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DNA damage and glutathione level in children with asthma bronchiale: Effect of antiasthmatic therapy

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When the production of reactive oxygen species (ROS) exceeds the capacity of antioxidant defences, a condition known as oxidative stress occurs and it has been implicated in many pathological conditions including asthma. Interaction of ROS with DNA may result in mutagenic oxidative base modifications such as 8-hydroxydeoxyguanosine (8-oxo-*d*Guo) and DNA strand breaks. Reduced glutathione (GSH) serves as a powerful antioxidant against harmful effects of ROS. The aim of this study was to describe DNA damage as level of DNA strand breaks and formamidopyrimidine DNA glycosylase (Fpg)-sensitive sites, which reflects oxidative DNA damage and GSH level in children with mild-to-moderate persistent asthma; and to examine the effect of antiasthmatic therapy on these DNA damage parameters and GSH level. Before and after 8 wk of antiasthmatic therapy blood samples were taken, DNA strand breaks and Fpg-sensitive sites in peripheral leukocytes were determined by comet assay, GSH level of whole blood was measured by spectrophotometric method. DNA strand breaks and Fpg-sensitive sites in the asthma group were found to be increased as compared with control group. GSH level in the asthma group was not significantly different from those in the control group. Levels of strand breaks, Fpg-sensitive sites and GSH were found to be decreased in the asthma group after the treatment. In conclusion, oxidative DNA damage (strand breaks and Fpg-sensitive sites) is at a high level in children with asthma. DNA damage parameters and GSH level were found to be decreased after therapy. Our findings imply that antiasthmatic therapy including glucocorticosteroids not only controls asthma but also decreases mutation risk in children with asthma bronchiale.

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Key words: asthma; comet assay; DNA strand breaks; formamidopyrimidine DNA glycosylase-sensitive sites; glutathione

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Recent studies have revealed the association between asthma and increased oxidative stress (1, 2). The relation between chronic inflammation and oxidative stress is well documented. Enormous amounts of reactive oxygen species (ROS) are produced during the respiratory burst. On the other hand, aerobic cells possess antioxidant defences, which trap and/or inactivate ROS. When the production of ROS exceeds the capacity of antioxidant defences a condition known as oxidative stress occurs and it has been implicated in many of pathological conditions including asthma (3). ROS are highly reactive molecules, they can easily oxidize cellular components such as lipid, protein and DNA. During inflammation, the generation of ROS may result in oxidative base modifications such as 8-hydroxydeoxyguanosine (8-oxo-*d*Guo) and strand

breaks (4). Reduced glutathione (GSH) serves as a powerful antioxidant against harmful effects of ROS and is abundant in all mammalian cells.

The single cell gel electrophoresis (known as comet assay) can be used for determination of DNA strand breaks and oxidized purines on DNA by using damage specific-repair endonuclease, formamidopyrimidine DNA glycosylase (Fpg). This rapid, sensitive fluorescence microscopic method for detection of damage in individual cell has been increasingly employed (5, 6). In the present study, we aimed to describe DNA damage as level of DNA strand breaks and Fpg-sensitive sites which reflects oxidative DNA damage in peripheral leukocytes and GSH level in whole blood; and to examine the effect of antiasthmatic therapy on DNA damage parameters and GSH level in children with asthma bronchiale.

Methods

Study groups

A total of 30 children with asthma bronchiale between 4 and 11 yr of age were recruited from Bakirkoy Dr Sadi Konuk Education And Research Hospital, Department of Pediatrics.

