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Effect of Buddleja officinalis eye drops to inflammatory factors of lacrimal gland cells of castrated male rabbit with dry eye

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Foundation items: National Natural Science Foundation of China (No. 81574031); Hunan Provincial High Level Health Talent "225" Training Project; Project Supported by Hunan Provincial Science and Technology Department (No. 2015SF2016-6); Project Supported by Hunan Provincial Development and Reform Commission (No. [2014] 658); Construction Project of Hunan Provincial Key Laboratory for Prevention and Treatment of Ophthalmology and Otolaryngology with Traditonal Chinese Medicine (No. 2017TP1018); Changsha Science and Technology Project (No. KC1704005).

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Received: 2017-05-12 Accepted: 2018-06-03

密蒙花滴眼液对去势雄兔泪腺细胞炎症因子的影响

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基金项目:国家自然科学基金面上资助项目(No. 81574031);湖南省高层次卫生人才"225"工程培养项目资助;湖南省科技厅科研基金资助项目(No. 2015SF2016-6);湖南省发展改革委科研基金资助项目(湘发改投资[2014] 658 号);中医药防治眼耳鼻咽喉疾病湖南省重点实验室建设项目(No. 2017TP1018);长沙市科技计划项目(No. KC1704005).

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摘要

目的:观察不同浓度密蒙花滴眼液对去势雄兔泪腺细胞炎症因子 TNF-α、IL-1β 的影响,探讨不同浓度密蒙花滴眼液对去势雄兔干眼症模型的疗效。

方法:将密蒙花原药材制备成低、中、高 3 种浓度滴眼液。将 42 只健康成年新西兰长耳白兔(雄性),随机分为 A:空白组;B:模型组;C:低浓度密蒙花滴眼液组;D:中浓度密蒙花滴眼液组;E:高浓度密蒙花滴眼液组;F:安慰剂滴眼液组;G:睾酮组等 7 组,每组 6 只。除 A 组外其余 6 组实验用兔行双侧睾丸及附睾切除术。从术后第 3 天开始 C、D、E、F 组分别予相应滴眼液滴双眼。各组实验用兔,分别于术前和术后第 4wk 各进行一次 Schirmer I 试验(SIT)和泪膜破裂时间(BUT)测定。采用免疫组化法对泪腺细胞炎症因子 TNF-α 和 IL-1β 的表达进行检测。

结果:1)SIT 与 BUT 值比较:术后 C 组与 D、E、G 组比较,有统计学意义($P<0.01$);D、E、G 组间相比较,无统计学意义($P>0.05$);2)术后各组炎症因子 TNF-α、IL-1β 比较:C 组与 D、E、G 组比较,有统计学意义($P<0.05$);D、E、G 组间相比较,无统计学意义($P>0.05$)。

结论:1)密蒙花滴眼液具有与雄激素相似的抑制细胞炎症因子表达的作用,但其作用弱于雄激素;2)中、高浓度密蒙花滴眼液对 TNF-α 和 IL-1β 的抑制作用要强于低浓度密蒙花滴眼液,但中、高浓度密蒙花滴眼液的抑制作用差别不明显。

关键词:密蒙花滴眼液;干眼;去势雄兔;泪腺细胞;炎症因子

引用:彭俊,欧阳云,李文娟,吴权龙,谭涵宇,彭清华. 密蒙花滴眼液对去势雄兔泪腺细胞炎症因子的影响. 国际眼科杂志 2018;18(8):1359-1364

Abstract

• AIM: To observe the effect of different concentrations Buddleja offcinalis eye drops (BOED) to inflammatory factors of lacrimal gland cells of castrated male rabbit with dry eye.

