

The Effect of Passive Smoking on Total Antioxidant Capacity of Serum and Saliva in Rats

¹Mina Motalebnejad ²Mahdi Pouramir ³Ali-Akbar Moghadamnia ⁴Leila Ghasemi *⁵Laleh Soleimani

¹Associate Professor, Dept. of Oral Medicine, Dental Materials research Center, School of Dentistry, Babol University of Medical Sciences, Babol, Iran.

²Professor, Dept. of Biochemistry, Cellular and Molecular Research Center, Babol University of Medical Sciences, Babol, Iran.

³Professor, Dept. of Pharmacology, Cellular and Molecular Research Center, Babol University of Medical Sciences, Babol, Iran.

⁴General Practitioner.

*⁵General Practitioner. E-mail: laleh_tulip2002@yahoo.com.au

Abstract

Objective: Active and passive smoking lead to the production of a number of oxidants and antioxidants with various adverse health effects compromising the immune system. Tobacco use increases the production of free radicals as well. The purpose of this study was to determine the effects of passive smoking on total antioxidant capacity (TAC) of serum and saliva in rats.

Methods: This experimental study was conducted on 18 rats with an age range of 7-11 weeks weighing 160-200 g; 9 of them were exposed to cigarette smoke 3 times daily for 8 minutes. The 9 controls were not exposed to cigarette smoke. After injection of 0.2 mg/kg midazolam and 0.5 mg/kg pilocarpine, serum and saliva samples were taken from subjects in the exposure and control groups at 0, 15 and 30 days. Serum cotinine was measured using ELISA kit. TAC of saliva and serum was measured using ferric reducing antioxidant power (FRAP) assay. TAC values at different time points were statistically analyzed in each group using Repeated Measures ANOVA and compared between the two groups using t-test.

Results: At baseline, no significant difference existed between the two groups in terms of serum cotinine concentration but at days 15 and 30, cotinine serum concentration significantly increased in the exposure group. At baseline and 30 days, no significant difference existed between the two groups of passive smoker and non-smoker in terms of serum TAC values but at day 15, serum TAC values were significantly higher in the exposure group. Also, TAC of saliva was significantly higher in the passive smoker group at baseline and at day 15 but at day 30, the difference in this respect between the two groups was not statistically significant.

Conclusion: Based on the obtained results, changes in TAC of serum and saliva in rats due to exposure to cigarette smoke were compensated by their immune system activity. However, further investigations are still required in this respect.

Key words: Free radicals, Passive smoking, Saliva, Cotinine, Immune system, Total antioxidant capacity,

Please cite this article as follows:

Motalebnejad M, Pouramir M, Moghadamnia AA, Ghasemi L, Soleimani L. The Effect of Passive Smoking on Total Antioxidant Capacity of Serum and Saliva in Rats. *J Dent Sch* 2013; 31(2): 117-124.

Received: 05.09.2012

Final Revision: 13.03.2013

Accepted: 07.04.2013

Introduction:

Passive smoking or passive inhalation of cigarette smoke in public places is a public health hazard. It is estimated that at least one billion adults are addicted to tobacco use worldwide and at least 700 million children

breathe in cigarette smoke-polluted air (1, 2). Exposure to cigarette smoke is almost inevitable since adults smoke in areas where children live or play (2, 3). Children are particularly more susceptible to passive smoking because they have smaller airways and their immune system has yet to be fully developed (4). Free radicals

are unwanted molecules that are physiologically produced in an organism. In some cases, their production increases as the result of active or passive smoking. It has been revealed that one puff of cigarette approximately produces 1,014 free radicals (5).

Cigarette smoke contains numerous (more than 4000) chemical compounds that spread in the form of gas. Many of these compounds are oxidants and pro-oxidants and are capable of producing various oxygen species (6). Increased production of reactive oxygen species due to tobacco use can lead to oxidative stress and result in lipid oxidation, DNA break down, inactivation of specific proteins and disintegration of biologic membranes (7, 8). Increased oxidative stress plays a critical role in the pathogenesis of some smoking-related diseases such as cancer, cardiovascular diseases and oral pathologies (9-11).

