• Basic Research •

Inhibitory effects of luteolin on TLR3-mediated inflammation caused by TAK/NF-κB signaling in human corneal fibroblasts

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Abstract

● **AIM:** To study the role of luteolin (LUT) in the expression of toll-like receptors 3 (TLR3) ligand polyI:C stimulated inflammatory factors in human corneal fibroblasts (HCFs).

● **METHODS:** HCFs cells were cultivated with or without LUT or polyI:C. The expression levels of interleukin (IL)-6, IL-8, monocyte chemotactic protein-1 (MCP-1), vascular cell adhesion molecule (VCAM)-1, as well as intercellular adhesion molecule (ICAM)-1 were measured using enzyme-linked immunosorbent assay (ELISA), immunoblotting or reverse transcription-quantitative polymerase chain reaction (PCR) analyses. Immunoblotting was used to assess toll-interleukin-1 receptor-domain-containing adapter-inducing interferon-β (TRIF), TLR3, transforming growth factor-b-activated kinase 1 (TAK1), tumor necrosis factor receptor-associated factor 6 (TRAF6), the transcription factor AP-1, as well as transcription factor nuclear factor (NF-κB)–inhibitory protein IκB-α degradation and phosphorylation. Immunofluorescence assays were used to localize the cellular location of the p65 subunit of NF-κB.

● **RESULTS:** Corneal fibroblasts exposed to polyI:C demonstrated decreased VCAM-1, ICAM-1, MCP-1, IL-6, and IL-8 expression levels upon exposure to LUT in a time-dependent and concentration-dependent manner. LUT was observed to suppress polyI:C-triggered expression of TLR3, the translocation of NF-κB p65 into cell nuclei, as well as the phosphorylation of TAK, c-Jun, and IκB-α, while no impact on the expression levels of TRIF and TRAF6 were observed.

● **CONCLUSION:** LUT suppress the expression of proinflammatory adhesion molecules, chemokines, and cytokines in polyI:C exposed HCFs. These effects are likely mediated through TAK/NF-κB signal attenuation. Therefore, LUT is a candidate molecule that can prevent the TLR3-mediated inflammation response associated with corneal viral infection.

● **KEYWORDS:** toll-like receptor; inflammation; corneal fibroblast; polyI:C

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INTRODUCTION

Cellular immunity has been recognized as the first-line immune defense mechanism against foreign pathogens. It is an indispensable part of the host response that prevents viral replication and disease[1]. Cellular immunity encompasses the activation of pathogen recognition receptors, including toll-like receptors (TLRs), C-type lectin receptors, and scavenger receptors[2]. In human corneal fibroblasts (HCFs), TLRs are essential for the recognition of a myriad of organisms, including fungi, bacteria, and viruses[3]. TLR3 functions as a double stranded viral RNA sensor found in HCFs and acts to initiate early innate immune defense responses against viruses through the activation of the Toll-IL-1 receptor domain-containing adapter-inducing IFN-β (TRIF) pathway[4].

Corneal viral infection causes corneal ulceration, progressive and irreversible corneal scarring, corneal neovascularization, and turbidity, and may progress to blindness[4]. An epidemiological study conducted in 2012 showed that there were approximately 1.5 million cases of herpes simplex virus keratitis worldwide. These cases included approximately 40 000 newly diagnoses of severe vision loss or blindness[5]. Cell viral

invasion is accompanied by TLR3 receptor recognition of the double-stranded viral RNA, which recruits TRIF and leads to activation of a series of terminal downstream reaction elements, including nuclear factor kappa B (NF-κB) as well as activator protein-1 (AP-1), which eventually culminate in the release of proinflammatory agents, including interleukin (IL)-6, IL-8, monocyte chemotactic protein-1 (MCP-1), vascular cell adhesion molecule 1 (VCAM-1) as well as intercellular adhesion molecule 1 (ICAM-1)[3,6]. High concentrations of local proinflammatory mediators in the cornea that are secreted in response to viral invasion, which results in irreversible corneal damage, such as in cases of herpetic stromal keratitis. Steroids are currently the mainstay method of treatment used to control inflammation in viral keratitis[7]. Nevertheless, serious adverse events, such as increased intraocular pressure, which results in secondary glaucoma, which limits their use as long-term agents to treat viral keratitis[8]. Therefore, there is a need to develop safer and efficacious local anti-inflammatory antiviral agents.

Luteolin (LUT) is a natural flavonoid that has been reported to be characterized by antioxidant, anti-microbial, anti-inflammatory, chemo-preventive, as well as chemotherapeutic properties[9]. In Iran, Brazil, and China, plants with high LUT content have been used to treat several inflammatory conditions[9]. Previously published reports have shown that LUT can reduce levels of IL-6 as well as MCP-1 release in human retinal pigment epithelial cells exposed to 4-hydroxynonenal and alleviate the adverse effects of viral inflammation caused by inhibiting cytokines and chemokines in dsRNA-induced macrophages[9]. Human umbilical vein endothelial cells treated with LUT demonstrated suppressed VCAM-1, ICAM-1, as well as MCP-1 expression levels[9]. However, the effects of LUT on TLR3-mediated inflammation in the cornea have yet to be completely elucidated. The current investigation delineates the impact of LUT on polyI:C-stimulated TLR3 ligand release from inflammatory components in HCFs as well as TLR3/TRIF/NF-κB, and c-Jun activation.

