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Full Length Article

Long-term exposure to fluoride as a factor promoting changes in the expression and activity of cyclooxygenases (COX1 and COX2) in various rat brain structures

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ABSTRACT

Background: : Sixty percent of the mammalian brain is composed of lipids including arachidonic acid (AA). AA released from cell membranes is metabolised in the cyclooxygenase (COX) pathway to prostanoids – biologically active substances involved in the regulation of many processes including inflammation. It has been shown that long-term exposure to fluoride in pre and neonatal period is dangerous because this element is able to penetrate through the placenta and to cross the blood-brain barrier. Exposure to fluoride during the development affects metabolism and physiology of neurons and glia which results in the impairment of cognitive functions but the exact mechanisms of fluoride neurotoxicity are not clearly defined.

Objective: : The aim of this study was to determine whether exposure to fluoride during the development affects COXes activity and the synthesis of prostanoids.

Material and methods: : Pre- and postnatal toxicity model in Wistar rats was used. Experimental animals received 50 mg/L of NaF in drinking water *ad libitum*, while control animals received tap water. In cerebral cortex, hippocampus, cerebellum and striatum were measured fluoride concentration, *COX1* and *COX2* genes expression, immunolocalization of the enzymatic proteins and concentration of PGE2 and TXB2.

Results: : of this study showed statistically significant changes in the concentration of fluoride in brain structures between study group and control animals. Moreover, significant changes in the expression level of COX1 and COX2, and in the concentration of PGE2 and TXB2 were observed.

Conclusion: : Exposure to fluoride in the prenatal and neonatal period result in the increase in COX2 activity and increase in PGE2 concentration in rats brain, which may lead to disturbances in central nervous system homeostasis.

1. Introduction

The central nervous system (CNS) is particularly sensitive to the effects of toxic substances such as fluoride (F-) accumulating in the human body. Both young and adult subjects exposed to high doses of F- experience a decreased learning ability, behavioural abnormalities,

Abbreviations: AA, arachidonic acid; Aβ, amyloid-beta; BBB, blood-brain barrier; BDNF, brain derived neurotrophic factor; cAMP, cyclic adenosine monophosphate; CNS, central nervous system; COX, cyclooxygenase (COX1, COX2); CREB, cAMP responsive element binding protein; DHA, docosahexaenoic acid; Il, interleukin (Il-1B, Il-6); IQ, intelligence quotient; MAPK, mitogen activated kinase; NF-kβ, nuclear factor kappa β; NSAID, non-steroidal anti-inflammatory drugs; PGE2/H2, prostaglandin E2/H2; PGES, prostaglandin E2 synthase; PKA, protein kinase A; PLA2, phospholipase A2; PTGS, prostaglandin-endoperoxide synthase; PUFA, spolyunsaturated fatty acids; qRT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; TNFα, tumour necrosis factor α; TXA2/B2, thromboxane A2/B2; WHO, World Health Organisation

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memory and cognitive functions impairment which in turn results in decreased quality of life (Banala et al., 2015). F- is particularly dangerous for juveniles during development due to weakened defense mechanisms and increased permeability of the blood-brain barrier (BBB) (Needham et al., 2011; Perumal et al., 2013). F- penetrates the placenta, can be found in maternal milk, and has the ability to penetrate the BBB (Fawell et al., 2006; Shalini et al., 2015; Niu et al., 2015). A progressive accumulation of F- in the brain results in brain damage and impairment of its functions (Wang et al., 2007). Epidemiological studies carried out in geographic regions where F- levels in drinking water significantly exceed WHO standards have shown that children living in these areas have significantly lower levels of intelligence quotient (IQ), compared to those living in areas not contaminated with this element (Choi et al., 2012; Rocha-Amador et al., 2007; Basha et al., 2011).

To date, researchers have investigated the effect of F- on neuron metabolism, neurotransmission, free radical processes, synthesis of proinflammatory factors, and the apoptosis of CNS cells (Akinrinade et al., 2015a; Scheff et al., 2005; Bhatnagar et al., 2006; Chirumari et al., 2007). Available literature data indicate that exposure to F- leads to the initiation of oxidative stress, inflammation and degeneration of neurons (Akinrinade et al., 2015b; Dec et al., 2017; Yan et al., 2016). At the cellular level, F- is mentioned as one of the factors inducing the inflammation within the brain (Yan et al., 2016). The mechanism of the proinflammatory effect of F- is based on an increase in the production of proinflammatory cytokines, dependent on the stimulation of transcription factors (Sharma et al., 2014). The mechanism of fluoride-dependent neuroinflammation also consists of an increase in peroxidation or the generation of reactive oxygen species (ROS), with the brain showing significant sensitivity to their increased concentrations. In addition to stimulating the oxidation reaction cascade, F- also reduces the relatively low antioxidant potential of the brain (Sharma et al., 2014). This is caused, among others, by F- reducing the activity of antioxidant enzymes not only by increasing ROS synthesis and the consumption of cofactors essential for enzyme action but also because it binds to metals present in the active center of enzymes as a competitive inhibitor (Chlubek, 2003). F- is a highly reactive element, and thus affects the activity of many enzymes whose cofactors are metal ions (Chlubek, 2003). The effect of F- on enzyme activity is one of the main mechanisms regulating the metabolism of neurons, being involved in the modulation of the activities of cyclooxygenases, (COX1, COX2) essential for the induction and development of inflammation among others (Ridley et al., 2009).

Sixty percent of the mammalian brain is composed of lipids. Development and functioning of the brain crucially depends on polyunsaturated fatty acids (PUFAs), which include arachidonic acid (AA) and docosahexaenoic acid (DHA). Intensive accumulation of AA in the brains of developing individuals is observed from the beginning of the third trimester of pregnancy up to the age of two (Hadley et al., 2016). AA is a component of the phospholipids that build cell membranes, and in the brain it is involved, inter alia, in the transmission of signals by synapses, regulation of neuronal stimulation, and the development and regeneration of axons (Burke et al., 2009; Hadley et al., 2016). The release of AA from the membrane is catalysed by phospholipase A2 (PLA2). The activation of cPLA2 in the brain occurs under the influence of oxidative stress and induction by proinflammatory factors. Activation of cPLA2 in neurons, astrocytes and microglia leads to a release of AA from membranes, which is metabolised in the COX pathway to eicosanoids – biologically active substances involved in the regulation of inflammation (Chuang et al., 2015; Ridley et al., 2009). Eicosanoids formed in the COX pathway include prostanoids such as prostaglandins, prostacyclins and thromboxanes (Hadley et al., 2016). COX, or prostaglandin-endoperoxide synthase (PTGS), exhibits oxygenase and peroxidase activity. There are two COX isoforms; constitutive COX1 and inducible COX2 (Ikeda-Matsuo, 2017; Vane et al., 2003). Both COX1 and COX2 catalyse the peroxidation and conversion of AA to biologically active prostanoids, which regulate the intracellular activity of protein kinases, calcium concentration, and the activity of voltage-gated and ligand-dependent ion channels (NMDA, AMPA, Na^+, K^+) in the CNS (Gu et al., 2015). Formerly it was thought that only COX2 participated in the development of inflammation in the CNS and neuronal degeneration, but the most recent analyses indicate the co-participation of COX1 in these processes (Bjork et al., 2016; Bonfill-Texidor et al., 2017; Luo et al., 2017). Increased COX2 expression is observed in most tissues during developing inflammation, which is accompanied by increased synthesis and influx of proinflammatory cytokines (e.g. interleukins). The increase in COX2 expression and activity is observed in neurodegenerative diseases, e.g. in patients with Alzheimer's disease (Luo et al., 2017). Furthermore, in vitro studies have shown the inhibition of amyloid beta protein dependent neurocytotoxicity (Aβ-protein) by using non-steroidal anti-inflammatory drugs (NSAID) whose mechanism of action is based on the inhibition of COX1 and COX2 (Lim et al., 2000; Netland et al., 1998).

