

Original article**Immune response in rats following administration of honey with sulfonamides residues****Evidențierea răspunsului imun la șobolani după administrarea de miere cu reziduuri de sulfonamide**

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Abstract

Introduction. The aim of this paper was to study the immune response in rats after honey intake containing sulfonamides residues, by analysis and profiling of 12 cytokines, immunoglobulin E (IgE) and plasma eosinophils. *Materials and methods.* In order to evaluate the changes in serum values of the parameters involved in allergic-type hypersensitivity reactions, the variation of serum levels of cytokines: interleukin (IL)-2, IL-4, IL-6, IL-8, IL-10, vascular endothelial growth factor (VEGF), interferon gamma (IFN γ), tumor necrosis factor alpha (TNF α), IL-1 α , IL-1 β , monocyte chemoattractant protein-1 (MCP-1), epidermal growth factor (EGF), before and after sulfonamide containing honey administration in Wistar rats, was analyzed. The study was conducted on rats, which were divided into three groups: group 1 – control, group 2 – rats receiving honey containing sulfonamide residues for 5 days, and group 3 - rats receiving honey containing sulfonamide residues for 14 consecutive days, respectively. Serum cytokine levels were evaluated in two groups and compared with controls. *Results and discussions.* In both rats groups that had received honey with sulfonamides the levels of IL-4, IL-6, IL-8, IL-10, VEGF, IFN γ , TNF α , MCP-1, EGF, IgE and eosinophils were significantly increased compared with controls. Correlation between cytokine levels, IgE and eosinophils levels was also determined and found positive. *Conclusions.* The levels of the cytokines IL-4 and IL-10 along with IgE and eosinophils were increased 5–30 fold with a maximum response after 14 days' exposure, the differences were statistically significant.

Keywords: cytokines, eosinophils, sulfonamides, hypersensitivity

Rezumat

Introducere. Scopul prezentului studiu a fost evidențierea răspunsului imun apărut la șobolani în urma administrării de miere de albine ce conține reziduuri de sulfonamide, prin determinarea unui profil de 12 citokine, imunoglobuline E (IgE) și eozinofile plasmatice. *Material și metodă.* S-a evaluat modificarea valorilor plas-

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matice ale parametrilor implicați în reacțiile de hipersensibilitate de tip alergic prin analizarea variației valorilor serice ale citokinelor: interleukina (IL)-2, IL-4, IL-6, IL-8, IL-10, factorul de creștere al endoteliului vascular (VEGF), interferon gamma (IFN γ), factor de necroză tumorală alfa (TNF α), IL-1 α , IL-1 β , MCP-1, factorul de creștere epidermică (EGF) la șobolanii Wistar înainte și după administrarea de miere de albine îmbogățită cu sulfonamide. Șobolanii rasa Wistar au fost împărțiți în 3 loturi: lotul 1 – lot martor, lotul 2 – șobolani cărora li s-a administrat miere cu reziduuri de sulfonamide timp de 5 zile și lotul 3 – șobolani cărora li s-a administrat miere cu reziduuri de sulfonamide timp de 14 zile consecutiv. Concentrațiile citokinelor au fost evaluate în cele 3 loturi și comparate cu valorile obținute pentru lotul martor. Rezultate și discuții. La șobolanii cărora li s-a administrat miere cu reziduuri de sulfonamide, nivelul citokinelor IL-4, IL-6, IL-8, IL-10, VEGF, IFN γ , TNF α , MCP-1, EGF, IgE și eozinofile a fost semnificativ crescut comparativ cu lotul martor. De asemenea, s-a efectuat analiza corelației dintre nivelurile de citokine, imunoglobulina E (IgE) și nivelurile de eozinofile la cele trei loturi și s-a determinat că există corelație pozitivă, directă. Concluzii. Nivelurile de citokine IL-4 și IL-10, împreună cu IgE și eozinofilele au crescut de 5-30 ori cu un răspuns maxim după o expunere timp de 14 zile diferențele fiind semnificative statistic.

