Perfluorooctanoic Acid and Perfluorooctane Sulfonate Induce Neuronal Cell Apoptosis via the FADD Ligand of the Death Pathway

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**ABSTRACT**

Per and polyfluoroalkyl substances (PFAS chemicals) have been prevalent since the 1940s. Yet, the impacts of these synthetic substances on human health still remain uncertain. In vivo studies exhibit cytotoxicity targets including: hepatotoxicity, reproductive toxicity, thyroid disruption, neurotoxicity, and many more. In this study, Perfluorooctane sulfonate (PFOS) and Perfluorooctanoic acid (PFOA) were tested for their potential effects on HTB-11 neuroblastoma cells, derived from bone marrow. Molecular docking, using the PYRX docking program, was additionally utilized to determine affinity linkages with death domain proteins. PFOS and PFOA prompted caspase activity on targeted neuronal cells, indicating prominent levels of apoptosis. PFOS and PFOA, moreover, reduced target neuronal cell survival in the Microculture Tetrazolium assay (MTT), with all concentrations significant. Evidence of high binding affinities with a death domain protein was also present. Based on this evidence, researchers should study more closely the health effects PFAS chemicals can instigate in the biological processes of humans.

**Introduction**

1.1 What are PFAS?

“Forever chemicals”- also known as PFAS (Per and polyfluoroalkyl substances) - have played a big impact worldwide on industrial processes, the manufacturing of electronics, and the production of other daily utensils and products that humans rely on. With over 4500 variants, there are two main components that all PFAS variations have in common: PFAS chemicals are man-made, and have a carbon-fluorine bond. As a result of their enduring molecular structure, these chemicals have a strong non-biodegradable nature and an adept resistance to water, oil, heat, stains, greases, etc. The physical and chemical properties of these substances enable them to travel through the environment easily, infiltrate drinking water systems, and bioaccumulate within the bodies of humans and other living organisms, even after exposure has stopped.

Two of the most widespread and extensively researched PFAS chemicals are: PFOS (Perfluorooctane sulfonate) and PFOA (Perfluorooctanoic acid). Although PFOS and PFOA substances are no longer produced in the United States, every-day objects being imported globally, transport these substances into the United States. Examples include: nonstick cookware, food packaging materials, cosmetics, textiles, cleaning products, fire-fighting foam, etc, and just recently, PFAS chemicals have been found in antifogging sprays and cloths for glasses, as well as in packaging of fast food products.

Currently there is little research on the molecular mechanisms and negative effect(s) of these chemicals on neuronal cells. The aim of this research is to investigate if these chemicals can induce neuronal cell death at low concentrations, and to identify its cell’s molecular targets.
1.2 Main cytotoxicity effects and cellular targets of PFOS & PFOA

Given that PFAS chemicals are widely distributed, researchers are growing more concerned of the possible health effects PFAS chemicals can cause in humans. Current studies have found associations with cytotoxicity results including:

1.2a -- Hepatotoxicity

PFOS was found to be more active in the liver than PFOA. Multiple studies have found signs and evidence of PFOS target bioaccumulation in the liver that have associated results “in hepatic steatosis, hepatomegaly, hepatocellular hyperplasia, and oxidative damage of hepatocytes” (Du et al., 2009; Wan et al., 2012; Huang et al., 2014; Fai Tse et al., 2016; Lai et al., 2017b; Xu et al., 2017). Through fat metabolism interference, the β-oxidation, a beta carbon of the fatty acid produced in the liver, has been found to decrease when exposed to PFOS, giving rise to a greater accumulation of triglycerides and fatty acids, disrupting the regular metabolism process. (Wan et al., 2012; Cheng et al., 2016; Jacobsen et al., 2018). Along with this, both chemicals also induce oxidative stress, which potentially triggers ROS (reactive oxygen species).

1.2b-- Neurotoxicity

PFOA and PFOS were found to be potentially neurotoxic in past studies, but to an unknown extent. In addition, PFOS displayed higher neurotoxic effects. In vivo studies from roundworms to frogs, toxicology professor Josh Cannon from Purdue university discovered a decrease in dopamine levels; dopamine is crucial to the movement and mobility of a human. An implication in dopamine is described as a “blueprint” for Parkinson’s disease (Shreesh Raj Sammi, Rachel M Foguth, Claudia Sofía Nieves, Chloe De Perre, Peter Wipf, Cynthia T McMurray, Linda S Lee, Jason R Cannon, 2019).

