The Effects of Estrogen and Androgen on Tear Secretion and Matrix Metalloproteinase-2 Expression in Lacrimal Glands of Ovariectomized Rats

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PURPOSE. Previous studies have shown that ovariectomy (OVX) induces lacrimal gland dysfunction and that androgens are implicated. This study explored the effects of estrogen and androgen on tear secretion and matrix metalloproteinase 2 (MMP-2) expression in lacrimal glands of ovariectomized rats.

METHODS. Sixty-four adult female Wistar rats were randomly divided into three groups (control, sham operated, and OVX). Bilateral OVX was performed in the OVX group. After 5 months, the OVX group was further divided into six subgroups receiving topical ophthalmic or systemic treatment with corn oil vehicle, estradiol, or testosterone for 6 weeks. Schirmer test (SIT), assessment of tear film breakup time (BUT), corneal fluorescein staining, and measurement of estradiol and testosterone levels were performed before OVX and 1, 2, 3, 4, and 5 months after OVX, as well as after 6 weeks of treatment. Lacrimal glands were assessed for MMP-2 mRNA and protein expression.

RESULTS. The mean (SD) tear film BUT decreased from 10.53 (0.79) to 9.98 (1.00) seconds (P < 0.01) in the first month after OVX, and the mean (SD) SIT result decreased by 50% from 7.32 (1.61) to 3.59 (1.15) mm (P < 0.01) in the third month after OVX. The mean (SD) corneal fluorescein staining score increased from 0.35 (0.11) to 6.02 (1.34) (P < 0.05) in the fourth month after OVX. The values increased or decreased in parallel with the time course (P < 0.01). In serum, ovariectomy resulted in a mean (SD) decline in estradiol levels from 44.38 (9.78) to 23.00 (3.78) pg/mL (P < 0.01), and the mean (SD) testosterone levels decreased from 2.42 (0.26) to 1.87 (0.15) ng/mL (P < 0.05). The mean (SD) estradiol level was elevated to 35.38 (3.34) pg/mL by systemic estradiol administration for 6 weeks, which also led to a further mean (SD) decrease in tear film BUT from 5.28 (0.81) to 3.65 (0.55) seconds (P < 0.01) and in SIT from 2.19 (1.01) to 1.47 (0.85) mm (P < 0.05), as well as a higher mean (SD) corneal fluorescein staining score from 7.59 (1.54) to 9.89 (1.27) (P < 0.05). However, the mean (SD) testosterone level was increased to 3.53 (0.67) ng/mL by systemic testosterone administration for 6 weeks. As a result, the mean (SD) tear film BUT increased from 5.08 (0.40) to 6.05 (1.48) seconds (P < 0.05), and the mean (SD) SIT result increased from 2.38 (1.20) to 3.66 (1.90) mm (P < 0.05). The mean (SD) corneal fluorescein staining score declined from 7.45 (0.73) to 4.56 (1.21) (P < 0.05). In the nontreated OVX group, the mean (SD) MMP-2 mRNA (0.66 [0.10]) and protein (0.55 [0.13]) expression in lacrimal glands was significantly increased compared with that in the sham-operated group (0.50 [0.09] and 0.40 [0.07], respectively) (P < 0.05). Systemic estradiol administration further increased the mean (SD) MMP-2 mRNA (0.85 [0.10]) and protein (0.69 [0.12]) expression (P < 0.05), while systemic testosterone administration decreased the mean (SD) MMP-2 mRNA (0.12 [0.04]) and protein (0.27 [0.07]) expression (P < 0.01). Topical ophthalmic administration of two sex hormones had no effect on the mean (SD) MMP-2 mRNA (0.59 [0.12] for estradiol and 0.57 [0.14] for testosterone) or protein (0.49 [0.11] for estradiol and 0.46 [0.13] for testosterone) expression (P > 0.05).

CONCLUSIONS. Ovariectomy-induced ocular surface impairment may be associated with androgen deficiency. A pathogenetic role for estrogen in dry eye may involve upregulation of MMP-2 expression, while androgen suppresses MMP-2 expression.