The clinical diagnosis and severity of asthma were determined by using the criteria defined in the Global Initiative for Asthma guidelines (GINA) (7). Children have mild-to-moderate persistent asthma. All of the children were newly diagnosed and none of them had taken antiasthmatic therapy previously. Subjects with anaemia, acute and/or chronic inflammatory disease, autoimmune disorder or infectious disease were excluded. Family history of atopy, seasonal alterations, smoking habit of the parents were inquired, complete blood count was performed, serum level of total IgE was measured, eosinophilia in peripheral blood and in nasal smear were examined, weight and height percentiles were determined, spirometric measurement was carried on and skin prick test (SPT) to aeroallergens was performed in all study subjects. The control group was constituted by age-matched 19 healthy children between 4 and 13 yr of age who are periodically attend same clinic for regular check-ups of their development. Children were included in the control group if they have no signs of atopic disorder and a personal or family history of atopy. Their spirometric test results were normal. Eosinophil level was in the normal range (< 5%) in whole blood and was lower than %5 in nasal smear in each control subjects. All control subjects were negative for SPT. Children with anaemia, acute and/or chronic inflammatory disease, autoimmune disorder and infectious disease were excluded. In both control and asthma group, none of the children was taking antioxidant vitamin supplementation. Children with asthma have treated with a same dosage of an inhaler corticosteroid + leucotriene receptor antagonist + systemic antihistaminic + inhaler beta2 mimetic, if necessary. After 8 wk of treatment, their disease had been controlled, none of them had any asthma exacerbation within the previous 4 wk. Bakirkoy Dr. Sadi Konuk Education And Research Hospital Ethical Committee approval was taken in accordance with the principles of Declaration of Helsinki and informed consent was obtained from parents of all subjects.

Blood sampling and measurements

Blood samples were taken before and after 8 wk of treatment. Venous blood samples were collected after an overnight fasting. Serum level of total IgE and complete blood count were determined in the routine analysis laboratory of the hospital. Five millilitre of venous blood was withdrawn into heparinized tube and used for determination of GSH and DNA damage parameters immediately.

Determination of GSH level in whole blood

GSH levels were measured by the method of Beutler et al. (8) with 5,5-dithiobis 2-nitro benzoic acid (DTNB) used as the sulfhydril reagent. A volume of 0.2 ml of whole blood was added to 1.8 ml of distilled water and 3 ml of the precipitating solution (1.67 g glacial meta phosphoric acid + 0.2 g EDTA + 30 g NaCl per 100 ml solution) was mixed with the haemolysate. The mixture was allowed to stand for *c.* 5 min and filtered. A volume of 2 ml of the filtrate was added to 8 ml of the phosphate tampon and 1 ml of the DTNB solution (40 mg DTNB per 100 ml of 1% sodium citrate) was added again. The optical density was measured at 432 nm using the spectrophotometer. GSH level was determined using the molar absorption coefficient of the GSH at 432 nm (1.36×10^4 l/mol/cm), and expressed as micromoles/gram haemoglobin (μmol/gHb).

Determination of basal level of DNA strand breaks and Fpg-sensitive sites

DNA damage parameters were detected in DNA of peripheral leukocytes with the comet assay

established by Singh et al. (9), and as decribed previously (10). Briefly, after embedding cells in a layer of agarose on a microscope slide, they are lysed with detergent and treated with high-salt solution, leaving 'nucleoids' containing intact supercoiled loops of DNA. If strand breaks are present, supercoiling is relaxed when placed in alkali. Electrophoresis is then carried out at high pH, and loops of DNA extend towards the anode, giving a appearance of the tail of comet when stained and viewed by fluorescence microscopy. Undamaged DNA remains within the head. In order to evaluate the degree of damage comet images were scored visually. Slides were duplicated for each case. Fifty cells per slide were examined and one hundred cells were examined per case by a single observer who was not aware of the subject's diagnosis. Each comet was classified to five categories (0–4) according to an extend of DNA migration. The comets with bright heads and no apparent tails were assigned to category 0, images with very little heads and long diffused tails to category 4. Comets displaying features intermediate between category 0 and 4 were divided and assigned to easily distinguishable categories 1, 2 and 3. The number of comets in each category was counted and average DNA damage in the case of strand breaks was expressed as arbitrary unit (au) which is related to the percentage of DNA in the tail (11) .

To examine Fpg-sensitive sites, DNA was incubated with Fpg, a bacterial protein which generates additional breaks at sites containing 8-oxo-*d*Guo, after the lysis step. Subtracting the per cent DNA in tail without Fpg incubation from the per cent DNA in tail with Fpg incubation gives the net amount of base damage represented by Fpg-sensitive sites (12).

Statistical analysis

Data were expressed as mean ± s.d. Data were analyzed using the SPSS for Windows computing program, version 10.0. Comparisons between groups were carried out by paired *t*-test and Wilcoxon rank test. When the asthma group was divided into subgroups, (i.e., children with family history of atopy and without family history of atopy, children had smoker parents and non-smoker parents, children with mild asthma and moderate persistent asthma) comparisons between those groups were performed by Mann–Whitney *U*-test. A value of $p < 0.05$ was considered statistically significant. Correlations between variables were assessed by Pearson test.

