Exp. Anim. 39(2), 223—229, 1990

Virucidal Effect of Ozone Treatment of Laboratory Animal Viruses

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(Received 19 September 1989/Accepted 15 November 1989)

An ozonization method was used to inactivate the viral pathogens of laboratory animals. Ozone at a concentration of over 100 ppm with high humidity was highly virucidal against 4 RNA viruses : HVJ, Theiler's murine encephalomyelitis virus (TMEV), Reo type 3 virus (RV) and murine hepatitis virus (MHV). For the ozone tests, 0. 1 ml of a virus suspension in deionized water or saline and was placed in 35-mm dishes. The titer of 10^6 plaque-forming units of TMEV in a liquid-phase, which was highly stable against physical treatments, was reduced within 1hr to a level of 0 by 300 ppm of ozone at 80% humidity and 22-25℃. HVJ and MHV were more susceptible than TMEV to the ozone treatment. RV was the most resistant of the 4 viruses. The ozonization method may be a good way to disinfect not only for the laboratory animal RNA-viruses (both of enveloped and unenveloped viruses) but also animal rooms, clean rooms and even safety cabinets.

In our previous studies, Theiler's murine encephalomyelitis virus (TMEV) and Reo type 3 virus (RV) was highly resistant to physicochemical virucidal treatments compared with Sendai virus (HVJ), canine distemper virus (CDV), hemorrhagic fever with renal syndrome virus and lymphocytic choriomeningitis virus [9, 13]. After 60-min exposures to UV irradiation and heat treatment at 22℃ or 45℃, 10^9 plaque forming units (PFU) of the former two viruses were never completely inactivated. Those results led us to this study to develop an effective, convenient, safe and easily applied way to inactivate the pathogens of laboratory animals.

Recently, an ozonization method has been developed for the microbial disinfection of water and wastewater [1, 2, 12], and for human viruses in dry-phase virus samples [4], but not for disinfecting laboratory animals.

Here we report on an ozone treatment with high virucidal efficacy against the enveloped viruses and even the unenveloped viruses which tend to be resistant to the physicochemical treatment previously reported [13].

Materials and Methods

Viruses : Four RNA viruses (TMEV, HVJ, RV and MHV) were studied. TMEV, propagated in BHK/21 cells, and was mainly studied because of its resistance to physicochemical treatment. The cells infected with a multiplicity of infection (m. o. i.) were cultured for 2 days at 37℃ and the virus was harvested after freezing/thawing 3 times. The virus titer was 10^8 PFU/ml. RV grown in LLC-MK_2 cells were treated by the same method as TMEV. All supernatants in Eagle's minimal essential medium (MEM) plus 2 % heat-inactivated fetal calf serum (FCS) was collected and stored at −80℃ until they were used. DBT and MA-104 cells were used for the propagation of MHV and HVJ, respectively [13].

Cell cultures and infectivity assay : BHK/21 cells (TMEV) and LLC-MK_2 cells (RV) were grown in MEM with 5 % FCS. The concentration of the FCS was reduced to 2 % for maintenance medium. Viruses inoculated onto monolayer cultures in 35-mm dishes (Coaster Co., Calif. U. S. A.) were allowed to be absorbed by the cells for 1hr at 37℃ in 5 % CO_2-air atmosphere. After aspiration of the

inoculum, a 1st layer (maintenance medium plus 0.6% agarose) was overlaid and cultured as above. The infectivity of HVJ and MHV were assayed as previously described [13]. Plaques were counted after staining with neutral red dye.

Lyophilization : Twenty five microliters of each virus in vials (Wheaton Co., New Jersey, U. S. A.) with MEM consisting of 0.5 % gelatin and 10% FCS was rapidly frozen in acetone-dry ice and lyophilized at −50℃ for 6 hr using a freeze-drier (Tokyo Rika Co., Tokyo, Japan).

