The opioid peptide biphalin modulates human corneal epithelial wound healing in vitro

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Abstract

Background: Analgesic drugs, including nonselective opioids and non-steroidal anti-inflammatory drugs, should be used with great precautions to relieve pain after physical damage of the corneal epithelium because of their unfavorable effects on the wound-healing process. Biphalin is a synthetic opioid peptide that has been demonstrated to possess a strong analgesic effect on rodents. The purpose of this study is to investigate the effects of biphalin on human corneal epithelium wound healing.

Methods: An immortalized human corneal epithelial cell (HCEC) culture was used to test the effects of biphalin on wound healing. The toxicity of biphalin in various concentrations was measured with the MTT assay. The effect of 1 μ M and 10 μ M biphalin were tested on wound closure in an in vitro scratch assay of HCECs and for cell migration and proliferation separately. Naloxone, a non-selective competitive antagonist of opioid receptors, was also used to inhibit the effects of biphalin in all experiments.

Results: Biphalin did not cause any toxic effect on HCECs in concentrations lower than 100 μ M at various incubation time points. Biphalin increased the wound closure process significantly at 1 μ M concentration in an in vitro scratch assay of HCECs ($p < 0.05$). It also increased the migration of HCECs significantly ($p < 0.01$). There was no significant difference between biphalin and control groups of HCECs in the Ki67 proliferation assay.

Conclusion: Biphalin, a synthetic opioid peptide, has a potential role as a novel topical analgesic agent that promotes corneal epithelial wound healing. This role should be evaluated in further in vivo and clinical studies.

Background

Ocular pain is an important symptom of inflammatory or traumatic disorders affecting anterior segment structures, such as the cornea, sclera, conjunctiva, and uvea. It is commonly relieved with ophthalmic topical anesthetic agents, such as tetracaine, procaine, benoxinate, and proxymetacaine (proparacaine), most of which have toxic side effects to the corneal epithelium, such as corneal epithelial erosion and delayed wound healing. [1, 2]

Opioids are considered potent and appropriate analgesics for moderate to severe acute and chronic pain and have been used to treat pain since early ages. They are used in ophthalmology by a systemic or periorbital (sub-Tenon's block, peribulbar, retrobulbar) administration route. [3, 4] In addition to their analgesic effect, opioids show cytoprotective, neuroendocrine regulatory, immunomodulatory, and behavioral-modification effects by affecting opioid receptors. [5] Opioids interact with three receptor classes of seven heterotrimeric inhibitory transmembrane G-protein-coupled opioid receptors (GPCRs): delta opioid (DOR), kappa-opioid (KOR), and mu-opioid (MOR). [6]

Biphalin is a dimeric nonspecific opioid analog [(Tyr-D-Ala-Gly-Phe-NH-)₂], which mainly activates DORs and MORs, resulting in a proven complete analgesic response and neuroprotective effect in the central

nervous system. [7] Biphalin exerts less dependence and tolerance compared to morphine, since DOR- and MOR-induced side effects are believed to be prevented by activation of KOR. [8] To date, biphalin has been shown to be one of the most potent peptide-based opioid analgesics and has been recently investigated as a potential treatment for abdominal pain associated with inflammatory bowel disease. [9] The analgesic potency of biphalin is seven times greater than that of etorphine and three orders of magnitude greater than morphine after intracerebroventricular administration. [10] Since common analgesic drugs used to relieve pain after physical damage to the corneal epithelium have unfavorable effects on the wound-healing process, the search for an analgesic that has no adverse effect on corneal wound healing continues.

To the best of our knowledge, the role of synthetic opioid peptides on human corneal epithelial cells has not been investigated in the published English literature. Since biphalin is a nonspecific opioid analog, this study aimed to evaluate the effects of biphalin on human corneal epithelial cells, particularly on wound healing, cell migration, and cell proliferation in vitro.

Methods

Synthesis of the Opioid Agonist

Biphalin was synthesized by Adriano Mollica at his laboratory in Università degli Studi G. d'Annunzio Chieti e Pescara, Department of Pharmacy, Chieti, Italy. The peptide's chemical properties were in full agreement with those already reported in the literature (Fig. 1). [11]

Corneal Epithelial Cell Culture

Human immortalized corneal epithelial cells (HCECs) were a generous gift from Dr. James Jester (Irvine, CA, USA). The HCECs were cultured in a keratinocyte serum-free medium (KSFM; Gibco, NY, USA) supplemented with bovine pituitary extract (BPE; 25 µg/mL), epidermal growth factor (EGF; 50 ng/mL), penicillin (100 IU/mL), and streptomycin (100 µg/mL). The cells were maintained in 75 cm² flasks until experimentation. The HCECs did not differentiate in the keratinocyte serum-free medium. To differentiate HCECs, we followed the protocol described by Sahin et al. [12] We changed the medium with DMEM with 10% FBS. In this way, the HCECs were differentiated and stratified.

