Dna damage and oxidative stress in passively smoking children

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ABSTRACT

Tobacco smoking is the greatest single cause of preventable illness and premature death in killing half of all people who continue to smoke for most of their life. Passive smoking causes lung cancer in non-smokers. Genetic markers are used to detect early biological responses in an attempt to link carcinogen exposure to initiating events in the carcinogenesis process.

The present study was conducted on 66 children. Their ages ranged 1-8 years. Twenty children were control, and 46 children were selected from the Outpatient Clinic of Mansoura University Children Hospital. Patients were classified into two test groups. Group 1 comprised of 22 children exposed to light passive smoking. Group 2 included 24 children exposed to heavy passive smoking. Urinary cotinine/creatinine ratio was determined as a marker of exposure to passive smoking for those children.

Heparinized blood samples were collected and used for separation of lymphocytes. Lymphocytes were used for the comet assay and blood sera were used for measurement of glutathione peroxidase activity (GPX), malondialdehde (MDA) and tocopherol fractions (α, γ, δ).

There is significant increase in the number of cases showing underweight, chest problems and other presentations as gastroenteritis in exposed children. Passive smoking increases clinical complications. There is significant increase in comet assay % (P<0.001), and MDA (P<0.001), and significant decrease in GPX activity (P<0.001) and tocopherol fractions (P<0.001) in group 1 and group 2 compared with control.

There is significant correlation between the comet assay and GPX activity (P<0.001), MDA (P<0.001), tocopherol fractions (P<0.001) and cotinine/creatinine ratio (P<0.001). Stepwise regression analysis showed that comet assay results could be explained by the changes of MDA, tocopherol and cotinine/creatinine ratio. In conclusion, exposure of children to tobacco smoke could cause oxidative stress with increased DNA damage which may have a role in certain diseases of children.

INTRODUCTION

In 1994, it was reported that approximately 15 million children were exposed to a second hand smoke. The Third National Health and Nutrition Examination Survey (NHANES III) confirms that 43% of
children live in a home with at least one smoker\cite{1}. In children, there is strong evidence that exposure to environmental tobacco smoke (ETS) is associated with increased risk of respiratory illnesses and asthma\cite{2}, anesthesia complications\cite{3}, sudden infant death syndrome\cite{4}, low birth weight\cite{5}, and adverse lipid profiles\cite{6}.

Tobacco smoke contains many carcinogens that exert their biological effects through interaction of reactive intermediates with DNA to form DNA adducts. Components of tobacco smoke, also, induce oxidative DNA damage. Passive smoking causes elevated levels of protein adducts and lung cancer in exposed non-smokers relative to none exposed non-smokers\cite{7}.

Among the different techniques measuring and analyzing DNA breakage in mammalian cells, the single cell gel electrophoresis assay (comet assay) is considered a rapid, simple, visual and sensitive technique\cite{8}. Smoking was reported to increase the extent of lymphocytes migration by comet assay. Individuals who quit smoking had returned to base levels of DNA damage one year after cessation. However, little data is available about the effects of passive smoking on the antioxidants and DNA damage in exposed non-smokers\cite{9}.

The aim of the current study is to evaluate DNA damage in passive smoking children and its relation to oxidative stress. Also, glutathione peroxidase activity and tocopherols, as antioxidants, were evaluated to throw a light on the pathogenesis accompanying DNA damage in those children.

\section*{SUBJECTS & METHODS}

\subsection*{Subjects}

The current study was conducted on 66 children with age range from one to eight years. Twenty children were considered as a control group. They belonged to non-smoker parents, and they revealed no clinical manifestations of chest or gastrointestinal diseases. In addition, 46 children were selected from the Outpatient Clinic in Mansoura University Children Hospital, and were classified into two subgroups. The first subgroup comprised 22 children whose fathers smoke less than 20 cigarettes/ day (light passive smokers). The second subgroup included 24 children whose fathers smoke more than 20 cigarettes/ day (heavy passive smokers)\cite{5, 10}. On history taking, all the cases were found to be exposed to paternal smoking. Clinical examination was done and included anthropometric measurements (weight, height), chest, heart, and abdominal examination.

