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Original Article

Effects of sex hormones on cell proliferation and apoptosis in the urinary bladder muscle of ovariectomized rat

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Abstract

Objective: To reveal the exogenous effects of sex hormones on cell proliferation and apoptosis in the detrusor muscle of ovariectomized rat urinary bladder.

Materials and Methods: Proliferating cell nuclear antigen (PCNA) and *in-situ* apoptosis detection kit were used to evaluate cell proliferation and death in the detrusor muscle of castrated female rats after 1 day, 3 days, and 7 days of supplementary sex steroid hormone administration, including estrogen, progesterone, and testosterone. The percentage of cells positive for PCNA (proliferative index) and for apoptosis (apoptotic index) in various groups was calculated.

Results: When compared to the group of bilateral ovariectomy without hormonal supplementation, the groups given 3 days and 7 days of estrogen supplementation ($p = 0.031$ and $p = 0.005$, respectively) and the group given 7 days of combined supplementation with estrogen and progesterone ($p = 0.044$) had a significant increase in the proliferative indices. A significant decrease of apoptotic index was found in the group given 7 days of estrogen supplementation when compared to bilateral ovariectomy without hormonal supplementation ($p = 0.035$).

Conclusions: Exogenous estrogen supplementation stimulates proliferation and slows down apoptosis in the detrusor muscle of ovariectomized rat urinary bladder, an effect not counteracted by concomitant use of progesterone. Our results may have clinical implications for estrogen supplementation in offering benefits to menopausal women with lower urinary tract syndromes, based on evaluating the relationship between cell apoptosis and cell proliferation.

Keywords: apoptosis; proliferation; rat; sex hormone; urinary bladder

Introduction

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 [1–3]. Hypoestrogenism is known for its relationship with the development of lower urinary tract syndromes in women, such as urgency, frequency, urinary incontinence, and detrusor overactivity [4,5]. In animal studies, hypoestrogenism caused by ovariectomy has been shown to induce urothelial atrophy [6], and result in significantly decreased smooth muscle density and increased connective tissue within the detrusor of urinary bladder [7–9]. Histologically, ovariectomy causes significant urothelial apoptosis and, with estrogen treatment, there would be significant urothelial growth and hyperplasia [10]. Hyperplasia and apoptosis are opposing cellular processes; subsequent estrogen treatment after ovariectomy increases bladder smooth muscle cell density, possibly owing to proliferation of smooth muscle cells [3,8], but the details about increased bladder smooth muscle cells are still vague. However, expression of the apoptosis in ovariectomized rodent detrusor

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muscle has rarely been investigated. Furthermore, not only estrogen, but also progesterone and testosterone have been reported in potentially exerting certain effects on the lower urinary tract [11–13]. So, the purpose of the present experiment is to reveal, after ovarian castration, the exogenous effects of estrogen, progesterone, and testosterone on cell proliferation and apoptosis of the detrusor muscle.

Materials and methods

Thirty mature female Sprague–Dawley rats (from the National Laboratory Animal Center, Taipei, Taiwan) weighing 250–300 g were divided into five experimental groups for analyzing the cell proliferation and the cell death of detrusor muscle. Prior to any sex hormone was supplied to experimental groups, bilateral ovariectomy was performed through a lower abdominal midline incision using sterile technique with 2% isoflurane in oxygen. The animals were allowed to live at an ambient temperature (21–23 °C) after recovery from the anesthesia. The sex steroid hormones supplementation started 2 weeks after castration in the following fashion: Group 1 received no hormonal supplementation after ovariectomy; Group 2 received an intramuscular injection of estrogen (estradiol benzoate, 50 μg/day; Wyeth-Ayerst , Saint Laurent, Quebec, Canada); Group 3 was given an intramuscular injection of progesterone (medroxyprogesterone acetate, 2 mg/day; Pharmacia NV, Puurs, Belgium); Group 4 was treated with an intramuscular injection of 50 μg estrogen and 2 mg progesterone per day, and Group 5 took an intramuscular injection of androgen (testosterone enanthate, 1.8 mg/day; Paddock Laboratories, Inc., North Minneapolis, MN, USA). With regard to hormone levels prior to and after ovariectomy and after hormonal supplementation, our preliminary data (unpublished) showed that average estrogen levels in preovariectomy, postovariectomy, 1 day hormonal treatment, 3 days hormonal treatment, and 7 days hormonal treatment were 55.9 pg/mL,14.6 pg/mL, 147.3 pg/mL, 162.5 pg/mL, and 171.1 pg/mL; average progesterone levels were 14.2 ng/mL, 3.3 ng/mL, 84.3 ng/mL, 118.2 ng/mL, and 122.4 ng/mL; and average testosterone levels were 0.33 pg/mL, 0.008 pg/mL, 19.6 pg/mL, and 20.5/22.1 pg/mL, respectively.

