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Androgen Support of Lacrimal Gland Function

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The effects of dihydrotestosterone (DHT) (1 mg/kg) on biochemical parameters related to lacrimal secretion, basal tear flow rate, and pilocarpine-stimulated lacrimal gland fluid secretion, in mature ovariectomized rabbits were studied. The effects of the synthetic estrogen, diethylstilbestrol (DES) (100 μg/kg), on lacrimal gland biochemical parameters in normal mature female rabbits was also studied. Ovariectomy decreased the total serum levels of testosterone (T) by 88.5% and androstenedione by 35.9%, without changing the levels of dehydroepiandrosterone (DHEA) or its sulfate. Ovariectomy caused a significant regression of the lacrimal glands, decreasing total DNA by 35%, and total protein by 22%. DHT treatment of ovariectomized animals prevented lacrimal gland regression, increasing total gland DNA (31%) and total protein (18%). DHT treatment also increases Na^+,K^+-ATPase activity (29%) and β-adrenergic receptor binding sites (23%) compared to the ovariectomized group. DHT increased pilocarpine stimulated lacrimal gland fluid secretion (13.26 ± 1.47 μL/min) compared to the ovariectomized group (7.72 ± 0.41 μL/min), but DHT treatment paradoxically decreased basal tear flow rate (1.02 ± 0.04 μL/min) as compared to the ovariectomized rabbits (1.96 ± 0.12 μL/min). DES decreased the total serum T from 59.33 ± 10.54 pg/mL to 21.5 ± 6.06 pg/mL. DES decreased total Na^+,K^+-ATPase by 12% and increased β-adrenergic receptor binding sites by 83.3%. These results suggest that androgens play a major role in supporting lacrimal gland secretory function. Additionally, they suggest that estrogens may influence certain aspects of lacrimal functions, although it is not clear to what extent those actions are elicited directly or indirectly.

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Introduction

Lacrimal gland insufficiency is the most common cause of dry eye conditions. Its incidence is gender related, in that it affects women much more frequently than men *(1)*. Sjögren's Syndrome, an autoimmune disease of the lacrimal and salivary glands, is characterized by progressive Helper T-cell and B-cell infiltration, degeneration of acinar and ductal cells, and severe impairment of glandular secretion; it affects women ten times more frequently than men, primarily women after menopause. Non-Sjögren's lacrimal insufficiency (currently referred to as primary lacrimal deficiency or PLD) *(2)* most frequently affects women after menopause or ovariectomy, and in other hormonally altered states, such as during pregnancy, lactation, and oral contraceptive use *(1,3)*. These observations implicate sex hormones in the regulation of the lacrimal gland's secretory function and its tendency to initiate autoimmune processes. Several characteristics of the lacrimal gland have been shown to be sexually dimorphic. There are reports of distinct morphological differences between the lacrimal glands of male and female rats, mice, guinea pigs, rabbits, and humans *(4,5)*, biochemical differences in rats and rabbits *(6–8)*, and functional differences in rats, Syrian hamsters, and rabbits *(9–11)*. Evidence has accumulated that implicates androgens as the major agents influencing these gender-related differences. Androgens have been shown to be responsible for the male-like morphological and functional characteristics of the gland, including larger acini, greater secretion of IgA *(12)*, and greater production of the polymeric IgA receptor, measured as secretory component, SC *(9)* in rats. When female rats are treated with androgens, the morphology of their lacrimal gland changes and resembles the male lacrimal gland *(13)*. More recently, it has been shown that dihydrotestosterone (DHT) increases total DNA above control values, and that it partially restores the amount of protein, Na^+,K^+-ATPase activity and β-adrenergic receptor binding activity in lacrimal

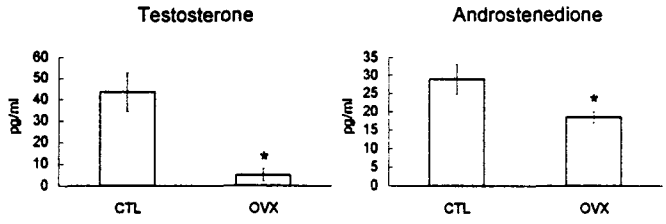

Fig. 1. Serum levels of testosterone (T) and androstenedione after 9 d of ovariectomy (OVX), or sham operation (CTL), of sexually mature female rabbits. $N = 9$. $*p < 0.05$.

