

Efeito do tratamento com melatonina associado ao paclitaxel em células de carcinoma ovariano

LETÍCIA BARBOSA GAIOTTE

Dissertação apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Mestre no Programa de Pós- Graduação em Biologia Geral e Aplicada, Área de concentração Biologia Estrutural e Funcional.

Orientador: Prof. Dr. Luiz Gustavo de Almeida Chuffa.

BOTUCATU – SP

2021

UNIVERSIDADE ESTADUAL PAULISTA
“Júlio de Mesquita Filho”
INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

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Melatonina; Paclitaxel; Sinalização celular.

Dedicatória

Foi pensando nas pessoas que executei este projeto, por isso dedico este trabalho a todos aqueles a quem esta pesquisa possa ajudar de alguma forma.

“Nenhuma pessoa deve ser encarada como uma abstração. Antes, é preciso enxergar em cada pessoa um universo com seus próprios segredos, com seus próprios tesouros, com suas próprias fontes de angústia e com certa dose de triunfo.”

*(Elie Wiesel The Nazi doctors and the
Nuremberg Code)*

Do livro A vida imortal de Henrietta Lacks

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AKT: Proteína quinase B;

Apaf-1: Fator de ativação 1 da protease apoptótica;

APCs: células apresentadoras de antígeno;

CDK2: Quinase 2 dependente de ciclina;

CDK4: Quinase 4 dependente de ciclina;

c-Jun: Fator de transcrição AP-1;

CO: Câncer de ovário;

DCs: células dendríticas;

ERK 1/2: Proteína quinase 1 ativada por mitógeno 3;

JNK: c-Jun N- terminal cinase

HER-2: Receptor tirosina quinase;

IFN- β : Interferon-beta;

I κ B- α : Inibidor alfa de NF- κ B;

IL-10: interleucina 10;

IL-6: interleucina 6;

IL-8: interleucina 8;

Mel: melatonina;

MT1: Receptor de melatonina 1;

MT2: Receptor de melatonina 2;

mTOR: Serina/treonina proteína quinase;

MyD88: fator de diferenciação mielóide 88;

NF- κ B: Fator nuclear kappa-B;

NK: célula exterminadora natural;

PAMPs: Padrões moleculares associados a patógenos;

PD-1: Proteína de morte celular programada-1;

PD-L1: ligante de morte celular programada 1;

P-gp: Glicoproteína-P;

PI3K: fosfatidil-inositol 3-quinase;

PTX: Paclitaxel;

SHP2: Proteína tirosina fosfatase;

TAAs: Antígenos associados a tumores;

TCD8+: célula T citotóxica positivo;

TCR: Receptor de células T;

TGF- β : Fator de transformação do crescimento beta;

Th1: resposta polarizada T helper 1;

TILs: Infiltrados linfocitários;

TLR: receptor celular do tipo Toll;

TLR2: receptor celular do tipo Toll 2;

TLR4: receptor celular do tipo Toll 4;

TNF- α : fator de necrose tumoral alfa;

Tregs: células T reguladoras;

VEGF: Fator de crescimento endotelial vascular;

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Resumo

O câncer de ovário (CO) é uma doença ginecológica com altas taxas de mortalidade entre as mulheres. Embora a maioria dos pacientes responda à quimioterapia convencional [por exemplo, paclitaxel (PTX)], muitas mulheres se tornam resistentes e a doença progride. A melatonina exerce funções antioxidantes, antitumorais e imunomoduladoras em uma variedade de tumores sólidos. Portanto, este estudo investigou os efeitos do PTX combinado com melatonina na via inflamatória mediada pelo receptor toll-like (TLR), sinalização celular e sobrevivência de células de carcinoma de ovário humano SKOV-3. O ensaio de citometria de fluxo mostrou que a melatonina associada ao PTX promoveu um aumento significativo nas taxas de apoptose/necrose. A migração celular foi atenuada apenas pela melatonina e, após sua associação com PTX, o número de células migradas e invasivas reduziu significativamente. Embora o tratamento combinado de PTX e melatonina não tenha sido capaz de reduzir TLR2, os níveis de TLR4, MyD88, TRIF e PD-L1 foram reduzidos após a combinação de terapia e até melatonina sozinha. Além disso, a terapia combinada reduziu significativamente os níveis de NF- κ B p65, p-PI3K, p-AKT, p38, ERK 1/2, JNK, CREB, p70s6K e STAT 5. Concluímos que o tratamento combinado foi eficaz na redução Viabilidade e capacidade invasiva das células OC enquanto aumenta as taxas de apoptose e necrose. Além disso, a combinação atenuou as moléculas a jusante da via inflamatória mediada por TLR4 e proteínas relacionadas à sinalização celular em células SKOV-3, mostrando-se, assim, capaz de promover alterações na resposta celular e em seu potencial maligno. A melatonina pode melhorar a quimiossensibilidade do PTX, servindo como uma terapia adjuvante eficaz contra OC.

Palavras-chave: Câncer de ovário, melatonina, paclitaxel, receptor toll-like, PD-L1, sinalização celular, células SKOV-3.

Abstract

Ovarian cancer (OC) is a gynecological disease with high mortality rates among women. Although most patients respond to the conventional chemotherapy [e.g., paclitaxel (PTX)], many women become resistant and the disease progresses. Melatonin exerts antioxidants, antitumor, and immunomodulatory functions in a variety of solid tumors. Therefore, this study investigated the effects of PTX combined with melatonin on the toll-like receptor (TLR) mediated inflammatory pathway, cell signaling, and survival of SKOV-3 human ovarian carcinoma cells. The flow cytometry assay showed that melatonin associated with PTX promoted a significant increase in apoptosis/necrosis rates. The cell migration was attenuated by melatonin alone, and after its association with PTX, the number of migrated and invasive cells reduced significantly. Although the combined treatment of PTX and melatonin was unable to reduce TLR2, the levels of TLR4, MyD88, TRIF, and PD-L1 were reduced following therapy combination and even melatonin alone. Furthermore, the combination therapy significantly reduced the levels of NF- κ B p65, p-PI3K, p-AKT, p38, ERK 1/2, JNK, CREB, p70s6K, and STAT 5. We concluded that the combined treatment was effective in reducing OC cell viability and invasive capacity while increasing apoptosis and necrosis rates. Moreover, the combination attenuated the downstream molecules of the TLR4-mediated inflammatory pathway and cell signaling-related proteins in SKOV-3 cells, thus proving to be capable of promoting changes in the cellular response and in its malignant potential. Melatonin may improve the chemosensitivity of PTX, serving as an effective adjuvant therapy against OC.

Keywords: Ovarian cancer, melatonin, paclitaxel, toll-like receptor, PD-L1, cell signaling, SKOV-3 cells.

Capítulo 1

1. Introdução

1.1. Câncer de ovário: prevalência e mortalidade

O câncer de ovário (CO) é a neoplasia ginecológica com uma das mais altas taxas de letalidade entre as neoplasias que acometem o sistema genital feminino (NEERJA; ANNWEN, 2018; WHO, 2021). Mundialmente há uma incidência de 6,6 mil novos casos a cada 100,000 mulheres em 2021 (WHO, 2021)(Tabela 1).

Estima-se que até o final de 2022, serão diagnosticados 19.880 novos casos e ocorrerão 12.810 óbitos em decorrência da doença nos Estados Unidos de acordo com *Surveillance, Epidemiology and End Results* (SEER, 2022). Segundo dados do (Instituto Nacional do Câncer (INCA) para o triênio 2020/2022 serão diagnosticados no Brasil 6,650, novos casos a cada 100, 000 mulheres classificando-o como o 7.º mais incidente (Tabela 2). Em 2017, ocorreram 3,879 óbitos por CO no país o equivalente a 3,75 a cada 100, 000 mulheres (INCA, 2021).

Em decorrência da inespecificidade dos sintomas e métodos de triagem iniciais escassos, muitas mulheres são diagnosticadas apenas quando a doença se encontra em estágio avançado (FEENEY *et al.*, 2020; JESSMON *et al.*, 2017). Os sintomas podem variar de inchaço abdominal, dor pélvica, dificuldade para se alimentar e aumento da frequência urinária (BETHESDA, 2002).

