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The Comparison of the Effects of Estrogen and Melatonin against Corneal Disorders in Ovariectomized and Pinealectomized Rats

Elif Taslidere (Corresponding author)
Department of Histology and Embryology,
Faculty of Medicine, Inonu University, Malatya, Turkey
E-mail: eliftaslidere@hotmail.com
ORCİD ID: 0000-0003-1723-2556

Nigar Vardi
Department of Histology and Embryology,
Faculty of Medicine, Inonu University, Malatya, Turkey

Seda Tasdemir
Department of Pharmacology,
Faculty of Medicine Inonu University, Malatya, Turkey

Azibe Yildiz
Department of Histology and Embryology,
Faculty of Medicine, Inonu University, Malatya, Turkey

Abstract
The aim of the current study was to evaluate the role of estrogen (E) and melatonin (M) in preventing corneal disorders in ovariectomized (Ovx) and pinealectomized (Px) rats. Rats were randomly grouped into seven as follows: Sham-operated, Px, Bilateral Ovx, Ovx+Px, Ovx+Px+M, Ovx+Px+E, Ovx+Px+EM. Rats with Px, Ovx or Ovx+Px were housed for 5 months before the beginning of treatment. Melatonin-treated animals were injected with 5 mg/kg melatonin for 28 days. Estro-gen-treated animals received subcutaneous injections of 250 µg/kg β-estradiol17-cypionate once a week for 4 weeks. At the end of the study eyes tissues were fixed in 10%formol and was embedded in paraffin. Sections of tissue were cut at 5 µm, mounted on slides, stained with hematoxylin-eosin. The mean thicknesses of the total cornea, corneal epithelium, stroma and descement membrane from three different areas of each cornea were measured. Pinealectomy and ovariectomy caused an increase in the thickness of the epithelium, stroma, descement membrane and total cornea. The corneal thicknesses was higher in Ovx group than Px group. Making of ovariectomy and pinealectomy together didn't more affect the corneal thicknesses according to the individual. In conclusions, **e**strogen administration was more effective than melatonin in respect to preservation of corneal structures.

Keywords: Ovariectomy, Pinealectomy, Cornea, Estrogen, Melatonin

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1. Introduction

The cornea is the outermost avascular and transparent part of the eye consisting of epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium (Mittanamalli *et al.,* 2018 Sridhar *et al.,* 2018) Each layer has a specific important function, and a defect in any of these layers can lead to corneal disorders.

Women experience instabilities in sex steroids hormonal changes throughout their life span in association with puberty, ovarian cycles, pregnancy, and aged menopause (Giuffré *et al.,* 2007)

Estrogens are steroid hormones long known for their profound effects on both male and female reproductive systems. Estrogens regulate growth, differentiation, and function of diverse tissues both within and outside the reproductive system. The effects of estrogens are mediated by specific nuclear receptors, the estrogen receptor α and β types, that act as hormone-inducible transcription factors (Kumar *et al.*, 87, Evans *et al.*, 88, King *et al.*, 94). The receptors for estrogen were found in the nuclei of corneal epithelial, stroma and endothelial cell thereby suggesting that these tissues may well be target sites for hormonal influences (Wickham *et al.*, 20006, Suzuki *et al.*, 2001, Tachibana *et al.*, 2000).
Melatonin (MT) is chemically known as *N*-Acetyl-5-methoxytryptamine which, is a hormone produced mainly by the pineal gland from the amino acid precursor, L-tryptophan. It plays a major role in regulating the internal clock that sets daily events and in controlling diverse physiological functions, such as regulation of the circadian rhythm of sleep and waking (Reiter *et al.*, 91), anti-inflammatory actions (Esposito *et al.*, 2010), anticancer effects (Mediavilla *et al.*, 2010), antioxidant activity (Reiter *et al.*, 2014), and reduction of intraocular pressure (IOP) (Samples *et al.*, 88). There are three membrane MT receptor subtypes (MT1, MT2, and MT3) in the ocular tissues comprising the retina, ciliary body, lens, lachrymal glands, and cornea (Wiechmann *et al.*, 2009).
The aim of the present study was to evaluate the effects of estrogen and melatonin therapy on corneal disorders in ovariectomized (Ovx) and pinealectomized (Px) rats.

