Perfluorinated compounds: a cause for neurodevelopmental concern in humans?

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Abstract

Many consumer products produced in the last century contain Perfluorinated compounds (PFCs). These chemicals have not been fully tested for their effect on human neuronal cells, and this study aimed to determine their toxicity levels and the cell death pathways induced by exposure.

In-vitro cell culture was used to grow neuroblastoma cells for exposure. Inhibitor studies were undertaken to determine cell death pathways and Western Blotting to identify any changes in protein levels. Fluorescent microscopy was also performed to count cell numbers.

The study concluded that both PFCs tested were toxic to human neuroblastoma cells, with LC_{50} values calculated at 733.38µM for PFOA and 292.71µM for PFDA. However the cell death pathway was not elucidated, as it appeared that both apoptosis and necroptosis may be partially occurring.

Introduction

Perfluorinated compounds (PFCs) such as Perfluorooctanoic acid (PFOA) and Perfluorodecanoic acid (PFDA) were once commonly used as processing aids in the production of Teflon® and Gore-Tex®[1]. With half-lives of around 4 years[2], they could potentially impact on human health, due to their tendencies to bioaccumulate in the environment. Quantities of these compounds have been found in human serum, as well as umbilical cord and breast milk samples worldwide[3]. Previous studies in mice have demonstrated a detrimental neurodevelopmental effect[2] of these PFCs and their ability to cross the placenta[4]. This study aimed to consider by *in-vitro* methods, whether these compounds are potential neurotoxins to developing human brain cells.

Methods

Cell culture:

Human SH-SY5Y cells were cultured in Growth Media + 10% foetal bovine serum up to a confluence of 70% before testing was done on the cells.

Toxicity study:

Cells were plated onto a 48 well plate and exposed to known concentrations of PFC or control (DMSO, Rotenone or Media) for 24 hours. 35µL of Alamar blue was added to each well and left to develop for 3-4 hours. A 96 well plate was then created and the fluorescence read at 530/ 590nm using a plate reader.

Inhibitor study:

Cell were plated onto 48 well plates then exposed to a combination of PFC and inhibitor for 24 hours. Alamar blue was added and left to develop, the plates were then analysed as in the toxicity study.

Western Blotting:

Cells were exposed to PFCs for 2, 4, 8, and 24 hours and DMSO for 24 hours, to observe the effect of exposure time on the levels of cellular proteins. The proteins observed were Poly ADP-Ribose Polymerase (PARP) an inducer of apoptosis, Inhibitors of Apoptosis (IAPs), LC3B a protein involved in autophagy, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control.

Fluorescent microscopy:

SY5Y cells grown on microscope slides were exposed to PFDA and inhibitor for 24 hours. The slides were fixed using formaldehyde, and dipped in DAPI. Glycerol solution was used to fix the cover slip to the slide. The slides were then visualised under a fluorescent microscope, and the cells counted.

Statistical analysis:

Statistical differences were analysed for using a Holm-Sidak analysis, using the SigmaPlot software package. Western Blotting images were analysed using Image-J.

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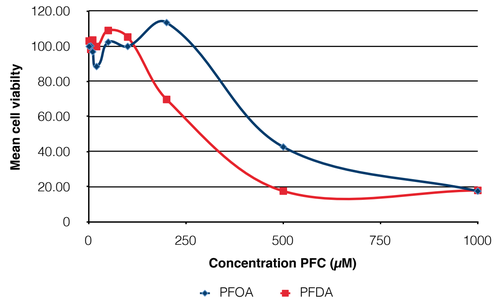
Results: Toxicity study

Statistically significant differences were found between 200 and 500µM for PFOA, and 100 and 200µM for PFDA, suggesting the limit of toxicity lies within these concentration ranges. This also suggests that PFDA is more toxic than PFOA, as the concentration at which cells die is lower. This was also shown by LC_{50} values of 733.38µM for PFOA and 292.71µM for PFDA.

Figure 1: PFC toxicity curve



Results: Inhibitor study

PFC and z-VAD-FMK

The addition of the apoptosis inhibitor z-VAD-FMK appeared to have no effect on cell viability. The lack of an increase in cell viability suggests that apoptosis is not occurring upon PFC exposure.

PFC and N-acetylcysteine

The addition of N-acetylcysteine, an inhibitor of oxidative stress, also appeared to have no effect on the viability of cells exposed to PFCs. The lack of increase in cell viability suggests that oxidative stress is also not the mechanism by which cells are dying after PFC exposure.

