Toxicity and Carcinogenicity of Ozone in Combination with 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone and Dibutyl Phthalate in B6C3F1 Mice for 16 and 32 Weeks[1]

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Objective To evaluate the toxic and carcinogenic potential of ozone alone or in combination with 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and/or dibutyl phthalate (DBP). **Methods** Male and female B6C3F1 mice were exposed, through inhalation, intravenous administration and diet, to 0.5 ppm of ozone, 1.0 mg/kg of NNK and 5000 ppm of DBP, individually and in combination for 16 and 32 weeks. **Results** No treatment-related death was seen, but significant differences in body and organ weights between control and treated mice were observed during the study. No incidence of lung tumor incidence was recorded in mice exposed to either ozone alone or combined treatment. Oviductal carcinomas were observed in female mice exposed to ozone or DBP alone for 16 weeks and ozone in combination with NNK and DBP for 32 weeks. **Conclusion** Although ozone alone and in conjunction with NNK and/or DBP does not induce lung cancer under our experimental conditions, they induce oviductal carcinomas in B6C3F1 mice.

Key words: Ozone; NNK; DBP; Combined treatment; Toxicity; Carcinogenicity

INTRODUCTION

Ozone, a common urban area pollutant, is formed by reactions of ambient nitrogen oxides and volatile organic compounds (VOCs) in the presence of sunlight and heat. Ozone is a potent oxidant of biomolecules. Inhaled ozone is degraded quickly into molecular oxygen and oxygen free radicals, which in turn combine with water to form highly oxidative hydroxyl radicals that damage nucleic acids, lipids, and proteins[1]. Ozone elicits inflammation, hyperreactivity and epithelial damage of airways, as well as altered ventilation and weakened pulmonary function[2]. Recent epidemiological studies have shown that increased ozone exposure is associated with a risk of developing cancer[3-4]. Animal studies have also demonstrated that ozone exposure can induce lung tumors[5-7]. However, the exact carcinogenic mechanism of ozone remains largely uncharacterized.

Several nitrosamines derived from tobacco alkaloids are carcinogenic to laboratory animals[8-9]. Among them, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is not only a potent lung carcinogen in rodents but also a potential causative factor in human lung carcinogensis[8-9]. Dibutyl phthalate (DBP) is mainly used as a plasticizer in polyvinyl chloride (PVC) plastics and to a lesser degree in paints, adhesives, cosmetics[10-11] and infant formula[12]. Recently, it has been suggested that DBP, a suspected endocrine disruptor, may contribute to the development of hormone-dependent cancers, such as breast and endometrial cancer[10].

Most toxicological studies have tested single chemical agents at relatively high doses, and fewer studies have addressed the toxic effects of chemical interactions. It is important to understand the toxicity of chemical mixtures in order to assess more realistic risks of environmental and occupational exposures. Although the respective toxicological action of ozone, NNK and DBP has been extensively studied, few *in vivo* studies are available on the cacinogenicity and

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toxicity induced by combination of these toxicants. Therefore, to elucidate the interactive pathological effect of NNK and DBP on ozone, we have examined the potential carcinogenicity of subchronic ozone exposure in the presence or absence of NNK and/or DBP in B6C3F1 mice.

MATERIALS AND METHODS

Chemicals and Diet

NNK was purchased from Chemsyn Science Laboratories (Lenexa, KS). Trioctanoin was obtained from Wako Chemical (Japan). DBP (CAS NO. 84-74-2) was acquired from Sigma Co. (St. Louis, MO).

Fresh diet containing DBP was prepared once every week. A predetermined amount of DBP was weighed, added to a small aliquot of ground basal diet, and hand blended. This stock was then added to a preweighed ground basal diet and blended in a mill for 30 min. A number of batches were analyzed by HPLC for actual incorporation levels of the test compound, its homogenous distribution and stability.

Animals

All methods used in this study were approved by the Animal Care and Use Committee at SNU and conform to the NIH guidelines (NIH publication No.86-23, revised 1985). Male and female B6C3F1 mice, 4-5 weeks of age, were purchased from Seoul National University (SNU) Laboratory Animal Facility (Seoul, Korea). The room temperature and humidity were 23±2 ℃ and 50%±20%, respectively, with a 12 h light/dark cycle. After acclimation for 7 days, animals were divided into groups with no statically difference in group-mean body weights and no significant lesions.

