Ascorbic Acid Promotes the Stemness of Corneal Epithelial Stem/Progenitor Cells and Accelerates Epithelial Wound Healing in the Cornea

JIALIN CHEN,[a] JIE LAN,[b] DONGLE LIU,[b] LUDVIG J. BACKMAN,[a] WEI ZHANG,[a] QINGJUN ZHOU,[b] PATRIK DANIELSON[a,c]

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[a]Department of Integrative Medical Biology, Anatomy, [c]Department of Clinical Sciences, Ophthalmology, Umeå University, Umeå, Sweden; [b]State Key Laboratory Cultivation Base, Shandong Provincial Key Laboratory of Ophthalmology, Shandong Eye Institute, Shandong Academy of Medical Sciences, Qingdao, People's Republic of China

Correspondence: Patrik Danielson, M.D., Ph.D., Department of Integrative Medical Biology, Anatomy, Umeå University, SE-901 87 Umeå, Sweden. Telephone: 46 (0)90 786 58 93; Fax: 46 (0)90 786 66 83; e-mail: patrik. danielson@umu.se; or Qingjun Zhou, Ph.D., Shandong Eye Institute, 5 Yan'erdao Road, Qingdao 266071, People's Republic of China. Telephone: 86-532-8589-9270; Fax: 86-532-8589-1110; e-mail: qjzhou2000@ hotmail.com

ABSTRACT

High concentration of ascorbic acid (vitamin C) has been found in corneal epithelium of various species. However, the specific functions and mechanisms of ascorbic acid in the repair of corneal epithelium are not clear. In this study, it was found that ascorbic acid accelerates corneal epithelial wound healing in vivo in mouse. In addition, ascorbic acid enhanced the stemness of cultured mouse corneal epithelial stem/progenitor cells (TKE2) in vitro, as shown by elevated clone formation ability and increased expression of stemness markers (especially p63 and SOX2). The contribution of ascorbic acid on the stemness enhancement was not dependent on the promotion of Akt phosphorylation, as concluded by using Akt inhibitor, nor was the stemness found to be dependent on the regulation of oxidative stress, as seen by the use of two other antioxidants (GMEE and NAC). However, ascorbic acid was found to promote extracellular matrix (ECM) production, and by using two collagen synthesis inhibitors (AzC and CIS), the increased expression of p63 and SOX2 by ascorbic acid was decreased by around 50%, showing that the increased stemness by ascorbic acid can be attributed to its regulation of ECM components. Moreover, the expression of p63 and SOX2 was elevated when TKE2 cells were cultured on collagen I coated plates, a situation that mimics the in vivo situation as collagen I is the main component in the corneal stroma. This study shows direct therapeutic benefits of ascorbic acid on corneal epithelial wound healing and provides new insights into the mechanisms involved. STEM CELLS TRANSLATIONAL MEDICINE 2017;6:1356–1365

SIGNIFICANCE STATEMENT

Vitamin C (ascorbic acid) is important for human health, the lack of which in diet would cause scurvy. A protective effect of vitamin C has been found in the repair of corneal injury. However, the direct effect of vitamin C on the repair of the corneal epithelium is not clear, as well as its inherent mechanism. This study reports the promotion effect of vitamin C on stem cell markers expression in corneal epithelial stem/progenitor cells, and its acceleration of corneal epithelial wound healing. The findings suggest a new mechanism in the therapeutic benefits of vitamin C, and describes a specific function of vitamin C in the repair of corneal epithelium.

INTRODUCTION

Ascorbic acid (vitamin C, VC) is an important water-soluble vitamin for humans and other species. Sailors and soldiers in the past faced a fatal disease called scurvy if they did not include fresh fruits and vegetables in their diet, due to lack of ascorbic acid. These days, high doses of ascorbic acid have been widely tried in the treatment of various conditions, including common cold, diabetes, cataracts, glaucoma, heart disease, and even cancer [1–4]. Despite the important role of ascorbic acid in humans, we cannot synthesize it due to the lack of the enzyme that is responsible for the final step in the ascorbic acid synthesis. Therefore, humans need ascorbic acid in their diet for survival. The intake of ascorbic acid is specifically transported in our body via Sodium Dependent Vitamin C Transporters 1 and 2 (SVCT1 and SVCT2) [5, 6]. High concentration of ascorbic acid has been found in corneal epithelium of the eye in various species [7], and the presence of SVCT2 has been shown in the deeper layers of rabbit corneal epithelium [6]. In humans, the concentration of ascorbic acid in corneal epithelium is 14 times higher than in the aqueous humor [8], indicating a potentially important role of ascorbic acid for the function of corneal epithelium.

Ascorbic acid has been shown to have protective effects in cornea disease repair in animals and in clinic, such as in UV irradiation [9], chemical corneal burns [10, 11], corneal

neovascularization [12], and inflammation [13, 14]. However, the mechanisms of the therapeutic benefits of ascorbic acid have not been extensively studied. The specific function of ascorbic acid in the repair of corneal epithelium is in need of clear and direct evidence.

The function of ascorbic acid in corneal epithelium could possibly be attributed to its role as an antioxidant that suppresses the intracellular reactive oxygen species (ROS) level [15, 16]. Ascorbic acid is also well known for its effect on enhancing cell proliferation [17] and extracellular matrix (ECM) production [18, 19]. Beneficial effects of ascorbic acid have been shown for the reprogramming efficacy in the formation of induced pluripotent stem cells (iPSCs), both in mice [20–22] and humans [20]. Recently, a promotion effect of ascorbic acid was reported on the stemness marker expression in adipose-derived stem cells (ASCs) [23] and gingival stem cells [24], which reveals a new possible role of ascorbic acid on the regulation of stem cells.

In the present study, we hypothesized that ascorbic acid accelerates corneal epithelial wound healing, at least partially through stemness enhancement of corneal epithelial stem/progenitor cells. To test this hypothesis, we evaluated the effect of L-ascorbic acid 2-phosphate (A2-P), a more stable derivative form of ascorbic acid that has been used in many studies [17, 23, 25–27], on the corneal epithelial wound healing in an in vivo mouse model. Furthermore, expression of different stemness markers in vitro in mouse corneal epithelial stem/progenitor cells (TKE2) was analyzed following A2-P exposure. Possible inherent mechanisms were studied, including cell proliferation, ECM production, and antioxidation.

