Development and pharmacodynamic evaluation of recombinant human lysozyme eye drops

Jin Shi
Shaanxi Huikang Bio-Tech Co., Ltd
Chang Shi
Beijing Rixin Technology Co., Ltd
Xiao-Ying Li
Shaanxi Huikang Bio-Tech Co., Ltd
En Gao
Shaanxi Huikang Bio-Tech Co., Ltd
Yuan Si
Shaanxi Huikang Bio-Tech Co., Ltd
Xiao-Lin Yang
Shaanxi Huikang Bio-Tech Co., Ltd
Jin-Li Zhao (zhjl999@163.com)
Shaanxi Huikang Bio-Tech Co., Ltd

Research Article

Keywords: dry eye disease (DED), artificial tears, recombinant human lysozyme (rhLYZ), pharmacodynamic evaluation, anti-inflammatory

Posted Date: March 13th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-287521/v1

Abstract

Dry eye disease (DED) is a multifactorial ocular surface disease. The commonest treatment is artificial tears. A novel artificial tears is urgently required to treat DED pathophysiologically, more than symptoms relief. In this study, a novel artificial tears with highly purified recombinant human lysozyme (rhLYZ), high homologous with natural tear, was obtained for DED treatment on a established rabbit DED model. 36 models were divided into 6 groups (n = 6), and treated with different concentration of novel artificial tears 180μl of drops daily, continuously for 4 weeks. To evaluate pathophysiologic therapeutic effect of the novel artificial tears, rabbit eyes were performed to the schirmer I test, tear ferning test, and corneal fluorescein staining test in 0, 1, 2, 3 and 4 weeks post-administration, respectively. The sacrificed models' corneas in 4 weeks were performed by pathological section staining. In the project, the quality of the artificial tears including rhLYZ was up to the quality standards of pharmacopoeia. RhLYZ eye drops, especially in the medium (0.15% of rhLYZ) and the high(0.30% of rhLYZ) concentration, enable to improve dry eye symptoms effectively, benefit to anti-inflammatory function and promote corneal repair. The novel artificial tears is a promising pathophysiological therapeutic agent in DED treatment.

Introduction

Dry eye disease (DED) is among the most common ocular surface disorders affecting the visual comfort and function of individuals worldwide [1]. DED, caused by various factors [2–5], is a complex, multifactorial disease and difficult to be characterized by a simple process, sign or symptom [6], thus, long-term treatment of DED is required [7]. Artificial tears are regarded as the main therapy for all severity grades of dry eye [7, 8]. Different artificial tears comprise a wide variety of products, which typically target one or more layers of the tear film [9–12], merely with relief of symptoms [13]. The reason may be that the component of existing agents is simple, not targeting underlying pathophysiology of DED [8].

Among the mechanism of pathophysiology of DED involving tear film instability, tear hyperosmolarity and inflammation at the ocular surface [2, 14], ocular surface inflammation is critical [15]. The inflammatory cascades lead to severe ocular surface damage, and develop a self-perpetuating inflammatory cycle [16]. It is detected that the expression of inflammation-related cytokines IL-2, IL-6, TNF-α, TGF-β are increased in tears or ocular surface of DED patients comparing with the normal ones [2, 17]. Anti-inflammatory therapy, rather than other treatment, is considered as the effective treatment due to its target of the underlying pathology [18]. It was documented that corticosteroids and cyclosporine A are performed as the anti-inflammatory therapies to moderate severe dry-eye symptoms, while, side-effects and restricted efficacy limit the treatment [7, 19]. In addition, multi-dose artificial lubricants, another treatment of DED, typically require a preservative, whereas, preservatives is well recognized to induce toxicity and adverse changes to the ocular surface, companying with dry eye symptoms progression [20–25].

Generally, natural tear protect the ocular surface and prevent DED development physiologically, thus, the artificial tears, homologous with nature tears, with fewer side-effect were targeted. Meanwhile, lysozyme

in natural tears with the high concentration of up to 2.07 mg/mL [26, 27], is well known for the muramidase activity [27], also possesses immunostimulating, anti-inflammatory, regenerative, and analgesic activity [28]. Recent decades of researches have implicated that accompanying with aqueous deficient, lysozyme level of natural tear is reduced in DED patients [29, 30]. Therefore, it is critical for OTC and clinical therapy to develop the novel artificial tears, analogue with nature tears, with antibacterial and anti-inflammatory function as well as no preservative to prevent and cure the DED in long-term,accompanying with low side effects.

Based on the reasons above, industrialized recombinant human lysozyme (rhLYZ), highly homologous with natural human lysozyme, was fermented and purified via biological genetic engineering technology. Furthermore, novel artificial tears containing prepared rhLYZ were designed and developed, termed as rhLYZ eye drops. The components and physicochemical properties of rhLYZ eyes drops were basically analogous with natural tears. The physiological and pathological effects of rhLYZ eye drops in different concentrations on DED symptoms was systematically evaluated via the establishment of a stable and reliable New Zealand rabbit dry eye model. This research of the novel eye drops may be pathophysiologically important and beneficial in DED treatment.

Methods

RhLYZ raw material

The high purity freeze-dried rhLYZ raw materials were obtained via biogenetic engineering technology, high density fermentation, fine purification, freeze-drying and other processes. The materials need up to the pharmacopoeia quality standards based on quality researches. Quality researches are including purity testing, content detection, activity assay, and western blot analysis, amino acid sequence analysis, isoelectric point detection, host protein residue detection, exogenous DNA residue detection, methanol residue, heavy metal residues detection, endotoxin detection, microbial limit test.

Artificial tears

RhLYZ eye drops were prepared and employed different concertation (0.075%-0.30%) of rhLYZ raw material and with the supplementary including: 0.10% sodium hyaluronate (National medicine approval word H20113379, Huaxi Furuida Biological Medicine Co., Ltd, China), 0.75% sodium chloride (National medicine approval word H32020718, Jiangsu Qinfen Pharmaceutical Co., Ltd, China), 0.15% disodium hydrogen phosphate (Sichuan medicine approval word F20080005, Chengdu huayi pharmaceutical accessories manufacturing Co., Ltd, China), 0.08% sodium dihydrogen phosphate (Sichuan medicine approval word F20080002, Chengdu huayi pharmaceutical accessories manufacturing Co., Ltd, China), and 98.845%-98.62% water for injection (self-production).

All the raw materials above were prepared into a transparent liquid according to the specific formula ratio and processes.

The prepared solution was filtered and sterilized with 0.22μm sterilizing filter, followed by pouring into the disposable eye drop vials via an all-in-one blowing and sealing machine under the protection of grade A

laminar flow. The filling specification was 0.8mL/vial. The aseptically filled products were stored under the condition of 2–8°C after quality inspection, involving load capacity examination, activity and sterility along with stability test, also the pH, content, character, visible particle, osmotic pressure detection, etc.

