Epigenetic toxicity and cytotoxicity of perfluorooctanoic acid and its effects on gene expression in embryonic mouse hypothalamus cells

Hun Kim, Min-Wook Hong, Yun-ho Bae, and Sung-Jin Lee

Kangwon National University College of Animal Life Sciences, Chuncheon, Korea

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Even though the endocrine-disrupting potential of perfluorooctanoic acid (PFOA) is well known, the mechanisms underlying its cellular and epigenetic toxicity at the critical stage of hypothalamic development are poorly understood. This is why we studied its effects on the embryonic mouse hypothalamic cell line N46 (mHypoE-N46) with a hope to shed more light on the mechanisms through which PFOA causes embryonic hypothalamic cell damage. To do that, we studied cell viability, global DNA methylation, and gene expression in cells exposed to PFOA. As the PFOA dose increased, cell viability decreased, while global DNA methylation increased. PFOA also significantly altered the expression of genes related to the apoptosis and cell cycle, neurotrophic genes, and the Tet, Dnmt, and Mecp2 genes. Our findings suggest that exposure to PFOA affects cell survival through the reprogramming of embryonic hypothalamic DNA methylation patterns and altering cell homeostasis genes. DNA methylation and changes in the Mecp2 gene expression induced by PFOA also imply wider ramifications, as they alter genes of other major mechanisms of the embryonic hypothalamus. Our study may therefore serve as a good starting point for further research into the mechanisms of PFOA effect of hypothalamic development.

KEY WORDS: cytotoxicity, DNA methylation, endocrine-disrupting chemicals, epigenetic toxicity, mHypoE-N46 cell line, PFOA

Perfluorooctanoic acid (PFOA) is a synthetic surfactant used in various industrial and consumer water- and oil-resistant products with the annual production of several hundred metric tonnes since the 1950s, which makes it one of the most abundantly produced endocrine-disrupting chemicals (EDCs) worldwide (1). Its strong carbon-fluorine bond renders it highly stable and prone to bioaccumulation in the environment (2). It can easily be found in water, food, house dust, stain-resistant carpets, cookware coatings, and industrial waste (3–6). This indicates a high risk of exposure, which has been confirmed by its discovery in human blood, breast milk, and umbilical cord serum (7–11). In addition, with its average half-life in humans of 2.4 years (12), PFOA has a potential of high bioaccumulation.

Exposure to EDCs such as PFOA during early development can have lifelong detrimental effects on metabolic homeostasis and endocrine physiology (13, 14). However, while studies demonstrate that development of the embryonic hypothalamus can be disrupted by PFOA, the underlying molecular mechanisms for the metabolic toxicity of PFOA are not yet clear. These involve epigenetic covalent modifications of histone tails (15, 16), small non-coding RNAs that regulate gene expression (17), and DNA methylation (18–20). Modifications in DNA methylation patterns lead to developmental reprogramming, and these epigenetic changes can be inherited through cell division (21). In addition, transgenerational exposure to EDCs can affect embryonic cell development through genomic imprinting (22). Previous research has shown that PFOA exposure induces changes in global DNA methylation in human umbilical cord serum (23), mouse embryonic fibroblasts (24), and human hepatocellular carcinoma cells (25), but there have been no studies on the effects of PFOA on global DNA methylation in mouse embryonic hypothalamic cells.

To fill this gap, our study focused on epigenetic toxicity and cytotoxicity of PFOA in mHypoE-N46 cells and its effects on gene expression and how it affects apoptosis, cell cycle, proliferation, and neurotrophic genes. In addition, it looked into its effects on gene modifications of ten-eleven translocation methylcytosine dioxygenases (Tets), DNA methyltransferases (Dnmts) and methyl-CpG binding protein 2 (Mecp2s).

MATERIALS AND METHODS

Cell culture and reagents

The mHypoE-N46 (Cellutions Biosystems, Burlington, Canada) cell line, derived from the mouse embryonic hypothalamus was immortalised by retroviral transfer of
SV40 T-antigen into embryonic mouse hypothalamic primary cell cultures (days 15, 17, and 18) (26). The cells were cultured as a monolayer in Dulbecco’s modified Eagle medium (DMEM) with 25 mmol/L glucose (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 % foetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and 1 % penicillin/streptomycin (Welgene, Daegu, Korea). The cells were incubated at 37 °C in 5 % CO₂.

