# *In Vivo* Examination of the Genotoxicity of the Urban Air and Surface Soil Pollutant, 3,6-Dinitrobenzo[e]pyrene, with Intraperitoneal and Intratracheal Administration

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**ABSTRACT:** 3,6-Dinitrobenzo[e]pyrene (3,6-DNBeP) was identified as a new potent mutagen toward *Salmonella* strains in surface soil and airborne particles. Because data of *in vivo* examination of the genotoxicity of 3,6-DNBeP are limited, micronucleus test was performed in peripheral blood and bone marrow, and comet assay in the lungs of mice treated with 3,6-DNBeP. In male ICR mice intraperitoneally (i.p.) injected with 3,6-DNBeP, the frequency of micronuclated polychromatic erythrocytes (MNPCEs) was increased in the peripheral blood and bone marrow after 24 h in a dose-dependent manner. Compared to controls, the highest dose of 3,6-DNBeP (40 mg/kg B.W.) induced 7.3- and 8.7-fold increases of MNPCE frequency in the peripheral blood and bone marrow, respectively. Furthermore, when 3,6-DNBeP was intratracheally (i.t.) instilled to male ICR mice, 3,6-DNBeP at the highest dose of 0.1 mg/kg body exhibited 3.1-fold increase of DNA tail moment in the lungs at 3 h after the instillation compared to controls. The values of DNA tail moment at 9 and 24 h after the instillation were increased up to 3.5 and 4.2-fold, respectively. These data indicate that 3,6-DNBeP is genotoxic to mammalians in *in vivo* and suggest that 3,6-DNBeP may be a carcinogenic compound present in the human environment. © 2011 Wiley Periodicals, Inc. Environ Toxicol 28: 588–594, 2013.

**Keywords:** 3,6-dinitrobenzo[e]pyrene; genotoxicity; surface soil; intratracheal instillation; nitro-PAHs

## INTRODUCTION

Many kinds of chemicals are released into the atmospheric environment from diesel exhaust, waste incineration plants, and so on and are adsorbed to atmospheric particles (Leiter et al., 1942; Siak et al., 1985; Lewtas et al., 1990). Some of them including polycyclic aromatic hydrocarbons (PAHs) and nitrated PAHs (nitro-PAHs) show mutagenic and/or carcinogenic activities (Stenberg et al., 1983). Atmospheric

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particles precipitate into the ground surface to some extent, and the surface soil is contaminated with these genotoxic compounds (Hasei et al., 2006). In fact, surface soils from parks (Nishimura et al., 1984; Watanabe et al., 2005b), agricultural land (Brown et al., 1985), roadsides (Arashidani et al., 1992), and residential sites (Knize et al., 1987; Watanabe et al., 2003) are reported to exhibit genotoxic activity.

Nitro-PAHs are released during combustion processes by diesel engines, wood-burning, (Rosenkrantz et al., 1982; Gibson, 1983) and so forth and are formed secondarily by the reaction of PAHs with nitrogen oxide in the atmosphere (Tokiwa et al., 1981). Nitro-PAHs are of enormous concern because of their genotoxicity (Tokiwa and Ohnishi, 1986) and ubiquity in the atmospheric environment (Gibson, 1983). Especially, 1,3-, 1,6-, and 1,8-dinitropyrene (DNP) isomers have been examined with great attention, because these compounds show highly mutagenic activity in bacteria (Tokiwa and Ohnishi, 1986) and cultured mammalian cell lines (O'Donocan et al., 1990) and have exhibited carcinogenicity in experimental animals (Takayama et al., 1985).

Recently, we found a new nitro-PAH as a novel mutagen from surface soil collected at Takatsuki city in Osaka Prefecture and four other cities, and the compound was identified as 3,6-dinitrobenzo[*e*]pyrene (3,6-DNBeP) (Watanabe et al., 2005a). Extracts of these surface soils exhibited potent mutagenicity in the Ames/*Salmonella* assay, and 3,6-DNBeP also showed significant mutagenic activity in *Salmonella typhimurium* TA98 without the metabolic activation system (S9 mix), inducing 285,000 revertants/nmol. The potency is equivalent to those of DNP isomers that have been classified as possible human carcinogens (group 2B) by IARC (1989; 1996). In addition, using a sensitive method with a reducer column and a fluorescence detector for quantifying 3,6-DNBeP in surface soil and airborne particles (Hasei et al., 2006), 3,6-DNBeP was detected in surface soil and airborne particles with the range of 347-5007 pg/g for soil and 137-1238 fg/m$^3$ for airborne particles. The contribution ratios of 3,6-DNBeP toward total mutagenicity of soil extracts in *S. typimurium* TA98 without S9 mix were from 16.5 to 30.6%, and the amounts of 3,6-DNBeP in both surface soil and airborne particles were comparable to those of DNP isomers. Therefore, it is important to evaluate the genotoxicity of 3,6-DNBeP in assays other than Ames/*Salmonella* assay. Kawanishi et al. (2009) reported that treatment of HepG2 cells with 3,6-DNBeP indicated mutagenic and genotoxic activity in *hprt* mutation assay, sister chromatid exchange test, and micronucleus test. Moreover, the DNA damaging potency of 3,6-DNBeP was observed in ICR mice intraperitoneally (i.p.) injected with this compound, as shown by comet assay (Kawanishi et al., 2009).

