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Involvement of Estrogen in the Pathogenesis of Cyclophosphamide-Induced Cystitis in Rats

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We examined the effects of ovariectomy and castration on the histology of cyclophosphamide (CP)-induced cystitis in rats. The animals were injected with CP (100 mg/kg) or saline intraperitoneally twice with an intervening 4 d and were euthanized at 1 or 2 wk after the initial CP administration. Saline treatment did not cause an apparent histological change in the bladder regardless of surgery, i.e., ovariectomy, castration, and sham-operation. CP treatment resulted in submucosal edema, urothelial damage, hemorrhage, and leukocyte infiltration in the bladder at 1 wk after the initial CP administration regardless of surgery. However, these histological changes were more severe in ovariectomized rats than in the sham-operated rats. In contrast, there were no significant differences in CP-induced histological changes in the bladder between castrated and sham-operated groups. We further examined the role of estrogen and progesterone on the CP-induced histology in the bladder by the replacement with estrogen only or estrogen plus progesterone for 2 wk in overiectomized rats. Estrogen treatment ameliorated CP-induced histological changes compared to oil treatment, whereas estrogen plus progesterone treatment did not produce any differences in the histology of the bladder compared to estrogen treatment. These results suggest that estrogen may play a role in the pathogenesis of bladder inflammation.

Key Words: Estrogen; progesterone; testosterone; ovariectomy; castration; inflammation; cyclophosphamide.

Introduction

The gonadal steroid hormones, such as estrogen, progesterone, and testosterone, are generally thought to play a pro-inflammatory role in the reproductive organs. In particular, estrogen stimulates epithelial cell proliferation, increases vascular permeability and infiltration of macrophages and eosinophils into the targeted reproductive organs *(1)*. Recently, several studies have revealed that estrogen may also exert anti-inflammatory activity in lung, vascular smooth muscle, and joints in a variety of animal species *(1–3)*. For instance, in the rat lung, ovariectomy influenced adversely granulomatous inflammation and exogenous ovarian steroid hormones suppressed inflammatory process *(1)*. In vitro studies have shown that estrogen blocks cytokine-dependent induction of inducible nitric oxide (NO) synthase in vascular smooth muscle and macrophages *(2,3)*. In addition, clinical studies have also revealed that estrogen is beneficial to rheumatoid arthritis and that clinical remission of the disease occurs during late pregnancy, which is associated with high levels of estrogen *(4,5)*.

The gonadal steroid hormones exert their biological actions by binding to their specific receptors such as the estrogen receptor (ER)-α, ER-β, progesterone receptor (PR), and androgen receptor (AR). Anatomical studies have revealed that these receptors are widely distributed in a variety of organs including bladder *(6)*.

With regard to the bladder, accumulating evidence suggests that estrogen plays a role in the regulation of the morphology and the function in the bladder among a variety of animals *(7–12)*. Gonadectomy results in an induction of mucosal atrophy and a decrease in smooth muscle density *(12)*. The replacement of estrogen in gonadectomized animals reverses these effects *(12)*. Recent studies have revealed that ovariectomy causes significantly higher levels of urothelial apoptosis in the rabbit bladder and in the rat urethra compared to sham-operated animals and ovariectomized animals treated with estrogen *(7,13)*. Despite considerable amounts of evidence showing the role of estrogen in the morphology and the function of the bladder, there is little information regarding the involvement of estrogen, progesterone, and testosterone in the pathogenesis of inflammation in the bladder. Previously, Bon et al. showed that the phase of the estrus cycle had little effect on the histological severity of cyclophosphamide (CP)-induced cystitis in rats, but that there was a more rapid onset of pain-related behavioral abnormalities in females compared to males *(14)*. Nevertheless, the involvement of gonadal steroid hormones in the regulation of bladder inflammation is still unclear. In the present

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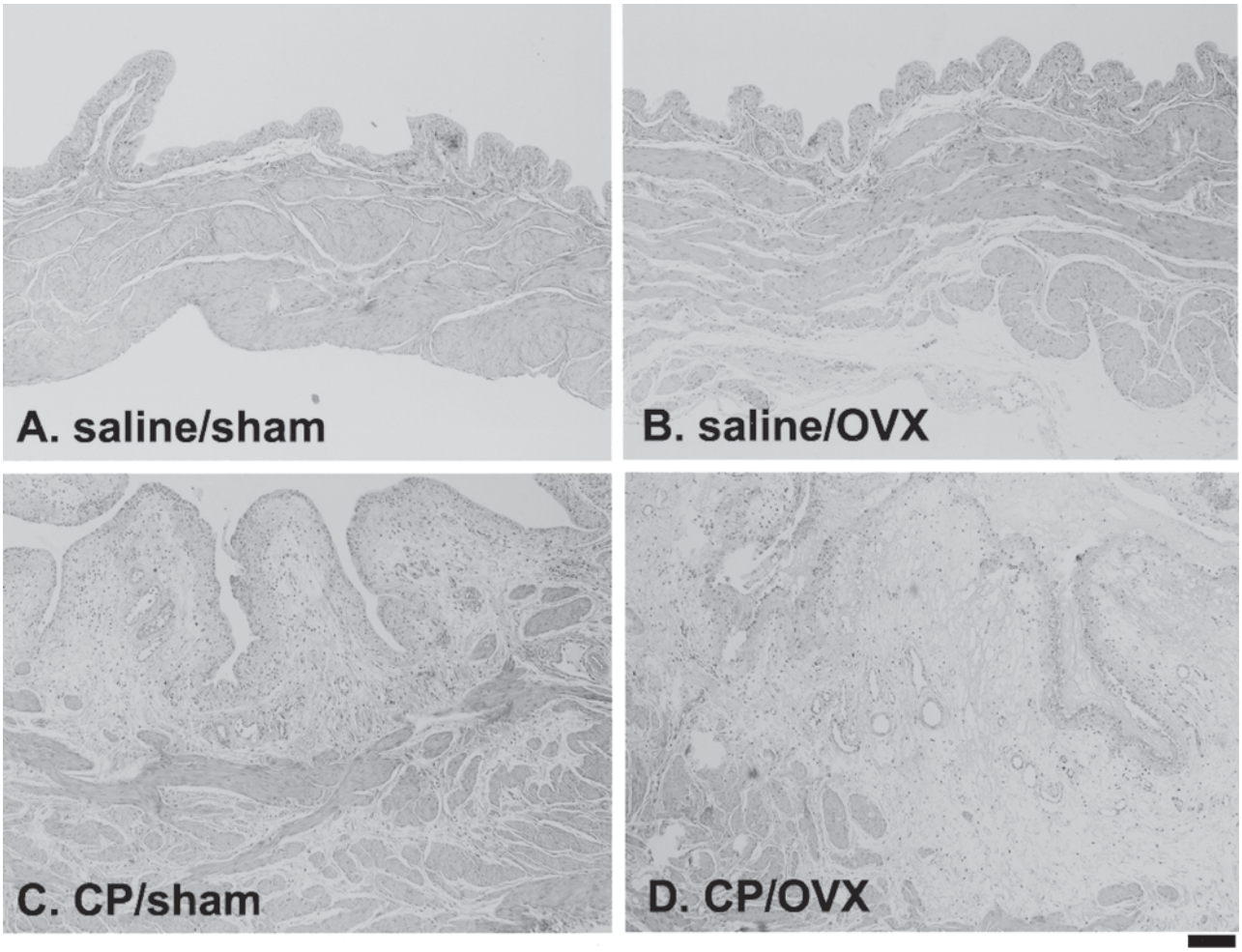