Cytotoxicity Assay

The HCECs were treated with different concentrations of biphalin (from 1 pM to 100 µM) in 96-well culture dishes (Corning, NY, USA) for 24 hours. The cytotoxicity of exposure was measured with the MTT (3-(4,5-dimethylthiazolyl)-2-2', 5'-diphenyltetrazolium bromide) assay (Thermo Fisher Scientific, MA, USA). The color of the MTT tetrazole salt was measured with a spectrophotometer at a wavelength of 570 nm.

In Vitro Scratch Assay

The HCECs were grown to confluence on 12-well culture dishes (Corning, NY, USA). On reaching confluence, the cells were rinsed with a phosphate-buffered saline solution (PBS) and exposed to a differentiation medium consisting of Dulbecco's Modified Eagle Medium (DMEM; Gibco, NY, USA) with 10% fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100 µg/mL) for one day. Two perpendicular linear scratches were made using a sterile 200 µL pipette tip, and the wells were washed with PBS. Immediately after the scratch, all groups were incubated in KSFM. Biphalin (in two different concentrations; 1 µM [10^{-6} M] and 10 µM [10^{-5} M]) or biphalin plus naloxone solution (in two different concentrations; 1 µM and 10 µM) or their vehicle (PBS) was added to the cultures. Naloxone, which is a selective opioid receptor antagonist, was added to inhibit the opioid receptor-related effects of biphalin. The scratch area was captured hourly for 24 hours using live-cell microscopy (DMI 8; Leica, Wetzlar, Germany). The relative wound area (RWA) was measured using ImageJ software (National Institutes of Health [NIH], MD, USA) (Fig. 2).

Transwell Migration Assay

After wounding, the cells were used to do a Transwell Migration Assay. This is because during confluency, gene expressions of cells are different from the wound-healing situation. By creating a wound, we simulated the wound process in epithelial cells. Immediately after the wounding process was complete, as described above, the HCECs were trypsinized, washed, and plated (2.5×10^5 cells per insert) in 8.0 µm pore size Transwell inserts (Corning, NY, USA) in KSFM.

The lower compartment was filled with DMEM with 10% FBS or DMEM with 10% FBS plus either biphalin (in two different concentrations; 1 µM and 10 µM) or biphalin plus naloxone solution (in two different concentrations; 1 µM and 10 µM) or their vehicle (PBS). After 24 hours, the cells on the upper side of the insert were removed by scraping, and the cells that had migrated through were fixed on the lower side of the membrane with 4% paraformaldehyde, then stained with hematoxylin-eosin and quantified by counting the number of cells in 10 separate fields. The data were expressed as the number of migrated cells per micrograph field for each sample well.

Ki67 Proliferation Assay

The effect of biphalin on in vitro proliferation was assessed by immunofluorescence staining for Ki67. The Ki67 protein is present during the G1, S, G2, and M phases of the cell cycle and is strictly associated with cell proliferation. 2.5×10^5 HCECs were plated in equal numbers in 24-well culture dishes (Corning, NY, USA). After reaching confluence, cells were rinsed twice with PBS and exposed to a stratification medium consisting of DMEM with 10% FBS. Two perpendicular linear scratches were made using a sterile 200 µL pipette tip, and the wells were washed three times with PBS and incubated with KSFM

