LABORATORY STUDY

Protective effects of estrogen and bortezomib in kidney tissue of post-menopausal rats: an ultrastructural study

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ABSTRACT

Purpose: Symptoms and disorders related to menopause and its associated estrogen deficiency have become a considerable health concern worldwide. Ovarian hormone depletion/estrogen deficiency can be usefully studied using animal models after removal of the ovaries (ovariectomy (Ovx)). This study assessed renal changes after Ovx-induced estrogen deficiency in a rat model.

Methods: Rats were randomly allotted into one control group (group I, healthy) and three study groups (group II, Ovx group; group III, Ovx + 17\textbeta-estradiol group; and group IV, Ovx + bortezomib group).

Results: In the Ovx group (group II), thickening of glomerular capillary walls, narrowing of Bowman’s capsular space, glomerular hypertrophy, atrophic tubules, and loss of the basal membranes of the tubules were observed. Mesangial cell proliferation was observed, particularly in the glomerulus. Immunohistochemical (IHC) staining studies in this group showed dense staining in the mesangial cells, tubular cell NF-kB/p65, and caspase-3. Groups III and IV (Ovx + 17\textbeta-estradiol and Ovx + bortezomib) showed decreased NF-kB/p65 and caspase-3 expression compared with the Ovx group ($\ p < 0.05$).

Conclusion: In renal failure related to estrogen deficiency caused by Ovx, 17\textbeta-estradiol and bortezomib have a protective effect on renal tissue.

Introduction

Estrogen is an important regulator of physiological processes in women. Estrogen deficiency has been acknowledged as causing disorders in postmenopausal women and is associated with metabolic effects and conditions that include osteoporosis, cardiovascular disease, chronic kidney disease (CKD), colon cancer, and dementia.\textsuperscript{1,2} 17\textbeta-estradiol (E2) and estrone (E1) levels have the greatest association with the onset of symptoms related to menopause.\textsuperscript{3}

Ovarian hormone depletion in an experimental animal model that has undergone ovariectomy (Ovx) is a useful model for studying the effects of hormone depletion in women.\textsuperscript{4–6} Ovx, or removal of the ovaries (called “oophorectomy”) when performed in women as a procedure, reduces estrogen levels and increases the incidence of different types of cardiovascular and renal disorders.\textsuperscript{7,8} Decreased synthesis of 17\textbeta-estradiol (E2) in experimental animals after Ovx is accompanied by an increased incidence of cardiovascular disorders and accelerated progression of renal disease.

Reduced estrogen levels can reduce the amount of information one can obtain about pathologic changes in kidney ultrastructure, immunohistochemistry (IHC), and stereologic analysis. Some studies have suggested that decreased synthesis of E2 causes renal changes and inflammatory cell infiltration.\textsuperscript{4,9} Additionally, several studies have demonstrated that decreased estrogen levels at menopause are associated with elevated oxidative stress throughout menopause.\textsuperscript{1,3,10,11}

Inflammatory cytokine production induced by reactive oxygen species (ROS) occurs with hormone depletion in postmenopausal women.\textsuperscript{12,13} Nuclear factor kappa-B (NF-kB) promotes the expression of a number of genes involved in inflammation, such as cytokines and adhesion molecules. NF-kB/p65 is important in the control of cell proliferation, more specifically in protecting cells from programmed cell death. Renal tubular epithelial cells may express a number of NF-kB/p65-dependent genes.

Recent studies have focused on using proteasome inhibitors to prevent the development of renal...
inflammation and fibrosis. Bortezomib, a ubiquitin proteasome pathway inhibitor (via ROS-induced deubiquitinase inhibition) that is used in kidney transplants, and other compounds are under investigation as inhibitors of renal fibrosis, since transforming growth factor beta (TGF-β), a key factor in renal fibrosis, is regulated by the ubiquitin proteasome inhibitors pathway through degradation of TGF-β-signaling molecules.

