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DNA Damage, DNA Repair and Carcinogenicity: Tobacco Smoke versus Electronic Cigarette Aerosol

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Abstract

The allure of tobacco smoking is linked to the instant gratification provided by inhaled nicotine. Unfortunately, tobacco curing and burning generates many mutagens including more than 70 carcinogens. There are two types of mutagens and carcinogens in tobacco smoke (TS): direct DNA damaging carcinogens and procarcinogens, which require metabolic activation to become

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DNA damaging. Recent studies provide three new insights on TS-induced DNA damage. First, two major types of TS DNA damage are induced by direct carcinogen aldehydes, cyclic-1,N^2-hydroxy-deoxyguanosine (γ- OH-PdG) and α-methyl-1,N^2- γ -OH-PdG, rather than by the procarcinogens, polycyclic aromatic amines and aromatic amines. Second, TS reduces DNA repair proteins and activity levels. TS aldehydes also prevent procarcinogen activation. Based on these findings, we propose that aldehydes are major sources of TS induced DNA damage and a driving force for carcinogenesis. E-cigarettes (E-cigs) are designed to deliver nicotine in an aerosol state, without burning tobacco. E-cigarette aerosols (ECAs) contain nicotine, propylene glycol and vegetable glycerin. ECAs induce O^6-methyl-deoxyguanosines (O^6-medG) and cyclic γ-hydroxy-1,N^2--propano-dG (γ -OH-PdG) in mouse lung, heart and bladder tissues and causes a reduction of DNA repair proteins and activity in lungs. Nicotine and nicotine-derived nitrosamine ketone (NNK) induce the same types of DNA adducts and cause DNA repair inhibition in human cells. After long-term exposure, ECAs induce lung adenocarcinoma and bladder urothelial hyperplasia in mice. We propose that E-cig nicotine can be nitrosated in mouse and human cells becoming nitrosamines, thereby causing two carcinogenic effects, induction of DNA damage and inhibition of DNA repair, and that ECA is carcinogenic in mice. Thus, this article reviews the newest literature on DNA adducts and DNA repair inhibition induced by nicotine and ECAs in mice and cultured human cells, and provides insights into ECA carcinogenicity in mice.

Keywords

DNA damage; DNA repair; tobacco smoke; E-cigarette; carcinogenesis

Introduction

Tobacco smoke (TS) delivers the stimulant nicotine, which can reach the brain within seconds and provide instant gratification (1). Unfortunately, TS contains carcinogenic compounds and TS has long been recognized as a human carcinogen (2– 4). TS is responsible for roughly one-third of human cancer incidence (5–7). Lung cancer is the most prevalent TS induced cancer; 85% of lung cancer is TS related (8, 9). Annually there are 1.8 million lung cancer deaths worldwide, with more than 130,000 in the US alone (7, 10). The lung cancer risk of life-long smokers is 23 times higher than non-smokers (7, 11, 12). The major cancer etiological agents in TS come from tobacco curing and burning. More than 7000 chemicals resulting from the incomplete combustion products of tobacco leaves, and among these over 70 chemicals have been proven to be potently carcinogenic in animal models (13–17).

TS compositions have been studied since the early 1950s (14). There has been extensive study on the effects of individual TS compounds. As well, total TS induced DNA damage and TS effects on cellular functions and tumorigenicity have been widely studied (14). Numerous excellent reviews on these topics have been published (2, 11, 16), and the topics of identifying TS carcinogens and their carcinogenic mechanisms have been well-covered. Therefore, this article does not address the known chemical nature of carcinogenic TS agents, such as polycyclic aromatic hydrocarbons (PAHs) and heterocyclic aromatic amines (AAs), DNA damage induced by these agents, or their carcinogenicity. Rather, this article

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focuses on the puzzling questions arising from recent results on DNA damage induced by TS in the lung tissues of mice and humans (18–21).

Nicotine is a major component of TS. It is known to be addictive, but it is believed to be non-carcinogenic (2, 12, 22, 23). The lack of strong evidence that nicotine is carcinogenic, as opposed to the 70 known carcinogens in TS, is probably one of the leading reasons, aside from profit, behind the development of stimulant nicotine delivery devices that do not involve burning tobacco. The most notable devices include nicotine patches, nicotine gum, and electronic-cigarettes (E-cigs) (24–26).

E-cigs are designed to deliver nicotine in an aerosol state. Nicotine is dissolved in relatively harmless organic solvents [propylene glycol (PG) and vegetable glycerin (VG)] and aerosolized by controlled electric heating (~250 oC) (27, 28). E-cigs do not use tobacco leaves, do not have curing byproducts, do not contain tobacco specific nitrosamines (TSNAs), and the process of generating E-cig aerosols (ECAs) (or vapor) does not involve burning, Therefore ECAs do not contain incomplete combustion byproducts (29). E-cig aerosol (ECA) contains only aerosolized nicotine and organic solvents, therefore, E-cigs are promoted as delivering a TS ‘high’ without its negative effects (30). The population of E-cig users is rising rapidly. Currently there are more than 11 million E-cig users in the US alone, among them 4 million are middle and high school students (31). The health effects of ECAs, particularly their potential carcinogenicity, deserves careful scrutiny. This article will focus on reviewing the current understanding of DNA damage, DNA repair inhibition and carcinogenic effect induced by nicotine and ECA in mice and in cultured human cells, as well as ECA carcinogenicity in mice.

Although many E-cigs on the market contain flavors besides nicotine and the PG and VG solvents, study on the effect of flavors in the ECA on the DNA damage at molecular level remains lacking (32), and the effect of flavors in E-cig on the cytotoxicity and endothelial dysfunction remain controversial (33, 34). Therefore, the topic of the role flavor on ECA chemistry and biological effect is beyond the scope of this review.

Paradox of tobacco smoke carcinogenicity in humans and mice

It is well established that only 10–15% of life-time heavy smokers go on to develop lung cancer and, on average, it takes two decades for smokers to develop lung cancer (35–37). This low lung cancer incidence among heavy smokers is in stark contrast to many occupational exposure related cancers with high incidence, such as the 40–50% bladder cancer rate among life-time printing shop workers who were exposed to aminobiphenyl (38), and mesothelioma in 23–90% of asbestos workers (39). In addition to the puzzling results of low incidence of cancer among heavy smokers are the findings that TS is not a strong carcinogen in animal models (40, 41). In fact, TS has been classified as a weak carcinogen because it does not significantly enhance spontaneous lung cancer incidence in mouse models (40, 41). Intriguingly, many TS compounds, such as benzo(a)pyrene (BP), and TSNAs, induce mutagenesis and carcinogenesis more effectively than TS in animal models (12, 42–44). To date, the underlying reasons why despite containing 70 carcinogens, TS as a whole is not a strong cancer-causing agent in animal models remain unclear, despite many attempts to address this question (12, 17, 45–48).

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TS induces DNA damage in humans and in mice

It is well established that TS contains more than 70 proven carcinogens (13–16).The relatively low lung cancer incidence (10–15%) among life-time tobacco smokers based on human epidemiological study results and from animal experiments (40, 41,49–52) raises three possible scenarios on TS carcinogenesis: first, the carcinogenicity of TS is not additive from the 70 or so carcinogens in TS; second, the bioavailable carcinogens in TS are low; and third, TS components may interfere with the metabolic activation of procarcinogens. To understand the reasons behind the low carcinogenicity of TS and to distinguish the hypothetic aforementioned scenarios, identification of DNA damage induced by TS *in vivo* is essential. This is a critical way to fully understand the mechanism of TS carcinogenesis and the role of individual TS carcinogens.

TS contains numerous chemicals such as aldehydes, which have been shown to directly bond with naked DNA and cellular genomic DNA to form different isomeric forms of DNA adducts (19, 53, 54). TS also contains numerous compounds, such as polycyclic aromatic hydrocarbons (PAHs), heterocyclic aromatic amines (AAs), nitrosamines and benzene, which via metabolic activation, can be transformed into derivatives that can covalently bond with naked DNA and cellular genomic DNA to form different isomeric forms of DNA adducts (38, 55, 56). Recently, we found that aflatoxin B1 (AFB1) following metabolism can induce oxidative stress and lipid peroxidation, consequently inducing abundant lipid peroxidation byproducts, aldehydes, that induce more DNA adducts than those induced by metabolically activated AFB1 (57). Although AFB1 is rare in TS, many enzymes that metabolize it, such as, CYP1A1, CYP1A2, CYP2A6, and CYP3A4, also have the capability to metabolize many TS compounds and lead to the same aldehyde-dependent outcome as AFB1 (55). Therefore, it is reasonable to assume that many TS components can induce secondary toxicants, which can form oxidative DNA damage and aldehyde-DNA adducts with cellular genomic DNA. On the other hand, it has been found that TS aldehydes, such as acrolein, can inhibit TS indirect DNA damaging agents, such as benzo(a)pyrene (BP) and nicotine-derived nitrosamine ketone (NNK) to induce DNA damage, presumably via their covalently modification of p450 enzymes (20).

