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Authors: Abdurrahim Kocyigit, Eray Metin Guler, Ersin Karatas, Hifa Caglar, Huri Bulut

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Dose-dependent Proliferative and Cytotoxic Effects of Melatonin on Human Epidermoid Carcinoma and Normal Skin Fibroblast Cells

Abdurrahim Kocyigit*, Eray Metin Guler, Ersin Karatas, Hifa Caglar, Huri Bulut

Bezmialem Vakif University, Medical Faculty, Department of Medical Biochemistry, 93034 Istanbul-Turkey

***Corresponding Author:** Prof. Dr. Abdurrahim Kocyigit (M.D.)
E –mail address: abdurrahimkocyigit@yahoo.com
Tel: +90 506 6599646
Fax: +90 2126217573

Highlight

- Melatonin acts either as proliferative or anti-proliferative in a dose dependent manner.
- Cytotoxic, genotoxic and apoptotic effects of melatonin depend on its ROS generating activity in both melanoma cancer and normal fibroblast cells.
- Therapeutic doses of melatonin may play a potential role in anticancer therapy and may act as an effective therapeutic agent in future.

Abstract

New *in vitro* studies have demonstrated that N-acetyl-5-methoxytryptamine (Melatonin) has cytotoxic and apoptotic effects on various cell types although most of the previous investigations document that it is a potent antioxidant. However, the precise molecular mechanism(s) of its effects are not fully elucidated. In this study, we examined dose-dependent cytotoxic, genotoxic, apoptotic and reactive oxygen species (ROS) generating effects of melatonin in human epidermoid carcinoma cells (A-431) and human normal skin fibroblastic cells (CCD-1079Sk). The cells were incubated with different doses of melatonin (0.031 to 5 mM) for 24 hours. Cell viability was assessed based on luminometric ATP cell viability assay. Intracellular ROS was detected using 2,7-dichlorodihydrofluorescein-diacetate (H_2DCF-DA) fluorescent probes. Genotoxicity was evaluated by alkaline single cell gel electrophoresis assay (Comet Assay). Apoptosis was evaluated by western blotting, DAPI staining, acridine orange/ethidium bromide and Annexin V-FITC/propidium iodide double staining

methods. Mitochondrial membrane potentials were measured by flow cytometry. Although lower doses of melatonin (0.031 to 0.06 mM) increased cell proliferation and decreased ROS generation, higher doses (0.125 to 5 mM) markedly inhibited the cell viability, induced DNA damage, apoptosis and ROS generation. Cytotoxic, genotoxic, apoptotic and ROS generating effects were significantly higher in cancer cells than those observed in normal cells. Melatonin-induced cell death, and ROS generating activity were effectively inhibited by N-acetyl-L-cysteine (NAC) In conclusion, at low doses, melatonin has proliferative effects on both cancer and normal cells, whereas high concentrations have cytotoxic effects. Cytotoxic, genotoxic and apoptotic effects at higher doses of melatonin may be due to its ROS production capacity.

Abbreviations

ROS: Reactive oxygen species

H_2DCF-DA: 2,7-dichlorodihydrofluorescein-diacetate

AO: Acridine Orange

EB: Ethidium Bromide

FCS: Melatonin, fetal calf serum

DMEM: Dulbecco's modified Eagle medium

DMSO Dimethyl sulfoxide

PBS: Phosphate buffered saline

DAPI: 40-6-diamidino- 2-phenylindole

MMP: Determination of Mitochondrial Membrane Potential

DiOC6(3): 3.3 - dihexyloxacarbocyanine iodide

Key Words: Melatonin, reactive oxygen species, genotoxicity, cytotoxicity, apoptosis

1. Introduction

Skin cancer is a main cause of death in humans. About 20% of the population will probably develop skin cancer during their life time [1]. Currently, the treatment for skin cancer includes surgery, radiotherapy and chemotherapy or combination treatments, but the rate of cure is unsatisfactory. For these reasons, studies are being conducted for more effective alternative treatments. Melatonin or N-acetyl-5-methoxytryptamine is an indolamine produced in the mammalian pineal gland and characterized as a hormone involved in regulation of physiological and neuroendocrine functions according to the circadian rhythm [2]. It is widely used for the treatment of various diseases from sleep disorders to cancer [3, 4]. Melatonin and

its metabolic derivatives have been shown to be potent antioxidants with strong free radical scavenging properties[5]. Experimental studies have documented that melatonin carries anti-carcinogenic properties for a various number of cancers [6], induces antioxidant enzymes and inhibits pro-oxidant enzymes besides its free radical scavenging function [7]. Other studies have also reported that it increases survival of normal tissue cells [8, 9], is highly effective in protecting DNA from oxidative damage [10] and, prevents cell death under pathological as well as physiological conditions [11]. It was even reported that melatonin does not enter the redox cycle and therefore does not show oxidant effects [12]. Although most of the studies have proved the antioxidant capacity of melatonin, some new *in vitro* studies have found that they may have pro-oxidant effects at pharmacological concentrations (μM to mM range). Therefore, it may be cytotoxic in tumor and non-tumor cells by promoting the production of reactive oxygen species (ROS) [13-15] increasing caspase [16], apoptotic [17] and anti-proliferative [18] activity especially in some cancer cells. However, the underlying mechanism(s) associated with these opposite effects of melatonin remains unclear. Different explanations are suggested, including the modulation of the endocrine or immune system or the oncostatic effect of melatonin directly on neoplastic cells. Surprisingly, recent findings indicate that melatonin can produce intracellular reactive oxygen species (ROS) [19, 20]. In addition, apoptosis can be induced by ROS in several cell types [21, 22]. However, there is no report available on the dose-dependent proliferative or anti-proliferative effects of melatonin and its relation to ROS production capacity in both cancer and normal cells. For this reason, the aim of this study is to investigate dose-dependent cytotoxic, genotoxic and apoptotic effects of melatonin in epidermoid cancer and normal fibroblastic cells and its relation to ROS production capacity.

2. Materials and Methods

2.1. Chemicals and Reagents

Melatonin, fetal calf serum (FCS), Dulbecco's modified Eagle medium (DMEM), 2,7-dichlorodihydrofluorescein-diacetate (H_2DCF-DA), penicillin-streptomycin, ethidium bromide (EB), acridine orange (AO), 4',6-diamidino-2-phenylindole (DAPI) and N-acetyl-L-cysteine (NAC) were obtained from Sigma-Aldrich (Seelze, Germany). Annexin V-FITC staining kit was obtained from Roche Life Sciences. Unless mentioned otherwise, all reagents used in the study are at analytical grade. Melatonin stock solution was prepared by dissolving in dimethyl sulfoxide (DMSO), then diluting with DMEM for obtaining required concentrations prior to use. The ultimate concentration of DMSO in the melatonin solution was less than 0.1%. The

level of DMSO was confirmed to not induce DNA damage in the cells before the experiments. Remaining reagents were prepared freshly before the experiment.

2.2. Cell Culture and Maintenance

A-431 cells (as standard cell line from human epidermoid carcinoma cells) and CCD-1079Sk cells (as standard human normal fibroblastic cells) were obtained from American Type Cell Culture Collection (ATCC, Germany). A-431 cells were cultured in DMEM and CCD-1079Sk cells were cultured in EMEM under 5% CO_2 atmosphere at 37ºC equilibrium. The medium was supplemented with 10% FCS, 100 U/ml of penicillin and 100 ng/ml of streptomycin. Trypan blue exclusion test was used for estimation of the number of viable cells.

2.3. Cytotoxicity Assay

Cytotoxic activity of melatonin on the cells were examined by ATP levels measured with luminescence test (Cell-Titer-Glo Luminescent Cell Viability Assay, Promega). Cells were seeded onto 96-well plates at a density of 1.5×10^4 cells per well and incubated overnight at 37ºC in 5% CO_2. The medium was then changed with fresh complete medium including varying concentrations of melatonin (0.031 to 5 mM). Control cells were treated with 0.1% DMSO. Cells were left for incubation under humidified 5% CO_2 and 95% O_2 at 37ºC for 24 hours. Afterwards, the cells were rinsed with the culture medium and examined for ATP. Each sample was supplemented with 100 µL of the reagent (Cell Titer-Glo Luminescent Cell Viability Assay, Promega), mixed for 2 minutes and incubated for 10 minutes at room temperature. The results were evaluated with a luminometer (Varioscan Flash Multimode Reader, Thermo, Waltham, MA). The light emitted in the presence of ATP was measured as relative light units (RLU). The intensity of the emitted light quantities is directly related to the ATP content in the sample being tested. Cell viability was expressed as a percentage relative to the negative control group indicated as 100%. Half maximal growth inhibitory concentration (IC50) values were calculated from non-linear regression analysis from concentration-response curves. Also, A-431 and CCD-1076Sk cells were treated with 2 mM melatonin in the presence of 0.5, 1 and 2 mM NAC for 24 hours. Live/dead assay was performed to quantify live and dead cells as described previously. All experiments were repeated three times and the standard deviation was 5%.

