Honors Research Thesis:
Effect of PFAS on Lipid Homeostasis and Cell Viability in RTL-W1 Cells

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Abstract

Perfluoroalkyl substances are compounds commonly used in waterproofing products such as Teflon and Scotch Guard. These compounds are pervasive in the environment and persistent in organisms, leading to negative health effects over time. PFAS have been shown to act as endocrine disruptors, induce cancer, and disrupt lipid metabolism; however, their mechanism of toxicity is still poorly understood. In order to study the mechanisms of action of these chemicals, we used an in vitro model of Rainbow Trout liver (RTL-W1) and measured cytotoxicity, qPCR, and in the future lipid droplet assays. We calculated dose-response curves for PFOS and PFOA across a gradient of fetal bovine serum (FBS) concentrations. Interestingly, PFOS showed lysosomal stimulation that was not shown by PFOA. We also noted a protective effect at higher FBS levels when we compared exposure media with 1%, 5%, and 10% FBS levels. Finally, our qPCR analysis showed no change in PPARγ expression at 50 nM PFOS and PFOA, though there was an inhibitory effect at 1 μM PFOS. This study would benefit from future explorations into additional exposure concentrations, lipid-regulating genes, chemicals, and the optimization/completion of the lipid droplet assays. It did serve as a foundation to begin comparing between exposure concentration, toxicity, and gene expression changes in the RTL-W1 cell line upon exposure to PFAS.

1. Introduction

Perfluoroalkyl substances are compounds such as perfluorooctanoic acid (PFOA) and perfluoroctane sulfonic acid (PFOS). These chemicals are known for their wide use in waterproofing products such as Scotch Guard and Teflon. These chemicals are found almost everywhere in nature. They are found in waterbodies at concentrations around 50 nM and built up in European perch (*Perca fluviatilis*), European eel (*Anguilla anguilla*), and freshwater mussels (*Dreissena bugensis* and *Corbicula fluminea*) at around 1 μM (Teunen et al., 2021). They have also been linked to chronic disease states in humans such as steatosis, nonalcoholic fatty liver disease (NAFLD), and eventually hepatocellular carcinoma (HCC). These chemicals trigger these disease states over a long period of time by acting as endocrine-disrupting chemicals (Foulds et al., 2017).

Endocrine-disrupting chemicals (EDCs) are known for mimicking the structure of natural endocrine hormones (Heindel, 2019). Because the body uses hormones for signaling and regulatory mechanisms, any molecule which replicates the hormone structure, and therefore effect, can cause serious imbalances in the homeostasis of the organism. Hormones are very tightly regulated and require very low concentrations to produce an effect. The low threshold for action allows very low doses (μM or nM) of EDCs to activate or inhibit reactions in the body (Heindel, 2019).

A link between EDCs and obesity was not investigated until 2002 when Paula F. Bailie-Hamilton published a review of recent studies showing that diet, exercise, and genetics were not the only factors affecting weight regulation. Her notice of weight regulation drugs used in the livestock industry led to her hypothesis that a class of EDCs was causing an unwanted weight gain. Weight gain was not the only discrepancy noticed; dysregulation of the body's natural weight-control mechanisms also caused unexplained changes in the body beyond just obesity such as disruption of appetite, food efficiency, metabolism, and desire for exercise (Bailie-Hamilton et al., 2002).

It wasn't until 2006 that this class of EDCs were classified as obesogens. Felix Grün and Bruce Blumberg coined the new name in a review discussing how tin-based compounds like tributyltin (TBT) can lead to adipogenesis in cells. This collected data and compared some of the first studies to use in vitro models as a way to analyze the mechanism of adipogenesis by obesogens. It also discussed the connection between retinoid X receptors (RXRα, RXRβ, and RXRγ) as well as peroxisome proliferator-activated receptors (PPARα, PPARβ, and PPARγ) and how they regulate lipid homeostasis in cells (Grün & Blumberg, 2006).

A large review looking at the sources of these obesogens highlighted the fact that though these chemicals share a common side-effect of hormone mimicry, they are used in many different settings and industries. This review listed about 85 different chemicals from 15 different applications, including; antimicrobials, biogenic compounds, byproducts/intermediate reactants, flame retardants, food additives and contact materials, household product ingredients, industrial additives, medical/veterinary research, metabolite/degradation, metal/metallurgy, personal care products/cosmetic ingredients, pesticide/fungicide and ingredients, plastics/rubbers, solvents, and air pollutants (Heindel, 2019). The endocrine disruption exchange website (the source of Heindel's list) includes 1482 distinct endocrine-disrupting chemicals. The high number of other EDCs that have been shown to have obesogenic properties means that many of these EDCs could potentially be obesogenic.

A paper by Giorgio Dimastrogiovanni focuses on the mechanism of how obesogens trigger these changes in cells. The author used qPCR analysis to confirm the presence and activation level of genes targeted by obesogens to track how exposure to obesogens affected gene expression. The genes of interest in this paper were ABCA1, LXR, CD36, PPARβ, PPARγ, PPARα, FAS, FATP1, and LPL; while EF1α was used as a reference gene. The author covers the specifics of how each obesogenic compound either up-regulates or down-regulates each specific gene, but the most important point of note is that various obesogens destabilized lipid homeostasis in different ways. (Dimastrogiovanni, 2015).

