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[1] Department of Veterinary Medicine, University of Bari Aldo Moro, S.P. per Casamassima km. 3 70010 Valenzano (BA), Italy
[2] Local Health Authority of Matera, Via Montescaglioso 75100 Matera (MT), Italy
* Correspondence: annalisa.rizzo@uniba.it (A.R.); vincenzo.cicirelli@uniba.it (V.C.); Tel.: +390805443881 (V.C.); Fax: +390805443883 (V.C.)

Simple Summary: *Alphaherpesviruses* cause genital lesions in both animals and humans. Ozone (O_3) has a strong virucidal action on enveloped and naked viruses. The aim of this study was to test the *in vitro* virucidal and antiviral activity of an Ozone/Oxygen (O_3/O_2) gaseous mixture against Caprine herpesvirus type 1 (CpHV-1). To test the virucidal activity the virus was exposed at different concentrations (20 and 50 µg/ml) of the gaseous mixture at different time, and a decrease in the viral titre by up to 2.0 log10 $TCID_{50}$/50 µl was observed. To test the antiviral activity, the virus was exposed to non-cytotoxic different concentrations of the gaseous mixture. When MDBK cell monolayers were treated with the gas mixture after infection with CpHV-1 at a concentration of 50 µg/ml, significant antiviral activity was observed with a decrease in viral titer of 2.0 log10 $TCID_{50}$/50 µl. These findings open perspectives to studies aimed at assessing if topical treatment of genital herpes lesions in vivo with O_3/O_2 gaseous mixture could be a valid and safe therapeutic option in the animal model, with possible translational applications for the therapy of Hu-man Herpes simplex virus 2 (HSV-2) which shares several biological similarities with CpHV-1.

Abstract: *Alphaherpesviruses* cause genital lesions and reproductive failure in both humans and animals. Their control is mainly based on prevention by hygienic prophylactic measures, due to the absence of vaccines and limitations of antiviral drug therapy. Ozone is an oxidating gas showing a strong microbicidal activity on bacteria, fungi, viruses, and protozoa. The present study assessed the *in vitro* virucidal and antiviral activity of ozone against Caprine herpesvirus type 1. Virucidal activity of a gaseous mixture containing O_3 at 20 and 50 µg /mL was assessed against the virus for different contact times (30 s, 60s, 90s, 120s, 180s and 300s). Antiviral activity of a gaseous mixture containing O_3 at 20 and 50 µg /mL was evaluated against the virus to for 30s and 60s. Ozone displayed significant virucidal activity when used at all the tested concentrations whilst significant antiviral activity was observed using ozone at 50 µg/ml. The gaseous mixture, tested in the present study, showed virucidal and antiviral activity against CpHV-1 with a dose- and a time-contact - dependent fashion. Ozone therapy could be evaluated in *vivo* for the treatment of CpHV-1-induced genital lesions in goats, through topical applications.

Keywords: Ozone; Caprine herpesvirus 1; *in vitro*; virucidal activity; antiviral activity

1. Introduction

Viral infections of the reproductive system are endemic in mammals and have negative repercussions on sexual and reproductive performances. Among them, the *Alphaherpesviruses* (family

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Herpesviridae, subfamily *Alphaherpesvirinae*) that cause genital lesions and abortus in both humans and animals [1,2]. *Alphaherpesviruses* are large, enveloped DNA viruses characterized by rapid, lytic growth cycles [3]. Some of them infect the genital tract and, subsequently, establish a lifelong latent infection in the lumbosacral sensory ganglia which could be recurrently reactivated by stress, immunosuppression or hormonal changes [4].

In humans, Herpes simplex virus type 2 (HSV-2) is a major cause of genital infection inducing painful genital ulcers with 13% of the population aged 15–49 years being infected [2]. HSV-2 mainly causes genital herpes which is the most common sexually transmitted ulcerative disease in the world and is considered a global health problem[5].

The control of HSV-2 is mainly based on prevention (by information and education) and on the use of viral DNA polymerase inhibitors [6]. These molecules can accelerate symptom resolution and lesion healing, but they cannot eradicate latent HSV infection, and they could select drug resistance [7]. Resistance to antiviral drugs is a major problem in the fight against contagious diseases such as influenza and hepatitis. The impact of resistance to antivirals can be important and fatal, as it can affect drastically the effectiveness of therapy. This has driven the research to find alternative therapies.

