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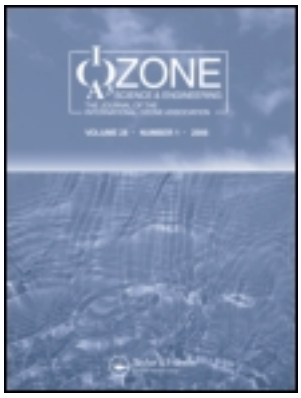

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Greici Petry[a], Luciana Grazziotin Rossato[b], Jaqueline Nespolo[c], Luiz Carlos Kreutz[c] & Charise Dallazem Bertol[a]

[a] Laboratório de Controle de Qualidade, Curso de Farmácia, Instituto de Ciências Biológicas, Universidade de Passo Fundo, Passo Fundo, RS, 99052-900, Brasil

[b] Departamento de Toxicologia, Faculdade de Farmácia, Universidade do Porto, Porto, 4050-313, Portugal

[c] Laboratório de Virologia e Imunologia, Faculdade de Agronomia e Medicina Veterinária, Universidade de Passo Fundo, Passo Fundo, RS, 99052-900, Brasil

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In Vitro Inactivation of Herpes Virus by Ozone

Greici Petry,[1] Luciana Grazziotin Rossato,[2] Jaqueline Nespolo,[3] Luiz Carlos Kreutz,[3] and Charise Dallazem Bertol[1]

[1]Laboratório de Controle de Qualidade, Curso de Farmácia, Instituto de Ciências Biológicas, Universidade de Passo Fundo, Passo Fundo, RS, 99052-900, Brasil
[2]Departamento de Toxicologia, Faculdade de Farmácia, Universidade do Porto, Porto, 4050-313, Portugal
[3]Laboratório de Virologia e Imunologia, Faculdade de Agronomia e Medicina Veterinária, Universidade de Passo Fundo, Passo Fundo, RS, 99052-900, Brasil

Viruses represent a major threat to human health and might be transmitted by direct and indirect contact. Reducing the viral load, either in the host or in the environment greatly reduces virus spreading. In this work we aimed to evaluate the virucidal activity of ozone against herpes virus of human (Herpes Simplex Virus 1 – HSV-1) and bovine (Bovine Herpes Virus 1 – BoHV-1) origin. The virucidal activity was measured by tittering aliquots of HSV-1 and BoHV-1 exposed for 1, 2, and 3 h to ozone generated by a domestic device. In addition, the possible cytotoxic effect of ozone to cultured MDBK cells was also assessed using the MTT method. MDBK cells exposed to ozone for 3 h and tested immediately after exposure, or after culturing for 24 h, had viability similar to non-exposed cells, indicating that ozone per se was not cytotoxic to the cells. Furthermore, a significant reduction in BoHV-1 (99.62%) and HSV-1 (90.0%) titer was observed after 3 h exposure to ozone. Our results indicate that ozone might be safely used to reduce environmental load of herpes virus.

Keywords Ozone, Air Purifier, Cytotoxicity, Virucidal Activity, Herpesviridae

INTRODUCTION

Viruses represent a major threat to human health and are responsible for approximately 60% of human infections worldwide; the most common viral illnesses are characterized by enteric and respiratory distress (Barker et al. 2001). Transmission of viruses from an infected to non-infected host can occur by direct contact or indirect routes such as contaminated surfaces or aerosolized viral particles. Transmission is dependent on many factors, including the amount of viral particles excreted by an infected organism, virus stability in the environment, and the interaction of the virus with the new host. In addition, a higher viral load increases the likelihood of transmission (Rzezutka and Cook 2004; Vasickova et al. 2010).

Infections caused by herpes virus are recurrent and lifelong (Beuman 2005). Herpes viruses in man and animals cause similar lesions, ranging from localized vesicular eruptions of surface epithelia to diffuse and widespread damage to the mucosa of the respiratory, digestive, and genital tracts (Burrows 1977). Beyond this unpleasant common symptom seen in the oral and genital herpes virus infections, other more severe complications might occur, such as giant cell proliferation in glandular epithelium, necrosis of liver, lymphoid and other tissues, and even neuron damage and encephalitis, which can result in fatal outcomes (Burrows 1977; Ibitoye et al. 2012).

