UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

PPG - BGA

Efeitos da melatonina sobre o metabolismo energético e estresse oxidativo em duas linhagens de células de câncer de ovário (SKOV-3 e CAISMOV-24)

Henrique Spaulonci Silveira

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

Orientador: Prof. Dr. Luiz Gustavo de Almeida Chuffa
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BOTUCATU – SP

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Palavras-chave: Câncer de ovário; Estresse oxidativo; Melatonina; Metabolismo energético; Processos metabólico.

A minha família e minha pequena filha Elis

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SUMÁRIO

LISTA DE ABREVIATURAS

AANAT: Aralkylamine N-Acetyltransferase α-tocopherol: Alpha-tocopherol (vitamin E)
AANAT: Aralkylamine N-Acetyltransferase
ASMT: Acetylserotonin O-Methyltransferase
CA-125: Cancer Antigen 125
CAT: Catalase
CO: Cancer of the Ovary
CREB: cAMP response element-binding protein
CS: Citrate Synthase
EC: Enzyme Commission (enzyme classification)
ERK1/2: Extracellular signal-regulated kinases 1 and 2
FR: Free radicals
G6PDH: Glucose-6-Phosphate Dehydrogenase
GLUT1: Glucose Transporter 1
GSH: Reduced Glutathione
GSH-Px: Glutathione peroxidase
GSH-Rd: Glutathione reductase
HIF-1α: Hypoxia-Inducible Factor 1-alpha
JNK1/2: c-Jun N-terminal kinases 1 and 2
LDH: Lactate Dehydrogenase
Mel: Melatonin
NADPH: Nicotinamide Adenine Dinucleotide Phosphate
NOX: NADPH oxidase
OC: Ovarian cancer
OS: Oxidative stress
PDC: Pyruvate Dehydrogenase Complex
PDH: Pyruvate Dehydrogenase
PDK: Pyruvate dehydrogenase kinase
PFK-1: Phosphofructokinase 1
ROS: Reactive Oxygen Species
RNS: Reactive Nitrogen Species
SOD: Superoxide dismutase
STAT3: Signal transducer and activator of transcription 3
STAT5: Signal transducer and activator of transcription 5

LISTA DE TABELAS

LISTA DE FIGURAS

RESUMO

O câncer de ovário (CO) é a segunda neoplasia ginecológica que mais afeta as mulheres e, por possuir sintomas genéricos, tem altas taxas de recorrência e rápida progressão. A proliferação celular descontrolada representa uma das principais características da doença neoplásica e, para tal, as células tumorais ajustam o seu metabolismo energético para promover o rápido crescimento e divisão celular; esse padrão metabólico é muito diferente daquele encontrado nas células saudáveis. A melatonina (Mel), é um hormônio produzido e secretado pela glândula pineal no período do escuro e, mais recentemente sua produção tem sido evidenciada nas mitocôndrias das células. No CO a concentração de Mel está bastante reduzida. Experimentos envolvendo modelos animais e cultura celular de CO já documentaram as propriedades antitumorais da Mel. Portanto, o objetivo do presente estudo foi avaliar a ação da terapia com Mel sobre o metabolismo energético e estresse oxidativo em células de carcinoma ovariano humano, linhagem SKOV-3 e CAISMOV-24. Os experimentos envolveram grupos controle, tratamento com Mel nas concentrações de 3,4 µM para SKOV-3 e 7 µM para CAIMOV-24 com base no cálculo da $IC_{50,}$ tratamento com Luzindole na concentração de 10^{-6} M para ambas as linhagens e Mel. Foram realizados os ensaios de citotoxicidade celular por MTT, e de migração e invasão com insertos. Para verificação dos efeitos sobre o metabolismo energético celular foram quantificadas as atividades de glicose-6 fosfato desidrogenase (G6PDH), fosfofrutoquinase 1 (PFK-1), complexo piruvato desidrogenase (PDH), citrato sintase (CS) e lactato desidrogenase (LDH). Também foram quantificados os níveis das proteínas HIF-1α, G6PDH, GAPDH e PDH por Western blot. A concentração de lactato foi analisada, por espectrofotometria, bem como a da glutamina. Em uma segunda abordagem, as enzimas ligadas ao estresse oxidativo como superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GSH-Px), bem como as concentrações de glutationa reduzida (GSH) e oxidada (GSSG), foram investigadas por espectrofotometria. Também foram avaliados os perfis de moléculas de sinalização celular envolvidas com agressividade tumoral através de ensaio multiplex e os níveis de Mel através de ensaio ELISA. Houve uma diminuição significativa nos níveis de HIF-1α, G6PDH, GAPDH e PDH após o tratamento com Mel, mesmo na presença de luzindole em ambas as células de CO. O tratamento com Mel também reduziu a atividade das enzimas metabolicamente relevantes como PFK-1, G6PDH, LDH e citrato sintase, enquanto a atividade de PDH aumentou em ambas as células. Os níveis de lactato e glutamina foram significativamente reduzidos após o tratamento com Mel. A Mel promoveu ainda a redução nas concentrações de CREB, JNK, NF-kB, p-38, ERK1/2, AKT, p70S6K e STAT em ambas as linhagens celulares. Houve também uma redução nos potenciais de migração e invasão celular em ambas as linhagens. O tratamento com Mel atenuou a capacidade migratória e invasiva das células CO de maneira independente do receptor, ao mesmo tempo que estimulou sua síntese intracelular. Além disso, as defesas enzimáticas antioxidantes foram atenuadas pela Mel, especialmente nas células CAISMOV-24. Coletivamente, a Mel regula os processos relacionados ao metabolismo energético que são alterados nas células de CO, revertendo o metabolismo do tipo Warburg e, potencialmente, reduzindo a glutaminólise. Essa regulação contribui para atenuar várias moléculas oncogênicas associadas à progressão e invasão do CO.

Palavras chaves: Câncer de ovário; melatonina; processos metabólicos; metabolismo energético; estresse oxidativo.

ABSTRACT

Ovarian cancer (OC) is the second gynecological neoplasm that most affects women and, as it has generic symptoms, it has high recurrence rates and rapid progression. Uncontrolled cell proliferation represents one of the main characteristics of neoplastic disease and, to this end, tumor cells adjust their energy metabolism to promote rapid cell growth and division; This metabolic pattern is very different from that found in healthy cells. Melatonin (Mel) is a hormone produced and secreted by the pineal gland during the dark period and, more recently, its production has been demonstrated in the mitochondria of cells. In CO the concentration of Mel is greatly reduced. Experiments involving animal models and CO cell culture have already documented the antitumor properties of Mel. Therefore, the objective of the present study was to evaluate the action of Mel therapy on energy metabolism and oxidative stress in human ovarian carcinoma cells, lineage SKOV-3 and CAISMOV-24. The experiments involved control groups, treatment with Mel at concentrations of 3.4 µM for SKOV-3 and 7 µM for CAIMOV-24 based on IC50 calculation, and treatment with Luzindole at a concentration of 10-6 M for both strains and Mel. Cellular cytotoxicity assays using MTT, and migration and invasion assays with inserts were carried out. To verify the effects on cellular energy metabolism, the activities of glucose-6 phosphate dehydrogenase (G6PDH), phosphofructokinase 1 (PFK-1), pyruvate dehydrogenase complex (PDH), citrate synthase (CS), and lactate dehydrogenase (LDH) were quantified. The levels of HIF-1α, G6PDH, GAPDH, and PDH proteins were also quantified by Western blot. The concentration of lactate was analyzed by spectrophotometry, as well as that of glutamine. In a second approach, enzymes linked to oxidative stress such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), as well as the concentrations of reduced (GSH) and oxidized glutathione (GSSG), were investigated by spectrophotometry. The profiles of cell signaling molecules involved in tumor aggressiveness were also evaluated using a multiplex assay and Mel levels using an ELISA assay. There was a significant decrease in the levels of HIF-1α, G6PDH, GAPDH, and PDH after Mel treatment, even in the presence of luzindole in both CO cells. Mel treatment also reduced the activity of metabolically relevant enzymes such as PFK-1, G6PDH, LDH, and citrate synthase, while PDH activity increased in both cells. Lactate and glutamine levels were significantly reduced after Mel treatment. Mel also promoted a reduction in the concentrations of CREB, JNK, NF-kB, p-38, ERK1/2, AKT, p70S6K, and STAT in both cell lines. There was also a reduction in cell migration and invasion potentials in both lineages. Mel treatment attenuated the migratory and invasive capacity of OC cells in a receptor-independent manner, while also stimulating their intracellular synthesis. Furthermore, antioxidant enzymatic defenses were attenuated by Mel, especially in CAISMOV-24 cells. Collectively, Mel regulates processes related to energy metabolism that are altered in CO cells, reversing Warburg-type metabolism and potentially reducing glutaminolysis. This regulation contributes to attenuating several oncogenic molecules associated with OC progression and invasion.

