Combining Runmu fengye tang preparation with hydroxysugar glycolic acid eye drops regulates Th17/Treg balance to treat dry eyes via inhibition of JAK2/STAT3 pathway

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INTRODUCTION

Dry eye disease (DED) is a multifactorial ocular surface disease characterized by tear film homeostasis, ocular surface inflammatory response and damage, and ocular discomfort [1]. The reported prevalence of DED ranges from 5 to 34 %, with a higher prevalence in women and...
older adults [2,3]. In recent years, with the changes in the living environment and the extensive use of video terminals, the incidence of dry eye disease is increasing year by year and there is a trend of rejuvenation [4].

The DED is often associated with symptoms such as itching, foreign body sensation and burning sensation in both eyes, which reduce the quality of life of affected individuals. The chronic inflammatory state involved in DE lead to vision loss or blindness if not treated promptly with appropriate therapies. However, treatment of DED is difficult and effective treatments are still lacking. Thus, an investigation into effective drugs required for the prevention and treatment of dry eye is a key scientific task that needs to be done, as it will have practical and effective implications on the patients and therapeutics in general. In recent years, the study of immunomodulation has gained increasing attention and researchers have found that regulatory T cells (Treg) and the helper T cell subset 17 cells (Th17) play a very important role in immune-inflammatory diseases [5]. It has been shown that the development of dry eye disease is significantly associated with disturbed Treg/Th17 cell homeostasis and that blocking IL-17 in vivo significantly reduces the severity and progression of the disease by restoring the Treg/Th17 cell ratio [6]. Therefore, protection of the homeostatic balance of Th17/Treg cells may contribute to the treatment of dry eye.

Traditional Chinese medicine may also play an important role in the treatment of dry eye disease. Therefore, the aim of this study was investigate the efficacy of a combined remedy using RMFS and HGA in the treatment of dry eye symptoms in model rabbits.

**EXPERIMENTAL**

**Animals and design**

A total of 30 healthy New Zealand rabbits (2 - 3 months old, males = females = 15, 1.5 kg ± 0.45 kg) were obtained from Key Laboratory of Medical Biotechnology and Translational Medicine, Embryo Engineering Laboratory, Guilin Medical University (Guilin, China). All animal experiments were approved by the Ethics Committee of Affiliated Hospital of Guilin Medical University for the use of animals (Grant no. GXZYC20220296) and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines [7]. All rabbits were housed in a single standard rabbit cage (2 rabbits/cage) at 21 ± 2 °C, 58 ± 9 % relative humidity and 12 h light/dark cycle. Water and standard feed were provided *ad libitum* and they were allowed to acclimatize for 5 days. Prior to the experiments, the animals were carefully examined and found to be free of ocular inflammation or obvious signs of abnormality.

The rabbits were randomly divided into 5 groups, with 6 rabbits in each group. The five groups were control group, model group, model + RMFS group, model + HGA group and model + RMFS + HGA group. Control group did not receive any treatment while the remaining four model groups were treated by subcutaneous injection of 0.2 mg/kg scopolamine (Sigma-Aldrich, St. Louis, MO, USA) four times a day (injected at 8:00, 11:00, 14:00 and 17:00 hours, respectively) for 28 consecutive days to induce dry eye. For the model + RMFS group, 51.85 g/kg of *Runmu Fengliang Tang* (RMFS) was administered by gavage, twice daily for 28 days. In the model + HGA group, Hypromellose 2910, Dextran 70 and Glycerol Eye Drops (HGA, Alcon Laboratories, Inc, TX, USA) was administered into the eyes of the rabbits at 1 to 2 drops (approximately 0.025 - 0.05 mL) 4 times daily, for 28 days. Rabbits in model + RMFS + HGA group were treated with a combination of RMFS and HGA for 28 days. Tear volume (Schirmer’s I test (SIt)) and tear film rupture break-up time (BUT) were measured on day 0, 7, 14, 17, 21 and 28 for each group of rabbits and the experimental animals were euthanized on the 28th day.

