

## In vitro effects of different concentrations of aqueous ozone on cells infected with herpes virus

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**Abstract:** Ozone therapy has unique therapeutic properties of non-tissue invasion, absence of side effects or adverse reactions, all of which are responsible for its widespread use. This study investigated the therapeutic potential of aqueous ozone in the treatment of herpes simplex virus infection. Aqueous ozone in different doses and concentrations were applied in vitro on Vero cells infected with HSV1 and on non-infected cells. The results showed complete inhibition of HSV1 infected cells at concentrations of 0.01, 0.02 mg/L respectively in all doses. The control normal Vero cells exposed to aqueous ozone with various concentrations showed no effects on their morphology and cell growth rate. It was concluded that ozone therapy had antiviral activity against HSV1 virus infections in vitro which was both dose and concentrations dependent. Future studies may show that aqueous ozone could become a safe therapeutic agent for herpes simplex virus infections.

[Abeer Gawish, Maha A. Bahammam. **In vitro effects of different concentrations of aqueous ozone on cells infected with herpes virus.** *Life Sci J* 2014;11(5):349-352]. (ISSN:1097-8135). <http://www.lifesciencesite.com>. 46

**Keywords:** ozone, ozone therapy, herpes simplex, viral disease

### 1. Introduction

Ozone has been used in medicine for over a hundred years because of its microbiological effects. Its use has been investigated in the treatment of several diseases such as ocular diseases, infectious diseases, dermatological disorders, and in pulmonary, renal, hematological and neurodegenerative pathologies (Stubinger et al., 2006).

In dental practice, ozone therapy had been evaluated since 1933 in the treatment of oral lesions and chronic periodontal infections (Azarpazhooch and Limeback, 2008). The bactericidal, fungicidal and virucidal properties of ozone are the result of its intense oxidizing capacity, with the formation of free radical and direct destruction of almost all microorganisms. Moreover, ozone favors tissue healing and increases blood perfusion of the wound. Intra-orally, ozone has been used to treat chronic periodontitis, caries, and infections after dental extractions, lesions caused by radiotherapy, aphthae and mycoses, and can be used for disinfecting root canals (Stubinger et al., 2006).

There is evidence of interest in the clinical application of ozone to treat virus infection in vivo because ozone is known to neutralize viruses in vitro. Most of the viruses are sensitive to ozone; yet differ widely in their susceptibility. Analysis of viral components showed damage to polypeptide chains and envelope proteins impairing viral attachment capability, and breakage of viral RNA (Roy and Engelbrecht, 1982).

Viruses have no enzymatic protection against oxidative confrontation. Lipid-enveloped viruses are especially sensitive to ozone challenge because lipid alteration is an important cause of viral death. Studies showed that viruses containing lipid envelopes (lipid coated) include the Hepadnaviridae (Hepatitis B), the Flaviviridae (hepatitis C, West Nile virus, yellow fever); the Herpes viridae, (a large family grouping the Simplex, Varicella-Zoster, Cytomegalovirus, and Epstein-Barr viruses); Orthomyxoviridae (influenza); the Paramyxoviridae (mumps, measles); Retroviridae (HIV), and Filoviridae (Gopalakrishnan, 2012). When virion's lipid envelope becomes fragmented, its DNA or RNA core cannot progress in its life cycle making it more susceptible to ozone therapy than viruses that do not have an envelope, the so-called "naked viruses." Ozone interacts with the viral proteins of naked viruses, forming protein hydroxides and peroxides, leading to viral death.

Its virucidal effects are based on its strong oxidation effect with the formation of free radicals as well as its direct destruction of almost all microorganisms. It is well established that ozone in the gaseous or aqueous phase can kill bacteria, fungi and viruses (Rice, 2002). The aim of the present investigation was to determine the effects of the ozone therapy on Herpes Simplex virus in vitro.

### 2. Materials and Methods

Herpes simplex virus strain (a titer of  $10^4$  tissue culture infectious dose 50/ml) was supplied by the Department of Virology at VACSERA (The Holding Company for Biological Products & Vaccines). Vero

cell culture line was used for propagation and titration of HSV1 (Sweet and Hilleman, 1960).

