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A novel concept of immunological and allergy interactions in autism spectrum disorders: Molecular, anti-inflammatory effect of osthole

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ABSTRACT

Background: Autism spectrum disorder (ASD) is a neurodevelopmental disorder defined by Diagnosis and Statistic Manual 5 (DSM-5) as persistent social interaction and communication deficient across multiple contexts. Various immunological findings have been reported in children with ASD, and co-existing allergic problems have been recorded in children diagnosed with ASD. Osthole, the effective component of Chinese traditional medicine, is reported to have anti-inflammatory effects. This study assessed the anti-inflammatory effect of osthole on the histamine-induced inflammatory responses in PBMC cells.

Methods: Peripheral blood mononuclear cells (PBMC's) from children with: (1) ASD group with co-existing allergies/asthma (n = 29); (2) ASD group without allergy/asthma (n = 29); (3) Allergy group (n = 30) and from typically developing age-matched control subjects (n = 28) were stimulated with either histamine, FXF, osthole or mixture of this substances. mRNA *COX-2* gene expression, COX-2 production and inhibitory effect of tested substances on COX-2 were assessed after stimulation.

Results: Children with ASD may show either an innate proinflammatory response or increased activity of COX-2 which could display more impaired behavioral profile than children with non-inflamed. This study indicated that COX-2 may be involved in pathogenesis of ASD and/or allergy, and osthole could be used to decrease the effects of COX-2 in inflammation and ASD development. High incidence of allergy in ASD patients may indicate immune dysregulation that could be of relevance to the pathophysiology, symptomatology or neuroimmunology of ASD.

Conclusions: This study shows that fexofenadine (FXF – antihistamine drug) and osthole exhibit selective COX-2 enzyme inhibitory activity. The selective COX-2 activity of osthole may explain further the anti-inflammatory properties of osthole in relieving congestion in allergic rhinitis, and as distinctive effects between FXF and osthole were observed, individual antihistamines may have different modes of action via the COX enzyme system.

1. Introduction

Recent research has established immune dysregulation in autism spectrum disorder (ASD) to be linked to genetic variation in leukotriene, prostaglandin and cytokine expression and function. Epidemiological studies have also associated ASD with autoimmunity and allergies [1,2]. Although the precise effect of these associations remains unclear, normal immune regulation is of particular importance for ASD, as Vargas et al. showed a link between immune gene dysfunction and the up-regulation of cytokines in brain and serum of ASD patients [3,4]. Furthermore, Gent et al. reported dysfunction in B, T and NK cells and increased production of pro-inflammatory cytokines and auto-antibodies [5] and immune mediated conditions in ASD [4,6]. Those Th1, Th2 lymphocyte and cytokine alterations also suggest that hyperreactivity against allergens contributes to ASD neuropathogenesis [7] what is new date suggested dysregulation of T cell immunoglobulin and mucin domain 3 (TIM-3) [8]. In addition, Jyonouchi and Jyonouchi et al. confirmed the prevalence of allergic diseases in children with ASD, and our study [Kordulewska et al., date unpublished] also found that allergies are under-diagnosed in ASD children and that these can exacerbate ASD symptoms [9,10].

Histamine is the main mediator of the early-phase response, which

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along with other inflammatory mediators, such as the kinins and arachidonic acid derivatives, is involved in eliciting the early-phase allergic reaction. The late phase of the allergic response mainly involves infiltration of the nasal epithelium by eosinophils, basophils, monocytes and T lymphocytes [11]. Minghetti, reported that growth factors, cytokines and proinflammatory molecules induce cyclooxygenase-2 (COX-2), which in turn produces prostanoids in acute and chronic allergic inflammation. This author also stated that not only the COX-2 is involved in normal neuronal functions, but also its expression may play a role in ischemia, seizures, synaptic plasticity and neurotoxicity [12]. Buki et al. supported this latter finding, stating that COX-2 is expressed in the forebrain, hippocampal, hypothalamic and amygdala neurons, which are altered in psychiatric disorders [13].

The involvement of COX-2 in the ASD pathogenesis can be supported by the following indirect evidence: (1) the possible relationship between COX-2 and Rett syndrome; a variant of ASD currently not included in DSM-5. In this disorder, the laminar pattern of cortical COX-2 immunoreactivity is disrupted and COX-2-positive neurons are decreased in number and are randomly distributed [14]. (2) COX-2 can play a role in long-term potentiation, which is a major model in synaptic plasticity and subsequent learning and memory formation. This theory emerged from a study where subjection of rats to selective destruction of basal forebrain cholinergic neurons in the first post-natal week resulted in decreased levels of hippocampal COX-2 in adulthood, a damage that led to impaired social memory, which in humans is a critical deficit in ASD [14]. (3) It has been hypothesized that dysregulated or abnormal immune response might be involved in some forms of ASD. Since cytokines and other products of immune activation have widespread effects on neuronal pathways and can alter behaviors such as mood and sleep, it is possible for COX-2 to be involved in these abnormal ASD immune processes [14].

We therefore utilized fexofenadine (FXF) and Osthole to stimulate in vitro peripheral blood mononuclear cells (PBMC's) isolated from children with diagnosed ASD to assess how pro-inflammatory factors such as COX-2 changed in the tested group. FXF is the active metabolite of terfenadine, a selective histamine H_1 receptor antagonist that does not cross the blood brain barrier, appears to display anti-inflammatory properties and is used in allergy therapies. Our previous study confirmed that osthole derived from natural sources possesses promising anti-allergic [14–18]. Other research suggested that osthole has antioxidant, anti-cancer, anti-inflammatory and immunomodulatory properties [14–18]. Therefore, it's reported multiple bioactivities may encourage the development of osthole- and its derivatives-based multi-target drugs, which could be applied to minimize ASD symptoms [16,18]. There is increasing evidence that selective induction of COX-2 activity by antigen challenge is suppressed by corticosteroids and selective COX-2 inhibitors [11,18]. These findings suggest that prostanoids play a pivotal role in the recurrence of allergic inflammation, and this recurrence is reduced following inhibition of inducible COX-2 activity. However, additional evidence is required to more thoroughly understand the inhibitory effects of antihistamines on COX-2 activity.

To further elucidate the potential anti-inflammatory mechanism of action of antihistamines on prostanoid formation, the selective activity of therapeutically relevant concentrations of FXF and osthole on COX-2 enzymes was investigated using a cell-free in vitro model, under allergic conditions in children with diagnosed ASD.

We observed PBMC reaction to FXF stimulation and compared this with osthole stimulation, thus identifying whether COX-*2* gene expression and protein level were altered in control and ASD groups. We also measured COX-2 concentration and inhibitory effect of FXF and osthole on COX-2 activity in cultured media.