Direct exposure of mucous membranes, abraded skin due to lesions, or mucosal secretions of an individual with active primary or recurrent infection, are the main routes of Herpes Simplex Virus 1 (HSV-1) infection in humans. The transmission might also occur by respiratory droplets or exposure to mucous-cutaneous secretions of an asymptomatic individual shedding the virus in the absence of clinical disease (Beuman 2005; Fatahzadeh and Schwartz 2007). HSV-1 has been found in the hands of individual with herpes simplex labialis indicating the likelihood of horizontal spread (Turner et al. 1982). HSV-1 can remain viable for brief periods on the skin, clothing or plastic, facilitating transmission through close nonsexual contact, such as kissing on the cheeks or sharing common utensils (Fatahzadeh and Schwartz 2007).

The use of air purifiers can increase the indoor air quality and reduce the environmental load of micro-organisms and, consequently, viral transmission by indirect routes. Among

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Address correspondence to Charise Dallazem Bertol, Laboratório de Controle de Qualidade, Curso de Farmácia, Instituto de Ciências Biológicas, Universidade de Passo Fundo, BR 285, Km 292, Bairro São José, Passo Fundo, RS, 99052-900, Brasil. E-mail: charibertol@yahoo.com.br

the air purifiers there are ozone generators. Ozone ions are produced by electrical discharges or through ionizing radiation of oxygen (FDA 2010).

Recently, we demonstrated that a 2-h period of ozonation at a concentration smaller than 0.05 ppm, which is considered safe for indoor environmental use (Ministério do Trabalho e Emprego, Brasil 2013; IEC 2002; OSHA 1992), reduced the amount of viable fungi and bacteria in the environment by more than 80% (Bertol et al. 2012).

The antiviral activity of ozone has already been demonstrated against enterovirus 71 (EV71), feline calicivirus (FCV), enteric adenovirus type 40, HSV-1 (with different exposure method and concentration proposed in this article), vesicular stomatitis, Indiana virus, vaccinia virus, adenovirus type 2, influenza virus, and norovirus (Hudson et al. 2007; Lin et al. 2007; Murray et al. 2008; Thurston-Enriquez et al. 2005). However, little is known about the virucidal activity of ozone against HSV-1 and Bovine Herpes Virus 1 (BoHV-1) and its effective concentration in the air. Considering the oxidant potential of ozone, this work aimed to evaluate the antiviral activity of an air purifier ozone generator against Herpes Virus type 1.

MATERIAL AND METHODS

The in vitro antiviral activity of ozone against HSV-1 and BoHV-1 was measured by a viral plaque assay and MDBK cells.

MDBK Cells, HSV1 and BoHV1 Virus

Cells (Madin-Darby bovine kidney – MDBK; ATCC CCL-22) were grown and maintained in Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) and 1.25 mg/mL of gentamicin in 75-cm^2 tissue culture flasks at 37 °C and 5% CO_2 atmosphere (Minitub, Porto Alegre/RS, Brazil). Cells were sub-cultured every 3 days using 1 mL of trypsin (2.5 mg/mL). HSV-1 (KOS Strain) and BoHV-1 (Cooper strain) were propagated and titered by end-point dilution (Reed and Muench 1938) in MDBK cells, and viral aliquots were kept frozen at -20 °C up to use.

The Effect of Ozone on MDBK Cells

The ozone was generated by a commercial air purifier (Brizzamar, Ronda Alta, RS, Brazil). Experiments were performed in a laminar flow chamber (VLPS18CL2A, Veco, Campinas/SP, Brazil). The flow was turned on for 30 min as a preparatory decontamination step. Afterwards, laminar flow was turned off and the ozone generator was turned on. The ozone generator was kept on for 3 h, and the ozone total concentration in the environmental was monitored through the sensor EcoSensor Model OS-4 (Ozone Switch TM, Newark, CA, USA). The conditions of temperature and humidity were also observed with thermo-hygrometer (ICEL/Manaus HT 7100, Brazil).

