Effect of Plasma-Activated Medium and Water on Replication and Extracellular Virions of Herpes Simplex Virus-1

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ABSTRACT: We use a surface-wave sustained discharge (SWD) in argon at atmospheric pressure (using a plasma torch) in these experiments. The plasma torch is sustained using a 2.45-GHz electromagnetic wave with applied microwave powers of 13, 15, and 20 W. At these discharge conditions, the length of the plasma torch outside of the quartz tube is ~1–1.5 cm, and the gas plasma temperature does not exceed 40°C. This allows direct treatment of samples using the active zone of the discharge. In the cytotoxicity study, only two of the experimental settings achieve up to 50% survival of the cell monolayer after adding plasma-treated medium. Examining the effect of the plasma torch treatment media on herpes simplex virus-1 replication, we found that none of the applied experimental assays show significant protection on the cell monolayer. In a study of the virucidal action of a plasma-treated viral suspension diluted with sterile water at a ratio of 1:2 that was treated for 300 s at 13-W wave power, a decrease in the viral sample titer occurred unlike in the 1.67 log₁₀ control. Using optical emission spectroscopy, we found that OH intensity increases at the contact point between plasma and the water surface. Intensity of NO-γ also increases to the contact point with applied power. We also monitored the amount of peroxide radicals in plasma-treated water and nutrient medium in the presence of lucigenin.

KEY WORDS: surface-wave discharge, HSV-1, active forms of oxygen, atmospheric, pressure plasma torch

I. INTRODUCTION

The Herpesviridae family includes a large number of viruses that can be isolated from different vertebrates. Representatives of this family cause diseases of varying strengths and clinical presentations, from asymptomatic infections and self-limiting skin lesions to severe generalized infections, encephalitis, meningitis, and malignancies.

Continuous development of technologies makes it possible to study the body's reactions to the application of various innovative therapies and to trace the technologies' impact on viral and bacterial infections *in vitro* and *in vivo*. Plasma-based technologies take an increasing place in new, advanced conceptions for wastewater management as a promising tool for treatment of persistent organic pollutants that have low biodegradability. Plasma's major advantage is its ability to produce high-efficiency synergy of active components with pollutant removal. Additional benefits include the disinfection

of treated water. Plasma's bactericidal effect can influence treatment effectiveness when the technology is used in combination with biological methods.¹ The plasma that we use in our study has specific characteristics that could allow its use in food disinfection.²

One of the fastest growing areas in plasma technology is so-called plasma medicine. Here, we discuss an emerging field of plasma medicine, in which nonequilibrium plasmas can initiate, promote, control, and catalyze various complex behaviors and responses in biological systems. Nonequilibrium plasmas are nondestructive to tissue, safe, and effective for inactivation of various parasites and foreign organisms.³ The effects of nonequilibrium atmospheric pressure plasma on representatives of both classes of bacteria (Gram-negative and -positive) have already been studied.⁴ Literary data have shown that cold atmospheric pressure plasma can exhibit virucidal properties against feline calicivirus,⁵ MS2 virus,⁶ and flu viruses.⁷

Here, we present results obtained from the study of the effect of cold plasma-treated culture media on replication of herpes simplex virus (HSV)-1 and the effects of plasma on a viral suspension diluted with sterile water.

II. MATERIALS AND METHODS

A. Materials

1. Plasma Source

For all experiments, we used a surface-wave discharge (SWD) in argon at atmospheric pressure.^{8–11} The experimental setup is schematically presented in Fig. 1. Plasma was generated by a surface electromagnetic wave of 2.45 GHz using the Surfatron S-80 wave launcher (Sairem, Décines-Charpieu, France) coupled to a solid-state microwave generator (Sairem, GMS 200 W). With constant gas flow of 5 L/min, a mass flow controller supplied 99.999% pure argon. We used a discharge quartz tube with an 8-mm outer diameter and 4-mm inner diameter (denoted “tube 8/4”). The SWD that was produced operated in atmospheric pressure and formed a torch in open space. Variation of gas-flow speed and/or applied microwave

FIG. 1: Schematic representation of the experimental setup. Microwave; UV-VIS, ultraviolet visible spectroscopy.

power led to different discharge properties including plasma column length, temperature of neutral particles in the plasma (called plasma gas temperature), and concentration of charged and excited particles. We used applied wave powers of 13, 15, or 20 W in the experiments, with reflected power kept under 1 W. Discharge conditions were chosen so that plasma torch length was sufficient to treat samples (12, 13, and 17 mm at wave powers of 13, 15, or 20 W, respectively) and plasma gas temperature did not exceed 40°C.

