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Antibiofilm and repair activity of ozonated oil in liposome

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Summary

The use of medical devices, such as contact lenses, represents a substantial risk of infection, as they can act as scaffolds for formation of microbial biofilms. Recently, the increasing emergency of antibiotic resistance has prompted the development of novel and effective antimicrobial drugs for biofilm treatment, such as oxidizing agents. The purpose of this study is to investigate the effects of Ozodrop® and Ozodrop® gel, commercial names of ozonated oil in liposomes plus hypromellose, on eradication and *de novo* formation of biofilms on different supports, such as plastic plates and contact lens. Our results demonstrate that ozonated liposomal sunflower oil plus hypromellose have an excellent inhibitory effect on bacterial viability and on both *de novo* formation and eradication of biofilms produced on plates and contact lens by *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Moreover, we show that Ozodrop® formulations stimulate expression of antimicrobial peptides and that Ozodrop® gel has a strong repair activity on human epithelial cells, suggesting further applications for the treatment of non-healing infected wounds.

Introduction

The use of contact lenses and the placement of intraocular lenses have enhanced the life quality of millions of patients with uncorrected refractive errors and cataracts. However, most infectious keratitis is associated with the use of soft contact lenses (Teo *et al*., 2011). Indeed, these devices represent a new surface on which many microbial pathogens can form biofilms (Bispo and Haas, 2015). Biofilms are communities of microorganisms attached to a substratum and enclosed in an extracellular polymeric matrix produced by themselves (Donlan and Costerton, 2002). They are involved in 80% of microbial infections affecting the human body, such as endocarditis, osteomyelitis, sinusitis, urinary tract infections, chronic prostatitis, periodontitis and ocular infections (Del Pozo and Patel, 2007; Veerachamy *et al*., 2014). Moreover, biofilms impact wound healing and can lead to the establishment of biofilm-based wound (Wu and Cheng, 2018). Antibiotics, the principal treatments used for bacterial infections, have often proven ineffective on biofilms, and considering the actual emergency of antibiotic resistance, new drugs are required to prevent bacterial colonization (Wolfmeier *et al*., 2018; Alter *et al*., 2019). New promising approaches for biofilm treatment are based on the use of oxidizing agents that can act against all microorganisms without the induction of antibiotic resistance. Ozone is the most powerful oxidizing agent and it is found in nature in the O_3 gas form (Travagli and Zanardi, 2009). Due to the high instability of ozone as a gas, ozonated derivatives resulting from the reaction of ozone with unsaturated substrates such as oil have found a number of applications (Valacchi and Fortino, 2005; Ugazio *et al*., 2020). Many studies have demonstrated the beneficial effects of ozonated oils in various infectious skin diseases (Zeng and Lu, 2018), including abscesses and tinia pedis (athlete's foot) (Bialoszewski *et al*., 2011; Zanardi *et al*., 2013; Ouf *et al*., 2016; Ozturk *et al*., 2017), wound healing (Kim *et al*., 2009; Valacchi *et al*., 2011; Patel *et al*., 2012; Lu *et al*., 2018), ulcer recovery (Solovəstru *et al*., 2015; Borges *et al*., 2017), psoriasis and palmoplantar pustulosis (Di Paolo and Gaggiotti, 2005; Bocci *et al*., 2015). In the ocular treatment, it is known that ozone has anti-inflammatory and bactericidal activity in some eye pathologies and promotes tissue repair (Spadea *et al*., 2018, 2021). However, since the topical application of ozonated oil could cause corneal tissue irritation (Ugazio *et al*., 2020), specific formulations based on ozonated liposomal sunflower oil plus hypromellose have been recently developed and commercialized in the form of eye drops (Ozodrop®; FB Vision, Ascoli Piceno, Italy), with reported antibacterial, antimycotic and antiviral

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properties (Re *et al*., 2008; Spadea *et al*., 2018; Cutarelli *et al*., 2019; Marchegiani *et al*., 2019; Cagini *et al*., 2020; Celenza *et al*., 2020; Rizzo *et al*., 2021). Recently, the tissue repair-promoting activity and the antimicrobial efficacy of Ozodrop® against *Staphylococcus aureus* and *Pseudomonas aeruginosa* were demonstrated (Cutarelli *et al*., 2019; Spadea *et al*., 2021), but its effect on microorganism communities that could infect ocular surface has not been tested so far. In the present study, we investigated the effect of gel and normal formulation of ozonated oil in liposomes plus hypromellose (Ozodrop® gel and Ozodrop®) on bacterial viability and on eradication and *de novo* formation of biofilms by the Gram-positive *S. aureus* and the Gram-negative *P. aeruginosa,* which are known to be involved in ocular keratitis (Urwin *et al*., 2020). We also analysed the repairing capacity of the Ozodrop® gel formulation *in vitro* on human immortalized keratinocytes and compared its efficacy in wound healing to Xanternet (SIFI SpA, Catania, Italy), an ophthalmic gel containing 0.15% sodium hyaluronate, 1% xanthan gum and 0.3% netilmicin, which is largely used after eye surgery or for the treatment of post-traumatic corneal abrasions (Faraldi *et al*., 2012; Kocatürk *et al*., 2015). Sodium hyaluronate and xanthan gum are high molecular weight polysaccharides that help maintain corneal hydration. Sodium hyaluronate also increases cell migration for rapid recovery of corneal epithelial lesions, while netilmicin is a third generation non-toxic aminoglycoside with antibacterial activity, which does not interfere with wound closure.

Overall, our report provides compelling evidence supporting the use of Ozodrop® formulations in the treatment of ocular infections associated with biofilms growing on contact lenses and paves the way for new applications of ozonated oil in liposomes plus hypromellose as an alternative treatment of epithelial lesions.