Based on previous reports, we hypothesized that the immune system and the nervous system are intricately interconnected, COX-2 is related to ASD pathogenesis and osthole can reduce inflammation and ensuing symptoms of ASD. This research focused on the following;

(I) investigating if histamine administration increases COX-2 expression, concentration and activity of COX-2 in cultured media,
(II) investigating whether the administration of the H_1-selective inhibitor FXF causes decrease in histamine levels, and if osthole has the same of better/worse results,
(III) investigating whether the anti-inflammatory effect of FXF and osthole could be due to the inhibition of COX-2,
(IV) assessment of the differences in *COX-2* expression, concentration and activity in healthy and ASD diagnosed children.
(V) determine whether increased/decreased COX-2 system elements occur to the ASD groups or to the allergy group.

2. Materials and methods

2.1. Subjects

Control group comprised of 28 healthy children with no history of behavioral disorders and the study groups consisted of: (1) 29 children diagnosed with ASD with co-existing allergies/asthma; (2) 29 children diagnosed ASD without allergy/asthma (ASD, ICD-10 - F84.0, DSM-5 code 299.00); (3) 30 children diagnosed with allergy/asthma without neuronal dysregulation. Children with fever, infections and skin problems, those taking steroids or antibiotics were excluded from the study. The patients were allowed β2 antagonists when necessary, but no medications were taken 24 h prior to blood collection and all other medications, including anti-histamines, were excluded during the study. Patients were selected based on established criteria: diagnosed asthma, skin prick test, means of cIgE and a-sIgE levels in the blood as well as blood eosinophilia. Demographic and clinical characteristic of the study population are shown in Table 1.

The patients were also recruited by specialists in the Centre for Diagnosis, Treatment and Therapy of Autism at the Regional Children's Hospital in Olsztyn, Poland. The diagnoses were based on the International Classification of Mental and Behavioral Disorders – ICD-10 and DSM-5. ASD in children was identified on the basis of interdisciplinary differential diagnosis including: psychiatric examination excluding mental illness; studies evaluating cognitive parameters in the respondents; neurological examination - EEG, evaluation of reflexes; speech therapy – evaluation study of the development of speech. Also, passive and participatory observation lasting from 6 to 12 months; and the analysis of the documentation: names of parents, the opinions of educational institutions, video. The IQ level was evaluated by the tests of cognitive development: the Leiter test and the Wechsler test (Leiter scale - standard IQ from 70 to 107; Wechsler – standard IQ from 90 to 104). This choice was dictated by the communication abilities of autistic children and their biological age. The Wechsler test was used in older children in full verbal contact with a recognized milder ASD (also known in ICD-10 as Asperger syndrome). The Leiter test was used for younger, unable to talk children with autism. ASD consisted mostly of children with IQ's of 70–104 and 9 children with IQ's of < 70, which indicated intellectual disability. This paper uses the term ASD as defined by DSM-5. Although in Poland, the official diagnostic and statistical classification in medical centers is ICD-10, DSM-5 is widely used to aid in making the diagnosis. Typically developing children were screened for history of neurological, psychiatric and developmental disorders and all were un-medicated (including no psychotropic medication) and in good health at the time of participation. Each patient also has a basic neurological examination and an EEG. As a result of the diagnostic process, patients were afflicted with F84.0 code of ASD in the (ICD-10). Exclusion criteria were known neurological disorders including fragile-X syndrome and tuberous sclerosis, congenital metabolic disorders, chronic infectious diseases such as tuberculosis, acute infectious disease within the last 4 weeks, immunization within the last 8 weeks and immune-modulating medication in the previous four weeks.

Informed consent was obtained from all children's parents and the

Table 1
Demographic data and clinical characteristics of the study population.

Characteristics	Control group	ASD group with co-existing allergy/asthma	ASD group without allergy/ asthma	Allergy/asthma group
Patient's samples, no.	28	29	29	30
Age (y), mean ± SD	7.60 ± 1.93	5.4 ± 1.95	1.06 ± 2.05	4 ± 1.75
Female sex, no. (%)	10 (36%)	8 (28%)	8 (28%)	8 (27%)
Diagnosed allergy, no. (%)	0 (0%)	29 (100%)	0 (0%)	30 (100%)
Moderate/severe asthma, no. (%)	0 (0%)	12 (41%)	0 (0%)	15 (50%)
Skin prick test positivity, no. (%)	0 (0%)	29 (100%)	0 (0%)	30 (100%)
cIgE [IU/ml] mean ± SD	90.13 ± 43.93	638.9 ± 177.2	120 ± 189.2	307.7 ± 130.0
a-sIgE [IU/ml] class	Negative predictive value, class 0	Positive predictive value, class 4–6	Negative predictive value, class 0	Positive predictive value, class 4–6
Blood eosinophilia (mean % of eosinophils in the blood smear) mean ± SD	3.668 ± 1.104	25.83 ± 5.027	4.253 ± 1.564	23.92 ± 5.587
ASD classification				
CARS (*Childhood Autism Rating Scale*) Scale	–	30.0–45.5	30.0–45.5	–
F.84.0 typical childhood ASD, according to the ICD-10	–	21 (73%)	21 (73%)	–
F.84.1 atypical ASD, according to the ICD-10	–	2 (7%)	2 (7%)	–
F.84.5 Asperger Syndrom (AS) according to ICD-10	–	6 (20%)	6 (20%)	–

study was approved by the Local Bioethics Committee (No. 19/2016; 18/5/2016).

2.2. Biological materials

Sample of 5–10 ml peripheral blood was collected from each patient by medical staff at the Regional Children's Hospital in Olsztyn. All biological materials were immediately transported to the laboratory and used in analysis or stored at −80 °C.

2.3. Examined substances

Samples of fexofenadine (FXF), osthole and histamine were obtained from Sigma-Aldrich, ST. Lois, MO, USA.

(1) FXF (PubChem CID: 63002) was dissolved in 8% dimethyl sulfoxide (DMSO, Sigma-Aldrich, ST. Lois, MO, USA) and when it was mixed with cells, the final concentration of DMSO was below 0.1%; thus, not affecting cell viability. For PBMC's stimulation in an in vitro model, we used concentration of 300 ng/ml FXF.
(2) Osthole (PubChem CID: 10228) was dissolved in 96% ethyl alcohol (AbChem, Poland). We used concentration of 300 ng/ml osthole to compare our results with FXF.
(3) Histamine (PubChem CID: 774) was dissolved in RPMI-1640 (Sigma-Aldrich, ST. Lois, MO, USA). For PBMC's stimulation in an in vitro model, we used concentration of 150 ng/ml histamine.
(4) DuP – 697 (PubChem CID: 3177) was obtained from Cayman Chemical (Company, USA). DuP – 697 was dissolved in 96% ethyl alcohol (AbChem, Poland) and we also used concentration of 300 ng/ml to compare our results with FXF and osthole.

