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**Whole body exposure of mice to secondhand smoke induces dose-dependent and persistent
promutagenic DNA adducts in the lung**

Running title: *Secondhand smoke & DNA adducts*

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Keywords: ³²P-postlabeling, lung cancer, DNA adducts, mutations, secondhand smoke

ABBREVIATIONS: BAL, bronchoalveolar lavage; CI, confidence intervals; DRZ, diagonal
radioactive zone; FTC, Federal Trade Commission; HEPA, high-efficiency particulate-air; RAL,
relative adduct labeling; SHS, secondhand smoke; TLC, thin-layer chromatography; TSP, total
suspended particulate.

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ABSTRACT

Secondhand smoke (SHS) exposure is a known risk factor for lung cancer in lifelong nonsmokers. However, the underlying mechanism of action of SHS in lung carcinogenesis remains elusive. We have investigated, using the ^{32}P -postlabeling assay, the genotoxic potential of SHS *in vivo* by determining the formation and kinetics of repair of DNA adducts in the lungs of mice exposed whole body to SHS for 2 or 4 months (5 hours/day, 5 days/week), and an ensuing one-month recovery period. We demonstrate that exposure of mice to SHS elicits a significant genotoxic response as reflected by the elevation of DNA adduct levels in the lungs of SHS-exposed animals. The increases in DNA adduct levels in the lungs of SHS-exposed mice are dose-dependent as they are related to the intensity and duration of SHS exposure. After one month of recovery in clean air, the levels of lung DNA adducts in the mice exposed for 4 months remain significantly higher than those in the mice exposed for 2 months ($P < 0.0005$), levels in both groups being significantly elevated relative to controls ($P < 0.00001$). Our experimental findings accord with the epidemiological data showing that exposure to smoke-derived carcinogens is a risk factor for lung cancer; not only does the magnitude of risk depend upon carcinogen dose, but it also becomes more irreversible with prolonged exposure. The confirmation of epidemiologic data by our experimental findings is of significance because it strengthens the case for the etiologic involvement of SHS in nonsmokers' lung cancer. Identifying the etiologic factors involved in the pathogenesis of lung cancer can help define future strategies for prevention, early detection, and treatment of this highly lethal malignancy.

1. INTRODUCTION

Ample epidemiologic evidence has linked secondhand smoke (SHS) exposure to lung cancer development in lifelong nonsmokers [1-3]. However, the mechanistic involvement of SHS in nonsmokers' lung cancer is unknown [4,5]. Elucidating the mechanism of action of SHS in the genesis of lung cancer is a high priority for research because this malignancy continues to remain the leading cause of cancer-related deaths worldwide [6]. The alarming situation in many developing countries in which the smoking trend is on the rise, compounded with the inexistence or ineffectiveness of laws against public smoking [5,7,8], highlights the importance of research on SHS and lung cancer [9]. Such investigations can provide proof-of-evidence data, which will raise public awareness against health consequences of exposure to SHS [8,10]. Identifying the etiologic factors involved in the pathogenesis of lung cancer will also enable the scientific community to devise preventive and therapeutic strategies against this highly lethal disease (*reviewed in Ref. [4]*).

SHS is a complex and dynamic mixture of several thousand chemicals, including particulate and (semi)- volatile compounds [11,12]. This aerosol consists of the exhaled mainstream smoke of active smokers, the sidestream smoke emitted from the smoldering cone of tobacco products and the smoke diffused through the wrapping materials, *e.g.*, cigarette paper [4]. Being produced at a lower temperature relative to mainstream smoke and having undergone aging and dilution in ambient air, SHS is different from mainstream smoke in terms of quantities of its constituents, and physicochemistry [11,13]. Qualitatively, however, SHS contains essentially the same toxicants and carcinogens as those found in mainstream smoke [11-13]. Many of the carcinogens present in mainstream smoke are known to exert their effects through a genotoxic mode of action, which is mainly based on their ability to

induce DNA damage and mutations [9,14], although an epigenetic mode of action, *e.g.*, through aberrant DNA methylation and histone modifications, is also beginning to emerge for a few of these carcinogenic compounds [4,15]. The genotoxicity of mainstream smoke carcinogens is mostly ascribed to the formation of covalently bound DNA adducts, which upon eluding repair, may cause mispairing during DNA replication and lead to mutagenesis [16,17]. The DNA adduct-driven mutagenicity of mainstream smoke carcinogens is best represented by the co-localization of preferential and repair-resistant DNA adducts in cells treated *in vitro* with smoke-derived carcinogens with lung cancer mutational hotspots found in the *TP53* tumor suppressor gene and *RAS* oncogene [18,19]. The correspondence between DNA adduction sites and hypermutated codons in the *TP53* and the *RAS* genes, which are frequently targeted in smoking-attributable lung cancer, has provided significant clues to the etiology of this disease [20,21].

