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Melatonin and cisplatin synergistically enhance apoptosis via autophagy-dependent alteration of P53 transcription in human colorectal cancer cells

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Abstract

Cisplatin is one of the most general chemotherapeutic agents used to treat various cancers, including colorectal and breast cancer. because cisplatin has some adverse effects including cardiotoxicity and hepatoxicity, it is usage limited. Melatonin is a natural product that responsible for regulator of circadian rhythms and has anti-cancer potential. However, its synergistic effects with melatonin and cisplatin, its efficacy in cancer cell death, its mechanisms and biological targets are not well understood. In this study, it was aimed to determine the synergistic activity of cisplatin with melatonin in colon cancer cell death through apoptotic and autophagic mechanisms. In the present study, we found that melatonin with cisplatin treatment did not affect the cytotoxicity, but cisplatin increased in 24 h incubation period. Melatonin and combined treatment of melatonin and cisplatin also increased the cytotoxicity in 48h incubation period. It was observed that cisplatin treatments used together with melatonin and melatonin inhibited the mitogen activity of colon cancer cells. In addition, combined treatment of cisplatin and melatonin and single treatment of cisplatin increased both apoptosis and autophagic cell death. The results revealed that the use of melatonin with combined cisplatin has been shown to increase the apoptosis and autophagic cell deaths via P53 gene activation.

Keywords: Melatonin, cisplatin, colorectal cancer, apoptosis, autophagy

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Introduction

Colorectal cancer is the third most common type of cancer worldwide and is the second leading cause of

cancer-related deaths. In addition, while the incidence of colon cancer is increasing with obesity worldwide (1) the effectiveness or synergistic treatment of natural products molecules in the treatment of cancer has been emphasized in recent years (2) Melatonin (N-acetyl-5-methoxytryptamine) is a hormone synthesized and secreted by the pineal gland in the body, and is an indolic compound that plays a central role in the regulation of circadian and seasonal biorhythms in humans (2). Melatonin has shown chemotherapeutic potential in many cancer types. It can also rise the efficacy of anticancer drugs (eg, cisplatin, epirubicin) by regulating many different signaling pathways (3). Thus, melatonin has been recognized as a potential complementary product in chemotherapy as well as to reduce the negative effects of anticancer drugs (2). Nevertheless, the mechanisms of melatonin synergize with anticancer drugs are still unclear. An occurring any DNA damage in the cell, many pathways make play role in the cell (4). These damages activate pathways that function at cell checkpoints and slow down the transition to S phase by inhibiting the progression of cells towards G1 and G2 phases. DNA Damages cause an excessive expression of the p53 tumor suppressor gene that regulates the cell cycle. TP53 is a very short-lived protein (5). The p53 gene, which ensures the stability of the genome, is activated in DNA damage, preventing the cycle from G1 to S, thus providing the cell with the time necessary for the repair of the damage. With the increase in p53 expression, transcription of many genes is also stimulated. If the damage is repaired, the Murine double minute 2 (mdm2) gene, which is an important regulator of the p53 tumor suppressor gene, is activated. With its E3 ubiquitin ligase activity, the MDM2 protein recognizes p53 from its N-terminal trans-activation domain, causing its proteosomal degradation and inhibiting the transcriptional activity of p53 (6). In this way, it regulates the cell cycle negatively. When DNA damage occurs in the cell, structural changes such as acetylation and phosphorylation occur at the binding site of MDM2 protein to p53, and MDM2 cannot bind p53. Therefore, free p53 cannot stop the cycle. If the damage cannot be repaired, p53 induced apoptosis cell death the in the cell (7). Additionally, cell cycle progression is controlled by cyclin-dependent kinases (Cdk), which are the catalytic partners of cyclins that maintain the cycle. Cdk activity that is not regularly controlled causes increased cell proliferation and genomic instability. Three different Cip/Kip (Cdk Inhibitory Protein / Kinase Inhibitory Protein) family CDKIs have been identified that regulate Cdk activity and stimulate cell cycle suppression. These genes are p21Cip1 (CDKN1A), p27Kip1 (CDKN1B) ve p57Kip2 (CDKN1C) (8). The first cloned Cip/Kip member, CDKI, is p21 and is responsible for G1 suppression in the response to DNA damage. p27 is an important molecule that regulates growth in response to antimitogenic signals. p57; It has 40% homology with p27 and is involved in proliferation and differentiation (8). According to the results of research conducted in recent years; It is known that p21 induces "growth arrest", p57 ensures the continuation of "growth arrest" and p27 stimulates differentiation genes. Mutational inactivation of Cip/Kip family CDKIs is very rare (9,10).

