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Research article

Effects of melatonin on uterine hypertrophy/hyperplasia: A preliminary experimental rat study

Mustafa Can Sivas [a], Omer Lutfi Tapisiz [a,*], Rasit Tan Ayik [a], Devrim Kahraman [b], Sadiman Kiykac Altinbas [a], Ozlem Moraloglu Tekin [a]

[a] Department of Obstetrics and Gynecology, University of Health Sciences, Etlik Zubeyde Hanim Women's Health Training and Research Hospital, Ankara, Turkey
[b] Department of Pathology, School of Medicine, The Union of Chambers and Commodity Exchanges of Turkey, University of Economics and Technology (TOBB ETU), Ankara, Turkey

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ABSTRACT

Endometrial hyperplasia is a process of endometrial proliferation that results in a thickening of the endometrial tissue. Melatonin might be able to change the pathophysiological process and prognosis into a positive way that might prevent and heal endometrial hyperplasia, which is the first stage of endometrial cancer.

For this perspective, we tried to investigate the effect of melatonin on uterine hypertrophy/hyperplasia in an experimental rat model. Forty Wistar-Albino rats were undergone bilateral oophorectomy and randomized into four groups. To create a model of uterine hypertrophy/hyperplasia in all groups, except the control group [C] (n = 10), 4 mg/kg/day estradiol hemihydrate were given for 14 days. The uterine hypertrophy/hyperplasia was evaluated histopathologically in the left uterine horns, then the groups were treated for 14 days as follows; melatonin (10 mg/kg/day/po) [M] (n = 10), melatonin + estradiol hemihydrate (10 mg/kg/day/po and 4 mg/kg/day/po) [M + E] (n = 10), and dark environment [D] (n = 10). Finally, the effects of the melatonin were examined histopathologically in the right uterine horns.

An uterine hypertrophy/hyperplasia model was established in all groups compared to the control group (p < 0.05). In the [M] and [M + E] groups, epithelial cell height and luminal epithelial cell height significantly decreased (41μm vs 12μm, p = 0.005; 14μm vs 10μm, p = 0.005, respectively for [M] group) and (32μm vs 14μm, p = 0.012; 17μm vs 10μm, p = 0.017, respectively for [M + E] group). The [D] group exhibited a significant decrease in epithelial cell height (33μm vs 20μm, p = 0.017).

With or without estrogen exposure, melatonin-treated and physiologically melatonin-released rats experienced a significant uterine hypertrophy/hyperplasia recovery. Melatonin may have protective effects on endometrial hyperplasia.

1. Introduction

Endometrial hyperplasia is a process of endometrial proliferation that results in a thickening of the endometrial tissue. Clinically, endometrial hyperplasia is crucial because it is the precursor lesion of endometrial adenocarcinoma [1, 2, 3, 4].

Some studies have compared the level of melatonin in endometrial hyperplasia, endometrial cancer stages 1–3 and advanced stage (stage 4) endometrial cancer and have shown that the level of melatonin is the lowest in advanced stage (stage 4) endometrial cancer [5]. Similarly, when endometrial hyperplasia is studied by itself, the level of melatonin is found to range from a high to a low level in simple hyperplasia (without

atypia), complex hyperplasia (without atypia), simple hyperplasia (with atypia) and complex hyperplasia (with atypia) respectively [6].

In light of these data, a lower melatonin level can be a reason for the progression of the disease in patients who have advanced-stage endometrial cancer. Melatonin might be able to change the prognosis and pathophysiological process into a positive one that might prevent and heal endometrial hyperplasia, which is the first stage of endometrial cancer.

To investigate whether such a relationship exists, we investigated the effects of melatonin by evaluating the histopathological differences in uterine hypertrophy/hyperplasia parameters using rats modelled for uterine hypertrophy/hyperplasia (UH/H) after they had been given melatonin. Additionally, we aimed to examine the effects of the release of

* Corresponding author.
 E-mail address: omertapisiz@yahoo.com.tr (O.L. Tapisiz).

physiological melatonin due to a dark environment on the UH/H model. Answers were sought to the following questions:

- Does melatonin affect UH/H parameters?
- What are the possible effects of physiological melatonin release due to exposure to a dark environment on UH/H parameters?

