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Research Article

Inhibition of Autophagy on Melatonin-Induced Apoptosis in MCF-7 and MDA-MB-231 Cell Lines

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Abstract

Aim: We looked at the connection between autophagy and apoptosis after our prior research indicated that melatonin could cause MCF-7 and MDA-MB-231 cells in the present study.
Material and Method: In order to investigate the autophagy inhibition's effect on the melatonin-induced BC cells' apoptosis, melatonin and/or 3-methyladenine (3-MA, autophagy inhibitor) have been utilized. Melatonin was applied to the cells following a 5-mM 3-MA pre-cultivation. Then, apoptosis was detected by the TUNEL method. The technique for double immunofluorescence labeling was used to identify the molecular alterations in Bax/Bcl-2 expression. To evaluate the cell viability, the MTT test was used.
Results: When an autophagy inhibitor, 3-MA, and melatonin treatment were co-administered in MCF-7 cells, apoptosis was decreased, compared to melatonin treatment alone, but it was not significant. In addition, 3-MA application downregulated Bax expression compared with melatonin alone treatment. Combined therapy markedly elevated apoptosis and significantly up-regulated Bax protein in MDA-MB-231 cells.
Conclusion: Taken together, in MCF-7 cells, autophagy's inhibition contributes to the downregulation of apoptosis, whereas increased apoptosis is seen in MDA-MB-231 cells. Inhibiting autophagy in these cells treated with melatonin could serve as a self-defense mechanism, and This might be a good strategy for breast cancer adjuvant treatment.

Keywords: Autophagy, breast cancer, melatonin, 3-MA

INTRODUCTION

Breast cancer (BC) has been categorized in two ways: as expressed carcinoma that expresses the receptor 2 for human epidermal growth factor and as BC that expresses the estrogen receptor (ER) or progesterone receptor (PR), respectively (1,2). Triple-negative BC is still challenging to treat due to its aggressive characteristics and few therapeutic choices (3). The most aggressive type of BC is called triple-negative BC, and it is more likely to spread among women and is challenging to treat. Few therapy options and a poor prognosis for triple-negative BC patients make it urgently necessary to develop novel therapeutics (4). In the search for treatments for BC, the focus is on testing curative agents for their effectiveness against cancer cell proliferation using diversified cell lines. MCF-7 is a BC cell line that is ER-positive and PR-positive (5). MDA-MB-231 cells are negative for ER, PR, and HER2, so they are called triple-negative BC cells. Both MDA-MB-231 and MCF-7 cells have epithelial characteristics. They came from carcinoma cells in the mammary gland. MCF-7 is BC's luminal type histologically, while MDA-MB-231 is a basal type. MDA-MB-231 cells are characterized by a high rate of metastasis, a poor prognosis, a propensity for relapse, and an insensitivity to therapy (6-10).

Serotonin is used for the production of melatonin, which is the pineal gland's primary hormone. Also, a number of peripheral tissues and immune cells produce melatonin (11). It possesses cytoskeleton modulatory and oncostatic capabilities, capable of reducing tumor growth and cancer cells' invasiveness (12). The mechanisms underlying melatonin's anticancer actions have been thought to be its antioxidant effects (13,14) and triggering apoptotic pathways in cancer cells. (15). The morphology

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of cells can be used to distinguish between two main kinds of cell death: autophagy- and apoptosis-cell death. Under normal physiological conditions, apoptosis acts as a targeted removal of undesirable or dangerous cells (16,17). Autophagy degrades damaged organelles and/or proteins and recycles them to maintain cellular components' quality. It is an evolutionarily preserved catabolic process (18). Autophagy's role in cancer is arguable because, depending on the tumor's form and stage, it may be able to operate as either a tumor promoter or a tumor suppressor (19).

3-MA is one of the autophagy inhibitors. It has been noted to suppress the event of PI3-kinase and stop autophagic vacuoles from forming (20). The studies showed that treatment with specific autophagy inhibitors, such as 3-MA, may increase the apoptotic effectiveness of chemotherapeutic drugs in lung cancer (21), prostate (22), colon (23), HeLa (24), and BC cells (25). Because of this, the therapeutic potential of possible drug candidates may be increased by identifying their apoptotic molecular targets as well as their autophagic responses. Therefore, this study's purpose was to determine how autophagy inhibition affects melatonin-induced apoptosis in both BC cell lines.

