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MECHANISMS



Alterations of serum and saliva oxidative markers in patients with bronchial asthma

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ABSTRACT

Backgrounds: The development of asthma is highly affected by exposure to exogenous and endogenous oxidative molecules, but the impact of this exposure on the pathophysiology of asthma has received little attention.

Objectives: Evaluating group of selective oxidative stress markers as a tool in the management of asthma disease.

Methods: In comparison with matched healthy controls, levels of the oxidant and antioxidant markers: lipid peroxidation malondialdehyde (MDA), Total glutathione (tGSH), Uric acid (UA), Glutathione peroxidase (GPx), Catalase (CAT) superoxide dismutase (SOD), and Total antioxidant capacity (TAC) were assessed in serum and saliva of different asthma groups.

Results: All oxidative markers in serum and saliva of asthma patients showed significant alterations from normal healthy controls ($P < 0.05$), except the salivary SOD ($P = 0.441$). Their levels in serum were significantly correlated with asthma severity ($P < 0.05$), and the distinguishing between childhood and adult asthma was significantly accomplished by GPx, SOD, TAC markers ($P < 0.05$). However, in patients with childhood asthma no significant differences were detected between the levels of GPx, CAT, UA, MDA in serum and saliva samples ($P > 0.05$).

Conclusion: Determination of the oxidative markers GPx, CAT, UA in serum or saliva can distinguish asthma from healthy states. The serum levels of UA and TAC are highly effective in monitoring asthma severity, while the salivary GPx, CAT, UA, MDA are beneficial in the management of childhood asthma. Discrimination of the age factor between asthma groups can be achieved by testing GPx, SOD, TAC in serum.

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Introduction

Asthma is a pulmonary chronic disease that attacks adults as well as children, and it is characterized by the clinical symptoms of airway obstruction and hyper-responsiveness (1). The development of asthma is enormously influenced by oxidative stress resulting from the excess formation of ROS inside the human body (1,2). These oxidative radicals are contributed by various exogenous and endogenous sources. The exogenous sources of ROS mainly come from exposure to environmental pro-oxidants such as cigarette smoking, ultraviolet light, ionizing radiation, air pollutants, ozone, organic solvents, metals, and some medical drugs (3). The cell organelles, various enzymes, immune cells, and metal ions are the main contributors to the endogenous sources of ROS (4).

Under physiological conditions, the human body usually ensures efficient protection to counteract this oxidative insult by generating antioxidant systems consisting of enzymes and non-enzymatic small molecules. The antioxidant enzymes include superoxide dismutase, catalase, and glutathione peroxidase, while the antioxidant non-enzymatic small molecules involve reduced glutathione, uric acid, alpha-tocopherol, and ascorbate (5). However, in asthma episodes, a collapse may occur in this antioxidant defense system due to the diminution of small antioxidant agents and the modification of defensive enzymes (6). When such a defect in the antioxidant protective system is combined with excessive production of ROS, it may lead to an imbalance in the body's redox state that produces a status of oxidative stress (7). The molecular

impact of this oxidative stress on asthma prognosis can trigger the accumulation of altered proteins, modified DNA, lipid peroxidation (8), and the hampering of airway smooth muscle cell contraction (9). Some of these modifications can also induce pro-inflammatory signaling pathways, leading to further oxidative damages which may aggravate asthma to a progressive stage of high severity (3).

A direct determination of ROS levels is rather complicated due to their very short half-life, high reactivity, and extremely low concentrations inside the cell (10). Thus, to assist the biological roles of ROS inside the human body, often their effects on various oxidative molecules are determined indirectly (11,12).

In the present study, a selective group of oxidative stress by-products was investigated as markers for the pathogenicity of asthma disease. The impact of asthma pathogenicity was deduced from the evaluation of serum and saliva levels of these oxidative markers under disease conditions, in comparison with healthy control subjects.

