

**The Effects of Penta-Brominated Diphenyl Ether (penta-BDE, BDE-99) on Human
Embryonic Kidney (HEK293) Cells**

By

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Abstract

Individuals are exposed to toxic chemicals on a daily basis. Some toxic chemicals produce reactive oxygen species (ROS), which contribute to protein modification and the accumulation of protein carbonyl groups. The antioxidant response to ROS involves the binding of nuclear factor-erythroid 2 subunit-related transcription factors (NFE2L or Nrf) to antioxidant response elements (AREs)/electrophile response elements (EpREs), located in the promoter and enhancer regions of antioxidant and detoxification enzymes.

Activation of the AREs/EpREs pathway by Nrf proteins, in response to penta-brominated diphenyl ether (penta-BDE, BDE-99) flame retardant, was observed. Protein damage (as assessed protein carbonyl assays), lipid damage (as assessed by oxidation assays), and the activation of the beta-galactosidase enzyme occurred as a results of penta-BDE treatment. These results provide insight into how penta-BDE a) initiates the antioxidant response, b) damages proteins and lipids through ROS and c) increases beta-galactosidase activity.

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List of Abbreviations

AChE – 8-isoprostane-acetylcholinesterase

ARE – Antioxidant response element

ATCC – American Type Culture Collection

ATP – Adenosine triphosphate

ATSDR – Agency for Toxic Substances and Disease Registry

Bach – BTB-basic leucine zipper transcription factors

bZIP – Basic leucine zipper domain

CAT – Catalase

CBP – CREB-binding protein

C₁₂FDG – 5-Dodecanoylamino fluorescein Di-β-D-Galactopyranoside

CNC – Cap'-n'-collar

CPRG – Chlorophenol red-β-D-galactopyranoside

CREB – cAMP response element-binding protein

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

DNPH – 2, 4-dinitrophenylhydrazine

DTT – Dithiothreitol

EpRE – Electrophile response element

ER – Endoplasmic reticulum

FBS – Fetal bovine serum

Gclc/GCLC – Glutamate-cysteine ligase catalytic subunit

Gclm – Glutamate-cysteine ligase regulatory subunit

GCS – glutamylcysteine synthetase

Gpx1 – Glutathione peroxidase 1

Gstp1 – Glutathione S-Transferase Pi 1

Gstp2 – Glutathione S-Transferase Pi 2

Gstm3 – Glutathione S-Transferase Mu 3

Gstm6 – Glutathione S-Transferase Mu 6

Gsta1 – Glutathione S-Transferase Alpha 1

HEK293T – Human embryonic kidney cells transfected

Hmox1 – Heme oxygenase 1

H₂O₂ – Hydrogen peroxide

Keap1 – Kelch-like ECH-associated protein 1

LOAEL – Lowest observed-adverse-effect level

Maf – Musculo-aponeurotic fibrosarcoma oncogene

Mt1 – Metallothionein 1

Mt2 – Metallothionein 2

NAD(P)H – Nicotinamide adenine dinucleotide phosphate

NCS – Newborn calf serum

NOAEL – No-observed-adverse-effect level

Nrf – Nuclear factor-erythroid 2 subunit-related transcription factors

NWRC – National Wildlife Research Centre

NQO1 – NAD(P)H:quinone reductase

PBDE – Polybrominated diphenyl ether

PCB – Polychlorinated biphenyl

PEI – Polyethyleneimine

Penta-BDE – Penta-brominated diphenyl ether

PMSF – Phenylmethanesulfonyl fluoride

RNA – Ribonucleic acid

ROS – Reactive oxygen species

SEM – Standard error of the mean

SOD1 – Superoxide dismutase

UGT – UDP-glucuronosyltransferases

Chapter 1: Introduction

1.1: Oxidative Stress, ROS, Free Radicals

The increase in oxygen levels in the Earth's atmosphere allowed for the evolution of aerobic organisms that use dioxygen as the final electron acceptor in the process of breaking down energy sources, allowing a higher energy yield in comparison to fermentation or anaerobic respiration (Scandalios, 1993). For instance, in aerobic metabolism, the total breakdown of one molecule of glucose yields 38 molecules of the energy molecule ATP, while the anaerobic breakdown of the same one molecule yields only 8 ATPs (Scandalios, 1993). In its ground state, molecular dioxygen is relatively stable and unreactive, but it is capable of producing lethal, reactive, excited states molecules in forms of free radicals (Scandalios, 1993).

The oxygen that we breathe is converted to water in a reaction catalyzed by cytochrome oxidase of complex IV in the electron transport chain of the inner mitochondrial membrane. Cytochrome oxidase is the last electron acceptor in the chain and must give up its reducing equivalents in order to allow continued electron movement down the chain; if electron movement stops, the proton motive force dissolves and ATP production halts. Thus, in aerobic species, the key function of oxygen is to act as a sink or dump for electrons (Cadenas & Davies, 2000).

Although this system is very efficient, the very nature of alternating oxidation reduction reactions allows each electron carrier to participate in side reactions with oxygen. For example, as ubiquinone cycles between its fully oxidized and fully reduced forms, there is a chance for an electron to pass to oxygen forming oxygen radicals instead

of passing to the next electron carrier in the electron transport chain. There are several enzymes within the electron transport chain that are prone to forming oxygen radicals via side reactions with oxygen, they include: NADH dehydrogenase, NADH oxidase, succinate dehydrogenase, succinate oxidase, and ATPase. These side reactions are the major source of oxygen radicals under normal intercellular conditions (Cadenas & Davies, 2000).

In addition to these toxic reactions caused by the electron transport chain of the inner mitochondrial membrane, the enzyme monoamine oxidase of the outer mitochondrial membrane also produces large amounts of H₂O₂. This enzyme catalyzes the oxidative deamination of biogenic amines and in turn generates large amounts of reactive oxygen species (ROS) within both the mitochondrial matrix and the cytosol (Cadenas & Davies, 2000). Furthermore, there are non-mitochondrial sources of ROS in the cell, such as the enzyme NADPH oxidase, which reduces oxygen to superoxide radical and in turn produces secondary reactive oxidants, making it the main source of oxidative stress during inflammation (Wu *et al.*, 2003).

When the body produces more ROS than it can neutralize, it is said to be under oxidative stress (Valavanidis *et al.*, 2006; Davies, 1995). ROS can go on to form free radicals, which are the main drivers behind the damage to important cellular and mitochondrial molecules (Sohal *et al.*, 2002). Free radicals cause this damage by removing electrons from the body's molecules in a process called oxidation (Finkel & Holbrook, 2000). Oxidation may alter the shape of molecules leading to loss of function. Different types of ROS, such as superoxide, hydrogen peroxide, nitric oxide,

peroxynitrite, and the hydroxyl radical, cause oxidative damage to DNA, lipids, and proteins (Finkel & Holbrook, 2000).

Some studies align the capacity to neutralize H₂O₂ with the capacity to deal with oxidative stress (Oliver & Brooke, 2016; Kaneuchi *et al.*, 2003). Other studies have shown that when bovine mammary epithelial cells were treated with H₂O₂, there was a time- and dose-dependent inhibition of cell survival, accompanied with sharp increases of ROS (Jin *et al.*, 2016).

Oxidative stress can lead to the production of markers of cellular aging or, more specifically, increased number of carbonyl groups on proteins. Oxidative damage to macromolecules has been quantified by measuring protein carbonyl content (Oliver & Brooke, 2016; Dalle-Donne *et al.*, 2003; Reznick, 1994). In mice studies, researchers have shown that oxidative stress can increase the amount of carbonyl group modifications on proteins (Tanase *et al.*, 2016). Furthermore, researchers observed, using fluorescence imaging data, an increase of oxidative stress in aging mice (Tanase *et al.*, 2016).

Other studies measured the protein carbonyl content, and concluded that older cells are associated with the greater accumulation of protein carbonyl groups, in both *in vitro* and *in vivo* studies (Adachi *et al.*, 1998). As well, these studies showed a link between oxidative stress-mediated protein oxidation and aging (Adachi *et al.*, 1998). Other *in vitro* evidence shows that the amount of oxidative damage to various macromolecules, such as lipids, proteins, and DNA, increases exponentially with age in a variety of tissues in different species (Sohal *et al.*, 2002; Sohal & Weindruch, 1996). Others have demonstrated that oxidative damage to proteins must be an important factor in aging, because oxidized proteins lose catalytic and structural integrity and are

substantially broken down (Sohal *et al.*, 2002; Berlett & Stadtman, 1997; Stadtman & Levine, 2000).

The addition of carbonyl groups to proteins causes them to aggregate. Protein aggregation increases autophagy which leads to apoptosis, resulting in aggregate-related cytotoxicity. Proteins also aggregate during normal physiological aging, when proteolytic degradation by the proteasome begins to break down and cellular proteins collect and become insoluble. These proteins have important biological function and as such their loss is detrimental, leading to disorders such as neurodegenerative diseases (Tanase *et al.*, 2016). Protein modifications caused by oxidative stress also include glycation, glycooxidation, lipoxidation and carbonylation, and these have been observed in diseases such as diabetes, metabolic syndrome, chronic inflammation and degenerative conditions (Tanase *et al.*, 2016).

1.2: Antioxidant Response Element

The antioxidant response to oxidative stress starts when specific transcription factors bind to an enhancer element known as the antioxidant-response element; also referred to as the electrophile response element (ARE/EpRE), which has the consensus sequence TGCTCAGTCAT (Chepelev *et al.*, 2011a). This enhancer element is located in the promoter and enhancer regions of genes of antioxidant and phase one and two detoxification enzymes. Genes that are expressed under the control of ARE/EpRE include, heme oxygenase-1, ferritin (light and heavy chains), metallothioneines, NAD(P)H quinone (oxido) reductase, glutathione S-transferases, β -globin, and subunits of the proteasome (Chepelev *et al.*, 2011a; Jung and Kwak, 2010). The family of

transcription factors known as the nuclear factor-erythroid 2 subunit-related transcription factors (Nrf1, Nrf2, and Nrf3) regulates gene expression through binding to ARE/EpRE (Chepelev *et al.*, 2013a). These transcription factors are members of the cap'-n'-collar (CNC) basic region leucine zipper (bZIP) family of proteins. These factors bind to small Maf (musculo-aponeurotic fibrosarcoma oncogene) proteins and then go on to either activate or repress gene expression (Chepelev *et al.*, 2013a; Bugno *et al.*, 2015). Other members of the CNC-bZIP family include, Bach1 and Bach2. These proteins also form heterodimers with Maf proteins, but act as dominant repressors of the ARE/EpRE genes (Chepelev *et al.*, 2011a; Bugno *et al.*, 2015).

1.3: Nuclear Factor-Erythroid 2 Subunit-Related-1 (Nrf1)

Nrf2 has been shown to be inhibited by Kelch-like ECH-associated protein 1 (Keap1), which it associates with in the cytosol and must be released from in order to be activated by cAMP response element-binding protein (CREB)-binding protein (CBP) (Kawai *et al.*, 2011). CBP acetylates two lysines on Nrf2, which releases it from Keap1 and allows it to travel to the nucleus (Kawai *et al.*, 2011; Cosgrove *et al.*, 2006; Zhang *et al.*, 2006). In the nucleus Nrf2 associates with co-activating small Maf proteins and then binds the ARE/ EpRE to express antioxidant genes. Genes specifically regulated by Nrf2 include NAD(P)H:quinone reductase (NQO1), glutamylcysteine synthetase (GCS), UDP-glucuronosyltransferases (UGT), epoxide hydrolase (Thimmulappa *et al.*, 2002).

The Nrf1 protein is an important activator when the cell is under oxidative stress (Kwong *et al.*, 1999). In its full length (p120) form, Nrf1 is anchored to the endoplasmic reticulum (ER) by its N terminal targeting sequence (Zhang *et al.*, 2007), and is released

by deglycosylation of its Asn/Ser/Thr-rich (NST) domain (Zhang *et al.*, 2007; Zhang *et al.*, 2009). Before entering the nucleus, Nrf1 must go through further positive regulation by proteolytic cleavage (Wang & Chan, 2006), and it is the fragments that bind the small Mafs, then bind to the ARE/EpRE and transactivate gene expression. The active nuclear form is p95 Nrf1, while the repressor nuclear form is the p65 Nrf1 (Chepelev *et al.*, 2013b). Other forms and their functionality include: activator p140, activator p120, partial repressors p55, repressor p46, p36, and p25 (Bugno *et al.*, 2015). These forms either need further processing to become the shorter nuclear active p95 form, or have gone through too much cleavage, where they have lost their transactivation domains. p65 Nrf1 has the DNA-binding domains and is able to bind the ARE/EpRE, but lacks one of the transactivation domains, causing it to act as a dominant negative inhibitor of full length Nrf1 and Nrf2, stopping them from binding to ARE (Chepelev *et al.*, 2013b).

