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Effects of Estrogen Replacement Therapy on the Expression of Apoptosis-Related Genes in Old Female Rats

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Effects of estradiol (E_2) on the urinary bladder of old female rats were investigated by morphological, immunohistochemical and apoptosis-related gene analysis. Sixteen-month-old female Wistar rats were divided into the following 5 groups: normal controls; rats replaced with low-dose E_2 for 2 and 4 weeks, respectively; and rats replaced with high-dose E_2 for 2 and 4 weeks, respectively. After treatment, the bladders were removed, weighed and stained with hematoxylin and eosin, and immunostained by monoclonal antibodies against Bcl-2 and Bax proteins. Further, the expression of apoptosis-related genes *bcl*-2 and *bax* were investigated by PCR analysis, and evaluated by computerized quantification. Immunostained bladders showed no difference between control and E_2-replaced rats in reactivity against Bcl-2 protein. In contrast, against Bax protein, control rats showed immunoreactivity, whereas E_2-replaced rats showed little reactivity. Upon mRNA analysis, the expression of *bax* mRNA was suppressed in the E_2-replaced rats as compared with the controls, though the expression of *bcl*-2 mRNA showed no difference among groups. These results suggest that E_2 administration affects the expression of apoptosis-related genes and suppressees apoptotic action, and that these changes could consequently alter voiding behavior.

Key words: apoptosis; bladder; estrogen; rat

The decrease in serum level of estrogen that occurs in menopause causes metabolic and atrophic alterations in many organs. Postmenopausal urogenital atrophy causes pollakiuria, urinary urgency, stress and urge incontinence, dysuria and so on (Versi, 1990). The symptoms show increasing incidence with age (Eika et al., 1990).

Previous investigations have demonstrated the presence of estrogen receptors in the human lower urinary tract. This supports the practice of giving estrogen treatment to postmenopausal women with lower urinary tract dysfunction. Estrogen has been found to improve lower urinary tract function in postmenopausal women (Batra et al., 1987; Iosif et al., 1981).

A decrease in serum estrogen results in an increase of collagen in the bladder. Large interspaces between the muscle cells of the bladder and smooth muscles with hypertrophy were observed in old female rats. The interspaces were occupied by collagen fibrils. The collagen content of the detrusor muscle, which could be affected by estrogen concentration in the serum, has been found to be significantly increased in women after the age of 50 years as compared to younger women and to men of the same age (Eika et al., 1990). Increased collagen deposits between the smooth muscle cells of the detrusor and the urethra might affect the functional properties of the lower urinary tract, and estrogen is considered to influence the lower urinary tract.

On the other hand, several studies have indicated that estrogen regulates cell survival and death factors. Estrogen administration decreases apoptosis. In breast cancer cells

Abbreviations: E_2, estradiol; HE,hematoxylin and eosin; RT, reverse transcription

(Teixeria et al., 1995; Kandouz et al., 1996; Huang et al., 1997) and brain cells (Dubal et al., 1999), respectively, estrogen was shown to have an effect on mRNA expression of *bcl*-2, *bad* and *bax*, and, ultimately, to prevent the preapoptotic actions of the members of the *bcl*-2 family such as *bax* and *bad*.

In the present study which uses female rats after menopause, the effects of estrogen replacement therapy on their bladders were investigated, including the effects on the expressions of *bcl*-2 and *bax*.

Materials and Methods

Animals

All animal experiments were performed in accordance with the guidelines set out by the Tottori University Committee for Animal Experimentation. Sixteen-month-old female Wistar rats were divided into 5 groups. In group I, no hormonal treatment was carried out (control group; $n = 8$). In group II, a silastic tube containing estradiol (E_2) dissolved in sesame oil at 10 μg was subcutaneously placed for 2 weeks from the age of 16 months, without an ovariectomy (low-dose E_2 replaced for 2 weeks; $n = 8$). In group III, E_2 was administered for 4 weeks in the same manner as in group II (low-dose E_2 replaced for 4 weeks; $n = 8$). In group IV, E_2 was replaced in the form of silastic capsules containing 2.5 mg β-E_2 for 2 weeks (high-dose E_2 replaced for 2 weeks; $n = 8$), and in group V, the same capsules as in group IV were given for 4 weeks (high-dose E_2 replaced for 4 weeks; $n = 8$). Rats in the control group received placebos by the same methods as E_2-replaced rats. 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 E_2

Plasma E_2 concentrations were measured when the rats were 16 months old in the control group, and in E_2-replaced groups, 2 weeks (groups II and V) or 4 weeks (groups III and V) after hormone replacement.

