·Basic Research·

Effects of extract of Buddleja officinalis on partial inflammation of lacrimal gland in castrated rabbits with dry eye

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Abstract

• AIM: To assess the effects of extract of Buddleja officinalis on tear secretion volume, tear film stability, expressions of TGF-β 1, IL-1β , TNF-α in lacrimal gland of castrated rabbits with dry eye.

• METHODS: A total of 30 victory rabbits were divided averagely into normal group (A), model group (B), therapy group with low dose extract of Buddleja officinalis (C), therapy group with high dose extract of Buddleja officinalis (D) and therapy group with genistein (E). The dry eye model was established with orchiectomy on Group B, C, D, E. Group C, D, E were administered intragastrically with corresponding dose extract of Buddleja officinalis or genistein for 30 days. All rabbits were detected with SIT. TGF-β 1, IL-1β , TNF-α were detected with immunohistochemistry and the ultrastructure of lacrimal gland was observed under transmission electron microscope.

• RESULTS: The SIT value of group C, D, E were respectively 13.167 ± 4.957, 14.667 ± 5.279, 8.667 ± 0.516, obviously higher than that of group B 5.667± 2.338 (P<0.01). The positive expression of IL-1β in acinar cell and glandular tube cell of group C, D were 0.470 ± 0.048, 0.510 ± 0.088, obviously lower than that of group B 0.770± 0.118 (P <0.01). The positive expression of TNF-α of group C, D were 0.498± 0.156, 0.435 ± 0.069, obviously lower than that of group B 0.769 ± 0.095 too (P <0.01). The positive expression of TGF-β 1 of group C, D were 0.406 ± 0.171, 0.497 ± 0.147, obviously higher than that of group B 0.222± 0.113(P<0.01). Any result of group C, D was positive compared with that of group E (P<0.05). Ultrastructure of the lacrimal gland of group C, D, E was well preserved, especially in D group it was remarkable.

• CONCLUSION: The extract of Buddleja officinalis can adjust lacrimal gland partial inflammation of dry eye.

• KEYWORDS: dry eye; lacrimal gland; extract of buddleja officinalis; androgen

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INTRODUCTION

The abnormal change of tear capacity or quality can be due to many reasons from eyes or the general body, which may result in abnormal lacrimal film. Its typical pathological changes are focal lymphocytic infiltration and hyperplasy of lacrimal gland, progressive destruction of glandular tissue, which lead to functional decline and even loss of glandular secretion. Recent studies indicate that androgen could regulate immunity and lacrimal appearance, growth, differentiation and secretion. The decreased sexual hormone will disregulate larimal function, which leads to inflammation aggravation and dry eyes. Studies indicate that 8 flavonoids, which have the simulated function of androgen and play an important part in dry eye due to decreased hormone, are separated from Buddleja officinalis [1]. The purpose that we made castrated rabbits and tested the changes of basic lacrimal secretion after administration and studied the inflammatory reaction, is to discuss the mechanism and effects of extract of Buddleja officinalis on

dry eyes due to decreased androgen.

MATERIALS AND METHODS

Materials A total of 30 Japanese macrotia male rabbits of 2 months old, 2.0 to 2.5kg without abnormalities of anterior segment of eyes and optic fundus, were supplied by animal lab of Hunan University of Traditional Chinese Medicine. Schirmer I test (SIT) was less than 10mm per 5 minutes; tear film break-up time (BUT) was more than 10 seconds. These rabbits were randomly divided into group A (blank group), group B (model group), group C (low dose group), group D (high dose group), and group E (genistein group), with 6 rabbits in each group. Experimental drugs included dry powder of extract of buddleja officinalis (produced by Hunan Yalong Biological Engineering Co, Ltd.), 98% pure dry powder of genistein (produced by Hunan Jiuhui Modern Traditional Medicine Co, Ltd.) Reagents involved were 5% BSA, anti-TGF-β1, IL-1β, TNF-α, polyresistin, anti-Rb IgG (H+L)/Bio, strept avidin-biotin complex, SABC, 3,3-diaminobenzidine, DAB (provided by Wuhan Boster Biological Technology Co, Ltd).