Cuvinte cheie: citokine, eozinofile, sulfonamide, hipersensibilitate

Introduction

The sulfonamides are used in beekeeping for the treatment of various bacterial diseases in bees, such as *Loca Americana* (AFB). Although the antimicrobial chemotherapy is effective for the treatment of those diseases, the drug residues may persist for a long time in tissues and the intake of honey containing such residues may be harmful for human health and safety. The drug residues contained in food may produce adverse effects like cutaneous effects (rash, itchiness) and respiratory, cardiovascular and gastrointestinal effects, respectively. Bio-tests made on rodents also demonstrated the sulfonamides' toxicity on the thyroid gland. Overall frequency of allergic reactions to drugs is around 10% (1). Allergic sensitization usually develops at 5-14 days after the first administration. Antibacterial sulfonamides are a class of drugs associated with hypersensitivity reactions and represent the model most widely studied to explore the chemical basis of immune activation. Hypersensitivity reactions to sulfamethoxazole occur in 3% to 8% of cases (2). T-cell receptor cross-reactivity with different nitroso sulfonamides reveals a potential mechanism for the development of hypersensitivity reactions to different drug structures through metabolite formation and protein bind-

ing. Those allergic effects may appear at very low drug doses as they require a preliminary sensibility to the drug, but also they may be genetically related. The drug allergic reactions are often mediated by immunoglobulins E (IgE), synthesized as a reaction to the aggression of some allergens or haptens. The cytokines increase and prolong the allergic response (3).

Cytokines are regulatory proteins that normally function as part of a complex interactive network. Their interactions with cells are mediated by specific surface receptors; many of them are also produced as secreted soluble forms and can be important regulators of cytokine function. Complex networks of cytokines interact in a dynamic way to regulate immune responses and other biological pathways. Since the delineation of type 1 (Th1) and type 2 (Th2) CD4 T-cells in mice and in humans, evidence has largely shown that activated Th2 lymphocytes and cytokines such as IL-4, IL-13, and IL-5 are responsible for the IgE production and eosinophil activation necessary for allergic inflammation (4). From the cytokines category, the most important are interleukins, TNF α , TNF β , the cell colony stimulating factors, the chemotactic factors and the growth factors, respectively. TNF- α , IL-1 α and IL-1 β are pro-inflammatory cytokines that regulate *in vitro* expression of MCP-1, leading to an in-

crease of the inflammatory response. Cytokines are involved in both enhancing and suppressing immune responses through their influence on T-cells and other immune effectors. IL-2, IL-12, INF- γ , and TNF- α activate Th-1 lymphocytes, whereas IL-4, IL-5 and IL-10 lead to Th2 cell activation. Cytokines Th2 derived may play a role in stimulating IgE production from B cells and increase target cell sensitivity to endothelial mediators, whereas these actions are inhibited by IFN- γ . Pro-inflammatory mediators such as IL-1 β , IL-6 and TNF- α may also act as amplification signals for immune cells, whereas the anti-inflammatory cytokine IL-10 it is involved in the systemic immune response. The chemokines IL-8 and eotaxin may be involved in recruiting proinflammatory cells to the sites of allergen exposure (5).

Hypersensitivity reactions to therapeutic levels of sulfonamides have been reported but there have been no cases that involved exposure to drug residues in foods. Because limited published data are available, we decided to perform a comprehensive evaluation on the effect of sulfonamides residues concerning the immune response in hypersensitivity reactions in rats.

The objective of the present study was to test the hypothesis that a combination of biomarkers (IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFN γ , TNF α , IL-1 α , IL-1 β , MCP-1, EGF, IgE and eosinophils) could be used to identify the risk of allergic adverse effects in the case of sulfonamides drug residues. The goals of our study were threefold: to define serum cytokine profiles of 3 different subsets of rats, to evaluate the effect of ingestion of honey with sulfonamide residues on serum cytokine levels and to correlate serum cytokine levels with IgE levels and eosinophils.