In the present study, in order to further evaluate the *in vivo* genotoxicity of 3,6-DNBeP, micronucleus test was performed using peripheral blood and bone marrow derived from ICR mice i.p. injected with 3,6-DNBeP. Moreover, because 3,6-DNBeP is present in atmospheric environment, DNA damaging activity of 3,6-DNBeP was examined using the lungs of mice intratracheally (i.t.) instilled with this compound. Genotoxic activity of 3,6-DNBeP was detected in both assay systems, and its potency was comparable to those of other air pollutants, DNP isomers. Possible carcinogenic potency of 3,6-DNBeP is also discussed.

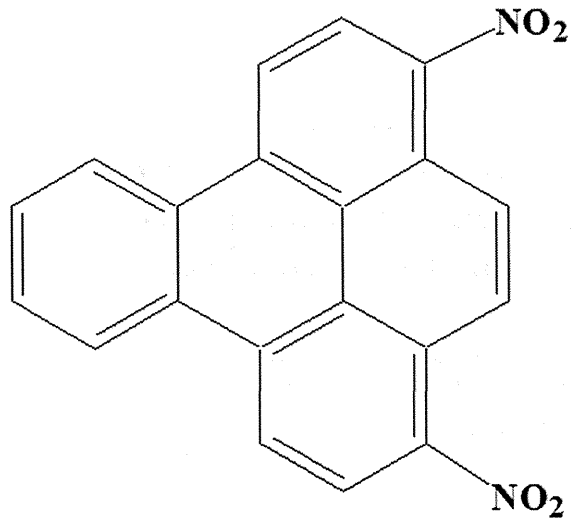


**Fig. 1.** Chemical structure of 3,6-DNBeP.

## MATERIALS AND METHODS

### Materials

3,6-DNBeP was synthesized as described previously (Watanabe et al., 2005a). Figure 1 shows the chemical structure of 3,6-DNBeP. Corn oil, glyceryltrioctanoate, low melting point (LMP) and normal melting point (NMP) agarose, dimethyl sulfoxide, and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). Halothane was from Takeda Pharmaceutical Company (Osaka, Japan). Fetal bovine serum (FBS) was taken from Gibco-BRL (Grand Island, NY). Ethidium bromide was obtained from Merck (Darmstadt, Germany), and all other chemicals were from Wako Pure Chemicals (Osaka, Japan).

### Animals

Eight-week-old male ICR mice were obtained from Japan SLC (Shizuoka, Japan) and used for experiment when 9 weeks old. Food (CE-2 commercial diet: Japan Clea Co., Tokyo, Japan) and water were given freely. The room in which mice were placed was air conditioned at 23°C with a light/dark (12-h) cycle. The experiments were conducted

according to the Guidelines for Animal Experiments in the National Cancer Center and the Guidelines of the University of Shizuoka for the Care and Use of the Laboratory Animals.

## Micronucleus Test Using Peripheral Blood and Bone Marrow

Two doses (20 and 40 mg/kg B.W.) of 3,6-DNBeP dissolved in corn oil were i.p. administrated to mice ($n = 5$). Only corn oil was also injected as vehicle control. The animals were sacrificed 24 and 48 h after administration, and peripheral blood and bone marrow were obtained. Peripheral blood and bone marrow were used for the micronucleus test.

The micronucleus test was performed according to the method of Hayashi et al. (Hayashi et al., 1994) with some modifications. Five microliters of peripheral blood were diluted with the same volume of FBS. Five microliters of diluted blood were spread on an acrydine orange-coated glass slide, covered with a coverslip, and supravitally stained. Within a few days of slide preparation, 1000 polychromatic erythrocytes (PCEs) were observed with a fluorescence microscope (Nikon ECLIPSE 80i fluorescence microscope: magnification, 1000×) and the numbers of micronuclated PCEs (MNPCEs) were recorded. Bone marrow samples were cleaned-out with 1 mL FBS, and cells were collected by centrifugation. The volumes of supernatants were adjusted to the same volume of deposition, and the supernatants were mixed with pipetting. Five microliters of cell suspension were smeared on a clean slide with a cover glass, and the slides were dehydrated with 70% ethanol to fixate. Then, the slides were stained with 40 μg/mL acridine orange just before microscopical observation, 1000 PCEs were observed with a fluorescence microscope (magnification, 400×), and the numbers of MNPCE were recorded. At the same time, the numbers of PCEs in 1000 normochromatic erythrocytes (NCEs) were counted.

## Alkaline Comet Assay Using the Lungs Obtained from Mice After i.t. Instillation with 3,6-DNBeP

Two doses (0.05 and 0.1 mg) of 3,6-DNBeP dissolved in 50 μL glyceryltrioctanoate were i.t instilled to mice ($n = 5$) using a polyethylene tube under anesthesia with 4% halothane. Only glyceryltrioctanoate also was i.t. instilled as vehicle control ($n = 5$). Mice were sacrificed at 3, 9, and 24 h after 3,6-DNBeP injection, and the lungs were circulated with saline and subsequently taken for the comet assay.

