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Melatonin induces antiproliferative activity through modulation of apoptotic pathway in H-*ras* oncogene transformed 5RP7 cells

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Abstract: In the present study, cytotoxic and apoptotic effects of melatonin were investigated on H-*ras* oncogene transformed rat embryo fibroblast 5RP7 cells. Melatonin inhibited cell growth and induced apoptosis in 5RP7 cells. We showed inhibition of cell proliferation that was time- and dose-dependent in 5RP7 cells for 24 and 48 h. Melatonin was not toxic in NIH/3T3 primary mouse embryonic fibroblast cells at low doses for 24 and 48 h. The IC_{50} (380 μM) value of melatonin for 48 h was chosen for advanced assays. The percentages of early/late apoptotic cells to which melatonin (IC_{50}: 380 μM) was administered were increased 6.2-fold in 5RP7 cells compared to controls for 48 h. The melatonin treatment resulted in increased caspase-3 activity and reduced the mitochondrial membrane potential in the 5RP7 cells for 48 h. The cell cycle arrest caused by melatonin was observed in G1 and G2 phases in the cell cycle analyses in 5RP7 cells for 48 h. These findings show that melatonin induces cell death and apoptosis in H-*ras* oncogene transformed 5RP7 cells. Melatonin may be an anticancer agent against H-*ras* oncogene activated cancer cells.

Key words: H-*ras* oncogene transformed 5RP7 cells, melatonin, apoptosis, caspase-3, cell cycle, mitochondrial membrane potential

1. Introduction

Oncogenes are important factors in the carcinogenesis process. Cancer cells may demonstrate an uncontrolled proliferation due to oncogenes. *Ras* is one of these oncogenes, and it is responsible for cell differentiation/proliferation. It is well known that *ras* mutation occurs in human tumors such as pancreatic, thyroid, and colon cancers. H-*ras*, K-*ras*, and N-*ras* proteins belonging to the ras family play a role in cancer diseases (Brunner et al., 2004). Thus, *ras* genes are effective targets for anticancer drug development (Weiwer et al., 2012). In this study, we used H-*ras* oncogene transformed 5RP7 cells. These cells were obtained from embryonic tissues of *Rattus norvegicus* and transformed by the H-*ras* oncogene.

Melatonin (N-acetyl-5-methoxytryptamine) is a methoxyindole secretory product as a natural compound, which is produced by the pineal gland and a variety of organs (such as the retina, Harderian glands, gut, ovary, testes, or bone marrow) during the dark phase (Leon-Blanco et al., 2003; Sainz et al., 2003; Espesito et al., 2008). There have been reports published on the pharmacological effects of melatonin recently. The anticancer and antioxidant capacities and the preventive effects on cell aging and the immune system of melatonin have been researched with in vitro and in vivo clinical investigations (Farriol et al., 2000). Reports have showed that melatonin decreases cell viability in a large number of cancer cell models (breast, non-small cell lung, metastatic renal cell carcinoma, skin, hepatocellular carcinoma, and brain tumors) (Petranka et al., 1999; Jung and Ahmad, 2006). There have been numerous reports on the apoptosis-inducing effects of melatonin in cancer cells, unlike in the protection of healthy cells (Sainz et al., 2003; Altun and Uğur-Altun, 2007; Bejarano et al., 2009).

Apoptosis regulates cell death by controlling the number and size of the cells (Sainz et al., 2003). Apoptosis resistance is one of the basic hallmarks in carcinogenesis (Sainz et al., 2003). The induction of apoptosis is significant for the development of cancer treatments (Martin-Renedo et al., 2008). Caspase-3 is a central protein in the triggering of apoptosis (Joo and Yoo, 2009). Sanchez-Hidalgo et al. (2012) showed that melatonin induced apoptosis via the caspase-3 and promoted cell cycle arrest in various cell lines. Furthermore, it has been observed that melatonin decreases the mitochondrial membrane potential (Yang et al., 2006; Yürüker et al., 2015).

The aim of our study was to detect the signal pathways that underlie the anticancer effects of melatonin against

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H-*ras* oncogene transformed rat embryo fibroblast 5RP7 cells. We researched the effects of melatonin on apoptosis using Annexin V/PI, caspase-3 activity, mitochondrial membrane potential, and the cell cycle arrest assays. Because the morphological features of apoptosis are cellular shrinkage, membrane blebbing, chromatin condensation, and nuclear fragmentation, we also aimed to study morphological changes induced by melatonin in 5RP7 cells.

2. Materials and methods

2.1. Chemicals

The melatonin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's Medium (DMEM) (Lonza, Switzerland), fetal bovine serum (FBS) (GIBCO Inc., İstanbul, Turkey), penicillin-streptomycin (GIBCO Inc.), Dulbecco's phosphate buffered saline (PBS) concentrate (10X) (Biological Industries, Beit-Haemek, Israel), trypsin/EDTA solution (Biochrom, Berlin, Germany), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Alfa Aesar, Karlsruhe, Germany), and dimethyl sulfoxide (DMSO) (Sigma Chemical Co.) were also obtained. The Annexin-V FITC/propidium iodide (PI) apoptosis detection kits were purchased from BD Biosciences (San Diego, CA, USA). The caspase-3 kit was obtained from BD Biosciences. The JC1 kit was obtained from BD Biosciences. The cell cycle kits were obtained from BD Biosciences. The Annexin-V FITC and acridine orange was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), 4,6-diamidino-2-phenylindole (DAPI) was purchased from Sigma Chemical Co., and Triton X-100 was obtained from Amresco (Solon, OH, USA).