The genes activated by Nrf1, when the cell is under oxidative stress, include but are not limited to: glutamate-cysteine ligase catalytic subunit (glutathione synthesis enzyme) (Gclc), glutamate-cysteine ligase regulatory subunit (glutathione synthesis enzyme) (Gclm), glutathione peroxidase 1 (selenium-dependent enzyme that catalyzes the breakdown of hydrogen peroxide) (Gpx1), heme oxygenase 1 (enzyme involved in heme degradation) (Hmox1), metallothioneins 1 and 2 (metal-binding proteins) (Mt1 and Mt2), and different forms of glutathione S-transferase (involved in detoxification) (Gstp1, Gstp2, Gstm3, Gstm6, and Gsta1) (Bugno *et al.*, 2015).

Studies show Nrf1 to be essential for development. Nrf1 but not Nrf2 global knockout mice, are embryonic lethal after embryonic day 12.5 (Bugno *et al.*, 2015). Other studies show an overlap between Nrf1 and Nrf2 function in liver development where

double mice mutants died before embryonic day 10 (Bugno et al., 2015). These effects are linked with impaired expression of antioxidant genes, oxidative stress, and increased sensitivity to death via oxidant generation (Bugno et al., 2015).

1.4: Cellular Senescence

Among many theories, the genetic theory of aging states that there are programmed signals with timed functional changes that cause the cell to age, through the shortening of telomeres and declines in hormonal and immunologic function (Tosato *et al.*, 2007). After a certain period of time, our bodies send out a signal to reduce the repair process and to start the degradation process (Tosato *et al.*, 2007). The specific time is different for different organisms; for example a fruit fly lives for 30 days (Carey *et al.*, 1998), a mouse for 3 years (Langston *et al.*, 1999), a turtle for 100 years (Gibbons, 1987), and a Redwood tree lives for 1000 years (Lewington & Parker, 1999). These signal genes are called genetic timers and they are specific sequences of DNA that govern each stage of the body's development and determine the next appropriate step in the aging process (Olovnikov, 2003). These genes keep track of the body's progress and thus control the age at which certain events occur (Hayflick Limit) (Tosato *et al.*, 2007). There are other genes that are called death genes, and these genes tell the body to deteriorate and die (Saltsman, 2005).

Chemical pollutants that produce more ROS than the body's antioxidant defenses can neutralize will cause oxidative stress leading to inflammation, tissue damage, disease, and aging. This can also contribute to protein modification and the accumulation of protein carbonyl groups, which is often used as an indicator of cellular aging (Sohal *et*

al., 2002). ROS can also go on to form free radicals, which are the main reason behind the damaging effects leading to aging and age-related disease (Sohal *et al.*, 2002). Because free radicals are highly reactive, they cause damage by reacting with the body's molecules and by extracting electrons, thus damaging the macromolecule and causing a chain reaction of radical formation (Finkel & Holbrook, 2000). This damage may cause excess blood clotting, cataracts, and atherosclerosis (Finkel & Holbrook, 2000).

Although mild amounts of oxidative damage, such as that experienced during exercise, can be easily combated by the body's antioxidant defenses, such toxic reactions contribute greatly to the aging process and form the central dogma of the Free Radical Theory of Aging (Finkel & Holbrook, 2000). This theory states that ROS produce free radicals, which go on to damage biomolecules via oxidation and cause aging and age-related diseases (Beckman & Ames, 1998). Cellular and mitochondrial DNA, RNA, and proteins are subject to modifications by oxidative stress, and these oxidatively damaged products are selectively degraded by nucleases and proteases. Apoptosis induced by oxidative stress involves an early break down of mitochondrial polynucleotides (both DNA and RNA), most likely by a calcium-activated mitochondrial nuclease (Finkel & Holbrook, 2000). This process comes before apoptotic nuclear DNA fragmentation but occurs at the same time as the increase of mitochondrial membrane permeability and the release of mitochondrial cytochrome *c*. Partially digested mitochondrial polynucleotides and released cytochrome *c* play similar roles in that they both activate pro-apoptotic enzymes (Finkel & Holbrook, 2000). Studies have shown that oxidative damage to mitochondrial enzymes, and to the mitochondrial genome itself, have a huge impact on many age-related degenerative processes (Finkel & Holbrook, 2000).

Several studies have shown that oxidatively damaged lipids tend to accumulate with age, due to decrease in functionality of antioxidant enzymes, weakened repair systems, and excess amounts of damaging ROS (Wang *et al.*, 1995; Pacifici & Davies, 1991). Growing evidence shows that oxidative damage is a factor that contributes to the aging process, though it is one of several factors thought to be involved in the process (Pacifici & Davies, 1991).

Studies done in marrow stromal cells examined changes in bone mass during the aging process and the effects that lipid oxidation might have on bone aging (Parhami, 2003). It was observed that oxidized lipids take part in the gradual impairment of premature bone cells' potential to differentiate. These damaging actions are pronounced with age and in the age-related disease osteoporosis. The agent isoprostane 8-isoprostaglandin E₂ (which is an isoprostane similar to 8-*iso* prostaglandin F_{2α}; both produced from the oxidization of arachidonic acid) has been implicated in the inhibition of osteoblastic differentiation (Parhami, 2003). It has been reported that an increase in age correlates with an increase in lipid peroxide levels, which also correlates with an increase in the levels of ROS, promoting the oxidative breakdown of cellular molecules (Beckman & Ames, 1998). Oxidative damage to cellular biomacromolecules may accelerate the aging process and this damage may promote the generation of more ROS in a forward feedback loop. In turn, oxidatively damaged biomolecules (ex. lipids), cause negative effects on bone development and reabsorption, leading to the reduction in bone mass and density (Parhami, 2003).

Other studies have shown that with senescence comes an accumulation of lipofuscin (partial lysosomal degradation of lipids), the alteration of antioxidant defense

systems, and the decrease of gene expression involved in antioxidant defenses (Jervis & Robaire, 2004). Jervis and Robaire (2004) looked at oxidative stress and aging in the rat epididymis and observed all these effects. They concluded that oxidative stress is associated with the aging process, that there is an age-related accumulation of oxidative damage, and that there is increased longevity with the lessening of oxidative stress (Jervis & Robaire, 2004).

1.5: Polybrominated Diphenyl Ethers

Polybrominated diphenyl ethers (PBDEs) are manufactured flame retardants that are used in many products including insulation, textiles, building materials, plastics, electronics, and foams (Frouin, *et al.*, 2010). Large quantities of these chemicals were produced and distributed in the past decades, resulting in widespread contamination of the environment, and bioaccumulation in foods, and are still persistent in the environment (Lefevre *et al.*, 2015). PBDEs and their metabolites have been found in blood, adipose tissue, and the liver of fish, birds and mammals, as well as in human blood, serum, adipose tissue, breast milk and placental tissue (Agency for Toxic Substances and Disease Registry (ATSDR), 2004). Constant exposure from house dust and breast milk leads to toddlers and infants having the highest body burdens of these chemicals, refer to Table 1, which shows the presence of these chemicals in placental tissue from various countries (Tagliaferrir *et al.*, 2010; Ma *et al.*, 2012). *In vitro* studies on neuronal cells show that PBDEs induce oxidative stress by altering the intracellular oxidant/antioxidant balance, and others have shown that they cause apoptotic cell death (Tagliaferrir *et al.*,

2010). *In vivo* studies show PBDEs-induced neurotoxic effects, in particular in the early stages of brain development (Tagliaferrir *et al.*, 2010).

Table 1: The presence of flame retardants (BDE-47 and BDE-99) in the placental tissues sampled from various countries (ng/g weight) (Ma *et al.*, 2012)

Country	N	BDE-47	BDE-99	Reference
China	130	4.6 x 10 ⁻³	3.5 x 10 ⁻³	Ma et al., 2012
Spain	100	0.25	0.12	Gomara et al., 2007
Denmark	50	0.32	0.23	Frederiksen et al., 2009
USA	5	0.49	0.46	Dassanayake et al., 2009

According to the ATSDR's report in 2004, PBDEs and their metabolites cause oxidative stress through the production of free radicals and ROS (ATSDR, 2004). These products of oxidative stress are the main reason behind the damage to biomolecules that may lead the cell to aging or may cause the organism to develop age related diseases. Organisms have low molecular weight antioxidants, as well as antioxidant enzymes, in their cells that protect from such damage, but over time, even these are depleted or damaged themselves, leading to cellular aging (ATSDR, 2004).

The ATSDR has no-observed-adverse-effect levels (NOAELs) and lowest observed-adverse-effect levels (LOAELs) values based on animal models for many PBDEs congeners. These are a NOAEL of 1 mg/kg/day and a LOAEL of 3 mg/kg/day (ATSDR, 2004).

1.6: Toxicological Background of PBDEs

PBDEs are widely distributed throughout the environment and have been found in all biotic and abiotic matrices worldwide (Letcher *et al.*, 2014). Higher molecular weight congeners are less bioactive and tend to persist in the environment, while lower molecular forms (tri- to hexa-BDEs) are almost completely absorbed and slowly eliminated (McDonald, 2002). Under UV light exposure, higher molecular weight PBDEs are easily degraded to lower molecular weight PBDEs and thus with time chemicals that have accumulated in the soil become bioactive and are leached into the water ways or into the air (Shih & Wang, 2009). There is evidence of PBDE transport via air, in similar manner as chlorinated dioxins and furans (McDonald, 2002).

Previous studies of PBDEs concentrations in humans reported levels of up to 400 ng/g in lipid tissues, and this was shown to be on the rise in some populations (McDonald, 2002). Based on toxicity data, structural similarities, and mechanistic similarities to polychlorinated biphenyls (PCBs), the toxic endpoints associated with PBDEs are likely to be thyroid hormone disruption, neurobehavioral toxicity, and for some congeners cancer (McDonald, 2002).

The mechanisms behind PBDE toxicity are still unclear, with some studies suggesting that the hydroxylated metabolites (OH-BDE) are the main cause of oxidative stress being associated with cellular damage and apoptosis (Ji *et al.*, 2011). These metabolites have also been connected to DNA damage via the blocking of replication and subsequent chromosomal breaks (Ji *et al.*, 2011).

As PBDEs are simple additives to polymers, they can easily leach out of the product and into the environment (Costa *et al.*, 2008). PBDEs have been shown to be persistent organic pollutants that bioaccumulate in the environment, biomagnify up the food chain, and can be detected in high amounts in animals and humans (Costa *et al.*, 2008). The main sources of chemical exposure are diet based, where PBDEs can leach out of plastic containers and into food, though indoor air and occupational exposure have been documented. The highest concentrations of contaminants among foods are in fish (3276 pg/g), meats (1378 pg/g), and dairy products (485 pg/g) (Costa *et al.*, 2008). Regardless of the type of food, exposure to PBDEs from foods is the highest in the U.S. (60- 84 ng/day), and Europe (38-97 ng/day) (Costa *et al.*, 2008).

Among humans, infants have the highest body burden of PDBEs. PBDEs have been detected in human breast milk, with levels of 956 ng/g in North America in 2005,

and 24.6 ng/g in Europe in 2003 (Costa *et al.*, 2008). As well as in breast milk, PBDE levels in serum have been increasing in the past few decades in the general population (Costa *et al.*, 2008).

PBDEs are distributed to the liver, kidneys, and the thyroid gland after long term exposure. Different PBDEs have different toxicities with the larger deca-BDE being the least potent and least bioactive, while the smaller congeners are more easily absorbed, metabolized, and eliminated. For example, the NOAEL for deca-BDE is in the grams/kg/day, while for penta-BDE it is in the milligrams/kg/day. (Costa *et al.*, 2008)

Toxicokinetic studies in animals have shown that absorption, metabolism and excretion of PBDEs depend on the specific congener, on the species, and on the gender of the animal. For example, the lower brominated forms are broken down into mono- and di-hydroxylated metabolites, which are toxic and tend to accumulate in the serum, while the larger deca-BDE is broken down into the lower brominated forms (Costa *et al.*, 2008).

Some evidence shows that PBDEs are endocrine disruptors, where they disrupt the levels of androgenic, estrogenic, and thyroid hormones activities (Darnerud, 2008). Studies in humans show exposure to PBDEs have adverse effects on male reproduction associated with alternations to steroid hormone levels (Johnson *et al.*, 2013; Turyk *et al.*, 2008), and decreases in sperm count and quality (Meeker & Stapleton, 2010; Abdelouahab *et al.*, 2011). Previous studies report that high levels of PBDEs detected in follicular fluid or in serum is associated with a longer time to conceive (Harley *et al.*, 2010) and with failure of embryo implantation after *in vitro* fertilization (Johnson *et al.*, 2012). Studies on PBDEs in animal models show adverse effects that include disruption

to the endocrine system (Darnerud, 2008; Legler, 2008) and other effects associated with diabetes, cancer, neurobehavioral and developmental disorders (Kim *et al.*, 2014). Studies have also shown that the time point at which animals are exposed to PBDEs is important because perinatal exposure results in clear disruptions in the onset of puberty and in gametes production in both male and female rats (van der Ven *et al.*, 2009; Ema *et al.*, 2008).

