BIOMEDICAL AND ENVIRONMENTAL SCIENCES **18**,43-47 (2005)

8-Hydroxydeoxyguanosine as a Biomarker of Oxidative DNA Damage Induced by Environmental Tobacco Side-stream Smoke and Its Mechanism

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Objective To study the genotoxicity effect of environmental tobacco side-stream smokes (ETSS) on oxidative DNA damage and its molecular mechanism. **Methods** DNA adduct 8-hydroxydeoxyguanosine (8-OHdG) was used as a biomarker of oxidative DNA damage. The level of 8-OHdG in DNA exposed to ETSS was detected by high performance liquid chromatography with electrochemical detection. Organic and inorganic components in ETSS were analyzed by gas chromatography-mass spectrum and atomic absorption spectrum respectively. **Results** Particle matters (PMs) and volatile organic compounds (VOCs) in ETSS could directly induce oxidative DNA damage and formation of 8-OHdG. There were 123 and 84 kinds of organic components in PMs and VOCs respectively, and 7 kinds of inorganic components in ETSS. Some components, especially quinones and polyphenols in ETSS, could produce free radicals *in vitro* by auto-oxidation without any biological activity systems, and with the catalytic reaction of metals, the DNA adduct 8-OHdG was produced. **Conclusion** ETSS have biological oxidative effect on DNA *in vitro* and *in vivo*, and expressed direct genotoxicity. 8-OHdG is a valuable biomarker of oxidative DNA damage.

Key words: Oxidative DNA damage; 8-hydroxydeoxyguanosine; Environmental tobacco side-stream smoke; Liquid chromatography with electrochemical detection

INTRODUCTION

There are about 1 billion people, 22% of the world population smoking cigarettes, and 6 000 billion cigarettes were consumed every year. In China, the number of smoking people is much greater than this percentage, and the age of smoking people has been getting younger in recent years. Environmental tobacco smoke has three types, namely environmental tobacco main-stream smoke, environmental tobacco side-stream smoke (ETSS), and smoke spread to the environmental atmosphere by smokers. ETSS is one of the important pollution sources of indoor air. It is hazardous to the health of smokers as well as non-smokers around them. The components of ETSS are complex. Many kinds of chemicals have been identified including stimulating gases, mixture of carcinogenic tar particles, or carcinogenic compounds[1]. It has been found that smoking can induce pulmonary cancers and respiratory diseases, and is related with heart diseases, but the fingerprint relationship of tobacco smoking with carcinogenesis has not yet been proved. Recent researches have shown that the risk of passive smoking is greater than that of active smoking[2]. In an attempt to study the genotoxicology of DNA induced by ETSS, we used DNA adduct 8- hydroxydeoxyguanosine (8-OHdG) as a biomarker of oxidative DNA damage. The levels of 8-OHdG in calf thymus DNA treated with ETSS extract *in vitro*, and in lung tissue DNA of rats exposed to ETSS *in vivo* were determined. Organic and inorganic components in ETSS were also analyzed. In addition, the molecular mechanism of DNA damages induced by ETSS was explored on the basis of its chemical components.

MATERIALS AND METHODS

Reagents

8-OHdG (Sigma), calf thymus DNA (type Ⅰ, Sigma), nuclease P1 (Sigma, 80 units/mg DNA), *E. coli* alkaline phosphatase (type Ⅲ-s, Sigma, 20 units/mg DNA), methanol (HPLC grade, Tianjin Siyou, China), other chemicals used were of analytical grade, and all were made in China.

[1]The research was supported and financed by brainstorm project and public good fund from the Ministry of Science and Technology, China (2001BA704B01& 2001DIA10001).

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0895-3988/2005
CN 11-2816/Q

ETSS Collection[3]

The cigarette holder was connected with a pump, and the cigarette (HengDa Brand, without filter was burned by inhalation of pump, thus producing tobacco smoke. Upon the head of the cigarette, particle matters of ETSS were collected through a glass fiber filter, and volatile organic compounds (VOCs) were captured by an activated carbon tube at the rate of 0.3 mL/min, which is approximate to the rate of people smoking.

