

DNA Damage in Children Exposed to Secondhand Cigarette Smoke and its Association with Oxidative Stress

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Received: October 05, 2011; Initial review: October 08, 2011; Accepted: March 05, 2012.

Objective: To compare oxidative status, total antioxidant capacity and values of DNA damage in peripheral blood lymphocytes in children exposed to secondhand cigarette smoke with healthy controls.

Design: Analytical, Observational.

Participants: 54 children without any chronic diseases, attending the healthy child monitoring polyclinic. These comprised 27 children who had been exposed to passive cigarette smoke and 27 children who had not been exposed to cigarette smoke.

Main Outcome Measures: Urine cotinine levels by the chemiluminescent technique; DNA damage by alkaline comet assay; and total oxidant status (TOS) using a novel automated measurement method.

Results: The mean urine cotinine, TOS, Oxidative Stress Index (OSI) and DNA damage values of the group exposed to cigarette smoke were determined to be at significantly higher level compared to the group not exposed to cigarette smoke ($P < 0.001$). No statistically significant difference was determined in the TAS level between the two groups ($P = 0.1$).

Conclusions: The results showed that TOS levels, OSI index and DNA damage in peripheral blood lymphocytes were significantly higher in children exposed to secondhand cigarette smoke than in those not exposed to secondhand cigarette smoke.

Key words: Antioxidant status, Children, DNA damage, Oxidant status, Secondhand cigarette smoke.

Published online: 2012, June 30. PII:S097475591100831-1

Cigarette smoke inhalation causes cancer in various organs, and smoking during pregnancy harms both mother and baby, initially retarding intrauterine development with several side-effects [1]. Various respiratory diseases can be seen in children, even at low-level exposure to environmental cigarette smoke [2].

Cigarette smoke contains several free radicals which may damage lipids, proteins, DNA, carbohydrates and other biomolecules [3]. Increased production of reactive oxygen species (ROS) leads to an imbalance between the oxidative forces and the antioxidant defence systems favoring an oxidative injury.

DNA is a particular target for oxidation as damage may lead to important alterations. Many oxidative footprints are thought to be the result of nonenzymatic reactions between reactive oxygen species and organic molecules, such as proteins, lipids, or DNA. It has been proposed that DNA damage induced by ROS may contribute to increased mutation rates, genome instability, apoptosis and associated tissue regeneration and cell proliferation [4].

Therefore, this study aimed to compare oxidative status, total antioxidant capacity and values of DNA damage in peripheral blood lymphocytes in children exposed to secondhand cigarette smoke with those of healthy controls who had not experienced secondhand cigarette smoke exposure.

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METHODS

A total of 54 children who had no chronic diseases and were attending the healthy child monitoring polyclinic at Harran University Practice and Research Hospital between July and September 2010 were enrolled into the study. These comprised 27 children who had been exposed to passive cigarette smoke and 27 children who had not been exposed to cigarette smoke. Those who had been exposed to environmental cigarette smoke, although they did not smoke themselves (daily exposure to at least 1 cigarette or at least 2 hours exposure to environmental cigarette smoke) and who had a urine cotinine level below 200 ng/mL were accepted as the passive smoking group and those whose

parents did not smoke and had not been exposed to environmental cigarette smoke and had a urine cotinine level below 30ng/mL, formed the control group [5].

Approval was obtained from the Local Ethics Committee for this cross-sectional, controlled study and informed consent was obtained from the parents of all the children. Data was collected by the researcher through face-to-face interviews.

Measurement of Urine Cotinine and Creatinine: A urine sample was taken from each child in a sterile and closed urine tube. At the same time a 5 cc blood sample was taken into a heparinized tube for examination of mononuclear leukocyte DNA damage. The assessment of urine cotinine levels was made by the chemiluminescent technique using DPC Immulite 2000 (Siemens USA). Cotinine levels were calculated as ng/ml. Variations may be seen because cotinine expression in the urine is dependent on the amount of creatinine, so the urine creatinine/cotinine ratio was calculated. Creatinine measurements were made from spot urine samples using the Jaffe colorimetric technique with the Abbott Architect C16000 autoanalyser commercial kit (Abbott Laboratories, USA).