Keywords: MMP-2, ovariectomy, estrogen, androgen, dry eye

T he ocular surface is an integrated unit comprising corneal and conjunctival epithelia, meibomian glands, main and accessory lachrymal glands, and trigeminal neurons; their dysfunction results in a scarce or unstable tear film that causes dry eye, with a higher incidence among postmenopausal women. The etiology of dry eye is complicated, but it is mainly associated with inflammation, cell apoptosis, abnormal neuronal regulation, and sex hormone imbalance. It has been
recognized that sex hormones may impact the incidence and course of dry eye syndrome, especially in postmenopausal women. In vivo estrogen level is associated with the development and progression of dry eye.\(^1\) Sullivan and Allansmith\(^2\) found that orchietomy increased precorneal tear volume and that androgen administration decreased it. Scott et al.\(^3\) concluded that systemic replacement treatment with combined esterified estrogen and methyltestosterone may be efficacious for dry eye syndrome of various etiologies. The relationship between sex hormone levels and tear production is complex, and it is unclear how sex hormones regulate the functional activity of these tissues.

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that have integral physiological roles in angiogenesis, inflammation, arteriosclerosis, malignant tumor invasion and metastasis, and wound repair by degrading extracellular matrix (ECM) components. There are many factors regulating MMP expression, including cytokines, growth factors, sex hormones, neurohormones, cell morphology changes, cellular interactions, and cell transformation. In many tissues, MMP-2 has been shown to be regulated by sex hormones. Quantities of MMP-2 and MMP-9 are increased in the tears and saliva of patients with and without Sjögren syndrome (SS).\(^4\)\(^5\) Suzuki and Sullivan\(^6\) demonstrated that estrogen administration significantly upregulated gene expression of proinflammatory cytokines and MMPs (MMP-2, MMP-7, and MMP-9) in human corneal epithelial cells, which was observed after 6 hours and 24 hours of sex hormone treatment. These findings suggest that sex hormone action may have an etiologic role in the ocular surface inflammation of dry eye.

It remains controversial whether dry eye is caused by estrogen excess or deficiency, androgen deficiency, or estrogen and androgen imbalance. The mechanism by which MMP-2 expression in lacrimal glands is regulated by sex hormones remains unclear.

In this study, we observed the effects of estrogen and androgen on tear secretion and MMP-2 expression in lacrimal glands in ovariectomized rats. Furthermore, we clarified the mechanism of MMP-2 in the pathogenesis of dry eye.

**Methods**

**Animals and Groups**

Sixty-four 3-month-old female Wistar rats (range, 230–250 g) were provided by the animal center of Hebei Medical University and raised in a pathogen-free environment. They were randomly divided into the following three groups: control (n = 8), sham operated (n = 8), and ovariectomy (OVX) (n = 48). In the sham-operated group, the abdominal cavity was opened, and only partial fat was cut and removed. Rats in the OVX group underwent bilateral OVX to simulate menopause. Five months after OVX, the OVX group was further subdivided into the following six treatment groups: nontreated OVX, corn oil vehicle, systemic estradiol administration OVX, topical ophthamal estradiol administration OVX, systemic testosterone administration OVX, and topical ophthalmic testosterone administration OVX. For systemic administration (once every 3 days), estradiol benzoate diluted in 100 μL corn oil to a final concentration of 200 μg/kg was subcutaneously injected, or 3.75 mg/kg testosterone propionate was intramuscularly injected. For topical ophthalmic administration (one drop four times per day), rats were treated with ophthalmic solutions containing estradiol benzoate (1 mg/1 mL solution in one ampule) or 2.5% testosterone propionate (25 mg/1 mL solution in one ampule). For vehicle control, 100 μL corn oil was subcutaneously injected once every 3 days. After treatment for 6 weeks, the animals were killed, and lacrimal glands were obtained and divided into portions for RNA extraction and Western blot analysis.

**Materials and Methods**

Tear secretion, tear film breakup time (BUT), and corneal fluorescein staining were measured before surgery. Measurements were also obtained 1, 2, 3, 4, and 5 months after OVX, as well as after 6 weeks of treatment.

