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Different Effects of Pro-Inflammatory Factors and Hyperosmotic Stress on Corneal Epithelial Stem/Progenitor Cells and Wound Healing in Mice

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ABSTRACT

Chronic inflammation and severe dry eye are two important adverse factors for the successful transplant of cultured limbal stem cells. The aim of this study was to investigate the effects of inflammation and hyperosmotic stress (a key pathological factor in dry eye) on corneal epithelial stem cells (CESCs) and corneal epithelial wound healing. We observed that the CESCs exhibited significant morphological changes when treated with interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), or hyperosmotic stress. Colony-forming efficiency or colony-forming size was decreased with the increasing concentrations of IL-1β, TNF-α, or hyperosmotic stress, which was exacerbated when treated simultaneously with pro-inflammatory factors and hyperosmotic stress. However, the colony-forming capacity of CESCs recovered more easily from pro-inflammatory factor treatment than from hyperosmotic stress treatment. Moreover, when compared with pro-inflammatory factors treatment, hyperosmotic stress treatment caused a more significant increase of apoptotic and necrotic cell numbers and cell cycle arrest in the G2/M phase. Furthermore, the normal ability of corneal epithelial wound healing in the mice model was suppressed by both pro-inflammatory factors and hyperosmotic stress treatment, and especially severely by hyperosmotic stress treatment. In addition, inflammation combined with hyperosmotic stress treatment induced more serious epithelial repair delays and apoptosis in corneal epithelium. Elevated levels of inflammatory factors were found in hyperosmotic stress-treated cells and mice corneas, which persisted even during the recovery period. The results suggested that pro-inflammatory factors cause transient inhibition, while hyperosmotic stress causes severe apoptosis and necrosis, persistent cell cycle arrest of CESCs, and severe corneal wound healing delay. STEM CELLS TRANSLATIONAL MEDICINE *2019;8:46–57*

SIGNIFICANCE STATEMENT

Chronic inflammation and dry eye are two risk factors that impair the success rate of limbal stem cell transplantation. This study found that, compared with an IL-1β- or TNF-α-induced inflammatory environment, sodium chloride-induced hyperosmotic stress caused persistent impairment of corneal epithelial stem/progenitor cells by inducing more severe cell apoptosis, necrosis, and G2/M arrest of cell cycle and caused more serious epithelial wound healing delay. These findings provide the cellular basis for the strict control of inflammation and dry eye before clinical limbal stem cell transplantation, and the effective control of hyperosmotic stress by dry eye is crucial.

INTRODUCTION

The homeostasis of the ocular surface is vital for the maintenance of healthy corneal epithelium, which is important for the preservation of corneal transparency and vision [1]. The integrity and health of corneal epithelial surface depend largely on proper corneal epithelial wound healing. Various chemical, physical, and pathological injuries and diseases could greatly damage the corneal epithelium, resulting in the disruption of its barrier function and wound formation ability. It is well known that limbal epithelial stem cells (LESCs), which is locate in the limbal region, play an essential role in corneal wound healing [2–4]. Severe ocular surface injury and disease usually cause a partial or

total deficiency of LESCs called limbal stem cell deficiency (LSCD), which is frequently accompanied by a lack of corneal epithelial regeneration and delayed wound healing, eventually resulting in chronic inflammation, neovascularization, ingrowth of conjunctival cells, and even blindness [5–7].

In the past decade, cultured limbal epithelial transplantation (CLET) has been used for the therapy of LSCD and promotion of corneal epithelial wound healing [8–10]. Although the culture and transplantation techniques and outcome parameters varied among the studies, the overall success rate was still around 70% as reported [9, 11–15]. However, the success rate may be varied according to the cause of LSCD and poor optimization of the ocular surface prior to transplantation [8, 16]. Eyes with severe cicatricial ocular surface disorders or persistent epithelium deficiency, such as Stevens-Johnson syndrome, toxic epidermal necrolysis, ocular cicatricial pemphigoid, and chemical/thermal burn of the cornea, have poorer final corneal epithelialization [17–19].

With autoimmune diseases, it is usually hard to restore a stable ocular surface because of the relentless inflammation and dry ocular surface. For instance, in patients with Stevens-Johnson syndrome, the eyes always exhibit complicated ocular surface abnormalities, including decreased tear secretion, persistent inflammation, squamous metaplasia of the conjunctival epithelium, trichiasis, and lid margin keratinization [17, 20]. Some studies suggested that decreased tear production, lid abnormalities, and inflammation may be important restricted factors for successful limbal stem cell transplantation, either for keratolimbal allograft or CLET [21–26]. Therefore, it can be considered that chronic inflammation and severe dry eye condition are two important adverse factors for the success of cultured limbal epithelium transplantation. In terms of inflammation, pro-inflammatory cytokines (interleukin [IL]-1α, IL-1β, tumor necrosis factor [TNF]-α, interferons [IFN]-γ, and IL-6) and chemokines (monocyte chemoattractant protein 1 [MCP]-1 and IL-8) have been extensively reported to be involved in mediating corneal inflammatory response [27–32]. In addition, the central mechanism in the pathogenesis of ocular surface damage from dry eye is thought to be tear hyperosmolarity, with studies demonstrating that hyperosmotic stress can induce cell cycle arrest, cell apoptosis, DNA damage, reactive oxygen species overproduction, and pro-inflammatory factor infiltration [33–38]. However, almost all research introduced above was done in acute conditions with relative higher osmolarity (≥450 mosm) and short-time exposure (≤48 hours). The different effects or coeffects of persistent inflammation and hyperosmolarity on the homeostasis of corneal epithelial stem cells (CESCs) and the ability of epithelial wound healing remains unclear. Here, we demonstrated that pro-inflammatory cytokines always cause transient inhibition, while sodium chloride-induced hyperosmosis causes more severe damage to CESCs by leading to apoptosis or necrosis process, which suggests that the impacts of these two risk factors vary considerably in the survival of CESCs.

Materials and Methods

CESC Culture and Treatment

Mouse corneal epithelial stem/progenitor cell line (TKE2, Public Health England, 11033107, http://www.phe-culturecollections.org.uk/) was presented by Dr. Tetsuya Kawakita of Keio University (Tokyo, Japan). The cells were cultured in a keratinocyte serum-free medium (KSFM, Gibco-Invitrogen, Carlsbad, CA) supplemented with 5 ng/ml human recombinant epithelial growth factor, 50 μg/ml bovine pituitary extract (BPE) at 37°C and 5% CO_2 [39]. The cell line has been characterized in our group [40, 41]. TKE2 cells were treated with 1, 5, 10, and 20 ng/ml of IL-1β (Millipore, Temecula, CA), TNF-α (R&D, Minneapolis, MN), or varying osmolarities (340, 360, 400, or 450 mosm) achieved by the addition of sodium chloride in a BPE-free KSFM medium. In some experiments, TKE2 cells were treated with 10 ng/ml of IL-1β or TNF-α combined with hyperosmotic stress (340, 360, or 400 mosm).