Histological examination of the rat bladder and reverse transcription-PCR analysis

Animals at the age of 16 months or after replacement therapy were all anesthetized by an intraperitoneal injection of sodium pentobarbital and then weighed. The bladder was removed and weighed after emptying the urine. Four bladders per group were placed in 10% formalin and embedded in paraffin. Each paraffin block was serially sectioned and routinely stained with hematoxylin and eosin (HE). The serial sections were also immunohistochemically stained by the monoclonal antibody against Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) or Bax (Santa Cruz Biotechnology) in a buffer diluted 1:100 with phosphate-buffered saline. Diaminobenzidine tetrahydrochloride was used as the final chromogen.

After weighing, 4 bladders per group were frozen in liquid nitrogen. Total RNA was extracted from the dissected tissues using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions and then quantified spectrophotometrically. Reverse transcription (RT) was carried out with Moloney murine leukemia virus reverse transcriptase (Roche Molecular Systems, Branchburg, NJ), and PCR was performed using AmpliTag gold DNA polymerase (Roche Molecular Systems). For *bcl*-2 and *bax* cDNA amplification, the primers were 5'-CACCCCTGGCATCTTCTCCTT-3' (sense) and 5'-AGCGTCTTCAGAGACAGCCAG-3' (antisense) for *bcl*-2 (Genbank accession number U34964, 519-bp DNA fragment), and 5'-CACCAGCTCTGAACAGATCATGA-3' (sense) and 5'-TCAGCCCATCTTCTTCCAGATGGT-3' (antisense) for *bax* (Genbank accession number RRU 49729, 540-bp DNA fragment) according to the method of Ananth et al. (2001). The PCR cycles consisted of denaturation at 94˚C for 30 s, annealing at 68˚C for 30 s and extension at 72˚C for 1 min for 25 cycles (*bcl*-2); and denaturation at 94˚C

Table 1. Body weight, bladder weight and serum E_2 levels

Group	Body weight (g)	Bladder weight (mg)	Serum E_2 levels (pg/mL)
I: control	353.5 ± 37.0	110.7 ± 10.4	7.3 ± 2.5
II: low-dose E_2 replaced for 2 wk	358.7 ± 28.0	116.1 ± 10.6	25.0 ± 4.8
III: low-dose E_2 replaced for 4 wk	339.0 ± 24.3	136.5 ± 24.8	29.4 ± 10.7
IV: high-dose E_2 replaced for 2 wk	332.2 ± 34.4	124.2 ± 9.3	258.1 ± 55.0
V: high-dose E_2 replaced for 4 wk	340.0 ± 23.2	128.3 ± 10.5	213.6 ± 67.3

E_2, estradiol; wk, weeks.
* Significant difference; $P < 0.05$.
** Significant difference; $P < 0.01$.

for 30 s, annealing 62˚C for 30 s and extension at 72˚C for 1 min for 25 cycles (*bax*). As an internal control, a 285-bp DNA fragment of rat β-actin was also amplified using the following primers: 5'-TCATGAAGTGTGACGTTG ACATCCGT-3' (sense) and 5'-CCTAGAA GCATTTGCGGTGCAGGATG-3' (antisense). The temperature cycling conditions were as follows: 10 min at 94˚C 18 cycles of (94˚C for 30 s, 65˚C for 30 s, 72˚C for 30 s) and a final extension at 72˚C for 30 s.

In this experiment, 5 μg of total RNA was reverse-transcribed in a 15-μL reaction volume. An 8-μL aliquot of each PCR product was size-separated by electrophoresis on a 2% cyber green-containing agarose gel and photographed. For quantification, photographs showing PCR products were scanned and analyzed using Densitograph version 4.0 software (ATTO Corp., Tokyo, Japan). For each sample, the PCR product values were normalized to β-actin PCR product values. Data were analyzed by Student's *t*-test.

Results

Plasma E_2 levels

Plasma E_2 levels ranged from 15 to 45 pg/mL in low-dose E_2-replaced groups (groups II and III), from 100 to 330 pg/mL in high-dose E_2-replaced groups (groups IV and V) and less than 10 pg/mL in the control group.