PFOA (Sigma-Aldrich) at final concentrations with a 10-fold difference from 0.25 to 250 µmol/L was dissolved in dimethyl sulphoxide (final concentration 0.1 %; Sigma-Aldrich). Dimethyl sulphoxide (0.1 %) was not toxic to the mHypoE-N46 cells and had no effect on cell survival or ability to divide (data not shown). To eliminate the effects of steroids contained in DMEM and FBS, PFOA treatment was performed in phenol red-free DMEM with 25 mmol/L glucose (Gibco), supplemented with 1 % charcoal-stripped FBS and 1 % penicillin/streptomycin.

Assessment of cell viability

The cells were seeded at 5x10³ cells per well in 96-well plates with 200 µL of fresh complete medium and incubated at 37 °C in 5 % CO₂ for 24 h. Cell culture medium (200 µL) was then replaced, and PFOA (at final concentrations of 0, 0.25, 2.5, 25, and 250 µmol/L) was added and incubated for 24 or 48 h. The CellTiter 96™ non-radioactive cell proliferation assay kit (Promega, Madison, WI, USA) was used according to the manufacturer’s protocol. The absorbance was read at 570 nm using an enzyme-linked immunosorben assay (ELISA) microplate reader (Molecular Devices, San Jose, CA, USA). The measured optical density values were normalised and cell viability analysed with the GraphPad Prism Software 7.0 (GraphPad Software, San Diego, CA, USA).

RNA isolation and quantitative real-time PCR

Neurons were harvested and total RNA extracted using the TRizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA purity and concentration were quantified with the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative real-time PCR was performed using the iTaq™ Universal SYBR® Green One-Step kit (Bio-Rad, Foster, CA, USA) with gene-specific primers (Table 1). Data were analysed using the 2-ΔΔCT method and transcript levels were normalised to those of the standardisation gene (β-actin).

Global DNA methylation assay

Genomic DNA from mHypoE-N46 was extracted immediately after PFOA treatment using the G-spin™ Total DNA Extraction Kit (iNtRON Biotechnology, Seongnam, Korea). Total DNA quantity and quality were tested with the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). The whole-genome DNA methylation profile was determined using the EpiGentek MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (Epigentek, Farmingdale, NY, USA) following the manufacturer’s protocols.

Statistical analysis

Results are presented as mean ± SEM (standard error of the mean) and analysed with the GraphPad Prism Software 7.0. Statistical significance at p<0.05 was determined with one-way ANOVA, followed by a Bonferroni post hoc test. For the calculation of the half maximal inhibitory concentration (IC₅₀), we relied on the Quest Graph™ IC₅₀ Calculator program from the AAT Bioquest website (27).

RESULTS

Cell viability

The MTT analysis showed a drop in cell viability in a dose-dependent manner in both 24 and 48-hour PFOA treatment (Figure 1). No statistically significant differences were found between the PFOA concentrations of 0.25 µmol/L and 2.5 µmol/L, but they started to show with 25 µmol/L (p<0.01) and 250 µmol/L (p<0.001). As 48-hour PFOA treatment showed little difference from the 24-hour treatment, all subsequent experiments were done in cells cultured with PFOA for 24 h. IC₅₀ for 24-hour exposure to PFOA was 27.5 µmol/L. Gene expression experiments were therefore performed on cells treated with 2.5 µmol/L and