The rabbits used for the primary corneal limbal epithelial cells culture was purchased from Qingdao Kangda Rabbit Industry Development Co., Ltd. Three rabbits were used in this assay. Rabbit corneal limbal epithelial cells were cultured as below procedure: the corneal limbal tissues from New Zealand rabbits were treated with 2.4 U/ml Dispase (Roche, Indi-anapolis, IN) in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) overnight at 4°C. The limbal epithelium was detached under a dissecting microscope and digested with 0.25% trypsin/0.02% EDTA for 15 minutes at 37°C. The acquired rabbit primary limbal epithelial cells were suspended in DMEM/F-12 medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), Insulin-Transferrin-Selenium Supplement (ITS, Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1 nM cholera toxin (Sigma, St. Louis, MO), 2 nM 3,305-Triiodo-L-thyronine Sodium salt (Sigma), 0.4 ng/ml hydrocortisone succinate (Wako, Osaka, Japan), 2 mM L-Glutamine (Invitrogen), penicillinestreptomycin (Hyclone, Logan, UT), and 10 ng/ml recombinant human epidermal growth factor (R&D Systems). NIH-3 T3 cells were treated with 4 mg/ml mitomycin C (Haiz-heng, Taizhou, China) for 2 hours at 37°C and reinoculated at a density of 3×10^5 cells in six-well plate as feeder layers. The rabbit primary limbal epithelial cells were seeded on NIH-3T3 feeder surface. Rabbit corneal limbal epithelial cells were treated with 10 ng/ml of IL-1β, TNF-α, or hyperosmotic stress at 400 mosm. All of the animal experiments were conducted in accordance with the Animal Care and Use Committee guidelines of the Shan-dong Eye Institute and the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Colony-Forming Assay

For the colony-forming assay, TKE2 cells were inoculated at a density of 1,000 cells per well of six well plate, incubated in KSFM overnight, and treated with IL-1β, TNF-α, or hyperosmotic stress for 8 days. The colonies were washed with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde for 10 minutes. Wells were rinsed once again with PBS and colonies were stained with 0.2% crystal violet (Sigma) for 10 minutes at room temperature. Excess stain was removed by washing three times with PBS and then the colony number was counted and evaluated using Image-J software. In this analysis, the colonies were divided into large (d > 1.5 mm), medium sized (1.0 mm ≤ d ≤ 1.5 mm), and small (d < 1.0 mm) colonies according to the diameter of the colony.

Table 1. Primers used for real-time PCR

Gene	Forward primer	Reverse primer
IL-1β	CTTTCCCGTGGACCTTCCA	CTCGGAGCCTGTAGTGCAGTT
TNF-α	ATGAGAAGTTCCCAAATGGC	CTCCACTTGGTGGTTTGCTA
IL-8	CAAGGCTGGTCCATGCTCC	TGCTATCACTTCCTTTCTGTTGC
MCP-1	GCCTCCTGCTCATAGCTACCA	GACACTGGCTGCTTGTGATTCTC
GAPDH	ATGCTGGCGCTGAGTACGT	AGCCCCAGCCTTCTCCAT

Immunofluorescence Staining

Eyeballs were snap-frozen in Tissue-Tek optimum cutting temperature compound (Sakura Finetechnical, Tokyo, Japan). For immunofluorescent staining, cultured cells or cryosections were fixed using 4% para-formaldehyde for 10 minutes at room temperature and permeabilizated with 0.1% Triton X-100 (Sigma) for 30 minutes. Nonspecific staining was blocked with 5% normal goat serum. The samples were incubated with ΔNp63 (Biolegend, SanDiego, CA), Ki67, importin 13, ck3/12, involucrin, or K12 (Abcam, Cambridge, MA) primary antibodies at 4°C overnight. The samples were then incubated with fluorescein-conjugated secondary antibodies (Invitrogen) at room temperature for 1 hour. Cell staining was examined under a Nikon confocal laser-scanning microscope. Secondary control was incubated with normal serum and the appropriate secondary antibodies. For the staining of TUNEL, cryosections were fixed with 4% para-formaldehyde and then performed using In SituCell Death Detection Kit (Roche) according to the instruction manual.

Cell Recovery Assay

For the analysis of recovery capacity, the IL-1β, TNF-α, and hyperosmotic stress-treated cells were harvested and reseeded at a density of 1,000 cells per well, and incubated in a normal medium without pro-inflammatory cytokines or hyperosmotic stress for another 8 days. Colony-forming efficiency was assessed as mentioned above.

Cell Apoptosis Analysis

The IL-1β, TNF-α, or hyperosmotic stress-treated cells were harvested and stained with Annexin V/propidium iodide (PI; BD Bioscience, San Jose, CA) according to the manufacturer's recommendations. In brief, the collected cells were suspended in a binding buffer and incubated with Annexin V-FITC and PI for 15 minutes at room temperature. The cells were examined by FACScalibur flow cytometry (BD Bioscience) with a minimum of 10,000 cells counted for each group, and data analysis was performed with FlowJo software.

Cell Cycle Analysis

The IL-1β, TNF-α, or hyperosmotic stress-treated cells were harvested, fixed in ice-cold 70% ethanol, and incubated in PBS, containing 50 μg/ml PI and 0.25 mg/ml RNase A in the dark at 37°C for 30 minutes. The measurements were made with a Becton Dickinson FACS Calibur machine. A total of 20,000 cells was collected by FACS and analyzed using Modifit software. On each occasion, at least three samples of each treatment were analyzed.

Corneal Epithelial Wound Healing

Adult male C57BL/6 mice purchased from the Beijing Pharmacology Institute (Beijing, China) were used in this experiment. Normal mice were anesthetized by an intraperitoneal injection of xylazine (7 mg/kg) and ketamine (70 mg/kg) followed by topical application of 2% xylocaine. The central corneal epithelium (~2.5 mm in diameter) was removed using algerbrush II corneal rust ring remover (Alger Co, Lago Vista, TX) and subsequently applied with ofoxacin eye drops to avoid infection. Physiological saline solution contains 10 ng/ml of IL-1β, TNF-α, 400 mosm of sodium chloride, 10 ng/ml of IL-1β combined with 400 mosm of sodium chloride, or 10 ng/ml of TNF-α combined with 400 mosm of sodium chloride were topically administered (5 μl, four times per day) starting the same day of corneal epithelial debridement and physiological saline was used as control. The corneal epithelium healing was monitored at 24, 36, and 48 hours by instilling 0.25% fluorescein sodium and photographed under slit lamp (BQ900, Haag-Streit, Bern, Switzerland). The staining area was analyzed by using image J software and calculated as the percentage of residual epithelial defect. All the animal experiments in this work were conducted in accordance with the Animal Care and Use Committee guidelines of the Shan-dong Eye Institute and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Reverse Transcription–Quantitative Polymerase Chain Reaction

Total RNA from TKE2 cells or mice corneas was extracted using Nucleospin RNA Kits (MACHEREY-NAGEL, Dűren, Germany). cDNAs were synthesized using the Primescript RT Master Mix (TaKaRa, Dalian, China) according to the manufacturer's protocol. Real-time PCR was carried out using EvaGreen 2× qPCR MasterMix and the Rotor-Gene Q (QIAGEN, Hilden, Germany). The cycling conditions were 10 minutes at 95°C followed by 40 two-step cycles (15 seconds at 95°C and 60 seconds at 60°C). The quantification data were analyzed with the Rotor-Gene Q software (QIAGEN), using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The specific primers used in this assay are listed in Table 1.

ELISA Assay

Supernatant from normal, 400 mosm osmolarity treated and recovery cells of 400 mosm osmolarity treated were collected and analyzed by ELISA kits including IL-1β and TNF-α (Proteintech, Wuhan, China) according to the manufacturer's procedures. The concentrations of total proteins were calculated using the Pierce BCA Protein Assay Kit (Pierce; Thermo Fisher Scientific, Inc.).

