

# Anti-inflammatory effects of hinokitiol on human corneal epithelial cells: an *in vitro* study

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## Abstract

**Purpose** This study assessed the anti-inflammatory effect and mechanism of action of hinokitiol in human corneal epithelial (HCE) cells.

**Methods** HCE cells were incubated with different concentrations of hinokitiol or dimethylsulfoxide (DMSO), which served as a vehicle control. Cell viability was evaluated using Cell Counting Kit-8 (CCK-8) assay. After polyriboinosinic:polyribocytidylic acid (poly(I:C)) stimulus, cells with or without hinokitiol were evaluated for the mRNA and protein levels of interleukin-8 (IL-8), interleukin-6 (IL-6), and interleukin-1 $\beta$  (IL-1 $\beta$ ) using real-time PCR analysis and an enzyme-linked immunosorbent assay (ELISA), respectively. Nuclear and cytoplasmic levels of nuclear factor kappa B (NF- $\kappa$ B) p65 protein and an inhibitor of NF- $\kappa$ B  $\alpha$  (I $\kappa$ B $\alpha$ ) were evaluated using western blotting.

**Results** There were no significant differences among the treatment concentrations of hinokitiol compared with cells incubated in medium only. Incubating with 100  $\mu$ M hinokitiol significantly decreased the mRNA levels of IL-8 to 58.77  $\pm$  10.41% ( $P < 0.01$ ), IL-6 to 64.64  $\pm$  12.71% ( $P < 0.01$ ), and IL-1 $\beta$  to 54.19  $\pm$  8.10% ( $P < 0.01$ ) compared with cells stimulated with poly(I:C) alone. The protein levels of IL-8, IL-6, and IL-1 $\beta$  had similar trend. Further analysis revealed that hinokitiol maintained the levels of I $\kappa$ B $\alpha$  and significantly reduced NF- $\kappa$ B p65 subunit translocation to the nucleus which significantly inhibiting the activation of the NF- $\kappa$ B signal pathway.

**Conclusion** Hinokitiol showed a significant protective effect against ocular surface inflammation through inhibiting the NF- $\kappa$ B pathway, which may indicate the possibility to relieve the ocular surface inflammation of dry eye syndrome (DES).

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## Introduction

Dry eye syndrome (DES) is a multifactorial disease of the tear ducts and ocular surface found both in humans and some domesticated animals, which results in symptoms of ocular discomfort (such as a burning sensation, itching, redness, and foreign body sensation), visual disturbance, and tear-film instability.<sup>1–3</sup> Epidemiologic studies have reported that > 15% of people suffer from DES worldwide<sup>4,5</sup> and it is becoming the most common reason for which patients seek ophthalmological care in the developed world.<sup>6</sup>

Although the exact mechanism of DES is not yet completely understood, there is increasing evidence to suggest that DES is the result of chronic inflammation and underlying cytokines.<sup>7–9</sup> Disease or dysfunction of the tear secretory glands leads to changes in tear composition, such as hyperosmolarity, which stimulate the production of inflammatory mediators on the ocular surface.<sup>10</sup> In turn, inflammation may cause dysfunction or disappearance of the cells responsible for tear secretion or retention.<sup>11</sup>

Hinokitiol is a natural component isolated from *Chamaecyparis taiwanensis* that exhibits antibacterial, antifungal, antiviral, antitumor, and insecticidal activities, with insignificant cytotoxicity.<sup>12–15</sup> Recent studies reported that Hinokitiol displayed a significant anti-inflammatory activity in a series of cells through different mechanisms.<sup>16,17</sup> Owing to the intense research interest surrounding new therapies for DES and the previous promising activity of hinokitiol in inflammatory conditions, it is of great interest to assess whether hinokitiol can display an anti-inflammatory effect on the ocular

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surface. To the best of our knowledge, previous reports investigating this issue in human corneal epithelial (HCE) cells have not appeared in the literature.

In this study, we examined the direct local anti-inflammatory effect of hinokitiol in HCE cells in order to evaluate whether it may be considered a possible agent to control the ocular surface inflammation.