In the present study, we examined the potential of melatonin to enhance the efficacy of cisplatin in Colorectal cancer cell lines. We demonstrate that the combination of melatonin and cisplatin remarkably induces autophagfy and apoptosis in the Colorectal cancer cells by altering P53 mRNA. We also investigated the synergic effects of melatonin and cisplatin in the colorectal cancer cells.

Materials and Methods

Cells and culture conditions: Human Colorectal cancer cells (HT-29) were purchased from the Tarım ve Orman Bakanlığı Şap Enstitüsü (Ankara, Turkey). The cells were maintained as mono-layer cultures in 75-cm2 plastic culture flasks in Dulbecco's modified

Eagle's medium (DMEM) (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, USA), 2%L glutamine (Gibco, USA), %1 penicillin (20 units/mL) and streptomycin (20 lg/mL) (Sigma-Aldrich, USA) at 37°C in a humid atmosphere containing 5% CO_2.

Drug treatment: Human Colorectal cancer cells (HT-29) were seeded in a 96-well plate at a density of 1500 cells/well in 100-µL of complete culture medium and left attach overnight. After 24 h and 48h, melatonin (dosing at 5-µM and 10-µM) (Sigma Aldrich, USA) and/or cisplatin 50-µM was added (KoçakFarma, Turkey). Melatonin was dissolved at 1M stock solution in ethanol (Sigma-Aldrich, USA) and the corresponding alcohol concentration (ethanol at a final concentration lower than 0.0001%) was added to control cells. Cisplatin was diluted with DMEM solution.

Measurement of cellular proliferation: After 24h and 48h of incubation, MTT reagent was added at a final concentration of 0.5 mg/mL and allowed to react for 3h. Then, MTT solution (100-µl) was added and incubated in the dark at 37 °C for 20 minutes. The absorbance was measured at a spectrophotometer microplate reader µ-Quant, BioTek Instruments (Winooski, Vermont, USA) at a wavelength of 570 nm using absorbance at 690 nm as reference wavelength.

Tunel assay: After the 24h and 48 h incubation of cell on poly-L-lysine coated slides in the 10 cm petri dishes, the slides were fixed with methanol solution at -20 °C for 10 min and then washed with PBS. The apoptotic cells were detected by terminal Transferase dUTP Nick End Labeling (TUNEL Promega G7130) method. For fixation, ethanol solution at -20° was used and then slides dropped in 3% hydrogen peroxide. Then, the cells were treated with 0.1% Triton X-100 for 10 min for permeabilization. After 3 times washing with PBS, 1% bovine serum albumin (BSA) in PBS solution was used for blocking of non-specific bindings. The cells were incubated with terminal deoxynucleotidyl transferase enzyme (Tdtenzyme) for 1 h at 37°C. Then cells were applied with Converted Pod solution for 30 min. The cells were stained with Harris hematoxylin for counterstaining. Cells were mounted with aqueous media. The apoptotic cells were counted using light microscope (Nikon eclipse i50, Japan).

Tunel positive cell evaluation: For the apoptotic (tunel positive cells), Stereological Optic Fractionator Frame method was used to compare the tunnel results between groups. These analyzes were performed under stereology workstation system (BioPrecision MAC 5000controller system) and stereology software (Stereo Investigator version 9.0, Microbrightfield, Colchester, VT) attached light microscope (Leica DM4000B, Tokyo, Japan). In our study, tunnel positivity on HT-29 cell preparations was calculated using the "Unbiased Counting Frame and Fractionator" method, and the positive cell density in each preparation belonging to all groups was calculated according to the following formula (Fig. 1): PHY = PHS/(CA x RS), PHY; Positive cell density per µm² area, PHS; positive cell count, CA; frame area (µm²) and RS; number of frames. The data obtained are based on duplicate measurements for each group, and 4 parallel preparations from each group were stained.

