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Short communication

Virion disruption by ozone-mediated reactive oxygen species

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A B S T R A C T

It is well documented in the scientific literature that ozone–oxygen mixtures inactivate microorganisms including bacteria, fungi and viruses (Hoff, J.C., 1986. Inactivation of microbial agents by chemical disinfectants. EPA 600 S2-86 067. Office of Water, U.S. Environmental Protection Agency, Washington, DC; Khadre, M.A., Yousef, A.E., Kim, J.-G., 2001. Microbiological aspects of ozone applications in food: a review. J. Food Sci. 66, 1242–1252). In the current study, delivery and absorption of precisely known concentrations of ozone (in liquid media) were used to inactivate virus infectivity. An ozone–oxygen delivery system capable of monitoring and recording ozone concentrations in real time was used to inactivate a series of enveloped and non-enveloped viruses including herpes simplex virus type-1 (HHV-1, strain McIntyre), vesicular stomatitis Indiana virus (VSIV), vaccinia virus (VACV, strain Elstree), adenovirus type-2 (HAdV-2), and the PR8 strain of influenza A virus (FLUAVA/PR/8/34/H1N1; FLUAV). The results of the study showed that ozone exposure reduced viral infectivity by lipid peroxidation and subsequent lipid envelope and protein shell damage. These data suggest that a wide range of virus types can be inactivated in an environment of known ozone exposure.

Ozone is best known for its role in the atmosphere's ecological balance. The unique biological properties of ozone are currently under investigation for their potential application in various medical disciplines. Since the latter part of the 19th century, ozone has been investigated for its reactivity with a spectrum of compounds containing unsaturated carbon–carbon double bonds (Razumovskii and Zaikov, 1984). Ozone (O_3) and molecular oxygen (O_2) are both reactive oxygen species (ROS) along with additional ROS and reactive nitrogen species (NOS) generated in water and buffered salt solutions. These include a vast number of primary ROS and NOS: singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet -}$), ozone radical ($O_3^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), nitrite (NO_2^-) and peroxynitrite ($ONOO^-$) among other radicals. Secondary ROS and NOS may be generated from the oxidation of bonds in lipids, proteins and amino acids, thereby yielding a variety of reactive free radicals ($RCOO^{\bullet}$). Through the oxidation of double bonds, ozone possesses the unique ability to destroy toxic or noxious industrial impurities (phenols, cyanides, tetraethyl lead, and others) and to inactivate biological (viral and bacterial) contaminants (Bruice, 2001). The virucidal and bactericidal properties of ozone have been recognized since the First World War, when ozone was applied topically to disinfect wounds and mustard gas burns (Rilling and Viebahn-Hänsler, 1987). Following the elucidation of the biochemical mechanisms involved in ozone-mediated detoxification, the potential applications for ozone have expanded greatly and include the disinfection of water (Burns et al., 2007).

Data suggest that ozone inactivation of viruses occurs primarily in two ways: by lipid peroxidation and by protein peroxidation (Friedman and Stromberg, 1993; Carbonneau et al., 1991; Dianzani, 1993). Lipid peroxidation, a mechanism of tissue damage initiated by a variety of ROS, generates free radicals that are responsible for many pathological sequelae (Esterbauer et al., 1991). The fatty-acid components of phospholipids contain many points of unsaturation along their hydrocarbon chains, and oxidation of these bonds leads to severe structural and functional damage to the lipid bilayer of the plasma membrane. A widely accepted standard for measuring lipid peroxidation is an assay for malondialdehyde (MDA), a stable peroxidation byproduct (Esterbauer et al., 1991).

Alternatively, protein peroxidation is defined as the covalent modification of proteins either directly through interaction with ROS or indirectly by interaction with secondary byproducts of oxidative stress (Shacter, 2000). Various ozone-mediated ROS including H_2O_2 can interact with proteins and cause oxida-