2.2. Cell culture

The monolayers cultures of the H-*ras* oncogene transformed rat embryo fibroblast 5RP7 cells and the NIH/3T3 primary mouse embryonic fibroblast cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The stock cells were maintained by DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL) in a humidified 5% CO_2 atmosphere at 37 °C. The cells were grown in 75 cm^2 flasks and subcultured every other day. For the subculturing, the 5RP7 cells were washed with PBS and trypsinized. Then the cells were centrifuged and cultured in new 75 cm^2 flasks in DMEM at 37 °C.

2.3. Cytotoxicity of melatonin

To investigate the cytotoxic effects of melatonin, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) analysis was used. The H-*ras* oncogene transformed rat embryo fibroblast 5RP7 cells and the NIH/3T3 primary mouse embryonic fibroblast cells were plated in 96-well dishes in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at a density of 3×10^3 cells/mL. The melatonin was dissolved in DMSO and diluted with medium at 1:10. Then the melatonin was added to each well at defined concentrations ranging from 1 to 1000 µM (this concentration range was determined after preliminary studies as 1-5-10-25-50-100-125-250-500-1000 µM) for 24 and 48 h. After 24 and 48 h of incubation, MTT dissolved in PBS was added to the cells as 20 µL and incubated for 2 h. Then the medium was removed and 100 µL of DMSO was added to each well, dissolving the sediment. The cells were measured at 540 nm using a microtiter plate reader (Bio. Tec. ELx808IU, USA). The cell viability was calculated as a percent ratio and compared with the control cells.

2.4. Analysis of early/late apoptosis by flow cytometry

The number of apoptotic cells was measured by Annexin V/PI (propidium iodide) analysis. The 5RP7 cells were seeded in 6-well plates at a concentration of 1×10^5 cells/well with DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. The inhibition concentration of melatonin (IC_{50}: 380 µM) was added to 5RP7 cells for 48 h. The plates were incubated at 37 °C in an atmosphere of 5% CO_2 for 48 h. After the incubation, the cells were trypsinized and centrifuged, and the cell pellets were twice resuspended in PBS. Then 100 µL of binding buffer solution (0.1 M HEPES/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$) (BD Pharmingen, San Diego, CA, USA) was added to each sample, and the cells were stained with Annexin V-FITC (5 µg/mL) and PI (5 µg/mL) for 15 min in a dark room temperature (20–25 °C). Then the samples were analyzed by BD FACSAria Cell Sorter Software version 6.1.1 flow cytometry. Cells showing early/late apoptosis were determined as a percentage of all cells.

2.5. Caspase-3 activity by flow cytometry

Caspase-3 activity has a key role and is one of the major cascade proteins in apoptosis. The H-*ras* oncogene transformed 5RP7 cells (100,000) were seeded per six-well plates. The IC_{50} (380 µM) inhibition concentration of melatonin was added to 5RP7 cells at 37 °C in an atmosphere of 5% CO_2 for 48 h. At the end of incubation, the cells were washed twice with PBS, and the cells were collected by centrifugation. The cells were suspended in 0.5 mL of BD Cytofix/Cytoperm (aqueous buffered solution containing paraformaldehyde and saponin) for 20 min at 4 °C. The BD Cytofix/Cytoperm was removed and cells were washed twice with 0.5 mL of BD Perm/Wash Buffer (aqueous buffered solution containing saponin, FBS, and ≤0.09% sodium azide). The cells were incubated in 100 µL of BD Perm/Wash Buffer and 20 µL of caspase-3 antibody solution (aqueous buffered solution containing BSA and ≤0.09% sodium azide) for 30 min. Then the cells were washed in 1 mL of BD Perm/Wash Buffer and centrifuged.

The supernatant was removed and 0.5 mL of BD Perm/Wash Buffer was added to the cells. Then the samples were analyzed by BD FACSAria Cell Sorter Software version 6.1.1 flow cytometry. The cells showing caspase-3 activation were recorded as a percentage of all cells.

2.6. Analysis of mitochondrial membrane potential (JC1) by flow cytometry

The H-*ras* oncogene transformed 5RP7 cells were seeded in six-well plates at a density of 10^5 cells/mL, and the IC_{50} dose of melatonin was added to cells. The cells were incubated in 5% CO_2 air-conditioned atmosphere at 37 °C. After 48 h of incubation, the cells were trypsinized, washed with PBS, and centrifuged at 400 × *g* for 5 min. 5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC1) dye solution (1X assay buffer + JC1 stock solution) was added to the cells. The stock solution was prepared by dissolving the DMSO. Then the samples were incubated at a temperature of 37 °C for 10–15 min. After incubation, the cells were washed twice with an assay buffer and analyzed by BD FACSAria Cell Sorter Software version 6.1.1 flow cytometry. The cells showing mitochondrial membrane potential disruption were determined as a percentage of all cells.

2.7. Analysis of cell cycle by flow cytometry

The percentage of apoptosis and the cells in G0/G1, G1/M, and S phases was measured. The cells were seeded at 5 × 10^5 cells/mL in 6 well-plates and the IC_{50} dose of melatonin was added for 48 h. After incubation, the cells were trypsinized and centrifuged for 400 × *g* for 5 min at room temperature. Solution A (trypsin buffer) was added to each sample and stirred gently by hand. The samples were incubated with Solution A for 10 min at room temperature. Then Solution B (trypsin inhibitor and RNAse buffer) was added and samples were incubated for 10 min at room temperature. Cold Solution C was added to the mixture and incubated for 10 min at 4 °C. The samples were analyzed by BD FACSAria Cell Sorter Software version 6.1.1 flow cytometry, and the cells in each cycle were determined as percentages of all cells.