Keywords: Ovarian cancer; melatonin; cellular metabolism; energy metabolism; oxidative stress.

1. INTRODUÇÃO

1.1. Câncer de Ovário: fatores de risco e variedades

O câncer de ovário (CO) é classificado entre os oito tipos de câncer mais comuns em mulheres e é o segundo mais fatal entre os tumores do sistema genital feminino, resultando em óbito para mais da metade das mulheres afetadas (SIEGEL et al., 2022; SUNG et al., 2021). A Tabela 1 apresenta um resumo da incidência e mortalidade do CO.

Tabela 1. Estimativas globais de incidência e mortalidade por câncer em mulheres,em todas as faixas etárias, para o ano de 2020.

Tipos de câncer	Incidência (números totais)	Mortalidade (números totais)
Mama	2.088.849	626.679
Colorretal	823.303	396.568
Pulmão	725.352	576.060
Cérvix uterina	569.847	311.365
Tireoide	436.344	25.514
Corpo do Útero	382.069	89.929
Estômago	349.947	269.130
Ovário	295.414	184.799
Fígado	244.506	233.256
Linfoma não-Hodgkin	224.877	102.755

Fonte: Global Cancer Observatory, World Health Organization, 2020

No Brasil, entre os anos de 2020 e 2021, foram registrados 6.650 novos casos de CO, representando uma incidência de 5,79 casos para cada 100 mil mulheres. Esses números posicionam o CO como o oitavo tipo mais comum de câncer entre as mulheres e a terceira principal causa de mortalidade por câncer (INCA, 2022). (Tabela 2)

Tabela 2. Distribuição proporcional dos dez tipos de câncer mais incidentes em mulheres, estimados para o ano de 2020

Tipos de câncer	Casos (números totais)	%
Mama	59.700	29.5%
Colorretal	18.980	9.4%
Cérvix uterina	16.370	8.1%
Pulmão	12.530	6.2%
Tireoide	8.040	4.0%
Estômago	7.750	3.8%
Corpo do Útero	6.600	3.3%
Ovário	6.150	3.0%
Sistema Nervoso Central	5.510	2.7%
Leucemias	4.860	2.4%

Fonte: INCA, Instituto Nacional de Câncer, 2020

Esse cenário pode ser atribuído ao desenvolvimento tumoral na cavidade peritoneal (ARMSTRONG et al., 2006; LENGYEL, 2010), cuja detecção precoce é de difícil diagnóstico. Apesar das terapias existentes, incluindo cirurgia e quimioterapia, os estágios iniciais da doença (I e II) apresentam uma taxa de cura que varia entre 70% e 90% dos casos (MATHIEU et al., 2018). Por outro lado, os pacientes nos estágios III ou IV da doença apresentam uma taxa de sobrevida de apenas cerca de 20% de (ARMSTRONG et al., 2006; KURMAN; SHIH, 2016). A incidência do CO aumenta após a menopausa, em parte, devido a fatores associados à senescência ovariana como depleção de oócitos, redução dos esteroides hormonais, e aumento dos níveis de gonadotrofinas circulantes (VANDERHYDEN, 2005). A utilização de contraceptivos orais, laqueadura tubária e amamentação também podem influenciar no desenvolvimento da doença, assim como o acúmulo de mutações nos genes BRCA1/2 e TP53, inicialmente associadas ao aumento do risco de câncer de mama (MAVADDAT et al., 2013; NAROD, 2010), e que ao longo do tempo podem aumentar o risco de desenvolvimento do CO (KOTSOPOULOS et al., 2015, 2018). Além disso, o processo ovulatório incessante, associado à inflamação crônica e a exposição a carcinógenos ambientais, contribuem para a oncogênese ovariana (NESS; COTTREAU, 1999; STEWART et al., 2004). Os sintomas do CO são muitas vezes inespecíficos e podem ser confundidos com outras condições, incluindo dores na região pélvica e abdominal, aumento na frequência urinária, indigestão, sensação de inchaço e dores nas costas (GABRA, 2019). A identificação diagnóstica do CO pode ser -realizada pelo exame físico, histórico médico da paciente, ultrassonografia (pélvica ou transvaginal), hemograma completo, tomografia computadorizada (TC), tomografia por emissão de pósitrons (PET), ressonância magnética (RM), radiografia de tórax. Além disso, os níveis do antígeno cancerígeno 125 (CA-125) e o exame histopatológico são essenciais no diagnóstico do CO (GABRA, 2019; PDQ ADULT TREATMENT EDITORIAL BOARD, 2002). Com base na classificação das lesões cancerosas originadas do epitélio ovariano ou de cistos de inclusão, são observadas as diferenças entre tumores indolentes limítrofes (tipo I) e com potencial invasivo (tipo II). Os tumores tipo I apresentam baixo grau de malignidade e incluem o carcinoma seroso micropapilar, carcinoma mucinoso, carcinoma endometrioide e carcinoma de células claras (LISIO et al., 2019; SHIH; KURMAN, 2005). Já os tumores do tipo II, que apresentam alto grau de malignidade, são representados pelos subtipos: carcinoma seroso e carcinoma mesotelial misto indiferenciado (KURMAN; SHIH, 2016; LISIO et al., 2019). A maioria dos CO serosos de alto grau tem origem nas tubas uterinas e em outras áreas de origem epitelial, com estudos apontando que esses tipos II podem surgir de lesões nas fímbrias das tubas uterinas (DUBEAU; DRAPKIN,