2. Materials and methods

2.1. Animals

Forty sexually mature, cycling, female Wistar-Albino rats weighing 250–300 g, were used. They were caged in a temperature-controlled (24
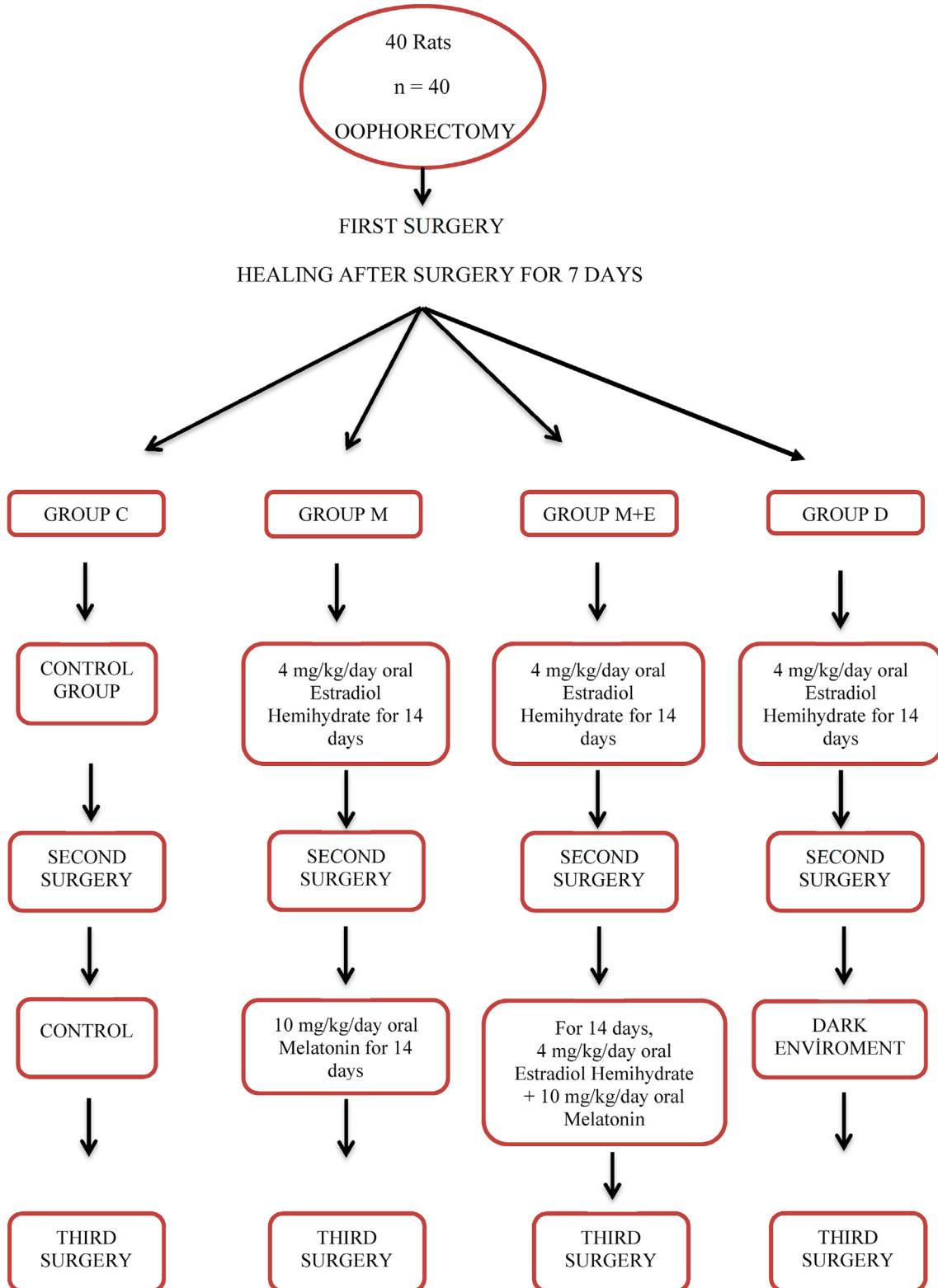

Figure 1. The algorithm for experimental model.

°C) environment maintained on a 12 h light (L)/12 h dark (D) photo-period. Only one experimental rat group [D] was exposed to the dark for 24 h [7, 8]. Standard rat feed and water were provided *ad libitum*. All rats were allowed to acclimate in the vivarium for two weeks period before the experimental research. The research was approved by the animal research ethics committee of Ankara Education and Research Hospital, Ankara, Turkey (meeting no: 0044 on 19.12.2017) and the local ethics committee of the Etlik Zubeyde Hanim Woman's Health Education and Research Hospital, Ankara, Turkey (number: 90057706-900/training, meeting no: 02 on 01.02.2018).