The lungs were minced and suspended with chilled homogenizing buffer [30 mM EDTA-2Na and 0.9% KCl (v/v)] and homogenized gently using a Dunce-type homogenizer on ice to gain the single-cell suspensions.

The alkaline comet assay was carried out by the method described previously with some modifications (Singh et al., 1988; Toyoizumi et al., 2008). Briefly, to make slide coated with a first agarose layer, 75 μL of 0.7% NMP agarose in phosphate buffer saline (PBS) was spread on a clear frosted slide and allowed to dry overnight. Seventy-five microliters of cell suspension were mixed with the same volume of 1.4% LMP agarose in PBS. The mixture (75 μL) was layered on a first agarose layer and covered with 75 μL of 0.7% LMP agarose. After slide preparation, the slide was immersed in lysing solution (2.5 M NaCl, 0.1 M EDTA-2Na, 0.01 M Tris base, 1% salcosinate-NaOH buffer, 10% dimethyl sulfoxide and 1% Triton X-100, and pH 10) and refrigerated at 0°C for 1 h. Each slide was then placed in alkaline electrophoresis buffer (0.3 M NaOH and 1 mM EDTA-2Na) for 10 min to allow for DNA unwinding. Electrophoresis was performed at 25 V, 300 mA for 15 min at 4°C. The slides were neutralized with Tris buffer (0.4 M Tris base, pH 7.5) for 5 min twice and dehydrated with 70% ethanol to fixate. The cells were stained with 50 μL of ethidium bromide solution (20 μg/mL). Comet images were analyzed using a fluorescence microscope (magnification 200×) equipped with a CCD camera. Fifty cells were examined per one plate. The tail moment of DNA was measured using Comet Analyzer from Youworks Co. (Ibaraki, Japan).

## Statistical Analysis

Dunnett's test after one-way ANOVA was used to evaluate the significance of differences of MN frequency in the micronucleus test and DNA tail moment in the comet assay between groups treated with 3,6-DNBeP and control groups; $P$ values lower than 0.05 were considered to indicate statistical significance.

# RESULTS

## Clastogenic Activity in Peripheral Blood and Bone Marrow by i.p. Administration of 3,6-DNBeP

To evaluate the clastogenic activity, the micronuclei induction by i.p. administration of 3,6-DNBeP was measured. Peripheral blood and bone marrow taken at 24 and 48 h after administration were subjected to the micronucleus test. Figure 2 and Table I show the frequency of MNPCEs in peripheral blood. The dose-dependent increase of frequency was observed at 24 h after administration of 3,6-DNBeP. Compared to vehicle control, frequencies increased 5.0 ($P < 0.05$) and 7.3 ($P < 0.01$) fold with 20 and 40 mg/kg, respectively. In the case of 48 h, MNPCE frequency decreased by 64% with high dose compared to that observed at 24 h after the administration, whereas no

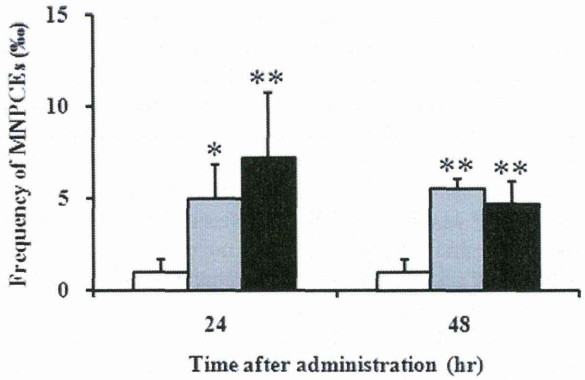


**Fig. 2.** Clastogenic activity of 3,6-DNBeP in peripheral blood. Mice were i.p. injected with 3,6-DNBeP at doses of 20 (gray bar) and 40 mg/kg B.W. (black bar). Control mice were injected with corn oil (open bar). One thousand PCEs were observed per mouse. The values represent the mean of five animals ± SD. *$P < 0.05$, ** $P < 0.01$ (vs. control).

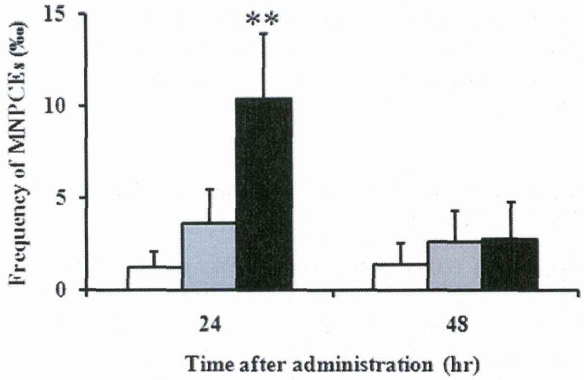


**Fig. 3.** Clastogenic activity of 3,6-DNBeP in bone marrow. Mice were i.p. injected with 3,6-DNBeP at doses of 20 (gray bar) and 40 mg/kg B.W (black bar). Control mice were injected with corn oil (open bar). One thousand PCEs were observed per mouse. The values represent the mean of five animals ± SD. *$P < 0.05$, ** $P < 0.01$ (vs. control).

notable changes of MNPCE frequencies were observed at low dose between two sampling times.