Schirmer test (SIT) was performed according to the improved method reported by Fujihara et al.\(^7\) The test paper was cut into 1 × 17-mm strips, and then a 2.5-mm fold was made at one end of the test paper and placed into the conjunctival sac. The eye was shut for 1 minute, and the length of the wet test paper was measured.

Tear film BUT was assessed. Fluorescein sodium was eyedropped into the conjunctival sac with a sterile glass rod. The appearance of fluorescein sodium in the tear film was observed after a blink by cobalt blue light using a slitlamp. The time when the first dark spot emerged on the cornea was recorded. The experiment was repeated three times to obtain an average value.

For corneal fluorescein staining, fluorescein sodium was eyedropped into the conjunctival sac with a sterile glass rod. The epithelial defect site was observed with a green stain using a slitlamp. The time when the first dark spot emerged on the cornea was recorded. The experiment was repeated three times to obtain an average value.

For corneal fluorescein staining, fluorescein sodium was eyedropped into the conjunctival sac with a sterile glass rod. The epithelial defect site was observed with a green stain using a slitlamp. The extent of corneal fluorescein staining was graded.
MMP-2 Expression in Ovariectomized Rats

Androgen and Estrogen Serum Measurements

Blood (2 mL) was collected by cardiac puncture before OVX, 5 months after OVX, and 6 weeks after sex hormone administration. The blood sample was centrifuged at 3000 rpm for 5 minutes, and the supernatant was collected for estradiol and testosterone measurement by radioimmunoassay.

Statistical Analysis

Data are presented as means (SDs) and compared with ANOVAs and Student’s t-tests using SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL). P < 0.05 was considered statistically significant.

Effect of Gonadal Hormone Supplementation on the SIT Result in Ovariectomized Rats

According to the following scoring system: absent (0 point), fewer than five spots (1 point), intermediate (2 points), and plaque or filament (3 points). The quadrant scores were summed, and the total score ranged from 0 to 12.

TABLE 2. Comparison of Tear Film BUTs in the Control Group, Sham-Operated Group, and Experimental Group (in Seconds)

<table>
<thead>
<tr>
<th>Group</th>
<th>Before OVX</th>
<th>1 mo After OVX</th>
<th>2 mo After OVX</th>
<th>3 mo After OVX</th>
<th>4 mo After OVX</th>
<th>5 mo After OVX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.94 (0.66)</td>
<td>10.88 (0.89)</td>
<td>10.80 (0.67)</td>
<td>10.97 (0.74)</td>
<td>10.90 (0.89)</td>
<td>10.74 (0.79)</td>
</tr>
<tr>
<td>Sham operated</td>
<td>10.98 (1.04)</td>
<td>10.99 (0.96)</td>
<td>10.87 (0.76)</td>
<td>10.90 (0.85)</td>
<td>10.95 (0.96)</td>
<td>10.49 (0.99)</td>
</tr>
<tr>
<td>Experimental</td>
<td>10.53 (0.79)</td>
<td>9.98 (1.00)*</td>
<td>8.46 (0.91)*</td>
<td>6.00 (1.22)*</td>
<td>5.18 (0.90)*</td>
<td>5.16 (0.83)*</td>
</tr>
</tbody>
</table>

All values are mean (± standard deviation). *
P < 0.01 versus before OVX.

Effect of Gonadal Hormone Supplementation on Tear Film BUT in Ovariectomized Rats

Ovariectomy decreased the tear film BUT in the first month after OVX from 10.53 (0.79) to 9.98 (1.00) seconds (P < 0.01); blocked in Tris-buffered saline containing 5% milk, incubated with MMP-2 antibody overnight, washed, and further incubated with secondary antibody. Blocking and secondary antibody incubation each lasted for 1 hour at 37°C. After several washes, the membrane was developed with enhanced chemiluminescence.

