



Pulmonary, gastrointestinal and urogenital pharmacology

Cytokine production by PBMC and serum from allergic and non-allergic subjects following in vitro histamine stimulation to test fexofenadine and osthole anti-allergic properties

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ABSTRACT

FXF is a third-generation antihistamine drug and osthole is assumed a natural antihistamine alternative. This paper compares peripheral blood mononuclear cell (PBMC) incubation with FXF and osthole, by studying FXF, osthole and histamine cytokine secretion in PBMC in vitro cultures. Mabtech kits determined the interleukins IL-1 β , IL-4, IL-10, IL-13 and TNF- α . The influence of the above active substances on cytokine secretion in PBMCs and serum was assessed: cytokines were IL-1 β , IL-4, IL-10, IL-13 and TNF- α ; and cytokine levels secreted by untreated PBMCs in pure culture medium formed the absolute control (ctrl).

We determined that osthole affects PBMC cytokine secretion to almost precisely the same extent as FXF (IL-1 β , IL-4, IL-10 and TNF). In addition osthole had greater IL-13 blocking ability than FXF. Moreover, we observed significantly decreased IL-4 level in histamine/osthole treatment compared to histamine alone. Meanwhile, FXF not significantly decrease the level of IL-4 increased by histamine. This data indicates osthole's strong role in allergic inflammation.

All results confirm our hypothesis that osthole is a natural histamine antagonist and therefore can be beneficially used in antihistamine treatment of conditions such as allergies.

1. Introduction

The link between allergic diseases and abnormal patterns of immune development has stimulated efforts to define the precise patterns of cytokine dysregulation associated with specific atopic phenotypes (Sismanopoulos et al., 2012). The immune system has developed efficient peripheral tolerance mechanisms to avoid chronic cell activation and inflammation against nonpathogenic antigens from ingestion, inhalation and through the skin (Yan and Hansson, 2007).

Allergic diseases are caused by an aberrant immune response mediated through a key effect for the Th2 cell and an associated cytokine pattern including interleukins (ILs) -4, -5 and -13 (Gabay et al., 1997; Hobbs et al., 1998; Mosmann, 1996; Ying et al., 1991). Clinical manifestations of mast cell- and basophil-dependent allergic reactions include not only rapidly evolving, immediate type reactions, but also more protracted "late-cutaneous" reactions which are mast cell dependent and associated with an influx of neutrophils, macrophages and activated lymphocytes and eosinophils (Bochner et al., 1995;

Hoekstra et al., 1997; Prescott et al., 1998).

Rapidly released mast cell mediators and numerous cytokines produced by these cells are reported to induce and sustain this response. Cytokines produced by human mast cells include molecules initiating and boosting the TH-2 type immunological reactions, and also a broad spectrum of pro-inflammatory, growth-promoting and chemotactic responses for diverse cell types. These mediators include ILs-1, 3, 4, 5, 6, 10, 13 and 16, tumor necrosis factor (TNF)- α , granulocyte macrophage colony stimulating factor (GM-CSF), transforming growth factor (TGF)- β , platelet derived growth factor (PDGF), special isoforms of vascular endothelial growth factor (VEGF), nerve growth factor (NGF), chemokines IL-8 and I 309, and macrophage chemotactic protein (MCP)-1: "regulated-upon-activation normal T cell expressed and secreted" (RANTES), macrophage inhibitory proteins (MIP)-1a and b and lymphotactin (Borish et al., 1996; Bradding et al., 1994; Gabay et al., 1997; Lummus et al., 1998; Zipperlen et al., 2005; Metcalfe et al., 1997). *In vitro* and animal studies suggest a cytokine role in initiation of allergic inflammation (Olsen et al., 2004).

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Osthole is a natural coumarin, first derived from the *Cnidium* plant, *Fructus Cnidii*, and it is reported to strengthen the immune system, enhance male sexual function and relieve rheumatic pain. (Hoult and Pava, 1996; Wang et al., 2007). Modern research suggests that osthole also has antioxidant, anticancer, anti-inflammatory and immunomodulatory properties (Fiorentino et al., 1991; Hampe et al., 1999; Pyo et al., 2003; Simpson and Jarvis, 2000). Hence, we presume that osthole inhibits secretion of pro-inflammatory IL after allergic inflammation.

To test this hypothesis, we measured cytokine IL-1 β , IL-4, IL-10, IL-13 and TNF- α secretion in medium and in the serum of allergic and non allergic patients after PBMC stimulation by histamine to determine fexofenadine (FXF) and osthole anti-allergic properties. We also compared the levels of measured IL between histamine and histamine/FXF and histamine/osthole to investigate FFX and osthole anti-histamine effects. A very important by-product of these results; was to discover if osthole has the same effect as fexofenadine in experimental conditions; and hence, can osthole equal beneficial effect of FFX and replace it in clinical practice?

2. Material and methods

2.1. Study participants

Peripheral blood mononuclear cells (PBMC's) were collected from 30 healthy people (age: $M=30.90$ years, $SD=4.95$ years) (control group) and 30 people diagnosed with allergy (age: $M=31.10$ years, $SD=4.94$ years). People with fever, infections and skin problems, those taking steroids or antibiotics and current or previous smokers were excluded from the study. The patients were allowed β_2 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. All patients underwent skin tests for allergies and had their blood tested for IgE antibodies; with all results positive in the study group and negative in the control group. All participants gave written informed consent, and our study was approved by the local Ethics Committee.

2.2. Chemicals

Fexofenadine (FXF; PubChem CID: 63002), osthole (PubChem CID: 10228) and histamine (PubChem CID: 774) were obtained from Sigma-Aldrich, and all were prepared as in Kordulewska et al. (2015).

2.3. PBMC isolation

Subject blood was collected in K_3 ETDA tubes (BD, Biosciences) and PBMC isolation began immediately. Cell collection by Histopaque reagent (Sigma) was as in Kordulewska et al. (2015). PBMC's were counted by Scepter automatic cell counter (Merck Millipore) and seeded for up to 3 days in 24-well plates in 1×10^6 /0.5 ml of RPMI-1640 (Sigma) containing 1% gentamicin, 1% human AB serum, and 0.25% phytohaemagglutinin (PHA, Roche); at 37 °C in humidified 5% CO_2 . PBMC's were in medium alone or with histamine, FFX and osthole in concentrations of 150 ng/ml histamine and 300 ng/ml FFX and osthole. These concentrations were chosen because this is the FFX human serum level following its administration; and similar osthole concentrations were required for comparison. The PBMC suspension was then centrifuged at 800g and 20 °C for 5 min and the cell residue was rinsed twice with Dulbecco's phosphate-buffered saline (DPBS, Invitrogen). The supernatant and plasma were collected and stored at -80 °C for further analysis.

2.4. Cytokine determination

Commercial ELISA Diaclone kits determined interleukin IL-1 β , IL-

10 and tumor necrosis factor (TNF- α) output and Mabtech kits determined interleukin IL-4 and IL-13, via quantitative sandwich immunoassay; with kits used according to manufacturer instructions. Triplicate samples were run and the results were equalized by comparison with standard curves expressed in pg/ml.

