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Melatonina e seus receptores MT1 e MT2: efeitos na apoptose, proliferação e potencial migratório de células de carcinoma ovariano (linhagem SKOV-3)

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Orientador

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

Profº Drº Luiz Gustavo de Almeida Chuffa

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Palavras-chave: Apoptose; Câncer de ovário; Efeito Warburg; Melatonina; Receptores MT1.

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Resumo

O câncer de ovário (CO) é a malignidade ginecológica que mais acomete mulheres e a quinta causa de morte por câncer devido a rápida progressão e resistência aos tratamentos. A melatonina, um neurohormônio secretado pela glândula pineal e tecidos extrapineais, tem mostrado propriedades antitumorais em ensaios *in vitro* e *in vivo*. Assim, o objetivo do estudo foi avaliar se a influência da terapia com melatonina sobre a apoptose, ciclo celular, migração/invasão, integridade mitocondrial, metabolismo energético e sinalização celular em células de carcinoma ovariano humano (SKOV-3) é dependente da ativação dos receptores MT1. Para isso, as células SKOV-3 foram expostas a diferentes concentrações de melatonina (1.6, 3.2 e 4 mM) em duas condições: células com expressão dos receptores MT1 e células silenciadas para os receptores MT1 através do RNA de interferência. A melatonina diminuiu a viabilidade celular, e aumentou as taxas de apoptose e necrose em todos os grupos tratados com melatonina, independente da ativação de MT1. A melatonina diminuiu o número de células com capacidade migratória e invasiva, e a marcação com PI mostrou parada do ciclo celular pela redução do conteúdo de DNA nas fases S e G2/M em células SKOV-3 tratadas com 3,2 e 4 mM. As concentrações das quinases Akt-ERK-PI3K, JNK, CREB e p38 diminuíram após o tratamento com melatonina. Os níveis intracelulares de melatonina foram reeestabelecidos após o tratamento com 3,2 mM. Houve uma diminuição no potencial de membrana mitocondrial nos grupos tratados com 4 mM. O consumo de glicose foi reduzido e a interconversão de lactato em piruvato aumentou após o tratamento com melatonina. Além disso, a atividade da enzima lactato desidrogenase diminuiu após tratamento com melatonina, mas aumentou significativamente após o silenciamento de MT1 em todas as doses. Análises usando GEPIA mostraram baixa expressão da enzima N-acetilserotonina O-metiltransferase (ASMT) em pacientes com CO. Com o auxílio da ferramenta UCSC XenaBrowser, mostramos uma correlação positiva entre o gene *ASMTL* humano e os genes da família transportadora de ATPases Na+/K+ (*ATP1A1* e *ATP1A3*), gene da succinato desidrogenase (*SDHD*) e genes da piruvato desidrogenase (*PDHA* e *PDHB*). Concluímos que a melatonina possui papel antitumoral nas células SKOV-3 na presença e ausência dos receptores MT1 e, além disso, o silenciamento dos receptores parece modular mecanismos antitumorais podendo ser considerado um possível alvo terapêutico. O entendimento desses efeitos poderão trazer novas perspectivas para o tratamento do CO associado ao papel pleiotrópico da melatonina.

Palavras chaves: câncer de ovário, melatonina, receptores MT1, efeito *Warburg*, apoptose.

Abstract

Ovarian cancer (OC) is a gynecological malignancy that most affects women and the fifth cause of cancer death due to rapid progression and resistance to treatments. Melatonin, a neurohormone secreted by the pineal gland and extrapineal tissues, has shown antitumor properties in *in vitro* and *in vivo* assays. Thus, the study aimed to evaluate whether the influence of melatonin therapy on apoptosis, cell cycle, migration/invasion, mitochondrial integrity, energy metabolism, and cell signaling in human ovarian carcinoma (SKOV-3) cells is dependent on the activation of MT1 receptors. To achieve this objective, SKOV-3 cells were exposed to different concentrations of melatonin (1.6, 3.2, and 4 mM) in two conditions: cells with MT1 receptors and cells silenced for MT1receptors through RNA interference (RNAi). Melatonin decreased cell viability and increased apoptosis and necrosis rates in all melatonin-treated groups regardless of the MT1. Melatonin decreased the number of cells with migratory and invasive capacity, and PI labeling showed cell cycle arrest by reducing the DNA content in S and G2/M phases in SKOV-3 cells treated with 3.2 and 4 mM. The concentration of Akt-ERK-PI3K, JNK, CREB, and p38 kinases decreased after melatonin treatment. Intracellular melatonin levels were re-established after treatment with 3.2 mM. There was a reduction in mitochondrial membrane potential after cell were treated with 4 mM. Glucose consumption was reduced, and interconversion of lactate to pyruvate increased after melatonin treatment. Furthermore, lactate dehydrogenase enzyme activity decreased after melatonin treatment, but significantly increased after MT1 silencing at all groups. By using GEPIA, we identified a low expression of N-acetylserotonin O-methyltransferase (*ASMT*) in patients with OC. Also, using the UCSC XenaBrowser tool, we showed a positive correlation between the human *ASMTL* gene and the genes of the Na+/K+ ATPase transporter family (*ATP1A1* and *ATP1A3*), succinate dehydrogenase gene (*SDHD*), and pyruvate dehydrogenase genes (*PDHA* and *PDHB*). We conclude that melatonin has an antitumor role in SKOV-3 cells in the presence or absence of MT1while MT1 silencing seems to modulate cellular mechanisms potentially being considered a therapeutic target. The understanding of these effects may bring new perspectives for OC treatment associated with the pleiotropic action of melatonin.

Keywords: ovarian cancer, melatonin, MT1 receptors, Warburg effect, apoptosis.

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Lista de abreviações

AA-NAT	- arilalquilamina N-acetiltranferase
AMPc	- monofosfato cíclico de adenosina
ASMT	- N-acetilserotonina O-metiltransferase
ATP	- adenosina trifosfato
BRCA 1 e 2	- gene do câncer de mama 1 e 2
CO	- câncer de ovário
ER	- receptor de estrógeno
RE	- retículo endoplasmático
FIGO	- Federação Internacional de Oncologia Ginecológica
GLUT1	- transportador de glicose 1
HIF	- fator induzido por hipóxia
LDH	- lactato desidrogenase
MMP	- metaloproteinase de matriz
MT1/2	- receptores de melatonina MT1 e MT2
PDK	- piruvato desidrogenase kinase
PEPT1/2	- transportador de peptídeo humano 1 e 2
PI	- Iodeto de propídio
ERNs	- Espécie reativa de nitrogênio
ROR/RZR	- receptor nuclear da família de receptor órfão
EROs	- Espécies reativas de oxigênio
Si-NC	- controle negativo do silenciamento
siRNA	- silenciamento gênico por RNA de interferência
TP53	- gene que codifica a proteína anti tumoral p53

Sumário

Capítulo 1

“So the problem is not so much to see what nobody has yet seen, as to think what nobody has yet thought concerning that which everybody sees.”

- Arthur Schopenhaue

1. *Introdução*

1.1 *Aspectos Gerais do Câncer de Ovário: incidência, fatores de riscos, classificação e tratamento*

O câncer de ovário (CO) é a malignidade ginecológica mais letal e representa a quinta causa de morte por câncer devido à falta de sintomas aparentes e resistência aos tratamentos, implicando em diagnóstico tardio e baixo prognóstico (Siegel *et al.*, 2022). Os fatores de risco mais comuns no aumento da incidência de CO estão relacionados ao número de gestações, menarca precoce, duração da menopausa, uso de contraceptivo hormonal, fatores genéticos e idade (Mok *et al.*, 2007; Flaum *et al.*, 2020). O prognóstico pode ser avaliado de acordo com a idade da paciente, o estadiamento proposto pela Federação Internacional de Oncologia Ginecológica (FIGO), volume residual após procedimento cirúrgico e *status* genético do tumor (análise de genes como o BRCA, por exemplo), entre outros (Jayson *et al.*, 2014; Zhong *et al.*, 2015).

O carcinoma ovariano primário pode ser de origem epitelial, mesenquimal, da célula germinativa, ou da camada da granulosa. Cerca de 90% dos subtipos de CO originam-se a partir da superfície epitelial que recobre o órgão ou de derivações do epitélio *mulleriano* (Jayson *et al.*, 2014; Chuffa *et al.*, 2017). Mais de 70% dos casos de CO são diagnosticados em estágios avançados da doença (estágios III ou IV). Esse é um tumor sólido e, diferentemente da maioria dos tumores, apresenta metástase hematogênica rara (Lengyel, 2010). O passo inicial para tal evento é a migração de células malignas para a cavidade peritoneal através da ação de proteases (Symowicz et al., 2007; Sawada et al., 2008; Öhlund et al., 2013).

Com base na taxa de crescimento e características moleculares da lesão originada do epitélio ovariano, podemos reconhecer o tipo I (com desenvolvimento lento e indolentes) e o tipo II (tumores agressivos). Os tumores do tipo I apresentam baixo grau de malignidade e abrange o carcinoma seroso micropapilar, carcinoma mucinoso, carcinoma endometrioide e carcinoma de células claras (Shih and Kurman, 2005; Stewart *et al.*, 2019). Já os tumores do tipo II possuem alto grau de malignidade e reúne o carcinoma seroso, carcinoma mesotelial misto (carcinossarcoma) e carcinoma indiferenciado (Bell and Scully, 1994; Stewart *et al.*, 2019).

Alterações somáticas no gene *TP53* estão presentes em grande parte dos tumores de ovário epiteliais de alto grau, e ainda, outros genes supressores de tumores como a *BRCA1* e *BRCA2*. A instabilidade genética presente nesse tipo tumoral auxilia no desenvolvimento de variantes, e assim

aumenta a diversidade genética e o desenvolvimento de subclones geneticamente distintos dentro do mesmo tumor. A instabilidade genética pode auxiliar no ganho de características que favoreçam essas células geneticamente distintas e assim, aumentando as taxas de sobrevivência desses tumores e resistência aos tratamentos padrões, como quimioterapia baseada em platina (Salomon-Perzyński *et al.*, 2017).

O tratamento convencional é feito através de cirurgia de redução da massa, com associação de quimoterapia ou neoadjuvantes (Vergote *et al.*, 2010). O câncer epitelial ovariano é considerado um tumor quimioresponsivo à terapia padrão com platina e/ou taxol, com aproximadamente 80% da taxa de resposta para o tipo seroso e endometrióide, e apenas 35% para o tipo mucinoso e de células claras (Cloven *et al.*, 2004; Ho *et al.*, 2004; Salomon-Perzyński *et al.*, 2017). Porém, com o diagnóstico tardio, as células tumorais podem adquirir características que a tornam quimioresistentes aos tratamentos convencionais sendo um fator limitante ao tratamento com frequente reincidência da doença e maior agressividade (Chuffa *et al.*, 2017). O desenvolvimento de quimiresistência leva a recorrência da doença e os pacientes tornam-se incuráveis (Kuroki and Guntupalli, 2020). Apesar das terapias antitumorais convencionais serem bem aceitas, há fatores limitantes na eficácia dessas terapias, além dos diversos efeitos colaterais, dessa forma pesquisas com novos agentes anticâncer estão sendo feitos com a finalidade de melhorar o prognóstico dessas pacientes (Talib, 2018).

Dentre os modelos de estudo do desenvolvimento tumoral, as análises *in vitro* através da cultura de células permite entender a biologia celular dos tumores e os mecanismos envolvidos na doença (Kapałczyńska *et al.*, 2018), sendo altamente eficientes e de alta reprodutibilidade. Por isso, modelos como a linhagem de carcinoma ovariano humano SKOV-3 (ATCC® HTB-77) são bem aceitas e amplamente utilizadas no estudo do CO. Essa linhagem é derivada de fluido de ascite peritoneal de uma paciente caucasiana de 64 anos com histórico de CO no ano de 1973. As células SKOV-3 são resistentes a drogas como cisplatina, adriamicina e fatores de necrose tumoral, além de apresentarem característica de células tumorais em estágios avançados da doença (Figura 1). Sendo assim, as células SKOV-3 são um ótimo modelo para testar uma substância neoadjuvante aos tratamentos convencionais e que pode potencializar a morte celular.

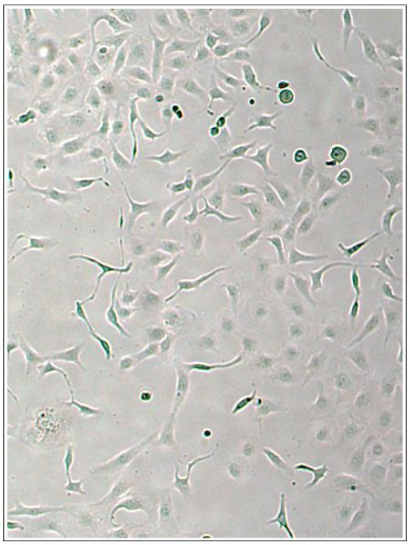

Figura 1. Células SKOV-3 em cultura apresentam características aderente, atipias morfológicas com predomínio do formato fusiforme e poligonal, e crescimento rápido. Acervo pessoal, 2020.

1.2 ***Melatonina e seus receptores: papel pleiotrópico no câncer de ovário***

A melatonina é uma pequena indolamina lipofílica (N-acetil-5-metoxitriptamina) secretada pela glândula pineal e em menor quantidade por outros tecidos, sendo principalmente responsável pela regulação do sono (Tosches *et al.*, 2014; Gandhi *et al.*, 2015; Bonmati-Carrion and Tomas-Loba, 2021). A melatonina apresenta função bem conhecida atuando como um potente antioxidante, removendo espécies reativas de oxigênio (EROs) e nitrogênio (ERNs) do organismo e aumentando a atividade de enzimas antioxidantes (Hu *et al.*, 2016; Reiter, S. Rosales-Corral, *et al.*, 2017). Além disso, a melatonina previne indução de apoptose em células normais (Reiter *et al.*, 2016). Entretanto, nas células tumorais, tem sido descrita com propriedades antiproliferativas, anti-angiogênica, pró-apoptótica e imunomoduladora, como mostrado na Figura 2 (Chuffa *et al.*, 2015, 2017; Zonta *et al.*, 2017; Talib, 2018; González-González *et al.*, 2020)(Chuffa *et al.*, 2015, 2017; Luiz Gustavo A. Chuffa, Lupi Júnior, Fábio R.F. Seiva, *et al.*, 2016; Zonta *et al.*, 2017; Talib, 2018; González-González *et al.*, 2020). Os mecanismos moleculares envolvidos com essas propriedades estão relacionados com a regulação da expressão de receptores de estrógeno, atividade de quinases, sinalização de cálcio, reorganização e função do citoesqueleto, sinalização celular através dos receptores de melatonina e metabolismo energético mitocondrial (Blask, Sauer and Dauchy, 2002; Gurunathan *et al.*, 2021a). A relação entre a ação da melatonina e o câncer vem sendo estudada

desde os anos 80 e estudos demonstraram atividades antitumoral em diversos tipos tumorais como mama, coloretal e próstata (Bonmati-Carrion and Tomas-Loba, 2021; Gurunathan *et al.*, 2021a). A atividade antitumoral da melatonina é mediada através da sua ação em diversos *hallmarkers* do câncer. A resistência aos tratamentos convencionais é um grande desafio no tratamento do câncer, particularmente nos carcinomas ovarianos (Kuroki and Guntupalli, 2020). A melatonina foi utilizada como terapia neoadjuvante na linhagem tumoral cervical HeLa, e mostrou aumento na sensibilidade ao tratamento com cisplatina pelo acúmulo de dano ao DNA e, consequentemente, aumento na taxa de apoptose (Pariente *et al.*, 2016).

