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The Effects of Aging and Estrogen Treatment during Aging on the Rat Epididymis

Kathryn Davis

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Committee Member: Dr. John Spitsbergen
April 21st, 2016

Abstract

Previous studies from our lab have demonstrated that a normal estrogen environment in the testis contributes to maintaining spermatogenesis in adult rats and that estrogen treatment can attenuate the age-associated decline in sperm production. The purpose of this study was to determine if the estrogen environment of the epididymis is altered with age and what effects estrogen treatment may have on the epididymis during aging. The study compared untreated rats at 15 months of age to 18 month old rats that were treated with either vehicle, or estradiol-valerate, once every third day from 15 to 18 months. Mean concentrations of testosterone were highest in the corpus compared to the initial segment/caput and proximal cauda. A similar trend was observed for mean estradiol concentrations with the highest concentration in the corpus. Even though there were significant regional differences in hormone concentrations, the concentrations were similar amongst the three groups. In all three groups, the tubular and luminal diameter of the cauda was significantly larger than the initial segment. In the 15 month old and 18 month old treated animals, the epithelial cell height of the cauda was significantly smaller than that of the initial segment. These regional differences were not seen in the 18 month old animals. Immunocytochemistry demonstrated that ER α was expressed in nuclei of principal cells throughout the epididymis even though the intensity of immunostaining did not appear to differ between treatment groups. However, transit time through the distal cauda was significantly different between treatment groups. Mean transit time was 3.8 days at 15 months of age and increased to 5.5 days at 18 months of age; transit time was 3.3 days in estradiol-valerate treated rats. This suggests that treatment with estradiol-valerate was able to speed up epididymal transit time and prevent the prolonged transit time associated with aging. Collectively these results suggest that the estrogen environment of the epididymis may not change during aging in a

manner similar to what was observed in the testis, but epididymal functions, and thus sperm maturation, are likely altered during aging and by estrogen treatment.

Introduction

The epididymis is a tightly regulated, highly specialized organ responsible for the process of sperm maturation, transport, and storage in the male reproductive tract. In the epididymis, immature spermatozoa from the testis gain motility and the ability to fertilize an egg (Belleannée, Thimon, and Sullivan, 2012). It links the efferent ductules to the vas deferens and is divided into four distinct regions, from proximal to distal: initial segment, caput, corpus, and cauda (Joseph, Shur, and Hess, 2010). Each region contains a unique microenvironment specialized for each step of sperm maturation. Maturation of sperm is a sequential process from proximal to distal regions of the cauda, and each modification must occur in a particular order (Belleannée et al., 2012). In the caput and corpus, sperm acquire both motility and fertilization ability. In the cauda, motility is suppressed, and sperm is stored until ejaculation. These compartmentalized functions result from highly regulated gene expression and secretions in each region (Belleannée et al., 2012). Regional variation in gene expression correlates to the various types of epithelial cells found along the length of the epididymis. While there are five primary epithelial cell types (principal, narrow, apical, clear, and basal), this study primarily focuses on principal cells, basal cells, and clear cells. Principal cells make up 65-80% of the epididymal epithelium and are responsible for synthesizing and secreting a plethora of proteins into the lumen of epididymis (Joseph et al., 2010b). Basal cells constitute 15-20% of the epididymal epithelium. While anchored to the basement membrane, these cells have cytoplasmic extensions that come in contact with the lumen and may regulate principal and clear cell function

(Belleannée et al., 2012; Joseph et al., 2010b). Clear cells are most active in the cauda. These cells have an endocytotic function that insures spermatozoa remain immobile by creating an acidic luminal environment (Belleannée et al., 2012). Regional differences and various cellular functions give rise to an organ with compartmentalized and specialized functions.

Hormonal factors play a significant role in regulating gene expression within epididymal epithelial cells. Testosterone from the testis regulates gene expression of many initial segment and caput specific genes. While the epididymis does not produce its own testosterone, it is crucial in regulating gene expression, as androgen receptor (AR) is present in every region of the epididymis (Zaya, Hennick, and Pearl, 2012). While it has traditionally been thought that androgens are primarily responsible for regulating the functions of the male reproductive tract, estrogens play a significant role as well. In the epididymis, the enzyme cytochrome P450 aromatase converts testosterone to estradiol (Joseph et al., 2010b). By binding to estrogen receptors (ER), estradiol and other estrogens play various functional roles within the epididymis. Estrogen regulates the aquaporin gene (*Aqp*) and, in turn, regulates fluid reabsorption within the lumen (Joseph et al., 2010b). Estrogen also regulates the contractility of smooth muscle in the epididymis by its interactions with oxytocin. Oxytocin receptor, through the release of endothelin-1, stimulates smooth muscle contraction. Estrogens increase the sensitivity of oxytocin receptors to oxytocin, effectively up-regulating epididymal motility (Filippi et al., 2002). Also pertinent to this study is the relationship between lactoferrin (Lf) and estrogen in the epididymis. Lactoferrin mRNA and subsequent Lf protein expression has been shown to be stimulated by oestrogen (Yu and Chen, 1993). While estrogens have historically been disregarded in the male reproductive tract, their importance in the epididymis cannot be denied.

Various protein factors are regulated by hormones and affect the processes of sperm maturation, transport, and storage in the epididymis. One such protein factor, lactoferrin (Lf), is an important secretory protein in the epididymis. In the male reproductive tract, Lf has antibacterial, antifungal, antiviral, and antioxidant activity (Pearl and Roser, 2014). It is a major sperm-coating antigen, and the epididymis is the primary producer of Lf in the male reproductive tract (Yu and Chen, 1993). Lactoferrin is found in highest concentrations in the principal cells of the corpus and cauda and is stored in vesicles near the apical membrane (Pearl and Roser, 2014). Lactoferrin is just one of many protein factors found in the epididymis.