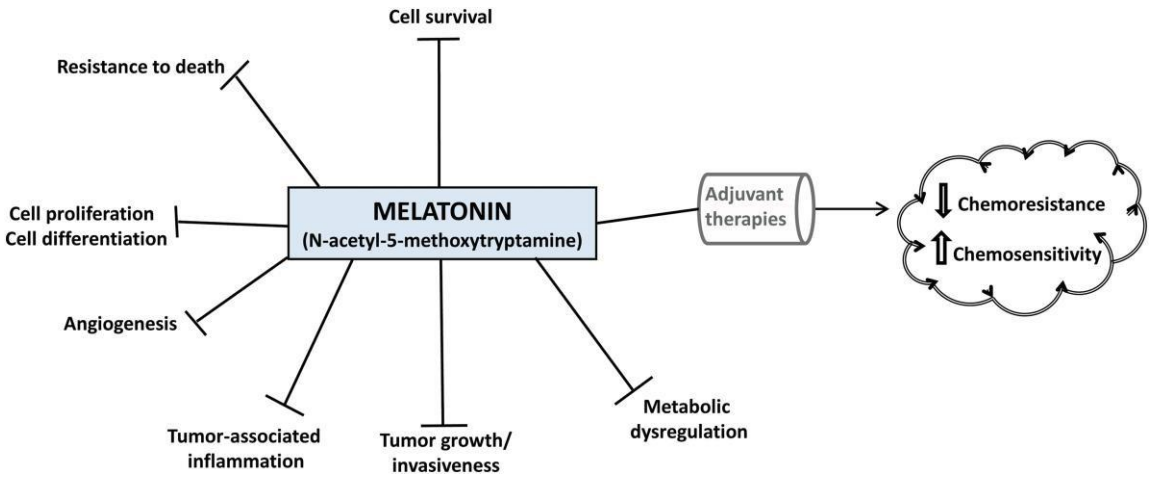
2013). A proximidade anatômica entre as fimbrias e o epitélio ovariano sugere a possibilidade de formação de aderências tubo-ovarianas, podendo levar ao desenvolvimento de neoplasias serosas intra-ováricas (DUBEAU, 2008). Aproximadamente 90% dos carcinomas serosos ovarianos são de alto grau, caracterizados por uma marcante proliferação celular, intensa atividade mitótica, núcleos pleomórficos, e estruturas papiliformes (KURMAN; SHIH, 2008; SMITH SEHDEV; SEHDEV; KURMAN, 2003). O CO responde bem à quimioterapia padrão utilizando compostos derivados de platina e/ou taxol como a Cisplatina, Carboplatina, Paclitaxel e o Topotecano, resultando em uma melhora significativa na sobrevida dos pacientes (CLOVEN et al., 2004; HO et al., 2004; KIKKAWA et al., 2006). Terapias-alvo, como inibidores de angiogênese (por exemplo, Bevacizumab) e inibidores de PARP (Poli ADP ribose polimerase) tem sido cada vez mais utilizadas, embora sua eficácia precise ser mais bem estabelecida (LISIO et al., 2019). Contudo, a maioria dos pacientes desenvolve quimioresistência, levando à rápida recorrência da doença e as pacientes se tornam incuráveis (BEREK et al., 1999). Os mecanismos subjacentes à quimioresistência ainda não estão completamente elucidados, mas incluem diminuição do acúmulo de fármacos, aumento da expulsão celular das drogas e maior tolerância aos tratamentos com taxol e platina (ITAMOCHI; KIGAWA; TERAKAWA, 2008).

1.2. Melatonina: Aspectos gerais e a relação com o Câncer de Ovário

A melatonina (Mel) é uma indolamina lipofílica também denominada de N-acetil-5metoxitriptamina, que é convertida a partir da serotonina pelas enzimas arylalquilamina Nacetiltransferase (AANAT) e acetilserotonina O-metiltransferase (ASMT). Sua principal secreção ocorre no período do escuro pela glândula pineal, embora possa ser sintetizada por outros tecidos em menor concentração (ZHAO et al., 2019). Embora sua função mais reconhecida seja a regulação do sono e do ritmo circadiano, a Mel desempenha importante função antioxidante, combatendo espécies reativas de oxigênio (ROS) e nitrogênio (RNS) no organismo. Além de neutralizar essas espécies radicalares, a Mel também aumenta a atividade de enzimas antioxidantes (GANDHI et al., 2015; REITER et al., 2016b; TAN et al., 2013; TOSCHES et al., 2014). Em células normais, a Mel tem ação protetora frente a apoptose, contudo, em células tumorais, ela tem demonstrado ter ações antiangiogênicas, antiproliferativas, pró-apoptóticas e imunomodulatórias (CHUFFA et al., 2015; REITER et al., 2016a; ZONTA et al., 2017a). Os níveis plasmáticos de Mel são baixos se comparados aos encontrados no líquido folicular ovariano pré-ovulatório e, quando ocorre uma suplementação

de Mel em meio contendo oócitos ovulados ou vitrificados, a Mel auxilia no desenvolvimento saudável e maturação dos mesmos (PAN et al., 2018; TAMURA et al., 2008). Já é conhecida a produção de Mel em diversas células, dentre elas, os oócitos (HE et al., 2016; TAN et al., 2013). De acordo com He et al. (2016), em estudos realizados em camundongos, a localização da AANAT foi identificada por imuno-histoquímica, revelando sua presença nas mitocôndrias dos oócitos em todos os estágios de maturação. Quando ocorre a suplementação do meio de cultura com serotonina, a concentração de Mel nos oócitos aumenta e isso não ocorre quando os oócitos são privados dessa suplementação (REITER; MA; SHARMA, 2020). A Mel já foi associada ao tratamento de diversos tipos de câncer, incluindo o de próstata, mama e ovário, e também tem sido considerada como um adjuvante terapêutico, aumentando a sensibilidade aos tratamentos quimioterápicos e reduzindo os efeitos adversos decorrentes (CALASTRETTI et al., 2018; GONZÁLEZ-GONZÁLEZ; MEDIAVILLA; SÁNCHEZ-BARCELÓ, 2018; ZARE et al., 2019). Estudos mostraram que os níveis usualmente elevados de Mel durante à noite ajudam na organização dos ritmos metabólicos homeostáticos do organismo. A desregulação do ritmo circadiano foi identificada como um dos fatores que contribuem para o desenvolvimento e progressão do câncer (SLOMINSKI et al., 2012; STEVENS et al., 2014). Os mecanismos moleculares associados aessas propriedades incluem a inibição da proliferação celular, angiogênese, e indução do processo de apoptose (MENÉNDEZ-MENÉNDEZ; MARTÍNEZ-CAMPA, 2018). A Mel ainda atua no controle da iniciação dos tumores, podendo ser utilizada como agente quimiopreventivo; devido a sua atividade de eliminação de radicais livres, exerce função protetora para o DNA. Essas injúrias cumulativas causadas ao material genético levam a um aumento do número de mutações e, consequentemente, podem resultar no surgimento de lesões malignas (ASCHAUER; MULLER, 2016; REITER et al., 2017). A propriedade de remoção de radicais livres e aumento da atividade de enzimas antioxidantes pela Mel foi observada no ovário. Estudos revelaram que a elevada concentração de ROS durante o processo ovulatório pode desencadear a transformação de células das fímbrias uterinas, podendo resultar no surgimento inicial do CO. Nesse sentido, a Mel pode interromper o efeito tumorigênico induzido por ROS e proteger o tecido original contra transformações moleculares malignas (HUANG et al., 2015; ZARE et al., 2019). De acordo com Reiter et al. (2019), a Mel está presente em maiores concentrações em células normais em comparação com células tumorais, e o tratamento de certos tumores com Mel demonstrou impacto na reversão do efeito Warburg, diminuindo a proliferação celular, invasão, migração e, consequentemente, a metástase. Em outro estudo, o

tratamento com Mel utilizando concentrações de 400 a 600 µM, reduziu a taxa de sobrevivência e proliferação das 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 (SHEN et al., 2016). Em algumas linhagens celulares de CO, a Mel demonstra efeitos inibitórios na sobrevivência celular, e potencializa o tratamento combinado com a cisplatina (ZEMŁA et al., 2017). Estudos *in vivo* investigaram os efeitos a longo prazo da terapia com Mel sobre proteínas anti-apoptóticas (Bcl-2 e survivina) e pro-apoptóticas (p53, BAX e caspase-3) em modelos experimentais de CO papilífero seroso; a Mel demonstrou aumentar a expressão de BAX, p53 e caspase-3, além de induzir a fragmentação do DNA, conforme observado no teste do TUNEL (CHUFFA et al., 2016). Recentemente, estudos *in vitro* e *in vivo* evidenciaram o papel pró-apoptótico da Mel em células tumorais, ao mesmo tempo em que promove a sobrevivência de células normais (CHUFFA; REITER; LUPI, 2017). Nosso grupo de pesquisa já investigou a interação entre a Mel e o metabolismo energético do CO em um estudo *in vivo,* envolvendo análise proteômica global. A Mel diminuiu a expressão de proteínas envolvidas em processos metabólicos, como a produção de energia celular e proteínas associadas à mitocôndria, além de afetar vias associadas ao estresse do retículo endoplasmático, ao processamento e apresentação de antígeno, e a proteoglicanos relacionados ao CO. Assim, esses resultados destacam a Mel como uma terapia adjuvante importante no tratamento do CO, controlando a proliferação de células malignas e auxiliando na preservação e atividade de moléculas com efeitos antitumorais (Figura 1). É ESSA REFERÊNCIA?