In order to fight oxidants, anti-oxidant mechanisms exist through which, antioxidant molecules inhibit the harmful reactions caused by oxidative stress (12). However, in some cases oxidants increase and antioxidants decrease or antioxidant mechanisms are not sufficient to completely prevent the damage caused by oxidants. Based on the available evidence, smoking is associated with an increase in free radicals, oxidative stress and antioxidant depletion (13). Oxidative stress is involved in etiopathogenesis of about 100 different diseases (14). Also, cigarette smoking decreases serum TAC and this reduction initiates oral inflammatory diseases, promotes precancerous transformations and destroys homeostasis of oral cavity (15).

Several methods are available for the measurement of exposure to passive smoke. Although serum or plasma concentration of different antioxidants can be separately measured in the laboratories, these measurements are very expensive and time consuming and require the use of advanced

techniques. Saliva is a relatively thick fluid that protects the oral cavity. Its constituents include enzymatic and non-enzymatic proteins, calcium, phosphorus, sodium, other salts, dissolved gases such as nitrogen, oxygen and carbon dioxide and cells (16). Salivary cotinine is also a product of decomposition of nicotine with a half-life of 20h. It is stable against thermal changes or infection. Furthermore, it has higher sensitivity and specificity for the measurement of exposure to passive smoke compared to nicotine (17). Determining the salivary cotinine concentration is a suitable method for the measurement of exposure to passive smoke because its collection is easy, its half-life is longer than the plasma nicotine and has specific properties against tobacco (2, 18).

Considering the growing use of cigarette and tobacco products, a large number of children are inadvertently exposed to cigarette smoke. It is particularly important to find out whether passive smoking has the same effects as active smoking or not. Thus, the present study aimed to determine the effect of passive smoking on TAC of saliva and serum in rats.

Methods:

This experimental animal model study was conducted on 20 rats that were divided into two groups of 10. During the first 15 days, one animal in the exposure group died. Thus, one rat was also excluded from the control group. The study was continued on 2 groups of 9. The rats were in the age range of 7-11 weeks and weighed 160-200 g. The rats were all males and of Albino race. Animals were kept under similar conditions at $22\pm 1^{\circ}\text{C}$ temperature and shared the same food and water source. At day 0 (baseline), 0.2 mg/kg midazolam and 0.5 mg/kg pilocarpine were administered and blood and saliva samples were obtained (blood sample was taken from the rats' corner of eyes). From the next day on, rats in the exposure group were exposed to cigarette

smoke of 10 cigarettes (Winston with nicotine=13 and tar=1mg) 3 times a day (8 a.m., 12 p.m. and 4 p.m.) for 8 minutes. Control rats at the mentioned time points were only transferred to another box with no exposure to cigarette smoke. Rats in the exposure group were placed in a box specifically designed for this purpose. After the injection of midazolam and pilocarpine, serum and saliva samples were obtained at 0, 15 and 30 days and the animals were sacrificed afterwards. Blood samples after each time of collection were centrifuged at 3000 rpm and serum and saliva samples were stored in a freezer. After centrifugation, the supernatant

was transferred to a test tube with a pipette and serum cotinine level was measured using ELISA kit (rat cotinine ELISA kit, Calbiotech Inc.). This way we ensured the exposure to cigarette smoke in rats in the exposure group and no exposure in the control group. Next, FRAP assay was carried out on serum and saliva samples and the reduction of a ferric 2,4,6-tripyridyl-s triazine complex (Fe^{3+} -TPTZ) by antioxidants to ferrous form was assessed using spectrophotometry at 593 nm wavelength against standard FeSO_4 to measure TAC of serum and saliva.