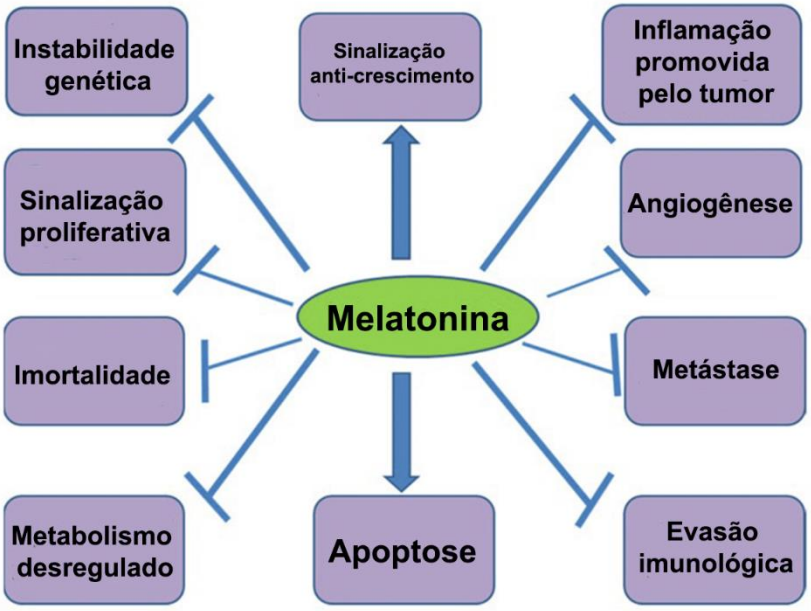

Figura 2. Efeito da melatonina nos diferentes *hallmarkers* do câncer. Seta: estimulação; Barra: inibição. Adaptado de Talib, 2018

Em ovários normais, a melatonina é secretada pelas células da granulosa do folículo pré-ovulatório e o tratamento adicional com melatonina pode influenciar na produção de hormônios essenciais na ovulação, qualidade oocitária e atividade antioxidante (Tamura *et al.*, 2009; Olcese, 2020). Interessantemente, na tentativa de explicar a origem primária do CO, estudos sugerem que células das fímbrias da tuba uterina poderiam sofrer transformações malignas pelo aumento de

EROs durante o processo ovulatório; neste contexto, a melatonina pode interromper o efeito tumorigênico induzido por EROs e proteger o tecido original (Huang *et al.*, 2015). O tratamento com melatonina, nas concentrações de 400 a 600 µM, reduziu a taxa de sobrevivência e proliferação das linhagens de células tumorais ovarianas OVCAR-429 e PA-1, aumentando o número de células na fase G1 do ciclo celular e diminuindo o número de células na fase S do ciclo (Shen *et al.*, 2016). Estudos *in vivo* reportaram os efeitos da terapia com melatonina em proteínas pro-apoptóticas (p53, BAX, caspase-3 total e clivada) e proteínas anti-apoptóticas (Bcl-2 e survivina) em modelos animais de CO papilífero seroso. E ainda, a melatonina promoveu aumento na expressão de p53, BAX e caspase-3 ativa, além de induzir a fragmentação do DNA observada pelo teste do TUNEL (Chuffa *et al.*, 2016; Chuffa *et al.*, 2017)

A melatonina pode atuar via receptores de membrana chamados de MT1/MT2, receptores nucleares (ROR/RZR) e ligantes de calmodulina (Hill *et al.*, 2015; Chuffa *et al.*, 2019). Estudos recentes mostraram o papel oncostático da melatonina responsável pela inibição da síntese de AMPc e deleção de proteínas kinases Akt/ERK/MAPK (Das and Samanta, 2021; Gaiotte *et al.*, 2022). Outros mecanismos deflagrados pela ativação de MT1 estão relacionados com a expressão de genes relacionados a angiogênese, proliferação celular e metástase (Hill *et al.*, 2015; Bhattacharya *et al.*, 2019; Kong *et al.*, 2020). A linhagem de células ovarianas normais IOSE 364 apresentam maior expressão de MT1 quando comparado à linhagens de células ovarianas neoplásicas como SKOV-3 e OVCAR-3 (Jablonska *et al.*, 2014)(Jablonska *et al.*, 2014b). Recentemente, foi identificado elevados níveis de MT1 em animais com CO após tratamento com melatonina (Chuffa *et al.*, 2017). Esses dados sugerem um importante, apesar de pouco compreendido, papel da melatonina associado a ativação da via mediada por MT1 na oncogênese do CO. A melatonina e seus análogos podem atuar ativando, simultaneamente, os heterodímeros MT1/MT2 em vários tipos celulares promovendo ação aditiva, sinérgica ou mesmo antagonista (Ayoub *et al.*, 2004). Agonistas dos receptores MT1/MT2 inibiram a proliferação da linhagem celular neoplásica de câncer de mama MCF-7, enquanto o efeito antiproliferativo da melatonina foi suprimido após tratamento com antagonista de MT1 e MT2 (Liu *et al.*, 2016). Desta forma, o melhor reconhecimento do potencial de interação entre a melatonina e seus receptores, frente as suas inúmeras respostas fisiológicas e moleculares na célula tumoral, poderá ser obtido através de estratégias de silenciamento genético de seus receptores MT1 e MT2 em linhagens bem estabelecidas de CO.

A propriedade anfipática da melatonina permite alcançar o meio intracelular independentemente do receptor MT1/2, ligando-se a receptores nucleares RZR/RORα e levando morte celular através da modulação da taxa BAX/BCL-2, alterando o metabolismo das células tumorais e inibindo a angiogênese (Hill *et al.*, 2015; Talib, 2018). Além disso, a melatonina pode ligar-se a calmodulina levando a parada no ciclo celular (Tan *et al.*, 2015; Reiter *et al.*, 2016), modulando genes responsáveis pela proliferação celular (Berchtold and Villalobo, 2014) e a via relacionada a stress celular via Sirt1 (Chuffa *et al.*, 2019). Zemla et al. (2017) tratou células da linhagem de carcinoma ovariano OVCAR-3 e SKOV-3 com melatonina (0,1-2mM) por 72h e observou redução da sobrevivência celular, em todas as linhagens celulares, independente do receptor MT1 (Zemła *et al.*, 2017). Petranka e colaboradores observaram efeito oncostático da melatonina em linhagem de células neoplásicas ovarianas BG-1; nesse estudo, a melatonina e o agonista do receptor RZR (CGP52608) foram efetivos na redução do crescimento celular e mostraram impacto significativo na apoptose (Petranka *et al.*, 1999).

Além das possiblidades já citadas, recentemente, foi descoberto outros dois transportadores capazes de interagir com a melatonina, sendo o transportador de glicose 1 (GLUT 1) e o transportador de peptídeo, PEPT1 e PEPT2 (Hevia *et al.*, 2015; Huo *et al.*, 2017), como mostrado na Figura 3. Dessa forma a melatonina é capaz de competir com a glicose, diminuindo o influxo de glicose para dentro da célula e contribuindo para redução do Efeito *Warburg* (Chuffa *et al.*, 2019). O transportador PEPT1/2 funciona, principalmente, como um facilitador da entrada de melatonina na mitocôndria, influenciando na resposta mitocondrial da célula tumoral (Chuffa *et al.*, 2019; Reiter, Ma and Sharma R, 2020).

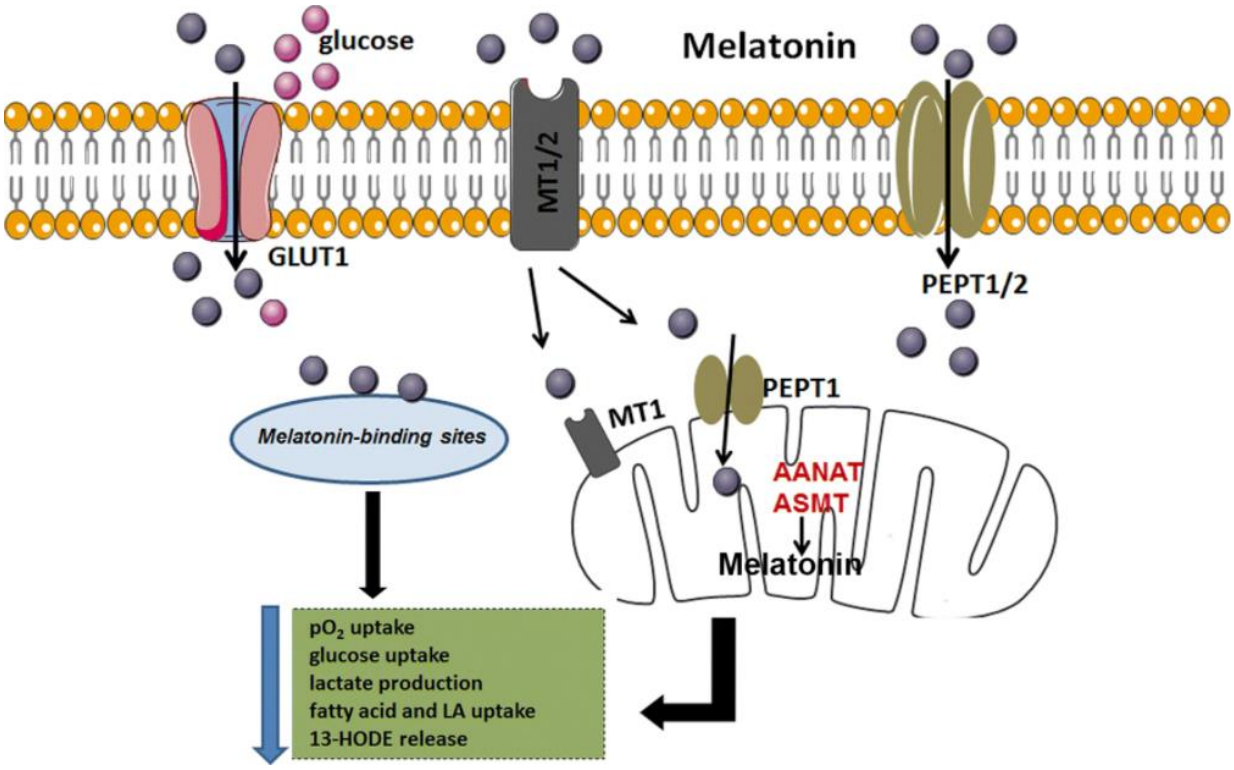

Figura 3. Vias de sinalização da melatonina nas células tumorais. A melatonina pode interagir com os receptores de membrana MT1/2, e também, com os transportadores GLUT1 e PEPT1/2. No citosol das células tumorais, a melatonina é capaz de alterar o metabolismo glicolítico através dos receptores MT1/2 e também de forma independente dos receptores levando a uma reversão do efeito *Warburg*. Adaptado de Reiter et al., 2020.

A interação da melatonina com o metabolismo energético da célula tumoral, em estudo realizado por nosso grupo de pesquisa, mostrou vias mitocondriais importantes em modelo *in vivo* do CO utilizando o emprego da estratégia proteômica global (Chuffa *et al.*, 2016). Outros estudos do nosso grupo de pesquisa mostraram o efeito da melatonina na diminuição da expressão de proteínas envolvidas em diversos processos metabólicos, como na geração de metabólitos e energia celular, vias associadas ao estresse do retículo endoplasmático e ao sistema imune e ainda proteoglicanos relacionados ao CO *in vitro* (Cesário *et al.*, 2022; Gaiotte *et al.*, 2022). Outras proteínas importantes relacionadas aos processos mitocondriais (ex: desidrogenases) e enzimas associadas ao metabolismo energético também tiveram baixa expressão após o tratamento com melatonina. Essas alterações metabólicas podem afetar a glicólise aeróbica (efeito *Warburg*), resultando na diminuição da proliferação e potencial metastático das células tumorais (Chuffa *et al.*, 2019; Reiter *et al.*, 2020). Tendo a propósito que a melatonina regula muitas funções

mitocondriais, existe a necessidade de se compreender qual tipo de relação é estabelecida na célula do CO, e se a síntese da melatonina na mitocôndria do CO é modulada pelos tratamentos.

1.3 Mitocôndria e melatonina: intimidade funcional dependente do "status" celular

Em células saudáveis, as mitocôndrias são organelas biossintéticas e bioenergéticas que exercem papel importante na regulação de processos fisiológicos, como proliferação, morte celular, adaptação metabólica e homeostase de Ca^{2+}. Além disso, são responsáveis pela oxidação de ácidos graxos, ciclo tricarboxílico, fosforilação oxidativa, entre outros processos bioenergéticos (Grasso *et al.*, 2020). EROs são formados como resultado da cadeia transportadora de elétrons, podendo ativar vias de transdução de sinal como as quinases MAPK/ERK e fator indutor de hipóxia (HIF) (Wallace, 2012). Em condições normais, o aumento na produção de EROs pode estar relacionado com a apoptose (Sullivan and Chandel, 2014). Na mitocôndria também ocorre a regulação do processo apoptótico através da modulação de proteínas pró- e anti-apoptóticas como BCL-2, BAX E BAK, através da liberação do citocromo *c* do espaço intermembrana da mitocôndria. Em síntese, o citocromo *c* estimula processos dependentes da ativação de caspases no citoplasma, levando a morte celular (Moldoveanu *et al.*, 2014). O desequilíbrio funcional na mitocôndria pode desencadear ou agravar uma série de doenças, incluindo o câncer.

As mitocôndrias sintetizam altas taxas de melatonina, podendo atingir concentrações na ordem de 100 vezes maior do que aquela encontrada no plasma (Acuña-Castroviejo, Escames, Venegas, Díaz-Casado, *et al.*, 2014). As enzimas limitantes na síntese da melatonina (AANAT e ASMT) e seus derivados foram encontrados na mitocôndria de pinealócitos e oócitos (Sakaguchi *et al.*, 2013; Coelho *et al.*, 2015), sugerindo que a mitocôndria exerce papel fundamental no catabolismo da melatonina (Tan *et al.*, 2016; Reiter, Ma and Sharma R, 2020).

A melatonina tem sido listada como um agente promissor que desempenha um papel efetivo na promoção da homeostase mitocondrial, regulando a fosforilação oxidativa e fluxo de elétrons, síntese de ATP, bioenergética, homeostase de cálcio e atividade do poro de transição de permeabilidade mitocondrial (mtPTP) (Martín *et al.*, 2002; López *et al.*, 2009; Farhood *et al.*, 2019; Mortezaee *et al.*, 2019). Além disso, a melatonina também demonstrou exercer a homeostase mitocondrial através da regulação do DNA nuclear e das atividades de transcrição do DNA mitocondrial (Acuña-Castroviejo, Escames, Venegas, Díaz-Casado, *et al.*, 2014). Essas atividades certamente ajudam a célula na prevenção de mutações do DNA e podem estar envolvida nos mecanismos responsáveis pela iniciação do câncer, resistência aos medicamentos e progressão da

doença. Na mitocôndria, a melatonina tem capacidade de capturar EROs diminuindo as suas concentrações e reforçando seu papel citoprotetor, inclusive nos ovários (Reiter *et al.*, 2000; Adriaens *et al.*, 2006). A ação da melatonina na mitocôndria foi reportada *in vivo*, onde ratos tratados com melatonina mostraram um aumento significativo na atividade dos componentes C-I e C-IV da cadeia respiratória de mitocôndrias isoladas (Martín *et al.*, 2000).

Na mitocôndria de células tumorais, existe maiores taxas de glicólise aeróbica, mecanismo bem conhecido como Efeito *Warburg*, gerando energia requerida para altas taxas de proliferação celular (Reiter *et al.*, 2000; Chuffa *et al.*, 2019). O Efeito *Warburg* permite que a célula tumoral prolifere rapidamente, evitando morte celular e promovendo características que levam a maior invasividade e metástase. Isso acontece, pois na glicólise ocorre maior liberação de lactato no microambiente tumoral, levando a maior instabilidade genética e ganhos de características que favorecem a progressão tumoral (Proietti *et al.*, 2017; Reiter, Ma and Sharma R, 2020). No desenvolvimento do câncer, a desregulação nas funções mitocondriais (Figura 4) está associada com a superprodução de EROs, que participa da sinalização oncogênica e progressão do tumor pela modificação irreversível do DNA e oxidação de proteínas (Ishii *et al.*, 1998, 2005). Neste contexto, após o desenvolvimento tumoral, a melatonina exerce um papel pró-oxidante e pró-apoptótico (Reiter, S. Rosales-Corral, *et al.*, 2017; Reiter, Ma and Sharma, 2020). No CO, os níveis de Bcl-2 parecem alterar a apoptose induzida por cisplatina, podendo até mesmo levar a quimioresistência via alteração na regulação do cálcio (Dai *et al.*, 2017).

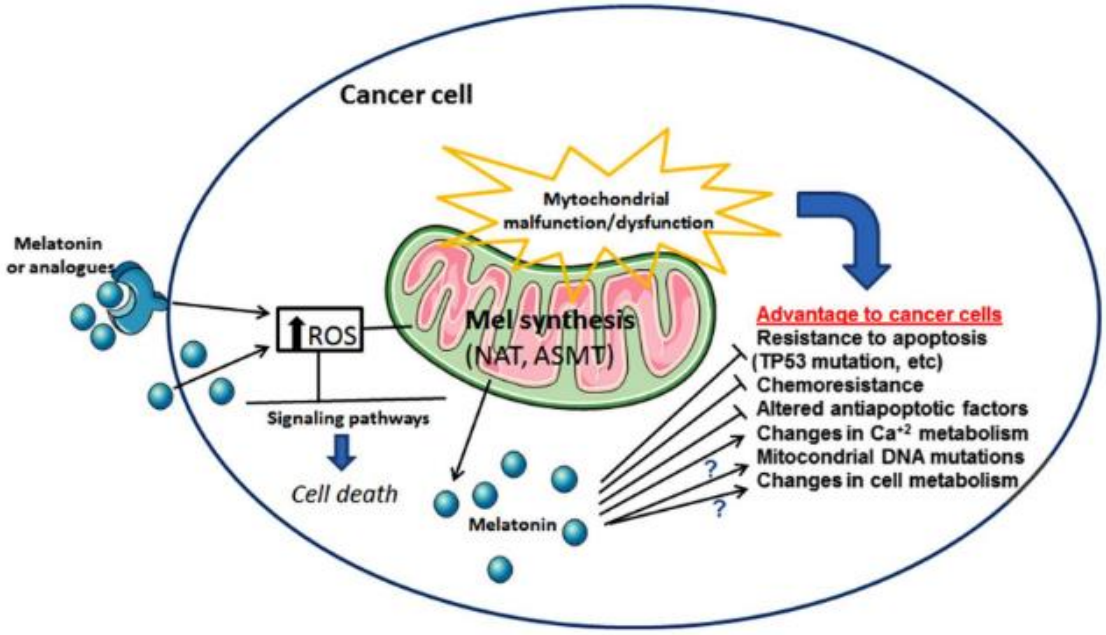

Figura 4. Disfunções mitocondriais em células tumorais podem levar ao aumento da malignidade dessas células. A melatonina afeta a produção de ROS e aumenta as taxas de morte celular pela via intrínseca da apoptose. Além disso, é capaz de reverter o metabolismo mitocondrial, contribuindo para a redução das vantagens das células tumorais. Adaptado de Chuffa et al. (2019).