However, with other factors, more research needs to be done in order to draw a conclusion for the neuropathology and neurological mechanisms deriving from contact with PFAS chemical(s). Additionally, there have been studies revealing neuroinflammation, damage in human cells like hippocampal cells, and interference in synaptogenesis and synaptic plasticity from PFOS (Chen et al., 2018a; Chen et al., 2018b, Bourgeron, 2015).

1.2c -- Immunotoxicity

There is limited knowledge on immuno-toxic effects from PFOS and PFOA. Existing in vivo and vitro studies show that PFOS could possibly disturb normal function of immune cells and promote cell proliferation.

In one vitro study, the IL-2 (cytokine responsible for balancing the activities of the immune system) located within human T cells was reduced. Although there was an association of immunotoxicity imposed by PFOS and PFOA, effects varied in different conditions (Qazi et al., 2009; Midgett et al., 2015).

1.2d Thyroid Disruption

PFAS chemicals have played a role in vitro disruption of thyroid cells/hormones. PFOA and PFOS have been associated with disrupting cell processes, homeostasis, and hormonal imbalance. In one study that took place in 2020, serum thyrotropin (TSH) levels were measured for 2, 4, and 6 year old children exposed to PFAS substances. Results denoted decreased TSH levels in their serum (Hwa Young Kim, Kyoung-Nam Kim, Choong Ho Shin, Youn-Hee Lim, Johanna Inhyang Kim, Bung-Nyun Kim, Yun-Chul Hong, and Young Ah Lee, 2020). It should be noted that higher concentrations of PFOS are more likely to induce cytotoxicity in thyroid cells via passive diffusion.
1.2e Reproductive toxicity

In a vivo study of mice, the weight of the testis decreased along with a reduction in sperm count after oral exposure. In another vivo study, the gonads of both female and male zebrafish significantly changed; estrogen levels of the female increased after exposure to PFAS (Chen et al. 2016, Zhang et al. 2015, Wang et al. 2018). These results demonstrate the potential harmful effects of PFOA and PFOS chemicals on the reproductive organs in both male and female zebrafish. Another study conducted found that the gene expression related to FSH, GnRH, LH, and androgen receptors were altered. The alteration of receptors interfere with endocrine systems interconnected in reproductive organ functions (López-Doval et al., 2016). Although many research studies have found varying possible hazardous outcomes, these problems were the most frequent.

1.3 Molecular docking

To study the biochemical process of PFAS chemicals, PFOA was used in molecular docking to observe the binding affinities with hERα (estrogen), hAR (androgen), and hTRβ (thyroid) receptors. Consistent with existing research studies, there was a high potency of PFOA binding. The order of the highest affinity binding was: hTRβ > hERα > hAR. PFOA seems to interact greatest with the thyroid receptor, and therefore likely interferes with the human endocrine system through a thyroid-mediated pathway. The high binding affinity (Yan Cheng, Huiming Chen, Wenlian Yu, Yuan Cui, Lili Zhou, Haishan Li, Naining Song, Lei Li, Xi Li, Jing Zhang, Ping Ma, Xin Sun, Zheng Wang, Lu Han 2010).

In another study involving molecular docking, PFOS was found to be bonded close to the center of the CAT enzyme (an enzyme fighting oxidative damage and stress); this specifies that PFOS has an effect in the reduction of CAT (catalase) activity. On the other hand, PFOA had little effect because it bonded more on the surface than the center, signaling little effect on the actual enzyme activity (Mengchen Xu, Zhaohao Cui, Lining Zhao, Shimeng Hu, Wansong Zong, Rutao Liu, 2018).

From suspected neurotoxicity to thyroid disruption, there is significant evidence correlating with various unpropitious effects of PFAS on different systems of the body. With limited research on targets within the human body, it’s more difficult to discover connections between diseases and illnesses that may or may not be caused by a combination of these forever chemicals.