2.2. Study design

Vaginal smears were performed daily to document the phase of the estrous cycle; this was started two week after acclimation. The five phases of the estrous cycle were defined as previously determined in the literature [9]. After that, to ensure hormonal standardization for the estrous cycle and to remove the effects of endogenous hormones; we performed bilateral oophorectomy (first surgery) on all rats [10]. Then, the rats were randomly allocated to four groups (n = 10 rats per group) blinded to the surgeons, using the card technique. The rats were then left to recover for a week.

Group [C] (control group, n = 10): The rats were not given any medication for two weeks; after that period, the second surgery was performed. In the second surgery, the first uterine horns (left horn) of all rats were resected. In this group [C], the rats were not given any medication, and after a second two-week period, a third surgery was performed.

Group [M] (n = 10): Rats were given 4 mg/kg/day oral estradiol hemihydrate for 14 days. After this period, the second surgery was performed. In the following 14 days, which covered three cycles, rats were given 10 mg/kg/day oral melatonin during the light phase of light/dark cycle (time between 08:00–09:00 AM) [11, 12, 13, 14], and after this process, the third surgery was performed. Melatonin tablets (Melatonina, 3 mg, Lek-Am S, Poland) dissolved in water were given by oral gavage.

Group [M + E] (n = 10): Rats were given 4 mg/kg/day oral estradiol hemihydrate for 14 days. After this period, the second surgery was performed. In the following 14 days, estradiol hemihydrate was given at the same dose simultaneously with 10 mg/kg/day oral melatonin, and the third surgery was then performed.

Group [D] (n = 10): Rats were given 4 mg/kg/day oral estradiol hemihydrate for 14 days. After this period the second surgery was performed. Rats were kept in a dark environment for the entire 14-day period, except to meet daily needs such as nutrition [7, 8]. After this, the third surgery was performed.

In the third surgery, the second uterine horns (right horn) of all rats were resected. These resected horns were taken for histopathological examination to observe the effects of the melatonin on UH/H in rats. The study model is shown as an algorithm in Figure 1.

2.3. Surgery

Xylazine (Alfazyne, Alfasan, Amsterdam) was given at 10 mg/kg/i.m. for neuromuscular blockage and ketamine (Ketalar Flakon, Pfizer, Istanbul) was given at 50 mg/kg/i.m. for anesthesia in all surgeries. Next, the rat was placed in a supine position. The abdomen was shaved, and the skin was cleaned. A midline laparotomy was performed with a 2–3 cm vertical incision. After the surgical procedures, the abdomen was closed by suturing in layers.

All surgeries were performed by same surgeons (MCS, OLT, RTA, SKA);

In the first surgery, bilateral ovarian tissues were ligated with a 3.0 polyglactin 910 suture (Vicryl, Ethicon, USA) from the proximal and distal zones and resected.

In the second surgery, the left uterine horns of all rats (in all groups) were resected by ligation with a 3.0 polyglactin 910 suture (Vicryl, Ethicon, USA) from the proximal zone.

In the third surgery, the right uterine horns of all rats (in all groups) were resected by using the same procedure as that used in the previous surgery. The animals were sacrificed by decapitation.