## Materials and methods

### Drugs and chemicals

Hinokitiol was purchased from Sigma–Aldrich (St Louis, MO, USA), polyriboinosinic:polyribocytidylic acid (poly (I:C)) was purchased from InvivoGen (San Diego, CA, USA), NF- $\kappa$ B p65 and  $\beta$ -actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-TATA-binding protein (TBP) antibody was purchased from Abcam (Cambridge, MA, USA).

### Cell culture

Simian virus (SV) 40-immortalized human corneal epithelial cells were kindly provided by Dr Zan Pan (New York University, New York City, NY, USA). The cell culture was performed according to a previously described method,<sup>18</sup> which was modified as follows. In brief, HCEs were cultured in Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM/F12; Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Gibco), 5 ng/ml human epidermal growth factor (Gibco), 5  $\mu$ g/ml insulin (Gibco), and 100  $\mu$ /ml penicillin–streptomycin, and cultured in 60-mm cell culture dishes at 37 °C in an atmosphere of 95% air and 5% CO<sub>2</sub>. Normal culture development was assessed daily by phase-contrast microscopy. Cells were passed with 0.25% trypsin–EDTA (Sigma–Aldrich, St Louis, MO, USA) and seeded into new dishes until a density of 90% confluency was reached. The medium was replaced in each well with serum-free medium 2 h before the experiment.

### Cell viability assay

The viability of cells was measured using a Cell Counting Kit-8 (CCK-8) assay. In brief,  $2 \times 10^4$  HCE cells/well were seeded into 96-well microplates and allowed to adhere overnight before treatment with different concentrations of hinokitiol dissolved in DMSO (100, 50, or 25  $\mu$ M) for 24 h. An equal volume of medium with 1% DMSO was used as a vehicle control, as well as medium as a blank control. After treatment, the medium was replaced with 10% CCK-8 agent (Dojindo, Kumamoto, Japan) and then cells were incubated for another 3 h at 37 °C in the dark. Absorbance at 450 nm was measured with an iMark

Microplate Reader (Bio-Rad, CA, USA) and the results were normalized to untreated cells.

### Real-time PCR analysis

For quantitative real-time PCR (qPCR) analysis, cells were pretreated with different concentrations of hinokitiol (100, 50, 25, or 0  $\mu$ M) for 1 h after they reached an appropriate density in 6-well plates, then the cells were not washed and treated with poly(I:C) at a dose of 25  $\mu$ g/ml, as previously described,<sup>19</sup> for 12 h before collecting. An equal volume of medium with 1% DMSO was used as a vehicle control, as well as serum-free medium, which was used as a blank control. Total RNA was extracted using a TRIzol RNA Kit (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. cDNA was synthesized from purified and concentrated 0.5  $\mu$ g RNA from each sample using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio, Inc., Shiga, Japan). qPCR was run on a CFX96 Real-Time PCR Detection System (Bio-Rad) using the SYBR Premix Ex Taq Kit (Takara Bio, Inc., Shiga, Japan) to evaluate interleukin-8 (IL-8), interleukin-6 (IL-6), and interleukin-1 $\beta$  (IL-1 $\beta$ ) mRNA expression levels. The PCR product calculations were made using the  $\Delta\Delta C_T$  method, as previously described.<sup>20</sup> Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control gene in order to normalize the PCR for the amount of RNA added to the real-time reactions.

