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ORIGINAL ARTICLE

17β-Estradiol affects the proliferation and apoptosis of rat bladder neck smooth muscle cells by modulating cell cycle transition and related proteins

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Abstract
Background Bladder outlet obstruction (BOO) is primarily a stromal disease. Smooth muscle cells are the major cellular components of stroma. Estrogen may directly stimulate Bladder Neck smooth muscle cells (BSMC). However, little information has been gathered on the mechanism of how the estrogen affects the BSMC in vitro. The purpose of this paper is to investigate the effect of 17β-estradiol (E_2) on the proliferation and apoptosis of Bladder Neck Smooth Muscle Cells (BSMC) and the potential mechanisms via cell cycle analysis and related protein detection.
Methods The synthetic rat BSMC were obtained through the enzyme-digesting method and exposed to gradient concentrations (0.1–100 nmol/l) of E_2 for different amounts of time. The progression of cell cycle, the apoptosis and the expressions of Cyclin D1 protein were examined by flow cytometry. Apoptosis-related proteins, Bcl-2 and Bax, were detected by western blot.
Results E_2 in the definite concentrations (0.1–10 nmol/l) promoted the BSMC growth in a concentration-dependent manner by accelerating cell cycle transition from G_1 to S phases, and up-regulating the expression of Cyclin D1. However, high doses of E_2 (10 and 100 nmol/l) increased the rate of apoptosis of the cells accompanied by a significant raise of Bax expression and the ratio of Bax/Bcl-2.
Conclusion The effect of E_2 on subcultured BSMC is bilateral; it promotes the cells proliferation by enhancing the expression of Cyclin D1, which accelerates G_1 to S phase transition, while on the other hand, it induces apoptosis of the cells by up-regulating the expression of Bax.

Keywords Estradiol · Bladder neck · Smooth muscle cell · Proliferation · Apoptosis · Cell cycle

Introduction

Benign prostatic hyperplasia (BPH) is the most common benign neoplasm in older men [1, 2]. It is considered a frequent cause of bladder outlet obstruction (BOO) and lower urinary tract symptoms (LUTS), although the physiopathologic mechanism through which BPH causes LUTS is not clear [3]. Several morphologic and functional modifications of the bladder detrusor have been described in patients with BPH and could play a direct role in determining symptoms [4]. The opinion is spreading that the enlarged prostates in patients with LUTS is nothing more than a mere bystander. Evidence has accumulated, however, supporting the role of BPH-related BOO as the direct cause determining bladder dysfunction and indirectly causing urinary symptoms [5].

Bladder hypertrophy is a general consequence of BOO and a typical phenomenon observed in clinical urologic diseases such as BPH and neurogenic bladder [5]. It is characterized by smooth muscle hyperplasia, altered extracellular matrix composition, and increased contractile function [6, 7]. Various growth factors are likely involved in hypertrophic pathophysiology, but their functions

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remain unknown. Imamura et al. [7] investigated the role of basic fibroblast growth factor (bFGF) using a rat bladder smooth muscle cell (BSMC) culture system and an original animal model, in which bFGF was released from a gelatin hydrogel directly onto rat bladders. bFGF treatment promoted BSMC proliferation both in vitro and in vivo. Estrada et al. [8] found urinary BOO results in sustained stretch of the detrusor muscle and can lead to pathological smooth muscle hyperplasia and hypertrophy. The epidermal growth factor receptor (EGFR) is a cognate receptor for mitogens implicated in bladder hyperplasia/hypertrophy.

Smooth muscle cells are the major cellular components of human bladder hyperplasia tissue [9]. The proliferation of smooth muscle cells results in the dynamic obstruction of the bladder outlet. Some evidence indicates that estrogens may stimulate bladder smooth muscle cells (BSMC) [10].

However, little information has been collected on the mechanism of how the estrogen affects the BSMC in vitro. We thus suggest that the relative increase of estrogen in old males may exert a launching effect in the pathology of BSMC proliferation which results in a stromal predominant BOO with the assistance of other factors.

