

Melatonin Sensitizes OVCAR-3 Cells to Cisplatin through Suppression of PI3K/Akt Pathway

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ABSTRACT

This study examined the effect of melatonin on oxidative stress, expression of pro-apoptotic protein, anti-apoptotic proteins, and the activity of the PI3K/Akt signaling pathway in the human ovarian cancer cell line (OVCAR3). OVCAR3 cells were treated with cisplatin, melatonin, cisplatin + melatonin, and siRNA Akt. Reactive oxygen species levels were assessed. The expression of the proteins was determined by Western blot. Melatonin administration significantly increased intracellular ROS generation, the cleavage of caspase 3 and decreased phosphorylation of Akt. Combination therapy of cisplatin and melatonin increases apoptosis in the OVCAR-3 cells by inhibiting of PI3K/Akt signaling pathway and exacerbating oxidative stress.

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Introduction

Ovarian cancer (OC) is the common cause of mortality from gynecologic tumors worldwide (1). This cancer is often diagnosed in advanced stages when cure rates are low, possibly due to late presentation, heterogeneous nature, and lack of effective screening (2). The main risk factors for OC are family history, early menarche, obesity, fertility treatments, diabetes, alcohol consumption, aging, and smoking (3-5). Available standard treatments for diagnosed patients are surgery as the initial choice and chemotherapy as the second treatment step. In the advanced phase of OC, most patients are likely to develop chemotherapy resistance, which is the leading cause of treatment failure (6).

Several molecular signaling pathways, including excessive accumulation of reactive oxygen species (ROS), inflammation, apoptosis cell death, and angiogenesis, are implicated in OC development. The PI3K/Akt pathway is one of the deregulated molecular pathways in ovarian cancer (7). Akt is a serine-threonine kinase that plays a crucial role in cellular survival, proliferation, cell growth, and drug resistance metabolism. Moreover, its biological activity depends on the phosphorylation of Thr-308

and Ser-473 residues (8). Accumulating evidence shows overexpression of Akt in OC; therefore, Akt ablation can be a successful approach in the treatment of advanced-stage OC (9). Among different Akt isoforms, Akt1, Akt2, and Akt3, Akt1 is a predominant isoform involved in ovarian cancer cell proliferation and protection against apoptosis (10).

Cisplatin is a DNA-damaging agent and the platinum analogs that have been successfully used to treat various types of cancer, including blood vessels, bone, muscle, soft tissue, and ovarian sarcoma (11). The main mechanisms underlying the anti-tumor activity of cisplatin are its ability to induce DNA damage and apoptosis cell death (12,13). However, cisplatin resistance often develops in clinical practice, which ultimately leads to treatment failure (14). The definitive mechanisms underlying chemoresistance remain to be elucidated. Previous studies have shown that activation of Akt contributes to resistance to cisplatin in several cancers, particularly OC, while inhibition of Akt sensitizes ovarian cancer cells to cisplatin, promotes the anti-tumor activity of cisplatin, and induces apoptosis (15-17). Thus, PI3K/Akt pathway inhibition can be considered as a potential therapeutic strategy either as monotherapy or in

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combination with chemotherapy drugs (18).

Melatonin (N-acetyl-5-methoxytryptamine) is an endogenous free-radical scavenger synthesized and secreted by the pineal gland and other organs such as the ovary, intestine, and testes (19). Although melatonin prevents apoptosis in healthy cells, in many cancer types, including OC, melatonin exerts antiproliferative, anti-migration, and pro-apoptotic properties (20-22). Moreover, melatonin induces apoptosis via activation of caspases and downregulation of the phosphorylation of Akt in various cancers (23). However, evidence shows that melatonin production is decreased in women with OC compared to healthy women (24).

Since there is an urgent need for the development of safe and efficient novel drugs for the management of OC, the purpose of this study was to investigate the knockdown of the Akt signaling pathway and the protective effects of melatonin and cisplatin therapies on apoptosis and cell survival in an *in vitro* model of OC cells.

Materials and Methods

Materials

Melatonin, cisplatin, and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were obtained from Sigma Chemical Co. (St. Louis, MO). Human ovarian cancer cells, OVCAR3 cell line (NCBI code: C209), were purchased from the National Cell Bank of Iran (NCBI), Pasteur Institute. Cell culture reagents were obtained from appropriate commercially available suppliers.

Cell culture

The OVCAR3 cells were cultured in RPMI 1640 (Sigma-Aldrich, USA) medium supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, USA) at 37°C in a 5% CO₂ humidified chamber.

Melatonin preparation

Melatonin was dissolved in dimethyl sulfoxide (DMSO, Merck, Germany) to prepare a 0.2 M (50 mg/ml) stock solution. This stock solution was diluted with RPMI-1640 to prepare different working solution concentrations immediately before use.

