The effect of inhaled corticosteroid on salivary cytokine level in relation to *Candida albicans* among asthmatic children

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ABSTRACT

Background: Asthma is a common chronic disease in children in which the body's airways swell, preventing the lungs from filling with air, there are many different inflammatory cells involved in asthma which can synthesize and release cytokines which are recognized to be important in chronic inflammation and play a critical role in the inflammatory response.

Objectives: to assess the local effect of ICS on oral tissue by measuring Interlukine-12 level and *Candida albicans* colony in saliva among12 year's old asthmatic children who were collected from AL- Zahra Center Advisory for Allergy and Asthma, and compares them with non-asthmatic children of the same age and gender.

Type of the study: Cross –sectional study.

Methods: The total sample composed of 60 children 30 asthmatic children who received medium dose of ICS/day for two years and thirty non-asthmatic children. The unstimulated saliva was collected under standard condition and then analyzed for assessment of IL-12 level and *Candida albicans* colony.

Results: Among asthmatic and non- asthmatic children; the mean rank of salivary IL-12 was found to be higher among *C. albicans* carrier group than without *C.albicans* with statistically highly significant difference (P< 0.01). Concerning each gender the same findings were recorded. Concerning IL-12 + and IL-12-, results reported that the mean rank differences were found statistically non-significant difference (P>0.05). Concerning each gender the same results were reported. Concerning gender differences, data analysis showed that there were no statistically significant differences. The results illustrated that the mean rank of salivary IL-12 was found to be higher among asthmatic than non- asthmatic children with statistically significant difference (P=0.016). Concerning each gender, similar results were recorded as the difference was not significant (P>0.05). For *C.albicans* carrier asthmatic and non- asthmatic children and total asthmatic and non-asthmatic children**,** the relation between the IL-12 concentration and *C.albicans* quantities were strong highly significant in positive direction

Conclusions: The findings of the present study showed that the asthmatic disease and oral candida colonization play an important role in elevation of the IL-12 level in saliva.

Keywords: asthma, *candida*, IL-12.

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Asthma is a common chronic disease in children in which the body's airways swell, preventing the lungs from filling with air [1]. Corticosteroids are anti-inflammatory medication that can be inhaled and delivered directly to the respiratory system to reduce the inflamed tissues and allow breathing to resume [2].

Saliva contains a large number of proteins that participate in protection of oral tissue in addition to several peptides with fungal killing activity which had been identified [3]. The cytokines are chemical messengers essential for communication among immune cells which are primarily derived from Antigen Presenting Cells (APCs) i.e. dendritic cells (DCs), mononuclear phagocytes and other APCs [4] , some of them are proinflammatory cytokines which are necessary to initiate an inflammatory response necessary to recruit granulocytes and later on lymphocytes to fight disease by enhancing the bactericidal capacity of phagocytes, recruit additional innate cell populations to sites of infection, induce dentritic cell maturation and direct the ensuing specific immune response to the invading microbes, among these cytokines which appear to play a major role not only as modulators of antifungal effect or functions but also as key regulators in the development of the different T-helper cells (Th) subsets from precursor Th cells which is IL-12 [5].

Interleukin-12 (IL-12) is a heterodimeric cytokine encoded by IL-12A /p35 and IL-12B /p 40 resulting in the only biologically active IL-12/P70 which has a potent immune activity [6] , it is a key immunoregulatory cytokine, it is the critical Th1-polarizing cytokine, and it is a cytokine with links to both innate and adaptive immunity[7].

Concerning IL-12 and *Candida*, the host response to *C.albicans* infection is a complex interplay between cellular and humoral immunity and usually provides a sufficient defense against the microorganism in the healthy individuals [8], the immunoregulatory cytokines released as a result of the initial contact of *Candida* with host monocytes/ macrophages which are the major factor that can regulate the innate immune response and determined the type of the acquired immunity through T-cell development [9]. Lilic et al. [10], showed that patients with chronic mucocatenous candidiasis (CMC) had impaired production of type 1cytokines, particularly IL-12 as well as IL- 2 and IFN-γ.

