Corneal Epithelial Wound Healing and Bactericidal Effect of Conditioned Medium From Human Uterine Cervical Stem Cells

Maria A. Bermudez,[1] Juan Sendon-Lago,[2] Noemi Eiro,[3,4] Mercedes Treviño,[5] Francisco Gonzalez,[1,6] Eva Yebra-Pimentel,[7] Maria Jesus Giraldez,[7] Manuel Macia,[8] Maria Luz Lamelas,[3] Jorge Saa,[3,9] Francisco Vizoso,[3,4] and Roman Perez-Fernandez[2]

[1]Department of Surgery, Center for Research in Molecular Medicine and Chronic Diseases (CIMUS), University of Santiago de Compostela, Santiago de Compostela, Spain
[2]Department of Physiology, Center for Research in Molecular Medicine and Chronic Diseases (CIMUS), University of Santiago de Compostela, Santiago de Compostela, Spain
[3]Unidad de Investigación, Fundación Hospital de Jove, Gijón, Spain
[4]Fundación para la Investigación con Células Madre Uterinas (FICEMU), Gijón, Spain
[5]Servicio de Microbiología, Complejo Hospitalario Universitario de Santiago de Compostela, Santiago de Compostela, Spain
[6]Service of Ophthalmology, Complejo Hospitalario Universitario de Santiago de Compostela, Santiago de Compostela, Spain
[7]Department of Applied Physics (Optometry Area), Optic and Optometry Faculty, University of Santiago de Compostela, Santiago de Compostela, Spain
[8]Department of Obstetrics and Gynecology, University of Santiago de Compostela, Santiago de Compostela, Spain
[9]Service of Ophthalmology, Fundación Hospital de Jove, Gijón, Spain

Correspondence: Francisco Vizoso, Unidad de Investigación, Fundación Hospital de Jove, Avenida Eduardo de Castro, s/n 33290, Gijón, Asturias, Spain; investigación@hospitaldejove.com.
Roman Perez-Fernandez, Department of Physiology, Center for Research in Molecular Medicine and Chronic Diseases (CIMUS; P1L3), Avenida Barcelona, s/n 15782, University of Santiago de Compostela, Santiago de Compostela, Spain; roman.perez.fernandez@usc.es.

PURPOSE. To evaluate the effect of conditioned medium from human uterine cervical stem cells (CM-hUCESCs) on corneal epithelial healing in a rat model of dry eye after alkaline corneal epithelial ulcer. We also tested the bactericidal effect of CM-hUCESCs.

METHODS. Dry eye was induced in rats by extraocular lacrimal gland excision, and corneal ulcers were produced using NaOH. Corneal histologic evaluation was made with hematoxylin-eosin (H&E) staining. Real-time PCR was used to evaluate mRNA expression levels of proinflammatory cytokines. We also studied the bactericidal effect of CM-hUCESCs in vitro and on infected corneal contact lenses (CLs) using *Escherichia coli* and *Staphylococcus epidermidis* bacteria. In addition, in order to investigate proteins from CM-hUCESCs that could mediate these effects, we carried out a human cytokine antibody array.

RESULTS. After injury, dry eyes treated with CM-hUCESCs significantly improved epithelial regeneration and showed reduced corneal macrophage inflammatory protein-1 alpha (MIP-1α) and TNF-α mRNA expression as compared to untreated eyes and eyes treated with culture medium or sodium hyaluronate ophthalmic drops. In addition, we found in CM-hUCESCs high levels of proteins, such as tissue inhibitors of metalloproteinases 1 and 2, fibroblast growth factor 6 and 7, urokinase receptor, and hepatocyte growth factor, that could mediate these effects. In vitro, CM-hUCESCs showed a clear bactericidal effect on both *E. coli* and *S. epidermidis* and CLs infected with *S. epidermidis*. Analyses of CM-hUCESCs showed elevated levels of proteins that could be involved in the bactericidal effect, such as the chemokine (C-X-C motif) ligands 1, 6, 8, 10, and the chemokine (C-C motif) ligands 5 and 20.

CONCLUSIONS. Treatment with CM-hUCESCs improved wound healing of alkali-injured corneas and showed a strong bactericidal effect on CLs. Patients using CLs and suffering from dry eye, allergies induced by commercial solutions, or small corneal injuries could benefit from this treatment.

Keywords: dry eye, corneal ulcers, *E. coli*, *S. epiderimidis*, secretoma

Corneal ulcers are a very common human ophthalmologic disease. They are characterized by a disruption of the corneal epithelial layer often accompanied by inflammation. The most common causes of corneal ulcers are trauma, chemical injuries, use of contact lenses (CLs), infections, and keratoconjictivitis sicca or dry eye syndrome.[1,2] Dry eye is characterized by dysfunctional tear production and/or an increased tear evaporation rate. Normal symptoms are blurred vision, photophobia, and pain. Age, hormonal changes, pollution, looking at computer screens for long periods, and CLs are some of the common causes of dry eye.[3] Contact lens use can affect the natural protective mechanisms of the cornea, augmenting adhesion of microbial cells, and progress to more serious problems such as infections or corneal ulcers.[4,5] The International Organization for Standardization (ISO/CD 14729) specifies that disinfecting solutions have to be able to reduce an

initial concentration of bacteria by log3. However, a common microorganism such as *Escherichia coli* is not included in the ISO standards, but it can be found frequently as an opportunistic pathogen human in ocular infections together with *Staphylococcus epidermidis*.[6]

Despite there being no consensus about the ideal treatment for corneal chemical burns, the usual procedure consists of topical application of anti-inflammatory or antiangiogenic artificial tears several times per day on the surface of the cornea. With this treatment, patients usually achieve temporary relief from symptoms. Recent studies, using animal models, have shown promising results with both mesenchymal stem cells and the medium in which these cells were cultured, that is, conditioned medium (CM).[7-9] These multipotent cells were originally isolated from bone marrow and later obtained from adipose tissue[10] and cord blood.[11] Even though some authors have attributed the therapeutic effect of stem cells to different factors secreted by them, further studies are required to elucidate the possible mechanisms of action and clinical use.

To do the experiments, we used CM from the culture of human uterine cervical stem cells (hUCESCs). The hUCESCs were obtained from healthy women when the endocervix was sampled for routine Pap cervical smear, as previously described.[12] The endocervix, like the cornea, has a constantly self-healing epithelium and is continuously exposed to microorganisms. Thus, it seems that CM-hUCESCs might be an ideal candidate for study with regard to regenerative medicine and specifically in cornea-related pathologies.

To evaluate the effect of CM-hUCESCs on wound healing we used rats with extraorbital lacrimal glands excised, which produced dry eye with few secondary effects. Then we performed a corneal alkali burn to induce a fast and controlled wound, both in size and in depth. These experimental models were previously described.[9,13] In addition, we also tested the bactericidal effect of CM-hUCESC against *S. epidermidis* and *E. coli* in CLs in vitro.

METHODS

Ethics Statement

This study adhered to national regulations and was approved by the regional Ethics and Investigation Committee (Comité Ético de Investigación Clínica Regional del Principado de Asturias). Cervical smears were obtained from patients who underwent surgery or a routine gynecologic checkup at Fundación Hospital de Jove, Asturias, Spain. All patients provided informed written consent.