Fonte: CHUFFA, L. G. DE A.; REITER, R. J.; LUPI, L. A (2017, p.948)

FIGURA 1 - Resumo dos Processos em que a Mel atua no CO.

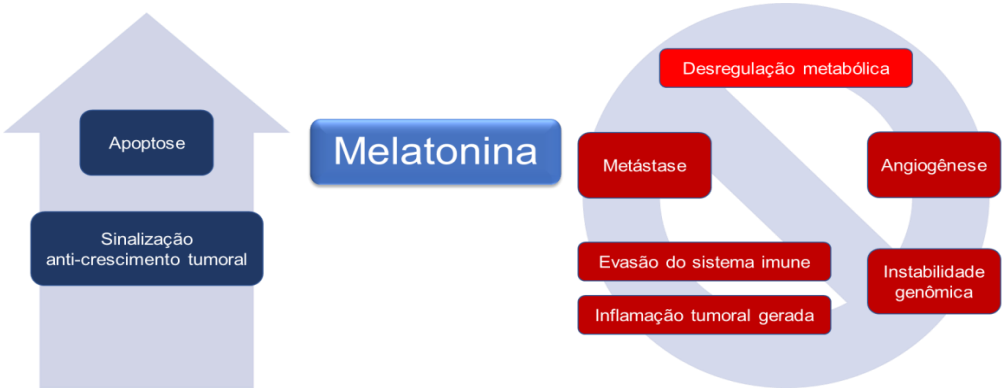
1.3. Câncer de ovário e metabolismo energético

No CO, ocorrem mudanças metabólicas devido à presença de células malignas que alteram as condições normais, promovendo a carcinogênese. A fosfofrutoquinase 1 (PFK1) é responsável pela conversão da frutose-6-fosfato em frutose1,6-bisfosfato, sendo o segundo passo irreversível da glicólise (YALCIN et al., 2014). Mutações nessa enzima alteram o fluxo glicolítico da célula tumoral, enquanto seu ativador mais potente, a frutose-2,6 bisfosfato, é altamente expresso no CO (ATSUMI et al., 2002; WEBB et al., 2015). O aumento na expressão de PFK-1 inativa o complexo piruvato desidrogenase (PDH), suprimindo o ciclo de Krebs e, consequentemente, a fosforilação oxidativa. Esse mecanismo desvia a produção de ATP para a glicólise favorecendo o efeito Warburg (ASSAILY; BENCHIMOL, 2006). A citrato sintase (CS) catalisa a primeira reação do ciclo do ácido tricarboxílico, que regula a geração de energia na respiração mitocondrial e desempenha um papel importante no metabolismo dos carboidratos (SCHLICHTHOLZ et al., 2005). Segundo Anderson et al. (2013), a atividade da CS está aumentada durante a progressão do CO induzido em modelo experimental de câncer epitelial da superfície ovariana (MOSE). Em estudo que envolveu experimento *in vitro* com linhagens SKOV-3 e A2780A, a expressão desregulada de CS no CO influenciou a invasão, migração, proliferação celular e quimiossensibilidade, sugerindo que a inibição da CS pode melhorar o prognóstico das pacientes, reduzindo metástases e quimioresistência (CHEN et al., 2014). A lactato desidrogenase (LDH), por sua vez, catalisa a conversão de piruvato em lactato. Quando sua atividade é estimulada na glicólise ocorre acidificação excessiva no microambiente tumoral, favorecendo a invasão e metástase (GATENBY; GILLIES, 2004). O aumento de LDH é observado no líquido peritoneal e soro de pacientes com CO, e está relacionado como um importante promotor da tumorigênese, estimulando o efeito Warburg (HANAHAN; WEINBERG, 2011).

1.4. Melatonina, metabolismo energético e Câncer de Ovário: Interações e perspectivas

As células malignas passam por reprogramações metabólicas para atender suas crescentes demandas de energia e controle dos níveis de ROS (DE BERARDINIS; CHANDEL, 2016). Essas adaptações metabólicas, caracterizadas como “Hallmarks” do câncer, incluem a capacidade de invasão e metastização, evasão do controle do crescimento celular, a proliferação descontroladaentre outros processos (HANAHAN; WEINBERG, 2011). Identificar quais dessas características um tumor apresenta pode facilitar sua classificação e orientar o tratamento de forma mais precisa (PAVLOVA; THOMPSON, 2016). A Mel está relacionada com essas assinaturas do câncer, pois suas propriedades antioxidantes

protegem o material genético, seja removendo ROS ou estimulando os sistemas de reparo do DNA, o que pode ajudar a prevenir a instabilidade genômica (TALIB, 2018) (Figura 2).



Fonte: Imagem elaborada pelo autor

FIGURA 2 - Impacto da Mel em diferentes “Hallmarks” do câncer.

No entanto, a Mel exerce uma ação antagônica nas células tumorais ao promover um efeito pró-oxidante, contribuindo para a morte celular dessas células (ZHANG; ZHANG, 2014). Estudos realizados em linhagens celulares de câncer de mama (MCF-7) e cólon (HCT-15) demonstraram que a Mel aumenta a capacidade de reparo do DNA, afetando os genes envolvidos nas vias de resposta a danos no DNA (LIU et al., 2013). Em relação às alterações metabólicas no câncer, o fenômeno conhecido como efeito Warburg ocorre devido à reprogramação metabólica das células tumorais, que direcionam a oxidação da glicose para o citoplasma pelo processo de glicólise aeróbica. A glicólise aeróbica resulta em produção de lactato e de ATP em menor quantidade, porém com maior velocidade de síntese se comparado com a fosforilação oxidativa (KALYANARAMAN, 2017; SPENCER; STANTON, 2019).

A capacidade das células tumorais de alternar entre substratos energéticos e vias metabólicas pode estar associada a um prognostico desfavorável (HAN et al., 2018a). A glicólise funciona como uma via precursora, com seus produtos sendo utilizados em diversas vias, incluindo a fosforilação oxidativa, a via das pentoses fosfato, a gliconeogênese e a síntese de ácidos graxos (HAY, 2016). Além da glicólise, a via das pentoses também se eleva no câncer, favorecendo o crescimento tumoral e a síntese de intermediários redutores, como o NADPH, que são essenciais para a proteção contra os danos oxidativos (CATANZARO et al., 2015). Essas alterações no fornecimento de energia garantem às células tumorais mais eficiência em termos de proliferação celular, proteção contra apoptose e um processo de metástase mais acelerado (REITER et al., 2020). O fator induzível por hipóxia 1-alfa (HIF-1α) é um fator transcricional que está envolvido no estabelecimento do efeito Warburg nas células tumorais, aumentando a expressão de enzimas glicolíticas e transportadores de glicose,