O bloqueio do metabolismo glicolítico representa uma estratégia contra o crescimento celular, sendo a melatonina um novo agente antitumoral capaz de modular as funções mitocondriais das células tumorais. Coletivamente, a melatonina é capaz de regular a cadeia transportadora de elétrons através da liberação de Ca^{2+}, alterar a via glicólica diminuindo o Efeito *Warburg*, aumentar taxas de morte celular, seja isoladamente ou potencializando a ação de outros agentes. Desta forma, a melatonina figura como um promissor agente neoadjuvante aos tratamentos padrões, com alta eficiência em células tumorais resistentes às terapias já existentes.

2. Relevância e justificativa do tema

Considerando que os mecanismos moleculares envolvidos na proliferação e diferenciação celular, apoptose e resistência aos tratamentos podem compartilhar eventos comuns na mitocôndria da célula do CO, e tendo a propósito a terapia funcional com melatonina sobre essas vias de regulação, o presente estudo avaliou o processo apoptótico, migração e sinalização celular. Adicionalmente, é relevante compreender como se processa a síntese endógena de melatonina pelas mitocôndrias dessas células frente a diferentes doses de tratamentos; como a melatonina exógena interefere na maquinaria de produção endógena da melatonina mitocondrial no CO? A relação antiga e harmonica da melatonia com a mitocôndria tem sido bastante estudada e, contrariamente, em algumas células tumorais, a melatonina exerce um papel pró-oxidativo e pró-apoptótico. Portanto, como esses eventos ocorrem na célula do CO frente à terapia com melatonina é o ponto norteador dessa investigação. Somado a isso, evidências já relataram que os efeitos da melatonina na célula tumoral são, na maioria das vezes, mediados pelos receptores de membrana MT1 e MT2. Seriam os efeitos decorrentes do tratamento dependentes da ativação desses receptores na célula do CO? Ou eles ocorrem através de outros mecanismos ainda desconhecidos? O entendimento desses efeitos poderão trazer novas perspectivas para o tratamento do CO associado ao papel pleiotrópico da melatonina.

3. Hipótese

Considerando a importância da interação mitocôndria-melatonina nas células tumorais, a hipótese do estudo é de que o tratamento com melatonina altera a biosíntese da melatonina mitocondrial, regulando positivamente a apoptose e a maquinaria de sinalização celular e negativamente a migração das células SKOV-3, sendo em partes esses efeitos biológicos dependentes da ativação dos receptores MT1 e MT2.

4. Objetivos

4.1 ***Gerais***

Avaliar o efeito da terapia com diferentes concentrações de melatonina sobre as células de carcinoma de ovário humano (SKOV-3), com ênfase no processo apoptótico, sinalização celular, metabolismo energético e sobre a produção da melatonina mitocondrial. Verificar, ainda, se esses efeitos biológicos são mediados pelos receptores MT1 e MT2.

4.2 ***Objetivos específicos***

- ✓ Investigar se a ação da melatonina é dependente dos receptores MT1 em células SKOV-3 através de RNA de interferência (RNAi)
- ✓ Avaliar o efeito da melatonina na proliferação, morte e ciclo celular, além do potencial de membrana mitocondrial nas células SKOV-3;
- ✓ Avaliar potencial metastático das células SKOV-3, através de ensaio de migração e invasão celular utilizando insertos *transwell*;
- ✓ Quantificar, por ensaio de ELISA, os níveis de melatonina intracelular produzidos pela mitocôndria;
- ✓ Avaliar o consumo de glicose, liberação de lactato presentes no sobrenadante da cultura celular e ainda, avaliar a atividade da enzima lactato desidrogenage no extrato celular;
- ✓ Quantificar quinases envolvidas na via de sinalização intracelular (Akt, ERK1/2, JNK, p38, CREB, p70S6K, STAT3 e STAT5) por ensaio multiplex;

Capítulo 2

"As consequências do analfabetismo científico são muito mais perigosas em nossa época do que em qualquer outro período anterior"

- Carl Sagan

Melatonin induces cell death while suppresses metastatic capacity of ovarian cancer cells through attenuation of kinases signaling

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Abstract

Ovarian cancer has a high mortality rate among women, and melatonin exhibits various antitumor properties. Herein, we investigated the influence of melatonin therapy on cell dynamics and kinases signaling in human ovarian carcinoma cells (SKOV-3) and verified whether their effects are dependent on MT1 receptor. SKOV-3 cells were exposed to different melatonin concentrations in the presence of MT1 receptors (melatonin groups) or after MT1 knockdown (siRNA MT1+melatonin). MTT assay showed that melatonin reduced cell viability while Annexin V/PI labeling revealed high apoptosis and necrosis rates in all melatonin-exposed groups. Melatonin attenuated the migratory and invasive capacity of cells, and PI labeling showed cell cycle arrest by reducing DNA content in S and G2/M phase in SKOV-3 cells treated with 3.2 and 4 mM. The Akt-ERK-PI3K, JNK, CREB, and p38 kinases involved in cell survival, proliferation, motility, and stress response, were decreased after melatonin treatment. The MT1 receptor's knockdown spotlighted the anti-migratory and anti-invasive effect and further contributed to decreasing kinase associated with cell survival. In conclusion, SKOV-3 cells showed an oncostatic effect after melatonin treatment, and MT1 knockdown did not abolish its antitumoral actions in OC, thus indicating other potential mechanisms of melatonin's actions regardless of MT1 activation. However, MT1 receptor knockdown can act unfavorably on tumor cell progression.

Keywords: ovarian cancer; melatonin; MT1; apoptosis; migration; kinase signaling; SKOV-3 cells.

1. Introduction

Ovarian cancer begins with an uncontrolled cellular proliferation and rapidly spreads to the abdominal cavity, being 90% of these tumors from the epithelial origin (Gurunathan *et al.*, 2021b). Besides being less prevalent than other gynecological malignancies, the mortality rate is high, making this tumor the fifth death cause among women (Siegel *et al.*, 2022). Due to the lack of more accurate diagnostic methods, ovarian cancer is usually diagnosed late with a worse prognosis (Lheureux *et al.*, 2019). The standard treatment for ovarian cancer is cytoreductive surgical debulking and platinum-based chemotherapy; however, the recurrence rate after the development of chemoresistance is high (Cortez *et al.*, 2017).

Melatonin, a neurohormone secreted by the pineal gland and extra-pineal tissues, regulates primordial functions such as the circadian cycle and has anti-oxidant, immunomodulatory, anti-inflammatory, and anti-tumor effects (Tan *et al.*, 2016; Gheban, Rosca and Crisan, 2019). The melatonin's action can be dependent or independent of its receptors, termed MT1 and MT2; in some tumor types, the melatonin oncostatic activity is mediated by MT1 receptor activation (Talib, 2018; Ostrin, 2019). The melatonin receptor belongs to the G protein-coupled receptors and is activated by ligands such as melatonin, N-acetylserotonin, and clinical drugs (Cardinali, 2019). Activation of melatonin receptors causes detachment of the heterotrimeric G-protein, and the subunits formed interact with effector molecules related to cell signaling. Among the downstream pathways, activation of the kinases may be responsible for cell survival and proliferation, such as mitogen-activated protein kinases (MAPKs), including Akt/PI3K/ERK pathway and CREB, JNK, and NF-κB (Nikolaev, Robeva and Konakchieva, 2021). Recently, the relationship between MT1 and MT2 receptors and the kinases was observed in ELT3 leiomyoma cells (Lin *et al.*, 2020).

In addition, receptor-independent activities of melatonin showed potential interaction with tumor cells via two different transporters, such as glucose transporter 1 (GLUT1) and human peptide transporter 1 and 2 (PEPT1/2) (Hevia *et al.*, 2015; Huo *et al.*, 2017; Reiter, Sharma, Rosales-Corral, *et al.*, 2021). The PEPT1/2 are members of the solute carrier family transporters and melatonin has been shown to binds to the active site of PEPT1/2 (Huo *et al.*, 2017). Furthermore, GLUT1 belongs to an integral membrane protein and are member of a major facilitator superfamily; melatonin seems to interact with the same glucose site, thus competing with glucose uptake by tumor cells (Hevia *et al.*, 2015, 2017). Uptake transporters in plasmatic membrane make up cell gatekeepers and facilitate molecules entering the cell (Li *et al.*, 2019).

Several studies with different cancer types, including ovarian cancer, have documented the antitumor and oncostatic effects of melatonin both in *in vivo* and *in vitro* experiments (Li *et al.*, 2017; Reiter, S. A. Rosales-Corral, *et al.*, 2017; Chuffa *et al.*, 2019). These include upregulation of p53, BAX, cleaved caspase-3 and DNA fragmentation resulting in tumor cell apoptosis (Chuffa *et al.*, 2016; Das and Samanta, 2021). Furthermore, melatonin downregulated CDK 2 and 4, leading to cell cycle arrest in the G1 phase (Shen *et al.*, 2016; Moloudizargari *et al.*, 2021). Signaling pathways mediated by tyrosine kinase receptors often increase metabolism and cell growth. ERK1/2, Akt, and JNK kinases are MAP kinase-related families and, when activated by tyrosine kinase receptors, lead to cell proliferation, growth, and survival. The p38, p70S6k, CREB, STAT3, and STAT5 are protein kinases associated with cell survival, drug resistance signals, and metastasis pathways. More recently, we showed that melatonin combined with paclitaxel reduced JNK, CREB, NF-κB, p38, ERK1/2, p70S6K, and STAT5 (Gaiotte *et al.*, 2022). The current study investigated the pro-apoptotic and anti-invasive potential of melatonin, using different concentrations, in SKOV-3 ovarian cancer cell line and verified whether their effects are dependent on the MT1 activation.

2. Material and Methods

2.1 Cell culture and reagents

The SKOV-3 cells (ATCC, Rockville, MD, USA) were cultured in RPMI 1640 medium (LGC Biotechnology, Brazil) supplemented with 10% Fetal Bovine Serum (FBS; Gibco, USA), 100U/ml Penicillin and 100μg/ml streptomycin (Gibco, USA). The cells were maintained in optimal conditions. The melatonin (Sigma-Aldrich, St. Louis, MO, USA) solution stock (1M) was dissolved in dimethyl sulfoxide (DMSO, Merck, Germany), and the working solution were dissolved in RPMI 1640 medium and incubated with SKOV-3 cells.

2.2 Experimental groups

SKOV-3 cells were seeded ($1x10^5$ cells) and reached the ideal confluence of 80%. Afterwards, cells were treated with three melatonin concentrations (1.6 mM, 3.2 mM, and 4.0 mM) or not (control group) for 48h. The cells were analyzed in two conditions based on the MT1 receptors: presence of MT1 receptors (melatonin-exposed groups) or knockdown of MT1 receptors

using RNAi (20 µg) (siRNA MT1+melatonin-exposed groups). The control groups either received DMSO (control group) or were MT1 silenced and received no treatment (siRNA MT1). All the experiments were assayed in three technical and biological replicates.

2.3 Oligonucleotides and transfection

The knockdown of *MTNR1A* and *MTNR1B* receptor was perfomed using RNA interference (RNAi) technology. Two oligonucleotides sequences (20 nM) (Silencer® Select siRNA; s224070 and s9051, Thermo Fisher) were diluted with Opti-MEM® Reduced Serum Medium (Thermo Fisher, USA). The transfection was carried out with RNAiMAX Lipofectamine (Thermo Fisher, USA) associated with oligonucleotides sequences for 24h. The negative control was performed according manufactures' indication (Silencer® Select Negative Control No. 1 siRNA, Thermo Fisher, USA). The silencing validation were done through RT-qPCR and Western Blot.

2.4 RNA isolation and RT-qPCR

The RNA extraction was performed using TRIzol ® and for RNA analysis were used NanoVue Plus Spectrophotometer (GE Healthcare, USA). The High Capacity RNA-to-cDNA Kit (Life Technologies) was used to transcribed cDNA. Amplification mRNAs was perfomed using SYBR Green Master Mix (Thermo Fisher Scientifica, USA) with 20 µL reaction and run on a QuantStudio 12K Flex System (Thermo Fisher Scientifica, USA), following the conditions: : 95º C for 10 min followed by 40 cycles of 95º C for 15 sec and 60º C for 1 min. We used National Center for Biotechnology Information (NCBI) to validated oligonucleotides and the mRNA used: *MTNR1A* (MT1) 5'-AGCTCAGGAACGCAGGAAAC-3' (forward) e 5'-CAGTGCAGATAGCCCAGGTT-3' (reverse); *MNTR1B* (MT2) 5'-CCGGAACGCAGGTAATTTGT-3'(forward) e 5'- GCCCAGCCGTCATAGAAGAT -3'(reverse); *RPS13* 5'-AGAAACGGCTACCACATCCA-3' (forward) e 5'-CACCAGACTTGCCCTCCA-3' (reverse); *GAPDH* 5'-GCTCCCTCTTTCTTTGCAGCAAT-3' (forward) e 5'-TACCATGAGTCCTTCCACGATAC-3' (reverse).

2.5 Cytotoxicity assay

SKOV-3 cells were seeded in a 96-well plate at a density of 1×10^4 cells/well. The cell survival was measured using a colorimetric method. After melatonin exposure, the MTT solution (5 mg/mL) was added for 4 h, and the presence of formazan crystals was determined by Epoch

microplate reader (BioTek Instruments, Highland Park, PO, USA) at 540 nm, being the reference curve fixed at 650 nm. The percentage of crystal formation was calculated by fixing the control group as 100%.

2.6 Cell Migration and Invasion Assay by Boyden Chamber

Transwell inserts BD BioCoat™ with 8 μm pores (BD Biosciences™) were used to assess the migratory cell potential. For the cell invasion assay, inserts were coated with Geltrex (Gibco, Paisley, UK) as determined by the manufacturer. In the upper part of the insert, SKOV-3 cells ($1x10^4$) were added in a medium, but without fetal bovine serum (FBS). In the lower part, a medium supplemented with 10% FBS was added. Afterward, the plates were incubated at 37°C with 5% CO_2 for 24h (the exposure time was determined based on the literature and according to the doubling time of the cells). After the incubation period, the cells were fixed in methanol for 10 min and those remaining on the top of the insert, were removed by scraping. The migrating cells were stained with hematoxylin solution for 10 seconds and photographed with a 20x objective under an inverted microscope (ZeissAxiovert®, Germany). The experiments were performed in triplicate using four fields and submitted to cell counting with ImageJ software.

2.7 Cell cycle assay

The cell cycle stages (G0/G1, S, and G2/M) were determined by flow cytometry by quantifying the amount of DNA by propidium iodide (PI). After treatments, SKOV-3 cells ($1x10^5$) were trypsinized, washed with PBS, and centrifuged for 5 min at 1500 rpm. After the cells were fixed with 70% ethyl alcohol at 4ºC for 1 h, the samples were incubated with a solution containing 50μg/mL of PI and 10 mg/mL of RNase A for 1 h at room temperature and protected from light. The assay was performed using FACSCanto cytometer (BD Biosciences, Clontech, CA, USA). Relative cell rates in G0/G1, S, and G2/M were calculated using FlowJo software (vX.10.6, Tree Star Inc.).

2.8 Cell death assay

After melatonin exposure, SKOV-3 cells were processed for flow cytometry analysis using apoptosis detection kit Annexin V (Becton Dickinson, Franklin Lakes, NJ). Cells were labeled with Annexin V and the vital dye PI to identify apoptosis and necrosis. For the analyses, viable cells are

negative for annexin V and PI, while cells in early apoptosis are positive for annexin V and negative PI. Cells in late apoptosis or necrosis stained positively for annexin V and PI. All data were acquired on the FACSCantoTM II cytometer (BD Biosciences) with FACSDiva software (BD Biosciences). The results were analyzed using the FlowJo software, version vX.10.6 (Tree Stars Inc.).

2.9 Cell signaling by multiplex immunoassay

Quantitative measurements of cellular signaling molecules were performed using MILLIPLEX enzyme-linked immunosorbent assay (48-681MAG kit, Millipore, Billerica, MA, USA). Samples were processed using cell lysis buffer with phosphatase and protease inhibitors and the protein concentration was obtained according to the Bradford assay (BioRad). The following probes were included: extracellular signal-regulated kinase/mitogen-activated protein kinase 1/2 (ERK/MAPK 1/2), protein kinase B (Akt), signal transducer and activator of transcription 3 (STAT3), c-Jun N-terminal kinase (JNK), ribosomal protein S6 kinase beta (p70S6K), signal transducer and activator of transcription 5 (STAT5A/B), cAMP-response element binding protein (CREB), and p38 mitogen-activated protein kinases (p38). The assay was carried out following the manufacturer's protocol, and fluorescence was assessed using xMAP technology on the MAGPIX platform (Luminex, Austin, TX). Mean fluorescent intensities (pg/mL) were obtained in triplicate for each sample and normalized to the total protein content.

2.10 Statistical analysis

Data were evaluated using analysis of variance (ANOVA) with independent factors complemented with Tukey's test for multiple comparisons. For non-parametric data, Kruskal-Wallis test was used complemented with Dunn's test. To assess the effects of MT1 silencing, we applied the Student's t-test between the silencing negative control group (si-NC) and the MT1-silenced group (siRNA MT1). Results were analyzed using GraphPad Prism software version 9.2, and data were expressed as the mean ± SD. Statistical significance was set at $P < 0.05$.