• METHODS: Forty-two healthy adult New Zealand rabbits were randomly divided into 7 groups, 6 rabbits each group. Group A: blank group; Group B: model group; Group C: low concentrations BOED group; Group D: medium concentrations BOED; Group E: high concentrations BOED; Group F: the placebo group; Group G: testosterone groups. All rabbits of groups B to G were cut off bilateral testis and epididymis except group A. The rabbits of groups C, D, E and F were dropped relevant eye drops except groups A and B. All rabbits were tested for Schimer I test (SIT) and break-up time (BUT) before operation and 4wk after operation. Expressions of inflammatory factors of TNF-α, IL-1β from lacrimal gland cells were checked in immunohistochemical staining method.

• RESULTS: 1) Comparison of SIT and BUT: Compared with the groups D, E and G, group C had statistical significance ($P<0.01$). Compared with groups D and E, G groups, there was no significant difference among those group after operation ($P>0.05$); 2) Comparison of inflammatory factors of TNF-α, IL-1β after operation: Compared with value of the average optical density of TNF-α and IL-1β of group C, those of groups D, E and G were obviously different after operation ($P<0.05$). Compared D with E, G groups, there was no significant difference between those group after operation ($P>0.05$).
• CONCLUSION: 1) Compared with androgen, BOED has the similar effect of depressing the expression of inflammatory factors. But its effect is slightly weaker than the androgen; 2) The depressing effect of medium and high concentration BOED are better than low concentration. Compared with medium and high concentration of BOED, there was no significant difference between two groups.
• KEYWORDS: Buddleja offcinalis eye drops; dry eye; lacrimal gland cell; inflammatory factors
DOI:10.3980/j.issn.1672-5123.2018.8.01

Citation: Peng J, Ouyang Y, Li WJ, Wu QL, Tan HY, Peng QH. Effect of Buddleja officinalis eye drops to inflammatory factors of lacrimal gland cells of castrated male rabbit with dry eye. *Guoji Yanke Zazhi*(*Int Eye Sci*) 2018;18(8):1359-1364

INTRODUCTION

Dry eye is also known as keratoconjunctivitis sicca, which is a variety of diseases characterized by abnormal quality or quantity of tear due to any reason, or drop the tear film stability caused by abnormal dynamics and accompanied ocular discomfort and/or ocular surface tissue disease[1]. Dry eye is a common ocular surface disease, most of which are binocular diseases. Epidemiological and clinical studies have been found that the incidence is far higher than what people think.

We have confirmed that Buddleja officinalis has a good curative effect on dry eye in previous clinical and animal experiments. In this experiment Buddleja offcinalis eye drops was made by Buddleja officinalis crude drug, for the treatment of animal model with dry eye induced by androgen reducing to investigate the effects of Buddleja offcinalis eye drops (BOED) on lacrimal gland cell inflammatory factor TNF-α and IL-1β of dry eye caused by androgen decreasing.

MATERIALS AND METHODS

Laboratory Animals Forty-two healthy adult New Zealand white rabbits (male) were chosen, body weight between 1.5-2.0 kg[Animal experimental center of Hunan University of Chinese Medicine, Laboratory animal quality certificate No. SCXK(Xiang)2009-0012].

Laboratory Equipment Slit lamp microscope, hand-held direct ophthalmoscope, GB11241-89 constant temperature water bath box, Leica Paraffin section machine, micro camera and computer image analysis system, *etc*. Relevant equipment used in the above experiments were provided by Hunan University of Chinese Medicine Laboratory of morphology and Laboratory of Ophthalmology.