Prostaglandin E2 (PGE2) is one of the main products of the COX pathway in the brain, and depending on the intensity of its synthesis and concentration, it participates in the regulation of physiological or pathological processes. PGE2, like other COX products, is synthesized mainly from AA. In the first stage of synthesis, AA is converted to prostaglandin H2 (PGH2) in a COX-catalyzed reaction; then prostaglandin E2 synthase (PGES) isomerizes PGH2 to PGE2 (Ikeda-Matsuo, 2017). There are three isoforms of PGES, microsomal - mPGES1 and mPGES2, and cytoplasmic - cPGES (Ikeda-Matsuo, 2017). Many factors influence PGES activity, including those that are involved in the initiation and regulation of inflammation such as NF-kβ or MAP kinases - ERK 1/2, JNK and p38 protein (Akundi et al., 2005; Bhatia et al., 2016). PGE2 synthesized in cells is secreted into the extracellular space directly by diffusion or by special membrane transporters – MRP4. The secreted PGE2 affects neighbouring cells, with the EP1-EP4 membrane receptors belonging to the G protein family (Andreasson, 2010; Chen et al., 2017; McCullough et al., 2004). Depending on the type of stimulated receptor, there is a change in intracellular cAMP concentration, calcium ion level (Ca^{2+}), or the activity of phosphatidylinositol 3-kinase (Chen et al., 2017). PGE2 in the CNS regulates the plasticity and stimulation of neurons, as well as the proliferation and differentiation of cells, and therefore participates in memory and learning processes (Cowley et al., 2009; Wong et al., 2016). It is also responsible for the course of inflammation, and an increased synthesis is often observed within lesion sites in the brain. It is worth noting that this compound, depending on its concentration and stage of inflammation, may stimulate or resolve the ongoing inflammatory process (Ikeda-Matsuo, 2017). Available literature data indicate that activation of the receptors EP1 and EP3 is associated with the neurotoxic effect of PGE2, whereas stimulation of receptors EP4 and less frequently, depending on the cell type - EP2, leads to the activation of neuroprotective mechanisms (Andreasson, 2010; McCullough et al., 2004).

Thromboxane A2 (TXA2), another AA derivative, is formed by the conversion of PGH2, catalyzed by thromboxane synthase (TS) (Sumimoto et al., 2015). TXA2 is mainly produced in platelets and endothelial cells and reacts with TP receptors belonging to the G protein family which are expressed on microglial membranes, astrocytes and oligodendrocytes (Sumimoto et al., 2015; Yang et al., 2016). There are two types of TP receptors – TPα (the dominant form in CNS) and TPβ. The stimulation of TP receptors in the CNS under physiological conditions, or as a result of damage to brain structures, is associated with the development and regeneration of neurons and has neuroprotective properties (Sumimoto et al., 2015). However, TP receptors on microglial cells are also involved in development of inflammation, and their stimulation leads to an increased synthesis of proinflammatory molecules (Yang et al., 2016).

Previous experimental and epidemiological studies have shown that exposure to increased F- concentrations during prenatal and postnatal development impairs the metabolism of neurons and glial cells, which results in subsequent memory and learning disorders. It was also found

that F- induces oxidative stress, activates glial cells and enhances the synthesis of proinflammatory cytokines involved in the initiation and development of inflammation in the brain. F- also influences the activity of enzymes responsible for the production of biologically active AA derivatives involved in the development of inflammation (Dec et al., 2017). Bearing this in mind, the aim of the study was to analyze the effect of F- on the development of inflammation in the brain in rats exposed to fluoride during prenatal and postnatal period.

2. Materials and methods

2.1. In vivo model of fluoride toxicity

Wistar rats were used to investigate *in vivo* F- toxicity. Procedures involving animals were carried out in strict accordance with international standards of animal care guidelines and every effort was made to minimize suffering and the number of animals used. Experiments were approved by the Local Ethical Committee on Animal Testing in Szczecin, Poland (approval No 32/2015). During the experiment the animals had free and continuous access to food (standard diet) and drinking water. The temperature in the room where the cages were placed was under control and a 12-h light/dark regime was maintained. Adult females (n = 6) were placed in the cages with sexually mature males for 7 days, after which the pregnant females were separated and randomly divided into two groups – study group (n = 3) and control (n = 3). Animals from the control group received tap water to drink, while those from the study group received water containing 50 mg/L of sodium fluoride (NaF). The experiment lasted from the day of fertilization until the maturity of the offspring (up to the 90th day after birth; PND 90). After the birth, the offspring were fed by the mothers up to the 21st day of life (PND 21), after which they were separated from the mothers and placed in new cages. After weaning, the young rats were kept under the same environmental conditions – the animals from the control group (males, n = 12) received tap water, while the animals from the test group (males, n = 12) were exposed to 50 mg/L NaF in the drinking water. After the end of exposure (PND 90), the animals were sacrificed by decapitation, their blood collected and immediately centrifuged to separate the serum. We also collected individual brain structures including cerebellum, prefrontal cortex, hippocampus and striatum (the collected material for biochemical analyses was placed in liquid nitrogen; for immunohistochemical analysis it was placed in 4% formalin solution, and the material for gene expression was placed in RNAlater buffer). The test material was stored at −80 °C until analysis.

We chose an oral administration of NaF as it reflects environmental exposure. A concentration of 50 mg/L NaF is based on commonly used models for the analysis of F- toxicity which show that in rats a 5-fold higher concentration of this element should be used to achieve an effect similar to that observed in the human body. According to this model, a dose of 50 mg/L in rats is equivalent to a human exposure in drinking water of 10 mg/L (Pereira et al., 2016). It should be noted, however, that rats consume about 30 ml–50 ml of water per day, which when given 50 mg/L results in an intake of about 1.5–2.5 mg of F- per day. The norms of consumption of this element according to the Polish standard SAI (Safe and Adequate Daily Intake) and ADI (Acceptable Daily Intake) are 3–4 mg/day for an adult (depending on gender), and retrospective studies have shown that the symptoms of fluorosis in an adult human weighing 70 kg appear with a consumption of F- at more than 10 mg per day (Flores-Mendez et al., 2014).

