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Abstract: Although 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is classified as a human carcinogen, TCDD only induced oxidative DNA damages. In our present study, we combined TCDD with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) to investigate their tumorigenic effects on lung tumor formation in A/J mice. Application of NNK at a tumorigenic dose (2 mg/mouse) induced lung adenoma in both male and female A/J mice. Neither application of NNK at a non-tumorigenic dose (1 mg/mouse) nor repeated application of TCDD alone increased tumor incidence. Following the single injection of NNK at a non-tumorigenic dose (1 mg/mouse), repeated application of TCDD significantly increased the lung tumor incidence in female, but not in male, A/J mice 24 weeks later. Utilizing the real-time RT-PCR array, we found that P16 mRNA was significantly reduced in female lung, but not male lung, of NNK/TCDD co-treated A/J mice. With immunohistochemical staining, we confirmed that nuclear P16 protein was reduced in the lungs of NNK/TCDD co-treated female mice. These data suggest that P16 reduction at least partially contributed to synergistic effects of TCDD in lung tumorigenesis.

Dear Editor:

Kindly consider the enclosed manuscript entitled “Synergism between 2,3,7,8-tetrachlorodibenzo-p-dioxin and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone on lung tumor incidence in mice”, by Wang et al., for publication in *Journal of Hazardous Materials*. The data presented in this manuscript have not been submitted for publication elsewhere. All authors are aware of and agree to the content of the paper and their being listed as an author on the paper. This manuscript contains 3438 words including text, figure and table legends.

Although 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is classified as a human carcinogen, its function in lung carcinogenesis is still uncertain. In our present study, we demonstrated a synergistic effect between TCDD and NNK (a tobacco-specific nitrosamine) on lung adenoma formation in female A/J mice. We further identified that TCDD reduced p16 expression in the lung of A/J mice. Our data suggest that p16 reduction at least partially contributed to the synergistic effects between TCDD and NNK in lung tumorigenesis.

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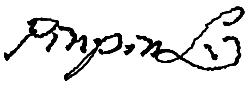

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***Novelty Statement**

Dear Editor:

Journal of Hazardous Materials is aimed to characterize the harmful effects of hazardous materials. Our present data characterized a synergistic harmful effect of two environmental pollutants, cigarette smoking and dioxin. The information for carcinogenic interaction of environmental pollutants in animal models is rare, especially in a lung tumor model. By publishing our data in this journal, more scientists will be aware of the importance of chemical-chemical interaction. Our data also offer new directions in understanding environmental factors-associated lung cancer.

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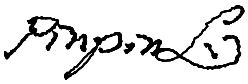

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Synergism between 2,3,7,8- tetrachlorodibenzo-p-dioxin and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone on lung tumor incidence in mice

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Abstract

Although 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is classified as a human carcinogen, TCDD only induced oxidative DNA damages. In our present study, we combined TCDD with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) to investigate their tumorigenic effects on lung tumor formation in A/J mice. Application of NNK at a tumorigenic dose (2 mg/mouse) induced lung adenoma in both male and female A/J mice. Neither application of NNK at a non-tumorigenic dose (1 mg/mouse) nor repeated application of TCDD alone increased tumor incidence. Following the single injection of NNK at a non-tumorigenic dose (1 mg/mouse), repeated application of TCDD significantly increased the lung tumor incidence in female, but not in male, A/J mice 24 weeks later. Utilizing the real-time RT-PCR array, we found that *P16* mRNA was significantly reduced in female lung, but not male lung, of NNK/TCDD co-treated A/J mice. With immunohistochemical staining, we confirmed that nuclear P16 protein was reduced in the lungs of NNK/TCDD co-treated female mice. These data suggest that P16 reduction at least partially contributed to synergistic effects of TCDD in lung tumorigenesis.

Keyword: 2,3,7,8- tetrachlorodibenzo-p-dioxin, P16, lung cancer

Introduction

The health impact of exposure to persistent organic pollutants, such as dioxins, is of great concern to the general public. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most potent dioxin congener. Epidemiological studies show that exposure to TCDD increases all cancer mortality, including lung cancer [1, 2]. Long-term treatment (2 years) of TCDD leads to the development of tumors of the liver, thyroid, lung, and other sites in female rats [3]. In experimental studies, TCDD activates aryl hydrocarbon receptors (AhRs), which change many gene expressions and possibly affect cancer development [4]. Based on epidemiological data and mechanistic studies, International Agency for Research on Cancer (IARC) has classified TCDD as a human carcinogen since 1997. However, Cole *et al*. [5] indicated that the increase in human cancer risk was only modest when people were exposed to TCDD. They believed that cancer risk increased when TCDD exposure was combined with other environmental factors, such as cigarette smoking.

