Research Article

Combination of melatonin with paclitaxel reduces the TLR4-mediated inflammatory pathway, PD-L1 levels, and survival of ovarian carcinoma cells

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ABSTRACT

Ovarian cancer (OC) has a high mortality rate. Although most patients respond to the conventional chemotherapy [e.g., paclitaxel (PTX)], some also develop drug resistance to make the treatment less effective. Since melatonin exhibits antioxidant, antitumor, and immunomodulatory functions in a variety of solid tumors, in this study the effects of a combination of PTX and melatonin on SKOV-3 human ovarian carcinoma cells were investigated and the focus was given to the Toll-like receptor (TLR)-mediated inflammatory pathway and cell signaling-related molecules. Flow cytometry showed that this combination significantly boosted the apoptosis/necrosis responses of the cancer cells. Cell migration was attenuated by melatonin alone, and the combination led to a reduced number of migrating and invasive cells. Melatonin alone and its combination also reduced the levels of TLR4, MyD88, TRIF, and PD-L1, but not TLR2. In addition, the combination significantly lowered the levels of NF-kB p65, PI3K, p-AKT, p38, ERK 1/2, JNK, CREB, p70s6K, and STAT5. The results suggested that this combination was effective in reducing the viability and the invasive capacity of SKOV-3 cells while increasing their apoptosis and necrosis rates. The potential mechanism of this combination is to attenuate the downstream molecules of the TLR4-mediated inflammatory pathway and cell signaling-related proteins in the cancer cells. Thus, melatonin improved the chemosensitivity of the cancer cells to PTX, serving as an effective adjuvant therapy against OC.

Key words: Ovarian cancer, melatonin, paclitaxel, Toll-like receptor, PD-L1, cell signaling, SKOV-3 cells.

1. INTRODUCTION

Ovarian cancer (OC) is the most common malignancy among women with relatively high mortality rate (1). Due to the lack of specific symptoms and initial screening methods, many women are usually diagnosed at an advanced stage, and most of them become incurable (2, 3). The current standard treatment for OC includes chemotherapy, radiation therapy, surgical removal, and immunotherapy, depending on the tumor stages (4, 5). In early stage, surgery and chemotherapy with platinum derivatives are the choice whereas in the advanced stage platinum derivatives plus taxols is the common therapy (6).

Paclitaxel (PTX) is designed for the treatment of several cancers, including OC (7, 8). Although many patients are responsive to the standard therapies, tumor relapse and recurrence are rather frequence (9), and mainly associated with chemoresistance (10). Chemoresistance may involve multiple mechanisms and one of them is associated with changes in specific receptors or even cell surface transporters, resulting in reduced drug influx, which decreases drug sensitivity (11). Also, it is known that PTX is a ligand of the Toll-like receptor 4 (TLR4) (12, 13); therefore, chemoresistance to PTX may occur through the mechanisms linked to TLR4 in OC (14).

TLRs function by promoting local inflammation in response to pathogens and/or related molecules, being predominantly expressed by immune cells and tumor cells (15, 16). Curtin *et al.* (17) reported that the activation of the TLR pathway promoted tumor growth. Upon activation, TLRs trigger inflammation via the molecular adapters called MyD88 or TRIF (18), which leads to the activation of nuclear factor-kB (NF-kB). NF-kB regulates the transcription of several genes related to immune response, cell adhesion, proliferation, angiogenesis, and apoptosis (19).

Immune checkpoint molecules are expressed in different types of immune and tumor cells. Among them, PD-L1 acts to inhibit T cell-mediated immune response (20). Therapies using monoclonal antibodies against PD-1/PD-L1 have been recently documented (21), including for treatment of the developmental stages of OC (22, 23). Since the mortality rate associated with OC is alarmingly high (24), it is essential to understand how these checkpoint molecules work in the context of the novel therapeutics.

Many signaling pathways are associated with tumor progression. These include the PI3K-AKT signaling pathway (25) and its activation is often found in the advanced stages of breast and ovarian cancers (26). The MAPK pathway includes protein kinases such as ERK1/2, p38, and c-Jun (JNK1/2/3) responsible for controlling cell proliferation, cell survival, and cell death (27, 28). JNKs are active through cellular stress, respond to extracellular stimuli, and are routinely involved with PTX chemoresistance in OC (29–31). More recently, Jiang *et al.* (32) reaffirmed that MAPK/ERK pathway was expressed in OC cells (e.g., SKOV-3 cells), and this pathway played a vital role in the development and progression of OC. The STAT family of transcription factors were involved in apoptotic processes, angiogenesis, cell proliferation, and suppression of antitumor immunity (33, 34). The STAT5 pathway has a significant correlation with OC recurrence and lower survival rates of the patients (35), and is also related to advanced tumor stage and resistance to chemotherapy (36, 37).

