Original Article

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Effects of Melatonin on Different Stages of Bladder Cancer Survival

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Abstract

Objective: This study investigated the impact of melatonin on bladder cancer cells, focusing on its influence on cell survival and apoptosis at various stages, and evaluated its potential as a therapeutic agent.
Materials and Methods: The research involved the use of the bladder cancer cell lines RT-112 (grade 1, non-invasive) and HTB-9 (grade 2, invasive) cultured in RPMI-1640 medium. The XTT assay was used to assess cell viability after treating cells with different concentrations of melatonin for various durations. Apoptotic activity was examined via annexin V/PI staining, followed by flow cytometry to quantify cell death. To explore the apoptotic mechanisms involved, Caspase 3/7 activity was also measured. Statistical evaluations were performed using GraphPad Prism, and two-way analysis of variance and Tukey's test were used to determine significance ($p<0.05$).
Results: Melatonin demonstrated a marked reduction in cell viability for both RT-112 and HTB-9 bladder cancer cell lines, with IC50 values determined at 1.115 mM for RT-112 and 2.111 mM for HTB-9. After 24 h of melatonin treatment, the rates of apoptotic cells increased to 47.85% for RT-112 and 38% for HTB-9, both showing statistical significance compared with the control groups ($p<0.0001$). Additionally, cells treated with melatonin showed increased caspase 3/7 activity, indicating the strong induction of apoptosis. Specifically, caspase 3/7 activities were recorded at 46.4% for RT-112 and 38.9% for HTB-9, both significantly exceeding those of control cells ($p<0.0001$). These findings suggest that melatonin effectively inhibits bladder cancer cell proliferation by enhancing apoptosis.
Conclusion: In summary, melatonin is a promising therapeutic agent for bladder cancer because it lowers cell viability and triggers apoptosis in cancer cell lines. The influence of caspase 3/7 activity underscores the importance of activating apoptotic pathways, opening new research possibilities for clinical applications.
Keywords: Annexin V/PI, caspase 3/7, HTB-9 (5637), melatonin, RT-112

Introduction

Bladder cancer ranks as the tenth most prevalent cancer globally, with the incidence of this malignancy steadily rising over recent decades (1). Despite improvements in diagnostic and treatment approaches, the high rates of recurrence and progression pose a substantial health challenge (2,3). Conventional treatment options for bladder tumors, such as transurethral resection, chemotherapy, and immunotherapy, often come with significant side effects and limitations, highlighting the need for new therapeutic agents (4,5). Recently, melatonin -a hormone mainly secreted by the pineal gland- has attracted interest for its potential anticancer properties (6). Apart from its established role in managing circadian rhythms and sleep-wake cycles, melatonin exhibits antioxidant, anti-inflammatory, and immunomodulatory characteristics, making it a promising contender in oncology (7-9).

Research has increasingly emphasized melatonin's capacity to influence multiple biological mechanisms that play a role in cancer progression, including oxidative stress, apoptosis, and the regulation of the cell cycle (10-13). In the context of bladder cancer, oxidative stress is a significant contributor to both carcinogenesis and tumor progression. Melatonin acts as an effective free radical scavenger, potentially mitigating these effects by lowering the levels of reactive oxygen species and bolstering antioxidant enzyme activity (14,15). Furthermore, melatonin has been shown to promote apoptosis and inhibit

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the proliferation of various cancer cell lines, particularly those originating from bladder tissue (16,17). Its engagement with melatonin receptors (MT1 and MT2) in cancer cells indicates its involvement in cell signaling pathways that may affect tumor dynamics (16,18).

Additionally, disturbances in circadian rhythms, commonly seen in shift workers or individuals with inconsistent sleep schedules, have been linked to an elevated risk of developing bladder cancer (19,20). Melatonin's role in preserving circadian balance suggests a potential preventive function in bladder carcinogenesis (14,21). Given these diverse actions, a deeper understanding of the relationship between melatonin and bladder cancer could pave the way for innovative therapeutic strategies. This study aimed to analyze existing knowledge regarding melatonin's impact on bladder cancer cells, specifically focusing on its effects on cell survival and apoptosis across different stages of the disease.

Materials and Methods

This study employed cell lines; thus, ethical approval was not deemed necessary. The bladder cancer cell lines RT-112 (ATCC) and HTB-9 (ATCC) were obtained commercially.

Cell Culture

RT-112 (grade 1, non-invasive) and HTB-9 (grade 2, invasive) bladder cancer cells were maintained in RPMI-1640 (Capricorn Scientific) medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Capricorn Scientific) at 37 °C using standard cell culture techniques in an incubator with %5 CO_2 and 95% humidity.