Ozone treatment :

1. Dry-phase : The lyophilized virus samples in the glass vials were exposed to various concentrations of ozone in a glove box (Fig. 1 ; ELIOS Ozonizer ; Shinryo Reinetsu Co., Tokyo, Japan). Ozone generation was regulated within 10% of the desired concentration. Also, humidity (50% to 90%) and temperature (22-25℃) conditions were monitored by a recorder and controlled by a sonic humidifier.
2. Liquid-phase. A 0.1 ml portion of hundred-fold virus sample was put into a 35 mm-dish (Nunclon, Nunc Co., Denmark) resulting in a 0.1-mm thickness of the liquid phase, and various concentrations of ozone gas were applied to the samples as in the dry-phase treatment. No dishes were rocked during the treatment.

Evaluation of ozone treatment : Virucidal efficacy was evaluated by calculating the virus reduction by dividing the $\log_{10}$ virus titer (treated with ozone) by the $\log_{10}$ virus titer (control ; control viruses were kept in a moisture chamber at almost 90% humidity for the same time period as the test viruses under with ozone treatment) as previously described for the physicochemical treatments [8, 13].

Results

Effect of relative humidity on dry-phase samples : A preliminary experiment was conducted with two viruses, HVJ and TMEV. Dry-phase samples of the viruses were exposed to 100 or 200 ppm of ozone gas at 50, 70, and 80% humidity (Fig. 2). A zero level of infectivity was obtained within 1 hr (HVJ) and 3 hr (TMEV) when the two virus samples were exposed to 200 ppm of ozone and 80% humidity. To determine precisely the effect of humidity

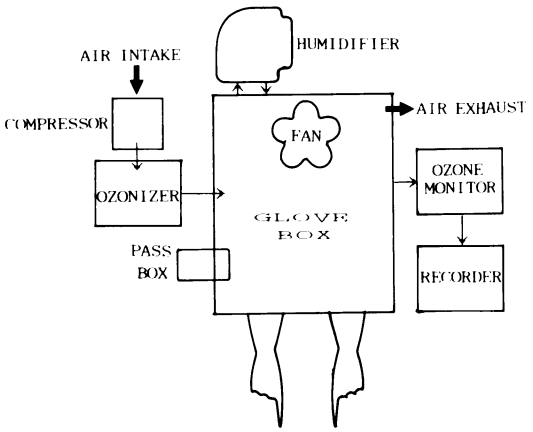

Fig. 1. Ozone generation system. The virus samples were put into a glove box through the pass-box, then treated with the gas for selected times. The ozone concentration and humidity were controlled and monitored by recorders.

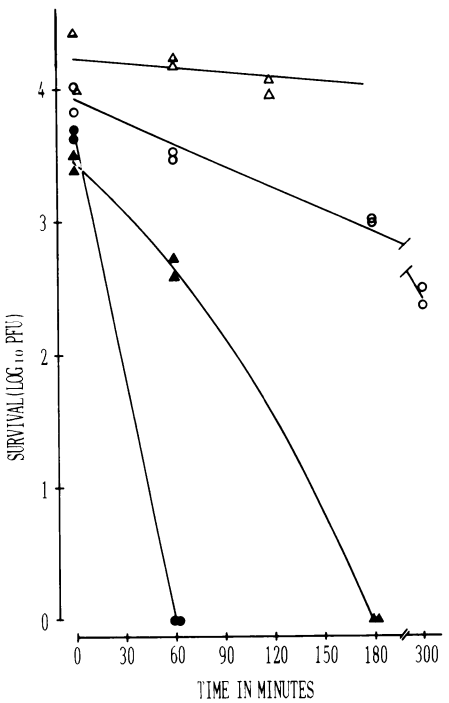

Fig. 2. Effect of ozonization on the dry-phase samples (HVJ and TMEV). HVJ samples were treated with 200 ppm of ozone at 50% (○) or 80% (●) relative humidity. TMEV samples were treated with 100 ppm of ozone at 70% (△) humidity and 200 ppm at 80% (▲) humidity.

