·Basic Research·

Emodin ameliorates lipopolysaccharides-induced corneal inflammation in rats

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Abstract

• **AIM: To investigate the effect of emodin on pseudomonas aeruginosa lipopolysaccharides (LPS) -induced corneal inflammation in rats.**

• **METHODS: Corneal infection was induced by pseudomonas aeruginosa LPS in Wistar rats. The inflammation induced by LPS were examined by slit lamp microscope and cytological checkup of aqueous humor. Corneal tissue structure was observed by hematoxylin and eosin (HE) staining. The activation of nuclear factor kappaB (NF-κB) was determined by Western blot. Messenger ribonucleic acid (mRNA) of tumor necrosis factor-α (TNF-α) and intercellular adhesion molecule-1 (ICAM-1) in LPS-challenged rat corneas were measured with reverse transcription-polymerase chain reaction (RT-PCR).**

• **RESULTS: Typical manifestations of acute corneal inflammation were observed in LPS-induce rat model, and the corneal inflammatory response and structure were improved in rats pretreated with emodin. Treatment with emodin could improve corneal structure, reduce corneal injure by reducing corneal inflammatory response. Emodin could inhibit the decreasing lever of inhibitor of kappaB alpha (IκBα) express, and the mRNA expression of TNF-α and ICAM-1 in corneal tissues was also inhibited by emodin. The differences were statistically significant between groups treated with emodin and those without treatment (P<0.01).**

• **CONCLUSION: Emodin could ameliorate LPS-induced corneal inflammation, which might *via* inhibiting the activation of NF-κB.**

• **KEYWORDS:** nuclear factor kappaB inhibitor alpha; therapy; inflammation
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INTRODUCTION

The cornea protects the eye from insults caused by various external factors. An avascular and transparent cornea is required for proper vision. Bacterial keratitis is still one of the important main causes of blindness and visual disability throughout the world [1,2]. Investigations show that bacterial infections can induce infiltration of inflammatory cells in pathological progress of bacterial keratitis and cause migration of inflammatory cells in anterior chamber resulting to severe reaction and development of hypopyon[3]. Bacterial keratitis hampers the transparency of the cornea and may cause permanent vision loss due to scarring and perforation. Therefore, inhibition of cytokines expression may be a therapeutic strategy for keratitis.

The nuclear factor kappaB (NF-κB) is a key transcription factor that is responsible for many key biological processes [4,5]. Numerous recent studies have investigated NF-κB in the context of ocular surface disorders, including microbial infections, chemical injury, ultraviolet radiation-induced injury, allergic eye diseases, dry eye, pterygium, and corneal graft rejection [5]. A number of cytokines and growth factors, *via* NF-κB pathway for signaling on ligand binding to cell surface receptors that are up-regulated in corneal cells further contribute to tissue inflammation.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone), an anthraquinone and is the major bioactive compound found in several Chinese herbs, has been shown to display a wide range of biological activities including antiviral, antimicrobial, anti-inflammatory, anti-tumor, anti-fibrosis and elimination of reactive oxygen species [6]. Recent studies suggest that emodin could weaken inflammation, and one of the mechanisms of anti-inflammatory effects has been shown to be the inhibition of pro-inflammatory transcription factor NF-κB [7,8]. In the present study, an animal model based on pseudomonas aeruginosa lipopolysaccharides (LPS)-induced

corneal inflammation was established to evaluate the influences of emodin as an anti-inflammatory agent.

MATERIALS AND METHODS

Materials

Animal and grouping All experiments were carried out in male Wistar rats, weighing between 230 g and 250 g (Animal Center of Shandong University). Rats were kept under the same laboratory conditions, and allowed to acclimatize for one week before experiment. All animals were maintained according to institutional guidelines and the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. All rats were divided into keratitis group and emodin group, and which received emodin subconjunctival injection in emodin group and its vehicle in the keratitis group 30min before LPS exposure.

