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ORIGINAL ARTICLE

ANTIOXIDANT ENZYMES ACTIVITY AND SERUM LEVEL OF OXIDATIVE STRESS PRODUCTS IN EGYPTIAN ASTHMATIC INFANTS AND YOUNG CHILDREN

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Background: ***The oxidant versus antioxidants balance (Redox balance) plays a major role in the response of the airways to injury. Disequilibrium in this balance may be implicated in airway diseases and allergic disorders (e.g. pediatric asthma).***

Objective: ***The aim of this study was to evaluate the oxidant/antioxidant status in early childhood asthma to detect its relation to various clinical and laboratory parameters of pediatric asthma process.***

Patients and Methods: ***This case-control study was conducted on 30 Egyptian asthmatic children who presented to the allergy clinic with early onset asthma, also 20 age and sex matched healthy children were included as control. All the included children were subjected to measurement of red blood cell Superoxide dismutase (SOD) and Glutathione peroxidase (GSH-Px) activities as antioxidant status of those children. Measurement of plasma concentration of Protein carbonyl (PC), detection of leukocyte – generated nitric oxide (NO) and measurement of plasma levels of nitrite were done as assessment of oxidant stress status of those children. These parameters were correlated to different clinical and laboratory data.***

Results: ***Our study showed that the mean level of SOD and GSH-Px activity were significantly lower in patients compared to controls, mean levels of generated NO, plasma nitrate and PC were significantly higher in patients compared to controls (P<0.001). A significant negative correlation was found between the level of SOD and absolute eosinophilic count, while a significant positive correlation was found between leukocyte-generated nitric oxide & total serum IgE. There was no significant correlation between severity of asthma and the various measured parameters in this study.***

Conclusion: ***These findings are consistent with evident increased oxidative stress in Egyptian infants and young children with asthma inflammation. On the other hand evident reduced activity of antioxidant system in Egyptian asthmatic children could be explained by overconsumption of antioxidants against oxidant stress or may spotlight an authentic inherited defect of those rescue tools. This result must be further tested by genetic studies of Egyptian children.***

Keywords: ***Asthma, children, nitric oxide, nitrite, superoxide dismutase, glutathione peroxidase, protein carbonyl.***

INTRODUCTION

The prevalence of asthma has been increasing dramatically over the last few decades particularly in infants and young children. The exact pathophysiological

mechanisms involved in the development of asthma early in life are still unclear. These children seem to have a peculiar response to environmental insults and infective agents.[1]

The airways are highly susceptible to oxidative injury and thus are equipped with strong antioxidant defenses to counteract the oxidant insult. An imbalance between the oxidative forces and the antioxidant defense system favoring oxidative injury has been implicated in many airway diseases including asthma.[2,3]

The production of reactive species during oxidative stress was supposed to contribute for the chronic inflammatory process characteristic of asthma and can produce many of the pathophysiological features of asthma including airway hyperresponsivness, smooth muscle contraction, mucus hypersecretion, and epithelial shedding.[4] The most important oxidant products are the reactive oxygen species (ROS) (such as superoxide radical, hydrogen peroxide, hypochlorous acid and hydroxyl radical) and the reactive nitrogen species (RNS) as nitric oxide (NO).[5-7]

In the human lung, the function of NO as toxic pro-inflammatory or protective anti-inflammatory agent is still unsolved. One of the potentially beneficial effects of NO is derived from its bronchodilating action through regulation of airway smooth muscle.[8] Excess NO and its insufficiency has been implicated in the pathogenesis of numerous lung diseases with an inflammatory component mainly bronchial asthma.[9]

Superoxide dismutase (SOD) enzyme is believed to exert an important protective function against the cytotoxicity of superoxide anion radicals (O2-) that are byproducts of oxidative processes.[10]

Also, the protective function of glutathione peroxidase enzyme is very important through reduction of H2O2 and free fatty acids to harmless compounds.[11]

Oxidative stress may increase lung permeability by up-regulation of matrix-metalloproteinase-9 (a type IV collagenase) that can disturb alveolar basement membrane; protein carbonyl may be associated with this up-regulation.[12]

Therefore, in the present study we aimed at assessing the various aspects of the redox balance in infants and young children with asthma through measurement of the activity of SOD and GSH-Px enzymes in red blood cells, plasma levels of PC, and the levels of nitric oxide generated from activated neutrophils and its metabolite (Nitrite) as markers of tissue injury. The correlation of the measured parameters to different clinical data (age, age of onset, severity, duration, and other atopic manifestations) and laboratory data (blood eosinophil count, total serum IgE) were assessed as well.

