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Polyoxyethylene and polypropylene emulsification enhances retinol palmitate efficacy in corneal wound healing

Haruki Horiuchi✉, Keisuke Watanabe, Hiroshi Iijima & Yoshiyuki Obayashi

Retinol palmitate (VApal), an active ingredient in ophthalmic solution, has been reported to repair corneal injuries. Additionally, it has been suggested that the efficacy of VApal is enhanced by a specific surfactant, polyoxyethylene-polypropylene [$EO_{100}PO_{70}EO_{100}$ (EOPO)]. We aimed to determine the efficacy of VApal in corneal wound healing in comparison to that of hyaluronic acid (HA), carboxymethyl cellulose (CMC) and hydroxypropyl methylcellulose (HPMC) used in over-the-counter ophthalmic solutions and whether the efficacy of VApal could be enhanced by EOPO compared to the widely used surfactant, polyoxyethylene hydrogenated castor oil 60 (HCO60). To evaluate the efficacy of VApal or VApal emulsified with a surfactant, we performed a wound healing assay using corneal epithelial cells in monolayer ($n = 4$) or 3D culture ($n = 6$). Wound closure rates were calculated each time, and the efficacy was compared using the time to reach a 50% wound closure rate (ET50). The ET50 values of VApal, HA, CMC and HPMC were approximately 17.31 h, 26.99 h, 28.98 h and 26.01 h respectively. The ET50 values of VApal emulsified with EOPO or HCO60 were 34.49 h and 43.31 h, respectively. In conclusion, VApal is more beneficial than other ingredients for corneal wound healing. Additionally, the efficacy of VApal can be enhanced using EOPO instead of HCO60.

Keywords Wound healing, Cornea, Retinol palmitate, Vitamin A, Surfactant, Drug delivery system

The cornea, which functions in visual information intake, tear fluid retention, and biological defense[1], comprises the epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium. The corneal epithelium is particularly vulnerable to external stimuli and can easily be injured[2]. Corneal epithelial wounds are commonly caused by contact lens wear and increased blink friction due to tear film destabilization[3]. These wounds are typically repaired through a self-healing process consisting of three phases: cell extension and migration (phase 1), division and proliferation (phase 2), and differentiation (phase 3)[4].

Retinol palmitate (VApal), a lipophilic derivative of vitamin A, is crucial for maintaining homeostasis in the eye, skin, and mucous membranes[5,6]. VApal is used to treat conditions such as night blindness and keratoderma and is formulated as an active ingredient in over-the-counter medicines in several countries, including ophthalmic solutions for eye fatigue, blurred vision and external preparations for the skin.

The biologically active form of VApal, retinoic acid, regulates various functions, such as cell growth, differentiation, and organogenesis[7], by binding to retinoic acid receptors, which are nuclear receptors. In the corneal epithelium, VApal increases hyaluronic acid (HA) production[8]. Moreover, retinoic acid has been reported to increase mucin expression, which is involved in tear fluid retention[9], and to upregulate lysyl oxidase-like 4, which promotes cell migration[10]. Additionally, Toshida et al.[11] demonstrated the topical application of VApal to repair corneal wounds in a mouse model.

In addition to VApal, several other ingredients used in ophthalmic solutions have been reported to promote corneal wound healing. For example, HA was found to promote the migration of corneal epithelial cells by binding to CD44[12] and accelerating corneal wound healing in a rabbit corneal alkali burn model[13]. Similarly, carboxymethyl cellulose (CMC) promotes epithelial cell migration by interacting with matrix proteins and has demonstrated efficacy in accelerating wound healing in human corneal epithelial cells[14]. Hydroxypropyl methylcellulose (HPMC), which is often used as a thickening agent, was found to accelerate corneal wound healing in a rat corneal detachment model[15]. Cyclosporine suppresses inflammation and improves ocular

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surface conditions. In a clinical phase II study involving patients with moderate-to-severe dry eye, cyclosporine significantly reduced the degree of corneal fluorescein staining compared to the vehicle[16].

Despite the availability of ingredients that accelerate corneal wound healing, previous studies comparing the efficacy of VApal and other ingredients are limited. In a study that compared the efficacy of VApal and cyclosporine in corneal wound healing, Reimondez-Troitiño et al.[17] reported that the efficacy of retinoic acid in corneal wound healing was equal to that of cyclosporine in a mouse injured by photorefractive keratectomy surgery. Their study compared the efficacy of ingredients with different mechanisms of action, including VApal (cell migration and water retention) and cyclosporine (anti-inflammatory). We did not find any previous studies that compared the efficacy of VApal in wound healing with ingredients that have mechanisms of action similar to those of VApal (e.g., HA). Therefore, when compared to HA, CMC, and HPMC, which have similar mechanisms of action as VApal, the relative value of VApal in corneal wound healing remains unclear.