MATERIAL AND METHOD

Cell Culture

The Genome and Stem Cell Center in Kayseri is where the BC cells were bought. At 37°C in humidified air with 5% CO_2, BC cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich, USA), supplemented with 1% L-glutamine (Thermo Fisher Scientific, USA), 10% fetal bovine serum (Gibco, South America), and 1% antibiotics (penicillin/streptomycin, Capricorn Scientific, Germany).

Chemicals

To dissolve melatonin, Dimethyl sulfoxide (DMSO, Sigma Aldrich, USA) was used. 5 mM stock solution a prepared. This was then kept in the dark. The DMSO content was 1% at the end. The autophagy inhibitor used was 3-MA (Sigma Aldrich, USA). Before use, 3-MA is freshly prepared as a 50 mM stock solution in dH2O. Melatonin and 3-MA were afterwards diluted in DMEM medium to reach the appropriate concentration. Melatonin concentrations for MCF-7 and MDA-MB-231 cells were 3.5 and 4 mM, respectively, as previously determined (26).

Autophagy Inhibition

On a circular coverslip, $5x10^4$ cancer cells from each of the two BC cells were planted into 12-well plates. To induce autophagy in both BC cell lines, the media were switched out for FBS serum-free medium for 24 hours. Thus, autophagy was activated in the cells. Then, to determine 3-MA's autophagy inhibition dose, different doses of 3-MA (1, 5, and 10 mM) were applied for 24 hours to the cells. The autophagy markers Beclin-1 (Novus Biologicals, Littleton, USA), LC3 (Cell Signaling Technology, Danvers, USA) and p62 (Novus Biologicals, Littleton, USA) were examined using the immunofluorescence staining method as previously published (26). As a result, the dose at which inhibition occurred was determined.

Treatment

Using the aforementioned experiment, cells were plated. After 24 hours of incubation, drugs were added by the experimental groups. Six groups were created for the experiment: control (no drug intervention), 3-MA (5 mM 3-MA treatment alone), melatonin (melatonin treatment alone), DMSO (DMSO treatment alone), 3-MA and MEL combined (3-MA and melatonin were added simultaneously), and 3-MA and DMSO combined (3-MA and DMSO were added simultaneously). Cells were incubated at 37°C with 5% CO_2 and humidity for a full day. Then, immunocytochemical assays were carried out on BC cells to verify apoptosis and the Bax and Bcl-2 protein's expression. In addition, the MTT method was also used to assess the effect of combining 3-MA and melatonin on cell viability.

Apoptosis Detected by TUNEL

According to the six experimental groups, The twelve-well plates were seeded with $5x10^4$ cells in each well and pretreated with 3-MA, melatonin, or DMSO for 24 hours. 10% formaldehyde was used to fix the cells. Cell apoptosis was assessed using the EMD Millipore's apoptosis detection kit (Darmstadt, Germany), named ApopTag Fluorescein (26). After successfully staining with a mixture of TUNEL reactions, the cells were immediately detected using a fluorescent microscope.

Analysis of the Expression of Bax and Bcl-2

To verify Bax (Novus Biologicals, Littleton, USA) and Bcl-2 (Thermo Fisher Scientific, Rockford, USA) expression profiles, a double immunofluorescence staining method was carried out (26). For the quantification of the proteins' expression, 10 images at 400x magnification were randomly obtained from microscopic fields. The immunoreactivity intensity was calculated using the Image J software program.

Cell Viability Assay

The dose-dependent effects of 3-MA, melatonin, and DMSO on BC cells were investigated using a colorimetric MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, Roche, Indianapolis, IN, USA). In 96-well plates, 1x104 cells were seeded of per well, and drugs were added by the experimental groups. 10 µl of MTT (5 mg/ml in PBS) was added after cells had been exposed to the relevant drug combinations and doses, and the cells were then incubated at 37°C for 4 hours in order to enable mitochondrial enzymes to convert the MTT to formazan crystals. The medium was aspirated after the allotted time had passed, and for each well 100 µl DMSO was added. An ELISA reader (Promega Glomax Multireader, Wisconsin, ABD) was used to measure 570 nm absorbance.

Statistical Analysis

The total immunoreactivity intensity of each antibody was measured using the Image J software program while analyzing the immunofluorescence staining results. For

every group, pictures were captured from ten different microscopic fields at a magnification of 400X. The data that was obtained was analyzed statistically with GraphPad Prism 8.0.2.. The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to assess how much the data conformed to a normal distribution. The values were compared using the Mann-Whitney U test based on drugs, dose groups, and percent viability. When comparing more than two groups, Kruskal-Wallis tests and one-way analysis of variance were used. The Tukey and Dunn-Bonferroni tests were applied for multiple comparisons. The significance limit of $p<0.05$ was accepted.