Usually in the DBD plasma jets the low gas temperature is only in the afterglow region of the discharge which is used for treatment.¹² In our case we are not using any afterglow discharge zone. The plasma torch produced in this way corresponds to the active discharge zone in whole its volume to its end. In all treatments the active discharge zone is in interaction with the treated sample that is why it is important the gas temperature inside the plasma torch to be lower than 40°C.

It is known that the properties of SWD at the same axial position measured from the plasma column end are similar.¹³ That is why for the plasma treatment we have used the last 2–3 mm of the plasma torch to be in contact with the sample or the liquid surface like in the previous work.¹⁴

2. Nutrient Media

Cells and viruses were cultured in DMEM (Dulbecco's modified Eagle's medium) (Biochrom, Berlin, Germany). Fetal bovine serum (FBS) was added to the medium, at 8% for growth media and 4% for maintenance media.

3. Cell Line

In all experiments, we used a Madin-Darby bovine kidney monolayer cell line.

4. Virus

The virus used was HSV-1 (human alphaherpesvirus 1), strain F.

5. Chemiluminescent System for Detection of Peroxide Radicals

The system used lucigenin (3 mg/mL) and NaOH (100 mm). To 10 mL of 100 mm Tris (pH 9.4), we added 0.2 mL of hemolysate (10 mL H₂O + 0.02 mL total blood).

B. Methods

1. Cytotoxic Effect of Plasma-Activated Nutrient Medium

We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method described by Mosmann¹⁵ to study the plasma's cytotoxic effects (CEs) and determine cell viability. When the cell monolayer in 96-well plates reached between 90%

and 100% confluency (usually after 24 h), the supernatant was decanted and the DMEM (4% FBS) treated by direct contact of the last 2–3 mm of the torch, at the surface of the liquid. The following experimental protocols were applied.

1. We treated the medium with plasma at 20-W power in the flame for 30, 60, 90, 120, 240, and 300 s. During one plasma treatment, 0.1 mL of medium was treated by direct contact of the last 2–3 mm of the torch at the surface of the liquid. Treated medium was added to each well at a volume of 0.1 mL followed by 0.1 mL of untreated support medium. At least three wells were collected for each treatment length.
2. Plasma parameters were the same as those described above. Treated medium volume for one plasma treatment was 0.2 mL. In each well of 96-well plates, the 0.2 mL of treated medium were added without adding untreated medium. Medium was treated at the same time intervals.
3. Medium was treated with plasma at a wave power of 15 W. For one plasma treatment, we added 0.2 mL of treated support medium to the cell monolayer in each well. Treatment intervals of the medium were the same.
4. Medium was treated with plasma at a wave power of 13 W. For one plasma treatment, we added 0.2 mL of treated medium to the cell monolayer in each well. Treatment intervals were the same as above (30, 60, 90, 120, 240, and 300 s).

Untreated media (0.2 mL each) were placed in several wells of the plate and used as cell controls.

2. Effect of Plasma-Treated Nutrient Medium on HSV-1 Replication

The virus that we used was titrated, and experiments were performed with a $10^{-6.5}$ cell culture infected dose of 50% (CCID₅₀)/mL. Survival of infected cells and those treated with activated nutrient medium was determined using the MTT assay¹⁵ and modified using the Pauwels et al.¹⁶ method for rapid screening of antiherpes compounds.

The medium was treated by direct contact with the last 2–3 mm of the torch at the surface of the liquid. Culture medium was treated for the same time intervals as above (30, 60, 90, 120, 240, and 300 s) and added to the virus-infected cells according to the following working protocols.

1. For consistent application, treated medium was added after adsorption of the virus with removal of nonadsorbed virus. After 1 h of virus adsorption, the viral suspension was withdrawn. This was followed by flushing three times with saline solution. For one plasma treatment, we added 0.2 mL of treated medium at appropriate intervals. After 5 and 20 min of contact, we removed the treated medium and replaced it with 0.2 mL of untreated medium.
2. For consistent application, treated medium was added after adsorption of the virus with removal of nonadsorbed virus, but 0.2 mL of the medium treated at appropriate intervals was added to each well, without subsequent removal and replacement with untreated maintenance medium. During one plasma treatment, 0.2 mL of the medium was treated.

