The effects of daylight exposure on melatonin levels, Kiss1 expression, and melanoma formation in mice

Aim To determine how daylight exposure in mice affects melatonin protein expression in blood and Kiss1 gene expression in the hypothalamus. The second aim was to assess the relationship between skin cancer formation, daylight exposure, melatonin blood level, and kisspeptin gene expression level.

Methods New-born mice (n=96) were assigned into the blind group or daylight group. The blind group was raised in the dark and the daylight group was raised under 12 hours light/12 hours dark cycle for 17 weeks. At the end of the 11th week, melanoma cell line was inoculated to mice, and tumor growth was observed for 6 weeks. At the end of the experiment, melatonin level was measured from blood serum and Kiss1 expression from the hypothalamus.

Results The blind group had significantly higher melatonin and lower Kiss1 expression levels than the daylight group. Tumor volume was inversely proportional to melatonin levels and directly proportional to Kiss1 expression levels. Tumor growth speed was lower in the blind than in the daylight group.

Conclusion Melatonin and Kiss1 were shown to be involved in tumor suppression. They were affected by daylight and were mutually affected by each other.
Melatonin is an endocrine hormone produced by the pineal gland and several body tissues, and its blood levels are inversely proportional to the amount of light received throughout the day (1,2). Melatonin alterations regulate the circadian rhythm of many bodily functions (3). It has been shown that circadian rhythm disruptions may lead to impaired thyroid-stimulating hormone (TSH) secretion, increase in nocturnal cortisol secretion, changes in lipid and glucose metabolism, changes in cytokine balance, and inhibition of antioxidant genes (4). Melatonin also regulates production of kisspeptin, a protein coded by Kiss1 and synthesized mostly in hypothalamic tissue and (5). It has been shown that kisspeptin levels vary depending on melatonin blood concentration (6).

Melatonin’s tumor suppressor properties are the subject of considerable research. Its antioxidant properties and DNA protective features (nuclear and mitochondrial) have been extensively confirmed (7,8), while cell culture and animal studies have emphasized its role in the suppression of different tumor types (9,10). For example, the incidences of breast cancer, stomach cancer, and skin cancer were lower in blind people, whose melatonin levels were consistently higher than those in sighted individuals (11,12).

Melatonin and kisspeptin synthesis are both affected by daylight exposure (13). Also, decreased melatonin blood levels lead to increased kisspeptin synthesis in the hypothalamus (14,15). Although kisspeptin’s primary function is the seasonal control of reproduction, various studies also showed its antimetastatic role (16,17).

The relationships between daylight exposure and melatonin, daylight exposure and kisspeptin, and kisspeptin and melatonin have been widely investigated, but there have been no detailed studies on their mutual effects. This study aimed to determine how daylight exposure in mice affected melatonin blood levels and the rate of kisspeptin synthesis in the hypothalamus. In addition, we investigated the relationship between skin cancer formation, daylight intake, melatonin blood level, and kisspeptin synthesis rate.

MATERIAL AND METHODS

The study, conducted in 2017, used 96 newborn BALB/c albino mice obtained from the Çukurova University Faculty of Medicine Experimental Medicine Research and Application Center. No inclusion or exclusion criteria other than age and sex were applied. This study was approved by the Ethics Committee of the Çukurova University Faculty of Medicine Experimental Medicine Research and Application Centre.

MICE GROUPS AND EXPERIMENTAL WORKFLOW

The mice were assigned to the blind group (n = 48) or the daylight group (n = 48). Each group was further divided into the control (n = 12) and melanoma (n = 36) subgroups. All subgroups had an equal number of male and female mice. The blind group was housed with their mothers in a dark room (0 lux) one week after birth. Since visual skills in mice develop 10-14 days after birth, the exposure to darkness was used to imitate blindness from birth (18). The daylight group was housed with their mothers in a room with normal daylight (4000 lux, 12 hours daylight, 12 hours dark) one week after birth. All mice were separated from their mothers at the end of week 3 and were raised under appropriate conditions (unlimited Laboratory Diet 5K52, unlimited water, 20°C, 50% humidity). At the end of week 11, the mice in the melanoma subgroups were subcutaneously injected with B16F10 cell line and raised for 6 more weeks (17-week old mice). The tumor sizes were measured weekly with a caliper. At the end of week 17, tumor sizes were measured, blood samples were taken, and the hypothalameses were removed.