3. Results

3.1 Validation of the MT1 receptor silencing

The knockdown of the melatonin receptor genes (*MTNR1A* and *MTNR1B*) were performed using RNAi technology. The *MTNR1A* gene expression was knocked down after cell incubation for 24h (Figure 1A). The MT1 protein concentration analysis confirmed the receptor knockdown compared to the si-NC group (Figure 1 B, C). *MTNR1B* receptor gene expression and MT2 protein levels was not detected in the SKOV-3 cells.

3.2 Melatonin reduces cell viability regardless of the MT1

The cytotoxic capacity of melatonin was performed using an MTT assay. The treatment with different concentrations of melatonin for 48h decreased SKOV-3 cell viability (about ~ 20-40%) (Figure 1D). Interestingly, MT1 knockdown provoked an even greater reduction in cell viability compared with other experimental groups. MT1 knockdown was more forcefully in decreasing ovarian cancer cell viability.

3.3 Melatonin induced cell death in SKOV-3 cells

Annexin V (FITC)/propidium iodide (PI) labeling was used to detect the rate of apoptosis/necrosis induced by melatonin using flow cytometry. We observed morphological alterations in size patterns (FSC-A, forward scatter area) and cell granularity (SSC-A, side scatter area) over experimental groups. The apoptosis and necrosis rates increased significantly with melatonin and siRNA MT1 + melatonin groups than in the control and siRNA MT1 groups (Figure 1E). The MT1 silenced group that received melatonin had higher rates of cell death when compared to the melatonin-treated groups at 1.6 and 3.2 mM in the presence of MT1. By contrast, the concentration of 4 mM of melatonin showed an increased apoptotic rate compared with siRNA MT1 + melatonin group (Figure 1E). Necrosis rates were higher after the treatment with 3.2 and 4 mM of melatonin compared to the group siRNA MT1 + melatonin at the same concentration; the MT1 knockdown increased necrosis after treatment with 1.6 mM of melatonin. After melatonin treatment, cell death rates were enhanced regardless of the MT1 receptor (Figure 1F). SKOV-3 cells presented a larger size than granularity, but there was an inverse relationship when exposed to melatonin, thus presenting a smaller size than granularity (Figure 1G).

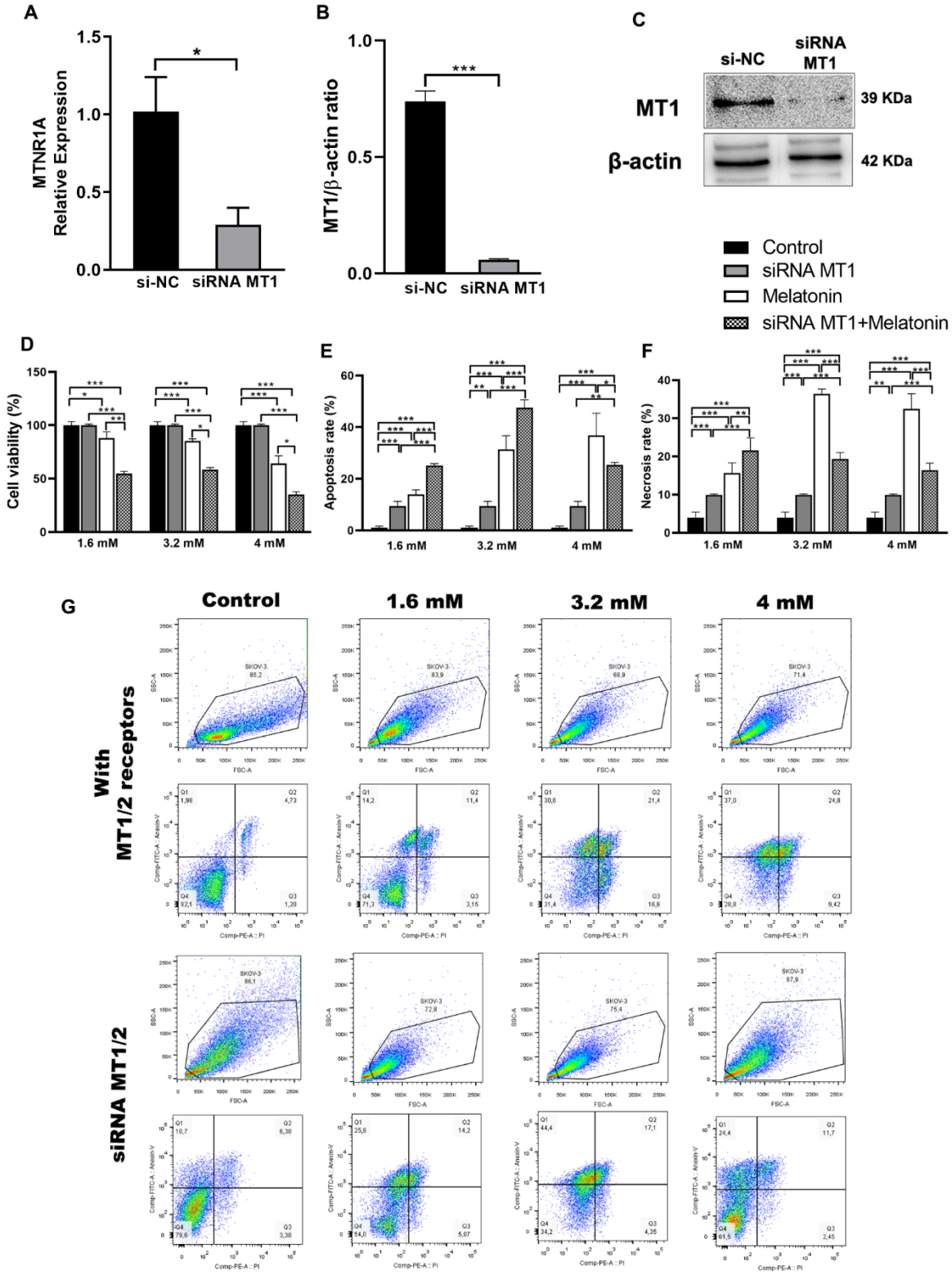
A
MTNR1A Relative Expression
si-NC
siRNA MT1
B
MT1/β-actin ratio
si-NC
siRNA MT1
C
si-NC
siRNA MT1
MT1
39 KDa
β-actin
42 KDa
Control
siRNA MT1
Melatonin
siRNA MT1+Melatonin
D
Cell viability (%)
1.6 mM
3.2 mM
4 mM
E
Apoptosis rate (%)
1.6 mM
3.2 mM
4 mM
F
Necrosis rate (%)
1.6 mM
3.2 mM
4 mM
G
Control
1.6 mM
3.2 mM
4 mM
With MT1/2 receptors
siRNA MT1/2

Figure 1. MT1 silencing, cell viability and apoptosis rate in SKOV-3 cells treated with melatonin. (A) Relative gene expression of the MTNR1A after silencing by RNAi for 24h. The RT-qPCR data were presented as fold-change (2-ΔΔCt) compared with the endogenous genes (RPS13 and GAPDH). (B) Densitometric analysis of the MT1 levels by western blotting was reduced with RNAi. (C) Representative profile of the MT1 protein after gene silencing. MT1 protein levels were normalized using endogenous protein (β-actin). (D) MTT assay showed a reduction in cell viability with melatonin treatment after 48h. (E) Rate of apoptosis and (F) Rate of relative necrosis in SKOV-3 cells detected by Annexin V/PI by flow cytometry. (G) Percentages of the representative cells in apoptosis and necrosis after exposure to melatonin for 48h. The samples were assayed in three technical and biological replicates. The data are expressed as the mean ± SD. *P<0.05, **p<0.01 ***P<0.001.

3.4 Melatonin has a prominent anti-migratory and anti-invasive effect in SKOV-3 cells

The migration and invasion assays were performed using transwell inserts to assess the role of melatonin. After MT1 receptor was knocked down, cells were seeded and treated with different concentrations of melatonin for 24h. The groups treated with melatonin at the 3.2 mM and 4 mM reduced migratory rate compared to the control group, while the 1.6 mM concentration showed a higher migratory potential. Importantly, the MT1 silenced groups, treated or not with melatonin, showed a low migratory rate than the control and melatonin-exposed groups at all concentrations (Figure 2 A, C).

For the cell invasion assay, the inserts were coated with Geltrex to mimic the basal membrane. Treatment with melatonin promoted lower cell invasion rates than the control group at all concentrations. The siRNA MT1 + melatonin group had a more significant reduction in the invasive rate than the control and melatonin-exposed groups. Since both siRNA MT1 and siRNA MT1 + melatonin-exposed groups presented lowest invasive rates, the lack of MT1 receptors could be responsible to mediate some of the anti-migratory and anti-invasive processes (Figure 2 B, D).

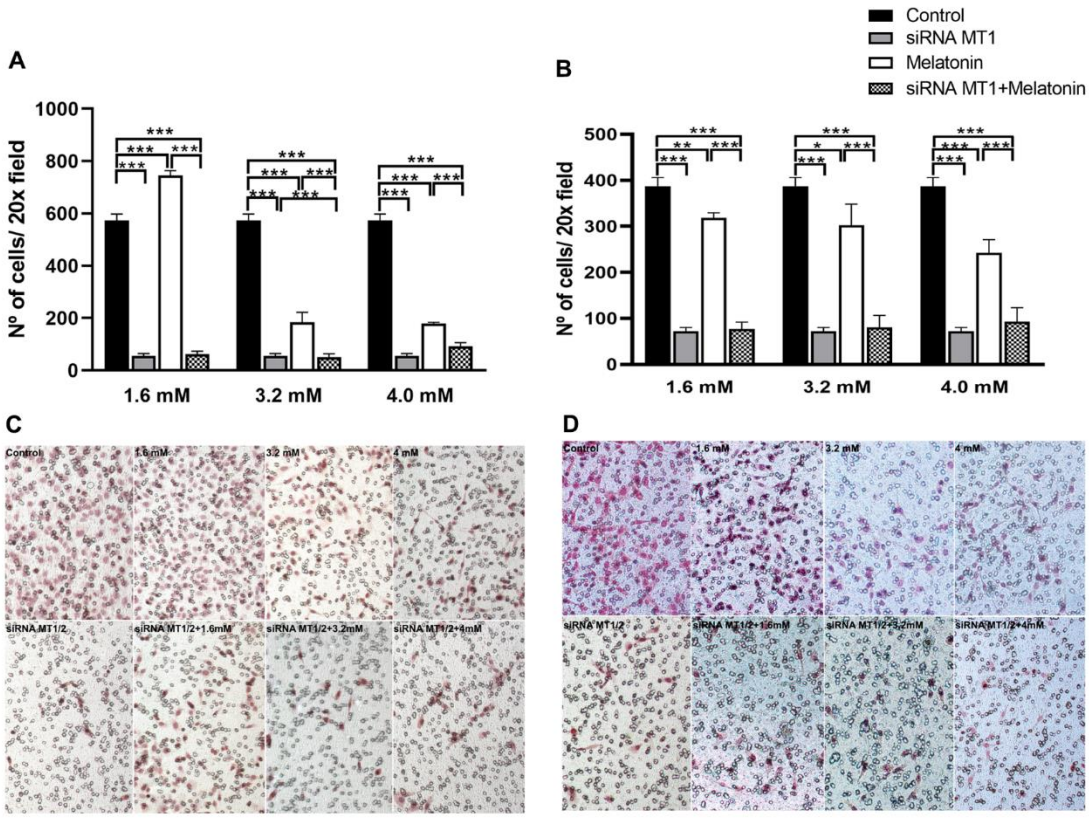

Figure 2. Melatonin changes the migration and invasion capacity of SKOV-3 cells. (A) Cell migration was calculated by the number of cells on the lower side of the chamber. (B) Cell invasion was measured by the number of cells in the lower chamber previously covered with Geltrex®. Representative images of migratory (C) and invasive (D) cells were obtained at 20X magnification. The assay was performed in three technical and biological replicates. The data are expressed as the mean ± SD. *P<0.05, ** p<0.01 and ***P<0.001.

3.5 Melatonin induces cell cycle arrest in ovarian cancer cells

SKOV-3 cells were PI labeled to determine the amount of DNA after melatonin treatment by flow cytometry. The siRNA MT1 + melatonin and siRNA MT1 groups increased DNA content in G1 phase compared to the control group; the group treated with 4 mM of melatonin also showed an increase in DNA content in the G1 phase compared to the control group (Figure 3A). Regarding the S phase, the groups treated with 3.2 and 4 mM of melatonin reduced the content of DNA whereas MT1 silencing associated with melatonin led to a profound decrease in DNA content compared with the controls and melatonin-treated cells (Figure 3B). The MT1 knockdown seems to be associated with reduced DNA content in S phase. In the G2/M phase, treatment with 3.2 and

4 mM of melatonin decreased the DNA content compared to the control and siRNA MT1 + melatonin-treated groups; by contrast, cells treated with 1.6 mM of melatonin increased the DNA content compared to the control and siRNA MT1 + melatonin-treated groups (Figure 3C). These data indicated that melatonin causes cell cycle arrest and the MT1 receptor knockdown seem to amplify its effects evidenced by a low DNA content in S and G2/M phases. Figure 3D shows the representative DNA content by PI labeled in G1, S, and G2/M after treatments and experimental conditions.

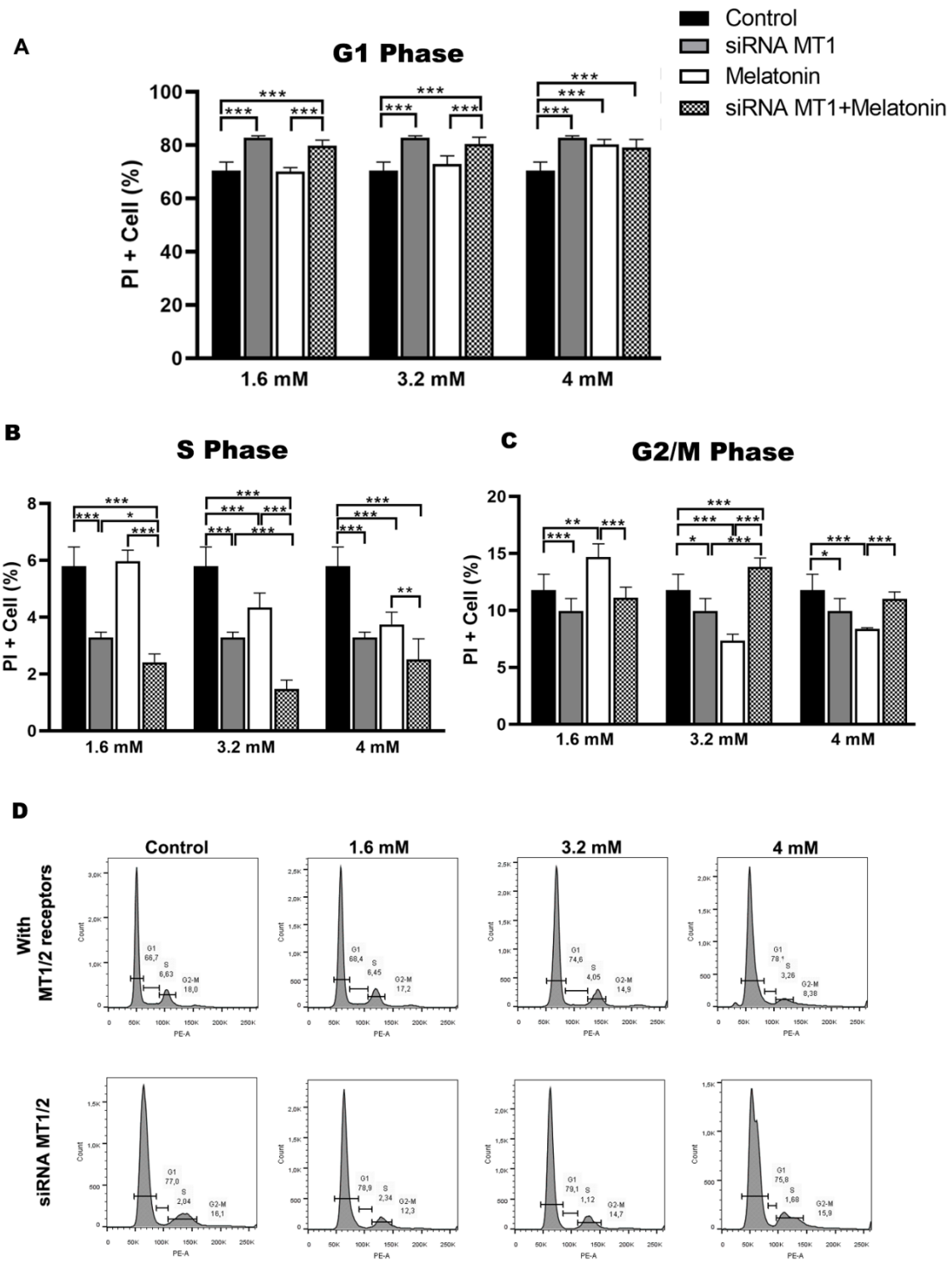
A
G1 Phase
Control
siRNA MT1
Melatonin
siRNA MT1+Melatonin
PI + Cell (%)
1.6 mM
3.2 mM
4 mM
B
S Phase
C
G2/M Phase
D
With MT1/2 receptors
siRNA MT1/2
Control
1.6 mM
3.2 mM
4 mM
PE-A
Count

Figure 3. Melatonin alters the DNA content in SKOV-3 cells. Percentage of PI+cells in G1 (A), S (B) and G2/M (C) phases. (D) Percentage of representative cell cycle analyses in SKOV-3 cells. The assay was performed in three technical and biological replicates. The data are expressed as the mean ± SD. *P<0.05, ** p<0.01 and ***P<0.001.

