Different Effects of Melatonin on X-Rays-Irradiated Cancer Cells in a Dose-Dependent Manner

Hao Zhu[1], Yong Chen[2], Liang-cai Bai[1] , Xiang-rong Cao[1], and Rui Xu[1]

Abstract

The purpose of this study is to investigate the effects of melatonin on the radiosensitivity of HeLa cells. Concentration from 10 to 1000 μM of melatonin was used on HeLa cells before X-rays irradiation (IR). The cellular inactivation effect was analyzed by clonogenic assay, and cell growth was measured by MTT assay at various concentrations. Ten micrometer melatonin promoted the cell-killing effects of IR, while 1000-μM melatonin prevented IR-induced cellular inactivation. Further analysis revealed that 1000-μM melatonin protected the cells from IR-induced reactive oxygen species damage, as the oxidative stress measured by fluorescent microscopy and fluorescence-activated cell sorting using 2,7-dichlorofluorescein diacetate staining. This is further confirmed by melatonin receptor agonist, which has no antioxidant capacity. A 10-μM melatonin, on the contrary, enhanced the cell-killing effects of IR by activating c-Jun NH2-terminal kinase (JNK) signaling. c-Jun NH2-terminal kinase signaling activation was indicated by Western blot of phosphorylated JNK. We used JNK inhibitor to further confirm the involvement of JNK signaling in the cell-killing enhancement of 10-μM melatonin administration. Our results suggest the importance of dose-dependent effects in melatonin application for radiotherapy.

Introduction

Melatonin is a natural indoleamine that regulates circadian rhythms in living organisms. Besides its role in light/darkness responses regulation, melatonin has been shown to exert anti-tumoral effects in many cancer cells.[1-3] Its effect on cancer cells varies depending on its concentration. At low concentrations, melatonin inhibits cancer cell proliferation.[4,5] At high concentration, it enhances apoptosis in some certain cancer types.[6-8]

The mechanism involved in the antitumoral property of melatonin in high and low concentrations is different. Low concentration of melatonin shows oncostatic effects on tumors, which works mainly via melatonin receptors on the membrane through multiple pathways.[9] The high concentration of melatonin induces apoptosis via the early increase in reactive oxygen species (ROS), as well as a reduction in intracellular glutathione (GSH).[10] The observed redox disturbance of high concentration of melatonin on cancer cells is counterintuitive since millimolar of melatonin is effective ROS scavenger that can protect normal cells from oxidative damage.

Melatonin can either activate or inhibit signal transduction in a receptor-dependent or receptor-independent manner. It binds with high affinity to the membrane receptors in the picomolar range, in the nanomolar range it can translocate to the nuclear receptors (RZR/ROR) as well as to calmodulin. At millimolar concentrations, it can serve as an antioxidant compound.[11] Many cancer types has been shown to be susceptible to inhibition by melatonin, including, but not limited, to breast cancer,[12] ovarian cancer,[13] pancreatic cancer,[14] and hepatic cancer.[15] Considering its antitumoral potential and its

[1] Radiology Department, Lanzhou University Second Hospital, Lanzhou, China
[2] MOE Key Laboratory of Cell Activities and Stress Adaptations, School of Life Sciences, Lanzhou University, Lanzhou, China

Corresponding Author:
Liang-cai Bai, Radiology Department, Lanzhou University Second Hospital, Cui ying Gate 82#, Chengguan District, Gansu Province, Lanzhou 730030, China.
Email: 16465115@qq.com

protection against ROS damage for normal cells, melatonin could be a good candidate for radiotherapy to enhance antitumoral effects, as well as to limit the collateral damage of ionizing radiation (IR), if the correct dosage can be applied. The c-Jun NH2-terminal kinase (JNK) pathway is one of the major signaling cassettes of the mitogen-activated protein kinase signaling pathway. It regulates several cellular processes, including proliferation and apoptosis. Irradiation can stimulate JNK cascade that leads to apoptosis.[16,17] Similarly, melatonin can also induce cell apoptosis through activation of JNK.[18] Coadministration of melatonin has been shown to improve the sensitivity of cancers to inhibition by conventional drugs. It is, therefore, interesting to investigate whether JNK signaling participates in the effect of cell death by the combination of melatonin and IR.