2.4. Histopathological evaluation of uterine horns

Middle segments of the rat uterine horns were prepared for histopathological evaluation [10]. Surgical patterns were fixed in 10%
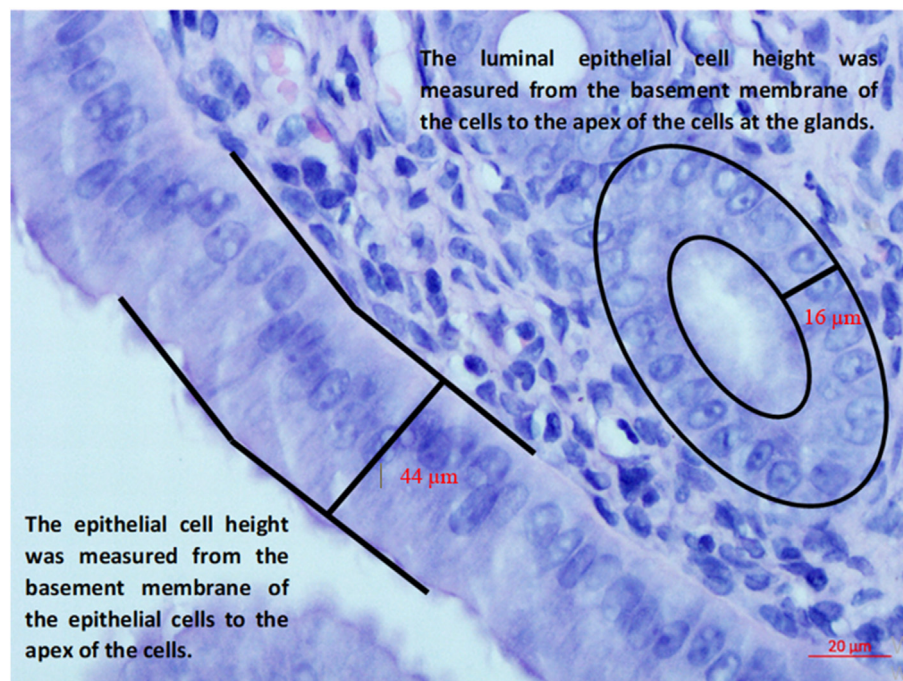

Figure 2. Histopathologic evaluation of the uterine horns in terms of uterine hypertrophy/hyperplasia.
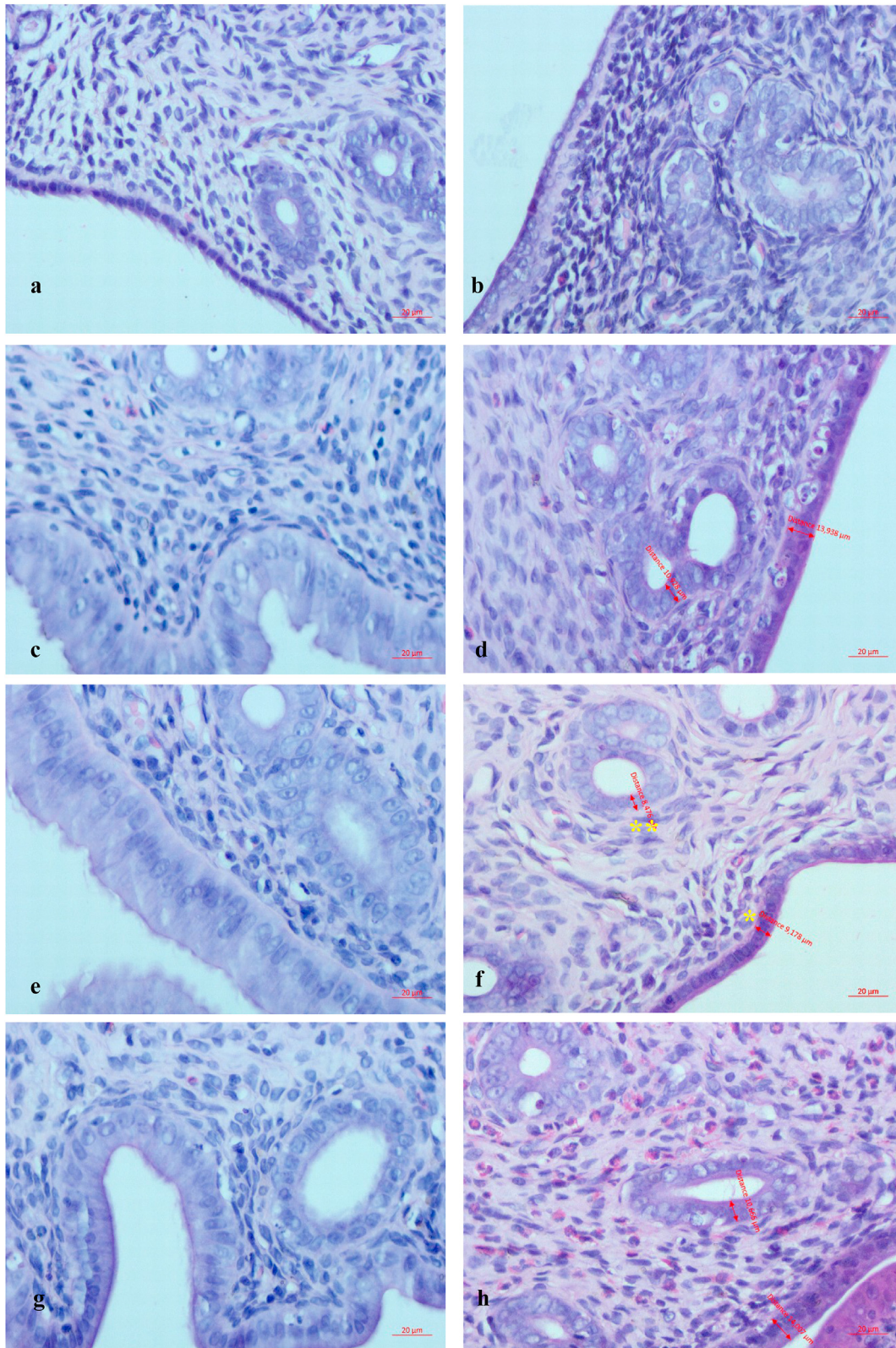
Figure 3. Histopathological images of all groups after surgeries a: Group [C]-Second surgery. b: Group [C]-Third surgery c: Group [M]-Second surgery d: Group [M]-third surgery e: Group [M + E]-second surgery f: Group [M + E]-third surgery g: Group [D]-second surgery h: Group [D]-third surgery.* Epithelial cell height, ** Luminal epithelial cell height.

