Perfluorooctanoic acid exposure and its neurodegenerative consequences

Azadeh Khosravi  
Isfahan University of Medical Sciences

Mahboobeh Kafi Mousavi  
Isfahan University of Medical Sciences

Masoud Soltani  
Isfahan University of Medical Sciences

Mehdi Aliomrani  
Isfahan University of Medical Sciences

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Abstract

Perfluorooctanoic acid (PFOA) is a member of Per- and polyfluoroalkyl substances (PFASs), an industrial pollutant that has been produced for decades and widely used in various industries. Accumulation of this compound in the environment and body of organisms led to increased concerns about this compound. The toxic effects of PFOA on the nervous system are unknown, yet. We aimed to assess the myelination and neurogenesis in brain tissue.

Method and Materials:

In this study, PFOA at doses of 1, 5, 10, and 20 mg/kg were injected intraperitoneally into C57BL/6J mice for 14 days, and the myelin content, CD4+ and CD8+ cell infiltration to brain regions were evaluated. Also, bromodeoxyuridine (BrdU) labeling was performed to compare neurogenesis among the groups.

Results

The LFB stating revealed a significant decrease in myelin content in both gender at high concentrations (p < 0.001). The impaired neurogenesis was observed in both genders especially females which was highly related to the dose and region of the brain. The infiltration rates of CD4+ and CD8+ cells to the brain were shown to be decreased meanwhile the lymphocyte count was not significantly changed among groups over time and vice versa for the monocyte and neutrophils.

Conclusion

Our results showed that PFOA had a negative impact on neurogenesis and the myelination process through the specific region of the brain depending on the dose and gender. Also, PFOA could disturb the number of CD4+ and CD8+ cells infiltrating the brain, which plays a crucial role in neurogenesis, leading to toxicity and neurological abnormalities. It seems that more research is needed to determine the exact mechanisms of PFOA neurotoxicity and its long-term behavioral consequences.

Introduction

Per- and polyfluoroalkyl substances (PFASs) are a class of manufactured chemicals in which perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) are the most abundant. Because of its stability and surface tension, PFOA is widely used in various industries and products (1–3). This group of chemicals includes about 4,000 compounds that are utilized in items such as household appliances, paints, waxes, and cleansers. Between 2001 and 2014, an average of 250 tons of PFOA were produced every year, peaking at 460 tons in 2007. Although many countries, including the United States,
have discontinued producing these chemicals, others, such as India and China, continued to use them yet (4–6). Because of its significant bioaccumulation and endurance due to carbon-fluorine bonding, this chemical has sparked widespread worry about its potential toxicity, especially after finding them in blood and breast milk (7). Several studies confirmed the PFASs residue in breast milk and shed a light on the possible infant toxicity (8).

Reduced fetal growth, immune system toxicity, genetic/epigenetic alteration, and decreased hepatic-renal balance have all been linked to the accumulation of these chemicals in the body. Surprisingly, the majority of the data acquired in this area suggests a link between PFOA and various malignancies (7, 9). The influence on the immune system is the most concerning of these side effects, as a malfunction of this defense mechanism is the root of many diseases. Many of these chemicals can cause immune system problems, even at very low amounts (9, 10). Following PFOA exposure, it has been demonstrated that the immune system was suppressed, and lymphocyte functions, as well as cytokine expression, were decreased (13).

The dentate gyrus (DG) is a part of the brain where neurogenesis takes place and continues to a degree throughout adulthood (19, 20). Numerous studies have demonstrated the importance of immune system activity in hippocampus neurogenesis, as well as the fact that immune system suppression affects neurogenesis (20, 21). Microglia are myeloid cells in the central nervous system that have been associated with Alzheimer’s disease and have been shown to decrease neurogenesis in DG when activated (20). In addition to microglia, CD4 + and CD8 + cells are involved in the neurogenesis process. Reduced CD4 + T lymphocytes inhibit neurogenesis in the hippocampus and diminish brain-derived neurotrophic factor (BDNF) expression (22). CD4 + T lymphocytes play a critical role in the plasticity of the brain. Deficiency in these cells causes memory and learning problems, as well as a reduction in long-term depression (LTP) (23, 24).

In this study, we investigated the relationship between PFOA exposure and the rate of CD4 + and CD8 + cell infiltration into the brain and the amount of neurogenesis in subventricular zone of C57BL/6J mice in the light of recent increasing contact with PFOA-containing contaminants and their effects on the immune system or probable neurotoxicity.