Methods

Preparation of extract of Buddleja officinalis Dry buds of 20kg from Buddleja officinalis were extracted twice with 700mL/L ethanol. At first eight times the amount of 700mL/L ethanol was added; secondly, 6 times the amount of 700mL/L ethanol was added. Combined with ethanol extract, they were decompressed and recovered until there was no alcohol taste. The concentrated liquid was dried in vacuum and then crushed. The powders were extracted with ethyl acetate three times. Each time six times the amount was added and combined with extract of ethyl acetate. Ethyl acetate would be recovered at normal pressure. The extracts were dried in vacuum and crushed for later use.

Preparation of animal model of dry eye due to reduction of androgen level Bilateral orchiectomy (ORX) before administration was as follows: rabbits in Group B, C, D, E were resected testicles and epididymis; rabbits in Group A were cut scrotum without excision of testicles. The infection after operation was prevented.

Drug delivery Group B, C, D and E started to be administrated after the model had been set. Group A and B: 8.5g/L normal saline (NS) 5mL was used for intragastric administration everyday; Group C: 50mg/kg extract of Buddleja officinalis was employed for intragastric administration everyday; Group D: 100mg/kg extract of Buddleja officinalis was used for intragastric administration everyday; Group E: 50mg/kg genistein was employed for intragastric administration everyday. The intragastric administration lasted for 4 weeks.

Disposal of specimens Rabbits of each group were taken away the lacrimal gland immediately after executed by air embolization. The diameter of the lacrimal gland was about 4mm, and the lacrimal gland was cut into two parts. The larger one was fixed by 40g/L PFA and embedded in paraffin, while the smaller one was fixed by 25g/L GA.

Schirmer I test (SIT) According to Liu *et al*[2], SIT was carried before and 4 weeks after the administration. Normal length should be no less than 10mm/5min. In model group the length was no more than 9mm, while in administration group the length was no less than 10mm.

Immunohistochemistry Referring to the method of Ji *et al* [3], every embedding was sliced by the machine Shandon325, normally dewaxed, washed by distilled water twice, 2 minutes each time. The slice was kept in 30mL/L H_2O_2 at room temperature for 10 minutes. Washed by distilled water twice, 2 minutes each time. The slice was immerged into 0.01mol/L citrate buffer, heated till 90℃ and electricity was cut off. With an interval of 5-10 minutes, the procedure was repeated once or twice. They were washed by phosphate buffer saline (PBS) after cooling down. 5% BSA was added and kept in room temperature for 20 minutes. Then it was kept in 37℃ incubator of DNP-9162 for 2 hours, washed by PBS for 3 times, 2 minutes each time. The biotin-labeled Goat Anti-Rabbit IgG was dripped and put into the wet box, kept in the room temperature for 30 minutes, washed by PBS 3 times, 2 minutes each time. Then the reagent SABC was added and placed into the wet box, kept in the room temperature for 30 minutes. PBS washing was carried out for 4 times, 5 minutes per time. Western blotting DAB was used. 1mL distilled water was added in reagent A, B, C, one drip for each, which was finally added to the slice. It showed color at room temperature and the microscopic time control was about 5-30 minutes, washed by distilled water. Hematoxylin afterstain, dehydrated, transparent and mounting. Structure of lacrimal gland and expressions of TGF-β1, IL-1β, TNF-α were observed under the microscope and videography was taken by Motic B_5. The immunohistochemistry measurement system in MIAS-1000 high-resolution color graphic analysis system was adopted. 2.105μm served as the pixel length. In an area of $1.271\times10^6\mu m^2$ window the average absorbance values of cytokine TGF-β1, IL-1β, TNF-α were measured, and then the semi-quantitative analysis was performed.