Altering the estrogen environment of the epididymis has a significant impact on its functional abilities. Animals without a functioning *Esr1* gene are infertile, and the cauda sperm are less motile (Joseph et al., 2010b). In some species, a delay in epididymal development is observed when endogenous estrogen is reduced (Pearl and Roser, 2008). Additionally, animals without the *Esr1* gene lack the ability to properly reabsorb fluid, leading to an accumulation of luminal fluid in the epididymis. Also in these animals, the pH of the lumen remains alkaline instead of acidic as in the normal animals (Joseph et al., 2010a). Therefore, estrogen is essential for male fertility.

Changes in the epididymis are also observed during aging. In some species, morphological changes, most obvious in the cauda, adversely affect sperm quality. There is a decrease in sperm motility and also a decrease in sperm concentration (Calvo, Pastor, Martinez, Vazquez, and Roca, 1999). Additionally, the process of sperm maturation in the epididymis occurs at an increased rate, which implies there is not proper time for maturation to occur (Calvo et al., 1999). However, the exact physiological causes of the impaired epididymal function have yet to be discovered.

Previous studies from our lab have demonstrated the importance of estrogen signaling during aging in the rat testis. During aging, there is a decrease in intra-testicular estradiol concentration and a corresponding decrease in daily sperm production (DSP). Estrogen treatment during aging prevented the decline in estradiol concentration and attenuated the decline in DSP. In rats, maintaining a normal estrogen environment in the testis is crucial for the process of spermatogenesis (Clarke and Pearl, 2014). However, it remains unclear if the estrogen environment is altered during aging in the rest of the male reproductive tract as observed in the testis. More specifically, it is unknown if a changing estrogen environment in the epididymis affects the process of sperm maturation during aging. The purpose of this study was to determine if the estrogen environment of the epididymis is altered with age and how treatment with estrogen may affect the epididymis during the aging process.

Methods

Animals

Nine male Sprague-Dawley rats were used for this study and obtained from Charles River Laboratories (Wilmington, MA, USA). Throughout the study, animals were individually housed in the Western Michigan University animal facility with *ad libitum* access to food and water. Animals were divided into three groups (n=3). The first group contained animals at 15 months of age, which did not receive any experimental treatment. Tissues were collected from these three animals at 15 months of age. The other two groups were given subcutaneous injections on the dorsal side of the animals once every third day from age 15 to 18 months. Three animals were injected with 1 μ g/kg estradiol valerate dissolved in isotonic saline with 1% ethanol. The other

three animals were injected with isotonic saline with 1% ethanol as a vehicle control. Once these animals reached 18 months of age, tissues were collected.

When the animals reached the appropriate ages, they were euthanized and epididymides were collected. Of each epididymis pair, one was fixed in 4% paraformaldehyde while the other was snap frozen in liquid nitrogen and stored at -80°C .

Immunocytochemistry

Fixed tissue was paraffin embedded and cut into sections $5\mu\text{m}$ in thickness. Tissue was de-waxed using Citrisolv and subsequently rehydrated step-wise in 100%, 95%, and 70% ethanol in H_2O . Endogenous peroxidase activity was quenched using 0.3% H_2O_2 in methanol for a period of 30 minutes. In the determination of the presence of $\text{ER}\alpha$ and AR, antigen retrieval was performed. Slides were placed in Coplin jars, submerged in a citrate buffer antigen unmasking solution, and steam heated to 93°C for 5 minutes. Tissue was then cooled to room temperature. Antigen retrieval was not performed when determining the presence of Lactoferrin. All tissue was incubated with a blocking serum for 20 minutes at room temperature. Tissue was then incubated overnight at 4°C with antibodies to $\text{ER}\alpha$ (1:2000), AR (1:2000), or Lactoferrin (1:500). This 16-hour primary antibody incubation was followed by a 30-minute biotinylated secondary antibody incubation at room temperature. Next, the tissue was incubated in an avidin-biotin-peroxidase solution for 30 minutes, again at room temperature. Between each incubation, tissue was washed in phosphate-buffered saline/tween (PBST) for 5 minutes. Immunostaining was visualized using DAB chromagen for $\text{ER}\alpha$ and NovaRed chromagen for Lactoferrin and AR. These tissues were then counterstained using ImmunoMaster Hematoxylin and evaluated using light microscopy.

Morphology

Fixed tissue was paraffin embedded and cut into sections 5 μ m in thickness. The tissue was stained with PAS-H and visualized using light microscopy. Lumen diameter, epithelial cell height, and tubule diameter were determined in each region of the epididymis (initial segment, caput, corpus, and cauda) using 5 tubes per animal per region. Measurements were made using NiS Elements imaging software (Nikon).

Steroid Concentrations

Snap frozen epididymides were thawed and separated by region (IS/caput, corpus, and cauda). Tissue was homogenized in a buffer containing 50mM Tris-Base, 10mM EDTA, 150mM NaCl, 0.1% Tween 20, and protease inhibitors. The homogenate was centrifuged to remove any insoluble tissue. The supernatant was collected and assayed for testosterone and estradiol by separate competitive ELISAs (ENZO Life Sciences) following manufacturer instructions. An extraction step was performed during the estradiol assay using ethyl ether. Steroid concentrations were normalized to the amount of protein present in the homogenate as determined by a BCA protein assay. Values reported are mean pg/mg protein for both testosterone and estradiol.