Analysis of Organic Components by Gas Chromatography-mass Spectrum (GC-MS)[4]

The organic components were extracted by Soxhlet extraction method. After sampling, the glass fiber filter was extracted in Soxhlet extracting tube with 120 mL of dichloromethane at 80℃ for 8 h. The organic components contained in the ETSS were very complex. Before analysis by GC-MS, they were separated into three parts, namely organic acids, organic bases and neutral substances by chemical methods. Further, the neutral substances were passed through a silica column and separated into aliphatic hydrocarbon, polycyclic aromatic hydrocarbons (PAHs) and polar compounds. Each part of them was condensed to 1 mL using a K-D condenser and then analyzed by GC-MS.

The activated carbon with VOCs of ETSS was dissolved in 1 mL CS_2, and treated with a ultrasonic device for 20 min, then filtered and the filtrate was analyzed by GC-MS.

GC-MS conditions: Analysis of the organic components in ETSS was carried out by using HP 5890 Ⅱ gas chromatography with 5971A mass spectrometry detector (Hewlett Packard, U. S.). GC conditions are shown in Table 1. MS conditions were as follows: ion source was electron ion (EI), 70eV, using auto tune mode, and MS library was NBS75k (America National Standard Bureau). The samples were qualified by full scan mode, the mass range of scanning was 30 to 450 amu (atomic mass units), and confirmed by the retention time of standards.

TABLE 1

GC Conditions

Components	Column	Temperature Program
Aliphatic Hydrocarbon	HP-5, φ0.22×25m Silica Capillary Column	60℃/2min 4℃/min ⟶ 300℃
PAHs	HP-5, φ0.22×25m Silica Capillary Column	100℃/2min 20℃/min ⟶ 200℃ 4℃/min⟶ 300℃
Organic Acids	BP-20, φ0.22×25m Silica Capillary Column	180℃ 4.8℃/min ⟶ 250℃
Organic Bases	BP-20, φ0.22×25m Silica Capillary Column	180℃ 4.8℃/min ⟶ 250℃
Polar Compounds	BP-20, φ0.22×25m Silica Capillary Column	210℃ 4.8℃/min ⟶ 250℃
VOCs	HP-5, φ0.22×25m Silica Capillary Column	30℃ 10℃/min ⟶ 180℃ 5℃/min ⟶280℃

Analysis of Inorganic Components by Atomic Absorption Spectrum (AAS)

The silica fibre filter was dissolved in 0.5 mol/L HNO_3 overnight, then washed 4-5 times with water, treated with HNO_3 and $HClO_4$ and condensed to near dryness, fixed volume to 1 mL with 0.01 mol/L HNO_3, then determined by AAS.

The activated carbon with VOCs was placed in a crucible ashed at 500℃ for 10 hours, then fixed volume to 1 mL with 0.01 mol/L HNO_3, and analyzed by AAS.

Analysis of 8-OHdG in DNA Calf Thymus and Lung Tissue of Rat DNA Exposed by ETSS[5]

The silica fibre with PMs and activated carbon with VOCs of ETSS were suspended in 10 mL and 3 mL phosphate buffer (50 mmol/L, pH 7.4) solution respectively, which contained 0.05% Tween 80 (emulsifier) and 0.9% sodium chloride, and were treated by ultrasound for 30 min, and the obtained extract was incubated with 100 μg calf thymus DNA at 37℃ for 10 h. The treated DNA was precipitated with 0.3 mL of 3.0 mol/L sodium acetate and twice volume of cooled ethanol, and then dissolved in 1 mL of 10 mmol/L NaH_2PO_4. A portion of the exposed DNA was hydrolyzed to deoxynucleosides by incubation first with nuclease P1 at 37℃ for 30 min and then with *E. coli* alkaline phosphatase at 37℃ for 1 hours, the deoxynucleosides mixture obtained was injected into an HPLC-EC apparatus for analysis of 8-OHdG. Another portion of the exposed DNA was used to measure the content of DNA by UV-Vis spectrophotometer.

Fifteen female Wistar rats were randomly divided into control group, low dose group and high dose group. After anaesthesia with urethane, the rats were exposed to the particle extract of ETTS by intratracheal instillation, and killed after 24 h. The

DNA was extracted from lung tissue by kits, and quantified with UV-Vis, and then the level of 8-OHdG was analyzed by liquid chromatography with electrochemical detection (HPLC-EC).

