Localization of the Androgen-Synthesizing Enzymes, Androgen Receptor, and Sex Steroids in the Vagina: Possible Implications for the Treatment of Postmenopausal Sexual Dysfunction

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ABSTRACT

Introduction. To better understand the mechanisms underlying the beneficial effects of the intravaginal administration of dehydroepiandrosterone (DHEA) observed in postmenopausal women on sexual dysfunction.
Aims. To identify the distribution of the androgen-synthesizing enzymes as well as androgen receptor (AR) and measure steroid levels in the monkey vagina.
Methods. The cynomolgus monkey (*Macaca fascicularis*), the closest model to the human, has been used to measure the expression levels of steroidogenic enzymes and androgen receptor by quantitative reverse transcription polymerase chain reaction (n = 4), confirmed by immunohistochemistry, and immunofluorescence (n = 3). DHEA and its androgenic metabolites were quantified by LC-MS/MS (n = 4).
Main Outcome Measures. The presence of SRD5A1, SRD5A2, HSD17B3, AR as well as nerve fibers (PGP 9.5) was investigated, and steroid levels were measured.
Results. AR is widely distributed within the vaginal epithelium and also in the lamina propria with a lower expression in the muscularis layer and blood vessel walls. Androgen-forming enzymes, on the other hand, are expressed in the vaginal stratified squamous epithelium at a relatively high level where they are uniformly distributed from the basal membrane up to the superficial keratinized cells. The enzymes are at a lower level in blood vessel walls and zona muscularis where nerve fibers are localized. DHEA and its androgen metabolites are present at biologically significant concentrations in the monkey vagina.
Conclusion. The enzymes responsible for androgen formation as well as AR are at the highest level in the superficial layer of the stratified epithelium and muscularis layers of the vagina. These data provide a potential explanation for the described role of androgens in regulating vaginal lubrication, smooth muscle activity, blood flow, and the neuronal activity potentially involved in the correction of sexual dysfunction. **Bertin J, Dury AY, Ouellet J, Pelletier G, and Labrie F. Localization of the androgen-synthesizing enzymes, androgen receptor, and sex steroids in the vagina: Possible implications for the treatment of postmenopausal sexual dysfunction. J Sex Med **;**:**–**.**

Key Words. DHEA; Androgens; Vagina; Sexual Dysfunction; Vulvovaginal Atrophy

Introduction

Sexual response in women includes mucosal lubrication, increased genital blood flow and muscle tonus. Hormonal dysregulations altering any of these physiological processes may contribute to diminished sexual performance. It is well recognized that 17-β estradiol (E_2) influences the physiology of the vagina [1–4]. However, E_2 secretion by the ovary stops at menopause, and dehydroepiandrosterone (DHEA), mainly of adrenal origin [5,6], becomes the exclusive source of sex steroids after menopause [5,7].

Vulvovaginal atrophy (VVA) is a typical example of hormone deficiency in postmenopausal women,

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and it strongly correlates with low serum DHEA while serum E_2 remains at biologically inactive levels [5,7–10]. In addition, sexual dysfunction is a frequent problem, especially among this population of postmenopausal women [11–14]. Indeed, sexual dysfunction can affect up to 50% of women [15,16], a complication with a prevalence that increases with age [17] and an effect most likely related to the hormone deficiency of menopause.

In women, androgens are essential for the development of reproductive function and are the immediate precursors for the synthesis of estrogens. An imbalance in androgen biosynthesis or metabolism may alter the female general health and sexual function [18,19]. In this context, it has been suggested that DHEA replacement in women with adrenal insufficiency improves sexual function [19,20]. In fact, androgens are believed to be critical for maintenance of sexual functioning and sexual desire in postmenopausal women [21]. Moreover, intravaginal administration of DHEA for 12 weeks in postmenopausal women has been found to exert beneficial effects on the four domains of sexual dysfunction [22]. However, the exact physiological and biochemical role of androgens in female sexual function remains poorly understood. Following intravaginal administration, DHEA acts exclusively in the vagina following its tissue-specific local conversion into androgens and/or estrogens by the mechanisms of intracrinology [23,24] with no meaningful change in serum levels of sex steroids, which remain below the threshold of biological activity [8–10].

Most studies on the influence of sex steroids in the vagina have focused on luminal epithelial cells, especially maturation index and pH [3,4]. Little is known about changes in the two other layers of the vagina, namely the lamina propria and muscle layers. DHEA, an inactive precursor of sex steroids, is secreted in relatively large amounts by the adrenals in the human and other primates and is transformed into either or both androgens and estrogens in peripheral tissues, including the vagina [23–25]. As mentioned above, recent data have shown positive effects of intravaginal DHEA on VVA [26] and sexual dysfunction [22].

The role of testosterone on the physiological structure and function of the vaginal mucosa and the consequences of changes in testosterone intracellular availability are largely unknown. Evidence accumulated in recent years, especially from animal studies, suggests that androgens may have a direct effect on vaginal structure and function, which are, at least partially, independent from estradiol [27]. Indeed, it has been shown that the 5α–reductases enzymes responsible for the transformation of testosterone into dihydrotestosterone (DHT) and the androgen receptor (AR) are expressed in the vaginal mucosa, stroma, smooth muscle, and vascular endothelial layers [28].

Testosterone action on target organs occurs either directly through binding to AR before or after conversion into DHT, or indirectly through the estrogen receptor (ER) after its aromatization into estrogens. Although aromatization of testosterone into E_2 occurs in vaginal tissue [29], androgens may also have a direct effect on vaginal tissue not mediated by E_2 but by testosterone or DHT (Figure 1) following activity of the 5α-reductase enzymes. Androgens may also induce rapid activation of kinase-signaling cascades while also modulating intracellular calcium levels in a nongenomic manner [30,31].

Studies in animals have suggested that androgens regulate different physiological functions in the vagina such as mucification, nerve fiber density, and vasodilatation [27,32–36]. Preclinical data have clearly shown the beneficial effects of the steroids made locally from DHEA in the vagina, not only in the superficial epithelial layer but most importantly on collagen fibers and nerve fibers of the lamina propria and on the muscularis on nerve fibers [34–36].