PATIENTS AND METHOD

Study Design: This is a cross-sectional case-control study.

Subjects:

Thirty infants and young children with recently diagnosed asthma recruited from the Allergy Clinic of the Allergy and Pulmonology Unit of the Specialized Children Hospital, Cairo University. The children's parents were informed on the purpose of this research and gave their consent. Asthma was diagnosed according to the international guidelines (Expert panel report-2, National Asthma Education and Prevention Program) (NAEPP); 1997.[13]

Patients were included if they were five years of age or younger, clinically stable with no history of treatment with controller medications including systemic steroids and maintained basically on short term quick relief medications (short-acting B2 agonist and/or aminophylline), and with different grades of asthma severity according to asthma severity classification of the international guidelines (1997).

Exclusion criteria of patients included if parents reported preterm delivery, the need for incubator care, ventilation and/or intubation in neonatal period or beyond. Other exclusion criteria were the use of systemic steroids or evidence of respiratory infection at the time of sampling or within the previous two weeks, and suspected or proven alternative causes for recurrent wheezing or other respiratory disease.

Twenty age and sex matched healthy children with no history of atopic disease nor chronic upper or lower airway diseases were included as controls. They fulfilled all exclusion criteria as patients and were subjected to the same investigation as well.

Methods:

All patients were subjected to the following:

Complete history taking with special attention to the onset of symptoms, the duration of the disease and the severity of asthma.

Thorough medical examination.

Sampling: Blood was obtained by venipuncture in heparinised tubes from patients and control.

Laboratory investigations including complete blood picture and total serum IgE. Measurement of red blood cell SOD and GSH-Px activity, plasma level of PC, production of NO and Nitrites by activated neutrophils.

Determination of red blood cell superoxide dismutase (SOD) activity:

Washed red blood cells were hemolysed by means of addition of ice-cold distilled water, and SOD was extracted in a chloroform/ethanol mixture. After centrifugation, the supernatant was analyzed; the assay was carried out according to the method of Abbe.[14] Ranox kits Ranod Cat No: 5D was used.

Determination of red blood cell Glutathione Peroxidase (GSH-Px) activity:

Washed red blood cells were lysed with ice-cold distilled water. Drabkin reagent was added to hemolysate, the assay was carried out by using the method of Little et al,[15] with cumene hydroperoxide as the substrate. The level of glutathione peroxidase was measured by using Ransel Kit.

Determination of plasma level of Protein Carbonyl (PC):

Protein carbonyl in plasma was measured at 380nm by using 2, 4-dinitrophenylhdrazine according to the method of Levine et al.[16]

Determination of Nitric Oxide (NO) and Nitrite generation in plasma and stimulated neutrophils:

Neutrophils were separated by dextran sedimentation. The production of NO by stimulated neutrophils was determined by griess reaction 30, 60, 90 minutes post stimulation with phorbol 12-myristate 13-acetate (PMA).[17]

The Griess reagent (1:1 mixture of 1% sulfanilamide in 5% orthophosphoric acid and 0.1% N-{1-naphthyl} ethylenediamine, Sigma) was added for the development of color.[18]

Statistical Analysis: Data were statistically represented in terms of mean, standard deviation (SD). Comparison of variables was performed using unpaired student's t-test (two-tailed). Spearman's correlation was used for correlation between different variables; correlation is significant at the 0.01 level (2-tailed). All statistical calculation were done using computer program Microsoft excel SPSS version (Statistical Package for Social Science).

RESULTS

Thirty asthmatic children were enrolled in the present study. Their age ranged from 1.5 to 5 years with a mean age of 3.44+0.97 years. Nineteen of them were males (63.3%) and eleven were females (36.7%), with a male to female ratio of 1.7:1.