Electron microscopy After taken out from 25g/L glutaraldehyde (GA), the sample was rinsed by 0.1mol/L PBS, 3 times, 10 minutes per time; then it was fixed by 10g/L osmic acid for 1-2 hours. It was taken out and rinsed by 0.1mol/L PBS for 3 times, 10 minutes each time; dehydrated by graded acetone respectively, 10 minutes for each concentration. The proportion of Epon812 and acetone was 1:1, soaked for 4 hours, Epon812 for 1 hour, embedded and determined by LKB 11800, sliced by LKB 8800, stained

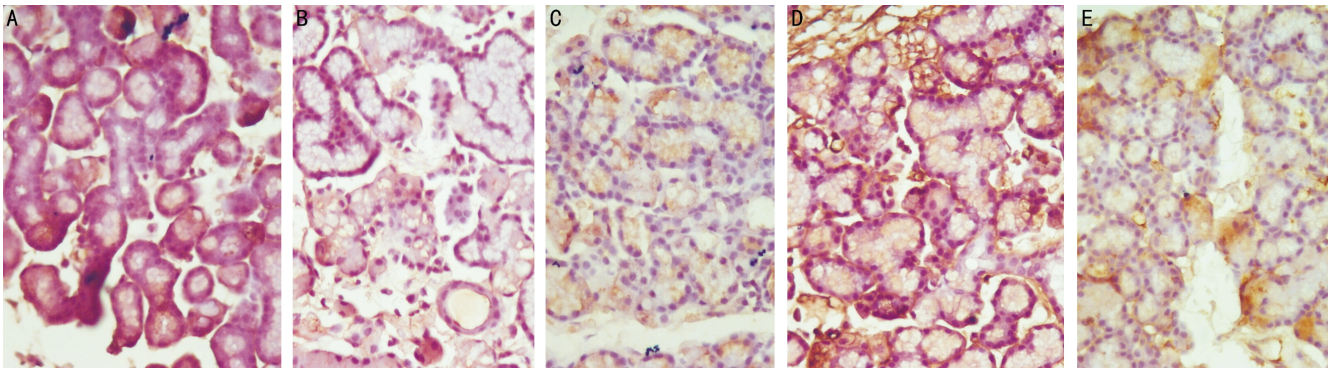

Figure 1 TGF-β1 expression lacrimal gland tissue of Group A, B, C, D, E

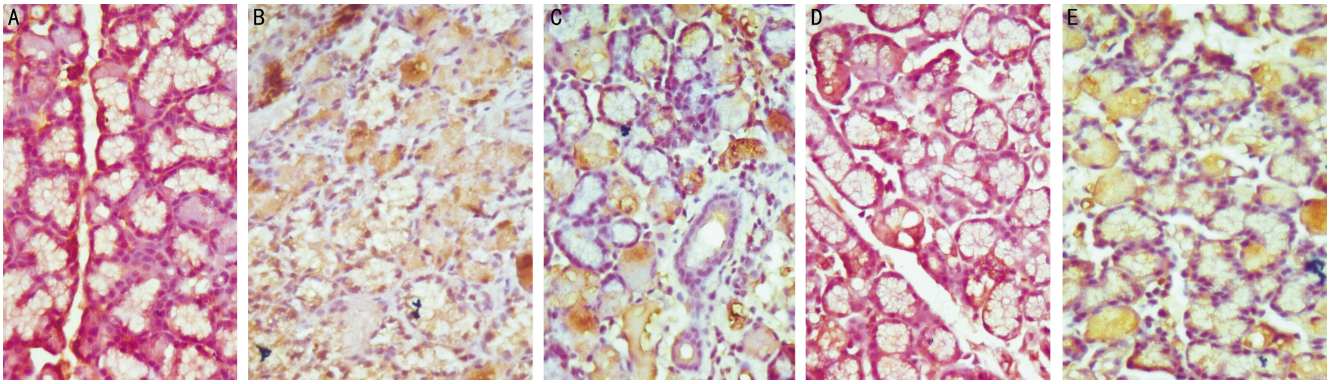

Figure 2 IL-1β expression in lacrimal gland of Group A, B, C, D, E

by uranyl acetate for 10 minutes, then stained via lead nitrate for 5 minutes. The lacrimal gland was observed under electron microscope HitachiH-600.