Cell Viability Assay

Cell viability for RT-112 and HTB-9 was assessed using the XTT (Biotium) assay to evaluate the cytotoxic effects of melatonin (22). Briefly, 1×10^4 cells were seeded in 96-well plates and allowed to attach overnight. Cells were then treated with various concentrations of melatonin (0, 0.5, 1, 2, and 5 mM) for 12, 24, or 48 h. Following treatment, an XTT solution was added, and the mixture was incubated for 3 h at 37 °C. Cell viability was measured using a microplate reader at an absorbance wavelength of 460 nm. Each experiment was repeated three times.

Annexin V/PI Analysis

To evaluate apoptosis, RT-112 and HTB-9 bladder cancer cells were plated in 6-well dishes. The cell lines were treated with melatonin at concentrations of 1.077 mM for RT-112 and 2.111 mM for HTB-9. After a 24-h incubation period, all cells were collected using trypsin-EDTA, washed with PBS, and the concentration was adjusted to 1×10^5 cells in 100 µL. The resulting cell suspension was transferred to 12x75 mm polystyrene tubes, and 5 µL of Annexin V-FITC and 5 µL of propidium iodide were added to 500 µL of 1X Annexin Binding Buffer. After incubating at room temperature for 15 min, cells were analyzed using an ACEA NovoCyte flow cytometer (Agilent). Based on flow cytometric measurements, cells were analyzed as described in our previous study (23).

Caspase 3/7 Analysis

As in our previous studies, caspase 3/7 analysis was performed to identify the apoptotic pathways utilized by RT-112 and HTB-9 cells (24). Cells were treated with the IC50 of melatonin and incubated overnight. After 24 h of treatment, the cells were trypsinized, collected, and assessed for caspase 3/7 activity using the Cell Meter™ Caspase 3/7 flow cytometry assay kit on an ACEA NovoCyte flow cytometer (Agilent). All measurements were repeated three times, and the average standard deviation was calculated for the data.

Statistical Analysis

Statistical evaluations were performed using GraphPad Prism version 8.4.2. The two-way ANOVA test was used for intergroup comparisons, with differences in means compared with the control group assessed using the Tukey test. Results are expressed as mean ± standard deviation, and a significance level of $p<0.05$ was established.

Results

Melatonin Reduces the Viability of Bladder Cancer Cells

The effects of melatonin were assessed at concentrations of 0, 0.25, 0.50, 1, 2, and 5 mM on the RT-112 and HTB-9 human bladder cancer cell lines, with treatment durations of 12, 24, and 48 h. As illustrated in Figure 1, melatonin exerted significant cytotoxic effects on the bladder cancer cell lines in a time-dependent manner, with IC50 values of 1.115 mM for RT-112 and 2.111 mM for HTB-9 after 24 h. The XTT assay results revealed that the viability of RT-112 cells after 24 h was 100%, 79.68%, 69.24%, 49%, 36.07%, and 20.50%. Similarly, for HTB-9 cells, the viability percentages were 100%, 88.6%, 82.80%, 63.32%, 50.30%, and 32.70% at the same concentrations (Figure 1). These findings indicate that melatonin reduces the viability of bladder cancer cells in a dose-dependent manner, suggesting its potential to inhibit cell proliferation. Increased melatonin concentrations correlated with a more pronounced decrease in cell viability for both RT-112 and HTB-9 cells.

Melatonin Induces Apoptosis in RT-112 and HTB-9 Cells

Treatment with melatonin at concentrations of 1.115 mM for RT-112 and 2.111 mM for HTB-9 for 24 h resulted in higher rates of apoptosis, with 47.85% and 38% of cells, respectively, undergoing apoptotic changes compared with the control group. This finding is consistent with the cytotoxicity data (Figure 2). The results confirmed that melatonin effectively inhibited the growth of RT-112 and HTB-9 bladder cancer cells by promoting apoptosis. Additionally, melatonin-treated RT-112 and HTB-9 cells exhibited a reduction in viable cell populations of 51.2% and 59.25%, respectively, compared with controls. Notably, non-invasive RT-112 cells exhibited a higher apoptosis rate than invasive HTB-9 cells.