Fig. 1. Representative photomicrographs showing the histological changes of the bladder in the hematoxylin and eosin staining section at 1 wk after the initial saline (**A,B**) or cyclophosphamide (CP, 100 mg/kg twice with 4-d interval) administration (**C,D**). **A** and **C** are sections from sham-operated rats. **B** and **D** are sections from ovariectomzed rats. Scale bars represent 100 μm.

study, therefore, we first examined the effects of ovariectomy and castration on the histological changes of the bladder using a CP-induced cystitis model. We further examined the role of estrogen and progesterone in the histological changes of CP-induced cystitis by replacement with estrogen only or estrogen plus progesterone in ovariectomized rats. Although the exact cause of CP-induced cystitis is not fully understood, it has been proposed that urothelial damage occurs through direct contact with acrolein, the hepatic aldehyde metabolite of CP, which causes edema, infiltration of polymorphonuclear leukocyte, ulceration, and hemorrhages. The CP-induced cystitis is reproducible and controllable, and no anesthesia and stimulating device are required.

Results

Effects of Ovariectomy on Histological Changes of CP-Induced Cystitis

In the saline-treated group, no apparent histological changes were observed in the bladder of both ovariectomized and sham-operated rats (Figs. 1A,B). There were no significant differences in the thickness of submucosal and mucosal layers between the both groups (Figs. 1A,B, 2). At 1 wk after the initial administration of CP, urothelial damage, edema, leukocyte infiltration, hemorrhage, fibrin deposition were observed in the bladder of both ovariectomized and sham-operated rats (Figs. 1C,D). The thickness of the submucosal layer in the bladder was significantly increased in the both groups due to submucosal edema compared to each saline-treated group (Figs. 1C,D, 2, $p < 0.01$). However, the thickness of the submucosal layer was significantly higher in CP-treated ovariectomized rats compared to sham-operated rats (Figs. 1C,D, 2, $p < 0.05$). These histological changes returned to normal levels by 2 wk after the initial CP administration and there were no significant differences in the thickness of submucosal and mucosal layers between saline and CP-treated groups regardless of surgery, indicating that CP-induced histological changes were transient (Fig. 2). There were also no significant differences in the thickness of the submucosal layer between ovariectomized and sham-

