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Effects of estrogen on the relaxation response of rabbit clitoral cavernous smooth muscles

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Abstract We investigated whether estrogen has a peripheral effect on the smooth muscle relaxation response of the clitoris, similar to that of androgens in cavernous tissues of the penis. Forty-two New Zealand White female rabbits (2.3–2.7 kg) were randomly divided into control (sham operation) and bilateral oophorectomy groups (acute and chronic groups with or without estradiol replacement). The acute and chronic groups were killed at 5 days and 12 weeks post-oophorectomy, respectively. Relaxation responses of the clitoral cavernous strips to endothelium-dependent and independent vasodilators and electrical field stimulation (EFS) were observed. Serum total cholesterol and low-density lipoprotein increased significantly and acetylcholine-induced relaxation of the clitoral strips was significantly attenuated in the oophorectomy group with chronic estrogen deficiency but not in the chronic oophorectomy group with estradiol replacement or in the acute estrogen deficiency group. No significant differences in the relaxation responses of the cavernous strips to sodium nitroprusside or to the non-adrenergic, non-cholinergic-selective relaxation response to the EFS were found among the control, acute and chronic estrogen deficiency groups. These findings suggest that estrogen may not have a direct peripheral effect on clitoral cavernous smooth muscle relaxation. Hypercholesterolemia might be a possible explanation for the impaired endothelium-dependent relaxation of clitoral smooth muscle in chronic estrogen deficiency.

Keywords Clitoris · Rabbit · Smooth muscle · Relaxation · Estrogen deficiency

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Introduction

During female sexual stimulation, sensory stimulation leads to central nervous system activation resulting in neurotransmitter-mediated vaginal and clitoral smooth muscle relaxation and increased genital blood flow with a subsequent increase in vaginal lubrication, wall engorgement and luminal diameter as well as increased clitoral length and diameter [6, 11, 16]. Using duplex Doppler ultrasound, Berman et al. [2] documented significant increases in the blood velocity to these structures following sexual stimulation. They found that older women had significantly lower baseline blood velocity than younger women. Within the menopausal group, women receiving hormone replacement therapy (HRT) had significantly higher baseline blood velocities than those who were not.

Although testosterone is known to affect spinal reflex activity [5], the primary site of its action on the control of sexual function is probably the brain. Recently, androgens have been reported to have a peripheral action in cavernous tissue itself and to control the erectile response. Seven days after orchiectomy, the electric field stimulation (EFS)-induced erectile response of rats was reduced by 40% in comparison with intact rats. Testosterone restored this reduction to normal [13].

The symptoms related to alterations in genital sensation and blood flow are partly secondary to declining estrogen levels, and there is a direct correlation between the incidence of sexual complaints and circulating estrogen levels. The aim of this study was to investigate whether estrogen has a direct action on the smooth muscle relaxation of the clitoris as androgens do in the cavernous tissues of the penis.

Materials and methods

Materials

A total of 42 New Zealand White female rabbits (2.3–2.7 kg) were randomly divided into control (sham operation) and bilaterally oophorectomized groups. The oophorectomized group was divided into two main groups: acute and chronic. Each was further divided into two nearly equal groups, one of which ($n=7$) received estradiol replacement (a single intramuscular injection, 1 mg/kg for the acute subgroup; a weekly intramuscular injection, 1 mg/kg for 12 weeks for the chronic subgroup), the other ($n=8$) did not. Thus, there were four experimental (acute and chronic groups with or without estradiol replacement) and two control (acute and chronic groups, $n=6$ in each) groups. The acute group was killed 5 days and the chronic group 12 weeks after oophorectomy. Blood samples for the measurement of estradiol, serum total cholesterol, triglyceride, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were obtained from an ear vein just before the oophorectomy and directly before death. Following the removal of the clitoris, its cavernous tissues were excised and dissected free from the tunica albuginea and surrounding connective tissue.

Measurement of serum lipid fractions and estradiol levels

Blood samples for the measurement of serum lipid fractions were collected in EDTA-treated tubes and processed immediately. After centrifugation at 3000 rpm at 4°C for 10 min, the plasma total cholesterol, triglyceride, LDL, and HDL were measured by a standard enzymatic method (Abbott, USA). Serum estradiol levels were measured by radioimmunoassay with standard commercially available kits (Diagnostic Products, USA).