Unlike to most carcinogens, TCDD does not directly produce DNA adducts or DNA damage. Nonetheless, TCDD increases oxidative stress [6, 7]. Oxidative stress is one of the mechanisms of tumor promotion [8, 9]. Currently, there are only three studies that investigated the promotion effects of TCDD in lung tumors, and one of them reported positive results [10, 11]. While both single treatment with N-nitrosodimethylamine (NDMA) and the combined treatment of NDMA plus TCDD induced lung tumors in 100% of animals, the multiplicity of lung tumors was increased in the lungs of NDMA/TCDD co-treated mice [11]. The other two studies utilized diethyl-N-nitrosamine (DEN) as the tumor initiator, which failed to show the growth promotion effect of TCDD for lung tumors in either mice or rats [10]. It appears that TCDD did not universally promote lung tumorigenicity, but varied

depending on the kind of tumor initiator used. Furthermore, neither DEN nor NDMA is present in the environment, the synergistic effect between TCDD and other chemicals to which humans are also exposed should be investigated [12]. Cigarette smoke is one of the major environmental risk factors for lung cancer development. A tobacco-specific *N*-nitrosamine, 4-(methylnitrosamino) -1-(3-pyridyl)-1-butanone (NNK) plays an important role in tobacco-related human lung cancer [13]. Furthermore, NNK induces lung adenoma/adenocarcinoma in A/J mice [14], which is often used as an animal model for lung carcinogenesis studies. Therefore, in our present study we evaluated the synergistic effects between TCDD and NNK in A/J mice.

Results generated from mechanistic studies are one of the reasons why TCDD is classified as a human carcinogen. For example, TCDD induces matrix metalloproteinase expression and invasion in melanoma cells [15]. TCDD modulated cell plasticity and mobility in a Jun NH2-terminal kinase dependent mechanism [16]. Ray and Swanson [17] reported that TCDD inhibited culture-induced senescence in keratinocytes. However, most (or all) of these data were obtained *in vitro*. In our present study, we planned to identify cancer-related genes modulated by TCDD *in vivo*.

Materials and Methods

Animals

A/J mice (6 weeks of age) , acquired from the animal center of the National Cheng Kung University Medical College, were housed five per cage at 24 ± 2°C and 50% ± 10% relative humidity and subjected to a 12-h light/12-h dark cycle. They were acclimatized for 1 week before use and fed with a Purina chow diet and water *ad*

libitum.

Female mice were randomly divided into five groups (groups I to V). Group I (n = 37) were given a single injection of 0.1 ml normal saline (vehicle) intraperitoneally per mouse as the negative control. Group II (n = 19) were given a single high dose of NNK (2 mg/0.1 ml saline/mouse intraperitoneally) as the positive control. Group III (n = 36) were given a single low dose of NNK (1 mg/0.1 ml saline/mouse intraperitoneally). Group IV (n = 22) were given a loading dose of 5 μg of TCDD/kg of body weight, followed by weekly maintenance doses of 1.42 μg of TCDD/kg of body weight administered intraperitoneally. Group V (n = 36) were given a low dose of NNK for 1 week, then a loading dose of 5 μg of TCDD/kg of body weight, followed by weekly maintenance doses of 1.42 μg of TCDD/kg of body weight administered intraperitoneally. The experiments were terminated 24 weeks after the first treatment. Male mice were randomly divided into four groups (groups I to IV), including vehicle control (n = 20), high dose of NNK (2 mg/mouse, n = 7), low dose of NNK (1 mg/mouse, n = 20) and low dose NNK plus TCDD (n = 19). Figure 1 showed the full Schedule for animal treatments.

All of the surviving mice were sacrificed under ether anesthesia. At autopsy, their lungs were excised and weighed, infused with 10% neutral buffered formalin, and inspected grossly. All of the lung tumors were macroscopically observed, and tumor-bearing lung lobes were examined histopathologically.

