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TOXICOLOGICAL DIFFERENCES BETWEEN PERFLUOROALKYL SUBSTANCES (PFAS) ISOMERS USING DEVELOPMENTAL BIOMARKERS

THESIS

Gabriel A. Cantu, Captain, USAF

AFIT–ENV–MS-17-M-178

DEPARTMENT OF THE AIR FORCE
AIR UNIVERSITY

AIR FORCE INSTITUTE OF TECHNOLOGY

Wright-Patterson Air Force Base, Ohio

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THESIS

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In Partial Fulfillment of the Requirements for the

Degree of Master of Science in Environmental Engineering and Science

Gabriel A. Cantu, BS

Captain, USAF

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AFIT-ENV-MS-17-M-178

Abstract

Perfluoroalkyl substances (PFAS) are metabolically stable synthetic chemicals that have been manufactured for commercial and industrial purposes since the 1950's (Brown, 2016; Buck et al., 2011). PFAS possess surfactant properties that make them ideal to fight hydrocarbon fires and are therefore present in aqueous film forming foams (AFFF) (Lau et al., 2007; Moody & Field, 2000). Furthermore, AFFF may contain blends of both linear and branched PFAS isomers. Research suggests that branched PFAS isomers have greater relative placental transfer efficiencies than their linear counterparts, but few studies have evaluated their toxicity (Beesoon et al., 2011; Beesoon & Martin, 2015; Gützkow et al., 2012). Therefore, the sustained use of AFFF in the U.S. Air Force presents an unquantified risk of branched PFAS exposure to pregnant females.

This study investigated the toxicological differences between branched and linear PFAS isomers *in vitro* using the JEG-3 human placental cell-line as a model. Cells were exposed to linear and branched perfluorohexane sulfonate (PFHxS) for 24 to 48 hours at concentrations ranging from 0.2 µM to 50 µM. Subsequently, changes in three specific biomarkers were examined. No significant statistical differences in cellular proliferation and cellular viability were highlighted in cells exposed to both compounds at equivalent concentrations; however, mean cell proliferation appeared greater when exposed to linear PFHxS. Reactive oxygen species (ROS) generation was statistically higher in JEG-3 cells exposed to branched PFHxS isomers at corresponding concentrations.

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Gabriel A. Cantu

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TOXICOLOGICAL DIFFERENCES BETWEEN PERFLUORALKYL SUBSTANCES (PFAS) ISOMERS USING DEVELOPMENTAL BIOMARKERS

I. Introduction

1.1 General Issue

Perfluoroalkyl substances (PFAS) are a family of synthetic organic chemicals that have been manufactured since the mid twentieth century, and have been employed extensively in a wide range of commercial and industrial settings (Brown, 2016; Buck et al., 2011). PFAS are unique in that they resemble fatty acids, and are characterized by a charged oleophobic moiety attached to a hydrophobic carbon chain. The carbon atoms on the chain have all had their hydrogen bonds replaced with highly electronegative fluorine atoms (Buck et al., 2011). These carbon-fluorine (C-F) bonds are considered the second strongest in organic chemistry, and render PFAS extremely stable in the environment and resistant to degradation (Andersen et al., 2008; Lau et al., 2007). The C-F bonds are also responsible for the low surface tension of PFAS, and for their hydrophobic and oleophobic properties (Lau et al., 2007). Because, of their unique properties, PFAS are considered ideal surfactants and have been employed in various consumer and industrial applications such as photographic emulsifiers, oil and stain-resistant coatings, paints, adhesives, and aqueous film forming foams (AFFF) that are used to fight hydrocarbon fires (Lau, et al., 2007; Moody & Field, 2000).

The detection of organic fluorine in human sera by Taves in 1968 began a series of investigations that led to the discovery of PFAS in water, wildlife, and in the general public around the world (Andersen et al., 2008; Giesy & Kannan, 2001; Lau et al., 2007). Consequently, the toxicological properties of PFAS have become of greater concern to

the regulatory and scientific communities. Three specific PFAS have become the subject of most environmental and toxicological studies, as well as the topic of regulatory scrutiny – perfluorooctanoic acid (PFOA), perfluorooctane sulfonate (PFOS), and perfluorohexane sulfonate (PFHxS). Both PFOS and PFOA are formed by a linear carbon backbone that is eight atoms in length, however, their moieties are different; PFOA contains a carboxylate moiety, whereas PFOS contains a sulfonate moiety. PFHxS also contains a sulfonate moiety; however, its backbone is formed by only six carbon atoms. All three of these perfluorinated alkyl substances have been detected in wildlife and in human matrices (Lau et al., 2007). PFHxS and PFOS have also been detected at abnormal levels in the sera of firefighters exposed to AFFF (Rotander, Toms, Aylward, Kay, & Mueller, 2015).

Although there has recently been a drive on behalf of industries and the United States Environmental Protection Agency (USEPA) to phase out the manufacture of certain PFAS such as PFOS, the production of other types of PFAS has increased (Lau et al., 2007; USEPA, 2016). Additionally, the use of legacy products containing PFOS continue to be employed in various industries, most notably, photographic emulsifiers and AFFF formulations that are employed by the Department of Defense (Brown, 2016; Moody & Field, 2000; USEPA, 2016).

There are two manufacturing processes that are used to create PFAS at an industrial scale, electrochemical fluorination (ECF) and telomerization. ECF was predominantly utilized over the first four decades of production, and generated mixtures of linear and branched PFAS isomers. AFFFs are known to contain blends of PFAS isomers and PFAS homologues with various carbon chain lengths. There are currently

few products that meet the required military specifications to suppress aircraft fires, and those that do, are manufactured using precursors that have the potential to oxidize or transform in-situ to a more persistent homologue (Nilsson et al., 2013; Place & Field, 2012). Therefore, there are presently no substitute AFFFs for use by the USAF that are completely free of PFAS.

The sustained use of PFAS has prompted many research studies from which a wide range of health effects in both animals and humans have been observed. Animal studies have suggested PFAS are predominantly found in plasma and in highly vascularized organs such as the liver and kidneys and that PFAS elimination rates are greater in females (J P Benskin et al., 2009; Ohmori, Kudo, Katayama, & Kawashima, 2003; Vanden Heuvel, Kuslikis, Van Rafelghem, & Peterson, 1991; Zhang, Beesoon, Zhu, & Martin, 2013). Studies have found that longer and linear forms of PFAS, are generally more toxic and have greater oral adsorption rates than shorter chained and branched isomers (Loveless et al., 2006; Ohmori et al., 2003). Furthermore; compounds with sulfonate moieties demonstrate greater toxicity than those with carboxylate moieties (Gorrochategui, Pérez-Albaladejo, Casas, Lacorte, & Porte, 2014). However, short and branched isomers, by and large, have higher clearances than their linear homologues (J P Benskin et al., 2009; Ohmori et al., 2003; Zhang et al., 2013). Epidemiological investigations have revealed that PFAS can persist in human serum for several years, and compounds with sulfonate moieties have longer blood half-lives than those with carboxylate moieties (Beesoon & Martin, 2015; Olsen et al., 2007). Furthermore, linear compounds with longer carbon backbones typically have longer blood half-lives than their branched and shorter homologues (Beesoon & Martin, 2015; Olsen et al., 2007).

PFHxS, while having a shorter chain length than PFOA and PFOS, was observed to have the longest mean half-life in human serum at approximately eight and a half years, almost twice as long as those of PFOA and PFOS (Olsen, et al., 2007). Additional epidemiological studies observed that PFHxS concentrations were greater than PFOS in umbilical cord blood than in maternal blood and suggested that its shorter chain-length was the determining factor in placental passage efficiency (Gützkow et al., 2012). Furthermore, Gützkow et al. (2012) observed greater relative placental transfer efficiencies of branched PFOS over its linear counterpart. Larger relative transplacental transfer efficiencies of branched PFAS isomers are also highlighted in other studies, and human cell-line research suggests that long-chained linear PFAS are more cytotoxic than their short-chained counterparts (Beesoon & Martin, 2015; Eriksen et al., 2010; Gorrochategui et al., 2014).

As previously mentioned, in-vitro human cell studies have been used to evaluate the cytotoxicity of PFAS. The results from animal studies are highly variable; therefore, human cell-line studies may produce more reproducible and consistent toxicological models. The human choriocarcinoma cell-line JEG-3 (ATCC® HTB36™) is a cancerous placental cell-line that is epithelial in nature and can be grown on a large scale (figure 1). JEG-3 has been previously used to determine the developmental toxicity of linear PFAS in an in-vitro study and may be used to evaluate the toxicity of branched PFAS isomers (Gorrochategui, Perez-Albaladejo, Casas, Lacorte, & Porte, 2014).

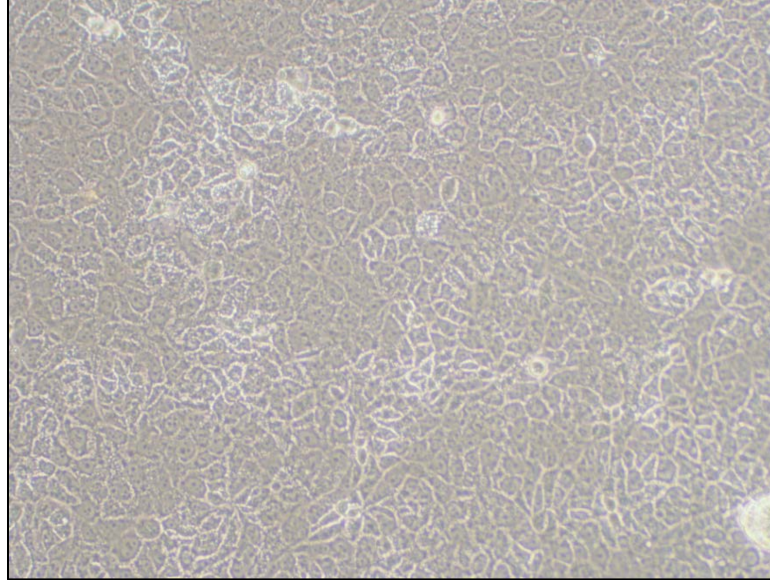

Figure 1: Image of JEG-3 choriocarcinoma cells captured using an Olympus CKX41 inverted microscope coupled with an Olympus DP71 camera at a 10x magnification (2016).

1.2 Problem Statement

Over the past decades, scientific research has focused on examining the toxicological properties of linear PFAS due to their greater manufactured abundance, more persistent in the environment, and because linear isomers have displayed greater retention times inside humans (Andersen et al., 2008; Olsen et al., 2007). Branched isomers, on the other hand, are more efficiently excreted in urine, but have been observed to have higher transplacental transfer efficiencies (Beesoon et al., 2011; Gützkow et al., 2012; Zhang et al., 2013). Beeson and Martin (2015) suggested that the higher affinity of linear isomers to human sera proteins was partly due to the fact that branched isomers were less hydrophobic than their corresponding linear isomers. Additionally, they suggested the larger size of branched isomers reduced the binding to albumin because of steric hindrance (Beesoon & Martin, 2015). Therefore, the higher concentration of the

unbound branched PFAS in human sera suggests greater transfer efficiencies from mothers to their fetuses. Monroy et al. (2008), observed serum levels of PFHxS in umbilical cords that were statistically greater than the levels in maternal serum and thus provided evidence of developmental exposures to this particular chemical.

Sustained use of PFAS-containing AFFFs in the USAF presents a risk of exposure to branched forms of PFAS to female personnel. Consequently, investigating the toxicological effects of the branched PFAS isomers and their modes of action would contribute to the overall toxicological knowledge.

1.3 Research Focus

The objective of this research is to clarify the hypothesis that linear and branched isomers of PFHxS interact with JEG-3 cells differently, and that this difference may lead to diverse cytotoxic effects in the cell cultures.

1.4 Scope and Approach

Three cell-line experiments were performed in the 711th Human Performance Wing's biological laboratories at Wright-Patterson Air Force Base (WPAFB). The human placental cell-line JEG-3 was selected to serve as the developmental toxicity model. The JEG-3 placental cell-line was exposed to both linear and branched isomers of PFHxS at specific dose ranges, and then incubated for a period of time. At the end of the experiment, the cells were analyzed for a set of biological endpoints using fluorescence microscopy and colorimetric assays.

1.5 Significance

The focus of this thesis is to compare the specific toxicity of linear and branched isomers of PFHxS on human placental cell-lines, and provide potential insights on the biochemical mechanisms of their toxicity using a series experiments.

1.6 Preview

This document was written using the AFIT style guide. Chapter II of this thesis describes the review of literature that was conducted for the purpose of this thesis. Chapter III presents the methodology used to perform the thesis experiments. All results and analyses from the experiments are revealed in Chapter IV, and a detailed discussion of the findings as well as the potential areas of future research are presented in Chapter V. Comprehensive data and sampling results are detailed in the appendices.

II. Literature Review

2.1 Chapter Overview

This chapter discusses and evaluates published scientific literature relevant to the thesis topic and to the related fields of study. The section provides the foundation and justification for our research.

2.2 Background

Perfluoroalkyl substances possess unique physicochemical characteristics that are the source of their remarkable stability under both natural and extreme environmental conditions. These distinctive properties allow PFAS to be highly resistant to biodegradation, and to be highly persistent in the environment. The perfluorinated substance family is constituted of many chemicals with individual configurations, moieties, and derivatives (Andersen, 2008). The toxicological effects of PFAS are dependent of the structure and size of the chemical isomer (Beesoon & Martin, 2015; Buhrke, Kibellus, & Lampen, 2013; Ohmori et al., 2003). Additionally, Perfluoroalkyl substances continue to be manufactured using the electrochemical fluorination (ECF) process, which produces mixtures of linear and branched forms of the compounds (Buck et al., 2011). The toxicological profiles of branched PFAS isomers have not been the focus of many research studies; however, there is evidence to suggest that branched PFAS have greater relative transplacental transfer efficiencies and are less toxic than their linear counterparts (Beesoon et al., 2011; Gützkow et al., 2012; Loveless et al., 2006). The JEG-3 human placental choriocarcinoma cell line has been used to examine the toxicity of linear PFAS and will be used to explore the toxicological potential of

branched forms of the chemicals (Gorrochategui et al., 2014; Tsang et al., 2013). JEG-3 is an epithelial cell-line that can be easily passaged and that forms adherent monolayers. These characteristics make JEG-3 cells ideal for fluorescence microscopy analyses and a suitable developmental toxicity model.

Cell metabolic activity and proliferation will be evaluated using a colorimetric assay at three PFAS exposure concentrations of 5µM, 10µM, and 50µM. Cellular viability, membrane integrity, and reactive oxygen species generation will be measured using two dye fluorescence assays at a concentration range between 0.2µM and 20µM. The concentrations and experimental parameters that were selected for this study are based on the conditions and results from similar work found in literature. A summary of the studies reviewed in literature can be found in Table 1.

