Title
COVID-19 ocular prophylaxis: the potential role of Ozonated-oils in liposome eyedrop gel
New Approaches for Personalized Medicine

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SARS-CoV-2 transmission through the ocular surface cannot be neglected. A barrier to prevent virus infection is thus needed. In vitro ozonated-oil in liposome eyedrop gel may prevent SARS-CoV-2 infection at 72 h after two applications daily.
Abstract

**Purpose:** To assess whether ozonated-oil in liposome eyedrop gel (OED) could be used to prevent the severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) infection in an *in vitro* infection model.

**Design:** Prospective, qualitative comparison of *in vitro* tissue.

**Participants:** First, we tested the efficacy of OED on *in vitro* cell regeneration and dry eye resolution in human corneal epithelial cells (hCE-2). Second, we assessed the *in vitro* anti-SARS-CoV-2 infection efficacy of OED using Vero E6 cells.

**Methods:** hCE-2 and Vero E6 cells were treated with two drops of OED or phosphate-buffered saline solution as the control. Tissues were examined to assess different parameters: morphology, histology, and mRNA expression at 24 h post-treatment. All experiments were performed in triplicates.

**Main Outcome Measures:** The main outcome was to assess the prophylactic potential of OED to reduce *in vitro* cell infection with SARS-CoV-2. The secondary outcome was to evaluate the repair and regeneration effect of OED on conjunctival microvilli defects such as in dry eye disease.

**Results:** OED could restore 50% of the scratch in the monolayer of hCE-2 cells *in vitro* compared with the 25% obtained with PBS. At 24 h post-treatment with OED, the number of microvilli and the mucin network were restored, as observed using scanning electron microscopy. In Vero E6 cells infected with a primary SARS-CoV-2 strain and treated with OED two times/day, viral replication was found to be inhibited, with a 70-fold reduction observed at 72 h post-infection compared with that under the untreated and PBS-treated conditions.

**Conclusions:** SARS-CoV-2 transmission through the ocular surface should not be ignored. Although the prevalence of coronavirus disease 2019 conjunctivitis infection is low, the need for a barrier to prevent possible viral infection is warranted. *In vitro* OED treatment may prevent the risk of SARS-CoV-2 infection after 72 h of two daily applications.
**Key words:** Ozonated-Oil in liposome Eyedrop Gel; Innovative Biotechnologies; Personalized Medicine; Prophylaxis Agent; SARS-CoV-2.

**Abbreviations/Acronyms:**

*COVID-19*: Coronavirus disease 2019

*DED*: Dry eye disease

*OED*: Ozonated-Oil in liposome Eyedrops Gel (Ozodrop® Gel)

*SARS-CoV-2*: Severe acute respiratory syndrome- coronavirus 2
Introduction

Owing to the global severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) pandemic, scientists are making numerous attempts to design new therapeutic strategies to fight invisible invaders of the human body. Several resources have thus been invested in the defense against infection and the search for an effective vaccine. However, one of the most powerful weapons is prevention, which is almost impossible with SARS-CoV-2. Recent studies have focused on viral interactions between viral glycoproteins and human host receptors to better understand the mechanism of virus entry into cells.\(^1\) One possible interference of a virus’ access to cells is ozone (O\(_3\)).\(^2\) O\(_3\) gas is a molecule consisting of three oxygen atoms in a dynamically unstable structure due to the presence of mesomeric states.\(^3\)

The potential effect of ozone is related to the control of inflammation, stimulation of immunity, and antiviral activity, suggesting a new methodology for immune therapy.\(^4\) Owing to its remarkable oxidative power, O\(_3\) has been recognized as one of the best antimicrobial agents.\(^5,6\) O\(_3\) is used medically to disinfect and treat infectious diseases and inactivate bacteria, viruses, fungi, yeast, and protozoa. The efficacy of O\(_3\) in enveloping viruses might be related to its ability to damage and enclose the viral capsid, thereby upsetting the reproductive cycle of the virus with peroxidation. Medications of a gaseous nature are somewhat unusual, and ozone in the form of O\(_3\) gas is extremely reactive and is not always suitable as a topical treatment. For this reason, special applications have been developed. Interestingly, despite its instability, the ozone molecule can be stabilized for topical use by creating ozonide through a reaction between ozone and the double bonds of a monounsaturated fatty acid, such as oleic acid,\(^7\) and the derived compounds are known as “ozonated oils” (Figure 1).
Figure 1. (A) Three oxygen atoms cause the ozone (O$_3$) molecule to be highly unstable. (B) The ozone molecule can only be stabilized via enveloping in micellar lipid solution called “ozonated oil”. (C) The lipid portion of the micelle binds to the lipid portion of the virus membrane, which releases O$_3$ when the viral capsid is disrupted.