Methods

Model of corneal infection For infection, rats were challenged with pseudomonas aeruginosa LPS. The right central corneas were scarified with three parallel abrasions using a 26-gauge needle that did not penetrate beyond the superficial stroma, as described previously [9], and a 10 μL aliquot containing 50 μg LPS was applied to each scarified cornea. At various times post infection, rats were examined with a slit lamp microscope (six-six visual Inc., Suzhou, Jiangsu Province, China), then were killed and the eyes were used for cytological checkup of aqueous humor, the corneas were processed for pathological examination, Western blot and reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Slit lamp examination The examination of anterior segment of the eye by a slit lamp microscope was performed. Rats' eyes were graded by using a scale previously described by Girgis *et al*[10]: 0, cornea was clear and normal; 1, readily detectable opacity; 2, dense opacity or opacity partially covering the entire corneal surface over pupil; 3, dense opacity covering entire corneal surface over pupil; 4, moderate to dense opacity covering entire corneal surface with corneal erosion. Corneal erosions were detected with fluorescein (Bei ruida Inc., Beijing, China).

Cytological checkup of aqueous humor After being examined with slit lamp microscope, rats were sacrificed and the eyes were used for cytological checkup of aqueous humor. The conjunctival sac liquid were dried with a piece of filter paper, then the aqueous humor was drew out by rapid puncture into the anterior chamber through transparent cornea near limbus, and were blended, smeared on the slides and routinely stained by hematoxylin and eosin (HE). Cells number and classification of aqueous humor were observed with optical microscope.

Pathological evaluation At various times after challenged with LPS, the corneas were excised from the eyeball at the limbus. After fixation, the tissues were washed three times with PBS and embedded in paraffin by a routine procedure. Consecutive 4 μm corneal sections were prepared, and HE staining was performed, then the changes of corneal pathological morphology were examined with light microscope.

Western blot analysis Proteins were extracted from frozen corneal tissue according to the manufacturer's instructions. Protein concentrations were determined and equivalent amounts of the samples (30 μg) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and followed by electrotransferring onto a nitrocellulose membrane according to the manufacturer's instructions (Millipore, Bedford, USA). The membranes were blocked with 5% fat free milk for 2h at room temperature, washed three times with PBS, and followed by an overnight incubation at 4℃ with an antibody to NF-κB inhibitor of kappaB alpha (IκBα, Neomarkers, USA). The membrane was then washed and probed with a secondary antibody conjugated with Horseradish peroxidase for 2h, and were washed twice with PBS at room temperature. The immune complexes were visualized by using of a Western blotting detection reagent (Kodak System, Japan).

Reverse transcription –polymerase chain reaction analysis Total RNA was prepared from corneal tissue and the samples were treated with DNase before the experiments in order to discharge any contaminant DNA. The messenger ribonucleic acid (mRNA) of tumor necrosis factor-α (TNF-α) intercellular adhesion molecule-1 (ICAM-1), and β-actin were measured according to the manufacturers' instructions. The primers used for PCR reactions were 5'-AAGATCCTGACCGAGCGTGG -3' and 5'- CAGCACTGT GTTGGCATAGAGG -3' for β-actin; 5'- GCCAATGGCAT GGATCTCAAAG -3' and 5'- CAGAGCAATGACTCCAAA GT -3' for TNF-α; 5'- GATGCTGACCCTGGAGAGCA -3' and 5'- CAGGGACTTCCCATCCACCT -3' for ICAM-1. The PCR products were separated by gelose-gelatine electrophoresis and photographed under UV light following ethidium bromide staining. Results were analyzed with gelatine, image analysis system (Kodak System, Japan) using β-actin as an internal control. The relative mRNA expression level of TNF-α and ICAM-1 in cornea was represented by the ratio of expression intensity of β-actin.

Statistical Analysis All data were expressed as mean± SD. All statistical analyses were performed using SPSS 16.0 for Windows software. The data were statistically evaluated by paired Student's t-test. A P value less than 0.05 was considered statistically significant, and extremely significant when $P < 0.01$.