Materials and Methods

Experimental groups

Fifty-six female Wistar rats weighing 150–200 g were kept at a constant temperature (21 ± 2 ºC) and humidity (60 ± 5 %) in a controlled room in which a 12:12 h light–dark cycle was maintained. The rats were divided into seven groups of eight animals each by simple randomization methods as follows: 1) Sham-operated 2) Px 3) Bilateral Ovx 4) Ovx+Px 5) Ovx+Px+Melatonin (M) 6) Ovx+Px+estrogen (E) 7) Ovx+Px+EM. Rats with Px, Ovx or Ovx+Px were housed for 5 months before the beginning of treatment. Melatonin (Sigma Chemical Co., St Louis, MO, USA) was dissolved in ethanol and further diluted in saline (vehicle) (0.09 % NaCl, w/v) to give a fi nal concentration of 1 %. Melatonin-treated animals were injected with 5 mg/kg melatonin (i.p.) for 28 days. All injections were administered at 17:00 h. Estro gen-treated animals received subcutaneous injections of 250 μg/kg β-estradiol
17-cypionate (Sigma) in corn oil once a week for 4 weeks. Animals from sham-operated and Ovx+Px group received an equal volume (0.5 mL/kg) of vehicle solution. The experiment was performed in accordance with the Guidelines for Animal Research from the National Institute of Health and was approved by the Committee on Animal Research at Inonu University, Malatya. For experimental Px and Ovx in the same season, the rats were preoperatively anesthetized with a mixture of ketamine hydrochloride (75 mg/kg) and xylazine hydrochloride (8 mg/kg) intraperitoneally (i.p.). According to anatomic localization of pineal gland and ovaries, the skin was shaved with electric clippers and prepared with povidone-iodine (Poviiodeks; Kim-Pa Ilac Lab. Tic. Ltd. Sti., Istanbul, Turkey). During the surgical procedure asepsis was maintained with a local sterile environment.

Pinealectomy

Pinealectomy was performed as described by Hoffman and Reiter (Hoffman *et al.*, 65). The skin on the top of the head was cut to expose the skull. The animal was fastened to the dissection table; an incision was made in the skin and the subcutaneous tissue, bringing the lambda into view. The skullcap was opened with the aid of a micromotor, bringing the cerebral hemispheres and the superior sagittal sinus into view. The pineal gland , located under the venous sinus, was removed in one piece using forceps. Next, the bone fragment was returned to its place and the surgical layers were sutured. After surgery, the animals received a single dose of prophylactic antibiotic. The procedure was completed within 15 min. Pinealectomy was confirmed by the histological evaluation of the gland for each animal. Rats in the sham-operated group underwent similar surgical procedures without the removal of the pineal gland.

Ovariectomy

It was performed bilateraly under the anesthesia 4 cm midline laparotomy was made through the flank skin of the rat, and the ovaries and ovarian fat were removed. Ovaries were isolated
by ligation of the most proximal portion of the oviduct before removal (Waynfort *et al.*, 92). Immediately after surgery, 5 mg/kg of carprofen was injected subcutaneously for analgesia.

Histolopathologic Examination
At the end of the study eyes tissues were fixed in 10% formol and was embedded in paraffin. Sections of tissue were cut at 5 µm, mounted on slides, stained with hematoxylin-eosin (H-E).

Semiquantitative Evaluation
The mean thicknesses of the total cornea, corneal epithelium, stroma and descement membrane from three different areas of each cornea were measured using a Leica Q Win Plus Image Analysis System (Leica Micros Imaging Solutions Ltd, Cambridge, United Kingdom) at 40X.

Results
The stratified squamous epithelium, stroma and descement membrane were normal histologic appearance in the sham group. A thin layer of simple squamous endothelium was observed under the descement membrane (Figures 1). The mean thicknesses for the sham group were as follows: epithelium, 87,85 ± 16,9 µm; stroma, 280,21 ± 68,4 µm; descement membrane, 21, 53 ± 4,67 µm and total cornea, 397,93 ± 79,3 µm. Pinealectomy and ovariectomy caused an increase in the thickness of the epithelium, stroma, descement membrane and total cornea. The corneal thicknesses was higher in Ovx group than Px group.