Tabela 1 – Taxa mundial estimada de incidência e mortalidade de todos os tipos de cânceres a cada 100, 000 mulheres em 2021.

Câncer	Incidência	Mortalidade
Mama	47,8	13,6
Colo retal	16,2	7,2
Pulmão	14,6	11,2
Cérvix uterino	13,3	7,3
Tireoide	10,1	0,50
Corpus uterino	8,7	1,8
Estômago	7,0	4,9
Ovário	6,6	4,2
Fígado	5,2	4,8
Linfoma não Hodgkin	4,8	2,1

Fonte: Global Cancer Observatory, World Health Organization, 2021

Tabela 2 - Incidência estimada de cânceres a cada 100, 000 mulheres no Brasil.

Câncer	Casos novos	%
Mama	66,280	29,7
Cólon e Reto	20,470	9,2
Colo do útero	16,710	7,5
Traqueia, Brônquio e Pulmão	12,440	5,6
Glândula Tireoide	11,950	5,4
Estômago	7,870	3,5
Ovário	6,650	3,0
Corpo do útero	6,540	2,9
Linfoma não-Hodgkin	5,450	2,4
Sistema Nervoso Central	5,230	2,3

Fonte: MS/ INCA/ Estimativa de Câncer no Brasil, 2021

O CO abrange grupos tumorais ovarianos divergentes sendo classificados com base nas células de origem (WHO, 2021) em três grandes grupos: origem epitelial, células germinativas e células estromais (KURMAN *et al.*, 2014; PRAT, 2012).

Os tumores provenientes de origem epitelial representam 90% dos diagnósticos (MATULONIS *et al.*, 2016) e são subdivididos em seroso, mucinoso, endometrióide e de células claras (KURMAN *et al.*, 2014). O subtipo seroso ainda é agrupado em carcinoma seroso de baixo grau ou carcinoma seroso de alto grau. Carcinomas de alto grau são diagnosticados em 80% das vezes enquanto os de baixo grau, apenas 5% (Figura 1) (PRAT, 2012; PRAT; D'ANGELO; ESPINOSA, 2018; WHO, 2021).

O carcinoma seroso de alto grau tem classificação tipo II e é o mais letal pois apresenta sintomas em estágios avançados (III ou IV) (KROEGER; DRAPKIN, 2017; LISIO *et al.*, 2019). Esses tumores evoluem a partir de lesões malignas *in situ* no epitélio da fímbria das tubas uterinas e se espalham posteriormente para os ovários (BETHESDA, 2002).

Os tumores serosos sendo de alto ou baixo grau, representam diferenças morfológicas, patogêneses, eventos moleculares e prognósticos distintos. Tumores de alto grau tem a localização na extremidade fimbrial distal da tuba uterina de uma lesão precursora denominada carcinoma intraepitelial tubário seroso, estes, estão relacionados com mutações TP53, deficiência de recombinação homóloga e mutações BRCA enquanto os de baixo grau se estabelecem dentro do ovário, formando tumores serosos benignos e limítrofes e apresentam mutações associadas a BRAF ou KRAS (DE LEO *et al.*, 2021; KURMAN *et al.*, 2014; KURMAN; SHIH, 2010).

O risco de desenvolvimento de CO aumenta em mulheres com mais de 40 anos e atinge o pico de incidência em mulheres com mais de 70 anos de idade (WEBB; JORDAN, 2017). Mulheres com histórico familiar de CO na família possuem três vezes

mais chances de ter a doença (STRATTON *et al.*, 1998). Os processos ovulatórios também se relacionam com o histórico de CO, menopausa tardia e menarca precoce representam altas chances de desenvolvimento CO (WENTZENSEN *et al.*, 2016).

Alguns eventos promovem efeitos protetivos no desenvolvimento tumoral, mulheres que passaram por algumas gestações, há uma estimativa de cada gestação reduzir em 10-20% o risco de CO (WENTZENSEN *et al.*, 2016), a amamentação reduz em até 25% as chances de CO (LUAN *et al.*, 2013), estudos demonstraram que mulheres que fazem o uso contínuo de contraceptivos orais tem 30% menos chances de desenvolver CO (BERAL *et al.*, 2008), a laqueadura tubária também parece estar envolvida com a redução dos riscos de CO em até 30% (SIEH *et al.*, 2013).

O CO conta com vários estágios tumorais, estes descrevem diversos aspectos do tumor como localização e disseminação. A classificação é importante para determinar a extensão tumoral, a disseminação para os órgãos linfáticos, a propagação para locais distantes e, assim, saber o quão avançado o câncer está. Os estágios podem variar de I a IV sendo o IV o estágio mais avançado (PRAT; OLAWAIYE; BERMUDEZ, 2017).

I Estádio: tumor restrito aos ovários;

II Estágio: tumor limitado a pelve;

III Estágio: tumor se estende ao peritônio e pelve;

IV Estágio: Metástase (INCA, 2021; PRAT; OLAWAIYE; BERMUDEZ, 2017).

Apesar dos sintomas iniciais serem inespecíficos, conforme há um crescimento tumoral, os estágios iniciais vão avançando e pode ocorrer relatos de pressão, dor ou inchaço no abdômen costas ou pernas, náuseas e cansaço constante (INCA, 2021);

O CO apresenta desenvolvimento rápido e acentuada proliferação na região próxima ao peritônio (WAGNER; DUDLEY, 2013). Células tumorais originadas da extremidade distal das tubas uterinas ou do epitélio superficial ovariano sofrem transição epitelial para mesenquimal, se destacam e espalham-se pela cavidade peritoneal (LENGYEL, 2010).

Os adipócitos secretam fatores de crescimento além de moléculas bioativas tornando o ambiente facilmente proliferativo e favorecendo a viabilidade tumoral (WAGNER; DUDLEY, 2013). A região peritoneal é rica em tecido adiposo e colabora com acentuada proliferação no CO. No processo de metástase, os adipócitos são recrutados pelas células neoplásicas e se associam ao microambiente tumoral (DIRAT *et al.*, 2011, 2010).

Dentre os modelos experimentais existentes, o método de cultivo de células tem diversas vantagens desde facilidade de propagação, grande estabilidade e alta reprodutibilidade (TUDREJ *et al.*, 2019). A linhagem de células SKOV-3 deriva do fluído de ascite peritoneal de paciente caucasiana, de 64 anos diagnosticada com CO em 1973, a linhagem representa alto potencial invasivo e proliferativo e é muito utilizada em experimentos envolvendo tumores de CO epiteliais.

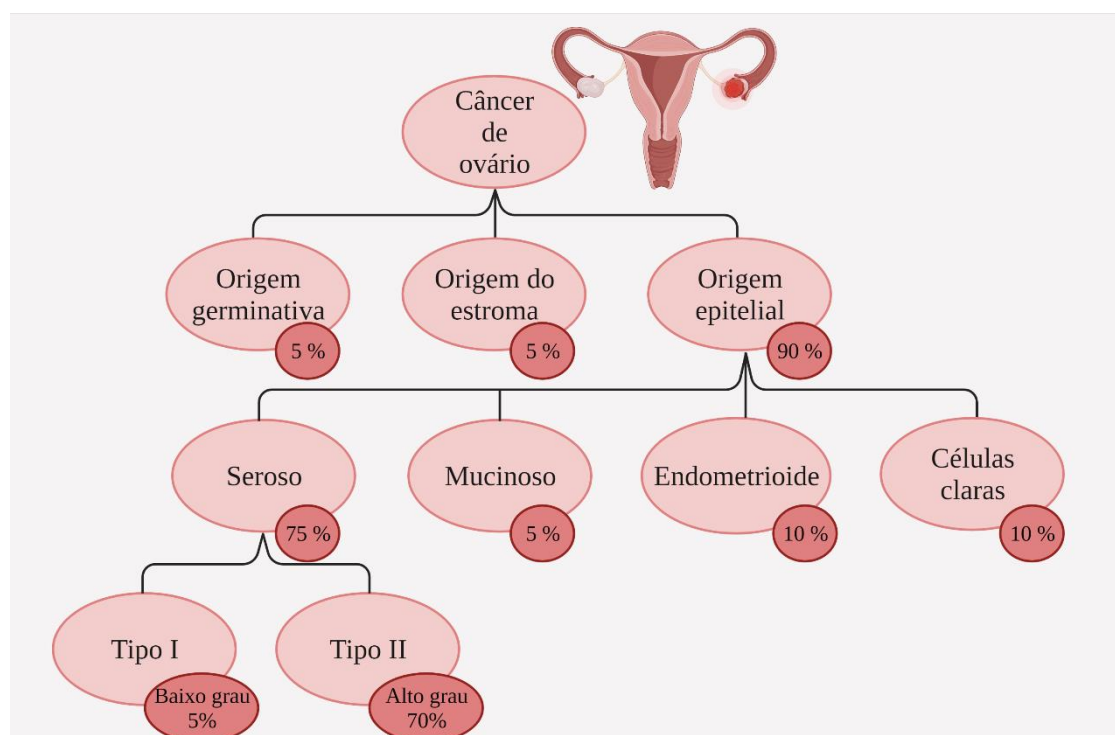


Figura 1- Subtipos histológicos do CO, classificação baseada na histologia e grau tumoral.

