen could be induced to a system failure including cells of the

immune system as the lymphocytes as well as not completely differentiated cells like lymphoblasts.

C. Cell Death Overview:

There are several programmed cell death mechanisms that can be named including: autophagy, necrosis, oncosis, and pyroptosis, but the most commonly known is apoptosis. The process of cell death is initiated by morphological and biochemical reactions which also help to differentiate the ongoing death mechanism.

Necrosis is a degrading process that occurs after cell death, but it is also described as a passive mechanism of energy independent death. Many times this term is used while referring to one of the cell death mechanisms known as oncosis. Oncosis is described as a pre-lethal pathway leading to cell death accompanied by cellular swelling, blebbing, and increased membrane permeability (Majno & Joris, 1995). The morphological features of oncosis include nuclear chromatin clumping in the absence of evident dense chromatin bodies, cytoplasmic swelling and vacuolation, with lysosomal disruption and leakage as well as mitochondrial swelling and disruption (Tang et al., 2008). This type of cell death mechanism occurs when ATP generation is attenuated or when cellular energy consumption becomes unregulated (Majno & Joris, 1995; Elmore, 2007).

Pyroptosis is a proinflammatory programmed cell death (Fink & Cookson, 2005) induced by infection and where caspase I has an important role in the activation process of the inflammatory cytokines IL-beta and IL-18.

Autophagy or type II cell death features degradation of cellular components within the dying cells in autophagic vacuoles (Fink & Cookson, 2005). This mechanism begins with the sequestration of cytoplasmic material by a membrane of unidentified

origin that eventually forms a double membrane autophagic vacuole (autophagosomes) (Galluzi, Vicencio, Kepp, Joza, Tajeddine & Kroemer, 2008). This type of cell death represents a prominent mechanism of cellular adaptation to stress initially induced to prolong cell survival (Reed, 2008) as well as a mechanism of cell immunity in response to invasion by extracellular pathogens. Autophagy is also related to the normal development process and has the role of maintaining genomic stability (Mathew, Kongara, Beaudoin, Karp, Bray, Degenhardt, Chen, Jin, & White, 2007). All these cell death mechanisms are less understood and studied than the cell death mechanism known as programmed cell death or apoptosis.

Apoptosis is a sophisticated, highly complex homeostatic mechanism of the organism that involves an energy-dependent cascade of molecular events (Elmore, 2007). Apoptosis also occurs as part of a defense mechanism against any offense to the organism. It is a biochemical cascade that activates proteases which destroy molecules required for cell survival and others that mediate a program of cell suicide (Friedlander, 2003) and may also be described as an active programmed process of autonomous cellular dismantling that avoids eliciting inflammation; a controlled physiologic process of removing individual components of an organism without destruction or damage to the organ systems (Fink & Cookson, 2005).

In early apoptosis, the cell shrinks and pyknosis (chromatin condensation) occurs, the most characteristic feature of apoptosis, followed by karyorrhexis (degeneration of the cell nucleus) and separation of cell fragments, the apoptotic bodies (Elmore, 2007).

The mechanism of apoptosis can be triggered by diverse external and internal stimuli. The external stimuli are signals that will go through the cell membrane receptors

which direct signals inside the cell, as the soluble cell death ligands. The intrinsic stimuli are the result of alterations in cellular function and metabolism (Tang, Mura, Seth, & Liu, 2008), but can also be triggered as part of the organism's homeostasis control or the exposure to exogenous agents that cause heavy damage inside the cell.

There are two principal apoptotic pathways: the Intrinsic Apoptotic Pathway and the Extrinsic Apoptotic Pathway. The intrinsic apoptotic pathway, also known as the mitochondria pathway, involves an array of non-receptor-mediated stimuli that produce intracellular signals. The events initiated in the mitochondrion are the result of the loss of the membrane potential and the release of pro-apoptotic proteins after the inner mitochondrial membrane suffers changes.

For the mitochondrial pathway to occur, the damage signal to the mitochondria must persist so other proteins in addition to cytochrome C are released. Several factors have to interfere such as the Bcl-2 family of proteins. This family participates in the regulation of the intrinsic apoptotic pathway (Brunelle & Letai, 2009) having control over the mitochondrial membrane permeability (Elmore, 2007) and in mediating the ER stress-induced apoptosis (Lai, Teodoro, & Volchuk, 2007). This family of proteins is composed of pro-apoptotic and anti-apoptotic proteins that share up to four conserved regions known as Bcl-2 homology (BH) domains and interact with the mitochondrial membrane and between each other to determine if the process occurs or not.

Proapoptotic Bcl-2 family proteins, such as the Bcl-2 associated X protein (Bax) and Bcl-2 antagonist/killer-1 (Bak), participate in the induction of mitochondrial outer membrane permeabilization (MOMP), causing the release of caspase activating proteins (cytochrome C) and other cell death mediators. Meanwhile, antiapoptotic proteins such

as Bcl-2 serve as guardians of the outer membrane (Reed, 2007) interacting with it and also with proapoptotic proteins of the same family.

If the mitochondrial membrane suffers permeability, the first group of proteins released is the cytochrome C, Smac/Diablo, and HtrA2/Omi (Elmore, 2007). Cytochrome C is a component of the mitochondrial electron transfer chain that initiates caspase activation when released from this organelle during apoptosis. Cytochrome c promotes the activation of the caspase cascade when it binds to the apoptotic protease-activating factor (Apaf-1), a cytosolic protein containing a caspase activation and recruitment domain (CARD), a nucleotide binding prodomain of the initiator caspases (Nhan, Liles, & Schwartz, 2006), and multiple WD-40 repeats. The CARD can recruit multiple procaspase 9 molecules and facilitate their auto activation forming the apoptosome, a complex composed of cytochrome C, the cytosolic protein Apaf-1 with ATP and the multiples procaspase 9 recruited. Caspase 9 activation is of great importance because it is the only caspase able to efficiently cleave and activate downstream executioner/effector caspases such as caspase 3.

The Smac/Diablo is a 25KD mitochondrial nuclei-encoded protein also released from the mitochondrion if the damage to mitochondria is severe and persistent. Its function is to remove Inhibitors of Apoptosis Proteins (IAP) inhibition and allow apoptosis to proceed. The Smac/Diablo at its N terminus contains a 55 amino acid mitochondrial targeting sequence that is removed when imported into the mitochondria and generates a new N terminus (Wang, 2001).

The release of the second group of proteins, AIF, Endonuclease G and CAD, is a late event that occurs after the cells are committed to death (Elmore, 2007). The

Apoptosis-inducing factor (AIF) is a 57KD flavoprotein with oxidoreductase activity that resides in the mitochondrial intermembrane space and translocates to the nucleus and causes DNA fragmentation (Elmore, 2007; Nhanet et al., 2006). The Endonuclease G (Endo G) is a 30kD nuclease that when released translocates to the nucleus and induces nucleosomal DNA fragmentation, its activity being independent of caspase activation. Endonuclease CAD is released and translocates to the nucleus after cleavage by caspase 3, and leads to oligonucleosomal DNA fragmentation with a more advanced chromatin condensation (Elmore, 2007).

As part of the intrinsic pathway after the release of mitochondrial proteins, an additional group is involved in the apoptosis process. It is the caspases (cysteine-dependent aspartate specific proteases), a family of proteases that are major executioners in the apoptosis program (Friedlander, 2003). Of the caspase family the 2, 3, 6, 7, 8, 9, and 10 are related to the apoptosis process (Fink et al., 2005). This group can be divided as the initiator caspases 2, 8, 9, and 10 that are responsible for the cascade initiation activated through its dimerization and the effectors caspases 3, 6, and 7 that perform the cell dismantling process and are converted to their active forms through proteolysis at internal Asp residues.

The caspases are also involved with the extrinsic apoptotic pathway or death receptor pathway, a way to initiate caspase activation independent of mitochondria. This pathway involves cell surface receptors (the death receptors) and their ligands. The sequence of events used to describe it is based on the FasL/FasR and the TNF- / TNFR1 trail receptor models, the best characterized at present. The binding between the transmembrane receptor and the ligand is followed by the recruitment of cytoplasmic

adapter proteins which bind to the corresponding death domain of the receptor. When the death receptor is activated, the death-inducing signaling complex (DISC) is formed which recruits and activates multiple procaspase 8+. When activated, caspase 8 can activate caspase 3 and with it the extrinsic pathway ends and the execution phase of apoptosis begins, but caspase 8 can also activate the BH-3 interacting domain (Bid) by its cleavage forming truncated Bid (tBid) (Brunelle & Letai, 2009). The tBid is known to act over the mitochondria external membrane provoking the permeability of this membrane and inducing the release of apoptogenic proteins. But the BH-3 interacting domain (Bid) can also be activated by calpain (calcium activated neutral proteases), a calcium vessel that can be found in the cytosol, (Bröker, Kruyt, & Giaccone, 2005) and inside the mitochondrion (Ozaki, Tomita, Tamai, & Ishiguro, 2007). The Bid activation by calpain could be caused by the Endoplasmic Reticulum (ER), an important sensor of cellular stress (Bröker et al., 2005), which when it suffers damage, its capacity to perform the protein synthesis function is compromised.

The activation of caspase 3 or any other execution caspase, like 6 and 7, is the starting point of the execution phase of apoptosis and the point where extrinsic and intrinsic pathways meet. At this step high levels of IAP molecules such as XIAP are able to abort the receptor pathway through the inhibition of caspase 3, but it is clear that apoptosis can proceed in the absence of caspase activity when mitochondria are damaged. The effector caspases activate cytoplasmic endonucleases and proteases. The cleaving caused by caspases 3, 6, and 7 produce the morphological and biochemical changes of apoptotic cells.

D. Risk Assessment:

Risk Assessment (RA) is a process that consists of four general parts: Hazard Identification, Exposure Assessment, Dose-Response Assessment, and Risk Characterization. The Hazard Identification phase implies the review of studies that indicate the effects of these compounds observed in animal and/or human research. Exposure Assessment searches for information about human exposure to these compounds. In the Dose-Response Assessment step, the information obtained in the previous part is evaluated to estimate the amount of a chemical that is likely to result in a particular health effect in humans. For the last step of the risk assessment process, the information obtained in all the past steps is put together to estimate the risk in certain populations.

The Risk Assessment process is closely related with toxicity studies. Toxicity is the property or properties of a material that produces a harmful effect on a biological system (Yu, 2005). To determine the effects caused by a substance over a biological system, toxicity studies examine the dose-response relationship that refers to the relationship between dose and biological effect (Yu, 2005). Experiments of concentration range and response of the biological form depict a dose-response curve in which the midpoint of the graph referred by several names thought for this work will be the Inhibitory Concentration 50 (IC_{50}), indicating the fifty percentile (50%) decrease of the growth rate. The key contribution of the environmental risk assessment process resides in the possibilities to prevent the exposure and health effect of phthalates or any other xenobiotics.

The data obtained from this *in vitro* cytotoxicity study serve as tools to help build a more complete risk assessment of the toxicity of Di ethylhexyl phthalate and Mono ethylhexyl phthalate on the immune system and contribute to the risk assessment process at the exposure and effect assessment steps.

Legal Approach

In the United States at the end of 2008 legislation was approved over the use of phthalates in consumer products and more research about these compounds was requested. With the Consumer Product Safety Improvement Act of 2008, the 110th Congress established safety standards and other safety requirements for children's products among other objectives at the 15 USC 2057c. Title I Children's Products Safety in section 108 prohibits the sale of certain products containing specific phthalates. Parts (a) and (b) request an appointment for a Chronic Hazard Advisory Panel to complete an examination of the full range of phthalates including their effects on children's health and of any other group that can be affected.