PFC and Necrostatin 1

Figure 2: PFOA and Necrostatin 1

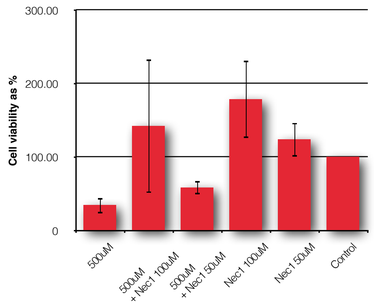
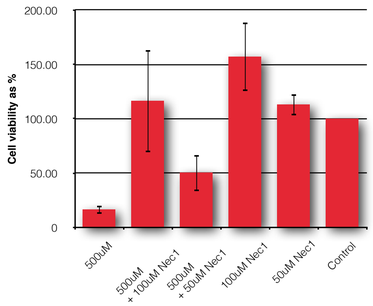


Figure 3: PFDA and Necrostatin 1

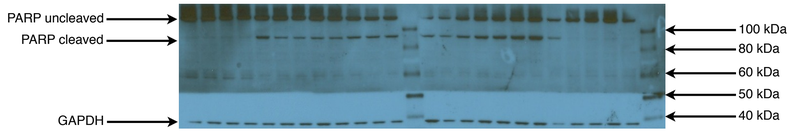


Figures 2 and 3 show the effect of Necrostatin 1 (Nec1), an inhibitor of necroptosis, on the viability of PFC exposed cells. These results suggest that there is an increase in cell viability when Nec1 is added to the cells. When PFC alone is added, the cell viability is around 20%. However upon the addition of Nec1 along with PFC cell viability returned to 100%, often surpassing this value and reaching 150%. This therefore suggests that the addition of the inhibitor is preventing cell death from occurring by blocking the necroptotic pathway.

Results : Western Blotting

Analysis of the Western Blot, Figure 4, showed an increase in the levels of cleaved Poly ADP-Ribose Polymerase (PARP), a marker of caspase dependent apoptosis, until 8 hours after PFOA exposure. This contradicted the findings of the inhibitor study, in that this suggests that apoptosis and/or DNA damage occur upon PFC exposure.

Figure 4: Western Blot of PFOA, showing PARP and GAPDH

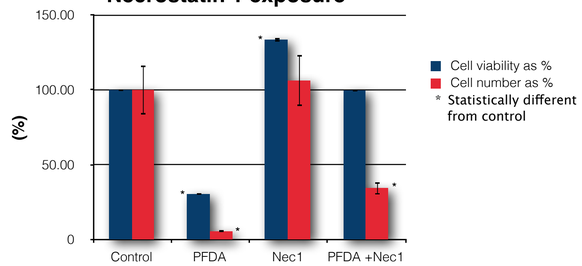


Levels of LC3B, IAPs, were also analysed and showed no statistical change in protein levels.

Results: Fluorescent microscopy

Figure 5 shows that cell death occurs after PFDA exposure (red), with cell numbers falling to 5% of the initial number. Upon the addition of Nec1, the cell number does not return to 100%, suggesting that although the necroptotic pathway may be occurring, it is not the only cell death pathway occurring.

Figure 5: Cell viability and number upon PFDA and Necrostatin 1 exposure



This contradicts the finding of the Nec1 inhibitor study (blue), which indicated a return of cell viability to 100% upon the addition of Nec1 and complete inhibition of cell death.

Nec1 appears to affect PFC exposed SY5Y cells, but not by eliminating cell death as suggested in Figures 2 and 3, but by possibly interfering with mitochondrial mechanisms. This highlights the need for further testing in the area and the development of new experimental methods to be used for the identification of the necroptotic pathway.

Conclusions

PFCs such as PFOA and PFDA are a cause for concern for human health, as both compounds were found to be toxic to human neuroblastoma cells. PFDA could be considered the more toxic of the two compounds as it has a lower LC_{50} value of 292.71µM, compared to that of PFOA of 733.38µM.

Oxidative stress was found not to be the cell death pathway induced by PFCs. The inhibitor studies and Western Blotting appear to show that the necroptotic and apoptotic pathways may partially occur upon PFC exposure, but neither appears to be the sole pathway of cell death. Therefore further testing needs to be carried out to establish the full death pathway.

References

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