Concerning IL-12 and asthma, there are many different inflammatory cells involved in asthma including

activated T-helper lymphocytes, eosinophils, mast cells and macrophages, these cells can synthesize and release cytokines which are recognized to be important in chronic inflammation and play a critical role in the inflammatory response[11]. However, these cytokines as well as their positive and negative regulatory network may play important roles in the airway remodeling that occur during asthma pathogenesis including airway wall thickening, matrix deposition, collagen deposition, sub epithelial fibrosis, airway smooth muscle hyperplasia and hypertrophy [12]. Wong et al [13], found that the plasma concentrations of proinflammatory cytokines IL-18 and IL-12, and T-helper cells type tow (Th2) cytokines IL-10 and IL-13 were significantly higher in allergic asthmatic patients than in healthy controls. In contrast Soferman et al [7], found no relationship between IL-12 peripheral blood levels and the course of established asthma in asthmatic children aged 3 to 16 years, however all the participants in this study were treated by inhaled for at least the two previous month, they concluded that there were no publications of a direct influence of inhaled corticosteroids on IL-12 blood levels. On the other hand Pukelsheim et al [14], found that the cytokine levels in asthmatic children with a mean age of 11 years show significantly elevation in IL-4, IL-5, and IL-12, and a decrease in IL-10 and TNF-α as compared to asthmatic adults.

Al-Quraishi [15], found decreased serum IL-12 concentration with no significant differences between asthmatic and healthy children, their ages ranged from 3-12 years with history of recurrent wheeze, cough and dyspnea in the previous 12 months. Later on, Zhang et al [12], reported that the level of serum IL-12 in the asthmatic children was drastically reduced compared to the budesonide-treated alleviated and healthy controls groups whereas the latter two groups showed no significant differences in their serum IL-12 levels.

The studies concerning the concentration of the IL-12 in relation to gender show conflicting results. Bouman et al [16], found that the IL-12 production in males was higher as compared with females.

Method. In the present research, the study group composed of 30 asthmatic children who received medium dose of ICS/day (200-400 microgram /day) aged 12 years old for two years, they were examined in AL- Zahra Center Advisory for Allergy and Asthma during the period from 20 December 2013 till the end of March 2013. The control group composed of 30 non asthmatic children who possess as much as similarity as possible to the study group with regard to gender, social structure, age, and geographic position except in disease condition. The collection of unstimulated saliva was performed under standard condition according to the instructions cited by Navazesh and Kumer [17], and immediately placed it in ice box until reach the microbiological laboratory.

The salivary samples at the Ministry of Science and Technology fungal laboratory, divided into two portions; 1st portion used for microbiological analysis and the second portion used for IL-12 level determination.

For Isolation of Candida albicans each salivary sample of study and control group was dispersed using vortex mixer for 1 minute and then tenfold dilutions were performed by transferring 0.1 ml of each suspension from each tube of the control and study to 0.9 ml of sterile phosphate buffer saline (pH 7.0), then from dilution 10^{-2} salivary samples, 0.1 ml was taken and spread on the Sabourauds dextrose agar (SDA), then the plates were incubated aerobically for 48 hr at 37°C.

For IL-12 Determination, the second portion of each salivary sample was centrifuged according to CUSABIO manufactures instruction at 4000 rpm at 2-8°C for 10 minute, the clear supernatant was separated by micropipette and then stored in a deep freeze at -20°C till further assessment for IL-12 level in saliva by Human Interleukin 12 (IL-12/P70) ELISA Kit; it's Catalog Number CSB-E04599h Figure (1), by using Enzyme Linked-ImmunoSorbent Assay (ELISA) machine. However, the principle of reagent preparation, procedure assay and calculation of results were all done according to CUSABIO manufacture procedure instructions.