Statistical Analysis All the data were analyzed by SPSS 13.0. SIT from same group would be compared preoperatively and postoperatively by paired t-test. If it can't meet the requirement, signed rank sum test was employed. SIT from different groups would be compared postoperatively by covariance analysis. Multivariate analysis of variance was resorted to compare average integral optical density (IOD) of TGF-β1, IL-1β, TNF-α in different groups.

RESULTS

Comparison of SIT Pre- and Post-treatment In group B, after 4 weeks of intragastric administration by normal saline, basic tears secretion volume were obviously reduced. SIT decreased significantly and the difference had statistical significance (t=17.033, P<0.01, Table 1). In group C and D, after castrated and then medicated for 4 weeks, basic tear secretion volume was stable. SIT value had no statistical significance compared with that before medication(t=1.195, t=0.349, P>0.01). After covariance analysis, post SIT in different groups had significant differences compared with group B (F=34.187, P<0.01). Among them group C and D's SIT values increased significantly, compared with group B (all P<0.01). After being emasculated, group E was medicated by isogenistein and basic tear secretion volume increased. SIT values rose remarkably compared with group B, but did not returned to the status before emasculation (all P <0.01). Compared with group A, it had statistical significance (P<0.01).

Comparison of Local Inflammation Among Groups Lacrimal gland of group A arranged in neat rows. No fibroplasia and inflammatory cell infiltration. The expressions of TGF-β1, IL-1β and TNF-α were not obvious (Figure 1A, 2A, 3A). Four weeks after castration of group B, lacrimal gland was disorganized, and necrosis and degeneration of acinar cells were observed. Necrosis fibrous tissue hyperplasia, infiltration of local inflammatory cell, expressions of TGF-β1 were not obvious and most IL-1β and TNF-α expressed in cell membrane and cytolymph, like brown granules (Figure 1B, 2B, 3B). After treated by extract of Buddleja officinalis, degeneration and infiltration of group C and D decreased, and expressions of TGF-β1 were enhanced in cell membrane and cytoplasm, like brown granules, while IL-1β and TNF-α expression weakened (Figure 1CD, 2CD, 3CD). The effect of group E after treated by genistein was not obvious though group B was observed by microscope to be improved; degeneration and necrosis exist. Infiltration inflammatory cells existed in some slices. TGF-β1 was expressed obviously. A large amount of IL-1β and TNF-α was expressed in cell membrane and cytoplasm, like brown granules (Figure 1E, 2E, 3E).

Through multivariate ANOVA the results of TGF-β1, IL-1β and TNF-α comparison among groups indicated that