Alphaherpesviruses also cause reproductive failure in farm animals and economic loss for livestock industry [1]. Caprine herpesvirus type 1 (CpHV-1) is a widespread virus in goat herds and causes vulvo-vaginitis and balanoposthitis, infertility, but also abortions and stillbirth [8]. Abortions associated with CpHV-1 occur during the second half of pregnancy and can be reproduced experimentally after intranasal and intravenous inoculation of pregnant goats [9]. CpHV-1 establishes latent infections but unlike other herpesviruses its reactivation is extremely difficult both in natural and experimental conditions and has been reported very rarely. In natural infections, CpHV-1 is reactivated during estrus, but only in animals with low neutralizing antibody titers. In previous studies latent CpHV-1 has been experimentally induced in adult goats by administration of a high dose of dexamethasone for several days [8]. Interestingly, after reactivation or experimental infection, even when the virus has been inoculated intranasally, elimination occurs via the genital route far longer than by the nasal route. The results of these studies indicate that CpHV-1 recognizes the genital tract as a target district [8].

On goat farms the control of CpHV-1 is based on prevention and eradication. Different types of vaccines have been experimented since the 2000s. However, vaccines for CpHV-1 have not been released as this pharmaceutical market is not economically profitable. Consequently, the control of this infection relies on hygienic prophylactic measures [10] and the research for alternative solutions is needed.

CpHV-1 has a significant biological similarity to HSV-2 inducing latent infection in the sacral ganglia and similar genital lesions [8]. This has suggested the use of CpHV-1 infection in goat as a model for the study of HSV-2 infection in humans [11,12].

The immunosuppressive drug Mizoribine, when combined with Aciclovir, has been evaluated *in vitro*, showing to be useful for treatment purposes against CpHV-1 [13]. The administration of Cidofovir has also raised interest in the treatment of genital lesions in the caprine species based on *in vivo* and *in vitro* tests [12]. In addition, PHA767491, an anti-tumor drug, has been used against both HSV-1 and HSV-2 [14] and CpHV-1 [15]. Some natural substances, like essential oils have been tested for their anti-infective properties: volatile oils of *Melissa officinalis Lamiaceae* effectively inhibited HSV-2 replication [16]. Ginger essential oil was found to be effective as virucide, inactivating CpHV-1 up to 100% [17]. Moreover, fig latex has also shown to be effective against CpHV-1 *in vivo* and *in vitro* [18]. In addition, several essential oils have been tested against human viruses [19]Anyway, the use of essential oils in veterinary medical practice is limited.

The Ozone (O_3) therapy is an alternative therapy that uses O_3 in mixture with Oxygen (O_2) for medical purposes [20]. O_3 is an allotropic form of Oxygen, composed by three Oxygen atoms, organized in a relatively unstable cyclic structure that makes it a powerful oxidant agent [21]. Due to this feature, it shows microbicidal and antimicrobial properties against bacteria, fungi, viruses, and protozoa [20,22,23]. Against viruses, O_3 determines a structural damage by protein and lipid

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peroxidation of envelope and capsid, respectively, and by destruction of nucleic acids [24,25]. Nucleic acid damage is determined by the disruption of specific regions of the viral genome. Some authors exposed poliovirus type 1 to ozonized water demonstrating a specific damage in the 5′-non-coding regions of the genome [25]. Protein peroxidation play a key role in the inactivation of non-enveloped viruses: Thurston-Enriquez et al. [26] inactivated feline calicivirus and adenovirus type 40 using ozonized water at 300 and 60 μg/L respectively. Encouraging results have been achieved by Dubuis et al. [27] on murine norovirus and phage viruses by using an O_3 in air treatment at low concentrations (0.23 ppm equal to 230 μg/L). Lipid peroxidation is the main procedure used to inactivate enveloped viruses; in a study conducted by Wells et al. [28], human immunodeficiency virus type 1 was inactivated *in vitro* by O_3 in a dose dependent manner. Severe Acute Respiratory Syndrome coronavirus type 2's (SARS-CoV-2) viral titre significantly decreased on different materials (fleece, gauze, wood, glass, plastic) after 30 minutes/2 hours of exposure in a plexiglass chamber to O3 (0.2-4 ppm equal to 200-4000 μg/L) [29]. A gaseous mixture of 21% O_3 in air for 80 minutes was able to effectively induce a 4-fold reduction of influenza A virus titre. Conversely, this mixture was ineffective against respiratory syncytial virus [30].

O_3 displayed *in vitro* virucidal activity on herpes simplex virus type 1 (HSV-1) and Bovid Herpesvirus type 1 (BoHV-1), inducing viral inhibition over 90% after 3 hours of exposure [31]. Nevertheless, data regarding the virucidal efficacy of O_3 against CpHV-1 and HSV-2 are not available.

In large animal veterinary medicine, O_3 has already been administered systemically, by auto-haemoadmistration [32,33], or topically [20]. Moreover, O_3 was used to treat postpartum pathologies [34], to improve reproductive parameters in postpartum dairy cows [35,36] and increased the fertility rate in cows affected by urovagina [37]. O_3 therapy could overlap or outperforms antibiotics treatments, avoiding the occurrence of antimicrobial resistance [35,38] and withdrawal times for meat and milk because it does not leave residues in biological tissue [38]. In goat medicine, few studies have been conducted on the application of O_3 therapy and they are mainly focused on reproductive [35] and milk production [39] performances.