Materials and methods

Reagents and equipment

The Evidence Investigator™ (Randox Laboratories, Crumlin, UK) and *High Sensitivity Cytokine Array I* (Randox Laboratories, UK)

were used for the simultaneous measurement of 12 cytokines IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFN γ , TNF α , IL-1 α , IL-1 β , MCP-1, EGF. In parallel with cytokine measurement, sample specimens were assayed for IgE using an ELISA kit (Diagnostic Automation, USA). The cell blood count (CBC) was performed on Sysmex KX21 autoanalyser.

The *High Sensitivity Cytokine Array I* kit contain ready to use, liquid reagents, multi-analyte calibrators, multi-analyte conjugates, assay buffer, signal reagent (luminol and peroxide), wash buffer and the biochips. Biochip array technology offers the advantage of multiplexing several specific antibodies on a single biochip surface to increase the number of detected analytes. The core technology is the biochip onto which a selection of antibodies are precisely immobilized and stabilized at separate X- and Y-coordinates, each biochip contain 23 distinct test regions and allows multiple assays to be performed simultaneously on a single sample. The biosensor system has been specifically developed as a microtitre plate assay combined with a purpose designed charged coupled device camera (CCD) and completed with dedicated software. The biochips are held in a carrier accommodating 9 biochips in a 3x3 format, totaling nine reaction wells with one biochip per well. One biochip contain 12 cytokine and growth factor assays. The assay is based on the sandwich immunoassay principles, namely the antibodies onto the reactive surface were designed to capture the cytokines present in the sample. Single samples and reagents were added to each well to perform the assay. Following the addition of a horseradish peroxidase enzyme-labeled multi-conjugate, signal reagent, incubation and washing steps, the intensity of the resulting chemiluminescent signal was detected using the highly sensitive CCD camera. The signal intensity was directly proportional to the concentration of the cytokines in the serum sample. A single biochip carrier was placed into the processing compart-

ment of the imaging module. The light signal generated from each of the test regions on the biochip was detected using digital imaging technology and compared to that from a stored calibration curve. The analyte concentration in the sample was calculated from the calibration curve. To check the accuracy and precision of the method multi-analyte controls (Randox Laboratories, UK) were used (6, 7).

All the cytokines were measured in picogram quantities with sensitivity ranging from 0.12 pg/ml for IL-6 (calibration range 0–400 pg/ml) to 2.12 pg/ml for IL-4 (calibration range 0–450 pg/ml). The intra-assay and inter-assay precision ($n = 20$) expressed as %CV were typically 12% for three multianalyte control levels.

Experimental protocol

Female Wistar rats weighing 180 - 220 g were used. Rats were divided into the following groups: group 1 - control – the rats were fed with standard food and received 2 ml of honey free of sulfonamides, daily, by gastric gavage, group 2 – the rats received honey containing a mixture of sulfonamides, for a period of 5 days (2 ml honey with a 100 µg/kg mixture of sulfonamides were administered daily by gastric gavage) and group 3 – the rats received 2 ml of honey with a 100 µg/kg mixture of sulfonamides by gastric gavage for 14 consecutive days. The mixture of sulfonamides was prepared from sulfamethoxazole and sulfamerazine in equal proportions. All honey samples were sourced from retail outlets in Romania. Honeys determined by LC/MS/MS to be free of drug residues were used. The study was conducted under approval issued by the Ethics Committee of the University of Medicine and Pharmacy “Gr. T. Popa”, Iasi. After the end of the experiment the rats were killed. The blood samples mixed with anticoagulant (EDTA Na 1%: blood - 1 volume anticoagulant to 9 parts whole blood) were used for cell blood count. Blood samples without any additives were centrifuged immediately and the serum was portioned for the determination of cytokines and IgE. The serum samples were kept frozen at -25°C until testing.

Serum cytokine levels were evaluated in 3 groups and compared with controls.