Melanoma cell line injection

The cell lines were prepared and injected according to the modified protocol by Overwijk and Restifo (19). B16F10 cells, which were in the active dividing state in the cell culture, were collected and diluted with DMEM to a concentration of 10^6 cells/mL. Melanoma cell solution of 100 μL (10^5 cells) was administered subcutaneously to the abdominal areas.

Tumor size measurement and volume calculation

The measurements were made between the longest transverse (width) and the longest longitudinal (length) sections. The short section was considered to be the tumor width and the long section was considered to be the tumor length. Tumor volume was calculated by the formula: tumor volume = width × width × length/2 (20,21).

Determination of melatonin concentration

Melatonin blood concentration was determined with ELISA kit (SunredBio Inc., Shanghai, China, detection range:
15.6-1000 pg/mL) according to the manufacturer’s protocol. Since the melatonin level was measured from the blood serum, no standardization was done.

**Determination of Kiss1 expression**

Expression was determined in the hypothalami. The expression level was determined with real time quantitative polymerase chain reaction by using the TaqMan Gene Expression Assay (ThermoFisher Scientific Inc, Waltham, MA, USA) containing the FAM stained probe designed for Kiss1 gene. RNA was isolated with TRIzol method (22). Complementary DNA was synthesized with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA, USA). The expression level of Kiss1 gene was determined with ΔCt method using β-actin gene as reference (23). One of the samples was accepted as "1" and the expression levels of other samples were determined relatively.

**Statistical analysis**

Normality testing was conducted with the Kolmogorov-Smirnov test. Significance of differences between the groups in melatonin and Kiss1 levels was assessed with the independent t test, while the significance of differences between the groups in the rate of tumor volume change was assessed with the two-way ANOVA. Correlations between melatonin and Kiss1 values and tumor volumes were assessed with the Pearson correlation analysis. The level of statistical significance was set to 0.05. The analysis was conducted with Graphpad Prism 6 software (GraphPad Software Inc, San Diego, CA, USA).

**RESULTS**

At the end of the experiment, 87 mice survived. Nine mice (1 healthy from the daylight group and 8 injected mice, 5 from daylight and 3 from blind group) died from unknown causes and were excluded from the study.

The blind group had significantly higher melatonin level (17.26 ± 0.97 ng/L vs 12.77 ± 0.53 ng/L, P ≤ 0.001, t = 3.980) and significantly lower Kiss1 expression level than the daylight group (5.89 ± 1.21 vs 13.00 ± 2.92, P = 0.024, t = 2.306 for Kiss1).

Healthy mice had significantly higher melatonin level (16.09 ± 1.26 ng/L vs 9.59 ± 0.98 ng/L, P = 0.002, t = 3.440) and significantly lower Kiss1 expression level than tumor-bearing mice (3.08 ± 1.15 vs 11.96 ± 3.07, P = 0.003, t = 3.280).

A tumor was formed in 12 of 72 mice injected with a melanoma cell line. Six of these were female and 6 were in the daylight group. There was no difference between the groups and sexes in the number of tumor-bearing mice. The weekly change of tumor volume from the injection to sacrifice is shown in Table 1. There was a strong inverse correlation (correlation coefficient = -0.766, P = 0.004) between melatonin levels and tumor volumes and a strong positive correlation (correlation coefficient = 0.849, P = 0.001) between Kiss1 expression levels and tumor volumes (Figure 1).