Materials and Methods

Cell Culture and IR

The human cervical cancer cell line HeLa was purchased from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 10% fetal bovine serum (FBS). Cells were maintained at 37°C using humidified air supplemented with 5% CO_2. Cells in exponential growth were irradiated with 250 kVp X-rays with a dose rate of 1 Gy/min. Different concentrations of melatonin were added to the medium 2 hours before IR.

Survival Assay

The sensitivity of cells to X-rays radiation was measured using a colony-formation assay. Confluent cultures were replated within 1 hour post-IR into 100-mm dishes in quadruplicate at numbers estimated to yield 50 to 100 clonogenic colonies per dish. After incubation for 2 weeks, cultures were fixed in methanol and stained with crystal violet. These stained colonies were observed with an Olympus light microscope. Colonies with 50 or more cells were scored as clonogenic colonies.

MTT Assay

HeLa cells were grown in 96-well culture plates and incubated for 1 to 3 days with DMEM/serum containing various concentrations of melatonin. MTT (5 mg/mL in water, 10 μL/well) was then added into each well. After incubation for 4 hours at 37°C, the supernatant was abandoned and 100 μL of dimethyl sulfoxide was added into each well to dissolve the produced formazan. After shaking the plates for 5 minutes, absorbance values of the produced purple formazan were recorded with a microplate reader at 490 nm.

Western Blot Assay

Cells were lysed in RIPA buffer (Beyotime, Nanjing, China). Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a methanol-activated polyvinylidene difluoride membrane. The membrane was blocked for 1 hour in Tris-buffered saline (TBST) containing 0.5% FBS and subsequently probed with Phospho-SAPK/JNK (Thr183/Tyr185) antibody (Cell Signaling, California) at 4°C overnight with shaking. After 1-hour incubation with HRP-conjugated secondary antibody (Santa Cruz), membranes were washed 3 times for 15 minutes each with TBST. Reactive proteins were visualized using a chemiluminescence kit (Santa Cruz) according to the manufacturer's instructions.

Reactive Oxygen Species Measurement

The fluorescent probe, 2,7-dichlorofluorescein diacetate (DCFH-DA, Sigma), was employed to quantify the level of ROS. Confluent cultures were washed with D-Hank buffer solution complemented with 1% FBS at 37°C for 2 minutes, stained with 2 M DCFH-DA for 40 minutes in the incubator and then washed with cold D-Hank twice. The staining was captured under microscopy.

For fluorescence-activated cell sorting (FACS) assay, cells were harvested by trypsin–EDTA solution to produce a single-cell suspension. The cells were pelleted by centrifugation and washed twice with phosphate-buffered serum to remove the background fluorescence. Stained cells were analyzed using a flow cytometer (Becton Dickinson, Heidelberg, Germany) in combination with Flowing (http://flowingsoftware.btk.fi/).

Statistical Analysis

Data represent mean (standard deviation) of 3 replicates. Independent samples t test was applied. Experiments were performed successfully at least 3 times. All experiment data were tested using GraphPad Prism 6.02 (GraphPad Software, La Jolla, California). Value of $P < .05$ was considered statistically significant.

Results

Different Effects of Melatonin on Cell Survival After IR in a Dose-Dependent Manner

