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Histological Effect of Nitrous Acid with Secondary Products of Nitrogen Dioxide and Nitric Oxide Exposure on Pulmonary Tissue in Mice

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Abstract

Numerous epidemiological studies of respiratory effects of nitrogen dioxide may have included exposures to nitrous acid, because conventional assays of nitrogen dioxide measure nitrous acid as nitrogen dioxide. A few epidemiological studies and human inhalation experiments of nitrous acid reported that nitrous acid is associated with decrements in lung function and possibly with respiratory symptoms. Moreover, our guinea pig exposure experiment of nitrous acid demonstrated that nitrous acid inducible the pulmonary emphysema-like disease and the architecture alterations of the alveolar duct centriacinar regions with the thickened interstitium. The purpose of this study was to assess the acute toxicity and the injury of exposure to nitrous acid with histopathological alterations in the respiratory tract in mice. We continuously exposed only filtered air and the filtered air with 8.4 ppm nitrous acid with secondary products of 2.8 ppm nitrogen dioxide and 7.2 ppm nitric oxide (24 hr/day) to two mice groups (n=5) for 3 weeks. We conducted histopathological analyses. We found the hyperplasia of the terminal bronchial epithelial cells with the meandering irregularly and without dysplasia in nitrous acid mice exposure group. In nitrous acid mice exposure group, we did not observe the architectural alterations such as the emphysema-like alterations which we have observed in our guinea pig exposure experiment of 3.6 ppm nitrous acid. Moreover, the collagen bundles and thickened interstitium were also indistinct in the alveolar duct centriacinar regions in this concentration of nitrous acid exposure to mice. These histopathological results suggest that the injury effects of HONO were weak.

Keywords: Nitrous acid; Nitrogen dioxide; Environmental pollutant; Asthma; Emphysema

Abbreviations: HONO: Nitrous Acid; NO_2: Nitrogen Dioxide; NO: Nitric Oxide

Introduction

Nitrous acid (HONO) exists as a gas in the atmosphere [1]. HONO is a primary product in a material combustion as well as nitrogen dioxide (NO_2) [2]. The significant positive correlation coefficients were found between HONO and NO_2 in homes and offices [3]. Moreover, conventional assays of NO_2 measure HONO as NO_2 [4]. For these reasons, previous studies of respiratory effects of NO_2 may have included exposures to HONO without independent measurement of exposure and effect [5]. Although numerous studies have shown the relationship between NO_2 and the alteration of respiratory function or histopathological changes [6-8], there are few examinations of the biological effects of HONO such as our guinea pig exposure experiment [9]. Our results demonstrated that HONO induced some similar effects with NO_2 at a lower concentration than NO_2 such as the pulmonary emphysema-like disease and the architecture alterations of the alveolar duct centriacinar regions with the thickened interstitium. Although the fibrosis in the alveolar duct centriacinar regions of the animals exposed to NO_2 have been described elsewhere [6], we could not observe the increased collagen bundles in the alveolar duct centriacinar regions in the HONO exposure group. Therefore we considered that the mechanism of similar biological effects of between HONO and NO_2 is different.

The purpose of this study was to assess the acute toxicity and the injuriousness of exposure to nitrous acid with histopathological alterations in the respiratory tract in mice. Because the clinical emphysema does not accompany with fibrosis, we consider that the collagen bundles is an important index in order to examine pulmonary emphysema effect of the environmental pollutant.

Materials and Methods

Chemicals

We purchased charcoal activated granular (a reagent prime class), soda lime No.2 (for CO_2 absorption), sodium nitrite (> 98.5% pure), lactic acid (> 85% pure), sodium hydrogen carbonate (> 99.5% pure), sodium carbonate (> 99.5% pure), glycerol (> 99% pure), methanol (> 99.8% pure), and eosin (> 85% pure) from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hematoxylin (Gill's Hematoxylin 2 for HE staining) was purchased from Muto Pure Chemicals Co., Ltd. (Tokyo, Japan).

Experimental animals

This study was performed with the approval of the committee on animal care of the Osaka Prefectural Institute of Public Health. Ten male ICR mice (about 50 g body weight and 6 weeks of age) were purchased from SLC, Shizuoka, Japan. The animals were divided into two groups (n = 5/group), and were preliminarily housed for 2 week in each chamber with filtered air before the exposure to HONO.