como o GLUT1. Sob baixas concentrações de oxigênio, o HIF-1α estimula a transcrição de genes responsáveis pela agressividade tumoral (VAUPEL; MAYER, 2007). O HIF-1α ainda estimula a expressão de piruvato desidrogenase quinase (PDK) que atua inativando o complexo piruvato desidrogenase (PDC). A inibição do PDC resulta na oxidação de piruvato em acetil-CoA e eleva os níveis de lactato na célula (BENSINGER; CHRISTOFK, 2012). Interessantemente, estudos *in vivo* demonstraram que células mamárias humanas malignas liberam quantidades consideráveis de lactato durante o dia, mas os níveis são significativamente mais baixos à noite, juntamente com outros parâmetros como síntese de DNA e captação de glicose (BLASK et al., 2014). Esses resultados sugerem que as células exibem o efeito Warburg durante o dia e revertem durante a noite, indicando uma associação entre a atividade metabólica e o ciclo circadiano. Quando ocorre supressão da Mel pela exposição a luz durante o período noturno, as células tumorais mantêm o efeito Warburg, evidenciando a relação entre a Mel e a atividade metabólica celular (BLASK et al., 2014). Mais recentemente, observou-se que a Mel nas mitocôndrias das células tumorais inibe a ação de PDK, permitindo que o piruvato seja convertido em acetil coa (REITER et al., 2020). Isso potencializa a eficiência da cadeia de transporte de elétrons, aumentando a síntese de ATP e eliminação de ERO na matriz mitocondrial (JOU et al., 2004; REITER et al., 2018). No entanto, são necessários mais estudos para entender completamente o mecanismo pelo qual a Mel regula o PDC e como isso afeta o metabolismo celular. O acetil é fundamental para síntese da enzima limitante na produção de Mel, aril-alquilamina N-acetiltransferase (AANAT). Estudos anteriores do nosso grupo demonstraram que o tratamento com Mel reduziu os níveis de HIF-1α e fatores angiogênicos em um modelo de carcinoma ovariano papilar seroso em ratas com preferência ao etanol (ZONTA et al., 2017b). Corroborando o achado, Park et al. (2010) observaram que a Mel, devido às suas propriedades antioxidantes contra ROS, inibiu a atividade do HIF-1α inibindo em células tumorais do cólon humano (linhagem HCT116). A glicose-6-fosfato desidrogenase (G6PDH) é uma enzima essencial para o metabolismo energético que tem como produto o NADPH para manter os níveis de GSH. Em células tumorais, essa enzima elimina ROS, protegendo as células contra danos oxidativos (JU et al., 2017). São necessárias mais investigações sobre como o tratamento com Mel pode afetar a via das pentoses, inibindo a produção de G6PDH em células de CO. Mais estudos são necessários para compreender o papel da Mel em diferentes concentrações na interação com o metabolismo celular do CO, a fim de verificar se ocorre diminuição da proliferação de células malignas e se a Mel exerce função antitumoral no CO. Esses achados

deverão prover fundamentos que possam auxiliar na compreensão dos seus efeitos terapêuticos.

2. RELEVÂNCIA E JUSTIFICATIVA DO TEMA

Os efeitos da terapia com a Mel sobre as vias de regulação no CO, que interagem com mecanismos envolvidos na proliferação, diferenciação, apoptose e resistência aos tratamentos são bem conhecidos (DE ALMEIDA CHUFFA et al., 2018). No entanto, pouco se sabe sobre os efeitos da Mel na mitocôndria de células tumorais quanto a reprogramação do metabolismo energético. O presente estudo investigou moléculas associadas ao metabolismo celular em duas linhagens de células de CO, bem como componentes do estresse oxidativo e sinalização celular frente ao tratamento com Mel. Assim, será possível obter um panorama de como a Mel interage com esses componentes e se essa interação varia conforme o grau de malignidade tumoral. Logo, sabendo que a Mel é uma molécula que pode atuar por vários mecanismos em diversos tumores, a compreensão desses efeitos poderá trazer novas perspectivas para o tratamento do CO, melhorando os resultados e abrindo novas perspectivas para serem exploradas em combinação com outros tratamentos na prática clínica.

3. HIPÓTESE

Uma vez que a melatonina tem demonstrado resultados promissores como terapia em diferentes classes de tumores, a hipótese do presente estudo é de que a melatonina redireciona a glicose para ser oxidada na mitocôndria das células de câncer de ovário, modulando a assinatura metabólica da célula tumoral e controlando sua disseminação, enquanto estimula a morte celular mediada pelo estresse oxidativo.

4. OBJETIVO GERAL

Investigar o efeito da terapia com melatonina sobre o metabolismo celular energético, sinalização celular e estresse oxidativo em células de carcinoma ovariano humano (SKOV-3) e (CAISMOV-24).

4.1 OBJETIVOS ESPECÍFICOS

1. Avaliar a viabilidade celular e citotoxicidade através do ensaio do MTT;

2. Realizar ensaio de migração e invasão celular;

3. Analisar o perfil das moléculas de sinalização celular e mediadores: CREB, JNK, AKT, NF-kB, p38, ERK1/2, STAT3, STAT5 e P70S6K relacionadas ao metabolismo energético de ambas as linhagens;

4. Quantificar os níveis de lactato desidrogenase, lactato, G6PDH, PFK, PDH, PFK, citrato sintase e glutamina através de análises bioquímicas para correlação com o tipo de metabolismo desempenhado pelas células;

5. Quantificar os níveis de melatonina nas linhagens celulares através de kit ELISA para validar a baixa produção na célula tumoral;

6. Quantificar por Western Blot, os seguintes alvos moleculares: HIF-1α, G6PDH, GAPDH e PDH relacionados com o metabolismo energético tumoral;

7. Analisar indiretamente o estresse oxidativo através dos níveis de moléculas antioxidantes SOD, CAT, GSH-PX**:**

8. Avaliar a razão GSH e GSSG

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CAPÍTULO 1

Artigo submetido ao periódico científico "*Life Sciences"*

Melatonin changes energy metabolism and reduces oncogenic signaling in two ovarian cancer cells.

Henrique Spaulonci Silveira[1], Roberta Carvalho Cesário[1], Renan Aparecido Vígaro[1], Leticia Barbosa Gaiotte[1], Maira Smaniotto Cucielo[1], Fernando Guimarães[2], Fábio Rodrigues Ferreira Seiva[1], Debora Aparecida P. C. Zuccari[3], Russel J. Reiter[4], Luiz Gustavo de Almeida Chuffa[1]*

[1]Department of Structural and Functional Biology, UNESP - São Paulo State University, Institute of Bioscences, Botucatu, 18618-689, São Paulo, Brazil.

[2] Hospital da Mulher "Professor Doutor José Aristodemo Pinotti" – CAISM, UNICAMP; Campinas; São Paulo; Brasil.

[3] Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, SP, 15090–000, Brazil.

[4] Department of Cellular and Structural Biology, UTHealth, San Antonio, TX 78229, USA.

*****Luiz Gustavo de Almeida Chuffa, Department of Structural and Functional Biology, Institute of Biosciences of Botucatu, UNESP - São Paulo State University, Botucatu, São Paulo, Brazil, Zip Code: 510; P.O Box: 18618–689, Rubião Júnior, s/n, Botucatu, SP – Brazil, Phone: +55 (14) 3880-0027. Email: luiz-gustavo.chuffa@unesp.br

Abstract: Ovarian cancer (OC) adjusts energy metabolism in favor of its progression and dissemination. Given the myriad of antitumor actions attributed to melatonin (Mel), we sought to explore its effects on energy metabolism and kinase signaling in human OC cells (SKOV-3 and CAISMOV-24). Cells were divided into a control group and experimental groups. They were treated with Mel in the presence or absence of the antagonist luzindole. Cell viability was reduced with Mel concentrations at 3.4 μM for SKOV-3 and 7 μM for CAISMOV-24 cells. There was a significant decrease in HIF-1α, G6PDH, GAPDH, and PDH levels after Mel treatment even in the presence of luzindole in both OC cells. Mel treatment also reduced the activity of OC-related enzymes such as PFK-1, G6PDH, LDH, and citrate synthase, whereas PDH activity was increased in both cells. The levels of lactate and glutamine significantly dropped after Mel treatment. Mel promoted a reduction in the concentrations of CREB, JNK, NF-kB, p-38, ERK1/2, AKT, P70S6K, and STAT in both cell lines. Collectively, Mel regulates energy-related processes that are altered in OC cells, thus reversing the Warburg-type metabolism and possibly reducing glutaminolysis thereby attenuating various oncogenic molecules associated with OC progression and invasion. Most of these changes were receptor independent.

Keywords: Ovarian cancer; melatonin; cellular metabolism; Warburg effect; kinase signaling; tumor progression; glutamine.