**Method And Materials**

**Animal**

In this study, 8 week-old C57BL/6J mice (60, both sexes, weight: 20-30 g) were obtained from the animal nest of the School of Pharmacy and Pharmaceutical Sciences, Isfahan, Iran, and housed at 22 ± 5 °C on a 12 hours light: 12 hours dark cycle and kept for three days in the research laboratory of the Department of Pharmacology and Toxicology in a propylene cage to get used to the environment with free access to water and food. The animal experimental procedures and protocols were accepted by Iran National Committee for Ethics in Biomedical Research (IR.MUI.RESEARCH.REC.1400.025). They were then sorted into six groups of five randomly. For 14 days, groups 1 to 4 were given PFOA at doses of 1, 5, 10, and 20
mg/kg intraperitoneally. Dexamethasone was given to Group 5 at a dose of 0.1 mg/kg. PFOA vehicle (distilled water and tween) were given to Group 6 as the vehicle group. All the mice were euthanized by inhaling CO2 gas at the end of the experiment.

**Myelin content**

After euthanasia, brain tissues were fixed in 10% formalin for 48 hours and coronally sectioned through the mid-brain region based on the Allen Mouse Brain Atlas. The myelin content in the median corpus callosum was measured using Luxol Fast Blue (LFB; Sigma) solution. Briefly, deparaffinized slides were subsequently immersed in an alcohol gradient and then immersed for 24 hours at 60 °C in a 0.1 percent LFB solution. The slides were counterstained, cleared with xylene, and mounted after immersing in 0.05% lithium carbonate solution and 70% alcohol. Finally, the slides were imaged by Nikon Eclipse 55i bright-field microscope (Nikon, Japan), and the images were quantitatively evaluated by Image J software.

**BrdU Immunofluorescent imaging**

Slides were dewaxed in xylene and rehydrated, then subjected to acid hydrolysis using 2 M HCL in 0.1% PBS-Tween for 20 min at room temperature to denature the DNA. They were then permeabilized with 0.1% Triton X-100 for 10 min and blocked with 1% BSA/ 10% normal goat serum/ 0.3 M glycine in 0.1% PBS-Tween for 1 h. The slides were then incubated overnight at 4 °C with a 1/100 dilution of the Anti-BrdU antibody (Alexa Fluor® 488) and quantified using a fluorescent microscope (excitation 495 nm, emission 519 nm). Imaging and quantification images were taken using a Nikon Eclipse 55i fluorescent microscope (Nikon, Japan) with a 40x objective magnification. Stained cells were quantified with NIH Fiji software (National Institute of Health, Bethesda, Maryland, USA) and expressed as cells/mm². All of the mentioned quantifications were done on three slides for each brain sample and analyzed in a blinded coding system to omit any disturbance from confounding factors.

**CD4+ and CD8+ infiltration**

First, the prepared samples with an 8 µm thickness were deparaffinized at 80°C for 20 minutes. After that, they were immersed in xylene three times for 20 minutes each, followed by an alcohol gradient. Slides were placed in Tris-EDTA buffer (pH = 9.1M, TE buffer) in the TintoRetriever Pressure Cooker for 10 minutes to improve tissue permeability and antigen recovery. The primary antibodies (Mouse monoclonal anti-CD4 (SP35) and mouse monoclonal anti-CD8 (SP16)) were incubated for 1 hour at room temperature as directed by the supplier (Abcam, UK), then rinsed in PBS buffer and blocked with 0.03 percent hydrogen peroxide solution. Finally, a substrate of 3,3'-diaminobenzidine was employed. The samples were dewatered and montaged after being stained with hematoxylin. The slides were obtained at a magnification of 40 with a Nikon Eclipse 55i bright-field microscope (Nikon, Japan). Color deconvolution plugin was used to segregate colored and unpainted cells, then the total number of cells was measured and the data were reported as mean±SEM.

**Blood Cell counting**
Blood was taken from the retroorbital route using a capillary tube (Fisher, St. Louis, Mo) on day 0 (at the start point of the study before injection), 7, and 14 (after the last exposure) and placed in vacationer tubes containing Ethylene diamine tetra acetic acid (EDTA). The Sysmex XT-400i automated hematology analyzer was used to count white blood cells.

**Statistical Analysis**

GraphPad Prism 8 software was used to analyze the data. The normality of the data was determined using the Shapiro-Wilk test. Statistical differences in CD4+ and CD8+ cell count, neurogenesis, and myelin percentage were examined using one-way ANOVA (Turkey's post hoc test, p < 0.05) and blood cell count using two-way ANOVA (Tukey's multiple comparison test) and were considered statistically significant (*/# p<0.05, **/## p< 0.01, ###/#### p<0.001). The data was provided as Mean±SEM.

