• Basic Research •

The anti-inflammatory effects of asiatic acid in lipopolysaccharide-stimulated human corneal epithelial cells

Hao Chen, Xiao-Min Hua, Bai-Chen Ze, Bin Wang, Li Wei

Special Medicine Department of Medical College, Qingdao University, Qingdao 266071, Shandong Province, China
Correspondence to: Bin Wang; Li Wei. Special Medicine Department of Medical College, Qingdao University, Qingdao 266071, Shandong Province, China. wangbintezhong@126.com; liweitezhong@126.com
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Abstract

• AIM: To investigate the anti-inflammatory effects of asiatic acid (AA) on lipopolysaccharide (LPS)-induced inflammatory response in human corneal epithelial cells (HCECs).

• METHODS: Cell viability was measured using a cell counting kit-8 (CCK-8) assay. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine the mRNA expression of interleukin-8 (IL-8), interleukin-6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), and transforming growth factor-β (TGF-β) in HCECs. Intracellular reactive oxygen species (ROS) was measured using the ROS assay kit. Glutathione (GSH) concentration was measured using the total GSH assay kit. Akt1 and Akt phosphorylation (p-Akt1) levels were measured by Western blotting and immunofluorescence.

• RESULTS: AA induced toxicity at high concentrations and significantly stimulated the proliferation of HCECs at concentrations of 20 μmol/L for 1h. LPS at concentrations of 300 ng/mL for 1h significantly stimulated the mRNA expression of IL-8, IL-6, IL-1β, TNF-α, and TGF-β in HCECs, while the stimulation effects were significantly inhibited by AA (20 μmol/L). In addition, AA was found to decrease the content of ROS, increase GSH generation, and also inhibit LPS-induced p-Akt in HCECs.

• CONCLUSION: AA decreases the generation of inflammatory factors IL-8, IL-6, IL-1β, TNF-α, and TGF-β in LPS-stimulated HCECs. AA significantly inhibites the intracellular concentrations of ROS and increases GSH generation. AA also inhibites LPS-induced p-Akt in HCECs. These findings reveal that AA has anti-inflammation effects in LPS-stimulated HCECs.

• KEYWORDS: asiatic acid; lipopolysaccharide; inflammatory factors; reactive oxygen species; glutathione; Akt phosphorylation

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INTRODUCTION

Initiated by a breakdown of the epithelial barrier, bacterial keratitis is a serious ocular infectious disease that can lead to severe visual disability and blindness[1-3]. After the occurrence of bacterial keratitis, even if antimicrobial agents were used to control infection clinically, secreted inflammatory medium will cause severe inflammation. Commonly used clinical hormonal anti-inflammatory agents have a strong effect, but can easily cause complications, such as cataract and high intraocular pressure. Non-steroidal anti-inflammatory drugs are often weak and have difficulty meeting the demands of a clinical anti-inflammatory. Therefore, many people have shown much interest in traditional Chinese medicine. Indeed, asiatic acid (AA), a triterpenoid isolated from Centella asiatica, has been reported to exhibit anti-oxidant, anti-tumor, and anti-inflammatory effects[4-6]. Previous studies have indicated that AA also has beneficial effects in decreasing the paw edema[4], inducing cancer cell inhibition and apoptosis[7-8], and improving metabolic and hemodynamic abnormalities by decreasing oxidative stress and inflammation[5].

Cells generate energy by reducing molecular oxygen to water. During this process, small amounts of partially reduced reactive oxygen forms are produced as an unavoidable by-product of mitochondrial respiration. Some of these forms are free radicals that can cause cell injury. They are referred to as reactive oxygen species (ROS). The toxic effect of these ROS and free radicals can be eliminated by enzymes such as superoxide dismutase (SOD) which eliminates O_2 to produce H_2O_2[9]. This is eliminated by glutathione (GSH) peroxidase or by catalase. Oxidative damage has also been reported to play a role in corneal diseases including corneal inflammation[10] and keratitis[11].

These ROS stimulate the activation of mediator signaling molecules as the transcription factor nuclear factor kappa-B (NF-κB)[12], that up-regulates the production of inflammatory

cytokines, such as interleukin-1β (IL-1β) or tumor necrosis factor-α (TNF-α)[13] and others mediators, as inducible nitric oxide synthase (iNOS) or cyclooxygenase-2 (COX-2)[14]. Moreover, ROS can damage cellular lipids, lipid peroxidation products, and lipid-derived aldehydes as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), and acrolein, which are implicated in numerous oxidative stress-induced inflammatory diseases with harmful effects[15]. Inflammation is a normal protective response to a variety of cell and tissue damage. Its role is to remove harmful cells and tissues, as well as repair them. Uncontrolled inflammatory response results in extensive cell and tissue damage, giving rise to normal cell and tissue destruction, which is associated with chronic inflammation and various human chronic diseases[16]. Ocular tissue and fluids, except the retina, possess little protection against superoxide radicals that would be formed during an inflammatory reaction. Oxidative stress has recently been linked to the corneal, conjunctival and lacrimal gland injury that is associated with certain ocular surface conditions.

