CELL PROLIFERATION AND APOPTOSIS IN URINARY BLADDER UROTHELIUM OF RATS FOLLOWING OVARIECTOMY AND CHRONIC ESTROGEN REPLACEMENT THERAPY

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Abstract

Aims. *In this study we followed the effect of menopause and estrogenic replacement therapy on the proliferative and apoptotic activity of the bladder urothelial cells.*

Methods. *The experimental model of menopause was reproduced using a standard protocol of bilateral ovariectomy in rats, estrogen replacement therapy being achieved by systemic administration of hexestrol diacetate for six weeks. Proliferative and apoptotic activity was monitored by quantifying the urothelium imunoexpression for PCNA antigen as a marker of S phase of the cell cycle and Cleaved Caspase 3 for monitoring apoptotic activity.*

Results. *Following ovariectomy, the main changes were urothelial atrophy associated with intensification of the apoptotic activity at these level. Estrogen therapy managed to improve the urothelium activity by reducing the Apoptotic Index and by increasing urothelial proliferative activity.*

Conclusions. *The results show the important role of estrogens in maintaining urothelial activities, highlighting their potential use in the treatment of urothelium atrophic and degenerative processes associated with menopause.*

Keywords: proliferation, apoptosis, urothelium, estrogen, replacement therapy.

Introduction

The estrogen deficiency or hypoestrogenism associated with menopause is one of many factors that influence the aging process in women. Although urothelium sensitivity to estrogenic hormone action has long been known, the urinary bladder being a target organ for the actions of estrogens [1], the pathophysiologic changes of the urotheluim resulting from menopause is poorly elucidated [2]. Because of this, the therapeutic usage of the estrogen-like hormone replacement therapy in the atrophic process of the urinary bladder associated with menopause is still controversial [3].

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Methods

Animals and experimental model

For the experiment we used thirty young adult Wistar female albino rats (weight 230±50 g) housed in a conventional animal facility. The animals underwent bilateral ovariectomy following the tehnique previously described by Waynforth and Flecknell [4]. Then the animals were randomly divided into three equal experimental groups: first group which underwent sham operation, representing the reference group (SHAM group), estrogen group (BOV+E) whice received estrogen hormones and untreated ovariectomized group (BOV) which received no medication after ovariectomy. In order to observe the effects of the hexestrol diacetate on the urinary bladder urothelium at 14 days after oophorectomy, time required for appearance of the postoperative ovarian hormonal insufficiency, estrogen replacement therapy was started.

The estrogenic replacement therapy was carried for six weeks by intramuscular administration of 10 μg hexestrol diacetate, twice weekly. After this interval the animals were euthanized and necropsied. During necropsy tissue samples were harvested and fixed in 10% neutral buffered formalin. All the experimental protocol used in this study had been approved by the Ethics Committee of the Iuliu Haţieganu University of Medicine and Pharmacy (number 62-1.04/2010).

Histology

Tissue samples after complete aldehyde fixation were routinely processed into paraffin embedded blocks. From each paraffin block multiple sections were cut and stained according to standard protocols by Hematoxylin-Eosin (HE) [5]. The tissue processing and analyses followed the international recommendation of the INHAND (International Harmonization of Nomenclature and Diagnostic Criteria For Lesions in Rats and Mice), both the body and neck of the urinary bladder being submitted to the histopatological examination. The histology slides were examined under an Olympus BX microscope and images were obtained with an Olympus SP 350 digital camera.

Immunohistochemistry

For the assessment of the cell proliferative activity of the bladder`s urothelium we used the immunohisto-chemical expression of the Proliferating Cell Nuclear Antigen (PCNA) (Dako Danmark, cat. Nr. M0879) and Caspase 3 (Thermo Scientific, UK, cat. Nr. PAK0488) as a immunohistochemical marker of apoptotic activity. After dewaxing and rehydration, the antigens from tissue sections were unmasked by boiling for 3 minutes in pH 6 sodium citrate buffer. The activity of the endogenous peroxidase and alkaline phosphatase were suppressed using the Dual Endogenous Enzyme-Blocking Reagent from Dako (product no. S200389). After blocking the antibody specific binding sites, slides were incubated with primary antibodies for 2 hour at room temperature in a humid chamber. The antigens expression was detected using the Universal LSAB-HRP Kit (Dako, product no. K069089), following a standard avidin-biotin detection method. The positive antibody reaction was observed by incubation of the slides with diaminobenzidine for 1-2 minutes. Finally slides were counterstained with Mayer`s hematoxylin (Dako, Product No. S330930) and mounted with an xylen compatible medium (Merck, Germany).