Histopathology

Lung tissues were sliced and immediately fixed in 10% neutral buffered formalin for 16–48 hours before being processed with standard histopathological tissue methods,

including ethanol dehydration and paraffin embedding. All lung tissues were sliced into 5-μm thick sections. Lung sections were then deparaffinized in xylene and rehydrated through graded ethanol solutions to distilled water. Initially, one section was stained with hematoxylin and eosin (H-E) for examination of the lung lesions including hyperplasia, adenoma, and malignancy diagnosed according to the criteria of *Tumors of the Mouse* [18]. When the inconsistent findings between macroscopic and microscopic examinations were observed, 10 serial sections were then cut and number-labeled. The odd numbered sections were stained with H-E for further confirmation of the presence of tumor formation and the enumeration of the lung tumors microscopically. The mouse without any tumor was defined as a negative tumor-bearing mouse. The rest of sections were subjected to performance of immunohistochemical assays for P16 protein.

Analysis of the real-time reverse transcription polymerase chain reaction (RT-PCR) array

Total RNA was prepared using TriReagent (Life Technologies, Rockville, MD, USA) and the phenol-chloroform extraction method. Synthesis of cDNA was performed using Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase, deoxynucleotides, and RNase inhibitor (Promega, Madison, WI, USA), with 2 μg of total RNA mixed with 250 ng of random primer (BioLabs, Beverly, MA, USA). Eight representative lung cDNA samples were selected from among the vehicle, NNK, TCDD, and NNK plus TCDD groups. Between groups, the relative gene expression was measured using the mouse Cancer PathwayFinder (PAMM-033, Superarray, Frederick, MD), which includes 84 genes involved in transformation and tumorigenesis.

Real-time RT-PCR assay

Quantitative PCR of cyclin-dependent kinase inhibitor 2A (*Cdkn2a*, *P16*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPHD*) were performed using the TaqMan Universal PCR Master Mix (Perkin-Elmer Applied Biosystems, Foster City, CA) and the ABI PRISM 7700 Sequence Detector System (Perkin-Elmer Applied Biosystems). The *P16* primers and probes were the Assay-on-Demand Gene Expression Assay Mix (Perkin-Elmer Applied Biosystems). Each data point was repeated four times. Quantitative values were obtained from the threshold cycle (C_T) number. The relative mRNA levels of *P16* = $2^{-\Delta Ct}$, $\Delta Ct = Ct_{P16}-Ct_{GAPDH}$.

P16 immunohistochemistry

In order to confirm the expression and location of P16 protein in the lung tissues, immunohistochemistry was performed as previously described [19]. The specific primary antibody for P16 (1:400 dilution; Clone F-12, Santa Cruz Biotechnology, Santa Cruz, CA), the Universal LSAB2 kit (DakoCytomation, Glostrup, Denmark), and the Chromogen DAB+ system (DakoCytomation) were used for detecting the immunoreactivity. The step of antigen retrieval was not essential for P16 antibody. The P16 staining was reactive to the nucleus and cytoplasm of lung tumor cells and respiratory epithelial cells. P16 was occasionally expressed in the nuclei of stroma cells in the lung sections, while the interstitial stroma was always P16-negative. When the staining intensity of nucleus and/or cytoplasm in stained cells was stronger than that of the stroma, the cells were defined as P16 nuclear and/or cytoplasmic positive cells. Furthermore, lung tumors having more than 10% P16-nuclear positive cells were regarded as P16-positive tumors. The human tonsil collected from Chung Shan Medical University Hospital was used as a positive control [20]. When primary antibody was replaced with normal serum and phosphate buffer in the procedure, the

results were used as negative controls.

Statistics

Student's *t*-test was used to compare the results of anchorage-independent growth assays and real-time RT-PCR assay among the groups. Fisher's exact test was used to compare the incidence of tumor formation between treated and control groups. The two-sided *P* values less than 0.05 were considered significant.

Results

Effects of TCDD and NNK on lung adenoma formation

Gross examination of almost all lung tumors showed that they were located in the subpleural areas. They were well-circumscribed, white in color, and ranged in size from 1 to 3 mm^3 at their greatest dimension. Microscopically, all of the lung tumors featured histology consistent with adenoma. No other pathological lesions including hyperplasia, dysplasia, or adenocarcinoma were identified in the sections examined. Tumor incidences for each treated and control group is listed in Table 1. The gender difference in TCDD-promoted lung tumorigenesis was verified in A/J mice. Both female and male A/J mice had 8% and 10% of spontaneous lung adenomas, respectively (Table 1). While treatment with high-dose NNK (2 mg/mouse) significantly increased tumor incidences in both genders (84% in females and 71% in males**,** both $P = 0.001$ and 0.005 respectively), treatment with low-dose NNK (1 mg/mouse) only slightly increased tumor incidences (19% in females and 15% in males, $P = 0.190$ and 1.000 respectively. However, combined treatment with low-dose NNK and TCDD significantly increased the incidence to 36% in females, but increased the incidence to 26% in males without significance (Table 1). Treatment with TCDD resulted in a low incidence (9%) of adenomas in females. It appears that

the synergistic effect between TCDD and NNK was significant in females, but not in males.