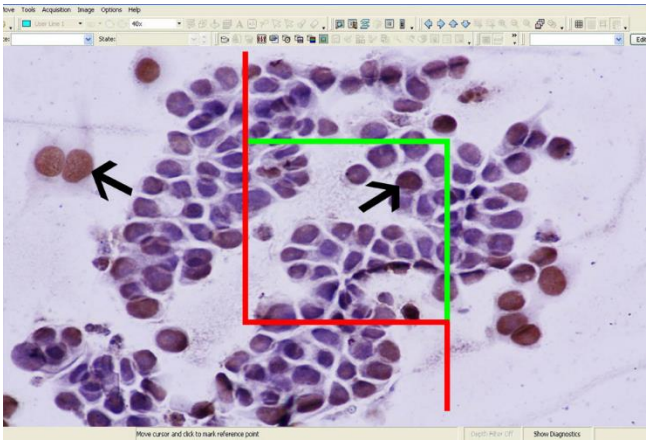

Fig 1. Evaluation of tunnel positivity with stereological "Optical Fractionator Frame" method.

Quantitative Real Time PCR (qRT-PCR) Analyzes

RNA isolation: The total cellular RNA was isolated from HT-29 cells and purified with the Nucleospin RNA Kit (Qiagen RNA Mini Kit, USA) following the manufacturer's instructions. The concentration and purity of the isolated total RNA were determined by spectrophotometric method. For this purpose, Nanodrop device designed for DNA/RNA measurements, capable of measuring in micro volume was used (µ-Biotek, USA) and RNA concentration and purity were calculated automatically with the software on the device according to 260/280 nm values, and the results were determined as ng/µl.

cDNA Synthesis: For cDNA Synthesis, 140 ng RNA was denatured at 65°C for 10 min and reverse transcribed for 50 min at 45°C with cDNA Synthesis kit (Qiagen, USA) in a final volume of 20-µL. After determination of obtained cDNAs concentrations by spectrophotometric method (µ-Biotek Nanodrop, USA), relative quantitation analyzes of cDNAs were started with qRT-PCR (Roche, German).

qRT-PCR analysis: For the mRNA expression levels of BECLIN1, ATG4, TP53, MDM2, CDKN1A, CDKN1B and LC3 genes were evaluated the using quantitative real time PCR (Roche Light Cycler 480 Real-Time, German). qRT-PCR was performed in 20 µl total volume (5µl cDNA, 8 µl ddH2O, 5 µl Probe Master mix 2 µl primers). In qRT-PCR, 4 standards were used to obtain the Costom Assay PCR program's principle Absolute Quantification value. Then, Absolute Quantitative analysis was performed and the values given by the device to the samples as BECLIN1, ATG4, TP53, MDM2, CDKN1A, CDKN1B and LC3 were obtained based on the standards.

Statistical analysis: Normal distributed values form MTT, tunel and qRT-PCR analyses were evaluated the Duncan Post hoc test after one-way analysis of variance (ANOVA) analysis. The significance value (P) was accepted as 0.05. in qRT-PCR analysis, Glucose 6-phosphate dehydrogenase (G6PD) was used as a housekeeping gene for the determination of BECLIN1, ATG-4, TP53, MDM2, CDKN1A, CDKN1B and LC3 gene expression levels and were normalized by taking the target Gene/reference gene ratio.

Results

Cytotoxicity evaluation: In the MTT analysis, it was determined that the cytotoxic effect of cisplatin treatment was significantly higher in the groups incubated for 24 hours ($p<0.05$). In the 48-h incubation groups, cisplatin (50µM) + melatonin (5µM) and Cisplatin (50µM) treatments were showed a similar effect on the cells. Therefore, the cytotoxic effect of melatonin doses was found to be similar to the control group (Table 1 and Fig. 2).