The formulation of VApal in water-based ophthalmic solutions requires surfactants. Polyoxyethylene-hydrogenated castor oil 60 (HCO60) is typically used to emulsify VApal. Previously, Miyake et al.[18] investigated a method for the formulation of VApal using polyoxyethylene-polypropylene ($EO_{100}PO_{70}EO_{100}$ [EOPO])[18]. They investigated the behavior of fluorescence-labeled VApal emulsified with EOPO in relation to cell-membrane interactions, focusing on surface chemistry[18]. Their study demonstrated that VApal emulsified with EOPO exhibited greater localization around the cell nucleus than did HCO60. Similarly, Toshida et al.[19] reported the effectiveness of VApal emulsified with EOPO in patients with dry eyes. In their study, both the objective signs and subjective symptoms of dry eye significantly improved with VApal emulsified with EOPO compared with placebo. However, regarding the efficacy of VApal in corneal wound healing, it remains unclear whether EOPO is a better surfactant than commonly used surfactants (e.g., HCO60).

In this study, we aimed to determine (1) the efficacy of VApal in corneal wound healing compared to ingredients with similar mechanisms of action (i.e., HA, CMC, and HPMC), and (2) whether the efficacy of VApal emulsified with EOPO was higher than that of HCO60.

Methods

Materials

VApal was purchased from DSM Nutrition Japan (Tokyo, Japan). HA was purchased from Bloomage Biotechnology (Tokyo, Japan). CMC and HPMC were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). EOPO and HCO60 were purchased from BASF Japan (Tokyo, Japan) and Nikko Chemicals (Tokyo, Japan), respectively.

Preparation of solutions for the wound healing assay

VApal (913,000 IU/ml), dissolved in dimethyl sulfoxide (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), was diluted 500-fold with a low-serum medium consisting of Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F-12; Thermo Fisher Scientific, MA, USA) supplemented with 1% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, MA, USA), 100 units/mL penicillin, and 100 mg/mL streptomycin (Thermo Fisher Scientific, MA, USA).

HA (3 mg/ml), CMC (5 mg/ml), and HPMC (3 mg/ml) were dissolved in water. These concentrations were matched with the following over-the-counter ophthalmic solutions: HA (Hyalein® ophthalmic Solution 0.3%; Santen, Osaka, Japan), CMC (Refresh plus®, Allergan, Marlow, UK), and HPMC (Genteal® Eye Drops, Novartis, Basel, Switzerland). Each solution was diluted 25-fold with a low-serum medium.

VApal emulsified with HCO60 and EOPO was prepared following the report by Miyake et al. The prepared formulations were sterilized by filtration using a 0.22 μm filter. VApal (50,000 IU/ml) emulsified with EOPO or HCO60 (0.2%) was diluted 25-fold with a low-serum medium. The concentration of EOPO or HCO60 was determined to be the minimum concentration necessary to emulsify VApal.

Cell culture and wound healing assay

SV40-immortalized human corneal epithelial cells (HCE-T: RCB2280, RIKEN BRC, Tokyo, Japan) were cultured in a growth medium consisting of DMEM/F-12 supplemented with 5% FBS, 5 mg/mL insulin (Thermo Fisher Scientific, MA, USA), 10 ng/mL epidermal growth factor (Thermo Fisher Scientific, MA, USA), 0.5% dimethyl sulfoxide, 100 units/mL penicillin, and 100 mg/mL streptomycin. The HCE-T cells were maintained at 37 ℃ in 5% CO_2, and the growth medium was replaced every other day. The HCE-T cells were seeded into 24-well plates (Sumitomo Bakelite, Tokyo, Japan) at a density of 2×10^5 cells/well. After 24 h, the growth medium was removed, and the cells were washed with phosphate-buffered saline (PBS) (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). After washing, the cells were treated with solutions containing each ingredient (as indicated in the figure legends) in a low-serum medium. After 24 h, wounds were induced on the confluent cell layer using a 200 μL pipette tip. Thereafter, the cells were treated with the same solution. The wound healing process from 0 to 60 h was photographed using a confocal microscope (ORCA-Flash 4.0 V3, Nikon, Tokyo, Japan) with an attached device to maintain 37 °C in 5% CO_2 (STX CO_2O_2, TOKAI HIT, Fujinomiya, Japan) or a monitoring system (Provi CM20, OLYMPUS, Tokyo, Japan). The wound areas at 0, 12, 24, 36, 48, and 60 h were calculated using ImageJ software, and the wound closure rates were calculated using the following formula:

Wound closure rates (%) = 100 × (1 - (wound area at each time/wound area at 0 h)).

The ET50 was defined as the time required to reach 50% of the total wound healing area. It was calculated using a quadratic curve approximation derived from a plot of the wound closure rates.

Use of 3D corneal epithelial cells

Before inducing the wounds, 3D corneal epithelial cells (Japan Tissue Engineering, Gamagori, Japan) were pre-incubated according to the manufacturer's protocol. The wound healing model using 3D corneas was based

on method by Kimiko[20]. To create wounds, we first prepared a filter paper (No.5 C, Toyo Roshi, Tokyo, Japan), which was punched into 3 mm diameter discs using a biopsy trepan (BP-30 F, KAI MEDICAL, Tokyo, Japan). Next, we placed a circular filter paper soaked in 1 N NaOH (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) on the 3D corneal epithelial cells for 1 min. The filter paper was then removed, and the cells were washed with PBS. The 3D corneal epithelial cells were then treated with solutions (as indicated in the figure legends) in a low-serum medium for 60 h. Wound healing was observed at 0, 12, 24, 36, 48, and 60 h using a digital microscope (VHX-7000; Keyence, Tokyo, Japan). The wound area was calculated using ImageJ software, and the wound closure rates were calculated as described above.