Figure 1 Cell viability of mHypoE-N46 cells following PFOA treatment for (A) 24 h and (B) 48 h (MTT assay). Data are shown as mean ± standard error of triplicate measurements. ** p<0.01 and *** p<0.001 compared to control.
| Gene     | Primer sequence (5’→3’)                  | Amplicon size |
|----------|------------------------------------------|--------------|
| Bax      | Forward: TGCAGAGGATGATGTTCTGGCA          | 173 bp       |
|          | Reverse: GATCAGCTCGGACACTTTAG            |              |
| Casp3    | Forward: CTGCCGGAGATCTGACTGGAA           | 97 bp        |
|          | Reverse: ATCGATCCCCTGTCTCTGAAATG        |              |
| Trp73 (p73) | Forward: AGCCTTGTGTTGACTCTCTACG    | 119 bp       |
|          | Reverse: ACCACGGTGTTACCTTGGTA          |              |
| Censa2   | Forward: ACCTGCTCCACTCATTTGCT           | 139 bp       |
|          | Reverse: TGGTGAAAGGTCCACAAGACA         |              |
| Ccnb1    | Forward: CAGTTGTTGCGCCAAGAAGA           | 216 bp       |
|          | Reverse: CTACGGAGGAAAGTGCAAGGA          |              |
| Ccne1    | Forward: CACCACCTGAGTCTCCAGAA           | 230 bp       |
|          | Reverse: CTGGTGCTGACAGTGGAAGGA          |              |
| Cdkn1a   | Forward: GGGATGGCGAGTTAGGGGAA           | 244 bp       |
|          | Reverse: GTGGGGCAAGATGCCTGATGA          |              |
| Cdk4     | Forward: CAATTGTGTACGGCTGTGGG          | 178 bp       |
|          | Reverse: CAGGCCGCTTAGAAGACGTA           |              |
| Rps6     | Forward: GTCCGCCGATATGTTGTGCA           | 103 bp       |
|          | Reverse: GTGCAAGGACAGGAGGTA            |              |
| Kitl     | Forward: TCATGCTGACCGTATCTTA           | 170 bp       |
|          | Reverse: CTTTGCCACGGTCTTCTGACT         |              |
| Bdnf     | Forward: GTATCCAAAGGCCAAGTA            | 183 bp       |
|          | Reverse: TTATGAATACGCGCAGCAAT          |              |
| Nrk2 (TrkB) | Forward: CCGCCTGAGCTCTGAGACTCTG      | 171 bp       |
|          | Reverse: CTGCCTTACTGGCTCGGAC           |              |
| Nrk1 (TrkA) | Forward: GAGGTCTCTGCTCAAGGTGCA          | 131 bp       |
|          | Reverse: TGGAAACCAAGGCGCAGA            |              |
| Ngf      | Forward: CAGACCCGGCAACATCAGTGTA        | 131 bp       |
|          | Reverse: CCATGCGGCCTGAGGCTTAG          |              |
| Tet1     | Forward: CGGAGAACAGCCAACAGAT           | 219 bp       |
|          | Reverse: TTGGTTCCTTCTCCCCATG          |              |
| Tet2     | Forward: GTGGCAAGAAGAAAGGGGA          | 229 bp       |
|          | Reverse: CTCTGCGCCTCTGGAAGGT           |              |
| Tet3     | Forward: TCAGGGATGAGAAGGCTACT         | 176 bp       |
|          | Reverse: CACGGGCCAGGATACAGA           |              |
| Dnmt1    | Forward: CCTAGTCTCTGCTGCAAGGAGA        | 136 bp       |
|          | Reverse: CTCTCTCTCTGCTGCAAGGCTCA      |              |
| Dnmt3a   | Forward: CAGCGGGCCGGTACTTTCTCTG        | 76 bp        |
|          | Reverse: TCCAGCTTACTCAGACTGGAT         |              |
| Dnmt3b   | Forward: TTCAGTGACCGTCTCTGACACAGA      | 144 bp       |
|          | Reverse: TCAGAGGCTGGAGACCTCTTCCTT       |              |
| Mecp2_e1 | Forward: AGGAGACAGTGGGAGAAGA          | 70 bp        |
|          | Reverse: CTTAACTTCTAGTGCTGTCT           |              |
| Mecp2_e2 | Forward: CCTCATAAAAAATACAGACTCTCAGAAGT | 183 bp       |
|          | Reverse: CTTAAACTTCTAGTGCTGTCT           |              |
| β-actin  | Forward: ATGTTGGAATGGGTCAGAAGG         | 157 bp       |
|          | Reverse: CACGCAGCTCATTTGAAGA            |              |
25 µmol/L of PFOA, whose viability was higher than the IC_{50}.

Effect of PFOA on apoptosis-, cell cycle-, and proliferation-related genes

Because of the previously reported effects of PFOA on cell cycle and proliferation (25, 28–30) and the strong effect on cell viability found in this study, we decided to continue by testing several genes related to apoptosis, cell cycle, and proliferation.