**TABLE 1.** Tumor volumes (mm³) of melanoma bearing mice by week

| Mouse* | Week 1† | Week 2 | Week 3 | Week 4 | Week 5 | Week 6 |
|--------|---------|--------|--------|--------|--------|--------|
| Blind group | | | | | | |
| F | - | 18.00 | 87.50 | 486.00 | 936.00 | 936.00 |
| F | - | 32.00 | 320.00 | 2560.00 | 7488.00 | 7488.00 |
| F | - | 22.50 | 245.00 | 1764.00 | 4630.50 | 4630.50 |
| F | - | 6.00 | 56.00 | 288.00 | 726.00 | 726.00 |
| M | - | 13.50 | 56.00 | 320.00 | 786.50 | 786.50 |
| M | - | 6.00 | 31.50 | 220.50 | 550.00 | 550.00 |
| Daylight group | | | | | | |
| F | - | 32.00 | 486.00 | 3240.00 | 3971.00 | 3971.00 |
| F | - | 40.00 | 936.00 | 6083.50 | 8125.00 | 8125.00 |
| M | - | 18.00 | 650.00 | 2601.00 | 3610.00 | 3610.00 |
| M | - | 13.50 | 550.00 | 1912.50 | 2432.00 | 2432.00 |
| M | - | 13.50 | 288.00 | 936.00 | 1470.00 | 1470.00 |
| M | - | 6.00 | 220.50 | 786.50 | 1352.00 | 1352.00 |

*F – female; M – male.
†Week numbers represent weeks after injection.
Tumor volumes measured each week (Table 1) were divided by the values at the week 2, when tumors were first spotted, and the growth rate was determined for every week after tumor formation (Table 2). The tumor volumes in the daylight group grew significantly faster than those in the blind group ($P=0.026$) (Figure 2).

**DISCUSSION**

In this study, mice kept in darkness (blind group) had a slower tumor growth rate in comparison with mice exposed to daylight conditions (daylight group). Furthermore, the blind group had significantly higher melatonin level and significantly lower Kiss1 expression level than the daylight group. One of the most important factors that regulate the melatonin cycle is the light stimulation of the retinal nerves (24,25). Individuals with partial visual impairment who could perceive light had slightly deviated melatonin cycle, whereas individuals with complete visual impairment, not able to perceive light, had an abnormal cycle during the day (26). In addition, individuals who had lost both eyes had disrupted circadian rhythm and a spontaneous melatonin cycle (27). The higher melatonin levels in the blind group observed in this study could be attributed to the irregular melatonin cycle in the blind, leading to higher melatonin levels during the day (26,27). Both groups were sacrificed during the daytime to detect the baseline blood melatonin levels.

**FIGURE 1.** The relationship between melatonin and Kiss1 and tumor volumes at the end of the experiment ($P<0.05$).

**TABLE 2.** Changes in rate of tumor growth (volumes) by week

| Mouse* | Week 2† | Week 3 | Week 4 | Week 5 | Week 6 |
|--------|---------|--------|--------|--------|--------|
| **Blind group** | | | | | |
| F      | 1.00    | 4.86   | 27.00  | 52.00  | 52.00  |
| F      | 1.00    | 10.00  | 80.00  | 234.00 | 234.00 |
| F      | 1.00    | 10.89  | 78.40  | 205.80 | 205.80 |
| F      | 1.00    | 9.33   | 48.00  | 121.00 | 121.00 |
| M      | 1.00    | 4.15   | 23.70  | 58.26  | 58.26  |
| M      | 1.00    | 5.25   | 36.75  | 91.67  | 91.67  |
| **Daylight group** | | | | | |
| F      | 1.00    | 15.19  | 101.25 | 124.09 | 124.09 |
| F      | 1.00    | 23.40  | 152.09 | 203.13 | 203.13 |
| M      | 1.00    | 36.11  | 144.50 | 200.56 | 200.56 |
| M      | 1.00    | 40.74  | 141.67 | 180.15 | 180.15 |
| M      | 1.00    | 21.33  | 69.33  | 108.89 | 108.89 |
| M      | 1.00    | 36.75  | 131.08 | 225.33 | 225.33 |

