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Original Article

Effects of Buddleja officinalis granules on apoptosis factors Bax, Caspase-3, Fas, and FasL in lacrimal gland cells of castrated male rabbits

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Abstract: Objective: The aim of this study was to observe the effects of Buddleja officinalis granules (BOG) on expression of Bax, Caspase-3, Fas, and FasL in lacrimal gland cells of castrated male rabbits. Methods: Thirty New Zealand white rabbits were randomly divided into 5 groups: group A: normal group, group B: model group, group C: BOG group, Group D: placebo group, and Group E: testosterone group. Except for group A, all rabbits were treated with bilateral testicular and epididymis resections before intervention. Schirmer I test (SIT) and break up times (BUT) were measured on the 1st day before modeling and 30th day after modeling. After 30 days of intervention, all rabbits were sacrificed. The removal lacrimal gland was stained in eosin staining, then the structure was observed. Expression of Bax, Caspase-3, Fas, and FasL in lacrimal gland tissues was checked via immunohistochemistry. Results: H&E staining showed that lacrimal glands of rabbits in the BOG group were neatly arranged. The cell structure was evident and there was a small amount of inflammatory cell infiltration and apoptosis. A small amount of Bax, Caspase-3, Fas, and FasL were expressed in cell membranes and cytoplasm. Immunohistochemistry showed that the average optical density of Bax, Caspase-3, Fas, and FasL in the BOG group was less than that in the model group ($P < 0.01$). Conclusion: BOG may alleviate apoptosis of lacrimal gland tissue by inhibiting expression of Bax, Caspase-3, Fas, and FasL in lacrimal gland tissues, treating dry eye.

Keywords: Buddleja officinalis granules, castrated male rabbits, lacrimal gland tissue, apoptosis factor

Introduction

International Dry Eye Working Group (DWES) defined dry eye, in 2007, as a multifactorial disease of tears and the surface of the eye [1]. Worldwide average incidence of dry eye is 14%-33%, increasing yearly [2, 3]. Dry eye has attracted wide attention in the medical field and has become a hot topic in ophthalmology. Therefore, attention should be paid to the prevention and treatment of dry eye. Apoptosis of lacrimal glands is one of the most important pathogenic factors in dry eye. Bcl-2-associated X protein (Bax), cysteinyl aspartate specific proteinase 3 (Caspase-3), Fas, and its ligand FasL are the key cells in apoptosis signaling pathways. In this study, the effects of Buddleja officinalis granules (BOG) on the histological structure of lacrimal glands and relative expression levels of apoptosis-related genes Bax, Caspase-3, Fas, and FasL in castrated male rabbits were observed, aiming to explore the mechanisms of BOG in treatment of dry eyes.

Materials and methods

Experimental animals

A total of 30 Healthy adult New Zealand white rabbits (male) were chosen, with body weights ranging from 1.5-2.0 kg (Animal Experimental Center of Hunan University of Chinese Medicine, Laboratory animal quality certificate number: SCXK (Xiang) 2009-0012).

Laboratory equipment

Slit lamp microscopes, hand-held direct ophthalmoscopes, GB11241-89 constant temperature water bath, Leica paraffin slicer, micro cameras, and computer image analysis sys-

Table 1. SIT values before and after modeling in each group of rabbits (n = 12 mm)

	Group A	Group B	Group C	Group D	Group E
Before Modeling	14.75 ± 2.26	14.83 ± 2.04	14.77 ± 1.68	14.67 ± 1.92	14.83 ± 1.59
After Modeling	14.50 ± 1.45	7.75 ± 1.54▲	14.27 ± 1.14	7.50 ± 1.62▲	14.33 ± 1.15

Note: After the operation, groups B and D were compared with groups A, C, and E, ▲$P < 0.01$.

tems were used. 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

Buddleja officinalis granules (composed of Buddleja officinalis, wolfberry, and chrysanthemum) were prepared in modern technology by the Department of Medicine of Hunan University of Chinese Medicine. Testosterone propionate injections included: Tianjin Jinyao Pharmaceutical Co., Ltd., (code number approved by SFDA: H12020531, specification: 1 mL: 25 mg), rabbit anti-rabbit Bax monoclonal antibody (provided by Wuhan Boster Bioengineering Co., Ltd., specifications: 200 μg/mL), rabbit anti-rabbit Caspase-3 monoclonal antibody (provided by Wuhan Boster Bioengineering Co., Ltd., specification: 200 μg/mL), rabbit anti-rabbit Fas monoclonal antibody (provided by Wuhan Boster Bioengineering Co., Ltd., specifications: 200 μg/mL), rabbit anti-rabbit FasL monoclonal antibody (provided by Wuhan Boster Bioengineering Co., Ltd., specification: 200 μg/mL).

