Sidestream tobacco smoke is a male germ cell mutagen

Francesco Marchetti[a,1], Andrea Rowan-Carroll[b], Andrew Williams[b], Aris Polyzos[a], M. Lynn Berndt-Weis[b], and Carole L. Yauk[b,1]

[a]Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720; and [b]Environmental Health Sciences and Research Bureau, Health Canada, Ottawa, ON, Canada K1A 0K9

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Active cigarette smoking increases oxidative damage, DNA adducts, DNA strand breaks, chromosomal aberrations, and heritable mutations in sperm. However, little is known regarding the effects of second-hand smoke on the male germ line. We show here that short-term exposure to mainstream tobacco smoke or sidestream tobacco smoke (STS), the main component of second-hand smoke, induces mutations at an expanded simple tandem repeat locus (*Ms6-hm*) in mouse sperm. We further show that the response to STS is not linear and that, for both mainstream tobacco smoke and STS, doses that induced significant increases in expanded simple tandem repeat mutations in sperm did not increase the frequencies of micronucleated reticulocytes and erythrocytes in the bone marrow and blood of exposed mice. These data show that passive exposure to cigarette smoke can cause tandem repeat mutations in sperm under conditions that may not induce genetic damage in somatic cells. Although the relationship between noncoding tandem repeat instability and mutations in functional regions of the genome is unclear, our data suggest that paternal exposure to second-hand smoke may have reproductive consequences that go beyond the passive smoker.

DNA mutation | micronuclei | germline | spermatogenesis

Despite years of intense public campaigns to limit and reduce tobacco consumption, smoking is still widespread, and its health effects remain a significant public concern. Approximately 35% of men of reproductive age in the United States smoke cigarettes (1), and there is extensive evidence to suggest that tobacco smoking can result in abnormal reproductive outcomes such as spontaneous abortions and birth defects (2). Men who smoke are at high risk for several semen abnormalities, including reduced motility, sperm DNA damage such as DNA breaks and adducts, and chromosomal abnormalities (3). In mice, exposure to mainstream tobacco smoke (MTS) induces germ-line mutations in expanded simple tandem repeats (ESTR) that can be transmitted to the progeny, causing irreversible modifications in the genome of the offspring (4). Recently, the International Agency for Research on Cancer concluded that there is enough evidence to link paternal smoking in humans with increased risk of childhood cancer, suggesting that tobacco smoking causes heritable germ cell mutation in humans (5).

Exposure to second-hand smoke also remains widespread, with an estimated 40% of nonsmokers being exposed to second-hand smoke (6). Much less is known about the health effects of second-hand (or passive) smoking, although evidence is rapidly accumulating that exposure to second-hand smoke can be as harmful and hazardous to human health as active smoking (6, 7), and that there may be no risk-free level of exposure (8). Sidestream tobacco smoke (STS), the main component of second-hand smoke, is a complex mixture of more than 4,000 chemicals, including at least 50 known carcinogens (8). Qualitative differences between MTS—the smoke inhaled by active smokers—and STS are small; however, some toxicants and carcinogens are present at higher concentrations in STS (9). Maternal exposure to second-hand smoke during pregnancy has significant pre- and postnatal consequences (10). The effects of paternal exposure to second-hand smoke on the sperm genome and reproductive outcomes remain largely unknown.

In this study, we investigated the induction of tandem repeat mutations in sperm after short-term exposures to MTS or STS by using a mouse model. Noncoding ESTRs are the most sensitive marker currently available to measure induced germline mutation because they have quantifiable baseline levels of mutations resulting from the intrinsic instability of repetitive DNA (11). As a result, mutation rates can be estimated in a relatively small number of cells and individuals. Furthermore, we analyzed the induction of micronuclei (MN) in red blood cells of exposed mice to compare and contrast the response of somatic versus germ cells to tobacco smoke. The in vivo rodent MN test is widely used to assess the genotoxicity of chemicals for risk assessment and regulatory purposes (12), and it is generally believed that evaluations based on somatic cell mutation assays are sufficient to identify agents that are likely to enhance the risk of germ cell mutation (13).