Effects of TCDD on p16 expression in lungs

To understand the mechanisms of the tumor growth promotional effect of TCDD, we screened for the differential expression of 84 cancer-related genes in female mouse lungs using a real-time RT-PCR array. Gene expression modulated by treatment with low-dose NNK (LNNK), TCDD, or LNNK/TCDD is shown in Table 1. Expression of 12, 10, or 9 cancer-related genes was modulated in LNNK, TCDD, or LNNK/TCDD-treated groups, respectively. *P16* is a tumor suppressor gene and its expression is commonly reduced in human lung cancer specimens (Belinsky, 2004). In our experiments, *P16* was modulated in the LNNK-treated group and was uniquely down regulated in the LNNK/TCDD-treated group (Table 2). Furthermore, the reduction of *P16* mRNA was only observed in female, but not in male, mice co-treated with NNK/TCDD (Figure 2).

The reduction of P16 protein levels was further confirmed by immunostaining. Most bronchiolar and alveolar cells displayed positive P16 immunostaining in the nuclei and cytoplasm of control mice (Figure 3, A and B). However, the results of immunostaining in lung tumor cells of NNK/TCDD co-treated groups were different in male and female mice. In the female NNK/TCDD co-treated group, P16 nuclear immunostaining occurred in 43% of lung tumors in which a weak P16 staining was observed in nuclei, cytoplasm or both (Figure 3C). In contrast, in the male NNK/TCDD co-treated group, P16 nuclear immunostaining occurred in 75% of lung tumors in which tumor cells showed a moderately cytoplasmic or strongly P16 nuclear staining (Figure 3D). It appears that TCDD reduced P16 expression in the lungs of A/J mice in a gender-dependent manner.

Discussion

Cigarette smoke and dioxin are classified as human carcinogens. NNK is the potent carcinogen in cigarette smoke and TCDD is the most potent AhR agonist among dioxins. Because NNK metabolites and TCDD are detectable in human specimens of the general population [21-24], it is possible that NNK may interact with TCDD and enhance cancer risks in the general population, such as lung cancer. In our present study, we demonstrated that the combined treatment of NNK and TCDD at non-carcinogenic doses significantly increased the incidence of lung adenoma in A/J mice. Furthermore, this carcinogenic effect was more common in female than in male mice.

Although low-dose NNK (1 mg/mouse) or TCDD alone failed to increase the incidence of lung adenoma in female mice, low-dose NNK or TCDD modulated expression of several cancer-related genes. Low-dose NNK reduced expression of nine genes and four of them (*Mta2*, *Mmp9*, *S100A4*, and *Plau*) involved invasion and metastasis. It is consistent with the phenomenon that NNK tends to induce adenoma in mice, which is a non-invasive phenotype. TCDD alone increased expression of nine genes with diverse functions, but only transformation related protein 53 (*P53*) was also induced in LNNK/TCDD co-treated mice. On the other hand, *P16* expression was only reduced in LNNK/TCDD co-treated mice. It appears that a synergistic interaction occurred between low dose NNK and TCDD. This interaction not only reduced *P16* expression, but also increased the tumor incidence in female mice.

Cellular senescence is proposed to be a tumor-suppressive mechanism that stops incipient cancer cells from proliferating. The hallmark of cellular senescence is an

inability to progress through the cell cycle. The P16- retinoblastoma protein (pRB) tumor suppressor pathway is one of the pathways that controls senescent growth arrest [25]. Some senescence-inducing stimuli, such as oncogenes and DNA-damage responses, activate the P16-pRB and P53 pathways. Previously, Ray and Swanson [26] demonstrated that TCDD attenuated senescence and repressed expression of P16 as well as P53 in primary human keratinocytes. In the present study, mRNA and protein levels of P16 were significantly reduced in the lungs of NNK/TCDD co-treated female mice. Furthermore, P16 reduction correlated with tumor incidence in a gender dependent manner. These results imply that P16 reduction might involve in the synergisitc effect of TCDD in the lungs of female mice. These data are also consistent with results reported by other studies that loss of P16 function in mice increased susceptibility to carcinogens [27, 28].