All solutions were sterilized through a 0.22 μg/ml filter and stored at 4 °C as stock solutions for later dilution.

2.4. PBMC isolation

Participants' blood was collected directly into tubes containing K_3ETDA (BD, Biosciences) and peripheral blood mononuclear cell (PBMC) isolation began immediately. Raw PBMC's were prepared as in Kordulewska et al. and counted by automatic cell counter – Scepter (Merck Millipore) [14,16–18]. The cells were seeded in 24-well plates at 1×10^6 per well with RPMI-1640 and supplemented with 1% heat-inactivated human AB serum, 1% gentamicin and 0.25% PHA (Roche). Active reagents were added to each well after 24-hour incubation and pure medium formed the control for each substance. Incubation with examined substances was conducted for three days and PBMC suspension was then centrifuged (800 × *g*, 20 °C, 5 min) and cell residue was rinsed twice with Dulbecco's Phosphate-Buffered Saline (DPBS, Invitrogen).

2.5. Analysis of COX-2 gene expression

RNA was isolated from PBMC's as in Kordulewska et al. with TRIzol Reagent (Life Technologies, UK) and the conditions for all Real-Time PCR analysis were optimized at 58–65 °C melting point and primer concentrations before commencing relevant experiments [14,16].

Gene expressions of *COX-2* and housekeeping human β-actin gene (*ACTB*) were examined and *ACTB* was used as the reference gene to normalize differences in total RNA amounts in each sample. Oligonucleotide primers specific to each gene were designed with Primer-BLAST and PCR primers are listed in Table 2.

2.6. Measurement of COX-2 concentration

COX-2 concentrations in medium were evaluated by commercial enzyme – linked Immunosorbent Assay Kit for Prostaglandin Endoperoxide Synthase 2 (PTGS2) (Cloud – Clone Corp; USA) according to the manufacturer's instructions. Briefly, we prepared all reagents, samples and standards according to the manual instructions of the kit. First of all, we added 100 μl of standard or medium with PBMC cells to each well and incubated 1 h at 37 °C. Then aspirated and added 100 μl of prepared Detection Reagent A, and incubated 1 h at 37 °C. After that time, we washed 3 times and added 100 μl of prepared Detection Reagent B. Then incubated 30 min at 37 °C; washed 5 times and incubated 20 min at 37 °C with 90 μl of Substrate Solution and added 50 μl of Stop Solution. The absorbance at 450 nm was read immediately by a microplate reader and the concentration of COX-2 was calculated according to the manufacturer's instructions.

2.7. Assay of COX-2 enzymatic activity

The in vitro inhibitory activity of histamine, FXF, osthole and DuP - 697 on purified COX-2 enzyme was determined using a colorimetric COX inhibitor screening assay kit according to the manufacturer's instructions (Cayman Chemical Company, USA). Briefly, 160 μl assay buffer and 10 μl heme were added to the background wells, while 150 μl assay buffer, 10 μl heme and 10 μl COX-2 enzyme were added to the 100% initial activity wells. Tested substances in 10 μl at final

Table 2
Primers used in PCR; with PCR primer nucleotide sequences used to assay gene expression by Real-Time quantitative PCR.

Gen	Forward primer	Revers primer	Primer depending temperature Real-Time PCR (annealing temperature)	Base pairs
ACTB NM-001101.3	5′-TCC CTG GAG GAA GAG CTA CGA-3′	5′-AGC ACT GTG TTG GCG TAC G-3′	60 °C	194 bp
COX-2 M90100.1	5′-TGG CTA CAA AAG CTG GGA AG-3′	5′-GCT GCT TTT TAC CTT TGA CAC C-3′	63 °C	110 bp

concentrations were added to the sample wells and 10 μl of medium RPMI was added to the background wells. The plate was carefully shaken for a few seconds and incubated for 5 min at 25 °C. The colorimetric substrate solution (20 μl) followed by arachidonic acid (20 μl) were added to each well. The plate was again shaken carefully for a few seconds and incubated for 5 min at 25 °C. The absorbance at 590 nm was read by a microplate reader and the inhibition ratio of COX-2 enzymatic activity was calculated according to the manufacturer's instructions.

2.8. Statistical analysis

All statistical analyses were performed in triplicate by GraphPad Prism version 6.0 (GraphPad Software, Inc., USA); with results presented as mean ± S.E.M. and the mean values between control and ASD groups were compared using:

(1) ANOVA test ($p < 0.05$, 95% confidence interval) *for HRH-1, IL-1RI, EP_2* and *COX-2* gene expression and IL-1β, COX-2 level.
(2) Unpaired *t*-test ($p < 0.05$, 95% confidence interval) with equal S.D. for COX-2 and IL-1β concentration and % COX-2 inhibition.

3. Results

3.1. Basal expression of COX-2 in tested groups

After three days of incubation, the lowest level of mRNA *COX-2* expression was observed in control group, and the highest in ASD group with co-existing allergy/asthma ($p < 0.0001$). The same result was noted in ASD without allergy/asthma ($p < 0.0001$), however the mRNA level was lower. In allergy group we identified the lowest level of all tested groups ($p < 0.001$). In addition, it was recorded that differences between levels of mRNA COX-2 expression in groups with ASD were significant ($p < 0.01$). ASD group with co-existing allergy/asthma had increased basic expression of COX-2 compared to allergy group (Fig. 1).

3.2. COX-2 gene expression

The *COX-2* mRNA effects on cultured cells are presented in Fig. 2A and B. A significant decrease in *COX-2* expression in cultured cells for histamine-treated group was observed compared to untreated ($p < 0.0001$). In ASD groups with and without allergy/asthma, it was noted that FXF and osthole treatment significantly decreased expression of *COX-2* mRNA compared to histamine-treated group ($p < 0.0001$).

In ASD group with allergy/asthma it was observed that mixture of histamine/FXF and histamine/osthole 1:2 (v/v) significantly decreased expression *COX-2* mRNA compared to the group stimulated with histamine ($p < 0.0001$). In addition, cells after incubation with histamine/FXF had significantly decreased expression of *COX-2* mRNA compared to untreated cells.

In ASD group without allergy/asthma we observed that after incubating the cells with histamine and histamine/FXF no significant differences were noted, but after the mixed stimulation the level was increased compared to untreated cells ($p < 0.01$). Incubation of the cells with histamine/osthole 1:2 (v/v) significantly decreased the *COX-2* mRNA expression level compared to cells treated histamine ($p < 0.01$).

