

Mouse Epididymal Sperm Contain Active P450 Aromatase Which Decreases as Sperm Traverse the Epididymis

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ABSTRACT: Recently we reported that mouse germ cells in the testis contain active P450 aromatase (P450arom), the enzyme that converts androgens to estrogens. This finding suggested that germ cells have the ability to produce estrogen. Further studies have shown that germ cells in the testis of several species contain P450arom. The goal of this study was to determine if epididymal sperm contain P450arom and if P450arom activity in sperm changes during traversal of the epididymis in the adult mouse. P450arom was localized in sperm present in the efferent ductules and epididymis by immunocytochemistry using an antiserum generated against purified human placental cytochrome P450arom. P450arom immunostaining in sperm was most prominent in sperm located in the proximal caput epididymis, decreased as sperm traversed the corpus epididymis,

and was only slightly apparent in sperm in the cauda epididymis. The immunolocalization of P450arom in epididymal sperm was supported by the measurement of P450arom activity in sperm by the $^3\text{H}_2\text{O}$ assay. We found that P450arom activity in sperm significantly decreases as sperm traverse the epididymis. Based upon these observations, we conclude that sperm can synthesize estrogen and that the synthesis of estrogen by sperm present in the efferent ductules and caput epididymis could be important in the process of sperm maturation.

Key words: Spermatozoa, estrogen, efferent ductules, cytoplasmic droplet, testicular excurrent ducts.

J Androl 1996;17:111-116

A novel discovery in our laboratories demonstrated that developing germ cells in the adult mouse testis contain active P450 aromatase (P450arom), the enzyme responsible for converting androgens to estrogens (Nitta et al, 1993). P450arom, localized by immunocytochemistry, was located primarily in the Golgi region of round spermatids, throughout the cytoplasm of elongating spermatids, and along the flagella of late spermatids. The localization of P450arom within the germinal epithelium was supported by Western blot analysis of isolated germ cells. Northern blot analysis indicated that P450arom mRNA is synthesized in testicular germ cells, and the $^3\text{H}_2\text{O}$ water assay demonstrated that P450arom is active in germ cells. Further studies have also shown that germ cells in the testis of several species contain P450arom (bear: Tsubota et al, 1993; rooster: Kwon et al, 1995; monkey: unpublished observations). From these studies, we have concluded that testicular germ cells are capable of estrogen synthesis.

However, we had yet to examine sperm in the epidid-

ymis for the presence of P450arom. The presence of active P450arom in epididymal sperm would suggest that sperm-synthesized estrogen would be available in the ductal lumen for entry into target cells of the epididymal epithelium. We suspected that sperm might synthesize estrogen, because estrogen is found in higher concentrations in epididymal luminal fluid and tissue than in the blood plasma of some species. For example, in rat caput epididymal tissues the concentration of estrogen is 25 times greater than that in the blood (Kumari et al, 1980). Notably, the highest concentrations of estrogen receptors in the adult male reproductive tract have also been reported to be in the efferent ductules of both the mouse (Schleicher et al, 1984) and the monkey (West and Brenner, 1990). Therefore, our aim was to determine if sperm in the epididymis are capable of estrogen synthesis by detecting the presence of active P450arom. We utilized immunocytochemistry to localize the P450arom protein in epididymal sperm and the tritiated water assay for P450arom to determine if the P450arom present in the sperm is active.

Supported in part by NIH grant HD07028 and USDA grant AG93-37203-9021.

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Received for publication August 17, 1995; accepted for publication November 17, 1995.

Materials and Methods

Immunocytochemistry

Epididymides were collected from 8-week-old ICR mice and fixed in Bouin's solution overnight. Epididymides were dehy-