RESULTS

Inhibition of Autophagy

To determine the dose at which 3-MA inhibited autophagy, 1 mM, 5 mM, and 10 mM 3-MA were applied to cell lines after exposure to serum-free medium for 24 hours. After the Beclin-1, LC3, and p62 expression analysis of these cells, the inhibition dose for both was determined to be 5 mM.

Cell Death in BC Cells Induced by 3-MA and Melatonin Treatment

To examine the apoptotic effect of 3-MA and melatonin on human BC cells, TUNEL assays were used. In MCF-7 cells, MEL induces nuclear shrinkage and fragmentation. Compared to the control group, DNA fragmentation was reduced in MCF-7 cells with the addition of 3-MA and MEL (Figure 1).

In MEL-treated MDA-MB-231 cells, apoptotic cell immunoreactivity was increased. In the 3-MA+MEL group, apoptotic cell immunoreactivity was observed to increase according to the MEL group (Figure 2).

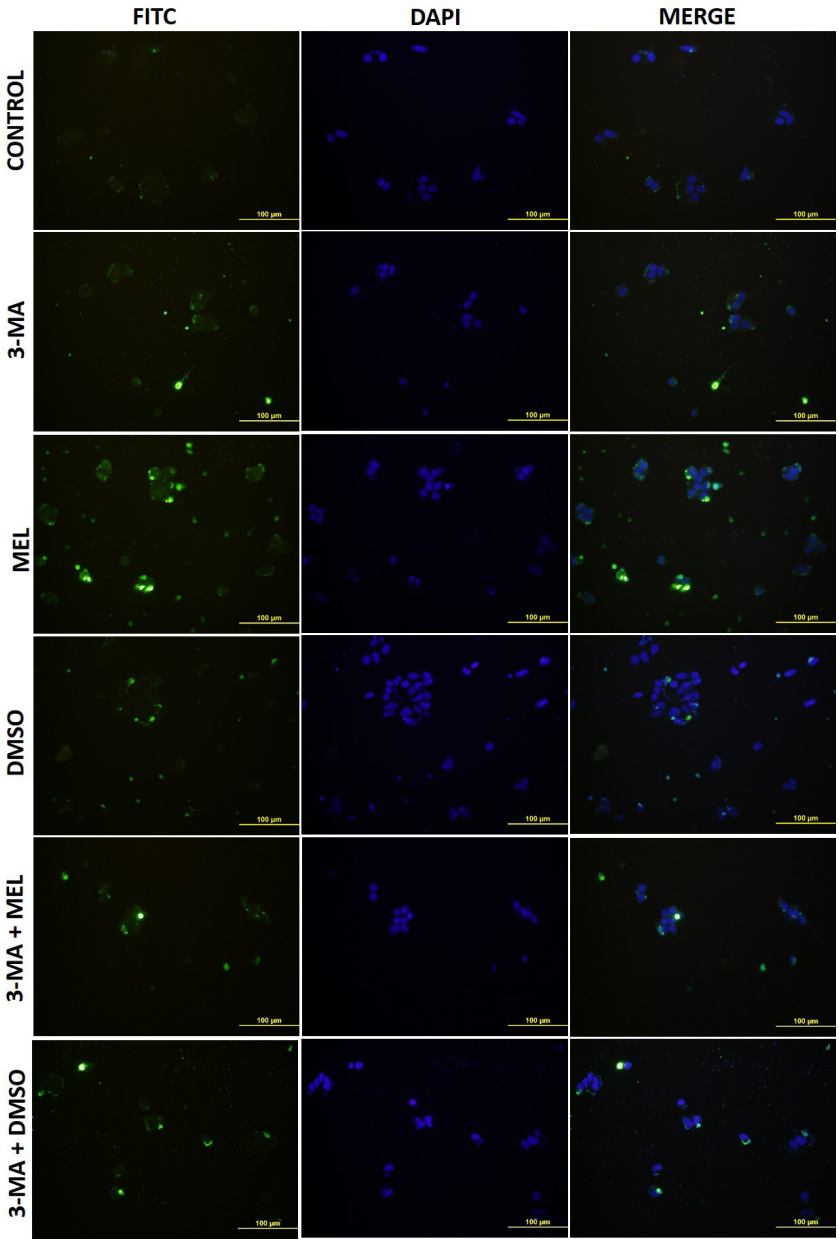

Figure 1. TUNEL images of the experimental groups using the MCF-7 cell line following the application of 3-MA and MEL. Apoptotic bodies that have formed in cells can be noticed as green fluorescent reflections under a fluorescent microscope (400X)