This was followed by another paper describing the main mechanisms of metabolism disruption: increased uptake of lipids, decreased efflux of lipids, increased fatty acid synthesis, and defects in oxidative metabolism of fatty acids (Franco et al., 2020). Franco tested several different endocrine disrupting compounds, such as: fenofibrate (FEN), rosiglitazone (ROS), perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS), di-2-ethylexylphthalate (DEHP), bisphenol A (BPA), and tributyltin (TBT) for cytotoxicity and lipid accumulation. The author showed that these compounds led to cellular toxicity, accumulation of lipids, but also a change in lipid profile within the cells; shifting lipids from the membrane into triglyceride forms within the cell. This reallocation of lipids was commonly seen in patients presenting with obesity related illnesses.

Exposure to obesogenic compounds can be simple disruption of lipid homeostasis in cells, but oftentimes progresses to a point of obesity and in some cases leads to disease states. Because of the liver's role in the distribution and regulation of lipids, it is one of the most studied areas of adipose tissue build up. Obesity and nonalcoholic fatty liver disease (NAFLD) are the primary diseases analyzed in studies of obesogen exposure. NAFLD is the name of a range of diseases all characterized by reversible hepatic steatosis (fatty liver deposits). This can remain constant (nonalcoholic steatohepatitis, NASH) or develop into harmful inflammation (steatohepatitis, SH). These conditions can progress further to life-threatening conditions such as fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (Foulds et al., 2017).

Throughout the history of obesogen testing, there have been a wide variety of models examined. Whole organism studies in fish and mice, observational studies in humans, fish cell lines (RTL-W1), mice cell lines (3T3-L1), and even human cell lines (HepaRG and HUH7). The cell line models have been effective in analyzing the mechanisms by which obesogens affect cells, while whole organism studies have helped classify obesogenic compounds. Human-based models provide practical information for health initiatives, but alternative organism cell line testing is still important for research into specific methods of dysregulation (Franco et al., 2020).

Much less research has been done on aquatic organisms at the cellular level and RTL-W1 remains a largely unstudied model. One study by Giorgio Dimastrogiovanni examined the gene expression of RTL-W1 cells when exposed to potential and known obesogens (though not PFOS or PFOA) and provides an avenue for further investigation using those models. This cell line remains important for these studies because of the liver's role in the synthesis and regulation of lipids and fatty acids. The liver is also the site of most lipid-related diseases, and as such is extremely relevant to obesogenic studies (Dimastrogiovanni, 2015).

RTL-W1 was selected as an effective model to study these chemicals due to various environmental, fiscal, and ethical reasons. Cell lines in general allow high throughput testing without the loss of animal life, without the maintenance cost of live animals, and are able to replicate themselves over time. RTL-W1, in particular, for cell lines is effective as a model because of the organ system and species. As a species, rainbow trout is completely genetically cataloged, well-studied in general, and as an aquatic species commonly exposed to these types of effluents. The liver cell line is important because the liver has a role in metabolizing chemicals taken into the organism as well as regulating free lipids. The liver's role in metabolizing these compounds and ultimately suffering the health effects make a liver cell line an effective model.

For this project, we hypothesize that an increase in the concentration of PFOS and PFOA will lead to decreased cell viability, increased intracellular lipid storage, and increased expression of the PPAR genes. There are studies measuring these chemicals in the environment and classifying their negative health effects, but more needs to be done to examine their mechanism of action. It will be the first study to examine these chemicals using the RTL-W1 cell line. We will examine these questions through the use of multi-endpoint cytotoxicity assays, lipid droplet assays, and qPCR analysis.

2. Methods

2.1 Cell Culture Maintenance

RTL-W1 are rainbow trout (*Oncorhynchus mykiss*) liver cells used in the current study. These cells are cultured in 75 cm^2 flasks of Leibovitz's L-15 complete media with 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) and stored in an incubator at 19°C. In order to encourage cell growth, the media is replaced about every seven days. This process consists of aspirating old media from each flask and replacing it with 12 mL of fresh L-15 complete media. This media is made in 1 L batches and kept refrigerated until use.

In order to maintain and grow the cells, they are cultured in Leibovitz's L-15 medium. This media contains a mixture of sugars, salts, amino acids, and vitamins and is supplemented with FBS necessary for the promotion of cell confluency (Ibrahim 2020). They are cultured in this media throughout their time of growth and are only kept in exposure media during an assay. Before exposure to the compounds under investigation, the L-15 complete is exchanged for L-

15/ex. The chemicals under investigation are dissolved in a solvent such as dimethyl sulfoxide (DMSO) and then added to the L-15/ex media for exposure. DMSO concentration is maintained at 0.5% in all exposure media. This L-15/ex media only contains the necessary sugars and salts to maintain cells throughout exposure, all reactive or confounding chemicals are removed to avoid the risk of complexation.

After initial testing, the initial exposure media was modified for the sake of providing cells with lipids to measure lipid dysregulation after exposure, a modified exposure solution was made with L-15 complete and 1%, 5%, or 10% FBS. After optimization, cytotoxicity and qPCR were both completed with L-15 media with 5% FBS to ensure the comparison between the two.