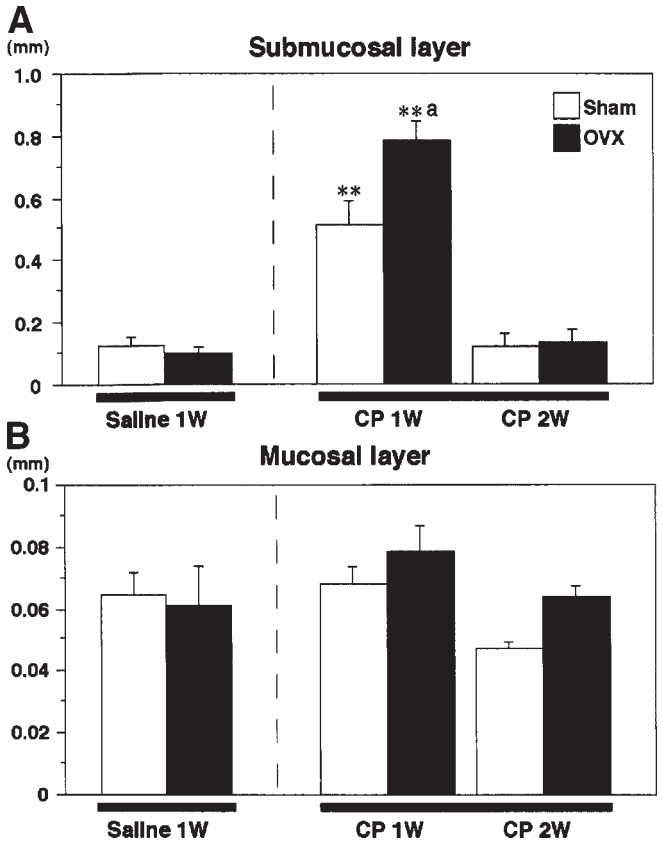

Fig. 2. Effects of ovariectomy on the thickness of submucosal (**A**) and mucosal (**B**) layers of the bladder in female rats following an intraperitoneal administration of saline or cyclophosphamide (CP, 100 mg/kg twice with 4-d interval). In saline-treated group, all animals were euthanized at 1 wk (Saline 1W) after the initial saline administration. In CP-treated group, the animals were euthanized at 1 (CP 1W) or 2 wk (CP 2W) after the initial administration of CP. Number of the rats used: sham-operated rats $n = 26$ (Saline 1W, $n = 8$; CP 1W, $n = 11$; CP 2W, $n = 7$), ovariectomized (OVX) rats $n = 31$ (Saline 1W, $n = 10$; CP 1W, $n = 14$; CP 2W, $n = 7$). Value represents the mean ± SEM. $^{**}p < 0.01$ vs saline-treated group, $^{a}p < 0.01$ vs sham-operated group.

operated groups at 2 wk after the initial CP administration (Fig. 2).

Although CP administration caused reactive hyperplasia in the mucosal layer of the bladder as previously reported *(15,16)*, there were no overall significant differences in the thickness of mucosal layer in the bladder between saline- and CP-treated groups. At 2 wk after the initial CP administration, the mucosal layer tended to be thicker in ovariectomized rats than in sham-operated rats ($p = 0.07$) (Fig. 2).

The state of bladder inflammation using the four-grade scale revealed that there were significant differences in the scores between ovariectomized and sham-operated rats at 1 wk after the initial CP administration (Table 1). The score was significantly higher in ovariectomized rats than in sham-operated rats (Table 1, $p < 0.01$). At 2 wk after the initial CP administration, the scores in CP-treated groups returned to the levels in saline-treated group regardless of surgery (Table 1). There were no significant differences in the scores between ovariectomized and sham-operated rats at 2 wk after the initial CP administration (Table 1).

Table 1
Urinary Bladder State Following
a CyclophosphamideAdministration in Female Rats

	Severity index of cyctitis	
	1 wk after an initial injection	2 wk after an initial injection
Sham operation	2.2 ± 0.2	1.4 ± 0.2
Ovariectomy	3.1 ± 0.2**	1.3 ± 0.2

$^{**}p < 0.01$ vs sham operation.

Effects of Castration on Histological Changes of CP-Induced Cystitis

Like females, an administration of saline did not cause any significant histological changes in both castrated and sham-operated rats (Figs. 3A,B). There were no significant differences in the thickness of submucosal and mucosal layers between the groups (Figs. 3A,B, 4). CP administration resulted in similar histological changes as found in female rats (Figs. 3C,D). A significant increase in the thickness of submucosal layer was observed in the bladder of both castrated and sham-operated rats compared to each saline-treated group at 1 wk after the initial CP administration (Figs. 3C,D, 4, $p < 0.01$). In contrast to females, there were no significant differences in the thickness of the submucosal layer between castrated and sham-operated rats at either 1 or 2 wk after CP administration (Fig. 4). CP administration did not cause any statistically significant effects in the thickness of the mucosal layer regardless of castration or sham-operation at 1 or 2 wk after the CP administration (Figs. 3C,D, 4).

The state of bladder inflammation using the four-grade scale revealed that unlike ovariectomy, there were no significant differences in the scores between castrated and sham-operated rats at 1 and 2 wk after the initial CP administration (Table 2).

Effects of Estrogen Replacement Therapy Both With and Without Progesterone on the Histological Changes of CP-Induced Cystitis in Ovariectomized Rats

In the saline-treated group, there were no apparent histological changes and the thickness of submucosal and mucosal layers among oil-, 17β-estradiol 3-benzoate (EB)-, and EB plus progesterone-treated animals (Figs. 5A,B,C, 6). At 1 wk after the initial administration of CP, histological changes such as urothelial damage, edema, leukocyte infiltration, and hemorrhages were found in the bladder regard-

