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Original Research

Effects of melatonin on apoptosis and cell differentiation in MCF-7 derived cancer stem cells

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Abstract: Melatonin is a hormone of the pineal gland that has a wide range of biological effects such as antioxidant, anti-inflammatory, and anti-tumor activity. Previous studies have shown that melatonin also affects survival, proliferation, and apoptosis of the cells. In this study, we investigated the effect of melatonin on apoptosis, self-renewal, and differentiation. For this purpose, MCF-7 and HEK293 cells were subjected to melatonin treatment. Expression of genes related to apoptosis (Bax and Bcl2) and self-renewal and differentiation (*Oct4*, *Sox2,* and *Nanog*) analyzed after the sorting of cancer stem cells from MCF-7 cells. Results showed that the effect of melatonin is dependent on the melatonin concentration and treatment periods. Melatonin treatment decreased the cell proliferation rate of MCF-7 in contrast to HEK293. Also, this treatment increased apoptosis in MCF-7 cells and decreased in HEK293 cells. Gene expression of *Nanog* was decreased and *Sox2* was increased in both cell groups after the melatonin treatment. Expression of *Oct4* was decreased in MCF-7 cells and increased in HEK293 cells. We determined that melatonin decreases apoptosis and differentiation of stem cells in normal HEK293 stem cells, but increases apoptosis and differentiation in the MCF-7 cancer stem cells.

Key words: Cancer stem cells; Melatonin; Apoptosis; Self-renewal.

Introduction

Detection of cellular heterogeneity in tumor body has led to hypothesis that a small subgroup of cell is responsible for the initiation, progression, and recurrence of cancer (1). These cells have stem cell-like characters and called cancer stem cells (CSCs) (1). The first evidence of CSCs was described in acute myeloid leukemia (2) and then identified in many solid cancers, such as breast, brain, prostate, colorectal, pancreas, and liver (3,4). These cells also show strong potency to initiate tumor formation *in vivo* (5) and *in vitro* (6). CSCs exhibit resistance to standard chemo- and radiotherapy (5). It has been shown that CSCs are playing roles in recurring and metastasis to distance in many types of cancers such as breast, pancreas, and colon (6,7). Therefore, CSCs may be exploited as a potential therapeutic target to overcome cancer recurrence or metastasis (7).

Despite advances in cancer treatment, there has not been a considerable development in the survival rate of patients with some cancers (8). Moreover, the experimental evidence indicates that most currently applied chemotherapeutics are actually insufficient against CSCs (9,10). This confers that alternative treatment approaches are needed for treatment. Different compounds and drugs that selectively targeting CSCs have been identified, recently (11-14). Some of these compounds comprise microbially and plant-derived biomolecules (15-20) and some classical current drugs, such as metformin (21-24), tranilast (13,25) and thioridazine (26) that have been used for the treatment of metabolic, allergic, and psychotic disorders, respectively. In advantage is most of them have fewer side effects.

Melatonin, N-acetyl-5-methoxytryptamine, is a hormone produced in the pineal gland and several organs such as skin, bone marrow and retina (27). Melatonin involves various physiological processes including circadian rhythm regulation, seasonal shifts, sleeping period, reproduction and cardiovascular functions (28). Melatonin also regulates the immune and hemopoietic systems (29). It was reported that melatonin has oncostatic and antiproliferative effects on breast carcinogenesis (30) and also induces the apoptotic pathway via increasing p53 and p21 expressions in MCF-7 cells (31). However, there is no any study about the effects of melatonin on CSCs in the literature. In the present study, we investigate the effect of melatonin on differentiation and apoptosis in CSCs that obtained from MCF-7 cells.

Materials and Methods

Cell culture

HEK293 and MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 1% L-glutamine and 1% penicillin (100 unit/ml) and 1% streptomycin (10 mg/ml) in a 5% CO2 humidified incubator at 37°C. After cells reached 80% confluence, they were trypsinized with 0.25% trypsin and harvested. In order to perform the experiments, cells were seeded as 2×10^4, 5×10^4, 10^5 and 5×10^5cells in 96, 24, 12 and 6-well plates, respectively. The experiments were triplicated for each one.