Oophorectomy

Bilateral oophorectomy was performed under anesthesia induced with an intramuscular injection of ketamine (30 mg/kg) and xylazine (0.15 mg/kg). On the day of surgery, ampicillin (2 mg/kg) was given intramuscularly.

Chemicals and solutions

Acetylcholine chloride, sodium nitroprusside (SNP), norepinephrine (NE), guanethidine, atropine sulfate, HEPES, potassium chloride (KCl), calcium chloride ($CaCl_2$), and magnesium chloride ($MgCl_2$) were purchased from Sigma (USA). The composition of the HEPES-buffered physiological salt solution (PSS) was as follows: 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5 mM HEPES, 11 mM glucose, pH titrated to 7.4 with 1 N NaOH.

Relaxation responses of the cavernous strips

The excised cavernous tissues were immediately placed in 100% oxygen-saturated HEPES-buffered PSS and studied within 1 h. Strips of cavernous smooth muscles were trimmed to 0.2× 0.2×0.9 cm and mounted. In order to record isometric tension, the strips were attached by a silk thread to a fixed support on one end and to a wire-connected force transducer (52-9545, Harvard, UK) and polygraph (50-8630, Harvard) on the other. The tissue was placed in a 25-ml organ chamber containing HEPES-buffered PSS which was bubbled with 100% O_2 and maintained at 37°C, pH 7.4. The resting tension for each strip was adjusted to the optimal isometric tension at which contraction by NE was maximal, and the developed tension was recorded.

Relaxation responses induced by the endothelium-dependent vasodilator acetylcholine and those induced by the endothelium-independent vasodilator SNP were then studied in the cavernous strips in which tone had been elicited with 5×10^{-5} M NE. Concentration-response curves were determined by adding successive logarithmic increments of the vasodilator agents from 10^{-9} to 10^{-4} M to the chamber. Inhibitory concentration (IC_{50}) values for each agent were determined from individual concentration-response curves using a nonlinear curve-fitting program (Origin software, version 6.0).

To assess NANC-selective neural relaxation, the remaining strips were similarly precontracted with NE and underwent EFS after muscarinic and prejunctional adrenergic blockade by preincubation with guanethidine and atropine sulfate (10^{-4} M each) for 30 min. Square wave pulses of 2 ms and 20 V at differing frequencies (1, 2, 4, 8, 16, 32, and 64 Hz) were delivered for 10 s at intervals of 10 min.

Contractile tension was expressed in grams. The results of the relaxation studies were expressed as a percent relaxation of the contraction induced by 5×10^{-5} M NE.

Statistical analysis

Data were expressed as the mean ± SD, with n representing the number of specimens. Statistical analysis of the data was performed by Student's unpaired t-test for two groups, and the analysis of variance (ANOVA) test was used for more than two comparisons. Results were considered statistically significant at $P<0.05$.

Results

Body weight, uterus weight, and blood levels of estradiol and lipid fractions

Acute group

There were no significant differences in the body and uterus weights of the rabbits between the acute and control groups. The blood level of estradiol was significantly lower ($P<0.01$) in the oophorectomy without estradiol replacement group (1.8 ± 2.0 pg/ml) than in the control (19.9 ± 6.4 pg/ml) and oophorectomy with estradiol replacement (27.6 ± 4.9 pg/ml) groups. There were no significant differences in the serum levels of total cholesterol, triglyceride, LDL, or HDL among the control, oophorectomy without estradiol replacement, and oophorectomy with estradiol replacement groups.

Chronic group

Body weights increased significantly ($P<0.01$) in the control, oophorectomy with and without estradiol replacement groups compared with the baseline, but there was no significant difference among the three groups (Table 1). In the oophorectomy without estradiol replacement group, uterus weights were significantly lower ($P<0.01$) than in the control and oophorectomy with estradiol replacement groups (Table 1).