### Enzyme-linked immunosorbent assay (ELISA)

HCE cells were preincubated with different concentrations of hinokitiol (100, 50, 25, or 0  $\mu$ M) for 1 h in 24-well plates, then cells were not washed and treated with 25  $\mu$ g/ml poly(I:C) for 24 h before the culture supernatants were collected. An equal volume of medium with 1% DMSO was used as a vehicle control and serum-free medium was used as a blank control. IL-8, IL-6, and IL-1 $\beta$  ELISA kits (RayBiotech, Norcross, GA, USA) were used to assess the levels of these cytokines. All the steps were performed according to the manufacturer's instructions. In summary, 100  $\mu$ l of each standard and 100  $\mu$ l of the study samples were pipetted into precoated 96-well plates, which were covered and incubated for 2.5 h at room temperature. Then, the solutions were discarded and the wells were washed with Wash Buffer. To each well, 100  $\mu$ l 1 $\times$  prepared biotinylated antibody was added and the plates incubated for 1 h at room temperature. After washing away any unbound biotinylated antibody, 100  $\mu$ l horseradish peroxidase (HRP)-conjugated streptavidin was added and, after 45 min, the plate was washed again, followed by the addition of 100  $\mu$ l TMB (3,3',5,5'-tetramethylbenzidine) One-Step Substrate Reagent and incubation for 30 min at

room temperature in the dark. After the addition of a stop solution, the color intensity was measured at 450 nm with an iMark Microplate Reader (Bio-Rad). The concentrations of the cytokines were calculated using Sigmaplot 12.0 software (IBM, Armonk, NY, USA).

#### Western blot

To assess I $\kappa$ B $\alpha$  in whole-cell lysate and NF- $\kappa$ B p65 protein levels in the cytoplasm and nucleus, HCE cells were preincubated with hinokitiol (100 or 0  $\mu$ M as a control) for 1 h, then cells were washed and treated with 25  $\mu$ g/ml poly(I:C) for 1 h before collecting. An equal volume of medium with 1% DMSO was used as a vehicle control and serum-free medium was used as a blank control. For I $\kappa$ B $\alpha$ , a total protein isolation kit (Abcam, Cambridge, MA, USA) was used according to the manufacturer's protocol. The separation of cytoplasmic and nuclear protein was performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific Pierce, Rockford, IL, USA). Protein concentrations were determined using a BCA assay. Proteins (60  $\mu$ g) from each sample were electrophoresed on a 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat milk for 1 h and were then incubated overnight at 4  $^{\circ}$ C with the following primary antibodies: I $\kappa$ B $\alpha$ ; NF- $\kappa$ B p65;  $\beta$ -actin; and TATA-binding protein (TBP). After four washings with Tris-buffered saline with Tween 20 (TBST), membranes were incubated with the secondary antibodies for 1 h at room temperature. After five washings with TBST, immunoreactive bands were developed by enhanced chemiluminescence and scanned via an ultraviolet spectrophotometer (Eppendorf, HH, Germany). Data were analyzed using BandsScan 5.0 software (BioMarin, San Rafael, UK).

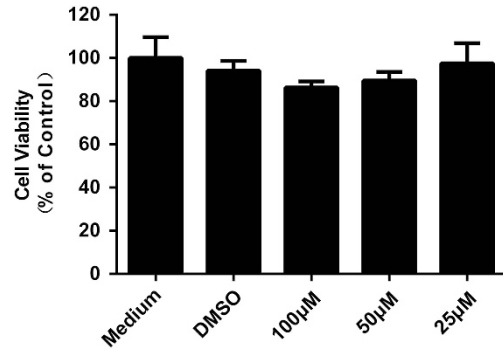
#### Statistical analysis

Data are shown as the mean  $\pm$  SE. Statistical analyses and multiple comparisons were performed by one-way analysis of variance (ANOVA) with a *post hoc* Tukey's test using the GraphPad Prism software version 6.0 (GraphPad Software Inc., San Diego, CA, USA). Differences between the variants were considered significant when  $P < 0.05$ .

## Results

#### Effects of hinokitiol on HCE cell viability

The viability of HCE cells following treatment with a series of concentrations of hinokitiol, as mentioned above, for 24 h was examined using the CCK-8 method. As



**Figure 1** HCE cell viability after hinokitiol treatment. Cells were treated with medium only, 1% DMSO or hinokitiol (100, 50, or 25  $\mu$ M) and cell viability was measured via a CCK-8 assay after 24 h incubation. Hinokitiol did not affect cell viability at the assessed concentrations. Data are shown as a percentage compared with cells cultured in medium only from three independent experiments.

shown in Figure 1, hinokitiol did not affect cell viability compared with the untreated control. In addition, there were no significant differences in cell viability between the different concentrations of hinokitiol (100, 50, and 25  $\mu$ M) and the vehicle control.