**Results**

**Weight**

The relationship between weight changes and the time of exposure was evaluated using Pearson's r, and it was observed that group received 20 mg/kg PFOA lost their weight during the study in both gender especially females (r:-.096, P<0.0001 vs, r:-0.85, P<0.001). Interestingly male mice received PFOA at doses 5 and 10 mg/kg lost their weight after two weeks of exposure (P<0.001 and <0.05, respectively). Although weight loss was not statistically significant in such administered doses in Female groups. Comparison of the groups based on the average food intake per day (g/g BW) was not statistically significant (p-value >0.05).

**Myelin content**

The density of myelin in the median part of the corpus callosum was examined using LFB staining. This staining revealed a decrease in myelin density in males at high concentrations. The PFOA (10) and PFOA (20) groups are considerably lower than the vehicle group, but the other groups did not differ significantly as compared with the control group. There was a significant difference in the dose of 20 mg/kg/day compared to the groups of 1, 5 mg/kg/day, and DEXA in the analyzed groups. Furthermore, the 10 mg/kg/day group was significantly different from the 1, 5 mg/kg/day and DEXA (like the 20 mg/kg/day group). However, no significant differences were observed in other groups. All PFOA groups showed significant changes compared to control and DEXA groups after LFB staining in the Female sex. Furthermore, the 20 mg/kg/day dose was substantially different from the 1, 5, and 10 mg/kg/day doses. There was no statistically significant difference.

**Neurogenesis**

With an increased dose of PFOA, a reduction in neurogenesis was found after labeling especially in female corpus callusom compared to the subventricular zone region. This type of reduction was higher in
subventricular zone areas in male groups by increasing the dose of exposure. Except for the PFOA 1 mg/kg group, higher doses decreased the number of BrdU positive cells in comparison to the vehicle group. This type of reduction shows that it is region and gender-dependent but it is not dose-dependent between groups of study.

With higher dosages of PFOA, a decrease in neurogenesis was found in the cells of the male corpus callosum, which were marked with BrdU in the same way as females. The PFOA (1) group differed considerably from the PFOA (20), PFOA (10), and PFOA (5) groups, while the PFOA (5) group differed only from the Vehicle group; the PFOA (20) and PFOA (10) groups differed significantly from the Vehicle and DEXA groups. Between the other groups, there was no statistically significant difference.

In the SVZ region, males labeled with the BrdU PFOA group (20) displayed noteworthy differences with PFOA (1), DEXA, and the Vehicle groups. While the PFOA (5) and PFOA (10) groups were significantly different only from the Vehicle and DEXA groups.

**CD8+ and CD4+**

Exposure to PFOA lowered the number of CD4+ and CD8+ lymphocytes in brain tissue. After counting CD8+ cells in females, the PFOA (1) group showed a significant difference with PFOA (10), PFOA (20), and DEXA groups, but not from the Vehicle group or the PFOA (5) group. The PFOA (5) group differed significantly from the PFOA (10), PFOA (20), and DEXA groups. Furthermore, the Vehicle group showed significant differences in PFOA (10), PFOA (20), and DEXA, but these groups were not significantly different from each other. The vehicle group differed significantly from the other groups in males. Only the PFOA group (20) revealed statistically significant differences between DEXA and PFOA (1) groups when the groups were compared.

Male CD4+ cell counts revealed that the PFOA (1) group was significantly different from the PFOA (5), PFOA (10), PFOA (20), and DEXA groups; the PFOA (5) group, on the other hand, was statistically distinct from the DEXA, PFOA (10), and PFOA (20) groups. This statistical difference was seen in all of the PFOA groups as well as the carriers. The PFOA (10) group was considerably different from the other groups in terms of females. Furthermore, there were substantial differences between the PFOA (1) group and DEXA. The other groups, on the other hand, did not exhibit a significant difference.

**Blood cell**

On days 0, 7, and 14 after the injection, we looked at the impact of PFOA on different subpopulations of white blood cells (WBCs). The number of neutrophils in PFOA (20) was substantially different in both sexes (p < 0.05) in the second week (comparison of days 7 to 14), but there was no significant difference in the first week (comparison of days 0 to 7). Furthermore, the number of neutrophils in other male groups did not differ statistically. In PFOA (10) in the first and second weeks, there was no significant difference in females, but the number of neutrophils on day 14 was considerably different (p < 0.05) from the first day. The number of neutrophils on days 0 and 7 compared to day 14 of injection at a dose of 5
mg/kg/day (p < 0.01 and p < 0.001), equivalent to DEXA (p < 0.01 and p < 0.05), indicated a statistically significant difference. During the injection period, however, the number of neutrophils in PFOA (1), which was similar to the Vehicle, did not alter.