All animal studies were approved by the University of Santiago de Compostela Ethics Committee for Animal Experiments. The research was conducted in full compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell Culture, CM-hUCESCs, and Bacterial Strains

Human UCESCs were cultured in 90-mm Petri dishes to 70% confluence with 5 mL Dulbecco's modified Eagle's medium-F12 (DMEM-F12) culture medium with 10% FBS (Gibco, Life Technologies, Paisley, UK) in air-CO$_2$ (95:5) atmosphere at 37°C during 48 hours. Afterward, the cells were washed three times in PBS and cultured again in DMEM-F12 without FBS. After 48 hours, the medium was centrifuged for 5 min at 300g, and the supernatant was collected and used immediately. Conditioned medium–hUCESCs were also lyophilized (25L Genesis 5 Q EL-85; SP Scientific, Gardiner, NY, USA) and then stored at −80°C until used. The lyophilized powder was then resuspended just before use in 5 mL deionized distilled water (ddH$_2$O) or 2.5 mL to obtain the concentrated CM-hUCESCs (2×).

The *S. epidermidis* (ATCC 12228) and *E. coli* (ATCC 25922) strains were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA).

Animals and Dry Eye Model

Female Sprague-Dawley rats weighing 200 to 250 g (Central Animal Facility, University of Santiago de Compostela, Santiago de Compostela, Spain) were used in this study. Animals were anesthetized by intraperitoneal injection of a mixture of ketamine (425 mg/kg) and xylazine (1.6 mg/kg) in NaCl.

To induce dry eye syndrome, rats were anesthetized and extraocular lacrimal glands were excised bilaterally to reduce the number of animals to three per group ($n = 6$ eyes). One week after surgery, tear production was measured using the Schirmer test (Laboratorios Cusí SA, Barcelona, Spain). Paper strips for the Schirmer test were adapted by cutting 2-mm strips. A 1-mm fold was made at the tip of the strip and introduced under the eyelid for 5 minutes. The length of the wet part of the strip was then measured.

Corneal Alkali Wound and Treatments

Rats were anesthetized as described above, and a central corneal alkali ulcer was produced in both eyes by applying a piece of Whatman paper (2 × 2 mm; Sigma-Aldrich Corp., Madrid, Spain) soaked in 2 μL NaOH (1 M) for 60 seconds. Immediately afterward, the cornea was rinsed with saline solution for 30 seconds, stained with fluorescein (Colircusí Fluoresceina; Alcon Cusí, S.A., Barcelona, Spain), and photographed under blue light with a digital camera (Nikon D200; Nikon, Tokyo, Japan) attached to a surgical microscope (Takagi OM-5 220-2; Takagi, Tokyo, Japan). Corneal injury was measured quantitatively offline on the photograph using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The number of fluorescein-colored pixels was calculated as a percentage of the total number of pixels of the surface of the cornea.

Percentage epithelial regeneration (%ER) was calculated using the formula %ER = 100 $(m_i - m_f)/m_i$, where m_i is the first measurement of the corneal ulcer (just after the alkali burn) and m_f is the final measurement 48 hours afterward.

Treatments were applied on each eye topically (one drop) four times per day for 5 days. Eighteen rats were divided into six groups of three rats each: (1) rats with dry eyes and corneal alkali burn treated with CM-hUCESCs; (2) rats with dry eyes and corneal alkali burn treated with ophthalmic drops with sodium hyaluronate (0.015 g/10 mL; Ophthalmic drops group); (3) rats with dry eyes and corneal alkali burn treated with culture medium (DMEM-F12, Medium group); (4) rats with dry eyes and corneal alkali burn untreated (NoTreat group); (5) rats with dry eyes but no lesion (No Lesion group); and (6) rats with normal eyes and no lesion (Normal group).

Histologic Evaluation, RNA Isolation, and Real-Time PCR Analysis

Five days after corneal alkali burn, one rat was randomly selected from each group and euthanized for histologic evaluation. Eyes of the other two animals were used for real-time PCR analysis.

For histologic evaluation, eyeballs were excised and immersion-fixed in 10% neutral buffered formalin. After 24 hours, corneas were dissected and immersed in ethanol (70%)

TABLE. Primer Sets for Real-Time PCR

Gene	Forward, 5′–3′	Reverse, 5′–3′
MIP-1α	ATGAAGGTCTCCACCACTGC	AAAGGCTGCTGGTCTCAAAA
MCP-1	ATGCAGTTAATGCCCCACTC	TTCCTTATTGGGGTCAGCAC
TNF-α	TCAGTTCCATGGCCCAGAC	GTTGTCTTTGAGATCCATGCCATT
β-actin	GGAGATTACTGCCCTGGCTCCTA	GACTCATCGTACTCCTGCTTGCTG

for 1 day, soaked in paraffin, cut in 20-μm sections, mounted, and stained with hematoxylin-eosin (H&E). Photographs of corneas were taken from equivalent center slides.

Macrophage inflammatory protein-1 alpha (MIP-1α), monocyte chemotactic protein-1 (MCP-1), and tumor necrosis factor-alpha (TNF-α) mRNA expression levels in cornea were evaluated by real-time PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Barcelona, Spain). Complementary DNA synthesis was performed as described elsewhere.[14] Real-time PCR was done with 2 μg cDNA in a 20-μL volume using Luminaris Color HiGreenqPCR Master Mix (Fisher Scientific, Rockford, IL, USA). Samples were denatured at 94°C for 10 seconds, annealed at 58°C for 10 seconds, and extended at 72°C for 10 seconds for a total of 35 cycles. The amount of PCR product formed in each cycle was evaluated based on SYBR Green fluorescence (Bio-Rad, Madrid, Spain) with β-actin as the endogenous control. The oligonucleotide sequences are detailed in the Table.

Bacterial Cultures and Antimicrobial Assays

The bactericidal effect of CM in culture was determined by incubating 300 colony-forming units (CFUs) of *E. coli* and *S. epidermidis* (made from serial dilutions from McFarland scale no. 0.5) in 600 μL of either CM or medium alone at 37°C for 6 hours. After this time, dilutions (1:500 and 1:1000) were made in Luria broth medium (LB), and 250 μL was spread on agar plates. After incubation at 37°C overnight (ON), CFUs were counted. Experiments were performed at least three times for each condition.

To evaluate the bactericidal effect of CM-hUCESCs on CLs, we used two commercial CLs made of different materials and tested in a previous study[15]: Omalficon A (CLs1; a 2-hydroxyethyl methacrylate copolymer; ProClear; Cooper Vision, Pleasanton, CA, USA) and Senolficon A (CLs2; a silicone hydrogel; Acuvue OASYS; Johnson & Johnson, Jacksonville, FL, USA). Contact lenses were soaked in 600 μL of the following solutions: LB, CM-hUCESCs; concentrated CM-hUCESCs (2×); and two commercial solutions for CLs, one preserved with DYMED (0.0001% polyaminopropyl biguanide) and another with POLYQUAD (0.001% polidronium chloride). Each CL was immersed convex side up in the above-mentioned solutions, each containing 9×10^8 CFU/mL (adjusted to McFarland scale no. 3) in a 24-multiwell plate. After incubation at 37°C and ON shaking at 15 revolutions per minute (rpm), each CL was carefully removed, washed in sterile saline solution, and placed in a tube with 1 mL saline. Tubes were then placed in ice and sonicated for 1 minute (Digital Sonifier 450; Branson Ultrasonics Corporation, Danbury, CT, USA). Then 250 μL of the suspension was spread on agar plates and incubated ON at 37°C, and the CFUs were counted. Experiments were performed at least three times for each solution.