Melatonin showed no significant inhibitory effect on cell growth, except a temporal growth inhibition at 24 hours at a concentration of 1 mM (Figure 1A). Dose-dependent cytotoxicity on clonal formation was observed with IR alone (Figure 1B). Melatonin has antitumoral effects by inducing apoptosis at a millimolar concentration.[10] To test whether melatonin can enhance the cell-killing effect of IR, various concentrations of melatonin were used. To our surprise, 10-μM melatonin administration markedly reduced the clonal formation after 4 Gy IR, and 1-mM melatonin protected HeLa cells from radiation-induced clonal formation inhibition (Figure 1C).
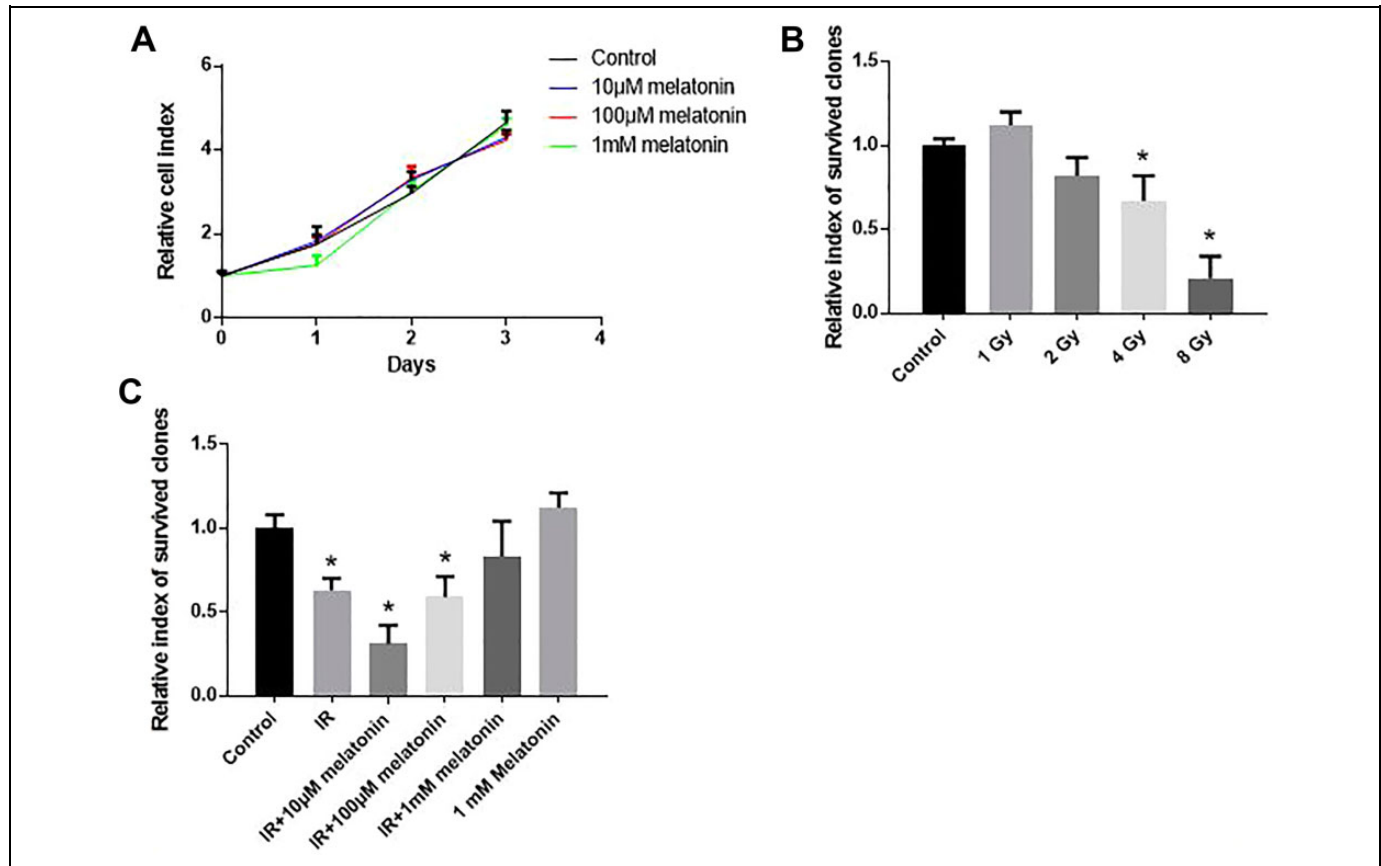
Figure 1. The dosage effects of melatonin on IR-induced cellular inactivation of HeLa cells. A, Cell growth measured by MTT from 1 to 3 days with a presence of melatonin from 10 to 1000 μM. B, Dose-dependent reduction of survived clones after IR. C, The effects of melatonin on clonogenic survival of irradiated cells, cells were irradiated by 4 Gy IR. * Significant difference versus control at *P* <.05. IR indicates irradiation.

