Role of Melatonin on Follicle Development and Apoptotic Changes in Pinealectomized Rat Ovary

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OBJECTIVE: The aim of this study is to determine the effect of pinealectomy and exogenous melatonin
STUDY DESIGN: Twenty-one Wistar rats were divided into 3 groups: (I) Sham-operated (Non-Px), (II) pinealectomized (Px), (III) pinealectomized and melatonin. Sham and Px groups had received vehicle whereas group III had received 4 mg/kg melatonin. Terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end-labeling activity (TUNEL) assessment was used to identify follicles that contain fragmented nuclei, an indication of apoptosis. TUNEL staining was evaluated semi-quantitatively and ovarian cysts were counted.
RESULTS: In Px group, TUNEL activity was significantly higher than sham group. Melatonin administration did not reduce the intensity of TUNEL positive follicles. The difference between pinealectomy and melatonin groups regarding TUNEL activity was insignificant. Significantly increased ovarian cysts were detected after pinealectomy and melatonin did not prevent this increase. Px and melatonin groups were not significantly different than each other regarding the TUNEL activities and ovarian cysts.
CONCLUSIONS: Pinealectomy increases ovarian cysts and apoptosis in the follicles that can not be prevented by melatonin which was probably a result of the dose and duration of melatonin.

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Key Words: Rat ovary, Apoptosis, Ovarian cyst, TUNEL, Pinealectomy, Melatonin

Introduction

Melatonin has been implicated in the control of physiological processes, including circadian rhythmicity and the photoperiodic control of seasonal breeding in mammals. Humans are not seasonal breeders. On the other hand, seasonal fluctuations have been described in human and melatonin also appears to exert an important role in the neuroendocrine regulation of human reproduction.[1] In mammalian ovary, small fraction of oocytes ovulates during the reproductive life, whereas the majority of ovarian follicles undergo atresia by a hormonally regulated apoptotic mechanism. Recent studies have demonstrated that apoptotic cell death is associated with follicular atresia in chicken, porcine, and rodent ovaries.[2,3] Melatonin is a chemical mediator produced mainly in the pineal gland.[4] It has also been shown to modulate immune functions, growth processes and oxidative reactions.[5,6] Apoptosis is a highly regulated and programmed suicide mechanism of the cell.[2,3] The most recently described property of melatonin is its antiapoptotic effect in thymocytes[7] and neuronal cells.[8] However, the mechanisms by which melatonin acts on the neuroendocrine systems are not known. Molecular study on sheep pars tuberalis, a major neuroendocrine target for melatonin, have shown that melatonin alone has no direct effect on a number of intracellular signal transduction processes.[9] While melatonin has a progonadotrophic effect in photoperiodic species, it has antigonadotrophic properties in rodents.[10,11] The down-regulation of gonadotropin-releasing hormone (GnRH) gene expression and secretion in GT1-7 cells by melatonin supports the hypothesis that the hypothalamus is a major target tissue for the antigonadal action of melatonin.[12] Moreover in vitro melatonin inhibits GnRH induced luteinizing hormone (LH) release by cultured rat pituitary glands.[13] Melatonin also inhibits GnRH induced increases in cAMP, diacylglycerol and c-Fos in neonatal rat gonadotrophs.[14] As a result, melatonin reduces the release of LH and follicle-stimulating hormone (FSH).[15] Implants or infusion of melatonin in the hypothalamus mimic or block photoperiodic responses in several species.[12,16,17] Deboleena et al.[12] showed the inhibitory nature of melatonin on GnRH secretion.[12] Because treatment with GnRH or its agonists antagonizes the gonadotropin stimulation of follicle development, GnRH has been suggested to induce atresia in the ovary.[18] Freeman et al.[19] demonstrate that GnRH may act as an atretogenic factor for follicles and increase atresia. In addition to antioxidant and antiapoptotic properties, inhibitory effect of melatonin on GnRH secretion prompt us possible clinical ap-

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plications of this chemical in the prevention of free radical damage and apoptosis in follicular atresia. The present study tests this hypothesis by assessing preventive effects of melatonin on apoptotic changes in pinealectomized rats ovary.

Material and Methods

Experimental conditions

Twenty-one female Wistar rats, aged 6-8 weeks, and 150-200 g of weight were kept in temperature (21-22 C) and humidity (60±5%) controlled conditions. A 12:12 hr light and dark cycle was maintained. Food and water were available ad libitum. Rats were divided into three groups of seven rats per each: (I) Sham-operated rats (Non-Px), (II) Pinealectomized and placebo-treated rats (Px), (III) Pinealectomized and melatonin-treated rats (Px+melatonin).