The fact that TS contains numerous carcinogens, which by themselves can cause DNA damage, mutations and tumorigenesis, yet TS, as a whole, is a weak mutagen and carcinogen in animal models (28,29), raises the possibility that TS-induced DNA damage is not a sum of potential DNA damage from all TS DNA damaging agents. If this is true, then determining the major DNA damage induced by TS, and not focusing on the minute novel DNA adducts, is crucial for understanding the mechanisms of TS carcinogenesis.

With this in mind this article focuses on reviewing the literature for TS that: 1) identifies the more abundant TS-induced DNA adducts rather than dwelling on finding new and minor DNA adducts; and 2) explores the potential mechanisms of the TS DNA adduct induction and their role in carcinogenesis.

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Quantify the total DNA adducts induced by TS

At least four important issues should be considered when interpreting the results from measuring TS-induced DNA damage. First, there are differences in p450 expression in different organs which determines the amount of procarcinogens (such as PAHs TSNAs, and AAs) that become DNA damaging agents (12, 58–60). Second, there are potential interactions among different TS components, which may attenuate DNA adduct formation compared to that observed in studies of individual TS components.Third, TS components may prevent the activation of procarcinogens becoming DNA binding agents (20, 61). Fourth, many DNA adducts can be formed in TS exposed animals and humans, which result from secondary toxicants rather than from TS components directly (57). Therefore, one cannot assume that TS induced DNA adducts are a sum of all DNA damaging agents in TS. To assess TS induced DNA damage *in vivo*, it is imperative to measure DNA adducts directly from different tissues of animals and humans following exposures to the mixture that is TS.

The analytical method for detecting TS-induced DNA adducts was originally developed by Randerath and his colleagues in the 1980's, and then improved in the 1990's. It involves ^{32}P labeling of adducted nucleotide monophosphates and two- dimensional thin layer chromatography/high performance liquid chromatography (2D- TLC/HPLC) separation of the adducted deoxyribonucleoside monophosphate (dNMP) from the unadducted ones. This method remains one of the best methods to detect the totality of the abundant DNA adducts induced by TS (62–67). This method uses various phosphodiesterase to digest DNA to dNMPs, then employs a kinase to label the dNMPs (including those that are adducted) with ^{32}P and nuclease P1 (NP1) to dephosphorylate the unadducted dNMPs. The ^{32}P-labeled and adducted dNMPs are then separated via 2D-TLC and form a so called diagonal radioactive zone (DRZ) (66, 67). The DRZ contains most, if not all, of the TS-induced DNA adducts including PAH-, alkyl-, AA- and aldehyde-dNMP adducts (62, 66, 67). The DNA adducts in the DRZ can be further separated by HPLC using elution conditions tailored based upon the nature of the DNA adducts (20, 68). The amount of DRZ formation has been shown to be related to the level of TS consumption (66). The quantification of DNA adducts is based on the assumption that ^{32}P labelling efficiency of DNA adducts versus deoxyguanosine monophosphate (dGMP) has a constant relationship (69, 70). It is foreseeable that this "constant relationship" could vary for different DNA adducts with different structures. Therefore, the calculation is not "one-size-fits-all" and trial and error are necessary steps for establishing the ^{32}P labelling efficiency of DNA adducts versus dGMP for better quantifications. This calculation is relative rather than absolute, and therefore the assay has been characterized as a "semiquantitative" method (71). Nonetheless, this method is ideal for detecting a broad range of TS induced DNA damage. Furthermore, this method requires less than a few μg of DNA and is able to detect DNA damage in the range of a few adducts per 10^{10} nucleotides (62). This method uses relatively mild conditions (neutral pH and low temperature) to digest DNA, therefore it is relatively non-destructive with respect to most DNA adducts.

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Identify and quantify the individual DNA adducts induced by TS

Two other powerful methods, besides the ^{32}P post-labeling 2D-TLC/HPLC method, have been used to identify individual DNA adducts induced by TS in biological samples, an immunochemical method and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (20, 68, 71). The immunochemical method uses primary antibody against specific DNA adducts to bind the DNA damage and then utilizes a secondary antibody labeled with a fluorescent dye to visualize the primary antibody-DNA damage complex. There are two ways to measure the fluorescent intensity of the antibody-DNA damage complex. In the ELISA protocol, the genomic DNAs that are fixed in individual wells are first reacted with the primary antibody. After unbound primary antibodies are washed away, a secondary antibody with a fluorescent dye is added. The unbound secondary antibodies are removed by washing and the fluorescence intensity of the complexes of DNA adducts bound to the primary and secondary antibodies are quantified by fluorescent spectrophotometry (72–75). In the slot blotting method, genomic DNAs are fixed onto a nitrocellulose membrane which is overlaid by the primary antibody followed by a reaction with the fluorescent secondary antibody. After washing away the excessive unbound antibodies, the fluorescence intensity of complexes of DNA adducts bound to the primary and secondary antibodies are quantified (20, 61, 76). This immunochemical method requires only a minute amount of DNA (<1 μg) to detect DNA adducts in the range of a few adducts per 10^8 nucleotides; it is a simple protocol that can be conducted in clinical settings. Although the fluorescent labeled antibody-DNA adduct complexes can be quantified using an ELISA method, this method requires relatively large amount of DNA compared to the membrane blotting method. One advantage of specific antibody-DNA adduct reaction methods is that they can also be used *in situ* at the cytological level to locate and visualize DNA adducts inside cells (77, 78). This unique capability of the immunochemical method has been applied to view the dynamics of the DNA damage process *in vivo* in real time (79–81). For example, this method has been applied to discover the dynamics of UV-induced photoproduct formation and repair in different cellular compartments (81). It should be noted, however, that certain DNA adduct specific antibodies are more specific and efficient in recognizing DNA adducts in single-stranded DNA, which can be generated by the denaturation of double-stranded DNA through heating. Therefore, this method is ideal for quantifying heat-stable DNA adducts in human samples (76, 82). It should be noted that very often the epitope that the antibodies recognize can be shared by different DNA adducts. For example, cyclobutane pyrimidine dimer (CPD) antibodies can recognize all CPD structures formed at -TT-, -TC-, -CT- and -CC- sequences (83, 84), and the monoclonal antibodies against acrolein (Acr)-DNA adducts can recognize all cyclic 1,N^2-propano-dG (PdG) with different length side chains (82).

For targeted DNA adduct analysis, the LC-MS/MS method provides unmatched specificity in identifying DNA adduct structures (71). This method uses non-radio- isotopic labeling and has the potential to be at least as sensitive as the ^{32}P post-labeling method, if not more sensitive. Currently, it has been reported that the most improved LC-MS/MS method is able to detect DNA adducts at the level of 10-^{11}, equivalent to the detection of one DNA adduct in 100 human cells (71). This method is widely used by many chemistry laboratories for the identification of DNA adduct structures, particularly for discovery of

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novel and minor DNA adducts. The LC-MS/MS method requires DNA to be digested into mononucleotide form by phosphodiesterase, the use of non-radioisotopes to label DNA adducts, followed by electro-spray HPLC and mass spectrometry (71). The super-sensitivity of adduct detection by this method requires a relatively large amount of DNA (>10 μg). LC-MS/MS has been useful for identifying the chemical nature of abundant DNA adducts and minor DNA adducts in TS (85–87). Currently, however, the information based on this method for finding the relationship between TS induced DNA damage and tumorigenesis is limited. Nevertheless, with greatly improved data analysis ability, coupled with new state-of-the-art powerful LC-MS/MS instruments, a new field of "tobacco smoke-induced adductomics" is emerging (86, 87).

TS induced DNA damage in human lung tissues

The formation and compositions of adducts identified in the DRZ using Randerath protocols on lung tissues of tobacco smokers have been extensively studied and assessed by many excellent review articles (88, 89). These studies conclude that the DRZ contains a mixture of DNA adducts induced by PAHs, AAs, nitrosamines, benzene, aldehydes and perhaps other reactive components from TS (18). Formation of DRZ adducts has been shown to correlate strongly with TS exposure (66). Since PAHs and AAs are powerful mutagens and are carcinogenic, the DRZ has been viewed for many years as a collection of DNA adducts, induced by PAHs and AAs (18). Consequently, PAHs and AAs have been believed to be the major TS carcinogens (18, 20). This belief was recently challenged by findings from Gupta's laboratory, specifically that the two most abundant and characteristic adducts in the TS-induced DRZ are not the result of PAHs and AAs, as is commonly believed. Instead they were found to be induced by aldehydes (18). Gupta et al.'s findings raise important questions. What are the major DNA adducts induced by TS that can lead to carcinogenesis? Is it possible that while the DNA adducts induced by PAHs and AAs are powerfully mutagenic and carcinogenic, the amounts of these DNA adducts in human lung are negligible due to the minute levels of these compounds in TS, and/or are these compounds not activated to become DNA damaging agents in human lung tissues? In this regard it is worth noting the results from Schoket's group (21) show that while the total bulky DNA adduct formation (total DNA adducts in the DRZ, detected by ^{32}P-postlabeling 2D TLC method) in lung tissues of smokers (n = 47) is higher than in nonsmokers (n = 38), the PAH-DNA adduct levels (2.6–6.2/10^8 nt), detected by polyclonal antibodies against BPDE-DNA, show no statistical difference between smokers and nonsmokers. These results are similar with the results from Tang's group (20) which show no statistical significance of the differences of the levels of BPDE-dG adducts (average 0.9–1.3/10^8 nt, detected by antibody against BPDE-DNA adduct, the same as Schoket) in lungs of smokers (n = 41) compared to nonsmokers (n =13).