Ozonated oils have the same properties and activities as gaseous ozone and are well tolerated by biological tissues. Further, their biological activities are related to oxygenated compounds.$^{8,9}$ In particular, ozonated oils are new products for the treatment of ocular pain and inflammation that occur during events, such as external ocular infections and inflammation, due to the related risk of blindness.

Ozonated oil can eliminate pathogens by direct oxidation mediated by hydrogen peroxide, lipoperoxidation, and selective cytotoxicity on fast-dividing cells. These actions occur through bacterial lysis and cell death, negative regulation of mitochondrial activity in bacteria, and disturbance of viral lithic enzymes in superimposable manners as compared to the action of phagocytic cells of the immune system.$^{10}$

Ozone allows “physiological” wound healing, which minimizes the risk of keloidal scarring and haze in the cornea. According to Marchegiani et al., liposomal ozone-dispersion is as effective as povidone iodine for reducing bacterial load on the ocular surface.$^{11}$ As a result, this nanoformulation has
recently been developed for ophthalmic use, and its potential antiseptic prophylactic effects against SARS-CoV-2 have been described by Mazzotta et al.\textsuperscript{12}

Concerning the ocular surface, the presence of small damage, such as in dry eye disease (DED), induces high vulnerability to microorganism penetration and infection.\textsuperscript{13} However, restoring the ocular surface may further prevent the entry of pathogens into cells.

Because the integrity of cells and the occurrence of coronavirus disease 2019 (COVID-19) are closely interconnected, we decided to divide the present study into two phases. In the first phase, we tested the \textit{in vitro} efficacy of ozonated-oil in liposome eyedrop gel (OED) on human corneal epithelial cells (hCE-2) affected with DED. In the second phase, we assessed the \textit{in vitro} anti-COVID-19 infection efficacy of OED in Vero E6 cells (these are cells isolated from kidney epithelial cells extracted from an African green monkey). Two different cell lines were assessed herein because of the limited proliferation of SARS-CoV-2 in hCE-2 cells compared with that in Vero E6 cells, which are the optimal cells for SARS-CoV-2 replication. The identification of Vero E6 cells as the optimum substratum for intracellular SARS-CoV-2 infection was recently demonstrated via ultrastructural analysis.\textsuperscript{14}

Based on such knowledge, the aim of the present study was to assess the prophylactic effect of OED against \textit{in vitro} cell infection with SARS-CoV-2. The secondary aim was to evaluate the repair and regeneration effect of OED on conjunctival microvilli defects, such as in DED.

**Materials and Methods**

The study was approved by the Catholic University/Fondazione Policlinico A. Gemelli IRCCS Institutional Ethics Committee (Protocol number: 0013008/20. ID: 3045). All authors reviewed the manuscript and declare the accuracy and completeness of the data and adherence to the study protocol.
The study was divided into two phases. In the first phase, we determined the *in vitro* efficacy of OED on hCE-2 with DED. In the second phase, we assessed the *in vitro* efficacy of OED in Vero E6 cells for the COVID-19 infection. All experiments were performed in triplicates.

