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Gender Difference in DNA Damage Induced by the Environmental Carcinogen Dibenzo[*def,p*]chrysene Individually and in Combination with Mouse Papillomavirus Infection in the Mouse Oral Cavity

Kun-Ming Chen, Yuan-Wan Sun, Jiafen Hu, Karla Balogh, Krishne Gowda, Cesar Aliaga, Dongxiao Sun, Neil Christensen, Shantu Amin, and Karam El-Bayoumy*

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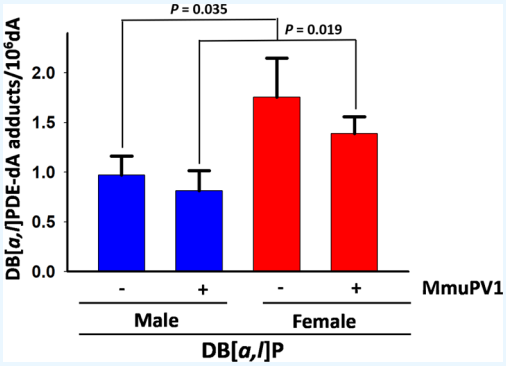
ABSTRACT: Tobacco smoking and human papillomavirus infection are established etiological agents in the development of head and neck squamous cell carcinoma (HNSCC). The incidence and mortality of HNSCC are higher in men than women. To provide biochemical basis for sex differences, we tested the hypothesis that carcinogen treatment using dibenzo[*def,p*]chrysene, which is an environmental pollutant and tobacco smoke constituent, in the absence or presence of the mouse papillomavirus infection results in significantly higher levels of DNA damage in the oral cavity in male than in female mice. However, the results of the present investigation do not support our hypothesis since we found that females were more susceptible to carcinogen-induced covalent DNA damage than males independent of the viral infection. Since DNA damage represents only a single-step in the carcinogenesis process, additional factors may contribute to sex differences in humans.



INTRODUCTION

The most common types of head and neck squamous cell carcinoma (HNSCC) include oropharyngeal squamous cell carcinoma (OPSCC) and oral squamous cell carcinoma (OSCC). Tobacco smoking is an established risk factor for HNSCC, including OPSCC, while the sexual behavior is now an established risk factor for human papillomavirus (HPV)-related OPSCC.[1] In the United States, it is estimated that 54,540 new cases and 11,580 deaths were reported in 2023.[2] Furthermore, the estimated new cancer cases by sex for oral and pharynx are 39,290 for males and 15,250 for females; the corresponding values for deaths are 8140 for males and 3440 for females. Although it had been established that women are more proficient in their immune system,[3] and they are likely to smoke less than men, the results of epidemiological studies that examined the contribution of several factors such as the role of sex hormones remain inconclusive,[4−7] and thus, elucidation of the mechanisms at the molecular level that may account for sex differences are urgently needed.

Preclinical animal models of HNSCC are essential in investigating the molecular targets during the multistep carcinogenesis process; the advantages and disadvantages of the various animal models have been reported.[8,9] Chemically induced HNSCC in immune-competent mice usually reflects better heterogeneity and the relatively more complex situations in the clinical setting.[10,11] Synthetic agents and tobacco smoke constituents have been used extensively to induce cancer in the oral cavity in rats, mice, and hamsters.[8] While male and female rodents were used in these previous studies, to our surprise, we could not find studies, including our own using the tobacco constituent dibenzo[*def,p*]chrysene (it is also named as dibenzo[*a,l*]pyrene, DB[*a,l*]P) (Figure 1) that compared the induction of oral cancer by chemical carcinogens in males and females side-by-side under identical conditions. However, we acknowledge that it was not possible to provide unequivocal data on sex difference to induce oral cancer with a limited sample size (n = 3 per sex) from a pilot study in 1954, using

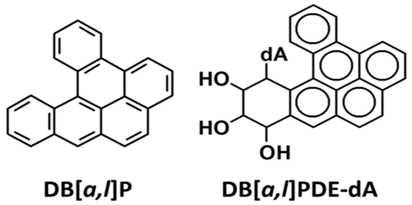


Figure 1. Structures of dibenzo[*a,l*]pyrene and its DNA adduct.

