Nonthermal Dielectric Barrier Discharge (DBD) Plasma Suppresses Herpes Simplex Virus Type 1 (HSV-1) Replication in Corneal Epithelium

Oleg Alekseev1*, Kelly Donovan1*, Vladimir Limonnik1, and Jane Azizkhan-Clifford1

1 Department of Biochemistry and Molecular Biology, Drexel University College of Medicine, Philadelphia, PA

Correspondence: Jane Azizkhan-Clifford, Department of Biochemistry and Molecular Biology, Drexel University College of Medicine, Philadelphia, PA 19102, USA. e-mail: jane.clifford@drexelmed.edu

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Introduction

Herpes keratitis (HK) is the most common cause of cornea-derived blindness and infection-associated blindness in the developed world. While acyclovir and its derivatives have been very successful in HK patients, this condition still presents a clinical problem, with an annual incidence of roughly 11.8 per 100,000 people and a total prevalence of over 500,000 in the United States alone.1–3 In addition, acyclovir-resistant strains of herpes simplex virus (HSV) are beginning to emerge, further emphasizing the need for new antiviral lines of defense in combating HK.4–10 Almost all of the current antiviral drugs target the same viral enzyme (HSV-1 DNA polymerase), which severely limits options for combination therapy and allows for the development of drug resistance. For these reasons, investigations into novel nonpharmacologic methods of suppressing HSV-1 infection in the cornea may be of therapeutic interest.

In this study, we have examined the antiviral properties of liquids treated with nonthermal dielectric barrier discharge (DBD) plasma. “Plasma” is a term used to refer to a partially ionized gas. Plasmas are normally generated in nature or in the laboratory by subjecting a gas to high voltage, which strips electrons from atoms to create ions.11 Man-made plasmas can be generally categorized into thermal and nonthermal. In thermal plasmas, the energized...
electrons are in equilibrium with the heavy particles, leading to energy transfer and rapid heating of the gas. Nonthermal plasmas, on the other hand, do not facilitate such equilibrium, which leaves the gas at ambient temperature. Due to this feature, nonthermal plasmas have recently attracted considerable attention in the biomedical field. Dielectric barrier discharge plasma is a type of nonthermal plasma that was originally introduced by Werner von Siemens in 1857. The medical applications of DBD plasma have been studied in the context of wound healing, blood coagulation, cancer therapy, surface sterilization, and many more. Specifically in the cornea, DBD plasma has been explored for its antibacterial, antifungal, and wound healing properties. Interestingly, however, the antiviral potential of DBD plasma remains largely unexplored.

In light of the few and contradictory reports, we set out to characterize the antiviral effects of DBD plasma specifically in the context of HSV-1 keratitis. We had previously described a system for “separated” DBD plasma treatment, in which an aqueous solution, such as cell culture medium, is first exposed to DBD plasma and then applied to cells. This is in contrast to the “direct” treatment, where DBD plasma is generated directly on the surface of cultured cells. In our experiments, plasma-treated growth medium was applied to cultured monolayers of human corneal epithelial cells and to intact ex vivo human corneas. This method offers several advantages as compared to the direct DBD plasma treatment of the cornea or the use of flow plasma, another common system for producing plasma. Generation of DBD plasma directly on the tissue strictly depends on the exposed surface being regular and parallel to the electrode. Since human corneas range in curvature and size and are known to have surface irregularities, direct exposure to the electrode would produce damaging arcs (localized and concentrated plasma streamers) instead of a uniform discharge. In addition, plasmas generate ozone and contain a minor ultraviolet (UV) component, both of which could be damaging to the cornea in the case of a direct treatment. Finally, the use of DBD plasma–treated liquids allows for a greater degree of consistency than either direct DBD plasma or flow plasma treatment, since treated liquids can be generated in a standardized and well-controlled manner and administered as eye drops consistent with standard clinical practice.

In this report, we demonstrate the antiviral potential of DBD plasma–treated liquids in the context of HK. Application of plasma-activated growth medium to HSV-1-infected corneal epithelial cells and ex vivo human corneas caused a significant suppression of infection. Importantly, the antiviral effect was produced in the absence of pronounced corneal toxicity or damage. We propose that the use of DBD plasma–treated liquids, which act through a mechanism different from that of the available antivirals, could be a useful addition to the current HK drug armamentarium. Further studies of the molecular mechanisms underlying this phenotype will allow for optimized use of DBD plasma in herpetic corneal disease.

