

Effects of estrogen replacement therapy on apoptosis and vascular endothelial growth factor expression in ocular surface epithelial cells: An experimental study

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Foundation items: Supported by the Research Fund of Adnan Menderes University, Aydın, Turkey. This study was presented at the 42nd National Congress of the Turkish Ophthalmology Society in Belek-Antalya, Turkey, November 19-23, 2008 and gained third of best research award

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Received: 2011-10-14 Accepted: 2012-01-10

Abstract

• **AIM:** To investigate the effects of estrogen replacement therapy (ERT) on apoptosis and vascular endothelial growth factor (VEGF) expression in ocular surface in an experimental rat model.

• **METHODS:** Forty female, Wistar rats were randomized in 4 groups in the study. Subcutaneous ERT (17 β -estradiol, 10g/kg/day) was administered to the first group without ovariectomy and to the second group with ovariectomy for three months. Third group had only ovariectomy and fourth group had sham operation. All rats were sacrificed in estrous cycles determined by vaginal smear test and their right eyes were enucleated at the end of the third month. Enucleated eyes were analyzed by immunohistochemical method for expressions of caspase-3, bcl-2, VEGF and TUNEL assay.

• **RESULTS:** Caspase-3 expression of conjunctival epithelium was significantly higher in group 3 than group 1 ($P=0.005$), and group 2 ($P=0.007$). TUNEL score of conjunctival epithelium was significantly higher in group 3 than group 1 ($P=0.006$). TUNEL score of corneal epithelium was significantly higher in group 3 than group 2 ($P=0.012$), and group 4 ($P=0.002$). There was no significant difference

between groups in that bcl-2 and VEGF expressions.

• **CONCLUSION:** We determined increased apoptosis in ocular surface epithelial cells in ovariectomized rats. ERT and endogen estrogen decreased the apoptosis, and did not result in difference in VEGF expression between the groups. Estrogen may be beneficial for the treatment of apoptosis-mediated ocular surface disorders such as dry eye. Further studies are needed on this subject for a better understanding of the role of estrogen and to provide a new insight for treatment and prevention of apoptosis-mediated ocular surface disorders.

• **KEYWORDS:** apoptosis; conjunctiva; cornea; estrogen; ocular surface; vascular endothelial growth factor

DOI:10.3980/j.issn.2222-3959.2012.01.13

Özcura F, Dündar SO, Çetin ED, Beder N, Dündar M. Effects of estrogen replacement therapy on apoptosis and vascular endothelial growth factor expression in ocular surface epithelial cells: An experimental study. *Int J Ophthalmol* 2012;5(1):64-68

INTRODUCTION

The ocular surface is defined as the primary interface between the eye and the atmosphere. The ocular surface is composed of tear film, corneal epithelium, and conjunctival epithelium. The ocular surface, main and accessory lacrimal gland and the interconnecting innervations are called as lacrimal functional unit. Ocular structure and optic quality of the eye is protected by means of healthy working lacrimal functional unit. Dry eye syndrome (DES) is the most common ocular surface disorder and arises from dysfunction of lacrimal functional unit. DES has also accompanied many of ocular surface disorders^[1].

The incidence of DES increases in both gender with age but has a higher incidence among women after menopause than in men^[2]. It was also reported that DES has a higher incidence with premature ovarian failure consisting of amenorrhea, elevated menopausal level gonadotropins, and sex steroid deficiency in women less than 40 years old^[3]. So, the effect of sex hormones especially estrogen on

pathophysiology of DES was investigated; however, the relation between the menopause and the DES was not clearly understood. The clinical studies, performed on postmenopausal women taking hormone replacement therapy (HRT) consist of estrogen, revealed conflicting results. Some of these reported that HRT increased DES sign and symptoms [4-6] but the others reported HRT decreased them [7-11].

The drastically aggravated ocular surface epithelial disorders were accompanied by an increase in apoptotic epithelial cell death [12,13]. It was reported that estrogen protects pancreatic beta [14], retina ganglion [15], neuronal [16], bone [17], and heart [18] cells via inhibition of apoptosis. However, estrogen increases apoptosis in other cells type, such as osteoclasts [19], breast cancer cells [20], and leukemia cells [21]. To our knowledge, there is no report in the literature about the effect of estrogen on apoptosis in ocular surface cells.

A wide range of inflammatory, infectious, degenerative, or traumatic disorders of cornea, and severe DES may induce corneal neovascularization. During corneal neovascularization, an up-regulation of angiogenic factors must be present, most likely in association with a down-regulation of anti-angiogenic molecules. It was shown that vascular endothelial growth factor (VEGF) was up-regulated in vascularized corneas in humans and in animal models, subconjunctival and topical anti-VEGF (bevacizumab) therapy regressed the corneal neovascularization [22-24]. It was reported that estrogen up-regulated VEGF in breast [25,26], and endometrium [27]. However, to our knowledge, there are no reports in the literature about the effect of estrogen on VEGF expression in ocular surface cells.

