Detection of In Vivo DNA Damage Induced by Very Low Doses of Mainstream and Sidestream Smoke Extracts Using a Novel Assay

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Background: Mainstream (MS) smoke, the main smoke inhaled by active smokers, and sidestream (SS) smoke, the main component of secondhand smoke, induce a wide range of DNA lesions. Owing to technical limitations, the in vivo levels of tobacco-induced DNA damage are unknown. Recently, the authors developed a highly sensitive primer-anchored DNA damage detection assay (PADDA) to quantify endogenous and induced DNA damage.

Purpose: To quantify the in vivo levels of DNA damage induced by MS and SS smoke extracts in human cells using PADDA and define the strand-specific patterns of DNA damage and repair following exposure to diverse doses of MS and SS smoke.

Methods: Human epithelial cells were exposed to escalating doses of hydrogen peroxide (H_2O_2), MS, or SS smoke. *TP53* gene DNA damage was quantified using PADDA at various time points. DNA double-strand breaks were detected by immunofluorescence analysis of phosphorylated histone H2AX (γ-H2AX). Cell viability was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Data were collected and analyzed by *t*-test in 2012–2014.

Results: A dose-dependent increase in DNA damage was detected in vivo with increasing doses of H_2O_2, MS, and SS smoke. Even 1 hour of exposure to very low doses of MS or SS smoke resulted in significant DNA damage ($p < 0.01$). MS and SS smoke induced distinctive strand-specific patterns of DNA damage and DNA repair kinetics.

Conclusions: Very low concentrations of MS and SS smoke induce significant DNA damage in human cells. Application of PADDA to population studies has major potential to establish biomarkers of susceptibility to tobacco-induced diseases.
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Introduction

Active and passive smoking constitute a significant public health problem, as tobacco use is the leading preventable cause of morbidity and mortality.[1] Tobacco smoking is a major risk factor for cardiovascular and chronic obstructive pulmonary disease, with many adverse reproductive and early childhood effects.[2] Tobacco use causes many types of cancer and is the primary risk factor for lung and head and neck cancers, two of the most common malignancies worldwide.[3] Although cigarette smoking is a major risk factor for cancer, individual susceptibility plays a significant role; for example, even among heavy smokers, only a relatively small fraction develops cancer.

Cigarette smoke comprises a complex mixture of more than 7,000 chemicals; hundreds of these are hazardous, and at least 69 are known to cause cancer.[4] Tobacco smoke also contains reactive oxygen species (ROS), which induce oxidative DNA damage.[4] Additionally, tobacco smoke reduces the antioxidant capacity of tissues[5] and the DNA damage repair rate.[6] When a

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cigarette is smoked, it results in a mixture of two types of smoke: mainstream (MS) smoke, the material drawn from the mouth end of a cigarette during puffing, and sidestream (SS) smoke, the material released into the air from the burning cigarette tip. SS smoke is the main component of secondhand smoke (which also includes exhaled MS smoke). MS smoke is the main component inhaled by smokers. MS and SS smoke vary in their specific chemical composition, but both are known carcinogens shown to induce DNA damage.[7–9]

Tobacco causes many types of DNA damage that are differentially recognized by diverse DNA damage repair and tolerance machinery. DNA damage is the main initiator of cancer and plays a key role in the pathogenesis of aging-related, neurodegenerative, pulmonary, and cardiovascular diseases.[10–14] DNA damage is also constantly generated endogenously in living cells. Consequently, the steady-state levels of DNA damage on a given tissue reflect the end result of specific genotoxic exposures, as well as individual and tissue-specific metabolic, genetic, and epigenetic variations. Therefore, a significant fraction of tobacco-induced disease is expected to occur in individuals with tissue-specific (or global) high levels of tobacco-induced DNA damage. Nevertheless, current prevention strategies for tobacco-associated diseases are based mainly on the levels of tobacco exposure. This is in light of the fact that, until recently,[15] DNA damage detection assays did not have sensitivity to quantify the overall levels of in vivo DNA damage[15,16] and were not practical for population screening.[16,17]

To precisely quantify the levels of in vivo DNA damage, an assay should measure both endogenous and induced damage. Recently, the authors developed a novel and highly sensitive primer-anchored DNA damage detection assay (PADDA). In contrast with available assays, PADDA is able to map and quantify endogenous and induced DNA damage.[15] PADDA does not require hazardous reagents, sophisticated equipment, or specialized skills and can be used in a real-time polymerase chain reaction (PCR) setting to ease population screenings.

It is hypothesized that PADDA accurately quantifies the in vivo levels of DNA damage induced by MS and SS smoke extracts and therefore has major potential to be used in population screenings to identify individuals at higher risk of developing tobacco-associated diseases.