Cell viability assay

The effects of cisplatin and melatonin therapies on cell viability were evaluated by a colorimetric assay using (3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) (Sigma-Aldrich, USA). Briefly, OVCAR3 cells were seeded in a 96-well plate and cultured in an RPMI-1640 medium containing 10% FBS, then the cells were exposed to different concentrations of cisplatin or melatonin for 24, 48, and 72 h in the same medium supplemented with 2% FBS. Subsequently, 20 µl of MTT solution (5 mg/ml) was added to each well and incubated for 4 h. All treatments were carried out in triplicate. The absorbance value was measured at a wavelength of 630 nm using a microplate reader (Biotek Instruments, USA) and the results were presented as a percentage of the viability of control cells. The drug concentration causing 50% cell growth inhibition (IC₅₀) values for each treatment were calculated from the dose-response curve of percent growth inhibition against test concentrations. To evaluate the synergizing function of melatonin and cisplatin, cells were co-treated with IC₅₀ value of 48 h of melatonin and various concentrations of cisplatin for 24, 48, and 72 h, and cell viability was determined.

siRNA transfection

For protein knockdown of Akt in OVCAR3 cells, small interfering RNA (Akt siRNA (h): sc-29195 siRNA) was used. Akt-3m-siRNA was used as the control siRNA for transfection: Briefly, in a six-well tissue culture plate, 2 x 10⁵ cells per well in 2 ml antibiotic-free standard growth medium were seeded, supplemented with FBS, and incubated at 37° C in a CO₂ incubator until the cells reached to 60–80% confluence. A 0.8 ml mixture (1:1) of Solution A (siRNA duplex in siRNA Transfection Medium (sc-36868) and Solution B (siRNA Transfection Reagent (sc-29528) in siRNA Transfection Medium (sc-36868) was added onto the washed cells and incubated for 5-7 h at 37° C in a CO₂ incubator. Fluorescein Conjugated Control siRNA was incubated for 5-7 h at 37° C in a CO₂ incubator and assessed by fluorescent microscopy. Then, the transfection mixture was removed and replaced with 1x normal growth medium and incubated for an additional 18–24 h. The expression level of Akt protein was confirmed

by Western blot analysis. Controls were transfected with non-specific siRNA under similar conditions.

Measurement of reactive oxygen species (ROS)

The intracellular ROS level was determined using a DCFH-DA (2',7'-dichlorodihydro fluorescein diacetate) assay kit. For this purpose, cells were seeded into a 96-well plate, washed with PBS twice, and then incubated with 100 μ M DCFH-DA in a fresh medium at 37°C for 30 min in the dark. Intracellular ROS production was detected by measuring the fluorescence intensity at 485 nm excitation and 530 nm emission wavelengths in a multiwell plate reader spectrofluorometer and photographed by fluorescence microscope (Olympus IX70, Tokyo, Japan). The measured fluorescence values were expressed as a percentage of the control.

Protein extraction and Western blot analysis

Cells were lysed in 100 μ l RIPA lysis buffer (25 mM HEPES, 1% Triton X-100, 2 mM EDTA, 0.1 M NaCl, 25 mM NaF, 1 mM Sodium Orthovanadate) containing a protease inhibitor cocktail for 30 min on ice. Then, cell lysates were centrifuged at 12000 g for 20 min at 4 °C, and the supernatant was collected. Protein concentration in the supernatant was determined using the Bradford protein assay. The equal amount of protein (~100 μ g) loaded at 12% SDS- acrylamide gel followed by transferring onto a polyvinylidene difluoride (PVDF) membrane (Roche, UK). Non-specific binding reactions in the membranes were blocked by bovine serum albumin (BSA) 3% in Tris-buffered saline (pH 7.5) at room temperature for 1 h. Subsequently, the membranes were incubated overnight with diluted (1:500 concentrations) primary antibodies (Santa Cruz Biotechnology, U.S.A) against phospho-Akt (sc-52940), Akt (sc-5298), X-linked inhibitor of apoptosis protein (sc-55550), Survivin (sc-17779), caspase 3 (sc-136219), HIF1 (sc-13515), VEGF (sc-7269), GSK3 β (sc-81462), p-GSK3 β (sc-373800), p53 (sc-126), and β -actin (sc-47778) overnight at 4 °C. Next, the membrane was incubated with appropriate horseradish peroxidase-conjugated (HRP) secondary antibody after washing three times with PBS at room temperature for 2 h. The antigen-antibody complexes

were detected using enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL). The density of each band was acquired by Image J software (version 1.62, National Institutes of Health, Bethesda, MD, USA) and normalized to β -actin (8).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 8 Scientific software (GraphPad Software, Inc., La Jolla, CA). The results were expressed as the mean \pm standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by a Tukey post-hoc test was performed for multiple comparisons. A P value of <0.05 was considered to indicate statistical significance.