2.8. Confocal microscopy analysis

The 5RP7 cells were plated onto sterilized coverslips in 6-well plates and exposed to the IC_{50} dose of melatonin for 48 h at 37 °C. After exposure, the cells were washed with PBS and stained with Annexin V/FITC and acridine orange fluorescent dyes. We also stained with 4.6-diamidino-2-phenylindole (DAPI) using a fixative 3.7% paraformaldehyde. Moreover, 0.2% Triton X-100 was used for cell membrane permeability. Then the structure of the cell membrane and nucleus morphologically was observed and the structural changes were detected. Morphology was scanned with a Leica TCS-SP5 II confocal microscopy and Leica Confocal Software version 2.00 was used.

3. Results

3.1. Cytotoxic effects of melatonin on 5RP7 cells

The melatonin showed cytotoxic effects in a time- and dose-dependent manner on H-*ras* oncogene transformed 5RP7 cells for 24–48 h when compared with the control cells (Figure 1A). The increasing concentrations of the melatonin induced cytotoxicity in the H-*ras* oncogene transformed 5RP7 cells ($P < 0.05$), but the cytotoxic effects of melatonin were not observed on the NIH/3T3 cells at low doses (Figure 1B). The cytotoxicity of the melatonin was less on the NIH/3T3 cells at high doses (250 or 500 μM) for 24–48 h ($P > 0.05$). However, the highest dose (1000 μM) of melatonin administered to the NIH/3T3 cells showed more significant reduction (30%–40%) in viability for 24–48 h ($P < 0.05$) (Figure 1B). The maximum

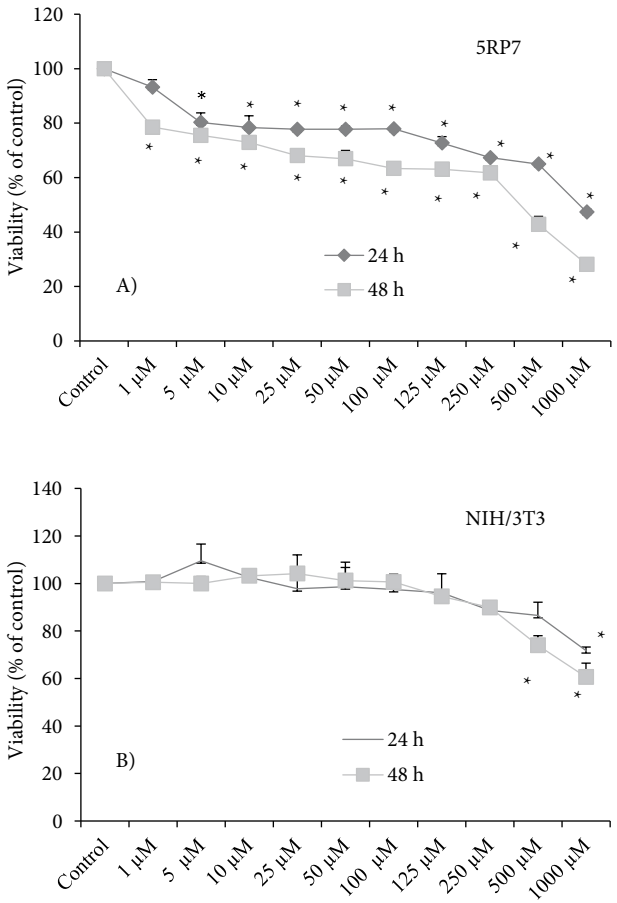

Figure 1. The time- and dose-dependent effects of melatonin in H-*ras* oncogene transformed 5RP7 cells and NIH/3T3 cells were evaluated by MTT analyses for 24 h and 48 h. **A**) Melatonin induced cell death in the H-*ras* oncogene transformed 5RP7 cells. **B**) The toxic effects of the melatonin were not more or less on the NIH/3T3 cells when compared to the 5RP7 cells. The statistical significance was detected by one-way ANOVA, followed by Tukey tests. It was significantly different from the control, *$P < 0.05$ ($n = 6$).

percentages of cell death were 53% for 24 h and 72% for 48 h in the 5RP7 cells. However, the maximum percentages of NIH/3T3 cell death were 28% for 24 h and 39% for 48 h. The IC_{50} value of the melatonin was found to be 380 μM in 5RP7 cells for 48 h (Figure 1A). We investigated the apoptotic effects of melatonin on 5RP7 cells at IC_{50} (380 μM) concentration. The administration of melatonin (IC_{50} concentration: 380 μM) to the NIH/3T3 cells did not cause significant cytotoxicity for either 24 or 48 h ($P > 0.05$).

3.2. Detection of early/late apoptotic cells via flow cytometry

After 48 h, the early/late apoptotic cell percentages were higher in melatonin (380 μM) treated 5RP7 cells compared to the control cells, as shown in Table 1 and Figure 2. The early/late apoptotic cells increased 6.2-fold compared to control cells.