2.2. The measurement of fluoride concentration in rat serum and brain structures

The brain structures – cerebellum, prefrontal cortex, hippocampus and striatum, were dried at 95 °C to obtain a dry mass (minimum 48 h, depending on the tissue weight), then homogenised in ceramic mortars. Test portions of 5 mg were prepared from each sample, then incubated with 1 ml of 1 M perchloric acid (Sigma Aldrich, Poland) for 1 h at 95 °C. F- content was then determined by potentiometric method using an ion-selective ORION electrode (Thermo Scientific, Pierce Biotechnology, USA), from the difference in the potentials of the sample before and after the addition of the appropriate NaF standard, the sample mass (in the case of brain structures) and the concentration of the added standard. For the assay, 0,5 ml was taken from each previously prepared sample, 2,5 ml of TISAB II (Thermo Scientific, Pierce Biotechnology, USA) and 2,0 ml of citrate were added, and the potential of the obtained solution was measured for 5 min. Then, 0,5 ml of the appropriate NaF standard was added and the potential was again measured for 5 min.

To determine the content of F- in the blood serum, 0,5 ml of the sample was taken, to which 0,5 ml of 5% TISAB III solution was added (Thermo Scientific, Pierce Biotechnology, USA) and the potential was measured for 10 m in. from 5 min before to 5 min after adding the appropriate NaF standard (0,1 ml).

2.3. Determination of protein concentration by spectrophotometry

The protein concentration in the samples was determined spectrophotometrically using a MicroBCA Protein Assay Kit (Thermo Scientific, Pierce Biotechnology, USA) according to the manufacturer's recommendations. Standards and BCA Working Reagent (WR) were prepared. Appropriate volumes of the standards and samples were applied to a 96-well plate, WR was added, followed by incubation for 1 h at 37 °C. After the incubation, measurement was performed using an ASYS UVM 340 spectrophotometer (Biogenet) and the results read using MicroWin software. The final results were calculated based on the obtained standard curve.

2.4. Analysis of the expression of COX in the brain structures by quantitative real-time polymerase chain reaction (qRT-PCR)

Analysis of *COX1* and *COX2* expression was performed using the qRT-PCR method. After the section, the collected brain structures were immediately placed in the RNAlater buffer (Qiagen, Poland) in order to inhibit the RNA degradation process. RNA from tissues was extracted using an RNeasy Lipid Tissue Mini Kit (Qiagen, Poland) according to the manufacturer's instructions. Next, 1 µg of cellular RNA was prepared for analysis using a FirstStrand cDNA synthesis kit and oligo-dT primers (Fermentas, USA). In order to quantify the level of mRNA, RT-PCR was performed using an ABI 7500Fast and Power Master SYBR Green PCR Master Mix. The following reaction conditions were used: 95 °C (15 s), 40 cycles at 95 °C (15 s) and 60 °C (1 min). Each sample was analyzed twice (2 technological replicates) and mean Ct values (the number of cycles when the sample reaction curve intersects the threshold line) were used for further calculations. In addition, the relative amount of product was recalculated with respect to *GAPDH* – a control gene showing constant expression. The following primer pairs were used: *GAPDH* forward: ATGACTCTACCCACGGCAAG, reverse: CTGGAAGAT GGTGATGGGTT; *COX1* forward: GTTCACAGGAGAGAAGGAGATG, reverse: GGAGCCCCCATCTCTATCATGC; *COX2* forward: AATGAGTACC GCAAACGCTTCT, reverse: AGCCATTTCTTTCTCCTGTAAG.

2.5. Immunolocalization of COX1 and COX2 in the brain using immunohistochemistry

The collected brain tissue materials were dehydrated by washing with a series of alcohols with increasing concentrations, and then fixing in paraffin. 4 µm sections were cut using a microtome, then dewaxed and rehydrated by washing in a series of alcohols of decreasing concentration. The recovered tissues were then boiled twice in a microwave oven (700 W, 4 min and 3 min) in 10 nM sodium citrate buffer (pH = 6.0) in order to reveal the epitope for the antibody to be used in

the next stage of analysis. The test material was incubated with a primary antibody (Santa Cruz Biotechnology, Inc.) (1:5000) against COX1 and COX2 overnight at 4 °C. A secondary antibody conjugated with horseradish peroxidase (HRP) and other reagents necessary for visualization of the reaction (DAKO LSAB + System-HRP, DakoCytomation, UK) were used according to the manufacturer's instructions. Photographs were taken using a Leica CTR 5000 optical microscope.

2.6. Determinations of the concentration of COX products PGE2 and TXB2 in the brain performed by ELISA immunoassay

The concentrations of PGE2 and TXB2 in the brain tissue samples were measured spectrophotometrically using a Prostaglandin E2 EIA Kit and a Thromboxane B2 EIA Kit (Cayman Chemical, USA) respectively. Physiologically active TXA2 synthesized by COX is characterized by a short half-life (37 s) and is rapidly hydrolyzed to a stable biologically inactive form – TXB2. Therefore, in order to analyze the synthesis of TXA2 in the examined brain structures, the levels of stable TXB2 were determined. The samples were suitably prepared for analysis as recommended by the reagent kit manufacturer. The material was homogenised with knife-mills in a cold stable PBS solution (Biomed, Poland) and centrifuged in an Eppendorf centrifuge (10 min/7000 rpm/4 °C). After centrifugation, the supernatant was collected and extracted on Bakerbond SPE columns (JT Baker, USA). The concentrations of PGE2 and TXB2 in the samples were converted into protein mass in each assay.

2.7. Statistical analysis

Statistical analysis of the obtained results was performed using Statistica 12.0 software. For each of the tested parameters, the arithmetic mean ± SD was calculated. In order to obtain a distribution of results for particular variables, a Shapiro-Wilk (W) test was used. Most of the data deviated from a normal distribution, therefore nonparametric tests were used for further analysis. To evaluate the differences between the control group and the study group, a Mann-Whitney U test was used, with differences considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Analysis of fluoride concentrations in serum and brain structures of rats prenatally and postnatally exposed to fluoride

The first stage of laboratory analysis was the measurement of the F- concentrations in blood serum and the cerebellum, prefrontal cortex, hippocampus and striatum samples from the control group receiving tap water and rats exposed to F- in drinking water (NaF 50 mg/L). The analysis did not show a difference in the concentration of F- in the serum of those from the study group (0.19 mg/L) compared to the control group (0.18 mg/L) (Fig. 1A). Statistically significant differences in F- content were observed in the brain structures. A statistically significantly higher F- concentration was observed in the hippocampus (15.8% higher, $p = 0.025$) and in the striatum (53.4% higher, $p = 0.013$) of the F- exposed group in comparison to the control (Fig. 1B). Higher F- concentration were also observed in the prefrontal cortex (+35.3%) and the cerebellum (+14.1%) of the F- exposed animals than the control group, but these differences were not statistically significant (Fig. 1B).