MATERIALS AND METHODS

Eagle's minimum essential medium (MEM; cat. C11095500BT), 10% fetal bovine serum (FBS; cat. 10270106), phosphate-buffered saline (PBS; cat. SH30256.01), HEPEs (cat. no. 15630080), as well as 0.25% trypsin-EDTA (cat. 25200072) were procured from Invitrogen-Gibco company (Rockville, MD, USA). The Corning company (Corning, NY, USA) supplied flasks, 6-well plates, and cell culture dishes. Luteolin (cat. L9283-50MG) and antibodies against cytokeratin (cat. C5301), vimentin (cat. V6389), and clpha-smooth muscle actin (α-SMA; cat. SAB1400414) were obtained from Sigma-Aldrich (Saint Louis, USA), while polyI:C (cat. tlrl-pic) was obtained from InvivoGen (San Diego, California, USA). Promega Corporation (Hollow Road, Madison, USA) supplied the CytoTox 96® Non-Radioactive Cytotoxicity Assay (cat. G1780) and CellTiter 96 Aqueous One Solution Cell Proliferation assay (cat. G3580). Enzyme-linked immunosorbent assay (ELISA) kits for IL-6 (cat. Q6000B), IL-8 (cat. Q8000B), and MCP-1 (cat. DCP00) identification were supplied by R&D Systems (Minneapolis, MN, USA). The Cell Signaling Technology company (Beverly, MA, USA) supplied the following antibodies: IκB-α (cat. 9242), p-IκB-α (cat. 9251), c-Jun (cat. 9165), p-c-Jun (cat. 9164), tumor necrosis factor receptor-associated factor 6 (TRAF6; cat. 8028), transforming growth factor-b-activated kinase 1 (TAK1; cat. 4505), and p-TAK-1 (cat. 4508). Santa Cruz Biotechnology (Delaware Avenue, Santa Cruz, USA) supplied the following antibodies: ICAM-1 (cat. sc-8439), VCAM-1 (cat. sc-13160), and the p65 subunit of NF-κB (cat. sc-8008). Enzo Life Sciences (Farmingdale, NY, US) supplied antibodies specific to TRIF (cat. ALX-215-016-R200). R&D system (Minneapolis, MN, USA) supplied antibodies specific to TLR3 (cat. AF1487). Protein Tech Group (Pearl Street, Rosemont, USA.) supplied the antibody against GAPDH (cat. CPA9067). Molecular Probes (Eugene, Oregon) was the source of 4'6-diamidino-2-phenylindole (DAPI, cat. 62247) as well as Alexa Fluor 488–labeled goat antibodies (cat. A30006) against mouse or rabbit IgG. All materials used for cell culture and reagents that possessed minimal cytotoxicity.

Cell Culture ScienCell Research Laboratories (Carlsbad, CA, USA) provided primary HCFs. The cells were cultivated in Eagle's MEM supplemented with 10% fatal bovine serun (FBS; 10% FBS-MEM), penicillin (100 U/mL), as well as streptomycin (100 μg/mL), and cultured in a humidified environment with CO_2 (5%) and at a temperature of 37°C. The cell medium was refreshed every three to four days. The experiments were performed using cells that were between passages four and seven.

Cytotoxicity Assay To quantify LDH activity in the supernatants of the HCF cell culture, 96-well plates were used to house the cells (3×10^4 cells/well) in MEM containing 10% FBS. The cell medium was replaced with serum-free medium for another 24h after 95% cell confluence was achieved. The starved cells were incubated for another 24h in serum-free MEM with or without LUT (0, 5, 10, 20 μmol/L). The cell supernatant was incubated with the reagent cartridge at room temperature for 30min and then detected using a 96-well microplate reader and at 490 nm. The experiments were performed in triplicate.

Cell Proliferation Assay and Cell Viability Assay For the MTS assay, the HCFs were added into 96-well plates (4×10^3 cells/well) for different durations (4, 8, 12, 24, 36, 48h). Subsequently, MTS assay solution (20 μL) was added into the 96-well plate for 4h. A microplate reader was used to

evaluate the optical density (OD) value at 490 nm. To assess the cell viability, the HCFs were cultured in 24-well plates (1.5×10^4 cells/well) in MEM containing 10% FBS for 7d. The cells were stained with Trypan blue and the number of cells was counted using a hematocytometer. The experiments were performed in triplicate.

MCP-1, IL-6, and IL-8 Assays Three immune mediators, MCP-1, IL-6, as well as IL-8 were quantified using ELISA kits. The serum-deprived HCFs were incubated whether or not with LUT (0, 5, 10, 20 μmol/L) overnight, and treated for another 24h with polyI:C (1 μg/mL)[10]. The cell supernatant was collected after centrifuged (120×g, 5min, 4℃), and frozen at the temperature of -80℃ to be used in subsequent ELISA analyses of the immune mediators. To determine the number of cells per well, the cells were exposed to trypsin-EDTA and isolated from the plates for culture and counted using a hemocytometer. Based on the cell counts and the cell morphology analyses, count data was obtained through cell protein measurements of the cell supernatant divided by the cell number[11]. The experiments were repeated thrice.