Table 1. Effect of Mel and/or Cis on Cytotoxicity in HT29 cells incubated for 24h and 48h were exposed to Mel (5µM and 10µM) and/or Cis (50µM) for Tunel staining.

Groups	24h	48h
Control	0.854±0.08[a]	0.751±0.11[a]
Mel-5µM	0.839±0.13[a]	0.696±0.23[b]
Mel-10µM	0.863±0.21[a]	0.728±0.19[a]
Cisplatin	0.728±0.14[b]	0.622±0.12[b]
Mel-5µM-Cis	0.847±0.17[a]	0.649±0.15[b]

[(a,b)] the Letters indicate statistical difference between columns.

Tunel Analysis Results: In the evaluation of apoptotic cells, there was no significant difference between tunel positive cell densities of Control, Mel-5µM and HT-29 cells incubated for 24 hours with Mel-10µM- Cisplatin (50-µM) treatments ($P>0.05$), while significant tunneling was observed in HT-29 cells only cisplatin applied. It was determined that there was a significant increase in positive cell density ($P<0.05$).

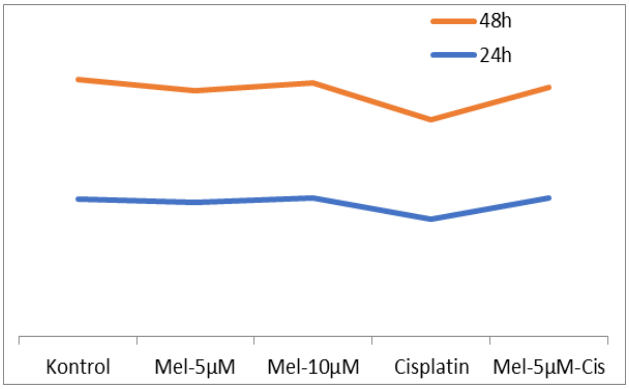

Fig. 2. Synergistic cytotoxicity of melatonin (Mel (5µM) and Mel (10µM)) and Cisplatin (Cis(50µM)) co-treatment (Mel (5µM) +Cis (50µM)) in HT-29 cell lines incubated for 24h and 48h.

In addition, while the tunel positive cell density of Cis-50 µM and Mel-5µM+Cis-50 µM groups was significantly higher than the other groups in the 48-hour incubation period ($P<0.05$), there was no found any statistical differences between Mel(5µM), Mel (10µM), and control groups ($P>0.05$). Tunel positive cell densities for all groups were presents in Table 1 and seen in Table 2 and Fig 3.

Table 2. Effect of Mel and/or Cis on Tunel-positive cells in HT29 cells incubated for 24h and 48h were exposed to Mel (5µM and 10µM) and/or Cis (50µM) for Tunel staining.

Groups	24h	48h
Control	0.101±0.04[a]	0.151±0.02[a]
Mel (5µM)	0.115±0.02[a]	0.167±0.03[a]
Mel (10µM)	0.146±0.03[a]	0.203±0.04[a]
Cis (50µM)	0.235±0.04[b]	0.426±0.07[b]
Mel (5µM) +Cis (50µM)	0.165±0.07[a]	0.278±0.06[c]

[(a,b,c)] the Letters indicate statistical difference between columns.

mRNA expression levels: In the TP53 mRNA expression analysis, the expression levels of Mel (5µM) and Cis (50µM) groups in HT-29 colon cancer cells incubated for 24 hours were higher than the other groups, while the expression levels of Mel (10µM) and Mel (5µM)+Cis(50µM) groups were found to be nearly similar to the Control group. In 48h incubation period, TP53 mRNA expression levels of Mel (5µM) and Mel (5µM)+Cis(50µM) groups were increased compared to control group. Other groups were found nearly similar to control group (Table 3 and Fig 4).