**Phase one: *in vitro* efficacy of OED on hCE-2 cells**

- **Scratch test**

  The scratch test is generally applied to cells *in vitro* to assess the regeneration capability of damaged cells after the use of a particular substance (in our case). The hCE-2 cell line (ATCC CRL-11135) was cultured in keratinocyte serum-free medium (Gibco 17005-042) supplemented with 0.05 mg/ml bovine pituitary extract (BPE), 5 ng/mL epidermal growth factor (EGF), 500 ng/mL hydrocortisone, and 0.005 mg/mL insulin (Gibco). The cells were cultured in a culture-insert 2 well (Ibidi GmbH) to obtain a scratch. After 24 h, the inserts were removed, and two drops of OED (Ozodrop-GEL®; FBVision) were added to the test sample. Images of the wounded areas were captured before the addition of the OED and after 24 h of treatment. The control group received 1x phosphate-buffered saline (PBS; Gibco). The wound area was assessed at 0 h and 24 h using Adobe Photoshop. The treated samples were compared to the controls.

- **Experimentally induced *in vitro* dry eye in human corneal tissues**

  Corneal tissues from “Eye Biobank: Fondazione Banca degli Occhi del Veneto” were placed under controlled environmental conditions (<40% relative humidity, 40 ± 5 °C temperature, and 5% CO₂) for 24 h to mimic dryness. The corneal tissues were then treated with two drops/day of OED. A similar culture was treated with two drops/day of PBS to serve as the control. Tissues were investigated to assess different parameters: *morphology, histology, and mRNA expression* of selected genes at 24 h post-treatment (**Table 1**).
**Table 1.** Corneal tissue analysis after 24 h of OED application two times/day. Morphology, histology, and mRNA expression were investigated.

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Corneal tissues were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 h at 4 °C. After the samples were washed three times for 5 min with 0.1 M phosphate buffer, they were placed in 1% OsO₄ in 0.1 M phosphate buffer. Samples were dehydrated using a graded series of ethanol and a graded series of hexamethyldisilane. Specimens were mounted on aluminum stubs with silver-conducting paint, Quorum Q 150RS, and observed under the scanning electron microscope, Zeiss Evo 40.

At the end of OED treatment, corneal tissues were fixed in 10% formalin solution (HT501128). After embedding in paraffin, vertical sections (4-μm thick) were cut with a microtome and stained with hematoxylin and eosin (H&E) following internal procedures. Histological samples were analyzed under a light microscope, and the overall morphology of the epithelium and its modifications were compared to those of the control samples.

mRNA was extracted from the corneal tissues using the RNeasy Mini Kit (Qiagen), according to the manufacturer’s protocol. Subsequently, the cDNAs were synthesized using the SuperScript kit (ThermoFisher). Real time polymerase chain reaction was performed in triplicate in a final reaction volume of 25 μL using the ABI PRISM 7500 Real Time PCR System (Applied Biosystems) with TaqMan® assay (Ambion-Applied Biosystems). The cDNA was amplified using TaqMan Universal PCR Master Mix and TaqMan gene expression assay provided as a 20x Assay mix (Human MMP9: TaqMan probe MMP9 Hs00234579_m1; Human IL-8: TaqMan probe IL-8 Hs00174103_m1; Human GAPDH as the calibrator gene: Taqman probe GAPDH Hs99999905_m1). The PCR conditions were 95 °C for 10 min (AmpliTaq Gold DNA Polymerase activation) followed by 40 amplification cycles (95 °C for 15 s, then 60 °C for 1 min). The relative gene expression was calculated using the 2 (-Delta C[T]) method.

**Phase 2: in vitro anti-SARS-CoV-2 infection efficacy of OED in Vero E6 cells**

**SARS-CoV-2 infection**

Vero E6 cells (ATCC CRL-1586) were grown as described previously. SARS-CoV-2 was isolated from a nasopharyngeal swab retrieved from a patient with COVID-19. The identity of the strain was verified in Vero E6 cells using real-time polymerase chain reaction (PCR) and metagenomic sequencing, from which the reads were mapped to nCoV-2019 (genomic data are available at EBI under study accession no. PRJEB38101). We propagated the clinical isolate in Vero E6 cells and determined the viral titer using a standard plaque assay. The infection experiments were carried out in a biosafety level-3 (BLS-3) laboratory at multiplicity of infections (MOIs) of 0.05 and 1.0. Vero E6 cells were seeded at a density of 5 x 10⁴ cells/well in a 24-well plate and infected for 1 h with the SARS-CoV-2 isolate at an MOI of 0.05. The infection was carried out in Dulbecco’s modified Eagle’s medium without fetal bovine serum (FBS). After virus removal and washing with warm PBS, the
cells were cultured in medium containing 2% FBS in the presence or absence of two drops of OED or PBS as the control. At 24, 48, and 72 h post-infection, both the cells and supernatants were collected for further viral genome quantification analysis.