\subsection*{Methods}

Five ml blood were collected from each child (after consent). Three ml of the blood were added to sodium heparin for separation of lymphocytes according to Boyum\cite{11}, and the other 2 ml were used to obtain sera for detection of Glutathione peroxidase activity (GPX), tocopherols levels (α, γ and δ) and malondialdehyde (MDA). In addition, urine samples were collected and stored at -20 °C to evaluate cotinine level.

\textbf{1-Comet assay (single cell gel electrophoresis)}\cite{12}
Lymphocyte pellets were mixed with 70 μl of 0.7 % low-melting – temperature agarose (Sigma) at 37°C and placed on microscope slide which has been already covered with a thin layer of 0.5 % normal – melting temperature agarose. After cooling at 4°C for 5 min, slides were covered with a third layer of low- melting agarose. After solidification at 4°C for 5 min, slides were dipped in a lysing solution containing 1 % sodium sarcosinate (Sigma) pH 10 for 60 min.

Slides were then removed and placed on a horizontal gel electrophoresis unit filled with fresh electrophoretic buffer (1 mM disodium EDTA and 200 mM sodium hydroxide, pH 13). Electrophoresis was conducted for 30 min. at 70 V and 300 mA.

Slides were then stained with ethidium bromide stain and examined with the fluorescent microscope. The migrated nuclear DNA was considered as a damaged DNA spot. It was evaluated in 100 randomly selected cells per sample.

2- Determination of Tochopherol (α, γ, δ)(13)

Tochopherols concentrations in serum were determined by high performance liquid chromatography (HPLC), which was performed using a system consisting of a Hewlett packard (1084 B liquid chromatography, USA), and variable wavelength detector adjusted at 284 nm (Hewlett packard, USA). The tochopherols (alpha, gamma and delta) were separated on a reversed phase column (RP – 18 lichrosorb, Hibar, Merck, Darmstadt, Germany). An isocratic eluent with 65% acetonitrile, 35% methanol at a flow rate of 1ml/min. was used. The serum was extracted with n-hexan (Sigma) for 5 minutes then the mixture was centrifuged at 3400 rpm for 8 minutes to separate the hexan layer. The extract was completely dried using a waterbath at 40°C. The extract was reconstituted with 300 μl of methanol.

3- Determination of Malonaldehyde (MDA)" Thiobarbituric acid reactive substances"(14)

Serum proteins were precipitated by addition of trichloroacetic acid (TCA), then, thiobarbituric acid (TAB) reacts with malondialdehyde (MDA) to form thiobarbituric acid reactive product, which is measured colorimetrically at 534nm.

4- Estimation of serum glutathione peroxidase activity:

Glutathione peroxidase activity was assayed by kit purchased from Randox (Randox Laboratories Ltd., UK) (Cat.No. RS 505). The method based on the fact that, glutathione peroxidase (Glu Px) catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP, then the decrease in absorbance at 340 nm is measured colorimetrically.

5- Cotinine assay (16):

Urine samples were refrigerated and cotinine concentrations were determined using an enzyme-linked immunosorbent assay (Solar Care Technologies, Bethesda, PA). In a previous study, the assay was found accurate for measuring cotinine.
concentrations ≥3 ng/mL\(^{(17)}\). Urinary cotinine concentrations were standardized to creatinine concentrations and were expressed as ratios of cotinine to creatinine. Creatinine was measured colorimetrically using picric acid in an alkaline environment\(^{(18)}\).

**Statistical Analysis**

Statistical analysis was performed using MedCalc\® program version 8.1.0.0\(^{(19)}\). The data were expressed as mean ± standard deviation. The qualitative data were presented in the form of number and percentage. Unpaired t-student test was used as a test of significance for comparison between two groups. Spearman rank correlation coefficient was done to study the relation between variables. Multiple regression was used to examine relationship between one dependent variable (Y) and one or more independent variables (Xi) by mean of regression equation, or regression model. P value was considered significant if less than 0.05.

