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Ten-week Whole-body Inhalation Toxicity Study of Chlorine Dioxide Gas in Rats

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Abstract

Chlorine dioxide (ClO_2) gas can protect against influenza virus infection at a concentration of 0.03 parts par million (volume/volume), which is believed to be nontoxic in humans. This suggests an opportunity for the development of a novel protective method against airborne infectious diseases. This method is especially applicable to the highly pathogenic H5N1 influenza virus, as there is currently no safe and effective protection against H5N1. However, concerns remain regarding the safety of ClO_2 gas for protection against virus infection, especially whether a concentration of 0.03 parts per million is really non-toxic when it is used for humans in closed or semiclosed spaces. In view of the importance of the usefulness and necessity of low concentration ClO_2 gas treatment, it is critical to determine the "no observed adverse effect level" (NOAEL) of ClO_2 gas. In 1972 Paulet and Desbrousses reported that the "lowest observed adverse effect level" (LOAEL) of ClO_2 gas was 1 part per million. We attempted to confirm their data under carefully designed experimental conditions. Here, we performed a rat hole-body inhalation toxicity study, where rats were exposed to 1 part per million ClO_2 gas for 5 hours per day and 5 days per week over a period of 10 weeks. The rats were exposed to a meticulously controlled low-concentration ClO_2 gas. No adverse effect was observed under these experimental conditions in contrast to the study by Paulet and Desbrousses. We conclude that the no observed adverse effect level of ClO_2 gas at these experimental conditions is 1 ppm. We hope this result will help develop a preventive method against airborne microbial infectious diseases of humans.

Keywords: Chlorine dioxide; ClO_2; Gas; Inhalation; Toxicity; Whole-body; Rat

Introduction

The advent of highly pathogenic H5N1-type avian influenza virus [1] has posed a serious threat to human health. This is because currently there is no effective method to prevent airborne virus floating in the air. This is especially true during long flights, where people are confined in a completely closed space for long period of time. It is impossible to fumigate the inside of an aircraft without evacuating passengers. A safe and highly effective virucidal agent that can be used without evacuating humans from closed or semi-closed areas is urgently required. Such an agent could be used in closed or semi-closed spaces, such as aircrafts, hospitals, theaters, and houses.

Ogata and Shibata recently demonstrated that low-concentration chlorine dioxide (ClO_2) gas could protect mice exposed to aerosolized influenza virus [2]. Ogata recently elucidated the mechanism of the protective effect of ClO_2 gas against influenza virus at a molecular level [3,4]. The concentration of ClO_2 gas in the study [2] was 0.03 ppm (v/v) (parts per million, volume/volume) (83 μg/m^3), and mice exposed to this concentration of gas were apparently healthy despite presence of fatal levels of influenza virus [2]. This suggests that ClO_2 gas at this concentration can be used safely, and can protect animals from infection by airborne pathogens. However, several studies have demonstrated that high-concentration ClO_2 gas is toxic to animals [5-8]. Thus, it is crucial to determine what concentration of ClO_2 gas can be used for humans to protect them from microbial infections without necessitating their evacuation. In particular, we were very interested in a toxicity study by Paulet and Desbrousses [6-8], and whether their result was reproducible by repeating their experimental conditions [7]. They reported that rats exposed to ClO_2 gas at a concentration of 1 ppm (2.8 mg/m^3) caused vascular congestion of blood vessels of all diameters in the lung and minimum interstitial edema of the tracheal mucosa and the bronchus wall in a 10-week inhalation toxicity study [7]. Based on their results, the "lowest observed adverse effect level" (LOAEL) of ClO_2 inhalation toxicity was set at 1 ppm by the US Environmental Protection Agency (EPA) (http://www.epa.gov/IRIS/subset/0496.htm). This EPA report may hinder the worldwide dissemination of use of ClO_2 gas for protection against airborne microbial infectious diseases. We demonstrate here, under meticulously controlled experimental conditions, that we could not reproduce their result [7]. Based on our present results, we report that the "no observed adverse effect level" (NOAEL), not LOAEL, of ClO_2 gas is 1 ppm. Our result will fortify the safe use of low-concentration (< 1 ppm) ClO_2 gas for protection against airborne microbial pathogens.