3.3. Melatonin induces caspase-3 activation in 5RP7 cells

The evaluation of caspase-3 activity induced by various stimuli is a significant subject in apoptosis. To determine the caspase-dependent apoptosis, we studied the caspase-3 activity in 380 μM melatonin treated 5RP7 cells (Figure 3). The caspase-3 activity was found to be 17-fold higher in 5RP7 cells compared to the control cells for 48 h (Table 2).

Table 1. Early/late apoptotic effects of melatonin (IC_{50}: 380 μM) on 5RP7 cells for 48 h. Q1: Necrosis, Q2: late apoptosis, Q3: viability, Q4: early apoptosis.

Groups	Q1	Q2	Q3	Q4
Control (%)	1.7	3.8	93.8	0.6
Melatonin (%)	2.4	1.6	70.2	11.2

3.4. Effects of melatonin on mitochondrial membrane potential

The 5RP7 cells were treated with melatonin (380 μM), labeled with JC1 dye, and analyzed by flow cytometry after 48 h (Figure 4). The mitochondrial membrane potential (ΔΨm) was disrupted 2.5-fold compared to the control cells for 48 h (Table 3).

3.5. Effects of melatonin on cell cycle

The analysis of the cell cycle check point was performed by flow cytometry for 48 h and melatonin was found to affect the cell cycle. The IC_{50} concentration of melatonin (380 μM) inhibited 5RP7 cell proliferation depending on cell cycle arrest for 48 h (Table 4). The percentage of the cells increased in the G0/G1 phase (18% versus the control) for 48 h in 5RP7 cells. A significant decrease in the number of cells in the S phase (25% versus the control) was detected and the progressive increase of cell percentage was observed in the G2/M phase (7% versus control) (Table 4). These results have showed that apoptosis was associated with cell cycle arrest in the G0/G1 and G2/M phases in the H-*ras* oncogene transformed rat embryo fibroblast 5RP7 cells.

3.6. Morphological evaluation by confocal microscopy

Apoptotic bodies were observed as a result of exposure of melatonin (IC_{50}: 380 μM) in 5RP7 cells. The cells stained with Annexin-V/FITC had apoptotic nuclei. The cytoplasms of the cells were stained with acridine orange. The morphological changes were nucleus condensation and DNA fragmentation after Annexin-V FITC/acridine orange staining. We also used DAPI staining and observed fragmented nuclei when compared to the untreated cells in melatonin treated cells (Figure 5).

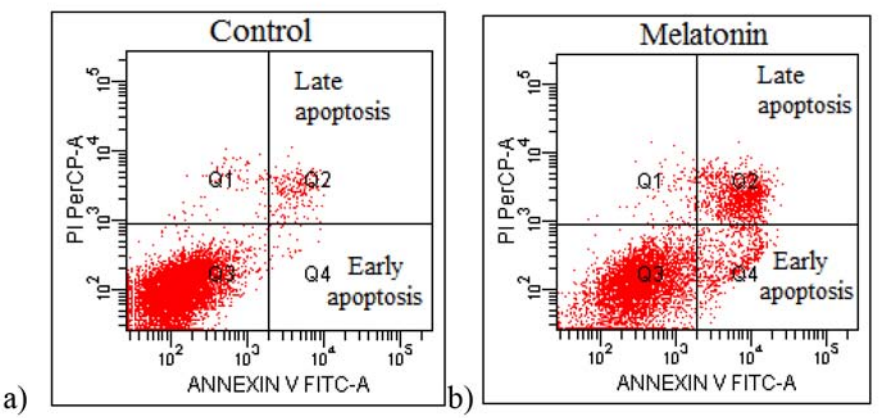

Figure 2. The quantification of early/late apoptotic cells treated with the indicated concentration of melatonin (IC_{50}: 380 μM) for 48 h by flow cytometry. The 5RP7 cells were stained with Annexin V/FITC and propidium iodide. Q1: Necrosis, Q2: late apoptosis, Q3: viability, Q4: early apoptosis. At least 10,000 cells were analyzed per sample, and quadrant analysis was performed.

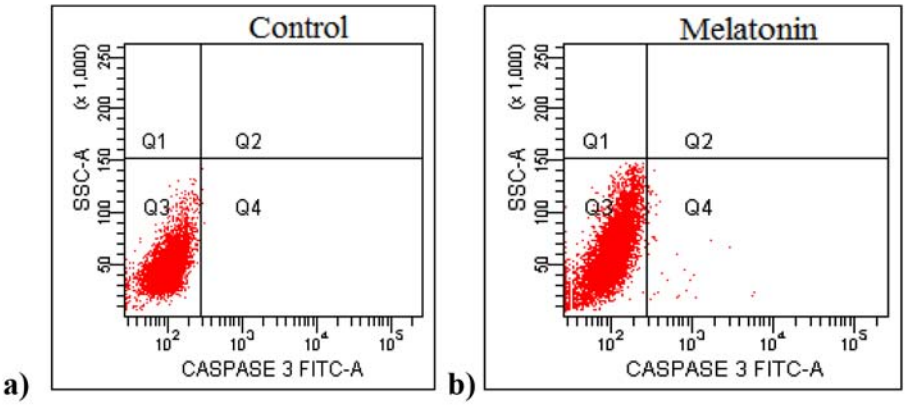

Figure 3. Caspase-3 activity after 48 h of inhibition concentration (IC_{50}: 380 µM) of melatonin treatment in 5RP7 cells. Left panel: Q1+Q3, intact cells; Q2+Q4, exhibiting caspase-3 activity. At least 10,000 cells were analyzed per sample, and quadrant analysis was performed.