Loss of P16 expression occurs in 30%–70% of human non-small cell lung cancers, and is more prevalent in smokers than in nonsmokers [29]. In human lung cancer, loss of P16 expression typically results from allelic loss in combination with hypermethylation of the *P16* promoter [30-33]. Some studies also reported that histone modifications regulated *P16* expression [34-36]. Belinsky et al. [37] reported that NNK treatment reduced *P16* expression in approximately half of lung tumor-bearing A/J mice. They further suggested that reduced P16 expression in some of these tumors was attributed to deletion of the p16 gene, but not hypermethylation of *P16* promoter [37, 38]. On the other hand, Ray and Swanson *et al*. [26] reported that TCDD induced methylation of the *P16* promoter in primary human keratinocytes. In our present study, we observed reduced P16 expression in NNK/TCDD co-treated female mice. The levels of P16 protein were reduced in both the nuclei and cytoplasm of the tumor cells of NNK/TCDD co-treated female mice. The most striking result

was that the P16 reduction by NNK/TCDD in the lung was gender specific. DNA methyltransferases catalyze DNA methylation. We examined the expression of DNA methyltransferase 1 (DNMT1) protein in these specimens, but DNMT1 expression did not correlate with the loss of P16 protein in these specimens (data not shown). The mechanism remains to be clarified in the future.

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Declarations of interest

The Author(s) declare that they have no competing interests.

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Table 1. The incidence of lung tumor formation in A/J mice treated with NNK and/or TCDD.

Gender	Treatment	Mice numbers per group	Number (%) of tumor-bearing mice
Female	Control	37	3 (8)
	HNNK	19	16 (84)*
	LNNK	36	7 (19)
	TCDD	22	2 (9)
	LNNK + TCDD	36	13 (36)*
Male	Control	20	2 (10)
	HNNK	7	5 (71)*
	LNNK	20	3 (15)
	LNNK + TCDD	19	5 (26)

Control, injected with sterile normal saline; low dose of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (LNNK), injected with 1 mg NNK/mouse; high dose of NNK (HNNK), injected with 2 mg NNK/mouse; 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), injected with 5μg/kg TCDD once and then 1.42 μg/kg TCDD for three times weekly.

*, $P < 0.05$, compared with control group by using two tailed Fisher's exact test.

Table 2. Gene expression modulated by NNK and/or TCDD in the lung of female mice.

Treatment	Symbol	Description	Fold
LNNK	*Twist1*	Twist gene homolog 1 (Drosophila)	3.43
	Brca1	Breast cancer 1	1.5
	Casp8	Caspase-8/FLICE	1.49
	Mta2	Metastasis-associated gene family, member 2	0.67
	Plau	Plasminogen activator, urokinase	0.67
	Birc5	Baculoviral IAP repeat-containing 5	0.56
	Jun	Jun oncogene	0.53
	S100a4	S100 calcium binding protein A4	0.53
	Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	0.51
	Fos	FBJ osteosarcoma oncogene	0.45
	Mcam	Melanoma cell adhesion molecule	0.4
	Mmp9	Matrix metallopeptidase 9	0.18
TCDD	*Twist1*	Twist gene homolog 1 (Drosophila)	4.09
	Ncam1	Neural cell adhesion molecule 1	2.37
	Tnfrsf10b	Tumor necrosis factor receptor superfamily,	1.93
	Casp8	Caspase-8/FLICE	1.82
	Trp53	Transformation related protein 53	1.82
	Egfr	Epidermal growth factor receptor	1.76
	Myc	Myelocytomatosis oncogene	1.55
	Nme4	Non-metastatic cells 4, protein expressed in	1.51
	Hgf	Hepatocyte growth factor	1.46
	Kiss1	KiSS-1 metastasis-suppressor	0.46
LNNK+TCDD	*Brca1*	Breast cancer 1	1.75
	Trp53	Transformation related protein 53	1.6
	Plau	Plasminogen activator, urokinase	0.67
	Birc5	Baculoviral IAP repeat-containing 5	0.64
	Jun	Jun oncogene	0.57
	Cdkn2a	Cyclin-dependent kinase inhibitor 2A	0.56
	Fos	FBJ osteosarcoma oncogene	0.46
	S100a4	S100 calcium binding protein A4	0.45
	Mmp9	Matrix metallopeptidase 9	0.11

Each group contained eight animals. Data of low dose of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (1 mg/0.1 ml saline/mouse intraperitoneally, LNNK), 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) or NNK/TCDD-treated group were compared with those of control group. The fold changes of list genes were statistical significant ($p < 0.05$).