Human Cytokine Antibody Array

Forty-eight-hour CM-hUCESCs and LCM-hUCESCs were subjected to profiling using RayBio Human Cytokine Antibody Array G-Series 2000 (RayBiotech, Norcross, GA, USA).

Culture medium (DMEM-F12; Gibco, Life Technologies) was processed in parallel as a negative control. Signal intensity values representing detected cytokines were subtracted from the background and normalized to positive controls on the same membrane. Experimental steps and analyses were conducted according to the manufacturer's instructions. Signal intensity values of each cytokine are presented as mean ± standard deviation (SD).

Statistical Analysis

Values are expressed as mean ± SD. Average values were compared using one-way ANOVA, with Tukey's range test for post hoc comparisons. *P* values less than 0.05 were considered statistically significant. MATLAB R2011a Version 7.1 (Math-Works, Inc., Madrid, Spain) software was used for all calculations.

RESULTS

CM-hUCESCs Enhance Corneal Wound Healing

In order to study the effect of the CM-hUCESCs on a corneal wound in a rat model of dry eye, the extraocular lacrimal glands were excised bilaterally, and tear production was measured 7 days after excision using the Schirmer test (Fig. 1A). All rats that underwent surgery showed dry eyes 7 days after the excision (Fig. 1B).

Ulcers covered between 85% and 95% of the corneal surface (Fig. 1C, top). Fifteen hours after injury, epithelial regeneration was greater in eyes treated with the CM-hUCESCs (62 ± 5% of total lesion) as compared with the other treatments (group treated with medium 34 ± 15%; group treated with commercial ophthalmic drops 32 ± 15%) and no treatment (36 ± 13%) (Fig. 1C, middle; Fig. 1D, *P* < 0.008). On day 5 after the alkali burn, the recovery of the corneal surface was almost complete in all treated groups (>74%), but rats treated with commercial ophthalmic drops (74 ± 15%) and medium (77 ± 15%) showed no significant differences from untreated rats (62 ± 16%). However, treatment with CM-hUCESCs (92 ± 4%) significantly (*P* = 0.005) enhanced wound healing as compared with no treatment (Fig. 1C, bottom; Fig. 1E).

CM-hUCESCs Recover Epithelial Cell Layer and Reduce Proinflammatory Cytokines in Cornea

Histologic evaluation of corneas showed that the group treated with CM-hUCESCs had completely regenerated the epithelium (Fig. 2A). However, in the remaining groups (Medium, Ophthalmic drops, and NoTreat) the regeneration of the epithelium was not complete, showing disruption in the epithelial layer at some point on the surface (Fig. 2A).