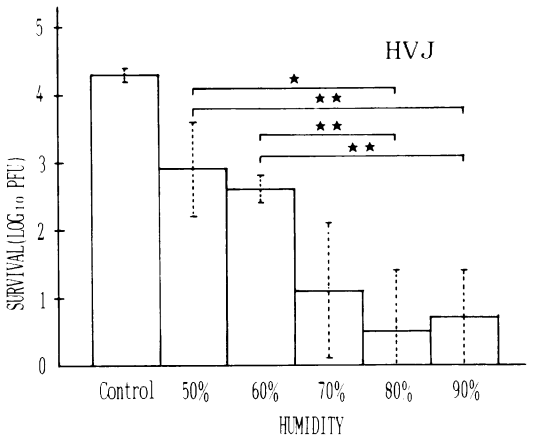

Fig. 3. Effect of humidity on dry-phase HVJ. Lyophilized samples were treated at 200 ppm for 30 min. The humidity was adjusted to 50, 60, 70, 80, or 90%. Control samples were kept in a moisture chamber at almost 90% humidity for 30 min at 22-25℃. After the treatment, the samples were diluted with 0.9 ml of MEM containing 0.1% albumin to make a 10-fold dilution and the survival was titrated in MA-104 cells. $^{*}p < 0.05$ $^{**}p < 0.01$

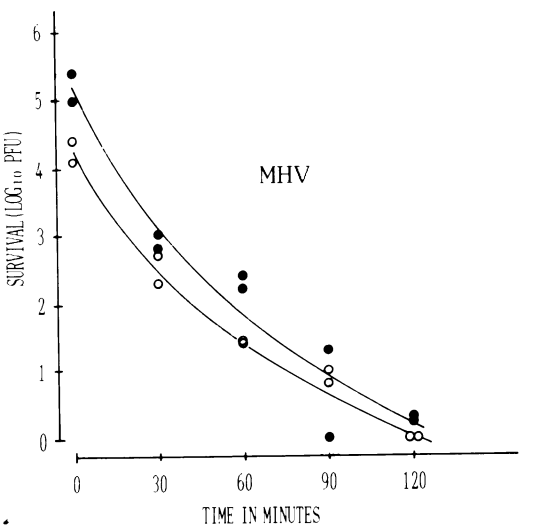

Fig. 4. Effect of ozonization on dry-phase MHV treated with 200 ppm (○) or 300 ppm (●) of ozone at 80% humidity.

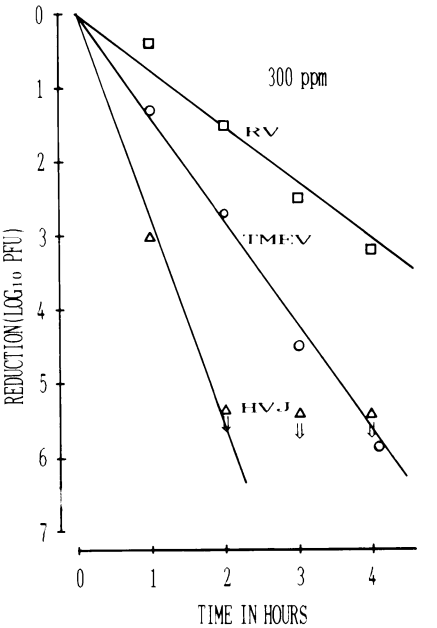

Fig. 5. Ozonization of three viruses (RV, TMEV, HVJ). The liquid-phase samples of three viruses were treated with 300 ppm of ozone at 80% humidity.

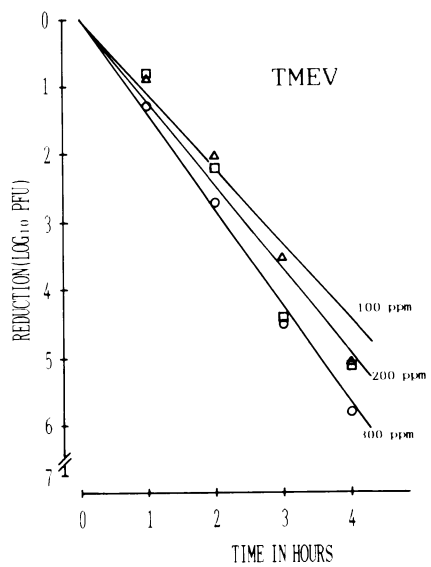

Fig. 6. Effect of ozone concentration on liquid-phase TMEV sample.The samples were ozonized with three different concentrations of ozone.

on dry-phase virus samples, the HVJ was treated with ozone at 200 ppm and at various humidities between 50% and 90%. Fig. 3 shows that over 80% humidity is significantly effective in the inactivation of HVJ.

