Activities of autonomic neurotransmitters in meibomian gland tissues are associated with menopausal dry eye[*]

Lianxiang Li[1], Dongling Jin[2], Jinsheng Gao[2], Liguang Wang[3], Xianjun Liu[2], Jingzhang Wang[4], Zhongxin Xu[1]

1 Department of Anatomy, Histology and Embryology, Laboratory of Basic Medicine, Medical College, Hebei University of Engineering, Handan 056002, Hebei Province, China
2 Department of Pathology, Medical College, Hebei University of Engineering, Handan 056002, Hebei Province, China
3 Department of Physiology, Medical College, Hebei University of Engineering, Handan 056002, Hebei Province, China
4 Department of Biochemistry, Medical College, Hebei University of Engineering, Handan 056002, Hebei Province, China

Abstract

The secretory activities of meibomian glands are regulated by the autonomic nervous system. The change in density and activity of autonomic nerves in meibomian glands during menopause play an important role in the pathogenesis of dry eye. In view of this, we established a dry eye rat model by removing the bilateral ovaries. We used neuropeptide Y and vasoactive intestinal polypeptide as markers of autonomic neurotransmitters. Our results showed that the concentration of estradiol in serum significantly decreased, the density of neuropeptide Y immunoreactivity in nerve fibers significantly increased, the density of vasoactive intestinal polypeptide immunoreactivity in nerve fibers significantly decreased, and the ratio of vasoactive intestinal polypeptide/neuropeptide Y positive staining significantly decreased. These results suggest that a decrease in ovary activity may lead to autonomic nervous system dysfunction, thereby affecting the secretory activity of the meibomian gland, which participates in sexual hormone imbalance-induced dry eye.

Key Words

ovariectomy; meibomian gland; meibomian gland dysfunction; neuropeptide Y; vasoactive intestinal polypeptide; autonomic nervous system dysfunction; dry eye; sexual hormone; rats; autonomic nerve; neural regeneration

Research Highlights

(1) The mechanism underlying the development of dry eyes that are caused by sexual hormone disorders remains unclear. Clinical results have shown that the incidence of dry eye in women is five to six times greater in men. The incidence of dry eye in menopausal or postmenopausal women significantly increases, and women with ovarian failure are more likely to show ocular surface damage and suffer from dry eye.
(2) In the ovariectomized dry eye rat model, the expression of neuropeptide Y increased, while the expression of vasoactive intestinal polypeptide decreased. These results indicate that the decreased activities of the ovaries may lead to autonomic nervous system dysfunction, thereby affecting the secretory activity of the meibomian gland, which participates in sexual hormone imbalance-induced dry eye.

Abbreviations

MGD, meibomian gland dysfunction; NPY, neuropeptide Y; VIP, vasoactive intestinal polypeptide

Lianxiang Li★, Master, Professor, Department of Anatomy, Histology and Embryology, Laboratory of Basic Medicine, Medical College, Hebei University of Engineering, Handan 056002, Hebei Province, China

Corresponding author: Lianxiang Li, Department of Anatomy, Histology and Embryology, Laboratory of Basic Medicine, Medical College, Hebei University of Engineering, Handan 056002, Hebei Province, China
LianxiangLm@sohu.com

INTRODUCTION

The meibomian glands are longitudinally located in both the upper and the lower tarsi, with approximately 30–40 meibomian glands in the upper eyelid and 20–30 in the lower lid. These glands appear faint yellow in color *in vivo*. Histologically, the meibomian glands are complex vesicular glands belonging to modified sebaceous glands. Each gland consists of a duct and a number of acini, with the main duct opening at the posterior lip of the lid margin, which facilitates the distribution of secretory lipids on the front surface of the tear film[1]. Meibomian gland acini are mostly spherical or elliptical in shape, and are wrapped by the tunica. The peripheral cells around the acini are cuboidal, and no fat droplets are visible in the cytoplasm. As epithelial cells differentiate and develop, they gradually move towards the inside of the acinus, increase in volume and change morphologically to a polygonal shape, after which fat droplets appear in the cytoplasm. When cells differentiate, develop and migrate to the acinar center or near to the catheter, the cell volume sharply increases along with the amount of fat in the cytoplasm, until the fat droplets completely occupy the cells. The fat droplets and other substances are excreted into the catheter by pulling pulp secretions. Meibomian glands secrete various lipids including cholesterol, cholesterol esters, wax esters, triglycerides, phospholipids, free cholesterol, and free fatty acids. The melting points of these lipids differ, which is very important for the even distribution and stability of the tear film. The lipids secreted by the meibomian glands serve to prevent the evaporation of liquid water, reduce ocular surface tension in the tear film and maintain the health of ocular surface tissue. Any factor disturbing the synthesis, concentration, secretion and excretion of lipids in the meibomian glands will lead to decreased secretion or a change in the composition of lipids, resulting in meibomian gland dysfunction (MGD)[2-5]. MGD is known to be one of the causes of ocular surface damage and dry eye[6-8], and its etiology can be found in chalazions and blepharitis[9] following an imbalance of hormone levels[10-11], or exposure to noxious chemical or physical stimuli[12]. The incidence of MGD and dry eye increases with age[13-14], however, it significantly shows an obvious gender difference, and women with premature ovarian failure[15] or those experiencing menopause have a higher incidence of disease[16-19]. The ovariectomized rats can develop meibomian gland tissue injury and dysfunction, and subsequent dry eye[20-21]. Neuroanatomical studies have shown that the autonomic nerves are abundant in meibomian gland tissue from different animals, which plays an important role in regulating the secretory activities of meibomian glands[22-25]. However, the pathogenesis of MGD and dry eye is not very clear. These diseases often occur before or after menopause, and they are often accompanied by symptoms that are usually caused by automatic dysfunction at menopause, such as hot flashes, sweating, irritability, palpitations, xerostomia, and nasal cavity dryness.