Drugs and Reagents Testosterone Propionate Injection: Tianjin Jin Yao Pharmaceutical Co., Ltd. (Code number approved by SFDA: H12020531; specifications: 1 mL: 25 mg); Benzylpenicillin Sodium for Injection: Guangzhou Baiyun Mountain Tianxin pharmaceutical Limited by Share Ltd (Code number approved by SFDA: H444022446; specifications: 400 thousand units); Sodium Chloride Injection: Jiangsu Yabang Shengyuan Pharmaceutical Co. Ltd. (Code number approved by SFDA: H32024531; specifications: 500 mL:4.5 g); chloral hydrate: analysis of pure Tianjin Kermel Chemical Reagent Co. Ltd. (specifications: 250 g); Sterile Water for Injection: Shanghai treeful Jinshan Pharmaceutical Co. Ltd. (Code number approved by SFDA: H31021935; specifications: 500 mL). Buddleja officinalis and placebo eye drops were prepared in modern technology by the Department of Medicine of Hunan University of Chinese Medicine (Specifications: 3 mL low concentration BOED was equivalent to 0.5 g original medicinal materials; each 3 mL medium concentration BOED was equivalent to 1 g original medicinal material; each 3 mL high concentration BOED was equivalent to 2 g original medicinal material).

Harris hematoxylin, eosin and goat anti rabbit TNF- antibody (specifications: 200 g/mL) were from Beijing Golden Bridge Biotechnology Co. Ltd. Rabbit anti rat IL-1 antibodies (specifications: 200 g/mL) and 3, 3-diaminobenzidine chromogenic agent were from Wuhan Boster Biological Technology., LTD.

Experimental Methods

Experimental Animal Group According to the random arrangement table method 42 rabbits were divided into 7 groups, each group of 6 rabbits. A: blank group; B: model group; C: low concentration BOED group; D: medium concentration BOED group; E: high concentration BOED group; F: placebo group; G: testosterone group.

The Establishment of Animal Model of Dry Eye All rabbits were treated by bilateral testicular and epididymis resection with reference to the relevant literature[2-3] except blank group.

Postoperative Administration In addition to A, B group, from the third day after the operation groups C, D, E were respectively treated with corresponding concentration BOED, 3 times one day. Group F was treated with placebo eyedrop, 3 times one day Group G was injected testosterone propionate injection in the thigh muscles by 0.5 mL/kg, once every 3d.

Dry Eye Routine Inspection Method Each test was completed by the same group of experimental personnel division. All the rabbits were made 1d before and the fourth week after operation for Schirmer Ⅰ test (SⅠt) and tear film BUT determination.

Specimen Collection and Processing After 4wk all rabbits after conventional breeding, were scarified by air embolism.

Table 1 Preoperative and postoperative S Ⅰ t values in different groups (Mean±SD; n=42; mm)

Time	Group A	Group B	Group C	Group D	Group E	Group F	Group G
Preoperative	14.75±2.26	14.83±2.04	14.92±2.07	14.75±1.66	14.58±1.51	14.67±1.92	14.83±1.59
Postoperative	14.50±1.45	7.75±1.54[a]	12.82±1.19[b]	14.17±1.11	14.17±1.19	7.50±1.62[a]	14.33±1.15

Compared with each group before the surgery, there was not statistically significant (P>0.05). They were comparable. Compared with group B and group F before and after operation, there were significant differences (P<0.01). Compared with the other groups before and after surgery, there was no statistical significance (P>0.05). There was no statistical significance between groups B and F after operation (P>0.05). Compared with the other groups, group B had statistical significance ([a]P<0.01). Compared with the groups D, E and G, group C had statistical significance ([b]P<0.01) Compared with groups E and G, the group D had no statistical significance.

Table 2 Preoperative and postoperative BUT values in different groups (Mean±SD; n=42; s)

Time	Group A	Group B	Group C	Group D	Group E	Group F	Group G
Preoperative	14.33±2.42	14.42±1.38	14.42±1.51	14.58±1.98	14.67±1.61	14.25±2.42	14.75±2.22
Postoperative	14.08±1.88	6.75±1.66[a]	12.92±1.08[b]	14.00±1.28	14.08±1.24	6.83±1.70[a]	14.50±1.24

Compared with each group before the surgery, there was not statistically significant (P>0.05). They were comparable. Compared with group B and group F before and after operation, there were significant differences (P<0.01). Compared with the other groups before and after surgery, there was no statistical significance (P>0.05). There was no statistical significance between groups B and F after operation (P>0.05). Compared with the other groups, group B had statistical significance ([a]P<0.01). Compared with the groups D, E and G, group C had statistical significance ([b]P<0.01). Compared with groups E and G, the group D had no statistical significance.

