Effects of eye drops of *Buddleja officinalis* Maxim. extract on lacrimal gland cell apoptosis in castrated rats with dry eye

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**Objective:** To explore the possible mechanism of eye drops of *Buddleja officinalis* extract in treating dry eye of castrated rats by analyzing the expressions of Bax and Bcl-2 proteins.

**Methods:** Forty-five Wistar male rats were randomly divided into sham-operated group, untreated group and eye drops of *Buddleja officinalis* Maxim. extract (treatment) group. The dry eye model was established with orchietomy in the untreated group and treatment group. Rats in the treatment group were treated with eye drops of *Buddleja officinalis* Maxim. extract, one drop once, three times daily. Eyes of rats in the sham-operated group and untreated group were instilled with normal saline. After one-, two-, or three-month treatment, five rats in each group were sacrificed respectively. Then samples were taken to detect related indices. Expressions of Bax and Bcl-2 of lacrimal gland were checked by immunohistochemical method and quantity of apoptotic cells was counted.

**Results:** After one-, two- or three-month treatment, the quantities of expressions of Bax in acinar epithelial cells and glandular tube cells were significantly lower, and those of Bcl-2 were significantly higher in the treatment group than in the untreated group, and the quantities of apoptotic cells of the treatment group were significantly lower than those of the untreated group (*P*<0.01).

**Conclusion:** The main components of extract of *Buddleja officinalis* Maxim. are flavonoids, which can significantly inhibit cell apoptosis in lacrimal gland.

**Keywords:** orchietomy; xerophthalmia; lacrimal apparatus; apoptosis; *Buddleja officinalis*; eye drops; rats
Dry eye has a high incidence and has been a top issue in the present research. Androgen level declining is the main pathogenesis of dry eye, which has gradually become a hot research. The effective parts of *Buddleja officinalis* Maxim. (Mimenghua) are flavonoids\(^1\). Androgen and flavonoids are all heterocyclic polyphenolic compounds. It has been proved that some flavonoids have androgen-like effects\(^3\), which can treat the diseases due to decreased androgen levels\(^4\). Study also showed that flavonoids are agonists of membrane androgen receptor (AR)\(^5\). We therefore propose the hypothesis that flavonoids extracted from *Buddleja officinalis* Maxim. should be combined with the AR to produce androgen-like effects in treating dry eye caused by decreased androgen levels. Our preliminary studies found that gavage of *Buddleja officinalis* Maxim. extract had good efficacy in treating experimental animal with declining androgen levels\(^6\) and could inhibit cell apoptosis of lacrimal gland\(^7\). The main form of drugs of ocular surface diseases is eye drops. So we made the eye drops of *Buddleja officinalis* Maxim. extract and used castrated rats with dry eye to investigate the therapeutic effect of the eye drops of *Buddleja officinalis* Maxim. extract on dry eye due to decreased androgen levels and whether it could inhibit apoptosis of lacrimal gland cells.

1 Materials and methods

1.1 Materials

1.1.1 Experimental animals Forty-five one-month-old healthy Wistar male rats were used, weighing from 0.18 to 0.2 kg (Slac Laboratory Animal Co., Ltd. SPF level, with license number 2007-0361, outbreeding). They were provided by Animal Experimental Center of Hunan University of Traditional Chinese Medicine. Rats with the following features were used: anterior segment and fundus were normal after being checked by slit lamp microscope and retinoscope; the Schirmer I test (SIT) value was no less than 10 mm in 5 min; the tear film break-up time (BUT) value was no less than 10 s after using 0.5% tetracaine eye drops.

1.1.2 Experimental medicines Eye drops of *Buddleja officinalis* Maxim. extract were provided by Department of Pharmacology, the First Affiliated Hospital of Hunan University of Traditional Chinese Medicine.

1.1.3 Main reagents The reference substance of linarin (purity: 98.3%; batch number: 111528-200606) was obtained from National Institute for the Control of Pharmaceutical and Biological Products. 5% bovine serum albumin (BSA), rat polyclonal anti-rat Bax and Bcl-2, fluorescein-conjugated goat anti-rabbit IgG, strepto-avidin-biotin complex (SABC) immunohistochemical kit and 3,3'-diaminobenzidine (DAB) were purchased from Wuhan Boster Biological Technology, Ltd.