The similar chemical compositions of mainstream smoke and SHS [4,6] suggests that SHS possesses a genotoxic mode of action based on the capacity of some of its components to induce DNA adducts. In the present study, we have tested this hypothesis by investigating the DNA adduct-inducing potential of SHS in an *in vivo* mouse model, under well-defined and controlled exposure conditions. We have investigated the formation and kinetics of repair of DNA adducts in the lungs of mice exposed to SHS, generated by a microprocessor-controlled smoking machine. We have determined, using the highly sensitive ³²P-postlabeling assay [22], the formation and persistence of lung DNA adducts in mice exposed whole body to SHS for a duration of 2 or 4 months (5 hours/day, 5 days/week), with an ensuing one-month recovery period.

2. MATERIALS AND METHODS

2.1. Animals. Eighty male C57BL/6 mice (6-8 weeks old) were randomly divided into two groups of (1) Experimental (SHS exposure; $n = 40$) and (2) Control (clean air sham-exposure; $n = 40$), each subdividing into four categories ($n = 10$), including (I) two months exposure, (II) two months exposure + one month recovery, (III) four months exposure, and (IV) four months exposure + one month recovery. The mice assigned to each experimental or control group ($n = 10$) were kept in polypropylene cages in groups of 3-4 animals per cage, and housed in an air-conditioned animal room with an ambient temperature of $21 \pm 1^\circ\text{C}$, and relative humidity of 55%, with 12-hours light/dark cycle. Throughout all experiments, including the exposure and recovery periods, the mice had access to food (PicoLab Rodent Diet 20, PMI Nutrition International, LLC.; Brentwood, MO), and water *ad libitum*. All animal experiments were conducted in the City of Hope Animal Resources Center, and approved by the Institutional Animal Care and Use Committee in accordance with the recommendations of the National Institutes of Health provided in the Guide for the Care and Use of Laboratory Animals. Of note, although C57BL/6 mice are less sensitive to pulmonary carcinogens as compared to other strains, such as A/J mice; the latter mice, however, develop spontaneously lung tumors, *e.g.*, adenomas, at very high frequency [23-26]. Work in our laboratory has shown that SHS is a comparatively weak mutagen, whose genotoxicity can be established in the C57BL/6 mice with very high specificity (*ongoing experiments*).

2.2. Smoking machine. We used a custom-made smoking machine (model TE-10; Teague Enterprises, Davis, CA) to generate SHS for experimental exposure of mice. The TE-10 smoking machine is a microprocessor-controlled unit, which can smoke up to ten cigarettes at

a time. The machine is loaded with 40 cigarettes, which are then moved to a smoking chamber and placed into a wheel. The cigarettes are lit and smoked for nine minutes after which they are ejected from the wheel into a water-containing bin, and new cigarettes are placed, ignited, and smoked, thereafter. A multi-component accessory system collects, ages, and dilutes the smoke generated in the smoking chamber, and converts the high levels of smoke to concentrations required for different applications in two exposure chambers. The machine can produce mainstream smoke, sidestream smoke or a combination of the two in varying proportions. Included in the TE-10 smoking machine are calibration and recording features, which document the number of smoked cigarettes at a given flow rate, and measure total suspended particulate (TSP) levels in each of the two exposure chambers. Cigarettes are smoked using the Federal Trade Commission (FTC) method, which consists of 2-seconds puffs of 35 cc, each, at one-minute intervals [27]. We programmed the TE-10 smoking machine to produce a mixture of sidestream smoke (89%) and mainstream smoke (11%), which is conventionally used to mimic SHS for experimental purposes [27-31]. We used the 3R4F Reference Kentucky cigarettes (University of Kentucky; Lexington, KY), which have a declared content of 11.0 mg total particulate matter, 9.4 mg tar, and 0.73 mg nicotine, individually. Each cigarette was smoked using the FTC method for a duration of nine minutes at a flow rate of 1.05 L/min [27]. As part of the FTC method, the cigarettes were stored at 4°C until needed. At least 48 hours prior to use, the cigarettes were placed in a closed chamber at 23°C along with a solution of glycerin/water (mixed in a ratio of 0.76/0.26) to establish a relative humidity of 60% [28].