**Methods**

**Cells and Viruses**

All cells were cultured at 37°C and 5% CO₂, and supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. Human corneal epithelial cells immortalized with hTERT (hTCEpi; a kind gift from Stephen Jennings at Drexel University College of Medicine) were used throughout, except for the plaque expansion experiment, which was performed with KOS-GFP strain (a kind gift from Nigel Fraser at University of Pennsylvania). All viral stocks were titered on CV-1 monolayers.

**Cell Culture Model**

Subconfluent monolayers of hTCEpi cells were grown in six-well plates. Infections with KOS strain of HSV-1 were carried out at multiplicity of infection (MOI) 0.1 in a 200-μL inoculum volume at 37°C for 1 hour with intermittent rocking.
layers were then exposed to DBD plasma–treated medium (as described below) and overlaid with fresh KGM-2 for the remainder of experiment. At 16 hours postinfection, phase-contrast images were taken; cells were collected for isolation of DNA, RNA, or protein; and culture medium was collected for plaque assays.

For plaque expansion experiments, hTCEpi monolayers were infected with KOS-GFP strain of HSV-1, which constitutively expresses green fluorescent protein (GFP) from a cytomegalovirus immediate early promoter. Infections were carried out at very low MOI to ensure that the viral plaques would be sufficiently sparse. Following exposure to DBD plasma–treated medium, cells were overlaid with fresh KGM-2 containing 1.25% wt/vol methocellulose. Infectious plaques were then allowed to develop and were imaged by fluorescence microscopy.

**Corneal Explant Model**

Human corneas were obtained from the Lions Eye Bank of Delaware Valley. Experimentation using human corneas was approved by the Drexel University College of Medicine Institutional Review Board and adhered to the tenets of the Declaration of Helsinki. Protocol established by Alekseev et al. for ex vivo corneal culture, infection, and treatment was followed closely. Briefly, corneoscleral buttons were rinsed in PBS containing 200 U/mL penicillin and 200 μg/mL streptomycin. The endothelial concavity was filled with culture medium containing 1% low melting temperature agarose. The corneas were cultured epithelial side up in KGM-2 medium supplemented with 200 U/mL penicillin and 200 μg/mL streptomycin. The next day, they were infected with 1×10⁴ plaque forming units (PFU)/cornea of strain KOS HSV-1 for 1 hour, exposed to DBD plasma–treated medium (as described below), and cultured for 24 hours. The epithelial cell layer was collected by scraping to isolate DNA for quantitative PCR (qPCR) analysis, and the culture medium was collected for viral titer quantification by plaque assay.

**Generation of DBD Plasma in Atmospheric Air**

To initiate uniform DBD in atmospheric air, we used a nanosecond-pulsed power system. The power supply (FID GmbH, Burbach, Germany) generates pulses with +15.5-kV pulse amplitude in a 50 Ω coaxial cable (31 kV on the high-voltage electrode due to pulse reflection), 10-ns pulse duration (90% amplitude), 2-ns rise time, and 3-ns fall time. Power measurements were performed using a high-voltage probe (P6015A, 75-MHz bandwidth; Tektronix, Beaverton, OR) and Pearson current monitor (model 6585, usable time 1 ns, 1 GHz bandwidth; Palo Alto, CA) connected to a 1-GHz oscilloscope (DPO4104B; Tektronix). Because the probe’s frequency response limits its ability to accurately detect the pulse shape, voltage was measured using back current shunts (BCS). For this purpose, pulses were delivered to the electrodes via 30 m of RG 393/U high-voltage coaxial cable, and BCS was mounted 6.7 m from the output of the power supply. The shunt comprised 10 carbon-composition 3 Ω resistors (OF30GJE-ND; DigiKey, Thief River Falls, MN) soldered into a gap within the shield of the cable. Amplitude calibration of the BSC was performed using a high-voltage probe. In order to account for the displacement current, which was later subtracted from the total current of the discharge, measurements were first done with a large electrode gap when the electric field in the gap was not sufficient to generate a discharge and therefore only displacement current could be measured. These measurements were also confirmed by a well-established technique for estimation of energy deposition based on comparison of the first incident and reflected voltage pulses in a long cable. These measurements estimate the resulting DBD pulse energy at 45 ± 4 mJ. Liquid treatment experiments were performed at a frequency of 1 kHz, corresponding to the total plasma discharge power of 1.88 ± 0.2 W/cm².