CHAPTER III

METHODOLOGY

Cellular Population under Study

The main purpose of this study is to determine the toxicity and the cell death mechanism of phthalates Di-ethylhexyl, phthalate (DEHP) and Mono ethylhexyl phthalate (MEHP) on the TK6 human lymphoblast cell line. The present study is based on cell culture techniques where human lymphoblast cells are treated with DEHP and MEHP to evaluate cytotoxic endpoints including growth inhibition, mitochondrial membrane damage, generation of reactive oxygen species and activation of caspases 3 and 7. The experimental part was performed in the facilities of the Chemical Environmental and Molecular Toxicology Laboratory (ChEMTox Lab) at the Research Building, Universidad Metropolitana (UMET).

Materials and Methods

A. Cell Cultures:

This *in vitro* study used the TK-6 cell line obtained from the American Type Culture Collection (ATCC) Human Cells with catalog number CRL-8015 identified as lymphoblasts and extracted from a human spleen. TK6 cells were cultured in RPMI 1640 1X culture media modified with 10% fetal bovine serum, 0.3% of glucose, 0.1% of penicillin-streptomycin solution stabilized and incubated at 37°C with 5% of CO_2 on a Sanyo model MCO-18AIC incubator. Before treating TK6 cells with the phthalates, cells

were cultured to assure a proper and stable cellular growth. A growth curve was generated for up to 72 hours (Figure 2) to confirm cell stability and replication rate.

B. Stock Solutions and Reagents:

Stock solutions of DEHP and MEHP were prepared at concentrations of 2mM using as dilution agent dimethyl sulphoxide (DMSO) biotechnology performance certified, kept on crystal vials and stored in a refrigerator at 4°C (Appendix 1).

To determine the IC_{50} (viability inhibition of 50 % of the treated cells) of DEHP and MEHP on human lymphoblast cells (TK6) when exposed for 48 and 72 hours.

Prior to cell treatments with DEHP and MEHP, TK6 were kept at a constant cell density to assure exponential growth and appropriate growth environment. The cells were kept in culture and treated in $25cm^2$ flasks. For each tested concentration, $1x\ 10^6$ TK6 in 3.5mL media were cultured. Treatments were performed for 48 and 72 hours in duplicates and the DEHP and MEHP solutions were added as needed per concentration (Appendixes 2 and 3).

Cell viability was accessed through Trypan Blue exclusion analysis, for which an aliquot of 100µL of the media/cells solution was diluted into 100µL of Trypan Blue solution at 0.4%. An analysis was performed with the cell counter Countess™ Automated Cell Counter (Invitrogen Corp. Carlsbad, California) for cell viability determination with a hemocytometer (Appendix 4) as recommended by the manufacturer.

The concentration that inhibits cell viability in 50% of treated cells (IC_{50}) was determined to evaluate the potency of these compounds to inhibit cellular growth (cell death) and also to determine the concentration to be applied on the cell death mechanism related endpoints to be measured such as mitochondrial membrane permeability; ROS generation and caspases 3 & 7 activation.

To determine if exposure of human lymphoblast cells (TK6) to DEHP and MEHP at the IC_{50} concentrations for 48 hours can induce mitochondrial membrane permeability.

TK6 cells in culture were exposed to the IC_{50} concentrations of DEHP and MEHP following a similar protocol as presented in the first part of this study with some modifications (Appendix 2). Dimethyl sulphoxide (DMSO) was used as negative control and Valinomycin (11µM), a commercially available antibiotic, was used as the positive control. Treatments where performed for 48 hours. This assay was performed to assess qualitative and quantitative data about the mitochondrial membrane permeability through fluorescence measurements in fluorescence standard units (FSU) with the application of the Modulus fluorometer (Promega, Sunnyvale, CA).

For apoptosis induction through mitochondrial damage, the Immunochemistry Apoptosis MitoPT^{TM} assay (Immunochemistry Technologies LLC, Bloomington, MN) was applied. Treatments where performed for 48 hours to evaluate the effect of phthalates on the permeability of the mitochondria. As indicated by the manufacturers, the intensity of the of the green fluorescence emitted indicated the number of apoptotic cells or the cells with mitochondrial membrane permeability as observed in apoptotic cells. The red fluorescence indicated healthy cells or cells without mitochondrial membrane damage. Both fluorescent indicators are produced by the fluorescence agent JC-1 reaction with the mitochondrial membrane (electrochemical charge) turning to green if the membrane charge is altered when the mitochondrial membrane is permeabilized (Appendix 5).

To determine if exposure of human lymphoblast cells (TK6) to DEHP and MEHP at the IC_{50} concentrations for 48 hours can induce reactive oxygen species generation (ROS).

To determine if exposure of TK6 to DEHP and MEHP at the IC_{50} concentrations could induce the generation of reactive oxygen species (ROS) cells were treated with DEHP and MEHP under similar conditions presented in the first part of this study with some modifications (Appendix 2). Dimethyl sulphoxide (DMSO) was applied as negative control and cis-diammine platinum (II) dichloride (Cisplatin) (13µM), an anticancer drug, as positive control for an exposure period of 48 hours. This assay was performed to assess quantitatively the generation of ROS through fluorescence emissions indicated by a chemical reaction with the 2,7- dichlorofluorescein diacetate (DCFH-DA) reactive agent. The generation of reactive oxygen species (ROS) inside the cells is caused by damage to the electron transport chain inside the mitochondria. The fluorescent green emissions from the cells are caused by the 2,7- dichlorofluorescein diacetate (DCFH-DA) reaction in the presence of ROS. An analysis was performed with a Modulus fluorometer (Promega, Sunnyvale CA). The intensity of the green fluorescent light is proportional to the level of ROS generated (Appendix 6).

To determine if exposure of human lymphoblast cells (TK6) to DEHP and MEHP at the IC_{50} concentrations for 48 hours can induce activation of caspases 3 and 7.

TK6 cells in culture were exposed to the IC_{50} concentration of DEHP and MEHP under similar conditions as those presented in the first part of this study with some modifications (Appendix 2). Dimethyl sulphoxide (DMSO) was used as negative control and staurosporine (1µM), a commercially available antibiotic, as positive control. The exposure period was for 48 hours with the exception of the positive control which had an

exposure period of three hours as indicated by the manufacturer. The applied assay known commercially as Magic Red^{TM} (Immunochemistry Technologies LLC, Bloomington, MN) is effective to detect caspases 3 and 7 activation measured by fluorescence intensity to assess quantitatively the phthalates' capacity to activate caspases 3 and 7.

Data Analysis

For the cell viability and IC_{50} determination of data representing the number of cells that survived the 48 and 72 hour, exposure per concentration was recorded in Excel for analysis. The percent survival per concentration was determined in comparison with the control (vehicle) applying the following equation: [(live cells c_i/ live cells control) x 100]. The percent of survival against concentration was then plotted to determine the IC_{50} applying the generated line for the TK6 lymphoblast cells. An analysis of mitochondrial membrane permeabilization, ROS generation and caspases 3 and 7 activation was based on the level of fluorescent emission as discussed in the protocol for each endpoint. Comparison of the fluorescent emission average of each compound against the control average was plotted in Excel. To assess if the difference between the averages observed values per endpoint among the tested compounds and the control was statistically significant, a one way ANOVA with fixed effect was performed. In case significant results were found in ANOVA, a Tukey Post Hoc Test honestly significant difference (HSD) was also performed. These statistical tests were realized with the SPSS Statistics 19 software.

CHAPTER IV

RESULTS

The overall purpose of the study was to determine the toxicity of two phthalate compounds: DEHP and MEHP on the human TK6 lymphoblast cell line. To determine their toxicity, several objectives were proposed and implemented. The first objective was to determine the concentration that decreases cell viability on 50% of the treated cells (IC_{50}). Objectives 2 through 4 were directed to determine cellular events that provide insights in their cell death induction mechanism. These cellular damages included: mitochondrial membrane permeability; generation of reactive oxygen species (ROS) and activation of caspases 3 and 7.

The TK6 cells were obtained from ATCC and the culture was established as recommended by ATCC. Prior to the DEHP and MEHP treatments, a cell growth curve was determined to assure healthy cells were grown in an exponential fashion to be used for the study. Figure 2 presents the TK6 cell growth curve.

To determine the IC_{50} (viability inhibition of 50 % of the treated cells) of DEHP and MEHP on human lymphoblast cells (TK6) when exposed for 48 and 72 hours.

For DEHP, the IC_{50} after 48 and 72 hours of treatment was 234µM (91.4mg/L) and 100µM (30.06mg/L), respectively. Figure 3 presents the TK6 cells viability after treatment with DEHP at concentrations ranging from 10 µM to 500 µM for 48 and 72 hours. In Figure 3 the X axis represents the tested concentrations and the Y axis represents the TK6 viability percentage.

For the MEHP compound, the IC_{50} for 48 hours was calculated as 196µM (54.55mg/L) and for the 72 hours period the IC_{50} was 80µM (22.27mg/L) (Figure 4). In

Figure 4 the horizontal axis represents the tested compound concentrations and the vertical axis represents the TK6 viability percentage.

To determine if exposure of human lymphoblast cells (TK6) to DEHP and MEHP at the IC_{50} concentrations for 48 hours can induce mitochondrial membrane permeability.

The mitochondrial membrane permeability on DEHP and MEHP treated cells was measured using the Mito PT^{TM} fluorescence. Figure 5 presents the comparison of the mitochondrial membrane permeability between treated and control (Valinomycin). Results indicated that cells exposed to DEHP, MEHP and Valinomycin undergo mitochondrial membrane damage. The cells exposed to Valinomycin presented higher mitochondrial membrane permeabilization based on the FSU (2327.65± 131.7 FSU). DEHP exposed cells presented mitochondrial damage at (2505.58± 84.6 FSU) close to those observed in the positive control. The MEHP exposed cells presented the lowest level of damage to the mitochondrial membrane (2210.09± 58.07 FSU) but when compared to the negative control DMSO (1065.73± 89.16 FSU), the level of mitochondrial damage was more than twice those of the negative control (Figure 5). An ANOVA single way with a Post Hoc Test Tukey (HSD) indicated a significant difference on the level of mitochondrial membrane permeability caused by DEHP and MEHP *(P*-value of 0.002 and 0.024, respectively) in comparison with the negative control. These phthalates clearly demonstrated the capacity to induce alterations to the mitochondrial membrane potential.

To determine if exposure of human lymphoblast cells (TK6) to DEHP and MEHP at the IC_{50} concentrations for 48 hours can induce reactive oxygen species (ROS) generation.

Comparison among the levels of reactive oxygen species (ROS) produced in cells after exposure to DEHP, MEHP, DMSO (negative) and Cisplatin (positive) was assessed by comparison to their respective FSU resulting from the reaction of the fluorescent marker 2, 7-dichlorofluoroscein diacetate (DCFH-DA) and the generated ROS. Figure 6 shows the relation between the generated fluorescence measured in FSU indicating the level of ROS generated by the cells per the tested compounds. The results of this experiment showed that the cells exposed to the positive control Cisplatin generated the highest quantity of ROS (1575.35± 108.4 FSU), a small difference over the cells exposed to MEHP (1518.75± 3.7 FSU), but almost twice the level generated by DEHP (793.27± 108.74 FSU) and 40 times greater when compared to the negative control DMSO (39.62± 8.14 FSU). DEHP exposed cells generated the lowest quantity of ROS but still much higher when compared to the negative control, DMSO.

An analysis of the observed similarities between the negative control and DEHP by an ANOVA single way with a Post Hoc Test Tukey (HSD) resulted in a P-value of 0 indicating a significant difference in the level of ROS generated by the cells exposed to DEHP, but also compared with the positive control and MEHP. Comparison of the level of ROS generated by the cells exposed to DEHP and MEHP was also analyzed generating a *P*-value of 0.009. No significant difference was observed between the positive control (cisplatin) and MEHP with a *P* value of 1 indicating similarities in their capacity to induce treated cells to produce ROS.