Estrogen has been noted as an effective treatment for the prevention of osteoporosis in postmenopausal women with normal renal function. In this study, our aim was to determine whether inhibiting NF-κB activation with bortezomib protects the kidneys from damage caused by E2 deficiency. We assessed the protective effects of 17β-estradiol in E2 deficiency by inducing deficiency with Ovx, with a focus on its protective effects against renal damage.

Materials and methods

Animals study

A total of 32 adult Wistar albino female rats (age, about 3 months; weight, 200 ± 50 g) were procured from the Ataturk University Animal Care and Research Unit, Erzurum, Turkey. The rats were housed in clean polypropylene cages with eight rats per cage. During the experimental period, all subjects were fed with pellets containing 21% crude protein (Purina; Nestlé Purina PetCare Company, St Louis, MO) and clean daily drinking water. All animals received humane care according to the criteria outlined in the guide for the care and use of laboratory animals prepared by the National Academy of Sciences and published by the National Institutes of Health. The study was approved by the Ataturk University Institutional Animal Ethical Committee. Our subjects were divided into four groups of eight rats each (total number of rats, N = 32) and had the same biological and physiological characteristics. The rats were randomly allotted into one of four groups: a control group (group I) or one of three experimental groups (group II, Ovx; group III, Ovx + 17β estradiol; group IV, Ovx + bortezomib).

Drug preparation and experimental protocols

For the three experimental groups, ovariectomies were performed in the operation room of Ataturk University’s experimental animals research branch. Rats were anesthetized with an intraperitoneal (IP) injection of 20 mg/kg sodium thiopental. A longitudinal slit (0.5–1 cm) was made in the midline area of the lower abdomen and the ovaries were removed. After Ovx, 25 mg/kg metamizole sodium was administered as an analgesic for 2 days. For the purpose of administration in group III, 17β-estradiol (Estrafem; Novo Nordisk, Bagsvaerd, Denmark) was dissolved in 0.9% NaCl. Approximately 8 weeks after Ovx, 17β-estradiol with 0.9% NaCl (group III), or 0.9% NaCl alone (group II) was administered 0.2 mg/kg by oral gavage once a day until the end of the experiment. At the same time interval after Ovx, group IV (Ovx + bortezomib) was given 0.4 mg/kg bortezomib administered IP until the end of the experiment.

Histological preparation

Kidney tissue samples were fixed in 10% formalin. After the fixation, specimens were dehydrated in an ascending series of ethanol, cleared in xylene and embedded in paraffin. The sections having thickness 4–5 μm were prepared for hematoxylin-eosin (H&E) staining (Applichem GmbH, Darmstadt, Germany).

IHC preparation

Kidney tissue samples were cut into 1–2 μm semi-thick sections and prepared for NF-κB/p65 and caspase-3 staining with Ventana benchmark GX (Ventana Medical Systems; Oro Valley, AZ). Following steps were performed for IHC staining: the sections were deparaffinized and treated with proteinase K solution (20 mg/mL in PBS), washed in distilled water, and immersed in 3% hydrogen peroxide. After several washes with PBS, the sections were immersed in an equilibration buffer. The secondary kit used was the ultraview universal DAB detection kit (Ventana Medical Systems, Oro Valley, AZ).

Stereologic analysis

For stereologic examination, stereo investigator 8 (MBF Science; Williston, VT) with a camera attachment was used. Kidney tissue samples for each rat were examined at a low magnification, with a pilot study used to estimate suitable grid size and an unbiased counting frame. The lined area was sampled systematically and randomly via fractionator probe (MBF Science; Williston, VT), and cells positive for NF-κB/p65 and caspase-3 were counted at high magnification. Sections were obtained without any randomness in their orientation, and determination of immuno-positive cells were applied as described by Selli and Kalkan.

Finally, the mean numerical density of cells positive for NF-κB/p65 and caspase-3 was estimated by the following formula:

$$N_v = \frac{Q}{S \times A}$$
where $N_v$ is the numerical density, $Q$ represents the total markers counted, $S$ is the number of sampling sides, and $A$ represents the counting frame area.