Millimolar Melatonin Prevent Radiation-Induced Cellular Inactivation by Its Antioxidant Capacity

Ionizing radiation is used to kill cells by inducing DNA damage via ROS generation. It is possible that the protective effect of melatonin against IR could be due to its antioxidant ability. The DCF signal is much weaker in 4 Gy-irradiated cells treated with 1-mM melatonin compared to untreated cells (Figure 2A). The FACS result also confirmed the reduction of ROS in 1-mM melatonin-treated group (Figure 2B). Agomelatine, a melatonin receptor agonist, was unable to protect the cells from IR-induced cellular inhibition, but instead enhanced the cell-killing effect of IR (Figure 2C).

Micromolar Melatonin Activated JNK Signaling to Promote IR-Induced Cellular Inactivation

The synergistic effect of IR and melatonin agonist suggests that MT-mediated signaling plays an important role in the boosted cell-killing effect by melatonin. Melatonin can activate JNK signaling through melatonin receptors on the membrane. Since JNK signaling pathway can drive cells to apoptosis, we postulate that the increased cellular inactivation by 10-μM melatonin

treatment after IR could be due to JNK activation. The JNK phosphorylation was detected with the presence of melatonin from the range of 10 to 1000 μM (Figure 3A). The induction of JNK phosphorylation was independent of IR (Figure 3B). SP600125, a specific p-JNK inhibitor, abrogated the enhancement of cell killing in cells treated with 10-μM melatonin and IR (Figure 3C).