Materials and methods

Patients and controls

The present study was conducted on one hundred and three asthma patients under the state of remission, for almost one year. The asthma patients comprised mixed-gender groups of 48 adults and 55 children. They were admitted as clinically suspected cases of asthma to the respiratory clinic unit at the Governmental Hospital of Al-Karak city in Jordan. Their definitive diagnosis with asthma disease was confirmed using the criteria described in the report of the Global Initiative for Asthma Program (13). These diagnostic criteria mainly involved the detection of abnormal changes in pulmonary function tests [Forced expiratory volume (FEV1) + Forced vital capacity (FVC)], the observation of breathlessness, wheezing, dyspnea episodes in addition to the pattern of response to treatment with the anti-asthma drug β_2 -agonist (14).

All cases of non-asthmatic pulmonary diseases, such as upper or lower respiratory tract infection, as well as smoking, were excluded from this study by default. A control was selected from a mixed-gender group of 102 matching healthy individuals, with no previous history of smoking, or pulmonary diseases, or other related risk factors.

Asthma severity was determined by FEV1 values and the frequency of asthmatic symptoms as described before (13). Using these criteria, the asthma severity

was divided into three groups of mild persistent, moderate persistent, and severe persistent.

Adults and parents of participating children have agreed to give fully informed consent regarding their acceptances to join this research study, in compliance with the ethical criteria issued by the Al-Karak Hospital in Jordan.

Sample collection

Blood (10 ml) or saliva (5 ml) samples were obtained from patients and controls, respectively. The processed serum samples either were used directly for the measurements of oxidative markers activity or kept in a storage compartment at -80°C , until used. The non-stimulated whole saliva samples were collected in sterile vials after prior rinsing of the patient's mouth with distilled water and excluding any oral intake for at least 20 min before the sample collection. The collected saliva was distributed into aliquots and stored at -80°C until used.

Assays of oxidative markers

The activities of glutathione peroxidase, catalase, copper-zinc superoxide dismutase, concentrations of uric acid, and malondialdehyde were assayed, using the manufacturer's instructions kits from the Abcam comp (www.abcam.com, Cambridge, UK). The total concentration of glutathione (reduced and oxidized glutathione) was assayed colorimetrically using the manufacturer's instructions of the OxiSelect™ assay kit from Cell Biolabs, Inc., San Diego, CA, USA. In this assay, the samples (serum or saliva) were processed immediately due to the instability of glutathione. Any interfering proteins or enzymes were removed from the samples by treatment with met- phosphoric acid. Total Antioxidant Capacity was determined in the sample by following the manufacturer's instructions of the OxiSelect™ kit from Cell Biolabs, Inc. This assay is based on allowing the reduction of copper (II) to copper (I), followed by the reaction of the reduced ion with a chromogenic reagent to produce pink color having maximum absorbance at 490 nm.

Statistical analysis

All data were expressed as mean $\pm$ SD. The comparison between the two groups was performed according to a one-way analysis of variance (ANOVA). The *p* values of less than 0.05 was considered statistically significant.

Results

Characteristics of asthma patients and control individuals

Data in Table 1 show the characteristics of 103 diagnosed asthma cases that participated in this study. They included 48 adults of mixed gender with ages ranging between 20–55 years and 55 children with ages ranging between 6–17 years. The control group comprised 102 matched healthy individuals of mixed gender, aged between 6–57 years subdivided into 50 adults (with ages ranging between 19–57 years) and 52 children (with ages ranging between 6–18 years). The highly significant decrease ($P < 0.001$) in the pulmonary function tests FEV1 (% predicted) and FVC (% predicted) among the group of asthma patients compared to the healthy group, emphasized the disease impact on these patients.

Alterations of oxidative markers level in serum and saliva samples of asthma patients

A significant decrease ($P < 0.05$) in the serum concentration of glutathione peroxidases (Gpx), catalase (CAT), Cu-Zn superoxide dismutase (SOD), uric acid (UA), and total antioxidant capacity (TAC) was observed in the whole asthma group (total adults and children) compared to the concentration of these markers in the matched control group (Table 2). The whole asthma patients also showed a significant increase in serum levels of total glutathione (tGSH) and malondialdehyde (MDA) relative to their serum concentrations in the control group. The most significant differences in the marker concentration between patients and controls were found with GPx, and UA ($P < 0.001$), while the least significant difference was noted with the marker tGSH ($P = 0.039$).