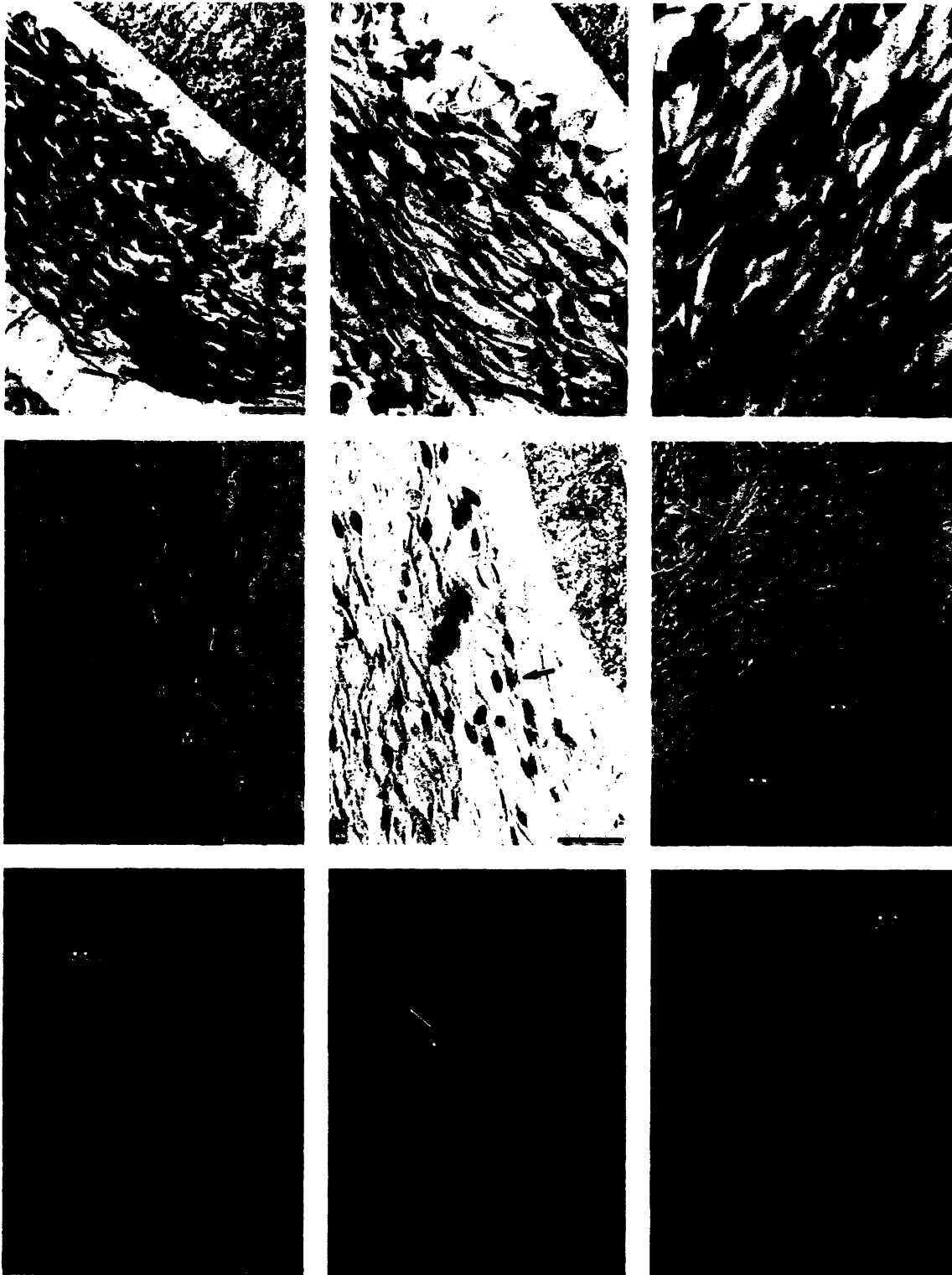


FIG. 1. (A–D) Immunocytochemical localization of P450arom in sperm present in the proximal caput epididymis. Sperm in this region of the epididymis were strongly stained for P450arom. (A) Proximal caput epididymis at low magnification. Spermatozoa in the lumen stained intensely for P450arom (brown-colored reaction product). No immunostaining is apparent in the epididymal epithelium (pce). (B) Immunostaining for P450arom appears concentrated in the cytoplasmic droplet (arrows) of the sperm. (C) Proximal caput epididymis at high magnification. Strong P450arom immunostaining is apparent in the cytoplasmic droplets of luminal sperm. (D) In this higher magnification image, immunostaining of the cytoplasmic droplet (arrows) is clearly apparent, because the axoneme of the tail extends through the center of the droplet. The head (H), mid-piece (M), and flagellum (F) are labeled and demarcated by arrowheads on a spermatozoa to show more clearly the localization of P450arom to the cytoplasmic