Table 1: Summary of PFAS studies reviewed and observed effects

Authors	Chemical	Model	Dose Level	Average Measured Concentration	Observed Effect
Steenland and Woskie (2012)	PFOA	Epidemiological: Cohort Occupational	N/A	350 ng/mL (0.845 µM)	Renal Disease
Barry, Winquist, and Steenland (2013)	PFOA	Epidemiological: Cohort Occupational	N/A	174 ng/mL (0.42 µM)	Kidney and Testicular Cancer
		Epidemiological: General Public		19.4 ng/mL (0.047 µM)	
Rotander et al. (2015)	PFOS	Epidemiological: Cohort Occupational	N/A	74 ng/mL (0.148 µM)	None
	PFHxS			33 ng/mL (0.066 µM)	
	PFOA			4.6 ng/mL (0.011 µM)	
Calafat et al. (2007)	PFOS	Epidemiological: General Population (U.S.)	N/A	21.1 ng/mL (0.0422 µM)	None
	PFOA			4 ng/mL (0.01 µM)	
	PFHxS			1.9 ng/mL (0.004 µM)	
	PFNA			1 ng/mL (0.002 µM)	

Authors	Chemical	Model	Dose Level	Average Measured Concentration	Observed Effect
Butenhoff et al. (2012)	PFOS	In-vivo: Sprague Dawley Rats	20 µg/g/day	N/A	Decreased cholesterol levels; Increases in hepatocellular adenomas **LOAEL: 20 ppm**
Lau et al. (2006)	PFOA	In-vivo: Rats In-vivo: Mice	1 to 40 mg/kg/day	N/A	Adverse developmental outcomes; Lower post-natal survival; Increased liver weight; Growth delays **LOAEL: 3 mg/kg/day**
Loveless et al. (2006)	Linear/ Branched APFO	In-vivo: Rats	0.3 to 30 mg/kg/day	N/A	Weight loss; Decrease lipid levels **LOAEL: 0.3 to 1 mg/kg/day**
		In-vivo: Mice			Weight loss; Lower HDL and cholesterol; Increased triglycerides **LOAEL: 0.3 mg/kg/day**
Seacat et al. (2003)	PFOS	In-vivo: Sprague Dawley Rats	0.5 to 20 ppm/day	N/A	Increased liver weight; Decreased cholesterol **LOAEL: 20 ppm**
Eriksen et al. (2010)	PFOA PFOS PFBS PFNA PFHxA	In-vitro: HepG2 Hepatocarcinoma	0.4 to 2000 µM	N/A	ROS production and DNA damage after PFOS and PFOA exposures **LOAEL: 0.4 µM**
Wielsøe et al. (2015)	PFHxS PFOS PFOA PFNA PFDA PFUnA PFDoA	In-vitro: HepG2 Hepatocarcinoma	0.2 to 200 µM	N/A	ROS production; DNA damage; TAC decrease **LOAEL: 0.2 µM (PFOS)**
Florentin et al. (2011)	PFOA PFOS	In-vitro: HepG2 Hepatocarcinoma	5 to 800 µM	N/A	Cytotoxicity **LOAEL: 200 µM (PFOA)**
Buhrke et al. (2013)	PFBA PFOA PFHxA PFHpA PFNA PFDA PFDDA	In-vitro: HepG2 Hepatocarcinoma	0.1 to 2000 µM	N/A	Cytotoxicity; Cell proliferation; PPARα activation **LOAEL: 0.3 µM (PFDDA)**

Authors	Chemical	Model	Dose Level	Average Measured Concentration	Observed Effect
Buhrke et al. (2015)	PFOA	In-vitro: HepG2 Hepatocarcinoma	0.1 to 100 µM	N/A	Cell proliferation; PPARα activation; Gene expression **LOAEL: 10 µM**
Gorrochategui et al. (2014)	PFBA PFHxA PFOA PFNA PFDoA PFBS PFHxS PFOS	In-vitro: JEG-3 Choriocarcinoma	3 nM to 500 µM	N/A	Cytotoxicity; Cell lipid alteration; Aromatase inhibition **LOAEL: 57 µM (PFOS)**
Tsang et al. (2013)	PFOA	In-vitro: JEG-3 Choriocarcinoma	0.01 to 100 µM	N/A	Spheroid attachment; B-catenin suppression; E-cadherin expression; PPAR activation **LOAEL: 10 µM**
O'Brien et al. (2011)	T-PFOS L-PFOS	In-vitro: Chicken Embryonic Hepatocytes	1 to 40 µM	N/A	Altered expression of transcripts from genes regulating lipid metabolism, cell growth and proliferation, and liver development **LOAEL: 10 µM**

2.3 Toxicology

During the last few decades, numerous sampling studies uncovered PFAS in air, water, soil, wildlife, as well as in individuals around the world (Giesy & Kannan, 2001). Furthermore, these surveys exposed the bioaccumulative and biomagnification potential of PFAS in mammals (Giesy & Kannan, 2001; Kannan, et al., 2001). The primary exposure pathway for perfluoroalkyl substances is through ingestion of contaminated water or food, but low levels of the compounds may also enter the body by means of inhalation and dermal contact (Haug, Huber, Becher, & Thomsen, 2011; Steenland, Fletcher, & Savitz, 2010). Therefore, the health effects, distribution, and modes of action of perfluorinated compounds are of significant interest to the scientific community.

There are currently no known deaths that have occurred from acute overexposures to PFAS; however, human epidemiological studies have investigated mortality and morbidity rates in chronically exposed individuals (ATSDR, 2009). Some studies have suggested links between PFAS exposures and cholesterol and uric acid levels in humans, others have observed slightly higher incidences of prostate, testicular, and kidney cancer in individuals that have been occupationally exposed to the chemicals (ATSDR, 2009; Barry, Winquist, & Steenland, 2013). Positive correlations between serum PFOA concentrations and both kidney and testicular cancer were found in residents of the Mid-Ohio Valley exposed to contaminated drinking water (Barry et al., 2013). Likewise, Steenland and Woskie (2012) examined death rates in workers at a West Virginia DuPont chemical plant whose median PFOA serum levels were two orders of magnitude greater than the general population (4 ng/mL or 0.01 μM). The authors found evidence to suggest positive associations between high PFOA exposures and renal diseases. Epidemiological studies have also positively associated mean serum PFOA concentration levels in humans to the years of residence in a contaminated environment (Seals, Bartell, & Steenland, 2011). Elevated mean serum levels of PFOS (74 ng/mL), PFHxS (33 ng/mL), and PFOA (4.6 ng/mL) were also measured in a more recent study conducted by Rotander et al. (2015) on a group of Australian firefighters working with 3M AFFF. In 3% of the firefighters that participated in this study, mean serum concentration levels of PFOS remained above 200 ng/mL a decade after 3M's phasing out of PFOS-containing AFFF. Commonly encountered perfluorinated compounds have mean serum half-lives that ranged up to eight and a half years and have been detected at low concentration levels in the general U.S. population (Calafat, Wong, Kuklenyik, Reidy, & Needham,

2007; Olsen et al., 2007; Steenland et al., 2010). Although epidemiological studies have correlated specific health outcomes to PFAS exposures, evidence of the potential toxicological and carcinogenic effects of PFAS in humans is often inconsistent, and their distribution inside human tissue or target organs is not fully understood (ATSDR, 2009; Chang et al., 2014; Lau et al., 2007; Steenland et al., 2010). There is little to no indication that PFAS are directly genotoxic, there is however evidence that suggests that exposures to PFAS may result in liver and developmental toxicity (ASTDR, 2009; Andersen et al., 2008).

Humans and animals respond differently to PFAS exposures. Animal studies have revealed associations between PFAS exposures and a range of health outcomes. In addition to liver and developmental toxicity, immunologic and endocrine disruptions have been identified (Andersen et al., 2008). Biochemical effects and morphological changes in response to PFAS exposures have been widely studied in rodents. Dietary PFOS intakes of 20 μg/g/day have demonstrated increases in liver size and tumorigenesis as well as growth delays, and decreased post-birth survival rates in rodents (Butenhoff, Chang, Olsen, & Thomford, 2012; Lau et al., 2006). Weight loss and lowered cholesterol and triglyceride levels in plasma have been observed in rodents orally exposed to PFOS at levels of 20 μg/g/day (Andersen et al., 2008; Seacat et al., 2003).

Few toxicological studies have been conducted on branched PFAS isomers. Loveless et al. (2006) compared the responses of linear/branched, linear, and branched ammonium perfluorooctanoate (APFO) exposures in mice and rats. Ammonium perfluorooctanoate is a precursor of PFOA that is used in the manufacturing process. The authors noted that the toxicity profile of linear/branched APFO mix had similar endpoints to the toxicity

profile of linear APFO. Furthermore, the rodents exposed to linear PFAS experienced greater weight losses than those exposed to their branched counterparts. In rats, all three forms of PFAS exposure reduced lipid levels. In mice, all three forms of PFAS exposure lowered HDL cholesterol but triglycerides increased at lower levels (Loveless et al., 2006). Exposures to branched PFAS isomers of PFOA appeared to be less effective than linear and mixed forms. An animal-based in vitro study conducted by O'Brien et al (2011) revealed that technical grade PFOS containing mixtures of branched and linear isomers affected the expression of a greater number of transcripts from genes involved in lipid metabolism, cell proliferation and growth, and liver development. The authors suggested that the greater structural diversity of branched PFOS isomers activated a more extensive number of receptors, affected more signaling pathways, and employed a greater number of transcription factors (O'Brien et al., 2011). The limited data that is currently available in literature indicated evidence that branched PFAS isomers may generate different toxicological effects that the linear forms (Beesoon & Martin, 2015).

Linear PFAS such as PFOS and PFOA have greater binding affinity to human serum proteins than their branched counterparts (Beesoon & Martin, 2015). That said, shorter and branched forms are generally more readily eliminated and are believed to be less toxic overall (Jonathan P Benskin et al., 2009; Loveless et al., 2006; Ohmori et al., 2003; Zhang et al., 2013). Evidence for the lower toxicity of branched isomers is supported by the Loveless et al. (2006) study previously discussed in which mean body weight reductions in rodents exposed to branched PFAS isomers that were four to six times lower than those exposed to linear compounds. Nonetheless, researchers have

measured higher relative transplacental transfer efficiencies of branched isomers than their corresponding linear forms (Beesoon et al., 2011; Gützkow et al., 2012). Beeson et al. (2011) observed median transplacental transfer efficiencies between 0.34 and 0.88 for branched PFOS isomers and 0.30 for linear PFOS. Additionally, the authors measured transplacental transfer efficiencies that were at times greater than one for several branched PFOA isomers. Therefore, it is of great interest to understand the toxicological properties of branched perfluorinated compounds in order to evaluate their effects on fetal development.

2.4 Toxicokinetics and Modes of Action

Perfluoroalkyl substances structurally resemble fatty acids and seem to share several of their characteristic behaviors inside biological systems. Additionally, PFAS appear to be metabolically stable; thus, the toxicity of their potential metabolites is of little or no concern (Andersen, et al., 2008). This remarkable stability coupled with the observed affinity of linear PFAS towards albumin and other human serum proteins may explain their relatively long half-lives and poor elimination rates in humans (Andersen et al., 2008; Beesoon & Martin, 2015).

There is evidence that perfluorinated compounds increase the production of intracellular reactive oxygen species (ROS) that may lead to DNA damage, and cell death (Eriksen et al., 2010; Wielsøe, Long, Ghisari, & Bonefeld-Jørgensen, 2015). However, some studies have indicated contradictory results that show no link between PFAS and DNA damage (Florentin, Deblonde, Diguio, Hautemaniere, & Hartemann, 2011). PFAS activation of various types of peroxisome proliferator-activated receptors (PPAR) has

been confirmed in mice and rats (Vanden Heuvel, Thompson, Frame, & Gillies, 2006). Activation of PPARα in rodents induced weight loss and lowered cholesterol and triglyceride levels in plasma due to increased mitochondrial and peroxisomal oxidation of fats (Andersen et al., 2008; Seacat et al., 2003).

Elimination rates vary significantly with each animal species and gender, and excretion primarily occurs via bile and urine. However, fecal excretion predominates in humans (Andersen, et al., 2008). With the exception of 1m-PFOS, branched isomers of PFOA and PFOS are preferentially excreted in urine compared to linear isomers (Zhang et al., 2013). Additionally, PFHxS has the lowest renal clearance efficiency out of the linear isomers (Zhang et al., 2013).

The focus of PFAS toxicological research has been on linear isomers. Branched forms of PFAS, however, have higher transplacental transfer efficiencies (Beesoon et al., 2011; Gützkow et al., 2012). Beeson and Martin (2011) observed that the placental transfer of branched PFOS increased as branching point moved closer to the sulfonate moiety. The authors used ultrafiltration devices to determine dissociation constants of PFOS and PFOA isomers with human serum albumin, and to examine relative binding affinities of isomers to human serum. Their results indicated that linear PFAS bind more tightly to human sera proteins than branched isomers (with the exception of 1m-PFOS). The authors suggested that the difference in affinity is due to the lower hydrophobicity and larger size of branched PFAS isomers with respect to their linear counterparts. They noted that larger ligands do not bind as readily to albumin and other serum proteins because of steric hindrance (Beesoon & Martin, 2015). Furthermore, the authors propose that the lower binding affinity of branched isomers results in their higher relative

concentrations in human sera and may potentially explain their higher transplacental transfer efficiencies and favored renal excretions (Beesoon & Martin, 2015; Zhang et al., 2013).

Animal studies, however, are often species and gender specific, and their relevance to humans is sometimes difficult to extrapolate (Lau et al., 2007; Steenland et al., 2010). Similarly, human and epidemiological studies may not be able explain the biological mechanisms from which a chemical can induce toxicity. Investigating toxicological effects using human cell-lines may therefore provide more applicable and reproducible data.

2.5 Human Cell-line Studies:

Human cell-line studies have been used to model the toxicological effects of PFAS in humans because they provide an easier, relevant, and more cost effective solution to animal studies and human epidemiological studies. Various cell-line studies have been conducted to characterize the toxicological effects of PFAS. Several studies suggested hepatotoxic effects from PFAS exposures using the HepG2 hepatocellular carcinoma cell-line, and hypothesized that the potential mode of action is through generation of oxidative stress (Wielsoe M. , Long, Ghisari, & Bonefeld-Jorgensen, 2014; Florentin, Deblonde, Hautemaniere, & Hartemann, 2011; Eriksen K. T., et al., 2010). HepG2 cells are often used in liver toxicological studies because they exhibit a significant metabolic activity and because their epithelial nature allows scientists to easily grow and observe their morphological changes (Florentin, Deblonde, Hautemaniere, & Hartemann, 2011). Eriksen *et al* (2010) investigated the potential of five different

perfluorinated compounds of different carbon chain lengths to generate ROS and induce oxidative DNA damage in hepatocellular HepG2 cells. The study confirmed modest increases in intracellular ROS production from exposures to PFOA and PFOS, but genotoxic effects were only observed in cells exposed to perfluorononanoic acid (PFNA) at concentrations capable of cytotoxicity (Eriksen et al., 2010). Similarly, Wielsøe *et al* (2015) examined the potential effects of seven long-chained PFAS commonly found in human sera using the HepG2 cell-line. The authors of this particular study tested ROS generation, genotoxicity, and total antioxidant capacity (TAC) disruption of PFAS after a 24 hour exposure to PFAS concentrations between 0.02 μM to 200 μM (Wielsøe et al., 2015). Their results mirrored Eriksen *et al* study in terms of the potential for PFAS to induce the production of ROS; however, four of the compounds that they tested (PFHxS, PFOA, PFOS, and PFNA) showed signs of genotoxicity due to a dose dependent increase in cellular DNA damage registered by comet assay. Furthermore, their results suggested that PFOA decreased the TAC 0.70 to 0.82 fold relative to their solvent control (Wielsøe et al., 2015). The concentrations used in this study were comparatively high to the levels commonly found in humans. Mean serum concentration levels of PFOA, PFOS, and PFHxS in the general U.S. population are in the 1.5 to 55.8 ng/ml range or approximately 3 to 135 nM (ATSDR, 2009). Florentin *et al* (2011) investigated the genotoxic and cytotoxic effects of PFOA and PFOS using human HepG2 cells after one hour and 24 hour exposures and determined direct mutagenic effects through DNA damage and indirect mutagenic effects though ROS generation. The cytotoxic effects were evaluated by MTT assay and the genotoxic potential was investigated using single cell gel electrophoresis assay and micronucleus assay. Cytotoxic effects were only observed after

24 hour exposures at concentrations over 200 µM for PFOA, and at concentrations greater than 300µM for PFOS. Moreover, above these concentrations intracellular vacuoles began to form and extracellular matrices disappeared which resulted in a substantial number of suspended cells. Despite the resulting cytotoxic effects, no significant genotoxic effects were observed during this study which supported Eriksen *et al* results, but differed from those of Wielsøe *et al*. Both PFOA and PFOS decreased ROS generation relative to a 2.5% DMSO solvent control in the Florentin *et al* study. The effect of different chain length PFAS was also explored by Buhrke *et al* (2013) using the HepG2 cell-line. This specific study showed no evidence of genotoxic potential, though cytotoxic effects increased with PFAS chain length.