Figure legends

Figure 1. The schedule for 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and/or 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) treatment in A/J mice. The control was intraperitoneally injected with phosphate-buffered saline on the first day. The HNNK group was intraperitoneally injected with 2 mg of NNK per mouse on the first day. The LNNK group was intraperitoneally injected with 1 mg of NNK per mouse on the first day. The TCDD was intraperitoneally injected with 5 μg/kg of TCDD once for the first week, followed by weekly injections of 1.42 μg/kg of TCDD for 3 weeks.

Figure 2. Effects of NNK/TCDD co-treatment on *p16* mRNA levels in male and female A/J mice. *P16* mRNA levels were determined with the real-time RT-PCR method. Each group contained eight animals, and each data point was repeated for four times. *$p < 0.05$, compared to the female control group.

Figure 3. Immunohistochemical staining of P16 in control and LNNK/TCDD-treated lung of mice. A and B. In female and male mice of control groups, many alveolar and airway epithelial cells demonstrated strong staining of P16 in the cell nucleus and/or cytoplasm. C. In LNNK/TCDD treated female mice, some

tumor cells demonstrated P16 staining in the cell cytoplasm and/or nucleus. D. In LNNK/TCDD-treated male mice, many tumor cells demonstrated P16 positive staining in both the cell nucleus and cytoplasm. Scale bar, 50 μm.

Figure 1

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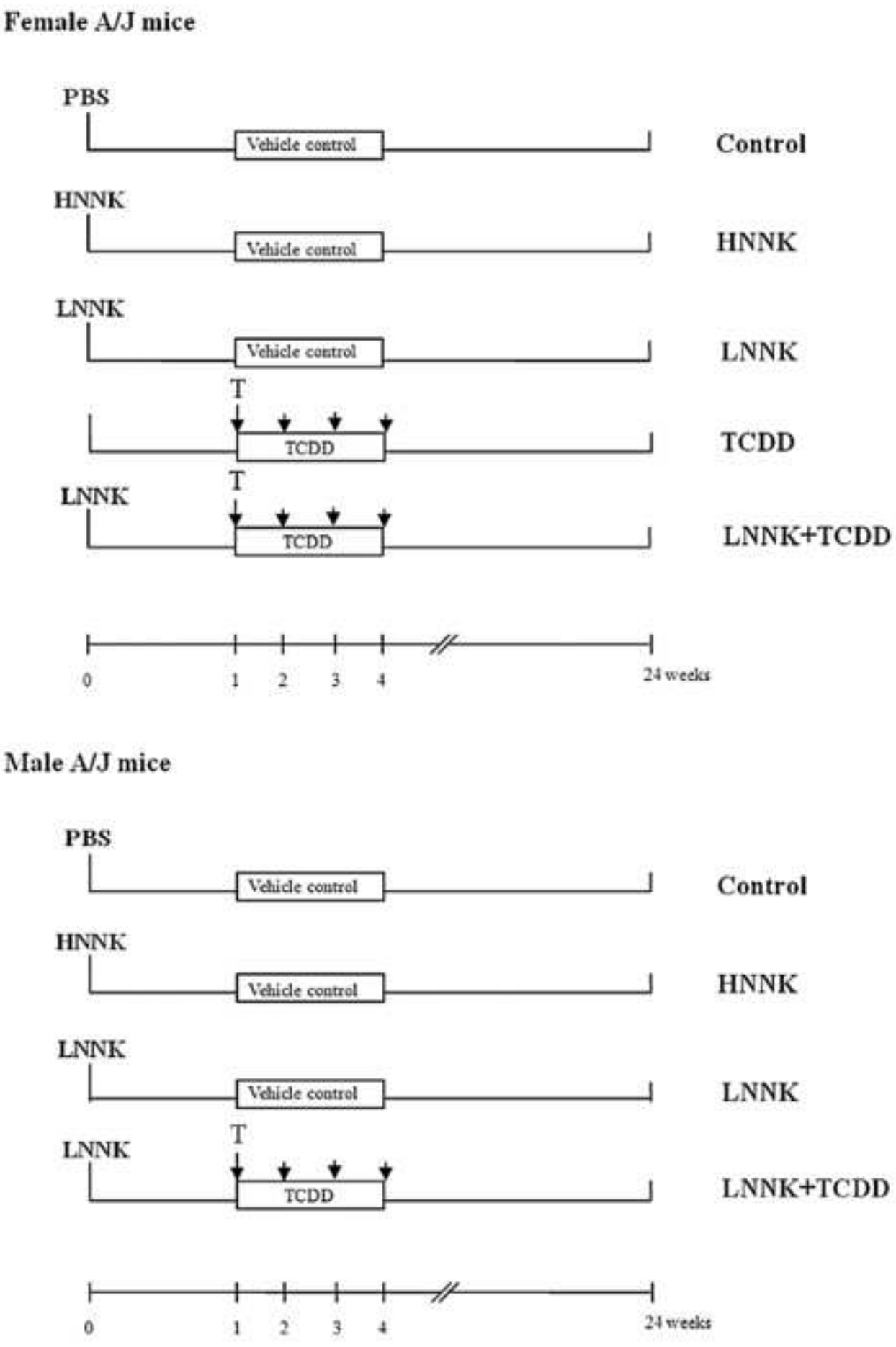