Dielectric barrier discharge optical emission spectrum was obtained using a fiber optic bundle (10 fibers, 200-μm core) connected to a spectrometer system (TriVista TR555) with a digital intensified charge-coupled device (ICCD) camera (PI-MAX), all purchased from Princeton Instruments (Trenton, NJ). The rotational temperature of nitrogen, which represents the gas temperature, was determined by fitting a synthetic spectrum to the experimental spectrum of the (0–2) transition emission bands of the N₂(3Π_g–B 3Π_u) transition (second positive system) in the range 360 to 381 nm, using the Specair 3.0 program (SpectralFit S.A.S., Antony, France). The measured rotational temperature of nitrogen was 343 ± 6 K.

**Nonthermal DBD Plasma Treatment of Culture Medium**

Dielectric barrier discharge plasma–treated liquid was generated by exposing 1 mL complete KGM-2
medium in a glass holder (Fig. 1B) to DBD plasma, as shown in the photograph (Fig. 1A). The potency of plasma-treated liquid was adjusted by varying the duration of treatment time (0–180 seconds). Once treated, the liquid was applied to cells or corneas (400 µL for cells, 800 µL for corneas) in six-well plates at exactly 1 hour after the start of HSV-1 infection. Cells were incubated with the DBD plasma–treated medium for 1 minute and corneas for 5 minutes before fresh KGM-2 (2 mL for cells, 6 mL for corneas) was added to each well to dilute the DBD plasma–treated medium. After 1 hour, cells or corneas were rinsed and overlaid with fresh KGM-2 for the remainder of the experiment (Fig. 1C).

**Corneal Toxicity Assessment**

Explanted human corneas not infected with HSV-1 were exposed to DBD plasma–treated medium in the same manner as described above and were subsequently cultured for 24 hours. For histology studies, corneas were fixed in 3% paraformaldehyde/2% sucrose solution, paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E). For assessment of epithelial toxicity, corneas were briefly stained with fluorescein (1% wt/vol in PBS), and epithelial defects were imaged with 464-nm-wavelength blue light (LDP LLC, Carlstadt, NJ).
Genotoxic Toxicity Assessment

Explanted human corneas were exposed to hydrogen peroxide (200 μM), UV light (20 J/m²), DBD plasma–treated KGM-2 (120 seconds), or mock treatment. The corneas were then incubated in fresh KGM-2 for 2 hours and flash-frozen in optimal cutting temperature (OCT) compound. Frozen tissue blocks were sectioned at 5-μm thickness, fixed, and processed for indirect immunofluorescence. Detection of cyclobutane pyrimidine dimers with the TDM-2 primary antibody (a kind gift from Toshio Mori at Nara Medical University, Japan) was performed according to a previously published protocol.29 Oxidative damage to nucleic acids was assessed by staining with the 8-OHdG primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), which detects 8-hydroxy-2′-deoxyguanosine, 8-hydroxyguanine, and 8-hydroxyguanosine. Standard immunofluorescence protocol was followed. Nuclei were counterstained with H¨ochst 33,258.

Viral Genome Replication and Transcription

Viral genome replication and transcription were measured by qPCR. Total DNA and RNA from infected cells were isolated using the DNeasy Blood & Tissue Kit and the RNeasy Mini Kit, respectively (Qiagen, Hilden, Germany). RNA was converted to cDNA using qScript (Quanta BioSciences, Gaithersburg, MD). Real-time qPCR was performed with SYBR Green (Bio-Rad, Hercules, CA). Target primers for UL30 (DNA polymerase catalytic subunit) and reference primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used to measure genome replication. Transcription of the three gene families was measured with primers for RL2 (ICP0), UL30 (DNA polymerase catalytic subunit), and UL44 (gC), with reference primers for the 18S rRNA (Table). All primer sequences have been previously published.40

Western Blot

Standard protocol was followed for Western blot analysis. Cell lysates were collected in 200 μL Laemmli buffer, vortexed, and boiled at 95°C for 5 minutes. Protein concentrations were measured by bicinchoninic acid assay. SDS–PAGE was followed by transfer onto a polyvinylidene fluoride membrane, which was then blocked in 5% BSA. Blots were stained with primary antibodies against glycoprotein C (rabbit polyclonal; a kind gift from Roselyn Eisenberg at University of Pennsylvania) and nucleolin (mouse monoclonal; Santa Cruz Biotechnology). Blots were stained with secondary antibodies and visualized with the Odyssey near-infrared system (LI-COR, Lincoln, NE).