To address these questions further, using the same ^{32}P post-labeling method with 2D-TLC separation, our laboratory has further separated the TS-induced DRZ adducts resulted from tobacco smokers' lung tissues by HPLC and found that Acr-dG and crotonaldehyde (Cro)-dG adducts are the two most abundant adducts in lung tissues of tobacco smokers (20). These results are consistent with previous reports that higher levels of aldehyde-DNA

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adducts were formed in buccal cells and sputum of tobacco smokers than non-smokers (20, 54).

Detection of tobacco smoke-induced DNA adducts – benzo(a)pyrene diol epoxide (BPDE)-dG adducts versus aldehyde-induced cyclic 1,N^2-propano-dG (PdG) adducts – by LC-MS/MS method.

While immunochemical and ^{32}P post-labeling 2D-TLC/HPLC can quantify adducted bases with great sensitivity and separation, both methods rely on comparison with standard materials for quantification and identification. Thus, both applications yield indirect measurements. It is well known that nonspecific agents which cross-react with presumably specific antibodies can occur, consequently introducing errors in immunochemical assay results. With the 2D-TLC/HPLC method, it is likely that different materials can have the same migration and elution profiles, therefore a single peak could contain more than one species of substance. The LC-MS/MS method avoids these potential sources of error; however, a relatively high quantity of sample DNA is needed for this method. For example, using 40 μg of genomic DNA, Hecht's laboratory was able to detect BPDE-dG adducts at the level of 1 BPDE-dG/10^{11} nucleotides in the lung tissues of nonsmokers and 3.1 BPDE-dG/10^{11} nucleotides in the lung tissues of smokers, which is equivalent to 1 and 3 BPDE-dG per 100 lung cells in lung tissues of non-smokers and smokers, respectively (71). It should be noted that this level of BPDE-dG in smokers' lungs is far lower than the level of 8-oxo-dG adducts and depurination sites that spontaneously occur at one to ten thousand per genome in normal human cells (90). It is difficult to imagine the impact that this low level of TS- induced BPDE-dG adducts would have in cells with a background of more than one thousand-fold higher levels of spontaneously occurring oxidative DNA damage and abasic sites.

Using both ^{32}P post-labeling/3D-TLC analysis and the immunochemical method, we found that there is no significant difference in BPDE-dG levels between lung tissues of smokers compared to non-smokers (20). Due to the strongly carcinogenic and mutagenic effect of BP in both cultured human cells and in animal models, BPDE-dG has long been accepted as a "poster child" for the DNA damage that induces lung carcinogenesis by TS (91–96). However, based on the conflicting results from different groups, along with the extremely low level of BPDE-dG detected by Hecht's group (71), the role of BPDE-dG in TS lung carcinogenesis needs to be carefully re-evaluated.

On the other hand, we found that the levels of both the Acr- and Cro-DNA adducts, γ -OH-PdG and α-methyl- γ -OH-PdG, in buccal cells, sputum and lung tissues of smokers are significantly higher than in non-smokers (20). We also found that PdG induces G to T and G to A mutations (Table 1), which are similar to the mutations found in the *TP53* gene in lung cancer. Furthermore, PdG distribution in the *TP53* gene is similar if not identical to the *TP53* mutational spectrum in lung cancer (97, 98) (Fig. 1). Based on these results, we have suggested that PdG adducts are the major cause for TS lung carcinogenesis (20). It should be noted that it has been found that Acr is carcinogenic in rodents (99). Acr has recently been classified as class 2 carcinogen by IARC (100). It has long been recognized

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that Cro can cause liver cancer in animal models (101), and Cro has also been classified as a group 2A carcinogen by IARC (100).

Measurement of cyclized propano-dG adducts can be problematic and many variables can affect the outcome. By adding glutathione to the solution for PdG adduct detection and using the sophisticated LC-MS/MS method, Hecht's laboratory found that, in general, the α-OH-PdG levels are higher than the γ -OH-PdG levels in human lung tissues and leukocytes, and that smokers and non-smokers have similar levels of both isoforms of PdG adducts (71, 102, 103). Two particular results reported by Hecht's group, the higher level of α-OH-PdG than γ -OH-PdG, and the lack of difference between smokers and non-smokers for these PdG adducts in lung tissues, are different from the findings of others (20, 54, 61, 68, 76, 97). It appears that differences in DNA isolation, DNA digestion, and the detection methods may contribute to these discrepancies. PdG is sensitive to ring-opening at high pH under reducing conditions (104, 105). Chung's group has addressed this important issue by a systematic analysis of the effect of different buffer systems used for genomic DNA isolation on the yield the two isomers of Acr-dG adducts and reported that 1) using phosphate buffer for genomic DNA isolation results in not only low yield of PdG but also disproportionally high ratio of α-OH-PdG to γ -OH-PdG; and 2) Tris buffer, buffer with amino acids, and cell lysates can eliminate these artifacts (106). Furthermore, we found that under normal physiological pH 7 conditions the major PdG adducts resulting from Acr-DNA interaction are γ -OH-PdG, with only a few percent of α-OH-PdG (105). Only under high pH conditions was more α-OH-PdG produced than γ -OH-PdG (105). It should be noted that TS contains relatively large quantity Acr which has been shown to induce γ -OH-PdG with a minor amount of α-OH-PdG in cultured human cells (76, 97, 105).

In summary, there are two conflicting results regarding the determination of the type and quantity of DNA adducts in lung tissues of tobacco smokers versus non-smokers. Using the immunochemical and ^{32}P post-labeling 2D-TLC/HPLC methods, the major DNA adducts found in the lung tissues of smokers are γ -OH-PdG and α-methyl- γ - OH-PdG in the range of a few adducts per 107 nucleotides (20, 54, 61, 68, 76, 97). Very low levels of αγ-OH-PdG were detected in lung tissues of smokers and non-smokers (20). There was no difference in the levels of BPDE-dG adduct in lung tissues of smokers versus nonsmokers (20). In contrast, using succinate buffer in the presence of glutathione for DNA isolation and LC-MS/MS analysis method, Hecht's group found similar levels of α-OH-PdG and γ -OH-PdG at a few adducts per 10^8 nucleotide range in human lung tissues, with no difference in the level of α- and γ -OH-PdG adducts between smoker and non-smokers (71, 102, 103).

Mainstream TS versus side-stream TS in DNA adduct induction in mice

Very few experiments have been conducted to date on determining the levels of DNA adducts and their chemical nature induced by real time TS exposure in animal models. This may be due to the mechanical and physiological difficulties of "making" animals smoke. Instead, the "tar" collection of "particulate matter" (PM) from tobacco smoke has been widely used as a surrogate for exposure to TS for assessing TS mutagenicity, tumorigenicity and other biological effects in animals (66, 107–111). It has been shown that when topically

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applied to mouse skin, TS-derived tar induces DRZ adducts in skin and lung tissues (69, 107–111).

When a smoker inhales a lit cigarette two types of cigarette smoke will be generated – mainstream tobacco smoke (MTS) and side-stream tobacco smoke (SSS). SSS results from passive burning of the lit cigarette. The temperature of SSS can be up to 400°C. When a smoker inhales from a lit cigarette, a stream of oxygen passes through the burning cigarette, which elevates the burning temperature up to 400–900°C. While the chemical compositions of MTS and SSS are similar, the ratio of components in each varies significantly (11, 112, 113). For example, SSS is much richer in aldehydes and BP than MTS (114, 115). It has been suggested that in a pound-by- pound comparison, SSS is much more toxic than MTS (114, 116). Epidemiological and animal studies also suggest that second hand smoke is more carcinogenic than MTS (117, 118). These results raise the possibility that SSS and MTS may have different efficiencies in DNA damage induction. This question was recently addressed in mice by determining the DNA damage induced by MTS and SSS using a whole-body exposure approach in mice (19, 20).