Experimental Design

Mice were exposed to ozone (0.50±0.02 ppm) in 1.5 m^3 whole body inhalation exposure chambers (Air Dynamics Inc., San Angelo, TX), lasting for 16 and 32 weeks , 5 days per week (6 h per day). Ozone was generated by pure oxygen using a silent electric arc discharge ozonator (Model KDA-8, Sam-Il Environment Technology, Pusan, Korea) and mixed with a main stream of filtered air before entering the exposure chamber. Ozone concentrations in the chamber were monitored through a gas detection system with O_3 gas sensor (Analytical Technology, Oaks, PA). Measurements were taken from 12 locations in each chamber to ensure the uniformity of ozone distribution, with mixing further enhanced using a recirculation device. Airflow in the chambers was maintained at 15 changes per hour. Ambient ozone was removed from the air entering all chambers using a potassium permanganate filter, along with charcoal and HEPA filters. The animals were individually housed in a suspended stainless steel wire-mesh cage which allowed observation of all individually housed animals during inhalation exposure, and provided with food and water *ad libitum*, except during the 6 h inhalation exposure. Each animal's location in the chamber was rotated on a weekly basis throughout the study.

During the experiment, mice were subcutaneously injected with 1.0 mg NNK per kg body weight in trioctanoin, three times a week. They also received diets containing DBP at a concentration of 5 000 ppm for 16 and 32 weeks. The concentration of each test material was determined based on the National Toxicology Program (NTP) Carcinogenesis Study (NTP, 1994, 1995). The experimental groups were as follows: (a) unexposed control group, (b) group exposed to 0.5 ppm ozone, (c) group exposed to 1.0 mg NNK/kg body weight, (d) group exposed to 5 000 ppm DBP, (e) co-treatment of ozone with NNK, (f) co-treatment of ozone with DBP, (g) co-treatment of ozone with NNK plus DBP. Each group consisted of 20 males and 20 females.

Clinical signs were monitored twice daily for morbidity and mortality while the body were measured once a week and just prior to sacrifice.

Histopathological Examination

Any animals died prior to study termination and those killed at terminal sacrifice were subjected to complete necropsy. Absolute and relative weights of liver, lung, kidney, adrenal gland, testis, and ovary were measured. Approximately 12 tissue samples and all gross lesions were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H & E) for histopathological examination under light microscope.

Statistical Analysis

Data were expressed as $\bar{x} \pm s$ for each parameter. Data from the unexposed control and treated groups were analyzed with ANOVA and Student's *t*-test using the SigmaStat and SPSS statistical software. $P<0.05$ was considered statistically significant.

RESULTS

Survival, Body Weight, and Clinical Findings

During the experiment, no treatment-related death occurred. The mean body weights of mice of

both genders exposed to ozone alone were not significantly different from the controls, while treatment with ozone in combination with NNK and DBP led to 32.5% and 28% of body weight loss at 32 weeks, as compared with the controls ($P<0.05$) (data not shown). Clinical observations in mice were generally unremarkable. Hypoactivity and ruffled fur were observed in mice of both genders during and immediately after ozone exposure.

Pathological Evaluation

The absolute and relative weights of those organs exhibiting significantly increased or decreased weights after treatments are listed in Tables 1-4. A prominent treatment-related weight increase of the lung and liver of both genders was seen. An increase in liver and lung weight, except absolute weight of liver, was observed in male and female mice of the treated groups.

TABLE I

Absolute and Relative Organ Weights of the B6C3F1 Male Mice Treated for 16 Weeks ($\bar{x} \pm s$, n=20)

