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ANTI-CANCER EFFECTS OF RESVERATROL ON ISHIKAWA CELL CULTURE

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ABSTRACT

Cancer is a serious problem with significant impacts on health systems. Combination therapy is the cornerstone of cancer treatment today. Our research aims to obtain specific data about the agents that can be used in combination with chemotherapeutics in cancer treatment.

In our study MTT cytotoxicity assessment, transmission electron microscopic analysis of ultrastructural changes, detection of phosphatidylserine translocation and caspase 3/7 analysis were performed on Ishikawa cancer cell lines.

Cytotoxicity test results showed that resveratrol has ant proliferative activity on Ishikawa cells in a dose-dependent manner. Transmission electron microscopy findings indicated important ultra-structural changes on Ishikawa cells caused by resveratrol. Apoptotic profiles of Ishikawa cells were investigated via the annexin-V technique. Compared to the control Ishikawa cells in the population of resveratrol treated cells, the percentage of total apoptotic cells were detected to be 27.75%. Activation of caspases is an indicator of apoptosis pathways that are triggered into the cells. The total percentage of activated caspases was detected to be 5.35% when compared to that of control cells.

The highlight of our research is the transmission electron microscope examination. With this examination, morphological changes in mitochondria caused by the anticancer effect of resveratrol were determined.

***Key Words**-Resveratrol, Ishikawa cell culture, Anti-cancer effects*

INTRODUCTION

Cancer is a serious problem with significant impacts on health systems. Despite advances in diagnosis and treatment, it still affects millions of people around the world. Endometrial cancer is the most common cancer encountered in developed countries and the second most common cancer in developing countries. The five-year survival of patients with endometrial cancer is quite low. Therefore, it is very important to develop an appropriate chemotherapeutic regimen for endometrial cancer[1].

Combination therapy can be defined as the combined use of two or more therapeutic agents. It is the cornerstone of cancer treatment today. Combined therapy increases the effectiveness of cancer drugs through synergy or similar ways. In addition, it decreases drug

resistance, stops tumor growth and metastatic effect, and induces apoptosis. Five-year survival for metastatic cancers is still low, and developing new cancer drugs is costly and time-consuming. Therefore, the development of agents to be used in cancer treatment by *in vitro* methods has been one of the issues that have been emphasized in recent years[2].

In vitro cell culture is a method used to study the behavior of cells. A controlled environment is created in cell culture and this environment is protected from possible variations. Today, a wide variety of cell cultures have been developed. Cell cultures are used in areas such as basic cell biology, the effects of drugs and other chemicals, vaccine production. *In vitro* cellular activity studies have several advantages and disadvantages. The most important advantage of cell cultures is the consistency and reproducibility of the results obtained. In addition, cell cultures are working environments where physiological conditions can be precisely controlled. In these environments, the results are pretty clear. Although it is accepted that cell cultures do not fully represent processes in living tissue, it is currently considered the most precise and controlled method for research projects. Cell cultures are the first choice in studies in areas such as pathophysiology and therapeutic targets against various diseases[3,4].

Resveratrol (3,4,5-trihydroxy-stilbene) is a phytoalexin from the stilbene group synthesized by the grape plant. It is found especially in the shell and seeds of the plant. Its chemical formula is $C_{14}H_{12}O_3$ (Figure 1).