2.5. Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). Results are presented as mean $\pm$ S.E.M., and mean values between the control and allergic groups were compared using:

1. ANOVA test ($P < 0.0001$) for cytokine secretion in PBMC
2. unpaired t test ($P < 0.0001$) with equal S.D. for IgE and cytokine secretion in plasma
3. Pearson's correlation ($P < 0.0001$) correlation between IgE and cytokine secretion in plasma.

3. Results

The influence of the above active substances on cytokine secretion in PBMC's and serum was assessed: cytokines were IL-1 β , IL-4, IL-10, IL-13 and TNF- α , and cytokine levels secreted by untreated PBMC's in pure culture medium formed the absolute control.(ctrl).

3.1. Secretion of cytokines in PBMC

3.1.1. IL-1 β secretion

The lowest levels of IL-1 β with all examined substances were observed in the controls (Fig. 1A). PBMC incubation with histamine had the greatest IL-1 β increases in the control and study groups (Fig. 1A and B). In contrast, PBMC incubation with FFX had the lowest IL-1 β in the control group (Fig. 1A and B - significance in control vs. study group $P=0.0006$). This same result was observed in cells cultured in the pure medium control; where osthole, histamine/FXF and histamine/osthole in the control vs. study group, ctrl in control vs. study group indicated significantly increased $P < 0.0001$, $P=0.0001$, $P=0.0002$ and $P=0.0001$; respectively).

No statistically significant differences in examined substances were established in the control and study groups (Fig. 1B); and no statistically significant differences between histamine/FXF vs. histamine/osthole were determined (Fig. 1C).

3.1.2. IL-4 secretion

Histamine, FFX and osthole caused statistically significant increase in PBMC IL-4 secretion in the study group compared to the control group (Fig. 2A). While no statistical significance was observed in IL-4 secretion in the control group (Fig. 2B), histamine had significantly increased study group IL-4 compared to controls (Fig. 2B). FFX and osthole significantly decreased IL-4 secretion in cultured cells compared to histamine ($P < 0.01$ and $P < 0.001$, respectively), and we also observed a similar result in combined histamine/osthole treatment where cells decreased IL-4 secretion compared to histamine ($P < 0.01$) (Fig. 2B and C).

3.1.3. IL-10 secretion

All examined substances caused statistically significant increase in PBMC IL-10 secretion in the study group compared to the control group ($P < 0.0001$ - Fig. 3A). PBMC incubation with osthole had the greatest IL-10 levels in the study group, compared to histamine which had the lowest at $P < 0.01$ (Fig. 3A and B). While no statistical differences were noted in the tested control group substances (Fig. 3B), histamine effect was noticeable in the study group but not in the pure medium control. Histamine statistically depressed the level of IL-10 (ctrl vs. histamine $P=0.0003$), but PBMC's cultured with FFX

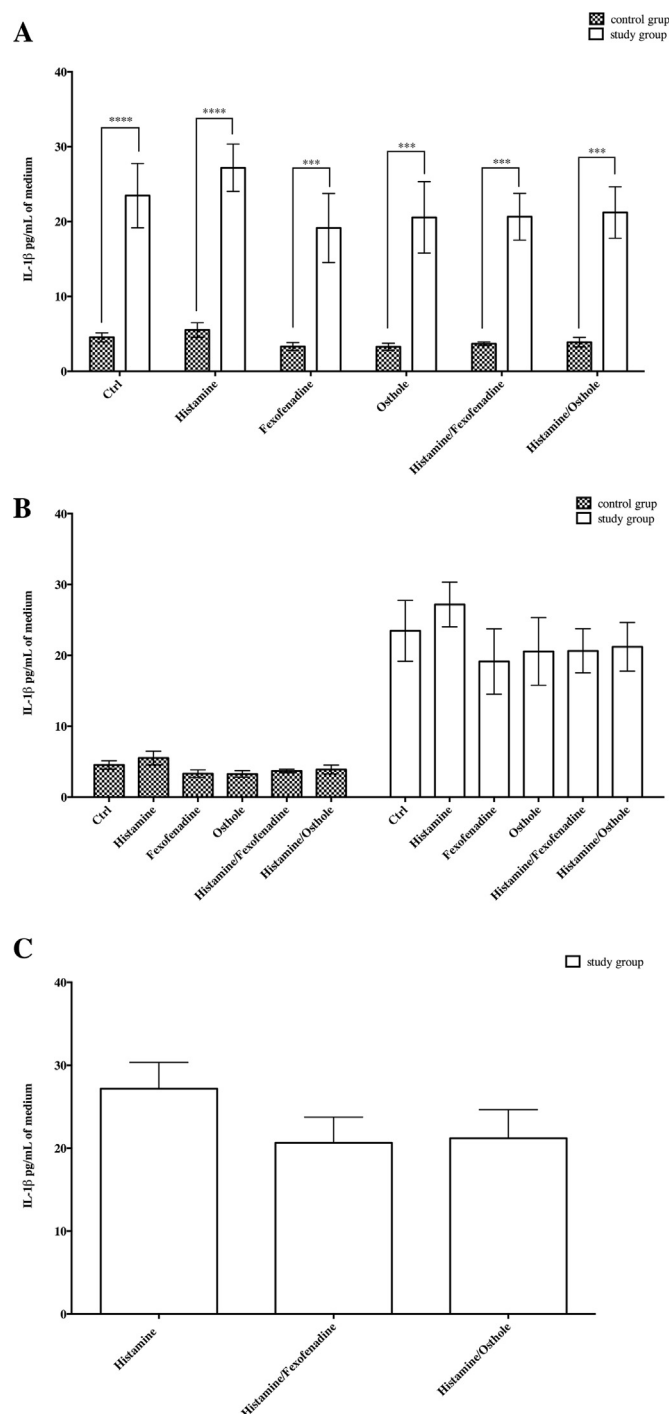


Fig. 1. The influence of tested substances on PBMC IL-1 β interleukin secretion; (A) between the study and control group; (B) within the group study and control and (C) histamine vs. histamine/FXF vs. histamine/osthole in the study group. Here, PBMC IL-1 β secretion in pure medium forms the control, and tested substance secretions are expressed as pg/ml $\pm$ S.E.M. ns $P > 0.05$, *** $P < 0.001$, **** $P < 0.0001$, $n=30$.

and osthole had increased IL-10 level compared to those cultured in histamine ($P=0.0041$ and 0.0024 , respectively).

Our most important results were (1) the very similar activity of osthole and FFX and (2) no statistically significant differences were recorded when PBMC's were incubated with the control and with histamine/FXF and histamine/osthole (Fig. 3B).