**Viral RNA extraction and quantitative reverse transcription-PCR**

RNA was extracted from clarified cell culture supernatants (16,000 × g for 10 min) and infected cells using a QIAamp Viral RNA Mini Kit and RNeasy Plus mini kit (Qiagen), respectively, according to the manufacturer’s instructions. RNA was eluted in 30 μL of RNase-free water and stored at −80 °C until use. Quantitative reverse transcription PCR (qRT-PCR) was carried out following previously described procedures with minor modifications. Briefly, reverse transcription and amplification of the S gene were performed using the one-step QuantiFast SYBR Green RT-PCR mix (Qiagen) and the following cycling profile: 50 °C for 10 min, 95 °C for 5 min; 95 °C for 10 s, 60 °C for 30 s (40 cycles) (primers: RBD-qF1: 5′-CAATGGTTTAACAGGCACAGG-3′ and RBD-qR1: 5′-CTCAAGTGTCTGTGGATCACG-3′). A standard curve was generated by determining the copy numbers derived from serial dilutions (10^3–10^9 copies) of the pGEM T-easy vector (Promega) containing the receptor-binding domain of the S gene (primers: RBD-F: 5′-GCTGGATCCCCCTAATATTACAAAACCTTGCC-3′; RBD-R: 5′-TGCCCTCGAGCTCAAGTGTCTGTGGATCAC-3′). Each quantification was performed in triplicate.

**Immunofluorescence analysis**

The expression of angiotensin-converting enzyme 2 (ACE2) and the SARS-CoV-2 nucleoprotein was analyzed using immunofluorescence with the anti-SARS-CoV-2 nucleocapsid protein (NB100-56576; Novus Biologicals, Centennial, 1:250 dilution) and anti-ACE2 (clone EPR4435-2; Abcam, 1:250 dilution) antibodies.
Statistical analysis

Statistical comparisons of the qRT–PCR data of the control and treatment groups were performed using the Student’s t-test as the data displayed a normal distribution based on the Kolmogorov–Smirnov test. Differences were considered significant at $p < 0.05$. Statistical analyses were performed using GraphPad Prism version 8.

Results

Effects of OED on hCE-2 cell growth

First, we assayed the effects of different concentrations of OED on the proliferation of hCE-2 cells, an established model system for the scratch test. After 72 h of culture, 2–6 drops of OED were found to have a negligible effect on the extent of growth (viability: $98 \pm 2\%$), which was assayed as the viable cell number detected using the trypan blue dye exclusion test. However, 8-10 drops were identified to be slightly cytotoxic (viability: $80 \pm 5\%$) (Figure 2a). As a result, we proceeded to assess the effect of 2 drops of the OED.

Scratch test

hCE-2 cells were cultured in a monolayer, and a scratch was obtained with culture-insert 2 well as previously reported. As shown in Figure 2b, the OED could restore $50 \pm 5\%$ of the scratch in the cell monolayer compared with the $25 \pm 3\%$ obtained after PBS treatment (Figure 2c, $p < 0.001$; Student’s t-test).
Figure 2 a) Effect of different doses of ozonated-oil liposome eyedrop gel (OED) on the proliferation of hCE-2 cells after 72 h of culture. From 2 to 6 drops of OED/day, a negligible effect on the extent of growth was observed. However, 8 to 10 drops of OED/day resulted in cytotoxic effects. b, c) Scratch assay: representative results in the hCE-2 cell line after 24 h of treatment with OED and phosphate-buffered saline (PBS).