Therefore, we hypothesized that autonomic nervous system dysfunction caused by a decrease in ovarian function may affect the secretory activity of the meibomian gland, which results in the pathogenesis of dry eye at menopause. In this study, we established a menopausal dry eye rat model by performing ovariectomy. We quantitatively studied the changes in peptidergic innervation and the activities of neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP), and determined the ratio in meibomian glands to confirm our hypothesis.

RESULTS

Quantitative analysis of experimental animals

Female Sprague-Dawley adult rats (*n* = 48) were randomly divided into a control group (normal feeding), a sham surgery group (only exposing the wound without the ovarian resection) and a model group (both ovaries removed by establishing a castrated rat model of xerophthalmia), with 16 rats in each group. All 48 rats were used in the final analysis.

Measurements of estradiol in serum from dry eye rat models

At 12 weeks after model establishment, the concentration of serum estradiol in rats in the model group was 15.22 ± 2.89 ng/L, which was significantly lower than the control group (38.04 ± 4.52 ng/L) and the sham surgery group (36.13 ± 6.11 ng/L; *P* < 0.01).

Expression of NPY and VIP immunoreactive nerve fibers in the dry eye rat models

At 12 weeks after model establishment, immunohistochemistry experiments showed that NPY and VIP immunoreactive nerve fibers in tissues of the meibomian glands were yellow or brown with various shapes, such as dots, cords and beads. In the control group and the sham surgery group, many positive NPY and VIP immunoreactive nerve fibers existed around the acini in the meibomian glands, and they were closely

attached to the acinar basal membrane. Uneven distribution of the NPY and VIP immunoreactive nerve fibers were observed. NPY and VIP immunoreactive nerve fibers were occasionally seen in the acinar interstitial space. In the model group, the distribution of NPY and VIP immunoreactive nerve fibers was similar to that in the control group and sham surgery group.

The density of the NPY immunoreactive nerve fibers in the model group was higher with a deeper color than that in the control and sham surgery groups (Figure 1), while the density of the VIP immunoreactive nerves in the model group was lower, with a weaker color than that in the other two groups (Figure 2).
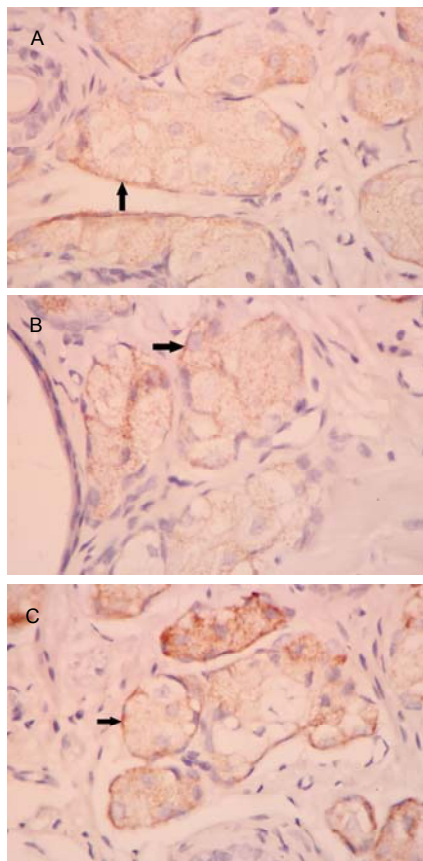

Figure 1 Distribution of neuropeptide Y immunoreactive nerve fibers in meibomian gland tissues (immunohistochemical staining, × 400).

At 12 weeks after model establishment, the density of neuropeptide Y immunoreactive nerve fibers in the model group (C) was higher with a deeper color than that in the control group (A) and the sham surgery group (B). The cross-sections of neuropeptide Y immunoreactive nerve fibers in the meibomian glands were yellow or brown, and shaped like dots or cords.

Arrows mean the neuropeptide Y immunoreactive nerve fibers around the meibomian gland acini.