2.3. SHS exposure. All mice assigned to various experimental groups underwent an acclimatization period, during which they were gradually exposed to incremental doses of SHS as follows: 1st day: one hour exposure to SHS produced through continuous smoking of 2 cigarettes simultaneously; 2nd day: two hours exposure to 3 cigarettes simultaneously; 3rd day: three hours exposure to 4 cigarettes simultaneously; 4th day: four hours exposure to 5 cigarettes simultaneously; and 5th day: five hours exposure to 6 cigarettes simultaneously. Following the acclimatization period, the mice were maintained on a SHS exposure regimen, which included 5 hours per day, 5 days per week, and 2 or 4 months whole body exposure to SHS produced through continuous smoking of 7-9 cigarettes. Throughout all SHS-exposure experiments, the mice were kept in their original cages, placed in the exposure chambers. The position of cages in the exposure chambers was rotated on a weekly basis. The concentrations of TSP in both exposure chambers were measured gravimetrically twice per day. All mice were monitored closely for development of any unusual symptoms during both the exposure and recovery periods, and body weights were charted once per week. Control mice were handled similarly to SHS-treated animals, and maintained in clean air following sham-exposure to filtered high-efficiency particulate-air (HEPA). At the end of all experiments, the mice were euthanized by CO₂ asphyxiation, and upon necropsy, lungs were harvested and preserved at -80°C until further analysis.

2.4. Genomic DNA isolation. Lung cellular DNA from SHS-treated and control mice was isolated using a standard phenol and chloroform extraction and ethanol precipitation protocol [32]. The DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), and kept at -80°C until further analysis.

2.5. ³²P-postlabeling of DNA adducts. DNA adducts were measured for each DNA sample using the nuclease P₁ version of the ³²P-postlabeling assay as described earlier [22]. For analysis, DNA samples (4 µg) were digested with micrococcal nuclease (120 mUnits) and calf spleen phosphodiesterase (40 mUnits), enriched, and labeled as reported elsewhere [22]. Chromatographic conditions for thin-layer chromatography (TLC) on polyethyleneimine-cellulose plates (Macherey-Nagel, Düren, Germany) were as follows: D1, 1.0 M sodium phosphate, pH 6.0; D3, 4.0 M lithium-formate, 7.0 M urea, pH 3.5; D4, 0.8 M LiCl, 0.5 M Tris, 8.5 M urea, pH 8.0 [33]. After chromatography, TLC sheets were scanned using a Packard Instant Imager (Dowers Grove, IL). For each sample, relative adduct labeling (RAL), which is representative of the level of DNA adducts, was calculated from adduct cpm, the specific activity of [γ -³²P]ATP, and the amount of DNA (pmol of DNA-P) used. As in prior studies [34,35], total DNA adduct levels were measured in the diagonal radioactive zone (DRZ) area of the TLC plates, and were considered representative of polycyclic aromatic hydrocarbon-DNA adducts and other aromatic/hydrophobic-DNA adducts resistant to nuclease P₁ digestion. The method provides a summary measure of a complex mixture of adducts present in the postlabeling chromatograms. The results were expressed as RAL/10⁸ nucleotides. An external benzo[a]pyrene-diol-epoxide-DNA standard was included with each batch as a positive control [36]. Each DNA sample was determined by two independent ³²P-postlabeling analyses.

2.6. Statistical Analysis. Given the small sizes of experimental/control groups, and relatively large intergroup data variability, all results are expressed as medians $\pm$ 95% confidence intervals (CIs), which give a better estimation of data distribution. For the same reason, non-

parametric tests were used throughout. Comparison of all variables between two separate groups was done using the Wilcoxon rank-sum test. All statistical tests were two-sided. Values of $P \leq 0.05$ were considered statistically significant. The S-Plus 7.0 for Windows software (Insightful Corp.; Seattle, WA) was used for all statistical analyses.