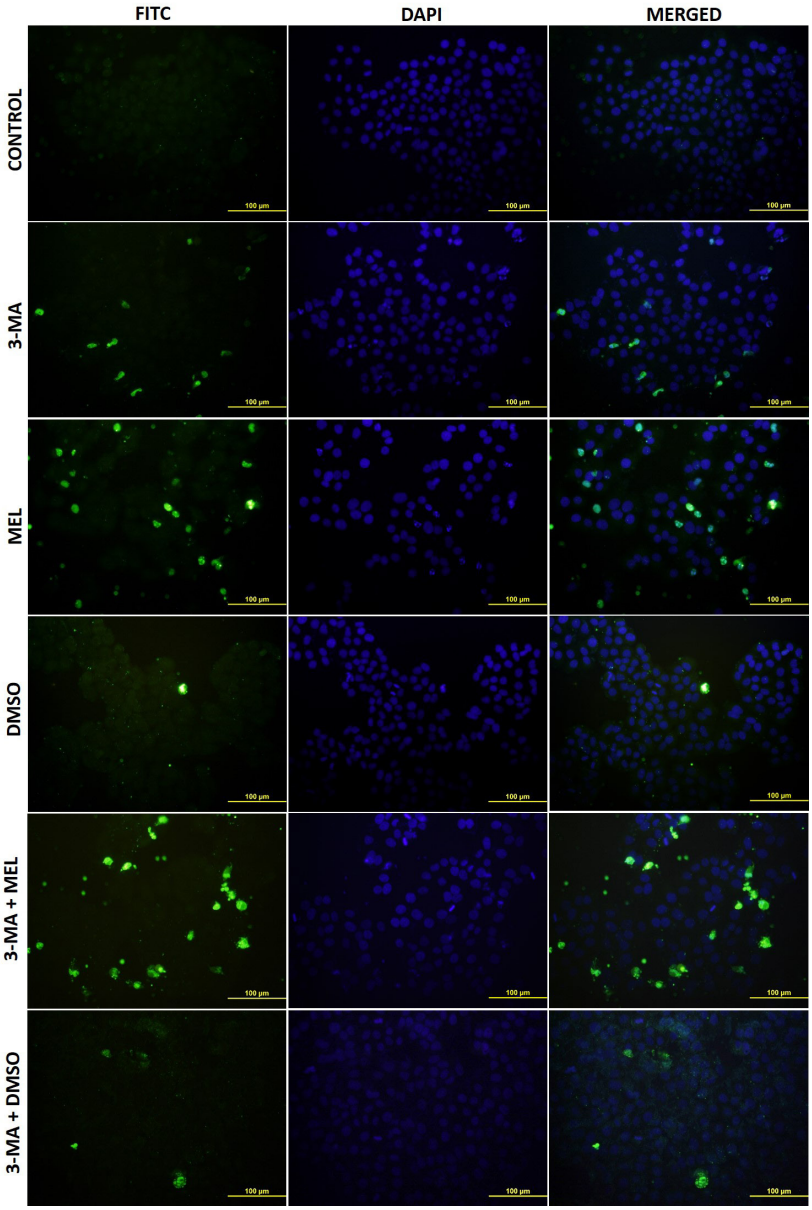

Figure 2. TUNEL images of the experimental groups using the MDA-MB-231 cell line following the application of 3-MA and MEL. Apoptotic bodies that have formed in cells can be noticed as green fluorescent reflections under a fluorescent microscope (400X)

The analysis of statistics of the immunoreactivities of apoptotic cells following 3-MA and MEL administration in the BC cell lines is shown in Table 1 according to experimental groups. It was found that the 3-MA group resembled the control group in MCF-7 cells and that between them, no statistically significant difference was seen. (>0.9999). The statistical significance of the difference between the MEL and the control group was determined (p=0.0061). TUNEL+apoptotic cell density was found to be lower in the 3-MA+MEL group than in the MEL group. In terms of statistics, there was no difference between them (>0.9999). When the experimental groups were examined, it was found that the control group's TUNEL+ cell immunoreactivity was the lowest in MDA-MB-231 BC cells. The MEL group demonstrated a statistically significant increase in comparison to the control group (p<0.0001). Comparing the 3-MA+MEL group to the MEL group, A statistically significant increase was observed (p=0.0029).