Until date, infectious keratitis poses a diagnostic dilemma due to the varied presentation and visual morbidity if adequate control measures are not instituted in time[17]. Newer drugs with an increased efficacy and less resistance could led to a decrease in the rates of blinding complications. Although a wide range of potentially therapeutic effects of AA have been reported, however, little is known about the effects of AA on bacterial keratitis. Thus, the present study investigated the anti-inflammatory effects of AA in lipopolysaccharide (LPS)-stimulated human corneal epithelial cells (HCECs).

MATERIALS AND METHODS

Materials AA (97%) and LPS (Escherichia coli 055:B5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The AA was diluted to the test concentration with DMEM-F12 supplemented with 5% (v/v) fetal bovine serum (FBS). Phosphate-buffered saline (PBS), DMEM-F12, and FBS were obtained from Gibco (Invitrogen, USA). Other chemicals and reagents used in this study were of analytical grade.

Cell culture HCECs lines were obtained from the Shandong Eye Institute. HCECs were propagated in DMEM-F12 supplemented with 10% (v/v) FBS and maintained at 37℃ in a humidified atmosphere containing 5% (v/v) CO_2, with the medium changed every two or three days.

Cell Proliferation Assay Cell viability was measured using a cell counting kit-8 (CCK-8) assay (SAB, USA) according to the manufacturer's instructions. HCECs (1.0×10^5 cells/mL) were seeded into 96-well plates with 200 μL of growth medium containing 2.5, 5, 10, 20, 40, 60 and 80 μmol/L of AA and 200 μL DMEM-F12 supplemented with 5% (v/v) FBS as a blank control group. The cells were incubated at 37℃ in CO_2 for 24, 48 and 72h, respectively. Of 100 μL of serum-free DMEM-F12 and 10 μL of CCK-8 solution were added and incubated at 37℃ for 4h. Optical density (OD) value was measured using a microplate reader (SUNRISE, Switzerland) at 450 nm. All experiments were conducted in triplicate.

Treatment of Human Corneal Epithelial Cells with E. Coli Lipopolysaccharide and Asiatic Acid The HCECs (1.0×10^5 cells/mL) were seeded into 6-well plates and allowed to attach and grow overnight. They were then replaced with 1 mL of growth medium containing 10, 50, 100, 200 and 300 ng/mL of E. Coli LPS and 1 mL of DMEM-F12 supplemented with 5% (v/v) FBS as a blank control group for 1 and 24h, respectively. The HCECs (1.0×10^5 cells/mL) were then seeded into 6-well plates and pretreated with serum-free DMEM-F12 for 12h, and then treated with E. Coli LPS and AA. The experimental groups are listed as follows: control, LPS (300 ng/mL) 1h, AA (20 μmol/L) 1h, and AA (20 μmol/L)+LPS (300 ng/mL) 1h.

RNA Extraction and Quantitative Real-time Polymerase Chain Reaction To investigate the change of the mRNA expression of inflammatory factors interleukin-8 (IL-8), interleukin-6 (IL-6), IL-1β, TNF-α, transforming growth factor-β (TGF-β) in HCECs, quantitative real-time polymerase chain reaction (qRT-PCR) analyses were performed. Total RNA was extracted using an MN-total RNA isolation kit (Macherey-nagel, Germany) according to the manufacturer's instructions. First-strand cDNA were synthesized using the transcriptor first strand cDNA synthesis kit (Roche, Switzerland). qRT-PCR was carried out using the SYBR Premix Ex TaqTM kit (TaKaRa) with β-actin as the reference gene. The primers used to amplify IL-8, IL-6, IL-1β, TNF-α, TGF-β, and β-actin are described in Table 1.

Measurement of Intracellular Reactive Oxygen Species To determine whether AA effect ROS generation, the content of ROS Intracellular ROS was measured using the ROS assay kit (Beyotime Biotechnology, Haimen, China). The oxidation of 2′ and 7′-dichlorofluorescein diacetate (DCFH-DA) to 2′ and 7′-dichlorofluorescein (DCF) was used to estimate the content of ROS. The cells of the experimental groups in 6-well culture dishes were incubated with DCFH-DA (1:5000, v/v) for 20min and washed three times by serum-free DMEM-F12, with group used as the positive control. The fluorescence intensity was measured using a confocal laser scanning microscope (OLYMPUS, Japan) at an excitation and emission wavelength of 488 nm and 525 nm, respectively.