MHV was treated at 200 ppm or 300 ppm of ozone and 80% humidity. There was no significant difference between the two ozone concentrations (Fig. 4).

Effect of ozone gas on liquid-phase samples : To determine the susceptibility of liquid-phase virus samples against the ozone gas, viral suspensions were diluted 100-fold with deionized water (DW) and 0.1-ml portions were placed in 35-mm plastic dishes. They were exposed to ozone gas (100 ppm to 300 ppm at 80% humidity). The results, Fig. 5, show distinct differences among the three viruses, RV, TMEV and HVJ. HVJ was the most susceptible. Treatment of HVJ with 300 ppm for 1 hr reduced the virus to 10^3 PFU. The infectivity was reduced to $>5.5 \log_{10}$ PFU within 2 hr. The RV was the most resistant, 0.1% of original virus dose survived after treatment for 3 hr. However, the 50% reduction time was estimated to be about 45 min. TMEV was intermediate in susceptibility.

In TMEV, the effect of the three concentrations of ozone, 100, 200 and 300 ppm, were not significantly different (Fig. 6).

Effects of various suspension media on liquid-phase samples : In preliminary experiments, when phosphate-buffered saline (PBS) was used instead of DW, virus inactivation by ozone was clearly blocked (data not shown). In additional experiments, various suspension media including 0.001-0.1% NaOH, 0.05-0.4% $NaHCO_3$, 0.1-0.2% bovine serum albumin (BSA) and 1/15 M PBS were used to determine the effectiveness of ozonization on TMEV. Hundred-fold dilutions of TMEV samples made with these media were exposed to ozone of 300 ppm for 1hr at 80% humidity and compared with non-exposed controls in a moisture chamber at almost 90% humidity at 22-25℃. As shown in Table 1, the alkaline solution and PBS partially blocked the effect of ozonization, but DW containing 0.1-0.2% BSA had no

Table 1. Effect of various suspension media on ozone treatment of TMEV*

Suspension media	Virus reduction** ($\log_{10}$ PFU)			
Deionized water (DW)	>5.3	>5.6	>5.8	>5.9
DW, 0.1% BSA	>5.9			
DW, 0.05% $NaHCO_3$	2.0			
DW, 0.1% $NaHCO_3$	1.8			
DW, 0.2% $NaHCO_3$	1.8	2.5		
DW, 0.2% $NaHCO_3$, 0.1% BSA	1.8			
DW, 0.4% $NaHCO_3$	3.1			
MEM, 0.2% $NaHCO_3$	2.3	2.4	2.8	
DW, 0.001% NaOH	3.7	5.2		
DW, 0.01% NaOH	1.7	1.9		
DW, 0.1% NaOH	1.8	2.2		
Saline	>5.2	>5.2		
PBS (7.2)***	3.8	4.7		
PBS (7.2), 0.01% Ca^{++} & Mg^{++}	3.5	4.0		

* A sample of the virus suspended in various media was transferred into a 35-mm plastic dish (0.1ml/dish), and exposed to ozone at 300 ppm for 1hr at 80% humidity. MEM (0.9ml, 10% FCS) was immediately added to the samples after the treatment. ** Reduction is as described in Materials and Methods. *** 1/15 M PBS

