

Effects of extract of *Buddleja officinalis* eye drops on androgen receptors of lacrimal gland cells of castrated rats with dry eye

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Abstract

• **AIM:** To evaluate the effects of the extract of *Buddleja officinalis* eye drops in basic tears secretory volume, tear film stability, expression of androgen receptors (AR) in castrated rats with dry eye, and to investigate the therapeutic effects of the extract of *Buddleja officinalis* on dry eye caused by gonadal hormones level imbalance.

• **METHODS:** Forty-five Wistar masculinity rats were divided at random into nine groups, including normal groups (A1, A2 and A3); model groups (B1, B2 and B3); therapy groups with extract of *Buddleja officinalis* eye drops (C1, C2 and C3). The "1" stood for being fed for 1 month, and "2" for 2 months, and "3" for 3 months. The dry eye model was established with orchietomy on groups B and C. Group C was treated with *Buddleja officinalis* extract eye drops for one month. All rats were checked with Schirmer I test (SIT) and tear film break-up time (BUT). Expression of AR was analyzed by flow cytometer (FCM).

• **RESULTS:** The SIT value of group C was significantly

higher than that of group B ($P < 0.01$) and the BUT value of group C was significantly longer than that of group B ($P < 0.01$), which indicated the eye drop could significantly keep basic tears secretory volume and tear film stability. And the expression of AR of group C was much higher than that of group B, which showed that available composition of the eye drops may display androgen-like activity.

• **CONCLUSION:** The main components of the extract of *Buddleja officinalis* is the flavonoids that can significantly inhibit happening of dry eye of rat after androgen level lowered. Its mechanism is like androgen's and it can display androgen-like activity to keep basic tears secretory volume and tear film stability

• **KEYWORDS:** castrate; dry eye; lacrimal gland; androgen receptors; androgen-like activity; the extract of *Buddleja officinalis*; eye drops

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INTRODUCTION

Dry eye could affect people's work and life by significant declining in sight. Although it may not threaten people's lives, it possesses a high incidence and has been a top issue in recent research. Androgen level declining is the main cause of dry eye. In present therapies, substitute therapy of androgen is the only therapy that could treat dry eye caused by gonadal hormones imbalance, but long use of this therapy will inevitably bring many side effects. There are some other therapies which also have certain flaws and limitations. *Buddleja officinalis* is a drug stored by Chinese Medicine, and it's often used to treat eyes with flavonoids as its effective parts. Androgen and flavonoids compounds are all heterocyclic polyphenols compounds, so the similarity in their chemical structures can be used to expound their endogenous androgen effect to treat some eye disease caused by gonadal hormones level imbalance including dry

eye caused by imbalance of gonadal hormones level. The extract of *Buddleja officinalis* eye drops are made to follow the characteristic of partial treatment for surface diseases of eyes, make model from dry eye of rat for research, and aim to investigate the broken effect mechanism and the therapeutic effects of *Buddleja officinalis* on dry eye caused by imbalance of gonadal hormones level.

MATERIALS AND METHODS

Materials Forty-five one-month-old healthy Wistar male rats weighing approximately 200g were purchased from animal experimental center of Hunan University of Traditional Chinese Medicine. Animals with following features were used: the front and ground eyes were normal after being checked by slit-lamp microscope and retinoscope, the SIT value was no less than 10mm/5min and the BUT value 10 seconds after the surface anaesthesia with 5g/L cocain eyedrops. The extract of *Buddleja officinalis* eye drops were made by the department of pharmacy of the first affiliated hospital of Hunan University of Traditional Chinese Medicine. The comparison products of linarin (Medical Biology Products Checking Bureau, China, containing 98.3%.)