Results and discussion

MTT assay

The IC₅₀ values of cisplatin or melatonin in single treatment after 24, 48, and 72 h, are shown in Figures 1A and B, respectively. The IC₅₀ values of cisplatin alone were 12.6 μ M at 24 h, 10.4 μ M at 48 h, and 8.3 μ M at 72 h. For melatonin alone, the IC₅₀ values were 4.8 mM at 24 h, 2.9 mM at 48 h, and 2.8 mM at 72 h. To evaluate the cytotoxicity of cisplatin in the presence of melatonin on OVCAR3 cells, the MTT assay in the presence of constant values of melatonin (4 mM) with different concentrations of cisplatin was conducted on OVCAR3 cells (Figure 1C). The results document that co-administration of melatonin with cisplatin significantly decreased IC₅₀ values of cisplatin to 4.1 μ M, 3.7 μ M, and 2.2 μ M at 24 h, 48 h, and 72 h, respectively, compared to the cisplatin alone ($p < 0.05$).

Intracellular ROS levels

As expected, cisplatin significantly elevated cellular ROS production compared with the untreated control cells ($p < 0.001$). Furthermore, combined treatment with cisplatin and melatonin markedly increased ROS levels compared with cisplatin alone ($p < 0.001$). Moreover, the production of ROS was significantly reduced in the melatonin-treated Akt siRNA-treated cells compared to those treated with cisplatin alone ($p < 0.001$; Figure 2).

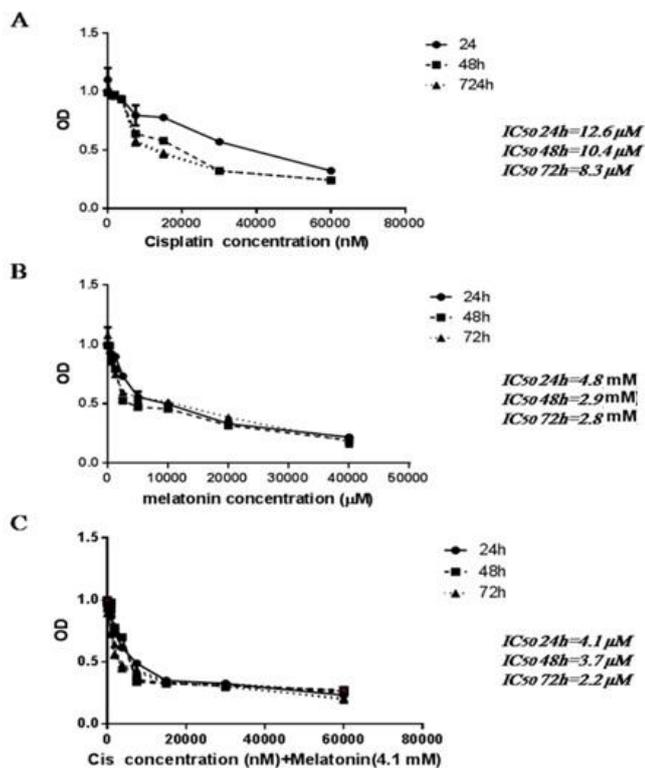


Figure 1. Effect of cisplatin and melatonin on cell viability of ovarian cancer cells alone or in combination. OVCAR3 cells were treated with different concentrations of A) cisplatin, B) melatonin, and C) cisplatin+ melatonin in combination for 24, 48 and 72 h, and analyzed by MTT assay. Data were obtained from triplicate tests and expressed as mean± SD.

Apoptosis markers

To determine the mechanisms underlying apoptotic induction, the expression of apoptosis-related proteins in the treated cells was investigated. The immunoblotting assay revealed that treatment with cisplatin did not influence the expression of XIAP.

Unexpectedly, the addition of melatonin to the OVCAR3 cells significantly elevated protein levels of XIAP compared to the Cis group ($p<0.05$, Figure 3B). However, the combination therapy of cisplatin and melatonin or Akt siRNA had no significant effects on the protein expression of XIAP.

The protein content of pro-caspase 3 was significantly decreased in a single treatment with cisplatin ($p<0.001$) or melatonin ($p<0.05$) compared to the untreated control cells. However, the combination therapy of cisplatin+melatonin or Akt siRNA did not further reduce pro-caspase 3 expressions compared to the cisplatin group. Furthermore, cisplatin alone ($p<0.001$) as well as combination therapy ($p<0.001$) markedly increased cleaved caspase 3 levels compared to the untreated control cells. Cleaved caspase 3 level was significantly lower in the melatonin alone, Akt siRNA, and control siRNA groups compared to the cisplatin-treated cells ($p<0.001$, Figure 3D).

Akt activity

As shown in Figure 4, none of the treatments had significant effects on the expression of PI3K (Figure 4B) and Survivin (Figure 4E). A significant drop in p-Akt levels was observed in the cisplatin-treated group versus untreated control cells ($p<0.05$, Figure 4C). Furthermore, melatonin alone ($p<0.05$), the combination therapy ($p<0.05$), or Akt siRNA ($p<0.01$) treatments significantly reduced p-Akt levels compared to the cisplatin-treated cells. As expected, Akt siRNA treatment significantly ($p<0.001$) reduced Akt protein expression.