Immunoblotting Analysis Immunoblotting analysis was adopted for the detection of HCF expression levels of ICAM-1, VCAM-1, as well as various phosphorylated signaling proteins[12]. The 6 cm petri dishes were used to house the HCFs (5×10^5 cells) in MEM containing 0.5% FBS. The cell medium was replaced with the media without serum for another twenty-four hours once 95% cell confluence was achieved. Then, the starved cells were incubated for another 24h in serum-free MEM whether or not with LUT (20 μmol/L). Thereafter, the cells were re-incubated with the same buffer whether or not with polyI:C (1 μg/mL) for an additional 90min (for signaling proteins) or 24h (for ICAM-1, VCAM-1 and TLR3). The treated cells were washed twice using chilled PBS. Then, protein lysate (100 μL) was added to each well, prior to collecting the proteins on ice. Lysate protein concentrations were evaluated using the BCA method. SDS-PAGE was performed using in-house produced 10% gels. The separated proteins were transferred onto PVDF membranes (0.45 μm) purchased from Merck Millipore (Carrigtwohill, Ireland) and were incubated with 5% skimmed milk dissolved in 1×TBS containing 0.3% Tween 20 to inhibit endogenous reactions. The membranes were then incubated with the blocking buffer-diluted primary antibodies (all at 1:1000) overnight at 4℃. After rinsed the following day using a Tris-HCl (pH 7.4) buffer (20 mmol/L) as well as Tween-20 (0.1%), membranes were incubated again at room temperature with the corresponding secondary antibodies bound to horseradish peroxidase (dilution 1:3000) for an hour. The protein blots were promptly visualized using a Tanon-5200 Multi-imaging System after treatment using an enhanced chemiluminescence (ECL) kit obtained from Tanon Science and Technology (Shanghai, China). The experiments were performed in triplicate.

Table 1 List of DNA primers sequences

Gene	Nucleotide sequence (5'-3')	Product length (bp)
GAPDH		187
F	ACTCCTCCACCTTTGACGCT	
R	GGTCTCTCTCTTCCTCTTGTGC	
IL-6		120
F	TTCGGTCCAGTTGCCTTCT	
R	GGTGAGTGGCTGTCTGTGTG	
IL-8		192
F	GGGTGGAAAGGTTTGGAGTAT	
R	TAGGACAAGAGCCAGGAAGAA	
MCP-1		173
F	ATCAATGCCCCAGTCACCT	
R	TCCTGAACCCACTTCTGCTT	
TLR3		690
F	CGCCAACTTCACAAGGTA	
R	GGAAGCCAAGCAAAGGAA	
ICAM-1		750
F	CACAGTCACCTATGGCAACG	
R	TCCTTGATCTTCCGCTGGC	
VCAM-1		108
F	CCTTCATCCCTACCATT	
R	GTGTTTGCGTACTCTGC	

F: Forward; R: Reverse.

Reverse Transcription-quantitative PCR Analysis Real-time RT-qPCR assays were conducted to evaluate the mRNA expression levels of IL-6, IL-8, MCP-1, ICAM-1, VCAM-1, and TLR3[13-14]. The HCFs were incubated in MEM containing 10% FBS for 24h, followed by another 24h of incubation in a medium without serum, another 24h of incubation whether or not with LUT (5, 10, or 20 μmol/L) in the MEM without serum, finally another 60min of incubation (for TLR3), or 6h (for IL-6, IL-8, MCP-1, ICAM-1, and VCAM-1) in the same solution whether or not with polyI:C (1 μg/mL). Thereafter, total cell RNA was extracted using a TIANGEN RNA Extraction Kit. cDNA was synthesized using 500 ng of RNA and a Reverse Transcription Kit (Takara, Japan) followed by real-time PCR assay using the Bio-Rad (Hercules, CA) CFX96 optical reaction. Table 1 shows the sequences of PCR primers. The RT-qPCR protocol used was as follows: 95℃ for 3min for primary denaturation, 95℃ of 15s for denaturation, 61℃ for 15s for annealing, and 72℃ for 15s for extension. A total of 40 cycles were conducted for each reaction. The relative protein expressions were normalized by the GAPDH expression level with the CT method used to calculate target change $=2^{-\Delta\Delta CT}$. All experiments were performed in triplicate, with the average calculated.

Immunofluorescence Staining For immunofluorescence staining[15], the HCFs (5×10^5 cells) were first seeded into 60 mm dishes and kept in MEM containing 0.5% FBS