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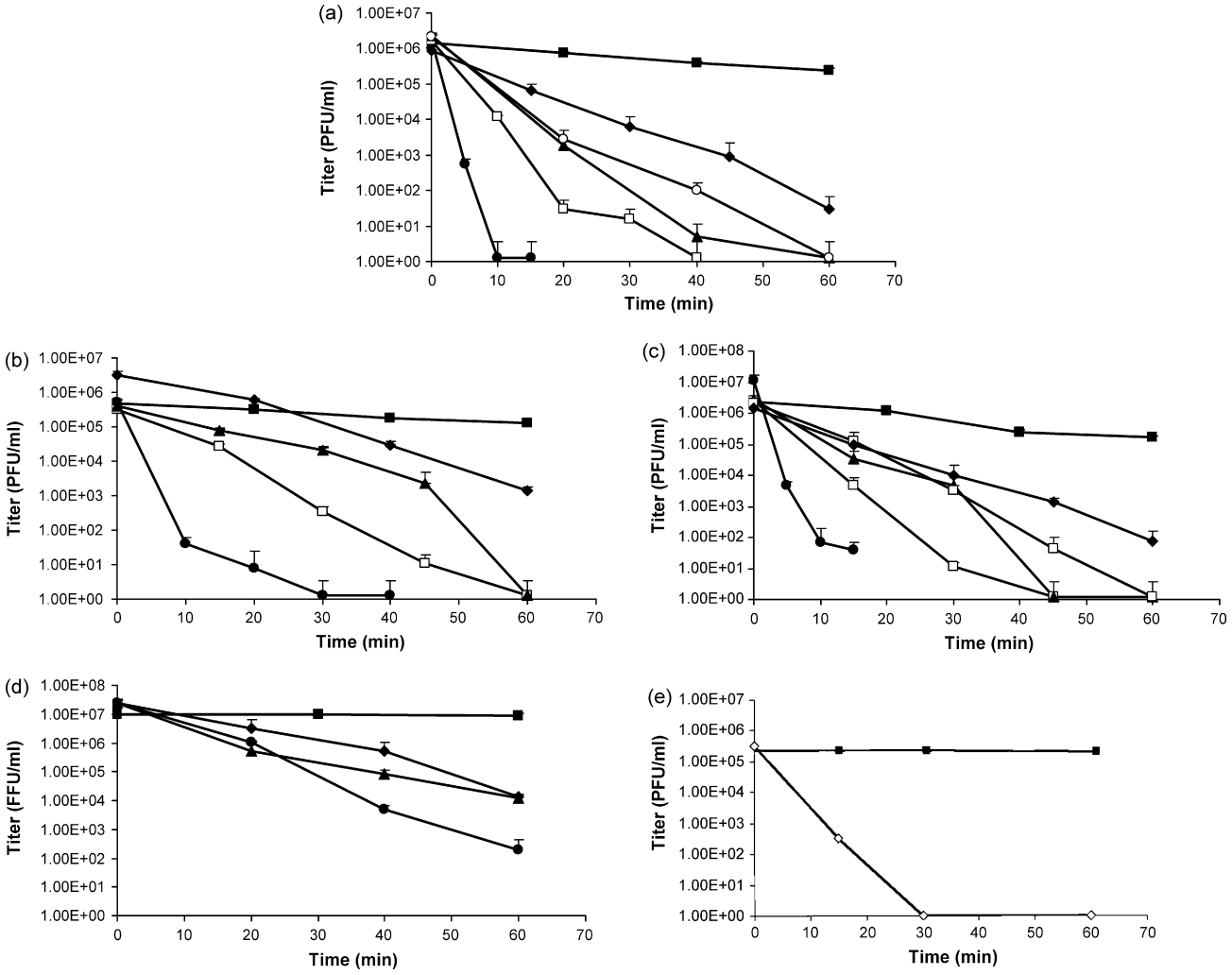

Fig. 1. Loss of virus infectivity following ozone exposure. (a) VSIV; (b) VACV; (c) HHV-1; (d) HAdV-2 infectivity data from two independent ozone treatments; (e) FLUAV infectivity data are representative of one ozone treatment. All *y*-axis error bars represent one standard deviation of the mean. Control treatment with O_2 gas in PBS (■;), ozone in PBS (●), 10% calf serum in PBS (□), 50% calf serum in PBS (▲), 80% calf serum in PBS (○), 100% calf serum (♦), and DME with 0.125% bovine serum albumin (BSA, ◊).

tive changes that inhibit normal cellular mechanisms. These include losses of aggregation and proteolysis control, changes in enzyme–substrate binding activities, and alterations in immunogenicity (Shacter, 2000). In particular, protein peroxidation may play a key role in the inactivation of non-enveloped viruses, such as adenovirus, poliovirus and other enteroviruses. In previous studies, enveloped viruses, such as human immunodeficiency virus type-1 (HIV-1) have demonstrated sensitivity to ozone treatment even at non-cytotoxic concentrations (Wells et al., 1991; Carpendale and Freeberg, 1991). Venezuelan Equine Encephalomyelitis (VEE) virus has also been successfully inactivated using a liquid-phase ozone application (Akey and Walton, 1985). In addition, intra- and extracellular enteroviruses and noroviruses have been inactivated by ROS (Lin and Wu, 2006; Hudson et al., 2007; Keswick et al., 1985; Roy et al., 1981; Shin and Sobsey, 2003). In 1997, a research team suggested a nebulization technique to inactivate viruses with ozone (Kekez and Sattar, 1997). In these studies, nebulized ozone was successful in the inactivation of viruses in large volumes of body fluids (e.g. plasma, partial blood and whole blood) in a relatively short period of time. Viral components necessary for productive infections, such as the membrane-associated antireceptors and lipid envelope, may have been the primary targets of ROS attack. The sensitivity of lipid-containing viruses to ozone in these studies suggests that enveloped viruses may lose infectivity through lipid peroxidation.

