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Assessment of Health Hazards of Passive Tobacco Smoking in School-age Children; Role of Oxidative Stress Biomarkers and Nitric Oxide Metabolites

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Abstract **Background:** Oxidative stress is thought to be produced by cigarette smoking whether by active or passive exposure. For assessment of smoking related health hazards, several oxidative stress biomarkers have been in use. **The aim of the present work** is to investigate the impact of passive smoking on the health of school age children who had first degree relative smokers at home. Assessment of smoking related health hazards was done by measurement of malondialdehyde (MDA) as a biomarker of oxidative stress, in addition to measurement of the total antioxidant capacity (TAC), nitric oxide (NO) metabolites and cotinine levels in urine. The state of children' exposure to tobacco smoke was assessed via parents' questionnaire. **Methods:** The current study involved participation of a total of 183 Egyptian school age children ranged from 6-15 years old in the period from September 2012 to June 2013. They were grouped into two groups group I cases; Second Hand Smoke (SHS) (n=132) were exposed to cigarette smoke at home (one of the first degree relatives was a heavy smoker; smokes 15-25 cigarettes per day. Group II control group (n=51) were not exposed to cigarette smoke at home. A morning urine sample was collected from all participants for colorimetric measurements of MDA, TAC, NO metabolites and cotinine levels. **Results**: MDA, NO metabolites and urinary cotinine levels were significantly higher in SHS cases compared to the control group ($p<0.0001$) while total antioxidant were significantly lower in SHS cases compared to control group ($p<0.0001$). TAC was significantly higher in males than in females ($p=0.003$). A negative correlation was found between NO metabolites (nitrite) and the TAC ($r =-0.34$, $P<0.0001$). Positive correlation was found between NO metabolites (nitrite) and MDA levels ($r =0.44$, $P<0.0001$), between MDA and urinary cotinine ($r=0.26$, $P<0.0001$) and between MDA levels and the age ($r =0.22$, $P<0.0001$). Grouping of the participated children according to their age group and studying its relation to the different studied biomarkers using one way ANOVA test followed by post hoc test the least significant difference (LSD); NO metabolites was statistically significant between the age group 1 (from 6 to 9 years) and age group 3 (from12 to 15years) ($p =0.05$). **Conclusion:** passive parental smoking is associated with significant changes in the balance between oxidant / antioxidant leading to oxidative stress and associated with elevated levels of NO but impaired its action.

Keywords: *MDA, TAC, NO, cotinine, school age children*

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1. Introduction

Tobacco is considered to be the single most preventable cause of death in the world today. Worldwide, tobacco use causes more than 5 million deaths per year, and current trends show that tobacco use will cause more than 8 million deaths annually by 2030. [1] Tobacco smoke contains approximately 4,000 toxic chemicals, including oxidative gases, heavy metals, cyanide, and at least 50 carcinogens. [2,3] Furthermore**,** cigarette smoking was reported to cause 30% of all cancer mortality in developed countries and passive smoking of tobacco was also proposed to be an important cause of cancer. [4]

Active smoking is a major and well-established modifiable risk factor for coronary heart disease. [5] Over recent years, the focus of interest has turned to the role of passive smoking in the etiology of cardiovascular diseases and evidence is now suggesting a causal relationship. [6] Furthermore, other health hazards have been suggested to occur as a result of exposure to tobacco smoke like lung cancer and aggravation of chronic obstructive pulmonary disease (COPD) in adults and asthma in children. [7,8]

Currently, 1.3 billion people smoke or use tobacco, and nearly 5 million worldwide die of diseases associated with tobacco smoke each year. [1] Environmental tobacco smoke (ETS) consists of particles much smaller than those in the mainstream smoke, and therefore has greater

penetrability to the airways of children. Exposure to the ETS among children in their homes has been reported to vary from 27.6% in Africa, 34.3% in Southeast Asia, 50.6% in Western Pacific, and up to 77.8% in Europe. [3,6]