After 14 days or when cells have reached about 90% confluency, the cells are split into separate flasks to grant them more space to grow. This process begins with aspiration of the media, a one-minute wash of the cells with 1 mL Versene, aspiration of the Versene, a rewash of 5-15 minutes of 1 mL Versene, and complete aspiration of the second wash to make sure that all liquid is removed. After this 1 mL of 0.25% Trypsin was washed back and forth over cells for less than five minutes and cells were examined under a light microscope to ensure proper cell detachment from the flask. 5 mL of L-15 complete media was added to the flask after the 5-minute period to neutralize the Trypsin. The 6 mL solution of cells and media is homogenized and split into 3 flasks of 2 mL apiece. Then fresh L-15 complete media is added to each flask to bring it back to 12 mL of media. Each flask is then labeled and returned to the incubator to continue growing.

When prepared for the assay, begin the process of seeding cells onto the well plates. First, aspirate the media from a flask of cells to be analyzed. Wash the flask with 1 mL of versene for 1 minute and aspirate. Wash the flask with 1 mL of versene for 3 minutes and aspirate. Add 1 mL trypsine to flask and swirl over cells for 3 minutes. Check cells under a microscope to ensure they are detaching. Neutralize the trypsine by adding 5 mL L-15 complete media to flask. Pipette cells in media and transfer to a 15 mL falcon tube. Balance against a falcon tube of water and centrifuge for 5 minutes. Aspirate media from around the clumped cells at the base of the falcon tube. Add 2 mL of media to the cells and homogenize the mixture. Fill a 0.2 mL PCR vial with 10 µL Trypan Blue stain and 10 µL cells and homogenize the mixture. Take 10 µL of this mixture and apply it to the A side of a slide and then take the remaining 10 µL of the mixture to apply to the B side of the slide. Analyze the slide in the Countess II automated cell counter and record the number of living cells for both sides of the slide and average to calculate cell density in the cell media (Ibrahim et al., 2020). Use the prepared excel spreadsheet to determine the ratio of cell media to L-15 complete to deposit 100,000 cells/mL in each well. Deposit 1 mL of solution in each well, excluding the top left control, and then incubate these plates for 48 hours to allow cells to reach confluency.

After verifying that cell viability is over 90%, the data collected is used to calculate the ratio of cell solution and fresh media to mix for seeding. The cell solution and additional exposure media is mixed in a 50 mL trough and a multichannel pipette is used to dispense 1 mL of cell solution into 23 of the 24 wells. The final well of each well plate is left empty as a negative control. These well plates are then left to incubate for 2-3 days to reach confluency before exposure.

During exposure, cells in the well plate are washed and immediately exposed to exposure media prepared with the compounds under investigation. Further exposure information can be reviewed below.

2.2 Exposure

For a cytotoxicity assay, a range of concentrations are used to determine the effective concentration 50% (EC50) of the compounds under investigation. These cytotoxicity assays involve exposing cells in multi-well plates to solutions of exposure media with the compounds of interest. In this study, an initial assay with concentrations of 1.588 μM, 3.175 μM, 6.25 μM, 12.5 μM, 25 μM, and 50 μM of PFOS and PFOA was completed. After further testing, this range was increased to 12.5 μM, 25 μM, 50 μM, 100 μM, 300 μM, and 600 μM. Controls consisted of an empty well, a well containing L-15 media, and a well containing L-15 with the 0.5% DMSO used as a solvent. Treatment concentrations were performed in triplicate. During exposure, cells in the well plate are washed and immediately exposed to exposure media prepared with the compounds under investigation, then incubated for 24 hours. A sample of each exposure was collected in order to analyze later by LC-MS/MS in order to verify concentration.

For the lipid droplet assay, prepare exposure solutions of PFOS at necessary concentrations in 0.5% DMSO and L-15 complete media with 5% FBS. For this assay, prepare concentrations at EC50, EC20, and EC10. These concentrations are at 284.3 μM for the EC50, 165.7 μM for the EC20, and 12.83 μM for EC10. In order to prepare these concentrations: dissolve 426.45 μL of the 10 μM stock solution of PFOS in 14.57 mL media, 248.55 μL in 14.75 mL, and 181.25 μL in 14.82 mL. When it is time for exposure, aspirate media in the control row and add appropriate controls (empty well control, L-15 complete media control, 0.5% DMSO solvent control, and oleic acid positive control). Aspirate media in other rows and replace with appropriate concentrations of exposure solution. Incubate plates for 24 hours after exposure.

For the qPCR assay, the various treatment groups were an L-15 complete control, L-15 complete w/ 0.5% DMSO solvent control, low PFOS (50 nM), high PFOS (1 μM), low PFOA (50 nM), and high PFOA (1 μM). These exposure concentrations were selected based on environmentally relevant concentrations in river systems (50 nM) and fish (1 μM) (Teunen et al., 2021). The cells were exposed to these solutions for 24 hours.

2.3 Cytotoxicity Assay

After this 24-hour period, exposure solutions were aspirated and washed once again with L-15/ex. Cells are then exposed to L-15/ex solutions containing fluorescent dyes (Ibrahim et al., 2020). Fluorescence is measured after a period (AB, CFDA-AM 30 minutes, NR 60 minutes) of dye exposure using a plate reader, and levels of fluorescence are used to quantify cell viability.

Three different dyes have been developed for use with fish cell lines: AlamarBlue (AB), 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), and Neutral Red (NR). Each dye gives insight into a different aspect of cellular activity; AB is an indicator of metabolic activity in the mitochondria, CFDA-AM is an indicator of cell membrane integrity, and NR is an indicator of lysosomal αactivity (Dayeh et al., 2005). After exposure fluorescence is measured after an incubation period of 30 minutes (AB and CFDA-AM) and 60 minutes (NR) using a plate reader. Levels of fluorescence are used to quantify cell viability.