#### mRNA levels of proinflammatory cytokines

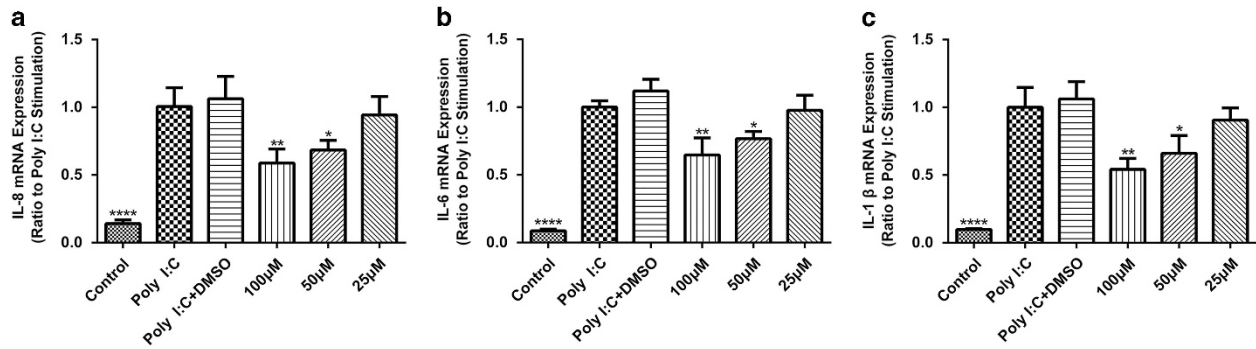
The mRNA level of IL-8 in HCE cells stimulated with poly(I:C) was up to 7-fold higher than untreated control cells, 11-fold higher for IL-6 ( $P < 0.01$ ), and 10-fold higher for IL-1 $\beta$  ( $P < 0.01$ ).

Hinokitiol treatment at a concentration of 100  $\mu$ M, significantly reduced the mRNA expression levels of IL-8 to  $58.77 \pm 10.41\%$  ( $P < 0.01$ ), IL-6 to  $64.64 \pm 12.71\%$  ( $P < 0.01$ ), and IL-1 $\beta$  to  $54.19 \pm 8.10\%$  ( $P < 0.01$ ) compared with cells stimulated with poly(I:C) alone. Similarly, 50  $\mu$ M hinokitiol showed a significant effect with regard to the inhibition of mRNA expression for these proinflammatory cytokines. However, the levels of IL-8, IL-6, and IL-1 $\beta$  decreased without statistically significant differences as compared with control cells following treatment with 25  $\mu$ M hinokitiol (Figures 2a–c).

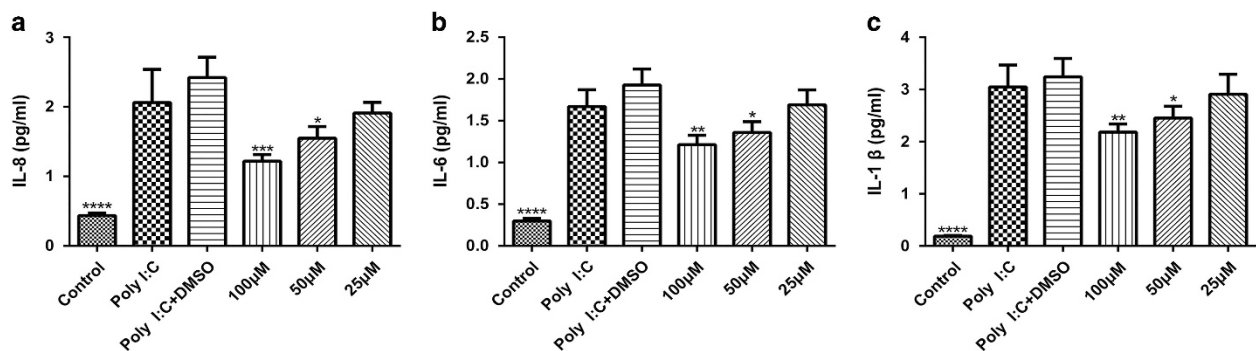
#### Protein levels of proinflammatory cytokines

The protein level of IL-8 in HCE cells stimulated with poly(I:C) was 2.062 pg/ml ( $P < 0.0001$ ), 1.669 pg/ml for IL-6 ( $P < 0.0001$ ), and 3.05 pg/ml for IL-1 $\beta$  ( $P < 0.0001$ ).