To evaluate the effect of CM-hUCESCs on inflammation, we assessed by real-time PCR the mRNA production of MIP-1α and MCP-1 chemotactic factors and the TNF-α immune-stimulatory cytokine. We found that MCP-1, MIP-1α, and TNF-α mRNA levels were significantly (*P* < 0.0001) higher in the group treated with culture medium as compared with the other
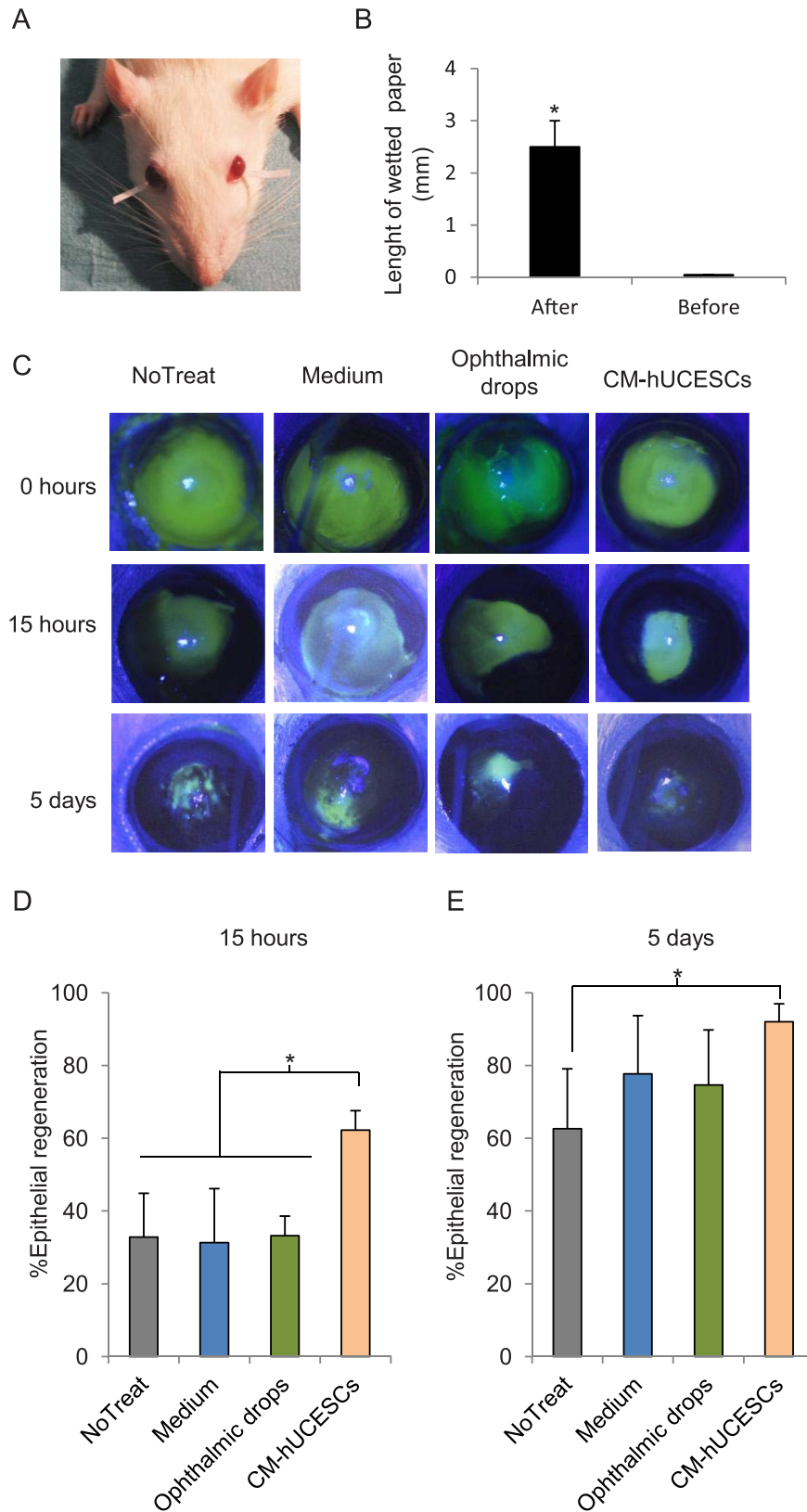
FIGURE 1. Model of dry eye and in vivo epithelial regeneration. (**A**) Schirmer test used to measure tear production. (**B**) Schirmer test results as millimeters of wetted paper measured in the same eye before (Before) and 7 days after (After) excision of the extraorbital gland (*$P < 0.0001$). (**C**) Representative images of cornea fluorescein staining after the alkali burn (0 hours, 15 hours, and 5 days). Each *column* represents the same eye with the same treatment. (**D**) Percent of epithelial corneal regeneration 15 hours after the alkali burn (*$P < 0.01$). (**E**) Percent of epithelial corneal regeneration 5 days after the alkali burn (*$P = 0.005$). Treatments were CM-hUCESCs, ophthalmic drops with sodium hyaluronate (Ophthalmic drops), culture medium (Medium), and no treatment (NoTreat) ($n = 6$ corneas in each group).
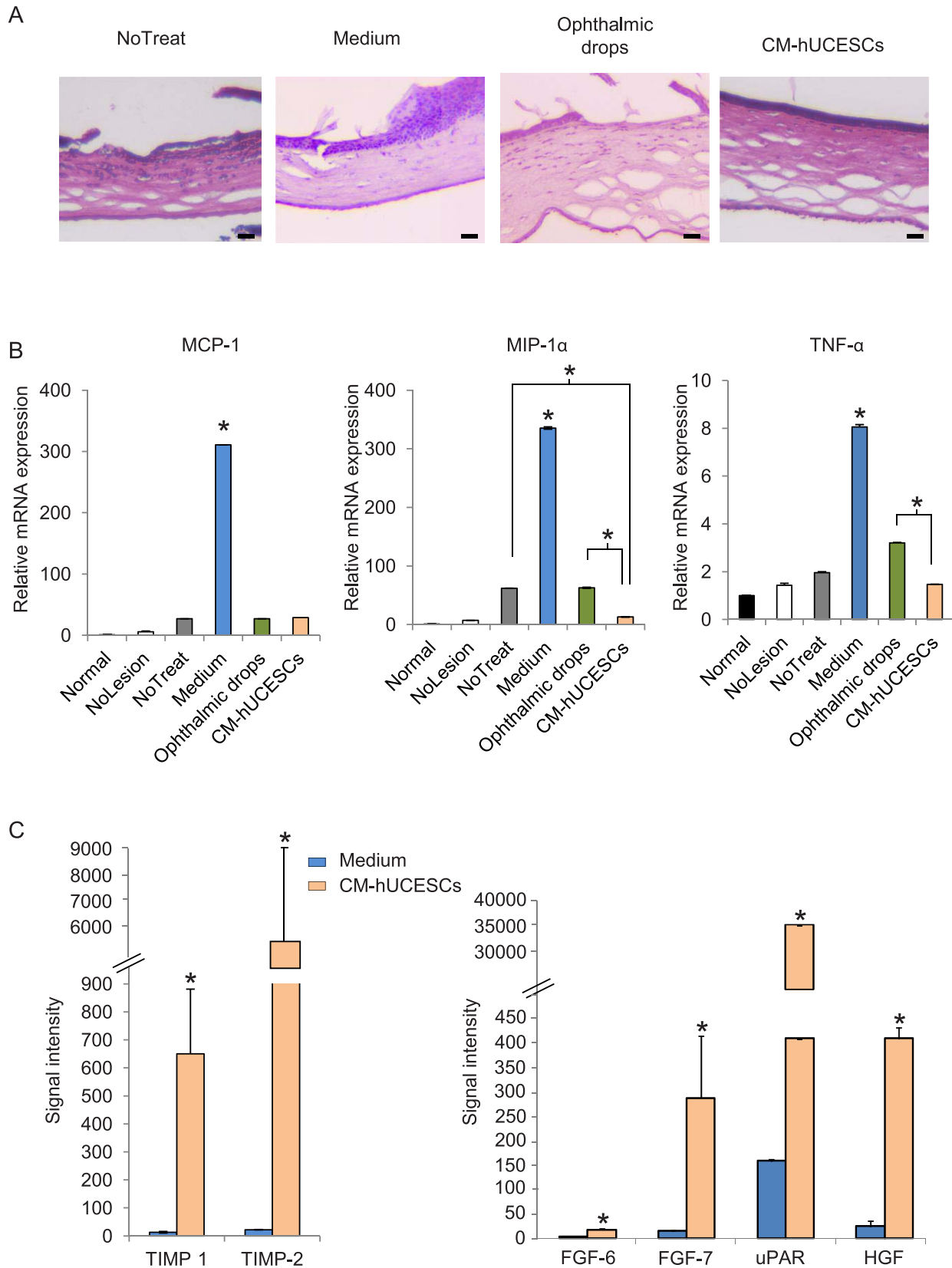
FIGURE 2. Conditioned medium from hUCESCs induce epithelial recovery and anti-inflammatory effects. (**A**) Representative images of hematoxylin-eosin (H&E) staining of untreated corneas (NoTreat) and treatment with culture medium (Medium), ophthalmic drops with sodium hyaluronate (Ophthalmic drops), and CM-hUCESCs 5 days after alkali burn (*scale bar*: 20 μm). All photographs were taken from equivalent center slides of the corneas. (**B**) Monocyte chemotactic protein-1, MIP-1α, and TNF-α mRNA in corneas 5 days after the alkali burn was evaluated by real-time PCR. Corneas were divided into the following groups: healthy corneas (without lesion, Normal), dry eye corneas without lesion (NoLesion), nontreated lesioned dry eye corneas (NoTreat), lesioned dry eye corneas treated with culture medium (Medium), lesioned dry eye corneas treated with

ophthalmic drops containing sodium hyaluronate (Ophthalmic drops), lesioned dry eye corneas treated with CM-hUCESCs (*$P < 0.05$). (C) TIMP-1, TIMP-2, FGF-6, FGF-7, uPAR, and HGF protein expression in culture medium (*blue bars*) and in CM-hUCESCs (*orange bars*) after 48 hours of culture. *Error bars* represent mean ± SD of signal intensity value as detected in RayBio human cytokine antibody array (*$P < 0.05$).

groups, including the NoTreat group (with lesion and without treatment) (Fig. 2B). Levels of MIP-1α mRNA in the group treated with CM-hUCESCs were similar to those in the normal rats and the rats without corneal lesion, and significantly ($P < 0.05$) lower than in the other groups (Fig. 2B).

In order to test other factors that could be involved in corneal epithelium wound healing, we carried out a human cytokine antibody array. We found that levels of tissue inhibitors of metalloproteinases (TIMP)-1 and -2, fibroblast growth factors (FGF)-6 and -7, urokinase receptor (uPAR), and hepatocyte growth factor (HGF) were significantly ($P < 0.05$) higher in CM-hUCESCs compared with the control medium (Fig. 2C).