RESULTS

Clinical Evaluation Rats were examined by slit lamp microscope at different period, and typical manifestations of acute corneal inflammation were observed. Mixed hyperemia in conjunctiva, edema and opaque in cornea of different degree could be observed at 1-24h and was most obvious at 6-12h after LPS challenged. Inflammatory response of anterior chamber could be observed at 3h and was most obvious at 6-12h when a little fibrinous exudation was shown in anterior chamber. All above signs and symptoms were improved in rats injected with emodin subconjunctivally before LPS challenged. Slit lamp examination scores of rats' corneas were shown in Figure 1.

Checkup of Aqueous Humor Smear of aqueous humor after HE staining showed as follows: at 3h postinfection, cells increased obviously in anterior chamber, and were most obvious at 6h as same as examined with slit lamp microscope in rats when a little fibrinous exudation was shown in anterior chamber. Most of the cells were neutrophils, and a few were lymphocytes and macrophages. Obviously decreased cell numbers in aqueous humor of emodin group than in keratitis group, and the difference was statistically significant (P<0.01; Figure 2).

Histological Observation of Corneas Eyes were enucleated at various time intervals and processed for pathological examination. Different degree of corneal edema, opacity and infiltration of inflammatory cells were observed in corneal tissues between 3 and 24h after LPS treatment, and it was most obvious at 6-12h. Most of the inflammatory cells were polymorpho-nuclear leukocytes (PMN), and a few were monocytes or lymphocytes. Compared with corneas in keratitis group, pretreated with emodin could decrease the infiltration of PMNs and lighten corneal edema and damages before LPS challenged (Figure 3).

Activation of Nuclear Factor KappaB NF-κB would be activated only after degradation of the inhibitory protein IκB, thus the expression of IκB protein in cytoplasmic could be used for evaluating the activation of NF-κB. Western blot showed that the expression of IκBα protein was decreased, especially at 3-6h after challenged with LPS. Compared with the keratitis group, the expression of IκBα was up-regulated in emodin group, and the difference between the groups was statistically significant (P<0.01; Figure 4).

Messenger Ribonucleic Acid of Tumor Necrosis Factor-α and Intercellular Adhesion Molecule -1 RT-PCR assays tested the effects of emodin on cytokine production in rats' corneas. LPS challenged could up-regulate the mRNA expression of TNF-α and ICAM-1. Significantly decreased mRNA levels of TNF-α and ICAM-1 in corneas of emodin group than in keratitis group, and the difference was statistically significant (P<0.01; Figures 5, 6).

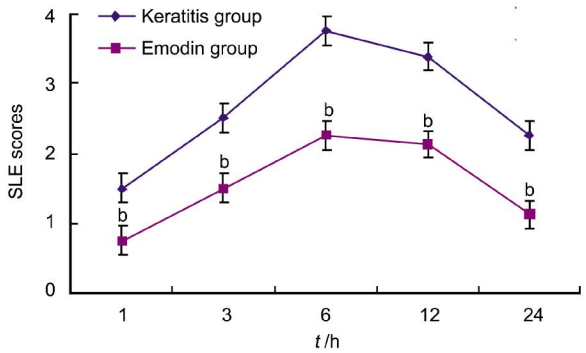

Figure 1 Slit lamp examination scores of corneas P< 0.01, [b]the comparison of emodin group versus keratitis group.

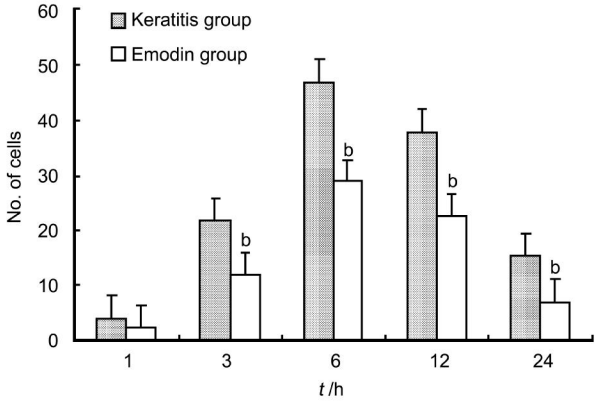

Figure 2 Checkup of the aqueous humor P< 0.01, [b]the comparison of emodin group versus keratitis group.