In the attempt to resist tumor growth and progression, melatonin was suggested as a powerful agent to be used in the early and late stages of the tumor processes (38). Melatonin is an indoleamine secreted by the pineal gland during darkness, but also produced in many cells in a non-circadian manner. This molecule presents a remarkably functional versatility with antioxidant, oncostatic, and immunomodulatory properties (39, 40). In a previous study we showed that melatonin reduced tumor mass and volume in a rat model of OC induced by chemicals (41, 42). We also observed that melatonin administered to OC animals suppressed the TLR4 expression and its downstream molecules, and furtherly altered the MAPK pathway

(10, 43). While these findings support an anti-cancer role of melatonin, additional details of these processes in the presence of PTX may indicate the novel mechanisms of molecular interaction. Since melatonin is a multitasking agent with many functions, we investigated whether the combination of PTX with melatonin regulates the TLR-mediated inflammatory pathway and cell survival-related signaling pathway in SKOV-3 cells.

2. MATERIALS E METHODS

2.1. Cell culture and reagents.

The SKOV-3 cell line (ATCC® HTB-77) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in RPMI medium (Gibco, Paisley, UK), supplemented with 10% fetal bovine serum (FBS) (Gibco) and penicillin at 100 IU/ml and 100 µg/ml streptomycin (Gibco) and incubated in a humidified atmosphere at 37°C with 5% CO_2. All cells were expanded in 75 cm^2 cell culture flasks (Costar, Cambridge, MA, USA), containing the basal culture medium which was changed periodically. After reaching 90% confluence, cell culture supernatant was aspirated, and cells were washed twice with 10% phosphate-buffered saline (PBS; Oxoid Limited, Hampshire, UK). The cells were then incubated with trypsin/EDTA (Gibco) to avoid any adherence to the flasks. After centrifugation, cells were washed in a culture medium, resuspended, used in the experiments.

2.2. Experimental design.

To assess the effects of the combination of PTX and melatonin, we initially identified the suitable concentration of melatonin and PTX which promoted greater apoptosis and cell necrosis evaluated by the flow cytometry assay (Annexin V, FITC/PI). The experiment was carried out with the evaluation of either melatonin alone or combination with PTX. The cells were exposed to pharmacological concentrations of melatonin (1.6, 3.2, and 4.0mM) and PTX (0.625µM), respectively for 48h in the culture medium. Next, eight experimental groups were formed, namely: Group 1) Control: standard medium containing 200µl DMSO solution as vehicle; Group 2) Melatonin at the concentration of 1.6mM; Group 3) Melatonin at the concentration of 3.2mM; Group 4) Melatonin at the concentration of 4.0mM; Group 5) PTX at the concentration of 0.625µM; Group 6) PTX + Melatonin (1.6mM); Group 7) PTX + Melatonin (3.2mM), and Group 8) PTX + Melatonin (4.0mM). Melatonin was dissolved in DMSO. Since the concentration of 3.2 mM of melatonin potentiated the effects of PTX on apoptosis (~ 50%), we used this concentration to carry out the subsequent experiments. All experimental assays were performed in biological and technical triplicate. The procedure was illustrated in Figure 1.

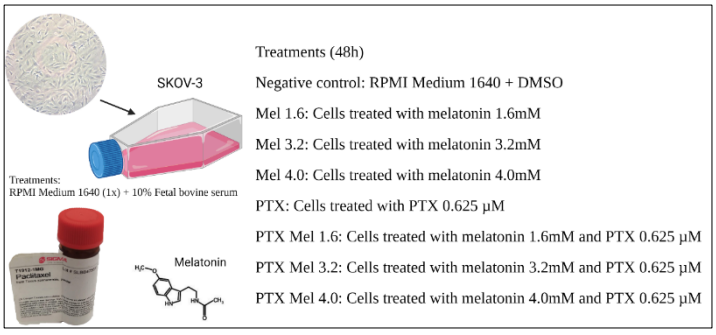

Fig. 1. Schematic representation of the experimental protocol.
Mel: melatonin; PTX: paclitaxel.

2.3. Analysis of apoptosis by flow cytometry.

After treatment, SKOV-3 cells were processed for flow cytometry analysis using apoptotic detection kit (Becton Dickinson, Franklin Lakes, NJ). Annexin V was conjugated with the vital dye propidium iodide (PI) which allowed the identification of apoptotic cells. We set (Annexin V and FITC+/PI-) as apoptosis, and (Annexin V+/PI+) and (PI+) as necrosis. The assay was performed using a FACSCanto II cytometer (Becton Dickinson, San Jose, CA, USA), and the analysis was made using the FlowJo software version 7.2.4 (Three Star).

2.4. Measurement of intracellular melatonin concentration.

After cells were washed to eliminate the outside melatonin, they were lysed and intracellular levels of melatonin was determined by human-specific commercial ELISA assays (EH3344, Fine Test), according to the manufacturer's instructions. The absorbance was read at 450 nm on a microplate reader (Epoch, BioTek Instruments, USA). Results were interpolated from standard curves generated by plotting the concentration of the standards against their absorbance. The concentrations are presented in pg/mL.

