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Short communication

Effects of ozone exposure on inactivation of intra- and extracellular enterovirus 71

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Abstract

In this study, the potential of ozone in inactivating enterovirus 71 (EV71) free particles was investigated using either various ozone flow rates of 100, 80 or 60 mg/h or a constant flow rate of 80 mg/h, given to culture medium or various pH culture media containing EV71, respectively. Results demonstrated that EV71 inactivation by ozone was related to the kinetics of ozone solubility, ~99% inactivation being obtained in the exponential phase of ozone solubility. However, the inactivation rate was dependent on the ozone input flow rate and positively enhanced at acidic pH. Inactivation of intracellular EV71 was also studied. At a constant ozone supply of 60 mg/h, a significant reduction of intracellular virus titer (≥99%, $p < 0.01$) was obtained after 45 or 60 min exposure but with low cell viability. Upon 30 min exposure, however, 45% cell viability was retained. The results indicate that the inactivating effect of ozone on intracellular EV71 virus is dependent on exposure duration.

Keywords: Enterovirus 71; Ozone; Virus inactivation

1. Introduction

Enterovirus 71 (EV71), a positive single stranded RNA virus, is a member of the Enterovirus genus of the family of *Picornaviruses*. This virus is the most common cause of child infections (Komatsu et al., 1999) and has been associated with several outbreaks of hand, foot-and-mouth disease (HFMD) (Hagiwara et al., 1978; Miwa et al., 1980), and severe neurological disease in Australia (Katzenelson et al., 1979; Gilbert et al., 1988), Europe (Chumakov et al., 1979; Nagy et al., 1982), Asia (Tagaya et al., 1981) and the United States (Alexander et al., 1994; Hayward et al., 1989). The central nervous system (CNS) complications associated with EV71 infection are being reported with increasing frequency. Most recently, large outbreaks of EV71 infection with severe and fatal cases of brain stem encephalitis have occurred in Malaysia in 1997 (AbuBakar et al., 1999) and in Taiwan in 1998 (Chang et al., 1999; Ho et al., 1999), causing death of more than 100 children. However, no efficient antiviral agent is available for the treatment of EV71 infections. The potential of EV71in causing severe disease underscores the need for therapy or prevention.

Ozone is the triatomic allotrope of oxygen and has been shown to possess broad-spectrum antimicrobial activity (Akey and Walton, 1985; Domingue et al., 1988). It has been widely used in sewage treatment, in water purification and in medicine (Clarke and Breman, 1983; Raung and Viebahn, 1987). Ozone also has been shown to be effective against a number of enveloped and non-enveloped viral species (Akey and Walton, 1985; Herbold et al., 1989; Carpendale and Freeberg, 1991; Wells et al., 1991; Arimoto et al., 1996; Khadre and Yousef, 2002; Shin and Sobsey, 2003). Studies on inactivation of HIV indicated ozone treatment provides a promising method for removing infectivity from human body fluids and blood product preparation (Wells et al., 1991; Carpendale and Freeberg, 1991). Studies carried out in influenza virus infection revealed that ozone exposure diminished the severity of disease in mice, as shown by decreased mortality and increased survival time (Wolcott et al., 1982; Jakab and Hmieleski, 1988; Selgrade et al., 1988). No information on the inactivation of EV71 by any disinfectant is available. In this study, we examined the effects of ozone on inactivation of EV71 in culture medium, as well as intracellularly. Information on the inactivation of enterovirus 71 in vitro may contribute to

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the development of therapeutic strategies for enterovirus 71 infections.

2. Materials and methods

2.1. Cell culture and virus preparation

Vero cells (green monkey kidney cells) were routinely maintained in M199 medium (GIBCO, Invitrogen Corporation) containing 10% fetal bovine serum (FBS). EV71 (kindly provided by Dr. M.S. Ho, IBMS Academic Sinica) was propagated in Vero cells by infection with an MOI of 1 in medium M199 with 2% fetal bovine serum and incubated at 37 °C in 5% CO_2. When 80% of the cells showed the typical enteroviral cytopathic effect (CPE), the infected cells were subjected to two freeze–thaw cycles for the virus harvest. The cellular debris was removed by centrifugation at 2000 × *g* for 6 min and the resultant supernatant was stored at −70 °C until use.