1.2. O desenvolvimento de quimiorresistência frente a terapia com paclitaxel

Atualmente existem muitos medicamentos e terapias alvos direcionadas para o tratamento de câncer de ovário (CORTEZ *et al.*, 2018). Entretanto, por apresentarem numerosas variações histológicas, a neoplasia conta com diferentes prognósticos o que torna ainda mais desafiador o tratamento (KUJAWA; LISOWSKA, 2015; KURMAN; SHIH, 2010).

O padrão atual de tratamento para CO inclui quimioterapia, radioterapia, remoção cirúrgica e imunoterapia, mas dependerá do estágio do tumor diagnosticado (JAYSON *et al.*, 2014; STEWART; RALYEA; LOCKWOOD, 2019). Em fases iniciais, o paciente é submetido a cirurgia e quimioterapia com derivados de platina

enquanto em fases avançadas é possível realizar uma terapia combinada com derivados de platina e taxol (ORR; EDWARDS, 2018).

O grupo dos taxanos compreende moléculas que apresentam em sua composição um anel complexo “taxano” e uma função amida (WALL, 1998). Faz parte dos membros da família dos taxanos o paclitaxel (PTX), um dos agentes antineoplásicos mais utilizados para tratar diversos tipos de câncer como: mama, pulmão, bexiga urinária, próstata, gástrico, de cabeça e pescoço e ovário (BERNABEU *et al.*, 2017; FU *et al.*, 2009).

O fármaco é um agente de polimerização que apresenta amplo espectro antitumoral em decorrência da capacidade de estabilizar microtúbulos celulares. Ao se ligar a subunidade β da proteína tubulina, proporciona uma polimerização que acarreta a formação de um fuso mitótico disfuncional. Ao suprimir o fuso dos microtúbulos, consequentemente, irá bloquear a transição metáfase-anáfase; uma vez inibida a mitose, a célula é induzida à morte por apoptose (DERRY; WILSON; JORDAN, 1995; HORWITZ, 1992; KHANNA; ROSENBERG; VAIL, 2015).

O mecanismo de ação do PTX ocorre por ativação de vias pró apoptóticas dependentes da cascata TLR4, MyD88, c-Jun, ERK1/2 e NF-kB (PFANNENSTIEL *et al.*, 2010). O fármaco por ativar citocinas inflamatórias tem como consequência a morte das células tumorais, entretanto, alterações nessas vias levam ao desenvolvimento de resistência a droga e ineficácia do tratamento (SZAJNIK *et al.*, 2009; YAKIREVICH *et al.*, 2006).

Muitas mulheres desenvolvem resistência ao PTX por diversos mecanismos entre eles, mudanças no mRNA tendo a presença de múltiplos genes ribossomais, alteração na síntese proteica, estresse oxidativo, glicólise e metabolismo da glutatona (AGARWAL; KAYE, 2003). Outro estudo demonstrou que em uma análise com 845 genes, 337 foram alterados em células neoplásicas resistentes a PTX (SHERMAN-BAUST *et al.*, 2011).

A resistência a quimioterapia também denominada quimiorresistência é uma adversidade na eficácia do tratamento de tumores humanos. Seja por mecanismos adquiridos ou naturais, células em estado metastático são resistentes ao tratamento com diversos agentes antitumorais em decorrência da alta adaptabilidade e instabilidade genômica das mesmas (GATTI; ZUNINO, 2005).

A quimiorresistência frente ao tratamento do CO é frequentemente observada e responsável por 90% das mortes de pacientes (SHERMAN-BAUST *et al.*, 2011). A

maquinaria celular para quimiorresistência é muito ampla, podemos citar como fator principal alterações farmacocinéticas da droga que irá modificar absorção, distribuição, metabolismo e eliminação do fármaco no organismo interrompendo o ciclo do tratamento antitumoral. Alterações no influxo e efluxo do fármaco, inativação e alteração no alvo da droga, sistema de reparo do DNA aprimorado, alterações nos checkpoints do ciclo celular, inibição da apoptose, transição epitélio mesenquimal também irão contribuir com a ineficácia do tratamento, escape das células tumorais e quimiorresistência (AGARWAL; KAYE, 2003; GATTI; ZUNINO, 2005; MOSCA et al., 2021).

A capacidade da célula tumoral de diminuir o influxo da droga acarreta ineficácia do tratamento, tal mecanismo é conhecido como resistência a múltiplas drogas do inglês multidrug resistance (MDR) e faz com que as células se tornem resistentes a quimioterapia devido a alteração no microambiente tumoral ou farmacocinética da droga (AGARWAL; KAYE, 2003). MDR pode ser intrínseco quando o paciente já apresenta modificações genéticas pré-existentes ou adquirida, quando a resistência é induzida por quimioterapia (KELDERMAN; SCHUMACHER; HAANEN, 2014; LIPPERT; RUOFF; VOLM, 2008) e é capaz de elevar metabolismo de xenobióticos bem como elevar o sistema de reparo de DNA (BUKOWSKI; KCIUK; KONTEK, 2020).

Existe uma profunda correlação entre membros de proteínas transmembranas da superfamília de transportadores ABC e MDR (BUSH; LI, 2002). ABCB1 é o transportador mais evidente dessa superfamília e é também conhecido como glicoproteína-P (P-gp) (FLETCHER et al., 2010; ZHANG et al., 2015). P-gp é altamente expressa na superfície apical de células tumorais epiteliais de CO (FOJO et al., 1987; GOLDSTEIN et al., 1989; SCHINKEL et al., 1997) e associada diretamente ao mau prognóstico (BERGER et al., 2005; DEXTER et al., 1998).

A recorrência do CO deve-se ao fato de tal mecanismo realizar efluxo ativo de moléculas pela membrana celular. O mecanismo envolvendo P-gp está bem elucidado no CO e é responsável por reduzir a concentração intracelular de PTX (DING et al., 2022). GUO et al., (2019) demonstraram que a P-gp está presente na mitocôndria de células de CO humanas associadas com a resistência ao taxol e é mediada pelo efluxo de drogas existentes na organela.

Muitas mulheres desenvolvem resistência ao PTX por diversos mecanismos entre eles, mudanças no mRNA tendo a presença de múltiplos genes ribossomais,

alteração na síntese proteica, estresse oxidativo, glicólise e metabolismo da glutathione (AGARWAL; KAYE, 2003). A resistência ao paclitaxel pode estar juntamente associada com múltiplas alterações no alvo intracelular, é possível encontrar modificações frequentes nos níveis de tubulina α ou β , na mobilidade eletroforética de isoformas e acetilação de α tubulina (ZUNINO et al., 1999). Outro estudo demonstrou que em uma análise com 845 genes, 337 foram alterados em células neoplásicas resistentes a PTX (SHERMAN-BAUST et al., 2011).