Experimental animal group

According to the random arrangement table method, thirty adult New Zealand white rabbits were randomly divided into 5 groups, each containing 6 rabbits. Group A: normal group; Group B: model group; Group C: BOG group; Group D: placebo group; and Group E: testosterone group. Except for the normal group, all rabbits were treated by bilateral testicular and epididymis resections.

Postoperative administration

In addition to A and B groups, from the third day after the operation, groups C and D were, respectively, treated with intragastric administration of BOG and saline for 100 mg/kg, 3 times a day for thirty days. Group E was injected with testosterone propionate in the thigh muscles by 0.5 mL/kg, once every 3 days.

Specimen collection and processing

After thirty days, all rabbits were scarified by air embolisms. Lacrimal glands of both eyes were removed and specimens were fixed in 4% paraformaldehyde for 24 hours. They were then embedded in paraffin sections.

Index detection

Schirmer I-tests (SIT) and tear film break-up times (BUT), of each group of rabbits, 1 day before modeling and 30 days after modeling, were checked. The removal lacrimal gland tissues were stained in eosin staining and detected via immunohistochemistry. Expression of apoptosis factors Bax, Caspase-3, Fas, and FasL in lacrimal gland cells was observed.

Statistical treatment

All experimental data were processed using SPSS 23.0 statistical software. Measurement data are expressed by means plus or minus standard deviation (s). Normality and homogeneity of variance and one-way ANOVA were tested. *P values* less than 0.05 indicate statistical significance, while *P values* less than 0.01 indicate that differences were significant.

Results

SIT values on the 1st day before modeling and 30th day after modeling

There were no significant differences in SIT between the groups before modeling ($P > 0.05$). There were significant differences between group B and D before and after modeling ($P < 0.05$). There were no significant differences between the other groups before and after modeling ($P > 0.05$). There were no significant differences between group B and group D after modeling ($P > 0.05$). There were no significant differences between group A, C, and E after modeling ($P > 0.05$). Compared with groups A, C, and E after modeling, group B and D showed significant differences ($P < 0.01$), as shown in **Table 1**.

Table 2. BUT values before and after surgery in each group of rabbits (n = 12 mm)

	Group A	Group B	Group C	Group D	Group E
Before Modeling	14.33 ± 2.42	14.42 ± 1.38	14.67 ± 1.89	14.25 ± 2.42	14.75 ± 2.22
After Modeling	14.08 ± 1.88	6.75 ± 1.66▲	14.30 ± 1.38	6.83 ± 1.70▲	14.50 ± 1.24

Note: After the operation, groups B and D were compared with groups A, C, and E, ▲$P < 0.01$.

Table 3. Average optical density values of Bax, Caspase-3, Fas, and FasL in lacrimal gland tissue of rabbits after the study (n = 12)

	Group A	Group B	Group C	Group D	Group E
Bax	0.14±0.08	0.55±0.31▲	0.21 ± 0.16	0.57±0.28▲	0.21±0.17
Caspase-3	0.35 ± 0.12	0.66±0.15▲	0.47±0.13	0.64±0.17▲	0.38 ± 0.16
Fas	0.27 ± 0.07	0.62±0.08▲	0.32 ± 0.12	0.66±0.18▲	0.30 ± 0.11
FasL	0.23 ± 0.06	0.59 ± 0.28▲	0.28 ± 0.09	0.50 ± 0.46▲	0.25 ± 0.09

Note: Comparison of Bax, Caspase-3, Fas, and FasL values: After the operation, groups B and D were compared with groups A, C, and E, ▲$P < 0.01$.

BUT values on the 1st day before modeling and 30th day after modeling

There were no significant differences in BUT between the groups before modeling ($P > 0.05$). There were significant differences between group B and D before and after modeling ($P < 0.05$). There were no significant differences between the other groups before and after modeling ($P > 0.05$). There were no significant differences between group B and group D after modeling ($P > 0.05$). There were no significant differences between groups A, C, and E after modeling ($P > 0.05$). Compared with groups A, C, and E after modeling, group B and D showed significant differences ($P < 0.01$), as shown in **Table 2**.