1.7: Current Concern and Regulations on PBDEs

As of 2015, the Government of Canada (Chemical Management Division) has documented that PBDE levels in Canadian biota are rising, with reports showing increases in tissue concentrations, with penta-, octa-, and deca-brominated diphenyl ethers being the most commercially available mixtures (Chemical Management Division (CMD), 2015). The highest levels in the environment are associated with industrialized regions; however, PBDEs are also found in the Arctic, which suggests long range atmospheric transport of these compounds (CMD, 2015).

In December 2006, Environment Canada and Health Canada promised to reduce the concentration of PBDEs in the environment to the lowest possible level and published the Risk Management Strategy for PBDEs (CMD, 2015). This strategy uses a multilevel approach to achieve the outcome and combines regulatory and voluntary methods, development of environmental quality controls, international cooperation, and ongoing national monitoring (CMD, 2015).

In July 2008, the Government of Canada in, a means to protect the Canadian environment, published the Polybrominated Diphenyl Ethers Regulations and restricted

the manufacture and use of these chemicals in Canada, thus reducing their release into the environment (CMD, 2015). Specifically, this document prohibits the manufacture of the following forms of PBDEs: tetraBDE, pentaBDE, hexaBDE, heptaBDE, octaBDE, nonaBDE, and decaBDE congeners. Under the Canadian Environmental Protection Act, 1999, the congeners tetra, penta, and hexa meet the criteria for elimination, and thus under the Regulations, they are prohibited from being used, sold, bought, or imported into Canada (CMD, 2015).

It is clear that even though PBDEs are highly regulated in Canada, they are still prevalent in the environment and their effects are still present. This is why I chose to work with PBDEs. I wanted to work with a chemical that is relevant and that was used in a variety of products that are important to consumers.

1.8: Cell Lines Used

Human Embryonic Kidney (HEK293T) cells were chosen for this study because they are a model cell line in which the presences of Nrf1 and Nrf2 have already been observed (Chepelev *et al.*, 2013a). They are metabolically active and immortalized, meaning they do not have a Hayflick Limit and should not show any signs of the aging process, in terms of morphology or proteomics (Xia *et al.*, 2008; Kavsan *et al.*, 2011), making them a great cell model to use in our studies. Lastly, they have the ability to accept external DNA by means of transfection with high efficiency rates (Thomas & Smart, 2005).

1.9: Hypothesis and Objectives

The focus of this Master's thesis is to show that penta-BDE causes oxidative stress and that this oxidative stress can lead to aging. It is predicted that increasing exposure to PBDEs will cause oxidative injury to the cell that will elicit the antioxidant response and cause the increased activity of senescence-associated beta-galactosidase. To test out this hypothesis we first used a positive control for the induction of oxidative stress and then tested to see if PBDE treatments lead to the same outcomes. Hydrogen peroxide (H_2O_2) (Figure 4) is a pro-oxidant and, when converted to the highly reactive hydroxyl radical, can induce the oxidation of DNA, RNA, and proteins (refer to Figure 1) (D'Orazio *et al.*, 2013).

Therefore, the objective of this study is to examine the effects of exposure to PDBEs (Figure 2), more specifically penta-brominated diphenyl ether (Figure 3). To show oxidative stress, we measured the antioxidant response, the amount of oxidized proteins, and the amount of oxidized lipids. To show that PDBE-derived oxidative stress leads to increased levels of markers of cellular senescence, we measured the levels of activity of the senescence-associated beta-galactosidase enzyme. Human embryonic kidney cells are used as a model system for testing due to their high metabolic activity and DNA transfectability.

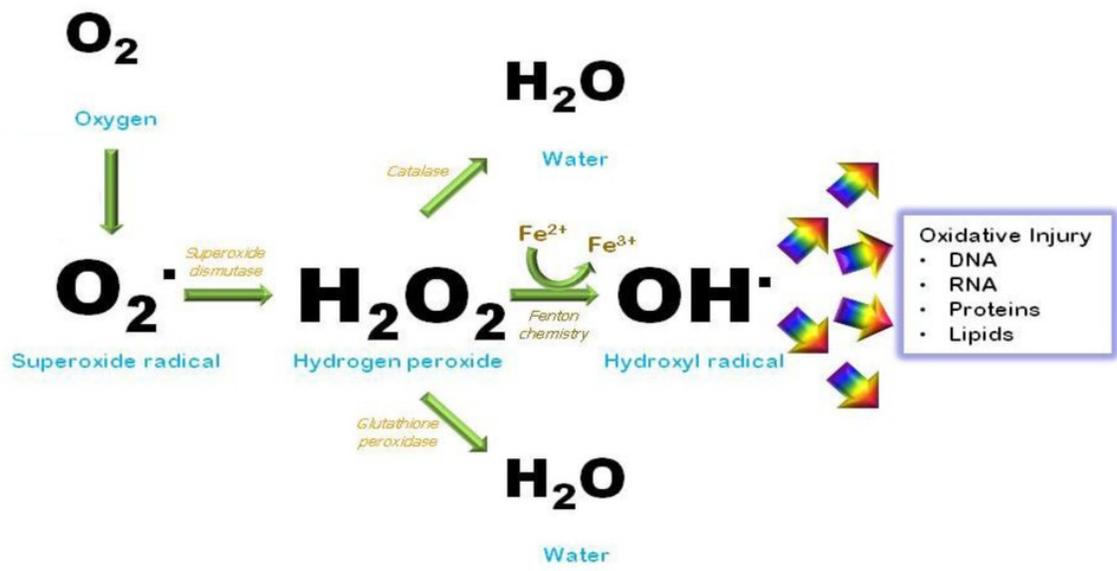


Figure 1: Elevated oxygen level is the first step in the process of forming free radicals and causing oxidative injury. Taken and modified from D’Orazio *et al.*, 2013.

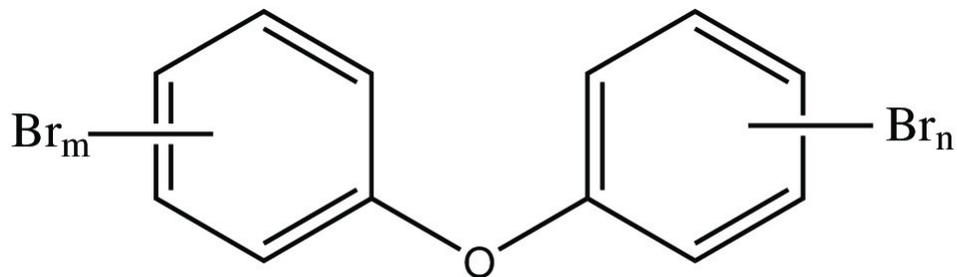


Figure 2: Chemical structure of polybrominated diphenyl ethers, where $m + n$ can range from four to ten bromines.

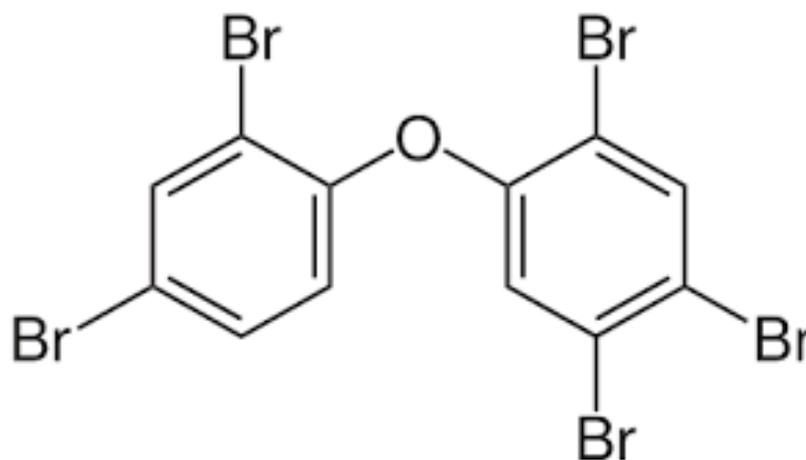


Figure 3: Chemical structure of penta-BDE, also commonly known as BDE-99, showing the total number of bromines on the two phenyl rings as five.

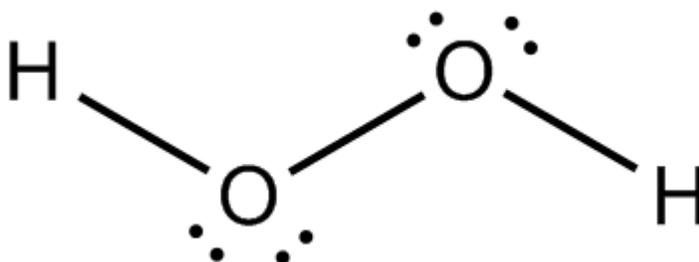


Figure 4: Lewis dot structure of hydrogen peroxide, showing its proposed shape and the reactivity of the oxygens due to the electron lone pairs.

Chapter 2: Materials and Methods

2.1: Cells

Human embryonic kidney cells (HEK293T) were purchased from the American Type Culture Collection (ATCC, Manasses, VA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 5% newborn calf serum (NCS) (Gibco, Grand Island, NY), incubated in 5% CO₂ at 37°C and 95% humidity.

2.2: Chemicals and Reagents

Hydrogen peroxide (30%) was purchased from Sigma-Aldrich (Oakville, Ontario) and diluted to working concentrations of 100 and 1000 µM using ultrapure water. When diluting the stock chemical, its concentration was confirmed using absorbance spectrum. Working concentrations were kept in dark 4°C fridge.

Penta-brominated diphenyl ether (penta-BDE) was received as a highly pure powder form from the National Wildlife Research Centre (NWRC; Environment and Climate Change Canada) and diluted to working concentrations of 1 and 10 mM using sterile dimethyl sulfoxide (DMSO) under sterile conditions (Stapleton *et al.*, 2004). Working concentrations were kept at room temperature in the dark.

Anti-Nrf1 (H4), anti-Nrf2 (C-20), goat anti-mouse-HRP and goat anti-rabbit-HRP were purchased from Santa Cruz Biotechnologies, Inc. (Dallas, Texas). Anti-beta-tubulin (E-7) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, Iowa).

2.3: MTT Assay

Cells were plated in a 96 well tissue culture plate at a volume of 200 μL per well and concentration of 50,000 cells/mL and left to grow overnight. The next day, cells were treated with varying concentrations of either H_2O_2 (0, 1, 5, 8, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80 μM ; 2 hours) or penta-BDE (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 μM ; 24 hours). The treatment durations were selected based on literature research, (2 hours for H_2O_2 from Jin *et al.*, 2016 and 24 hours for penta-BDE from Tagliaferri *et al.*, 2010). The treatment durations were also based on MTT viability assays showing low cell survival rates at longer treatment times (data not shown). After the treatment period, the treatment media was removed replaced with fresh media. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Bio-Rad, Hercules, California) reagent was added at a volume of one tenth of that of the fresh media and concentration of 0.5 mg/mL, and the mixture was left to incubate for 1-2 hrs at 37°C and 5% CO_2 and 95% humidity. After the incubation period, the media containing the MTT was removed and 100 μL of DMSO was added to each well. Once the DMSO dissolved the precipitate, the plate was read in a microplate reader at the wavelength of 530 nm with a background subtraction of 630 nm. This MTT assay was repeated three times (on independent days) for both hydrogen peroxide and penta-BDE.

2.4: Western Blotting

Cells were plated at a concentration of 5.0×10^5 per well in a 6 well plate and allowed to grow overnight. The next day, each well was treated with the 0, 1, 5, 10, 15, or 20 μM of either H_2O_2 or penta-BDE and left to incubate for 2 hours (H_2O_2) or 24 hours

(penta-BDE). After the treatment period, cells were harvested and lysed using RIPA buffer (Thermo Fisher Scientific, Ottawa, Ontario), supplemented with 100 and 500 μ M dithiothreitol (DTT). Protein concentration of samples was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Ottawa, Ontario). Samples were loaded onto an SDS-PAGE gel (150 V, 300 mA, 1.5 hours at room temperature) with 50 μ g of total protein per well.