Human cell-line studies have been conducted to determine toxicity of PFAS using placental choriocarcinoma cells (JEG-3). Gorrochategui *et al* (2014) examined the cytotoxicity of eight PFAS using the JEG-3 cell-line. The cell's chemical uptake was analyzed to confirm that PFAS were being absorbed by the cells, the cell's P450 aromatase activity to observe signs of endocrine disruption, and the cell's lipid content to examine for lipid metabolism disturbance. The authors noted that long-chained PFAS such as PFOA, PFOS, perfluorododecanoic acid (PFDoA), and perfluorononanoic acid (PFNA) were cytotoxic to JEG-3 cells, whereas short-chained PFAS such as PFHxS showed no cytotoxicity even at the highest concentration of 500µM. Short-chained PFAS did, however, appear to inhibited aromatase activity suggesting a potential disruption of the endocrine regulatory system. Additionally, the authors observed that PFAS with sulfonated moieties had relatively higher toxicity than perfluorinated compounds with carboxylated moieties of the same length. This difference in

cytotoxicity was most apparent between PFOS and PFOA where the latter was observed to have an EC_{50} that was five times greater. Moreover, Gorrochategui *et al* (2014) distinguished that PFAS altered the patterns of cellular lipids at concentrations below those associated with cytotoxicity and hormonal disruption. The most pronounced changes in cellular lipidome were recorded at an exposure concentration of 0.6µM which was the lowest concentration that was evaluated. Another study performed on JEG-3 cells showed evidence that PFOA at a concentration equal or greater that 10 µM also disrupted cell to cell adhesion by means of PPARα activation (Tsang et al., 2013). Cell to cell adhesion in placental cells is critical to the initial attachment of an embryo to the uterine wall (Tsang et al., 2013).

The results from the Tsang *et al* study and the higher transplacental transfer efficiencies observed in branched PFAS isomers support the need for further toxicological studies in the field. Thus, the objective of this thesis is to confirm the hypothesis that linear and branched isomers affect cells differently, and that this difference will lead to diverse cytotoxic effects in cell cultures. The research may also provide proof that higher transplacental transfer efficiencies of branched isomers lead to increased developmental toxicity by investigating cell viability, cell morphology, and ROS generation of JEG-3 cells under PFAS exposure.

III. Methodology

3.1 Chapter Overview

The purpose of this chapter is to describe the materials, experiments, and analytical procedures that were used to investigate the toxicological differences between branched and linear isomers of PFHxS on a human cell line. The JEG-3 choriocarcinoma placental cells were treated with different concentrations of both linear and branched PFHxS isomers and then observed for changes in biomarkers. Three distinct experiments were performed to analyze the cytotoxicity of PFAS exposures.

Cell viability was examined using a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay at PFHxS concentrations of 5 µM, 10 µM, and 50 µM. MTT is a dye that is metabolized by living cells into the purple compound formazan whose peak light absorbance is near 570 nm. Exposures to PFAS will either promote or inhibit cell proliferation. At higher cell densities the MTT assay will result in higher light absorbencies that can be read by a microplate reader.

Cell death and cell membrane damage was investigated using the Molecular Probes™ LIVE/DEAD® fluorescence-based cell viability assay at five concentrations between 0.2 µM and 20 µM. JEG-3 cells were treated with a calcein AM and ethidium homodimer (EthD-1) fluorescent dye solution that measures intracellular esterase activity and plasma membrane integrity, respectively. Intact living cells are discerned from dead cells by a green fluorescence, whereas dead cells are characterized by a red fluorescence. This assay is used to determine the cytotoxicity of PFAS via fluorescence microscopy

under a blue excitation light through a longpass filter. Higher red to green fluorescence ratios indicate greater cell toxicity.

Intracellular reactive oxygen species (ROS) generation was studied using a 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) based fluorescence assay at four concentrations between 0.2 μM and 10 μM. Cells are treated with both carboxy-H2DCFDA fluorescent dye and Hoechst 33342 nuclear counterstain. The carboxy dye interacts with intracellular esterases and oxidation from ROS and is transformed into a homologue that fluoresces green under a blue excitation light. The counterstain reacts with nuclear DNA and fluoresces blue under UV light. Subsequently, green fluorescence is normalized by cell number and quantified via software. A greater presence of ROS will result in more intense green fluorescent signals.

3.2 Materials and Equipment

3.2.1 Chemicals

Linear and branched forms of PFHxS (MW = 400.12 g/mol; pKa = 0.14) were purchased from Wellington Laboratories (ON, CANADA) and dissolved in dimethyl sulfoxide (DMSO) to a one millimolar (mM) stock solution. Figure 2 displays the structural diagrams of all the PFHxS isomers that were employed in this study. The LIVE/DEAD® (Molecular Probes™, OR, USA) cell viability assay was ordered from Thermo Fisher Scientific (MA, USA). Reactive oxygen species generation assay dyes and MTT dye were obtained from Sigma-Aldrich (MO, USA).

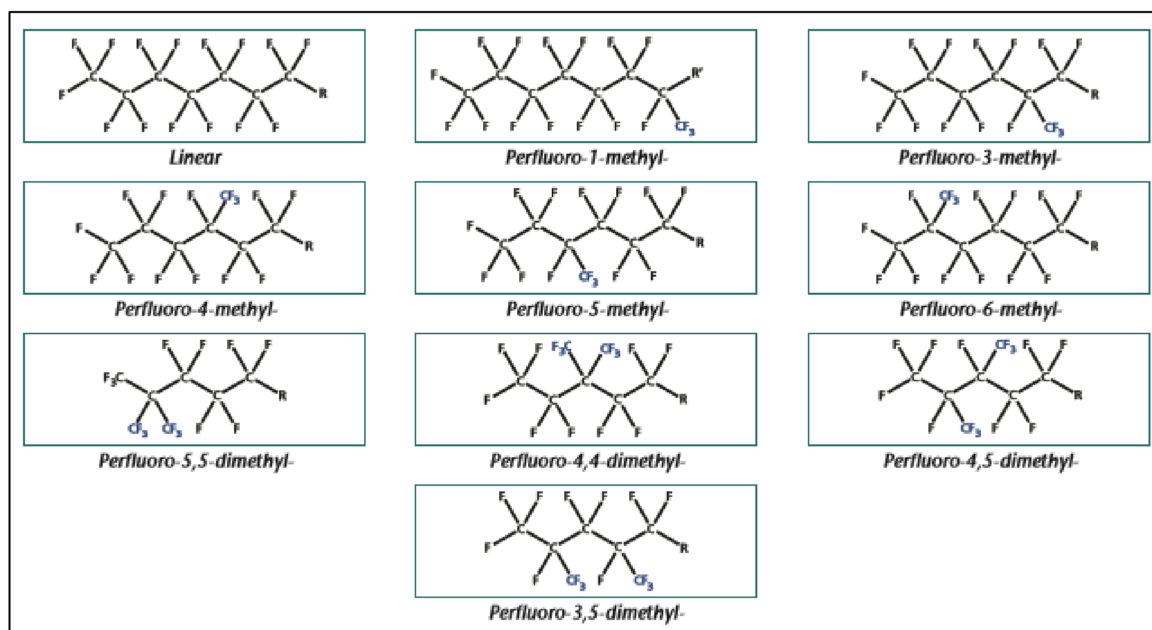

Figure 2: Structural diagrams of linear and branched PFAS isomers tested (R = $CF_2SO_3^-$ and R'= SO_3^-). The image was retrieved from the 2016-2018 Wellington Laboratories catalog on 5 February 2017.

3.2.2 Cell Cultures

The JEG-3 cell line was acquired from American Type Culture Collection (ATCC, MD, USA), and cultured in 75 cm^2 culture flasks (Corning, NY, USA) at 37°C and 5% CO_2 in a humidified environment with Eagle's Minimum Essential Medium (EMEM) (ATCC, VA, USA) supplemented with 10% fetal bovine serum (ATCC, VA, USA) and 1% and penicillin-streptomycin (Sigma-Aldrich, MO, USA). The cells were passaged every three to four days at 70-90% confluency. Cell densities were selected and adjusted based on comparable research.

3.2.3 Equipment and Software

Fluorescence imaging for the LIVE/DEAD® cell viability assay was conducted with an Olympus CKX41 inverted microscope coupled with an Olympus DP71 camera. Images were taken at 10X or 20X magnification and processed with the DP71 proprietary software. Cell counting was accomplished with Fiji image analysis software (Schindelin et al., 2012). Absorbance measurements for the MTT assays were collected using a BioTek® Synergy 2 high-performance multi-mode reader. Absorbance data was processed with BioTek®'s Gen5 microplate reader and imager software. Fluorescence images for the ROS generation assay were obtained with a Molecular Devices ImageXpress Micro high capacity imager and analyzed with Fiji image analysis software (Schindelin et al., 2012).

3.3 Experimental Procedures

3.3.1 Metabolic Activity and Cell Proliferation Examination

JEG-3 cells were seeded in a 96-well culturing plate at a density of 5,000 cells per well in 200 µL of EMEM media and then incubated for 24 hours at 5% CO_2 and 37°C. Subsequently, the cells were treated with 5 µM, 10 µM, and 50 µM of each chemical and then incubated for another 48 hours. Three wells were left unseeded and served as blank samples. An additional well was treated with 5% DMSO to serve as a negative control and another well was treated with 10 ng/mL of epidermal growth factor (EGF) to serve as a positive control. A 48 hour exposure to PFAS was specifically selected for this assay based on logistical constraints; however, the exposure time is consistent with similar

studies found in literature. After the 48 hour exposure to both linear and branched PFHxS, the cells were incubated for four additional hours with a 5mg/ml MTT solution in sterile phosphate-buffered saline (PBS) solution. Following the incubation period, 50 µL of DMSO was mixed into each well and the culturing plate was incubated for another ten minutes at 5% CO_2 and 37°C in a humidified environment. Upon completion, the contents of each well were then mixed using a plate shaker and their absorbance was measured at a wavelength of 540 nm using the microplate reader. Three technical replicates were conducted simultaneously.

3.3.2 Cell Viability and Membrane Damage Examination

JEG-3 cells were seeded in a 8-well chamber slide at a density of 5,000 cells per well with 200 µL of EMEM media and incubated for 24 hours at 5% CO_2 and 37°C. Five wells were then treated with different concentrations of PFAS and incubated at 5% CO_2 and 37°C for 24 hours. After the 24 hours, the cells were rinsed with PBS and two wells were treated with 70% ethanol for ten minutes to serve as negative controls. The negative controls were rinsed three times with PBS, and the fluorescent dyes were then mixed into each well. The slide was then incubated for an additional 45 minutes. The staining solution was then removed from each well and 100 µL of PBS was added to each well. Subsequently, the cells were imaged under a fluorescent microscope and then counted. A series of three independent experiments were performed for both linear and branched PFHxS to ensure reproducibility.

3.3.3 Intracellular ROS Generation Examination

JEG-3 cells were seeded in a 8-well chamber slide at a density of 5,000 cells per well with 200 µL of EMEM media, and incubated for 24 hours at 5% CO_2 and 37°C. The cells were then treated with 0.2 µM, 2 µM, 5 µM, and 10 µM of both linear and branched PFHxS and incubated at 5% CO_2 and 37°C for 24 hours. One well was treated with 0.1% DMSO to serve as our solvent control. After the 24 hours, the cells were rinsed with PBS and an additional well was treated with a 100 µM solution of tert-butyl hydroperoxide (tBuOOH) for one hour to serve as our positive control, and then rinsed again with PBS. The cells were subsequently labeled with carboxy-H2DCFDA fluorescent dye, and incubated for 30 minutes under the previous conditions. After 25 minutes, the nuclear counterstain was applied to each well and the cells were then rinsed three times with PBS prior to being imaged under fluorescence microscopy.

3.4 Statistical Analysis

Statistical analysis was accomplished using GraphPad Prism version 5.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Statistical differences in the MTT, LIVE/DEAD®, and ROS generation assays were evaluated using non-parametric Kruskal-Wallis tests with Dunn's post hoc test ($p < 0.05$). Mann-Whitney U tests ($p < 0.05$) were also used to compare statistical differences between isomers at specific concentrations as well as the differences between different concentrations. Data could not be tested for normality due to the small sample numbers; however, parametric analyses (one-way ANOVA with Bonferroni multiple comparison test, and Student's *t*-tests) were conducted in parallel under the assumption of normality,

and compared to our results ($p < 0.05$). F-tests were performed to compare variances between sample sets and Welch's corrections were applied to comparisons between samples sets with unequal variances.

IV. Analysis and Results

4.1 Chapter Overview

This chapter presents and discusses the results from the three experiments that were designed and conducted for to test the toxicological differences between linear and branched isomers of PFHxS. Assay development and improvement are not addressed in this section.

4.2 Experimental Results

4.2.1 Metabolic Activity and Cell Proliferation

The results of the MTT assay are presented in Figure 2 as relative percent absorbance to untreated cells (control). After the 48 hours of exposure to linear and branched PFHxS, no statistically significant differences were observed between linear and branched PFHxS at any of the concentrations that were tested (Kruskal-Wallis one-way ANOVA with Dunn's post hoc test and Mann-Whitney U tests, $p < 0.05$). However, the arithmetic mean absorbance of branched PFHxS was measured to be 20.25% lower than that of its linear homologue at a concentration of 50 μM. Similarly, no statistically significant differences were found between untreated cells and exposed cells, though the mean absorbance for linear PFHxS at 50 μM was 20.4% higher than that of untreated cells. Lastly, no statistically significant differences were perceived between all three concentrations in both linear and branched forms of PFHxS. Arithmetic means do suggest, however, a slight dose-dependent increase in cell proliferation induced by the linear form of PFHxS. Parametric analyses of our data found similar outcomes.

Results from this experiment are consistent with similar human cell-line studies. The negative control successfully decreased metabolic activity but the positive control showed no changes with respect to untreated cells. Buhrke et al. (2013) measured cellular proliferation stimulation in HepG2 cells when exposed to low PFAS concentrations. PFOA, most noticeably, induced metabolic activities in cells at concentrations between 5 μM and 25 μM, and inhibited (IC_{50}) cell proliferation at approximately 47 μM (Buhrke et al., 2013).