Table 2. Caspase-3 activity of melatonin (IC_{50}: 380 µM) in 5RP7 cells for 48 h. Q1+Q3 = living cells, Q2+Q4 = exhibiting caspase-3 activity.

Groups	Q1	Q2	Q3	Q4
Control (%)	0.4	0.3	99.3	0.1
Melatonin (%)	5.1	6.2	88.1	0.6

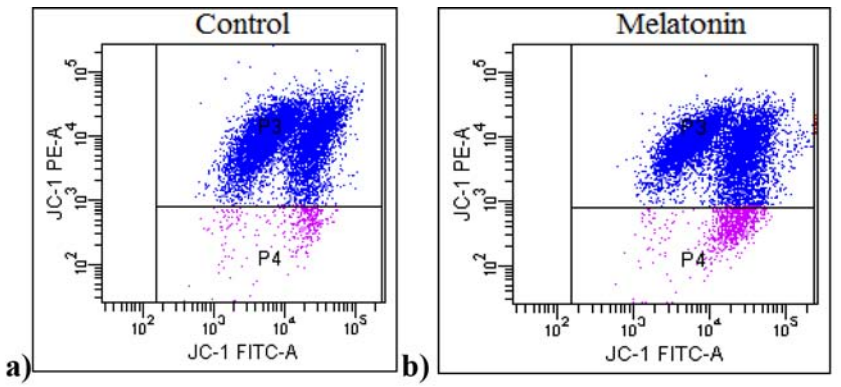

Figure 4. The reduction of the mitochondrial membrane potential in 5RP7 cells by melatonin. The cells treated with the IC_{50} (380 µM) dose of melatonin or untreated for 48 h were stained with the mitochondrial-selective JC1 dye and analyzed by flow cytometry. P3: Mitochondrial membrane polarized cells, P4: mitochondrial membrane depolarized cells. At least 10,000 cells were analyzed per sample, and quadrant analysis was performed.

Table 3. Effects of melatonin (IC_{50}: 380 µM) on mitochondrial membrane potential of 5RP7 cells for 48 h.

Groups	P3	P4
Control (%)	96.3	3.8
Melatonin (%)	90.8	9.5

Table 4. Effects of melatonin (IC_{50}: 380 μM) on the cell cycle of 5RP7 cells for 48 h.

Groups	G0/G1	S	G2/M
Control (%)	42.89	57.11	0.00
Melatonin (%)	60.96	32.17	6.87

The cell cycle analysis of the 5RP7 cells was read by flow cytometry. The melatonin provided the arrest of the 5RP7 cells in the G1 phase.

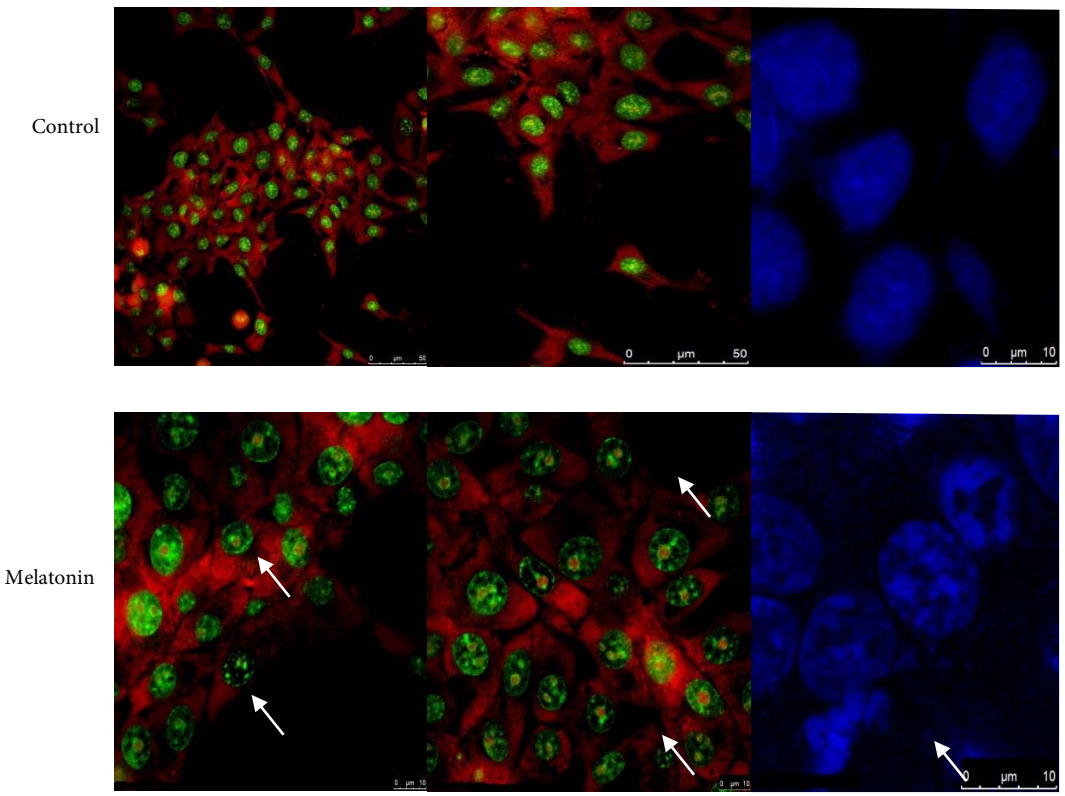

Figure 5. Melatonin induced apoptotic nuclei in H-*ras* oncogene transformed 5RP7 cells stained with Annexin FITC/acridine orange and DAPI. Morphologic changes were observed at 48 h of exposure to melatonin (IC_{50}: 380 μM). The apoptotic structures were labeled with red dye, and the cytoplasm was stained with green dye in the melatonin treatment for Annexin FITC/acridine orange. The nuclei of the control cells were not stained red. DNA staining with DAPI was observed as a blue color. DAPI identified morphological changes in nuclei. The arrows show apoptotic bodies.