Results

Demographic and clinical data of the study groups are shown in Table 1. DNA strand breaks and Fpg-sensitive sites in the asthma group before the treatment were found to be increased as compared with control group, but GSH level was not significantly different from those in the control group (Table 2). In the group of children with asthma, DNA strand breaks level before the treatment was higher in the children with family history of atopy (80 ± 8 au) au, than those in the children without family history of atopy (75 ± 4 au) (Fig. 1), there was not significant difference between those groups for level of Fpg-sensitive sites (37 ± 5 au and 40 ± 5 au, respectively) and GSH (2.89 ± 0.74 μmol/g Hb and 2.54 ± 0.52 μmol/g Hb, respectively). In the group of children with asthma, there was no significant difference between the children who had smoker parents (DNA strand breaks = 78 ± 7 au, Fpg-sensitive sites = 37 ± 6 au, GSH = 2.90 ± 0.50 μmol/g Hb) and non-smoker parents (DNA strand breaks = 81 ± 8 au, Fpg-sensitive sites = 38 ± 4 au, GSH = 2.63 ±

Table 1. Demographic and clinical data of the study groups

	Asthma group n = 30	Control group n = 19
Mean age (yrs)	8 ± 3	9 ± 3
Gender		
Male	18	5
Female	12	14
Weight percentile	50 ± 27	51 ± 29
Height percentile	53 ± 26	52 ± 26
Total IgE (IU/ml)	244 ± 240*	120 ± 57
Atopy in family (%)	73	0
Smoking in parents (%)	60	58
Severity of asthma (%)		
Mild	53.3	
Moderate	46.7	

*$p < 0.05$ vs. control group.

Table 2. DNA damage parameters and GSH level in the study groups

	Children with asthma group		
	Before the treatment	After the treatment	Control group
DNA strand breaks (au)	79 ± 7	69 ± 7*	72 ± 9†
FPG-sensitive sites (au)	37 ± 5	28 ± 6*,‡	32 ± 7§
Blood GSH (μmol/g Hb)	2.79 ± 0.60	2.40 ± 0.73¶	2.52 ± 1.22

*$p < 0.001$ vs. before the treatment.
†$p < 0.01$ vs. before the treatment.
‡$p < 0.004$ vs. control.
§$p < 0.002$ vs. before treatment.
¶$p < 0.05$ vs. before treatment.

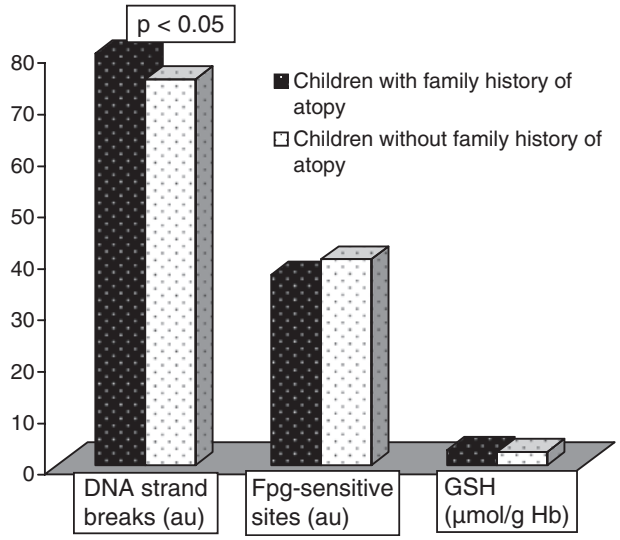


Fig. 1. DNA damage parameters and GSH level in children with asthma as respect with the family history of atopy.

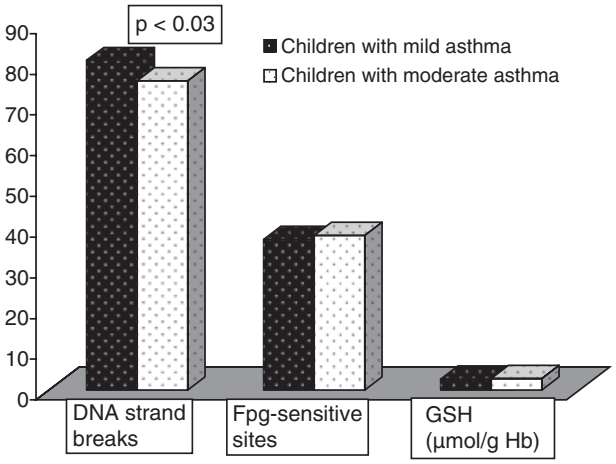


Fig. 2. DNA damage parameters and GSH level in children with asthma as respect with the severity of disease.