To determine if exposure of human lymphoblast cells (TK6) to DEHP and MEHP at the IC_{50} concentrations for 48 hours can induce activation of caspases 3 and 7.

To determine if caspases 3 and 7 were activated during the death mechanism process after the exposure to the IC_{50}'s of DEHP and MEHP, the assay known

commercially as Magic Red TM (Immunochemistry Technologies LLC, Bloomington, MN) was implemented. The assay measures intensity the activation of caspases 3 and 7 through fluorescence. DMSO was used as the negative control and Staurosporine as the positive control. The results of this fluorescence assay are shown in Figure 7 where comparison of the fluorescence measures of the cells exposed to the positive control Staurosporine (1322.55± 77.13 FSU), DEHP (1406.9± 28.3 FSU), MEHP (1209.72± 97.15 FSU) and DMSO (33.21± 48.04 FSU) are presented. The results indicate that both phthalate compounds promote the activation of caspases 3 and 7 similarly to the positive control. An ANOVA single way with a Tukey Post Hoc Test (HSD) for comparison of the negative control with DEHP reported a P-value of 0.357 and 0.455 for MEHP. The comparison of the positive control with DEHP produced lack of significance with a value of 1 while with MEHP the value was 0.999. The results of the p-values demonstrated the lack of significance between the tested compounds and the controls in their capacity to activate caspases 3 and 7.

DISCUSSION

The cytotoxicity of compounds DEHP and MEHP on TK6 lymphoblast cells *in vitro* was examined. In this work the IC_{50} at 48 and 72 hours of DEHP as 234µM (91.4mg/L) and 100µM (39.06mg/L) was determined (Figure 3). The MEHP IC_{50} at 48 hours was 196µM (54.55mg/L) and 80µM (22.27mg/L) for the 72 hour period (Figure 4).

The results of the TK6 cells exposure to the parent compound DEHP can be observed in Figure 3 where it illustrates the relation between the cells' viability percentage and the DEHP concentration in micro molars (µM). The results for both exposure periods are illustrated in the same plot for comparative analysis. IC_{50}'s for

DEHP at 48 and 72 hours differ approximately by 100µM, with a more toxic effect after longer exposure periods. In Figure 3 at the 48 hours period the cell viability at 50µM was similar to the controls but, as the concentration was increased, the cells' viability began to diminish considerably. The exposure period of DEHP definitely influences the effect over the TK6 cells' viability.

The experimentally determined IC_{50}'s for DEHP in this study are comparable with the concentrations found in sludge from several sewage treatment plants (STPs) that range from 15 to 346mg/Kg in Quebec, Canada (Beauchesne, Barnabe, Cooper, and Nicell, 2008) and in some blood patient samples ranging between 50 to 350µM (Plonait, Nau, Maier, Wittfoht, & Obladen, 1993; Tickner, Schettler, Guidotti, McCally, & Rossi, 2001).

The second compound under study, MEHP, is the principal metabolite of DEHP. Figure 4 shows the relation between the cells' viability percentage and MEHP concentrations in micro molar (µM) units. The IC_{50} for MEHP in TK6 at 48 and 72 hours exposure periods differs by more than 100µM. The cell viability inhibition concentration for 48 hours was 196µM (54.55mg/L) and for the 72 hour period was 80µM (22.27mg/L). In comparison with the negative control culture (DMSO), the cells' viability was stimulated when treated with 20µM of MEHP resulting in an increase of over 120% for both exposure periods. This stimulus could be explained by the capacity of the immune system to respond and proliferate in the presence of exogenous agents. For higher concentrations, however, a clear dose response was observed for both exposure periods resulting in higher cell death as concentrations increased. MEHP exposure period

definitely is an important factor in the effect over the TK6 cells as observed with DEHP exposure.

Toxicity increases for both compounds as the exposure periods increase since a decrease in the IC_{50}'s was observed when cells where incubated for 72 hours in comparison to the 48 hour exposure period. It is also of great importance to point out that the metabolite compound resulted more harmful to the cells at both exposure periods. Other studies indicated that phthalates seem to exploit different pathways to evoke multiple and independent effects in human leukocytes, each potentially contributing to an inflammatory reaction through inappropriate cell activation (Palleschi, Rossi, Diana, & Silvestroni, 2009).

The implications of these results are that both compounds can affect cells from the immune system with concentrations even lower than the IC_{50}'s determined in this study over longer exposure periods. Studies explain that cells of the immune system are affected by phthalates and their metabolites because their PPAR agonist activity and specified PPARγ agonist can induce apoptosis in lymphocytes (Schlezinger, Howard, Hurst, Emberley, Waxman, Webster, & Sherr, 2004). In 2009, Eveillard, Lasserre, Tayrac de, Polizzi, Claus, Canlet, Mselli-Lakhal, Gotrdi, Paris, Guillou, Martin, and Pineau indicated that high levels of nuclear receptors (PPAR) in lymphocytes could explain why DEHP and /or its major metabolite MEHP induce toxicity, modulating the activities of three nuclear receptors PPARα, PPARγ, and PXR, making lymphocytes a good target for phthalates.

The first objective was directed to assess the cytotoxicity of DEHP and MEHP on TK6 lymphoblast cells *in vitro* and the second objective was to determine if the TK6 cells

exposed to DEHP and MEHP induced mitochondrial membrane permeability, reactive oxygen species generation and activation of caspases 3 and 7.

These three assays were performed to provide insights into the death mechanism. The mitochondrial membrane permeability was determined through a fluorescence assay (Mito PT™) and the results can be observed in Figure 5. The cells exposed to the positive control, the Valinomycin (antibiotic), induced high levels of mitochondrial damage (2327.65± 131.7 FSU) as expected. The cells exposed to DEHP (2505.58± 84.6 FSU) and MEHP (2210.09± 58.07 FSU) suffered mitochondrial damage similar to the positive control and showed double the level of mitochondrial permeability in comparison to the cells exposed to the negative control DMSO (1065.73± 89.16 FSU).

The results show that the difference in mitochondrial damage induction between both compounds was small (less than 300 FSU) implying similarities in their toxicity mechanism. These results coincide with previous studies, where DEHP and MEHP exposure induced mitochondrial membrane permeability (Bissonnette, Teague, Sherr, & Schlezinger, 2008; Palleschi et al., 2009; Melnick & Schiller, 1982; Pant, Shukla, M., Kumar Patel, Shukla, Y., Mathur, Kumar Gupta, & Krishna Saxena, 2008).

Reactive oxygen species generation assay results as presented in Figure 6 illustrate the relation between the FSU values that are proportional to the ROS generated by the cells after the exposure to each of the compounds. For this particular assay the cells were exposed to each of the two phthalates, to the positive control cis-Diammine platinum (II) dichloride (Cisplatin) and the negative control DMSO. The Cisplatin exposed cells generated ROS fluorescence measures of 1575.35± 108.4 FSU, followed by MEHP 1518.75± 3.7 FSU, DEHP 793.27± 108.74 FSU, and DMSO 39.62± 8.14 FSU.

The cells exposed to the metabolite generated nearly the same quantity of ROS than the cells exposed to the positive control and almost twice the production of the cells exposed to DEHP. Clearly both DEHP and MEHP promoted the generation of ROS in TK6 cells at least 10 times the level of ROS generated by the negative control.

This outcome concurs with previous studies that indicated how DEHP is able to trigger ROS production as well as its metabolite MEHP in other cell types (Kasahara, Sato, Miyoshi, Konaka, Hiramoto, Sasaki, Tokuda, Nakano, & Inoue, 2002; Palleschi et al., 2009; Pant et al., 2008). But when both phthalates are compared, the metabolite (MEHP) generated was approximately twice the level generated by the parent compound.

The third and last assay was performed in the search to identify the death mechanism suffered by the cells was the activation of caspases 3 and 7. The level of activation of caspases 3 and 7 in TK6 cells after being exposed to the tested compounds were as follows: DEHP (1406.9± 28.3 FSU) with the highest activation level > positive control Staurosporine (1322.55± 77.13 FSU) > MEHP (1209.72± 97.15 FSU) > DMSO (negative control) 33.21± 48.04 FSU. These results concurred with other studies that indicate how MEHP can induce classic terminal features of apoptosis, including caspase 3 cleavage and activity (Bissonnette et al., 2008).

The results obtained suggest that DEHP and MEHP induced toxicity in the lymphoblast cells and the exposure to these compounds can cause mitochondrial membrane permeability, as well as ROS generation and activation of caspases 3 and 7. These three events are recognized (among others) as hallmark events in the apoptosis process. Apoptosis is a very complex process and can occur through multiple sequences of events generating differences in signal and/or activation intensities. This study

provides evidence that DEHP can induce mitochondrial membrane permeability, ROS generation and caspase 3 and 7 activation in the TK6. To better understand how the complete apoptosis process is induced by these compounds, additional studies are needed as well as complementation of previously reported studies and results.

Palleschi et al. (2009) identified DEHP capacity to stimulate Ca^{2+} influx inside human granulocyte cells and its relation with the ROS generation. Ozaki et al. (2007) indicated how the increase of the intracellular calcium ions may cause an influx of Ca^{2+} into the mitochondria and how this can affect different mitochondrial enzymes. Ca^{2+} serves as a physiological stimulus that influences the mitochondrial membrane permeability transition (MTP) and activates the mitochondrial permeability transition pore (MPTP) that regulates the inner membrane permeabilization leading to energetic failure causing oxidative stress (Armstrong, 2006; Ozaki et al., 2007; Tsujimoto & Shimizu, 2007). This Ca^{2+} influx can be the caused by DEHP influence or the interaction with the mitochondrial proteins NADPH Oxidase, NADPH-cytochrome c reductase, and cytochrome P450, as indicated by Rusyn, Kadiiska, Dikalova, Kono, Yin, Tsuchiya, Mason, Peters, Gonzalez, Segal, Holland, and Thurman (2001); and Ganning, Klasson, Bergman, Brunk, and Dallner (1982) causing not only the permeabilization but promoting also the ROS generation and the activation of diverse caspases. The Ca^{2+} influx inside human granulocyte cells stimulated by both compounds could also affect the calpains at the cytoplasm and the endoplasmic reticulum (ER) internal homeostasis (balance) with a calcium influx or efflux. Bax, Bak and Bcl-2 proteins which are associated to the mitochondria and the ER membrane have a role in mediating ER stress-induced apoptosis (Lai, Teodoro, & Volchuk, 2007). Bax and Bak when present in the

ER membrane can suffer conformational changes causing calcium release to the cytosol leading to the activation of m-calpain (Nakagawa & Yuan, 2000; Zang, Li, Hatzivassiliou, Lindsten, Yu, Yuan, Thompson, 2003). Bax can also be translocated to the mitochondria membrane promoting the membrane permeabilization with the possible activation of Bid through caspase 8 which can be activated by caspase 3 and 6 and/or through caspase 2, which can be activated by caspase 3. Both caspases 2 and 3 can activate Bid (Bissonnette et al., 2008; Cowling & Downward, 2002; Zhivotovsky & Orrenius, 2005; Slee, Keogh, & Martin, 2000) amplifying the mitochondrial permeabilization signal.

The ROS generation on TK6 cells exposed to DEHP is almost ten times more than the negative control, but it was almost half the ROS generated by the TK6 cells exposed to MEHP. The generation difference can be explained through the compounds' interaction with several mitochondrial proteins and the calcium influx to the mitochondria. Interactions of these two compounds and their metabolites with several mitochondrial proteins like NADPH Oxidase, NADPH-cytochrome c reductase, and cytochrome P450, are established as the reasons for the mitochondrial ROS generation by cells exposed to DEHP and partially to MEHP (Rusyn et al., 2001; Ganning et al., 1982).