The purpose of this paper is to investigate the effect of 17β-estradiol (E_2) on the proliferation and apoptosis of BSMC via cell cycle analysis and related protein detection.

Estrogen receptors α and β will be detected and more interference will be applied including the treatment with estrogen receptor antagonists.

Materials and methods

Animals

Thirty adult male Sprague-Dawley rats aged 3 months, weighing 272 ± 34 g, and bred in Wuhan University's experimental animal center were used for these studies and were maintained in a controlled environment with free access to food and water. The animal use protocol is approved by the Institutional Animal Care and Use Committee of Wuhan University.

Materials

Phenol red-free RPMI 1640, charcoal/dextran treated fetal bovine serum (FBS) and standard FBS were obtained from HyClone Laboratories Inc., (Logan, UT). E_2 (E4389) and a soybean trypsin inhibitor were purchased from Sigma-Aldrich Co. (St. Louis, MO). Mouse or rabbit monoclonal antibodies to Cyclin D1 (sc-8396), Bax (sc-526) and Bcl-2 (sc-783) were obtained from Santa Cruz Biotechnology (CA, USA). The α-smooth muscle actin (α-SMA) and desmin antibodies were purchased from Bioss Co. (Beijing, China) and Boster Co. (Wuhan, China), respectively.

An ELISA Cell Death Detection kit was obtained from the Roche Diagnostics Corporation (Basel, Switzerland).

BSMC culture

BSMC were enzyme-dispersed using a modified method originally described by Ricciardelli et al. [11]. Briefly, aseptically dissected ventral bladder neck was placed in cold D-Hank's balanced salt solution. After removing the connective tissue, the bladder neck was cut into small pieces (about 1–3 mm^3) and incubated in 2 g/l collagenase II (Invitrogen, Carlsbad, CA) with 0.5 g/l soybean trypsin inhibitor. After digestion, the tissues were transferred into a centrifuge tube containing 3 ml medium with 10% FBS and centrifuged at 70*g* for 5 min. The cell pellet was resuspended and plated at a density of 1×10^4/ml into a 50-ml culture flask. The preferential adhesion technique was used to reduce contaminating fibroblasts at this stage. Because of the known estrogenic effects of phenol red, the cells were cultured in phenol red-free RPMI 1640 containing 10% FBS, 1×10^5 Units/l penicillin, 100 ng/l streptomycin and 4 mmol/l L-glutamine at 37°C in 5% CO_2. The α-SMA and desmin antibodies were used to identify the cells by immunocytochemistry passages of 3–4 were used for this study.

Analysis of BSMC growth

The effect of E_2 on BSMC growth was determined through cell counting. The cells were plated into 6-well plastic plate at a seeding density of 3×10^5 cells/well, and cultured in medium with or without 10 nmol/l E_2, respectively, in the existence of 10% charcoal/dextran treated FBS [12]. On the indicated days, triplicate wells were trypsinized and resuspended in 10 ml isotonic saline solution. Duplicate samples from each well were counted with a hemocytometer under a phase-contrast microscope. To determine whether the effect of E_2 on BSMC growth is in a dose-dependent manner, the cells were cultured in medium with gradient concentrations of E_2 (0–100 nmol/l) for 3 days in the existence of 10% charcoal/dextran treated FBS.