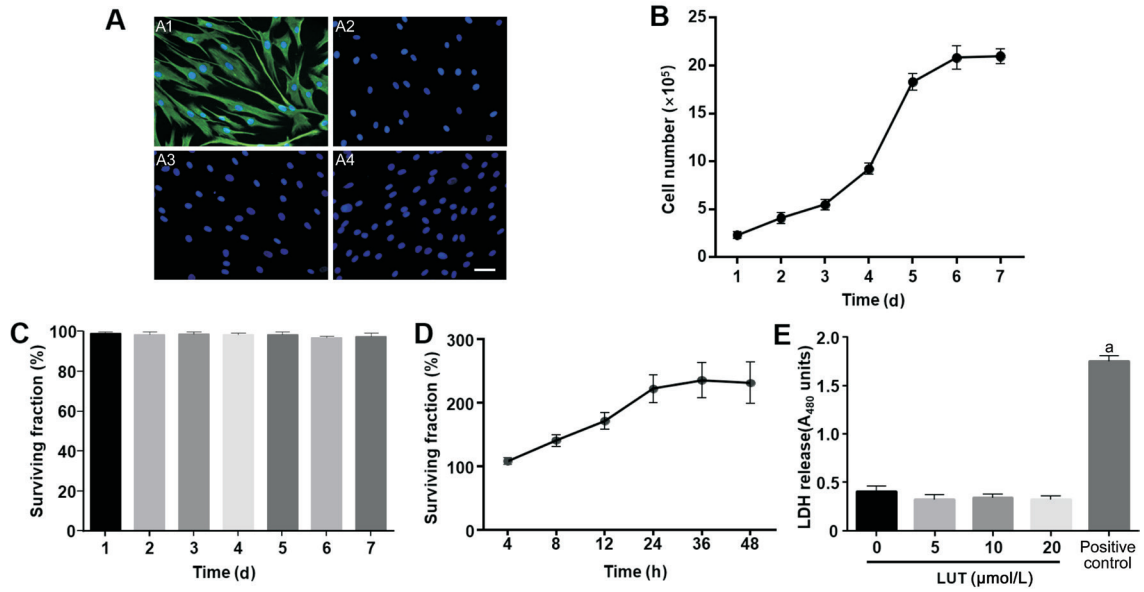

Figure 1 The characteristic and viability of HCFs A: Immunofluorescence staining (green) of cultured HCFs using antibodies against vimentin (A1), cytokeratin (A2), α-SMA (A3) or with control mouse immunoglobulin G (A4). The cell nuclei were stained with DAPI (blue). Scale bar, 100 μm. B-D: The viability of HCFs was assessed by cell counting (B), Trypan blue staining (C) and MTS assay (D). E: The HCFs were grown in serum-free media using the indicated doses of LUT for 24h prior to the quantification of the LDH activity of the culture medium. [a]*P*<0.05 *vs* treatment group.

until 60%-70% confluence was achieved. The cell medium was replaced with serum-free medium for an additional 24h. Then, the serum-starved cells were incubated for another 24h in serum-free MEM with or without LUT (20 μmol/L), followed by another 90min of incubation in the same solution in the presence or absence of polyI:C (1 μg/mL). 4% paraformaldehyde was used to fix the cells, which were then permeabilized for 15min with Triton-X-100 (0.2%) at ambient room temperature. The slides were rinsed with PBST (0.1% Tween in PBS) between each step and were then immersed in bovine serum albumin (3%) for fifteen minutes at room temperature to inhibit non-specific antibody binding before incubation with the primary antibody. Then, mouse monoclonal antibody against NF-κB (diluted using 1% bovine serum albumin: 1:50) was adopted for cell incubation for an hour at room temperature, followed by the incubation with the corresponding Alexa Fluor 488-conjugated secondary antibodies (diluted 1:500) for 1h at room temperature followed by DAPI incubation. A Zeiss fluorescence microscope (Oberkochen, Germany) was used to obtain the cell images. The experiments were performed in triplicate.

Statistical Analysis SPSS 20.0 (IBM Corp., USA) was adopted to perform all data analyses. All data were depicted as mean±standard error (number of observations). Comparisons between variables were carried out by Student's *t* test; Comparisons among multiple datasets were perfomed by one-way analysis of variance (ANOVA) test. *P* values less than 0.05 indicate statistically significant.

RESULTS

Characteristic and Viability of Cultured HCFs The purity of the corneal fibroblast cultures was determined based on both cell morphology and immunoreactivity using antibodies against cytokeratin (epithelial cell marker), vimentin (non-epithelial cell marker), as well as α-SMA (myofibroblast marker). All cells used in this study were positive for vimentin but negative for both cytokeratin and α-SMA (Figure 1A). The results of Trypan blue staining showed that the percentage of viable cells was >99% (Figure 1C). The cell growth curve (Figure 1B) and the MTS assay (Figure 1D) demonstrated the good proliferation ability of the cultured HCFs. Cell exposure to various concentrations (5, 10, and 20 μmol/L) of LUT for 24h had no effect on the release of LDH, indicating a lack of cytotoxicity of this drug (Figure 1E).

LUT Inhibits polyI:C-stimulated IL-6, IL-8, and MCP-1 Secretion in HCFs To investigate the way LUT affects polyI:C-induced inflammatory cytokine secretion, the protein expressions of IL-6, IL-8, as well as MCP-1 were evaluated using ELISA. LUT (5, 10, and 20 μmol/L) pretreatment noticeably suppressed the polyI:C induction of IL-6, IL-8, and MCP-1 in a dose-dependent manner, *vs* the corresponding controls (Figure 2). Additionally, the RT-qPCR analysis revealed that polyI:C induced the upregulation of mRNA expression levels of IL-6, IL-8, and MCP-1. Similar to the results of the ELISA analyses, LUT suppressed mRNA levels of the aforementioned inflammatory cytokines. Moreover, preconditioning cells for 24h with LUT (5, 10 and 20 μmol/L) further inhibited the

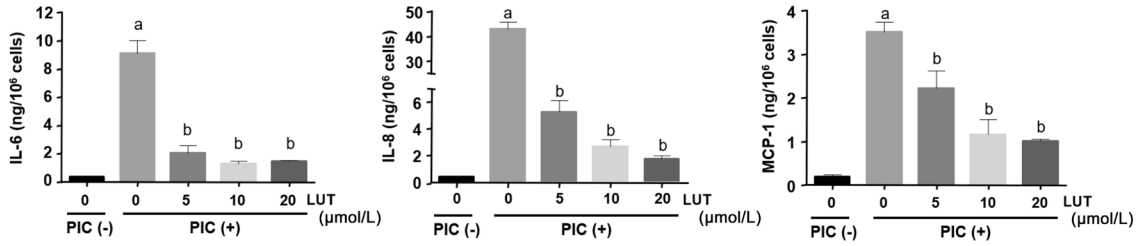

Figure 2 LUT inhibits polyI:C-stimulated secretions of IL-6, IL-8, as well as MCP-1 in HCFs Serum-deprived HCFs were exposed to predetermined concentrations of LUT for 24h followed by 24h of exposure to polyI:C (1 μg/mL). Enzyme-linked immunosorbent assay (ELISA) was conducted to quantify HCF secretion levels of IL-6, IL-8, and MCP-1. [a]*P*<0.05 *vs* cells incubated without the treatment; [b]*P*<0.05 *vs* cells incubated with polyI:C alone.

