Effects of Melatonin Deprivation on Vaginal Squamous Cell Maturation of Pinealectomized Rats*

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Abstract

Objective: The pineal secretory product, melatonin is known to exhibit free radical scavenging ability. The integrity of the pineal gland and the presence of endogenous melatonin seem to be necessary to maintain ovarian function. Using Papanicolau method, the present study investigated the effect of pinealectomy on the vaginal epithelial maturation.
Materials and Methods: Twenty-one pinealectomized rats were randomly assigned into 3 groups with seven rats in each group. The groups consisted of sham-operated (control), pinealectomized only, pinealectomized animals treated with melatonin. Melatonin administration started at the 60th day following pinealectomy and continued for 21 days. At the end of the entire course, smears were obtained from each rats. Smears were stained with usual Papanicolau method, and observed with a light microscope by an experienced cytopathologist. Cytological grading was made according to the extend of parabasal, intermediate, superficial and anuclear squamous cells (Grade 1, 0-25% of cells; Grade 2, 25-50% of cells; Grade 3, 50-75% of cells; Grade 4, more than 75% of cells).
Results: Pinealectomized rats had similar scores for superficial and anuclear cells when compared to sham operated animals. Melatonin group had lower scores for superficial and anuclear cells than those of sham and pinealectomized group. All of three groups had same scores for intermediate and parabasal cells.
Discussion: The results of this study demonstrated that melatonin attenuates the improvement in vaginal stratification generally observed after pinealectomy. Pinealectomy may maintain the levels of superficial cells similar to conventional hormone replacement agents. Potential trophic action of pinealectomy on the vagina may improve the menopausal urogenital symptoms.

Keywords: vaginal smear, Papanicolau, pinealectomy, melatonin

Özet

Pinealektomiye Bağlı Melatonin Eksikliğinin Vajinal Skuamöz Hücre Maturasyonu Üzerine Etkileri

Amaç: Pineal bez ürünü melatonin serbest radikal süpürücü bir antioksidan olup, endojen melatonin varlığı ve pineal bezin bütünlüğü normal ovaryen fonksiyon için gereklidir. Bu çalışma, pineal bezin çıkartılmasına bağlı vajen epitelinde meydana gelen sitolojik değişikliklerinin Papanicolau yöntemiyle saptanması amacıyla planlandı.
Materyal ve Metot: Pinealektomi yapılmış 21 sıçan, her grupta 7 hayvan olmak üzere rasgele 3 gruba ayrıldı: Grup 1; kontrol (sham), Grup 2; pinealektomi ve Grup 3; pinealektomi+melatonin. Grup üçteki hayvanlara pinelaktominin 60. günü melatonin tedavisi başlandı ve 21 gün boyunca devam edildi. Tedavi sonrası tüm sıçanlardan vajinal "smear" alındı. "Smear"ler Papanicolau yöntemiyle boyanıp ışık mikroskobunda değerlendirildi. Sitolojik derecelendirme parabazal, intermediyer, yüzeyel ve nukleussuz skuamöz hücrelerin yaygınlığına göre yapıldı (hücrelerin %0-25'i Grade 1; %25-50'si Grade 2; %50-75'i Grade 3; >%75'i Grade 4).
Sonuçlar: Kontrol grubuyla karşılaştırıldığında, pineal bezi çıkartılan hayvanlar süperfisyel ve nükleussuz hücreler açısından benzer gradelere sahipti. Ancak melatonin verilen grupta süperfisyel ve anüklear hücreler kontrol ve pinealektomi grubundan daha düşüktü. İntermediyer ve parabazal hücreler bakımından gruplar arasında fark yoktu.
Tartışma: Pinealektomi vajinal hücre matürasyonunu artırırken, eksojen olarak verilen melatonin, matürasyonu baskılar. Pineal bezin çıkartılması klasik hormon replasman tedavisine benzer şekilde süperfisyel hücreleri artırır. Melatoninin antagonizmasının vajinal epitel üzerine olan potansiyel olumlu etkisi menapoz sonrası dönemde ürogenital semptomları önleyebilir.