Limitation/gaps of current research

Multiple studies have focused on more in-vivo models than in-vitro models. Given that there is a colossal amount of different mutants, it’s arduous and time-costly to figure out what each individual one strand can do, and what impact they can have as a whole mixture. The reactions PFAS chemicals have on molecular mechanisms and cellular processes on numerous human biological systems are still not understood presently. Furthermore, there’s unconfirmed evidence on whether or not PFAS chemicals in the bloodstream can be a root cause for neurodegenerative diseases, cancer, etc. There are many unknowns that build a barrier for the performance of more advanced studies on PFAS chemicals.

Material Methods

3.1 Cell Culture
Old media was poured out of the container. 4 mL of trypsin (human gut enzyme) was added into the sample. Sample was placed in an incubator at 37 degrees Celsius with 5% CO₂ for 4 minutes. Cells (from ATCC) in the flask are tapped in order to detach them. 5mL of fresh media (Gibco) was added to neutralize trypsin; suspended cells were picked up and then injected into tube(s). Tubes were centrifuged at 3000 rpm for 4 minutes. Supernatant was poured out, so there was only a cell pellet at the bottom of the tube.

3.2 Sample Preparation

Chemicals were weighed out: (14mg for PFOS x1, 16mg PFOA x1). 10 mL of water was added to each tube, and the tubes were vortexed to fully dissolve the chemicals. Following one-tenth series dilution, the concentrations of PFOA 193μM, 19.3μM, 1.93μM, and 0.193μM were made. Following the same dilution factor, the concentrations of PFOS 140μM, 14μM, 1.4μM, and 0.14μM were made. All dilutions were stored at 4 degrees Celsius.

3.3 MTT (Microculture Tetrazolium Assay):

Following cell culture procedures, cells were seeded in a 96-well plate and were treated with PFOA and PFOS chemicals at various concentrations. After cell treatment and incubation, 10 μl of MTT solution was added into each of the 96 wells. After adding MTT into the wells, place the plate in the incubator at 37 degrees Celsius and 5% CO₂ for 2 hours. After 2 hours, add 80μl of DMSO (Dimethyl Sulphoxide) into all wells. Cells were incubated at room temperature for ten minutes. After 10 minutes, the plate was read using a microplate reader at 595 nanometers wavelength to measure the absorption for each sample. Outliers were removed and an average absorbance was calculated for each treatment group. Percent survival was calculated through the equation:

\[
\frac{(\text{Treatment sample average abs})}{(\text{control average abs})} \times 100 = \% \text{ survival}
\]

3.4 LDH (Cy QUANT lactate dehydrogenase) Assay:

Following cell culture procedures, cells were seeded in a 96-well plate and were treated with PFOA and PFOS chemicals at various concentrations. After treatment and incubation, two fresh 96 well plates were obtained and one was labeled as released LDH and the other as total LDH. 30 μl of media was transferred from the treated 96-well plate to the fresh 96-well plate labeled as released LDH. 30 μl of LDH substrate solution was added into each well of the released LDH plate. The released LDH plate was placed in a box at room temperature away from light for 30 minutes. In the released LDH plate, 30 μl of stop solution was added into each well to end the reaction. The plate was read in a microplate reader (Microplate Manager 6 software) and was read at 490 nanometers and 655 nanometers wavelengths. The 490nm reading gives the absorbance values related to the degree of color change as a result of the LDH reaction. The 655nm reading assesses background interference to ensure the reading is accurate. Corrected values were calculated by subtracting the 655nm reading from the 490nm readings to remove background interference.

For the total LDH, 10 μl of lysis buffer was added to the treated plate with the cells and was incubated at 37 degrees celsius and 5% CO₂ for 45 minutes. After 45 minutes, 30μl of media from the lysed cells was transferred to the fresh plate labeled as total LDH. 30 μl of LDH substrate solution was injected into the total LDH plate wells and the plate was placed in a box at room temperature away from light for 30 minutes. 30 μl of stop solution was added into each well to stop the reaction. The plate was read following the same steps as for the released LDH plate. The % cytotoxicity was calculated using the following equation:
(Treatment sample released abs − spontaneous released abs)  