1. Introduction

Ovarian cancer (OC) is a common cancer of the female reproductive system and has the highest mortality rate of any reproductive cancer [1]. This high death rate is especially due to the generic and silent symptoms in addition to its recurrence rate and aggressiveness [2,3]. Among the risk factors associated with OC development are late menopause, high number of

pregnancies, hormone replacement therapy, incessant number of ovulations, and genetic factors [4]. The heterogeneity of OC subtypes makes conventional treatments with chemotherapy and surgical removal difficult and contributes to disease recurrence and chemoresistance [5,6]. Changes in cellular metabolism occur as tumors reprogram nutrient acquisition and metabolism pathways to meet the demands for bioenergy, replication, and regulation of reactive oxygen species (ROS) levels in these malignant cells [7]. These alterations are considered hallmarks of cancer metabolism, having as main characteristics of tissue invasion and metastasis, avoidance of cell growth suppression, proliferation, replicative immortality, resistance to cell death, induction of the angiogenesis process, immune evasion, and altered cellular energy [8,9].

Melatonin (Mel) is a lipophilic indoleamine, also known as N-acetyl-5-methoxytryptamine. It is synthesized from serotonin by the enzymes arylalkylamine N-acetyltransferase (AANAT) and acetylserotonin O-methyltransferase (ASMT) [10]. Although Mel production occurs in the pineal gland at night, many other tissues and cells (perhaps all) also produce the indole for local actions [11,12]. In healthy cells, Mel protects the genetic material by removing destructive ROS or by stimulating DNA repair systems, both of which reduce genomic instability [13]. However, in tumor cells, Mel has an antagonist action by promoting a prooxidant effect which contributes to cancer cell death [14].

Aerobic glycolysis (or the Warburg effect), which many tumor cells display, consists of enhanced glucose uptake and lactate production, resulting in a rapid energy supply required to support tumor growth and metastatic processes [15–18]. Because it reverses Warburg type metabolism, Mel has been hypothesized to inhibit the activity of pyruvate dehydrogenase kinase (PDK), which in turn normally activates the pyruvate dehydrogenase complex (PDH) thereby allowing the conversion of pyruvate to acetyl-CoA and redirecting glucose for oxidation in the mitochondria [19]. It has been recently reported that Mel, at pharmacological concentrations, reduced the levels of lactate and lactate dehydrogenase in SKOV-3 cells, corroborating the idea of an alternative route for pyruvate metabolism [20]. However, it has not yet been documented which mechanisms and signaling pathways are involved with these processes. Additional emphasis on different pathways related to glycolysis, pentose phosphate pathway, and citric acid cycle will help reveal which biochemical intermediates are influenced by Mel; many of these processes are examined in the current report.

Altered cell signaling is usually linked to the progression of different tumors. The overexpression of cAMP response element-binding protein (CREB) induces apoptosis in T

cells and stimulates cancer growth; in OC, CREB functions as an important regulator of tumor initiation, progression, and metastasis [21,22]. The signal transducer and activator of transcription 3 (STAT3) and 5 (STAT5) activation play important roles in metastasis including cell proliferation, invasion, migration, and angiogenesis [23]. Also, nuclear factor-kB (NF-kB) promotes to the transcription of several genes involved in immune response, cell proliferation, adhesion, and apoptosis [24]. Protein kinase B (PKB), also known as Akt, and extracellular signal-regulated kinases 1 and 2 (ERK1/2) are signaling pathways associated with cell motility which are also regulated by Mel [25]. Mel exerts an inhibitory effect on breast cancer cell invasion through downregulation of the mammalian p38 mitogen-activated protein kinase (p38) pathway [26]. Another factor to be investigated is the ribosomal protein S6 kinase (P70S6K), which plays a pivotal role in regulating cell growth by stimulating the synthesis of proteins associated with cancer progression [27].

As Mel exhibits diverse mechanisms of action in different tumor types, our study aimed to elucidate its regulatory mechanisms in glycolysis-related energy metabolism and cell signaling pathways in two OC cell lines, SKOV-3 and CAISMOV-24 cells. An understanding of these mechanisms from a metabolic standpoint may bring new perspectives for patients suffering with deadly OC.

2. Materials and Methods

2.1. Cell culture and reagents

SKOV-3 cell line (ATCC® HTB-77) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and the CAISMOV-24 cell line was obtained from the Hospital da Mulher Prof. Dr. José Aristodemo Pinotti Caism (UNICAMP, Campinas, SP, BRAZIL). The CAISMOV-24 cell line was derived from ascites obtained from a 60-year-old patient with recurrent disease. It is classified as a low-grade serous OC [28]. SKOV-3 cells were grown in RPMI medium (Gibco, Paisley, UK) whereas CAISMOV-24 cells were grown in DMEM HAM'S F12 (LGC, Cotia, BR). Both cell types were supplemented with 10% fetal bovine serum (FBS; Gibco) and penicillin at 100 IU/ml and 100 µg/ml streptomycin (Gibco) and incubated in a humidified atmosphere at 37°C with 5% CO2. The cells were expanded in 75 and 25 cm2 cell culture flasks (Costar, Cambridge, MA, USA) containing the basal culture medium, which was changed periodically. Once the cells reached 90% confluence, the culture supernatant was aspirated, and cells were washed twice with 10% phosphate-buffered saline (PBS; Oxoid Limited, Hampshire, UK). Next, cells were incubated with trypsin/EDTA

(Gibco) to avoid any adherence to the flasks. After centrifugation, cells were washed in culture medium, resuspended, and used in all experiments.

2.2. Experimental design and treatments

To assess the effects of Mel and its combination with luzindole, a Mel receptor blocker, on OC cells, we initially determined effective concentrations using the IC_{50} method (MTT cytotoxicity assay). Concentrations of 3.4 µM and 7 µM of Melatonin (Mel, catalog number M5250-1G, Sigma-Aldrich, Saint Louis, MO, USA) were effective in inhibiting SKOV-3 and CAISMOV-24 cells over a period of 24 h. This time period corresponds to a single circadian cycle of Mel action, influencing the choice for a 24-hour exposure. The concentration of the Mel receptor antagonist luzindole (L2407, Sigma CO, Saint Louis, MO, USA) was used at a concentration of 1 µM for both cell lines. Subsequently, to assess the combined effect of Mel and luzindole on cancer cells, each cancer cell line was divided into three experimental groups: Control: cells treated with culture medium containing 100 µL of DMSO solution as vehicle; Melatonin: cells treated with Mel at concentrations of 3.4 for SKOV-3 and 7 µM for CAISMOV-24, plus vehicle; Mel + luzindole: cells treated with a combination of Mel and luzindole, both dissolved in DMSO. For the combination group, luzindole was first added to the incubation medium, and after 30 min, the pre-established concentration of Mel was added. Mel and luzindole were dissolved in DMSO to maintain the molar concentrations specified by the manufacturers. All experimental assays were performed in biological and technical triplicates (Figure 1).

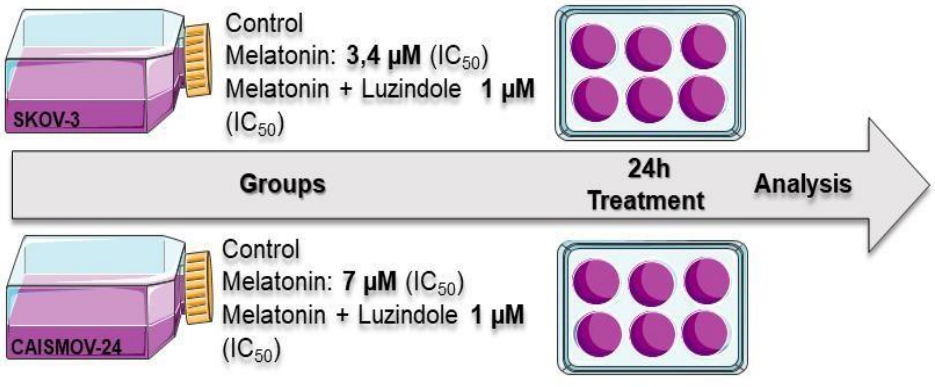

Figure 1. Schematic representation of the treatment groups and experimental procedures.