Hinokitiol treatment at a concentration of 100  $\mu$ M significantly reduced the protein expression levels of IL-8 to 1.218 pg/ml ( $P < 0.001$ ), IL-6 to 1.214 pg/ml ( $P < 0.01$ ), and IL-1 $\beta$  to 2.183 pg/ml ( $P < 0.01$ ) compared with cells stimulated with poly(I:C) alone. Similarly, 50  $\mu$ M



**Figure 2** Effect of hinokitiol on the mRNA expression of poly(I:C)-induced proinflammatory cytokines in HCE cells, as detected by qPCR (a–c). Cells were treated with serum-free medium as a control, 25 μg/ml poly(I:C), poly(I:C) with 1% DMSO, poly(I:C) with 100 μM hinokitiol, poly(I:C) with 50 μM hinokitiol, and poly(I:C) with 25 μM hinokitiol. Hinokitiol was added 1 h before the poly(I:C) stimulus and the total incubation period was 12 h. Data are shown as ratios with the poly(I:C) treatment group from three independent experiments. \*\*\*\* $P < 0.0001$ , \*\* $P < 0.01$ , and \* $P < 0.05$  compared with the poly(I:C) treatment group.



**Figure 3** Effect of hinokitiol on the protein expression of poly(I:C)-induced proinflammatory cytokines in HCE cells, as detected by ELISA (a–c). Cells were treated with serum-free medium as a control, 25 μg/ml poly(I:C), poly(I:C) with 1% DMSO, poly(I:C) with 100 μM hinokitiol, poly(I:C) with 50 μM hinokitiol, and poly(I:C) with 25 μM hinokitiol. Hinokitiol was added 1 h before the poly(I:C) stimulus and the total incubation period was 24 h. Data from three independent experiments are shown. \*\*\*\* $P < 0.0001$ , \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , and \* $P < 0.05$  compared with the poly(I:C) treatment group.

hinokitiol showed a significant effect with regard to the inhibition of the expression of these proinflammatory cytokines. However, although 25 μM hinokitiol resulted in a reduction in the protein expression of these cytokines, the changes were not statistically significant (Figures 3a–c).

