The Protective Effect of Aesculin Eye Drops on Dry Eye Syndrome Mice

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Abstract

Background: The objective of this study was to test the effectiveness of the novel aesculin (AES) eye drops on treating dry eye syndrome (DES), using murine model.

Methods: Dry eye was induced in murine eye with the use of an intelligent controlled environmental system (ICES). High-flow air which had been desiccated within the ICES, induced the DES. Treatment included Aesculin eye drops for 14 days. After expiration, corneal fluorescein staining was examined and stained with an endothelial marker created for tracking of lymph: Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1). Subsequently, real-time PCR and ELISA were completed to quantify secretion of TNF- α , IFN- γ , IL-1 β and IL-8 in serum, ocular surface and lacrimal gland.

Results: Aesculin eye drops significantly repaired ICES-induced corneal injury in murine models. Statistically significant increase in lymph angiogenesis was observed at day 14 of observation. Consequently, DES in treatment group was reduced using aesculin eye drops. aesculin eye drops can significantly inhibit lymphangiogenesis and reduce inflammatory cytokines levels in mice. The percentage of Th1 and Th17 cells were decreased after treating with aesculin eye drops, while the percentage of Treg was increased. Injection of VEGF-C into mice can reduce the efficacy of aesculin eye drops in ICES-induced DES.

Conclusion: as treatment group of murine models indicated significant reduction of inflammation in the cornea associated with DES, treatment of DES with aesculin eye drops, should be considered a viable treatment method for DES.

Background

Keratoconjunctivitis sicca, commonly known as dry eye syndrome (DES), refers to a progressive multifactorial disorder ocular surface, which results in patient discomfort and disruption in visual acuity (McDonald et al., 2016). Dry eye syndrome is a medical condition in which those affected experience chronic lack of moisture within the eye. Although, cases of DES in singular eyes are reported, most patients are infected in both eyes (Pflugfelder and de Paiva, 2017). When patients contract DES, they are likely to present with an array of symptoms.

When individuals are affected by DES, patients' eyes are often easily fatigued, and patients present with redness, lacrimal stringy discharge, and blurred vision (Barabino et al., 2016). Blurred vision is frequently accompanied by pressure behind the eye and sensitivity to light (Tsubota et al., 2017). Additional symptoms of DES include a burning sensation and feelings of sandy irritation, or dust in the eye (McDonald et al., 2016). When DES infection is novel, symptoms are generally mild. Mild symptomology is often easily resolved, with little or no long-term effects (Yamanishi et al., 2019). However, if DES advances untreated, patients are likely to experience increased damage of the eye, including scarring on the cornea, resulting in permanent impairment to vision and in rare cases, loss of vision (Song et al., 2018).

Dry eye syndrome occurs for three main reasons, inadequate tear production, rapid tear evaporation or inadequate blinking reflexes (Tsubota et al., 2017). Underperformance, or overperformance, of blinking reflex can create a variety of issues for persons with DES (Tsubota et al., 2017). Underperformance of the blink reflex is often caused by overuse of the eye (Song et al., 2018). Typically, individuals that have occupations that require prolonged focus of the eyes are prone to DES (Lambert, 2018). Conversely, when eyes are irritated tears are created at an elevated rate, creating an overabundance of tears (Lambert, 2018). Although irritated eyes create an excess of tears, increased tear production counterintuitively facilitates drier eyes. Dry eye syndrome is accelerated with persistent tearing, as reflexive tears of this nature does not have the lubricating quality of tears created when irritated (Tsubota et al., 2017). Environmental factors also impact the prevalence of DES (Lambert, 2018). When individuals reside in environments that boast elevated wind, dust or smoke, DES is more apt to occur (Tsubota et al., 2017). Moreover, environments that are higher in altitude, lower in humidity or the climate is artificially regulated are also more prone to exacerbate symptoms associated with inadequate tear production (Lambert, 2018).

