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Original Article

Electroacupuncture modulates the TLR4-NF-κB inflammatory signaling pathway to attenuate ocular surface inflammation in dry eyes of type 2 diabetic rats

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Abstract

Bioinformatics analysis was performed to reveal the underlying pathogenesis of type 2 diabetes (T2DM) dry eye(DE) and to predict the core targets and potential pathways for electroacupuncture (EA) treatment of T2DM DE, in which key targets such as Toll-likereceptor4 (TLR4), NF-κB and Tumor necrosis factor-α (TNF-α) may be involved. Next, streptozotocin and a high-fat diet were used to generate T2DM-DE rats. Randomly picked EA, fluorometholone, model, and sham EA groups were created from successfully modelled T2DM DE rats. Six more rats were chosen as the blank group from among the normal rats. The results of DE index showed that EA improved the ocular surface symptoms.HE staining showed that EA attenuated the pathological changes in the cornea, conjunctiva and lacrimal gland of T2DM DE rats. EA decreased the expression of TLR4, MyD88, P-NF-κB P65, and TNF-α in the cornea, conjunctiva, and lacrimal gland, in accordance with immunofluorescence and Western blot data. Thus, EA reduced ocular surface symptoms and improved pathological changes of cornea, conjunctiva, and lacrimal gland induced by T2DM DE inT2DM DE rats, and the mechanism may be related to the inhibition of overactivation of the TLR4/NF-κB signaling pathway by EA and thus attenuating ocular surface inflammation.

Keywords: Type 2 diabetes; Dry eye; Electroacupuncture; TLR4/NF-κB Inflammatory signaling pathway; Inflammation

1. Introduction

Type 2 diabetes mellitus (T2DM) is frequently accompanied by dry eye (DE), and TFOS DEWS II has explicitly identified T2DM as one of the risk factors for DE[1]. T2DM DE is closely related to inflammation[2]. Inflammation on the ocular surface of DE patients with T2DM will lead to destruction of corneal epithelial barrier, nerve damage[3], apoptosis of conjunctival goblet cells[4], and atrophy of lacrimal gland acinifphys[5] causing discomfort symptoms such as dryness, burning sensation, foreign body sensation in the patient's eyes, and even leading to visual dysfunction[6], seriously affecting the life quality of patients..Currently, the eye drops commonly used clinically for T2DM DE include artificial tears and steroid drops[7]. Artificial tears do not have a direct anti-inflammatory effect, treating the symptoms but not the root cause; steroid hormone eye drops will produce increased intraocular pressure, cataracts and other adverse reactions after long-term use[8].Therefore, effective and safe treatment strategies need to be explored for T2DM DE.

Because EA is safe and reproducible, it is commonly used to treat diabetes and its complications[9-10]. Our previous study found that EA relieved ocular surface inflammation and increased tear production in an animal model of DE[11-12]. Nonetheless, most studies have focused mainly on DE-related studies, and the effect of EA on T2DM DE has rarely been reported.

Based on the fact that inflammation is an important pathogenesis of T2DM DE, we applied bioinformatic analysis for validation and screening of key targets and potential pathways, and then further explored the role and mechanism of EA in T2DM DE in rats with T2DM DE induced by a high-sugar and high-fat diet combined with streptozotocin. Although further studies are still needed, our findings demonstrate that EA for T2DM DE is a safe and effective potential therapeutic strategy.

2. Material and methods

2.1. Exploration of key targets and pathways of T2DM

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DE 3. Proteins associated with T2DM DE

Target proteins associated with T2DM DE were identified in the Online Mendelian Inheritance in Man (OMIM) (http://omim.org/), GeneCards (http://www.Genecards.org/), PharmGkb (https://www.pharmgkb.org/), DrugBank (https://go.drugbank.com/), and TherapeuticTarget Database (TTD) (http://db.idrblab.net/ttd/) databases using the keywords 'dry eye' and 'type 2 diabetes mellitus'.

2.2. Protein-protein interaction network analysis

Using the STRING database, protein-protein interaction (PPI) analysis of the intersection targets was performed, and Cytoscape 3.7.2 was implemented to illustrate the results. The network topology metrics of targets, among them betweenness, proximity, and degree, were analysed using the CytoNCA plugin.

2.3. Function and pathway enrichment analyses

In ClusterProfiler (Version: 3.14.3), the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) were searched for additional analysis of the shared protein targets between DE and T2DM. In these studies, the criterion for substantial enrichment was set at $P < 0.05$.