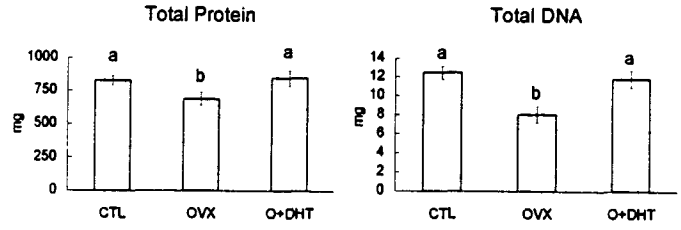

Fig. 2. Total lacrimal gland protein and DNA 9 d after ovariectomy (OVX), ovariectomy, and simultaneous treatment with 1 mg/kg/d of dihydrotestosterone (O + DHT) or sham operation (CTL), of sexually mature female rabbits. $N = 9$. Letters on the top of the bars indicate significant differences between groups with different letters. $p < 0.05$.

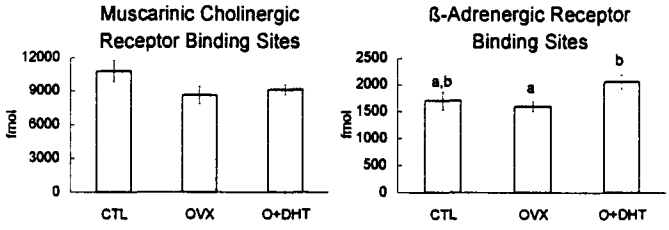

Fig. 3. Total lacrimal gland muscarinic cholinergic and β-adrenergic receptor binding sites 9 d after ovariectomy (OVX), ovariectomy, and simultaneous treatment with 1 mg/kg/d of dihydrotestosterone (O + DHT) or sham operation (CTL), of sexually mature female rabbits. $N = 9$. Letters on the top of the bars indicate significant differences between groups with different letters. $p < 0.05$.

glands of hypophysectomized female rats *(8)*. It was thus hypothesized that androgens are necessary to maintain the optimal function of the gland, and that reduction of androgen levels leads to decreased lacrimal gland secretory function.

The present experiments had several purposes. First, to determine whether parameters related to gland secretion regress after ovariectomy with consequent decreases in lacrimal gland fluid and tear flow rate. Second, to ascertain if administration of DHT prevents this regression. Third, because many women are treated with estrogen after menopause or ovariectomy, the authors wanted to determine if the administration of DES would suppress pituitary function enough to decrease ovarian androgen production and lead to lacrimal gland regression. In the postmenopausal human, ovarian androgens are produced by the interstitium. In intact diestrus rabbits, androgens are also of interstitial origin, and thus this animal model can yield insight into the human condition. The effects of ovariectomy, DHT, and DES on the activities of the enzymes alkaline phosphatase and acid phosphatase was also determined. These enzymes have been used routinely as convenient membrane markers in the authors' laboratory. The role of these enzymes in lacrimal secretion is not known, but it was recently found that their activities in the lacrimal gland are androgen and prolactin dependent *(8)*.

Results

Effects of Ovariectomy and DHT Treatment

Ovariectomy significantly decreased the serum levels of androgens T and androstenedione by 88.5 and 35.9%, respectively (Fig. 1), without changing the levels of the adrenal androgen DHEA (630 ± 190 pg/mL) compared with control (720 ± 140 pg/mL).

Lacrimal gland fluid secretion depends on the size of the gland (measured by its total content of protein and DNA), its total content of Na^+,K^+-ATPase (the sodium pump that drives fluid secretion), and its total content of receptors for autonomic secretomotor neurotransmitters (which regulate the activity of the secretory machinery). It was previously found that total gland protein is directly proportional to total gland weight and that gland weight within a treatment group exhibits fairly small variance (unpublished data). Therefore, the authors are expressing their results as total gland content of each parameter (e.g., enzyme activity or receptor binding sites).