Dry eye assay

The corneal tissues were tested for their ability to restore the physiological status of DED. The corneal tissues were maintained at 43 °C for 24 h to simulate a dry eye condition. Thereafter, the tissues were treated with one drop of the gel every 10 h for 24 h. The corneal tissues were then harvested and used in SEM analysis, H&E staining, and real-time PCR to detect the inflammatory marker (interleukin [IL]-8) and matrix metallopeptidase-9 (MMP-9).18,19

Figure 3 shows the overall morphology of the control corneal tissue, control dry eye, and the dry eye treated with OED for 24 h, as observed under a light microscope. The tissue morphology of the control was preserved (Figure 3a, b, c), with a flattened layer of non-keratinized superficial cells, an intermediate cell layer, and cells displaying lateral cytoplasmic extensions similar to wing cells. Further, the basal layer of the regular column cuboidal cells was clearly visible.
Figure 3. Histo-morphological analysis of a, b, c) control group corneal tissues; d, e, f) control dry eye, and f, g, h) dry eye corneal tissues treated with ozonated-oil liposome eyedrop gel. Magnifications: 4x, 10x, and 20x.

A remarkable reduction in the thickness of the epithelium was observed in the dry eye condition compared with that in the control (Figure 3 d, e, f). Notably, the epithelium in the corneal tissues (experimentally induced dry eye in vitro) was viable, as assessed by the expression of the housekeeping gene using qRT-PCR (Figure 5a). The reduction in thickness appears to be related to the loss of water due to the severe dry experimental conditions. Therefore, we concluded that low humidity and high temperature could reproduce the dry environmental conditions that cause ocular discomfort and inflammation. Treatment with OED restored the basal condition, ultimately preserving the corneal cells from dehydration (Figure 3 g, h, i).
Microvilli analysis using SEM

SEM analysis revealed that the control tissue was rich in microvilli and mucin networks (Figure 4a, b). A significant reduction in the number of microvilli and mucin networks was observed following the induction of the dry eye condition (Figure 4c, d). At 24 h post-treatment with OED, the number of microvilli and the mucin network were restored (Figure 4e, f).

**Figure 4.** Scanning electron microscopy images of human corneal microvilli and mucin network in a, b) control; c, d) dry eye; and e, f) ozonated-oil liposome eyedrop gel-treated dry eye condition. Different magnifications are reported.
Quantification of MMP9 and IL-8 mRNAs

We tested duplicate corneal tissue samples in the gene expression studies. Transcriptional analysis using qRT–PCR confirmed the viability of the cells in all culture conditions. Further, the housekeeping gene, GAPDH, was found to be equally expressed in all experimental settings (Figure 5a). However, the expression of the inflammatory cytokine, IL-8, was increased by 2-fold at 24 h following the induction of the dry eye condition (p < 0.001; Student’s t-test) (Figure 5a). Treatment with OED restored the basal levels of IL-8. Further, a 9-fold overexpression from the basal level of MMP-9 was observed at 24 h following dry eye induction (Figure 5b). However, treatment with OED restored the basal levels of MMP-9.

Figure 5. Quantitative reverse transcription polymerase chain reaction results of a) GAPDH, b) IL-8, and c) MMP9 levels in corneal tissues under normal conditions (37 °C) and dry eye condition (Dry eyes) without (phosphate-buffered saline [PBS-treated]) or with ozonated-oil liposome eyedrop gel (OED) treatment (OED treated). The results are expressed as the mean of triplicate experiments after 24 h of treatment.

Effects of OED on Vero E6 cell growth
First, we assayed the effect of different concentrations of the OED gel on the proliferation of Vero E6 cells, an established model system for SARS-CoV-2 replication. After 72 h of culture, 2 to 6 drops of OED were found to have a negligible effect on the extent of growth, which was assayed as the viable cell number detected using the trypan blue dye exclusion test (viability: 94 ± 3%). However, 8 to 10 drops resulted in cytotoxicity (viability: 81 ± 5%) (Figure 6a). As a result, we proceeded with the use of two drops of OED.