A cell viability assay based on MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction was performed to evaluate the potential damage induced by ozone onto MDBK cells. Cells (4.5×10^4 cells/well) were exposed to ozone for 1, 2, and 3 h ($n = 3$). Control cells were kept for the same periods of time, temperature, and humidity conditions in the absence of ozone. Cell viability was evaluated by MTT right after exposure to ozone and also after exposure to ozone followed by a 24-h growth period (37 °C/5% CO_2). For the MTT assay, cell medium was removed and fresh cell medium containing 50 μL of MTT (1 mg/mL) was added and the cells were incubated (37 °C/5% CO_2) for 4 h. After this period, the medium was removed and the formazan crystals were dissolved in 100 μL of DMSO. The absorbance was measured at 550 nm in an ELISA multi-well plaque reader (Anthos 2010). The percent of the MTT reduction relative to control cells was used as viability measure. Results are expressed as: % viability = ($OD_t \times 100/OD_c$), where "OD_t" is the mean optic density of treated cells (in the presence of ozone) and "OD_c" is the mean optic density of control cells. In this assay, the viability of control, non-exposed cells was considered 100% and the concentration of ozone that reduced cell viability by more than 80% was considered cytotoxic (Garré et al. 2007).

Ozone Antiviral Activity

The effect of ozone on herpes virus was evaluated in similar conditions as described for evaluating cell cytotoxicity. Viral aliquots in 35-mm petri dishes were placed on the laminar flow and exposed to ozone for 1, 2, and 3 h. Control viral samples were incubated at the same conditions in the absence of ozone. After each time period (1-, 2-, and 3-h) viral aliquots were collected and tittered in MDBK cells (4.5×10^4 cells/well) by limiting dilution in 96-well plates. Viral titer was calculated as previously described (Reed and Muench 1938).

Statistical Analyses

The results were analyzed by ANOVA followed by Tukey's test ($p < 0.05$).

RESULTS AND DISCUSSION

Spreading of viruses relies on direct contact between and infected and non-infected host, or by indirect contact by means of contaminated materials and aerosolized viral particles. Poorly ventilated indoor environments with continuous flux of individuals might easily become overloaded with airborne pathogens. Reducing environmental micro-organism contamination using disinfectant agents and air purifiers is therefore crucial for reducing new infections. In this work, we use an ozone generator device to reduce herpes virus viability. Herpes viruses comprise a large family of enveloped DNA viruses that cause several syndromes characterized mostly by respiratory and genital distress (Boehmer and Lehman 1997).

TABLE 1. Inactivation of Herpes Virus by Ozone

Time of exposure	HSV-1 titer (TCID50)			BoHV-1 titer (TCID50)		
	Control	Exposed to ozone	Inhibition (%)	Control	Exposed to ozone	Inhibition (%)
1 h	$10^{4,66}$ ($\pm$0.1)^{a}	$10^{4,16}$ ($\pm$0.3)^{b}	68.4	$10^{7,08}$ ($\pm$0.4)^{a}	$10^{7,08}$ ($\pm$0.4)^{a}	0.0
2 h	$10^{3,82}$ ($\pm$0.3)^{d}	$10^{3,07}$ ($\pm$0.5)^{e}	82.2	$10^{6,82}$ ($\pm$0.3)^{b}	$10^{4,90}$ ($\pm$0.2)^{d}	98.8
3 h	$10^{3,99}$ ($\pm$0.3)^{c}	$10^{2,99}$ ($\pm$0.3)^{f}	90.0	$10^{6,5}$ ($\pm$0)^{c}	$10^{4,07}$ ($\pm$0.5)^{e}	99.6

Note: Human and bovine herpes viruses were exposed to ozone for 1, 2, and 3 h, and the remaining virus was titered on MDBK cells and compared to viruses treated at the same conditions except for ozone exposure. Results are expressed by means $\pm$ standard deviation ($n = 3$).

Superscripted letters indicate statistical differences between groups (Tukey's test, $p < 0.05$).

A progressive inhibition of HSV-1 was observed after 1-, 2-, and 3-h exposure to ozone (68.37%, 82.21%, and 90.00%, respectively; Table 1). And, for BoHV-1, although no viral inhibition could be detected after 1-h exposure, a 3-h exposure time also inhibited viral viability by greater than 99%. The ozone promoted a significant reduction of more than 90% of viral replication for both viruses tested.

