Доклади на Българската академия на науките
Comptes rendus de l'Académie bulgare des Sciences
Tome 78, No 10, 2025

BIOLOGY
Cell biology

INVESTIGATION OF THE EFFECTS OF MELATONIN ON GRANULOSA CELL PROLIFERATION AND DNA METHYLATION

Ervanur Turkmen[1], Gul Ipek Gundogan[2,6], Halil Ibrahim Arslan[3,6], Asiye Gok Yurttas[4], Tugba Elgun[5,6]✉

Received on July 8, 2025
Presented by N. Atanassova, Corresponding Member of BAS, on September 30, 2025

Abstract

Melatonin, a pineal hormone with antioxidant and regulatory functions, has emerged as a key modulator of ovarian physiology. Its presence in follicular fluid suggests important roles in granulosa cell function, follicle development, and reproductive outcomes. However, its effects on granulosa cell tumour (GCT) biology and epigenetic regulation remain insufficiently defined. This study aimed to investigate the effects of melatonin on proliferation and global DNA methylation in human granulosa tumour cells (COV434) compared with healthy endothelial controls (HUVECs). COV434 and HUVEC cells were treated with melatonin at 1, 10, 100, and 1000 µM. Cell viability and proliferation were assessed using the MTT [3-(4.5-dimethylthiazol-2-yl)-diphenyl tetrazolium bromide] (Cambridge, UK) assay and xCELLigence RTCA system (Roche), while DNA methylation was quantified with a 5-mC ELISA kit (Epigentek Group Inc, USA). Experimental groups included negative, sham, melatonin-treated, and positive controls. Melatonin showed a cell type-dependent effect. In COV434 cells, proliferation was significantly inhibited, with an IC_{50} of 10.55 µM, whereas HUVECs displayed increased proliferation at higher doses. DNA methylation levels decreased in both cell types in a dose-dependent manner, reaching the highest significance in COV434 cells at 1000 µM ($p < 0.001$). In conclusion, melatonin demonstrated a dose-dependent inhibitory effect on COV434 cell proliferation while simultaneously reducing global DNA methylation levels.

Key words: melatonin, granulosa cell, proliferation, DNA methylation

https://doi.org/10.7546/CRABS.2025.10.05

Introduction. Folliculogenesis is a tightly regulated process that depends on granulosa cell proliferation and differentiation [1]. These cells provide paracrine and structural support to the oocyte, synthesize estrogens via aromatase, and secrete Anti-Müllerian Hormone (AMH), thereby coordinating follicle dynamics. Beyond these essential functions, granulosa cells are increasingly recognized as critical targets for paracrine and endocrine modulators, including melatonin, which influence ovarian physiology and pathology [2, 3].

Melatonin (N-acetyl-5-methoxytryptamine), classically known for circadian regulation, is present at high concentrations in follicular fluid and plays multifaceted roles in reproduction. It reduces oxidative stress, improves oocyte competence, and supports follicular development. In clinical and experimental studies, melatonin supplementation enhanced oocyte quality and ART outcomes in women with diminished ovarian reserve, largely by protecting granulosa and cumulus cells against ferroptosis and oxidative injury [3]. Mechanistically, melatonin acts through MT1 and MT2 G-protein-coupled receptors in granulosa and luteal cells, while at higher concentrations, nuclear orphan receptors of the RZR/ROR family may mediate additional effects [4, 5]. Emerging evidence also highlights melatonin as an epigenetic regulator. It can modulate DNA methyltransferase activity and reshape methylation patterns, thereby influencing gene expression and cell fate [6, 7]. In reproductive models, melatonin was shown to activate the PI3K/Akt/mTOR pathway and inhibit autophagy, thereby preserving granulosa cell function and ovarian reserve [8].

Granulosa cell tumours (GCTs) represent 2–5% of ovarian cancers and are characterized by late recurrence and limited treatment options beyond surgery [9]. Given melatonin's dual actions on proliferation and epigenetic regulation, this study aimed to evaluate its effects on proliferation and global DNA methylation in human granulosa tumour cells (COV434) compared with healthy endothelial controls (HUVECs).