Measurement of Glutathione To determine whether AA effect GSH generation, GSH concentration was measured using the total GSH assay kit (Beyotime Biotechnology, Haimen, China) according to the manufacturer's instructions. This kit utilizes an enzymatic recycling method based on a reaction between GSH and 5, 5-Dithiobis (2-nitrobenzoic acid) (DTNB) that produces a yellow-colored compound (TNB). The absorbance at A412 was measured on a microplate reader (SUNRISE,

Table 1 The primers used for amplification of gene by PCR

Genes	Productions (bp)	Genbank	Sequences of primers
IL-8	150	NM_000584.3	F: 5'-TTGGCAGCCTTCCTGATTTC-3' R: 5'-TGGTCCACTCTCAATCACTCTCA-3'
IL-6	150	NM_000600.3	F: 5'-TGGCTGAAAAAGATGGATGCT-3' R: 5'-TCTGCACAGCTCTGGCTTGT-3'
IL-1β	150	NM_000576.2	F: 5'-CCTGTCCTGCGTGTTGAAAGA-3' R: 5'-GGGAACTGGGCAGACTCAAA-3'
TNF-α	154	NM_000594.3	F: 5'-TGTAGCCCATGTTGTAGCAAACC-3' R:5'-GAGGACCTGGGAGTAGATGAGGTA-3'
TGF-β	129	NM_000660.5	F: 5'-CGCCAGAGTGGTTATCTTTTGA-3' R: 5'-CGGTAGTGAACCCGTTGATGT-3'
β-actin	154	NM_001101.3	F: 5'-TGGAACGGTGAAGGTGACAG-3' R: 5'-GGCTTTTAGGATGGCAAGGG -3'

IL-8: Interleukin-8; IL-6: Interleukin-6; IL-1β: Interleukin-1β; TNF-α: Tumor necrosis factor-alpha; TGF-β: Transforming growth factor-β.

Switzerland). The cells of the experimental groups in the 6-well culture dishes were washed by PBS, centrifuged, and treated with assay solution. The cell lysates were frozen and thawed two times rapidly and centrifuged. The liquid supernatant was measured. The GSH concentrations were determined by comparison with standards. Absorbance measurements were obtained at 5min intervals with six measurements per sample.

Western Blot Analysis To determine the relevance of Akt phosphorylation (p-Akt) in HCECs, Western blot analysis were performed. Cells were harvested in 1× SDS sample buffer and sonicated briefly. Fifty micrograms of protein was resolved on 10% SDS-PAGE gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The blots were blocked in 5% non-fat dry milk dissolved in TBST buffer for 1h, and then incubated with primary antibodies (anti-Akt1/p-Akt1 antibody, ABclonal, MD, USA) in TBST for 1h, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1h. The blots were developed with SuperSignal West Femto Maximum Sensitivity substrate (Pierce Biotechnology, Rockford, IL, USA) and exposed to X-ray film (Kodak, Rochester, NY, USA).

Immunofluorescence Staining Analysis To determine the relevance of p-Akt in HCECs, immunofluorescence (IF) analysis were performed. The HCECs were grown on coverslips to 70%-80% confluence and treated with AA (20 μmol/L) and LPS (300 ng/mL) for 1h, then fixed with 4% paraformaldehyde for 15min, washed with PBS for 5min, permeabilized with 0.1% Triton X-100 for 10min, and washed three times for 15min. Blocking was performed with goat serum for 25min. Phospho-Akt1 polyclonal antibody (ABclonal) was used as the primary antibody in a 1:100 dilution overnight at 4℃, followed by several rinses with PBS. Finally, the cells were incubated with a secondary antibody (Bioss, Inc.). After washing with PBS, the cells were counterstained with DAPI (C1006, Beyotime, Shanghai, China) and evaluated using a laser scanning confocal microscope (Laser Scanning Confocal Microscope LEICA TCS SP2). Images were collected and processed using the imaging software provided by the Leica TCS system.

Statistical Analysis Statistical data were analyzed using SPSS 17.0. All the experiments were performed in triplicate with data shown as mean±SD based on three separate experiments. Variance analysis between groups was performed using a one-way ANOVA and the significance of difference between the control and treatment groups was analyzed using the Dunnett multiple comparison test. $P<0.05$ was considered statistically significant.

RESULTS

Cell Viability After Treatment of Asiatic Acid Figure 1 shows the CCK-8 assay of AA on the proliferation of HCECs at concentrations of 2.5, 5, 10, 20, 40, 60 and 80 μmol/L for 24, 48 and 72h, respectively. AA increased the proliferation of HCECs at low concen trations in 24, 48 and 72h and induced toxicity at high concentrations. Significant differences were shown at 10 μmol/L after 48 and 72h, and 20 μmol/L after 24h compared to the control.