3.1.4. IL-13 secretion

The following analyses were confirmed; (1) while no statistical

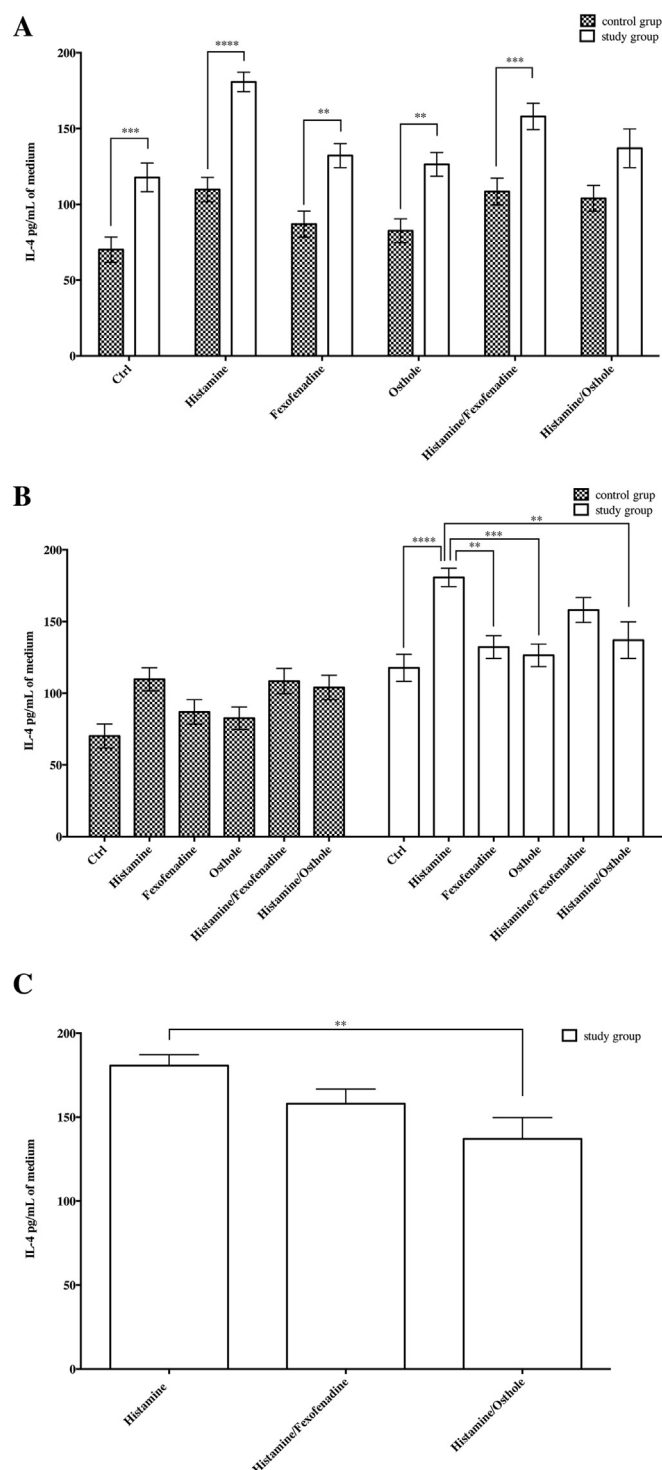


Fig. 2. The influence of tested substances on PBMC IL-4 interleukin secretion; (A) between the study and control group; (B) within the group study and control and (C) histamine vs. histamine/FXF vs. histamine/osthole in the study group. Here, PBMC IL-4 secretion in pure medium forms the control, and tested substance secretions are expressed as pg/ml $\pm$ S.E.M. ns $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, $n=30$.

differences were noted between tested substances in the control group (Fig. 4B), statistically significant increased IL-13 secretion was observed in study group PBMC's compared to controls ($P < 0.0001$) (Fig. 4A); (2) study group histamine, FFX and their combination statistically increased IL-13 secretion compared to controls ($P < 0.0001$ – histamine and histamine/FXF while $P < 0.001$ FFX) (Fig. 4B) and (3)

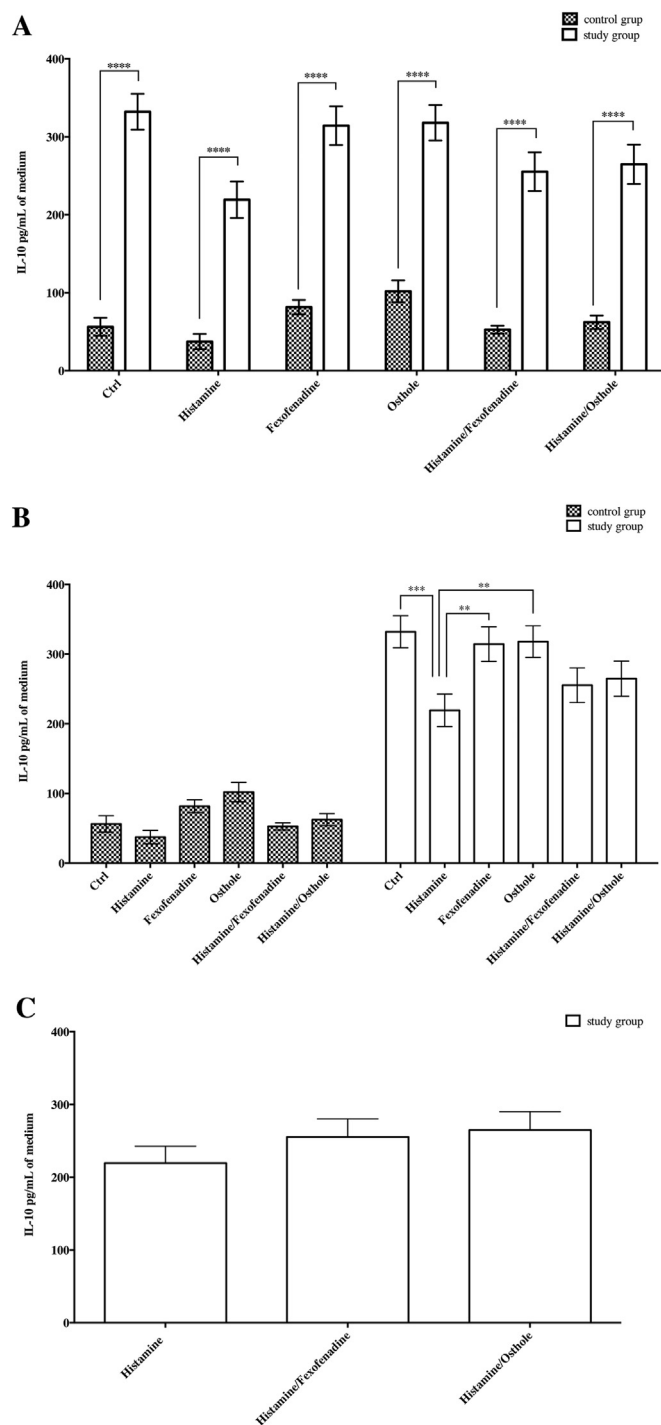


Fig. 3. The influence of tested substances on PBMC IL-10 interleukin secretion; (A) between the study and control group; (B) within the group study and control and (C) histamine vs. histamine/FXF vs. histamine/osthole in the study group. Here, PBMC IL-1 β secretion in pure medium forms the control, and tested substance secretions are expressed as pg/ml $\pm$ S.E.M. ns $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, $n=30$.

in contrast, FXF and osthole statistically decreased the level of IL-13 compared to histamine ($P < 0.01$ and $P < 0.0001$, respectively). It is interesting here that only combined histamine/osthole statistically decreased IL-13 secretion compared to histamine ($P < 0.0001$).