Table 3 Value of the average optical density of TNF-α and IL-1β in each rabbit lacrimal gland cells after operation (Mean±SD, n=42)

Time	Group A	Group B	Group C	Group D	Group E	Group F	Group G
TNF-α	0.18±0.10	0.68±0.14	0.35±0.14	0.24±0.09	0.24±0.11	0.65±0.13	0.23±0.14
IL-1β	0.24±0.12	0.59±0.17	0.41±0.16	0.30±0.08	0.24±0.13	0.57±0.28	0.17±0.21

Comparison of TNF-alpha, IL-1 beta value: compared with groups B and F after operation, there was no statistical significance (P>0.05). Compared with the other groups , groups B and F had statistical significance (P<0.01). Compared with groups D, E and G, group C had statistical significance (P<0.05). Compared with groups D, E and G, there was no statistical significance (P>0.05).

Lacrimal gland of both eyes were removed, and specimens were fixed in 4% paraformaldehyde for 24h, embedded in paraffin sections.

Index Detection The removal lacrimal gland tissue were stained in eosin staining and immunohistochemical staining, then inflammatory factors of lacrimal gland cells after staining were observed in 400 times optical microscope, then taken photos and preserved.

Statistics Treatment All the experimental data were processed by SPSS16.0 statistical software. The measurement data were expressed by mean plus or minus standard deviation (s), and the normal distribution and homogeneity of variance were tested, and the variance analysis was used to meet the normality and homogeneity of variance. Non parametric multiple comparisons were used to satisfy the normality and variance homogeneity. Enumeration data were analyzed with χ^2 test, and other homogeneity of variance with rank sun test. P less than 0.05 was considered to be statistically significant. P less than 0.01 means the difference had a significant meaning.

RESULT

The Results of Routine Examination of Dry Eye

Value of Schirmer Ⅰ test Compared with the placebo group and the model group, the value of SIT decreased significantly (P<0.01). Compared with the model group, the values of S Ⅰ t in each concentration BOED group and the testosterone group did not significantly decreased post-operatively (P>0.01). Compared with the low concentration BOED group, the S Ⅰ t values of the medium, high concentration BOED group and testosterone group did not significantly reduced postoperatively (P>0.01). Compared with the placebo group, the S Ⅰ t values of the testosterone group did not decrease significantly postoperatively (P>0.01).

Tear Break-up Time Compared with the placebo group and the model group, the value of BUT decreased significantly (P<0.01). Compared with the model group, the values of BUT in each concentration BOED group and the testosterone group did not significantly decreased postoperatively (P>0.01). Compared with the low concentration BOED group, the BUT values of the medium, high concentration BOED group and testosterone group did not significantly reduced postoperatively(P>0.01). Compared with the placebo group, the BUT values of the testosterone group did not decrease significantly postoperatively (P>0.01).

The Expression of TNF-α, IL-1β were Observed in Rabbit Lacrimal Gland Cells after Operation Compared with the blank group, the expression of the model group and the placebo group increased significantly (P<0.01). Compared with the model group, the expression of each concentration BOED group and the testosterone group did not increase significantly (P>0.01). Compared with the low concentration BOED group, the expression of medium, high concentration BOED group and testosterone group did not