**Statistical analysis**

Data from the statistical analysis of the numerical density of cells positive for NF-kB/p65 and caspase-3 are expressed as means ± SEM (standard error of the mean). Statistical analysis was performed using one-way analysis of variance followed by Duncan’s test for each paired experiment value; $p < 0.05$ was considered significant. All statistics were calculated and analyzed using IBM SPSS 20 software (IBM/SPSS; Chicago, IL).

**Results**

**Light microscopy results**

The histological samples obtained for the control group were of the normal glomerulus, the glomerular capillary wall and typical podocyte, and mesangial cells. A typical Bowman’s capsular space is also shown (Figures 1(A) and 2(A)).

The Ovx group histological samples demonstrated hypertrophy and focal glomerulosclerosis, narrowing of Bowman’s capsular space, and glomerular capillary basement membrane thickening, with hemorrhage shown in the kidney medulla. Additionally, atrophy is shown in the proximal and distal tubules. Light microscopy samples show dense mesangial cells. The basement membrane is slightly thickened, with mild mesangial proliferation (Figures 1(B) and 2(B)).

For the Ovx + bortezomib group, some thickening is shown in the glomerular capillary wall, with typical podocytes and mesangial cells. Bowman’s capsule is typical; some capillary expansion also manifests. With respect to the proximal and distal tubule basal membrane structure, the histological samples were typical (Figures 1(C) and 2(C)).

The Ovx +17β-estradiol group histological samples demonstrated normal thickening of the glomerular capillary wall with typical podocytes and mesangial cells, along with typical proximal and distal tubule basal membrane structure (Figures 1(D) and 2(D)).

**IHC results**

NF-kB/p65 is more or less extensively expressed in immunostaining in nuclear and/or cytoplasmic staining. The IHC studies showed Ovx group samples with densely stained mesangial cells, tubular cell NF-kB/p65, and caspase-3 (Figures 3(B) and 4(B)).

**Electron microscopy results**

The micrographs from electron microscopy showed typical Bowman’s capsules in the control group, along with normal glomerular basement membrane ultrastructure. Podocyte foot processes also appear clearly (Figure 5(A)).

For the Ovx group, micrographs showed basement membrane thickening; not visible are the basement membrane lamina densa, lamina rara interna, and externa. Podocyte foot processes and foot process infolding are reduced (Figure 5(B)).

In the histological samples for the bortezomib group, the micrographs show some thickening of the glomerular capillary wall, with typical podocytes and mesangial cells. Some capillary expansion is shown along with typical podocyte foot processes (Figure 5(C)).

**Figures 1.** H&E staining. (A) Control group. Light microscopy of a glomerulus demonstrating typically glomerular (g) structures. Bowman’s capsular space (c) × 40. (B) O VX group. Glomerular hypertrophy with capillary dilation (arrow). Atrophic tubules (t). Bowman’s capsule narrowing (arrow head). × 40. (C) Ovx + bortezomib group. Typical glomerulus (g). Bowman’s parietal line cell (arrow head). × 40. (D) O VX +17β-estradiol group. Typical glomerulus (g). Bowman’s parietal line cell (arrow head). × 40.
The histological samples for the 17β-estradiol group demonstrate normal thickening of the glomerular capillary wall and typical podocyte ultrastructure (Figure 5(D)).

**Statistical analysis**

In the Ovx group, the results revealed greater immunostaining for NF-kB/p65 and caspase-3 in the renal cortex.
compared to the control group \((p < 0.05)\). In the Ovx + 17β-estradiol group, NF-κB/p65 and caspase-3 expression was significantly decreased compared to the Ovx group \((p < 0.05)\). The location and amount of immunostained NF-κB/p65 and caspase-3 positive cells were similar between the 17β-estradiol and bortezomib groups \((p > 0.05)\) (Table 1).