When these markers were assessed in the saliva sample of whole asthma patients, they depicted a similar significant change to their measurement in the serum, except the SOD marker ($P = 0.441$) (Table 3).

The salivary concentration of GPx scored the highest significant difference ($P < 0.001$) while the salivary TAC marked the lowest significant difference ($P = 0.046$) between asthma patients and healthy controls.

A step chart of ROC (Receiver Operating Characteristic) plot expresses the sensitivity versus 1-specificity, was used to test the efficiency of serum and salivary oxidative markers to discriminate asthma disease (Figure 1). The central dotted line on the ROC curve (reference line) represents the middle distance between the disease and non-disease areas, at which the sensitivity = 1-specificity. An improvement in the accuracy of test diagnosis should result in a close shift of the ROC graph to the top left-hand border, and more expansion in the area under the curve (AUC) to approach a maximum value of 1.

Figure 1 reveals that in whole asthma patients the graphs of serum (A) and salivary (B) GPx, UA, and CAT markers behave together as a clustering group separated from the graphs of remaining markers. The graphs of this group are characterized by their farthest shift to the upper left corner and the greater expansion in AUC. The remaining SOD, tGSH, MDA, and TAC markers produce another group with clustering graphs, which remain close to the reference line and endure a pronounced reduction in their AUC.

Correlation of oxidative markers level in serum with asthma severity

To test the correlation of oxidative markers with asthma severity, the serum level of each marker in moderate and severe asthma was compared relative to its corresponding concentration in mild asthma, as a control. Data in Table 4 demonstrate that in severe asthma all markers except GPx show such significant alterations from the control, but the maximum changes were scored by uric acid and TAC ($P < 0.001$). In moderate asthma, a similar relative deviation in the concentration of the markers from the control ($P > 0.05$)

Table 1. Characteristics of asthma patients and control subjects.

Characteristics	Whole asthmatic † (Mixed gender)	Healthy control (Mixed gender)	P value
Number	103	102	
Age group (years)	6–55	6–57	
Adults: Number	48	50	
Age range (years)	20–55	19–57	
Children: Number	55	52	
Age range (years)	6–17	6–18	
FEV1 (% predicted)*	58.3 ± 2.6	89.6 ± 7.5	<0.001
FVC (% predicted)**	71.6 ± 8.2	92.3 ± 3.5	<0.001

*FEV1 (% predicted) = forced expiratory volume in 1 s of predicted.

**FVC (% predicted) = Percent predicted forced vital capacity

†whole asthmatics (children + adults) individuals

P values < 0.05 significant

Table 2. Alterations of oxidative markers level in serum sample of whole asthma patients.

Oxidative marker	Serum sample		P value
	Control (n = 102)	Asthmatics† (n = 103)	
	Mean ± SD	Mean ± SD	
GPx (mU/mL)	174.36 ± 28.3	37.01 ± 6.2	< 0.001
CAT (mU/ml)	6.00 ± 0.7	3.82 ± 0.6	0.028
SOD (ng/ml)	63.53 ± 11.2	46.35 ± 8.1	< 0.01
tGSH (μM)	3.01 ± 0.60	4.51 ± 0.7	0.039
UA (nmol/ml)	312.09 ± 40.7	234.49 ± 51.3	< 0.001
MDA (nmol/ml)	3.78 ± 0.8	6.86 ± 1.5	0.012
TAC (mM)	0.42 ± 0.03	0.21 ± 0.02	0.032

n number of individuals

†whole asthma patients (children + adults)

SD standard deviation

SOD: Cu-Zn superoxide dismutase, Gpx: glutathione peroxidases, MDA: Malondialdehyde, tGSH: total glutathione, UA: uric acid, TAC: total antioxidant capacity. CAT: catalase.

Table 3. Alterations of oxidative markers level in saliva sample of whole asthma patients.