2.2. Exposure of viral solution to ozone

Twenty-five milliliter of virus solution (10^6 $CCID_{50}$ ml^{-1}, pH 6.7 ± 0.2) was placed in a roller bottle. Ozone produced from a generator (Tenco, XV1043CA, Taiwan) was bubbled through a gas diffusing stone into the solution at different flow rates of 100, 80 and 60 mg/h. The input flow rate of ozone in mg/h used in this study represented a substantial mass of ozone provided to avoid the influence of temperature or pressure, causing the complexity of chemical reaction calculation. Virus solution subjected to filtered air served as control. Samples of the exposed virus solution were taken at each time point and immediately neutralized with 0.1N sodium thiosulfate for the determination of viral titer. All procedures were performed at room temperature (25 °C).

2.3. Effect of pH on ozone disinfection

A virus solution with pH 6.5 ± 0.3 was adjusted by adding appropriate volume of sodium hydroxide (1N) or hydrochloric acid (1N) to reach a particular pH value of 3, 5, 7, 9 and 11. For ozone exposure, 15 ml of adjusted virus solution (10^5 $CCID_{50}$ ml^{-1}) was immediately exposed to filtered air or ozone (80 mg/h), following the method described above.

2.4. Exposure of infected cells to ozone

Monolayers of Vero cells (10^6 per dish) growing in dishes (40 mm × 10 mm) were infected with EV71 at an MOI of 1. The virus was allowed to adsorb for 1 h at 37 °C, and the cells were then grown in 1 ml of M199 containing 2% FBS. When ∼45% of cells showed cytopathic effect, the whole dish with medium was exposed to ozone in a chamber fitted with a pair of tubes for ozone inlet and outlet. Both tubes were fitted with an air filter. A flow rate of 60 mg/h of ozone was obtained through an air filter into the chamber at 37 °C, 5% CO_2. Multiple identical dishes were set up for different exposure periods (15, 30, 45 and 60 min). At the end of exposure time, dishes taken immediately served as time-zero samples, others were washed with PBS and replaced with fresh growth medium and then incubated at 37 °C, 5% CO_2 for recovery. Samples were taken at each time point for determination of the viral titer, cell viability and LDH release. Cells without EV71 infection as control were exposed to ozone using the same method described above.

2.5. Determination of ozone concentration

The concentration of ozone in culture medium or virus solution was determined using a colorimetric method, as described in *Standard Methods* (American Public Health Association, 1992). Spectrophotometric reading at 600 nm was obtained to calculate the difference between the absorbances in sample and blank.

2.6. Viral titration

Virus titration was performed with the controls and the ozone-treated samples. Serial dilutions of virus in M199 containing 2% FBS were inoculated onto confluent Vero cell monolayers in 96-well plates and incubated at 37 °C. Endpoint titers were determined by Reed and Muench method (1938) as the reciprocal of the lowest viral dilution that resulted in CPE in more than 50% of cells.

2.7. Cell viability

Cell viability was determined by the ability of the cells to metabolically reduce 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to a formazan dye. The MTT test was carried out according to the manufacturer's instructions with minor modifications. Briefly, a 50 μl volume of MTT (Roche) solution was added to each dish containing test cells. The dishes were then incubated at 37 °C for 4 h. At the end of the incubation, 500 μl of the solubilization solution (10% SDS in 0.01 M HCl, Roche) was added to each dish, and the dishes were allowed to stand overnight at 37 °C. A 100 μl volume of the resulting solution was removed from each dish and the spectrophotometrical absorbance was measured at 570 nm using a microtiter plate reader. Results were expressed as % control.

2.8. Lactate dehydrogenase (LDH) release assay

The release of lactate dehydrogenase from cells was measured using the cytotoxicity detection kit (Boehringer, Mannheim), according to the commercial protocol with some modifications. Briefly, a 100 μl aliquot of medium from each sample was added to a 96-well plate, in duplicate, followed by adding 100 μl reaction mixture to each well and incubation for 30 min at room temperature. Absorbance was read at 490 nm. The maximum LDH release and spontaneous LDH release were determined according to the commercial protocol. Results were expressed as % cytotoxicity [((experimental value − low control)/(high control − low control)) × 100].