2.4. Measurement of ROS Generation

ROS production was evaluated using a cell-permeable fluorescent probe dihydrodichlorofluorescein diacetate (H_2DCF-DA) as an indicator of ROS. H_2DCF-DA is oxidized to green fluorescent dichlorofluorescein (DCF) at a high rate with ROS formation. A-431 and CCD-1079Sk cells were pretreated with melatonin (0.031 to 5 mM) at various concentrations for 24 hours. After the incubation period, cells were rinsed with cold PBS and incubated with 100 μM H_2DCF-DA for 30 minutes at 37^0C. Fluorescent plate reader (Varioskan Flash Multimode Reader, Thermo, Waltham, Mass.) was used to measure DCF fluorescence intensity in Ex / Em. = 488/525 nm. Also, both cancer and normal cells were treated with 2 mM melatonin in the presence (0.5, 1 and 2 mM) or absence of NAC for 24 hours and ROS generation was performed as described previously. Estimates were performed three times each time that the number of cells per treatment group was the same to ensure reproducibility. Values were expressed as % relative fluorescence when compared to the control.

2.5. Genotoxic Activity Assay

Alkaline single cell gel electrophoresis assay (Comet Assay) was performed for evaluation of genotoxic effects of melatonin on cell lines according to Singh et al. [23] with slight modification. Two different cells were seeded onto 6-well cell culture plates (approximately $2x10^5$ cells per well) containing cell culture medium and incubated at 37ºC in 5% CO_2 for 24 hours for determination of the genotoxic potential of melatonin. Following 24 hours, under IC_{50} concentrations of melatonin (0.031 to 2 mM) in 1% DMSO were added to the medium and incubated for another 24 hours at 37ºC. DMSO (0.1%) was applied for negative control. The cells were rinsed with phosphate buffered saline (PBS) following incubation, harvested using trypsin/EDTA and collected for centrifugation at 400 x g for 5 min at 4ºC. Cells were rinsed with phosphate buffered saline (PBS) after incubation, harvested using trypsin / EDTA and collected for centrifugation at 400 x g for 5 minutes. The supernatant was drained and the cell density was adjusted to $2x10^5$ cells / ml using cold PBS. 90 μL of 0.6% low melting agarose and 10 μL cell suspension were mixed and placed on 1% normal-melt agarose pre-coated slides. The slides were immersed in lysis solution (2.5M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100 and 10% DMSO, pH 10) at 4 ° C for 1 hour following agarose solidification. The slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100 and 10% DMSO, pH 10) at 4 °C for 1 hour. After agarose solidification. The slides were removed from the lysis solution, rinsed with cold PBS, and placed side by side in a horizontal electrophoresis tank. The DNA was allowed to unwind in the alkaline electrophoresis buffer

containing the freshly prepared 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 minutes. After unwinding, electrophoresis was performed at 0.72 V/cm (26 V, 300 mA) for 25 min at 4°C. The slides were rinsed for 5 minutes at 4 °C with neutralization buffer (0.4 M Tris, pH 7.5), then dehydrated with ethanol before staining. Then, the slides were stained with EB (2 μg / mL in distilled H2O; 70 μL / slide) covered with a coverslip and analyzed using an epifluorescence-equipped 200 x magnification fluorescence microscope (Leica DM 1000, Solms, Germany) equipped with a rhodamine filter (excitation wavelength of 546 nm and a barrier of 580 nm). Computerized image analysis system (Comet Assay IV; Perceptive Instruments) was used. The percentage of DNA in the tail was used as the primary criterion for DNA damage according to Hartmann et al. [26]. All steps were performed three times.

2.6. *Measurements of Apoptosis Indicators*

2.6.1. Acridine Orange/Ethidium Bromide Double Staining Test

Acridine orange/ethidium bromide (AO/EB) double staining for evaluation of morphological changes in cells was performed as described by McGahon et al. [24]. The cells undergoing apoptosis are differentiated from the viable cells by the morphological changes of apoptotic nuclei. AO and EB are DNA intercalating dyes. AO is taken up by both living and dead cells and stains double-stranded [25] and single-stranded nucleic acids. AO spreads green fluorescence upon stimulation at 480-490 nm from live cells while being diffused into dsDNA [25]. EB is taken up by dead cells and stains DNA in orange. Briefly, the cells were cultured in six-well plates (2×10^5 cells/well) and incubated for 24 hours. After 24 hours, melatonin in 0.1% DMSO was added and incubated for another 24 hours at 37 °C. DMSO (0.1%) was applied for control. The cells were rinsed with phosphate buffered saline (PBS) after incubation, harvested using trypsin/EDTA and collected for centrifugation at 400 x g for 5 min at 4ºC. The supernatant was drained and the cell density was adjusted to $2x10^5$ cells/ml using cold PBS. Finally, the AO/EB solution was added to the cell suspension and the nuclear morphology was assessed by fluorescence microscopy (Leica DM 1000, Solms, Germany). Multiple images were obtained in randomly selected areas and a minimum of 100 cells were counted. Live cells have normally visible green nuclei, apoptotic cells contain lysed chromatin and green nuclei, and dead cells have orange / red nuclei according to the method. All experiments were performed three times.

2.6.2. DNA fragmentation by DAPI fluorescence staining

Apoptotic bodies were stained with 40-6-diamidino-2-phenylindole (DAPI) staining-intact nuclei and labeled by observation with DAPI. The melatonin-treated A-431 and CCD-1079Sk cells were washed once in PBS in 6-well plates, fixed in methanol for 10 minutes, then stained with 1 μg/ml DAPI for 10 minutes in the dark and then washed three times. The unstained and stained cells were observed under inverted fluorescence microscope (Carl Zeiss, Göttingen, Germany).

2.6.3. Western Blotting Assay

A-431 and CCD-1079Sk cells (1.5×10^5 cells/well) were seeded onto six well plates for 24 hours and then treated with melatonin concentrations below IC_{50}. After incubation for 24 hours, the cells were harvested and prepared in NP-40 cell lysis buffer (2 mM Tris–Cl pH 7.5, 150 mM NaCl, 10% glycerol and 0.2% NP-40 plus a protease inhibitor cocktail) for 30 min on ice. Centrifuged at 14,000 x rpm (Beckman Coulter, Krefeld, Germany) for 10 min at 4°C, and the final supernatant was used as the cytosolic fraction. Then, 5 × loading buffer was added to the above obtained supernatant, and the mixture was boiled at 100°C for 15 min. Protein concentrations were determined using the Bradford protein assay method [26]. Protein was then separated on 8-12% sodium SDS-PAGE, transferred onto a PVDF membrane, and then incubated with primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology). Protein bands were visualized with Pierce ECL Western blotting substrate (Thermo Scientific) on secondary antibodies against P-21, P53, p-NF-κB, NF–κB, Caspase-3, Bax; Bcl-2 and β-actin (Santa Cruz, Biotechnology).

2.6.4. Apoptosis by Flow Cytometry

Annexin V-FITC staining kit (Roche Applied Science, Penzberg, Germany) was used according to the manufacturer's instructions for detection of apoptosis. Briefly, A-431 and CCD-1079Sk cells were seeded (1.5×10^5 cells/well) onto six-well plates and allowed to adhere overnight for treatment with melatonin concentrations below IC_{50} for 24 hours. Trypsin-digested cells were centrifuged at 200 x *g* for 5 minutes. The cell pellet was re-suspended in 100 μL Annexin V-FITC labeling solution and incubated for 10–15 minutes at 15–20°C and immediately analyzed with flow cytometry (Becton Dickinson, FACS Canto II) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

2.6.5.. Determination of Mitochondrial Membrane Potential (MMP)

After different treatments, cells were incubated with 40 nM 3.3 dihexyloxacarbocyanine iodide (DiOC6(3)) for 15 min. at 37^0C then washed with ice-cold PBS, and collected by centrifugation at 500 x g for 10 min. Collected cells were re-suspended in 500 ml of PBS containing 40 nM DiOC6(3) [27]. In living cells, this cyanine dye can accumulate in the mitochondrial matrix under the influence of the mitochondrial transmembrane potential. Fluorescence intensities of DiOC6(3) were analyzed on a flow cytometry (Becton Dickinson, FACS Canto II) with excitation and emission settings of 484 and 500 nm, respectively. Experiments were performed five times.