The four specifications (quantity: content: activity) of the rhLYZ eye drops applied for animal experiments were produced according to the processes above.
The administration group was graded as 5: base solution group (0.8mL:0.0mg:0U, excluding rhLYZ), low concentration rhLYZ group (0.8mL:0.60mg:60000U, rhLYZ 0.075%), medium concentration rhLYZ group (0.8mL:1.20mg:120000U, rhLYZ 0.15%), high concentration rhLYZ group(0.8mL:2.40mg:240000U, rhLYZ 0.30%). HYCOSAN®-Sodium Hyaluronate Eye Drops (0.1% Sodium Hyaluronate, URSAPHARM Arzneimittel GmbH, Germany) was treated as a positive control.

Establishment and evaluation of DED model

All animals in this study were required to be performed and managed in accordance with the *Guide for the Care and Use of Laboratory Animals*. The experiment organization was accredited by the *Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)* and fully complied with the relevant regulations of the People's Republic of China on the management of experimental animal welfare. This protocol was submitted and approved by the *Institutional Animal Care and Use Committee (IACUC)*. All methods described in this study were performed in accordance with *ARRIVE guidelines*.

36 New Zealand rabbit's (n = 18 male, n = 18 female, 3–4 month, Beijing xinglong laboratory animal breeding factory) were performed and suffered from the complete excision of lacrimal gland, harderian gland and the third eyelid in left eyes, following cauterized with 30% trichloroacetic acid to left conjunctivas. The right eye of each rabbit was served as blank control. Both eyes of 36 rabbits were respectively subjected to the schirmer I test [6, 31], tear ferning test [6, 32, 33], and the corneal fluorescein staining test [6, 34, 35] pre-surgery and 1,2 weeks post-surgery to determine the model establishment.

Pharmacodynamic evaluation

The established 36 DED models were randomly divided into 6 groups (n = 6, 3male and 3 female): sham group, base solution group, low concentration group, medium concentration group, high concentration group, positive control. The right eye of each group serves as blank control. The left conjunctival sac was treated with eye drops of 60μl, 3 times per day continuously for 4 weeks. The schirmer I test, the tear ferning test, and the corneal fluorescein staining test were conducted to the 36 models pre-treatment and 1, 2, 3, 4 weeks post-treatment. After 4 weeks, all the models were sacrificed and the corneas were deparaffinizated for H&E stain as well as immunohistochemical (IHC) to evaluate the therapeutic impact of rhLYZ eye drops and locate the expression of inflammation-related cytokines. IHC reaction was quantitatively assessed via computer-assisted microscopic image analysis.

Statistic analysis

The mean value and standard deviation of measurement data were calculated according to the group. The analysis of variance was carried out, and the t-test was conducted. $p < 0.05$ indicated the statistical significance, while, $p < 0.01$ was as extremely significant difference.

Results

Quality test of rhLYZ

The rhLYZ raw materials were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and reversed-phase high performance liquid chromatography (RP-HPLC) C18 column detection, following analysis of gray scan and area normalization, the purity of rhLYZ was greater than 98% (Fig. 1, a-b). The highly consistent expression of rhLYZ was detected by western blotting (anti-lysozyme antibody, Abcam ab91653) (Fig. 1, c). Then the molecular weight of 14697.56Da were identified by MALDI-TOF-MS (Fig. 1, d), which was identical with the theoretical molecular weight [36]. The amino acid composition of rhLYZ was precisely quantified via phenyl isothiocyanate (PITC) derivative method, and it was coincident with that of human lysozyme (Fig. 1, e) [36]. In addition, the isoelectric point of 9.24 was detected via capillary isoelectric focusing electrophoresis (CIFE) (Fig. 1f). Furthermore, the N-terminal and C-terminal amino acid sequence of rhLYZ were determined and the results were analogous with the theoretical amino acid sequences. The others quality inspection of rhLYZ was performed, according to the *Pharmacopoeia of the People's Republic of China (2015)*. The acquired rhLYZ was white dry powder-like substances with the antibacterial activity of 100,000U/mg and the characteristic ultraviolet absorption wavelength of 280nm. Besides, host protein residues were less than 0.1% and the exogenous DNA remains were less than 0.01%. Furthermore, methanol residue was less than 0.002% and heavy metal residues were less than 0.1mg/kg. The endotoxin content was less than 10EU/mg.

Quality test of rhLYZ eye drops

According to the *Pharmacopoeia of the People's Republic of China (2015)*, the produced rhLYZ eye drops was colorless transparent liquid. No visible foreign body was detected. The quantity of each unit was 0.8mL with pH value of 6.5. The osmotic pressure of eye drops measured by the freezing point osmotic device was 285–310 mOsmol/kg. The eye drops contained 0.1% sodium hyaluronate. All the rhLYZ eye drops were qualified via sterility test. The efficiency of rhLYZ eye drops stored at 2–8℃ were stable within 24 months.

The establishment of DED model

Based on the previous publication [37], the multiple severe dry eye model with insufficient tear secretion were produced. The 36 rabbits' eyes were subjected to the schirmer I test (Fig. 2, a1-a2). No significant difference was observed between the operation eye (the left eye) and the normal eye (the right eye) prior to operation. After 1 and 2 weeks post-op, the volume of the tear of operation eyes was significantly less comparing with the normal eyes (Fig. 2, a3). According to Rolando's classification method, tear ferning patterns were graded four levels (Ⅰ-Ⅳ). The eyes pre-op and the normal eyes were identified as level Ⅰ with even and dense ferning dentate branch of crystallization, as well as the small spaces (Fig. 2, b1). The tear ferming level of 1 or 2 weeks post-op was markedly increased and classified as level Ⅱ-Ⅳ(Fig. 2, b2-b4), appearing less crystallization and larger spaces (Fig. 2, b5). Referring to the grading criteria, no significant difference in the corneal fluorescein staining test was discovered between the operation eyes and the normal eyes prior to surgery (Fig. 2, c1). However, after 1 and 2 weeks post-operation, the corneal

fluorescein staining score of the operation eye was remarkably raised (Fig. 2, c2-c4). There was a high discrimination in the score between the operation eyes and the normal eyes (Fig. 2, c5). Referring to the DED-related test, more notable deterioration of DED was presented on the dry eye model in 2 weeks post-op, comparing with 1week post-op. It indicated that the novel dry eye model of New Zealand rabbits with insufficient tear secretion in 2 weeks post operation was successfully established.

Evaluation of rhLYZ eye drops

According to the data of schirmer I test, tear ferning test, and corneal fluorescein staining test with treatment of novel rhLYZ eye drops on produced dry eye model within 4 weeks, there was no markedly difference among the low concentration group, medium concentration group, high concentration group and positive control. The difference between the four groups and the base solution group as well as the sham group was statistically remarkably (Fig. 3, a-c). During the 2–4 weeks of treatment, the four groups highly increased tear secretion on dry eye model. And after 4 weeks of treatment, the amount of tear secretion in the operation eye was basically recovered as the normal eye (Fig. 3, a). During the 3–4 weeks of treatment, tear composition and quality were significantly improved in the four groups (Fig. 3, b). Besides, the staining score of corneal fluorescein sodium decreased markedly after 2 weeks of administration (Fig. 3, c). Although treatment ones were not restored as normal level, the dramatically repair effect of agents on the damaged eyes was obvious.