The DEHP metabolite, MEHP, causes mitochondria membrane permeability, ROS generation and caspase 3 and 7 activation as reported by past studies (Bissonnette, et al., 2008) that indicated MEHP induced mitochondrial membrane permeability on B cells through a combination of factors in addition to the cytochrome c release, a detail also confirmed by Kasahara et al. (2002). MEHP induces the translocation of Bax to the mitochondrial membrane inducing pore formation and the transformation of Bid to tBid

possibly through the single or combined activation of caspase 8 activated as result of a downstream event of the intrinsic pathway (Bissonnette et al., 2008; Zhivotovsky & Orrenius, 2005; Cowling & Downward, 2002).

MEHP caused TK6 to generate ROS, but at higher concentrations than DEHP. Studies with these compounds have identified factors that contribute to ROS generation on other cells. These factors could be the cause of the quantitative difference on TK6 when exposed to DEHP and MEHP. Two redox-sensitive mitochondrial proteins, the peroxiredoxin 3 (Prx3) and cyclo oxygenase -2 (COX-2), were identified in germinal cells as factors affected by the MEHP exposure, but not by DEHP (Onorato, Brown, & Morris, 2008). It was also found in isolated rat testicular cells, that MEHP and not DEHP increased the fluorescence intensity of DCFH-DA (Kasahara et al., 2002), the same oxidative stress detector used in this study and with Sertoli cells. Neither compound caused an increase in oxidative stress (Kasahara et al., 2002) which could indicate the sensitivity to MEHP and DEHP among cell types.

The presence of MEHP exerts influence over Prx3, an antiapoptotic protein that increases its presence in the mitochondria caused by oxidative stress and mitochondrial insults and such increase reflects changes in the cellular redox homeostasis and/or inhibition of apoptosis following short-term MEHP exposure (Onorato et al., 2008). But acute and chronic oxidative injuries can lead to reduced Prx3 levels (Wood-Allum, Barber, Kirby, Heath, Holden, Mead, Higginbottom, Allen, Beaujeux, Alexson, Ince, & Shaw, 2006) resulting in diminished protective responsiveness and increased susceptibility to oxidative stressors (Onorato et al., 2008). COX-2 is also affected by the exposure to MEHP and not DEHP. This protein protects the cells against oxidative stress-

induced apoptosis. It is also known that cytochrome c oxidase can be stimulated by MEHP and ethyl hexanol (EH) (75-91%), but not by DEHP in hepatocytes (Ganning et al., 1982, 1983; Ganning & Dallner, 1981). These three enzymes had been found affected with the presence of MEHP, but not with DEHP. This can be a significant factor for the difference in ROS generation.

The results indicate that TK6 lymphoblasts treated with DEHP and MEHP can undergo apoptosis as a death mechanism, but the specific pathway (intrinsic and extrinsic) cannot be confirmed. A combination between both apoptosis pathways may be present.

CHAPTER V

CONCLUSION AND RECOMMENDATIONS

The phthalate compounds DEHP and MEHP are known environmental polluters with a constant presence in human life and confirmed by several studies relating their concentrations in human tissues with numerous human health conditions. The constant presence of both compounds in the environment and in human tissues can be seen as a public health risk.

The main purpose of this cytotoxicity study was to understand the adverse effect of DEHP and MEHP on the human immune system and their mechanism of action involved. The results obtained through the use of the TK6 cell line show that MEHP is more toxic for these cells than DEHP, but it also demonstrated that toxicity increases as the exposure time is prolonged. Basically, MEHP toxicity over the TK6 cells indicates that the IC_{50} of this compound gets lower with a longer exposure period.

Even though DEHP is not the most toxic compound, it is more widely used and the one to which more humans might be exposed. Its biotransformation results in an even more toxic molecule, MEHP. Its constant exposure and absorption into the organism is a factor that could compromise the human immune defense system in a similar way to the one observed *in vitro* where the cells become more fragile with the constant presence and a long exposure period.

The IC_{50} of the compounds in relation with the exposure period and their effects on the mitochondrial membrane permeability compares with the antibiotic Valinomycin

(positive control). Their capacity to induce generation of reactive oxygen species is similar to Cisplatin, an anticancer drug and the capacity to activate caspases 3 and 7 is similar to Staurosporine another antibiotic.

It is recommended to further perform this study on cells freshly isolated from humans and compare them with the observed results with cell lines. The purpose is to corroborate and determine if the concentrations and sensitivity observed apply to freshly isolated cells from the immune system when exposed to DEHP, MEHP and 2-ethyl-1-hexanol (the second metabolite product of the DEHP hydrolysis).

Future work is necessary to better assess the environmental and health risks of phthalates. Future research could include the determination of the concentration of these compounds in the blood of the general population in Puerto Rico. Only one study has been performed (Colon et al, 2000) where DEHP concentrations in the blood and the thelarche in young Puerto Rican girls have been related. An additional series of studies may include the monitoring of DEHP and MEHP from environmental samples including indoor air, ground and water samples, as in urban rivers specifically near water treatment plants, work areas air samples and terrain samples near landfills in Puerto Rico. There is very little known about levels of phthalates in air, water or soil in Puerto Rico. It is the responsibility of environmental managers and scientists to provide the necessary information to estimate the potential public health risks and impact of pollutants such as phthalates as well as to develop ways to prevent and manage such risks.

CITED LITERATURE

Andersen, M. H., Schrama, D., Straten, P., & Becker, J. C. (2006). Cytotoxic T cells. *Journal of Investigative Dermatology*, 126. Doi: 10.1038/sj.jid5700001.

Armstrong, J. S. (2006). The role of the mitochondrial permeability transition in cell death. *Mitochondrion*, 6: 225–234.

Beauchesne, I., Barnabe, S., Cooper, D. G. & Nicell, J. A. (2008). Plasticizers and related toxic degradation products in wastewater sludges. *Water Science & Technology*, 57(3): 367-374. Doi: 10.2166/wst.2008.001.

Bissonnette, S. L., Teague, J. E., Sherr, D. H., & Schlezinger, J. J. (2008). An endogenous prostaglandin enhances environmental phthalate-induced apoptosis in bone marrow B cells: Activation of distinct but overlapping pathways. *Journal of Immunology*, 181, 1728 – 1736.

Bröker, L. E., Kruyt, F. A. E., & Giaccone, G. (2005). Cell death independent of caspases: A review. *Clinical Cancer Research,* 11(9), 3155 – 3162.

Brunelle, J. K., & Letai, A. (2009). Control of mitochondrial apoptosis by the Bcl-2 family. *Journal of Cell Science*, 122: 437-441. doi:10.1242/jcs.031682.

Calafat, A. M., & McKee, R. H. (2006). Integrating biomonitoring data into the risk assessment process: Phthalates [Diethyl Phthalate and Di(2-ethylhexyl) Phthalate] as a case study. *Environmental Health Perspectives*, 114: 1783-1789. Doi: 10.1289/ehp.9059.

Carter, J. E., Roll, D. B., & Petersen, R.V. (1974). The *In Vitro* hydrolysis of Di Ethylhexyl Phthalate by rat tissues. *Drug Metabolism and Disposition*, 2(4): 341-344.

Chou, K., & Wright, R. O. (2006). Phthalates in Food and Medical Devices. *Journal of Medical Toxicology*, 2: 126-135.

Cobellis, L., Latini, G., De Felice, C., Razzi, S., Paris, I., Ruggieri, F., Mazzeo, P., & Petraglia, F. (2003). High plasma concentration of di-(2-ethylhexyl)- phthalate in women with endometriosis. *Human Reproduction*, 18: 1512-1515. Doi: 10.1093/humrep/deg254.

Colon, I., Caro, D., Bourdony, C. J., & Rosario, O. (2000). Identification of phthalate Esters in the Serum of young Puerto Rican girls with Premature Breast Development. *Environmental Health Perspectives,* 108(9): 895-900.

Cowling, V., & Downward, J. (2002). Caspase-6 is the direct activator of caspase-8 in the cytochrome c induced apoptosis pathway: Absolute requirement for removal of caspase-6 prodomain. *Cell Death and Differentiation*, 9: 1046-1056. doi:10.1038/sj.cdd.4401065

Elmore, S. (2007). Apoptosis: A review of programmed cell death. *Toxicologic Pathology*, 35(4): 495-516. Doi: 10.1080/01926230701320337.

Environmental Protection Agency (2002). Documentation of Environmental Indicator Determination, RCRA Corrective Action Environmental Indicator (EI) RCRA Info code (CA725) Current Human Exposure Under Control, Chevron Phillips Chemical Puerto Rico Core, Inc. U.S. Environmental Protection Agency.

Eveillard, A., Lasserre, F., Tayrac de, M., Polizzi, A., Claus, S., Canlet, C., Mselli-Lakhal, L., Gotrdi, G., Paris, A., Guillou, H., Martin, P. G. P., and Pineau, T. (2009). Identification of potential mechanism of toxicity alter di-(2-ethylhexyl)-phthalate (DEHP) adult exposure in the liver using a system biology approach. *Toxicology and Applied Pharmacology*, 236: 282-292. Doi:10.1016/j.taap.2009.02.008.

Fabja, E., Hulzebos, E., Mennes, W., & Piersma, A. H. (2006). A category approach for reproductive effects of phthalates. *Critical Reviews in Toxicology*, 36: 695-726. DOI: 10.1080/10408440600894914.

Fink, S.L., & Cookson, B.T. (2005). Apoptosis, pyroptosis, and necrosis: Mechanistic description of dead and dying eukaryotic cells. *Infection and Immunity*, 73:1907-1916. Doi: 10.1128/IAI.73.4.1907-1916.2005.

Frauwirth, K.A., & Thompson, C. B. (2004). Regulation of T lymphocytes metabolism. *The Journal of Immunology*, 172: 4661- 4666.

Friedlander, R.M. (2003). Apoptosis and caspases in neurodegenerative diseases. *The New England Journal of Medicine*, 348: 1365-1375.

Galluzi, L., Morselli, E., J. M. Vicencio., O. Kepp., N. Joza., N. Tajeddine, & Kroemer, G. (2008). Life, death and burial: Multifaceted impact of autophagy. *Biochemical Society Transactions*, 36(5): 786-790. doi:10.1042/BST0360786.

Ganning, A. E., & Dallner, G. (1981). Induction of peroxisomes and mitochondria by di-(2-ethylhexyl) phthalate. *FEBS Letters*, 130(1): 77-79.

Ganning, A. E., Klasson, E., Bergman, A., Brunk, U., & Dallner, G. (1982). Effect of phthalate ester metabolites on rat liver. *Acta Chemica Scandinavica,* B36: 563-565. Doi: 10.3891/acta.chem.scand.36b-0563.

Ganning, A. E., Brunk, U., & Dallner, G. (1983). Effects of dietary di-(2-ethylhexyl) phthalate on the structure and function of rat hepatocytes. *Biochimica et Biophysica Acta,* 763(1): 72-82. Doi: 10.1016/0167-4889(83)90027-7.

Garrido Frenich, A., Barco Bonilla, De las Nieves, M., López, Martínez, J. C., Martínez, Vidal, J. L., & Romero-González, R. (2009). Determination of di-(ethylhexyl) phthalate in environmental samples by liquid chromatography coupled with mass spectrometry. *Journal of Separation Science*, 32: 1383-1389. Doi: 10.1002/jssc.200900020.

Gustaf Bornehag, C., Sundell, J., Weschler, C. J., Sigsgnard, T., Lundgren, B., Hasselgren, M., & Hägerhed-Engman, L. (2004). The Association between asthma and allergic symptoms in children and phthalates in house dust: A nested case-control study. *Environmental Health Perspectives*, 112(14); 1393-1397. Doi: 10.1289/ehp.7187.

Hauser, R. Meeker, J. D. Park, S., Silva, M. J., & Calafat, A. M. (2004). Temporal variability of urinary phthalate metabolite levels in men of reproductive age. *Environmental Health Perspectives*, 112: 1734-1740. Doi: 10.1289/ehp.7212.