MATERIALS AND METHODS

Animal Experiments

Twenty-four 6- to 8-weeks-old male C57BL/6 mice for each treatment group were used in the experiments. The study protocol was performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and was approved by Shandong Eye Institute. The same researcher performed all the animal experiments. Under general and topical anesthesia, 3 mm in diameter of corneal epithelium was scraped by using algerbrush II corneal rust ring remover (Alger Co, Lago Vista, TX, http://alger-company.com/), with some verified remaining epithelial cells in the limbal region. Subsequently, 10% solution of A2-P (Sigma-Aldrich, St. Louis, MO, A8960, https://www.sigmaaldrich.com/) was used as eye drop in the experimental group and in the control group saline was used. After 24, 48, and 72 hours, the defect area of the corneal epithelium was evaluated using 0.25% fluorescein sodium under a BQ900 slit lamp (Haag-Streit, Bern, Switzerland, https://www.haag-streit.com/). The staining area was quantified by Image J and shown as the percentage of residual epithelial defect.

Cell Culture and Reagents

Mouse corneal epithelial stem/progenitor cell line (TKE2, Public Health England, 11033107, http://www.phe-culturecollections.org.uk/) was cultured in keratinocyte serum-free medium (KSFM, Life technologies, Grand Island, NY, http://www.thermofisher.com/) supplemented with bovine pituitary extract and epidermal growth factor. The cell line has been characterized in our lab [28]. The

Table 1. Antibodies used for immunofluorescence staining and Western blot

Antibody	Company	Code
ΔNP63	Santa Cruz	sc-8609
p63	Thermo	MA1-21871
ABCG2	Santa Cruz	sc-130933
TCF4	Abcam	ab32873
β-Actin	Cell Signal	4967
SOX2	Abcam	ab92494
OCT4	Abcam	ab27985
Bmi-1	Santa Cruz	sc-8906
Ki67	Abcam	ab15580
PCNA	Cell Signal	2586
p-Akt	Epitomics	2118-1
p-Akt	Cell Signal	4060
Akt	Cell Signal	4691
COL I	Abcam	ab34710
COL IV	Abcam	ab6586
Laminin	Abcam	ab11575
Fibronectin	Abcam	ab2413
Anti-mouse IgG, HRP-linked Antibody	Cell Signal	7076
Donkey anti-goat IgG-HRP	Santa Cruz	Sc-2020
Anti-rabbit IgG, HRP-linked Antibody	Cell Signal	7074
Polyclonal Swine Anti-Rabbit Immunoglobulins/TRITC	Dako	R0156
Polyclonal Rabbit Anti-Mouse Immunoglobulins/TRITC	Dako	R0270
Alexa Fluor 488, donkey anti-goat	Invitrogen	A-11055

Abbreviations: COL I, collagen type I; COL IV, collagen type IV; HRP, horseradish peroxidase; p-Akt, Akt phosphorylation.

cultured cells were treated with 50 μg/ml A2-P supplemented culture medium for different time periods. The medium was changed every 2 days in long-term experiments. Medium without A2-P served as control group.

For Akt inhibition, cultured TKE2 cells were pretreated with 40 μM Akt inhibitor (Calbiochem, #124012, La Jolla, CA, http://www.merckmillipore.com/) for 30 minutes before A2-P exposure. Cell proliferation was measured at day 0 and day 2 and protein samples were collected for Western blot analysis.

For antioxidation experiments, TKE2 cells were treated with antioxidants L-Glutathione reduced (GMEE, 1.5 mg/l; Sigma-Aldrich, G6013) or N-Acetyl-L-cysteine (NAC, 1 mM; Sigma-Aldrich, A9165) for 24 hours, as compared to 50 μg/ml A2-P treatment and medium alone. Intracellular ROS generation and glutathione content was measured and protein samples were collected for Western blot analysis.

For ECM component experiments, TKE2 cells were treated with collagen synthesis inhibitors L-Azetidine-2-carboxylic acid (AzC, 300 μM; Sigma-Aldrich, A0760) or cis-4-Hydroxy-D-proline (CIS, 300 μM; Sigma-Aldrich, H5877) together with 50 μg/ml A2-P medium. Following 3 days of exposure the total collagen content was assessed by Sircol Soluble Collagen Assay (Biocolor, Newtownabbey, U.K., S1000, , http://www.biocolor.co.uk/) and performed according to the manufacturer's instructions. Protein samples were collected for Western blot analysis. Furthermore, TKE2 cells were cultured on collagen I coated 6-well plates (Life technologies, A11428-01) with normal culture medium for 3 days, cells cultured on regular culture plates served as control. Protein samples were collected for Western blot analysis.