3. The third protocol was consistent application without removal of the nonadsorbing virus. After 1 h of adsorption of the virus, 0.1 mL of the treated culture medium (treated at appropriate intervals as described above) was added to each well. During one plasma treatment, 0.1 mL of the medium was treated.
4. For parallel application, the viral suspension was added at the same time as the treated medium at appropriate intervals, without waiting for viral adsorption. After removal of growth medium over the cell monolayer, viral suspension and treated support medium were added in each well in equal volumes (0.1 mL each). During one plasma treatment, 0.1 mL of the medium was treated.

All working protocols for each plate were subject to appropriate controls. Cell control – contains uninfected cells and not treated with activated medium. Virus control – virus-infected cells but untreated with activated medium.

3. Effect of Plasma-Activated Sterile Water on Extracellular Virions

To investigate the effect of plasma-activated sterile water on extracellular virions, we used the following working protocols.

1. After determining the viral titer ($10^{-6.5}$ CCID₅₀/mL), the viral suspension was diluted 1:1 with distilled water (dH₂O), with final volumes of 3 mL. The diluted suspension in this volume was treated with the plasma source at 13 W and gas flow of 5 L/min for treatment intervals of 30, 60, 90, 120, 180, and 240 s. During one plasma treatment, 3 mL of the diluted suspension was treated. As soon as the suspension was treated at the appropriate intervals, a cell monolayer was infected with it to determine the virus titer using the Reed–Muench method.¹⁷
2. After infection of the cell monolayer with the treated diluted suspension, the viral titer was determined using the Reed–Muench final dilution method.¹⁷
3. The test setup was the same, but the viral suspension was diluted 1:2 with sterile water. After infection of the cell monolayer with the treated diluted suspension, viral titer was determined.

4. Determination of Reactive Oxygen Species Obtained by Low-Temperature Nonequilibrium Gas-Discharge Plasma

a. Plasma Diagnostics Using Optical Emission Spectroscopy

We used optical emission spectroscopy (OES) to identify reactive oxygen and reactive nitrogen species (RONS) present in the plasma column. Although we used pure argon plasma, such particles are still produced at the plasma-water interface during plasma-liquid interaction. Due to the axial inhomogeneity of this type of plasma source, we measured axial profiles of the intensity up to the contact point between the plasma and the water surface (Fig. 1).

b. Tracking Peroxide Radicals Obtained by Low-Temperature Nonequilibrium Gas-Discharge Plasma

The amount of hydrogen peroxide was tested with the participation of lucigenin-register reactive oxygen species (ROS). The signal was initially measured when H_2O_2 (0.001 mL; 50 mM) and culture medium of 0.05 mL was present in the lucigenin system (lucigenin + H_2O_2 + culture medium; see the Materials and Methods section above). After 1 min, we measured the output signal. This signal serves as a kind of control for the amount of peroxide radicals. In the next experiment, we added plasma-activated sterile water at 0.001 mL (treated at appropriate intervals of 0–60 s) to the lucigenin system, instead of H_2O_2 . Lucigenin was present in the system, and nutrient medium was in the same volume of 0.05 mL and water. After 1 min, we again measured the received signal. Treatment time intervals of water were 0–60 s, with plasma at powers of 13 and 20 W.

III. RESULTS AND DISCUSSION

A. CE of Plasma-Activated Nutrient Medium

The values that we obtained after studying the CE of plasma-activated nutrient medium are presented in Table 1; we determined there to be a CE of 50 (CE_{50}). Only two of the experimental formulations produced results that measured < 50% cell monolayer survival, maintaining the cells with 0.2 mL treated medium, 20 W plasma source power, and 0.2 mL treated medium at 15 W.

B. Effect of Plasma-Treated Nutrient Medium on HSV-1 Replication

The MTT test determined the survival of virus-infected cells and the addition of medium that was treated with low-temperature nonequilibrium gas-discharge plasma. The results obtained are summarized in Table 2.

In none of the experimental formulations did a significant amount of protection occur on the cell monolayer. The highest amount of protection was achieved with plasma at a power of 13 W after removing nonabsorbing virus and adding nonremovable treated media at 0.2 mL.