The aim of this study was to evaluate the *in vitro* virucidal and antiviral effects of a medical O_3/O_2 gaseous mixture against CpHV-1.

2. Materials and Methods

2.1. O_3 Generator

An O_3 medical generator (Vet-Ozone Medica srl-Italy) was used to produce an Ozone/Oxygen (O_3/O_2) gas mixture: after being connected to an electrical source and to an O_2 cylinder, the generator produces electrical discharges which, acting on the O_2 (substrate), convert part of it into O_3. The generator can produce a gas mixture containing 20 and 50 μg of O_3/ml.

2.2. Hermetic Box for Gas Flow

An in-house method to expose the Petri dishes to the O_3 gas flow was developed, as previously described [23].

Two silicon tubes were assembled on the cover of a polypropylene hermetic box. The tube for the incoming flow was connected to the O3 generator and the output tube to a drainpipe.

After placing the uncovered Petry dishes inside the box, the box was hermetically sealed, and the Ozone generator was switched on. The ozonized gas mixture generated entered the box through tube 1; subsequently, the gas mixture came into contact with the Petri dishes, and exited through tube 2, allowing a continuous gas flow (Figure 1). The box was disinfected between each test by sodium hypochlorite (1%) with a label contact time for at least 1 min, as suggested by the guidelines for "Disinfection and sterilization in healthcare facilities" [40].

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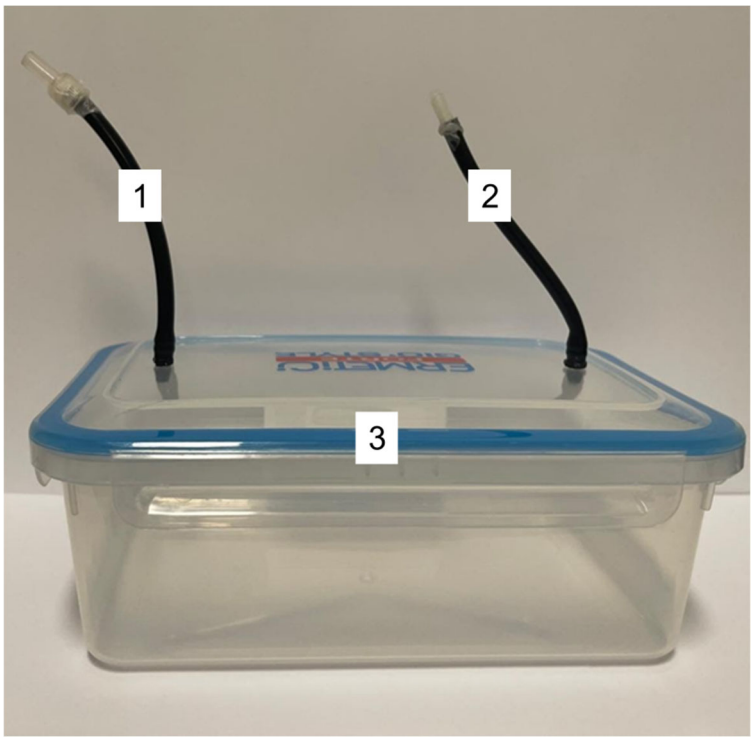

Figure 1. Modified hermetic box for continuous gas flow. The device is composed by two silicone tubes [one tube for gas entry (1) and one tube for gas exit (2)] and by a polypropylene hermetic box (3).

2.3. Cells and Virus

Madin Darby Bovine Kidney cells (MDBK) were kindly provided by dr. Maura Ferrari responsible for the Cell Substrate Center of the Experimental Zooprofilattic Institute of Lombardy and Emilia–Romagna. The cells were cultured at 37 °C in a 5% carbon dioxide (CO_2) atmosphere in Dulbecco Minimum Essential medium (D-MEM) supplemented with 10% foetal bovine serum, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 2 mM l-glutamine. The same medium was used for the antiviral assays. The CpHV-1 strain Ba-1, previously isolated from vaginitis in goat, was cultured and titrated in MDBK cells. The virus stock with a titre of 7.25 $\log_{10}$ Tissue Culture Infectious Dose ($TCID_{50}$)/50µl was stored at −80 °C and used for the experiments. The CpHV-1 viral suspension used in the experiments underwent a preliminary centrifugation at 4000 xg for 15 min to separate cellular debris. .