As shown in Figure 3, MNPCE frequency increased more markedly in bone marrow than in peripheral blood ($P < 0.01$) with high dose 24 h after the administration. No significant increase was detected with both doses after 48 h.

## Cytotoxicity of 3,6-DNBeP Against Hematopoietic Tissues

Concomitantly with micronucleus test, the numbers of PCEs in 1000 NCEs, as a cytotoxicity against hematopoietic tissues induced by i.p. injection with 3,6-DNBeP, were counted. The number of PCEs in 1000 NCEs decreased in a dose- and time-dependent manner (Table I). The decrease of the number of PCEs was most prominent at 48 h after the administration with high dose of 3,6-DNBeP ($P < 0.01$).

**TABLE I. The frequency of MNPCEs per 1000 PCEs and PCEs per 1000 NCEs in peripheral blood**

| | Frequency of MNPCEs (‰) | | Frequency of PCEs (‰) | |
|---|---|---|---|---|
| | 24 h | 48 h | 24 h | 48 h |
| Control | 1.0 ± 0.7 | 1.0 ± 0.7 | 58.6 ± 5.7 | 57.4 ± 4.7 |
| 3,6-DNBeP (20 mg/kg) | 5.0 ± 1.9[a] | 5.5 ± 0.6[b] | 51.4 ± 4.9 | 46.8 ± 4.1[a] |
| 3,6-DNBeP (40 mg/kg) | 7.3 ± 3.5[b] | 4.7 ± 1.2[b] | 42.4 ± 8.1[b] | 16.8 ± 3.7[b] |

The PCEs/NCEs ratios were obtained concomitantly with the micronucleus test. The PCEs/NCEs ratios reflect the cytotoxicity of chemicals against hematopoietic systems. These values represent the mean of five animals ± SD.

[a] $P < 0.05$.

[b] $P < 0.01$ (vs. control).

## DNA Damaging Potency in the Lungs by i.t. Instillation of 3,6-DNBeP

DNA damaging potency of 3,6-DNBeP in the lungs of mice was evaluated using the comet assay under alkaline conditions. Figure 4(a) shows the mean values of DNA tail moment in the lungs of mice treated with 3,6-DNBeP at 0.05 and 0.1 mg/mouse for 3 h. The DNA damages were dose-dependent, and the values of DNA tail moment were significantly increased as compared with that from control mice. DNA damage against vehicle control was increased 2.4 ($P < 0.01$) and 3.1 ($P < 0.01$) fold with the low and high doses of 3,6-DNBeP, respectively. Furthermore, we examined the DNA damage in the lungs at three time points after i.t. instillation, i.e., 3, 9, and 24 h at 0.1 mg of 3,6-DNBeP/mouse. As shown in Figure 4(b), the highest DNA tail moment was detected when the tissue was removed at 24 h after the instillation, whereas no time-dependent increase of the tail moment was shown in the control animals.

## DISCUSSION

In the present study, we found that 3,6-DNBeP, which is isolated from surface soil and airborne particles as one of the major mutagens in *Salmonella*, showed *in vivo* genotoxic activity in micronucleus test and comet assay in mice.

The micronucleus test is widely used as a screening method for detecting the clastogenicity of chemicals. Hayashi et al. (1994) introduced a method for the observation of micronucleated young erythrocytes using supravital staining with acridine orange. This method can detect very young micronucleated erythrocytes in the peripheral blood