ETS has been regarded as one of the most important public health issues. It has been estimated that approximately 700 millions of children in the world are exposed to ETS. Children are especially at high risk of toxicity from inhaled toxins because of differences in their pulmonary physiology and higher minute ventilatory rate. [10] This explains the increasing restrictions on smoking in public places in the past decade worldwide. Many workplaces in Britain have introduced smoking policies, in which smoking has been banned, such as on buses and the underground and in banks, cinemas, post offices, and shops. Unfortunately, smoking at home is less easily regulated. Much of the public health burden from passive smoking falls on children at home, with clear evidence of causal effects for several diseases. [11,12] During 2007-2008 in the United States, approximately 88 million non-smokers aged 3 years and above were reported to be exposed to Second Hand Smoke (SHS). [13]

For Egypt, smoking is prevalent with 19 billion cigarettes smoked annually, making it the largest market in the Arab world. [14] Egypt is also ranked as one of the top ten per capita consumers of tobacco by the World Lung Foundation. In Egypt, cigarettes are the most common form of tobacco consumption followed by shisha water-pipes. A national survey conducted by the Egyptian Smoking Prevention Research Institute showed that waterpipe smoking was inversely related to educational level, and that most users believed that using a waterpipe is less harmful than cigarettes. The survey also showed that more than 70% of male waterpipe smokers smoked at homes in the presence of their children and wives, calling attention to the unfortunate lack of knowledge regarding indoor environmental tobacco smoke exposure. [15] There are approximately 34,000 tobacco-related deaths each year in Egypt according to the statistics by CDC's Morbidity and Mortality Weekly Reports.

Although exposure patterns differ significantly from one person to another and these differences are attributed to variations in tobacco products, room ventilation, proximity of smokers to non-smokers, and many other environmental factors. [9] Nonetheless, several epidemiological studies have determined specific biomarkers and proposed their cutoff points to be used for differentiation between active and non-smokers. [16,17]

Oxidative stress is thought to be produced by cigarette smoking whether by active or passive exposure. For assessment of smoking related health hazards, several oxidative stress biomarkers have been in use. [6,18] Moreover, accurate quantitative measurement of second hand smoker (SHS) is important when considered as a primary risk factor. In addition, understanding determinants of exposure is useful in designing SHS-reduction policies and interventions. [19]

Determination of nitrite and nitrate in body fluids like plasma and urine is widely used as a marker of NO production which plays an important role both in maintaining normal homeostasis and in the pathogenesis of various disorders. [20,21] NO has a short biological half-life and is rapidly converted into its stable metabolites; nitrite and nitrate. [22,23]

Cotinine is the major degradation product of nicotine metabolism and has a serum half-life of about 17 hours compared to two hours for the parent compound. Therefore, measurement of cotinine levels can provide a sensitive estimate of tobacco smoke exposure. [24]

Thus, the current study aimed to investigate the impact of passive smoking on the health of school age Egyptian children who had first degree relative smokers at home. This is the first study to investigate oxidative stress biomarkers (MDA and TAC), NO metabolites and cotinine levels in urine of school-age children. The state of children' exposure to tobacco smoke was assessed via parents' questionnaire.

2. Subjects and Methods

2.1. Samples Collection

The study involved participation of a total of 183 Egyptian school age children ranged from 6-15 years old in the period from September 2012 to June 2013. They were grouped into two groups group I cases; Second Hand Smoke (SHS) (n=132) were exposed to cigarette smoke at home (one of the first degree relatives was a heavy smoker; smokes 15-25 cigarettes per day. Group II control group (n=51) were not exposed to cigarette smoke at home.

Exclusion criteria; children suffering from asthma, fever, renal disease and heart disease were excluded from the study. The history of smoking was assessed by questionnaire.

A morning urine sample was collected from all participants for measurements of MDA, TAC, NO metabolites (nitrite) and cotinine levels.

Urine samples were collected in clean bottles and were preserved by adding a small crystal of thymol and freezed. The study was conducted in accordance with the provisions of the Declaration of Helsinki and Good Clinical Practice guidelines. [25] Ethical approval and permissions were obtained from the Ethics Committee at Faculty of Medicine, Alexandria University, Egypt. An informed consent was obtained from parents of all the children included in the study.

2.1.1. Assay of Lipid Peroxidation (MDA) in Urine Samples by Colorimetric Method: [26]

The kit was supplied by Biodiagnostic (Giza, Egypt). Analysis was performed according to manufacturer's instruction.

2.1.2. Assay of Urinary T AC by Colorimetric Method. [27]

The kit was supplied by Biodiagnostic (Giza, Egypt). Analysis was performed according to manufacturer's instruction.