Results

Biofilm prevention of ozonated oil in liposome eye drops on contact lenses

We evaluated the capacity of Ozodrop® and Ozodrop® gel in preventing *P. aeruginosa* and *S. aureus* biofilm formation on daily disposable contact lenses composed of 31% nelfilcon A and 69% water. Ozodrop® and Ozodrop® gel were used at a final concentration of 20% and biofilm formation following 30 and 50 h of exposure to these two formulations was evaluated through a crystal violet assay. Biofilms formed by bacteria that did not undergo any treatment were used as controls. As shown in Fig. 1, contact lens treated with 20% of Ozodrop® or Ozodrop® gel display a notable reduction in the rate of both *P. aeruginosa* and *S. aureus* biofilm formation. The antibiofilm effect of Ozodrop® is more effective on *S. aureus* in comparison to *P. aeruginosa* (Fig. 1A-D). Moreover, MTT assays carried out in the same conditions confirmed that both Ozodrop® and Ozodrop® gel reduce bacterial viability in biofilms formed on contact lenses. In particular, we found a cell viability of $37.05 \pm 3.72\%$ and $29.51 \pm 0.85\%$ in *P. aeruginosa* and a cell viability of $18.57 \pm 1.43\%$ and $46.67 \pm 2.15\%$ in *S. aureus* treated with Ozodrop® and Ozodrop® gel respectively (Fig. 1E).

In addition, the antibacterial activity of 20% Ozodrop® and 20% Ozodrop® gel on both *P. aeruginosa* and *S. aureus* has been further confirmed by CFU assays (Fig. S1).

Biofilm eradication of ozonated oil in liposome eye drops on contact lenses

Next, we tested the biofilm eradication capacity of Ozodrop® and Ozodrop® gel mimicking the application of eye drops on the eye. The contact lenses were incubated with bacteria for 72 h at 37°C to allow biofilm attachment and growth. Then, we carried out three 10 min-treatments at 37°C, each spaced two hours apart, with Ozodrop® and Ozodrop® gel formulations, dissolved at 20% in Nutrient Broth. After each 10 min-application, contact lens were washed and re-incubated in Nutrient Broth. Biofilms formed by bacteria that undergo the treatment with only Nutrient Broth were used as controls. Crystal violet colorimetric analysis shows a significant reduction of both *P. aeruginosa and* of *S. aureus* biofilms deposited on contact lens treated with Ozodrop®($34.91 \pm 0.67\%$ and $44.64 \pm 1.26\%$ respectively) and Ozodrop® gel ($36.79 \pm 3.33\%$ and $32.14 \pm 1.26\%$ respectively) (Fig. 2 A). According to such evidences, MTT assay showed that Ozodrop® and Ozodrop® gel also reduce bacterial viability within *P. aeruginosa* biofilms respectively to $16.30 \pm 2.82\%$ and $8.69 \pm 9.41\%$ and within *S. aureus* biofilms to $35.29 \pm 3.82\%$ and $5.88 \pm 14.35\%$ respect to controls (Fig. 2B).

Biofilm prevention and eradication of ozonated oil in liposome eye drops on plastic plates

To confirm the antibiofilm and antibacterial activity of Ozodrop® and Ozodrop® gel, we tested the antimicrobial effect of these formulations on biofilms generated on alternative supports, such as plastic plates for tissue culture. Biofilm formation on tissue culture plates was significatively prevented by either 20% Ozodrop® or 20% Ozodrop® gel for both *P. aeruginosa* and *S. aureus* (Fig. 3A). Moreover, after three cycles of 10 min-applications of 20% Ozodrop® or 20% Ozodrop® gel, each followed by wash out, biofilms generated by both *P. aeruginosa* and *S. aureus* were consistently reduced (Fig. 3B), confirming the results obtained on contact