Basic demographic, clinical and laboratory data of patients included in the study were shown in Table 1.

Table 1. Basic demographic, clinical and laboratory data of patients.

Variables	Range	Mean±SD
Age (years)	1.5-5	3.44±0.97
Age of Onset (months)	0-48	11.97±11.84
Duration (years)	1-4.6	2.48±0.99
Absolute eosinophil count (cell/ml)	0-756	264.7±210.6
Total serum IgE (IU/dl)	10-1159	334.23±342.05
Superoxide dismutase(unit/ml)	93-195	134.5±25.7
Glutathione peroxidase(unit/ml)	1.1-4.2	2.48±0.82
Protein carbonyl(nm/ml)	2.2-9.86	6.13±2.16
Nitric oxide(nmol/ml)	1.2-4.6	2.99±0.91
Nitrite(nmol/ml)	0.9-2.9	1.79±0.47

Included patients had different degrees of asthma severity as shown in Table 2.

Table 2. Degree of asthma severity among included patients.

Degree of Asthma	Number (no)	Percent (%)
Intermittent	14	47%
Mild	9	30%
Moderate	4	13%
Severe	3	10%
Total	30	100%

Individual and mean values of SOD, GSH-Px, PC, NO and Nitrite in studied patients and controls were of high statistical significance, P<0.001 as shown in Table 3 and (Figs. 1-5) respectively.

Table 3. Mean levels of SOD, GSH-Px, protein carbonyl, NO and nitrite in patients and controls.

Parameter	Patients N=30	Controls N=20	P-value
SOD (unit / ml)			
Range	93-195	177-265	
Mean ± SD	135±25.7	211.4±28.46	P < 0.001**
GSH-Px (unit / ml)			
Range	1.1-4.2	3.4-9.4	P < 0.001**
Mean ± SD	2.48±0.98	6.79±1.8	
PC (nm / ml)			
Range	2.2-9.86	1.2-3.5	
Mean ± SD	6.1±2.2	2.02±0.63	P < 0. 001**
NO (n mol / ml)			
Range	1.2-4.6	1.2-2.4	
Mean ± SD	2.99±0.97	1.72±0.27	P < 0.001**
Nitrite (n mol / ml)			
Range	0.9-2.9	0.1-0.9	
Mean ± SD	1.79±0.46	0.44±0.18	P < 0.001**

**P-value < 0.001.

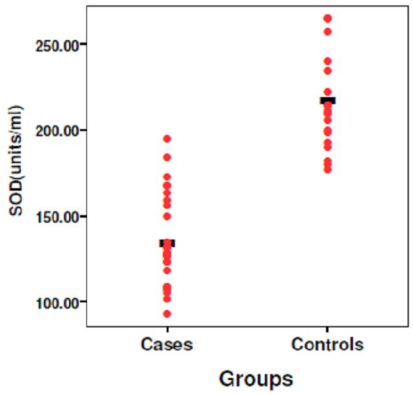

Fig 1. Individual and mean values of SOD in cases and controls P<0.001 Horizontal bars represent the mean values.

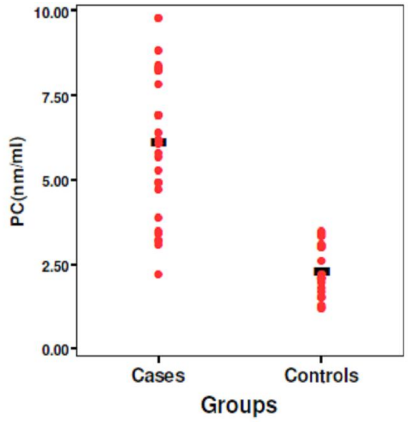

Fig 3. Individual and mean values of PC in cases and controls P<0.001 Horizontal bars represent the mean values.

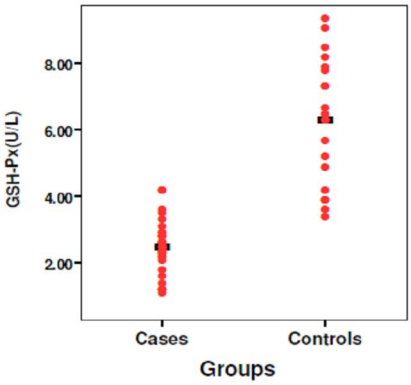

Fig 2. Individual and mean values of GSH-Px in cases and controls P<0.001 Horizontal bars represent the mean values.