MTT assay

HEK293 and MCF-7 cells were seeded as 10^4 cells per 96-well plate and cultured for 48 hr. Then, melatonin applied to wells at 10, 20, 40 and 80 µM concentrations, one group stayed as control group without any treatment. Melatonin-treated and untreated cell lines subjected to Cell Proliferation Kit (MTT, Sigma-Alrich, St. Louis, MO), according to the manufacturer instructions. Viability tests were done three times for each group. IC50 dosage determined by MTT assay.

Apoptosis analysis

Effect of melatonin on apoptosis was carried out by Annexin V-FITC Apoptosis Detection Kit (APOAF; Sigma-Aldrich, St. Louis, USA). The assay was performed according to manufacturer instructions. HEK293 and MCF-7 cells were treated with melatonin and washed twice with PBS. Then, $1x10^6$cells re-suspended in annexin-V binding buffer and incubated for 1 hour at room temperature after adding the FITC conjugated annexin-V and 10 µl of propidium iodide solution. Applicated cells washed and re-suspended in annexin-binding buffer and about 10,000 events were acquired in the FACS machine (BD FACSAriaTM III cell sorter). Data were analyzed by using BD FACSAria TM III software (BD Biosciences, CA, USA).

Flow cytometry assay

CSCs with CD44+/CD24- were sorted by using BD FACSAriaTM III cell sorter and the effect of melatonin on the CSCs were evaluated on these cells. To isolate and evaluate of CSC with CD44+/CD24- phenotype, cells were detected by staining with APC-conjugated anti-CD44 surface marker and FITC-conjugated anti-CD24 IgG antibodies. Also, to isolate and evaluate the CSCs in the HEK293 with SSEA-4 (+)/SSEA-1(-) phenotype, cells were detected by staining with PE-conjugated anti-SSEA-4 surface marker and FITC-conjugated anti-SSEA-1IgG antibodies.

Real-time quantitative PCR analysis

Expression of *Oct4, Sox2, Nanog* and *Bax, Bcl2* genes, which are stem cell and apoptosis markers, were assessed by using real-time quantitative PCR (qPCR) assay. Total RNA isolated from the treated cells and their sorted CD44+/CD24-/low and SSEA-4 (+)/SSEA-1(-)/low phenotype cells by using TRIzol reagent (Invitrogen, Waltham, USA). Synthesis of cDNA from the total RNA was carried out by Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland). cDNAs were obtained by using OligodT primers. cDNA specific primers of studied genes are given in Table 1. Primer sequences were obtained by using DNA Technology (IDT) PrimerQuest online software (available at http://eu.idtdna.com/primerquest/Home/Index) and synthesized by Biomers Inc. (Ulm, Germany). qPCR reactions were done by using SyberGreen universal PCR master mix in LightCycler 480 II (Roche, Germany). The cycle thresholds results were normalized with GAPDH as endogenous control. The expression levels were calculated by $2^{-\Delta\Delta ct}$.

Western blot analysis

Expressions of *Bax* and *Bcl2* proteins assessed by western blot analysis. Total protein extraction carried out by ReadyPreb protein extraction kit (Bio-Rad Laboratories, Berkeley, USA) from the melatonin-treated cells. Protein samples were incubated at 95°C for 5 minutes after the addition of the same volume of 4x Laemmli buffer. An amount of 40 µg of total protein was loaded onto each well of the 4-15% gradient gel and run at 40 mA electric current for an hour. After the electrophoresis, proteins transferred onto nitrocellulose membrane by using a semi-dry system for 1.5 hours. Blocking of the membrane was done with 5% non-fat milk solution for one hour in 50 rpm agitation. The immune blotting was accomplished by the incubation of the membrane with 1 µg/ml of primary antibody solution and then 0.1 µg/ml of secondary antibody for 2 hours at room temperature. At the end of the blotting, the membrane was incubated in Immune-star WesternCChemiluminescent (Bio-Rad Laboratories, Berkeley, USA) substrate solution for 10 minutes and then exposed to X-ray film (Fujifilm, Tokyo, Japan) for one minute and imaged using an X-ray film processor (Ecomax, Germany).