Analysis of cell cycle progression

To investigate the effect of E_2 on the cell cycle progression of the BSMC, the cells were seeded into a 6-well plastic plate at 3×10^5 cells/well and incubated for 2 days in the medium with 10% standard FBS. After a 24 h of serum deprivation to synchronize their cell cycles, the BSMC were re-stimulated with 10% charcoal/dextran FBS and gradient concentrations of E_2 (0–100 nmol/l)for a further

3 days. The cells were then harvested by trypsinization and fixed in 70% cold ethanol at 4°C for 10 h. The cells were washed twice with ice-cold PBS buffer, and incubated with RNase (100 mg/l) and DNA intercalating dye propidium iodide (50 mg/l) for half an hour in a 37°C aqueous bath before analysis. The cell cycle phases were analyzed using a FC500 flow cytometer (Beckman Coulter, Mountain View, CA, USA) and CXP software. A minimum of 1×10^4 events were analyzed. Triplicate samples were assessed for each group and each assay was repeated twice. The proliferative index (PI) was calculated with the following formula: PI (%) = $(S + G_2/M)/(G_0/G_1 + S + G_2/M) \times 100\%$.

Detections of apoptosis

The apoptotic rate represented by the percentage of sub-G_1 peak in flow cytometry histogram with propidium iodide stain was used to estimate the number of apoptotic cells. To retrieve the discrepancy of the above assay in discriminating the apoptotic cell and corpuscle fragment, the Cell Death Detection ELISA Plus kit was used to measure histone-bound DNA fragments (nucleosome) in an ELISA format. The cells were treated for 3 days with different concentrations (0–100 nmol/l) of E_2. Samples were prepared according to the protocol provided by the manufacturer and analyzed on a microplate spectrophotometer at 405 nm. Data was expressed as means of absorbance from duplicate experiments performed in each sample [13].

Protein assay for Cyclin D1

To investigate the latent mechanisms of the cell cycle promoting effect of E_2, a regulator for G_1 checkpoint, Cyclin D1, was analyzed through flow cytometry. The BSMC treated with or without 10 nmol/l E_2 for 1–5 days were fixed in suspension in 37 g/l paraformaldehyde, washed with PBS, and treated with 2 g/l Triton-X100 and 50 g/l block serum for 15 min on ice. After washing, the cells were incubated with the primary antibody to Cyclin D1 for 45 min on ice, followed by the staining with the corresponding FITC conjugated second antibodies for 45 min. Then washed samples were placed in tubes and read on the FC500 flow cytometer. The control cells were incubated in the absence of the primary antibody. The relative expression levels of the tested proteins were expressed by FITC fluoresce intensity [14].

Protein assays for Bcl-2 and Bax

To investigate the mechanisms underlying E_2-induced BSMC apoptosis, two apoptosis-related proteins, Bax and Bcl-2, were examined. The cells treated as above were collected and lysed in ice-cold RIPA lysis buffer (Beyotime, Shanghai, China). Following centrifugation at 12,000*g* for 5 min at 4°C, the supernatants were collected and stored at −70°C until use. Equal amounts of protein extracts (20 mg/lane) were subjected to SDS-PAGE on a 10% separating gel and electrophoretically transferred onto PVDF membrane. After being blocked with 50 g/l skim milk powder, 1 g/l Tween-20 in TBS buffer for 1 h, the membranes were then incubated with either anti-Bax or anti-Bcl-2 antibody for 10 h at 4°C followed by the corresponding horseradish peroxidase-conjugated second antibody for another 1 h. Anti-β-actin antibody was used as an internal standard for protein concentration and integrity. The reaction was visualized by DAB staining. Quantitative analysis for all the pixels in each band was carried out with the GeneTools software (Syngene, Cambridge, UK). The relative expression levels of the proteins were expressed as ratio of Bax or Bcl-2 raw volumes divided by corresponding β-actin value [15].

Statistical analysis

Data are expressed as mean ± standard deviation (SD). SPSS 13.0 software (SPSS Inc. IL, USA) was used in the process. Comparison of data in more than two groups was analyzed by one-way ANOVA followed by the SNK test. Independent samples *t* test was used for a comparison of data between the two groups. The difference was statistically considerably significant at $P < 0.05$.