4. Discussion

The aim of this experiment was to explain the cytotoxic and apoptotic actions of melatonin in H-*ras* oncogene transformed embryo fibroblast 5RP7 cells because the literature suggests that there are no studies on the oncostatic effects of melatonin on H-*ras* oncogene transformed 5RP7 cells. To detect the apoptotic pathways activated by melatonin in 5RP7 cells, we analyzed the early/late apoptotic cell numbers, caspase-3 activity, depolarized mitochondrial membrane potential, cell cycle arrest, and nuclear morphology. Melatonin was administered to different cell lines and found to have oncostatic effects. Reports suggest that melatonin treatment reduced cell growth in MCF7 (human breast carcinoma cell line) (Liburdy et al., 1993; Furuya et al., 1994; Cos et al., 1996, 2002; Leon-Blanco et al., 2003; Cucina et al., 2009; Martinez-Campa et al., 2009; Chottanapund et al., 2014), BG1 (ovarian carcinoma cell line) (Petranka

et al., 1999), SK-N-MC (human neuroblastoma cell line) (Garcia-Santos et al., 2006), B65 (rat dopaminergic neuroblastoma cell line) (Pizarro et al., 2008), AR42J (rat pancreatic tumor cell line) (Uguz et al., 2012), HepG2 (hepatocarcinoma cell line) (Martin-Renedo et al., 2008; Fan et al., 2010), LnCaP (prostate cancer cell line) (Joo and Yoo, 2009), HL60 (human leukemia cell line), Jurkat (human T lymphoblastic leukemia cell line), MOLT-4 (human T lymphoblastic leukemia cell line), Daudi (human B lymphoblastic cell line), K562 (erythroleukemia cell line) (Büyükavcı et al., 2006), SNG-II (human uterine endometrial adenocarcinoma cell line), and Ishikawa (human endometrial adenocarcinoma cell line) (Kanishi et al., 2000) cell lines.

Our results showed that melatonin inhibited cell viability in 5RP7 cells, and growth inhibition of the melatonin (380 μM) was both dose- and time-dependent for 24 and 48 h. However, this cytotoxic effect was not observed in the NIH/3T3 cells at this concentration. On the other hand, the reduction in cell viability with the melatonin was found to be maximal at 1000 μM in both cell lines. This dose (1000 μM) was much more effective in 5RP7 cells to reduce cell viability. Pizarro et al. (2008) showed that the same doses of melatonin (0.1 and 1 mM) inhibited cell proliferation in dopaminergic neuroblastoma B65. The apoptotic mechanism that melatonin induces has not been clarified in H-*ras* oncogene transformed 5RP7 cells. Apoptotic death is a significant phenomenon for a large number of diseases, and particularly in cancer. Apoptosis has some characteristics including both biochemical and morphological changes. Phosphatidylserine redistribution is seen from the inner face to the outer face of the cell membrane in apoptotic cells. This apoptotic pathway activates proteolytic enzymes called caspases that mediate the rapid dismantling of cellular organelles and architecture. Among them, the most important and well recognized is caspase-3. Caspase-3 is a frequently activated death protease that catalyzes the specific cleavage of many key cellular proteins (Akalın Çiftçi et al., 2014). In our study, percentages of early and late apoptotic cells caused by phosphatidylserine redistribution in melatonin (380 μM) treated cells were increased 6.45-fold compared to control cells (Table 1). Garcia-Santos et al. (2006) also suggested that the percentage of early/late apoptotic cells increased by treatment with 1 mM melatonin. Furthermore, they suggested that caspase-3 activity was 2.5-fold higher than in control cells after 48 h. In our study, caspase-3 activity was 6.8% in melatonin administered cells (IC_{50}: 380 μM) and was higher 17-fold compared to control cells after 48 h. Similar results were obtained from the studies of Joo and Yoo (2009). They showed that induction of apoptosis by melatonin in prostate cancer cells (LNCaP) was observed via early/late apoptosis and caspase-3 activation. Xu et al. (2013) and Martin-Renedo et al. (2008) showed the apoptotic effects of melatonin in SW-1990 (pancreatic cancer) and HepG2 cells respectively by similar mechanisms. Martin-Renedo et al. (2008) also showed that treatment with melatonin caused marked caspase-3 activation in HEPG2 cells (236%–362%). However, our results showed moderate increases in caspase-3 activation. These results show that different cells have different sensitivities to melatonin administration. Another hallmark of apoptosis is identifying the changes in the mitochondrial membrane potential. In a previous study, melatonin reduced the mitochondrial membrane potential (Fischer et al., 2008). In our study, melatonin also disrupted the mitochondrial membrane potential in the 5RP7 cells, showing a triggering of the mitochondrial apoptotic pathway.