Statistical Analysis

Statistical significance was determined using Student’s t-test and is indicated by ns (P > 0.05), * (P < 0.05), ** (P < 0.01), or *** (P < 0.001).

Results

DBD Plasma–Treated Medium Suppresses HSV-1 Infection in Human Corneal Epithelial Cells

In order to obtain a general characterization of the effect of DBD plasma on HSV-1 infection, we conducted experiments using hTCEpi corneal epithe-
**Figure 2.** DBD plasma-treated medium suppresses the cytopathic effect of HSV-1 in human corneal epithelial cells. hTCEpi cells were infected at MOI 0.1 and exposed to KGM-2 medium treated with DBD plasma for 0 to 40 seconds. Control cells were neither infected nor treated. Phase-contrast images were taken at 16 hours postinfection. Microphotographs are representative of at least three independent experiments.

**Figure 3.** DBD plasma–treated medium limits the expansion of HSV-1 plaques in human corneal epithelial cell monolayers. hTCEpi monolayers were infected with KOS-GFP strain of HSV-1 at a low MOI, exposed to KGM-2 medium treated with DBD plasma for 0 to 40 seconds, and overlaid with methocellulose-containing medium to allow for plaque development. Plaques were visualized by fluorescence microscopy. A representative plaque from each treatment group is shown. Bar: 400 μm. n = 2.
Monolayers of hTCEpi cells were infected with HSV-1 at low MOI (0.1) to simulate physiologically relevant viral titers. KGM-2 growth medium was treated with DBD plasma for 0 to 40 seconds and then applied to the infected cells as described in Methods (Fig. 1). This range of treatment times was chosen based on our previous studies of biological effects of DBD plasma (data not shown). The cytopathic effect produced by HSV-1 infection was suppressed by DBD plasma–treated medium in a dose-dependent manner, with the maximal antiviral activity achieved at 35 to 40 seconds of DBD plasma treatment (Fig. 2). To gain better understanding of this antiviral effect, we sought to monitor the spread of HSV-1 infection within the hTCEpi monolayers. To this end, we infected confluent hTCEpi cells with KOS-GFP strain of virus, which constitutively expresses GFP allowing for easy visual detection of infected cells. The monolayers were overlaid with methocellulose-containing medium, limiting viral infection to direct spread. Examination of the infectious plaques by fluorescence microscopy revealed that DBD plasma greatly limited HSV-1 plaque expansion (Fig. 3).

To provide a quantitative evaluation of the antiviral effect of DBD plasma, we used a qPCR assay for the measurement of viral genome replication. hTCEpi monolayers that had been exposed to DBD plasma–treated medium contained significantly lower HSV-1 genome copies than control monolayers. The inhibition of genome replication was greater than 90% at the 40-second treatment intensity (Fig. 4A). Culture media from the same monolayers were analyzed by plaque assay, revealing a concomitant inhibition of infectious viral particle production, which reached 150-fold reduction at the 40-second

Figure 4. DBD plasma–treated medium reduces viral replication in human corneal epithelial cells. hTCEpi cells were infected at MOI 0.1 and exposed to KGM-2 medium treated with DBD plasma for 0 to 40 seconds. (A) Total DNA was collected at 16 hours postinfection for analysis by qPCR with primers against HSV-1 polymerase and GAPDH. Bars represent relative $\Delta \Delta C(t)$ values ± SEM. (B) Supernatants were collected at 16 hours postinfection for analysis by plaque assay. A representative experiment is shown. $n = 3$ for all.
treatment intensity (Fig. 4B). Consistent with the initial assessment of the cytopathic effect (Fig. 2), the maximal effect on both the genome replication and the viral titers was achieved at the 35- to 40-second treatment intensity.

