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# Effect of Estrogen Replacement Therapy on Bladder Circulation in Old Female Rats

**Katsuya Hikita, Naoto Kobayashi and Ikuo Miyagawa**

*Division of Urology, Department of Surgery, School of Medicine, Tottori University Faculty of Medicine, Yonago 683-8504 Japan*

**The effect of estradiol (E2) on the urinary bladder of old female rats was investigated by examining the histology and blood flow in the bladders. A total of 20 female Wister rats aged 16 months were divided into 4 groups: Group I, 5 normal controls; Group II, 5 rats treated with E2 for 4 weeks; Group III, rats treated with E2 for 8 weeks; and Group IV, 5 rats receiving a placebo for 8 weeks. After the treatment, we removed the bladders, then weighed and stained them with hematoxylin and eosin. The muscle content was analyzed with the Elastica-van Gieson method, and the number of blood vessels with the Masson's trichrome method. Blood circulation in the bladders was also measured. The E2-replaced groups showed higher levels than the other groups in terms of blood flow in the bladder (20.6 ± 1.8 mL/min for Group II and 23.4 ± 1.5 mL/min for Group III, both $P < 0.05$ versus Groups I and IV), muscle content (2.33 ± 0.47 and 3.11 ± 0.48 for Groups II and III, respectively, both $P < 0.05$ versus Groups I and IV) and bladder weight (185.3 ± 6.2 mg and 193.2 ± 23.5 mg for Groups II and III; Group III showed $P < 0.05$ versus Groups I and IV). Differences in body weight and number of blood vessels among groups were not significant. We observed an increase in blood circulation, muscle content and weight of the bladder: E2-replacement therapy positively affected bladder functions.**

**Key words:** bladder; blood flow; estrogen; rat; smooth muscle

Urinary incontinence is one of the most difficult problems for elderly women to maintain a decent quality of life. Such changes in bladder function are produced postmenopausally as detrusor instability, stress urinary incontinence and a propensity toward urinary tract infection: the dysfunctions are attributed in part to estrogen deficiency (Stenberg et al., 1995; Thom and Brown, 1998). The tissues of the lower urinary tract and pelvic floor are known to be estrogen-sensitive because estrogen receptors are located throughout the bladder and urethra (Batra and Iosif, 1983; Klutke and Bergman, 1995). It is hypothesized that estrogen replacement therapy may be useful for urinary incontinence for several reasons. In women with stress incontinence, estrogen might reverse the effects of urethral atrophy, improve coaptation and, therefore, increase urethral closure pressure. Because estrogen has also been linked in increasing the sensory threshold of the bladder to cholinergic stimulation, estrogen replacement therapy may be equally useful for treating detrusor instability (Ekstrom et al., 1993; Fantl et al., 1996). To date, research on the success of estrogen replacement therapy for postmenopausal

Abbreviations: E2, estradiol; HE, hematoxylin and eosin; NIH, National Institutes of Health

bladder dysfunction has been quite controversial because many studies were based on subjective reports. More than 60 years ago, Salmon et al. (1941) reported successful treatment of postmenopausal women with intramuscular estrogen therapy. Similar results have been reported elsewhere (Rud, 1980).

Animal studies have helped to shed light on the role of estrogen in bladder function. Longhurst et al. (1992) found that estrogen treatment actually increases detrusor sensitivity and improves bladder constriction. One study proposed that estrogenic effects may also increase urethral blood flow (Batra and Iosif, 1983). In that study, the effects were examined after several days of treatment with relatively high amounts of estrogen. We decided to quantitatively assess the effect of long-term estrogen replacement on the smooth muscle, vesical vessels and blood flow, and muscle content of the bladder in old female rats.

## Materials and Methods

All animal experiments were performed in accordance with the Guidelines established by the Tottori University Committee for Animal Experimentation. We divided 20 female Wister rats aged 16 months into 4 groups each with 5 animals. A silastic tube (inside diameter = 2.5 mm, length = 30 mm) (Kaneda Medix, Osaka, Japan) containing sesame oil (Kadoya, Tokyo, Japan) was subcutaneously inserted in the rats of 3 groups (not in the control group). Ovariectomy was not performed in any group. Group-I rats received no treatment (control group). Group-II rats were administered 2.50 mg of estradiol (E2) dissolved in sesame oil through a silastic tube for 4 weeks (4-week E2-replaced group). Group-III rats were E2-administered for 8 weeks in the same manner as for Group II (8-week E2-replaced group). Group-IV rats (placebo group) were treated only with sesame oil without E2 for 8 weeks by the same methods as for Groups II and III (Ohata, 2002). All animals were kept in an air-conditioned room lighted 12 h a day, and allowed access to food and water ad libitum.