Table 1. TUNEL statistical analysis results of MCF-7 and MDA-MB-231 breast cancer cell lines treated with 3-MA and melatonin

	Groups						
	CONTROL	3-MA	MEL	DMSO	3-MA+MEL	3-MA+DMSO	p
MCF-7	(7.03±1.00)^{a}	(7.04±1.00)^{a}	(11.05±1.00)^{b}	(6.18±1.05)^{a}	(10.09±1.01)^{bc}	(7.15±1.03)^{ac}	.0003
MDA-MB-231	(4.41±0.10)^{a}	(6.8±0.10)^{b}	(11.5±0.62)^{c}	(5.56±0.15)^{ab}	(13.62±1.00)^{d}	(5.73±0.11)^{ab}	<0.0001

Data are expressed as mean±standard deviation. The same lowercase letters on the same line indicate similarity between groups, and different letters indicate difference. (3-MA: 3-methyl adenine, MEL: melatonin, DMSO: dimethyl sulfoxide)

Effects of MEL with or without 3-MA on Bax/Bcl-2 Protein Expression

The double immunofluorescence staining protocol was done to examine the effect of administering MEL with or without 3-MA on the Bax and Bcl-2 proteins' expression in both BC cell lines. Figure 3 shows images of the MCF-7 cell line, and Figure 4 exhibits images of the MDA-MB-231 cell line.

Comparing the MEL group to the control group, an important increase was observed when the Bax expression profile was compared between groups in MCF-7 BC cells ($p<0.0001$). Comparing the 3-MA+MEL group to the MEL group, it was found that there was an increase ($p=0.0002$). This rise was not statistically significant. A statistically insignificant change ($p<0.05$) was found when Bcl-2 expressions were analyzed (Table 2). In MDA-MB-231 BC cells, an increase in the MEL group relative to the control group was seen when the Bax expression profile was examined between groups. There was no statistically significant growth in this ($p=0.0708$). Comparing the 3-MA+MEL group to the MEL group, there was a statistically significant increase in Bax expression ($p=0.0034$). A statistically insignificant change ($p<0.05$) was found when Bcl-2 expressions were analyzed (Table 2).