2.4. Cytotoxicity Assay

A cytotoxicity assay was carried out in order to determine the conditions of cell exposure to O_3 (O_3 concentration in the gas mixture and exposure time) for the antiviral activity tests. For the purpose, confluent 24-h monolayers of MDBK cells grown in 35mm Petry dishes and maintained in D-MEM were exposed to O_3/O_2 gas mixture containing different concentrations of O_3 (20 and 50 µg/mL), at room temperature, for 30 s (T1), 60 s (T2), 90s (T3), 120 (T4)s, 180s (T5) and 300s (T6). Negative controls were prepared putting cells inside the hermetic box at the same temperature and for the same time intervals without O_3/O_2 gas mixture exposition. Cytotoxicity was assessed both by direct microscopic examination of cell morphology (loss of cell monolayer, granulation, cytoplasmic vacuolization, stretching and narrowing of cell extensions and darkening of the cell borders)[41], and by indirect measurement of cell viability using an in vitro Toxicology Assay Kit (Sigma–Aldrich Srl, Milan, Italy) based on 3-(4,5-dimethylthiazol-2 yl)-2,5-diphenyl tetrazolium bromide (XTT). The XTT test was carried out as previously described[41], following the manufacturer's instructions, and the obtained optical density (OD) values were used to calculate the percentage of cytotoxicity (percentage of dead cells) according to the formula: % Cytotoxicity = [(OD of control cells−OD of treated cells)

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×100] / OD of control cells. The assay was performed in triplicate and data were expressed as mean ± SD. The exposure conditions that did not reduce the viability of the treated MDBK cells by more than 20% (cytotoxicity threshold) were considered as non-cytotoxic and were selected for subsequent antiviral tests.

2.5 Cytophatic Effect

The cytophatic effect of CpHV-1 was evaluated on MDBK cells using an inverted microscope by live-cell imaging and hematoxylin eosin staining.

2.6. Virucidal Activity Assay

The virucidal activity of O_3/O_2 gaseous mixture against CpHV-1 was assessed at 20 and 50 µg/mL O_3 concentration.

One ml of CpHV-1 stock virus was poured in a 35 mm Petri dishes and directly exposed to the O_3/O_2 gas mixture in the modified hermetic box at room temperature. At different time intervals (T1 to T6), 100 µl of the treated viral suspension were collected for subsequent viral titration.

A 1 ml aliquot of CpHV-1 stock virus was left untreated at room temperature and similarly sampled for viral titration, serving as virus control.

The experiments were performed in triplicate.

2.7. Antiviral Assays

On the basis of the cytotoxicity assay results, the antiviral activity against the CpHV-1 strain Ba-1 was evaluated using the O_3/O_2 gaseous mixture containing O_3 at 20 and 50 µg/mL for different exposure times (T1 and T2). To identify the step of viral inhibition by O_3 against CpHV-1, two different protocols (A and B) were carried out as detailed below. All the experiments were performed in triplicate.

2.7.1. Protocol A: Virus Infection of Cell Monolayers before Treatment with O_3

Confluent monolayers of MDBK cells of 24 h in 24-well plates were used. Cells were infected with 100 µl of viral suspension containing 100 $TCID_{50}$ CpHV-1. After virus adsorption for 1h at 37 °C, the viral inoculum was removed and cell monolayers were washed once with D-MEM before adding 1 ml of maintenance medium (D-MEM). Then, cell monolayers were treated with the O_3/O_2 gaseous mixture. Untreated infected cells were used as virus control. After 72 hours, aliquots of the supernatants were collected for subsequent viral titration.

2.7.2. Protocol B: Virus Infection of Cell Monolayers after Treatment with O_3

Confluent monolayers of MDBK cells of 24 h in 24-well plates were used. Cells were treated with the O_3/O_2 gaseous mixture. Then, the monolayers were washed once with D-MEM and infected with 100 µl viral suspension containing 100 $TCID_{50}$ CpHV-1. After virus adsorption for 1 h at 37 °C, the inoculum was removed and the monolayers were washed with D-MEM before adding 1 ml of maintenance medium (D-MEM). Untreated infected cells were used as virus control. After 72 h, aliquots of each supernatants were collected for subsequent viral titration.

2.8. Viral Titration

Ten-fold dilutions (up to 10^{-8}) of each supernatant, were titrated in quadruplicates in 96-well plates containing MDBK cells. The plates were incubated for 72 h at 37 °C in 5% CO_2. Cytopathic effect of CpHV-1 on MDBK cells was evaluated using an inverted microscope by live-cell imaging or using hematoxylin eosin staining. Based on cytopathic effect, $TCID_{50}$/50 µl was calculated following the Reed–Muench method [42]

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2.9. Data Analysis

All data were expressed as mean ± SD and analyzed by GraphPad Prism (v 9.5.0) program (Intuitive Software for Science, San Diego, CA, USA). To assess normality of distribution, Shapiro-Wilk test was performed. Two-way factorial ANOVA, with concentration * time as factors and Tukey test as *post hoc* test were applied to cytotoxicity results. T student test for independent samples were performed on virucidal and antiviral activity results ($p < 0.05$).