Methods

Cell Culture

The human epithelial squamous cell carcinoma (SCC-1) cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics under standard conditions.[18]

Hydrogen Peroxide Exposure

SCC-1 cells were treated with different doses of hydrogen peroxide (H_2O_2; 1, 10, 100, 1,000 and 10,000 μM) in DMEM without growth factors. After 1 hour, media was removed; cells were rinsed twice with phosphate-buffered saline (PBS) and harvested for DNA extraction at various time points (0, 3, and 24 hours post-exposure).

Mainstream and Sidestream Exposure

MS and SS smoke extracts were prepared as described elsewhere.[19] MS and SS extracts (0.3, 1.5, 3, 15, and 30 μg/mL) were added to the culture media, and 1 or 16 hours later, SCC-1 cells were used for the cell viability assay, immunofluorescence analysis of phosphorylated histone H2AX (γ-H2AX), and DNA extraction for the q-PADDA assay.

Cell Viability Assay

To determine cell viability after exposure to MS or SS extracts, 1.25 μg/μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Invitrogen, Carlsbad CA) were added to each well and incubated for 4 hours at 37°C. Then, a 1:1 volume of 20% sodium dodecyl sulfate (SDS) in 50% dimethyl formamide (DMF) solution was added as a solubilizer. The optical density was read at 570 nm using a BioTek Synergy HT reader (Winooski VT).

Immunofluorescence

The presence of phosphorylated histone H2AX (γ-H2AX), a marker for double-strand breaks (DSBs), was determined as previously described.[20] Digital fluorescent images (400X) were taken with an Olympus DP70 camera using fluorescein isothiocyanate (FITC)/tetramethylrhodamine (TRITC) filters under an Olympus microscope IX701 (Tokyo, Japan).

DNA Extraction

Total genomic DNA was isolated according to the protocol of Mullenbach et al.[21] and by following previously described steps to reduce artifactual DNA damage.[15]

Primer-Anchored DNA Damage Detection Assay

PADDA was performed as the authors previously described,[15] except for the following modifications: (1) primer extension was performed within the genomic region of the *TP53* gene, the most frequently mutated gene in cancers associated with tobacco-smoke[4]; (2) DNA damage was quantified in the transcribed strand (TS) and non-transcribed strand (NTS) of *TP53* in a targeted area of approximately 700 base pairs (oligonucleotides listed in Appendix Table 1); and (3) 200 ng of human genomic DNA were used in primer extension. Briefly, to screen for DNA damage, a single non-cycled primer extension was performed in the region of interest with a biotinylated primer. After several purification steps, the extended single-stranded products were resuspended in *tris(hydroxymethyl) aminomethane* (*Tris*)-*ethylenediaminetetraacetic acid (EDTA)* buffer and processed for high-throughput damage quantification (q-PADDA).[15] Real-time PCR reactions were carried out in a CFX96 real-time PCR system with a 10-μL final volume using SsoAdvanced™ SYBR® Green Supermix (Bio-Rad, Hercules CA), 0.3 μM
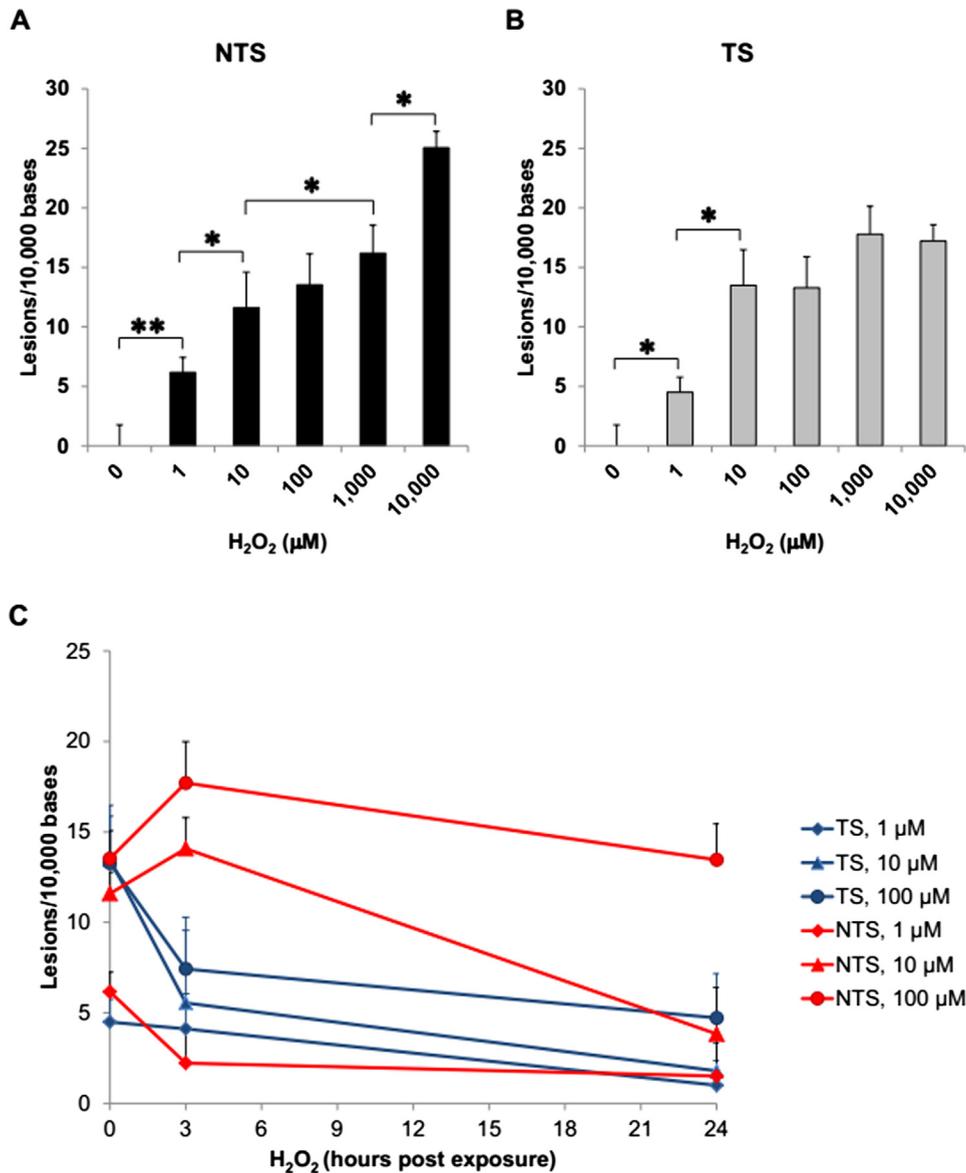
Figure 1. Dose-dependent increase in DNA damage in cells exposed to H_2O_2.