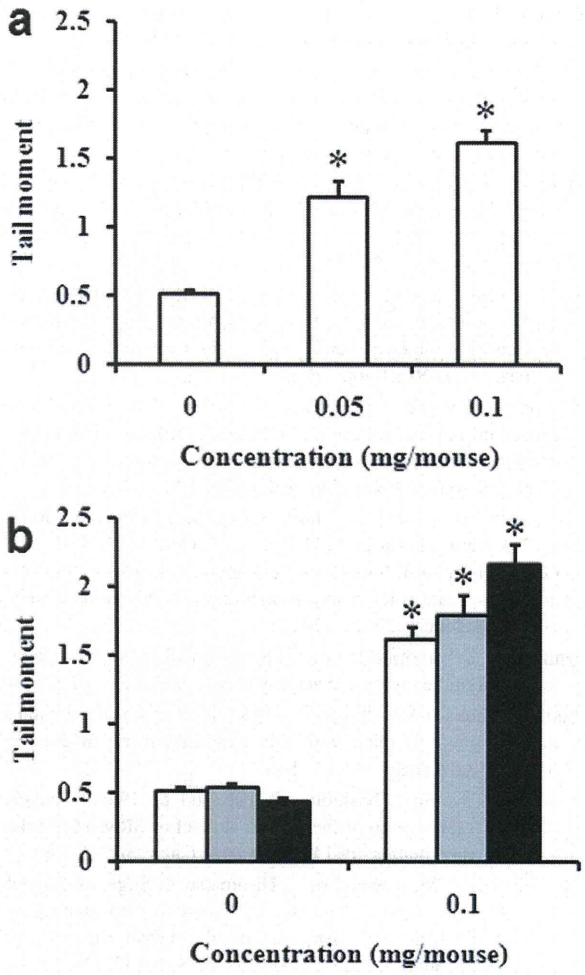


**Fig. 4.** DNA damaging potency of 3,6-DNBeP in the lungs by i.t. instillation. Mice were i.t. instilled with 3,6-DNBeP at doses of 0.05 and 0.1 mg/mouse. Control mice were treated with glyceryltrioctanoate. Mice were sacrificed at 3 (open bar), 9 (gray bar), and 24 h (black bar) after instillation. The tail moment is shown in a and b with doses and time course, respectively. The values represent the mean of five animals ± SEM. *$P < 0.01$ (vs. control).

stream before being trapped and destroyed by the spleen. Two doses (20 and 40 mg/kg B.W.) of 3,6-DNBeP were i.p. injected to ICR mice, and the mice were dissected at 24 and 48 h after injection. The dose-dependently increased MNPCEs were observed at 24 h after injection in both peripheral blood and bone marrow. In the peripheral blood, the MNPCEs at higher dose decreased at 48 h when compared with 24 h, whereas those at lower dose remained unchanged. According to the data of Vrzocand and Petras, when *N*-methyl-*N*-nitrosourea (MNU) was i.p. administrated at extremely high dose of 150 mg/kg B.W. in male BALB/cBtJ mice, MNPCEs were hardly observed in the peripheral blood of mice at 48 h after administration (Vrzoc et al., 1997). Because almost no observation of MNPCEs was thought to be due to the damages caused by MNU in hematopoietic systems, the decrease of MNPCEs with higher dose of 3,6-DNBeP may also be due to the same damages. In fact, our data showed that the number of PCEs in 1000 NCEs was decreased by i.p. injection at dose of 40 mg/kg B.W. at 48 h after the administration, suggesting that 3,6-DNBeP may show strong toxicity to hematopoietic tissues. Consistent with these observations, in bone marrow, the MNPCEs were dominantly increased in higher dose for 24 h and reduced until spontaneous level at 48 h after instillation. Previous studies showed that treatment of CHL cells with 0.25 $\mu$g/mL of 1,8-DNP and 0.2$\mu$g/mL of 3,6-DNBeP increased the frequencies of micronucleated cells about 11.5 and 2.5 times higher than the background level, respectively (Watanabe et al., 1994; Kawanishi et al., 2009). Moreover, 1,8-DNP exhibited carcinogenicity in BALB/c mice (Otohuji et al., 1987).

Because 3,6-DNBeP is thought to be mainly present in ambient air, we injected by i.t. instillation to investigate the genotoxicity of 3,6-DNBeP in the lungs by using the comet assay. The comet assay was developed by Singh et al. (1988), and this method has a high sensitivity for detecting low levels of DNA damage in individual cells (Speit et al., 2004). Moreover, it requires small numbers of cells per sample and short time to complete a study. Inhalation experiments are the best way to estimate the effects of inhaled gas, dust, and chemicals, but require not only considerable amounts of materials, but also a safe and well-controlled system for exposure and maintenance of animals (Nagy et al., 2005). Thus, i.t. instillation is a useful method as a model for inhalation. The increased tail moment values were observed by i.t. instillation of 3,6-DNBeP, and the highest genotoxic activity was found at 24 h after instillation. It has been reported that DNA damage in the liver, kidneys, lungs, and bone marrow of ICR mice was induced by i.p. injection of 3,6-DNBeP (20, 40, and 80 mg/kg B.W.), and the values of Tail moment in lungs were about 1.5 (Kawanishi et al., 2009). In the present study, almost the same DNA damaging potency was observed in the lungs even though the dose of 3,6-DNBeP was quite low (about 2.5 mg/kg B.W.). It is thought that the administration route could influence the distribution (concentration) of 3,6-DNBeP in the lungs. Because 3,6-DNBeP could be spread into several tissues by i.p. injection, the amount of 3,6-DNBeP reaching the lungs might be only a part, whereas almost the whole amount of 3,6-DNBeP could be retained in the lungs by i.t. instillation. Thus, the DNA damaging potency in the present study may be the same as that of the previous report. Moreover, we determined the DNA damaging potency induced by i.p. injection of 3,6-DNBeP at the doses of 20 and 40 mg/kg B.W. in the same manner of Kawanishi et al. (2009) and observed similar data (data not shown). Another previous study showed that

the increased levels of DNA adducts derived from 1,6-DNP were observed at 24 h after direct administration to the lungs, followed by a decline. The comet assay can detect alkaline sensitive sites, in which DNA adducts are formed (Tice et al., 2000); therefore, the results of our comet assay, in which the highest genotoxic activity who observed at 24 h after injection, could correspond to the data of DNA adduct formation by 1,6-DNP.