3.1.5. TNF- α secretion

We observed statistically higher level of TNF- α secretion in the study group compared to the control group ($P < 0.0001$ - Fig. 5A).

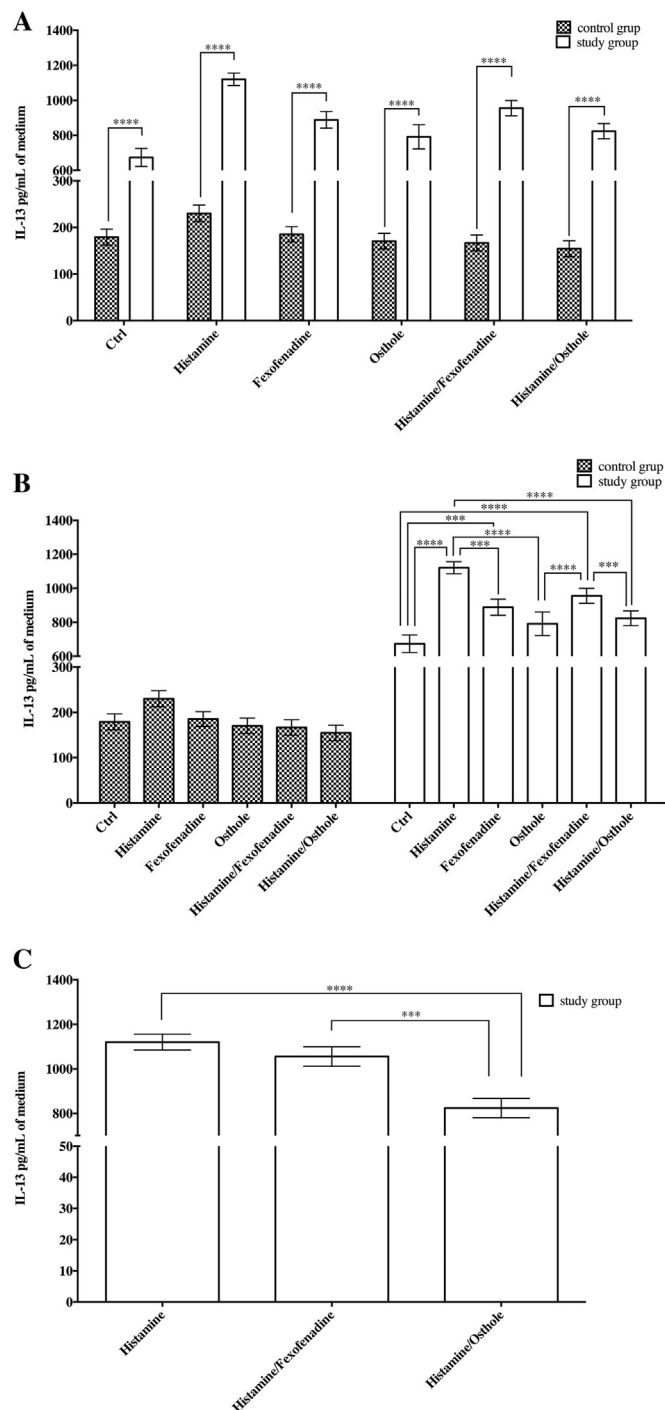


Fig. 4. The influence of tested substances on PBMC IL-13 interleukin secretion; (A) between the study and control group; (B) within the group study and control (C) histamine vs. histamine/FXF vs. histamine/osthole in the study group. Here, PBMC IL-1 β secretion in pure medium forms the control, and tested substance secretions are expressed as pg/ml $\pm$ S.E.M. ns $P > 0.05$, *** $P < 0.001$, **** $P < 0.0001$, $n=30$.

We noted the highest level of TNF- α in the medium control; and control group decreased significantly in the presence of both FXF and osthole ($P=0.0021$ and $P=0.0022$, respectively - Fig. 5B). However, the study group exhibited no statistical differences between the tested substances (Fig. 5B); with TNF- α level maintained at a similar level in each incubated substance. Again, no statistically significant differences were recorded between histamine/FXF and histamine/osthole in TNF- α secretion (Fig. 5C).

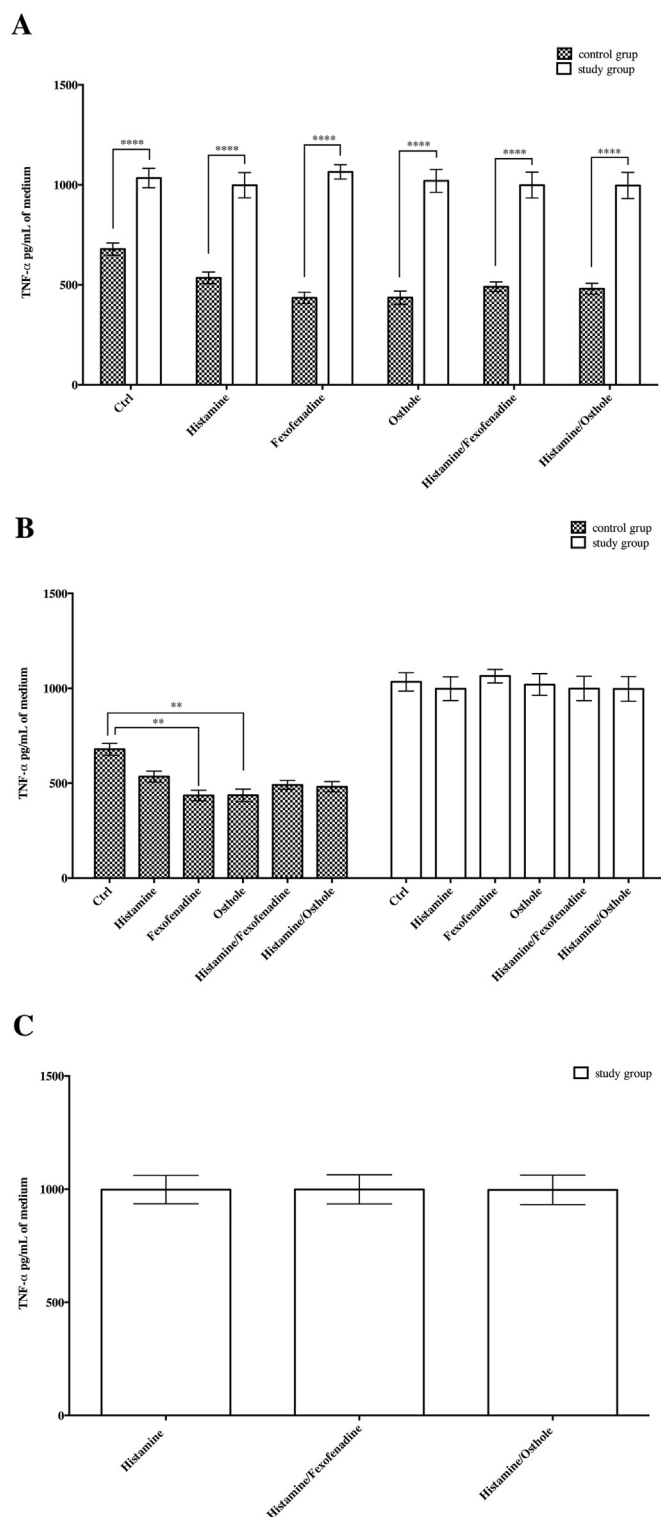


Fig. 5. The influence of tested substances on PBMC TNF- α interleukin secretion; (A) between the study and control group; (B) within the group study and control (C) histamine vs. histamine/FXF vs. histamine/osthole in the study group. Here, PBMC IL-1 β secretion in pure medium forms the control, and tested substance secretions are expressed as pg/ml $\pm$ S.E.M. ns $P > 0.05$, ** $P < 0.01$, **** $P < 0.0001$, $n=30$.