Results

The effect of E_2 on BSMC growth

As shown in Fig. 1, the average numbers of the control and E_2-treated cells were very similar on Day 1. On Days 2–4, the average cell numbers in the E_2-treated group were significantly higher than those in control cultures. By Day 5, E_2-treated and control cells became confluent. The result indicates that E_2 had a transient growth-promoting effect on the cells. Figure 2 shows that the effect of E_2 on the BSMC is dose-dependent at the concentrations from 0.1 to 10 nmol/l; however, when the E_2 concentrations were higher than 10 nmol/l, the effect was decreased.

The effect of E_2 on BSMC cell cycle progression

Cell cycle analysis (Table 1; Fig. 3.) shows that at the concentrations from 0.1 to 10 nmol/l, the rates of the BSMC at G_0/G_1 phase were significantly decreased, while those at the S and G_2/M phase increased in a concentration-dependent manner, which resulted in a significant increase of

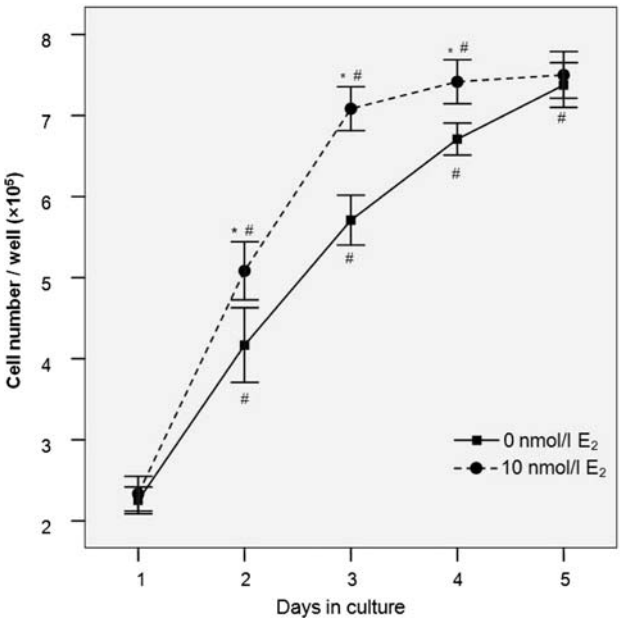

Fig. 1 Cell count for growth of BSMC treated with or without 10 nmol/l E_2 on different days.*$P < 0.05$, compared with control group; #$P < 0.05$, compared with the previous adjacent group. $N = 6$

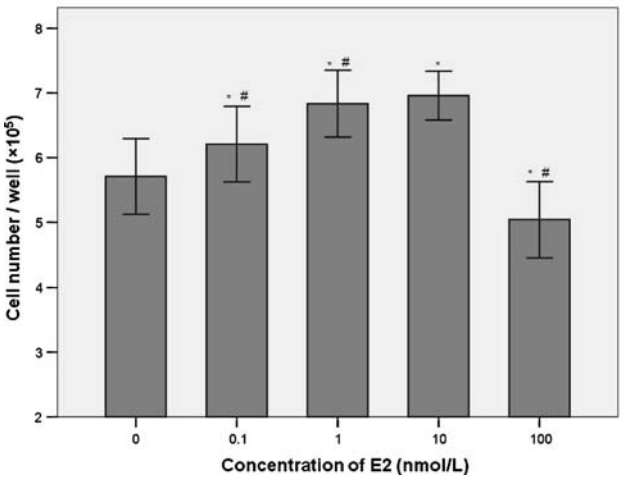

Fig. 2 Cell count for growth of BSMC treated with different concentrations of E_2 for 72 h.*$P < 0.05$, compared with control group; #$P < 0.05$, compared with the previous adjacent group. $N = 6$

proliferative index. It indicates that E_2 stimulates the growth of the BSMC by accelerating their cell cycle progression from G_1 to S and G_2 phases. When the concentration of E_2 reached 100 nmol/l, a self-inhibition of the hormone's action was also observed. The sub-G_1 population appeared and increased along with accruement in concentrations of E_2 with the increased concentrations of E_2 (Fig. 3).