The vagina is the key organ of female sexual response, and better understanding of the androgenic effects in this tissue is crucial to the development of an efficient and physiological strategy to treat sexual dysfunction, which could poten-

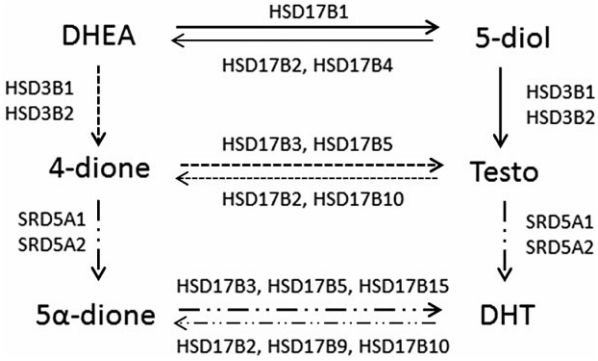


Figure 1 Schematic representation of the "frontdoor" and "backdoor" pathways leading to androgen formation from dehydroepiandrosterone (DHEA) in peripheral target tissues. Dehydroepiandrosterone, DHEA; androst-5-ene-3α,17β-diol, 5-diol; androstenedione, 4-dione; testosterone, testo; androstanedione, 5α-dione, and dihydrotestosterone, DHT.

tially be accomplished by locally administering prasterone. As a step toward that goal, we have investigated the route of androgen formation from DHEA by LC-MS/MS, as well as the AR distribution in the vagina quantitative RT-PCR, immunohistochemistry (IHC) and immunofluorescence (IF) in nonhuman primate vaginal tissue, the closest model to the human.

Material and Methods

Animals and Tissues

All animals in this study have been maintained and handled in accordance with the policies of the Canadian Council on Animal Care and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Tissues from four cynomolgus monkeys aged between 6 and 7 years were collected for quantitative RT-PCR and LC-MS/MS assays following perfusion with a saline solution. With regard to microscopy, three different animals aged between 5 and 7 years old were sacrificed under isoflurane anesthesia followed by intracardiac perfusion with 4% paraformaldehyde (PFA) neutral buffer instead of a saline solution. The vagina collected from PFA-perfused animals were then soaked in 4% PFA neutral buffer for 12 hours, routinely processed and embedded in paraffin for IF and IHC analysis. Alternatively, the vagina collected from the animals perfused only with the saline solution was immediately snapped frozen in liquid nitrogen for quantitative RT–PCR and LC-MS/MS assays.

Antibodies

To develop antibodies against SRD5A1, we overexpressed the peptide sequence 28–151 as described in [37]. Antibodies against HSD17B15 were developed using the peptide sequence 67–143 of the enzyme overproduced in E. Coli B2-21 using PET23a vector (EMD Biosciences, San Diego, CA, USA). The purified proteins were subsequently diluted in phosphate saline buffer containing 50% complete Freund's adjuvant and injected sc with 1 mL at multiple sites on New Zealand rabbits. The animals were then treated twice with the same amounts of proteins in 50% of Freund's adjuvant at a 1-month interval. Antiserum was obtained by decantation and separation by centrifugation and was stored at –80°C. Antisera were analyzed by immunoblot using HEK-293 cells nontransfected and stably transfected as negative and positive controls, respectively, with SRD5A1 or HSD17B15. Antisera were analyzed by immunoblot separately by 12% SDS-PAGE and transferred into a nitrocellulose gel for analysis with the protein A–purified antibody to 17β-HSD-15 (0.4 mg/mL). Horse anti-rabbit IgG antibody conjugated with horseradish peroxidase was used at a secondary antibody (1:15,000 dilution) and visualized using the enhanced chemiluminescence kit (Perkin Elmer Life Sciences, Waltham, MA, USA) and exposed on a X-Omat blue film for 30 seconds. Expression of HSD17B3 and SRD5A2 was determined with antibodies obtained from Aviva Systems Biology (San Diego, CA, USA) while AR was detected using an antibody from Santa Cruz Biotechnology Inc (Dallas, Texas, USA). PGP 9.5 was purchased from Abcam (Cambridge, MA, USA). The red-fluorescent Cy3-coupled goat anti-rabbit antibody was purchased from Abcam while the goat anti-mouse Alexa488-coupled antibody was obtained from Life Technologies (Burlington, ON, Canada).

Immunohistochemistry

Paraffin sections were deparaffinized, hydrated, and treated with 3% H2O2 in methanol for 20 minutes. The sections were then blocked before incubation overnight at 4°C with antibodies (SRD5A1 at a dilution of 1/500, SRD5A2 at 1/10, HSD17B3 at 1/20, HSD17B15 at 1/100, and AR at 1/250). Negative controls were run by incubating tissues with normal rabbit isotypic control (Life Technologies, 08-6199) at concentrations similar to those used for primary antibodies. A commercial detection system kit (Covance Research Products Inc, Deham, MA, USA) using the streptavidin-biotin peroxidase amplication method was then used. Finally, the antigen–antibody complex was visualized with a solution of PBS 1X containing 5 mg/mL of 3,3-diaminobenzidine and 0.012% H2O2. Sections were lightly counterstained with Gill modified hematoxylin (EMD, Billerica, MA, USA). Images were generated utilizing a 10X objective on the Leica DMRB.

Immunofluorescence

Deparaffinized sections were blocked (0.5% BSA, 0.4% triton-X, and 10% normal goat serum) and incubated overnight at 4°C with antibodies (SRD5A1 at a dilution of 1/250, SRD5A2 at 1/10, HSD17B3 at 1/20, HSD17B15 at 1/100, AR at 1/50, and PGP 9.5 at 1/2,000 and then incubated for 1 hour with a red-fluorescent Cy3-coupled goat anti-rabbit antibody (1/250; Abcam) and combined with the Alexa 488 complex-goat anti-mouse (1/500) in coimmuno-stainings. Negative controls were studied by incubating tissues with normal

rabbit isotypic control (Life Technologies, 08-6199) at concentrations similar to those of primary antibodies, respectively. Tissue slides were then incubated with the blue-fluorescent 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies) nucleic acid stain (300 nM) for 1 minute at room temperature and thoroughly washed in PBS 1X. Negative controls were run by incubating tissues with normal rabbit serum. The specificity of the antibodies used within this study was assessed using HEK293 cells transfected, or not, with vectors encoding for the full length transcripts of the appropriate enzymes (data not shown) and further supported by qRT-PCR data. Images were generated utilizing a 20X objective on the Leica DMRB (TRITC/DiI/Cy3 filter set; D540/25x excitation, D605/55 m emission, and the UV filter set 358/461 nm).