Hokanson, R., Hanneman, W., Hennessey, M., Donnelly, K.C., McDonald, T., Chowdhary, R., & Busbee, D.L. (2006). DEHP, bis(2)-ethylhexyl phthalate, alters gene expression in human cells: possible correlation with initiation of fetal developmental abnormalities. *Human & Experimental Toxicology*, 25: 687-695. Doi: 10.1177/0960327106071977.

Jen, J.F., & Liu, T.C. (2006). Determination of phthalate esters from food-contacted materials by on-line microdialysis and liquid chromatography. *Journal of Chromatography A*, 1130: 28-33. Doi: 10.1016/j.chroma.2006.06.029.

Kasahara, E., Sato, E.F., Miyoshi, M., Konaka, R., Hiramoto, K., Sasaki, J., Tokuda, M., Nakano, Y., & Inoue, M. (2002). Role of oxidative stress in germ cell apoptosis induced by Di(2-ethylhexyl) phthalate. *Biochemical Journal,* 365: 849-856. Doi:10.1042/BJ20020254.

Kato, K., Silva, M. J., Needham, L. L., & Calafat, A.M. (2005). Determination of 16 phthalate metabolites in urine using automated sample preparation and on-line preconcentration/high-performance liquid chromatographyt/Tandem mass spectrometry. *Analytical Chemistry*, 77(9): 2985-2991. Doi: 10.1021/ac0481248.

Kicheva, Y.I., Richter, H., & Popova, E. (2004). Evaluation of the effect of the concentration of the Plasticizer Di (2-ethylhexyl) phthalate on the quantity of residual monomer vinyl chloride in PVC chest drainages tubes. *Journal of Material Science: Materials in Medicine*, 15: 909-914.

Koike, E., Inoue, Ken-ichiro., Yanagisawa, R., & Takano, H. (2009). Di-(2-ethylhexyl) phthalate affects immune cells from atopic prone mice *in vitro*. *Toxicology*, 259: 54-60. Doi: 10.1016/j.tox.2009.02.002.

Koo, H. J., & Lee, B. M. (2004). Estimated exposure to phthalates in cosmetics and risk assessment. *Journal of Toxicology and Environmental Health, Part A,* 67: 1901-1914. Doi: 10.1080/15287390490513300.

Lai, E., Teodoro, T., & Volchuk, A. (2007). Endoplasmic reticulum stress: Signaling the unfolded protein response. *Physiology*, 22: 193-201. doi:10.1152/physiol.00050.2006.

Lippolis, J. D. (2008). Immunological signaling networks: Integrating the body's immune response. *Journal of Animal Science*, 86: E53-E63. Doi: 10.2527/jas.2007-0620.

Majno, G., & Joris, I., (1995). Apoptosis, oncosis and necrosis: An overview of cell death. *American Journal of Pathology*, 146(1): 3-15.

Martini, F. H. (2006). Fundamentals of anatomy and physiology. Seventh Edition. San Francisco, CA: Pearson Education, Inc.

Mathew, R., Kongara, S., Beaudoin, B., Karp, C. M., Bray, K., Degenhardt, K., Chen, G., Jin, S., & White, E. (2007). Autophagy suppresses tumor progression by limiting chromosomal instability. *Genes & Development*, 21: 1367-1381. Doi: 10.1101/gad.1545107.

Marttinen, S. K., Kettunen, R. H., & Rintala, J. A. (2003). Occurrence and removal of organic pollutants in sewages and landfill leachates. *The Science of the Total Environment*, 301; 1-12.

Melnick, R. L., & Schiller, C. M. (1982). Mitochondrial toxicity of phthalate esters. *Environmental Health Perspectives*, 45: 51 – 56.

Nakagawa, T., & Yuan, J. (2000). Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *The Journal of Cell Biology*, 150(4): 887-894. Doi: 10.1083/jcb.150.4.887

Nakamiya, K., Takagi, H., Nakayama, T., Ito, H., Tsuruga, H., Edmonds, J. S., & Morita, M. (2005). Microbial production and vaporization of Mono-(2-ethylhexyl) phthalate from di-(2-ethylhexyl) phthalate by microorganisms inside houses. *Archives of Environmental and Occupational Health*, 60: 321-325.

Nhan, T. Q., Liles, W. C., & Schwartz, S. M. (2006). Physiological functions of caspases beyond cell death. *The American Journal of Pathology*, 169: 729-737. doi:10.2353/ajpath.2006.060105.

Onorato, T. M., Brown, P. W., & Morris, P. L. (2008). Mono (2-ethylhexyl) phthalate increases spermatocyte mitochondrial Peroxiredoxin 3 and Cyclooxygenase 2. *Journal of Andrology*, 29(3): 293-303. Doi: 10.2164/jandrol.107.003335.

Ozaki, T., Tomita, T., Tamai, M., & Ishiguro, S. (2007). Characteristics of mitochondrial calpains. *Journal of Biochemistry*, 142 (3): 365-376. doi: 10.1093/jb/mvm143.

Palleschi, S., Rossi, B., Diana, L., and Silvestroni. (2009). Di(2-ethylhexyl) phthalate stimulates Ca^{2+} entry, chemotaxis and ROS production in human granulocytes. *Toxicology Letters,* 187: 52-57. Doi: 10.1016/j.toxlet.2009.01.031

Pant, N., Shukla, M., Kumar Patel, D., Shukla, Y., Mathur, N., Kumar Gupta, Y., & Krishna Saxena, D. (2008). Correlation of phthalates exposures with semen quality. *Toxicology and Applied Pharmacology*, 231; 112-116. Doi: 10.1016/j.taap.2008.04.001.

Plonait, S. L., H. Nau, R. F. Maier, W. Wittfoht, and M. Obladen. (1993). Exposure of newborn infants to di-(2-ethylhexyl)-phthalate and 2-ethylhexanoic acid following exchange transfusion with polyvinylchloride catheters. *Transfusion,* 33: 598 – 605.

Reed, J. C. (2008). Bcl-2 family proteins and hematologic malignancies: History and future prospects. *Blood*, 111(17): 3322-3330. doi:10.1182/blood-2007-09-078162.

Ross, M. H., Kaye, G. I., & Pawlina, W. (2003). Histology A text and atlas with cell and molecular biology. Fourth Edition. Lippincott Williams & Wilkins, Baltimore, MD.

Rusyn, I., Kadiiska, M. B., Dikalova, A., Kono, H., Yin, M., Tsuchiya, K., Mason, R. P., Peters, J. M., Gonzalez, F. J., Segal, B. H., Holland, S. M., & Thurman, R. G. (2001). Phthalates rapidly increase production of reactive oxygen species *in vivo*: Role of Kupffer cells. *Molecular Pharmacology*, 59(4): 744-750.

Sathyanarayana, S., Karr, C. J., Lozano, P., Brown, E., Calafat, A. M., Liu, F., & Swan, S.H. (2008). Baby care products: Possible sources of infant phthalate exposure. *Pediatrics*, 121;e260-e268. DOI: 10.1542/peds.2006-3766.

Schlezinger, J. J., Howard, G. J., Hurst, C. H., Emberley, J. K., Waxman, D. J., Webster, T., & Sherr, D. H. (2004). Environmental and endogenous peroxisome proliferator-activated receptor γ agonist induced Bone Marrow B Cell Growth Arrest and Apoptosis: Interactions between Mono (2-ehtylhexyl) phthalate, 9-cis-Retinoic Acid, and 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2. *The Journal of Immunology,* 173: 3165-3177.

Scott, F. L., Denault, J. B., Riedl, S. J., Shin, H., Renatus, M., & Salvesen, G. S. (2005). XIAP inhibits caspase-3 and -7 using two binding sites: Evolutionarily conserved mechanism of IAPs. *The European Molecular Biology Organization Journal*, 24(3): 645-655. Doi: 10.1038/sj.emboj.7600544.

Shea, K. M. & the Committee on Environmental Health. (2003). Pediatric exposure and potential toxicity of phthalates plasticizers. *Pediatrics*, 111: 1467-1474. DOI: 10.1542/peds.111.6.1467.

Silva, M. J., Samandar, E., Preau Jr, J. L., Reidy, J. A., Needham, L. L., & Calafat, A. M. (2007). Quantification of 22 phthalate metabolites in human urine. *Journal of Chromatography B*, 860: 106-112. Doi: 10.1016/j.jchromb.2007.10.023.

Silva, M. J., Slakman, R., Reidy, J. A., Preau Jr, J. L., Herbert, A. R., Samandar, E., Needham, L. L., & Calafat, A. M. (2004). Analysis of human urine for fifteen phthalate metabolites using automated solid-phase extraction. *Journal of Chromatography B*, 805: 161-167. Doi: 10.1016/j.jchromb.2004.02.038.

Slee, E. A., Keogh, S. A., & Martin, S. J. (2000). Cleavage of BID during cytotoxic drug and UV radiation-induced apoptosis occurs downstream of the point of Bcl-2 action and is catalyzed by caspase-3: A potential feedback loop for amplification of apoptosis-associated mitochondrial cytochrome c release. *Cell Death Differentiation*, 7(6): 556-565.

Tang, P. S., Mura, M., Seth, R., & Liu, M. (2008). Acute lung injury and cell death: How many ways can cells die? *American Journal of Physiology Lung Cell Molecular Physiology*, 294: L632-L641. Doi: 10.1152/ajplung.00262.2007.

Tickner, J. A., T. Schettler, T. Guidotti, M. McCally, & M. Rossi. 2001. Health risks posed by use of di-2-ethylhexyl phthalate (DEHP) in PVC medical devices: A critical review. *Am. J. Ind. Med.* 39: 100 –111.

Tsujimoto, Y., & Shimizu, S. (2007). Role of the mitochondrial membrane permeability transition in cell death. *Apoptosis*, 12: 835-840. Doi: 10.1007/s10495-006-0525-7.

Wahl, H. G., Hong, Q., Hildenbrand, S., Risler, T., Luft, D., & Liebich, H. (2004). 4-Heptanone is a metabolite of the plasticizer di-(2-ethylhexyl) phthalate (DEHP) in haemodialysis patients. *Nephrology Dialysis Transplantation*, 19: 2576-2583. Doi: 10.1093/ndt/gfh425.

Wang, X. (2001). The expanding role of mitochondria in apoptosis. *Genes and Development*, 15: 2922-2933.

Wichert Grande, S., Andrade, A. J. M., Talsness, C. E., Grote, K., & Chahoud, I. (2006). A dose-response study following in utero and lactational exposure to di(2-ethylhexyl) phthalate: Effects on female rat reproductive development. *Toxicological Sciences*, 91: 247-254. Doi: 10.1093/toxsci/kfj128.

Wittassek, M., and Angerer, J. (2008). Phthalates: Metabolism and exposure. *International Journal of Andrology,* 31: 131-138. Doi: 10.1111/j.1365-2605.2007.00837.x.

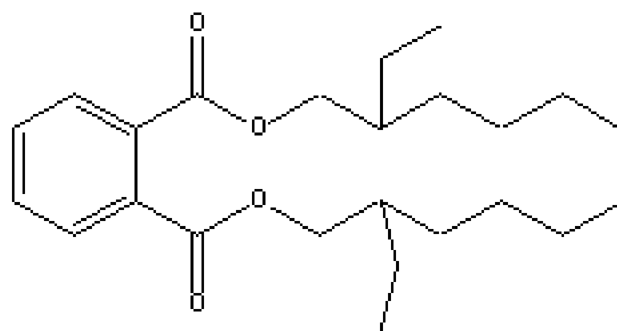
Wood-Allum, C. A., Barber, S. C., Kirby, J., Heath, P., Holden, H., Mead, R., Higginbottom, A., Allen, S., Beaujeux, T., Alexson, S. E., Ince, P. G., & Shaw, P.J. (2006). Impairment of mitochondrial anti-oxidant defence in SODI- related motor neuron injury and amelioration by ebselen. *Brain,* 129: 1693 – 1709. Doi: 10.1093/brain/awl118.