	Control	Ozone	NNK	DBP	Ozone + NNK	Ozone + DBP	Ozone + NNK + DBP
Liver							
Absolute (g)[a]	1.62±0.26	1.51±0.18	1.41±0.07*	1.21±0.22*	1.31±0.11*	1.11±0.12*	1.24±0.19*
Relative (%)[b]	3.948±0.679	4.112±0.420	4.212±0.721*	4.041±0.242	4.121±0.511	4.109±0.410	4.120±0.326
Lung							
Absolute (g)	0.21±0.02	0.25±0.03*	0.23±0.01	0.25±0.03*	0.29±0.070*	0.23±0.02	0.29±0.06*
Relative (%)	0.674±0.068	0.727±0.072*	0.714±0.109	0.712±0.034	0.732±0.104*	0.712±0.041	0.730±0.107*
Kidney (L)							
Absolute (g)	0.29±0.03	0.34±0.03*	0.35±0.02*	0.37±0.03*	0.34±0.01*	0.38±0.02*	0.34±0.01*
Relative (%)	0.812±0.119	0.967±0.081*	0.971±0.126*	0.954±0.098*	0.902±0.104*	0.972±0.091*	0.944±0.109*
Kidney (R)							
Absolute (g)	0.30±0.04	0.31±0.03	0.32±0.02	0.34±0.07*	0.32±0.08	0.29±0.05	0.32±0.02
Relative (%)	0.836±0.130	0.843±0.02	0.821±0.027	0.841±0.01	0.926±0.097*	0.941±0.07*	0.957±0.023*
Adrenal (L)							
Absolute (g)	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.000	0.01±0.00	0.01±0.00	0.01±0.00
Relative (%)	0.029±0.001	0.031±0.001*	0.030±0.002	0.028±0.001	0.028±0.002	0.030±0.001	0.031±0.002*
Adrenal (R)							
Absolute (g)	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
Relative (%)	0.029±0.001	0.028±0.001	0.031±0.008	0.030±0.001	0.031±0.003	0.029±0.002	0.031±0.004
Testis (L)							
Absolute (g)	0.20±0.03	0.18±0.03	0.18±0.02	0.18±0.02	0.18±0.02	0.19±0.03	0.18±0.02
Relative (%)	0.380±0.075	0.394±0.036*	0.378±0.051	0.369±0.071	0.368±0.012*	0.361±0.041*	0.372±0.032*
Testis (R)							
Absolute (g)	0.2±0.01	0.17±0.04	0.17±0.02	0.15±0.03*	0.19±0.02	0.18±0.01	0.19±0.02
Relative (%)	0.339±0.079	0.371±0.046*	0.351±0.038*	0.351±0.066*	0.348±0.035*	0.347±0.061*	0.332±0.030*

Note. [a]Absolute weight in grams. [b]Relative weight in %=organ weight (g)/body weight (g)×100. *Significantly different from control at $P<0.05$.

TABLE 2

Absolute and Relative Organ Weights of the B6C3F1 Female Mice Treated for 16 Weeks ($\bar{x} \pm s$, n=20)

	Control	Ozone	NNK	DBP	Ozone + NNK	Ozone + DBP	Ozone + NNK+ DBP
Liver							
Absolute (g)[a]	1.3±1.325	1.21±0.12	0.99±0.11*	1.10±0.18	1.12±0.22	1.12±0.19	1.32±3.12
Relative (%)[b]	3.234±0.451	3.610±0.541*	3.210±0.168	3.642±0.125*	3.542±0.342*	3.331±0.441	3.651±3.10*
Lung							
Absolute (g)	0.18±0.01	0.20±0.03	0.24±0.04	0.31±0.02*	0.34±0.02*	0.41±0.01*	0.38±0.03*
Relative (%)	0.756±0.147	0.704±0.109	0.843±0.164*	0.842±0.361*	0.776±0.331	0.810±0.167	0.824±0.188
Kidney (L)							
Absolute (g)	0.17±0.02	0.20±0.02*	0.20±0.02*	0.21±0.02	0.18±0.01	0.19±0.03	0.19±0.01
Relative (%)	0.621±0.110	0.725±0.086*	0.708±0.076*	0.741±0.056*	0.761±0.041*	0.721±0.021*	0.0741±0.033*

(to be continued)