2.5. Colorimetric assay for cell viability.

The cell viability was analyzed using the MTT colorimetric method. After reaching 90% confluence, SKOV-3 cells were trypsinized, seeded in 96-well plates at a density of $1x10^4$ cells per well, and cultured in RPMI medium supplemented with 10% FBS. After cell adherence, melatonin and PTX were added into culture medium. Viability curves were estimated after 48h of treatment using the MTT solution (5mg/mL). A microplate reader (Epoch, BioTek Instruments, USA) was used to detect the presence of formazan crystals. The percentage (%) of cell viability was evaluated based on the control group of each experiment.

2.6. Western blot analysis.

At the termination of the experiments, cells (5×10^5) were washed with PBS and homogenized with RIPA lysis buffer containing protease inhibitors (Sigma CO, Saint Louis, MO, USA). Protein quantification was performed using a NanoVue® (GE Healthcare) spectrophotometer. Samples with 40 µg of protein cell extract were solubilized and applied on 4-20% polyacrylamide gel (SDS-PAGE). After performing electrophoresis, the proteins were electrotransferred (35 mA) to 0.2 µm nitrocellulose membrane (BioRad, California, USA). The membranes were blocked with 3% milk diluted in TBS-Tween and incubated with the primary antibodies: TLR2 (ab191458, abcam, 1:500), TLR4 (ab22048, abcam, 1:500), MyD88 (ab28763, abcam, 1:500), TRIF (ab13810, abcam, 1:500), PD-L1 (ab238697, abcam, 1:500), p-PI3K (ab182651, abcam, 1:500), p-AKT (ab81283, abcam, 1:500) diluted in 1% TBS-Tween. Next, the membrane was washed in basal solution (1% TBS-Tween) and incubated with secondary antibody (1:10,000) diluted in 1% milk. After washing, the reaction was generated using the chemiluminescent substrate ECL® Selected Western Blotting Detection Reagent (GE Healthcare, Uppsala, Sweden). The analysis evaluated the presence or absence of bands in a G-Box transilluminator. The intensity of bands was quantified using Image J, based on the optical densitometry and corrected by the endogenous β-actin. Three replicates from each group were used.

2.7. Measurement of cell signaling molecules.

The levels of different molecules associated with cell signaling were determined after the treatments. The protein extraction (n= 24 samples / group) was performed by using a MilliPlex® immunoassay Map Kit (EMD Millipore, Darmstad, Germany) with a standard detection kit following the manufacturer´s protocol. The profile of cell signaling (Cat # 48-681 MAG) included the following molecules: CREB, JNK, NF-kB, p38, ERK1/2, STAT5, and p70S6K. The levels of these molecules ranged from 2.4 to 15000 pg/mL. Fluorescence intensity was read at 575 nm and measured using the MAGPIX system (Luminex® Corporation, Austin, TX, USA).

2.8. Cell invasion and migration assays.

Evaluation of SKOV-3 cell invasiveness was performed using 24-well plates. A thin membrane of Geltrex® was added to each well, occluding the lower polyethylene terephthalate (PET) membrane. SKOV-3 cells ($1x10^5$) were added to the top of the insert and received standard medium without FBS. The invasive potential was analyzed based on the ability of cells to cross the Geltrex® barrier and the PET membrane through the pores, being attracted chemotactically by inferior coverage of culture medium containing 5% FBS. The plates were placed in a CO_2 atmosphere at 37ºC for 24 h. After the incubation period, cells were fixed in methanol for 10 min, and the remaining cells were removed by scraping. Migrated cells were stained with a 0.1% toluidine blue solution and photographed with a 5X objective in an inverted microscope (ZeissAxiovert®). For the migration assay, a similar experimental procedure was used, with the exception of Geltrex® which was not added to the transwell chamber. All experiments were performed in triplicate based on four fields and submitted to cell count.

2.9. Statistical analysis.

The data were analyzed by analysis of variance (One-way ANOVA) complemented with Tukey's multiple comparisons test. For non-parametric data, the Kruskal-Wallis test was used, complemented by Dunn's test. The results were expressed as the mean ± SEM and presented in tables and graphs, considering 5% of significance (P value < 0.05).

3. RESULTS

3.1. Effects of combination of melatonin and PTX on SKOV-3 cell apoptosis

Flow cytometry of cells labeled with annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay was performed to analyze the percentage of cells undergoing either apoptosis or necrosis induced by melatonin combined with PTX (Figure 2). The results have identified the early (Annexin V-FITC+/PI-) and late apoptosis (Annexin V+/PI+) which were designed as apoptosis as well as necrosis (Annexin V-/PI+). Based on previous studies, the increased concentrations of melatonin were used to promote tumor cell death. As shown in Figure 2 A-D, apoptosis and necrosis rates were enhanced by the melatonin treatment following 1.6, 3.2, and 4.0 mM and PTX 0.625 µM alone for 48 h. Notably, the most effective combination was melatonin at 3.2mM with PTX which significantly increased the apoptosis and necrosis rate to near the IC50 values; the percentages of apoptosis and necrosis rates increased to 45% and 42%, respectively, in comparison to the control group. Of special interest, this combination significantly enhanced the apoptosis (~ 35%) and necrosis (~24%)

rates compared to PTX alone (Figure 2 A-D). Thus, the rest of experiments were carried out with this combination.