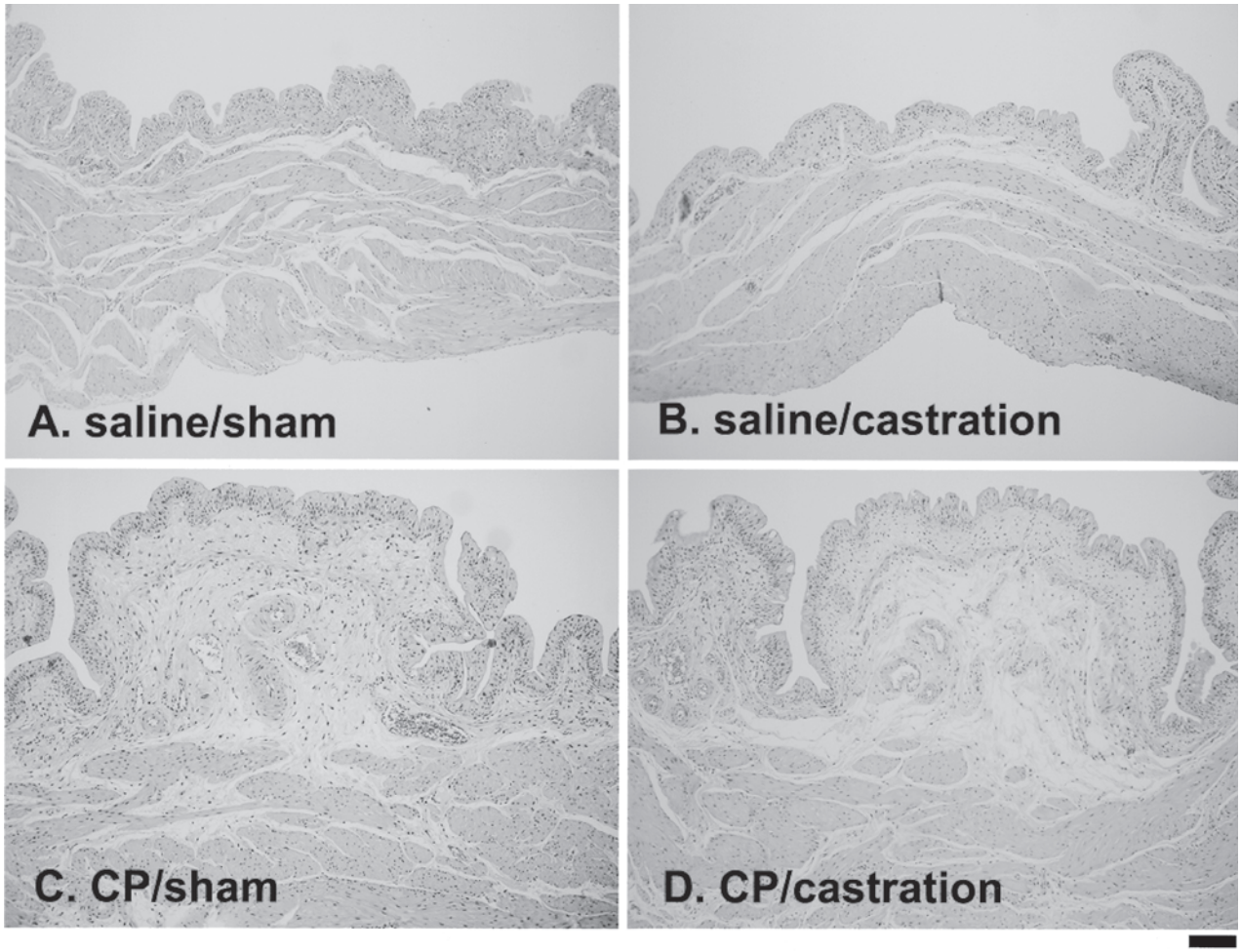

Fig. 3. Representative photomicrographs showing the histological changes of the bladder in the hematoxylin and eosin staining section at 1 wk after the initial saline (**A,B**) or cyclophosphamide (CP, 100 mg/kg twice with 4-d interval) administration (**C,D**). **A** and **C** are sections from sham-operated rats. **B** and **D** are sections from castrated rats. Scale bars represent 100 μm.

less of replacement therapy. However, the thickness of the submucosal layer in the bladder was significantly decreased in estrogen-treated group compared to oil-treated group (Figs. 5D,E,F, 6; $p < 0.01$). EB plus progesterone replacement did not provide any significant effects on the thickness of submucosal layer of CP-induced cystitis compared with estrogen-treated animals (Fig. 6). Although the mucosal layer in oil-treated group tended to be thicker than in EB- and EB plus progesterone-treated group following a CP administration, there were no significant overall differences among the groups ($p = 0.07$) (Fig. 6).

The state of the bladder inflammation using the four-grade scale revealed that the score was significantly lower in EB- and EB plus progesterone-treated rats than in oil-treated rats (Table 3, $p < 0.01$). There were no significant differences between the scores of estrogen- and estrogen plus progesterone-treated rats after the initial CP administration (Table 3).

Effects of Gonadectomy on the Histological Changes in the Mucosal Layer

In ovariectomized rats, histological changes such as chromatin condensation and cell shrinking were sparsely observed in the mucosal layer of the bladder. These histological changes were rarely observed in castrated male and sham-operated male and female rats (Fig. 7).

Expression of Gonadal Steroid Hormone Receptors and Gap Junction Proteins in the Bladder

Immunohistochemistry revealed that ERα- and ERβ-labeled cells were present mainly in the submucosal layer and sparsely in the mucosal layer. The expression level and pattern of ERα and ERβ were unchanged regardless of hormone condition. Few PR-labeled cells were observed in the bladder regardless of hormone condition.

The weak immunoreactivity (IR) for both connexin 43 and 26 were present in the mucosal layer, whereas connexin