Ovariectomy caused a significant regression of the lacrimal gland. Ovariectomy significantly decreased total gland protein by 22% and DNA by 35% (Fig. 2). DHT treatment prevented the decrease of total gland protein and DNA observed after Ovariectomy (Fig. 2). Ovariectomy also caused a decrease in the mean number of muscarinic cholinergic receptor binding sites, however, this decrease was not significant (Fig. 3). DHT had no significant effect on muscarinic cholinergic receptor binding sites. Ovariectomy also failed to significantly change the β-adrenergic receptor (β-AR) binding sites (Fig. 3). However, DHT treatment significantly increased β-AR binding sites by 23% over the ovariectomized group.

Whereas ovariectomy appeared to decrease the total Na^+,K^+-ATPase activity, this change was not statistically significant ($p = 0.11$) (Fig. 4). However, DHT significantly increased Na^+,K^+-ATPase activities by 29% over the ovariectomized group (Fig. 4). Ovariectomy had no effect on the activity of acid phosphatase (Fig. 4), but DHT treatment increased acid phosphatase activity by 37.5% compared to the ovariectomized group. Ovariectomy decreased alkaline phosphatase activity by 33%. DHT had no significant effect on alkaline phosphatase activity (Fig. 4).

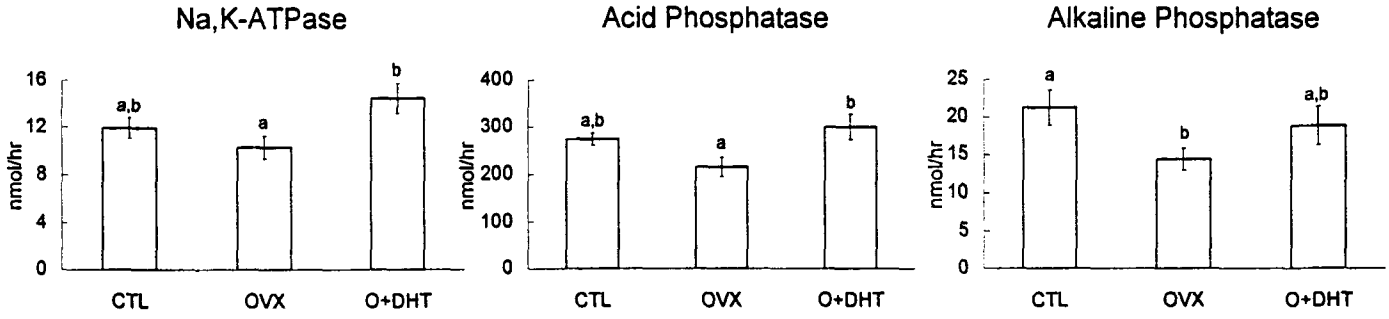

Fig. 4. Total lacrimal gland Na^+,K^+-ATPase, acid phosphatase, and alkaline phosphatase activities 9 d after ovariectomy (OVX), ovariectomy, and simultaneous treatment with 1 mg/kg/d of dihydrotestosterone (O + DHT), or sham operation (CTL) of sexually mature female rabbits. $N = 9$. Letters on the top of the bars indicate significant differences between groups with different letters. $p < 0.05$.