Inadequate tear production occurs primarily from lacrimal hyposecretion, while rapid evaporation of tears does not allow lacrimal secretion to coat the eye properly. In both cases, the aqueous tear layer (ATL) is impacted. The ATL is a discrete layer that, along with two other layers, comprise the tear film. The tear film is amalgamation of three discrete layers the mucin layer, lipid layer. and aqueous layer (Sridhar, 2018). The most proximal layer of the tear film is the mucin layer, which abuts the cornea (Werkmeister, 2017). The primary function of the mucin layer is to nourish the cornea, and to aid in cornea functioning. The mucin layer of the tear film also allows the tears to slide evenly over the ocular surface and allows for even distribution of tears (Werkmeister, 2017). The mucin layer of the tear film also allows the tears to slide evenly over the ocular surface and allows for even distribution of tears (Sridhar, 2018). The middle layer of the tear film is the aqueous layer (Rolando and Zierhut, 2001). The aqueous tear layer lubricates the eye and allows the clearing of particles and prevent infection (Sridhar, 2018). Finally, the most distal layer of the tear film is lipid layer (Cwiklik, 2016). The lipid layer allows for the sealing of the tear film, which reduces evaporation and ensure the eye remains hydrated (Sridhar, 2018).

The positioning of the film layers is uniform, however the composition of the three layers of the tear films are unique amongst individuals (Bai et al., 2019). Thickness of mucin, aqueous, and lipid layers are dependent on environment, diet and biological factors, creating fluctuations in ocular tear film efficacy. Thickness of mucin, aqueous, and lipid layers are dependent on environment, diet and biological factors, creating fluctuations in ocular tear film efficacy (King-Smith et al., 2000). The thickness of the human precorneal tear film: evidence from reflection (Rolando and Zierhut, 2001). Other risk factors for dry eye syndrome. In addition to tear film thickness, and proper blink response, other factors likely contribute to the presentation of DES (Lambert, 2018). Other risk factors for dry eye syndrome. In addition to tear film thickness, and proper blink response, other factors likely contribute to the presentation of DES (Song et al., 2018). Menopausal women are most at risk for contracting DES, provided environmental (Song et al., 2018).

In addition to environmental factors, DES is also associated with ocular surface inflammation caused by immune response (Stern and Pflugfelder, 2004; Stern et al., 2013). Until relatively recently, the eye was believed to lack all lymphatic vessels, except for the conjunctiva (Schroedl et al., 2014). Advancements in ophthalmic instruments allowed for the classification of a lymphatic network using endothelial markers especially Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), as well as lymphangiogenic factors such as vascular endothelial growth factor c (VEGF-C). Even more recent studies have established that the lacrimal gland, orbital meninges, extraocular muscles and the corneal limbus all are connect to the lymphatic system (Nakao et al., 2012). Although little is known about lymphatic fluid of the ocular region, research has established that lymphatic pathway might contribute to overall ocular fluid homeostasis.

The identification of the lymphatic pathway in fluid homeostasis generated data that indicated that lymphangiogenesis influences a variety of pathological conditions within the eye. Lymphangiogenesis has been associated with corneal transplant complication and subsequent corneal rejection, ocular tumor growth and various ocular infection (Cursiefen et al., 2003). The role of lymphangiogenesis in DES remains unknown. As such, studies on corneal lymphangiogenesis using a murine model could create better understanding of the role of the lymphangiogenesis in ocular inflammation and DES.

A better understanding of lymphatic and lymphangiogenesis in the eye will open new therapeutic opportunities to prevent vision loss in ocular diseases. Previous research suggests that T-cells are present within both murine and human models of DES, especially within proximal lymph nodes. The acquisition of specific chemokine markers near the ocular regions allow for the congregation of T-cells into the inflamed and irritated ocular surfaces, commonly found in mice with DES in laboratory studies. Autoimmunity was also demonstrated within the murine model within the context of DES when lymphatic vessels were drained (Chauhan et al., 2009). Aesculin (Figure. 1A), a natural product from the traditional and widely-used Chinese medicine named Qinpi, *Fraxinus rhynchophylla*. Previous studies found that aesculin have effectiveness in anti-inflammation and analgesia. Aesculin eye drops have already been put to clinical use for curing conjunctivitis from last century. Within this study, we attempt to determine the growth of lymphatic vessels in the cornea with the presentation and progression of DES. The expression levels of regulatory T cells (Tregs) and T helper cells Th1 and Th17 were also investigated after aesculin eye drops treatment. We pleasantly found that aesculin eye drops might have significant effectiveness on treating DES.