The corneas of 4 weeks models were performed H&E staining, the hyperplasia and disorder of corneal epithelial cells was observed in the sham group and the base solution group. Moreover, the matrix layer was shriveled and the regular elastic fiber structure was disorder comparing with the blank group (Fig. 4, a1-a3). A large number of inflammatory cells were detected in the sham group (Fig. 4, a2), similarly, in the base solution group (Fig. 4, a3). In the low concentration group, corneal epithelial cells exhibited modest hyperplasia and the cell arrangement was virtually normal, meanwhile, the inflammatory cells were hardly found (Fig. 4, a4). The morphology of corneal cells in the medium concentration group, high concentration group and positive control was similar with that in the blank control (Fig. 4, a1, a5-a7). The results suggested that the eye drop performed a notable recovery impacts on the damaged corneal epithelial cells.

To reveal the eye drops mechanism, the results of immunohistochemical analysis of cornea were analyzed. TNF-α and TGF-β1 were significantly increased in the base solution group and sham group compared to the blank control physiologically (Fig. 4, b1-b3; c1-c3; Fig. 5). The appearance of TNF-α in the low concentration group, medium concentration group, high concentration group and positive control was decreased in comparison with that in the sham group and base solution group (Fig. 4, b2-b7; Fig. 5). Especially, TNF-α expression in high concentration group was lower in comparison to that in positive control (Fig. 4, b6-b7; Fig. 5). Higher TGF-β1 expression was obtained in the low concentration group, medium concentration group, high concentration group, and positive control relative to the sham group and the base solution group (Fig. 4, c2-c7; Fig. 5). Even, the expression of TGF-β1 in the medium and high concentration groups was up-regulated than that in the positive control (Fig. 4, c5-c7; Fig. 5). It was

implied that the novel rhLYZ eye drop could inhibit the expression of TNF-α, on the contrary, activate the expression of TGF-β1 in the tissues.

All in all, the novel rhLYZ eye drops, especially in the medium (0.15%) and high concentration (0.30%) enable to improve dry eye symptoms effectively, increase tear secretion, promote tear ingredients, recover corneas injury, benefit to anti-inflammatory function and promote corneal repair. The overall therapeutic was more effective than that in the positive control group.

Discussion

DED animal models

DED is a complex disease with multiple and variable symptoms. Despite more advances were discovered in understanding of DED, significant knowledge gaps remained. The limitation is due to the lack of typical and informative animal models [38]. A variety of dry eye models was produced [38–40], but it is difficult to obtain a stable dry eye animal model. In some models suffered with simple operations, such as atropine eye drop method and low-vitamin A diet administration, dry eye symptoms were not visible and significant individual differences were indicated. Furthermore, a new dry eye model was acquired by fixing upper and lower eyelids to reduce blink times with obvious dry eye symptoms, but it remained only for a few hours and unable to satisfy long-term research.

Theoretically, tears are mainly secreted by lacrimal gland [41]. Harderian gland is a peripheral immune organ, and also secretes tear fluid to lubricate the transient membrane, playing a mechanical protective role on eyes. Transient membrane, as the "third eyelid", enables to cover the cornea and moist the eyeball consequently. Meanwhile, it was documented that burning the conjunctiva of rabbit bulb with 50% trichloroacetic acid contributed to the angular conjunctiva injury, a common syndrome in dry eye [37]. Collectively, in this study, an improved and stable dry eye model with severe dry eye symptoms were established successfully via excision of the lacrimal gland, harderian gland and the third eyelid completely, in addition to searing with 30% trichloroacetic acid in conjunctiva. During research period, no adverse reactions and well-tolerated were detected. Actually, it was observed that the slight existence of tears via schirmer tear test probably due to the compensatory pathway in organism, such as the accessory lacrimal glands as well as the plasma leakage from conjunctival vessels [42–44]. It was likely to be positive impact to partly maintain the physiological function of ocular surface, as complete dry eye symptom model with full injury of cornea was not a desired DED model [38]. Summarily, the established stable severe DED model caused by water deficiency in this project may bring a promising support for the further research on DED and pharmaceutical development.

Anti-inflammatory treatment

To date, the anti-inflammatory agents for DED, including topical Corticosteroid, Cyclosporine, and Tacrolimus, were common clinical medication to modulate anterior segment inflammation, referring to the ocular surface inflammation plays a critical role in the pathophysiology of DED [15]. Multiple studies

have documented the clinical value of the agents was in short-term treatment of DED [8]. It appeared that topical corticosteroids accompanying with the potential complications may be not an ideal administration in the long-term management of DED, even in the short term, as it may induce ocular hypertension, cataracts and opportunistic infections [45]. Cyclosporine, an immunomodulators, was understood to be as an immunomodulatory treatment with anti-inflammatory properties to relieve DED. The mechanism is to inhibit T-cell activation and stimulate inflammatory cytokines. It has been available as eye drops in concentrations of 0.05% and 0.1% [46–48], whereas, some patients suffered discomfortable in long-term administration, due to the overdose may cause immune imbalance [49]. Tacrolimus, a macrolide produced by streptomyces tsukubaensis, significantly improved dry eye symptoms and signs with administration of 0.03% [50]. In addition, lymphocyte function-associated antigen 1 (LFA-1) antagonist, tetracycline, macrolide were also performed as the DED agents, but the exogenous agents may lead to more or less adverse effects with long-term treatment [8]. Therefore, an endogenous anti-inflammatory agent for DED treatment was urgently required.

Lysozyme, a component in tissues and secretions, are focused. Lysozyme was widely distributed in plants and animals, even in human natural tears with the concentration of 2.07 mg/mL [26, 27]. Besides the well-known antibacterial function, lysozyme served anti-inflammatory and antiviral function were revealed, rather than suppression of innate immune response of macrophages [28, 51]. Lysozyme significantly repressed the expression of IL-6 and TNF-α inflammatory cytokines via depression of the phosphorylation of JNK in a dose-dependent manner [51]. Meanwhile, lysozyme was not cytotoxic to peritoneal macrophages or RAW264.7 cells even at the concertation of 1,000 μg/mL [51]. Although, in this project, a significant difference was implied between the basic solution group with sodium hyaluronate and the positive control, the overall therapeutic rhLYZ in the medium and high concentration groups was more effective than that in the positive control group. RhLYZ exerted compensatory impact to cover the deficiency of self-produced base solution. It indicated that lysozyme perform an important potential in anti-inflammatory effect and promotion of damaged cornea repair, even in the treatment of DED. The mechanism of rhLYZ eye drops is related to the regulation of inflammatory responses. Therefore, we applied lysozyme as a beneficial candidate for DED treatment has been supported by solid theoretical and experimental data.