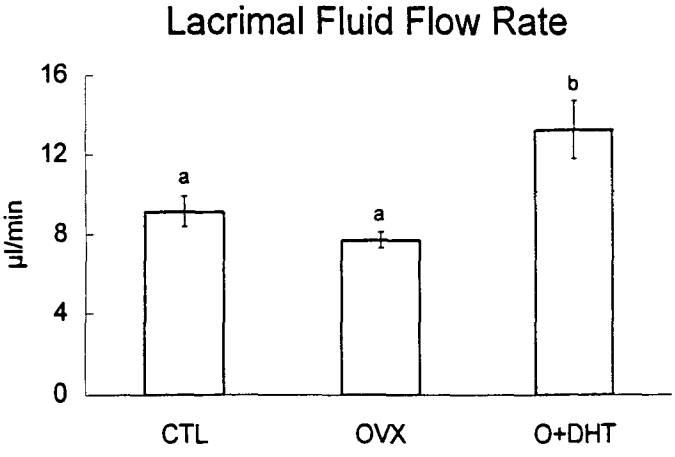

Fig. 5. Stimulated lacrimal fluid flow rate from sexually mature female rabbits, 9 d after ovariectomy (OVX), ovariectomy, and simultaneous treatment with 1 mg/kg/d of dihydrotestosterone (O + DHT) or sham operated (CTL) animals. $N = 6$. Letters on the top of the bars indicate significant differences between groups with different letters. $p < 0.05$.

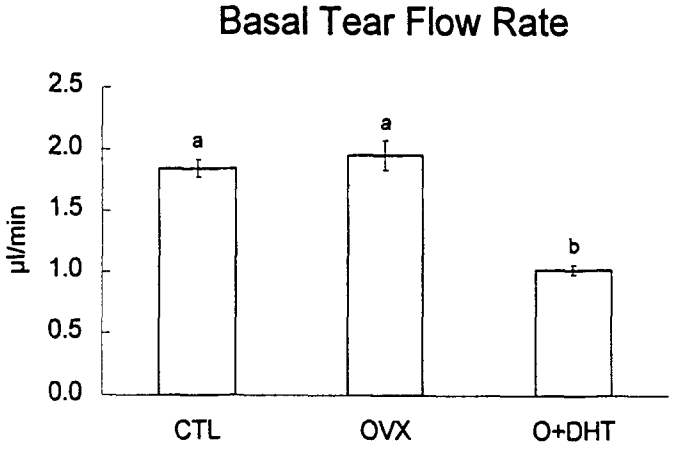

Fig. 6. Basal tear flow rate from sexually mature female rabbits, after 9 d of ovariectomy (OVX), ovariectomy, and simultaneous treatment with 1 mg/kg/d of dihydrotestosterone (O + DHT) or sham operated (CTL) animals. $N = 9$. Letters on the top of the bars indicate significant differences between groups with different letters. $p < 0.05$.

Peak stimulated lacrimal gland fluid flow rate, measured by collecting the fluid after cannulation of the lacrimal excretory ducts, showed no change after ovariectomy (Fig. 5). However, DHT significantly increased the stimulated lacrimal gland fluid flow rate compared to ovariectomized rabbits (13.26 ± 1.48 µL/min and 7.72 ± 0.41 µL/min, respectively) ($p < 0.01$) (Fig. 5). After ovariectomy, no changes were observed in basal tear flow rate, measured by collection from the tear meniscus by capillary pipets (1.96 ± 0.12 µL/min) compared to sham-operated controls (1.84 ± 0.07 µL/min) (Fig. 6). In contrast to its effect of pilocarpine-stimulated lacrimal gland fluid production, DHT significantly decreased the basal tear flow rate (1.02 ± 0.04 µL/min) compared to ovariectomized animals (1.96 ± 0.12 µL/min) (Fig. 6).

Effect of DES Treatment

DES treatment significantly decreased the testosterone level (21.5 ± 6.1 pg/mL) compared to control (59.3 ± 10.5 pg/mL). DES treatment did not cause general regression of the lacrimal gland since total protein (323.19 ± 27.94 mg), and total DNA (8.88 ± 0.39 mg) did not change significantly from control values (299.58 ± 21.61 mg and 8.41 ± 0.44 mg, respectively). However, DES decreased Na^+,K^+-ATPase activity by 11% ($p < 0.05$) (Fig. 7). DES also decreased acid phosphatase activity by 40% ($p < 0.01$), but had no effect on alkaline phosphatase activity (Fig. 7). DES had no effect on muscarinic cholinergic receptor binding sites, but increased the β-AR binding sites by 43% ($p < 0.05$) (Fig. 8).