Cell viability assay

Cell viability assays were performed using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Tokyo, Japan) according to the manufacturer's protocol. HCE-T cells were seeded into 96-well plates (Sumitomo Bakelite, Tokyo, Japan) at 2×10^4 cells/well, or 3D corneal epithelial cells were treated with solutions containing the ingredients indicated in the figure legends for 24, 48, and 72 h in a serum-free medium (DMEM/F-12 supplemented with 100 units/mL penicillin and 100 mg/mL streptomycin). Each solution was removed, and the cells were incubated with the CCK-8 reagent for 2 h. The absorbance was measured using a microplate reader (SYNERGY H1; Agilent Technologies, CA, USA).

Statistical analysis

The wound closure rates of the VApal group ($n = 4$) and other ingredients commonly used in over-the-counter ophthalmic solutions ($n = 4$) at each time point were compared using Dunnett's test (vs. VApal group). The ET50 values of VApal and other ingredients were compared using Dunnett's test (vs. VApal group). The wound closure rates in the VApal ($n = 4$) and EOPO + VApal ($n = 4$) groups at each time point and the ET50 of these groups were compared using Student's t-test. The cell viabilities of the EOPO or HCO60 group ($n = 3$ or 4) and control ($n = 3$ or 4) groups at each time point were compared using Student's t-test. The wound closure rates in the EOPO + VApal group ($n = 6$) and HCO60 + VApal ($n = 6$) at each time point and the ET50 of these groups were compared using Student's t-test. Every experimental result is presented as mean ± standard deviation. The level of statistical significance was set at $p < 0.05$. All statistical analyses were performed using JMP ® 17.0.0 (JMP Statistical Discovery LLC, Tokyo, Japan).

Results

Efficacy of VApal in human corneal wound healing compared to other ingredients

To investigate the efficacy of VApal in corneal wound healing, we conducted a wound healing assay using SV40-immortalized human corneal epithelial cells (HCE-T cells). We compared the efficacy of VApal with that of HA, CMC, and HPMC, which are commonly used as active ingredients in ophthalmic solutions for corneal wounds. Figure 1a shows representative images of the wound healing process in the control, VApal, HA, CMC, and HPMC groups after 24 h. The wound area was noticeably smaller in the VApal group than in the other groups.

Figure 1b shows the wound closure rates in each group from 0 to 60 h. The VApal group had higher wound closure rates than the HA and CMC groups at all time points. Compared to the HPMC group, the VApal group had significantly higher wound closure rates at all time points, except at 36 h (Table 1). To quantify the efficacy of each treatment, we defined the time required to reach 50% of the total wound healing area (ET50). The ET50 of the VApal group was significantly shorter than that of the HA, CMC, and HPMC groups ($p = 0.0002$, $p < 0.0001$, and $p = 0.0005$, respectively) (Table 2).

Efficacy of VApal emulsified with EOPO in human corneal wound healing

We investigated the efficacy of VApal emulsified with surfactants in corneal wound healing. Prior to this, we assessed the toxicity of EOPO on corneal epithelial cells using a cell survival assay (Fig. 2a). The exposure of corneal epithelial cells to EOPO for up to 72 h did not affect cell viability, indicating that EOPO was not toxic to corneal epithelial cells. Next, we examined whether EOPO could increase the efficacy of VApal and compared the efficacy of human corneal wound healing between the VApal and EOPO + VApal groups. Figure 2b shows representative images of the wound healing process in the VApal and EOPO + VApal groups at 24 h. The wound area in the EOPO + VApal group was smaller than that in the VApal group. Figure 2c shows the wound closure rates in each group from 0 to 60 h. At 24, 36, and 48 h, the wound closure rates in the EOPO + VApal group were significantly higher than those in the VApal group ($p = 0.0002$, $p = 0.0186$, and $p = 0.0183$, respectively). Moreover, the ET50 of the EOPO + VApal group was significantly shorter than that of the VApal group ($p = 0.0013$) (Table 3).

Finally, we compared the efficacy of VApal emulsified with EOPO to that of VApal emulsified with HCO60 in promoting corneal wound healing. HCO60 is a surfactant commonly used as an emulsifier for VApal in ophthalmic over-the-counter solutions. Before conducting the wound healing assay, we assessed the toxicity of HCO60 in corneal epithelial cells. The cell viability rates in the HCO60 group were significantly lower than those in the control group at 48 and 72 h (Fig. 2d) ($p = 0.0004$ and $p = 0.0004$, respectively). Therefore, we concluded that a monolayer culture of corneal epithelial cells may not be suitable for investigating the efficacy of VApal emulsified with HCO60.