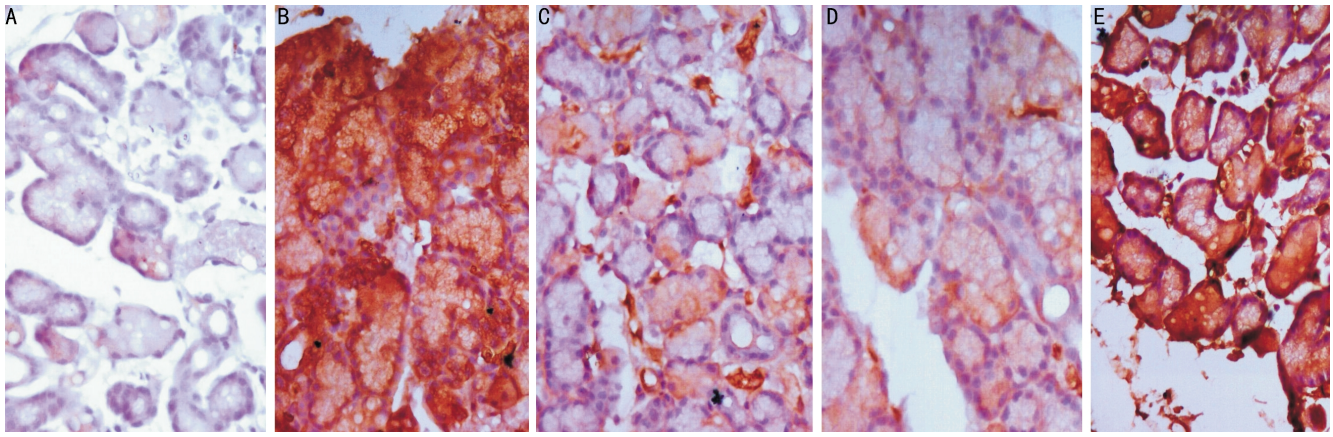

Figure 3 TNF-α expression in lacrimal gland of Group A, B, C, D, E

TGF-β1, IL-1β and TNF-**α** had significant differences among groups (F=6.777, F=36.805, F=34.403, all P<0.01). IL-1β and TNF-α of group B was enhanced 4 weeks after castration. Compared with group A there were signigicant differences (all P<0.01). Group C and D were treated by extract of buddleja officinalis after castration and expression of TGF-β1 was enhanced compared with Group B (P<0.05, P<0.01), whereas the expressions of IL-1β and TNF-α weakened (all P<0.01). After treated by genistein of castrated group E, the expressions of TGF-β1 did not show evident increase compared with group B and the expressions of IL-1β and TNF-α remained strong compared with group B (Table 2).

Transmission Electron Microscope Blank group (A): clear chondrinosome, no obvious ridge loss, nucleus rotundus, no crimple of karyotheca, no inflammatory cells (Figure 4A). Model group (B): Degeneration of lacrimal epithelium, focal necrosis, the electric density of endocrine granule in cytoplasm reduced or dissolved, degranulation, ridge loss, vacuolar degeneration, endoplasmic reticulum mild expansion. Some vacuole occurred in lacrimal epithelium, karyotheca crimpled, many inflammatory cells (Figure 4B), karyotin clumped distribution (Figure 4C). Low dosage group (C): mild lacrimal edema, no necrosis and cecullar edema. Abundant endocrine granule in lacrimal epithelium but no filament change. Vacuole in mitochondria alleviated, karyotheca crimpled and unclear edge set (Figure 4D). Interstitial substance extended and collagen fibers increased, a few inflammatory cells was discovered. High dosage group (D): clear structure, no necrosis cells and cecullar edema in lacrimal epithelium. Abundant endocrine granule in lacrimal epithelium, no filament change, vacuole in mitochondria alleviated, round karyon and mild crimple, uniform distribution of karyotin, but none inflammatory cells (Figure 4E). Genistein group (E): clear structure, loss of cytoplasmic mitochondria reduced compared with model group. The density of endocrine granule was low. Nuclear membrane was still mild shrinkage, chromatin uniformly distributed (Figure 4F). A few inflammatory cells were in interstitial substance.

Table 1 SIT comparisons of pre- and post-treatment among groups (mean±SD)

Group	SIT(mm)	
	pre-treatment	4 weeks after medication
A	15.333±5.716	15.417±5.869[d]
B	12.417±2.290	5.667±2.338[b]
C	12.417±3.412	13.167±4.957[d]
D	14.500±4.123	14.667±5.279[d]
E	13.333±2.858	8.667±0.516[b,d]

[b]P<0.01 *vs* pre-treatment in the same group, [d]P<0.01 *vs* model group

Table 2 TGF-β1, IL-1β and TNF-α comparisons among groups after treatment (mean±SD)

group	TGF-β1	IL-1β	TNF-α
A	0.195±0.036	0.228±0.060	0.203±0.087
B	0.222±0.113	0.770±0.118[d]	0.769±0.095[d]
C	0.406±0.171[b]	0.470±0.048[b]	0.498±0.156[b]
D	0.497±0.147[b]	0.510±0.088[b]	0.435±0.069[b]
E	0.274±0.088	0.734±0.109[f]	0.737±0.038[f]