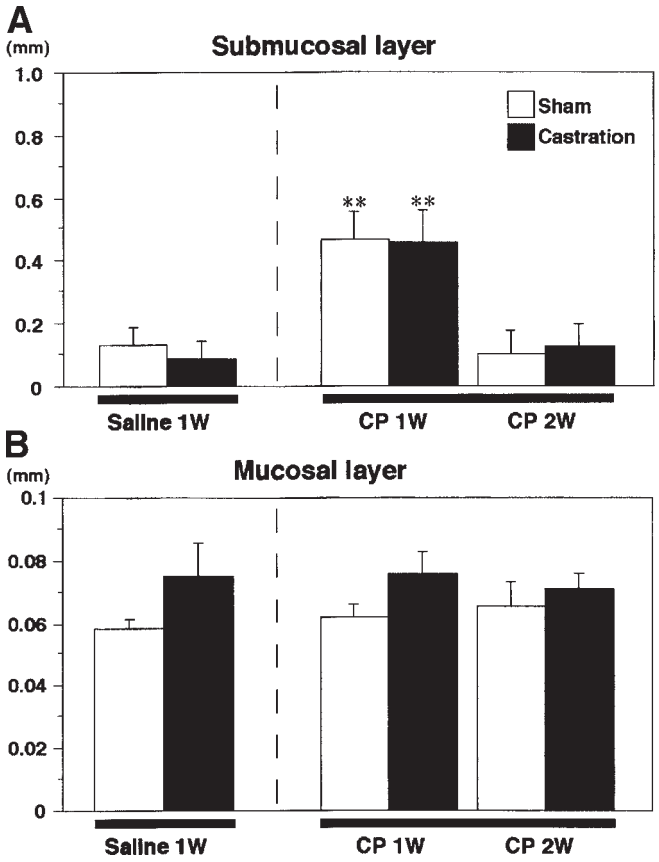

Fig. 4. Effects of castration on the thickness of submucosal (**A**) and mucosal (**B**) layers of the bladder in male rats following an intraperitoneal administration of saline or cyclophosphamide (CP, 100 mg/kg twice with 4-d interval). In saline-treated group, all animals were sacrificed at 1 wk (Saline 1W) after the initial saline administration. In CP-treated group, the animals were euthanized at 1 (CP 1W) or 2 wk (CP 2W) after the initial administration of CP. Number of the rats used: sham-operated rats $n = 20$ (Saline 1W, $n = 6$; CP 1W, $n = 8$; CP 2W, $n = 6$), castrated rats $n = 20$ (Saline 1W, $n = 6$; CP 1W, $n = 6$; CP 2W, $n = 8$). Value represents the mean ± SEM. $^{**}p < 0.01$ vs saline-treated group.

43-IR was expressed abundantly in the muscle layer. The levels of both connexin 43- and 26-IR were unchanged regardless of hormone condition.

Effect of Estrogen on the Permeability of the Bladder

There were no significant differences in the intensity of the Trypan blue staining in the bladder among oil-, EB-, and EB plus progesterone-treated ovariectomized rats.

Discussion

The present study showed that ovariectomy resulted in more severe CP-induced histological changes in the bladder with regard to submucosal edema, urothelial damages, and hemorrhage as compared to sham-operation. Furthermore, estrogen replacement ameliorated CP-induced inflammatory changes in the bladder compared with oil treatment,

Table 2
Urinary Bladder State Following
a Cyclophosphamide Administration in Male Rats

	Severity index of cyctitis	
	1 wk after an initial injection	2 wk after an initial injection
Sham operation	2.1 ± 0.2	1.2 ± 0.2
Castration	2.0 ± 0.1	1.3 ± 0.2

whereas estrogen plus progesterone replacement therapy did not provide any difference in the histology of CP-induced cystitis compared with estrogen treatment. These findings suggest that estrogen may be involved in the pathogenesis of bladder inflammation.

The estrogenic regulation of the CP-induced histological changes in the bladder may depend on the levels of serum estrogen. Previously, Bon et al. showed that the estrus cycle had little effects on the histological changes of CP-induced cystitis *(14)*. These findings suggest that the fluctuation of estrogen levels during the estrus cycle may fail to cause any effects on the histology in CP-induced bladder. In contrast, complete deprivation of ovarian steroid hormones for 3 wk caused significant histological changes in CP-induced cystitis of female rats and estrogen replacement for 3 wk ameliorated the histology in response to CP.

Effects of estrogen on the bladder histology may be obvious in the presence of active inflammation. Following saline administration, there were no significant differences in histology regardless of surgery and replacement. At 2 wk after the initial CP administration, CP-induced histological changes in the bladder returned to almost normal levels regardless of whether the animals had had an ovariectomy, castration, or sham-operation. There were also no significant differences in the bladder histology among oil-, estrogen-, and estrogen plus progesterone-treated animals following an ip administration of saline. These results suggest that estrogen has little effect on the bladder histology in the absence of inflammatory chemicals.

The precise mechanisms by which estrogen modulates the degree of bladder inflammation remain unclear, although estrogen has also been shown to play an anti-inflammatory role in several organs such as lungs, vascular smooth muscles, and joints *(1–3)*. The expression of ER-α and ER-β in the bladder, particularly in the mucosal layer as well as submucosal layer led us to the hypothesis that estrogen may play a role in maintaining the normal mucosal cells and that the deprivation of estrogen may cause altered permeability in the mucosal layers *(6,17–19)*. It is possible that apoptosis may affect the permeability of the mucosal layer. Recent studies have consistently shown that ovariectomy results in a significant increase in apoptosis of the bladder mucosal