Quantitative RT-PCR

Fifty-micrometer thick slices (up to 0.2 g total) of the tissue covering the superficial to the deeper layers of the vagina were dissolved in homogenizing tubes containing stainless metal beads and lysis buffer (Illustra™ RNAspin Isolation Kit, GE Healthcare, Mississauga, ON, Canada) and then homogenized utilizing the Omni Bead Ruptor 12 (Omni International, GA, USA) at high speed for 1.7 minutes. RNA was then purified as indicated by the manufacturer (Illustra™ RNAspin Isolation Kit). Oligoprimer pairs were designed with NCBI primer-BLAST, and their specificity was verified by Blast in the GenBank database. The synthesis was performed by IDT (Integrated DNA Technology, Coralville, IA, USA) (Table 1). The reverse transcription was performed with the qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). cDNA corresponding to 10–20 ng of total RNA was used to perform fluorescent-based Realtime PCR quantitation using the LightCycler Nano (Roche Diagnostics, Mannheim, Germany). Amplification of cDNA was performed using the Perfecta SYBRGreen FastMix (Quanta Biosciences, Gaithersburg, MD, USA) as described by the manufacturer. The conditions for PCR reactions were: 50 cycles, denaturation at 95°C for 10 seconds, annealing at 57°C for 10 seconds, elongation at 72°C for 14 seconds, and then at 74°C for 5 seconds (reading). A melting curve was done to assess nonspecific signal. Calculation of the number of copies of each mRNA was performed according to Luu-The et al. [38] using the second derivative method and a standard curve of Cp vs. logarithm of the quantity. The standard curve was established

Table 1 Primer description

Gene Symbol	Description	GenBank	Size (bp)	Primer sequences 5′ → 3′ S/AS
HSD17B3	Macaca fascicularis 17-beta hydroxysteroid dehydrogenase 3 (HSD17B3)	DQ078722	220	ATCAGCCGGATGCTGGAA/GAATTTCATCTGGCGCGTTC
HSD17B15*	Prostate short-chain dehydrogenase reductase 1 (PSDR1)/17-beta hydroxysteroid dehydrogenase 15 (HSD17B15)	AF167438.1	172	TCTGAACTGGTTCGGCACT/CAGACACCCATGCCACATGA
SRD5A1	Macaca fascicularis steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1) (SRD5A1)	S77162	213	GGCCATGTTCCTCGTCCACTA/AACCACACAAGCCAAAACCTATTAGA
SRD5A2	Homo sapiens steroid-5-alpha-reductase, alpha polypeptide 2 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 2) (SRD5A2)	S77165	144	GGTTTTGCTTGGGTGTCTTCTTA/TGGCTCCAGAAACATACGTAAACAAG
AR	Macaca fascicularis androgen receptor (AR)	U94179.1	294	AGGCGTTTCTACCAGCTCAC/GGCACTGCAGAGGAGTAGTG
Atp5o	Macaca fascicularis ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	AB179481	195	CGGCTCTTTATTCTGCTGCATCAA/GGTTGGTGGTGAGGGGGAGAGAA
Hprt1	Macaca fascicularis hypoxanthine phosphoribosyltransferase 1	AB169539	266	CTTTGCTGACCTGCTGGATTACAT/AGCTTGCGACCTTGACCATCTT
18S	Homo sapiens 18S ribosomal RNA	NR_003286	226	ACGGACCAGAGCGAAAGCATT/TCCGTCAATTCCTTTAAGTTTCAGCT
ADNg	Macaca mulatta Uox gene for urate oxidase, intron 4, partial sequence (Uox)	AB074108	250	CACGCTGCTACCCATTTATGAA/TGGCCAACTCACCTCATGTATTT

*Luu-The V, Labrie F. The intracrine sex steroid biosynthesis pathways., in: L. Martini, Chrousos GP, Labrie F, Pacak K, D.e. Pfaff (Eds.), Neuroendocrinology, Pathological Situations and Diseases, Progress in Brain Research., Elsevier, 2010. pp. 177–192.

using known amounts of purified PCR products (10, 10^2, 10^3, 10^4, 10^5, and 10^6 copies) using the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Mississauga, ON, Canada). PCR amplification efficiency was verified and used as a normalization criteria for each targeted amplicon. Further normalization was performed using the reference genes shown to have stable expression levels from embryonic life through adulthood in various tissues [39]: ATP synthase O subunit (ATP5O), hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1) and 18S ribosomal RNA (18S). Genomic DNA was also monitored for possible interference.

LC-MS/MS

Approximately 0.4 g of snap frozen tissue (–80°C) was collected from each vagina on dry ice, placed in tubes containing four stainless metal beads and immediately homogenized with the Bead Ruptor (Omni International, Kennesaw, GA, USA) along with 0.1 mL of appropriate deuterated internal standards (DHEA-d5, Testo-d1, 4-dione-d7, 5-diol-d3, 3α-diol-3G-d5, 3α-diol-17G-d3, ADT-G-d4, and DHEA-S-d5) and containing their respective analyte for endogenous quality controls. Samples were then homogenized twice with methanol. The solvent was collected, snap-cooled in an acetone/dry ice bath to precipitate proteins, and the supernatant was then evaporated under gas nitrogen. Samples were resuspended in MeOH containing 1% formic acid and split into two equal volumes to extract free steroids and conjugated steroids separately. Phospholipids were removed from each part by a solid phase extraction (Phree, Phenomenex, Torrance, CA, USA). Eluted volumes were then evaporated and resuspended with 1-chlorobutane or 2% formic acid in water for free steroids and conjugated steroids, respectively. Free steroids were then further purified on conditioned SI-1 silica columns (Phenemonex), with washes done with pure hexane solvent and finally eluted with ethyl acetate/hexane (1:1, v/v). Evaporated samples were then selectively derivatized with dansyl chloride in acetone.