Methods And Materials

Animals for Murine Modeling

This animal experiment was approved by the Animal Care and Ethics Committee of Shanghai University of Traditional Chinese Medicine. All care for murine models adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research to reinforce ethical behavior and care. Six female mice (C57BL) aged 4–6 weeks were used within the study.

The Intelligently Controlled Environmental System

The intelligent controlled environmental system (ICES), included one box. The box utilized for this study was a dry and sealed box (500L capacity) with an adjustable temperature humidifier, as well as an intelligent humidifier. Dual humidifiers were used to create a two-step process to reduce moisture within study environment.

Industrial temperature humidifier (ZD-890c, Ouvi Company, Hangzhou, China) was used first to lower humidity 40% – 50%. Once humidity was reduced within the sealed box, an intelligent dehumidifying device will be used. The intelligent dehumidifying device (MS, 13X; Hengye Company, Shanghai, China) was used to lower humidity within study environment further. The MS humidifier was selected as it promotes a new environmentally friendly material, referred to molecule filter. Molecular filter is toxic free and allows for the active control of moisture within the ICE.

The last component of the ICE is a noise free ventilator (LFA-40, 539; Jiaxin Company, Wenzhou, China). Ventilator was placed 20 cm from subjects (speed 0-5m/s). Noise free ventilator was chosen to allow for ambient noise to reflect the same level of the noise within the ICE. With the noise free ventilator and two humidifiers, researchers were able to control temperature, humidity and din within the ICE.

Finally, an anemometer used to measure air flow, (AR816; Haoxin Company, Shanghai, China) was used to stabilize airflow between 0 to 30 m/s. The anemometer had was selected as it boasted measures within 5% accuracy. Thus, the humidity and temperature in the ICES were monitored and subsequently displayed by a control system found within the intelligent dehumidifying device.

All mice were placed into one of three groups which were randomly assigned: control, environmental control and treatment group. The control group was housed in RH 60–80%, 21–23 degrees Celsius. Mice within the experimental groups are sealed within container in noise, humidity and temperature controls.

Dry Eye Model Testing

Aesculin (purity > 98.5%) was purchased from Meilune (Dalian, China). Distilled water was added to the powder to create the eye drop used in the experiment. The eyes of mice were treated twice a day with 80 µg of aesculin (8 µL of 10 g/L solution) or vehicle (saline). Daily treatment with aesculin started 2 days before ICES and continued up until the time animals were euthanized and cornea tissue was collected for testing. All mice were humanely slain with a lethal overdose of ketamine and xylazine. Area of the superior conjunctivas were marked by researchers with black ink after the animals expired. Each eye was fixed in 10% formalin for dehydration.

Taking out the right eyeball after death, remove cornea at 2 mm and placed in 10% in formaldehyde. Tear production in murine model was measured in 6 eyes using the phenol red thread test. Testing lasted 60 seconds per eye. Phenol red threads were held by researchers using one pair of forceps throughout the entirety of testing. Forceps were applied to the lateral cantus of the conjunctival fornix 6 right eyes for 60

seconds under slit lamp biomicroscopy. The length of the wet cotton thread was measured using a millimeter scale and recorded.

Corneal Fluorescein Staining

Corneal Fluorescein (CF) Staining was used to measure new lymph growth on the ocular surface. Corneal Fluorescein staining was evaluated in 6 right eyes by injecting 0.5 L of five percent fluorescein solution into the inferior conjunctival sac using a sterilized micropipette. The cornea of each eye was examined utilizing slit-lamp microscopy under cobalt blue light 10 minutes subsequent after fluorescein instillation. The corneal surface was then split into five distinct regions and then graded on a system from 0 to 3. New lymph growth electronic microscope observation: sterile condition, put sample in 2.5% Glutaraldehyde Fixative Solution for 2 h. 1% Potassium osmate $H_2[OsO_4(OH)_2]$ solution for 2 h dehydrate, ultra-thin slice. Uranyl acetate and citrate trihydrate double dye. Then observation is done.