2.4. Animals

Five healthy male Sprague Dawley rats per cage, weighing 180–220g, were kept in the Basic Pharmacology Laboratory of the Affiliated Hospital of Nanjing University of Chinese Medicine. Their license number is SCXK (Su) 2019-0001, Nantong University. They were free to eat and drink. The rats were housed in a climate-controlled chamber with a 12-hour light-dark cycle, 21 ± 2°C temperature, and 40–60% relative humidity. The Affiliated Hospital of Nanjing University of Chinese Medicine's ethical committee accepted the animal trials, which complied with all applicable national experimental animal welfare ethical regulations (batch number: 2022NL-KS093). The People's Republic of China's Ministry of Science and Technology's Guiding Opinions on Treating Experimental Animals were followed in all experimental protocols.

2.5. Establishment of models: T2DM rat with DE

For a total of four weeks, rats were provided with a high-fat, high-carbohydrate (HFHC) diet containing 10% lard, 20% sucrose, 2.5% cholesterol, 1% cholate, and 66.5% ordinary feed. After that, 1% streptozotocin (STZ) in citric acid buffer (S0130, Sigma Aldrich, USA) was administered intraperitoneally into them. The dose of STZ was 30mg/kg, and the injection was completed within 15 minutes[13]. After injection, it was considered that the T2DM rat models with DE were successfully established when the random blood glucose ≥16.7mmol/L, phenol red thread test <7mm/20s, the tear break up time (BUT) <5s, and the corneal fluorescein staining was positive[14-15].

2.6. Electroacupuncture

In accordance with the rules for experimental acupuncture and moxibustion, the disposable sterile acupuncture needles (0.18 mm * 13 mm, Huatuo Medical Instruments Co., Ltd., China) were inserted into the bilateral "Jingming" (BL1), "Cuanzhu" (BL2), "Sizhukong" (SJ23), "Tongziliao" (GB1), and "Taiyang" (EX-HN5) of the rats. Following that, ipsilateral "Cuanzhu" and "Taiyang" were connected to a pair of electrodes from an EA instrument (SDZ-II B-type, Huatuo Medical Instruments Co., Ltd., China) via dilatational wave with a frequency of 4 Hz/20 Hz, pulse width of 0.2 ms, output intensity of 1 mA, and EA for 15 minutes, once a day[12].

2.7. Experimental design

Injection was employed to establish the model in the four extra groups following a 4-week HFHC diet. To create T2DM rat models with DE, 35 rats received injections of STZ. A successful induction rate of 24 rats (68.6%) was achieved. Four of the seven rats (11.4% mortality rate) died, and the model could not be established. The five groups—model (n = 6), EA (n = 6), sham EA (n = 6), and fluorometholone (n = 6)—had been assigned at random to these rats.The normal feed was given to the blank group (n = 6). The 12-week period saw the start of the 2-week interventions in the fluorometholone, sham EA, and EA groups. EA was administered once a day to the rats in the EA group. For fifteen minutes per day, the rats in the sham EA group received blunt-head acupuncture treatment[16]. Fluorometholone eye drops were administered to the rats in the pranoprofen group three times a day, at 8:00, 14:00, and 17:00, one drop per eye. On weeks 0, 4, 8, 12, and 14, ocular surface tests and radom blood glucose levels were assessed and documented, in that order. All tests were performed by the same tester, who was not aware of the rats' groupings at all times. After the experiment's fourteenth week, all of the rats were subjected to death, and tissues were gathered.

2.8. Detection indicators and methods

2.8.1. Radom blood glucose (RBG)

Rats' tail veins were utilized for obtaining blood samples so that a glucometer could measure their glucose levels.

2.8.2. Corneal fluorescence staining (FL)

The saline-moisturized portion of the infiltrated sodium fluorescence ophthalmic test paper was gently applied to the inferior fornix of the rats' eyes, prompting the rats to blink several times and facilitating the uniform distribution of sodium fluorescein in the cornea. A portable slit lamp was used to observe the corneal epithelial lesion. Every corneal quadrant received a score between 0 and 3: 0, no staining; 1, staining in less than 30 spots; 2, staining in more than 30 spots but not in fused patches; and 3, staining in fused patches. Ultimately, a monocular fluorescence staining score was calculated by adding the scores for each quadrant[17].

2.8.3. Phenol red thread test (PRT)

The phenol-red-attached end of the thread was folded back and inserted into the outside third of the rat's lower eyelid. Rats were given 20 seconds to close their eyes. After the thread was wetted with tears, the thread was taken out while the length of the phenol red immersion was measured[18].

2.8.4. Tear break-up time (BUT)

A slit lamp was employed to record the tear break-up time. After monitoring corneal fluorescence staining, observe how long it takes for the tear film to remain intact until the first dry patch on the cornea appears after the eyes

are reopened.