Materials and Methods

Animals and reagents

Male and female Wistar rats (5 week old) were purchased from Charles River (Yokohama, Japan). They were acclimatized for one week before the commencement of the experiment. Two animals of the same gender were put in a 25 × 38 × 15 (height) cm stainless steel-mesh cage. This was then put in a larger "exposure chamber" for exposure to ClO_2 gas mixed with air or air only. Each exposure chamber contained 16 rats (8 male and 8 female rats) in 8 cages. The exposure chambers

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were placed in an animal room, and maintained at 24 ± 3°C, 50 ± 10% relative humidity, and a 12 hours light/12 hours dark cycle. Animal diet (CRF-1, Oriental Yeast, Tokyo, Japan) and tap water were given to the rats *ad libitum*. All animal experiment procedures were conducted in strict adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Taiko Pharmaceutical Ethical Committee of Animal Experiments.

ClO_2 gas generator

A ClO_2 gas generator was made in our laboratory as described previously [9]. The generation of gas was carefully controlled to keep the concentration of gas constant in the exposure chamber. The exposure chamber consisted of a box made of transparent plastic, whose dimensions were 90 × 120 × 45 (height) cm. The chamber had five holes of 12 mm diameter in the top (Figure 1) to monitor the concentration of ClO_2 gas inside the chamber. The concentration of ClO_2 gas in the chamber was measured twice a day by introducing a sampling tube into the chamber through the hole using a ClO_2 analyzer (models 4330-SP and 4330-1, Interscan Corporation, Simi Valley, CA). The chamber was ventilated constantly at a rate of 32 ± 1 m^3/h (n=9) by aspirating air by air pump. To homogenize the ClO_2 concentration in the chamber a cylindrical (30 cm wide and 6 cm diameter) laminar-flow electric fan (model MF930B-BC, Oriental Motor, Tokyo, Japan) was put inside.

Exposure of ClO_2 gas to rats

The rats were exposed to ClO_2 gas at 1 ppm for 5 hours per day, 5 days per week over a period of 10 weeks. Body weights of the rats were measured twice per week. The day after the end of the exposure period of 10 weeks, rats were anesthetized by inhalation of isoflurane gas using a veterinary anesthetizer (model V1, VetEquip Inc., Pleasanton, CA) and oxygen as carrier gas. Next, blood was aspirated from the abdominal aorta, followed by the excision of organs. The excised organs were photographed, weighed, visually observed, and fixed in 1.3 mol/L formaldehyde in 75 mmol/L phosphate buffer (pH 7.4). The formaldehyde-fixed organ was embedded in a paraffin block, sliced and stained by hematoxylin-eosin or by silver impregnation for collagen fibers and orcein for elastic fibers. Broncho-alveolar lavage fluid (BALF) was obtained from the excised lung by introducing 0.5 ml of phosphate-buffered saline into the left bronchus. Saline was introduced in and out of the bronchus three times, and a smear of a 0.1-ml aliquot was made for cell analysis. The histology of the organs and the BALF analysis were observed and recorded by a single person, and results were graded from – to 5 + depending upon the degree of the findings. The blood was subjected to biochemical and hematological analyses.

Statistical analysis

Differences of analytical values of two groups (ClO_2- *vs.* air-exposed) were evaluated statistically by a two-tailed Student *t* test for parametric data and by two-tailed Mann-Whitney *U* test for non-parametric data. The differences were considered significant if $p<0.05$.