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The exposure chamber system

The exposure chamber system has already been described [9]. In briefly, mice were exposed to filtered air or HONO with NO_2 and NO in hand-made acrylic chambers for stainless cages-hanging structures. The air for the chambers was supplied by the filtration of room air with charcoal activated granular and American air filter for vinyl isolator (Clea Japan, Inc.; Tokyo, Japan). The filtered air flow was controlled by using a mass flow controller (Kojima Instruments Inc. model 8350MC-0-1-1; Kyoto, Japan) with an air flow of 20 L/min. These chambers were operated +1 mm H_2O relative to atmospheric pressure. The exhaust gas of the blower was passed through a tank of soda lime, and it was discharged to the outdoors.

HONO generation system

A HONO generation system has already been described [9,10]. In briefly, two tubing pumps (Nikko Engineering Concept, Inc. MRP-1X; Kanagawa, Japan) added two milliQ water solutions of 150 mMol sodium nitrite and 150 mMol lactic acid into one poreflon tube (Sumitomo Electric Fine Polymer, Inc. Osaka, Japan. TB-0201 (of 2 meters length)) in which the gas penetrates to the outside of the tube. The outside gas of the poreflon tube was send into the exposure chamber with the filtered air.

HONO was continuously supplied by passing the filtered air through the HONO generator for 3 weeks. The HONO exposure was stopped during about 3 hours in every 1 week for the exchanging cages and the cleaning chambers.

Measurement of nitrogen oxides

We used a noncontinuous method of measuring HONO employing the Harvard EPA Annular Denuder System [11,12] with two denuders in series sampling. The concentration of nitrite ion in the denuder extract was measured by the Saltzmann reagent method [13]. The difference of the measured value in the first denuder and second denuder was allowed determination of the total amount of HONO that passed through the denuder system. The chamber HONO concentration was calculated from the sampled volume. Table 1 shows the concentrations of exposure HONO.

The concentrations of the contaminated NO_2 and NO were measured by a NOx analyzer (J-science, Inc. ECL-77A; Kyoto, Japan) after passing into the sodium carbonate annular denuders. Table 2 shows the concentrations of the contaminated NO_2 and NO. NOx concentration of the filtered air chamber was confirmed under the limit of detection by the using a NOx analyzer.

Histopathological analysis

Tissue sections were affixed to microscope slides and deparaffinized. The slides were stained with hematoxylin and eosin and Elastica van Gieson, and examined under light microscopy.

Statistical analysis

Differences between the two experimental groups were examined for statistical significance using the student's t-test. Differences associated with P values less than 0.05 were considered significant.

Results

Effects of HONO exposure on histopathology

At 3 weeks before and after exposure to 8.4 ppm HONO with secondary products of 2.8 ppm NO_2 and 7.2 ppm NO, no significant differences were observed in the body weight between the animals in the filtered air group and the HONO exposure group. Macroscopic examination revealed that the hairs of the mice in the HONO group had a deep yellowish orange pigmentation (Figure 1).

Architectural alterations were not clear in all mice in the HONO exposure group such as the diffuse emphysemalike alterations in guinea pigs (Figure 2). The collagen bundles and the thickened interstitium were indistinct in the alveolar duct centriacinar regions in both the HONO exposure group and the filtered air group (Figure 3). However, we observed the slight multi-layering of the bronchial epithelial cells in the HONO exposure group with the meandering irregularly and without dysplasia (Figure 4-6). However, we could not observe cilia damage, goblet cell hyperplasia, and alterations in bronchi smooth muscle cells in the central airways (Figure 7).

Discussion

The effect of environmental HONO on the health of humans is not well-understood. The effects of environmental HONO may have been considered the effects of environmental NO_2, since the measurements of environmental HONO and environmental true NO_2 are difficult. Harris et al. suggested that the effects of HONO may have been ignored, because the outdoor ambient concentrations of HONO are less than those of NO_2 [14]. However, the indoor HONO concentrations

Concentration of nitrogen oxides (ppm: mg/L)†			
Sampling Period*	1st Denuder	2nd Denuder	1st Denuder–2nd Denuder
First half	10.9	2.0	8.9
Latter half	9.7	1.9	7.9
Estimate of HONO concentration (ppm:mg/L)			(8.4)

*The total sampling periods was under 2% in all exposure period.
†The collection efficiency of sodium carbonate annular denuder is different according to type of nitrogen oxides. HONO is perfectly trapped by the coated walls, while nonacidic gases such as NO_2 or NO passed through the coated walls. The first denuders trap all HONO and approximately 2–3% of NO_2 or NO. The second denuders trap only approximately 2–3% of NO_2 or NO

Table 1: Concentrations of exposure HONO.