2.3. Cell viability (MTT assay)

Cell viability was analyzed using the MTT colorimetric method based on IC_{50} to determine proper Mel concentrations. After the confluence rate, SKOV-3 and CAISMOV-24

cells were trypsinized, seeded in 6-well plates at a density of 5x105 cells per well, and cultured with appropriate medium supplemented with 10% FBS. After cell adherence, Mel and luzindole were added to the culture medium following the experiment design with specific concentrations (2.0, 3.4, 5.0, and 7.0 µM). These concentrations are close to the physiological levels of Mel. Viability curves were estimated after a 24h of treatment period using the MTT solution (5mg/mL). To assess cytotoxicity, the reactions were performed using a microplate reader (Epoch, Bio Tek Instruments, USA).

2.4. Western blot analysis

At the end of the experiments, SKOV-3 and CAISMOV-24 cells (5 x 10^4) were washed with PBS and homogenized with RIPA lysis buffer containing protease inhibitors (Sigma CO, Saint Louis, MO, USA). Protein quantification was performed using a Nano-Vue® (GE Healthcare) spectrophotometer. Samples with 50 µg of protein cell extract were solubilized and applied on 4-20% polyacrylamide gel (SDS-PAGE). After performing electrophoresis, the proteins were electro transferred (35 mA) to a nitrocellulose membrane (Bio-Rad, California, USA). The membranes were blocked with 3% milk diluted in TBS-Tween and incubated with the primary antibodies: glucose-6-phosphate dehydrogenase (G6PDH) (ab87230, abcam, 1:500), (GAPDH) (ab9485, abcam, 1:500), hypoxia-inducible factor 1-alpha (HIF-1α) (ab2185, abcam, 1:500), and pyruvate dehydrogenase (PDH) E1 subunit (ab110416, abcam, 1:500), diluted in 1% TBS-Tween. Next, the membrane was washed in basal solution (1% TBS-Tween) and incubated with secondary antibody (1:10,000) diluted in 1% milk. After washing, the reaction was generated using the chemiluminescent substrate ECL® Selected Western Blotting Detection Reagent (GE Healthcare, Uppsala, Sweden). The analysis detected the presence or absence of bands in G-Box transilluminator. The intensity of bands was quantified using Image J software through optical densitometry and normalized to the endogenous α-tubulin (ab4074, abcam, 1:500). Three replicates from each group were used.

2.5. Biochemical analysis of energy metabolism

To analyze enzymatic activities or the level of the molecules that participate in the glycolytic pathway, pentose phosphate pathway, citric acid cycle, and glutaminolysis, the assays were carried out on specific biochemical kits for each analyte. For standardization, we used 40 µg of proteins that were extracted with a lysis buffer containing protease inhibitors

(Sigma CO, Saint Louis, MO, USA) according to the experimental design. The biochemical kits were as follows: Glucose-6-Phosphate Dehydrogenase (G6PDH) Assay Kit (MAK015), Phosphofructokinase 1 (PFK-1) Activity Colorimetric Assay Kit (MAK093), Lactate Dehydrogenase (LDH) Assay Kit (MAK0661KT), Lactate Assay Kit (MAK064), Citrate Synthase (CS) Assay Kit (MAK193), Pyruvate Dehydrogenase (PDH) Activity Assay Kit (MAK183), and Glutamine Assay Kit (MAK438). All biochemical kits were manufactured by Sigma CO (Saint Louis, MO, USA). After the experiments, readings were performed on a microplate reader (Epoch, Bio Tek Instruments, USA).

2.6. Measurement of cell signaling molecules

Multiple molecules associated with tumor-related cell signaling were determined after the treatments. The protein extraction (n= 24 samples/group) was performed using a MilliPlex® immunoassay Map Kit (EMD Millipore, Darmstadt, Germany) according to the manufacturer's protocol. The profile of cell signaling (Cat # 48–681 MAG) included the following factors: CREB, JNK, AKT, NF-kB, p38, ERK1/2, STAT3, STAT5, and P70S6K. The levels of these molecules ranged from 200 to 10000 pg/mL according to the manufacturer's instructions. Fluorescence intensity was read at 575 nm and measured using the MAGPIX system (Luminex® Corporation, Austin, TX, USA).

2.7. Statistical analysis

Data were processed using an analysis of variance (One-way ANOVA) for one factor and presented as the mean ± standard deviation (SD). Significant results were complemented by Tukey's test. Statistical analysis was performed using GraphPad Prism 9.0 software (GraphPad Software, San Diego, CA) and significant differences between groups were determined with a p-value <0.05.

3. Results

3.1. Cell viability is significantly reduced with melatonin concentrations in both OC cells.

Initially, we identified the cytotoxic effect of Mel at different concentrations, with IC50 was pre-established at 3.4 µM for SKOV-3 cells and 7 µM for CAISMOV-24 cells; Notably, CAISMOV-24 cells, derived from malignant ascites, exhibited greater resistance to Mel's treatment (Figure 2A). To determine whether the effect of Mel was receptor-dependent or independent, luzindole, acting as an antagonist of Mel receptors MT1 and MT2 Mel

receptors, was employed. Subsequently, cell viability was assessed using different concentrations of luzindole ranging from 100 µM to 0.0001 µM. After 24 hours of treatment, viable cells were counted, and the results were expressed as cell growth relative to the control group; the effective concentration of luzindole was found to be 1 µM for both cell types (Figure 2B).

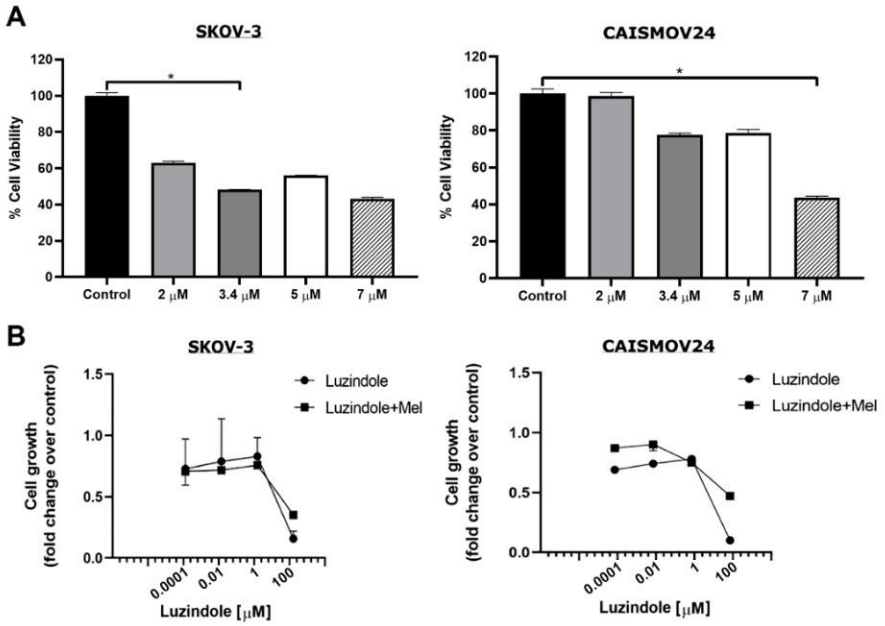

Figure 2. Cell viability after Mel and luzindole exposures. A) MTT reduction assay after treatment with different concentrations of Mel (2 µM, 3.4 µM, 5 µM, and 7 µM) in SKOV-3 and CAISMOV-24 cells to determine optimal concentration based on IC50. B) MTT assay after treatment with different concentrations of luzindole (100 µM, 1 µM, 0,01 µM, and 0,0001 µM.) in SKOV-3 and CAISMOV-24 cells. Data are expressed as the mean ± SD. Experiments were performed using three technical and biological replicates. *P<0.05.