FIGURE 2. The results of tear film BUT before and after treatment (in seconds). (A) Nontreated OVX group. (B) Vehicle group. (C) Systemic estradiol administration OVX group. (D) Topical ophthalmic estradiol administration OVX group. (E) Systemic testosterone administration OVX group. (F) Topical ophthalmic testosterone administration OVX group.
Because both sex hormones are generated in women. Many factors are involved in androgen shortages produced some degree of improvement. Our experiments indicated that OVX decreases testosterone levels by 88.5% in animal models.5 Because both sex hormones are generated from ovaries in females, this condition could lead to a reduction in estrogen availability.6,7 Generally, decreased estrogen is associated with a reduction in androgen availability in women. Many factors are involved in androgen shortages such as menopause, consenescence, autoimmune disease, and androgen drug use. The data in the present study indicate that OVX provokes ocular surface impairment, and systemic estradiol administration further aggravates ocular surface injury after OVX. Conversely, systemic testosterone administration produced some degree of improvement. Our experiments suggest that lacrimal gland secretory function depends on circulating androgen levels and that OVX causes functional and biochemical atrophy of the lacrimal gland.

A previous article13 and abstract (Mathers WD, et al. IOVS 1997;38:ARVO Abstract 1022) have demonstrated a correlation between the corneal fluorescein staining and the time course (P < 0.01) (Table 2). Systemic estradiol administration caused a further decrease in tear film BUT from 5.28 (0.81) to 3.65 (0.55) seconds (P < 0.01), and systemic testosterone administration improved the tear film BUT from 5.08 (0.40) to 6.03 (1.48) seconds (P < 0.05). Topical ophthalmic administration of estradiol or testosterone had no significant effect on tear film BUT (P > 0.05) (Fig. 2).

**Effect of Gonadal Hormone Supplementation on Corneal Fluorescein Staining**

Ovariectomy dramatically increased the corneal fluorescein staining score at the fourth month after OVX from 0.35 (0.11) to 6.02 (1.54) (P < 0.05). The values increased in parallel with the time course (P < 0.01) (Table 3). Systemic estradiol administration caused a further corneal fluorescein staining score increase from 7.39 (1.54) to 9.89 (1.27) (P < 0.05), and systemic testosterone administration induced a decrease from 7.45 (0.73) to 4.56 (1.21) (P < 0.05). Ophthalmic administration of estradiol or testosterone did not significantly affect corneal fluorescein staining (P > 0.05) (Fig. 3).

**Effect of OVX on Sex Hormone Levels in Serum**

As shown in Figure 4 at 5 months after OVX, the serum estradiol level had decreased from 44.38 (7.98) to 23.00 (3.78) pg/mL (P < 0.01), and the serum testosterone level had decreased from 2.42 (0.26) to 1.57 (0.35) ng/mL (P < 0.05). After systemic estradiol or testosterone supplementation for 6 weeks, the estradiol level was elevated to 35.38 (3.49) pg/mL, and the testosterone level was elevated to 3.53 (0.67) ng/mL.

**Effect of Gonadal Hormone Supplementation on MMP-2 mRNA and Protein Expression in Ovariectomized Rats**

In the nontreated OVX group, MMP-2 mRNA (0.66 [0.10]) and protein (0.55 [0.13]) expression in lacrimal glands was significantly increased compared with that in the sham-operated group (0.50 [0.09] and 0.40 [0.07], respectively) (P < 0.05). Systemic estradiol administration further increased the expression of MMP-2 mRNA (0.85 [0.10]) and protein (0.69 [0.12]) compared with that in the nontreated OVX group (0.66 [0.10] and 0.55 [0.13], respectively) (P < 0.05 for both), while systemic testosterone administration decreased the expression of MMP-2 mRNA (0.83 [0.10]) and protein (0.34 [0.05]) compared with that in the nontreated OVX group (0.63 [0.10] and 0.55 [0.13], respectively) (P < 0.01 for both). Topical ophthalmic administration of two sex hormones had no effect on the expression of MMP-2 mRNA (0.59 [0.12] for estradiol and 0.57 [0.14] for testosterone) or protein (0.49 [0.11] for estradiol and 0.46 [0.13] for testosterone compared with the nontreated OVX group) (P > 0.05) (Figs. 5, 6).