Immunofluorescence

Frozen sections were briefly blocked by goat serum for 60 minutes. When blocking the specimen, prepare the primary antibody in the antibody dilution buffer according to the dilution ratio recommended in the data sheet (LYVE-1: ab10278, 1:100; VEGFC: ab83905, 1:100; VEGFR-3: ab10284, 1:100, Abcam Inc.). Aspirate the blocking buffer and add the diluted primary antibody. Incubate at 4 ° C overnight. Rinse three times with 1X PBS for 5 minutes each. After diluting the fluorescently labeled secondary antibody with antibody dilution buffer, incubate the specimen in the dark at room temperature for 1–2 hours. Rinse three times with 1X PBS for 5 minutes each. Use Prolong® Gold Antifade Reagent with DAPI (CST, 8961) and cover the section with a coverslip. Sections were imaged with a confocal microscope.

RNA isolation and quantitative real-time PCR (qRT-PCR)

The RNA from the tissue was isolated using TRIzol reagent (Invitrogen) according to manufacturer's instructions and reverse-transcribed using an miScript Reverse Transcription Kit (QIAGEN). The qRT-PCR was performed using a SYBR Premium Ex Taq II kit (TaKaRa) on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). All reactions were performed in triplicate and the mean value was used to calculate the relative level of expression after normalization against β -actin.

Corneal epithelial OGD staining

Corneal epithelial staining with Oregon Green dextran (OGD; 70,000 MW; Invitrogen Inc., Grand Island, NY) was assessed in the 5 different groups. Briefly, 0.5 μ l of 50 μ g/ml OGD was instilled in the ocular surface 1 min before euthanasia. Corneas were rinsed with 2 ml PBS and photographed with a stereoscopic zoom microscope (V20; Zeiss with kryptonargon and He-Ne laser; Carl Zeiss Meditec, Ltd., Thornwood, NY) under fluorescence excitation at 470 nm. Images were obtained 2 h after instillation of the last treatment drop and were processed. The fluorescein solution contained 1 mg fluorescein sodium in 0.5 ml PBS. The severity of corneal OGD staining shown in digital images was graded by two masked observers, using the Baylor grading scheme for corneal fluorescent staining. The number of fluorescein staining dots were graded in the 1-mm central cornea zone of each eye, on a standardized five-point scale (0 dot,

grade 0; 1–5 dots, grade 1; 6–15 dots, grade 2; 16–30 dots, grade 3; 30 dots, grade 4). One point was added to the score if there was one area of confluent staining, and two points were added for two or more areas of confluence.

Statistical analysis

Data are expressed as the mean $\pm$ standard error of the mean (SEM). Significant differences were determined by One-Way ANOVAs with LSD post hoc tests; and $P < 0.05$ is considered to be statistically significant.

Results

Aesculin eye drops significantly repaired ICES-induced corneal injury in murine models

ICES were used for induce DES. Corneal fluorescein staining was applied for assessing the corneal integrity. It is commonly accepted that fluorescein staining represents compromised corneal epithelium (Dundas et al., 2001). The mean corneal staining results of mice in the NC, ICES1W, ICES2W, Aesculin eye drops and vehicle groups were quantified and compared. Results showed that ICES time-dependently led to higher corneal staining score (Figure. 1B). Aesculin eye drops topical treatment significantly reduced corneal staining score compared to ICES groups and control groups (Figure. 1A). To further confirm the corneal staining results, corneal epithelial staining with OGD was then carried out to detect the injury levels in 5 groups. Similar results were obtained after OGD staining, the specific ICES environment directly increased corneal damage while Aesculin eye drops treatment noticeably inhibit the level of corneal injury (Figure. 1C). These results suggested that Aesculin eye drops have remarkable recovery effects on curing corneal injury in ICES-induced DES murine models.