Density of NPY and VIP immunoreactive nerve fibers in the meibomian gland acini

The density of NPY immunoreactive nerve fibers in the model group was significantly higher than that in the control and sham surgery groups ($P < 0.01$). In contrast, the density of VIP immunoreactive nerve fibers in the model group was significantly lower than that in the control and sham surgery groups ($P < 0.05$; Table 1).
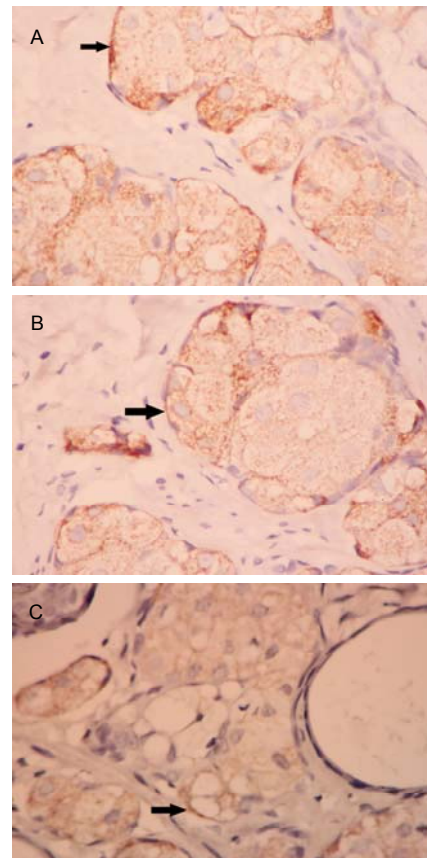

Figure 2 Distribution of vasoactive intestinal polypeptide immunoreactive nerve fibers in meibomian gland tissues (immunohistochemical staining, × 400).

At 12 weeks after model establishment, the dot or cord-like vasoactive intestinal polypeptide immunoreactive nerve fibers stained brown were observed in the acinar basal membrane around the acini in the meibomian glands. The density of vasoactive intestinal polypeptide immunoreactive nerve fibers (arrows) in the model group (C) was lower and had a weaker color than that in the control group (A) and sham surgery group (B).

Staining intensity of NPY and VIP immunoreactive fibers in meibomian glands

In the model group, the staining intensity of NPY immunoreactive fibers was significantly higher than that in the control and sham surgery groups ($P < 0.01$), while that of VIP immunoreactive fibers was significantly lower ($P < 0.05$), and the VIP/NPY ratio of the immunoreactive fibers was significantly lower ($P < 0.05$; Table 2).

Table 1   The density of neuropeptide Y and vasoactive intestinal polypeptide immunoreactive nerve fibers in meibomian gland acini

Group	Neuropeptide Y	Vasoactive intestinal polypeptide
Control	0.26±0.03[a]	0.33±0.02[b]
Sham surgery	0.25±0.03[a]	0.35±0.02[b]
Model	0.40±0.03	0.26±0.02

Each complete acinar cross-section is taken as a unit of observation. All the immunoreactive nerve cross-sections that were discontinuously distributed in the basement membrane of each acinus are summarized. The sum (μm) of the immunoreactive nerve fibers was then divided by the perimeter length (μm) of the indicated acinus, and the result was the density of the immunoreactive nerve fibers (× 400).

[a]$P < 0.01$, [b]$P < 0.05$, *vs.* model group. Data were expressed as mean ± SD (n = 16). One-way analysis of variance was used for multi-group comparison, and the Students-Newman-Keuls test was used to compare both groups.

Table 2   The staining intensity of neuropeptide Y and vasoactive intestinal polypeptide immunoreactive nerve fibers in meibomian gland acini

Group	Neuropeptide Y	Vasoactive intestinal polypeptide	Vasoactive intestinal polypeptide/ neuropeptide Y
Control	0.58 (0.55–0.62)[a]	0.69 (0.66–0.71)[b]	1.20 (1.17–1.23)[b]
Sham surgery	0.57 (0.54–0.61)[a]	0.66 (0.64–0.69)[b]	1.16 (1.13–1.20)[b]
Model	0.88 (0.84–0.92)	0.42 (0.39–0.45)	0.49 (0.46–0.51)

[a]$P < 0.01$, [b]$P < 0.05$, *vs.* model group. Data were expressed as median (range), n = 16. The Kruskal-Wallis test was used for multi-group comparisons. Staining positive index = ratio of immunoreactive nerve fibers in the indicated acinus × staining intensity of the immunoreactive nerve fibers in the indicated acinus.

DISCUSSION

The meibomian gland was first described by Heinrich Meibom in 1666. People have only come to realize the role of the meibomian gland in dry eye, blepharitis and ocular surface diseases in the past 20 years. Dry eye is a common eye disease induced by many factors, such as reduced tear production, mucus secretion abnormalities, and meibomian gland dysfunction, and involves the absolute or relative lack of the tear film, abnormal composition of tears, and increased evaporation of tears. The tear film covers the eye and the conjunctiva surface, and is the special product secreted by the ocular surface tissue, which maintains its health. The tear film is divided into an external lipid layer, a water layer, and a mucus layer. The mucus layer is secreted by the conjunctiva goblet cells and the corneal epithelial cells,

which are attached to the surface of the corneal epithelium. The main components of the mucus layer is mucin and trefoil factor-1, which plays an important role in maintaining the stability of the tear film, lubricating the eye, participating in intracellular signal transduction, and building the ocular surface tissue barrier to defend against microbial invasion. The water layer is secreted by the lacrimal and accessory lacrimal glands, and is mainly composed of water, with a certain amount of inorganic salt, urea, glucose, lactoferrin protein, lysozyme, IgA, and complement. The lipid layer is secreted by the meibomian gland, which is a transparent grease-like substance, and has the function of preventing the evaporation of liquid water, reducing ocular surface tension, and increasing the stability of the tear film.