Discussions

The physiological concentration of melatonin, which is found in the plasma in animals and the human body, is within the nanomolar range. However, our initial experiments found no changes in HeLa cells if melatonin was administrated at nanomolar (data not shown). This could be due to its short half-life estimated (20-50 minutes in the body). Sainz et al reported that 1-mM melatonin does not promote IR-induced cell death due to increased GSH content.[19] Similarly, we found that 1-mM melatonin prevented IR-induced cell death. The protective effect is due to enhanced antioxidant capacity by the presence of a high concentration of melatonin. Also, we found a low concentration of melatonin (10 μM) efficiently enhanced the cell-killing effect of IR, with the induction of JNK signaling. This is the
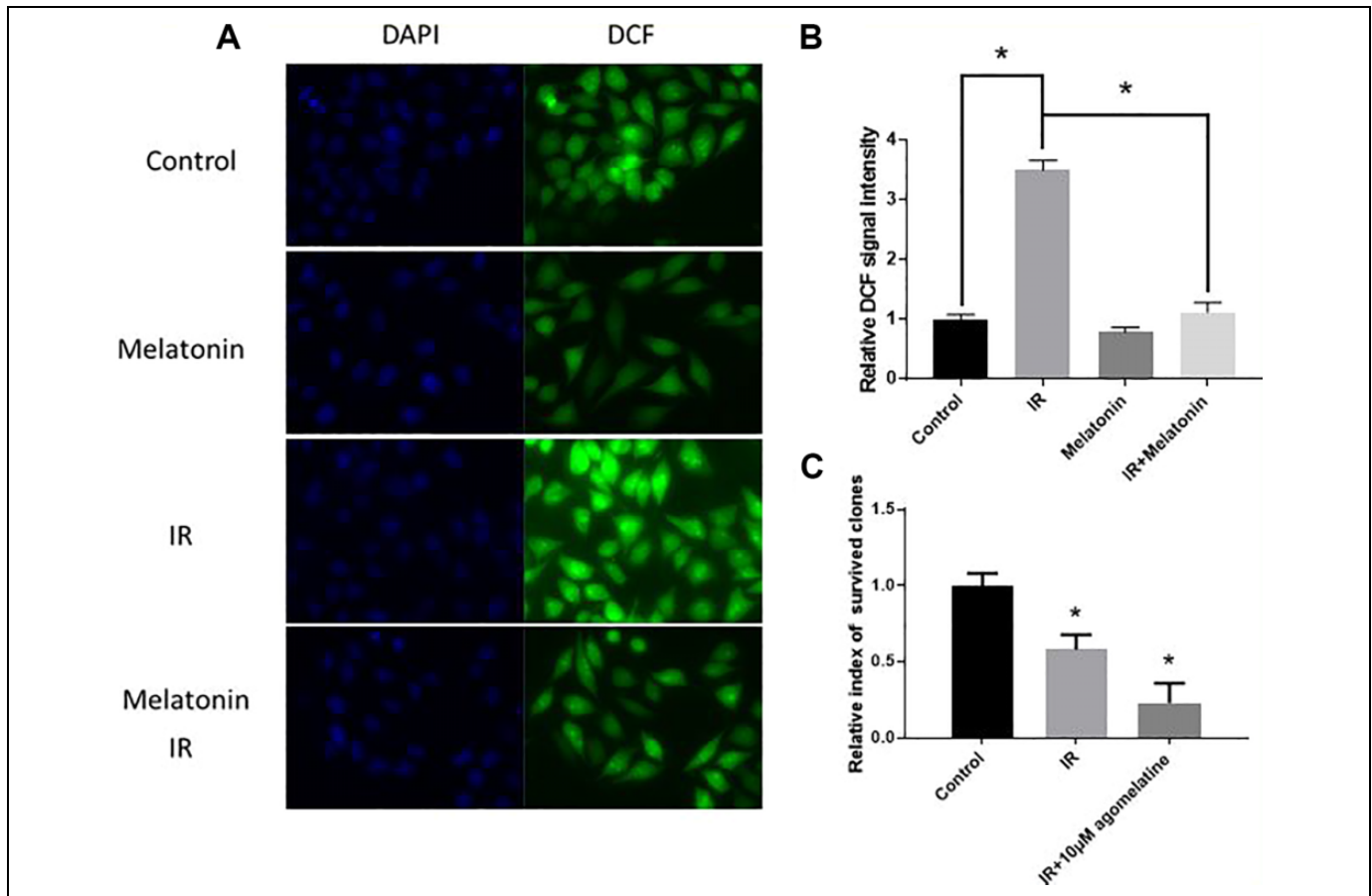
Figure 2. The antioxidant capacity of high concentration melatonin protected HeLa cells from IR-induced cellular inactivation. A, Representative images of cells stained by DCFH-DA 2 hours after 4 Gy IR. HeLa cells were treated with IR and/or 1000-μM melatonin. B, FACS measurement of DCF signal 2 hours after IR with/without 1000-μM melatonin after 4 Gy IR. C, The relative index of survived clones after 4 Gy IR with/without melatonin receptor agonist agomelatine. * Significant difference between 2 groups at $P<.05$. DCFH-DA indicates 2,7-dichlorofluorescein diacetate; FACS, fluorescence-activated cell sorting; IR, irradiation.

first report showing that melatonin at a relative lower concentration can enhance the efficiency of cellular inactivation by IR.

The researchers reported that melatonin-induced cell death is associated with an early increase in ROS at a concentration of 1 mM.[20-25] The cell growth was not significantly affected by melatonin up to 1 mM in HeLa cells, indicating the cellular inhibition effect of melatonin could be cell-type specific. Another explanation is that HeLa cells can tolerate the early increase in ROS induced by melatonin. But, since we are looking for the long-term effect of radiotherapy, this ROS generation in the early exposure to melatonin was not investigated.