Materials and methods. The effects of melatonin on COV434 cell proliferation and DNA methylation levels were investigated.

Cell culture. COV434 (ATCC, VA, USA) and HUVEC (ATCC: CRL-1730, USA) cells were used; COV434s were grown in DMEM/F12 with 10% FBS at 37 °C, 5% CO_2. Melatonin (1, 10, 100, and 1000 µM; Solgar) was applied in DMEM/F12 containing 1% DMSO.

Cell viability and proliferation analysis using the MTT assay. Cell viability was assessed using the MTT assay (Abcam Kit AB2110911000TEST), which measures NAD(P)H-dependent reduction of MTT to formazan [11]. Cells (1×10^4/well) were seeded in 96-well plates (150 µL/well), allowed to adhere for 24 h at 37 °C, and then treated with melatonin (1–1000 µM) following the manufacturer's protocol. Experimental groups were:

- **Negative control (NC–COV434/HUVEC):** Standard culture medium.

- **Sham group (S–COV434/HUVEC):** Medium with 1% DMSO.
- **Melatonin test group (MT–COV434/HUVEC):** 1% DMSO medium with melatonin (1–1000 µM).
- **Positive control (PC–COV434/HUVEC):** Medium with 500 µM H_2O_2.

Melatonin's IC_{50} and effects on proliferation were determined. Real-time proliferation of COV434 and HUVEC cells was monitored using the xCELLigence RTCA System (Roche) with impedance-based analysis on gold-coated E-plates [10].

DNA methylation analysis. After 72 h ± melatonin, DNA was extracted with Qiagen DNeasy kits, quantified on a NanoDrop 2000c, and global 5-mC methylation was measured using the MethylFlash™ ELISA kit (Epigentek) per the manufacturer's protocol.

Statistical analysis. Statistics in GraphPad v9.1.1 used t-tests or Two-way ANOVA for normal data and Mann–Whitney U or Kruskal–Wallis for non-normal data; DNA methylation was analyzed by two-way ANOVA with Tukey's post hoc. Results (mean ± SD) from triplicate assays with three independent replicates were deemed significant at $p < 0.05$.

Results. Since no statistically significant difference was observed between the NC-COV434/HUVEC and S-COV434/HUVEC, their mean values were calculated and presented as a single control data. In the PC-COV434/HUVEC group, no cell viability was observed due to the cytotoxic effect of H_2O_2 (Fig. 1–3) ($p < 0.05$). The results obtained indicate statistically significant differences among the study groups concerning MTT and xCELLigence variables ($p < 0.05$).