Biochip arrays incorporating multiple specific antibodies to proteins involved in inflammation were used to evaluate the plasma profile of pro- and anti-inflammatory cytokines and of cellular adhesion proteins and compared with levels in healthy controls. Biochip array technology enables determination of multiple analytes with a single sample.

Statistical analysis

The statistical analysis of obtained data was performed using Student's test or Mann-Whitney rank test (Microsoft Excel). Variables were calculated as mean (M) and mean standard error (MES). The significance of differences between groups was determined on the basis of confidence intervals. Correlation between cytokine levels and IgE and eosinophils levels was determined. Relationships between eosinophils levels, serum IgE or cytokines concentrations on the three studied groups were examined using Pearson's correlation for parametric data (p values less than 0.05 were considered significant).

Results

The cytokines analyzed were divided into three categories based on their primary cell source; Th1 type cytokines included IL-2 and IFN- γ ; Th2 type cytokines were represented by IL-4, IL-6, and IL-10 and APC-derived cytokines included IL-1 α , IL-1 β , IL-8, and TNF- α . Mean serum cytokines, growth factors, IgE and eosinophils levels in the examined groups are shown in *Table 1*. We found that group 2 and group 3 rats had higher mean serum levels of both Th1 cytokines tested (IL-2 and IFN- γ), and all three Th2 cytokines tested (IL-4, IL-6, and IL-10) compared with controls. The differences between mean levels of IL-1 α and IL-1 β in group 2 and group 3 were not statistically significant compared with controls.

Table 1. Mean serum cytokine, growth factors, IgE and eosinophils levels in the studied groups

	Primarily T _H -1 derived		Primarily T _H -2 derived		Primarily APC derived					Growth factors			IgE	Eosinophils
	IL-2 (pg/ml)	IFN- γ (pg/ml)	IL-4 (pg/ml)	IL -10 (pg/ml)	IL-1 α (pg/ml)	IL-1 β (pg/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)	TNF- α (pg/ml)	VEGF (pg/ml)	MCP1 (pg/ml)	EGF (pg/ml)	UI/ml	%
Group 1 (n=10)	4.96±0.73	4.06±0.45	5.39±0.72	2.84±0.28	3.21±0.46	4.67±1.24	5.23±0.49	4.58±0.66	3.03±0.23	2.91±0.35	137.1±1.26	4.76±0.62	2.05±0.33	2.42±0.41
Group 2 (n=15)	6.56±0.72	6.79±0.85	14.19±2.35	9.27±2.92	3.16±0.35	3.84±1.28	9.85±2.49	9.34±2.06	4.65±0.48	8.53±1.91	150.51±4.02	6.71±0.81	14.42±2.51	7.17±0.89
Group 3 (n=15)	6.75±0.72	6.71±0.77	16.14±2.87	8.36±2.13	2.56±0.20	5.99±2.07	8.15±1.39	8.56±2.07	4.93±0.53	8.58±1.91	148.31±3.61	6.84±0.99	16.02±3.08	8.32±0.96

Values are Mean \pm MES

Table 2. Comparison of mean serum cytokine levels, IgE and between controls and the subsets of studied groups

	IL2	IL4	IL6	IL8	IL10	VEGF	IFN γ	TNF α	IL1 α	IL1 β	MCP1	EGF	IgE	Eosinophils
Group 1 vs Group 2	0.06	0.001	0.04	0.02	0.02	0.005	0.005	0.003	0.46	0.32	0.002	0.03	0.0001	0.00006
Group 1 vs Group 3	0.04	0.001	0.03	0.04	0.01	0.053	0.003	0.002	0.11	0.29	0.004	0.04	0.0002	0.00001
Group 2 vs Group 3	0.42	0.301	0.27	0.39	0.40	0.491	0.470	0.354	0.07	0.19	0.343	0.46	0.3454	0.1950

Significant p values ($p < 0.05$) are in boldface type.