3. Results

3.1. Cytotoxicity Assay

Direct exposure of MDBK cells to O_3/O_2 gas mixture containing O_3 at 20 and 50 µg/mL did not produce any changes in cell morphology at T1 and T2 whereas morphological signs of cytotoxicity were consistently observed in the cells exposed to O_3 at 20 and 50 µg/mL for longer time intervals (i.e., at T3 to T6).

Morphological observations overlapped indirect measurements of cytotoxicity by XTT test. Cell exposure to O_3 at 20 and 50 µg/mL at different time intervals (T1 to T6) resulted in an increasing cytotoxicity with a dose-dependent and a time-contact fashion (Figure 2). O_3 at 20 µg/mL at T1 and T2 induced cytotoxicity of 0.53% (± 0.15) and 3.64% (± 0.8), respectively, below the cytotoxic threshold. Higher cytotoxicity of 31.03% (±1.1), 36.78% (±1.2), 40.10% (±1.3) and 81.52% (±2.3) was observed at T3, T4, T5 and T6, respectively (Figure 2A).

O_3 at 50 µg/mL at T1 and T2 produced cytotoxicity of 0.51 % (±0.13) and 3.61 % (±0.95), respectively, below the cytotoxic threshold. Higher cytotoxicity of 59.77% (±1.3), 65.51% (±1.6), 82.57% (±1.8) and 85.39% (±2.6) was observed at T3, T4, T5 and T6, respectively (Figure 2B).

The ANOVA model showed statistically significant decrease in cytotoxicity in MDBK cells treated with O_3 at 20 ($F = 1517$, $p < 0.0001$) and 50 ($F = 1822$, $p < 0.0001$) µg/mL between different time intervals (T1-T6). By a two-by-two comparison of cytotoxicity induced by O_3 at 20 and 50 µg/mL statistically significant decrease in cytotoxicity was consistently observed at different time intervals (T1-T6). Conversely, the comparison between O_3 at 20 µg/mL at T4 and T5 and O3 and between O_3 at 50 µg/mL at T5 and T6 lacked of statistical significance ($P > 0.05$).

On the basis of these results, the antiviral activity assays were carried out using O_3 at 20 and 50 µg/mL at T1 and T2, below the cytotoxicity threshold.

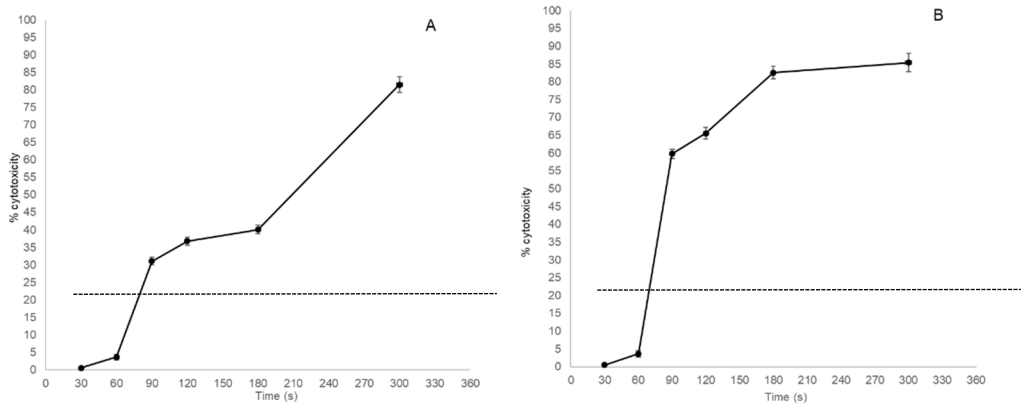

Figure 2. Cytotoxicity of MDBK cells treated with O_3/O_2 gas mixture containing O_3 at 20 µg/mL (A) and 50 µg/mL (B) plotted against time of exposure. The horizontal dotted line indicates the threshold of cytotoxicity (20% of cell death).

3.2. Cytophatic Effect

Cytopathic effect of CpHV-1 on MDBK cells is displayed in Figure 3.

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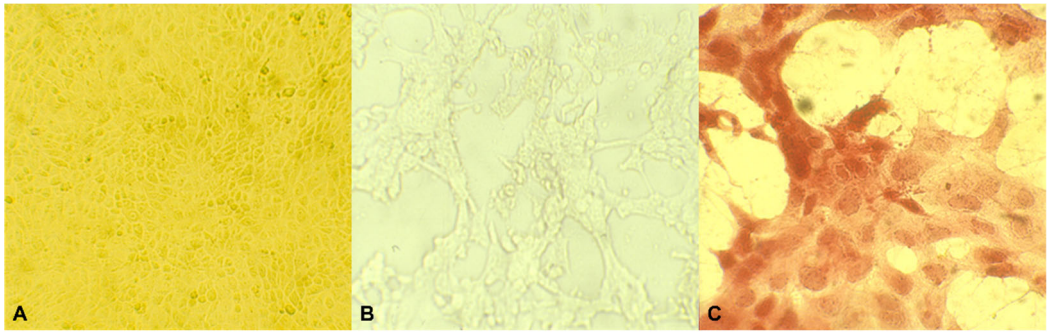

Figure 3. 24-hours monolayer of Madin Darby Bovine Kidney (MDBK) cells (magnification 10x) (Panel A); Cytopathic effect of CpHV-1 on MDBK cells by live-cell imaging (magnification 40x) (Panel B); Cytopathic effect of CpHV-1 on MDBK cells hematoxylin eosin stained (magnification 40x) (Panel C).