TABLE 1: CEs of low-temperature nonequilibrium gas-discharge plasma

Experimental production	CE_{50} (s)
20 W; 0.1 mL treated medium + 0.1 mL untreated medium	n.d.
20 W; 0.2 mL treated medium	232
15 W; 0.2 mL treated medium	258
13 W; 0.2 mL treated medium	n.d.

CE_{50} , cytotoxic effect of 50; n.d., no data.

TABLE 2: Protection of infected cells using different experimental settings

Experimental conditions	Cell protection (%)	CE ₅₀ (s)
13 W; 0.2 mL treated medium withdrawn after 5 min and replaced with untreated medium. Medium added after virus adsorption	33, 63	n.d.
13 W; 0.2 mL treated medium withdrawn after 20 min and replaced with untreated medium. Medium added after virus adsorption	30, 60	n.d.
13 W; 0.2 mL treated medium without removal. Medium added after virus adsorption	50, 24	n.d.
13 W; 0.1 mL treated medium without removal of nonadsorbing virus and medium. Medium added after virus adsorption	37, 95	n.d.
13 W, 0.1 mL treated medium + 0.1 mL viral suspension added simultaneously	n.d.	n.d.

CE₅₀, cytotoxic effect of 50; n.d., no data.

C. Effect of Plasma-Activated Sterile Water on Extracellular Virions

The virus used was titrated and the experiments were performed with $10^{-6.5}$ CCID₅₀. For the experiments, we prepared two- and three-time dilutions of the viral suspension in water (with final volumes of 3 mL), in accordance with the working protocols described in the Materials and Methods section above. Dilutions thus obtained were treated by direct contact of the last 2–3 mm of the torch with the liquid surface at a power of 13 W for time intervals of 60, 120, 240, and 300 s.

Results obtained from our experiments are presented in the tables. The change in virus titer at 1:1 dilution of the viral sample with distilled water is presented in Table 3. From the data, we can see that after treatment of the viral suspension diluted with dH₂O at a ratio of 1:1, plasma at a power of 13 W, and exposure time of 300 s, viral titer decreased by 1.23 log₁₀.

Data show that the first results are established after 120 s of treatment at a dilution of 1:2, reducing the viral titer by 0.5 log₁₀. During plasma treatment for 300 s, viral titer decreased by 1.67 log₁₀. These results are presented in Table 4.

TABLE 3: Change in virus titer at 1:1 dilution of viral sample with distilled water

Time intervals (s)	Virus titer in control	Virus titer in sample	Δlog
60	6.23	6.23	0
120	6.23	6.23	0
240	6.23	5.75	0.48
300	6.23	5.00	1.23

TABLE 4: Change in virus titer at 1:2 dilution of viral sample with distilled water

Time intervals (s)	Virus titer in control	Virus titer in sample	$\Delta\log$
60	6.00	6.00	0
120	6.00	5.50	0.50
240	6.00	5.23	0.77
300	6.00	4.33	1.67

D. Determination of ROS Obtained by Low-Temperature Nonequilibrium Gas-Discharge Plasma

From the emission spectra, we can conclude that RONS are present in this plasma source, and OH has very high intensity on the plasma-water contact point due to H_2O dissociation (Fig. 2).

Distance between the Surfatron launcher and the distilled water sample was fixed at 13.5 mm, and the quartz tube was 1 mm out of the launcher, so by increasing applied power, which also increased the length of the plasma torch, we augmented the contact area between the plasma and the water surface. From OES, the following results (presented in Fig. 3) were obtained: OH intensity increased at the contact point of the plasma-water surface and at the applied power for the given conditions (Fig. 3[a]). Previous studies with this plasma device have shown that for the same set of applied powers in air, no significant change in electron temperature occurred,^{9,10} so this effect must only be due to the increase in contact area. Atomic oxygen does not seem to be influenced by applied power and stays relatively constant for the given conditions (Fig. 3[b]). Intensity of NO- γ also increased toward the contact point and at the applied power (Fig. 3[c]).

FIG. 2: Optical emission spectrum of the plasma-water interface. SPS, second positive system.

FIG. 3: OH intensity as a function of power: (a) atomic oxygen intensity as a function of power (b) and NO- γ intensity as a function of applied power (c). The zero point denotes the end of the plasma column.

The amount of hydrogen peroxide was tested with the participation of lucigenin registers. When applying the test setup (lucigenin system), as described in the Materials and Methods section, the start signal was measured immediately after the H_2O_2 (0.001 mL; 50 mM) and nutrient media were present in the system (lucigenin system + H_2O_2 + nutrient media). After 1 min, we measured the output signal, and the results are presented in Table 5.