2.1.3. Assay of NO in Urine Samples. Colorimetric Determination of Nitrite [28]

The kit was supplied by Biodiagnostic (Giza, Egypt). Analysis was performed according to manufacturer's instruction.

2.1.4. Determination of Urinary Cotinine [29]

Chemicals were purchased from Sigma-Aldrich. Frozen urine samples were thawed at room temperature. In a

small test tube, one ml urine was pipetted, and 0.2 ml acetate buffer, 0.2 ml aqueous potassium cyanide 10%, 0.2 ml chloramine T solution and 1 ml 1% DETBA were added successively and mixed after each addition. The tubes were left to stand at room temperature for 20 minutes. The pink red color, that developed in positive cases was extracted using 2 ml ethyl acetate and centrifuged. 1.5 ml ethyl acetate extract was pipetted in a dry test tube and 0.2 ml ethanol was added then was shaken. A blank that contains all reagents plus water that replaces urine was prepared. The absorbance of blank, aqueous cotinine standard and the samples were measured at 532 nm. (The concentration of urinary cotinine was estimated from standard curve and was expressed in ng/ml).

2.2. Statistical Analysis

The collected data were tabulated and analyzed through computer facilities using the Statistical Package for Social Science (SPSS) version 20.0. [30] Descriptive statistics were calculated (e.g. frequency, percentage, mean and standard deviation). Quantitative continuous data was compared using Student t-test or ANOVA F test, as appropriate. Qualitative variables were compared using chi-square test. Correlations between variables were analyzed by Pearson's correlation coefficient. A p value of less than 0.05 was considered significant. All calculated P values were two-tailed.

3. Results

Our results revealed no statistical difference between group I and group II as regards age and sex (Table 1). Comparison of biomarkers between group I and group II showed significant differences (p<0.0001) (Table 2 and Figure 1 - Figure 4). Our results showed negative correlation between NO metabolites (nitrite) and the TAC (r=-0.34, P<0.0001). Positive correlation was found between NO metabolites (nitrite) and MDA levels (r=0.44, P<0.0001), between MDA and urinary cotinine (r=0.26, P<0.0001) and between MDA levels and the age (r=0.22, P<0.0001) (Table 3). TAC was significantly higher in males than in females (p=0.003) (Table 4).

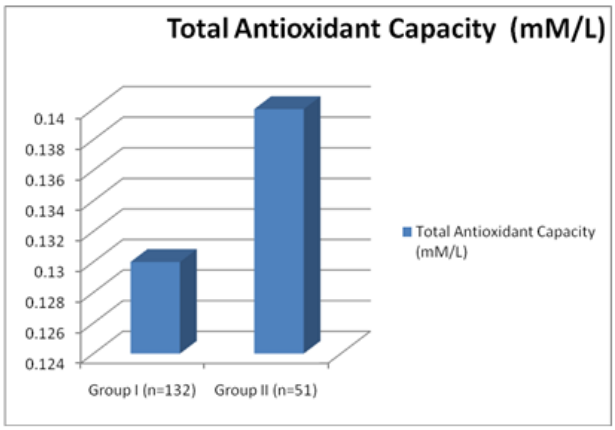

Figure 1. Bar chart showing the significant difference between group I and group II as regards TAC as revealed by student T- test

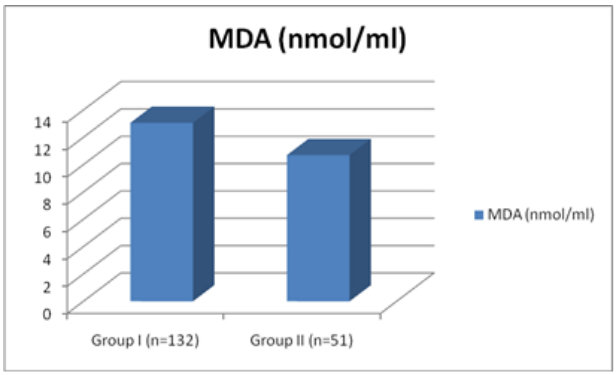

Figure 2. Bar chart showing the significant difference between group I and group II as regards MDA as revealed by student T- test

Table 1. Comparison Of Age and Sex Distribution Between Group I& Group II

Cases versus control group	Group I (n=132)	Group II (n=51)	P value
Age (mean ±SD)	11.15±2.71	11±1.79	P=0.71*
Sex Female n (%)/Male n(/%)	76(57.6%)/56(42.4%)	26(51%)/25(49%)	P=0.42**

*p value of T -test
**p value of Chi-Square Test (X^2)
SD: standard deviation.