Sperm Counts

Sperm count of the distal cauda was evaluated through a determination of detergent-resistant spermatids. Briefly, the distal cauda was homogenized in 0.9% saline with 0.05% Triton X-100 at room temperature. The homogenate was stored for 24 hours at 4°C. The following day, the number of detergent-resistant spermatids was counted using a hemocytometer (two counts for each sample) and phase contrast microscopy. Using these sperm counts, both the number of

sperm per distal cauda and sperm per gram of tissue were determined. Epididymal transit time was calculated by dividing the number of sperm in the distal cauda by the daily sperm production (DSP) for the corresponding testis. DSP was previously calculated and published for these animals (Clarke and Pearl, 2014).

Data Analysis

Data were analyzed by ANOVA using SAS. If the overall ANOVA was significant, differences between 15 month, 18 month, 18 month treated groups were analyzed by Tukey's multiple comparison test. To ensure normality, steroid concentration data was analyzed using log-transformed data. All values reported are mean \pm SEM.

Results

Epididymal Steroid Concentrations

To determine the effect of aging and estrogen treatment on steroid levels in the epididymis, concentrations of testosterone (Table 1a) and estradiol (Table 1b) were measured in 15 month, 18 month, and 18 month estradiol-valerate treated animals. In the 15 month old animals, the concentration of testosterone was 188 ± 51 pg/mg of protein in the caput, 599 ± 132 pg/mg in the corpus, and 265 ± 58 pg/mg in the cauda. This increase in concentration from the caput to the corpus was statistically significant. In the 18 month old animals, the concentration of testosterone was 242 ± 41 pg/mg of protein in the caput, 529 ± 103 pg/mg in the corpus, and 233 ± 58 pg/mg in the cauda. While the testosterone concentration in the caput and corpus were no longer significantly different, the decrease in concentration from the corpus to the cauda was statistically significant. A similar trend was seen in 18 month old estradiol-valerate treated

animals, with the testosterone concentration being 184 ± 24 pg/mg of protein in the caput, 452 ± 99 pg/mg in the corpus, and 171 ± 7.9 pg/mg in the cauda. Treatment with estradiol-valerate restored the previously observed difference between the caput and corpus, and the testosterone concentration was also significantly different between the corpus and cauda as seen in the 18 month old control animals. When comparing single epididymal regions, no differences were observed between treatment groups.

When quantifying the epididymal estradiol concentrations, a trend was observed throughout all treatment groups; estradiol concentration was highest in the corpus and lowest in the caput. In the 15 month old animals, the concentration of estradiol was 12.6 ± 1.5 pg/mg of protein in the caput, 34.4 ± 4.4 pg/mg in the corpus, and 26.9 ± 2.4 pg/mg in the cauda. In the 18 month old animals, the concentration of estradiol was 14.1 ± 1.7 pg/mg of protein in the caput, 30.5 ± 5.1 pg/mg in the corpus, and 25.1 ± 2.1 pg/mg in the cauda. In the 18 month old estradiol-valerate treated animals, the estradiol concentration was 13.2 ± 0.97 pg/mg of protein in the caput, 37.6 ± 5.8 pg/mg in the corpus, and 23.7 ± 2.7 pg/mg in the cauda. In all treatment groups, the estradiol concentration in the caput was significantly lower than that of the corpus and cauda. When comparing single regions, no differences were observed between treatment groups.

Epididymal Morphology

To determine the effect of aging and estrogen treatment on the structure of the epididymis, morphology of the epididymis was studied in 15 month old, 18 month old, and 18 month old estradiol-valerate treated animals. In the 15 month old animals, the lumen diameter (Table 2a) was the narrowest in the initial segment (127.1 ± 5.4 μm) and widest in the cauda (460.4 ± 30.8 μm). The diameter was significantly different between each region of the

Table 1a. Epididymal Steroid Concentrations- Testosterone. Testosterone concentrations were highest in the corpus and lower in the caput and cauda. While regional differences in testosterone were observed within groups, there were no significant differences in concentration between treatment groups (a vs. b indicates $p < 0.05$ within age/treatment group).

	Testosterone (pg/mg protein)		
	IS/Caput	Corpus	Cauda
15 month	188 ± 51 (a)	599 ± 132 (b)	265 ± 58 (ab)
18 month	242 ± 41 (ab)	529 ± 103 (a)	233 ± 42 (b)
18 month EV	184 ± 24 (a)	452 ± 99 (b)	171 ± 7.9 (a)

Table 1b. Epididymal Steroid Concentrations- Estradiol. Estradiol concentrations were highest in the corpus and lowest in the caput. While regional differences in estradiol were observed within groups, there were no significant differences in concentration between treatment groups (a vs. b indicates $p < 0.05$ within age/treatment group).

	Estradiol (pg/mg protein)		
	IS/Caput	Corpus	Cauda
15 month	12.6 ± 1.5 (a)	34.4 ± 4.4 (b)	26.9 ± 2.4 (b)
18 month	14.1 ± 1.7 (a)	30.5 ± 5.1 (b)	25.1 ± 2.1 (b)
18 month EV	13.2 ± 0.97 (a)	37.6 ± 5.8 (b)	23.7 ± 2.7 (b)

epididymis. In the 18 month old control animals, a similar trend was observed, with the lumen diameter narrowest in the initial segment ($111.8 \pm 10.4 \mu\text{m}$) and widest in the cauda ($520.2 \pm 53.6 \mu\text{m}$). While the lumen diameter was significantly different between the initial segment, caput, and cauda, the lumen diameter of the corpus was not different from the initial segment or caput. The same trend was observed in the 18 month old estradiol-valerate treated animals with the lumen diameter narrowest in the initial segment ($98.1 \pm 12.7 \mu\text{m}$) and widest in the cauda ($433.7 \pm 32.8 \mu\text{m}$). The diameters of the caput and corpus were not different from each other, but they were significantly different when compared to the initial segment and cauda. While regional differences were noted within age and treatment groups, no significant differences were observed between groups.