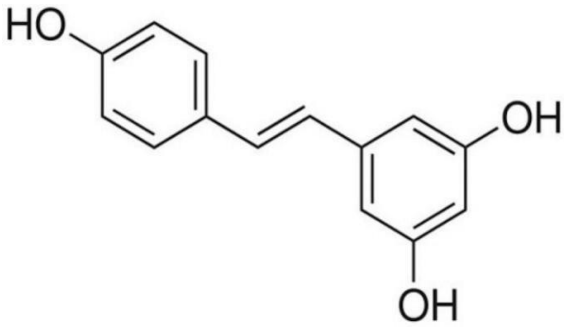

Fig. 1: Chemical structure of resveratrol

It has two phenol rings connected by an ethylene bridge. Today, it is used in the treatment of some diseases. It has a very high antioxidant potential. Studies have shown that resveratrol also has antifungal and antibacterial properties. The main interesting effect of resveratrol is its antitumor activity. Cancer researches constitute the majority of studies examining the effects of resveratrol. It is thought to be a potential therapeutic for various types of cancer. Its anticancer properties have been demonstrated by *in vitro* and *in vivo* studies. It has been noted that resveratrol obtained from grapes reduces the risk of breast cancer and reduces the frequency of esophageal cancer. In addition, resveratrol is also effective on cancer types such as colorectal, lung, breast, ovarian, and prostate cancer[5-8].

The mechanism of resveratrol's anticancer action has been studied in many studies. It has been reported that it prevents the formation of colorectal cancer by decreasing oncogenic KRAS (Kirsten rat sarcoma virus) expression. Resveratrol also prevented glycolysis by inhibiting hexokinase II expression and inhibited the growth of non-small cell lung cancer cells. In addition, it acts by inducing cyclooxygenase (COX)-2 expression and decreasing α5β1 integrins. It has also been shown to inhibit the growth of HeLa cells by inhibiting the expression of phospholipid scramblase 1 (PLSCR1). Resveratrol increases p53 expression through activation of caspase-3 and caspase-9. In this way, it shows apoptosis-inducing effects[9-12].

The transmembrane asymmetry of the distribution of phospholipids is an important marker of cell activity, including phosphatidylserine (PS). PS is a membrane phospholipid whose distribution is completely asymmetrical. In normal cells, it forms only the inner cytomembrane layer. Mechanisms that maintain PS distribution include translocation enzymes and cytoskeletal elements. PS exposure in cells plays a role in many biological processes. One of them is apoptotic processes. PS exposure is known to transform critical signals for apoptotic cell clearance. PS translocation is recognized as one of the early apoptotic markers. Exposure to PS in cells is induced by activation of scrambles, the protein responsible for the displacement of phospholipids between the lipid layers of the cell membrane, and inactivation of P4-ATPases[13,14].

One of the biochemical features of cells undergoing apoptosis is loss of plasma membrane asymmetry. As a result of this loss, a high amount of phosphatidylserine is released on the outer cell surface. These cells can be recognized by staining with Annexin V, which binds with a high affinity to phosphatidylserine. Caspases are a large family of cysteine proteases required for the initiation and

execution of apoptosis. Among these, caspase-3 plays a key role[15,16].

Our research aims to examine the anti-cancer effects of resveratrol, a phytoalexin from the stilbene group, in Ishikawa cell culture. For this purpose, the effects of the agent on cells were examined by various analysis methods and electron microscopy. Within the scope of the results obtained, it is expected to obtain specific data about the agents that can be used in combination with chemotherapeutics in cancer treatment.

MATERIALS AND METHODS

Human endometrial adenocarcinoma Ishikawa cells were purchased from the American Type Culture Collection (Manassas, USA). Resveratrol was obtained from Akcan Kimya (Turkey), fetal bovine serum (FBS), penicillin/streptomycin, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium bromide (MTT), and Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) were purchased from Sigma-Aldrich (St. Louis, USA). Caspase 3/7 and Annexin-V Kits were from (Merck, Millipore, USA).

Ishikawa cell culture

Ishikawa cells were cultured in DMEM/F-12 medium with 100 units/mL-100 µg/mL of penicillin-streptomycin and 10% fetal bovine serum in routine incubator conditions of 37°C and 5% CO_2.

Cytotoxicity assessment

Resveratrol was diluted in DMSO and a stock solution was prepared. Serial dilutions (1-100µM) of resveratrol were obtained in freshly prepared DMEM/F-12 medium. The prepared concentrations of resveratrol were applied to Ishikawa cells seeded at a density of 5 × 103/well in 96 well culture plates in triplicates and incubated for 24 and 48 hours at cell culture conditions 37°C and 5% CO_2. At the end of the incubation time, 20 µL/well of MTT (5 mg/mL) was added and further incubated for 4 hours. DMSO of 200 µL/well was added to the plates and read on an ELISA reader (HTX Synergy, BioTek, USA) at a wavelength of 570 nm (n=3). Viability percentages and IC_{50} values were detected from the absorbances[17].