#### Inhibition of the NF-κB signal pathway

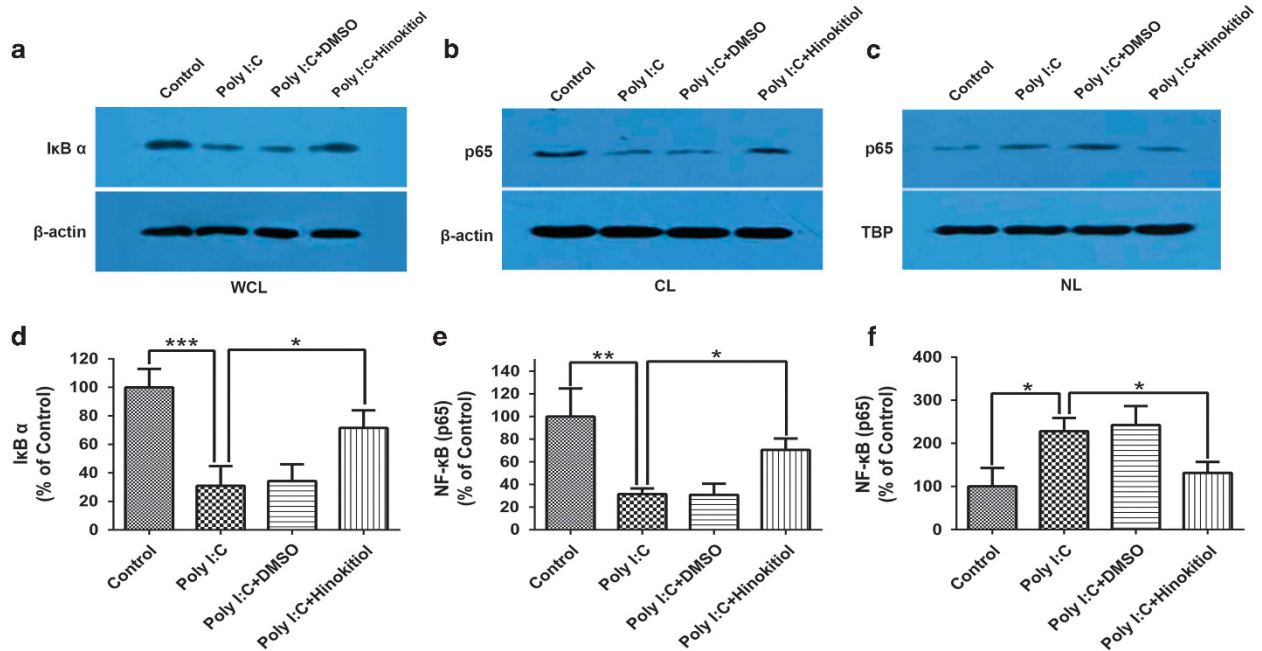
NF-κB is a central molecule in the inflammatory cascade. To study whether the anti-inflammatory effect of hinokitiol could be related to the inhibition of NF-κB activation, NF-κB translocation was examined. As shown in Figures 4a–f, in nuclear fractions from cells treated with poly(I:C), NF-κB p65 increased to  $228.2 \pm 30.93\%$  ( $P < 0.05$ ) compared with untreated cells. However, following preincubation with 100 μM hinokitiol, the p65 nuclear translocation level reduced to  $131.3 \pm 25.41\%$  ( $P < 0.05$ ). The cytoplasmic levels of NF-κB

p65 in the hinokitiol-treated group were significantly higher than that of the poly(I:C) only group ( $P < 0.05$ ). We also monitored the IκBα expression of the whole-cell lysate in the presence or absence of hinokitiol. HCE cells treated with poly(I:C) alone had significantly decreased levels of IκBα; however, hinokitiol pretreatment attenuated this effect. These results indicated that hinokitiol administration could significantly inhibit the activation of NF-κB through maintenance of IκBα levels and reduction of the nuclear translocation of NF-κB p65 after poly(I:C) stimulation.

#### Discussion

In our study, hinokitiol displayed a remarkable anti-inflammatory effect in HCE cells without a detrimental effect on cell viability. It significantly inhibited the inflammatory reaction induced by poly(I:C) and markedly reduced the expression of proinflammatory





**Figure 4** Effect of hinokitiol on the inhibition of the NF-κB signal pathway in a poly(I:C)-induced inflammation model. Cells were treated with serum-free medium as a control, 25 μg/ml poly(I:C), poly(I:C) with 1% DMSO, and poly(I:C) with 100 μM hinokitiol. Hinokitiol was added 1 h before the poly(I:C) stimulus and the total incubation period was 2 h. The levels of IkBα (a, d) and cytosolic (b, e) and nuclear (c, f) NF-κB p65 were detected by western blot. Data are shown as a ratio compared with untreated cells from three independent experiments. WCL, whole-cell lysate; CL, cytosolic lysate; NL, nuclear lysate. \*\*\**P*<0.001, \*\**P*<0.01, and \**P*<0.05 compared with the poly(I:C) treatment group.

cytokines, IL-8, IL-6, and IL-1β, at both the mRNA and protein levels in a dose-dependent manner. Furthermore, the translocation of NF-κB p65 protein from the cytoplasm to the nucleus after poly(I:C) stimulation was significantly inhibited by hinokitiol administration, which suggested that the anti-inflammatory effect of hinokitiol may act through the inhibition of the NF-κB signal pathway.