Ozone is considered an effective agent against fungi, bacteria, and virus (Bertol et al. 2012; Velano et al. 2001). It has been demonstrated that even residual ozone concentrations (<0.01 mg/L) are effective to deactivate FCV virus (Thurston-Enriquez et al. 2005). As demonstrated here, ozone has a time-dependent anti-herpectic effect, similar to the virucidal effect against EV71 virus (Lin and Wu 2006). Moreover, the production of cytokines induced by ozone before the EV71 infection caused antiviral effects that were beneficial in suppressing subsequent EV71 infections (Lin et al. 2007). An ozone-oxygen generator system was also effective in reducing the viral infectivity of enveloped and non-enveloped viruses through lipid peroxidation and protein damage (Murray et al. 2008). Additionally, Hudson et al. (2007) demonstrated that ozone can deactivate viruses even in dry surfaces.

Here, ozone concentration was monitored during the experiments and was kept between 0.02 and 0.05 ppm. The temperature and humidity conditions were also monitored and they were kept at 26.2 °C and 37% humidity for the HSV-1 and 29.2 °C and 30% humidity for the BoHV-1 experiments. The ozone concentration used is considered safe to humans in accordance with current regulations (IEC 2002; Ministério do Trabalho e Emprego, Brasil 2013; OSHA 1992), demonstrating that an ozone generator can be used indoors, such as medical and dental offices, hospitals, and schools, aimed at environmental disinfection. Ozone might be especially important on premises with increased risks for micro-organism dissemination, such as nursery rooms and oral health care offices (Fatahzadeh and Schwartz 2007).

We also demonstrated that ozone is harmless to cultured MDBK cells in that the viability of cells exposed to ozone for 3 h was higher than 80%, a threshold considered crucial for measuring cytotoxicity (Garré et al. 2007). After 1, 2, and 3 h of ozone exposure, the viability was 101.9%, 107.4%, and 94.8%, respectively, in comparison to control values. Interestingly, however, cells exposed to ozone for 1, 2, and 3 h and further cultured for 24 h had higher viability than cultured cells non-exposed to ozone (see Figure 1), with the viability values of 115.5%, 127.2% and 122.8%, respectively. After statistical analyses, there was no significant difference between control cells and ozonated cells in both conditions.

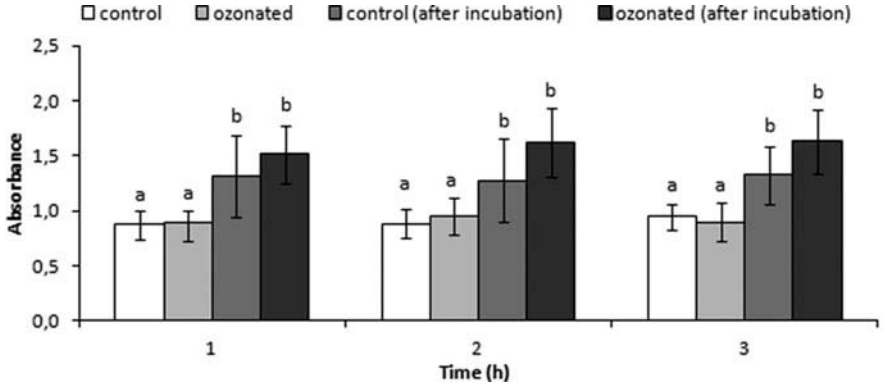

FIGURE 1. Cell viability after exposure to ozone for 1, 2, and 3 h. After exposure cells were assayed for viability by the MTT method, or cultivated for 24 h and then tested. The results represent the mean $\pm$ SD ($n = 3$). Different letters indicate significant diference within each time point (Tukey's test, $p < 0.05$).

Ozone is known to induce cytokine expression on cells (Lin et al. 2007), and it might be speculated that the pool of cytokine expressed by ozonated cells has antiviral activity and even might increase cell viability or induce proliferation. Ozone has been widely used as a therapeutic agent in medicine, even to improve would healing and regenerate tissue (Viebahn-Hänsler et al. 2012). Further studies will demonstrate if differences in cell viability after culturing for longer times are statistically significant.

CONCLUSION

In our study we demonstrated that non-cytotoxic concentrations of ozone, generated by a domestic device, had a significant virucidal effect on human and bovine herpes viruses. Exposure of viruses for 3 h leads to greater than 90% in virus inhibition, indicating the potential use of ozone generators to improve air quality and reduce environmental load of infecting micro-organisms.

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