Every group with 6 rats in each subgroup was given a different dosage of hormone; the rats underwent analysis of apoptosis and proliferating cell nuclear antigen (PCNA) after 1 day, 3 days, and 7 days of hormonal supplementation. Each animal was sacrificed with pentobarbital injection, and the total urinary bladder was removed and placed in iced balanced salt solution. For immunohistochemical study, fresh-frozen bladder sections were used. The dissected bladders were frozen in powdered dry ice and stored at −70 °C. Then, they were subjected to cryosections (10 μm) at −18 °C, with the sections transferred to glass microscope slides coated with saline. The sections were air dried for 30 minutes at room temperature and fixed in 3.7% formaldehyde solution for 10 minutes. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996).

To detect proliferation, the sections were treated with 3% hydrogen peroxidase in methanol for 5 minutes at room temperature and then washed with phosphate buffered saline buffer. After blocking with 10% normal horse serum for 1 hour at room temperature, the sections were rinsed and incubated overnight at 4 °C with a mouse monoclonal antibody against PCNA (PC10, IG2a, DAKO, Glostrup, Demark) diluted in the buffer (1:500). Then again washed and incubated for 2 hours with biotinylated anti-mouse IgG made in horse in the buffer (1:200), followed by incubation for 30 minutes with avidin–biotin–horseradish peroxidase complex. Staining was developed with diaminobenzidine (DAB) in the presence of 0.03% H_2O_2. A 1% methyl green solution was used for the counterstaining (Fig. 1).

To detect apoptotic cells in the detrusor muscle layer of urinary bladder, the TACS terminal deoxynucleotidyl transferase (TdT)-DAB *In Situ* Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA) was applied to perform TdT-mediated dUTP nick end labeling (TUNEL) staining (Fig. 2). Briefly, the sections were incubated with proteinase K (20 μg/mL) for 15 minutes at room temperature, washed in distilled water, and then treated with 3% hydrogen peroxidase in methanol for 5 minutes at room temperature to quench endogenous peroxidase activity. The sections were immersed in TdT labeling buffer for 5 minutes at room temperature, and then TdT and biotinylated dNTP in TdT labeling buffer were dropped into the sections. After incubation at 37 °C for 2 hours, they were transferred into TdT stop buffer for 5 minutes at room temperature, followed by rinsing with phosphate buffered saline and later treatment with streptavidin–horseradish peroxidase for 10 minutes at room temperature. Finally, the sections underwent wash with phosphate buffered saline and were then developed with DAB in the presence of 0.03% H_2O_2. A 1% methyl green solution was used for the counterstaining.

The numbers of apoptotic and proliferating cells were calculated by counting at least 1000 cells per urinary bladder in 10 randomly chosen field stained nuclei of cells under a magnification of ×400 [14]. The proliferative index for each sample was the percentage of cells positive for PCNA. The apoptotic index was counted in the same fashion as was described for PCNA-positive cells. The statistics was done by Student *t* test or one-way analysis of variance, as appropriate. A p value <0.05 was considered statistically significant. Data are presented as the mean ± standard error of the mean.

Results

In Fig. 3, when compared to the group of bilateral ovariectomy without hormonal supplementation, the groups given 3 days and 7 days estrogen supplementation demonstrated a significant improvement of proliferative index ($p = 0.031$ and $p = 0.005$, respectively). The increase was also significant in the group given 7 days combined supplementation with estrogen and progesterone ($p = 0.044$). In Fig. 4, according to the apoptotic index, the apoptosis after castration did not seem