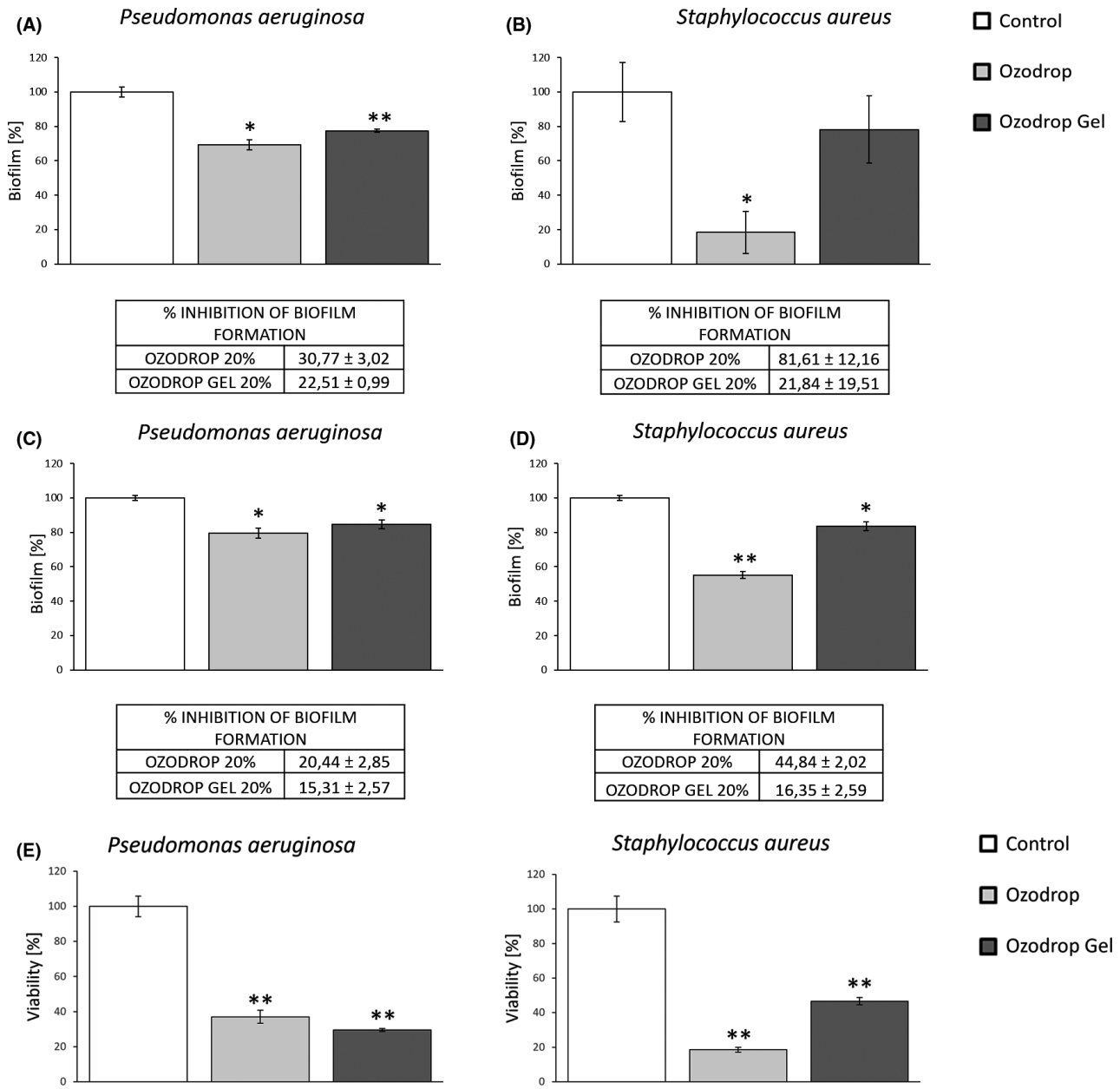


Fig. 1. *P. aeruginosa* and *S. aureus* antibiofilm activities of 20% Ozodrop® and 20% Ozodrop® gel at 30 h (A–B) and 50 h (C–D) on contact lens. Total biomass was quantified by crystal violet staining. To each value was subtracted the blank value (Nutrient Broth without bacteria). E. Bacterial viability measured by MTT assay on biofilms formed for 30 h on contact lenses by *P. aeruginosa* (left) and *S. aureus* (right). Data shown represent the mean ± SD ($n = 3$). Statistical analysis was performed with *t* test (*$P < 0.05$; **$P < 0.01$).

lenses. Interestingly, the biofilm inhibition effect exerted by formulations of Ozodrop® is higher on plates than on contact lenses, probably due to a background staining that is observed on lenses treated with crystal violets (Figs 1–3). The effect of Ozodrop® and Ozodrop® gel on bacterial viability in both biofilm prevention and eradication assay on plastic plates was also evaluated by MTT. Confirmingly, both formulations show a strong antibacterial activity in all conditions tested (Fig. 3C–D).

Ozonated oil in liposome eye drops stimulates expression of antimicrobial peptides

Next, we investigated whether Ozodrop® formulations could also stimulate an antibacterial response in human epithelial cells. Thus, we analysed the gene expression levels of typical antimicrobial agents produced by human immortalized keratinocytes HaCat upon bacterial infection, such as calprotectins (S100A8