Statistical analysis

The statistical analyzes were done by applying two-tailed student's t-test and analysis of variance (ANOVA) by using GraphPad Prism® V.5.00 software (GraphPad-software Inc., La Jolla, USA). Data were expressed as means ± SEM. Fold changes in gene expression, com-

Table 1. Primer sequences used in real-time qPCR gene expression analysis.

Primers	Primer sequence
BAX - forward	5'- CCCGAGAGGTCTTTTTCCGAG -3'
BAX - reverse	5'-CCAGCCCATGATGGTTCTGAT -3'
BCL-2 – forward	5'- GGTCATGTGTGTGGAGAGC -3'
BCL-2 – reverse	5'- GATCCAGGTGTGCAGGTG -3'
OCT4 – forward	5'- GGGAGATTGATAACTGGTGTGTT -3'
OCT4 – reverse	5'- GTGTATATCCCAGGGTGATCCTC -3'
SOX2 – forward	5'- GAGAGAAAGAAGAGGAGAGAGAAAG -3'
SOX2 – reverse	5'- GCCGCCGATGATTGTTATTATT -3'
NANOG – forward	5'- AATACCTCAGCCTCCAGCAGATG -3'
NANOG – reverse	5'- TGCGTCACACCATTGCTATTCTTC -3'
GAPDH – forward	5'- AGCCACATCGCTCAGACAC -3'
GAPDH – reverse	5'- GCCCAATACGACCAAATCC -3'

parative CT method and statistical analysis were determined by using the freely available Relative Expression Software Tool (REST 2009, Qiagen). The tests considered a basic significance level of $p < 0.05$.

Results

Melatonin inhibits MCF-7cell proliferation through activation of apoptosis

Effects of melatonin on cell proliferation were analyzed with different melatonin concentrations and treatment periods, by using MTT assay. Results indicated an opposed alteration pattern between HEK293 and MCF-7 cells. There was an increased proliferation rate of HEK293 and a decreased proliferation rate of MCF-7 cells, both depending on melatonin concentration and treatment periods (Figure 1A). Regression analysis revealed that melatonin stimulated the proliferation of HEK293 cells. In contrast, it had a negative effect on MCF-7 cell proliferation. Increased concentration and also exposing period of melatonin inhibited the MCF-7 cell proliferation (Figure 1B). The mean growth slope of HEK293 cells was 0.043 (sd = 0.0027; $p<0.01$) while it was determined as – 0.034 (sd = 0.0068; $p<0.01$) for MCF-7 cells. These results revealed that, based on the concentration and treatment period, melatonin was decreased the proliferation of MCF-7 cells while it was increased HEK293 ($p<0.0001$; Figure 1B).

Apoptosis analysis showed that melatonin treatment significantly increased apoptosis in MCF-7 cells ($p<0.01$; Figure 2A) while decreased in HEK293 cells. To confirm these findings, *Bax* and *Bcl2* expression analysis were done by Western blotting in melatonin-treated MCF-7 and HEK293 cells. In MCF-7 cells, results showed that the expression of *Bax* gene was up-regulated ($p<0.001$) and the expression of *Bcl2* gene was down-regulated ($p<0.0001$). As for HEK293 cells, results showed that the expression of *Bax* gene was decreased ($p<0.01$) while *Bcl2* gene expression was increased ($p< 0.01$) when compared to negative controls (Figure 2B and C). These findings indicate that melatonin suppresses cell proliferation and induces cell death by activation of apoptosis merely in MCF-7 cells.