The next parameter of epididymal morphology that was studied was epithelial cell height (Table 2b). In the 15 month old animals, the epithelial cell height was tallest in the initial segment ($23.4 \pm 0.9 \mu\text{m}$) and shortest in the cauda ($11.7 \pm 1.4 \mu\text{m}$). The cell height of the cauda was significantly shorter than the other regions of the epididymis. Again, in the 18 month old animals, the epithelial cell height was tallest in the initial segment ($25.4 \pm 3.9 \mu\text{m}$) and shortest in the cauda ($13.2 \pm 0.99 \mu\text{m}$). Unlike the 15 month old animals, however, there was no significant difference in epithelial cell height between the epididymal regions. As observed in the other groups, the epithelial cell height of the 18 month old estradiol-valerate treated animals was tallest in the initial segment ($28.5 \pm 3.2 \mu\text{m}$) and shortest in the cauda ($12.1 \pm 1.3 \mu\text{m}$). Regional differences in epithelial cell height were restored in the treated animals, with the cell height of the cauda being significantly shorter than that of the initial segment and caput. Thus, it appears that estrogen treatment is able to maintain regional differences during aging.

Lastly, tubule diameter was studied in all 3 groups (Table 2c). In the 15 month old

animals, the tubule diameter was narrowest in the initial segment ($174.0 \pm 5.26 \mu\text{m}$) and widest in the cauda ($483.8 \pm 28 \mu\text{m}$). The diameter was significantly different between each region of the epididymis. A similar trend was observed in the 18 month old animals. The tubule diameter of the initial segment was narrowest ($162.7 \pm 2.74 \mu\text{m}$), and the cauda was the widest ($546.5 \pm 54.8 \mu\text{m}$). While the tubule diameter was significantly different between the initial segment, caput, and cauda, the tubule diameter of the corpus was not different from the initial segment or caput. In the 18 month old estradiol-valerate treated animals, the tubule diameter was narrowest in the initial segment ($155.1 \pm 10.2 \mu\text{m}$) and widest in the cauda ($458.1 \pm 30.6 \mu\text{m}$), as observed in other groups. While the diameters of the initial segment and cauda were different from every other region, the tubule diameters of the caput and corpus were not different from each other. Again, while regional differences existed within age and treatment groups, no significant differences were noted between groups.

In addition to morphological characteristics of the epididymis, the weight of the epididymis was also recorded. In 15 month old animals, the weight of a single epididymis was $0.81 \pm .05\text{g}$. In 18 month old animals, this weight increased to $0.84 \pm 0.03\text{g}$. Similarly, in 18 month old estradiol-valerate treated animals, the weight of a single epididymis was $0.83 \pm 0.02\text{g}$. No significant differences in epididymal weight between groups were noted.

Epididymal Sperm Counts

To determine the effect of aging and estrogen treatment on sperm maturation and storage in the epididymis, sperm counts were done in 15 month old, 18 month old, and 18 month old estradiol-valerate treated animals. First, the number of sperm per distal cauda was counted (Figure 1). The number of sperm in the distal cauda of the 15 month old animals was 133.7×10^6

Table 2a. Epididymal Morphology- Lumen Diameter. In each group, the lumen diameter was narrowest in the initial segment and widest in the cauda. Regional differences were noted within each animal group, while there were no significant differences between groups (a vs. b indicates $p < 0.05$ within age/treatment group).

	Lumen Diameter			
	Initial Segment	Caput	Corpus	Cauda
15 month	127.1 ± 5.4 (a)	317.9 ± 14.8 (b)	231.8 ± 15.1 (c)	460.4 ± 30.8 (d)
18 month	111.8 ± 10.4 (a)	283.4 ± 13 (b)	242.9 ± 14.4 (ab)	520.2 ± 53.6 (c)
18 month EV	98.1 ± 12.7 (a)	301 ± 14.7 (b)	231.3 ± 4.6 (b)	433.7 ± 32.8 (c)

Table 2b. Epididymal Morphology- Epithelial Cell Height. In each group, the cell height was shortest in the cauda and tallest in the initial segment. Regional differences were noted within the 15 month old group, but not the 18 month old group. Regional differences were restored in 18 month old treated animals. There were no significant differences between groups (a vs. b indicates $p < 0.05$ within age/treatment group).

	Cell Height			
	Initial Segment	Caput	Corpus	Cauda
15 month	23.4 ± 0.9 (a)	18.4 ± 0.59 (a)	20.9 ± 1.4 (a)	11.7 ± 1.4 (b)
18 month	25.4 ± 3.9	19.8 ± 2.4	22.1 ± 2.9	13.2 ± 0.99
18 month EV	28.5 ± 3.2 (a)	22.5 ± 2.1 (a)	21.1 ± 0.97 (ab)	12.1 ± 1.3 (b)

Table 2c. Epididymal Morphology- Tubule Diameter. In each group, the tubule diameter was narrowest in the initial segment and widest in the cauda. Regional differences were noted within each animal group, while there were no significant differences between groups (a vs. b indicates $p < 0.05$ within age/treatment group).