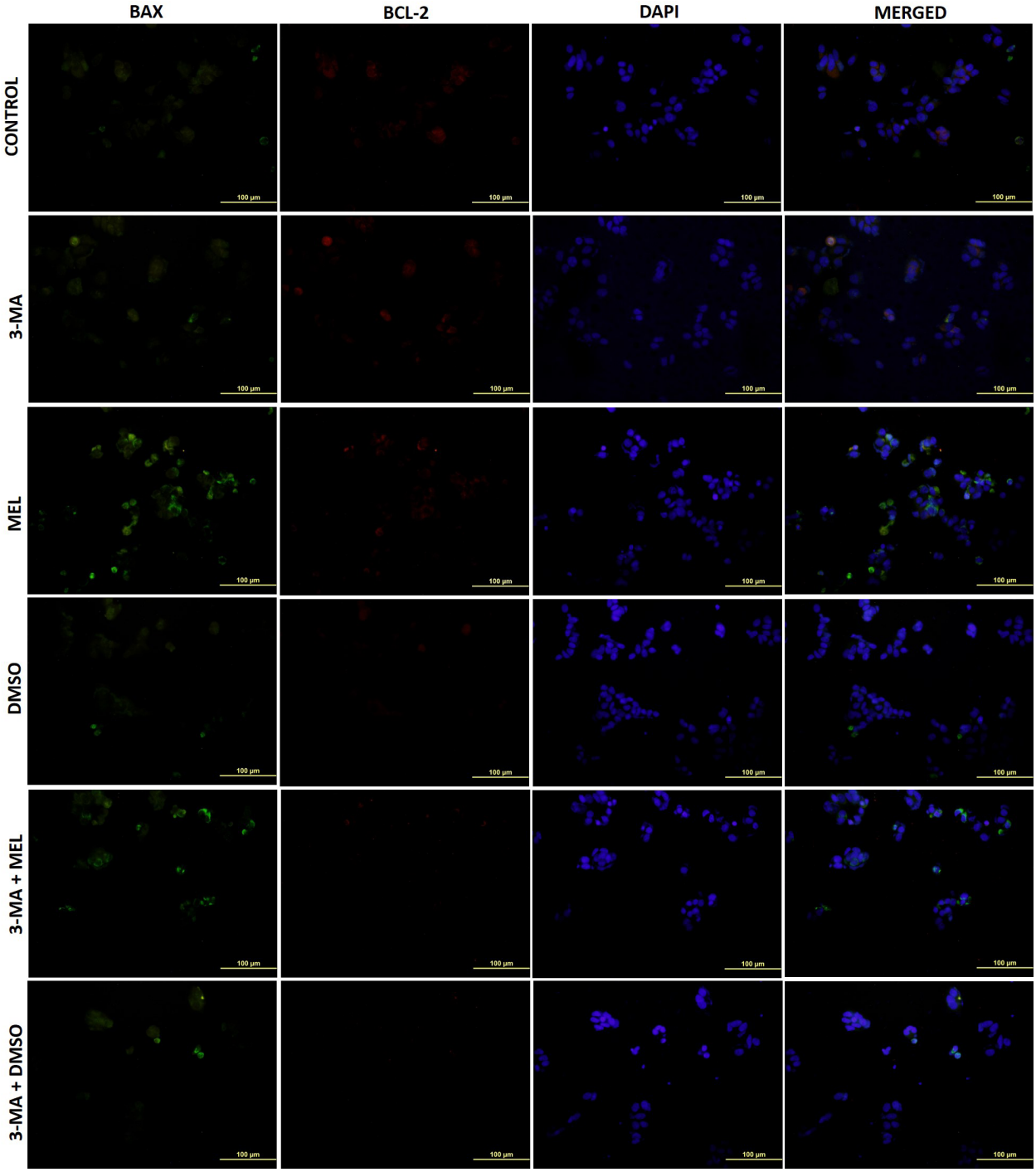

Figure 3. Expressions of Bax/Bcl-2 in the 3-MA- and melatonin-treated MCF-7 cell line Bax expression is demonstrated by bright green reflections in the first column, and Bcl-2 expression is displayed by bright red fluorescent reflections in the second column (400X)