Na maioria dos casos, o CO é diagnosticado tardiamente (BOWTELL et al., 2015; HOWLADER et al., 2018) e, embora muitos pacientes respondam ao tratamento padrão (cirurgia e quimioterapia), com o tempo, ocorre recidiva tumoral (BOWTELL et al., 2015; LUO et al., 2016) associada a quimiorresistência (CHUFFA et al., 2015). Assim, a neoplasia conta com um prognóstico ruim tendo uma taxa de sobrevida de 47,6% em 5 anos (HOWLADER et al., 2018).

Como a resistência a fármacos antitumorais é um fenômeno multifatorial, é necessário abordagens terapêuticas que atuem em diversos mecanismos (GATTI; ZUNINO, 2005).

Portanto, tem-se tentado obter resultados melhores com drogas combinadas ao PTX na tentativa de reduzir a quimiorresistência e aumentar sua sensibilidade ao tratamento. Contudo, os resultados são controversos e não foram adotados como padrões de tratamento (MARTH; REIMER; ZEIMET, 2017).

1.3. Mediadores inflamatórios e progressão tumoral

Receptores Toll like (TLR) são moléculas transmembranas capazes de promover inflamação local em resposta a infecções por patógenos e/ou moléculas relacionadas, sendo predominantemente expressos por várias células imunes e algumas células tumorais (GIRLING; HEDGER, 2007). Segundo (CURTIN et al., 2009), a via TLR pode promover o crescimento tumoral. KELLY et al., (2006) sugere que a quimiorresistência ao PTX deve ocorrer através de mecanismos ligados ao receptor TLR4 no CO.

Em decorrência da inflamação crônica gerada pelo processo de quimiorresistência, receptores TLR são ativados (KELLY et al., 2006), estes por sua vez estimulam proteínas quinases MAPKs, p38 e JNK bem como fatores de transcrição como o NF- κ B e outras STATs (LIM; STAUDT, 2013; ZHAO et al., 2021). Por conseguinte, a via composta por MAPK/ERK1/2 regula os fatores de transcrição JNK,

FOS, NF-kB e STATs sendo altamente expressos na progressão tumoral e responsáveis por processos decorrentes de proliferação, diferenciação, crescimento, migração e morte celular (DHILLON et al., 2007; SCHULZE-OSTHOFF et al., 1997).

Uma vez expressa, a via mediada por TLR, frequentemente alterada nos tecidos ovarianos, ativa MyD88 que contribui com a progressão tumoral e metástase além de estimular vias de resistência pró apoptóticas (ZHU et al., 2012). A atuação de TLR4 juntamente com a via de sinalização seguida de MyD88 colabora com a inflamação crônica local (HUANG et al., 2005). Sendo assim, a expressão reduzida e até bloqueio da sinalização de TLR4/MyD88 resulta em atenuação tumoral (CHEN et al., 2007).

Enquanto o TLR4, ativado em células imunes, apresenta atividade antitumoral, sua ativação em células neoplásicas promove secreção de moléculas pró-inflamatórias (IL-6, IL-8, TNF, NO, TGF- β , VEGF), resultando em progressão tumoral, resistência apoptótica, supressão do sistema imune, escape imunológico e resistência a terapias convencionais (LUPI et al., 2020). Estudos realizados em nosso laboratório mostrou aumento da ativação da via de sinalização mediada por TLR4, além da elevação dos níveis de IFN- β , IL-6 e TNF- α pelas células do CO induzido quimicamente (CHUFFA et al., 2015, 2018).

De maneira semelhante ao TLR4, diversos estudos associam o aumento do receptor TLR2 em CO, este estimula mediadores da cascata PI3K e promove progressão tumoral e metástase (PARK; CHUNG; KIM, 2017; ZHANG et al., 2015). Células neoplásicas apresentam, na maioria das vezes, alterações na via de sinalização PI3K e Akt, sendo frequentemente ativada (YUAN; CANTLEY, 2008). Estudos mostram que Akt atua na patogénia do câncer, sendo capaz de modificar a sobrevivência, proliferação e crescimento celular (VIVANCO; SAWYERS, 2002).

CHEFETZ et al., (2013) apontaram, em estudos *in vivo* e *in vitro* de CO, que a via inflamatória TLR2-MyD88-NF-kB, quando deflagrada no momento da cirurgia ou quimioterapia, favorece a liberação de diversos detritos celulares formando um processo de lesão e resultando em reparo tumoral e recidiva da doença. Novas abordagens são esperadas para o reconhecimento da ativação dessa via de sinalização.

A cascata sinalizadora induzida por MAPK é super expressa tanto nos tecidos quanto em linhagens celulares de CO (JIANG et al., 2019), esta transmite sinais extracelulares para alvos intracelulares desempenhando papel fundamental na regulação de processos básicos (KESHET; SEGER, 2010; SABIO; DAVIS, 2014). A via é

composta proteínas quinases como ERK1/2, p38 e c-Jun (JNK1/2/3) responsáveis pelo controle da proliferação celular, sobrevivência celular e morte celular (DAVIS, 2000; JOHNSON; LAPADAT, 2002). As JNKs são ativas através do estresse celular, respondem a estímulos extracelulares e estão constantemente presentes na quimiorresistência do PTX em OC (DHANASEKARAN; REDDY, 2008; HUANG et al., 2016).

A sinalização ERK1/2 é ativada por meio de fatores mitogênicos, e sua redução pode auxiliar no tratamento de OC (ROBERTS; DER, 2007). Mais recentemente, JIANG et al., (2019) reforçaram que a via de sinalização MAPK/ERK é expressa em células OC (por exemplo, células SKOV-3), e tal via desempenha um papel vital no desenvolvimento e progressão da OC. Da mesma forma, o CREB, importante fator de transcrição, pode ser fosforilado por diversas quinases, incluindo ERK 1/2, Akt e p38, e sua ativação está relacionada à proliferação celular, apoptose, angiogênese e metástase (STEVEN et al., 2020).

A família STAT de fatores de transcrição se comunica com a membrana celular até o núcleo (JAMES E. DARNELL, 1997) e assim estão envolvidos em processos apoptóticos, proliferação celular angiogênica e supressão da imunidade antitumoral (LEVY; DARNELL, 2002; YU; PARDOLL; JOVE, 2009). Ativação de STATs principalmente 5 tem importante participação oncogênica envolvida no comportamento maligno do CO (SILVER et al., 2004). Estudos indicam que a ativação de STATs relaciona-se com avanço tumoral, maior resistência à quimioterapia e recorrência (CHEN et al., 2017; JINAWATH et al., 2010).

É comum as células tumorais expressarem níveis de PD-L1, alta expressão do *checkpoint* imunológico corrobora com um ambiente imune adaptativo e favorece o escape imunológico frente ao tratamento quimioterápico (OHAEBULAM et al., 2015). PD-L1 é expresso em macrófagos, linfócitos e algumas células epiteliais em perfil inflamatório (SHARPE et al., 2007). A expressão de PD-L1 se relaciona com um pior prognóstico em pacientes com CO (HAMANISHI et al., 2007). Ao se ligar com o receptor PD-1 presente em células T, o eixo PD-L1/PD-1 é capaz de inibir a ativação dos linfócitos corroborando com um aumento de citocinas e mau prognóstico (TAUBE, 2014; TUMEH et al., 2014).

A ativação da cascata inflamatória promovida por PI3K/AKT tem como consequência a sinalização de PD-L1 (CHEN et al., 2016). A expressão de PD-L1 também pode ser induzida por TLR via NF- κ B (HAN; LIU; LI, 2020). PENG et al.,

(2015) demonstraram que a sinalização de NF- κ B promovida por quimioterapia induz expressão de PD-L1 em células de CO, dessa maneira, outras combinações terapêuticas são necessárias uma vez que a quimioterapia provoca alterações imunológicas importantes no combate ao CO.

A produção desregulada de mediadores inflamatórios em neoplasias ovarianas segue associado com baixa sobrevida de pacientes (SAVANT; SRIRAMKUMAR; O'HAGAN, 2018). Dessa forma, a melhor compreensão da patogênese molecular do CO fornecerá subsídios para que novos alvos terapêuticos possam ser identificados, favorecendo a detecção precoce e a descoberta de novos tratamentos.