**OED strongly inhibits SARS-CoV-2 replication**

Vero E6 cells were either infected at a low MOI (0.05) or high MOI (1.0). Quantification of the released RNA after 48 h indicated that viral production (100,000 ± 1,200 copies/mL) was very similar at both MOIs (Figure 6b). Therefore, in the subsequent experiments, an MOI of 0.05 was employed. We proceeded to determine whether OED could affect the replication of SARS-CoV-2. Briefly, Vero E6 cells were infected with a primary SARS-CoV-2 strain isolated in Brescia, Italy. After 1 h, the cells were cultured in the absence or presence of OED, with 2 drops administered every 10 h, as recommended for the product. OED efficiently inhibited viral replication (Figure 6c). In fact, OED abolished the SARS-CoV-2 cytolytic effects on Vero E6 cells, with only limited detectable cytopathic effects observed at 72 h post-infection; this finding may be due to cell senescence (Figure 6c). Quantification of viral RNA copy number in the cell culture supernatants confirmed the potent inhibitory effect of OED on viral particle production, with a reduction of nearly 4 logarithms at 72 h post-infection relative to that observed in untreated and PBS-treated infected cells (Figure 6d). qRT-PCR quantification of intracellular SARS-CoV-2 RNA in SARS-CoV-2-infected cells confirmed the inhibition of almost 4 logarithms of the intracellular SARS-CoV-2 genome expression in the presence of OED compared with that in the untreated and PBS-treated cells (Figure 6e).
Figure 6. **a**) Effect of different doses of ozonated-oil liposome eyedrop gel (OED) on the proliferation of Vero E6 cells after 72 h of culture. From 2 to 6 drops of OED/day, a negligible effect on the extent of growth was observed; however, from 8 to 10 drops of OED/day, a cytotoxic effect was indicated. **b**) Vero E6 cells were infected with SARS-CoV-2 at a multiplicity of infection (MOI) of 0.05 or 1.0 for 1 h at 37 °C. Thereafter, the cells were washed and cultured for 48 h. Viral yield was quantified in the cell supernatant using quantitative reverse-transcription PCR (qRT-PCR). At least three independent replicates were tested. Data are representative of three independent experiments. Vero E6 cells were infected with SARS-CoV-2 at an MOI of 0.05 in the absence or presence of OED. **c**) Images of cells were captured with an optical microscope to detect the typical SARS-CoV-2-induced cytolytic effects. **d**) Viral yield was quantified in the cell supernatant using qRT-PCR. At least three independent replicates were tested. Data are representative of three independent experiments (*p < 0.001). **e**) Quantitation of SARS-CoV-2 genome at the intracellular level using qRT-PCR. At least, three independent replicates were analyzed. Data are representative of three independent experiments. (*p< 0.001).
**SARS-CoV-2 replication was not assessed in hCE-2 cells**

To determine whether OED interfered with SARS-CoV-2 replication in corneal cells, we infected hCE-2 cells with the virus. Further, to confirm that hCE-2 cells presented the main SARS-CoV-2 receptor, ACE2, we performed an immunofluorescence assay. A slight expression of ACE2 was observed in hCE-2 cells (**Figure 7a**), supporting the possibility of SARS-CoV-2 infection. However, quantification of the released RNA after 24, 48, and 72 h of infection indicated that viral production was very low in hCE-2 cells (**Figure 7b**). The highest SARS-CoV-2 RNA expression was observed at 24 h, as revealed using immunofluorescence analysis, with low expression of the viral nucleoprotein (**Figure 7a**). These results suggest an unsuccessful SARS-CoV-2 infection in hCE-2 cells. We proceeded to determine the possible efficacy of OED on corneal cells within the 24 h post-infection. In the presence of OED, SARS-CoV-2 RNA expression was found to decrease at 24 h post-infection compared with that observed in untreated cells or cells treated with PBS. The low SARS-CoV-2 replication in hCE-2 cells was counterbalanced by the expression of inflammatory molecules. By assessing the expression levels of IL-8 and MMP9, we found that during the first 24 h of infection, an increased expression of IL-8 and MMP9 occurred in hCE-2 cells which was restored to basal level in the presence of OED (**Figure 7e, f**).
**Figure 7.** a) Immunofluorescence analysis of the expression of angiotensin-converting enzyme 2 (ACE2) (middle panel) and the SARS-CoV-2 nucleoprotein (right panel) on the surfaces of hCE-2 cells infected with SARS-CoV-2 at a multiplicity of infection (MOI) of 1.0 for 24 h. A Nikon Eclipse TE2000S system equipped with 621 was used as the digital camera; original magnification, 10×. b) hCE-2 cells were infected with SARS-CoV-2 at an MOI of 1.0 for 1 h at 37 °C. Thereafter, the cells were washed and cultured for 48 h. Viral yield was quantified using the cell supernatant with quantitative reverse-transcription PCR (qRT-PCR). At least three independent replicates were assessed. Data are representative of three independent experiments. c) Viral yield was quantified in the cell supernatant using qRT-PCR. At least three independent replicates were analyzed. Data are representative of three independent experiments. (*p < 0.001). qRT–PCR results for d) GAPDH, e) IL-8, and f) MMP9 in hCE-2 after 24 h of infection with SARS-CoV-2 at an MOI of 1.0 without (PBS-treated) or with OED (OED-treated). The results are expressed as the mean of triplicate experiments after 24 h of treatment (*p < 0.001).
Discussion