**RESULTS**

By application of the Egyptian centile growth curve\(^{(20)}\), there was significant increase in the number of underweight cases when group 1 and group 2 were compared to control (P<0.05, P<0.001 respectively). However, there was insignificant increase in the number of underweight cases between group 2 and group 1 (table 1).

There was high significant increase in the number of cases presented with chest problems in groups 1 and 2 than control (P<0.001), but there was no significant difference between group 1 and group 2 (table 1). In the same time, there was significant increase in the number of cases complaining of gastroenteritis, failure to thrive, pharyngitis, and dysphagia in group 1 (P<0.01) and group 2 (P<0.001) compared to control, but there was no significant difference in the number of these cases between group 2 and group 1 (P=0.05).

The percentage of the comet assay was significantly higher in groups 1 and 2 than control (P<0.0001), in addition the comet assay % in group 2 was found to be significantly higher compared to group 1 (P<0.0001) (table 2 and figures 1&2).

Table (2) shows highly significantly increased level of MDA in group 1 and group 2 compared to control (P<0.001). Also, the level of MDA was found to be significantly higher in group 2 than group 1 (P=0.001). Furthermore, glutathione peroxidase activity and the levels of alpha, gamma, and delta tocopherols were significantly lower in group 1 and group 2 compared to control (P<0.05, P<0.001 respectively).

Meanwhile, significant decrease was encountered when both groups were compared to each other (P<0.05). Figures (3,4) showed HPLC chromatograms of standard mixtures and patient sample extracted for tocopherols (\(\alpha, \gamma, \delta\)).

There was highly significant increase in cotinine/creatinine ratio in groups 1 and 2 compared to control (P<0.0001). In the same time, high significant increase in cotinine/creatinine ratio was found
when group 2 was compared to group 1 (p<0.0001) (table 2).
There was a positive correlation between the comet assay and MDA and cotinine/creatinine ratio. Meanwhile, comet assay was negatively correlated to GPX activity and alpha, gamma, and delta tocopherols (P<0.0001) (table 3). By stepwise multiple regression analysis of these data, it was found that comet assay was mostly affected by MDA (P<0.0001), alpha tocopherol (P<0.05) and cotinine/creatinine ratio (P<0.05) (table 4).

Figure (1): A photomicrograph of the comet assay from a control child showing no tailing of the normal lymphocytes.

Figure (2): A photomicrograph of the comet assay from a patient showing tailing of lymphocytes.
Figure (3) show HPLC chromatogram of standard mixtures of tocoferols (α, γ, δ).

Figure (4) show HPLC chromatogram of patient sample for tocoferal (α, γ, δ).
Table (1): Demographic and clinical presentations in different groups:

<table>
<thead>
<tr>
<th></th>
<th>Control N=20</th>
<th>group 1 N=22</th>
<th>group 2 N=24</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Under weight %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Centile growth &lt; 5%)</td>
<td>0% (0/20)</td>
<td>31.8 (7/22)</td>
<td>58.3% (14/24)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical presentations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chest problems</td>
<td>0% (0/20)</td>
<td>54.6 (12/22)</td>
<td>62.5 (15/24)</td>
<td>P1 =0.0004</td>
<td>P2 =0.001</td>
<td>P3 =0.0807</td>
</tr>
<tr>
<td>Others (pharyngitis, gastroenteritis, failure to thrive and dysphagia)</td>
<td>0% (0/20)</td>
<td>45.4 (10/22)</td>
<td>37.5 (9/24)</td>
<td>P1 =0.002</td>
<td>P2 =0.0007</td>
<td>P3 =0.807</td>
</tr>
</tbody>
</table>