Note: Strand-specific DNA damage was quantified by q-PADDA in NTS (A) and TS (B) of the *TP53* gene. (C) DNA damage induced by exposure to H_2O_2 is repaired quicker in the TS than in the NTS of *TP53*. Data are represented as mean ± SEM.

NTS, non-transcribed strand; PADDA, primer-anchored DNA damage detection assay; TS, transcribed strand.

*$p < 0.05$; **$p < 0.01$.

of each oligomer, and 1.5/100 of total extended product pool. Each sample was assayed in three independent experiments. The relative amount of undamaged template was derived using the $2^{-\Delta\Delta CT}$ method.[22] To assess the amount of induced damage per strand, data were normalized to the respective strand in the DNA obtained from control cells. Lesion frequency was estimated using the Poisson equation $n = -\ln(o)$.[15]

Statistical Analysis

Data collection and analysis occurred between 2012 and 2014. Data were compiled in Microsoft Excel files and statistical analyses were performed using SAS/STAT, version 9.1. Independent means were compared using unpaired Student's *t*-tests with corrected df, when appropriate, for inequality of variance. The authors considered $p < 0.05$ to be statistically significant.

Results

Primer-Anchored DNA Damage Detection Assay Detected a Dose-Dependent Increase in DNA Damage in Cells Exposed to H_2O_2