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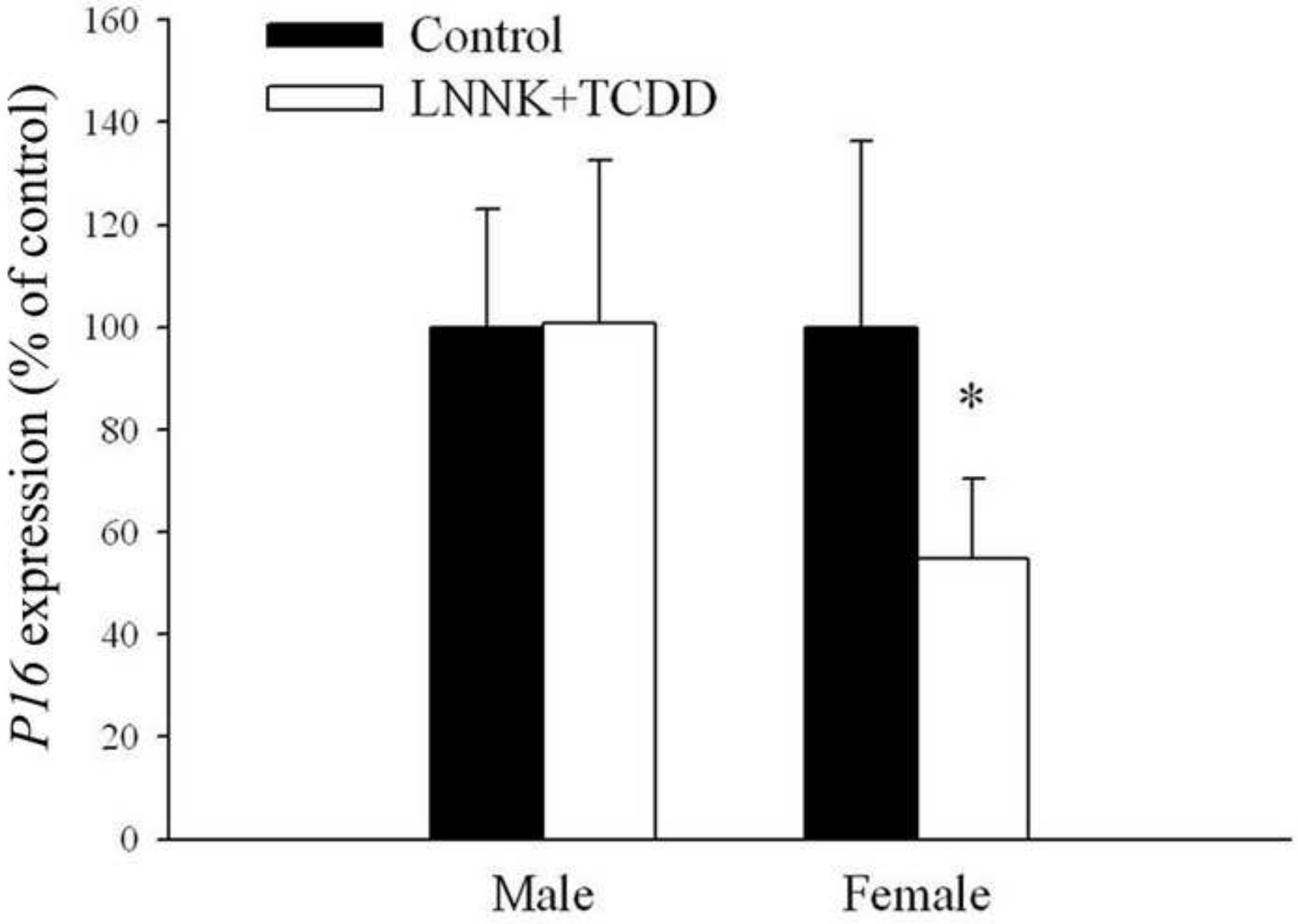