We found that SSS induces γ -OH-PdG adducts in the lung and bladder tissues in mice after 16 weeks of chronic exposure (19). Interestingly, under the same experimental conditions, no significant DNA damage was found in different organs of mice exposed to SSS for 8 weeks (19). In contrast, both γ -OH-PdG and α -methyl- γ -OH- PdG adducts were detected in lung tissues of mice exposed to MTS after 12 weeks of exposure (20). However, only γ -OH-PdG adducts, not α -methyl- γ -OH-PdG adducts, were detected in bladder tissues (20). It has been established that γ -OH-PdG is formed by Acr-DNA interaction and that α -methyl- γ -OH-PdG is formed by Cro-DNA interaction (54, 119, 120). Recently, it has been reported that acetaldehyde, via dimerization producing crotonaldehyde, can induce α -methyl- γ -OH-PdG (121). Acetaldehyde is the most abundant aldehyde in TS (2, 11, 16, 122–124). These results raise the possibility that the TS acetaldehyde in the blood stream may be further metabolized into acetates, in which case acetaldehyde might not be excreted in the urine. The level of Cro is one tenth that of Acr in TS; the low concentration of Cro in TS, and lack of validation of acetaldehyde in urine, may explain why only γ -OH-PdG was detected in bladder tissues of MTS exposed mice (2, 11, 16, 20, 60, 68). On the other hand, acetaldehydes and Cro in TS may directly damage lung cells to induce α -methyl- γ -OH-PdG. This scenario may account for the formation of two different PdG in the lungs of MTS exposed mice (20).The boiling point of acetaldehyde is 16 °C, which is lower than ambient temperature (20, 125). Therefore, the concentration of acetaldehyde may be greatly reduced in the process of collecting of SSS. Hence, SSS does not induce α -methyl- γ -OH-PdG in the lung tissues of mice. Consistent with this explanation is the observation that both types of PdG are found in lung tissues of human smokers and that the level of α -methyl- γ -OH- PdG is higher than γ -OH-PdG (20).

It should be noted that the levels of the BPDE-dG adduct in different organs including lungs show no significant difference between TS (MTS and SSS) exposed and control mice (one BPDE per 10^7 nt) (20). These results are consistent with the findings that there is no significant difference of the level of BPDE-dG adduct in lung tissues of tobacco smokers and non-smokers (20). BP is a potent carcinogen, and it has been found that BPDE, the

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metabolic activated BP, preferentially causes G to T mutations and binds to the mutational hotspots in the *TP53* gene often mutated in lung cancer (91– 96). Therefore, BP is generally believed to be the most important carcinogen in TS (91– 96) (Fig. 1). The observations that MTS and SSS exposure do not enhance BPDE-dG in mouse lung tissues, and that lung tissues of tobacco smokers and non-smokers have similar levels of BPDE-dG are important, albeit negative findings. It is worth noting, however, that the amount of BP per cigarette is relatively minute (~25 ng/cig) (2, 11, 16), compared to the amount of BP in the air from fuel (0.3–9300 ng/m^3), heating (2–490 ng/m3) and cooking (6–24 ng/m3) (126, 127). Therefore, it is likely that the amount of BP intake from ambient air and diet is much higher than the BP intake from TS, which consequently offsets the sensitivity of detection of BP from TS. We believe the same rationale could explain the lack of difference in BPDE-dG level in different tissues including lungs between tobacco smokers and non-smokers (21, 128–130).

TS effect on DNA repair

It has been long recognized that aldehydes through their carbonyl group and olefin double bond can react with many amino acid moieties, such as, lysine, histidine and arginine, which are found in most proteins (105). These aldehyde modifications very often affect protein functions and structural integrity, and lead to protein degradation (105). At high concentrations, aldehydes can cause protein crosslinks (131, 132). Since TS contains abundant aldehydes it is conceivable that TS may interfere with many cellular functions via interactions of TS aldehydes with various cellular proteins. Two possible effects of aldehyde perturbations associated with cellular functions related to TS carcinogenesis have recently been tested: DNA repair functions and the activation of procarcinogens (19, 20, 53, 61, 76, 98). It has been found that aldehydes can instantaneously inhibit both nucleotide excision repair (NER) and base excision repair (BER) functions in *in vitro* assay systems (19, 20, 53, 61, 76, 98). In cultured human cells, aldehyde treatments not only can impair three major DNA repair mechanisms, NER, BER and mismatch repair (MMR), but can also cause degradation of repair proteins and enhancement of mutational susceptibility (19, 20, 53, 61, 76, 98). In mice, TS causes a reduction of NER and BER activity and reduces the levels of repair proteins such as, XPC, OGG1, Ref1 and MHL1 in lung tissues (19, 20, 61). Since it has been well-established that aldehydes can cause protein dysfunction and degradation via aldehyde-protein interactions, and that aldehydes are major components in TS, these results, together, strongly suggest that aldehydes contribute greatly to the TS-induced reduction of DNA repair proteins and repair activity.

Aldehydes play a dominant and negative effect in TS-induced DNA adduct formation

It is well established that upon uptake, many procarcinogens in TS, such as PAHs, AAs, benzene, and heterocyclic hydrocarbons, will be “detoxified” and become electrophilic via O-acetylation, N-hydroxylation, N-oxidation, N-acetylation, epoxidation, sulfation and esterification (133–140). It is these electrophilic derivatives that can covalently interact with DNA to form mutagenic and carcinogenic DNA adducts (86, 141–143). These detoxification reactions are mainly carried out by numerous cellular enzymes such as cytochrome p450s, sulfotransferases, and acetyl esterases (133–140). We have recently found that adding Acr to cultured human cells can prevent activation of procarcinogens BP and NNK from becoming DNA damaging agents (20). We have also found that in mouse models the major DNA

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adducts induced by both MTS and SSS are aldehyde-DNA adducts, such as Acr-dG and Cro-dG adducts, and not the PAH-diol epoxide (PAHDE)-dG and O^6-medG adducts (19, 20). These results suggest that the abundant aldehydes in TS not only induce DNA damage but also inhibit the activation of procarcinogens such as PAHs, AAs, and nitrosamines. The same reasoning may explain the fact that aldehyde-DNA adducts are the major types of DNA damage found in buccal cells, sputum and lung tissues of tobacco smokers, and that the levels of BPDE-dG and O^6-medG are no different between tobacco smokers and nonsmokers (20).

In summary, most recent findings show that the major DNA adducts induced by TS are the consequence of interactions of direct DNA damaging agents in TS, such as Acr, acetaldehyde, and Cro, and the DNA adducts are not induced by procarcinogens such as PAHs, AAs, and nitrosamines as conventionally believed (19, 20). One important concept derived from the recent findings is that TS induced DNA adducts are not a simple arithmetic sum of all TS DNA damaging agents. Simply put, TS aldehydes are major driving forces to induce TS carcinogenesis via three mechanisms: induction of DNA adducts, inhibition of procarcinogen activation, and lastly, inhibition of DNA repair activity, and enhancement of mutagenesis susceptibility via interactions with activation enzymes and repair proteins (Fig. 2). We wish to point out that our results show that in a short period of TS exposure (8 weeks) in mice, while no significant amounts of Acr-DNA adduct were found in the lung tissues, an elevated level of BPDE-dG was found in bronchial lavage and bladder tissues ($p=0.035$) (19). These results suggest that the TS aldehyde effect can potentially be greatly reduced at a given stage, and that there are abundant aldehyde-neutralizing agents such as glutathione in the upper aerodigestive system. In this case PAHs, perhaps AAs, and NNK also, may be activated and able to induce DNA adducts.

Electronic cigarettes

Following the recognition of the harmful effects of tobacco smoke, most notably the carcinogenic nature of the products of the incomplete combustion of the tobacco leaves, alternative devices to deliver nicotine emerged. One of the most notable examples is Herbert Gilbert's 1963 development of a device that utilizes battery power to heat and aerosolize the "flavor" materials, including nicotine, that are dissolved in propylene glycol (PG) and vegetable glycerin (VG) (144). From 1970 to late 1990 there was a flurry of activity involving similarly designed devices intended to heat and aerosolize nicotine (145). However, Gilbert's patented design never reached market and the tobacco companies did not seriously market their similar devices (145). The electronic-cigarettes (E-cigs) in current use became popular more than a decade ago only when a pharmacist Han Li started marketing his reinvented device using inhalation- riggered-battery-powered electricity, which allows aerosolization of nicotine containing organic solvents of PG and VG. Han Li's E-cig gained popular acceptance almost instantaneously (24). For the past decade literally scores of similar devices have emerged on the market, most notably the trademarked JUUL and "Pod" brands, which currently occupy more than 70% of the market (146). The essential design and operation of all these E-cig devices are similar, that is, they contain two major components: a compartment to store E-liquid, or E-juice – (nicotine dissolved in the organic solvents, PG and VG), and a battery powered device that is triggered by inhalation to heat

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up and aerosolize the E-liquid. The sources of PG and VG are from chemical synthesis, while nicotine is extracted from tobacco leaves, because tobacco leaf nicotine is abundant and nicotine extraction is a simple process. Various flavorants such as spice, fruit and candy-flavorings are typically added to the E-liquid to enhance the product's appeal (24). Since the boiling points of the solvents (PG,188.2°C and VG, 290°C) and nicotine (247°C) are relatively low, the battery heating temperature is usually designed so it does not cause burning. Consequently, negligible amounts of incomplete combustion products are generated (147, 148). The E-cig is therefore marketed as a device for the delivery of a "nicotine high" without the ill effects of TS incomplete combustion products (24, 30, 149, 150). The focus of the remainder of this review is on the induction of DNA damage and the carcinogenic effect of E-cig aerosols (ECAs) and its major component, nicotine.