HPLC-EC analysis was performed using a Model 490E instrument with a 464-electrochemical detector (Waters company, U.S.). The column used was μ Bondapark C18 4.6 mm×300 mm, the fluent used was buffer solution: methanol=3:1. The buffer solution was 30 mmol/L citric acids-50 mmol/L sodium acetate-50 mmol/L NaOH-20 mmol/L acetic acids, the flow rate was 0.6 mL/min. The working electrode of electrochemical detector was glassy carbon electrode and the working voltage was set to +800 mV, using Hg/Hg_2Cl_2 as reference electrode.

RESULTS

Organic Components in ETSS

There were 123 kinds of organic components and their isomers were detected in PMs and 84 kinds in VOCs of ETSS (Table 2).

TABLE 2

Types of Organic Components in PMs and VOCs of ETSS

	Alkane	Alkene	Alkyne	SAH	PAH	Heterocycle	Aldehyde	Ketone	Alcohol	Acid	Ester	Phenol	Polyphenol	Quinone	Amide	Nicotine
PMs	26	18	4	12	9	0	1	0	1	37	7	2	0	0	4	1
VOCs	4	9	1	24	3	18	1	12	0	4	3	3	1	1	0	1

Some PAHs, heterocyclic compounds including nitrogen and sulfur element etc., were detected. Many of them were proved to have carcinogenicity and mutagenicity, but the toxicity of these compounds could be emerged only through biological metabolism with some specific enzymes involved. Moreover, there were many kinds of phenol compounds which had been detected, mainly phenols and their replacements, which had no carcinogenicity themselves but pro-carcinogenicity. In VOCs, small molecule organics were chiefly present. The number of phenol compounds detected in VOCs was more than that in PMs of ETSS, especially including polyphenols and quinone compounds.

Inorganic Components in ETSS

The metals present in PMs of ETSS with relatively high amounts were Fe, Cu, Cd, Pb, and in VOCs were Fe, Cu (Table 3).

TABLE 3

Contents of Metals in PMs and VOCs of ETSS*

Metals	Fe	Cu	Cr	Cd	Ni	Co	Pb
PMs (μg/g)	314.2	81.5	0.7	132.8	5.6	3.3	32.4
VOCs (μg/g)	140.1	18.0	1.6	7.0	2.9	1.2	5.2

Note. *All values are the average of replicated experiments.

Oxidative DNA Damage Induced by ETSS

Some free radicals including oxygen ($\cdot OH, O_2^{\bullet -}$) attacked DNA base guanosine to produce DNA adduct 8-OHdG, which are widely used as a biomarker of DNA and protein oxidative damage at present. In this study, we observed the original oxidative damage to calf thymus DNA *in vitro* by PMs and VOCs in ETSS, i.e. their effects on the formation of 8-OHdG. The levels of 8-OHdG in each DNA sample were expressed as the molar ratio of 8-OHdG to deoxyguanosine, i.e. 8-OHdG/10^5dG (Fig. 1).

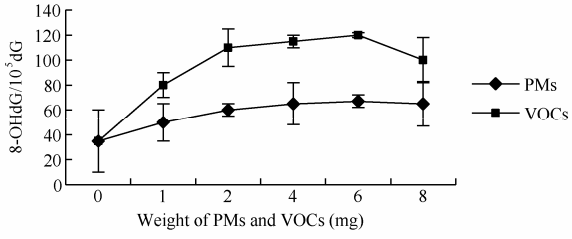

FIG. 1. Formation of 8-OHdG in calf thymus DNA after treatment with PMs and VOCs. 100 μg calf thymus DNA was treated with different weights of PMs and VOCs in ETTS at 37℃ for 10 h. Points and bars represent average values and standard deviations for 5 independent analyses. The comparison of the fifth points of the two curves was statistically significant ($P<0.01$) by *t* test.

As shown in Fig. 1, with the weight of PMs and VOCs increasing, the levels of 8-OHdG in DNA also increased, but smoothed down as the weight increased further. The contents of 8-OHdG induced by VOCs were significantly higher than those by PMs. The main interpretation was that, when cigarette burned, high temperature caused VOCs to carry more polyphenol and quinone compounds, which induced more severe oxidative DNA damage *in vitro*.