Statistical Analysis

The data were presented as means ± SD. The differences between control and experimental groups were tested with one-way ANOVA analysis of variance using SPSS10.0. $p < .05$ was considered to be statistically significant.

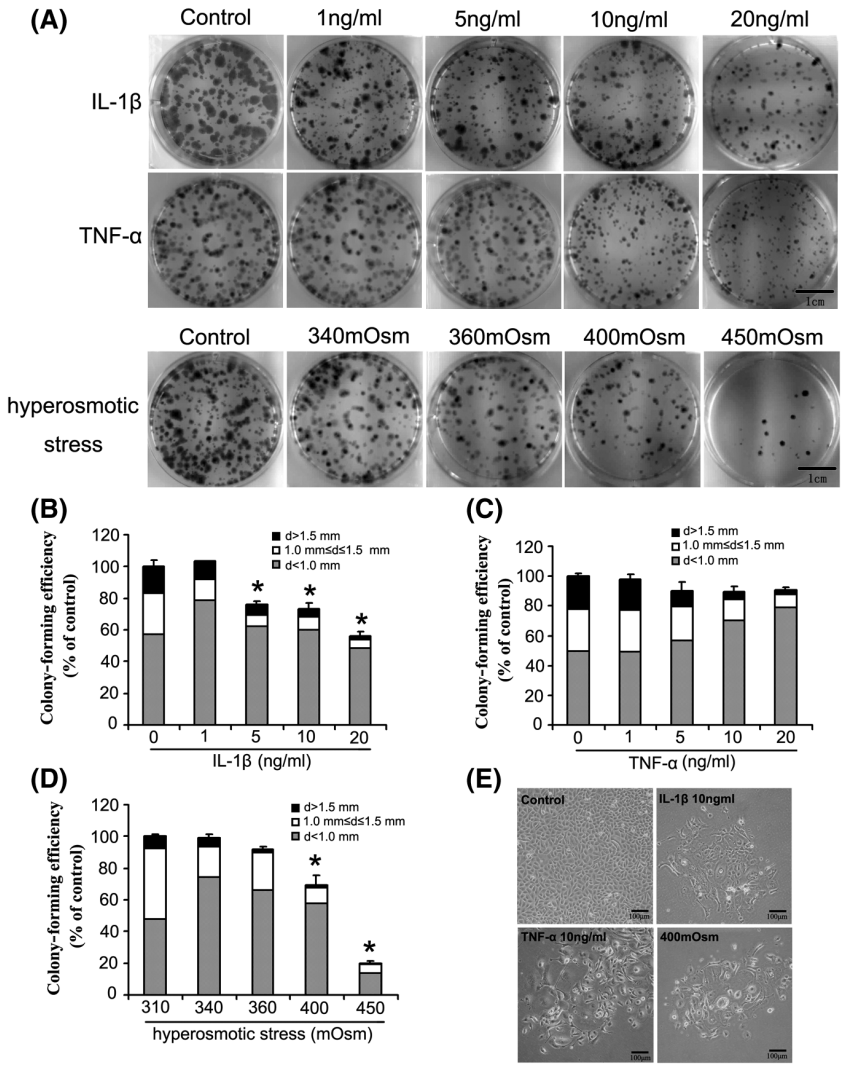

Figure 1. Pro-inflammatory cytokines and hyperosmotic stress inhibited the colony-forming capacity of TKE2 cells. TKE2 cells were incubated with 1, 5, 10, and 20 ng/ml of IL-1β, TNF-α, or varying osmolarities (340, 360, 400, or 450 mosm) induced by sodium chloride for 8 days and stained with crystal violet **(A)**, then the colony-forming efficiency (% of control) was calculated **(B–D)**. The colonies were divided into large (d > 1.5 mm), medium (1.0 mm ≤ d ≤ 1.5 mm), and small sized clones (d < 1.0 mm) according to the diameter of the colonies. *p < .05, the total colony-forming efficiency compared with control. The morphological alteration of TKE2 cells after pro-inflammatory factors and hyperosmotic stress treatment for 8 days were shown **(E)**. Abbreviations: IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor alpha.

RESULTS

Inhibitory Effects of Pro-Inflammatory Cytokines and Hyperosmotic Stress on Colony-Forming Capacity of CESCs

The effects of different concentrations of IL-1β or TNF-α on colony-forming efficiency in mouse corneal stem/progenitor cells (TKE2 cells) were investigated, as revealed by crystal violet staining (Fig. 1A). As shown in Figure 1B, colony density was decreased by IL-1β treatment in a dose-dependent manner. The total colony-forming efficiency (% of control) was 73.1% ± 4.0% in 10 ng/ml IL-1β-treated TKE2 cells. While the colony density was decreased slightly by TNF-α treatment, showing 89.3% ± 3.6% total colony-forming efficiency (% of control) in 10 ng/ml TNF-α-treated TKE2 cells (Fig. 1C). However, the colony sizes were decreased markedly both in IL-1β- and TNF-α-treated TKE2 cells in a concentration-dependent manner. The colony-forming efficiencies (% of total control) for large colonies (d > 1.5 mm) were 4.9% ± 0.3% and 4.8% ± 0.8% in 10 ng/ml IL-1β and 10 ng/ml TNF-α-treated TKE2 cells, respectively. And the colony-forming efficiencies (% of total control) for medium sized colonies (1.0 mm ≤ d ≤ 1.5 mm) were 8.2% ± 1.6% and 14.5% ± 1.7% respectively in 10 ng/ml IL-1β and 10 ng/ml TNF-α-treated TKE2 cells (Fig. 1B, C).

The TKE2 cells were treated with sodium chloride in order to simulate the hyperosmotic environment in dry eye. To determine the effects of hyperosmotic stress on colony-forming efficiency of CESCs, TKE2 cells were incubated with various osmolarities for 8 days. Also, as presented by crystal violet staining (Fig. 1A), the colony-forming efficiencies and colony sizes were obviously reduced. A quantitative analysis of the colony density showed that the total colony-forming efficiencies (% of control) were 99.2% ± 2.9%, 91.7% ± 2.2%, 69.4% ± 7.4% and 19.8% ± 1.7% for the 340, 360, 400, and