3.6 Melatonin attenuates molecules associated with cell survival and proliferation

Protein kinases, which are essential for cell signaling related to cell proliferation, survival, and substrate anchorage, were studied using Luminex xMAP technology. Melatonin treatment reduced the levels of Akt, ERK 1/2, STAT3, and STAT5 at all concentrations; in addition, CREB JNK, p38, and p70s6k levels were reduced only at 3.2 and 4 mM of melatonin (Figure 4). The siRNA MT1 + melatonin group showed the lowest levels of cell signaling molecules compared to their controls and melatonin-exposed groups. The reduced protein concentration in the siRNA MT1 + melatonin group may be due to the fact that melatonin receptors are G protein-coupled tyrosine kinase receptors, responsible for initiating the signaling cascade of kinases such Akt, ERK, and CREB; the MT1 receptor knockdown is capable of attenuating the activation of important pathways in ovarian cancer cell (Figure 4).

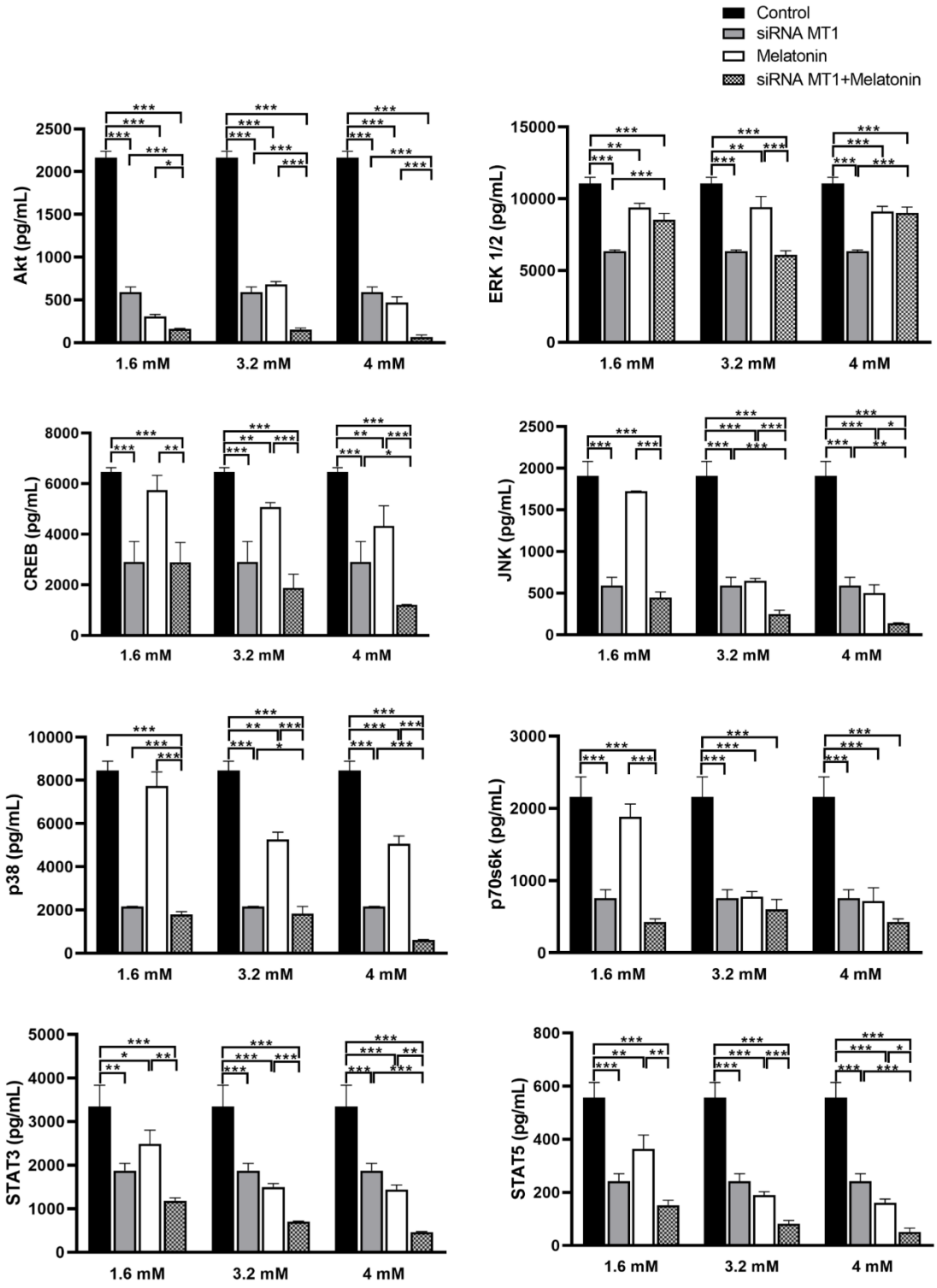
Control
siRNA MT1
Melatonin
siRNA MT1+Melatonin
Akt (pg/mL)
ERK 1/2 (pg/mL)
CREB (pg/mL)
JNK (pg/mL)
p38 (pg/mL)
p70s6k (pg/mL)
STAT3 (pg/mL)
STAT5 (pg/mL)
1.6 mM
3.2 mM
4 mM

Figure 4. Evaluation of the protein kinases involved with cell survival and proliferation-related signaling pathways after melatonin treatment. Treatment with melatonin reduced the levels of ERK1/2, Akt, STAT3, JNK, p70S6K, STAT5, CREB and p38 showing the most prominent effect after MT1 knockdown. The assay was performed in three biological replicates. The data are expressed as the mean ± SD. *P<0.05, ** p<0.01 and ***P<0.001.

4. Discussion

We reported that melatonin promotes apoptosis while inhibits proliferation and invasion of ovarian cancer cells which may be mainly attributed to the reduced levels of kinases related to cell signaling. These cellular mechanisms were shown to be strengthened by the MT1 silencing. The potentiating effect of melatonin combined with other therapies such as cisplatin, doxorubicin, and laser therapy has already been shown to be efficient for cell death (Kim *et al.*, 2012; Akbarzadeh *et al.*, 2016; Menéndez-Menéndez and Martínez-Campa, 2018). Our findings demonstrate that melatonin decrease the viability of the SKOV-3 cells at pharmacological doses while increasing apoptosis and necrosis rates. Melatonin enhances BAX, a pro-apoptotic protein, and downregulates BCL-2, an anti-apoptotic protein, thereby activating caspases to promote cell death (Chuffa *et al.*, 2016; Alonso-González *et al.*, 2018; Sánchez *et al.*, 2018). The increase in apoptosis seems to affect the wild type and MT1 knockdown cells, showing that melatonin signals via alternative mechanisms to perform its antitumor action regardless of its MT1 receptor (Huo *et al.*, 2017; Chuffa *et al.*, 2019; Reiter, Sharma, Pires De Campos Zuccari, *et al.*, 2021)

Melatonin has been documented to inhibit cell cycle by activating p53 (Wang, Selth and Callen, 2017). In the current study, treatment with the highest concentrations of melatonin (3.2 and 4 mM) significantly reduced the DNA content in S and G2/M phases. Consistently, a previous study reported that melatonin at 400, 600 and 800 µM for 4h increased the DNA content in the G1 phase and decreased the S phase in the ovarian cancer cells (OVCAR-429 and PA-1 cell lines) (Shen *et al.*, 2016). The same effect was observed in cisplatin-sensitive lung carcinoma SK-LU-1 cells after treatment with 1 and 2 mM of melatonin for 48h (Plaimee *et al.*, 2014). We further showed that SKOV-3 cells silenced for the MT1 receptor showed an increase in DNA content of the G1 phase compared to the control. The groups treated with 3.2 and 4 mM of melatonin, in the absence of MT1 receptors, showed decreased S phase DNA content and an augment in the G2 phase compared to the melatonin-treated group.

The mechanisms involved in cell motility are numerous and dependent on cell-to-cell and cell-extracellular matrix (MEC) adhesion, MEC remodeling by matrix metalloproteinases (MMPs),

cytoskeleton reorganization, and epithelial-mesenchymal transition (EMT) (Stuelten, Parent and Montell, 2018). The results found in our study showed that 3.2 and 4 mM of melatonin attenuated the cell migration rate, being all concentrations able to inhibit cell invasion. The MT1 knockdown showed a remarkable reduction in cell migration and invasion, thus evidencing alternative mechanisms whereby melatonin potentially acts when it is available and MT1 unbinding. Several kinases responsible for cell motility such as Akt, P70S6K, STAT3 and STAT5 were lowered by melatonin treatment. Past studies showed that melatonin increased the expression of anchoring proteins such as E-cadherin and integrins-β1 and further decreased proteins and transcription factors related to EMT (vimentin, ZEB1, Snail, Slug), thus inhibiting cell motility and metastatic potential of ovarian cancer cells (Akbarzadeh *et al.*, 2017). Nevertheless, melatonin regulates the expression and activity of MMP genes, inhibiting the catalytic activity of this enzyme (Rudra *et al.*, 2013), and in addition, melatonin was able to inhibit MMP-9 transcription and cell invasion via the Akt/Erk/JNK signaling pathway (Lin *et al.*, 2016). The action of melatonin on cell motility is receptor-independent, but the receptor knockdown appears to be involved in several regulatory mechanisms through protein kinases and second messengers. These data pointed to the MT1 expression as a potential therapeutic target for invasive tumors

Melatonin downregulated intracellular signaling pathways responsible for cell survival and proliferation and potentiated the action of anti-cancer drugs in several tumors types such ovary, lung, prostate, pancreatic (Moloudizargari *et al.*, 2021). The Akt and ERK/MAP proteins are two families of serine-threonine kinases activated in response to signals triggered by the serine-threonine receptors; by activating other kinases such as p70S6, STAT3, and CREB, they further stimulate p58 and JNK (D'yakonov *et al.*, 2021). Akt is an essential component of the phosphatidylinositol-3-kinase (PI3K) signaling pathway, especially involved in cell growth and differentiation (Reiter *et al.*, 2020b), and may modulate cell migration and invasion through the β-catenin/SLUG axis activation (Bu *et al.*, 2020). Melatonin inhibits ERK 1/2 and Akt via inducing cell cycle arrest, and the downregulation of Akt and ERK1/2 signaling is restored with the suppression of MT1 receptor activity (Lin *et al.*, 2020). Moreover, melatonin has been shown to prevent the elevation of p38 levels in ovarian cancer cells, corroborating our findings (Ferreira *et al.*, 2014; Menéndez-Menéndez and Martínez-Campa, 2018). Since these kinases have a pro-oncogene role, its reduction may control the oncogenic cell transformation we showed that melatonin decreased the levels of protein kinases, and MT1 knockdown led to enhanced

suppression of their levels. These results may be attributed to the MT1, which are of the serine-threonine type, essential for signal transduction involving kinases activation and second messengers (Song *et al.*, 2017; Nikolaev, Robeva and Konakchieva, 2021).

The receptor-independent effects can also be seen, with multiple signaling from different cascades (Mayo *et al.*, 2019). Several studies have indicated that the PI3K/Akt cascade is associated with melatonin-mediated antiproliferative action (Zhang *et al.*, 2017). Furthermore, the independent effect of MT1 and MT2 activation can open cyclic nucleotide-gated channels and elevate calcium levels; these levels trigger the extrinsic apoptotic pathway by PKCα (Samanta, 2020). Besides, melatonin competes with glucose uptake at a specific low-binding site of GLUT-1, allowing melatonin to cross the plasma membrane (Ren *et al.*, 2017). The lower entry of sugar into the cell leads to decreased proliferation and Warburg effect (preference for aerobic glycolysis) in tumor cells (Reiter *et al.*, 2020). Melatonin seems to be transported via PEPT1/2 , a new route for facilitating melatonin entry to the mitochondria (Huo *et al.*, 2017).

Melatonin shows receptor-dependent and independent oncostatic effects, corroborating the findings that classify it as an antitumor agent. However, the knockdown of the MT1 receptors showed a prominent antitumoral effect, probably due to the other existing melatonin entryways. In this study, we observed that the MT1 knockdown had a greater anti-migratory and anti-invasive effect, pro-apoptotic effect, and showed greater reduction in kinases responsible for cell survival and proliferation.. Figure 5 summarizes the main findings of the current study.

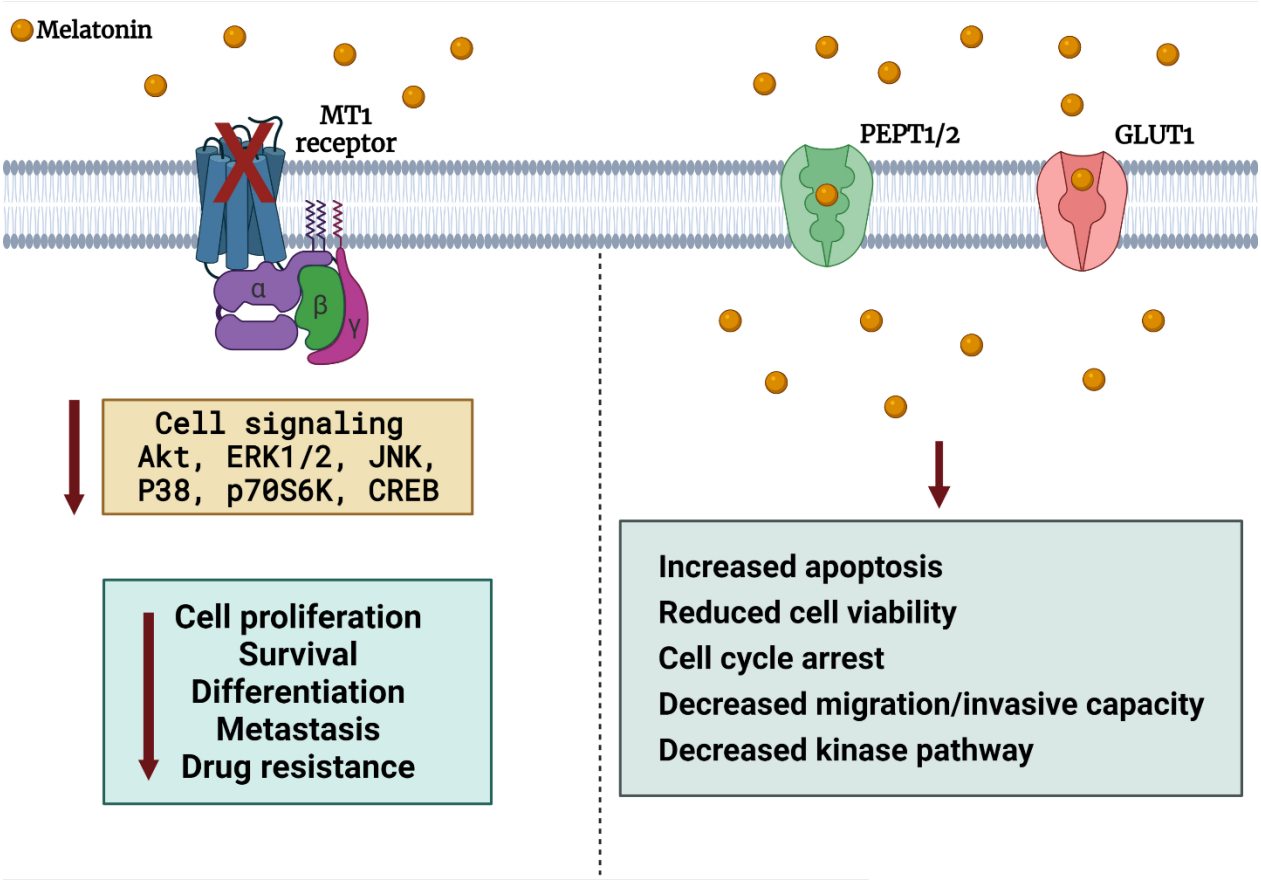

Figure 5. *This figure illustrates the main melatonin's effects in SKOV-3 cells. Melatonin showed an antitumoral effect in cells with MT1 receptors and MT1 knockdown. MT1 silencing seems to promote higher melatonin effects related to the attenuation of kinases signaling and significant reduction of the migratory and invasive potential of SKOV-3 cells. Melatonin can alternatively enter tumor cells using other membrane transporting such as PEPT1/2 and GLUT1.* Adapted from BioRender.com (2022).