**Schirmer’s I test (SIt)**

As previously reported, one end of the Color Bar Schirmer strips (EagleVision, Memphis TN) was folded and placed into the conjunctival sac in the outer 1/3 of the rabbit’s lower eyelid. After 5 minutes, the filter paper was collected and the length of wetting was measured from the point of folding [8].

**Tear break up time (BUT)**

Placing 1 μL of 0.1 % liquid sodium fluorescein into the conjunctival sac caused 3 blinks, which were recorded over several seconds and this was observed under a slit-lamp microscope with cobalt blue light (CKX41, Olympus, Tokyo, Japan) until the first dryness of the tear film spot appeared. The time from eyelid opening to the appearance of the first dry spot was recorded and measured. The experiment was repeated three times and the average calculated.

**Hematoxylin and eosin (H&E)**

The lacrimal glands of rabbits in each group were removed and treated with 4 % paraformaldehyde...
immediately and embedded in paraffin. Then, tissues were cut into 4 μm slices and subsequently de-paraffinized. Thereafter, the samples were rehydrated and stained with hematoxylin and eosin (H&E). The images were examined under a light microscope and three pathologists who were unaware of the grouping of the animals were asked to evaluate each group of sections.

**Flow cytometry**

For apoptosis detection experiments, rabbit corneal tissues from each group were removed and homogenates were prepared (one homogenate was made from each group of collected tissues). Then they were digested with enzymes and undigested sections were filtered out using strainers to form single cell suspensions. Next, the cells were stained with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) reagent (Vazyme, Nanjing, China) under dark conditions for 15 minutes. Subsequently, flow cytometry (BD, FACSCalibur, USA) was used to analyze the percentage of apoptosis.

For experiments to measure Th17 and Treg cells, lacrimal glands of rabbits in each group were taken and dried for 5 days and then homogenized (one homogenate was made from each group of collected tissues). They were digested with pancreatic enzymes and undigested sections were filtered out using strainers to form single cell suspensions. Then, single cell suspensions of the lacrimal gland were subjected to flow cytometry using the following specific antibodies: anti-CD4 antibody, fluorophore-conjugated anti-IL17 antibody and anti-Foxp3. The negative control samples were stained with the appropriate isotype-matched antibodies. Data was analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

**Western blotting (WB)**

The lacrimal gland tissues isolated from the rabbits were lysed using 1X radioimmunoprecipitation (RIPA) lysis buffer (20-188, Millipore, USA). The concentration of total protein was determined using a BCA protein assay kit (Beyotime, Shanghai, China). Protein lysates were separated by electrophoresis on a 10 % sodium SDS-polyacrylamide gel (SDS-PAGE), then transferred to PVDF membranes (Millipore, China) and treated with 5 % non-fat milk for 1 hour at room temperature. The protein was identified by incubating with specific primary antibodies overnight at 4 °C.

Information on the specific primary antibodies used in this study is as follows: anti-BCL2 (ab196495, 1:1000, Abcam, Cambridge, MA, USA), anti-Cleaved caspase3 (ab2302, 1:1000, Abcam, Cambridge, MA, USA), anti-BAX (ab104156, 1:1000, Abcam, Cambridge, MA, USA), anti-ROTY (ab91187, 1:1000, Abcam, Cambridge, MA, USA), anti-Foxp3 (ab75763, 1:1000, Abcam, Cambridge, MA, USA), anti-p-STAT3 (ab30647, 1:1000, Abcam, Cambridge, MA, USA), anti-p-STAT3 (ab31370, 1:1000, Abcam, Cambridge, MA, USA), anti-p-JAK2 (ab195055, 1:1000, Abcam, Cambridge, MA, USA), anti-JAK2 (ab245303, 1:1000, Abcam, Cambridge, MA, USA) and anti-β-actin (ab8227, 1:1000 dilution, Abcam, Cambridge, MA, USA). The following day, PVDF membrane were washed 3 times in TBST and incubated with HRP-conjugated secondary IgG antibody ((ab6734, 1:2000 dilution, Abcam, Cambridge, MA, USA) for 1 hour at room temperature. Image J was used for quantification.