Transmission electron microscopic analysis of ultrastructural changes

Ishikawa cells were treated with IC_{50} value of resveratrol for 24 hours. A group of untreated cells was used as control cells. All cell groups were fixed with glutaraldehyde (2.0%). Secondary fixation was realized in osmium tetroxide (2.0%). Fixed cells were dehydrated in graded ethanol and embedded in Epon 812 epoxy. Embedded samples were kept at 60°C incubators for 8 hours for polymerization. Thin sections of 100 nm were taken on an ultramicrotome (Leica EMUC6) and placed in copper grids. Sections were stained in uranyl acetate and lead citrate and imaged under a TEM (FEI Tecnai BioTWIN)[18].

Detection of phosphatidylserine translocation

Phosphatidylserine translocation was tested in order to detect the apoptosis-inducing capability of resveratrol on Ishikawa cells. Briefly, Ishikawa cells were cultured in six-well culture plates with a density of 5×10^5 cells/well and treated with IC_{50} value of resveratrol for 24 hours under cell culture conditions. After the incubation, Ishikawa cells were harvested by trypsinization. Harvested cell samples were washed in PBS. 100 µL of annexin-V reagent was added to each test tube and samples were incubated for 15 minutes at dark at room temperature (Muse®Annexin-V and Dead Cell Assay Kit). At the end of incubation, samples were read on Muse™ Cell Analyzer (Merck, Millipore, Hayward, California, USA) [19].

Caspase 3/7 Analysis

The intracellular cell death pathway was investigated by the caspase 3/7 technique. Briefly, Ishikawa cells were seeded in six-well plates at a density of 5×10^5 cells per well. The IC_{50} concentration was administered to Ishikawa cells for 24 hours at 37°C in 5% incubator conditions. After the incubation, Ishikawa cells were trypsinized and washed with PBS. Ishikawa cells were incubated with caspase 3/7 working solution and 7-ADD solution according to the instructions of the manufacturer of the caspase 3/7 kit. All cell samples were read on a cell analyzer (Muse TM Cell Analyzer Merck, Millipore, Hayward, California, USA)[20].

Statistics

One-way variance analysis for multiple comparisons of GraphPad Prism 6.0 for Windows was used for analyzing the statistical significances of the obtained data.

RESULTS

Cytotoxicity results

Resveratrol significantly reduced the viability of Ishikawa cells in an application time of 24 hours (Figure 2). The viability of Ishikawa cells is decreased by an increase in the applied resveratrol concentration. The highest growth suppression was detected at the highest resveratrol dose. Half maximal inhibitory concentration of resveratrol on Ishikawa cells for 24 hours was detected to be 45 µM. In the application period of 48 hours a significant decrease in the viability of Ishikawa cells was not detected (Figure 2). Cytotoxicity test results showed that resveratrol has anti-proliferative activity on Ishikawa cells in a dose-dependent manner. Additionally, the findings underline the short-term application efficacy of resveratrol in the manner of causing cytotoxicity on Ishikawa cells.