The effects of SARS-CoV-2 are becoming increasingly well-known and are causing the global population to panic. No-one seems to be spared from this virus, and the predisposing factors for a more aggressive infection are still unknown. As we await a vaccine that can reduce the spread of the virus, there are few defenses that can be deployed to reduce the risk of contagion.

The role of the ocular surface as a possible portal of entry, reservoir for replication, and site of transmission of SARS-CoV-2 RNA has been explored extensively. Recently, live virus has been identified in ocular fluids based on the cytopathic effects observed in Vero E6 cells. As contagion through the eye can occur, strategies to reduce the risk of virus entry should be evaluated. The ocular contagion risk increases in cases of tear film abnormalities, such as in DED. In these cases, the epithelial conjunctival microvilli have incipient epithelial damage, and the ocular surface is more vulnerable to infectious microorganisms.

Although new molecules are being explored for their potential defensive effects against SARS-CoV-2, we opted to focus on O₃. Ozone-mediated virus inactivation occurs primarily in two ways: lipid peroxidation and protein peroxidation. Herein, OED was found to efficiently inhibit viral replication at the post-entry stages of SARS-CoV-2 infection. The in vitro administration of OED may thus reduce the ability of SARS-CoV-2 to replicate relative to that observed in the untreated state. By creating a more "oxidized" environment on the ocular surface using ozone eye drops, viral infection could be alleviated. Topical OED administration might reduce the risk of SARS-CoV-2 infection through the ocular surface and could be an essential safeguarding procedure not only for healthcare professionals but also for the entire population. The potential prophylactic and antiviral effects of O₃ on SARS-CoV-2 infection could also be applied to cover the different entry routes that the virus could utilize to access the host, starting with the respiratory route.
The OED gel efficiently restored cell regeneration and controlled cell inflammation during the dry eye condition. Furthermore, the presence of the ACE2 receptors and TMPRSS2 protein on the corneal limbal stem cells may theoretically allow the betacoronavirus to cross the ocular surface and subsequently spread from the eye to other parts of the body through the blood stream or the nervous system (ophthalmic branch of trigeminal nerve).\textsuperscript{25} Although there is no current evidence to suggest that the COVID-19 virus, in humans, can enter the eye or spread to the brain through the corneal nerves, in some animal models (feline and murine), betacoronaviruses caused several ocular infections (e.g., conjunctivitis, uveitis, retinitis, and optic neuritis), thereby suggesting that they could penetrate the ocular globe in some mammals.\textsuperscript{26}