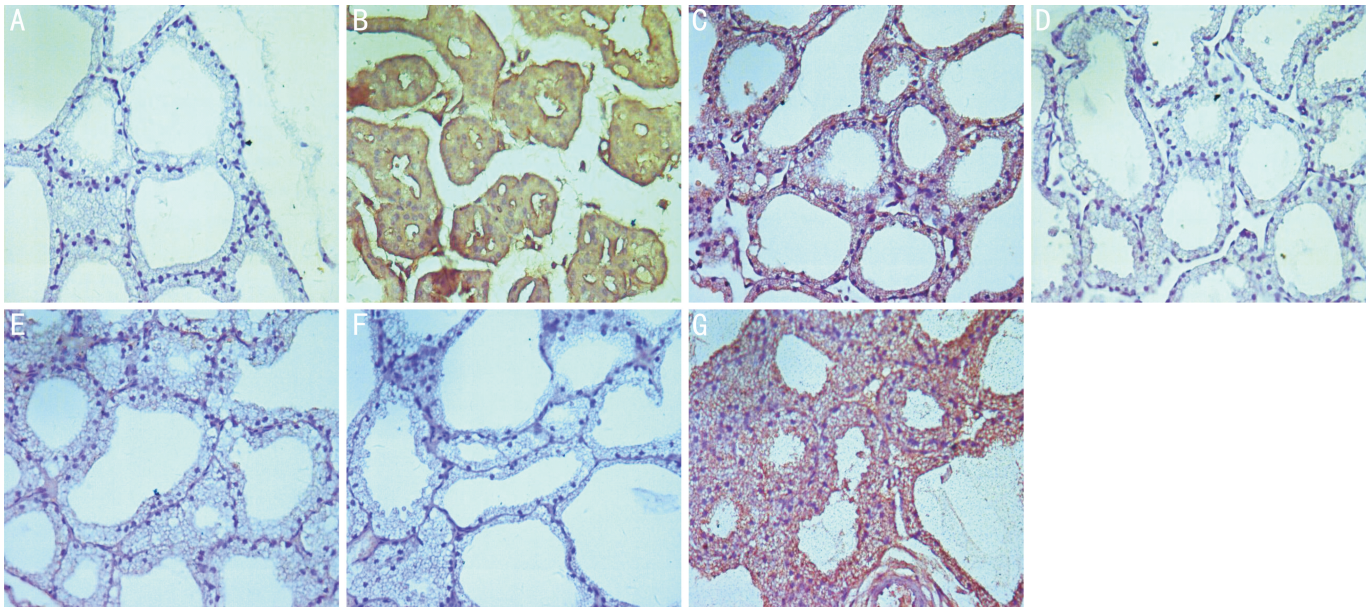

Figure 1　Expression of TNF-α in each group after operation (200 times)　A (Group A): Lacrimal gland cells arranged orderly, clear structure, no inflammatory cell infiltration, no apoptotic cells, no expression of TNF-α; B (Group B): Lacrimal gland cells arranged irregularly, large structure degeneration, inflammatory cell infiltration and cell apoptosis, lots of TNF-α expression in cell membrane and cytoplasm showing brownish yellow granules; C,D and E (Groups C,D and E): Lacrimal gland cells arranged orderly, clear structure, a small amount of inflammatory cell infiltration and apoptosis, TNF-α expression in cell membrane and cytoplasm showing brownish yellow granules; F (Group F): Lacrimal gland cells arranged orderly, clear structure, the infiltration of scattered inflammatory cells and cell apoptosis, TNF-α expression scattered in the cell membrane and cytoplasm showing brownish yellow granules; G (Group G): Lacrimal gland cells arranged irregularly, large structure degeneration, a lot of inflammatory cell infiltration and cell apoptosis, lots of TNF-α expression in cell membrane and cytoplasm showing brownish yellow granules.