Exposure to cigarette smoke significantly increases the cellular content of pro-oxidants such as superoxide (O_2^-); H_2O_2; and hydroxyl (OH^-) radicals, all of which can
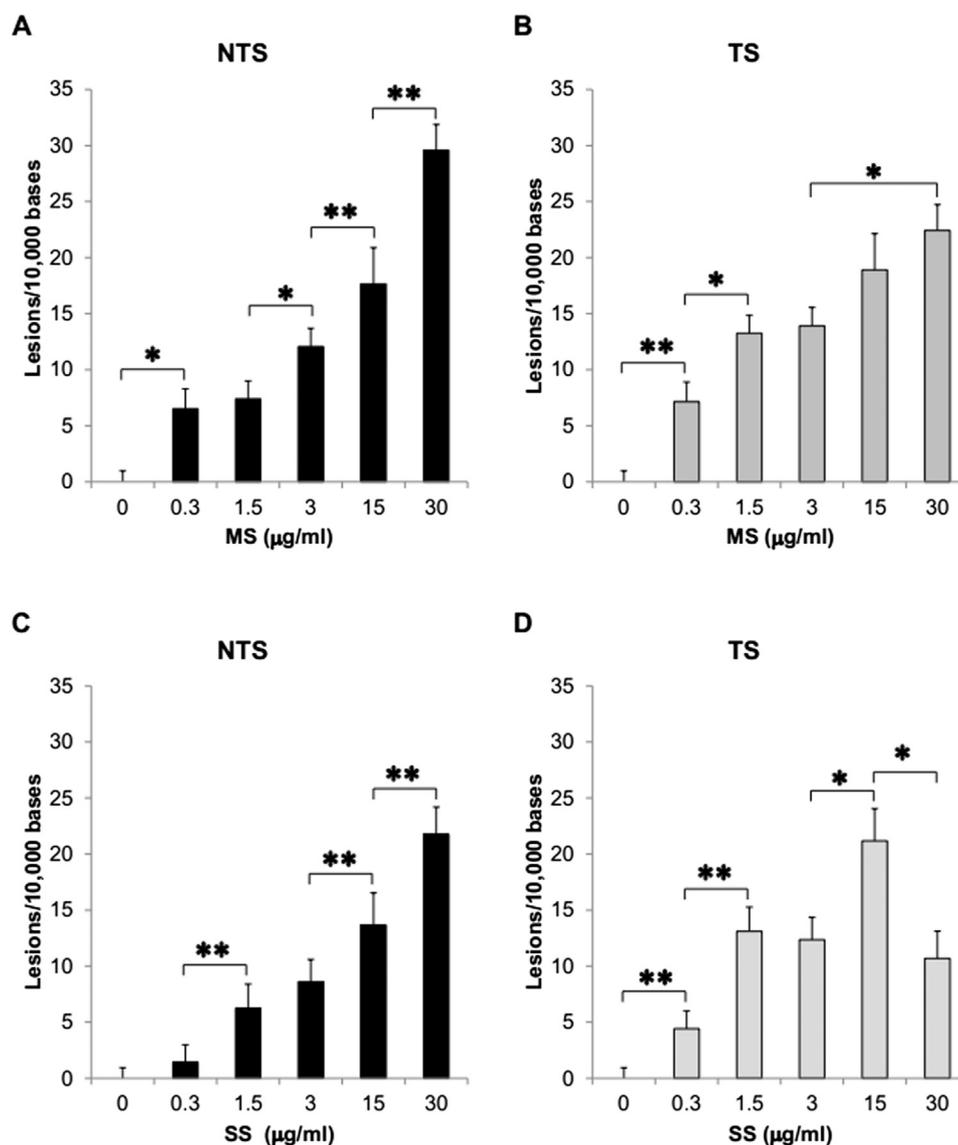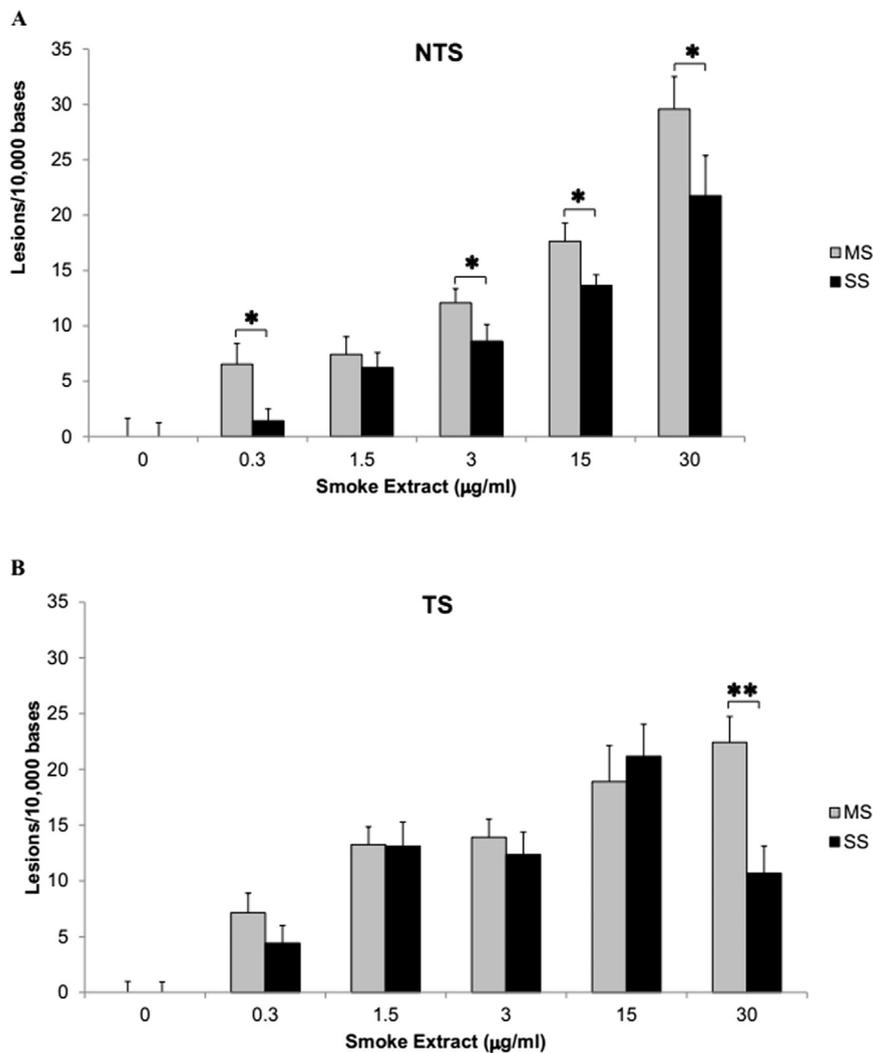
Figure 2. MS and SS smoke induce a dose-dependent increase in DNA damage.
Note: Increase in DNA damage was observed in NTS (A) and TS (B) of *TP53* in the cells exposed to increasing doses of MS smoke for 1 hour. SS smoke also induced an increase in DNA damage in both NTS (C) and TS (D) of *TP53*. Damage was quantified by q-PADDA. Data are represented as mean $\pm$ SEM. MS, mainstream smoke; NTS, non-transcribed strand; PADDA, primer-anchored DNA damage detection assay; SS, sidestream smoke; TS, transcribed strand.
*$p < 0.05$; **$p < 0.01$.