The components in ECAs

The compositions of ECAs have been extensively analyzed (147, 148, 151). Due to the lack of regulation, the compositions of E-liquids are not necessarily uniform, and the mechanical designs of the E-cig devices vary among different products (152, 153). The compositions of ECAs and the proportionality of the different components have been found to vary across brands (153). Many brands of E-cig contain a variety of flavors ranging from menthol, peppermint, tobacco, or coffee to fruit flavors such as mango, kiwi, apple and strawberry and much more. The purpose of adding flavors in E-cig juice is for enticing E-cig usage. The health effect of these flavors remains largely unknown. It has been reported that the different methods of generating ECAs, such as the sources of air, ambient air versus filtered air, and the range of voltage, greatly affect the compositions of the ECAs (153). Assessments of the impact of ECA generating methodology on the chemical compositions of ECAs are well documented (151, 153). Findings reported in all publications on ECAs are consistent in four aspects: first, ECA is rich in nicotine, PG and VG; second, the chemicals found commonly in conventional TS, such as PAHs, nitrosamines, carbonyls, aromatic amines and metals are occur at extremely low levels in ECA; third, elevating the voltage of an E-cig device to higher than that recommended (<4.2 V) for aerosolizing the E-liquid results in carbonyls in ECAs; and fourth, adding flavor substances to E-liquid may or may not change the ECA composition (151, 153). Therefore, our discussion on the ECA biological effects will be limited to ECA generated by the basic process recommended for most marketed E-cig brands, that is, aerosolized PG or VG (50:50 ratio) containing nicotine (1 to 3.6%) at low voltage heating (4.0 V,1.96 amp), excluding flavorants.

Methodology for assessing the biological effects of ECA

The methodology for assessing ECA's biological effects is similar, if not identical, to those used for assessing TS effects (154–162). The most often used technique is collecting the ECA on membranes (158–162). This method can collect ECA particulate materials larger in size than the pore size of the membrane and that originate from puffs at different times, such as early puffs after lighting and later end puffs. The collected materials, often referred as ECA extracts, were then used to treat cultured cells or animals in order to assess their effects. While this method is convenient, there are likely limitations since it is conceivable that the ECA extracts are subjected to aging, and chemical interactions that may not occur in the aerosol state. Furthermore, the total aerosols and condensate do not encompass all the

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constituents of whole aerosols because some gases and vapors pass through the collection system (i.e., the filter pads). Exposure of animals to ECA generated from E-cig mimic devices in real time is a preferable procedure for assessing ECA effects. To accomplish this, animals are usually housed in a controlled chamber, and inhale only the ECA injected into the chamber. It should be noted that the body of the animals is also exposed to ECA under most exposure conditions using current available exposure chambers. Therefore, the effects measured by this method represent the sum of inhaled ECA, ECA absorbed by the skin and ECA taken in through the digestive system via licking.

ECA cytotoxicity

Compared to many elements in TS, such as aldehydes, PAHs, AAs, and metals, both the solvents found in E-cig, PG and VG, and the nicotine in E-liquid are significantly less cytotoxic (147, 156, 157, 159, 160). Therefore, it was not surprising to find that ECA extracts are much less cytotoxic than TS extracts towards cultured cardiomyocytes and fibroblasts (158, 159, 161, 162). Furthermore, the concentrations of solvents and nicotine seem to have negligible effect on ECA cytotoxicity (161, 162). These studies confirm the non-cytotoxic nature of PG and VG. However, it has been found that the ECA generated by high voltage and electric power were much more cytotoxic than the ECA generated by regular usage settings, and even more cytotoxic than TS extracts (160–165). It has been found that high voltage generates carbonyls, including acrolein, acetaldehyde, and formaldehyde, similarly to TS (151, 163, 164). Since carbonyls are relatively cytotoxic, it is not surprising that the high-voltage- generated ECA is very cytotoxic (53, 76, 98). It should be noticed that acrolein is a potent apoptotic agent that induces release of caspases from mitochondria (105, 166– 168), and that the cytotoxicity of TS is mainly via induction of apoptosis (169). Because of its low cytotoxicity, E-cigs have been suggested to replace tobacco for smoking cessation and therapeutic purposes (170). However, caution is warranted given that the carbonyl content of ECA can be increased easily by slight increase of the E-cig voltage and electric power (163–165).

Induction of DNA damage by ECA

The finding that ECA is much less cytotoxic than TS strengthens the argument that ECA is less carcinogenic than TS to humans. Many studies focused on measuring the harmful materials present in TS, such as PAHs, AAs, TSNAs, benzene, and aldehydes in ECA and rendered the obvious results that these harmful and carcinogenic materials are at significantly lower levels in ECAs than in TS (171–173). In fact, the levels of these materials in ECAs is equivalent to ambient air (171). These results emboldened many studies to conclude that E-cigs are harmless and to recommend E-cigs as an alternative to regular cigarettes (171, 174). However, all these studies conveniently avoid discussing the implications of the crucial fact that there is as much nicotine in ECA as in TS, if not more (175–177). Nicotine by itself has been found to induce sarcomas and leiomyomas in animal models (22, 178). Therefore, measuring ECA’s harmful and carcinogenic effects, rather than measuring the TS carcinogenic components in ECAs, is the proper way to address the question of whether or not ECA is harmful and carcinogenic.

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It is well established and generally accepted that induction of DNA damage and mutations are necessary steps for genotoxic carcinogens and procarcinogens to initiate carcinogenesis (140–143, 179–181). Therefore, it is reasonable that the first step toward addressing the carcinogenicity of ECAs is to determine whether or not ECAs can induce DNA damage in cells. Since under normal operation conditions (≤4 volts, 1.96 amp), E-cigarette vaping does not generate significant amounts of aldehydes (151, 163, 164, 182), and PG and VG by themselves have never been shown to be able to damage DNA directly, if ECA can induce DNA damage, it is most likely due to nicotine.Therefore, the answer to these questions resides in the results of treating cells and animals with ECA and nicotine. To date, it has been found that ECA extracts can induce DNA strand breaks and oxidative DNA damage (ODD), such as 8-oxo-dG, in cultured cells (183, 184). ECA can induce O^6-medG and cyclic γ -OH-PdG adducts in lung, heart and bladder tissues of exposed mice (61). And, nicotine can induce the same type of DNA adducts as ECA in cultured human bronchial epithelial cells and bladder urothelial cells (61). The review will next focus on the causes of the variety of DNA damage induced by ECAs and nicotine.

DNA strand break and oxidative DNA damage induced by ECA

To date, DNA strand breaks and ODD, 8-oxo-dG in particular, are the most often reported DNA damage in cultured human cells resulting from ECA exposure. Based on comet assay results, it has been reported that ECA extracts induced DNA strand breaks (183, 185, 186). Using the ELISA assay, Ganapathy et al., (184) reported that ECA extracts caused an increase of 8-oxo-dG in human oral squamous sarcoma cells. ECA extracts also cause blockage of DNA primer extension, as detected by quantitative PCR (QPCR) indicating that ECA induces ‘bulky” DNA damage. However, the mechanisms by which ECA extracts cause DNA strand breaks and 8-oxo-dG are not clear, and the types of DNA damage that block DNA synthesis were not investigated in these studies. It has been speculated that ECA extracts induce oxidative stress responses and suppression of cellular antioxidant defense mechanisms (184). The underlying mechanisms that lead to induction of oxidative DNA damage by ECA extracts seem to be that ECA extracts negatively affect the cellular homeostasis of oxidative potential and, consequently, excessive ROS are generated from cellular activity causing strand breaks and oxidative DNA damage. It is unlikely that the metabolites of ECA extracts can directly interact with DNA causing DNA strand breaks and 8-oxo-dG. In this regard, it is worth noting that the Fenton reaction is necessary for induction of ROS, which can induce 8-oxo-dG and DNA strand breakage. It has been found that nicotine treatment causes reduction of cellular ROS (187) and that nicotine is a chelating agent for divalent cations, which are necessary for Fenton reactions to generate ROS (188). These contradictory results on 8-oxo-dG and ROS production by ECA extracts and nicotine are difficult to reconcile and indicate that unknown components in ECA extracts cause oxidative stress in cells, which leads to 8-oxo-dG formation. It is worth noting that Yu et al., (183) found that ECA extracts generated from E-liquid with and without nicotine have the same cytotoxicity and clastogenic effect. These results raise the possibility that the steps of condensing ECA may introduce unknown chemical reactions producing new compounds that can cause both cytotoxicity and DNA strand breaks. These results highlight the necessity of using real time ECA for determining E-cig effects.

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ECA induces O6-medG and cyclic γ -OH-PdG adducts, inhibits DNA repair, and reduces repair protein level in mice

It is well established that during tobacco curing and burning a small portion of nicotine (0.1%) is converted into so-called tobacco specific nitrosamines (TSNAs) due to nitrosation of nicotine mediated by nitronium ions (189–191). Many of the TSNAs, such as NNK (nicotine-derived nitrosamine ketone) and NNN (N-nitrosonornicotine), are potently carcinogenic in animal models and have been recognized as human carcinogens (12, 17, 192).