Discussion

In the mammalian ovary, androgen production is an essential intermediate for estrogen synthesis. Thus, ovariectomy decreases the production of the androgens T and androstenedione as well as the estrogens estradiol (E_2) and estrone (E_1). In this experimental rabbit model, the female animals are at the stage of diestrous, where steroid production is low. Circulating androgens were significantly

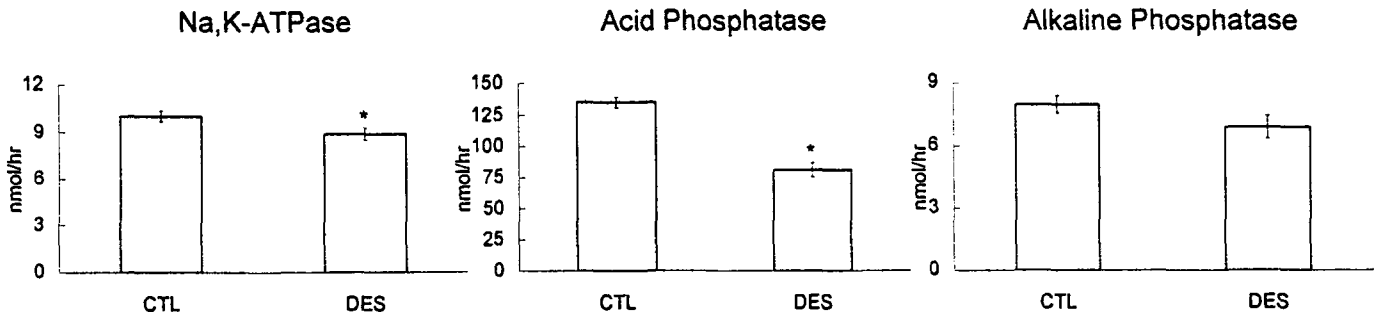

Fig. 7. Total lacrimal gland Na^+,K^+-ATPase, acid phosphatase, and alkaline phosphatase activities from intact sexually mature female rabbits receiving 100 μg/kg/d of diethylstilbestrol (DES) or vehicle (CTL) for 8 d. $N = 6$. $*p < 0.05$.

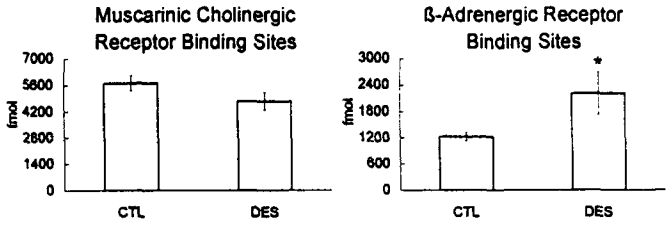

Fig. 8. Total lacrimal gland muscarinic cholinergic and β-adrenergic receptor binding sites from intact sexually mature female rabbits receiving 100 μg/kg/d of diethylstilbestrol (DES) or vehicle (CTL) for 8 d. $N = 6$. $*p < 0.05$.

decreased after ovariectomy, and the remaining levels of T and androstenedione as well as DHEA were most likely from adrenal sources.

Ovariectomy also caused a regression of the lacrimal glands, manifested by a decrease in total gland protein and DNA. These decreases were similar but smaller in magnitude than the decreases observed when rats were hypophysectomized (40% for gland protein and 50% for DNA) *(8)*. These two animal models differ in several respects, and several possible explanations for the quantitative difference in the responses can be visualized. One explanation could be that hypophysectomy, in addition to removing the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and eliminating the production of the ovarian androgens, also removes ACTH and eliminates the production of the adrenal androgens (predominantly DHEA and its sulfate DHEAS, but also some T and androstenedione), whereas ovariectomy removes only ovarian androgen production without altering adrenal androgen production. Thus, a greater decrease in the total circulating levels of androgens could account for the greater regression observed after hypophysectomy.

Another factor could be that hypophysectomy also removes prolactin (PRL), in addition to other pituitary and pituitary-dependent hormones. PRL receptors have been found in both male and female rat lacrimal gland acinar cells *(14)*, and PRL has been implicated in regulating certain aspects of lacrimal gland function *(8)*. PRL has been found to exert general trophic actions on the lacrimal gland and specifically on lacrimal Na^+,K^+-ATPase, alkaline phosphatase, and muscarinic cholinergic receptors *(8)*. Thus, the combined lack of androgens, PRL and perhaps other hormones, such as thyroid hormone and glucocorticoids, in the hypophysectomized rat model might impair the gland's function more than a lack of androgens alone.