Quantification of the PCNA and Caspase 3 expression

For quantification of the PCNA and Caspase 3 urothelial expression, we followed the protocol previously used by Thiruchelvam et al. [6] for immunohistochemical assessment of the proliferative and apoptotoc activity in the urinary bladder. Briefly, nuclei were counted on 10-20 microscopic fields at 20x amplification so that on each analyzed bladder sample 1,000 nuclei being counted. The Proliferative and Apoptotic Index of the urotelium results from the percentage of immunolabeled cells positive for PCNA and respective for Caspase 3.

Statistics

Group results were expressed as the mean±SD. The statistical test used in the calculation and interpretation of our results is the mono-factorial analysis system ANOVA, P value of <0.05 being considered statistically significant.

Results

Histopatology

The histopathological aspect observed in the urothelium of the BOV group was the marked atrophy, aspect suggested by the decreased thickness of urothelium, presence of cells with intensely eosinophilic cytoplasm, hyperchromatic nuclei and loss of intercellular junctions (Figure 1). The morphologic changes were also observed in the urothelium of the BOV+E group, but the atrophic phenomenon was present in a lesser form than those observed in the BOV group. Interesting in the BOV+E group was the alternation between thickened, hyperplastic areas of urothelium and of normal-looking urothelium were noticed.

Proliferative and apoptotic activity of the urothelium

Regarding the urothelium proliferative activity, a state highlighted by immunohistochemical expression of PCNA, in all the studied groups we observed cells with division activity (cells in the S phase of the cell cycle). Important differences between groups were reflected primarily in the number of cells that have proliferative activity as well as in the arrangement of these cells within the three areas of the urothelium. Thus, if the Proliferation Index for the reference group was 34±11%, for the BOV+E group it had a tendency to increase ($p<0.05$) to values of 43±14% (Figure 2A). For the BOV group, the divisional activity of urothelium did not change significantly from the Sham group.

Distribution of PCNA positive cells in the control group was predominant in the basal layer of the urothelium, uniformly distributed in the basal layer of cells and in a lesser amount in the intermediate layer of urothelium. The PCNA positive cells in the BOV group were unevenly distributed, areas of intense immunolabeled cells alternating with negative groups of cells in both basal and intermediate layers of urothelium. For the BOV+E group the distribution of the PCNA positive cells within the urothelial layers was uniform, positive cells being observed both in the basal layer and the intermediate one.

Significant changes were observed in the urothelium apoptotic activity for the studied groups. Thus, if in the control group the apoptotic activity was observed almost exclusively in the outer cell layers of the urothelium, with an apoptotic index of 0.5±0.1% for the ovariectomized groups, the Apoptotic Index had a significant increase