Branched PFHxS exposures resulted in a decrease in cell proliferation with increasing concentration. The drop in cell metabolic activity was most noticeable between 5 μM and 10 μM (-16.9%), however, the mean relative absorbance at 50 μM was negligibly higher than at 10 μM (9.3%) and lower than at 5 μM (-9.2%). After an extensive review of literature, there are no scientific studies that have specifically examined the effects of branched PFAS on cell proliferation of human cell-lines. Therefore the results from this experiment are new to the field and cannot be compared to other research.

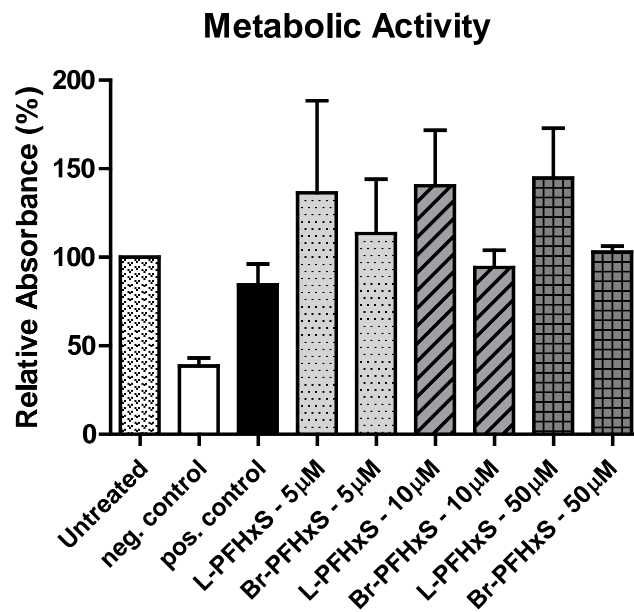

Figure 3: Metabolic activity induced by linear and branched PFHxS. JEG-3 cells were exposed to three concentrations for 48 hours. The metabolic activity was subsequently measured via MTT assay and normalized to the untreated control set to 100%. A 5% DMSO solution served as negative control and 10 ng/mL of EGF served as positive control. Overall data was analyzed using Kruskal-Wallis non-parametric test followed by Dunn's post hoc test ($p<0.05$). Mann-Whitney U tests were used to compare data sets to the control ($p<0.05$). Error bars indicate ± SD from the mean (n=3).

4.2.2 Cell Viability and Membrane Damage

Results from the cell-viability experiments are presented in Figure 3 as percent viability of total cells. After 24 hours of exposure to both linear and branched PFHxS, no statistical differences were observed between linear and branched PFHxS exposures at any of the concentrations that were tested (Kruskal-Wallis one-way ANOVA with Dunn's post hoc test and Mann-Whitney U tests, $p< 0.05$). Mean cellular viability, however, was negligibly higher in JEG-3 cells exposed to the branched form of PFHxS at all five concentrations 0.2 µM (3.74%), 2 µM (3.79%), 5 µM (2.12%), 10 µM (3.42%), and 20 µM (1.1%).

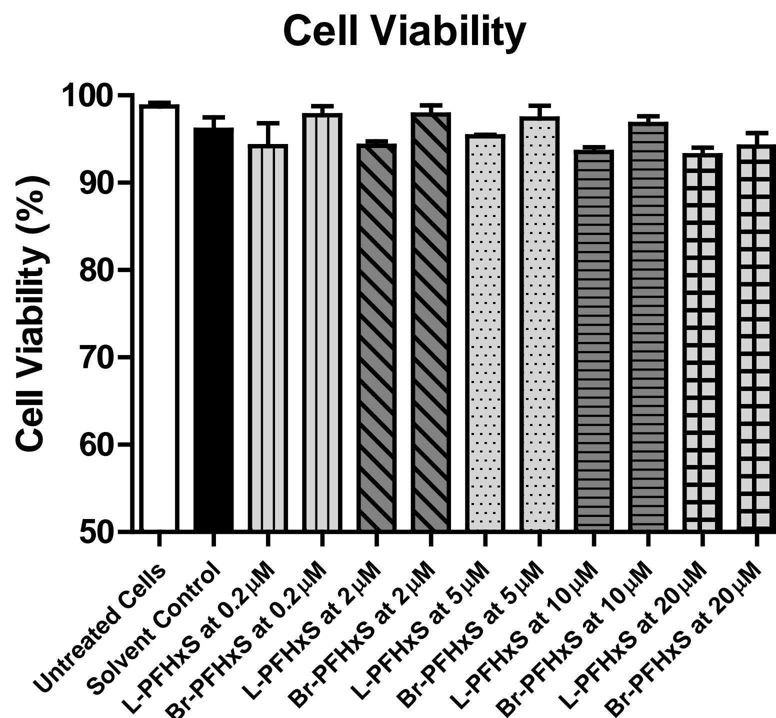

Figure 4: Cellular viability after exposure to linear and branched PFHxS. JEG-3 cells were exposed to five concentrations for 24 hours. The experiment was conducted using Thermo Fisher Scientific's LIVE/DEAD® fluorescence-based cell viability assay. Results are presented as percent viability of total cells. A 0.1% DMSO solution served as solvent control. Kruskal-Wallis one-way ANOVA was used to compare all data ($p<0.05$). Mann-Whitney U tests were used to compare two data sets ($p<0.05$). Means ± SD (n=3).

Furthermore, no statistically significant differences in cell viability were measured between untreated cells and cells exposed to both linear and branched PFHxS isomers at any of the concentrations that were tested.

Nevertheless, parametric analyses (one-way ANOVA with Bonferroni's multiple comparison test and unpaired Student's *t*–tests with Welch's corrections when applicable, $p < 0.05$) showed statistically significant differences between branched and linear isomers at 2 µM ($p = 0.0061$), and at 10 µM ($p = 0.0058$). No differences were found at any of the other concentrations. Compared to untreated cells, viability was statistically lower in

cells exposed to linear PFHxS at 2 μM ($p = 0.0003$), 5 μM ($p = 0.0002$), 10 μM ($p = 0.0002$), and 20 μM ($p = 0.0006$). Similarly, cells exposed to branched PFHxS showed statistically lower viabilities than untreated cells at 10 μM ($p = 0.0234$) and at 20 μM ($p = 0.0074$).

The data was subsequently normalized to the control (untreated cells) in order to discern any potential effects on the cells from the solvent and plotted as relative percent viability (Figure 4). Although no statistical differences were observed between untreated cells and the solvent control after a Mann Whitney U test, an unpaired Student's *t*-test found a difference between the two that was statistically significant ($p = 0.0365$). Additionally, the mean viability of cells exposed to the solvent was 2.72% lower than the viability of untreated cells. This difference is expected but does not appear to have a significant impact on the results of the experiment given that the cells exposed to branched PFHxS exhibited generally higher relative mean viability to the solvent control whereas those exposed to linear PFHxS displayed lower relative mean viability.

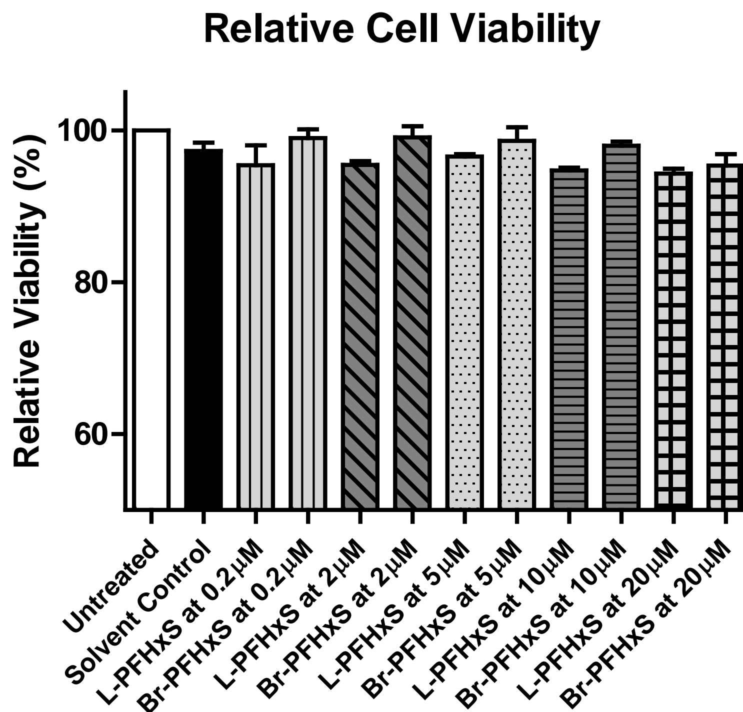

Figure 5: Cellular viability after exposure to linear and branched PFHxS. JEG-3 cells were exposed to five concentrations for 24 hours. The experiment was conducted using Thermo Fisher Scientific's LIVE/DEAD® fluorescence-based cell viability assay. Results are presented as percent viability relative to control (untreated cells). A 0.1% DMSO solution served as solvent control. Kruskal-Wallis one-way ANOVA was used to compare all data ($p < 0.05$). Mann-Whitney U tests were used to compare two data sets ($p < 0.05$). Means ± SD ($n = 3$).

There are no studies that have specifically used Thermo Fischer's LIVE/DEAD® assay to examine the effects of PFAS on human cell-lines. However, Gorrochategui et al. (2014) evaluated the cellular viability of JEG-3 cells exposed to 500 µM of eight different linear PFAS using a similar two fluorescent dye assay (Alamar Blue, and 5-carboxyfluorescein diacetate). The authors did not notice any significant changes in cellular viability upon exposure to linear PFHxS at 500 µM. The results obtained by Gorrochategui et al. (2014) are in line with those highlighted in this thesis and suggest that linear PFHxS at low concentrations is not cytotoxic to JEG-3 placental cells.

Morphological differences in JEG-3 cells were visually examined under fluorescence microscopy. Figure 6 presents representative images of cells exposed to different concentrations of linear PFHxS for 24 hours.

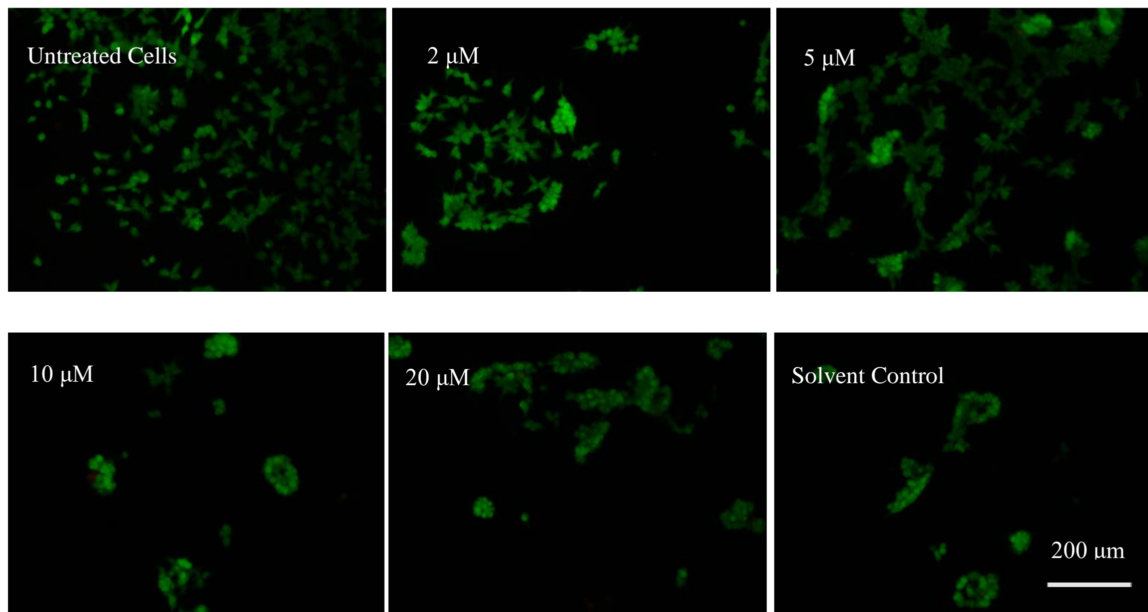

Figure 6: Morphological differences in JEG-3 cells exposed to linear PFHxS for 24 hours. Images were captured at 10x magnification.

The disappearance of oblong spindle shapes and the formation of small clusters of rounded cells was observed at exposures greater or equal to 2 µM. Negligible cell lysis was noticed at exposures of 10 µM and above, though no significant amount of cell detachment was detected. The solvent control in our experiment appeared to have a measurable impact on the cells, and induced cell lysis and similar cluster formations as linear PFHxS. Changes in cell morphology in our experiment may therefore be the result of the solvent used (0.1% DMSO).

The effects of branched PFHxS on JEG3 cellular morphology were also investigated and are presented in figure 6.

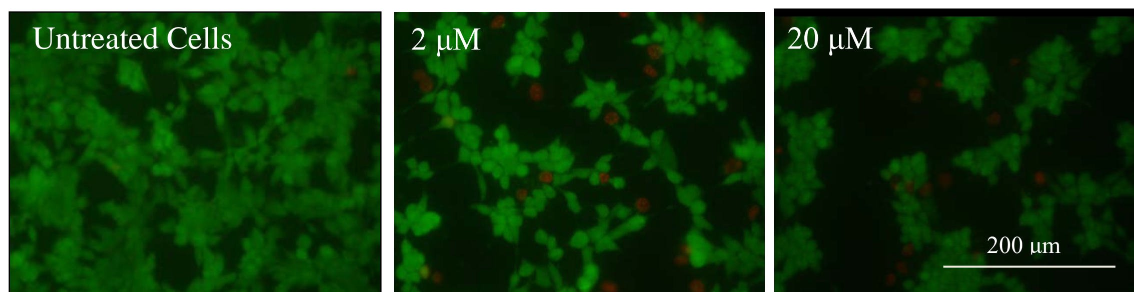

Figure 7: Morphological differences in JEG-3 cells exposed to branched PFHxS for 24 hours. Images were obtained at a 20x magnification.

There were no significant morphological changes detected in JEG-3 cells that were exposed to branched PFHxS isomers at any of the concentrations tested. These observations are consistent with the higher relative cellular viability that was previously measured, but contradicts the cellular effects that were observed in the solvent control. Unfortunately, no research that was reviewed has examined the morphological effects of branched PFAS on human cell-lines. Consequently, our results cannot be compared or validated by other studies.

4.2.3 Intracellular ROS Generation

The results from the ROS generation assay are presented in Figure 8 as fold increases in fluorescence intensity with respect to the control (untreated cells).

