OBJECTIVES
To investigate the roles of steroid hormones, including estrogen, progesterone, and testosterone, in the morphology and vascularization of the female bladder.

METHODS
A total of 70 adult, female Sprague-Dawley rats were divided into 7 groups: group 1, sham; group 2, ovariectomized rats without treatment; group 3, low-dose estradiol; group 4, high-dose estradiol; group 5, progesterone; group 6, estradiol combined with progesterone; and group 7, testosterone. All were given for 4 weeks. The serum steroid hormone levels were determined by radioimmunoassay. The total weight and thickness of the bladder were measured. Morphologic changes were observed under light and electron microscopy. The expression of vascular endothelial growth factor (VEGF) in the bladder was evaluated by immunochemistry and Western blotting.

RESULTS
The ovariectomized rats had a thinner bladder wall compared with the sham group (0.97 ± 0.11 mm vs 1.10 ± 0.10 mm, P < .05) and widened spaces between the detrusor muscle fascicles with collagen deposit. Estrogen reversed these changes, and testosterone increased the thickness of the bladder wall to 1.26 ± 0.12 mm (P < .05). VEGF staining was mainly located in the urothelium and endothelial cells, with weak staining in the smooth muscles. VEGF was almost absent in the urothelium after ovariectomy. In the estrogen- and androgen-treated groups, although the expression of VEGF was significantly greater than that in the nontreated ovariectomized group, it was still lower than normal.

CONCLUSIONS
Our findings suggest the importance of steroid hormones in maintaining the integrity of the bladder structure and regulating the expression of VEGF in the female urinary tract. Both estrogen and androgen can reverse the bladder muscle atrophy induced by ovariectomy. However, the decline in VEGF expression in the bladder cannot be fully recovered with either estrogen or androgen replacement.
Vascular endothelial growth factor (VEGF) is a critical angiogenic factor known to be required for the normal development of the vasculature, as well as for pathologic angiogenesis. VEGF exerts its effects on the vascular endothelium through binding to 2 high-affinity receptors, R1 (fms-related tyrosine kinase [Flt-1]) and R2 (kinase insert domain-containing receptor/fetal liver kinase [KDR/Flk-1]). This binding, in turn, activates the intrinsic tyrosine kinase activity of their cytodomains, initiating intracellular signaling. VEGF-, VEGFR1-, and VEGFR2-positive cells were detected in E14 and E18 bladders. Exogenous VEGF has been observed to enhance embryonic bladder growth in organ culture in both the urothelial and the detrusor smooth muscle compartments. It has been demonstrated that VEGF might be regulated by steroid hormones. With the decline of ovarian function, serum levels of VEGF were diminished. In ovariectomized rats that received estrogen treatment, the serum VEGF concentration was significantly greater than in those not receiving estrogen. A few studies have been done on the relationship of steroid hormones and VEGF and VEGF receptors in some sex steroid-responsive tissues, but none have yet studied this relationship in the bladder. Hervé et al. reported on an increase in KDR/Flk-1 expression in the uterus of ovariectomized mice treated with estradiol or estradiol combined with progesterone. However, KDR expression was not regulated by estradiol in vitro. In contrast, it was upregulated by VEGF itself in a time- and dose-dependent manner, suggesting that estradiol upregulates KDR expression mainly through the paracrine mechanism of VEGF. Häggström et al. found that VEGF mRNA and protein levels were significantly decreased by castration, and testosterone treatment induced VEGF synthesis in the rat ventral prostate epithelium. However, the expression of the Flt-1 and KDR/Flk-1 receptors was unaffected. According to these previous studies, it seems that steroid hormones can directly modulate the expression of VEGF.

It is known that the ovary is also a critical organ for producing testosterone in peri- and postmenopausal women. Of the circulatory levels of androgen, 50% are contributed equally from the ovaries and the adrenal glands. Davison et al. measured the testosterone levels in 595 female patients aged 18-75 years and found that the testosterone levels declined by around 55% with aging. Although estrogen and progesterone are being studied widely, the effects of androgen on the female urinary tract have barely been investigated and are poorly understood.

In the present study, we investigated the morphologic changes in ovariectomized rats induced by androgen and compared these with rats treated estrogen and/or progesterone. To further understand the regulation of steroid hormones on angiogenesis in the female urinary tract, we analyzed the expression profiles of VEGF in the bladders of rats undergoing different hormonal treatments.

