Apigenin ameliorates ocular surface lesions in a rat model of dry eye disease

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Abstract
To study the effects of apigenin on dry eye disease (DED) in rats. Rats were divided into six groups: (I) normal control group, (II) DED control group, (III) vehicle control group, (IV) DED + apigenin 10 mg/kg, (V) DED + apigenin 20 mg/kg, and (VI) DED + apigenin 50 mg/kg. Schirmer test, tear film break-up time (BUT), and corneal fluorescein staining were used to evaluate the effects of apigenin on the ocular surface. The related inflammatory cytokines were detected by enzyme-linked immunosorbent assay (ELISA). Histopathological examination and inflammatory index were also performed. The results showed that administration of apigenin was shown a significant effect on the recovery of ocular surface function. Compared to the control group, apigenin treatment in DED rats significantly decreased the level of the tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6); however, the interleukin-10 (IL-10) level was increased. Histopathological examination further verified the anti-inflammatory effects of apigenin on DED rats. The results demonstrated that apigenin could protect DED rats via inhibition of inflammation, suggesting that it may have potential as a therapy for DED.

Keywords
apigenin, dry eye disease, inflammatory, rats

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Introduction
Dry eye disease (DED), also known as keratoconjunctivitis sicca (KCS), is one of the most common ocular diseases affecting population worldwide.1 With the multifactorial disorder characterized by the status of the tear film, DED can lead to ocular discomfort and visual impairment.2 DED has become a major public health issue in industrial societies.3

There is emerging evidence showing that DED is a multifactorial and complex disease, involving chronic inflammatory infiltration, interruption of neuronal stimulation for tear secretion, and meibomian gland dysfunction.4 Numerous animal models have been developed to reflect the multiplicity of pathophysiological mechanisms involved in DED.5 Among them, main lacrimal gland extirpation has been recognized as a potential way to establish DED model. So, in this study, we established DED model by main lacrimal gland extirpation.

Apigenin (4,5,7-trihydroxyflavone) can be found in many plants.6 It has shown multiple functions including anti-cancer, antioxidant, and anti-inflammatory effects.7 Therefore, in this study, we studied the effects of apigenin on experimental DED model in rats.
Materials and methods

Animals and ethics statements

Male Sprague–Dawley (SD) rats (6 weeks old, 180–200 g) were obtained from the Experimental Animal Center of Suzhou Aiermaite Technology Co. Ltd. (SPF grade, Certificate No. SCXK20140007). Rats with the following features were used: anterior segment and fundus were normal after being checked by slit-lamp microscope and retinoscope; the Schirmer test value was not less than 10 mm in 1 min; the tear film break-up time (BUT) value was not less than 10 s. All rats were housed in 23°C ± 2°C, with relative humidity 50% ± 10% in 12 h light/dark cycle.

All animals were used according to the Association of Research and Vision in Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research. All animal experiments were performed in accordance with the Institutional Animal Care Committee of Dezhou People’s Hospital.

Experimental procedure and grouping

Rats were anesthetized with 10% chloral hydrate (300 mg/kg), made longitudinal incision of 1 cm under the ears and transverse incision of 1 cm under eyes laterally, exposing the lacrimal gland and removed it completely. After establishment of DED, rats were randomly assigned to six groups (n = 10): (I) normal control group, (II) DED control group, (III) vehicle control group, (IV) DED + apigenin 10 mg/kg, (V) DED + apigenin 20 mg/kg, and (VI) DED + apigenin 50 mg/kg.

Apigenin (Chengdu Must Biotechnology Co., Ltd., Chengdu, China) was dissolved in 0.1 M sodium hydroxide; 7 days after surgery, the rats in IV, V, and VI groups received apigenin by gavage, once daily for 8 weeks. I and II groups received physiologic saline and III group received 0.1 M sodium hydroxide instead. The related indexes were detected 8 weeks after treatment.

Schirmer test

A modified Schirmer test was performed to measure tear fluid secretion. A Schirmer tear test strip (1 × 15 mm; cobalt chloride paper; Toyo Roshi Kaisha, Tokyo, Japan) was placed on the temporal side of the lower eyelid margin for 1 min after scraping of the corneal epithelium. After 8 weeks, tear fluid secretion of the right eye of each rat was measured three times after the eye had been open for 15 s. The length of the moistened area from the edge was measured.

BUT scoring

After 1 μL of 0.1% liquid sodium fluorescein was dropped into the conjunctival sac, BUT was measured as the time lapse between the last blink and the appearance of the first random dry spot appearing in the cornea.

Corneal fluorescein staining

A volume of 2% sodium fluorescein solutions was coated in the conjunctival sac of the rat, and corneal epithelial damage was graded with a cobalt blue filter under a slit-lamp microscope (Kanghua Science & Technology Co., Ltd., Chongqing, China). The corneal fluorescein staining was recorded using a score from 0 to 4, where 0 is equal to no staining, 1 is less than 25% surface staining, 2 is 25%–50% surface staining, 3 is 50%–75% surface staining, and 4 is greater than 75% surface staining.