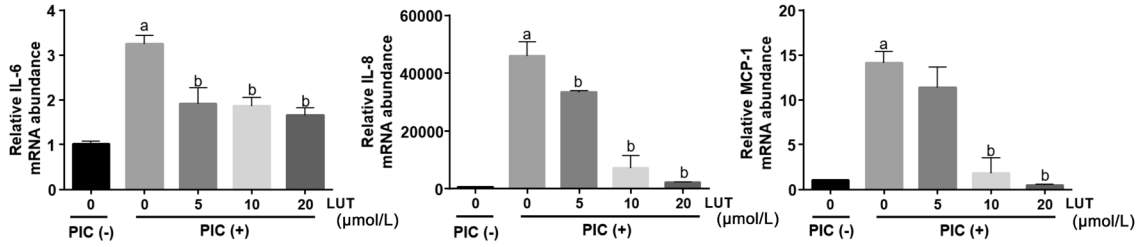

Figure 3 LUT inhibits polyI:C-stimulated upregulation of IL-6, IL-8, as well as MCP-1 mRNA expression levels in HCFs Serum-starved HCFs were incubated with LUT (5, 10, and 20 μmol/L) for 24h, followed by 6h whether or not with polyI:C (1 μg/mL). RT-qPCR was used to quantify the IL-6, IL-8, and MCP-1 mRNA expression levels. Data is depicted as the mean±SD of the experiment performed in triplicate. [a]*P*<0.05 *vs* cells incubated without the treatment; [b]*P*<0.05 *vs* cells incubated with polyI:C alone.

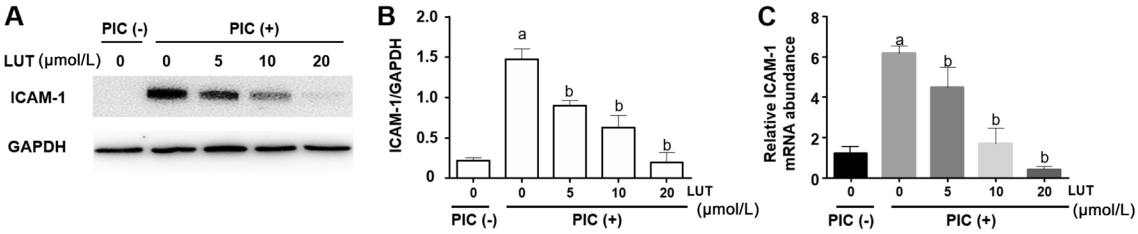

Figure 4 LUT exerts concentration-dependent effects on ICAM-1 expression by HCFs Both immunoblotting (A) and qualitative (B) analyses indicate that HCF secretes ICAM-1 upon exposure to polyI:C in a concentration-dependent manner. Error bars represent SD. [a]*P*<0.05 *vs* cells incubated without pretreatment; [b]*P*<0.05 *vs* cells incubated with polyI:C alone. C: Serum-starved HCFs were incubated for 24h whether or not with LUT (5, 10, and 20 μmol/L), followed by 6h whether or not with polyI:C (1 μg/mL). RT-qPCR was used to assess ICAM-1 mRNA expression levels. [a]*P*<0.05 *vs* cells incubated without the treatment; [b]*P*<0.05 *vs* cells incubated with polyI:C alone.

proinflammatory effect exerted by polyI:C. LUT also reduced the basal quantities of IL-6, IL-8, as well as MCP-1 mRNA among HCF cells in a dose-dependent manner (Figure 3).

Inhibition by LUT of polyI:C-induced ICAM-1 and VCAM-1 Expressions in HCFs RT-qPCR as well as Western blotting were adopted to assess the impact of LUT on polyI:C-induced expression of adhesion molecules in the HCFs. PolyI:C remarkably enhanced ICAM-1 (Figure 4A) and VCAM-1 (Figure 5A) expression levels, *vs* the normal group, while LUT exposure (5, 10, 20 μmol/L) significantly reduced ICAM-1 and VCAM-1 expression levels of polyI:C-stimulated cells in a concentration-dependent manner. The results were further analyzed using grayscale analysis (Figures 4B and 5B). Similar findings were also obtained from the RT-qPCR analysis, showing that polyI:C upregulated the mRNA expression levels of ICAM-1 (Figure 4C) as well as VCAM-1 (Figure 5C), while LUT pretreatment (20 μmol/L) for 6h re-established the baseline levels of the adhesion molecules.

Effects of LUT on TLR3 Expression in polyI:C treated HCFs PolyI:C resulted in upregulated the protein (Figure 6A) and mRNA (Figure 6B) levels of TLR3, compared with the controls. LUT exposure abolished the effects exerted by polyI:C on TLR3, resulting in normal or even downregulated TLR3 levels.