2.9. Statistics

All experiments were performed at least three times and standard errors of means (S.E.M.) of all data were computed.

Comparisons between exposures were performed by Student's paired *t*-test. Tests were performed two-tailed and a value of $p < 0.05$ was considered as significant.

3. Results

3.1. Effect of ozone on inactivation of EV71 in free particle form

The kinetics of dissolving ozone for three different flow rates applied were plotted in the form of an exponential phase followed by a steady state, as shown in Fig. 1. Ozone solubility rate increased with high input flow rate, showing an input flow rate-dependent exponential phase and steady state. The steady states for flow rates of 100, 80 and 60 mg/h were reached after 10, 20 and 30 min of aeration, with an ozone concentration of 5.4, 4 and 2.8 mg l^{-1}, respectively.

When EV71 virus particle solution was exposed to ozone, significant efficiency of virus inactivation evidenced by a 4 $\log_{10}$ $CCID_{50}$ ml^{-1} (99.98%, $p < 0.01$) and a 2 $\log_{10}$ $CCID_{50}$ ml^{-1} (99%, $p < 0.01$) viral titer reduction after 10 min of exposure for 100 and 80 mg/h, respectively (Fig. 2). At 60 mg/h, a notable decrease (99% reduction) in virus titer was not seen until after 20 min of exposure. For all flow rates tested, a lagging efficiency of inactivation appeared after reaching the steady level of ozone concentration (Figs. 1 and 2). The time for 100% virus inactivation occurred at 30, 50 and 60 min of exposure at 100, 80 and 60 mg/h, respectively.

3.2. Influence of medium pH on effect of EV71 inactivation by ozone

The influence of the culture medium pH on ozone solubility was examined over a range of pH values, using a constant flow rate of 80 mg/h. The solubility curve of ozone for each pH value tested was similar to Fig. 1 (see Fig. 3). The rate of ozone solubility increased with decreasing pH value, exhibiting a pH-dependent reaction. The best pH value for ozone solubility was pH 3, which allowed the highest solubility of 11.3 mg l^{-1}.

Prior to the experiment, the influence of pH adjustment on viral titer was examined to rule out the possible reduction of viral titer by this procedure. The results indicated that sudden variations in pH of culture medium did not affect the virus activation (data not shown). Fig. 4 shows the inactivation data of EV71 virus particles in various pH culture media by ozone. Among the various pH values applied, the most efficient viral titer reduction

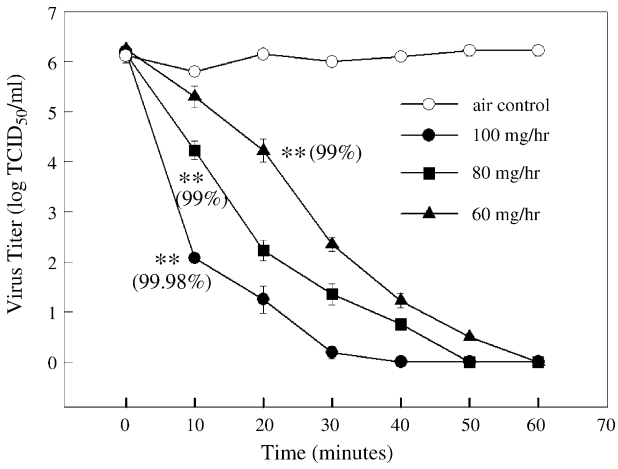

Fig. 2. Inactivating effect of ozone on EV71 free particles in culture medium using different input flow rates. Twenty-five milliliter of virus solution (10^6 $CCID_{50}$ ml^{-1}) was exposed to ozone at a flow rate of 100 mg/h (●), 80 mg/h (■) or 60 mg/h (▲), or subjected to filtered air (5 ml/h, ◯) for 60 min. Virus titer of exposed solution was determined by $CCID_{50}$. Data are means ± S.E.M., $n = 4$. ** Indicates a statistically significant ($p < 0.01$) difference in virus titer between filtered air and O_3 exposure. 99.98% virus reduction occurred within 10 min at 100 mg/h, ~ 99% virus reduction occurred within 10 min at 80 mg/h or within 20 min at 60 mg/h.