This assay employs the quantitative sandwich enzyme immunoassay technique [Enzyme-linked immunosorbent assay (ELISA) which is a test that uses antibodies and color change to identify a substance]. Antibody specific for IL-12/P70 had been pre-coated onto a microplate. The standards and samples were pipetted into the wells and any IL-12/P70 present was bounded by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for IL-12/P70 was added to the wells. After washing, avidin conjugated Horse Radish Peroxidase (HRP) was added to the wells which followed by a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and color was developed in proportion to the amount of IL-12/P70 bound in the initial step. The color development was stopped and the intensity of the color was measured.

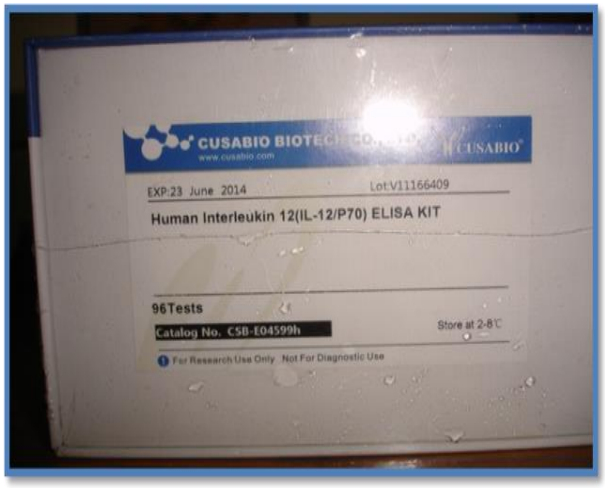

Figure 1: Human Interleukin 12 (IL-12/P70) ELISA Kit

Results. Table (1) shows the difference in salivary IL-12 concentration (pg/ml) between *C.albicans* carrier group and without *C.albicans* group in asthmatic children.

Results reported that the mean rank of salivary IL-12 was significantly higher among *C. albicans* carrier group than without *C.albicans* group of asthmatic children (P< 0.01). Concerning each gender the same finding was recorded with statistically highly significant difference (P< 0.01).

Table (2) shows the difference in salivary IL-12 concentration (pg/ml) between *C.albicans* carrier group and without *C.albicans* group in non- asthmatic children.

Results reported that the mean rank of salivary IL-12 of *C. albicans* carrier group was significantly higher than without *C. albicans* group (P< 0.01). For each

gender the same finding was recorded with statistically highly significant difference (P< 0.01).

Table (3) shows the differences in salivary IL-12 + concentration (*C. albicans* carrier group) and salivary IL-12- concentration (without *C. albicans* group) between asthmatic and non- asthmatic children.

Concerning IL-12 + and IL-12-, results reported that the mean rank was found to be higher among asthmatic than non- asthmatic children with statistically non-significant difference (P>0.05). Concerning each gender for both IL-12 + and IL-12-, the same results were reported with statistically non -significant difference (P>0.05).

Table (4) shows comparison between genders. Concerning asthmatic children, results reported that the mean rank of IL-12+ was found to be equal between boys and girls. While, the mean rank of IL-12- was found to be higher among boys than girls with statistically non-significant difference (P>0.05).

Concerning non-asthmatic children, the results showed that the mean rank of all IL-12+, IL-12- were found to be higher among girls than boys with statistically non-significant difference (P>0.05).

Differences in salivary IL-12 concentration (pg/ml) between asthmatic and non- asthmatic children for total sample are illustrated in Table (5).

Data analysis showed that the mean rank of salivary IL-12 was found to be higher among asthmatic than non- asthmatic children with statistically significant difference (Mann Whitney =287, Z= -2.410, P=0.016). Concerning each gender, similar results were recorded as the difference was not significant (P>0.05).

The relation of the salivary IL-12 concentration with *C.albicans* quantities among *C.albicans* carrier group and with the total sample (with and without C. albicans groups) of asthmatic and non- asthmatic children are illustrated in Table (6).

For *C.albicans* carrier asthmatic and non-asthmatic children, the relation between the IL-12 concentration and *C.albicans* quantities was strong highly significant in positive direction.