Figure 2A shows the expression levels of the apoptotic genes Bax, Casp3, and Trp73. The expression of the Bax gene, which acts as a pro-apoptotic regulator, increased significantly (p<0.001) upon PFOA exposure in a dose-dependent manner, while the Casp3 gene expression in apoptotic cells significantly decreased at 2.5 µmol/L (p<0.01) and 25 µmol/L (p<0.05). The mRNA level of the Trp73 gene, which encodes for the p73 protein involved in cell-cycle regulation and induction of apoptosis, significantly decreased after exposure to 2.5 µmol/L (p<0.05) and 25 µmol/L (p<0.01) of PFOA.

Figure 2B shows the expression patterns of three cell cycle-related genes, Ccna2, Ccnb1, and Ccne1. PFOA had no significant effect on Ccna2, but the expression of Ccnb1, which encodes for G2/mitotic-specific cyclin-B1, increased significantly in dose-dependent manner upon exposure to both 2.5 µmol/L (p<0.01) and 25 µmol/L (p<0.001) of PFOA. Ccne1 gene expression, on the other hand, was downregulated at both PFOA concentrations (p<0.01).

Figure 2C shows the expression levels of Cdkn1a, Cdk4, Rps6, and Kit1, which are genes related to cell proliferation. The expression of the cyclin-dependent kinase (CDK) inhibitor 1-encoding Cdkn1a significantly increased in a dose-dependent manner (p<0.05 at 2.5 µmol/L and p<0.001 at 25 µmol/L), but other proliferation gene expressions were not significantly affected.

Effect of PFOA on nerve growth factor-related genes

In the nerve growth factor – neurotrophic tyrosine kinase receptor type 1 (NGF-NTRK1) neurotrophin system, the gene expression level of the ligand Ngf significantly increased (p<0.05) with exposure to 25 µmol/L of PFOA, unlike the gene expression of receptor Ntrk1 (Figure 3A).

In the BDNF-NTRK2 neurotrophin system, 25 µmol/L of PFOA significantly (p<0.01) increased the expression of the ligand Bdnf gene, while both concentrations significantly increased the expression of Ntrk2, a high-affinity catalytic receptor for several neurotrophins and BDNF (p<0.001) (Figure 3B).

Epigenetic toxicity of PFOA

As PFOA showed dose-dependent effects on some genes, we decided to assess global DNA methylation to better understand its effect on genome-wide gene expression. Genome-wide 5-methylcytosine content showed a significant dose-dependent increase compared to control at both PFOA concentrations (p<0.01 at 2.5 µmol/L and p<0.001 at 25 µmol/L) (Figure 4A).

To better understand the mechanism of how methylation levels change, we also analysed relative gene expression of Tet1, Tet2, Tet3, Dnmt1, Dnmt3a, and Dnmt3b. Tet1 and Tet3 increased significantly with both PFOA concentrations (p<0.001), whereas the mRNA expression of Tet2 significantly decreased at PFOA concentration of 2.5 µmol/L (p<0.05) (Figure 4B). The expression of Dnmt1 (p<0.05), Dnmt3a, and Dnmt3b (p<0.001) significantly decreased at 25 µmol/L of PFOA, while the Dnmt3b gene

![Figure 2](image-url) Expression of (A) apoptosis-related genes, (B) cell cycle-related genes, and (C) cell proliferation-related genes after 24 h of exposure to PFOA (qPCR). Data are shown as mean ± standard error of three independent measurements. * p<0.05, ** p<0.01, and *** p<0.001 compared to control.
expression also significantly decreased at 2.5 µmol/L (p<0.01) (Figure 4C). Finally, in order to investigate the effect of PFOA on CpG-DNA-mediated gene transcription and the involvement of methyl-CpG binding protein, we analysed the changes in gene expression of Mecp2 isoforms e1 and e2, which are important readers of DNA methylation. The expression of both isoforms, significantly decreased at 2.5 µmol/L (p<0.01 and p<0.05, respectively; Figure 4D).