Oxidative marker	Saliva sample		P value
	Control (n = 60)	Asthmatics † (n = 60)	
	Mean ± SD	Mean ± SD	
GPx (mU/mL)	79.79 ± 13.3	14.62 ± 3.8	< 0.001
CAT (mU/ml)	5.58 ± 1.1	3.14 ± 0.66	0.031
SOD (ng/ml)	51.55 ± 8.6	47.60 ± 9.1	0.441
tGSH (μM)	1.1 ± 0.3	1.61 ± 0.4	0.043
UA (nmol/ml)	200.7 ± 32.6	171.05 ± 26.7	0.026
MDA (nmol/ml)	0.41 ± 0.07	0.63 ± 0.12	0.011
TAC (mM)	0.34 ± 0.06	0.24 ± 0.08	0.046

was detected with SOD, UA, MDA, and TAC, respectively.

Comparison of oxidative markers concentration in serum and saliva samples of children with asthma

Due to the variation in the control value of each marker, an arbitrary expression of relative index value was used to present the proportional level of each marker in the asthmatic patients compared to its corresponding value in the control. Figure 2 on childhood asthma indicates that only the relative index values of SOD, that, and TAC showed significant variations ($P < 0.05$) between serum and saliva samples of asthma patients. In contrast, no such significant differences in GPx, CAT, UA, and MDA markers were found in asthmatic children, when these markers were determined in serum or saliva samples ($P > 0.05$).

Age factor contribution to the oxidative stress of asthma

The same expression of relative index value in Figure 2 was used to examine the effect of oxidative markers on the age factor of asthma groups. The markers GPx, SOD, and TAC exhibited significant differences in their relative index values ($P < 0.05$) between adult

and childhood asthma (Figure 3). In contrast, the serum values of the remaining markers CAT, UA, and MDA failed to discriminate the age factor between the two asthma groups ($P > 0.05$).

Discussion

The serum analysis of the investigated oxidative markers revealed significant alterations of their levels in asthma patients ($P < 0.05$) when they were compared with normal healthy controls. Of these alterations, a very significant decrease was observed in the enzyme activities of GPx ($P < 0.001$), SOD ($P < 0.01$) and CAT ($P = 0.028$). These enzymes play important antioxidant roles in neutralizing the primary superoxide radicals inside the human body. The neutralization process involves a dismutation of superoxide radical by SOD to give H_2O_2 , which undergoes a further reduction by CAT and GPx activities to yield water as a final product (11). Thus, by demonstrating a selective decline in the concentration of these three enzymes among the asthma patients; we reinforce the correlation suggested between the clinical manifestation of asthma development and the defect in the intracellular antioxidant protection system (15–18).

We also showed significant asthmatic changes in serum levels of the non-enzymatic antioxidants uric acid and total antioxidant capacity. It is worth noting