Cytoperm (5g/L saponin solution, BD company). Rat anti-human AR and FITC-IgG (Wuhan Boshide Biology and Engineer Ltd Co.). Liquid 1525-2996 high performance liquid chromatography (USA Water Company, containing 2996 test machine of two polar tubes, 1525 and 717 automatic sample machine); Empower Chinese Chromatographic Work Station (USA Water Company); Photometer of ultraviolet visible light of double beams (Beijing Limited Liability Corporation of Puxin General Equipment); Flow cytometer (FACS type, USA BD company)

Buddleja officinalis 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 elution fluid were collected, dried and smashed to be extract of *Buddleja officinalis*. Columns: Phenomenex × Gemini C₁₈, Mobile phase: Methanol-0.1% phosphoric acid 55:45, v/v); Velocity: 0.8mL/min; Detection wavelength: 326nm; Column temperature: 30; Injection volume: 10μL. Linarin standard solution was prepared as a control, using external standard method to calculate. Linarin content was prepared as a control to establish the standard curve of UV and total flavonoids of *Buddleja* was measured in UV determination.

Extract of *Buddleja officinalis* dissolved in distilled water extract, quality ratio of water : extracts was 1:0.1, adding eye lubricant carboxymethyl cellulose, the concentration of

control of 1.5% , by adding potassium bicarbonate and potassium chloride as buffer system, the concentration of control at 0.1% or less. At this time physical and chemical properties testing pH, osmotic pressure, specific gravity and refractive index were adjust to achieve the following criteria:(1)pH7.3-7.8; (2)osmotic:311-350mOsm; (3)weight: approximately equal to 1; (4)refractive index: 1.336. Finally adding preservatives benzalkonium bromide, the concentration was 0.005%.

Methods Forty-five big male rats were randomly divided into nine groups: A1, B1, C1; A2, B2, C2; A3, B3, C3; with five rats for each group. The A stood for fake operation normal group (short for normal group as follows), the B stood for operation control group (short for control group), and the C stood for therapy group with *Buddleja officinalis* extract eye drops (short for therapy group); The "1" stood for being fed for 1 month, the "2" stood for being fed for 2 months and the "3" stood for being fed for 3 months. Ma^[1] method was referred to make animal model: the orchietomy of double sides: experimental rats from B1, B2, B3, C1, C2, C3 groups were fed by either food or water 12 hours before the experiment, their outside muscle of back thigh were disinfected by iodide, and they were anesthetized with 30mg/kg infection of ketamine hydrochloride through abdomen. As to male rats, they were on their back with four limbs open and fixed, the hair in their lower abdomen were faded by special medicament, and their hypescrotum were partially anesthetized with 20g/L lidocaine, and were disinfected by iodide. Then they were put on a germfree sheet, and one testicle was squeezed into scrotum through peritoneum to be prevented from slipping. After that, the scrotum was cut a hole in with a disinfecting knife, and the testicle was squeezed out with force, then the vein and tube of sperm duct were tightly pricked and the testicle and attaching testicle were cut off. After the scrotum skin partial iodine was sewed and disinfected in case of infection. The other testicle and the attaching testicle were cut in the same way. As to the animals in A1, A2, and A3 groups, only scrotum was cut, and the scrotum skin was constantly sewed after the operation. 200 000 U penicillin was injected into the muscle drop before the operation, and 200,000 U penicillin was injected with continuous three days muscle infection after the operation in case of infection. Groups A1 and B1 were dropped with saline eye drops everyday, three drops per day and group C1 was dropped with *Buddleja officinalis* eye drops for everyday, 1 drop per day. All of the three lasted one month, and after one month's normal feeding, animals in these three groups were killed. Groups

A2 and B2 were dropped with saline eye drops everyday, three drops per day and for group C2 was dropped with Buddleja officinalis eye drops everyday, 1 drop per day. All of the three lasted two months, and after two months' normal feeding, animals in these three groups were killed. Groups A3 and B3 were dropped with saline eye drops for everyday, three drops per day and group C3 was dropped with Buddleja officinalis eye drops everyday, 1 drop per day. All of the three lasted three months, and after three months' normal feeding, animals in these three groups were killed.