The blood level of estradiol was significantly lower ($P<0.01$) in the oophorectomy without estradiol replacement group and significantly higher ($P<0.01$) in the oophorectomy with estradiol replacement group compared with the control group (Table 2). There were no significant differences in the serum levels of total

Table 1. Changes in total body and uterus weights after 12 weeks in control and experimental groups

	Body weight (kg)		Uterus weight (g)
	Baseline (*n*)	12 weeks (*n*)	12 weeks (*n*)
Control	2.34 ± 0.23 (6)	3.50 ± 0.32[a] (6)	10.8 ± 0.9 (6)
Oophorectomy without estradiol	2.44 ± 0.10 (8)	3.35 ± 0.28[a] (8)	1.7 ± 0.4[b] (8)
Oophorectomy with estradiol	2.37 ± 0.10 (7)	3.69 ± 0.26[a] (7)	14.4 ± 0.6 (7)

[a] Significantly different from baseline data ($P < 0.01$)
[b] Significantly different from control data ($P < 0.01$)

Table 2. Changes in serum levels of estradiol and lipid fractions after 12 weeks in control and experimental groups. *HDL* high-density lipoprotein, *LDL* low-density lipoprotein

	Estradiol (pg/ml)	Total cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Control					
Baseline	20.3 ± 4.5	51.3 ± 32.5	61.5 ± 19.9	25.0 ± 8.1	14.0 ± 25.6
12 weeks	19.9 ± 6.4	47.0 ± 15.7	58.0 ± 24.1	23.5 ± 9.7	11.9 ± 24.2
Oophorectomy without estradiol					
Baseline	19.9 ± 7.2	51.0 ± 13.6	57.9 ± 18.4	28.3 ± 16.3	11.1 ± 14.3
12 weeks	0.1 ± 0.04[a]	176.7 ± 51.4[a]	144.1 ± 32.7[a]	26.7 ± 4.5	121.2 ± 52.3[a]
Oophorectomy with estradiol					
Baseline	19.7 ± 4.6	62.6 ± 18.3	67.8 ± 18.9	27.0 ± 6.3	22.1 ± 15.2
12 weeks	83.3 ± 39.5[a]	68.0 ± 14.6	86.1 ± 16.4	30.4 ± 8.0	20.4 ± 10.1

[a] Significantly different from baseline data ($P < 0.01$)

cholesterol, triglyceride, LDL, or HDL between the oophorectomy with estradiol replacement and control groups. In the oophorectomy without estradiol replacement group, however, the serum levels of total cholesterol, triglyceride and LDL increased significantly ($P < 0.01$) compared with the baseline data (Table 2).

Relaxation responses of cavernous strips

Acute group

There were no significant differences in the relaxation responses of the cavernous strips to acetylcholine among the control (mean IC_{50} value: 5.2×10^{-6} M), oophorectomy without estradiol replacement (mean IC_{50}: 5.9×10^{-6} M), and oophorectomy with estradiol replacement (mean IC_{50}: 3.8×10^{-6} M) groups. There were also no significant differences in the relaxation responses of the cavernous strips to SNP among the control (mean IC_{50}: 3.1×10^{-6} M), oophorectomy without estradiol replacement (mean IC_{50}: 2.5×10^{-6} M), and oophorectomy with estradiol replacement (mean IC_{50}: 1.7×10^{-6} M) groups. No significant difference was found in the NANC-selective relaxation response to EFS among the three groups, although a higher frequency of stimulation produced substantially greater relaxation in all three groups.

Chronic group

Acetylcholine-induced relaxation of the clitoral strips was significantly attenuated ($P < 0.05$) in the oophorectomy without estradiol replacement group (mean IC_{50} value: 2.4×10^{-4} M) compared with that of the control (IC_{50}: 5.6×10^{-6} M) or oophorectomy with estradiol replacement (IC_{50}: 8.0×10^{-7} M) groups (Fig. 1). There were no significant differences in the responses between the acute and chronic estradiol replacement groups. SNP- (Fig. 2) and NANC (Fig. 3)-induced relaxations were not significantly different among the three groups.

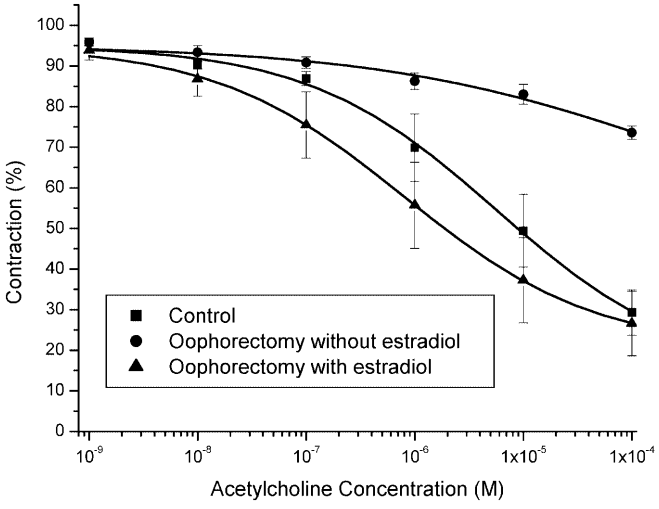

Fig. 1. Comparison of the relaxation responses of clitoral cavernous smooth muscles to acetylcholine after 12 weeks. The response is significantly ($P < 0.05$) impaired in the oophorectomy without estradiol replacement group (*circle*) compared to the control (*square*) and oophorectomy with estradiol replacement groups (*triangle*)