After overnight fasting, venous blood was withdrawn into heparinized tubes and citrated tubes. One mL of heparinized blood was pipetted into another tube immediately to measure lymphocyte DNA damage. The remaining blood was centrifuged at 1300 g for 10 min to separate the plasma. The plasma samples were stored at -80°C until analysis of total antioxidant status (TAS) and total oxidant status (TOS).

Lymphocyte separation: An amount of 1 mL heparinized blood was carefully layered over 1 mL Lymphoprep (Sigma and Aldrich, Oslo, Norway) and centrifuged for 35 min at 500 g and 25°C . The interface band containing lymphocyte was washed with phosphate-buffered saline (PBS) and then collected by 15 min centrifugation at 400 g. The resulting pellets were resuspended in PBS. Membrane integrity was assessed by means of Trypan Blue exclusion method.

Measurement of lymphocyte DNA damage: The endogenous lymphocytes DNA damage was analyzed by alkaline comet assay according to Singh, *et al.* [6] with minor modifications. Ten mL of fresh lymphocyte cell suspension (around 20,000 cells) was mixed with 80 mL of 0.7% low-melting-point agarose (LMA) (Sigma) in PBS at 37°C . Subsequently, 80 μL of this mixture was layered onto slides that had previously been coated with 1.0% hot (60°C) normal melting point agarose (NMA), covered with a cover-slip at 4°C for at least 5 min to allow the agarose to solidify. After removing the cover-slips, the slides were submerged in freshly prepared cold (4°C) lysing solution (2.5 M NaCl, 100 mM EDTA-2Na; 10 mM Tris-HCl, pH

10-10.5; 1% Triton X-100 and 10% DMSO added just before use) for at least 1 hr. Slides were then immersed in freshly prepared alkaline electrophoresis buffer (0.3 mol/l NaOH and 1 mmol/l Na₂EDTA, pH > 13) at 4°C for unwinding (40 min) and then electrophoresis is done (25 V/300 mA, 25 min). All of the above steps were conducted under red light or without direct light in order to prevent additional DNA damage. After electrophoresis, the slides were stained with ethidium bromide (2 $\mu\text{g}/\text{mL}$ in distilled; 70 $\mu\text{l}/\text{slide}$), covered with a coverslip and analyzed using a fluorescence microscope (Nikon, Japan) fitted with epifluorescence and equipped with rhodamine filter (excitation wavelength, 546 nm; barrier filter, 580 nm) The images of 100 randomly chosen nuclei (50 cells from each of two replicate slides) were analyzed visually from each subject, as described elsewhere. Each image was classified according to the intensity of the fluorescence in the comet tail and was given a value of either of 0, 1, 2, 3, or 4 (from undamaged class 0 to maximally damaged class 4), so that the total scores of the slides could be between 0 and 400 arbitrary units (AU). All procedures were completed by the same biochemistry staff and DNA damage was detected by a single observer who was not aware of the subject's diagnosis.

Plasma TAS levels were determined using a novel automated measurement method, developed by Erel [7]. Plasma TOS levels were determined using a novel automated measurement method, developed by Erel [8].

Statistical analysis: Data were analyzed using the SPSS for Windows (Version 11.5). All the values are expressed as mean \pm SD. For a comparison of differences between the children exposed to secondhand cigarette smoke and the control group, chi-squared test and Student's *t*-test or Mann Whitney U-test were used for non-continuous and continuous variables, respectively. Correlation analyses were performed using Pearson's correlation test and Spearman's rank correlation. Statistical significance was defined at $P < 0.05$.

RESULTS

A total of 54 children were enrolled in the study, comprising 27 passive smoking and 27 not exposed to cigarette smoke. There was no statistical significant difference between the groups in terms of gender, age, weight, height and body mass index (**Table I**).