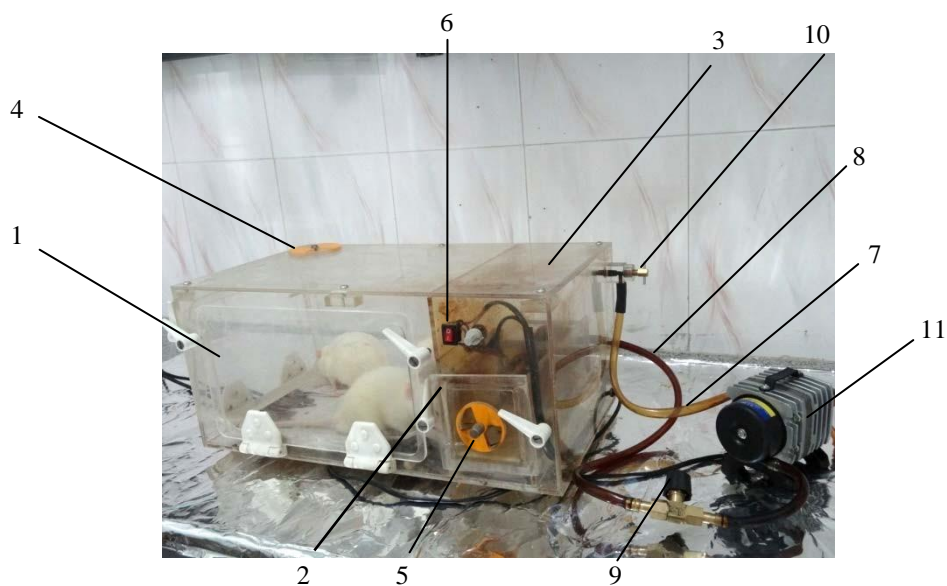


Figure 1- 1. Room for placement of rats, 2. Location of cigarettes, 3. Smoke accumulation area, 4. Air exit way, 5. Valve for the exit of excess smoke, 6. Holes to allow transfer of smoke from the smoke container to the rats container, 7. Pipe passing the smoke from the engine to the upper compartment, 8. Tube suctioning the smoke from the cigarette container, 9. Adjustment knob, 11. Engine

FRAP assay

The FRAP assay was carried out according to the method described by Benzie and Strain (1996)(19). This assay is based on the reduction of a ferric 2,4,6-tripyridyl-s triazine complex (Fe^{3+} -TPTZ) by antioxidants to ferrous form at low pH. As a result, ferrous tri-pyridyl-triazine complex is produced in presence of antioxidants; which is a colored product. FRAP reagent containing 10 mM TPTZ (Merck) in 40 mM

hydrochloric acid, 20 mM FeCl_3 (Merck) and 300 mM/lit acetate buffer in 10:1:1 ratio was prepared for the assay. FRAP values were obtained by the comparison of absorbance changes at 593 nm between the understudy specimen and the standards containing ferrous ions in specific amounts. The standards used contained FeSO_4 (Merck) at different concentrations (125, 250, 500 and 1000 mM). A total of 1.5 ml of a freshly prepared FRAP solution with 1:1:10 ratio of acetate buffer,

FeCl₃ and TPTZ reagent was poured into the test tubes and placed in a bain-marie at 37°C for 5 min. Afterwards, 50 µl of the specimen was added to the tube and stored in a bain-marie at 37°C for 10 min. Absorbance values were read at 593 nm with a spectrophotometer versus a blank reading (1.5 ml FRAP and 50 µl distilled water). Finally, by using standard samples and drawing a standard chart, antioxidant concentration of understudy specimens was calculated. Measurements were done in triplicate.

Repeated Measures ANOVA was used to evaluate changes in understudy parameters at different time points in each group. Independent t-test was applied to compare the parameters at

different time points between the two groups.

Results:

Serum cotinine concentration in the exposure and non-exposure groups at 0, 15 and 30 days is demonstrated in Table 1. ANOVA showed a significant increase in serum cotinine levels at different time points in the exposure group compared to the non-exposure group (both $p < 0.0001$). T-test showed that at baseline, no significant difference existed between the two groups in terms of cotinine concentration ($p = 0.693$). But at days 15 ($p < 0.001$) and 30 ($p < 0.0001$), cotinine concentration significantly increased in the exposure group (Table 1).

Table 1. The mean and standard deviation of cotinine concentration in rats in the two groups of exposure and non-exposure at different time points.

Group	Day 0	Day 15	Day 30	P value* (intragroup)
Exposure	2.54 ± 1.26	47.51 ± 25.35	32.31 ± 11.75	P < 0.0001
Non-exposure	2.79 ± 1.32	2.87 ± 0.6	7.1 ± 3.26	P < 0.0001
P value**	P = 0.693	P < 0.001	P < 0.0001	

*Repeated Measures ANOVA

**T-test

Serum TAC in the two groups at different time points is demonstrated in Table 2. ANOVA showed a significant increase in serum TAC in the passive smoker group at all understudy time points ($p < 0.0001$). But, the difference in serum TAC at the mentioned time points was not significant in the non-smoker group ($p = 0.6$).

Furthermore, t-test revealed that no significant difference existed at baseline in serum TAC between the two groups of exposure and non-exposure but at day 15, TAC values significantly increased in the passive smoker group ($p < 0.0001$) (Table 2).