Table 2. Blocking effect of PBS (pH 7.0) on ozonization of TMEV*

Mol	Survival (log_{10} PFU/ml) control	treated	Virus Reduction (log_{10} PFU)**
Cont. (DW)	6.4, 5.4	0.4, 0	6.0, >5.4(>5.7)***
1/15M	6.0, 5.3	2.0, 0	4.0, >5.3(>4.7)
1/30M	6.0, 5.0	1.9, 1.3	4.1, 3.7 (3.9)
1/60M	5.7, 5.4	2.4, 1.7	3.7, 3.7 (3.7)
1/120M	5.8, 5.2	3.0, 1.4	2.8, 3.8 (3.3)
1/240M	5.9, 5.3	3.0, 2.0	2.9, 3.3 (3.1)
1/480M	5.6, 5.3	3.0, 1.8	2.6, 3.5 (3.1)
1/960M	5.8, 5.3	3.1, 1.4	2.7, 3.9 (3.3)
1/1,920M	5.7, 5.3	1.9, 0	3.8, >5.3(>4.6)
1/3,840M	5.5, 5.2	1.1, 0	4.4, >5.2(>4.8)

* A 0.1ml portion of the virus samples diluted 100-fold with various concentrations of PBS were put into 35-mm plastic dish (0.1ml/dish) and exposed in the chamber to ozone gas at 300 ppm for 1hr at 80% humidity and 22-25℃. MEM (0.9 ml, 10% FCS) was immediately added to the virus samples after the ozone treatment. ** Reduction is as shown in Materials and Methods. *** Numbers in parentheses indicate the mean virus reduction.

Table 3. Blocking effect of PBS (pH 7.0) on ozonization of RV*

Mol	Survival (log_{10} PFU/ml) control	treated	Virus Reduction (log_{10} PFU)**
Cont. (DW)	4.9, 4.7	0, 0	>4.9, 4.7 (>4.8)***
1/15 M	4.8, 4.7	2.7, 3.7	2.1, 1.0 (1.6)
1/150 M	4.8, 4.8	3.5, 3.8	1.3, 1.0 (1.2)
1/1,500 M	4.9, 4.8	0, 2.4	>4.9, 2.2 (>3.6)
1/15,000 M	4.9, 4.7	0, 0.4	>4.9, 4.3 (>4.6)

* The ozonization procedure was similar to the experiment in Fig. 2. ** Reduction is as shown in Materials and Methods. *** Number in parentheses indicate the mean virus reduction.

effect.

Blocking effect of PBS: Various concentrations were tested to determine the inhibitory effect of PBS. PBS in the range between 1/30M and 1/960M gave almost the same blocking effect, showing the maximum effect at 1/240M to 1/480M (Table 2). These results were reproducible. The same blocking effect of these PBS dilutions also appeared in RV samples (Table 3). However, when Tris-HCL buffer solution (pH 7.1) was used instead of PBS to maintain a neutral pH, it was ineffective against the ozone treatment (Table 4).

Effect of thickness of liquid-phase samples: To determine the permeability of ozone gas to the liquid-phase samples, TMEV was diluted with DW and 0.1 ml and 0.5 ml of the dilution were put into 35-mm dishes. The thickness of the samples were 0.1mm and 0.5mm, respectively, ignoring the meniscuc. They were ozonized at 300 ppm for 1 to 4 hrs.

The results are shown in Fig. 7. The infectivity of samples with 0.1 mm thickness was reduced over 10^4 PFU by ozonization within 1 hr, although the reduction rate in samples 0.5mm thick was lower than that in 0.1mm.

Table 4. Ineffectiveness of Tris-HCl buffered solution (pH 7.1) on ozonization of TMEV*

Mol	Survival (log_{10} PFU/ml) control	treated	Virus Reduction (log_{10} PFU)**
Cont. (DW)	5.5	0	>5.5
1/20 M	5.1	0	>5.1
1/80 M	5.4	0	>5.4
1/320 M	5.2	0	>5.2
1/1,280 M	5.3	0	>5.3
1/5,120 M	5.4	0	>5.4

* The ozonization procedure was similar to the experiment in Fig. 2. ** Reduction is as described in Materials and Methods.