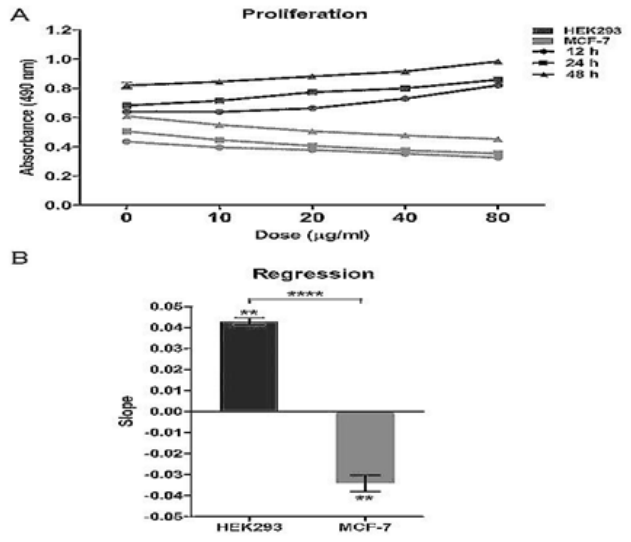

Figure 1. The MTT assay results. Stimulation and inhibition of cell proliferation in HEK293 cells and MCF-7 cells, respectively, in a dose-dependent manner (A). Effect of melatonin on cell proliferation in HEK293 cells and MCF-7 cells (B).

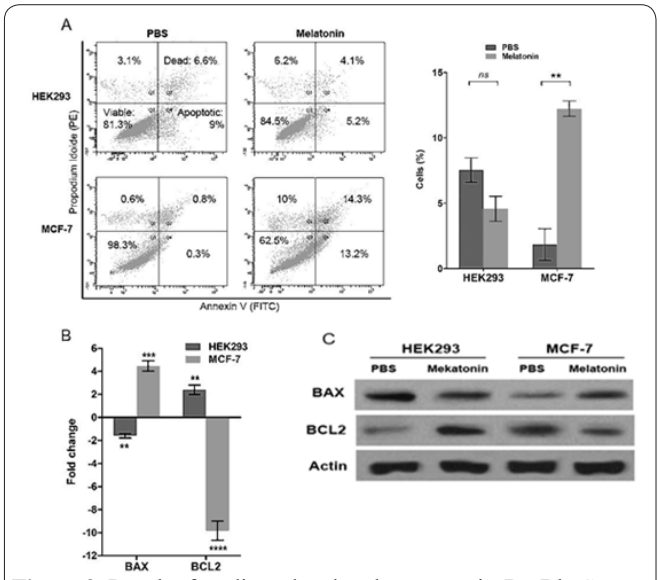

Figure 2. Result of studies related to the apoptosis. DotBlotScatter and Bar Chart results of Annexin V analysis that conducted to confirm effective dose determined by MTT assay (A). Results of the gene expression of *Bax* and *Bcl2* genes (B). Results of western blot analysis of *Bax*, *Bcl2,* and *actin* genes (C). Data displays the mean ± standard deviation of three independent experiments. **p<0.01; ***p<0.001. Abbreviations: FITC, fluorescein isothiocyanate; PI, propidium iodide; Q, quadrant.

Melatonin suppresses CD44+/CD24-/low phenotype cell subpopulation

Effects of melatonin on the population of CSCs were analyzed in MCF-7 and HEK293 cells with CD44+/CD24- and SSEA-4 (+)/SSEA-1(-) marker by using flow cytometry. Cells treated with melatonin (as final concentration of 25 µM) for 48 hours were analyzed. Results showed that the mean percentage of CSCs with CD44+/CD24- was significantly lower in MCF-7 treated cells when compared with untreated control cells ($p=0.02$) (Figure 3). However, in HEK293, there were no significant differences between melatonin-treated HEK293 and untreated control cells (1.05 and 0.83, respectively) ($p>0.05$) (Figure 3).

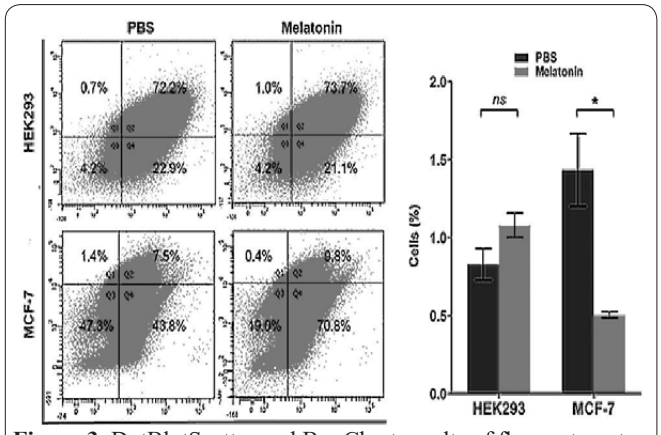

Figure 3. DotBlotScatter and Bar Chart results of flow cytometry. CSCs percentage with CD44+/CD24- was significantly lower in MCF-7 cells when compared melatonin-treated and untreated control group. There was no any difference between melatonin-treated HEK293 and untreated control cells.