Lipopolysaccharide Induced the Expression of Interleukin-8, Interleukin-6, Interleukin-1β, Tumor Necrosis Factor-alpha, and Transforming Growth Factor-β in Human Corneal Epithelial Cells As seen in Figure 2, HCECs were treated with E. Coli LPS at concentrations of 10, 50, 100, 200 and 300 ng/mL for 1h, and the expression of inflammatory factors IL-8, IL-6, IL-1β, TNF-α, TGF-β were increased, especially within the 300 ng/mL concentration. The incubation of HCECs with the LPS (300 ng/mL) elicited up to about 3-fold higher levels of IL-8 and IL-6 ($P<0.001$), 2.8-fold IL-1β ($P<0.001$), 2.5-fold TNF-α ($P<0.001$), and 1.5-fold TGF-β

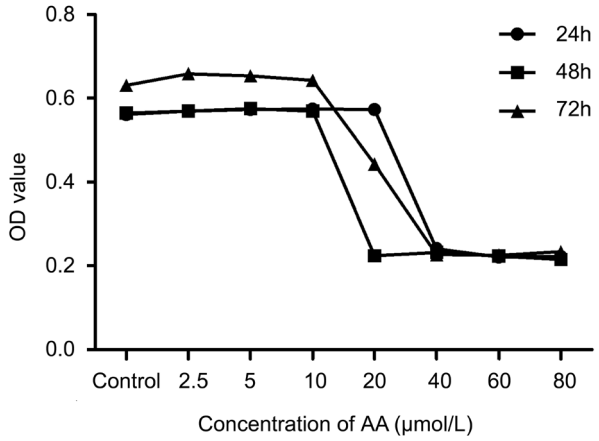

Figure 1 CCK-8 assay of AA on the proliferation of HCECs at concentrations of 2.5, 5, 10, 20, 40, 60 and 80 μmol/L for 24, 48 and 72h.

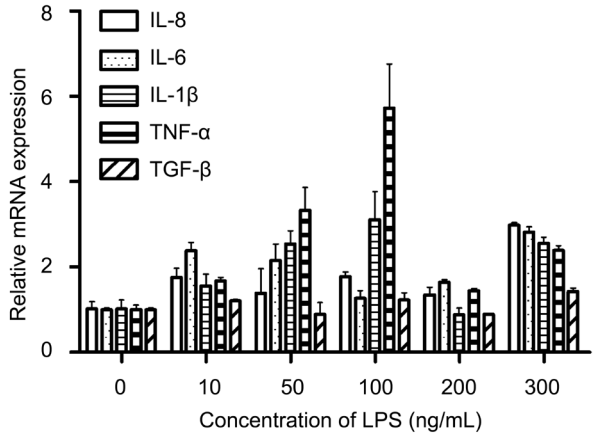

Figure 2 Relative mRNA expression of inflammatory factors IL-8, IL-6, IL-1β, TNF-α, and TGF-β in LPS-stimulated HCECs.

(P<0.01) compared with the corresponding levels from cells incubated in medium alone. Therefore, LPS at a concentration of 300 ng/mL for 1h was used in the subsequent studies. The expression of IL-8, IL-6, IL-1β, TNF-α, and TGF-β mRNA was determined by qRT-PCR.

Asiatic Acid Reduced Lipopolysaccharide-induced Inflammatory Factor Production We treated HCECs with AA (20 μmol/L) and LPS (300 ng/mL) for 1h. As shown in Figure 3, the qRT-PCR results revealed that treatment with LPS could significantly increase the mRNA expression of inflammatory factors IL-8, IL-6, IL-1β, TNF-α, and TGF-β. AA treatment in HCECs after stimulation with LPS elicited a significant reduction of IL-8 mRNA levels to 26.9% (P<0.001), IL-6 to 16.7% (P<0.001), IL-1β to 48.85% (P<0.01), TNF-α to 6.53% (P<0.001), and TGF-β to 57.1% (P<0.01), compared with incubation of the cells with LPS.

Asiatic Acid Reduced Lipopolysaccharide-induced Reactive Oxygen Species Production To determine whether AA exhibits its effects by reducing ROS generation, we detected the content of ROS. LPS (300 ng/mL) increased the intracellular level of ROS compared to the control (Figure 4A, 4B). Only treatment with AA and treatment with AA and LPS for 1h significantly