*F – female; M – male.
†Week numbers represent weeks after injection.
with the increase in \( K \) on melanoma formation. Tumor volumes also increased, which indicates the protective effect of melatonin, whereas it strongly directly correlated with \( K \)iss1 expression, and melanoma in mice. However, despite the difference in tumor volume, there was no difference between the daylight and blind group in the number of tumor-bearing mice, which makes this possibility less probable.

Melatonin levels were very low in tumor-bearing mice compared with healthy mice. Grinevich and Labunetz (30) also found very low melatonin levels in melanoma patients compared with healthy individuals. Low melatonin levels in tumor-bearing mice may be related to the circadian rhythm disruption. Another possibility is that mice with lower melatonin levels developed melanoma, while mice with higher melatonin levels were able to protect themselves from tumor formation. However, despite the different melatonin levels, there was no difference between the daylight and blind group in the number of tumor-bearing mice, which makes this possibility less probable. Kiss1 expression level was much higher in tumor-bearing than in healthy mice. If we take into account kisspeptin’s antimetastatic and anticancer properties, it can be concluded that the hypothalamic synthesis of kisspeptin was increased because of tumor formation. Contrary to our findings, Shirasaki et al (31) reported that Kiss1 expression was reduced in metastatic melanomas. This difference can be explained by the fact that our mice did not have metastases. In addition, tumor volume strongly inversely correlated with Kiss1 expression, and melanoma formation. Tumor volumes also increased with the increase in Kiss1 expression level, and considering the fact that the mice had no metastases, this observation may be explained by potential effect of changed kisspeptin synthesis on metastasis inhibition. However, this interpretation has to be confirmed by kisspeptin assessment in tumor tissues.

The main limitation of the study was the fast melanoma growth. In addition, the study did not analyze both protein and gene expression of melatonin and kisspeptin – we analyzed melatonin protein expression in blood and kisspeptin gene expression level in the hypothalamic tissue.

Our results showed that melatonin and Kiss1 were important tumor suppressors and were highly affected by daylight. In addition, these two tumor suppressors were mutually affected by each other. Our results indicate that melatonin and kisspeptin are highly affected by daylight and involved in tumor suppression. Further investigation is needed in order to clarify impact of other factors on melatonin and kisspeptin role in tumor growth and suppression. Future studies should analyze both protein and gene expression of melatonin and kisspeptin in tumor tissues to answer the remaining questions, particularly how to generate more slowly progressing cancers in mice.

**Funding** This research was supported by Çukurova University Research Fund (TDK-2015-2966).

**Ethical approval** given by Ethics Committee of the Çukurova University Faculty of Medicine Experimental Medicine Research and Application Centre (4-1, 2016).

**Declaration of authorship** PP, DA, MBY, ES, and HK conceived and designed the study; PP, HMK, and AAY acquired the data; PP, UL, AP, and HK analyzed and interpreted the data; PP, DA, MBY, ES, and HK critically revised the manuscript; PP, DA, MBY, ES, and HK critically revised the manuscript for important intellectual content; all authors gave approval of the version to be submitted; all authors agree to be accountable for all aspects of the work.

**Competing interests** All authors have completed the Unified Competing Interest form at [www.icmje.org/coi_disclosure.pdf](http://www.icmje.org/coi_disclosure.pdf) (available on request from the corresponding author) and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

**References**

1. Mack JM, Schamne MG, Sampaio TB, Pertilie RA, Fernandes PA, Markus RF, et al. Melatoninergic system in Parkinson’s disease: from neuroprotection to the management of motor and nonmotor symptoms. Oxid Med Cell Longev. 2016;2016:3472032. Medline:27829983 doi:10.1155/2016/3472032

2. Rusanova I, Martínez-Ruíz L, Florido J, Rodríguez-Santana C, Guerra-Libreo A, Acuna-Castroviejo D, et al. Protective effects of melatonin on the skin: future perspectives. Int J Mol Sci. 2019;20.