Table 2. Comparison Between Group I& Group II As Regards The Studied Different Biomarkers

Biomarker (unit)	Group I versus group II	Mean±SD	T-test
TAC (mM/L)	Group I (n=132)	0.13±0.016	P=<0.0001**
	Group II (n=51)	0.14±0.02	
MDA (nmol/ml)	Group I (n=132)	13.06±1.59	P=<0.0001**
	Group II (n=51)	10.7±1.80	
Nitric oxide (nitrite) (µmol/L)	Group I (n=132)	23.53±1.2	P=<0.0001**
	Group II (n=51)	22.52±0.55	
Urinary cotinine (ng/ml)	Group I (n=132)	213.61±158.56	P=<0.0001**
	Group II (n=51)	19.16±6.04	

NS: non significant
*: Statistically significant (p≤0.05).
**: Highly significant (p≤0.01).
SD: standard deviation.

Table 3. Pearson's Correlation Study Between The Studied biomarkers and The Age

		TAC (mM/L)	Urinary cotinine (ng/ml)	MDA (nmol/ml)	NO(nitrite) (µmol/L)	Age
TAC (mM/L)	r	1	-0.1	0.05	-0.34	0.00
	P		0.26 ^{NS}	0.6 ^{NS}	<0.0001**	0.98 ^{NS}
Urinary Cotinine (ng/ml)	r	-0.1	1	0.26	0.08	0.09
	P	0.26 ^{NS}		<0.0001**	0.35 ^{NS}	0.32 ^{NS}
MDA (nmol/ml)	r	0.05	0.26	1	0.44	0.22
	P	0.59 ^{NS}	0.003**		<0.0001**	0.012*
Nitric oxide (nitrite) (µmol/L)	r	-0.34	0.08	0.44	1	0.004
	P	<0.0001**	0.35 ^{NS}	<0.0001**		0.97 ^{NS}
Age	r	0.00	0.086	0.22	0.00	1
	P	0.98 ^{NS}	0.33 ^{NS}	0.01*	0.97 ^{NS}	

r: Pearson correlation coefficient.
NS: non significant
*: Statistically significant (p≤0.05).
**: Highly significant (p≤0.01).

Table 4. Comparison Of The Studied Parameters By Sex Among SHS Children Using Student T- Test

biomarkers	Sex	Mean ±SD	T-test p value
TAC (mM/L)	Female (n=56)	0.126±0.015	0.003**
	Male (n=76)	0.134±0.015	
MDA (nmol/ml)	Female (n=56)	12.89±1.72	0.28 ^{NS}
	Male (n=76)	13.19±1.49	
NO (nitrite) (µmol/L)	Female (n=56)	23.43±1.05	0.44 ^{NS}
	Male (n=76)	23.6±1.3	
Urinary cotinine (ng/ml)	Female (n=56)	219.8±61.47	0.34 ^{NS}
	Male (n=76)	209.04±56.47	

*: Statistically significant (p≤0.05).
**: Highly significant (p≤0.01).
NS: non significant.
SD standard deviation.

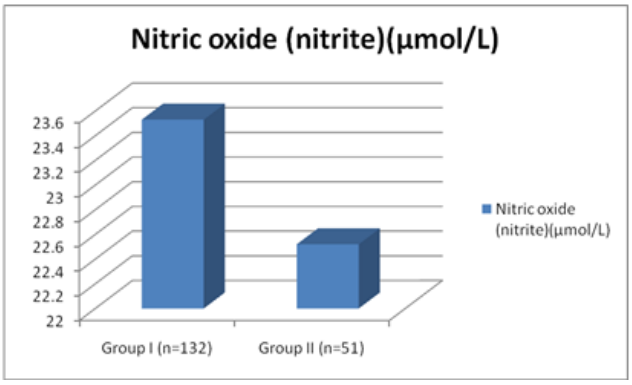

Figure 3. Bar chart showing the significant difference between group I and group II as regards urinary cotinine levels as revealed by student T-test