Based on the effects of estrogen on apoptosis and VEGF expression in extraocular organ or tissue, our hypothesis is that estrogen may alter the apoptosis and VEGF expression in ocular surface. So, we investigated for the first time the effects of estrogen replacement therapy (ERT) on apoptosis and VEGF expression in ocular surface cells of the rats; ovariectomized (mimic as postmenopausal period) and non-ovariectomized (mimic as reproductive period).

MATERIALS AND METHODS

Materials Forty female, 4-month-old Wistar rats were included in the study. All protocols performed on experimental rats were approved by the Animal Ethics Committee of Adnan Menderes University, Aydin, Turkey and conformed to the standards in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were randomized to four groups: Group 1: ERT without ovariectomy; Group 2: ERT with ovariectomy; Group 3: only ovariectomy; Group 4: sham-operated.

Methods Intraperitoneal injection of Xylazine Hydrochloride (5mg/kg) and ketamine (25 mg/kg) were used for the anesthesia in ovariectomy and sham operations. Bilateral

ovariectomy was performed by dorsal flank approach as described previously [28]. Ovariectomy (group 2 and 3) and sham operations were performed on the same day. 17 β -estradiol(10 μ g/kg/day) was administered subcutaneously. Beta estradiol 3-benzoate, Sigma Cat: E8515 was used for the ERT. Estrogen was injected on the same time everyday. ERT was started one day after the ovariectomy and continued for 3 months. Four rats in group 1 were dead during the experiment. At the end of the third month, all rats were sacrificed in estrous cycles determined by vaginal smear test and their right eyes were enucleated. Posterior segment (retina-koroid) [29] and lens [30] parts of our experimental study were published earlier.

Immunohistochemical evaluation Avidin-biotin complex method was used for the immunohistochemical evaluation. Five μ m-thick sections obtained from formalin-fixed, paraffin-embedded tissue were placed on coated slides. Sections were treated twice for 5 minutes in citrate buffer (0.01mol/L, pH 6.0) in a microwave oven at 700 W after deparaffinization and dehydration. The slides were then cooled to room temperature for 1h. Endogenous peroxidase activity was blocked by immersing the sections in 3% hydrogen peroxide in methanol for 30 minutes. Sections were then incubated with primary antibody for 1 hour at room temperature. Biotinylated goat anti-rabbit secondary antibody was applied for 60 minutes at room temperature. Bound antibody was visualized with avidin-biotin- peroxidase complex (HISTOSTAIN-PLUS KITS, Zymed, San Francisco, USA, code no: 85-9843) for 1 hour at room temperature. The color was developed by 3,3'-diaminobenzidine tetrahydrochloride. Between steps, the slides were rinsed three times for 10 minutes in tris-buffered saline (pH 7.6). The antibodies used were as follows: VEGF (VEGF Ab-1, Neomarkers, USA, Dilution 1/100), Caspase-3 (pro-apoptotic, Caspase-3 Ab3, Neomarkers, USA, Dilution 1/100), Bcl-2 (anti-apoptotic, Bcl-2alpha Ab3, RTU-ready to use solution-, Neomarkers, USA). Apoptosis was examined using the terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling (TUNEL) method. For staining by TUNEL method, the recommended protocol for In Situ Cell Death Detection Kit, POD (code number 1684817, Roche, Fremont, Mannheim, Germany), was applied. The sections were finally counterstained with hematoxylin.

In the immunohistochemical staining a tonsil tissue sample was used for Caspase-3 and Bcl-2 and a hemangiosarcoma tissue sample was used for VEGF as positive control tissues. As negative control the primary antibody phase was skipped and staining process continued. All samples were examined by a light microscope (Olympus BX51). In addition, fluorescent attachment was used in one stage in TUNEL stain procedure. Interpalpebral zones of bulbar conjunctiva and entire corneal epithelium were sampled for examination.

