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Estrogen in the Adult Male: from a Curiosity to Absolute Necessity

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Abstract

Hess RA. Estrogen in the Adult Male: from a Curiosity to Absolute Necessity. ARBS Ann Rev Biomed Sci 2004; 6:1-12. Androgens are considered the male hormone. However, estrogen's presence and potential importance in the male reproductive system was noted as early as the 1940's and 1950's. Reference to estrogens in the male by most scientists prior to the 1990's appeared to be more of a curiosity than serious suggestion of endocrine function in the male. One of the turning points was the discovery that P450 aromatase was present and very active in the testis. Another major discovery was the effects of diethylstilbestrol treatment during pregnancy on male mice. These discoveries opened the door to serious investigations into the potential role of estrogen in the adult male and the potential long-term effects of exposure to estrogenic compounds during development. The decade of the 90's brought new discoveries that have led us to hypothesize that estrogen not only has important functions in the adult male reproductive tract, but that estrogen and its a-receptor are "essential" for normal fertility. Among these discoveries are the following: testicular germ cells and epididymal sperm synthesize estrogen; estrogen receptors are found in high concentration in the efferent ductule epithelium of all species examined; the ERa knockout (ERaKO) male is infertile and the efferent ductules are abnormal; ERa regulates fluid reabsorption by the efferent ductule epithelium; a pure antiestrogen inhibits sodium and water transport in the male tract.

Key words: Testis, Epididymis, Efferent Ductules, Estrogen, Testosterone, Aromatase.

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Contents

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Introduction

Testosterone is associated with the male and estrogen with the female. The primary sex steroid for function of the testis and accessory sex glands is testosterone, while its metabolite, 5a-dihydrotestosterone, is recognized as the primary hormone regulating epididymal functions (Orgebin-Crist *et al.*, 1975; Robaire & Viger, 1995). Nevertheless, estrogen is synthesized in the testis and is found in high concentrations in rete testis fluid and semen. Estrogen receptors are also expressed abundantly in the adult male tissues. However, the significance of estrogen's presence and function in the male reproductive system has only been recognized since the 1990's. It was known as early as the 1940's that developmental exposure to high doses of estrogens caused malformation of the male reproductive tract. Thus, data collected during the early formative years of the discipline of reproductive biology suggested that estrogen may be important in the male. At first, we did not know what to think of the presence of estrogen and its receptors in the male reproductive tract. Was estrogen a left over product of an abundance of testosterone synthesis? Did the male estrogen only function if it entered semen and then the female reproductive tract? Maybe estrogen receptors were required for development of the testis; could they be vestigial protein left over from the indifferent stage of embryological differentiation? How could estrogen have a function in the midst of an overload of androgens? These and many other questions were raised regarding estrogen presence and function in the male.

Reference to estrogen production by the testis began as early as the 1950's and 60's, but it was more of a curiosity, an effort to determine what types of testosterone metabolites were being produced in the testis (Baggett *et al.*, 1959; Staffieri *et al.*, 1965; Bedrak & Samuels, 1969). During the 1970's, the prediction of an estrogen receptor in testis or epididymis became a reality as estradiol binding was discovered (Baggett *et al.*, 1959; Staffieri *et al.,* 1965; Bedrak & Samuels, 1969; Danzo *et al.*, 1975; Danzo *et al.*, 1977; Danzo *et al.*, 1978; Danzo & Eller, 1979). However, it was clear from subsequent publications that most scientists did not consider estrogen to be a major steroid hormone in the male reproductive tract in the adult (Danzo *et al.*, 1983; Hendry *et al.*, 1985; Danzo, 1986; Toney & Danzo, 1988). At best, it was thought that estrogen binding was left over from developmental processes and that estrogen played only a small role in the adult male (Greco *et al.*, 1991; Greco *et al.*, 1992). Most interesting was the discovery that cytochrome P450 aromatase, which is capable of converting androgens into estrogens, was present in the testis (Tcholakian *et al.*, 1974; Dorrington *et al.*, 1978; Pomerantz, 1979; Valladares & Payne, 1979).