Clone Formation Assay

Cultured TKE2 cells were trypsinized by TrypLE Express Enzyme (Thermo Fisher Scientific, 12604-013, http://www.
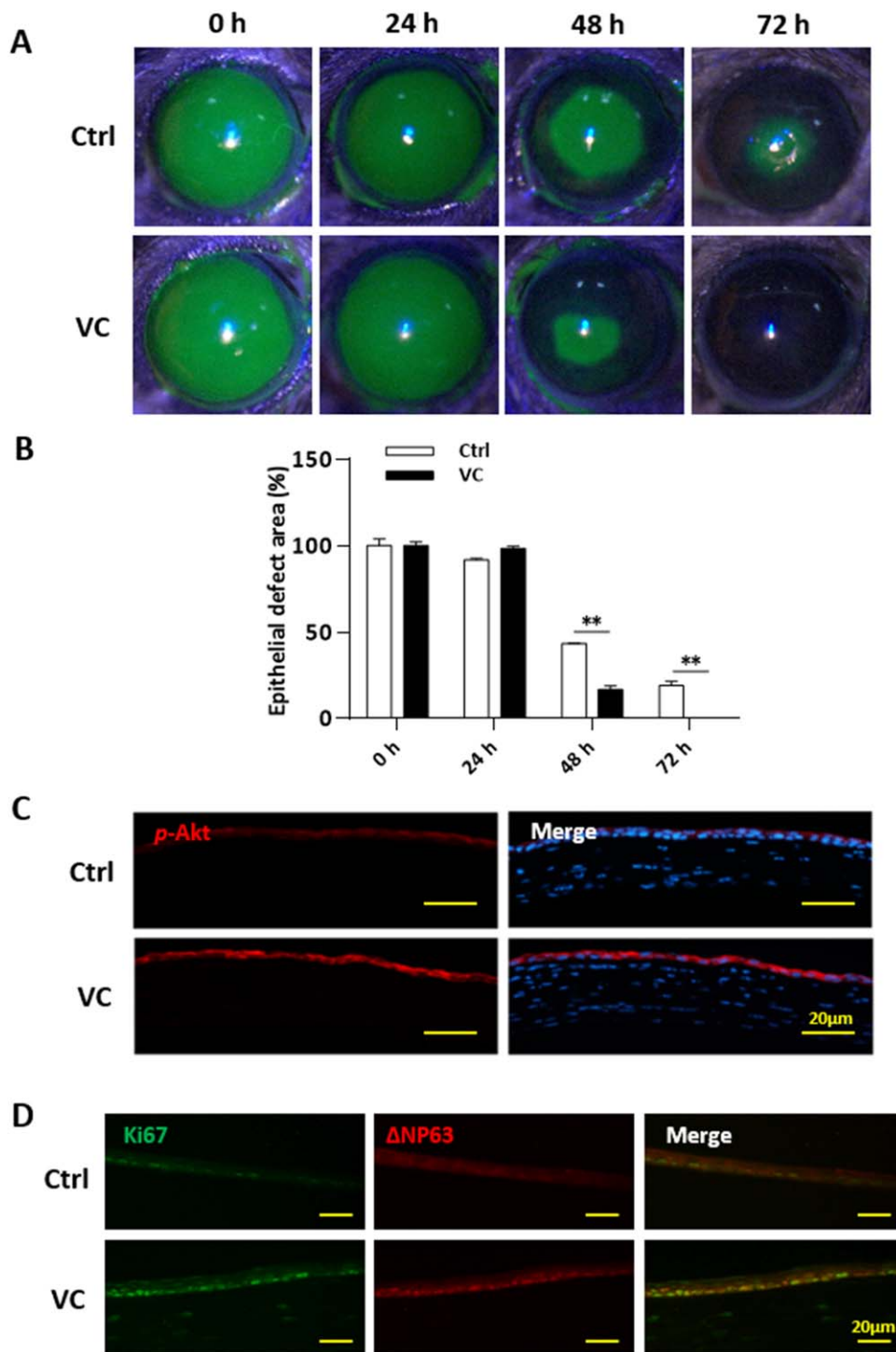
Figure 1. Ascorbic acid (i.e., VC) accelerates corneal epithelial wound healing and increases ΔNP63 expression in vivo. 10% A2-P eye drops were applied after the scrape of corneal epithelium. **(A)**: The defect area of corneal epithelium was evaluated after 24, 48, and 72 hours by 0.25% fluorescein sodium under a BQ900 slit lamp. **(B)**: Residual epithelial defect is presented as the percentage of the original wound size. **(C)**: Immunofluorescence staining of *p*-Akt between A2-P treated group and Ctrl at 48 hours after epithelial debridement. The right column is the merged picture of left column and DAPI staining. **(D)**: Immunofluorescence staining was carried out to show the expression of Ki67 and ΔNP63 in both groups at 48 hours after epithelial debridement. The right column is the merged picture of left column and middle column. Ascorbic acid treatment was performed using the vitamin C derivate A2-P (VC). **Significant difference between two groups at $p < .001$. Abbreviations: Ctrl, control group; VC, vitamin C; *p*-Akt, Akt phosphorylation.