The effect of E_2 on apoptosis

FCM analysis on sub-G_1 rate reveals that the absorbance of BSMC in 10 nmol/l group and 100 nmol/l group were 2.22 ± 0.32 and 4.49 ± 0.98 on Day 3, respectively. Both of them present significant differences to the control group (1.08 ± 0.32), $P < 0.01$. The other two groups (0.1 and 1 nmol/l) did not demonstrate significant differences to the control. It indicated that the apoptotic rates raised significantly in cells of 10 nmol/l group and 100 nmol/l group. This result was compatible with the ELISA data (as shown in Fig. 4). A time course study through FCM analysis demonstrated that at the same concentration (10 nmol/l), E_2-induced apoptosis was time-dependent (Table 2).

The effect of E_2 on Cyclin D1 protein expressions

Administration of 10 nmol/l E_2 to the BSMC cultures induced a significant increase in the expression levels of Cyclin D1 in comparison with the control cells in all culture duration (Table 3.). However, Cyclin D1 showed a tendency of decrease on Day 5.

Effects of E_2 on expressions of Bax and Bcl-2

Western blot analysis showed that after the exposure of the BSMC to 10 nmol/l E_2, the levels of Bcl-2 had no obvious changes in comparison with the control cells (Table 4; Fig. 5), but the expression of Bax was significantly increased, leading to a corresponding increment in the ratio of Bax/Bcl-2. Moreover, Bax protein augmented in a time-dependent manner.

Table 1 Cell cycle progression of BSMC treated with different concentrations of E_2 for 3 days (%, mean ± SD)

Concentrations of E_2 (nmol/l)	G_0/G_1 (*N*)	S (*N*)	G_2/M (*N*)	PI (*N*)
0	74.57 ± 5.32 (6)	12.19 ± 2.98 (6)	13.23 ± 3.36 (6)	25.43 ± 5.32 (6)
0.1	69.10 ± 2.47 (6)	13.40 ± 2.11 (6)	17.49 ± 2.42 (6)	30.90 ± 2.47 (6)
1	42.79 ± 5.56*·** (6)	17.06 ± 1.96* (6)	40.15 ± 6.83*·** (6)	57.21 ± 5.56*·** (6)
10	43.01 ± 6.08* (6)	22.6 ± 5.5*·** (6)	34.39 ± 9.52* (6)	56.99 ± 6.18* (6)
100	80.63 ± 2.65*·** (6)	8.30 ± 2.16*·** (6)	11.06 ± 2.48** (6)	19.37 ± 2.65*·** (6)

* $P < 0.05$, compared with control group; ** $P < 0.05$, compared with the previous adjacent group