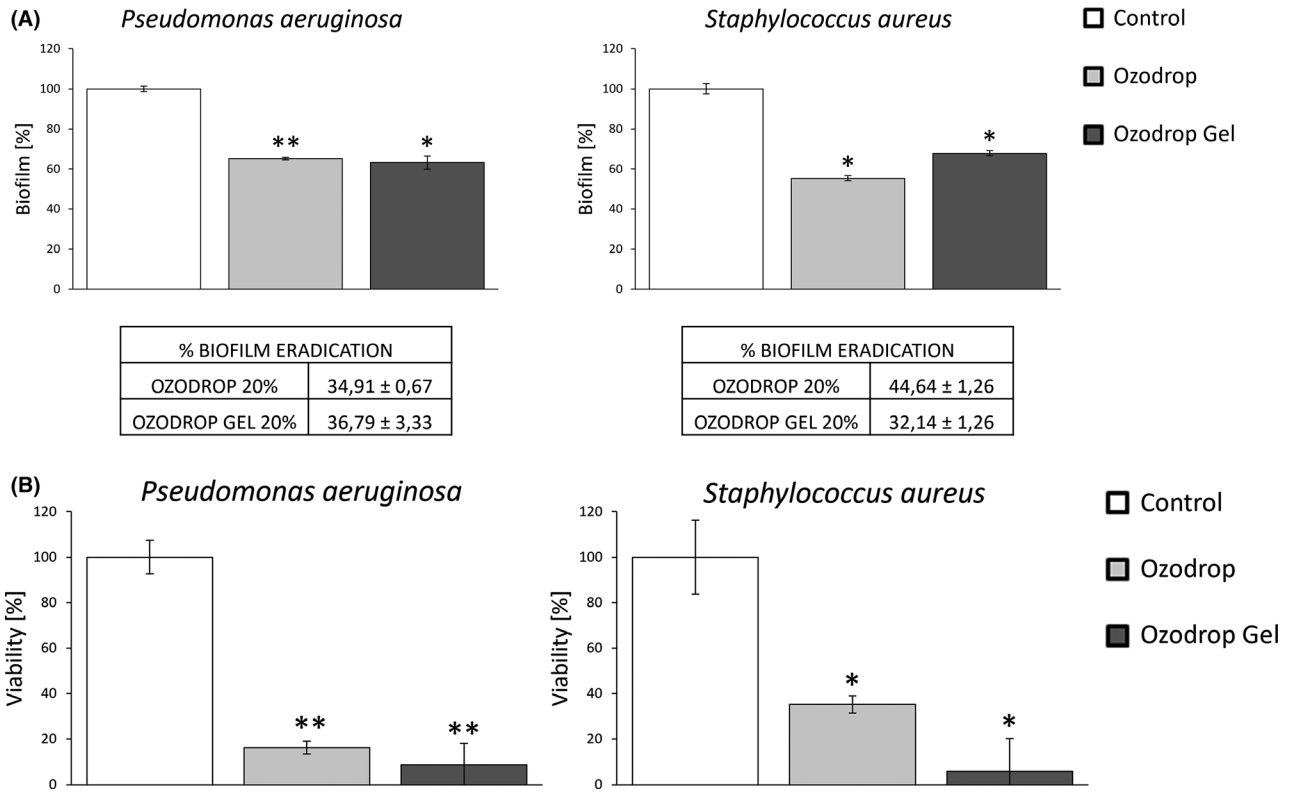


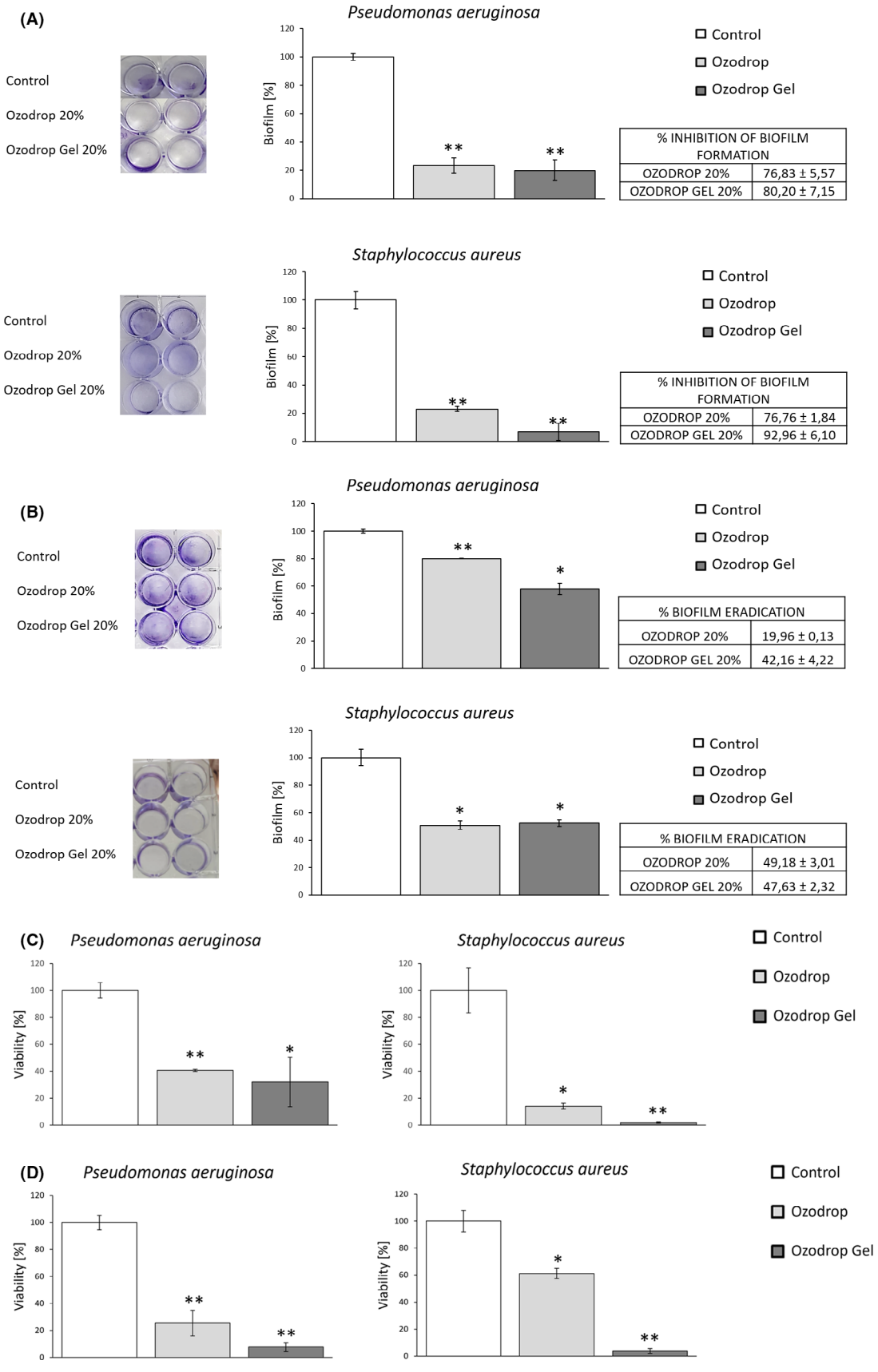
Fig. 2. A. *P. aeruginosa* and *S. aureus* eradication biofilm activities of 20% Ozodrop® and 20% Ozodrop® gel on contact lens. The total biomass was quantified using crystal violet staining. To each value was subtracted the blank value (Nutrient Broth without bacteria).
B. Bacterial viability measured by MTT assay on biofilms formed for 72 h on contact lenses by *P. aeruginosa* (left) and *S. aureus* (right). Data are presented as the mean ± SD ($n = 3$). Significance respect to control: $^{*}P < 0.05$, $^{**}P < 0.01$.

and S100A9) and calgranulin C (S100A12). The results of these experiments indicate that Ozodrop® alone is sufficient to induce antimicrobial peptides and such induction is notably enhanced for calgranulin C/S100A12 when cells are co-stimulated with the bacterial cell wall component lipopolysaccharide (LPS), which is specifically recognized by Toll-like Receptors 2/4 (Fig. 4A). On the other hand, Ozodrop® gel is more effective in up-regulating calprotectins S100A8 and S100A9 following exposure to LPS, suggesting that different Ozodrop® formulations may promote differentiated responses (Fig. 4A). On the other hand, both Ozodrop® and Ozodrop® gel have detrimental effect on inflammation, as observed by inhibition of the expression of LPS-induced pro-inflammatory chemokine CCL20 (Fig. 4B).