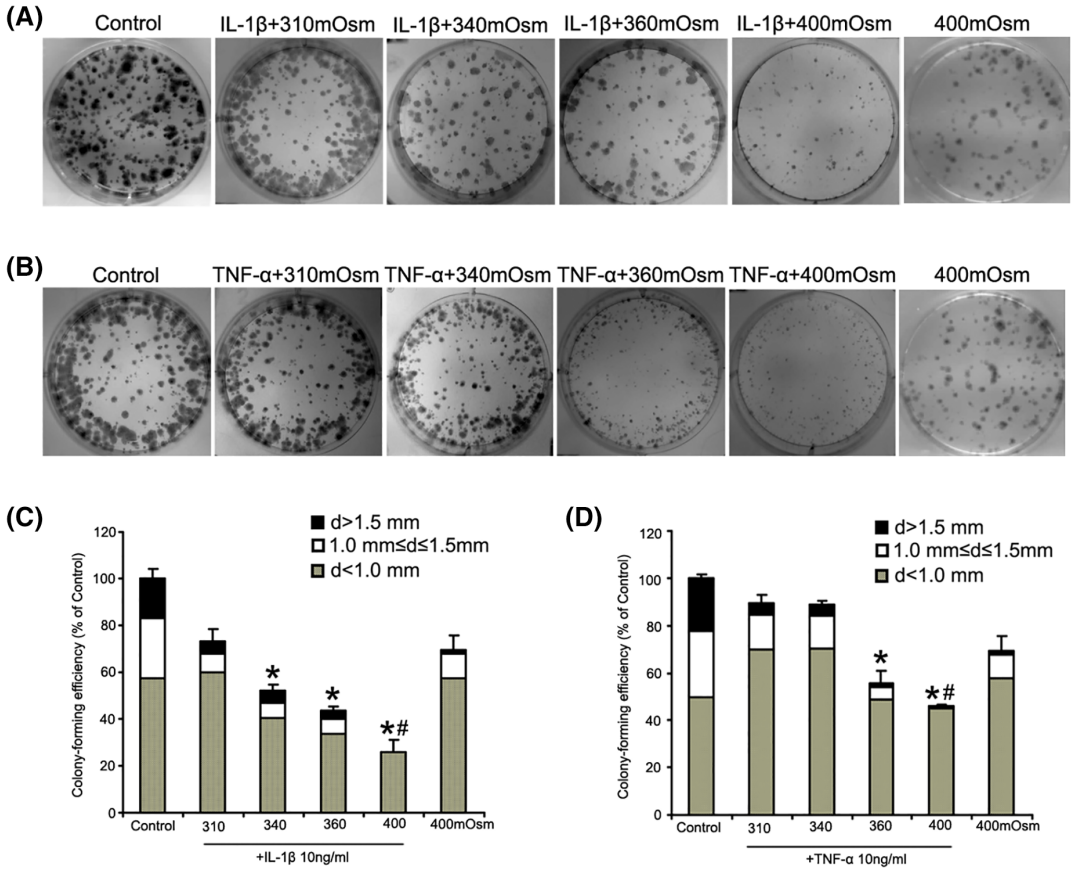

Figure 2. Effects of pro-inflammatory cytokines combined with hyperosmotic stress on the colony-forming of TKE2 cells. TKE2 cells were treated with IL-β **(A)** or TNF-α **(B)** combined with 340, 360, or 400 mosm osmolarities for 8 days and stained with crystal violet. A quantitative analysis of the colony-forming efficiency showed that the colony-forming capacity was decreased more severely in combined administration group than that of IL-β- **(C)** or TNF-α- **(D)** treated groups. *p < .05, the total colony-forming efficiency compared with IL-β- or TNF-α-treated group. #p < .05, the large colony-forming efficiencies of combined administration groups compared with that of 400 mosm osmolarity-treated group. Abbreviations: IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor alpha.

450 mosm osmolarity-treated TKE2 cells, respectively (Fig. 1D). The colony-forming efficiencies (% of total control) for large colonies (d > 1.5 mm) and medium sized colonies (1.0 mm ≤ d ≤ 1.5 mm) were decreased to 1.6% ± 0.1% and 10.3% ± 2.5%, respectively, under 400 mosm osmolarity treatment (Fig. 1D).

From the morphological observation, pro-inflammatory factors (10 ng/ml IL-1β or 10 ng/ml TNF-α) and hyperosmotic stress (400 mosm osmolarity) treatment for 8 days both could cause similar enlarged, irregular, and detached morphological changes in TKE2 cells (Fig. 1E). Which was also confirmed by the experiments performed in pro-inflammatory factors (10 ng/ml IL-1β or 10 ng/ml TNF-α) or hyperosmotic stress (400 mosm osmolarity)-treated cultured rabbit CESCs, showing that pro-inflammatory factors and hyperosmotic stress have similar colony size inhibition and morphological alteration capacities in rabbit CESCs as in TKE2 cells (Supporting Information Fig. S1A). Especially, large increase of cell size was observed in IL-1β-treated rabbit CESCs. Considering of the inhibition effects of pro-inflammatory factors and hyperosmotic stress on the colony-forming efficiencies and sizes, and the morphological alteration in CESCs, we chose 10 ng/ml IL-1β, 10 ng/ml TNF-α, and 400 mosm osmolarity as the treatment concentration for the remaining studies.

Effects of Pro-Inflammatory Cytokines Combined with Hyperosmotic Stress on Colony-Forming Capacity of CESCs

The combined effects of pro-inflammatory cytokines (10 ng/ml IL-1β or TNF-α) and varying osmolarities (340, 360, and 400 mosm) on the colony-forming capacity of corneal epithelial cells were also detected in the present study (Fig. 2). Compared to the treatment with 10 ng/ml IL-1β or TNF-α alone, combined administration with hyperosmotic stress and pro-inflammatory cytokine cause significant decline in TKE2 cells and presenting an osmolarity-dependent manner (Fig. 2C, D). In addition, even the low osmolarity at 340 mosm combined with 10 ng/ml IL-1β treatment brought about a significant decline of colony-forming efficiency compared with only 10 ng/ml IL-1β-treated cells (Fig. 2C). However, the colony-forming efficiency in TNF-α combined hyperosmotic stress-treated group was decreased more significantly than TNF-α treated alone when osmolarity was above 360 mosm (Fig. 2D). Also, the colony sizes were decreased more markedly when TKE2 cells were treated with pro-inflammatory cytokines combined with hyperosmotic stress. Moreover, there was almost no large or medium sized colony formation when the combined administration of osmolarity at 400 mosm with IL-1β or TNF-α. The large colony formation efficiency between the

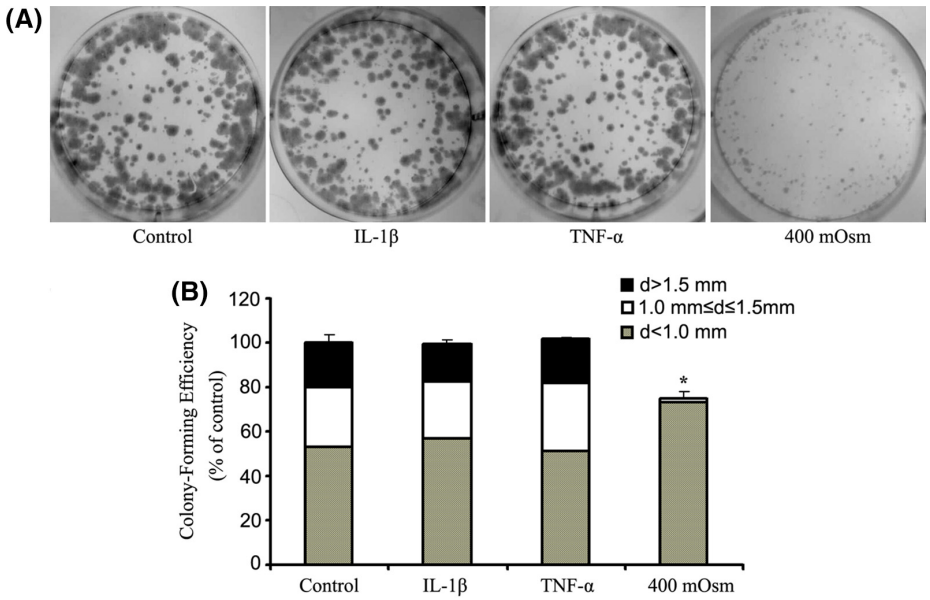

Figure 3. The recovery capacity of TKE2 cells from IL-1β, TNF-α, or hyperosmotic stress treatment. TKE2 cells were replated in normal medium after the treatment of 10 ng/ml IL-1β, 10 ng/ml TNF-α, or 400 mosm osmolarity, and then the colony-forming capacity was detected **(A)**. The quantitative analysis of colony-forming efficiency showed that TKE2 cells were easily recovered from IL-1β or TNF-α treatment; however, TKE2 cells were hard to recover from hyperosmotic stress treatment **(B)**. *$p < .05$, the total colony-forming efficiency compared with control. Abbreviations: IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor alpha.

combined treatment group and only 400 mosm osmolarity treatment group had significant statistic differences ($p < .05$; Fig. 2C, D).