Results

ClO_2 gas concentration

In a preliminary experiment, we found that the ClO_2 gas concentration varied markedly at several locations in the exposure chamber, although the rate of ClO_2 gas generation from the gas generator [9] was finely controlled and was extremely constant during a long period. Thus, it appeared that the gas concentration in the chamber was not homogeneous in terms of time and location despite constant introduction of ClO_2 gas from the generator into the chamber. To overcome this, we put a low-flow rate and laminar-flow electric fan in each exposure chamber to homogenize distribution of the gas in the chamber. As expected, we found a fairly constant gas concentration at many locations in the chamber (Figures 1 and 2, Table 1) over a long period (Figure 2). The gas concentration 1 hour after the commencement of gas exposure was 1.02 ± 0.07 ppm (2.81 ± 0.19 mg/m^3, n=50, data obtained during the entire period of the study) when ClO_2 gas concentration was measured at the center of the chamber (sampling port 5 in figure 1).

Body weight and general vital conditions

The body weight of male and female rats did not change significantly

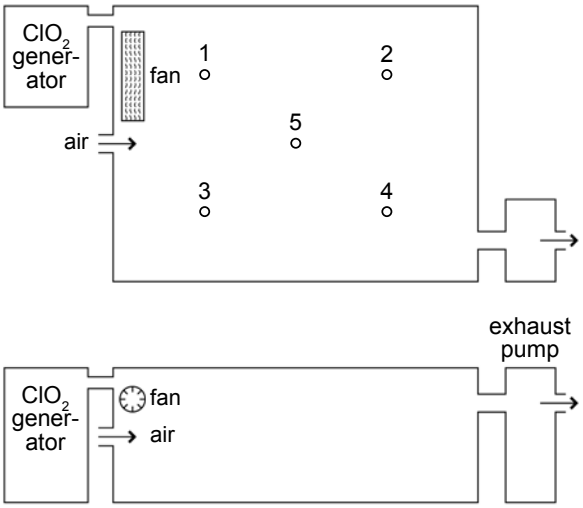

Figure 1: Experimental setup for the exposure of rats to ClO_2 gas or air using an "exposure chamber". The top-down view (upper) and lateral view (lower) of the ClO_2-exposure chamber are shown. ClO_2 gas sampling ports are shown and numbered 1 through 5. An "air-exposure chamber" had the same construction as the ClO_2-exposure chamber except that a ClO_2 generator was omitted.

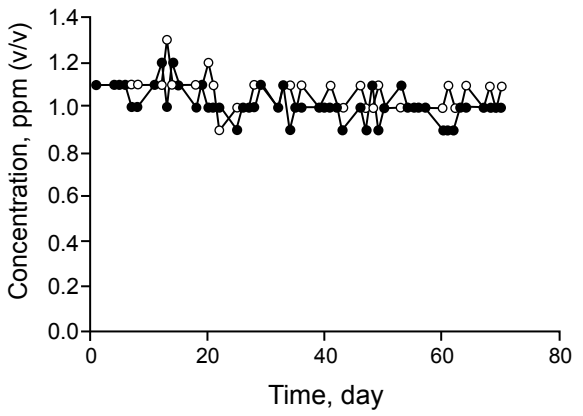

Figure 2: Time course of ClO_2 gas concentration in the ClO_2-exposure chamber. The concentration of ClO_2 gas was measured at the center of the ClO_2-exposure chamber (sampling port 5 in figure 1) 1 hour (filled circles) and 4 hours (open circles) after commencement of the ClO_2 gas delivery into the exposure chamber each day. Each point represents one measurement.

	ClO_2 gas sampling port				
Measurement	**1**	**2**	**3**	**4**	**5**
First	1.1	1.3	1.1	1.2	1.1
Second	1.2	1.2	1.1	1.2	1.0
Third	1.2	1.2	1.2	1.2	1.1

Table 1: ClO_2 gas concentration at various locations in the ClO_2-exposure chamber. ClO_2 gas was measured 1 hour after the commencement of gas exposure at various locations (sampling ports) in the exposure chamber as indicated in figure 1. Each value represents gas concentration in ppm (v/v) of a single measurement.