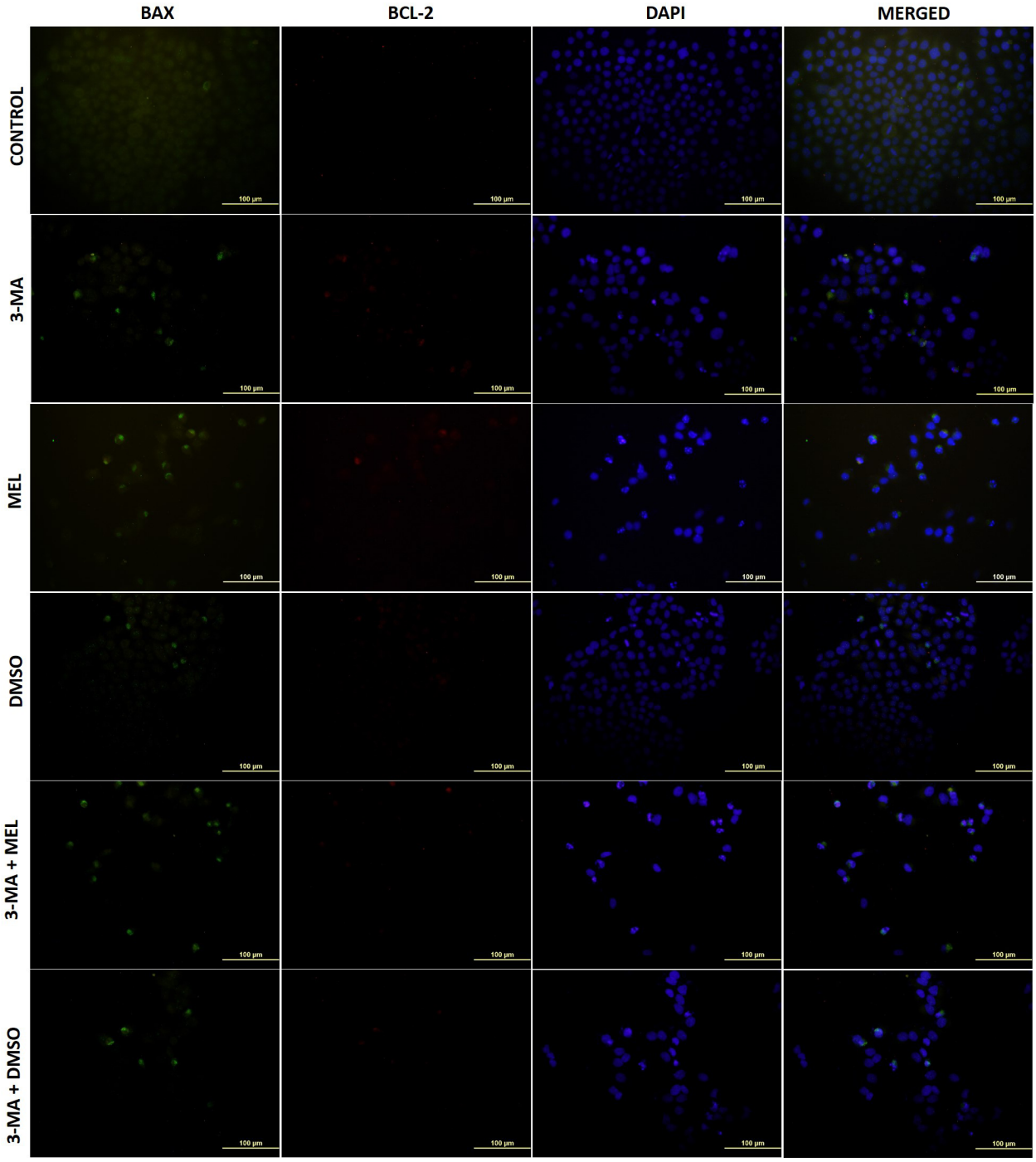

Figure 4. Expressions of Bax/Bcl-2 in the 3-MA- and melatonin-treated MDA-MB-231 cell line. Bax expression is demonstrated by bright green reflections in the first column, and Bcl-2 expression is displayed by bright red fluorescent reflections in the second column (400X)

Table 2. Statistical analysis results of Bax/Bcl-2 expression in the MCF-7 and MDA-MB-231 breast cancer cell line treated with 3-MA and melatonin

		Control	3-MA	MEL	DMSO	3-MA+MEL	3-MA+DMSO	p
MCF-7	Bax	(0.51±0.10)^{a}	(0.62±0.11)^{a}	(1.40±0.10)^{b}	(0.51±0.10)^{a}	(0.80±0.10)^{a}	(0.63±0.11)^{a}	.0001
	Bcl-2	(0.42±0.11)^{a}	(0.51±0.10)^{a}	(0.42±0.11)^{a}	(0.32±0.10)^{a}	(0.23±0.11)^{a}	(0.23±0.11)^{a}	.0355
MDA-MB-231	Bax	(0.42±0.11)^{a}	(0.62±0.10)^{a}	(0.75±0.13)^{ac}	(0.50±1.10)^{a}	(1.23±0.11)^{b}	(1.03±0.11)^{bc}	<0.0001
	Bcl-2	(0.19±0.01)^{a}	(0.30±0.10)^{a}	(0.33±0.11)^{a}	(0.16±0.05)^{a}	(0.19±0.01)^{a}	(0.18±0.02)^{a}	.0325

Data are expressed as mean±standard deviation. The same lowercase letters on the same line indicate similarity between groups, and different letters indicate difference. (3-MA: 3-methyl adenine, MEL: melatonin, DMSO: dimethyl sulfoxide)

Effects of MEL with or without 3-MA on Cell Viability

After the experimental groups were formed, the effect of the application of MEL with or without 3-MA on cell viability was demonstrated by applying MTT. The findings obtained after MTT analysis in MCF-7 cells are shown in Figure 5A. Examining the graph, it was discovered that the MEL group's cell viability was lower than the control group (p=0.0003). It was found that the cell viability in the 3-MA+MEL and MEL groups was similar (p>0.9999).

The findings were obtained after MTT analysis in MDA-MB-231 BC cells (Figure 5B). When the graph was examined, it was observed that cell viability decreased in the MEL group according to the control group. This result was statistically significant (p<0.001). Compared to the MEL group, The results showed a statistically significant decrease in cell viability in the 3-MA+MEL group (p=0.0004).