It is well known that the metabolites of nitro-PAHs by cellular enzymes bind to DNA to form DNA adducts. Nitro-PAHs form *N*-hydoroxylamines by nitroreduction, followed by activation by *O*-esterification (Arlt et al., 2003). In human, these metabolisms are mediated by phase II enzymes such as *N*-acetyltransferase and sulfotransferase and so on (Arlt et al., 2003). Moreover, the DNA adducts derived from nitro-PAH can induce gene mutation associated with carcinogenesis (Ritter et al., 2002). 3,6-DNBeP might be activated by those enzymes in a similar manner, however, the mechanisms of genotoxicity of 3,6-DNBeP are still unclear. Therefore, further studies regarding the mechanisms of genotoxicity of 3,6-DNBeP are necessary.

In conclusion, several epidemiological studies have demonstrated that environmental air pollution has a tendency to be associated with the incidence of lung cancer and cardiopulmonary mortality (Pope et al., 2002; 2004). Therefore, it is important to monitor such compounds in our environment and evaluate their effects on human health. We demonstrated that 3,6-DNBeP was genotoxic in *in vivo* assay systems. Moreover, 3,6-DNBeP showed equivalent or further genotoxic activity when compared with other nitro-PAHs such as DNP isomers. DNP isomers show carcinogenicity in experimental animals and are thought to be human carcinogens in the environment. Thus, 3,6-DNBeP could be carcinogenic in experimental animals and also in humans. Further studies on genotoxicity mechanisms including DNA adduct formation, carcinogenicity, and exposure levels of 3,6-DNBeP should be conducted.

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Review Article

# Epidemiology of Esophageal Cancer in Japan and China

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## ABSTRACT

In preparation for a collaborative multidisciplinary study of the pathogenesis of esophageal cancer, the authors reviewed the published literature to identify similarities and differences between Japan and China in esophageal cancer epidemiology. Esophageal squamous cell carcinoma (ESCC) is the predominant histologic type, while the incidence of esophageal adenocarcinoma remains extremely low in both countries. Numerous epidemiologic studies in both countries show that alcohol consumption and cigarette smoking are contributing risk factors for ESCC. There are differences, however, in many aspects of esophageal cancer between Japan and China, including cancer burden, patterns of incidence and mortality, sex ratio of mortality, risk factor profiles, and genetic variants. Overall incidence and mortality rates are higher in China than in Japan, and variation in mortality and incidence patterns is greater in China than in Japan. During the study period (1987–2000), the decline in age-adjusted mortality rates was more apparent in China than in Japan. Risk factor profiles differed between high- and low-incidence areas within China, but not in Japan. The association of smoking and drinking with ESCC risk appears to be weaker in China than in Japan. Genome-wide association studies in China showed that variants in several chromosome regions conferred increased risk, but only genetic variants in alcohol-metabolizing genes were significantly associated with ESCC risk in Japan. A well-designed multidisciplinary epidemiologic study is needed to examine the role of diet and eating habits in ESCC risk.

***Key words:*** esophageal cancer; epidemiology; risk factor

## INTRODUCTION

Since the signing in November 2008 of a memorandum between the Ministry of Health (China) and the Ministry of Health, Labour and Welfare (Japan), the National Cancer Center (Tokyo) and Chinese Academy of Medical Sciences (Beijing) have each assembled a research group to facilitate collaboration on cancer epidemiology, prevention, and control. Areas of potential or ongoing collaboration include cancer registries, tobacco control, cancer epidemiologic studies and prevention, and environmental exposure assessment. After literature review and mutual field visits, the 2 research groups have reached an agreement on conducting a multidisciplinary study of the pathogenesis of esophageal and gastric cardia cancer in Hebei Province, China, a region that contains areas with some of the highest incidences of esophageal cancer in the world. To prepare for this collaborative work, the authors reviewed the published literature to identify similarities and differences in esophageal cancer epidemiology between Japan and China and generate hypotheses for further study.

Two major histologic types of esophageal cancer have been defined: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC).[1] While EAC has emerged as the major type in some Western countries, in Asia ESCC is the predominant type and EAC remains rare.[2–5] ESCC and EAC share biologic features and some common risk factors

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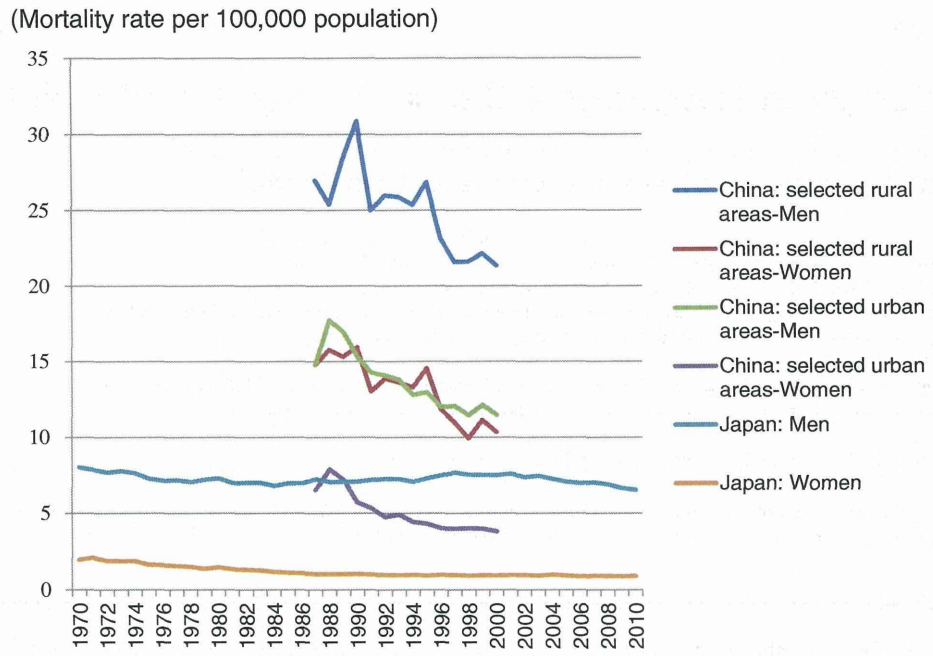