### *Measurement of plasma E2*

Plasma E2 concentrations were assessed by radioimmunoassay with a kit from SRL (Tokyo) when rats were 16 months old in Group I, 17 months old in Group II and 18 months old in Groups III and IV. After the rats were anesthesized with subcutaneous injection of 1.0 g/kg urethane, an amount of blood (2.5 mL) was taken from the heart and placed in a cyclone separator to measure the plasma E2 concentration levels.

### *Measurement of blood flow in the urinary bladder*

We measured blood flow in the rat bladder with a laser Doppler flowmeter (BRL-100, Bioresearch, Nagoya, Japan). After incision of the abdomen under anesthesia by subcutaneous injection of 1.0 g/kg urethane, we identified the bladder, and emptied and attached a probe to the dome of the bladder (Saito and Miyagawa, 2001) to measure blood flow for 5 min. Data used were mean ± SE for 1 min.

### *Histological examination of the rat bladder*

After the animals were anesthetized with subcutaneous 1.0 g/kg urethane injection, the bladder was surgically removed from the surrounding tissues and weighed. Each bladder was transected at the urethra level, sectioned into sagital slices, immediately fixed with 10% formalin, and then embedded in paraffin. From these paraffin blocks, 10-$\mu$m-thick tissue sections were prepared in vertical cross slices. The tissue sections were deparaffinized with xylene, and rehydrated with a graded series of ethanol. All specimens were stained either with hematoxylin and eosin (HE), Elastica-van Gieson or Masson's trichrome. The Elastica-van Gieson method distinguishes the smooth muscle area density from the connective tissue area density, staining smooth muscle yellow and collagen red. Each section was viewed under a light microscope at a magnification of 400 times. To analyze the ef-

fect of estrogen replacement on the smooth muscle content, color-assisted quantitative image analysis was used with the Elastica-van Gieson method. The Masson's trichrome method was applied to observe each section of blood vessels in the smooth muscle of the bladder linings. Data were obtained from the 5 animals in each group and averaged.

### *Color-assisted quantitative image analysis*

The stained tissue sections were viewed under a microscope (BX50, Olympus, Tokyo). The image of the histologic sections was displayed on a color monitor (PVM-20M4V, Sony, Tokyo) and printed with a digital color printer (CP700DSA, Mitsubishi, Tokyo). It was then digitized with a scanner (GT-8000, Epson, Tokyo) operated by a Macintosh Powerbook G3 (Apple Computer, Inc., Cupertino, CA), and automated image analysis was performed with National Institutes of Health (NIH) Image 1.55 (Research Service Branch, NIH, Bethesda, MD). The NIH Image 1.55 system discriminates color differences in stained tissue sections: We applied the system to discriminate differentially stained components of smooth muscle and connective tissues, which were calculated per full screen. At least 10 different fields were examined from each tissue section. The ratio of smooth muscle area/connective tissue area was determined for each animal.

### *Statistical analysis*

Statistical significances of differences among groups were compared with analysis of variance (ANOVA) and Fisher's multiple comparison test. Probability levels less than 0.05 were regarded as significant.

## Results

### *Plasma E2 levels*

Plasma E2 levels ranged from 7.5 to 28.3 pg/mL in Group I, 35.0 to 57.5 pg/mL in Group II, 35.0 to 57.4 pg/mL in Group III and 9.9 to 19.7 pg/mL in Group IV. Differences in plasma E2 levels were significant between all pairs of Groups, except between Groups I and IV (Table 1).

### *Bladder and body weight*

Bladder weight was significantly higher in Group III than in Groups I and IV. Group II showed a higher tendency of mean bladder weight than Groups I and IV, but there was no statistical significance (Table 1). Differences in body weight were not significant among all groups, while E2-supplied groups showed a lower tendency of mean body weight.