After the gel was run, proteins were transferred to PVDF (polyvinylidene fluoride) membrane overnight at 4°C. Cell extracts on PVDF membranes were probed with the following antibodies diluted in Tris-buffered saline with Tween-20 (TBST): Nrf1 (H4) (1:1000) with goat anti-mouse-HRP (1:2000); Nrf2 (C-20) (1:1000) with goat anti-rabbit-HRP (1:2000); and beta-tubulin (E-7) (1:1000) with goat anti-rabbit-HRP (1:2000). This Western blot was repeated three times (on independent days) for both hydrogen peroxide and penta-BDE.

2.5: Protein Carbonyl Assay

Cells were plated at a concentration of 5.0×10^5 per well in a 6 well plate and allowed to grow overnight. The next day, the media was replaced with fresh media and cells were treated with the following concentrations 0, 1, 5, 10, 15, or 20 μ M of either H₂O₂ or penta-BDE and left to incubate for 2 hours (H₂O₂) or 24 hours (penta-BDE). The cells were harvested and the protocol according to the Protein Carbonyl Colorimetric Assay Kit, (Cayman Chemical, Ann Arbor, Michigan) was followed precisely. In short, this assay uses the 2, 4-dinitrophenylhydrazine (DNPH) to measure the protein carbonyl content in cell lysate sample. The amount of protein-hydrozone product formed is

measured spectrophotometrically at an absorbance between 360-385 nm. No changes were made to the protocol and each chemical treatment was repeated three times on independent days.

2.6: 8-Isoprostanes Assay

Cells were plated at a concentration of 5.0×10^5 per well in a 6 well plate and allowed to grow overnight. The next day, the media was replaced with fresh media and cells were treated with the following concentrations 0, 1, 5, 10, 15, or 20 μM of either H_2O_2 or penta-BDE and left to incubate for 2 hours (H_2O_2) or 24 hours (penta-BDE). The cells were harvested and the protocol according to the 8-Isoprostane ELISA Kit (Cayman Chemical, Ann Arbor, Michigan) was followed precisely. In short, this assay is based on the competition between 8-isoprostane and 8-isoprostane-acetylcholinesterase (AChE) conjugate (8-Isoprostane Tracer) for a limited number of 8-isoprostane specific rabbit antiserum binding sites. The concentration of 8-isoprostane tracer is constant while the concentration of 8-isoprostane varies according to the sample, giving the relationship of the amount of tracer bound is inversely proportional to the concentration of 8-isoprostane present. The distinct yellow product of the enzymatic reaction can be measured at 412 nm, and the intensity of the colour is proportional to the amount of tracer bound and inversely proportional to the amount of 8-isoprostane present in each sample. No changes were made and each chemical treatment was repeated three times on independent days.

2.7: Luciferase Reporter Assay

Cells were plated at a concentration of 3.0×10^5 cells per well in a 6 well plate and left to grow overnight. The next day, the culture media was replaced with fresh media 30 mins prior to transfection. Ten μL of the 10x stock of transfection reagent polyethyleneimine (PEI) was diluted in 100 μL Opti-MEM (Gibco, Grand Island, New York). The following plasmids were also diluted in 100 μL Opti-MEM: 0.4 μg beta-galactosidase in pCR3.1 vector, 6 μg GCLC in pCR3.1 vector, and 4 μg pCR3.1 empty vector. The GCLC-ARE-luciferase reporter plasmid was a gift from Dr. Dale A. Dickinson (University of Alabama) and its construction has been described elsewhere (Mulcahy *et al.*, 1997). This plasmid, with three copies of the GCLC AREs/EpREs, provides information on the activity of the Nrf transcription factors when they bind to the ARE/EpRE and the amount of luminescence is measured. The diluted PEI was added to the diluted DNA and the mixture was vortexed 5 times for 5 seconds every 3 minutes for a total of 15 minutes. The mixture was then added drop wise to each well, leaving one well with just cells as the untransfected control.

After 24 hours incubation, the transfection media was replaced with fresh media and left for another 24 hours. The cells were then harvested and lysed using Luciferase Lysis Buffer (25 mM glycylglycine, 15 mM KH_2PO_4 , 15 mM MgSO_4 , 4 mM EDTA, 1 mM DTT, 1% Triton X-100, 1x Protease Inhibitor (Bioshop, Burlington, Ontario)). The lysates were centrifuged at the max speed for 15 minutes, and the protein supernatant was used in the subsequent steps.

Ten μL of cell lysates were combined with 76 μL of Luciferase Assay Buffer (25 mM glycylglycine, 15 mM KH_2PO_4 , 15 mM MgSO_4 , 4 mM EDTA, 2 mM DTT, 2 mM ATP) in a 96 well white bottom plate. Each well was injected with 50 μL Luciferin

Solution (25 mM glycylglycine, 15 mM KH₂PO₄, 15 mM MgSO₄, 4 mM EDTA, 2 mM DTT, 200 μM Luciferin) and the luminescence was measured using a FLUOstar OPTIMA (BMG Labtech) microplate reader.

Afterwards, 50 μL of the luciferase lysate was transferred to a clear bottom 96-well plate and combined with 85 μL of Beta-Galactosidase Assay Buffer (60 mM Na₂HPO₄, 10 mM KCl, 1 mM MgCl₂, 1 mM DTT, 0.2 mg/mL chlorophenol red-β-D-galactopyranoside (CPRG)). The plate was incubated at 37°C for 30 minutes to 1 hr and then read at 570 nm with a background subtraction at 630 nm.

The luciferase readings were divided by the beta-galactosidase readings and the ratio was graphed against the concentrations of either H₂O₂ or penta-BDE treatment.

2.8: Flow Cytometry for Measuring Senescence Associated Beta-Galactosidase Activity

Cells were plated at a concentration of 5.0×10^5 per well in a 6 well plate and allowed to grow overnight. The next day, the media was replaced with fresh media and cells were treated with the following concentrations 0, 1, 5, 10, 15, or 20 μM of either H₂O₂ or penta-BDE and left to incubate for 2 hours (H₂O₂) or 24 hours (penta-BDE). Whole cell collection was performed and the protocol according to Debacq-Chainiaux et al. (2009) was followed precisely. The lysosomal alkalization in the procedure was removed as it was found to be an unnecessary step (Noppe *et al.*, 2009). According to the authors of this modification, pH modulation had no apparent effect on senescence-associated beta-galactosidase activity quantification via flow cytometry (Noppe *et al.*, 2009). The substrate used was C₁₂FDG, a fluorescent substrate of the beta-galactosidase

enzyme. This fluorescent dye produces a green fluorescent product (Miao *et al.*, 1993), and thus using Filter 1 (for measuring green fluorescence) in the BD Accuri™ C6 Flow Cytometer (San Jose, California), the amount of fluorescence was measured. This assay was repeated three times (on independent days) for both hydrogen peroxide and penta-BDE.

2.9: Statistical Analysis

All values displayed in the Results Section represent the mean \pm the standard error of the mean (SEM) for three independent experiments performed on three independent days. All statistical analyses were made using a one-way ANOVA followed by the Dunnett test, for comparisons to controls (0 μ M treatments), with SigmaStat software. Values were considered to be significantly different from one another if the p value was less than or equal to 0.05.

Chapter 3: Results

3.1: Determination of Chemical Concentrations

Using human embryonic kidney (HEK293T) cells as the model system and hydrogen peroxide as the positive control for all experiments, cell viability with treatment was tested using the MTT assay. At varying concentrations of chemical treatments, the absorbance was measured, with higher absorbance values being directly proportional to the more viable cells. The treatment duration was 2 hours for hydrogen peroxide and 24 hours for penta-BDE. As the concentration of hydrogen peroxide or penta-BDE increased past 25 μM , the absorbance decreases below 0.2 units, which shows that fewer cells are viable (Figures 5 and 6). Using this information, the following concentrations were chosen: 0, 1, 5, 10, 15, and 20 μM . With this, we conducted the rest of the experiments in this study.

3.2: Increased Chemical Treatment Results in Increased Nrf Protein Expression

Using the chemical concentrations determined from the MTT assay (0, 1, 5, 10, 15, and 20 μM) we performed Westerns to show the amount of Nrf1 and Nrf2 protein expression in response to increasing chemical treatment. There was an increase in protein expression with increasing chemical treatment from 0 μM (no chemical, just cells) to 20 μM of either chemical (Figures 7b and 8b). Both hydrogen peroxide (2 hour treatment) and penta-BDE (24 hour treatment) resulted in increases in both Nrf1 and Nrf2 proteins in HEK293T cells.

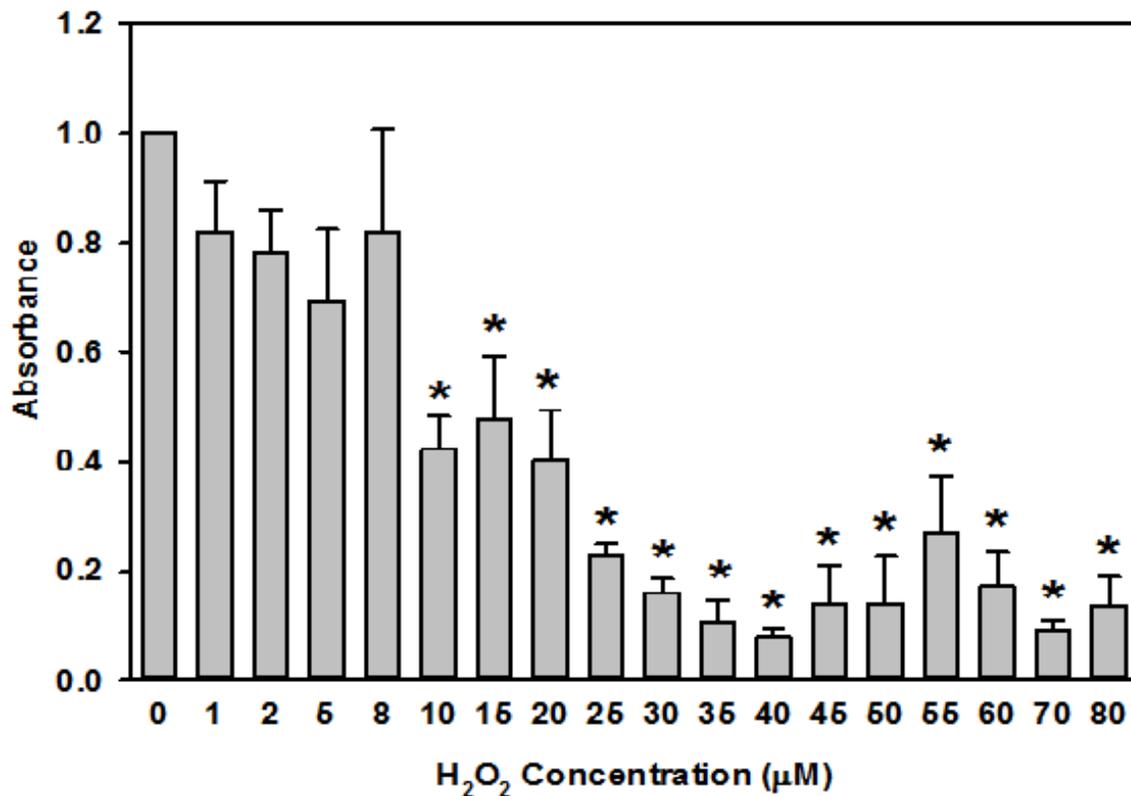


Figure 5: MTT assay for cell viability using hydrogen peroxide on HEK293T cells.

With increasing concentrations of hydrogen peroxide (after 2 hour treatment), viability of HEK293T cells decreases with I.C.₅₀ of approximately 20 µM. Values shown are the means normalized to the control (0 µM) ± SEM. N=3, (*) indicates significance (p<0.05) when compared to the control concentration of 0 µM. Concentrations of 10 to 80 µM show significant decreases in cell survival. Results from one-way ANOVA show significant decreases between means of concentrations. (I.C.₅₀ is the concentration of the inhibitor, in this case the chemical, where the response of the cells is reduced by 50%).