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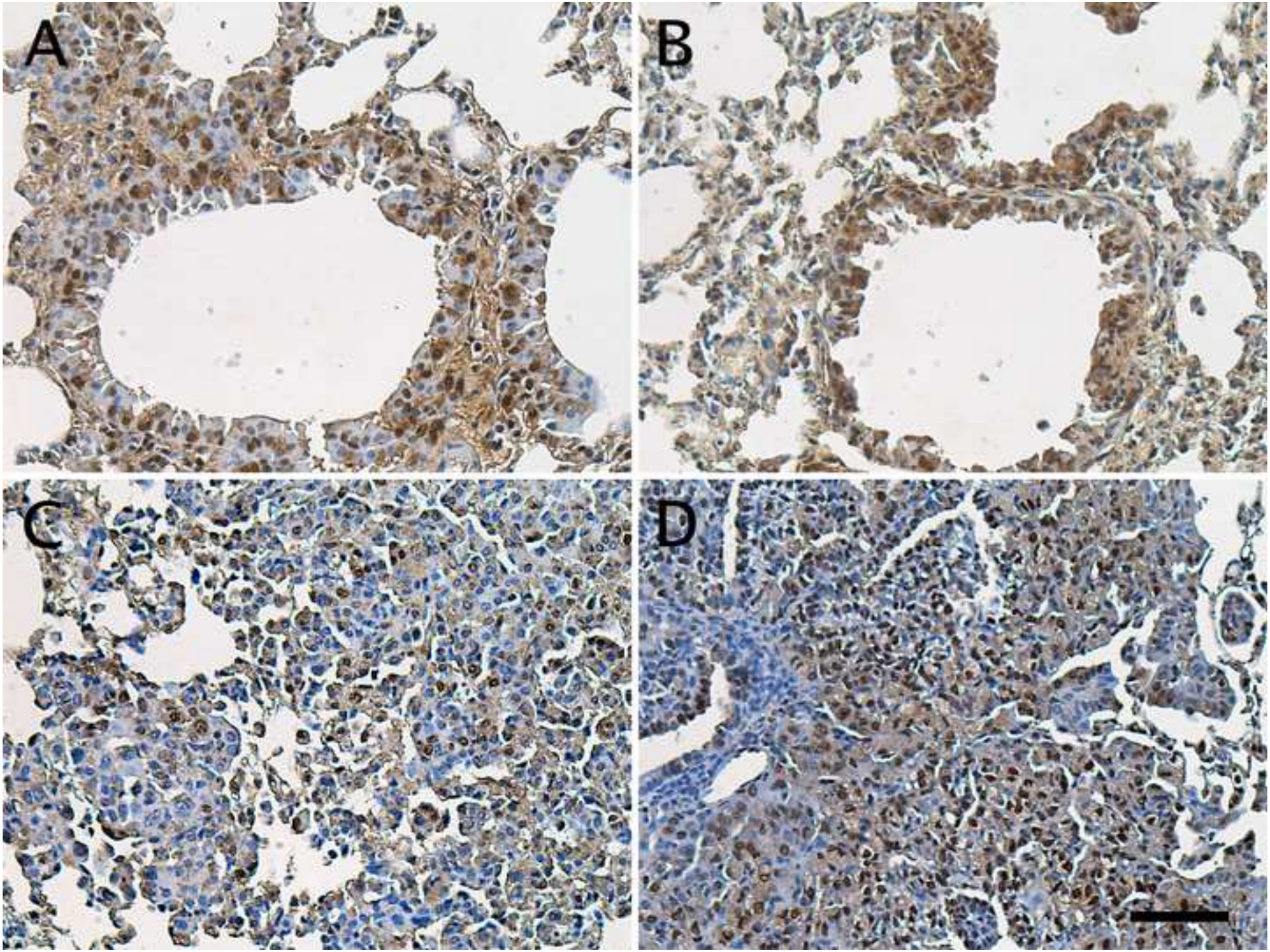

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