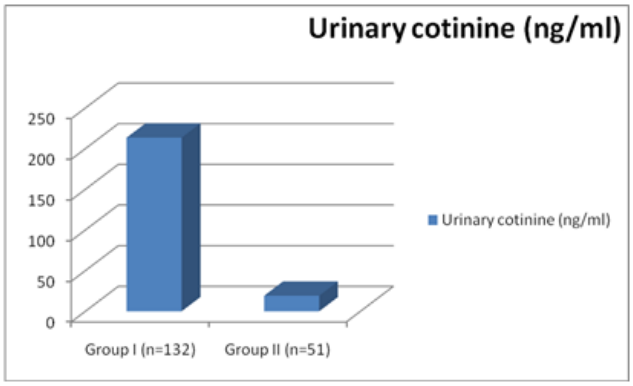

Figure 4. Bar chart showing the significant difference between group I and group II as regards urinary cotinine levels as revealed by student T-test

Table 5. Grouping Of The Participated Children According To Their Age Group And Its Relation To The Different Studied biomarkers Using One Way ANOVA Test Followed By Post Hoc Test LSD (least significant difference)

Biomarkers	Grouping by age			One way ANOVA P
	Group1 aged from 6 to 9 years	Group 2aged from >9 to 12years	Group 3 aged from >12 to 15 years	
	Mean ± SD	Mean ± SD	Mean ±SD	
TAC (mM/L)	0.13±.22	0.13±.01	0.13±.12	0.93 ^{NS}
MDA (nmol/ml)	13.31±1.51	12.91±1.96	12.95±1.25	0.44 ^{NS}
NO(nitrite) (µmol/L)	23.22±1.35	23.50±1.09	23.84±1.07	0.05* LSD between group 1&3
Urinary cotinine (ng/ml)	201.82±48.76	216.57±73.05	222.17±51.59	0.24 ^{NS}

*: Statistically significant (p≤0.05).
**: Highly significant (p≤0.01).
NS: non significant.

Grouping of the participated children according to their age group and studying its relation to the different studied biomarkers using one way ANOVA test followed by post hoc test the least significant difference LSD; NO metabolites was statistically significant between the age group 1 (≥6-9ys) and age group 3 (>12-15ys) ($p = 0.05$) (Table 5).

4. Discussion

The main adverse effect of active or passive smoking is a result of numerous compounds emitted in gases, many of which are oxidants and pro-oxidants; increased production of reactive oxygen species by smoke is related to increased free radical production. The increase in oxidative state can result in the lipid oxidation, induction of DNA single ribbon breakage, the inactivation of certain proteins and rupture membranes that are associated with numerous adverse effects to the health of fetuses, infants, children and adults. [31]

In our study urinary cotinine levels were significantly higher in SHS cases compared to the control group this in accordance with Yıldırım *et al* 2011, [32] who found urinary cotinine levels were significantly higher in passive smoking preschool children compared to the control group. Cotinine, which is the major metabolite of nicotine, is an indicator commonly used to reflect the level of exposure to smoke, although its half-life is less than 24 hours, the urinary cotinine is a good indicator as it represents the magnitude, duration and frequency of exposure and it can be measured simply and accurately and is detectable in urine samples at low concentration. [33]

Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation in the cells. An increase in free radicals causes overproduction of MDA. Malondialdehyde level is commonly used as a marker of oxidative stress and the antioxidant status in many diseased conditions. [34] In this study MDA were significantly higher in SHS cases compared to the control group this is in accordance with Aycicek et al 2005, [35] who found increased markers of oxidative stress in passive smoker infants and their mothers than those of non-smokers.

In our study there is a significant positive correlation between the MDA levels and the age which means that as the SHS child is getting old, the MDA levels is increased. Some reports showed increased plasma levels of MDA with age in healthy subjects. [36,37] others confirmed that peroxidative damage increases with the increasing age. [38] In this study, there is a positive correlation between MDA (nmol/ml) and urinary cotinine (ng/ml).This indicates that SHS are exposed to potent oxidative stress. The increased oxidative stress found in this study in SHS can represent a risk factor for the development of chronic disease in school age children.