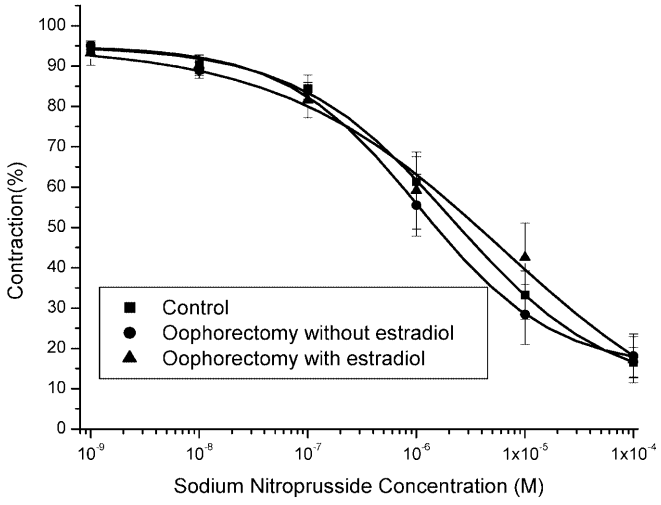

Fig. 2. Comparison of the relaxation responses of clitoral corpus cavernosal smooth muscles to sodium nitroprusside after 12 weeks. There were no significant differences in relaxation responses among the three groups

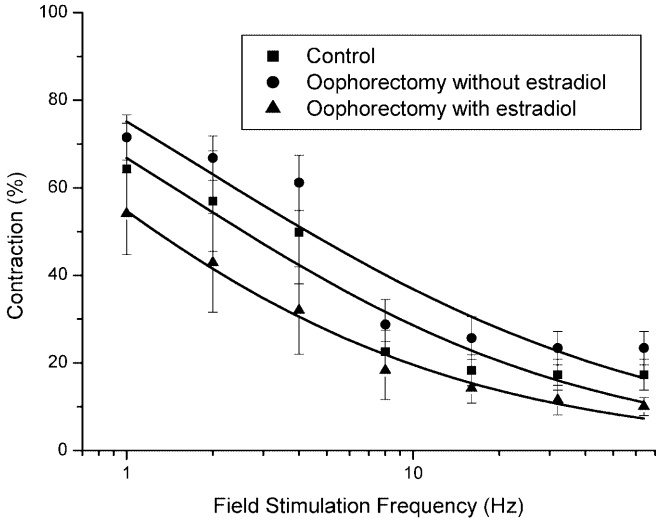

Fig. 3. Comparison of the relaxation responses of clitoral cavernous smooth muscles to electrical field stimulation of nonadrenergic noncholinergic nerves after 12 weeks. The nerve stimulation evokes a frequency-dependent increase in the relaxation response. There were no significant differences in the relaxation responses among the three groups

Discussion

The clitoris may play a major role in enhancing the female sexual response to coitus during sexual activity. Clitoral stimulation may induce local autonomic and somatic reflexes causing vaginal vasocongestion, engorgement, and subsequent lubrication of the introital canal making the sexual act easier, more comfortable, and more pleasurable. The more stimulation, the higher the level of arousal and the easier it is to further increase stimulation [18].

Investigators interested in the pathophysiology of female sexual dysfunction have suggested that in some women this may be associated with abnormal arterial circulation into the vagina or clitoris during sexual stimulation. One of the most interesting current subjects of research is the sexual arousal disorder showing symptoms such as a lack of or diminished vaginal lubrication, decreased clitoral and labial sensation and engorgement, and lack of vaginal smooth muscle relaxation. Abnormal arterial circulation into the vagina or clitoris during sexual stimulation may be considered a disorder of arousal. Park et al. [12], using an atherosclerotic animal model, demonstrated that vaginal engorgement and clitoral erection depend on increased blood inflow and that atherosclerosis is associated with vaginal engorgement insufficiency and clitoral erectile insufficiency.