There was a significant difference in the number of lymphocytes in males in the second week (p < 0.05) after injection but no significant difference in females during the same period. In females, the number of monocytes in doses 5 (p < 0.001) and 20 (p < 0.0001), similar to DEXA (p < 0.0001), revealed a significant difference in the first week and during the period, whereas the dose 1 mg/kg/day only represents a substantial difference in the second week (p < 0.05). Only the DEXA group displayed a significant difference in the second week in males (p < 0.05), whereas the other groups showed no difference.

**Discussion**

The neurological system and brain tissue are two of the most critical sections of the body, and even small levels of pollutants like PFOA and PFOS can cause permanent nerve damage in the long run. In this study, we aimed to investigate how these chemicals affected CD4+ and CD8+ cell infiltration, myelination, and neurogenesis in different areas of the brain that were exposed to them. Previous research on the effects of these chemicals on body weight has yielded mixed results. As stated in the results, PFOA induced weight loss, confirming the findings of prior investigations(25). However, several prior studies have found that high dosages of PFOS cause weight loss(26). Exposure to PFASs can cause endocrine disruption, which can lead to weight loss(27). Furthermore, these chemicals can disrupt weight management by acting on hormone receptors involved in metabolism and weight control (alpha receptors triggered by the peroxisome proliferator)(28).

Calcium (Ca) plays a key role in brain functions as a cellular messenger. Memory and learning can be harmed by a disruption in Ca homeostasis. Ca influences synaptic ductility and flexibility, as well as the signal transduction route and neurotransmitter release(29). In previous studies, Purkinje cells and Ca homeostasis in hippocampus neurons have been demonstrated to be affected by PFOA and PFOS(30). Furthermore, PFOS can disrupt the Ca membrane flow of the cell membrane and cause neurotoxicity via modifying the levels of norepinephrine in the rat brain(31). The Ca signaling cycle can also be influenced by glutamate. The neuron-astrocyte cycle uses glutamate as the most abundant neurotransmitter in processes like synaptogenesis and synaptic plasticity(32). By disturbing calcium homeostasis, PFASs decrease neurite development and impede synaptogenesis in the hippocampus. Previous research has found calcium to play a role in oxidative damage. Neurotoxicity is caused by abnormal calcium balance and neurotransmitters. In studies, PFASs have been found to induce neurotoxicity in neurons through oxidative damage and apoptosis(16, 33, 34). PFASs can cause neurotoxicity by interfering with calcium homeostasis and related signaling pathways, synaptogenesis, neurotransmitters, and cell death(35).

In this study, it was observed that high doses of PFOA diminish myelin content in the brain as well as neurogenesis. In the long run, this alteration in neurogenesis could contribute to neurodegenerative diseases. Previous research has demonstrated that PFOA has been demonstrated to be hazardous to the
developing nervous system(36). These chemicals are thought to inhibit myelination and neurogenesis by acting on various regions and pathways involved in myelination and neurogenesis.

Memory, learning, and motor function are all affected by PFASs(37). Blood-brain barrier (BBB) problems and brain malformations result from exposure to chemicals in this group. Furthermore, the findings revealed that PFOS damages the nervous system and neurons by causing breakage and swelling of the myelin sheath in neurons(38). It was suggested that high levels of PFAS in maternal blood have been linked to a decrease in cerebellar and frontal brain volume in adolescents(39). Thyroid hormone imbalances can also influence myelin oligodendrocyte synthesis, radial glial cell maturation in the hippocampus' CAI area, and cerebellar astrocytes(40). PFOA can damage memory by affecting synapse density and distorting the hippocampus(41).

Persistent organic pollutants (POPs) including PFOA and PFOS exposure could impair thyroid hormone signaling, which inhibits the nerve growth. Thyroid hormones play a critical role in the differentiation of oligodendrocytes and the myelination process. Previously it was observed that polychlorinated biphenyls (PCB), polybrominated diphenyl ether (PBDE), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) have all been linked to lower myelin levels in the corpus callosum as well as re-myelination dysfunction(42).