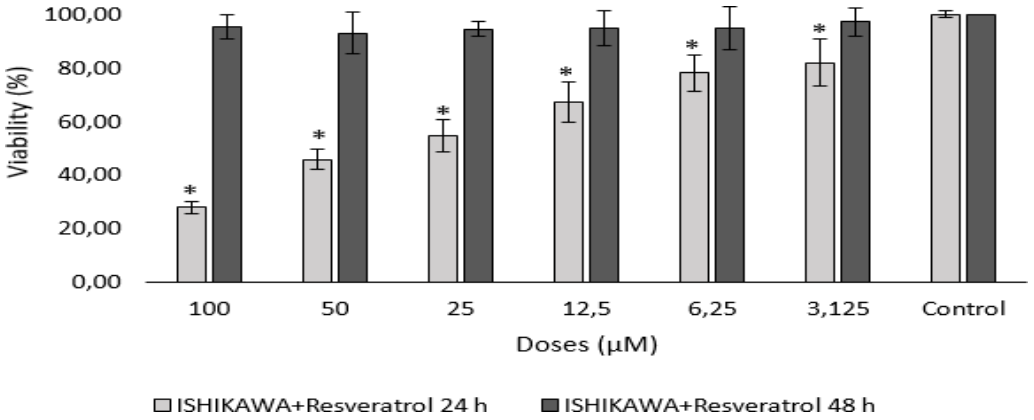

Fig. 2: Cytotoxicity of resveratrol on Ishikawa cells. IC_{50} concentration of resveratrol was detected to be 45 µM for 24 hours. (*: p<0.05)

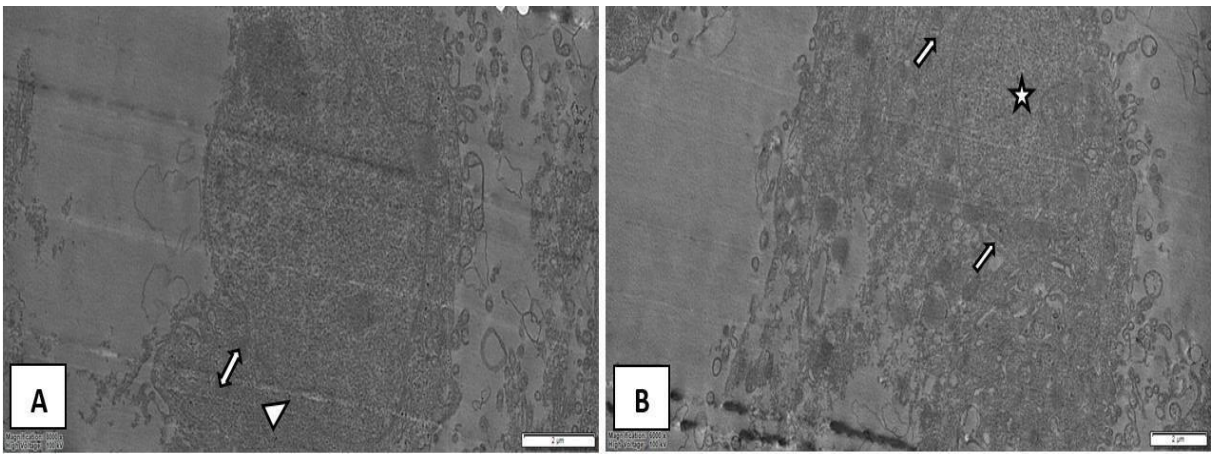

Fig. 3: Transmission electron micrographs of Ishikawa cells

Note: A: Resveratrol treated Ishikawa cells; arrowhead-compact nucleus, double-headed arrow- Undamaged mitochondria.

B: Control cells: Arrow-disintegrated mitochondria without cristae, asterisk-condensed horseshoe-shaped nucleus

Transmission electron microscopy findings indicated important ultra-structural changes on Ishikawa cells caused by resveratrol after 24 hours of application of IC_{50} concentration. When compared to untreated Ishikawa cells (Figure 3A) that were with the compact nucleus and membranous organelles, the ultrastructure of resveratrol treated Ishikawa cells was significantly changed. Detected ultra-structural changes were loss of cristae of mitochondria and chromatin condensation that caused the horseshoe nucleus (Figure 3B). These ultra-structural changes were clear structural signs of apoptosis.

Annexin-V assessment findings

Apoptotic profiles of Ishikawa cells were investigated *via* the annexin-V technique that evaluates the translocation of phosphatidylserine as an indicator of apoptotic cell death. Compared to the control Ishikawa cells in the population of resveratrol treated cells, the percentage of total apoptotic cells were detected to be 27.75% (Figure 4). This underlines the proapoptotic activity of resveratrol after short-term treatment of 24 hours.