The potential importance of estrogen during development of the male reproductive system was made popular by the report that diethylstilbestrol (DES) treatment during pregnancy induces cryptorchidism and epididymal cysts in male mice (McLachlan *et al.*, 1975). The discovery of DES effects opened the door to serious investigations into the potential long-term effects of exposure to estrogenic compounds during development on male reproduction, an area of inquiry that continues to this day (Sharpe *et al.*, 2003; Wistuba *et al.*, 2003). Although developmental effects of estrogen in the male are important, such studies did not actually prove that estrogen has a role in adult male reproductive function. Therefore, efforts to understand estrogen's presence in the testis continued in the 1980's with further discoveries related to estrogen concentrations in semen (Jensen, 1974; Ganjam & Amann, 1976; Eiler & Graves, 1977; Free & Jaffe, 1979; Waites & Einer- Setchell *et al.*, 1983; Adamopoulos *et al.*, 1984; Claus *et al.*, 1985; Claus *et al.*, 1992; Bujan *et al.*, 1993) and localization of aromatase in testicular cells (Valladares *et al.*, 1979; Rommerts & Brinkman, 1981; Nozu *et al.*, 1982; Rommerts *et al.*,1982; Tsai-Morris *et al.*, 1984; Kurosumi *et al.*, 1985; Tsai-Morris *et al.*, 1985; Papadopoulos *et al.*, 1986; Payne *et al.*, 1987; Tsai-Morris *et al.*, 1987; Carreau *et al.*, 1998).

Thus, up to the 1990's it appears that most scientific inquiry into estrogen's presence in the male was more of a curiosity and a worry that estrogen exposure during development was harmful. Then, the decade of the 90's brought new discoveries in the male that led to hypotheses proposing that estrogen not only has important functions in the adult male reproductive tract, but that estrogen and its a-receptor are "essential" for normal fertility. This flood of new concepts for estrogen's role in the male, began with the discovery that testicular germ cells and epididymal sperm synthesize estrogen (Nitta *et al.*, 1993). This discovery explained the presence of high concentration of estradiol in rete testis of the rat (Free & Jaffe, 1979) and provided a source of estrogen for the high concentration of receptors that were subsequently found to populate the head of the male reproductive tract (Cooke *et al.*, 1991; Iguchi *et al.*, 1991; Hess *et al.*, 1995; Fisher *et al.*, 1997; Goyal *et al.*, 1997[a]; Hess *et al.*, 1997[a]). However, the most important discoveries came with the production of the knockout mice. The ERa knockout (ERKO) that was generated by Dennis Lubahn and colleagues (Lubahn *et al.*, 1993) showed for the first time that ERa was essential for fertility in the male (Lubahn *et al.*, 1993; Eddy *et al.*, 1996; Dupont *et al.*, 2000). This animal model was further developed by my laboratory and colleagues to show that estrogen provides a physiological function in regulating fluid dynamics in the male reproductive tract, a function that is "essential" (Hess *et al.*, 1997[b]; Lee *et al.*, 2000; Hess & Nakai, 2000; Nakai *et al.*, 2001; Zhou *et al.*, 2001; Lee *et al.*, 2001; Oliveira *et al.*, 2001; Oliveira *et* al., 2002).