without EGF and BPE. Immediately after the scratch, biphalin (in two different concentrations; 1 μ M and 10 μ M) or biphalin plus naloxone solution (in two different concentrations; 1 μ M and 10 μ M) or their vehicle (PBS) were added to the cell culture medium. The cells were incubated for 6 hours at 37°C. After the treatment, the cells grown on 24-well culture dishes were fixed in 4% paraformaldehyde for 20 min. After three washes with PBS, the cells were incubated with 0.1% TritonX-100 in PBS for 8 min. The cells were incubated with Superblock (Thermo Fisher Scientific, MA, USA) for 10 min at room temperature and then overnight at 4°C with the rabbit anti-Ki67 primary antibody (Abcam, MA, USA) at optimal dilutions in a blocking solution. After three washes with PBS, the cells were incubated with the FITC-conjugated secondary antibody (Abcam, MA, USA) for 90 min at 37°C, then washed, counterstained with 406-diamidino-2-phenylindole (DAPI), and mounted. Negative controls were stained in a similar fashion (DMI 8; Leica, Wetzlar, Germany). The Ki67 proliferation index is the proportion of Ki67 stained cell nuclei to DAPI stained cell nuclei from 20 different micrograph areas. The Ki67 proliferation index is calculated by dividing the number of Ki67 stained cell nuclei by the number of DAPI stained cell nuclei using ImageJ software (National Institutes of Health [NIH], MD, USA)

Gene Expression Analysis of Opioid Receptors with Quantitative Reverse Transcription PCR

The presence and expression levels of MOR, DOR, and KOR in the HCECs were measured quantitatively using a real-time polymerase chain reaction (qRT-PCR). The presence of OPRM1, OPRD1, and OPRK1 mRNAs, which are related with mu, delta, and kappa opioid receptor proteins, respectively, was studied in differentiated and undifferentiated HCECs and in SH-SY5Y cell lines. SH-SY5Y cell lines are human neuronal cancer cell lines that have demonstrated expressions of these three opioid receptors in previous literature.[13] To show the effect of the serum on HCECs, HCECs cultured in KSFM and in DMEM+10% FBS were used in qRT-PCR analysis. The qRT-PCR primers for the genes that are responsible for the opioid receptor expressions selected is shown in Table 1. RNA isolation from cells was performed via a Quick-RNA MicroPrep Kit (Zymo Research, Irvine, CA, USA). Extracted RNAs were quantified with Nanodrop 2000 spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA), and 1000 ng cDNA was prepared using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative real-time expressions of mRNAs were detected and compared with a Light Cycler 480 SYBR Green I Master (Roche, Basel, Switzerland).

Statistical Analysis

Each experiment was performed at least two times. For blind analysis, collection of images was made by E.Y., and each image was assigned a number. Then images were analyzed anonymously by K.K. Values were displayed as a mean $\pm$ standard deviation. For the in vitro scratch assay, we measured time-dependent change in the wound area. For other assays, we collected data only at one time point. Since two-way ANOVA is used to examine the interaction between two independent variables (treatment and

time), and one-way ANOVA tests the effect of one independent variable (treatment), statistical analysis was performed using two-way ANOVA for in vitro scratch assay results, and one-way ANOVA with a Tukey's Honest Significant Difference test was used for other results to determine the degree of significance (R; R-Project, Vienna, Austria).

Results were considered statistically significant when the p-value was less than 0.05.

Results

MTT Toxicity Assay

First, we measured the cytotoxic effect of biphalin on HCECs with the MTT toxicity assay. Biphalin has no cytotoxic effects at doses lower than 100 μM after a 24-hour drug incubation (Fig. 3). Because of this, we selected two doses, 1 μM and 10 μM , to apply on HCECs during in vitro experiments.

The In Vitro Scratch Assay

We observed a statistically significant decrease in RWA in biphalin-treated cells at 1 μM concentration compared to the vehicle group (at the 6th hour, biphalin RWA = 0.02126 ± 0.02299 , biphalin plus naloxone RWA = 0.06015 ± 0.06286 , vehicle RWA = 0.1552 ± 0.16940 , $n = 8$, $p < 0.05$) (Fig. 4a). However, there was no statistically significant difference in RWA in 10 μM biphalin, biphalin plus naloxone, or the vehicle groups (at the 6th hour, biphalin RWA = 0.05838 ± 0.06159 , biphalin plus naloxone RWA = 0.06015 ± 0.06897 , vehicle RWA = 0.01507 ± 0.02983 , $n = 8$) (Fig. 4b). It shows the positive wound-healing effect of biphalin in the in vitro wound-healing model of HCECs. But this effect could be because of proliferation or migration. To understand which cell behavior leads to the positive effect, we performed a Transwell Migration Assay and a Ki67 proliferation assay.