**Statistical analysis**

Mean ± standard deviation (SD) are used to express all the data. Each set of experiments were repeated three times. GraphPad Prism (version 7.04) was used for statistical analysis. Student’s t-test was used for comparison between two groups, while one-way ANOVA was used for comparisons between multiple groups. P < 0.05 was considered statistically significant.

**RESULTS**

**Treatment with RMFS and HGA repairs ocular surface damage**

In the model group, the SIt value decreased significantly with time compared to control group (p < 0.001), whereas treatment with RMFS or HGA alone restored the SIt value. Interestingly, the increase in SIt was more when combined treatment of RMFS and HGA was used (Figure 1 A). Furthermore, as demonstrated in Figure 1 B, the BUT of the model group was significantly shorter than that of control group and the decrease was inversely proportional to incubation time, while treatment with RMFS or HGA alone enhanced the BUT of the model group. The BUT of the model group treated with a combination of RMFS and HGA was longer than the single-administration group. Next, pathological sections of the lacrimal gland tissues were made on each group of animals. The results showed that, compared to control group, the lacrimal gland structure was unclear, and the vesicles and lacrimal epithelium were of different sizes and disorganized in the model...
This observation was better modified when combined RMFS and HGA treatment was used (Figure 1 C). The combination of Runmufengye soup and hydroxysugar glycolic acid eye drops could improve dry eye symptoms and repair ocular surface damage in a rabbit dry eye model.

Figure 1: Combined treatment with RMFS and HGA repairs ocular surface damage in the rabbit dry eye model. (A) The Schirmer’s test to evaluate tear secretion in the respective groups; (B) Break-up time (BUT) and (C) Pathological sections of the lacrimal gland tissue was performed by H&E in the various groups. *P < 0.05, **p < 0.01, ***p < 0.001, compared with control group; #p < 0.05, ##p < 0.01, ###p < 0.001, vs. model group; δp < 0.05, δδp < 0.01, δδδp < 0.001, vs. model + RMFS group; ωp < 0.05, ωωp < 0.01, ωωωp < 0.001 vs. model + HGA group; χp < 0.05, χχp < 0.01, χχχp < 0.001 vs. model + RMFS + HGA group.

Treatment with RMFS and HGA inhibits cell apoptosis

As depicted in Figure 2 A, apoptosis rate in the cornea tissues significantly increased in model group compared with control group (p < 0.001). However, the rate of apoptosis was found to decrease significantly in both treatment groups with a more significant reduction observed in the co-administration group. This trend was also observed in the regulation of apoptosis and anti-apoptotic proteins as revealed in the Western blot results. The protein levels of Bax and cleaved caspase3 were significantly increased, while that of BCL2 was significantly decreased in the model groups (Figure 2 B). Interestingly, this observation was significantly reversed by the combination of RMFS and HGA. Taken together, the combined treatment with RMFS and HGA inhibited cell apoptosis of cornea tissues in rabbit dry eye model.

Treatment with RMFS and HGA regulates Th17/Treg related cytokine balance

Flow cytometry results show that the dry eye model group had a significantly increased proportion of Th17 cells in the lacrimal gland, while RMFS combined with HGA significantly suppressed this effect (Figure 3 A). The proportion of Treg cells in the lacrimal gland was slightly increased in the model group (p < 0.05), significantly increased in the RMFS or HGA group (p < 0.001), with the most significant increase in Treg cell population seen in the co-treatment group (p < 0.001; Figure 3 B).