3.2. Analysis of the expression of COX in the brain of rats subjected to prenatal and postnatal exposure to fluoride

The analysis of the expression of *COX1* and *COX2* showed that long-term exposure to F- during prenatal and postnatal development significantly influenced the expression of these genes of the studied

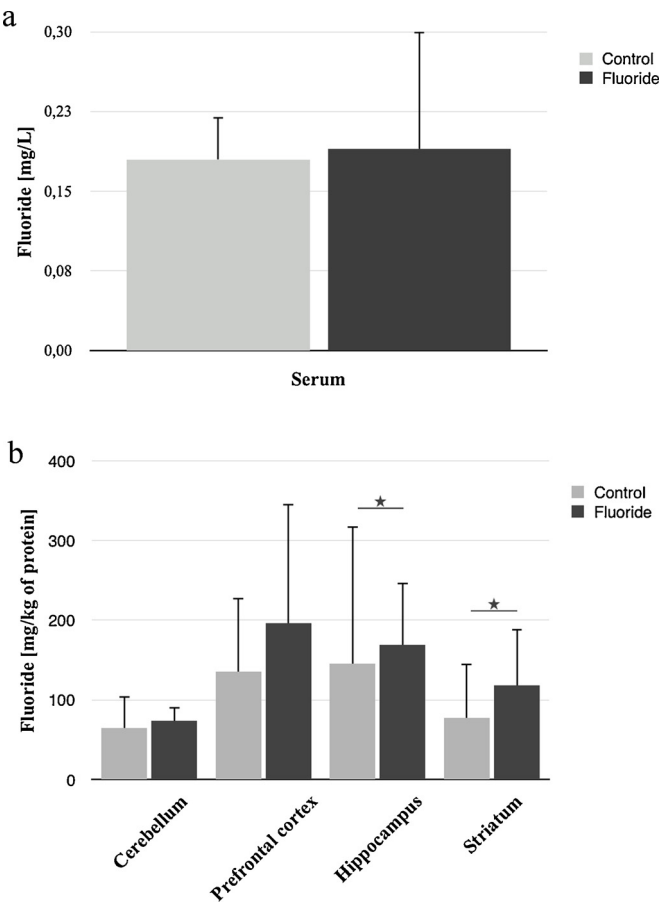

Fig. 1. The cocncentration of fluoride (F-) in serum (A) and brain structures (B) (prefrontal cortex, cerebellum, hippocampus and striatum) in control (n = 6) and F-exposed group (F)(n = 6). *$p \leq 0.05$, for the significance of difference between the groups (Mann-Whitney test).

enzymes. The cerebellum, hippocampus and striatum of those from the F- exposed group showed significantly lower expressions of both *COX1* and *COX2* (Figs. 2A, B), whereas in the prefrontal cortex only *COX1* expression was significantly lower (Fig. 2A)

3.3. Immunolocalization of COX1 and COX2 in the brain of rats exposed to fluoride during development

To assess protein expression and the cellular location of COX1 and COX2, immunohistochemistry was performed on the brain structures. In the cerebellum, no change in COX1 or COX2 expression was observed between the group receiving F- in drinking water and the control group (Fig. 3) In the cerebral cortex, a slightly higher expression of both COX1 and COX2 was observed in the control subjects compared to those exposed to F- (Fig. 4). In the hippocampus, comparable expressions of COX1 were observed in both individuals from the F- exposed group and the control group, and a significantly higher expression of COX2 was observed in those exposed to F- compared to the control group (Figs. 5 and 6). In striatum COX immunoexpression was higher in the control group, in which COX1 was mainly observed in glial cells (white arrows) while COX2 was observed in neurons (black arrows). Lower COX expression was observed in fluoride exposed animals (Fig. 7).

3.4. Analysis of the concentration of COX products, PGE2 and thromboxane B2, in the brain of rats exposed to fluoride

The assessment of COX activity in the studied brain structures was

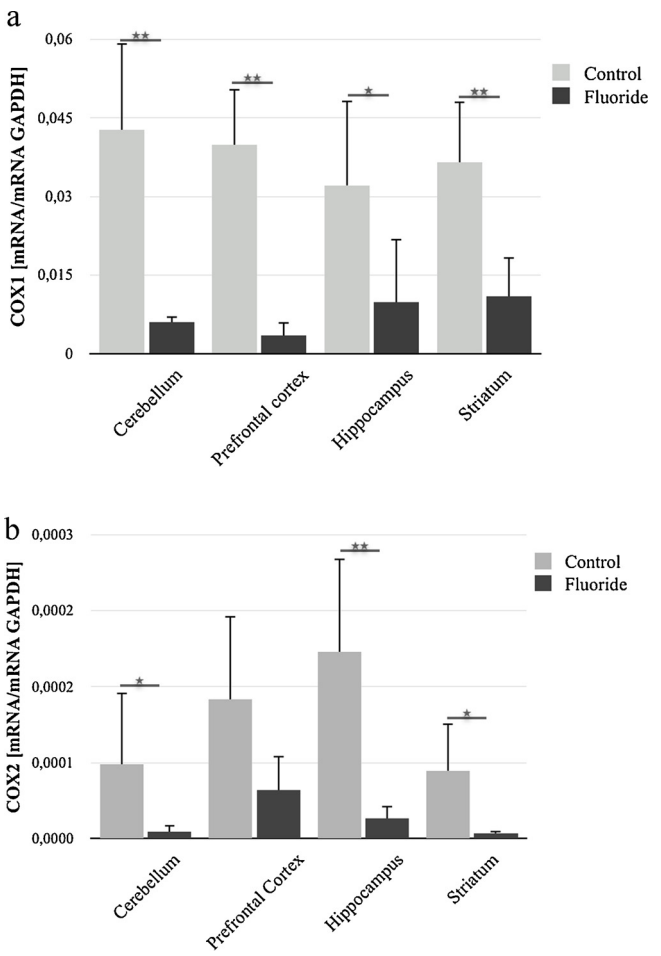

Fig. 2. The level of cyclooxygenase 1 (COX1) (A) and cyclooxygenase 2 (COX2) (B) mRNA expression in brain structures (prefrontal cortex, cerebellum, hippocampus and striatum) in control (n = 6) and F-exposed group (F)(n = 6). *p ≤ 0.05; **p ≤ 0.01, for the significance of difference between the groups (Mann-Whitney test).

made by measuring the concentration of their reaction products – PGE2 and thromboxane B2. The results of the analysis showed statistically significant differences in the concentration of PGE2 in the hippocampus (increase by 42.2%, p = 0.02) and cerebellum (decrease by 18.9%, p = 0.01) (Fig. 7A), and TXB2 in the striatum (decrease by 36.3%, p = 0.007) (Fig. 7B) in the F- exposed animals compared to the controls. Statistically non-significant differences in PGE2 concentration were observed in the prefrontal cortex and striatum (Fig. 7A) and a decrease in TXB2 concentration in the hippocampus and prefrontal cortex (Fig. 7B) between the animals exposed to F- and the controls.