3. Zisapel N. New perspectives on the role of melatonin in human sleep. Medline:31597233
sleep, circadian rhythms and their regulation. Br J Pharmacol. 2018;175:3190-9. Medline:29318587 doi:10.1111/bph.14116

4 Potter GD, Skene DJ, Arendt J, Cade JE, Grant PJ, Hardie LJ. Circadian rhythm and sleep disruption: causes, metabolic consequences, and countermeasures. Endocr Rev. 2016;37:584-608. Medline:27763782 doi:10.1210/er.2016-1083

5 Kim TH, Cho SG. Melatonin-induced KISS1 expression inhibits triple-negative breast cancer cell invasiveness. Oncol Lett. 2017;14:2511-6. Medline:28781689 doi:10.3892/ol.2017.6434

6 Pinilla L, Aguilar E, Dieguez C, Millar RP, Tena-Sempere M. Free radical-mediated molecular damage. Mechanisms for the protective actions of melatonin in the central nervous system. Ann N Y Acad Sci. 2001;939:200-15. Medline:11462772 doi:10.1111/j.1749-6632.2001.tb03627.x

7 Reiter RJ, Acuna-Castroviejo D, Tan DX, Burkhardt S. Free radical-mediated molecular damage. Mechanisms for the protective actions of melatonin in the central nervous system. Ann N Y Acad Sci. 2001;939:200-15. Medline:11462772 doi:10.1111/j.1749-6632.2001.tb03627.x

8 Guerrero JM, Reiter RJ. Melatonin-immune system relationships. Curr Top Med Chem. 2002;2:167-79. Medline:11899099 doi:10.2174/1568026023394335

9 Wang TH, Huhse C, Chen CC, Li WS, Yeh CT, Lian JH, et al. Melatonin Inhibits the progression of hepatocellular carcinoma through microRNA Let7i-3p mediated RAI1 reduction. Int J Mol Sci. 2018;19. Medline:30201903

10 Yun CW, Kim S, Lee JH, Lee SH. Melatonin promotes apoptosis of colorectal cancer cells via superoxide-mediated ER stress by inhibiting cellular prion protein expression. Anticancer Res. 2018;38:3951-60. Medline:29970517 doi:10.21873/anticancers.12681

11 Coleman MP, Reiter RJ. Breast cancer, blindness and melatonin. Eur J Cancer. 1992;28:501-3. Medline:1591073 doi:10.1016/0014-0294(92)90087-5

12 Reiter RJ, Tan DX, Korkmaz A, Erren TC, Piekar斯基 C, Tamura H, et al. Light at night, chronodisruption, melatonin suppression, and cancer risk: a review. Crit Rev Oncog. 2007;13:303-28. Medline:18540832 doi:10.1615/CritRevOncog.v13.i4.30

13 Walton JC, Weil ZM, Nelson RJ. Influence of photoperiod on hormones, behavior, and immune function. Front Neuroendocrinol. 2011;32:303-19. Medline:21561879 doi:10.1016/j.yfrne.2010.12.003

14 Bohlen TM, Silveira MA, Buonfiglio DDC, Ferreira-Neto HC, Cipolla-Neto J, Donato J Jr, et al. A short-day photoperiod delays the timing of puberty in female mice via changes in the kisspeptin system. Front Endocrinol (Lausanne). 2018;9:44. Medline:29515520 doi:10.3389/fendo.2018.00044

15 Boafo A, Greenham S, Alenezi S, Robillard R, Pajer K, Tavakoli P, et al. Could long-term administration of melatonin to prepubertal children affect timing of puberty? a clinician's perspective. Nat Sci Sleep. 2019;11:1-10. Medline:30774488 doi:10.2147/NSS.S5181365