Hormones, especially estrogen, seem to play a significant role in regulating female sexual function. Plasma estrogen levels affect cells throughout the peripheral and central nervous system and influence nerve transmission. In animal models, estradiol administration results in expanded touch receptor zones along the distribution of the pudendal nerve, suggesting that estrogen affects sensory thresholds [10]. Female reproductive systems, especially the clitoris and vagina, are the target organs of estrogens. In postmenopausal women, estrogen replacement restores clitoral and vaginal vibration and pressure thresholds to levels close to those of premenopausal women. Estrogens also have vasoprotective and vasodilatory effects that result in increased vaginal, clitoral, and urethral arterial flow [15]. Using duplex Doppler ultrasound, Berman et al. [2] found that older women had a significantly lower baseline blood velocity to these structures than younger women. Within the menopausal group, women receiving HRT had significantly higher baseline blood velocities than those who were not.

There is a significant difference between men and women in the incidence of cardiovascular disease. Before menopause, the incidence of coronary artery disease in women is less than that of men, but postmenopausal women show an increasing incidence which becomes similar to that of men [14]. For postmenopausal women, estrogen deficiency raises plasma total cholesterol and LDL, which leads to atheroma formation. Studies have shown that estrogen deficiency consequently leads to an increased risk of coronary heart disease [7].

In this study, the serum levels of total cholesterol, triglyceride, LDL and HDL in the oophorectomized group of rabbits with acute estrogen deficiency were not significantly different from those of the control and oophorectomy with estradiol replacement groups. However, serum total cholesterol and LDL increased significantly in the oophorectomized group of rabbits with chronic estrogen deficiency compared with the baseline data. Acetylcholine-induced relaxation of the clitoral strips was significantly attenuated in the oophorectomized group with chronic estrogen deficiency but not in the oophorectomized group with acute estrogen deficiency. We could not find any abnormal

histologic changes on light microscopic examination of the clitoral cavernous arteries and smooth muscles of the oophorectomized group with either acute or chronic estrogen deficiency, although this does not rule out arteriosclerotic changes of the clitoral cavernosal artery that might have developed with a continued deficiency of estrogen. These results suggest that estrogen may not have a direct peripheral action on clitoral cavernous smooth muscle to control the relaxation response, unlike the action of androgens in the cavernous smooth muscle of the penis.

There are several reasons to postulate nitric oxide (NO) as a mediator of clitoral cavernosal smooth muscle relaxation. First, embryologically the clitoris is the homologue of the penis. Second, endothelial and neuronal NO synthase immunoreactivities were detected in vascular endothelium and nerve bundles in human clitoral tissue [3]. Third, organ bath analysis of rabbit clitoral cavernosal smooth muscle strips demonstrated an enhanced relaxation in response to SNP and L-arginine, which are NO donor and NO precursor, respectively [1, 4]. One of the major findings of this study was that acetylcholine, SNP and NANC-selective electrical stimulation caused the concentration- or frequency-dependent relaxation of cavernous smooth muscle strips in the control, acute, and chronic groups. This finding corroborates the conclusions of previous studies, and points to a potential role of NO as a mediator of clitoral cavernosal smooth muscle relaxation.

In our previous studies [8, 9], we found a functional impairment of penile cavernous smooth muscle relaxation in response to endothelium-mediated stimuli in hypercholesterolemic rabbits, and that oxidized LDL inhibits the endothelium-dependent relaxation of cavernous smooth muscles, while relaxation in response to endothelium-independent agents and NANC-selective neural relaxation was preserved. The functional impairment of endothelial NO synthase (eNOS) was proposed as a possible cause of the decrease in cavernous smooth muscle relaxation response in the early stage of hypercholesterolemia [17]. In this context, the hypercholesterolemia in a state of chronic estrogen deficiency may be one of the major causative factors of the impaired endothelium-dependent relaxation of rabbit clitoral smooth muscle.

In conclusion, the results of this study suggest that estrogen may not have a direct peripheral action on the ability of the clitoral cavernous smooth muscle to control the relaxation response and that secondary hypercholesterolemia might be a possible explanation for the impaired endothelium-dependent relaxation of clitoral smooth muscle in chronic estrogen deficiency.

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