3.2. IgE and serum cytokine levels

3.2.1. IgE

The IgE level was significantly higher in the study group than in the control group $P < 0.0001$, $R=0.3973$ (Fig. 6A); a 4-fold increase.

3.2.2. IL-1 β

Significant difference was recorded between the study group and control group at $P < 0.0001$, $R=0.6220$ (Fig. 6B).

3.2.3. IL-4

Significant difference existed between the study group and control group at $P < 0.0001$, $R=0.3763$ (Fig. 6C).

3.2.4. IL-10

Significant difference was determined between the study group and control group at $P=0.0012$, $R=0.1678$ (Fig. 6D).

3.2.5. IL-13

Significant difference was recorded between the study group and control group at $P < 0.0001$, $R=0.5373$ (Fig. 6E).

3.2.6. TNF- α

No statistical difference was detected between the study group and control group at $P=0.5905$, $R=0.005022$ (Fig. 6F).

3.2.7. Correlation between serum IgE levels and cytokine levels

While the study group total IgE level positively correlated with serum IL-10 level ($P < 0.0001$, $R=0.4294$ - Fig. 7C), no further correlations were established between the study and control groups (Fig. 7A, B); and none were observed between age or gender and serum cytokine levels in either group.

4. Discussion

4.1. Osthole

New research suggests that osthole has antioxidant, anticancer, anti-inflammatory and immunomodulatory properties (Fiorentino et al., 1991; Hampe et al., 1999; Pyo et al., 2003; Simpson and Jarvis, 2000); hence its reported multiple bioactivities encourage the development of osthole and its derivatives as potential multi-target drugs. While it remains expedient to summarize pharmacological and biological research on this coumarin, to review the mechanisms behind its effects and obtain a comprehensive picture of its miscellaneous functions, it was most important in this work to precisely compare osthole and fexofenadine (FXF) effects. FXF is a non-sedating anti-histamine drug indicated in the treatment of chronic urticaria and seasonal allergic rhinitis; so future clinical practice for osthole as an FXF replacement may be determined by our experimental results (Salamonsen et al., 2007).

4.2. Immune System - allergic and inflammatory responses

Three discrete findings indicate that dysregulated IL-1 β , IL-4, IL-10, IL-13 and TNF- α production plays a critical role in the pathogenesis of allergic inflammation. (1) IgE in the serum of allergic patients positively correlates with the IL-10 level in our study group (Fig. 7C); (2) patient PBMC's produce 5-times more IL-1 β than healthy people (Fig. 1A) and (3) treatment with FXF statistically decreases cytokine (IL-4 and IL-13) levels compared to stimulated with histamine (Figs. 2B and 4B).

Allergen-induced IgE synthesis triggers eosinophils, basophils and mast cells to release cytokines for differentiation of Th cells into Th2 cells to secrete IL-4, IL-5, IL-10 and IL-13. Moreover, basophils, mast cells and eosinophils act as effectors of allergic inflammation through the release of pro-inflammatory, vasoactive and fibrogenic factors including histamine, peptide leukotrienes, platelet activating factor tryptase and chymase. These are responsible for allergy and inflammatory response symptoms (Marone, 1998). Th2 cytokines, including IL-4 and IL-5 are crucially involved in local infiltration and activation of eosinophils (Kaminuma et al., 1999), while other Th2 cytokine IL-10