The Recovery Capacities of Corneal Epithelial Cells after IL-1β, TNF-α, or Hyperosmotic Stress Treatment

To detect the recovery capacities of corneal epithelial cells after the treatment of pro-inflammatory cytokines or hyperosmotic stress, TKE2 cells were first treated with 10 ng/ml IL-1β, 10 ng/ml TNF-α, or 400 mosm osmolarity for 8 days, and then digested and replated in normal medium for another 8 days to perform the examination of colony-forming efficiency. As shown in Figure 3, the colony-forming density or size was easily recovered from the treatment of IL-1β or TNF-α. However, both the colony-forming density and the colony size were hard to be recovered to normal levels after the treatment with hyperosmotic stress at the first passage.

Pro-Inflammatory Cytokines or Hyperosmotic Stress Regulated the Differentiation of TKE2 Cells

To further evaluate the impairment of pro-inflammatory cytokines or hyperosmotic stress on CESCs, the expression of Ki67, ΔNp63, importin 13, ck3/12, and involucrin were measured by immunofluorescence staining. As shown in Figure 4, normal TKE2 cells exhibited the characterizations of CESCs, manifesting as high expression levels of cell proliferation marker Ki67 and the CESCs marker ΔNp63, importin 13, and low expression levels of the corneal epithelial differentiation marker ck3/12 and involucrin. However, Ki67, ΔNp63, and importin 13 in TKE2 cells were downregulated, and ck3/12 and involucrin were upregulated significantly by IL-1β, TNF-α, or hyperosmotic stress treatment.

Effects of Pro-Inflammatory Cytokines or Hyperosmotic Stress on Cell Apoptosis and Cell Cycle of TKE2 Cells

The effects of pro-inflammatory cytokines or hyperosmotic stress on the apoptosis and cell cycle of corneal epithelial cells were investigated in this study by FACS analysis. The apoptotic profiles of different treatment were shown in Figure 5A. The late apoptotic cells were visible in the upper right and early apoptotic cells in the lower right quadrants, while the necrotic cells were visible in the upper left and live cells in the lower left quadrants, respectively. The apoptotic cells were counted as the percent of Annexin V+ cells, and the necrotic cells were counted as Annexin V-/PI+ cells (Fig. 5B). Exposure of 400 mosm osmolarity caused a significant increase in the percentage of apoptotic cells (24.49% ± 5.41%) compared with that of untreated (4.79% ± 0.48%), IL-1β-treated (6.79% ± 1.88%), and TNF-α-treated (7.79% ± 2.57%) groups. While IL-1β- or TNF-α-treated cells have no significant effect on the amount of apoptotic cells as compared to the untreated cells. IL-1β, TNF-α, and 400 mosm osmolarity treatment increased the number of necrotic cells to 5.71% ± 0.30%, 4.23% ± 0.17%, and 8.93% ± 1.66%, respectively, as compared to untreated control (0.37% ± 0.08%). And 400 mosm osmolarity treatment also increased the number of necrotic cells significantly, compared with that of IL-1β or TNF-α treatment. Following the treatment of TKE2 cells with 10 ng/ml IL-1β, 10 ng/ml TNF-α, or 400 mosm osmolarity for 8 days, the cell population at G2/M phase increased to 12.40% ± 0.95%, 13.53% ± 1.39%, and 21.07% ± 2.09%, respectively, compared with that of untreated control (6.92% ± 1.36%), which was accompanied with a marked number decrease in S phase (Fig. 5D). In addition, 400 mosm osmolarity treatment induced more severe G2/M-arrest in TKE2 cells than that of IL-1β or TNF-α treatment. These results

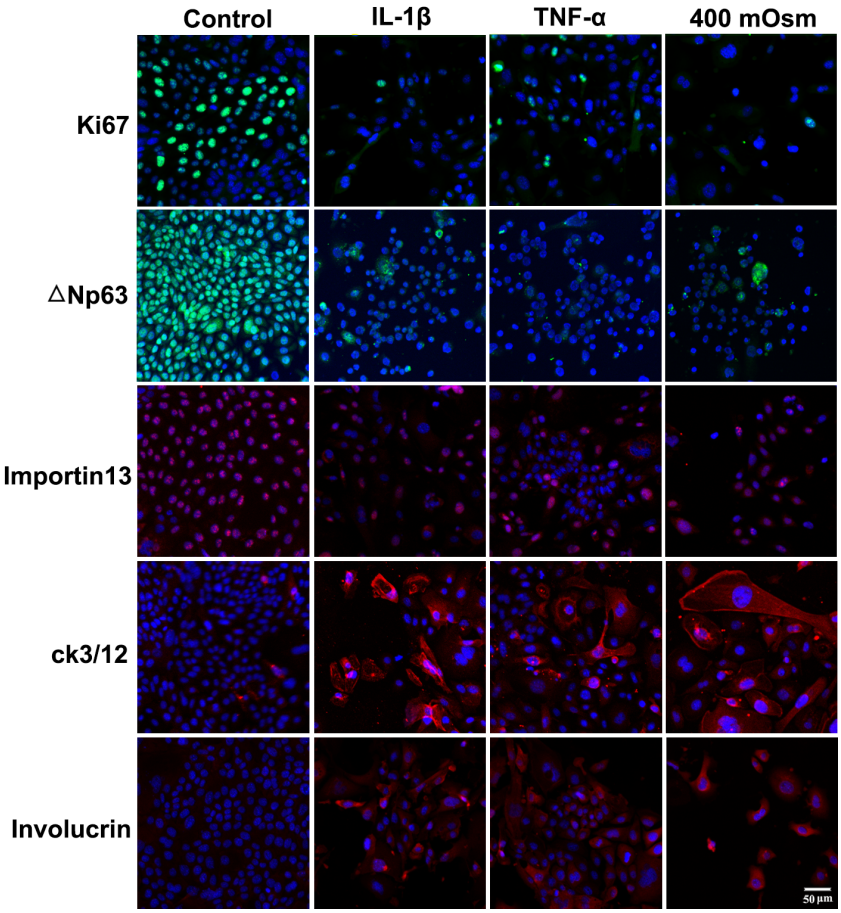

Figure 4. Pro-inflammatory cytokines and hyperosmotic stress promoted the differentiation of TKE2 cells. IL-1β, TNF-α, or 400 mosm osmolarity treatment decreased the expression of cell proliferation marker Ki67 and the corneal epithelial stem cells marker ΔNp63 and importin 13; however, the corneal epithelial differentiation marker ck3/12 and involucrin were increased significantly. Abbreviations: IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor alpha.

clearly indicated that, compared with IL-1β or TNF-α treatment, hyperosmotic stress treatment had more significant damage on the survival of corneal epithelial cells, at least partially through inducing cell apoptosis, necrosis, and G2/M-arrest. As well, the effects of pro-inflammatory cytokines or hyperosmotic stress on the apoptosis and cell cycle of cultured rabbit corneal epithelial cells was examined and similar induction of apoptosis and G2/M arrest as in TKE2 cells were found by FACS assay (Supporting Information Fig. S1B–E).