In allergy group we noted that cells incubated with FXF showed increased level of COX-2 in comparison to untreated cells ($p < 0.01$) (Fig. 2A).

The statistical differences after active substance stimulation in groups are shown in Fig. 2B. A significant increase of mRNA expression after histamine stimulation was observed compared to control-healthy group. Also, the differences between ASD groups with and without allergy/asthma ($p < 0.01$), and allergy group with ASD with co-existing

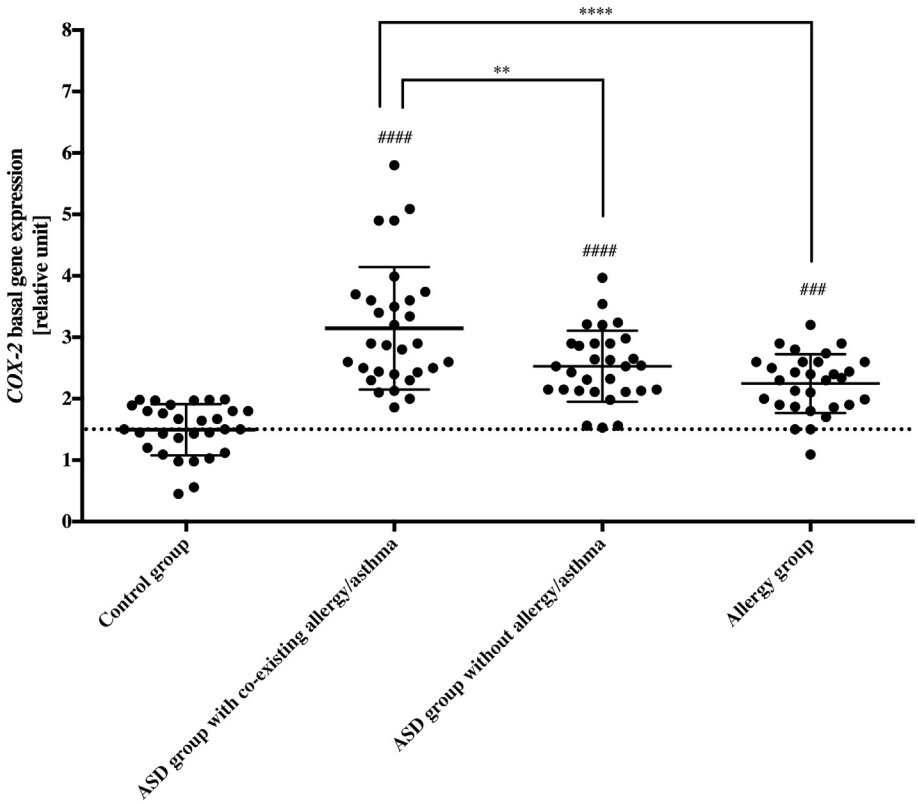


Fig. 1. Basal expression of *COX-2* mRNA in cultured PBMC from control group, ASD group with co-existing allergy/asthma, ASD group without allergy/asthma and allergy group. Statistically significant differences between the control and tested sample are directly above the error bar: $^{###}p < 0.001$, $^{####}p < 0.0001$ vs. control; $^{**}p < 0.01$, $^{****}p < 0.0001$ vs tested group.

allergy ($p < 0.0001$) were noted. After FXF stimulation, the differences were only noted in ASD group with co-existing allergy/asthma ($p < 0.05$) and allergy group ($p < 0.01$) in comparison to healthy-control children. After 3 days incubation osthole indicated differences in allergy group compared to healthy-control participants. Histamine/FXF 1:2 (v/v) showed significantly differences in ASD groups ($p < 0.001$ and $p < 0.0001$, respectively co-existing allergy and without allergy) and allergy group compared to healthy-control children. Histamine/osthole 1:2 (v/v) indicated differences only in ASD group without allergy/asthma in comparison to healthy group ($p < 0.05$).

3.3. Concentration of COX-2 in medium

Fig. 3A shows COX-2 concentrations in media for the control and ASD groups. A statistically significant decrease in cells stimulated histamine in all tested groups compared to control group was observed ($p < 0.0001$). In control-healthy group it was shown that the following treatments resulted in decreased concentration of COX-2 compared to untreated cells: FXF ($p < 0.01$), histamine/FXF 1:2 (v/v) and histamine/osthole 1:2 (v/v) ($p < 0.0001$). In significantly differences FXF and osthole increased COX-2 level compared to histamine stimulation cells ($p < 0.01$ and $p < 0.0001$, respectively).

In ASD with co-existing allergies the cells after all active compound-treatments in this study showed significantly increased COX-2 concentration levels compared to untreated cells ($p < 0.0001$). Furthermore, FXF and osthole caused significant decrease of COX-2 after incubation with histamine (0.05 and $p < 0.0001$, respectively). Similar results were noted in ASD group without allergy/asthma.

In allergy group we noted that osthole and a mixture of histamine/osthole 1:2 (v/v) statistically decreased COX-2 level in comparisons to untreated cells ($p < 0.001$).

In Fig. 3B we showed statistical differences in tested groups after active substances tested. We noted only statistical differences in COX-2 levels in all tested groups compared to healthy-control participants, no differences between analyzed tested groups had been found.

3.4. The inhibitory effects of COX-2

Data presented in Fig. 4A show the inhibitory effects of FXF, osthole and DuP – 697 in RPMI medium without PBMC cells. Human COX-2 activity was significantly inhibited compared to pure enzyme by FXF ($p < 0.001$), osthole, DuP – 697 ($p < 0.0001$) and the mixture of histamine /FXF, /osthole and /DuP – 697 1:2 (v/v) ($p < 0.05$).

A similar situation was observed for PBMC cells with incubated substances in control and ASD groups (Fig. 4B). It was noted that all active, tested substances significantly inhibited COX-2 activity compared to pure medium. Furthermore, FXF and osthole significantly decreased COX-2 activity in comparison to histamine stimulation. Moreover, the inhibitive action of osthole was more potent than that of the FXF stimulation. These results demonstrated that osthole significantly inhibited the activity of histamine, which may be responsible for secretion of proinflammatory mediators and be responsible for anti-inflammatory effect.