Currently, two main applications for ozone-mediated viral inactivation are known. First, there is an abundance of research performed during the latter half of the 20th century which involves the ozone-mediated inactivation of many water-contaminating viruses. Second, there were many attempts at ozone-based therapy modalities for the treatment of viremias. However, a precision ozone delivery system has not been available, and the toxicity produced by excessive ROS exposure has limited the progression of ozone applications in medicine. The objective of this study was to determine if the precision delivery of an ozone–oxygen gas mixture measured and recorded in real time could inactivate a variety of viruses (enveloped, non-enveloped, DNA, RNA, simple, and complex) by using a minimal amount of ozone gas, thereby possibly maintaining the biological integrity of the treated fluids. Such a precision-delivery protocol may allow widespread applications of ozone treatments, including improved water sanitation and serum product disinfection, and serve as a primer for clinical studies involving ozone-mediated viremia treatments.

To identify the mechanism for virus inactivation, we first inactivated viruses using a Gas-Fluid exchange device (Lipidviro Tech, Inc., Salt Lake City, UT) which produces ozone gas from medical

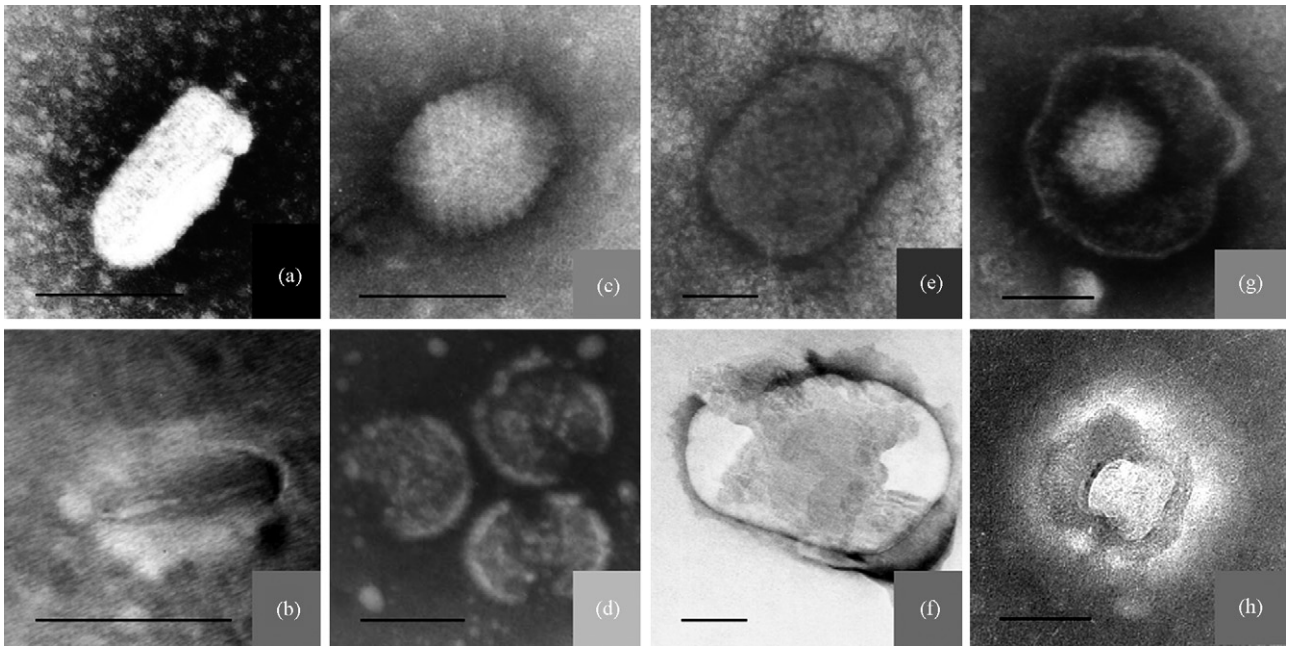

Fig. 2. Negatively stained electron micrographs of virus preparations exposed to ozone in PBS. (a) VSIV control; (b) VSIV ozone-exposed; (c) HAdV-2 control; (d) HAdV-2 ozone-exposed; (e)VACV control; (f) VACV ozone-exposed; (g) HHV-1 control; and (h) HHV-1 ozone-exposed. Bars represent 100-nm length. Images of virus particles in ozone-treated micrographs (b, d, f and h) show that the nucleocapsids have been disrupted corresponding to loss of virion infectivity.