P1= comparison between control and group 1
P2= comparison between control and group 2
P3= comparison between group 1 and group 2

Table (2): Comparison between comet assay (%), MDA (nmol/mL), GPX activity(U/L), alpha tocopherol (ug/mL), Gamma tocopherol (ug/mL), delta tocopherol(ug/mL), and cotinine/creatinine ratio in different groups (mean± SD):

<table>
<thead>
<tr>
<th></th>
<th>Control N=20</th>
<th>group 1 N=22</th>
<th>group 2 N=24</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comet (%)</td>
<td>7.46 ± 2.44</td>
<td>11.81 ± 2.49</td>
<td>17.46 ± 4.36</td>
<td>P1 &lt; 0.0001</td>
<td>P2 &lt; 0.0001</td>
<td>P3 &lt; 0.0001</td>
</tr>
<tr>
<td>MDA (nmol/mL)</td>
<td>2.42 ± 0.68</td>
<td>4.16 ± 1.0</td>
<td>5.94 ± 1.41</td>
<td>P1 &lt; 0.001</td>
<td>P2 &lt; 0.001</td>
<td>P3 &lt; 0.001</td>
</tr>
<tr>
<td>GPX activity (U/L)</td>
<td>1311.3 ± 219.2</td>
<td>1177.4 ± 127.9</td>
<td>922.4 ± 121.3</td>
<td>P1 &lt; 0.0188</td>
<td>P2 &lt; 0.0001</td>
<td>P3 = 0.0001</td>
</tr>
<tr>
<td>Alpha-tocopherol (ug/mL)</td>
<td>13.9 ± 3.28</td>
<td>11.79 ± 2.18</td>
<td>9.31 ± 2.23</td>
<td>P1 = 0.0114</td>
<td>P2 = 0.0001</td>
<td>P3 = 0.001</td>
</tr>
<tr>
<td>Gamma-tocopherol (ug/mL)</td>
<td>2.13 ± 0.43</td>
<td>1.75 ± 0.63</td>
<td>1.08 ± 0.36</td>
<td>P1 = 0.0294</td>
<td>P2 = 0.0001</td>
<td>P3 = 0.0003</td>
</tr>
<tr>
<td>Delta-tocopherol (ug/mL)</td>
<td>1.26 ± 0.46</td>
<td>1.12 ± 0.36</td>
<td>0.75 ± 0.33</td>
<td>P1 = 0.0275</td>
<td>P2 = 0.0006</td>
<td>P3 = 0.0252</td>
</tr>
<tr>
<td>Cotinine / creatinine ratio (ng/mg)</td>
<td>4.4 ± 1.8</td>
<td>15.9 ± 3.7</td>
<td>27.2 ± 8</td>
<td>P1 &lt; 0.0001</td>
<td>P2 &lt; 0.0001</td>
<td>P3 &lt; 0.0001</td>
</tr>
</tbody>
</table>

P1= comparison between control and group 1
P2= comparison between control and group 2
P3= comparison between group 1 and group 2
Table (3): Correlation between Comet assay and MDA (nmol/L), GPX (U/L), alpha tocoopherol, gamma tocoopherol, delta tocoopherol and cotinine/creatinine ratio (n=66)

<table>
<thead>
<tr>
<th></th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/L)</td>
<td>+0.83</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>GPX (U/L)</td>
<td>-0.63</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Alpha tocopherol (ug/mL)</td>
<td>-0.70</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Gamma tocopherol (ug/mL)</td>
<td>-0.56</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Delta tocopherol (ug/mL)</td>
<td>-0.47</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Cotinine / creatinine ratio</td>
<td>+0.74</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Table (4): Stepwise regression analysis of comet assay with other variables

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Coefficient</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Constant)</td>
<td>9.25405</td>
<td></td>
</tr>
<tr>
<td>MDA (nmol/mL)</td>
<td>1.38930</td>
<td>0.0001</td>
</tr>
<tr>
<td>Alpha tocopherol (ug/mL)</td>
<td>-0.41050</td>
<td>0.0068</td>
</tr>
<tr>
<td>Cotinin/Creatine</td>
<td>0.12355</td>
<td>0.0145</td>
</tr>
</tbody>
</table>