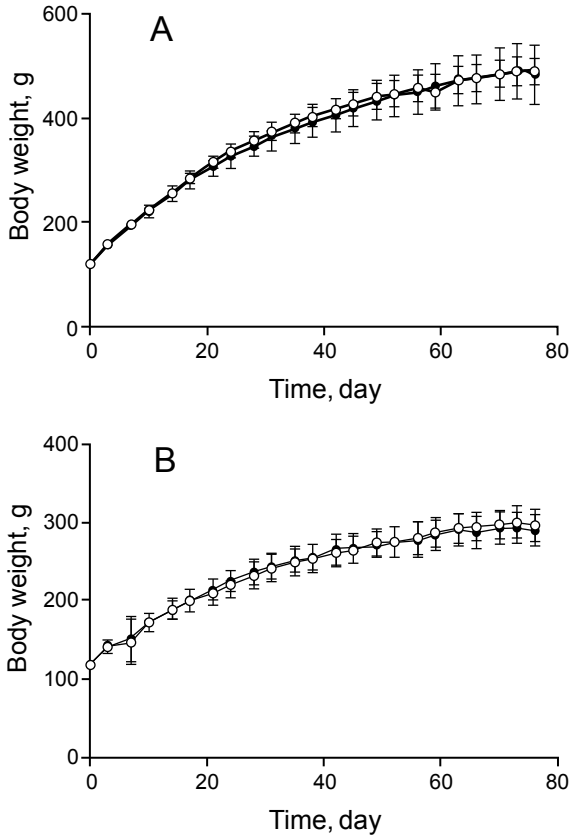

Figure 3: Body weight changes of rats exposed to ClO_2 gas and air. Male (A) and Female (B) rats exposed to 1 ppm ClO_2 (filled circles) or air (open circles) are shown. Each point represents mean ± standard deviation of eight rats.

whether they were exposed to ClO_2 gas or air (Figure 3). The general conditions of the rats from both groups (ClO_2- and air-exposed), as assessed visually, were healthy during the entire period of the study.

Pathological findings at necropsy

At necropsy, the organs observed (tongue, pharynx, larynx, trachea (both outside and inside), lungs, liver, kidneys, heart, thymus, spleen, stomach, small and large intestines, mesenteric lymph nodes, ovaries, and uterus) appeared normal visually in both groups (ClO_2- and air-exposed) (data not shown). However, there was a small pulmonary atelectasis in one male rat exposed to ClO_2 gas. This appeared to not be caused by inhalation of ClO_2 gas, because pulmonary atelectasis of a similar size was also found in one female rat in the air-exposed group. Pulmonary atelectasis is sometimes found in laboratory rats, and we speculate that it is caused by inhalation of dust particles originating from the animal diet. Weights of excised organs (lungs, liver and kidneys) were almost the same in both groups of rats (Supplementary Table 1).

Hematological and biochemical analyses

Both hematological and biochemical analyses were within normal limits in the ClO_2- and air-exposed groups (Supplementary Tables 2 and 3). The results indicated that inhalation of ClO_2 gas does not affect metabolism and bone marrow function of rats.

BALF analysis

BALF smears were analyzed microscopically (Figure 4). There were

Figure 4: Microscopic pictures of cells in bronchoalveolar lavage fluid. Representative cells obtained from bronchoalveolar lavage fluid of female rat exposed to air (left) or 1 ppm ClO_2 gas (right) and stained with Giemsa solution are shown.