From the fluorescence data collected of the different concentrations, it is possible to create a dose-response curve and calculate the effective concentration 50% (EC50). EC50 concentrations were determined using non-linear regression sigmoidal dose-response curves following the Hill slope Equation These show the concentrations at which cell viability diminished.

Having a multiple endpoint viability assay allows more information to be gained from each test. Having a single dye allows the investigation of cell viability, but multiple dyes allow the comparison of dose-response curves to investigate what the compound of interest is mechanistically doing to cells.

The fluorescence results were graphed through GraphPad software version 7.0 to create dose-response curves highlighting each endpoint for each treatment group.

2.4 Lipid Drop Assay

This assay was modified for live staining from the fixed staining protocol of Qiu & Simon in 2016.

Prior to the assay, an oleic acid, BODIPY stain, and plate reading protocol must be prepared. The oleic acid is thawed from a closed glass ampule. 14.123 mg are pulled from the ampule for use and the remainder are stored in a sealed glass vial. The 14.123 mg of oleic acid are dissolved in 5 mL DMSO to produce a 10 mM stock solution. 10 µL of the stock solution are dissolved in 9.99 mL L-15 media with 0.05% DMSO to produce a 10 µM exposure solution.

The BODIPY stain is prepared by dissolving 1 mg BODIPY in 1 mL DMSO to make a 3.8153 mM stock solution. Twenty 50 µL aliquots are prepared in 0.2 mL PCR tubes. Each PCR tube is wrapped in foil and stored in ziplock bags in the freezer to protect them from environmental degradation. Each aliquot is dissolved into 12 mL L-15 exposure medium to produce a 4 µM staining solution. This solution was diluted in half with more L-15 exposure medium to produce the final 2 µM stain.

The plate reading protocol will be programmed according to the specifications described by the manufacturer's instructions (BioTek). It will include a step to quantify viability with fluorescence, as well as a step to photograph the lipid droplets while stained.

Perform the seeding as described earlier in the cell maintenance section.

In the meantime, prepare exposure solutions of PFOS at necessary concentrations in 0.5% DMSO. (PFOS 10%: EC50=284.3 µM, EC20=165.7 µM, EC10=12.83 µM) (284.3 µM=426.45 µL in 14.57 mL, 165.7 µM=248.55 µL in 14.75 mL, 120.83 µM=181.25 in 14.82 mL) When it is time for exposure, aspirate media in the control row and add appropriate controls (empty, L-15 complete media, DMSO media, and oleic acid). Aspirate media and replace with appropriate concentrations of exposure solution. Incubate plates for 24 hours after exposure.

Prepare 2 µM BODIPY staining solution and prepare the Lipid Assay program on the plate reader. Discard the exposure solution and wash each well with 1 mL L-15/ex. Discard wash solution and add 50 µL BODIPY staining solution to each well. Cover plate in foil to prevent light exposure and incubate for 20 minutes. Add 1 drop of Nuclear Blue to each well and incubate for 10 minutes covered in foil. Wash plate and replace with L-15 without phenol red. Use plate reader to collect images of lipid droplets and record fluorescence (450-490 excitation, 50-550 emission, GFP filter).

2.5 Quantitative Polymerase Chain Reaction

In order to measure the expression of lipid-regulating genes, perform a quantitative polymerase chain reaction (qPCR). The two types of qPCR are absolute quantification and relative quantification. Absolute quantification involves the direct measurement of replicates

produced, while relative quantification compares the quantity of the target gene in controls compared to treated cells.

To prepare the qPCR assay we designed primers for specific target gene. The genes of interest are PPARα, PPARβ, and PPARγ; and so sequencing of those genes is collected from the NCBI Nucleotide Blast database. From these sequences, forward and reverse primers, both internal and external, are selected. The external primers produce an amplicon between 500 and 1000 base pairs, while the internal primers produce an amplicon of about 200 base pairs. These internal primers can lower the risk of dimer formation, hairpins, or pairing with random nucleotides. Elongation Factor 1 alpha (EF-1α) was also selected for use as a reference gene due to its ubiquity and stability. The EF-1α primer sequence was previously described in Ibrahim et al., (2020).

PPARα Primers:
External F1: TGATGGCCAAGCTGGTAGGT
External R1: CTATGCATCCGTCATAGCTGC
Internal F1: CAGTGCCTGGACCCTGAATGA
Internal R1: GGTAATGAACCCTCCTCCGC

PPARγ Primers:
External F1: CACTGCCTGTCAACACATTGG
External R1: ACACTTGTTGCGGTCTTCT
Internal F1: CCACAGCCAGGTTCAGGAG
Internal R1: TGTTGAGTAGGGGAAGCGGTG

After receiving the manufactured primers from Integrated DNA Technologies, test and optimize them prior to qPCR use. This involves testing the primers at various annealing temperatures in a thermocycler and then using gel electrophoresis to visualize whether or not the genes were expressed using those primers. After testing primers, the PPARα primers were not leading to an expressed gene so we were unable to use them. The PPARγ primers were 93% efficient though, and so were usable for testing.

After protocol optimization and collection of materials, cells are trypsinized and pulled from maintenance flasks and seeded on 6-well plates. They are seeded at 100,000 cells/cm^2 and incubated for 48 hours to reach confluency. At this point, the media was aspirated and a prepared exposure media was added to the wells. The exposure media was prepared as discussed in the exposure section of the methods.