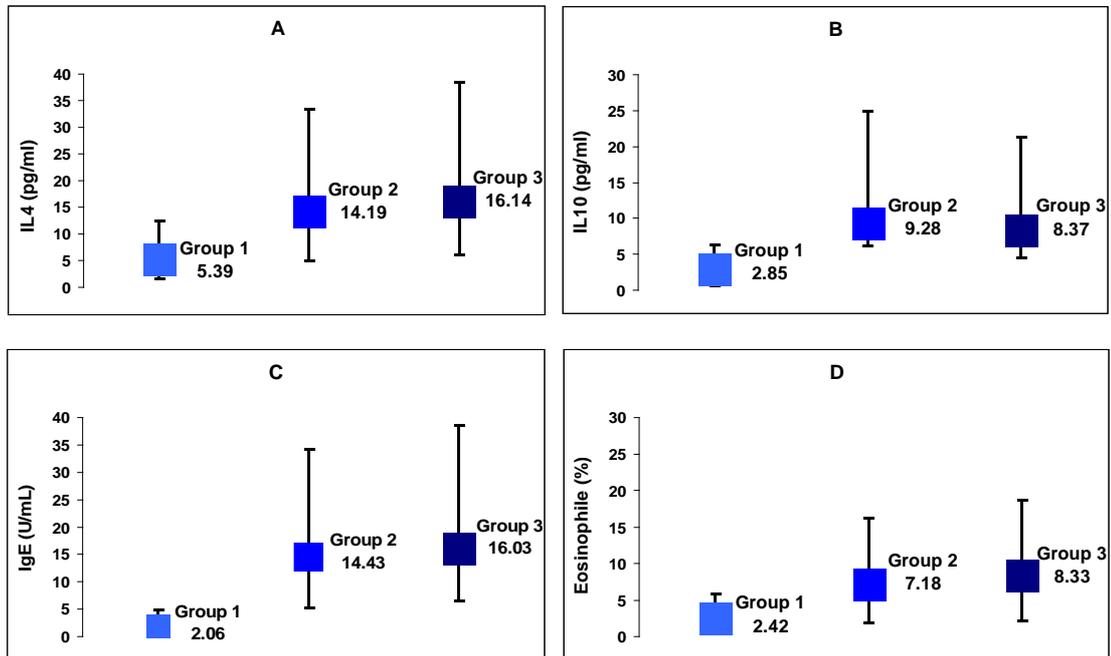


Figure 1. Confidence intervals for mean values of IL4 (A), IL10 (B), IgE (C) and eosinophils (D) for the studied groups

Comparison of mean serum cytokine levels, IgE and eosinophils between controls and the studied groups is indicated in *Table 1* and *Table 2*.

Based on confidence intervals for mean values of IL-4 for the studied groups (*Figure 1*) we found that IL-4 values for group 2 are significantly higher than group 1, IL-4 values for group 3 are significantly higher than group 1 and IL-4 values for group 3 are not significantly higher than in group 2. Confidence intervals for mean values of IL-10 for the studied groups (*Figure 1*) also revealed that IL-10 values in group 2 are significantly higher than in group 1, IL-10 values in group 3 are significantly higher than in group 1 and IL-10 values in group 3 are not significantly lower than IL-10 values in group 2. IgE confidence intervals for mean values for the studied groups emphasize the fact that IgE values on group 2 are significantly higher than group 1 values and IgE values in group 3 are significantly higher compared with

those in group 1 but IgE values in group 3 are not significantly higher than in group 2. Group 2 eosinophils levels are significantly higher compared with those in group 1, group 3 eosinophils levels are significantly higher than in group 1 but IgE values in group 3 are not significantly higher compared with those obtained in group 2 rats. The mean serum levels of these biomarkers (IL-4, IL-10, IgE and eosinophils) were not significantly different when group 2 was compared with group 3 (*Figure 1, Table 2*).