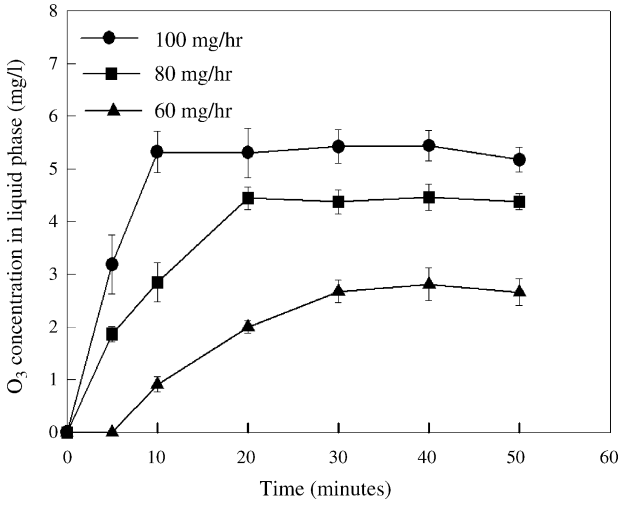

Fig. 1. The kinetic curves of ozone solubility under different input flow rates. A flow rate of 100 mg/h (●), 80 mg/h (■) or 60 mg/h (▲) was bubbled into 25 ml of culture medium for 50 min. Samples were taken and ozone concentration immediately determined using *Standard Methods*. Data are means ± S.E.M., $n = 4$.

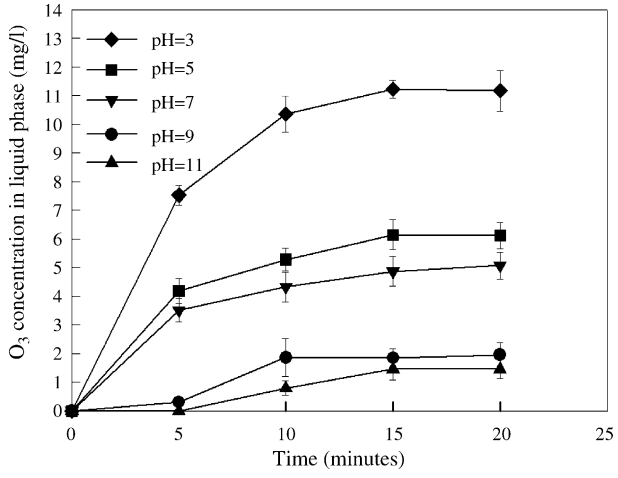

Fig. 3. The kinetic curves of ozone solubility at different pH values of culture medium. A flow rate of 80 mg/h was bubbled into 15 ml of culture medium with pH 3 (♦), pH 5 (■), pH 7 (▼), pH 9 (●) or pH 11 (▲) for 20 min. Samples were taken and ozone concentration immediately determined using *Standard Methods*. Data are means ± S.E.M., $n = 4$.

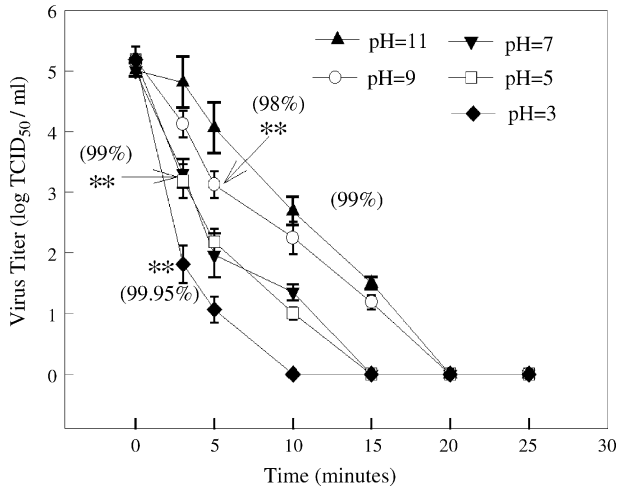

Fig. 4. Inactivating effect of ozone on EV71 free particles at different pH values of culture medium. Fifteen milliliter of virus solution (10^5 $CCID_{50}$ ml^{-1}) with pH 3 (♦), pH 5 (□), pH 7 (▼), pH 9 (◯) or pH 11 (▲) was exposed to ozone at a flow rate of 80 mg/h. Virus titer of exposed solution was determined by $CCID_{50}$. Data are means ± S.E.M., $n = 4$. **Indicates a statistically significant ($p < 0.01$) difference in virus titer between filtered air and O_3 exposure. 99.95% virus reduction occurred within 3 min at pH 3, ~ 98% virus reduction occurred within 3 min at pH 5 and pH 7, within 5 min at pH 9 or within 10 min at pH 11.