	Tubule Diameter			
	Initial Segment	Caput	Corpus	Cauda
15 month	174.0 ± 5.26 (a)	354.6 ± 14.1 (b)	273.7 ± 12.5 (c)	483.8 ± 28.0 (d)
18 month	162.7 ± 2.74 (a)	323.0 ± 14.8 (b)	287.1 ± 1.7 (ab)	546.5 ± 54.8 (c)
18 month EV	155.1 ± 10.2 (a)	346.1 ± 10.5 (b)	273.4 ± 4.23 (b)	458.1 ± 30.6 (c)

$\pm 3.96 \times 10^6$. The number of sperm in the distal cauda of the 18 month old animals was $136.5 \times 10^6 \pm 18.4 \times 10^6$. In the 18 month old treated animals, the number of sperm in the distal cauda was $100.6 \times 10^6 \pm 13.8 \times 10^6$. Despite this slight decline, the differences between groups were not significant.

The number of sperm per distal cauda was then normalized by weight, as shown in Figure 2. In the 15 month old animals, the weight of the ground epididymis was $0.193 \pm 0.01\text{g}$, and the number of sperm per gram of tissue was $696.9 \times 10^6 \pm 31.4 \times 10^6$. Similarly, in the 18 month old animals, the weight of the ground epididymis was $0.199 \pm 0.03\text{g}$, and the number of sperm per gram of tissue was $697.2 \times 10^6 \pm 70.7 \times 10^6$. In the 18 month old treated animals, the weight of the ground epididymis was $0.15 \pm 0.01\text{g}$, and the number of sperm per gram of tissue was $665.8 \times 10^6 \pm 55.3 \times 10^6$. Again, there were no significant differences between groups.

To calculate the transit time through the epididymis, the number of sperm in the distal cauda was divided by the daily sperm production (DSP) of the corresponding testis. The DSP of the 15 month old rats was $36 \times 10^6 \pm 0.98 \times 10^6$ sperm/g testis. In the 18 month old rats, the DSP decreased to $25 \times 10^6 \pm 1.00 \times 10^6$ sperm/g testis. With estradiol-valerate treatment, the DSP of the 18 month old treated animals increased to $30 \times 10^6 \pm 0.28 \times 10^6$ sperm/g testis. The transit time (Figure 3) through the epididymis of the 15 month old animals was 3.79 ± 0.21 days. The transit time slowed through the epididymis of the 18 month old animals to 5.47 ± 0.47 days. In the 18 month old estradiol-valerate treated animals, the transit time sped up to 3.29 ± 0.44 days, similar to the 15 month old animals. The increase in speed between the 18 month old control animals and the 18 month old treated animals was significant. Thus, estrogen appears to regulate epididymal transit time and likely exposure to the luminal contents of the epididymis.

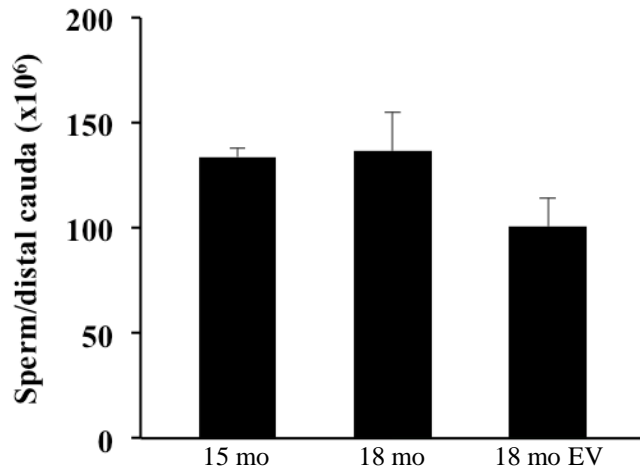


Figure 1. Sperm per distal cauda. No significant differences were observed in sperm/distal cauda between the three groups groups (n = 3 animals per group).

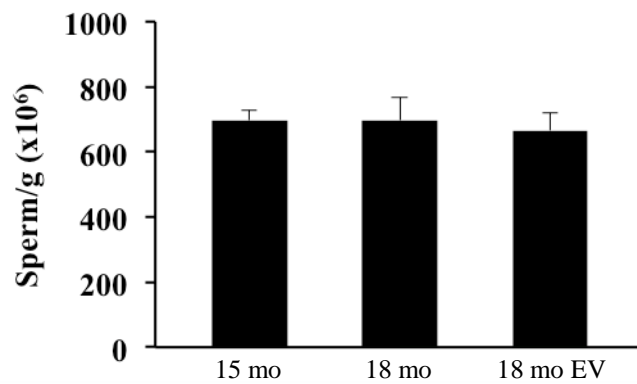


Figure 2. Sperm per gram of Epididymis. No significant differences between groups were observed when sperm counts were normalized by weight (n = 3 animals per group).

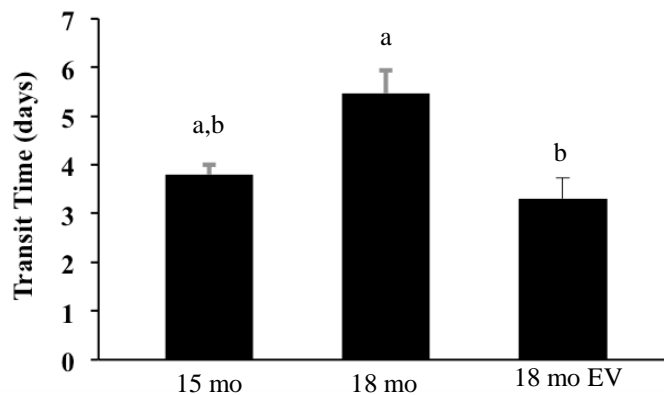


Figure 3. Epididymal Transit Time. The transit time was significantly slower in the 18 month old animals as compared to the animals of the same age treated with estradiol-valerate (a vs. b indicates $p < 0.05$).