4. Discussion

Uncontrolled inflammation is the basis for many neurological disorders and dementia diseases (Di Meco et al., 2017). Numerous analyses have demonstrated the relationship between inflammation in the CNS and the etiopathogenesis of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Dec et al., 2017; Shalini et al., 2015). The development of inflammation in the brain is influenced by a variety of factors such as increased synthesis and release of ROS, NO, proinflammatory cytokines, glutamate, and the induction of PLAs and metalloproteinases which lead to the activation of M1 microglia cells and the expansion of this process (Chuang et al., 2015). Activated microglia occur in the CNS in two phenotypes – M1 and M2. It is believed that the M1 microglia have cytotoxic activity and are associated with pathological processes occurring in the brain, whereas the M2 have neuroprotective properties and are activated in the repair phase (Chuang et al., 2015; Tang et al., 2016). The microglial cells are activated by factors including proinflammatory cytokines, lipopolysaccharides, β-amyloid, and exogenous toxins such as lead or F-. These neurotoxins lead to the induction of NADPH oxidase (NOX) and NO synthase, and hence to increased ROS and NO synthesis, thus causing oxidative stress (Sheng et al., 2011). Neuronal damage and loss are often a result of permanent inflammation in the brain, where oxidative stress and lipid derivatives formed in the COX pathway play an important role in the initiation, development and suppression of inflammation (Grandjean et al., 2006; Pereira et al., 2011).

4.1. Evaluation of fluoride concentrations in serum and brain of rats subjected to prenatal and postnatal exposure to fluoride

The conducted analysis showed that the concentration of F- in the serum of rats receiving 50 mg NaF/L drinking water did not differ significantly from the control group receiving pure tap water. A higher concentration of F- was observed in all the examined brain structures in subjects exposed to F- compared to the control group, with statistically significant higher levels found in the striatum and hippocampus. The obtained results confirm other studies that the excess of F- ingested by the body is removed from the blood and can accumulate in the brain.

F- is known to penetrate the blood-brain barrier and enter the CNS. An analysis carried out by Tsunoda et al. showed that even short-term exposure to F- leads to an accumulation in the CNS, where a significant increase in F- concentration in the brain of mice exposed to this element for one month was observed at 25 mg/L and 125 mg/L in the drinking water (Tsunoda et al., 2005). Tsunoda et al. observed the penetration of F- across the blood-brain barrier and its accumulation in the brain after 30 days exposure to this element, at a concentration twice lower than the concentration used in our analysis. Han et al. observed a significant increase in the concentration of F- in the hippocampus of mice subjected to a 180-day exposure to 50 mg/L and 100 mg/L of F- in drinking water (Han et al., 2014). Similar results were obtained by Long et al. who observed a significant increase in the concentration of F- in the urine and brain of adult rats exposed to F- (30 mg/L and 100 mg/L) for 7 months, which confirms the argument that this element when delivered to the body accumulates in the CNS. In addition, the experiment conducted by Long et al. showed that an excess of F- is removed from the body in the urine, although with a long-term exposure to F- this mechanism may not be sufficiently efficient (Long et al., 2002).

4.2. Effect of prenatal and postnatal fluoride exposure on the expression and activity of COX in the rat brain

COX1 was hitherto regarded as a constitutive isoform of the enzyme, whereas COX2 was considered an inducible isoform, stimulated mainly in pathological conditions (Kirkby et al., 2016, 2012; Nandakishore et al., 2014). However, recent analyses show that in tissues such as the thymus, intestines, kidneys and brain *COX2* also shows a constant expression at a relatively low level and is responsible for regulating the physiological processes and maintaining homeostasis in these organs (Chung et al., 2010; Kirkby et al., 2012). The increased *COX2* expression observed in neurological disorders is mainly seen in the early stage of inflammation, where intensification of the inflammation by the action of this enzyme may lead to degenerative changes (Varnum et al., 2012). COX1 also participates in the regulation of inflammation and an increased expression is mainly observed in acute inflammations (Shukuri et al., 2016; Yermakova et al., 1999). Changes in *COX1* expression have also been shown in the course of degenerative diseases. In the histopathological picture of patients with Alzheimer's disease, there is an increase in *COX1* expression in microglial cells surrounded by amyloid plaques, and analyses carried out in an animal model of Alzheimer's disease have shown that the COX1 is

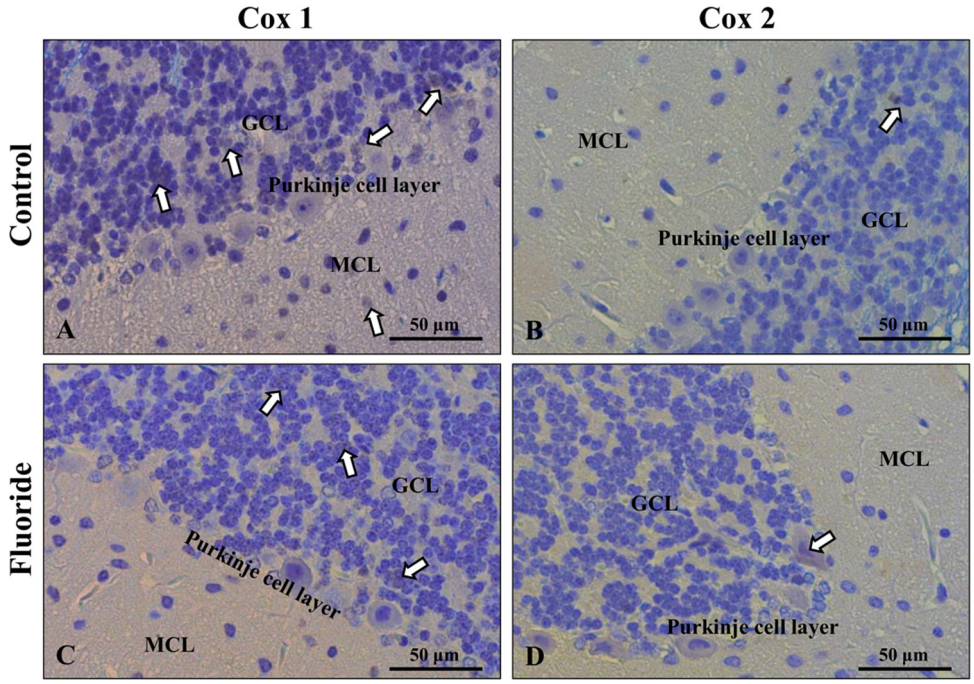

Fig. 3. Immunoexpression of cyclooxygenases COX1 and COX2 in neurons of the cerebellum of rats from the control group and rats exposed to fluoride. White arrows indicate the positive expression of COX in cells. GCL – granular cell layer; MCL – molecular cell layer. Objective magnification: x 40..

induced only in microglia cells, while its activity does not change in astrocytes (Shukuri et al., 2016). In addition, in vitro and in vivo studies have shown that silencing COX1 expression protects neurons from damage by inhibiting degenerative processes (Choi et al., 2008).

The results of our analysis show that the highest level of *COX2* expression in physiological conditions (in the control group) occurred in the hippocampus and prefrontal cortex of the rats. The obtained results confirm the observations of Kirkby et al. who demonstrated that under physiological conditions the highest level of *COX2* expression in the CNS is observed in the cerebral cortex (Kirkby et al., 2012). Our analysis also confirms reports on the expression of *COX2* in physiological conditions in the brain. Expression of *COX2* was observed in all examined brain structures of the rats from the control group – cerebellum, prefrontal cortex, hippocampus and striatum. In addition, in the control subjects, the expression of *COX2* was approximately two hundred times lower than the *COX1*.