2.7. Statistical Analysis

The results are presented as the mean ± standard deviation (Mean ± SD) of three replicates. Data in all experiments were analyzed for statistical significance using analyses of variance (One-way ANOVA). Post hoc analyses were made to compare parameters of different groups. The *p* value <0.05 was considered as statistically significant. IC_{50} values of melatonin over the cell lines were calculated by nonlinear regression analysis. *p* value <0.05 was considered statistically significant. SPSS for Windows package program (Version 20, Chicago, IL) was used.

3. Results

3.1. Cell Viability against Melatonin Administration on Cancer and Normal Cells

Concentration response cell viability assays were performed with A-431 and CCD-1079Sk cells for 24 hours to evaluate the effect of melatonin on cell growth. Following incubation, the effects of melatonin was determined by the ATP cell viability assay. Cell viability in all cell cultures were greater than 95% before all experiments. The control cell viability was used as 100 for reference. After addition of melatonin, the cell viability increased up to 130% relative to the control (0.062 mM concentration). Cell viability level begun to decrease at this concentration and, cell viability was the same as the control in the concentration of 0.125 mM melatonin. With upper levels of this concentration, the percentage of cytotoxic activity progressively increased (p<0.001) in a concentration dependent manner. Higher doses of melatonin resulted in greater cellular death in cancer cells than in normal cells (p<0.05), (Fig 1).

FIGURE 1

The half maximal growth inhibitory concentration value (IC_{50}) of melatonin for A-431 and CCD-1079 Sk cells at 24 hours were calculated with concentration-response curve and found

as 1.2 and 1.9 mM, respectively. These data indicate that melatonin has a proliferative effect at lower concentrations and cytotoxic effect at higher concentrations. However, no significant toxicity was observed in these cells treated with melatonin, at concentrations of 0.25 mM or less for cancer cells and at 1 mM or less for normal cells (Fig. 1). For this reason, as shown in Figure 1, the physiological concentration for cancer cells was selected to be 0.25 mM, while 1 mM was selected for normal cells. As shown in Figure 1, the cytotoxic effect of melatonin at high doses was greater in cancer cells than in normal cells. Treatment of cells with 0.5, 1 and 2 mM NAC reversed the cytotoxic effect of melatonin, indicating that high doses are associated with pro-oxidant potential of melatonin-induced cell death (Figure 3A).

3.2. Concentration-dependent Reactive Oxygen Generating Activity

We evaluated intracellular ROS generation by using H_2DCF-DA as a fluorescence probe as shown in Figure 2.

FIGURE 2

Treatment of cells with lower doses of melatonin (0.031 to 0.062 mM) for 24 hours decreased intracellular ROS production in cancer and normal cells ($p<0.001$). However, ROS production increased by higher doses of melatonin exposure (0.125 to 5 mM). Melatonin, depending on concentration, increased ROS production in cancer cells compared to normal cells. Treatment of cells with 0.5, 1 and 2 mM NAC reversed the ROS generating effect of melatonin in both cancer and normal cells. Figure 3B shows the inhibitory effect of NAC as an antioxidant on ROS production caused by melatonin at higher concentrations.

FIURE 3A/B

3.3. Melatonin Causes DNA Strand Breaks

DNA damage caused by melatonin treatment was measured to evaluate the DNA damaging effect of ROS. For the analysis of genotoxic activity, cells were treated with different dosages of melatonin for 24 hours and DNA damage was evaluated by the comet assay. Nuclei with damaged DNA had a comet feature with a bright head and a tail, whereas nuclei with undamaged DNA appeared to be round without a tail. Each figure represents a typical comet tail of the observed cells (at least 100 cells) from two slides in each experiment. Typical micrographs of comet assays are shown in Figure 4. After 24 hours of melatonin treatment,

cancer cells showed different size, fragmentation and comet structures with increased % tail intensity when compared to normal cells. These findings show that melatonin induced DNA damage occurs in a dose-dependent manner ($p<0.001$), and there were significant changes in the tail % of DNA between the normal cells and cancer cells at higher doses of melatonin (Figure 4).

FIGURE 4

3.4. Melatonin Increases Apoptosis

Apoptosis is critical in tumorigenesis and resistance to therapy. DAPI nuclear staining, AO/EB double staining, Western Blotting and Annexin-V-FITC staining and MMP assay methods were performed to clarify whether melatonin induced cell apoptosis in cancer and normal cells. To testify the morphological features of apoptosis, A-431 and CCD-1079Sk cells were exposed to melatonin at different doses for 24 hours, stained with DAPI and AO/EB dual staining and examined under fluorescence microscopy (Figure 5).

FIGURE 5

With DAPI nucleus staining, higher doses of melatonin treated cells showed typical apoptotic signs, including chromatin condensation, karyopyknosis, and nuclear fragmentation, which are characteristic features of apoptotic cells. With AO/EB double staining, as the concentration of melatonin increased, the number of uniformly green viable cells decreased and yellow-orange apoptotic cells increased after 24 hours in both cancer and normal cells. The higher doses of melatonin resulted in greater apoptosis in cancer than normal cells (Figure 6).

FIGURE 6

We also investigated the relationship between proliferative and cytotoxic effects of melatonin and apoptosis signaling pathways. Expressional analysis of P-21, P53, p-NF-κB, Caspase-3, Bax and Bcl-2 proteins as well as β-actin in cancer and normal cells have been studied by western blotting method. Melatonin treatment regulates pro-apoptotic and anti-apoptotic pathways. A-431 and CCD-1079Sk cells were treated with different concentrations of melatonin for 24 hours. DMSO was used as a negative control. Cell extracts were used for

western blotting to detect apoptotic protein expression. The β-actin level was used as control. The results revealed that 24 hours exposure of melatonin on the cells increased the density of P-21, P53, Caspase-3 and Bax protein, whereas the density of Bcl-2 decreased compared to the control in a dose-dependent manner in both cancer and normal cells. However, expression levels were higher in cancer cells than those of normal fibroblastic cells at higher doses of melatonin (Figure 7).

FIGURE 7

We also determined both p-NF-κB and NF-κB expression level to show the effect of melatonin on NF-κB activation. The results revealed that while 24 hours exposure of melatonin on these cells did not change phosphorylated NF-κB protein expression levels, NF-κB expression levels decreased significantly according to the controls in a dose dependent manner. Expression levels in cancer cells were significantly lower in the higher melatonin concentrations than in normal cells (Figure 8).

FIGURE 8

We performed Annexin V-FITC double-staining method to measure melatonin-induced apoptosis. After 24 hours of treatment, flow cytometry was used to detect the cells. Annexin V-FITC double staining represented early and late apoptosis or necrosis (Figure 9).

FIGURE 9

Higher melatonin concentration resulted in a detection of a higher proportion of Annexin-V- FITC double staining in the epidermoid carcinoma and normal fibroblastic cell lines. When cancer and normal cells were exposed to melatonin (0.031 to 2 mM) there was a significant increase in both early and late apoptosis in a concentration dependent manner according to the control (Figure 10).

FIGURE 10

The mitochondrial apoptotic pathway was investigated to demonstrate mechanisms underlying apoptotic induction in cancer and normal fibroblastic cells. Since loss of DΨ leads to apoptosis via mitochondrial pathways, levels of DΨm were determined. Results from flow cytometric analysis indicated that according to the control, melatonin increased loss of DΨm significantly (p<0.05) in a concentration dependent manner in cancer cells (Figure 11).

FIGURE 11

However, loss of DΨm was not significantly higher in normal cells. Results indicate that melatonin increased loss of DΨm and apoptotic induction, supported by linear correlation between cytotoxicity or apoptotic induction and DΨm in cancer cells.

4. Discussion

Most of the previous studies have shown that melatonin and its derivatives exhibit antioxidant and free radical scavenging properties [28, 29], or pro-oxidant properties alone [30, 31]. However, the mechanisms of these adverse effects of melatonin are not fully understood and no comparative studies have been conducted for the effects on cancer and normal cells. To clarify these points, we investigated the cytotoxic, genotoxic, apoptotic and ROS generating effects of melatonin on epidermoid carcinoma cells at different doses compared with normal fibroblast cells.