In Fig. 4C significant differences in activity of COX-2 in all tested groups in comparison to healthy-control participants were noted. Additionally, differences between ASD ($p < 0.001$) groups and allergy group ($p < 0.0001$) were observed. After incubation with FXF significant differences between ASD with co-existing allergies/asthma and control-healthy children ($p < 0.0001$), and between ASD groups tested ($p < 0.01$) were seen. Osthole indicated significantly differences between ASD without allergy/asthma and ASD with co-existing allergy/asthma ($p < 0.0001$) and allergy group ($p < 0.001$). Besides that, differences between ASD group with allergy/asthma and allergy group in comparison to control-healthy children ($p < 0.0001$) were noted. Histamine/FXF mixture 1:2 (v/v) indicated differences in ASD group with co-existing allergy/asthma compared to healthy participants ($p < 0.0001$) and ASD group without allergy/asthma ($p < 0.01$). The same result was obtained after histamine/osthole stimulation, furthermore the differences in levels of activity of COX-2 in allergy group

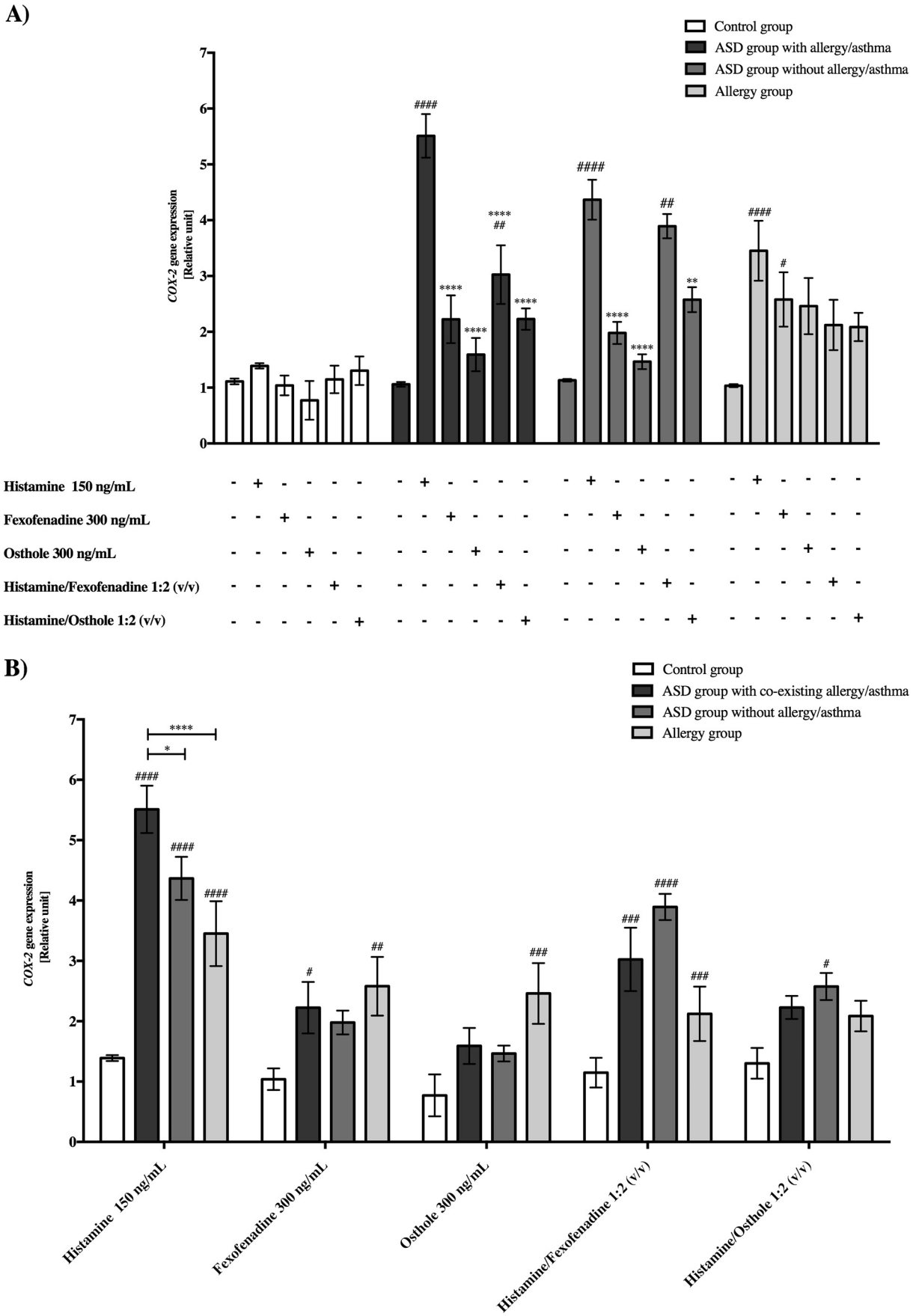


Fig. 2. Comparison of mRNA *COX-2* gene expression changes in control, ASD groups and allergy group. PBMC's under the influence of histamine, FXF, osthole, histamine/FXF and histamine/osthole. Control is COX-2 gene expression in native cells; presented as 1. Data are presented as the mean ± S.E.M. $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ vs. control; $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. treated histamine cells.