World Health Organization. (2000). IARC monographs on the evaluation of carcinogenic risk to humans, 77, 41.

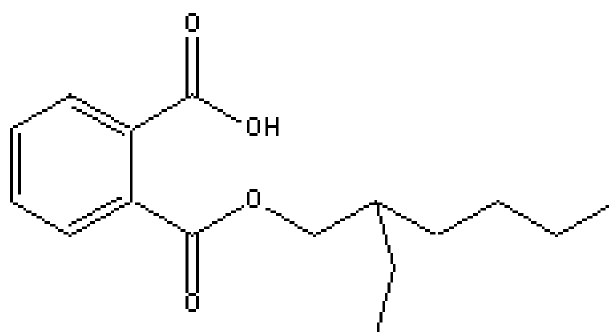
Zong, W.X., Li, C., Hatzivassiliou, G., Lindsten, T., Yu, Q. C., Yuan, J., & Thompson, C. B. (2003). Bax and bak can localize to the endoplasmic reticulum to initiate apoptosis. *The Journal of Cell Biology*, 162(1): 59-69. Doi: 10.1083/jcb.200302084.

Zhivotovsky, B., & Orrenius, S. (2005). Caspase-2 function in response to DNA damage. *Biochemical and Biophysical Research Communications*, 331: 859-867. Doi: 10.1016/j.bbrc.2005.03.191.

FIGURES



Di-ethylhexyl phthalate



Mono ethylhexyl phthalate

Figure 1. Diagrams of the di-ethylhexyl phthalate (DEHP) and mono ethylhexyl phthalate (MEHP) molecules.

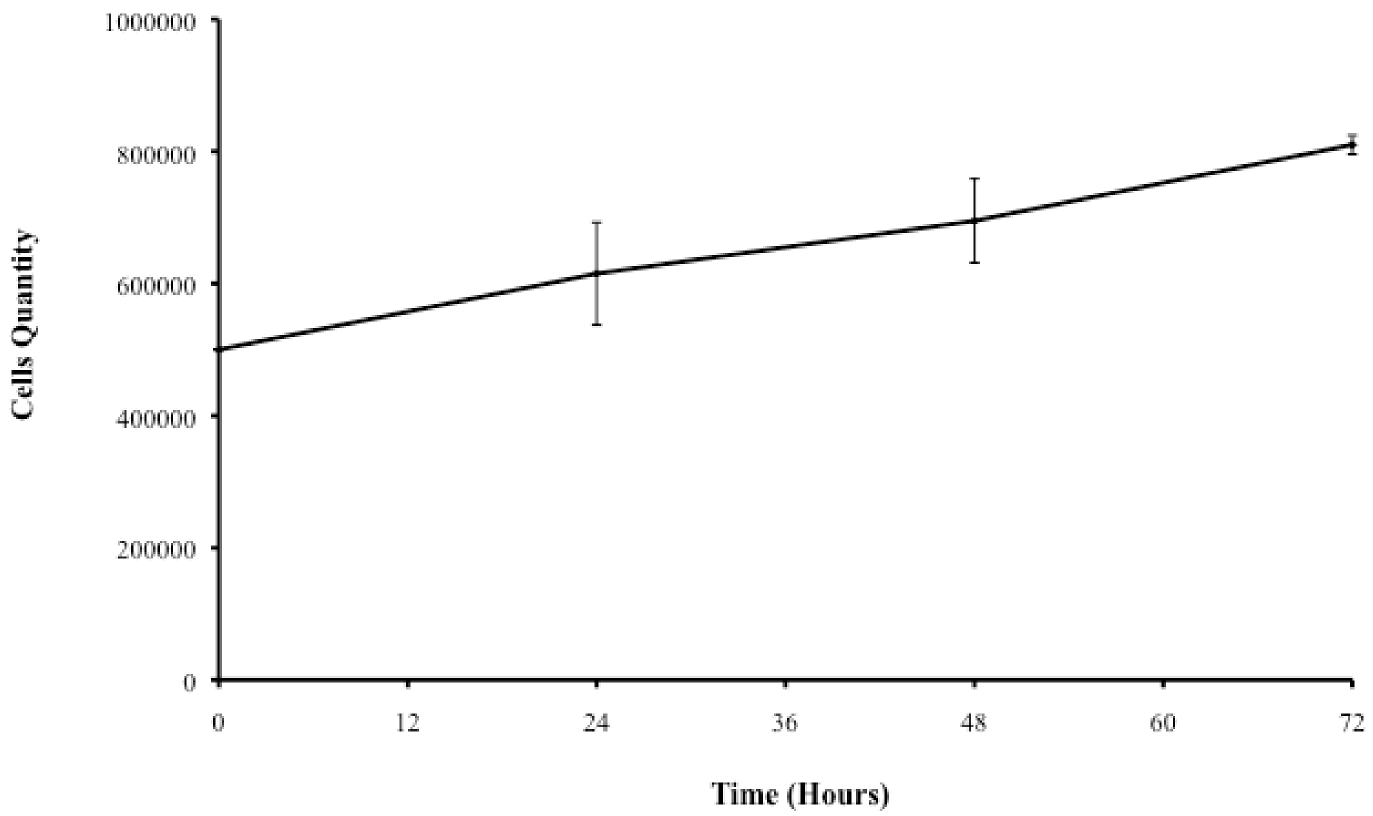


Figure 2. TK6 cells growth curve.

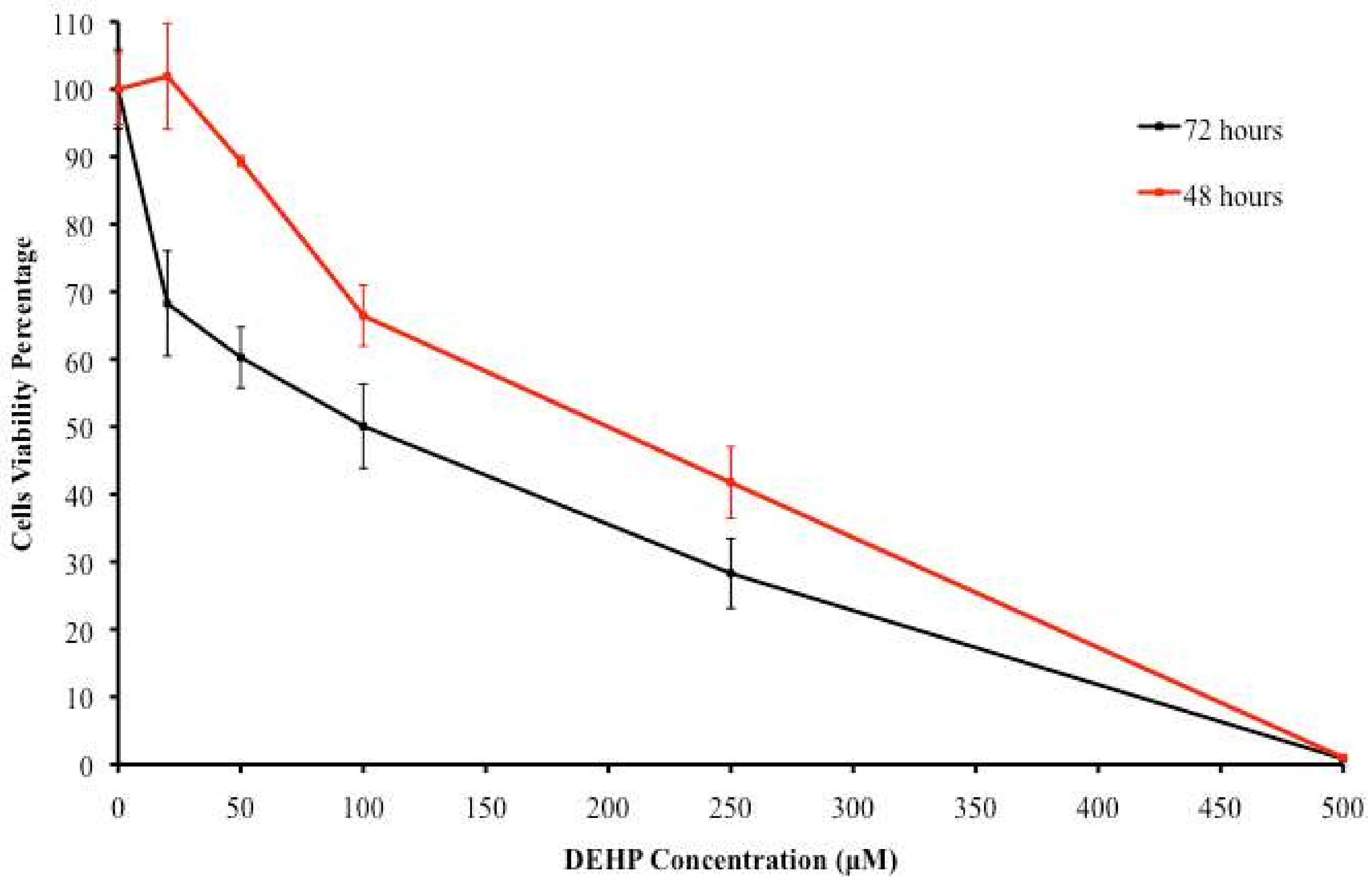


Figure 3. TK6 cells viability after exposure to di-ethylhexyl phthalate.

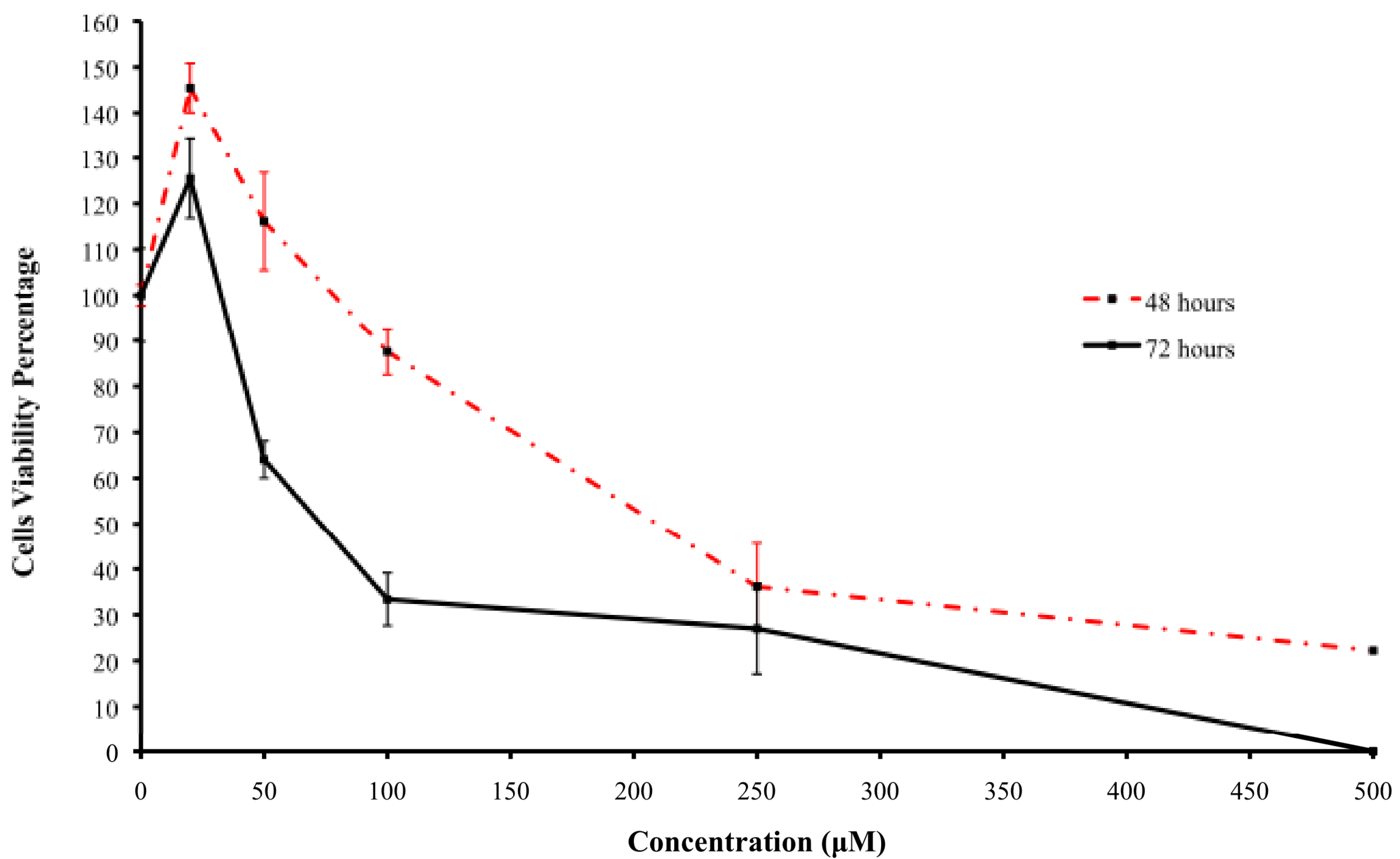


Figure 4. TK6 cells viability after exposure to mono ethylhexyl phthalate.