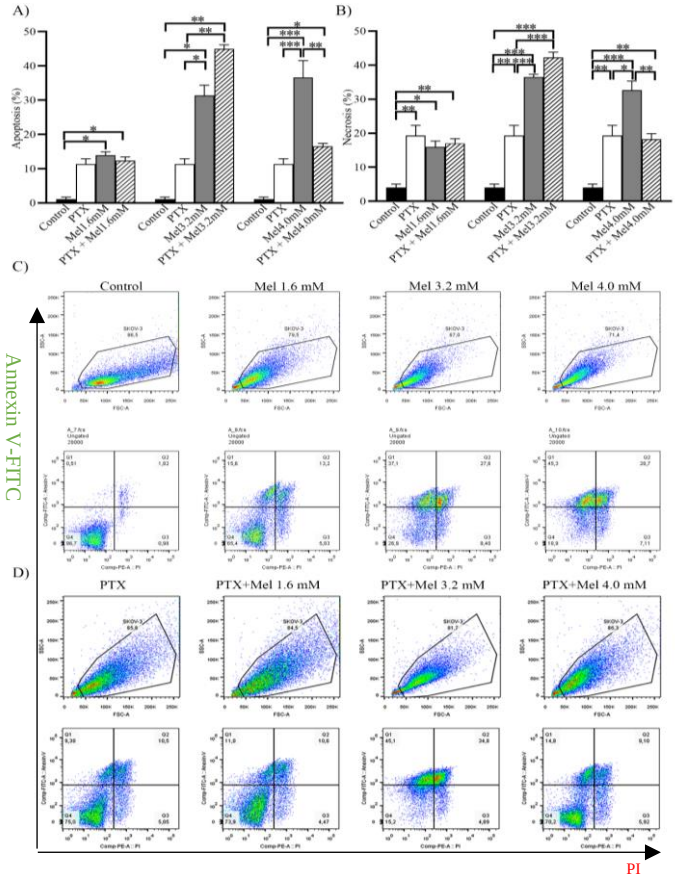

Fig. 2. Effects of the combination of melatonin with PTX on apoptosis in SKOV-3 cells.

*SKOV-3 cells were treated with melatonin and PTX + melatonin for 48h. A) Percentage of apoptotic cells. B) Percentage of necrotic cells. C and D) Representative scatter plots of Annexin V (y-axis) vs. PI (x-axis) after administration of PTX and Melatonin. Q1: apoptosis, Q2: necrosis, Q3: necrosis and Q4: viable cells. Annexin can be detected in both early and late stages of apoptosis, whereas PI stains cells can only be detected in late apoptosis or necrosis. Data were expressed as mean ± SEM of triplets. *p<0.05; ** p<0.01; *** p<0.001. One-Way ANOVA complemented by Tukey's test.*

3.2. Effects of melatonin and PTX on the mitochondrial viability in OC cells.

First, the cellular melatonin levels were measured by the enzyme linked immunosorbent assay (ELISA). The results showed that SKOV-3 cells presented significantly lower melatonin level than that in healthy ovarian cells. Both PTX and melatonin treatments elevated the intracellular concentration of melatonin in the tumor cells. More importantly, the combination treatment of melatonin and PTX restored the concentration of melatonin in SKOV-3 cells close to that in healthy ovarian cells (Figure 3 A).

To better understand whether the combination of melatonin and PTX reduced mitochondrial activity in tumor cells, we performed the MTT reduction assay. Cell viability was markedly reduced after treatment with the combination for 48 h (reduced by ~ 96% vs. control; Figure 3 B); this reduction was even more pronounced when compared with PTX alone (~ 50% vs. control) or melatonin alone (reduced by ~ 87% vs. control). The combination of PTX with melatonin efficiently reduced cell viability compared to PTX alone (~ 45%).

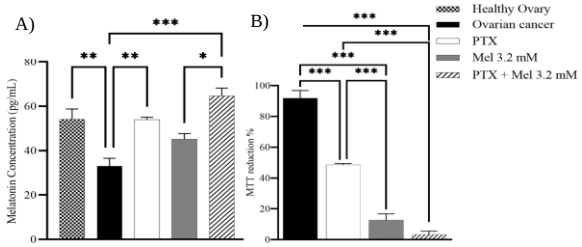

Fig. 3. Melatonin levels and cell viability.