grade pure oxygen and continually measures and records precise concentrations of ozone delivered and absorbed in the sample. Stocks of HHV-1, VSIV, and VACV were prepared in Vero cells and titered using standard plaque assays. Titers were reported as plaque-forming units (PFU) per ml. HAdV-2 preparations were assayed in cultures of HeLa cells using a polyclonal antibody specific for HAdV-2 in a histochemical focus forming unit (FFU) assay (Luker et al., 1991; Johnson and Visick, 1992). Titers were presented as FFU per ml. FLUAV was propagated and assayed in MDCK cells using an immunofluorescent FFU assay and titers were also reported as FFU per ml.

Virus pools were exposed in solution in a thin liquid layer using an ozone–oxygen mixture with ozone concentrations ranging from 800 parts per million by volume (ppmv) to 1500 ppmv. Data presented here are from experiments using 1200 ppmv of ozone which were representative of the ozone concentrations used. Confirmation that viral inactivation was ozone dependent was established in control cultures by exposing viruses to O_2 rather than ozone

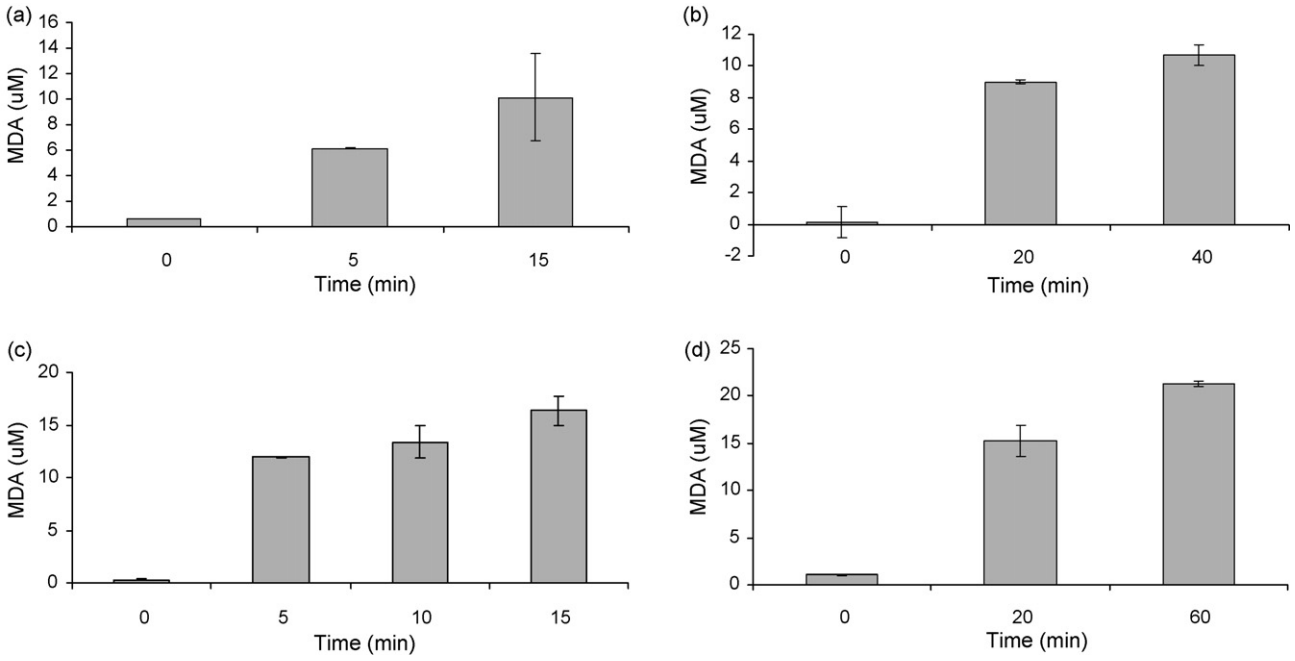

Fig. 3. MDA concentrations following ozone exposure (a) VSIV; (b) VACV; (c) HHV-1; and (d) HAdV-2. Virus pools were suspended in PBS, exposed to ozone, and assayed at various times for MDA content expressed in μM. Data are representative of two replicates and error bars represent one standard deviation from the mean.