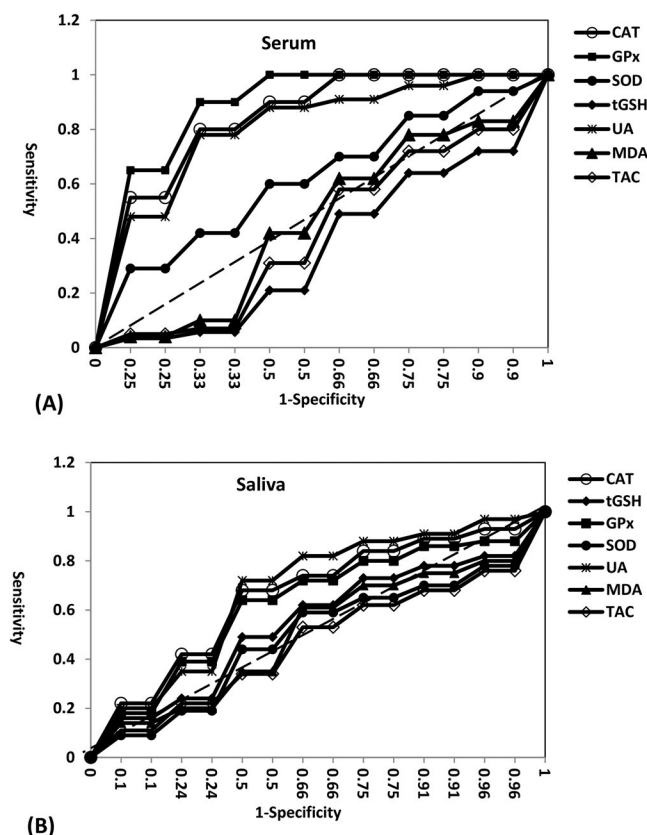


Figure 1. Step chart of ROC plot for the oxidative markers in whole asthma patients. Levels of all seven markers were determined either in serum (A) or in saliva (B) of whole asthma patients. Sensitivity and specificity were determined for each marker. A plot of sensitivity against 1- specificity was performed at different cutoff points. The central dotted line (reference line) is the mark of equality on the curve at Which: the sensitivity = 1- specificity .

Table 4. Correlation of oxidative markers level with asthma severity.

Oxidative marker	Whole Asthma (n=60)		
	Mild (n=13)	Moderate (n=20)	Severe (n=27)
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
GPx (mU/mL)	35.16 $\pm$ 6.4	35.08 $\pm$ 5.9 [§]	34.10 $\pm$ 4.0 [§]
CAT (mU/ml)	4.17 $\pm$ 0.2	3.82 $\pm$ 0.04 [§]	3.35 $\pm$ 0.7*
SOD (ng/ml)	55.22 $\pm$ 9.5	48.32 $\pm$ 8.5*	41.84 $\pm$ 7.9*
tGSH (μ M)	3.25 $\pm$ 0.6	3.23 $\pm$ 0.77 [§]	3.79 $\pm$ 0.68*
UA (nmol/ml)	284.23 $\pm$ 24.9***	237.45 $\pm$ 39.4*	172.55 $\pm$ 34.9
MDA (nmol/ml)	6.21 $\pm$ 0.9	7.25 $\pm$ 1.5*	7.72 $\pm$ 1.5*
TAC (mM)	0.31 $\pm$ 0.06	0.22 $\pm$ 0.03*	0.15 $\pm$ 0.01***

[§]P > 0.05 insignificant.

*P < 0.05 significant.

***. P < 0.001 highly significant.

The serum level of each marker in moderate or severe asthma groups was compared with mild asthma as a control.

that uric acid is a very strong scavenger of ROS, capable of boosting serum total antioxidant capacity (11,19). Other non-enzymatic oxidative markers such as MDA and total glutathione likewise displayed similar asthmatic changes in the current study, in support of the reported data that linked their serum alterations with the burden of asthma pathogenesis (20,21).

The loss of GPx protective effects on cell membrane integrity during an asthma episode exposes this membrane to a high risk of increased lipid

peroxidation, leading to the generation of excess MDA (22). Besides, the dominant generation of oxidized over the reduced form of glutathione favored by the redox shift in asthma disease (23); may contribute to the elevation of total glutathione.

We presented a clear association between asthma severity and the changes in concentrations of the tested oxidative markers, except GPx. The increase in ROS levels (24,25) and the persistent provocation of airway inflammation (26,27), altogether play important

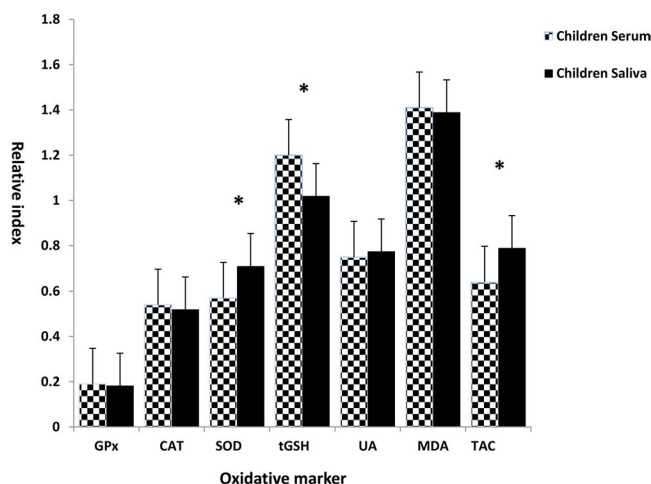


Figure 2. Comparison of oxidative markers level in serum and saliva samples of asthmatic children. Relative index = the marker level in asthmatic patient divided by its level in the control. *The difference is significant.