The inhibition of genome replication caused a subsequent reduction in the accumulation of viral gene products. Levels of viral transcripts from all three kinetic families—immediate early, early, and late—were reduced, as measured by qRT-PCR with primers against RL2 (ICP0), UL30 (DNA polymerase catalytic subunit), and UL44 (glycoprotein C) (Fig. 5A). There was a consistent reduction in the accumulation of glycoprotein C protein product as detected by Western blot (Fig. 5B). Interestingly, the decrease of glycoprotein C protein levels was more pronounced than the decrease of its mRNA transcript, which could point to an unexplored translational effect of DBD plasma.

Taken together, our experiments in the corneal tissue culture model reveal a potent antiviral effect of DBD plasma. The 35- to 40-second treatment intensity resulted in pronounced reduction of the cytopathic effect, infectious plaque expansion, viral genome replication, production of infectious progeny, and accumulation of viral gene products.
In order to extend our experiments to a more physiologically relevant model of corneal HSV-1 infection, we followed the method of Alekseev et al.\textsuperscript{36} for ex vivo corneal culture, infection, and treatment (inset in Fig. 6B). Intact human corneoscleral buttons were infected with HSV-1 and exposed to DBD plasma–treated medium similarly to the in vitro experiments. Due to the inherent differences between cell monolayers and explanted corneas, a new dose–response curve was generated (data not shown); based on the ex vivo dose response, 120 seconds was chosen as the optimal treatment intensity to be used in subsequent experiments. A set of 16 donor-matched human corneas was infected and exposed to medium treated with DBD plasma or mock (DBD plasma power source turned off). We achieved a substantial (over 80%) reduction in HSV-1 genome replication in treated corneas compared to matched mock-treated controls (Fig. 6A). This decrease was accompanied by a similar reduction of the viral load in the culture medium (Fig. 6B). Thus, our initial in vitro findings (Fig. 4) are supported by our ex vivo experiments in intact human corneas.

**DBD Plasma–Treated Medium Exhibits Low Toxicity in Explanted Human Corneas**

Brun et al.\textsuperscript{22} have performed comprehensive and extensive toxicity studies, demonstrating a lack of pronounced or lasting detrimental effects of nonthermal plasma to the human cornea. However, since the method of plasma treatment utilized in the present study is different from the helium-flow plasma used by Brun et al., additional toxicity assessment was...
necessary. Explanted human corneas were exposed to DBD plasma–treated medium (120 seconds) and subsequently cultured under conditions identical to those in virus-inhibition experiments (Fig. 6). At 24 hours posttreatment, the integrity of corneal epithelium was assessed by fluorescein staining, with surgically de-epithelialized corneas serving as a positive staining control. \( n = 2 \). (B) Histologic assessment of toxicity was performed by examining H&E-stained tissue sections from 12 matched corneas obtained from 6 donors. Representative images from a matched pair are shown \( (n = 6) \).

Plasmas have been shown to produce reactive oxygen and nitrogen species,\(^{11,12}\) as well as a minor amount of UV energy.\(^{31}\) These entities are known to be damaging to cells and can be particularly deleterious to the nucleic acids (especially DNA) by catalyzing mutagenic structural changes. In particular, UV exposure promotes the formation of aberrant structures known as cyclobutane pyrimidine dimers (CPDs),\(^{41,42}\) and oxidation of nucleic acids can promote inappropriate nucleotide substitutions in the genome.\(^{43}\) For this reason, we examined the potential toxic effects of DBD plasma–treated medium on the nucleic acids of corneal epithelial cells. Explanted human corneas were exposed to DBD...
plasma–treated medium, mock-treated medium, or damaging agents for positive control (UV and H2O2). Not surprisingly, DBD plasma–treated medium did not induce the formation of CPDs, as detected by staining with an antibody specific to 8-OHdG, a common marker of oxidative damage (Fig. 8B).

Taken together, these experiments demonstrate that DBD plasma–treated medium suppresses HSV-1 infection in explanted human corneas without producing appreciable toxicity, as monitored by fluorescein staining, histologic assessment, and detection of genotoxic damage.