After 24 hours of exposure, RNA is extracted from each well. This is done using a 900 μL TRIzol solution to lyse cells, but preserve nucleic acids. The TRIzol solution with the cellular components is then moved to a 1.5 mL Eppendorf tube and frozen in the -80° freezer. Once ready, remove the tubes to thaw for 10 minutes and add 180 μL of chloroform and vortex well. Once this is done, the RNA, DNA, and proteins of each tube are allowed to separate according to density and the RNA is carefully pipetted from the other phases into new Eppendorf tubes. It is then centrifuged until the RNA forms a pellet in the base of the tube. RNA quality was evaluated using electrophoresis of 1 μg of RNA (*Fig. 1*). RNA quantification and purity evaluation was performed using spectrophotometrically using the Cytation 5 plate reader. All samples showed a 260/280 ratio above 1.8. Complementary DNA was synthesized from 1 μg of RNA using the

Superscript III Reverse Transcriptase (Invitrogen) according to manufacturer instructions. For qPCR, we use the TBGreen Master Mix (Takara).

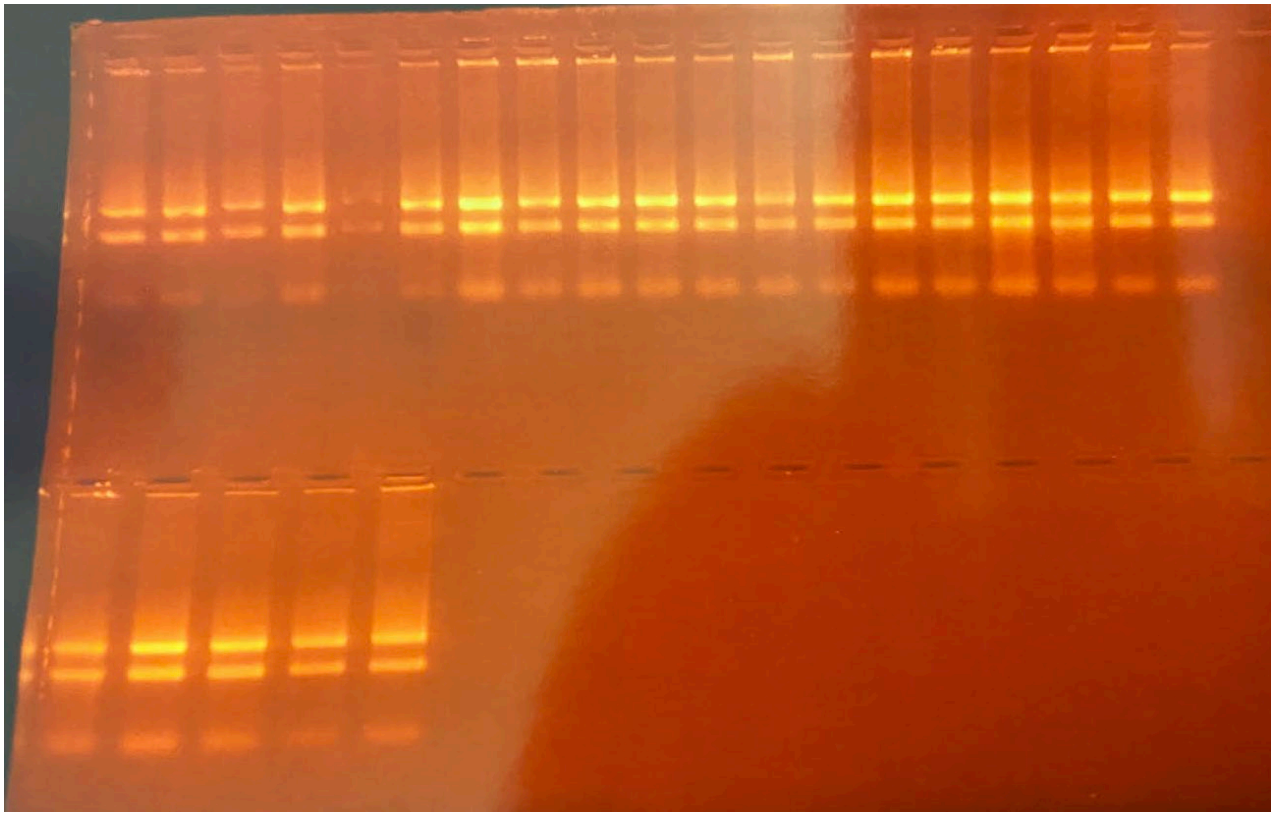

Fig 1. Gel showing each sample after checking RNA for quality before DNA synthesis step. Note the lack of fluorescence of sample 5 (one of the 1 μM PFOS samples). *Due to this, sample 5 was excluded from use at this step, though all others were sufficient.*

A qPCR machine is used with qBase software to compare the expression of a target gene (PPARγ) to the expression of a reference gene (ELF-1). Results of the qPCR were able to be measured as Ct (crossing threshold) for each sample and ultimately converted to fold change for final results.