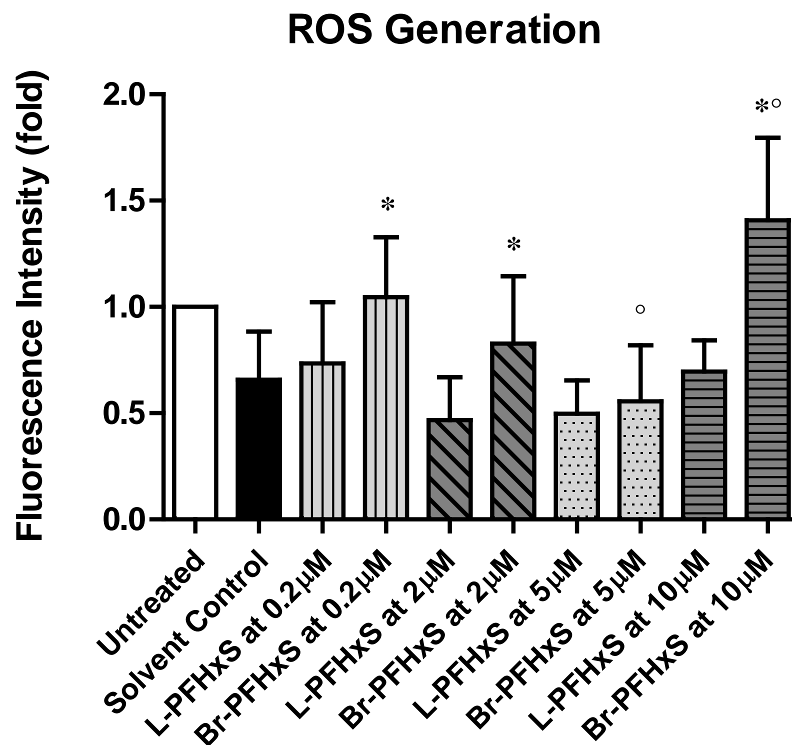

Figure 8: ROS generation in JEG-3 cells induced by linear and branched PFHxS. Cells were exposed to four concentration for 24 hours. ROS generation was subsequently measured using a carboxy-H2DCFDA based fluorescence assay. Results are presented as the fold ratio compared to untreated cells (control) and expressed as means ± SD ($p<0.05$). A 0.1% DMSO solution served as negative control. * Statistically significant differences between cells exposed to linear and branched PFHxS isomers at equal concentrations. ° Statistically significant differences between cells exposed to linear or branched PFHxS and untreated cells.

After a 24 hour exposure to both linear and branched PFHxS, we observe statistical differences in the production of ROS between linear and branched isomers (Mann-Whitney U tests, $p < 0.05$) at 0.2 µM ($p = 0.0009$), 2 µM ($p = 0.0011$), and 10 µM ($p = < 0.0001$). Variations in mean fluorescence intensity were 31.7% greater for branched PFHxS at 0.2 µM, 43.4% greater at 2 µM, 7.3% greater at 5 µM, and 50.5% greater at 10 µM. No statistical difference between linear and branched isomers were found at 5 µM ($p = 0.6949$). Parametric analyses of the data resulted in similar outcomes.

Results suggest that branched PFHxS generated higher rates of ROS generation than its linear counterpart at equal concentrations. Mean fluorescence intensities of cells exposed to linear PFHxS were statistically lower than those from untreated cells (Mann-Whitney U tests, $p < 0.05$) at 0.2 µM ($p = 0.0471$), 2 µM ($p < 0.0001$), 5 µM ($p < 0.0001$), and 10 µM ($p = 0.0417$). Results from this experiment suggest negligible ROS generation in JEG-3 cells after exposure to linear forms of PFHxS. Representative images obtained during our experiment are presented in figures 8 through 13.

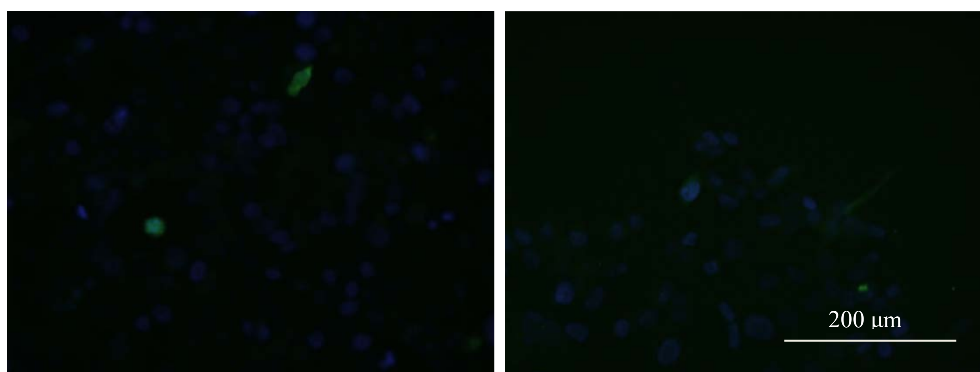

Figure 8: Untreated cells (left) and solvent control (right). Images were captured at 20x magnification.

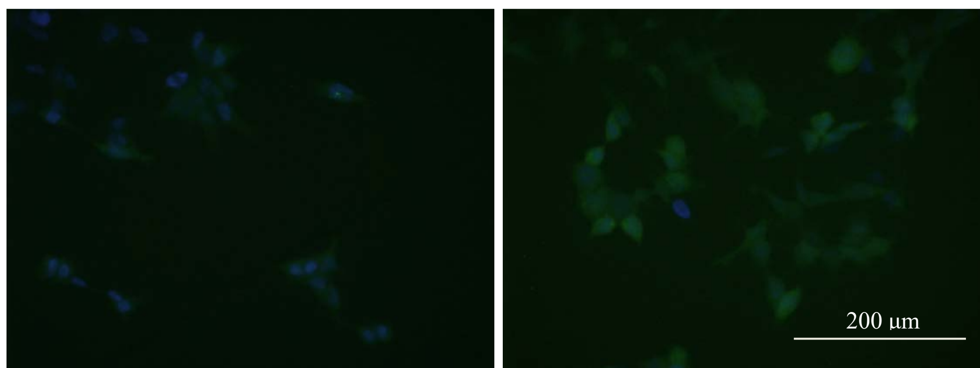

Figure 9: ROS generation for linear (left) vs branched (right) PFHxS at 0.2 µM. Images were captured at 20x magnification.

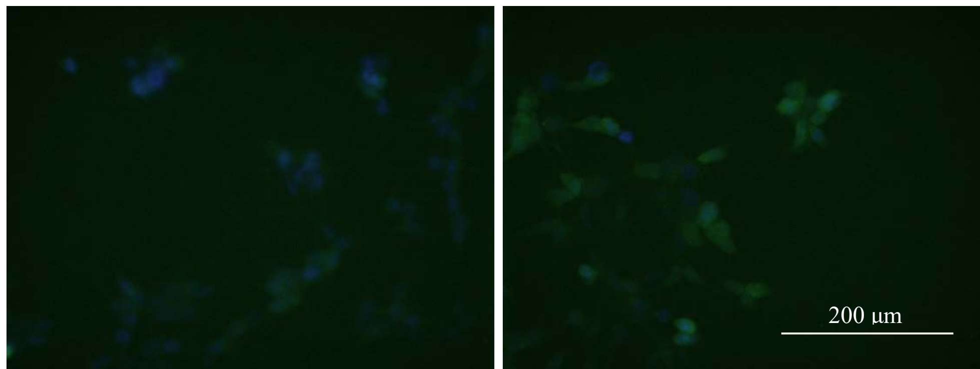

Figure 10: ROS generation for linear (left) vs branched (right) PFHxS at 2 µM. Images were captured at 20x magnification.

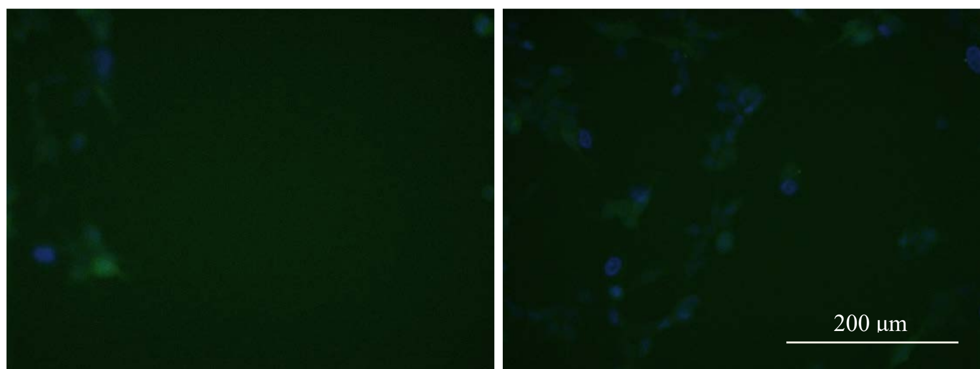

Figure 11: ROS generation for linear (left) vs branched (right) PFHxS at 5 µM. Images were captured at 20x magnification.

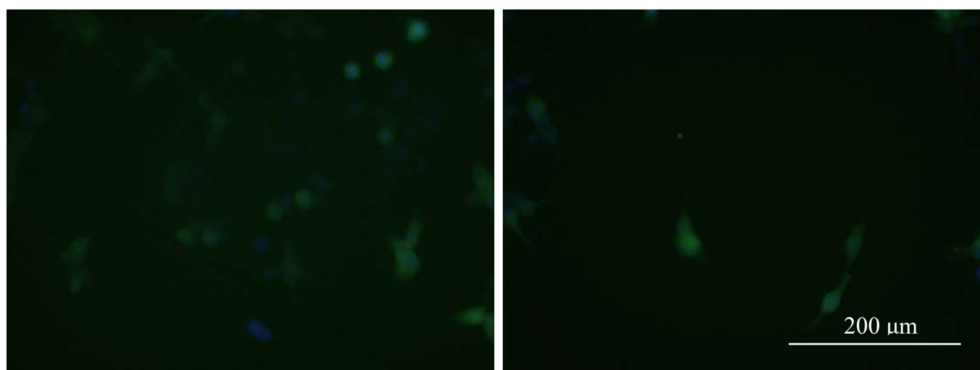

Figure 12: ROS generation for linear (left) vs branched (right) PFHxS at 10 µM. Images were captured at 20x magnification.

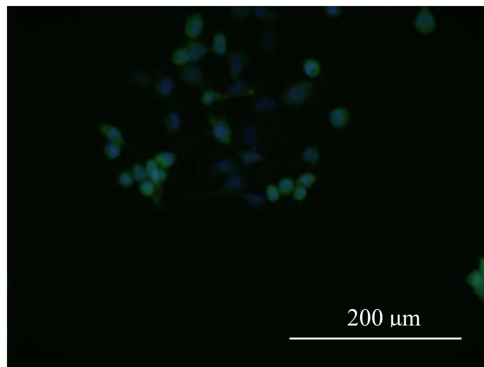

Figure 13: ROS generation for positive control (tBuOOH). Images were captured at 20x magnification.

Florentin et al. (2011) obtained similar results when exposing HepG2 human liver cells to linear forms of PFOS and PFOA at concentrations between 5 μM and 400 μM for a period of 24 hours, though the authors of this study employed a different procedure to detect ROS. Under similar conditions, however, Eriksen et al. (2010) observed minor increases in ROS generation in HepG2 exposed to PFOA (1.52 fold) and PFOS (1.25 fold) compared to untreated cells. Florentin et al. (2011) suggested that the difference in results from these two studies was potentially due to differences in procedures; more specifically, Florentin et al. (2011) avoided re-suspending cells after trypsinization and pelleting so as not to induce further cellular stress. Cells were not re-suspended after trypsinization and pelleting in our experiment. A third study conducted by Wielsøe et al. (2015) exposed HepG2 cells to seven different linear PFAS at concentrations between 0.2 μM to 200 μM, and reported an increase a ROS generation in six of them — PFOA, PFOS, PFHxS, PFNA, PFDA, and PFUnA. Furthermore, the authors described an ROS production that was dose dependent for exposures to PFHxS and PFUnA. Results from our study did not show a dose-dependent ROS generation for either linear or branched PFHxS.

There were no statistical differences that were observed between untreated cells and cells exposed to branched PFHxS at 0.2 µM, and 2 µM. Nonetheless, statistically significant differences were measured between untreated cells and cells exposed to branched PFHxS at 5 µM ($p = 0.0002$) and 10 µM ($p = 0.0023$), though mean ROS generation was lower at 5 µM (-46.41%), and higher at 10 µM (40.71%).

4.3 Synthesis

The results from our research suggest negligible toxicological differences between linear and branched PFHxS isomers on JEG-3 cells at the concentrations investigated. The MTT assay highlighted a slight concentration-dependent increase in cellular metabolic activity in JEG-3 cells exposed to linear PFHxS. Conversely, the experiment indicated a potential decrease in cell proliferation upon exposure to branched PFHxS. These results were statistically insignificant. The LIVE/DEAD® assay, however, indicated slightly higher mean relative viability in JEG-3 cells exposed to branched PFHxS isomers, though the results were statistically insignificant. Additionally, observations of fluorescence images suggested dose-dependent morphological changes in JEG-3 cells exposed to linear PFHxS. Finally, the ROS generation assay suggested a statistically higher ROS production in JEG-3 cells exposed to branched PFHxS than cells exposed to linear PFHxS at equivalent concentrations.

The higher ROS production in cells exposed to branched PFHxS suggests greater cytotoxic and possibly genotoxic potential of this compound in comparison to its linear counterpart. A possible explanation for the lower toxicity of branched isomers observed in previous studies could be a result of more effective branched isomer clearance rates.

Earlier studies such as the one performed by Zhang et al. (2013) noticed that branched isomers of PFOS and PFOA have mostly higher renal clearance rates in humans than their linear homologues. Loveless et al. (2006) reached a similar conclusion by observing a decline in serum concentration levels of branched APFO in rats upon dosing disruptions. This study however was not in-vivo and could not control for renal clearances. Therefore, the toxicological differences between branched and linear PFAS at equal concentrations that were observed in the experiments were most likely the result of structural and steric effects. An in vitro study performed by O'Brien et al. (2011) using chicken embryonic hepatocytes revealed that PFOS mixtures containing branched isomers had an effect on genes responsible for cell growth and proliferation. The authors suggested oxidative stress response as one of the possible pathways for these effects. Results from this thesis appear to corroborate the authors' conclusions given that branched PFAS isomers induced greater ROS production compared to their linear counterparts. Nevertheless, the toxicological effects of PFAS vary among species and any correlations between the studies should be considered with caution. The results from this study and those of similar research have been summarized and tabulated in Table 2.

Table 2: Result comparison

Author	Chemical	Model	Dose Level/ Exposure time	Biomarker Tested	Effective Dose	Observed Outcome
Cantu, et al. (2017)	Linear and Branched PFHxS	In vitro: JEG-3 Choriocarcinoma	5, 10, and 50 μM 48 hours	Metabolic Activity/Cell Proliferation	None	No effect
Buhrke et al. (2013)	PFOA	In vitro: HepG2 Hepatocarcinoma	0.1 to 2000 μM 48 hours	Metabolic Activity/Cell Proliferation	47 μM	IC_{50}
Cantu et al. (2017)	Linear and Branched PFHxS	J In vitro: EG-3 Choriocarcinoma	0.2 to 20 μM 24 hours	Cell viability	None	No effect
				Morphology	2 μM	Morphological changes in cells exposed to linear PFHxS at ≥ 2 μM

Author	Chemical	Model	Dose Level/ Exposure time	Biomarker Tested	Effective Dose	Observed Outcome
Gorrochategui et al. (2014)	PFHxS	In vitro: JEG-3 Choriocarcinoma	500 µM 24 hours	Cell viability	None	No statistical difference between exposed cells and control
Cantu et al. (2017)	Linear and Branched PFHxS	In vitro: JEG-3 Choriocarcinoma	0.2 to 10 µM 24 hours	ROS generation	10 µM (branched PFHxS)	Higher ROS production in cells exposed to branched PFHxS compared to the linear form at all concentrations. Lower ROS production in cells exposed to linear PFHxS compared to untreated cells Higher ROS production in cells exposed to 10 µM of branched PFHxS compared to untreated cells
Florentin et al. (2011)	PFOA	In vitro: HepG2 Hepatocarcinoma	5 to 400 µM 1 and 48 hours	ROS generation	None	No effect
	PFOS				None	
Eriksen et al. (2010)	PFOA	In vitro: HepG2 Hepatocarcinoma	0.4 to 2000 µM 3 hours	ROS generation	None	Higher ROS generation in cells exposed to PFOA (1.52 fold) and PFOS (1.25 fold) compared to untreated cells No dose dependency was observed
	PFOS				None	
	PFBS				None	
	PFNA				None	
	PFHxA				None	
Loveless et al. (2006)	Linear/Branched APFO	In-vivo: Rats	0.3 to 30 mg/kg/day 14 days	Body weight; Liver weight; Mortality; Serum lipids	LOAEL: 0.3 to 1 mg/kg/day	Weight loss; Decrease lipid levels
					LOAEL: 0.3 mg/kg/day	Weight loss; Lower HDL and cholesterol; Increased triglycerides
O'Brien et al. (2011)	Linear/Branched PFOS	In vitro: Chicken Embryonic Hepatocytes	1 to 40 µM 24 hours	Transcriptional profiles; Gene functional analysis	LOAEL: 10 µM	Altered expression of transcripts from genes regulating lipid metabolism, cell growth and proliferation, and liver development

V. Conclusions and Recommendations

5.1 Chapter Overview

Chapter five presents the conclusion of the thesis and discusses the significance of the research, the limitations of the study, and the potential areas for future research related to the discussion topic.