2.8.5. Corneal mechanical sensitivity

The rat corneal mechanical perception threshold, which has a negative relationship with corneal mechanical sensitivity, has been determined using the Cochet-Bonnet corneal perception instrument. With the nylon thread tip adjusted to a total length of 60 mm, the tester softly contacted the middle region of the rat's cornea vertically. The thread's mechanical perception threshold—the distance at which two of the three repeated stimuli caused the rat's cornea to blink—was indicative of the cornea's threshold. The nylon thread (5 mm) was gradually pulled shorter, and this step was repeated until the rat showed signs of blinking.

2.8.6. HE staining

The conjunctiva, LG, and PFA-fixed eyeballs were cut, embedded, dewaxed, and hydrated. After rinsing and staining the slices with hematoxylin, proceed to stain them with eosin, dehydrate, and seal the slices. Images were examined with a Nikon Eclipse E200 microscope (Japan).

2.8.7. Paraffin immunofluorescence staining

After rinsing, block the cut slices. The slices underwent thirty minutes of blocking following antigen repair. Toll-likereceptor4 (TLR4) (1:500; GB11519, Servicebio, China) and phospho-NFKB p65 (Ser536) (1:200; bs-0982R, Bioss, China) were added to the slices and left to sit at 4°C overnight so that TLR4 and phospho-NFKB immunofluorescence could be done. After adding goat anti-rabbit IgG (1:300; GB21303, Servicebio, China) tagged with CY3, the slices were incubated for fifty minutes on the following day. Add the DAPI staining solution, and let it sit for ten minutes in the dark at room temperature. The tissue slices were sealed with an anti-fluorescence quenching chemical and dried after the tissue's spontaneous fluorescent activity was extinguished. Under a fluorescence microscope (Nikon Eclipse C1, Japan), pictures were taken and examined.

2.8.9. Western blot

The bicinchoninic acid method was used to quantify the protein isolated from the eyeball, conjunctiva, and LG. Following electrophoresis, equivalent protein samples were wet-transferred on a PVDF membrane. Following a 2-hour blocking period, the PVDF membranes were incubated overnight at 4°C with the addition of primary antibodies such as TLR4 (1:1000; GB11519, Servicebio, China), MyD88 (1:1000; bs-1047R, Bioss, China), phospho-NFKB p65 (Ser536) (1:1000; bs-0982R, Bioss, China), and Tumor necrosis factor-α (TNF-α) (1:1000; 60291-1-IG, Proteintech, China). The membranes were incubated for two hours after secondary antibodies (1:3000; # S0001, # S0002, Affinity, China) were added. Chemiluminescence detection revealed the membranes. Following the elution of proteins from the membranes using an antibody, β-Tubulin (1:10000; 10094-1-AP, Proteintech, China) was used for detection during the subsequent re-exposure process. Using ImageJ image analysis software, the grayscale values of the bands were examined, and the relative expression level was indicated by the ratio of the target protein to β-Tubulin.

2.9. Statistical analysis

Version 26.0 of SPSS software was utilized for data processing and analysis. The means ± standard errors of the data are given as x ± SEM. For comparisons within each group, single-factor analysis of variance was employed, whereas for multiple comparisons, the LSD approach was employed. If the data did not follow a normal distribution or homogeneity of variance, nonparametric tests were chosen. $P<0.05$ denotes a comparative difference, while $P<0.01$ denotes a significant difference.

3. Results

3.1. TLR4-NF-κB inflammatory signaling pathway: a key target and potential signaling pathway predicted in T2DM DE

The above databases were searched, screened, and de-emphasized to obtain 390 disease-related targets of dry eye and 18, 805 diabetes-related targets, and then 223 intersecting targets were obtained by Wayne's analysis(Fig.1.a). All 223 intersecting targets were imported into STRING and the T2DM DE PPI network was constructed by Cytoscape(Fig.1.b). The top 13 targets were selected by screening based on the calculation of Betweenness, Closeness, Degree, Eigenvector, LAC, and Network median(Fig.1.c). Based on 223 cross-targets, GO enrichment analysis was performed, including biological process (BP), cellular composition (CC), and molecular function (MF), and the top ten with the smallest p-value in each section were selected as the prominent biological processes, and the results were mainly closely related to the inflammatory response, the immune response, and the positive regulation of MAPK cascade(Fig.1.d).KEGG functional enrichment analysis showed that these targets were closely related to the MAPK signaling pathway, IL-17signaling pathway, and PI3K-Aktsignaling pathway(Fig.1.e). These results suggest that T2DM DE pathogenesis is regulated through the regulation of multiple pathways and