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	Control	Ozone	NNK	DBP	Ozone + NNK	Ozone + DBP	Ozone + NNK+ DBP
Kidney (R)							
Absolute (g)	0.18 ±0.03	0.20 ±0.02	0.20 ±0.02	0.20 ±0.01	0.22 ±0.01*	0.24 ±0.02*	0.20 ±0.03
Relative (%)	0.610 ±0.105	0.692 ±0.068*	0.719 ±0.093*	0.724 ±0.061*	0.721 ±0.054*	0.708 ±0.046*	0.731 ±0.061*
Adrenal (L)							
Absolute (g)	0.03 ±0.00	0.02 ±0.00	0.01 ±0.00	0.01 ±0.01	0.02 ±0.01	0.03 ±0.01	0.03 ±0.01
Relative (%)	0.035 ±0.001	0.031 ±0.001	0.036 ±0.002	0.035 ±0.02	0.036 ±0.01	0.036 ±0.00	0.036 ±0.01
Adrenal (R)							
Absolute (g)	0.03 ±0.00	0.01 ±0.00*	0.01 ±0.00*	0.02 ±0.01	0.03 ±0.01	0.03 ±0.01	0.02 ±0.02
Relative (%)	0.035 ±0.001	0.035 ±0.001	0.036 ±0.002	0.036 ±0.01	0.035 ±0.01	0.035 ±0.01	0..036 ±0.02
Ovary (L)							
Absolute (g)	0.01 ±0.01	0.02 ±0.00	0.02 ±0.01	0.02 ±0.001	0.02 ±0.00	0.01 ±0.01	0.02 ±0.01
Relative (%)	0.056 ±0.017	0.060 ±0.017	0.064 ±0.016*	0.062 ±0.015	0.071 ±0.021*	0.064 ±0.013*	0.060 ±0.012
Ovary (R)							
Absolute (g)	0.01 ±0.01	0.02 ±0.00	0.02 ±0.00	0.02 ±0.00	0.02 ±0.01	0.02 ±0.01	0.02 ±0.01
Relative (%)	0.049 ±0.011	0.064 ±0.015*	0.064 ±0.016*	0.051 ±0.018	0.053 ±0.019	0.052 ±0.021	0.053 ±0.019

Note. [a]Absolute weight in grams. [b]Relative weight in %=organ weight (g)/body weight (g) ×100. *Significantly different from control at $P<0.05$.

TABLE 3

Absolute and Relative Organ Weights of the B6C3F1 Male Mice Treated for 32 Weeks ($\bar{x} \pm s$, n=20)

	Control	Ozone	NNK	DBP	Ozone + NNK	Ozone + DBP	Ozone + NNK+ DBP
Liver							
Absolute (g)[a]	1.56 ±0.32	1.71 ±0.31*	1.62 ±0.24*	1.72 ±0.34*	1.74 ±0.33*	1.64 ±0.28*	1.66 ±0.31*
Relative (%)[b]	3.917 ±0.511	4.110 ±0.502	3.819 ±0.487	4.221 ±0.841*	3.841 ±0.386	4.123 ±0.446	4.871 ±0.369*
Lung							
Absolute (g)	0.26 ±0.03	0.34 ±0.02*	0.31 ±0.01	0.24 ±0.06	0.33 ±0.03*	0.41 ±0.02*	0.36 ±0.02*
Relative (%)	0.674 ±0.068	0.691 ±0.046*	0.698 ±0.033*	0.702 ±0.042*	0.709 ±0.041*	0.771 ±0.039*	0.721 ±0.0510*
Kidney (L)							
Absolute (g)	0.27 ±0.03	0.33 ±0.02*	0.30 ±0.01	0.26 ±0.04	0.31 ±0.02	0.40 ±0.04*	0.35 ±0.03*
Relative (%)	0.807 ±0.124	0.702 ±0.034*	0.691 ±0.022*	0.681 ±0.021*	0.710 ±0.035*	0.761 ±0.046*	0.718 ±0.043*
Kidney (R)							
Absolute (g)	0.28 ±0.02	0.31 ±0.02*	0.29 ±0.01	0.27 ±0.02	0.28 ±0.03	0.26 ±0.01	0.27 ±0.02
Relative (%)	0.824 ±0.110	0.831 ±0.126*	0.831 ±0.224*	0.814 ±0.221*	0.824 ±0.261	0.831 ±0.261*	0.834 ±0.316*
Adrenal (L)							
Absolute (g)	0.02 ±0.00	0.02 ±0.01	0.03 ±0.01*	0.03 ±0.01	0.02 ±0.00	0.02 ±0.01	0.03 ±0.01*
Relative (%)	0.032 ±0.003	0.028 ±0.002	0.029 ±0.004	0.031 ±0.002	0.030 ±0.010	0.038 ±0.005*	0.027 ±0.003*
Adrenal (R)							
Absolute (g)	0.02 ±0.01	0.02 ±0.01	0.03 ±0.01	0.02 ±0.00	0.02 ±0.01	0.02 ±0.00	0.03 ±0.01
Relative (%)	0.034 ±0.001	0.031 ±0.002	0.029 ±0.01*	0.036 ±0.002	0.034 ±0.001	0.033 ±0.003	0.029 ±0.002*
Testis (L)							
Absolute (g)	0.12 ±0.01	0.13 ±0.01	0.14 ±0.02*	0.12 ±0.01	0.13 ±0.02	0.12 ±0.03	0.13 ±0.01
Relative (%)	0.370 ±0.022	0.369 ±0.021	0.354 ±0.034*	0.364 ±0.02	0.391 ±0.04*	0.371 ±0.02	0.389 ±0.02*
Testis (R)							
Absolute (g)	0.14 ±0.06	0.13 ±0.04	0.14 ±0.02	0.13 ±0.01	0.14 ±0.05	0.13 ±0.02	0.14 ±0.03
Relative (%)	0.314 ±0.071	0.302 ±0.069*	0.319 ±0.03	0.310 ±0.02	0.326 ±0.03*	0.310 ±0.02	0.322 ±0.04*