Results and Discussion

Male B6C3F1 mice were exposed in inhalation chambers to MTS or STS generated from three or 16 cigarettes per day (~20 or 90 min per day, respectively) for 2 wk. This exposure regimen produced plasma cotinine levels similar to those in light smokers and passive smokers for MTS and STS, respectively (14). Sperm were collected from the cauda epididymis 6 wk after the end of the exposure period. ESTR mutations require DNA replication, and this interval permits sperm that were at the spermatogonial cell stage during exposure, which is the last stage at which DNA replication occurs during spermatogenesis (15), to reach the epididymis. ESTR mutation frequencies in two independent sham-exposed groups were 1.5% (range, 0.8–2.9%) and 1.3% (range, 0.9–1.7%), respectively. As shown in Table 1, we found that both doses of MTS resulted in a significant, greater than 2.6-fold increase in ESTR mutations in sperm. Mean mutation frequencies were 4.0% (range, 3.4–4.7%; $P = 0.01$) and 4.7% (range, 4.1–5.4%; $P = 0.007$) for the low and high doses of MTS, respectively. More importantly, we found that exposure to STS also induced a significant increase in ESTR mutations. Mean mutation frequencies were 4.6% (range, 3.0–7.2%) and 2.6% (range, 1.8–3.8%) for the low and high doses of STS, respectively (Table 1). The low dose of STS produced a level of ESTR mutations that was comparable with those observed after MTS exposure, whereas the high dose of STS produced only a 2.1-fold increase in ESTR mutations that was at the borderline of statistical significance ($P = 0.06$).

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[1]To whom correspondence may be addressed. E-mail: francesco.marchetti@hc-sc.gc.ca or carole.yauk@hc-sc.gc.ca.

Table 1. Sperm mutation data for the *Ms6-hm* locus after exposure to MTS or STS

Treatment*	Mice	Mutants	Progenitors (95% CI)†	Mutation frequency (95% CI), %‡	Ratio relative to sham (95% CI)	*P* value
Control	6	8	514 (469–559)	1.52 (0.78–2.91)	—	—
MTS–3	6	17	418 (378–459)	4.01 (3.39–4.74)	2.63 (1.35–5.13)	0.0112§
MTS–16	6	24	499 (455–544)	4.67 (4.06–5.37)	3.07 (1.58–5.94)	0.0074§
Control	4	4	295 (261–329)	1.26 (0.91–1.74)	—	—
STS–3	5	20	418 (377–458)	4.65 (3.00–7.20)	3.69 (2.14–6.35)	0.0218§
STS–16	5	17	626 (576–675)	2.62 (1.80–3.79)	2.08 (1.27–3.40)	0.0625

*Experimental groups are identified by the type of smoke (MTS or STS) and the number of cigarettes (three or 16) used for each daily exposure.
†Estimation of the number of progenitor molecule was determined from the number of positive PCR products generated by using Poisson distribution, pooling across samples; prediction and 95% CI are shown.
‡Average mutation frequency is given. Numbers in parentheses indicate the uncertainty resulting from the estimate derived for the number of progenitors scored.
§Significant after Bonferroni–Holm correction.