1.1.4 Main apparatus Waters 1525-2996 high-performance liquid chromatography (HPLC) (Water Company, America) was equipped with 2996 photodiode array, delivery pumps (Waters 1525), an automatic injector (Waters 717 autosampler). Empower chromatographic workstation was produced by Water Company, America. Double-beam ultraviolet-visible spectrophotometer was produced by Beijing Pesse General Equipment Co., Ltd. LEICA DM LB2 binocular microscope was produced by LEICA Company, Germany. Shandon325 paraffin-cutting machine was produced by Shandon Company, England. DNA-9162 Series Electric Thermostatic Incubator was produced by Shanghai Jing Hong Laboratory Instrument Co., Ltd. Shanghai. Motic B5 microscope system was produced by Motic Fair Co., Ltd.

1.2 Methods

1.2.1 Preparation of eye drops of *Buddleja officinalis* Maxim. extract

1.2.1.1 Extraction and quality control of *Buddleja officinalis* Maxim. effective parts *Buddleja officinalis* Maxim. dry buds were extracted two times in alcohol for centrifugal filtration. The filtrate was on HPD100 macroporous resin column, and eluted with ethanol. 70% ethanol eluates were collected, dried and smashed to be extract of *Buddleja officinalis* Maxim.

Quality control of *Buddleja officinalis* Maxim. was measured with HPLC. Conditions of HPLC were as follows. Columns: Phenomenex Gemini C\(_{18}\); mobile phase: Methanol-0.1% phosphoric acid (55:45, V/V); velocity: 0.6 mL/min; detection wavelength: 326 nm; column temperature: 30 °C; injection volume: 10 μL. Linarin standard solution was as a control, and the contents of linarin were calculated with external standard method.