Immunocytochemistry

To determine the effect of aging and estrogen treatment on the expression of androgen receptor (AR), estrogen receptor alpha (ER α), and lactoferrin (Lf), immunocytochemistry procedures were followed in 15 month old, 18 month old, and 18 month old estradiol-valerate treated animals. AR expression (Figure 4) was observed in the nuclei of both principal cells and basal cells throughout the length of the epididymis. Receptor expression appeared higher in the initial segment when compared to the caput, corpus, and cauda. Although this regional difference was observed, there did not appear to be significant differences between ages and treatment groups. ER α expression (Figure 5) was observed in both the nuclei and cytoplasm of principal cells in each region of the epididymis. The presence of ER α was relatively constant between the initial segment, caput, corpus, and cauda. As with AR, ER α expression was similar between ages and treatment groups. As seen in other species, the expression of Lf (Figure 6) increased in the more distal regions of the epididymis. The protein expression was absent in the initial segment and caput. Positive staining was observed in the cytoplasm of principal cells in the corpus with the strongest presence in the cauda. Clear cells in the cauda were negative for Lf expression. Again, significant differences were not observed between ages or treatment groups.

Discussion

The purpose of this study was to determine if the estrogen environment of the epididymis is altered with age and how treatment with estrogen may affect the epididymis during the aging process. Based on the results of this study, receptor expression, protein expression, and hormone concentration were not altered with age. However, regional differences in epithelial cell height deteriorated with age, and treatment with estrogen during aging helped to maintain these regional

Androgen Receptor

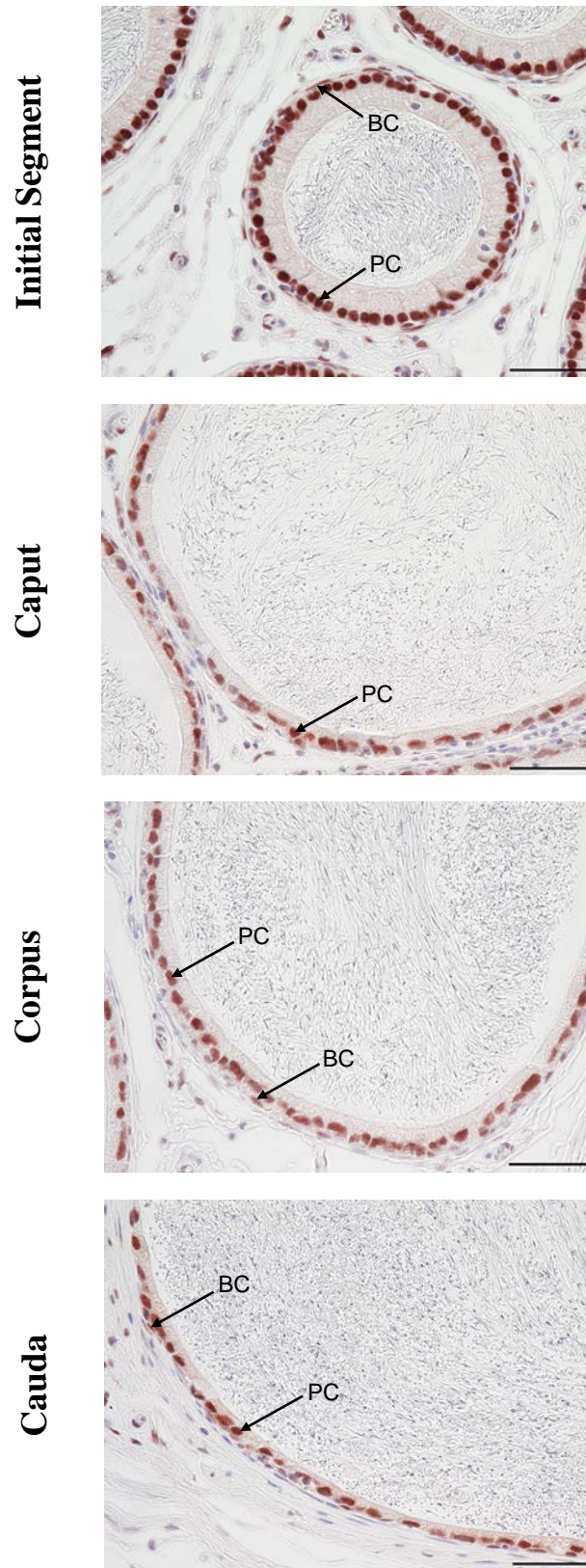


Figure 4. Representative Immunolocalization of Androgen Receptor (AR). Positive nuclear staining for AR was observed in principal (arrow, PC) and basal cells (arrow, BC) of the initial segment, caput, corpus, and cauda. AR expression appeared similar between ages and treatment groups. Scale Bar = 50 μ m.