DES is a heterogeneous disease that is related with aging, hormonal changes, inflammatory, environmental influence, and autoimmune states.<sup>21</sup> Supplementation with artificial tears is regarded as a mainstay for mild to moderate dry eye at present. However, it often requires frequent application and turns out only symptomatic relief.<sup>22</sup>

Recently, two novel agents—Diquafosol and Rebamipide—with different mechanisms have entered dry eye treatment clinical trials, which brought out promising results. Diquafosol is a P2Y<sub>2</sub> purinergic receptor agonist which can stimulate both fluid secretion from conjunctival epithelial cells and mucin secretion from conjunctival goblet cells directly on the ocular surface independently of lacrimal gland.<sup>23–25</sup> Several studies have reported the clinical efficacy of diquafosol in treating objective signs and subjective symptoms of dry eye.<sup>26–30</sup> Rebamipide is a quinolinone derivative. At the end of 2011, rebamipide ophthalmic suspension (Otsuka Pharmaceutical, Co, Ltd, Tokyo, Japan) was approved for treating dry eye.<sup>31</sup> It has been reported that rebamipide

increases the amount of mucin-like substances on the conjunctiva and cornea of rabbits whose mucin in the cornea and conjunctiva were decreased by *N*-acetylcysteine.<sup>32</sup> A series prospective studies have demonstrated that rebamipide suspension has significant effect of improving fluorescein corneal staining, lissamine green conjunctival staining, Schirmer's test, tear-film breakup time, and dry eye-related ocular symptoms without serious adverse reaction.<sup>31,33–35</sup>

Meanwhile, there is increasing evidence that the excessive expression of inflammatory mediators, such as IL-8, IL-6, IL-1β, and TNF-α, on the ocular surface may have an important role in DES pathogenesis and clinical symptoms.<sup>36–38</sup> These cytokines promote the activation and migration of leukocytes, the expansion of pathogenic T cells and the production of other proinflammatory factors that mediate DES.<sup>39</sup> Regardless of the initiating cause, a vicious cycle of inflammation resulting from activation of an inflammatory cascade on the ocular surface may lead to eye damage.<sup>40</sup>

In light of research findings suggesting that inflammatory mediators are involved in DES, the use of anti-inflammatory therapy has been gaining popularity.<sup>41</sup> The major anti-inflammatory agents currently in use include topical corticosteroids and immunomodulatory agents.<sup>42</sup> However, despite the rapid and significant effect of corticosteroids, questions surround their potential

toxicity following long-term use and they have been linked to an increase in intraocular pressure and cataracts. Such side effects limit the use of corticosteroids in the chronic treatment of DES.<sup>40</sup> Meanwhile, some topical immunomodulatory agents have been approved by the FDA for use on the ocular surface, such as Restasis, which is based on CsA. However, the controversial adverse effects, such as burning and stinging eyes, conjunctival hyperemia, cataracts, and eye pain, have led to the discontinuation of treatments based on CsA.<sup>43</sup> As such, the development of safe and efficient treatments for DES remains of great importance.

Poly(I:C) is a synthetic dsRNA that strongly induces a series of proinflammatory cytokines, including IL-8, IL-6, IL-1 $\beta$ , and interferons, probably through binding to Toll-like receptor (TLR)-3, as has been shown in a rabbit kidney cell line<sup>44</sup> and an HCE cell line.<sup>19</sup> Thus, we applied poly(I:C) to induce the secretion of inflammatory mediators in HCE cells in order to study the innate ocular surface immunity after hinokitiol treatment.