**Table 1. Plasma E2 levels, Bladder weight and Body weight**

| Group | Plasma E2 levels (pg/mL) | Bladder weight (mg) | Body weight (g) |
|---|---|---|---|
| I | 19.3 ± 8.4 | 164.5 ± 9.1 | 355.5 ± 32.0 |
| II | 38.5 ± 6.1 | 185.3 ± 6.2 | 338.6 ± 27.0 |
| III | 48.8 ± 9.1 | 193.2 ± 23.5 | 344.8 ± 34.2 |
| IV | 13.9 ± 3.8 | 164.3 ± 14.0 | 349.7± 25.6 |

Each value is mean ± SE.
* Significant difference; $P < 0.05$.

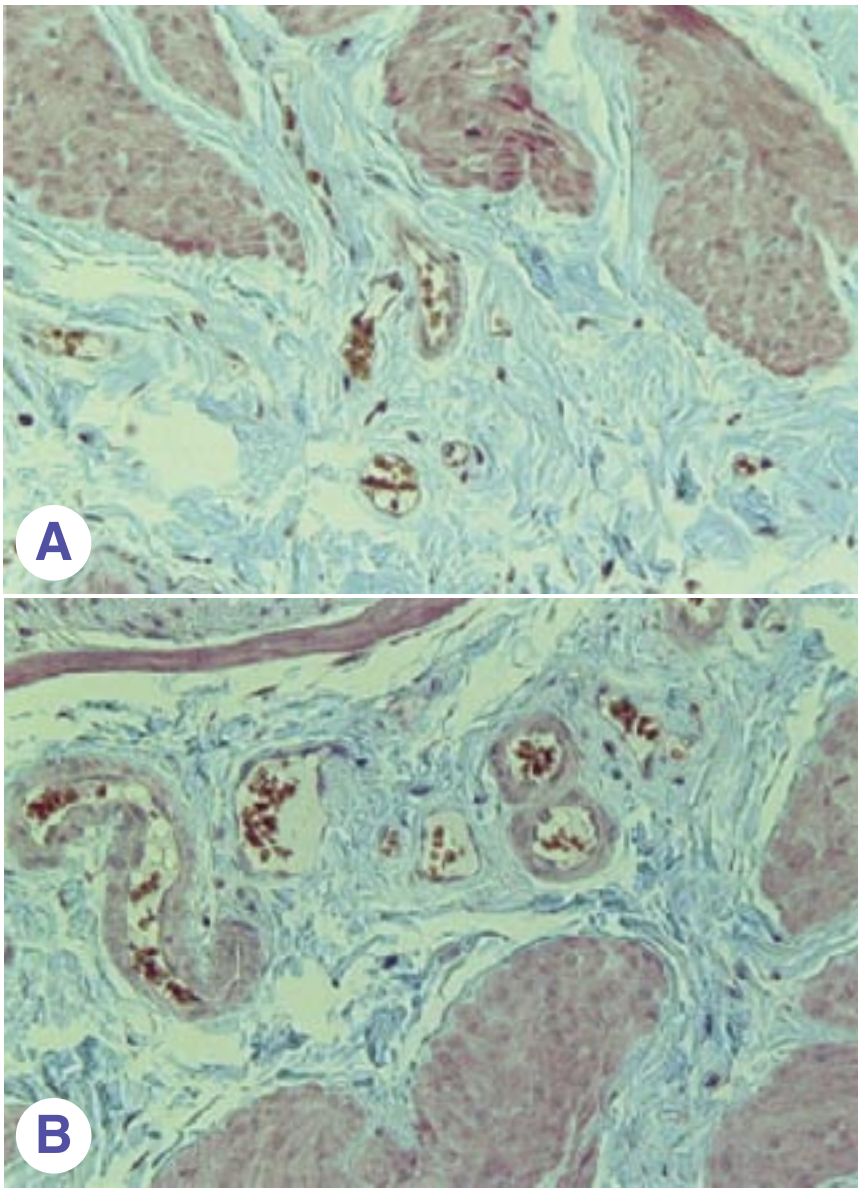



**Fig. 1.** Sections of rat bladders stained with the Masson's trichrome method (original magnification, × 400).
**A:** A control rat (Group I).
**B:** A rat treated with E2 for 4 weeks (Group II).
**C:** A rat treated with E2 for 8 weeks (Group III).
**D:** A placebo rat (Group IV).