Serum E_2 levels in the replaced groups were significantly higher than those in the control group. Compared in terms of the E_2 dose, serum E_2 levels were significantly higher in the high-dose groups (groups IV and V) than in the low-dose groups (groups II and III) (Table 1).

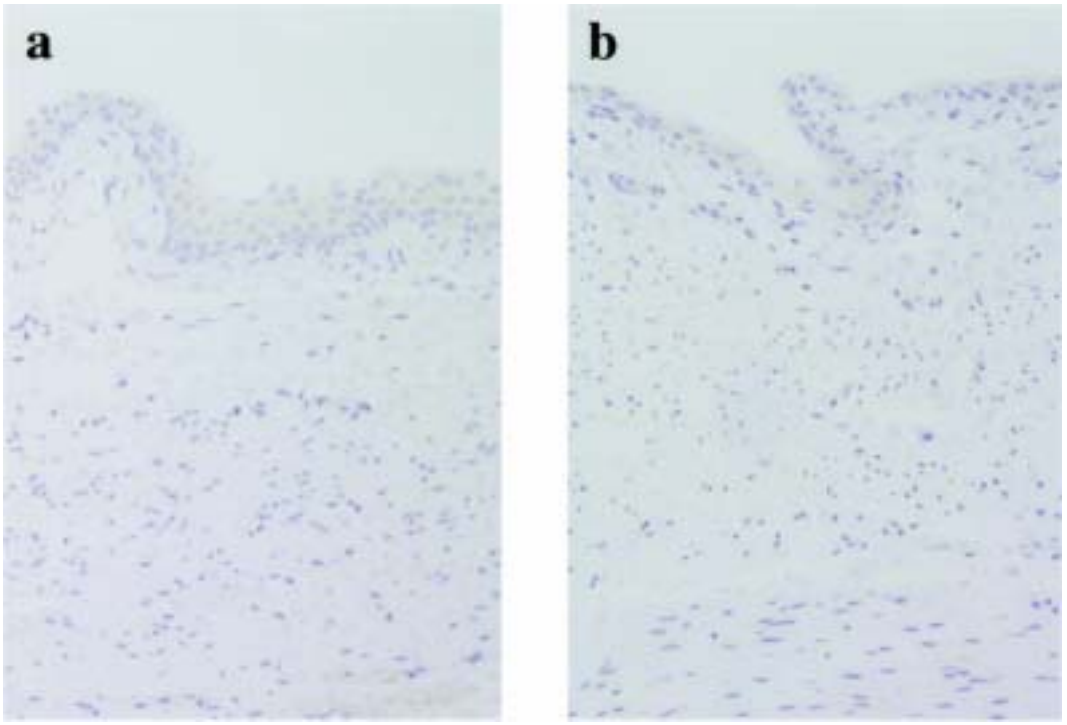
Body and bladder weight

There were no significant differences among groups in body weight. However, E_2-replaced groups showed a lower tendency in mean body weight than the control group: body weight tended to be higher as the E_2 dose was increased.

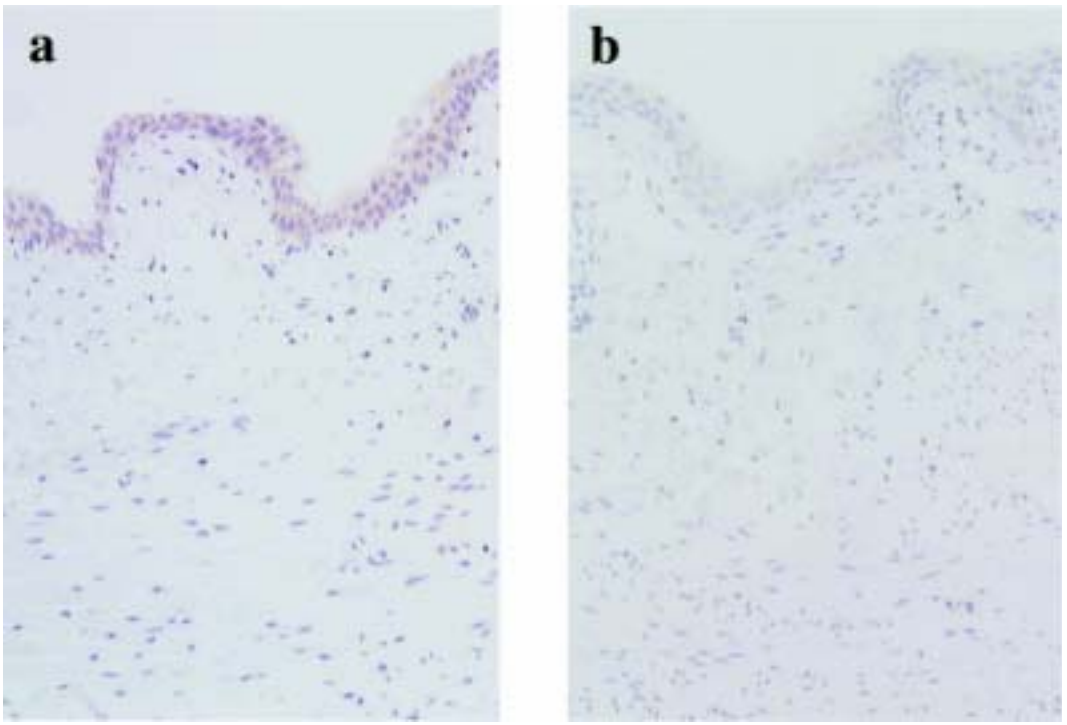
On the other hand, bladder weight was significantly heavier in the E_2-replaced groups than in the control group, except for group II replaced with low-dose E_2 for 2 weeks. The mean bladder weight showed no significant differences among the low- and high-dose replaced groups (Table 1).