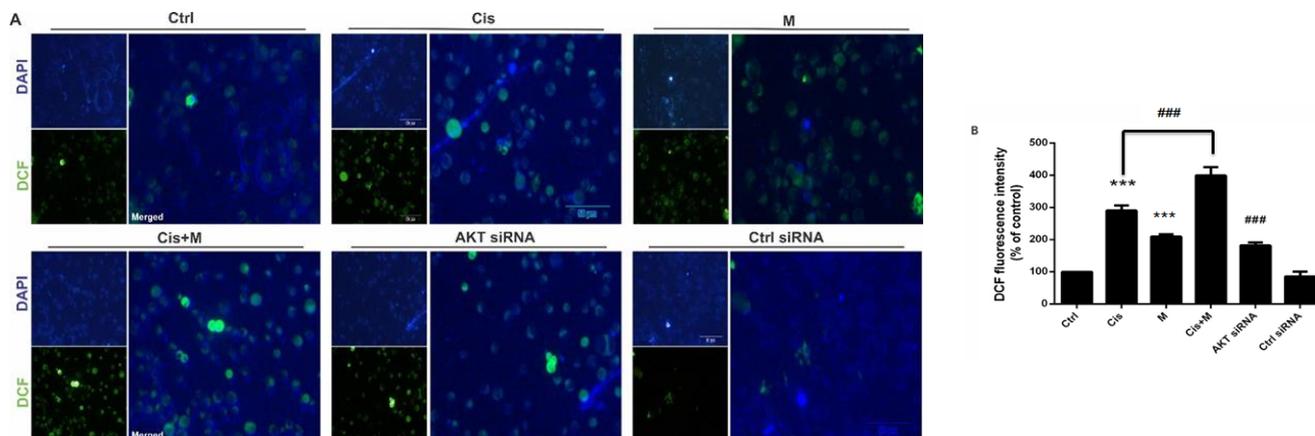


Figure 2. Effect of different interventions on reactive oxygen species (ROS) production in OVCAR3 cells. Data are presented as means ± SEM: *** $p<0.001$ vs. Ctrl group; ### $p<0.001$ vs. Cis group. (Ctrl: control; Cis: cisplatin, M: melatonin, Cis+M: Cisplatin + melatonin).

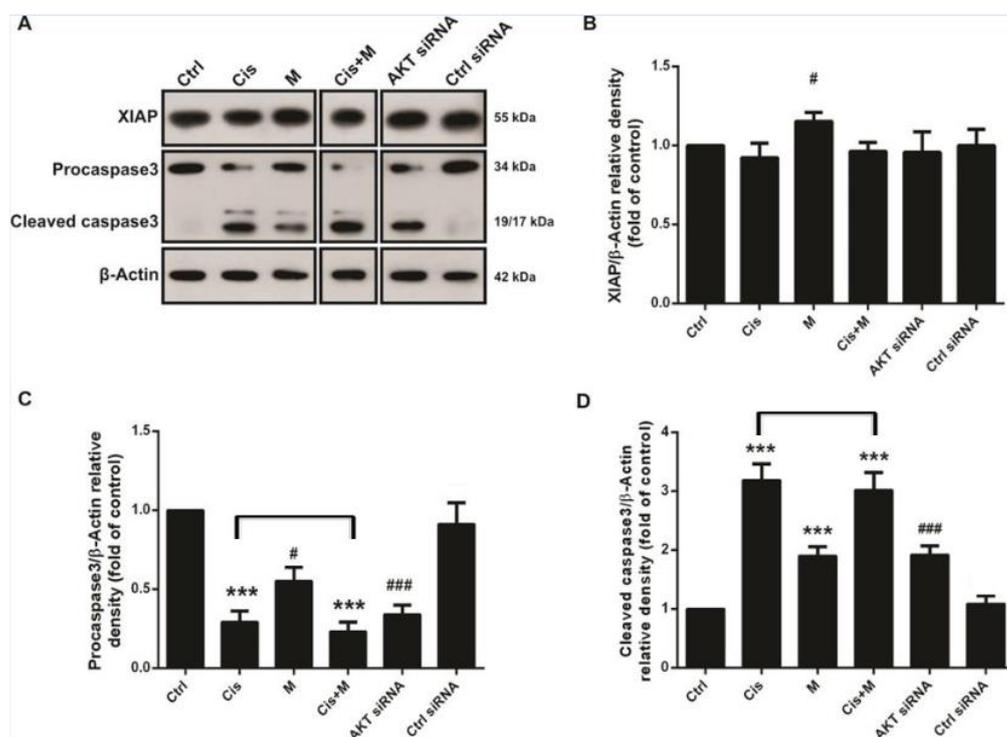


Figure 3. The effect of different treatments on the protein expressions of XIAP, caspase 3, and cleavage of caspase 3 in the OVCAR3 cells. A) Immunoblotting images of XIAP, pro-caspase 3, cleaved caspase 3, and β-actin proteins detected by Western blot. Quantification of band densities of B) XIAP, C) pro-caspase 3, and D) cleaved caspase 3 in different experimental groups. Data are presented as means ± SEM (triplicate): *** $p < 0.001$ vs. Ctrl group; # $p < 0.05$, ### $p < 0.001$ vs. Cis group. (Ctrl: control; Cis: cisplatin, M: melatonin, Cis+M: Cisplatin + melatonin).