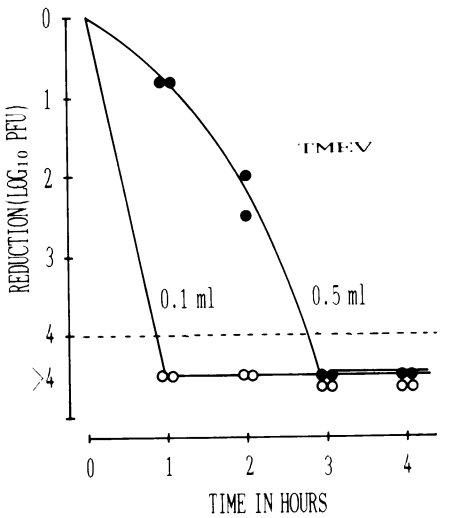

Fig. 7. Permeability of ozone to liquid-phase sample. TMEV samples diluted 100-fold with DW were put in 0.1 ml or 0.5 ml aliquots into 35-mm plastic dishes and ozonized with 300 ppm and at 80% humidity.

Discussion

Ozonization has been used to disinfect water and wastewater in Europe and America [5]. There are several reports concerning the effects of ozone treatment on human viruses [1-3, 5-7, 12]. However,its effect on dry-phase samples have been described in only one report [4]. Here we report the first application of ozone gas to laboratory animal viruses. We determined the effect of the ozone treatment on 4 laboratory animal viruses (enveloped and unenveloped RNA viruses) using dry-and liquid-phase samples. Our data indicated that more than 100 ppm ozone and 80% humidity was strongly virucidal, comparable to the chemical disinfectants iodophor or sodium hypochlorite [13]. The relative humidity was certainly a critical factor in the dry-phase virus tests. When 60% humidity was compared with 80% and 90% humidity, there was significantly higher inactivation at the higher humidity. So, it is important to maintain 80% humidity during the ozonization of dry-phase samples. RV and TMEV (unenveloped virus) were more resistant than HVJ or MHV (enveloped virus) in both dry-and liquid-phase samples. This character also appears with chemical disinfectants [13].

Although the mechanism of the ozone action is still unclear, Shinriki et al. reported that ozone attacked both the coat protein and the RNA of Tobacco mosaic virus, so the virus lost its infectivity because it could not uncoat [10, 11].

Alkaline pH and PBS (pH 7.2) partially blocked the efficacy of ozonization of TMEV liquid-phase samples. But adding BSA or diluting with Tris-HCl (pH 7.0) had no blocking effect. Roy et al. reported that the inactivation of human enteroviruses by ozone was influenced by a change of pH in the water [7]. The effect of PBS was a mol-dependent phenomenon which showed the highest blocking effect of ozone in the range of 1/240M to 1/480M PBS. This blocking effect may be due to a phosphate substance, since Tris-HCl gave no effect. The mechanism of this blocking effect by the phosphate should be clarified in detail.

Ozonizing may be a good method to use in laboratory animal centers in the following 3 ways : its high inactivation efficiency, its safety compared with formaldehyde fumigation, and third, it leaves no residual substance to be eliminated.

We wish to thank Dr. K. Maejima, Keio University school of Medicine,for his kind advice in the initiating this experiment.

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オゾンによる実験動物ウイルスの不活化

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欧米で汎用されている上水殺菌法の一つであるオゾン殺菌法を実験動物由来のセンダイウイルス (HVJ), マウス脳脊髄炎ウイルス (TMEV), マウス肝炎ウイルス (MHV), レオ3型ウイルス (RV) の4種RNAウイルスに対して作用させ, その不活化効果を検討した。その結果, 凍結乾燥状態のウイルス材料には80%以上の高湿度条件が必須であることが解明され, また液体状態のウイルス材料に対してもオゾンは不活化効果が高く, オゾン濃度100ppm以上, 湿度80%以上で高い有効性を示した。TMEVは物理化学的処理に対し比較的抵抗性が高いウイルスであるが, このウイルスの液体材料でもオゾン100ppm, 湿度80%, 温度22-25℃, 1時間処理により 10^4 PFU以上の不活化効果を示した。オゾン燻蒸法は浸透性にやや難点を持つものの上記条件を整備することにより, 乾燥及び液体状態の実験動物ウイルス材料に対し高い有効性を示し, 且つ使用上の安全性や残留性においてホルムアルデヒド燻蒸法に優っていると考えられる。これらのメリットを生かした殺菌・消毒法の一つとして動物実験施設の飼育室やクリーンルームあるいは安全キャビネット等に適用できる可能性がある。