Estrogen Receptor α

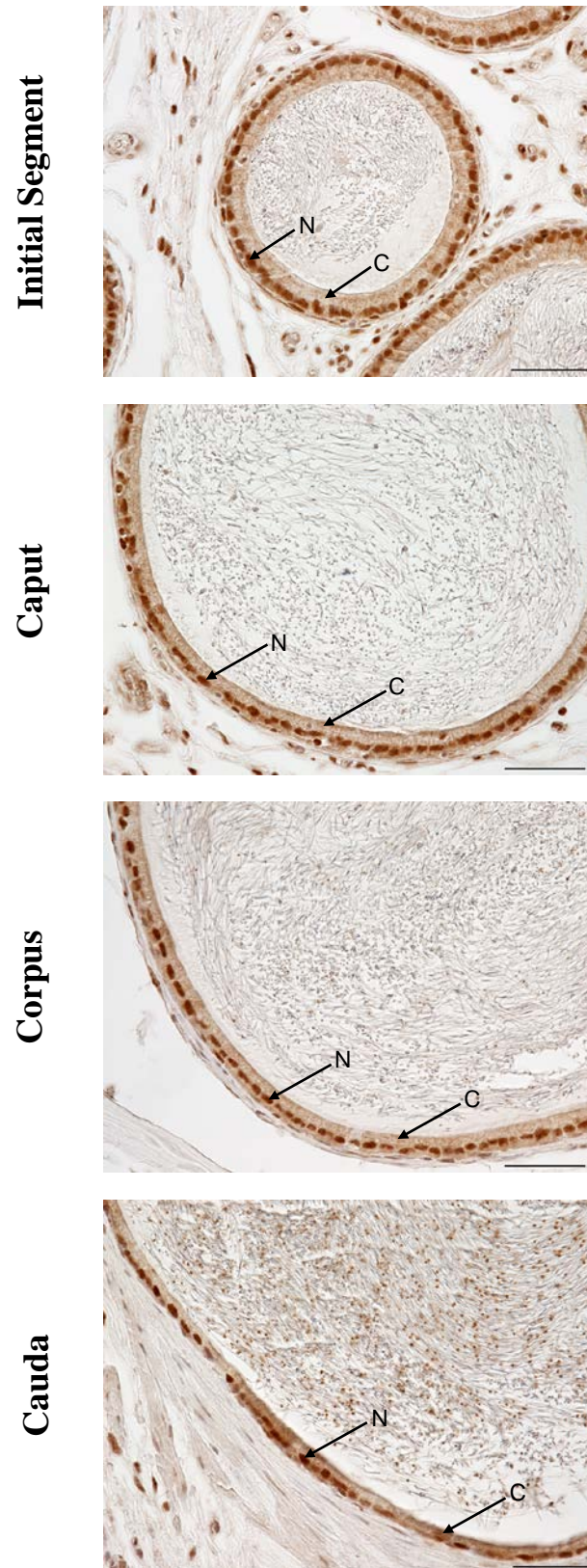
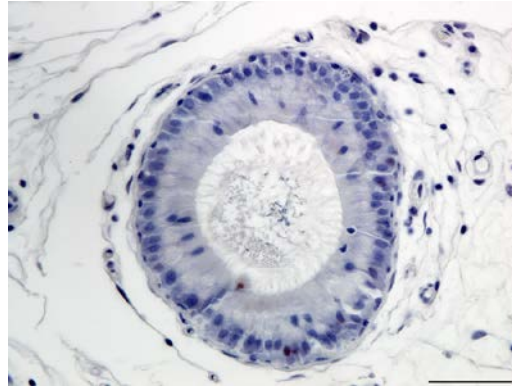


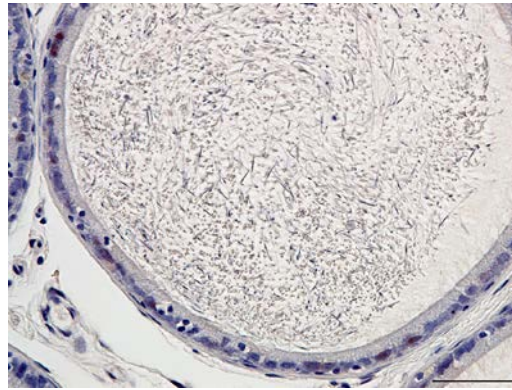
Figure 5. Representative Immunolocalization of Estrogen Receptor alpha ($ER\alpha$). Positive nuclear (arrow, N) and cytoplasmic (arrow, C) staining was observed in principal cells of the initial segment, caput, corpus, and cauda. $ER\alpha$ expression appeared similar between ages and treatment groups. Scale Bar = 50 μ m.

Lactoferrin

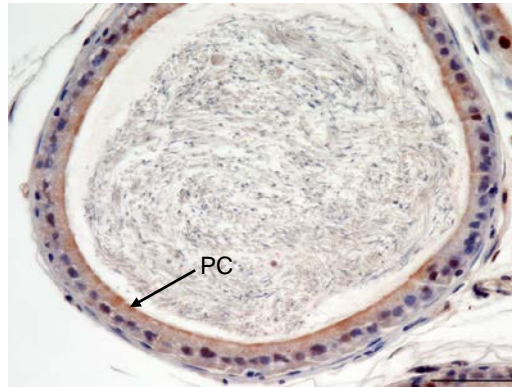
Initial Segment



Caput



Corpus



Cauda

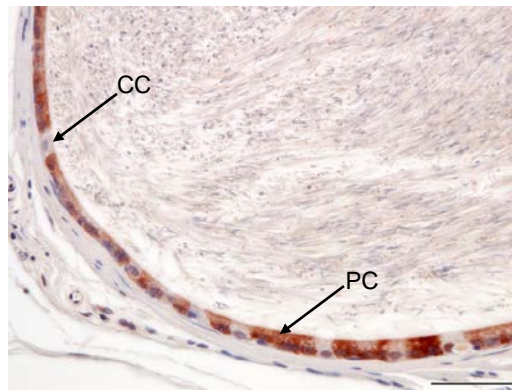


Figure 6. Representative Immunolocalization of Lactoferrin (Lf). Positive cytoplasmic staining for Lf was observed in principal cells (arrow, PC) of the corpus and cauda. Clear cells (arrow, CC) in the cauda appeared negative. Lf expression appeared similar between ages and treatment groups. Scale Bar = 50 μ m.

differences. Additionally, transit time significantly increased with age, and treatment with estrogen prevented this increase associated with aging.