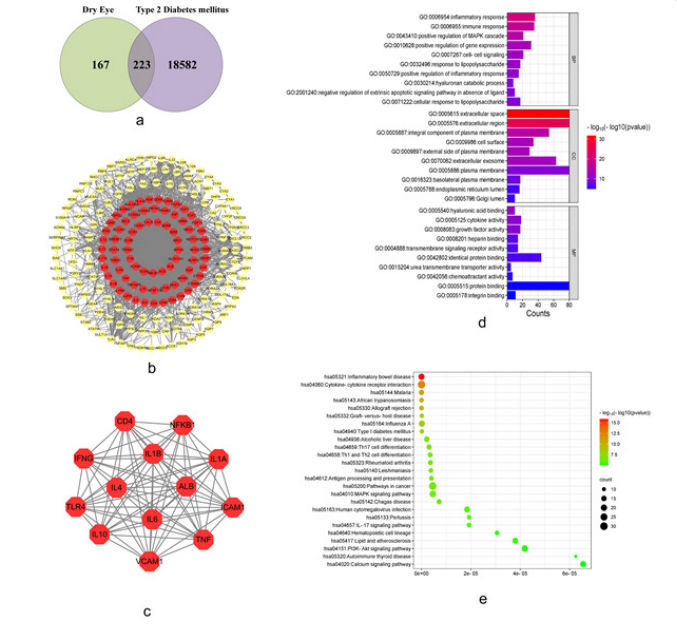

Fig. 1. a. Venn diagram showing the intersection of candidate targets in DE and T2DM. b. A PPI network of proteins to identify targets of T2DM DE. c. The top 13 targets of the PPI network are based on CytoNCA. d. GO enrichment analysis of protein targets for T2DM DE. e. KEGG pathway analysis of protein targets for T2DM DE.

that multiple risk genes for T2DM DE function in multiple pathways simultaneously.TLR4 as one of the core risk genes, it is involved in PI3K-Akt signaling, MAPK signaling pathway, and IL-17signaling pathway, and can stimulate downstream NF-κB phosphorylation and promote its nuclear translocation, and promote the expression of downstream cellular inflammatory genes (e.g. TNF-α). From this, we hypothesized that the TLR4-NF-κB pathway may be involved in the development of T2DM DE, and EA may achieve therapeutic effects by reducing ocular surface inflammation through inhibiting TLR4-NF-κB inflammatory signaling. However, this hypothesis requires subsequent experimental verification.

3.2. Electroacupuncture effectively relieves ocular surface symptoms in T2DM DE rats

Eight weeks after STZ injection, the randomized blood glucose and ocular surface signs of the rats had met the criteria for T2DM DE rats. After two weeks of EA treatment, FL, BUT, PRT and CTT were expectedly improved in diabetic dry eye rats ($P<0.05$). In addition, CTT and PRT increased in the EA group compared with the fluorometholone group, suggesting that the effect of EA in improving corneal perception and promoting tear secretion in T2DM DE rats may be superior to that of the fluorometholone group, reflecting the advantageous aspects of EA in the treatment of T2DM DE (Fig. 2).

3.3. EA ameliorates pathological damage to the cornea, conjunctiva and lacrimal glands in T2DM DE rats

The results of corneal HE staining (Fig. 3.a)showed that the cornea of rats in the model group was thickened, the surface was not smooth, and the epithelial cells were arranged in a disordered manner; in the EA group, the surface of the cornea was smooth, the corneal epithelial cells were arranged in a neat manner, and the thickness of the cornea was also returned to the normal level ($P<0.05$), (Fig. 3.b).

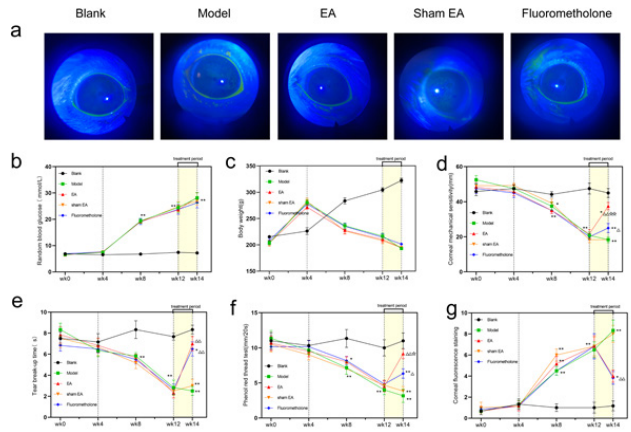

Fig. 2. a. Corneal fluorescence staining photographs of rats taken under slit lamp examination. b.Comparison of random blood glucose levels in each group of rats. C. Comparison of Body weight in each group of rats. d.Comparisons of the corneal fluorescence staining in each group of rats. e. Comparison of the tear break-up times in each group. f. Comparison of phenol red thread test results in each group. g. Comparison of the corneal mechanical sensitivity in each group. All data are expressed as means ±standard error, n=12 (eyes). $^{*}P<0.05$, $^{**}P<0.01$ versus the blank group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ versus the model group;$^{\star}P<0.05$, $^{\star\star}P<0.01$ versus the EA group.