5.2 Review of Findings

Both MTT and LIVE/DEAD® assays did not show statistical differences in cytotoxicity between linear and branched PFHxS; however, branched PFHxS isomers induced statistically greater intracellular ROS.

The MTT assay indicated that a slight cellular proliferation was induced by the linear form of PFHxS with respect to untreated cells at concentrations up to 50 μM; however these results were not statistically significant. Similar results have been discerned by other cell-line studies using hepatocytes. Buhrke et al. (2013) suggested that linear forms of PFOA and PFOS can stimulate cell proliferation at concentrations below 50 μM prior to inducing cytotoxicity. This study did not find any statistically significant cell proliferation induced by branched forms of PFHxS.

Likewise, the LIVE/DEAD® fluorescence assay did not record any significant statistical differences in viability between JEG-3 cells exposed to linear and those exposed to branched PFHxS isomers at equivalent concentrations. That said, mean cellular viability was greater in cells exposed to branched forms of PFHxS than in the cells exposed to their linear homologues. Parametric analyses of the data under the

assumption of normality did indicate a statistically significant difference between the two isomers at 2 μM and 10 μM, but not at any of the other concentrations. Fluorescence image observations suggest morphological changes in cells exposed to linear PFHxS at concentrations equal or greater than 2 μM. Branched PFHxS isomers did not appear to alter the morphology of JEG-3 cells. Although the cellular viability between cells exposed to linear and those exposed to branched PFHxS was statistically indistinguishable, morphological observations suggest a difference in effects between the two compounds.

ROS production in JEG-3 cells was statistically greater for branched PFHxS isomers relative to their linear counterparts at equal concentrations. Variations in mean fluorescence intensity were 31.7% greater for branched PFHxS at 0.2 μM ($p = 0.0009$), 43.4% greater at 2 μM ($p = 0.0011$), 7.3% greater at 5 μM ($p = 0.6949$), and 50.5% greater at 10 μM ($p = < 0.001$). Nevertheless, no dose-dependent effects were observed in cells exposed to either linear or branched PFHxS. To the best of our knowledge this is the first time that the ROS generation from exposures to branched PFAS isomers has been examined.

5.3 Limitations

The cell proliferation and viability assays were only repeated three times (n=3) due to the limited amount of PFHxS available and, therefore, do not guarantee the accuracy and reproducibility of the results. Additional experiments may need to be conducted to reveal statistical differences between isomers. Also, JEG-3 cells did not respond to EGF even though they possess corresponding receptors. The lack of an

effective positive control for the MTT assay may have prevented an adequate assessment of cellular proliferation. Fluorescence measurements were performed by hand using Fiji and may be prone to inconsistencies due to human error. Evaluating cellular viability and ROS generation using flow cytometry or automated software may have improved the accuracy and precision of the results. Lastly, the solvent control that was used for this experiment (0.1% DMSO) had observable effects on JEG-3 cells in both the LIVE/DEAD® and ROS generation assays that may have skewed the experimental results.

5.4 Significance of Findings

This study contributes to the overall understanding of PFAS toxicity and presents evidence that linear and branched PFAS isomers have different toxicological effects on human cell-lines. Furthermore, the outcomes discussed in this thesis reveal that branched PFAS isomers may also have a negative effect on JEG-3 human placental cell-lines and may have the potential to induce developmental toxicity.

5.5 Future Research

The continued use of AFFFs in the USAF and the potential for female personnel to be exposed to PFAS highlights the need to perform further toxicological studies on branched PFHxS isomers. Future research should undertake additional cellular proliferation, cellular viability, and ROS generation assays at broader concentration ranges in order to determine the half maximal effective and half maximal inhibitory concentrations of branched PFHxS isomers on the JEG-3 cell-line. Moreover, research

will have to investigate the different modes of action in which branched PFAS induce cell toxicity such as PPAR activation and DNA damage.

Appendix

Table A1: MTT assay absorbance measurements (540 nm)

	L-PFHxS Concentration (µM)			Br-PFHxS Concentration (µM)						
	5	10	50	5	10	50	Blank with Cells	DMSO Negative Control	EGF Positive Control	Blank without Cells
Sample 1	0.28	0.359	0.408	0.398	0.344	0.354	0.34	0.181	0.332	0.086
Sample 2	0.348	0.353	0.284	0.196	0.203	0.239	0.232	0.119	0.186	0.059
Sample 3	0.353	0.32	0.37	0.312	0.252	0.254	0.255	0.171	0.23	0.106
Average	0.327	0.344	0.354	0.302	0.266	0.282	0.275	0.157	0.249	0.0836
Standard Deviation	0.03329	0.0171	0.051	0.0827	0.0584	0.0510	0.0464	0.0271	0.0611	0.0192

Table A2: MTT Kruskal-Wallis test with Dunn's post hoc test analysis

Table Analyzed	Metabolic Activity		
Kruskal-Wallis test			
P value	0.0235		
Exact or approximate P value?	Gaussian Approximation		
P value summary	*		
Do the medians vary signif. (P < 0.05)	Yes		
Number of groups	9		
Kruskal-Wallis statistic	17.71		
Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
neg. control vs pos. control	-4.667	No	ns
neg. control vs L-PFHxS - 5µM	-16	No	ns
neg. control vs Br-PFHxS - 5µM	-13.67	No	ns
neg. control vs L-PFHxS - 10µM	-20.33	No	ns
neg. control vs Br-PFHxS - 10µM	-9	No	ns
neg. control vs L-PFHxS - 50µM	-20.67	No	ns
neg. control vs Br-PFHxS - 50µM	-12.67	No	ns
neg. control vs control	-11	No	ns

pos. control vs L-PFHxS - 5µM	-11.33	No	ns
pos. control vs Br-PFHxS - 5µM	-9	No	ns
pos. control vs L-PFHxS - 10µM	-15.67	No	ns
pos. control vs Br-PFHxS - 10µM	-4.333	No	ns
pos. control vs L-PFHxS - 50µM	-16	No	ns
pos. control vs Br-PFHxS - 50µM	-8	No	ns
pos. control vs control	-6.333	No	ns
L-PFHxS - 5µM vs Br-PFHxS - 5µM	2.333	No	ns
L-PFHxS - 5µM vs L-PFHxS - 10µM	-4.333	No	ns
L-PFHxS - 5µM vs Br-PFHxS - 10µM	7	No	ns
L-PFHxS - 5µM vs L-PFHxS - 50µM	-4.667	No	ns
L-PFHxS - 5µM vs Br-PFHxS - 50µM	3.333	No	ns
L-PFHxS - 5µM vs control	5	No	ns
Br-PFHxS - 5µM vs L-PFHxS - 10µM	-6.667	No	ns
Br-PFHxS - 5µM vs Br-PFHxS - 10µM	4.667	No	ns
Br-PFHxS - 5µM vs L-PFHxS - 50µM	-7	No	ns
Br-PFHxS - 5µM vs Br-PFHxS - 50µM	1	No	ns
Br-PFHxS - 5µM vs control	2.667	No	ns
L-PFHxS - 10µM vs Br-PFHxS - 10µM	11.33	No	ns
L-PFHxS - 10µM vs L-PFHxS - 50µM	-0.3333	No	ns
L-PFHxS - 10µM vs Br-PFHxS - 50µM	7.667	No	ns
L-PFHxS - 10µM vs control	9.333	No	ns
Br-PFHxS - 10µM vs L-PFHxS - 50µM	-11.67	No	ns
Br-PFHxS - 10µM vs Br-PFHxS - 50µM	-3.667	No	ns
Br-PFHxS - 10µM vs control	-2	No	ns
L-PFHxS - 50µM vs Br-PFHxS - 50µM	8	No	ns
L-PFHxS - 50µM vs control	9.667	No	ns
Br-PFHxS - 50µM vs control	1.667	No	ns

Table A3: MTT Mann-Whitney U tests

Table Analyzed	Metabolic Activity	
Column C	L-PFHxS - 5μM	
vs	vs	
Column D	Br-PFHxS - 5μM	
Mann Whitney test		
P value	0.7	
Exact or approximate P value?	Exact	
P value summary	ns	
Are medians signif. different? (P < 0.05)	No	
One- or two-tailed P value?	Two-tailed	
Sum of ranks in column C,D	12 , 9	
Mann-Whitney U	3	

Table Analyzed	Metabolic Activity	
Column E	L-PFHxS - 10μM	
vs	vs	
Column F	Br-PFHxS - 10μM	
Mann Whitney test		
P value	0.1	
Exact or approximate P value?	Exact	
P value summary	ns	
Are medians signif. different? (P < 0.05)	No	
One- or two-tailed P value?	Two-tailed	
Sum of ranks in column E,F	15 , 6	
Mann-Whitney U	0	

Table Analyzed	Metabolic Activity	
Column G	L-PFHxS - 50μM	
vs	vs	
Column H	Br-PFHxS - 50μM	
Mann Whitney test		
P value	0.1	
Exact or approximate P value?	Exact	
P value summary	ns	
Are medians signif. different? (P < 0.05)	No	
One- or two-tailed P value?	Two-tailed	
Sum of ranks in column G,H	15 , 6	
Mann-Whitney U	0	

Table A4: LIVE/DEAD® assay - total percent viability

	0 uM	Solvent	Positive	L0.2 uM	B0.2 uM	L2 uM	B2 uM	L5 uM	B5 uM	L10 uM	B10 uM	L20 uM	B20 uM
Experiment 1	99.159	96.999	0.057	93.322	98.276	94.150	96.589	95.480	95.715	94.127	97.747	93.573	95.277
Experiment 2	98.738	96.771	0.058	97.147	96.507	94.763	98.373	95.196	98.575	93.108	96.232	93.722	92.406
Experiment 3	98.324	94.389	0.045	92.139	98.381	93.812	98.478	95.270	97.741	93.366	96.203	92.104	94.750

Table A5: LIVE/DEAD® assay – relative percent viability to untreated cells

Solvent	Positive	L0.2 uM	B0.2 uM	L2 uM	B2 uM	L5 uM	B5 uM	L10 uM	B10 uM	L20 uM	B20 uM
97.822	0.058	94.113	99.110	94.948	97.408	96.290	96.527	94.925	98.575	94.366	96.085
98.007	0.059	98.389	97.740	95.974	99.630	96.413	99.835	94.298	97.462	94.920	93.586
95.999	0.046	93.710	100.059	95.411	100.157	96.894	99.407	94.958	97.843	93.674	96.366

Table A6: LIVE/DEAD® Kruskal-Wallis with Dunn's post hoc test analysis

Kruskal-Wallis test			
P value	0.0047		
Exact or approximate P value?	Gaussian Approximation		
P value summary	**		
Do the medians vary signif. (P < 0.05)	Yes		
Number of groups	13		
Kruskal-Wallis statistic	26.93		
Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
Untreated Cells vs Solvent Control	13.33	No	ns
Untreated Cells vs Pos. Control	33.67	No	ns
Untreated Cells vs L-PFHxS at 0.2μM	22.67	No	ns
Untreated Cells vs Br-PFHxS at 0.2μM	6	No	ns
Untreated Cells vs L-PFHxS at 2μM	22.33	No	ns
Untreated Cells vs Br-PFHxS at 2μM	4.667	No	ns
Untreated Cells vs L-PFHxS at 5μM	17.33	No	ns
Untreated Cells vs Br-PFHxS at 5μM	7	No	ns
Untreated Cells vs L-PFHxS at 10μM	27	No	ns
Untreated Cells vs Br-PFHxS at 10μM	10.67	No	ns
Untreated Cells vs L-PFHxS at 20μM	28.33	No	ns
Untreated Cells vs Br-PFHxS at 20μM	22.67	No	ns
Solvent Control vs Pos. Control	20.33	No	ns
Solvent Control vs L-PFHxS at 0.2μM	9.333	No	ns
Solvent Control vs Br-PFHxS at 0.2μM	-7.333	No	ns
Solvent Control vs L-PFHxS at 2μM	9	No	ns
Solvent Control vs Br-PFHxS at 2μM	-8.667	No	ns
Solvent Control vs L-PFHxS at 5μM	4	No	ns
Solvent Control vs Br-PFHxS at 5μM	-6.333	No	ns
Solvent Control vs L-PFHxS at 10μM	13.67	No	ns
Solvent Control vs Br-PFHxS at 10μM	-2.667	No	ns
Solvent Control vs L-PFHxS at 20μM	15	No	ns
Solvent Control vs Br-PFHxS at 20μM	9.333	No	ns
Pos. Control vs L-PFHxS at 0.2μM	-11	No	ns
Pos. Control vs Br-PFHxS at 0.2μM	-27.67	No	ns
Pos. Control vs L-PFHxS at 2μM	-11.33	No	ns

Pos. Control vs Br-PFHxS at 2μM	-29	No	ns
Pos. Control vs L-PFHxS at 5μM	-16.33	No	ns
Pos. Control vs Br-PFHxS at 5μM	-26.67	No	ns
Pos. Control vs L-PFHxS at 10μM	-6.667	No	ns
Pos. Control vs Br-PFHxS at 10μM	-23	No	ns
Pos. Control vs L-PFHxS at 20μM	-5.333	No	ns
Pos. Control vs Br-PFHxS at 20μM	-11	No	ns
L-PFHxS at 0.2μM vs Br-PFHxS at 0.2μM	-16.67	No	ns
L-PFHxS at 0.2μM vs L-PFHxS at 2μM	-0.3333	No	ns
L-PFHxS at 0.2μM vs Br-PFHxS at 2μM	-18	No	ns
L-PFHxS at 0.2μM vs L-PFHxS at 5μM	-5.333	No	ns
L-PFHxS at 0.2μM vs Br-PFHxS at 5μM	-15.67	No	ns
L-PFHxS at 0.2μM vs L-PFHxS at 10μM	4.333	No	ns
L-PFHxS at 0.2μM vs Br-PFHxS at 10μM	-12	No	ns
L-PFHxS at 0.2μM vs L-PFHxS at 20μM	5.667	No	ns
L-PFHxS at 0.2μM vs Br-PFHxS at 20μM	0	No	ns
Br-PFHxS at 0.2μM vs L-PFHxS at 2μM	16.33	No	ns
Br-PFHxS at 0.2μM vs Br-PFHxS at 2μM	-1.333	No	ns
Br-PFHxS at 0.2μM vs L-PFHxS at 5μM	11.33	No	ns
Br-PFHxS at 0.2μM vs Br-PFHxS at 5μM	1	No	ns
Br-PFHxS at 0.2μM vs L-PFHxS at 10μM	21	No	ns
Br-PFHxS at 0.2μM vs Br-PFHxS at 10μM	4.667	No	ns
Br-PFHxS at 0.2μM vs L-PFHxS at 20μM	22.33	No	ns
Br-PFHxS at 0.2μM vs Br-PFHxS at 20μM	16.67	No	ns
L-PFHxS at 2μM vs Br-PFHxS at 2μM	-17.67	No	ns
L-PFHxS at 2μM vs L-PFHxS at 5μM	-5	No	ns
L-PFHxS at 2μM vs Br-PFHxS at 5μM	-15.33	No	ns
L-PFHxS at 2μM vs L-PFHxS at 10μM	4.667	No	ns
L-PFHxS at 2μM vs Br-PFHxS at 10μM	-11.67	No	ns
L-PFHxS at 2μM vs L-PFHxS at 20μM	6	No	ns
L-PFHxS at 2μM vs Br-PFHxS at 20μM	0.3333	No	ns
Br-PFHxS at 2μM vs L-PFHxS at 5μM	12.67	No	ns
Br-PFHxS at 2μM vs Br-PFHxS at 5μM	2.333	No	ns
Br-PFHxS at 2μM vs L-PFHxS at 10μM	22.33	No	ns
Br-PFHxS at 2μM vs Br-PFHxS at 10μM	6	No	ns