thermofisher.com/) and seeded at very low density (300 cells per well in a 6-well plate) to form colonies. After 10–12 days, the formed colonies were stained with 1% crystal violet (Sigma, C3886) in methanol for 10 minutes. The number of all colonies with diameters >0.5 mm was counted.
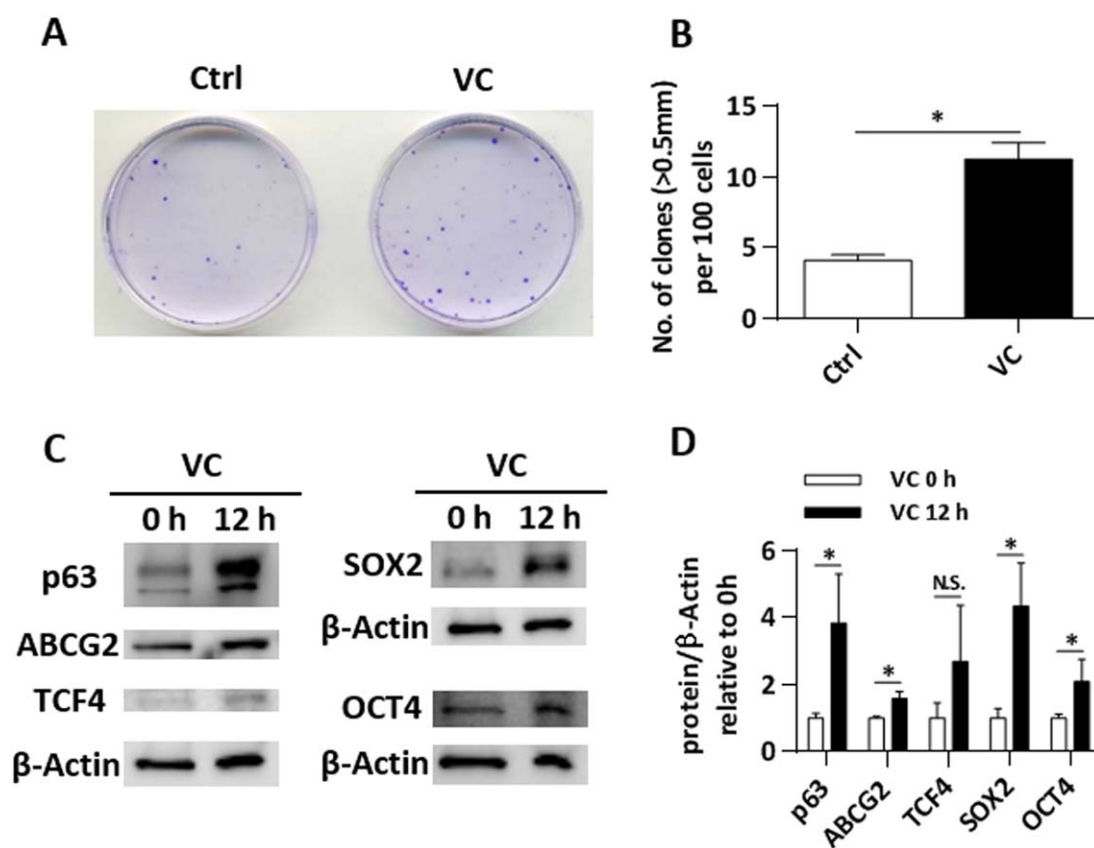
Figure 2. Ascorbic acid (VC) promotes the stemness of corneal epithelial stem/progenitor cells. **(A)**: Mouse TKE2 cells were incubated with or without 50 μg/ml A2-P for 10–12 days. The colonies formed were stained with 1% crystal violet. **(B)**: The number of all colonies with diameters >0.5 mm was counted and compared between the two groups. **(C, D)**: TKE2 cells were treated with 50 μg/ml A2-P for 12 hours. The expression of stemness markers for corneal epithelial stem cells (p63, ABCG2, and TCF4) and pluripotent stem cells (SOX2 and OCT4) was analyzed by Western blot (C) and quantified relative to β-Actin (D). *Significant difference between two groups at $p < .05$. N.S,'No significant difference between two groups at $p \geq .05$. Abbreviations: Ctrl, control group; VC, vitamin C.

Western Blot Analysis

Protein was extracted using RIPA buffer and quantified using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, http://www.bio-rad.com/). Proteins were loaded and run for 1 hour at 150 V on a precast polyacrylamide gel and later transferred to a PVDF Blotting Membrane (GE Healthcare, Little Chalfont, Buckinghamshire, U.K., http://www3.gehealthcare.co.uk/) for 1 hour at 100 V. The membrane was blocked in 5 % bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (TBST) for 1 hour, and incubated with primary antibodies (Table 1) at 4°C overnight. The blots were incubated with an horseradish peroxidase (HRP)-linked secondary antibody (Table 1) for 1 hour after the membranes had been washed in TBST. After an additional wash, the blots were incubated with ECL (GE Healthcare) to visualize the bands using Odyssey Fc Dual-Mode Imaging System (LI-COR, Lincoln, NE, https://www.licor.com/). Densitometry was performed using ImageJ analysis software (NIH, https://www.nih.gov/).

Immunofluorescence

Frozen mouse corneal sections or cultured cells were fixed in 3.7% (vol/vol) paraformaldehyde. The samples were permeabilized and blocked, and then immunostained with the primary antibody (Table 1) overnight at 4°C. Subsequently, fluorescein-conjugated secondary antibody (Table 1) was used to incubate the samples and DAPI was used to reveal the nuclei of the cells.

Cell Proliferation Assay

Cell proliferation was measured using the CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay (Promega, Fitchburg, WI, https://www.promega.com/). Cells cultured for 1, 3, 5, and 7 days with or without 50 μg/ml of A2-P were incubated in CellTiter 96 AQ$_{ueous}$ One Solution Reagent in a 5% CO$_2$ incubator for 1 hour at 37°C. The absorbance of the culture medium was measured at 490 nm. The data was shown relative to the control group.

Intracellular ROS Generation and Glutathione Content Detection

To detect intracellular ROS generation and glutathione content, cultured TKE2 cells were exposed to 10 μM fluorescence probe 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA; Molecular Probes, Eugene, OR, C2938, http://www.thermofisher.com/) and 50 μM monochlorobimane (MCB; Sigma-Aldrich, 69899), respectively, at 37°C for half an hour. The staining was captured under microscopy.