Hinokitiol is a bioactive tropolone-related compound found in the wood of cupressaceous plants. In recent years, accumulative studies have focused on the potential protective effect of hinokitiol against inflammation in different research fields. Jayakumar *et al*<sup>45</sup> reported the inhibitory effect of hinokitiol on inflammatory responses (ie, HIF-1 $\alpha$  and induced NO synthase expression) and apoptosis (ie, TNF- $\alpha$  and active caspase-3) in rats with cerebral ischemia, resulting in a reduction of infarct volume and an improvement in neurobehavior. Byeon *et al*<sup>16</sup> discovered that hinokitiol inhibited lipopolysaccharide (LPS)-activated TNF- $\alpha$  secretion and NO synthase in macrophage-like (RAW264.7) cells. Furthermore, hinokitiol also exhibited anti-inflammatory activation in MG-63 cells, which led to the downregulation of inflammatory gene mRNA levels for COX-2 and HIF-1 $\alpha$ .<sup>17</sup>

In rodent models, administration of an IL-1 receptor antagonist provided a significant improvement in ocular surface integrity, increased tear secretion and restored the normal glycosylation pattern of goblet cell mucins.<sup>46</sup> Moreover, the proinflammatory cytokine blocking agent such as Anakinra also provided a novel therapeutic effect in a randomized clinical trial, where it improved corneal fluorescein staining, complete bilateral corneal fluorescein staining (CFS) clearance, and dry eye-related symptoms as measured by the Ocular Surface Disease Index, tear-film breakup time, and meibomian gland secretion quality, with no reports of a serious adverse reaction.<sup>47</sup>

In this study, hinokitiol inhibited IL-8, IL-6, and IL-1  $\beta$  at both the mRNA and protein levels, indicating that it has anti-inflammatory potential and may be an efficient agent for the treatment of DES. It should be noted, however, that while doses of 100 and 50  $\mu$ M resulted in statistically significant reductions in the mRNA and

protein levels of IL-8, IL-6, and IL-1  $\beta$ , 25  $\mu$ M hinokitiol resulted in only slight decreases, which were not statistically significant. This suggests that there may be a threshold concentration of hinokitiol that results in a therapeutic anti-inflammatory effect and a dose of 25  $\mu$ M may be close to or below this threshold value.

NF- $\kappa$ B is a key regulator of the inflammatory cascade and has had a pivotal role in the onset and resolution phases of inflammation, acting as a primary transcription factor responding rapidly to the inflammatory stimulus by controlling the transcription of inflammatory cytokine genes (ie, IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$ ).<sup>48,49</sup> Activation of NF- $\kappa$ B involves the phosphorylation and subsequent proteolytic degradation of the inhibitory protein I $\kappa$ B $\alpha$  kinases, which leads to NF- $\kappa$ B translocation to the nucleus and initiation of the transcription of downstream inflammatory mediators. Consequently, targeting NF- $\kappa$ B is one crucial strategy for the suppression of inflammation. Our data show that hinokitiol maintains the levels of I $\kappa$ B $\alpha$  and significantly reduces NF- $\kappa$ B p65 subunit translocation to the nucleus, suggesting that the anti-inflammatory activity may take effect through inhibition of the NF- $\kappa$ B signal pathway.

## Conclusion

This study provides evidence to support the anti-inflammatory activity of hinokitiol in HCE cells and, importantly, indicates that this activity may involve the inhibition of the NF- $\kappa$ B cascade and its downstream proinflammatory mediators which may indicate the possibility of relieving the ocular surface inflammation and regulating the innate immune response system status inducing TLR3 of DES.

## Summary

### What was known before

- Dry eye syndrome (DES) is a multifactorial disease, which results in symptoms of ocular discomfort. Although the exact mechanism of DES is not yet completely understood, there is increasing evidence suggesting that DES is the result of chronic inflammation and underlying cytokines. However, to date, there has been a lack of efficient anti-inflammatory agents with proven long-term safety.

### What this study adds

- Our study demonstrates the significant protective effect of hinokitiol against poly(I:C)-induced inflammation by decreasing IL-8, IL-6 and IL-1 both on mRNA and protein levels in human corneal epithelial cells. Our research suggests that this anti-inflammatory effect may be the result of inhibition of the NF- $\kappa$ B signal pathway. Consequently, our findings indicate the possibility of hinokitiol to relieve the inflammation of DES.

### Conflict of interest

The authors declare no conflicts of interest.

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