Conjugated steroids were isolated by solid phase extraction on conditioned Strata-X-CW 33 µ, 60 mg 96-well plates (Phenomenex). Washing was performed with 2% formic acid in water and then with MeOH : water (2:8; v/v) followed by elution with pure MeOH. After evaporation, samples were reconstituted with MeOH : water (2:8; v/v). Free steroid extractions were injected on the UPLC (LC-30AD system; Shimadzu; Columbia, ON, Canada)-MS/MS (Qtrap 6500 system; AB Sciex, Concord, ON, Canada) and followed by a multiple reaction monitoring (MRM) analysis. The analytical column used was an Agilent Poroshell 120 EC-C18 (3.0 × 50 mm; 2.7 µm) with an in-line filter (Waters, Milford, MA, USA) with 0.2% formic acid in water and MeOH as weak and strong mobile phases, respectively, for free steroid analytes. Conjugated steroids were injected on the Accuity LC system (Waters)-MS/MS (API5000; AB Sciex) for MRM analysis. The analytical column used for glucuronidated steroids was a Zorbax Eclipse Plus C18 (50 × 2.1 mm, 1.8 µm; Agilent, Santa Clara, CA, USA) with ammonium formate (1 mM) in tetrahydrofuran/water (4:96; v/v) and ammonium formate (1 mM) in tetrahydrofuran/water (4:4:92; v/v/v) as weak and strong mobile phases, respectively. The Zorbax SB-C18, 3.5 µm, 50 × 2.1 mm analytical column (Agilent) was used for sulfated steroids with 3 mM ammonium formate in water and MeOH as mobile phases.

Statistical Analysis

Data are presented as mean ± SEM or mean ± SD, as indicated in Table 2.

Results

Quantification of DHEA and Its Androgen Metabolites in the Monkey Vagina

We first quantified DHEA and its androgen metabolites in the vagina (n = 4) using highly spe-

Table 2 Steroid levels in the monkey vagina

	DHEA (ng/g)*	DHEA-S (ng/g)*	TESTO (ng/g)*	5-diol (ng/g)*	4-dione (ng/g)*	3α-diol-3G (ng/g)*	ADT-G (ng/g)*	ETIO-G (ng/g)*
Monkey 1	10.6 ± 0.9	385.7 ± 9.8	0.6 ± 0.04	2.1 ± 0.3	0.8 ± 0.2	5.8 ± 0.3	244.2 ± 31.3	44.31 ± 5.1
Monkey 2	19.5 ± 0.3	384.7 ± 45.0	1.6 ± 0.1	3.5 ± 0.4	0.9 ± 0.2	29.1 ± 6.4	2131.4 ± 328.2	239.7 ± 41.7
Monkey 3	13.1 ± 0.5	255.4 ± 118.1	0.5 ± 0.1	2.2 ± 0.2	0.4 ± 0.09	17.2 ± 1.7	818.8 ± 66.23	110.9 ± 10.2
Monkey 4	57.1 ± 6.7	333.4 ± 61.4	0.9 ± 0.2	6.3 ± 0.05	0.7 ± 0.2	29.8 ± 0.5	1048.4 ± 33.1	99.2 ± 7.6
Average ± SEM†	25.1 ± 21.6	339.8 ± 61.3	0.90 ± 0.5	3.5 ± 1.9	0.7 ± 0.2	20.4 ± 11.4	1060.7 ± 789.9	123.5 ± 82.7

*Mean ± SEM for individual values
†Mean ± SEM for group values

cific and sensitive LC-MS/MS assays. As described in Table 2, the quantifications show that the high levels of DHEA and DHEA-S present in the tissue are metabolized into testosterone. Interestingly, the elevated levels of glucuronidated androgens (3α-diol-3G, ATD-G, and Etio-G) measured confirm androgen formation and inactivation in the vagina according to the mechanisms of intracrinology. 3α-diol-3G was below quantification limits and its measured concentrations is extrapolated.

Quantification of mRNA Transcripts of Steroidogenic Enzymes and AR in the Monkey Vagina

We have quantified the mRNAs encoding localized enzymes involved in androgen formation from DHEA as well as the AR transcripts in the monkey vagina. Real-time quantitative RT-PCR in total tissue extracts revealed that low levels of mRNAs encoding SRD5A2, HSD17B3, and HSD17B15 are present (Figure 2). The expression of SRD5A1 is at moderate levels while AR expression, however, is relatively high with no significant correlation with the thickness of the squamous epithelium (Figure 2). A complete description of the primers used to measure all of the mRNAs is presented in Table 1.

Localization of Androgen-Forming Enzymes in Monkey Vagina

IF and IHC were performed to determine the spatial distribution of the steroidogenic enzymes in different layers of the vagina. To better demonstrate both signal intensity and morphology that are often favored by IF and IHC, respectively, we have used both of these somewhat similar techniques to better understand the enzyme distribution. Immunolabeling of the enzymes was especially localized in the stratified squamous epithelial cells, around blood vessels and in the muscularis (Figures 3 and 4).

Expression of the AR in Monkey Vagina

IF and IHC reveal that immunostaining of the AR is specifically restricted to the nuclei of epithelial cells localized in the vaginal stratified squamous epithelium, lamina propria, around blood vessels, and in the muscularis layer (Figure 5).