Repair activity of ozonated oil in liposome eye drops on HaCaT

Since biofilm eradication is critical for the effective treatment of chronic non-healing infected wounds, we evaluated the repair activity of ozonized oil in liposome eye drops. Thus, we analysed the expression levels of proliferation and migration markers in HaCat cells treated or not with Ozodrop® gel, Ozodrop® and Xanternet, a

Fig. 3. A. Prevention of *P. aeruginosa* and *S. aureus* biofilms by 20% Ozodrop® and 20% Ozodrop® gel after 30 hours on tissue culture plastic plates.
B. Eradication of *P. aeruginosa* and *S. aureus* biofilms generated for 72 h on tissue culture plastic plates by 20% Ozodrop® and 20% Ozodrop® gel. The total biomass in (A) and (B) was quantified using crystal violet staining, followed by absorbance measurement at 570 nm by spectrophotometer. The blank value (Nutrient Broth without bacteria) was subtracted to each value. Data are presented as the mean ± SD ($n = 2$) and are representative of three independent experiments performed in duplicates.
C. Bacterial viability measured by MTT assay in the prevention experiment on biofilms formed for 30 h on plastic plates by *P. aeruginosa* (left) and *S. aureus* (right).
D. Bacterial viability measured by MTT assay in the eradication experiment on biofilms formed for 72 h on plastic plates by *P. aeruginosa* (left) and *S. aureus* (right). Data are presented as the mean ± SD ($n = 3$). Significance respect to control: $^{*}P < 0.05$, $^{**}P < 0.01$.



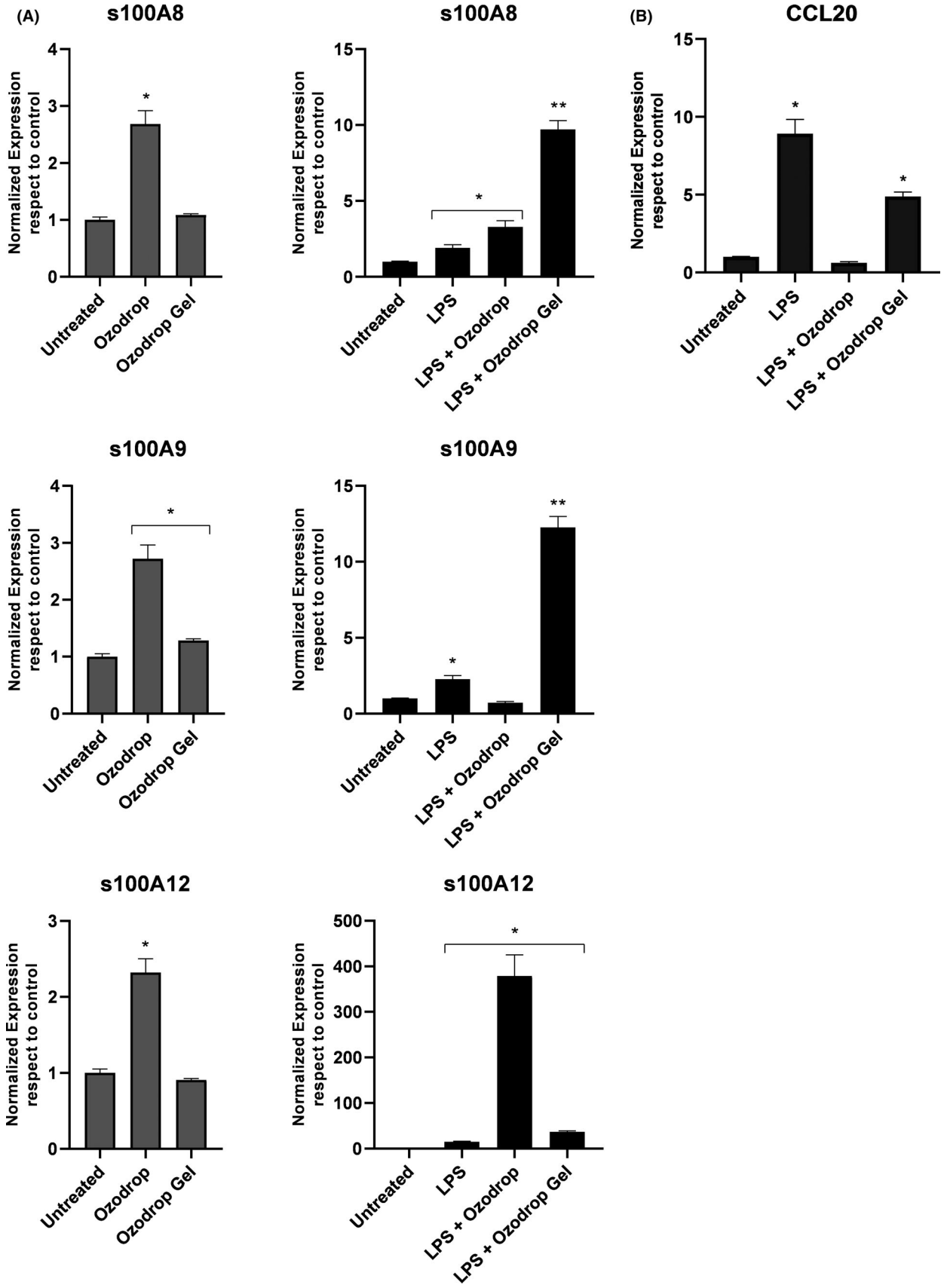

(A)
s100A8
Normalized Expression respect to control
Untreated
Ozodrop
Ozodrop Gel
s100A8
LPS
LPS + Ozodrop
LPS + Ozodrop Gel
(B)
CCL20
s100A9
s100A12

Fig. 4. A. Antimicrobial peptide expression in HaCat cells upon Ozodrop® and Ozodrop® gel treatment, with or without 100 ng ml^{-1} of LPS for 24 h.
B. CCL20 gene expression in HaCat cells upon Ozodrop® and Ozodrop® gel treatment with 24 h of 100 ng ml^{-1} LPS stimulation. Data are representative of three independent experiments performed in duplicates. Statistical significance was assessed with unpaired *t* test: $^{*}P \leq 0.05$; $^{**}P \leq 0.005$.

hyaluronic acid-containing eye drop formulation, which is known to promote tissue repair. RT-qPCR experiments show a slight up-regulation of CyclinD1 and MMP9, but not of MMP2 gene expression in HaCat cells treated with Ozodrop®, when compared with either control or Xanternet-treated cells (Fig. 5A).