5. Conclusions

Melatonin treatment allowed SKOV-3 ovarian cancer cells to lost their tumor progression advantages. Melatonin was able to inhibit kinases related to cell signaling and motility, thereby leading to increased apoptosis and necrosis rates while lowering the proliferative, migratory and invasive potential of cells. Our data showed the antitumor and oncostatic effect of melatonin at pharmacological concentrations, being most of them receptor independent. However, melatonin seems to regulate their mechanisms differently in ovarian cancer cells in the presence or absence of MT1 receptors. Knocking down MT1 receptors in SKOV-3 cells favored the inhibition of important kinases involved in tumor aggressiveness. Future studies are needed to understand the alternative pathways by which melatonin exerts its antitumor action in ovarian cancer.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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Capítulo 3

“Celebre a mulher que você está se tornando
Não tape os ouvidos
Ela está te chamando
Ela dança com o fogo
Ela é pancada
Mas também é doce
Ela sempre foi sua melhor escolha
Ela é tudo aquilo que sobreviveu”

- Ryane Leão

Melatonin reverses the Warburg-type metabolism and reduces mitochondrial membrane potential of ovarian cancer cells independent of MT1 receptor activation

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Abstract

Ovarian cancer (OC) is the most lethal gynecologic malignancy, and melatonin, an indoleamine secreted by the pineal gland during darkness, has shown various antitumor properties. It has been documented that intracellular melatonin alters the metabolic profile of tumor cells. Herein, we investigated the influence of melatonin therapy on energy metabolism and mitochondrial integrity in human ovarian carcinoma cells (SKOV-3) and tested whether its biological effects depended on the presence of the MT1 receptor. SKOV-3 cells were exposed to different concentrations of melatonin, and experimental groups were divided as to the presence of MT1 receptors (melatonin groups) or receptor absence by RNAi silencing (siRNA MT1+melatonin). Melatonin levels were reduced in SKOV-3 cells and its intracellular levels were increased after treatment with 3.2 mM of melatonin independent of the MT1; its knockdown actually enhanced melatonin in OC cells. Using GEPIA bioinformatic tool, we observed that OC patients' samples had a low rate of acetyl serotonin methyl transferase (ASMT) expression; ASMT is the melatonin forming enzyme. The mitochondrial membrane potential of SKOV-3 cells was decreased in the group treated with 4 mM of melatonin and in the siRNA MT1+melatonin group treated with 3.2 and 4 mM of melatonin. Cellular glucose consumption was reduced after melatonin treatment while MT1 knockdown increased its consumption. Interconversion of lactate to pyruvate increased after treatment with 3.2 mM melatonin and in all siRNA MT1+melatonin groups when compared with their controls. Moreover, the activity of lactate dehydrogenase was decreased with melatonin but significantly increased after MT1 silencing at all concentrations. By using UCSC XenaBrowser tool, we showed a positive correlation between the human *ASMTL* gene and the ATP synthase genes (*ATP5A1, ATPAF2, ATP5B, ATPAF1, C16orf7, ATP5C1, ATP5D and ATP5E*), succinate dehydrogenase gene (*SDHD*) and pyruvate dehydrogenase genes (*PDHA* and PDHB). We conclude that melatonin changes the glycolytic phenotype and mitochondrial integrity of SKOV-3 cells independent of the MT1 receptor, thus decreasing the survival advantage of OC cells.

Keywords: ovarian cancer; melatonin; mitochondrial metabolism, glucose, Warburg effect; SKOV-3 cells

1. Introduction

Ovarian cancer (OC) is the most lethal gynecologic malignancy and the fifth leading cause of death worldwide (Siegel *et al.*, 2022). OC can originate from mesenchymal cells, granulosa cell layer, germinative cells, and epithelial cells, the latter being responsible for about 90% of all OC types (Chuffa *et al.*, 2017; Zare *et al.*, 2019). The standard treatment includes cisplatin-taxol-based chemotherapy; however, many tumors often relapse with aggressive features and become chemoresistant to conventional therapies (Talib, 2018; Lheureux *et al.*, 2019).

Melatonin is secreted by the pineal gland and is produced, but not secreted, by extra-pineal tissues such as the retina, intestine, and ovary (Tan *et al.*, 2016). Melatonin's actions are mediated by melatonin receptors (MT1 and MT2), or they are receptor-independent (Hill *et al.*, 2015; Ostrin, 2019). In addition to the regulatory role on circadian cycle, seasonal reproduction, immune response, etc. (Gheban, Rosca and Crisan, 2019; Zhao *et al.*, 2019), evidence has shown its antitumor actions in several solid tumors such as prostate, breast, and colorectal cancers (Bonmati-Carrion and Tomas-Loba, 2021). Its oncostatic effects include cell cycle arrest, pro-apoptotic, anti-proliferative, and anti-metastatic actions, in addition to interfering in mitochondrial physiology (Samanta, 2020). Some of antitumor characteristics of melatonin are dependent on activation of the MT1 receptor; however, melatonin also exerts intracellular effects which are independent of MT1 (Talib, 2018). Previous studies demonstrated that melatonin reaches specific subcellular compartments through the glucose transporter (GLUT1) and human peptide transporter 1 and 2 (PEPT1/2) (Huo *et al.*, 2017; Chuffa *et al.*, 2019; Reiter, Sharma, Rosales-Corral, *et al.*, 2021a).

Melatonin biosynthesis depends on biochemical processes such as the transformation of tryptophan into melatonin through the action of two limiting enzymes, arylalkylamine N-acetyltransferase (AANAT) and N-acetylserotonin-O-methyltransferase (ASMT) (Reiter, Sharma, Rosales-Corral, *et al.*, 2021a; Tan and Hardeland, 2021). Both enzymes are present in mitochondria, strengthening the idea that melatonin is produced by perhaps all cells (Suofu *et al.* 2017, Tan and Hardeland, 2021). As a result, the maintenance of mitochondrial integrity is influenced by local melatonin production in healthy cells and, in tumor cells, mitochondrial dysfunction may be a result of altered melatonin synthesis which contributes to a defective cell phenotype (Gaiotte *et al.* 2022).

Mitochondria are the organelles responsible for cell metabolic functions such as energy production, pyruvate metabolism, and cellular respiration (oxidative phosphorylation or OXPHOS)

(Reiter *et al.*, 2020; Reiter, Sharma, Rosales-Corral, *et al.*, 2021). Metabolic reprogramming which induces high glycolytic rates even in the presence of oxygen (*Warburg* effect) is a hallmark of cancer (Talib, 2018; Reiter *et al.*, 2020). In tumor cells, pyruvate is converted to lactate instead of mitochondrial acetyl-CoA, compromising the electron transport chain (ETC) and decreasing OXPHOS (Rodríguez *et al.*, 2021). In addition, acetyl-CoA is a cofactor to the AANAT enzyme which is rate limiting in mitochondrial melatonin synthesis. The reduction in acetyl-CoA levels results in less melatonin for tumor cells and depresses the tricarboxylic acid cycle which normally supports ATP production (Rodriguez *et al.*, 2013; Reiter, Sharma, Rosales-Corral, *et al.*, 2021a). Against this background, the present study investigated the influence of melatonin therapy on energy metabolism and mitochondrial physiology in human ovarian carcinoma cells (SKOV-3 cell line) and further examined whether these biological effects are mediated by the MT1 melatonin receptor.

2. Material and methods

2.1 Cell line and cell culture

SKOV-3 cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI 1640 (LGC Biotechnology, Brazil) supplemented with 10% Fetal Bovine Serum (FBS; Gibco, USA), 100U/ml Penicillin and 100µg/ml streptomycin (Gibco, USA). The cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

2.2 Melatonin preparation

Melatonin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO, Merck, Germany) to prepare a 1M stock solution. Then, different concentrations of working solution were prepared in RPMI 1640 medium and incubated with SKOV-3 cells.

2.3 Experimental groups

SKOV-3 cells ($1x10^5$ cells) were seeded and incubated with melatonin (1.6 mM, 3.2 mM and 4.0 mM concentrations) or not (control group), with or without siRNA for MT1 receptor (20nM). In individual sets of experiments, SKOV-3 cells were incubated with melatonin in the presence of MT1 receptors (melatonin-exposed groups) or after MT1 silencing (siRNA MT1+melatonin-exposed groups) for 48 h. The control groups either received DMSO (control group) or were silenced for MT1 and received no treatment (siRNA MT1). All the experiments were assayed in three technical and biological replicates.

2.4 Oligonucleotides and transfection

Post-transcriptional silencing of the MT1 gene was performed using RNA interference (RNAi). Two Silencer® Select siRNA sequences (s224070 and s9051, Thermo Fisher) formed a complex with Opti-MEM® Reduced Serum Medium (Thermo Fisher, USA) before transfection. SKOV-3 transfections were performed with RNAiMAX Lipofectamine (Thermo Fisher, USA) combined with 20 nM of each oligonucleotide for 24 h when the cells reached 80% of confluence. The respective negative control was used (Silencer® Select Negative Control No. 1 siRNA, Thermo Fisher, USA). The time and concentration of the oligonucleotides were previously determined in a pilot study. The silencing validation was done through RT-qPCR.

2.5 RNA isolation and RT-qPCR

After transfection, cells were washed with D-PBS, and the RNA isolation was performed using TRIzol ®. RNA concentration and quality were assessed using a NanoVue Plus Spectrophotometer (GE Healthcare, USA). To validate MT1 receptor silencing, reverse transcription-quantitative PCR (RT-qPCR) was used. Total RNA samples were reverse transcribed into cDNA using the High Capacity RNA-to-cDNA Kit (Life Technologies). mRNAs qPCR analysis was performed in 20 μL reaction (SYBR Green Master Mix; Thermo Fisher Scientific, USA), according to the manufacturer's instruction, and run on a QuantStudio 12K Flex System (Thermo Fisher Scientific, USA), using the following conditions: 95º C for 10 min followed by 40 cycles of 95º C for 15 sec and 60º C for 1 min. The oligonucleotides for mRNAs were analyzed through the National Center for Biotechnology Information (NCBI) and the sequences used: *MTNR1A* (MT1) 5’-AGCTCAGGAACGCAGGAAAC-3' (forward) and 5'-CAGTGCAGATAGCCCAGGTT-3' (reverse); *RPS13* 5’-AGAAACGGCTACCACATCCA-3' (forward) and 5’-CACCAGACTTGCCCTCCA-3' (reverse); *GAPDH* 5’-GCTCCCTCTTTCTTTGCAGCAAT-3' (forward) and 5’-TACCATGAGTCCTTCCACGATAC-3' (reverse). Representative profile of the MT1 after gene silencing. The RT-qPCR data were presented as fold-change (2-ΔΔCt) compared with the endogenous genes (RPS13 and GAPDH). MT1 protein levels were normalized using endogenous protein (β-actin).

2.6 Cytotoxicity assay

The SKOV-3 cells were seeded in a 96-well plate at a density of 1×10^4 cells/well. The cell survival was measured using a colorimetric method. After melatonin exposure, the MTT solution

(5 mg/mL) was added for 4 h, and the presence of formazan crystals was determined by Epoch microplate reader (BioTek Instruments, Highland Park, PO, USA) at 540 nm, being the reference curve fixed at 650 nm. The percentage of crystal formation was calculated by fixing the control group as 100%.

2.7 Melatonin quantification by ELISA assay

Intracellular melatonin concentration was measured using the Human melatonin ELISA Kit (FineTest, catalog number EH3344). To compare melatonin concentration in normal and tumor cells, we used transformed ovarian cancer cells and cells from normal ovarian tissue. The protein concentration used in all samples was 3µg. For this purpose, a 96-well plate coated with antibody to melatonin received 50 µL of sample or standard solution per well, and then 50 µL of biotin was added followed by incubation for 45 minutes at 37ºC. Subsequently, the plate was washed, and 100 µL of HRP-streptavidin solution was added for 30 min at 37ºC. After this procedure, the plate was washed and incubated with 90 µL of TMB solution for 20 min at 37ºC, and finally, 50 µL of stop solution was added and read at 450 nm in a microplate reader (Epoch, BioTek Instruments, USA). All analyzes were performed in triplicate, and the standard curve was generated according to the manufacturer's instructions. Data were expressed in pg/mL.

2.8 Metabolic status of the OC cells

To evaluate the effect of melatonin on the energy metabolism of SKOV-3 cells, biochemical assays were used to detect lactate release, glucose consumption, and lactate dehydrogenase (LDH) activity. Cells were seeded in 12-well plates for glucose and lactate analysis ($5x10^4$ cells/well) and in six-well plates for LDH analysis ($1x10^5$ cells/well). After reaching 80% of cell confluence, the supernatant was collected for analysis (t0), and the cells were trypsinized, and viable cells were counted (x0). Next, the cells with or without the MT1 receptor were exposed to melatonin for 48h. After the exposure period, the supernatant was collected for analysis (t1) and the cells were trypsinized, with only the viable cells counted (x1). The supernatants were analyzed using a Bioclin Quibasa Química Básica Ltda enzyme kit (Belo Horizonte, MG, Brazil). Specific events involved in the Warburg effect including glucose consumption, lactate release, and LDH activity were analyzed by enzymatic assay after 48 h of melatonin exposure. The specific rate of substrate consumption and metabolite production was calculated using the formula $Q = 2\,x\,10 - 3\,(Ct0 - Ct1\,)/[(Xt1\, + Xt0)x\,t]$; Q is the specific consumption rate (mmol cell h^{-1}); C refers to

metabolite concentration (mmol cell^{-1} h^{-1}); t_0 and t_1 are the plated seeded times of cells (t_0=0h) and supernatant collection (t_1=48h); X_{t0} and X_{t1} are the number of viable cells at both times (cell ml^{-1}) (Slivac *et al.* 2010). The experiments were performed in biological triplicate.

2.9 Target prediction and bioinformatics analyses

GEPIA 2 database (http://gepia.cancer-pku.cn/.) provides an essential interactive function including differential expression analysis, correlation analysis, and patient survival analysis (Tang *et al.*, 2017). We used this web-based tool to compare the gene expression of melatonin receptors (*MT1* and *MT2*) and the genes of the limiting enzymes for melatonin synthesis (*AANAT* and *ASMT*) in normal ovarian tissue (88 samples) and ovarian cancer (426 samples).

We also analyzed the correlation between differential gene expression of *ASMTL* with ATP synthase related genes (*ATP5A1, ATPAF2, ATP5B, ATPAF1, C16orf7, ATP5C1, ATP5D and ATP5E*), pyruvate dehydrogenase genes (*PDHA1* and *PDHB*), and succinate dehydrogenase complex gene (*SDHD*) available from the Cancer Genome Atlas [TCGA; ovarian cancer dataset (n= 634 samples)] using the UCSC Xena browser (http://xenabrowser.net/) (Goldman *et al.*, 2020). The data were assessed in March, 2022.

2.10 Measurement of mitochondrial membrane potential

Flow cytometry was used to determine the mitochondrial membrane potential integrity using MitoStatus Red Kit (BD Pharmingen™, catalog number 564697). According to the manufacturer's recommendations, cells were incubated with 50 nM of MitoStatus Red at 37°C for 30 min and then washed using D-PBS. The assay was performed using the FACSCanto cytometer (BD Biosciences, Clontech, CA, USA). The relative rates of cells with low membrane potential were calculated using FlowJo software (vX.10.6, Tree Star Inc.).

2.11 Statistical analysis

Data were evaluated using analysis of variance (ANOVA) with independent factors complemented with Tukey's test for multiple comparisons. For non-parametric data, Kruskal-Wallis test was used complemented with Dunn's test. To assess the effects of MT1 silencing, we applied the Student's t-test between the silencing negative control group (si-NC) and the MT1-silenced group (siRNA MT1). Results were analyzed using GraphPad Prism software version 9.2, and data were expressed as mean ± SD. Statistical significance was set at $P < 0.05$.

3. Results

3.1 Validation of the MT1 receptor silencing and cell viability

To better understand whether melatonin's function in OC is dependent on the MT1 receptor, RNA interference (RNAi) was used to knockdown the melatonin receptor gene (*MTNR1A* and *MTNR1B*). After silencing, *MTNR1A* showed low relative gene expression (0.28±0.10) compared to the negative silencing control group (si-NC) (1.01±0.22) (~73% RNAi efficiency). The Western blot assay also showed a significant reduction in MT1 protein levels compared to the si-NC group. The expression of the *MTNR1B* receptor was not detected by RT-qPCR since it appeared to be very low or absent in the SKOV-3 cells (data not shown).

MTT assay was used to select a melatonin concentration with cytotoxic capacity and its further application in the cell culture. The melatonin concentrations showed ~ 20-45% cytotoxicity in SKOV-3 cells after 48h. After melatonin treatment, SKOV-3 cells showed a statistically significant reduction in cell viability in the melatonin-treated groups at all concentrations (1.6, 3.2 and 4 mM) (Figure 1). Interestingly, the siRNA MT1 + melatonin group exhibited a reduction in cell viability compared to the MT1 siRNA group and melatonin-exposed group at all concentrations. Although melatonin significantly reduced cell viability, this effect was accentuated when MT1 was knocked down.

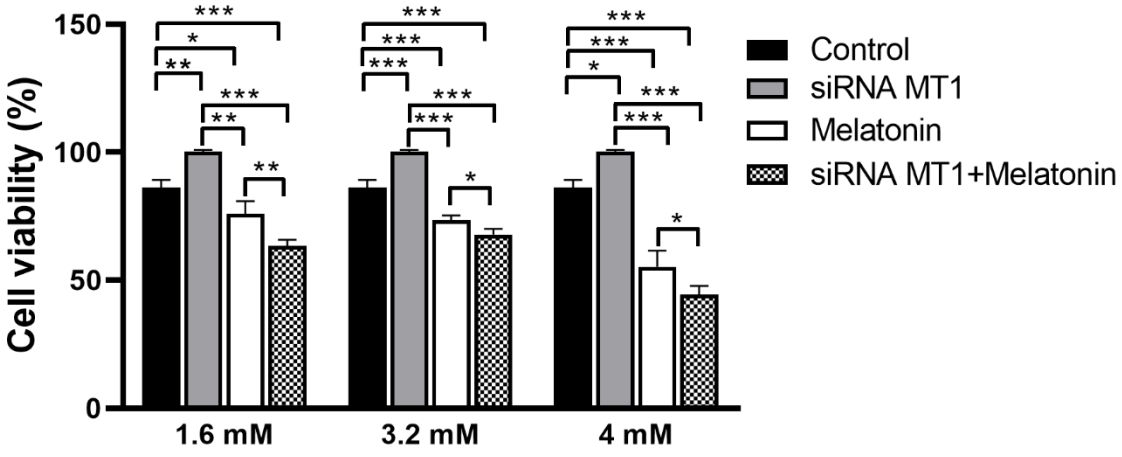

Figure 1. MTT assay showed that cell viability was reduced after melatonin treatment with different concentrations for 48h. The samples were assayed in three technical and biological replicates. The data are expressed as the mean ± SD. *P<0.05, **P<0.01, and ***P<0.001.