Forty-five male rats were respectively made SIT and BUT tests. The first time was before the rats were divided, and the other was after the last prescription was taken by the rats. The standard of the tests referred to the diagnoses standard of SIT and BUT. Animals' heads were cut down as soon as the SIT and BUT tests were finished, and lacrimal gland were removed the at once, then fixed with over 4% formaldehyde for the checking of flow cytometer.

The lacrimal gland tissues were washed with normal saline, cut into pieces, washed again with saline. The cell suspension were collected, then centrifugally washed for twice with normal saline, centrifugally washed again and the supernatant were abandoned. The tubes were added cytoperm with 500µL PBS-5g/L paraformaldehyde to break film. The supernatant was absorbed after centrifugation. Then added mouse anti-human1:40 AR (I resistance), and control tubes were added man equivalent IgG1Kappa I resistance with the same type. After the mixture was shook to make homogeneous, put in a warm room, 30 minutes in shadow. Then it was centrifuged, the supernatant was abandoned, washed with PBS for twice. Two tubes were added respectively into 1:80 FITC-IgG100 (II resistance), and after the mixture was shook to make homogeneous, put in a warm room, 30 minutes in shadow, and then it was centrifuged, the supernatant was abandoned, washed with PBS for twice. Again centrifugation, the supernatant was abandoned and the cells were suspended 500µL PBS-5g/L paraformaldehyde for test.

The flow cytometry of GACS type made in American BD company was applied to measure. Before the test the chicken erythrocytes were taken as standard sample to adjust the apparatus' coefficient of variation. 1 000 000 cells of each sample were tested. The quantitative analysis for androgen receptor flow cytometer uses dependent on the intensity of specific fluorescence. And this number varied with the combined digit capacity of the androgen receptor on each cell. Analyze Relative Fluorescence Intensity (RFI), (RFI equals the average fluorescence intensity in the test tube

Table 1 SIT values before and after the use of eye drops

Group		(mean±SD, mm)		
		1 month	2 months	3 months
Normal	Before	15.3±5.7	14.8±5.2	15.3±4.2
	After	15.4±5.9	15.3±5.0	16.1±5.9
Model	Before	13.4±2.3	15.5±4.3	14.5±3.1
	After	6.7±2.3 ^d	4.7±1.0 ^{a,d}	2.7±2.0 ^{b,d}
Therapy	Before	13.4±3.3	14.7±3.7	14.5±3.0
	After	14.7±5.3 ^f	13.3±5.7 ^f	12.7±5.2 ^f

^a $P<0.05$, ^b $P<0.01$ vs 1 month; ^d $P<0.01$ vs before castrated; ^f $P<0.01$ vs model group

mining the average fluorescence intensity in the control tube), and the result showed with positive expression rate.

Statistical Analysis Data were analyzed the patches statistically with SPSS13.0 software, and tested both sides. $P\leq 0.05$ means difference has statistical significance.

RESULTS

Basic Tear Secretion The comparison of SIT measured value in normal groups (A1, A2, and A3): according to the variance analysis, there was no significant difference among groups A1, A2 and A3 (Table 1, $P>0.05$), and there was also no obvious difference for rats' SIT of its pre and post self-match t test, before and after the use of physiologic brine eye drops ($P>0.05$). The comparison of SIT measured value in model groups (B1, B2, and B3): the model group was castrated respectively for 1, 2, and 3 months both pro and post self-match t test, the basic tears secretion volume had decreased obviously and the wetness length of filter paper had shortened. The changes were significant ($P<0.01$, $P<0.01$, $P<0.01$). Moreover, by variance analysis, after being castrated, the tears secretion volumes of groups B2, B3 were even lower ($P<0.05$, $P<0.01$), compared with that of group B1. The comparison revealed that the basic tears secretion volume was on the decline with the increase of time.

The comparison of SIT measured value in therapy groups (C1, C2, and C3): the castrated rabbits were treated with extract of Buddleja officinalis eye drops. The results were through variance analysis and indicated that there was no obvious difference between C1, C2 and C3 ($P>0.05$). Moreover, through variance analysis, the SIT values of group C had apparent difference ($P>0.05$, $P>0.05$, $P>0.05$), which showed the eye drop significantly kept basic tears secretion volume.