3. RESULTS AND DISCUSSION

To verify a consistent exposure of mice to SHS throughout all experiments, we measured the concentrations of TSP in both exposure chambers of the smoking machine twice daily. As shown in Figure 1A, after the acclimatization period, the average concentrations of TSP in the exposure chambers, wherein mice were exposed to SHS for 2 or 4 months were 224.9 ± 21.6 and $233.0 \pm 15.4 \text{ mg/m}^3$, respectively, which are not significantly different from one another. The respective concentrations of TSP correspond to SHS generated through continuous smoking of 7.5 ± 0.7 and 8.0 ± 0.5 cigarettes during the 2- and 4-months SHS exposure periods, respectively (*see*, Fig. 1B). The fluctuations of TSP concentration in the exposure chambers of the smoking machine were mostly due to temporal variations in airflow, which were adjusted after each TSP measurement by modulating the number of smoked cigarettes.

All mice from both experimental and control groups tolerated the SHS/sham-exposure regimens well, without exhibiting any sign of stress or discomfort throughout. The survival rate in all groups was 100% at the end of both SHS/sham-exposure period and the ensuing recovery time. Whereas mice in the control group gained body weight steadily throughout the sham-exposure and recovery periods, the mice in experimental groups showed a nearly flat pattern of body weight during both the 2- and 4-months SHS exposure periods, although they then gained weight progressively during the recovery period (*see*, Fig. 2).

Qualitatively, the formation of DNA adducts in the lungs of all 2- and 4-months SHS-exposed mice was confirmed by the detection of DRZ, which is an indicator of covalent modification of DNA by a complex mixture of chemicals [34,35,37-40]. The persistence of DNA adducts was also verified by the presence of DRZs in the lungs of any 2- and 4-months SHS-exposed mice after one month recovery in clean air. Figure 3 shows representative chromatograms of the ^{32}P -postlabeled DNA adducts in the lungs of 2- and 4-months SHS-exposed mice (panels 'B' and 'E', respectively), and the counterpart mice after one month recovery in clean air (panels 'C' and 'F', respectively). In all cases, highly intense DRZs were readily detectable in the chromatographic profiles of DNA adducts in the lungs of all SHS-exposed mice.

Quantitatively, we determined the levels of DNA adducts in the lungs of experimental and control mice. Because the background levels of DNA adducts in the lungs of various control groups, including (I) 2 months sham exposure; (II) 2 months sham exposure plus 1 month recovery; (III) 4 months sham exposure; and (IV) 4 months sham exposure plus 1 month recovery, did not differ significantly from each other (*data not shown*), we used the data only from control group (IV) for all comparative analyses. As shown in Figure 4, the background level of DNA adducts in the lungs of control mice ($1.9 \pm 0.8 / 10^8$ nucleotides) was significantly increased 12.8-fold to $24.4 \pm 4.4 / 10^8$ nucleotides by 2-months exposure and 23.8-fold to $45.2 \pm 11.8 / 10^8$ nucleotides by 4-months exposure ($P < 0.00001$; both cases). The levels of lung DNA adducts in mice exposed to SHS for 4 months were significantly higher than those in mice exposed to SHS for 2 months ($P < 0.00001$). After one month of recovery, the levels of DNA adduct still remained significantly increased relative to controls (9.4-fold to 17.8 ± 3.5 for 2-months exposure and 13.4-fold to $25.4 \pm 4.6 / 10^8$ nucleotides for 4-months exposure; $P < 0.0001$ and $P < 0.00001$, respectively). We note that although after

one month of recovery, the levels of lung DNA adducts in mice exposed to SHS for 2 months were significantly lower than those in mice exposed to SHS for 4 months ($P < 0.0005$), a higher percentage of adducts were lost during the recovery period in the later group (*i.e.*, 4 months SHS-exposed mice).