3.2 Melatonin treatment stimulates intracellular melatonin synthesis

SKOV-3 cells showed a significant reduction in melatonin concentration compared with healthy ovarian cells (Figure 2A). Intracellular concentrations of melatonin increased in the OC cells exposed to 3.2 mM of melatonin compared to the control group (Figure 2B). Curiously, the siRNA MT1+melatonin group showed a further increase in intracellular melatonin concentration compared to the control and melatonin-treated groups (Figure 2B). We used GEPIA web-based tool to verify gene expression related to melatonin receptors and limiting enzymes of melatonin synthesis in OC patients. Although not statistically significant, the samples from OC patients had a higher expression rate of *MTNR1A* and *AANAT* genes but a reduced expression of *ASMT* gene compared with healthy ovaries (Figure 2C), thus indicating an impairment in melatonin synthesis by these pathological cells. The expression of the *MTNR1B* receptor was not observed in either tumor and normal tissue. Furthermore, there is a superior survival rate in patients who had a higher *ASMT* expression when compared with lower *ASMT* expression (Figure 2D).

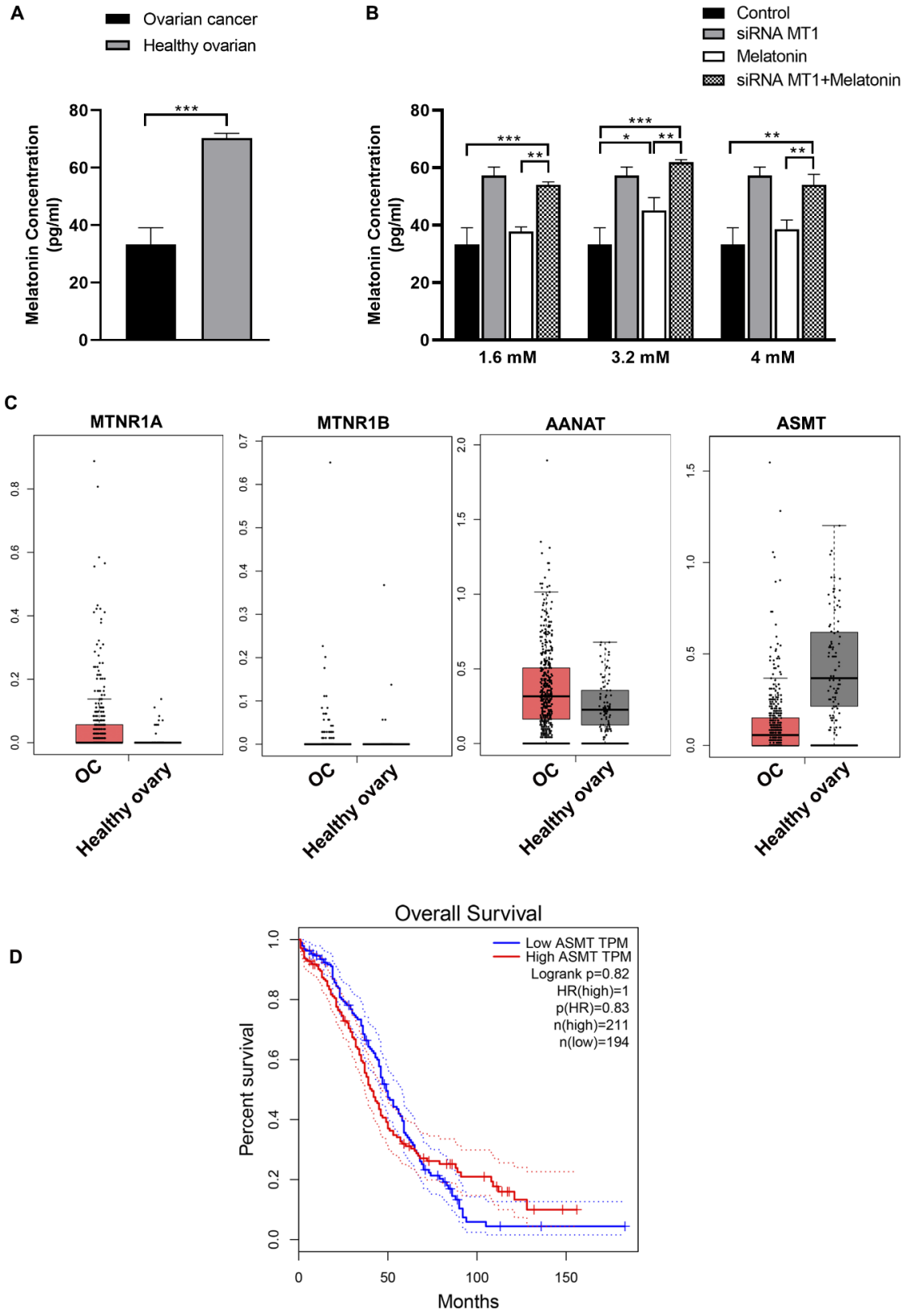
A
Ovarian cancer
Healthy ovarian
Melatonin Concentration (pg/ml)
***
B
Control
siRNA MT1
Melatonin
siRNA MT1+Melatonin
Melatonin Concentration (pg/ml)
1.6 mM
3.2 mM
4 mM
C
MTNR1A
MTNR1B
AANAT
ASMT
OC
Healthy ovary
D
Overall Survival
Low ASMT TPM
High ASMT TPM
Logrank p=0.82
HR(high)=1
p(HR)=0.83
n(high)=211
n(low)=194
Percent survival
Months

Figure 2. Intracellular melatonin concentration. (A) The melatonin concentration in SKOV-3 cells and healthy ovarian tissue. (B) Intracellular melatonin concentration in SKOV-3 cells, in the presence of MT1 receptor or not after melatonin for 48 h. (C) Expression levels of MT1 and MT2 melatonin receptors, and the limiting enzymes of melatonin synthesis (AANAT and ASMT) in patients with OC (red box) and healthy tissue (gray box) from the TCGA datasets. (D) The overall survival of OC patients with low *ASMT* (blue line) and high *ASMT* (red line). Images were obtained from the GEPIA online database. These analyses were made using different experimental conditions and their respective control groups. Data were expressed as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

3.3 Melatonin alters the mitochondrial membrane potential and glycolytic metabolism of SKOV-3 cells

After melatonin exposure, MitoStatus Red labeling was performed to determine the mitochondrial membrane potential in SKOV-3 cells, in the presence or absence of an MT1 receptor, using flow cytometry. Mitochondrial membrane potential was lower after treatment with 4 mM of melatonin when compared to the control group. Moreover, MT1-silenced cells that received melatonin at concentrations of 3.2 mM and 4 mM exhibited a reduced membrane potential compared to the control, siRNA groups, and melatonin-treated groups, showing that melatonin exerts MT1-independent actions in the mitochondria (Figure 3A). Figure 3B shows the representative analysis of mitochondrial membrane potential in SKOV-3 cells.

The rate of cellular glucose consumption was reduced after exposure to 4 mM melatonin. The siRNA MT1+melatonin groups showed low rates of glucose consumption when compared with the control group. Furthermore, MT1-silenced SKOV-3 cells that received 1.6 mM and 3.2 mM of melatonin showed lower glucose consumption than their respective melatonin-treated groups (Figure 3C). On the contrary, glucose consumption in MT1-silenced SKOV-3 cells treated with 4 mM of melatonin was enhanced compared to the melatonin-treated group in the presence of MT1 (Figure 3C). LDH activity, an enzyme that catalyzes the pyruvate to lactate interconversion, was reduced in SKOV-3 cells treated with 3.2 mM and 4 mM of melatonin compared to the control group. However, siRNA MT1 + melatonin groups showed an increase in LDH activity compared to control and melatonin-treated groups (Figure 3D). Analyzing the biological context, we observed that lactate was consumed rather than released to the extracellular medium. Thus, 3.2 mM of melatonin significantly reduced lactate levels during the treatment period compared to the control group. Interestingly, lactate consumption increased with MT1 knockdown considering all melatonin concentrations compared to the control and melatonin-treated groups (Figure 3E). It was suggested that MT1 receptors are essential to mediate glucose consumption in SKOV-3 cells

treated with 4 mM melatonin showing an anti-Warburg effect in SKOV-3 cells. In the presence of MT1, the group treated with 3.2mM of melatonin exhibited a reduction in lactate rates, but the knockdown of MT1 stimulated the oxidation of lactate to pyruvate by LDH as an alternative biochemical pathway. This enzyme showed a higher activity after MT1 knockdown compared to the melatonin-exposed group.

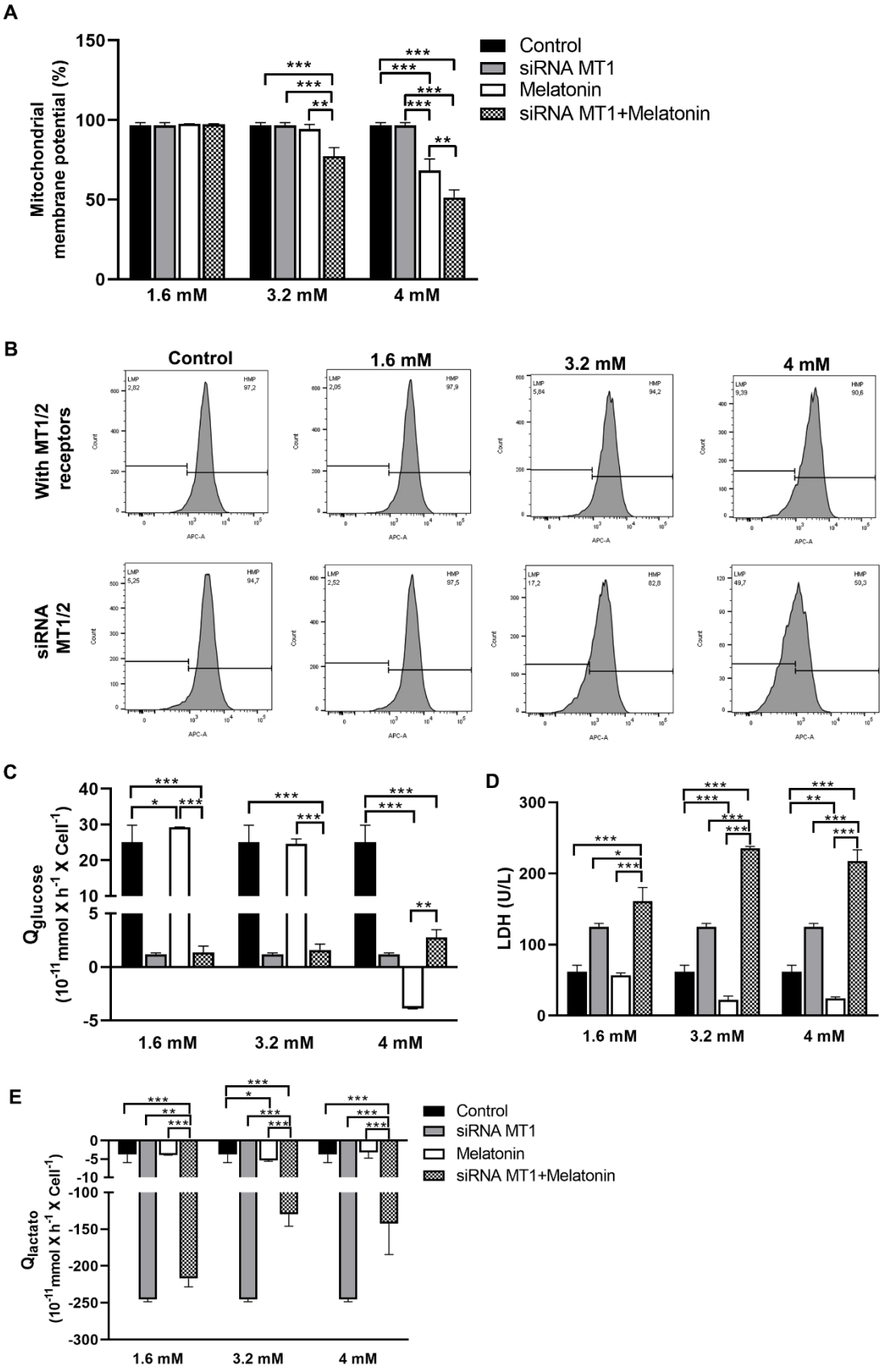
A
Mitochondrial membrane potential (%)
150
100
50
0
1.6 mM
3.2 mM
4 mM
Control
siRNA MT1
Melatonin
siRNA MT1+Melatonin
B
Control
1.6 mM
3.2 mM
4 mM
With MT1/2 receptors
siRNA MT1/2
C
Qglucose (10-11mmol X h-1 X Cell-1)
D
LDH (U/L)
E
Qlactato (10-11mmol X h-1 X Cell-1)

Figure 3. Melatonin alters mitochondrial integrity and reduces the Warburg effect in SKOV-3 cells. *(*A) Mitochondrial membrane potential rate in SKOV-3 cells, in the presence or absence of MT1, after melatonin treatment for 48 h. MitoStatus Red was used to detect cells by flow cytometry. (B) Representative analysis of mitochondrial membrane potential in SKOV-3 cells. (C) Glucose consumption. (D) LDH activity. (E) Lactate consumption in SKOV-3 cells after melatonin treatment, in the presence or absence of MT1. The analysis was made using different experimental conditions and the respective control groups. Data were expressed as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

3.4 ASMTL expression is positively correlated with specific mitochondrial enzymes-related genes in OC

By using publicly available data in TCGA platform, we performed a correlation analysis between the gene expression of the key limiting enzyme for melatonin synthesis, *ASMTL*, and the genes related to the molecules necessary for energy metabolism and mitochondrial respiration by comparing samples of OC patients and normal ovaries. Interestingly, *ASMTL*, which is a homologous gene that is highly expressed in OC, showed positive correlations with *PDHA1*, *PDHB*, *SDHD genes* and with ATP synthase subunits genes (*ATP5A1, ATPAF2, ATP5B, ATPAF1, C16orf7, ATP5C1, ATP5D and ATP5E*) (Figure 4).

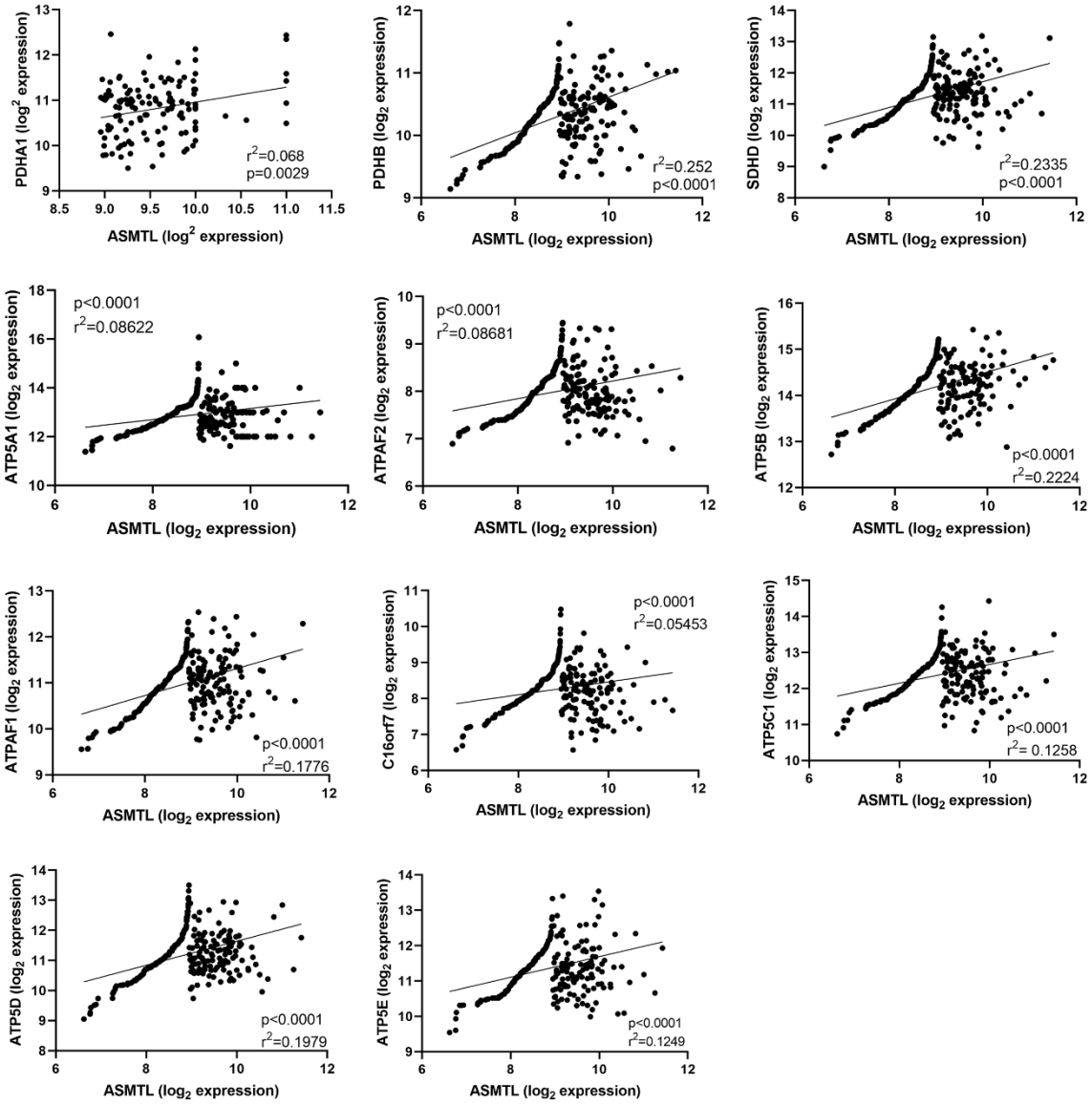

Figure 4. Correlation between *ASMTL* gene expression and the genes associated with mitochondrial energy metabolism and ATP production in human OC. Pearson's correlation coefficient was used individually for each comparison. UCSC Xena Browser was used to identify the relative gene expression profile in OC samples available from the TCGA database.