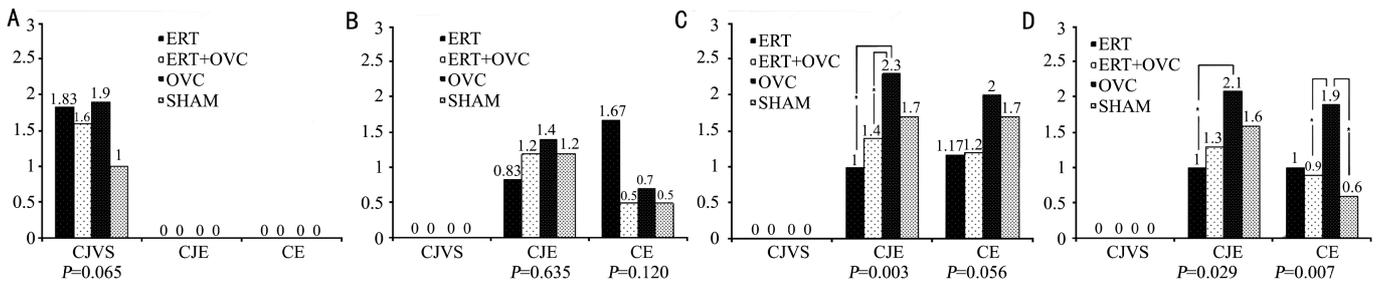


Figure 1 Vascular endothelial growth factor A: VEGF; B: Bcl-2; C: Caspase-3; D:TUNEL; scores of the conjunctival vascular structure (CJVS), conjunctival epithelium (CJE), and corneal epithelium (CE) (*statistically significant).

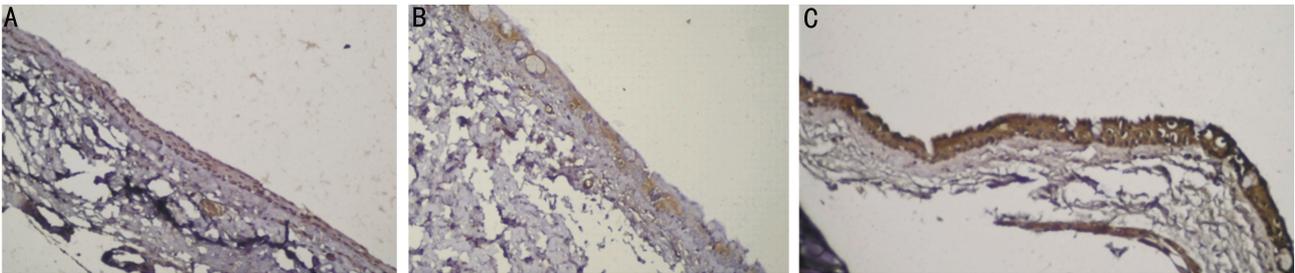


Figure 2 Immunohistochemical staining of caspase-3 in CJE A: Absent (DABx200); B Moderate(DABx400); C: Intense(DABx400).

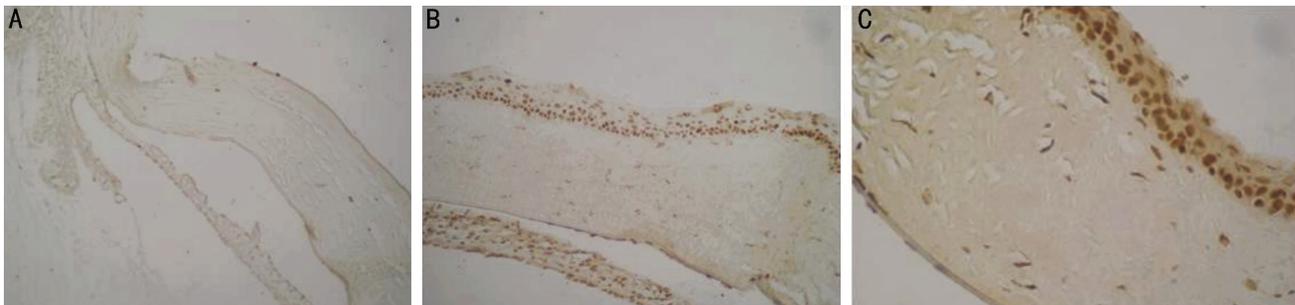


Figure 3 TUNEL staining in CE A: Absent (DABx200); B: Moderate (DABx400); C: Intense (DABx800).

Conjunctival vascular structure (CJVS), conjunctival epithelium (CJE), and corneal epithelium (CE) evaluated in the samples. The intensity of staining was scored by the same experienced investigator (EDC) semi-quantitatively on a scale of 0 to 3 as follows: (0); absent, (1); weak, (2); moderate, and (3); intense. The investigator was blind to the samples of the study groups.

Statistical Analysis Statistical analyses were performed with SPSS for Windows version 15.0 (SPSS, Chicago, IL, USA). Mean staining scores between the groups were compared via Kruskal-Wallis test ($P < 0.05$ as considered statistically significant). Mann-Whitney U test with Bonferroni correction ($P < 0.0125 = 0.05/4$ as considered statistically significant) were applied on significant results after Kruskal-Wallis test.

RESULTS

VEGF, TUNEL, caspase-3, and bcl-2 staining scores of the CJVS, CJE, and CE are shown in Figure 1. VEGF reactivity was observed in only CJVS; however, CJE and CE were stained by antibodies of caspase-3, bcl-2, and TUNEL procedure.