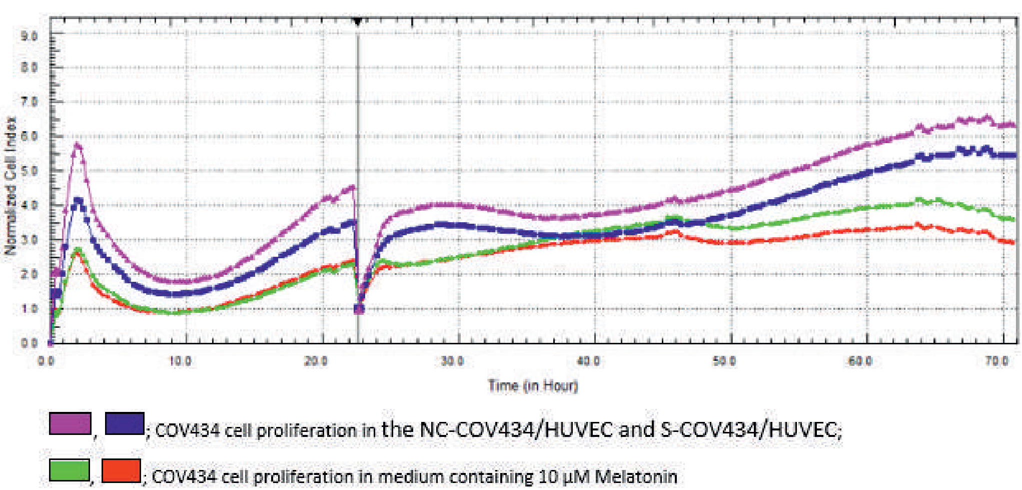

Fig. 1. Cell index values from real-time xCELLigence analysis for HUVEC and COV434 cells treated with melatonin

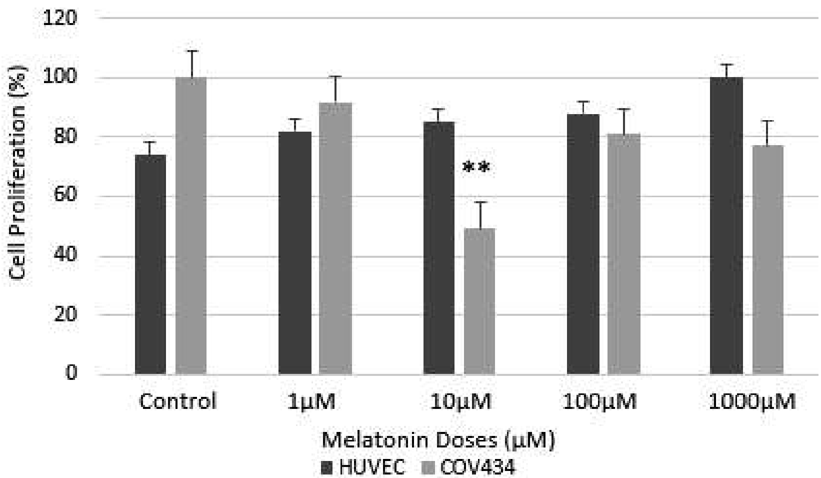

Fig. 2. Cell viability of HUVEC and COV434 cells treated with melatonin (1–1000 µM). Data are expressed as mean ± SD ($n = 3$). $^{***}p < 0.001$; Two-way ANOVA with Tukey's post hoc test. Both xCELLigence and MTT results (Fig. 1, 2) consistently showed cell type-dependent effects of melatonin. In COV434 cells, increasing doses reduced proliferation, with the strongest inhibition at 10 µM, while HUVECs displayed a modest, dose-related increase

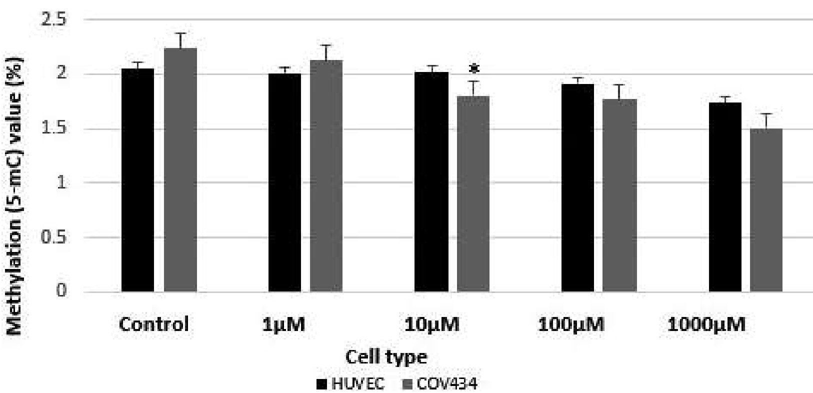

Fig. 3. Global DNA methylation levels in HUVEC and COV434 cells following melatonin treatment at different concentrations (1–1000 µM). Methylation percentages were calculated relative to untreated control samples and expressed as percent change. Data are presented as mean ± SD ($n = 3$). $^{*}p < 0.05$; Two-way ANOVA followed by Tukey's post hoc test