induce oxidative DNA damage.[23] The authors have previously shown that PADDA detects several types of oxidative DNA damage in vitro and in yeast cells.[15] To determine the ability of PADDA to quantify the in vivo levels of induced oxidative DNA damage in human cells, SCC-1 cells were exposed to increasing doses of H_2O_2 for 1 hour, and DNA damage was measured in the TS and NTS of *TP53*. A significant increase in DNA damage ($p < 0.01$) in *TP53* was observed in both DNA strands for all the doses of H_2O_2 tested when compared to the control (Figure 1). A dose-dependent increase in DNA damage was observed in the NTS of *TP53* (Figure 1A). Interestingly, the increase in DNA damage observed in

the TS of *TP53* after H_2O_2 exposure (Figure 1B) was linearly dose dependent only for the lower tested doses (1 and 10 μM). The authors have previously reported that, in yeast cells, oxidative damage is repaired preferentially in the TS than in the NTS.[15] To evaluate whether this also occurs in human cells, SCC-1 cells were exposed to different doses of H_2O_2 and allowed to recover for 3 and 24 hours. After recovery, DNA damage in the TS was significantly lower ($p < 0.001$) than in the NTS (Figure 1C). This was consistent with the authors' previous report[15] and showed that, in human cells, induced oxidative DNA damage was repaired preferentially and more quickly in the TS than in the NTS.

A

B

Figure 3. MS and SS smoke induce distinct levels of DNA damage.
Note: Quantification of DNA damage by PADDA damage in NTS (A) and TS (B) of *TP53* after 1 hour exposure to escalating doses of MS and SS smoke extracts. Data are represented as mean ± SEM. MS, mainstream smoke; NTS, non-transcribed strand; PADDA, primer-anchored DNA damage detection assay; SS, sidestream smoke; TS, transcribed strand.
*p < 0.05; **p < 0.01.

Primer-Anchored DNA Damage Detection Assay Detected a Dose-Dependent Increase in DNA Damage in Cells Exposed to Mainstream and Sidestream Smoke

MS and SS smoke induce a wide range of DNA lesions, which, if overwhelming for the cells, can cause cell death. Even so, owing to technical limitations, the effects of MS and SS smoke on human cells are most frequently shown as cytotoxic endpoints or partial genotoxic effects, such as the presence of induced single-strand breaks or mutations.[24] To determine the sensitivity of PADDA for the quantification of the in vivo levels of tobacco-induced DNA damage in human cells, SCC-1 cells were exposed to increasing doses of MS and SS smoke extracts and DNA

damage was quantified in *TP53* after 1 hour. To confirm that smoke extract–induced cytotoxicity is not a confounding variable in this study, cell viability was measured. No significant cell death was observed for any of the tested doses of MS or SS smoke (Appendix Figure 1). A significant increase in DNA damage ($p < 0.01$) was measured by PADDA in both *TP53* strands after exposure to all doses of MS and SS smoke, including the lowest dose (0.3 µg/mL), which roughly represents the tar concentration of 0.1 cigarettes/5 L or 0.00002 cigarettes/mL (Figure 2). Also, a dose-dependent increase in DNA damage in the NTS of *TP53* in cells exposed to SS and MS smoke was observed (Figure 2A and 2C). Smoke-induced DNA damage was generally higher in the TS than in the NTS (Figure 2), although this difference only attained significance ($p = 0.01$) at 1.5 µg/mL of SS and MS smoke exposure. A dose-dependent increase in DNA damage in the TS of *TP53* after exposure to MS or SS smoke extracts was not observed (Figure 2B and 2D). This observation most likely reflects the early activation of repair mechanisms, such as basic excision repair[15] and nucleotide excision repair,[25] which selectively remove DNA lesions from the TS of actively transcribed genes.