*A) Intracellular melatonin after treatment with PTX and melatonin. B) MTT reduction after treatment with PTX and PTX/melatonin. Ovarian cancer: SKOV-3 cells, PTX: paclitaxel, Mel: melatonin. Data were expressed as mean ± SEM of triplet. *p<0.05; ** p<0.01; *** p<0.001.*

3.3. Effects of melatonin and PTX on TLR4-mediated inflammatory pathway and PD-L1 in ovarian cancer cells.

The levels of TLR2, TLR4, MyD88, TRIF and PD-L1 were measured in the ovarian cancer cells. After melatonin treatment, the levels of TLR4, MyD88, and TRIF were significantly reduced (Figure 4). Although the TLR2 levels were increased with PTX, the combination did not reverse this rise. The combination of PTX and melatonin led to a significant decrease in TLR4 levels compared to PTX alone. The levels of MyD88 and TRIF were significantly reduced by both treatments; however, no additive effect was observed with the combination. To assess the role of melatonin and PTX treatment on the immune checkpoint mechanism, we estimated the levels of PD-L1. Notably, the combined treatments were effective in significantly reducing the PD-L1 levels (Figure 4 A, B).

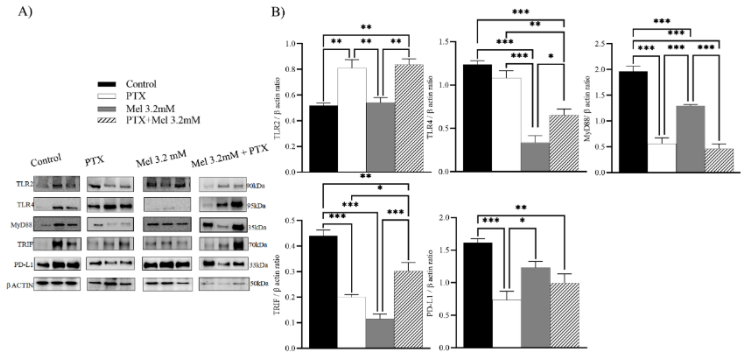

Fig. 4. Effects of PTX and PTX + Melatonin on TLR-mediated inflammatory pathway.

*A) Representative profile of the TLR2, TLR4, MyD88, TRIF, and PD-L1. B) Optical densitometric analysis of TLR2, TLR4, MyD88, TRIF and PD-L1 levels in ovarian cancer cells after normalization with β-actin. Mel: melatonin. Data expressed as mean ± SEM of triplet. *P<0.05, **P<0.01, and ***P<0.001.*

3.4. The regulatory effects of PTX or melatonin on the expressions of PI3K and AKT levels.

To examine cell survival-related proteins in response to PTX and melatonin, we evaluated the levels of p-PI3K and p-AKT in the cells. Treatment with melatonin or PTX caused a significant reduction in p-AKT levels in comparison to the control group. The combination therapy induced a reduced expression levels of p-PI3K and p-AKT compared to control and melatonin groups (Figure 5 A and B).

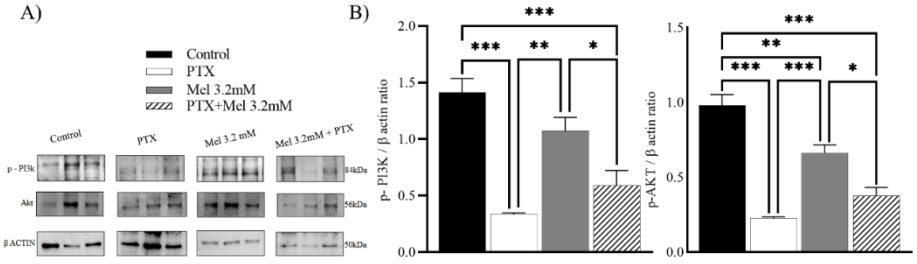

Fig. 5. **The effects of PTX or melatonin on the expressions of PI3K and AKT levels.**

*A) Representative profile of the proteins p-PI3K and p-AKT in cellular extracts of 40 µg of proteins using technical and biological triplicates. B) Optical densitometric analysis of p-PI3K and p-AKT levels in ovarian cancer cells after normalization with β-actin. Mel: melatonin. Data expressed as mean ± SEM of triplets. *P<0.05, **P<0.01, and ***P<0.001.*

3.5. Effects of melatonin with PTX on the signaling pathways for cell growth and proliferation.

MilliPlex® Map Kit (Cat # 48-681 MAG) assay was used to assess the total amount of protein per cell volume. The results showed that seven kinases, namely NF-kB p65, STAT5, ERK1/2, p70S6K, CREB, p38 and JNK were measured simultaneously. The PTX significantly reduced the levels of JNK, NF-kB, p38, P70s6K, and STAT5 compared to the control group. Melatonin significantly reduced the cellular concentration of NF-kB, p70S6K, and STAT5. Importantly, the combination of PTX with melatonin resulted in depressed levels of JNK, CREB, NF-kB, p38, ERK1/2, p70S6K, and STAT5 (Figure 6). These results proved the role of the combination against SKOV-3 cell aggressiveness, thereby revealing its potential in the control of tumor growth.