In our study, it has been shown that low dose melatonin (0.03 to 0.125 mM) increases cell proliferation while decreasing ROS production; however high dose melatonin (0.125 to 5 mM) leads to increased ROS production while decreasing cell viability. Similar to our findings, researchers have shown that melatonin at low doses has a proliferative effect [32-34] and, pharmacologic doses of melatonin decreased cell viability [35, 36]. However, the mechanism of proliferative and cytotoxic effects of melatonin has not been fully elucidated yet. In order to investigate the mechanisms of proliferative and cytotoxic effects of melatonin on these cells, the relationship between intercellular ROS and cell viability levels and the effect of adding NAC as an antioxidant on ROS and cell proliferation were studied. Our results indicated that the proliferative effect of low doses of melatonin may be due to its antioxidant action. As NAC supplementation together with melatonin was detected to decrease ROS levels and increase cell viability. The proliferative effects of melatonin on various cells have recently been shown to depend on various mechanisms [33, 37, 38]. Studies suggest that antioxidant enzymes are stimulated by the receptor and that free radical scavenging is done by non-melatonin receptor-

mediated processes [42], including up-regulation of antioxidant and down-regulation of pro-oxidant enzymes, and suppression of mitochondrial radical formation [43, 44]. However, no study has found the direct association between cell proliferation and ROS generation effects of melatonin on cancer and normal cells. Several studies have also shown that some antioxidant compounds increase cell proliferation with different mechanisms [39-41]. Antioxidants are agents that destroy free radicals; protecting proteins, enzymes, carbohydrates, DNA, lipids and thus the cells against oxidative damage [42]. It has been demonstrated that low doses of vitamin C can promote the proliferation of marrow mesenchymal stem cells from aging mice, possibly by increasing the cellular telomerase activity [43]. As a result, our findings together with the findings of other researchers show that antioxidant compounds can cause a decrease in ROS production or an increase in antioxidant levels, leading to an increase in cell proliferation.

Despite the proliferative effects of melatonin on cancer and normal cells at low physiological doses, we have also shown that high pharmacologic concentrations have a cytotoxic effect and, this effect is greater in cancer cells than in normal cells. The accepted physiological concentrations of melatonin for humans are considerably lower than in cell cultures. It is generally assumed that the physiological levels of melatonin are in picomolar or low nanomolar concentrations in humans, although it differs significantly in different body fluids and tissues [44]. In cell culture studies, although at low concentrations melatonin have been shown to inhibit significant cell proliferation in some cell types, some of them show little or no sensitivity to nanomolar levels while being sensitive to high concentrations of melatonin. For example, androgen sensitive (LNCaP) and androgen independent (PC3) prostate cancer cells are insensitive to nanomolar levels, while mM melatonin concentrations are induce cytotoxicity [45]. For this reason, non-toxic doses of melatonin were generally selected as physiological concentrations in cell culture studies. For instance, Li at al.[46] have selected 2 mM as a physiological concentrations in their study on the effects of melatonin in gastric cancer cells. We also accepted that physiological concentrations of 0.25 mM for melanoma cancer cells and 1 mM for normal cells in our study. Melatonin could also stimulate production of intracellular ROS especially at higher concentrations. Similar results with these opposite effects of melatonin are available in the literature. Osseni et al. [47] reported that melatonin enhances the viability of cells and increases intracellular glutathione (GSH) concentrations at low pharmacological concentrations (10^{-6} –10^{-8} M); however, it can be pro-oxidant at high (10^{-3} – 10^{-4} M) concentrations in human liver cancer cells. Buyukavci et al. [48] also demonstrated that melatonin induces production of ROS that may be associated with cytotoxicity depending on its concentration in some leukemia cells. These observations are consistent with our results as

shown in Figures 1, 2 and 3. All these findings demonstrated that ROS has major effects on cell death activity.

The apparent contradiction that a well-known antioxidant, such as melatonin, may also behave as a pro-oxidant in cancer cells, though reported by several studies in recent years [19, 20, 49], is not a widely accepted notion. However, it has been shown that antioxidants such as tocopherol, ascorbate or gallic acid may sometimes show pro-oxidant and ROS generating activities in some cases [50-52]. There are different *in vitro* time and concentration dependent studies about melatonin and ROS production in the literature. Radogna et al. [20] showed that melatonin (1mM) induces ROS generation at a time as early as 1 minute after addition. However, production of ROS was temporary, for only 5-6 hours. Osseni et al. [47] also demonstrated that melatonin (1-10 mM) initiated ROS production at high concentrations within 15 min. of addition to the medium of human hepatocellular carcinoma HepG2 cells. In Jurkat cells increased ROS production was measured 30 minutes after the addition of melatonin [53]. Other studies observed that ROS increased markedly from 2 to 48 hours after exposure to melatonin, depending on cell type [33]. These findings might be depending on the type of cell and the timing and duration of ROS elevation induced by melatonin [54]. Unlike other studies, we first compared cancer cells with normal cells and found that ROS production was greater in cancer cells than in normal cells. It is already known that cancer cells are under greater oxidative stress, compared to normal cells, due to oncogenic transformation, alterations in metabolic activity, and increased generation of ROS [55]. The increased production of ROS in cancer cells was observed in *in vitro* and *in vivo* studies [56, 57]. The cumulative effect of ROS in cancer cells might cause higher cytotoxicity than those of normal cells. As cancer cells with increased oxidative stress are likely to be more vulnerable to damage by further ROS insults induced by exogenous agents [58].

Genotoxicity and signaling pathways leading to apoptosis were investigated in cancer and normal cells in order to understand the mechanisms by which ROS produced by melatonin at high doses cause cytotoxicity. We used the comet assay test to assess the genotoxic effects of melatonin on these cells. The alkaline comet assay is one of the most important tools for the assessment of DNA damage in a wide variety of cell lines [59]. There are many studies about melatonin and DNA damage and the vast majority of these studies are about the protective effects of melatonin on DNA damage [60, 61]. Recently, several studies demonstrated that melatonin induced DNA damage in a concentration dependent manner in cancer cell lines [62-64]. They also found that melatonin acts as a pro-oxidant by activating ROS-dependent DNA damage and thus leading to apoptosis in cancer cells. Our results are consistent with these

observations. However, there is no available report to compare cancer and normal cells to show the DNA damaging effect of melatonin. Melatonin may act as a pro-oxidant at higher concentrations and increased ROS production may cause DNA damage in these cells. According to cancer cells, decreased DNA damage levels in normal cells may be due to low endogenous ROS production in normal cells. It is known that induction of DNA damage in dividing cells results in the activation of the cell cycle control point and the arrest of the cell cycle to repair the DNA damage [65]. When the repair is successfully completed, the cell can go into its own cell cycle. Alternatively, if the repair process fails, the cell cycle can be blocked permanently, leading to cell senescence or apoptosis [66].

Treatment with high doses of melatonin induced apoptosis in both epidermoid cancer and normal fibroblastic cells and apoptotic effects were significantly higher in cancer cells than in non-cancerous cells. Apoptosis can be measured by a number of methods by taking advantage of the morphological, biochemical and molecular changes occurring in a cell during this process. In this study we evaluated apoptosis in four different ways: detection of DAPI and AO / EB by fluorescence microscopy, detection of surface expression of phosphatidylserine by flow cytometry and western blotting. Apoptotic, necrotic and live cells can be distinguished using AO/EB staining. After staining, living cells are distinguished with a normal appearing green nucleus, apoptotic cells show orange-stained nuclei with chromatin condensation or fragmentations, while necrotic cells are clearly observed with uniformly orange-stained cell nuclei with no condensed chromatin. The results of the present work revealed that pharmacological concentrations of melatonin treated cancer cells exhibit more apoptotic events (chromatin condensation and nuclear fragmentation) with a significant decrease in cell viability than normal cells in a dose dependent manner. Qin et al. [67] also demonstrated by AO/EB method that melatonin could directly inhibit the growth of H-22 hepatocellular carcinoma cells by inducing apoptosis of the tumor cells. Fluorescence microscopy analysis of DAPI stained cells was undertaken to study nuclear alterations and apoptotic body formation, both of which are also features of apoptosis [68]. Morphological changes characterized by cytoplasmic and nuclear shrinkage, chromatin condensation and apoptotic body formation were observed in response to treatment of both epidermoid cancer and normal fibroblast cells with melatonin. In the untreated cells, the stained nuclei were rounded and homogeneously stained with DAPI, whereas treated cancer cells from both cell lines showed an altered nuclear DNA staining pattern with condensed chromatin and apoptotic bodies that are hallmarks of early and late apoptosis in a dose dependent manner. Our results are in concordance with previous reports [63, 69]. Melatonin treatment caused a decrease in the expression of proteins NF-κB and Bcl-

2 and an increase in the expression of P-21, P53, Bax, Caspase-3, which are apoptotic cell death markers and these expressions are more pronounced in cancer cells, especially at higher doses of melatonin. A variety of apoptosis-stimulatory agents cause over-expression and translocation of the pro-apoptotic Bax protein to mitochondria leading to release of mitochondrial cytochrome-c and activation of caspase-3 [70]. It is also well known that the ratio of Bax/Bcl-2 is involved in maintaining DΨm in mitochondria and mitochondrial dysfunction could activate a mitochondrial apoptotic pathway to cause translocation of Bax from cytosol to the mitochondria. Melatonin increased Bax, however, decreased Bcl-2 protein levels (Fig. 8). It was reported that anti-cancer agents induce apoptosis in tumor cells, which was correlated with the ability to decrease expression of Bcl-2 [71]. Activation of P53 pathway by DNA damage leads to G1/S checkpoint control, which causes reduction and delay of cell cycle progression and induction of apoptosis [72]. The protein P53 is known as the DNA guardian and its activation can cause cell cycle arrest at G1 [72]. In fact, it has been previously described that Bax and other Bcl-2 pro-apoptotic proteins are transcriptional targets of P53 [73]. It has been previously reported that melatonin is able to increase P53 and P21 expression in MCF-7 human breast cancer cells [74].