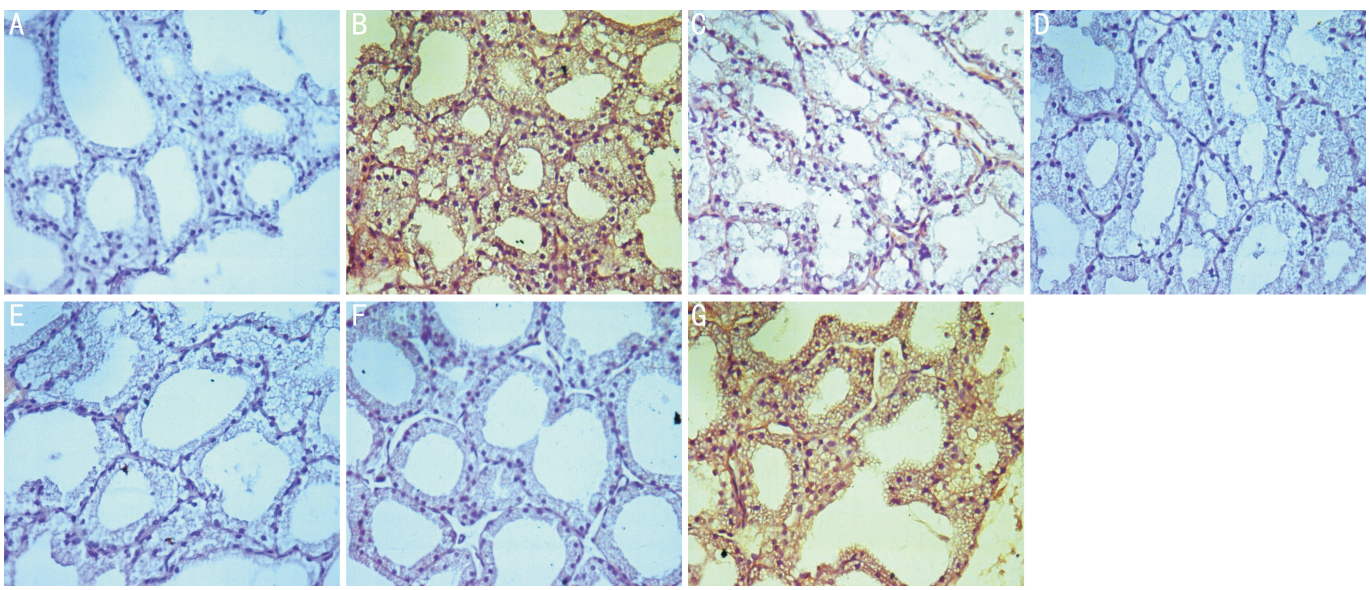

Figure 2　Expression of IL-1β in each group after operation (200 times)　A (Group A): Lacrimal gland cells arranged orderly, clear structure, no inflammatory cell infiltration, no apoptotic cells, no expression of IL-1 beta; B (Group B): Lacrimal gland cells arranged irregularly, large structure degeneration, inflammatory cell infiltration and cell apoptosis, lots of IL-1 beta. Expression in cell membrane and cytoplasm showing brownish yellow granules; C, D and E (Groups C, D and E): Lacrimal gland cells arranged orderly, clear structure, a small amount of inflammatory cell infiltration and apoptosis, IL-1 beta. Expression in cell membrane and cytoplasm showing brownish yellow granules; F (Group F): Lacrimal gland cells arranged orderly, clear structure, the infiltration of scattered inflammatory cells and cell apoptosis, IL-1 beta. Expression scattered in the cell membrane and cytoplasm showing brownish yellow granules; G (Group G): Lacrimal gland cells arranged irregularly, large structure degeneration, a lot of inflammatory cell infiltration and cell apoptosis, lots of IL-1 beta. Expression in cell membrane and cytoplasm showing brownish yellow granules.

significantly increased ($P>0.05$). There was a significant increase in the placebo group ($P<0.01$), and compared with placebo group, the expression of testosterone group did not increase significantly ($P>0.01$).

Observation of TNF-α and IL-1β　Groups A and G: lacrimal gland structure was clear and no expression of TNF-α

and IL-1β; groups B and F: lacrimal gland structure blurred, lots of TNF-α and IL-1β expressed in cell membrane and cytoplasm showing brown yellow granules; groups C, D and E: lacrimal gland structure was clear, scattered expression of TNF-α and IL-1β were observed.