The contrast of the SIT value between group C and group B (C1 vs B1, C2 vs B2, C3 vs B3): contrasted with the former wetness length of filter paper, the basic tears secretion volume of group C had greatly increased through the therapy

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of eye drops. By the variance analysis, the wetness length of filter paper of group C was apparently longer than that of group B ($P < 0.01$, $P < 0.01$, $P < 0.01$).

Tear Film Stability The contrast of BUT values of sham group (A1, A2, and A3): by variance analysis, there were no apparent differences among A1, A2 and A3 ($P > 0.05$). Furthermore, by t investigation, the BUT values of various groups showed no obvious changes after the use of natural saline ($P > 0.05$). The contrast of BUT values of model groups (B1, B2 and B3): through t investigation, the tear film stability of model group was on the decrease after being castrated respectively 1, 2 and 3 months later. The BUT value was longer than before ($P < 0.01$, Table 2). Moreover, compared with the tear film stability of group B1, the BUT value of group B3 was apparently even lower ($P < 0.05$) after being castrated, showing that the tear film stability was on the decline with the time. The comparison of BUT values of therapy groups (C1, C2 and C3): through variance analysis, there were no obvious differences in BUT values among groups C1, C2 and C3 after the treatment of eye drops ($P > 0.05$). And through t investigation, the BUT values of various groups showed no difference after the use of eye drops ($P > 0.05$, $P > 0.05$, $P > 0.05$), indicating that the extract of *Buddleja officinalis* eye drops significantly kept tear film stability. The comparison of BUT values between therapy group and model groups (C1 ν B1, C2 ν B2, C3 ν B3): compared with the former BUT, the tear film stability of groups C1, C2 and C3 had increased after the treatment of eye drops by variance analysis. The BUT has increased apparently contrasted with the corresponding model groups B1, B2 and B3 ($P < 0.01$, $P < 0.01$, $P < 0.01$). Furthermore, by variance analysis, compared with the tear film stability of group C1 castrated 1 month later, the BUT of group C3 castrated 3 months later was relatively lower ($P < 0.05$). The results proved that the eye drops' capability for keeping tear film stability was on the decrease with the time.

Lacrimal Gland's Androgen Acceptor Investigated by FCM, the AR positive expression values of various groups were listed in Table 3. The contrast of AR positive expression values of sham groups (A1, A2 and A3): by variance analysis, there were no obvious differences among groups A1, A2 and A3.

The contrast of AR positive expression values of model groups (B1, B2 and B3): by variance analysis, compared with the AR positive expression values of groups B1 and B3, the value of group B2, castrated 2 months later, is relatively higher ($P < 0.05$, $P < 0.05$). The results indicated that the AR positive expression values had the tendency of

Table 2 BUT values before and after the use of eye drops (mean \pm SD, s)

Group		1 month	2 months	3 months
Normal	Before	A1 14.3 \pm 3.2	A2 15.3 \pm 0.3	A3 14.8 \pm 5.7
	After	14.5 \pm 2.6	14.1 \pm 6.0	15.3 \pm 4.4
Model	Before	B1 13.6 \pm 1.8	B2 14.5 \pm 4.1	B3 14.5 \pm 4.1
	After	7.4 \pm 1.1 ^b	6.7 \pm 2.3 ^b	4.7 \pm 2.1 ^{a,b}
Therapy	Before	C1 14.6 \pm 2.8	C2 14.6 \pm 2.2	C3 13.8 \pm 1.7
	After	14.9 \pm 4.4 ^d	12.8 \pm 2.7 ^d	11.7 \pm 4.4 ^{a,d}

^a $P < 0.05$ ν 1 month; ^b $P < 0.01$ ν before castrated; ^d $P < 0.01$ ν model group

Table 3 Positive expression of AR values (mean \pm SD, RFI)