Mainstream and Sidestream Smoke Induced Distinctive, Differentially Repaired DNA Lesions

It is well accepted that both MS and SS smoke cause genotoxicity.[2] However, it is unclear whether the levels of genotoxicity induced by MS and SS smoke vary significantly.[7,26,27] Additionally, it is unknown whether MS and SS smoke effects differ between the DNA strands. Therefore, the authors compared the levels of DNA damage induced by MS and SS after 1 hour of exposure in both DNA strands (Figure 3). MS smoke induced
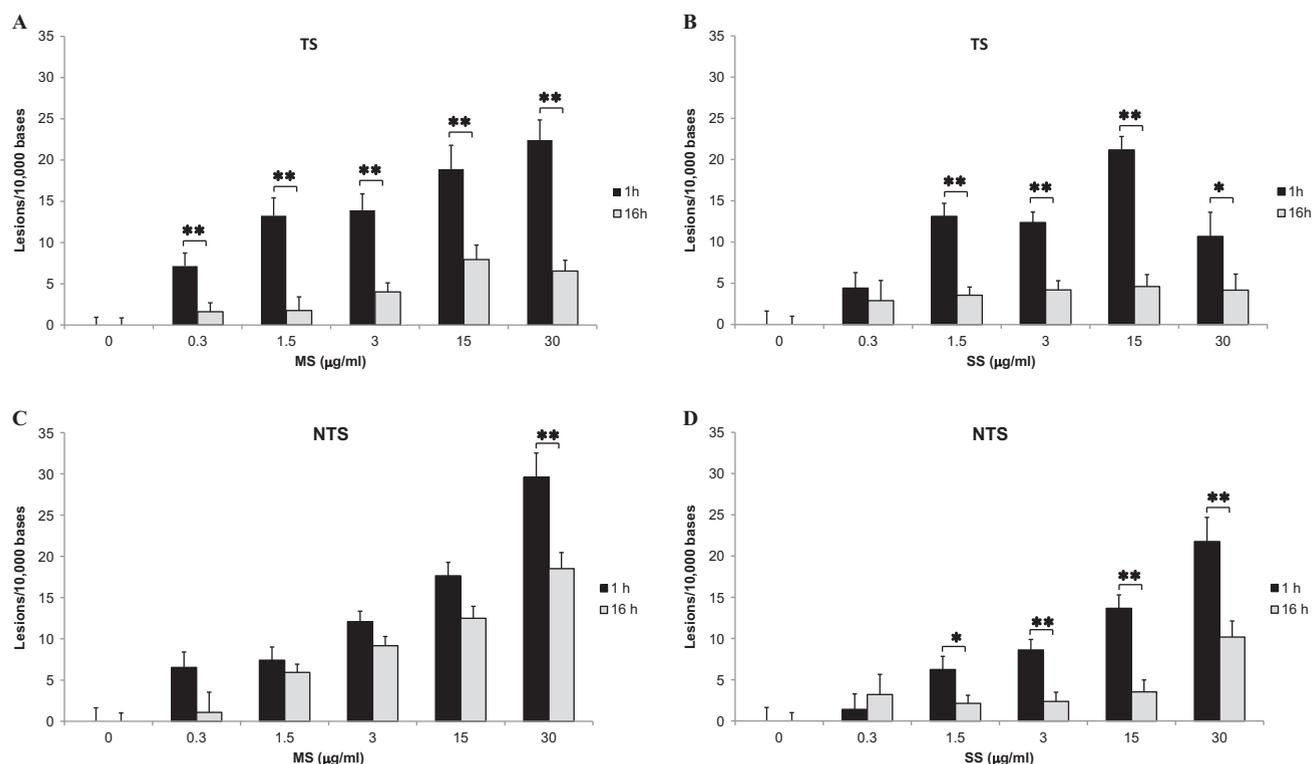
Figure 4. MS and SS smoke induce distinct DNA lesions that are repaired differentially.

Note: DNA damage induced by MS smoke (A and C) is repaired more slowly than DNA damage induced by SS smoke (B and D) as determined by q-PADDA. Data are represented as mean ± SEM.

MS, mainstream smoke; NTS, non-transcribed strand; PADDA, primer-anchored DNA damage detection assay; SS, sidestream smoke; TS, transcribed strand.

*$p < 0.05$; **$p < 0.01$.

significantly higher ($p < 0.05$) levels of DNA damage than SS smoke in the *TP53* NTS (Figure 3A). Interestingly, similar levels of DNA damage were observed in the TS at 1 hour for both MS and SS exposure at all doses except the highest, where the DNA damage was significantly higher for MS exposure (Figure 3B). These data showed that MS and SS extracts induced distinct levels of DNA damage and suggested they each induced distinctive DNA lesions. To investigate whether DNA damage induced by MS and SS smoke was repaired differentially, the damage induced by smoke extracts was quantified 16 hours after the initial exposure. A significant reduction ($p < 0.001$) in the levels of DNA damage in the TS of *TP53* was observed 16 hours after the initial exposure for both MS and SS (Figure 4A and 4B). Despite significant repair, the levels of DNA damage in the TS were persistently higher than control for all levels of MS and SS exposure (Figure 4A and 4B). Within the same time period, no significant repair of DNA damage induced by MS smoke in the NTS of the *TP53* gene was observed (Figure 4C) except for the highest dose of MS smoke ($p < 0.001$). By contrast, a significant decrease in DNA damage in the NTS of *TP53* at 16 hours was observed for

all but the lowest dose of SS smoke (Figure 4D). These data were consistent with the TS being repaired quicker than the NTS. Most importantly, these data showed that DNA damage induced by SS smoke in the NTS was repaired more rapidly than DNA damage induced by MS. Overall, these data showed that MS and SS smoke induced distinct DNA lesions that were differentially repaired.