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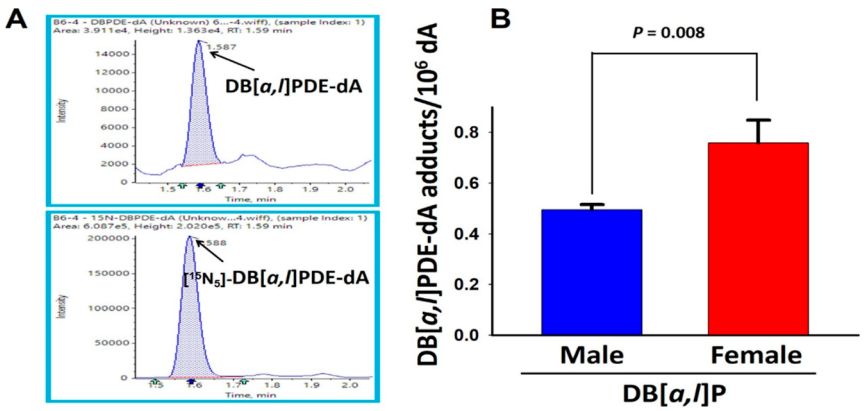

Figure 2. (A) Representative chromatogram of the analysis of the DB[*a,l*]P-dA adduct in the mouse oral cavity by LC−MS/MS following the oral application of DB[*a,l*]P. (B) Levels of covalent DNA adduct formation in the mouse oral cavity following multiple doses of the oral application of DB[*a,l*]P.

several chemical carcinogens in the hamster cheek pouch.[12] In a follow-up study in 1961,[13] also using the hamster cheek pouch model with an increased number of animals, the authors were not able to determine the sex difference because of multiple variables (the age of the animals, dose, and frequency of carcinogen used in treatment). Nevertheless, it is worth noting that humans lack an analogous cheek pouch, which limits the translational relevance of this model.

The progression of HNSCC involves sequential acquisition of both genetic and epigenetic alterations in genes that are encoding tumor suppressors and oncogenes.[14,15] In the process of multistep carcinogenesis, the formation of DNA adducts derived from chemical carcinogens including those found in tobacco smoke (benzo[*a*]pyrene [B[*a*]P], DB[*a,l*]P, *N*-nitrosonornicotine [NNN]) is a prerequisite but not a sufficient step; however, adduct formation remains a valid biomarker for cancer risk.[8] Therefore, the first goal of this study (Bioassay I) is to test our hypothesis by comparing the levels of DNA damage that can be induced by DB[*a,l*]P under identical conditions in immuno-competent male and female mice. DB[*a,l*]P is known to induce oral cancer as demonstrated in our previous report that included only female mice.[16]

It is generally accepted that HPV-positive OPSCC patients are a subgroup with deferential etiological factors and clinical behaviors when compared to HPV-negative cases.[17] The International Agency for Research on Cancer classified HPV16 as oncogenic to a number of cancer sites.[18] In contrast to cervical cancer,[19] cofactors, such as tobacco smoking, for HPV-associated OPSCC are not fully defined.[20] Our knowledge of the carcinogenesis process from HPV infection to the OPSCC is limited and mostly extrapolated from cervical cancer models. Using our established mouse papillomavirus (MmuPV1) infection model, no tumor was observed in the oral cavity of mice infected with the virus alone during the duration of the bioassay but a very low tumor incidence was observed in mice treated with DB[*a,l*]P at the dose used in that study.[21] However, tumor incidence was significantly increased in mice which was infected and also treated with DB[*a,l*]P as compared with carcinogen alone or infection only; these results suggest that DNA damage induced by DB[*a,l*]P cannot be completely ignored in the carcinogenesis process. However, in the above study the number of mice per sex was not sufficient to observe any sex difference.[21] Therefore, future studies should explore the mechanisms that can explain the role of DB[*a,l*]P, and MmuPV1 infection individually and in combination in the development of oral cancer. Thus, the second goal in our study (Bioassay II) is to compare the effects of MmuPV1 infection on levels of DB[*a,l*]P-induced DNA damage in immune-deficient male and female mice; the rationale of employing immune-deficient mice is to ensure that the viral infection was retained at the time of DNA adduct analysis.