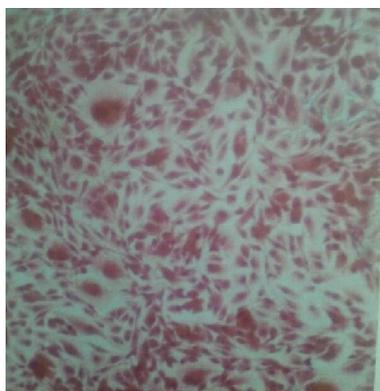
The isolated virus was propagated from  $10^5$ -  $10^7$  in Vero cells, which seeded at  $1 \times 10^5$  cells/ well on 96 well culture plate with MEM (minimal essential media) used with 10% new born calf serum, 1% glutamine, 100 U/ ml penicillin and 100  $\mu$ g/ml streptomycin at a pH 7 and incubated at 37° C, 5% CO<sub>2</sub> for growth purpose on Vero cells. The infected cells that showed the typical HSV-1 cytopathic effect were subjected to 2 freeze thaw cycles. The supernatant was stored at -70° C until used (Chen and Chen, 2009, Miyamura et al., 1974).

Aqueous ozone was prepared by exposing deionized water to ozone concentration range (0.003-0.02 mg/L) using Humazon® PM unit with different flow rates (30,50,70mg/ h) in constant; time (10 min), voltage (220 v) and current consumption (120A) (Viebahn, 1985).

The Vero cell culture line was divided into two groups based on the intended application of aqueous ozone. Group I: Ozonated water was applied on uninfected Vero cells (without HSV1), and in Group II, aqueous ozone was applied on infected Vero cells (with HSV1). A Normal Vero cell without treatment was used as negative control. Vero cells ( $1 \times 10^5$  cells/ well) were seeded in 96 well lat bottomed tissue culture plates with application of aqueous ozone with different concentrations (0.003-0.02mg/L) at different doses (0.1,0.2 and 0.3 mg/L). Cell viability was assessed 48 hours post treatment under inverted microscope for cytopathic effect (CPE). Data were tabulated and statistically analyzed using SPSS soft ware program for non-parametric tests.

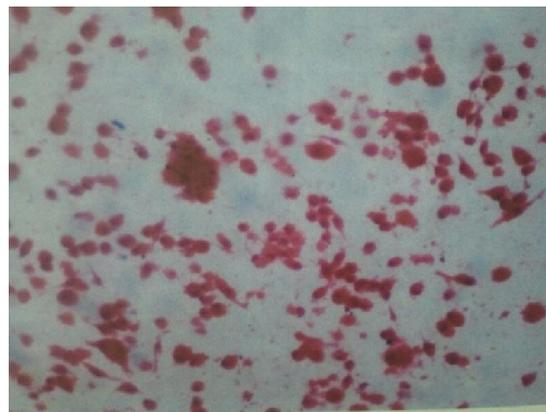
### 3. Results

The normal Vero cells showed elongated diamond shapes that aligned in a layer without nuclear overlaps (monolayer) Fig. 1.



**Figure 1:** Photomicrograph showing normal Vero cells (H&E.100X)

The observed cytopathic effects of herpes infection on the Vero cells included change in the morphology and viability of the cells. Some cells became rounded, shrunk while some completely disappeared (or were lysed). Other infected cells formed grape – like clusters, and some underwent enlargement and budding Fig.2.



**Figure 2:** Photomicrograph showing rounding up and enlargement of infected Vero cells with isolated HSV-1 (H&E.100X)

#### *The effect of aqueous ozone on Vero cells*

Table 1 showed that exposure of normal Vero cell culture (negative control cells) for 48 hours to different concentrations of aqueous ozone (0.1,0.2 and 0.3 mg/L) did not cause any morphological changes in the cells and did not affect the growth rate of the cell which were still viable as non treated cells.

**Table 1.** Effect of ozone on normal Vero cells

Ozone dose Residual ozone conc.	Cell viability		
	0.1 ml	0.2 ml	0.3 ml
0.003	No morphological change in Vero cells		
0.004			
0.008			
0.009			
0.01			
0.02			
P value	NC**	NC**	NC**

NC not computed

The results of the effect of aqueous ozone using different doses in different concentrations on HSV1 infected Vero cell is shown in Table 2.

At dose 0.1 ml, there was a statistically significant difference between the effects of the different concentrations on cell viability. There was complete inhibition of replication at 7 log<sub>10</sub> TCID<sub>50</sub> ml and 100% reduction with p<0.001 found at

0.01&0.02 mg /L, while at 0.004, 0.008 & 0.009 mg/L, there was notable decrease to 6 log 10TCID 50 ml, and 99.5 % reduction ( $p < 0.001$ ), and inhibition of viral replication to 6 log 10TCID 50 ml and 99% reduction ( $p < 0.001$ ) found at 0.003mg /L.