3.3. Virucidal Activity Assay

Data obtained were analyzed by Shapiro-Wilk test, confirming normality of distribution ($W=0.8137$, $p>0.05$). Data from the virucidal activity assay showed that the O_3/O_2 gaseous mixture containing O_3 at 20 µg/mL significantly reduced CpHV-1 titre of 1.25 $\log_{10}$ $TCID_{50}$/50 µl ($p<0.05$) at T1 and T2, of 1.50 $\log_{10}$ $TCID_{50}$/50 µl ($p<0.05$) at T3 to T5, and of 2.00 $\log_{10}$ $TCID_{50}$/50 µl at T6 ($p<0.0001$) as compared with the untreated control.

Data from the virucidal activity assay showed that the O_3/O_2 gas mixture containing O_3 at 50 µg/mL significantly reduced CpHV-1 titre of 1.25 $\log_{10}$ $TCID_{50}$/50 µl ($p<0.05$) at T1 and T2, of 1.50 $\log_{10}$ $TCID_{50}$/50 µl ($p<0.05$) at T3 to T4, of 1,75 $\log_{10}$ $TCID_{50}$/50 µl ($p<0.05$) at T5 and of 2.00 $\log_{10}$ $TCID_{50}$/50 µl at T6 ($p<0.0001$) as compared with the untreated control.

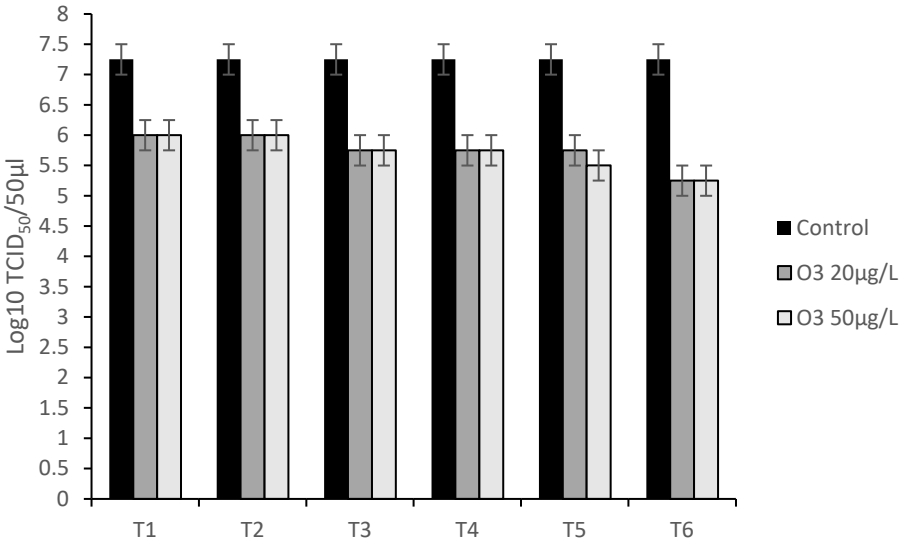

Figure 4. Viral titration on Madin Darby Bovine Kidney (MDBK) cells inoculated with Caprine herpes virus 1 (CpHV-1) and not treated (Control) or treated with Ozone/Oxygen (O_3/O_2 20 and 50 µg/mL) at room temperature for 30s (T1), 60s (T2), 90s (T3), 120s (T4), 180s (T5), 300 (T6).

3.4. Antiviral Assays

3.4.1. Protocol A: Virus Infection of Cell Monolayers before Treatment with O_3

Comparing the viral titre of the untreated infected cells (7.25 ±0.25 $\log_{10}$ $TCID_{50}$/50 µl) with the viral titre of the infected cells treated with the O_3/O_2 gas mixture containing O_3 at 20 µg/mL at T1 and T2 (7.00±0.25 $\log_{10}$ $TCID_{50}$/50 µl), a slight decrease of viral titre (0.25 $\log_{10}$) was induced although

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without statistical significance ($p > 0.05$). Comparing the viral titre of the untreated infected cells (7.25±0.25 $\log_{10}$ $TCID_{50}$/50 µl) with the viral titre of the infected cells treated with O_3 at 50 µg/mL at T1 and T2 (6.00 ±0.25 $\log_{10}$ $TCID_{50}$/50 µl), a significant decrease in the viral titre (1.25 $\log_{10}$) was induced ($p < 0.05$) (Figure 5).