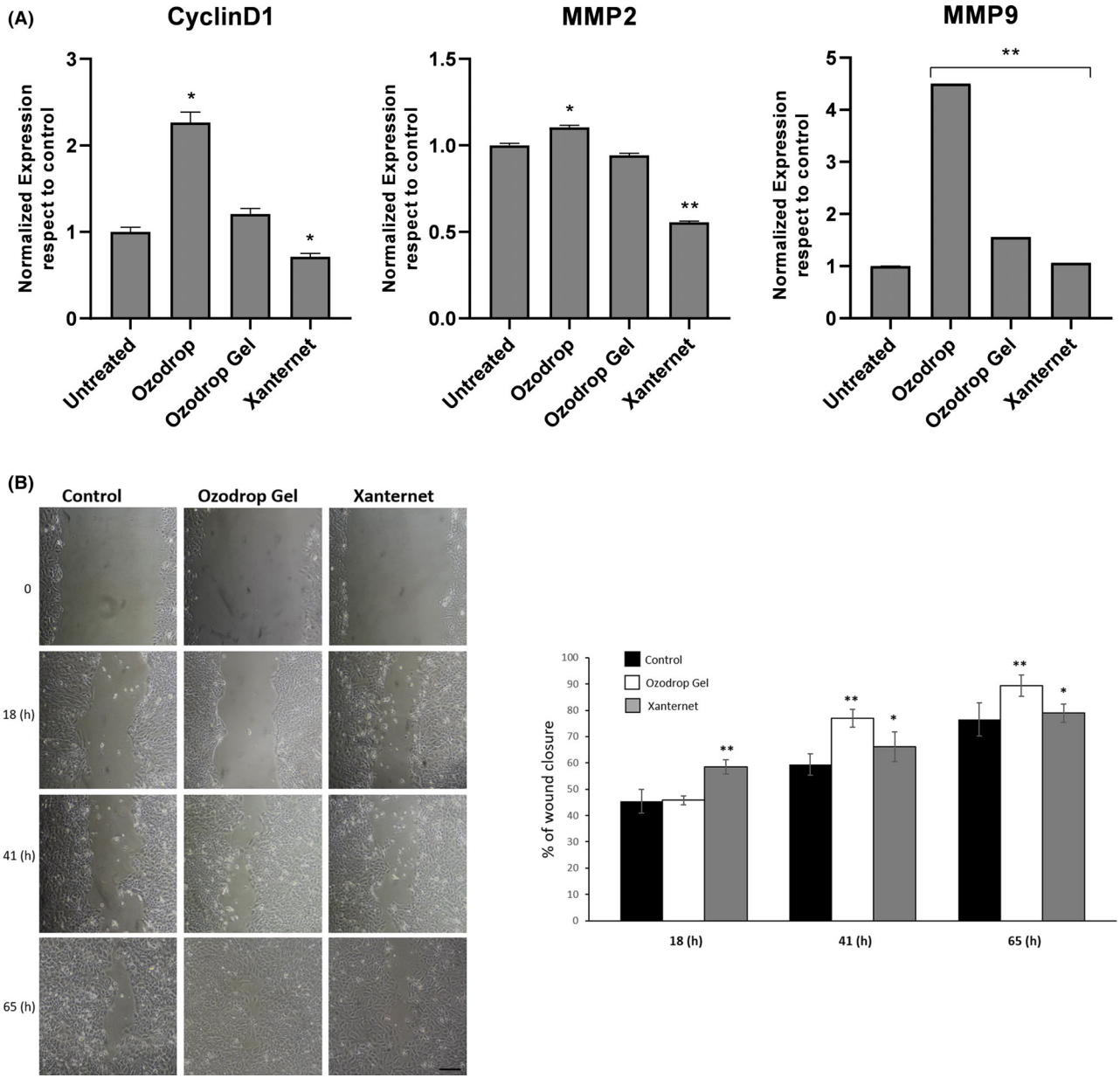


Fig. 5. A. Expression levels of CyclinD1 and migration markers (MMP2 and MMP9) in HaCat cells treated or not with Ozodrop® gel, Ozodrop® and Xanternet for 24 h. Data are representative of three independent experiments performed in duplicates. Unpaired *t* test was carried out to determine statistical significance: $^{*}P \leq 0.05$, $^{**}P \leq 0.005$.
B. Left panel: representative images of wound healing assay on HaCaT cells treated with 2.2% Ozodrop® gel, 2.2% Xanternet, or untreated at different time points. Scale bar is 200 μm. Right panel: Percentage of scratch closure in wound healing assay at different time points. Data are presented as the mean ± SD ($n = 3$). Significance respect to control: $^{*}P < 0.05$; $^{**}P < 0.01$.

To further corroborate whether Ozodrop® gel induce repair activity, we performed an in vitro scratch wound healing assay in HaCat cells. To this purpose, a monolayer of cells at 90–100% confluence was scratched with a sterile tip to create a cell-free zone in each well, washed with sterile PBS and incubated with either Ozodrop® gel 2.2% or Xanternet 2.2% in 2% FBS-containing medium. Wound closure due to the proliferation and migration of cells was observed 18, 41 and 65 h later using a phase-contrast microscope (Fig. 5B). The percentage of scratch closure by cultured keratinocytes treated with Ozodrop® gel 2.2% at 41 and 65 h (76.94 ± 3.38% and 89.37 ± 4.07% respectively) is significantly higher than both controls and Xanternet-treated cells, indicating a remarkable tissue-repair activity of Ozodrop® formulations (Fig. 5B).