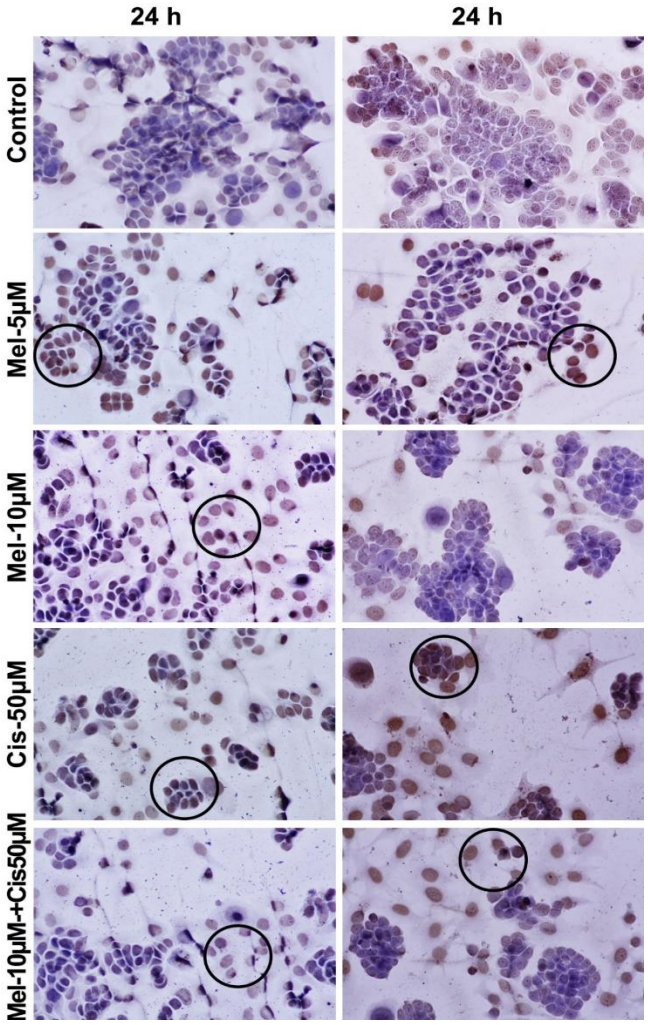

Fig 3. Illustrations of Mel and/or Cis on Tunel-positive cells in HT29 cells incubated for 24h and 48h were exposed to Mel (5µM and 10µM) and/or Cis (50µM) for Tunel staining.

In the MDM2 mRNA expression, the expression of Mel(5µM) and Cis(50µM) groups were higher in HT-29 colon cancer cells incubated for 24 hours, also the expression level was found higher in the Mel-10µM group than the control group, but lower in the Mel (5µM)+Cis(50µM) group than the control group. In the 48h incubation groups, the highest mRNA expression was observed in the Mel (5µM)+Cis(50µM) group, while the mRNA expression levels in the other groups decreased (Table 3 and Fig 4).

Table 3. Effect of Melatonin and/or Cisplatin on gene expression levels in HT29 cells incubated for 24h and 48h were exposed to Mel (5µM and 10µM) and/or Cis (50µM).

	Groups	TP53/ G6PD	MDM2/ G6PD	CDKN1A/ G6PD	CDKN1B/ G6PD	BECLIN1/ G6PD	ATG-4/ G6PD	LC3/ G6PD
24 h	Control	0.184	1.636	0.154	0.189	0.349	0.095	0.013
	Mel (5µM)	0.472	3.317	0.748	0.399	0.678	0.202	0.032
	Mel (10µM)	0.241	2.694	0.398	0.188	0.548	0.124	0.065
	Cis (50µM)	0.412	4.313	2.720	0.164	0.537	0.139	0.061
	Mel (5µM) + Cis (50µM)	0.159	1.184	0.220	0.108	0.220	0.051	0.011
48 h	Control	0.175	1.924	0.350	0.365	0.473	0.140	0.037
	Mel (5µM)	0.160	0.777	0.096	0.295	0.295	0.074	0.019
	Mel (10µM)	0.232	1.427	0.210	0.308	0.368	0.107	0.021
	Cis (50µM)	0.402	1.699	1.139	0.337	0.570	0.203	0.163
	Mel (5µM) + Cis (50µM)	0.300	2.797	1.234	0.222	0.483	0.193	0.140

The values of relative mRNA expression levels were normalized with internal control (G6PD)