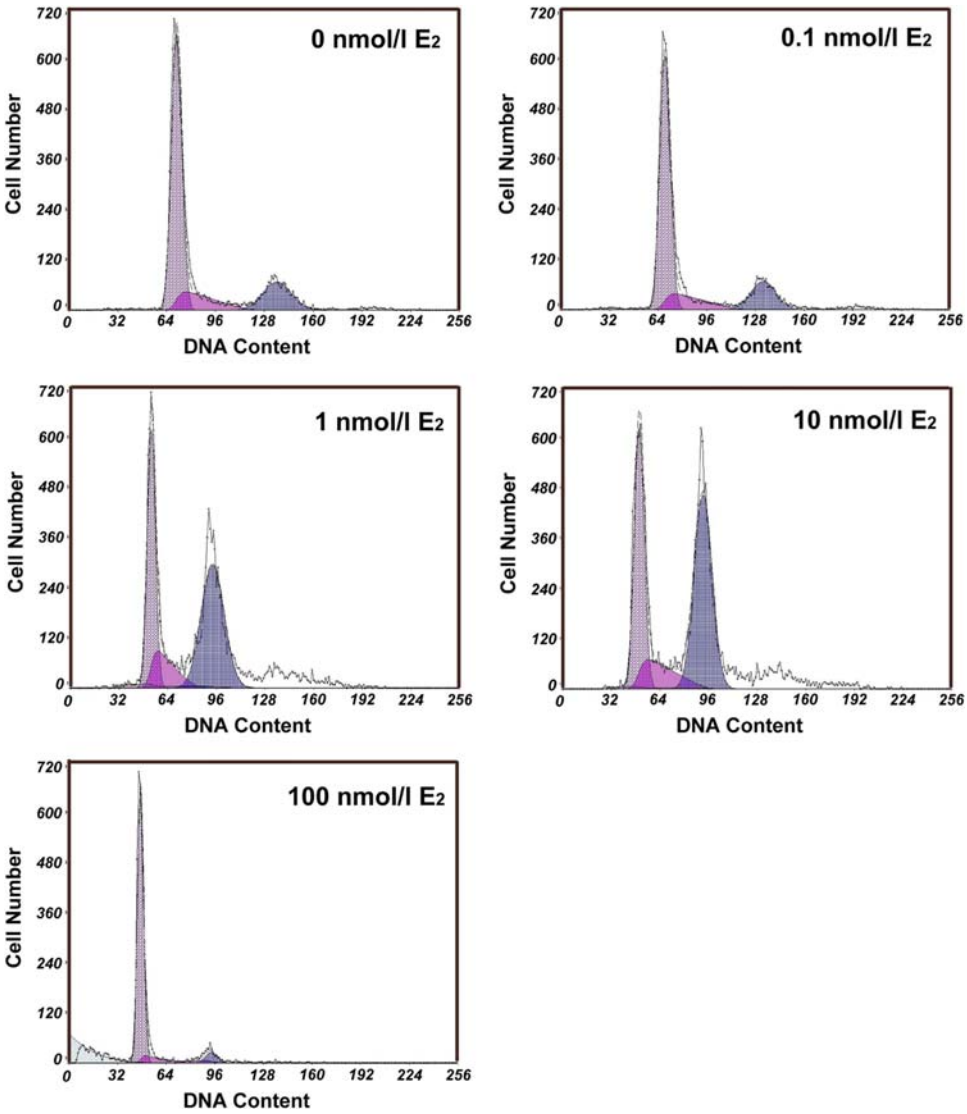

Fig. 3 Representative histograms of flow cytometric analysis for cell cycle distribution of synthetic BSMC treated with different concentrations of E_2. The cells were labelled with PI. Triplicate samples of 1×10^6 cells for each E_2 concentrations were analyzed under the excitation light of 488 nm and detected at 610 nm. $N = 6$

Data points in Fig. 1–4, the means and standard deviations, were calculated from replicated experiments for eight times.

Discussion

A variety of cell types have been verified to respond to mechanical overload that could be caused by different pathologic conditions [16]. Smooth muscle cells (SMC) are particularly responsive to overload stress on different pathological conditions [17, 18]. For example, hypertrophy or hyperplasia is seen in vascular smooth muscle cells (VSMC) in hypertension. The SMC present phenotypic transformation; the structural alteration can be also named remodeling [19, 20].The apparent functional diversity of the SMC response to abnormal or higher mechanical load has prompted interest in the possibility that there is plasticity in its function, which may be related to the severity of the tissue remodeling process during chronic overload stimulation. This effect of overload pressure on SMC resembles that of bladder or detrusor to outlet obstruction [21]. Detrusor smooth muscle cell (DSMC) may be one of the principal cells causing pathologic changes when the bladder is subjected to abnormal mechanical stress under the conditions of BOO [21]. Mechanical stretch stress is considered the trigger inducing these responses in the urodynamically overloaded bladder [22, 23]. Several studies using an in vitro model of mechanical stress demonstrate that repetitive stretch stimulation of bladder smooth muscle cells results in increased expression of a variety of growth factors and other specific proteins [24]. In the bladder smooth muscle cell, stretch-activated ion channels (SACs) and protein kinase C (PKC) sarcolemmal proteins may function as sensors for external mechanical forces [25].