For total asthmatic and non- asthmatic children, the same relation was found between the IL-12 concentration and *C.albicans* quantities, which was strong highly significant in positive direction.

Table 1: Difference in salivary IL-12 concentration (pg/ml) between *C. albicans* carrier group and without *C.albicans* group in asthmatic children

Variables	Genders	With *Candida*(carrier)		Without *Candida*		Difference		
		No.	Mean rank	No.	Mean rank	U test	z-value	p-value
IL-12 (pg/ml)	Boys	10	10.9	6	4.5	6	-2.603	0.009 **
	Girls	8	10.25	6	3.83	2	-2.840	0.005**
	Total	18	20.67	12	7.75	15	-3.937	0.000 **

** HS: Highly Sig. at P<0.01 between asthmatic and non- asthmatic children

Table 2: Difference in salivary IL-12 concentration (pg/ml) between carrier group with *C. albicans* and group without *C.albicans* in non-asthmatic children

Variables	Genders	With *Candida*(carrier)		Without *Candida*		Difference		
		No.	Mean rank	No.	Mean rank	U test	z-value	p-value
IL-12 (pg/ml)	Boys	7	13	9	5	0	-3.337	0.001**
	Girls	3	13	11	6	0	-2.572	0.01**
	Total	10	25.5	20	10.5	0	-4.403	0.000**

** HS: Highly Sig. at P<0.01 between asthmatic and non- asthmatic children

Table 3: Difference in salivary IL-12+ concentration (pg/ml) and salivary IL-12- concentration (pg/ml) between asthmatic and non- asthmatic children.

Variables	Genders	Asthmatic				Non- asthmatic				Difference		
		No.	Median	Mean± S.D	Mean rank	No.	Median	Mean± S.D	Mean rank	U test	z-value	p-value
IL-12+ (pg/ml)	Boys	10	72.54	94.55±55.13	10.2	7	64.11	64.37±10.39	7.29	23	-1.171	0.242
	Girls	8	74.62	93.81±58.22	6.25	3	65.14	69.62±14.19	5.33	10	-0.408	0.683
	Total	18	72.54	94.22±54.8	15.9	10	64.63	65.94±11.0	11.9	64.	-	0.22

				2	2			9	5	5	1.223	1
IL-12- (pg/ml)	Boys	6	38.62	41.6±12.25	10.08	9	30.28	32±9.53	6.61	14.5	-1.470	0.140
	Girls	6	30.82	33.73±5.12	9.33	11	34.87	33.99±9.92	8.82	31	-0.201	0.841
	Total	12	31.84	37.66±9.85	19.38	20	31.57	33.09±9.54	14.78	85.5	-1.344	0.179

(Non Sig. at P>0.05;*S: Sig. at P<0.05 between asthmatic and non- asthmatic children; IL-12 + (*C. albicans* carrier group); IL-12- (without *C. albicans* group).

Table 4: Genders differences for asthmatic and non-asthmatic children

Variables	Genders	Asthmatic		Difference			Non- asthmatic		Difference		
		No.	Mean rank	U-test	z-value	p-value	No.	Mean rank	U-test	z-value	p-value
IL-12+ (pg/ml)	Boys	10	9.5	40	0	1	7	5	7	-0.803	0.422
	Girls	8	9.5				3	6.67			
IL-12- (pg/ml)	Boys	6	7.17	14	-0.641	0.522	9	10.11	46	-0.27	0.79
	Girls	6	5.83				11	10.82			

(Non Sig. at P>0.05 between asthmatic and non- asthmatic children)

Table 5: Difference in salivary IL-12 concentration (pg/ml) between the total asthmatic and non- asthmatic children

Variables	Genders	Asthmatic				Non- asthmatic				Difference		
		No.	Median	Mean± S.D.	Mean rank	No.	Median	Mean± S.D.	Mean rank	U test	Z-value	p-value
IL-12 (pg/ml)	Boys	16	59.67	74.69±50.74	19.5	16	44.78	46.17±19.14	13.5	80	-1.810	0.070
	Girls	14	42.06	68.06±52.79	16.89	14	39.24	41.63±18.35	12.11	64.5	-1.540	0.124
	Total	30	55.87	71.59±50.92	35.93	30	40.57	44.05±18.59	25.07	287	-2.410	0.016 *