Table 2- The mean and standard deviation of serum TAC in rats in the two groups of passive smoker and non-exposure at different time points

Group	Day 0	Day 15	Day 30	P* value (intragroup)
Passive smoker	1723.604 (148.9893)	964.282 (65.8632)	880.1311 (39.9747)	0.0001
Non-smoker	1939.462 (213.8529)	769.3984 (63.9050)	815.6989 (62.30618)	0.6
P**	0.38	0.0001	0.086	

*Repeated Measures ANOVA

**T-test

Table 3 shows TAC of saliva in the two groups at different time points. ANOVA revealed a significant increase in TAC of saliva in the passive smoker group ($p < 0.009$) at three different time points compared to the non-

smoker group ($p < 0.0001$). T-test indicated a significant increase in TAC of saliva at baseline ($p < 0.024$) and day 15 ($p < 0.01$) in the passive smoker group compared to the control group. But, at day 30, the difference in this regard

between the two groups was not statistically significant ($p=0.897$).

Table 3. The mean and standard deviation of TAC of saliva in rats in the two groups of passive smoker and non-smoker at different time points

Group	Day 0	Day 15	Day 30	P* value (intragroup)
Passive smoker	743.052 (110.003)	772.8556 (190.579)	732.4253 (93.1909)	0.009
Non-smoker	628.8056 (42.1389)	527.9102 (80.1925)	707.5218 (125.2086)	0.0001
P**	0.024	0.01	0.897	

*Repeated Measures ANOVA

**T-test

Discussion:

Based on the obtained results, serum cotinine concentration in the exposure group at day 15 and 30 was significantly higher than in the control group. The difference in this regard between the two groups at baseline was not significant; which indicates the reliability of testing. In a study by Polidori *et al.* in 2003 all participants experienced a reduction in their cotinine serum concentration after cigarette smoking cessation; this finding is in accord with our study results (13). Based on the obtained results, TAC of serum and saliva in animals of the passive smoker group significantly increased compared to the control samples. However, the increase in TAC values at day 30 was not significant in the exposure group. At baseline, the difference in TAC of saliva between the two groups was significant but no such difference was noted in serum TAC between the two groups. It seems that after an increase in TAC of serum and saliva at day 15, rats' immune system was able to somehow compensate the adverse effects of passive smoking and reduce the TAC of serum and saliva. Also, it seems that if this trend had continued, greater reduction in TAC of saliva and serum would have been occurred in the passive smoker group.

In a study by Charalabopoulos *et al.* in 2005 no significant differences were found between smokers and non-smokers in TAC of saliva (20). However, in the mentioned study, TAC of plasma in smokers before and after smoking was

greater than in control subjects (20). There are evidence indicating that young smokers produce compensatory blood levels of glutathione after the inhalation of cigarette smoke that effectively prevent peroxidation of plasma lipids (in contrast to old smokers)(21). Thus, young smokers may be able to confront the oxidative stress caused by smoking via increasing the production of glutathione or its release into the plasma. Aside from the increased production of glutathione, this increase may be due to the release of intracellular antioxidants into the bloodstream (plasma).

Despite having an innate tendency for oxidative invasion, body has a natural anti-oxidant system in which a series of enzymes, vitamins and other antioxidants act as regulatory mechanisms. The superoxide dismutase and glutathione peroxidase enzymes are at the frontline of immune system against reactive oxygen species and are usually referred to as primary antioxidants (22).

Increased TAC of serum and saliva in passive smoker rats in the present study may be attributed to the activation of their immune system against the passive cigarette smoke. TAC of saliva participates in salivary antioxidant enzymatic mechanisms like glutathione peroxidase (23) and is also associated with presence of different antioxidants such as urate and ascorbate (24). These factors can prevent the effects of radicals produced following oxidative stress in the saliva and plasma (25) and salivary proteins like glucoproteins (26). There are some food products that may play a role in TAC of

saliva. Polyphenol and its metabolites such as flavonoids, ligin and tannin are among these compounds (27). These factors can all affect the TAC of saliva. On the other hand, increased production of reactive oxygen species following cigarette smoke exposure in some cases may overcome the immune system and cause oxidative damage to select proteins, lipids and DNA (28, 29).