MATERIAL AND METHODS

Rats
In the present study, we used 4-month-old adult female Sprague-Dawley rats (180-200 g). Bilateral ovariectomy or sham surgery was performed by low abdominal midline incision under pentobarbital anesthesia (40 mg/kg body weight intravenously). A total of 70 rats were divided into 7 groups. Each group was composed of 10 rats: sham group, rats with sham surgery; group OVX, ovariectomized rats; group OVX+E, ovariectomized rats treated with 0.25 mg/kg estradiol benzoate; group OVX+E1mg, ovariectomized rats treated with 1 mg/kg estradiol benzoate; group OVX+E+P, ovariectomized rats treated with 1 mg/kg progesterone; group OVX+E+P, ovariectomized rats treated with 0.25 mg/kg estradiol benzoate combined with 1 mg/kg progesterone; group OVX+E+P, ovariectomized rats treated with 3 mg/kg testosterone propionate. The solutions were diluted in sesame oil and given by intramuscular injection every 2 days for 4 weeks. The sham and OVX groups were only injected with sesame oil. Estradiol benzoate and testosterone propionate were purchased from Shanghai Tongyong Pharmaceutical (Shanghai, China). Progesterone was from Zhejiang Xianju Pharmaceutical (Zhejiang, China). All the procedures were performed under the guidelines of the Institutional Animal Care and Use Committee for the care and use of laboratory animals.

Sample Harvest and Preparation
At 4 weeks after bilateral ovariectomy or sham operation, deep anesthesia was induced. Blood samples were collected from the portal vein immediately before the rats were killed and centrifuged at 3000 rpm for 10 minutes before being stored at −20°C. Serum estradiol, progesterone, and testosterone levels were determined using a radioimmunoassay. The radioimmunoassay kit was purchased from Detu (Tianjin, China). The bladder and urethra were dissected and weighed. One half was fixed in 10% formalin, and the other half was immediately put into liquid nitrogen and stored at −80°C.

Hematoxylin-Eosin Staining and Thickness of Bladder Wall Measurement
Formalin-fixed specimens were embedded in paraffin in a vertical position, and serial 5-μm tissue sections were placed on microscope slides, dehydrated, and rehydrated. Hematoxylin-eosin-stained slides were used to determine the bladder thickness at equatorial cross-sections. Images were captured using a Carl Zeiss digital camera with an image definition of 1024 × 768 pixels, at 100× magnification. Each pixel corresponded to 2 μm. The computer program Image-Pro Plus (Media Cybernetics, Bethesda, MD) was used to analyze the digital images, and 10 consecutive points of each specimen were calculated.

Electron Microscopy
Bladder biopsy specimens were immediately immersed in freshly made 2.5% glutaraldehyde precooled to 4°C. Two blocks from each group were trimmed to a size of 1 mm². They were processed by osmification and dehydration and embedded in araldite. Ultrathin silver to silver-gold sections were obtained from the blocks and mounted on uncoated 150-mesh grids and stained by the standard uranyl nitrate/lead citrate sequence. The samples were observed under an EM300 electron microscope (Philips, Hamburg, Germany), and the images were cap-
was clarified by centrifugation at 10,000 × g for 20 min at 4°C. The supernatant was recovered, and the protein concentration was determined using the BCA kit. Equal amounts of protein were separated by 4%-20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blotting was performed using the primary antibody anti-VEGF-A (1:200 dilution) or anti-tubulin, mouse monoclonal antibody (1:1500 dilution, Santa Cruz Biotechnical). The intensity of the bands was determined by scanning video densitometry (Ultra Violet Products, Cambridge, England) and expressed as arbitrary densitometric units normalized to tubulin expression.

**Statistical Analysis**

The quantitative data are presented as the mean ± standard deviation and were analyzed according to the analysis of variance using computer software Statistical Package for Social Sciences, version 14.0. P < .05 was used as the level of significance.

**RESULTS**

**Serum Hormone Levels and Lower Urinary Tract Wet Weight in Animal Model**

The mean ± standard deviation serum hormone levels after treatment in each group are listed in **Table 1**. After ovariectomy, the serum estradiol and progesterone levels declined significantly, and 0.25 mg/kg estradiol benzoate with or without progesterone replacement recovered serum estradiol to normal levels efficiently. High-dose estradiol benzoate and testosterone propionate induced high concentrations of estradiol and testosterone after 4 weeks.