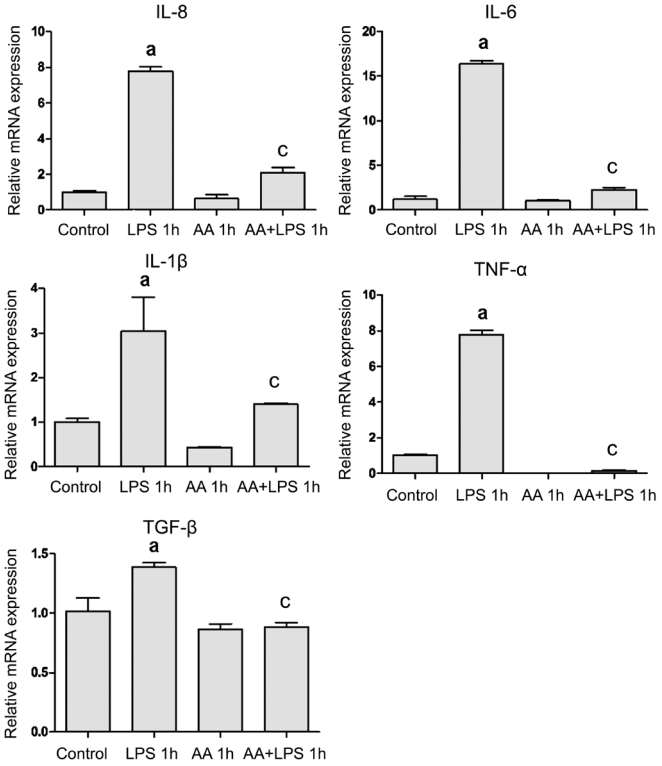

Figure 3 AA reduced LPS-induced production of inflammatory factors IL-8, IL-6, IL-1β, TNF-α and TGF-β in HCECs [a]P<0.05 compared to the control; [c]P<0.05 compared to the LPS 1h group.

reduced the intracellular concentration of ROS (Figure 4C, 4D).

Asiatic Acid Increased Glutathione Generation in Lipopolysaccharide-stimulated Human Corneal Epithelial Cells The results indicated that total GSH production decreased to 82.7% (P<0.05) after LPS treated for 1h compared to the control, and that there was an increase of 36% (P<0.05) in total GSH concentration after incubation with AA (20 μmol/L) (Figure 5). However, no significant difference was found in the AA (20 μmol/L) 1h group compared to the control.

Asiatic Acid Reduced Lipopolysaccharide-induced Akt Phosphorylation Akt plays an important role in the processes of cell survival and apoptosis. To determine the relevance of p-Akt in the anti-inflammatory property of AA in response to LPS challenge, we investigated whether AA could affect LPS-induced p-Akt *via* IF staining analysis and Western blotting. Our results showed that AA reduced LPS-induced p-Akt (Figure 6).

DISCUSSION

This study demonstrated a highly potent anti-inflammatory effect of AA. AA dramatically reduced the LPS stimulated production of the pro-inflammatory cytokines IL-8, IL-6, IL-1β, TNF-α, and TGF-β in cultured HCECs. AA significantly inhibited the intracellular concentrations of ROS compared to the control. Our findings indicated that AA was beneficial to GSH generation. Furthermore, our results showed that AA significantly inhibited p-Akt induced by LPS, suggesting that the anti-inflammatory effects of AA involve regulatory

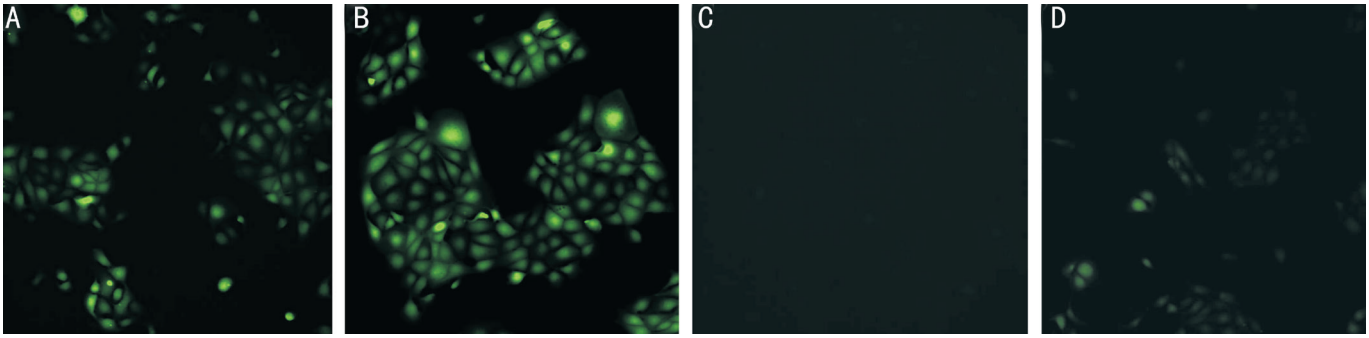

Figure 4 AA reduced the intracellular ROS production of HCECs A: Control; B: LPS (300 ng/mL) 1h; C: AA (20 μmol/L) 1h; D: AA (20 μmol/L)+LPS (300 ng/mL) 1h (400×).

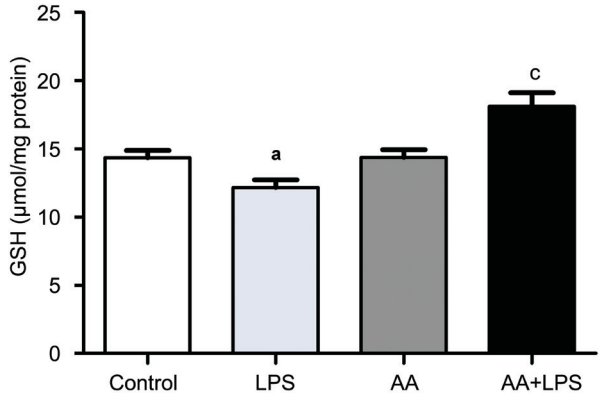

Figure 5 AA increased the intracellular GSH levels $^{a}P<0.05$ compared to the control; $^{c}P<0.05$ compared to the LPS group.