Pinealectomy

Pinealectomy was performed as described by Hoffman and Reiter.[20] Rats were anesthetized preoperatively by intraperitoneal (i.p.) application of a mixture consisting of ketamine hydrochloride (75 mg/kg) and xylazine hydrochloride (8 mg/kg). The entire procedure was completed within 15 min. Rats in sham-operated group underwent similar surgical procedures with no removal of pineal gland.

Melatonin Treatment

Melatonin (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in ethanol and diluted in saline to give a final concentration of 1% ethanol. Rats in sham and Px groups had only received vehicle whereas group III had received 4 mg/kg melatonin. This dose was chosen as it was previously used for the blocking of the production of reactive oxygen species.[21] Pinealectomy in the rat induces a significant increase of the arterial blood pressure within 15 days from the surgical procedure and this increase is still present 30 and 60 days after pinealectomy.[22] To avoid from hypertension, melatonin administrated after the 60th day of pinealectomy on a daily basis for 21 days. At the end of study, rats were sacrificed and the ovaries were removed.

Histopathological analysis

All harvested ovarian specimens were fixed in 10% formaldehyde solution for 24 h. After fixation they were processed in usual manner, and embedded in paraffin. Five-micrometer thick sections were taken onto polylysine-coated slides. Then the slides were deparaffinized in usual manner (oven at 65 C for 1 hour, xylene treatment, and through graded alcohols to water). In the present study, TUNEL assay was used to identify cells containing fragmented nuclei, an indication of apoptosis. After deparaffinization, TUNEL (in situ cell death detection kit, Roche Applied Science, Indianapolis, USA) kit was used according to the manufacturer's instructions. The steps taken were as follows: Inhibition of endogenous peroxidase in 0.3 % H2O2 in methanol for 30 min; rinse in tap water and soaking in 50 mM Tris-buffered saline with a pH of 7.6; pretreatment with microwave for 15 min in sodium citrate buffer; rinse in tap water and soaking in TdT buffer for 5 min; incubating with TdT mixture at 37 ^{O}C for 60 min. Tdt mixture contains dUTP which is biotin-labelled, allowing binding of peroxidase-labeled streptavidin. And then, rinse in 10mM phosphate-buffered saline (PBS), pH 7.2; incubating with peroxidase-labeled streptavidin for 15 min; rinse in 10mM PBS; reacting in the diaminobenzidine solution for 10 min; rinse in in tapwater; staining with Mayer's hematoxylin for 1 minute; rinse in tap water; dehydrating with xylene and mounting. TUNEL stained slides were observed using a Nikon Labophot microscope by a pathologist. Staining was evaluated semi-quantitatively as follows: (0) no staining, (+) mild, (++) moderate, and (+++) intense staining according to the dissemination of the damage. The biochemical hallmark of apoptosis was internucleosomal DNA fragmentation. TUNEL positive follicles contained condensed nuclei, which was a typical feature of cells undergoing apoptosis. Follicular atresia and apoptotic changes were evaluated in the oocyte and granulosa cells and cysts were counted at a light microscopic examination.

Data analysis

Statistical analyses were performed using The Statistical Package for Social Sciences version 10.0 (SPSS Inc, IL, USA). Results are given in the text as mean ± standart error (SE). Difference between the experimental groups with respect to TUNEL activities and ovarian cysts were tested using one-way ANOVA and post-hoc multiple comparisons (Least significant difference, LSD). The level of significance was $p<0.05$.

Results

The semi-quantitative evaluation of TUNEL activities are shown in Table 1.

*Table 1: Semi-quantitative evaluation of TUNEL activity**

Groups	Grade of Staining			
	0	+	++	+++
Non-Px (n=7)	7	0	0	0
Px (n=7)	0	0	4	3
Px + Melatonin (n=7)	0	1	3	3

**:Light microscopy was used to evaluate dissemination of follicular damage. Staining was graded semi-quantitatively as follows: (0) no staining, (+) mild, (++) moderate, and (+++) intense staining. Non-Px: Sham operated rats, Px: Pinealectomized rats, Px + melatonin: Pinealectomized rats with exogenous melatonin treatment.*

Sham group had normal oocyte nuclei that were counterstained blue by hematoxylin and were not stained for TUNEL. Px group, however, had extensive TUNEL positive apoptotic oocytes which were stained brown, indicating the presence of

DNA fragmentation. Pinealectomy group had also some apoptotic inflammatory cells (Fig. 1) which are presumably apoptotic granulosa cells. Consequently, compared to sham group, significantly higher TUNEL activities were observed in the Px group (0.00±0.00 vs. 2.42±0.20, p=0.00). Surprisingly, melatonin treatment did not reduce TUNEL positive oocyte and granulosa cells (2.28±0.28). According to sham group, significantly increased ovarian cysts were detected in the Px rats (1.42±0.20 vs. 5.57±0.48), (Fig. 2a) and melatonin did not prevent this increase (5.28±0.42), (Fig. 2b). Finally, Px and melatonin groups were not significantly different than each other regarding the ovarian cysts (p=0.608) and TUNEL activities (p=0.623) at the end of study.