The results of the effect of aqueous ozone at dose 0.2 ml demonstrated a statistically significant difference between the different concentrations. It showed a high percentage of complete inhibition (7 log 10 TCID 50 ml) of 100% reduction ( $p < 0.001$ ) at concentration 0.008, 0.009, 0,01 and 0.02 mg/L. There was notable decrease to 6 log 10TCID 50 ml (99.5 % reduction  $p < 0.001$ ) at concentration 0.004, 0.008 & 0.009 mg/L.

However at dose 0.003mg /L there was no statistically significant difference between the different concentrations. There was complete inhibition (7 log 10 TCID 50 ml 100% reduction  $p < 0.001$ ) with all concentrations of (0.003, 0.004, 0.008, 0.009, 0,001 and 0.02 mg/L) respectively.

**Table 2.** Percent reduction of virus titer in Vero cells following ozone therapy at different doses and concentrations

Ozone dose Residual ozone conc.	Cell viability		
	0.1 ml	0.2 ml	0.3 ml
0.003	99	99.5	100
0.004	99.5	99.5	100
0.008	99.5	100	100
0.009	99.5	100	100
0.01	100	100	100
0.02	100	100	100
P value	<0.001*	<0.001*	NC*

$P \leq 0.05$  significant

#### 4. Discussion

Ozone has become a treatment modality option in comprehensive treatment plan necessary for successful management of infectious diseases. Vero cells which were used in this study were derived from the kidney of an African green monkey, and are one of the more commonly used mammalian continuous cell lines in microbiology, and molecular and cell biology research (Shin and Sobsey, 2003). This anchorage-dependent cell line has been used extensively in virology studies, but has also been used in many other applications, including the propagation and study of intracellular bacteria and parasites, and assessment of the effects of chemicals, toxins and other substances on mammalian cells at the molecular level.

Among the advantages of ozone in aqueous phase is its potency, easy preparation and handling, lack of mutagenicity and its rapid microbicidal effects (Kim et al., 1999). The results of the present

study demonstrated that the aqueous ozone did not affect the viability or the morphology of Vero cells. Aqueous ozone in its different doses and concentrations appear to produce complete inhibition of HSV 1 on virally infected cells. These results were in accordance with data from several in vivo and in vitro studies which showed the antiviral activity of ozone therapy (Chen and Chen, 2009, Khadre and Yousef, 2002, Riley, 2002, Shin and Sobsey, 2003), demonstrated the role of 0.2mg/L aqueous ozone in adenovirus inactivation which was reduced by 99.99%. Others reported an inactivation of adenovirus by 4 log after treatment with 0.07mg/L ozone concentration (Shin and Sobsey, 2003).

The antiviral activity of ozone appears to be explained through different mechanisms: Ozone concentration plays an important role in exerting different response on cell membrane which may induce injury to it (Dumler et al., 1994, Kafoury et al., 1999). It may induce inactivation of the intracellular virus leading to disruption of cell membrane and to cell death (Chen and Chen, 2009). The lipid enveloped viruses are inactivated in aqueous ozone media via the oxidation of their lipoprotein glycoprotein and envelope (Girard, 2001). The mechanism of denaturation of virions is through direct contact with ozone, lipoprotein, lipid and glycoproteins. The presence of numerous double bonds in these unsaturated molecules makes them vulnerable to the oxidizing effect of ozone. When virions lack envelope, they cannot sustain or replicate themselves (Girard, 2003). Different concentrations and doses of ozone have an effect on cellular functions of different cell type (Fu et al., 2002, Gornicki and Gutsze, 2000, Koike et al., 2001) and do modulate the immune response (Koike et al., 2001). Ozone appear to increase CD4 with relative increase in the CD4/CD8 (Rattan, 2006), which is known to interact with constant regions of MHC class I or II. This interaction increases the binding affinity of the T cell being the main protection against viral infections. Ozone exhibits a strong germicidal effect at a higher concentration range via its oxidative power which has proven to be effective in destroying lipid enveloped viruses (Saul, 2007).

In conclusion, ozone therapy has antiviral activity against HSVI virus infections in vitro which is dose and concentrations dependent. It can be considered a safe therapeutic agent in vitro. Further investigations are needed to evaluate its efficacy clinically in patients with more attention to evaluate its effectiveness in infected patients.

#### Acknowledgements

The authors gratefully acknowledge the valuable contribution and continuous support of Prof.

Mohammad Khodeir and the team from Department of Virology at VACSERA for their assistance in the accomplishment of this research.

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3/15/2014