CM-hUCESCs Have a Bactericidal Effect on *E. coli* and *S. epidermidis*

To test the effect of CM-hUCESCs on *E. coli*, 300 CFUs were incubated in CM-hUCESCs for 6 hours. Conditioned medium–hUCESCs significantly ($P < 0.0001$) reduced the number of CFUs as compared with controls (Figs. 3A, 3B). The same procedure, but using 300 CFUs of *S. epidermidis*, was carried out and yielded similar results (Figs. 3C, 3D; $P < 0.05$). In summary, our results seem to indicate that CM-hUCESCs have a bactericidal effect on both strains.

In order to explore the bactericidal effect of CM-hUCESCs on CLs, two commercial CLs (CLs1 and CLs2) were infected with *S. epidermidis*, incubated with LB medium, CM-hUCESCs, concentrated CM-hUCESCs (2×), and two commercial contact lens solutions (solutions A and B), and CFUs were counted. Both CLs1 and CLs2 treated with CM-hUCESCs had significantly ($P < 0.01$) lower CFUs as compared with those treated with LB medium (Figs. 4A, 4B). The bactericidal effect of concentrated CM-hUCESCs (2×; Figs. 4A, 4B) was higher ($P < 0.01$) than that of nonconcentrated CM-hUCESCs. Results showed a greater effect of the CM-hUCESCs on CLs2 as compared with CLs1 ($P < 0.01$).

The human cytokine antibody array showed significantly higher levels ($P < 0.0001$) of CXCL10/IP10, CXCL8/IL-8, CXCL1/GRO-α, CXCL6/GCP-2, CCL20/MIP-3α, and CCL5/RANTES chemokines in CM-hUCESCs and in lyophilized CM-hUCESCs (LCM-hUCESCs) as compared with the control medium (Fig. 4C).

DISCUSSION

This study shows that treatment of alkali-injured corneas with CM from hUCESCs enhances epithelial wound healing. In addition, CM-hUCESCs showed anti-inflammatory and antibacterial properties. Taken together, our data suggest that CM-hUCESCs could be a good candidate for use as treatment for corneal ulcers and as a contact lens solution.

Dry eye is a condition characterized by changes in tear film quality, quantity, or both. A variety of dry eye animal models have been developed over the past decades, including desiccating stress conditions,[16,17] mechanical inhibition of blinking,[18] pharmacologic blockade of cholinergic receptors with atropine sulfate or scopolamine,[19] adiponectin,[20] injection of botulinum toxin into the lacrimal gland,[21] and surgical excision of tear-producing glands.[22] In this study, we chose the excision of the extraorbital lacrimal gland model given that it is the main lacrimal gland in rats and is easy to access (located on

the surface of the temporal area) and given that the procedure has no secondary effects.[23,24] As demonstrated, this procedure induced a clear dry eye syndrome.

Chemical burns of the cornea frequently lead to persistent epithelial defects, which can result in ulceration. In fact, alkali burns are commonly used for studying corneal ulceration in laboratory animals.[9,25] The corneal epithelium is self-renewing, and this process is essential for normal vision. It has been suggested that limbal stem cells migrate and proliferate to the injured area to induce the corneal regeneration.[26,27] In this study, we show that CM-hUCESCs enhance wound healing and reconstitute the corneal surface. Several studies have been previously performed in rats and rabbits, treating corneal wounds with mesenchymal stem cells from both bone marrow and umbilical cord blood.[8,9,28,29] These studies have reported an improvement of the corneal surface, probably due to the differentiation of exogenous mesenchymal cells into epithelial cells, as well as mobilization of endogenous mesenchymal stem cells to the injured area. The CM-hUCESCs used in the present study show faster regenerative effects than commercial ophthalmic drops when applied to the injured epithelium. Thus, factor/s secreted by hUCESCs and not the cells themselves seem to be responsible for wound healing. Our data also seem to suggest that TIMP-1 and TIMP-2, both inhibitors of matrix metalloproteinases (MMPs) and present at higher concentrations in CM-hUCESCs, could be involved in this process. In fact, it has been demonstrated that TIMPs in cornea protect against extensive corneal tissue destruction after a bacterial infection by protecting extracellular matrix components from active MMPs, promoting resurfacing of the corneal epithelium and wound healing.[30,31] Fibroblast growth factor-6, FGF-7, uPAR, and HGF, which are present at higher levels in CM-hUCESCs than in control medium, have also been related with wound healing. In fact, fibroblast growth factor-binding protein (FGF-BP) expression increases after injury, stimulating both FGF-7 and FGF-6 activity at the wound site, thus enhancing the epithelial repair process.[32–34] Lack of uPA and its receptor, uPAR, disturbs physiological tissue remodeling in mice,[31] and HGF has shown a chemokinetic effect, enhancing migration of keratinocytes.[33,35] However, we cannot discard other factors secreted by the hUCESCs with regenerative activity. In fact, other studies have suggested that microvesicles or exosomes released by both injured tissue and mesenchymal stem cells could be involved in this process.[36,37]