Experimental procedures

Biofilm prevention

*P. aeruginosa (*ATCC 9027) and *S. aureus* (ATCC 6538) were grown overnight in Nutrient Broth (VWR, Avantor, Radnor, PA) at 37°C. OD was measured at 600 nm and adjusted to 1×10^7 CFU ml^{-1} for *S. aureus* and 1×10^8 CFU ml^{-1} for *P. aeruginosa*. To each 24-well plates with or without a contact lens were transferred 500 μl of solutions composed of 100 μl aliquot of bacteria and Ozodrop® or Ozodrop® gel at 20% dissolved in Nutrient Broth, and as a control, a 100 μl aliquot of bacteria dissolved in Nutrient Broth. Biofilms formed by bacteria that did not undergo any treatment were used as controls. Daily disposable lenses composed of 31% nelfilcon A and 69% water (Dailies AquaComfort PLUS; Alcon Laboratories, Fort Worth, TX, USA) were used in the experiments and briefly rinsed in sterile phosphate-buffered saline (PBS; VWR- Avantor, Radnor, PA, USA) before every assay. Contact lenses or plates were incubated at 37°C for 30 or 50 h. After treatment, culture fluid was discarded and plate wells or contact lens were gently washed with PBS to remove planktonic bacteria. The biofilm deposition was assessed by evaluating the amount of biomass formed on different supports through a crystal violet assay and bacterial viability was measured by MTT assay.

Biofilm eradication

Bacteria were grown overnight in Nutrient Broth at 37°C. OD was measured at 600 nm and bacteria titres were adjusted to 1×10^7 CFU ml^{-1} for *S. aureus* and 1×10^8 CFU ml^{-1} for *P. aeruginosa*. A 100 μl aliquot of bacteria and 400 μl of Nutrient Broth were transferred to each 24-well plate with or without the contact lens. After 72 h of incubation at 37°C, medium was discarded from the well, and Ozodrop® or Ozodrop® gel was added to 500 μl of Nutrient Broth to a final concentration of 20% and incubated at 37°C for 10 min. At the end of application, each well was washed with Nutrient Broth and incubated with 500 μl of Nutrient Broth at 37°C. The treatment was repeated three times at a gap of 2 h from the previous application. Biomass of biofilms formed by bacteria that undergo the treatment with only Nutrient Broth was used as controls. The biofilm quantification and the bacterial viability were carried out through crystal violet assay and MTT respectively.

Crystal violet (CV) staining

The GRAM Stain P Crystal Violet P-1 was purchased by Condalab (Torrejon de Ardoz, Madrid, Spain). After the treatments previously described on plate wells or contact lenses, culture fluid was discarded. Both supports were gently washed with PBS to remove planktonic bacteria and air-dried for 10 min at room temperature. Plate wells were stained with 500 μl of a 10% CV solution for 10 min, whereas contact lenses were stained for 15 min with 500 μl of a 0.1% CV solution, to reduce the background staining observed on contact lenses without bacteria. After staining, each support was gently washed with PBS to remove the excess of CV. Stained biofilms were allowed to dry for 15 min at room temperature and dissolved in 400 μl of 95% ethanol. Optical Density at 570 nm was read with Nanodrop One (Thermo Scientific, Waltham, MA, USA) using Nutrient Broth without bacteria as blank. T-test was used to assess statistical significance, and a P value <0.05 was considered significant.

MTT

After treatments, culture fluid was discarded and plate wells with or without contact lens were gently washed with PBS to remove planktonic bacteria. MTT reagent was prepared by dissolving 25 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, Saint Louis, MO, USA) in 5 ml PBS. MTT reagent was added to all wells and plates containing PBS at 10% final concentration. The plates with or without the contact lens were incubated for 50 min at 37°C in the dark. After 50 min, an equal volume of isopropanol was added per well and plates were incubated for 10 min at room temperature in the dark. Cell viability was evaluated by subtracting background absorbance at 630 nm to signal absorbance at 505 nm measured with a microplate reader Seac Sirio S. Experiments were performed in triplicate and data are represented as mean ± SD. T-test was used to assess statistical significance, and a P value <0.05 was considered significant.

CFU Assay

P. aeruginosa (ATCC 9027) and *S. aureus* (ATCC 6538) were grown overnight in Nutrient Broth at 37°C. OD was measured at 600 nm and bacteria titres were adjusted to 1×10^3 CFU ml^{-1} for *S. aureus* and to 1×10^4 CFU ml^{-1} for *P. aeruginosa*. Two ml-aliquots of the diluted bacterial suspension were put into different conical sterile tubes together with Ozodrop® or Ozodrop® gel at 20%. Bacteria in Nutrient Broth were used as control. After incubation at 37°C for 1 h with shaking at 140 rpm, bacteria were seeded onto Nutrient Broth agar plates and incubated at 37°C overnight. CFU were counted the next day on three replicas. Data are represented as mean ± SD and statistical significance was measured by *T*-test.

Cell line

HaCat cells were purchased by CLS Cell Lines Service GmbH (Eppelheim, Germany) and cultured at 37°C, 95% humidity and 5% CO_2 in DMEM (Biowest, Stockumer Kirchstraße, Düsseldorf, Germany) supplemented with 10% FBS (Biowest; Stockumer Kirchstraße) and 1x solution of glutamine–penicillin–streptomycin (Biowest; Stockumer Kirchstraße). For cell treatment, after attaining confluence, culture medium was removed and fresh medium with 100 ng ml^{-1} of Lipopolysaccharide (Sigma Aldrich) was added to cells for 24 h with or without the tested substances.

Real-time RT-PCR

Gene expression analysis was performed as previously described (Vessichelli *et al.*, 2012; Reale *et al.*, 2016). Briefly, total RNA was isolated from HaCat cells using MicroElute® Total RNA Kit (Omega Bio-Tek, Norcross, GA, USA). The reverse transcriptase reaction was performed on 1 μg of RNA with iScript™ Advanced cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Then, 30 ng of cDNA were used in the subsequent amplification step along with 300 nM of each primer in a total volume of 15 μl. Real-time PCR reactions were performed in duplicate using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc.) in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) with the following cycling conditions: 25 s/95°C; 37 cycles of 10 s/95°C and 25 s/60°C (with Plate Read); and a melting curve analysis to confirm amplification of single products.