Ricciadelli et al. [26] put forward that smooth muscle cells were the target of estrogen by studying the effect of E_2

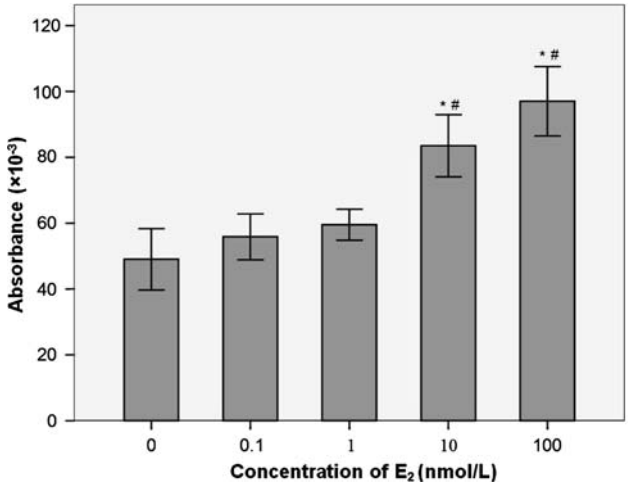

Fig. 4 ELISA analysis for nucleosomes in BSMC treated with different concentrations of E_2 for 3 days. The relative levels of nucleosomes were expressed as average values of absorbance at 405 nm. *$P < 0.05$, compared with control group; #$P < 0.05$, compared with the previous adjacent group. $N = 6$

Table 2 Apoptosis rate of BSMC treated with different concentrations of E_2 in different days determined by flow cytometry (%, mean ± SD)

Concentrations of E_2 (nmol/l)	1 day (*N*)	3 day (*N*)	5 day (*N*)
0	0.85 ± 0.39 (6)	1.08 ± 0.32 (6)	1.18 ± 0.32 (6)
10	1.24 ± 0.20 (6)	2.22 ± 0.32* (6)	4.41 ± 1.72*·** (6)

* $P < 0.05$ compared with control group; ** $P < 0.05$ compared with the group of previous adjacent checkpoint at the same concentration

Table 3 Expressions of Cyclin D1 by BSMC treated with different concentrations of E_2 in different days (fluorescence channel, mean ± SD)

Concentrations of E_2 (nmol/l)	1 day (*N*)	3 day (*N*)	5 day(*N*)
0	2.70 ± 0.37 (6)	2.68 ± 0.28 (6)	2.55 ± 0.27 (6)
10	3.89 ± 0.44* (6)	5.56 ± 0.50*·** (6)	4.58 ± 0.46*·** (6)

* $P < 0.05$ compared with control group; ** $P < 0.05$ compared with the group of previous adjacent checkpoint at the same concentration

on guinea-pig prostate smooth muscle cells in vitro. As far as the mechanism is concerned, Ricciardelli suggested that E_2 stimulates proliferation of guinea-pig prostate smooth muscle cells in vitro by an estrogen receptor-dependent mechanism. Hong et al. [27] found that estrogen could stimulate the growth of prostatic stromal cells and increases smooth muscle cell markers, which may be achieved through a pathway involving TGF-beta 1.