Histological changes in the rat bladders

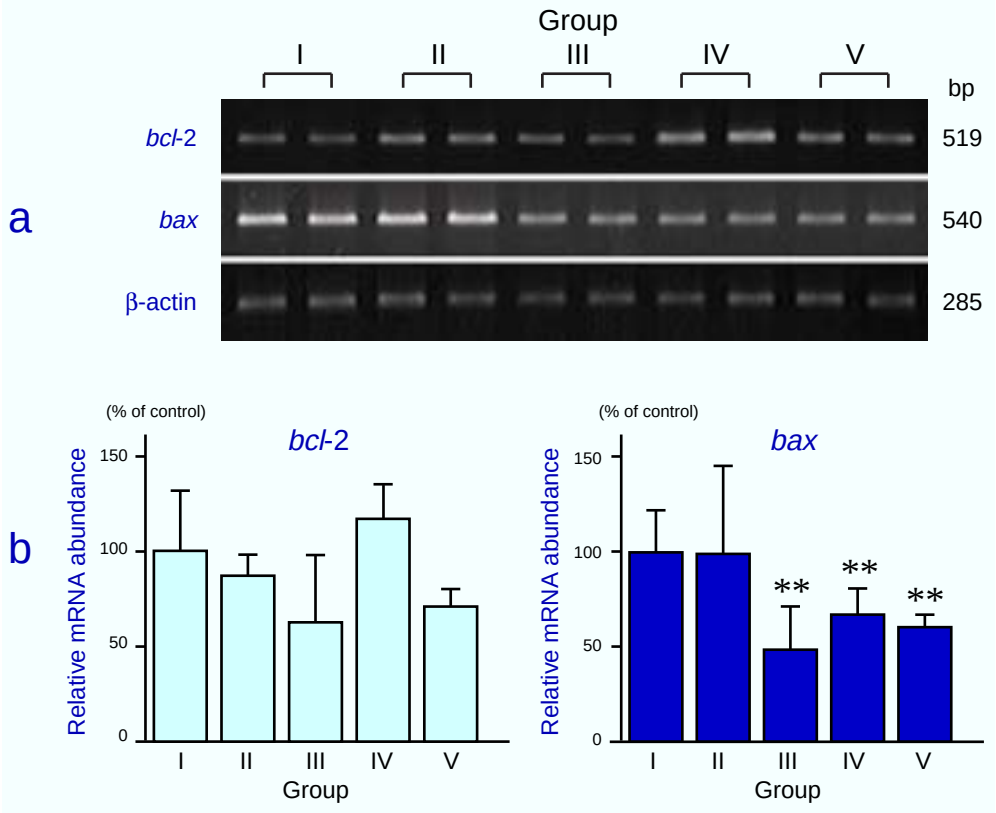
There were no significant differences between control and E_2-replaced rats in the HE stains of the bladder.



Figs. 1ab. Immunohistochemical stains of rat bladders by an antibody against Bcl-2 protein. **a:** A control rat (× 100). **b:** A group-V rat replaced with high-dose E_2 for 4 weeks (× 100).