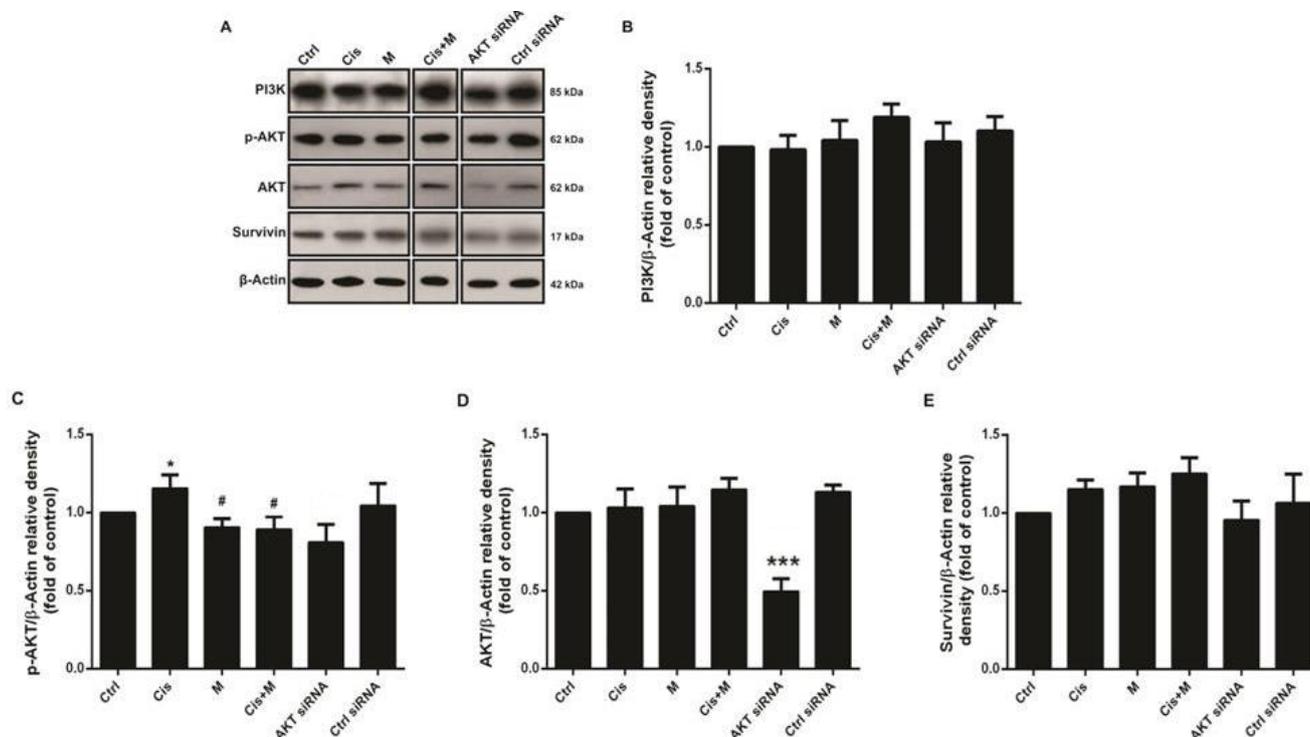


Figure 4. The effect of different treatments on the protein expressions of PI3K, Akt, and Survivin, as well as p-Akt levels in the OVCAR3 cells. A) Immunoblotting images of PI3K, p-AKT, Akt, Survivin, and β-actin proteins detected by Western blot. Quantification of band densities of B) PI3K, C) p-Akt, D) Akt, and E) Survivin in different groups. Data are presented as means ± SEM (n=3): * $p < 0.05$ vs. Ctrl group. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ vs. Cis group. (Ctrl: control; Cis: cisplatin, M: melatonin, Cis+M: Cisplatin + melatonin)

Angiogenesis and cancer proliferation

Expression of HIF1, VEGF, p-GSK3β, GSK3β and p53 as the main factors in angiogenesis and cancer cell proliferation were evaluated using immunoblotting (Figure 5). The results demonstrated a significant rise in HIF1 expression after cisplatin treatment compared to the controls (p<0.05). In contrast, melatonin alone and Akt siRNA treatments significantly attenuated the expression of HIF1 (p<0.001). HIF expression showed a marked drop in the cisplatin+melatonin treatment group compared to the cisplatin alone treatment (p<0.001).