Figure 1. Age-standardized mortality rates in Japan and China. Source: World Health Organization mortality database.

such as cigarette smoking; however, they differ in geographic and demographic characteristics, risk factors, and pathogenesis.[6] Because of the rarity of EAC, ESCC has been the subject of most studies in Japan and China. Although numerous studies have been conducted in both countries, very few have comprehensively compared the characteristics of esophageal cancer. We believe that such a comparison would better address unresolved questions in the field and provide new ideas for further studies.

In this review article, the term "esophageal cancer" is used to refer to ESCC unless EAC is specified. We compare the burden of esophageal cancer between Japan and China in terms of patterns of incidence and mortality, address factors associated with ESCC risk, based on epidemiologic studies conducted in each country, discuss prevention strategies, and propose 3 avenues for future studies of esophageal cancer pathogenesis.

## ESOPHAGEAL CANCER: INCIDENCE, MORTALITY, AND TRENDS

Overall incidence and mortality rates for esophageal cancer are higher in China than in Japan. According to the Globocan,[7] esophageal cancer is the tenth most common malignancy and the seventh most common cause of cancer death in Japan, with an estimated 17 497 new cases and 11 746 deaths in 2008. The estimated overall age-adjusted incidence rate (standardized for world population) in 2008 was 5.7 per 100 000 population. Data provided by the Center for Cancer Control and Information Services show that the age-adjusted incidence rate (per 100 000 population) increased from 8.3 to 11.7 during the period 1975–2006 among Japanese men but changed little among Japanese women, who had an estimated rate of approximately 1.5 during that period.[8] During the period 1950–2010, the annual number of deaths continued to increase among Japanese men but did not significantly change among Japanese women (data not shown; available from the World Health Organization [WHO] mortality database). The male-female ratio of esophageal cancer mortality is approximately 6:1. However, according to the WHO mortality database,[7] during the period 1970–2010, there was a gradual decrease in age-adjusted mortality among men after 1996 and a gradual decrease among women throughout the entire period (Figure 1).

In contrast, esophageal cancer is the fourth most frequently diagnosed cancer and the fourth leading cause of cancer death in China, with an estimated 259 235 new cases and 211 084 deaths in 2008. The estimated age-adjusted incidence rate in 2008 was 16.7 per 100 000 population. As compared with Japan, crude and age-standardized incidence rates for esophageal cancer markedly vary by geographic variation in China (Table 1). Throughout China, incidence rates are generally higher in rural areas than in urban areas. In particular, certain rural areas in Henan, Hebei, and Shanxi in Central North China have among the highest incidence rates in the world (>100 per 100 000 population). For example, Cixian has an incidence rate 18 times that of Beijing or Shanghai (Table 1). Previous mortality studies showed that areas with age-adjusted rates greater than 30 per 100 000 population were distributed in Sichuan, Anhui, Jiangsu,