Expression of Nerve Fibers in Monkey Vagina

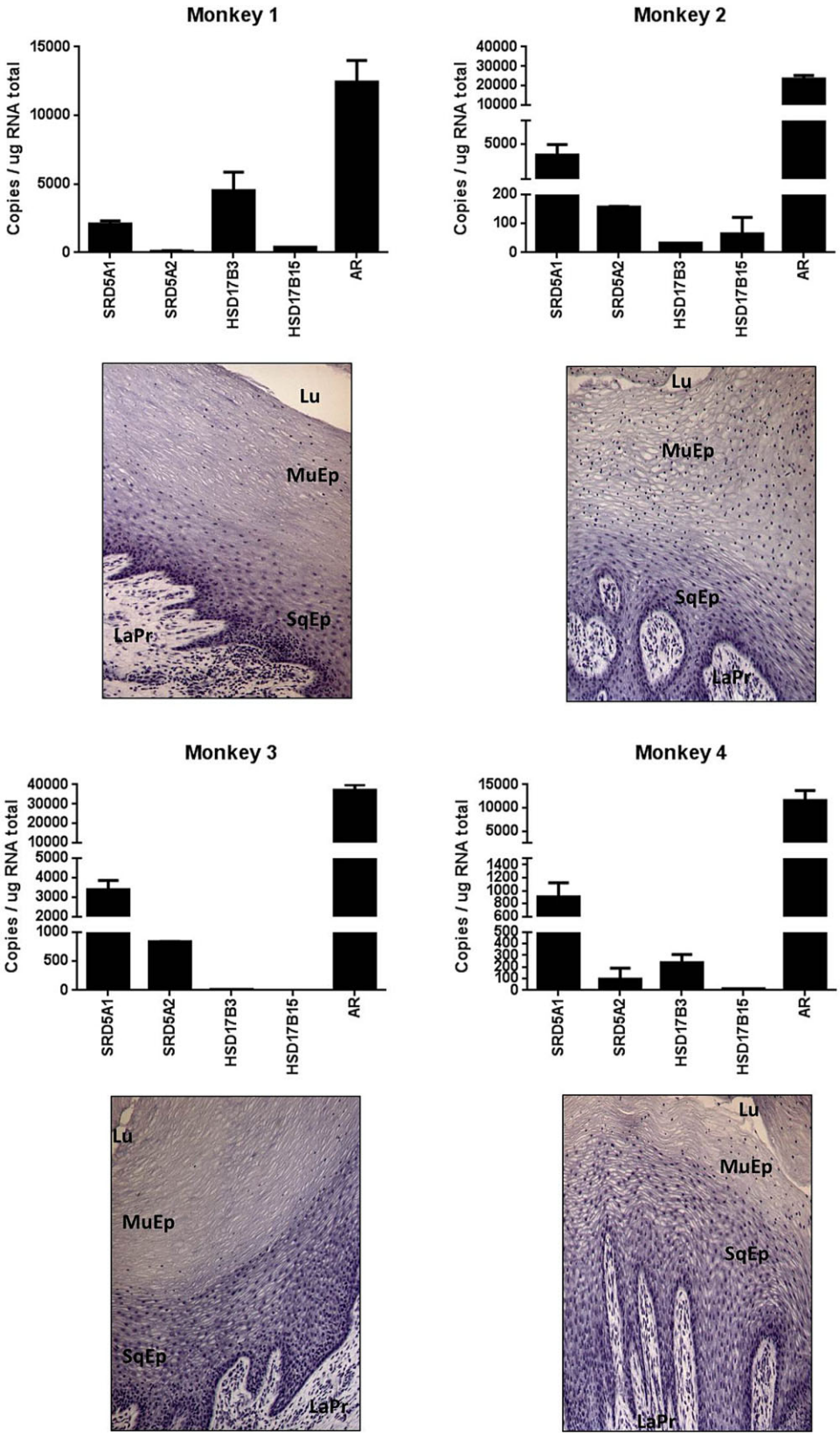
IF reveals that immunostaining of the nerve endings and AR are found in the same regions of the vagina, namely the lamina propria and muscularis. Denser networks of PGP 9.5 (protein gene product 9.5, a widely used panneuronal marker) were visualized in the lamina propria and at proximity of the blood vessels. Isolated fibers were found in the muscularis of the vagina conjointly with AR, while no nerve fiber labeling was detected in the epithelial layer (Figure 6).

Discussion

The present study investigates the expression and spatial distribution of androgen-forming enzymes and AR in the monkey vagina (*Macaca fascicularis*), the closest model to the human. Most importantly, the adrenal glands of rodents and any species below primates do not secrete significant amounts of DHEA [40]. Moreover, the cynomolgus monkey paralogs of the human enzymes involved in steroid formation and inactivation are much more closely related in sequence, tissue distribution and activity than the rodent enzymes [41,42].

Low serum testosterone levels in aging women are associated with a decline in sexual arousal, genital sensation, libido, and orgasm, all of which are not restored by estrogen replacement alone [43]. On the other hand, exogenous testosterone administration in these women improves different aspects of the sexual function such as desire, libido, and arousal [44–47]. Indeed, the AR has been visualized in the human vagina by IHC, thus suggesting a direct effect of testosterone on the vaginal tissue [28]. In fact, the labia majora, labia minora, and vagina stain positive for the AR [48,49]. Various animal studies have investigated modulation of AR expression under physiological conditions and after sex steroid administration [48–51].

Figure 2 Expression of AR and androgen-forming enzyme mRNA levels in the monkey vagina. Quantitative RT-PCR reveals that HSD17B3, HSD17B15, and SRD5A2 are weakly present in the vagina while higher levels of SRD5A1 and much higher levels of AR expression are measured. Sections not homogenized were counterstained with Gill modified hematoxylin. Monkeys 1 and 2 displayed sexual skin and no red liquid discharge before tissue collection. Monkey 3 displayed sexual skin and red liquid discharge 1 week before sample collection. Monkey 4 displayed no sexual skin and no red liquid discharge before tissue collection. Images are generated from one monkey representative of three separate experiments done with a distinct animal. Means ± SD calculated from triplicate samples for each amplified target. Each panel represents a distinct animal. Lu = lumen; MuEp = mucified epithelium; SqEp = squamous epithelium; LaPr = lamina propria.