of ~3 $\log_{10}$ $CCID_{50}$ ml^{-1} (99.95%, $p < 0.01$) was observed at pH 3 within 3 min. At pH 5 and pH 7, a virus titer decrease of 2 $\log_{10}$ $CCID_{50}$ ml^{-1} (99%, $p < 0.01$) was obtained after an initial exposure of 3 min. However, for ozone inactivation at pH 9 or pH 11, ≥98% inactivation efficiency ($p < 0.01$) required as long as 5 or 10 min of exposure. As seen in Fig. 2, upon reaching the steady state of ozone solubility the efficiency of inactivation tended to lag (Figs. 3 and 4). EV71 virus was completely inactivated within 10 min at pH 3, 15 min at pH 5 and pH 7, and 20 min at pH 9 and pH 11. Although a similar level of virus inactivation was seen between pH 5 and pH 7, the virus inactivation efficiency of ozone was diminished when the pH became alkaline.

3.3. Effect of ozone on cells

Vero cells without virus infection as control cells were exposed to ozone for various durations of 15, 30, 45 and 60 min to examine the susceptibility of cells to ozone exposure. Samples for cell viability were taken immediately (0 h), 24 or 48 h after exposure. As shown in Table 1, 15 min of ozone exposure had no effect on the viability of Vero cells. Cell viability was reduced by 55–90% as the exposure time was extended from 30 to 60 min, showing an ozone exposure time-related effect on cell viability. When the exposed cells (30–60 min) were returned to normal growth conditions, viability remained stable by 48 h, showing no evident cell growth (Table 1).

3.4. Effect of ozone on intracellular virus

To examine the effect of ozone inactivation on intracellular virus during the infection course, one set of culture dishes containing EV71-infected Vero cells exposed to filtered air were used as infected controls. Virus growth was evaluated by LDH activity to indicate the correlation between viral replication and cytotoxicity (Watanabe et al., 1995). Table 2 shows the virus titer and cytotoxicity in infected cells exposed to filtered air or ozone. The air-exposed infected control cells showed that virus production was not affected for any particular exposure time points, while increasing cytotoxicity of cells and decreasing cell viability were observed in response to the virus growth.

When EV71-infected cells were exposed to ozone, similar percentages of cell viability loss as in the no infection control were observed for each exposure time (Table 1). There seemed no additive effects on cell death induced by EV71 virus infection during ozone exposure. Immediately after ozone exposure (0 h), or upon 15 or 30 min exposure, there was no notable reduction of intracellular viral titer (Table 2). However, a statistically significant reduction was found upon both 45 min (99% inactivation, $p < 0.01$) and 60 min (99.96% inactivation, $p < 0.01$) exposure. Because ozone does not affect the infected Vero cell viability within 15 min exposure, virus growth must have continued within the 48 h recovery period, evidenced by the decreasing

Table 1
Effects of ozone exposure on the viability of Vero cell with or without EV71 infection

Exposure time[a] (h)	Vero cell viability (%)[b,c]				
	0 min	15 min	30 min	45 min	60 min
Cells without EV71 infection					
	98.21 ± 0.65				
0		98.01 ± 1.08	51.29 ± 1.09	16.60 ± 1.45	7.45 ± 1.03
24		97.36 ± 1.59	54.37 ± 2.19	12.45 ± 1.03	6.31 ± 0.62
48		98.75 ± 2.10	58.02 ± 2.06	13.19 ± 1.09	5.35 ± 0.62
Cells with EV71 infection					
	62.15 ± 1.36				
0		55.76 ± 1.32	45.32 ± 1.68	12.52 ± 1.31	7.04 ± 2.70
24		14.32 ± 1.45	44.40 ± 0.78	11.67 ± 0.58	7.22 ± 1.11
48		6.26 ± 1.31	46.82 ± 1.02	11.94 ± 2.65	6.34 ± 0.53

[a] Cells with/without infection were exposed to ozone at a flow rate of 60 mg/h for various duration.
[b] Cell viability was measured using MTT and expressed as % control.
[c] Data are means ± S.E.M., $n = 5$.