Figs. 2ab. Immunohistochemical stains of rat bladders by an antibody against Bax protein. **a:** A control rat (× 100). **b:** A group-V rat replaced with high-dose E_2 for 4 weeks (× 100).



Figs. 3ab.

a: Semiquantitative RT-PCR analysis of mRNA expression of *bcl*-2, *bax* and β-actin in rat bladders. RT, reverse transcription.

b: Relative densities of *bcl*-2 and *bax* mRNA signals. The signals are normalized with corresponding β-actin signals. Each bar represents the mean ± SEM. Statistical significances were examined in comparison to the control level expressed as 100%: **Significant difference; $P < 0.01$. Group I, control; group II, replaced with low-dose E_2 for 2 weeks; group III, replaced with low-dose E_2 for 4 weeks; group IV, replaced with high-dose E_2 for 2 weeks; group V, replaced with high-dose E_2 for 4 weeks; E_2, estratiol.

Figures 1a and b show representative light microscopic photographs of the bladders immunostained by the antibody against Bcl-2: Fig. 1a for a control rat and Fig. 1b for a group-V rat replaced with high-dose E_2 for 4 weeks. Both stains of the bladders demonstrate low immunoreactivity against Bcl-2, not different in appearance.

Figures 2a and b show bladders similarly stained by the antibody against Bax in a control rat and a group-V rat. Bax was detected in the mucosal region of the bladder of the control rat (Fig. 2a), but not in the bladder of the E_2-replaced rat (Fig. 2b).

RT-PCR analysis of expression of bcl-2 and bax mRNA

The expressions of *bcl*-2 mRNA were not significantly different among groups. In contrast, the expressions of *bax* mRNA were significantly decreased in groups III to V as compared with the control group. In group II, the expression of *bax* mRNA was almost the same level as that in the control group (Fig. 3).

Discussion

In the present study, plasma E_2 levels were lower than 10 pg/mL in the control group. It is well known that rats have a fertility period that lasts until the age of approximately 15 months, at which time the estrous cycle ceases. The values of plasma E_2 in the control group reflected this time frame, and the estrous cycle disappeared in control rats. In the E_2-replaced groups, the mean plasma E_2 levels reached over 20 pg/mL in low-dose groups and 200 pg/mL in high-dose groups. In high-dose groups, the plasma E_2 level was quite over the peak level of E2 throughout the usual estrous cycle (Handa et al., 1987; Shulman et al., 1987; Albert et al., 1991). Albert et al. (1991) found that a plasma E_2 level of 15 pg/mL was slightly below the mean level of E_2 present throughout the estrous cycle in rats maintaining normal body weight. Therefore, the method of E_2 replacement in this experiment is adequate in studying the effect of E_2 on the rat bladder.

Previous studies demonstrated that ovariectomized rats showed an increase in body weight and a decrease in bladder weight as compared with control rats (Lonhurst et al., 1992). In the present study, no significant difference in mean body weight was observed among groups; however, there was a linear correlation between the E_2 dose and rat body weight. When the E_2 dose was increased, body weight tended to be higher. Further, mean bladder weight was significantly lower in control rats than E_2-replaced rats, except for those receiving low-dose E_2 for 2 weeks (group II). These results suggest that E_2 replacement after menopause increases bladder weight while decreasing body weight in rats. The explanation for these changes is not clear, but it is suspected that the lack of E_2 causes a reduction in physical activity and E_2 replacement abolishes this reduction.

On the other hand, in E_2-replaced rats, the bladder might gain weight as the collagen content of the bladder increases and the bladder epithelium thickens (Suguita et al., 2000; Eika et al., 1990). Suguita et al. (2000) performed E_2 replacement in female rats: they compared the obtained results with the parameters in the progesterone-replaced group, and found an increase in thickness of the mucosa of the bladder and the proximal urethra. The present study, however, showed no significant difference in the HE stains of bladders between control and E_2-replaced rats. It might have been difficult to detect differences in the bladder mucosa or collagen content by HE staining due to the fact that the bladder weight increased 10% to 20%.

In the present histological study, immunoreactivity with the Bcl-2 antibody was weak in the bladder of control and E_2-replaced rats. However, the immunoreactivity with the Bax antibody was stronger in the control rats than in E_2-replaced rats.