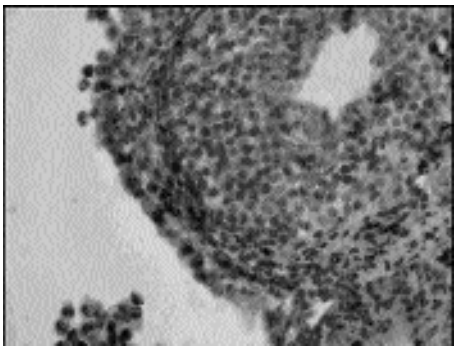

Figure 1: Intense nuclear staining in the follicle after pinealectomy (TUNEL staining x 40)

Figure: 2a

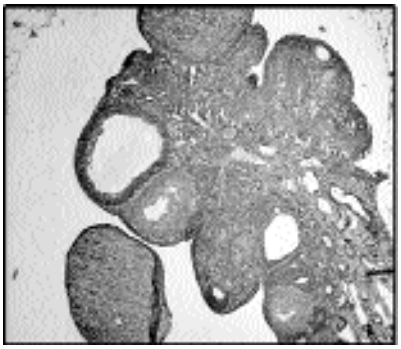

Figure: 2b

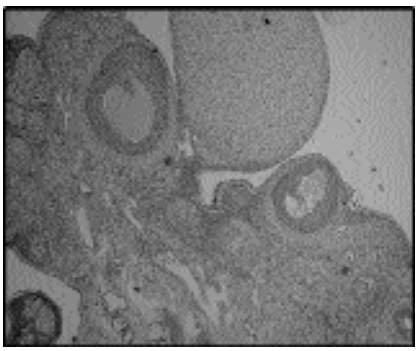

Figure 2: Morphological aspects of the ovarian polycystosis of the pinealectomized rats (a). Exogenous melatonin did not cause regression of ovarian cysts (b) (H&E x10).

Discussion

Recent study we showed the disruptive effects of pinealectomy on rat brain were prevented by melatonin treatment.[23] We have extended our series of study on melatonin to cover its in vivo effects on follicular development, atresia and programmed cell death. The molecular mechanism which underlies apoptotic DNA fragmentation with associated follicular atresia is unknown. The vast majority of ovarian follicles never ovulate and form corpora lutea, but become atretic at different stages of follicular development.[24] Ovarian apoptosis can be regulated in vivo through different pathways. Previous studies demonstrate that sex steroids and gonadotropins have been shown to modulate the incidence of atresia in the ovary .[25,26] In rats, GnRH has been shown to act through specific ovarian receptors [27] to block many of the trophic actions of gonadotropins.[28] Estrogens and FSH inhibit, whereas androgens [29] and GnRH enhance internucleosomal DNA fragmentation. A specific endonuclease responsible for digesting DNA is present in the ovary.[30,31]

Melatonin acts directly by affecting the hypothalamic functions involved in the inhibitory regulation of GnRH [4,21] Theoretically, melatonin should communicate either directly or indirectly to the neuronal GnRH to regulate seasonal changes in reproduction. One possible explanation of increased follicular atresia after pinealectomy is that melatonin deprivation increases the extent of apoptotic cell by leading increase in GnRH secretion. A recent study had showed that melatonin attenuated the GnRH-induced increase in LH secretion from the ovine pars tuberalis.[32] It is possible that the action of pinealectomy on the ovarian endonuclease activity is mediated through a Ca2+-dependent pathway, because GnRH has been shown to increase intracellular Ca2+ concentrations in granulosa cells.[33,34] Study, in prepubertal rats, reported that GnRH-a treatment produces an increase in ovarian follicle DNA fragmentation by interfering with the FSH, cAMP and growth factors.[35] Billig et al.[36] demonstrated that GnRH acts as an atretogenic factor on granulosa cells and it increases apoptotic DNA fragmentation in a time and dose dependent manner. Another explanation of the high apoptotic cells in Px rats most likely are the result of decreased antioxidant capacity. Recently, data have been provided to suggest that the generation of reactive oxygen species in cells plays a fundamental role in the initiation of cell death.[37] Csaba et al.[38] reported that pinealectomy causes atrophy in the thymus gland. Administration of antioxidants such as melatonin inhibits apoptotic cell death in the thymus gland[7,39] Similarly melatonin counteracted bone marrow toxicity which was caused by chemotherapy.[40] Tilly et al.[41] showed that granulosa cell apoptosis in rat ovarian follicles deprived of tropic hormone support is prevented by treatment with inhibitors of oxidative free

radical formation.