In our study we also investigated cell cycle arrest in melatonin treated 5RP7 cells because caspases, which are activated to trigger apoptosis, affect cell cycle regulation and signaling pathways together with the morphological manifestations of apoptosis, such as DNA condensation and fragmentation and membrane blebbing (Mancini et al., 1998). The percentage of cells were increased in G1 and G2 phases in comparison with control cells, but the percentages of cells in the S phase decreased. Cos et al. (1996) suggested that melatonin increased the duration of cell cycle arrest of human breast cancer cells at the G0/G1 phase. In another study, melatonin increased the percentage of cells in the G1 phase of the cell cycle in B65 cells (Pizarro et al., 2008). The growth inhibition of melatonin appeared in the G0-G1 and G2-M phases in HepG2 cells (Martin-Renedo et al., 2008). Melatonin accumulated in human neuroblastoma cells in the G2/M cell cycle phase (Garcia-Santos et al., 2006). Hong et al. (2014) showed that melatonin blocked the S phase percentage of the cells at 48 h and induced G1 phase arrest. These differences may be attributed to doses of melatonin used in the treatment of cells or to the types of cells.

Because apoptosis is morphologically characterized by cytoplasmic contraction, chromatin condensation, plasma membrane blebbing, and DNA fragmentation (Sainz et al., 2003), we also made microscopic evaluations. Our results showed that the apoptotic effects of the melatonin were nuclear condensation and DNA fragmentation in the H-*ras* oncogene transformed 5RP7 cells. Similar results were obtained from different studies. Garcia-Santos et al. (2006) showed condensed and fragmented nuclei by DNA staining with DAPI in SK-N-MC human neuroblastoma cells to which 1 mM melatonin was administered. Apoptotic bodies of SK-LU-1 human lung adenocarcinoma after treatment with melatonin (1 mM and 2 mM) were also detected by DAPI staining (Plaimee et al., 2015).

All of these assessments suggest that melatonin has oncostatic and antiproliferative effects against 5RP7 cells. These effects are dose- and time-dependent. Furthermore, melatonin caused apoptotic effects in this H-*ras* oncogene transformed cells. The most likely mechanisms of action are the arrest of cell cycle and the induction of apoptosis by caspase-3 activation, with decreased mitochondrial membrane potential. This study suggests that melatonin may be a useful pharmacological inhibitor agent against H-*ras* oncogene activated cancer cells.

References

Akalın Çiftçi G, Ulusoylar Yıldırım S, Altıntop, MD, Kaplancıklı ZA (2014). Induction of apoptosis in lung adenocarcinoma and glioma cells by some oxadiazole derivatives. Med Chem Res 23: 3353–3362.

Altun A, Uğur-Altun B (2007). Melatonin: therapeutic and clinical utilization. J Clin Pract 61: 835–845.

Bejarano I, Redondo PC, Espino J, Rosado JA, Paredes SD, Barriga C, Reiter RJ, Pariente JA (2009). Melatonin induces mitochondrial-mediated apoptosis in human myeloid HL-60 cells. J Pineal Res 46: 392–400.

Brunner TB, Hahn SM, McKenna WG, Bernhard EJ (2004). Radiation sensitization by inhibition of activated ras. Strahlenther Onkol 180:731–40.

Büyükavcı M, Özdemir Ö, Buck S, Stout M, Ravindranath Y, Savaşan S (2006). Melatonin cytotoxicity in human leukemia cells: relation with its pro-oxidant effect. Fundam Clin Pharmacol 20: 73–79.

Chottanapund S, Van Duursen MBM, Navasumrit P, Hunsonti P, Timtavorn S, Ruchirawat M, Van den Berg M (2014). Anti-aromatase effect of resveratrol and melatonin on hormonal positive breast cancer cells co-cultured with breast adipose fibroblasts. Toxicol In Vitro 28: 1215–1221.

Cos S, Mediavilla MD, Fernandez R, Gonzalez-Lamuno D, Sanchez-Barcelo EJ (2002). Does melatonin induce apoptosis in MCF7 human breast cancer cells in vitro? J Pineal Res 32: 90–96.

Cos S, Recio J, Sanchez-Barcelo EJ (1996). Modulation of the length of the cell cycle time of MCF7 human breast cancer cells by melatonin. Life Sci 58: 811–816.

Cucina A, Proietti S, D'Anselmi F, Coluccia P, Dinicola S, Frati L, BizzarriM (2009). Evidence for a biphasic apoptotic pathway induced by melatonin in MCF7 breast cancer cells. J Pineal Res 46: 172–180.

Espesito E, Lacono A, Muia C, Crisafulli C, Mattace Raso GM, Bramanti P, Meli R, Cuzzocrea S (2008). Signal transduction pathways involved in protective effects of melatonin in C6 glioma cells. J Pineal Res 44: 78–87.

Fan LL, Sun GP, Wei W, Wang ZG, Ge L, Fu WZ, Wang H (2010). Melatonin and Doxorubicin synergistically induce cell apoptosis in human hepatoma cell lines. World J Gastroenterol 16: 1473–1481.

Farriol M, Venereo Y, Orta X, Castellanos JM, Segovia-Silvestre T (2000). In vitro effects of melatonin on cell proliferation in a colon adenocarcinoma line. J Appl Toxicol 20: 21–24.

Fischer TW, Zmijewski MA, Wortsman J, Slominski A (2008). Melatonin maintains mitochondrial membrane potential and attenuates activation of initiator (casp-9) and effector caspases (casp-3/casp-7) and PARP in UVR-exposed HaCaT keratinocytes. J Pineal Res 44: 397–407.