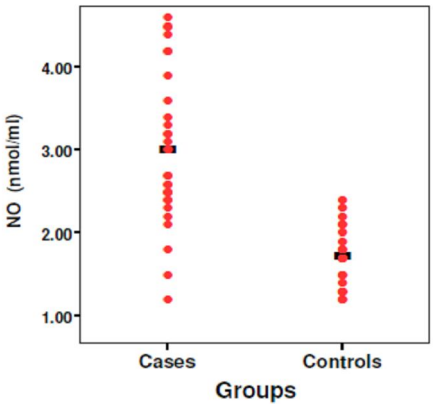

Fig 4. Individual and mean values of NO in cases and controls P<0.001 Horizontal bars represent the mean values.

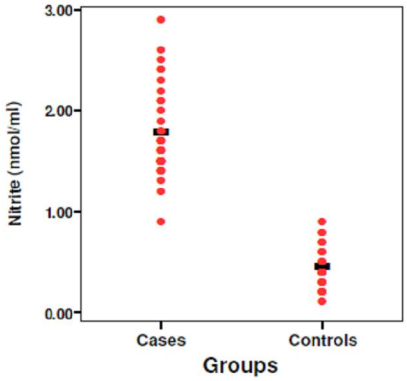

Fig 5. Individual and mean values of Nitrite in cases and controls P<0.001 Horizontal bars represent the mean values.

Spearman's correlation coefficient was used for correlation between age, onset, duration of the disease, severity of airway obstruction, absolute eosinophil count, total serum IgE and SOD, GSH-Px, PC, NO and nitrite levels as shown in Table 4. There was a statistically significant positive correlation between total serum IgE and serum level of generated NO (P 0.004) (Fig. 6), also a significant negative correlation between absolute eosionphil count and SOD (P 0.004) (Fig. 7). While, non-significant correlations were found between other variables and all measured parameters.

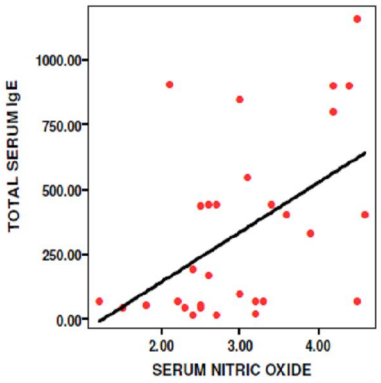

Fig 6. Correlation between NO and total serum IgE.

Fig. 6 shows a significant positive correlation between leukocyte-generated NO and total serum IgE.

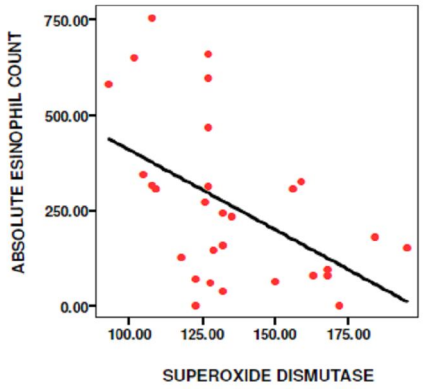

Fig 7. Correlation between SOD and absolute eosinophilic count.

Fig. 7 shows a significant negative correlation between level of SOD and absolute eosinophil count.

Table 4. Spearman's Correlation between clinical and laboratory patient variables and the measured parameters for all patients.

	Age	Onset	Duration	Severity	NO	Nitrites	SOD	GSH-Px	PC	IgE	AEC
Age											
Onset	0.335										
Duration	0.555	0.510									
Severity	0.094	0.514	0.587								
NO	0.650	0.059	0.073	0.258							
Nitrites	0.595	0.699	0.974	0.998	0.460						
SOD	0.942	0.942	0.918	0.629	0.583	0.151					
GSH-Px	0.607	0.159	0.285	0.974	0.440	0.159	0.202				
PC	0.165	0.237	0.029	0.537	0.110	0.266	0.313	0.093			
IgE	-0.196	0.022	00.151	0.966	0.004*	0.083	0.375	0.208	0.802		
AEC	-0.102	0.239	-0.035	0.852	0.663	0.316	0.004*	0.086	0.180	0.402	

* P<0.01.