Pro-Inflammatory Cytokines and Hyperosmotic Stress Inhibit Corneal Epithelial Wound Healing

To determine the effects of pro-inflammatory cytokines and hyperosmotic stress on corneal epithelial wound healing, topical IL-1β, TNF-α, 400 mosm osmolarity, IL-1β combined with 400 mosm osmolarity, and TNF-α combined with 400 mosm osmolarity solutions were respectively administrated to wounded corneas and wound closure was evaluated over 48 hours. 10 ng/ml of IL-1β, TNF-α, 400 mosm osmolarity, IL-1β combined with 400 mosm osmolarity, and TNF-α combined with 400 mosm-treated mice exhibited significant delay of re-epithelialization rate at 24 hours after corneal epithelium scrape compared to vehicle treatment (Fig. 6A, n = 6/each group). The defects of corneal epithelium in vehicle-treated normal mice were completely closed within 48 hours, while there are still 4.2% ± 2.1%, 9.0% ± 2.1%, 15.8% ± 2.2%, 27.7% ± 0.4%, and 27.1% ± 5.5% of remaining epithelial defect area, respectively, in 10 ng/ml of IL-1β, TNF-α, 400 mosm osmolarity, IL-1β combined with 400 mosm osmolarity, and TNF-α combined with 400 mosm osmolarity-treated mice (Fig. 6B). These results also showed that 400 mosm osmolarity treatment induced even more obvious delay of epithelial wound healing than that of IL-1β or TNF-α treatment, while IL-1β or TNF-α combined with 400 mosm osmolarity treatment caused even more severe delay of wound closure rate than 400 mosm osmolarity treatment alone. We further detected the proliferation, differentiation and apoptosis during wound healing process by the staining of Ki67, K12, and TUNEL. All these treatments decreased the Ki67 expression (Fig. 6C) and increased the K12 expression (Fig. 6D) obviously, implying the suppression effects to the cell proliferation and promotion effects to cell differentiation in corneal epithelium. In addition, TUNEL staining showed that IL-1β and TNF-α treatment induced slight apoptosis in corneal epithelium; however, 400 mosm osmolarity treatment resulted in stronger apoptosis in corneal epithelium. Moreover, the apoptosis situation was more aggravated in corneal epithelium when combined administration with pro-inflammatory cytokines and hyperosmotic

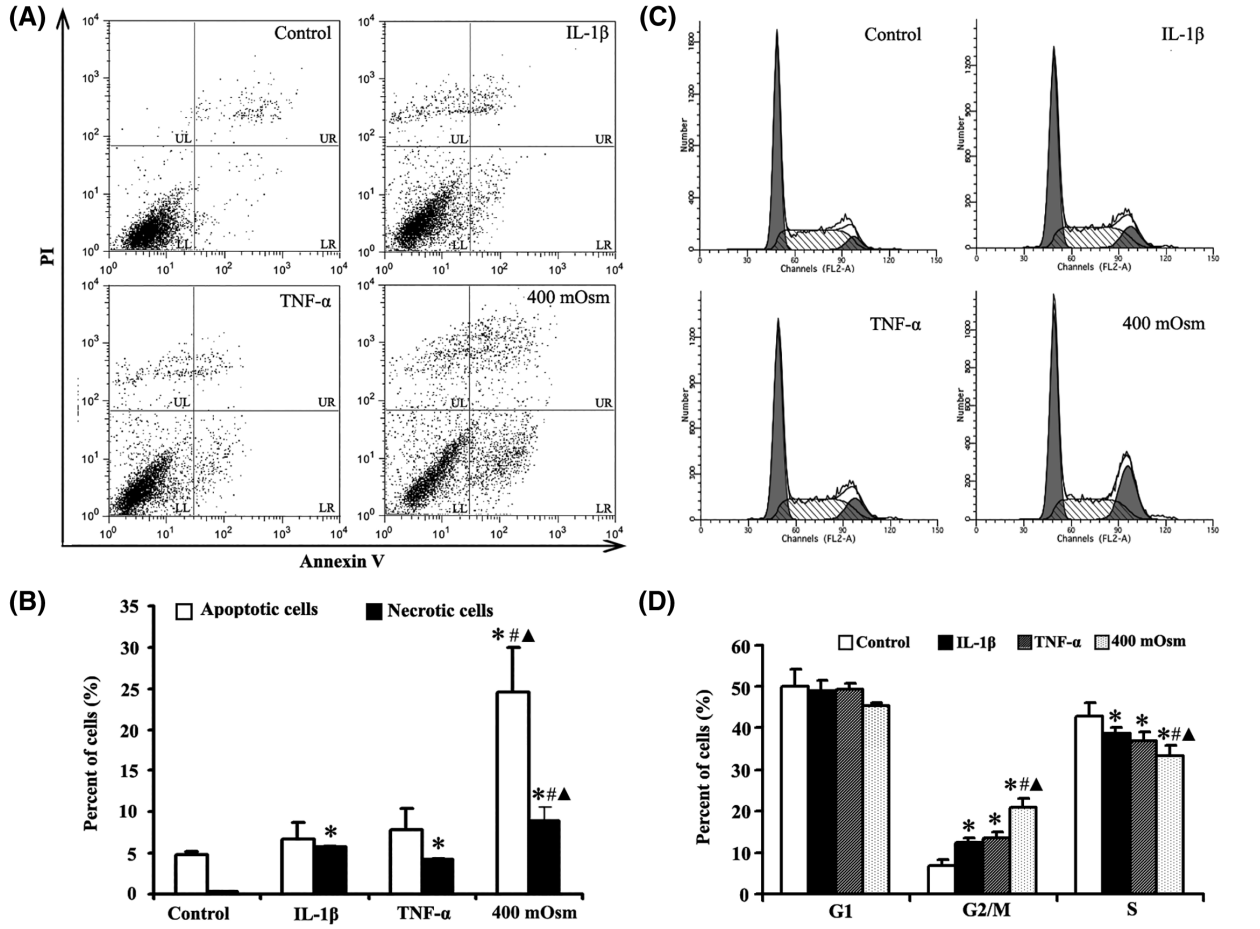

Figure 5. Effects of pro-inflammatory cytokines and hyperosmotic stress on the apoptosis and cell cycle of TKE2 cells. Following the treatment of TKE2 cells with 10 ng/ml IL-1β, 10 ng/ml TNF-α, or 400 mosm osmolarity for 8 days, the cell apoptosis were detected by FACS analysis **(A)**. The results showed that hyperosmotic stress treatment induced more severe cell apoptosis and necrosis in TKE2 cells than that of IL-1β or TNF-α treatment **(B)**. Following the treatment of TKE2 cells with 10 ng/ml IL-1β, 10 ng/ml TNF-α, or 400 mosm osmolarity for 8 days, the cell cycle were detected by fluorescence-activated cell sorting analysis **(C)**. The results showed that hyperosmotic stress treatment induced more severe G2/M arrest in TKE2 cells than that of IL-1β or TNF-α treatment **(D)**. *$p < .05$ compared with control; #$p < .05$ compared with IL-1β treatment; ▲$p < .05$ compared with TNF-α treatment. Abbreviations: IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor alpha.

stress. Besides, apoptosis level of corneal epithelial cells was more pronounced at 36 hours than at 48 hours after the scraping of corneal epithelium (Fig. 6E).