DISCUSSION

Since the human gene pool is our most precious heritage, it is vital to keep the mutagenic pressure as low as possible. The correlation between mutagenic and carcinogenic properties of chemical substances and the inability to cure mutations urgently push scientists to detect mutagenic chemicals. (21)

Parental tobacco use is a serious health issue for all family members. Exposure of children to ETS is a major concern because of its long-term consequences in terms of increased disease risk and morbidity. (22)

The present study showed that exposure of children to tobacco smoking was associated with decrease in weight gain and increase in chest problems and other presentations as gastroenteritis. These findings are in agreement with those of Goncalves et al., (23) who found that exposure of children to tobacco smoking was associated with low stature in children less than five years of age. Also, passive smoking was associated with increased risk of respiratory illnesses and asthma (24), that could be explained by the fact that prenatal exposure to immuno-toxic polycyclic aromatic hydrocarbons (PAHs), a component of ETS, might impair the immune function of the fetus and subsequently might be responsible for an increased susceptibility of newborns and young infants to respiratory infections. Exposure to PAHs, also, had impact on the duration of respiratory symptoms. (24)

In the present work, the significant increase in oxidative stress in the form of increased level of MDA and decreased GPX activity and tocoopherol fractions in exposed non smoking children were consistent with other researches as Dietrich et al. (25) Who found significantly lower plasma
antioxidant status in cigarette smokers and exposed nonsmokers than do unexposed nonsmokers, independent of differences in dietary antioxidant intakes. The mechanism of decreased α-tocopherol in smokers could be related to the increased oxidative stress accompanied by lower plasma ascorbic acid concentrations. Thus, smokers have an increased requirement for both α-tocopherol and ascorbic acid (26).

Prior evaluations of effects of ETS exposure on DNA damage are limited and most have not seen an association (27). However, the current study showed a significant increase in comet assay and hence DNA damage in exposed non-smoking children, a result in harmony with that of Crawford et al., (28) who reported a significant increase in PAHs albumin adducts (a surrogate for PAHs DNA adducts) in children exposed to ETS. In the same time, Fredrica et al. (29) found higher DNA adducts in newborns than in their non-smoking exposed mothers, a finding which was explained by increased susceptibility of the fetus to DNA damage and reduced ability to clear ETS constituents.

However, Whyatt and colleagues (30) found that active and passive smoking status of the mother was significantly associated with PAHs-DNA adduct levels in maternal, but not newborn, WBCs. This was explained by the fact that cigarette smoke might induce the metabolism of PAHs in the maternal tissues more than in the fetal ones. Exposure to PAHs during childhood might, also, be associated with development of cancer during adulthood (31).

In the same time, Phillips (7), showed DNA damage as measured by comet assay in human lymphoid cells treated in vitro with water soluble compounds from cigarette smoke.

In the present study, the significant correlation between the comet assay, and both MAD and antioxidant variables could explain induced DNA damage, as well as the formation of DNA adducts and 8-hydroxydeoxyguanosine associated with environmental tobacco sidestream smokes (32).

The changes of comet assay was found to be correlated with MDA, a tocopherol and cotinine/creatinine ratio, confirming the concept that DNA damage is directly related to oxidative stress.

Active and passive smokers are exposed to the reactive free radicals present in cigarette smoke (33). Since free radicals could lead to oxidative damage to macromolecules such as lipids, proteins, and DNA, they are believed to be involved in the pathogenesis of cardiovascular diseases and cancer (34).

In human, newborns of smoking mothers have elevated frequencies of mutations, translocations, and DNA strand breaks. In rodents, cigarette smoke induces sister chromatid exchanges (SCEs) and micronuclei in bone marrow and lung cells (35).