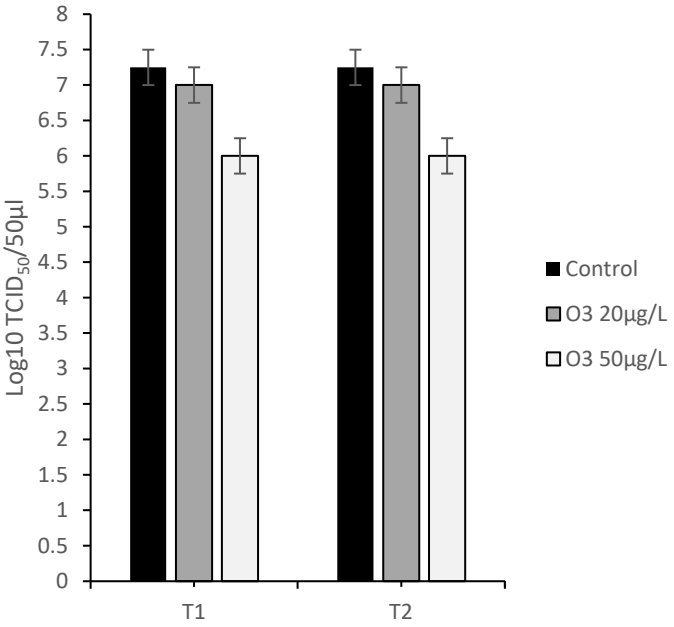

Figure 5. Viral titration on Madin Darby Bovine Kidney (MDBK) cells inoculated with Caprine herpes virus 1 (CpHV-1), treated after to inoculation with Ozone/Oxygen (O_3/O_2 20 and 50 µg/mL) at room temperature for 30s (T1), 60s (T2), and not treated (Control). .

3.4.2. Protocol B: Virus Infection of Cell Monolayers after Treatment with O_3

Comparing the viral titer of the untreated infected cells (7.25 ±0.25 $\log_{10}$ $TCID_{50}$/50 µl) with the viral titer of the infected cells pre-treated with the O_3/O_2 gas mixture containing O_3 at 20 and 50 µg/mL at T1 and T2 (7.25±0.25 $\log_{10}$ $TCID_{50}$/50 µl), no decrease in viral titer was observed. (Figure 6).

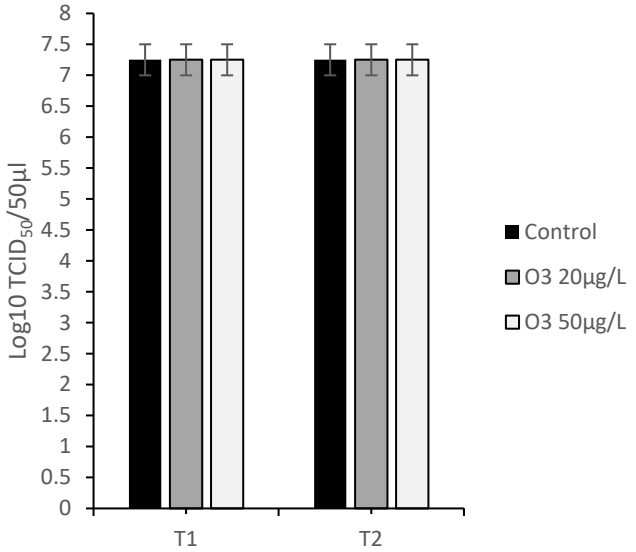

Figure 6. Viral titration on Madin Darby Bovine Kidney (MDBK) cells inoculated with Caprine herpes virus 1 (CpHV-1), treated before inoculation with Ozone/Oxygen (O_3/O_2 20 and 50 µg/mL) at room temperature for 30s (T1), 60s (T2), and not treated (Control). .

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4. Discussion

O_3 therapy is largely used in veterinary medicine for its disinfectant, anti-inflammatory, immunostimulant and antimicrobial effects [20].

In this study we have dealt with the activity of ozone therapy against the genital herpesvirus of the goat (CpHV-1), in view of a possible in field application in veterinary medicine, as well as in human medicine. Indeed, CpHV-1and human HSV-2 share important biological characteristics and the infection by CpHV-1 in goats is considered a valid animal model for the study of infection by HSV-2 and its therapy in humans, [15].

There are several *in vivo* and *in vitro* studies published in the literature addressing/demonstrating the therapeutic potential of O_3 in treating genital infections of farm animals [23,36].

The disinfectant, immunomodulatory and anti-inflammatory actions of O_3 have been reported. The inoculation of O_3 by foams into the vagina and uterus of cows affected by urovagina has shown a reduction in "open days" and in the number of artificial inseminations up to the onset of pregnancy in cows affected by urovagina. Moreover, the beneficial role of O_3 on the repair process of the vaginal and cervical mucosa was observed [37]. The interest of clinical researchers in new therapies, such as ozone therapy, opens new study perspectives for the treatment of infectious pathologies involving the use of antibiotics.