Here, we demonstrated that while melatonin did not change phosphorylated NF-κB levels at all concentrations in both cancer and normal cells, dephosphorylated NF-κB levels were decreased in a dose dependent manner. However, inhibition degree was found significantly higher in cancer cells than in normal cells especially at higher concentrations of melatonin. This is the preliminary study to show the effect of melatonin on expression levels of both p-NF-kB and NF-kB. NF-kB plays an important role in the signal transmission and a variety of gene expressions in the nucleus [75]. It consists of five members, including rel A (p65), rel B, c-rel, p105/50 and p100/52, which associate as homo or heterodimers and is regulated by diverse transduction cascade [76]. Activation of NF-kB results from the release of inhibitory IkB subunits (such as IkBa and IkBb) from the heterotrimeric complex followed by translocation of dimmer to the nucleus [77]. IkB subunits are phosphorylated by the IKK complex, which leads to ubiquitination and subsequent degradation [78]. Activated NF-κB is released from the IκB complex and translocated to the nucleus, where it induces the expression of genes encoding various proteins involved in suppressing apoptosis and inducing cellular proliferation, invasion and inflammation [79]. These target genes are important for the development of invasive tumors and include those encoding cell-cycle regulatory proteins, such as cyclin D1, and apoptosis-suppressor proteins, such as Bcl-2 and Bcl-XL [80]. Exceptional NF-κB activation is associated with the stimulation of proliferation and protection against

apoptosis in malignant cells [80]. The effect of melatonin on NF-κB activation is not well described in the literature. Studies focus on the inhibitory effect of melatonin on injury/inflammatory-induced NF-κB activation [81, 82] rather than on the possible effect of melatonin as an inducer of NF-κB activation. These results are in concordance with previous findings. However, Cristofanon et al [83] showed that melatonin can trigger NF-κB activation and claimed that this activation depends on the pro-oxidant activity of melatonin. Their different results may depend on their experimental procedure because they exposed cells to melatonin for a short period of time (5 hours) and added TNF-α in the culture medium to induce NF-κB activation.

Apoptotic induction via the mitochondrial pathway is an important mechanism initiated by chemotherapeutic agents [84]. Depolarization of DΨm leads to release of pro-apoptotic proteins, such as cytochrome c, from inner membranes of mitochondria, leading to activation of executioner caspase-3 to induce apoptosis [84]. In this study, melatonin significantly reduced DΨm in epidermoid carcinoma cells compared to fibroblastic cells. As previously reported, melatonin induces pro-apoptotic signaling pathways in human pancreatic carcinoma cells [85], and decreases in DΨm in human malignant lymphoid cell lines [16]. Although a large number of studies have demonstrated that melatonin prevents mitochondria from oxidative damage in physiological concentrations, some studies in recent years have reported that higher concentrations of melatonin initiate ROS production in mitochondria isolated from rat liver cells [86]. Zhang and Zhang et al. [86] demonstrated that 1 mM melatonin induces ROS formation via mitochondrial respiratory complex III in the region sensitive to calcium-independent antimycin A. However, melatonin continues to determine whether there is a direct interaction with complex III to increase ROS production.

5. Conclusion

Melatonin has a proliferative effect at lower concentrations and a cytotoxic effect at higher concentrations on both cancer and normal cells probably related to its pro-oxidant capacity. Excessive doses of melatonin can cause ROS production leading to DNA damage and cell cycle arrest and expression of key proteins that trigger apoptosis. All these effects are higher in cancer cells than in normal cells in a dose dependent manner. These findings show that, in contrast to the antioxidant and proliferative effects of melatonin at low doses, therapeutic doses may play a potential role in anticancer therapy and may act as an effective therapeutic agent in the future.

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Figure Captions

Fig. 1. Effect of melatonin on cell viability. A-431 and CCD-1079Sk cells were treated with melatonin at different concentrations for 24 hours. The ATP test was used to determine cell viability. Percent cell viability was calculated by normalizing with a control panel. Data are expressed as the mean ± SD. Significant difference compared to the control is indicated by *$p < 0.05$ and ** $p < 0.01$. Significant differences between cancer and normal cells were indicated by $p < 0.05$.

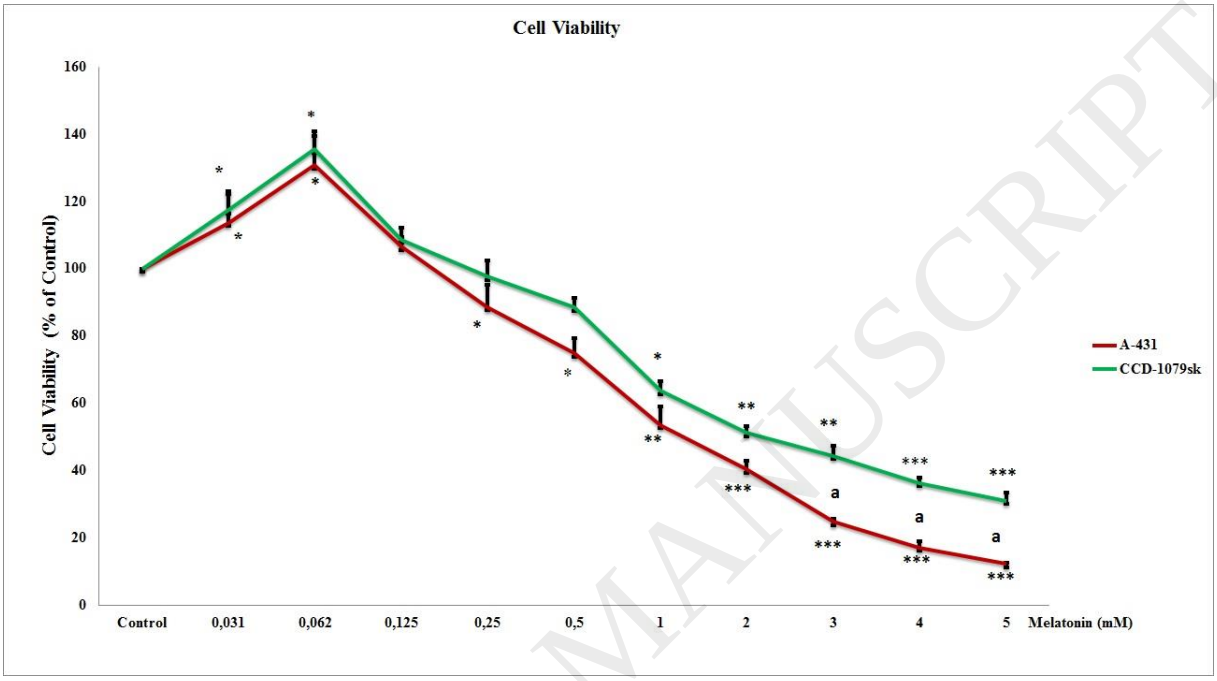

Fig 2. Reactive oxygen species (ROS) generating effect of melatonin on A-431 cancer cells and CCD-1079Sk normal cells. Melatonin reduced ROS generation at low concentrations (up to 0,062 mM) and increased ROS generation (0,062 to 5 mM). Results are presented as Mean ± SD. Significant difference compared to the control is indicated by *$p < 0.05$ and ** $p < 0.01$. Significant differences between cancer and normal cells were indicated by $p < 0.05$.