Cytotoxicity of HCO60 in a reconstructed human corneal epithelium model

Owing to the observed toxicity of HCO60 in the monolayer cultures of corneal epithelial cells, we were unable to demonstrate a difference in efficacy between VApal emulsified with EOPO and VApal emulsified with HCO60. Consequently, we opted to use a reconstructed human corneal epithelium model (3D corneal epithelial cells) that is more similar to the human corneal epithelial cells[21]. To evaluate the cytotoxicity of HCO60 in the 3D corneal epithelial cells, a cell survival assay was performed. The exposure of corneal epithelial cells to HCO60 for

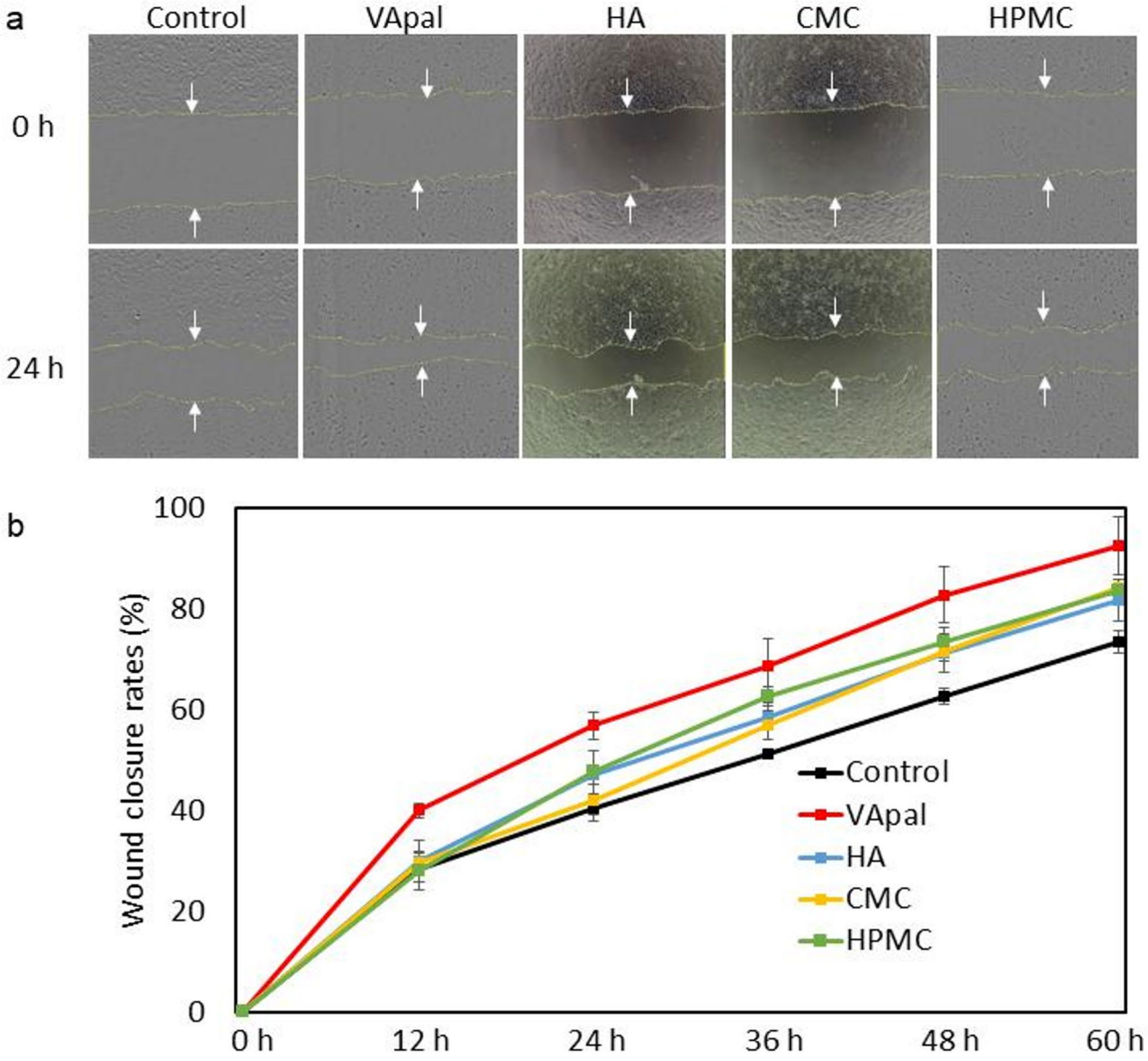

Fig. 1. Efficacy of VApal, HA, CMC, and HPMC in the wound healing assay using HCE-T cells. (**a**) Representative images of the wound healing process in each group. The wound area is delimited by yellow lines. (**b**) Wound closure rates of each group from 0 to 60 h. The wound area was monitored every 12 h. HCE-T cells were treated with VApal (2000 IU/ml), HA (0.12 mg/ml), CMC (0.2 mg/ml), and HPMC (0.12 mg/ml). The data represent mean + standard deviation ($n = 4$). The wound closure rates in the VApal and other ingredient groups at each point in time were compared using Dunnett's test (vs. VApal group). *VApal* retinol palmitate, *HA* hyaluronic acid, *CMC* carboxymethyl cellulose, *HPMC* hydroxypropyl methylcellulose, *HCE-T* human corneal epithelial cells.

up to 72 h did not affect cell viability (Fig. 3). Therefore, HCO60 was less toxic to 3D corneal epithelial cells than to monolayer cultures of corneal epithelial cells.

Comparison of the efficacy of VApal emulsified with EOPO vs. VApal emulsified with HCO60 in corneal wound healing in 3D corneal epithelial cells

To investigate the difference in efficacy between VApal emulsified with EOPO and VApal emulsified with HCO60 for corneal wound healing, we performed a wound healing assay using 3D corneal epithelial cells. Figure 4a shows representative images of the wound healing process in the EOPO + VApal and HCO60 + VApal groups at 36 h. The wound area was smaller in the EOPO + VApal group than that in the HCO60 + VApal group. Figure 4b shows the wound closure rates in each group from 0 to 60 h. After 36 h, the wound closure rates in the EOPO + VApal group were significantly higher than those in the HCO60 + VApal group ($p = 0.0007$, $p = 0.0004$, and $p = 0.0004$, respectively). Comparing the EOPO + VApal and HCO60 + VApal groups, the ET50 of the EOPO + VApal group was significantly shorter than that of the HCO60 + VApal group ($p = 0.0005$) (Table 4).