Upon uptake, nitrosamines (such as NNK and NNN) are subjected to metabolism by cytochrome p450s, which are abundant in animal and human cells, and the metabolites are eventually degraded into formaldehyde, methyldiazohydroxides (MDOH), and pyridylic-butylic derivatives (PBDs) (12). While all these final products are potential DNA damaging agents, nitrosamine carcinogenicity has been attributed to MDOH, which have been shown to induce mutagenic and carcinogenic O6-medG adducts with minor adducts of medT and medC (193). Because of these findings, the level of NNK, NNAL [(4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol] and NNN in TS, as well as in body fluids, have been widely used to assess the carcinogenic potency of TS from various cigarette brands and to estimate the carcinogenic risks of human exposure (193, 194). Commercially available E-liquid contains a relatively large quantity of nicotine (1 to 5%), which can be converted into carcinogenic nitrosamines by a single nitrosation (Fig. 3). Since the nitronium ions necessary for the nicotine nitrosation reaction are being generated constantly during normal cellular function (195), intuitively, one would think that nicotine nitrosation must take place *in vivo*. It is surprising, this question has not been carefully scrutinized. Hecht's group reported the detection of minute amounts of NNAL in saliva of animals exposed to nicotine (196). The lack of finding NNAL and the low level of NNAL detected in animals exposed to relatively large quantities of nicotine raises two questions. Are only minute amounts of exogenous nicotine subjected to nitrosation *in vivo*? Or, does nicotine nitrosation occur *in vivo* at all? What is the cause of low NNAL detection in nicotine exposed mice? One possible scenario is that during uptake by cells nicotine is nitrosated and becomes various nitrosamines, which are ultimately metabolized and degraded into DNA damaging agents. Consequently, very low levels of nitrosated nicotine, if any at all, exist inside the cells and the levels are even lower in body fluids. Since animal and human cells have an abundance of P450s which can metabolize nitrosamines resulting in their spontaneous degradation into DNA damaging agents, we propose that the DNA damage induced by these processes can initiate mutagenesis and carcinogenesis. If this is the case, then measuring DNA damage induced by ECA is a direct way to address the carcinogenic effect of nicotine and ECA and measuring nitrosamine levels may underestimate the extent of the nicotine induced carcinogenic effect. The seriousness of this issue has recently surfaced. Shahab et al., (197) have reported that the NNAL level in the urine and saliva in E-cig users is 5% of that in tobacco smokers. Subsequently, Hecht's group also detected NNAL in the urine samples of E-cig users at much lower levels than in the urine samples of regular tobacco smokers (198, 199).These findings have lead British medical organizations to conclude that E-cig is only 5% as harmful as TS, and to recommend E-cig as a substitute for regular cigarettes and for tobacco cessation therapeutic purposes (200, 201).

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However, a different picture emerges with the approach of detecting nitrosamine related DNA adducts in different organs of mice exposed to nicotine containing ECA versus ECA without containing nicotine. Real time ECA exposure induced three clear effects in mice: induction of mutagenic DNA adducts, γ -OH-PdG and O^6-medG, in lung, heart, and bladder tissues; inhibition of nucleotide and base excision repair (NER and BER) function; and reduction of the level of repair proteins XPC and OGG1 in lungs (61). Furthermore, our research also shows that nicotine and NNK induce the same types of DNA adducts and same inhibitory effect on DNA repair in cultured human bronchial epithelial and bladder urothelial cells (61). These results indicate that nicotine, upon uptake by cells, can be nitrosated into nitrosamines, such as NNK (61). These results together indicate that ECA and nicotine are DNA damaging agents in mice and potentially in humans. We wish to point out that these mice were exposed to ECA generated under the vaping conditions of 4.0 volts and 1.96 amp of the E-cig juice containing nicotine 10 mg/ml dissolved in solvents of PG and VG at 1 to 1 ratio without any flavors (57).

Carbonyl production in E-cigarette.—Although the amounts of aldehydes generated from E-cig vaping are minute, some of these aldehydes, particularly acrolein, can induce cyclic propano-dG adducts, the same type of DNA adducts which are also induced by the nitrosamines, the metabolites of nicotine. Therefore, a careful evaluation of the level of aldehydes generated from E-cig vaping, and their possible roles in DNA damage induction, by comparison to the level of nicotine and nicotine's role on DNA damage induction in ECA is worthwhile. It is well established that ECA contain minute amount of carbonyls such as formaldehyde, acetaldehyde and acrolein (202, 203). Because many aldehydes in ECA are carcinogenic, ample researches have investigated the origin of these carbonyls and the amounts of these carbonyls generated under different vaping conditions. Two major factors that have been found to determine the types and amount of carbonyl production are organic solvents, particularly the ratio of PG versus VG, and the heating coil temperature, which is proportional to the voltage and watt used in the E-cig (163, 204). Results reported from different laboratories, in general, are in a good agreement that the amounts of formaldehyde, acetaldehyde and acrolein generated from E-cig vaping conditions of voltage <4.5 volts, are minute. For example, Kosmider et al (163), report that under vaping conditions of total watt 10 W, the amounts of formaldehyde and acetaldehyde generated are 72 ng/puff and 25 ng/puff, respectively. The average amount of acrolein in an E-cig puff is 3.2 ng (205). It worth noting that the amounts of nicotine generated from regular E-cig are from 0.033 – 1 mg/puff (203), which are $10^4 - 10^5$ -fold of the aldehyde levels in ECA. While acrolein-DNA adducts are detected in the lung tissues of mice exposed to ECA, no acrolein-DNA adducts were detected in lung tissues of mice expose to PG and VG solvent without nicotine. Furthermore, crotonaldehyde-DNA adducts, the acetaldehyde -induced DNA adducts (121) were not detected in these lung tissues (61). Based on these results we have concluded that the acrolein-DNA adducts in these lung tissues are resulted from the nitrosamines of nicotine nitrosation products and not derived from the carbonyls generated in ECA (61).

ECA induces lung adenocarcinoma and bladder urothelial hyperplasia.

The carcinogenicity of ECA in mice has recently been discovered. Our lab recently reported that long-term (54 weeks) exposure to ECA induces lung adenocarcinoma and

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bladder urothelial hyperplasia in mice (206). While the number of mice used in the tumor experiment is less than ideal (40 for ECA, 20 each for filtered air and solvent aerosol exposure), the results are nonetheless statistically significant (206). The biochemical results and tumorigenesis results individually leave room for doubt about the potency of the carcinogenic effect of ECA and nicotine, but together they constitute a strong case that ECA and nicotine are carcinogens in mice, and thus potentially for humans.

Necessity of using cultured human cells and mouse models to determine ECA carcinogenesis.

The ultimate answers as to whether or not ECA is a human carcinogen will come from epidemiological research. It usually takes between one to two decades for tobacco smokers to develop cancer, including lung cancer (35). Given that E-cig use has only become popular in the past eight years, epidemiological data may not be able to accurately indicate a relationship between ECA and human lung cancer for another decade. Therefore, animal models are necessary as surrogates to study the ECA carcinogenic effects and the molecular changes induced by ECA. Mouse models are the best choice for three reasons. First, most human lung carcinogens, including NNK and PAHs, can induce lung cancer in mice (2, 12, 207, 208). Therefore, if ECA causes lung cancer in mice, it is likely that it will also cause lung cancer in humans. Second, based on the findings that NNK and PAHs induce similar types of DNA damage in mice and humans (2, 12, 207, 209), it is reasonable to expect that the metabolism and processing of ECA in mice and humans are similar. Third, genes that are frequently mutated in human lung cancer, such as *TP53* and *RAS*, are also frequently mutated in mouse lung cancer, indicating that lung carcinogenesis in humans and mice are similar (210). These results suggest that the carcinogenic effects of ECAs can be unraveled, similarly to many human carcinogens, in cultured human cell models and mouse models. Unfortunately, to date only a few such studies on the tumorigenicity of ECA in animal models have been performed.

Hypothetic role of nicotine in TS and ECA carcinogenesis

The role of nicotine in ECA and TS carcinogenicity is a logical conundrum. On one hand, it appears that nicotine and TSNAs in TS do not induce significant amounts of nitrosamine-specific DNA adducts (O^6-medG) in lung tissues of mice exposed TS or in the lungs of tobacco smokers, indicating that aldehydes in TS inhibit nicotine nitrosation and subsequent activation of nitrosamines to become DNA damaging agents (20). On the other hand, nicotine in ECA induces not only O^6-medG but also γ -OH-PdG adducts, the aldehyde DNA adduct signature in mice (61). Nicotine alone also induces the same types of DNA adducts in cultured human cells (61). Based on these results, we hypothesized that the overwhelming amount of aldehydes, generated during incomplete combustion of tobacco leaves, exerts a dominant and negative role among different TS components in DNA adduct induction and procarcinogen activation (20).However, due to the absence of incomplete combustion of tobacco generated aldehydes, nitrosated nicotine can be metabolized in cells to generate DNA damaging products such as, formaldehyde, MDOH and PBDs, which induce DNA adducts, inhibit DNA repair, and modify DNA repair proteins (Fig. 3).

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Which is worse, TS vs ECA?