Our results indicate that the test nutrient media had a strong quenching effect on peroxide radicals, with a stronger effect at 4% DMEM. The amount of peroxide radicals significantly decreased after 1 min. A possible reason for this strong quenching activity may be the rich component composition of each of the nutrient media. The presence of vitamins, minerals, and antioxidants especially in the medium containing calf serum is probably the reason for the high quenching effect and the reduction of peroxide radicals

TABLE 5: Comparison of chemiluminescent signal of DMEM and 4% DMEM in presence of H_2O_2 at 20 W

Experimental production (lucigenin, media, H_2O_2)	Start signal (mV)	Output signal (mV)
DMEM	1700	80
4% DMEM	1700	20

DMEM, Dulbecco's modified Eagle's medium.

TABLE 6: Effect of dH₂O on chemiluminescent signal

Exposure time (s)	Received signal at 13 W (mV)	Received signal at 20 W (mV)
m	50	50
5	208	298
10	110	164
20	90	120
30	90	113
40	87	110
60	85	105

in the media. Most likely, the lack of a significant effect on HSV-1 replication was also due to the strong quenching effect of the nutrient medium on the resulting radicals, also due to the rich component composition and the presence of vitamins and antioxidants in the environment used.

We determined the effect of low-temperature nonequilibrium gas-discharge plasma on the amount of peroxide radicals in the treatment of distilled water. The received signal was measured after 1 min at 13 and 20 W. Results, presented in Table 6, show generation of peroxide radicals in the treated sterile water. The received signal was weaker than the start signal obtained in the experimental setup, in which the lucigenin system, H₂O₂, and nutrient media were present, but it was stronger than the output signal (measured after 1 min) obtained in the presence of lucigenin, H₂O₂, and the culture medium (Table 5). This confirms the results obtained by other authors for generation of peroxide radicals in plasma-treated water, despite differences in plasma source.¹⁸ This is probably due to the stronger virucidal effect on dilution of the viral suspension at a ratio of 1:2 with sterile dH₂O.

IV. CONCLUSIONS

When studying the CEs of low-temperature nonequilibrium gas-discharge plasma, only two experimental formulations achieved cell monolayer survival of < 50%, maintaining cells with 0.2 mL of treated medium, by plasma at wave power of 20 W, and at 0.2 mL of treated medium at a power of 15 W. These values were reached, respectively, at 232 and 258 s of treatment.

In the study of the effect of plasma on HSV-1 replication, none of the applied experimental assays showed a significant amount of protection on the cell monolayer. The highest percentage of protection was achieved by treatment with plasma at a wave power of 13 W and successive treatment (after virus adsorption) without removal of medium.

In the study of the effect of plasma-activated sterile water on extracellular virions, we found that when treating a viral suspension diluted with sterile water in a 1:2 ratio, a significant decrease in viral titer occurred, with 1.67 log₁₀ compared to the viral control. Experimental data on the presence of the virucidal effect of plasma-treated water

correlate with data published by other authors using different types of plasma sources, gases for plasma, and types of viruses.^{5–7,12,19–22}

From the emission spectra, we can conclude that RONS are present in this plasma source, and OH is very high in intensity on the plasma-water contact point due to H₂O dissociation. From OES, the following results were obtained: OH intensity increases at the contact point of the plasma-water surface and increases with applied power for given conditions. Atomic oxygen does not seem to be influenced by applied power, staying relatively constant for the given conditions. Intensity of NO-γ also increases toward the contact point and with applied power. This confirms the results obtained by other authors for generation of radicals in plasma-treated water, despite differences in plasma source.¹⁹

A strong quenching effect of DMEM medium and DMEM medium with 4% FBS was detected by examining the amount of peroxide radicals obtained by treatment with low-temperature nonequilibrium gas-discharge plasma in the presence of H₂O₂ and using an experimental setup including lucigenin.

In monitoring the effect of plasma treated at appropriate intervals, sterile dH₂O on the chemiluminescent signal after application of a test setup in the presence of lucigenin and nutrient media and in the absence of hydrogen peroxide, we found that the presence of peroxide radicals occurred in greater quantities than those obtained when lucigenin, medium, and hydrogen peroxide were present in the system.

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