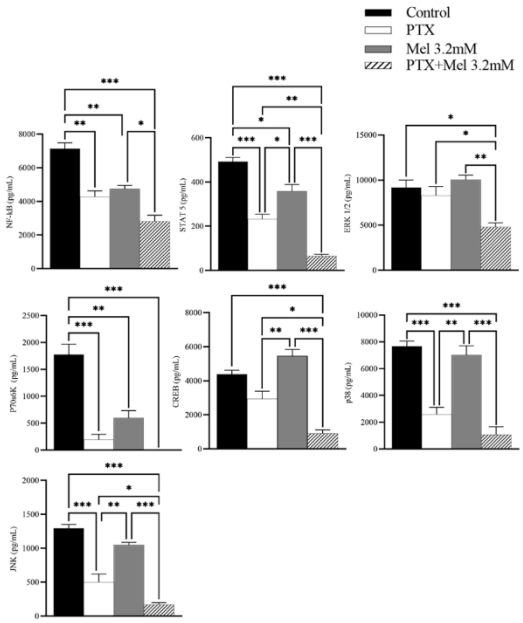

Fig. 6. **Effects of melatonin and/or PTX on the cell signaling-related molecules in the SKOV-3 cells**.

*Concentrations of CREB, JNK, NF-kB p65, p38, ERK1/2, p70S6K, and STAT5 were evaluated in the supernatants of cell culture. Mel: melatonin. Data expressed as mean ± SEM of triplets. *P<0.05, **P<0.01, and ***P<0.001.*

3.6. Effects of melatonin and PTX on the migratory and invasive capacity of SKOV-3 cells.

To functionally investigate the effects of PTX and melatonin on the migratory and invasive potential of SKOV-3 cells, tumor behaviors were examined using transwell inserts. After SKOV-3 cells were seeded in 24-well plates, they were treated with different doses of melatonin alone or with PTX. The results showed that melatonin alone significantly reduced cell migration by 35% whereas PTX dramatically reduced its rate by 85% compared to the control. When melatonin was combined with PTX, an even greater decrease in cell migration was observed compared to melatonin alone (Figure 7 A and B). The invasive potential of SKOV-3 cells was markedly reduced by PTX alone compared to the control. In these cells, melatonin did not potentiate the effects of PTX against cell invasiveness (Figure 7 C and D).

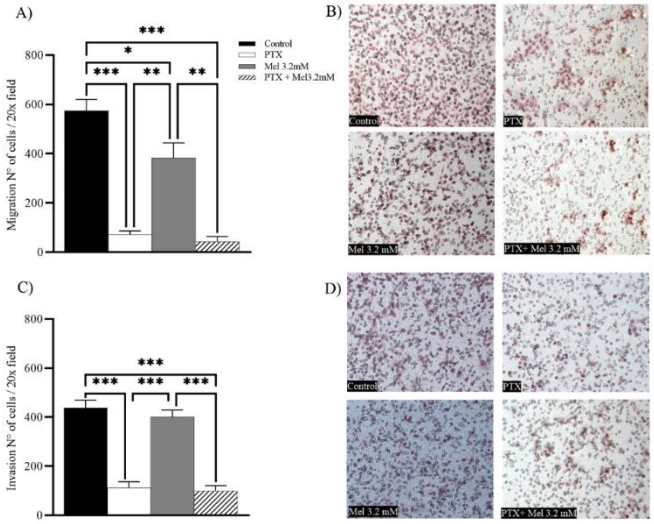

Fig. 7. **Effects of melatonin and PTX on the migration and invasion of SKOV-3 cells.**

*A) Effect of melatonin and PTX on the migratory capacity of SKOV-3 cells. B) Images of migrated cells after treatments. C) Effect of melatonin and PTX on the invasive potential of SKOV-3 cells. D) Images of invasive cells after treatments. Mel: melatonin. Data were expressed as mean ± SEM of triplets. *P<0.05, **P<0.01, and ***P<0.001.*

4. DISCUSSION

This study provides new information related to the combined effect of melatonin and PTX on OC cells, with a focus on the TLR-mediated inflammatory pathway, cell signaling and cell survival.