Hyperosmotic Stress Upregulated the Expression of Inflammatory Factors

The mRNA and protein production levels of inflammatory factors were examined after hyperosmotic stress treatment as well as after 8 days recovery. It can be seen in Figure 7A, the mRNA expression of IL-1β, TNF-α, MCP-1, and IL-8 were upregulated significantly after hyperosmotic stress treatment, and the levels of IL-1β, TNF-α, and MCP-1 still remained stable after the 8 days recovery. As well, the protein secretion of IL-1β and TNF-α were increased markedly after hyperosmotic stress treatment, and their high expression levels still remained stable during the 8 days recovery period (Fig. 7B). In mouse model with scraping epithelium, we also found more pronounced mRNA expression levels of IL-1β, TNF-α, MCP-1, and IL-8 in corneas after topical application of 400 mosm osmolarity solution (Fig. 7C).

Discussion

Transplantation of cells cultured from the corneal limbus is a clinical procedure to treat LESC deficiency and healing of corneal epithelium [12]. However, chronic inflammation and dry eye are two risk factors that impair the success rate of limbal stem cell transplantation. In this study, we explored the effects of persistent inflammation and hyperosmosis on fate-decision of corneal epithelial stem/progenitor cells in vitro and corneal epithelial wound healing in vivo. These findings show that, compared with an IL-1β- or TNF-α-induced inflammatory environment, sodium chloride-induced hyperosmotic stress caused persistent impairment on corneal epithelial stem/progenitor cells by inducing more severe cell apoptosis, necrosis, and G2/M arrest of cell cycle and caused more serious epithelial wound healing delay.

The mice corneal epithelial stem/progenitor cells used in this study have high expression levels of limbal progenitor cells markers ΔNp63 and importin 13 and have low expression levels of differentiated markers ck3/12 and involucrin, implying

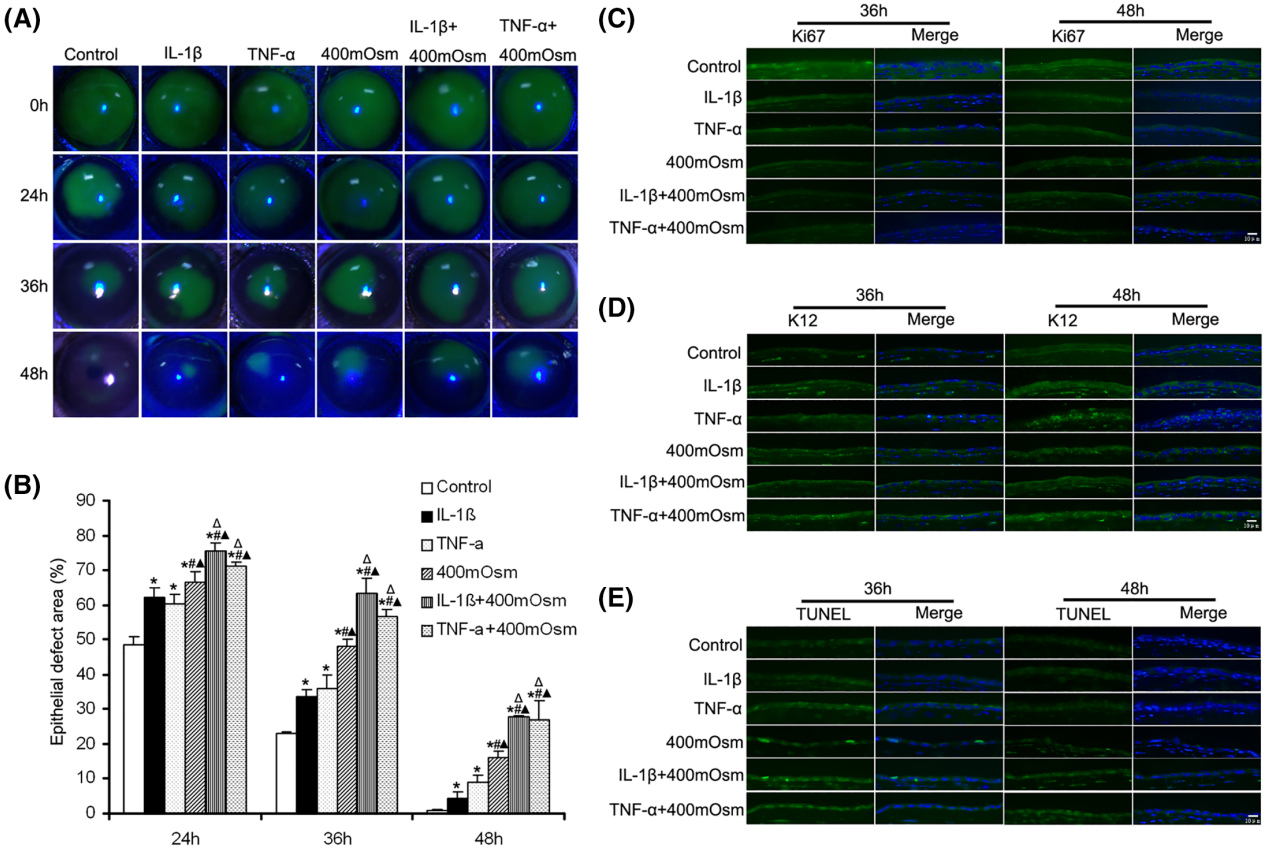

Figure 6. Pro-inflammatory cytokines and hyperosmotic stress suppressed the corneal epithelial wound healing in normal mice. Topical application of physiological saline solution with or without 10 ng/ml IL-1β, 10 ng/ml TNF-α, 400 mosm osmolarity, IL-1β combined with 400 mosm osmolarity, and TNF-α combined with 400 mosm osmolarity were used to examine the influence of pro-inflammatory cytokines and hyperosmotic stress on corneal wound healing. The corneal epithelial defect was stained with fluorescein sodium at 24, 36, and 48 hours after the corneal epithelium scrape **(A)**. Histogram of residual epithelial defect is presented as the percentage of the original wound size (n = 6 per group) **(B)**. *p < .05 compared with control; #p < .05 compared with IL-1β treatment; ▲p < .05 compared with TNF-α treatment; Δp < .05 compared with 400 mosm osmolarity treatment. And the proliferation, differentiation, and apoptosis were detected by Ki67 **(C)**, K12 **(D)**, and TUNEL **(E)**, staining respectively. Abbreviations: IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor alpha.