xCELLigence analysis data. xCELLigence results for COV434 and HUVEC cells treated with melatonin (1–1000 µM) are shown in Table 1. In HUVECs (control impedance 1.43), doses ≥ 100 µM significantly increased impedance ($^{*}p < 0.05$), indicating enhanced adhesion or growth. In contrast, COV434 cells (control 1.98) showed a sharp decrease at 10 µM (0.64) and 100 µM (0.69) ($^{**}p < 0.001$), reflecting growth inhibition. These findings confirm that melatonin acts in a dose- and cell type-dependent manner, stimulatory in HUVECs but inhibitory in COV434s.

Table 1

xCELLigence analysis data

XCELLigence average impedance values	**Control (without melatonin)**		**1 µM Melatonin**		**10 µM Melatonin**		**100 µM Melatonin**		**1000 µM Melatonin**		***p* value**
For HUVEC	1.43	1.60	1.72	1.66	1.76	1.87	**1.95***	**2.11***	**2.15***	**2.18***	$^{*}p < 0.05$
For COV434	1.98	2.10	1.28	1.35	**0.64****	**0.69****	0.75	0.84	1.05	1.16	$^{**}p < 0.001$

Two Way ANOVA

The graph of cell impedance data of HUVEC and COV434 cells in XCELLigence e-plate wells in melatonin-free medium and in medium containing 10 µM melatonin is shown in Fig. 1.

MTT analysis data. MTT analysis showed opposite effects of melatonin on HUVEC and COV434 cells. In HUVECs, melatonin significantly increased proliferation at higher doses, with the strongest effect at 1000 µM ($^{*}p < 0.05$). In contrast, COV434 cells exhibited the lowest viability at 10 µM ($^{**}p < 0.001$), with an IC_{50} of 10.55 µM, indicating a clear antiproliferative effect. These data highlight melatonin's dose- and cell type-specific actions.

DNA methylation analysis data. DNA methylation analysis results of 1–1000 µM melatonin doses applied to HUVEC and COV434 cells are given in Fig. 3. In HUVEC cells, a significant decrease in DNA methylation level was observed at 1000 µM melatonin dose ($p < 0.05$).

In COV434 cells, a decrease in DNA methylation level was detected at 10 and 100 µM melatonin doses at $p < 0.05$ level and at 1000 µM melatonin dose at $p < 0.001$ level with a higher significance. These results show that melatonin decreases DNA methylation level in both HUVEC and COV434 cells and this effect is dose responsive.

Discussion. The present study provides novel insights into the dual effects of melatonin on human granulosa tumour cells, showing a dose-dependent inhibition of proliferation accompanied by significant reductions in global DNA methylation. These findings support the notion that melatonin acts not only as an antioxidant and circadian regulator but also as an epigenetic modulator in ovarian cell biology.

Our proliferation assays demonstrated that melatonin exerted a marked inhibitory effect on COV434 cells at micromolar concentrations, with an IC_{50} of approximately 10.5 µM. These results are consistent with previous studies reporting that melatonin suppresses tumour growth by interfering with proliferative signalling pathways, modulating ROS homeostasis, and altering cell cycle dynamics [6]. In contrast, melatonin stimulated proliferation in HUVEC control cells, reinforcing the idea that its biological effects are cell type-specific and context-dependent.

The observed decrease in DNA methylation levels following melatonin treatment further underscores its potential role as an epigenetic regulator. Melatonin has been shown to modulate the activity of DNA methyltransferases (DNMTs), thereby reshaping methylation landscapes that influence gene expression in reproductive cells [7]. Our data suggest that this epigenetic modulation may be particularly relevant in granulosa tumour biology, where aberrant methylation contributes to dysregulated proliferation and survival. Importantly, the findings align with recent work demonstrating melatonin's capacity to preserve ovarian function by activating the PI3K/Akt/mTOR pathway and inhibiting autophagy [8].