Transwell Migration Assay

We recorded a statistically significant increase ($n = 20$, $p < 0.01$) in the number of cells passing through the Transwell membrane at 1 μM concentration of biphalin (82.35 ± 42.96) in respect of vehicle (34.35 ± 7.081) and biphalin plus naloxone (52.75 ± 23.76) (Fig. 5a). However, there was no statistically significant difference between the 10 μM concentration of biphalin (56.80 ± 42.36), biphalin plus naloxone (37.20 ± 13.23), or the vehicle (39.85 ± 12.35) groups in terms of the number of cells passing through the membrane ($n = 20$) (Fig. 5b).

Ki67 Proliferation Assay

To examine the proliferative activity of HCECs during wound healing, Ki67 expression was observed in the wound areas ($n = 20$). After image acquisition, for a more precise comparison of Ki67 expression between

the groups, the Ki67 labeling index was calculated. On the Ki67 proliferation index, there was no statistically significant difference between experimental groups in either concentration of biphalin, 1 μ M and 10 μ M, at 3 and 6 hours (Fig. 6). These findings suggest biphalin has no significant proliferative effect on HCECs.

Gene Expression Analysis with qRT-PCR of Opioid Receptors

On qRT-PCR, we demonstrated the presence of OPRM1, OPRD1, and OPRK1 mRNAs in both differentiated and undifferentiated HCECs compared to the SH-SY5Y cell lines. MOR, DOR, and KOR mRNA expression was significantly lower ($p < 0.001$) in HCECs than in the SH-SY5Y cell lines (Fig. 7).

Discussion

In this study, we demonstrated that biphalin, which acts mainly via MORs and DORs, increases cell migration at the wound area and accelerates wound closure without affecting cell proliferation. Partial inhibition of the wound-healing effect of biphalin with naloxone can suggest MOR- and DOR-dependent mechanism of action. Our findings on biphalin are consistent with the wound-healing accelerator effects of MOR and DOR stimulations in cell cultures, which have been described previously. [14, 15] We also demonstrated that all three types of opioid receptors are present in HCECs using the qRT-PCR, which is also in line with previous reports.[16] To the best of our knowledge, this is the first study on the effects of a synthetic dimeric opioid peptide on human corneal epithelium cells in the literature.

Opioid receptors are distributed in the anterior segment tissues as well as in various layers of the retina and the optic nerve. They play important roles in the regulation of iris function, accommodation power, aqueous humor dynamics, corneal wound healing, retinal development, inhibition of neuroinflammation, neuroprotection against glaucomatous damage, and reduction of intraocular pressure. [17-20] Although various pharmacological mechanisms were proposed, exact processes underlying the effects induced by opioid receptors have not been clearly defined. Regarding opioid receptor types, both DOR and MOR were determined in the corneal tissues of certain animals. [21]

Limited data is available regarding the effects of opioids on corneal wound healing. Peyman et al. examined the analgesic and toxic effects of topical morphine on corneal abrasion in a rabbit corneal abrasion model. [22] They showed that morphine sulphate had a desirable analgesic property without irritating or causing any adverse effect on the cornea. Later, Stiles et al. studied the effect of topical application of a 1% morphine sulfate solution on signs of pain and wound healing in dogs with corneal ulcers and examined normal corneas immunohistochemically for the presence of MORs and DORs. [21] They showed that both MORs and DORs were present in normal canine corneas. They also demonstrated that topical morphine sulfate provided analgesia and did not interfere with normal wound healing. A recent study by Bigliardi et al. studied the effect of DORs in an in vitro scratch wound model, which we

also used in our study. [14] They found that migration and wound recovery were enhanced in human keratinocyte monolayers overexpressing DORs in vitro. They concluded that opioid receptors affect intercellular adhesion and wound-healing mechanisms, underlining the importance of a neuroendocrine system in wound healing and homeostasis. In another study, Wang et al. showed the role of delta and mu opioid receptors on wound healing and migration in DOR and MOR knockout mice. [15]