Melatonin affect the expression of stem cell marker genes in MCF-7 cells

To confirm the results with each other, expression levels of *Nanog*, *Oct4* and *Sox2* genes were evaluated by quantitative real-time PCR and western blot analysis in melatonin-treated MCF-7 and HEK293 cells. These genes are key factors in the regulation of stem cell self-renewal and maintaining pluripotency. Results showed a decrease in the transcriptional expression level of *Nanog* gene in both MCF-7 and HEK293 cells treated with melatonin (25 μM) when compared with control (PBS treated ones; $p<0.05$). However, *Oct4* gene expression level was decreased in MCF-7 treated cells ($p<0.05$) while it was increased in HEK293 treated cells ($p<0.05$) (Figure 4). Moreover, both MCF-7 and HEK293 cells treated with melatonin showed an increased expression level of the *Sox2* gene, when compared with untreated controls ($p<0.05$) (Figure 4). These results indicated that melatonin had a considerable effect on the cell differentiation, self-renewal, and pluripotency of MCF-7 CSCs via *Nanog* and *Oct4* pathway.

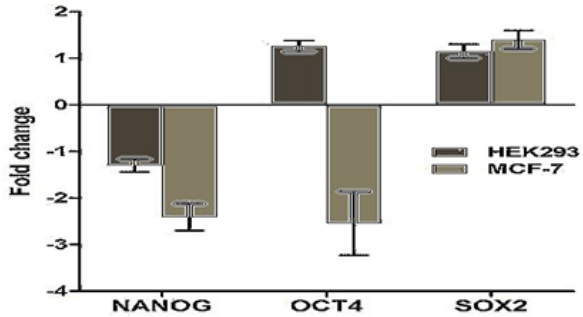

Figure 4. Comparison of the gene expression results of *Sox2*, *Oct4* and *Nanog* genes in the melatonin-treated and untreated cell lines. *Nanog* gene expression was decreased in both MCF-7 and HEK293 cells after the melatonin treatment. *Oct4* gene expression was decreased in MCF-7 cells and increased in HEK293 cells. *Sox2* gene expression was increased both MCF-7 and HEK293 cells when compared with untreated control groups.

Discussion

Melatonin plays an important role in the regulation of intermediary metabolism and cancer (28). Eck et al. indicated that the concomitant use of melatonin and retinoic acid on MCF-7 cells were able to promote apoptosis in MCF-7 breast cancer cells via activation of the RARα in a manner consistent with the decreased expression of TGFβ1 and Bcl2 (anti-apoptotic protein) and increased expression of the pro-apoptotic protein Bax (32). Follow-up studies made by Teplitzky et al. and Melancon et al. showed that co-administration of melatonin and retinoic acid was capable of inhibiting the development of N-nitroso-N-methylurea (NMU)–induced mammary tumors in rats in a proportion above of 90% and also induce a decline of 78% of recognized NMU-mammary tumors, with 54% complete regression (33,34). The interaction between the melatonin and vitamin D/VDR pathway was demonstrated to interact with each other in a report by Proietti et al., which showed that melatonin could promote VDR transcriptional activity and force MCF-7 breast tumor cells to apoptosis (35).

Mediavilla et al. showed that physiologic concentrations of melatonin inhibit the proliferation of breast cancer cells by the p53/p21 pathway *in vitro* (31). Some evidence related to the melatonin-induced apoptosis was observed *in vivo* studies in rat mammary cancers, which showed a significant increase in caspase-3 activity and DNA fragmentation in tumors (36). However, Cucina et al. reported that two different apoptotic processes could be induced by melatonin under appropriate conditions in MCF-7 cells, an early apoptotic response that is independent from TGFβ1 and caspase activity and both TGFβ1- and a later caspases-dependent response (37).