A wide range of oxidants and free radicals are released following tobacco use. In addition to oxidative injury, these factors can cause pulmonary conditions, cardiovascular diseases and cancer in the smoker individual (30). Although the mechanisms of involvement in smoking-related pathologies are still a matter of debate, it appears that free radicals play a critical role in the pathogenesis of these conditions (30). Free radicals are capable of inducing oxidative stress directly or indirectly. Bolisetty *et al.* in 2002 reported that infants exposed to free radicals had less antioxidant vitamins in comparison to control subjects (31). Furthermore, oxidative stress is a consequence of low levels of antioxidant vitamins in passive smokers. It has been reported that in infants exposed to passive smoke, some products of the antioxidant system in the body become defective (29). However, possible injury caused by free radicals is minimized via the antioxidant system. Chelchowska *et al.* in 2005 reported that TAC significantly decreased in blood samples of infants of smoker mothers (32). Also, Fayol *et*

al. in 2005 showed that TAC values decreased in blood samples of infants of passive smoker mothers while these changes were observed in the infants of active smoker mothers (33). Based on the results of Aycicek and Ipek in 2008, TAC of blood in passive and active smokers was significantly lower than that of control subjects (30). Also, Aycicek *et al.* in 2005 revealed that plasma TAC in passive smokers was significantly lower than control subjects (12). These findings are in contrast to our obtained results. Despite the fact that the nicotine level as an indicator of passive smoking has high specificity, its biologic half life in bloodstream is short (20-30 min). Thus, due to the importance of time in collection of samples after smoking, it cannot be used for research purposes. However, the clinical half life of cotinine, a nicotine metabolite, is longer (about 30 h) and thus, measurement of cotinine level is a good marker for the assessment of changes due to passive smoking (34).

Conclusion:

Based on the obtained results, changes in TAC of serum and saliva in rats due to exposure to cigarette smoke were compensated by their immune system. However, further investigations are still required in this respect. Due to various adverse effects, passive smoking should be avoided as much as possible.

References:

1. Ekerbicer HC, Celik M, Guler E, Davutoglu M, Kilinc M. Evaluating environmental tobacco smoke exposure in a group of Turkish primary school students and developing intervention methods for prevention. *BMC Public Health* 2007; 7: 202.
2. Hwang SH, Hwang JH, Moon JS, Lee DH. Environmental tobacco smoke and children's health. *Korean J Pediatr* 2012; 55: 35-41.
3. Jarvis MJ, Feyerabend C, Bryant A, Hedges B, Primates P. Passive smoking in the home: plasma cotinine concentrations in non-smokers with smoking partners. *Tob Control* 2001; 10: 368-374.