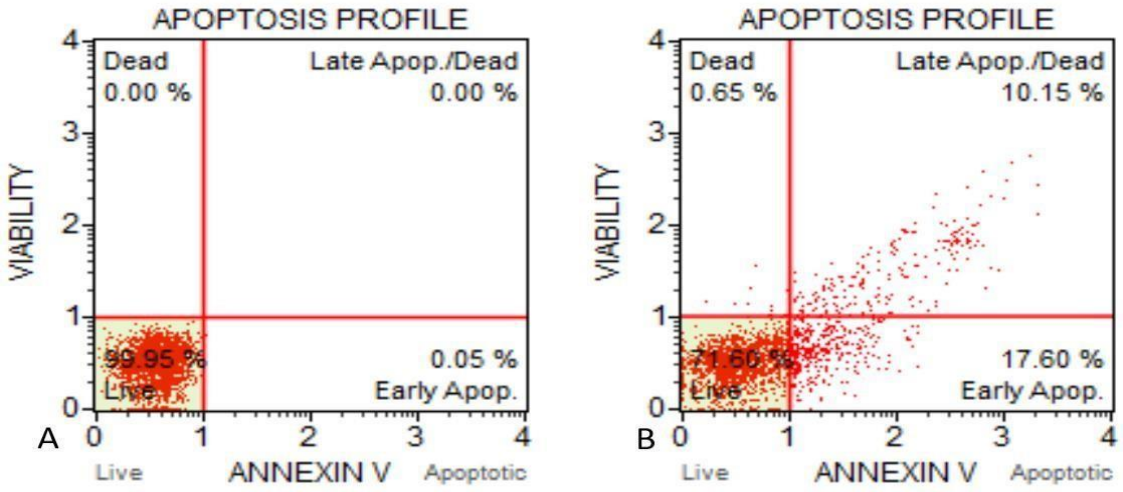

Fig. 4: Apoptosis-inducing percentages of resveratrol on Ishikawa cells

Note: A. Untreated Ishikawa cells; Live cells 99.95% and 0.05% early apoptotic cells were recorded in control cells. Late apoptotic and dead cells were not detected.B. Ishikawa cells treated with IC_{50} concentration of resveratrol for 24 hours. 71.60% of cells were alive. The percentages of the dead, early apoptotic, and late apoptotic cells were detected to be 0.65%, 17.60%, and 10.15% respectively

Caspase 3/7 analysis findings

Activation of caspases is an indicator of apoptosis pathways that are triggered into the cells. The total percentage of activated caspases was detected to be 5.35% when compared to that of control cells in which no activated caspases were present (Figure 5). This finding might show the different apoptotic pathways induced after resveratrol treatment in Ishikawa cells. From flow cytometric analysis is clearly showed that resveratrol triggered apoptosis in Ishikawa cells with a short-term application of two hours.