**TABLE 3.** Comparison of Corneal Fluorescein Staining Scores in the Control Group, Sham-Operated Group, and Experiment Group

<table>
<thead>
<tr>
<th>Group</th>
<th>Before OVX</th>
<th>1 mo After OVX</th>
<th>2 mo After OVX</th>
<th>3 mo After OVX</th>
<th>4 mo After OVX</th>
<th>5 mo After OVX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.31 (0.14)</td>
<td>0.38 (0.10)</td>
<td>0.33 (0.15)</td>
<td>0.39 (0.24)</td>
<td>0.36 (0.12)</td>
<td>0.40 (0.16)</td>
</tr>
<tr>
<td>Sham operated</td>
<td>0.36 (0.18)</td>
<td>0.33 (0.17)</td>
<td>0.30 (0.14)</td>
<td>0.38 (0.15)</td>
<td>0.35 (0.17)</td>
<td>0.39 (0.13)</td>
</tr>
<tr>
<td>Experimental</td>
<td>0.35 (0.11)</td>
<td>0.73 (0.47)</td>
<td>2.46 (1.91)</td>
<td>4.52 (1.83)</td>
<td>6.02 (1.34)†</td>
<td>7.39 (1.52)†</td>
</tr>
</tbody>
</table>

All values are mean (± standard deviation).
* P < 0.05 versus before OVX.
† P < 0.01 versus before OVX.

**Discussion**

Dry eye occurs more frequently in postmenopausal women. Dry eye morbidity is two to four times greater in women than in age-matched men.9 Impaired lacrimal secretion regulation seems to have an important role in postmenopausal ophthalmic symptomatology.10 In the present study, menopause was simulated by OVX, and plasma estradiol and testosterone levels were decreased after 5 months. Previous research has indicated that OVX decreases testosterone levels by 88.5% in animal models.11 Because both sex hormones are generated from ovaries in females, this condition could lead to a reduction in estrogen availability.12 Generally, decreased estrogen is associated with a reduction in androgen availability in women. Many factors are involved in androgen shortages such as menopause, consenescence, autoimmune disease, and antiandrogen drug use. The data in the present study indicate that OVX provokes ocular surface impairment, and systemic estradiol administration further aggravates ocular surface injury after OVX. Conversely, systemic testosterone administration produced some degree of improvement. Our experiments suggest that lacrimal gland secretory function depends on circulating androgen levels and that OVX causes functional and biochemical atrophy of the lacrimal gland.

**FIGURE 3.** Corneal fluorescein staining before and after treatment. (A) Corneal fluorescein staining before and after treatment in different groups: (a) Nontreated OVX group. (b) Vehicle group. (c) Systemic estradiol administration OVX group. (d) Topical ophthalmic estradiol administration OVX group. (e) Systemic testosterone administration OVX group. (f) Topical ophthalmic testosterone administration OVX group. (B) Corneal fluorescein staining in the control group. (C) Corneal fluorescein staining 5 months after OVX in the OVX group. (D) Corneal fluorescein staining in ovariectomized rats after 6 weeks of systemic estradiol administration. (E) Corneal fluorescein staining in ovariectomized rats after 6 weeks of systemic testosterone administration.
reported the various relationships between sex hormone serum levels and tear production in premenopausal and postmenopausal women. In premenopausal women, serum testosterone levels were negatively correlated with tear production, and estrogen levels were positively correlated; the correlations were reversed in postmenopausal women. Administration of topical androgen appears to be a novel treatment for dry eye and was found to restore the thickness and function of the tear film lipid layer 3 months after therapy. However, our results in ovariectomized rats treated with topical ophthalmic testosterone showed no significant changes after 6 weeks of therapy.

The MMPs may be implicated in the pathogenesis of dry eye. In the NRTN−/− mouse dry eye model, MMP-9 concentration in the corneal epithelium and tears was significantly higher than that in NRTN+/− or NRTN+/+ mice. The levels of MMP-2 and MMP-9 are increased in the tears and saliva of patients with SS and dry eye, similar to observations in the lacrimal gland and cornea in a lacrimal gland inflammation–induced rabbit dry eye model. It has been confirmed that MMP expression is promoted by estrogen in other tissues, including glomerular mesangial cells, lutein cells in the granular cell layer, RPE cells, and corneal epithelial cells.