DHT treatment simultaneous with ovariectomy was used to verify the importance of androgen in maintaining lacrimal gland secretion. DHT was used instead of testosterone, because it can not be converted to estrogens. The dose of DHT used in these experiments (1 mg/kg) maintains secondary sexual characteristics in castrated male rats *(15)*, and a similar dose stimulates castrated male rabbit accessory sex glands *(16,17)*, but is clearly pharmacologic in female rabbits. DHT given at the time of ovariectomy prevented the regression of the gland and maintained or increased the expression of several specific biochemical markers of lacrimal secretory capacity. These results agree with previous findings in hypophysectomized female rats, in which the same dose of DHT partially restored the Na^+,K^+-ATPase activity, β-AR binding sites and other markers. The observation that DHT had no effect on muscarinic cholinergic binding sites is also in accord with the results obtained with hypophysectomized rats *(8)*.

The authors' results are also consistent with the effects of the hormonal milieu on alkaline phosphatase and acid phosphatase activities observed previously *(8)*. Whereas the roles of these enzymes in the lacrimal gland still need to be investigated, the removal of the ovarian sex hormones caused significant decreases in alkaline phosphatase activity, and treatment with DHT increased the activity of acid phosphatase.

Ovariectomy and DHT significantly increase maximally stimulated lacrimal fluid flow rate over the value in ovariectomized rabbits. Thus, maximal secretory capacity of the lacrimal gland seems to be androgen dependent.

Ovariectomy did not change the basal tear fluid flow rate, but a significant decrease was observed after DHT treatment as compared to the ovariectomized group. Sullivan and Allansmith *(18)* found similar results, showing that castration of rats increased basal levels of tear

volume, whereas chronic testosterone treatment after castration decreased basal tear volume. The effects of androgens on maximally stimulated lacrimal gland fluid production and basal tear flow rate might seem inconsistent. However, parameters related to basal tear volume and flow and not to lacrimal gland fluid flow may provide an imperfect index of the lacrimal gland secretory capacity. One problem is that tear flow is also controlled by factors that affect eye lid dynamics and the drainage system. Another relates to our incomplete understanding of the variety of signals that are integrated to determine the rate of lacrimal gland fluid production. It is of particular interest that androgens increase β-adrenergic receptor binding sites with no effect on muscarinic cholinergic receptor binding sites. Botelho et al. *(19)* have shown that sympathetic nerve impulses inhibit secretion of lacrimal gland fluid when the gland is stimulated with a cholinergic agonist. In addition, a large body of evidence has recently accumulated in vascular and nonvascular smooth muscle, that indicate that cross talk between the cAMP and the polyphosphoinositide (PPI) signaling cascade plays an important role in the functional antagonism between the sympathetic and parasympathetic nervous system *(20)*. Thus, agents that elevate intracellular cAMP concentrations, such as β-adrenergic agonist, inhibit agonist-stimulated hydrolysis of the PPI system, such as muscarinic cholinergic agonist *(20)*. Therefore, it might be speculated that an increase in β-AR binding sites without a concomitant increase in muscarinic cholinergic receptor binding sites, as is the case in the DHT treated, ovariectomized rabbits, could shift the balance between stimulatory and inhibitory intracellular signals generated in response to basal autonomic output, resulting in a decrease of basal lacrimal gland fluid production and precorneal tear fluid volume even though the levels of cellular elements responsible for fluid production in the lacrimal glands are increased. When maximal stimulation with a cholinergic agonist occurs, the stimulatory effect of the cholinergic pathway presumably overrides the inhibition of the cAMP driven pathways.

A pharmacological dose of DES significantly decreased testosterone levels, probably through negative feedback to the hypothalamic–pituitary axis, thus decreasing LH stimulated-androgen production by the ovary. Negative feedback action of E_2 has been shown to occur in both the hypothalamic and the pituitary components of the hypothalamic–adenohypophyseal axis *(21)*. The decrease in testosterone caused by DES is of interest, because it mimics, in part, the effect of estrogen replacement therapy in postmenopausal women, when it is not supplemented with androgen *(22)*. Together with a decrease in testosterone, DES produced a significant decrease in Na^+,K^+-ATPase activity.