Estrogen in the Male

Estrogen is produced in sizable quantities in the testis, as well as the brain (Roselli *et al.*, 1997). It is also present in very high concentrations in the semen of several species (Claus *et al.*, 1992; Free & Jaffe, 1979; Ganjam & Amann, 1976; Bujan *et al.*, 1993; Waites & Einer-Jensen, 1974; Eiler & Graves, 1997; Claus *et al.*, 1985; Setchell *et al.*, 1983; Adamopoulos *et al.*, 1984). Early studies reported that the primary source of estrogen in the immature male was the Sertoli cell (van der Molen *et al.*, 1981). In the adult testis, Leydig cells express aromatase (P450arom) and actively synthesize estradiol at a rate much greater than that seen in the adult Sertoli cell (Payne *et al.*, 1987; Levallet *et al.*, 1998; Carreau *et al.*, 1999; Levin, 2002). Currently, a growing body of evidence indicates that germ cells also synthesize estrogen, and possibly serve as the major source of this steroid in the male reproductive tract. In 1993, in collaboration with the laboratories of Bahr and Bunick (Nitta *et al.*, 1993), we reported for the first time that P450arom is present in testicular germ cells of the adult male mouse. The enzyme was localized in the Golgi of round spermatids and throughout the cytoplasm of elongating and late spermatids. Its presence was confirmed by Western and Northern blot analysis to demonstrate its mRNA in isolated developing germ cells. Its activity in germ cells was equal to or exceeded the activity found in the interstitial cells.

The presence of P450arom in male germ cells has now been demonstrated in several species, including mouse, rat, brown bear and rooster (Nitta *et al.*, 1993; Hess *et al.*, 1995; Kwon *et al.*, 1995; Janulis *et al.*, 1996[a]; Janullis *et al.*, 1996[b]; Janulis *et al.*, 1998; Hess *et al.*, 2001). The enzyme is located in cytoplasmic droplets of the sperm tail, but the staining becomes less intense as sperm traverse the epididymis (Janulis *et al.*, 1996[b]). Its presence in germ cells and spermatozoa was recently confirmed and shown to represent approximately 62% of the total testicular aromatase (Levallet & Carreau, 1997; Levallet *et* al., 1998; Carreau *et al.*, 1999). Others have shown the absence of aromatase in the epididymis (Schleicher *et al.*, 1989); thus, the conversion of androgens to estrogens by sperm remains the primary source of estrogen in the lumen of the tract. This observation raises new and exciting hypotheses regarding the potential for estrogen to regulate functions in the efferent ductules, epididymis and vas deferens.

The concentration of estrogens in peripheral blood is typically low in the male, but ranges from 2-180 pg/ml depending upon the species. The horse is an exception, where estrone sulfate is found as high as 2,447 pg/ml (Setchell, 1982; Claus *et al.*, 1992). Estrogen concentrations are typically higher in the testicular vein and lymph than in the general circulation. Also, in the reproductive tract, estrogen concentrations can reach relatively high concentrations. In one report, estrogen concentration in rete testis fluid of the rat was approximately 250 pg/ml (Free & Jaffe, 1979), which is higher than the average serum concentration of estradiol in the female (Overpeck *et al.,* 1978; Roibare & Fan, 1998). Estrogens are also abundant in semen and depending upon the species, their concentration can range from 14 to nearly 900 pg/ml (Einer-Jensen, 1974; Ganjam & Amann, 1976; Waites & Eiler & Graves, 1977). Estrone-sulfate is found as high as 4000 pg/ml in the horse (Claus *et al.*, 1992).