formalin. Paraffin-embedded blocks and 5 μm sections were taken. Patterns were stained with hematoxylin and eosin. Preparations were examined under a light microscope.

In the histopathological evaluation, the epithelial cell height and luminal epithelial cell height were measured. While the epithelial cell height was measured from the basement membrane of the epithelial cells to the apex of the cells, the luminal epithelial cell height was measured from the basement membrane of the cells to the apex of the cells at the glands (Figure 2). We calculated an average height by assessing the lowest and the highest regions. The measurements were taken under 40x magnification as previously described in the literature in micrometer units by a single-blinded pathologist (DK) (Figure 3) [15, 16, 17].

2.5. Statistical analysis

For statistical analysis, SPSS Version 20.00 (IBM Corporation Armonk, NY: USA. Released 2011) was used. To evaluate the distribution of the data, the Shapiro-Wilk test was used. The median (interquartile range: IQR 25–75) value for descriptive statistics and nonparametric tests for defining the differences among groups were used. Nonnormally distributed metric variables were analyzed by the Kruskal–Wallis test for comparison of variables among groups. If the difference was significant, the binary comparisons were done with the Mann–Whitney U test, and Spearman's correlation test was used to ascertain the correlation between two groups. A p-value of 0.05 was taken as the threshold level for statistical significance.

3. Results

The research began with 40 rats with the median weights of 275 g (min 250g-max 300g). One of the rats in the group [C] died from unknown causes on the fourth day after the first surgery. Two rats; one from group [M] and one from group [M + E], died from unknown causes on the fifth and seventh days after the second surgery, respectively. The research was completed with 37 rats.

3.1. Analysis of the UH/H model

After the second surgery, the epithelial cell height and luminal epithelial cell height of groups [M], [M + E] and [D] were compared with group [C]. A statistically significant increase was found in the epithelial cell height and luminal epithelial cell height of all groups ([M], [M + E], [D]) given estrogen when compared to group [C] which had not been given estrogen (p < 0.05) (Table 1).

3.2. Analysis of group [M]

In the histopathological evaluation of the uterine horns resected in the third surgery after being exposed to melatonin for 14 days following the second surgery, a statistically significant decrease was found in both the epithelial cell height and luminal epithelial cell height in group [M] (p = 0.005 and p = 0.005, respectively) (Table 2).

3.3. Analysis of group [M + E]

According to the values following the second surgery after exposure to estrogen during the first half of the examination, a statistically significant decrease was found in the epithelial cell height and luminal epithelial cell height, measured after the third surgery, in those rats simultaneously given melatonin and estrogen in the second half of the examination (p = 0.012 and p = 0.017, respectively) (Table 2).

3.4. Analysis of group [D]

For rats that were exposed to estrogen in the first half of the experiment and then kept in a dark environment in the second half, the epithelial cell height measured after the third surgery was found to be statistically significantly lower than the epithelial cell height measured after the second surgery (p = 0.017); however, there was no statistically significant difference in the measurement of the luminal epithelial cell height between the second and the third surgeries in group [D] (p = 0.674) (Table 2).