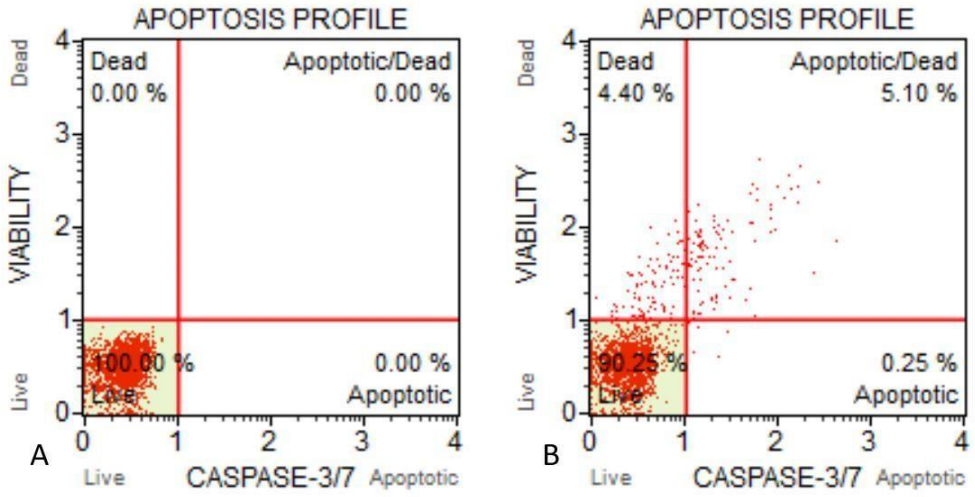

Fig. 5: Caspase activation percentages of resveratrol on Ishikawa cells

Note: A. Ishikawa control cells (100% live). B. Ishikawa cells treated with IC_{50} value of resveratrol for 24 hours (90.25% Live, 4.4% dead, 5.1% apoptotic/dead, and 0.25% apoptotic cells)

DISCUSSION

Based on all experimental findings of this study, the cytotoxic, anti-proliferative, and proapoptotic activities of resveratrol on human endometrial adenocarcinoma, Ishikawa cells were shown. Consecutively, resveratrol can be suggested for further investigations for anticancer potency in order to be used as an alternative therapeutic agent against human endometrial adenocarcinoma.

Resveratrol decreased the viability of cells in proportion to the increase in concentration after 24 hours of administration. When the duration was extended to 48 hours, no significant decrease in the viability of the cells was observed. These results show that the anti-proliferative effects of resveratrol are dose and time dependent. In studies on the subject, similar data were obtained on the dose-related anti-proliferative effects of resveratrol. In a study, the effect of resveratrol on tumor cell cultures and endothelial cell culture was examined. Cells were treated *in vitro* with various doses (0.1-100.0 µg/mL) of resveratrol. Cell number, apoptotic and mitotic index were measured 24 and 48 hours after treatment. While cell proliferation increased at low doses, apoptosis-induced mitotic activity decreased at doses of 10 µg/mL and above. In the same study, the possible role of formaldehyde in the mechanism of action of resveratrol was discussed. The dose-dependent effect detected in this study is in line with the results of our study. However, there was no finding supporting the time-dependent effect of resveratrol determined in our study. The results of another study also emphasized the dose-dependent effect. While the anti-proliferative effect was not observed at the dose of 0.1 µg/mL, a significant anti-proliferative effect was observed at the doses of 5 and 10 µg/mL. It has been observed that this effect increases over time. The findings of another study support the findings of our study. Accordingly, the effect of resveratrol at the end of 24 hours is greater than at the end of 48 hours[21-23].

Abnormal metabolic functions develop in cancer cells as a result of morphological changes in mitochondria. Ultra-structural mitochondrial changes in human tumors are not specific to neoplasms. Irregularity of mitochondria, cristae and cristolysis is observed when visualized by transmission electron microscopy, regardless of tumor histogenesis. The nucleus undergoes extensive deformation in response to environmental conditions. Examples of said deformation can be seen in the electron microscope. The cells in which this deformation takes place are large pleomorphic cells that histologically involve abundant cytoplasm. The nuclei are distant from the center, in the shape of a horseshoe or kidney, usually containing numerous small basophilic nucleoli. Electron microscopy is considered the gold standard for identifying apoptotic cells. However, its routine use is limited due to costs, equipment and specialist personnel needs. In electron microscopy, apoptosis markers are listed as nuclear irregularities, chromatin condensation, and diffuse mitochondrial changes[24-27].

In this study, transmission electron microscopy, which is accepted as the gold standard for the identification of apoptosis, was performed by the researchers. Significant ultra-structural changes were observed in Ishikawa cells after 24 hours of resveratrol exposure. The structure of their cells has changed considerably. Chromatin condensation in mitochondria causing loss of cristae and horseshoe-shaped nucleus has been identified. These ultra-structural changes are clear structural manifestations of apoptosis. These data are in agreement with those obtained in other studies. In the literature, it is not common to examine apoptosis in cancer cell lines with transmission electron microscopy. This may be due to the fact that the method is costly and requires equipment and specialized personnel. The fact that apoptosis was examined by the transmission electron microscope method increases the original value of our research.