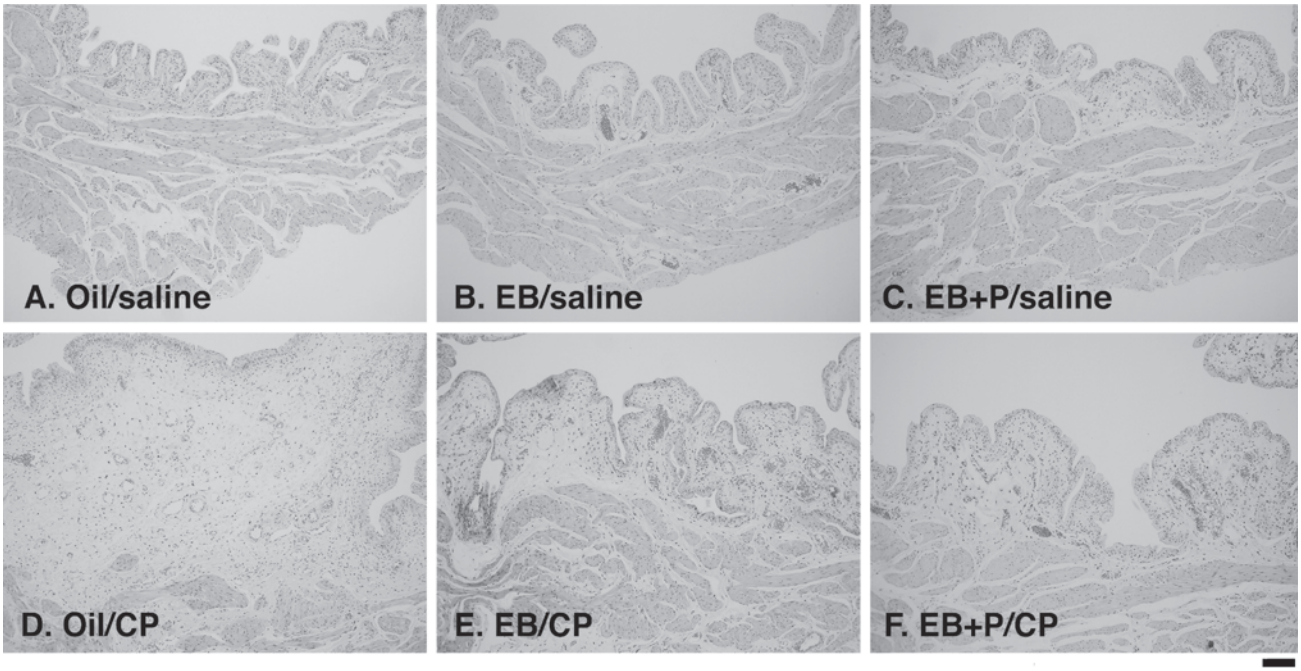

Fig. 5. Representative photomicrographs showing the effects of 17β-estradiol 3-benzoate (EB) alone or EB plus progesterone on the histological changes of the bladder of ovariectomized female rats in the hematoxylin and eosin staining section at 1 wk after the initial saline (**A,B,C**) or cyclophosphamide (CP, 100 mg/kg twice with 4-d interval) administration (**D,E,F**). **A** and **D** are sections from rats treated with oil. **B** and **E** are sections from the rats treated with EB (10 μg/body, daily) alone. **C** and **F** are sections from the rats treated with EB (10 μg/body, daily) plus progesterone (500 μg/body, every 4 d). Scale bars represent 100 μm.

cells in rabbits and the urethra in rats, and that estrogen treatment prevented these effects *(7,13)*. The present study also showed that histological changes such as cell shrinkage and chromatin condensation, which are often observed in apoptotic cells, were present sparsely in the oil-treated ovarietomized rats, whereas such changes were rarely found in castrated and sham-operated rats. It remains to be determined whether mucosal apoptotic changes observed after estradiol deprivation may affect mucosal permeability, because the present study using an intravesical administration of Trypan blue failed to reveal significant differences in mucosal permeability of the bladder among oil-, EB-, and EB plus progesterone-treated rats.

It is also possible that altered cell-to-cell adhesion of the mucosal cells may affect permeability of the mucosal layer. Gap junction proteins such as connexin-43 and -26, which enhance cell-to-cell adhesion, are expressed in the bladder *(20)*. A number of studies have revealed that estrogen stimulates the formation of gap junction and the expression of connexin-43, a predominant gap junction protein in the myometrium *(21)*. Immunocytochemical data revealed that connexin 43- and 26-IRs were very weak in the mucosal layer compared to muscle layer and the levels were unchanged regardless of hormone conditions. It is unlikely that estradiol may change the levels of connexins in the bladder in contrast to myometrium.

We also cannot exclude the possibility that estrogen altered metabolism of CP in the liver, which might affect inflammatory responses to CP since some studies have provided the evidence showing that revealed that estrogen induced the expression of hepatic cytochrome P450 2B mRNAs which is a responsible isoform for the metabolism of CP *(22,23)*.

Previously, Raz and Stamm have demonstrated in a double-blind, placebo-controlled clinical trial that intravaginal estriol cream prevents recurrent urinary tract infection (UTI) in postmenopausal women *(24)*. They have suggested that the effects of estriol on the prevention of recurrent UTI may be due to modifying vaginal flora *(26)*. Although it remains to be determined whether ovarian steroid hormones play an anti-inflammatory role in the development of bacterial cystitis, it is speculated that the estrogen replacement–induced prevention of UTI in postmenopausal women might be partly due to anti-inflammatory effects of estrogen in the bladder.

In contrast to estrogen, no apparent effects of progesterone on inflammatory changes were observed in the bladder because there were no differences in the CP-induced histology between estrogen- and estrogen plus progesterone-treated groups. In our preliminary study, we also showed no significant differences in the histological changes of CP-induced bladder between oil- and progesterone-treated animals and few PR-expressing cells in the bladder. It is well established that in the central nervous system, progesterone plays a facilitatory role in estrogen-induced female sexual behavior. However, the role of progesterone in bladder inflammation as well as function is obscure.