Our research indicates that long-term exposure to F- during the development leads to the decrease in the expression of *COX1* and *COX2* in the brain. A significant reduction in *COX1* expression was observed in all examined brain structures of the rats from the study group. *COX2*

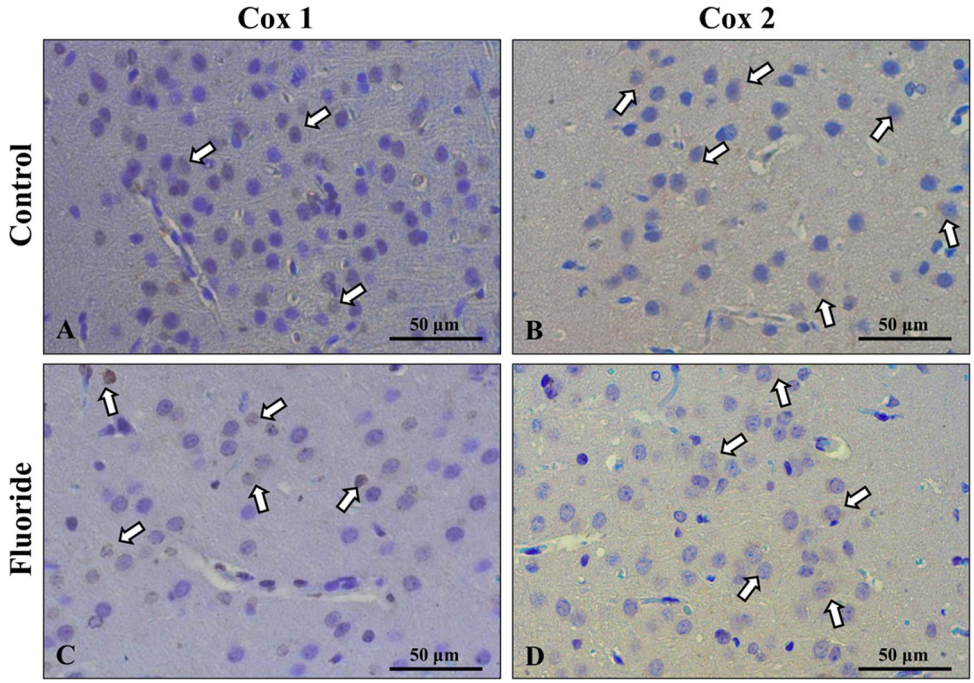

Fig. 4. Immunoexpression of cyclooxygenases COX1 and COX2 in neurons of the cerebral cortex of rats from the control group and rats exposed to fluoride. White arrows indicate the positive expression of COX in cells. GCL – granular cell layer; MCL – molecular cell layer. Objective magnification: x 40..

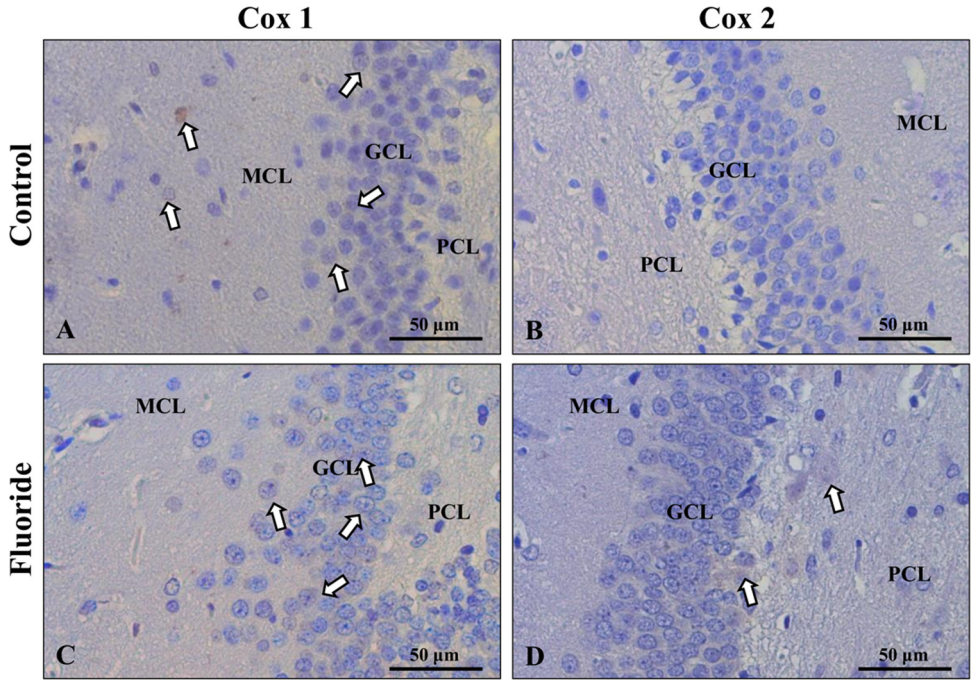

Fig. 5. Immunoexpression of cyclooxygenases COX1 and COX2 in neurons of the *gyrus dentatus* of rats from the control group and rats exposed to fluoride. White arrows indicate the positive expression of COX in cells. GCL - granular cell layer; MCL – molecular cell layer; PCL - polygonal cell layer. Objective magnification: x 40.

expression was also significantly reduced in the cerebellum, hippocampus and striatum due to the influence of F-. We have already mentioned that both COX1 and COX2 are involved in the regulation of physiological processes and inflammatory processes in the brain (both in the initiation and in the silencing of the inflammatory process). In the early phase of inflammation, COX2 is involved in the response to external factors, and an increased expression may lead to an intensification of the inflammatory process (Astakhova et al., 2015). An increase in concentration of COX2 products in the late phase of inflammation has an impact on the healing process and the inflammation resolution in the brain, and a reduction in enzyme expression at this stage may lead to a transformation of acute inflammation into chronic inflammation (Astakhova et al., 2015; Rajakariar et al., 2006). Thus, a decrease in the expression of either *COX1* or *COX2* in the brain of the rats exposed to F- may interfere not only with physiological processes but may also be associated with chronic inflammation.

Although the mRNA levels of *COX1* and *COX2* in the rat hippocampus from the F- exposed group were significantly reduced, a statistically significant elevated PGE2 concentration was observed in this structure, as well as an increase in concentration of COX2 enzyme