RT-PCR analysis for *bcl*-2 showed no significant difference among the groups. Upon *bax* analysis, however, the expression was stronger in the control group than in the E_2-replaced groups, except for group II (low-dose E_2 replaced for 2 weeks). Considering the histological results, it seems that E_2 replacement suppresses the *bax* gene expression in the mucosa of the rat bladder. In group II replaced with low-dose E_2 for 2 weeks, the E_2 dose might have been too low or the administration period might have been too short to cause a difference in gene expression.

It is clear from previous studies that the *bcl*-2 gene prolongs cell survival without affecting proliferation and that the *bcl*-2 gene is able to prevent apoptosis induced by growth factor deprivation. The mechanism by which *bcl*-2 exerts its antiapoptotic effect is not fully clear, although it has been speculated that *bcl*-2 acts either as a regulator of an antioxidant pathway that prevents oxidative damage such as lipid peroxidation caused by the generation of free radicals (Tsujimoto et al., 1986; Hockenbery et al., 1993) or as a regulator of intracellular Ca^{2+} compartmentalization (Kane et al., 1993). *bcl*-2 is a member of a large family of proteins, called the *bcl*-2 family, which is involved in the regulation of apoptosis. Among its members, *bax* has a unique effect and plays an important role. It is able to heterodimerize with *bcl*-2 and alter the latter's antiapoptotic action (Garcia et al., 1992). *bcl*-2 blocks the preapoptotic actions of

other members of the *bcl*-2 family such as *bax* and *bad* (Dubal et al., 1999). *bcl*-2 and other members of the *bcl*-2 gene family have been identified, in addition to being regulators of cell death, as estrogen-responsive genes (Teixeira et al., 1995; Kandouz et al., 1996). E_2 may directly upregulate the survival factor through receptor-mediated interactions with regions of the *bcl*-2 promoter or by indirect pathways (Teixeira et al., 1995). The antiapoptotic activity of *bcl*-2 correlates with its intracellular ratio to another protein called Bax. High levels of Bax have been shown to favor apoptosis in cells subjected to growth factor deprivation, whereas high levels of Bcl-2 prolong cell survival under the same conditions (Bafy et al., 1993). Teixeria et al. (1995) suggested that in the absence of E_2, the *bax*/*bcl*-2 ratio was increased and the cells with estrogen receptors underwent apoptosis.

In the present study, it was suggested that E_2-replacement therapy might prevent apoptosis mainly in the bladder mucosa through its effect on the *bcl*-2 family in the bladder of old female rats. Although the reason why the activity was demonstrated chiefly in the mucosa is not clear, one explanation might be the regulation of the apoptotic reaction in the sensory receptors or nerve fibers in the bladder. In fact, recent studies have allowed conjecture that the sensory nerve receptors against capsaicin (Lecci et al., 1998; Shea et al., 1999), bradykinin (Wotherspoon and Winter, 2000) and ATP (Cockayne et al., 2000; Vlaskovska et al., 2001) exist in the epithelial cells, and that the activation of the sensory nerves is related to urinary incontinence, voiding and the sensations of bladder fullness, discomfort and pain.

In this study, findings demonstrated that estrogen administration to menopausal rats suppresses the expression of apoptosis-related genes; *bax* mRNA was suppressed in the bladder of old female rats by estrogen administration, especially in the bladder mucosa, while the addition of estrogen prevented a decrease in contractile response induced by the absence of estrogen (Longhurst et al., 1992; Diep and Conatantinou, 1999). Sensory nerve receptors exist in the mucosa, and it might follow that suppression of *bax* mRNA in the mucosa would influence voiding behavior.

References

1 Albert DJ, Jonik RH, Gorzalka BB, Newlove T, Webb B, Walsh ML. Serum estradiol concentration required to maintain body weight, attractivity, proceptivity, and receptivity in the ovariectomized female rat. Physiol Behav 1991;49:225–231.

2 Ananth C, Thameem DS, Gopalakrishnakone P, Kaur C. Domoic acid-induced neuronal damage in the rat hippocampus: change in apoptosis-related genes (Bcl-2, Bax, Caspase-3) and microglial response. J Neurosci Res 2001;66:177–190.

3 Batra SC, Iosif CS. Progesterone receptors in the female lower urinary tract. J Urol 1987;138: 1301–1304.

4 Bafy G, Miyashita T, Williamson JR, Reed JC. Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3-dependent hematopoetic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncoprotein production. J Biol Chem 1993;268: 6511–6519.