Average optical density of Bax, Caspase-3, Fas, and FasL in the lacrimal glands of each group of rabbits

There were no significant differences in the average optical density values of apoptosis factors Bax, Caspase-3, Fas, and FasL between groups B and D after the study ($P > 0.05$). There were no significant differences between groups A, C, and E. ($P > 0.05$); Compared with groups A, C, and E after modeling, group B and D showed significant differences ($P < 0.01$), as shown in **Table 3**.

Expression of lacrimal gland tissue and apoptosis factors in rabbits after the study

At the end of the study, eosin staining and immunohistochemistry were performed in the lacrimal gland tissues of each group. Lacrimal gland tissues of group A were arranged orderly, with clear tissue structure, no infiltration of inflammatory cells, no apoptotic cells, and no expression of apoptotic factors. The lacrimal glands of group B were irregularly arranged, with large structure degeneration, high inflammatory cell infiltration, and apoptosis. Many apoptosis factors were expressed in cell membranes and cytoplasm, showing brown-yellow granules. Lacrimal gland tissues of group C were neatly arranged, with clear tissue structure, low inflammatory cell infiltration, and apoptosis. Scattered apoptosis factors were expressed in cell membranes and cytoplasm, showing brown-yellow granules. Lacrimal glands tissue of group D were neatly arranged, with clear tissue structure, low cell infiltration, and apoptosis. Many apoptosis factors were expressed in cell membranes and cytoplasm, showing brown-yellow granules. Lacrimal gland tissues of group E were neatly arranged, with clear tissue structure, low inflammatory cell infiltration, and apoptosis. Few apoptosis factors were expressed in cell membranes and cytoplasm, showing brown-yellow granules, as shown in **Figure 1**.

Discussion

Professor Peng Qinghua founded BOG based on many years of clinical experience and the pathogenesis of dry eyes. Previous clinical studies have proven that it is effective in the treatment of dry eyes. In this experiment, the lacrimal glands of castrated male New Zealand

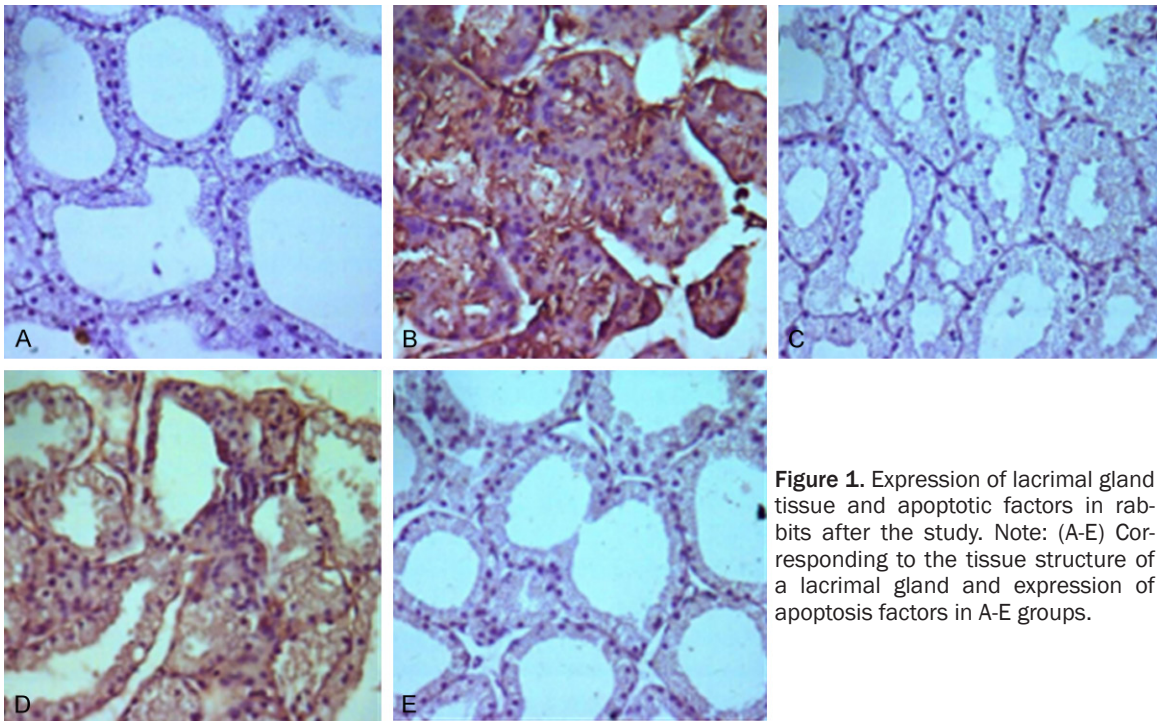

Figure 1. Expression of lacrimal gland tissue and apoptotic factors in rabbits after the study. Note: (A-E) Corresponding to the tissue structure of a lacrimal gland and expression of apoptosis factors in A-E groups.