It had been shown that long-term exposure (e.g., 12 wk) to MTS increased mutation frequencies in simple tandem repeats in mice (4). Our results show that even a short exposure to MTS (e.g., 2 wk) can induce ESTR mutation in sperm and demonstrate that STS is also capable of inducing these mutations. Two notable findings of our study relate to the similarity in mutation frequency between MTS and STS exposures and the lack of a dose–response relationship in mutation induction, especially for STS. ESTR mutations do not arise from direct damage to the DNA at the repeat locus (16–18). The current accepted model supports an indirect mutation mechanism that involves polymerase pausing at ESTR loci in response to DNA damage elsewhere in the genome, formation of secondary structures within the repeated sequences, and replication errors in the repeat array when polymerase extension resumes (4, 16–18). Such an indirect mechanism is less likely to exhibit a linear dose–response relationship than mutation events arising from a direct hit to the DNA, because instability may occur at ESTR sites that were not damaged by the exposure. The similar mutation frequencies observed after exposure to the low and high doses of MTS indicate that mild exposure to MTS is sufficient to trigger the indirect mutational process that leads to formation of ESTR mutations. As MTS and STS share extensive similarities in chemical composition (9), the significant increase in ESTR mutation seen after the low dose of STS is in line with this hypothesis.

We found that the high dose of STS resulted in a lower mutation frequency than observed after the low dose of STS. A nonlinear relationship between dose and ESTR mutation frequencies has been reported in the germ line of male mice exposed to DNA damaging agents (19, 20). A decrease in mutation frequency with increasing dose may be the consequence of increased cytotoxicity, with the higher dose inducing higher rates of cell death precluding the recovery of mutated cells (17, 21). Whether this cytotoxicity is related to higher carbon monoxide content of STS or to another (or others) of its components remains to be established. Nevertheless, these findings show that passive exposure to tobacco smoke induces germ-line mutations in mammalian sperm and that the STS dose–response relationship is not linear.

We also determined whether the same exposures to MTS and STS would result in genetic damage in somatic cells. For this purpose, we exposed separate groups of B6C3F1 male mice ($n = 6$ per group) to MTS or STS at the same time as the mice used for the analysis of ESTR mutations in sperm. Mice were euthanized at the end of the 2-wk exposure period and analyzed for the induction of MN in reticulocyte (RET) cells in bone marrow and normochromatic erythrocytes (NCEs) in blood by using a flow cytometry-based assay (22). RET cells represent newly produced red blood cells that are detectable for approximately 24 h before becoming NCE cells, which in turn persist in the blood stream for approximately 30 to 40 d in the mouse (23). Therefore, MN in RETs represent damage induced during the last day of tobacco exposure, whereas MN in NCEs represent damage induced throughout the exposure period.

We analyzed more than 700,000 RETs and 47 million NCEs. As shown in Table 2 and Fig. 1, only the high dose of MTS produced slight, but statistically significant, increases in the frequencies of MN-RETs (0.68% vs. 0.52% in controls; $P = 0.03$, unadjusted) and MN-NCEs (0.16% vs. 0.15% in controls; $P = 0.04$, unadjusted). These results confirm that MTS induces MN in the hematopoietic system, as previously shown (3, 24). Conversely, neither dose of STS significantly increased the frequencies of MN-RETs or MN-NCEs with respect to control values in bone marrow and blood, respectively (using a generalized score test or ANOVA). These results appear to contrast with previous reports of significant increases in MN-RETs after exposure to STS (reviewed in ref. 7). However, we are aware of only one previous study that investigated the induction of MN in the bone marrow of mice exposed to STS in inhalation chambers (25). Differences between the two studies in the dilution of STS, duration of exposure, and time of analysis after exposure may account for the different outcomes. Our results show that, with the conditions used in this study, 2 wk of exposure to STS did not significantly increase chromosomal damage in the hematopoietic system of exposed mice. More importantly, our results show that exposure to levels of MTS and STS that induce germ-line mutations do not increase chromosomal damage in somatic cells as measured by the MN assay. These results parallel the findings that N-hydroxymethyl acrylamide induced dominant lethal mutations in male germ cells without increasing MN in peripheral blood erythrocytes in mice (26), and challenge a widely accepted tenet in genetic toxicology that somatic cell tests alone are sufficient to identify agents that induce mutations in germ cells (27).