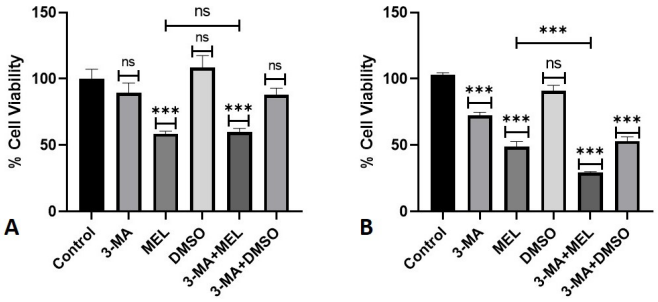

Figure 5. A. The MCF-7 cell lines cell viability after combined administration of 3-MA and MEL. **5B.** Cell viability of the MDA-MB-231 cell line after combined administration of 3-MA and MEL (ns: non-significant p>0.999, *p<0.05, **p<0.01, ***p<0.001

DISCUSSION

It is yet unknown how autophagy affects cancerous cell death or survival. According to studies, autophagy helps cancer cells survive by protecting them from hypoxia, malnutrition, and oxidative stress and by making established tumors resistant to chemotherapy (27-29). On the other hand, some studies have shown that apoptosis and autophagy can work together to induce cancer cells to die (30). However, it is currently uncertain what molecular switch or process determines whether a cell will survive through autophagy. Furthermore, the numerous processes of autophagy might be influenced by different cell types, the environment around the tumor, and the length of the period of therapy. The effectiveness of the associated drug in cancer cells, however, depends on studies into the connection between apoptosis and autophagy. In this study, the aim was to investigate the molecular pathways involved in the relationship between autophagy and apoptosis in BC cells following melatonin administration.

Suppression of autophagy by autophagy inhibitors demonstrates functional autophagy's importance in cancer cells (23,25). The effect of 5-FU and 3-MA's combination on apoptosis has been studied in a study using HT 29 and colon 26, two colon cancer cell lines. It has been showing that suppression of autophagy with 3-MA can increase the level of apoptosis caused by 5-FU. Therefore, it has been determined that autophagy occurs as a resistance mechanism to apoptosis, and when autophagy is inhibited, 5-FU increases its effectiveness on cancer cells (23). In a study with the MCF-7 cancer cell line, the effect on apoptosis and autophagy was investigated by applying oridonin treatment after autophagy inhibition with 3-MA. Consequently, it was found that autophagy contributed to the rise in apoptosis since autophagy inhibition reduced the level of apoptosis (25). Tran et al., in their study, investigated the anticarcinogenic effect of Tocomin, which contains tocopherols, on MCF-10A, MCF-7, and MDA-MB-231 cells. They stated that it potentiated apoptosis induced by Tocomin (31). In their research on BC, Chen et al. pre-treated both BC cells with wortmannin and 3-methyladenine, which are autophagy inhibitors, before anlotinib therapy to demonstrate the function of autophagy in anlotinib-induced death. Their research showed that anlotinib promoted autophagy in both cells, which at least partially, if not completely, caused apoptosis (32). Han et al. determined the anticancer specificity of myricetin in human BC SK-BR-3 cell lines. In cells treated with myricetin, 3-MA was used to assess the connection between autophagy and cell viability. The study's conclusions showed that inhibiting autophagy causes apoptosis and reduces cell viability. In addition, co-treatment with 3-MA and myricetin has been demonstrated to stimulate BC cell apoptosis (33). In the current study, we evaluated apoptosis, proapoptotic Bax, and anti-apoptotic Bcl-2 expressions after autophagy inhibition to show how the combined administration of 3-MA and melatonin affected BC cells. After all, it was shown that, when co-treatment with 3-MA, and melatonin treatment were co-administered in MCF-7 cells, there was no significant change in apoptosis compared to melatonin treatment alone. Nevertheless, it was shown that pro-apoptotic Bax expression was downregulated. The expression of Bcl-2 did not significantly differ from the other groups. On the other hand, it was determined that co-administration with 3-MA and melatonin, as opposed to melatonin therapy alone, notably enhanced the apoptotic cell numbers in MDA-MB-231 cells. Additionally, it was shown that Bax was upregulated. Bcl-2 expression among the other groups did not differ significantly.

CONCLUSION

In conclusion, according to these data, in MCF-7 cells, autophagy inhibition did not affect apoptosis triggered by melatonin, suggesting that the two processes proceed independently of each other. Also, it was determined that autophagy inhibition with 3-MA increased apoptosis in MDA-MB-231 cells; therefore, autophagy suppressed apoptosis in treatment with melatonin. As a result, it was suggested that melatonin treatment evaded MDA-MB-231 cells from cell death through autophagy.

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Conflict of interest: *The authors have no conflicts of interest to declare.*

Ethical approval*: The existence of an ethics committee is not necessary.*

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