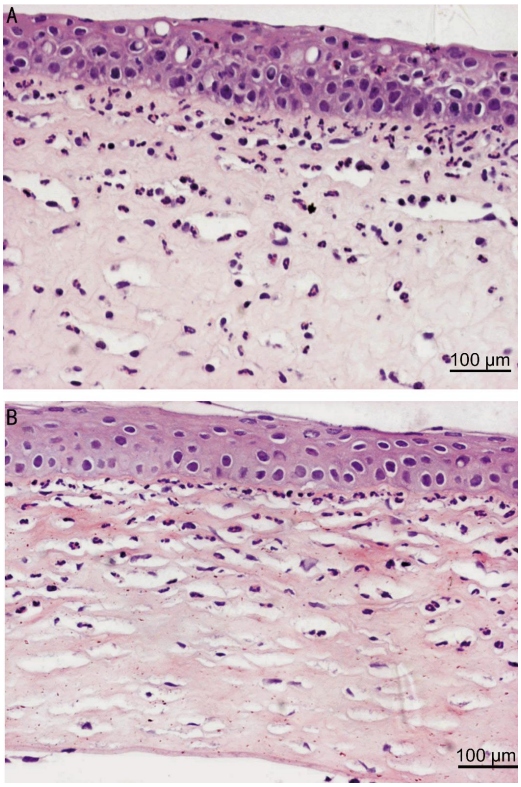

Figure 3 Histological observations of corneas A: Obvious tissue edematous, disarrangement collagen fibers, and a large number of inflammatory cells were present in corneal sections of keratitis rat; B: Most of the inflammatory cells were PMN, and a few were lymphocytes or monocytes. Pretreated with emodin could reduce the inflammatory cells and improve corneal tissue damage.

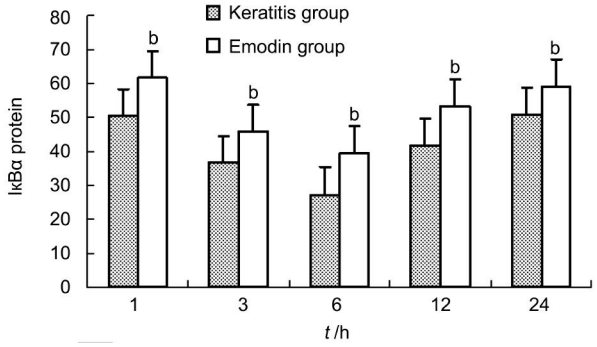

Figure 4 The expression of IκBα protein in corneas $P<0.01$, [b]the comparison of emodin group versus keratitis group.

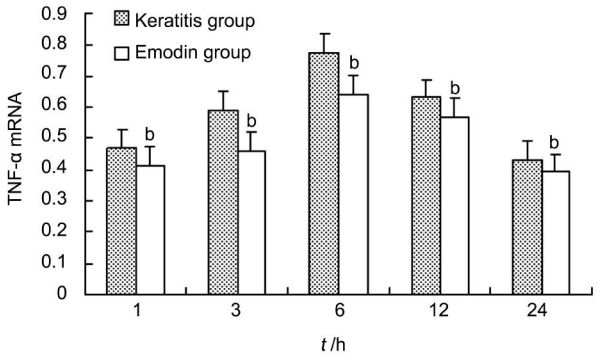

Figure 5 The mRNA expression of TNF–α in corneas $P<0.01$, [b]the comparison of emodin group versus keratitis group.

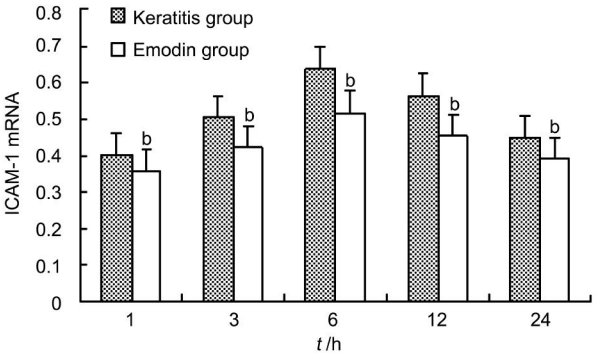

Figure 6 The mRNA expression of ICAM–1 in corneas $P<0.01$, [b]the comparison of emodin group versus keratitis group.