Total flavonoids of *Buddleja officinalis* Maxim. were measured with ultraviolet standard curve method. Ultraviolet standard curve was established with...
prepared linarin standard solution as a control.
1. 2. 1. 2 Preparation of eye drops of Buddleja officinalis Maxim. extract Eye drops of Buddleja officinalis Maxim. extract were made by the Pharmacy of the First Affiliated Hospital of Hunan University of Traditional Chinese Medicine. Extract of Buddleja officinalis Maxim. was dissolved in distilled water, and the quality ratio of water to extract was 1 : 0.1. Eye lubricant carbadoxymethyl cellulose was added with the concentration controlled at 1.5%. Potassium bicarbonate and potassium chloride were added as buffer system with concentration at 0.1%. Then physical and chemical properties including pH, osmotic pressure, specific gravity and refractive index were adjusted to the following criteria: pH value 7.3 to 7.8; osmotic pressure 311-350 mOsm/L; specific gravity approximately equal to 1; refractive index 1.335. Finally preservative benzalkonium bromide was added with concentration at 0.005%
1. 2. 2 Grouping of experimental animals The 45 male rats were divided into sham-operated group, untreated group and eye drops of Buddleja officinalis Maxim. extract group (abbreviated as treatment group) by using random number. There were 15 rats in each group. Each group was subdivided into three groups (five rats in each group) according to the sacrificed time after treatment: one, two or three months.
1. 2. 3 Animal model with dry eyes due to decreased androgen level Referring to Ma’s method[2], rats in the untreated group and the treatment group underwent bilateral orchietomy (ORX) to induce dry eyes.
1. 2. 4 Drug delivery Eyes of rats in the sham-operated group and the untreated group were instilled with normal saline, one drop once, three times daily for two eyes. Eye drops of Buddleja officinalis Maxim. extract were instilled into two eyes of rats in the treatment group, one drop once, three times daily. The course of treatment should last one month, two and three months according to the sacrificed time.
1. 2. 5 Sample collecting and parameter detecting 1. 2. 5. 1 Sampling Dye eye was confirmed by SIT and BUT values[4]. Rats were sacrificed by decapitation as soon as the SIT and BUT tests were finished, and lacrimal glands were removed from both sides at once. Then the tissues were fixed in 4% formaldehyde and embedded with paraffin packing for immunohistochemical assay.
1. 2. 5. 2 Immunohistochemical method Every paraffin was continuously cut into pieces by Shandon325 paraffin-cutting machine. Normally, the paraffin was dewaxed and rehydrated, and then washed with distilled water 2 minutes twice. The procedure of immunohistochemical method strictly followed the instruction book. Apoptosis of lacrimal duct cells and acinar epithelial cells and the immunohistochemical staining situation of lacrimal gland were observed under a microscope, and pictures were taken with Motic B5 microcamera system. Immunohistochemistry measurement of MIAS-1000 high-resolution color graphic analysis system was applied. In a window of 2.105 μm for the pixels long and an area of 1.271 × 106 μm² the average optical density values of apoptosis-related proteins of Bax and Bcl-2 were measured for semi-quantitative analysis. And the total numbers of apoptotic cells of each group in 5 to 7 high-power fields (×400) were randomly analyzed. And the average cell numbers per high-power field were calculated.
1. 3 Statistical analysis SPSS 13.0 software was used for statistical analysis. P value less than 0.05 was considered significant difference in statistics. Data were represented as x±s. And firstly data were conducted a normal distribution and homogeneity of variance test, if dada showed the normal distribution and homogeneity of variance, comparison of multi-group data would carry out by using analysis of variance. And comparison of multiple group data after treatment (pre-treatment values as covariates) were carried out by analysis of coviance. Data of non-normal distribution were analyzed by using Wilcoxon rank sum test.
2 Results
2. 1 Results of quality control of Buddleja officinalis Maxim. flavones A total of 223 g Buddleja officinalis Maxim. flavones were obtained from 3 000 g Buddleja officinalis Maxim. The parallel samples were chosen for HPLC detection. Contents of linarin were calculated 17.292% on average in external standard method. Then linarin was used as the standard to establish ultraviolet standard curve. Detected with established ultraviolet standard curve, contents of total flavonoids were 75.49%.
2. 2 Lacrimal gland cell apoptosis The quantity of apoptotic cells in the untreated group increased apparently after castration as compared with the sham-operated group (P<0.01). Some apoptosis-positive cells were found in acinar epithelial cells and glandular tube. Moreover the quantity of apoptotic cells in the untreated group 3 months after castration was even larger than 1 month (P<0.05). Under the microscope, the nucleus of apoptotic cells took on brownish-yellow color and some dyed substances in nucleus concentrated under nuclear membrane formed irregular ring-form or semi moon-like shapes. Some splitting nuclei were observed. Quantity of apoptotic cells decreased with the treatment of eye drops respectively for 1 month, 2 months, and 3 months. Compared with the corresponding untreated group there was significant difference (P<0.01, Table 1 and Figure 1).
Expressions of Bax and Bcl-2 in the sham-operated group were not apparent. After being castrated, Bax expression, which appeared as brownish-yellow granules, was seen strongly
expressed in lacrimal duct and acinus in the untreated group, while the expression of Bcl-2 was not obvious. The expression of Bax in the treatment group was decreased as compared with the untreated group \((P < 0.01)\). Expression of Bcl-2 appearing as brownish-yellow granules was increased in the treatment group as compared with the untreated group \((P < 0.01)\) and was observed in cell membrane and cytoplasm. The results were seen in Table 1, Figure 1 and 2.

**Table 1. Expressions of Bax and Bcl-2 proteins and quantity of apoptotic cells in lacrimal gland in different groups after treatment \((\bar{x} \pm s)\)**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Bax ((% \pm %))</th>
<th>Bcl-2 ((% \pm %))</th>
<th>Quantity of apoptotic cells ((\bar{x} \pm s))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>5</td>
<td>0.125 ± 0.044</td>
<td>0.314 ± 0.047</td>
<td>11.34 ± 4.67</td>
</tr>
<tr>
<td>After 1-month treatment</td>
<td>5</td>
<td>0.154 ± 0.044</td>
<td>0.321 ± 0.031</td>
<td>12.01 ± 5.55</td>
</tr>
<tr>
<td>After 2-month treatment</td>
<td>5</td>
<td>0.145 ± 0.058</td>
<td>0.353 ± 0.034</td>
<td>11.87 ± 5.22</td>
</tr>
<tr>
<td>Untreated</td>
<td>5</td>
<td>0.541 ± 0.134(\AA)</td>
<td>0.274 ± 0.074</td>
<td>45.45 ± 9.27(\AA)</td>
</tr>
<tr>
<td>After 1-month treatment</td>
<td>5</td>
<td>0.751 ± 0.084(\AA)</td>
<td>0.245 ± 0.055</td>
<td>58.22 ± 10.32(\AA)</td>
</tr>
<tr>
<td>After 2-month treatment</td>
<td>5</td>
<td>0.795 ± 0.038(\AA)</td>
<td>0.232 ± 0.021</td>
<td>70.41 ± 11.58(\AA)</td>
</tr>
<tr>
<td>Treatment</td>
<td>5</td>
<td>0.241 ± 0.091(\AA)</td>
<td>0.505 ± 0.114(\AA)</td>
<td>12.77 ± 7.91(\AA)</td>
</tr>
<tr>
<td>After 1-month treatment</td>
<td>5</td>
<td>0.255 ± 0.150(\AA)</td>
<td>0.435 ± 0.024(\AA)</td>
<td>21.27 ± 8.82(\AA)</td>
</tr>
<tr>
<td>After 2-month treatment</td>
<td>5</td>
<td>0.344 ± 0.073(\AA)</td>
<td>0.525 ± 0.055(\AA)</td>
<td>31.89 ± 5.67(\AA)</td>
</tr>
</tbody>
</table>