*Figs. 1A and B on p. 84*
*and*
*Figs. 1C and D on p. 85*

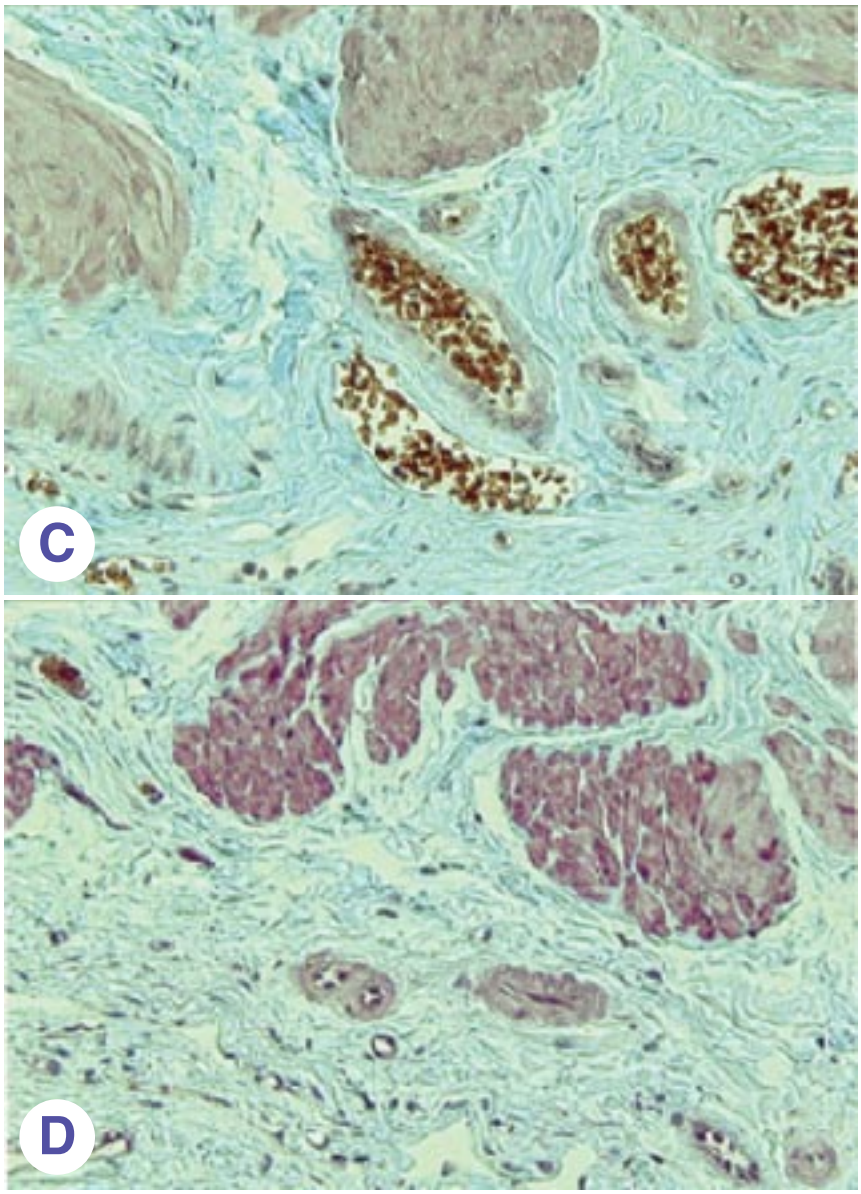
**Table 2. Blood flow and average number of bladder vessels**

| Group | Blood flow (mL/min) | Number of vessels |
|---|---|---|
| I | 16.6 ± 1.8 | 3.6 ± 1.1 |
| II | 20.6 ± 1.8 | 4.8 ± 1.3 |
| III | 23.4 ± 1.5 | 5.0 ± 1.2 |
| IV | 18.2 ± 0.4 | 3.6 ± 1.3 |

Each value is mean ± SE.
* Significant difference; $P < 0.05$.

### *Blood flow and histological examination of the rat bladder*

Blood flow levels of the bladder in Groups II and III were significantly ($P < 0.05$) higher than in Groups I and IV. A longer period of E2 supply showed an increasing tendency toward blood flow: blood flow level was higher in the 8-week-supplied Group III than the 4-week-supplied Group II (Table 2). The difference in number of blood vessels of the bladder was not significant, but Groups II and III tended to show a higher number than Groups I and IV (Table 2).