DISCUSSION

Bacterial keratitis, a common and severe ocular infection, may lead to permanent scarring and loss of vision. Pseudomonas aeruginosa keratitis is a rapidly progressive ocular disease, characterized by severe corneal infections usually present as a rapidly progressing, suppurative stromal infiltrate with marked mucopurulent exudates, distinctive inflammatory epithelial edema and stromal destruction, and is also a global cause of blindness and visual impairment[1,2]. Animal models of bacterial keratitis continue to be of value in study of this disease and these models have led to increased understanding of the mechanisms of corneal inflammation during bacterial keratitis [11-13]. Therefore, to probe into the pathogenesis and mechanism of corneal damage caused by inflammation has great clinical significance for preventing and treating keratitis and reducing scar formation.

LPS, a major component of the cell wall of gram negative bacteria and is a key factor in causing inflammatory response in various tissues, has also been suggested to play a direct role in severe ocular surface pathological processes such as delayed corneal wound healing, complications after corneal surgery, and aggravation of certain infectious situations. Moreover, the successful treatment of Gram-negative bacteria is being challenged by increasing antibiotic resistance. LPS activates complement and initiates the production of numerous cytokines [4,13,14]. Activation of these various responses, either individually or concurrently, may cause systemic and/or localized pathology. It is often used to produce various inflammatory models and has been set animal model of inflammation [15,16]. In this study, corneas were scratched and treated with LPS to induce acute keratitis in Wistar rats. A severe corneal inflammatory response, such as hyperemia，corneal edema and opacity were observed, and many inflammatory cells, mainly leukocyte, appeared in corneal tissue. The changes of the infiltration degree were in accordance with the ocular manifestations such as the edema and opacity of cornea.

Emodin could decrease the activation of NF-κB and the levels of proinflammatory cytokines TNF-α and interleukin (IL)-1β in myocarditis [17,18]. TNF-α is a potent chemoattractant for neutrophils, which is produced chiefly by activated neutrophils and macrophages, although it can be produced by other cell types as well. ICAM-1, an important adhesion molecule, takes part in cells signal transduction, activation, growth, differentiation, inflammation, immune response, *etc.* plays an important role in the inflammatory process. Administration of LPS could initiate the production and release of various cytokines, such as TNF-α, IL-6, ICAM-1, *etc.*[3,19]. In this study, we observed an increase in expression of TNF-α and ICAM-1 in LPS-treated corneas, which were correlated with the infiltration of inflammatory cells and damaged degree of keratitis in rats, and were considerably reduced in emodin pretreated corneas.

NF-κB is a multi-subunit transcription factor which rapidly activates the transcription of various cytokines and chemokines, playing key roles in the inflammatory response [18,20]. Its inhibitors intended to block NF-κB activity may be useful as anti-inflammatory agents [21]. The NF-κB dimers (p65 and p50) bound to its inhibitor, IκB, was found mainly in the cytoplasm in unactivated cells. The activation of NF-κB is dependent on the phosphorylation and degradation of IκB, an endogenous inhibitor that binds to NF-κB in the cytoplasm [19]. Upon activation, IκB is degraded; allowing NF-κB to translocate into the nucleus where it can regulate transcription of NF-κB target genes[5],

thus the expression of IκB protein in cytoplasmic could reflect the activation of NF-κB. Recent studies suggest that emodin could suppress the nuclear translocation of NF-κB and the degradation of cytoplasmic IκBα and regulate the productions of cytokines[22]. The present study indicated that emodin played a role in inhibiting NF-κB activation induced by LPS. Therefore, anti-inflammatory effects of emodin are exerted, at least in part, through the inhibition of NF-κB.

In summary, our studies provide some evidence that emodin, the major bioactive compound found in several Chinese herbs, has a protective effect related to the inhibition of local corneal inflammation by suppressing neutrophil infiltration and inflammatory cytokine production in LPS-induced keratitis rats, and the role may be due to inhibiting NF-κB pathway.

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