The formulation of supplementary

On the other side, the component of eye drops of artificial tears used in the treatment of DED should be extremely close to natural tears, since natural human tears, containing a variety of proteins, small molecule metabolites, electrolytes, gel-forming mucin, protect ocular surface and maintain the eye function naturally [26]. Hyaluronic acid, an endogenous substance in natural tears, is highly contained in the lens of the eye, which is capable of maintain the moisture and lubrication of eye surface, also be metabolized via itself [52]. Meanwhile, hyaluronic acid enables to be assembled and cover on corneal epithelial surface in order to maintain the content of rhLYZ on eye surface for anti-inflammation, reduce the microbial invasion, and promote the cell regeneration. Therefore, in this project hyaluronic acid is regarded as the mainstream of moisturizing materials in eye drops.

Also, concentration of sodium chloride was closely relative to the solubility and stability of rhLYZ in eye drops. During the preparation and experiment processes, in lower 0.6% of sodium chloride of regent, the solubility, stability, content and activity of rhLYZ was reduced accompanying with the decreasing concentration of sodium chloride. While, the solubility and stability of rhLYZ were maintained stable within the sodium chloride concentration of 0.6%-6%. Therefore, the 0.75% sodium chloride was performed in the novel eye drops, to sustain the characteristic of rhLYZ and osmolality of 300–310 mOsmol/kg, as the increasing tear osmolality is considered as the hallmark of DED pathophysiologically [18].

PH value is also a critical control index in artificial tears, related with the stability, effectivity and ocular irritation of ophthalmic preparation. The PI of rhLYZ is 9.24 and activity pH range is 5.0–8.0, with the optimum pH of 6.5. The pH of artificial eye drops was adjusted at 6.5 ± 0.1 by disodium hydrogen phosphate (0.15%) - sodium dihydrogen phosphate (0.08%) to maintains the activity of rhLYZ, even, the stability, effectiveness and eye nonirritant of eye drops preparation.

With the aging, high pressure, and the development of electronics, the risk of dry eye and dry eye syndrome is increasing. A potential pharmacological strategy to develop of artificial tears, especially high quality artificial tears, may prevent and treat DED effectively and bring a great social significance. In the future, based on the novel rhLYZ eye drops, more components included in natural tears will be added to improve our artificial tears on efficiency and function for various grades of DED patients. Development of novel artificial tears, highly homologous with natural tears is our target.

Conclusions

In conclusion, the novel artificial tears is developed with the characteristics of sterile, preservative-free, daily dose packaging, pH value of 6.5 ± 0.1, osmotic molality range of 285–310 mOsmol/kg, sodium hyaluronate content of 0.1%, rhLYZ content of 0.075%-0.30%. The significantly effective concentration of rhLYZ were performed with 0.15% and 0.30% on dry eye model pathologically, resulting in relief of DED symptoms, repair of corneas, and anti-inflammation. In detail, during the administration with rhLYZ eye drops, it was observed of the increased tear secretion via schirmer I test, promoted tear ingredients based on the results of tear ferning test, recovered corneas injury according to fluorescein staining test and H&E staining, meanwhile, inflammation-related factor TNF-α expression was down-regulated while TGF-β1 expression was up-regulated in the corneal epithelium. In a word, the novel artificial tears containing rhLYZ is a promising pathophysiological therapeutic agent in DED treatment. Furthermore, to develop the rhLYZ eye drops as a novel biological angent, subsequent studies should be required on tissue distribution, pharmacokinetics, toxicology, clinical safety as well as clinical pharmacology.

Declarations

Data Availability

All data generated or analysed during this study are included in this published article.

Acknowledgments

The authors would like to thank all the teachers and colleagues from Shaanxi Huikang Biotechnology Co., Ltd and Beijing Rixin Technology Co., Ltd for their efforts in the development of this new agent. The authors also would like to express their special thanks Mr. Ye Yuan and Mr. Qi Guo for their outstanding contributions to the study of drug efficacy evaluation.

Author contributions

J.S., C.S. and J.L.Z. participated in the study design. J.S., C.S., X.Y.L., E.G. and X.L.Y. conducted the experiments and performed data analysis. J.S. and Y.S. wrote and revised the manuscript. X.L.Y. and J.L.Z. supervised the study. All authors critically reviewed and edited draft versions of the paper and approved the final version.

Competing interests

The authors declare no competing interests.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

1. Stapleton, F. *et al.* TFOS DEWS â…¡ epidemiology report. *Ocul. Surf.***15**, 334–365 https://doi.org/10.1016/j.jtos.2017.05.003 (2017).
2. Bron, A. J. *et al.* TFOS DEWS â…¡ pathophysiology report. *Ocul. Surf.***15**, 438–510 https://doi.org/10.1016/j.jtos.2017.05.011 (2017).
3. Bron, A. J. *et al.* Rethinking dry eye disease: a perspective on clinical implications. *Ocul. Surf.***12**, S1–31 https://doi.org/10.1016/j.jtos.2014.02.002 (2014).
4. Kim, J. H. *et al.* Oral alcohol administration disturbs tear film and ocular surface. *Ophthalmology.***119**, 965–971 https://doi.org/10.1016/j.ophtha.2011.11.015 (2012).
5. Thomas, J., Jacob, G. P., Abraham, L. & Noushad, B. The effect of smoking on the ocular surface and the precorneal tear film. *Australas. Med. J.***5**, 221–226 https://doi.org/10.4066/AMJ.2012.1035 (2012).
6. Wolffsohn, J. S. *et al.* TFOS DEWS â…¡ diagnostic methodology report. *Ocul. Surf.***15**, 539–574 https://doi.org/10.1016/j.jtos.2017.05.001 (2017).
7. Messmer, E. M. The pathophysiology, diagnosis, and treatment of dry eye disease. *Dtsch. Arztebl. Int.***112**, 71–81 quiz 82 https://doi.org/10.3238/arztebl.2015.0071 (2015).