drated using a series of ethanol solutions and embedded in Paraplast (Brunswick Co., St. Louis, MO). Sections (5- μ m thick) were placed on glass slides, deparaffinized, and treated with 10% normal goat serum to reduce background staining. Sections were then stained for P450arom by immunocytochemistry, using a polyclonal anti-human placental P450arom. This antibody, raised in a rabbit, was generated against human placental P450arom that had been previously immunoaffinity purified using a monoclonal antibody to P450arom (Kitawaki et al, 1989; Yoshida and Osawa, 1991). Control sections were treated with normal rabbit serum in place of the primary antiserum at the same concentration (1:200). Additional control sections were treated with antibody that was preabsorbed with affinity-purified human placental P450arom, the same protein used to generate the antibody. All sections were then reacted with a biotinylated goat anti-rabbit IgG and an avidin-biotin-peroxidase complex (Rabbit Extr-Avidin Staining Kit; Sigma, St. Louis, MO). Peroxidase was visualized with a diaminobenzidine (DAB)-H₂O₂ solution.

Epididymal Sperm Isolation

Epididymides with attached efferent ductules were removed from adult (10- to 16-week-old) ICR male mice. The tissues were then placed in sterile Petri dishes containing ice-cold RPMI-1640 medium (GIBCO, Grand Island, NY) containing sodium bicarbonate and lactate, adjusted to pH 7.2 by gassing with CO₂. Epididymides were then dissected into three sections: efferent ductules with caput epididymides, corpus epididymides, and cauda epididymides. Sperm were then isolated separately from each of the three sections by gently shearing the tissues and rupturing the tubules, allowing sperm to float out into the medium. Sperm were filtered on ice through fine nylon mesh to remove epididymal tissue. Sperm were sonicated, and microsomal preparations were obtained using differential centrifuga-

³H₂O Assay for P450 Aromatase Activity

P450arom activity was measured by the release of one mole of ³H₂O from the aromatization of one mole of 1 β [³H]androst-4-ene-3,17-dione (1 β [³H]A) (New England Nuclear, Boston, MA) as described previously by Ackerman et al (1981) with the following modifications. 1 β [³H]A (6.11 disintegrations per minute [dpm]/ μ mol) and various cofactors (NADP [10 mM], NADPH [10 mM], glucose-6-PO₄ [100 mM], glucose-6-PO₄ dehydrogenase [10 U/ml], and MgCl₂ [8.1 mg/ml]; Sigma, St. Louis, MO) were prepared in 20 mM potassium phosphate buffer at pH 7.4 and added to sperm microsomal preparations. Microsomal frac-

tions of sperm were used because this procedure reduced the amount of endogenous androgens, allowing sufficient conversion of 1 β [³H]A to ³H₂O and estrone. Microsomal fractions from homogenates of chicken ovary and mouse leg muscle were used as positive and negative controls, respectively. Blank values were obtained from identical incubations in the absence of microsomes. Microsomal fractions were incubated in a shaking water bath at 34°C for 1 hour, after which samples were treated with an equal volume of charcoal dextran to remove unconverted 1 β [³H]A. Samples were centrifuged at 1,800 \times g for 10 minutes, and supernatants were removed and mixed with Aquasol-2 (New England Nuclear). Samples were counted for 10 minutes in a liquid scintillation counter (Packard, Downers Grove, IL). P450arom activity was calculated as fmol/mg protein/hour. This assay was previously validated for the measurement of P450arom activity in mouse germ cells (Nitta et al, 1993). Protein concentrations in microsomal fractions were determined with the Bradford assay (Bradford, 1976).

Statistical Analysis

Mean P450arom activities of sperm isolated from different regions of the epididymis were evaluated by *F*-test analysis using Statmost for Windows. Differences at a probability of *P* < 0.05 were considered statistically significant.