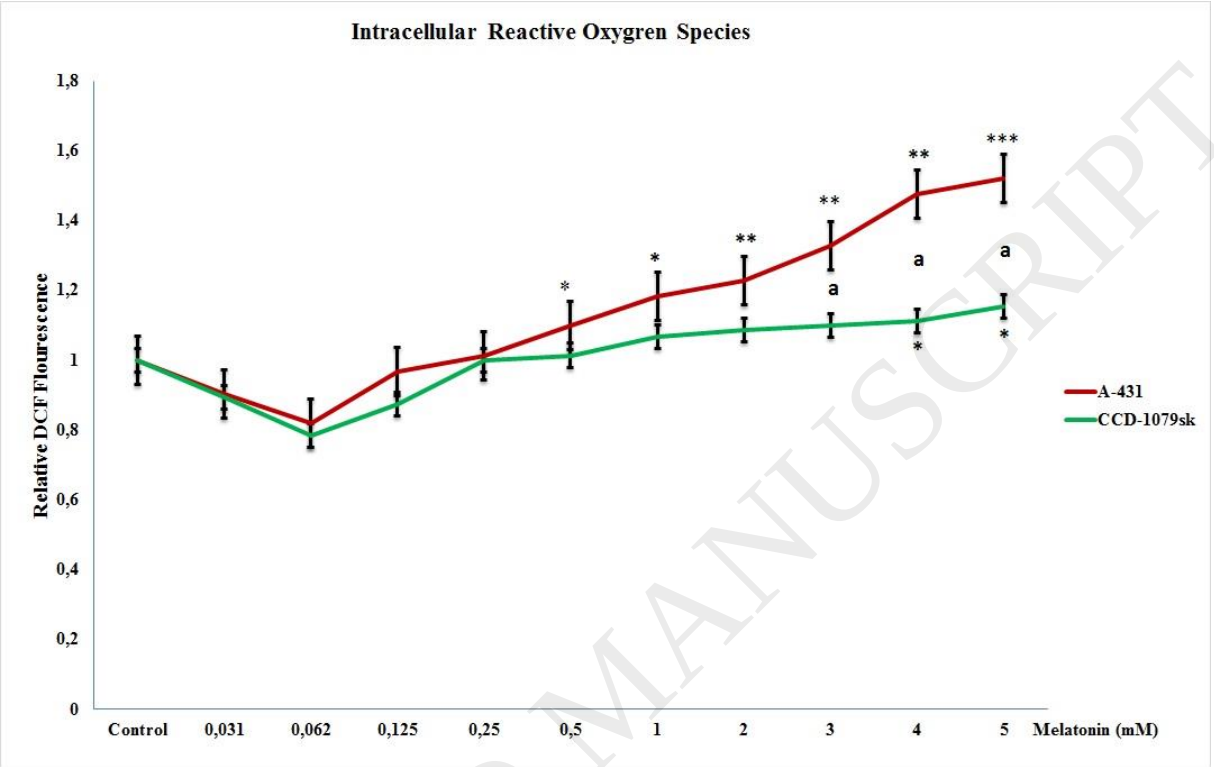

Fig. 3. Both cancer and normal cells were seeded in 96-well plates (1.5x10 ^{3} cells / well). After 24 hours, the cells were treated with both NAC (0.5, 1 and 2 mM) and melatonin. After 24 hours incubation, cell viability and ROS formation activity was assessed using the ATP cell viability test and the H_2DCFH-DA test. Figure 3A shows cell viability and Figure 3B shows ROS production activity. The data are expressed as mean ± SD. Significant differences in cancer and normal cells *, p <0.05 and **, p <0.01.

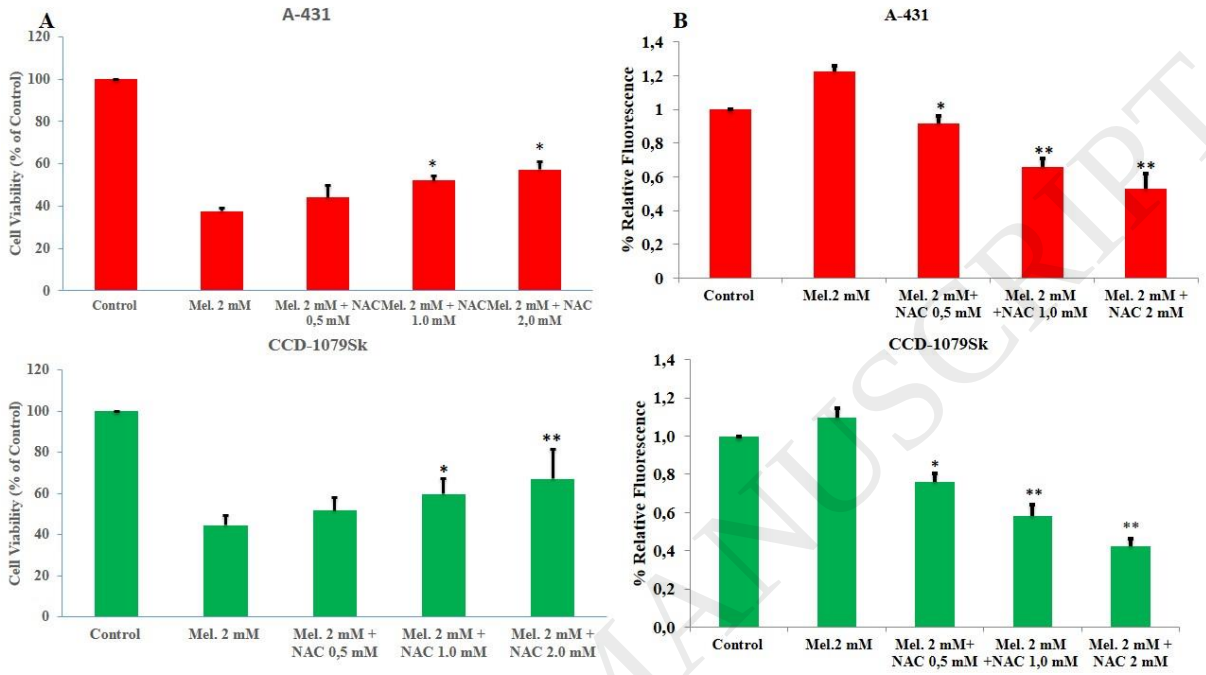

Fig. 4. Melatonin induces DNA damage. Cancer and normal cells were treated with different doses of melatonin for 24 hours and there were significant changes in the tail % of DNA between the normal and cancer cells except 0.031 and 0.062 mM of melatonin. Data presented were Mean ± SD (n = 3). Significant differences according to the control indicated by *p < 0.05, ** p < 0.01. and *** p<0.001. Significance differences between normal and cancer cells were indicated by "a".

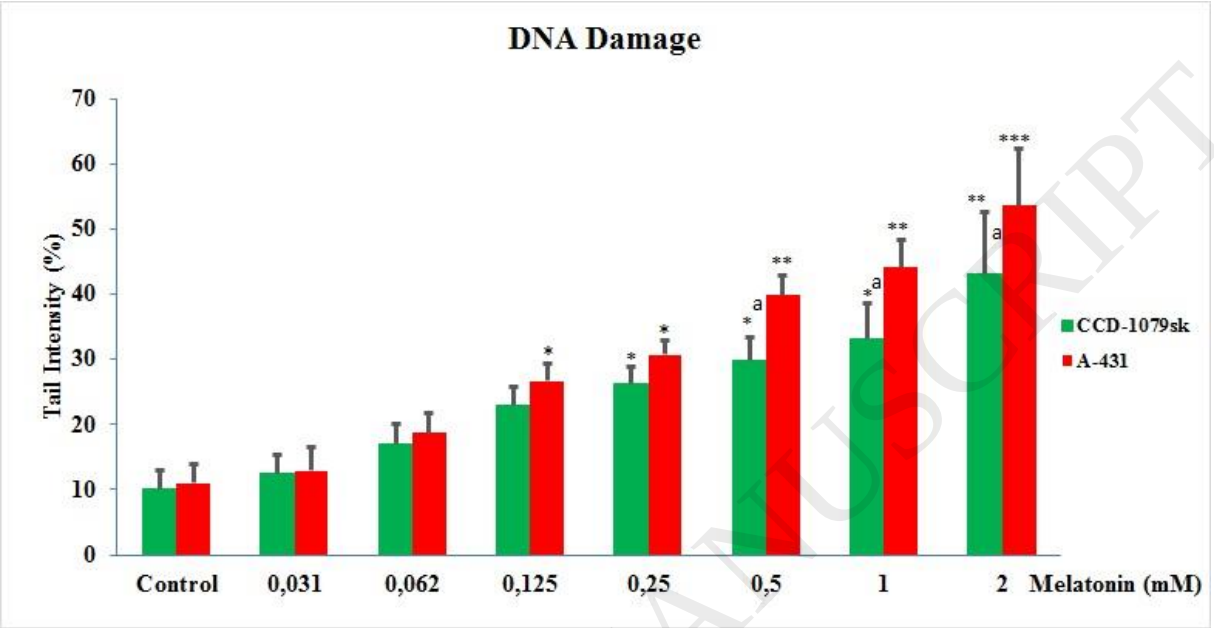

Fig. 5. Morphological observation on A-431 cells by Acridine Orange/ Ethidium Bromide (AO/EB) double staining and DAPI staining. Viable cells have uniform green nuclei (V) with organized structure while apoptotic cells would also have bright-red (or yellow-orange) nuclei (A) with condensed or fragmented chromatin by AO/EB staining. Apoptotic chromatin condensation was observed under fluorescence microscope by DAPI staining.