Aesculin eye drops inhibited lymphangiogenesis caused by ICES in mice

It has been revealed that corneal lymphangiogenesis played an important part in DES (Goyal et al., 2010; Ji et al., 2018). Lymphatic growth within the vascularized vessels is illustrated by facilitation and promotion of blood vessels. Corneal were stained with lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), vascular endothelial growth factor c (VEGF-C) and its receptor VEGFR-3 for indicating lymphangiogenesis of murine model. It is assumed lymphatic vessels are facilitated from the limbal vascular arcade proximal to the cornea, especially during early time point measurements (day 0, day 1, and day 7) in all three groups, which was shown to increase throughout the trial. Advanced growth was observed more typically towards the median of the murine cornea, especially with DES progression.

Immunofluorescence results of the frozen sections from all 5 groups showed that the expression of LYVE-1 significantly increased and continued to progress exponentially until day 14 (Figures. 2A and B). With Aesculin eye drops treatment, LYVE-1 was reduced remarkably compared to the ICES group (Figures. 2A

and B). Development of lymphatic vessels is regulated by factors common to lymphangiogenesis. Significant overexpression of VEGF-C and VEGFR-3 was observed in ICES group by day 14 (Figures. 2C - F). Topical Aesculin eye drops treatment reduced the mean fluorescence intensity (Figures. 2C - F). These results combinedly indicated that ICES-induced DES promoted the growth of lymphangiogenesis while Aesculin eye drops can inhibit the promoting effects.

Aesculin eye drops topical treatment reduced the expression of inflammatory factors

To further detect the ocular and systemic inflammation, samples from serum, ocular surface and lacrimal gland were collected and investigated. The detect objectives were TNF- α , IFN- γ , IL-1 β and IL-8, which had been regarded as cell signaling cytokines involved in systemic inflammation response (Chung and Benveniste, 1990; Doherty et al., 1992). ELISA results revealed that all 4 factors increased significantly in ICES groups, and Aesculin eye drops treatment reversed the expression to levels similar with NC groups while vehicle treatment had no exceptional influence (Fig. 3A). In addition, RT-PCR results showed that the gene expression of TNF- α , IFN- γ , IL-1 β and IL-8 saw a rapid rise in ICES groups by day 14, both in ocular surface and in lacrimal gland (Figures. 3B and C). However, treatment with Aesculin eye drops significantly inhibited the overexpression of all inflammatory factors, especially IL-1 β (Figures. 3B and C). It has been reported in many researches that the core mechanism of DES is inflammation (Hessen et al., 2014; Stern and Pflugfelder, 2004; Wei et al., 2014). These results suggested that Aesculin eye drops topical treatment significantly inhibited ICES-induced inflammation, which implied that Aesculin eye drops might have great effectiveness in controlling DES-induced ocular inflammation.

Cellular compositional changes of draining lymph nodes by dry eye induction in ICES mice

The immune response to foreign antigens requires a perfect coordination between sensor and effector cells. CD4 + T cells, also known as Th cells, play a central role in immune protection (Pflugfelder et al., 2013). IFN- γ production in T cells induce CD4 + Th precursor cells to differentiate into Th1 cells (Stern et al., 2005). Th1 cells secrete IFN- γ that activates macrophages that functioning eradicating intracellular microorganisms, such as mycobacteria (Pflugfelder et al., 2013). Th17 cells secrete IL-17, which induces production of proinflammatory molecules (Bettelli et al., 2006).

Flow cytometry analysis of the Th1-type cytokines IFN- γ in CD4 + T cells revealed that the percentage of cells expressing IFN- γ increased rapidly in ICES groups, from 5.38 to 15.1 (Fig. 4A). A dramatic reduction in the proportion of IFN- γ -expressed Th cells starting from 15.1 to 7.99 in Aesculin group was detectable (Fig. 4A). Analysis of IL-17A-expressed CD4 + cells presented results similar with IFN- γ groups. The proportion of Th cells expressing IL-17A saw a rapid rise after ICES day 14 (from 0.21 to 2.6) while Aesculin eye drops treatment significantly suppressed the expression (Fig. 4B). These results indicated that Th1 cells and Th17 cells increased in DES condition and Aesculin eye drops had great inhibitory effects on these cells.