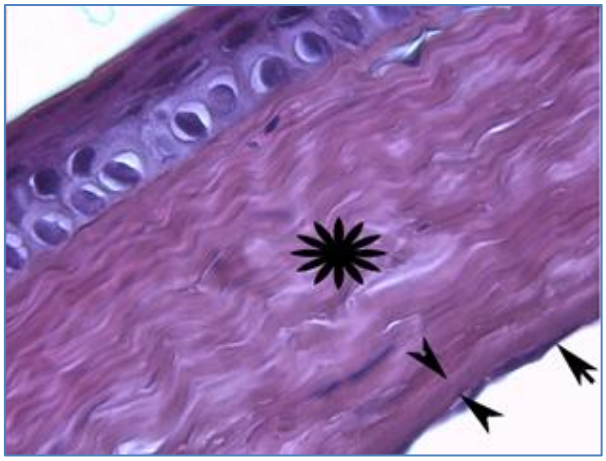

Figure 1: Sham group; the stroma (star), descement membrane (between two arrow heads) and endothelium (arrow) appear normal. H-E X100

The mean thicknesses for the Px and Ovx groups were as follows: epithelium, 99,58 ±29,7 and 144,55 ± 17,2 µm; stroma, 319,69 ± 64,7 and 396,69 ± 83,9 µm; descement mebrane 24,05 ± 7,26 and 24,89 ± 2,62 µm; total cornea, 439,16 ± 101,6 and 562,79 ± 97,6 µm (respectively). Making of ovariectomy and pinealectomy together didn't more affect the corneal thicknesses according to the individual. The mean thicknesses for the Ovx+Px group are as follows: epithelium, 100,12 ± 15,5 µm; stroma, 393,61 ± 40,2 µm; descement membrane 26,97 ± 4,33 µm, total cornea µm, 521,91 ± 45,2 µm. In Px, Ovx and Px+Ovx groups, the stroma was highly edematous. Wide detachment in the stroma were observed (Figures 2A, B and C).

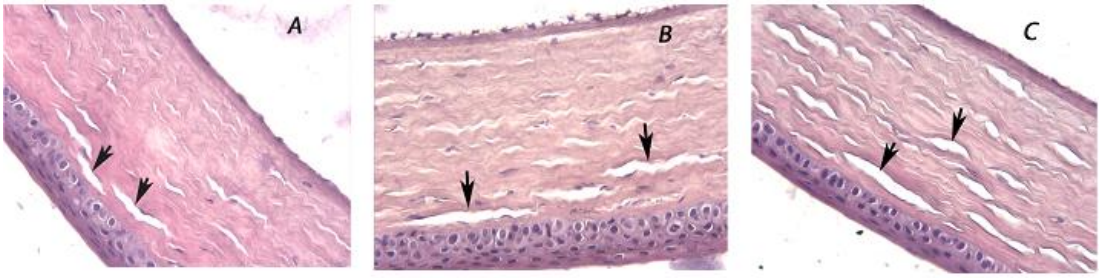

Figure 2: Px (A), Ovx (B) and Ovx+Px (C) groups; stromal detachment is prominent (arrows). H-EX40.

In addition, the lining of endothelial cells and shapes were destroyed in this groups. Also, vacuolization was seen in the cytoplasma of endothelium cells. The border of descement membrane distruption was detected especially in Ovx and Ovx+Px groups (Figures 3A, B and C).

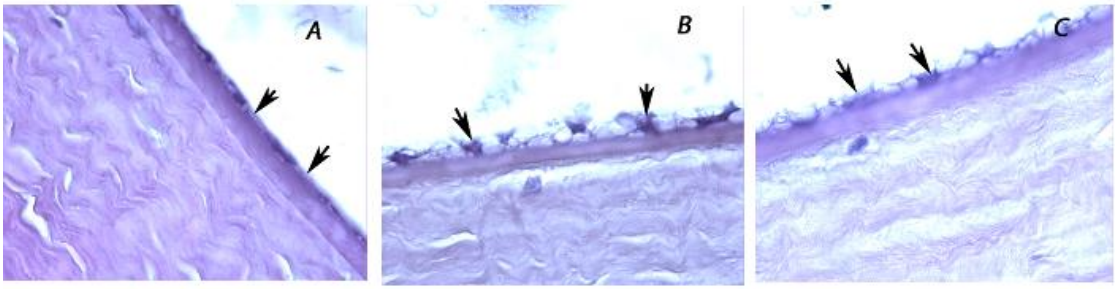

Figure 3: Px (A) group; vacuolisation (arrows), **Ovx (B) and Ovx+Px (C)**; distribution of shape in endothelium (arrows). H-EX100.