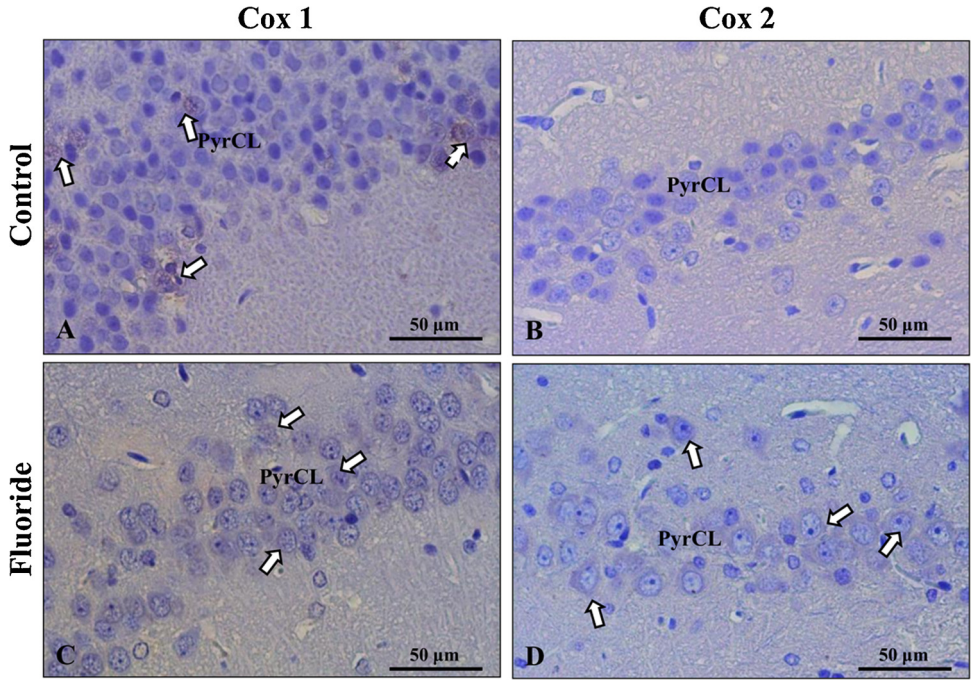

Fig. 6. Immunoexpression of cyclooxygenases COX1 and COX2 in neurons of the *cornus ammoni* of rats from the control group and rats exposed to fluoride. White arrows indicate the positive expression of COX in cells. PyrCL pyramidal cell layer. Objective magnification: x 40.

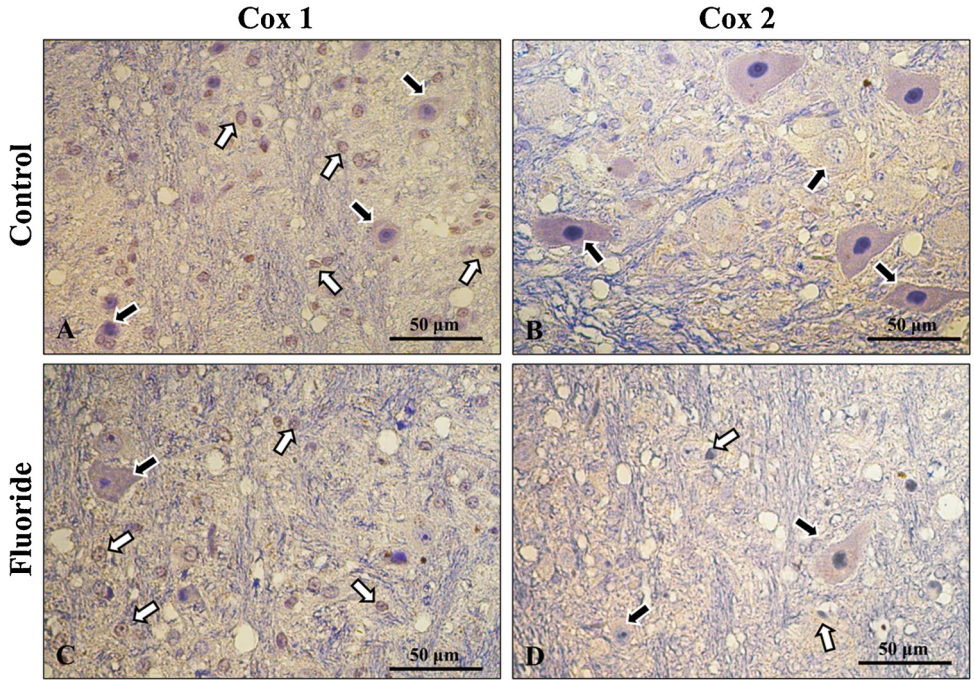

Fig. 7. Immunoexpression of COX1 and COX2 within the striatum of control and fluoride exposed animals. Black arrows show immunopositive neural cells; white arrows show immunopositive glial cells. Objective magnification: x 40.

proteins (increased number of immunopositive cells) in comparison to the control group. In the hippocampus, subjects from the study group showed the presence of COX2 positive cells which were not visible in the hippocampus of the control group, while the expression of COX1 in this brain structure was similar in both groups. The results obtained by means of immunohistochemical analysis indicate that the increase in PGE2 synthesis in the hippocampus may have been due to COX2 protein expression. PGE2, one of the main COX2 products, has both neuroprotective and neurotoxic properties, and the type of reaction it induces in cells depends on the type of receptor (EP1-EP4) to which it binds (Cowley et al., 2008). Under physiological conditions, PGE2 affects the plasticity of neurons by regulating the synthesis of brain derived neurotrophic factor (BDNF) in neurons, microglia and astrocytes, and regulation of long-term synaptic enhancement (LTP), and thus is a significant factor affecting learning and memory processes (Hutchinson et al., 2009). The neurotoxic mechanism of PGE2 is associated with the stimulation of EP3 receptors occurring on the membranes of the hippocampal neurons (Milatovic et al., 2011). Analyses carried out on a mouse model of Alzheimer's disease show that removal of EP3 receptors from CNS cells leads to the inhibition of expression of genes encoding proinflammatory factors and decrease in lipid peroxidation intensity, and consequently to an improvement of cognitive functions (Chen et al., 2013). In addition, studies conducted by Ying Luo et al. showed that an increase in PGE2 concentration in the hippocampus and interaction with the EP3 receptor results in a decrease in intracellular cAMP concentration in neurons, and inhibition of the cAMP/PKA/CREB/BDNF cascade which controls the plasticity of neurons (Fig. 8) (Luo et al., 2017). In this cascade, protein kinase A (PKA) activates the CREB transcription factor, which in turn induces the expression of BDNF – a neurotrophic factor responsible for nerve cell viability, regulation of plasticity, as well as the formation of long-term memory (Calabrese et al., 2014; Yan et al., 2016). Proper expression of BDNF is particularly important for immature nerve cells, because this factor affects their maturation and differentiation (Luo et al., 2017). The same mechanism – COX2/PGE2/EP3/cAMP/CREB/BDNF – is associated with the dysfunction of neurons of the cerebral cortex (Chen et al., 2017). Bonfill-Texidor et al. showed that the increase in PGE2 synthesis and activation of the EP4 receptor on microglial cells is anti-inflammatory (Bonfill-