The mean urine cotinine, TOS, OSI and DNA damage values of the group exposed to cigarette smoke were at a statistically significantly high level compared to the group not exposed to cigarette smoke, but no difference was determined in the TAS level (**Table II**). Children exposed to cigarette smoke were allocated into two groups according to the number of cigarettes exposed to daily; 22 children

(85%) were exposed to 1-10 cigarettes per day; 5 children (15%) were exposed to >10 cigarettes per day. A statistically significant difference was determined between these two groups in terms of urine cotinine, TOS, OSI and DNA damage levels but there was no difference in the TAS levels (**Table III**).

There was a significant degree of correlation between the mean cotinine level of the group exposed to cigarette smoke and TOS, OSI and DNA damage (**Fig. 1**). A statistically significant correlation was found between TOS and OSI values and DNA damage of the group exposed to cigarette smoke (**Fig. 2**)

DISCUSSION

In common with worldwide trends, Turkey is facing the significant health problem of children exposed to cigarette smoke. According to the European Tobacco Control Report 2007, the prevalence in Turkey of the passive effects of cigarette smoke on the 13-15-year old age group is 81.6% at home and 85.9% outside the home [9].

The harmful effects of direct exposure and passive smoking have been made known in several studies [10]. A study by Kocyigit, *et al.* [11] determined that smoking filter-cigarettes and hand-rolled cigarettes both strongly increase DNA damage and oxidative stress in humans. However, both DNA and lipids are more negatively affected by the smoke from hand-rolled cigarettes. These findings indicate a correlation between the extent of exposure to cigarette smoke and DNA damage and OS. Measuring the degree of passive smoking is of critical importance regarding the carcinogenic effect and various health problems which occur in children [12].

The results of the present study show that TOS levels, OSI index and DNA damage in peripheral blood lymphocytes were significantly higher in children exposed to secondhand cigarette smoke than in those not exposed to secondhand cigarette smoke. In the only published study of passive smoking and DNA damage in children [13], serum MDA concentration as an indicator of oxidative stress and DNA damage was found to be high in passive smoking children.

In the current study, a significant correlation was determined between OSI, TOS and DNA damage in children exposed to cigarette smoke. It has been demonstrated that oxidative stress can lead to DNA damage, including DNA adducts, strand breaks and other lesions [14]. In addition, the correlation between OS and DNA damage determined in various studies indicates that DNA damage is related to OS [15].

However, in the current study, no difference was determined between the TAS levels of the 2 groups and no

TABLE I A COMPARISON OF MEAN AGE, HEIGHT, WEIGHT AND BMI VALUES OF THE CHILDREN IN THE STUDY

	<i>Exposed to cigarette smoke (n=27)</i>	<i>Not exposed to cigarette smoke (n=27)</i>	<i>P</i>
Age (y)	5.1 ± 0.8	5.4 ± 0.7	0.15
Weight (kg)	17.7 ± 3.1	18.1 ± 2.8	0.59
Height (cm)	110.5 ± 7.9	112.5 ± 7.0	0.34
BMI (kg/m ²)	14.3 ± 1.1	14.2 ± 1.3	0.77

Values in mean ± SD; TAS: Total anti-oxidant status; TOS: Total oxidant status; OSI: Oxidative stress index.

TABLE II URINE COTININE, TAS, TOS, OSI AND DNA DAMAGE LEVELS OF THE GROUPS

	<i>Exposed to cigarette smoke (n=27)</i>	<i>Not exposed to cigarette smoke (n=27)</i>	<i>P</i>
Cotinine (ng/mL)	71.55±39.86	18.81±13.55	<0.001
TAS (mmolTroEqv/L)	0.95±0.16	1.02±0.13	0.10
TOS (µmolH ₂ O ₂ Eqv/L)	32.39±10.19 ^a	19.61±6.26 ^a	<0.001
OSI (AU)	3.21±1.39 ^a	1.93±0.67 ^a	<0.001
DNA damage (AU)	62.14±56.31	6.14±5.51	<0.001

Values in mean ± SD; TAS: Total anti-oxidant status; TOS: Total oxidant status; OSI: Oxidative stress index.