RESULTS AND DISCUSSION

We found that the DB[*a,l*]P-induced covalent DNA damage levels in immune competent mice measured by liquid chromatography−mass spectrometry LC−MS/MS as dA adducts (Figure 2A,B) are significantly (p = 0.008) higher in the oral cavity in females (0.76 ± 0.09 adducts/10^6 dA) than in males (0.50 ± 0.06 adducts/10^6 dA). Despite the limited number of mice (n = 3), the covalent adduct levels in DB[*a,l*]P treated immune competent mice remain significantly higher than those observed in dimethyl sulfoxide (DMSO)-treated mice and are comparable to those reported in numerous previous studies conducted in our lab using more than three female immune competent mice per group.[8,22]

Independent of the viral infections in nude mouse (Figure 3), the DB[*a,l*]P-dA adduct levels in females are significantly higher than in males (p < 0.05). Specifically, in females with

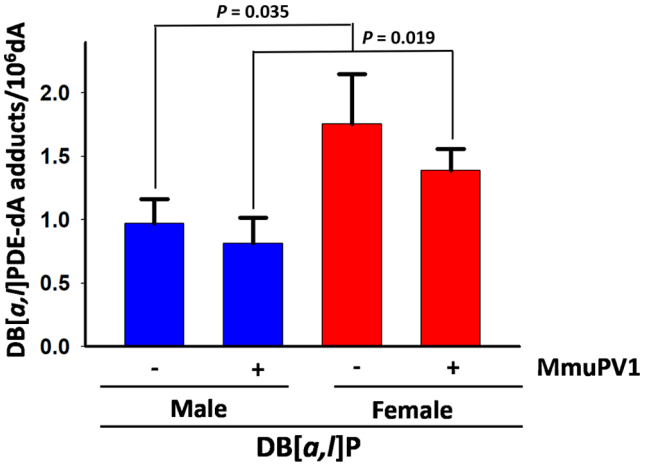

Figure 3. Observed effects of DB[*a,l*]P alone and in combination with the MmuPV1 on covalent DNA adducts induced by DB[*a,l*]P in the mouse oral cavity.

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and without virus, the levels of DB[*a*,*l*]P-dA adducts are 1.39 ± 0.17 and 1.76 ± 0.39 DB[*a*,*l*]PDE-dA adducts/10^6 dA, respectively; in males with and without virus, the levels of DB[*a*,*l*]P-dA adducts are 0.82 ± 0.20 and 0.97 ± 0.19 DB[*a*,*l*]PDE-dA adducts/10^6 dA, respectively. Upon close examination of the results presented in Figure 3, we found that viral infection resulted in lower levels of DNA adducts, but the effect was not statistically significant, and the mechanistic basis that can account for this observation requires further investigation.

The detection of DB[*a*,*l*]P has been described in environmental sources including soil and sediment samples as well as in cigarette smoke and human exposure to this carcinogen has been reported.[23,24] More recently, we reported the detection of DB[*a*,*l*]P-induced DNA damage in smokers' buccal cells suggesting the potential role of DB[*a*,*l*]P in the development of OSCC in smokers.[25] We have previously estimated that human exposure to this carcinogen via numerous environmental pollutants and lifetime cigarette smoking is comparable to the dose that can induce OSCC in mice.[8,16,26] In fact, we previously showed that DB[*a*,*l*]P-induced covalent DNA damage can account for the mechanisms of oral cancer induction in mice.[8]

The results of our studies clearly demonstrate that the levels of covalent DNA damage induced by DB[*a*,*l*]P are significantly higher in female mice than in male mice uninfected or infected with mouse papillomavirus. In animal models, gender-based differences were previously recognized since 1932 when female rats were reported to require only half the dose that was needed by male rats to induce sleep following the administration of barbiturate.[27] Gender-dependent metabolism in rodents is usually the consequence of differences in hepatic enzymes expression;[28] it is also true that gender dependence is existed in a number of fundamental pharmacokinetic parameters in a wide variety of animal species.[29] Several intriguing questions that arise from our results remain to be answered. Literature data show that other than the major risk factors (tobacco smoke, alcohol, and HPV infection) for HNSCC, female hormones may also contribute to disease development.[4] However, the molecular mechanism that can account for the role of hormones on the multistep carcinogenesis process of DB[*a*,*l*]P-induced OSCC in males and females remains to be explored.