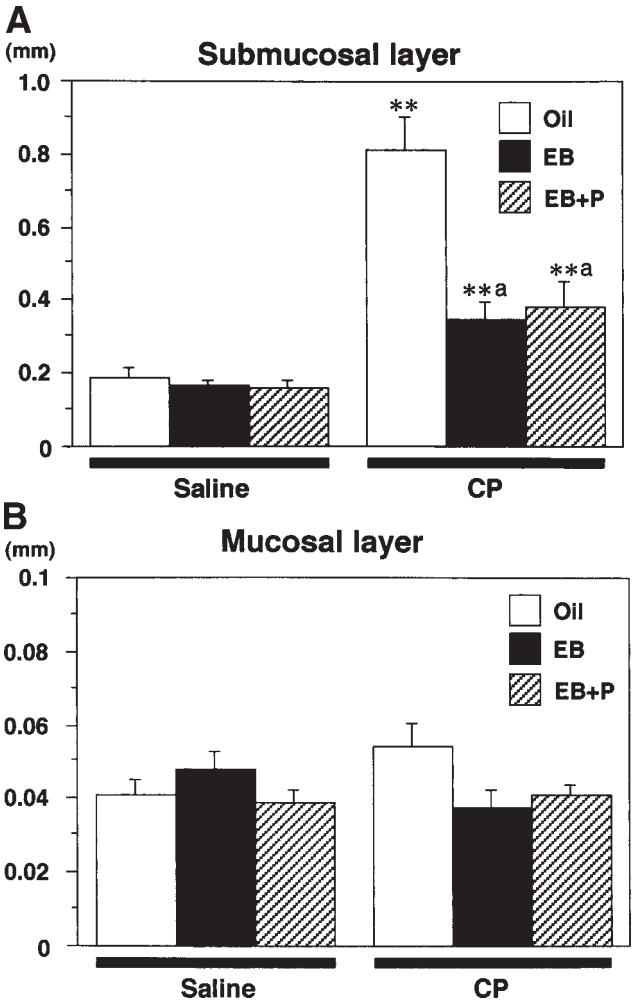

Fig. 6. Effects of 17β-estradiol 3-benzoate (EB) alone or EB plus progesterone (P) on the thickness of submucosal (**A**) and mucosal (**B**) layers of the bladder in ovariectomized female rats following an intraperitoneal administration of saline or cyclophosphamide (CP, 100 mg/kg twice with 4-d interval). All animals were euthanized at 1 wk after the initial saline or CP administration. Number of the rats used: saline-injected rats $n = 24$ (oil, $n = 7$; EB, $n = 9$; EB plus P, $n = 8$), CP-injected rats $n = 28$ (oil, $n = 10$; EB, $n = 8$; EB plus P, $n = 10$). Value represents the mean ± SEM. $^{**}p < 0.01$ vs saline-treated group, $^{a}p < 0.01$ vs oil-treated group.

Castration did not cause any significant effects on the histological changes in CP-induced cystitis compared to sham-operation. This result suggests that testosterone may not be involved in the pathogenesis of bladder inflammation. Some anatomical studies have shown that AR is present in the bladder and pelvic autonomic ganglion cells *(25)*. Keast and Saunders have revealed that castration results in a significant decrease in the size of noradrenergic pelvic neurons that supply the urinary bladder in male rats suggesting that testosterone may have effects on the function of bladder *(26)*. An in vitro study has revealed that testosterone and dihydrotestosterone inhibit interleukin-6 production by human gingival fibroblasts *(27)*. However, the roles of AR in the regulation of bladder function and the pathogenesis of bladder inflammation are largely unknown. Taken together, our findings raise a possibility that circulating levels of estradiol may contribute to the gender differences in the histological changes in CP-induced bladder.

Table 3
Urinary Bladder State Following a Cyclophosphamide Administration in Ovariectomized Female Rats

Treatments	Severity index of cyctitis
Oil	3.8 ± 0.4
Estradiol bezoate	2.2 ± 0.2**
Estradiol bezoate + progesterone	1.9 ± 0.2**

$^{**}p < 0.01$ vs oil.

In conclusion, the present study showed that ovariectomy caused more severe inflammatory changes in CP-induced cystitis and estrogen treatment ameliorated these histological changes. These results suggest that estrogen may play an anti-inflammatory role in CP-induced cystitis.

Materials and Methods

Animals

Adult male and female Sprague–Dawley rats weighing 240–260 g were used. They were group-housed (3–4 rats/cage) in plastic cages. A 12/12h light/dark cycle (light on at 7:00) and a constant temperature (22°C) were maintained throughout the studies. Food and water were available *ad libitum*. The ethics committee of animal care and experimentation of our university approved all procedures.

Experimental Procedure

We performed histological examination of CP-induced bladder in (i) gonadectomy (ovariectomy or castration) vs sham-operation (gonadally intact) and (ii) hormone replacement (oil-, estradiol-, or estradiol plus progesterone treated ovariectomized female rats). In the first experiment, 97 female and male rats were deeply anesthetized with pentobarbital sodium (50 mg/kg, ip) and were ovariectomized ($n = 31$), castrated ($n = 20$), or were given sham-operations (female $n = 26$, male $n = 20$). Two weeks after the surgery, a solution of 0.9% NaCl [0.4 mL/100 g body weight (bw)] containing CP (100 mg/kg bw) was administered intraperitoneally to rats twice with a 4-d interval. The same volume of 0.9% NaCl was administered ip into saline-treated control rats. The animals were decapitated and the bladders were then removed at 1 or 2 wk after the initial administration of CP or saline. At the time of euthanasia, the animals were allowed to urinate so that their bladders were empty at removal. Because we confirmed in the preliminary study that there were no remarkable histological changes of the bladder at both 1 or 2 wk after saline administration, the bladders were removed only at one week after the initial saline administration in saline-treated control group.