Figure 2: Combined treatment with RMFS and HGA inhibited cell apoptosis of ocular surface in the rabbit dry eye model. (A) Cell apoptosis as determined by flow cytometry and (B) Protein levels of Bax, Cleaved caspase3 and Bcl2 was measured by WB in the model group treated with the indicated drug. *p < 0.05, **p < 0.01, ***p < 0.001, vs. control group; #p < 0.05, #p < 0.01, ###p < 0.001, vs. model group; δp < 0.05, δδp < 0.01, δδδp < 0.001, vs. model + RMFS group; ωp < 0.05, ωωp < 0.01, ωωωp < 0.001 vs. model + HGA group; χp < 0.05, χχp < 0.01, χχχp < 0.001, vs. model + RMFS + HGA group.
Th17 and Foxp3, the transcription factor associated with Treg, were upregulated in the model group compared to control group, while the combination of RMFS and HGA significantly inhibited RORγt expression but caused a further increase in Foxp3 expression ($p < 0.001$; Figure 3 C).

**Treatment with RMFS and HGA inhibited the JAK2/STAT3 pathway**

As depicted in Figure 4, the protein levels of p-JAK2 and p-STAT3 were upregulated in the model group, while the combination of RMFS and HGA treatment decreased the protein levels of p-JAK2 as well as p-STAT3 ($p < 0.001$). Taken together, the combination of Runmufengye soup and hydroxsugar glycolic acid eye drops inhibited the JAK2/STAT3 pathway.

**DISCUSSION**

Dry eye disease is a multifactorial ocular surface disease characterized by tear film homeostasis, ocular surface inflammatory response and damage and ocular discomfort. It is highly prevalent and with symptoms such as foreign body sensation, burning and stinging, seriously affect an individual's quality of life and may lead to loss of vision or blindness if not treated promptly and appropriately [1]. However, the clinical therapy of DED is difficult and effective treatments are still lacking.

**Figure 3:** Combined treatment with RMFS and HGA regulates Th17/Treg related cytokine balance in the rabbit dry eye model. The proportion of (A) Th17 and (B) Treg cells in lacrimal gland tissues of the model group treated with RMFS and HGA alone or combined was measured by flow cytometry (C) The protein level of RORγt and Foxp3 was measured by Western blotting. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, vs. control group; $p < 0.05$, $p < 0.01$, $p < 0.001$, vs. model group; $p < 0.05$, $p < 0.01$, $p < 0.001$, vs. model + RMFS group; $p < 0.05$, $p < 0.01$, $p < 0.001$, vs. model + HGA group.

**Figure 4:** Combined treatment with RMFS and HGA inhibited the JAK2/STAT3 pathway. The protein levels of p-JAK2, JAK2, p-STAT3 and STAT3 in the various groups as shown by Western Blot. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$, vs. control group; $p < 0.05$, $p < 0.01$, $p < 0.001$, vs. model group; $p < 0.05$, $p < 0.01$, $p < 0.001$, vs. model + RMFS group; $p < 0.05$, $p < 0.01$, $p < 0.001$, vs. model + HGA group.
Recently, Chinese medicine has also played an important role in the treatment of dry eye disease. For example, Astragaloside IV is a single compound extracted from *Astragalus membranaceus*. It has a protective effect on the dry eye model induced by benzalkonium chloride (BAC) and showed clinical amelioration effected by regulating the MUC1-ErbB1 pathway [9]. *Buddleja officinalis* Maxim. Eye Drops downregulated the expressions of apoptotic factors Bax, Fas and FasL in the lacrimal gland of the dry eye rabbit model, thereby inhibiting lacrimal cell apoptosis and maintaining the basal amount of lacrimal secretion [10]. In this study, the efficacy of RMFS in treating dry eye disease was investigated. RMFS is composed of *Lilium Bulbus* (Baihe), *Rehmanniae Radix Praeparata* (Shudì), *Angelicae Sinensis Radix* (Danggui), *Atractylodes Macrocephala Koidz.* (Baizhu), *Rhizoma Dioscoreae Atraci* (Shudi), *Bulbus Lilii membranaceae* (JAK2/STAT3) signaling pathway regulates the Th17/Treg cell ratio. Therefore, maintaining the homeostatic balance of Th17/Treg cells may contribute to the development of dry eye disease [3-5]. The apoptosis of the corneal and conjunctiva epithelium caused by immune inflammatory response in the lacrimal gland is the main cause of the deterioration of the dry eye disease [12]. As reported in the present study, the corneal epithelial tissue in the rabbit dry eye model had more apoptotic cells than control group and combined treatment with RMFS and HGA reduced the number of apoptotic cells of cornea tissues in the rabbit dry eye model. In recent years, regulatory T cells (Treg) and helper T cell subset 17 (Th17) cells have been found to play a very important role in immune inflammatory diseases [5]. Among them, Th17 cells promote inflammatory responses by secreting IL-17, a cytokine, and retinoic acid-related orphan receptor γt (RORγt), a specific transcription factor whose expression directly affect the differentiation and function of Th17 cells [13,14].