The ΔΔCt method (Pfaffl et al., 2002) was used to calculate fold change. Ct of the reference gene was subtracted from the Ct of the target gene for each replicate of each sample to get a ΔCt. The ΔCt of each replicate was averaged together so that there was one ΔCt for each sample. The ΔCt of each replicate was then subtracted from the average ΔCt for the control to get a ΔΔCt for each sample. Values are then calculated from 2^-ΔΔCt for each sample. These values are compared to produce a fold change bar graph of each treatment group as it compares to the DMSO (solvent) control. Statistical significance is calculated using a student's T-test ($p<0.05$).

3. Results

3.3 Cytotoxicity Assay Results

The first iteration of the cytotoxicity assay was only done with treatment concentrations of 1.588 µM, 3.175 µM, 6.25 µM, 12.5 µM, 25 µM, and 50 µM of PFOS and PFOA. At the time, very little change in viability was seen and so tests were continued with treatments of 12.5 µM, 25 µM, 50 µM, 100 µM, 300 µM, and 600 µM. Toxicity was induced at around 100 µM for both PFOS and PFOA. An interesting point to note is that metabolic activity (AlamarBlue) and membrane integrity (CFDA-AM) measurements were as expected and showed a decrease in viability as concentration increased in both PFOS and PFOA (*Fig. 2*). The two chemicals differed in the lysosomal activity assay (Neutral Red) though. PFOA showed the standard decrease in viability, but PFOS had stimulation of lysosomal activity. This was especially true at higher concentrations, with a very large jump between 100 µM and lower as compared to the 300 and 600 µM concentrations.

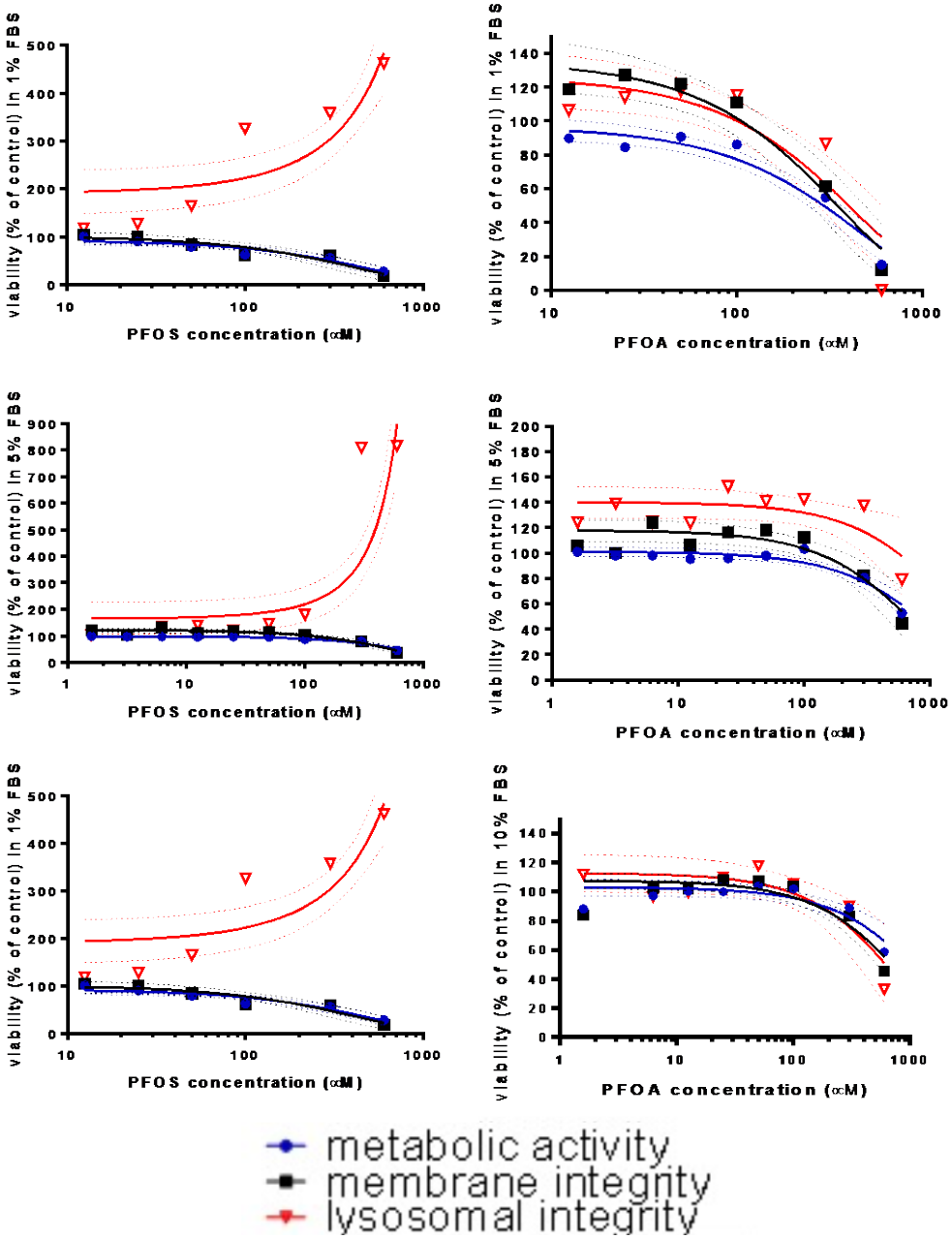

Fig. 2. Dose-response curves showing the relationship of PFOS (left column) and PFOA (right column) concentration to cell viability. Each row represents L-15 complete media with differing levels of fetal bovine serum. The y-axis of the graphs are not to scale with each other, but were sized for maximum intelligibility.