Br-PFHxS at 2μM vs L-PFHxS at 20μM	23.67	No	ns
Br-PFHxS at 2μM vs Br-PFHxS at 20μM	18	No	ns
L-PFHxS at 5μM vs Br-PFHxS at 5μM	-10.33	No	ns
L-PFHxS at 5μM vs L-PFHxS at 10μM	9.667	No	ns
L-PFHxS at 5μM vs Br-PFHxS at 10μM	-6.667	No	ns
L-PFHxS at 5μM vs L-PFHxS at 20μM	11	No	ns
L-PFHxS at 5μM vs Br-PFHxS at 20μM	5.333	No	ns
Br-PFHxS at 5μM vs L-PFHxS at 10μM	20	No	ns
Br-PFHxS at 5μM vs Br-PFHxS at 10μM	3.667	No	ns
Br-PFHxS at 5μM vs L-PFHxS at 20μM	21.33	No	ns
Br-PFHxS at 5μM vs Br-PFHxS at 20μM	15.67	No	ns
L-PFHxS at 10μM vs Br-PFHxS at 10μM	-16.33	No	ns
L-PFHxS at 10μM vs L-PFHxS at 20μM	1.333	No	ns
L-PFHxS at 10μM vs Br-PFHxS at 20μM	-4.333	No	ns
Br-PFHxS at 10μM vs L-PFHxS at 20μM	17.67	No	ns
Br-PFHxS at 10μM vs Br-PFHxS at 20μM	12	No	ns
L-PFHxS at 20μM vs Br-PFHxS at 20μM	-5.667	No	ns

Table A7: LIVE/DEAD® Mann-Whitney U tests

Table Analyzed	**Live Dead Assay - Cell Viability (%)**	**Table Analyzed**	**Live Dead Assay - Cell Viability (%)**
Column D	**L-PFHxS at 0.2μM**	**Column F**	**L-PFHxS at 2μM**
vs	**vs**	**vs**	**vs**
Column E	**Br-PFHxS at 0.2μM**	**Column G**	**Br-PFHxS at 2μM**
Mann Whitney test		Mann Whitney test	
P value	0.2	P value	0.1
Exact or approximate P value?	Exact	Exact or approximate P value?	Exact
P value summary	ns	P value summary	ns
Are medians signif. different? (P < 0.05)	No	Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed	One- or two-tailed P value?	Two-tailed
Sum of ranks in column D,E	7 , 14	Sum of ranks in column F,G	6 , 15
Mann-Whitney U	1	Mann-Whitney U	0
Table Analyzed	**Live Dead Assay - Cell Viability (%)**	**Table Analyzed**	**Live Dead Assay - Cell Viability (%)**
Column H	**L-PFHxS at 5μM**	**Column J**	**L-PFHxS at 10μM**
vs	**vs**	**vs**	**vs**
Column I	**Br-PFHxS at 5μM**	**Column K**	**Br-PFHxS at 10μM**
Mann Whitney test		Mann Whitney test	
P value	0.1	P value	0.1
Exact or approximate P value?	Exact	Exact or approximate P value?	Exact
P value summary	ns	P value summary	ns
Are medians signif. different? (P < 0.05)	No	Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed	One- or two-tailed P value?	Two-tailed
Sum of ranks in column H,I	6 , 15	Sum of ranks in column J,K	6 , 15
Mann-Whitney U	0	Mann-Whitney U	0

Table Analyzed	Live Dead Assay - Cell Viability (%)
Column L	**L-PFHxS at 20μM**
vs	**vs**
Column M	**Br-PFHxS at 20μM**
Mann Whitney test	
P value	0.4
Exact or approximate P value?	Exact
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
Sum of ranks in column L,M	8 , 13
Mann-Whitney U	2

Table A8: LIVE/DEAD® one-way ANOVA with Bonferroni's comparisons test

Table Analyzed	Live Dead Assay - Cell Viability (%)				
One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	12				
F	7.263				
R squared	0.769				
ANOVA Table	SS	df	MS		
Treatment (between columns)	119.6	11	10.87		

Residual (within columns)	35.93	24	1.497		
Total	155.5	35			
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary	95% CI of diff
Untreated Cells vs Solvent Control	2.687	2.69	No	ns	-1.231 to 6.606
Untreated Cells vs Pos. Control	98.74	0	No	ns	0.0000 to 0.0000
Untreated Cells vs L-PFHxS at 0.2μM	4.538	4.542	Yes	*	0.6192 to 8.456
Untreated Cells vs Br-PFHxS at 0.2μM	1.019	1.02	No	ns	-2.900 to 4.938
Untreated Cells vs L-PFHxS at 2μM	4.499	4.503	Yes	*	0.5802 to 8.417
Untreated Cells vs Br-PFHxS at 2μM	0.927	0.9279	No	ns	-2.992 to 4.846
Untreated Cells vs L-PFHxS at 5μM	3.425	3.428	No	ns	-0.4935 to 7.344
Untreated Cells vs Br-PFHxS at 5μM	1.397	1.398	No	ns	-2.522 to 5.315
Untreated Cells vs L-PFHxS at 10μM	5.207	5.212	Yes	**	1.288 to 9.125
Untreated Cells vs Br-PFHxS at 10μM	2.013	2.015	No	ns	-1.906 to 5.932
Untreated Cells vs L-PFHxS at 20μM	5.607	5.613	Yes	***	1.689 to 9.526
Untreated Cells vs Br-PFHxS at 20μM	4.596	4.6	Yes	**	0.6775 to 8.515
Solvent Control vs Pos. Control	96.05	0	No	ns	0.0000 to 0.0000
Solvent Control vs L-PFHxS at 0.2μM	1.85	1.852	No	ns	-2.068 to 5.769
Solvent Control vs Br-PFHxS at 0.2μM	-1.668	1.67	No	ns	-5.587 to 2.250
Solvent Control vs L-PFHxS at 2μM	1.811	1.813	No	ns	-2.107 to 5.730
Solvent Control vs Br-PFHxS at 2μM	-1.76	1.762	No	ns	-5.679 to 2.158
Solvent Control vs L-PFHxS at 5μM	0.7377	0.7384	No	ns	-3.181 to 4.656
Solvent Control vs Br-PFHxS at 5μM	-1.291	1.292	No	ns	-5.209 to 2.628
Solvent Control vs L-PFHxS at 10μM	2.519	2.522	No	ns	-1.399 to 6.438
Solvent Control vs Br-PFHxS at 10μM	-0.6743	0.675	No	ns	-4.593 to 3.244
Solvent Control vs L-PFHxS at 20μM	2.92	2.923	No	ns	-0.9985 to 6.839
Solvent Control vs Br-PFHxS at 20μM	1.909	1.911	No	ns	-2.010 to 5.827

Pos. Control vs L-PFHxS at 0.2μM	-94.2	0	No	ns	0.0000 to 0.0000
Pos. Control vs Br-PFHxS at 0.2μM	-97.72	0	No	ns	0.0000 to 0.0000
Pos. Control vs L-PFHxS at 2μM	-94.24	0	No	ns	0.0000 to 0.0000
Pos. Control vs Br-PFHxS at 2μM	-97.81	0	No	ns	0.0000 to 0.0000
Pos. Control vs L-PFHxS at 5μM	-95.32	0	No	ns	0.0000 to 0.0000
Pos. Control vs Br-PFHxS at 5μM	-97.34	0	No	ns	0.0000 to 0.0000
Pos. Control vs L-PFHxS at 10μM	-93.53	0	No	ns	0.0000 to 0.0000
Pos. Control vs Br-PFHxS at 10μM	-96.73	0	No	ns	0.0000 to 0.0000
Pos. Control vs L-PFHxS at 20μM	-93.13	0	No	ns	0.0000 to 0.0000
Pos. Control vs Br-PFHxS at 20μM	-94.14	0	No	ns	0.0000 to 0.0000
L-PFHxS at 0.2μM vs Br-PFHxS at 0.2μM	-3.519	3.522	No	ns	-7.437 to 0.3998
L-PFHxS at 0.2μM vs L-PFHxS at 2μM	-0.039	0.03904	No	ns	-3.958 to 3.880
L-PFHxS at 0.2μM vs Br-PFHxS at 2μM	-3.611	3.614	No	ns	-7.529 to 0.3078
L-PFHxS at 0.2μM vs L-PFHxS at 5μM	-1.113	1.114	No	ns	-5.031 to 2.806
L-PFHxS at 0.2μM vs Br-PFHxS at 5μM	-3.141	3.144	No	ns	-7.060 to 0.7775
L-PFHxS at 0.2μM vs L-PFHxS at 10μM	0.669	0.6697	No	ns	-3.250 to 4.588
L-PFHxS at 0.2μM vs Br-PFHxS at 10μM	-2.525	2.527	No	ns	-6.443 to 1.394
L-PFHxS at 0.2μM vs L-PFHxS at 20μM	1.07	1.071	No	ns	-2.849 to 4.988
L-PFHxS at 0.2μM vs Br-PFHxS at 20μM	0.05833	0.05839	No	ns	-3.860 to 3.977
Br-PFHxS at 0.2μM vs L-PFHxS at 2μM	3.48	3.483	No	ns	-0.4388 to 7.398
Br-PFHxS at 0.2μM vs Br-PFHxS at 2μM	-0.092	0.09208	No	ns	-4.011 to 3.827
Br-PFHxS at 0.2μM vs L-PFHxS at 5μM	2.406	2.408	No	ns	-1.513 to 6.325
Br-PFHxS at 0.2μM vs Br-PFHxS at 5μM	0.3777	0.378	No	ns	-3.541 to 4.296
Br-PFHxS at 0.2μM vs L-PFHxS at 10μM	4.188	4.192	Yes	*	0.2692 to 8.106
Br-PFHxS at 0.2μM vs Br-PFHxS at 10μM	0.994	0.995	No	ns	-2.925 to 4.913

Br-PFHxS at 0.2μM vs L-PFHxS at 20μM	4.588	4.593	Yes	**	0.6698 to 8.507
Br-PFHxS at 0.2μM vs Br-PFHxS at 20μM	3.577	3.58	No	ns	-0.3415 to 7.496
L-PFHxS at 2μM vs Br-PFHxS at 2μM	-3.572	3.575	No	ns	-7.490 to 0.3468
L-PFHxS at 2μM vs L-PFHxS at 5μM	-1.074	1.075	No	ns	-4.992 to 2.845
L-PFHxS at 2μM vs Br-PFHxS at 5μM	-3.102	3.105	No	ns	-7.021 to 0.8165
L-PFHxS at 2μM vs L-PFHxS at 10μM	0.708	0.7087	No	ns	-3.211 to 4.627
L-PFHxS at 2μM vs Br-PFHxS at 10μM	-2.486	2.488	No	ns	-6.404 to 1.433
L-PFHxS at 2μM vs L-PFHxS at 20μM	1.109	1.11	No	ns	-2.810 to 5.027
L-PFHxS at 2μM vs Br-PFHxS at 20μM	0.09734	0.09743	No	ns	-3.821 to 4.016
Br-PFHxS at 2μM vs L-PFHxS at 5μM	2.498	2.5	No	ns	-1.421 to 6.417
Br-PFHxS at 2μM vs Br-PFHxS at 5μM	0.4697	0.4701	No	ns	-3.449 to 4.388
Br-PFHxS at 2μM vs L-PFHxS at 10μM	4.28	4.284	Yes	*	0.3612 to 8.198
Br-PFHxS at 2μM vs Br-PFHxS at 10μM	1.086	1.087	No	ns	-2.833 to 5.005
Br-PFHxS at 2μM vs L-PFHxS at 20μM	4.68	4.685	Yes	**	0.7618 to 8.599
Br-PFHxS at 2μM vs Br-PFHxS at 20μM	3.669	3.673	No	ns	-0.2495 to 7.588
L-PFHxS at 5μM vs Br-PFHxS at 5μM	-2.028	2.03	No	ns	-5.947 to 1.890
L-PFHxS at 5μM vs L-PFHxS at 10μM	1.782	1.783	No	ns	-2.137 to 5.700
L-PFHxS at 5μM vs Br-PFHxS at 10μM	-1.412	1.413	No	ns	-5.331 to 2.507
L-PFHxS at 5μM vs L-PFHxS at 20μM	2.182	2.184	No	ns	-1.736 to 6.101
L-PFHxS at 5μM vs Br-PFHxS at 20μM	1.171	1.172	No	ns	-2.748 to 5.090
Br-PFHxS at 5μM vs L-PFHxS at 10μM	3.81	3.814	No	ns	-0.1085 to 7.729
Br-PFHxS at 5μM vs Br-PFHxS at 10μM	0.6163	0.6169	No	ns	-3.302 to 4.535
Br-PFHxS at 5μM vs L-PFHxS at 20μM	4.211	4.215	Yes	*	0.2922 to 8.129
Br-PFHxS at 5μM vs Br-PFHxS at 20μM	3.199	3.202	No	ns	-0.7192 to 7.118
L-PFHxS at 10μM vs Br-PFHxS at 10μM	-3.194	3.197	No	ns	-7.112 to 0.7248

L-PFHxS at 10μM vs L-PFHxS at 20μM	0.4007	0.4011	No	ns	-3.518 to 4.319
L-PFHxS at 10μM vs Br-PFHxS at 20μM	-0.6107	0.6113	No	ns	-4.529 to 3.308
Br-PFHxS at 10μM vs L-PFHxS at 20μM	3.594	3.598	No	ns	-0.3242 to 7.513
Br-PFHxS at 10μM vs Br-PFHxS at 20μM	2.583	2.586	No	ns	-1.336 to 6.502
L-PFHxS at 20μM vs Br-PFHxS at 20μM	-1.011	1.012	No	ns	-4.930 to 2.907