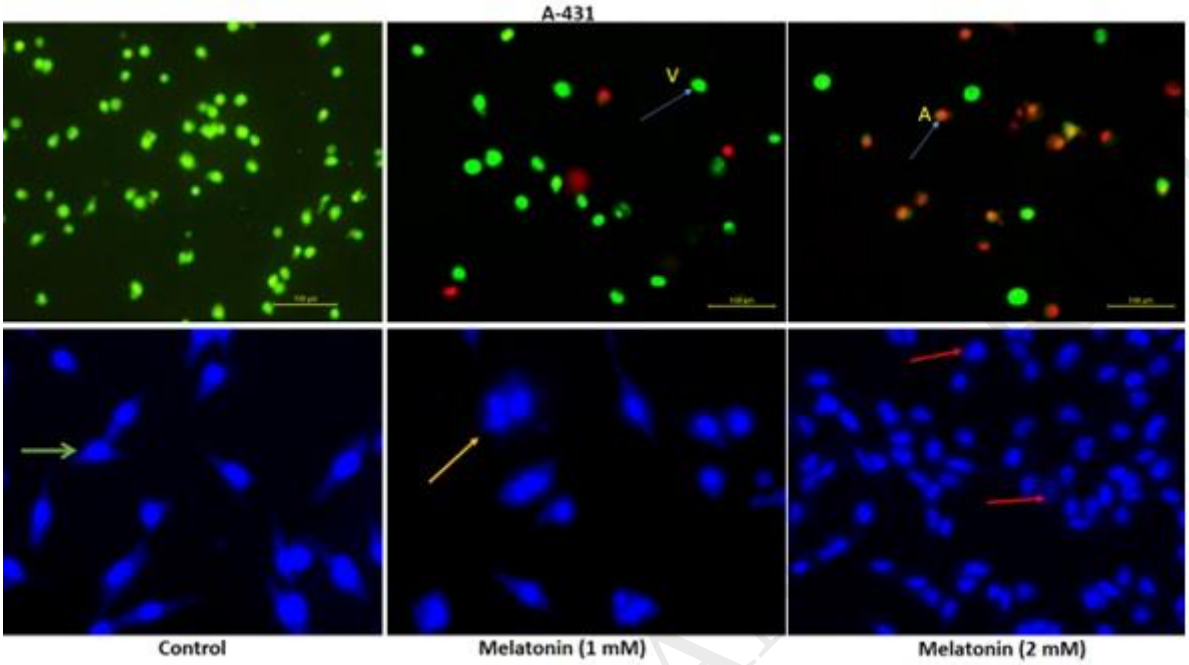

Fig. 6. Apoptotic activity of melatonin on A-431 and CCD-1079 Sk cell lines. Cells were treated with different concentrations of melatonin (0.031 to 5 mM) for 24 hours, Acridine Orange/ Propidium Iodide (AO/PI) double staining and measured by fluorescence microscopy. Data presented were Mean ± SD (n = 3). Significant differences according to the control indicated by *p < 0.05, ** p < 0.01. and *** p<0.001. A: Apoptosis, N: Necrosis.

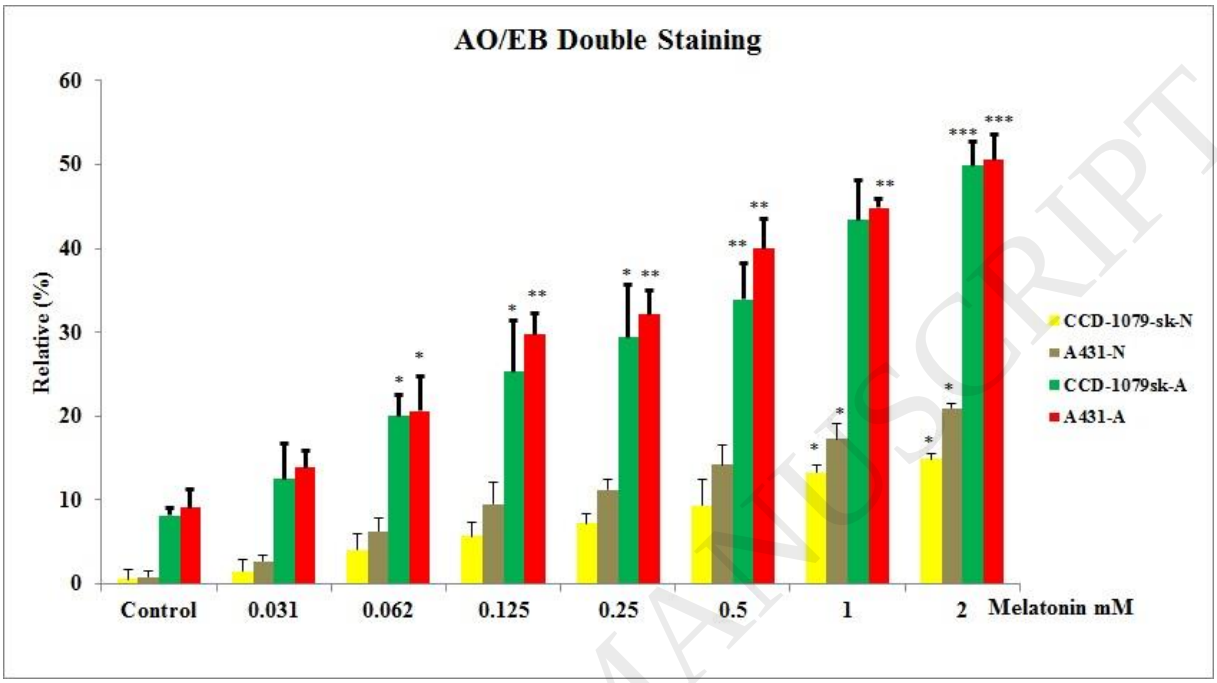

Fig. 7. Melatonin treatment regulates pro-apoptotic and anti-apoptotic pathways. A-431 and CCD-1079Sk cells were treated with different concentrations of melatonin for 24 hours. DMSO treatment was used as a negative control. Cell extracts were handled to western blotting to determine apoptotic protein expression. The β-actin was used as control. Significant differences according to the control indicated by *$p < 0.05$, ** $p < 0.01$.

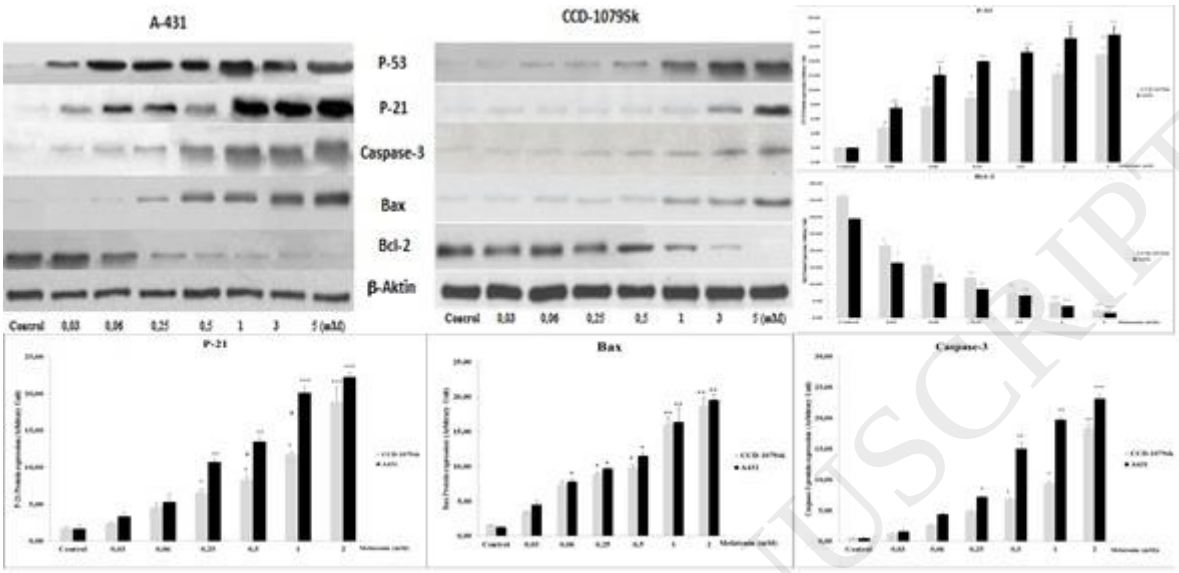

Fig. 8. Effects of melatonin on the phosphorylation of NF-κB and NF-κB pathway component. Data represent the mean ± SD of at least three independent experiments. *$p < 0.05$; **$p < 0.01$ vs. control cells. Significant differences between cancer and control levels shown by "a".