Importantly, secretions from the meibomian glands comprise the lipid layer. The content, composition, physical properties, and viscosity of these secretions can change the stability of the tear film. Once the quality or quantity of the lipid component is abnormal, it will result in increased evaporation of the tear film, leading to dry eye. The secretions from meibomian glands can be divided into four types according to their appearance: (1) egg white-like, transparent, colorless or light yellow secretions; (2) milk yellow-like, evenly turbid, creamy yellow secretions; (3) granular, liquid secretions with small white or yellowish granules; (4) toothpaste-like secretions that secrete out from the meibomian gland opening similar to squeezed toothpaste. The first type of secretion is normal, while the others are abnormal, positively correlating to MGD[26]. According to the changes in meibomian gland lipid secretions, MGD can be divided into three types: (1) hypersecretion, (2) hyposecretory and (3) obstructive MGD. The latter is considered to be the most common type[27], and is the main type observed in clinical and animal studies.

To study the relationship between MGD and dry eye as well as the pathophysiological changes of MGD, studies have blocked the excretion of meibomian gland secretions by means of locally administering adrenaline to induce keratosis of the meibomian gland ducts[28], or burning the meibomian gland openings[29], resulting in an obstructive MGD model. As a result, all model animals had evaporative dry eye, reflecting the importance of lipids secreted by the meibomian glands in maintaining ocular surface health.

The main histopathological change of the obstructive MGD meibomian gland is hyperkeratosis of the

meibomian gland ducts, resulting in clogged conduit opening, and consequently catheter dilatation and acinar disuse atrophy[27]. The pathological changes in meibomian gland tissues in the MGD caused by aging and evaporative xerophthalmia are very similar to obstructive MGD, with acinar atrophy, catheter keratosis, cystic dilatation, and reduced lipid synthesis[30-31]. Recent studies also showed that the pathological changes of the meibomian gland also included reduced alveolar epithelial cell proliferation, acinar volume decreases, and inflammatory cell infiltration[32].

Currently, the classification of dry eye obeys the dry eye taxonomy described by the U.S. National Eye Institute in 1995. This taxonomy suggests that dry eye includes tear deficiency and strong evaporative dry eye[33]. The factors inducing evaporative dry eye include MGD, blepharitis, frequent blinking, hot and dry climate, and seriously polluted air. Among these factors, MGD is the main reason for evaporative dry eye. According to previous literature[34], MGD is very common, and occurs in 39–50% of the U.S. population.

With the application of the infrared instrument for the examination of meibomian gland and our increasing understanding of MGD, it has been shown that the incidence of MGD significantly increases and that aging is a risk factor for MGD[35-37]. However, gender-related dry eye studies reveal that the elderly are prone to dry eye, especially postmenopausal women. The incidence of dry eye in menopausal women is significantly higher than premenopausal women[18], which may be because of menopausal women having ovarian endocrine dysfunction, disruption in sex hormone levels that cause autonomic nervous system dysfunction, and reduced secretion of the lacrimal gland, conjunctiva and meibomian gland. In pre- or postmenopausal women, the occurrence of dry eye is often accompanied with menopausal autonomic function disorders and other symptoms and signs. After treatment with Nylestriol, it was found that the secretion of tears and the stability of the tear film were both enhanced, and dry eye and other symptoms of menopausal syndrome also improved. However, the pathogenesis of sex hormone disorders and the structural and functional changes to meibomian gland tissues are unclear. Lin *et al*[20] identified stratified squamous metaplasia in meibomian gland acini, epithelial cell atrophy, and apoptotic acceleration after the removal of both ovaries in rats, which leads to meibomian gland dysfunction.

To further investigate the histopathological relationship

between sex hormone disorders and meibomian gland tissues, the transmission electron microscopy was used to study ultrastructural changes of the meibomian gland acini in the ovariectomized xerophthalmia rat model. We found that the volume of the meibomian gland acinar epithelial basal cells decreased, and that the nucleus had diverse morphological variation, such as irregular-shaped, kidney-shaped or long rod-shaped nuclei. The volume of transitional cells decreased, with reduced lipid droplets, swelling of mitochondria, structural damage to mitochondria, and pathological changes such as vacuolization and changes in morphology to a cloud-, mist-like appearance. In addition, pathological changes, such as round or irregularly shaped electron-dense lamellar bodies that consisted of eosinophilia osmium substances, and myelin-like structures, were also observed[38]. Sudan III staining revealed that lipids in acinar epithelial cells were reduced[21]. The lipids were synthesized in meibomian gland acinar epithelial cells. The integrity of epithelial cell structure in the meibomian gland is a prerequisite to ensure normal physiological metabolism. The damage and destruction of tissue structures will inevitably lead to meibomian gland dysfunction, and subsequent evaporative dry eye.