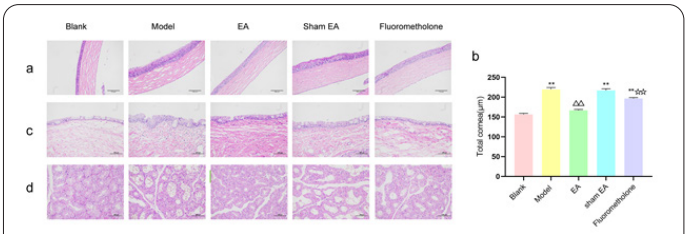

Fig. 3. a. HE staining of corneal slices in each group(200x). b. Comparison of total corneal thickness of rats in each group. All data are expressed as means ±standard error, n=3 $^{*}P<0.05$, $^{**}P<0.01$ versus the blank group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ versus the model group;$^{\star}P<0.05$, $^{\star\star}P<0.01$ versus the EA group. c. HE staining of conjunctival slices in each group(200x). d.HE staining of lacrimal slices in each group (200x).

HE staining of the conjunctiva showed that the conjunctival epithelium of the rats in the model group was hyperplastic, with partial epithelial detachment, sparse distribution of cup cells, and infiltration of inflammatory cells into the mucous layer and submucosal layer; the conjunctival epithelium in the EA group was more neatly arranged, and infiltration of inflammatory cells was not seen (Fig. 3.c).

HE staining of the lacrimal gland showed atrophy of the lobules of the lacrimal gland, vacuolization of glandular cells, and infiltration of local inflammatory cells around the glandular follicles in the model group of rats; the atrophy of the glandular follicles in the EA group improved, but vacuolization of the glandular cells and infiltration of local inflammatory cells were still observed in the fluorometholone group (Fig.3. d).

These results suggest that EA ameliorates pathological damage to the cornea, conjunctiva, and lacrimal gland in T2DM DE rats and may be more effective than fluorometholone in improving corneal thickness and lacrimal gland atrophy.

3.4. Electroacupuncture ameliorates inflammation on the ocular surface of diabetic dry eye rats by modulating the TLR4-NF-κB inflammatory signaling pathway

To deeply investigate the molecular basis of the efficacy of EA in the treatment of T2DM DE, we investigated the expression of TLR4, MyD88, p-NFκB p65, and TNF-α in the cornea, conjunctiva, and lachrymal glands. IF assay was used to obtain the expression of TLR4 (Fig. 4) and p-NF-κB p65 in the cornea, conjunctiva, and lachrymal tissues of the rats of the various groups (Fig. 5). TLR4 and p-NFκB p65 expression was reduced in the EA group, whereas a significant signal was observed in the model group. We also assessed the expression of TLR4, MyD88, p-NFκB p65, and TNF-α in corneal, conjunctival, and lacrimal gland tissues by protein blotting. We found that TLR4, MyD88, p-NFκB p65, and TNF-α protein levels were significantly reduced in the EA group compared with the model group(Fig. 6). Our study suggests that EA can reduce inflammation on the ocular surface of T2DM DE eye rats by inhibiting the TLR4-NF-κB inflammatory signaling pathway and thereby reducing inflammation.

4. Discussion

In this study, we revealed that inflammation is closely related to the progression of T2DM DE by bioinformatics analysis. By screening key target genes, biological processes and signaling pathways, the TLR4-NFκB signaling