The most obvious improvement among the treatment groups occurred in the Ovx+Px+E group (epithelium, 84,48 ± 19,7 µm; stroma, 343,74 ± 62,4 µm; descement membrane 19,01 ± 2,48 µm, total cornea µm, 455,62 ± 56,1 µm) followed by the Ovx+Px+EM (epithelium, 87,89 ± 20,6 µm; stroma, 385,96 ± 46,0 µm; descement membrane 21,88 ± 2,1µm, total cornea µm, 513,68 ± 54,9 µm) and Ovx+Px+M (epithelium, 109,77 ± 12,4 µm; stroma, 468,23 ± 35,0 µm; descement membrane 24,13 ± 4,80 µm, total cornea µm, 598,94 ± 42,9 µm) groups (Figures 4A, B and C).

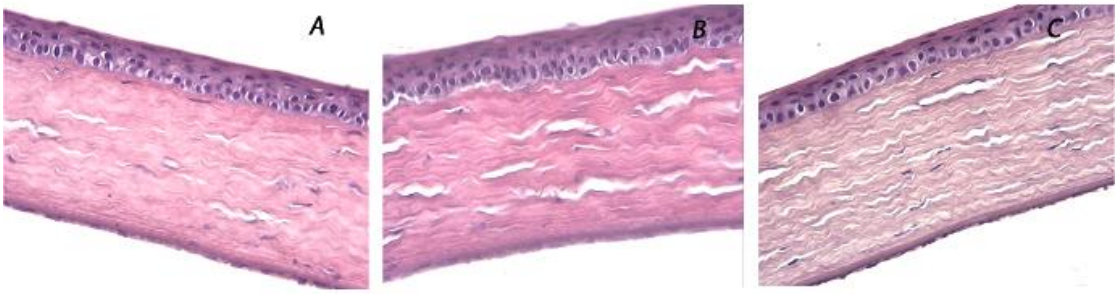

Figure 4: Ovx+Px+E(A), Ovx+Px+EM (B) and Ovx+Px+M (C) groups; the histological appereance of corneal structures. H-EX40.

Estrogens administration was more effective than melatonin in respect to preservation of corneal structures. Stroma detachment was not extensive in the estrogens-administered groups. Moreover, the layer of corneal endothelium was almost intact and more arranged except slight vacuolisation in the Ovx+Px+E and Ovx+Px+EM groups (Figures 5B and 6B). On the other hand, degenerative changes were still present in endothelium such as rupture between cells and desquamation in the Ovx+Px+M group (Figure 5C). The mean thicknesses of the corneal epithelium, stroma, descement membran and total cornea were statistically significantly different between the groups as shown in Table I.

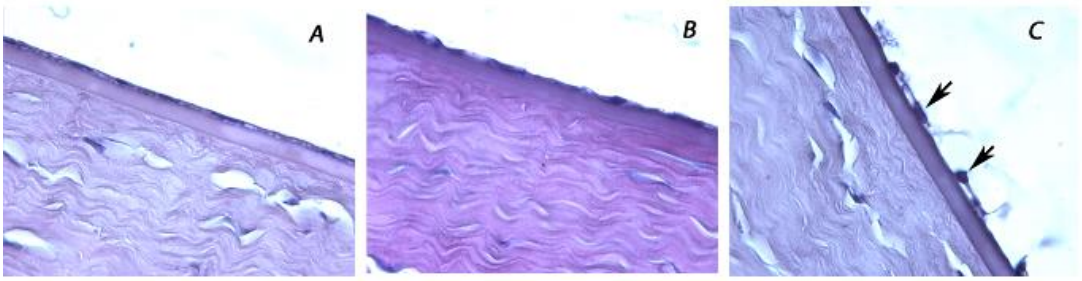

Figure 5: Ovx+Px+E(A), Ovx+Px+EM (B) groups; endothelial cells show almost normal histological structures except slight vacuolization, **Ovx+Px+M (C) group;** ruptered endothelial cells (arrows). H-E X100.

Table I. The mean thicknesses of the corneal epithelium, stroma, descement membran and total cornea of all groups.

Groups	Epithelium	Stroma	Descement membrane	Corneal thickness
Sham	87,85 ± 16,9	280,21 ± 68,4	21, 53 ± 4,67	397,93 ± 79,3
Px	99,58 ±29,7	319,69 ± 64,7	24,05 ± 7,26	439,16 ± 101,6
Ovx	144,55 ± 17,2[a,b]	396,69 ± 83,9[a,b]	24,89 ± 2,62[a]	562,79 ± 97,6[a,b]
Ovx+Px	100,12 ± 15,5[c]	393,61 ± 40,2[a,b]	26,97 ± 4,33[a]	521,91 ± 45,2[a]
Ovx+Px+M	109,77 ± 12,4 [a,c]	468,23 ± 35,0[a,b,f]	24,13 ± 4,80	598,94 ± 42,9[a,b,f]
Ovx+Px+E	84,48 ± 19,7[d,e]	343,74 ± 62,4[e]	19,01 ± 2,48[d,e,g]	455,62 ± 56,1[c,g,e]
Ovx+Px+EM	87,89 ± 20,6[d,e]	385,96 ± 46,0[a,b,e]	21,88 ± 2,15[g]	513,68 ± 54,9[a,e]