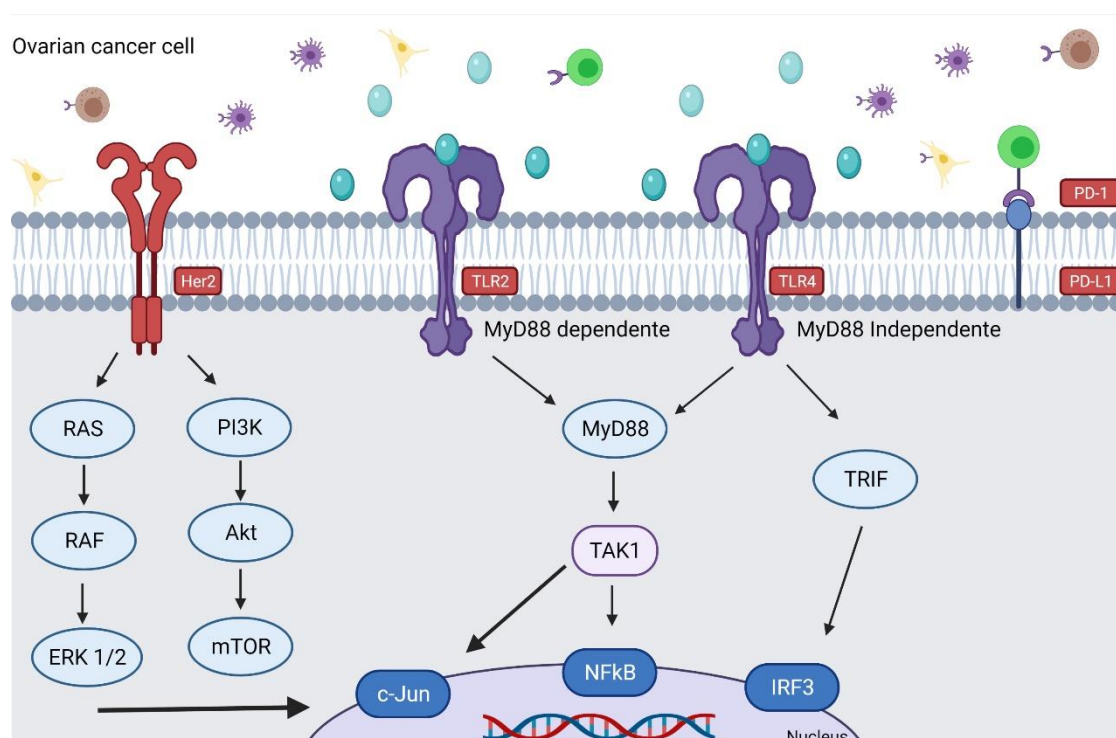


Figura 2 - Esquema representativo das vias de sinalização dos receptores HER2, TLR2, TLR4 e PD-L1. HER2 inicia a cascata sinalizada por RAS-RAF-ERK e PI3K-Akt e mTOR vias altamente expressas no câncer de ovário envolvidas na proliferação e sobrevivência celular. O receptor TLR2 ativa a via canônica enquanto o TLR4 ativa a via canônica e a não canônica, envolvidos na expressão moléculas inflamatórias. O ligante de morte programada PD-L1 é uma proteína altamente expressa em câncer de ovário que ao se ligar no receptor PD-1 tem capacidade de desativação de células T.

1.4. Melatonina: propriedades antitumorais e sobrevivência no CO.

A melatonina (Mel), hormônio secretado pela glândula pineal durante a fase de escuro, possui marcante versatilidade funcional com propriedades antioxidantes, oncostáticas e imunomoduladoras (REITER et al., 2017; REITER, 2004). Várias pesquisas têm chamado atenção para a relação entre o controle neuroimunomodulatório e o aparecimento e progressão do câncer. CONTI et al., (2000) relataram um duplo papel da Mel atuando como agente imunomodulador: 1) por ativação do sistema imune

seguido de um aumento da atividade de macrófagos, linfócitos T helper (Th1) e produção de citocinas; 2) por reajuste a longo-prazo das funções imunológicas devido à regulação circadiana, afetando hematopoiese e divisão celular. Um estudo anterior verificou que a administração de Mel suprimiu a expressão de TLR4 e, consequentemente, reduziu os níveis dos fatores MyD88, TRIF (IRF3), I κ B- α citosólica e NF- κ B/p65 (Figura 2) nuclear em modelo experimental *in vivo* de CO (CHUFFA et al., 2015). Além disso, vários registros apontam a Mel como atuante na prevenção das etapas de iniciação, promoção e progressão tumoral. Foi demonstrado que a eficácia da Mel em limitar a proliferação de células tumorais depende do período de administração, sendo o final da fase clara e início da fase escura mais indicada (SAUER; DAUCHY; BLASK, 2001). Os efeitos oncostáticos da Mel são mais pronunciados em tumores hormônio dependentes (mama e ovário), provavelmente por antagonizar a mitose mediada por estrógeno (HILL et al., 1992). Em adição aos efeitos oncostáticos, a Mel protege os precursores hematopoiéticos de possíveis efeitos tóxicos causado pela quimioterapia (SRINIVASAN et al., 2008).

Estudos mostram que a expressão do receptor de Mel em células ou tecidos tumorais, via ativação de MT1 e MT2, promoveu efeitos antiproliferativos e protetores em vários tipos de câncer (JABLONSKA et al., 2014). SHEN et al., (2016) observaram importantes efeitos na inibição do crescimento tumoral com a administração de Mel nas doses de 400, 600 e 800 μ M em células OVCAR-429 e PA-1; como consequência, houve atraso das fases G1/S e regulação negativa de CDK2 e CDK4. O uso da Mel como adjuvante terapêutico atenuou resposta inflamatória em células CO por meio da inibição de vias de sinalização canônicas (NF- κ B, p38 MAPK) e não canônicas (IRF3), logo, pode-se reduzir a resistência dos efeitos colaterais causados por quimioterapia (CHUFFA; REITER; LUPI, 2017).

Estudos em modelo *in vivo* de CO utilizando doses de 200 μ g de Mel por 60 dias evidenciaram redução na massa e volume tumoral e incidência de adenocarcinomas em ratas adultas, bem como indução de apoptose (CHUFFA et al., 2013, 2016). Esses achados demonstraram a efetividade da Mel contra o CO, porém um melhor detalhamento desses processos em modelo de célula humana frente ao tratamento com PTX pode apontar outros mecanismos envolvidos.

FERREIRA et al., (2014) demonstraram que em ratas tratadas com Mel na dose de 200 μ g/100g peso corpóreo/dia mostraram regulação negativa de Her-2 e de moléculas relacionadas com a via MAPK, Akt e mTOR. Portanto, a diminuição de Her-

2 pode auxiliar na melhora tumoral em CO resistente a quimioterapia. No entanto, é necessário o reconhecimento da ativação desta via pela Mel na presença de PTX.

AKBARZADEH et al., (2017) evidenciaram que a Mel também inibiu a proliferação de células tumorais isoladas de SKOV-3 por meio da modulação de PI3K e MAPK, agindo como um importante mecanismo na supressão tumoral. (WANG et al., (2012) constataram uma inibição por meio da Mel na fosforilação de NF- κ B e Akt/PI3K em células de câncer de mama MDA-MB-361, o que levou, consequentemente, a uma ativação da via apoptótica Apaf-1. Contudo, são necessários mais estudos que examinem as vias da Mel associadas ao PTX, para alcançar maior compreensão dos seus benefícios.