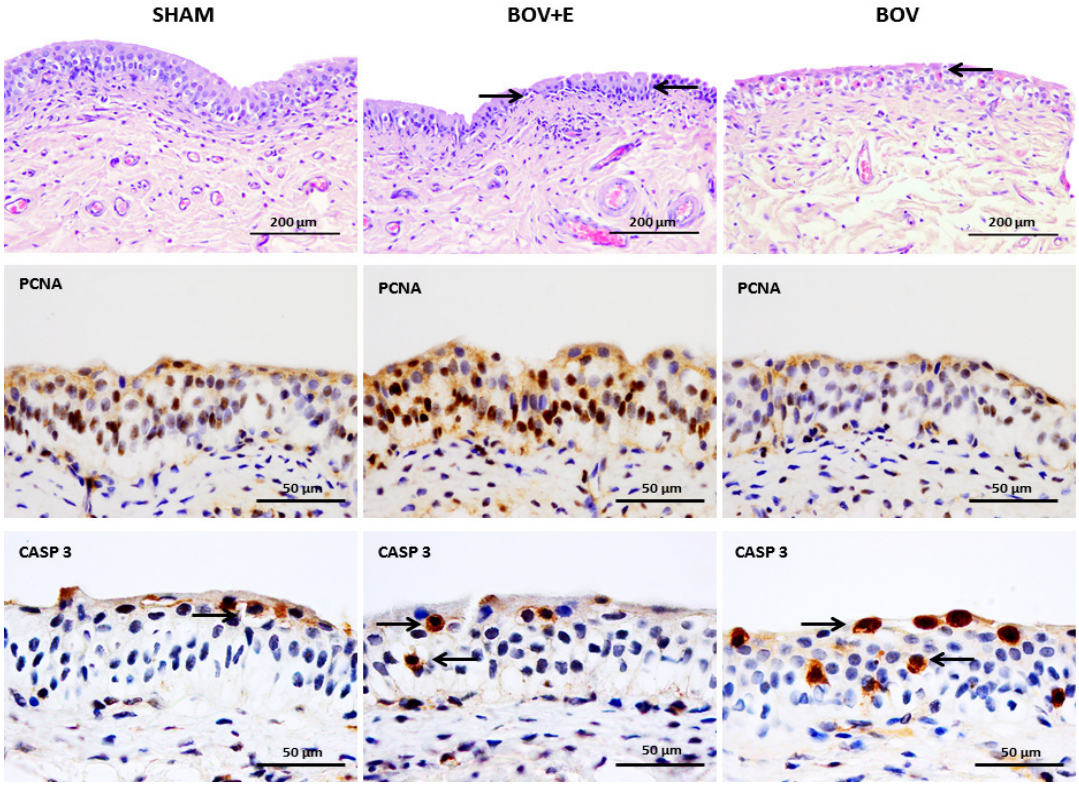

Figure 1. Histopathological findings and immune expression of PCNA and Caspase 3 in studied groups. On the first line of images arrows are indicating the apoptotic cells and the atrophy of the urothelium. In the second and third lines arrows indicates the immunoexpresion for PCNA and Caspase 3.

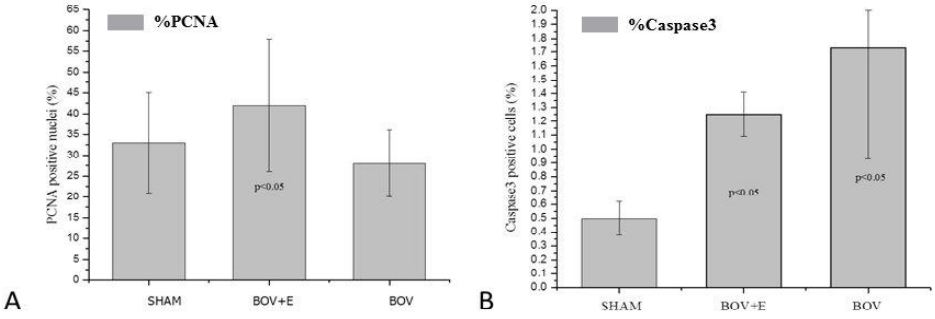

Figure 2. Image A represents the Proliferative Index, while the image B represents the Apoptotic Index of the urothelium.

reaching 1.25±0.1% for BOV+E group and 1.76±0.8% for BOV group. Significant differences were observed between BOV+E and BOV groups, the number of cells following apoptosis being significantly reduced for BOV+E group compared with the BOV group. For both ovariectomized groups the distribution of cells that undergo apoptosis is different from the reference group. Thus, for both BOV+E and BOV groups Caspase 3 positive cells were observed in the superficial and intermediate layers or even in the baseline.

Discussion

Estrogen hormones play a key role in maintaining the normal morphology and function of the lower urinary tract, role which is modulated directly through estrogen receptors (ERα and ERβ) found on the urothelium [7] or indirectly folowing alternative route through a rapid pathway, unmediated by estrogen receptor [8]. Urothelium shows an increased sensitivity to the level of circulating estrogens. Significant decreases in the level of estrogens consecutive to spontaneous or surgically induced menopause results in important urothelial atrophy [9,10,11], atrophy which is based mainly on an increase of apoptotic activity in the urothelium [1,12]. As in the study of Aikawa [1], we also found that substitutive administration of estrogens led to a decrease of apoptotic

cells in the urothelium and also a reduction of the atrophic effects in the urothelium secondary of the surgically induced menopause. Anti-apoptotic effects of estrogens on the urothelium had several possible sources. Here we include the effect of estrogens on the expression of the proteins of bcl2 family, especially on the expression of bax protein [12]. In the urothelium of rats a reduced amount of bax protein, one of the most important proapoptotic proteins in eukaryotic cells [13], was observed after estrogen supplementation in a surgically induced model of menopause [12]. Another mechanism by which estrogen contributes to the integrity of the urothelium is that estrogens promote, in normal and tumoral cells, the cell survival [14,15], this mechanism being important especially in pathological situation in which the cellular stress grows [16]. The protective effect of estrogens on the urothelium is mediated also by increasing the blood amount in the bladder`s wall [17] and by raising the amount of glycosaminoglycans from the superficial layers of the urothelium [18].

One of the most important factors which modulate urothelial cell proliferation and differentiation are estrogens, especially 17b-estradiol, by activating the two estrogenic cell receptors ERα and ERβ [15,19,20]. A growth in the number of cell in division from the urothelium found in our study could be linked to the activation of the Cyclin D1 by estrogens [15,21], Cyclin D1 having a prime role in triggering the cellular division in the urothelium [22].

The important role of estrogen in maintaining the urothelium integrity is emphasized by the fact that hormone replacement therapy reduces the atrophy and apoptotic phenomena at this level, while promoting the urothelium proliferative activity.

Conclusions

The presented findings demonstrate that proliferative and apoptotic activity of the urinary bladder urothelium is significantly affected the systemic level of estrogen hormones. In addition, systemic administration of the hexestrol diacetate has an important effect on bladder urothelium turnover by modulating the proliferative and apoptotic activity of the urothelial cells.

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