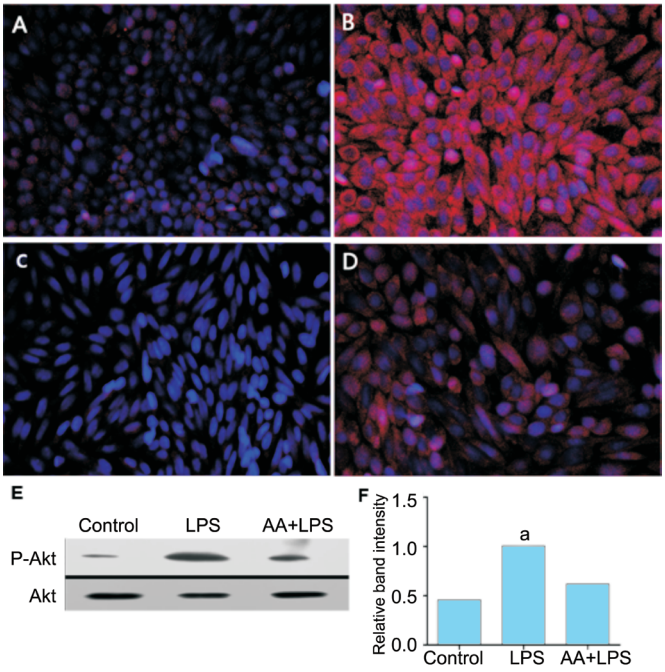

Figure 6 AA reduced the LPS-stimulated p-Akt in HCECs A: Control; B: LPS (300 ng/mL) 1h; C: AA (20 μmol/L) 1h; D: AA (20 μmol/L)+ LPS (300 ng/mL) 1h; E and F: AA inhibits LPS-induced p-Akt. $^{a}P<0.05$.

effects of the Akt pathway. This is the first in vitro study that demonstrates the anti-inflammatory effects of AA at the molecular level on a variety of cytokines that are involved in ocular surface inflammation. These findings have significant therapeutic implications.

The inflammation inducers frequently used were LPS and polyinosinic: polycytidylic acid (poly I:C). LPS is a component of the outer membranes of gram negative bacteria, while poly I:C is a molecular stimulant which mimics viral infection. Both molecules induce inflammation by stimulating host innate immune system response[18-19]. LPS binds to intramembranal complex of CD-14 and toll-like receptors (TLR), and induces the release of multiple pro-inflammatory cytokines, including IL-6, IL-8, and TNF-α and an activation of NF-κB in various cells throughout the body, including HCECs and fibroblasts[20-21]. Poly I:C works by triggering of TLR-3, which results in increased production of pro-inflammatory cytokines, including IL-6 and IL-8, and activation of NF-κB in HCECs[18]. Because of their potent effects on the expression and secretion of a large number of inflammatory mediators, LPS and poly I:C were frequently chosen as inflammation inducers[18-21]. Pseudomonas aeru-ginosa (gram-negative bacteria) is the common cause of bacterial keratitis. LPS are the best characterized as an immune stimulatory molecule in gram-negative bacteria that play a significant role in provoking a variety of immunostimulatory responses, such as the production of inflammatory factors in many cell types[22-23]. LPS was chosen as inflammation inducers in this study.

Several trials have demonstrated significant associations between AA and a systemic anti-inflammatory effect. Centella asiatica has been used in traditional Chinese medicine to treat various ailments. AA is a pentacyclic triterpene found in Centella asiatica. AA was found to exhibit anti-inflammatory properties by inhibiting the production of iNOS, COX-2, IL-6, and IL-1β. AA could ameliorate dextran sulfate sodium-induced murine experimental colitis *via* suppressing mitochondria-mediated NLRP3 inflammasome activation[24]. And re-infection administration of AA retards parasitaemia induction in plasmodium berghei murine malaria infected Sprague-Dawley rats[25]. Whether AA has a systemic anti-inflammatory effect in keratitis is still an open question.