After the initial assays with standardized 5% fetal bovine serum, another round of testing was completed with 1%, 5%, and 10% FBS in the exposure solution. Comparing the EC50s of each treatment group, the EC50 increases as FBS concentration increases (*Fig.3*). This trend holds true for each of the endpoints. EC50s averaged around 300 μM for the 1%, 539 μM for the 5%, and 647 μM for the 10% exposure solutions. Note the results of lysosomal activity differ between PFOS and PFOA, due to PFOS being measured from a stimulation curve and PFOA being measured from an inhibition curve. PFOA with 5% FBS reached ~80% viability at the highest concentration and so an accurate EC50 was unable to be calculated for this treatment group.

EC50 Values	**Metabolic Activity**	**Membrane Integrity**	**Lysosomal Activity**
PFOS 1%	265.9 μM (n=4)	256.1 μM (n=4)	79.18 μM (n=5)
PFOA 1%	285.38 μM (n=4)	353.58 μM (n=4)	337.87 μM (n=4)
PFOS 5%	626.3 μM (n=5)	502.3 μM (n=5)	130.3 μM (n=5)
PFOA 5%	538.75 μM (n=6)	487.95 μM (n=6)	nc
PFOS 10%	795.3 μM (n=5)	543.8 μM (n=5)	284.3 μM (n=5)
PFOA 10%	618.6 μM (n=5)	770.0 μM (n=5)	507.7 μM (n=5)

Fig. 3. EC50 values for each exposure solution and each endpoint, calculated from GraphPad software. Shown numerically for ease in comparison between groups.

3.4 Lipid Droplet Assay Results

Though a protocol was created for the lipid droplet assay, as of this time it still remains to be optimized and completed.

3.5 Quantitative Polymerase Chain Reaction Results

After testing primers, the PPARα primers were not leading to an expressed gene so we were unable to use them. The PPARγ primers were 93% efficient though, and so were usable for testing.

When comparing fold change of the various treatment groups for the qPCR assay, there appears to be little change in expression between the DMSO solvent control and the low dose of both PFOS and PFOA. Both of these remained close to the control results and had large variability. The high doses held more consistent results and both showed inhibition of PPARγ expression. The high dose PFOS was statistically significant from control according to the student T-test performed (*Fig. 4*).

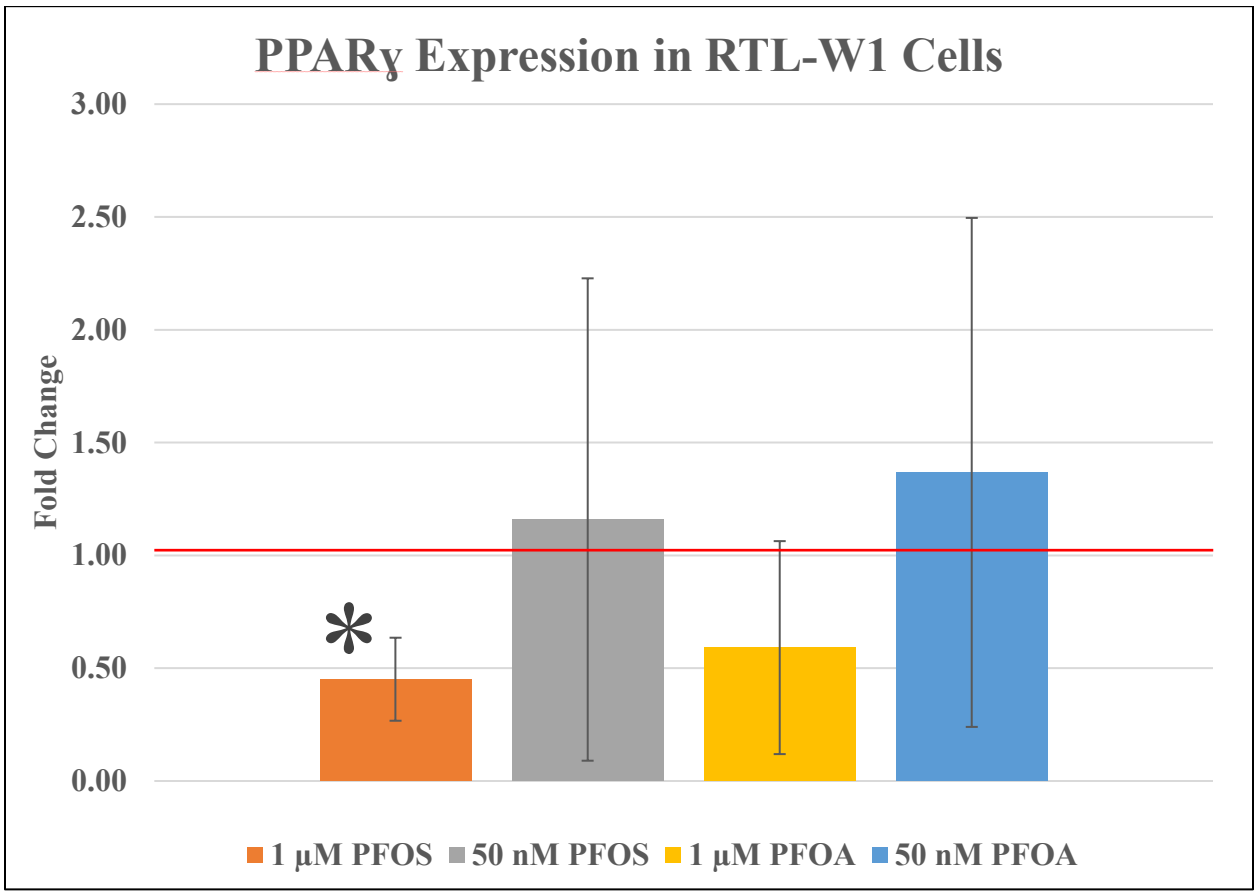

Fig. 4. Fold change in expression of PPARγ normalized against reference gene elongation factor 1. Treatment groups fold change when compared to DMSO control group.