Melatonin Promotes Caspase 3/7 Activity in RT-112 and HTB-9 Cells

To elucidate the mechanism by which melatonin induces apoptosis, we measured the levels of caspase 3/7, a known

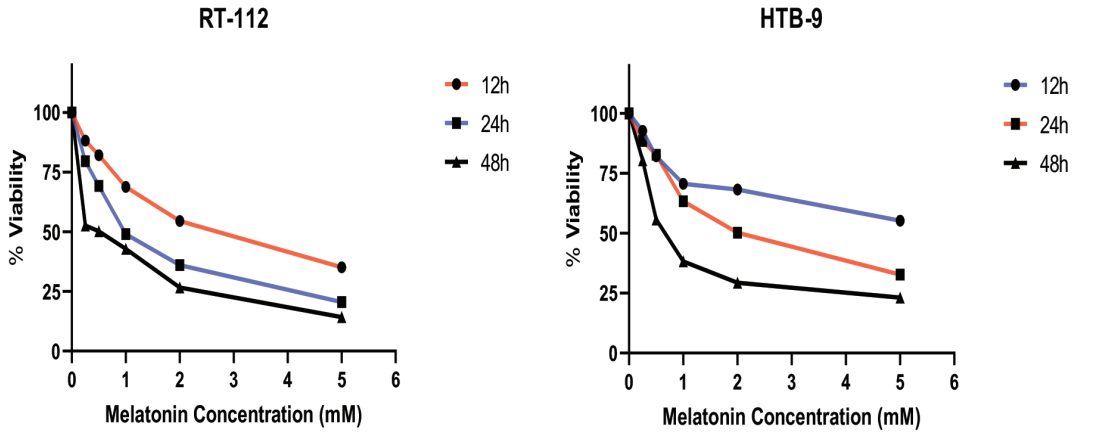

Figure 1. XTT analysis demonstrating the cell viability of RT-112 and HTB-9 cells following treatment with increasing concentrations of melatonin over 12, 24, and 48 h