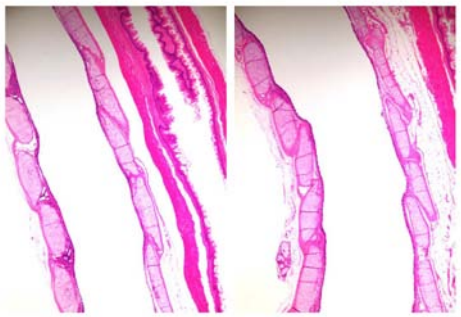

Figure 5: Microscopic pictures of tracheas. Representative pictures of trachea of male rat exposed to air (left) or 1 ppm ClO_2 gas (right) and stained with hematoxylin-eosin are shown.

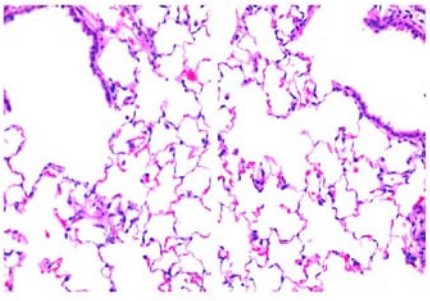

Figure 6: Microscopic pictures of lungs. Representative pictures of lung of female rat exposed to air (left) or 1 ppm ClO_2 gas (right) and stained with hematoxylin-eosin are shown.

no statistically significant differences, namely $p>0.05$, between the ClO_2- and air-exposed groups when analyzed for severity by Mann-Whitney *U*-test (Table 2), suggesting that inhalation of the ClO_2 gas does not affect the respiratory tract of rats.

Histological examination

The histological findings of organs were all within normal limits, and there were no statistically significant differences, namely $p>0.05$, between the ClO_2- and air-exposed groups when analyzed for severity by Mann-Whitney *U*-test (Table 3). Pharynx, trachea (Figure 5), bronchus, bronchiole and lung (Figure 6) were all normal. Collagen fibers and elastic fibers, each stained by a specific method as mentioned above, were observed in lung specimens, and appeared completely normal (data not shown). In one female rat exposed to ClO_2 gas, dilatation of the proximal renal tubule was observed. This finding may not be attributable to the effect of ClO_2 gas, since dilatation of the proximal renal tubule is sometimes found in the strain of rat used in this experiment. Taken together, we conclude that exposure of ClO_2 gas at 1 ppm under the experimental conditions described has no toxic effects on rats. Thus, NOAEL for ClO_2 gas in rats is 1 ppm.

Discussion

Paulet and Desbrousses reported that 1 ppm gas of ClO_2 was toxic to rats: Wistar rats (gender not specified) were exposed to the gas for 5 hours per day and 5 days per week during a period of 10 weeks [7] as in the present study. They reported that body weight, peripheral blood counts of erythrocyte and leukocyte, and parenchyma of the liver and lung did not change [7]. We observed similar results in the current study. However, they reported that "congestion of blood vessels of all diameters in the lung and minimum interstitial edema affecting tracheal mucosa and the wall of bronchus, without noticeable alteration of epithelium, were found" [7]. This observation is in strong contrast to our results (Table 3). We did not observe congestion of blood vessels of any diameter in the lungs. Furthermore, we did not observe interstitial edema in the mucosa of trachea and bronchus. The reason for these differences in results between studies is unknown.

We speculate that the differences might be due to fluctuation of the concentration of ClO_2 gas exposed to rats. Paulet and Desbrousses did not describe the details of their ClO_2 gas generation system and the method of measurement of ClO_2 gas concentration in their exposure