Monkey 1
Copies / ug RNA total
15000
10000
5000
0
SRD5A1
SRD5A2
HSD17B3
HSD17B15
AR
Monkey 2
Copies / ug RNA total
40000
30000
20000
10000
5000
200
100
0
SRD5A1
SRD5A2
HSD17B3
HSD17B15
AR
Lu
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LaPr
Lu
MuEp
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Monkey 3
Copies / ug RNA total
40000
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SRD5A1
SRD5A2
HSD17B3
HSD17B15
AR
Monkey 4
Copies / ug RNA total
15000
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1200
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800
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SRD5A1
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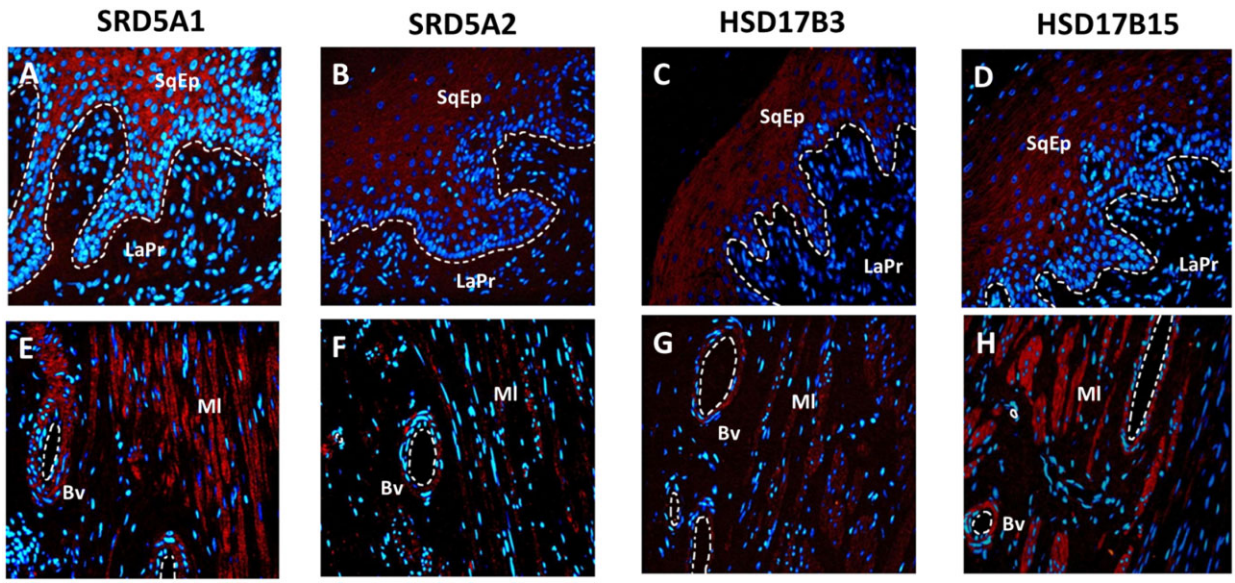

Figure 3 Immunofluorescence (IF) labeling shows that the steroidogenic enzymes are especially localized in the stratified epithelium of the vagina. IF thus reveals that the stained cells are localized mainly in the epithelium layer for SRD5A1 (A), SRD5A2 (B), HSD17B3 (C), and HSD17B15 (D) with no detected expression under the conditions used in the stromal region. SRD5A1 (E), SRD5A2 (F), HSD17B3 (G), and HSD17B15 (H) are also expressed around blood vessels and in the muscularis layer of the vagina. DAPI staining (blue) of the tissue confirms that the immunodetection of the AR is restricted to the nuclei. Images are generated from one monkey representative of three separate experiments done with a distinct animal. SqEp = squamous epithelium; LaPr = lamina propria; Bv = blood vessel; Ml = muscularis.

Circulating levels of serum testosterone somewhat decline with age in women [52,53] as a consequence of the age-related decline in DHEA secretion [6], although serum testosterone is not a reliable marker of total androgenic exposure or pool in women [54]. Sublingual intake of testosterone in women causes an increase in genital responsiveness and genital vasocongestion, 3–4.5 hours after reaching peak testosterone level [55]. Moreover, it has been shown that transdermal testosterone improved sexual function in women after surgically induced menopause [56]. However, the precise role of testosterone on maintaining vaginal structure, function, and sexual arousal is not clearly understood.

It is now well established that serum DHEA becomes the exclusive source of sex steroids after menopause [5–7,25], following is transformation into androgens and estrogens in specific peripheral tissues, including the vagina. In fact, VVA is the first clear example of the high efficacy and safety of DHEA (prasterone) to treat sex steroid deficiency at postmenopause [22,26]. In recent years, the addition of testosterone to hormone replacement therapy or testosterone administration alone has been proposed for the treatment of postmenopausal women with hypoactive sexual desire [57]. Several preparations of androgens have been used in studies on treatment of female hypoactive sexual desire disorder and positive effects on various aspects of sexual function have been reported [58]. However, due to the rather small amplitude of these effects and to uncertain long-term safety, there is a lack of consensus on whether or not this treatment should be proposed to women with hypoactive sexual desire disorder.

The present data describes the expression of the steroidogenic enzymes (namely HSD17B3, HSD17B15, SRD5A1, and SRD5A2) responsible for androgen formation from DHEA, as well as AR distribution in the monkey vagina.

Modest absolute levels of mRNA concentrations have been measured for most steroidogenic enzymes responsible for the formation of androgens. These findings can correspond to the fact that the local formation of sex steroids, according to the mechanisms of intracrinology [5,7,23,59], requires only small amounts of active steroids to exert efficient local action. Accordingly, the enzymes involved in the formation of active hormones in intracrine tissues are expressed at much lower than those found in the classical endocrine tissues [60]. Moreover, although low levels of