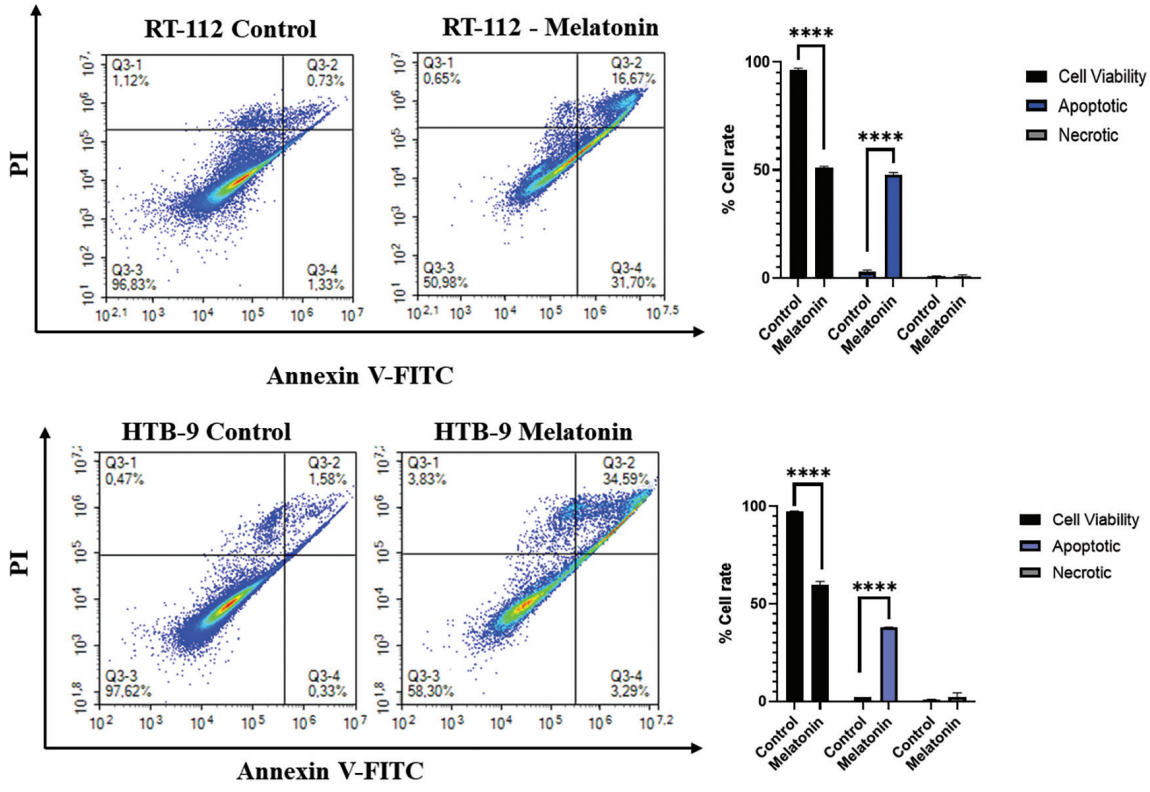

Figure 2. Representative dot plot graphs show RT-112 and HTB-9 cells treated with melatonin at their IC50 concentrations for 24 h, used to assess apoptotic cell death through Annexin V/FITC-PI staining. Additionally, the percentages of viable, apoptotic, and necrotic cells were analyzed statistically, with values representing three independent experiments (****p<0.0001)

marker of apoptotic activity, using flow cytometry. The results showed a significant increase in caspase 3/7 activation in both RT-112 and HTB-9 cells treated with melatonin compared with controls after 24 h at IC50 concentrations (p<0.0001) (Figure 3). Specifically, caspase 3/7 activity was 46.4% in RT-112 cells and 38.9% in HTB-9 cells in the melatonin-treated group, which was significantly higher than that in the control group (p<0.0001). These findings corroborate the apoptosis assay results.

Discussion

Bladder cancer remains a significant health challenge because of its increasing incidence and high rates of recurrence and

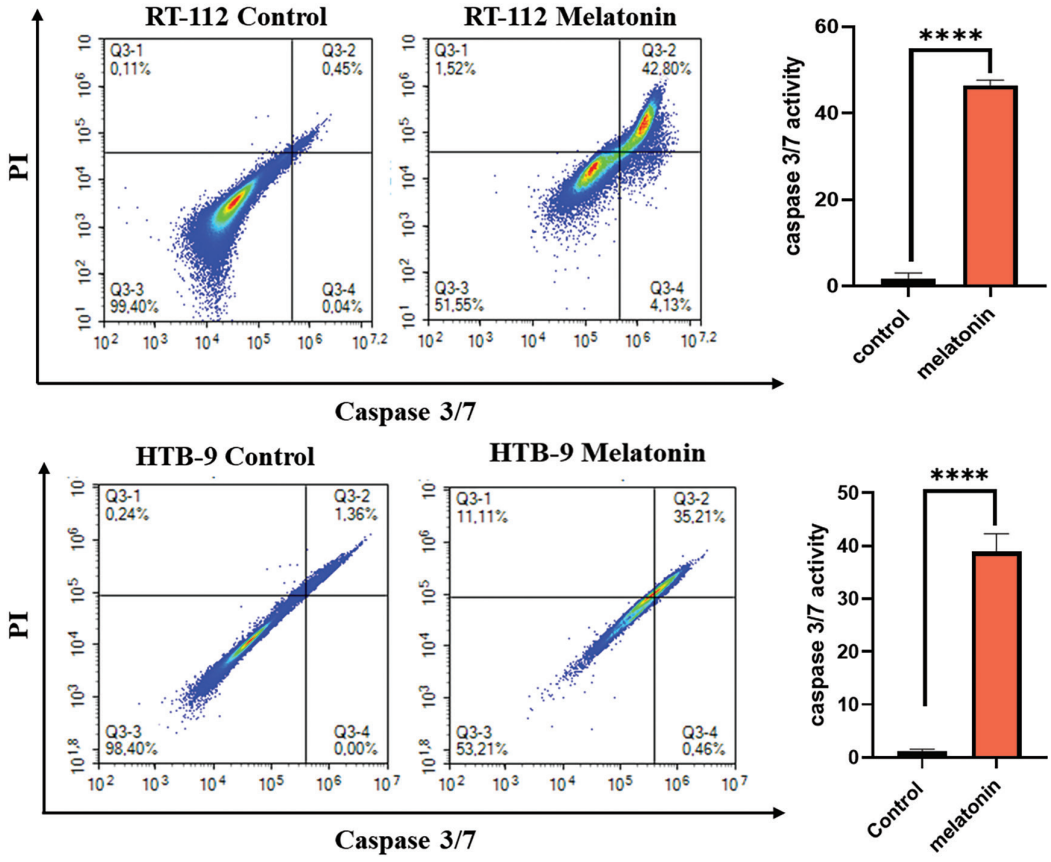

Figure 3. Caspase 3/7 activity measured by flow cytometry in RT-112 and HTB-9 cells treated with IC50 melatonin for 24 h. Bar graphs show the comparison of melatonin treated cells and control group of caspase 3/7 activity. Data are displayed as the mean ± standard deviation. Significant differences are shown compared with control and theobromine-treated cells (****p<0.0001)

progression. In this study, we examined the effects of melatonin on the bladder cancer cell lines RT-112 (non-invasive) and HTB-9 (invasive), specifically focusing on its influence on cell survival and apoptosis.