***Figs. 1C and D.***
*Legends, see the previous page.*

The area density of the bladder was compared between smooth muscles and connective tissues. The 18-month-old placebo group (Group IV) showed a lower tendency of smooth muscle area than the 16-month-old control group (Group I). The increase in smooth muscle area density was significant ($P < 0.05$ both) in E2-supplied Groups II and III compared to Groups I and IV, and the increase was significantly higher ($P < 0.05$) in the 8-week-supplied Group III than in the 4-week-supplied Group II (Table 3). Examples of bladder specimens are shown in Figs. 1 and 2.

**Table 3. Ratio of smooth muscle area/connective tissue area**

| Group | Smooth muscle area/connective tissue area |
|---|---|
| I | 1.45 ± 0.39 |
| II | 2.33 ± 0.47 |
| III | 3.11 ± 0.48 |
| IV | 1.14 ± 0.08 |

Each value is mean ± SE.
*Significant difference; $P < 0.05$.

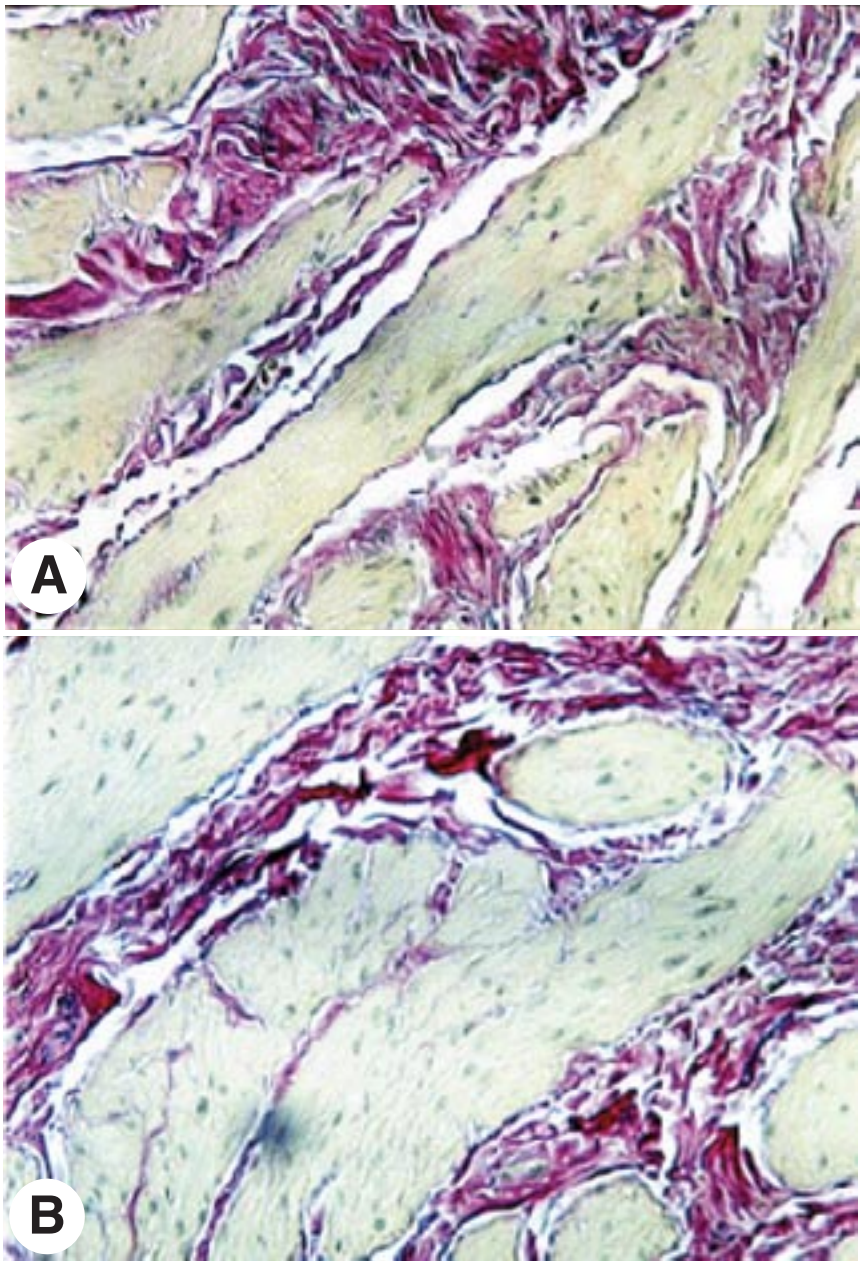