The virucidal effect of O_3 was reported on different viruses. This gas has a potent oxidant action on microorganisms [24–30] damaging the lipidic envelope and protein capsid of enveloped and non-enveloped viruses [24]. In addition, O_3 could inactivate viruses also by destroying guanine residues of nucleic acids [43] as demonstrated for poliovirus type 1 [25,44].

O_3 is a gas with equal or superior efficacy to iodine and chlorine *in vitro*. Ozone therapy has been reported as a successful therapeutic option also in dentistry and in the obstetrics field its use has not shown negative effects on spermatozoa allowing its use also in the reproductive field [37].

In this study, the *in vitro* virucidal activity of an O_3/O_2 gas mixture containing O_3 at 20 and 50 µg/mL, against CpHV-1 was evaluated at different time points (T1 to T6). The *in vitro* antiviral activity of an O_3/O_2 gas mixture containing O_3 at 20 and 50 µg/mL, against CpHV-1 was evaluated at T1 and T2. The concentrations of 20 and 50 µg/mL were chosen based on the cytotoxic activity obtained by XTT test on MDBK cells for different times (T1-T6). Both O_3 concentrations were regarded as non-cytotoxic (below the cytotoxicity threshold of 20%) at T1 and T2. At later time points, starting from T3, an increase in cytotoxicity was observed chiefly at the concentration of 50 µg/mL (over 60%).

In other studies concentrations from 10 to 20 µg/mL of O_3 in O_3/O_2 gas mixture, (generated with a medical ozone generator as in our study) were assessed on other cell lines, i.e., HeLa [45] and SH-SY5Y cells (a human neuroblastoma cell line) without displaying cytotoxic effect [46]. These concentrations did not induce significant alterations in cell viability, and cellular mortality was observed only when cells were treated with O_3 at 100 µg/mL [46].

Eukaryotic cells demonstrate *in vitro* a certain resistance to the prooxidant effect of O_3 because they are protected by the presence of albumin which, with its reducing group -SH, is one of the most protective compounds [47]. Of course, the O_3 concentration adopted is crucial as high concentrations could overwhelm this protective mechanism leading to cell damage and death [48].

In the virucidal activity assay, the exposure of CpHV-1 to the gas mixture was able to significantly reduce the viral titer in a time-dependent manner, leading to a decrease in viral titer of up to 2.00 $\log_{10}$ $TCID_{50}$/50 µl at T6.

To evaluate the antiviral activity at maximum non-cytotoxic dose of O_3 at 20 and 50 µg/mL at T1 and T2, in order to identify the phase in which viral replication might be inhibited, cells were infected with CpHV-1, before (protocol A) and after (protocol B) the treatment with O_3.

In protocol A, when O_3 was used at concentration of 20 µg/ml, we observed a very slight and non-statistically significant reduction in viral titer (0.25 $\log_{10}$ $TCID_{50}$/50 µl), suggesting that O_3 was not able to inhibit virus replication. O_3 at concentration of 50 µg/ml, induced a statistically significant reduction of viral titer (1.25 $\log_{10}$ $TCID_{50}$/50 µl).

Pretreatment of the cells with O_3 at 20 and 50 µg/ml (protocol B), did not reduce viral titer, hinting a lack of inhibition of O_3 in virus uptake and replication.

Overall, since significant results were obtained with short exposure times, use of O_3 *in vivo* could be appliable, especially in the veterinary field. Future studies could address the use of O_3 in CpHV-1-infected goats to gain more translational information for human herpesvirus genital infection. In a previous report the inactivation of herpes viruses (HSV-1 and BoHV-1) with O_3 was achieved by applying a long exposure time (1 to 3 hours) [31]. Compared to other studies [15,17,29,31–33,49], the contact time of the O_3/O_2 gas mixture required to trigger significant effects against CpHV-1 was lower, and this could be an advantage for *in vivo* experiments. Long treatment times would not be ideal due to excessive stress induced to animals, chiefly for animal containment.

5. Conclusions

We reported the *in vitro* virucidal and antiviral activity of a medical O_3/O_2 gaseous mixture against CpHV-1. A short exposure of the virus to O_3 at low concentration (20 µg /mL) was required to achieve partial virus inactivation. This study represents the first step to assess the clinical efficacy of O_3 therapy for the treatment of genital herpes infection. Further essential steps will be the evaluation of the *in vitro* effects on vulvar and vaginal epithelial cells, as well as of the efficacy at treatment of CpHV-1-associated genital lesions in infected goats *in vivo*. Furthermore, it might be interesting to test whether O_3 is also effective on HSV-2 given the close biological similarity with CpHV-1.

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