A previous study demonstrated decreased B and T cells by feeding PFOA and PFOS (w/w 0.02%-0.001%) to C57BL/6J male mice over 10 days. Leukopenia and lymphopenia were observed in the presence of PFOA and PFOS due to thymus and spleen shrinkage, but the decrease in neutrophils was mediated solely by PFOA(43–45). By activating PPAR, PFASs may decrease T-cell-dependent antibody response (TDAR) and T-cell-independent antibody response (TIAR)(46). Furthermore, Dewitt et al. (2008) found that when the immune system was exposed to PFOA concentrations greater than 3.75 mg/kg/day, IgM antibodies were reduced (for 10–15 days)(47).

The infiltration rates of CD4 + and CD8 + cells were shown to be lower in the study, which is consistent with earlier research. Different species and strains are more or less susceptible to PFOA toxicity, and the dose and period utilized can have an impact on the results(48–50). By accumulating PFOA in the spleen and lowering the immune system's resilience, PFOA and PFOS have previously been proven to have severe suppressive effects on the immune system(48).

T cells have been seen to impact brain function, including memory and learning. Because these substances are involved in the response of microglia, these cells play a significant role in brain plasticity, cognitive abilities, and hippocampus neurogenesis. Researchers have shown a relationship between cell proliferation in the hippocampus and the ratio of CD4 + to CD8 + cells in prior investigations employing genetic approaches. Reduced or suppressed CD4 + T cells diminish hippocampal neurogenesis, memory and learning impairment, and cognitive impairment(22, 23, 51).

Glial cells, such as astrocytes and microglia, play a vital role in a variety of neurological disorders. Astrocytes have a role in the development of the nervous system and neuronal connections, as well as in support of neurons, detoxification, and the healing of damaged tissue. However, pathological situations
can cause astrocytes to become activated and release inflammatory cytokines, harming neurons and contributing to nerve damage. By producing cytokines like TNF-a and 1L-1B, TCDD can induce neurological problems. TNF-a is a cytokine that, in addition to being involved in inflammatory responses in the CNS, is also involved in synaptic development and cognitive function. On the other hand, over-activation of this cytokine might result in nerve injury. Furthermore, PFOS has been shown to affect the rat hippocampus by activating astrocytes and releasing inflammatory chemicals(52, 53). In previous research, PFOS has been demonstrated to impact neurons and microglia, as well as generate inflammatory responses(53).

Microglia-induced inflammation and oxidative stress can harm healthy tissue. According to a previous study, microglia produce NO and ROS after exposure to these substances. Furthermore, through signaling to astrocytes and escalating inflammatory reactions, microglia produce an increased buildup of neurotoxins and the death of nerve cells and neurons(38, 54–56). Because neurons require a lot of energy, mitochondria have been found to play a role in brain illnesses. PFOA can disrupt brain and liver function by acting on mitochondria, creating reactive oxygen species (ROS), and activating apoptotic cell pathways(57, 58).

Moreover, numerous environmental compounds with similar qualities to Aryl hydrocarbon receptor (AhR) can impact AhR. AhR can influence neurogenesis. TCDD inhibits neurogenesis by interacting with AhR(59–62). Aromatic hydrocarbons (TCDD, PCB) can also affect the brain by interfering with thyroid hormones involved in myelination. According to previous research, TCDD exposure during pregnancy has been shown to impair the production of adult oligodendrocytes and astrocytes, as well as affect myelin repair potential in adulthood. Some of these brain alterations and problems last into adulthood. The cerebellum is affected by TCDD and PCB exposure, resulting in a variety of neurobehavioral abnormalities (63).

**Conclusion**

Our results showed that PFOA had a negative impact on neurogenesis and the myelination process through the specific region of the brain depending on the dose and gender. Also, PFOA could disturb the number of CD4 + and CD8 + cells infiltrating the brain, which plays a crucial role in neurogenesis, leading to toxicity and neurological abnormalities. It seems that more research is needed to determine the exact mechanisms of PFOA neurotoxicity and its long-term behavioral consequences.

**Declarations**

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**Conflicts of interest/Competing interests:**
All authors declare no conflict of interest.

**Ethics approval:**

All procedures were approved by the Iran National Committee for Ethics in Biomedical Research (IR.MUI.RESEARCH.REC.1400.025) which was performed in accordance with the Guidelines for the Care and Use of Laboratory Animals.

**Author's contribution**

AK and MAO conceived of the presented idea, AK and MK carried out the experiment, MS processed the results and prepared the manuscript, MAO supervise the project, all authors provided critical feedback and helped shape the research, analysis and manuscript.

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Figures

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