Although both TS and ECA induce PdG adducts, the mechanisms that lead to PdG formation by these two agents are diametrically different. Aldehydes in TS are the major cause for PdG formation, whereas in ECA nicotine induces PdG. These findings raise a curious but nonetheless serious question of interest to the general population: TS versus ECA, which one is more carcinogenic? The answer to this question requires a well-designed experimental approach using animal models, and only epidemiological studies will render the final conclusion. Nonetheless, biochemical results from TS and ECA exposed mice of the same strain, offer some clues. In the aforementioned studies, the nicotine consumption in TS exposed mice is roughly equivalent to an individual who smokes 2.8 packs per day of cigarette (17,18). The nicotine consumption in ECA exposed mice is roughly equivalent to an individual who smokes 0.4 pack of regular cigarettes per day (56). We found that the amount of PdG formed in lung and bladder urothelial tissues in mice exposed to ECA is higher than in mice exposed to TS (Table 2) (19, 20, 61). While nicotine in ECA induces O6-medG in addition to PdG, nicotine in TS does not. It appears that this nicotine effect is inhibited by aldehydes in TS exposed mice. However, TS contains thousands of chemicals that foreseeably can induce detrimental effects that ECA does not. We found that ECA induces lung adenocarcinoma by 5-fold, which is similar if not higher than the lung cancer induced by TS (Table 2) (206). Based on current results we propose that E-cig can cause lung cancer in mice. However, more animal experiments and epidemiological studies are needed to establish whether or not E-cigs are carcinogenic for humans. On the other hand, epidemiological results have firmly established TS causes cancer in humans (3, 4).

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Abbreviations

Acr	acrolein
Acr-dG	Acr-deoxyguanosine
Acr-DNA	Acr adducted DNA
AFB1	aflatoxin B1
AAs	aromatic amines
BER	base excision repair
BP	benzo(a)pyrene
BPDE	benzo(a)pyrene diol epoxide
BPDE-dG	BPDE-deoxyguanosine
Cro	crotonaldehyde

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Cro-dG	crotonaldehyde-dG (the same as α-methyl-γ-OH-dG)
dGMP	deoxyguanosine 5'-monophosphate (dGMP)
dNMP	deoxyribonucleoside 3'-monophosphate
DRZ	diagonal radioactive zone
E-cig	Electronic-cigarette
E-cigs	Electronic-cigarettes
ECA	E-cigarette aerosol
ECAs	E-cigarette aerosols
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry (LC- MS/MS)
medC	methylated cytosine
medT	methylated thymine
MDOH	methyldiazohydroxides
NER	nucleotide excision repair
NNAL	[(4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol]
NNK	nicotine-derived nitrosamine ketone
NP1	nuclease P1
nt	nucleitide
ODD	oxidative DNA damage
O^6-medG	O^6-methyl-deoxyguanosines
PAHs	polycyclic aromatic hydrocarbons
PBDs	pyridylic-butylic derivatives
PdG	cyclic 1,N^2-propano-dG
PG	propylene glycol
QPCR	quantitative PCR
TS	tobacco smoke
TSNAs	tobacco specific nitrosamines
VG	vegetable glycerin
α-methyl- γ -OH-dG	α-methyl- γ -hydroxy-1,N^2-propano-dG

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α-OH-PdG	cyclic α−hydroxy-1,N^2--propano-dG
γ -OH-PdG	cyclic γ −hydroxy-1,N^2--propano-dG
2D-TLC/HPLC	two-dimensional thin layer chromatography/high performance liquid chromatography
8-oxo-dG	8-oxo-deoxyguanosine

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Highlights

- Tobacco smoke induces mainly acrolein- and crotonaldehyde-DNA adducts in lungs.
- Tobacco smoke reduces DNA repair in lungs and aldehydes do the same in human cells.
- Aldehydes in tobacco smoke are the major driving forces of inducing DNA damage.
- E-cigarettes induce acrolein-DNA and methyl-dG adducts in the lungs and bladder.
- E-cigarettes induce lung adenocarcinoma and bladder urothelial hyperplasia in mice.

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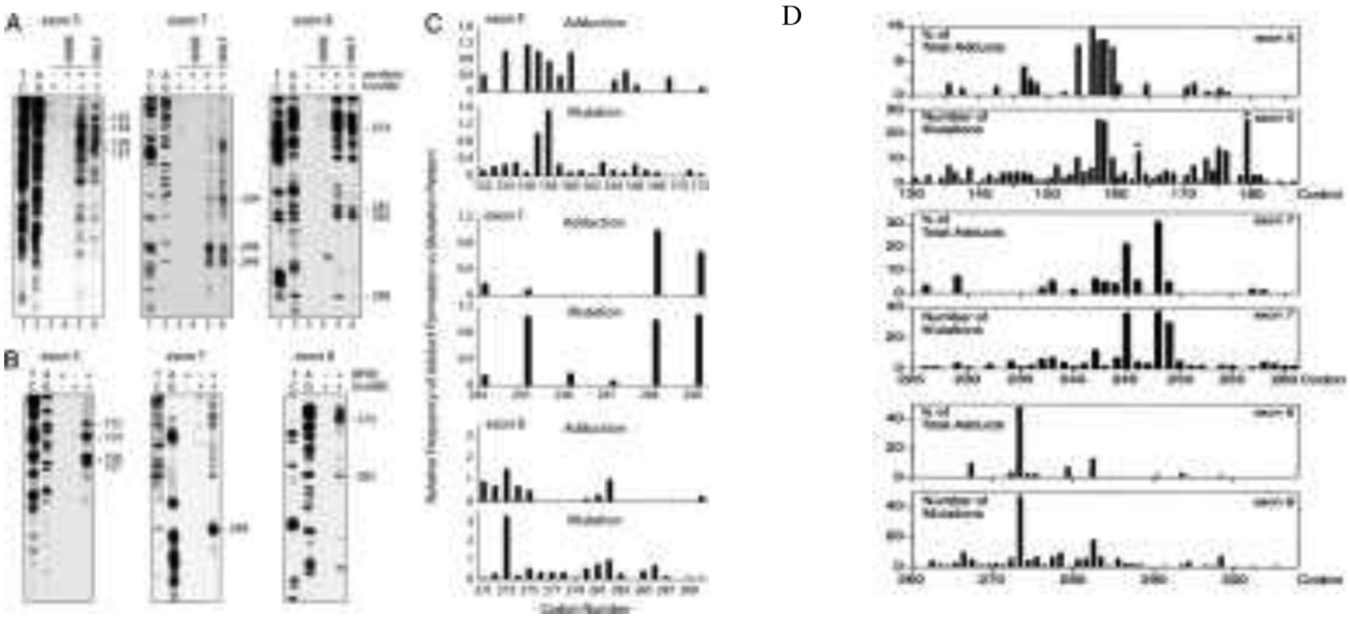

Fig. 1.

Acr– dG and BPDE– dG distributions in the *TP53* gene. (*A* and *B*) Acr– dG and BPDE– dG adduct distribution in exons 5, 7, and 8 of the *TP53* gene of normal human lung cells treated with Acr (*A*) and BPDE (*B*). In *A*, normal human bronchial epithelial (NHBE) cells and normal human lung fibroblasts (NHLF) were treated with 20 μM Acr for 6 h, and in *B*, NHBE cells were treated with 1 μM BPDE for 30 min. Genomic DNA was then isolated, the DNA adduct distribution was mapped by the UvrABC-ligation- mediated PCR (LMPCR) method, and the DNA was separated by electrophoresis (211). AG and TC are Maxam and Gilbert sequencing reaction products. (*C and D*) Comparisons of the frequency of Acr– dG (C) and BPDE-dG distribution along the *TP53* gene in NHBE cells with the frequency of the *TP53* mutations in TS-related lung cancer (International Agency for Research on Cancer *TP53* Mutation database, http://www-p53.iarc.fr) (Adapted from Feng et al.(98, 211),).