Anahtar sözcükler: vajinal smear, Papanicolau, pinealektomi, melatonin

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Introduction

Melatonin, the pineal secretory product, is a lipophilic agent, and it easily passes through biological membranes within minutes after its peripheral administration. This gives an advantage to melatonin over other antioxidants which has a slower penetration (1,2). The mechanisms by which melatonin acts on the neuroendocrine systems to affect reproduction are not known. The *pars tuberalis* is a morphologically distinct and highly vascularised region of the pituitary, which connects the median eminence of the hypothalamus with the *pars distalis* of the gland (3). Importantly, melatonin attenuated the gonadotrophin-releasing hormone (GnRH) induced increase in luteinizing hormone (LH) secretion from the ovine *pars tuberalis* (4). Changes in the duration of melatonin secretion constitute a signal to the neural structures controlling the secretion of gonadotrophins from the pituitary gland. In a number of rodent species and other mammals, the release of pituitary gonadotrophic hormones, follicle-stimulating hormone (FSH) and LH, often occurs on a rhythmic basis (5,6). Melatonin has marked anti-gonadotrophic properties, such as inhibition of gonadal development, spermatogenesis and androgen production in males and absence of follicles in female rats (5,6). Melatonin has an inhibitory effect on the incidence of vaginal estrus in the rat because circulating melatonin is taken up by the brain and is concentrated by the ovary and pituitary (7). After pinealectomy, the vagina of female rats cornifies, with the animals experiencing constant estrus (8,9). There is only isolated references in the literature where melatonin has been administered to pinealectomized rats with the aim of observing the effects on vaginal cytology (8). Therefore, the aim of this study was to examine the effect of melatonin deprivation on the development of cytological changes in the vagina, as well as to identify the effects of exogenous melatonin administration to pinealectomized rats.

Materials and Methods

Experimental conditions

Twenty-one female Wistar rats aged 6-8 week and weighting 150-200 g were placed in temperature (21±2°C) and humidity (60±5%) controlled room in which a 12:12 hours light:dark cycle was maintained. Food and water were available *ad libitum*. Aseptic procedures were initiated five days before the surgical procedures. The rats were divided into three groups of 7 rats each; pinealectomy: received diluted 1% ethanol with saline (vehicle), pinealectomy+melatonin (4 mg/kg) and sham (control)+vehicle. Rats in sham-operated group underwent similar surgical procedures with no removal of pineal gland. Since it was reported that pinealectomy may cause hypertension beyond 60 days, we used rats that were pinealectomised two months before smear evaluation to eliminate any possible effect of pinealectomy-induced hypertension. Rats were pinealectomised or sham operated 2 months before the beginning of the all injections. Melatonin or vehicle was administrated daily intraperitoneally (i.p.) by injection for 21 days after 60 days of pinealectomy. After the treatment with melatonin or vehicle, smears were taken from all rats. Melatonin (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in ethanol and diluted in saline to give a final concentration of 1% ethanol. It has been shown that small doses (1-10 mg) melatonin administration depress the growth of the immature rat ovary and the subsequent incidence of estrous vaginal smears in the adult rat (8,9). Because of the very variable melatonin dosage schemes reported in literature, we administrated melatonin at the dose of 4 mg/kg which concentration was previously used for blocking production of reactive oxygen species successfully.

Pinealectomy

Pinealectomy and sham-operation was achieved essentially as described by Hoffman and Reiter (10). Rats were anesthetized preoperatively by an intraperitoneal (i.p.) injection of a mixture consisting of ketamine hydrochloride (75 mg/kg) and xylazine hydrochloride (8 mg/kg). Pinealectomy was completed within 15 min. All experiments in this study were performed in accordance with the guidelines for animal research from the National Institutes of Health and were approved by the Committee on Animal Research at Inönü University.

Smear collection and slide preparation

Smears were taken from rats using a cotton swab; swab was inserted into the vagina and rotated 360° clockwise direction,

Table 1. Summary table for means ± standard error and significance levels according to univariate analysis of variance for superficial, anuclear, parabasal and intermeadiate cells for each of the experimental groups (n=7 rats per group)

Groups	Parabasal	Intermediate	Superficial	Anuclear
1-Sham	1.14±0.14	1.00±0.00	1.71±0.18	1.85±0.14
2-Pinealectomy alone	1.14±0.14	1.14±0.14	1.71±0.18	1.85±0.14
3-Pinealectomy+melatonin	1.28±0.18	1.28±0.18	1.00±0.00	1.14±0.14
	p values*			
1 vs 2	1.000	0.463	1.000	1.000
1 vs 3	0.530	0.151	0.004	0.002
3 vs 2	0.530	0.463	0.004	0.002

(1) Sham-operated control group, (2) Melatonin group (pinealectomized rats treated with melatonin 4 mg/kg/day) (3) Pinealectomized alone group (pinealectomized rats treated with 1% ethanol with saline).

* The mean difference is significant at the 0.05 level.