TABLE III URINE COTININE, TAS, TOS, OSI VALUES ACCORDING TO THE NUMBER OF CIGARETTES SMOKED BY PARENTS

	<i>1-10 cigarettes/d (n=22)</i>	<i>>10 cigarettes/d (n=5)</i>	<i>P</i>
Cotinine (ng/mL)	32.55±20.30	66.53±37.26	0.005
TAS (mmolTroEqv/L)	0.97±0.16	0.96±0.15	0.950
TOS (µmolH ₂ O ₂ Eqv/L)	24.27±5.12	26.12±7.36	0.061
OSI (AU)	2.22±0.55	3.76±1.16	0.009
DNA damage (AU)	11.20±7.56	75.09±54.96	0.01

Values in mean ± SD; TAS: Total anti-oxidant status; TOS: Total oxidant status; OSI: Oxidative stress index.

correlation was determined between TAS and DNA damage. Antioxidant level of smokers was determined to be low in a previous study [13]. It has been proposed that the development of the antioxidant system following increased OS from exposure to smoke could be a metabolic self-defence adaptation. Several published studies have put forward the idea that when the oxidant system increases, there is a decrease in the antioxidant system [16,17]. In contrast, a study of asthmatic children by Zeyrek, *et al.* [15] determined the TAS level to be high.

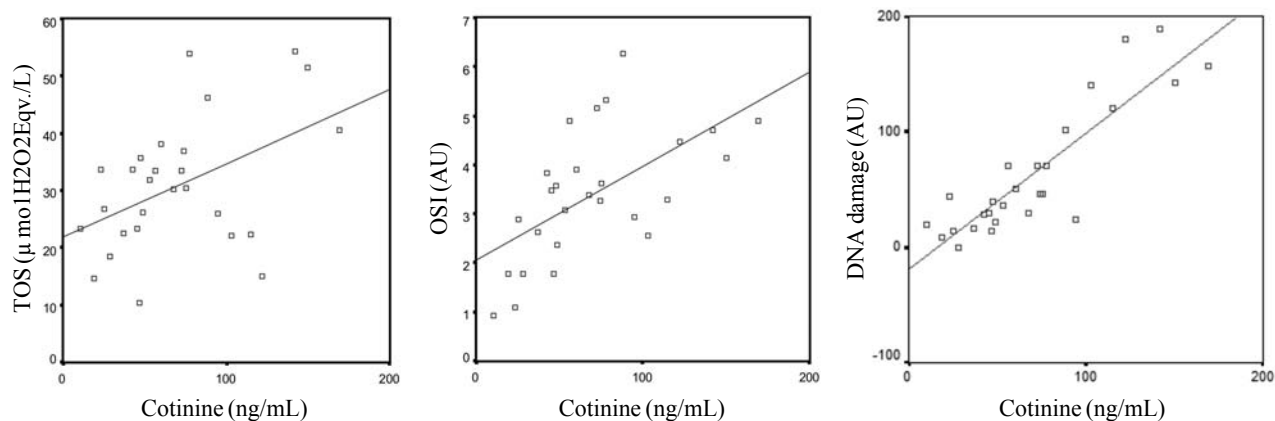


FIG. 1 Relation between cotinine level and Total oxidant status TOS ($r=0.38$); Oxidative stress index; OSI ($r=0.65$); and DNA damage ($r=0.84$) levels in the group exposed to cigarette smoke.