Exposure	Gender	Rat	Columnar epithelial cell	Macrophage	Neutrophil	Lymphocyte
Air	Male	1	2+	3+	-	-
		2	+	4+	-	-
		3	+	2+	-	-
		4	+	4+	-	+
		5	+	+	-	-
		6	+	-	-	-
		7	-	+	-	-
		8	+	3+	+	-
Air	Female	9	2+	4+	+	-
		10	+	2+	-	+
		11	+	+	-	-
		12	+	3+	+	-
		13	+	2+	-	-
		14	+	2+	-	-
		15	+	+	-	-
		16	+	2+	-	-
ClO_2	Male	17	+	4+	-	-
		18	+	4+	-	-
		19	2+	5+	-	-
		20	-	5+	-	-
		21	+	2+	-	-
		22	+	3+	-	+
		23	-	+	-	-
		24	+	3+	-	-
ClO_2	Female	25	+	-	-	-
		26	+	2+	-	-
		27	+	2+	-	-
		28	+	3+	-	-
		29	+	2+	-	-
		30	-	2+	-	-
		31	+	2+	+	-
		32	+	2+	-	-

The extent of inflammatory changes observed was graded from – to 5+ by a single examiner. Differences between the ClO_2- and air-exposed groups, evaluated by Mann-Whitney U-test, were statistically not significant

Table 2: Analysis of broncho-alveolar lavage fluid (BALF) of the ClO_2- and air-exposed rats obtained at necropsy.

Exposure	Gender	Rat	Secretion in trachel mucosa	Debris in tracheal	Vacuolization of hepatocytes	Lymphocyte mobilization in liver	Increase of intra-hepatic bie ducts	Secretion in nasal cavity	Macrophages in lung	Lymphocytes in lung
Air	Male	1	-	-	-	+	-	-	+	+
		2	+	-	-	+	-	+	+	+
		3	+	-	-	-	-	+	+	-
		4	+	-	-	+	-	+	+	-
		5	2+	-	-	+	-	-	+	+
		6	-	-	-	-	-	+	+	+
		7	+	-	-	+	-	+	+	-
		8	+	+	-	+	-	-	+	+

Air	Female	9	-	-	+	-	-	2+	+	+
		10	-	-	-	+	+	+	+	+
		11	-	-	-	+	-	+	+	-
		12	2+	-	-	-	-	2+	+	+
		13	-	-	+	+	-	+	2+	+
		14	+	-	-	-	-	+	+	+
		15	+	+	+	-	+	-	+	2+
		16	+	-	-	+	-	+	+	+
ClO_2	Male	17	-	-	-	+	-	2+	2+	+
		18	+	-	-	+	-	2+	+	+
		19	-	-	-	-	-	+	+	+
		20	-	-	-	+	-	+	+	+
		21	-	-	-	+	-	+	+	-
		22	+	-	+	+	-	+	2+	-
		23	+	+	+	-	+	-	2+	+
		24	+	-	-	2+	-	-	+	+
ClO_2	Female	25	+	-	+	+	-	-	+	-
		26	-	-	-	+	-	+	+	-
		27	-	-	-	+	-	+	2+	+
		28	-	-	-	-	+	2+	+	+
		29	+	+	-	+	-	+	+	+
		30	-	-	-	+	-	-	+	+
		31	+	+	-	+	-	-	+	+
		32	+	+	+	-	-	-	+	+

Table 3: Histological analysis was graded from – to 5+ by a single examiner. Differences between the ClO2- and air-exposed groups, evaluated by Mann-Whitney U-test, were statistically not significant.

apparatus, which was not described in detail [7]. In addition, data of the actual measurement of gas concentrations are not presented in their report. Consequently, it is unclear how precisely they maintained the 1 ppm concentration during the entire exposure period. As we described above, we originally found it quite difficult to keep homogeneous concentrations of ClO_2 gas in the exposure chamber. In addition, it was quite difficult to produce a ClO_2 gas generator that could generate ClO_2 at a constant rate until we developed an advanced device in our laboratory [9] where the generation rate of ClO_2 gas was very constant for a long period. Consequently, we were unable to evaluate their data in detail. We, therefore, repeated their experiment as exactly as possible, following their experimental conditions, to rigorously evaluate their data. As shown in our results, we could not reproduce their data. Thus, we suggest that the LOAEL of 1 ppm reported by Paulet and Desbrousses [7] should be interpreted as NOAEL.

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