(Treatment sample total abs − spontaneous released abs) * 100 = % cytotoxicity

3.5 Caspase Assay:

Following cell culture procedures, cells were seeded into a 6-well plate and were treated with PFOA and PFOS at various concentrations. The 6-well plate was incubated overnight in the incubator at 37 degrees Celsius and 5% CO₂. After 24 hours, the media was removed from each well and the cells were detached from the plate using 500μl of trypsin for 4 minutes. The trypsin was neutralized using 500μl of MEM. The suspended cells were collected in labeled microcentrifuge tubes. The tubes were centrifuged at 3000rpm for 4 minutes to obtain a cell pellet. The supernatant was decanted and a 50 μl of caspase assay lysis buffer was added to each cell pellet. The cells were homogenized in the lysis buffer and the tubes were stored at -20 degree Celsius. After sample collection, a 96 well plate was labeled with the various treatments where each sample was to be placed into four wells. 50 μl of caspase assay buffer was added into each well, then 45 μl of caspase assay lysis buffer was injected into each well. 5 μl of each treatment sample/lysed sample was inserted into their respective 4 wells. After that, 5 μl of caspase substrate solution was added into all wells. The plate was placed into a reading machine (Microplate Manager 6). The 1st reading was taken at 0 minutes, readings were taken every 15 minutes for 2 hours using the wavelength of 415 nanometers. Delta T (ΔT) was then calculated for all the readings. The average ΔT was found for all the values. To find the % change in caspase activity, the following equation was used to solve the % change in the various concentrations:

\[
\frac{\text{treatment average } \Delta T \text{ minute} - \text{control avg } \Delta T \text{ minute}}{\text{control } \Delta T \text{ minute}} \times 100 = \% \text{ change in caspase activity}
\]

Statistical Analysis:

A t-test inference procedure was performed for the MTT, LDH, and caspase assays to compare the results at the 0.05 significance level. If the p-value is less than 0.05, the data is unusual/abnormal, so it is statistically significant. If the p-value is greater than 0.05, the data is not significant. In the graphical displays in this paper, any significant sample has a black star symbol on top.

Results

4.1 PFOS and PFOA’s effect on neuronal cell proliferation (24 and 48 hours)

In the MTT cell viability assay, HTB-11 neuronal cells were used to measure the survival rates of both PFOS and PFOA chemicals after 24 and 48 hours.
Among all PFOA survival percentages, the lowest concentration of 19.3μM had the highest percent (%) reduction in survival of 30.82%. The 1.93μM concentration had a 35.02% reduction in survival, and the 0.193μM concentration had a 37.62% reduction in survival. This reveals a relationship between an increasing % reduction in survival of HTB-11 cells after 24 hours with increasing PFOA concentrations. All values were significant at the 0.05 significance level (Figure 1).

**Figure 1**
Among all PFOA survival percentages, the lowest concentration of 19.3μM had the highest percent (%) reduction in survival of 30.82%. The 1.93μM concentration had a 35.02% reduction in survival, and the 0.193μM concentration had a 37.62% reduction in survival. This reveals a relationship between an increasing % reduction in survival of HTB-11 cells after 24 hours with increasing PFOA concentrations. All values were significant at the 0.05 significance level (Figure 1).
After 48 hours, the PFOA 1.93μM concentration had the highest % reduction survival of 12.63%. The 0.193μM concentration had a 11.71% reduction survival, and the 19.3μM concentration had a 10.43% reduction survival. Overall, this data demonstrates that the % survival of neuron cells is high after 48 hours. No values were significant (Figure 2).

**PFOA Neuronal Cell Proliferation (24 and 48 hours)**

All the various concentrations of PFOA, from both 24 and 48 hour treatments, had at least a 50% survival rate. After 48 hours, the % reduction survival decreased, compared to the % reduction survival after 24 hours. (Figures 1 and 2).
Among all PFOS survival percentages, the lowest PFOS concentration of 0.14μM resulted in the highest percent reduction survival of 36.99%. Additionally, concentrations of PFOS 1.4μM had a 27.93% reduction survival, and PFOS 14μM had a 19.3% reduction survival in HTB-11 neuron cells. Contrary to PFOA % reduction survival, the % reduction survival outcome of PFOS reveals a decreasing % reduction survival with greater concentrations. All p-values were significant at the 0.05 significance (Figure 3).
After 48 hours, the PFOS 1.4μM concentration had the highest % reduction survival rate of 22.38%. The lowest concentration of PFOS (0.14μM) has a 13.11% reduction survival and the largest concentration of PFOS (14μM) has a 19% reduction survival. Only 1.4μM and 14μM concentrations were significant in their % reduction survival in HTB-11 cells (Figure 4).