	NO_2 (ppm)	NO (ppm)
Average of all measured value*	2.81 ± 0.78	7.17 ± 0.96
Estimate of NOx concentration(ppm)†	(2.8)	(7.2)

*One measured value was an average of the sampling period (30 minutes)
†Since the total sampling periods was under 2%, we estimated the concentrations of NOx

Table 2: Concentrations of the contaminated NO_2 and NO by the NOx analyzer.

Figure 1: Macroscopic examination on the hairs of the mice. (Left) The filtered air group. (Right) The HONO exposure group. Note the hairs of the guinea pigs had a deep yellowish orange pigmentation.

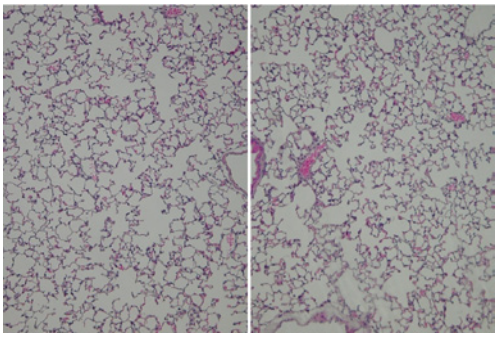

Figure 2: Hematoxylin and eosin stain for alveolus. (Left) The filtered air group. Note the usual air space distribution in the alveolus regions. (Right) The HONO exposure group. Note the similar air space distribution to the filtered air group in the alveolus regions.

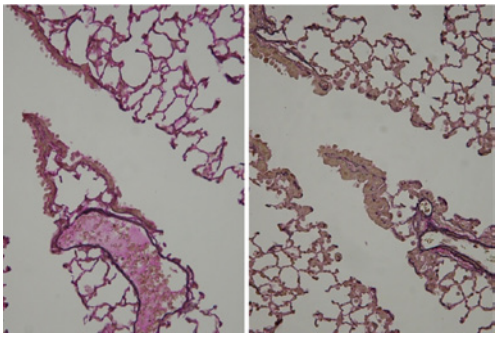

Figure 3: Elastica van Gieson stain for the collagen bundles and the interstitium of alveolar duct region. (Left) The filtered air group. Note the centriacinar regions of alveolar ducts were generally straight and less collagen bundles. (Right) The HONO exposure group. Note the similar architecture to the filtered air group in the alveolar duct centriacinar regions. However, we observed the replication of elastic fibers, and the terminal bronchial epithelial cells extended to the alveolar ducts. The collagen bundles were indistinct in both the HONO exposure group and the filtered air group.

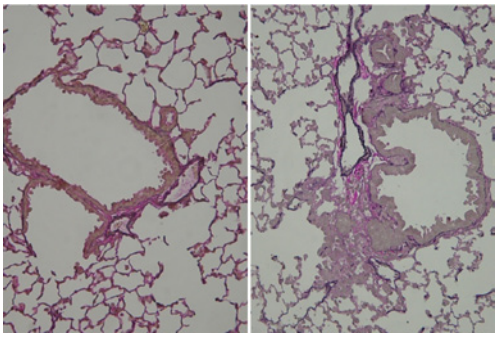

Figure 4: Elastica van Gieson stain for the alteration of the bronchial epithelial cells. (Left) The filtered air group. Note the slight invasion of the bronchial epithelial cells to alveolar regions. (Right) The HONO exposure group. Note the intense invasion of the bronchial epithelial cells to alveolar regions. The elastic fibers were distinct in around the invaded epithelial cells.

are not extremely different from those of NO_2. For example, in an epidemiological study on HONO, the median indoor concentration of HONO (3.10 ppb) was approximately one-fourth that of NO_2 (12.76 ppb), and the maximum indoor concentration of HONO (20.55 ppb) was approximately one-third that of NO_2 (59.12 ppb) [15].

We have demonstrated that 3.6 ppm HONO exposure to guinea pigs with secondary products of 0.3 ppm NO_2 and 1.6 ppm NO induced pulmonary emphysema-like alterations in the alveolar duct centriacinar regions, distortion of the centriacinar regions of alveolar ducts with extension of the bronchial epithelial cells and smooth muscle cells, and expansion of bronchial lumen [9]. However, we could not observe cilia damage, alterations in goblet cells, and collagen bundles

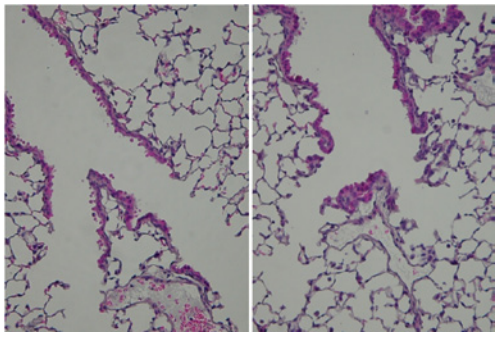

Figure 5: Hematoxylin and eosin stain for the alteration of the epithelial cells of terminal bronchioles. (Left) The filtered air group. Note the centriacinar regions of alveolar ducts were generally straight and thin alveolar walls. (Right) The HONO exposure group. Note the hyperplasia of the terminal bronchial epithelial cells with the meandering irregularly and without dysplasia.