Table 2
Cytotoxicity and intracellular virus titer in Vero cells following ozone exposure

Exposure time[a] (h)	% Cytotoxicity (LDH release)[b]					Intracellular virus titer (log_{10} $TCID_{50}$) ml^{-1} [b]				
	0 min	15 min	30 min	45 min	60 min	0 min	15 min	30 min	45 min	60 min
	62.65 ± 1.89					6.5 ± 0.22				
Filtered air										
0		60.45 ± 1.09	62.28 ± 2.03	61.32 ± 0.75	61.95 ± 2.91		6.0 ± 0.17	6.3 ± 0.15	6.1 ± 0.21	6.1 ± 0.31
24		81.85 ± 2.87	80.25 ± 3.09	78.41 ± 2.81	80.05 ± 3.44		7.2 ± 0.27	7.3 ± 0.32	7.4 ± 0.22	7.1 ± 0.35
48		98.75 ± 0.25	98.00 ± 0.41	95.20 ± 1.86	98.02 ± 0.41		8.1 ± 0.36	7.9 ± 0.11	8.0 ± 0.13	8.1 ± 0.11
Ozone										
0		58.27 ± 2.08	22.76 ± 1.24	17.69 ± 1.12	2.46 ± 1.45		6.0. ± 0.30	5.1 ± 0.11	4.5 ± 0.21**,c	3.1 ± 0.66**,d
24		78.52 ± 3.17	20.85 ± 1.49	16.45 ± 2.29	1.24 ± 0.47		7.3 ± 0.21	5.0 ± 0.32	4.0 ± 0.31	2.2 ± 0.22
48		95.54 ± 2.02	21.53 ± 3.71	15.25 ± 2.95	4.60 ± 2.09		7.8 ± 0.16	4.9 ± 0.10	3.0 ± 0.31	2.1 ± 0.18

** $p < 0.01$.
[a] Cells with infection were exposed to ozone at a flow rate of 60 mg/h for various duration.
[b] Data are means ± S.E.M., $n = 5$.
[c] 99% viral titer reduction.
[d] 99.96% viral titer reduction.

viability of cells and increasing cytotoxicity of cells. Intracellular virus titer in the cells exposed to ozone for 45 or 60 min slightly decreased (not statistically significant) when measured at 24 or 48 h of recovery, and cell viability appeared to be low (<20%, Table 1). In contrast, 30 min-exposed infected cells retained ~45% viability (Table 1) and an unchanged intracellular virus titer within the 48 h recovery period (Table 2). Cytotoxicity during subsequent recovery of 48 h was regarded as the consequence of virus growth rather than ozone effect. However, low cytotoxicity in the time immediately after 30–60 min ozone exposure was considered to be the secondary effect of ozone in inactivating LDH activity after LDH was released into medium.

4. Discussion

In the present work, we demonstrated the effects of ozone exposure on EV71 inactivation in culture medium and intracellularly. The results described herein demonstrate that ozone has potent anti-EV71 activity. EV71 virus in free state showed a higher inactivation sensitivity in response to ozone than intracellular virus. The efficiency of inactivating free viral particles in culture medium was related to the kinetics of ozone solubility and was ozone concentration-dependent. The inactivating effect of ozone on intracellular EV71 virus during the infection course, however, was dependent on ozone exposure duration.

Studies on virus inactivation by ozone in culture medium have been reported (Herbold et al., 1989; Carpendale and Freeberg, 1991; Wells et al., 1991; Kim et al., 1999; Khadre and Yousef, 2002; Shin and Sobsey, 2003), and involved a range of ozone dosages (0.081–5.2 mg l^{-1}), contact time (0.1 s–120 min) and log_{10} unit inactivation (2–11). Our results indicate that for free particles of EV71, the use of 100 mg/h of ozone input flow rate could cause a 4 log_{10} $CCID_{50}$ ml^{-1} (99.98%, $p < 0.01$) viral titer reduction within 10 min, and a flow rate of 80 mg/h applied to pH 3 culture medium could achieve ~3 log_{10} $CCID_{50}$ ml^{-1} viral titer reduction within 3 min (99.95%, $p < 0.01$). The inactivating effect of ozone on EV71 was related to the kinetics of ozone solubility. An efficient stage of ~99% inactivation was observed in the exponential phase, and a complete virus inactivation was achieved after a steady level of ozone concentration was reached. Additionally, ozone solubility played a significant part in viral inactivation. Ozone solubility in liquid was related to input flow rate and the pH of culture medium (Facile et al., 2000; Cho et al., 2003). By a high ozone input flow rate or acidic pH of the viral suspension, ozone solubility was increased (Figs. 1 and 3), leading to a higher inactivating effect of ozone on extracellular EV71 (Figs. 2 and 4).