The above findings demonstrate that the formation of DNA adducts in the lungs of SHS-exposed mice is directly related to the duration of exposure. To specifically determine whether the intensity of SHS exposure (concentration) can also modulate the induction of DNA adducts in our experimental system, we performed a complementary experiment in which subgroups of mice were exposed to SHS at TSP concentrations of 69.0 ± 5.5 or 90.4 ± 5.6 mg/m³ for a duration of two months relative to controls (*i.e.*, clean air sham-exposure) using the same protocol as described in ‘Materials and Methods’ (*see*, section 2.3). As shown in Figure 5, a concentration-dependent formation of DNA adducts was found in the lungs of SHS-exposed mice. More specifically, the intensity of DRZs and the levels of DNA adducts in the lungs of mice exposed to SHS at TSP concentration of 90.4 ± 5.6 mg/m³ were significantly higher than those in counterpart mice exposed to SHS at TSP concentration of 69.0 ± 5.5 mg/m³ ($P = 0.0074$) (*see*, Fig. 5B). In both cases, DNA adduct levels in the lung of SHS-exposed mice were significantly increased relative to controls ($P < 0.0001$; both cases) (*see*, Fig. 5B).

Izzotti *et al.* [41] have treated Sprague-Dawley rats with SHS at TSP concentrations of 73 – 93 mg/m³ for 6 hours/day, 5 days/week for a duration of 4-5 weeks. The authors have investigated the organ/tissue-selective formation and persistence of DNA adducts in the SHS-exposed rats by quantifying DNA adducts in the lung, heart, liver, bladder, testis, dissected tracheal epithelium, and isolated bronchoalveolar lavage (BAL) cells, using the butanol

version of the ^{32}P -postlabeling assay. A time-related increase of DNA adduct formation was detectable by autoradiography, in the form of massive DRZs and individual spots. Top levels were reached after 4-5 weeks of exposure. The ratio of SHS-induced DNA adducts to the background levels detected in sham-exposed rats was 11.2 in the tracheal epithelium, 10.4 in BAL cells, 7.3 in the heart, 6.3 in the lung, 5.1 in the bladder, 1.9 in the testis, and 1.1 in the liver. One week after discontinuing SHS exposure, the levels of DNA adducts significantly decreased in the lung, tracheal epithelium, heart, and bladder. The decrease was appreciable but not statistically significant in BAL cells, and was negligible in the heart, however. Specifically, the levels of DNA adducts in the lungs of SHS-exposed rats reached $18.8 \pm 8.2 / 10^8$ nucleotides (mean $\pm$ SD) after 4 weeks of treatment, and remained 2.0-fold over the background one week after discontinuing the exposure ($P < 0.01$) [41]. These DNA adduct dosimetry data in the lungs of SHS-exposed rats are in good agreement with our data despite differences in the study designs, *e.g.*, the use of different animal models, treatment protocols, SHS dose (duration and TSP concentration), recovery periods, and enrichment methods of the ^{32}P -postlabeling assay.

Organs of the respiratory tract are known targets of tumorigenesis in smoking-related malignancies [6] with the lung being the target organ for SHS-associated carcinogenesis [5]. Epidemiologic studies have shown that the incidence of lung cancer in smokers depends upon exposure intensity (*e.g.*, number of smoked cigarettes) and duration (*e.g.*, smoking years), which comprise the aggregate dose for smoke-derived carcinogens. Generally, carcinogen-exposed individuals have a higher risk of developing cancer relative to non-exposed individuals depending on carcinogen dose [2]. Elimination of carcinogen exposure can reduce the risk of exposed individuals to develop cancer - albeit never equating to that of non-

exposed individuals - and with increasing years of exposure, risk reduction lessens after discontinuation of exposure [2]. This is best exemplified by the case of smoking and lung cancer in which smokers have a significantly higher risk of developing lung cancer relative to nonsmoker; whereas former smokers have a reduced risk for lung cancer development relative to active smokers (but never equal to that of nonsmokers), late quitters have a higher risk of developing lung cancer than early quitters [42]. Our experimental data in SHS-exposed mice perfectly recapitulate the above-mentioned epidemiologic findings as we demonstrate a dose-dependent increase in the formation of DNA adducts in the lungs of mice exposed to SHS. More specifically, SHS genotoxicity in the lungs of experimental mice intensifies after four months of exposure relative to two months of exposure ($P < 0.00001$); however, after discontinuation of exposure, the reversibility of the genotoxic response (as reflected by the level of DNA adducts) remains inversely related to SHS dose ($P < 0.0005$) (*see*, Fig. 4 and Fig. 5). In other words, more prolonged SHS exposure results in a higher DNA adduct level after termination of exposure.