Note. [a]Absolute weight in grams. [b]Relative weight in %= organ weight (g)/body weight (g) ×100. *Significantly different from control at $P<0.05$.

TABLE 4

Absolute and Relative Organ Weights of the B6C3F1 Male Mice Treated for 32 Weeks ($\bar{x} \pm s$, n=20)

	Control	Ozone	NNK	DBP	Ozone + NNK	Ozone + DBP	Ozone + NNK+ DBP
Liver							
Absolute (g)[a]	1.13 ±0.23	1.24 ±0.36*	1.42 ±0.44*	1.33 ±0.46*	1.20 ±0.39	1.43 ±0.21*	1.53 ±0.44*
Relative (%)[b]	2.987 ±0.312	2.681 ±0.441*	2.991 ±0.484	3.120 ±0.512	3.221 ±0.610	3.108 ±0.550	3.264 ±0.612
Lung							
Absolute (g)	0.36 ±0.06	0.41 ±0.09*	0.42 ±0.10*	0.56 ±0.18*	0.51 ±0.14*	0.49 ±0.18*	0.32 ±0.17
Relative (%)	0.756 ±0.147	0.816 ±0.112*	0.824 ±0.133*	0.832 ±0.107*	0.814 ±0.120*	0.802 ±0.112*	0.789 ±0.120*
Kidney (L)							
Absolute (g)	0.18 ±0.03	0.17 ±0.02	0.18 ±0.02	0.18 ±0.04	0.19 ±0.01	0.18 ±0.01	0.17 ±0.01
Relative (%)	0.482 ±0.080	0.492 ±0.061	0.421 ±0.032*	0.431 ±0.024*	0.552 ±0.031*	0.542 ±0.066*	0.530 ±0.034*
Kidney (R)							
Absolute (g)	0.17 ±0.01	0.18 ±0.01	0.19 ±0.02	0.18 ±0.01	0.17 ±0.02	0.16 ±0.01	0.18 ±0.02
Relative (%)	0.511 ±0.213	0.481 ±0.203*	0.502 ±0.310	0.513 ±0.331	0.512 ±0.121	0.521 ±0.03*	0.531 ±0.06*
Adrenal (L)							
Absolute (g)	0.01 ±0.00	0.01 ±0.00	0.01 ±0.02	0.01 ±0.00	0.01 ±0.00	0.02 ±0.01	0.01 ±0.09
Relative (%)	0.035 ±0.001	0.031 ±0.002*	0.034 ±0.002	0.032 ±0.001*	0.036 ±0.002	0.033 ±0.003	0.040 ±0.001*
Adrenal (R)							
Absolute (g)	0.01 ±0.01	0.01 ±0.00	0.02 ±0.01	0.01 ±0.00	0.01 ±0.01	0.01 ±0.01	0.01 ±0.00
Relative (%)	0.035 ±0.001	0.034 ±0.002	0.034 ±0.001	0.036 ±0.002	0.035 ±0.001	0.036 ±0.02	0.031 ±0.03*
Ovary (L)							
Absolute (g)	0.01 ±0.01	0.01 ±.00	0.01 ±0.01	0.01 ±0.00	0.01 ±0.00	0.01 ±0.01	0.01 ±0.00
Relative (%)	0.060 ±0.021	0.061 ±0.031	0.058 ±0.034	0.064 ±0.028	0.068 ±0.054*	0.057 ±0.036	0.061 ±0.040
Ovary (R)							
Absolute (g)	0.01 ±0.01	0.01 ±.00	0.01 ±.01	0.01 ±0.00	0.02 ±0.01	0.01 ±0.00	0.02 ±0.01
Relative (%)	0.051 ±0.018	0.054 ±0.019	0.049 ±0.017	0.048 ±0.021	0.052 ±0.018	0.046 ±0.016*	0.048 ±0.017