Effects of LUT on polyI:C-induced TAK/NF-κB Signaling Activation in HCFs Several intracellular signaling pathways were investigated using immunoblotting, and it was found that LUT (20 μmol/L) suppressed polyI:C-induced phosphorylation of c-JUN and TAK-1 in HCFs, while exerting no significant inhibitory effects on other key factors in selected MAPKs

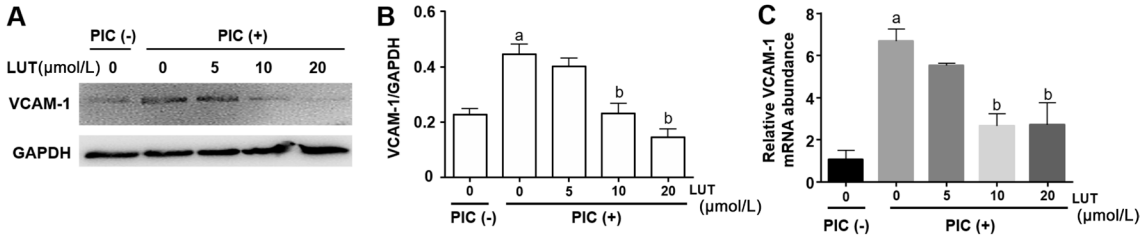

Figure 5 LUT exerts concentration-dependent effects on VCAM-1 expression in HCFs Both immunoblotting (A) and qualitative (B) analyses indicate that HCF secretes VCAM-1 upon exposure to polyI:C in a concentration-dependent manner. Error bars represent SD. [a]*P*<0.05 *vs* cells incubated without pretreatment; [b]*P*<0.05 *vs* cells incubated with polyI:C alone. C: Serum-starved HCFs were incubated for 24h whether or not with LUT (5, 10, and 20 μmol/L), then for 6h whether or not with polyI:C (1 μg/mL). RT-qPCR was used to assess VCAM-1 mRNA expression levels. [a]*P*<0.05 *vs* cells incubated without the treatment; [b]*P*<0.05 *vs* cells incubated with polyI:C alone.

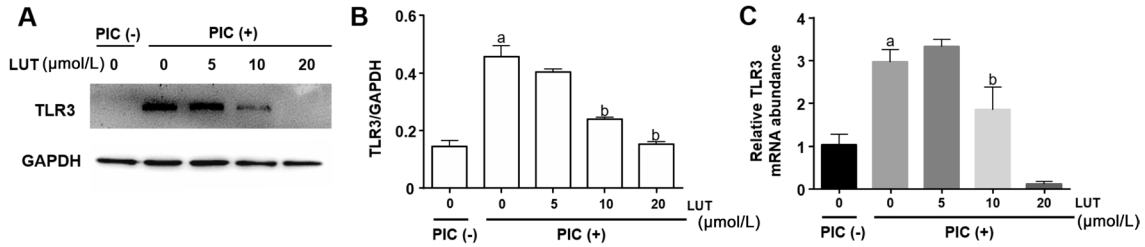

Figure 6 LUT inhibits polyI:C-stimulated upregulation of TLR3 mRNA expression in HCFs Serum-starved HCFs were incubated for twenty-four hours whether or not with LUT (5, 10, and 20 μmol/L), followed by 6h whether or not with polyI:C (1 μg/mL). The results of the immunoblot analysis (A), greyscale analysis (B) and RT-qPCR (C) showed that LUT inhibited the polyI:C-stimulated upregulation of TLR3 mRNA expression in HCFs. [a]*P*<0.05 *vs* cells incubated without the treatment; [b]*P*<0.05 *vs* cells incubated with polyI:C alone.

(Figure 7A). To investigate whether LUT (20 μmol/L) modulates polyI:C-induced NF-κB activation, we estimated the expression level of IκB-α and p-IκB-α and quantified the nuclear translocation of NF-κB p65. LUT (20 μmol/L) inhibited the polyI:C-stimulated IκB-α phosphorylation (a NF-κB inhibitor) of HCFs (Figure 7A). Greyscale analyses confirmed the statistically significant effect of LUT on the phosphorylation of TAK-1, c-JUN and IκB-α (Figure 7B). The immunofluorescence analysis showed that under control conditions, NF-κB p65 was predominantly situated in the cell plasm, whereas it was translocated into the cell nuclei after HCF cells were exposed to polyI:C for 90min, as assessed by immunofluorescence staining. Poly(I:C)'s effect was inhibited by LUT partially (Figure 8). Taken together, our findings suggest that LUT (20 μmol/L) attenuated NF-κB signaling pathway activation, which was stimulated by polyI:C treatment of the HCFs (Figures 7 and 8).

DISCUSSION

Viral corneal infection is characterized by immunoinflammatory lesions that arise from complex interactions between resident corneal cells and infiltrating immune cells, which include macrophages, T cells, and polymorphonuclear leukocytes[7]. In keratitis, direct viral invasion of the host cell results in local TLR3 production *via* viral double stranded RNA, which acts as a TLR3 ligand[16]. In an effort to eliminate the offending pathogen, this phenomenon activates the downstream TRIF/NF-κB signaling pathway, which subsequently induces corneal fibroblasts to enter an inflammatory state in which they release a myriad of cytokines to promote the inflammation (including IL-6, IL-8, as well as MCP-1), and cell adhesion molecules (including ICAM-1 as well as VCAM-1[6]). However excessive inflammation inevitably leads to an imbalance in corneal homeostasis, marked by the overproduction of vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP), which ultimately breaks down corneal infrastructure. Inflammation-induced corneal degeneration manifests clinically as progressive corneal ulcers, corneal turbidity and thinning, and may even lead to blindness[12,17]. Based on the above, it can be postulated that targeting the TLR3/NF-κB signaling pathway may be critical for abolishing the deleterious effects of viral keratitis on the cornea.