Estrogen Receptors

It has been known for at least 25 years that an estrogen receptor-like protein exists in epididymal tissues (Danzo *et al.*, 1975). However, those early studies lead to the conclusion that estrogen was more important during development of the epididymis than in adult function. Estrogen binding in epididymal tissues has been noted in many species, including the dog (Younes *et al.*, 1979; Younes & Pierrepoint, 1981), human (Murphy *et al.*, 1980), turtle (Dufaure *et al.*, 1983), monkey (Kamal *et al.*, 1985; West & Brenner, 1990), ram (Tekpetey & Amann, 1998), guinea pig (Danzo *et al.*, 1981), and the rat (Kuiper *et al.*, 1997). Autoradiography was also used to show estrogen binding throughout the male reproductive system (Schleicher *et at.*, 1984; Hess *et* al., 1997[a]). Schleicher and coworkers (Schleicher *et at.*, 1984) found very strong labeling of the efferent ductules and initial segment epididymis, with lesser binding in the distal tract. However, binding assays do not differentiate between ERa and b. Therefore, other methods, such as immunocytochemistry (ICC), *in situ* hybridization and Northern blot analysis, have been used to separate the two ER subtypes. However, these techniques do not always provide identical results, and there are disagreements between laboratories and between species. Using ICC, ER has been localized primarily in the epithelium of efferent ductules (Fisher *et al.*, 1997; Hess *et al.*, 1997[a]; West & Brenner, 1990; Goyal *et al.*, 1998; Goyal *et al.*, 1997[b]; Kwon *et al.*, 1997; Ergun *et al.*, 1997; Sato *et al.*, 1994; Saunders *et al.*, 2001). However, in the goat and monkey, only nonciliated cells of the efferent ductal epithelium stained ER positive (West & Brenner, 1990; Goyal *et al.*, 1997[a]). After the discovery of ER subtypes and the production of specific antibodies, ERa localization in the epididymis has also given confusing results (Fisher *et al.*, 1997; Hess *et al.*, 1997[b]; Goyal *et al.*, 1997[b]; Kwon *et al.*, 1997; Goyal *et al.*, 1998; Nie *et al.*, 2002; Zhou *et al.*, 2002). In the mouse at 90 days of age, the efferent ductule epithelium was strongly positive for ERa immunostaining, using the H222 antibody (Iguchi *et al.*, 1991). Other epithelia along the epididymis were only slightly positive. Using a different antibody, the mouse epididymis showed strong ERa staining in principal cells and other cell types, but in a region specific manner (Zhou *et al.*, 2002). This immunostaining is somewhat similar to the autoradiography data previously shown by Schleicher (Schleicher *et al.*, 1984). However, ^{3}H-E$_{2}$ showed binding in clear cells of the cauda and vas and in fibroblasts along the entire tract.

In the rat, ERa localization has been more controversial. In one study, using a mouse monoclonal antibody (6F11) against the A/B region of the human ERa, positive staining is found only in epithelial cells of the efferent ductules (Fisher *et al.*, 1997). The epididymal tissues were negative. Our laboratory repeated this study using the 6F11 antibody (Novocastra, UK) and the data are in complete agreement with the Fisher study, show staining only in epithelia of the efferent ductules (Hess *et al.*, 2002). In another study using frozen sections and the ER21 antibody, which is made against a peptide containing the first 21 amino acids of the rat and human ERa (does not cross-react with ERb), we also found predominant staining in efferent ductules (Hess *et al.*, 1997[b]), as shown for all species examined to date. However, the initial segment epididymis was also strongly positive and the remaining regions of the epididymis were moderately positive. This study was also repeated but using antigen retrieval methods, instead of frozen sections. The results differed only slightly (Hess *et al.*, 2002). The major difference was in staining that is now observed in the epithelium of the vas deferens, which is negative using frozen sections. This difference in staining in the rat between the two antibodies, 6F11 and ER21, raises serious questions regarding the literature's description of ER localization in the male reproductive tract using ICC alone. Autoradiography and estradiol binding assays indicate that ER is present in the rat epididymis. RT-PCR data also show that ERa is present in epididymal tissues (Hess *et al*., 1997[b]; Saunders *et al*., 2001). Therefore, future studies should focus on *in situ* hybridization methods for localizing the mRNA in specific regions and cell types of the epididymis.

Evidence for ER in the human efferent ductules and epididymis has been reported by several laboratories (Murphy *et al*., 1980; Palacios *et al*., 1993; Misao *et al*., 1997; Saunders *et al*., 2001). However, in some cases the principal cells were negative, while the basal cells and stromal cells were positive. The epididymis in nonhuman primates is also ER positive by RT-PCR, but there was no distinction between the a and b subtypes (Heikinheimo *et al*., 1995).