The anti-inflammatory potential of OED might be important in the control of SARS-CoV-2 inflammation, which is reported to be the basis of viral pathogenicity. SARS-CoV-2 conjunctivitis has been described as a mild follicular conjunctivitis, otherwise indistinguishable from other viral threats, that can be transmitted via aerosol contact with the conjunctiva.\textsuperscript{27} Other features of ocular surface involvement include unilateral or bilateral bulbar conjunctiva hyperemia alone or in association with chemosis, follicular reaction of the palpebral conjunctiva, watery discharge, epiphora, and mild eyelid edema. The prevalence of conjunctivitis in patients with COVID-19 remains controversial. Although only 0.9% of patients were found to develop signs of conjunctivitis,\textsuperscript{28} another report indicated that up to 31.6% of hospitalized patients had conjunctivitis.\textsuperscript{29} However, in the latter study, only about 5% of patients with positive findings for COVID-19 based on RT-PCR using nasopharyngeal swabs had a positive conjunctival swab. Moreover, only one of the 38 patients displayed conjunctivitis as their first symptom. Patients with ocular symptoms are more likely to have higher white blood cell and neutrophil counts, as well as higher levels of procalcitonin, C-reactive protein, and lactate dehydrogenase than patients without ocular symptoms. In one patient, the RT-PCR assay revealed the presence of viral RNA in the conjunctival specimen 13 days after disease onset. Further, the conjunctival swab specimens remained positive for SARS-CoV-2 at days 14 and 17 following onset. However, on day 19, the RT-PCR result was negative for SARS-CoV-2.\textsuperscript{18} In
another report, one hospitalized patient had SARS-CoV-2-positive conjunctival swabs up to day 21 from symptom onset; however, after a few days, the virus was undetectable in nasal swabs. Five days later, the virus was undetectable in the conjunctival swab but was detected on day 27, suggesting sustained replication of the virus in the conjunctiva.\textsuperscript{30}

Although the acquiring of COVID-19 infection through ocular transmission is remarkably concerning, its underlying mechanism has not been clarified.\textsuperscript{20} Similar to other betacoronaviruses,\textsuperscript{31(p2)} SARS-CoV-2 enters target cells via the binding of the viral Spike (S) protein to the specific cell-surface receptor, ACE2, which is then primed by the host cell transmembrane protease serine 2 (TMPRSS2).\textsuperscript{32} Although much remains to be learned about the factors involved in SARS-CoV-2 infection of human cells, ACE2 and TMPRSS2 are currently believed to represent the major players of its entry into cells.\textsuperscript{32,33} The presence of ACE2, which normally helps to regulate blood pressure, is widespread throughout the body, and the eye is not excluded.\textsuperscript{34,35(p2)} The human ocular globe has its own intraocular renin-angiotensin system, which is present not only on the surface of the eye (e.g., conjunctiva and cornea) but also inside the eye (trabecular meshwork, aqueous humor, iris, ciliary body, non-pigmented ciliary epithelium, and retina).\textsuperscript{36} Similarly, TMPRSS2 is highly expressed in various tissues, including the cornea limbal stem cells. As a result, both ACE2 and TMPRSS2 are co-expressed in the same cell (i.e., the cornea).\textsuperscript{37} To understand the “ocular route” from a clinical and molecular point of view, the following two main elements should be considered: the dynamism of the ocular surface system\textsuperscript{38} and the distribution of the ACE2 receptors and TMPRSS2 protein. First, the dynamism of the tear film is the factor supporting SARS-CoV-2 transport from the infected ocular surface to the respiratory and digestive tract through the lacrimal canaliculi (which drains tears from the eye surface into the nasal cavity), regardless of a more or less significant presence of the ACE2 receptors on the cornea and conjunctiva.\textsuperscript{39,40(p2)} Although the opposite passage of viruses from the nasal mucosa to the conjunctiva seems unlikely, it cannot be excluded. In addition, hematogenous infection of the lacrimal gland must also be considered. Evidently, lacrimal drainage from the conjunctival sac into the nasal cavity is not or is only partially
operative in individuals with dry eye. As a result, lacrimal substitutes are highly recommended for these individuals. However, the obstruction (complete or partial) of the lacrimal drainage pathways may play a role in the retention of the coronavirus on the ocular surface, regardless of its presence in the nasal cavity, thereby promoting periocular/face skin contamination by means of epiphora (i.e., excessive watering of the eye). Altogether, dry eye condition might be a risk factor for SARS-CoV-2 infection.

**Study Strengths and Limitations**

Our study has several limitations. Herein, in vitro systems were employed and no in vivo confirmation was carried out. Additionally, to overcome the difficulty encountered in assessing SARS-CoV-2 replication in the hCE-2 cell line model, infection of Vero E6 cells was conducted. The indirect effect of COVID-19 infection on the levels of inflammatory molecules was evaluated. Based on our result, both IL-8 and MMP9 were overexpressed. In fact, during the first 24 h of infection, their expression levels were increased in hCE-2 cells. In the future, we will test the effects of OED in vivo to better understand its preventive role.

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