4. Delphisheh A, Kelly Y, Brabin BJ. Passive cigarette smoke exposure in primary school children in Liverpool. *Public Health* 2006; 120: 65-69.
5. Church DF, Pryor WA. Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ Health Perspect* 1985; 64: 111-126.
6. Alberg A. The influence of cigarette smoking on circulating concentrations of antioxidant micronutrients. *Toxicology* 2002; 180: 121-137.
7. Durak I, Elgün S, Kemal Bingöl N, Burak Cimen MY, Kaçmaz M, Büyükoçak S, *et al.* Effects of cigarette smoking with different tar content on erythrocyte oxidant/antioxidant status. *Addict Biol* 2002; 7: 255-258.
8. Liu X, Lu J, Liu S. Synergistic induction of hydroxyl radical-induced DNA single-strand breaks by chromium (VI) compound and cigarette smoke solution. *Mutat Res* 1999; 440: 109-117.
9. Michael Pittilo R. Cigarette smoking, endothelial injury and cardiovascular disease. *Int J Exp Pathol* 2000; 81: 219-230.
10. Reibel J. Tobacco and oral diseases. Update on the evidence with recommendations. *Med Princ Pract* 2003; 12(Suppl): 22-32.
11. Therriault MJ, Proulx LI, Castonguay A, Bissonnette EY. Immunomodulatory effects of the tobacco-specific carcinogen, NNK, on alveolar macrophages. *Clin Exp Immunol* 2003; 132: 232-238.
12. Aycicek A, Erel O, Kocyigit A. Decreased total antioxidant capacity and increased oxidative stress in passive smoker infants and their mothers. *Pediatrics Int* 2005; 47: 635-639.
13. Polidori MC, Mecocci P, Stahl W, Sies H. Cigarette smoking cessation increases plasma levels of several antioxidant micronutrients and improves resistance towards oxidative challenge. *Br J Nutr* 2003; 90: 147-150.
14. Halliwell B. Antioxidants in human health and disease. *Annu Rev Nutr* 1996; 16: 33-50.
15. Greabu M, Totan A, Battino M, Mohora M, Didilescu A, Totan C, *et al.* Cigarette smoke effect on total salivary antioxidant capacity, salivary glutathione peroxidase and gamma-glutamyltransferase activity. *Biofactors* 2008; 33: 129-136.
16. Showkatbakhsh AR, Outad A, Samimi P. *Oral Biology*. 1st Ed. Shayan Nemozar Publishing Co. 2004; Chap 5: 73-89 [Persian].
17. Benowitz NL. Cotinine as a biomarker of environmental tobacco smoke exposure. *Epidemiol Rev* 1996; 18: 188-204.
18. Delphisheh A, Kelly Y, Rizwan S, Brabin BJ. Salivary cotinine, doctor-diagnosed asthma and respiratory symptoms in primary school children. *Matern Child Health J* 2008; 12: 188-193.
19. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* 1996; 239: 70-76.
20. Charalabopoulos K, Assimakopoulos D, Karkabounas S, Danielidis V, Kiortsis D, Evangelou A. Effects of cigarette smoking on the antioxidant defence in young healthy male volunteers. *Int J Clin Pract* 2005; 59: 25-30.
21. Duthie SJ, Hawdon A. DNA instability (strand breakage, uracil misincorporation, and defective repair) is increased by folic acid depletion in human lymphocytes in vitro. *FASEB J* 1998; 12: 1491-1497.
22. Kondo T, Tagami S, Yoshioka A, Nishimura M, Kawakami Y. Current smoking of elderly men reduces antioxidants in alveolar macrophages. *Am J Respir Crit Care Med* 1994; 149: 178-182.
23. Pereslegina IA. [Superoxide dismutase activity of digestive secretions]. *Ukr Biokhim Zh* 1990;

- 62: 53-58. [Article in Russian]
24. Moore S, Calder KA, Miller NJ, Rice-Evans CA. Antioxidant activity of saliva and periodontal disease. *Free Radic Res* 1994; 21: 417-425.
 25. Kohen R, Tirosh O, Kopolovich K. The reductive capacity index of saliva obtained from donors of various ages. *Exp Gerontol* 1992; 27: 161-168.
 26. Chapple IL, Mason GI, Garner I, Matthews JB, Thorpe GH, Maxwell SR, *et al.* Enhanced chemiluminescent assay for measuring the total antioxidant capacity of serum, saliva and crevicular fluid. *Ann Clin Biochem* 1997; 34: 412-421.
 27. Ericson T, Bratt P. Interactions between peroxide and salivary glycoprotein; protection by peroxidase. *J Oral Pathol* 1987; 16: 421-424.
 28. Fraga CG, Motchnik PA, Shigenaga MK, Helbock HJ, Jacob RA, Ames BN. Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. *Proc Natl Acad Sci USA* 1991; 88: 11003-11006.
 29. Yoshie Y, Ohshima H. Synergistic induction of DNA strand breakage by cigarette tar and nitric oxide. *Carcinogenesis* 1997; 18: 1359-1363.
 30. Aycicek A, Ipek A. Maternal active or passive smoking causes oxidative stress in cord blood. *Eur J Pediatr* 2008; 167: 81-85.
 31. Bolisetty S, Naidoo D, Lui K, Koh TH, Watson D, Montgomery R, *et al.* Postnatal changes in maternal and neonatal plasma antioxidant vitamins and the influence of smoking. *Arch Dis Child Fetal Neonatal Ed* 2002; 86: F36-40.
 32. Chelchowska M, Laskowska-Klita T, Leibschang J. [The effect of tobacco smoking during pregnancy on concentration of malondialdehyde in blood of mothers and in umbilical cord blood]. *Ginekol Pol* 2005; 76: 960-965. [Article in Polish]
 33. Fayol L, Gulian JM, Dalmaso C, Calaf R, Simeoni U, Millet V. Antioxidant status of neonates exposed in utero to tobacco smoke. *Biol Neonate* 2005; 87: 121-126.
 34. Etter JF, Vu Duc T, Perneger TV. Saliva cotinine levels in smokers and nonsmokers. *Am J Epidemiol* 2000; 151: 251-258.