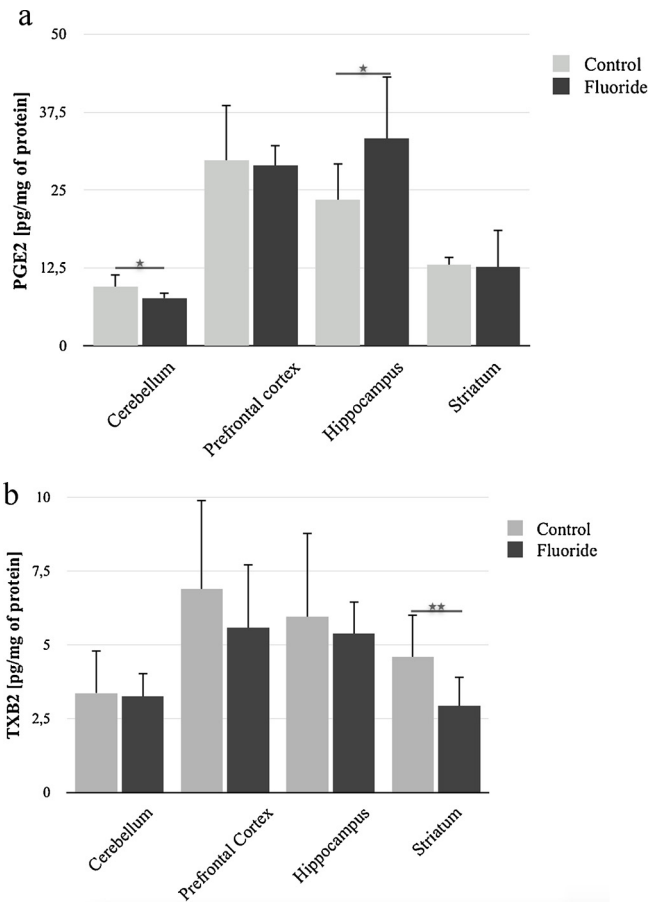

Fig. 8. The concentration of prostaglandin E2 (PGE2) (A) and thromboxane B2 (TXB2) (B) in brain structures (prefrontal cortex, cerebellum, hippocampus and striatum) in control (n = 6) and F-exposed group (F)(n = 6). *p ≤ 0.05, for the significance of difference between the groups (Mann-Whitney test).

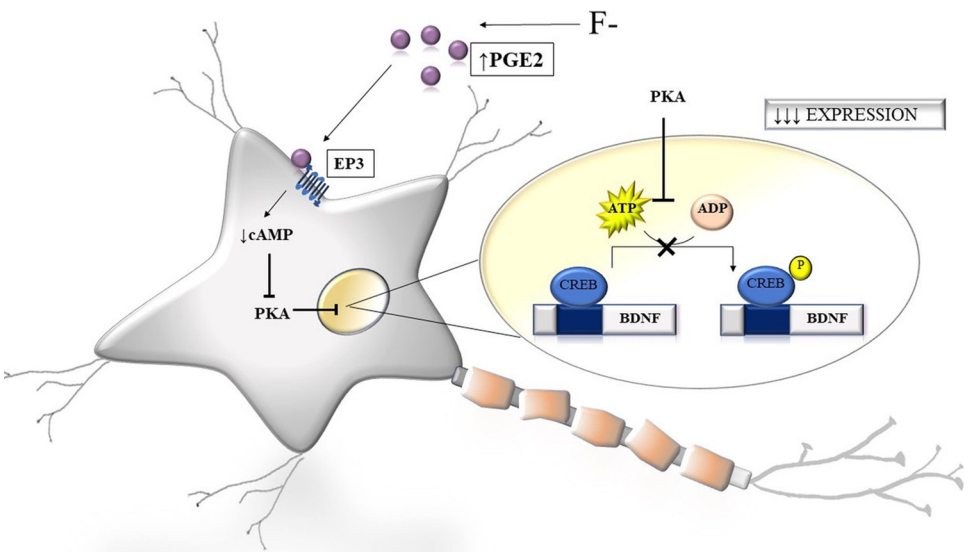

Fig. 9. Possible mechanism of the neurotoxic role of fluoride on hippocampal neurons. Fluoride causes an increase in the synthesis and secretion of prostaglandin E2 (PGE2) into the extracellular space, which binds to EP3 receptors on the neuronal cell membranes and leads to a decrease in the synthesis of cyclic AMP (cAMP) in the cell. A consequence of inhibition of cAMP synthesis is protein kinase A (PKA) inhibition, which leads to the inhibition of the CREB transcription factor and inhibition of brain-derived neurotrophic factor (BDNF) expression (Luo et al., 2017).

Texidor et al., 2017). In their study, induction of the EP4 receptor on microglial cells led to a decrease in the synthesis of proinflammatory cytokines, including TNFα (Bonfill-Texidor et al., 2017) (Fig. 9).

Our study also showed that exposure to F- during development phases led to a decrease in TXB2 concentration in the striatum and prefrontal cortex, with changes observed in the cortex not statistically significant. Analyses made by Sumimoto et al. showed that by stimulating TP receptors TXA2 induces neurite growth and development in the cerebral cortex (Sumimoto et al., 2015). TXA2, through the stimulation of TP receptors on oligodendrocyte precursors, stimulates the proliferation, differentiation, remielination and development of neural networks, and these processes are significant for restoration processes following the damage to the CNS (Harel et al., 2006; Sumimoto et al., 2015). However, TXA2, as well as PGE2, can have both a protective and pro-inflammatory effect at the same time. The stimulation of TP receptors on microglia may cause their activation and increased synthesis of proinflammatory cytokines such as Il-1β, Il-6 and induce NO synthesis (Yang et al., 2016). Microglia themselves may also be a significant source of cerebral TXA2, and its secretion may disrupt physiological processes occurring in other types of cells of the nervous system and affect the permeability of the blood-brain barrier (Yang et al., 2016). If a reduction in the synthesis of TXA2 is modulated by regulating COX activity via negative feedback, this may indicate that in the brain of rats exposed to F- an inflammatory process developed and protection mechanisms were activated to prevent long-term activation of microglia. However, it should be mentioned that TXA2 affects the viability of neurons and stimulates the restoration of neuronal networks after injury. Thus, if the inflammation caused by the neurotoxic effects of F- persists in the brain long enough to lead to neuronal degradation and structural disorders, the final reduction in TXA2 concentration may have a negative effect on the repair processes.

5. Conclusion

Exposure to F- in the prenatal and neonatal period of rats may result in the development of inflammation in the brain, and this neurotoxic mechanism of F- may be associated with an increase in COX2 activity and an increase in PGE2 concentration in the hippocampus.

The increase in PGE2 concentration in the hippocampus observed in this study may be related to the progression of the inflammatory process or with the inhibition of inflammation. The obtained results, however, did not allow a determination of the direction of changes. That would require analysis of the expression of individual receptors for PGE2. Based on the results, we can only suspect that an increase in PGE2 concentration in the hippocampus is indicative of inflammatory processes, and contributed to a decrease in the expression of COX2 via negative feedback. Changes in PGE2 and TXA2 concentrations in the brain caused by F- exposure are particularly dangerous for developing individuals, because these compounds affect, among other things, the regulation of BDNF synthesis in the hippocampus or development of neural networks in the cerebral cortex, and this mechanism may be the basis of memory and learning disorders observed in children living in areas contaminated with this element.

It is worth noting that the model of long-term exposure to F- used in our analysis during the development period influenced the concentration of this element in the studied brain structures, with no differences between the concentration of F- in the serum of subjects from the study group compared to individuals from the group control. This indicates that serum F- is not a good marker of the toxicity of the element. Both the exposure time and the concentration of F- in drinking water affect the changes in its concentration in serum and hard skeletal and soft tissues, and the results obtained by us are evidence that long-term exposure to even relatively low concentrations of F- has a neurotoxic effect.

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Conflicts of interest

The authors declare they have no actual or potential competing financial interests.

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