Correlations between IL-4 and IgE in group 2 (*Figure 2A*), IL-4 and IgE in group 3 (*Figure 2B*), IL-4 and eosinophils in group 2 (*Figure 2C*) and IL-4 and eosinophils in group 3 (*Figure 2D*), respectively were analyzed. The obtained value of $p = 0.00004$ indicate the existence of a correlation between IL-4 and IgE in group 2 rats; Pearson's correlation coefficient $r = 0.8579$ shows a strong, positive, direct correlation. The correlation coefficient $R^2 = 0.7359$, which returns the percentage values of which

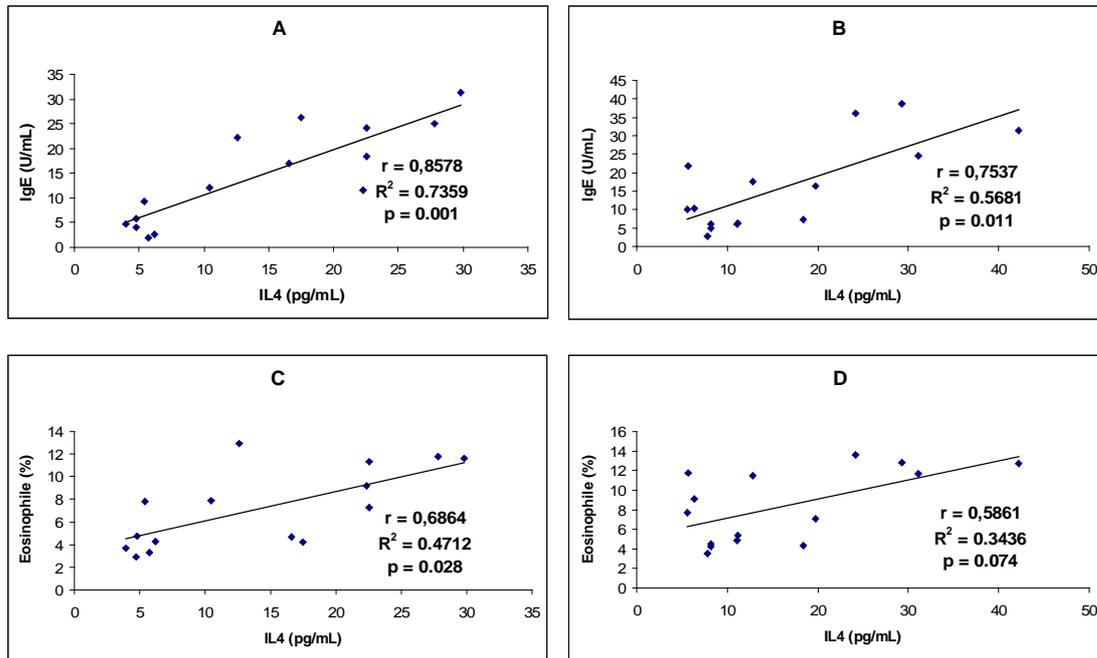


Figure 2. Correlation analysis between IL4 and IgE in group 2 (A), IL4 and IgE in group 3 (B), IL4 and eosinophils in group 2 (C) and IL4 and eosinophils in group 3 (D), respectively

the two variables analyzed are actually correlated, in group 2 rats it is 73.59%. Pearson's correlation coefficient value $r = 0.7534$ and p value < 0.05 shows a strong, positive, direct correlation between IL-4 and IgE in group 3. The correlation coefficient $R^2 = 0.5681$, which returns the percentage values of which the two variables analyzed are actually correlated, in group 3 subjects it is 56.81 % (Figure 2B).

The obtained value of $p = 0.0047$ indicates the existence of a correlation between IL-4 and eosinophils in group 2 subjects. Correlation analysis between IL-4 and eosinophils in group 2 subjects it is 47.12%. Pearson's correlation coefficient $r = 0.6865$ shows a moderate, positive, direct correlation between IL-4 and eosinophils in group 2 subjects. The correlation coefficient, showing the correlation between the values of two variables it is $R^2 = 0.4712$ (Figure 2C). Correlation analysis between IL-4 and eosinophils in group 3 rats was 34.36%. Pearson's correlation coefficient $r = 0.5857$ shows a moderate, positive, direct cor-

relation between IL-4 and eosinophils in group 3 subjects. The correlation coefficient it is $R^2 = 0.3436$ (Figure 2D). The obtained value of $p = 0.0217$ indicates the existence of a correlation between IL-4 and eosinophils in group 3 subjects.