16 Lee JH, Miele ME, Hicks DJ, Phillips KK, Trent JM, Weissman BE, et al. KISS-1, a novel human malignant melanoma metastasis-suppressor gene. J Natl Cancer Inst. 1996;88:1731-7. Medline:8944003 doi:10.1093/jnci/88.23.1731

17 Stathaki M, Stamatiou ME, Magioris G, Simantiris S, Syrigos N, Dourakis S, et al. The role of kisspeptin system in cancer biology. Crit Rev Oncol Hematol. 2019;142:130-40. Medline:31401420 doi:10.1016/j.critrevonc.2019.07.015

18 Aerts J, Nys J, Ackrens L. A highly reproducible and straightforward method to perform in vivo ocular enucleation in the mouse after eye opening. J Vis Exp. 2014:e51936. Medline:25350746

19 Overvijk WW, Restifo NP. B16 as a mouse model for human melanoma. Curr Protoc Immunol. 2001;Chapter 20.Unit 20.1.

20 Jensen MM, Jorgensen JT, Binderup T, Kjaer A. Tumor volume in subcutaneous mouse xenografts measured by microCT is more accurate and reproducible than determined by 18F-FDG-microPET or external caliper. BMC Med Imaging. 2008;8:16. Medline:18925932 doi:10.1186/1471-2342-8-16

21 Tomayko MM, Reynolds CP. Determination of subcutaneous tumor size in athymic (nude) mice. Cancer Chemother Pharmacol. 1989;24:148-54. Medline:1568026023394335

22 Rio DC, Ares M, Jr., Hannon GJ, Nilsen TW. Purification of RNA using TRizol (TRI reagent). Cold Spring Harb Protoc. 2010;2010:pdb prot5439.

23 Pfaff MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001;29:e45. Medline:11328886 doi:10.1093/nar/29.9.e45

24 Schomerus C, Korf HW. Mechanisms regulating melatonin synthesis in the mammalian pineal organ. Ann N Y Acad Sci. 2005;1057:372-83. Medline:16399907 doi:10.1196/annals.1356.028

25 Carrillo-Vico A, Guerrero JM, Lardone PJ, Reiter RJ. A review of the multiple actions of melatonin on the immune system. Endocrine. 2005;27:189-200. Medline:16217132 doi:10.1385/ENDO:27:2:189

26 Lockley SW, Skene DJ, Arendt J, Tabandeh H, Bird AC, Defrance R. Relationship between melatonin rhythms and visual loss in the blind. J Clin Endocrinol Metab. 1997;82:3763-70. Medline:9360538

27 Skene DJ, Arendt J. Circadian rhythm sleep disorders in the blind and their treatment with melatonin. Sleep Med. 2007;8:651-5. Medline:17420154 doi:10.1016/j.sleep.2006.11.013

28 Kauffman AS, Clifton DK, Steiner RA. Emerging ideas about kisspeptin- GPR54 signaling in the neuroendocrine regulation of reproduction. Trends Neurosci. 2007;30:504-11. Medline:17904653 doi:10.1016/j.tins.2007.08.001

29 Morgan PJ, Hazlerigg DG. Photoperiodic signalling through the melatonin receptor turns full circle. J Neuroendocrinol. 2008;20:820-6. Medline:18601705 doi:10.1111/j.1365-2826.2008.01724.x

30 Grinevich YA, Labunetz IF. Melatonin, thymic serum factor, and cortisol levels in healthy subjects of different age and patients with skin melanoma. J Pineal Res. 1986;3:263-75. Medline:3772725
Shirasaki F, Takata M, Hatta N, Takehara K. Loss of expression of the metastasis suppressor gene KiSS1 during melanoma progression and its association with LOH of chromosome 6q16.3-q23. Cancer Res. 2001;61:7422-5. Medline:11606374