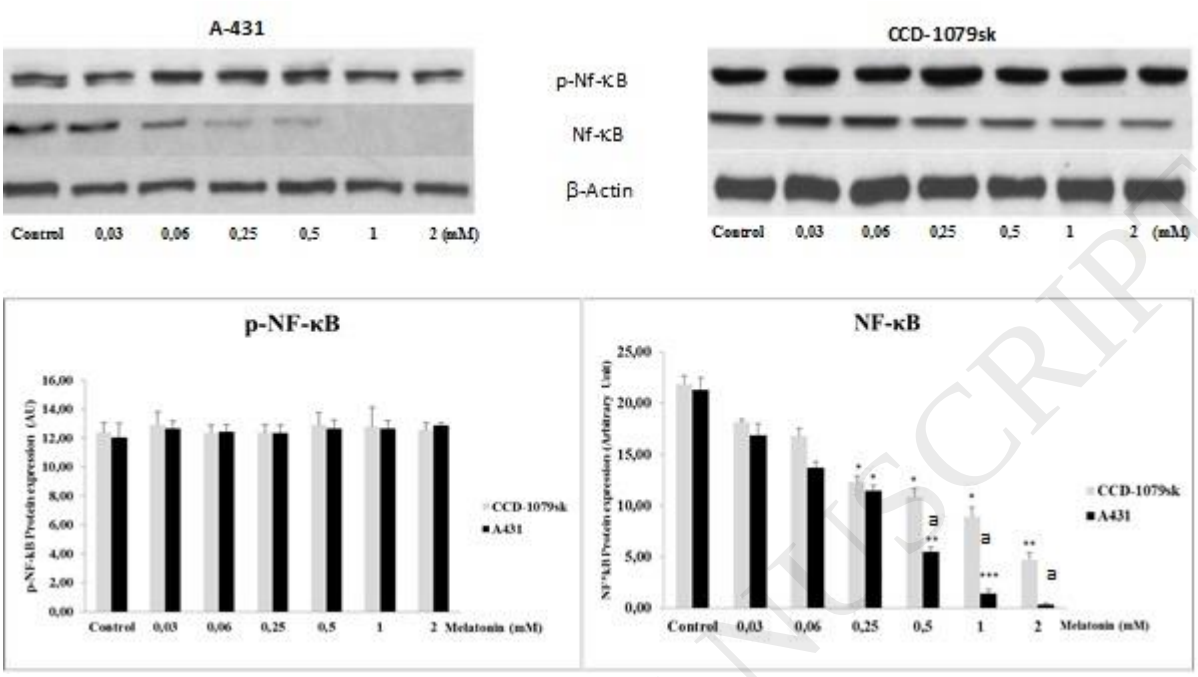

Fig. 9 . Annexin-V-FITC dual staining to assess apoptotic activity of melatonin on A-431 and CCD-1079 Sk cell lines. Cells were (A) untreated or treated with (B) 1 mM or (C) 2 mM melatonin for 24 hours.

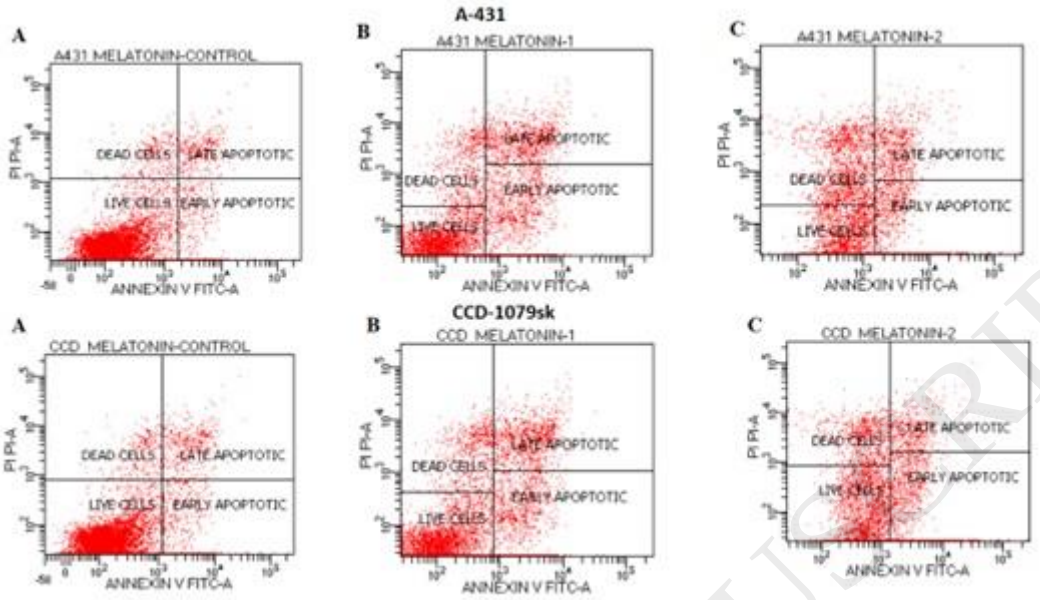

Fig. 10 . Annexin-V-FITC dual staining to assess apoptotic activity of melatonin on A-431 and CCD-1079 Sk cell lines. Cells were treated with different concentrations of melatonin (0.031 to 2 mM) for 24 hours, stained with Annexin- V and measured by a flow cytometry. Significant differences according to the controls indicated by *$p < 0.05$, ** $p < 0.01$.

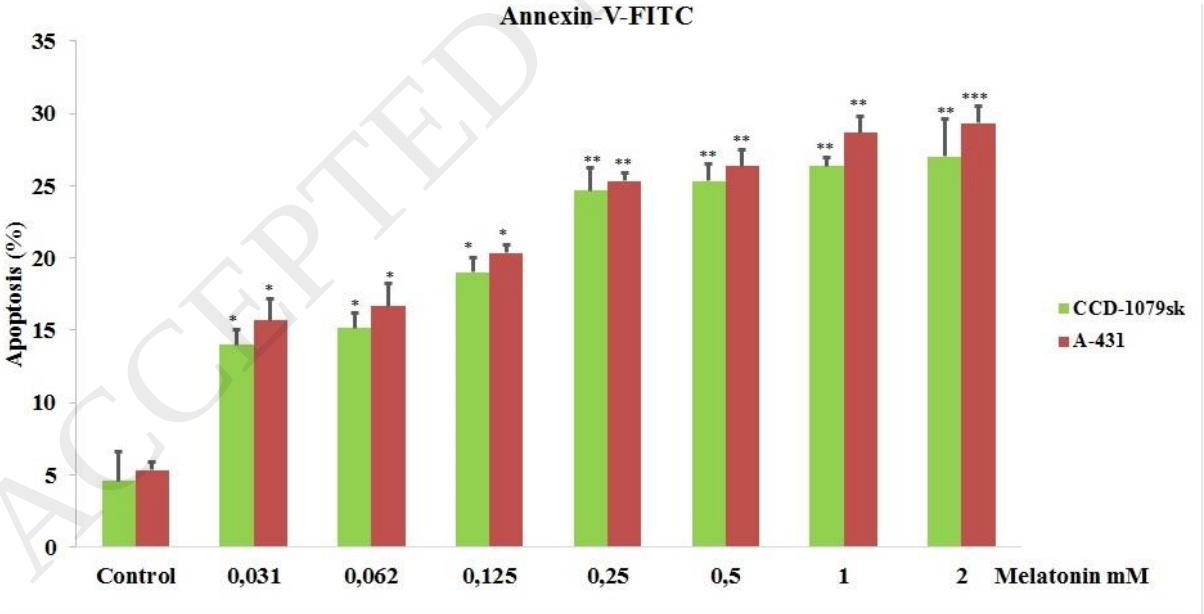

Fig. 11. Effects of melatonin on mitochondrial membrane potential in A-431 and CCD- 1079Sk cell lines. Loss of mitochondrial membrane potential (MMP) occurred in response to melatonin treatment of cancer and normal cells. Disruption of MMP was estimated by flow cytometry. The relative signal intensities of DiOC6 (3) are shown as a percentage of cancer cells compared with normal cells. Significant differences according to the control are indicated by *p < 0.05, ** p < 0.01. *** p<0,001. Significant differences between cancer and normal cells indicated by "a", (n = 3)