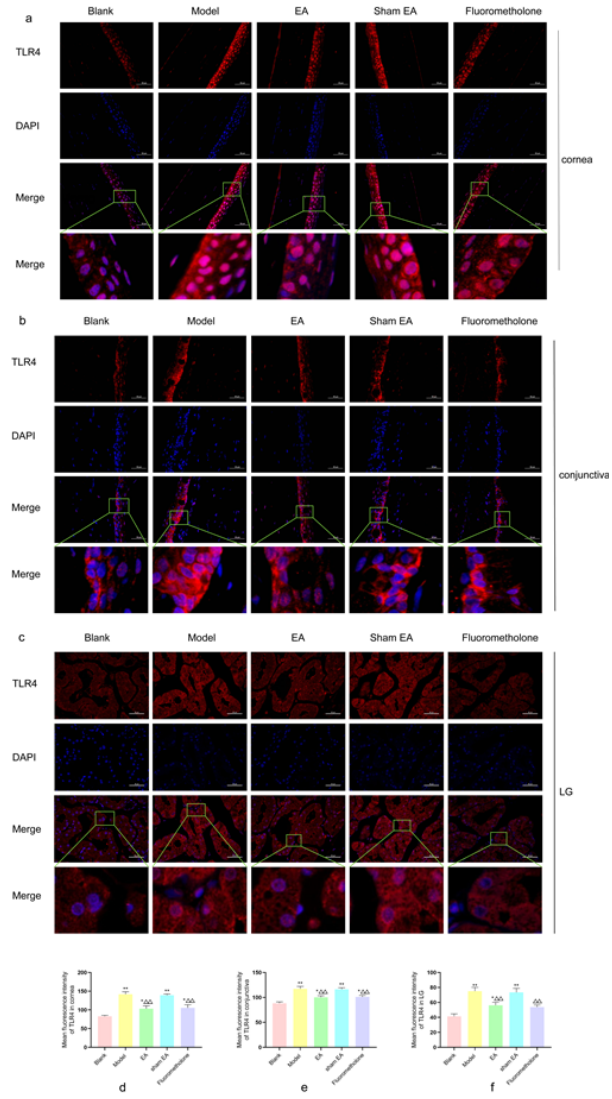

Fig. 4. a-c.Positive TLR4 expression in cornea, conjunctiva and LG(400x Red: TLR4; Blue: DAPI). d. Graph showed mean fluorescence intensity of TLR4 in cornea. e. Graph showed mean fluorescence intensity of TLR4 in conjunctiva. e. Graph showed mean fluorescence intensity of TLR4 in LG. Data are expressed as means ± standard error, n=3; *P<0.05, **P<0.01 versus the blank group; △P<0.05, △△P<0.01 versus the model group.

pathway was hypothesized to play an important role. After subsequent experimental validation, we found that EA can effectively improve the ocular surface symptoms of T2DM DE, and the mechanism may be related to the inhibition of the over-activation of TLR4/NF-κB signaling pathway by EA to reduce the ocular surface inflammation.

Inflammation plays a key role in T2DM DE progression. Effective inflammation is necessary for host defense against injury and tissue repair[19]. However, excessive or chronic inflammation can damage ocular surface tissues and decrease tear film stability, e.g. corneal epithelial defects, decreased corneal nerves[3] decreased conjunctival cuprocytes[4], and atrophy of lacrimal gland alveoli[5]. Numerous prospective and retrospective cohort studies have highlighted the relationship between pro-inflammatory cytokines (e.g., IL-1β, TNF-α, IL-6, etc.) and T2DM DE, as well as the harm that inflammatory cell infiltration and inflammatory factor release cause to ocular surface cells[20-23]. Increased activation of nuclear factor-κB and other pro-inflammatory transcription factors, along with the rapid triggering of inflammatory pathways and increased expression of pro-inflammatory cytokines and a sustained pro-inflammatory response, are the results of hyperglycemia and its associated late glycosylation end products[24-25]. Furthermore, hyperglycemia induces tear film hyperosmolarity, and when corneal structures—such as the corneal limbus and corneal epithelium—are exposed to this condition, a series of inflammatory reactions follow, culminating in a vicious cycle of inflammation[26]. Immune and structural cells in particular organs and tissues cause and sustain these inflammations[2]. The cells stimulate innate immunity primarily through pattern recognition receptors (PRRs); of these PRRs, Toll-like receptors (TLRs) have been the subject of the most research, particularly with regard to TLR4's function in inflammation[27]. Therefore, targeting inflammation is very relevant for the treatment of T2DM DE.

In our study, rats developed characteristic symptoms of T2DM with hyperglycemia and weight loss within 2 weeks after STZ injection[28]. After 2 weeks of suc-