Results

Immunocytochemical Localization of P450 Aromatase

P450arom in epididymal sperm of adult mice was localized using immunocytochemistry. Strong P450arom immunoreactivity (brown reaction product) was detected in sperm present in the proximal caput region of the mouse epididymis (Fig. 1A-D). More specific localization of P450arom was observed at higher magnification of sperm (Fig. 1C,D), which revealed that immunostaining was primarily found in the cytoplasmic droplet. There was a lack of staining on the axoneme of the tail that protrudes through the cytoplasmic droplet (Fig. 1D, arrows). Immunostaining for P450arom appeared to be less intense in sperm present in the distal caput, corpus, and cauda epididymis (Fig. 1E,F,G, respectively) and was absent in the vas deferens (Fig. 1H). No immunostaining was observed in the epididymal epithelium except in the clear

droplet (arrow). (E-H) Immunostaining for P450arom in sperm present in the distal caput (E), corpus (F), and cauda (G) epididymis and vas deferens (H). (E) Distal caput epididymis. Staining is dramatically reduced in the cytoplasmic droplets of sperm (arrows) in this region of the epididymis. No staining is present in the epithelium (dce). (F) Corpus epididymis. Cytoplasmic droplets are reduced in size and exhibit reduced immunostaining for P450arom (arrows). Immunostaining is apparent in the clear cells (cc); this may be due to either a nonspecific peroxidase reaction or the possible absorption of the P450arom protein in the cytoplasmic droplet. No other staining is present in the epididymal epithelium (coe) or sperm. (G) Cauda epididymis. Staining is similar to that seen in the corpus epididymis. No P450arom immunostaining is present in the epithelium (cde) except in clear cells (not shown), as is seen in the corpus epithelium. (H) Vas deferens. Cytoplasmic droplets have disappeared from sperm in this region. Immunostaining is lacking and appears similar to that of the control tissue. No staining is present in the vas deferens epithelium (vde). (I) Proximal caput epididymis (control). No reaction product is present in sperm or epithelium (pce). Abbreviations: pce, proximal caput epithelium; dce, distal caput epithelium; cc, clear cells; coe, corpus epithelium; cde, cauda epithelium; vde, vas deferens epithelium; H, head; M, mid-piece; F, flagellum. A, B, E-I: bar = 12.5 μ m; C, D: bar = 6.25 μ m.

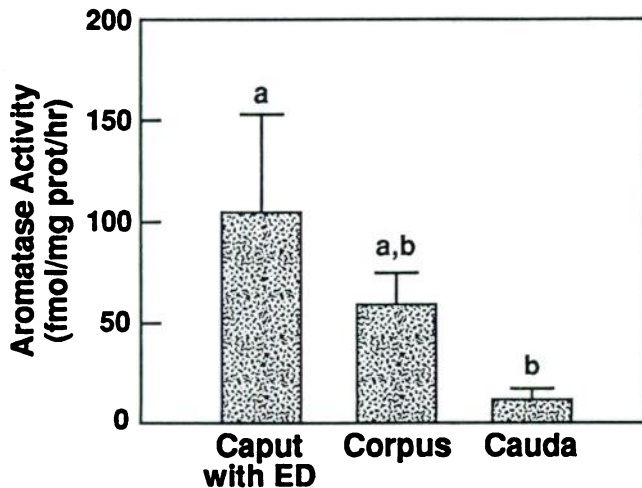


FIG. 2. P450arom activity in sperm decreased as sperm traversed from the efferent ductules (ED) and caput epididymis to the cauda epididymis. Data represent means \pm SEM from duplicate determinations of three separate experiments using eight mice each. The values without a common letter are significantly different ($P < 0.05$).

cells of the corpus (Fig. 1F) and cauda epididymis (not shown); this may be due to either a nonspecific peroxidase reaction or the possible endocytosis of the P450arom protein from the cytoplasmic droplet that is shed from the sperm as they traverse the epididymis. However, control sections from the corpus and cauda epididymis also exhibited immunostaining in the clear cells (not shown), but sections were not specifically pretreated with a block for the peroxidase reaction. Therefore, in the current study we cannot rule out nonspecific peroxidase staining of the clear cells. Other control tissues showed no reaction product when normal rabbit serum was substituted for the primary antibody (Fig. 1I), nor in sections treated with antibody that was preabsorbed with affinity-purified human placental P450arom (not shown).