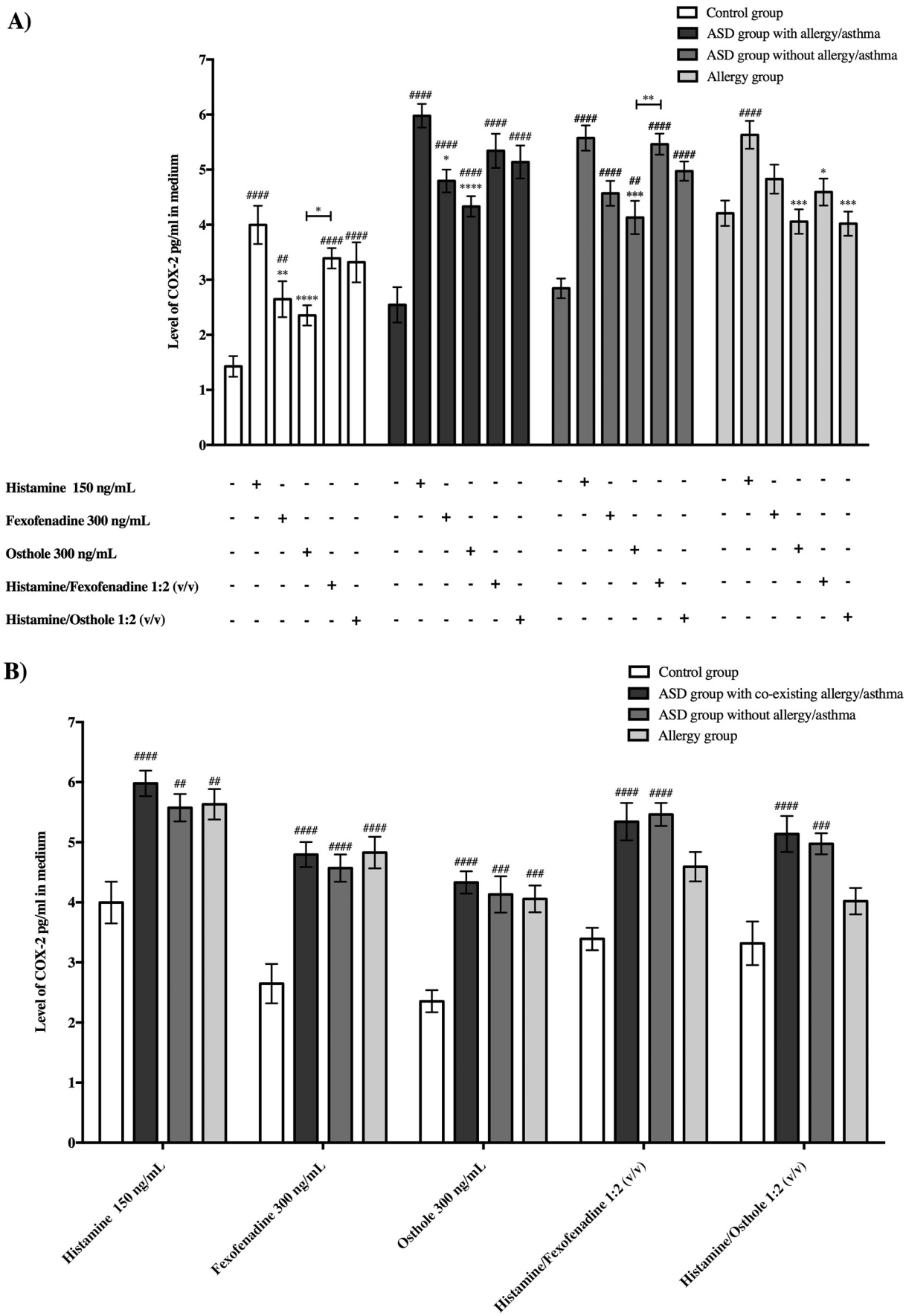
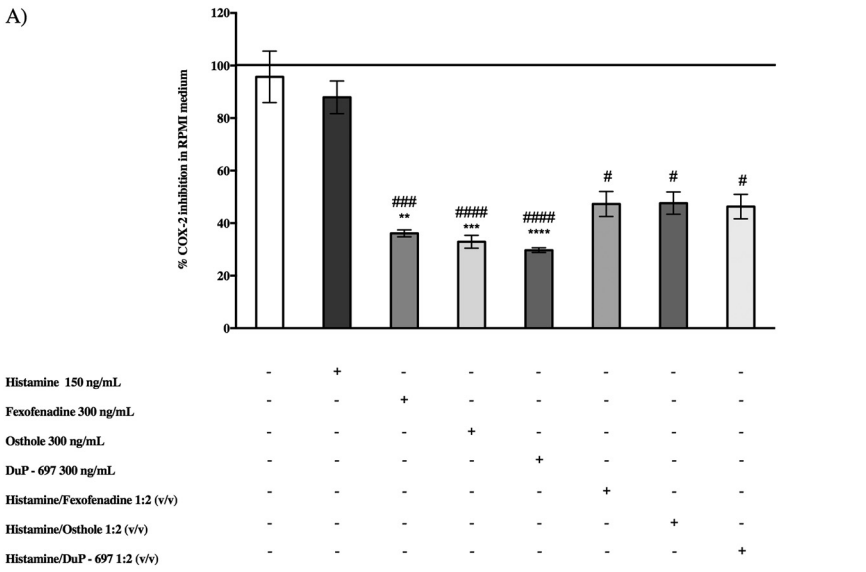
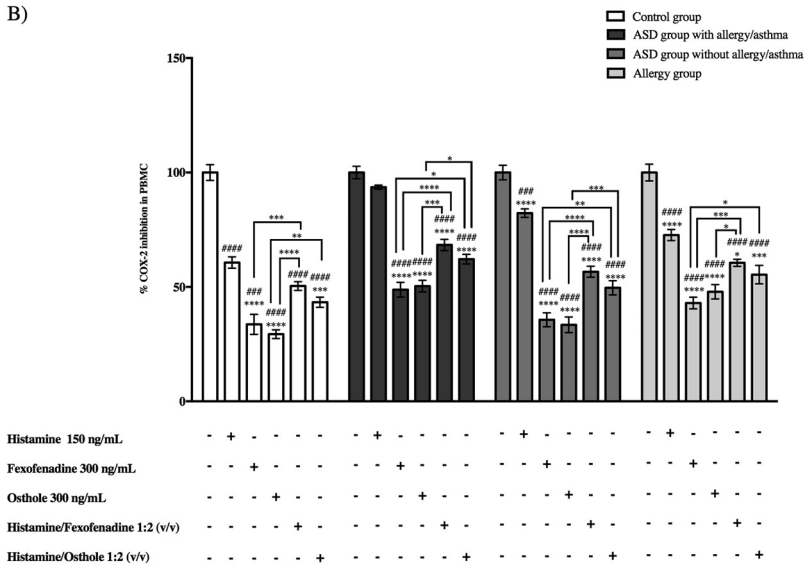


Fig. 3. Influence of tested substances on PBMC COX-2 secretion. COX-2 secretion from PMBC was measured in pure medium as the control and media after the treatment with tested substance. Data are presented as the mean ± S.E.M. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.0001$, $^{\#\#\#\#}p < 0.0001$ vs. control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. treated histamine cells.





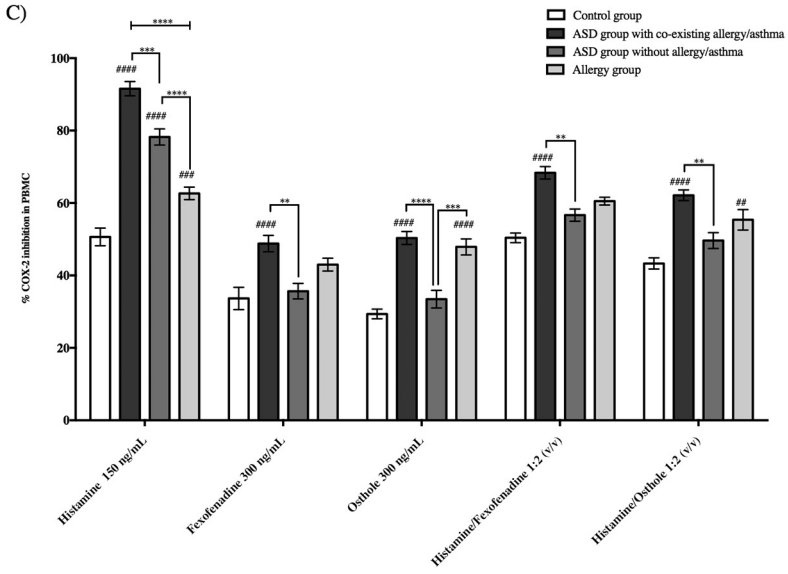


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Fig. 4. Inhibitory effect of tested substances. A) In RPMI medium. B & C) In PBMC from control and ASD group, respectively. Data are presented as the mean ± S.E.M. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ vs. control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. treated histamine cells.

compared to control-healthy participants ($p < 0.01$) were noted.