4. Discussion

The novelty of this study comes from two factors: it will be one of the first to use RTL-W1 as a model with obesogenic compounds (Dimastrogiovanni, 2015), as well as being one of the only studies to directly connect the toxicity of PFAS to lipid accumulation and gene activation. Examining the patterns in the results of these three things is useful in mechanistically understanding the effects of obesogens on cells. It examines many different aspects of change in the cell. It also allows a more start-to-finish approach; showing the gene activation at the

beginning of exposure, the lipid accumulation as a result of this activation, and the eventual time and method of cell death in comparison to past studies which mainly focused on gene activation (start) and obesity or liver disease (end). The use of a cell line to study these chemicals allowed a higher throughput and more ethical alternative to studies done with whole organism testing.

While the cytotoxicity assays moved beyond the initially selected environmentally relevant doses, the increased range of testing allowed for a better mechanistic analysis of PFOS and PFOA. Because this was an acute study with a time point of 24 hours, it is difficult to directly compare toxicity to chronically toxic situations. The acute study with higher doses did allow for a review of how the chemicals affected the various cellular structures within the cell, leading to the discovery of the lysosomal stimulation of PFOS. It remains to be seen exactly why this stimulation effect occurs and whether it is an attempt to protect the cell or just a byproduct of PFOS's toxic effect.

The protective effect of FBS was another interesting and unexpected result. Because of the lipid content of FBS, it was decided to run all assays with media containing FBS so that the cells would have a lipid supply to pull from. The assays with higher and lower concentrations were completed to determine if this would affect toxicity in any way. When it was seen that increased FBS led to decreased toxicity, we hypothesized that complexation with the amphipathic chemicals PFOS and PFOA by FBS was the primary reducer of toxicity and increased access to nutrients was a secondary effect that increased the cell's ability to survive. When the effect of FBS was noted, continuing with the cytotoxicity assays, lipid droplet assay, and qPCR was approved with L15 media containing 5% FBS in place of L15-ex.

While preparing for the qPCR testing, primers were designed for the genes PPARα and PPARγ, though only the qPCR for PPARγ was able to be completed. After performing this assay and noting an inhibitory effect due to PFOS and PFOA, we reviewed literature for an explanation. An inducing effect was seen in both Jacquet et al. 2012 and Takacs & Abbott 2007 which contradicts the results we found. Both studies exposed cells to PFOS and found an inducing effect in all PPAR genes, including PPARγ. However, they used alternative human and hamster cell lines that vary from the RTL-W1 cells in this study. Another study by Dimastrogiovanni 2015, used RTL-W1 cells and obesogens, but not specifically PFOS. That study found an induced PPARγ effect on cells exposed to similar obesogenic compounds, but had a different exposure methodology than what was conducted by our team. The previous study performed an exposure under which the cells were exposed for up to 7 full days and exposure solutions contained up to 100 μM of the obesogenic compounds. This would have allowed seven times as long for the compounds to affect the cells and one hundred times the toxic concentration. When we conducted the initial cytotoxicity study, we found very little response and were forced to increase the concentration range. I believe that continuing this study at higher ranges of concentrations would allow us to determine if there truly is an inhibitory effect or whether a higher dose would reveal an inductive effect. There may be a possible hormetic effect due to the low dosage and exposure time.

In the future, this study would benefit from the optimization and completion of the lipid droplet assay. To continue with this pathway, an analysis of the various lipid types would also be useful. Continuing from the Franco study that performed this previously, a more intensive look at specific lipid types related to disease endpoints, such as triglycerides, would be useful. An extension of the qPCR testing as well would be useful to examine the roles of PPARα, PPARβ, and other lipid-regulating genes alongside PPARγ. With additional time to design primers and

continue genetic testing, a suite of genes would allow a more holistic understanding of the genetic effects of PFOS and PFOA exposure.

Overall, this study has sought to act as a bridge of other more specific studies, bringing together the cytotoxic, genetic, and structural changes brought about by these chemicals of interest. It has not dived as deep into a larger group of obesogens, but should provide a jumping-off point that allows for comparison between the qualities examined that could be replicated with these additional compounds. It should also add in comparison between studies measuring different endpoints. For example, a study only having performed genetic testing may be able to postulate on cytotoxicity or lipid storage based upon the information shown here. Overall, it has revealed interesting places to delve deeper into the mechanisms of PFOS and PFOA. The structural differences between PFOS and PFOA that lead to the lysosomal stimulation should be examined further for insight into how these obesogenic compounds operate. The protective effects of FBS are also useful for taking into consideration differences in methodology between labs. The genetic effects of these chemicals are the most important pathway to learn about though, and so understanding the activation of the PPAR genes is essential for future studies in the field.

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