Ocular surface tissue (cornea, conjunctiva, meibomian gland), the main lacrimal gland and the nerves within these tissues jointly form a complete functional unit. Structural damage and dysfunction of any part of the functional unit will lead to the disorder of the ocular surface microenvironment, resulting in an abnormal ocular surface or the occurrence of dry eye. The secretory activities of the meibomian glands were modulated by both humoral regulation and autonomic nervous system regulation. According to the release of different neurotransmitters, the classic autonomic nervous system is divided into the sympathetic and the parasympathetic systems. In-depth studies on autonomic neurotransmitters reveal that some neurons in the autonomic nervous system can synthesize peptides, which coexist with classical neurotransmitters. All of them can be released by nerve endings, which play different roles in effector or target tissue cells.

The neuropeptides NPY and VIP are third-class neurotransmitters. Therefore, NPY and VIP peptidergic nerve fibers belong to the autonomic nervous system. NPY and VIP immunoreactive nerve fibers in the meibomian glands are postganglionic fibers that are generated from NPY-positive neurons in the superior cervical ganglion and VIP-positive neurons in the

sphenopalatine ganglion[39]. It has been shown that NPY neurotransmitters in sympathetic nerve endings and VIP neurotransmitters in parasympathetic nerve endings are related to the activities of blood vessels, conjunctiva, and meibomian glands[40-41]. It is well known that stimulation of sympathetic nerves induces the release of norepinephrine and NPY, resulting in the excitation of sympathetic nerves, while stimulation of parasympathetic nerves induces the release of VIP, resulting in the excitation of parasympathetic nerves. Normally, sympathetic nerves weaken the secretory activity of a gland, while parasympathetic nerves strengthen it. The functions of sympathetic and parasympathetic nerves are united as well as opposite, and the combination of them maintains the steady state and health of ocular surface tissues. Toshida *et al* [42] showed that this leads to xerophthalmia in rabbits by depriving the parasympathetic preganglionic fibers in the sphenopalatine ganglion. This study provides valuable evidence of the effects of autonomic nerve dysfunction during menopause on the pathogenesis of dry eye. NPY and VIP neurotransmitters are associated with sympathetic and parasympathetic nerves, respectively. Thus, the immune-positive reactions detected by immunohistochemical methods can reveal the amounts and densities of NPY and VIP neurotransmitters in meibomian glands. The higher the density, the more immunoreactive nerve fibers, and *vice versa.* The staining intensities revealed the concentrations and activities of NPY and VIP neurotransmitters in nerve fibers. The greater the staining intensity, the higher the concentrations and activities of NPY and VIP neurotransmitters, and vice versa. Thus, the densities and the intensities of VIP and NPY immunoreactive nerves indicate the morphology and functional status of the parasympathetic and sympathetic fibers, respectively. The VIP/NPY ratio of the positive index to some extent indicates the decrease or increase in excitability and the function of the parasympathetic/sympathetic nerves. Our results show that after ovariectomy, estradiol levels in serum significantly decreased, and the VIP/NPY ratio of the positive staining index in the model group significantly lowered when compared with the control group and the sham-surgery group, which indicated an increase in excitability of sympathetic nerves, a decrease in excitability of parasympathetic nerves, weakened secretory activities of meibomian glands, reduced secretion of lipids, decreased stability of the tear film, decreased tear film break time, and enhanced fluorescein staining of the corneal epithelium. These results suggest that decreased activities of the ovaries may lead to autonomic nervous system dysfunction,

thereby affecting the functional status of the meibomian gland, which participates in the pathogenesis of dry eye.

Because of the special localization, shape and distribution of the meibomian gland, it is difficult to calculate the density of innervation in the meibomian gland. Herein, we calculated nerve density using an image analysis system, which not only facilitated our research on the densities of nerve fibers in the meibomian gland, but also provided a new method for the analysis of densities of nerve fibers in other similar glands, such as the lacrimal gland, the submandibular gland, and the greater vestibular gland.

Until now, the pathogenesis of dry eye caused by an imbalance of sex hormones remained unclear. Menopausal dry eye occurs during the period of ovarian dysfunction. During this time, endocrine dysfunction and autonomic nervous system dysfunction coexist, and pathological changes to the immune system and physiological changes to the eyes coexist. Therefore, studies investigating the relationship between these dysfunctions and changes will be greatly beneficial to understanding the pathogenesis of postmenopausal dry eye.

MATERIALS AND METHODS

Design
A randomized, controlled animal experiment.

Time and setting
This study was performed at the Department of Anatomy, Histology and Embryology, Laboratory of Basic Medicine, Medical College, Hebei University of Engineering, China, between August 2010 and May 2011.