5 Cockayne AD, Hamilton GS, Zhu MQ, Dunn MP, Zhong Y, Novakovic S, et al. Urinary bladder hyporeflexia and reduced pain-related behavior in P2X3-deficient mice. Nature 2000;407: 1011–1015.

6 Diep N, Conatantinou EC. Age dependent response to exogenous estrogen on micturition, contractility and cholinergic receptors of the rat bladder. Life Sci 1999;64:279–289.

7 Dubal BD, Shughrue JP, Wilson EM, Merchenthaler L, Wise MP. Estradiol modulates bcl-2 in cerebral ischemia: a potential role for estrogen receptors. J Neurosci 1999;19:6385–6393.

8 Eika B, Salling LN, Christensen LL, Andersen A, Laurberg S, Danielsen CC. Long-term observation of the detrusor smooth muscle in rats. Urol Res 1990;18:439–442.

9 Garcia I, Martinou I, Tsujimoto Y, Martinou J-C. Prevention of programmed cell death of sympathetic neurons by the Bcl-2 proto-oncogene. Science 1992;258:302–304.

10 Handa RJ, Stadelman HL, Resko JA. Effect of estrogen on androgen receptor dynamic in female rat pituitary. Endocrinology 1987;121:84–89.

11 Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsmeyer SJ. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 1993; 75:241–251.

12 Huang Y, Ray S, Reed JC, Ibrado AM, Tang C, Nawabi A, et al. Estrogen increases intracellular p26Bcl-2 to p21Bax ratios and inhibits taxol-induced apoptosis of human breast cancer MCF-7 cells. Breast Cancer Res Treat 1997;42:73–81.
13 Iosif CS, Batra S, Ek A, Astedt B. Estrogen receptors in the human female lower urinary tract. Am J Obstet Gynecol 1981;414:817–820.
14 Kandouz M, Siromachkova M, Jacob D, Marquet CB, Therwath A, Gompel A. Antagonism between estradiol and progestin on bcl-2 expression in breast-cancer cells. Int J Cancer 1996;68:120–125.
15 Kane DJ, Sarafin TA, Auton S, Hahn H, Gralla FB, Valentine JC, et al. Bcl-2 inhibition of neural cell death: decreased generation of reactive oxygen species. Science 1993;262:1274–1276.
16 Lecci A, Giuliani S, Tramontana M, Santicioli P, Criscuoli M, Dion S, et al. Bladder distention and activation of the efferent function of sensory fibers: similarities with the effect of capsaicin. Br J Pharmacol 1998;124:259–266.
17 Longhurst AP, Kauer J, Leggett ER, Levin MR. The influence of ovariectomy and estradiol replacement on urinary bladder function in rats. J Urol 1992;148:915–919.
18 Shea VK, Cai R, Crepps B, Mason L, Prel ER. Sensory fibers of the pelvic nerve innervating the rat's urinary bladder. J Neurophysiol 2000;84: 1924–1933.
19 Shulman DI, Sweetland M, Duckett G, Root AW. Effect of estrogen on the growth hormone (GH) secretory response to GH-releasing factor in the castrated adult female rat in vivo. Endocrinology 1987;120:1047–1051.
20 Suguita M, Girão MJBC, Simões MJ, Sartori MGF, Baracat EC, Rodrigues G. Amorphologic and morphometric study of the vesical mucosa and urethra of castrated female rats following estrogen and/or progestogen replacement. Clin Exp Obstet Gynecol 2000:27:176–178.
21 Teixeira C, Reed CJ, Pratt CAM. Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. Cancer Res 1995;55:3902–3907.
22 Tsujimoto Y, Croce CM. Analysis of the structure, transcripts and protein products of Bcl-2, the gene involved in human follicular lymphoma. Proc Natl Acad Sci USA 1986;83:5214-5218.
23 Versi E. Incontinence in the climacteric. Clin Obstet Gynecol 1990;33:392–398.
24 Vlaskovska M, Kasakov L, Rong W, Bodin P, Bardini M, Cockayne AD, et al. P2X3 knock-out mice reveal a major sensory role for ureothelially released ATP. J Neurosci 2001;15:5670–5677.
25 Wotherspoon G, Winter J. Bradykinin B1 receptor is constitutively expressed in the rat sensory nervous system. Neurosci Lett 2000;294:175–178.

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