Group		1 month	2 months	3 months
Normal	A1	60.1 \pm 2.1 ^b	A2 62.8 \pm 4.1 ^b	A3 59.8 \pm 4.3 ^b
Model	B1	38.1 \pm 3.3	B2 42.6 \pm 4.9 ^a	B3 33.3 \pm 7.1
Therapy	C1	52.1 \pm 5.9 ^b	C2 47.5 \pm 1.9 ^b	C3 49.3 \pm 3.4 ^b

^a $P < 0.05$ ν 1 month; ^b $P < 0.01$ ν model group

decreasing, then rising followed by decreasing again with the time. The contrast of AR positive expression values of therapy groups (C1, C2 and C3): by variance analysis, compared with the AR positive expression value of group C1 castrated 1 month later, the value of group C3 castrated 3 months later was relatively lower ($P < 0.05$). Maybe the tissue adapt gradually to the androgen-like effects of the flavonoids of *Buddleja officinalis* and the easing feedback adjustment caused the results. The contrast of the AR positive expression values between therapy group and model groups (C1 ν B1, C2 ν B2 and C3 ν B3): by variance analysis, the AR positive expression values of groups C1, C2 and C3, receiving the treatment of *Buddleja* extract eyedrops after being castrated, increased apparently compared with the corresponding model groups B1, B2 and B3 ($P < 0.01$, $P < 0.01$, $P < 0.01$). The results indicated that the flavonoids of *Buddleja* had pretty good androgen-like effects.

DISCUSSION

Chinese medicine is one of ideal source for treating dry eye because Chinese medicine provides a large variety of natural herbs to choose from as well as the high safety of herbal hormones in clinic use. However, the researches are new from the perspective of Chinese medicine to treat of dry eye. Meanwhile, these researches are lack of depth and extent, no drugs for special treating dry eye, no further modern study of dry eye which combines theories and practices of Chinese medicine, no safety measurements and evaluations for dosage of eye drops and no consideration for the fake signs of worsening dry eye caused by the eye drops. It has been shown in the previous researches that the flavonoids in *Buddleja officinalis* had sound experimental effects to the

animal models to treat dry eye caused by castrate, which may relate to the androgen-like effects of the flavonoids in *Buddleja officinalis*, but further researches are needed to make clear whether the mechanism is similar and how it act^[2-5]. We aim at proving the androgen-like effects of eye drops made from extract of *Buddleja officinalis* in lacrimal gland and the curative effects of dry eye caused by the declining of androgen level via the research of androgen-like effects of eye drops made from extract of *Buddleja officinalis* in lacrimal gland.

Dry eye hasn't got a definitive name in TCM. According to the degree of syndromes it can be classified into slight level "baisezheng", and the heavy level "Less Aqueous Humor" and "waizhangyizheng". The dry eye caused by gonadal hormones level imbalance should be classified into "Less Aqueous Humor", "waizhangyizheng" is the result of deterioration. Milun Yanke Lunmulun states "What is Dry Eye? It is caused by the heat of zhang and fu organs. The waterway is dry, the heat transfer to the defense, the genuine qi cannot nourish the eye. This is the reason." Therefore, "Less Aqueous Humor" is caused by damage of yin by the inner heat, and the heat transfer into Wind-heat externally. Suwen xuanmingwuqioian says "fivezhang organs transfer into fluid, and liver into tear." Yin Haijingwei also says "tear is the fluid of liver". From these we can see that "shenshuijiangku" is rooted in liver, so the principle for treatment should focus on clearing the liver heat, nourishing the liver yin and relieving the Wind-heat externally.

Buddleja officinalis can clear and nourish the liver, it is the right drug for liver meridian of jueyin^[6]. Yin Haijingwei Yaixinlun says "*Buddleja officinalis* acts on liver meridians and brighten the eyes" The representative dosage collected is Mimenghuasan with *Buddleja officinalis* as its main herb. Ying Haijingwei Juanzhixia says "Mimenghuasan: for treatment the lingering internal and external zhangyi, fear of the sunshine qi stagnation caused by Windheat." Mizhuan Yanke Lunmulun has some statement for treat Dry Eye: "Mimenghuasan: for treat the attack of wind, dizziness and fear of the sunshine. The eye is hard to open, itching or aching dizziness, dry, dull pain with red swelling and aching eye can be treated by it." From this we can see that *Buddleja officinalis* can clear the liver heat and supplement liver yin internally, and relieve the Windheat of the defense externally. This is the mechanism of Dry Eye in TCM. With the theory of TCM, it is an ideal drug for Dry Eye.