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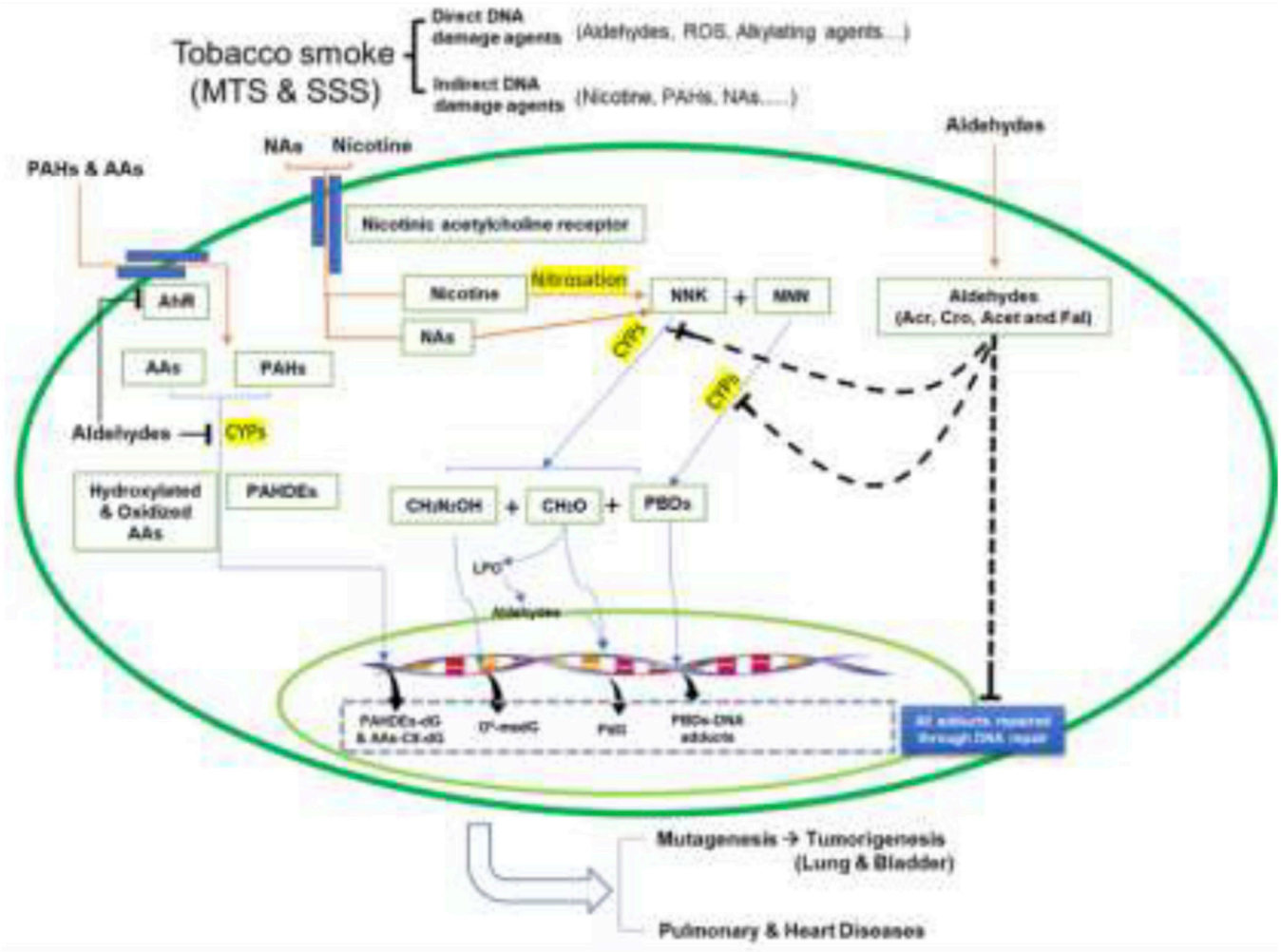

Fig. 2. Summary of TS induced DNA damage and effects on DNA repair.

This scheme is based on the results from DNA adduct detection in lung and bladder tissues of mice exposed to MST and SSS, DNA adduct analysis in lung tissues of tobacco smokers versus non-smokers, and results of analysis of aldehyde effect on DNA adduct induction, DNA repair activity, repair protein levels, and mutagenesis susceptibility in cultured human lung and bladder epithelial cells (19, 20, 61). Symbols: NNK, nicotine- derived nitrosamine ketone; NNN, N-nitrosonornicotine; AhR, aromatic hydrocarbon receptor; AAs, aromatic amines; CYPs, cytochrome p450s; NAs, nitrosamines; Acr, acrolein; Cro, crotonaldehyde; Fal, formaldehyde; LPO, lipid peroxidation; PBDs, pyridylic-butylic derivatives; PAHs, polycyclic aromatic hydrocarbons; PAHDEs, PAH diol epoxides.

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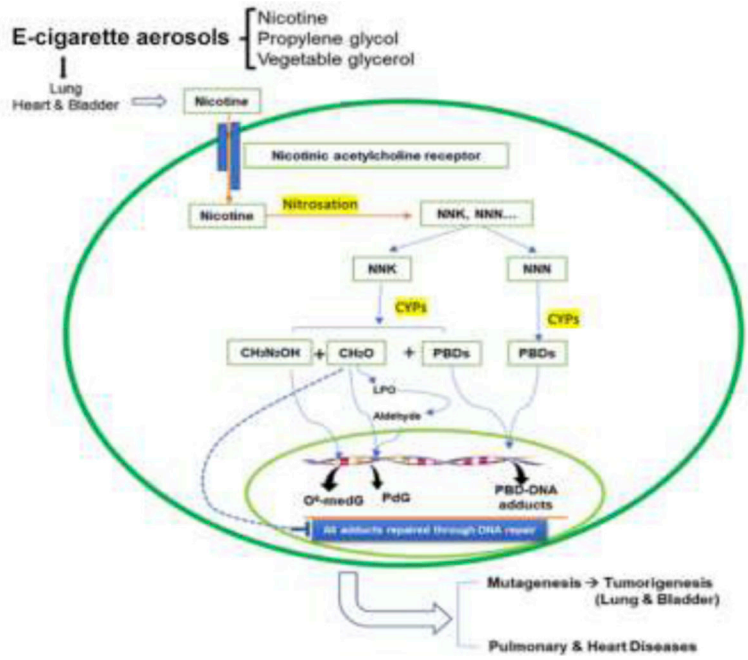

Fig. 3. Schematic representation of the uptake and nitrosation of nicotine in E-cig aerosols, the metabolism, subsequent degradation, the induction of DNA damage that follows, and the inhibition of DNA repair.

This scheme is based on the results from the DNA adduct analysis, DNA repair analysis, and tumorigenesis in lung and bladder tissues of mice exposed to ECAs (61, 105, 206). Symbols are the same as in Fig. 2.

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Table 1.

Identification of types of mutations induced by Acr or BPDE in the CpG methylated and unmethylated *supF* gene compared with types of mutations that occur at CpG mutational hotspots in the *TP53* gene in tobacco smoke–related lung cancer (Adapted from Wang et al.(97)).

Mutations at CpG site	Control		Acr[b]		BPDE[c]		p53[d]
	UM[a]	M[a]	UM	M	UM	M	
G to T	2 (40%)	1 (6%)	9 (36%)	15 (54%)	10 (48%)	20 (71%)	296 (49%)
G to A	2 (40%)	17 (94%)	7 (28%)	11 (39%)	1 (4%)	3 (11%)	219 (36%)
Others[e]	1 (20%)	0 (0%)	9 (36%)	2 (7%)	10 (48%)	5 (18%)	96 (15%)

[a] UM: unmethylated *supF*; M: methylated *supF*.

[b] Acr: 0.5 mM at 37°C for 24 h.

[c] BPDE: 2μM at 37°C for 1 h.

[d] CpG sites at codons 157, 158, 175, 245, 248, 273 and 282 of p53 in TS-related lung cancer.

[e] Other types of mutations including G to C, insertion, deletion or tandem mutations.

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Table 2.

A summary of the quantification of γ-OH-PdG,α-methyl-γ-OH-PdG, O^6-medG and BPDE-dG adduct formed in the lungs, heart, liver and bladder of mice exposed to MTS[1], SSS[2] and E CA[3].

Aerosol	Exposure	Nicotine In the chamber (μg/m^3)	Source of Aerosol	Aerosol Level (mg/m^3)	Organ	Exposed (n=10)/unexposed (n=10)			
						γ-OH-PdG/10^5 dG	α-methyl-γ −OH-PdG/10^5 dG	O^6-me-dG/10^7 dG	BPDE-dG/10^7 dG
MTS[1]	3 cig[4] 6 h/d 5 d/wk 12 wks	871.5	2R4F cig 2 puffs/min 35 ml/puff	75	Lung	0.55/0.05****	0.19/ND	0.05/0.25 ns	0.75/0.75 ns
					Heart	0.15/0.15 ns	-----	0.3/0.4 ns	0.4/0.4 ns
					Liver	0.4/0.3 ns	-----	0.075/0.075 ns	0.6/0.6 ns
					Bladder	0.3/0.05***	ND/ND	0.2/0.1 ns	0.2/0.1 ns
SSS[2]	3 cig[4] 6 h/d 5 d/wk 16 wks	36	2R4F cig 2 puffs/min 35 ml/puff	0.454	Lung	0.5/0.3*	ND		2/2 ns
					Heart	0.05/0.05 ns	-----		2/2 ns
					Liver	0.4/0.35 ns	-----		2/1.75 ns
					Bladder	0.7/0.4**	ND		1.8/1.7 ns
ECA[3]	E-liquid (Nicotine 10 mg/ml +PG and VG) 3 h/d 5 d/wk 12 wks	129	E-juice (nicotine, PG and VG) puffs/min 35 ml/puff	130	Lung	1/0.2***	ND	4/0.2****	-----
					Heart	0.3/0.05****	-----	0.5/0.1***	-----
					Liver	0.04/0.035 ns	-----	1/1 ns	----
					Bladder	0.4/0.05***	ND	1.25/0.2**	----

[1]Results from Weng et al (20).

[2]Results from Lee et al., (19).

[4]3 cig: smoke generated from 3 cigarettes burning at the same time.

[3]results from Lee et al., (61).

ND: not detectable; _____: not detected.

P value:

*p<0.05,

**p<0.01,

***p<0.001,

****p,0.0001, ns= not significant.