Discussion

The use of nonthermal plasmas in biomedical applications holds significant promise and has generated much interest in recent years. In the field of ophthalmic research, nonthermal plasmas have been investigated for potential use in wound healing and bacterial and fungal sterilization, and are already in use for ocular surgery (yttrium aluminium garnet laser and Fugo plasma blade). The work presented here contributes to these investigations by demonstrating the antiviral potential of DBD plasma in the treatment of HSV-1 corneal infection. The advantage of plasma technology is its high degree of versatility and adaptability. Nonthermal plasmas can be generated at a wide range of energy settings, in various customized gaseous media, and using a growing variety of electrodes. This multitude of parameters involved in plasma generation allows for fine-tuning of the nature, quality, and intensity of the produced plasma in order to fit a specific biomedical need. Dielectric barrier discharge plasma electrodes can be manufactured in different shapes and sizes, and the use of microelectrodes holds great potential for novel methods of targeted intervention within precise anatomical locations on the ocular surface as well as in the internal structures of the eye.

The mechanisms underlying the biological activities of nonthermal plasmas remain to be described and are likely multifactorial and highly dependent on plasma type and delivery method. The chemical species generated using our DBD plasma source are currently under investigation, but our prior studies point to the possible reactive oxygen species–mediated production of stable amino acid hydroperoxides in the treated medium. However, the DBD plasma source used in those investigations operates at substantially different frequencies from the one employed in the present work, making such extrapolations speculative. Our preliminary measurements performed using Fourier transform infrared absorption spectroscopy (D. Dobrynin, unpublished data)
2013) show that DBD plasma in atmospheric gas phase generates ozone \((1.7 \times 10^{17} \text{ cm}^{-3})\), \(\text{H}_2\text{O}_2\) \((4.2 \times 10^{17} \text{ cm}^{-3})\), and \(\text{N}_2\text{O}\) groups \((2 \times 10^{15} \text{ cm}^{-3})\), whereas the main component in liquid is \(\text{H}_2\text{O}_2\), with generation rate of approximately \(4 \mu\text{M/J}\). However, it is currently unclear what minor species may be generated from the various organic components present in the treated cell culture medium.

A significant obstacle presented by the current state of the field is the absence of a reliable reference system for the standardization and measurement of plasma. While the electrical power delivered to the insulated electrode can be easily quantified, it does not directly translate to a comparable universal measurement of the energy actually received by the treated tissue. Various biological parameters have been proposed as surrogate markers of treatment intensity (e.g., pH, reactive oxygen/nitrogen species, lipid peroxidation, and DNA damage), but they tend to be inconsistent and limited strictly to their biological meaning. Thus, the development of a convention for the measurement of biological activity of plasmas is necessary for comparison and reproducibility of data across plasma source devices, disease models, and laboratories, and would facilitate the elucidation of the cellular mechanisms affected by plasma.

The use of DBD plasma–treated liquids may provide useful therapeutic advantages for the treatment of corneal herpetic infections. Since this is a nonpharmacologic agent with a mechanism of action unrelated to the inhibition of HSV-1 DNA polymerase, it may serve as a unique option for patients with drug-resistant infection. It could also be used in combination therapy with established antiviral agents. The common target of the majority of current antitherpetic medications allows for the development of multidrug resistance. This is a growing concern in the immunocompromised population, where suppression of infection relies entirely on therapeutic intervention. Thus, the addition of DBD plasma to the accepted drug armamentarium in these patients may counteract the development of resistance. In addition, recent interest in the use of plasmas for the enhancement of wound healing, including corneal ulceration, could point to a possible 2-fold effect of DBD plasma whereby the reduction of viral load is accompanied by expedited resolution of the epithelial ulcer.

An intriguing concept that has not been addressed in this study is that DBD plasma may have analogous antiviral activity against other members of the herpesviridae family, which includes such prominent ocular pathogens as varicella zoster virus, herpes simplex virus type 2, Epstein-Barr virus, and cytomegalovirus.

In summary, this work provides seminal evidence for the antiviral effect of DBD plasma–treated medium. Human corneal epithelial cells and explanted corneas treated in this study experienced greatly reduced levels of HSV-1 infection without developing overt toxicity. The results of this study warrant necessary further investigations to characterize the underlying molecular mechanisms, optimize treatment parameters, detail the toxicity profile of DBD plasma, and advance these investigations into in vivo models of herpes keratitis.

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* Oleg Alekseev and Kelly Donovan contributed equally to this work.

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