8. Jones, L. *et al.* TFOS DEWS â…¡ management and therapy report. *Ocul. Surf.***15**, 575–628 https://doi.org/10.1016/j.jtos.2017.05.006 (2017).
9. Tong, L., Petznick, A., Lee, S. & Tan, J. Choice of artificial tear formulation for patients with dry eye: where do we start? *Cornea.***31 Suppl** (1), S32–36 https://doi.org/10.1097/ICO.0b013e318269cb99 (2012).
10. Murube, J., Paterson, A. & Murube, E. Classification of artificial tears. â… : Composition and properties. *Adv. Exp. Med. Biol.***438**, 693–704 (1998). 10.1007/978-1-4615-5359-5$499
11. Dogru, M., Nakamura, M., Shimazaki, J. & Tsubota, K. Changing trends in the treatment of dry-eye disease. *Expert. Opin. Investig. Drugs.***22**, 1581–1601 https://doi.org/10.1517/13543784.2013.838557 (2013).
12. Dogru, M. & Tsubota, K. Pharmacotherapy of dry eye. *Expert. Opin. Pharmacother.***12**, 325–334 https://doi.org/10.1517/14656566.2010.518612 (2011).
13. Massie, I., Spaniol, K., Geerling, G. & Schrader, S. Cryopreservation and hypothermic storage of lacrimal gland: towards enabling delivery of regenerative medicine therapies for treatment of dry eye syndrome. *J. Tissue. Eng. Regen. Med.***11**, 3373–3384 https://doi.org/10.1002/term.2251 (2017).
14. Pflugfelder, S. C. & de Paiva, C. S. The pathophysiology of dry eye disease: what we know and future directions for research. *Ophthalmology.***124**, S4–S13 https://doi.org/10.1016/j.ophtha.2017.07.010 (2017).
15. Craig, J. P. *et al.* TFOS DEWS â…¡ definition and classification report. *Ocul. Surf.***15**, 276–283 https://doi.org/10.1016/j.jtos.2017.05.008 (2017).
16. Belmonte, C. *et al.* TFOS DEWS â…¡ pain and sensation report. *Ocul. Surf.***15**, 404–437 https://doi.org/10.1016/j.jtos.2017.05.002 (2017).
17. Roda, M. *et al.* Dry eye disease and tear cytokine levels-a meta-analysis. *Int. J. Mol. Sci.***21**, 3111–3117 https://doi.org/10.3390/ijms21093111 (2020).
18. Dadapeer, K. *eye in Essentials of Ophthalmology (ed. Dadapeer, K.) 532–536 (Jaypee Brothers Medical Publishers Private Limited*, 2015).
19. McCabe, E. & Narayanan, S. Advancements in anti-inflammatory therapy for dry eye syndrome. *Optometry.***80**, 555–566 https://doi.org/10.1016/j.optm.2009.02.010 (2009).
20. Baudouin, C. The pathology of dry eye. *Surv. Ophthalmol.***45** (Suppl 2), S211–220 https://doi.org/10.1016/s0039-6257(00)00200-9 (2001).
21. Noecker, R. Effects of common ophthalmic preservatives on ocular health. *Adv. Ther.***18**, 205–215 https://doi.org/10.1007/BF02853166 (2001).
22. Stewart, W. C., Stewart, J. A. & Nelson, L. A. Ocular surface disease in patients with ocular hypertension and glaucoma. *Curr. Eye. Res.***36**, 391–398 https://doi.org/10.3109/02713683.2011.562340 (2011).
23. Mantelli, F., Tranchina, L., Lambiase, A. & Bonini, S. Ocular surface damage by ophthalmic compounds. *Curr. Opin. Allergy. Clin. Immunol.***11**, 464–470

https://doi.org/10.1097/ACI.0b013e32834a95c9 (2011).

24. Stalmans, I. *et al.* Preservative-free treatment in glaucoma: who, when, and why. *Eur. J. Ophthalmol.***23**, 518–525 https://doi.org/10.5301/ejo.5000270 (2013).
25. Anwar, Z., Wellik, S. R. & Galor, A. Glaucoma therapy and ocular surface disease: current literature and recommendations. *Curr. Opin. Ophthalmol.***24**, 136–143 https://doi.org/10.1097/ICU.0b013e32835c8aba (2013).
26. Zhou, L. *et al.* In-depth analysis of the human tear proteome. *J. Proteomics.***75**, 3877–3885 https://doi.org/10.1016/j.jprot.2012.04.053 (2012).
27. Tiffany, J. M. The normal tear film. *Dev. Ophthalmol.***41**, 1–20 https://doi.org/10.1159/000131066 (2008).
28. Romanovskaya, I. I., Dekina, S. S., Sotnikova, E. P. & Abramova, A. B. Preparation and properties of lysozyme-containing eye drops for tear-substitutive therapy. *Pharm. Chem. J.***53**, 755–758 https://doi.org/10.1007/s11094-019-02074-0 (2019).
29. deLuise, V. P. & Tabbara, K. F. Quantitation of tear lysozyme levels in dry-eye disorders. *Arch. Ophthalmol.***101**, 634–635 https://doi.org/10.1001/archopht.1983.01040010634022 (1983).
30. Mihaela, M. C. *et al. Spin probe method of electron paramagnetic resonance spectroscopy-a qualitative test for measuring the evolution of dry eye syndrome under treatment.Anal. Methods.11,965–972*; 10.1039/c8ay02783h (2019).
31. Kallarackal, G. U. *et al.* A comparative study to assess the clinical use of fluorescein meniscus time (FMT) with tear break up time (TBUT) and schirmer's tests (ST) in the diagnosis of dry eyes. *Eye.***16**, 594–600 https://doi.org/10.1038/sj.eye.6700177 (2002).
32. Masmali, A. M., Purslow, C. & Murphy, P. J. The tear ferning test: a simple clinical technique to evaluate the ocular tear film. *Clin. Exp. Optom.***97**, 399–406 https://doi.org/10.1111/cxo.12160 (2014).
33. Masmali, A. M. *et al.* Application of a new grading scale for tear ferning in non-dry eye and dry eye subjects. *Cont. Lens. Anterior. Eye.***38**, 39–43 https://doi.org/10.1016/j.clae.2014.09.007 (2015).
34. Peterson, R. C., Wolffsohn, J. S. & Fowler, C. W. Optimization of anterior eye fluorescein viewing. *Am. J. Ophthalmo.***142**, 572–575 https://doi.org/10.1016/j.ajo.2006.04.062 (2006).
35. Whitcher, J. P. *et al.* A simplified quantitative method for assessing keratoconjunctivitis sicca from the sjogren's syndrome international registry. *Am. J. Ophthalmol.***149**, 405–415 https://doi.org/10.1016/j.ajo.2009.09.013 (2010).
36. *Lysozyme [Homo sapiens]. National Center for Biotechnology Information*. https://www.ncbi.nlm.nih.gov/protein/1335210*(* 2021*).*
37. Li, N. *et al.* Establishment of the mild, moderate and severe dry eye models using three methods in rabbits. *BMC. Ophthalmol.***13**, 50 https://doi.org/10.1186/1471-2415-13-50 (2013).
38. Honkanen, R. A., Huang, L., Huang, W. & Rigas, B. Establishment of a severe dry eye model using complete dacryoadenectomy in rabbits. *J. Vis. Exp.***155**, 1–12 https://doi.org/10.3791/60126 (2020).