Treg has an immunosuppressive function, preventing the activation of autoimmune T cells, regulating the body’s immune response, maintaining immune homeostasis and suppressing the development of immune diseases [11,15]. Thus, Th17 and Treg cells exhibit functional antagonism towards each other, which may be an important factor in the homeostasis of immune function. It has been found that the development of dry eye disease was associated with a disruption in the homeostatic balance of Treg/Th17 cells, and that blocking IL-17 significantly reduced the severity and progression of the disease by restoring the Treg/Th17 cell ratio [6].

A number of subsequent studies have confirmed that the inflammatory response mediated by an imbalance in the Th17/Treg cell ratio was an important cause of dry eye and that drugs increased tear film rupture time and tear production by modulating the Th17/Treg cell ratio, thereby restoring the histological changes in tissues such as cornea, conjunctiva and lacrimal glands caused by dry eye [16-18]. Therefore, maintaining the homeostatic balance of Th17/Treg cells may contribute to the treatment of dry eye. In the present study, an imbalance in Th17/Treg ratio was found in the lacrimal gland of the model group and co-treatment with of RMFS and HGA regulated the balance of Th17/Treg related cytokine in the rabbit dry eye model.

inflammatory mediators involved in extracellular signaling pathways and plays an overall regulatory role in inflammatory responses and cell survival [19]. Most importantly, the JAK2/STAT3 signaling pathway is also involved in the regulation of Th17/Treg homeostasis [20]. In T lymphocytes, activated STAT3 mediates its downstream signaling pathway to enhance RORγt expression in initial CD4+ T lymphocytes, thereby contributing to the differentiation of CD4+ T cells to Th17 [21]. Previous reports have established that the expression levels of JAK2 and STAT3 are significantly increased in the lacrimal gland of a dry eye inflammation model and tear secretion in this model is promoted by inhibiting the activation of the JAK2/STAT3 signaling pathway [22,23]. As reported in this study, the combination treatment with RMFS and HGA remarkably decreased the protein levels of the p-JAK2 and p-STAT3 in the model group.

CONCLUSION

The combination of RMFS and HGA regulates Th17/Treg balance to inhibit inflammatory response and reduces cell apoptosis by inhibiting the JAK/STAT3 pathway. This treatment could be a potential therapeutic strategy for the management of dry eye disease. However, this study lacks clinical data to show the efficacy and safety of combined treatment with RMFS and HGA. Furthermore, there may be other molecular mechanisms of action for the combination of RMFS and HGA in treatment of dry eyes that are yet to be identified. Therefore, future studies should focus on clinical data collection to investigate the efficacy and safety of the combination. In addition, other pathogenic mechanisms of dry eye disease need to be investigated.

DECLARATIONS

Acknowledgements

This work was supported by Self-funded research subject for the Chinese Medicine Administration Bureau of Guangxi Zhuang Autonomous Region (Grant no. GXZYC20220296).

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Zhonghua Wen and Shulin Li designed the study and carried them out. Zhonghua Wen, Shulin Li, Jia Tian, Wei Wang, Liu Zheng and Ping Tao supervised the data collection, analyzed and interpreted the data. Zhonghua Wen and Shulin Li prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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Trop J Pharm Res, November 2023; 22(11): 2279