Another key mechanistic link lies in melatonin's capacity to modulate oxidative stress. Previous studies have established that melatonin reduces ROS through both direct scavenging activity and indirect enhancement of antioxidant enzymes such as SOD and GPx [12]. In the context of COV434 cells, decreased proliferation in response to melatonin may be partly mediated by its redox-regulatory functions, which intersect with signalling pathways controlling apoptosis and cell cycle arrest.

Thus, reduced ROS is due to both melatonin's direct antioxidant action and its indirect effects on signalling. Melatonin limits ROS by enhancing SOD and GPx activities and modulating MAPK/PI3K-Akt signalling [13]. It also suppresses ROS at membranes and within mitochondria, preserving mitochondrial membrane potential [12], reflecting both direct antioxidant and indirect signalling effects.

Melatonin regulates granulosa cell steroidogenesis through receptor-dependent and -independent mechanisms. At ovulation its follicular fluid levels ($\sim 3\times$ serum) modulate cAMP, cGMP, and PKC to stimulate proliferation via MAPK, but excessive melatonin inhibits MAPK and proliferation, disrupting oocyte maturation and fertilization [14]. FSH-driven aromatase produces estradiol pre-ovulation, while LH induces luteinization and progesterone release. Cadmium lowers DNMT1/3B, modulates DNMT3A, and elevates miR-92a-2-5p to suppress Bcl2 and trigger apoptosis [15].

While this study provides evidence for melatonin's antiproliferative and epigenetic actions in granulosa tumour cells, some limitations should be acknowledged. The use of HUVECs as a non-tumour control cell line may not fully reflect ovarian physiology; therefore, future investigations should employ normal granulosa or luteal cells to strengthen translational relevance. Furthermore, exploring cadmium-driven methylation alterations in granulosa cells could shed light on the interplay between environmental toxicants, epigenetic dysregulation, and melatonin's protective effects.

Overall, our results suggest that melatonin may act through complementary mechanisms – including ROS modulation, receptor-mediated signalling, and epigenetic reprogramming – to inhibit granulosa tumour cell growth. This multifaceted role highlights melatonin as a promising candidate for adjunctive strategies in reproductive oncology.

Conclusion. In conclusion, melatonin demonstrated a dose-dependent inhibitory effect on granulosa tumour cell proliferation while simultaneously reducing global DNA methylation levels. These findings indicate that melatonin's actions extend beyond its antioxidant properties to include direct modulation of epigenetic regulation in granulosa cells. The dual impact on proliferation and methylation underscores melatonin's potential as a supportive therapeutic agent in the management of granulosa cell tumours. Future studies should further dissect the molecular mechanisms underlying these effects, particularly the interaction between ROS signalling, DNA methyltransferase regulation, and melatonin receptor pathways, in order to evaluate its translational potential in reproductive medicine.

REFERENCES

[1] Terranova P. F. (2023) The female reproductive system. In: Part X, Reproductive Physiology, Chap. 38, 668–682, https://fac.ksu.edu.sa/sites/default/files/smch38.pdf.

[2] He J., T. Ye, K. Xu et al. (2025) Melatonin: a potential target for regulating ovarian function, Arch. Gynecol. Obstet., **312**, 721–731, https://doi.org/10.1007/s00404-025-08079-3.

[3] Sadeghpour S., M. Ghasemnejad-Berenji, F. Maleki et al. (2025) The effects of melatonin on follicular oxidative stress and art outcomes in women with diminished ovarian reserve: a randomized controlled trial, J. Ovarian Res., **18**(1), 5, https://doi.org/10.1186/s13048-024-01584-0.

[4] Cruz M. H. C., C. L. V. Leal, J. F. Cruz et al. (2014) Essential actions of melatonin in protecting the ovary from oxidative damage, Theriogenology, **82**(7), 925–932, https://doi.org/10.1016/j.theriogenology.2014.07.011.