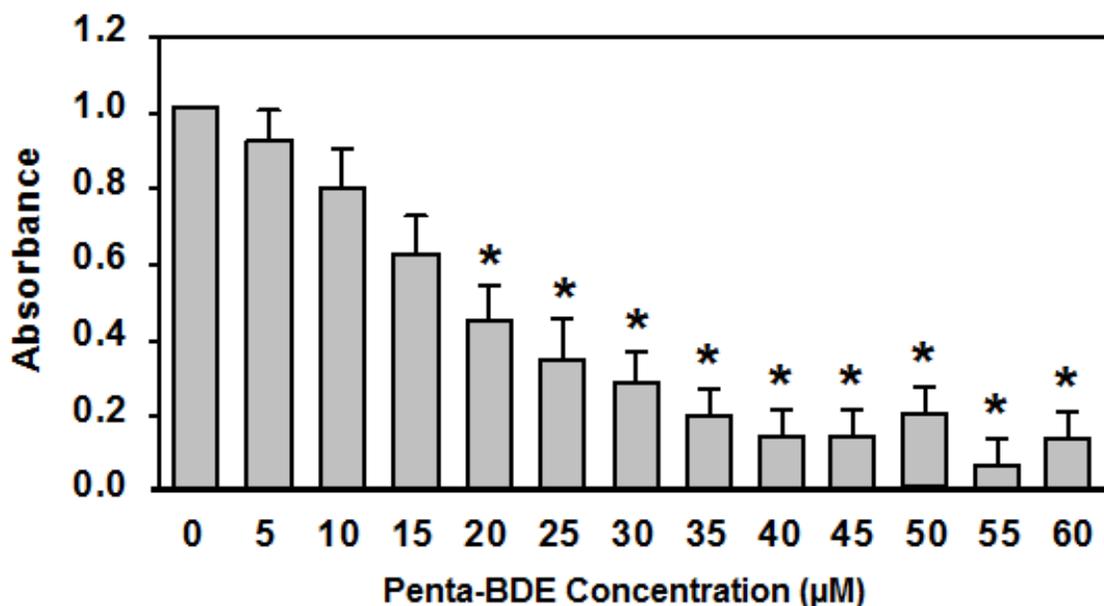
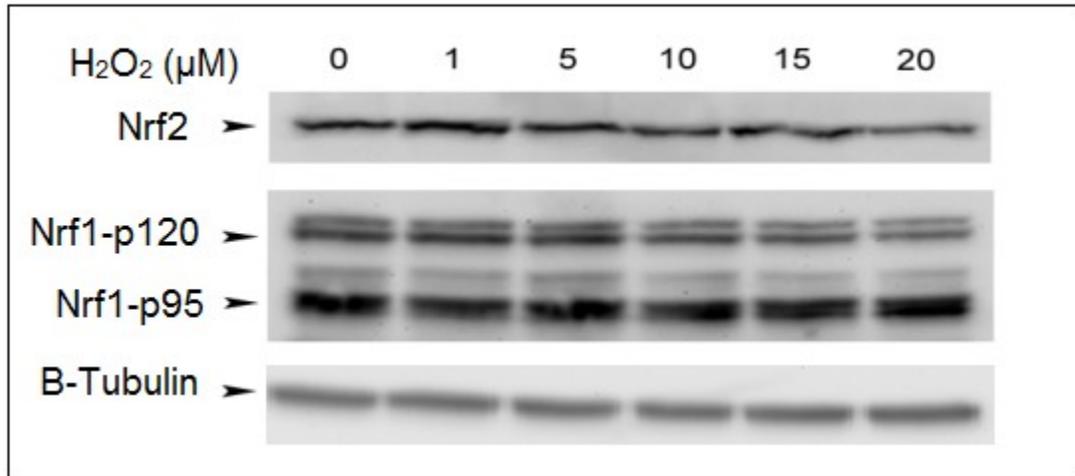


Figure 6: MTT assay for cell viability using penta-BDE on HEK293T cells. With increasing concentrations of penta-BDE (after 24 hour treatment), viability of HEK293T cells decreases with $I.C_{.50}$ of approximately 20 μM . Values shown are the means normalized to the control (0 μM) \pm SEM. $N=3$, (*) indicates significance ($p < 0.05$) when compared to the control concentration of 0 μM . Concentrations of 20 to 60 μM show significant decreases in cell survival. Results from one-way ANOVA show significant decreases between means of concentrations. ($I.C_{.50}$ is the concentration of the inhibitor, in this case the chemical, where the response of the cells is reduced by 50%).

(A)



(B)

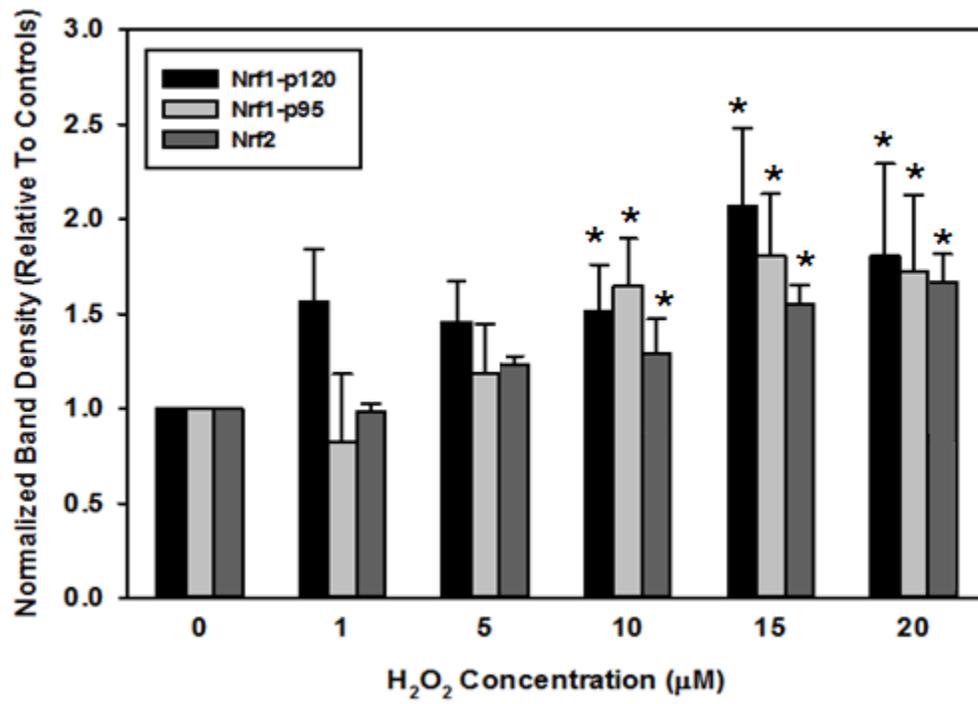
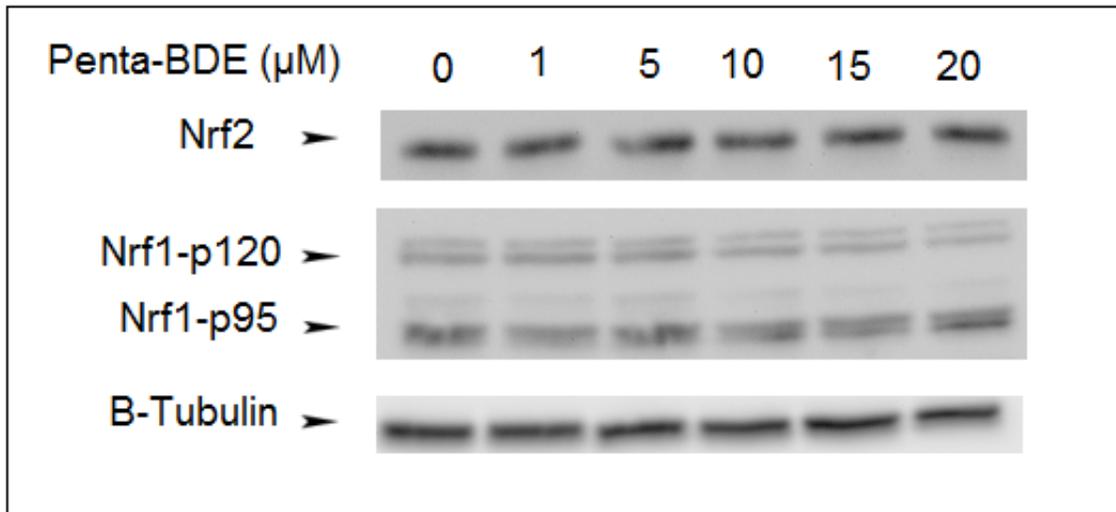


Figure 7: (A) Protein bands of p120 Nrf1, p95 Nrf1, and Nrf2 after H₂O₂ treatment. Beta-tubulin was used as a loading control. **(B) Protein levels of p120 Nrf1, p95 Nrf1, and Nrf2 after H₂O₂ treatment.** As chemical concentration increases so does the amount of all three proteins. Values shown are the means normalized to the control (0 μ M) \pm .SEM. N=3, (*) indicates significance (p<0.05) when compared to the control concentration of 0 μ M. Concentrations from 10 to 20 μ M show significant increases in protein levels when compared to the control (0 μ M). Results from one-way ANOVA show significant increases between means of concentrations.

(A)



(B)

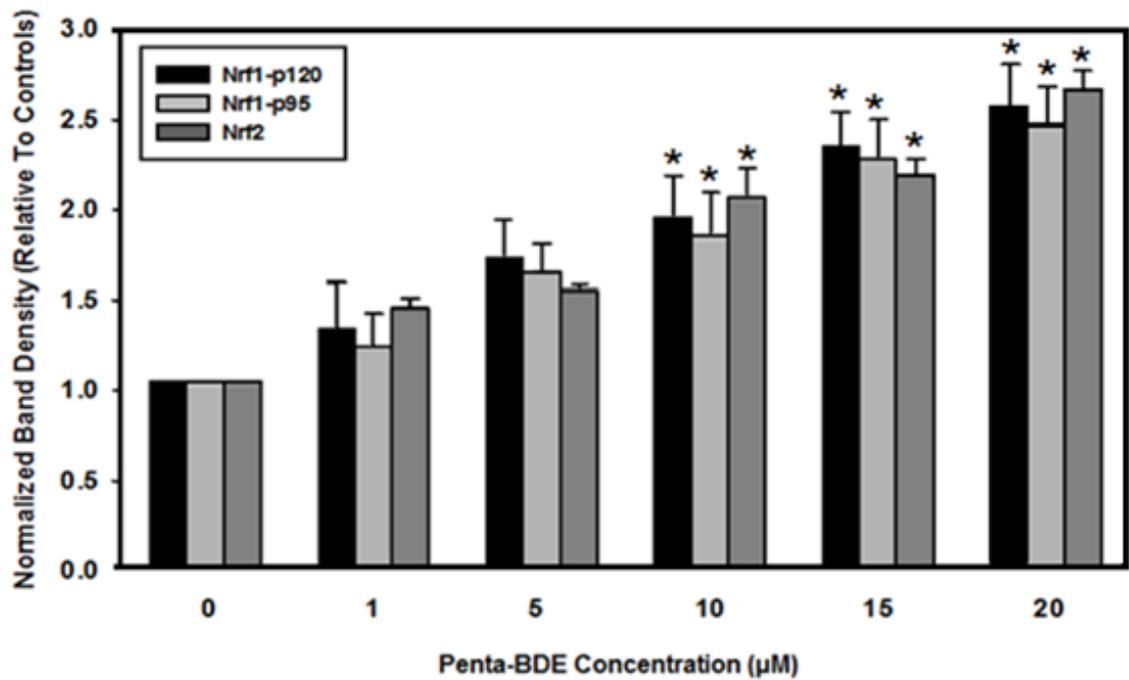


Figure 8: (A) Protein bands of p120 Nrf1, p95 Nrf1, and Nrf2 after penta-BDE treatment. Beta-tubulin was used as a loading control. **(B) Protein levels of p120 Nrf1, p95 Nrf1, and Nrf2 after penta-BDE treatment.** It is shown that as chemical concentration increases so does the amount of all three proteins. Values shown are the means normalized to the control (0 μ M) \pm SEM. N=3, (*) indicates significance ($p < 0.05$) when compared to the control concentration of 0 μ M. Concentrations from 10 to 20 μ M show significant increases in protein levels when compared to the control (0 μ M). Results from one-way ANOVA show significant increases between means of concentrations.

3.3: Increased Chemical Treatment Results in Increased Levels of Protein Carbonyl Groups

To explore the connection between oxidative stress to aging, we conducted a protein carbonyl assay, where the higher amount of carbonyl groups is used as an indicator of the aging process. From Figures 9 and 10, we see that as the concentration of chemical increases, so does the amount (nmol/ml) of carbonyl groups. After 2 hours of the cells being in hydrogen peroxide treatment, there was a significant increase in protein oxidation levels at 1 to 20 μM concentrations (Figure 9) when compared to the control treatment of 0 μM . After 24 hours of the cells being in penta-BDE treatment, there was a significant increase in protein oxidation levels at 10 to 20 μM concentrations (Figure 10) when compared to the control treatment of 0 μM .