(Fig. 1). Minimal loss of infectivity occurred even after 60 min of O_2 exposure. The inactivation kinetics for VSIV, VACV, HHV-1, HAdV-2, and FLUAV following ozone exposure are also shown in Fig. 1. While ozone was effective in inactivating all of the viruses studied, virus inactivation was most marked for VSIV, VACV and HHV-1, and least for HAdV-2. Virus pools suspended in PBS were rapidly inactivated (Fig. 1a–e). When virus pools were suspended in PBS supplemented with varying concentrations of calf serum, the virus-inactivating effects of ozone were diminished, presumably by competition with the abundant proteins in serum, which decreased the rate of viral inactivation (Fig. 1a–d). Enveloped viruses, such as VSIV (Fig. 1a), HHV-1 (Fig. 1c), and FLUAV (Fig. 1e), showed extreme sensitivity to ozone. The complex VACV virions showed similar sensitivity (Fig. 1b). However, the non-enveloped HAdV-2 was most resistant to ozone treatments (Fig. 1d). These data support the idea that the rate of viral inactivation depends on direct ozone contact with lipids (Ivanova et al., 1983).

Virus inactivation by ozone delivery strongly suggests damage and destruction of the lipid viral envelope and protein capsid. To confirm that the integrity of the viral envelope and protein coat had been compromised, samples were prepared for electron microscopy and negatively stained with phosphotungstic acid. Images in Fig. 2 show the disruption of the VSIV nucleocapsid, severe disruption of VACV morphology, and complete destruction of the HSV-1 nucleocapsid. HAdV-2 samples shown in Fig. 2d also show visible disruption of HAdV-2 protein capsids.

To address the question as to whether lipid peroxidation was involved in the observed deterioration of the viruses, MDA assays were conducted using a lipid peroxidation assay kit (Oxford Biomedical Research, Oxford, MI). The tests were performed following the manufacturer's instructions. After ozone treatment, increases in MDA concentrations compared to O_2-treated controls were observed for the viruses studied (Fig. 3). Elevated lipid peroxidation levels, as indicated by higher MDA concentrations, suggested that an abundance of primary and secondary ROS may be the cause of viral inactivation. As expected, MDA concentrations increased with ozone treatment time (Fig. 3a–d), and were inversely related to infectious virus titer. The concentration of MDA also increased in media controls (data not shown) suggesting that ROS species were also generated from components in the media. Potential targets for ROS attack (most probably through lipid and protein peroxidation) include the envelopes of viruses such as HHV-1, VSIV, FLUAV, and VACV, and the protein shells of HAdV-2.

Currently in the United States, ozone-mediated viral inactivation applications are limited to disinfection at water treatment municipalities (Environmental Protection Agency, 1999). Thus far, FDA approval of clinical ozone treatment protocols for viremia has not been granted. Although ozone treatment protocols have been successful in the inactivation of several enteroviruses, clinical experiments have not yielded equivalent success (Carpendale and Freeberg, 1991; Finch and Fairbairn, 1991; Katzenelson et al., 1979; Kekez and Sattar, 1997). The major insufficiency with current ozone-mediated inactivation protocols lies in the inability to precisely measure and to control ozone–oxygen gas delivery. The cytotoxicity of ozone-treated liquids creates an enormous obstacle for the clinical applications of ozone (Kekez and Sattar, 1997). Although success has been reported in the inactivation of some pathogens such as VEE virus, an arthropod-borne alphavirus endemic to northern South America, Trinidad, Central America, Mexico, and Florida, these applications were limited to biological safety cabinet and laboratory equipment sterilization (Akey and Walton, 1985). However, the present data suggest that precision delivery, monitoring and recording of ozone concentration is possible and that the toxic effects of ozone can be minimized while maximizing the beneficial virucidal and bactericidal properties of ozone. Real-time monitoring of ozone gas flow, ozone absorption, and ozone–oxygen gas exposure time provide for an ozone–oxygen delivery system with an unprecedented level of control. Significant improvements could be made for water disinfection protocols, serum product sterilization methods, and treatments for atherosclerosis and viremia (Babaei et al., 2002). These results provide a basis for further studies involving ozone-mediated inactivation of viruses and offer an opportunity to investigate the inactivation of other infectious agents in biological materials.

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