Significant changes in the structure and function of the epididymis were noted. Various morphological changes in the epididymis during aging can be indicative of functional changes. For example, previous studies have noted that an increase in basal membrane thickness through the length of the epididymis and a narrowing of the luminal diameter in the cauda during aging coincided with decreased functional capabilities (Serre and Robaire, 1998). In this study, epithelial cell height in the epididymis during aging was used as a marker of cellular function. Regional differences in cell height were observed in 15 month old rats, but during aging, these regional differences were lost. With estrogen treatment, however, regional differences in epididymal epithelial cell height were maintained during aging. Thus, estrogen treatment maintained regional differences and potentially maintained some of the functional capabilities of these epithelial cells as well.

In addition to structural changes, significant functional changes in the epididymis were observed as well with aging and estrogen treatment. Specifically, transit time through the epididymis was affected by aging and with estrogen treatment. As the animals aged, the transit time through the epididymis slowed considerably. With estrogen treatment, however, the increase in transit time in the aging animals was prevented, and the transit time through the epididymis of the 18 month old treated animals remained similar to the 15 month old animals. Estrogen plays a significant role in regulating the contractility of the epididymal smooth muscle via two pathways. Estrogen increases the sensitivity of oxytocin receptor to oxytocin, and, in turn, promotes smooth muscle contraction (Filippi et al., 2002). Additionally, in some species, estrogens up-regulate calcium sensitivity of smooth muscle cells via the RhoA/ROCK pathway.

This causes smooth muscle cells to contract with greater force. This pathway also interacts with the oxytocin pathway in the epididymis via a positive feedback loop (Fibbi et al., 2009). It is likely that estrogen treatment up-regulated these pathways, preventing the increase in transit time typically observed during aging.

Surprisingly, the concentrations of testosterone and estradiol in epididymal tissues were unaffected by aging or estrogen treatment. It is known that, during aging, both serum testosterone levels and testosterone production in the testis decreases (Zirkin and Chen, 2000). In the same animals used for this study, testicular testosterone concentration decreased with age (Clarke and Pearl, 2014). Also, in the same animals, testicular estradiol concentration decreased with age when comparing 15 and 18 month old rats (Clarke and Pearl, 2014). These same changes in steroid hormone concentrations were not reflected in the epididymis during aging.

The expression of both androgen receptor (AR) and estrogen receptor α (ER α) did not appear to be affected by aging or treatment with estrogen. This is not consistent with findings in other tissues. Studies have suggested that, in the periphery, there is an overall decline in AR expression in various rodent species with aging (Morley, 2001). In human males, there is a decrease in ER α mRNA expression in the tissues of the prostate during aging (Hermann, Untergasser, Rumpold, and Berger, 2000). In the testis of the same animals used for this study, ER α expression decreased during aging, and treatment with estrogen prevented this decline (Clarke and Pearl, 2014). It is clear that the expression of AR and ER α in other tissues is potentially altered with age, but this is not what was observed in the epididymis. However, it is probable that our study simply did not capture these changes in the epididymis. It is possible that the time frame was too short to observe these changes in receptor expression. For example, if comparisons were made between 6 month old tissues and 18 month old tissues, they would be

more likely to show changes in receptor expression, as opposed to comparing 15 and 18 month old tissues as was done in this study.

Lactoferrin (Lf) was consistently expressed in the epididymis during aging and with estrogen treatment. As previously stated, Lf expression is regulated by estrogens in the epididymis (Yu and Chen, 1993). Because of this relationship, Lf expression was used as an indicator of changes in estrogen concentration or responsiveness of tissues to estrogen. The unaltered expression of Lf in the epididymis could suggest that the estrogen environment of the epididymis was not altered during the process of aging. However, as was observed with receptor expression, it is possible that the time frame used for comparison (15 and 18 months) was too narrow to observe changes in protein expression.

While some results may seem counterintuitive, it is important to keep in mind that the epididymis is supplied with estrogen from a variety of sources. The epididymis receives a large amount of estrogen from testicular fluid (Hess, 2003). There is also a production of estrogen within the tissue from the actions of aromatase (Joseph et al., 2010b). Lastly, there is peripheral estrogen circulating in the blood, which is likely the source of estrogen for smooth muscle cells in the epididymis (Hess, 2003). This study only investigated the concentration of estradiol within epididymal tissue, including estrogens from the testis and estrogens produced within the epididymis. The estrogen concentration is much lower in the epididymis as compared to the testis. It is possible that, while the hormone concentration decreased in the testis, it was still high enough to maintain epididymal concentrations. It is also likely that there were variations in estrogen concentration during aging and with estrogen treatment, but the variation was in the periphery and was not investigated as part of this study. For these reasons, it is possible that

estrogen environment of the epididymis was altered with age, but measurements of estrogen concentration was not a good measure of this like it was in the testis of these animals.

In summary, based on the results of this study in particular, the estrogen environment of the rat epididymis was not altered with age or with estrogen treatment. However, this does not mean that the estrogen environment was, in fact, unaltered. It is possible, for various reasons, that our study did not accurately capture these changes. On the other hand, there were significant structural changes during aging, which could indicate functional deterioration as well. Treatment with estrogen prevented these morphological changes in aging rats. Functional capabilities of the epididymis were also altered with age, and estrogen treatment again prevented these changes. While it is still unclear what mechanisms within the epididymis caused the structural and functional changes during aging, the significant findings of this study were that estrogen treatment during aging could prevent these changes and help to maintain normal epididymal functions.

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