Synthetic opioids have been known to mimic the cytoprotective effects of endogenous opioidergic ligands and induce their physiological effects by the activation of DORs, KORs, and MORs, which were further subclassified into delta1 and delta2; kappa1, kappa2, and kappa3; and mu1, mu2, and mu3 opioid receptors, respectively. [6] Biphalin (PubChem CID: 5487663) was first synthesized by Lipkowski et al. in 1982 and then resynthesized by Mollica et al. with a modification in which two identical enkephalin-like tetrapeptides (Tyr-DAla-GlyPhe) were connected “head to head” by a hydrazide bridge (Fig. 1). [11, 23] In this modification, the natural residues of the dimeric opioid peptide biphalin were replaced by the corresponding homo- β amino acids. The derivative 1 containing h β Phe in place of Phe showed good MOR and DOR affinities and antinociceptive activity in vivo together with an increased enzymatic stability in human plasma. Biphalin has been shown to be one of the most potent peptide-based opioid analgesics. It crosses the blood-brain-barrier, resists enzymatic degradation, and exerts high metabolic stability in serum and the brain with half-lives of 87 and 193 min, respectively. [10] Biphalin exerts a higher binding affinity to DOR and MOR, greater analgesic potency than morphine and etorphine, and less dependence, excitatory hypersensitivity, and tolerance compared to morphine during chronic use. These DOR- and MOR-induced side effects of morphine are believed to be prevented by activation of KOR, and biphalin shows reduced MOR-related side effects due to the high potential of simultaneous MOR and DOR interactions and thus synergistic effects. [7, 8, 24]

Although the potential mechanisms of biphalin on promoting migration and accelerating wound healing in the corneal epithelium have not been evaluated in this study, inhibition of the influx of Ca²⁺, inhibition of PKC-dependent Na-K-Cl cotransporter, activation of MAP kinases, and inhibition of reactive oxygen species production or DORs may play a role in the mechanisms of biphalin-induced effects. [19; 25-27]

The antinociceptive effect of biphalin has been suggested to have a potential role in the treatment of cancer pain, abdominal pain associated with inflammatory bowel disease, and in neuropathic pain via different routes of administration, such as subcutaneous, intravenous, intrathecal, intracerebroventricular, and intraperitoneal. [9,28,29] Furthermore, recent studies have revealed the beneficial role of biphalin on cell viability and neuroprotection against excitotoxic and ischemic damage by inhibiting protein kinase C-dependent sodium potassium-chloride cotransporter expression in focal brain ischemia by activating downstream survival mitogen-activated protein kinases, and by inhibiting reactive oxygen species production in an opioid receptor-dependent manner, which was challenged by naltrexone. [19,25,30] Biphalin also acts as an immunomodulatory agent by stimulating human T cell proliferation, natural killer cell cytotoxicity in vitro, and interleukin-2 production and diminishes pro- and anti-inflammatory factors in lipopolysaccharide-treated microglial cells. [28, 31]

We proposed that the lack of a significant effect on proliferation of biphalin may be the result of its failure in binding to opioid growth factor receptors (OGFR), since OGFRs bear no resemblance to classical opioid receptors, and blockade of OGFR interaction with naltrexone accelerates cell growth. In the biphalin plus naloxone groups, both proliferation and wound healing were partially increased. We believe this effect was due to the binding of naloxone to OGFRs as a nonselective competitive opioid antagonist. [32]

In our study, there was no statistically significant difference between biphalin-treated cells at 10 μ M concentration and control groups on wound healing and cell migration. Biphalin shows approximately 100 times lower affinity to KOR than MOR and DOR. [33] Therefore, it is possible that at higher concentrations of biphalin, KORs are also activated and reverse the healing and migration process.

Many studies have demonstrated the cytoprotective and reformatory effects of DORs and MORs separately, and their synergistic activation may have strengthened their positive effects. [34] In further experiments, the exact effects of specific opioid receptor subgroup activation on human corneal epithelial cells should be explored with specific opioid peptides. Additionally, the effect of biphalin on the wound-healing process in the cornea and changes in keratinocytes, neural fibers, dendritic cells, the basement membrane, and extracellular matrix elements and their interactions should also be investigated.

The main limitation of the present study was the lack of data on the effect of biphalin on the intracellular signaling mechanisms and cell metabolism. In future experiments, we plan to examine the effects of biphalin and its derivatives on corneal healing in experimental animals, which will allow us to determine receptor-down signaling pathway interactions. A second limitation of our study that must be addressed is the lack of an *in vivo* animal model. We plan to conduct studies in transgenic mice in order to shed light on the effects of opioids in corneal wound healing.

Conclusion

In the corneal epithelium, biphalin accelerates wound healing and cell migration without affecting cell proliferation. Since the analgesic and antinociceptive effects of biphalin have been demonstrated in previous studies, it can be used in the near future as an analgesic after corneal traumas and surgeries to modulate corneal epithelial wound healing. We also propose that biphalin may become a new treatment alternative not only for corneal traumas but also for other neuropathic pain syndromes affecting the ocular surface, such as herpetic or diabetic neuropathy.