In our study, we assessed the effect of melatonin on total cancer cells by Annexin V assay. As a result of this analysis, it was observed that apoptosis in the HEK293 cell line was significantly decreased ($p=0.0324$). This result indicates that melatonin has an anti-apoptotic effect on HEK293 cell line (Figure 2A) and it is consistent with the study that was reported the suppression of EGF-mediated proliferation by melatonin (38). Besides, as a result of Annexin V analysis, we determined that melatonin leads to a statistically significant increase of apoptosis in MCF-7 cells (Figure 2A). It was shown in previous studies, use of melatonin alone or combined, induce apoptosis in MCF-7 cell line through the p21 and p53 pathways (31,39,40). Similar effects of melatonin have shown in different cancer types such as endometrium, over and colorectal cancers (41,42).

We have tried to determine the quantitative changes of stem cells in the cell lines by flow cytometry after the melatonin treatment. This analyzes showed that the proportion of stem cells significantly increased in the HEK293 cell line ($p=0.0034$), while significantly decreased in MCF-7 cell line ($p=0.002$) (Figure 3). A significant increase in normal stem cells was found, that is compatible with the study that reported melatonin decreased the differentiation and apoptosis in neural stem cells (43). When evaluated together with the results of previous studies, we saw that melatonin increased apoptosis and stem cell proportion in the HEK293 cell line. The proportional increase of stem cell population in HEK293 cell line may be due to the decrease of apoptosis or stem cell differentiation. When results obtained from MCF-7 cell line were considered, we thought that this may be associated with an increase of differentiation and apoptosis in cancer stem cells. Although there is no any publication about the effect of melatonin on cancer stem cells, this result seems to be consistent with the previous best practices (Indatraline, Thioridazine, Azathioprine, and Mefloquine) obtained on cancer stem cells (44).

We tried to confirm the results of previous studies by qPCR analysis. Results showed that the *Bax* gene expression, in the melatonin applied HEK293 stem cells was significantly decreased while the *Bcl2* gene expression was increased. The change of the expression of *Bax* and *Bcl2* genes in HEK293 stem cells were evaluated as anti-apoptotic effects of melatonin (Figure 2B). This data is coherent with a few previous publications that studied the effect of melatonin on the normal cancer cells (43). Also, we determined that melatonin was significantly increased *Bax* gene expression in MCF-7 cancer stem cells while decreased *Bcl2* (Figure 2B). This result may be interpreted as an apoptotic effect of melatonin on MCF-7 cancer stem cells. We could not compare the results, due to lack of similar studies in the

literature. However, this result seems to be consistent with the previously successful applications (Indatraline, Thioridazine, Azathioprine, and Mefloquine) obtained on the cancer stem cells (44).

Effect of melatonin on the differentiation of cancer stem cells is evaluated by investigation of expression levels of genes defined as cancer stem cell markers; *Nanog, Sox2, Oct4*. As a result of this analysis, it was determined that *Oct4* and *Sox2* gene expressions significantly increased in melatonin-treated HEK293 stem cells. But, the *Nanog* gene expression was decreased (Figure 4). These results were interpreted as melatonin inhibit cell differentiation by increasing expression of these genes. These data were consistent with the literature that reported melatonin reduces differentiation in the neural stem cells (43).

However, while *Nanog* and *Oct4* gene expressions significantly decreased in the melatonin-treated MCF-7 cell, *Sox2* gene expression minimally increased (Figure 4). Despite the limited increase in *Sox2* gene, significant increases in *Nanog* and *Oct4* genes indicates that melatonin may induce differentiation of MCF-7 cancer stem cells by reducing the expression of effective genes associated with maintaining the stem cell characters. We could not able to compare the results because there was no any study in the literature about the effect of melatonin on cancer stem cells. However, the obtained results were found to be consistent with the results of successful applications (Indatraline, Thioridazine, Azathioprine, and Mefloquine) performed on cancer stem cells (44).

In our study, we determined that melatonin decreases apoptosis and differentiation of stem cells in normal HEK293stem cell, but increases apoptosis and differentiation in the MCF-7 cancer stem cells. The pathways that are associated with these determined effects are not yet clear. Further studies are needed to determine these pathways.

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