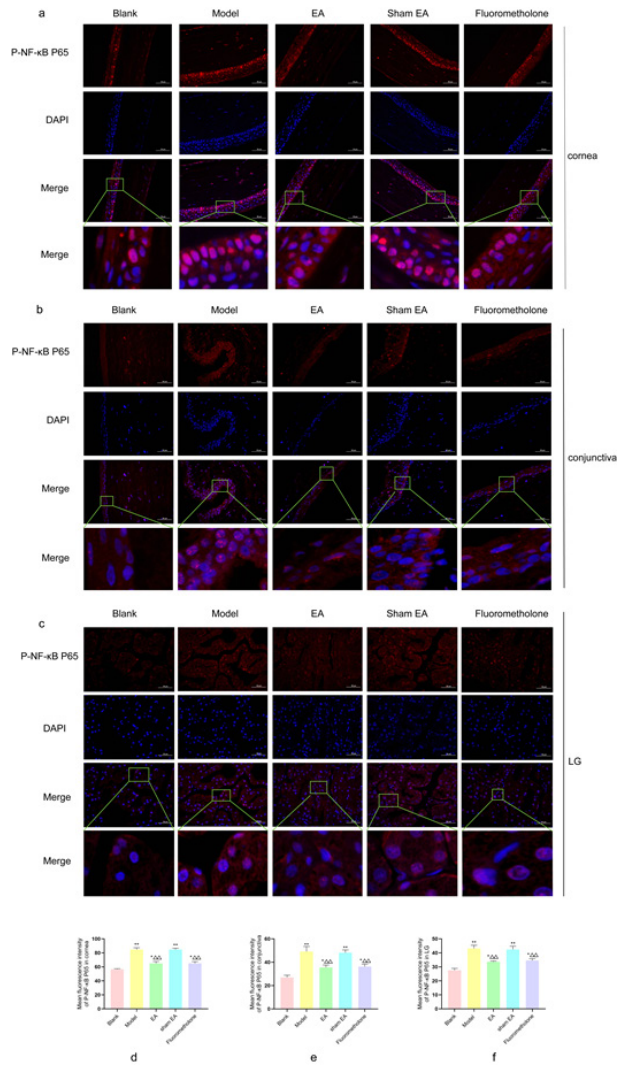

Fig. 5. a-c. Positive p-NFκB p65 expression in cornea, conjunctiva and LG(400x Red:TLR4; Blue: DAPI). d. Graph showed mean fluorescence intensity of p-NFκB p65 in cornea. e. Graph showed mean fluorescence intensity of p-NFκB p65 in conjunctiva. f. Graph showed mean fluorescence intensity of p-NFκB p65 in LG. Data are expressed as means ± standard error, n=3; *P<0.05, **P<0.01 versus the blank group; △P<0.05, △△P<0.01 versus the model group

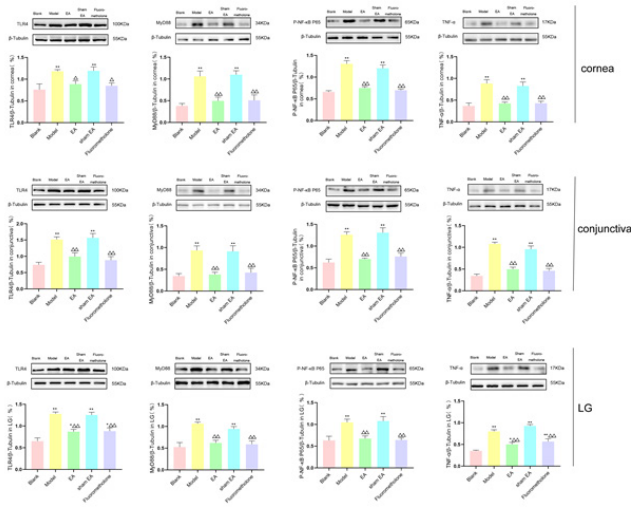

Fig. 6. Protein expression levels of TLR4, MyD88, p-NF-κB p65, TNF-α in the cornea, conjunctiva and LG by western blot and semi-quantitative analyses. Data are expressed as means ± standard error, n=3; *P<0.05, **P<0.01 versus the blank group; △P<0.05, △△P<0.01 versus the model group.

cessful diabetes modeling, the rats began to successively show changes such as decreased corneal perception and decreased tear production, which were similar to the diabetic dry eye symptoms described in humans[29]. Though there is still dispute regarding the most accurate and/or reproducible diagnostic approach for screening patients with DE, the PRT test was chosen for this study in place of the Schirmer I test since it is faster, less painful, and more repeatable[30]. We used the Cochet-Bonnet aesthesiometerto to assess corneal perception in rats, which is considered one of the standards for corneal sensitivity assessment[31], but studies have shown that non-contact Belmonte aesthesiometerto is more reproducible and reliable[32].In corneal changes in diabetic dry eye rats, JinH et al. observed perinuclear hyaline zone and interstitial edema in the corneal epithelium of diabetic rats, and the diabetic cornea's entire thickness was noticeably greater than the normal cornea's[33], which is consistent with our results.It is worth noting that T2DM damage to the cornea involves the entire corneal layer, especially the corneal nerve, which reduces corneal sensitivity, and the patient's blinking frequency reduces the tear evaporation too fast thus exacerbating DE[34]. The presence of excess conjunctival folds has been found to correlate with dry eye symptoms[35], and we observed an increase in the number of conjunctival epithelial cell layers in T2DM DE rats, and the above study may partially shed light on the cause of dry eye symptoms in T2DM DE rats. Just as studies have identified a number of atrophic features of the lacrimal gland in late diabetic mice (16 weeks of diabetes induction), including fibrosis and inflammatory cell infiltration, we also observed atrophic changes in rat lacrimal gland alveoli[36]. It has been shown that STZ-induced diabetic mice have reduced intraorbital lacrimal gland weight and decreased tear secretion, so the effect of T2DM on the outer lacrimal gland of the rat box also deserves our attention[37].