In lung tissue, vitamin E is one of the first lines of defense against the free radicals generated by cigarette smoke. Some researchers believe that vitamin E is being depleted from smoker's tissue concentrations in order to keep up its levels in the blood, leaving the tissues – including those of the lungs – particularly
vulnerable to attack by toxins and free radicals\textsuperscript{(26)}.

Non-smoking children exposed to paternal tobacco smoke at home compared with none exposed children showed average higher nicotine and cotinine concentrations. There were increased rates of detectable biomarkers in urine with increasing numbers of smoked cigarettes in the household. However, children exposed to paternal smoke showed much higher biomarker levels than the non-smoking spouse of an adult smoker. Therefore, children need specifically protection from ETS at home\textsuperscript{(36)}.

It could be concluded that, exposure of children to passive smoking might cause oxidative stress with increased DNA damage that might have a role in the pathogenesis of certain diseases in children. Since children are unable to complain or remove themselves from tobacco smoke exposure, parents should make sure that their children live in a smoke free environment.

REFERENCES


تلف الحامض النووي والإجهاد التآكسدي في الأطفال المعرضين للتدخين

عائشة زلة **، سهير يحيى ***، أمال البقري ***

كلية الطب - جامعة المنصورة

الهدف من هذه الدراسة هو تقييم تلف الحامض النووي للأطفال المعرضين للتدخين السليلي وعلاقته

بالإجهاد التآكسدي وأيضا دراسة نشاط إنزيم الجلوتاتابيون بروكساز وتركيزات التوكوفيرولات (الفا - جاما - دلتا) لقاح الضروء على أسباب تلف الحامض النووي في هذه المجموعة من الأطفال.

شملت الدراسة 62 طفلا تتراوح أعمارهم من 6-8 سنوات منهم عشرون طفلا كمجموعة ضابطة و42 طفلا في المجموعة الأولى (أباءهم يدخنون أقل من 20 سيجارة في اليوم) والمجموعة الثانية وشمل 24 طفل (أباءهم يدخنون أكثر من 20 سيجارة في اليوم).

وتم تجميع عينات من الدم مضاف إلى الهويارين للصل الخلايا الليفافية وذلك لاستخدامها في عمل اختبار خلايا المفردة الكهربائية بالجيل (اختبار المذنب)، وتم أخذ 2 مل من الدم للحصول على المصل لدراسة نشاط إنزيم الجلوتاتابيون بروكساز ونسبة الدهيد المالون وفصل أنواع التوكوفيرولات بجهاز الكروماتوغرافي السائل على الكفاءة. وتم قبض نسبة الكودين إلى الكرياتين في البول كنواة للتعرض للتدخين السليلي، وقد وجدت زيادة ذات دالة إحصائية في حالات نقص الوزن وأمراض الصدر والزائدة المعوية من بين أطفال المجموعة الأولى والثانية. وقد وجدت زيادة ذات دالة إحصائية في متوسط ثاني الدهيد المالون، وخصص المذنب للحاسوب النووي ونقص ذا دالة إحصائية في نشاط إنزيم الجلوتاتابيون بروكساز وأنواع التوكوفيرولات في المجموعة الأولى والثانية مقترنة بالمجموعة الضابطة وقد وجدت علاقات ارتباط طردي ذات دالة إحصائية بين اختبار المنتج للحاسوب النووي ونشاط إنزيم الجلوتاتابيون ونسبة الدهيد المالون وأنواع التوكوفيرولات ونسبة الكودين إلى الكرياتين في البول. وجدت بين من التحليل الإحصائي أن نتائج اختبار المنتج للفحص النووي يمكن تفسيرها بالاختلافات المصاحبة لذات الدهيد المالون والفاكوفيرول نسبا الكودين إلى الكرياتين في البول.

وذلك يمكن الاستنتاج بأن تعرض الأطفال للتدخين السليلي يعرضهم إلى أضرار صحية جسيمة.