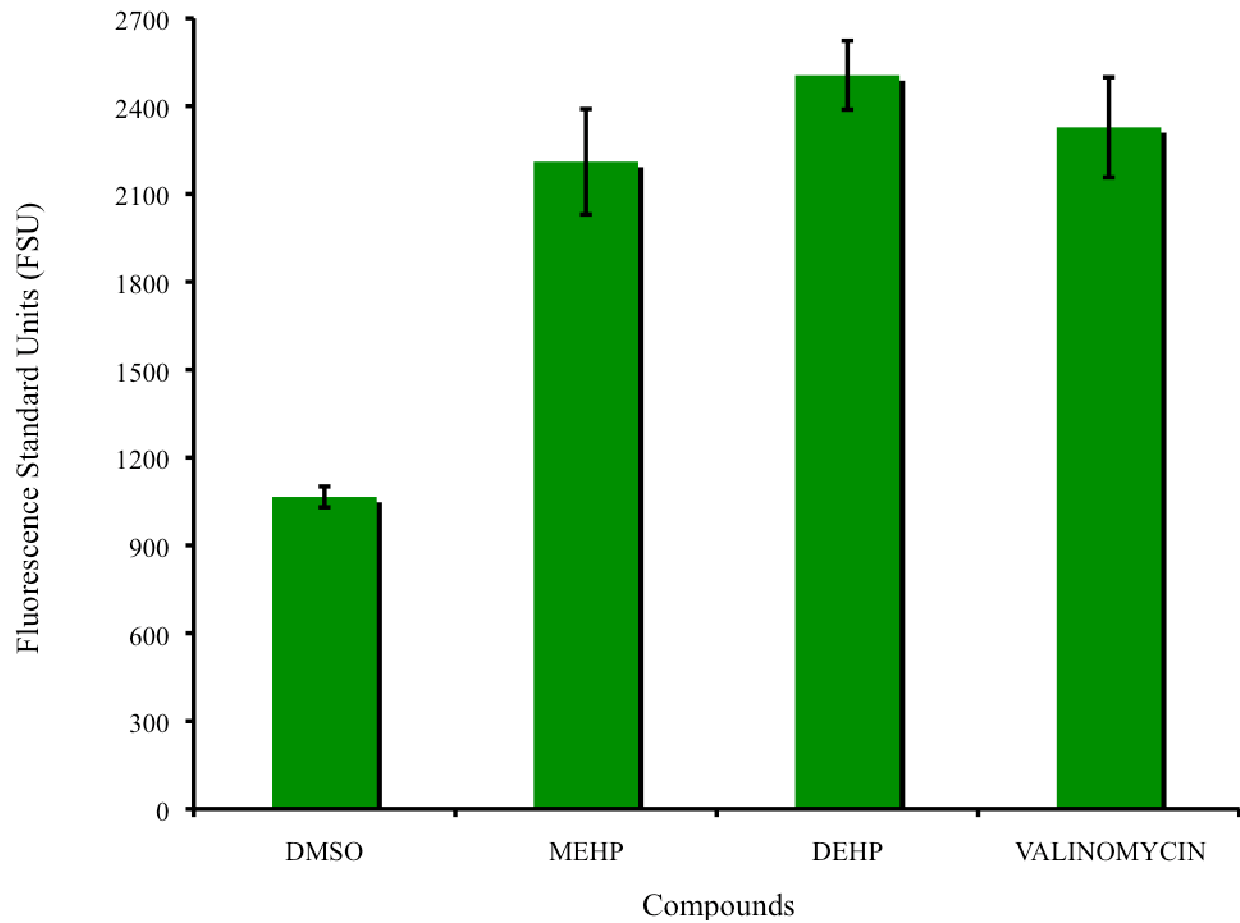


Figure 5. Mitochondrial membrane permeabilization on TK6 cells after exposure to DEHP and MEHP.

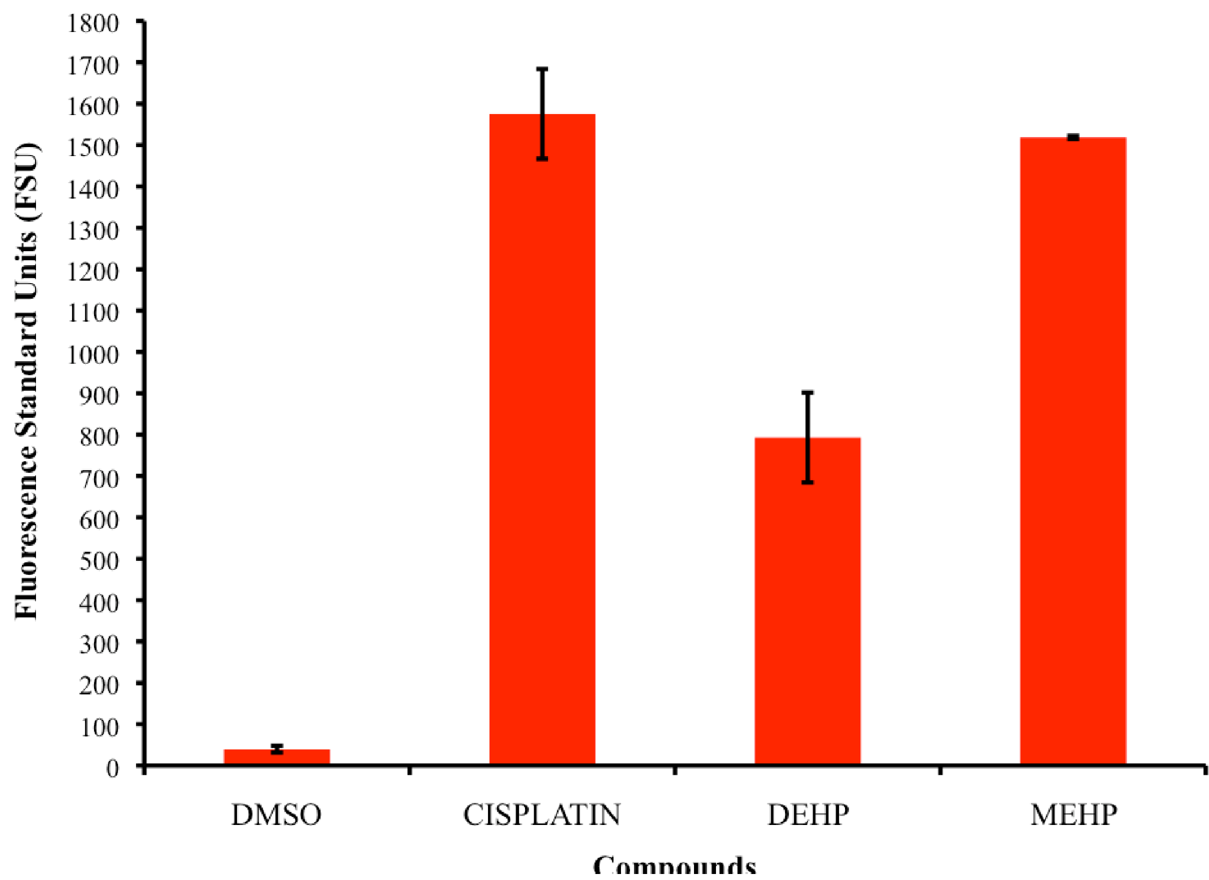


Figure 6. Reactive oxygen species generation on TK6 cells after exposure to DEHP and MEHP.

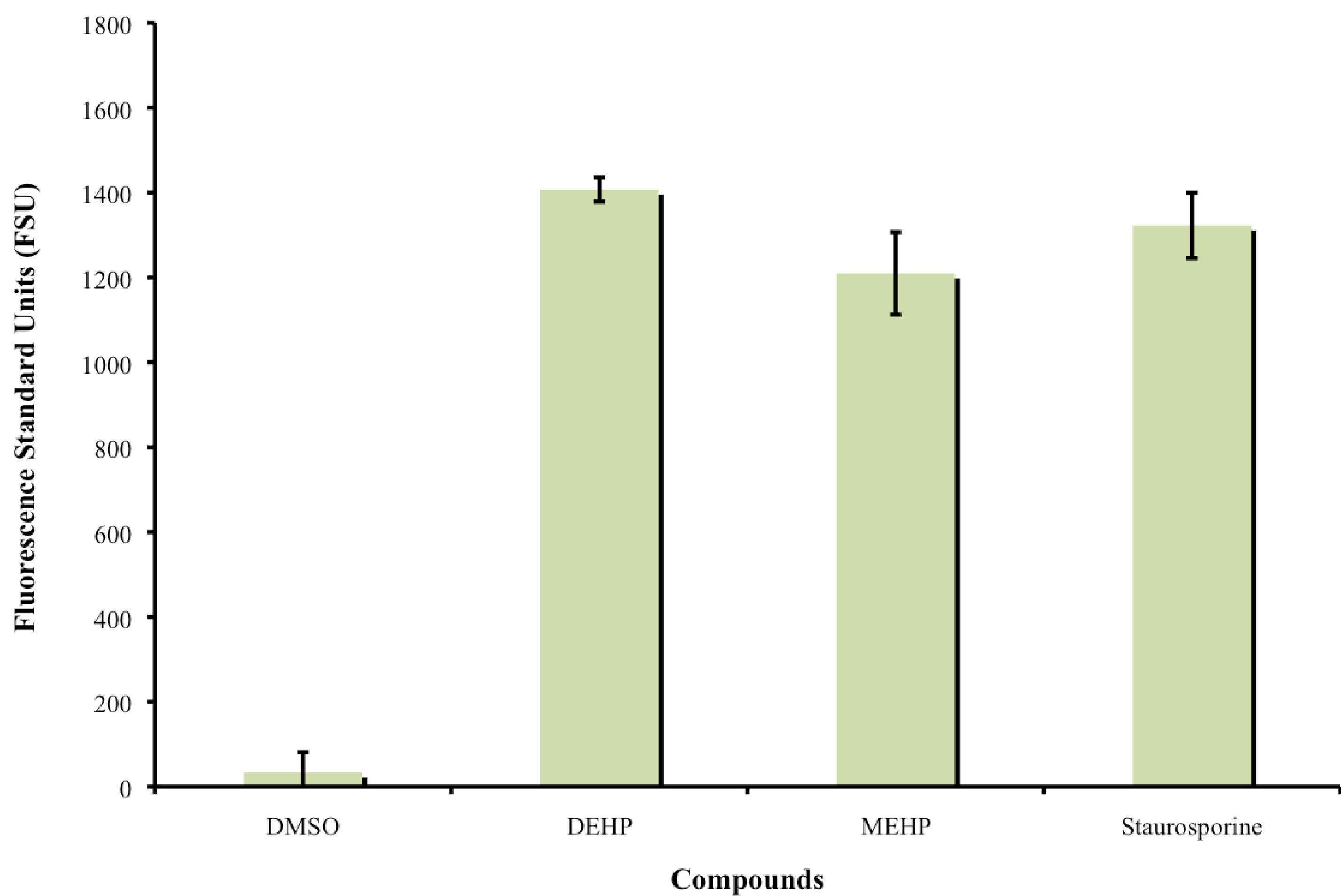


Figure 7. Caspase 3 and 7 activation on TK6 cells after exposure to DEHP and MEHP.