All treatments except for control siRNA notably exhibited reduced VEGF protein expression as compared to the untreated control group. Cisplatin treatment had the least impact (p<0.05) on VEGF expression, while combination treatment of cisplatin+melatonin had the highest impact (p<0.001) with approximately 75% decline. Moreover, combination treatment caused a marked decrease in VEGF expression compared to treatment of cisplatin alone. Also, cisplatin, melatonin, and Akt siRNA

treatment caused a significant decrease (p<0.001). Interestingly, combination therapy promoted a significant drop in p-GSK3β compared to cisplatin alone (p<0.05).

Cisplatin alone, Akt siRNA, and control siRNA treatment demonstrated a minor decrease, while melatonin alone and cisplatin+melatonin combination therapy showed an insignificant increase in GSK3β expression compared to the control group (p>0.05). In this regard, there were no differences in the single treatment of cisplatin compared to combination therapy (p=0.19)

Among all treatment groups, combination therapy with cisplatin+melatonin significantly increased the expression of p53 by about 80% (p<0.001) compared with the cisplatin alone treatment. Also, the melatonin-treated group caused a substantial increase in p53 levels by 30% (p<0.01) as compared to the untreated control. However, treatments of cisplatin alone (p=0.60) and Akt siRNA (p=0.12) did not significantly alter the expression of p53.

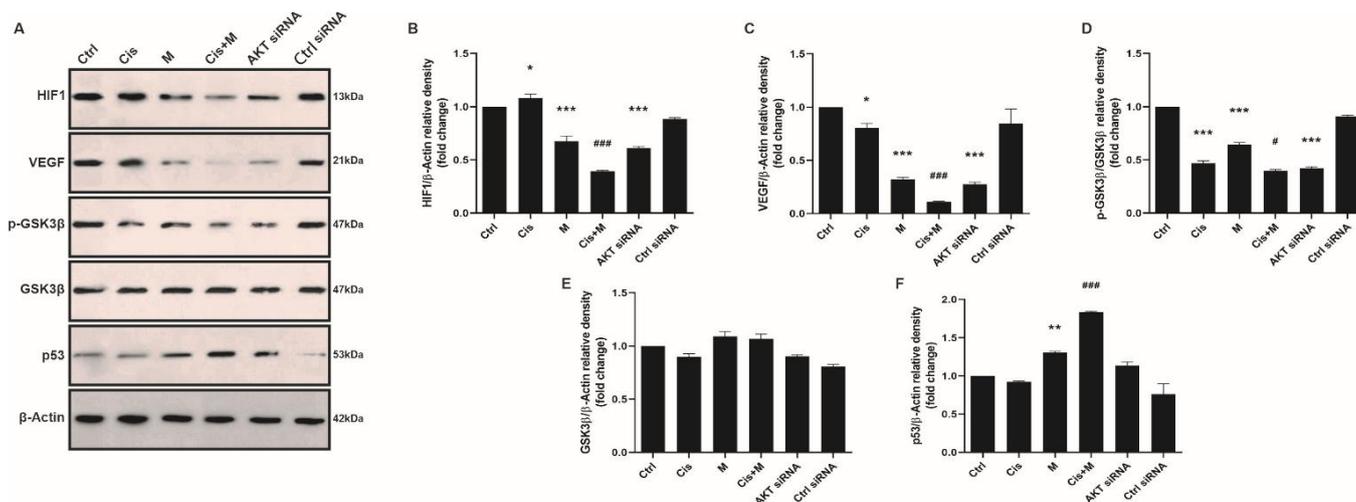


Figure 5. Evaluating the effect of different treatments on the protein expressions of HIF1, VEGF, p-GSK3β, GSK3β and p53 in the OVCAR3 cells. A) Immunoblotting images of the proteins detected by western blot after various treatments on OVCAR3 cell line. Quantification of band densities of HIF1 (B), VEGF (C), p-GSK3β (D), GSK3β (E) and p53 (F) in different experimental groups. Data are presented as means ± SEM (triplicate): ***p<0.001 vs. Ctrl group; # p<0.05, ### p<0.001 vs. Cis group. (Ctrl: control; Cis: cisplatin, M: melatonin, Cis+M: cisplatin + melatonin).

Apoptosis or programmed cell death is a critical physiological phenomenon playing an essential role in ovarian tissue homeostasis, the developmental processes of organs, and the deletion of potentially dangerous or defective cells (25). However, any flaw in the control of apoptosis could give rise to pathologies such as various types of ovarian cancer

(26). Therefore, strategies targeting apoptosis-related molecules have a great value in the treatment of OC. Moreover, the failure of cells to undergo apoptosis may lead to chemotherapy resistance, which is the main cause of treatment failure in OC (27,28). Inhibition of PI3K/Akt signaling and induction of oxidative stress have been known as two therapeutic

strategies to sensitize the resistant tumoral cells to apoptosis (29).