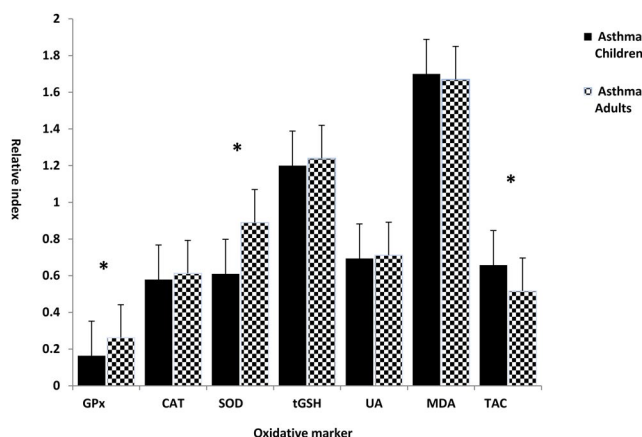


Figure 3. Comparison of oxidative markers level in serum sample of children and adults asthma. Relative index expressed as in Figure 2.

roles in the pathophysiology of asthma. A rise in oxidative stress may promote the progression of airway inflammation through the induction of hyper-responsiveness, production of mucin secretion, and the stimulation of various pro-inflammatory chemical mediators (28,29). Overall, these physiological transformations may result in the enhancement of asthma severity (21,30), which might be mediated by the augmentation of airway inflammation. This capability of the oxidative markers to sensitize inflammation highlights their importance in the assessment of monitoring the severity of asthma disease (31). However, it seems that such sensitization property might be hampered in the case of GPx enzyme action.

Our analysis of the saliva markers indicated that their alterations in asthma patients; almost followed a parallel pattern to their corresponding changes in serum. The exception of these asthmatic alterations was observed with the salivary SOD enzyme activity

($P=0.441$). An analogous relationship between the changes in salivary markers GPx, uric acid, TAC, MDA, tGSH, and the compliance of asthma disease was reported in several studies (32,33).

Since saliva represents the first line of defense in pulmonary airways against ROS-mediated oxidative stress (34), an investigation of salivary markers may help to explore the oxidative injury in airway tissues. Besides, the evaluation of their profile can offer an appropriate tool to track the course of asthma disease; especially when there is difficulty in collecting a blood sample (34).

In accord with this innovation, we showed that the serum and salivary GPx, CAT, UA, and MDA markers were exchangeable in their ability to distinguish asthmatics from non-asthmatic children. This property of the four salivary markers to discriminate the pathological and healthy states of asthma underscores their potential as a differential diagnostic tool in saliva to diagnose childhood asthma. On the contrary, the wide

differences between serum and saliva concentrations of SOD, tGSH, and TAC markers decrease their opportunity as a possible salivary test in the diagnosis of this asthma group.

ROC data have verified the results of Tables 2 and 3 by stressing the accuracy of GPx, CAT, and UA markers to discriminate the pathology of asthma disease. On top of this list emerged GPx as the most powerful discriminating agent, which is characterized by its consistent diagnostic performance in all types of analysis. Overall, the discrimination potential of asthma seems to be most pronounced, when the markers are determined in serum rather than in saliva samples.

Our findings on the substantial variations in serum GPx, SOD, TAC between childhood and adult asthma, highlighted the capability of these three markers to distinguish the age factor among various asthma groups. It has been suggested that characteristic physiological differences occur in the disease severity, duration of symptoms, lung function compliance, and mode of treatments between the groups of childhood and adult asthma (35,36).

Summary

Endogenous oxidative stress molecules can act as markers for tracking the progression of asthma pathogenesis when their levels are determined in serum and saliva samples. A shift in the homeostasis of these oxidative markers in serum is age-dependent and offers an appropriate tool to monitor the severity of asthma disease. Furthermore, the alterations of these markers in saliva samples provide an alternative tool in the management of childhood asthma, particularly when a blood sample is difficult to obtain. Of all promising oxidative markers, GPx emerged as the most accurate oxidative agent in the diagnosis of asthma.

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