PFOS Neuronal Cell Proliferation (24 and 48 hours)

All concentrations of PFOS had at least a 50% survival rate after 24 and 48 hours; this was the same conclusion with PFOA concentrations. Both PFOS and PFOA chemicals provide some evidence of a vast influence on cell proliferation in neuronal cells. (Figure 3 and 4).

4.2 PFOA and PFOS effect on LDH release

In the Cy QUANT LDH cytotoxicity assay, HTB-11 neuronal cells were used to measure the % cytotoxicity (measure of necrosis) for PFOA and PFOS chemicals after 24 hours.
Figure 5
The PFOA concentration of 0.193μM induced the highest % cytotoxicity (necrosis) of 3.35%; this is the greatest % cytotoxicity in both PFOA and PFOS findings. At 1.93μM, there’s 0.95% cytotoxicity, along with 19.3μM having a -0.18% cytotoxicity. PFOA at 0.193μM concentration is significant with a p-value of about 0.0027 at the 0.05 significance level (p<0.05). This concentration of PFOA most likely connotes the significant level of necrosis occurring in the cells. As the bars imply, there seems to be an overall decreasing % cytotoxicity with increasing concentrations of PFOA (Figure 5).

PFOA % cytotoxicity from LDH release

When compared to the control, the lowest concentration of PFOA (0.193μM) is significant. In this concentration, there is a 3.35% cytotoxicity effect. This is an unusual outcome. The PFOA (0.193μM) concentration contributes to cell damage/death, signifying toxicity to neuron cells (Figure 5).
Figure 6
PFOS 14μM concentration had the greatest % cytotoxicity of 1.85%. PFOS 0.14μM had 1.66% cytotoxicity, and PFOS 1.4μM had a 1.02% cytotoxicity in the HTB-11 neuronal cells. PFOS 14μM concentration is significant with a p-value of about 0.043 at the 0.05 alpha level (p<0.05). This concentration of PFOS indicates the significant level of necrosis occurring in the cells (Figure 6).

PFOS % cytotoxicity from LDH release

The highest concentration of PFOS (14μM) was significant when compared to control (Figure 6). As observed, the % cytotoxicity was approximately 1.85%. This concentration yields proof of cell damage/death to htb-11 cells from high PFOS concentrations; however, the lowest concentration of PFOS (0.14μM) had the second highest % cytotoxicity of 1.66% (Figure 6).

4.3 PFOS and PFOA’s effect on caspase activity (cell apoptosis)

In the Caspase assay, HTB-11 neuron cells were used to measure the % change in cell apoptosis after each treatment was added (PFOA and PFOS) after 24 hours.
PFOA 19.3μM had the highest % change in Caspase activity of 1075.85%, and PFOS 1.4μM had the lowest % change of 247.36% in Caspase activity. All p-values were significant. These results indicate that both PFOA and PFOS can stimulate at least 100% caspase activity in htb-11 neuron cells after 24 hours. It is evident that cell apoptosis levels are very high (Figure 7).

**PFOS and PFOA inducing (cell apoptosis)**

Since all % change in caspase activity was at least 100%, all concentrations of PFOA and PFOS confirmed high activity of cell apoptosis in these neuronal cells. In this display, PFOA and PFOS concentrations prove a predominant result in programmed cell death. Because percentages were so influential, PFOA and PFOS chemicals most likely harm HTB-11 cells through apoptosis (Figure 7).

**4.4 Molecular docking of PFOA and PFOS**

Molecular Docking (using PYRX)

Death Receptors website: RCSB PDB: Homepage
3D Ligand Models (PFOA and PFOS): PubChem (nih.gov)
PFOA MORT 1 Ligand

Figure 8.
Binding site of PFOA (Perfluorooctanoic acid) to the FADD/MORT 1 Death Domain from Human

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Figure 9.