\(\* P < 0.05, \quad \text{vs after 1-month treatment in the same group,} \quad \n_{\othemark P < 0.05, \quad \text{vs sham-operated group in same time point,} \quad \n_{\othertwo P < 0.01, \quad \text{vs untreated group in same time point.}} \)

**Figure 1. Expression of Bax protein in lacrimal gland in different groups observed by immunohistochemical method (Light microscopy, \(\times 400\))**

A, Sham-operated group after 1-month treatment; B, Sham-operated group after 2-month treatment; C, Sham-operated group after 3-month treatment; D, Untreated group after 1-month treatment; E, Untreated group after 2-month treatment; F, Untreated group after 3-month treatment; G, Treatment group after 1-month treatment; H, Treatment group after 2-month treatment; I, Treatment group after 3-month treatment.
3 Discussion

Dry eye hasn’t got a definitive name in traditional Chinese medicine. According to the degree of syndromes it can be classified into slight level “white dry eye”, and the heavy level “xerophthalmia” and “external ocuolopathy with cataract”. The dry eye caused by gonadal hormone level imbalance should be classified into “Less Aqueous Humor”, “Walizhangyizheng” is the result of deterioration.

The principle for treatment should focus on clearing the liver heat, nourishing the liver yin and relieving the wind-heat externally.

_Buddleja officinalis_ Maxim. is the right drug for jueyu meridian. It also can clear the liver heat and supplement liver yin internally, and relieve the wind-heat externally. According to the theory of traditional Chinese medicine, it may be a perfect drug for dry eye.

It has been reported by some studies that apoptosis seldom happens in normal conjunctiva epidermis and lacrimal gland tissue, while apoptosis increases in conjunctiva epidermis and lacrimal gland in patients with dry eye and keratoconjunctivitis sicca (KCS) animal models. Numerous studies have confirmed that Bcl-2 gene product could significantly inhibit apoptosis, thus prolong cell life, and increase the number of cells, while Bax protein could promote apoptosis.

Toda et al have found that besides lightened soaking of the lymph cells the Bax mRNA of MRL/lpr female rats had apparently declined after taking gonadal hormones. While Bcl-2, c-myc and AR mRNAs increased significantly. What’s more, Azzaro et _al_ found that gonadal hormones had the effects of preventing lacrimal gland apoptosis and soaking of the lymph cells, proving that the level of gonadal hormones have close relations with lacrimal gland cell apoptosis of dry eye and inflammation.

Our study shows that the eye drops of Buddleja officinalis Maxim. extract could prevent the cell apoptosis. Expression of Bcl-2 in lacrimal gland in the treatment group had apparently increased and expression of Bax significantly decreased. The cells in early apoptosis stage will draw back from
the process of apoptosis. As for male castrated rats, it is likely that the flavonoids contained in *Buddleja officinalis* Maxim. play as a substitute of gonadal hormones, causing the apparent declination of Bax mRNA expression and increase of Bcl-2, c-myb and AR mRNAs in lacrimal gland cells. Our results are in accordance with the experiment results Toda et al[10]

Based on the results of this experiment, we hold the view that extract of *Buddleja officinalis* Maxim. can inhibit apoptosis of lacrimal gland acinar and ductal cells, which conform to our preliminary studies using the extract of *Buddleja officinalis* Maxim. intragastrically[5,6]. The mechanism may be related to androgen-like effect of *Buddleja officinalis* Maxim. flavonoids, which is worth further study.

REFERENCES