Correlation analysis between IL-10 and eosinophils in group 2 (A), IL-10 and Eosinophils in group 3 (B), IL-10 and IgE in group 2 (C) and IL-10 and IgE in group 3 (D), was performed. After correlation analysis between IL-10 and eosinophils in group 2, Pearson's correlation coefficient $r = 0.4300$ shows a weak but positive, direct correlation. $R^2 = 0.1849$ correlation coefficient indicates a rate of 18.49% correlation between IL-10 levels and eosinophils in group 2 (Figure 3A). However after applying the statistical calculation for correlation, the p value obtained was 0.1096, which cancels the hypothesis that there is a correlation between IL-10 and eosinophils in group 2 rats.

In the correlation analysis between IL-10 and eosinophils in group 3, Pearson's cor-

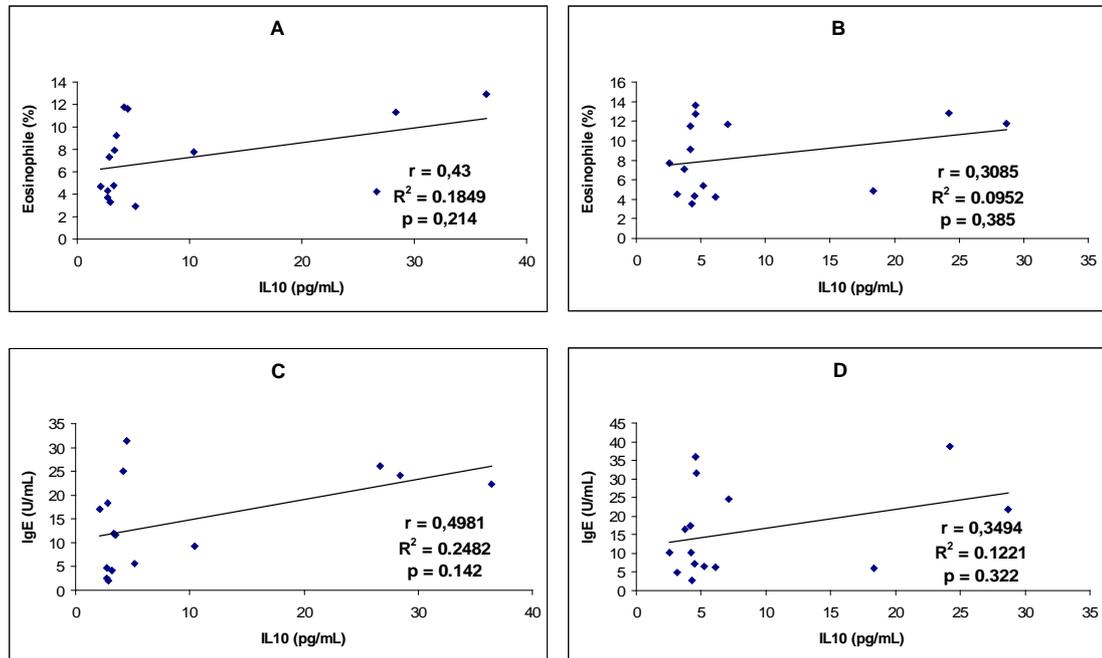


Figure 3. Correlation analysis between IL10 and eosinophils in group 2 (A), IL10 and eosinophils in group 3 (B), IL10 and IgE in group 2 (C) and IL10 and IgE in group 3 (D), respectively

relation coefficient $r = 0.3087$ shows a weak but positive, direct correlation. $R^2 = 0.0952$ correlation coefficient indicates a rate of 9.52% correlation between IL-10 levels and eosinophils in group 3 subjects (*Figure 3B*). After correlation analysis between IL-10 and IgE in group 2 rats, Pearson's correlation coefficient $r = 0.4982$ shows a weak but positive, direct correlation. $R^2 = 0.2482$ correlation coefficient indicates a rate of 24.82% correlation between IL-10 levels and IgE in group 2 (*Figure 3C*). As a result of correlation analysis between IL-10 and IgE in group 3, Pearson's correlation coefficient $r = 0.3372$ shows a weak but positive, direct correlation. $R^2 = 0.1221$ correlation coefficient indicates a rate of 12.21% correlation between IL-10 levels and IgE in group 3 (*Figure 3D*).