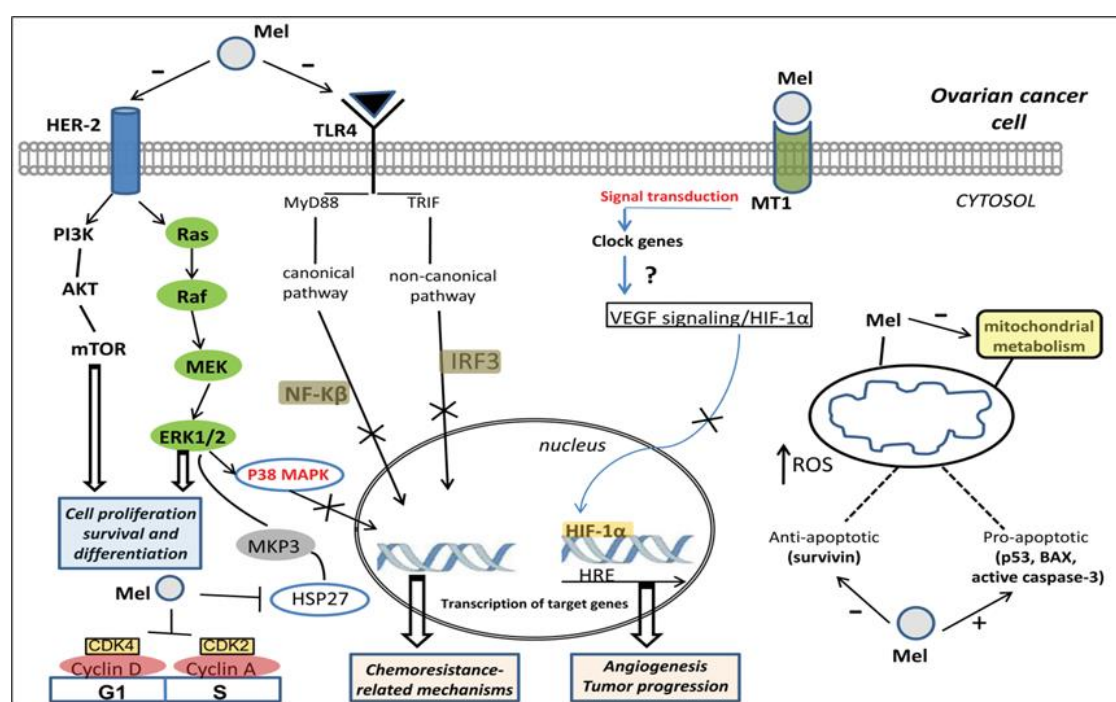


Figura 3 - Representação das vias atuantes da Mel no câncer de ovário. O hormônio tem capacidade de inibir proliferação, sobrevivência, migração celular, inflamação e angiogênese. Mel foi capaz de atenuar vias relacionadas a proliferação e quimiorresistência tais como Her2 e TLR4, ademais, inibiu ERK1/2 e NF- κ B responsáveis por aumentar genes de transcrição que facilitam o mau prognóstico. Adaptado de Chuffa et al. 2017.

2. Justificativa

Mecanismos desconhecidos que participam do desenvolvimento e progressão do CO podem estar diretamente associados à atividade inflamatória e resistência à apoptose. Quimioterápicos desempenham atividades fundamentais no controle do crescimento tumoral; no entanto, de forma súbita, muitas mulheres desenvolvem quimiorresistência e a progressão tumoral avança drasticamente. O mecanismo de quimiorresistência pode estar ligado ao padrão de resposta particular resultando em óbito da paciente. Tendo a propósito as terapias funcionais como ferramentas úteis no

entendimento dos aspectos moleculares que controlam a carcinogênese do CO, o estudo visa explorar os efeitos *in vitro* da melatonina associado ao PTX sobre a via inflamatória mediada por TLRs e sobrevivência celular em linhagem de carcinoma ovariano. Portanto, o presente estudo investigará os seguintes aspectos: 1) Poderia a melatonina reverter a quimioresistência frente ao tratamento com PTX? 2) Qual seria o efeito associado da melatonina com PTX sobre a ação tumoral no CO? 3) Qual a participação das moléculas de *checkpoint* nesse cenário?

Além da terapia com melatonina não promover citotoxicidade em tecidos e órgãos saudáveis, tem mostrado importante ação moduladora sobre alguns mediadores do processo imune em tumores sólidos, podendo favorecer o prognóstico da doença, auxiliar no bem-estar do paciente e, principalmente, no entendimento dos mecanismos ligados a quimioresistência. A avaliação do CO, sob o ponto de vista inflamatório, a sobrevivência da célula tumoral, e o papel da melatonina como adjuvante terapêutico apontam como novas estratégias de combate ao CO.

3. Hipótese

O tratamento com melatonina potencializa os efeitos do PTX, diminui a taxa de migração e invasão celular enquanto promove apoptose. Além disso, a associação dos agentes poderá atenuar os efeitos ligados a quimioresistência e sobrevivência das células SKOV-3.

4. Objetivo Geral

Avaliar o efeito do tratamento com a melatonina associado ao PTX sobre o padrão de resposta inflamatória mediada por TLR e a sobrevivência das células de carcinoma de ovário humano (SKOV-3).

4.1. Objetivos Específicos

1. Avaliar os efeitos dos tratamentos com melatonina associado ao PTX na linhagem de células SKOV-3;
2. Avaliar a viabilidade celular, citotoxicidade, migração e invasão celular, apoptose e necrose frente aos tratamentos específicos;
3. Quantificar os receptores TLR2 e TLR4, e os fatores imunológicos MYD88, TRIF e PD-L1 envolvidos no processo de resposta imune;
4. Quantificar os p-PI3K, p-Akt envolvidos na progressão tumoral;
5. Quantificar os mediadores de sobrevivência celular NF- κ B, ERK1/2, STAT5, CREB, p38 e JNK.

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Capítulo 2

Melatonin Research

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

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Running title: Melatonin and paclitaxel in the OC cell

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- κ B 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 stage (4, 5). In early stages, surgery and chemotherapy with platinum derivatives are the choice whereas in the advanced stages 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 frequent (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 (TLR)4 (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- κ B (NF- κ B). NF- κ B 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% CO₂. All cells were expanded in 75 cm² cell culture flasks (Costar, Cambridge, MA, USA), containing the basal culture medium which were 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 (1.6mM, 3.2mM, 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.

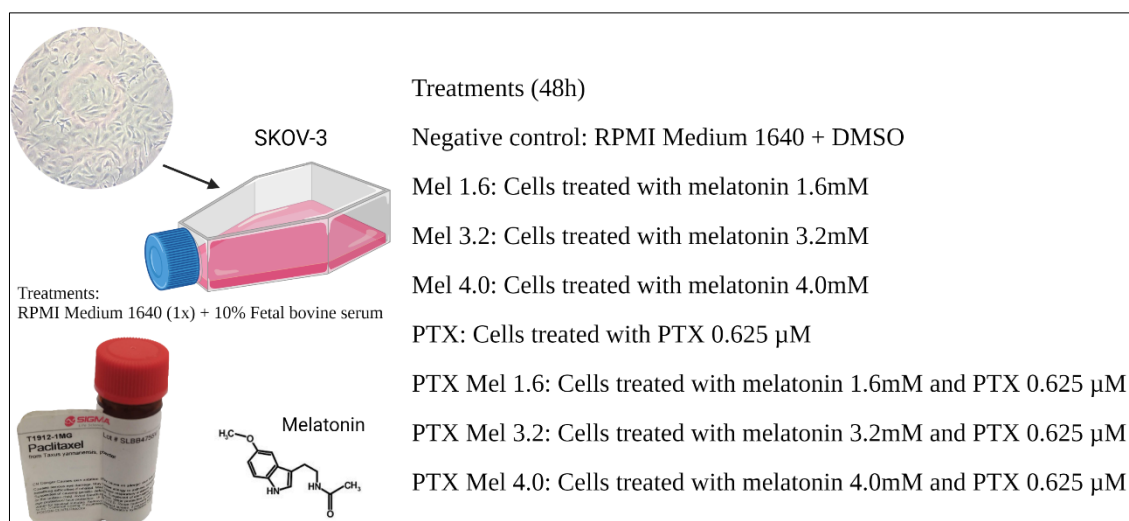


Fig. 1. Schematic representation of the experimental protocol.

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 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 1×10^4 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, Darmstadt, 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-κB, 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 (1×10^5) 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 CO₂ atmosphere at 37°C 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 $\pm$ 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 and necrosis (Annexin V-/PI+). Based on previous studies, the increasing concentrations of melatonin were used to promote cell death. As shown in Figure 2 A-D, apoptosis and necrosis rates were enhanced by the melatonin treatment following 1.6mM, 3.2mM, and 4.0mM and PTX 0.625 μ M alone for 48 h. Notably, the most effective combination was melatonin at 3.2mM and 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.