Note. [a] Absolute weight in grams. [b] Relative weight in %=organ weight (g)/body weight (g)×100. *Significantly different from control at P<0.05.

There was no test materials-related incidence of lung neoplasms during the exposure period. We neither found any lesions of lung in 16 weeks after treatment. However, microscopic examination of the lungs in 32 weeks after treatment revealed partial congestion and hemorrhage (10% of males treated with ozone only), peribronchial mononuclear cell infiltration (10% of males treated with ozone + NNK + DBP), focal bronchiolar alveolar hyperplasia (10% of males and 10% of females treated with ozone only, 20% of females treated with NNK only, and 30% of females treated with ozone + NNK + DBP), bronchiolar epithelium hyperplasia (10% of females treated with NNK only) and alveolar fibrosis (10% of males treated with NNK alone and 10% of females treated with ozone + NNK + DBP) (Fig. 1).

There was no incidence of tumor formation in the liver. Hepatocyte vacuolation (10% of males treated with ozone only, 10% of males and 20% of females treated with NNK only, and 20% of males and 10% of females treated with ozone + NNK + DBP) was seen after 32 weeks in the test groups.

Oviductal carcinoma occurred in 16 weeks after treatment with ozone (10%) and DBP (10%) individually and in 32 weeks after treatment with ozone in combination with NNK and DBP (10%) (Fig. 2). Oviductal hyperplasis occurred in 32 weeks after treatment with ozone in combination with NNK and DBP (10%) (Fig. 2).

Other microscopic findings in kidney, brain, testis, and uterus in 32 weeks after treatment were vacuolar degeneration in kidney (10% of males treated with ozone + NNK + DBP), gliosis and congestion in cerebrum (10% of males and 20% of females treated with ozone + NNK + DBP, respectively), and meningoencephalitis with focal hemorrhage (10% of females treated with ozone + NNK) in brain, dysspermia in testis (10% of males treated with ozone, and 20% of females treated with ozone + NNK + DBP), and mucosal emphysema alteration in uterus (10% of males treated with ozone + NNK).

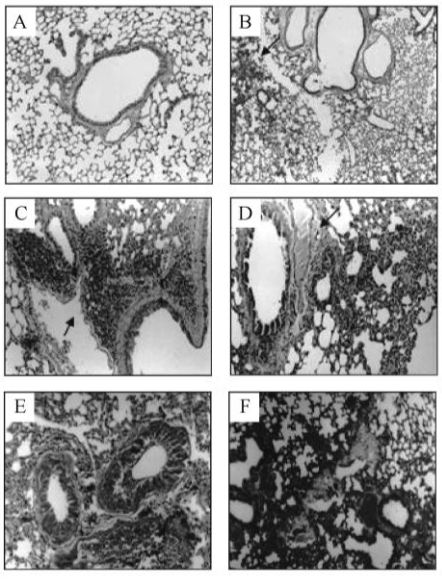

FIG. 1. Histopathology of the lung in mice exposed to test materials for 32 weeks showing normal lung in the control group (A), partial congestion and hemorrhage (B), peribronchial mononuclear cell infiltration (C), focal bronchiolar alveolar hyperplasia (D), bronchiolar epithelium hyperplasia (E), alveolar fibrosis (F) in the test groups (H & E stain, ×100).