Oxidative DNA Damage Induced by Fe^{2+} and Polyphenol

In order to prove the reason why ETSS could

damage DNA to produce a great amount of 8-OHdG *in vitro*, we designed this experiment to observe the change of 8-OHdG levels in DNA exposed to metal Fe^{2+} or polyphenol hydroquinone alone, or the combination of both without any biological activity system and enzymatic metabolism (Fig. 2).

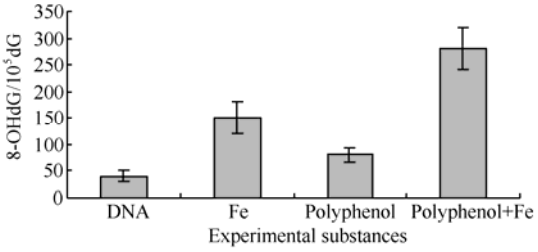

FIG. 2. Formation of 8-OHdG in calf thymus DNA after treatment with Fe^{2+} and polyphenol 100 μg calf thymus DNA was treated with 0.5 mmol/L Fe^{2+}, 20 mmol/L hydroquinone, and the mixture of them at 37℃ for 10 h.

As shown in Fig. 2, hydro-quinone alone caused a slightly increased 8-OHdG level, and Fe^{2+} induced a higher 8-OHdG level, while hydro-quinone + Fe^{2+} induced a more higher level of 8-OHdG than the sum of both compounds used alone, indicating that metals could catalyze polyphenols to increase the oxidative DNA damage.

Formation of 8-OHdG in Rat Lung Tissue DNA Induced by PMs of ETSS

As shown in Fig. 3, with the increasing of PMs, the amounts of 8-OHdG in DNA of lung tissues increased. The comparison of the low dose group and high dose group with the control group, or between the high dose group and the low dose group, were statistically significant (P<0.05). The results suggested that ETSS could induce oxidative DNA damage with a dose response relationship, and it might be the possible mechanism of the carcinogenicity of ETSS.

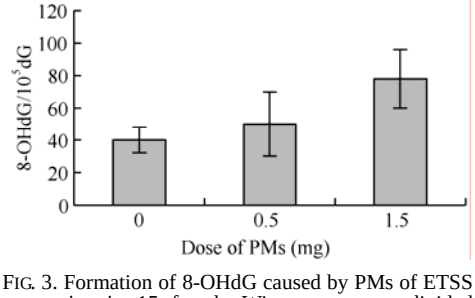

FIG. 3. Formation of 8-OHdG caused by PMs of ETSS *in vivo*.15 female Wistar rats were divided randomly into control group, low dose group and high dose group. Points and bars represent mean values and standard deviations for 5 rats. The comparison of the low-dose group and high-dose group with the control group, the comparison of high-dose group with low-dose group, were statistically significant (P<0.05) by t test, respectively.

DISCUSSION

There were various kinds of chemicals in ETSS, including many organics, such as PAHs, heteroclylic compounds, quinones, phenols, aldehyde, ketone, acids, ester and aliphatic hydrocarbon, etc., as well as trace metals, such as Fe, Cu, Cr, Cd, Ni, Co, Pb, etc. ETSS could produce a great amount of superoxide ($O_2^{\bullet -}$) and hydroxyl radicals (·OH) *in vitro*, which could damage calf thymus DNA to produce large amounts of DNA adduct 8-OHdG. We previously established an oxidative DNA model, using Fenton type hydroxyl free radical-generating system (such as chelator-Fe^{2+}-H_2O_2 system), as the oxidative source to react with calf thymus DNA to form 8-OHdG. ETSS could produce a great amount of active oxygen free radicals without any biological activity system, and enzymatic metabolism, because quinone and phenol compounds present in ETSS have auto-oxidation functions to generate a large amout of superoxide ($O_2^{\bullet -}$) and hydroxyl radicals (·OH) to attack DNA bases, forming DNA adduct 8-OHdG.