3.4: Increased Chemical Treatment Results in Increased Levels of 8-Isoprostane

The levels of 8-isoprostane (8-*iso* prostaglandin $\text{F}_{2\alpha}$), a product of lipid peroxidation resulting from oxidative stress, was measured. As chemical concentrations increased, so did the amount of the oxidized phospholipid (Figures 11 and 12). After 2 hours of hydrogen peroxide treatment, there was no significant increase in 8-isoprostane levels (Figure 11) when compared to the control treatment of 0 μM . After 24 hours of penta-BDE treatment, there was a significant increase in 8-isoprostane levels at 5 to 20 μM concentrations (Figure 12) when compared to the control treatment of 0 μM .

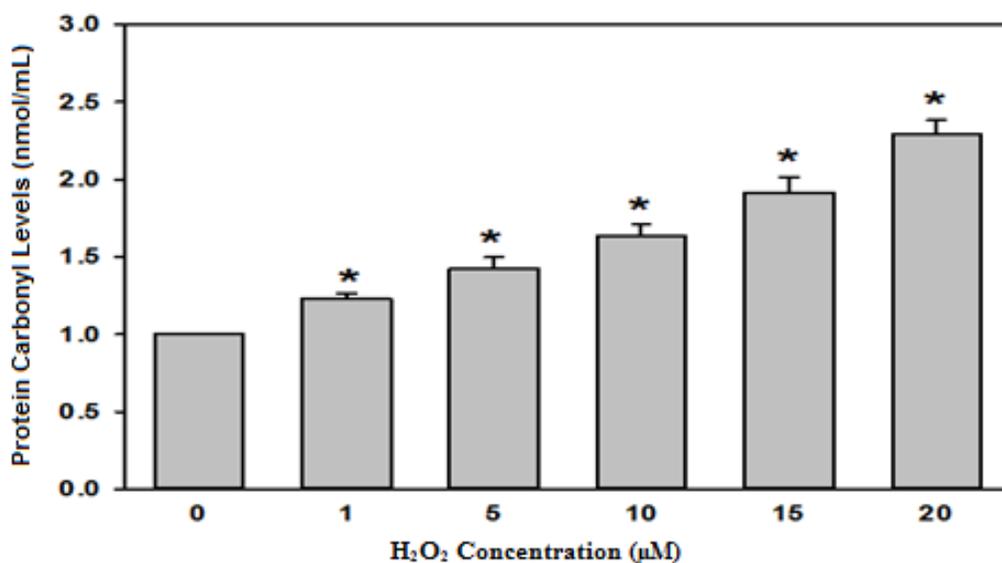


Figure 9: Protein carbonyl assay of HEK293T cells treated with H₂O₂. Results show increased levels of total carbonyl groups on proteins in HEK293T cells occurs with increased chemical treatment after 2 hours. Values shown are the means normalized to the control (0 µM) ± SEM. N=3, (*) indicates significance (p<0.05) when compared to the control concentration of 0 µM. Concentrations of 1 to 20 µM show a significant increase in protein oxidation levels. Results from one-way ANOVA show significant between increases means of concentrations.

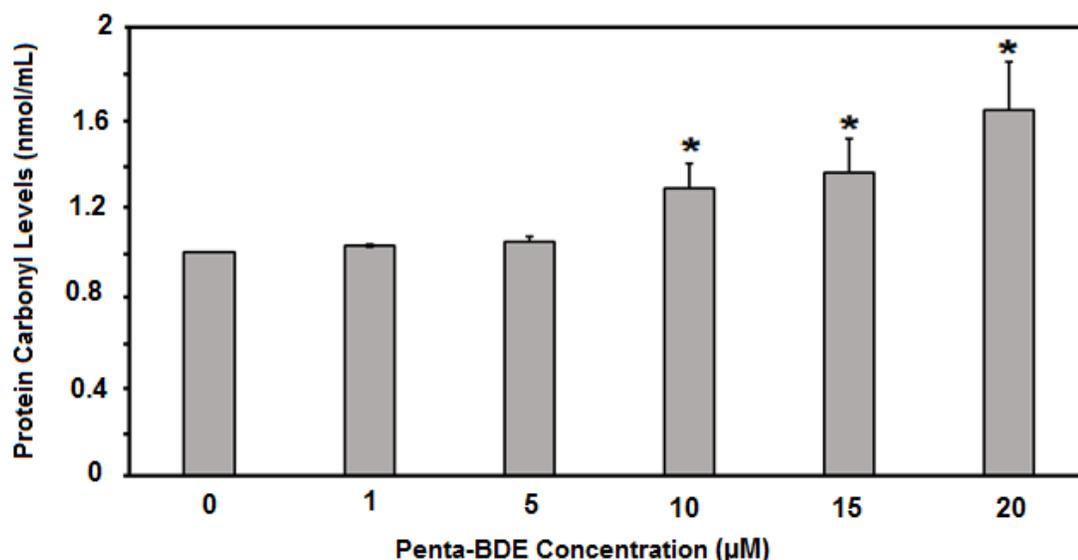


Figure 10: Protein carbonyl assay of HEK293T cells treated with penta-BDE. Results show increased levels of total carbonyl groups on proteins in HEK293T cells with increased chemical treatment after 24 hours. Values shown are the means normalized to the control (0 µM) \pm SEM. N=3, (*) indicates significance ($p < 0.05$) when compared to the control concentration of 0 µM. Concentrations of 10 to 20 µM show a significant increase in protein oxidation levels. Results from one-way ANOVA show significant increases between means of concentrations.

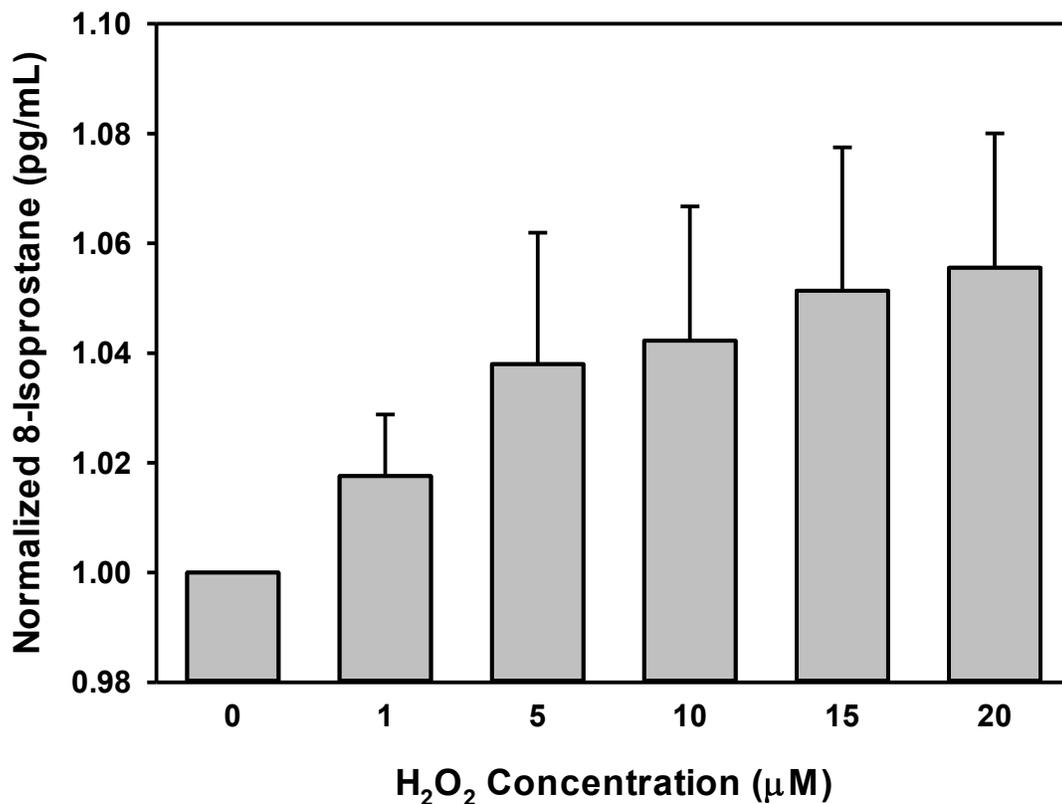


Figure 11: 8-Isoprostane assay of HEK293T cells treated with H₂O₂. Results show an increase in the concentration (pg/mL) of oxidized lipids with an increase in chemical treatment. Values shown are the means normalized to the control (0 µM) ± SEM. N=3. The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; therefore there is no statistically significant difference (p = 0.428).

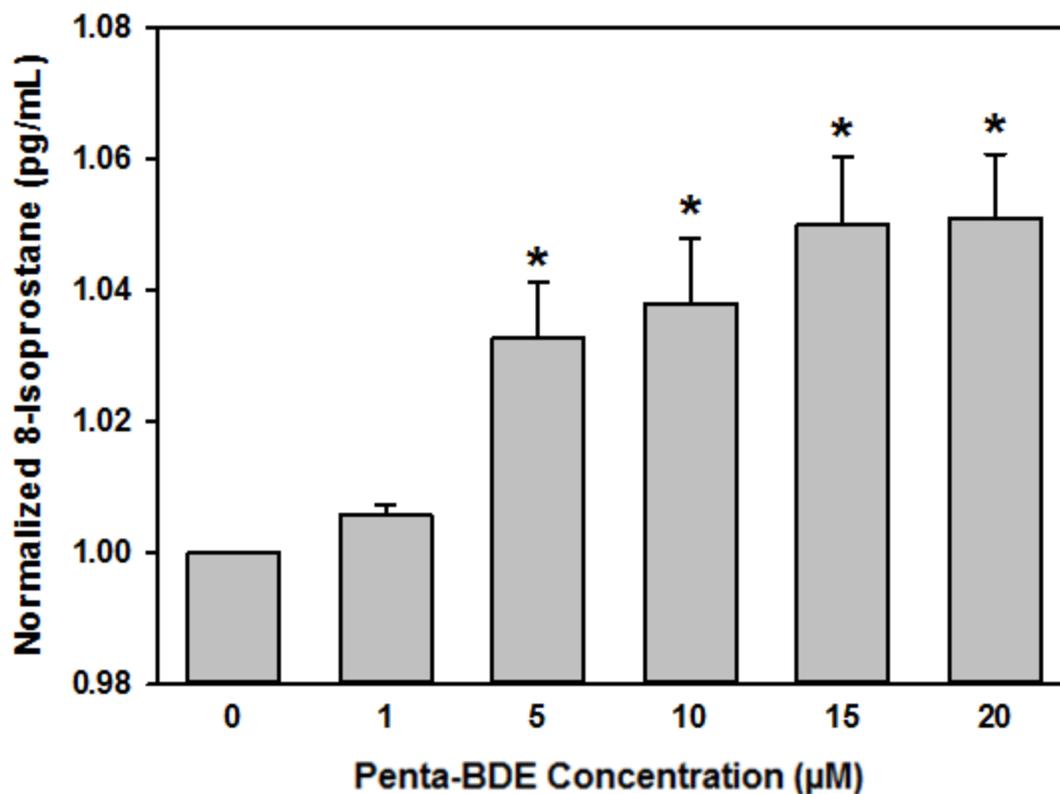


Figure 12: 8-Isoprostane assay of HEK293T cells treated with penta-BDE. Results show an increase in the concentration (pg/mL) of oxidized lipids with an increase in chemical treatment. Values shown are the means normalized to the control (0 µM) ± SEM. N=3, (*) indicates significance (p<0.05) when compared to the control concentration of 0 µM. Concentrations of 5 to 20 µM show a significant increase in lipid oxidation levels. Results from one-way ANOVA show significant increases between means of concentrations.

3.5: Increased Chemical Treatment Results in Increased Nrf1 Transactivation Activity

The luciferase reporter is an important tool widely used in research to measure the transcriptional levels of regulated sequences of DNA (Brasier *et al.*, 1988). In our experiments, the GCLC promoter, containing copies of the ARE/EpRE consensus sequence, was utilized. Results showed an increase in reporter assay activity with increasing chemical concentration (Figures 13 and 14). This observation reflects the oxidative damage that the cell is under and its antioxidant response to such harm. The cell responds via the antioxidant response pathway where the Nrf proteins, specifically Nrf1 and Nrf2 are activated and bind to DNA to transcribe antioxidant genes and phase II detoxification proteins.

3.6: Increased Chemical Treatment Results in Increased Levels of Beta-Galactosidase Activity Using Flow Cytometry

During the normal physiological aging process, cells accumulate higher levels of the beta-galactosidase enzyme and its activity is commonly used to measure cellular aging (Dimri *et al.*, 1995; Beattie *et al.*, 1994; Hall *et al.*, 2016). We measured beta-galactosidase activity in cells with increasing concentrations of either hydrogen peroxide (after 2 hour treatment) or penta-BDE (after 24 hour treatment). The results showed that as chemical concentration increases, the number of cells with active beta-galactosidase enzyme also increases (Figures 15 and 16). We used flow cytometry for counting fluorescent cells after treating them with chemicals and after treating them with C₁₂FDG (5-dodecanoylamino fluorescein di-β-D-galactopyranoside). C₁₂FDG is a fluorogenic

substrate that once broken down by beta-galactosidase, produces a green fluorescent product that remains within the cell (Miao *et al.*, 1993).

