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ARTICLE**

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Levels of Serum M30 and M65 Proteins as Biomarkers of Apoptosis in Children Exposed To Passive Smoking

ABSTRACT

Objective: DNA damage associated with passive smoking has been shown in children. Severe DNA damage can change the apoptosis process of the cell. M30 and M65 biomarkers are signals showing the apoptosis process and can be quantitatively measured. We aimed to determine M30 and M65 levels as apoptosis biomarkers in children exposed to passive smoking.

Methods: A total of 79 children were included in the study. Urine cotinine level was measured with the chemiluminescence method and the levels of apoptosis biomarkers M30 and M65 were measured in peripheral venous blood with PEVIVA/ALEXIS ELISA kits.

Results: The mean serum M30 level of the group exposed to smoking was found to be significantly low compared to that of the control group ($p=0.01$). The serum M65 value was not significant in either group ($p=0.1$). No correlation was determined between the mean cotinine level and M30 ($r=0.25$, $p=0.02$) or M65 ($r=0.16$, $p=0.15$).

Conclusions: The results of this study showed that the apoptosis biomarker M30 levels of the children exposed to passive smoking were low compared to those of the control group which can be interpreted as apoptosis inhibition in exposure to cigarette smoke.

Keywords: Apoptosis, M30, M65, Cotinine, Nicotine

Pasif Sigara İçen Çocuklarda Apoptozis Biyobelirteci Olarak Serum M30 Ve M65 Protein Düzeyleri

ÖZET

Amaç: Pasif sigara içiciliğine bağlı DNA hasarı çocuklarda gösterilmiştir. Ciddi DNA hasarı, hücrenin apoptoz sürecini değiştirebilir. M30 ve M65 biyobelirteçleri apoptoz sürecini gösteren sinyallerdir ve kantitatif olarak ölçülebilir. Pasif sigara içiciliğine maruz kalan çocuklarda M30 ve M65 düzeylerini apoptoz biyobelirteçleri olarak belirlemeyi amaçladık.

Gereç ve Yöntem: Çalışmaya toplam 79 çocuk dahil edildi. İdrar kotinin düzeyi kemilüminesans yöntemiyle ölçüldü ve periferik venöz kanda apoptoz biyobelirteçleri olan M30 ve M65 düzeyleri PEVIVA / ALEXIS ELISA kitleri ile ölçüldü.

Bulgular: Sigaraya maruz kalan grubun ortalama M30 düzeyi kontrol grubuna göre anlamlı derecede düşük bulundu ($p = 0.01$). Serum M65 değeri her iki grupta da anlamlı değildi ($p = 0.1$). Ortalama kotinin düzeyi ile M30 ($r = 0.25$, $p = 0.02$) veya M65 ($r = 0.16$, $p = 0.15$) arasında korelasyon saptanmadı.

Sonuç: Bu çalışmanın sonuçları, pasif sigara içiciliğine maruz kalan çocukların apoptoz biyobelirteci olan M30 düzeylerinin, kontrol grubuna göre kıyaslandığında sigara dumanına maruziyette apoptoz inhibisyonu olarak yorumlanabilecek şekilde düşük olduğunu gösterdi.

Anahtar Kelimeler: Apoptoz, M30, M65, Kotinin, Nikotin.

INTRODUCTION

Passive smoking can be encountered everywhere and is a pollutant which is extremely harmful to human health. Cigarette smoking is a heavy social burden as a risk factor for the development and progression of upper respiratory tract infection and asthma, chronic obstructive pulmonary disease (COPD), lung cancer, coronary heart disease, hypertension and osteoporosis. Even at low levels of exposure to environmental tobacco smoke, harmful effects may be seen in children such as respiratory problems, sudden infant death syndrome, low birth weight, impaired lipid profile and reduced hemoglobin concentration [1, 2].