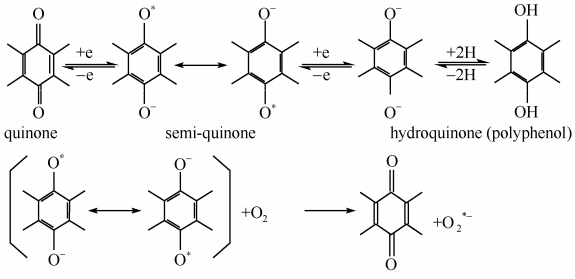

FIG. 4. Autooxidation of quinone and polyphenol compounds.

It has proved that Fe^{2+} can be gradually oxidized to Fe^{3+} by oxygen in the air, and in the meantime O_2 is changed to superoxide $O_2^{\bullet -}$ In the presence of Fe^{2+} or Fe^{3+}, superoxide $O_2^{\bullet -}$ can become hydroxyl radical through Fenton reaction.

$$Fe^{2+} + O_2 \rightleftharpoons Fe^{2+} - O_2 \rightleftharpoons Fe^{3+} - O^- \rightleftharpoons Fe^{3+} + O_2^{\bullet -}$$

$$Fe^{3+} + O_2^{\bullet -} \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \bullet OH + OH^-$$

$$O_2^{\bullet -} + H_2O_2 \longrightarrow \bullet OH + OH^- + O_2$$

Furthermore, these trace metal ions present in ETTS also could catalyze the auto-oxidation and Fenton reaction to form active oxygen free radicals $O_2^{\bullet -}$ and · OH, which might directly attack DNA to generate 8-OHdG or other DNA damages[7,8], such as

DNA single strand breakage, DNA double strand breakage and strand crosslink, etc.

The levels of 8-OHdG can reflect the extent of DNA damage. The molecule of phenol and quinine compounds is small, and easy to volatile. When cigarette was burned, fairly higher temperature contributed to volatilization of these compounds, so these two kinds of compounds captured in VOCs were more than in PMs of ETSS, which may initiate more significant DNA damage. In living organisms, PAHs, heterocyclic compounds, quinones etc. in the smoke may produce much larger amounts of superoxide through enzymatic metabolism to form significant DNA adduct and other DNA damage *in vivo*. This may be one of the molecular mechanisms of potential carcinogenicity of passive smoking. The results also provide a basis for the study of hazards from ETSS on non-smoker *in vivo*.

REFERENCES

1. Albert, A., Anton, J., Janet, H., and West, M. (1999). 8-Hydroxydeoxyguanosine in DNA from leukocytes of healthy adults: relationship with cigarette smoking, environmental tobacco smoke, alcohol and coffee consumption. *Mutation Research* **439**, 249-257.
2. Gan, D. K., Jiao, L., Bu, W. H., Ma, L., and He, X. J. (2000). The research progress of health effects of environmental tobacco smokes on non-smoking population. *Journal of Environmental and Health* **17**, 125-128. (In Chinese)
3. Zhao, X. S. and Zhao, H. X. (1998). Analysis of the organic components in the smoke of different kinds of cigarettes. *Shanghai Environmental Science* **17**(6), 21. (In Chinese)
4. Wang, Z. L., Zou, D. Z., and Yan, Y. P. (1995). Determination of organic pollutant in airborne particulate in Shanghai. *Shanghai Environmental Science* **14**(7), 34. (In Chinese)
5. M., Nagashima, H., Tsuda, S., Takenoshita, Y., Nagamachi, S., Hirohashi, J., Yokota, and H., Kasai (1995). 8-Hydroxydeoxyguanosine levels in DNA of human breast cancers are not significantly different from those of non-cancerous breast tissues by the HPLC-ECD method. *Cancer Letters* **90**, 157-162.
6. H., Kassi (1997). Analysis of a form of oxidative damage, 8-hydroxy-2'-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. *Mutation Research* **3887**,147-163.
7. H. G., Claycamp (1992). Phenol sensitization of DNA to subsequent oxidative damage in 8-hydroxydeoxyguanosine assays. *Carcinogenesis* **13**, 1289-1292.
8. T., Takeuchi, M., Nakajima, Y., Ohta, K., Mure, T., Takeshita, and K., Morimoto (1994). Evaluation of 8- Hydroxydeoxyguanosine, a typical oxidative DNA damage, in human leukocytes. *Carcinogenesis* **15**, 1519-1523.

(*Received December* 26, 2003 *Accepted June* 26, 2004)