Statistical Analysis

All the main in vitro experiments of the study were carried out using at least three replicates and were repeated successfully in a total number of experimental runs of at least three. Representative data are shown as mean ± SD. Statistical analysis was
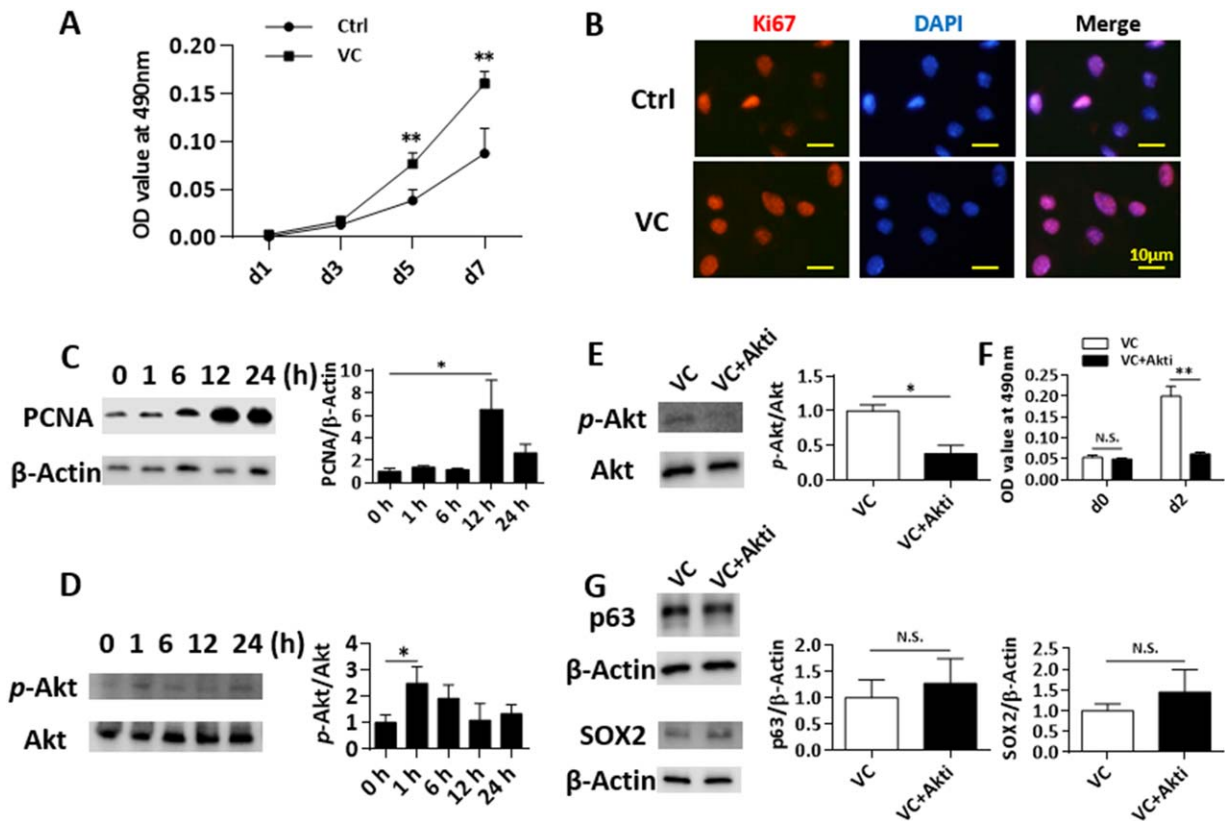
Figure 3. Ascorbic acid (VC) activates *p*-Akt and enhances cell proliferation. **(A)**: The proliferation rate was compared after TKE2 cells were cultured for 1, 3, 5, and 7 days with or without 50 μg/ml of A2-P. **(B)**: Immunofluorescence staining was performed to analyze the expression of Ki67. The right column is the merged picture of left column and middle column. **(C)**: The expression of PCNA was analyzed by Western blot after A2-P treatment for 0, 1, 6, 12, and 24 hours, and quantified relative to β-Actin. **(D)**: The *p*-Akt level was analyzed by Western blot after A2-P treatment for 0, 1, 6, 12, and 24 hours, and quantified relative to Akt. In (C, D), 0 hours was set as 1 in the quantified data, and all time points were compared to 0 hours in the statistical analysis; only statistically significant differences are marked. **(E)**: The *p*-Akt level was analyzed by Western blot after Akti treatment, and quantified relative to Akt. The A2-P treated group was set as 1 in the quantified data. **(F)**: The proliferation rate was compared between A2-P+Akti treated group and A2-P group after 0 and 2 days. **(G)**: The expression of p63 and SOX2 was evaluated by Western blot after Akti treatment, and quantified relative to β-Actin. A2-P treated group was set as 1 in the quantified data. *Significant difference between two groups at $p < .05$. **Significant difference between two groups at $p < .001$. N.S.: no significant difference between two groups at $p \geq .05$. Abbreviations: Akti, Akt inhibitor; Ctrl, control group; VC, vitamin C; *p*-Akt, Akt phosphorylation.

performed using Student's *t* test for two groups' comparison. One-way analysis of variance (ANOVA) with Bonferroni post hoc test was performed for more than two groups' comparison. Values of $p < .05$ were considered statistically significant.

RESULTS

Ascorbic Acid Accelerates Corneal Epithelial Wound Healing and Increases ΔNP63 expression In Vivo

To evaluate the effect of ascorbic acid for corneal epithelial wound healing, 10% A2-P eye drops were topically applied to the mouse cornea with epithelial debridement. The epithelial defect area was significantly decreased in the A2-P treated group as compared to the control group after 48 hours (16.6% ± 1.7% vs. 43.4% ± 0.2%, $p < .001$) and 72 hours (0 vs. 19.0% ± 2.0%, $p < .001$) after epithelial debridement (Fig. 1A, 1B). Elevated Akt phosphorylation (Fig. 1C), proliferation marker Ki67 expression (Fig. 1D), and corneal epithelial stem cells marker ΔNP63 (Fig. 1D) were found after A2-P treatment, which indicates a multi-effect of ascorbic acid for corneal epithelial would healing.

Ascorbic Acid Promotes the Stemness of Corneal Epithelial Stem/Progenitor Cells

The clone formation ability of the in vitro cultured mouse corneal epithelial stem/progenitor cell line (TKE2) was compared with or without A2-P treatment for 10 days. By crystal violet staining, more clones were found in the A2-P-treated group (11.2% ± 1.2%) than in the control group (4.1% ± 0.4%) (Fig. 2A, 2B). The size of the clones was not different between the two groups.