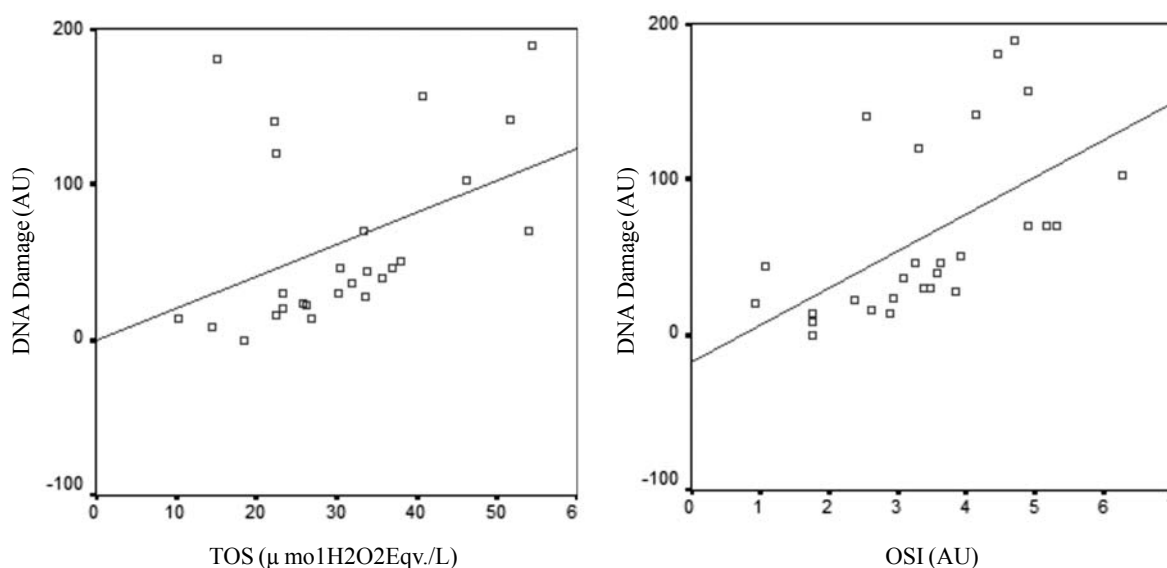


FIG. 2 Relation between the level of DNA damage and Total oxidant status TOS ($r=0.53$); Oxidative stress index OSI ($r=0.72$) levels of the group exposed to cigarette smoke.

Nadeem, *et al.* [16-18] recorded that when there was an increase in the oxidant system there was also an increase in the antioxidant system. Host antioxidant systems are generally activated in response to an oxidant attack, but individuals have different capacities of antioxidant defence, which are in part genetically determined [19]. A study by Ercan, *et al.* [20] showed that there were genetic differences in the antioxidant response. Various other studies have shown that as oxidative stress increases, so the antioxidant capacity increases as a protective mechanism [21].

DNA damage and OSI were determined to be at a significantly high level in the group exposed to more than 10 cigarettes per day (although the statistical value was low because the number in the group was low). Also, a

positive correlation was determined between DNA damage, OSI and cotinine level. The study by Zalata, *et al.* [13] determined a statistically significant relationship between the degree of exposure and DNA damage and oxidative stress. These findings indicate that the severity of exposure is important.

In conclusion, this study of passive smoking children has shown DNA damage and OSI by measuring the level of urine cotinine as an objective criteria of exposure to cigarette smoke. Despite the findings having been determined by reliable methods, a limitation of the study is that the number included in the study was low and because there is widespread exposure to secondhand cigarette smoke in our study population, the number of control cases was insufficient.

WHAT IS ALREADY KNOWN?

- Exposure to passive smoking in children is reported to cause DNA damage and increased oxidative stress.

WHAT THIS STUDY ADDS?

- We document similar findings in Turkish children.

Many studies of adult smokers and passive smokers have reported various substances in cigarette smoke which show a genotoxic effect by damaging the cellular DNA structure [22,23]. Various studies have shown a relationship between cancer and exposure to cigarette smoke in both adults and children [24]. It is thought that future studies of varied cohorts aimed at determining the relationship between DNA damage occurring in children exposed to cigarette smoke and the development of cancer, will increase the importance of these findings.

Contributors: All the authors have contributed, designed and approved the study; *Funding:* The research was funded by the Research Fund of Harran University School of Medicine; *Competing interests:* None stated.

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