**Table 1. Crude and age-standardized incidence rates of esophageal cancer in Japanese and Chinese populations**

| Area | Period | Men | | | | | Women | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Crude | ASR | MV (%) | DCO (%) | MI (%) | Crude | ASR | MV (%) | DCO (%) | MI (%) |
| **Japan** | | | | | | | | | | | |
| Aichi Prefecture | 1998–2002 | 9.2 | 6.4 | 82.1 | 15.5 | 69.0 | 1.4 | 0.8 | 64.9 | 27.0 | 70.3 |
| Fukui Prefecture | 1998–2002 | 12.2 | 6.0 | 89.4 | 3.7 | 56.9 | 2.9 | 1.1 | 82.0 | 9.8 | 55.7 |
| Hiroshima | 1996–2000 | 18.4 | 12.1 | 95.4 | 1.4 | 56.9 | 3.7 | 2.0 | 92.5 | 1.9 | 53.8 |
| Miyagi Prefecture | 1998–2002 | 28.6 | 15.4 | 85.2 | 8.4 | 57.7 | 5.4 | 2.2 | 79.5 | 12.8 | 56.6 |
| Nagasaki | 1998–2002 | 21.7 | 10.7 | 90.3 | 4.8 | 66.0 | 3.1 | 1.2 | 85.4 | 7.3 | 68.3 |
| Osaka Prefecture | 1998–2002 | 19.0 | 10.8 | 76.3 | 12.0 | 76.7 | 3.7 | 1.7 | 71.1 | 16.8 | 72.9 |
| Saga | 1993–1997 | 14.7 | 8.2 | 85.0 | 9.0 | 51.0 | 2.1 | 0.9 | 84.0 | 11.0 | 56.0 |
| Yamagata Prefecture | 1998–2002 | 29.7 | 13.0 | 87.1 | 8.4 | 68.5 | 4.7 | 1.6 | 76.0 | 14.0 | 62.7 |
| **China** | | | | | | | | | | | |
| Beijing | 1993–1997 | 14.6 | 10.2 | 74.0 | 2.0 | 71.0 | 6.4 | 4.0 | 67.0 | 3.0 | 70.0 |
| Changle[a] | 1993–1997 | 21.0 | 30.1 | 55.0 | — | 87.0 | 8.2 | 8.9 | 47.0 | — | 87.0 |
| Cixian[a] | 1993–1997 | 133.9 | 183.8 | 75.0 | 3.0 | 72.0 | 105.0 | 123.1 | 70.0 | 6.0 | 72.0 |
| Guangzhou | 2000–2002 | 9.2 | 9.3 | 71.0 | 0.2 | 86.0 | 2.3 | 1.8 | 70.8 | 0.0 | 75.8 |
| Qidong County | 1993–1997 | 13.7 | 13.2 | 57.0 | 0.0 | 92.0 | 5.6 | 3.9 | 54.0 | 0.0 | 92.0 |
| Shanghai | 1998–2002 | 14.4 | 9.2 | 63.4 | 0.6 | 70.4 | 6.2 | 3.0 | 55.8 | 0.8 | 72.0 |
| Zhongshan | 1998–2002 | 16.0 | 16.5 | 95.5 | 0.0 | — | 1.8 | 1.9 | 95.0 | 0.0 | — |

Source: Cancer Incidence in Five Continents Vol. VIII and Vol. IX, IARC Scientific Publications No. 155 and No. 160.
ASR: age-standardized rate, per 100 000 population; MV: morphologic verification of diagnosis; DCO: death certificate only; MI: ratio of mortality to incidence registered.
[a]These 2 areas were defined as "high-incidence areas" (ASR >30 per 100 000 population).

Hubei, Fujian, Guangdong, and Xinjiang provinces.[9] In the present study, we use this rate (>30 per 100 000 population) as the definition of a "high incidence area".

As compared with rural areas such as Cixian,[10] cities like Shanghai and Beijing have experienced a greater decrease in esophageal cancer incidence over the past several decades. Using well-developed Shanghai cancer registry data, Zheng et al showed that the incidence of esophageal cancer had significantly decreased, by 59%, between 1975 and 1988.[11] Notably, a comparison of cancer registry data from Osaka Prefecture, Japan and Shanghai showed that by 1998–2002, these areas had comparable incidence rates (Figure 2).

According to the WHO mortality database,[7] age-adjusted mortality rates were higher in some rural areas than in urban areas. Both rural and urban areas showed a decline in mortality rates during the period 1987–2000 (Figure 1). The male-female ratio for esophageal cancer mortality in China is approximately 2:1.

## FACTORS ASSOCIATED WITH ESCC

### Squamous dysplasia

*Japan:* In Japan, controversy remains as to whether dysplasia should be recognized as a precursor lesion. Data are lacking on the prevalence of dysplasia in asymptomatic Japanese people, but a recent study of 1345 individuals who underwent a screening endoscopy during a health check-up found that 3% had dysplasia.[12] The association between dysplasia and ESCC risk is unknown because no prospective data are available.

*China:* Cytologic and endoscopic screening in high-risk areas of China showed that it was possible to detect precursor lesions such as dysplasia in asymptomatic individuals with early-stage cancer.[13] High-grade dysplasia is the principal precursor lesion and was shown to be closely associated with ESCC. In a prospective follow-up study in Linxian, China, squamous dysplasia was strongly associated with ESCC risk; the relative risk (RR) was 28.3 for individuals with severe dysplasia as compared with those with normal mucosa.[14] Other studies estimated that SCC developed in 9% of patients with squamous dysplasia during a 15-year period[15] and in 30% of patients with high-grade dysplasia during an 8-year period.[16]

### Alcohol consumption and cigarette smoking

*Japan:* The prevalence of current drinking was 36.4% among men and 6.9% among women, according to the 2009 National Health and Nutrition Survey.[17] The prevalence of current smoking was 38.2% in men and 10.9% in women, according to the same survey. Alcohol consumption and cigarette smoking are 2 major risk factors for esophageal cancer in the Japanese population. A 2011 meta-analysis of 4 cohort studies and 8 case-control studies published between 1990 and 2010 showed that drinkers had a 3.3-fold increased risk of developing ESCC as compared with nondrinkers (Table 2).[18] Moreover, all the included cohort studies reported a dose-response relationship between the amount of alcohol consumed, frequency of consumption, and ESCC risk.[18]

A meta-analysis of 4 cohort studies and 11 case-control studies showed that the RR for current smokers relative to never smokers was 3.73 (95% CI, 2.16–6.43).[19] A dose-