Our data showed that melatonin, as well as the receptor agonist, can activate the melatonin receptor-mediated JNK pathway, which can enhance the cell-killing effect of IR. These data indicate the potential role of melatonin receptor-mediated signaling pathway in radiotherapy. Clinical applications of melatonin for cancer, such as breast cancer, have shown promising results. However, the potential application of melatonin

in radiotherapy is still under its way. Especially, the dosage effects of melatonin in combination with IR has not been studied. It will be ideal that an appropriate dose of melatonin can be administrated on cancer and its adjacent normal tissue during radiotherapy. The high concentration of melatonin presented in the normal tissue can abrogate the side effects of IR, while the presence of nanomolar melatonin can activate signaling pathway that enhances the killing effect of IR. The plasma concentration of melatonin is negatively related to age.[26] It will be interesting to investigate whether a supplement of melatonin to the aged patient could enhance the efficiency of radiotherapy.

In summary, we found that the melatonin can enhance the cell-killing effect of IR at low concentration, while the high dosage of melatonin protected cells from IR-induced death at high dosage. These different effects of melatonin are due to the versatile nature of melatonin. The MT-mediated JNK signaling accounts for the enhanced cell-killing effect, but melatonin can also prevent DNA damage induced by radiation by its ROS scavenging ability at higher concentration. These results indicate that melatonin can be used to promote radiotherapy
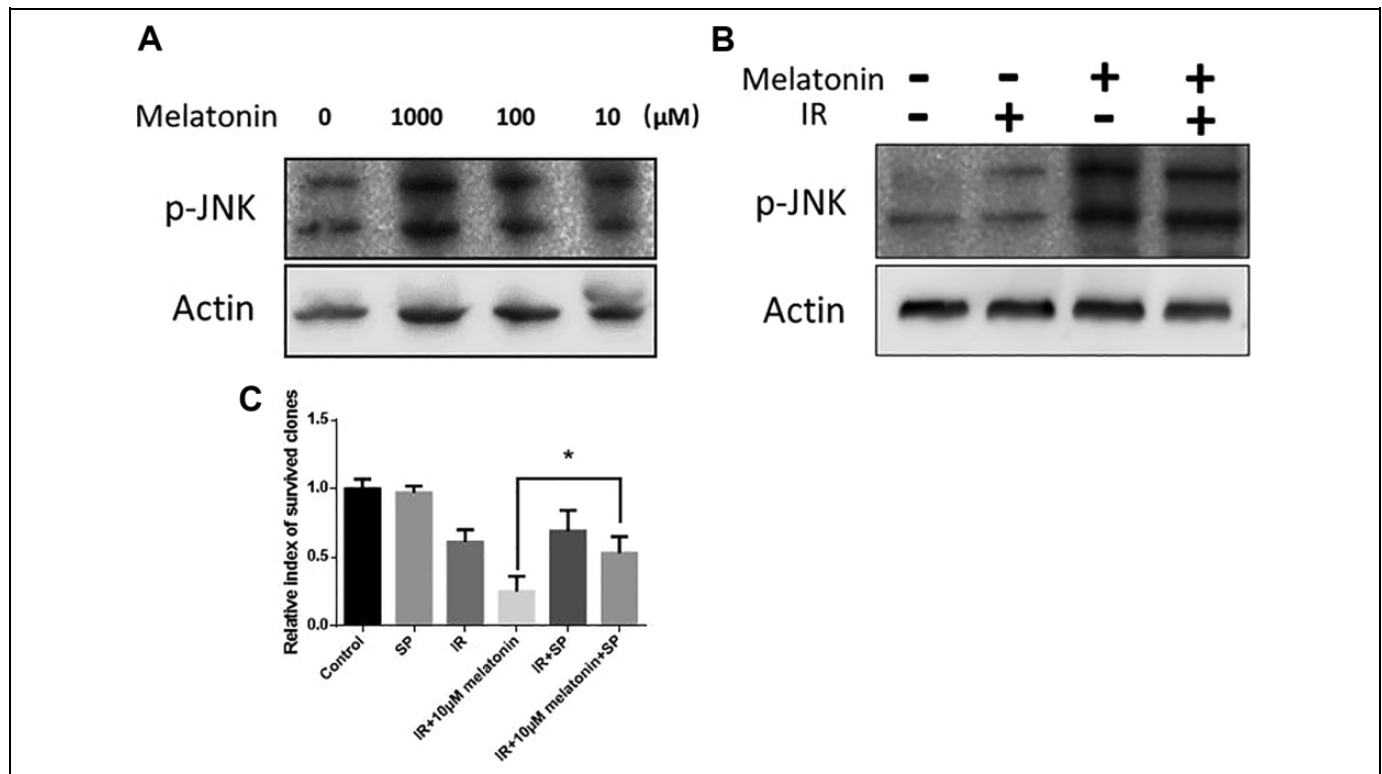
Figure 3. A 10-μM melatonin promoted IR-induced cellular inactivation via JNK signaling. A, Representative image of p-JNK blotting with different concentrations of melatonin. The cells were collected 4 hours with melatonin treatment. B, Representative image of p-JNK blotting after treatment of 10-μM melatonin with/without IR. The cells were collected 2 hours after 4 Gy IR. A 10-μM melatonin was added 2 hours prior to the IR. C, Relative survived clones treated with 10-μM melatonin with/without 100-μM JNK inhibitor SP600125 (SP) after 4 Gy IR. * Significant difference between 2 groups at $P < .05$. JNK indicates c-Jun NH2-terminal kinase; IR, irradiation.