Materials
Adult female Sprague-Dawley rats (*n* = 48; 56–60 days old, weighing 180–220 g) were obtained from the Experimental Animal Center of Hebei Province, China (license No. SCXK (Ji) 2010-1-012). Animals were housed at a temperature of 25 ± 1°C. The treatment of animals obeyed the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[43].

Methods
Establishment of dry eye model in ovariectomized female rats
In the model group, the tergal skin of the rats was cut

along the spine at 1 cm below the twelfth rib. The muscles were separated, and the pink ovaries that appeared soybean-like under the kidneys were removed from the abdominal cavity. In addition, the contralateral ovary was removed. After the bilateral ovaries were removed, and it was confirmed that the incision was not bleeding, the muscle and the skin were sutured. The rats in the sham surgery group were similarly operated on but without the removal of the ovaries. After surgery, the incision was rubbed with iodine to prevent infection. The rats in the control group received no treatment.

At 12 weeks before and after operation, 2% fluorescein sodium (Shanghai Yiji Industrial Co., Ltd., Shanghai, China) was dropped into the conjunctiva of each rat, and the breakup time of the tear film and corneal epithelial fluorescence staining were measured using cobalt blue light scanning and a model-YZ5B ophthalmic slit lamp (Suzhou Medical Instrument Factory, Suzhou, Jiangsu Province, China). Successful establishment of the rat xerophthalmia model was identified based on objective diagnostic criteria as previously described[44-46]. In brief, after surgery, rats that had reduced serum estradiol levels, flaky corneal epithelium staining, and a short breakup time of the tear film were considered to be successful models. Heart blood (2–3 mL) was obtained and centrifuged at 1 500 r/min for 20 minutes. Blood serum was saved at –20°C and serum estradiol levels were examined by radioimmunoassay[47] according to the kit instructions (Tianjin Jiuding Medical Bioengineering Co., Ltd., Tianjin, China).

Preparation of meibomian gland tissue samples
All rats were killed by cervical dislocation after anesthesia. Dissected tarsus tissues containing the meibomian gland were fixed in 10% (v/v) formalin for 48–72 hours, and were then embedded in paraffin blocks. The blocks were serially sliced into 4–5 μm sections, and used for hematoxylin-eosin staining and immunohistochemical staining.

Expression of NPY and VIP in meibomian gland tissues as detected by immunohistochemical staining
To eliminate the activity of intrinsic catalase, paraffin sections were incubated in 3% (v/v) H_2O_2 for 10 minutes at room temperature, and microwave oven antigen retrieval was carried out for 10 minutes. The sections were incubated in rabbit anti-rat NPY or VIP monoclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA; 1:100) overnight at 4°C, then sections were washed with PBS (0.1 M, pH 7.4) three times each for

3 minutes. Biotin-conjugated goat anti-rat IgG secondary antibody (1:50; Beijing Zhongshan-Golden Bridge Biotech Co., Ltd., Beijing, China) was added for 15 minutes at room temperature, then the sections were washed with PBS (0.1 M, pH 7.4) three times each for 3 minutes. The sections were incubated in horseradish peroxidase-labeled streptavidin solution for 15 minutes at 37°C, and were then washed with PBS (0.1 M, pH 7.4) three times each for 3 minutes. 3,3-diaminobenzidine was added as a chromogen for 5–10 minutes. After re-staining with hematoxylin, the sections were dehydrated, mounted, and viewed and imaged under a BX-50 Olympus microscope (Olympus, Tokyo, Japan). The NPY and VIP immunoreactive nerve specimens from the rat heart were used as positive controls, and samples treated with PBS instead of primary antibody were used as negative controls.

Immunohistochemical staining and image analysis
The yellow or brown dot, cord or bead-like substances in the interstitial tissue and the basement membranes of the meibomian gland acini were NPY or VIP immunoreactive nerve fibers. Each section was placed under the microscope, and the image was displayed on the monitor. The HPIAS-2000 colorful pathology image analysis system (Tongji Qianceng Image Company, Wuhan, Hubei Province, China) was applied to analyze the staining results, including the density of NPY and VIP immunoreactive nerve fibers, and staining intensity.

NPY and VIP immunoreactive nerve fibers were mainly distributed around the meibomian gland acinar epithelium, and were closely attached to the acinar basal membrane. To facilitate the quantitative analysis of the densities of the nerve fibers, we took each complete acinar cross-section as a unit of observation, then summarized all the immunoreactive nerve cross-sections that were discontinuously distributed in the basement membrane of each acinus. The sum (μm) of the immunoreactive nerve fibers was then divided by the perimeter length (μm) of the indicated acinus, and the ratio of the NPY and VIP immunoreactive nerve fibers to the perimeter length of the indicated acinus was an indicator of the density of the nerve fibers.

Staining intensity of NPY or VIP immunoreactive nerve fibers
As previously described[48], the staining intensity of immunoreactive nerve fibers was estimated: no color, score 0; light yellow, score 1; brown, score 2; sepia, score 3. For each specimen, five acini were randomly selected, and were observed and measured under high

magnification (400 × magnification). A positive index for NPY and VIP immunoreactive nerve fibers was calculated as the following:

Positive index = the ratio of immunoreactive nerve fibers in the indicated acinus × the staining intensity of the immunoreactive nerve fibers in the indicated acinus.