Acute inflammatory response is associated with the production of ROS such as superoxide anions, hydrogen peroxide, and peroxynitrite[4]. Previous findings suggest that Centella asiatica

plays an important role in reducing the activity of ROS in the body, protecting against oxidative stress, and reducing the extent of mitochondrial damage[26]. AA protected against spinal cord injury *via* activation of Nrf2 and HO-1 and inhibition of ROS and the NLRP3 inflammasome pathway[27]. Two reports document the involvement of tissue damage through oxidative processes in cornea. In one report, cationic glucose oxidase injected into rabbit corneas, with the resulting sustained release of hydrogen peroxide, yielded corneal opacification through attack on corneal glycol conjugates by ROS and infiltration of phagocytes, further compromising corneal integrity through the oxidative sequelae of their respiratory burst[28]. This result suggests that oxidative stress can cause inflammation and result in corneal tissue damage. But the exact mechanism of procedure is still not clear. Ulcerative keratitis can be treated successfully with topical antioxidants, demonstrating the importance of oxidative stress in ulcer pathogenesis. This is the case in ulcers with alkali burn as a cause[10] and in those with an infectious cause[11]. Oxidative stress has recently been linked to the corneal, conjunctival and lacrimal gland injury that is associated with certain ocular surface conditions. Whether it is a primary cause or merely a downstream consequence of the inflammatory process is still an open question.

Akt, also known as protein kinase B (PKB), is a serine/threonine kinase that is recruited to the plasma membrane in cells when stimulated by various stimulants. Akt plays a critical role in multiple cellular processes, such as cell-cycle regulation, cell survival, NF-κB activation, and cell apoptosis, depending on the nature of the stimuli. Previous findings have shown that p-Akt influences and promotes NF-κB activation, which is crucial for inflammatory processes[29-30]. AA exerted anticancer potential in human ovarian cancer cells *via* suppression of PI3K/Akt/mTOR signalling[31]. AA also improved glucose response by increasing GLUT4 in skeletal muscle through Akt and antioxidant defense in plasma and it also improved glucose homeostasis[32].

Taken together, the studies demonstrate significant local anti-inflammatory effects of AA. However, none of these studies have investigated the molecular mechanisms in HCEC. In summary, our study demonstrates several significant direct effects of AA in reducing inflammation in HCECs, partially *via* inactivation of the PI3K/Akt pathway, inhibition of the intracellular concentrations of ROS and increased GSH generation. AA may represent a potential therapeutic agent for bacterial keratitis.

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REFERENCES

1 Herretes S, Wang X, Reyes JM. Topical corticosteroids as adjunctive therapy for bacterial keratitis. *Cochrane Database Syst Rev* 2014;10: CD005430.

2 Song HY, Qiu BF, Liu C, Zhu SX, Wang SC, Miao J, Jing J, Shao YX. Identification of causative pathogens in mouse eyes with bacterial keratitis by sequence analysis of 16S rDNA libraries. *Exp Anim* 2015;64(1):49-56.

3 Rachwalik D, Pleyer U. Bacterial keratitis. *Klin Monbl Augenheilkd* 2015;232(6):738-744.

4 Huang SS, Chiu CS, Chen HJ, Hou WC, Sheu MJ, Lin YC, Shie PH, Huang GJ. Antinociceptive activities and the mechanisms of anti-inflammation of asiatic Acid in mice. *Evid Based Complement Alternat Med* 2011;2011:895857.

5 Pakdeechote P, Bunbupha S, Kukongviriyapan U, Prachaney P, Khrisanapant W, Kukongviriyapan V. Asiatic acid alleviates hemodynamic and metabolic alterations via restoring eNOS/iNOS expression, oxidative stress, and inflammation in diet-induced metabolic syndrome rats. *Nutrients* 2014;6(1):355-370.

6 Kavitha CV, Agarwal C, Agarwal R, Deep G. Asiatic acid inhibits pro-angiogenic effects of VEGF and human gliomas in endothelial cell culture models. *PLoS One* 2011;6(8):e22745.

7 Cho CW, Choi DS, Cardone MH, Kim CW, Sinskey AJ, Rha C. Glioblastoma cell death induced by asiatic acid. *Cell Biol Toxicol* 2006;22 (6):393-408.

8 Tang XL, Yang XY, Jung HJ, Kim SY, Jung SY, Choi DY, Park WC, Park H. Asiatic acid induces colon cancer cell growth inhibition and apoptosis through mitochondrial death cascade. *Biol Pharm Bull* 2009;32(8):1399-1405.

9 Valentine JS, Doucette PA, Zittin Potter S. Copper-zinc superoxide dismutase and amyotrophic lateral sclerosis. *Annu Rev Biochem* 2005;74:563-593.

10 Alio JL, Ayala MJ, Mulet ME, Artola A, Ruiz JM, Bellot J. Antioxidant therapy in the treatment of experimental acute corneal inflammation. *Ophthalmic Res* 1995;27(3):136-143.

11 Alio JL, Artola A, Serra A, Ayala MJ, Mulet ME. Effect of topical antioxidant therapy on experimental infectious keratitis. *Cornea* 1995;14 (2):175-179.

12 Buelna-Chontal M, Zazueta C. Redox activation of Nrf2 & NF-κB: a double end sword? *Cell Signal* 2013;25(12):2548-2557.