The discovery of a second form of ER (ERb) further complicates the interpretation of earlier data from estrogen binding studies. ERb has now been found in testis, efferent ductules, epididymis and prostate (Kuiper *et al*., 1996; Hess *et al*., 1997[a]; Kuiper *et al*., 1997; Prins *et al*., 1997; Prins *et al*., 1998; Krege *et al*., 1998; van Pelt *et al*., 1999; Saunders *et al*., 2001; Prins *et al*., 2001). However, a function for ERb in the male reproductive tract awaits further investigation, as the ERb knockout mouse has been shown to be fertile and appears to have a normal testis and epididymis (Krege *et al*., 1998). ERb is more widely distributed in the male tract than ERa (Hess *et al*., 2002). ERb has strong reactivity in efferent ductules, similar to ERa. In the remainder of the tract, ERb appears to be weaker in initial segment epididymis but stronger in the corpus, cauda and vas deferens. The stromal tissue cells also stain strongly positive for ERb throughout the male reproductive tract. Thus, there is a large potential for estrogen binding in the epididymis and vas deferens, either through ERa or ERb.

Estrogen Functions in Efferent Ductules

The efferent ductules are a series of tubules that connect rete testis to the epididymis. One-third or more of the head of the epididymis in man and other mammals contains these ducts and it was once thought that they simply transported sperm from testis to the epididymis. However, it is now known that efferent ductules have an important function in the reabsorption of over 90% of the rete testis fluid and thereby concentrate sperm prior to entering the epididymal lumen (Clulow *et al*., 1998). Nonciliated cells of the epithelium are reabsorptive, similar to proximal tubules of the kidney, having a brush border of microvilli connecting in the apical cytoplasm to a profusion of apical canaliculi, vesicles, tubules and membrane-bound bodies, which constitutes an elaborate endocytotic/ lysosomal system (Hermo *et al*., 1994). In the basal region, rough endoplasmic reticulum, mitochondria and lipid droplets are common (Ilio & Hess, 1994). The efferent ductules express an abundance of both androgens and estrogen receptors (Nie *et al*., 2002; Zhou *et al*., 2002; Hess *et al*., 2002).

Much of what we know about estrogen's function in efferent ductules has been derived from the study of the ERKO mouse and the use of antiestrogen treatment models. The male ERKO mouse was found to be infertile (Lubahn *et al*., 1993), raising the possibility that ERa is required for normal function of the male reproductive system. Although the ERKO testis appeared normal before puberty, after the onset of spermatogenesis, the testis began to degenerate and eventually became atrophic (Eddy *et al*., 1996). By 150 days, cauda sperm from the ERKO male were abnormal and sperm concentrations were significantly reduced (Eddy *et al*., 1996), suggesting that the reproductive tract was also abnormal. A later study by the Eddy's lab showed that ERKO germ cells transplanted into a normal testis (treated with busulphan to remove native germ cells) were capable of fertilization (Mahato *et al*., 2000). That study clearly pointed to extra-testicular regions, such as the efferent ductules and epididymis, being the major source of pathological alterations in ERKO males (Eddy *et al*., 1996; Hess *et al*., 1997[b]).

The rete testis in ERKO males is dilated and protrudes into the testis (Eddy *et al*., 1996; Hess *et al*., 1997[b]). Based upon these data, we hypothesized that the efferent ductules were either a) occluded due to excessive reabsorption, or b) dilated due to an inhibition of fluid reabsorption. After careful examination, we found the second hypothesis to be true, as the efferent ductule lumen was dilated markedly (Hess *et al*., 1997[b]). There appeared to be an inhibition of fluid reabsorption and possibly a net inward flux of water into the ductal lumen. Thus, the excessive accumulation of fluid in the lumen was overloading the funnel-like ductal system found in the rodent. As predicted, the accumulation of fluid caused a transient increase in testis weight in ERKO males between 32-81 days of age and then a steady decrease in weight out to 185 days of age, when total atrophy was observed. These data suggested that long-term atrophy of testes in the knockout mouse was caused by backpressure of the accumulating luminal fluids, a well-recognized pathogenesis found after exposure to various toxicants (Hess *et al.*, 1997[b]; Hess *et al.*, 2000). However, atrophy was not induced by antiestrogen treatment in adult mice (unpublished data), suggesting that in ERKO mouse, this pathological event is due to a developmental anomaly.