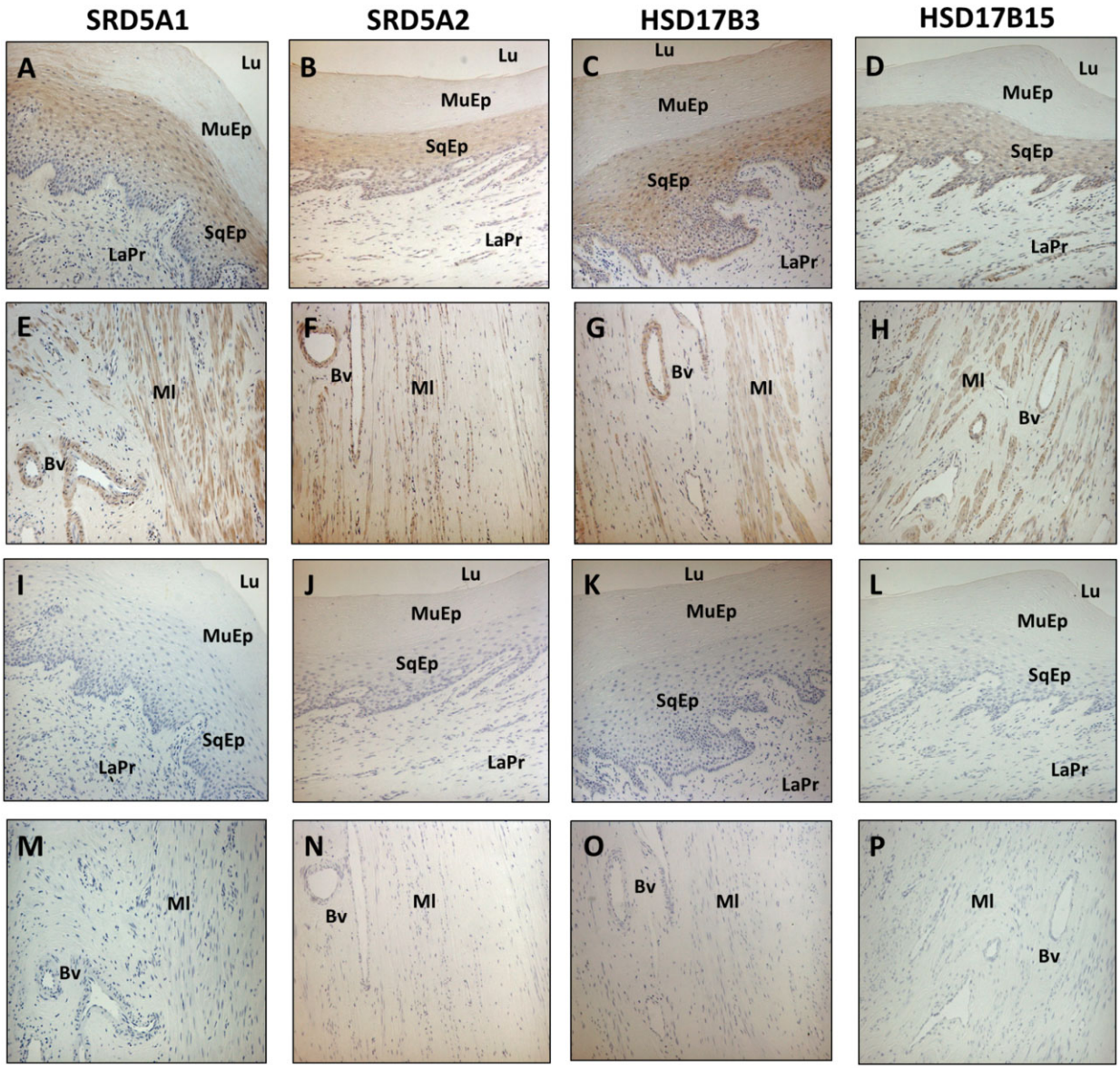

Figure 4 Immunohistochemical (IHC) staining of steroidogenic enzymes is mainly observed in the epithelial layer of the vagina. IHC reveals that the stained cells are localized exclusively in the epithelium for SRD5A1 (A), SRD5A2 (B), HSD17B3 (C), and HSD17B15 (D) with no detected expression under the conditions used in the stromal region. SRD5A1 (E), SRD5A2 (F), HSD17B3 (G), and HSD17B15 (H) are also expressed around blood vessels and in the muscularis layer of the vagina. Panels I to P show respective negative controls of primary antibodies used in panels A to H. Images are generated from one monkey representative of three separate experiments done with a distinct animal. Lu = lumen; MuEp = mucified epithelium; SqEp = squamous epithelium; LaPr = lamina propria; Bv = blood vessel; Ml = muscularis.

androgen-forming enzyme mRNA levels were measured in whole tissue homogenates, the enzymes are most likely expressed at a much higher level in specific cells underevaluated by qRT-PCR performed on total tissue. This explanation is well supported by immunostaining localized in specific cells as well as by the absolute quantification of steroids and androgen metabolites. The data presented herein clearly indicate that all of the machinery is well in place in the vagina to metabolize DHEA into active sex steroids, including androgens. In fact, the present study shows that the 5 alpha reductase as well as HSD17beta types 3 and 15 are highly expressed both in the superficial and deeper layers of the tissue, thus explaining why such important concentrations of testo and DHT are measured in the vaginal homogenate.

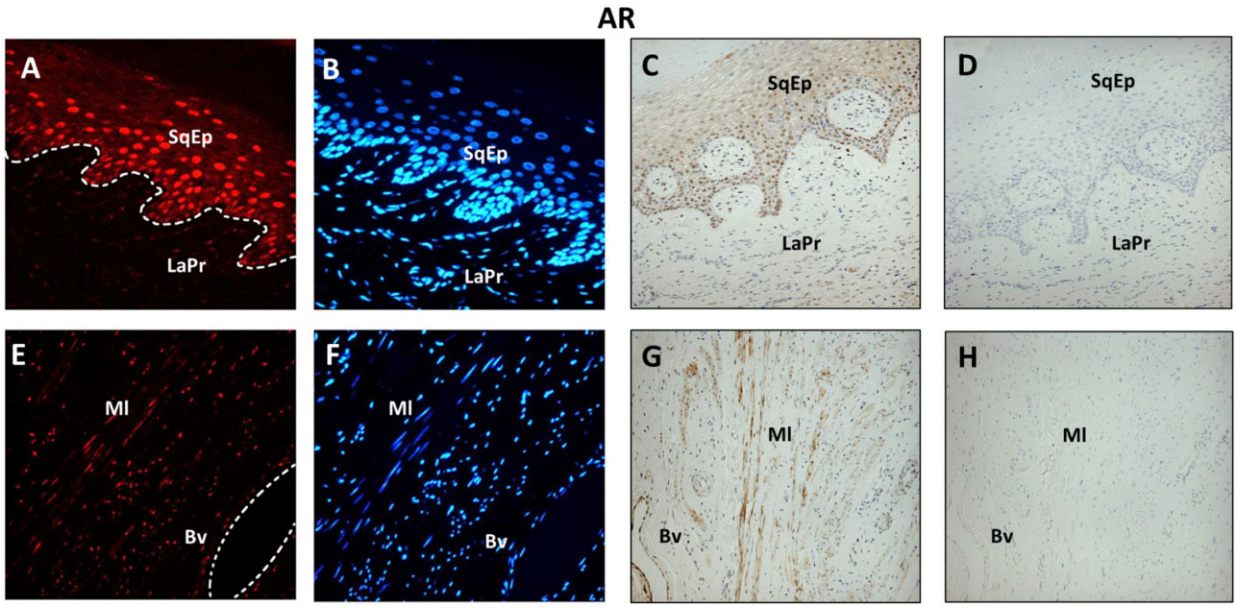

Figure 5 IF and IHC reveal that the androgen receptor is expressed at a higher level in the nuclei of squamous stratified epithelial cells (A and C), while its presence is weaker in the muscularis and around blood vessels (E and G). DAPI staining (blue) of the tissue confirms that the immunodetection of the AR is restricted in the nuclei (B and F). IHC negative controls show the specificity of the AR staining in the superficial and deeper layers of the vagina, respectively (D and H). Images are generated from one monkey representative of three separate experiments done with a distinct animal. Lu = lumen; MuEp = mucified epithelium; SqEp = squamous epithelium; LaPr = lamina propria; Bv = blood vessel; MI = muscularis.