DISCUSSION

In asthmatic patients the plasma shows increased production of lipid peroxidation products and protein carbonyls (which can be used as an indicator of cellular damage), increased isoprostanes, enhanced generation of ROS, increased oxidized glutathione in bronchoalveolar lavage, and increased nitric oxide (NO) in exhaled air.[19] Shcock and colleagues in 2003[12] found a significant correlation between the level of protein carbonyl and metalloproteinase-9 (which increases the lung permeability) concentration in bronchoalveolar lavage samples from newborn babies having respiratory diseases.

The lung and the blood are loaded with antioxidant enzymes which are crucial for protection of the airways against oxidant stress and these include superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase, vitamin E and vitamin C.[20,21]

Serum protein carbonyl level was significantly elevated in our patients compared to controls and this was in consistent with a study performed by Winter Bourn and colleagues 2000[22] who reported similar results of elevated plasma concentration of protein carbonyl in critically ill asthmatic patients compared to healthy controls, and suggested the use of PC as a marker of increased oxidative stress in asthma.

Nitric oxide (NO), a biological mediator as well as a simple free radical, elicits a wide range of physiological and pathophysiological effects and its role in the development of airway inflammation is increasingly being recognized. Nitrites in plasma are the products of NO metabolism and provide one of the most useful methods to quantify systemic NO production. Several lines of evidence indicate that NO is importantly involved in the regulation of airway smooth muscle tone.[9]

Recently, the finding of no significant difference was reported in serum NO levels of patients with non-acute asthma and controls. It is suggested that asthma patients in this "steady-state" appear to have normal serum NO levels. However in the present study the level of generated NO from blood leukocytes was significantly higher in patients compared to controls, which was in agreement with Paleeve and colleagues 2003[23] who conducted a study on 17 patients with stable asthma (in remission) and 23 healthy controls and reported increased leukocyte generated NO in patients.

Another study performed by Ramesh and colleagues 2001[24] to assess the production of NO by neutrophils in 30 asthmatic patients with different degrees of asthma severity compared to 10 healthy controls, they reported NO production by neutrophils which was significantly higher in asthmatics compared to healthy controls. In the present study plasma nitrite levels were found to be higher in patients compared to controls and the difference was of high statistical significance. This was in contrast to Ekmekci and colleagues 2003[25] who conducted a study on 32 asthmatic patients compared to 30 healthy controls where they reported increased plasma nitric oxide and nitrate in patients compared to controls while nitrite levels did not show any difference.

All the previously mentioned studies had comparable results to our work in that leukocyte-generated NO are increased in asthmatic patients and thus can be used as an inflammatory marker in asthma.

In our study the level of leukocytes generated NO was significantly correlated with an important marker of atopy namely serum IgE. This findings suggest the use of NO as a proinflammatory marker in asthma especially in young children where measurement of NO and nitrites in exhaled air may be tedious.

Several studies have shown decrease in SOD and GSH-Px activity within asthmatic airway epithelial cells, bronchoalveolar lavage fluid and bronchoalveolar lavage cells.[10,26,27] Such techniques can provide information about local antioxidant enzyme activity but they are invasive and unpractical especially in asthmatic infants and young children.

Evidence on a disturbed redox balance in the circulation of adult asthmatics has been presented by previous studies suggesting an ongoing systemic oxidative stress and impaired antioxidant defenses,[28,29] however much fewer studies discuss that in asthmatic children.

Moreover; a number of epidemiological studies suggest that individuals with lower antioxidant intake are at higher risk of development of asthma[30] and reduced pulmonary function.[31]

In the present study we have chosen to measure the levels of blood antioxidant enzymes (SOD, GSH-Px) and oxidatively damaged proteins to evaluate the overall redox balance and oxidative stress in children with early onset asthma.