### Discussion

The recent research shows that Ovx (leading to E2 deficiency) results in hypertension and associated cardiovascular diseases due to the loss of the cardioprotective effect of 17β-estradiol.\(^{20}\) Hypertension occurs due to the increase in reabsorption of sodium through the proximal and distal tubules, as the reduction in 17β-estradiol leads to activation between the nuclei in the hypothalamus, central nervous system, and in the renin–angiotensin system (RAAS). Hypertension causes expression of endothelin-1, which has a significant effect on kidney pathophysiology. Endothelin-1 has a vasoconstrictive effect and also causes inflammation and oxidative stress. Overexpression of the endothelin-1 gene leads to acute renal failure, followed by mesangial cell proliferation and inflammation.\(^{3,4,21–25}\)

It has also been reported in the literature that oxidative stress caused by E2 deficiency can reduce the activity of superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) enzymes, which cleanse free oxygen radicals in cells.\(^{3,21,23–26}\) Reduction of these radical cleaning enzymes causes the accumulation of free radicals in cells (ROS). Recent studies have demonstrated that ovarioctomized rats may strengthen the antioxidant defense system by reducing lipid peroxidation, and therefore they may play a role in preventing renal disorders.\(^{3,23}\)

NF-κB/p65 is activated by free oxygen radicals, which can be released upon the release of interleukins as a result of inflammation. NF-κB/p65 has an important role in inflammation, cell proliferation, and apoptosis and also plays a key role in renal damage. Some studies have shown that tubular cells (especially mesangial cells) can cause NF-κB/p65 activation by infiltration of leukocytes in kidneys. Angiotensin I and II also activate NF-κB/p65.\(^{10,11,14}\)

This study is in accord with other studies in demonstrating that expression of NF-κB/p65 increases with changes in mesangial and tubular cells (Table 1). In the Ovx + 17β-estradiol group, the expression of NF-κB/p65 decreased compared to Ovx group. Our thought is that 17β-estradiol inhibits the formation of free oxygen radicals by regulating the RAAS system. The decrease of NF-κB/p65 expression supports our idea that estrogen reduces visceral fat accumulation caused by the RAAS system or the lack of 17β-estradiol. In the

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**Table 1.** NK-κB and caspase-3 positive cell numerical density \((\text{mm}^2)\).

| Treatment                  | NK-κB        | Caspase-3   |
|----------------------------|--------------|-------------|
| Control group \((\text{group} 1)\) | 8.37 ± 3.14\(^b\) | 6.11 ± 2.25\(^b\) |
| Ovx group \((\text{group} 2)\)    | 95 ± 3.3\(^a\) | 97 ± 4.4\(^a\) |
| Ovx + bortezomib group \((\text{group} 3)\) | 11.72 ± 2.89\(^ab\) | 12.68 ± 3.02\(^ab\) |
| Ovx + 17β-estradiol group \((\text{group} 4)\) | 9.42 ± 2.09\(^ab\) | 8.89 ± 2.03\(^ab\) |

\(^a p < 0.05\) versus control group. \(^b p < 0.05\) versus Ovx group.

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**Figures 5.** Transmission electron microscopic micrographs. Uranyl acetate and Reynolds lead citrate stain. (A) Control group. Electron micrographs showing typical glomerular capillary with normal basal lamina (arrow). Normal podocytes with foot process. ×3000. Erythrocytes (e) ×3000. (B) Ovx group. Electron micrographs showing glomerular basement membrane thickening. Capillary basement membrane lamina densa, lamina rara interna and externa disappear (asterisk). Reduction in podocyte foot process and foot process infolding (arrow head). ×6000. (C) Ovx + bortezomib group. Podocytes (P). Normal podocytes foot process (arrow head). Typical glomerular capillary basement membrane (arrow). ×5000. (D) Ovx + 17β-estradiol group. Normal podocytes foot process (arrow head). Typical glomerular capillary basement membrane (arrow). ×10,000. Erythrocytes (e). Capillary lumen (c). Bowman’s capsular space (b).
Ovx + bortezomib group, expression of NF-kB/p65 decreased compared to the Ovx group. Our thought is that bortezomib ubiquitin (IkB), which is a proteasome inhibitor, prevents NF-kB/p65 expression by suppression, a finding supported by the study of Roberti et al. (Figures 3(A–D)).