CONCLUSION

The new definition of dry eye has been reported by the Tear Film and Ocular Surface Society Dry Eye Workshop (TFOS DEWS) in 2017 that is a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles. The disease was accompanied by inflammation of the ocular surface and increase of tear film permeability[4]. Compared with the traditional definition of dry eye, the definition emphasized the important role of inflammation in the pathogenesis of dry eye disease. Although the initial cause of dry eye caused by different mechanisms, once it enters the stage, inflammation became the key factor in the pathogenesis of dry eye. Compared with normal people, a variety of cytokines in tear and conjunctival epithelium of patients with dry eye including IL-1, IL-6, IL-8, alpha TNF-alpha and TGF-beta level changed significantly, and were related with the severity of dry eye[5].

In recent years, especially the sex hormone androgen has played an important role in tear secretion. Androgen exerts its immunosuppressive effect by stimulating the synthesis of TGF-beta to decrease TNF-alpha, IL-1 beta in lacrimal gland level, effectively protect the lacrimal gland from inflammation and degeneration, accelerate metabolism, regulate morphology and secretion function of lacrimal and meibomian gland.

Dry eye belongs to "Bai Se Zheng" of traditional Chinese medicine, is also called "Ganse Hunhua Zheng", "Shen Shui Jiang Ku Zheng", "Shen Qi Ku Cui". Generally dry eye is caused by deficiency of liver and kidney, yin blood in-sufficiency, being lack of nourishment. Buddleja officinalis granules is consisted of Buddleja officinalis, Chinese wolfberry, Chrysanthemum and so on. Buddleja officinalis is sweet, slightly cold, belonging to liver meridian, which has effect of clearing heat-fire, nourishing liver, and removing nebula for improving eyesight[6]. It is drug to treat red eye, blurred vision, eye dryness, cloudy vision due to liver deficiency and so on[7-8]. According to modern research the active ingredients of Buddleja officinalis are flavonoids[9], and some flavonoids have androgen like effect[10] which can be used to treat certain diseases induced by sex hormone levels decreased, such as bone loss[11].

We have confirmed previous experimental research of Buddleja officinalis, the main components of Buddleia flavonoids extract could play the androgenic like effect, and reduce the occurrence of dry eye in rats after androgen levels decreased, inhibit apoptosis of lacrimal gland cell, and maintain the stability and volume basic secretion of lacrimal gland[12-15].

In this study, the effects of 3 kinds of concentration eye drops on tear secretion, tear break-up time, the expression of inflammatory cytokines TNF-alpha and IL-1beta in the male rabbits were studied. Then we have drawn some conclusions: BOED has been androgen-like inhibition of expression of TNF-alpha and IL-1 beta, could inhibit the inflammatory reaction induced by androgen deficiency in rabbit lacrimal gland cells, but the effect has been weaker than that of androgen. The inhibitory effect of medium and high concentration of BOED on TNF-alpha and IL-1beta was better than that of low concentration of BOED, but there was not obvious difference in the inhibitory effect of medium and high concentration of BOED.

At present the dry eye is treated mainly by drugs. The clinical commonly used drug is artificial tears, which mainly relieve eye discomfort, and cannot change the pathological state of dry eye fundamentally. This study has been confirmed that BOED has had hormone like effect, could inhibit expression of inflammatory factor TNF-alpha and IL-1 induced androgen deficiency in rabbit lacrimal gland cells, maintained the stability and volume of basic secretion of lacrimal gland. A new natural drug has been provided for the treatment of dry eye disease, at the same time it has avoid the side effects caused by long-term use of and rogen, which has been broad application prospects.

ACKNOWLEDGEMENT

This paper was published on Journal of Hunan University of Chinese Medicine in Chinese version. And the journal of the university anthorized us to publish it in English on International Eye Science.

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