The body weights and lower urinary tract weights after treatment in each group are listed in **Table 1**. At the end of the treatments, no statistically significant difference was found in the body weights among the 7 groups. The mean lower urinary tract weights were decreased in group OVX and group OVX + P compared with the sham group (P < .001, P = .023, and P < .05 respectively). In the OVX + E, OVX + E1mg, and OVX + E + P groups, the lower urinary tract weights were close to that in the sham group and significantly greater in the testosterone-treated group (P = .001 and P < .01, respectively).

**Quantitative Morphometric Analysis of Bladder Wall**

Figure 1 shows that ovariectomy induced smooth muscle atrophy. The total bladder wall thickness of the OVX group was much thinner than that of the control group.
(0.97 ± 0.11 vs 1.10 mm ± 0.10 mm, P = .035 and P < .05, respectively). In the testosterone-treated groups, the thickness of the bladder wall increased to 1.26 ± 0.12 mm (P = .016, P < .05; Fig. 1C). No significant change was seen in the estrogen- or progesterone-treated group compared with the control group.

**Ultrastructural Morphology of Bladder Wall**

Electron microscopy was used to evaluate the ultrastructural changes in bladder detrusor cells (Fig. 2). After ovariectomy, smooth muscle compartments appeared mildly decreased with markedly widened spaces between individual muscle cells and their arrangement seemed less
well organized compared with other groups. Under high magnification, abundant collagen was deposited in the space between muscle fascicles, which did not show in other groups. A large number of caveolae were revealed in the detrusor muscles in the high-dose estrogen treatment group, but no obvious change was seen in the testosterone-treated group compared with the sham group.

Expression of VEGF in Bladder Tissue

Figure 3 shows that receptor patterns as evaluated with immunohistochemical staining and Western blotting. Brown staining in the investigated cells was considered as a positive immunoreaction. VEGF positive staining was mainly localized in the urothelium and endothelial cells, with weak staining in the smooth muscles. After ovariectomy, VEGF immunostaining was mostly absent in the urothelium and endothelial cells. With estrogen administration, very little was recovered. In the testosterone group, most of uroepithelial cells were positive for VEGF, and the expression of VEGF in the endothelial cells was weaker than normal.

Quantitative VEGF-A in each group was evaluated by Western blotting (Fig. 3D). The protein level of VEGF-A decreased significantly in group OVX compared with the sham group (16.6% ± 4.3% vs 87.8% ± 7.8%, P < .001). Estrogen treatment slightly increased the VEGF level (23.6% ± 5.7%, P = .027). High-dose estrogen and estrogen combined with progesterone resulted in better improvement (42.2% ± 8.1% and 30.1% ± 5.6% respectively, P < .001 vs group OVX). Testosterone increased the expression of VEGF to 61.6% ± 10.6%, although it was still significantly lower than that of the sham group (P < .001). Progesterone alone did not show a noticeable effect.

COMMENT

According to our animal study, ovariectomy induced a thinner bladder wall with an impaired ultrastructure of detrusor cells. Elbadawi et al.17 examined the biopsy specimens from patients with LUTS by electron microscopy and noted that changes in the detrusor tissue were characterized by collagenosis, with quite a bit of collagen plus some elastic fibers in the markedly widened spaces between muscle fascicles, suggesting that these changes in detrusor structure might account for the voiding dysfunction. In our present study, we observed a similar pattern of structural changes in ovariectomized rats. Therefore, the deficiency of steroid hormones is likely associated with bladder dysfunction.17,18 Hormone replacement with estrogen or androgen can reverse these effects induced by hormonal deficiency. Under the electron microscope, we observed a number of caveolae in the cytoplasm of the detrusor cells in the high-dose...
This unexpected finding might help explain the failure of estrogen therapy for menopausal LUTS. Although it has been well known that estrogens have important physiologic effects in the female lower urinary tract, the efficacy of estrogen replacement therapy in treating menopausal LUTS is still controversial. Meta-analyses have failed to show estrogen therapy had an effect greater than that of a placebo. However, it is still too early to conclude that high-dose estrogen has adverse effects on the function and morphology of the bladder from our current data.