	Dunnett's test (vs. VApal) *P*-value				
	12 h	24 h	36 h	48 h	60 h
Control	0.0002	< 0.0001	< 0.0001	< 0.0001	< 0.0001
HA	0.0009	0.0050	0.0109	0.0033	0.0053
CMC	0.0006	< 0.0001	0.0029	0.0040	0.0099
HPMC	< 0.0001	0.0084	0.1836	0.0178	0.0418

Table 1. Statistical analysis of the wound closure rates at each time. *VApal* retinol palmitate, *HA* hyaluronic acid, *CMC* carboxymethyl cellulose, *HPMC* hydroxypropyl methylcellulose.

	ET50 (h)	Dunnett's test (vs. VApal) *P*-value
Control	32.16 ± 1.50	< 0.0001
VApal	17.31 ± 1.27	–
HA	26.99 ± 1.82	0.0002
CMC	28.98 ± 2.06	< 0.0001
HPMC	26.01 ± 2.19	0.0005

Table 2. ET50 of the control, VApal, HA, CMC, and HPMC groups. *ET50* time required to reach 50% of the total wound healing area, *VApal* retinol palmitate, *HA* hyaluronic acid, *CMC* carboxymethyl cellulose, *HPMC* hydroxypropyl methylcellulose.

Discussion

In this study, we assessed the efficacy of VApal in corneal wound healing compared to other ingredients with a mechanism of action similar to that of VApal. The results indicated that the efficacy of VApal in corneal wound healing was higher than that of other ingredients, such as HA. These results may be attributed to the differences in the mechanisms of action of VApal and the other ingredients.

Previous reports have shown that HA and CMC promote cell migration through their binding to extracellular substrates[12,14]. HPMC is used in ophthalmic solutions because of its water retention properties. In contrast, VApal is metabolized into retinoic acid within the corneal epithelial cells, which regulates the expression of target genes mediated by retinoic acid receptors. VApal has also been reported to enhance HA production by increasing the expression of hyaluronic acid synthetase 3[22]. Retinoic acid has also been reported to increase the expression of lysyl oxidase-like protein 4, which is involved in cell migration[10]. In a study using a mouse dry eye model lacking aquaporin 5 in the corneal epithelium, retinoic acid enhanced cell proliferation and reduced apoptosis by inducing the expression of B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax) and Bcl-2[23]. As described above, ingredients such as HA promote corneal wound healing by enhancing cell migration, whereas VApal promotes cell proliferation and inhibits apoptosis. Considering these differences in the mechanisms of action, it is likely that the efficacy of VApal in corneal wound healing is higher than that of the other ingredients. However, the wound healing assay utilized in this study was an evaluation model in which there was no extracellular matrix. Therefore, it is possible that the efficacies of HA and CMC were lower because extracellular substrates affected their efficiency. In addition, because the cells are always exposed to the medium, it may be difficult to determine the efficacy of wound healing mediated by water retention. In the future, it will be necessary to evaluate the efficacy in in vivo studies to accurately understand the drug effects.

Toshida et al.[19] demonstrated the effectiveness of VApal emulsified with EOPO in a clinical study; however, it remains unclear whether the effectiveness of VApal emulsified with EOPO is higher than that of VApal emulsified with HCO60. We showed that the ET50 of VApal emulsified with EOPO was shorter than that of VApal alone. This suggests that EOPO can enhance the efficacy of VApal in corneal wound healing. We hypothesize that this enhancement may be attributed to differences in the transition mechanism of VApal at the cell membrane. Generally, VApal is transported into cells via transporters in the plasma membrane. Kawaguchi et al.[24] reported that VApal binds to retinol-binding proteins (RBPs) in the extracellular space, and VApal-RBP complexes are taken up into the cell by STRA6, a plasma membrane receptor that specifically recognizes RBPs. Miyake et al.[18] reported that VApal emulsified with EOPO may be transported into cells by endocytosis. They examined the interactions between VApal emulsified with EOPO and plasma membrane-mimicking giant unilamellar vesicles (GUVs). They demonstrated that VApal emulsified with EOPO induced morphological changes in GUVs and was taken up by GUVs in a manner similar to endocytosis. This implies that VApal emulsified with EOPO is transported not only by the usual transport pathway mediated by STRA6 but also by endocytosis. Furthermore, we assessed whether the efficacy of VApal emulsified with EOPO was higher than that of VApal emulsified with HCO60. We experimentally confirmed that the efficacy of VApal could be enhanced using EOPO instead of HCO60. Miyake et al.[18] suggested that EOPO emulsions increase the efficacy of VApal; however, it has not been confirmed biochemically whether the efficacy is higher than that of widely used surfactants, such as HCO60. To the best of our knowledge, this is the first biochemical study to investigate the enhancement of VApal's efficacy by EOPO.