Corneal avascularity is required for optical transparency. However, in corneal infections, chemical burns, and inflammation, neutrophils, monocytes, and macrophages infiltrate the cornea, releasing inflammatory factors and inducing corneal neovascularization.[38–41] Our results showed that CM-hUCESCs had anti-inflammatory effects on injured corneas as compared with the culture medium. However, no significant differences were found in MCP-1 and TNF-α mRNA levels between rats treated with CM-hUCESCs and NoTreat rats. It has been previously shown that administration of a synthetic inhibitor of MMPs in mice after alkali burn induces faster corneal epithelial regeneration compared with control. However, levels of TNF-α did not differ between treated and control groups, suggesting a slight upregulation of TNF-α after the burn.[42] In addition, it is known that stress increases some proinflammatory factors such as TNF-α,[43–45] and therefore the management of animals during treatment (four times per day)
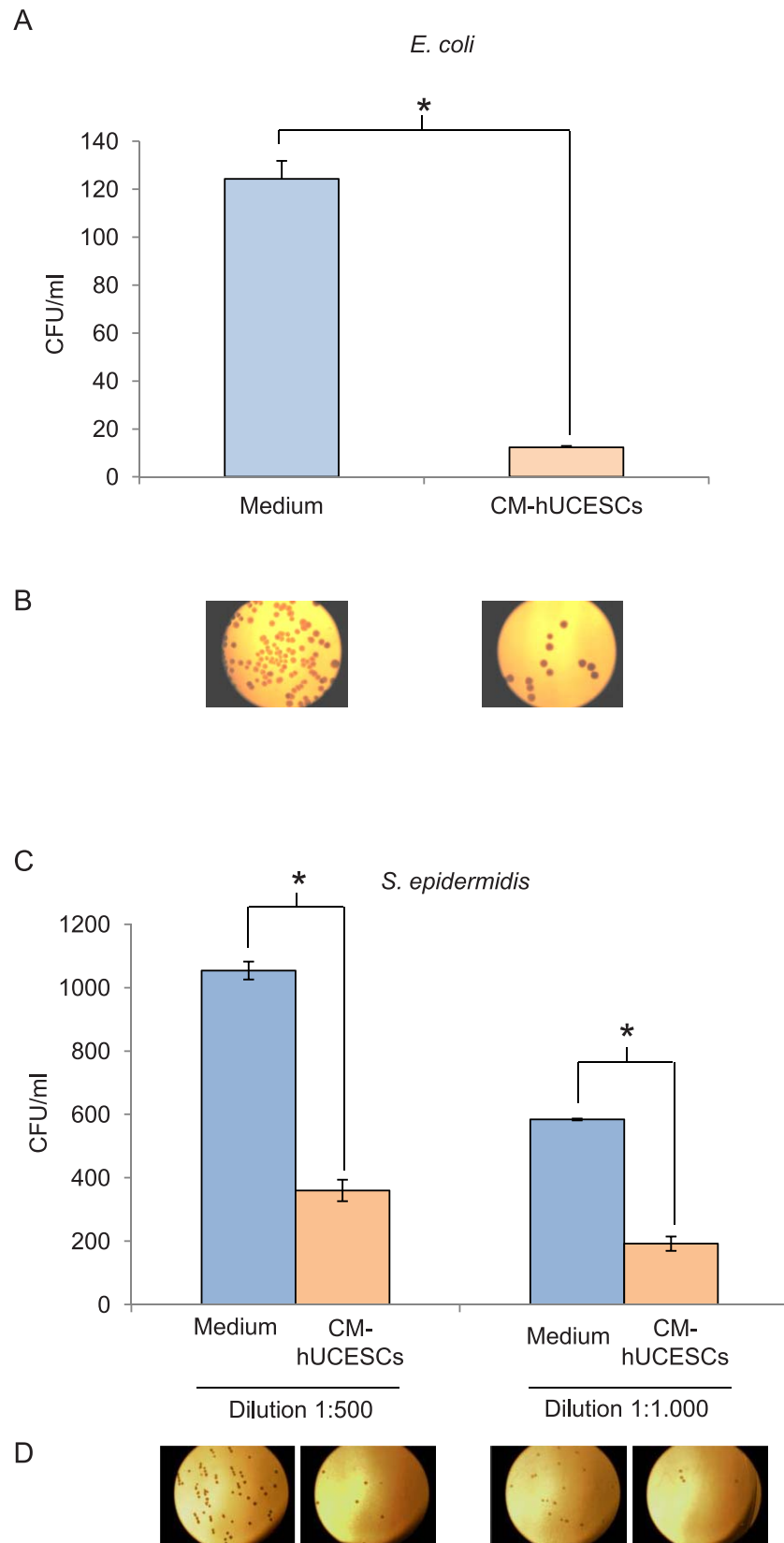
FIGURE 3. Antibacterial effect of CM-hUCESCs. (**A**) Quantitative analysis of *E. coli* after overnight growing of 300 CFUs with culture medium (Medium) and conditioned medium from hUCESCs (CM-hUCESCs). (**B**) Representative images of (**A**) on agar plates. (**C**) Quantitative analysis of *S. epidermidis* after overnight growing of 300 CFUs with culture medium (Medium) and CM-hUCESCs. (**D**) Representative images of (**C**) on agar plates ($n = 3$; *$P < 0.0001$).
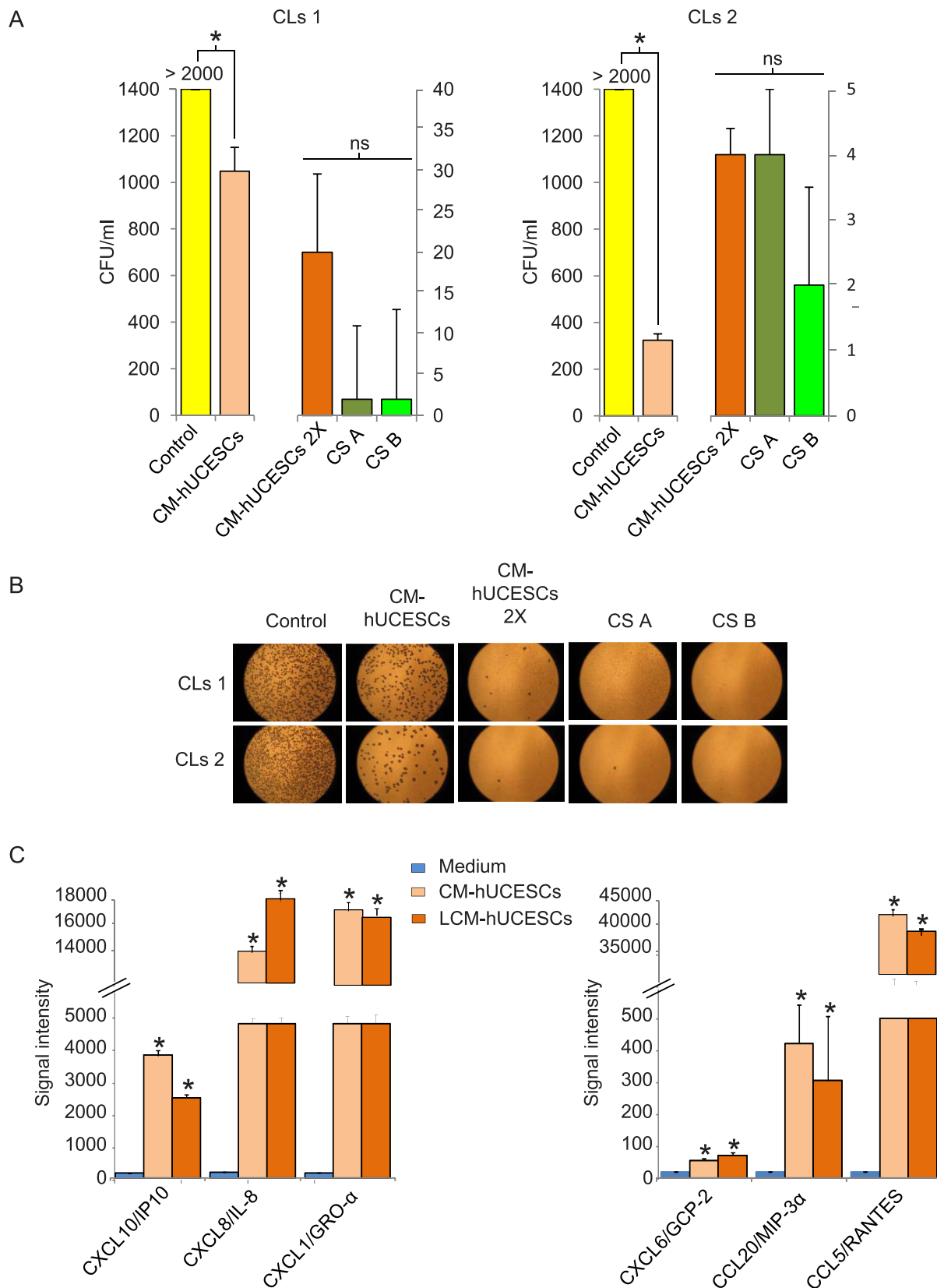
FIGURE 4. Antibacterial effect of CM-hUCESCs on CLs. (**A**) Quantitative analysis of CFUs after immersing the two types of CLs tested (CLs1 and CLs2) with *S. epidermidis* during 24 hours in LB (Control), CM-hUCESCs, CM-hUCESCs concentrated twice (CM-hUCESCs 2×), and two commercial contact lens solutions (CS A and CS B) (*$P < 0.0001$; ns: $P > 0.05$; $n = 3$). (**B**) Representative images of (**A**) showing the CFUs on agar plates. (**C**) CXCL10/IP10, CXCL8/IL-8, CXCL1/GRO-α, CXCL6/GCP-2, CCL20/MIP-3α, and CCL5/RANTES protein expression in culture medium (*blue bars*), CM-hUCESCs (*orange bars*), and lyophilized CM-hUCESCs (LCM-hUCESCs; *dark orange bars*) after 48 hours of culture. *Error bars* represent mean ± SD of signal intensity value as detected in RayBio human cytokine antibody array (*$P < 0.0001$).

could induce higher basal levels of these cytokines compared with those in NoTreat animals.