Interestingly, AR positive cells are not only present in the squamous epithelium of the vagina but also in the lamina propria and muscularis, often at a proximal distance of nerve endings. Indeed, it is known that the vagina is innervated by efferent and sensory fibers through both hypogastric and pelvic nerves [61,62].

We have hypothesized that low sex steroid activity observed after menopause could cause sexual dysfunction due to a deficient vaginal innervation and/or lack of nerve sensitivity [35,36].

While the effect of testosterone on vaginal nerve fibers was known [63], we have shown that the androgenic component of DHEA can increase the density of nerve fibers in the vagina [35,36]. In fact, our group has recently put forward the suggestion that the increase in PGP 9.5 density in the vaginal lamina propria and muscularis regions induced by locally administered DHEA as an effect mediated by androgens [36] in agreement with the findings of Pessina et al. [63], on the effect of testosterone while estrogens have no effect [27,36,63].

Conclusion

The present study shows that nerve endings and positively labeled androgen sensitive (AR positive) cells in the vagina are indeed found in the same regions, particularly in the lamina propria and muscularis of the tissue. Furthermore, it may be speculated that androgen stimulation in the vagina can induce axonal growth and increased sensitivity effects that may be mediated through the androgenic component of prasterone administration to restore sexual function in postmenopausal women [22].

With the likely lack of activity in the vaginal tissue of the very low levels of circulating testosterone [5,8,9], expression of the enzymes involved in the androgen biosynthetic pathway indicate the sites of intracellular conversion of DHEA into sex steroids, especially androgens, in the vaginal epithelium, muscularis and around blood vessels. Such data point toward a likely mechanism where intravaginal local administration of prasterone can restore normal vaginal physiology, including both androgenic and estrogenic components. It is pertinent to remember that. DHEA is being the exclusive source of intracellular sex steroids after menopause [7]. The decreased levels of circulating DHEA with age during postmenopause could provide an explanation for the impaired sexual response in postmenopausal women. It should be considered that sexual dysfunction occurs in the presence of estrogen-based hormone replacement

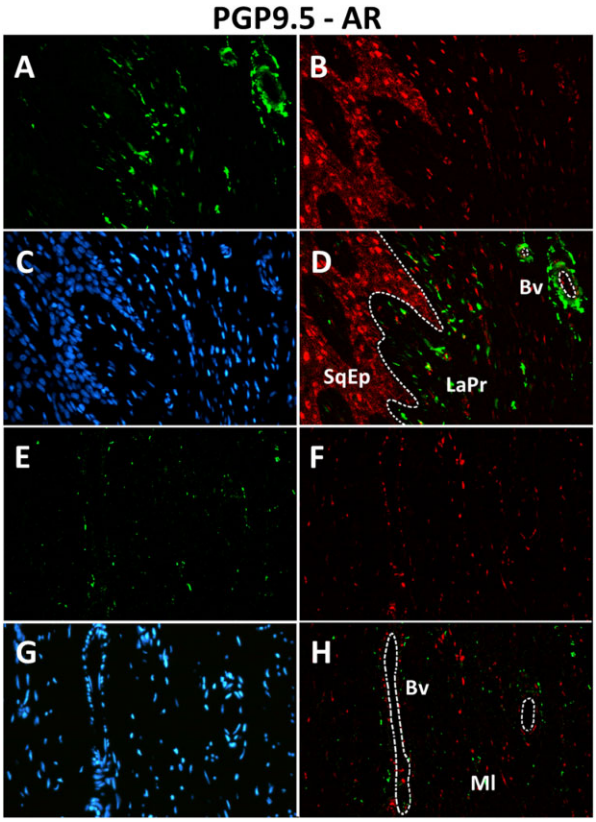

Figure 6 Nerve endings and AR are conjointly measured in the vagina. IF reveals that the PGP 9.5 immunostaining in the vagina is localized especially in the lamina propria (A), where AR is also modestly expressed (B). The specificity of the AR immunostaining and delimitation between the squamous epithelium and the lamina propria can be distinguished by the nuclear DAPI staining (C). The coimmunostainings of AR and PGP 9.5 illustrated in the merge image (D) shows that both proteins are often expressed at proximal sites in the lamina propria. Nerve fibers (E) and AR (F) are also found around blood vessels and in the muscle fibers of the deeper layer of the vagina with weaker PGP 9.5 signal intensity possibly due to the abundance of transversal orientation of nerve endings. Specificity of the AR staining and delimitation between blood vessels and muscle fibers can be distinguished by the nuclear DAPI staining (G). The costaining of AR and PGP 9.5 illustrated in the merged image (H) shows that both proteins are often expressed at proximal sites around blood vessels and in the muscularis. Images are generated from one monkey representative of three separate experiments done with a distinct animal. SqEp = squamous epithelium; LaPr = lamina propria; Bv = blood vessel; Ml = muscularis.

therapy [64–67] while estrogens do not appear to affect vaginal nerve density [27,36,63]. It has even been reported that AR expression in the human vagina is increased by testosterone administration [63,68].

Potential Clinical Value

While androgens have been shown to increase mucification of the vaginal superficial cells [34] and androgen [27] as well as DHEA [35,36] but not estrogens [27,36] have been shown to increase nerve density in the rat vagina, the presence of the androgen-forming enzymes in proximity with AR in the areas occupied by nerve endings could provide the basis for the beneficial androgenic effects of DHEA on sexual dysfunction in women [22].

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