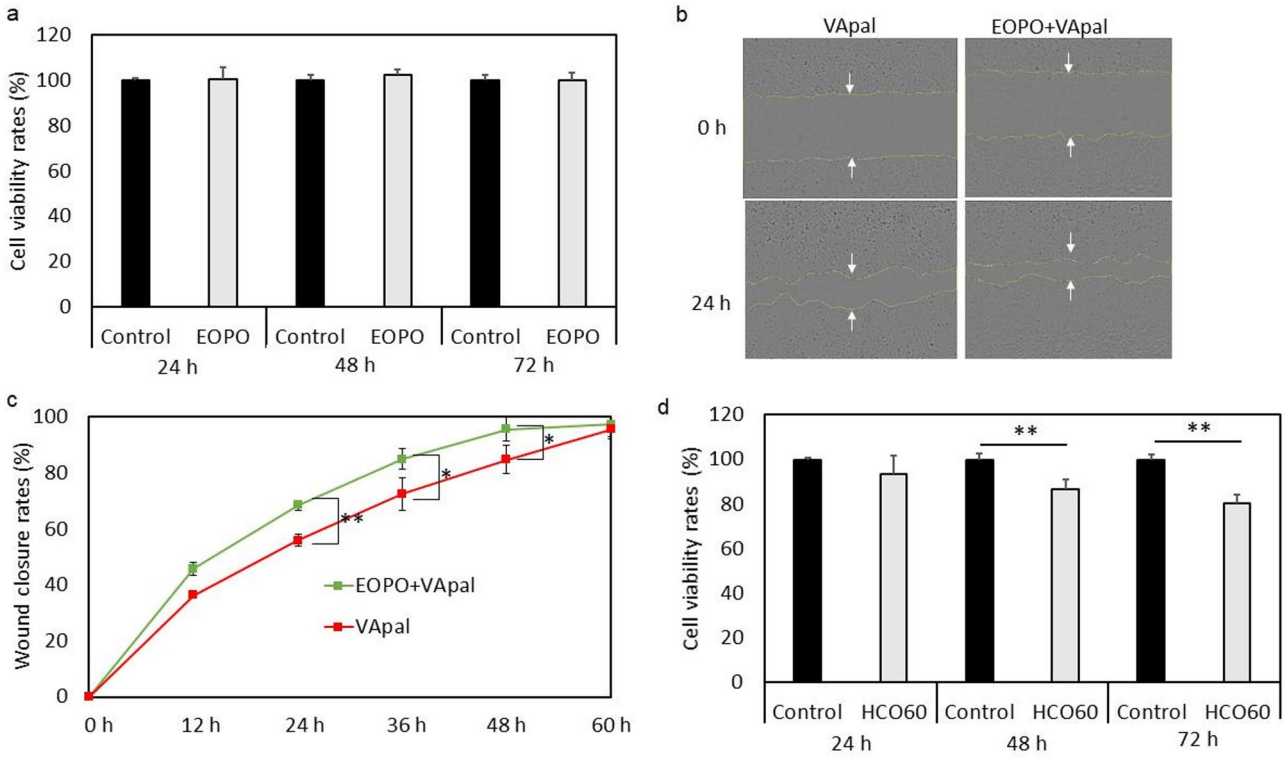

Fig. 2. Efficacy of VApal emulsified with EOPO in the wound healing assay and the cytotoxicity of EOPO and HCO60 in HCE-T cells. (**a**,**d**) Cytotoxicity of EOPO and HCO60 in HCE-T cells. HCE-T cells were exposed to EOPO (0.008%) (**a**) or HCO60 (0.008%) (**d**) for 24, 48, and 72 h. The cell viability was assessed using CCK-8, and these data were normalized to control (PBS-treated) values (100%). Each bar shows mean ± SD. Student's t-test; $^{**}p < 0.01$. (**b**) Representative images of the wound healing process in the VApal and EOPO + VApal groups. The wound area is delimited by yellow lines. (**c**) Wound closure rates of each group from 0 to 60 h. The wound area was monitored every 12 h. HCE-T cells were treated with VApal (2000 IU/ml) and EOPO + VApal (2000 IU/ml). The wound closure rates in the VApal and EOPO + VApal groups at each point in time were compared using Student's t-test ($^{*}p < 0.05$, $^{**}p < 0.01$). All the data are expressed as the mean ± SD ($n = 4$). *VApal* retinol palmitate, *EOPO* polyoxyethylene-polypropylene [$EO_{100}PO_{70}EO_{100}$], *HCO60* polyoxyethylene-hydrogenated castor oil 60, *HCE-T* human corneal epithelial cells, *SD* standard deviation, *PBS* phosphate-buffered saline, *CCK-8* cell counting kit-8.

	ET50 (h)	Student's t-test *P*-value
VApal	20.92 ± 1.59	–
EOPO + VApal	15.57 ± 0.35	0.0013

Table 3. ET50 of the VApal and EOPO + VApal groups. *ET50* time required to reach 50% of the total wound healing area, *VApal* retinol palmitate, *EOPO* polyoxyethylene-polypropylene [$EO_{100}PO_{70}EO_{100}$].