[a]Significant increase (P<0.05), vs. Sham group, [b]Significant increase (P<0.05), vs. Px group.[c]Significant decrease (P<0.05), vs. Ovx group, [d]Significant decrease (P<0.05), vs. Px group, [e]Significant decrease (P<0.05), vs. Ovx+Px+M group, [f]Significant increase (P<0.05), vs. Ovx+Px group. [g]Significant decrease (P<0.05), vs. Ovx+Px group

Discussion

The eye is a highly sensitive organ that is known to be impacted by changes in physiological conditions of the body (eg, menopause, pregnancy, menstrual cycle). Menopause has been reported to impact the structural integrity of cornea through changes in its curvature and thickness (Aydin *et al.*, 2007, Affinito *et al.*, 2003). Numerous studies have indicated that sex steroids (i.e. androgens, estrogens and progestins) may modulate the structural characteristics, functional attributes and/or pathological features of ocular tissues, and that these hormone actions may account for many of the gender-related differences in the eye. However, despite this endocrine influence, very little information exists concerning the precise mechanism(s) underlying sex steroid effects (Wickham *et al.*, 2000). Animal models have been used for investigation of pathogenic mechanisms of diseases due to menopause as well as for treatment modalities. The experimental model for cornea disease induced by ovariectomy and pinealectomy in female rats is also commonly used and is very useful for evaluation of problems related to cornea damage in postmenopausal women. The present study was to expose rats to one aspect of the ageing process via surgical pinealectomy (Px) and ovariectomy (Ovx) and evaluate the effect of melatonin and estrogen replacement against Ovx and Px rat model through the identification of histochemical alterations of the cornea tissue.

Sex steroid hormones, namely estrogen, progesterone, and testosterone, are produced by ovaries in females and testes in males. Although they circulate through blood, their effects rely on the receptors present in specific tissues and organs. These receptors are widely expressed in different ocular tissues, including the cornea. Corneal tissues express estrogen receptors types α and β progesterone receptors, and androgen receptors (Gupta *et al.*, 2005).

Corneal thickness varies throughout development to adulthood and from person to person (Mishima *et al.*, 86). A major contributor to corneal thickness has been associated with altered hormone levels with varying effects on corneal thickness occurring during pregnancy (Weinreb *et al.*, 88) and aging (Niederer *et al.*, 2007). Hormones mediate changes in cell function via binding to their respective receptors with both the androgen receptor (Rocha, *et al.*, 2000, Suzuki *et al.*, 2001). and estrogen receptor (Wickham *et al.*, 2000) being expressed within the human cornea suggesting that hormones may influence corneal function directly. Based on the expression pattern of α and β estrogen receptors in corneal cells, it has been postulated that estrogen is supplied through tears and aqueous humor at concentrations that are approximately half the concentrations found in plasma (Tachibana *et al.*, 2000). Hormone replacement therapies have been associated with a decreased incidence of ocular diseases, such as glaucoma (Sator *et al.*, 97) AMD (Smith *et al.*, 97) and cataract (Cumming *et al.*, 97, Klein *et al.*, 94) further suggesting a central role for estrogens in ocular physiology. The proposed mode of action of these steroidal hormones is via the regulation of gene expression in the nucleus, leading to changes in the concentration of ECM proteins, which are critical to the maintenance of corneal integrity (Khaled,*et al.*, 2017)

In this study, we found that pinealectomy and ovariectomy caused an increase in the thickness of the epithelium, stroma, descement membrane and total cornea. Also in Px, Ovx and Px+Ovx groups, the stroma was highly edematous. Wide detachment in the stroma were observed. In order that, these histopathological changes were improved significantly by estrogen treatment with compare to melatonin treatment. It is plausible that estrogen may be responsible for weakening the cornea via the stimulation of matrix metalloproteinases and the release of prostaglandins, causing activation of proteolytic enzymes for collagen and reduction in corneal-stiffness (Spoerl *et al.,* 2007).
In conclusions, we demonstrated that estrogen administration was more effective than melatonin against corneal disorders in ovariectomized (Ovx) and pinealectomized (Px) rats.

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