TLR4 is one of the important targets to overcome diabetes and its complications[38].TLR4 was found to be present in the cornea and conjunctiva, and the level of TLR4 protein expression was significantly elevated under hyperglycemic conditions, which is consistent with our results[39-40]. According to our research, the EA group's TLR4 expression in the cornea, conjunctiva, and lacrimal gland was considerably lower than that of the model group, indicating that TLR4 targeting offers a potentially effective intervention technique for the treatment of T2DM DE.MyD88 is a crucial bridge protein of the NF-κB signaling pathway and a downstream signaling ligand of the TLR4 receptor complex, which both contribute to the activation of inflammatory genes[41]. Our findings showed that EA lowered the TLR4 and MyD88 protein levels in the diabetic rats' cornea, conjunctiva, and lacrimal gland, indicating that EA may have an anti-inflammatory impact by blocking the TLR4/MyD88 signaling pathway. TLR4 signals by binding to MyD88, which stimulates downstream NF-κB phosphorylation and promotes its nuclear translocation, followed by upregulation of proinflammatory cytokine expression[42]. Our findings indicate that EA intervention reduces TLR4 and MyD88 protein levels, which in turn inhibits P-NF-κB P65, thereby reducing TNF-α expression. This suggests that blocking the TLR4/MyD88/NF-κB signaling pathway is an essential phase in the mechanism by which EA reduces ocular surface inflammation in T2DM DE rats.

In addition to inflammation, GO enrichment analysis also indicated that immune response was strongly associated with T2DM DE. Many scholars believe that T2DM may be an immune inflammatory disease[43], where activation of inflammatory vesicle-mediated immune response is associated with various secondary diabetic complications[44], including diabetic vascular complications, diabetic nephropathy, and diabetic retinopathy[45]. These scholars' studies are consistent with our findings.KEGG functional enrichment analysis showed that MAPK signaling pathway, IL-17 signaling pathway, and PI3K-Aktsignaling pathway were also involved in T2DM DE. Insulin resistance is induced by pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) produced by the PI3K/Akt, p38 MAPK, and IL-17 signaling pathways[46-47]. These pathways also reliably direct future research into the pathophysiology of T2DM DE and EA therapy.

We recognize several limitations of the current study. First, the experiments were in an exploratory phase, with modeling time of only three months, which did not allow for long-term observation of the disease process. Second, we did not use agonists and antagonists of TLR4 to further validate the role of EA in regulating the TLR4/NF-κB signaling pathway. In addition, we did not perform PAS staining of conjunctival tissues to further observe the effect of EA on the number of conjunctival cup cells in T2DM DE rats.

5. Conclusion

In conclusion, this study provides useful new insights to advance our understanding of the mechanism of action of EA for T2DM DE. EA reduced ocular surface symptoms and improved the pathological changes of cornea, conjunctiva, and lacrimal glands induced by T2DM DE rats, and the mechanism may be related to the inhibition of overactivation of the TLR4/NF-κB signaling pathway by EA to attenuate the ocular surface inflammation, which makes EA an effective and safe-acting strategy for the treatment of T2DM DE in the future.

Conflict of interest
All authors have to declare their conflict of interest.

Consent for publications
All authors have to write this sentence that they read and approved the final manuscript for publication.

Ethical Approval
The animal experiments conformed to the relevant provisions of the national experimental animal welfare ethics and were approved by the Ethics Committee of the Affiliated Hospital of Nanjing University of Chinese Medicine (batch number: 2022NL-KS093).

Availability of data and materials
The data used to support the results of this study can be obtained from the corresponding author upon reasonable request. All the images are consented to be published.

Authors' contributions
WPG & XYS conceptualized and designed the experiments. ZYTF and MMW executed the animal experiments. MMW and ZYTF performed the statistical analyses, while TJ interpreted the data. ZYTF wrote the initial draft of the manuscript. All authors approved the final version of the manuscript.

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