Mainstream and Sidestream Smoke Induced Significantly Different Levels of DNA Strand Breaks

Tobacco smoke has been shown to induce DSBs.[28] To determine whether MS and SS smoke differed in their ability to induce DSBs, SCC-1 cells exposed to increasing doses of smoke extracts for 1 hour were processed for immunofluorescence staining of γ-H2AX. The number of γ-H2AX foci per cell is an indirect measure of the number of DSBs in a cell.[29] There was a significant increase ($p < 0.05$) in the number of foci induced by all doses of MS and SS smoke; however, there was no significant increase in DSBs among increasing doses of genotoxic chemicals (Figure 5A). Overall, the levels of DSBs observed were higher with MS than with SS smoke (Figure 5).
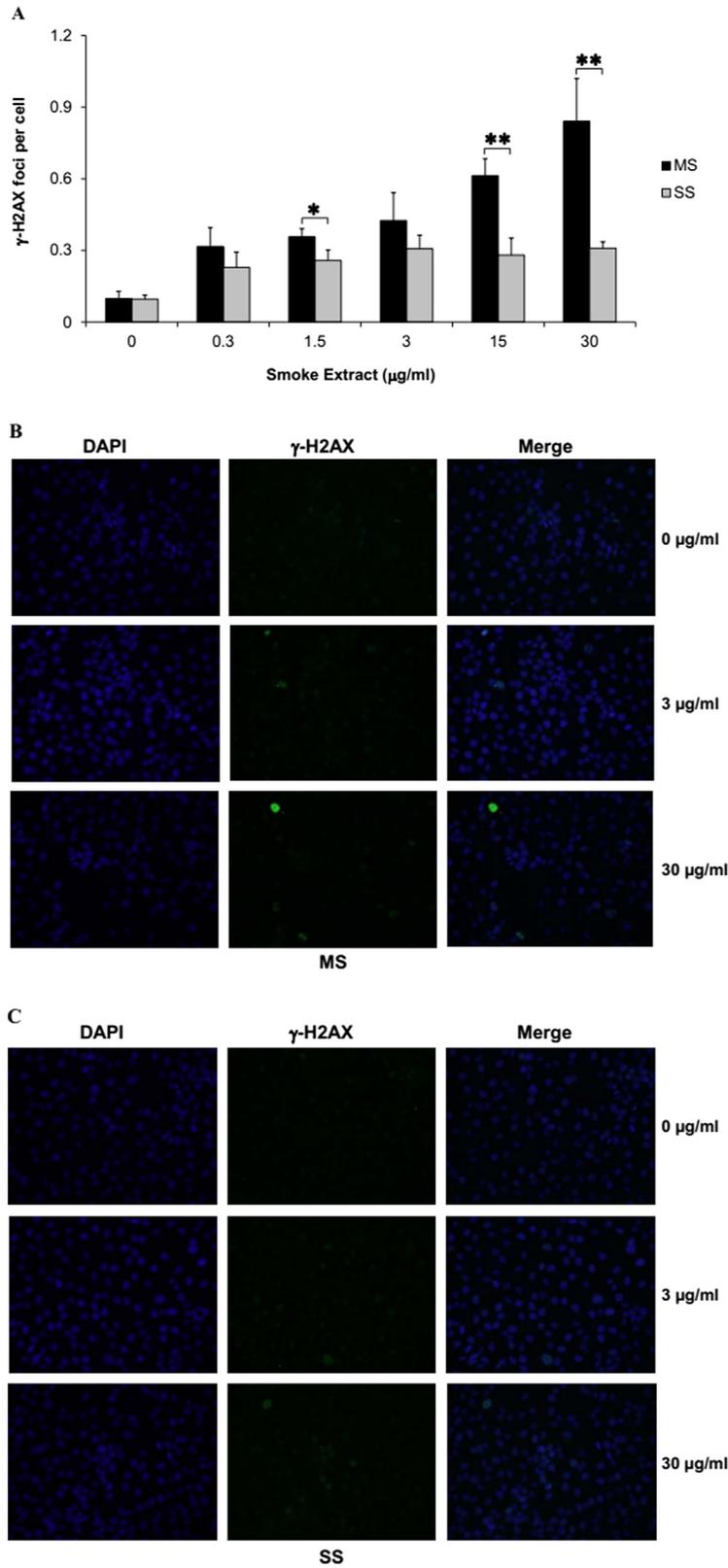
Figure 5. MS induces higher levels of DSBs in human cells than SS smoke, as determined by immunofluorescence staining for phosphorylated histone γ-H2AX.