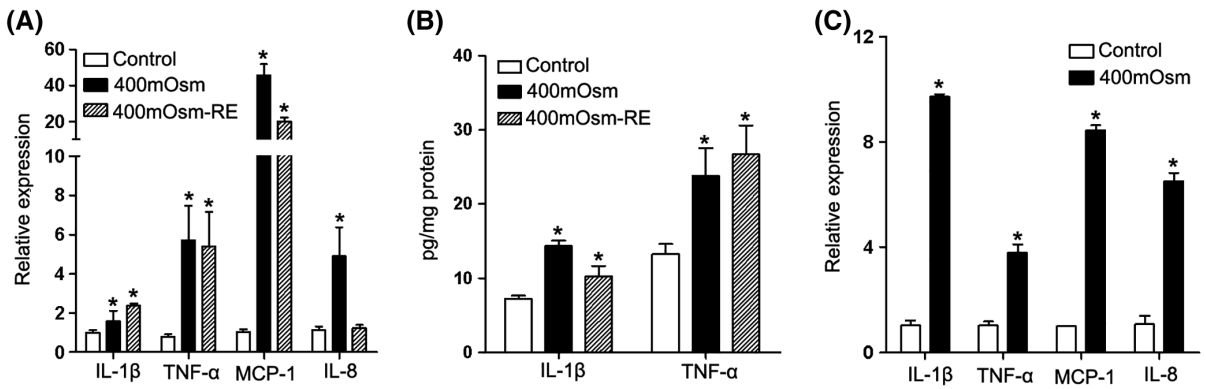

Figure 7. Hyperosmotic stress upregulated the expression of inflammatory factors. The inflammatory factors expression in TKE2 cells after 400 mosm osmolarity treatment, as well as after 8 days recovery were examined both in mRNA levels **(A)** and in protein secretion levels **(B)**. The elevated levels of inflammatory factors were detected in the 400 mosm osmolarity treatment condition and persisted during the 8 day recovery period. In addition, the elevated mRNA expression of IL-1β, TNF-α, MCP-1, and IL-8 were found in corneas with topical application of 400 mosm osmolarity solutions **(C)**. *p < .05 compared with control. Abbreviations: IL-1β, interleukin-1 beta; IL-8, interleukin-8; TNF-α, tumor necrosis factor alpha.

that these cells still retain stem cell properties. The TKE2 cells were cultured in keratinocyte serum-free low-Ca^{2+} medium to maintain the undifferentiated state, and this cell line has already been characterized and used for the study of CESC and corneal wound healing in other reports [40–43]. In addition, in order to validate our results from the TKE2 cells, we used primary cultured rabbit corneal epithelial cells for key experiments such as cell cycle and apoptosis detection.

Inflammatory cell infiltration was observed in corneal pannus specimens obtained from patients with total LSCD, indicating that ocular surface inflammation plays a major role in the development or maintenance of LSCD. Chronic inflammation exerts negative influences on the survival of transplanted LESCs [44]. Nubile et al. examined the expression of stem cell markers in corneoscleral specimens from healthy and inflamed donor eyes; they found marked downregulation of laminin 5, integrin α6, β1, β4, and desmoglein 3 in inflamed eyes, and only a few stem cell markers were still present [45]. It was already known that pro-inflammatory cytokines/chemokines, such as IL-1α, IL-1β, TNF-α, IFN-γ, MCP-1, IL-6, and IL-8, are critical in mediating corneal inflammation [27–32]. In this work, we chose IL-1β and TNF-α to mimic the chronic inflammation in the niche of corneal limbal stem cells in vitro. We found that IL-1β or TNF-α significantly affected the homeostasis of TKE2 cells, as reflected by the inhibition of colony-forming efficiency or colony size, the downregulation of stem cell marker (ΔNp63, importin13), and the augmentation of differentiated markers (ck3/12 and involucrin). Dry eye is another key factor that influences the effectiveness of limbal stem cell transplantation. Elevated tear osmolarity is thought to be the central mechanism in the pathogenesis of ocular surface damage in dry eye. The osmolarity in normal eyes is 300–310 mosm [46–48], while in patients with dry eye, the osmolarity values measured from the lower meniscus of tears could reach up to 360 mosm [49, 50]. As a physiologically relevant solute of the tear film [51], sodium chloride was usually used in corneal epithelial cells for the purpose of increasing osmolarity [38, 52, 53]. Here, our results demonstrated that hyperosmotic stress (340, 360, 400, or 450 mosm) generated by sodium chloride significantly decreased the colony-forming efficiency in an osmolarity-dependent manner, reduced stemness, induced apoptosis and promoted cell cycle arrest at G2/M phase. Similar to our results, previous studies also have shown that hyperosmotic stress can induce DNA damage and cell cycle arrest [54, 55], along with cell death and apoptosis [55–58]. This series of changes in corneal epithelial cells induced by hyperosmotic stress is possible under the regulation of fundamental signal molecules, including p38/MAPK [59, 60], JNK [61, 62], Akt [63], c-Jun [64], and the activated γH_2AX mediated by Plk3 [55]. Usually, the pathological inflammation and dry eye are coexistent in the eyes of patients. In order to persuasively mimic the ocular environment in patients, we further investigated the effects of combined administration with pro-inflammatory cytokines (IL-1β or TNF-α) and hyperosmotic stress on the colony-forming capacity. The combined administration results showed that the colony-forming capacity of corneal epithelial cells decreased more dramatically than cells treated alone with pro-inflammatory cytokines or hyperosmotic stress. These findings suggested that it was hard for CESCs to survive and maintain stemness when being transplanted to the ocular surface with severe inflammation and dry condition.

The recovery capacity of corneal epithelial cells subjected to IL-1β, TNF-α or hyperosmotic stress treatment was evaluated in this assay; these results indicated that the colony-forming efficiency was recovered quickly from the treatment of IL-1β or TNF-α. However, the colony-forming efficiency was hard to recover from the treatment of hyperosmotic stress at the first passage, suggesting that hyperosmotic stress induced persistent impairment on corneal epithelial stem/progenitor cells compared with IL-1β or TNF-α. The reason may be that the pro-inflammatory cytokines IL-1β and TNF-α just caused transient inhibition of stemness and the cell cycle, while hyperosmotic stress not only caused the suppression of stemness but also induced more severe cell apoptosis, necrosis, and a cell cycle arrested at the G2/M phase in CESCs, all of which are physiologic changes that cannot be recovered in a short time after treatment. In addition, several studies have demonstrated that hyperosmotic stress can raise the production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, TGF-β1, and IL-23) and chemokines (IL-8, CCL2, and CCL20) at mRNA and protein levels in human corneal epithelial cells [34, 65]. Our findings also confirmed that hyperosmosis treatment promoted the expression of TNF-α, IL-1β, MCP-1, and IL-8 both in cultured corneal epithelial stem/progenitor cells and corneal injury mouse model. And the elevated levels of TNF-α, IL-1β, and MCP-1 persisted even after 8 days recovery in corneal epithelial stem/progenitor cells. These findings also explained why hyperosmotic stress can induce more severe and persistent impairment on CESCs. LESCs play an essential role in wound healing [2-4]; while considering the negative effects of pro-inflammatory factors and hyperosmotic stress on LESCs, we detected the influence of IL-1β, TNF-α, and inflammation combined with hyperosmotic stress treatment on mouse corneal epithellial wound healing. We found that the normal corneal epithelial wound healing ability was significantly delayed by topical application of IL-1β, TNF-α, and sodium chloride solution (400 mosm); the impairment of wound healing ability was especially severe in the group with hyperosmotic stress treatment. Moreover, as expected, inflammation combined with hyperosmotic stress treatment induced more serious delay of corneal epithelial repair, and this may due to the increased corneal epithelial cell apoptosis after scraping of the corneal epithelium.

Conclusion

In the present study, we systematically compared the effects of persistent inflammation and hyperosmosis on CESCs. Our findings provide the cellular basis for the strict control of inflammation and dry eye before clinical limbal stem cell transplantation, and the effective control of hyperosmotic stress by dry eye is more crucial.

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Author Contributions

L.Y.: conception and design, financial support, provision of study material or patients, collection and/or assembly of data, data

analysis and interpretation, manuscript writing. S.Z.: provision of study material or patients, collection and/or assembly of data, data analysis and interpretation. H.D., M.D.: provision of study material or patients, collection and/or assembly of data. X.H.: manuscript writing. Z.Z., Y.W., X.Z.: collection and/or assembly of data. W.S.: conception and design. Q.Z.: conception and design, financial support, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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