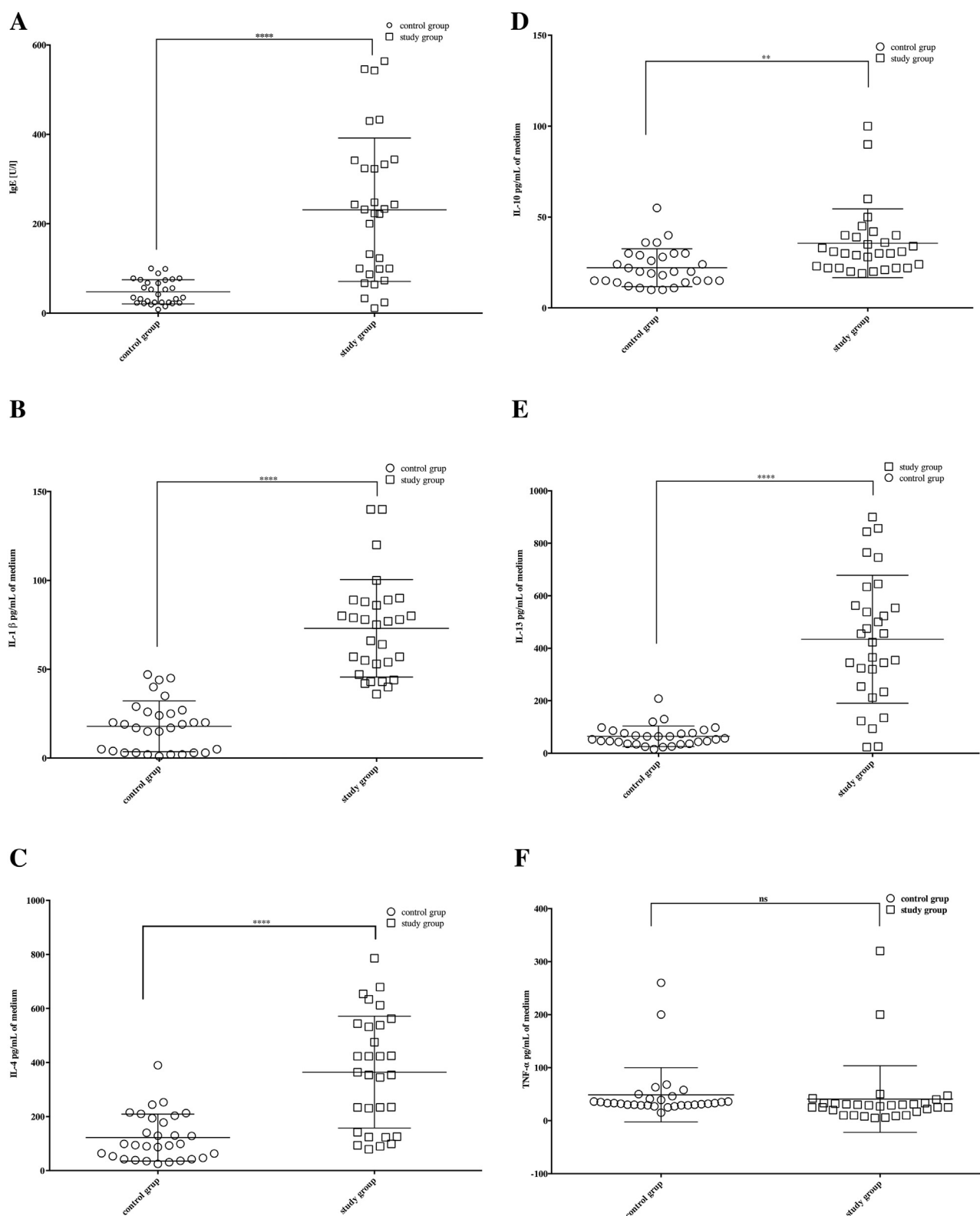


Fig. 6. (A) IgE, (B) IL-1 β , (C) IL-4, (D) IL-10, (E) IL-13, (F) TNF- α , serum levels of studied and controls groups. Statistically significant differences between the control and the tested sample is shown directly above the bar. Statistically significant differences between all the tested samples are placed on the bracket connecting the bars. Statistical significant differences: ns $P > 0.05$, ** $P < 0.01$, **** $P < 0.0001$, $n=30$.

and IL-13 are important in inducing airway hyper-reactivity and allergic inflammation. The release of cytokines as soluble messengers is a fundamental mechanism in cell–cell communication and immune system regulation.

In addition, macrophages and granulocytes release cytokines to

activate and recruit other cells during inflammation, or as direct killing agents (Chung, 2001; De Waal Malefyt et al., 1991). Signals from pattern-recognition motif receptors and other surface receptors trigger cytokine synthesis and release by host cells in response to microbial invasion and other stimuli. Cytokines also link immune system cells to

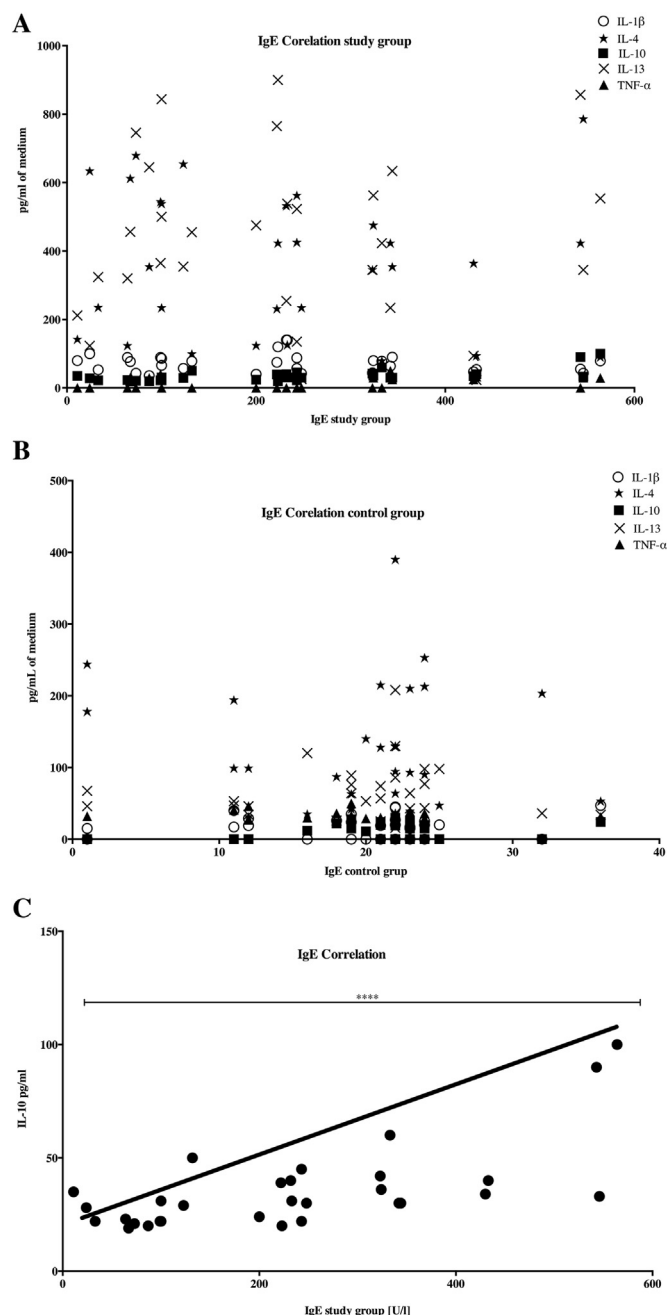


Fig. 7. Correlation between (A) study group and (B) control group in IgE levels and serum cytokines levels evaluated by Pearson rank correlation. (C) Correlation between IgE levels vs. IL-10 serum levels in study group evaluated by Pearson rank correlation. ns $P > 0.05$, **** $P < 0.0001$, $n=30$.

those in surrounding tissues, and they convey reparative or destructive signals to other cells during development, after injury and in tumor growth (Ying et al., 1991; You et al., 2009). After binding to their cognate receptors on target cells, cytokine downstream-effects activate or inhibit cellular functions in autocrine or paracrine fashion (Groux et al., 1996) and can therefore down-regulate allergies. Current knowledge increasingly highlights that many immune system cells have devised clever routes and regulatory systems to control targeting and timing of cytokine release. Understanding how cytokines are trafficked and how their secretion is managed provides vital insight into basic immunity, and into the many diseases associated with aberrant or excessive cytokine release (Robinson et al., 1996).

4.3. IL-1 β cytokine activity

IL-1 β is a pleiotropic cytokine with a broad spectrum of biological activities involving promotion of T and B cell proliferation, activation of neutrophils, increased expression of endothelial adhesion molecules, induction of prostaglandin and collagenase synthesis and stimulated synthesis of several cytokines, including itself (Cao et al., 2006; Lummus et al., 1998; Ying et al., 1991). Pascual et al. (2005) revealed that activated PBMC's from patients with systemic onset juvenile idiopathic arthritis (SoJIA) produce high levels of IL-1 β compared to control subjects. In addition, Shoham et al. (2003) reported that the monocytes in patients with auto-inflammatory diseases produce higher IL-1 β levels than control monocytes.

Our study produced similar results, where PBMC's from allergic people produced 5-fold IL-1 β compared to healthy people (Fig. 1A-B), and a similar result was obtained from serum analysis (Fig. 6B). Jablonska et al.'s. (2001) research also provided similar results to ours when they measured IL-1 β levels in culture supernatants of polymorphonuclear leukocytes (PMN) and PBMC's and also in the serum levels of patients with inflammation and cancer. Their IL-1 β concentrations were significantly higher in PBMC's cultured from the oral cavity in cancer patients than those in controls; and while IL-1 β serum levels in cancer patients were significantly higher than controls ($P < 0.05$), the higher IL-1 β serum levels in inflammation patients were not statistically significant. Researchers report that IL-1 β serum levels can be elevated in inflammatory and non-inflammatory conditions as different as sepsis, chronic rheumatoid disease, allergic inflammation and non-inflammatory tissue injury (Ulrich et al., 2001); and also that PBMC IL-1 β secretory changes in various conditions lead to changes in their body fluid IL-1 β concentrations (Seitz et al., 1995).