Although the most plausible mechanism of DES in inhibiting androgen concentration is probably through suppression of pituitary LH release, the significant increase in β-AR binding sites observed after DES treatment also suggests a possible direct effect of estrogens on rabbit lacrimal glands. However, other mechanisms are known by which DES could have affected the gland indirectly. These relate to alterations of other hormone(s) that influence the lacrimal gland. One of the hormonal candidates could be PRL, since estrogen is a potent stimulus for PRL release, and it was shown that PRL affects the lacrimal gland. PRL in the present experiments was not measured, but the data are not consistent with this hypothesis. In the study of hypophysectomized rats, PRL increased Na^+,K^+-ATPase, alkaline phosphatase activities and muscarinic cholinergic receptors, but had no effect on β-AR binding sites. DES has also been found to increase the levels of glucocorticoids in humans *(23)*, and glucocorticoids have been shown to increase the number of the β-adrenergic receptors in several tissues *(24–27)*. Such indirect mechanisms may be the sole mechanisms of estrogen action in rat lacrimal glands, since studies in the authors' laboratory have shown that DES has no effect in hypophysectomized rats (unpublished results), and previous research has shown that estrogen treatment alone does not alter the structure or function of the rat exorbital gland *(18,28,29)*. In addition, estrogen receptors have been found to be undetectable or present in negligible quantities in lacrimal tissue *(30)*.

Apart from the effect of androgens in supporting lacrimal gland structure and secretory function, they also seem to play a role in the generation of suppressor responses *(31–33)* that restrain T-cell activation and B-cell proliferation. From such data, Ariga et al. *(33)* suggested a beneficial effect of androgens on the local immune system of the lacrimal gland and a possible use of androgens to treat autoimmune Sjögren's lacrimal insufficiency.

The present experiments support the thesis that lacrimal gland secretory function depends for the most part on the action of androgens. This observation leads to the suggestion of androgen supplementation in hormone replacement therapy to treat and prevent primary lacrimal deficiency, which comprises the majority of the dry eye cases. Recent studies in humans have demonstrated a correlation between dry eye and decreased testosterone levels in women *(34)*. In addition, a theoretical argument has been presented *(35)* in which prevention of lacrimal gland regression may block progression from non-Sjögren's to autoimmune Sjögren's lacrimal insufficiency. This theory maintains that as the lacrimal gland regresses following withdrawal of hormonal support, the level of autonomic secretomotor stimulation increases in an attempt to maintain the tear film. This increased secretomotor stimulation is predicted to cause both induction of Class II major histocompatibility molecule expression and perturbation of intracellular membrane traffic that alter the spectrum of potential autoantigens that lacrimal epithelial cells display and secrete into the interstitium, connecting therefore the loss of hormonal support with autoimmunity.

Materials and Methods

Animals and Surgical Procedure

Sexually mature female New Zealand white rabbits (4–4.5 kg) were obtained from Myrtle's Rabbitry (Thompson Station, TN) or Irish Farms (Norco, CA).

Rabbits were anesthetized with an intramuscular injection of 40 mg/kg ketamine and 10 mg/kg xylazine, and ovariectomized through a midline abdominal incision. The analgesic Buprenex (0.02 mg/kg im) was given after 24 h of surgery. In addition, animals were monitored daily for eating, drinking, wound healing, and incision status after surgery was performed.

These experiments were approved by our Institutional Animal Care and Use Committee.

Experimental Design

Ovariectomy and DHT Treatment

1. Study of correlates of lacrimal secretion and basal tear flow rate: Eighteen female rabbits were ovariectomized. At the time of surgery, nine were implanted with DHT pellets from Innovative Research (Toledo, OH), formulated to deliver the hormone at the constant rate of 1 mg/kg/d for 10 d, and nine were implanted with placebo pellets. Nine sham operated animals were used as controls.
2. Study of stimulated lacrimal gland fluid rate: Twelve female rabbits were ovariectomized. Six of them were treated with DHT (1 mg/kg/d in corn oil) sc for 9 d. Six sham operated animals were used as controls.