The effective part of *buddleja* is flavonoids and eight kinds of flavonoid compounds are available after being detached^[7]. Eyes have proven to be the target organs of sex hormone. It

is confirmed that androgen receptor is widely present in the lacrimal gland, cornea and other ocular tissues of human^[8], rabbit and mouse; Lacrimal gland epithelial cells are target cells of androgen; AR exists in the acinar and tubular epithelial cell's nucleus. Androgen and flavonoids are polyphenolic heterocyclic compounds, which can combine with the AR taking advantage of the similarity of its chemical structure.

The current study has shown that some flavonoid compounds have the androgen-like effects^[9] and can be used to treat certain diseases due to the decrease of androgen levels, such as osteopenia^[10] and so on. Study using radioactive tracer marking method also shows that flavonoids is the LATs of cell AR and have biological effects combined with cell AR^[11]. It shows that the flavonoids contained in *Buddleja* should also be combined with AR to produce androgen-like effects to treat diseases due to the decrease of androgen level, including the disease of dry eye. This treatment is in line with Western medical pathogen and pathology based on Western medical theory. Therefore the flavonoids contained in *Buddleja* combined with cell AR is the ideal alternative to androgen for treatment of dry eyes.

Animal-model adopts the castrate method. Considering the inevitable trend of declining of androgen level, androgen-like effects cannot be evaluated by the androgen level. But there are androgen-receptors in eye tissues and by analyzing the situation of them; the androgen-like effects can be evaluated by the upward trend of androgen receptor. AR extensively exist in various organs of human body, and their declining can result in the declining of AR level in old men's body and low reaction to androgen^[12]. Measurements are carried out to AR by using the fiber cells from old man's genitalia, it did turn out that AR were declining in quality and quantity, which makes the syndromes of androgen declining more obvious. This experiment found the mouse in model groups (B1, B2, and B3) after castrating, AR (positive) were lower than blank group by examining of FCM. Similar to the AR situation with old men mentioned above, this means this model is successful. It is reported that androgen has double effects to AR: less concentration^[13] testosterone (1-10nmol/L) will increase the level of AR, androgen can increase the proteins, or changes in AR-transcriptional modification, or increase the stability of the proteins; Intermediate dosage will increase the gene expression of AR, high intensivetestosterone (100nmol/L (1-10nmol/L and above)^[14] will decrease the level of AR. From this we can see that androgen can adjust from various

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levels. We found the mouse in treating groups (C1, C2, and C3) treated by eye drops made from extract of *Buddleja officinalis* in lacrimal gland after castrating and examined by FCM, there was a pronouncing increase in positive AR rate compared with model groups(B1, B2, and B3). The reason may be the sound androgen-like effects of *Buddleja officinalis*, whose structures are similar to androgen which combined with AR. Yet they are not real androgen, they cannot take place to meet the high concentration level of testosterone, so they can only take effects to form low or intermediate level, so that to increase the level of AR. Besides, positive AR rate castrating treating of 3m in C3 were comparatively lower than castrating treating of 1m in C1, which may caused by the tissue gradually adapt to the androgen-like effects of *Buddleja officinalis* the feedbacks were mitigated.

Recently, androgen are considered to be more and more important in treating dry eye and considered to be a new way. But to use androgen alone may cause some inevitable side effects such as masculine, acnes and so on. Meanwhile, sex hormones have bi-effects and bi-control each other, to use androgen alone may cause imbalance. Therefore, to find a substitute of androgen to treat dry eye is of special significance. The unique direct or indirect effects of androgen-like effects of *Buddleja officinalis* in treating dry eye deserve further study.

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