39. Bucolo, C. *et al.* Antioxidant and osmoprotecting activity of taurine in dry eye models. *J. Ocul. Pharmacol. Ther.***34**, 188–194 https://doi.org/10.1089/jop.2017.0008 (2018).
40. Ziniauskaite, A. *et al.* Efficacy of trabodenoson in a mouse keratoconjunctivitis sicca (KCS) model for dry-eye syndrome. *Invest. Ophthalmol. Vis. Sci.***59**, 3088–3093 https://doi.org/10.1167/iovs.18-24432 (2018).
41. Conrady, C. D., Joos, Z. P. & Patel, B. C. Review: the lacrimal gland and its role in dry eye. *J. Ophthalmo.***4**, 1–11 https://doi.org/10.1155/2016/7542929 (2016).
42. Shiue, M. H. *et al.* Pharmacological modulation of fluid secretion in the pigmented rabbit conjunctiva. *Life. Sci.***66**, 105–111 https://doi.org/10.1016/s0024-3205(99)00638-4 (2000).
43. Li, Y. *et al.* Rabbit conjunctival epithelium transports fluid, and P2Y2(2) receptor agonists stimulate Cl(-) and fluid secretion. *Am. J. Physiol. Cell. Physiol.***281**, C595–602 https://doi.org/10.1152/ajpcell.2001.281.2.C595 (2001).
44. Dartt, D. A. Regulation of mucin and fluid secretion by conjunctival epithelial cells. *Prog. Retin. Eye. Res.***21**, 555–576 10.1016/s1350-9462(02)00038 – 1 (2002).
45. Marsh, P. & Pflugfelder, S. C. Topical nonpreserved methylprednisolone therapy for keratoconjunctivitis sicca in Sjogren syndrome. *Ophthalmology.***106**, 811–816 https://doi.org/10.1016/S0161-6420(99)90171-9 (1999).
46. Matsuda, S. & Koyasu, S. Mechanisms of action of cyclosporine. *Immunopharmacology.***47**, 119–125 https://doi.org/10.1016/s0162-3109(00)00192-2 (2000).
47. Donnenfeld, E. & Pflugfelder, S. C. Topical ophthalmic cyclosporine: pharmacology and clinical uses. *Surv. Ophthalmol.***54**, 321–338 https://doi.org/10.1016/j.survophthal.2009.02.002 (2009).
48. Barbarino, J. M., Staatz, C. E., Venkataramanan, R., Klein, T. E. & Altman, R. B. PharmGKB summary: cyclosporine and tacrolimus pathways. *Pharmacogenet. Genomics.***23**, 563–585 https://doi.org/10.1097/FPC.0b013e328364db84 (2013).
49. Leonardi, A. *et al.* Efficacy and safety of 0.1% cyclosporine A cationic emulsion in the treatment of severe dry eye disease: a multicenter randomized trial. *Eur. J. Ophthalmol.***26**, 287–296 https://doi.org/10.5301/ejo.5000779 (2016).
50. Moscovici, B. K. *et al.* Treatment of sjogren's syndrome dry eye using 0.03% tacrolimus eye drop: prospective double-blind randomized study. *Cont. Lens. Anterior. Eye.***38**, 373–378 https://doi.org/10.1016/j.clae.2015.04.004 (2015).
51. Tagashira, A., Nishi, K., Matsumoto, S. & Sugahara, T. Anti-inflammatory effect of lysozyme from hen egg white on mouse peritoneal macrophages. *Cytotechnology.***70**, 929–938 https://doi.org/10.1007/s10616-017-0184-2 (2018).
52. Inoue, M. & Katakami, C. The effect of hyaluronic acid on corneal epithelial cell proliferation. *Invest. Ophthalmol. Vis. Sci.***34**, 2313–2315 (1993).

Figures

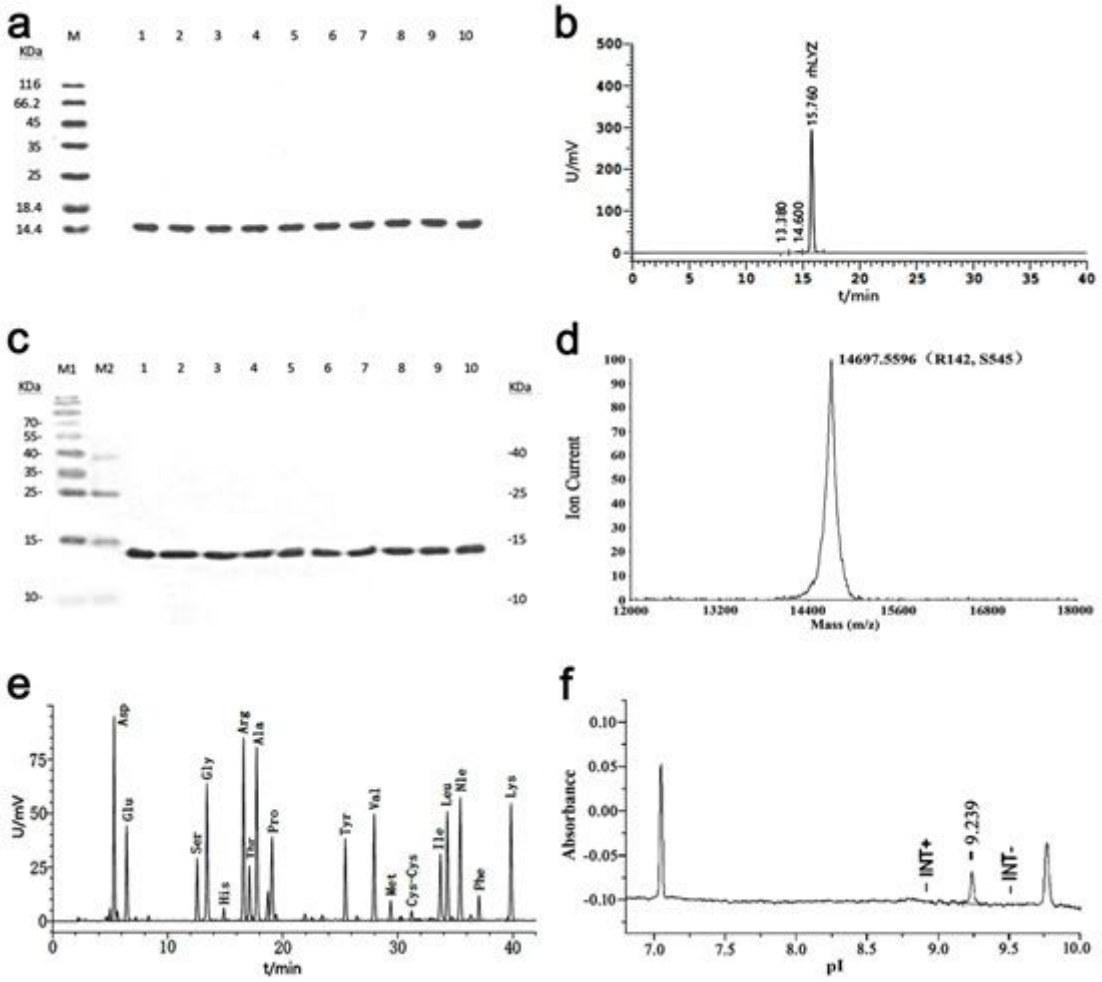

Figure 1

Quality test of raw rhLYZ. (a) Purity test of rhLYZ via SDS-PAGE (M:Marker; 1-10: rhLYZ). (b) Purity test of RhLYZ by RP-HPLC. (c) Specific expression analysis of rhLYZ via western blot (M1: marker 1; M2: marker 2; 1-10: rhLYZ). (d) Molecular weight identification of rhLYZ by MALDI-TOF-MS detection. (e) Amino acid composition analysis of rhLYZ by PITC. (f) Isoelectric point detection of rhLYZ via CIFE.