In the current study, we demonstrated that melatonin sensitized the OVCAR3 cells to cisplatin, a well-known chemotherapy agent, via induction of oxidative stress and PI3K/Akt signaling inhibition. Overexpression and activation of PI3K/Akt signaling, a critical cell process regulator of cell proliferation, have been demonstrated in OC (30), developing cisplatin resistance (31). Hence, inhibition of PI3K/Akt signaling activation could be a therapeutic target for epithelial OC (29). Many previous studies have also demonstrated that the PI3K/Akt signaling regulates ROS production and aberrant PI3K/Akt signaling contributes to the overproduction of ROS (32,33). Moreover, excessive ROS levels can potentiate PI3K/Akt signaling activation mainly through inhibition of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (12).

In the current study, we used Akt siRNA to inhibit Akt protein expression. We observed that Akt knockdown was associated with a reduction in Akt phosphorylation, increased ROS generation, and apoptosis induction in the Akt siRNA group compared to the control siRNA. In line with our results, previous reports have also shown that PI3K/Akt pathway inhibition is accompanied by excessive ROS production (33,34). It seems that Akt counteracts ROS generation in the oxidative stress pathway and apoptosis in cancer cells. Several studies have reported the beneficial effects of melatonin on the apoptosis of cancer cells by focusing on oxidative stress (35). ROS plays an important role in activating the apoptosis pathway, so that elevation in ROS generation leads to an alteration in mitochondrial membrane potential (MMP) and defects in the respiratory chain, consequently initiating the apoptotic process (36,37). Our data demonstrated that co-treatment of OVCAR3 cells with melatonin sensitized the cells to cisplatin treatment via increased ROS production in this cell line. Consistent with these findings, several studies have established that the anti-tumor activity of melatonin on cancer cells is ROS-dependent activation of apoptotic cell death (38,39).

We also report that cisplatin, a clinically proven drug to fight various cancer types, caused an increase in ROS production, and this elevation in combination with therapy with melatonin was much more

prominent than cisplatin alone. Recently, many researchers reported that cisplatin-induced cytotoxicity is strongly associated with increased ROS generation (40). Moreover, we observed a significant reduction in the p-Akt levels in the melatonin, combination therapy, and Akt siRNA-treated cells versus cisplatin-treated cells. It seems that the simultaneous reduction of Akt phosphorylation and ROS production is the basis for the sensitization of OVCAR3 cells to cisplatin. Moreover, activated Akt has the ability to phosphorylate one of its targets, GSK3 β (41). Multiple consequences may occur in the presence of the activated (non-phosphorylated) or inactivated (phosphorylated) form of GSK3 β . GSK3 β positively regulates NF- κ B activity; thus, the aberrated Akt pathway may lead to a high level of GSK3 β , increased NF- κ B function, and finally, OC proliferation (42).

As expected, we observed a decreased level of p-GSK3 β in the Akt siRNA treatment and combination therapy cells. Notably, our results demonstrated that neither cisplatin nor melatonin treatments could alter the expression level of GSK3 β . In concordance with other reports, we demonstrated the OVCAR3 cells sensitized to cisplatin and melatonin by regulating the activity of GSK3 β (43). Akt signaling pathway may be activated by integrin ligation, which triggers integrin-linked kinase activation and subsequently leads to elevation of Hypoxia-Induced Factor (HIF)-1 and Vascular Endothelial Growth Factor (VEGF) expression (44). HIF1 is a crucial factor in angiogenesis expressed in response to low oxygen concentration and contributes to the survival and proliferation of cancer cells (45). Following the phosphorylation of Akt, HIF1 enters the nucleus, acts as a transcription factor, and triggers the transcription of the VEGF gene. This signaling pathway leads to angiogenesis and tumor proliferation (46). Conversely, p53 negatively modulates this pathway through expression induction of MDM2, which subsequently triggers the degradation of HIF1 by the proteasome pathway (47).

Ai et al. hypothesized that downregulating HIF-1 would be an efficient strategy for overcoming the cisplatin resistance of human OC cells (48). They demonstrated that cisplatin can down-regulate HIF1 expression, thus inhibiting the proliferation of cancer cells by induction of ROS production (48).

Accumulating studies revealed the potential of cisplatin to abolish the expression of HIF1 and VEGF in OC (49,50). Furthermore, increasing evidence indicated that melatonin potentiates to affect angiogenesis by targeting HIF-1 under hypoxic conditions (51). These findings were in line with our results, in which we showed the combination therapy significantly down-regulated both HIF1 and VEGF expression levels compared to the untreated control group and even cisplatin alone and melatonin alone treatment. The information on HIF-1 α is very interesting in that melatonin also suppresses HIF-1 α , which converts cancer cells from aerobic glycolysis into mitochondrial oxidative phosphorylation, which help is overcoming chemoresistance. The change in glycolysis is mentioned in the report by Ai and colleagues (48) and more extensively discussed in a report which published recently: Melatonin and Pathological Cell Interactions: Mitochondrial Glucose Processing in Cancer Cells (52).