PTX represents the gold standard treatment for OC; however its use often leads to chemoresistance (44, 45). As a natural molecule, melatonin exerts antitumor activity in a variety of cancers including OC. Its potential mechanisms are involved in reducing cell proliferation, migration, inflammation and stimulation of the immunity (39, 46). These mechanisms have been confirmed in the current study. Considering the beneficial effects of melatonin on chemoresistance in different cancers (47–50) it was selected to potentiate the antitumor effects of PTX on OC in the study. Herein, we first analyzed the intracellular melatonin concentration since melatonin can promote apoptosis in SKOV-3 cells. This has been reported previously that melatonin at 2.5 mM increased the tumor cell death (51). Especially, melatonin treatment at 3.4 mM level significantly reduced the viability of SKOV-3 cancer stem cells after 48 h of incubation (52). In the current study, we also observed that the

major activity of melatonin on OC cells was to reduce their proliferation with the high efficiency at a concentration of 3.2 mM. At these respects, a combination of PTX and melatonin was used to treat SKOV-3 cells. The results showed that this combination significantly increased apoptosis and necrosis along with reduced tumor cell viability. This observation is consistent with previous report by Kim *et al.* (53) in which melatonin (2 mM) combined with cisplatin (80 µM) promoted apoptosis by activating caspase-3 in SKOV-3 cells or in type II A549 alveolar epithelial cells (54) and in HepG2 liver cells (55). The combination was effective against cell migration and invasion and these actions of melatonin and PTX might inhibit the metabolic activity of SKOV-3 cells since both molecules reduced the mitochondrial activity in the tumor cells. We have previously demonstrated that PTX lowers the viability and promotes cell death even after considerably lower levels of PTX (56). In addition, melatonin attenuated cell migration in several tumor types (57–59). Akbarzadeh *et al.* (52) also showed that melatonin at 3.4 mM reduced migration in SKOV-3 cells. Given that PTX alone caused a significant drop in the cell dynamics, this molecule might take a major role to attenuate tumor cell migration/invasion.

The promising effects of melatonin in the hormone-dependent cancers are related to its activities on apoptotic regulation, angiogenesis inhibition, tumor metabolism and cell survival (58). When combined with chemotherapeutic agents, it can potentiate chemosensitivity while mitigating the toxicity of the chemotherapies (60, 61). Women with OC often show a reduced plasma concentration of melatonin (50 pg/mL in OC versus 100 pg/mL in control subjects) (62). We verified that the OC cells had lower levels of melatonin than that of healthy ovarian cells. Moreover, the intracellular levels of melatonin were restored after PTX and melatonin treatments. Although there may be cellular uptake of melatonin in melatonin-treated tumor cells, the rise in intracellular melatonin after PTX treatment alone is of particular interest. One implication of these findings is that intracellular melatonin synthesis is inducible in animal cells as is well documented in plant cells (63).

TLR activation in the tumor microenvironment is related to drug resistance and disease progression (64–66). Our results documented that melatonin and PTX regulated TLR activation, specifically via TLR4 in the OC cell. TLRs, especially TLR2 and 4, are important members involved with OC prognosis (67). Particularly, activation of the TLR4/MyD88/NF-kB pathway is directly related to tumor progression and a worse prognosis (68, 69). In the current study, PTX did not decrease TLR4 expression and this is not surprising since the taxol class drugs are known to be a TLR4 agonist (70, 71). This feature of TLR4 may result in increased chemoresistance and poor prognosis in OC patients (69, 72). It has been reported that PTX promotes TLR4-mediated immunogenic cell death in OC (73) and stimulates the TLR4/NF-kB/ABCB1 signaling pathway in taxol-resistant SKOV-3 cells (67). By contrast, melatonin alone or combined with PTX significantly reduced the TLR4 expression in addition to the levels of MyD88 and TRIF, thus revealing it to be an effective agent acting against chemoresistance. The combination of PTX and melatonin also reduced downstream molecules of the TLR4 pathway, including NF-kB.

In previous study, by using the DMBA-induced OC animal model, we showed that long-term (60 days) of melatonin (200µg/100g b.w.) treatment attenuated the TLR4-mediated inflammatory response (10). Although melatonin did not reduce the TLR2 levels in SKOV-3 cells, it depressed TLR2 level in OC rats (10). This difference may be due to the cell types, melatonin concentrations or the duration of treatment. Melatonin combined with PTX attenuated the NF-kB p65 levels. In OC, NF-kB is associated with drug resistance (74), and DNA damage (75); thus, the inhibition of p65 transactivation is of great value. Melatonin has been proven to alter p65 subunit of the NF-kB, thus preventing its translocation to the nucleus and blocking the production of pro-inflammatory cytokines (68). The reduction of NF-kB activity by melatonin has already been demonstrated in experimental models of ovarian and

breast cancers (10, 76). The combination also reduced the expression of PD-L1 in SKOV-3 cells. PD-L1 is highly expressed in OC cells, suppressing the immune system by binding to the PD-1 receptor on T lymphocytes, which results in tumor progression (77, 78). Women who exhibit high expression of PD-L1 in OC are often diagnosed at more advanced stages of the disease, with a high recurrence rate (79). Therapies targeting PD-1 and PD-L1 have been investigated to evaluate the proper immune response and destruction of neoplastic cells (80, 81). To date, this is the only study that has identified the action of melatonin on the PD-L1 levels in OC, which could be considered for the regulation of immunological checkpoints of melatonin.