Table A9: LIVE/DEAD® unpaired t-tests

Table Analyzed	**Live Dead Assay - Cell Viability (%)**	**Table Analyzed**	**Live Dead Assay - Cell Viability (%)**
Column D	**L-PFHxS at 0.2μM**	**Column F**	**L-PFHxS at 2μM**
vs	**vs**	**vs**	**vs**
Column E	**Br-PFHxS at 0.2μM**	**Column G**	**Br-PFHxS at 2μM**
Unpaired t test		Unpaired t test	
P value	0.0969	P value	0.0061
P value summary	ns	P value summary	**
Are means signif. different? (P < 0.05)	No	Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed	One- or two-tailed P value?	Two-tailed
t, df	t=2.160 df=4	t, df	t=5.306 df=4
How big is the difference?		How big is the difference?	
Mean ± SEM of column D	94.20 ± 1.511 N=3	Mean ± SEM of column F	94.24 ± 0.2783 N=3
Mean ± SEM of column E	97.72 ± 0.6079 N=3	Mean ± SEM of column G	97.81 ± 0.6129 N=3
Difference between means	-3.519 ± 1.629	Difference between means	-3.572 ± 0.6732
95% confidence interval	-8.041 to 1.003	95% confidence interval	-5.440 to -1.703
R squared	0.5384	R squared	0.8756
F test to compare variances		F test to compare variances	
F,DFn, Dfd	6.180, 2, 2	F,DFn, Dfd	4.849, 2, 2

P value	0.2786	P value	0.3419
P value summary	ns	P value summary	ns
Are variances significantly different?	No	Are variances significantly different?	No

Table Analyzed	**Live Dead Assay - Cell Viability (%)**	**Table Analyzed**	**Live Dead Assay - Cell Viability (%)**
Column H	**L-PFHxS at 5μM**	**Column J**	**L-PFHxS at 10μM**
vs	**vs**	**vs**	**vs**
Column I	**Br-PFHxS at 5μM**	**Column K**	**Br-PFHxS at 10μM**
Unpaired t test with Welch's correction		Unpaired t test	
P value	0.1406	P value	0.0058
P value summary	ns	P value summary	**
Are means signif. different? (P < 0.05)	No	Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed	One- or two-tailed P value?	Two-tailed
Welch-corrected t, df	t=2.377 df=2	t, df	t=5.371 df=4
How big is the difference?		How big is the difference?	
Mean ± SEM of column H	95.32 ± 0.08506 N=3	Mean ± SEM of column J	93.53 ± 0.3059 N=3
Mean ± SEM of column I	97.34 ± 0.8492 N=3	Mean ± SEM of column K	96.73 ± 0.5099 N=3
Difference between means	-2.028 ± 0.8534	Difference between means	-3.194 ± 0.5946
95% confidence interval	-5.701 to 1.644	95% confidence interval	-4.844 to -1.543
R squared	0.7385	R squared	0.8782
F test to compare variances		F test to compare variances	
F,DFn, Dfd	99.66, 2, 2	F,DFn, Dfd	2.779, 2, 2
P value	0.0199	P value	0.5292
P value summary	*	P value summary	ns
Are variances significantly different?	Yes	Are variances significantly different?	No

Table Analyzed	**Live Dead Assay - Cell Viability (%)**
Column L	**L-PFHxS at 20μM**
vs	**vs**
Column M	**Br-PFHxS at 20μM**
Unpaired t test	
P value	0.3785
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.9892 df=4
How big is the difference?	
Mean ± SEM of column L	93.13 ± 0.5163 N=3
Mean ± SEM of column M	94.14 ± 0.8824 N=3
Difference between means	-1.011 ± 1.022
95% confidence interval	-3.849 to 1.827
R squared	0.1966
F test to compare variances	
F,DFn, Dfd	2.921, 2, 2
P value	0.5101
P value summary	ns
Are variances significantly different?	No

Table A10: ROS generation fluorescence measurements (normalized to untreated cells)

L 0.2 uM	B0.2 uM	L2 uM	B2 uM	L5 uM	B5 uM	L10 uM	B10 uM	Positive	Solvent
1.106079	0.878175	0.265598	0.708099	0.45615	0.412995	0.508274	0.845686	4.295303	0.974644
0.564781	1.081097	0.312238	0.37637	0.30138	0.70293	0.750188	1.388254	7.657951	0.388654
0.318836	0.693065	0.213478	0.742176	0.609241	0.808794	0.750188	1.416256	3.418062	0.760892
0.53828	0.855882	0.519651	0.51065	0.786741	0.447518	0.516907	1.811651	8.701681	0.511671
0.623363	0.991587	0.677219	0.302314	0.319608	0.187145	0.435327	0.747003	11.25131	0.2959
0.818691	0.73233	0.689641	0.765183	0.458495	0.643191	0.579223	1.049879	11.50349	0.534379
0.711556	1.423214	0.268917	1.359548	0.436687	0.750996	0.821365	1.597424	10.3012	0.477338
0.618925	1.430503	0.223497	1.224296	0.544703	1.020619	0.828789	1.665387	11.87792	0.420869
1.541623	0.970441	0.297516	0.985783	0.340491	0.57684	0.664902	1.898219	6.600985	0.57894
0.628616	0.732919	0.20569	0.667004	0.380192	0.208903	0.972844	0.615296	13.41031	0.826741
0.462258	1.087644	0.523197	0.813994	0.55065	0.580344	0.691711	1.451101	13.42293	0.919793
0.853875	1.377816	0.457399	1.145632	0.7624	0.540177	0.721714	1.379209	3.838649	1.014013
0.945566	0.884229	0.726881	1.149244	0.456415	0.560756	0.699488	1.822648	6.5512	0.838456
0.80954	1.500391	0.429764	0.827154	0.620152	0.275681	0.810955	1.505206	15.7898	0.904788
0.634761		0.722694		0.239286	0.138745		2.154487	17.71175	0.737371
0.562997		0.811722		0.584305	0.16196		1.464479	3.308274	0.542314
		0.345525		0.770995	0.570165		1.428249	13.58754	0.366412
		0.579646		0.425366	0.715162		1.10581	6.312169	0.744378
		0.624776		0.407853	0.378307		1.478368	10.43895	
				0.486123	1.036778		1.317866	14.65764	
					0.909378			10.58312	
					0.589309			4.828789	
								14.29817	
								6.50153	
								25.61981	
								11.57253	

Table A11: ROS Kruskal-Wallis with Dunn's post hoc test analysis

Table Analyzed	ROS Generation		
Kruskal-Wallis test			
P value	< 0.0001		
Exact or approximate P value?	Gaussian Approximation		
P value summary	***		
Do the medians vary signif. (P < 0.05)	Yes		
Number of groups	10		
Kruskal-Wallis statistic	132.1		
Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
Untreated vs L-PFHxS - 0.2μM	27.82	No	ns
Untreated vs Br-PFHxS - 0.2μM	-17.52	No	ns
Untreated vs L-PFHxS - 2μM	67.9	Yes	**
Untreated vs Br-PFHxS - 2μM	14.38	No	ns
Untreated vs L-PFHxS - 5μM	64.78	Yes	**
Untreated vs Br-PFHxS - 5μM	56.78	Yes	*
Untreated vs L-PFHxS - 10μM	29.16	No	ns
Untreated vs Br-PFHxS - 10μM	-39.77	No	ns
Untreated vs Positive	-82.12	Yes	***
L-PFHxS - 0.2μM vs Br-PFHxS - 0.2μM	-45.33	No	ns
L-PFHxS - 0.2μM vs L-PFHxS - 2μM	40.09	No	ns
L-PFHxS - 0.2μM vs Br-PFHxS - 2μM	-13.44	No	ns
L-PFHxS - 0.2μM vs L-PFHxS - 5μM	36.96	No	ns
L-PFHxS - 0.2μM vs Br-PFHxS - 5μM	28.96	No	ns
L-PFHxS - 0.2μM vs L-PFHxS - 10μM	1.348	No	ns
L-PFHxS - 0.2μM vs Br-PFHxS - 10μM	-67.59	Yes	*

L-PFHxS - 0.2μM vs Positive	-109.9	Yes	***
Br-PFHxS - 0.2μM vs L-PFHxS - 2μM	85.42	Yes	***
Br-PFHxS - 0.2μM vs Br-PFHxS - 2μM	31.89	No	ns
Br-PFHxS - 0.2μM vs L-PFHxS - 5μM	82.29	Yes	***
Br-PFHxS - 0.2μM vs Br-PFHxS - 5μM	74.29	Yes	**
Br-PFHxS - 0.2μM vs L-PFHxS - 10μM	46.68	No	ns
Br-PFHxS - 0.2μM vs Br-PFHxS - 10μM	-22.26	No	ns
Br-PFHxS - 0.2μM vs Positive	-64.61	Yes	*
L-PFHxS - 2μM vs Br-PFHxS - 2μM	-53.53	No	ns
L-PFHxS - 2μM vs L-PFHxS - 5μM	-3.126	No	ns
L-PFHxS - 2μM vs Br-PFHxS - 5μM	-11.13	No	ns
L-PFHxS - 2μM vs L-PFHxS - 10μM	-38.74	No	ns
L-PFHxS - 2μM vs Br-PFHxS - 10μM	-107.7	Yes	***
L-PFHxS - 2μM vs Positive	-150	Yes	***
Br-PFHxS - 2μM vs L-PFHxS - 5μM	50.4	No	ns
Br-PFHxS - 2μM vs Br-PFHxS - 5μM	42.4	No	ns
Br-PFHxS - 2μM vs L-PFHxS - 10μM	14.79	No	ns
Br-PFHxS - 2μM vs Br-PFHxS - 10μM	-54.15	No	ns
Br-PFHxS - 2μM vs Positive	-96.5	Yes	***
L-PFHxS - 5μM vs Br-PFHxS - 5μM	-8	No	ns
L-PFHxS - 5μM vs L-PFHxS - 10μM	-35.61	No	ns
L-PFHxS - 5μM vs Br-PFHxS - 10μM	-104.6	Yes	***
L-PFHxS - 5μM vs Positive	-146.9	Yes	***
Br-PFHxS - 5μM vs L-PFHxS - 10μM	-27.61	No	ns
Br-PFHxS - 5μM vs Br-PFHxS - 10μM	-96.55	Yes	***

Br-PFHxS - 5μM vs Positive	-138.9	Yes	***
L-PFHxS - 10μM vs Br-PFHxS - 10μM	-68.94	Yes	*
L-PFHxS - 10μM vs Positive	-111.3	Yes	***
Br-PFHxS - 10μM vs Positive	-42.35	No	ns

Table A12: ROS assay Mann-Whitney U tests

Table Analyzed	**ROS Generation**	**Table Analyzed**	**ROS Generation**
Column B	**L-PFHxS - 0.2μM**	**Column D**	**L-PFHxS - 2μM**
vs	**vs**	**vs**	**vs**
Column C	**Br-PFHxS - 0.2μM**	**Column E**	**Br-PFHxS - 2μM**
Mann Whitney test		Mann Whitney test	
P value	0.0009	P value	0.0011
Exact or approximate P value?	Gaussian Approximation	Exact or approximate P value?	Gaussian Approximation
P value summary	***	P value summary	**
Are medians signif. different? (P < 0.05)	Yes	Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed	One- or two-tailed P value?	Two-tailed
Sum of ranks in column B,C	187 , 443	Sum of ranks in column D,E	233 , 328
Mann-Whitney U	51	Mann-Whitney U	43

	ROS Generation	Table Analyzed	ROS Generation
Column F	**L-PFHxS - 5μM**	**Column H**	**L-PFHxS - 10μM**
vs	**vs**	**vs**	**vs**
Column G	**Br-PFHxS - 5μM**	**Column I**	**Br-PFHxS - 10μM**
Mann Whitney test		Mann Whitney test	
P value	0.6949	P value	< 0.0001
Exact or approximate P value?	Gaussian Approximation	Exact or approximate P value?	Gaussian Approximation
P value summary	ns	P value summary	***
Are medians signif. different? (P < 0.05)	No	Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed	One- or two-tailed P value?	Two-tailed
Sum of ranks in column F,G	395 , 425	Sum of ranks in column H,I	122 , 473
Mann-Whitney U	185	Mann-Whitney U	17

Table Analyzed	ROS Generation	Table Analyzed	ROS Generation
Column B	**L-PFHxS - 0.2μM**	**Column F**	**L-PFHxS - 5μM**
vs	**vs**	**vs**	**vs**
Column D	**L-PFHxS - 2μM**	**Column H**	**L-PFHxS - 10μM**
Mann Whitney test		Mann Whitney test	
P value	0.0057	P value	0.0025
Exact or approximate P value?	Gaussian Approximation	Exact or approximate P value?	Gaussian Approximation
P value summary	**	P value summary	**
Are medians signif. different? (P < 0.05)	Yes	Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed	One- or two-tailed P value?	Two-tailed
Sum of ranks in column B,D	372 , 258	Sum of ranks in column F,H	263 , 332
Mann-Whitney U	68	Mann-Whitney U	53

Table Analyzed	ROS Generation	Table Analyzed	ROS Generation
Column D	**L-PFHxS - 2μM**	**Column C**	**Br-PFHxS - 0.2μM**
vs	**vs**	**vs**	**vs**
Column F	**L-PFHxS - 5μM**	**Column E**	**Br-PFHxS - 2μM**
Mann Whitney test		Mann Whitney test	
P value	0.5646	P value	0.0362
Exact or approximate P value?	Gaussian Approximation	Exact or approximate P value?	Gaussian Approximation
P value summary	ns	P value summary	*
Are medians signif. different? (P < 0.05)	No	Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed	One- or two-tailed P value?	Two-tailed
Sum of ranks in column D,F	359 , 421	Sum of ranks in column C,E	381 , 180
Mann-Whitney U	169	Mann-Whitney U	75

Table Analyzed	ROS Generation	Table Analyzed	ROS Generation
Column E	**Br-PFHxS - 2μM**	**Column G**	**Br-PFHxS - 5μM**
vs	**vs**	**vs**	**vs**
Column G	**Br-PFHxS - 5μM**	**Column I**	**Br-PFHxS - 10μM**
Mann Whitney test		Mann Whitney test	
P value	0.0101	P value	< 0.0001
Exact or approximate P value?	Gaussian Approximation	Exact or approximate P value?	Gaussian Approximation
P value summary	*	P value summary	***
Are medians signif. different? (P < 0.05)	Yes	Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed	One- or two-tailed P value?	Two-tailed
Sum of ranks in column E,G	319 , 276	Sum of ranks in column G,I	223 , 597
Mann-Whitney U	66	Mann-Whitney U	13

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14. ABSTRACT Perfluoroalkyl substances (PFAS) are metabolically stable synthetic chemicals that have been manufactured for commercial and industrial purposes since the 1950's. PFAS possess surfactant properties that make them ideal to fight hydrocarbon fires and are therefore present in aqueous film forming foams (AFFF). Furthermore, AFFF may contain blends of both linear and branched PFAS isomers. Research suggests that branched PFAS isomers have greater relative placental transfer efficiencies than their linear counterpart, but few studies have evaluated their toxicity. Therefore, the sustained use of AFFF in the U.S. Air Force presents a risk of branched PFAS exposures in pregnant females. This study investigated the toxicological differences between branched and linear PFAS isomers *in vitro* using the JEG-3 human placental cell-line as a model. Cells were exposed to linear and branched perfluorohexane sulfonate (PFHXS) for 24 to 48 hrs. at concentrations ranging from 0.2 μM to 50 μM. Subsequently, changes in three specific biomarkers were examined. No significant statistical differences in cellular proliferation and cellular viability were highlighted in cells exposed to both compounds at equivalent concentrations; however, mean cell proliferation appeared greater when exposed to linear PFHxS. Reactive oxygen species (ROS) generation was statistically higher in JEG-3 cells exposed to branched PFHxS isomers at corresponding concentrations.

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