efficiency by enhancing cancer cell killing and protecting the normal tissue from radiation damage.

ORCID iD

Liang-cai Bai 🔟 https://orcid.org/0000-0003-1459-2775

References

1. Vijayalaxmi, Thomas CR Jr, Reiter RJ, Herman TS. Melatonin: from basic research to cancer treatment clinics. *J Clin Oncol*. 2002;20(10):2575-25601.
2. Reiter RJ. Melatonin: clinical relevance. *Best Pract Res Clin Endocrinol Metab*. 2003;17(2):273-285.
3. Reiter RJ, Tan DX, Tamura H, Cruz MH, Fuentes-Broto L. Clinical relevance of melatonin in ovarian and placental physiology: a review. *Gynecol Endocrinol*. 2014;30(2):83-89.
4. Hill SM, Blask DE. Effects of the pineal hormone melatonin on the proliferation and morphological characteristics of human breast cancer cells (MCF-7) in culture. *Cancer Res*. 1988; 48(21):6121-6126.
5. Blask DE, Sauer LA, Dauchy RT, Holowachuk EW, Ruhoff MS, Kopff HS. Melatonin inhibition of cancer growth in vivo involves suppression of tumor fatty acid metabolism via melatonin receptor-mediated signal transduction events. *Cancer Res*. 1999; 59(18):4693-4701.
6. Wenzel U, Nickel A, Daniel H. Melatonin potentiates flavone-induced apoptosis in human colon cancer cells by increasing the level of glycolytic end products. *Int J Cancer*. 2005;116(2):236-242.
7. Martin V, Garcia-Santos G, Rodriguez-Blanco J, et al. Melatonin sensitizes human malignant glioma cells against TRAIL-induced cell death. *Cancer Lett*. 2010;287(2):216-223.
8. Casado-Zapico S, Rodriguez-Blanco J, Garcia-Santos G, et al. Synergistic antitumor effect of melatonin with several chemotherapeutic drugs on human Ewing sarcoma cancer cells: potentiation of the extrinsic apoptotic pathway. *J Pineal Res*. 2010;48(1):72-80.
9. Hill SM, Frasch T, Xiang S, Yuan L, Duplessis T, Mao L. Molecular mechanisms of melatonin anticancer effects. *Integr Cancer Ther*. 2009;8(4):337-346.

10. Rodriguez C, Martin V, Herrera F, et al. Mechanisms involved in the pro-apoptotic effect of melatonin in cancer cells. *Int J Mol Sci*. 2013;14(4):6597-6613.