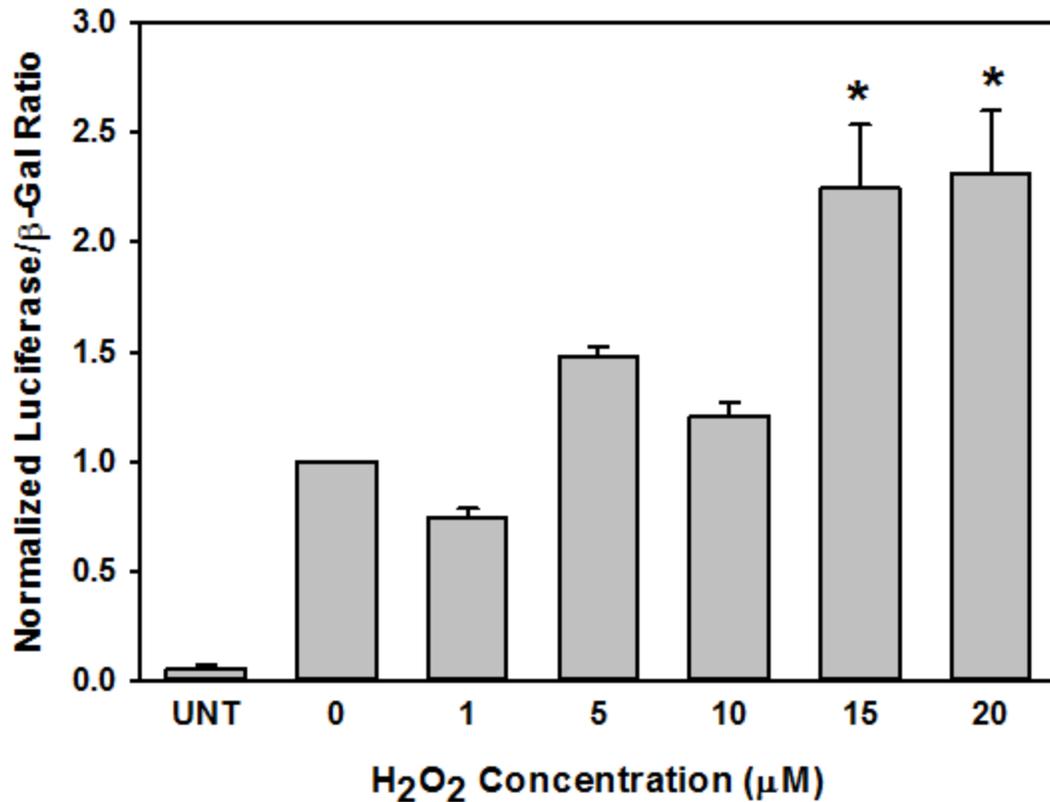


Figure 13: Luciferase reporter assay of HEK293T cells treated with H₂O₂. Results show an increase ARE/ EpRE transcription levels with increasing chemical concentrations. This means that the proteins Nrf1 and Nrf2 have an increase in activity level with an increase in chemical treatment. When the cell experiences greater levels of oxidative stress, it will initiate the antioxidant response pathway via Nrf1-ARE and Nrf2-ARE. Values shown are the means normalized to the control (0 μM) ± SEM. N=3, (*) indicates significance (p<0.05) when compared to the control concentration of 0 μM. Concentrations of 15 to 20 μM show a significant increase in luciferase reporter levels. Results from one-way ANOVA show significant increases between means of concentrations. (UNT = untransfected).

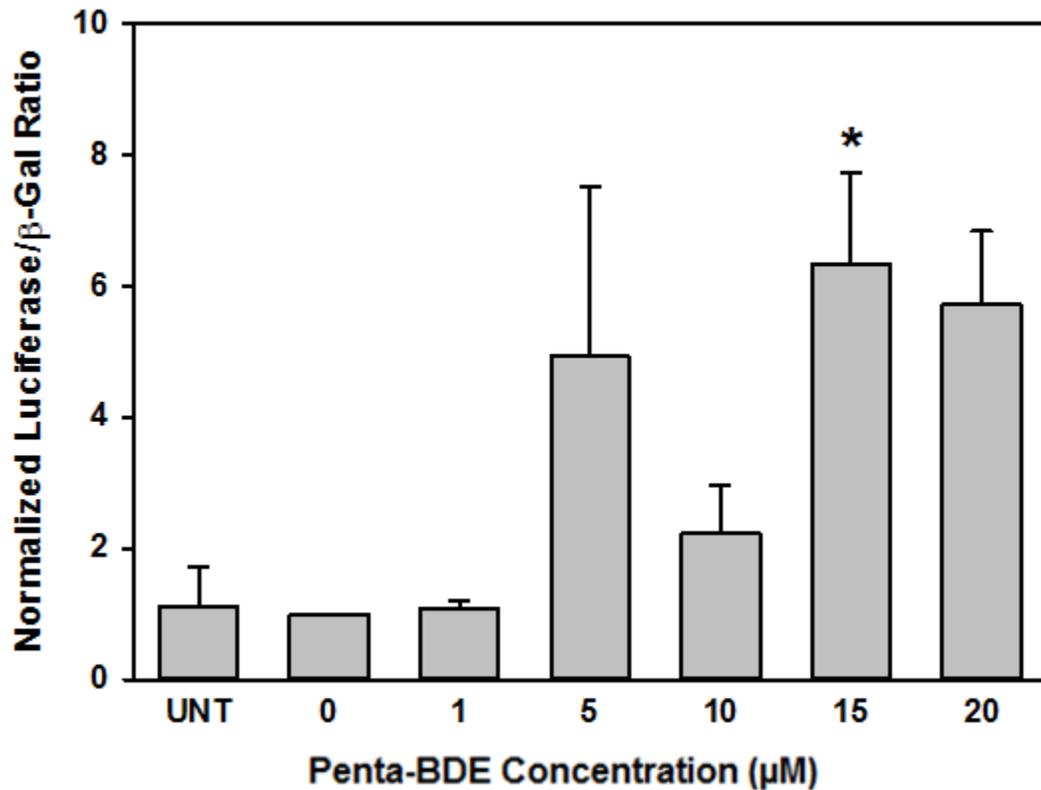


Figure 14: Luciferase reporter assay of HEK293T cells treated with penta-BDE. Results show an increase ARE/ EpRE transcription levels with increasing chemical concentrations. This means that the proteins Nrf1 and Nrf2 have an increase in activity level with an increase in chemical treatment. When the cell experiences greater levels of oxidative stress, it will kick start the antioxidant response pathway via Nrf1-ARE and Nrf2-ARE. Values shown are the means normalized to the control (0 μM) ± SEM. N=3, (*) indicates significance (p<0.05) when compared to the control concentration of 0 μM. The second highest concentration, 15 μM, was the only data point that showed a significant increase in luciferase reporter levels. Results from one-way ANOVA show significant decreases between means of concentrations. (UNT = untransfected).

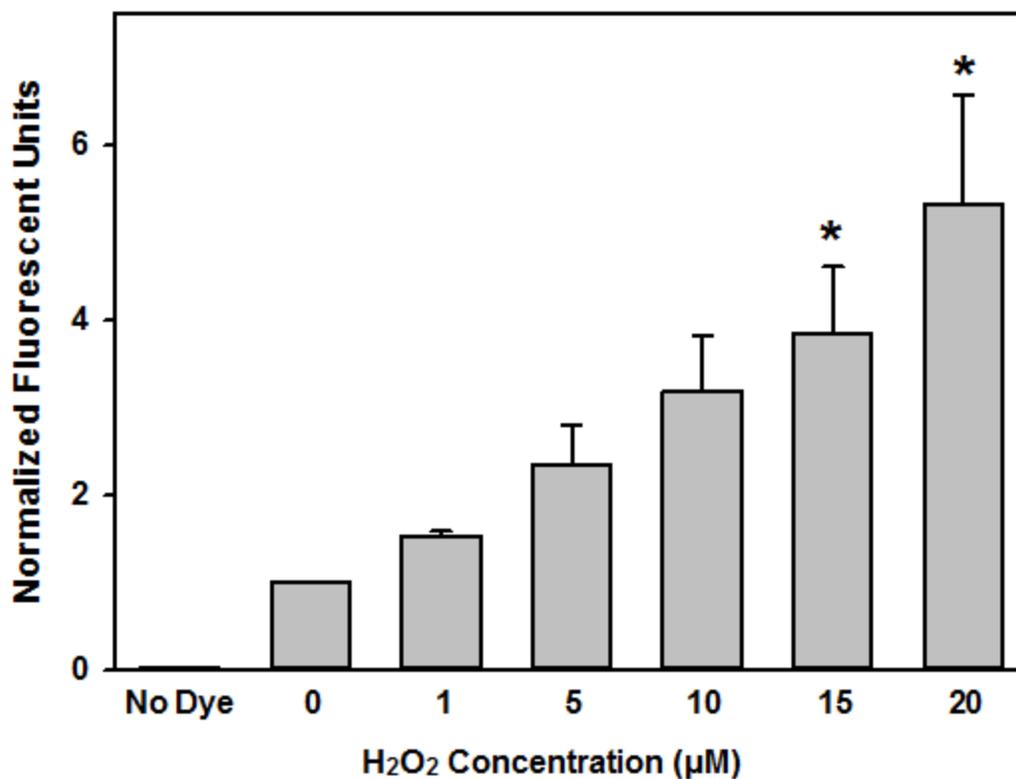


Figure 15: Activity level of beta-galactosidase enzyme in HEK293T cells after H₂O₂ treatment. As hydrogen peroxide concentration increases (after 2 hour treatments), so does the activity of the beta-galactosidase enzyme. Values shown are the means normalized to the control (0 µM) ± SEM. N=3, (*) indicates significance (p<0.05) when compared to the control concentration of 0 µM. Concentrations of 15 to 20 µM show a significant increase in enzyme activity levels. Results from one-way ANOVA show significant increases between means of concentrations.

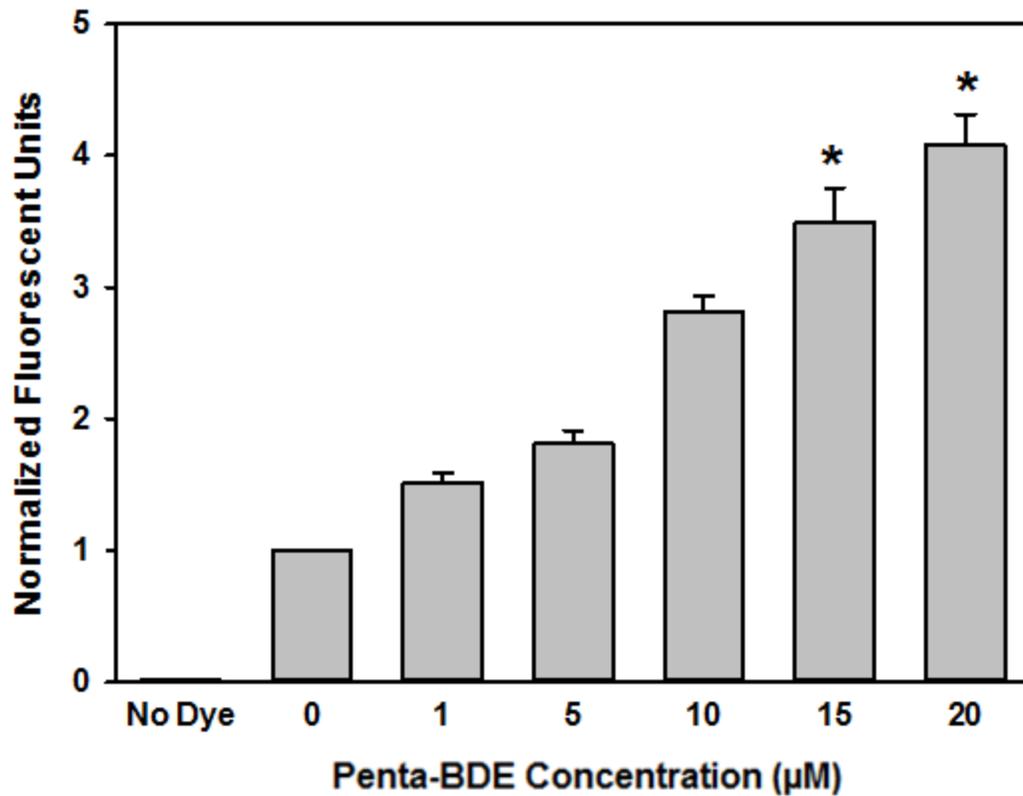


Figure 16: Activity level of beta-galactosidase enzyme in HEK293T cells after penta-BDE treatment. As penta-BDE concentration increases (after 24 hour treatments), so does the activity of the beta-galactosidase enzyme. Values shown are the means normalized to the control (0 µM) ± SEM. N=3, (*) indicates significance (p<0.05) when compared to the control concentration of 0 µM. Concentrations of 15 to 20 µM show a significant increase in enzyme activity levels. Results from one-way ANOVA show significant increases between means of concentrations.