4. Discussion

ASDs are a group of a developmental disorder which usually manifests itself in the first years of life. Children with ASD exhibit abnormal behavior in social interaction and communication problems, and global statistics indicate that ASD incidence is increasing annually and approaching epidemic proportions [19–21].

Baio recorded that ASD prevalence is strongly associated with male gender [22]. This was further confirmed by our research group (Table 1). Although the causes of ASD still remain obscure, it is clear that its development is affected by genetic and autoimmune factors, metabolic disorders, and epigenetic changes, and dependent on environmental and nutritional factors [23] or even after maternal infections during pregnancy [24], or maternal immune activation [25]. New published date also link environmental effects on mTOR pathway and possible contribution to ASD symptoms. Maternal illness or immune activation during pregnancy is one of the best-characterized environmental exposures that increase the risk of ASD [25]. Although there are several plausible mechanisms, inflammatory pathways can signal through mTOR. In animal models, mTOR activity is also acutely responsive to seizures [26]. Furthermore, rapamycin treatment in an animal model of neonatal seizures could prevent subsequent development of behavioral abnormalities [27].

4.1. ASD and allergy – antiallergic therapies (FXF – osthole)

Retrospective analysis of ASD children evaluated in the pediatric allergy/immunology clinic indicated that allergic diseases are prevalent in ASD children, with frequency equivalent to the general population [10]. Unfortunately, due to their impaired expressive language, abnormal behavior and limited tolerance to diagnostic measures compared to typically developing children, diagnosing allergic diseases is more challenging in ASD children. One author's experience is that ASD children are generally under-diagnosed and under-treated for allergic and common non-allergic childhood diseases [9]. In our study we also examined how anti-allergic FXF medication affected PBMC's isolated from ASD children with diagnosed allergy/asthma or without and the changes in *COX-2* gene expression, concentration and inhibition of COX-2 in medium. Numerous findings have recently confirmed that a great number of ASD children have accompanying allergies, and we propose that anti-allergy treatment can alter gene expression and secretion of inflammatory factors in those children and thus improve their mental health and general well-being.

Our previous studies compared osthole and FXF anti-histamine/anti-allergic effects in allergic and non-allergic patients [14,16–18] and results inspired us to examine these two compounds in the ASD group.

4.2. COX-2 gene expression

Wei and Hemmings, reported that hippocampal COX-2 level correlates with neuronal activity and influences memory consolidation [28], and Claycomb et al., demonstrated that COX-2 contributes to synaptic connectivity changes associated with epileptogenic by up-regulating hippocampal mRNA and protein expression during the development [29].

COX-2 is readily induced as an immediate early gene in response to cytokines, growth factors, phorbol esters and bacterial lipopolysaccharides, and is therefore thought to be the source of the prostaglandins that mediate the inflammatory response [11]. As previously data indicated cytokine aberrations in ASD have highlighted a possible relationship between cytokine aberration and ASD. Altered cytokine levels may facilitate the identification of ASD subtypes that share similar traits and profiles, as well as provide biological markers that facilitate monitoring of the benefits of active treatments over the time-course of clinical trials [4]. What is more, new data indicated abnormal regulation of the autocrine loop components that regulate the COX-2 pathway in allergy children. Abnormal regulation of the autocrine loop regulating the COX pathway may increase PGE2 production in allergic patients. This loop includes IL-1RI, COX-2, EP2 and PGE2. High EP2 receptor expression therefore has a central role in the dysregulated autocrine loop [18]. COX-2 is an inducible enzyme and we suggest that children with ASD affect biosynthesis of inflammatory prostaglandins which influence ASD susceptibility and progression. We also considered that aberrant COX-2 activity affects dysfunctional learning and memory and is quite likely related to the high prevalence of seizures in autism. However, this requires confirmation in larger research populations and elucidation of the relationship between COX-2 and ASD-afflicted children.

Firstly, our results demonstrated that the basal expression levels of *COX-2* mRNA were lower in control group compared to ASD and allergy groups. The highest basal expression of COX-2 mRNA was observed in ASD group with co-existing allergy/asthma but obtained results were significantly different from allergy group. This might mean that not only allergic patients have their connection in overexpression of COX-2, but also ASD patients. The results obtained from ASD group without allergy/asthma clearly supported this hypothesis, as we observed higher expression level of COX-2 in this ASD group compared to allergy group. There is no documented analysis of *COX-2* mRNA expression in controls and ASD-afflicted following stimulation by the tested substances, but COX-2 plays a key role in inflammation. O'Banion's study provided direct evidence that COX-2-dependent production of prostanoids, especially PGE_2, is prominent in inflamed tissues [30]. Our analyses confirmed O'Neill and Ford-Hutchinson's assertion that *COX-2* mRNA gene expression is normally co-expressed at detectable levels in human tissues [31] and we demonstrated that ASD group cells cultured with histamine registered increase *COX-2* expression compared to controls. Furthermore, children with ASD without allergy/asthma showed similar results. This suggests that ASD-afflicted children have dysfunctional histamine response, which subjects them to allergic reaction (Fig. 2).

It is interesting that cells cultured with FXF and osthole exhibit no statistically significant differences between controls and test subjects (Fig. 2). Moreover, FXF and osthole decreased *COX-2* expression compared to histamine ($p < 0.0001$). This was also established in cells stimulated by combined 1:2 (v/v) histamine/FXF and histamine/osthole, thus confirming stronger effect of FXF and osthole than histamine.