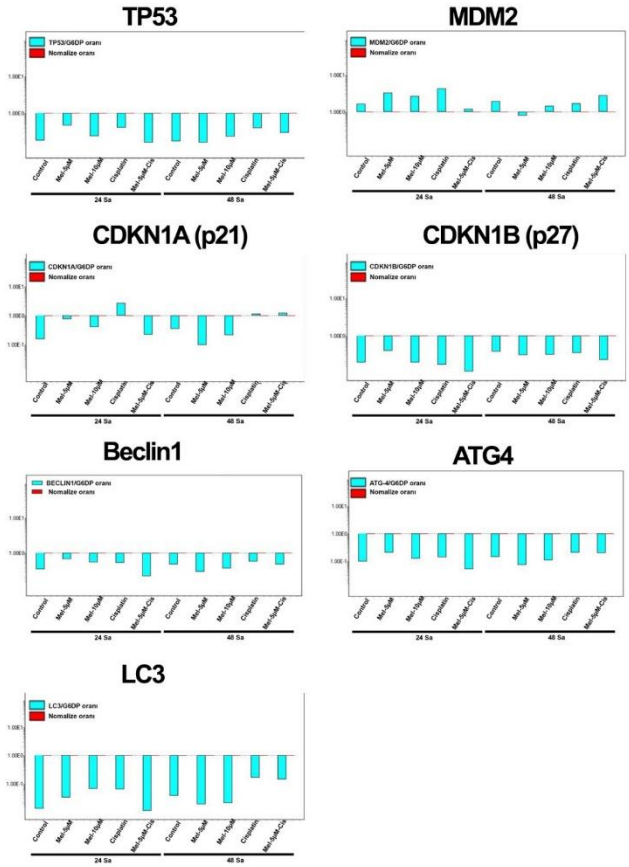

Fig 4. Effect of Melatonin and/or Cisplatin on relative gene expression levels in HT29 cells incubated for 24h and 48h were exposed to Mel (5µM and 10µM) and/or Cis (50µM).

In the analysis of CDKN1A mRNA expression, the highest expression level was found in the Cis (50µM) group in the HT-29 cells incubated for 24h. Although, it was observed an increase in the Mel (5µM) group, the increase in the other groups was close to the control group incubated for 24h. Also, the highest values were found in Cis (50µM) and Mel (5µM) +Cis (50µM) groups in 48 hours incubation groups. It was observed that the increase in the other groups was close to the control group value (Table 3 and Fig 4).

In CDKN1B mRNA analysis, an increase was observed in the expression level in the Mel (5µM) group with 24-h incubation, while the expression levels of the other groups were found to be close to the Control group. Also, mRNA levels of CDKN1B of all groups incubated for 48 h were decreased compared to the Control group (Table 3 and Fig 4).

In the mRNA expression analysis of BECLIN1 gene, had the highest expression value was found in Mel-5µM, Mel-10µM and Cis groups in HT-29 cells incubated for 24 hours. Also, mRNA expression level of BECLIN1 was decreased in Control and Mel (5µM) + Cis (50µM) groups. On the other hand, mRNA

expression level of BECLIN1 was increased in Control, Cis (50μM) and Mel (5μM) + Cis (50μM) groups in HT-29 colon cancer cells incubated for 48h, while the expression value of Mel (5μM) and Mel (10μM) groups was found to be lower than in the Control (Table 3 and Fig 4).

In the analysis of ATG-4 mRNA expression, Cis (50μM) and Mel (10μM) groups were higher than the Control and other groups in colon cancer cells incubated for 24 hours. The lowest value for incubated 24 h groups was found Mel (5μM) + Cis (50μM) group. In the ATG-4 mRNA expression levels of HT-29 colon cancer cells incubated for 48 hours, the highest expression was observed in Cis (50μM) and Mel (5μM) + Cis (50μM) groups, while the lowest value was found in the Mel (5μM) group (Table 3 and Fig 4).

In the LC3 mRNA expression analysis, the expression levels were found higher in the Mel (10μM) and Cis (50μM) groups in the 24-hour incubation groups. The mRNA expression levels of other groups were close to the control group. Also, In the group incubated for 48 hours, an increase was determined in the Cis (50μM) and Mel (5μM) +Cis (50μM) groups. In the Mel (5μM) and Mel (510μM) groups, a decrease in their expression was determined compared to the Control group (Table 3 and Fig 4).