Chapter 4: Discussion

4.1: Hydrogen Peroxide Is A Strong Pro-oxidant That Increases Nrf1 and Nrf2 Protein Levels

To show the possible effects of oxidative stress caused by PBDEs, we first used a well-known pro-oxidant, hydrogen peroxide, as a positive control in order to compare results between the two chemicals. Hydrogen peroxide is highly reactive and excessive amounts can cause oxidative stress by creating ROS and oxidizing proteins (Oliver & Brooke, 2016). It has been shown that hydrogen peroxide induces oxidative stress in cultured bovine mammary epithelial cells and results in reduced cell growth (Jin *et al.*, 2016).

The cellular response to oxidative stress is to activate transcription factors that control the expression of antioxidant and phase two enzymes. The transcription factors Nrf1 and Nrf2 are best known to regulate antioxidant and detoxification enzyme expression through AREs/EpREs and the protein levels of both factors increased with increased hydrogen peroxide treatment (Figure 7b). This revealed that HEK293 cells are capable of inducing both of these factors in response to oxidative stress.

4.2: Penta-Brominated Diphenyl Ether (penta-DBE) Treatment Increases Nrf1 and Nrf2 Protein Levels

Experiments were repeated with penta-DBE as the chemical of choice ($C_{12}H_5Br_5O$), and similar results were observed. With increasing PDBE concentration, Nrf1 and Nrf2 protein levels also increase (Figure 8b). As similar results were obtained

from both H₂O₂ and penta-BDE chemical treatments, this suggested that PBDEs are also pro-oxidants.

Studies have shown PBDEs cause oxidative stress when their metabolites react with cellular molecules to produce ROS in excess of what the cell's defenses can neutralize, leading to a shift in redox homeostasis and causing large amounts of cellular damage (Lefevre *et al.*, 2015). Researchers found that the genes encoding for two antioxidant enzymes, *Cat* and *Sod1* had decreased expression levels in the ovaries of female mice exposed to high levels of PBDEs (Lefevre *et al.*, 2015). These treatments lasted two to four weeks, according to the study, and thus prolonged exposure to PBDEs was found to cause the impairment of the antioxidant response. Schang and colleagues showed that the metabolism of organophosphate flame retardants is very similar to that of PBDEs, in that both produce ROS, which drives the cell towards apoptosis (Schang *et al.*, 2016). In their studies, increased levels of lipid peroxidation with chemical treatment contributed to the cytotoxicity (Schang *et al.*, 2016).

Our study examined the effects of acute exposure to PDBEs with a maximum treatment time of 24 hours. The results show an increased antioxidant response with a subsequent decrease in antioxidant response with prolonged treatment times. When the cell is faced with acute stress, it is able to respond and elicit the activity of Nrf1 and Nrf2 in order to activate production of antioxidant and phase 2 enzymes. With chronic exposure to PBDEs, the cell's ability to respond to oxidative stress is reduced. PBDEs are present in household material, such as insulation, textiles, building materials, plastics, electronics, and foams (Frouin, *et al.*, 2010). Over time, their break down products cause long-term exposure to PDBE metabolites in humans.

4.3: Protein Carbonyl Group Production and Aging

Following a very simple procedure, we attempted to show that the oxidative stress produced by PDBEs contribute to the cellular aging process (as predicted by the Free Radical Theory of Aging), by measuring carbonyl groups on cellular proteins. Carbonyl groups are formed on oxidatively modified proteins and have been used as an indicator of cellular aging (Sohal *et al.*, 2002). Both hydrogen peroxide and PBDE treatments caused an increase in the concentration of protein carbonyls groups, with a statistically significant difference between the controls (0 μM of chemical) and the highest concentration of chemicals (20 μM) (Figures 9 and 10 respectively).

Our results show that there is a significant amount of oxidized proteins produced in the highest treatment level of penta-BDE. Therefore, the cell is experiencing oxidative stress causing damage to proteins. Because our study was acute, we cannot tell if the damaged proteins would be cleared by the cell, or if they will cause excessive loss of function and cause the cell to die. This is one limitation encountered in this study, where other studies observed cellular senescence with long term chemical exposure. Prolonged oxidative damage and excessive loss of protein function due to oxidation has been the onset of the aging process in other studies (Smith *et al.*, 1991; Stadtman, 2006; Stadtman & Berlett, 1997).

Though short-term exposure studies, such as this one, show effects from a single snap shot in time, there are still dose-dependent effects being observed. The increasing concentrations from 0 to 20 μM show an increase in the amount of carbonyl groups, showing a dose-related trend. This means that the more chemical cells are exposed to, the more protein carbonyl groups are formed.

4.4: Lipid Oxidation and Aging

To further connect oxidative stress generated by PDBEs to aging, we examined phospholipids oxidation after chemical treatment. Cells exposed to increased concentrations of both hydrogen peroxide and PDBEs had increased concentration of 8-isoprostane (also known as 8-*iso* prostaglandin F_{2α}) (Figures 11 and 12 respectively). The presence of oxidized phospholipids in our studies suggested that chemical treatment times and concentrations were sufficient enough to produce oxidative damage to biomolecules. This phospholipid is a potent renal vasoconstrictor and has been implicated as a mediator of oxidative stress-derived toxicity (Banerjee *et al.*, 1992). Therefore, since the trend shows an increase in 8-isoprostane, the effects implied are those of extensive oxidative stress from increasing chemical concentrations (Vacchiano & Tempel, 1994). Other studies have also shown that 8-isoprostane tends to increase with the age of the subject (Wang *et al.*, 1995).

Our results show a significant amount of oxidized lipids produced in the highest treatment level of penta-BDE. Therefore, the cell is experiencing oxidative stress causing the production of ROS that go on to react with lipids and cause oxidative damage. Due to the short time duration of treatments in this study, it could not be determined if the accrued lipid damage was cleared from or repaired by the cell or not. Prolonged oxidative damage and excessive loss of important biomolecules that are energy sources for the cell (Cooper, 2000), can cause the cell to deteriorate and die. Even though our study was short term, the chemical treatments still caused an effect similar to long-term exposure to other oxidizing agents.

4.5: Activation of the AREs/EpREs by Penta-BDE

To further support evidence that PDBEs generate oxidative stress, we measured Nrf1 and Nrf2 protein and activity levels. To do this, we used an ARE-luciferase reporter which contained three ARE/EpRE binding sequences from the GCLC promoter (Brasier *et al.*, 1988). The activity and protein levels of both Nrf1 and Nrf2 increased with both H₂O₂ and penta-BDE treatments (Figures 13 and 14). Nrf2 is a well-known regulator of antioxidant and cellular protective genes, (Chepelev *et al.*, 2013b), and likewise, Nrf1 is important for cell survival when the cell is under oxidative stress (Kwong *et al.*, 1999). Both Nrf1 and Nrf2 protein levels increased with increasing treatment concentration (Figures 7b and 8b respectively). As well as their binding to the AREs/EpREs, cell treatment with PDBEs resulted in an increase in the content of the Nrf proteins within the cell (Figures 8a and 8b). It can therefore be concluded that PDBEs induce Nrf1 and Nrf2 activity in the antioxidant response to oxidative stress.

The time of treatments utilized in this study (2 hours and 24 hours) may be considered to have placed the cell under acute stress as HEK293T cells were able to elicit an antioxidant response and to recover from the treatments. More prolonged or chronic exposure to PDBEs may cause excessive build-up of oxidatively damaged proteins, which may compromise cellular function and direct the cell towards autophagy, apoptosis or cellular aging and senescence.

4.6: Senescence-associated beta-galactosidase (SA-β-gal) activity increases with H₂O₂ and penta-BDE exposure

As seen from the results (Figures 15 and 16), as treatment concentration increased (either H₂O₂ or PDBEs), so did the number of fluorescent cells. These results show that intracellular levels of β -galactosidase enzyme increased due to chemical treatment, allowing more substrate to be converted into fluorescent product. Aging cells, and cells with the potential to differentiate and mature, tend to express higher levels of beta-galactosidase activity, a trend that has been termed senescence-associated beta-galactosidase activity (SA- β -gal) (Dimri *et al.*, 1995; Beattie *et al.*, 1994). Therefore, we have shown that the highest concentrations of PDBEs used in the experiments in this study causes cells to produce more β -galactosidase enzyme activity and breakdown the C₁₂FDG substrate, an indication that they are experiencing a similar effect to that of a cell that is undergoing senescence.

The flow cytometry utilized in this study was developed for the detection of β -galactosidase activity in single cells (Miao *et al.*, 1993). The fluorogenic substrate, C₁₂FDG (5-dodecanoylamino-fluorescein di- β -D-galactopyranoside), contains a lipophilic group, allowing it to cross the cell membrane (Miao *et al.*, 1993). When this substrate is hydrolyzed by intracellular β -galactosidase, it produces a green fluorescent product that is retained in the cell. In turn, the positively stained cells display fluorescence, which is measured by flow cytometry (Miao *et al.*, 1993). The activity of β -galactosidase increases with aging in cell lines and the assay for its activity is a common measure of cellular senescence (Miao *et al.*, 1993). This assay is a novel way of examining cell senescence response to chemical exposure and has only been utilized in a few other studies (Behnia *et al.*, 2015). Due to its novelty, the use of this assay is in itself a limitation, since there is little literature to support the findings.

Chapter 5: Conclusion and Future Direction

In conclusion, we believe that this Master's Thesis was able to demonstrate that PDBEs cause oxidative stress to a human embryonic kidney cell. Furthermore, such oxidative stress increases the activity of the senescence-associated β -galactosidase. We used H_2O_2 as our positive pro-oxidant control and compared the results of BDE to that of H_2O_2 , to see if PBDE produces similar effects.

HEK293T cells responded, in a similar manner to both H_2O_2 and PBDE, in the following ways: (1) cells produced more of the antioxidant gene regulating proteins Nrf1 and Nrf2 (Figures 7 and 9), (2) cells increased the activity levels of antioxidant gene regulating proteins Nrf1 and Nrf2 (Figures 15 and 16), (3) cells accumulated oxidized biomolecules, specifically proteins and lipids (Figures 11 to 14), and (4) cells produced more β -galactosidase enzyme, a well-known biomarker of cellular senescence (Figures 17 and 18).

Evidence shows that cells tend to age when faced with prolonged and extensive oxidative stress and impaired antioxidant response mechanisms (Finkel & Holbrook, 2000). With our experiments having a maximum treatment time of only 24 hours, the cell still produced effects from chemical treatment. The PDBE concentrations used in our experiments (maximum of 20 μ M) were comparable to ranges of H_2O_2 concentrations (100 μ M with endothelial cells; Coyle & Kader, 2007 and 1000 μ M with epithelial cells; Jin *et al.*, 2016), and PBDE concentrations (12 μ M with immune cells; Frouin *et al.*, 2010 and 30 μ M with neuroblastoma cells; Tagliaferri *et al.*, 2010) used in other studies. Short-term treatment (24 hours or less) of human cells with PBDE was sufficient to produce markers of oxidative stress in this study.

Though this study has its limitations, it is new and provides insight on the cellular response with exposure to flame retardants used in homes and many consumer products. Specifically, we show that penta-BDE produces the biomarkers of oxidative stress and cellular senescence. These findings are novel to this study, and though future studies are needed for further support, this study provides a starting point for future researches.

Future studies should focus on prolonged chemical exposure, with or without recovery periods, since it is prolonged exposure to oxidative stress that results in cellular senescence. These results provide supplementary evidence that PDBEs cause oxidative stress which leads to an increase in the markers of cell senescence. Furthermore, studies with PDBEs using models that do not age, such as cancer cells, can provide additional evidence into the chemical's potential to induce marker enzymes of cell senescence. Also looking at animals models to compare *in vitro* to *in vivo* results will provide more insight onto how these chemicals are metabolized and distributed throughout the body. Lastly, cells originating from different tissues should be tested to determine if all of the tissues respond in a similar manner. For example, studies have shown that PBDEs cause neurotoxic effects (Tagliaferri *et al.*, 2010), and therefore performing such tests in neuronal cells can show if neurons are more or less sensitive to such effects.

Chapter 6: References

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