In this study, we constructed an experimental system using 3D corneal epithelial cells to compare the efficacy of VApal emulsified with EOPO to that of VApal emulsified with HCO60. Wound healing assays using 3D cells have been reported; Fallacara et al.[25] evaluated the efficacy of cross-linked HA through hematoxylin and eosin (HE) staining in surgically wounded 3D corneal epithelial cell, whereas Smith et al.[26] assessed wound healing by HE staining of 3D gingival and skin keratinocytes. However, both reports involved only qualitative examinations. Therefore, we developed an in vitro experimental system similar to the in vivo alkali burn model based on the report by Kato et al.[27]. In this system, we created a circular wound using 1 N NaOH treatment and evaluated the wound healing. Using this experimental system, we demonstrated that the efficacy of VApal emulsified with EOPO was higher than that of VApal emulsified with HCO60 in corneal wound healing. This experimental system may enable more extrapolated in vitro wound healing assays. The problem with this evaluation system is that it is difficult to consider the depth of the wound and the corneal tissue structure. Moreover, the wound healing time may differ depending on the depth of the wound. To improve the extrapolation of these evaluation systems, it may be necessary to measure and equalize the depth of wounds using confocal laser microscopy or tissue staining.

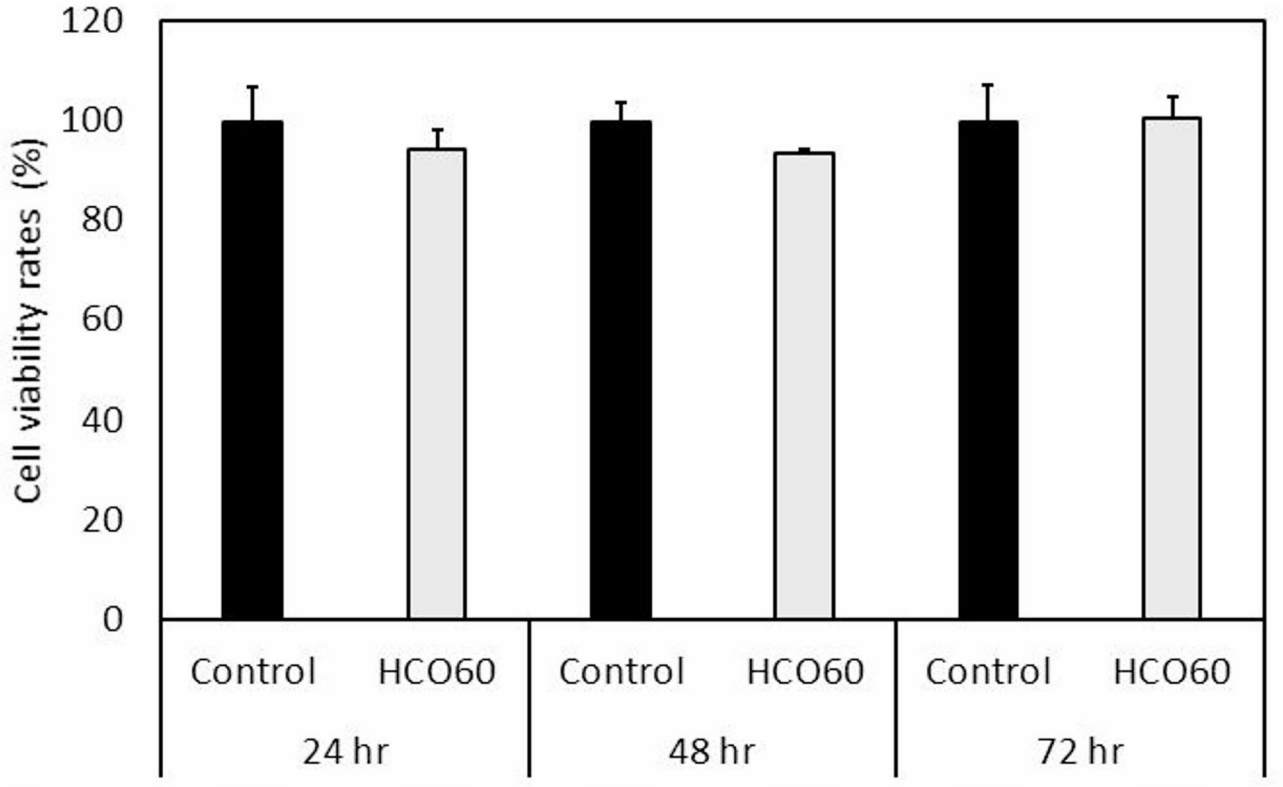

Fig. 3. Toxicity of HCO60 on the 3D corneal epithelial cells. The 3D corneal epithelial cells were exposed to HCO60 (0.008%) for 24, 48, and 72 h. Cell viability was assessed using CCK-8, and the data were normalized to control (PBS-treated) values (100%). All the data are expressed as the mean + standard deviation ($n = 3$). *HCO60* polyoxyethylene-hydrogenated castor oil 60, *PBS* phosphate-buffered saline, *CCK-8* cell counting kit-8.