Thus, CM-hUCESCs seem to have factors that block the proinflammatory effect of the medium alone. Some of the proinflammatory factors blocked by CM-hUCESCs could be the macrophage chemokine MIP-1α (also named CCL3), which is known to induce corneal neovascularization in an alkali-injured cornea by regulating the vascular endothelial growth factor produced by the macrophages.[41] Our results are in line with previous studies using stem cells (rather than CM) showing attenuated inflammation in the cornea as a result of inhibition of the production of proinflammatory cytokines and infiltration of inflammatory cells.[9,11,46]

Recent in vivo studies have shown beneficial effects of treatment with mesenchymal stem cells in bacterial-induced sepsis.[47–50] Some of these studies have hypothesized immunomodulatory properties in stem cells by enhanced phagocyte activity. Our data show that concentrated CM-hUCESCs (2×) have at least the same efficacy on *S. epidermidis* as the commercial CL solutions tested. Given that our experiments were carried out in vitro, the immune responses of the host cells are not involved in the process. Some substance or substances secreted by hUCESCs into the medium must be responsible for this antibacterial effect. We found that CXCL10/IP10, CXCL8/IL-8, CXCL1/GRO-α, CXCL6/GCP-2, CCL20/MIP-3α, and CCL5/RANTES chemokines were present in higher levels in CM-hUCESCs as compared with the control medium. It has been demonstrated that these chemokines have antibacterial effects against *E. coli* and other strains of staphylococcus,[51–55] suggesting that a complex paracrine signaling network could be implicated in the antibacterial potential of hUCESCs. However, we cannot discard other factors such as lysozymes or antimicrobial peptides that have been found to be involved in this process.[56]

According to our data, we may speculate that the beneficial effect of CM-hUCESCs on corneal wound healing, together with the anti-inflammatory and bactericidal properties, could make them a clinical alternative for people with dry eye, corneal injuries, or allergies. In addition, CM-hUCESCs could be used as a CL solution to prevent infections and corneal damage derived from daily use of CLs. The source of hUCESCs provides several advantages: It is less invasive and less painful than other sources, such as bone marrow or adipose tissue; it is available throughout the fertile life of women; and high quantities with a high proliferation rate can be isolated. In addition, from a clinical point of view, administration of lyophilized CM-hUCESCs rather than CM-hUCESCs could represent an important methodological advantage because they can be kept ready to use without loss of any of their properties. Further clinical trials should be performed to corroborate our data.

References

1. Cheng KH, Leung SL, Hoekman HW, et al. Incidence of contact lens-associated microbial keratitis and its related morbidity. *Lancet*. 1999;354:181–185.

2. Amescua G, Miller D, Alfonso EC. What is causing the corneal ulcer? Management strategies for unresponsive corneal ulceration. *Eye*. 2012;26:228–236.

3. Schultz C. Safety and efficacy of cyclosporine in the treatment of chronic dry eye. *Ophthalmol Eye Dis*. 2014;6:37–42.

4. Buehler PO, Schein OD, Stamler JF, Verdier DD, Katz J. The increased risk of ulcerative keratitis among disposable soft contact lens users. *Arch Ophthalmol*. 1992;110:1555–1558.

5. Catalanotti P, Lanza M, Del Prete A, et al. Slime-producing Staphylococcus epidermidis and S. aureus in acute bacterial conjunctivitis in soft contact lens wearers. *New Microbiol*. 2005;28:345–354.

6. Kuzman T, Kutija MB, Kordic R, et al. Comparative study of antibacterial and antifungal effects of rigid gas permeable contact lens disinfecting solutions. *Coll Antropol*. 2013;1:127–131.

7. Oh W, Kim DS, Yang YS, Lee JK. Immunological properties of umbilical cord blood-derived mesenchymal stromal cells. *Cell Immunol*. 2008;25:116–123.

8. Jiang TS, Cai L, Ji WY, et al. Reconstruction of the corneal epithelium with induced marrow mesenchymal stem cells in rats. *Mol Vis*. 2010;16:1304–1316.

9. Yao L, Li ZR, Su WR, et al. Role of mesenchymal stem cells on cornea wound healing induced by acute alkali burn. *PLoS One*. 2012;7:e30842.

10. Zannettino AC, Paton S, Arthur A, et al. Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo. *J Cell Physiol*. 2008;214:413–421.

11. Oh JY, Kim MK, Shin MS, Lee HJ, Ko JH. The anti-inflammatory and anti-angiogenic role of mesenchymal stem cells in corneal wound healing following chemical injury. *Stem Cells*. 2008;26:1047–1055.

12. Eiro N, Sendon-Lago J, Seoane S, et al. Potential therapeutic effect of the secretome from human uterine cervical stem cells against both cancer and stromal cells compared with adipose tissue stem cells. *Oncotarget*. 2014;5:10692–10708.

13. Stevenson W, Chen Y, Lee SM, et al. Extraorbital lacrimal gland excision: a reproducible model of severe aqueous tear-deficient dry eye disease. *Cornea*. 2014;33:1336–1341.

14. Ben-Batalla I, Seoane S, Garcia-Caballero T, et al. Deregulation of the Pit-1 transcription factor in human breast cancer cells promotes tumor growth and metastasis. *J Clin Invest*. 2010;120:4289–4302.

15. Giraldez MJ, Resua CG, Lira M, et al. Contact lens hydrophobicity and roughness effects on bacterial adhesion. *Optom Vis Sci*. 2010;87:E426–E431.

16. Chen W, Zhang X, Zhang J, et al. A murine model of dry eye induced by an intelligently controlled environmental system. *Invest Ophthalmol Vis Sci*. 2008;49:1386–1391.

17. Li Z, Woo JM, Chung SW, et al. Therapeutic effect of topical adiponectin in a mouse model of desiccating stress-induced dry eye. *Invest Ophthalmol Vis Sci*. 2013;54:155–162.

18. Fujihara T, Nagano T, Nakamura M, Shirasawa E. Establishment of a rabbit short-term dry eye model. *J Ocul Pharmacol*. 2000;11:503–508.

19. Dursun A, Arici MK, Dursun F, et al. Comparison of the effects of bevacizumab and ranibizumab injection on corneal angiogenesis in an alkali burn induced model. *Int J Ophthalmol*. 2012;5:448–451.

20. Li Z, Woo JM, Chung SW, et al. Therapeutic effect of topical adiponectin in a mouse model of desiccating stress-induced dry eye. *Invest Ophthalmol Vis Sci*. 2013;54:155–162.