(Non Sig. at P>0.05;*S: Sig. at P<0.05 between asthmatic and non- asthmatic children)

Table 6: Correlation between salivary IL-12 concentration (pg/ml) and *C. albicans* X 10^2 quantities (CFU /ml) in asthmatic and non-asthmatic children

	Asthmatic				Non-asthmatic			
	C.albicans x10^2 (CFU/ml)				*C.albicans* x10^2 (CFU/ml)			
	Carrier		Total		Carrier		Total	
	rho	p-value	rho	p-value	rho	p-value	rho	p-value
IL-12 (pg/ml)	0.991	0.000 **	0.910	0.000**	0.930	0.000 **	0.712	0.000**

(*S: Sig. at P<0.05; ** HS: Highly Sig. at P<0.01 between asthmatic and non- asthmatic children), rho= (Spearman's correlation coefficient)

Discussion. In the present study, sandwich Enzyme-Linked Immuno Sorbent Assay (ELISA) was used for salivary IL-12 concentrations measurement as it is a diagnostic tool in medicine because of its advantages as compared to other immunoassay methods, which is more accurate, sensitive, specific, fast since many samples can be processed at once–about 90 samples per plate in 2-3 hours, very little sample volume is needed less than 100 µl in most cases, easy to learn and simple procedures [7].

To correlate the course of *Candida* infection with the pattern of cytokine production that observed in response to different doses of the *Candida*, the salivary IL-12 concentration was assessed in the current research. However, no previous study measured IL-12 concentration in the saliva; the

present study represents the first study, therefore it is difficult to compare the salivary IL-12 of the present study with those studies in literatures because these studies measured it in the serum of asthmatic patients and because of the wide variation in the studies design, inclusion criteria, number of patients, the drug type and patient age [14].

The mean rank of salivary IL-12 of the current study was significantly higher among *C.albicans* carrier group than without *C.albicans* group of both asthmatic and non-asthmatic children that could be explained by the fact that the development of the protective immunity against *C.albicans* was linked to the production of endogenous IL-12 [18], and because the initial phagocytosis of *C. albicans* by monocytes and neutrophils as innate immunity induces the synthesis of proinflammatory cytokines such as IL-18, IL-12, and IL-1b [19].

In addition, Mencacci et al [20] and Elahi et al [21], concluded that the high levels of IL-12 was correlated with higher levels of colonization patterns for both blastospore and hyphal forms of *C.albicans* which supports the concept of a balanced Th1 and Th2 response as being an efficient host defense mechanism in clearing oral mucosal infection that give further explanation to the highly significant correlation between IL-12 level and *Candida.*

For total sample, data analysis of the current study showed that the mean rank of salivary IL-12 was found to be higher among asthmatic than non-asthmatic children with statistically significant difference. However these results were in agreement with Wong et al [13], and Pukelsheim et al [14], and were in disagreement with the results that concluded by Soferman et al [7], Al-Quraishi [15], and Zhang et al[12], this higher concentrations of IL-12 might be attributed to the steroid treatment, however there were several evidences and studies on the steroid effect on IL-12 expression (Wright et al [22], Wang et al [23], and Zhang et al [12]) these studies concluded that the steroid treatments might result in improved lung function by down regulating the serum level of IL-13 and up regulating the serum level of IL-12 and redress the imbalance of IL-12/IL-13 in asthmatic patients, thus the steroids might be beneficial for asthma or allergy by exerting at least part of its anti-inflammatory activity through the modulation of the cytokine output [24].

The lack of the genders differences in the current study was disagree with Bouman et al [16], this might due to a small proportion of asthmatic and healthy children resulting in a loss of power to discriminate between health and disease or due to children ages.

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