Menopause has been linked with elevated oxidative stress. Caspase-3 inhibits ROS and is required for efficient apoptosis. To focus on caspase-3, our kidney samples were stained with caspase-3 immunostaining. Recent studies show that caspase-9 can prevent cytochrome c release and (indirectly) the formation of ROS. Currently available research has made clear that the triggering of caspase-3 and caspase-7 by caspase-9 is an irreversible step that leads to caspase-3 and caspase-7 apoptosis. Cell death is more efficient in the presence of caspase-3, which is the primary executioner of apoptotic death. In accordance with other studies, our research shows that (especially in mesangial and tubular cells) expression of caspase-3 increased meaningfully in the Ovx group. In the Ovx +17β-estradiol group and Ovx + bortezomib group, the expression of caspase-3 decreased compared to Ovx group (Table 1) (Figures 4(A–D)).

Estrogens exert ROS-scavenging chain-breaking antioxidant activity as hydrogen donors from their phenol-hydroxyl ring. Estrogens can induce antioxidant enzyme expression by stimulating the antioxidant defense system. Estrogens inhibiting the formation of lipid peroxides in plasma and liver tissues in vitro.

Bortezomib is a proteasome inhibitor and, as such, is part of a therapeutic class that is being studied for potential use in kidney diseases and especially for treatment in cases of kidney transplantation. However, in research, it has been shown that especially high doses can cause neurotoxicity and nonselective toxicity. Due to its success in treatment and its nonselective toxicity at lower doses, a great number of phase II studies are ongoing. Protocols are also being developed for a comparison between the side effects and benefits of proteasome inhibitors during use for early treatment of renal disease or of CKD (such as permanent proteinuria and glomerular diseases) without causing damage. Additionally, proteasomes may have utility in addressing the antibodies that cause rejection of transplanted kidneys. Research has shown that bortezomib can help prevent the occurrence of renal fibrosis mediated by ubiquitin proteasomes. In our research, symptoms of renal fibrosis were not found in the Ovx + bortezomib group.

Research has revealed that the effect of E2 deficiency on kidney tissue is widespread and includes infiltration of tubulointerstitial inflammatory cells, tubular atrophy and dilation, glomerulosclerosis, adhesion of Bowman’s capsule, and glomerular hypertrophy with tubulointerstitial sclerosis.

In our research, thickening of glomerular capillary walls, narrowing of the Bowman’s capsular space, glomerular hypertrophy, atrophic tubules, and loss of the basement membrane of tubules were observed. Mesangial cell proliferation is particularly observed in glomerular cells. Glomerular hypertrophy and narrowing of Bowman’s capsular space are consistent given the assumption that obesity causes hypertension. Along these lines, we believe that obesity causes mesangial cell proliferation in the glomerular area and tubulointerstitial inflammation by causing inflammation through activation of NF-kB/p65. A dense presence of NF-kB/p65 receptors was associated with losses in tubular cells (Figures 3(A–D)).

Transmission electron microscopy provided less information related to Ovx-related E2 deficiency. Verlander et al. observed losses in the apical parts of tubular cells. We believe the thickening of capillary wall is caused by the accumulation of cells from activation of the immune system in the subendothelial area, and that the increase of inflammation from NF-kB/p65 causes this activation. A significant decrease in podocyte foot processes was also observed. The mechanism of renal damage from this remains to be defined (Figure 5).

Conclusions
Our results support the concept that 17β-estradiol and bortezomib have a protective effect against renal failure related to Ovx. The 17β-estradiol protects the kidney tissue by NF-kB/p65 expression, whereas bortezomib (mediated with ubiquitin [IkB]) protects kidney tissue by repressing NF-kB/p65 expression.

Disclosure statement
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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