Note: (A) All doses of MS and SS smoke induced significantly higher levels of DSBs than control. Data are represented as mean ± SD. *$p < 0.05$, **$p < 0.01$. (B) and (C) show immunofluorescence images of the cells exposed to different doses of MS and SS smoke, respectively, demonstrating dramatic accumulation of phosphorylated histone γ-H2AX. Magnification, 400X.

MS, mainstream smoke; SS, sidestream smoke.

Although significant differences ($p < 0.01$) in the number of DSBs were observed between low (0.3–1.5 μg/mL) and high (15–30 μg/mL) doses of MS smoke, no such difference was observed for SS smoke. These results documented that MS and SS smoke induced different levels of DSBs and showed that PADDA was significantly more sensitive than γ-H2AX to documenting DNA damage induced by smoke extracts.

Discussion

Tobacco smoke is a major public health problem. Biomonitoring of exposure indicates that almost one half of nonsmokers and more than 60% of young children are exposed to secondhand cigarette smoke.[4] MS and SS smoke have qualitatively similar chemical compositions but the respective quantities of individual smoke constituents can be quite different.[4,30] However, the impact of these differences in smoke-induced cell cytotoxicity and genotoxicity is poorly understood. DNA damage has detrimental effects on human health. Nevertheless, little progress has been made in the development of assays that can quantify in vivo DNA damage. The currently available and most reliable strategies to detect induced DNA damage are not practically feasible for population screening.[16,17,31] In this study, using a highly sensitive DNA damage detection assay and a wide range of smoke extract doses (from approximately one tenth of a cigarette to ten cigarettes/5 L), the authors show that both MS and SS smoke induce a dose-dependent increase in DNA damage. A main difference between this study and previously published studies is the relatively low doses of smoke extracts used; most studies used smoke extracts corresponding to doses higher than three cigarettes in 5 L.[24] Lower doses were selected in this study because doses that are not cytotoxic are particularly relevant for the purpose of studying cancer risk. Remarkably, this study documents that even very low doses of MS and SS smoke induce significant amounts of DNA damage detectable by PADDA. These data suggest that PADDA can quantify DNA damage induced by very low levels of secondhand smoke.

Of potential clinical significance, although MS causes more DNA damage than SS smoke in the NTS, there is no difference between the amount of DNA damage induced by MS and SS smoke in the TS. Furthermore, the DNA damage induced by MS is repaired with slower kinetics than damage induced by SS smoke. By performing immunofluorescence of γ-H2AX, the authors document that MS induces significantly higher DSBs than SS levels of smoke. These findings are consistent with previous reports that MS smoke causes breaks and alteration on chromatin integrity, whereas SS smoke causes only alterations in chromatin structural integrity in sperm DNA,[32] and might contribute for the slower kinetics of damage repair

observed for MS smoke. Taken together, these data demonstrate that MS and SS smoke each lead to distinct types and frequencies of DNA damage. Most importantly, this study shows for the first time that the damage induced by MS and SS smoke is repaired with different kinetics and in a strand-specific manner.

Data from this study also suggest that at least some of the apparently contradictory data in the field could be explained by the dose of the smoke extract, sensitivity of the method, and type of genotoxicity analyzed in each study. For example, the sensitivity of the comet assay used to detect DNA strand breaks varies significantly between laboratories.[16,31] Therefore, although some studies have shown that the comet assay is able to detect DNA damage induced by short exposure to cigarette smoking,[32] others have failed to document the same.[33] The fact that DNA damage induced by SS smoke is repaired quicker than damage induced by MS smoke could also explain the previously reported lack of a dose–response relationship in mutation induction by SS smoke.[24] Finally, the observation that DNA damage induced by very low doses of SS smoke persists on both DNA strands for at least 16 hours suggests that DNA repair is not activated for this level of damage or is not able to repair the damage. Further studies are needed to clarify this observation, but the existence of a potential threshold before which DNA repair is not activated pinpoints another probable risk of low exposure to secondhand smoke.

Technical limitations and analytical complexity have hampered identification of predictive markers of susceptibility to tobacco exposure or tobacco-associated disease risk. Here, the authors show that PADDA quantifies in vivo DNA damage induced by very low levels of exposure to MS and SS smoke and detects a dose-dependent increase in DNA damage following exposure to smoke extracts, a crucial test for PADDA's accuracy and a prerequisite for its use in biomonitoring. These observations are clinically important and reinforce the need for additional tobacco regulation to minimize the use of tobacco products and reduce the exposure of nonsmokers to secondhand smoke. Application of this assay to large series of smokers, former smokers, and people exposed to secondhand smoke has major potential to establish biomarkers of susceptibility to tobacco-induced disease and provide a model to guide preventive and diagnostic strategies tailored to individual risk level.

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Appendix

Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.1016/j.amepre.2014.08.017.