It has been revealed that the resistance of Th17 to Treg suppression played an important role in the autoimmune mechanisms of DES (Chauhan et al., 2009). We analyzed FOXP3-expressing CD25 cells to determine the proportion of Treg cells. Flow cytometry showed that ICES effectively suppressed the level of Treg cells from 4.85 to 1.39 (Fig. 4C). In Aesculin group, the percentage of Treg cells increased from 1.39 to 3.45 (Fig. 4C). These results suggested that ICES-induced dry eye gave a rise to the proportion of Th1 cells and Th17 cells while inhibited the level of Treg cells. Additionally, Aesculin eye drops treatment significantly suppressed the effects of ICES on all 3 kinds of T cells.

Aesculin eye drops treatment suppressed inflammation, Th1 and Th17 cells via inhibiting lymphangiogenesis in ICES-induce dry eye mice

To determine whether the therapeutic effects of Aesculin eye drops was related with its ability to suppress lymphangiogenesis, the mice were injected with VEGF-C through tail vein. The serum samples were collected and used for ELISA. Results showed that even though Aesculin eye drops treatment inhibited the expression of inflammatory factors TNF- α , IFN- γ , IL-1 β and IL-8, injection with VEGF-C totally reversed the inhibitory effects and gave a rise to the factors (Figure. 5A).

As for the cellular compositional changes draining lymph nodes, flow cytometry analysis confirmed that the percentage of Th1 and Th17 cells rose rapidly in ICES group and fell dramatically after Aesculin eye drops treatment (Figures. 5A and B). VEGF-C injection wiped out the inhibitory effects of Aesculin eye drops on Th1 and Th17 cells (Figures. 5A and B). A similar result was obtained from Treg groups: Aesculin eye drops treatment gave a rise to the decreased percentage of Treg cells in mice (from 1.54 to 3.56), while VEGF-C reversed the promoting effect. These results indicated that the therapeutic effects of Aesculin eye drops on suppressing inflammation might come from its function in inhibiting lymphangiogenesis.

Discussion

Previous studies have shown that DES was associated with lymphangiogenesis, inflammation and changes in T cell composition. Lymphatic vessels which indicated the presence of DES were present in the cornea of both the environmentally controlled and experimental murine models. Statistically significant increase in lymphangiogenesis was observed ($p < 0.001$) at 14 days of observation. DES was observed in 4 in control group, 4 in environmental control group. Dry eye syndrome was also observed in 4 subjects in treatment group with Aesculin eye drops. Consequently, DES in treatment group was reduced ($p < 0.001$) through the use of Aesculin eye drops. Study was completed on murine model in an ICES which was controlled for temperature, humidity, airflow and noise. These control variables were selected as DES is commonly associated with higher temperature, reduced humidity and increased airflow (Barabino et al., 2016). Additionally, ambient noise was kept at a minimum not to stress the mice.

The use of the ICES allows for maintenance of environments, and the experimental treatment. Moreover, use of ICES allowed for the establishment of DES in both environmental control and experimental groups. Mice within the ICES environment in both the experimental and environmental control group experienced

reduced tear loss and development of DES within approximately 14 days when compared to control group. Dry eye syndrome increased in symptomology throughout the course of experiment.

Mechanisms involved in DES in the present study is not exhaustive of DES in other models. However, DES symptomology including inflammation and cellular compositional changes were reduced within experimental group. Treatment with Aesculin eye drops was associated with reduced defects in ocular surface. We firstly detected the potentially role of lymphangiogenesis signaling pathway in cellular compositional changes of T cells. Moreover, Aesculin eye drops treatment allowed for mitigation of hyperproliferation in conjunctival epithelium.

Conclusion

Aesculin eye drops should be considered for use within future studies on other models, including in vivo trials and is a promising novel treatment for DES.

Abbreviations

AES aesculin

DES dry eye syndrome

ICES intelligent controlled environmental system

LYVE-1 lymphatic vessel endothelial hyaluronan receptor 1

VEGF-C vascular endothelial growth factor c

CF Corneal Fluorescein

OGD Oregon Green dextran

Declarations

- **Ethics approval and consent to participate**

This animal experiment was approved by the Animal Care and Ethics Committee of Shanghai University of Traditional Chinese Medicine. All care for murine models adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research to reinforce ethical behavior and care.