4.4. IL-4 cytokine activity

IL-4 is critical to development of allergic inflammation. It is associated with the isotype switch to IgE production and induces differentiation of naive helper Th0 cells to Th2 cells. The Th2 cells produce additional IL-4 in a positive feedback loop following IL-4 activation, and this IL-4 overproduction is associated with allergies. Animal models and human clinical studies indicate Th2 cells' important role in producing IL-4 and IL-13 in the pathogenesis of allergic asthma and allergy inflammation (Yssel and Groux, 2000). In addition, induction of Th1 cell response appears to aggravate the inflammatory process (Marone, 1998).

Neutralizing the biologic activities of excess IL-4 specifically targets the underlying cause of asthma and allergies by blocking multiple inflammation pathways at the key point of TH2 differentiation, thereby preventing the allergic response. Studies with IL-4 knockout mice have demonstrated that IL-4 is critical in development of allergen-specific IgE, airway eosinophilia and hyper-response (Coyle et al., 1995).

Kimura et al. (2000) measured PBMC IL-4, IL-13, and IFN- γ production in house dust mite stimulation (HDM) in children with bronchial asthma (BA). Similar to our results, they found that PBMC production of IL-4 and IL-13 was significantly higher in children with BA than in non-atopic control subjects. Here, we determined a significantly higher level of IL-4 secretion in PBMC from allergic patients than in the non-allergic controls (Fig. 2B); and Ying et al. (1997) and Hashimoto et al. (1993) also obtained the same results. Moreover, we observed significantly decreased IL-4 level in histamine/osthole treatment compared to histamine alone (Fig. 2C). Meanwhile, FFX not significantly decrease the level of IL-4 increased by histamine. This data indicates osthole's strong role in allergic inflammation.

Further research by Matsumoto et al. (1991) measured IL-4 serum levels in children with allergic diseases and found that the levels were significantly elevated in children with allergic diseases; with all the clinical manifestations, including atopic eczema, bronchial asthma and food anaphylaxis compared to non-allergic control children of the same

age and sex. Our results were similar, with a significantly higher IL-4 level in allergic patients than in non-allergic controls (Fig. 6C). Additional support is provided by; (1) Hashimoto et al. (1993) who determined elevated serum IL-4 in patients with allergic asthma compared to normal control subjects; (2) Pawankar et al. (1997) reported that nasal mast cells (NMC) from patients with perennial allergic rhinitis (PAR) expressed significantly greater IL-4 levels than cells of patients with chronic infective rhinitis (CIR) and (3) Fujishima et al. (1995) found that while their control group had low mean IL-4 levels in their tears, patients with seasonal allergic conjunctivitis and vernal keratoconjunctivitis had significantly elevated IL-4.

4.5. IL-10 cytokine activity

IL-10 is reported to have a major role in inducing and maintaining the anergic state (Pascual et al., 2005; Shoham et al., 2003; Van Deuren et al., 1997). This is a pleiotropic cytokine which exerts immunostimulatory or suppressive effects on a variety of cell types (Prescott et al., 1998). Despite releasing increased macrophage chemotactic protein (Holgate, 2000), IL-10 is a potent inhibitor of monocyte/macrophage function and suppresses the production of a number of pro-inflammatory cytokines including TNF- α , IL-1 β , IL-6, MIP-1 α and IL-8 (Costa et al., 1997; Holgate, 2000; Robinson et al., 1993; Hawrylowicz and O'garra, 2005). Thomas (2001) and Gosset et al.'s. (1992) studies into human asthma and allergy revealed increased TNF- α generation in macrophages and peripheral blood monocytes after antigen challenge, and also increased TNF- α mRNA in asthmatic airway lavage (Zhou and Liu, 2005). In addition, Lummus et al. (1998) recorded increased TNF- α and IL-1 β release from peripheral blood monocytes ex-vivo in diisocyanate-sensitive and other occupational asthmas. These studies support our results, where IL-1 β and TNF- α levels were higher in the PBMC study group than in controls and our serum IL-1 β levels were high. Further, our increased plasma IL-10 in allergy patients (Fig. 6D) concurs with Robinson et al.'s. (1996) observation that IL-10 mRNA expression is increased in allergy and atopic asthma.

It has been suggested that increased IL-10 expression in atopic skin contributes to aggravated skin lesions from suppressed expression of cathelicidin hBD-2 and β -defensin -LL-37 anti-microbial peptides. Studies conducted on animals suggest that IL-10 promotes poisonous nitric oxide development, and this relationship has also been observed in human atopic asthma (Heaton et al., 2005).

Our findings indicated a tendency towards increased IL-10 expression in asthma patient airways, rather than the reverse, and Borish et al. (1996) also reported this contrasting result of deficient macrophage IL-10 production in asthma and allergy. Therefore, T cell IL-10 production may be unregulated in asthma and allergy; with autoregulatory IL-10 produced after allergen challenge, but with reduced macrophage induced IL-10 production. Hobbs et al. (1998) have reported IL-10 promoter polymorphisms, so IL-10 effector functions may be reduced in atopic asthmatics.

Our research results of increased plasma cytokine level confirms that Th2 cytokine IL-10 plays a central role in allergic asthma. While we have shown surprising correlation between serum IL-10 level in the study group and the total IgE level other studies have reported that IL-10 effect on IgE synthesis is very complex; with IL-10 inhibiting IL-4 stimulated mRNA transcription of ϵ chain and IgE production but increasing IgE synthesis by "directed" B-lymphocytes (Jeannin et al., 1998).

4.6. IL-13 cytokine activity

Although it has similar effect on immune cells to those of the closely related IL-4 cytokine, IL-13 is considered a more central mediator of the physiological changes induced by allergic inflammation in many tissues. While it also has similar effect on human B cells as IL-4, but

less potent, no synergistic effects were observed when both cytokines were added at optimal concentrations. IL-13 is associated primarily with the induction of airway disease, has anti-inflammatory properties (Huang et al., 1995) and it is critical in pathogenesis of allergen-induced asthma, although operating through mechanisms independent of IgE and eosinophils. Interestingly, IL-13 deletion in mice does not markedly affect either Th2 cell development or antigen-specific IgE responses induced by potent allergens, and IL-4 deletion abrogates these responses. IL-13 therefore acts more prominently as a molecular bridge linking allergic inflammatory cells to the non-immune cells they contact; altering physiological function, rather than exerting lymphoid cytokine action (Katagiri et al., 1997). It also down-regulates macrophage activity, thereby inhibiting production of pro-inflammatory cytokines and chemokines.