Cytochrome P450 1B1 (the major enzyme) along with 1A1 and 3A4 are known to catalyze the metabolism of several tobacco smoke constituents including DB[*a*,*l*[P and those derived from 17β-estradiol to active metabolites that can damage DNA.[29,30] Consistent with our current results, exposure to estrogen was found to induce the protein expression of *CYP1B1* in the human oral leukoplakia cells; these studies provide important insights in mechanisms underlying the sex differences in susceptibility of head and neck carcinogenesis.[5] CYP1A1 and CYP1B1 are the major enzymes which catalyze the formation of DB[*a*,*l*]PDE (ultimate carcinogenic metabolite) derived from DB[*a*,*l*]P.[9] However, the relative contribution of phase I and II enzymes to metabolically activate DB[*a*,*l*]P leading to DNA damage in male and female mice remains to be determined. Furthermore, literature data, including our own, have demonstrated the induction of CYP1A1 and CYP1B1 expressions by cigarette smoke condensate and E-cigarette aerosol in oral cells through the activation of the aryl hydrocarbon receptor.[27,31,32] Taken together, estrogen could make the female oral cavity more susceptible to damage in response to cigarette smoke by acting as inducers of cytochrome P450 (CYP) enzymes to metabolize cigarette smoke components to active metabolites that can lead to the induction of DNA damage in oral tissues.

In the multistep carcinogenesis process, DNA adducts formed from various chemical carcinogens (including DB[*a*,*l*]-P) is a known prerequisite step; therefore, adduct formation is considered as a valid biomarker to assess cancer risk. Therefore, prior to the long-term and costly carcinogenesis bioassay, the goal of this study was to initially test our hypothesis in a short-term animal study by comparing, under identical conditions in male and female mice, the levels of DB[*a*,*l*]P-induced DNA damage, which is known to induce OSCC as demonstrated in our previous report that included only female mice.[16] The results presented in this report do not support our hypothesis since we clearly demonstrated that independent of the virus infection, female mice are more susceptible to DNA damage-induced by DB[*a*,*l*]P than males. Since DNA damage represents only a single step in the multistep carcinogenesis process, our future studies still focus on examining additional factors beyond DNA damage such as epigenetic and immunological regulation that may contribute to sex difference in HNSCC in humans.

METHODS

Our animal studies were conducted according to ARRIVE guidelines 2.0 and the NIH, USA, and AAALAC International Regulations. The Institutional Animal Care and Use Committee of the Penn State College of Medicine reviewed and approved all experiments prior to their initiation. As described in our previous study,[16] at 6 weeks of age (Bioassay I), the oral cavity of male and female immuno-competent mice (B6C3F1, n = 3/sex, body weights in males and females were comparable) were topically treated with DB[*a*,*l*]P that had been dissolved in DMSO as the vehicle (24 nmol, three times per week for a period of 7 weeks); DMSO was used in the treatment of control mice (n = 3/sex). Three hours after the last dose of the carcinogen treatment, these mice were sacrificed. Methods of oral tissue collection, DNA isolation, and assessment of DNA damage using LC−MS/MS were identical to those reported by us.[8,22,25,33] Briefly, we conducted the analysis of the DB[*a*,*l*]P-dA adduct by LC−MS/MS using a QTRAP 6500+ mass spectrometry coupled with an EXion HPLC system (Sciex), and a 1.7 μm Acquity UPLC BEH C18 column was used to separate analytes. The DB[*a*,*l*]P-dA adducts were monitored using the transitions of m/z 604 → m/z 335 and m/z 609 → m/z 335 for targeted adducts and the internal standard, [$^{15}N_5$]-DB[*a*,*l*]PDE-dA (150 pg, with all five ^{15}N-labeled in the adenine moiety), respectively. The fragmentation patterns of the molecular ions derived from the targeted adducts (m/z 604) includes fragments of m/z 488, 353, 335, 317, and 307, with fragment 335 (after the loss of a deoxyadenosyl fragment and a molecule of H_2O) given the strongest signal.[33] All samples in each study were analyzed side by side on the same day.

We infected athymic mouse NU/J mice (Jackson Laboratory, Farmington, CT) with MmuPV1 in the oral tissues (Bioassay II) using procedures previously described by us.[34] We treated male or female mice by topical application of DB[*a*,*l*]P in DMSO (24 nmol, 3 times per week for 7 weeks) into the oral cavity at 2 weeks after virus infection as described in Bioassay I.[16] Briefly, four cohorts of mice per sex (n = 3 each) were used including: (1) mice which were infected with