Stemness protein expression of TKE2 cells was evaluated by Western blot (Fig. 2C, 2D) and immunofluorescence staining (Supporting Information Fig. S1) to show the effect of A2-P treatment. As an important marker of corneal epithelial stem/progenitor cells, the expression of p63 was increased 3.8-fold ($p < .05$) after A2-P treatment. Results were similar for ABCG2 (1.6-fold; $p < .05$) and TCF4 (2.7-fold; $p > .05$); two other markers of corneal epithelial stem/progenitor cells. The expression of pluripotent stem cell markers SOX2 and OCT4 were also studied. Significant increase was found in SOX2 expression (4.3-fold; $p < .05$) and OCT4 expression (2.1-fold; $p < .05$) after A2-P treatment. Immunofluorescence staining showed similar results in the expression of ABCG2, Bmi-1, TCF4, p63, SOX2 and OCT4 (Supporting Information Fig. S1). As
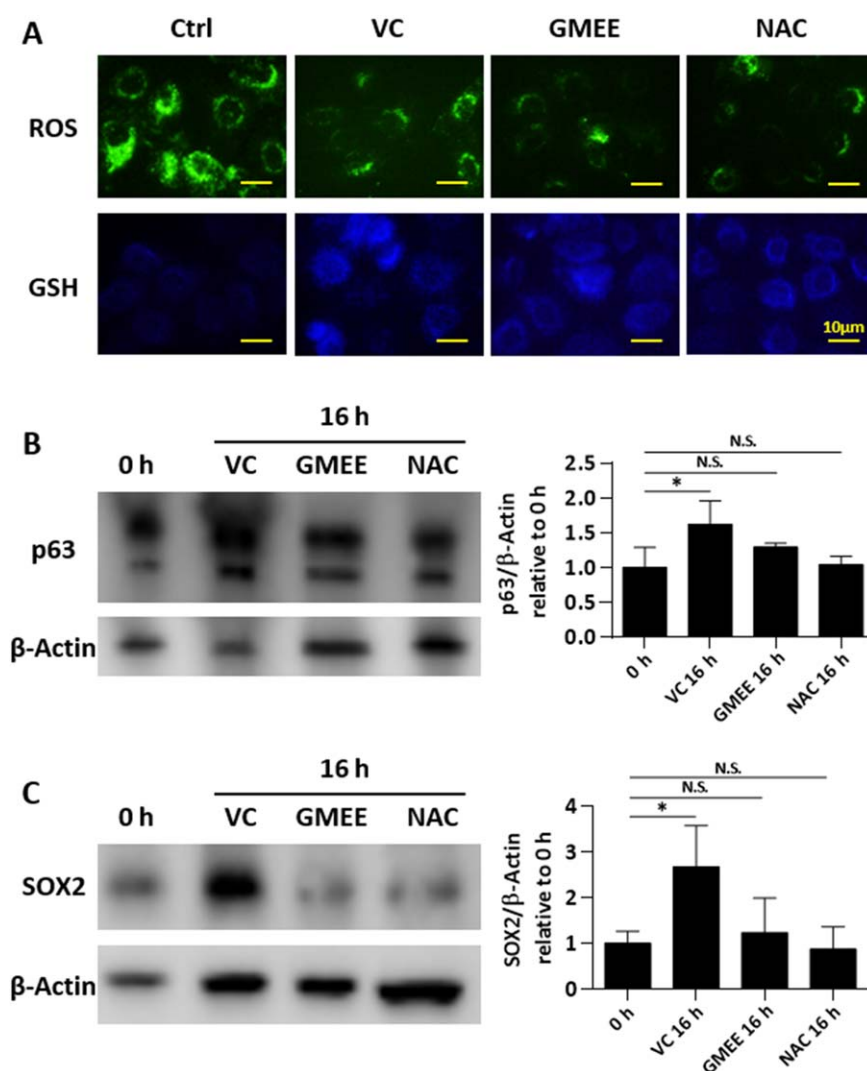
Figure 4. Ascorbic acid (VC) acts as an antioxidant in corneal epithelial stem/progenitor cells, but other antioxidants do not promote stemness in these cells. **(A)**: Mouse TKE2 cells were treated with 50 μg/ml of A2-P, or two other antioxidants (GMEE and NAC), for 24 hours. The staining density of intracellular ROS (green) and GSH (blue) was compared to the Ctrl. **(B)**: The expression of p63 was analyzed by Western blot after treatment with A2-P, GMEE or NAC and quantified relative to β-Actin. **(C)**: The expression of SOX2 was analyzed by Western blot after treatment with A2-P, GMEE, or NAC and quantified relative to β-Actin. *Significant difference between two groups at $p < .05$. N.S, no significant difference between two groups at $p \geq .05$. All experimental groups were compared to 0 hours. Abbreviations: Ctrl, control group; ROS, reactive oxygen species; GSH, glutathione; VC, vitamin C.

the expression of p63 and SOX2 had the highest increase among all the stemness markers examined after A2-P treatment (Fig. 2D), and since they belong to the markers of corneal epithelial stem/progenitor cells and pluripotent stem cells, respectively, they were chosen for evaluation in the following mechanism studies.

Ascorbic Acid Activates Akt Phosphorylation and Enhances Cell Proliferation

Akt phosphorylation and cell proliferation promotion are well-known effects of ascorbic acid. Therefore, we aimed at confirming these effects in TKE2 cells, as well as studying the possible role of ascorbic acid in promoting corneal epithelial stemness expression. Cell proliferation was compared at day 1, 3, 5 and 7 with or without A2-P treatment (Fig. 3A). Significant increase of cell proliferation was found in the A2-P treated group at day 5 ($p < .001$) and day 7 ($p < .001$), as compared to in the control group. Immunocytochemistry showed that the expression of

proliferation marker Ki67 was elevated after A2-P treatment (Fig. 3B). By Western blot, the expression of another proliferation marker, PCNA, was found to be significantly increased after A2-P treatment for 12 hours (6.5-fold; $p < .05$) (Fig. 3C). The level of Akt phosphorylation was elevated 1 hour after A2-P treatment (2.5-fold; $p < .05$) (Fig. 3D).