Despite the ubiquitous presence of known mutagens and carcinogens in our day-to-day environment, extensive animal data showing induction and transmission of germ cell mutations (27), human studies showing increased genetic and chromosomal damage in sperm of older men (28) and patients receiving chemotherapy (29), and reports on increases in mutations rates in the children of parents exposed to radioactive contamination (30–33), no germ cell mutagen has been identified conclusively in humans. In addition, a number of studies have failed to detect an increase in mutations among the children of Chernobyl cleanup workers (34–37). This may be caused largely by the lack of useful tools and approaches to evaluate the potential for agents to produce heritable effects. The recent International Agency for Research on Cancer determination that paternal exposure to cigarette smoking causes cancer in their descendants represents a groundbreaking decision and implies that tobacco smoking should be considered a human germ cell mutagen (5). Our data add to the weight of evidence in favor of the classification of

Table 2. Micronucleus frequency in mouse bone marrow RETs and circulating NCEs in blood after exposure to MTS or STS

	Bone marrow				Blood			
Treatment*	Total RET	Mean MN-RET ± SE, %	Ratio relative to sham (95% CI)	*P* value	Total NCE	Mean MN-NCE ± SE, %	Ratio relative to sham (95% CI)	*P* value
Control	119,378	622 (0.52 ± 0.04)	—	—	7,838,336	11,568 (0.147 ± 0.003)	—	—
MTS–3	119,432	618 (0.51 ± 0.08)	0.99 (0.73–1.34)	0.96	12,689,144	18,092 (0.142 ± 0.002)	0.97 (0.92–1.02)	0.22
MTS–16	119,188	813 (0.68 ± 0.05)	1.30 (1.08–1.58)	0.03†	9,498,095	14,999 (0.158 ± 0.002)	1.07 (1.02–1.13)	0.04†
STS–3	119,431	569 (0.47 ± 0.06)	0.91 (0.70–1.2)	0.52	10,381,885	14,931 (0.144 ± 0.001)	0.98 (0.93–1.02)	0.28
STS–16	119,283	717 (0.60 ± 0.04)	1.15 (0.97–1.37)	0.15	7,313,457	11,347 (0.155 ± 0.003)	1.05 (0.99–1.11)	0.12

*Experimental groups are identified by the type of smoke (MTS or STS) and the number of cigarettes (three or 16) used for each daily exposure, with six mice per group.
†Significant after Bonferroni–Holm correction.

tobacco smoke as a human germ cell mutagen, and also implicate second-hand smoke as a human germ cell mutagen.

Germ cell mutation is critically important to public health because increases in heritable mutations may result in an increased burden of genetic disease in subsequent generations. Previous studies have shown that paternal exposures to chemical mutagens (20), ionizing radiation (38, 39), and particulate air pollution (40) increase ESTR mutation frequencies in sperm, and, more importantly, induce persistent genomic instability in the F1 and F2 offspring of exposed mice (32, 38, 41). Although not directly assessed in the present study, the observation that the mutated alleles are already present in the sperm of exposed mice at frequencies that match those observed in their offspring (16, 20) lends support to the notion that the tobacco smoke-induced germ-line mutations reported here would be passed on to the next generation. The impact of these mutations on the health of the offspring remains uncertain, as the evidence linking increased mutation rates in noncoding tandem repeat regions to a phenotypic effect or to increased mutations rates in protein-coding regions is not well established. However, ESTR mutations show a temporal pattern of induction during spermatogenesis similar to that observed for mutations in DNA coding regions after exposure of mice to ionizing radiation (18) and alkylating agents (19). There is also growing evidence for an important role of tandem repeats in genome structure, organization, and function (42). Therefore, ESTR mutation may serve as a biomarker of genomic instability, which has been associated with oncogenic mutations and malignant transformation in somatic cells (43), and heritable disease.

Our finding that exposure of mice to second-hand smoke induces ESTR mutations in sperm provides compelling evidence in support of the argument that passive smoking should be regarded as a germ cell mutagen in humans. Consistent with data for first-hand smoke, male exposure to second-hand smoke before fertilization is likely to have detrimental reproductive consequences that go beyond the passive smoker.