We also demonstrated that androgen plays a potentially important role in maintaining the integrity of the bladder structure in female rats. Testosterone administration reversed the atrophy and disorganization of detrusor muscles induced by bilateral ovariectomy. A general consensus has been reached that androgens produce anabolic effects on skeletal muscle. Testosterone administration is associated with a dose- and concentration-dependent increase in fat-free muscle mass, muscle size, and maximal voluntary strength. Davison et al. have shown that the muscles in the pelvic floor and lower urinary tract are particularly sensitive to androgen. Moreover, androgen receptors have been detected in various female urogenital tissues. In rabbits, androgen receptors were found in the urethral and trigonal epithelium, detrusor muscle, and smooth muscle of the urethra. The widespread co-localization of androgen receptors with estrogen receptors and progesterone receptors in the female lower urinary tract suggests that androgen might play an important role in the maintenance of the normal function of female lower urinary tract. However, it is interesting that we did not see a reduction of serum testosterone after ovariectomy such as was shown for estrogen or progesterone. This could have been because the detection method of androgen has limitations under certain low levels. The other possibility is that the adrenal glands might produce more androgen to recover the depletion of ovary-derived androgen after ovariectomy. Moreover, the serum concentrations of testosterone appeared much greater than that of the controls after testosterone replacement. We used 3 mg/kg testosterone propionate in our study according to previously published data (1-5 mg/kg testosterone propionate was usually used in female rats); however, a lack of studies has been published on the relationship of androgen treatment and female bladders. Thus, the functions of physical level testosterone need additional study, although we did see some significant positive effects with high-dose androgen.

**Figure 3.** Immunostaining of vascular endothelial growth factor (VEGF) in bladder. Note, larger amount of stained (dark brown) cells in normal rats, especially in (A) uroepithelium. (B) VEGF almost absent in ovariectomized (OVX) group. (C) In testosterone-treated group, most uroepithelial cells were VEGF positive, but staining in endothelial cells was weaker than that in normal rats. Original magnification ×100. (D) Representative Western blotting of VEGF-A of bladder tissues and semiquantitative analysis. Protein levels determined by evaluating intensity of bands by scanning video densitometry, normalized to tubulin expression (i.e., tested protein/tubulin). Each bar represents mean value ± standard deviation. **P < .01 vs sham group. bP < .05 vs OVX group. cP < .01 vs OVX group. Abbreviations as in Figure 1.**
To our knowledge, this is the first report on the regulation of steroid hormones, including estrogen, progesterone, and androgen, in the expression of VEGF in the female bladder. We found that the expression of VEGF was extremely low after ovariectomy. Either estrogen or androgen can only partially recover this decline. Our finding is in accordance with those of previous studies on the genital system. Soares et al.\(^2\) evaluated the effects of estrogen and progesterone in the expression of VEGF mRNA and protein levels using a human breast cancer cell line, MCF-7, and observed that estrogen upregulated VEGF expression in both mRNA and protein. Fluctuations in the expression of VEGF in the genital tract occur with the menstruation cycles, as well as reproductive phenomena, such as ovulation, endometrial growth, implantation, and placentation.\(^2\) The presence of estrogen-response elements in the VEGF gene promoter region\(^2\) has also been reported, indicating that estrogens are, in fact, involved in VEGF regulation. According to our immunochemistry staining, VEGF-positive cells were mainly localized in the urothelium and endothelial cells. The expression of VEGF was particularly low in the endothelial cells even after hormone replacement. VEGF promotes endothelial cell proliferation and maintains the viability of immature blood vessels. The depletion of VEGF could lead to a decrease in angiogenesis, a reduction in the number of blood vessels, and, consequently, a weakening of the musculature and connective tissue. In addition, Yousif et al.\(^2\) found that VEGF not only enhanced smooth muscle formation and angiogenesis, but also increased nerve fiber generation in bladder grafts. The partial improvement of VEGF expression level by hormone replacements might also explain why estrogen therapy has not shown satisfactory efficacy in treating menopausal LUTS. Considering the positive effect of androgen in the female bladder, combined estrogen and testosterone therapy might be a promising strategy.

**CONCLUSIONS**

Although the most obvious and well-known consequences of menopause are due to the decline of estrogen levels, the effects of androgen are by no means less significant. Our findings suggest the importance of steroid hormones in maintaining the integrity of the bladder structure and imply a potential relationship between VEGF and urinary tract physiology that is partially regulated by steroid hormones.

**References**