Statistical analysis

Data were expressed as mean ± SD and the median (range from minimum value to maximum value). SPSS 11.5 statistical software (SPSS, Chicago, IL, USA) was used for statistical analysis. One-way analysis of variance and the Students-Newman-Keuls test were used to analyze the difference among groups. The Kruskal-Wallis test was used to analyze the median of the groups. A $P < 0.05$ value was considered statistically significant.

Author contributions: Lianxiang Li participated in the study concept and design, data analysis, and manuscript writing. Dongling Jin, Jinsheng Gao, Liguang Wang and Xianjun Liu were responsible for the integrity of the data, technical support and statistical analysis. Jingzhang Wang and Zhongxin Xu participated in experimental observations. Lianxiang Li is the master of the item. All authors approved the final version of the manuscript.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee at Medical College, Hebei University of Engineering, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

REFERENCES

[1] Knop N, Knop E. Meibomian glands. Part I: anatomy, embryology and histology of the Meibomian glands. Ophthalmologe. 2009;106(10):872-883.

[2] Li LX, Li P, Yang YH, et al. Study on ultrastructure and junction apparatus of acinar cell of the meibomian gland in rat. Hebei Gongcheng Daxue Xuebao. 2007;24(3):52-55.

[3] Mathers WD. Ocular evaporation in meibomian gland dysfunction and dry eye. Ophthalmology. 1993;100(3): 347-351.

[4] Knop E, Knop N, Schirra F. Meibomian glands. Part II: physiolohy, characteristics, distribution and function of meibomian oil. Ophthalmologe. 2009;106(10):884-892.

[5] Butovich IA. On the lipid composition of human meibum and tears: comparative analysis of nonpolar lipids. Invest Ophthalmol Vis Sci. 2008;49(9):3779-3789.

[6] Foulks GN, Borchman D. Meibomian gland dysfunction: The past, present, and future. Eye Contact Lens. 2010; 36(5):249-253.

[7] Blackie CA, Korb DR, Knop E, et al. Nonobvious obstructive meibomian gland dysfunction. Cornea. 2010; 29(12):1333-1345.

[8] Viso E, Gude F, Rodriguez-Ares MT. The association of meibomian gland dysfuction and other common ocular diseases with dry eye: a population-based study in Spain. Cornea. 2011;30(1):1-6.

[9] Bukhari A, Ajlan R, Alsaggaf H. Pevalence of dry eye in the normal population in Jeddah, Saudi Arabia. Orbit. 2009;28(6):392-397.

[10] Schirra F, Seitz B, Knop N, et al. Sex hormones and dry eye. Ophthalmologe. 2009;106(11):988-994.

[11] Knop E, Knop N. Meibomian glands. Part IV: functional, interactions in the pathogenesis of Meibomian glands dysfunction (MGD). Ophthalmologe. 2009;106(11): 980-987.

[12] Finis D, Schrader S, Geerling G. Meibomian gland dysfunction. Klin Monbl Augenheilkd. 2012;229(5):506-513.

[13] Nien CJ, Massei S, Lin G, et al. Effects of age and dysfunction on human meibomian gland. Arch Ophthalmol. 2011;129(4):462-469.

[14] Cueva M, Gonzalez-Garcia MJ, Castellanos E, et al. Correlations among symptoms, signs, and clinical tests in evaporative-type dry eye disease caused by meibomian gland dysfunction (MGD). Curr Eye Res. 2012;37(10): 855-863.

[15] Nelson LM, Covington SN, Rebar RW. An update: spontaneous premature ovarian failure is not an early menopause. Fertil Steril. 2005;83(5):1327-1332.

[16] Sullivan DA, Sullivan BD, Evans JE, et al. Androgen deficiency, Meibomian gland dysfunction, and Evaporative dry eye. Ann N Y Acad Sci. 2002;966:211-222.

[17] Peterson CM, Lesher JL Jr, Davis LS. Dry eye syndrome in postmenopausal women. J Am Med Assoc. 2002; 287(5):585-586.

[18] Pan LL, Jia H. The clinical characteristics of elderly patients with dry eye. Guoji Yanke Zazhi. 2006;6(5):1203-1206.

[19] Versura P, Campos EC. Menopause and dry eye. A possible relationship. Gynecol Endocrinol. 2005;5(20):409-416.

[20] Lin J, Wang CF, Wang LH, et al. Association between epithelial cell apoptosis in meibomian glands and tissue injury in ovariectomized rat models of xerophthalmia. Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu. 2007;29(1):5773-5776.

[21] Li LX, Li P, Wei LS, et al. Lipid changes of meibomian gland acinar epithelium in ovariectomized rats. Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu. 2008; 12(15):2854-2858.

[22] Chung CW, Tigges M, Stone RA. Peptidergic innervation of the primate meibomian gland. Invest Ophthalmol Vis Sci. 1996;37(1):238-245.