Tobacco smoke, contains more than 6,000 components, many of which can lead to DNA damage. Studies which have been conducted on DNA damage of cigarette smoking and secondary exposure have shown that passive smoking in children certainly leads to DNA damage [3-9]. Severe DNA damage caused by cigarette smoking or secondary exposure can change the natural apoptosis process of cells. If DNA damage in the cell is not repaired, it can cause permanent mutation resulting in genomic instability, ageing and malignancy. Non-completion of the repair process of damaged DNA is a highly significant factor in the pathogenesis of cancer [10, 11].

Apoptosis is genetically programmed cell death, which provides the safe removal of cells which have lost their function, aged, developed irregularly, formed from DNA damage or proliferated uncontrollably [12]. Cells which have formed with damage, for whatever reason, in the DNA are removed with apoptosis. As mutations occurring in the DNA may have the potential to develop malignancy, the death of these damaged cells by apoptosis is important. Cytokeratines (CK) are expressed from apoptotic cells or those in a state of proliferation [13, 14]. Fragmentation of CK proteins with caspase in the cell facilitates the formation of apoptotic bodies and increases apoptotic signals. In vitro studies have shown that the suppression of CK-18 with caspase during apoptosis resulted in the expression of the formed fragments to the extracellular area [15].

During apoptosis, the CK-18 molecule can cleave with caspase at aspartate-238 and aspartate-396 points. The cleaved fragment of CK-18 in aspartate 396 is known as M30 antigen biomarker and this biomarker can be quantitatively measured. In addition, the M65 biomarker measures both uncleaved and cleaved CK-18. Thus antigens of CK fragments can be used as biomarkers of the apoptotic process [16]. In invitro studies, it was shown that neoepitop fragments formed by caspase digestion of CK-18 during apoptosis were released into the extracellular field [17]. Neoepitop formation occurs in the early stages of apoptosis before the onset of DNA fragmentation. Neoepitope formation is not observed in living cells except in

the apoptosis process [17]. The harmful effects of nicotine which is one of the major toxic components of tobacco can be caused by itself or its metabolites such as cotinine [18, 19]. By measuring the urine cotinine and the M30 and M65 levels as apoptosis biomarkers in children exposed to passive smoking, this study aimed to determine whether or not passive smoking had any effect on apoptosis.

MATERIAL AND METHODS

The study comprised 51 children, aged 6 months-7 years, who had no chronic disease but were exposed to environmental tobacco smoke and 28 age-matched children who were not exposed to environmental tobacco smoke, all of whom presented at the pediatric clinic of Harran University Hospital between December 2012 and September 2013. Children who did not smoke themselves, were allocated to the passive smoking group or the control group. The status was determined by questionnaire as families where at least one cigarette per day was smoked or there was exposure to environmental cigarette smoke for at least two hours per day for the passive smoking group exposed to cigarette smoke and families where cigarettes were not smoked and there was no exposure to environmental cigarette smoke for the control group. The study was planned as a controlled, cross-sectional study. Approval for the study was granted by the Ethics Committee of Harran University Medical Faculty. Informed consent was obtained from all the parents of the children. Data were collected by face-to-face interviews conducted by the researcher. The study was explained to the parents and they were informed that they would be asked some questions and blood and urine samples would be taken from the children. All the children were weighed and their height was measured. Urine samples were taken from all the children into sterile containers for measurement of cotinine levels. At the same time, for measurement of the apoptosis biomarkers, 5 ml venous blood samples were taken into tubes washed with heparin. The blood samples were centrifuged at 3000 rpm for 10 minutes and the separated serum was stored at -80°C until the study. The urine cotinine level tests were made with a ROCHE HITACHI 912 device with the enzyme immunoassay (EIA) method. The cotinine levels were calculated as ng/ml. The passive smoking group was formed of those exposed to environmental cigarette smoke with a urine cotinine level below 200 ng/ml and the control group comprised those with a urine cotinine level below 30ng/ml whose parents did not smoke. Serum Cytokeratine-18 M30 and M65 biomarkers were measured by the ELISA method. Serum CK-18 biomarkers were measured using the PEVIVA/ALEXIS Human M30-Apoptosense ELISA kit (CH 4415 Lausen, Sweden) and PEVIVA M65 EpiDeath ELISA kit (CH 4415