Our findings revealed that this combination further attenuated the levels of JNK, ERK 1/2, and p38 MAPK in SKOV-3 cells compared with their respective controls. The JNK pathway may be one of the mechanisms associated with cell migration, invasion, and metastasis (82) and, consequently, it may promote drug resistance and poor prognosis in OC (83). By inhibiting the JNK pathway, a reduction in cell growth with an increase in apoptosis and cell cycle arrest in OC cells has been observed (84). Likewise, CREB, an important transcription factor, is phosphorylated by several kinases, including ERK 1/2, Akt and p38, and its activation is related to the cell proliferation, apoptosis, angiogenesis, and metastasis (85). Our study demonstrated the effectiveness of the treatment in reducing CREB levels in SKOV-3 cells. It also reduced the transcription factor STAT5 and p70s6K ribosomal protein. The STAT family participate in oncogenic transformation and progression of the OC (86), and consequently, high levels of the kinases may be related to poor prognosis and OC development (87).

PI3K/Akt pathway is altered in OC (88), and melatonin attenuated PI3K and Akt levels in both animals and cells (43, 57). During tumor invasion and metastasis, Akt/p70s6K pathway is often activated, serving as a potential prognostic marker for tumor chemoresistance (89). Melatonin reduced the MAPK signaling intermediates in different cancers (42, 59, 90–92), which reinforces its pivotal role against tumor cell survival. Overall, downregulation of these signaling molecules may negatively affect the biological activity of OC cells resulting in lowered proliferative and invasive capacity while inducing a higher apoptotic rate.

In conclusion, the study found that the combination of melatonin with PTX increased apoptosis and necrosis in SKOV-3 cells while also reducing the tumor cell migration and invasion. Moreover, this combination reduced the TLR4-mediated inflammatory pathway, PD-L1 levels, and cell survival-related signaling molecules in OC (Figure 8). This combination may overcome chemoresistance to serve as an additional strategy in the treatment of OC. Since melatonin has very low or even no cellular toxicity, it may improve PTX sensitivity while reducing the adverse effects of chemotherapy.

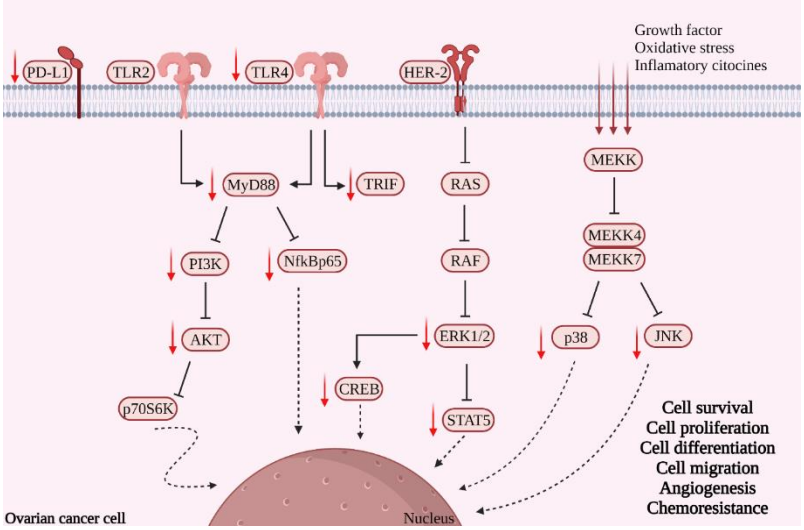

Fig. 8. **Schematic representation of the melatonin and PTX actions in different targets related to inflammation and survival of ovarian cancer cells.**

The combined treatment attenuated different intracellular molecules and membrane receptors potentially resulting in inhibition of cell proliferation and chemoresistance while enhancing apoptosis. Red arrows indicate downregulated targets by treatment combination. TLR2, Toll-like 2 receptor; TLR4, Toll-like 4 receptor; MyD88, myeloid differentiation factor 88; NF-kB, Nuclear Transcription Factor kappa B; PD-L1, Programmed Cell Death binding protein 1; TRIF, TIR-domain-containing adapter-inducing interferon-β; ERK 1/2, extracellular signal regulated protein kinase; CREB, Cyclic AMP response-element binding protein; STAT5, signal transducer and activators of transcription 5; p70S6K, 70 kDa ribosomal protein S6 kinase; PI3K, phosphatidylinositol 3- kinase; Akt, protein kinase B; p38, mitogen activated protein kinase; JNK, c-Jun N-terminal kinases.

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AUTHORSHIP

LBG, LGAC: conceived the hypothesis of the study, collected and analyzed the data, and drafted the manuscript. RCC, MSC, HSS, DAMO, GGR, DAPCZ, RK: participated in the design, intellectual conception of the study, and in the acquisition of data. RJR: participated in critical revision of the manuscript. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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