Our results indicate that melatonin effectively decreases the viability of bladder cancer cells in a dose-dependent manner. The IC50 values of RT-112 and HTB-9 after 24 h of treatment were 1.115 and 2.111 mM, respectively. These outcomes are consistent with previous studies that documented the cytotoxic properties of melatonin across various cancer cell types (25). Prior studies have also suggested that melatonin can hinder cancer cell proliferation by inducing apoptosis, a finding that aligns with our observations of significant apoptosis rates of 47.85% in RT-112 cells and 38% in HTB-9 cells (26).

The mechanism by which melatonin exerts its effects appears to be associated with its capacity to activate apoptotic pathways. The results of the caspase 3/7 assay revealed a substantial increase in caspase activity in both cell lines following treatment with melatonin, indicating that melatonin not only diminishes cell viability but also facilitates apoptotic cell death (26). This result is in agreement with prior reports that highlighted the role of melatonin in augmenting caspase activity in cancer cells (27).

Additionally, our findings highlight the differential responses of bladder cancer cell lines to melatonin, with noninvasive RT-112 cells demonstrating a higher rate of apoptosis compared with invasive HTB-9 cells. These findings suggest a potential role for melatonin as a therapeutic agent specifically targeting early-stage bladder cancer although further research is necessary to clarify the underlying mechanisms. The existing literature suggests that the invasive characteristics of cancer cells may contribute to a certain degree of resistance to apoptosis, potentially accounting for the lower apoptosis rate observed in HTB-9 cells (2,21).

Another crucial factor in our discussion was the relationship between circadian rhythms and cancer progression. Alterations in circadian rhythms have been associated with an increased risk of bladder cancer (19). Melatonin's role in maintaining circadian homeostasis might play a part in its preventive effects against bladder carcinogenesis, as highlighted in recent studies emphasizing the significance of circadian regulation in tumor biology (20). Therefore, incorporating melatonin into treatment protocols could provide dual advantages by directly targeting cancer cells and restoring circadian rhythms.

Our findings indicate that melatonin has considerable antitumor effects against bladder cancer cells. The pronounced apoptotic

response in RT-112 cells suggests the potential of melatonin as a therapeutic agent for early-stage bladder cancer treatment. These results demonstrate that melatonin inhibits cancer cell survival and activates apoptotic pathways. The reduced apoptotic response in HTB-9 cells, which can be attributed to their invasive traits, supports the idea that the aggressive nature of certain cancer cells may contribute to their treatment resistance.

Study Limitations

Although our study underscores the promise of melatonin in bladder cancer management, it is crucial to acknowledge its limitations. The findings are derived from in vitro studies, necessitating further research, including in vivo studies and clinical trials, to validate our results and evaluate the therapeutic efficacy of melatonin in patients with bladder cancer.

Conclusion

In conclusion, our study provides compelling evidence of the antiproliferative effects of melatonin in bladder cancer cell lines. The ability of melatonin to reduce cell viability, induce apoptosis, and modulate oxidative stress positions makes it a potential novel therapeutic agent for bladder cancer management. These findings add to the growing body of literature advocating for the integration of melatonin into cancer treatment strategies, particularly for individuals at elevated risk of developing bladder cancer.

Ethics

Ethics Committee Approval: This study employed cell lines; thus, ethical approval was not deemed necessary.

Informed Consent: Patient consent is not required.

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Publication: The results of the study were not published in full or in part in form of abstracts.

Contribution: There is not any contributors who may not be listed as authors.

Footnotes

Authorship Contributions

Concept: T.O-S., A.S., E.A., A.N.K., Design: T.O-S., A.S., E.A., A.N.K., Data Collection or Processing: T.O-S., A.S., E.A., A.N.K., Analysis or Interpretation: T.O-S., A.S., E.A., A.N.K., Literature Search: T.O-S., A.S., E.A., A.N.K., Writing: T.O-S., A.S., E.A., A.N.K. .

Conflict of Interest: One author of this article, (Ahmet Nihat Karakoyunlu) is a member of the Editorial Board of the Bulletin of Urooncology. However, he did not take part in any stage of the editorial decision of the manuscript. The editors who evaluated this manuscript are from different institutions. The other authors declared no conflict of interest.

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