Immune system homeostasis is dependent on a delicate balance of pro-inflammatory and anti-inflammatory cytokines[18]. Disorders of cytokine production can lead to severe inflammation[19]. A variety of interleukins, including IL-6 as well as IL-8, participate in inflammation regulating by modulating natural and acquired immunity. A myriad of immune cells, such as fibroblasts as well as activated T and B cells, have the ability to secrete IL-6. IL-6 can activate neutrophil chemotaxis by producing chemokines that mediate the occurrence of chronic inflammation[20]. As a member of

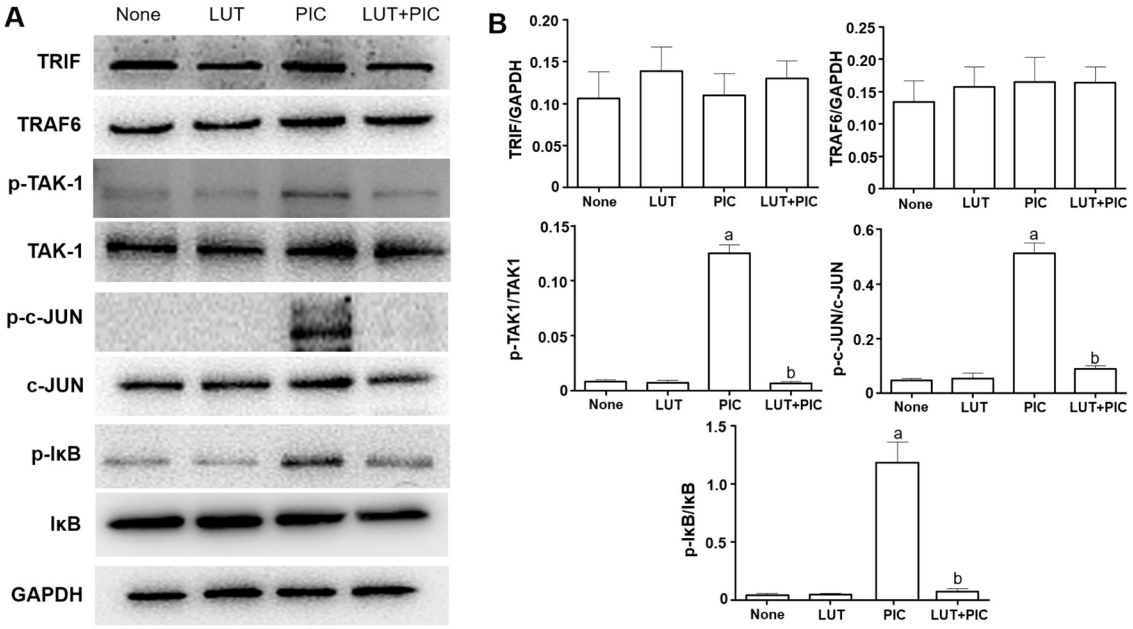

Figure 7 Effects of LUT on polyI:C-stimulated TAK/NF-κB signaling pathway activation among HCFs Serum-starved HCFs were incubated for twenty-four hours whether or not with LUT (20 μmol/L), then for another 90min whether or not with polyI:C (1 μg/mL). Western blotting (A) and greyscale analyses (B) were performed to determine the expression levels of TRIF, TRAF6, p-TAK1, TAK1, p-c-JUN, c-JUN, p-IκB, IκB and GAPDH protein. [a]*P*<0.05 *vs* cells incubated without the treatment; [b]*P*<0.05 *vs* cells incubated with polyI:C alone.

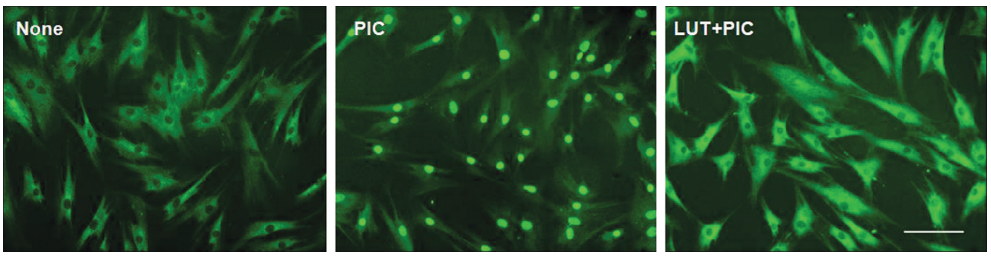

Figure 8 Effect of LUT on the polyI:C-related activation of NF-κB signaling among HCFs Serum-starved HCFs were incubated for twenty-four hours whether or not with LUT (20 μmol/L), followed by 90min whether or not with polyI:C (1 μg/mL). Immunofluorescence staining was performed and paraformaldehyde (4%) was adopted for cell fixation, while Triton-X-100 (0.2%) was adopted for cell permeabilization. Then, 3% bovine serum albumin was used to blocking the cells. NF-κB p65 antibodies (green fluorescence) were added to visualize the cells and DAPI (blue fluorescence) was used to visualize the nuclei under a Zeiss fluorescence microscope. LUT (20 μmol/L) interdicted the translocation of the NF-κB p65 subunit from the cytoplasm into the nucleus, after the cells were exposed to polyI:C for 90min. Scale bar, 20 μm.