Lausen, Sweden). M30 monoclonal antibody is against the serum ccK-18Asp396 neo-epitope and the M65 monoclonal antibody is against the serumccK-18 epitope. Binding was performed by adding serum CK-18 antigen onto the sample in the plate well of human CK-18 covered with mouse (clone M6, IgG2a) monoclonal antibody. Then HRP (Horseradish Peroxidase) conjugated with M30 or M65 monoclonal antibody was added onto the plate well. Solid phase/antigen/signalled antibody complex formed. Following incubation, the non-bound conjugates were removed with the washing process. Then the staining TMB (tetramethyl-benzidine) substrate was added in correct proportions to the M30 or M65 antigen amount. Following incubation, the colour reaction was halted by adding acidic solution (1M H₂SO₄) onto the plate wells at 450nm and absorbancy was measured. The measured absorbancy values were changed to U/L values with a standard curve graph. Thus the amount of M30 and M65 antigen in the samples was defined quantitatively.

Statistical Analysis: For statistical analysis, the SPSS v.20 (Statistical Package for the Social Sciences, version 20 for Windows, SPSS® Inc, Chicago, IL) software was used. Distribution of parameters was examined with the one-sample

Kolmogorov-Smirnov test, and apart from the cotinine results, the other distribution results were normal. Results for the parameters were stated as mean±standard deviation. In the comparison of the parameters, except for cotinine, the Independent Samples t-test and the Chi-square test were used. For cotinine not showing normal distribution, the Mann-Whitney U-test was used. Pearson correlation tests were applied to analyse relationships. A value of $p < 0.05$ was accepted as statistically significant.

RESULTS

A total of 79 children were included in the study. The children were divided into two groups according to exposure to environmental tobacco smoke. The group not exposed to environmental tobacco smoke (Control group) comprised 28 children; 14 (50%) girls and 14 (50%) boys. The group exposed to environmental tobacco smoke (passive smoking group) comprised 51 children; 26 (51%) girls and 25 (49%) boys. No statistically significant difference was determined between the groups in respect of gender ratios ($p > 0.05$) (Table 1). No statistically significant difference was determined between the groups in respect of age, weight, height and BMI ($p > 0.05$) (Table 1).

Table 1. Comparison of the mean age, weight, height and BMI values of the children

	Passive smoking group (n:51)	Control group (n:28)	P**
Gender (M/F)	25/26	14/14	>0.5
Age (years)	2.5±1.7 ^a	2.4±1.8 ^a	>0.8
Weight (kg)	10.6±3.6 ^a	10.7±4.7 ^a	>0.9
Height (cm)	83.6±14.9 ^a	82.7 ±19.2 ^a	>0.8
BMI (kg/m ²)	14.8±0.8 ^a	15±0.5 ^a	>0.3

^a Given as mean± standard deviation (SD); **: Independent Samples t-test

The mean urine cotinine level of the passive smoking group was determined to be statistically significant high compared to the control group ($p < 0.05$) (Table 2).

The mean M30 antibody levels of the passive smoking group were measured as

statistically significantly low compared to the control group ($p < 0.05$) (Table 2). No statistically significant difference was determined between the groups in respect of the mean M65 antibody levels (Table 2).