List Of Abbreviations

bovine pituitary extract: BPE; delta opioid receptor: DOR; 406-diamidino-2-phenylindole: DAPI; 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide: MTT; Dulbecco modified eagle medium: DMEM; epidermal growth factor: EGF; fetal bovine serum: FBS; G-protein-coupled opioid receptors: GPCRs; human immortalized corneal epithelial cells: HCECs; kappa-opioid receptor: KOR; keratinocyte serum-free

medium: KSFM; mu-opioid receptor: MOR; National Institutes of Health: NIH; phosphate-buffered saline solution: opioid growth factor receptors: OGFR; PBS; real-time polymerase chain reaction:qRT-PCR; relative wound area: RWA.

Declarations

Ethics approval and consent to participate: Since the study was a laboratory- based study not using patients or patient material, ethical approval was not required.

Consent for publication: Not applicable

Availability of data and material: Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Competing interests: The authors declare that they have no competing interests.

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Author Contributions: All authors have made substantial contributions to all of the following: (1) the conception and design of the study: OMGT, AS, EY (2) Performed the experiments: EY, KK (3) Analysis and interpretation of data: EY, KK (4) Contributed reagents/materials/analysis tools: AM, AS, EY (5) drafting the article or revising it critically for important intellectual content: OMGT, AS, EY, AM (3) Final approval of the version to be submitted: OMGT, AS, EY, AM, KK.

All authors read and approved the final manuscript.

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Titles And Legends For Supplementary Files

Supplement 1. Video of *in vitro* scratch assay model showing the effect of 1 μ M biphalin on relative wound healing area.

Supplement 2. Video of *in vitro* scratch assay model showing the effect of vehicle on relative wound healing area.

Table

Table 1. The primers of genes used in the study

Gene	Sequence	
GAPDH	F	5'-ACAAC TTTGGTATCGTGGAAGG-3'
	R	5'-GCCATCACGCCACAGTTTC-3'
OPRD1	F	5'-CGTCCGGTACACTAAGATGAAGA-3'
	R	5'-GCCACGTCTCCATCAGGTA-3'
OPRK1	F	5'-ATCATCACGGCGGTCTACTC-3'
	R	5'-ACTCTGAAAGGGCATGGTTGTA-3'
OPRM1	F	5'-CAGTGCTCATCATTACCGTGT-3'
	R	5'-CTGGGATTGTAACCAAGGCTTTA-3'

Figures

Figure 1

Molecular structure of biphalin.

Figure 2

Examples of in vitro live cell migration assays for each experimental group at various time points and example of the relative wound area analysis in ImageJ.

Figure 3

MTT cytotoxicity assay on HCECs for biphalin at various doses after 24-hour of drug incubation (n=4).

Figure 4

Effects of 1 μ M (a) and 10 μ M (b) biphalin and biphalin plus naloxone, and vehicle on in vitro scratch assay model on relative wound healing area (%). Both of the graphs show the results of two independent experiments ($n = 8$, * $p < 0.05$).

Figure 5

Effects of 1 μM (a) and 10 μM (b) biphalin and biphalin plus naloxone, and vehicle on the movement of corneal epithelial cells in the transwell migration assay. Both of the graphs show the mean number of migrated cells in ten different micrograph area (3.6 μm^2) in two independent experiments ($n = 20$, ** $p < 0.01$, *** $p < 0.001$).

Figure 6

Effects of 1 μ M biphalin and biphalin plus naloxone, and vehicle at 3 hours (a) and 6 hours (b); and those of 10 μ M biphalin and biphalin plus naloxone, and vehicle at 3 hours (c) and 6 hours (d) on cell proliferation in Ki67 proliferation assay. The graphs show the percentage of Ki67-labeled cells to DAPI-labeled cells in ten different micrographs in two independent experiments (n = 20).

Figure 7

Gene expression analysis with real-time polymerase chain reaction (qRT-PCR) of OPRM1 (a), OPRD1 (b), and OPRK1 (c) mRNAs, which are related with mu, delta and kappa opioid receptor proteins, in HCECs with KSFM or with serum (10% FBS) and compared with in SH-SY5Y cell lines.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplement1Biphalin.mp4](#)
- [Supplement2Vehicle.mp4](#)