[b]P<0.01: IOD (integrated optical density) had significant difference between buddleja officinalis groups and model group 4 weeks after emasculation; [d]P<0.01: IOD had significant difference between model group and blank group; [f]P<0.01: IOD had significant difference between genistein group and Buddleja officinalis groups

DISCUSSION

In all the available treatment, androgen substitutive therapy is the only etiological treatment. Long-term use will bring a lot of side effects, which include liver function damage, disorder of lipid metabolism and hyperplasia of prostate[4]. Referring to other unavailable etiological treatment, the only treatment is alleviation. Thus it is urgent subject for searching for a new treatment of traditional Chines e medicine, and a safe phytoestrogen alternative medicine.

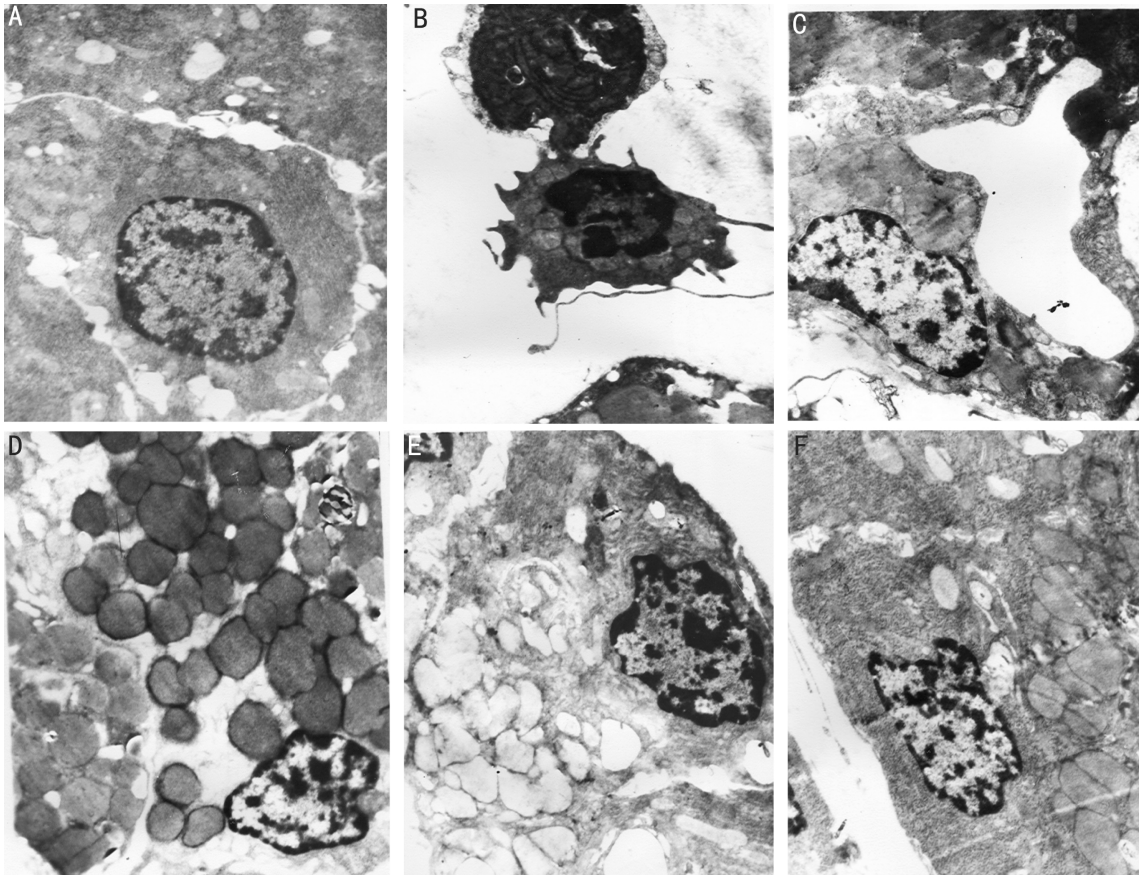

Figure 4 Ultrastructure of lacrimal gland (TEM×8 000)