In the ERKO efferent ductule epithelium, the endocytotic apparatus was nearly lost and other cytoplasmic organelles appeared reduced and scattered randomly (Hess *et al.*, 1997[b]; Lee *et al.*, 2000; Hess *et al.*, 2000; Nakai *et al.*, 2001; Zhou *et al.*, 2001). The endocytotic pathway includes apical vesicles and PAS+ lysosomal granules, which are prominent in nonciliated cells of normal efferent ductules (Hermo & de Melo, 1987; Ilio & Hess, 1994; Clulow *et al.*, 1998). The ERKO epithelium was also flattened and the microvillus border was shortened and even absent in some cells. All of these changes are consistent with a decrease in fluid reabsorption, which was observed in the ERKO male (Hess *et al.*, 1997[b]). Thus, in the absence of a functional ERa, the apical surface of this reabsorbing epithelium appeared to be transformed into a non-absorbing structure.

The ERKO mouse provided the first strong evidence that estrogen, or more specifically, a functional ERa, is involved in the regulation of fluid transport in the male reproductive tract, and responsible for increasing the concentration of sperm as they enter the epididymis. Subsequent studies have shown that the major Na+ transporter in the efferent ductule epithelium (NHE3) is down regulated in the ERKO male reproductive tract). Both the mRNA and NHE3 protein were decreased substantially in ERKO tissue, and Na+ uptake by the epithelial cell *in vitro* was negligible (Nakai *et al.*, 2001). However, the ERKO mouse lacks a functional ERa throughout development. Therefore, the morphological and physiological abnormalities observed could represent developmental defects, rather than adult dysfunction. To test this hypothesis, adult mice were treated with a pure antiestrogen, ICI 182,780 (AstraZeneca, Macclesfield, Cheshire, UK). This collaborative study with David Bunick and Janice Bahr showed conclusively that ERa is important for adult function of the efferent ductules, as ICI induced pathological changes that were nearly identical to those seen in the ERKO mouse (Lee *et al*, 2000). A second species, the adult male rat, also responds in a similar manner to ICI treatment over a 125-day period (Oliveira *et al.*, 2001; Oliveira *et al.*, 2002). The two major response variables, dilation of efferent ductule lumen and decreased expression of NHE3, show identical responses in rats and mice (Zhou *et al.*, 2001; Oliveira *et al.*, 2002). Although the rats become infertile, they do show greater variation in response overall than is seen in the ICI-treated mice. Long-term treatment in the rat resulted in a transient increase in testicular weight, eventual testicular atrophy at the time of infertility, whereas in the ICI-treated mouse there was no change in testicular weight. The rat efferent ductule epithelium also showed a transient increase and redistribution of PAS-positive lysosomal granules in the nonciliated cells after ICI treatment (Oliveira *et al.*, 2001; Oliveira *et al.*, 2002). However, with continued treatment the rat epithelium showed a decrease in the number of lysosomes to nearly undetectable levels (Hess *et al.*, 1997[b]), similar to ERKO and mice treated with ICI. Lysosomes are more numerous in rat than in mouse efferent ductules (Ilio & Hess, 1994); therefore, this intriguing interspecies difference in response to the antiestrogen must be examined in future studies involving other species. Overall, it was shown that ICI promotes adult dysfunctional changes in rat efferent ductules similar to those of ERKO and ICI treated mice, with luminal dilation, decreases in epithelial height, loss of cytoplasmic organelle and decreases in the expression of NHE3 protein and mRNA (Oliveira *et al.*, 2001; Oliveira *et al.*, 2002).

Conclusions

In conclusion, estrogen is important in the regulation of the male reproductive tract, with clear evidence pointing to a direct effect on the function of the efferent ductule epithelium. Estrogen is responsible for fluid reabsorption in this region of the male tract and also provides maintenance of epithelial morphology associated with endocytosis. These function are essential for fertility.

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