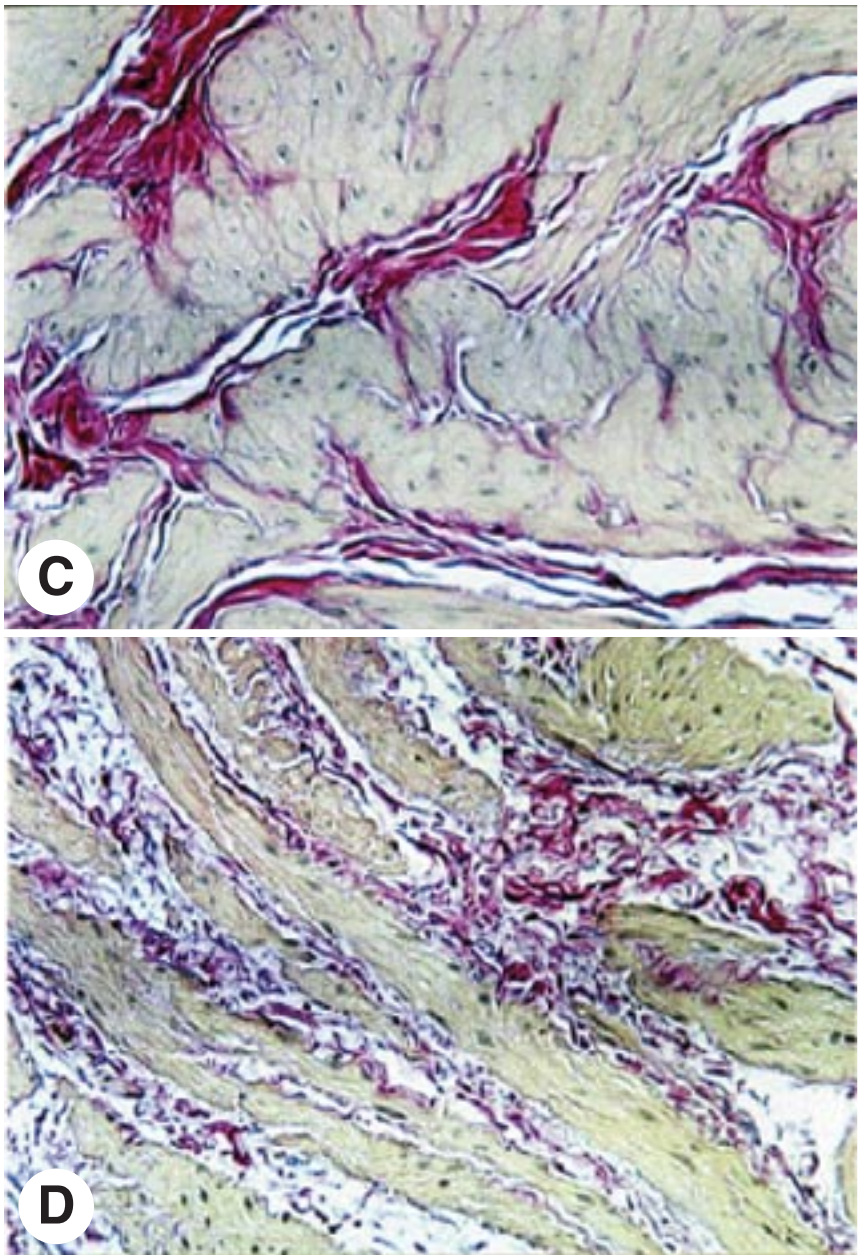

**Fig. 2.** Sections of rat bladders stained with the Elastica-van Gieson method (original magnification, × 400).
**A:** A control rat (Group I).
**B:** A rat treated with E2 for 4 weeks (Group II).
**C:** A rat treated with E2 for 8 weeks (Group III).
**D:** A placebo rat (Group IV).

*Figs. 2A and B on p. 86 and Figs. 2A and D on p. 87*

## Discussion

Past experiments in both humans and animals have shown that estrogen receptors are present in the urethra and bladder. Estrogen influences lower urinary tract function and continence with various mechanisms: estrogen increases cell activity in the bladder and urethra, increases blood flow in the urethral submucosa and urethral sphincter, sensitizes urethral sphincter α-adrenargic receptors and stimulates periurethral connective tissue metabolism (Screiter et al., 1976; Versi and Cardozo, 1986; Bergman et al., 1990; Blakeman et al., 1996; Jackson et al., 1996).