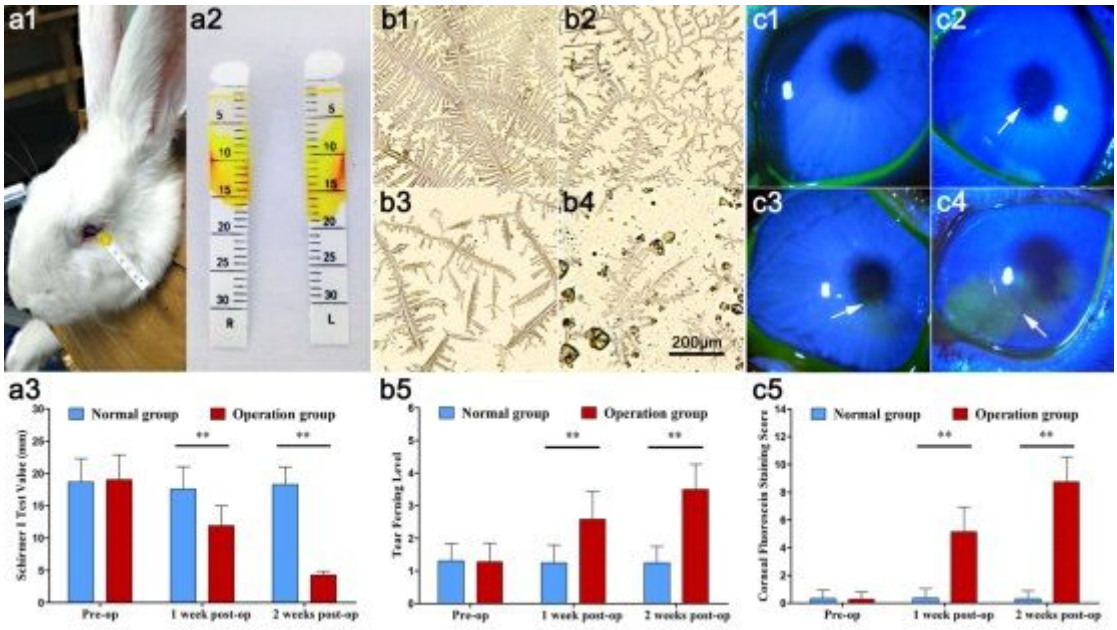

Figure 2

The establishment of dry eye model. (a1) The schirmer I test. (a2) The length of the schirmer tear test strips soaked by tears. (a3) The schirmer I test result in pre-op and 1, 2 weeks post-op (mm/5min). (b1-b4) The four levels of tear ferning test (scale bar: 200μm). (b1) Level Ⅰ. (b2) Level Ⅱ. (b3) Level Ⅲ. (b4) Level Ⅳ. (b5) The tear ferning level in pre-op and 1, 2 weeks post-op. (c1-c4) The corneal fluorescein staining test. (c1) Normal corneal with no staining each quadrant. (c2) Corneal of 1~30 spot staining each quadrant. (c3) Corneal of >30 spot staining but no staining fusion each quadrant. (c4) Corneal of spot staining fusion filaments and ulcers each quadrant. (c5) The score of corneal fluorescein staining in pre-op and 1, 2 weeks post-op . *p <0.05, **p <0.01.

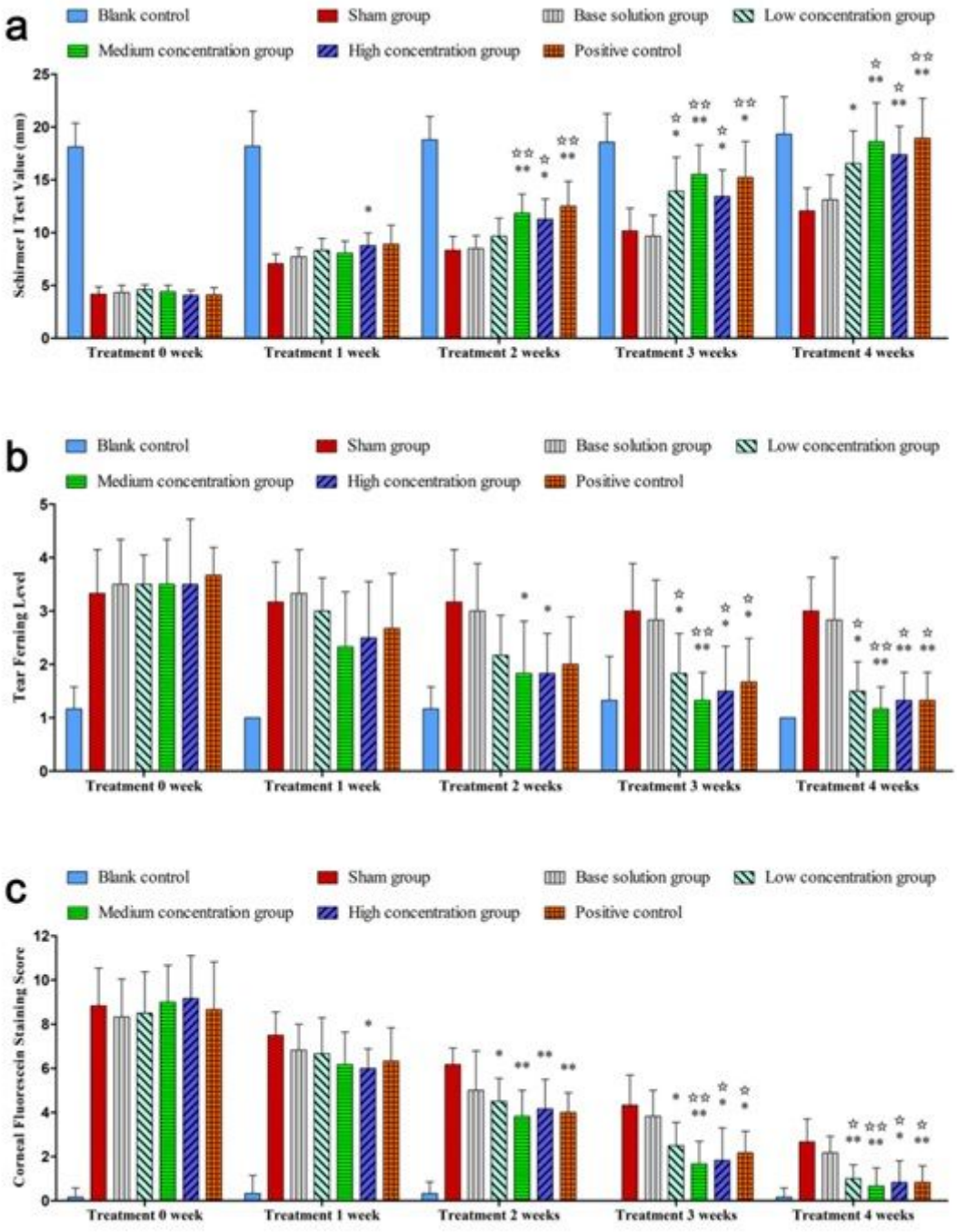

Figure 3

The results of eye drops potency test. (a) The results of schirmer I test. (b) The data of tear ferning level. (c) The corneal fluorescein staining score. Compared with the sham group, *p <0.05, **p <0.01; Compared with the base solution group, ▯p <0.05, ▯▯p <0.01.