DISCUSSION

To determine the effects of PFOA on embryonic hypothalamic cell metabolism, this study evaluated the expression of apoptosis, cell cycle, and cell proliferation genes that play an important role in the development and survival of early neurons. Our results confirmed earlier reports of PFOA cytotoxicity, epigenetic toxicity, and its effects on gene expression and pointed to the mechanisms behind them. The most prominent outcomes of PFOA treatment on mHypoE-N46 cells were a dose dependent drop in cell viability, changes in the expression of some genes related to cell cycle, proliferation, and NGFs, and the activation of apoptosis-related genes. There was a dose-dependent increase in global DNA methylation, and a change in the expression of the Tet and Dnmt genes. The expression of the Mecp2s gene, an important reader of DNA methylation, was reduced.

One of the most important findings of this study is the low PFOA IC_{50} value (27.52 µmol/L), which clearly suggests that embryonic hypothalamic cells are more sensitive to PFOA toxicity than other tissue cells. In previous studies (24, 25, 31, 32), the PFOA IC_{50} in other cell types was mostly above 200 µmol/L.

Among different genes involved in the neuronal apoptosis mechanism, here we focused on Bax, Casp3 and Trp73 genes. Bax acts as a pro-apoptotic regulator (33). Inactivation of Casp3 causes a delay in neuronal apoptosis (34), while activation of Trp73 has been implicated in apoptosis induced by abnormal cell proliferation and DNA damage (35, 36). We found that exposure to PFOA significantly increased the Bax gene expression in embryonic hypothalamus cells in a dose-dependent manner,

Figure 3 Expression of (A) ligand (Ngf) and receptor (Ntrk1) genes of the NGF-NTRK1 neurotrophin system and (B) ligand (Bdnf) and receptor (Ntrk2) genes of the BDNF-NTRK2 neurotrophin system after 24 h of exposure to PFOA (PCR). Data are shown as mean ± standard error of three independent measurements. * p<0.05, ** p<0.01, and *** p<0.001 compared to control.

Figure 4 Epigenetic toxicity of PFOA: (A) relative global methylation levels; (B) relative expression of ten-eleven translocation methylcytosine dioxygenase-encoding Tet1, Tet2, and Tet3; (C) relative expression of DNA methyltransferase-encoding Dnmt1, Dnmt3a, and Dnmt3b, and (D) relative expression of methyl-CpG binding protein 2 genes. Data are shown as mean ± standard error of three independent measurements. * p<0.05, ** p<0.01, and *** p<0.001 compared to control.
while the expression of Casp3 and Trp73 genes significantly decreased. This decrease in the expression of Casp3 and Trp73 was consistent with the reports for bisphenol A, another EDC that interferes with developmental processes in the foetal brain (37). Our results confirmed Casp3 and Trp73-mediated mechanism of PFOA inhibiting apoptosis in embryonic hypothalamus cells, which was consistent with BPA. However, the finding that PFOA led to a dose-dependent increase in the Bax gene expression, suggests that Bax-mediated mechanism of apoptosis prevails, despite the delay and suppression mechanisms mediated by Casp3 and Trp73.

We were also interested in how PFOA affects the cell cycle. We already know that CCNA2 regulates cell cycle progression by interacting with CDK during both the G1/S and G2/M transition phases (38). CCNB1 and CCNE1 are essential for the G2/M and G1/S transition, respectively (39, 40). Previous studies have shown that exposure to PFOA alters the expression of cyclin-related genes in the human hepatoma cell line (HepG2) and human primary hepatocytes cells (41). With PFOA exposure, such a change in cyclin encoding gene may point to the disturbance in the cell cycle. CDKN1A regulates cell proliferation indirectly at the transcriptional level (42–44) or directly through proliferating cell nuclear antigen (45) and binding to CDKs (46, 47). Our results showed that the expression of Cdkn1a significantly increased in all PFOA-treated groups. Overexpression of p21/CDKN1A can inhibit cell proliferation (48) and may be involved in apoptosis (49, 50). Overall, since there was no clear association between PFOA exposure and decreased cell viability, it is likely that the change in the expression of the cell homeostasis gene impaired cell viability.