To test the role of Akt phosphorylation and cell proliferation, an Akt inhibitor was used to treat TKE2 cells in the presence of A2-P. The effect of the Akt inhibitor was confirmed by Western blot (Fig. 3E). It was found that the level of Akt phosphorylation induced by A2-P decreased by 62% after Akt inhibitor treatment. Meanwhile, TKE2 cells proliferated much more slowly after Akt inhibitor treatment, as compared to A2-P alone (Fig. 3F). The protein expression of the stemness markers of interest, p63 and SOX2, were evaluated after Akt inhibitor treatment as compared to A2-P treatment alone. No significant difference was found by Western blot between the two groups (Fig. 3G).
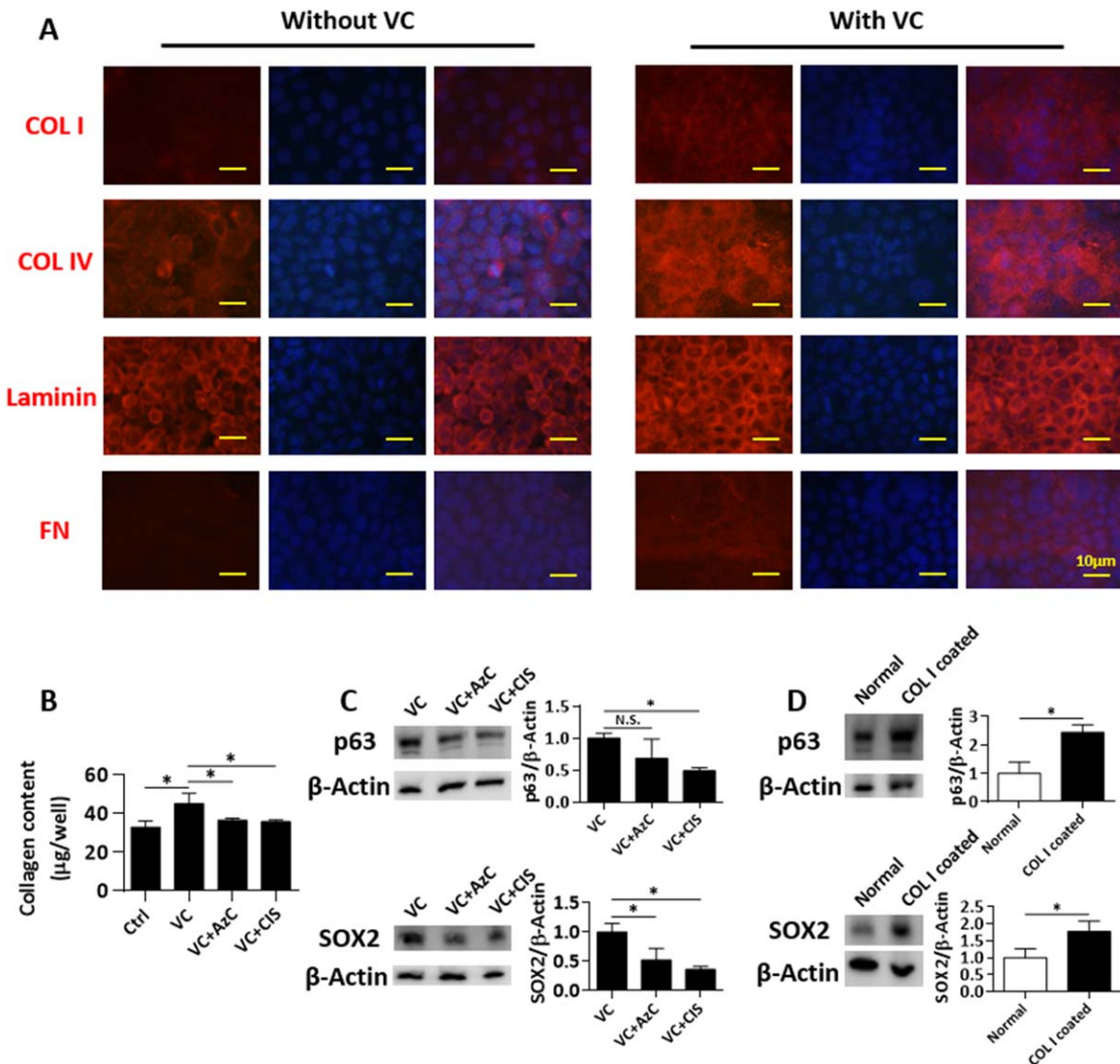
Figure 5. Ascorbic acid (VC) regulates extracellular matrix (ECM) components which accounts for the stemness enhancement. **(A)**: Immuno-fluorescence staining was performed to analyze the expression of ECM components, with or without 50 μg/ml of A2-P treatment. "COL I" indicates collagen type I; "COL IV" indicates collagen type IV; "FN" indicates fibronectin. **(B)**: TKE2 cells were not treated (Ctrl) or treated with 50 μg/ml of A2-P, alone or together with one of two collagen synthesis inhibitors (AzC and CIS) for 3 days. Collagen content was assessed for all groups and compared to the VC group. **(C)**: The expression of p63 and SOX2 was analyzed by Western blot after treatment with A2-P alone or together with AzC or CIS, and quantified relative to β-Actin. VC group was set as 1 in the quantified data, and all other groups were compared to the VC group in the statistical analysis. **(D)**: TKE2 cells were cultured on collagen I coated plates or normal culture plates for 3 days. The expression of p63 and SOX2 was analyzed by Western blot and quantified relative to β-Actin. Group with cells cultured on normal culture plates was set as 1 in the quantified data. *Significant difference between two groups at $p < .05$. N.S, no significant difference between two groups at $p \geq .05$. Abbreviations: COL I, collagen type I; COL IV, collagen type IV; FN, fibronectin.

Ascorbic Acid Acts as an Antioxidant in Corneal Epithelial Stem/Progenitor cells, but Other Antioxidants do not Promote the Stemness of These Cells

Antioxidative property is another well-known function of ascorbic acid. Therefore, the mechanism of enhanced corneal epithelial stemness was studied by using two antioxidants (GMEE and NAC). Decreased intracellular ROS level and increased glutathione staining was detected after A2-P, GMEE or NAC treatment, as compared to the control group (Fig. 4A). However, GMEE or NAC treatment did not enhance the expression of p63 and SOX2 (Fig. 4B and 4C), which implies that the ascorbic acid induced effect on corneal epithelial stemness is not due to its antioxidative properties.