Table 2. Urine cotinine, M30 and M65 values of the groups

	Passive smoking group (n:51) mean±SD	Control group (n:28) mean±SD	P value
M30 (U/L)	173.4±42.9	204.5±54.2	<0.01*
M65 (U/L)	400.5±135.4	455.3±158.4	<0.1*
Cotinine (ng/ml)	622.27±600.6	4.25±7.5	<0.001**

SS: Standard deviation; *: Independent Samples t-test; **: Mann-Whitney U-test

In the passive smoking group, there was no significant correlation between age and M30 ($r = 0.67$, $p = 0.56$) and M65 ($r = 0.55$, $p = 0.62$). Similarly, in the control group there was no significant correlation between age and M30 ($r = -0.29$, $p = 0.80$) and M65 ($r = -0.19$, $p = 0.91$). In the

passive smoking group, a negative correlation was determined between the mean cotinine level and the mean M30 level ($r = -0.23$, $p = 0.03$). In the passive smoking group, no correlation was determined between the mean cotinine level and the mean M65 level ($r = 0.11$, $p = 0.29$).

DISCUSSION

The evaluation of exposure to cigarette smoke was made in this study by questionnaire and the measurement of urine cotinine levels. Various previous studies have revealed the necessity of examining the information given by the family with the objective criteria of cotinine level when determining the rates of passive smoking in children [20, 21]. Cobanoğlu et al. [22] determined that the urine cotinine levels of children whose fathers smoked cigarettes were statistically significantly higher than those of children whose fathers did not smoke. Irvine et al. [23] reported that a positive correlation between cotinine levels and the number of cigarettes smoked in the home and the number of people smoking in the home. In our study, the urine cotinine levels of the group exposed to cigarette smoke were measured as significantly high compared to those of the group not exposed to cigarette smoke.

Apoptosis plays a significant role in the pathogenesis of several diseases and it can be used as a biomarker for diagnosis and clinical evaluation [23]. Many different methods have been developed to determine changes in the apoptosis process. To determine the apoptosis process in clinical trials, the most appropriate immunological methods are ELISA (DNA fragmentation, M30 and M65 antigen levels) or fluorometric measurements based on caspase activation in cell culture [24]. In our study, the apoptosis process was evaluated with the ELISA method as fluorometric measurement could not be taken for technical reasons. We found the M30 antibody as a biomarker of apoptosis was significantly low in the group exposed to cigarette smoke compared to the group not exposed. This can be interpreted as apoptosis inhibition in the passive smoking group exposed to cigarette smoke. In addition, there was a negative correlation between urine cotinine levels and M30 antibodies in our study. It suggests that greater exposure to environmental tobacco smoke can cause suppression of the apoptotic process. The effects of cigarette smoke on apoptosis is controversial. In

several studies, it has been reported that the apoptotic process in the bronchial epithelial cells, fibroblasts, alveolar macrophages, the placenta and endothelial cells is increased with the effect of tobacco smoke [25, 26]. However, there are also several studies reporting the exact opposite results [27-33]. These contradictory results can be ascribed to different mechanisms of apoptosis induction, the region where apoptosis occurs and the diversity in determining apoptosis.

Present study is open to various interpretations. The results may be different due to actual differences between the study population. The family situation or parents' behaviour may show a difference in the children. The lack of comparable studies and results such as great differences in the health status of the children exposed to environmental cigarette smoke and those not exposed should be interpreted with great care. The number of subjects in the control group was lower than the passive smoking group due to ethical reasons (due to the fact that the control group was selected only from healthy children) and financial resource limitations. The small number of cases in the control group could be considered as the limitation of this study.

In conclusion, to the best of our knowledge, this is the first study to use M30 and M65 biomarkers to evaluate the apoptosis process in children exposed to cigarette smoke. This study is important because it demonstrates the relationship between exposure to cigarette smoke and apoptosis in children. In this study, as the M30 levels as an apoptosis biomarker were low in children exposed to environmental tobacco smoke compared to those of children not exposed to environmental tobacco smoke. The results of our study suggested that environmental tobacco smoke may encourage a pro-carcinogenic occasion. There is a need for further studies to show the relationship between the extent of exposure to environmental tobacco smoke and apoptosis.

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