3.5. Comparing the efficiency of the treatment groups

To determine the relative superiority of the groups, the differences in the measurements of groups [M], [M + E] and [D] made after the second and third surgeries were compared (Table 3).

There were no statistically significant differences between the differences of the measurements after the second and third surgeries in terms of epithelial cell height among the groups (p = 0.068). However, it was found that the difference in the epithelial cell height of group [M] was higher than those of groups [M + E] and [D], but it was not statistically significant. As distinct from epithelial cell height, there were statistically significant differences between the differences of the measurements after the second and third surgeries in terms of luminal epithelial cell height among the groups (p = 0.02). In binary comparisons, there were no statistically significant differences between the group [M] and the group [M + E], and the group [M] and the group [D] in terms of the difference in luminal epithelial cell height (p > 0.05). However, it was found that the difference in the luminal epithelial cell height of the group [M + E] was higher than those of the group [D] (p = 0.018).

4. Discussion

In the present study, it was determined that melatonin had positive effects on UH/H in rats. UH/H significantly regressed in the presence of melatonin. Similarly, regression was found in the presence of melatonin while proceeding estrogen exposure. Additionally, UH/H regression was determined, due to the release of physiological melatonin, which occurred as a result of the rats being placed in a dark environment.

There are studies showing that melatonin may have more than one impact in aiding the recovery of precancerous/cancerous lesions in different tissue types and, mostly, in preventing cancer from progressing from the earliest stages [18, 19, 20, 21, 22, 23, 24]. Melatonin slows the growth of tumor cells by inhibiting uncontrolled cell division and

Table 1. Comparison of the histopathological parameters between the groups after second surgery.

	Groups				
	[C] (n = 9)	[M] (n = 10)	[M + E] (n = 10)	[D] (n = 10)	p values
Epithelial cell height	4 (3–4)	41 (35–42)	32 (26–40)	33 (30–35)	<0.001[a,b]
Luminal epithelial cell height	7 (6–9)	14 (13–17)	17 (12–19)	13 (9–14)	0.001[a,b]

[C], control group; [M], melatonin group; [M + E], melatonin + estradiol hemihydrate group; [D], dark environment group.
Values given as median (IQR 25–75), ([a] Kruskal–Wallis test was used for comparison of variables among groups; [b] Mann-Whitney U test was used for binary comparisons).

Table 2. Comparison of the histopathological parameters between the second and third surgery in groups [M], [M + E], and [D].

	Second Surgery	Third Surgery	p values
Group [M] (n = 9)			
Epithelial cell height	41 (35–42)	12 (10–13)	0.005[a]
Luminal epithelial cell height	14 (13–17)	10 (7–11)	0.005[a]
Group [M + E] (n = 9)			
Epithelial cell height	32 (26–40)	14 (9–20)	0.012[a]
Luminal epithelial cell height	17 (12–19)	10 (7–12)	0.017[a]
Group [D] (n = 10)			
Epithelial cell height	33 (30–35)	20 (14–24)	0.017[a]
Luminal epithelial cell height	13 (9–14)	12 (9–16)	0.674[a]

[M], melatonin group; [M + E], melatonin + estradiol hemihydrate group; [D], dark environment group.
Values given as median (IQR 25–75), ([a] Mann-Whitney U test was used).

Table 3. Comparison of the differences in the histopathological parameters after the second and third surgeries between the groups [M], [M + E], and [D].

	[M] (n = 9)	[M + E] (n = 9)	[D] (n = 10)	p values
Epithelial cell height	29 (20–33)	22 (15–30)	11 (6–21)	0.068[a]
Luminal epithelial cell height	5 (3–8)	10 (4–11)[b]*	-1 ((-2)-(3))	0.020[a]

[M], melatonin group; [M + E], melatonin + estradiol hemihydrate group; [D], dark environment group.
Values given as median (IQR 25–75), ([a] Kruskal Wallis test was used for three and more groups).
*p:0.018, ([b] Mann-Whitney U test was used for comparing group [M + E] and group [D]).