13 Naik E, Dixit VM. Mitochondrial reactive oxygen species drive proinflammatory cytokine production. *J Exp Med* 2011;208(3):417-420.

14 Lee KM, Kang BS, Lee HL, Son SJ, Hwang SH, Kim DS, Park JS, Cho HJ. Spinal NF-κB activation induces COX-2 upregulation and contributes to inflammatory pain hypersensitivity. *Eur J Neurosci* 2004;19(12):3375-3381.

15 Yadav UC, Ramana KV. Regulation of NF-kappaB-induced inflammatory signaling by lipid peroxidation-derived aldehydes. *Oxid Med Cell Longev* 2013;2013:690545.

16 Oyinloye BE, Adenowo AF, Kappo AP. Reactive oxygen species, apoptosis, antimicrobial peptides and human inflammatory diseases. *Pharmaceuticals (Basel)* 2015;8(2):151-175.

17 Solanki S, Rathi M, Khanduja S, Dhull CS, Sachdeva S, Phogat J. Recent trends: Medical management of infectious keratitis. *Oman J Ophthalmol* 2015;8(2):83-85.

18 Ueta M, Hamuro J, Kiyono H, Kinoshita S. Triggering of TLR3 by polyI:C in human corneal epithelial cells to induce inflammatory cytokines. *Biochem Biophys Res Commun* 2005;331(1):285-294.

19 Chow JC, Young DW, Golenbock DT, Christ WJ, Gusovsky F. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem* 1999;274(16):10689-10692.

20 Kumagai N, Fukuda K, Fujitsu Y, Lu Y, Chikamoto N, Nishida T. Lipopolysaccharide-induced expression of intercellular adhesion molecule-1 and chemokines in cultured human corneal fibroblasts. *Invest Ophthalmol Vis Sci* 2005;46(1):114-120.

21 Song PI, Abraham TA, Park Y, Zivony AS, Harten B, Edelhauser HF, Ward SL, Armstrong CA, Ansel JC. The expression of functional LPS receptor proteins CD14 and toll-like receptor 4 in human corneal cells. *Invest Ophthalmol Vis Sci* 2001;42(12):2867-2877.

22 Sirota M, Willemsen G, Sundar P, Pitts SJ, Potluri S, Prifti E, Kennedy S, Ehrlich SD, Neuteboom J, Kluft C, Malone KE, Cox DR, de Geus EJ, Boomsma DI. Effect of genome and environment on metabolic and inflammatory profiles. *PLoS One* 2015;10(4):e0120898.

23 Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. *Annu Rev Biochem* 2002;71:635-700.

24 Guo W, Liu W, Jin B, Geng J, Li J, Ding H, Wu X, Xu Q, Sun Y, Gao J. Asiatic acid ameliorates dextran sulfate sodium-induced murine experimental colitis via suppressing mitochondria-mediated NLRP3 inflammasome activation. *Int Immunopharmacol* 2015;24(2):232-238.

25 Mavondo GA, Mkhwananzi BN, Mabandla MV. Pre-infection administration of asiatic acid retards parasitaemia induction in Plasmodium berghei murine malaria infected Sprague-Dawley rats. *Malar J* 2016;15:226.

26 Hashim P, Sidek H, Helan MH, Sabery A, Palanisamy UD, Ilham M. Triterpene composition and bioactivities of Centella asiatica. *Molecules* 2011;16(2):1310-1322.

27 Jiang W, Li M, He F, Bian Z, He Q, Wang X, Yao W, Zhu L. Neuroprotective effect of asiatic acid against spinal cord injury in rats. *Life Sci* 2016;157:45-51.

28 Carubelli R, Nordquist RE, Rowsey JJ. Role of active oxygen species in corneal ulceration. Effect of hydrogen peroxide generated in situ. *Cornea* 1990;9(2):161-169.

29 Wang QB, Sun LY, Gong ZD, Du Y. Veratric acid inhibits LPS-induced IL-6 and IL-8 production in human gingival fibroblasts. *Inflammation* 2016;39(1):237-242.

30 Lo JY, Kamarudin MN, Hamdi OA, Awang K, Kadir HA. Curcumenol isolated from Curcuma zedoaria suppresses Akt-mediated NF-kappaB activation and p38 MAPK signaling pathway in LPS-stimulated BV-2 microglial cells. *Food Funct* 2015;6(11):3550-3559.

31 Ren L, Cao QX, Zhai FR, Yang SQ, Zhang HX. Asiatic acid exerts anticancer potential in human ovarian cancer cells via suppression of PI3K/Akt/mTOR signalling. *Pharm Biol* 2016;54(11):2377-2382.

32 Ramachandran V, Saravanan R. Glucose uptake through translocation and activation of GLUT4 in PI3K/Akt signaling pathway by asiatic acid in diabetic rats. *Hum Exp Toxicol* 2015;34(9):884-893.