- **Consent to publish**

Not applicable.

- **Availability of data and materials**

Please contact corresponding authors for data requests.

- **Competing interests**

The authors declare that they have no competing interests.

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- **Authors' Contributions**

Xinquan Liu, Jing Su and Yue Fang: performed the research. Dan Jiang and Zhiguo Dong: designed the research study. Yue Fang and Dahu Wang: contributed essential reagents or tools. Xinquan Liu and Jing Su: analysed the data. Xinquan Liu and Jing Su: wrote the paper.

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Figures

Figure 1

Aesculin eye drops significantly repaired ICES-induced corneal injury in murine models. Corneal Fluorescein (CF) Staining was used to measure new lymph growth on the ocular surface. A. Structural formula of Aesculin. The molecular formula and weight of API is C₁₅H₁₈O₁₀ and 358.297 g/mol. B. CF staining results of murine eyes from control, ICES1W, ICES2W, ICES2W+Aesculin and ICES2W+vehicle

groups and the quantified results. C. CF staining with OGD of murine eyes from all 5 groups and the quantified results. Data are presented as the mean $\pm$ SD. $P < 0.001$ versus control group.

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Figure 2

Aesculin eye drops inhibited lymphangiogenesis caused by ICES in mice Immunofluorescence assay were applied for the detection of LYVE-1, VEGF-C and VEGFR-3 in all 5 groups. A. Immunofluorescence results of LYVE-1 in control, ICES1W, ICES2W, ICES2W+Aesculin and ICES2W+vehicle groups. B. Quantified results of LYVE-1 mean fluorescence intensity. C. Immunofluorescence results of VEGF-C in all 5 groups. D. Quantified results of VEGF-C mean fluorescence intensity. E. Immunofluorescence results of VEGFR-3

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Figure 3

Aesculin eye drops topical treatment reduced the expression of inflammatory factors. Samples from serum, ocular surface and lacrimal gland were collected and investigated for the level of TNF- α , IFN- γ , IL-1 β and IL-8. A. ELISA results of TNF- α , IFN- γ , IL-1 β and IL-8 in serum in groups with different treatment. B. RT-PCR results of TNF- α , IFN- γ , IL-1 β and IL-8 expression level on ocular surface in all 5 groups. C. RT-PCR results of TNF- α , IFN- γ , IL-1 β and IL-8 expression level in lacrimal gland in all 5 groups. Data are presented as the mean $\pm$ SD. $P < 0.001$.

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Figure 4

Cellular compositional changes of draining lymph nodes by dry eye induction in ICES mice Th cells play a central role in immune protection. We used flow cytometry for assessment of the proportion of Th1, Th17 and Treg cells. A B. Flow cytometry analysis and quantified results of the CD4+ cells expressing IFN- γ or IL-17A in negative control, ICES2W, ICES2W+Aesculin and vehicle groups, which indicated the percentage of Th1 and Th17 cells, respectively. C. Flow cytometry analysis and quantified results of the CD25 cells expressing FOXP3 in all 4 groups. Data are presented as the mean $\pm$ SD. $P < 0.001$.

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Figure 5

Aesculin eye drops treatment suppressed inflammation, Th1 and Th17 cells via inhibiting lymphangiogenesis in ICES-induce dry eye mice. The mice were injected with VEGF-C to determine whether the therapeutic effects of Aesculin eye drops was related with its ability to suppress lymphangiogenesis. A. The expression of TNF- α , IFN- γ , IL-1 β and IL-8 in serum of mice before and after VEGF-C injection. B C. Flow cytometry analysis and quantified results of the CD4+ cells expressing IFN- γ

or IL-17A in groups with or without VEGF-C injection. D. Flow cytometry analysis and quantified results of the CD25 cells expressing FOXP3 in all 4 groups. Data are presented as the mean $\pm$ SD. $P < 0.001$.

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