P450 Aromatase Activity in Epididymal Sperm

To determine if the P450arom immunolocalized in epididymal sperm is active, P450arom activity was measured using the $^3\text{H}_2\text{O}$ assay in sperm isolated from different regions of the epididymis (Fig. 2). P450arom activities (fmol/mg protein/hour) measured in sperm isolated from various regions of the epididymis were: caput epididymis with attached efferent ductules, 105 ± 48 ; corpus epididymis, 59 ± 7 ; and cauda epididymis, 12 ± 6 . These results are the mean of three separate experiments using eight mice each. P450arom activity of sperm present in the efferent ductules and caput epididymis was consistently greater than P450arom activities of sperm isolated from the other regions of the epididymis and was significantly ($P < 0.05$) greater than P450arom activity of sperm isolated from the cauda epididymis.

Discussion

This is the first report that P450arom is present and active in sperm located in the efferent ductules and epididymis of the mouse. We have shown that the ability of testicular germ cells to synthesize estrogen is retained when sperm are released into the excurrent ductal system. In the current study we have shown that P450arom is also present and active in luminal sperm of the efferent ductules and epididymis. However, P450arom activity decreased as sperm traversed the epididymis, corresponding with the reduction and shedding of the cytoplasmic droplet (Herme et al, 1988). Immunostaining for P450arom was localized in the sperm within the cytoplasmic droplet, which has been suggested to contain remnants of the endoplasmic reticulum and Golgi apparatus (Bloom and Nicander, 1961; Phillips, 1975; Oko et al, 1993). P450arom activity in sperm isolated from the efferent ductules and caput epididymis was significantly greater than P450arom activity in sperm isolated from the distal region of the epididymis. There was a reduction in both P450arom immunostaining as well as P450arom activity in sperm located in the corpus and cauda epididymis. All of these data support the current finding that P450arom immunostaining is localized to the cytoplasmic droplet of the sperm. At this time, we can only hypothesize that this reduction in P450arom immunostaining and activity may be the result of the shedding of the cytoplasmic droplet. In the rat, the cytoplasmic droplet moves along the mid-piece of the sperm tail from the neck region to the annulus and is shed in the distal corpus and cauda epididymis, where its remnants are taken up by epithelial clear cells (Herme et al, 1988; Robaire and Herme, 1988). It is also possible that the antibody is no longer recognizing the P450arom protein due to degradation of the P450arom enzyme. Thus, we have proposed a model that demonstrates that P450arom immunoreactivity and activity in sperm decreases as sperm traverse the epididymis (Fig. 3).

Furthermore, these results indicate that the high concentration of estradiol present in rete testis fluid (Free and Jaffe, 1979) may be partly attributed to the production of estrogen by testicular germ cells (Nitta et al, 1993), because active P450arom enzyme is present in germ cells. Nevertheless, we cannot disregard the possibility that the Leydig cells have synthesized some of the estradiol present in luminal fluid. However, it is our hypothesis that the concentration of estrogen in the luminal fluid is dependent on the number of maturing spermatozoa; this would provide a more precise regulation of epididymal function than could be provided by a constant high concentration of androgen. Additionally, the concentration of estrogen produced by sperm would also be dependent upon the