prevents metastasis in rats [18, 19, 20, 21, 22]. Melatonin inhibited cervical/vaginal carcinogenesis induced by 7,12-dimethylbenz[a]anthracene in female rats [25]. By decreasing the expression of Estrogen Receptor a (ERa), melatonin prevented the ovarian cancerous process that had occurred as a result of the increase in expression of ERa with the effects of cadmium on the ovarian tissue of rats [26]. Our results may be explained as the effects of melatonin on estrogen receptors as mentioned in this study. By decreasing the expression of ki67, melatonin decreased the increase in ovarian cancer stem cells by 23% [27]. In the literature, there is only one study that examines the possible protective effects of melatonin on precancerous/cancerous lesions in the endometrium [28]. In this study, the endometrial hyperplasia model was used on rats. Hyperplasia was shown with an increase of endometrial thickness via ultrasonography and histopathological evaluations, and it was reported that melatonin had a role in preventing hyperplasia [28]. However, the hyperplasia model was not evaluated in terms of epithelial cell height and luminal epithelial cell height, as recommended in the literature. Our study is thus superior to this study in terms of the parameters used to evaluate the endometrial hyperplasia (UH/H) histopathologically. Besides, the effects of increasing physiological melatonin as a result of a dark environment on UH/H were examined in our study.

There are many studies in the literature on the release of melatonin in the dark environment in rats. In different studies, pineal gland and plasma blood melatonin levels of rats were measured after different exposure periods (2–6 weeks). The common feature of all studies was the significantly higher levels of melatonin in the darkness group [7, 8]. Besides, not only increased levels of melatonin but also increased melatonin receptors (MT1) in darkness group was reported [8]. In our study, it was found that after rats had been kept in the dark environment, there was a significant decrease in the epithelial cell height. If the study took longer, more significant regression in UH/H could have been expected. On the other hand, it was shown that melatonin could up-regulate the expression of its receptors and decrease the estrogen and progesterone receptors in rat endometrium, which might protect the cells from oxidative stress, control the apoptosis and, therefore affect the endometrial homeostasis [29, 30, 31, 32]. In an important study conducted by Ferreira et al., the authors reported that melatonin influenced the mechanism and decreased the apoptosis in uterus of rats exposed to continuous light [33]. However, there is still need for more studies of

rat/human models with circadian rhythm disorders. With the studies on this subject, the answers for the following questions can be found in the future: Could regular circadian sleep rhythms be important for women's genital health? Could women with circadian rhythms disrupted by working hours be more prone to endometrial hyperplasia/cancer?

To discuss the limitations of this experimental model, first and most importantly, it is not appropriate to adapt animal experiments directly to humans. Melatonin may react differently in different living creatures. The second limitation was the shortness of the follow-up period. However, in a-15-day follow-up period (the estrous cycle of rats was 4–5 days), a length of 3–4 cycles, was considered to be analogous to a similar 3–4 month follow-up period in humans. The limited number of rats in each group, due to ethical principles is another limitation that may affect the statistical significance. Furthermore, the differences between the histopathological evaluation criteria of rat-derived preparations and the histopathological evaluation criteria of human-derived preparations might limit the definite conclusions on the issue. Several factors such as a gland/stroma ratio, the presence/absence of nuclear atypia, a high nuclear cytoplasmic ratio are examined in the evaluation/classification of the presence of endometrial hyperplasia in human-derived preparations [34, 35]. However, our study was a rat study, and the histopathological evaluation of the preparations was performed by epithelial cell height and luminal epithelial cell height measurements based on the previous studies reported [15, 36, 37, 38, 39, 40]. In our opinion, this evaluation gave us quantitative data creating the opportunity for statistical comparisons in the experimental models. Last but not least, we used a control group mainly to reveal whether UH/H model occurred with estrogen administration rather than comparing with the study groups after the third surgery, and we did not create a 24-hour darkness environment for the control group.

To discuss the strength of this experimental model, a study that reports that melatonin has not only preventative effects but also healing effects is not available in the literature. In addition, the aspect of our study "the effects of increasing physiological melatonin as a result of a dark environment on UH/H" is a new concept that is not found in the literature, and this is the first time being discussed; if the blood melatonin level was also measured, it would have been a much stronger study.

In conclusion, with or without estrogen exposure, melatonin-treated rats experienced a significant UH/H recovery. Melatonin may affect

endometrial hyperplasia in a positive way. The melatonin, which is released physiologically in dark environments, may have protective effects on endometrial hyperplasia. In the light of this information and following the results of further studies, new treatment modalities, involving the use of melatonin to treat endometrial hyperplasia, could be developed.

Declarations

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