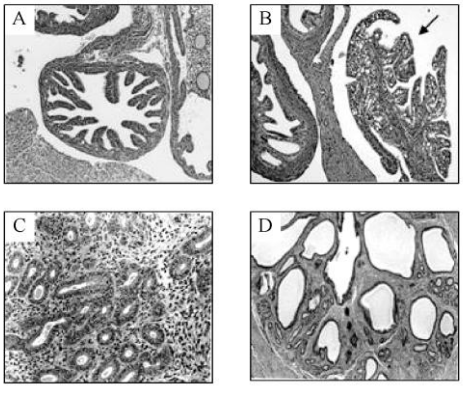

FIG. 2. Histopathology of the oviduct in mice exposed to test materials for 16 and 32 weeks showing normal oviduct in control group (A), hyperplasia (B), adenocarcinoma (C and D) of oviduct in treated groups (H & E stain, ×200).

DISCUSSION

Although a growing number of chemicals are introduced into the market on a weekly basis, risk assessment activities are largely concerned with individual chemical exposures, rather than with multiple chemical exposures. This study evaluated whether exposure to NNK and/or DBP would influence the carcinogenic responses in male and female B6C3F1 mice when exposed to ozone via inhalation for 16 and 32 weeks. No ozone-related death occurred in our study, though decreases in mean body weights of mice of both genders exposed to 0.5 ppm ozone were sporadically documented (data not shown), which is consistent with our previous result[13]. Catalano *et al.*[14] reported that the survival rate and mean body weight of male and female mice exposed to 0.5 ppm ozone were similar to those of the controls. However, treatment with ozone in combination with NNK or in combination with NNK and DBP resulted in 30% body weight loss in this study, indicating that combined treatment with NNK and/or DBP could modulate the adverse clinical effects of ozone.

Several studies are available on the carcinogenic potential of ozone[6,15-18]. However, no conclusive evidence exists to link ozone exposure to lung cancer in experimental animals. It was reported that ozone only gave a weak carcinogenic response in A/J and B6C3F1 mice[6,15-16]. However, other studies showed that ozone did not show its carcinogenic potential in A/J and C57BL/6 mice[17-18], indicating that ozone could slightly increase the incidence of lung tumor depending on the mouse strain used and exposure time. Previous studies showed that ozone inhalation increased the incidence of metaplasia in the nose and lung of mice exposed to ozone only or combined ozone and NNK[15-16], which is in line with our results. In this study, similar pathological lesions were observed after treatment with ozone or NNK alone (Fig. 1). Treatment with ozone in combination with NNK and DBP led to the incidence of these lesions, which were presumably associated with the toxicological interaction between ozone and NNK.

Oviductal carcinoma was observed in 16 weeks after treatment with ozone or DBP alone and in 32 weeks after treatment with ozone in combination with NNK and DBP (Fig. 2). Neoplastic cells were large and cuboidal, with abundant cytoplasm, large nuclei that were often vesicular, and mitotic figures were rare. Some tumors appeared as stroma (Fig. 2). We have recently reported the incidence of oviductal carcinoma in B6C3F1 mice in 12 weeks after exposure to 0.5 ppm ozone[13]. In addition, few carcinomas have been reported in the oviduct of B6C3F1 mice after treatment with DBP or ozone and/or NNK[16,19]. Therefore, it is necessary to elucidate the mechanisms of toxicity and carcinogenesis of the oviduct as a specific target organ for ozone alone or in combination with NNK and/or DBP.

The behavior of chemical mixtures may differ greatly from that of individual chemicals. Interactions between the mixture components may alter the

toxicity and carcinogenicity through mechanisms such as potentiation or inhibition. Biomarkers have been introduced as indicators of disease progression or therapeutic effects. Although ozone or ozone in combination with NNK and/or DBP does not induce lung tumor, biomarkers of complex mixtures can be used to show the mutagenic and genotoxic effects[20-23]. The level and frequency in chromosome aberration, micronucleated reticulocytes, and *HPRT* mutations were significantly higher after treatment with ozone in combination with NNK and/or DBP than after treatment with ozone alone in our study, suggesting that ozone in combination with NNK and DBP could increase the genotoxic effects of ozone[20-21]. Moreover, ozone in combination with NNK and DBP increases toxicity by changing cell cycle control[22] and modifying NF-kB and AP-1 in lung and liver[23]. Mutations of p53 gene, which has been found and widely used as a molecular biomarker in search for etiological factors in lung carcinogenesis[24], also increase more clearly after treatment with ozone in combination with NNK and DBP[22]. A better understanding of the mechanism or pathophysiologic state would therefore help establish criteria for environmental epidemiology studies and allow a better assessment of populations and individuals at risk.

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