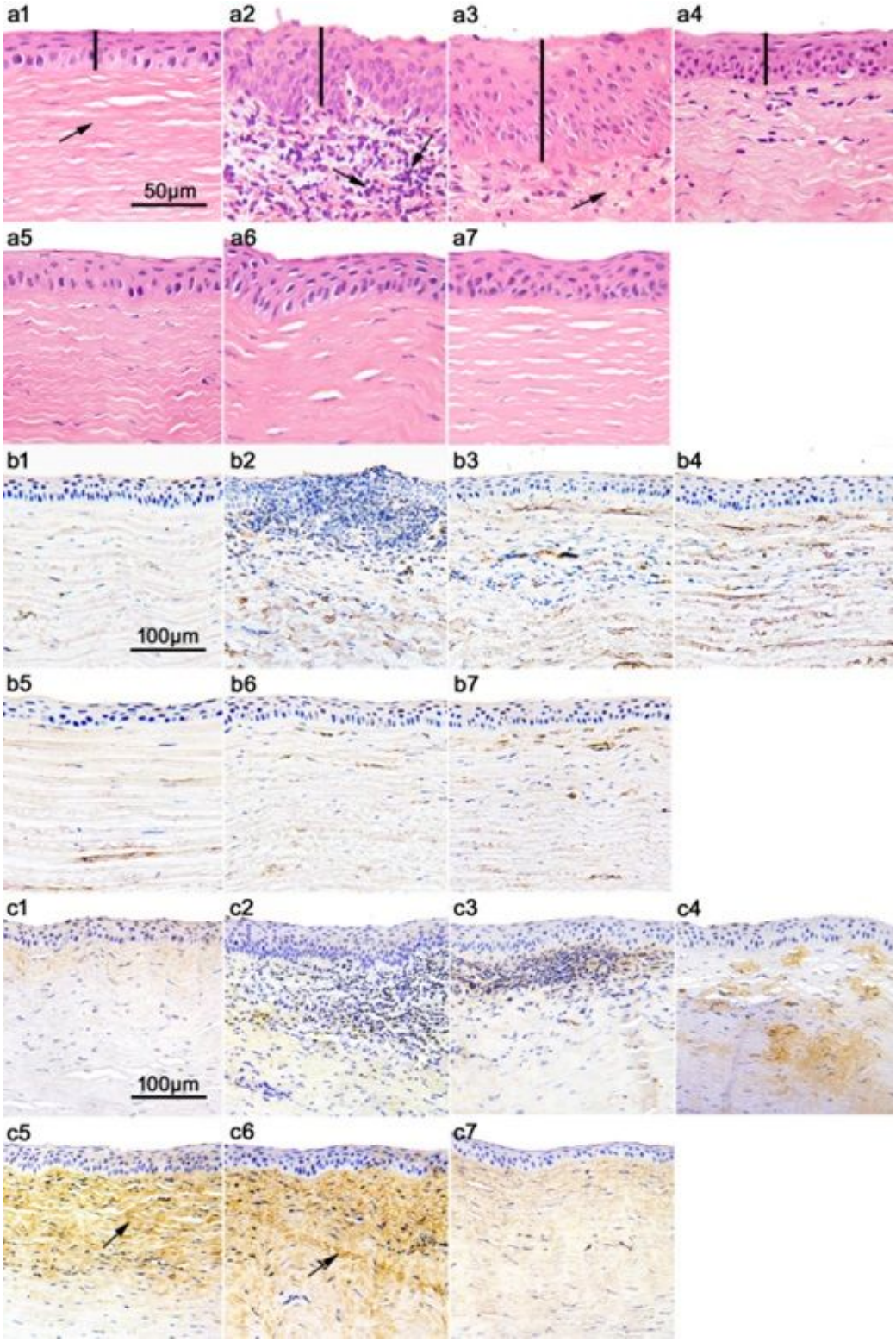

Figure 4

The histopathological evaluation of corneal epithelial. (a) H&E staining of corneal epithelial. Scale bar: 50μm. (b) Immunohistochemical detection of TNF-α in cornea. Scale bar: 100μm. (c) Immunohistochemical detection of TGF-β1in cornea. Scale bar: 100μm. (a1,b1,c1) Blank control. (a2,b2,c2) Sham group. (a3,b3,c3) Base solution group. (a4,b4,c4) Low concentration group. (a5,b5,c5) Medium concentration group. (a6,b6,c6) High concentration group. (a7,b7,c7) Positive control.

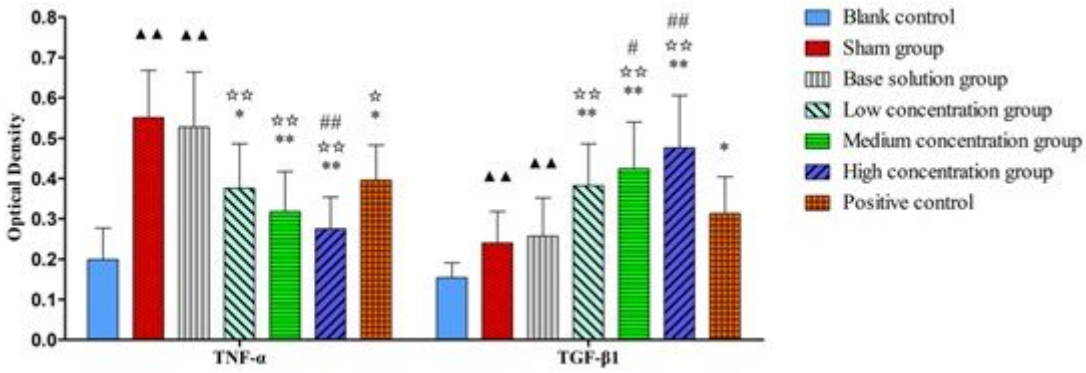

Figure 5

The quantitative assessment of the expression of TNF-α and TGF-β1 via immunohistochemical detection of cornea. Compared with the sham group, *p <0.05, **p <0.01; Compared with the base solution group, ☆p <0.05, ☆☆p <0.01; Compared with the positive control, #p <0.05, ##p <0.01; Compared with the blank control, ▲p <0.05, ▲▲p <0.01.