The present study shows that the effect of E_2 on subcultured BSMC is bilateral; it promotes the cells proliferation by enhancing the expression of Cyclin D1 which accelerates G_1 to S phase transition, and on the other hand, it

Table 4 Expressions of Bax and Bcl-2 by BSMC treated with or without 10 nmol/l E_2 in different culture duration (integrated intensity ratio, mean ± SD)

Group (time and concentration of E_2)	bax (*N*)	bcl-2 (*N*)	Bax/bcl-2 (*N*)
1day			
0 nmol/l	0.95 ± 0.09 (6)	0.26 ± 0.04 (6)	3.74 ± 0.67 (6)
10 nmol/l	1.00 ± 0.08 (6)	0.30 ± 0.05 (6)	3.43 ± 0.69 (6)
3 day			
0 nmol/l	0.93 ± 0.14 (6)	0.31 ± 0.12 (6)	3.32 ± 1.33 (6)
10 nmol/l	1.15 ± 0.08*·** (6)	0.24 ± 0.04 (6)	4.79 ± 0.48*·** (6)
5 day			
0 nmol/l	1.00 ± 0.11 (6)	0.29 ± 0.06 (6)	3.51 ± 0.84 (6)
10 nmol/l	1.28 ± 0.07*·** (6)	0.27 ± 0.03 (6)	4.71 ± 0.67* (6)

* $P < 0.05$, compared with control group. ** $P < 0.05$, compared with the group of previous adjacent checkpoint at the same concentration

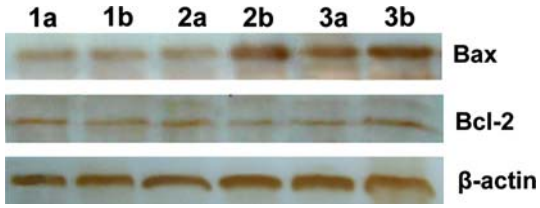

Fig. 5 Representative graphs of western blot analysis for Bax and Bcl-2 proteins from BSMC samples treated without (**a**) or with (**b**) 10 nmol/l E_2 in different culture duration. *Lane 1, 2* and *3* represent that the cells were treated for 1, 3 and 5 days, respectively. The equal loading of the sample was confirmed by β-actin as an internal control

induces apoptosis of the cells by up-regulating the expression of Bax at high concentration. These results agree with the study of Scarano et al. [28] who showed that hypertrophy of the smooth muscle cells of prostate was observed in the estradiol treated guinea pig through histological and histochemical procedures, while the bladder neck smooth muscles are anatomically extending from prostate smooth muscles according to McNeal [29], therefore, they may react similarly.

It is well established that Cyclin D1 is one of the key regulators that drives a cell from G_1 to S phases [30]. Estrogens, which activate Cyclin D1 gene expression with estrogen receptor-α, inhibit expression with estrogen receptor-β [31]. Our results revealed the modulating role of Cyclin D1; however, we could not display the change of estrogen receptor subtype from this study.

In our experiment, the E_2 treatment did not affect the expression of Bcl-2, but resulted in an up-regulation of Bax, leading to an increased ratio of Bax/Bcl-2 which was accepted as a crucial factor to trigger apoptosis. When the hormone reached a critical concentration, Bax induced apoptosis overwhelmed the proliferation-promoting effect

of E_2 and the growth of BSMC demonstrated a self inhibition character. This observation may be related to the fact that complex interactions of hormones were inhibited after treatment of activated charcoal and dextran to the serum for culture, thus the use of E_2 alone manifested a common character of the hormones, i.e., low concentrations of a hormone can stimulate a tissue, while high concentrations have the opposite effect. vom Saal et al. [32] found that when fetal mice were exposed to estradiol or diethylstilbestrol, prostate weight first increased then decreased with every dose, resulting in an inverted-U dose–response relationship. Our result supports their result in vivo, although the curve was not obvious. Arguably, a much greater range of doses of estradiol was required to show the inverted-U dose–response relationship.

Furthermore, we found that with the increase of culture time in a fixed concentration (10 nmol/l) of E_2, the proliferative index did not increase infinitely and the growth of cells slowed down after 5 days. Similarly, the increased expression of Bax might be responsible for the phenomenon.

Despite the anatomical differences between the humans and rats, our experiment provides an appropriate foundation for the further study of human prostatic cells.

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Conflict of interest statement There is no conflict of interest.

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