The results of a similar study examining apoptosis in cancer cell lines with a transmission electron microscope are consistent with this study. Accordingly, ultra-structural changes such as swollen mitochondria, loss of cristae and chromatin condensation were observed in HeLa cells treated with Quercetin[28].

In our study, apoptotic profiles of Ishikawa cells were investigated by annexin-V technique. Phosphatidylserine translocation is investigated in this technique as an indicator of apoptotic cell death. Apoptotic cells were found to increase by approximately 30% in cells treated with resveratrol compared to control. This demonstrates the apoptotic activity of resveratrol after 24 hours of short-term treatment.

In a study, apoptosis was investigated by Annexin V method in U937, MOLT-4, MCF-7 and HepG2 cancer cell lines treated with resveratrol. Cells were divided into four quadrants by flow cytometry analysis of stained cells. These are living cells, early apoptosis, late apoptosis and necrotic cells. U937 and MOLT-4 cells were treated with resveratrol at doses of 50 and 100 µM for 24 hours. A significant increase in the late apoptotic cell population was observed in a concentration-dependent manner. Early apoptosis was induced in MCF 7 and HepG2 cells treated with the same treatment[29].

Another study on the subject investigated anti-tumor activities in ovarian carcinoma treated with resveratrol. Cell viability analyzes were performed with MTT. Significant dose-dependent cytotoxicity was observed in the ovarian cancer cell lines SKOV3 and A2780 cells. Similarly, colony formation was largely inhibited by resveratrol at a dose of 25 µM or 50 µM. In the study, the apoptotic response was also examined and annexin staining was used for this. Staining results showed that apoptosis was significantly increased in both SKOV3 and A2780 cells with c treatment[30].

There is no controversy over the anticancer effect of resveratrol. However, the mechanism of resveratrol-induced anticancer effect at the molecular level has not been fully elucidated. In a study on the subject, the mechanism of resveratrol's anti-cancer effect was investigated in ovarian cancer cells (OVCAR-3 and Caov-3). The research focused on the role of autophagy in resveratrol-induced apoptotic cell death. Cell viability was performed using MTT analysis. Apoptotic cell death was investigated by staining with annexin V and measuring caspase 3 activity. Autophagy has been analyzed by examining ATG5 expression forming autophagosomes. These analyzes demonstrated that resveratrol triggers autophagy and subsequent apoptotic cell death with an increase in caspase 3 activity. It was concluded that there is a common pathway between autophagy and apoptosis in resveratrol-induced cell death in OVCAR-3 cells[31].

In a study designed similar to our study, in which Ishikawa cells were treated with resveratrol, the relationship between autophagy and anticancer effects was investigated. Cell proliferation, cell cycle and apoptosis were investigated by MTT assays, flow cytometry and annexin V staining. Autophagy was investigated by measuring expression levels of light chain 3-II (LC3-II), a marker of autophagy. Chloroquine has been used to inhibit autophagy. Resveratrol treatment inhibited cell proliferation and increased apoptosis in Ishikawa cells in a dose-dependent manner. When chloroquine and resveratrol are used in combination, it has been observed that the effects are stronger compared to resveratrol treatment alone. The result is that autophagy will reduce the antitumor effect of resveratrol in Ishikawa cells. It has been determined that combination therapy with an autophagy inhibitor such as chloroquine may be an option in the treatment of endometrial cancer[32].

CONCLUSION

The methods used in our research and the results obtained are parallel to these studies. In this study, unlike some of the other studies, it was observed that the anti-cancer effect of resveratrol was time-dependent. The effect decreased as the treatment time of cells with resveratrol was prolonged. There are studies in the literature that support this data. The time-dependent change of the anti-cancer effect of resveratrol should be examined with the studies to be planned. The highlight of our research is the transmission electron microscope examination. With this examination, morphological changes in mitochondria caused by the anticancer effect of resveratrol were

determined. Although it is accepted as the gold standard for the identification of apoptotic cells, electron microscope examination is not frequently encountered in the literature. Transmission electron microscope examination makes our study stand out among other studies. It increases the original value of our work.

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