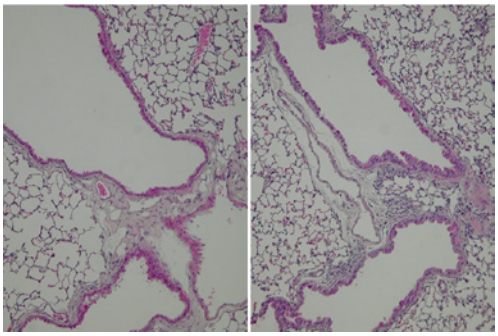

Figure 6: Hematoxylin and eosin stain for the alteration of the bronchial epithelial cells. (Left) The filtered air group. Note the bronchial epithelial cells were comparatively single-layering and the compact cell size. (Right) The HONO exposure group. Note the slight multi-layering of the bronchial epithelial cells with the meandering irregularly and without dysplasia.

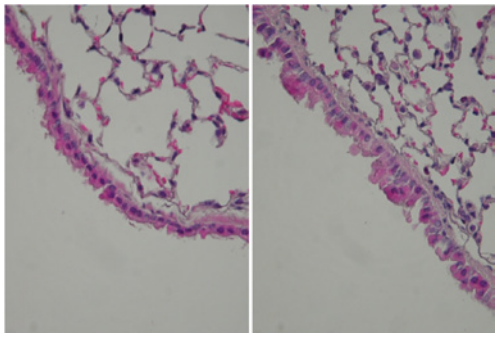

Figure 7: Hematoxylin and eosin stain for the alteration of the bronchial epithelial cells. (Left) The filtered air group. Note the bronchial epithelial cells were comparatively single-layering and the compact cell size. (Right) The HONO exposure group. Note the slight multi-layering of the bronchial epithelial cells with the expanded cytoplasm and without dysplasia.

in alveolar ducts in the guinea pigs HONO exposure experiment. In this mice experiment, we could not observe not only alterations in goblet cells and collagen bundles but also pulmonary emphysema-like alterations in spite of a higher concentration of HONO than the guinea pigs experiment.

These inflammatory alterations of mice exposed to NO_2 have been described elsewhere. For example, in C57BL/6 mice exposed to 20 ppm NO_2 for 14 h each day for up to 25 days, there was a marked progression in the extent of emphysema-like lesions with goblet cell hyperplasia and increased collagen deposition in the central airways [16].

Present indistinct results of alterations of pulmonary emphysema and collagen bundles suggest that the injury effects of HONO were weak. Mouse has no smooth muscle in the peripheral bronchus and few eosinophil granulocytes, and guinea pig has peripheral bronchi smooth muscle and rich eosinophil granulocytes. Therefore we considered that the effects of the HONO on the smooth muscle cells or eosinophil granulocytes might induce the architectural alterations in the exposure HONO.

Some epidemiologic studies have documented that outdoor NO_2 is associated with decreased lung function [17], and an increased number of hospital admissions for asthma [18-22]. However, it has been demonstrated that the emphysema by the animals NO_2 exposure experiments was accompanied with the fibrosis in the centriacinar regions of alveolar ducts. We consider that the NO_2 could induce the pulmonary emphysema by only high concentration such as the accompanying by the fibrosis. In human, clinical pulmonary emphysema has been defined as not accompanying by the fibrosis. Therefore, our results of mice and guinea pigs suggest that the environmental HONO is more effective in the human pulmonary emphysema than the environmental NO_2.

In the present experiment, the alterations of bronchial epithelial cells might be dependent on HONO and NO_2. The histopathological alterations have not been recognized in the concentrations of NO which are contaminated by the exposure to HONO gas. We want to discuss the effects of HONO on the bronchial epithelial cells by the purified HONO exposure experiment using improved HONO generator in the future.

In conclusion, the present results suggested that the injury effects of HONO were weak. We consider that the pulmonary emphysema effect of the environmental HONO is a more important than that of the environmental NO_2 in the human. There is still room for improvement on the HONO generator system. We will try to improve HONO generator for the purified HONO exposure experiments in the future.

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