There were significant differences in CJE with regard to caspase-3 score and in CE with regard to caspase-3 and

TUNEL score via Kruskal-Wallis test (Figure 1). Caspase-3 expression of CJE was significantly higher in group 3 than group 1 ($P = 0.005$), and group 2 ($P = 0.007$). TUNEL score of CJE was significantly higher in group 3 than group 1 ($P = 0.006$). TUNEL score of CE was significantly higher in group 3 than group 2 ($P = 0.012$), and group 4 ($P = 0.002$). There was no significant difference between the groups with regard to bcl-2 and VEGF expressions. Figure 2 shows immunohistochemical staining of caspase-3 in CJE, and Figure 3 shows TUNEL staining in CE.

DISCUSSION

Pathogenesis of many ocular surface disorders has not been clearly understood, however apoptosis has an important role in pathogenesis of ocular surface disorders [12,13,31]. Yeh *et al* [31], evaluated the effect of experimental dry eye on ocular surface apoptosis and found that apoptosis significantly increased central corneal, peripheral corneal, bulbar conjunctival, and tarsal conjunctival epithelium; tarsal stoma; and lid margin. So, they stated that apoptosis may play a key role in the pathogenesis of keratoconjunctivitis sicca and may be a therapeutic target for this condition. Topical cyclosporine A is the first drug approved by FDA and increases tear production property in the therapy of

DES. Topical cyclosporine A It has been thought that the effectiveness of cyclosporine A was derived from inhibition of apoptosis in ocular surface epithelial cells [32-34]. Pathogenetic mechanisms of many chronic ocular surface disorders have some similarities with DES. Because of this, it has been reported successful result with topical cyclosporine A for the treatment of many ocular surface disorders such as atopic keratoconjunctivitis, ocular rosacea, ligneous conjunctivitis, and superior limbic keratoconjunctivitis [35-37].

Laboratory and experimental studies investigated the effects of estrogen on tear lipocalin [38], metalloproteinase-2 and -9 [39], lacrimal fluid peroxidase activity [40], mucin gene expression in the ocular surface epithelium [41], and proinflammatory cytokine (interleukin 1 β , 6, 8) gene expression in corneal epithelial cells [42]. However, there is no study in the literature about the effect of estrogen on apoptosis that plays a key role in the pathogenesis and treatment of ocular surface disorders. We found increased apoptosis in ocular surface epithelial cells after the experimental menopause rat model, and ERT decreased the apoptosis significantly. Our results suggest that estrogen may be beneficial for the treatment of apoptosis-mediated ocular surface disorders.

Nevertheless, in the clinical studies, performed on postmenopausal women taking HRT consist of estrogen, revealed conflicting results; some of these reported that HRT increased DES sign and symptoms [4-6] but the others HRT decreased them [7-11]. These conflicting results may be caused by polymorphism of estrogen receptor gene [43] or effects of the other factors apart from apoptosis on pathogenesis of ocular surface disorders. For example; Zylberberg *et al* [39]. reported that metalloproteinase-2 and -9 levels increased in tear of patients with DES and estrogen up-regulated metalloproteinase-2 and -9 expressions in rabbit lacrimal glands. Also, some HRT drugs may lead to controversy in results because estrogen combined with progesterone or androgen in some HRT drugs.

Another objective of our study was to investigate the effect of ERT on VEGF expression in ocular surface and we found no statistically significant difference between the groups. Cornea is an avascular tissue unlike conjunctiva, but it includes VEGF receptor. Corneal neovascularization is one of the most common problems encountered to ocular surface disorders. It was reported that VEGF was up-regulated in vascularized corneas like pathogenesis of retinal vascular disorders such as diabetic retinopathy [22]. It was known that estrogen up-regulated VEGF in extraocular tissues such as breast, and endometrium [25-27]. So ERT may be considered as a risk factor for corneal neovascularization. However, our results did not support this idea.

There are some limitations about our study. Four of 10 ovary-intact rats, given ERT, were dead before the end of

the experiment. This may affect the statistical results. We did not measure estrogen level in serum; absence of this data was considered as another limitation. However, we taken vaginal smears of all rats including sham-operated rats to control menstrual cycle and all rats were sacrificed in estrus of the estrous cycles.

Management of many ocular surface disorders is a chronic and difficult process. In the present study, we determined increased apoptosis in ocular surface epithelial cells in ovariectomized rats. Estrogen decreased the apoptosis, and did not change the VEGF expression between the groups significantly. Our results suggest that estrogen may be beneficial for the treatment of apoptosis-mediated ocular surface disorders. Further studies are needed on this subject for a better understanding of the role of estrogen and to provide a new insight for treatment and prevention of ocular surface disorders.

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