APPENDIXES

PROTOCOLS

Appendix 1. Phthalate's Solution Preparation and Calculation Protocol

Phthalates	Molecular Weight (MW)
DEHP	390.56g/mol
MEHP	278.34g/mol

2 x 10^{-3} mol/L (MW g/mol) = mg/mL

i. DEHP 2x 10^{-3} mol/L (390.56g/mol) =

ii. MEHP 2x 10^{-3} mol/L (278.34g/mol =

Appendix 2. Cell treatment protocol

1. Fill culture flasks with 2.0mL of media.
2. Calculate the required volume of cell aliquot in order to seed the nearest amount of $1x10^6$ cells per flask (Appendix 4).
3. Add the calculated cell aliquot volume (μL), and the volume necessary from the stock solution to obtain the desired concentration of the tested compound.
4. Add media up to a final volume of 3.5mL per each $25cm^2$ flasks.

Appendix 3. Pelleting Protocol

1. Place the conical tubes in the centrifuge at 800 rpm for 3 minutes.
2. Remove from centrifuge and check for a semitransparent pellet at the bottom of the tube.
3. Carefully remove the media (supernatant), with a pipette without disturbing the pellet.
4. Add 1ml of PBS to the pellet without disturbing the pellet. This step is to wash any residue of old media.
5. Again place the conical tubes in the centrifuge at 800 rpm for 3 minutes.
6. Carefully remove the supernatant without disturbing the pellet.
7. Re-suspend the pellet with X*mL of media to dissolve the pellet uniformly.
8. Vortex the re-suspended pellet until it is visually dissolved.
9. Count the cells in the aliquot following the cell viability protocol.

* = Amount might change depending the size of the pellet.

Appendix 4. Cell Viability Protocol (Trypan Blue Cell Exclusion)

1. From previously prepared cellular solution transfer with a micropipette 100μL of cell suspension to a well of a 96 well plate.
2. Add 100uL of Trypan Blue stain to the same well for a final 1 to 1 dilution.
3. Wash the hemocytometer and the cover slip with methanol and dry it with a Kimwipe. Transfer 10μL of the Trypan blue-cell mixture to each of the sides of the hemocytometer.
4. Count the viable cells using the Countess™automated cell counter (Invitrogen Corp. Carlsbad, California) following the equipment intructions.

Mean of cells counted in the hemocytometer =

i) (Mean of side one + Mean of side two)/2

ii) From the result of the previous equation, determine then the cell population that was originally in the flasks.

Cells in the aliquot = [(Mean of cells) x 10^4 x 2 x (X* mL)]

* = Amount might change depending on the volume used to re-suspend the aliquot.

Appendix 5. Immunochemistry's Apoptosis (MitoPT^{TM}) Adjusted Staining Protocol

Preparation of cell cultures:

1. **Reagents Pre-Preparation and Storage:**

   i) Mix 500μL DMSO with the dry concentrate of the kit dye and vortex to dissolve it.

   ii) Store the reconstituted dye in aliquots of 10μL, cover them with foil paper and keep them frozen for future use.

   iii) Store the 10 X buffer solution in aliquots of 2mL. Each aliquot will be good to work with 4 samples.

2. **Cell Culture Preparation:**

   i) Follow the cell viability protocols.

   ii) Expose cells to the phthalates following the exposure protocol. Valinomycine 11μM was used as positive control

3. **Reagent Preparation Procedure:**

   i) Warm the 10 X buffer solution at 37°C in a water bath.

   ii) Mix the warmed 10 X buffer solution with DI water in order to dilute it 1/10. Keep the 1X buffer solution warm at 37°C in a water bath.

   iii) In a small eppendorf vial mix 10μL of Mito PT dye with 990μL of the 1X buffer solution. This will be the ready-to-use Mito PT dye and it will be good to stain two samples.

   iv) Centrifuge the contents at 1,000 rpm for 15 minutes. This will prevent bubbles and will mix evenly the buffer and dye.

v) Vortex the ready-to-use Mito PT dye until completely dissolved.

4. **Staining the cells:**

i) Count cells following the cell viability protocol.

ii) Once the number of cells for treatment is estimated, calculate the volume needed to prepare aliquots of 5 x 10^5 cells following the culture count protocol. For each exposed aliquot, two aliquots of 5 x 10^5 cells will be prepared; one will be stained and the other one will not be stained.

iii) To stain the cells, centrifuge and pellet the cells and extract the culture media and re-suspend the cells in 500μL of the ready-to-use Mito PT dye solution and incubate for twenty (20) minutes and 495μL of 1X buffer solution with 5μL of DI water to the non-stained samples. (Keep the rest of the unused buffer warm since it will be used now for a series of three washes).

5. **Quantitative Fluorescence Procedure:**

i) Ten minutes before the end of the incubation time turn on the Modulus fluorometer with the green and red filter adapters. The green filter will capture the fluorescence of the healthy cells while the red filter will capture the fluorescence of the apoptotic cells.

ii) Access the settings menu in order to perform three measurements in 1/3 of a second each time.

(1) Measure fluorescence on the mini-cell cuvette adaptor (200μL working volume):

(a) Place the mini-cell adapter inside the cavity of the filter that will be used.

(b) Take the non-stained cells suspended with 500μL of 1X buffer solution and transfer 5μL of each sample contents to individual glass mini-cell curvettes with 195μL of 1X buffer solution and measure the fluorescence of all the non-stained samples with the green filter, then measure the fluorescence of the non-stained samples with the red filter. After the incubation centrifuges the stained cells, discard the supernatant and re-suspend the stained cells in 500μL of 1X buffer solution. (Repeat this three times)

(c) Place the glass mini-cell curvettes in the mini-cell adaptor and measure fluorescence of the stained cells with 195μL of 1X buffer solution and 5μL of each sample contents.

iii) Once all samples have been measured in the green filter, switch to the red filter adapter and turn on the equipment. Wait ten minutes and record samples readings.

Appendix 6. Reactive Oxygen Species Generation Determination Protocol

1. **Cell Culture Preparation:**

   i) Follow the cell viability protocols.

   ii) Expose the cells to the phthalates IC_{50} following the exposure protocol (for 48 hours) with the positive control 13µM Cisplatin.

2. **Staining the cells:**

   i) Count cells following the cell viability protocol.

   ii) Once the number of cells to be exposed is estimated, calculate the volume needed to prepare aliquots of 5×10^5 cells following the culture count protocol. For each exposed aliquot, two aliquots of 5×10^5 cells will be prepared; one will be stained and the other one will not be stained.

   iii) To stain the cells, suspend the 5×10^5 cells pellet in 500µL of culture media of 2,7 –dichlorofluorescein diacetate (DCFH-DA) with 40µM (10µL to each 500µL) and incubate for 30 minutes.

3. **Qualitative Fluorescensce Microscope Procedure**

   i) Ten minutes before the end of the incubation time turn on the microscope light with the green and red filters to be used.

   ii) Start the QCapture program.

   iii) Take 10µL of the stained cells after washing them three times and place them on a glass slide and cover it with an slide cover and put it under the microscope with the G filter with excitation between 480-550nm.

4. **Quantitative Fluorescensce Procedure**

i) Ten minutes before the end of the incubation time turn on the Modulus fluorometer.

ii) After incubation with DCFH-DA wash with PBS (Two-Three times) and re-suspend in 500µL of PBS. The non-stained cells are also suspended in 500µL of culture media, washed with PBS and re-suspended in 500µL of PBS.

iii) To measure the fluorescence in the glass mini-cell curvettes, re-suspend 5µL of the cell suspension (stained cells or not stained cells) in 195µL of PBS then place the glass minicurvette in the mini-cell adaptor and measure the fluorescence using the green filter.

Appendix 7. Immunochemistry's Adjusted Qualitative and Quantitative Caspases 3 & 7 Evaluation Protocol

Preparation of cell cultures:

1. **Reagents Pre-Preparation and Storage:**

   i) Reconstitute the Magic Red dye by adding 50µL of culture grade DMSO to the vial (for the 25 test). Vortex to mix properly and store it in aliquots of 10uL (5 samples each aliquot). Aliquots will be good up to 6 months.

2. **Cell Culture Preparation:**

   i) Once the number of cells in the aliquots is estimated, calculate the volume of aliquot needed to seed $1x10^6$ cells following the culture count protocol.

   ii) Seed cells and expose cells to the respective phthalate compound following the exposure protocol and place samples in the incubator at 37°C and 5% CO_2. The positive control use is one of the recommended by the manufacturer of the Assay, Staurosporine 1µM for three hours.

3. **Reagent Preparation Procedure:**

Perform all of the following without light and protecting reagents with foil paper.

   i) Warm the 10µL of concentrated Magic Red dye at 37°C in a water bath.

   ii) Mix the warmed 10µL Magic Red dye with 40uL of MS-Water and vortex for 2-5 seconds in order to dilute it. (This will now be the ready to use Magic Red solution).

iii) Vortex the ready to use Magic Red solution until completely dissolved.

iv) Centrifuge the contents at 1,000g for 15 minutes. This will prevent bubble formation and will mix evenly the solution.

v) Let it warm for 10 minutes and vortex again until mixture becomes evenly distributed. This will work for 4 samples.

vi) Prepare an aliquot of 50mL of PBS and keep it warm at 37°C in a water bath.

4. Staining the cells:

i) Pellet and count cells following the pellet and cell viability protocols.

ii) Once the number of cells of the exposed aliquots is estimated, calculate the volume needed to prepare aliquots of $5x10^5$ cells following the culture count protocol. For each exposed aliquot, two aliquots of $5x10^5$ will be prepared; one will be stained and the other one will not be stained.

iii) Centrifuge and remove the supernatant.

iv) Add 300µL to each one of the eppendorf tubes and vortex for 2-5 seconds to distribute the cells evenly.

v) Add 10µL of the diluted dye to the samples and vortex again for 2-5 seconds.

vi) Incubate the samples for an hour. Vortex each of the samples 20 minutes to assure that the dye is well distributed.

vii) Once the staining is completed, vortex and centrifuge. Remove and discard the supernatant.

ix) Add 300µL of pre-warmed PBS and vortex each sample for 2-5 seconds.

Centrifuge at maximum speed (13,400 rpm) for one minute. Remove and discard the supernatant. Repeat this step once more.

5. Quantitative Fluorescence Procedure:

Measure the fluorescence of the Magic Red (Caspases 3 & 7) in the Modulus flourometer by using the green filter. Make sure that the readings are performed three consecutive times by selecting continuous samplings from the Main Menu.

i) To Measure fluorescence on the mini-cell curvette adaptor (200μL working volume):

(1) Add 300μL of PBS and mix well in the eppendorf by slowly pipeting up and down. Transfer 195μL of PBS to the mini- cell curvettes and 5μL of the cell samples. Place the mini-cell adaptor on the green filter. Measure continuously each sample three times.