In conclusion, we have demonstrated a genotoxic mode of action for SHS of relevance for lung carcinogenesis, which manifests as persistent DNA adduct formation in the lungs of mice treated with SHS. The formation of lung DNA adducts in the SHS-exposed mice is dose-dependent as it directly relates to the intensity and duration of SHS exposure. These experimental findings are in accord with the epidemiological data showing that exposure to smoke-derived carcinogens is a risk factor for lung cancer; not only does the magnitude of risk depend upon carcinogen dose (*i.e.*, intensity and duration of exposure) [2], but it also becomes less reversible as the duration of exposure increases [42]. These experimental findings are of importance because they reinforce the case for the etiologic involvement of

SHS in nonsmokers' lung cancer. Prospectively, understanding the underlying mechanism of action of SHS in lung carcinogenesis can help define future strategies for prevention, early detection, and treatment of this malignancy.

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CONFLICT OF INTEREST

None of the authors have conflicts of interest that might be construed to influence the results or interpretation of the data presented in this manuscript.

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FIGURE LEGENDS

Figure 1

External dosimetry of SHS exposure in mice.

(A) TSP concentration (mg/m^3)

(B) Number of smoked cigarettes

Results are expressed as medians $\pm$ 95% CIs. Horizontal dashed lines represent the median values in each exposure group.

Figure 2

Mice body weight chart.

(A) 2-months SHS exposure

(B) 4-months SHS exposure

(C) Control (clean air sham-exposure)

Results are expressed as medians + 95% CIs. For better visualization, data from an ongoing experiment in which the recovery period is extended to several months are also included.

Figure 3

Qualitative determination of DNA adducts in the lungs of SHS-exposed mice and controls. Representative chromatograms of the ^{32}P -postlabeled DNA adducts in the lungs of SHS-exposed mice and controls:

(A) Control group

(B) 2-months SHS-exposure group

(C) 2-months SHS-exposure + one month recovery group

(D) Benzo[a]pyrene-diol-epoxide-DNA standard (on average around 75 adducts/ 10^8 nucleotides).

(E) 4-months SHS-exposure group

(F) 4-months SHS-exposure + one month recovery group

Figure 4

Quantitative determination of DNA adducts in the lungs of SHS-exposed mice and controls. The nuclease P₁ version of the 32 P-postlabeling assay was performed on lung cellular DNA from SHS-exposed mice and controls, and relative adduct labeling (RAL), which is representative of the level of DNA adducts, was calculated as described in ‘Materials and Methods’ (*see*, section 2.5).

* Statistically significant as compared to Control; $P < 0.00001$

† Statistically significant as compared to Control; $P < 0.00001$

§ Statistically significant as compared to Control; $P < 0.0001$

** Statistically significant as compared to Control; $P < 0.00001$

‡ Statistically significant as compared to SHS ^(2 months) + Recovery ^(1 month); $P < 0.0005$

§§ Statistically significant as compared to SHS ^(2 months); $P < 0.00001$

Results are expressed as medians $\pm$ 95% CIs.

Figure 5

Qualitative and quantitative determinations of DNA adducts in the lungs of SHS-exposed mice and controls. Mice were exposed to SHS at TSP concentrations of 69.0 ± 5.5 or 90.4 ± 5.6 mg/m³ relative to controls (*i.e.*, clean air sham-exposure) using the same protocol as described in the text (*see*, Materials and Methods: section 2.3).

(A) Representative chromatograms of the ³²P-postlabeled DNA adducts in the lungs of SHS-exposed mice and controls:

(1) Control group

(2) SHS-exposure group (TSP: 69.0 ± 5.5 mg/m³)

(3) SHS-exposure group (TSP: 90.4 ± 5.6 mg/m³)

(B) The nuclease P₁ version of the ³²P-postlabeling assay was performed on lung cellular DNA from SHS-exposed mice and controls, and relative adduct labeling (RAL), which is representative of the level of DNA adducts, was calculated as described in ‘Materials and Methods’ (*see*, section 2.5).

* Statistically significant as compared to Control; $P < 0.0001$

† Statistically significant as compared to Control; $P < 0.0001$

§ Statistically significant as compared to SHS (TSP: 69.0 ± 5.5 mg/m³); $P = 0.0074$

Results are expressed as medians $\pm$ 95% CIs.

(A)

(B)

Fig. 1

(A)

(B)

(C)

Fig. 2

Fig. 3

Fig. 4

(A)

(B)

Fig. 5