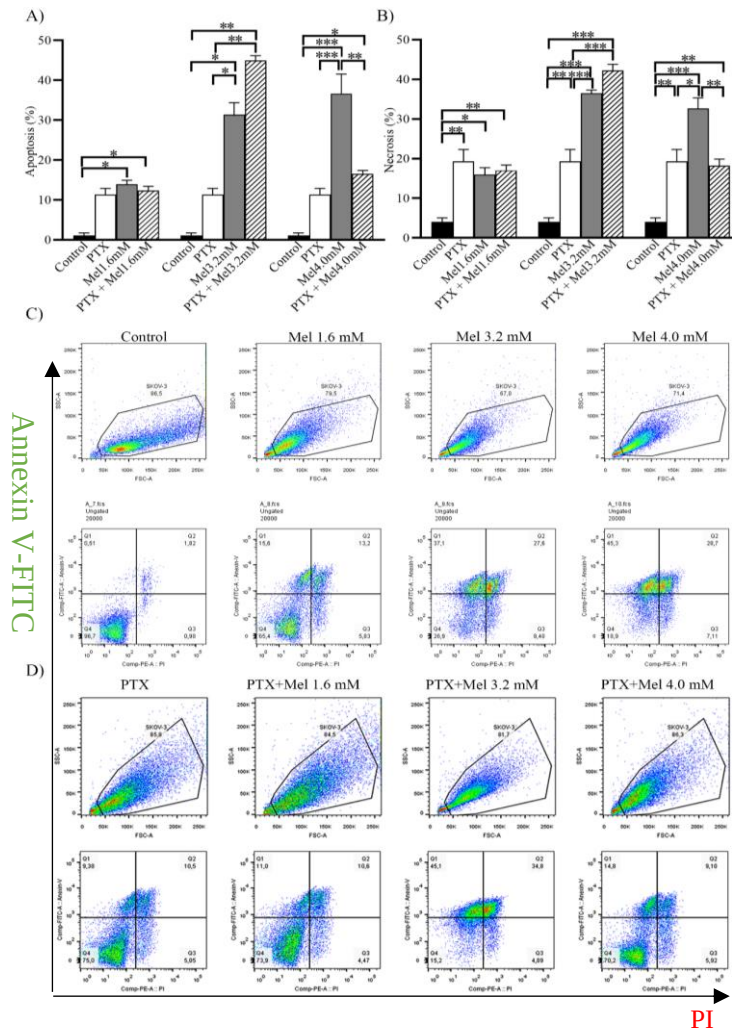


Fig. 2. Effects of the combination of melatonin with PTX induce apoptosis in SKOV-3 cells.

SKOV-3 cells were treated with melatonin and PTX + melatonin exposure 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 $\pm$ 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. More importantly, the combination treatment of melatonin and PTX restored the concentration of melatonin close to that in healthy ovarian cells (Figure 3 A).

To better understand whether the combination of melatonin and PTX reduced mitochondrial activity, 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%).

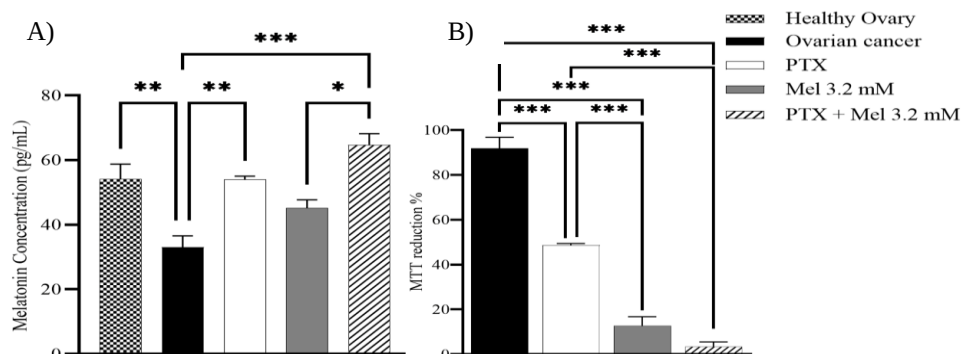


Fig. 3. Melatonin levels and cell viability.

A) Intracellular concentration of melatonin after treatment with PTX and PTX/melatonin. Healthy ovary, ovarian cancer (control), PTX, Mel 3.2 mM and PTX + Mel 3.2 mM. B) MTT reduction after treatment with PTX and PTX with melatonin. Ovarian cancer: control SKOV-3 cells, PTX: paclitaxel, Mel: melatonin. Data were analyzed using three technical and biological replicates and expressed as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. One-Way ANOVA complemented by Tukey's test.

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

To determine whether the treatments impact the TLR-mediated inflammatory signaling pathway, 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).

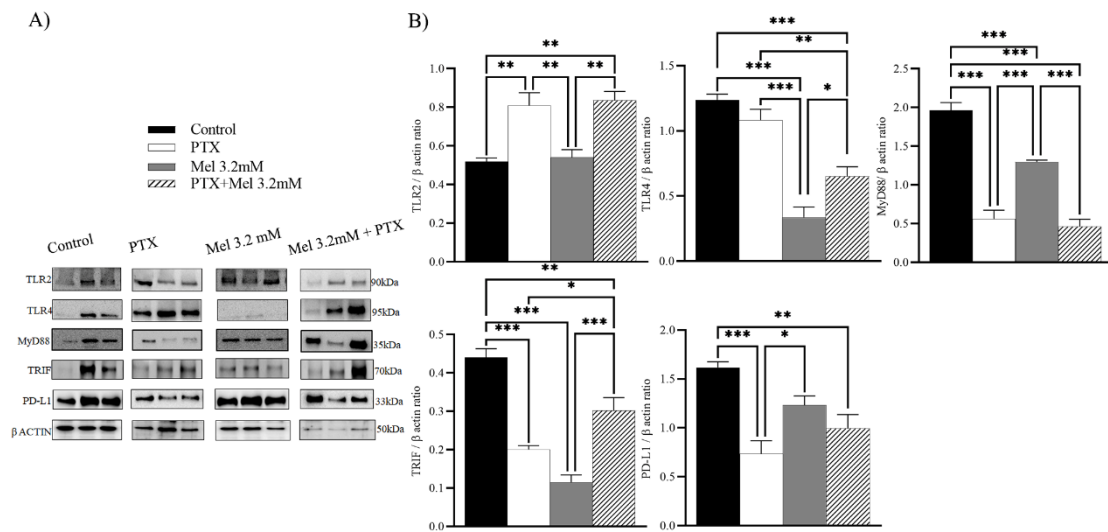


Fig. 4. Effects of the PTX, and PTX + Melatonin on TLR-mediated inflammatory pathway.

A) Representative profile of the TLR2, TLR4, MyD88, TRIF, and PD-L1 in the cells. B) Optical densitometric analysis of TLR2, TLR4, MyD88, TRIF, and PD-L1 levels in ovarian cancer cells after normalization with β -actin. Control, Mel: melatonin. Data expressed as mean $\pm$ 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).

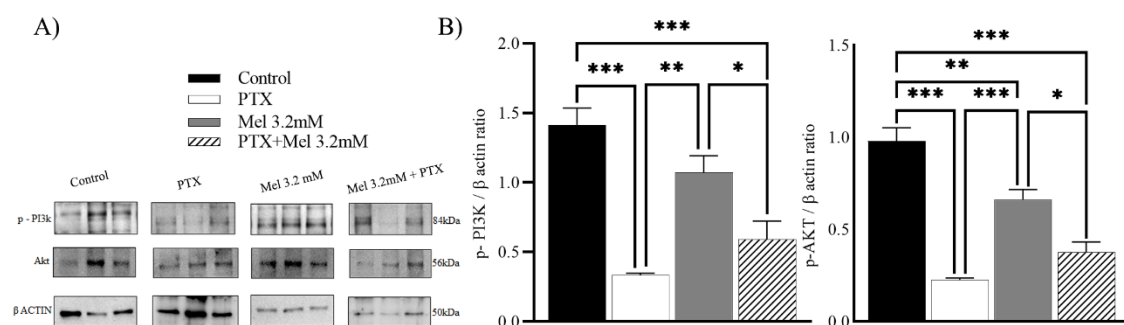


Fig. 5. The regulatory effects of PTX or melatonin on the expressions of PI3K and AKT levels.