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MmuPV1 and orally treated with DMSO-saline; (2) mice which were infected with MmuPV1 and orally treated with the DB[*a*,*l*]P; (3) mice which were not infected and orally treated with DMSO-saline; and (4) mice which were not infected and orally treated with DB[*a*,*l*]P. Although we did not anticipate any histological changes under our short-term duration of the bioassay,[16] mice were monitored weekly for any morphological abnormalities around the mouth and muzzle area as well as body weights. At 8 weeks postinfection, we terminated the experiment and collected oral tissues of mice 48 h after the last carcinogen treatment to analyze DNA damage and viral genomes by Q-PCR as previously described by us.[34] We isolated DNA from the above-mentioned mouse oral tissues and the major deoxyadenosine adducts (dA) derived from DB[*a*,*l*]P was analyzed by LC−MS/MS using the method reported previously by us.[8,22,25,33]

The levels of viral DNA in MmuPV1 infected mice were measured among the various groups defined by gender and DB[*a*,*l*]P treatment status at the time of sacrifice. In male mice infected with MmuPV1, the average viral DNA copy number is $11.33 \pm 11.56 \times 10^5$, and in infected females, the average viral DNA copy number is $16.18 \pm 10.03 \times 10^5$. In contrast to those infected mice, we did not detect viral genomes in any of the mice that did not receive MmuPV1 infections. We used the lmer function of the lmer R package[35] to fit linear mixed-effects models by the factors of gender, treatment status, and time. Nested models were used to compare the impact of factors of interest, as described above. DNA damage was statistically analyzed by Student's *t*-test.

AUTHOR INFORMATION

Corresponding Author

Karam El-Bayoumy − *Department of Biochemistry & Molecular Biology, Penn State College of Medicine, Hershey, Pennsylvania 17033-2360, United States;* orcid.org/0000-0002-4198-0696; Email: kee2@psu.edu

Authors

Kun-Ming Chen − *Department of Biochemistry & Molecular Biology, Penn State College of Medicine, Hershey, Pennsylvania 17033-2360, United States;* orcid.org/0000-0002-3820-6149

Yuan-Wan Sun − *Department of Biochemistry & Molecular Biology, Penn State College of Medicine, Hershey, Pennsylvania 17033-2360, United States;* orcid.org/0000-0002-2028-3533

Jiafen Hu − *The Jake Gittlen Laboratories for Cancer Research and Department of Pathology, Penn State College of Medicine, Hershey, Pennsylvania 17033-2360, United States*

Karla Balogh − *The Jake Gittlen Laboratories for Cancer Research, Penn State College of Medicine, Hershey, Pennsylvania 17033-2360, United States*

Krishne Gowda − *Department of Pharmacology, Penn State College of Medicine, Hershey, Pennsylvania 17033-2360, United States;* orcid.org/0000-0002-6042-0520

Cesar Aliaga − *Department of Biochemistry & Molecular Biology, Penn State College of Medicine, Hershey, Pennsylvania 17033-2360, United States*

Dongxiao Sun − *Department of Pharmacology, Penn State College of Medicine, Hershey, Pennsylvania 17033-2360, United States*

Neil Christensen − *The Jake Gittlen Laboratories for Cancer Research and Department of Pathology, Penn State College of Medicine, Hershey, Pennsylvania 17033-2360, United States*

Shantu Amin − *Department of Pharmacology, Penn State College of Medicine, Hershey, Pennsylvania 17033-2360, United States;* orcid.org/0000-0003-2780-2567

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c09611

Author Contributions

Karam El-Bayoumy's contribution included conceptualization, data analysis and interpretation, design and supervision of the study, and wrote the first draft of the manuscript. **Neil D. Christensen's** contribution included conceptualization, design, analyzed and interpreted the data, and assisted in writing the manuscript. **Kun-Ming Chen** performed the laboratory experiments regarding the analysis of carcinogen-induced covalent DNA damage in the mouse oral cavity. **Jiafen Hu's** contribution included conceptualization, writing, analyzing, and interpreting the data. **Yuan-Wan Sun** performed the laboratory experiments which focuses on assessing the effect of the carcinogen on DNA damage. **Cesar Aliaga** performed all animal experiments, sacrifice, and oral tissue collections. **Karla K. Balogh** was involved in animal treatment with the mouse virus. **Krishne Gowda** synthesized the carcinogen. **Shantu Amin's** contribution included design of the synthesis and structural confirmation of the carcinogen used in this study. **Dongxiao Sun** was involved in the analysis of DNA damage using LC/MS−MS.

Notes

The authors declare no competing financial interest.

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