DES Treatment

Normal female rabbits were injected subcutaneously with a pharmacological dose of the potent estrogen DES dissolved in corn oil (100 μg/kg/d), (n = 6), for eight consecutive days. Another group was injected with corn oil and used as control (n = 6).

Tear Collection

Unanesthetized rabbits were placed in a restraint cage and tear fluid was collected from the lateral canthus for 5 min using a 5- or 10-μL calibrated, flame polished micropipet. Results are expressed in μL/min. Basal tear flow is referred to as minimally tactile stimulated, but without cholinergic agonist stimulation.

Lacrimal Gland Fluid Collection

Rabbits were anesthetized as described above and the lacrimal gland ducts cannulated using polyethylene tubing (Intramedic PE-10). Pilocarpine, a cholinegic agonist, was injected through the ear vein at the dose of 0.3 mg/kg. This dose was found to maximally stimulate flow rate *(36)*. The effect of pilocarpine at the dose of 0.1 mg/kg was not significantly different compared with the nonstimulated flow rate. Lacrimal gland fluid was collected in preweighed micro centrifuge tubes for three consecutive 10 min collections and measured gravimetrically. Results are expressed in μL/min.

Tissue Collection and Fractionation

At the end of the experimental period the rabbits were anesthetized with an intramuscular injection of 40 mg/kg ketamine and 10 mg/kg xylazine. Blood was collected from the central ear artery into borosilicate glass tubes using a 19G needle. The rabbits were then killed with a lethal dose of sodium pentobarbital (80 mg/kg) through the marginal ear vein. The superior and inferior lobes of the lacrimal gland were obtained, snap frozen in liquid nitrogen, and stored at –70°C until processed. Differential centrifugation was performed as described in detail by Azzarolo et al. *(7)*. The low-speed pellet (P_o), contained mostly nuclei and cell debris; the high-speed pellet (P_i), represented a crude membrane sample, and the supernatant (S_i), contained the soluble phase. These samples were frozen in liquid nitrogen and kept at –70°C until analyzed.

Analytic Methods

Protein was measured using the Bio-Rad (Richmond, CA) assay kit. The sum of protein values in P_i, P_o, and S_i represented total gland protein, whereas P_i protein represented the membrane-bound protein. DNA was measured using the Pierce (Rockford, IL) DNA assay kit. No DNA was found in the soluble fraction, and total DNA per gland was obtained by summing DNA values in the P_i and P_o fractions. All of the enzymes and receptors studied were measured in the P_i fraction. Na^+,K^+-ATPase was measured using the K^+-dependent p-nitrophenylphosphatase (K^+-PNPPase) reaction described previously, Mircheff *(37)*. Acid phosphatase and alkaline phosphatase were assayed as already described *(38)*. Muscarinic cholinergic and β-adrenergic receptor ligand binding were determined as described by Bradley et al. *(39)*.

Hormone Analysis

The total serum levels (which include free and bound) of testosterone (T), androstenedione, and dehydroepiandrosterone (DHEA) were determined using previously described radioimmunoassay procedures *(40–43)*. Prior to the RIA, the steroids were first extracted with diethyl ether, and then subjected to Celite column partition chromatography. Assay sensitivity was defined as the smallest amount of steroid per RIA tube above the zero level that could be detected. The sensitivities were 4 pg/tube. The practical assay sensitivity of each assay after correction for dilutions and procedural losses was 10 pg/mL. Intra-assay and interassay coefficients of variation were 5–10% and 10–15%, respectively, in the four different assays.

Statistical Analysis

In experiments comparing rabbits that were sham-operated, ovariectomized or ovariectomized, and treated with DHT, analysis of variance (ANOVA) was utilized followed by evaluation using Duncan's New Multiple Range Test. In experiments comparing DES treated female rabbits to controls, the Student's t-test for unpaired samples was used. Significance was set at $p < 0.05$.

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