There was a statistical difference between sham and Px groups regarding the number of apoptotic cells. On the other hand if Px causes an increase in GnRH and estrogen levels and these agents cancel each others effects on cell apoptosis then there should be no difference between the Px and the sham groups. Although this observation seem contradictory, a careful consideration of the events that happens after the removal of the pineal gland may give a possible explanation to these findings. Px causes a decrease in melatonin level which subsequently causes an increase in GnRH and estrogen. Exogenous melatonin administration may attenuate the GnRH-induced increase in FSH, LH and estrogen secretion .[42] Another plausible possibility is that melatonin acts directly by affecting the hypothalamic functions involved in the inhibitory regulation of GnRH.[4,21] Studies demonstrated that, in rodents, melatonin has marked anti-gonadotrophic properties, such as absence of follicles and corpora lutea.[43] Tilly et al.[41] showed that granulosa cells within antral follicles collected rat ovaries exhibited extensive apoptosis after a 24-h incubation in the absence of tropic hormone support. Inclusion of FSH in the culture medium markedly reduced the extent of apoptosis [41,44] Other supporting findings indicate that circulating estrogens increase in pinealectomized rats. Teixeira et al.[45] observed that the endometrium of rats submitted to pinealectomy presented hyperplasia, which was reversed with the use of melatonin. However, in present study, melatonin replacement did not reverse the events causing cell death as there was no statistical difference between Px and melatonin groups. Pharmacologically this means that the effects that are caused by the removal of the pineal gland are not the consequences of the induced lack of melatonin.

There was a statistical difference between sham and Px regarding the ovarian cysts. Pinealectomy induced melatonin deprivation possibly produced gonadotrophic alterations, leading to ovarian cyst development. The decreased levels of of melatonin after pinealectomy may have modified gonadotrophin secretion, increasing the synthesis of LH. Prata et al.[46] reported a strong relationship between melatonin and the rodent equivalent of PCOS. However, in the present study, Px and melatonin groups were not significantly different than each other regarding the ovarian cysts. This may be result of the dose and duration of melatonin administration. Another possibility is that occurence of ovarian cysts after the pinealectomy may not the consequences of the decreased melatonin levels.

Pinealectomy induced melatonin deprivation indirectly induces apoptotic cell death in the ovary. Pinealectomy related follicular atresia may be initiated, at least in part, as a consequence of increased GnRH secretion and inadequate protection of maturing granulosa cells from the damaging effects of reactive oxygen species. This apoptosis cannot be prevented by exogenous melatonin. This study have some limitations, but this findings provide a basis for future studies on the mechanism underlying follicular atresia and on the signal transduction pathway involved in melatonin regulation of the apoptotic changes responsible for follicular damage. However this hypothesis must be carefully tested on several occasions where varying levels and durations of melatonin administration are used.

Melatoninin Pinealektomize Rat Overinde Folikül Gelişimi ve Apopitotik Değişikliklere Etkisi

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Bu çalışma ovaryan morfoloji, oosit ve granuloza hücre apopitozisine pinealektomi ve ekzojen melatonin replasmanının etkisini saptamak amacıyla planlandı. Yirmibir adet Wistar rat üç guruba ayrıldı: (I) Sham grup (Non-Px); (II) pinealektomize grup (Px); ve (III) pinealektomize + melatonin grup. Sham ve Px gruba etanol verilirken, III. gruba 4mg/kg melatonin verildi. Foliküllerdeki apopitotik değişiklikleri saptamak için terminal deoksinukleotidil transferaz mediated deoksiuridin 5- trifosfat (TUNEL) yöntemi kullanıldı. TUNEL ile boyanmış alanlar semiquantatif olarak sayıldı ve ovaryan kist sayıları kaydedildi. Pinealektomize grupta, TUNEL aktivitesi sham grubunda gözlenenden belirgin olarak daha yüksekti. Melatonin uygulaması TUNEL ile boyalı folikül sayısını azaltmadı. Pinealektomi ve melatonin grupları arasında TUNEL aktivitesi bakımından belirgin bir fark yoktu. Pinealektomi sonrası ovaryan kist oluşumunda belirgin artış oldu ve melatonin uygulaması bu artışı önlemedi. Px ve melatonin gruplarında TUNEL aktivitesi ve ovaryan kist oluşumu benzerdi.
Pinealektomi ovaryan kist oluşumu ve foliküler apoptozisi artırır. Eksojen melatonin uygulanımı pinealektomi etkilerini geri çevirmez.

Anahtar Kelimeler: Rat overi, Apoptosis, Ovarian kist, TUNEL, Pinealektomi, Melatonin

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