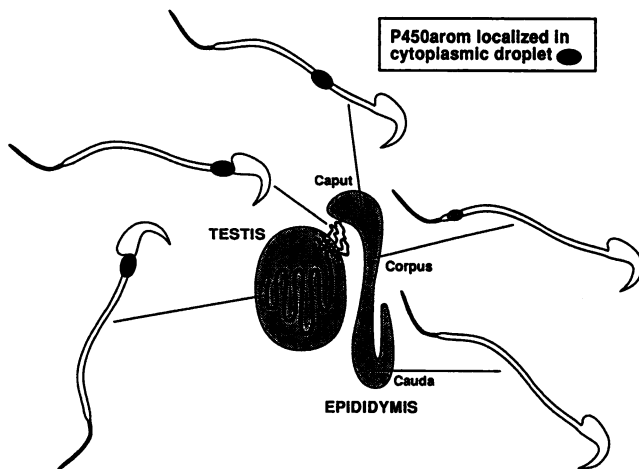


FIG. 3. Schematic representation of the decrease in P450arom immunostaining and activity as sperm traverse the efferent ductules and epididymis. P450arom is first detected in germ cells in the testis (Nitta et al, 1993). As sperm undergo maturation within the epididymis there is a decrease in P450arom immunostaining and activity, associated with the loss of the cytoplasmic droplet, that occurs in the corpus and cauda epididymis. It is our hypothesis that sperm-synthesized estrogen is important in proximal epididymal regulation.

location of sperm in the rete testis, efferent ductules, and epididymis.

The supposition that estrogen may participate in the regulation of epididymal function is supported by a number of studies. First, the head of the epididymis is not maintained by androgens alone (Fawcett and Hoffer, 1979). Castration followed by androgen replacement did not restore epididymal morphology. It has been hypothesized that a non-androgen sperm-associated factor(s) may help maintain epididymal integrity (Douglass et al, 1991). Second, the intramuscular injection of estradiol into adult rats resulted in increased concentrations of calcium and decreased concentrations of phosphorous in rete testis fluid, as well as increased concentrations of sodium and chloride and decreased concentrations of phosphorous, calcium, magnesium, and sulfur in the luminal fluid of the caput epididymis (Jenkins et al, 1983). Thus, it appears from this study that estrogen had a predominant effect on the luminal fluid in the proximal ducts, which are known for their importance in sperm maturation. Increasing the concentration of sperm, a major epididymal function, occurs through fluid absorption transpiring primarily in the efferent ductules and initial segment of the caput epididymis (Turner, 1984; Clulow et al, 1994). Third, estrogen may also help to regulate the rate of luminal fluid flow. Treatment with daily injections of estradiol caused an increase in the transit rate of sperm through the mouse epididymis (Meistrich et al, 1975). Finally, the highest concentration of estrogen receptors in the male reproductive tract is found in the efferent ductules and the head

of the epididymis in the few species studied thus far (Schleicher et al, 1984; West and Brenner, 1990). In our studies, it is this region that contains sperm with the greatest P450arom activity and the most intense immunostaining. In addition, cells reported to contain estrogen receptors (Schleicher et al, 1984) are also the cells implicated in the absorption of ions, water, and/or proteins (principal and apical cells) and cytoplasmic droplets (clear cells) (Robaire and Hermo, 1988). Thus, the role of estrogen in the regulation of epididymal function may be related to fluid and ion transport, which is maintained proportionally to the number of sperm in transit.

Estrogen receptors appear to be vital to normal male reproductive function. Lubahn et al (1993) first reported that the insertional disruption of the estrogen receptor gene (ERKO) in mice resulted in males with decreased fertility. In their study, ERKO mice showed decreased testis weights and very low sperm counts (10% those of control mice). However, subsequent study of ERKO mice showed that this mutation results in sterile males with seminiferous tubule dystrophy (Korach, 1994). Although estrogen's function in the male reproductive system is still unknown, the incidence of estrogen receptors in many parts of the male reproductive tract suggests a possible physiological role for estrogen in spermatogenesis and sperm maturation.

In conclusion, efferent ductule and epididymal sperm are capable of producing estrogen in the male reproductive system. Further study of the role of estrogen in the function of the epididymis may provide several benefits. The head of the epididymis, an estrogen target, is frequently implicated in cases of male infertility (Hendry et al, 1983). Knowledge of the role of estrogen in this region may provide solutions in the treatment of male infertility and could lead to the development of male contraception. Finally, an understanding of how estrogen functions in the epididymis could elucidate the mechanism by which neonatal exposure to diethylstilbestrol (DES) causes lesions of the adult epididymis (Gill et al, 1977). Currently, studies are being performed to explore the function of estrogen in the epididymis.

Acknowledgments

We thank Lesley Howell-Skalla and Dr. Shelley Tischkau for their help in the collection of tissue for the experiments and Dr. Charles Graves for his generous donation of the mice.

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