11. Carlberg C. Gene regulation by melatonin. *Ann N Y Acad Sci*. 2000;917(1):387-396.

12. Yun SM, Woo SH, Oh ST, et al. Melatonin enhances arsenic trioxide-induced cell death via sustained upregulation of Redd1 expression in breast cancer cells. *Mol Cell Endocrinol*. 2016;422: 64-73.

13. Ferreira GM, Martinez M, Camargo ICC, Domeniconi RF, Martinez FE, Chuffa LGA. Melatonin attenuates Her-2, p38 MAPK, p-AKT, and mTOR levels in ovarian carcinoma of ethanol-preferring rats. *J Cancer*. 2014;5(9):728-735.

14. Ruiz-Rabelo JF, Vazquez R, Perea MD, et al. Beneficial properties of melatonin in an experimental model of pancreatic cancer. *J Pineal Res*. 2007;43(3):270-275.

15. Fan LL, Sun GP, Ma T, Zhong F, Wei W. Melatonin overcomes apoptosis resistance in human hepatocellular carcinoma by targeting Survivin and XIAP. *J Pineal Res*. 2013;55(2):174-183.

16. Chen YR, Wang XP, Templeton D, Davis RJ, Tan TH. The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation—duration of JNK activation may determine cell death and proliferation. *J Biol Chem*. 1996;271(50): 31929-31936.

17. Hara T, Namba H, Yang TT, et al. Ionizing radiation activates c-Jun NH2-terminal kinase (JNK/SAPK) via a PKC-dependent pathway in human thyroid cells. *Biochem Bioph Res Co*. 1998; 244(1):41-44.

18. Li WM, Fan MD, Chen YN, et al. Melatonin induces cell apoptosis in AGS cells through the activation of JNK and P38 MAPK and the suppression of nuclear factor-kappa B: a novel therapeutic implication for gastric cancer. *Cell Physiol Biochem*. 2015;37(6): 2323-2338.

19. Sainz RM, Reiter RJ, Tan DX, et al. Critical role of glutathione in melatonin enhancement of tumor necrosis factor and ionizing radiation-induced apoptosis in prostate cancer cells in vitro. *J Pineal Res*. 2008;45(3):258-270.

20. Bejarano I, Espino J, Barriga C, Reiter RJ, Pariente JA, Rodriguez AB. Pro-oxidant effect of melatonin in tumour leucocytes: relation with its cytotoxic and pro-apoptotic effects. *Basic Clin Pharmacol Toxicol*. 2011;108(1):14-20.

21. Sanchez-Hidalgo M, Lee M, de la Lastra CA, Guerrero JM, Packham G. Melatonin inhibits cell proliferation and induces caspase activation and apoptosis in human malignant lymphoid cell lines. *J Pineal Res*. 2012;53(4):366-373.

22. Garcia-Santos G, Antolin I, Herrera F, et al. Melatonin induces apoptosis in human neuroblastoma cancer cells. *J Pineal Res*. 2006;41(2):130-135.

23. Osseni RA, Rat P, Bogdan A, Warnet JM, Touitou Y. Evidence of prooxidant and antioxidant action of melatonin on human liver cell line HepG2. *Life Sci*. 2000;68(4):387-399.

24. Wolfler A, Caluba HC, Abuja PM, Dohr G, Schauenstein K, Liebmann PM. Prooxidant activity of melatonin promotes fas-induced cell death in human leukemic Jurkat cells. *FEBS Lett*. 2001;502(3):127-131.

25. Buyukavci M, Ozdemir O, Buck S, Stout M, Ravindranath Y, Savasan S. Melatonin cytotoxicity in human leukemia cells: relation with its pro-oxidant effect. *Fundam Clin Pharmacol*. 2006; 20(1):73-79.

26. Sharma M, Palacios-Bois J, Schwartz G, et al. Circadian rhythms of melatonin and cortisol in aging. *Biol Psychiatry*. 1989;25(3): 305-319.