Our study also focused on the neurotrophins, which are important for the survival, development, and function of neurons (51, 52), while in embryonic neurons they can regulate apoptosis (53, 54). We analysed relative changes in the expression of Ngf, Ntrk1, Bdnf, and Ntrk2 and found that PFOA exposure affected the BDNF-NTRK2 neurotrophin system, as the expression levels of both the ligand Bdnf and the receptor Ntrk2 gene significantly increased. In related studies (37, 55), exposure to BPA altered the expression of Ngf and Bdnf genes, but not of Ntrk1 and Ntrk2. This suggests that changes in ligands of both neurotrophin systems caused by PFOA may follow a mechanism similar to that of BPA. On the other hand, the change in the expression of Ntrk2 suggests that PFOA disrupts the neurotrophin system through a specific mechanism, different from that of BPA. Additionally, these neurotrophin systems can interact with each other to regulate cell survival and death (53). We, therefore, believe that changes in the neurotrophin system caused by PFOA may affect not only growth and development but also survival of embryonic hypothalamic cells.

Our findings have also confirmed that PFOA increases overall DNA methylation in embryonic hypothalamic cells in a dose-dependent manner, unlike previous studies showing reduced global DNA methylation in PFOA-treated HepG2 and human breast epithelial cells (MCF7) (25, 31). This can be explained by the reported differences in the patterns of gene expression and epigenetic changes between tissues and cells (56, 57).

To evaluate the initiators of the changes in the patterns of DNA methylation after PFOA treatment we focused on Tets (mouse homologue of human TETs) and Dnmts (mouse homologue of human DNMTs). Dnmts and Tet2 expression decreased, while Tet1 and Tet3 expression and global DNA methylation increased. This points to a correlation between the level of PFOA exposure and the expression of cell homeostasis and nerve growth factor genes, and suggests that PFOA-induced epigenetic changes may play an important role in altering cell homeostasis and expression of neuronal growth factor genes.

Our findings of significantly downregulated expression of Mecp2_e1 and Mecp2_e2 genes may have wide ramifications, as MeCP2 not only aids in transcriptional silencing and acts as long-range regulator of methylated genes (58) but it also affects the expression of thousands of genes in the hypothalamus (59).

CONCLUSION

Overall, our findings suggest that exposure to PFOA affects cell survival through the reprogramming of embryonic hypothalamic DNA methylation patterns and altering cell homeostasis genes. They also suggest that DNA methylation and Mecp2 gene expression changes induced by PFOA could alter genes of other major mechanisms of the embryonic hypothalimus. All this may serve as a good starting point for further research into the mechanisms of PFOA effects on hypothalamic development.

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Epigenetska toksičnost i citotoksičnost perfluorooktanske kiseline i njezino djelovanje na gensku ekspresiju u stanicama hipotalamusa mišjeg embrija

Premda je perfluorooktanska kiselina (PFOA) dobro znana kao endokrini disruptor, i dalje se malo zna o mehanizmima u pozadini njezina djelovanja na stanice i njezine toksičnosti u kritičnoj fazi razvoja hipotalamusa. Stoga smo istražili njezino djelovanje u staničnoj liniji N46 hipotalamusa mišjeg embrija (mHypoE-N46) da bismo saznali o mehanizmima kroz koje ih PFOA oštećuje. S tom smo svrhom analizirali vijabilnost stanica, globalnu metilaciju DNA i gensku ekspresiju izloženih stanica. Porastom koncentracija PFOA padala je stanična vijabilnost, a globalna DNA metilacija rasla. Usto je PFOA značajno utjecala na ekspresiju gena povezanih s apoptozom i staničnim ciklusom, neurotrofnih gena te Tet, Dnmt i MeCP2 gena. Naše istraživanje ukazuje na to da izloženost PFOA utječe na preživljavanje stanica hipotalamusa mišjeg embrija reprogramiranjem obrazaca metilacije DNA te promjenama u genima zaduženim za održavanje homeostaze. Metilacija DNA i promjene u ekspresiji MeCP2 gena izazvane djelovanjem PFOA također imaju široki spektar implikacija, budući da utječu na promjene u genima zaduženim za druge važne mehanizme u embrijskom hipotalamusu. Stoga naše istraživanje može poslužiti kao dobra polazna točka za daljnje istraživanje mehanizama djelovanja PFOA na razvoj hipotalamusa.

KLJUČNE RIJEČI: citotoksičnost; DNA metilacija; endokrini disruptori; epigenetska toksičnost; mHypoE-N46 stanična linija; PFOA