The mean GSH-Px and SOD enzyme activities were significantly lower in asthmatic children compared to that observed in healthy controls. These findings are consistent with greater oxidant stress in asthma leading to greater inactivation of SOD, which likely amplifies inflammation and progressive airflow obstruction. This was supported by Sackesen and colleagues 2008[32] who reported a significant lower level of GSH-Px and SOD in 164 asthmatic children compared to 173 healthy controls. Also, this was in agreement with Raj and colleagues 2003[33] who reported a significantly reduced glutathione peroxidase activity in asthmatic children compared to

healthy controls.

Our study spotlights this imbalance in asthmatic children with evidence of reduced antioxidants activity. However; this may be explained against increased oxidant stress, also inherited genetic defect of those antioxidants could not be excluded.

Few studies suggested that variations in genes involved in antioxidant defense could be a contributing factor for asthma. Lee and colleagues, 2005[34] studied the genetic polymorphisms in the glutathione S-transferase P1 gene (GSTP1) and the glutathione S-transferase M1 gene (GSTM1) and they found that GSTP1-105 was a predictor for childhood asthma, whereas GSTM1 polymorphism might modify the risk. Also Ercan and colleagues, 2006[35] conducted a study on 312 asthmatic children who were genotyped for null variants of glutathione S transferase (GST) T1 and GSTM1, and ile105val variant of GSTP1 and they found that val/val genotype at GSTP1 ile105val locus may be an important factor in determining the degree of oxidant injury, so these children may be good candidates for supplemental antioxidant therapy. On the other hand Kinnula and colleagues, 2004[36] studied the functional polymorphisms in the genes encoding superoxide dismutases (SOD) and their study showed that it is highly unlikely for the functionally important genetic variants Ala16Val and Arg213Gly of SODs to play a major role in the genetic susceptibility of asthma.

Neverthless; much fewer pilot studies have tried to examine that in Egyptian children.

On the contrary, in some studies antioxidants' increase in response to oxidative stresses has been noticed. Nadeem and colleagues 2003[37] performed a study on 38 patients with an age range from 15 to 40 years and reported increased SOD activity and decreased GSH-Px activity in red blood cells, increased PC in plasma, and increased generation of total nitrates and nitrites in patients compared to 23 control subjects. This may reflect an initial compensatory response to the impaired redox balance followed by exhaustion of the antioxidant enzyme activity. Also this was reported by Jacobson and colleagues 2007[38] who reported increased levels of GSH-Px in 15 patients with acute severe asthma who presented to emergency unit compared to 15 healthy controls.

The measures of oxidative stress and antioxidant status did not differ significantly between patients with different asthma severity in our study which may be attributed to the small number of patients in each severity grade. But; a significant correlation between absolute eosionphil count and SOD was detected in the present study. However our results agreed with that of Nadeem and colleagues 2003[37] who found the same findings. In contrast Perisic and colleagues 2007[39] performed a study on 28 children with two degrees of asthma severity (11 with mild asthma and 17 with moderate persistent asthma), and reported an increased Cu Zn SOD enzyme activity in moderate asthma, but not in mild asthma, compared to 10 healthy controls.

In studies that have found differences related to severity, patients with acute exacerbation have been included, thus giving a wider spectrum of severity.[40]

In conclusion, our observations of reduced levels of SOD and GSH-Px enzymatic activity, increased plasma level of PC, and increased generated NO and nitrites reflect disequilibrium in the redox balance in asthmatic children with increased oxidative stress and evidently reduced antioxidant system. Either consumed or genetically defective antioxidant system in Egyptian asthmatic children was supposed.

Nevertheless, evident redox imbalance was found in all the asthmatic children, this was without any differentiations between different asthma severities. These findings imply that the presence of the disease itself is the most dominant factor determining the extent of the oxidant/antioxidant balance. However; measurement of blood levels of antioxidants and oxidatively modified proteins can serve as a valuable tool to assess the magnitude of oxidative injury in young children with early asthma.

Such findings have great implications regarding possible future therapeutic interventions through augmentation of antioxidant defenses. Further studies are extremely required for testing the genetic background of antioxidant system in asthmatic children and proposal for the delivery of the antioxidants to the airways, as means of novel specific treatment of pediatric asthma.

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