Furuya Y, Yamamoto K, Kohno N, Ku Y, Saito Y (1994). 5-Fluorouracil attenuates an oncostatic effect of melatonin on estrogen-sensitive human breast cancer cells (MCF7). Cancer Lett 81: 95–98.

Garcia-Santos G, Antolin I, Herrera F, Martin V, Rodriguez-Blanco J, Carrera MP, Rodriguez C (2006). Melatonin induces apoptosis in human neuroblastoma cancer cells. J Pineal Res 41: 130–135.

Hong Y, Won J, Lee Y, Lee S, Park K, Chang KT, Hong Y (2014). Melatonin treatment induces interplay of apoptosis, autophagy, and senescence in human colorectal cancer cells. J Pineal Res 56: 264–274.

Joo SS, Yoo YM (2009). Melatonin induces apoptotic death in LNCaP cells via p38 and JNK pathways: therapeutic implications for prostate cancer. J Pineal Res 47: 8–14.

Jung B, Ahmad N (2006). Melatonin in cancer management: progress and promise. Cancer Res 66: 9789–9793.

Kanishi Y, Kobayashi Y, Noda S, Ishizuka B, Saito K (2000). Differential growth inhibitory effect of melatonin o two endometrial cancer cell lines. J Pineal Res 28: 227–233.

Leon-Blanco MM, Guerrero JM, Reiter RJ, Calvo JR, Pozo D (2003). Melatonin inhibits telomerase activity in the MCF7 tumor cell line both in vivo and in vitro. J Pineal Res 35: 204–211.

Liburdy RP, Sloma TR, Sokolic R, Yaswen P (1993). ELF magnetic fields, breast cancer and melatonin: 60 Hz fields block melatonin's oncostatic action on ER + breast cancer cell proliferation. J Pineal Res 14: 89–97.

Mancini M, Nicholson DW, Roy S (1998) The caspase-3 precursor has a cytosolic and mitochondrial distribution: implications for apoptotic signaling. J Cell Biol 140: 1485–1490.

Martinez-Campa C, Gonzalez A, Mediavilla MD, Alonso-Gonzalez C, Alvarez-GarciaV, Sanchez-Barcelo EJ, Cos S (2009). Melatonin inhibits aromatase promoter expression by regulating cyclooxygenases expression and activity in breast cancer cells. Brit J Cancer 101: 1613–1619.

Martin-Renedo J, Mauriz JL, Jorquera F, Ruiz-Andres O, Gonzalez P, Gonzalez-Gallego J (2008). Melatonin induces cell cycle arrest and apoptosis in hepatocarcinoma HepG2 cell line. J Pineal Res 45: 532–540.

Petranka J, Baldwin W, Biermann J, Jayadev S, Barrett JC, Murphy E (1999). The oncostatic action of melatonin in an ovarian carcinoma cell line. J Pineal Res 26: 129–136.

Pizarro JG, Yeste-Velasco M, Esparza JL, Verdaguer E, Pallas M, Camins A, Folch J (2008). The antiproliferative activity of melatonin in B65 rat dopaminergic neuroblastoma cells is related to the downregulation of cell cycle-related genes. J Pineal Res 45: 8–16.

Plaimee P, Weerapreeyakul N, Barusrux S, Johns NP (2015). Melatonin potentiates cisplatin-induced apoptosis and cell cycle arrest in human lung adenocarcinoma cells. Cell Prolif 48: 67–77.

Sainz RM, Mayo JC, Rodriguez C, Tan DX, Lopez-Burillo S, Reiter RJ (2003). Melatonin and cell death: differential actions on apoptosis in normal and cancer cells. Cell Mol Life Sci 60: 1407–1426.

Sánchez-Hidalgo M, Lee M, Lastra CA, Guerrero JM, Pachham G (2012). Melatonin inhibits cell proliferation and induces caspase activation and apoptosis in human malignant lymphoid cell lines. J Pineal Res 53: 366–373.

Uguz AC, Cig B, Espino J, Bejarano I, Naziroglu M, Rodríguez AB, Pariente JA (2012). Melatonin potentiates chemotherapy-induced cytotoxicity and apoptosis in rat pancreatic tumor cells. J Pineal Res 53: 91–98.

Weiwer M, Bittker JA, Lewis TA, Shimada K, Yang WS, MacPherson L, Dandapani S, Palmer M, Stockwell BR, Schreiber SL et al. (2012). Development of small-molecule probes that selectively kill cells induced to express mutant RAS. Bioorg Med Chem Lett 22: 1822–1826.

Xu C, Wu A, Zhu H, Fang H, Xu L, Ye J, Shen J (2013). Melatonin is involved in the apoptosis and necrosis of pancreatic cancer cell line SW-1990 via modulating of Bcl-2/Bax balance. Biomed Pharmacother 67: 133–139.

Yang QH, Xu JN, Xu RK, Pang SF (2006). Inhibitory effects of melatonin on the growth of pituitary prolactin-secreting tumor in rats. J Pineal Res 40: 230–235.

Yürüker V, Nazıroğlu M, Şenol N (2015). Reduction in traumatic brain injury-induced oxidative stress, apoptosis, and calcium entry in rat hippocampus by melatonin: possible involvement of TRPM2 channels. Metab Brain Dis 30: 223–231.