Gene expression analysis was carried out with Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Inc.) using β2 microglobulin as reference for normalization (fwd 5'-CCA CTG AAA AAG ATG AGT ATG CCT-3'; rev 5'-CCA ATC CAA ATG CGG CAT CTT CA-3'). Target genes were amplified with the following primers:

S100A8 fwd 5'-GGG ATG ACC TGA AGA AAT TGC TA-3'

S100A8 rev 5'-TGT TGA TAT CCA ACT CTT TGA ACC A-3'

S100A9 fwd 5'-GTG CGA AAA GAT CTG CAA AAT TT-3'

S100A9 rev 5'-GGT CCT CCA TGA TGT GTT CTA TGA-3'

S100A12 fwd 5'-CTG CTT ACA AAG GAG CTT GCA A-3'

S100A12 rev 5'-GGC CTT GGA ATA TTT CAT CAA TG-3'

CCL20 fwd 5'-TTG TCT GTG TGC GCA AAT CC-3'

CCL20 rev 5'-CCA ACC CCA GCA AGG TTC TT-3'

CyclinD1 fwd 5'-TCCTCTCCAAAATGCCAGAG-3'

CyclinD1 rev 5'- TGAGGCGGTAGTAGGACAG-3'

Mmp2 fwd 5': AGAACCTGGATGCCGTCGT-3'

Mmp2 rev 5': TCACGCTCTTCAGACTTTGG-3'

Mmp9 fwd 5': TTCAGGGAGACGCCCATTTC-3'

Mmp9 rev 5': AACCGAGTTGGAACCACGA-3'

Statistical analysis to assess the significance of gene expression differences was performed on GraphPad Prism 8.0.1 (GraphPad Software, San Diego, CA, USA).

Wound healing assay

For wound healing assay, HaCaT cells were seeded in 6-well plates. 24 h later, confluent cells were uniformly scraped with a sterile tip across. Subsequently, cells were washed twice with PBS, and treated or not with either 2.2% Ozodrop® gel or 2.2% Xanternet in 2% FBS-containing media. Incubation was carried out in a humidified environment with 5% CO_2 at 37°C. The scratched region was photographed at 18, 41 and 65 h after wounding at 10× magnification. The scratch area was analysed using ImageJ software (U. S. National Institutes of Health, Bethesda, MD, USA) and the percentage of wound closure was calculated according to the following equation:

$$\%\,Wound\ closure = \left[\frac{(Surface\ area\ at\ time\ point\ 0 - Surface\ area\ at\ time\ point\ X)}{Surface\ area\ at\ time\ point\ 0}\right] x\ 100$$

Discussion

Due to tolerance to immune responses and antimicrobials, biofilms are difficult to treat and represent a major issue in the use of medical devices. Moreover, the emergence of antibiotic resistance makes it mandatory the discovery of new drugs for biofilm treatment (Wolfmeier *et al.*, 2018; Alter *et al.*, 2019). Ozone is well known to

have antiseptic and sanitizing properties, indeed, it is used for food production, disinfection of room air and tap water (Ugazio *et al*., 2020).

In our research work, we show that ozonated liposomal sunflower oil plus hypromellose (Ozodrop®; FB Vision) has an excellent effect on both inhibition of *P. aeruginosa* and *S. aureus* biofilm formation and removal of *P. aeruginosa* and *S. aureus* preformed biofilm on different supports, such as plastic plates and contact lens. Then, since not the biofilm, but viable bacteria infect the eye, it is important to point out that the formulations tested in this work also show a direct effect on inhibiting bacterial viability, as assessed by the MTT and CFU assays. In particular, the use of ozonated liposomal sunflower oil plus hypromellose could have a great impact in the prevention of post-operative endophthalmitis, whose risk is associated with the intraocular lens implantation (Garzozi and Harris, 2000; Suzuki *et al*., 2005). In addition, the effect of the substances tested on contact lenses is particularly relevant since many ocular infections are caused by the prolonged use of soft contact lenses and/or by poor hygiene and care habits in contact lens handling (Wilson *et al*. 1990; Hou *et al*., 2012). Previous studies addressed the issue of biofilms associated with contact lenses, mainly describing the capacity of natural compounds added to care solutions to prevent biofilm formation and reduce the risk of eye infections respect to commercially available disinfecting solutions (Maciejewska *et al*., 2016; El-Ganiny *et al*., 2017). To our knowledge, the current study is the first showing an antibiofilm and antibacterial activity of ozonated liposomal sunflower oil plus hypromellose as eye drops and as gel on contact lenses. However, since contact lens materials seem to affect the adherence of bacteria (Dutta and Willcox, 2013; Willcox, 2013), it could be interesting to analyse the effect of Ozodrop® formulations on different lens types.

We also observed that in human immortalized keratinocyes (HaCat cells), stimulated or not with LPS, the tested substances induce expression of antimicrobial peptides (S100A8, S100A9 and S100A12), that are highly efficient in reducing bacterial biofilms formed on contact lenses as assessed by Maciejewska *et al*. (2016). Thus, the antibiofilm and antibacterial activity could be even stronger *in vivo* with respect to our observations *in vitro* due to an activation of cell defence response. The observed increase of antimicrobial peptides gene expression upon treatment with Ozodrop® formulations could also counteract the chronic inflammation and the neutrophil accumulation associated with residual bacteria trapped under the soft lens (Patel *et al*., 2018). Indeed, the effect of Ozodrop® and Ozodrop® gel on HaCat cells does not exert a consequent up-regulation of the inflammatory chemokine CCL20. Such evidence suggests that the application of the tested substances should not enhance corneal tissue irritation, confirming previous findings about the anti-inflammatory and repairing effect of Ozodrop® eye drops *in vivo* (Spadea *et al*., 2018).

Finally, our results indicate that gel formulation of ozonated oil in liposomes induce repair activity as well as up-regulation of proliferative and migration genes, suggesting that it can find applications in the treatment of chronic non-healing wounds, which is often unsuccessful due to the presence of pathogen biofilms (Wu *et al*., 2018).

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Relative numbers (mean ± SD) of colony-forming units (CFU) in a culture of P. aeruginosa (left) and S. aureus (right) seeded on agar plates after incubation for 1 hour at 37 °C with 20% Ozodrop or 20% of Ozodrop gel. Untreated bacteria for each strain have been considered as controls. Experiments were carried out in triplicates.