Eyes are the target organ of the sex hormone. It has been proved the androgen receptor exist in lacrimal epithelium of people, rabbits and mouse [5]. Phytoestrogen could make biological effects by combining with the receptors. Flavone was one kind of phytoestrogen [6], which is also abundant in the extract of buddleja officinalis, so could make biological effects of receptor-binding. Someone proved [7] that flavones were the stimulator of androgen receptor by radioisotope tracing assay, and could play biological effect by combining with androgen receptors. Androgen and flavone are all heterocyclic polyphenol compounds. The character of the chemical structure could maintain the level of androgen and treat some androgen decreased disease. The study has improved that some flavones had androgen-like effect [8]. McVey *et al* [9] has proved that androgen increased in testicle and semen of F1 mouse after 120 days soybean was flavone intragastric administration. Moyad MA[10] discovered that flavone was effective on both menopausal osteoporosis and androgen deprivation therapy induced osteoporosis. From this we can say, the large amount of the extract of buddleja officinalis directly play androgen-like effect, so as to treat dry eyes duo to decreased androgen.

TGF-β1 was one kind of multifunction growth factor, which had biological effects on many organs and could regulate the growth, differentiation and immunity of cells, and inhibit the production of IL-1, IL-6 and TNF-α, promote the tissue repair [11]. IL-1 and TNF-α were the most inflammatory factors [12]. IL-1β could stimulate the monocytes and macrophages to produce IL-6 and TNF. IL-8 could mediate chemotaxis of neutrophilic granulocyte, and stimulate the release of inflammatory medium which could induce focal inflammation. TNF was one kind of non-glycosylated transmembrane protein, whose molecular weight was 25kD. In 1985 TNF produced by mononuclear macrophage was named as TNF-α [13]. Recently study indicated that, not only TNF-α played a role of cellular toxicity and growth inhabitant on tumor cell, but also could promote aggregation, activation and inflammatory factor release. TNF-α could enhance neutrophilic granulocyte's phagocytic activity during inflammatory reaction, increase superoxide anions, stimulate degranulation and excrete catalase, and increase expressions of MHC Ⅰ and ICAM-1 to promote the productions of IL-1, IL-6, IL-8, GM-CSF and PGE2, which had effect on pathological and physiological procedure of body injury. Solomon *et al*[14] found that IL-1β amount was much higher in dry eye patient's lacrimal film and conjunctiva than that in normal person. Sullivan *et al*[15] discovered that the decrease of androgen could promote Sjögren's syndrome and lacrimal reaction. Toda *et al*[16] found that androgen therapy could increase mRNA in

TGF-β1 in lacrimal of dry eyes from model group, and also promote the aggregation of TGF-β1, whose increase could decrease mRNA level of IL-1β and TNF-α. Those indicated androgen could be regulated by immunity reaction of TGF-β1 on eyes, but also inhibit lacrimal immunity damage[17], to reduce inflammation of lacrimal gland.

This research indicated that through genistein's intervention, expressions of TGF-β1 increased obviously in group C, D, C2, D2 and E2, while IL-1βand TNF-α obviously decreased to alleviate lacrimal inflammatory reaction. The inflammatory infiltration disappeared, normal lacrimal cells were protected and damaged tissues were repaired. For male rabbit it might relate to quasi-androgen effect. After flavonoids combining with androgen receptor in lacrimal, mRNA from TGF-β1 was improved and aggregation of TGF-β1 was promoted. Thereby further the productions of IL-1, TNF-α were inhibited to promote tissue repair. In group E, the rabbits were intervened by genistein, which belonged to isoflavone in phytoestrogen. Its androgen-like effect haven't been reported and is ineffective. The extract of buddleja officinalis plays an important effect on dry eyes duo to decreased androgen, alleviate lacrimal inflammatory reaction and prevent damage to maintain basic tears secretion to. It maybe relate to androgen-like effect, which is worth further study.

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