Histopathological analysis

Rats were sacrificed 8 weeks after gavage, and the eye of each animal was enucleated for histological analysis. All experimental procedures were performed under anesthesia of rats with overdose pentobarbital. The cornea tissues were fixed in 10% buffered formalin overnight and then dehydrated in ascending series of ethanol, cleared in methyl benzoate, and embedded in paraffin wax. Paraffin sections of 5 μm in thickness were prepared. The sections were stained with hematoxylin and eosin (HE) and then examined and photographed with transmission electron microscopy (TEM; JEM2100HC; JEOL, Tokyo, Japan).

Evaluation of inflammation

The inflammatory index was graded based on the following parameters. Central corneal edema (found with no visible pupil, 3; found with no visible iris details, 2; found with visible iris details, 1; none, 0), peripheral corneal edema (found with no visible iris, 3; found with no visible iris details, 2; found with visible iris details, 1; none, 0), and
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Ciliary hyperemia (more than 2 mm, 3; found between 1 and 2 mm, 2; less than 1 mm, 1; none, 0). The final inflammatory index was the sum of different parameters divided by nine.

Biochemical analysis

The cornea tissues were homogenized using a glass homogenizer in a 100-mM phosphate-buffered solution (PBS, pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF; proteinase inhibitor, 1 mM), and phosphatase inhibitor cocktail (1:100 dilution). The mixture was centrifuged 12,000×g for 30 min at 4°C. The levels of interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-1β (IL-1β), and tumor necrosis factor-alpha (TNF-α) in supernatant were detected by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s instructions (Nanjing Jiancheng Co., Nanjing, China).

Figure 1. Effects of apigenin on the recovery of ocular surface function in rats. Apigenin notably increases the (a) mean tear volume and (b) BUT scores suppressed by DED and reduces the (c) corneal epithelial fluorescein elicited by DED. Data were shown as mean ± SD (*P < 0.05 vs normal group, #P < 0.05 vs DED group, and &P > 0.05 vs DED group).

Statistical analysis

Statistical analysis was performed by using SPSS 17.0. Data were reported as the mean ± SD, and P < 0.05 was considered to indicate statistical significance. Differences between groups were analyzed by one-way analyses of variance (ANOVAs), and Dunnett’s test was used for multiple comparisons.

Results

Effect of apigenin on ocular surface function

The tear volume declined in the DED groups compared with the blank group. There were statistically significant differences between the groups (P < 0.05, respectively, Figure 1(a)). In the DED groups, BUTs...
were significantly shortened compared with the blank group ($P<0.05$, respectively, Figure 1(b)), while scores of fluorescein sodium (Figure 1(c)) significantly increased in DED groups ($P<0.05$, respectively). Mean tear volume and BUT scores of apigenin-treated group significantly increased and were significantly higher than those of control group after 8 weeks of therapy. Corneal epithelial fluorescein of apigenin-treated group was significantly decreased. The protective effect of apigenin on DED in a dose-dependent manner was noted.

Effect of apigenin on the inflammatory cytokines

In this study, we tested related inflammatory cytokines in cornea tissue. As shown in Figure 2, compared to the control group, apigenin treatment significantly prevented DED-induced elevation of TNF-$\alpha$, IL-1$\beta$, and IL-6 in a dose-dependent manner ($P<0.05$). However, compared to the DED group, the level of IL-10 in apigenin treatment groups marked increase in a dose-dependent manner ($P<0.05$, respectively).

Histopathological evaluation and inflammatory index

Figure 3(a) showed representative imaging of H&E-stained cornea tissues in different groups. There were no pathological changes in cornea of the control group. In contrast, diffuse leukocyte infiltration (primarily, polymorphonuclear leukocyte infiltration) was observed in the corneas of DED and vehicle groups. However, DED-induced pathological damages were attenuated by apigenin treatment. Inflammatory index further showed that DED rats developed profound inflammation in the cornea, and apigenin treatment could significantly
suppress the inflammation in the cornea (Figure 3(b)).

**Discussion**

In this study, we used main lacrimal gland extirpation-induced DED model to study the effect of apigenin on DED. Apigenin has been shown to have anti-inflammatory effects and have been successfully used to treat a variety of diseases. The Schirmer test is regarded as a routine method to assess tear volume. Objective studying on the BUT to find out the affecting factors related to the stability of tear film. Corneal damage in animals is known to cause a marked increase in fluorescein staining. The results showed that the mean tear volume and BUT scores of apigenin-treated group significantly increased and corneal epithelial fluorescein of apigenin-treated group was significantly decreased. These findings indicate that apigenin potential could be a therapeutic agent against DED.

Inflammation plays a major role in DED and perpetuates DED symptoms. Previous study has demonstrated increased levels of inflammatory cytokines, such as IL-1β, TNF-α, IL-6, and IL-8 in DED. Several of these cytokines such as IL-6 and IL-10 have been reported to influence the growth of epithelial cells and to promote hyperproliferation of the epidermis in psoriasis. In order to assess the anti-inflammatory effects of
apigenin on DED, we measured the level of IL-6, IL-10, IL-1β and TNF-α in the cornea tissues. The results showed that apigenin caused a marked reduction in the level of TNF-α, IL-1β, and IL-6. Apigenin treatment in DED rats also resulted in a marked increase in the level of anti-inflammatory cytokine IL-10. Thus, our finding suggested the anti-inflammatory actions of apigenin in DED.

In conclusion, this study is the first to demonstrate that apigenin could protect DED rats via its testosterone regulation and anti-inflammatory activities, suggesting that it may have potential as a therapy for DED.

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