[5] Ma J., L. Liu, H. Yang et al. (2024) Melatonin regulates the antioxidant capacity of sheep granulosa cells through a novel uORF-Nrf2aa mediated Nrf2/KEAP1 pathway, Life Sci., **349**, 122693, https://doi.org/10.1016/j.lfs.2024.122693.

[6] Linowiecka K., A. T. Slominski, R. J. Reiter et al. (2023) Melatonin: A potential regulator of DNA methylation, Antioxidants (Basel), **12**(6), 1155, https://doi.org/10.3390/antiox12061155.

[7] Asma A., S. Marc-André (2022) Melatonin signaling pathways implicated in metabolic processes in human granulosa cells (KGN), Int. J. Mol. Sci., **23**(6), 2988, https://doi.org/10.3390/ijms23062988.

[8] Liu Y., Y. Fang, J. Wei et al. (2022) Melatonin protects against primary ovarian insufficiency by activating the PI3K/Akt/mTOR pathway and inhibiting autophagy, Ann. Clin. Lab. Sci., **52**(6), 895–903, https://www.annclinlabsci.org/content/52/6/895.long.

[9] Şahin M., T. Arslanca, Y. Ö. Uçar et al. (2024) The experience of tertıary center for adult granulosa cell tumor: which factors predict survival?, J. Ovarian Res., **17**(1), 127, https://doi.org/10.1186/s13048-024-01453-w.

[10] Güralp O., T. Bese, G. Bildik et al. (2019) The mammalian target of rapamycin protein expression in human granulosa cell tumors, J. Turk. Ger. Gynecol. Assoc., **20**(4), 247–254, https://doi.org/10.4274/jtgga.galenos.2018.2018.0140.

[11] Elgun T., B. Sirinoglu Capan, C. Duman et al. (2023) In vitro evaluation of the cytotoxic effects of alkasite restorative material on human dental pulp stem cells, Ann. Clin. Anal. Med., **14**(11), 1044–1048, https://doi.org/10.4328/ACAM.21846.

[12] Reiter R. J., D. X. Tan, S. Rosales-Corral et al. (2018) Mitochondria: Central organelles for melatonin's antioxidant and anti-aging actions, Molecules, **23**(2), 509, https://doi.org/10.3390/molecules23020509.

[13] Taketani T., H. Tamura, A. Takasaki et al. (2011) Protective role of melatonin in progesterone production by human luteal cells, J. Pineal Res., **51**(2), 207–213, https://doi.org/10.1111/j.1600-079X.2011.00878.x.

[14] Tamura H., A. Takasaki, T. Taketani et al. (2013) Melatonin as a free radical scavenger in the ovarian follicle, Endocr. J., **60**(1), 1–13, https://doi.org/10.1507/endocrj.ej12-0263.

[15] Sun Y., C. Zong, J. Liu et al. (2021) C-myc promotes miR-92a-2-5p transcription in rat ovarian granulosa cells after cadmium exposure, Toxicol. Appl. Pharmacol., **421**, 115536, https://doi.org/10.1016/j.taap.2021.115536.

[1] *Department of Clinic Embryology, Istanbul Yeni Yüzyıl University, Istanbul, Türkiye*
e-mail: nur.erva2528@gmail.com

[2] *Department of Histology and Embryology, Faculty of Medicine, Biruni University, Istanbul, Türkiye*
e-mail: ggundogan@biruni.edu.tr

[3] *Department of Basic Medical Sciences, Faculty of Medicine, Biruni University, Istanbul, Türkiye*
e-mail: halila@biruni.edu.tr

[4] *Department of Biochemistry, Faculty of Pharmacy, Istanbul Health and Technology University, Istanbul, Türkiye*
e-mail: asiye.yurttas@istun.edu.tr

[5] *Department of Medical Biology, Faculty of Medicine, Biruni University, Istanbul, Türkiye*
e-mail: telgun@biruni.edu.tr

[6] *Biruni University Research Center (B@MER), Biruni University, 34015, Istanbul, Türkiye*