Researchers reported increased IL-13 production in allergic and atopic patients. Huang et al. (1995), Humbert et al. (1997), Koning et al. (1997) and Kimura et al. (2000), and we also determined lower PBMC IL-13 in control PBMCs than in the study group. Here, histamine induced the highest level in all tested variants in both control and study groups (Fig. 4A–B). It is also interesting that combined histamine/osthole significantly reduced IL-13 secretion compared do histamine alone (Fig. 4C), and that this combination was more effective than FFX/histamine in inhibiting PBMC IL-13 secretion.

Katagiri et al. (1997) analysed RT-PCR IL-13 mRNA expression in freshly isolated PBMCs of healthy adults and psoriasis and atopic dermatitis patients (AD). This revealed that PBMC IL-13 mRNA increased in AD patients compared to healthy controls, and hence elevated levels combined with undetermined factors are responsible for regulating IgE in vivo synthesis in AD and allergy patients. In addition, increased PBMC IL-13 expression has also been observed in the acute phase of eczema and in apparently healthy skin (Antunez et al., 2004).

4.7. TNF- α cytokine activity

TNF- α is the most widely studied pleiotropic cytokine of the TNF superfamily. It is an important cytokine in the innate immune response, with a key role in immediate host defense against invading microorganisms before activation of the adaptive immune system (McInnes and Schett, 2007; Roux-Lombard, 1998; Rumsaeng et al., 1997). TNF- α is also produced by other proinflammatory cells, including monocytes, dendritic cells, B cells, CD4+ cells, neutrophils, mast cells, eosinophils, structural fibroblasts, and epithelial and smooth muscle cells (Neaville et al., 2003). Chou et Panay (2001) implicated TNF- α role in the pathophysiology of chronic inflammatory bowel disease and rheumatoid arthritis (Tremelling et al., 2006). Olsen et Stein (2004) also reported that TNF- α antagonism by treatment with recombinant soluble receptors or neutralizing antibodies leads to improved disease-activity scores in rheumatoid patients, and positive results have been recorded in other conditions considered mediated by TNF- α . Its contribution to the inflammatory response in asthmatic airways is supported by increased TNF- α mRNA (Zhou and Liu, 2005) and protein (Möller et al., 1998) in asthma patients' airways, and also by Sun et al.'s. (1991) research which reported increased TNF- α in asthmatic and allergic airways (Cazzola and Polosa, 2006). This concurs with our results where TNF- α increase in the study group was statistically significant (Fig. 3A). In addition, Zipperlen et al. (2005) indicated over-expression of TNF- α in both normal and inflamed intestinal mucosa in patients with inflammatory bowel disease.

However similarly to other authors, we observed no differences in allergic and control serum TNF- α (Hughes et al., 2001; Yoshizawa et al., 2002) and no IFN- γ serum elevation in patients with allergic disease compared to normal control subjects. This does not exclude cytokine participation in allergic diseases. Increased TNF- α expression has been registered in atopic patients' peripheral blood (Antunez et al.,

2004, 2006) in asthmatic patient airways, even in remission periods (Brown et al., 2003; Obase et al., 2003), and it is accepted that TNF- α also increases expression of adhesive molecules and ecotaxin, resulting in eosinophil activation and recruitment at allergic inflammation sites.

4.8. Specific IgE activity

Specific IgE is an allergic response mediator and allergic inflammation marker in asthma (Groux et al., 1996; Simpson and Jarvis, 2000). Our experiment established significant IgE increase in study group serum over the control group (Fig. 6A). This is supported by Wong et al.'s. (2001) results; and our findings confirmed that all allergic patients were atopic and manifested inflammatory response with statistically higher IL-4 and IL-13 in patient's serum than in controls. This is most likely because IL-13-induced IgE synthesis is preceded by germline ϵ transcription induction; the prerequisite for subsequent IgE switching and production. Although IgE is not spontaneously produced by B cells in early fetal tissues, it is detectable in cord blood, and elevated IgE levels there are associated with a family history of atopy and increased risk of allergies early in life. This suggests that maternal IL-4 and IL-13 crosses the placenta. In contrast to IL-13 IL-4 directly acts on pre-B cells so IL-4, and not IL-13, induces enhanced IgE synthesis during intrauterine life and explains increased IgE production in neonates of atopic mothers. Although IL-4 and IL-13 both induce IgE synthesis in vitro, the relative contribution of each cytokine to IgE production in vivo remains undetermined because IL4, and not IL13, acts on murine B cells. Although initial research found IL-4-deficient mice unable to produce IgE in vivo, studies are hampered by lack of suitable animal models.

4.9. Histamine and anti-histamine effect of osthole

Interaction of histamine and histamine H₁-receptor in allergic disease mediates a variety of classic pathophysiologic effects, including vascular permeability, smooth muscle contraction, vasodilatation and flushing, mucus secretion and pruritus. Individual and combined histamine/osthole effect leads to asthmatic bronchial obstruction, nasal blockage, sneezing, rhinitic itching and discharge and the itchy skin wheals and flares in urticaria.

Although the classic effects of histamine at the organ level are generally well documented, and emphasized in allergic disease, there is increasing evidence that it directly or indirectly influences inflammatory, effector and immunology cells in allergic pathogenesis. It is therefore likely that histamine has a wider and critical role as a pro-inflammatory mediator in allergic disease than presently accepted. Studies have also demonstrated that histamine receptors are expressed on basophils, mast cells, neutrophils, eosinophils, lymphocytes, macrophages, epithelial cells and endothelial cells, and that histamine likely modulates these cells' function (Bachert, 2002).

Our research investigated osthole's anti-histamine effect and compared this to FFX. We determined that IL-4, IL-13 and other Th2 cytokines are involved in airway inflammation in allergic asthmatics, and our combined osthole/histamine significantly decreased secretion of PBMC IL-4 ($P < 0.01$) and IL-13 ($P < 0.0001$) compared to histamine alone. This inhibited IL-4 and IL-13 secretion confirms osthole's anti-allergic/anti-histamine clinical propensity.

5. Conclusion

We investigated PBMC and serum cytokine production in allergic and non-allergic individuals after in vitro stimulation by histamine, and FFX and osthole were compared for anti-allergic properties. Here, we determined that osthole affects PBMC cytokine secretion to almost precisely the same extent as FFX (Figs. 1–3A–C, 5A–C), with no statistically significant differences between histamine/FFX and histamine/osthole treatments. Additional results include; (1) IL-13 is a

recognised major mediator of physiological changes induced by allergic inflammation in many tissues, and our Fig. 4C highlights that osthole had greater IL-13 blocking ability than FFX and (2) osthole PBMC stimulation of cytokine IL-1 β , IL-10 and TNF- α secretion was as efficacious as that of FFX (Pyo et al., 2003).

The immune system has developed efficient peripheral tolerance mechanisms to avoid chronic cell activation and inflammation against nonpathogenic antigens from ingestion, inhalation and through the skin. (Yan and Hansson, 2007); and, the link between abnormal immune development and allergic diseases has stimulated efforts to define precise cytokine dysregulation associated with specific atopic phenotypes (Sismanopoulos et al., 2012). Finally, all results confirm our hypothesis that osthole is a natural histamine antagonist, and can therefore be efficacious in clinical antihistamine allergy treatment.

Conflict of interest

The authors declare they have no competing interests.

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