In our study total antioxidant capacity were significantly lower in SHS cases compared to control group this is in accordance with Yıldırım *et al* 2011, [32] who studied the effect of passive smoking on preschool children. It has been argued that the increased production of reactive oxygen species associated with smoking may exceed the capacity of the oxidant defense system, resulting in oxidative damage. [39,40] In our study TAC was significantly higher in males than in females; evidence is mounting that females metabolize some constituents of tobacco smoke differently than men and therefore may be more susceptible to chronic respiratory diseases such as COPD and lung cancer. Smoke exposure can also affect women in sex specific ways that are dependent on both biological and social factors. For example, exposure to secondhand smoke during adolescence is a risk factor for later development of premenopausal breast cancer in women. Sex-specific factors result in women being susceptible to the damaging effects of SHS, while gender dynamics influence women's exposure to second-hand smoke and capacity to negotiate smoke-free spaces. [41,42]

To assess the generation of NO; measurement of NO production in vivo is difficult because of its short half-life. Consequently, its metabolites has been used as surrogate markers for estimating NO production. [43] Comparing between smokers and non smokers Ghasemi *et al* 2010, [44] found Serum NO metabolites was significantly higher in the active smokers men compared to nonsmokers. Based on our knowledge this is the first study to demonstrate the effect of passive smoking on urinary NO. In our study NO metabolites were significantly higher in SHS cases in comparison to the control group this could be attributed to high concentrations of inhaled NO from smoke. We found a significant negative correlation between NO metabolites levels and the TAC and a significant positive correlation between MDA levels and NO metabolites levels. The correlation between cigarette smoking and high oxidative stress levels can be attributed in part to the associated increase NO levels and an impaired oxidant defense system.

Actually endogenously generated NO is an important cellular signaling molecule involved in many physiological and pathological processes. It is a powerful vasodilator with a short half-life of a few seconds. In the blood Nitric oxide is generated inside our body from the oxidation of L-arginine to L-citrulline by three isoenzymes of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent NO synthases (NOS). It has been documented that the enzymes responsible for NO synthesis constitute a family of at least two distinct types: a calcium dependent form which is constitutively expressed in brain and endothelial cells and a second type which can be induced in endothelial cells and macrophages by cytokines and endotoxin. [45] Inducible NO-forming enzyme is calcium-independent. Its expression is upregulated by IFNα, IFNγ, IL-1 and TNF-α as well as other pro-inflammatory mediators, Adams *et al* 2015, [46] found Expression of eNOS reduced and expression of (markers of inflammation) NF-κB was similarly increased in passive and active smokers compared with control subjects. The elevated levels of NO but impaired action of NO may be attributed to decreased activity of nitric oxide. The underlying molecular mechanisms for the reduced action of NO has been attributed to a decrease in NO bioavailability which may be caused by decreased expression of eNOS, alteration in the signaling pathways activating eNOS and /or accelerated degradation of NO by reactive oxygen species [47], NO may react with O2 to form ONOO-, leading to reaction with glutathione, cysteine, deoxyribose, and other thiols/thioethers. [48] This can result in formation of

reactive nitrating species in the presence of metal ions or complexes increased inflammation-induced inducible NO synthase (iNOS) expression and thus NO. Syngle *et al* 2010, [49] suggested that there is increased NO bioavailability but not the sensitivity of vascular smooth muscle to NO. In our study NO metabolites were significantly higher in children aged from 12 to 15 years than children aged from 6 to 9 years this could be attributed to long duration of exposure.

The relative risk for cardiovascular diseases in passive smokers is similar to that of active smokers despite almost a 100-fold lower dose of inhaled cigarette smoke. [46] Exposure to secondhand smoke is very common and has been implicated as a significant risk factor for health and as a habit that brings adverse consequences for the establishment and progression of various diseases. Children are especially vulnerable to the risk of such exposure on health, including upper and lower respiratory infections, acute and chronic ear infections, asthma exacerbation, changes in neurodevelopment and behavioral problems. Oxidants are increased and antioxidants are decreased in SHS than those of non-smokers; SHS are exposed to potent oxidative stress.

We conclude that the passive parental smoking is associated with significant changes in plasma balance oxidant / antioxidant and potent cause oxidative stress. The only way to protect non-smokers fully is to eliminate smoking in indoor spaces, including workplaces, public places (e.g., restaurants and bars), and private places (e.g., homes and vehicles) through smoke-free laws and policies and through decreased smoking prevalence.

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