the interleukin family, IL-8 can specifically chemoattract neutrophils into inflamed tissues, promote their degranulation, activate inflammatory cells, participate in inflammatory pathological damage, promote the release of inflammatory mediators, and promote fibroblast proliferation chronic inflammation[21]. MCP-1, otherwise known as monocyte chemotactic and activating factor, is a member of the C-C subfamily (β subfamily). MCP-1 has been confirmed both *in vivo* and *in vitro* to activate monocytes and macrophages, enhance monocyte chemotactic activity, release lysozymes, produce and release superoxide anions, increase cytoplasmic Ca^{2+} concentrations, produce cytokines IL-1 and IL-6, and upregulate macrophage and monocyte-specific adhesion molecules, including those of the integrin family, such as β2 and α4[22]. The entry and exit of inflammatory cells into inflamed corneal cells are guided by a variety of factors, including the adhesion molecules, VCAM-1 and ICAM-1[23]. Our study demonstrates that LUT inhibits the expression of ICAM-1 and VCAM-1 and the release of IL-6, IL-8, and MCP-1 in HCFs treated with polyI:C, in a concentration-dependent manner. Therefore, LUT may function as a therapeutic anti-inflammatory agent against corneal viral infection. These findings are similar to the results of previous experiments, in which LUT was found to suppress MCP-1 and IL-6 secretion levels as well as inhibit the surface expression of VCAM-1 and ICAM-1 in various cell types[10].

Recent research has provided convincing evidence that TLRs, which are widely expressed in various cells, function as pattern recognition receptors (PRR)[24]. TLR3 belongs to the TLR family that plays a fundamental role in pathogen

recognition on the ocular surface. It has been shown that TLR3 recognizes and binds to double-stranded RNA, resulting in enhanced TLR3 expression that overstimulates downstream signaling pathways and overall inflammatory mediation[6]. Activated TLR3 recruits TRIF and TRAF6, which is followed by the ubiquitination of the TAB2/TAB3/TAK1 complex that promotes TAK1 activation. In comparison, inhibitor of NF-κB (IκB) phosphorylation is known to be modulated by IKKα/β, which is controlled by upstream factors, such as TAK1. TAK1 phosphorylation binds it to inhibitor of nuclear factor kappa B kinase beta (IKKβ), leading to IκB phosphorylates degradation followed by NF-κB nuclear translocation which activates the transcription and consequent induction of inflammatory cytokines[25]. These findings demonstrated that the expression levels of polyI:C induced proinflammatory chemokines, cytokines, and adhesion molecules were abrogated in HCFs upon LUT exposure, likely due to the inhibitory effect exerted by LUT on TLR3 overexpression and TAK1 phosphorylation. Furthermore, previous studies have provided similar findings on other types of cells, including RAW 264.7 macrophages. Luteolin-7-O-Glucuronide (L7Gn), an active ingredient of LUT, has been shown to prevent inflammatory mediators (including IL-6, IL-1β, as well as cyclooxygenase-2) overproduced. This occurrence was found to be a result of TAK1 inhibition, which results in the suppressed activation of c-Jun N-terminal kinase (JNK), NF-κB, and p38 among RAW 264.7 macrophages[26]. In addition, experiments conducted on bone-derived macrophages have found that LUT inhibits TLR3 or TLR4-target gene expression levels, which include TNF-α, IL-6, IL-12, and IL-27[27]. Our findings suggest that LUT may be an effective for the treatment of severe inflammation and oxidative stress under other conditions apart from ocular disease.

To clarify the anti-inflammatory mechanisms of LUT, we sought to characterize the proinflammatory signaling pathways involved. NF-κB and AP-1 signals are the primary pathways though which HCFs regulate intraocular inflammation[4]. PolyI:C can be recognized by TLRs, providing it with the ability to trigger host defense mechanisms through NF-κB and AP-1 signaling pathway activation[28]. It has been established that the phosphorylation of TAK-1, an upstream component of TLR signaling, triggers the AP-1 pathway, in which c-jun exerts its regulatory effect as a result of heterodimerization-triggered AP-1 gene activation[3]. In relation, we confirmed that LUT suppressed polyI:C-induced upregulation of the AP-1 component, c-Jun, while interestingly inducing no significant change in other complexes of the adaptor proteins, such as TRIF, TRAF6, and TAK1. This implies that LUT inhibits AP-1 phosphorylation, resulting in suppressed initiation and propagation of inflammation.

NF-κB belongs to a family of heterodimeric transcription factors that exert widespread effects on the immune system[29]. In most resting cells, the inhibitory effect exerted by IκB proteins can sequester NF-κB proteins into the cytoplasm, which ensures the immune homeostasis of cells in their resting state. NF-κB is essential for stimulating proinflammatory gene expression. NF-κB is activated by the degradation of the phosphorylated form of its inhibitor, IκBα. Once activated, NF-κB is translocated into the nucleus to stimulate target gene expression[30]. Excessive NF-κB gene expression is a central component of several inflammatory diseases, since NF-κB signaling is essential in maintaining cellular immune homeostasis[31]. This study found that LUT can block NF-κB p65 translocation into the cell nuclei induced by the TLR3 agonist, polyI:C, which leads to the upregulation of inflammatory cytokines. Further studies are warranted to determine if there are other pathways besides the TRIF/NF-κB signaling pathway that modulate the effect of LUT on polyI:C-stimulated HCFs. However there are certain limitations in this study and more in-depth animal experiments, such as an alkali injury model to test that whether LUT can properly function during an inflammatory event, which should be the focus of further research in the future.

In conclusion, we showed that LUT can effectively abolish the inflammatory process activated by corneal keratitis *in vitro* through inhibiting TLR3/TAK/NF-κB signaling pathway. Our study is the first to demonstrate the anti-inflammatory properties of LUT and to provide insights into its potential use as a therapeutic agent against viral keratitis.

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