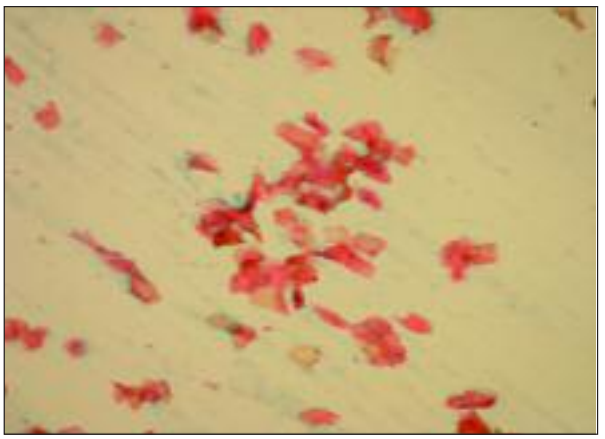

Figure 1. Vaginal smear of sham operated animal. Anuclear squames and superficial cells are seen (Papanicolau staining, x40).

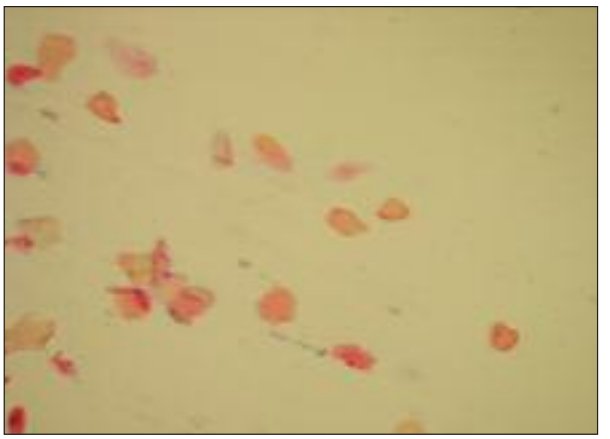

Figure 2. Vaginal smear of pinealectomy alone animal. Anuclear squames and superficial cells are seen (Papanicolau staining, x40).

then the swab was smeared onto a glass slides and fixed with ethanol. Smears were stained with usual Papanicolau method, and observed with a Nikon labophot microscope by an experienced cytopathologist uninformed of the rat groupings. A cytological grading was made according to the extent of parabasal, intermediate, superficial and anuclear squamous cells. Each slide was evaluated for each cell type. The grading was as follows: Grade 1, 0-25% of cells; Grade 2, 25-50% of cells; Grade 3, 50-75% of cells; Grade 4, more than 75% of cells.

Statistical analysis

The Statistical Package for Social Sciences (SPSS), version 10.0 was used for statistical analysis. The normality of the distribution was assessed with Kolmogorov Smirnov Z test and found to be normal. One way ANOVA and *post-hoc* multiple comparisons were performed by LSD on the parabasal, intermediate, superficial and anuclear cells to reveal any significant difference between the groups. The results are given in the text as means ± standart error (SE). For all tests, statistical significance was defined as $p<0.05$.

Results

The mean cytological scores of all groups were shown in Table 1. Smears of sham-operated rats revealed dominance of superficial and anuclear cells. Pinealectomized rats had similar scores for superficial and anuclear cells when compared to sham operated animal. Pinealectomy+melatonin group had predominantly intermediate and parabasal cells with scattered superficial and anuclear cells. Pinealectomy+ melatonin group had lower scores for superficial and anuclear cells when compared to sham and pinealectomy alone ($p<0.005$). It is interesting to note that melatonin administration was capable of preventing the changes in vaginal cellularity of pinealectomized rats (Figures 1, 2 and 3).

Discussion

Theoretically, melatonin should communicate either directly or indirectly to the neuronal GnRH to regulate seasonal changes in reproduction (11,12). One possible explanation of unchanged superficial and anuclear cells in pinealectomised rats is that pinealectomy-induced melatonin deprivation increases the incidence and extent of vaginal stratification by leading to increase in GnRH secretion. A recent study had showed that melatonin attenuated the GnRH-induced increase in LH secretion from the ovine *pars tuberalis* (4).