0.93 μmol/g Hb) for measured parameters. When data were compared with respect to the severity of asthma, DNA strand breaks level in the mild asthma group (81 ± 7 au) was found to be increased as compared to moderate persistent asthma group (76 ± 5 au) (Fig. 2), but no significant difference was determined for level of neither Fpg-sensitive sites (37 ± 5 au and 38 ± 6 au respectively) nor GSH (2.75 ± 0.72 μmol/g Hb and 2.85 ± 0.70 μmol/g Hb respectively). Levels of DNA strand breaks. Fpg-sensitive sites and GSH were found to be decreased in the asthma group after the treatment (Table 2). In the asthma group, a weak positive correlation was determined between serum level of total IgE and the level of Fpg-sensitive sites (r:0.39; $p < 0.05$).

Discussion

It is well documented that inflammation in respiratory track are the source of increased ROS formation in asthma patients (13). ROS can be produced spontaneously or after stimulation of fagocytic cells by environmental factors such as air pollutants and various allergens in asthma patients (14). Increased DNA strand breaks in lymphocytes of children with asthma bronchiale has been shown recently (15). In the present study, we have found increased DNA strand breaks and Fpg-sensitive sites in children with asthma as compared to control group. Strand breaks are produced either during the direct interaction of ROS with DNA or during the repair process of damaged DNA as an intermediate step. Increased Fpg-sensitive sites more dangerous condition than increased strand breaks. As Fpg is a bacterial protein that generates additional breaks at sites containing 8-oxo-*d*Guo. 8-oxo-*d*Guo is highly mutagenic lesion. It has the propensity to mispair with A residues, leading to an increased frequency of spontaneous G:C → T:A mutations (16). Increased frequency of Fpg-sensitive sites indicates increased risk of mutation in children with asthma.

Level of DNA strand breaks in the children with asthma who have a family history of atopy was higher than those in the children without family history of atopy. This increase may indicate a probable association between inheritance and oxidative/antioxidative system. As a matter of fact, a genetic determinant of antioxidant defence in childhood asthma has been reported by Ercan et al. (17) recently. According to findings of the present study, smoking in parents seems uneffective on DNA damage parameters in children with asthma. The degree of inflammation in the airway varies according to the severity of the asthma (18). However, in the contrary, DNA strand breaks level in the mild asthma group was found to be higher than those in the moderate persistent asthma group, no significant difference was determined for level of Fpg-sensitive sites. High level of DNA strand breaks in the mild asthma group may be attributed to compensatory increased DNA repair processes. Glucocorticosteroids control airway inflammation by their inhibiting effect on immune system. Various studies have shown that oxidative stress has decreased after the glucocorticosteroid treatment in patients with asthma who have a high degree of oxidative stress (19,

20). In the present study, frequency of DNA strand breaks and Fpg-sensitive sites were found to be decreased after a 8 wk of an antiasthmatic therapy. This finding gives rise to thought that antiasthmatic terapy also decreases mutation risk in children with asthma bronchiale.

Increased GSH level in erythrocyte haemolysates and bronchoalveoler lavage fluid from patients with asthma have been shown previously (15, 21, 22). This increase may be an adaptive response to increased oxidative stress. In the present study, although GSH level was higher in the asthma group as compared with control group, it did not reach at a statistically significant level. However, it has been found to be significantly decreased after the antiasthmatic therapy. This gives rise to thought that in the case of glucocorticosteroid treatment, inflammatory response is inhibited and oxidative stress decreases, in turn cellular demand for GSH decreases, and finally, synthesis of GSH decreases. In agreement with our data, Rahman et al. have reported decreased GSH level in alveolar epithelial cells after the glucocorticosteroid treatment (23).

In conclusion, to the best of our knowledge, this is the first study that reveals increased Fpg-sensitive sites, which is a highly mutagenic oxidative DNA lesion in children with asthma. Our findings imply that antiasthmatic therapy including glucocorticosteroids not only controls asthma but also lowers cellular demand for GSH and decreases mutation risk in children with asthma bronchiale.

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