Binding site of PFOA (Perfluorooctanoic acid) to the Fas/FADD Death Domain Complex

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PFOS FADD Ligand

Figure 10.
Binding site of PFOS (Perfluorooctane sulfonate) to the Fas/FADD Death Domain Complex

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PFOS MORT 1 Ligand

Figure 11.

Binding site of PFOS (Perfluorooctane sulfonate) to the FADD/MORT 1 Death Domain from Human

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Molecular docking, using PYRX, evaluated the binding locations and binding affinities of both PFOA and PFOS substances. The FADD/MORT 1 ligand was downloaded from the Protein Data Bank, and the chemical models (PFAS chemicals) were from PubChem. This data presents values measuring -5 and greater in binding affinity (kcal/mol). PFOA positioned close to the center of the receptor. PFOS ligand-target complex binding activity also showed similar affinities with the FADD/MORT 1 protein. PFOA and PFOS advance via the FADD/MORT 1 ligand of the death pathway.
Discussion

PFOA and PFOS can induce cytotoxicity cell damage/death to neuronal cells

When examining the importance of these results, the lowest PFOA concentration was found to be the most significant with a 3.35% (0.0027 p-value) cytotoxicity effect, indicating a high toxicity percentage towards neuronal cells. For PFOS, the highest concentration had a 1.86% cytotoxicity effect, significant at the 0.05 alpha level (0.043 p-value). The properties of PFOA were capable of causing cell death in neuronal cells at low concentrations. This is a big concern, because small concentrations can possibly generate unsafe reactions in humans.

PFOA and PFOS produce high levels of apoptosis in HTB-11 neuronal cells

Evidently, all concentrations of both PFOA and PFOS chemicals had at least a 100% increase in caspase activity. PFOA and PFOS chemicals give rise to high rates of apoptosis in cells, which could deteriorate distinct networks and activities of the human complex. This is another issue since all concentrations were significant at the significance level for apoptosis.

PFOA and PFOS prompt apoptosis via Fas/FADD and FADD/MORT 1 ligand pathways

Molecular docking, using PYRX, was utilized to evaluate the binding locations and affinity of both PFOA and PFOS. The two death receptors used were downloaded from the Protein Data Bank, and the chemical models (PFAS) were from PubChem. Each PFAS chemical was tested on both Fas/FADD (Death Domain Complex) and FADD/MORT 1 (Death Domain from Human) receptors.

The binding affinities (kcal/mol) of PFOA to Fas/FADD and FADD/MORT 1 proteins are essential, with values measuring -5 and greater in kcal/mol. PFOA positioned close to the center of the receptor. This was the same for PFOS to Fas/FADD and FADD/MORT 1 proteins. The ligand interaction with the death receptors convey some form of preliminary biomolecular interplay. It’s apparent that both substances have a direct role in advancing via death receptors, and should be studied more in depth.

Conclusion

In this project, PFOA and PFOS compounds did show indications of cell proliferation in neuronal cells. The greater the % reduction in survival in a concentration, the lower the % survival. This can be interpreted from figures 1, 2, 3 and 4. Nevertheless, concentrations of PFOS and PFOA highlighted the intensity of toxicity these substances had on the cells. These findings support retrospective studies on different cytotoxic targets in the human body (figures 5 and 6). All concentrations of PFOA and PFOS demonstrated the pinnacle degree of apoptosis. With over 200% increase in caspase activity of all samples, there is a clear link between cell programmed death induced by any concentration of PFOA and PFOS. To further investigate this, death receptors that were responsible for roles of protein adaptors in apoptosis, were used to bind both chemicals. These compounds were located near the center with relatively large values displayed under binding affinity (figures 8, 9, 10, 11). Thus, PFOA and PFOS show great amplification in programmed death, through the pathway of both Fas/FADD and FADD/MORT 1 death receptors. PFAS chemicals are environmentally persistent, lurking in drinking waters across the country. It’s pivotal that more attention is drawn to the intermolecular interactions of PFAS chemicals and death pathway proteins via apoptosis.
Acknowledgments

I am grateful to have worked with Wei Zhi.

References