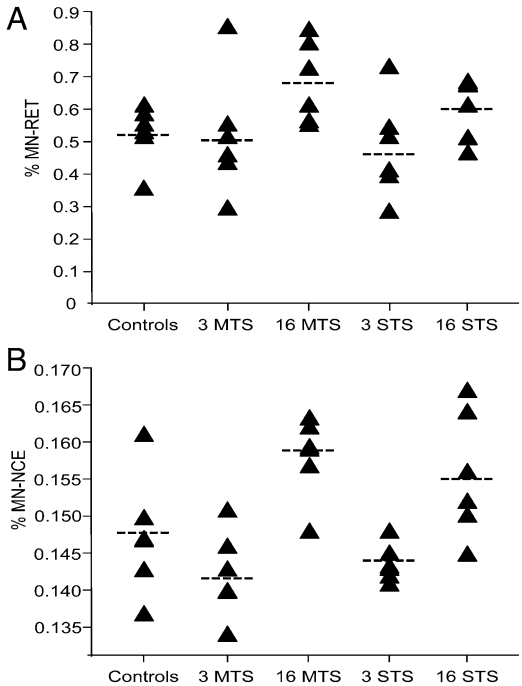

Fig. 1. Frequencies of MN-RETs in bone marrow (*A*) and MN-NCEs in blood (*B*) of mice exposed to MTS or STS. Each triangle represents one mouse. Dashed horizontal bars indicate the mean value for each group.

Materials and Methods

Animals and Tobacco Smoke Exposure. The research methods and procedures used in this study were approved by the Lawrence Berkeley National Laboratory Institutional Animal Care and Use Committee. B6C3F1/Crl mice were exposed to MTS or STS for 14 consecutive days using 2R4F research-grade cigarettes (University of Kentucky) and a cigarette smoking machine (CH Technologies) that delivered diluted MTS or STS to the inhalation chambers for whole-body exposure of mice. For each type of tobacco smoke, we used two levels of daily exposure: three or 16 cigarettes per day. Exposure to MTS and STS was conducted as described previously (14).

Sample Isolation. At the end of the 2 wk of exposure, we euthanized one group of male mice the day after the last daily tobacco exposure, and we collected blood from the heart and bone marrow by flushing the femurs. Blood and bone marrow were analyzed for the presence of MN in RETs and erythrocytes by using a flow cytometry-based assay (22). One advantage of the flow cytometry-based method, with respect to the standard analysis of MN in red blood cells or in binucleated bone marrow cells, is that it allows the analysis of a large number of cells (~20,000 RETs and several million NCEs per mouse vs. 1,000–2,000 cells normally scored with standard assays). Therefore, it has a higher power to detect small increases.

Another group of male mice was euthanized 6 wk after the last daily tobacco smoke exposure, and sperm were collected from the epididymis and processed for DNA isolation and analysis of mutations at the ESTR locus (*Ms6-hm*) locus as previously described (40).

Statistical Analyses. For calculating ESTR mutation frequencies, the rate parameter was estimated to determine the number of progenitor molecules by equating the probability (assuming a Poisson distribution) of observing no product in a single well to the proportion of wells with no product to obtain the expected number of progenitor molecules. The number of positive PCR products, mutation frequencies, and ratios were determined as previously described (4). A generalized score test was used to examine treatment effects through the SAS PROC GENMOD procedure (SAS/STAT software; SAS Institute) with a logit link function for the Poisson distribution with the estimated number of progenitor molecules as the offset. The generalized score test was used because each sample was treated as a cluster with repeated measurements. Similarly, for the micronucleus assay, a generalized score test was used to test for differences in mutation frequency between treated and control, assuming Poisson error with over-dispersion, whereby the logarithm of the number of cells scored was used as the offset.

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