Ganey et al. reported that *COX-2* gene expression is induced by inflammatory and transcription factors, and that this is inhibited by IL-4 and IL-10 [31,32]. Our results support these assertions, where histamine, as an activator of allergic inflammation, increased *COX-2* gene expression in the both ASD groups. Ariasnegrete et al. also observed this relationship, where macrophages had 8-fold increased *COX-2* expression after stimulation by lipopolysaccharides (LPS). These authors also studied dexamethasone (DEX), an effective corticosteroid for rheumatic abnormalities, a number of skin diseases, and severe allergies and asthma. Their reported decrease in *COX-2* mRNA expression in DEX stimulated cells is comparable to our results where FXF and osthole decreased PBMC COX-2 expression compared to histamine-treated cells [33]. Similar results are presented by Payvandi et al., where PBMC cells stimulated by LPS, IL-1β and TNF-α had increased *COX-2* expression

[34]. Moreover, Kordulewska et al. observed the same tendency in cultured PBMC's in allergic patients [14].

Although histamine increased *COX-2* mRNA expression in our study groups compared to controls, FXF and osthole inhibited this increased expression. A similar result was observed in cells cultured with histamine/osthole, where the combination decreased *COX-2* mRNA expression compared to the effect from histamine alone.

4.3. Concentration of COX-2 in medium

There is no available literature data describing COX-2 secretion in PBMC's isolated from healthy and ASD children, but our study confirmed COX-2 concentration in the media (Fig. 3). PBMC's stimulated by histamine also registered significant statistical increases in both tested group and Cianchi et al. reported that prostaglandin E2 (PGE2), the main product of COX-2 activity, incites the molecular machinery of allergic inflammation [35]. To the best of our knowledge there is no previous study of histamine and COX-2 activity interaction in allergy and we provide the first demonstration that exogenous histamine increases COX-2 protein secretion in PBMC from both control and ASD groups. Our results also indicate that osthole prevents histamine-induced COX-2 overexpression in all groups and that the selective H_1 receptor antagonist, FXF, suppresses it. In addition, Sousa et al. reported that non-steroidal anti-inflammatory drugs inhibit COX-2 activity [36]. This supports our conclusions that osthole statistically significantly decreases COX-2 secretion in cultured cells and that this will benefit anti-inflammatory therapy.

4.4. The inhibitory effects of COX-2

Using a cell-free in vitro COX-2 screening assay, it was found that COX-2 was significantly inhibited by FXF, osthole and the reference compound DuP – 679. DuP – 679 is a member of the diaryl heterocycle group of selective COX-2 inhibitors. Furthermore, we did not observe statistical differences between inhibition of DuP – 679 and FXF or osthole (Fig. 4A, B, C). These findings indicated that there may be the same models of action of osthole, FXF like DuP – 673 on the COX-2 inhibitory effect. This finding supports the additional pharmacological activity of FXF and confirms our earlier hypotheses about the anti-allergic effect of osthole.

Arachidonic acid metabolism gives rise to various eicosanoids (prostaglandins and leukotrienes), which can mediate several important physiological and pathological functions, and are also involved in the pathogenesis of many inflammatory diseases, including asthma, allergic rhinitis, chronic hyperplastic rhinosinusitis and nasal polyposis. Evidence has shown that COX inhibition is due to inhibition by an antiallergic prostaglandin, most likely PGE_2, which has been shown to downregulate inflammation by inhibiting the expression of both inflammatory mediators and their receptors [11].

It is acknowledged that COX-2 is induced in inflammatory cells such as monocytes and macrophages following stimulation by cytokines, mitogens, serum and endotoxins [37]. Moreover, O'Banion reported that COX-2 is expressed in many central nervous system cell types (CNS) including spinal cord neurons, and other authors indicated that inflammation initiates COX-2 induction leading to release of prostanoids which sensitize peripheral nociceptor terminals and produce localized pain hypersensitivity [30]. Peripheral inflammation also generates pain hypersensitivity in neighboring uninjured tissue because of increased spinal neuron excitability and this induces a syndrome with diffuse muscle and joint pain, fever and lethargy. Samad et al. also showed that COX-2 is involved in CNS responses by establishing widespread induction of COX-2 expression in spinal cord neurons and other CNS regions which elevates prostaglandin E2 (PGE2) levels in cerebrospinal fluid [38].

Animal studies have shown that after allergic stimulus, COX enzymes (particularly COX-1), play a protective role in regulating airway function and airway inflammation, which may be elicited through inhibition of the development of T-helper-2-mediated airway inflammation [39]. Drugs that are more potent inhibitors of COX-1 than COX-2 precipitate asthma attacks in sensitive patients, whereas drugs that inhibit COX-2 more than COX-1 can cause rhinorrhea and mild asthma at high doses [40]. However, high specificity COX-2 inhibitors are well tolerated by patients with aspirin-sensitive asthma and are used in the treatment of inflammation in these patients [11].

Animal studies have shown the role of COX-2 in allergic diseases and the importance of selective COX-2 inhibitors in the relief of allergic inflammation. In an allergic inflammation model, expression of COX-2 mRNA was induced after allergen provocation in the lungs of sensitized guinea pigs and was followed by an increase in the level of COX-2 protein and enzymatic activity [41].

4.5. Correlation between COX-2 gene expression and concentration of COX-2 in medium

We observed a correlation between control group *COX-2* gene expression and its concentration in medium. This relationship is reported for the first time, and it highlights abnormal COX expression, concentration and activation in autistic children. It also underlies our suggestion that activation of *COX-2* expression mediates COX-2 oversecretion by histamine in healthy children's PBMC's.

5. Conclusions

The hallmark heterogeneity of ASD is a key reason for the focus of researchers on the identification of potential biological measures as a means of describing subsets within ASD, and thereby facilitating the targeting of more individualized therapies. This study shows that FXF and osthole exhibit selective COX-2 enzyme inhibitory activity. The selective COX-2 activity of osthole may explain further the anti-inflammatory properties of osthole in relieving congestion in allergic rhinitis, and as distinctive effects between FXF and osthole were observed, individual antihistamines may have different modes of action via the COX enzyme system.

List of abbreviations

ASD	autism spectrum disorder
DSM-5	Diagnosis and Statistic Manual 5
PBMC's	peripheral blood mononuclear cells
FXF	fexofenadine
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
NK cells	Natural Killers cells
ICD-10	International Classification of Mental and Behavioral Disorders
EEG	neurological examination
HRH-1	histamine receptor 1

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Availability of data and materials

The figures datasets analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

Formal analysis, Natalia Kordulewska and Małgorzata Moszyńska; Investigation, Natalia Kordulewska; Methodology, Natalia Kordulewska; Supervision, Elżbieta Kostyra, Barbara Chwała and Beata Jarmołowska; Writing – original draft, Natalia Kordulewska; Writing – review & editing, Anna Cieślińska and Ewa Fiedorowicz.

Ethical approval and content to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Competing interests

None of the authors have reported any financial interests or potential conflicts of interest. None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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