21. Suwan-apichon O, Rizen M, Rangsin R, et al. Botulinum toxin B-induced mouse model of keratoconjunctivitis sicca. *Invest Ophthalmol Vis Sci*. 2006;47:133–139.

22. Maitchouk DY, Beuerman RW, Ohta T, Stern ME, Varnell RJ. Tear production after unilateral removal of the main lacrimal gland in squirrel monkeys. *Arch Ophthalmol*. 2000;118:246–252.

23. Herzog V, Miller F. The localization of endogenous peroxidase in the lacrimal gland of the rat during postnatal development. *J Cell Biol*. 1972;58:662–680.

24. Nemet A, Belkin M, Rosner M. Transplantation of newborn lacrimal gland cells in a rat model of reduced tear secretion. *Isr Med Assoc J*. 2007;9:94–98.

25. Shahriari HA, Tokhmehchi F, Reza M, Hashemi NF. Comparison of the effect of amniotic membrane suspension and autologous serum on alkaline corneal epithelial wound healing in the rabbit model. *Cornea*. 2008;10:1148–1150.

26. O'Sullivan F, Clynes M. Limbal stem cells, a review of their identification and culture for clinical use. *Cytotechnology*. 2007;53:101–106.

27. Ebrahimi M, Taghi-Abadi E, Baharvand H. Limbal stem cells in review. *J Ophthalmic Vis Res*. 2009;4:40–58.

28. Ye J, Yao K, Kim JC. Mesenchymal stem cell transplantation in a rabbit corneal alkali burn model: engraftment and involvement in wound healing. *Eye (Lond)*. 2006;20:482–490.

29. Joyce NC, Harris DL, Markov V, Zhang Z, Saitta B. Potential of human umbilical cord blood mesenchymal stem cells to heal damaged corneal endothelium. *Mol Vis*. 2012;18:547–564.

30. Kernacki K, Barrett R, Hazlett LD. Evidence for TIMP-1 protection against P. aeruginosa-induced corneal ulceration and perforation. *Invest Ophthalmol Vis Sci*. 1999;40:3168–3176.

31. Gordon GM, Austin JS, Sklar AL, Feuer WJ, LaGier AJ, Fini ME. Comprehensive gene expression profiling and functional analysis of matrix metalloproteinases and TIMPs, and identification of ADAM-10 gene expression, in a corneal model of epithelial resurfacing. *J Cell Physiol*. 2011;226:1461–1470.

32. Lund IK, Nielsen BS, Almholt K, et al. Concomitant lack of MMP9 and uPA disturbs physiological tissue remodeling. *Dev Biol*. 2011;358:56–67.

33. Beer HD, Bittner M, Niklaus G, et al. The fibroblast growth factor binding protein is a novel interaction partner of FGF-7, FGF-10 and FGF-22 and regulates FGF activity: implications for epithelial repair. *Oncogene*. 2005;24:5269–5277.

34. Werner S, Peters KG, Longaker MT, Fuller-Pace F, Banda MJ, Williams LT. Large induction of keratinocyte growth factor expression in the dermis during wound healing. *Proc Natl Acad Sci U S A*. 1992;89:6896–6900.

35. Peplow PV, Chatterjee MP. A review of the influence of growth factors and cytokines in in vitro human keratinocyte migration. *Cytokine*. 2013;62:1–21.

36. Fierabracci A, Del Fattore A, Luciano R, Muraca M, Teti A, Muraca M. Recent advances in mesenchymal stem cell immunomodulation. The role of microvesicles [published online ahead of print November 21, 2013]. *Cell Transplant*. doi:10.3727/096368913X675728.

37. Madrigal M, Rao KS, Riordan N. A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods. *J Transl Med*. 2014;12:260.

38. Wilson SE, Mohan RR, Mohan RR, Ambrosio R Jr, Hong J. The corneal wound healing response: cytokine-mediated interaction of the epithelium, stroma, and inflammatory cells. *Prog Retin Eye Res*. 2001;20:625–637.

39. Epstein RJ, Stulting RD, Hendricks RL, Harris DM. Corneal neovascularization. Pathogenesis and inhibition. *Cornea*. 1987;6:250–257.

40. Chang JH, Gabison EE, Kato T, Azar DT. Corneal neovascularization. *Curr Opin Ophthalmol*. 2001;12:242–249.

41. Peirong L, Longbiao L, Wu Y, Mukaida N, Zhang X. Essential contribution of CCL3 to alkali-induced corneal neovascularization by regulating vascular endothelial growth factor production by macrophages. *Mol Vis*. 2008;14:1614–1622.

42. Sotozono C, He J, Tei M, Honma Y, Kinoshita S. Effect of metalloproteinase inhibitor on corneal cytokine expression after alkali injury. *Invest Ophthalmol Vis Sci*. 1999;40:2430–2434.

43. Maes M. Psychological stress and the inflammatory response system. *Clin Sci (Lond)*. 2001;101:193–194.

44. Kubera M, Obuchowicz E, Goehler L, Brzeszcz J, Maes M. In animal models, psychosocial stress-induced (neuro)inflammation, apoptosis and reduced neurogenesis are associated to the onset of depression. *Prog Neuropsychopharmacol Biol Psychiatry*. 2011;35:744–759.

45. Maes M. Psychological stress, cytokines and the inflammatory response system. *Curr Opin Psychiatry*. 1999;12:695–700.

46. Ma Y, Xu Y, Xiao Z, et al. Reconstruction of chemically burned rat corneal surface by bone marrow-derived human mesenchymal stem cells. *Stem Cells*. 2005;24:315–321.

47. Gupta N, Su X, Popov B, et al. Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. *J Immunol*. 2007;179:1855–1863.

48. Gonzalez-Rey E, Anderson P, Gonzalez MA, et al. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. *Gut*. 2009;58:929–939.

49. Nemeth K, Leekahavanichkul A, Yuen PS, et al. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med*. 2009;15:42–49.

50. Mei S, Haitsma J, Dos Santos C, et al. Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. *Am J Respir Crit Care Med*. 2010;182:1047–1057.

51. Yang D, Cheng Q, Hoover DM, et al. Many chemokines including CCL20/MIP-3alpha display antimicrobial activity. *J Leukoc Biol*. 2003;74:448–455.

52. Egesten A, Eliasson M, Johansson HM, et al. The CXC chemokine MIG/CXCL9 is important in innate immunity against Streptococcus pyogenes. *J Infect Dis*. 2007;195:684–693.

53. Yount NY, Waring AJ, Gank KD, et al. Structural correlates of antimicrobial efficacy in IL-8 and related human kinocidins. *Biochim Biophys Acta*. 2007;1768:598–608.

54. Collin M, Linge HM, Bjartell A, et al. Constitutive expression of the antibacterial CXC chemokine GCP-2/CXCL6 by epithelial cells of the male reproductive tract. *J Reprod Immunol*. 2008;79:37–43.

55. Linge HM, Collin M, Nordenfelt P, et al. The human CXC-chemokine granulocyte chemotactic protein 2 (GCP-2)/CXXL6 possesses membrane disrupting properties and is antibacterial. *Antimicrob Agents Chemother*. 2008;7:2599–2607.

56. Krasnodembskaya A, Song Y, Fang X, et al. Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells*. 2010;28:2229–2238.