A) Representative profile of the proteins p-PI3K and p-AKT in cellular extracts of 40 μ g 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 $\pm$ 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.

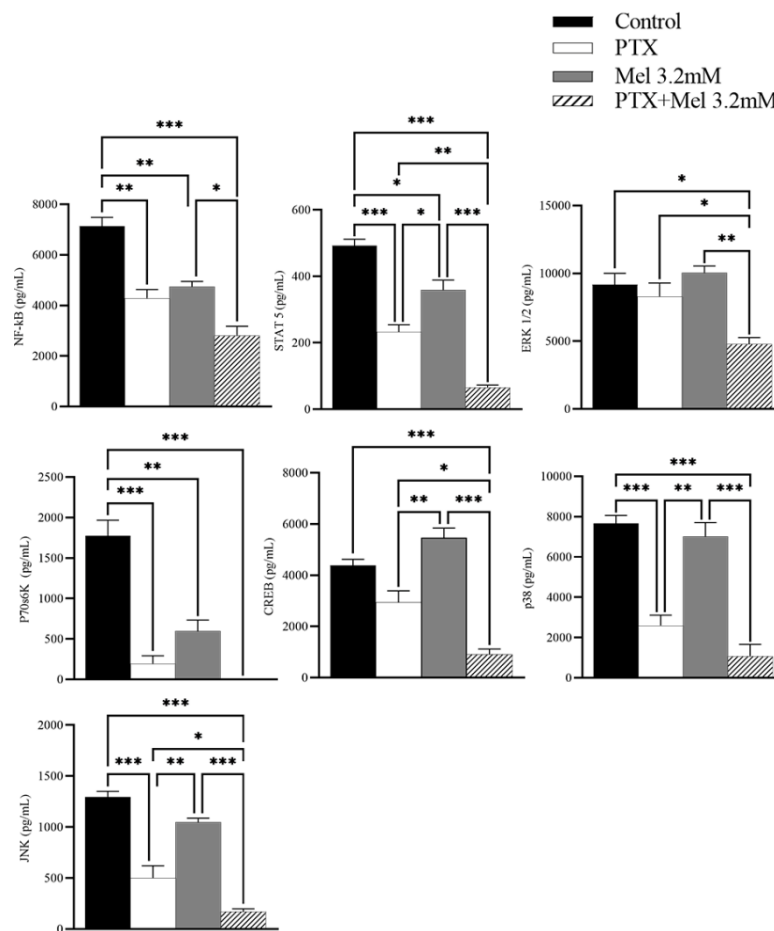


Fig. 6. Effects of melatonin and/or PTX on the cell signaling-related molecules in the SKOV-3 cells. Concentrations of CREB, JNK, NF-kB p65, p38, ERK1/2, p70S6K, and STAT5 were evaluated in the supernatants of cell culture. Mel: melatonin. Data expressed as mean $\pm$ SEM of triplets. * P <0.05, ** P <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).

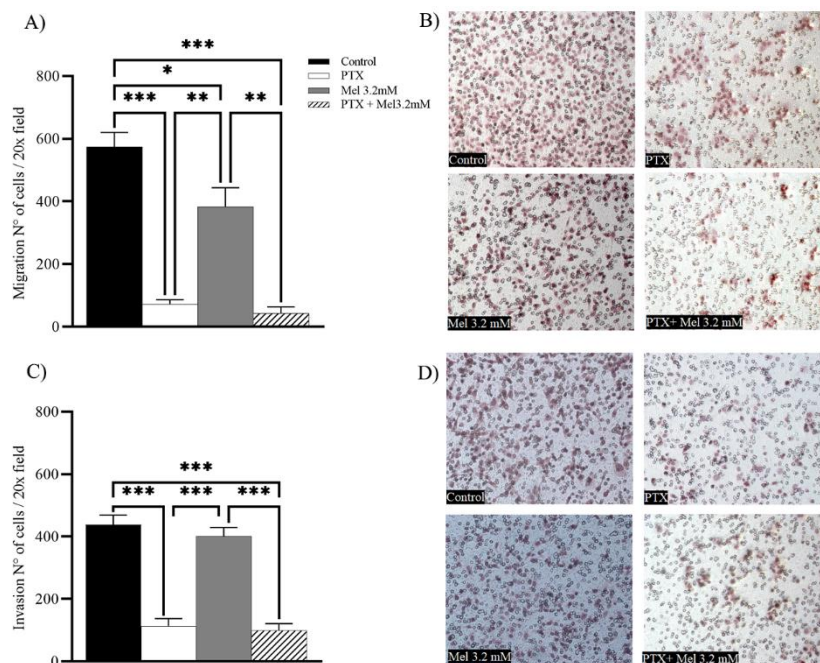


Fig. 7. Effects of melatonin and PTX on the migration and invasion capacity of SKOV-3 cells.

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 $\pm$ 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, inflammatory molecules and stimulating the immunomodulatory capacity (39, 46). These mechanisms have been confirmed in the current study. Considering the beneficial effects of melatonin on chemoresistance in different cancer types (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 μ M) 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 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 ($\sim$ 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- κ B 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- κ B/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- κ B.

In previous study, by using the DMBA-induced OC animal model, we showed that long-term (60 days) of melatonin (200 μ g/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- κ B p65 levels. In OC, NF- κ B 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- κ B, thus preventing its translocation to the nucleus and blocking the production of pro-inflammatory cytokines (68). The reduction of NF- κ B 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 combination treatment 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 as well as the p70s6K ribosomal protein. The proteins of 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 animal and cell levels (43, 57). During tumor invasion and metastasis, the pathway of Akt/p70s6K is often activated, serving as a potential prognostic marker for tumor chemoresistance (89). Melatonin reduced the MAPK signaling intermediates in different cancer types (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.

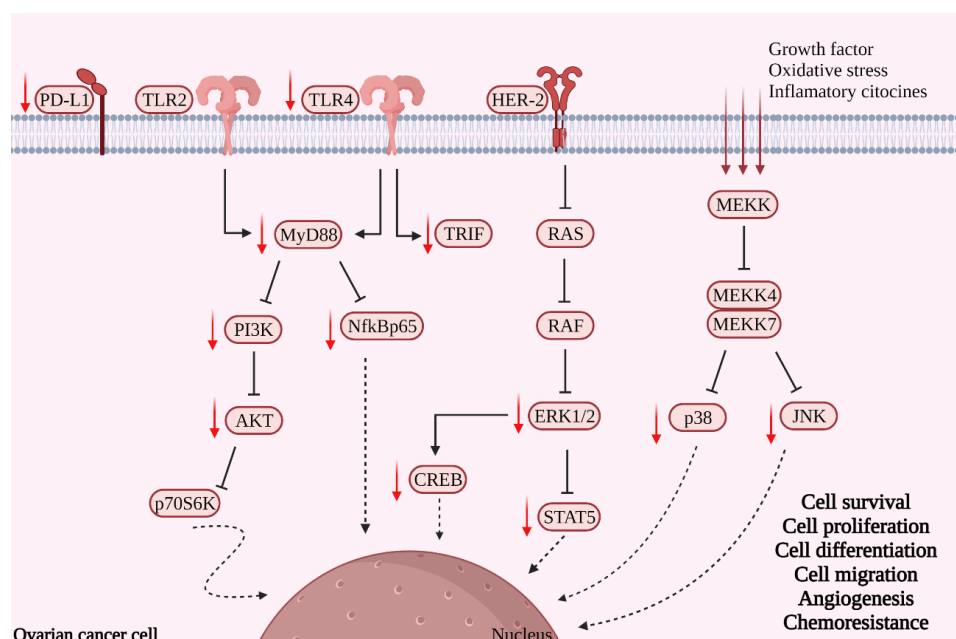


Fig. 8. Schematic representation of the melatonin and PTX actions in different targets related to inflammation and survival of ovarian cancer cells.

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-κB, 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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