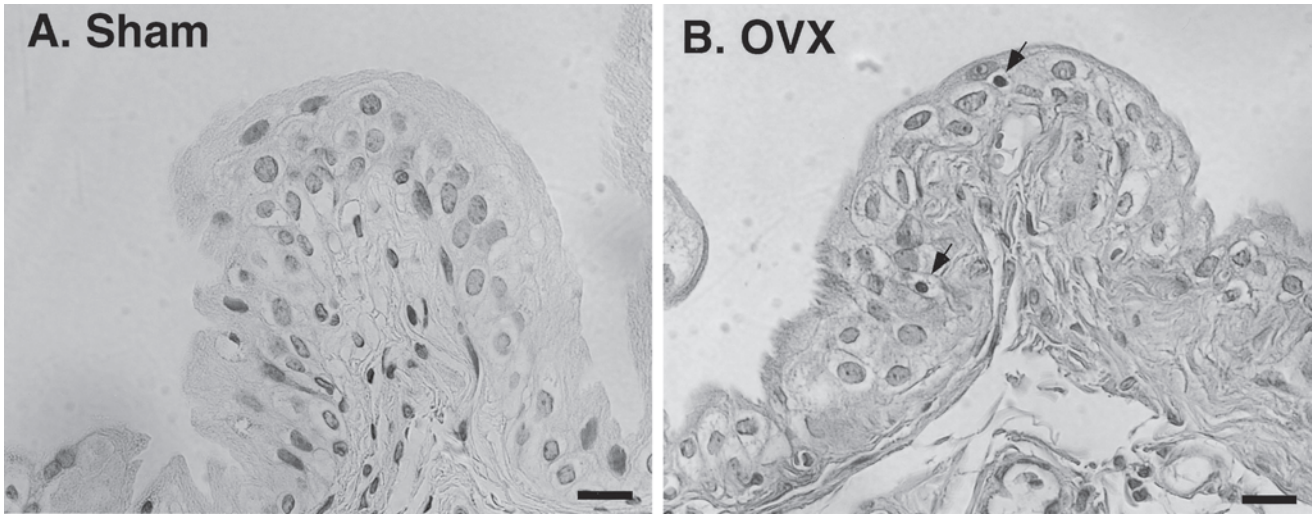

Fig. 7. Effect of ovariectomy on histological changes in the mucosal layer. Arrows in ovariectomized rats indicate chromatin condensation and cell shrinking. The scale bars represent 10 μm.

In the second experiments, 52 female rats were deeply anesthetized with pentobarbital sodium (50 mg/kg, ip) and were ovariectomized. Two weeks after the surgery, oil (n = 17), 17β-estradiol 3-benzoate alone (EB; 10 μg/body daily, n = 17), or EB (10 μg/body daily) plus progesterone (500 μg/body every 4 d) (n = 18) was administered subcutaneously to rats for 21 d until the day of euthanasia. Fourteen days after the hormone replacement, a solution of 0.9% NaCl (0.4 mL/100 g bw) containing CP (100 mg/kg bw) was administered ip to rats twice with a 4-d interval. The same volume of 0.9% NaCl was administered intraperitoneally into saline-treated control rats. The animals were decapitated and the bladders were then removed at 1 or 2 wk after the initial administration of CP or saline.

Histological Study

The bladders were fixed in 4% formaldehyde for about 12 h, processed, and embedded in paraffin blocks. Sections of 5 μm thickness were cut transversally from each block with a microtome, mounted on slides, deparaffinized in xylene, and dehydrated with graded ethanol. The bladder sections were created at the maximum diameter to minimize region-specific differences in the CP-induced histological changes. The sections were then stained with hematoxylin and eosin (H&E).

Immunocytochemistry was performed using universal Vecstain elite ABC kit (Vector Laboratories, Burlingame, CA, USA). The primary antibodies used were ER-α (1:1000, C1355, Upstate, NY, USA) , ER-β (1:5000, Z8P, Zymed Laboratories Inc., CA, USA), and PR (1:1000, A0098, Dako-Cytomation, CA, USA).

Histological Evaluation of CP-Induced Cystitis

The slides were scanned using an Olympus Digital Camera (Olympus, Tokyo, Japan). Image analysis was performed using a MCID Imaging Analysis System (Imaging Research Inc., Ontario, Canada). The bladder was divided equally into 12 sections. The maximum thickness of the submucosal and the mucosal layers within each section was measured at 12 points. The thickness of the submucosal and the mucosal layers was averaged at these points and calculated in each rat.

Another histological examination of the bladder was used to estimate the severity of the inflammation. As described in the previous study *(14)*, the state of bladder inflammation was assessed by an expert pathologist blind to the experimental condition of the rats, using a four-grade scale. Grade 1 was the control with no bladder lesion. Grade 2 consisted of simple edema without infiltration of polymorphonuclear leukocyte (presence of excessive submucosal fluid due to exudation of plasma, corresponding to early features of the inflammatory response as a sign of mild cystitis). Grade 3 consisted of edema associated with epithelial cleavage and thinning, resulting in mucosal abrasion, fibrin deposit, and the beginning of polymorphonuclear leukocyte infiltration as signs of intermediate cystitis. Grade 4 consisted of increased severity and spread of all of the above signs plus petechial hemorrhaging as a sign of severe cystitis. The pathologist diagnosed the grade of cystitis (grade 1, 2, 3, or 4) in each rat. The results in tables were expressed as mean numbers of the grade (± SEM) in each group. The pathologist also examined the apoptotic changes such as cell shrinking and chromatic condensation in ovariectomized, castrated, and sham-operated rats treated with saline.

Evaluation of Permeability in the Bladder

Ovariectomized female rats treated with oil, EB (n = 5; 10 μg/body, daily) or EB (10 μg/body, daily) plus progesterone (500 μg/body, every 4 d) for 2 wk were used in this experiment (n = 5 in each group). Under anesthesia with

pentobarbital sodium (50 mg/kg, ip), 0.5 mL of 2% solution of Trypan blue in 0.9% NaCl solution was instilled and left in the rat bladder for up to 1 h. After 1 h the dye was removed and the bladder washed with four 0.5 mL saline washes to remove all an absorbed dye. Full-thickness samples were taken from bladder. Specimens were fixed in buffered 10% formalin. The intensity of the staining in the bladder was compared among groups as describe previously *(28)*.

Statistics

All data are presented as mean ± SEM. In the first experiment, we initially compared the thickness of submucosal and mucosal layers between saline 1 wk and CP 1 wk using a two-way factorial analysis of variance (ANOVA) for the main effects of ip administration (saline vs CP) and surgery (sham-operation vs ovariectomy or castration). We then compared the thickness of submucosal and mucosal layers between CP 1 wk and CP 2 wk using a two-way factorial ANOVA for the main effects of time (1 wk vs 2 wk) and surgery (sham-operation vs ovariectomy or castration). In the second experiment, we used a two-way factorial ANOVA for the main effect of ip administration (saline vs CP) and hormone replacement (oil, estradiol, or estradiol plus progesterone). The statistical analysis was followed by the Bonferroni post-hoc test. The severity of cystitis was analysed statistically using the Wilcoxon's rank sum test. A $p < 0.05$ was considered statistically significant.

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