Early puberty, ovarian atrophy, chronic anovulation, permanent estrous condition, and hyperprolactinemia have been described in rodents submitted to pinealectomy (13). Pinealectomized rats had similar scores for superficial and anuclear cells when compared to sham operated animals. This finding observed in the pinealectomized group was probably a result of the anti-gonadotrophic effects of melatonin (11,12). Pinealectomy probably produced gonadotrophic alterations, leading to vaginal stratification (8). The absence of melatonin may have modified gonadotrophin secretion, increasing the synthesis of LH and reducing the synthesis of FSH (5,6,8). The LH

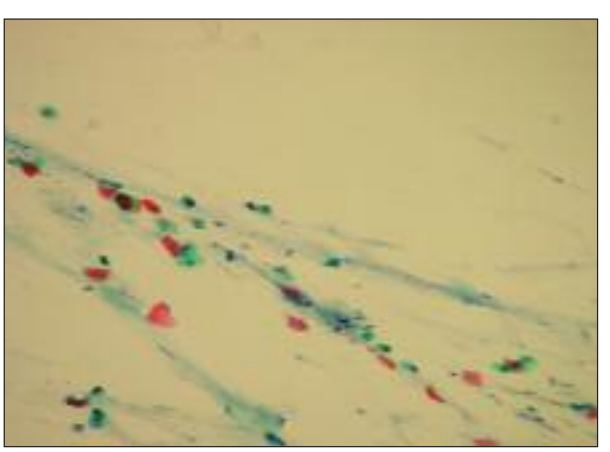

Figure 3. Vaginal smear of pinealectomy+melatonin treated rat. Parabasal epithelial and intermediate cells are seen (Papanicolau staining, x40).

increase may have been responsible for the superficial and anuclear cells proliferation observed in the pinealectomized animals. Wurtman et al. (14) demonstrated that, pinealectomy in the immature rat is also followed by enhanced ovarian growth; this hypertrophy is blocked by the administration of bovine pineal extracts. Recent study showed that after pinealectomy the ovaries developed a polycystic aspect at 4 months (15). Other supporting findings indicate that circulating estrogens increase in pinealectomized rats. Teixeira observed that the endometrium of rats submitted to pinealectomy presented hyperplasia, which was reversed with the use of melatonin (16).

Melatonin administration prevents the vaginal maturation attributed to pinealectomy, and this is accompanied by decrease in superficial and anuclear cells compared to that observed after pinealectomy. The reduction in superficial and anuclear cells in the melatonin group may be explained as a partial compensation by exogenous melatonin administration due to the fact that plasma melatonin levels are reduced by pinealectomy. Melatonin acts directly by affecting the hypothalamic functions involved in the inhibitory regulation of GnRH (11,12). Exogenous melatonin administration may attenuate the GnRH-induced increase in FSH, LH and estrogen secretion. Recent study in adult female rat demonstrated that, injection of large doses of melatonin in the afternoon of proestrus can inhibit the preovulatory LH and can block the expected ovulation (6). Similarly, in rodents, melatonin has marked anti-gonadotrophic properties, such as absence of follicles, *corpora lutea* and interstitial tissue proliferation (5,6). When rats are kept in constant light there is an increase in the incidence of estrous phases. A single dose of melatonin counteracts this effect of light. Chu et al. (8) have found that melatonin inhibits the increase in the incidence of estrous phases which follows pinealectomy. Another possibility is that melatonin acts directly by affecting the hypothalamic functions involved in the inhibitory regulation of GnRH (11,12). The observations that pinealectomy is associated with an increased incidence of estrus, and that this increase is inhibited by exogenous melatonin administration, indicate that endogenous melatonin release may play a role in the estrous cycle.

In conclusion, melatonin is a potent antioxidant without the undesired stimulatory effects of estrogens on uterine tissues. The integrity of the gland and the presence of endogenous melatonin seem to be necessary to maintain ovarian function. The effects that are caused by the removal of the pineal gland reversed by the exogenous replacement of the gland's product, the melatonin. For that reason, melatonin antagonism may benefit some women in alleviating menopausal symptoms or at least for a potential trophic action on the vagina. Vaginal atrophy and dryness is a common symptom among women in the late menopause. Symptoms of vaginal atrophy include dryness, itching, vaginitis, and dyspareunia. The association between vaginal dryness and low estrogen levels is clear (17). Systemic administration of estrogen, including oral and transdermal preparations, has been shown to be effective in treating vaginal atrophy. Locally released estrogen in the form of vaginal rings, vaginal creams, pessaries, and slow-release estradiol tablet also has been shown to be effective (18). Phytoestrogens have been studied for their effect on vaginal dryness and atrophy, and they appear to have no effect (19). Nonhormonal products for vaginal dryness and difficulty with intercourse are commercially available (20). Pinealectomy may maintain the levels of superficial cells that is similar to conventional hormone replacement agents. Further experiments are required to complete the agonist profile of this procedure and to test its efficacy in animal models of vaginal cytology, before its use in the treatment of atrophic vaginitis in humans.

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