Ascorbic Acid Regulates ECM Components Which Accounts for the Stemness Enhancement

Ascorbic acid is also well known for its property to enhance ECM synthesis. By treating TKE2 cells with A2-P for 3 days, more ECM components were accumulated, including collagen type I (COL I), collagen type IV (COL IV), laminin, and fibronectin (FN) (Fig. 5A). To study the possible role of ECM synthesis after ascorbic acid treatment in the corneal epithelial stemness promotion, collagen synthesis inhibitors AzC and CIS were used during A2-P treatment. Total collagen synthesis was increased by 38% after A2-P treatment, an effect that could be eliminated by AzC or CIS treatment together with A2-P (Fig. 5B). Western blot evaluation of stemness markers showed that protein level of p63 and SOX2 were

decreased by around 50% after AzC or CIS treatment, as compared to A2-P alone (Fig. 5C). Interestingly, when TKE2 cells were cultured on COL I coated plates, higher expression of p63 (2.5-fold; $p < .05$) and SOX2 (1.8-fold; $p < .05$) were found by Western blot as compared to TKE2 cells cultured on normal, uncoated, plates (Fig. 5D).

DISCUSSION

This study shows a therapeutic effect of ascorbic acid on mouse corneal epithelial wound healing in vivo. Ascorbic acid was furthermore found to increase clone formation ability of mouse TKE2 cells in vitro, with upregulation of markers for corneal epithelial stem/progenitor cells (especially p63) and pluripotent stem cells (especially SOX2). Although effects of ascorbic acid on Akt phosphorylation, cell proliferation, and antioxidation were found, only the promotion of ECM production was shown to contribute to the stemness enhancement. The results of this study show a specific function of ascorbic acid on the repair of corneal epithelium and suggest a new mechanism of these therapeutic effects of ascorbic acid.

The effect of ascorbic acid on ECM production has been widely reported on different cell types, including fibroblasts [29, 30], mesenchymal stem cells (MSCs) [18, 31, 32], ASCs [19, 23, 33], and tendon-derived stem cells [34, 35]. Because of that, ascorbic acid has been used to construct cell sheet for tissue engineering application [18, 19, 23, 29, 31–36]. Ascorbic acid has also been introduced into the culture of corneal cells, mainly on the ECM production of keratocytes [37–40]. It has been reported that human keratocytes cultured with ascorbic acid for 5 weeks could automatically assemble organized ECM with parallel arrays of fibrils, which are morphologically similar to the corneal stroma [39]. Our current study shows the effect of ascorbic acid on ECM remodeling in mouse corneal epithelial stem/progenitor cells. The typical ECM components of corneal epithelial basement membrane [41, 42], COL IV, laminin and FN, as well as COL I which is the main ECM component in corneal stroma, were synthesized to a significantly higher extent after ascorbic acid treatment. This would explain the accelerated corneal epithelial wound healing caused by ascorbic acid in vivo, with possible reconstruction of basement membrane by the resynthesis of collagen-like basement membrane components.

It has been widely accepted that unique ECM niches both maintain and regulate the stemness of different stem cells [43–45]. Plenty of attempts have been reported to maintain or regulate stemness by reconstruction of the ECM niche of corneal stem cells for in vitro cell amplification or tissue engineering purpose, usually through fabrication of materials to mimic the native tissue specific ECM niche [46–48]. Ji et al. prepared a 3D amniotic membrane as a natural microcarrier for epidermal stem cells [48]. This engineered amniotic membrane mimicked the niche of epidermal stem cells, which not only enhanced its ex vivo culture and amplification, but also benefited in vivo skin repair as a dermal scaffold. McMurray et al. reported a nanoscale growth substrate to address the stem cell niche, which can maintain the stem cell phenotype and growth of MSCs over 8 weeks [49]. Our current study found that ascorbic acid, a small chemical molecule, could promote the endogenous ECM production of mouse corneal epithelial stem/progenitor cells. This could possibly be followed by self-assembly of the endogenously produced ECM to reconstruct the unique niche in vitro, in order to maintain and enhance the stemness of corneal epithelial stem/progenitor cells. It was further shown that the expression of

stemness markers p63 and SOX2 was elevated when TKE2 cells were cultured on COL I coated culture plates. This mimics the in vivo situation in the cornea, as the epithelium is in close relation to the stroma in which the main component is COL I. It would be interesting for our future work to focus on TKE2 cells cultured on plates coated by other more unique and specific corneal epithelial ECM components, such as COL IV, laminin or FN, and examine the expression of stemness markers by the TKE2 cells. In fact, COL IV has been used to culture corneal epithelial stem cells [50, 51].

Besides the ECM production, alleviating cell senescence would be another reason for the stemness enhancement caused by ascorbic acid. It has been reported that ascorbic acid could enhance the generation of iPSCs from somatic cells, at least partially by alleviating cell senescence [20]. However, in the attempts of Yu et al. on ASCs, higher expression of senescence marker p21 was observed after ascorbic acid treatment [23], which is opposite to that in iPSCs generation [20]. In our current study, we also evaluated the possible role of cell senescence in the ascorbic acid induced stemness enhancement. However, no expression difference of senescence markers p21 and p53 was observed after ascorbic acid treatment (data not shown). The difference in the cell types used in the different studies, is a possible reason for the contradictory results. The ASCs used by Yu et al. are primary cells, while the TKE2 cells we used in our study are cells of a cell line. Generally, cells of cell lines are much less prone to become senescent, as compared to primary cells. Therefore, higher expression of senescence markers was not observed after ascorbic acid treatment in our system.

CONCLUSION

In summary, the current study explored the role and mechanism of ascorbic acid in promoting stemness of corneal epithelial stem/progenitor cells in vitro and repair of corneal epithelial wound in vivo in mouse. The expression of stemness markers (especially p63 and SOX2) in corneal epithelial stem/progenitor cells were upregulated after ascorbic acid treatment, which was mediated by the promoting effect of ascorbic acid on ECM production. The influence of ascorbic acid on Akt phosphorylation, cell proliferation and antioxidation, was also confirmed, but was found not to contribute to the stemness enhancement. This study shows direct evidence of the therapeutic benefits of ascorbic acid on the repair of corneal epithelial wounds, and provides new insights into the mechanisms involved.

AUTHOR CONTRIBUTIONS

J.C.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; J.L. and D.L.: collection and assembly of data, data analysis and interpretation; L.J.B.: data analysis and interpretation, manuscript writing; W.Z.: data analysis and interpretation; Q.Z. and P.D.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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