4. Discussion

Mitochondria are responsible for cellular bioenergetic regulation and respond to microenvironment changes (Grieco *et al.*, 2021). It is well-known that melatonin is capable of increasing apoptosis rates and promoting mitochondrial damage in tumor cells, mainly by stimulating ROS production and pro-apoptotic activity, in addition to reducing the mitochondrial membrane potential (Chuffa *et al.*, 2019). The cytotoxicity assay showed a decrease in cell viability after melatonin treatment at all concentrations (1.6 to 4mM), being more pronounced at the highest concentration, consistent with the literature data (Carpentieri *et al.*, 2012; Akbarzadeh *et al.*, 2016; Zemła *et al.*, 2017; Gaiotte *et al.*, 2022). Our present data revealed that melatonin application reduced the potential of the mitochondrial membrane, and this process was independent of MT1 activation. The siRNA MT1+melatonin groups exhibited a decrease in mitochondrial membrane potential compared to the group that received melatonin at 3.2 and 4 mM concentrations. Under the pro-oxidant effect of melatonin in tumor cells, reactive oxygen species (ROS) may be produced and programmed cell death signaling initiated, thus causing damage to mitochondrial membrane integrity (Waseem *et al.*, 2017).

The presence of key enzymes for melatonin synthesis has been documented in many cell types other than those in the pineal gland so that tissues continuously express *AANAT* and *ASMT* regardless of the prevailing light:dark cycle (Acuña-Castroviejo, Escames, Venegas, María, *et al.*, 2014). In non-tumor cells, these enzymes were found within the mitochondria to produce intracellular melatonin (Suofu *et al.*, 2017, Reiter *et al.*, 2018). We observed a significant reduction in melatonin levels in OC cells compared to healthy ovarian cells; however, intracellular melatonin levels were restored after treatment with 3.2 mM regardless of the presence of the MT1 receptor. As already reported, higher intracellular melatonin concentrations may contribute to pro-oxidative, pro-apoptotic, and anti-angiogenic processes and further change the metabolic profile of tumor cells (Talib, 2018; Reiter *et al.*, 2020). We showed that melatonin treatment increased *de novo* intracellular melatonin concentration, and these findings seemed to be receptor-independent since the siRNA MT1 group actually exhibited higher intracellular melatonin levels compared to the control group. A plausible explanation for the low intracellular levels of melatonin in tumor cells is due to the limited availability of acetyl-CoA in pathological cells exhibiting Warburg-type metabolism, acetyl-CoA is a necessary co-substrate for the limiting enzyme in melatonin synthesis, AANAT. In tumor cells the pyruvate dehydrogenase complex (PDC) enzyme, which catalyzes the

conversion of pyruvate to acetyl-CoA, is inhibited by the pyruvate dehydrogenase kinase (PDK) enzyme (Reiter, Sharma, Rosales-Corral, *et al.*, 2021a). Melatonin may disinhibit PDC activity by reducing HIF-1α, a factor highly expressed in tumors due to hypoxic conditions, which stimulates PDK. After PDC disinhibition, melatonin is synthesized in mitochondria and OXPHOS is re-established (De Lima Mota *et al.*, 2019).

Although tumor cells present high glycolytic rates, most cells are not capable of the non-cytosolic lactate conversion cycle, which is known mainly as the aerobic glycolysis or the "Warburg effect" (Cutruzzolà *et al.*, 2017). To assess this energy imbalance, we investigated the concentration of glucose, lactate, and LDH after exposure to melatonin, and the results showed that these molecules are susceptible to melatonin treatment. The rate of glucose consumption per time (Q) by SKOV-3 cells showed that 4 mM melatonin regulated glucose consumption differently in the experimental groups; there was a reduction in glucose uptake when compared to the control group in the presence of melatonin receptors. Conversely, the siRNA MT1 group treated with 4 mM of melatonin showed elevated glucose consumption; OC cells treated with 3.2 mM of melatonin showed higher lactate consumption per time (Q) compared to the control group. Cells with MT1 knockdown exhibited even higher lactate consumption at all melatonin concentrations compared to MT1 containing-cells treated with melatonin. Furthermore, melatonin treatment at concentrations of 3.2 and 4 mM reduced the LDH activity in SKOV-3 cells. Our data show that melatonin influences energy metabolism, with 3.2 mM and 4 mM melatonin being most effective. MT1 receptors seem to be related to glucose consumption, while the MT1-independent action may occur at the level of LDH activity by reverse metabolizing lactate to pyruvate in OC cells.

Treatment with melatonin alters the metabolic advantages of tumor cells by reversion of numerous tumor hallmarks associated with cell proliferation and survival. Previous studies indicate that, under specific pharmacological concentrations, melatonin affects cell proliferation by reducing glucose uptake, thereby altering the glucose metabolism of tumor cells (Gobbo *et al.*, 2015; Hevia *et al.*, 2015, 2017; Sanchez-Sanchez *et al.*, 2015). To ensure the oxidative profile of cells, optimal regulation of pyruvate metabolism is necessary by activating LDH (Zheng, Li and Luo, 2012; Mishra and Banerjee, 2019). The LDH gene encodes for two isoforms of the enzyme, LDHA and LDHB, the latter being able to convert lactate into pyruvate making it available to enter the mitochondria and influence the TCA cycle (Zhou *et al.*, 2011; Mishra and Banerjee, 2019). The oxidation of lactate to pyruvate leads to an increase in protons H^+, acidification of lysosomes, and

autophagy signaling by up-regulation of SIRT5; as a result, there is lactate uptake and cytotoxicity effects, as shown in the MTT assay (Shi *et al.*, 2019). This could explain the highly elevated LDH activity and lactate consumption in the MT1 silenced cells. This data showed that melatonin acts via the MT1 receptor, decreasing glucose consumption and reversing the Warburg phenotype. In the absence of MT1, however, melatonin utilizes other possible mechanisms possibly oxidizing lactate to pyruvate resulting in an anti-Warburg effect associated with a poorer cell fate. In addition, in the mitochondria, melatonin inhibits the PDK enzyme, which allows the conversion of pyruvate into acetyl-CoA, a component of the TCA cycle, causing a possible restoration of the normal metabolic phenotype of cells which use OXPHOS for energy generation (Reiter *et al.*, 2020). Melatonin can also modulate several mitochondrial pathways in SKOV-3 cells and redirect cells to the oxidative mitochondrial phenotype, leading to a reversal of the malignant phenotype of cells (Reiter *et al.*, 2020; Cesário *et al.*, 2022). These receptor-independent findings might be associated with other transport routes, such as involving GLUT1 and PEPT1/2.

Correlation analysis performed using GEPIA showed differences in the gene expression of *AANAT* and *ASMT* between OC samples and non-tumor tissue. The expression of these enzymes-encoding genes occurs naturally in the ovaries for melatonin production to maintain tissue integrity by scavenging ROS release during ovulation (Tamura *et al.*, 2009). We observed a decrease in the expression of *ASMT* in OC, corroborating the present findings where tumor tissue had a lower concentration of melatonin. The ASMT enzyme exhibits an important dimorphism related to melatonin synthesis, such as ASMTL, being essential for females, which are assumed to have a higher reserve capacity for melatonin production than males (Tan and Hardeland, 2021). When analyzing the survival curve of patients with high and low expression of *ASMT*, we observed a decrease, by several months, in the survival rate of patients with lower *ASMT* expression. Tran et al. (2021) analyzed breast cancer patients, and those with increased *ASMT* levels showed longer survival rate and were metastasis-free. These data support that tumor cells with gene mutations that depress melatonin synthesis have advantages for tumor progression.

Additional investigation of *ASMTL* expression correlated with particular genes related to TCA cycle and the electron transport chain (ETC). We observed that *ASMTL* showed positive correlation with *PDHA* and *PDHB*, *SDHD,* and the ATP synthase related genes. Downregulation of PDC enzymes, including pyruvate dehydrogenase E1 component subunit alpha and beta (PDHA and PDHB) causes a slowdown of OXPHOS rate while increasing the glycolysis rate to obtain

ATP. In turn, melatonin favors the formation of acetyl-CoA from pyruvate due to the PDC disinhibition, with acetyl-CoA being an essential intermediate for the TCA cycle and a cofactor for the AANAT enzyme. TCA plays a central role in the catabolism of carbohydrates, fatty acids, and amino acids via aerobic respiration. Succinate dehydrogenase (SDHD), an enzyme belonging to the mitochondrial complex II, plays an essential role in the ETC and the TCA cycle. Treatment of uterine endometrial cancer cells with melatonin halts tumor progression through SDH inhibition (Gu *et al.*, 2020). Moreover, we previously reported that female reproductive organ tumors exhibit a positive correlation of *ASMT* with OXPHOS-related enzymes including *PDHA1* and *SDHB* (Chuffa *et al.*, 2021). Mitochondria have the machinery responsible for the conversion of redox energy into an electrochemical gradient and the production of ATP formed in ETC through ATP synthase (Sharafati-Chaleshtori *et al.*, 2017). ATP synthase has a structure divided into two fractions, F0 in the mitochondrial membrane, and F1 in the mitochondrial matrix. In addition, each fraction has subunits transcribed by different genes and functionalities such as catalytic or motor (Senior, 2002). Through the proteomic profile, Chuffa *et al.* (2016) showed overexpression of the beta subunit ATP synthase after melatonin treatment in a rat model of ovarian cancer. The ability of melatonin to alter the characteristics of the glycolytic metabolism of tumor cells has been recently reported, especially by upregulating proteins related to TCA cycle and ETC (Cesário *et al.*, 2022). Our findings document a positive correlation between the enzyme-related genes responsible for melatonin synthesis and the alpha and beta subunits, corroborating other previous findings. Figure 5 summarizes the main cellular mechanisms after melatonin treatment in SKOV-3 cells.

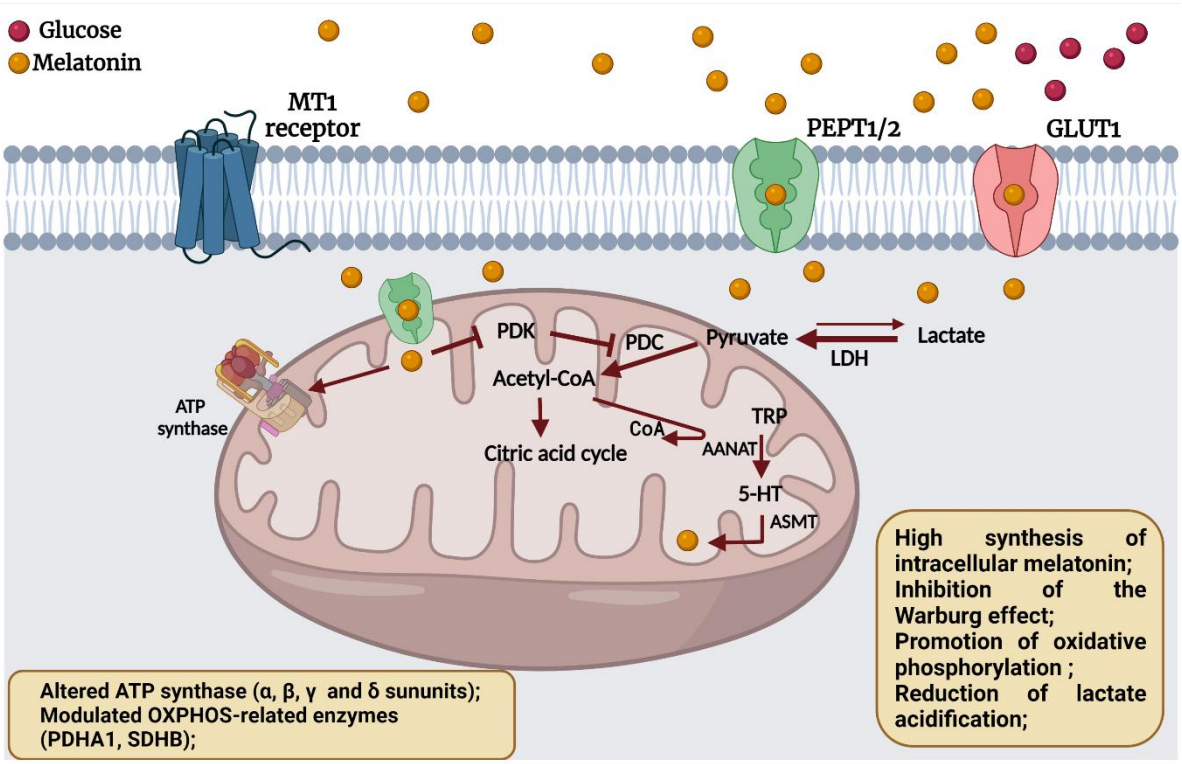

Figure 5. The diagrammatic representation shows potential mechanisms modulated by melatonin. Melatonin inhibits pyruvate dehydrogenase kinase (PDK), an enzyme that suppresses the dehydrogenase complex, responsible for converting pyruvate into acetyl-Coenzyme A (acetyl-CoA). The concentration of pyruvate can be increased by the enzyme lactate dehydrogenase (LDH) reverse conversion from lactate. The increased availability of pyruvate for conversion to acetyl-CoA for the citric acid cycle leads to the reversal of the Warburg effect; acetyl-CoA also is a limiting factor for the functioning of the AANAT enzyme, an intermediary in the synthesis of intramitochondrial melatonin. Furthermore, melatonin alters the function of ATP synthase, compromising the integrity of the mitochondrial membrane and inducing cell death. TRP: tryptophan; 5-HT: 5-hydroxytryptophan. Arrow: activation; bar-headed lines: inhibition. Adapted from BioRender.com (2022).

5. Conclusions

In summary, we demonstrated that melatonin is an essential antitumor agent and possesses a critical role in energy metabolism and mitochondrial integrity. At the highest concentrations, melatonin reversed glucose uptake through the MT1 receptor in SKOV-3 cells. In addition, an increase in lactate consumption was apparent possibly due to the LDH reverse conversion of lactate to pyruvate in a MT1-independent manner. Additional studies will provide further details on the role of melatonin in the signaling pathways that control cellular energy generation and metabolism. OC cells usually present lower enzyme levels for melatonin synthesis, and treatment with

melatonin augments its intracellular concentration. Moreover, our results reinforce the evidence that melatonin has both MT1-dependent and independent anti-cancer effects in OC.

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1. Author Contributions

MSC, LGAC: conceived the hypothesis of the study, collected and analyzed the data, and drafted the manuscript. LBG, SAAS, RCC, HSS, FRFS, DAPCZ: 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 with the submitted version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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5. Conclusão

Os resultados do presente trabalho reforçam a ação oncostática da melatonina no câncer de ovário, com efeitos dependentes e independentes dos receptores de melatonina. Foi observado diminuição da viabilidade celular, evidenciada pelo aumento nas taxas de morte e diminuição do potencial de membrana mitocondrial. Observou-se ainda diminuição da migração e invasão celular, e parada do ciclo celular na fase S e G2/M. Fica evidente que células de CO apresentam baixa concentração de melatonina devido às baixas taxas da enzimaa chave responsável pela síntese dessa indolamina, e que o tratamento com melatonina é capaz de reverter este quadro. O uso de ferramentas de bioinformática mostrou a correlação entre o gene *ASMTL* e proteínas relacionadas a integridade mitocondrial, mostrando que a síntese de melatonina aumenta a concentração de enzimas relacionadas ao metabolismo energético. Além disso, a melatonina foi capaz de diminuir o consumo de glicose e aumentar a interconversão de lactato em piruvato através da enzima LDH, levando a reversão do efeito Warburg. E ainda, as kinases responsáveis pela sinalização de proliferação e sobrevivência apresentaram diminuição após tratamento com melatonina. A ação da melatonina dependente do receptor parece estar associada apenas ao consumo de glicose, porém, o silenciamento dos receptores MT1 mostraram estar relacionado com a diminuição acentuada do potencial migratório e invasivo, e a redução dos níveis de kinases responsáveis pela sinalização intracelular. Estudos já revelaram que a melatonina pode atuar por outras vias, porém a modulação através dos receptores MT1 parece ter um papel importante na atividade das células tumorais, podendo até ser um possível alvo para tratamentos. Os dados apontam para ação benéfica da melatonina contra as vantagens adquiridas pelas células tumorais SKOV-3. Investigações adicionais são fundamentais para o entendimento dos mecanismos da ação da melatonina na mitocôndria, quanto ao seu papel efetivo nas moléculas relacionadas com a reversão do estado glicolítico, e os receptores MT1 como alvos para tumores com alto potencial metastático e desregulação em proteínas relacionadas com sinalização celular.

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