We studied the effects of estrogen replacement therapy on mature rats whose estrous cycles have discontinued. Our specific attention was paid to any result of estrogen replacement therapy: bladder changes in blood flow, number of blood vessels, tissues and weight. Furthermore, to study the effects of




***Figs. 2C and D.***
*Legends, see the previous page.*

the duration of estrogen replacement, estrogen was introduced for 4 or 8 weeks to 2 different groups for any consequence stemming from longer duration of treatment.

We observed plasma E2 levels lower than 15 pg/mL in Group IV. The fertility period of rats lasts approximately until the age of 15 months, at which time the estrous cycle ceases (Handa et al., 1987; Shulman et al., 1987; Albert et al., 1991). According to Albert et al. (1991), rats with normal body weights maintain the plasma E2 level slightly below the mean of 15 pg/mL throughout the estrous cycle. In the present study, we gave no estrogen replacement to Groups I and IV. The plasma E2 levels obtained from 16-month-old rats in Group I exceeded 15 pg/mL, slightly higher than expected at such an age. In Group IV, as expected, 18-month-old rats' levels were slightly lower than 15 pg/mL. Groups II and III received estrogen replacement, and their plasma E2 levels were significantly increased to more than 30 pg/mL and 40 pg/mL, respectively. Therefore, our E2-replacement method

provided adequate levels for studying its effects on rat bladders.

In a comparison to normal control rats, ovariectomized rats had a decreased bladder weight and increased body weight (Longhurst et al., 1992). Also in our observations, we found that bladder weight was significantly heavier in Group III than Groups I and IV, while there were no significant differences in Group II compared to the other groups. However, Group II had a higher tendency of mean bladder weight than Groups I and IV. Thus, the bladder tended to gain weight when the E2 level was increased, which suggests that E2 replacement after menopause increases bladder weight. The bladder had been thought to gain weight in muscle content and epithelium thickness (Rocha et al., 2002). We observed no significant difference in mean body weight among groups, but estrogen-replaced rats lost some weight. The mechanism for how estrogen protects the bladder against collagen formation is not yet known. Some evidence suggests it has a cardioprotective action preferably through nitric oxide from endothelial release: estrogen replacement may prevent the proliferation of fibroblasts and collagen deposition in the blood vessels of the heart (Blacher et al., 2000). Whether a similar mechanism exists in the bladder is a subject of further study.

The number of blood vessels influences intraurethral pressure (Versi and Cardozo, 1986). Estrogen replacement therapy was reported to increase the number of periurethral and bladder vessels (Madeiro et al., 2001). In ovariectomized rats, however, the number of bladder vessels was decreased (Rocha et al., 2002). We observed no significant differences in the number of bladder vessels among groups, but only a little increase in Groups II and III. Estrogen acts directly on the number of bladder vessels in the urethra and periurethral tissue. This action could explain the increase in the number of bladder vessels due to estrogen replacement. Even with estrogen replacement, however, the increase in the number of bladder blood vessels was slight in our experiments: the increase in muscle content and cell cycle activity might have acted together.

Estrogen treatment inhibits tissue weight gain and cardiac output, and so blood flow of the urinary tract is also increased by estrogen (Batra et al., 1985, 1986). In this study, there was a significant difference in blood flow between E2-replaced groups and the control and placebo groups. Estrogen increases blood flow as previously reported (Batra et al., 1986) as well as in the present study. The slight increase in bladder blood flow we observed may be due to the increase in bladder weight and overall blood volume (not measured in the present study). According to the literature, the compliance functions of the bladder and urinary pressure are also improved with estrogen. These improved functions also help improve circulation (Fleischmann et al., 2002). Our estrogen replacement therapy for mature rats resulted in an increase in bladder muscle content and blood circulation, similarly as reported (Rocha et al., 2002).

In medical trials conducted since the 1960s predominantly in the United States, menopausal women have been given progesterone concurrently with estrogen to protect against increased risks of cancer. Studies in Japan have also shown estrogen useful in the treatment of stress incontinence (Suzuki et al., 1997). Since the present study has shown the benefits and advance effects of estrogen replacement therapy in rats, it should be advocated as a treatment in general practice.

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*Corresponding author: Ikuo Miyagawa, MD*