[23] Seifert P, Spitznas M. Immunocytochemical and ultrastructural evaluation of the distribution of nervous tissue and neuropeptides in the meibomian gland. Graefes Arch Clin Exp Ophthalmol. 1996;234(10): 648-656.

[24] LeDoux MS, Zhou Q, Murphy RB. et al. Parasympathetic innervation of the meibomian glands in rats. Invest Ophthalmol Vis Sci. 2001;42(11):2434-2441.

[25] Kirch W, Horneber M, Tamm ER. Characterization of meibomian gland innervation in the cynomolgus monkey (Macaca fascicularis). Anat Embryol (Berl). 1996;193(4): 365-375.

[26] Liu JN, Dong GX. Nursing of patients with evaporative dry eye caused by meibomian gland dysfunction. Jiefangjun Huli Zazhi. 2011;28(10B):58-60.

[27] Foulks GN, Bron AJ. Meibomian gland dysfunction: a clinical scheme for description, diagnosis, classification, and grading. Ocul Surf. 2003;1(3):107-126.

[28] Jester JV, Nicolades N, Kiss-Palvolqyi I, et al. Meibomian gland dysfunction II. The role of keratinization in a rabbit model of MGD. Invest Ophthalmol Vis Sci. 1989;30(5): 936-945.

[29] Wang XD, Sheng MJ, Lin AJ. Evaluation of estabishing meibomian gland dysfunction dry eye model by cauterizing the meibomian gland orifices excising the tertiary eyelid. Yanke Yanjiu. 2010;28(9):827-431.

[30] Hykin PG, Bron AJ. Age-related morphological changesin lid margin and meibomian gland anatomy. Cornea. 1992; 11(4):334-342.

[31] Obata H. Anatomy and histopathology of human meibomian gland. Cornea. 2002;21(7):S70-74.

[32] Chyong JY, Nien CJ, Massei S, et al. Effects of age and dysfunction on human meibomian glands. Arch Ophthalmol. 2011;129(4):426-469.

[33] Lemp MA. The 1998 castroviejo lecture. new strategies in the treatment of eye states. Cornea. 1999;18(6):625-632.

[34] Lemp MA, Nichols KK. Blepharitis in the united states 2009: a survey-based perspective on prevalence and treatment. Ocul Surf. 2009;7(2):S1-14.

[35] Hom MM, Martinson JR, Knapp LL, et al. Prevalence of meibomian gland dysfunction. Optom Vis Sci. 1990;67(9): 710-712.

[36] Guillon M, Mäissa C. Tear film evaporation: effect of age and gender. Cont Lens Anterior Eye. 2010;33(4):171-175.

[37] Mäissa C, Guillon M. Tear film dynamics and lipid layer characteristics: effect of age and gender. Cont Lens Anterior Eye. 2010;33(4):176-182.

[38] Li LX, Jin DL, Gao JS, et al. A correlation study of the expression of estrogen receptors α, β of Meibomian gland with its histopathology changes in ovariectomized rats. Jiepou Xuebao. 2012;42(4):549-554.

[39] Simons E, Smith PG. Sensory and autonomic innervation of the rat eye: neuronal origins and peptide phenotypes. J Chem Neuroanat. 1994;7(1-2):35-47.

[40] Chanthaphavong RS, Murph SM, Anderson CR. Chemical coding of sympathetic neurons controlling the tarsal muscle of the rat. Auton Neurosci. 2003;105(2):77-89.

[41] Fan Q, Smith PG. Decreased vasoactive intestinal polypeptide-immunoreactivity of parasympathetic neurons and target innervation following long-term sympathectomy. Regul Pept. 1993;48(3):337-343.

[42] Toshida H, Nguyen DH, Beuerman RW, et al. Evaluation of novel dry eye model: preganglionic parasympathetic denervation in rabbit. Invest Ophthalmol Vis Sci. 2007; 48(10):4468-4475.

[43] The Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006.

[44] özcura F, Dündar SO, Cetin ED, et al. Effects of estrogen replacement therapy on apoptosis and vascular endothelial growth factor expression in ocular surface epithelial cell: An experimental study. Int J Ophthalmol. 2012;5(1):64-68.

[45] Lin J, Wang CF, Yang SS. Impaiment of corneal epithelium in estrogen deficient rat. Yanke Yanjiu. 2007; 25(11):814-817.

[46] Peng QH, Yao XL, Wu QL, et al. Establishment of dry eye model on perimenopause female rabbits with low sexual hormone. Yanke Xin Jinzhan. 2009;29(1):5-11.

[47] Qiu HM, Li X. Analysis of serum sex hormones in female patients with rheumatoid arthritis. Shiyong Quanke Yixue. 2007;5(6):543-544.

[48] Yang RC, Zhou RX, Wang P. Inveravation of the female human fetus urethral sphincter by immunohistochemical studies. Xiandai Miniao Waike Zazhi. 2007;12(4):252-254.

(Edited by Yang Y/Song LP)