In the present study, semiquantitative RT-PCR and Western blot analysis indicated that MMP-2 expression in the lacrimal gland was affected by sex hormones at both gene and protein levels in ovariectomized rats. Ovariectomy increased MMP-2 expression by Western blot analysis in the lacrimal gland and cornea in a lacrimal gland inflammation–induced rabbit dry eye model.
expression in the lacrimal gland compared with the sham-operated group. Six weeks of systemic estradiol treatment caused a significant increase in MMP-2 gene expression in lacrimal glands of ovariectomized rats, and systemic androgen treatment had the opposite effect. The results were associated with decreased cell proliferation in protein levels. Collectively, our results suggest that MMP-2 expression in lacrimal glands of ovariectomized rats is upregulated by estrogen and downregulated by androgen. Estrogen modulates the activity of various molecules that are important for ECM turnover, including MMP-2 and MMP-9. On the other hand, MMP-2 expression in ovary is decreased in rats with polycystic ovary that was induced by dehydroepiandrosterone.22 Suzuki and Sullivan6 reported that the expression of proinflammatory cytokines (IL-1α, IL-6, and IL-8) and MMPs (MMP-2, MMP-7, and MMP-9) increased 6 hours and 24 hours after 17β-estradiol administration to corneal epithelium cells. Our findings suggest that MMP-2 expression is upregulated in lacrimal glands of estrogen-treated ovariectomized rats. These results indicate that sex hormones affect the ocular surface and have important roles in the pathogenesis of dry eye.

Lacrimal gland secretion seems to depend on circulating levels of androgens. We demonstrated that estradiol upregulates MMP-2 expression and reduces both tear secretion and tear film stability. Conversely, androgen inhibits MMP-2 expression and improves tear secretion and tear film stability. Lacrimal glands are target organs of androgen, and androgen exerts effects by binding its receptor.22,23 Androgen receptors have been identified in acinar ductal epithelial cells in the lacrimal tissue of MRL/lpr mice.25 Previous investigations have demonstrated that androgens upregulate mRNA levels of key lipogenic enzymes in cholesterol and fatty acid pathways of the meibomian gland.24 Gene and protein expression in an ovariectomized rabbit model decreased, together with changes in catalytic activities or receptor numbers related to the capacity of the gland to secrete in response to autonomic stimulation, and dihydrotestosterone partially or totally reversed or prevented the decreases in most of these parameters.21 The cellular mechanism of androgen is control of gene expression via nuclear androgen receptors bound to sex hormone-response elements on DNA.25 Treatment of a mouse model of SS with testosterone increased androgen receptor expression and reduced lymphocyte infiltration.25 Estrogen action may occur through its classic estrogen nuclear receptors, but only negligible quantities of estrogen receptors have been identified in lacrimal epithelial tissue.26 In human RPE cells, regulation of MMP-2 expression by estrogen has been suggested to be mediated through the nuclear factor-κB pathway.27 Experimental dry eye stimulates IL-1β, TNF-α, and MMP-9 production and activates mitogen-activated protein kinase (MAPK) signaling pathways on the ocular surface. The MAPKs are known to stimulate the production of inflammatory cytokines and MMPs, and they could have an important role in reducing factors implicated in the pathogenesis of dry eye.28 Azzarolo et al.29 showed that glandular atrophy observed after OXV likely proceeds by necrosis of acinar cells rather than apoptosis. These results suggest that a critical level of androgen is necessary to maintain lacrimal gland structure and function and that a decrease in available androgen below this level could trigger lacrimal gland apoptosis and necrosis and an autoimmune response. Therefore, replacement of androgens in states of low-androgen levels such as after menopause might help to cure primary lacrimal deficiency and prevent SS autoimmunity.

In conclusion, the results of this study suggest that OXV provoked ocular surface impairment. These changes were ameliorated by systemic testosterone administration, suggesting that they might be associated with androgen deficiency. The role of androgen may be mediated via MMP-2 downregulation. A possible role for estrogen in the pathogenesis of dry eye is through upregulation of MMP levels. The precise role of MMPs in the pathogenesis of dry eye and the mechanism by which sex hormones regulate MMPs in the lacrimal gland remain to be determined. Our results suggest that androgen therapy is useful to treat dry eye in postmenopausal women. However, it may also induce considerable adverse reactions such as hirsutism and masculinization, and we propose that this subject deserves further research.

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References