In natural environment, viruses may be adsorbed or embedded within organic materials, resulting in a completely different response to inactivation in comparison with viruses in the free state (Emerson et al., 1982). Few studies investigate the effects of ozone exposure on viral infectivity in infected cells. The present study reveals that significant effect of inactivating intracellular EV71 was observed in the case of 45 or 60 min exposure and correlated with high cell death rate. However, upon 30 min exposure 45% cell viability was retained concomitantly with a non-elevated virus titer (Tables 1 and 2). Contact time with ozone

played an important role in exerting different response of cells to ozone exposure, involving virus inactivation and cell death. Ozone can cause membrane injury to cultured cells (Dumler et al., 1994; Kafoury et al., 1999). It is therefore inferred that inactivation of intracellular virus may result from the disruption of cell membranes induced by ozone leading to cell death, followed by inactivation of the intracellular virus.

It has been demonstrated that by different exposure concentrations and exposure duration, ozone exposure can affect cellular functions of a number of cell types (Leikauf et al., 1995; Pearson and Bhalla, 1997; Gornicki and Gutsze, 2000; Koike et al., 2001; Fu et al., 2002) and modulate some of them to produce mediators related to immune response (Jakab et al., 1995; Jaspers et al., 1997; Koike et al., 2001; Nichols et al., 2001). Study on inactivation of HIV by ozone indicated that ozone could exert several antiviral effects, including viral particle disruption, reverse transcriptase inactivation, and/or a perturbation of the ability of the virus to bind to its receptor on target cells (Wells et al., 1991). Ozone exposure also caused damage to viral RNA of poliovirus 1 leading to poliovirus inactivation (Roy et al., 1981). Ozone has been suggested to be of therapeutical use in viral infection (Wolcott et al., 1982; Jakab and Hmieleski, 1988; Selgrade et al., 1988; Carpendale and Freeberg, 1991; Friedman and Stromberg, 1993; Bocci, 1996, 1999; Cohen et al., 2001). Effects of ozone in the therapy of infectious diseases varied with ozone exposure concentration and exposure duration, and biological effects such as immune response of host mediated by ozone greatly contributed to the effects observed in disease therapy (Jakab et al., 1995; Bocci, 1999; Cohen et al., 2001).

This study was the first in an overall study of the effects of ozone on enterovirus 71 clearance. Although it is unclear to which extent ozone-mediated changes in cell functions are responsible for EV71 virus inactivation during the infection process, the results described herein provide information on the feasibility of inactivating intracellular EV71 virus by ozone during the infection course. Although the present concentration of ozone used in the study was found to be cytotoxic to Vero cells, ozone dose-dependent injury to lung-derived cells has been demonstrated in the studies of cultured cells and animals (Castlemam et al., 1977; Alpert et al., 1990), and indicated that cytotoxicity due to ozone exposure is variable among different cell types, different species and different exposure systems (Alpert et al., 1990; Ritter et al., 2001).

The presence of EV71 in the environment has become a danger for children's health. The results of this study indicate that the virus is substantially inactivated by ozone exposure. The application of different input ozone flow rates or various pH culture media showed that the EV71 inactivation by ozone was related to the kinetics of ozone solubility. Ozone has potential effects on the course of EV71 infection and may even result in decreased virus-induced injury. To prevent EV71 infections, inactivation of EV71 in free particle form by ozone may provide a means of eliminating environmental contaminated source of EV71, leading to the control of the transmission risk of EV71. Furthermore, inactivation of intracellular EV71 virus by ozone may be of some value in the therapy of EV71 infections.

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