rabbits were well arranged and organized after 30 days of intragastric administration of BOG. The average optical density and expression of apoptotic factors Bax, Caspase-3, Fas, and FasL was better than those of the model group, indicating that BOG could inhibit apoptosis of rabbit lacrimal gland cells induced by androgen deficiency. It showed similar effects of inhibiting expression of apoptotic factors Bax, Caspase-3, Fas, and FasL as androgens.

At present, treatment of dry eye is mainly through administration of drugs. Artificial tears are commonly used in clinic, which relieve discomfort. However, they cannot fundamentally change the pathological state of dry eyes. This study confirmed that BOG had a hormone-like effect, inhibiting expression of apoptosis factors Bax, Caspase-3, Fas, and FasL in the lacrimal glands of rabbits induced by androgen deficiency, maintaining the basic secretion of lacrimal glands, and maintaining the stability of lacrimal glands. It not only provides a new natural medicine for treatment of dry eye disease, but also avoids the side effects caused by long-term use of androgens, with broad application prospects.

Apoptosis of glandular tissue is an important factor in the pathogenesis of dry eyes. Apoptosis is a process of autonomous and orderly cell death. It is controlled by genes and divided into three stages: initiation, effector, and execution of apoptosis. The completion of each stage is also affected by activation, expression, and regulation of related genes [4]. Therefore, the key to curing dry eye is to delay or inhibit the apoptosis of lacrimal glands by effective methods.

Bax is one of the members of the Bcl-2 family. When apoptosis is induced by external stimuli, Bax is activated by death signals, transfers from the cytoplasm to the mitochondrial membrane, and binds to the membrane, rapidly forming homologous dimers to promote cell apoptosis [5]. Caspase is a protease in the cytoplasm. It participates in the regulation of cell growth, differentiation, and apoptosis. It is closely related to apoptosis of eukaryotic cells, but only after caspase activation can it play its role in promoting apoptosis. Activation of caspase is a multi-step hydrolysis and sequencing process in the process of apoptosis, including homologous and heterologous activation. Heterologous activation is a classical way to activate the zymogen of apoptotic protease. It activates from one caspase to another caspase. Caspase-3 is one of the Caspase of hetero-activation, which can directly induce apop-

tosis [6]. Over the years, the pro-apoptotic effects of Caspase-3 have been confirmed by many studies [7].

The Fas/FasL system-mediated receptor pathway is one of the classical pathways of apoptosis [8]. The location of Fas expression *in vivo* is very limited, usually distributed in immune exemption organs (eyes and testis) [9]. FasL is a natural ligand of Fas. When FasL binds to FasL, it can activate death receptor pathway directly, through a series of reaction processes, eventually leading to the activation of executive cysteine aspartase and inducing apoptosis [10, 11]. In the past ten years, Fas/FasL has been the focus of research on apoptosis. The Fas/FasL system can induce apoptosis of many kinds of cells. For example, interferon-γ can affect leukemia cells by regulating Fas/FasL signaling pathways [12]. Anti-tumor effective B-cells can directly kill tumor cells through apoptosis pathways of the Fas/FasL system [13]. Marginatoxin induces apoptosis of human hepatoma BEL-7402 cells, *in vitro* and *in vivo*, by activating Fas/FasL-mediated apoptosis pathways [6]. Apoptosis mediated by Fas/FasL can also induce apoptosis in cerebral ischemia/reperfusion injuryies.

In conclusion, BOG can inhibit expression of apoptotic factors Bax, Caspase-3, Fas, and FasL in lacrimal gland tissue, thereby inhibiting the apoptosis of lacrimal gland tissue and improving the function of lacrimal glands. This may be one of the possible mechanisms of BOG in the treatment of dry eye disease.

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Disclosure of conflict of interest

None.

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