Also aligned with our results, p53 expression is associated with cisplatin sensitivity in OC (16). We showed that combination treatment of cisplatin and melatonin had a significant positive association with p53 expression level compared to untreated control and cisplatin-treated cells. In concordance with our results, several studies revealed that p53 facilitates apoptosis during cisplatin treatment (53-55).

Like the current findings, other studies have shown that melatonin reduces phosphorylation of Akt in different cancer cells such as hepatoma cells (56), lung cancer (57), and breast cancer (58). Activation of caspase 3, an irreversible step of apoptosis, plays a vital role in the apoptosis process, and any defect in its function or downregulation of its expression may lead to the development of carcinogenesis (26). Moreover, studies have shown that the expression of caspase-3 is decreased or undetectable in OC cells, which is one of the causes of resistance to chemotherapy agents (59). However, its expression is increased in response to anti-cancer therapy leading to apoptosis in cancer cells (60). As shown by Henkels et al. (59), we also found that cisplatin increased the cleavage of caspase 3 in OVCAR3 cells and combined therapy with melatonin-mediated caspase 3 activation, indicating that melatonin sensitized OVCAR3 cell to the cytotoxic effect of cisplatin and promoted cell

death. Based on the results of MTT (Figure 1C), 24 h treatment with cisplatin and melatonin lowered the IC₅₀ of cisplatin by more than 50% compared to cisplatin alone (4.1 μ M vs. 12.6 μ M). XIAP, a direct inhibitor of caspase 3 and 7, and Survivin proteins are inhibitors of apoptosis, promoting cell cycle progression (61). Evidence shows that cancer cells have elevated expression levels of apoptosis inhibitory proteins, which promote cell survival and tumor growth and consequently chemoresistance (62). Therefore, therapies targeting inhibitors of apoptosis protein in cancer may improve the sensitivity of cancer cells to chemotherapies and hence potentiate cell death.

In the current study, we observed an insignificant reduction in XIAP protein expression in the cisplatin and combined therapy with melatonin. Surprisingly, melatonin-treated cells displayed elevated expression of XIAP compared to the treatment with cisplatin alone. Greater expression of XIAP along with elevated caspase 3 activation is an inconsistent observation in our results which should be further investigated for clarification. Asselin et al. noted that cisplatin decreases XIAP protein levels, which induces the cleavage of pro-caspase 9, pro-caspase 3, and Akt and, therefore, induces apoptosis of ovarian cancer cells (63). Conversely, overexpression of XIAP prevents cisplatin-induced Akt cleavage and increases p-Akt content, which in turn protects OC cells by inhibiting apoptosis (62).

Survivin is another apoptosis inhibitory protein that controls cell proliferation and suppresses apoptosis-induced cell death (64). Accumulating studies have shown that cancer cells have a higher expression level of Survivin (65,66). Overexpression of Survivin in normal and tumor cells prevents cell death that is promoted by apoptotic stimuli, such as caspases and anti-cancer drugs. Importantly, down-regulation of Survivin is accompanied by overexpression of caspase 3 which could have therapeutic benefits in OC cells (67). The blockade of Survivin expression has also been reported to improve the anti-tumor activity of chemotherapeutic drugs in OC (68). In this study, unexpectedly, melatonin did not influence Survivin protein expression. Moreover, only a reduction in Survivin expression was observed in the Akt siRNA group, indicating the critical role of Akt in cell proliferation. In contrast to our results, Fan et al. have

demonstrated that melatonin reverses apoptosis resistance in human hepatocellular carcinoma by inhibiting Survivin and XIAP (66).

Many earlier studies have confirmed pro-apoptotic and oncostatic properties of melatonin (67). Pro-apoptotic effects of melatonin in cancer cells are mainly through modulation of the release of pro-apoptotic (BAX, caspase 3, and cytochrome c) and anti-apoptotic (Bcl-2) proteins, as well as oxidative stress (69,70). Yun et al. also found that melatonin attenuated phosphorylation of Akt and induced caspase 3 activity and increased apoptosis in lung cancer cells (57). These differences highlight the need for more detailed investigations of these processes. In conclusion, the present study showed that melatonin increased cisplatin efficiency in inducing apoptosis in OVCAR3 cells. This effect may be partially mediated by elevated ROS production and PI3K/Akt signaling down-regulation.

Ethics approval and consent to participate

Not applicable.

Availability of data and materials

The data used to support the findings of this study are included in the article. The data and materials in the current study are available from the corresponding author on reasonable request.

Interest conflict

The authors declare that they have no conflict of interest.

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