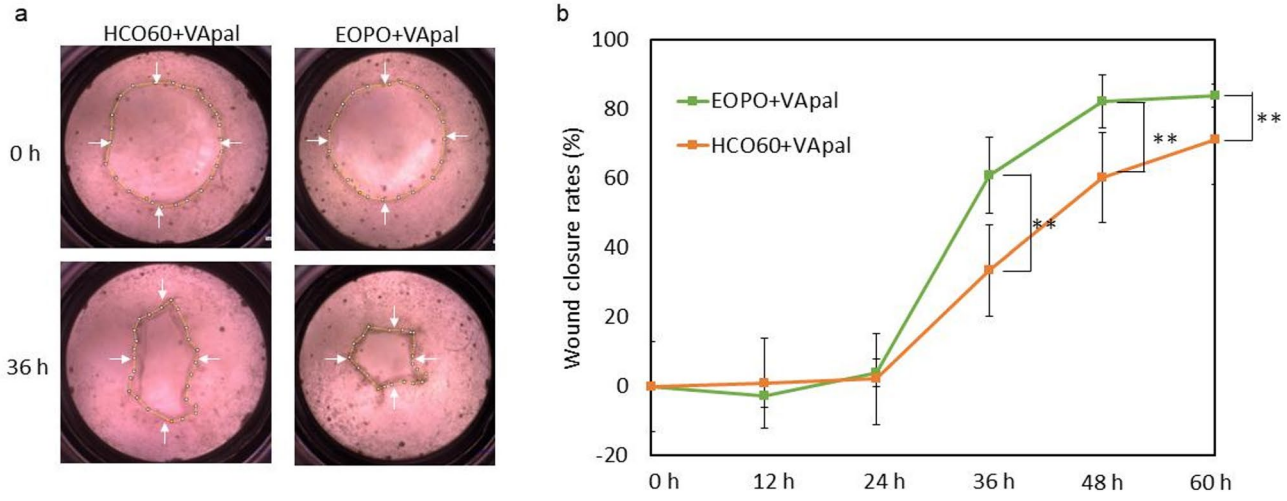

Fig. 4. Efficacy of VApal emulsified with EOPO and VApal emulsified with HCO60 in the 3D corneal epithelial cells. (**a**) Representative images of the wound healing process in each group. The wound area is delimited by yellow lines. (**b**) Wound closure rates of each group from 0 to 60 h. The wound area was monitored every 12 h. 3D epithelial cells were treated with EOPO + VApal (2000 IU/ml) and HCO60 + VApal (2000 IU/ml). The wound closure rates of the EOPO + VApal and HCO60 + VApal groups at each point in time were compared using the Student's t-test (**$p < 0.01$). The data represent mean + standard deviation ($n = 6$). *VApal* retinol palmitate, *EOPO* polyoxyethylene-polypropylene [$EO_{100}PO_{70}EO_{100}$], *HCO60* polyoxyethylene-hydrogenated castor oil 60.

	ET50 (h)	Student's t-test *P*-value
HCO60 + VApal	43.31 ± 4.56	–
EOPO + VApal	34.49 ± 1.08	0.0005

Table 4. ET50 of the HCO60 + VApal and EOPO + VApal groups. *ET50* time required to reach 50% of the total wound healing area, *VApal* retinol palmitate, *EOPO* polyoxyethylene-polypropylene ($EO_{100}PO_{70}EO_{100}$), *HCO60* polyoxyethylene-hydrogenated castor oil 60.

In conclusion, our findings suggest that VApal may be more useful than other ingredients used in ophthalmic solutions for corneal wound healing. For the treatment of dry eye disease, anti-inflammatory medications such as cyclosporine and artificial tears are often used[28]. Elvan et al.[27] reported on a comparison between the combined use of cyclosporine A with artificial tears and the use of artificial tears alone in patients with dysfunctional tear syndrome. In this report, they demonstrated that the combination of an anti-inflammatory and artificial tears (0.05% cyclosporine and 0.3% hydroxypropyl methylcellulose) showed statistically significantly better break up time, corneal fluorescein staining than the use of artificial tears alone. Because VApal has been shown to have significant efficacy for corneal wound healing in comparison with artificial tear ingredients, VApal may be a more effective ingredient to use in combination with cyclosporine for patients with dry eye disease. Additionally, EOPO was found to enhance the efficacy of VApal for corneal wound healing. By utilizing EOPO, the cellular permeability and effectiveness of other lipophilic ingredients, such as vitamin E, may be enhanced. However, it should be noted that this research is limited to in vitro results. Generally, the retention of drugs on the ocular surface is critical for the efficacy of ophthalmic solutions. The wound healing assay employed in this study was conducted under conditions where cells and tissues were continuously exposed to the drug. These conditions may not accurately reflect the actual retention time of the drug in the eye. Therefore, the constant exposure of cells and tissues to the drug without accounting for retention warrant caution in interpreting the efficacy results obtained from this study. For future studies, it will be necessary to develop models that accurately replicate the actual conditions of ophthalmic solution usage as well as experimental designs that can appropriately assess ocular drug retention. For instance, using in vivo animal models or developing in vitro systems that replicate human tear fluid dynamics are promising strategies. By combining these experimental techniques, more extrapolatable and reliable data should be able to be obtained.

Conclusion

In this study, we found that VA pal might be more useful in corneal wound healing than other ingredients used for OTC ophthalmic solutions. We also found that EOPO can enhance the efficacy of VA pal on corneal wound healing.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

H.H, K.W, H.I and Y.O contributed to the conception and design of the study.H.H wrote the main text of the manuscript and H.H prepared Figs. 1, 2, 3 and 4.

Declarations

Competing interests

All authors are current employees of Lion Corporation. All experiments were performed at the Well-Being Research Laboratories.

Additional information

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