Discussion

The goal of chemotherapy is to induce cancer cell death without damaging non-cancerous cells or tissues. Cisplatin is an essential chemotherapeutic reagent for the treatment of many tumors including colon cancer as well as many other types of cancer, but its many side effects limit its indication. Therefore, researchers are seeking to improve the therapeutic efficacy of cisplatin therapy for minimizing side effects of cisplatin. Melatonin has also been reported to show protective effects against various anti-cancer drugs (11). Thus, melatonin seems to be one of the most effective complementary components that can meet this requirement and increasing the sensitivity of cancer cells to cisplatin while providing protection against toxicity caused by cisplatin (12).

Colorectal cancer is one of the most common types of cancer (13). The study was investigated the effects of melatonin and/or cisplatin on cell cytotoxicity, apoptotic cell death and autophagy on colorectal cancer. In the study, HT-29 cell line was used and different concentrations (5, 10 nM) of melatonin and cisplatin (50 μM) were treated for 24 and 48 hours. In the result, administration of melatonin and/or cisplatin significantly decreased cell viability. Previous studies supports the therapeutic properties of melatonin on cancer cells (14). Also, some studies suggested that melatonin affects mitochondrial function by reducing ATP synthesis, triggering OS to encourage death in cancer cells, and blocking telomerase activity (15).

In the results of the tunnel analysis, it was observed that the melatonin and cisplatin treatments were therapeutically effective on HT-29 cells and increased the number of apoptotic cells. Combined administration of melatonin and cisplatin has been shown to be effective in osteosarcoma cells (16). In our study, the administration of melatonin and/or cisplatin was also found to be more effective when combined to use.

Expression levels of BECLIN1, ATG4, TP53, MDM2, CDKN1A, CDKN1B and LC3 genes, which are related to apoptotic and autophagy, were analyzed by quantitative PCR method. The BECLIN1 gene is one of the important genes involved in autophagy (17). In our study, mRNA expression level of Beclin1 was found higher in the 24 and 48h incubated HT-29 colorectal cancer cells treated with melatonin and/or cisplatin. Although ATG4 and LC3 genes are also genes involved in autophagy, autophagic cell death in the cancer (18). In the present study, the expression levels of ATG4 and LC3 were found increased in the 24 and 48 h melatonin /and or cisplatin incubated HT-29 cancer cells.

In the study, an increase was observed in the mRNA level of the p21 (CDKN1A) gene in the melatonin and cisplatin incubated groups compared untreated groups. The p21 gene is synthesized by the p53 gene and provides the synthesis of CDKs that control the cell cycle (19). Inhibitors that control cell division are called cyclins and one of cyclins is p27 (CDKN1B) gene, which is also a suppressor of TGFB, which controls the G2/M transition, which controls the cell cycle. In our study, it was observed that melatonin and cisplatin treatment increased the mRNA levels of p27.

The p53 gene is an important protein that is responsible for preventing tumor growth (20). In our study, an increase was observed in the treatment of melatonin and cisplatin in the level of the p53 gene. This shows that the treatment of melatonin and cisplatin trigger the colorectal cancer cell to apoptosis. Melatonin and cisplatin applications were also found to decrease at the level of MDM2, which is known as an inhibitor of TP53 proteins.

As a result, it was observed that the separate and combined treatment of melatonin and cisplatin provided a cytotoxic effect in HT-29 cells with colorectal cancer, it stimulated apoptotic cell death and increased autophagic gene expressions. therefore, it has been shown that melatonin and cisplatin applications can have therapeutic properties for colorectal cancer.

Declaration of Interest: The author declares that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions: SP, HT, EE and NAC, AK contributed to the study conception, design and laboratory work. Writing the article (SP, HT, AK). All authors read and approved the final manuscript. SP; Süleyman Polat, HT; Halime Topal, NAC: Nevra Aydemir Celep EE: Elif Erbaş, AK; Adem Kara.

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