The correlation analysis was also applied between IgE, eosinophils and the measured cytokines and growth factors in group 2 and group 3 rats, but no correlations have been found.

Discussions

On the basis of their respective cytokine profiles, responses to chemokines, and interactions with other cells, T-cell subsets can promote different types of inflammatory responses (8). During the development of allergic disease, effector Th2 cells produce IL-4. These cytokines are involved in production of specific IgE, eosinophilia and mucus. IFN- γ produced by Th1 cells plays a role in activation-induced death of skin keratinocytes, mucosal epithelial cells and T cells. IL-10 promotes human B cells differentiation and proliferation, inhibits the expression of many pro-inflammatory cytokines, increases IgG4 production and mediates allergen tolerance in allergen-specific immunotherapy (9, 10). Several mouse models demonstrate the importance of IL-10 in regulation of the inflammatory response (11). Type IV Hypersensitivity Reaction/Delayed, mediated by cytokines results in activation of immune cascade leading to tissue damage.

Serum cytokine measurements have some limitations, such as increased clearance and presence of receptors leading to falsely low levels (12). Coexisting inflammatory processes may also elevate serum levels of certain cytokines (7). However, the consistent pattern of 12 cytokine elevations in different subgroups leads us to conclusion that such considerations do not significantly affect the serum levels of the cytokines analyzed. We conclude that serum cytokines in studied groups have dual pro-inflammatory and anti-inflammatory profiles. Twelve different components, both pro- and anti-inflammatory, were analyzed: IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFN γ , TNF α , IL-1 α , IL-1 β , MCP-1 and EGF. However, confirmatory studies are required before one can consider the clinical use of this profile of biomarkers. Furthermore, particular mediators and mechanisms identified in animal models of hypersensitivity may not be relevant to humans. Human mast cells differ from rats mast cells in cytokine production, immunoglobulin receptor expression, degranulation and release of mediators.

In addition to IL-4 and IL-10 there are other cytokines that may be measured (IL-5 and IL-13) that reflect or are indices of hypersensitivity and which have not been evaluated in this context.

Conclusions

Assessment of changes in cytokine profile after administration of honey with sulfonamides led to results that highlight the levels of determined cytokines were significantly increased in rats receiving honey with sulfonamides for 5 - 14 consecutive days compared with controls. The IL-4 and IL-10 cytokines levels along with eosinophils and IgE were increased 5–30 fold with a maximum response at 14 days after allergen exposure. Other pro-inflammatory cytokines and chemokines were elevated at the time-points analyzed but without a reliable correlation with hypersensitivity indicators. By contrast, no significantly increased concentrations of IL-1 α and

IL-1 β were registered. The blood concentrations of cytokines, which are indicators of hypersensitivity, are elevated and correlate with the presence of eosinophilia in rats after ingestion of honey with sulfonamides residues.

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Abbreviations

APC - Antigen-presenting cell
 EGF - epidermal growth factor
 IFN γ - Interferon-gamma
 IgE - immunoglobulin E
 IL-2 - interleukin 2
 IL-4 - interleukin 4
 IL-6 - interleukin 6
 IL-8 - interleukin 8
 IL-10 - interleukin 10
 IL-1 α - interleukin 1 alpha
 IL-1 β - interleukin 1 beta
 MCP-1 - monocyte chemotactic protein-1
 TNF α - tumor necrosis factor-alpha
 VEGF - Vascular endothelial growth factor

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