3.2. Melatonin attenuates the levels of proteins related to the Warburg effect in OC cells.

To investigate the efficiency of Mel on glycolytic intermediates, we analyzed the levels of glucose-6-phosphate dehydrogenase ***(***G6PDH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate dehydrogenase (PDH), and hypoxia-inducible factor 1-alpha (HIF-1α) in both OC cell lines after treatment for a period of 24 h (Figures 2 and 3). Mel alone significantly decreased HIF-1α levels, (1.72-fold and 2.38-fold decrease vs control groups in SKOV-3 and CAISMOV-24 cells). In CAISMOV-24 cells, HIF-1α was significantly reduced by the Mel (1.55-fold decrease vs control group) and also after Mel and luzindole exposure (2.03-fold decrease vs control group). GAPDH is involved not only in the glycolysis but also in other signaling pathways that favor tumor expansion; Mel significantly suppressed its increase (1.15fold and 2.11-fold decreased vs control group in SKOV-3 and

CAISMOV-24 cells). In the presence of luzindole, Mel also reduced the levels of GAPDH in the two OC strains (1.81-fold and 1.96-fold decrease vs control groups in SKOV-3 and CAISMOV-24 cells). The Mel treatment also caused a significant reduction in G6PDH levels in both OC strains (1.88-fold and 1.75-fold decrease vs control group in SKOV-3 and CAISMOV-24 cells). PDH levels were also reduced in the presence of Mel alone (1.51-fold and 1.9-fold decrease vs control group in SKOV-3 and CAISMOV-24 cells). For CAISMOV-24 cells, a significant reduction in PDH levels was observed after a combination of Mel and luzindole (1.58-fold decrease vs control group). The effects in SKOV-3 cells mostly depended on receptors, whereas in CAISMOV-24 cells these effects were independent of Mel receptors. In general, these results confirm that Mel reverses the Warburg effect in OC cells, reducing proteins associated with tumor progression (Figure 3 and 4).

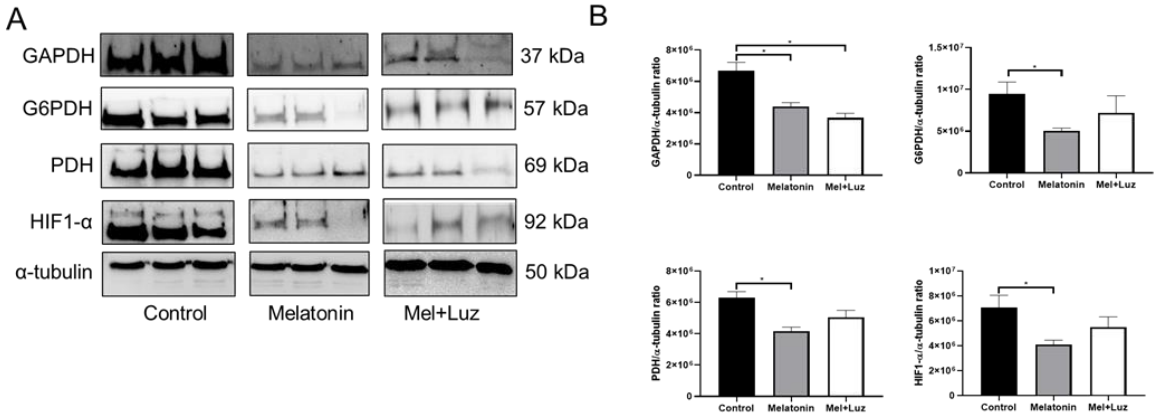

Figure 3. Mel reduces molecules involved with energy metabolism in SKOV-3 cells. A) Representative profile of the proteins GAPDH, G6PDH, PDH, and HIF-1α in cellular extracts of 50 µg of proteins using three technical and biological replicates. B) Optical densitometric analysis of selected protein levels in SKOV-3 cells after normalization with α-tubulin. Data are expressed as the mean ± SEM of triplets. * P<0.05. Mel: melatonin, GAPDH: glyceraldehyde 3-phosphate dehydrogenase; G6PDH: glucose-6phosphate dehydrogenase; PDH: pyruvate dehydrogenase complex; HIF-1α: hypoxia-inducible factor 1-alpha.

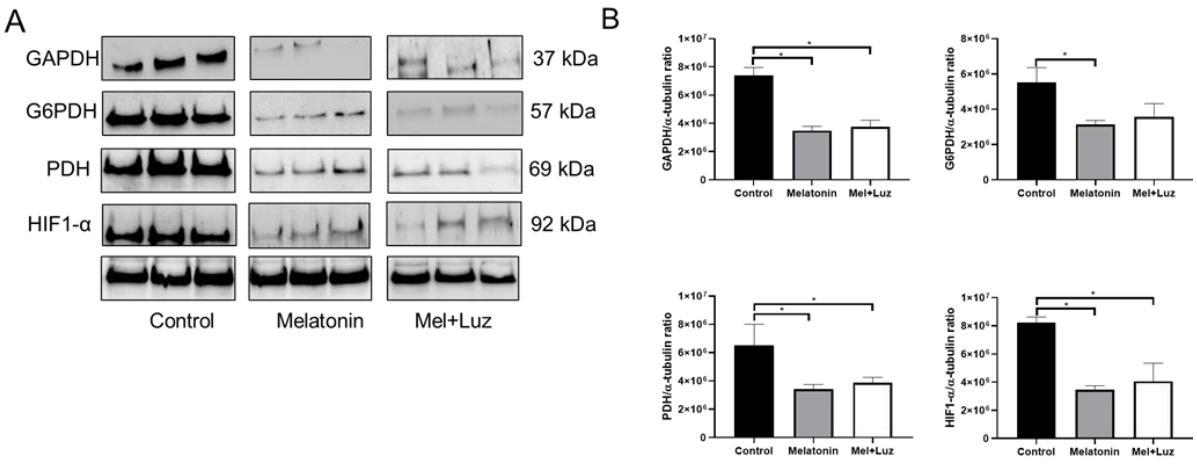

Figure 4. Mel reduces molecules involved with energy metabolism in CAISMOV-24 cells. A) Representative profile of the proteins GAPDH, G6PDH, PDH, and HIF-1α in cellular extracts of 50 µg of proteins using three technical and biological replicates. B) Optical densitometric analysis of selected protein levels in CAISMOV-24 cells after normalization with α-tubulin. Data are expressed as the mean ± SEM of triplets. *P<0.05. Mel: melatonin, GAPDH: glyceraldehyde 3-phosphate dehydrogenase; G6PDH: glucose-6-phosphate dehydrogenase; PDH: pyruvate dehydrogenase complex; HIF-1α: hypoxia-inducible factor 1-alpha.

3.3. Melatonin affects the activity of key molecules involved with glycolysis, Krebs cycle, and glutaminolysis in OC cells.

To explore the activities of regulatory enzymes associated with energy metabolism and the Warburg effect, we individually assayed G6PDH, PFK-1, LDH, CS, and PDH in addition to measuring lactate and glutamine levels (Figures 5 and 6). Mel combined or not with luzindole reduced the activity of CS and the levels of lactate in SKOV-3 cells (p=0.003, Mel group vs Control) and (p=0.031, Mel+Luz vs Control). In this cell line, treatment with melatonin alone reduced G6PDH (p=0.019), PFK-1 (p=0.015), and LDH (p=0.032) activities in addition to decreasing the levels of glutamine (p=0.004). In the CAISMOV-24 cells, Mel combined or not with luzindole, reduced the activities of G6PDH (p=0.008), PFK-1 (p=0.015), LDH (p<0.001) while lowering the levels of glutamine (p=0.008). Also, there was a significant reduction in the CS activity (p<0.001) and in the levels of lactate (p=0.033), but only when Mel was administered alone. Considering PDH, treatments with Mel significantly stimulated its activity in SKOV-3 (p=0.004) and in CAISMOV-24 cells (p=0.013), which may favor mitochondrial metabolism. While in SKOV-3 cells this increase was promoted by Mel alone (receptor dependent), in the CAISMOV-24 strain it was receptor independent (Figures 5 and 6). These results are the first to demonstrate the involvement of Mel in enzymatic activities and key substrates of OC-related energy metabolism, disfavoring aerobic glycolysis and its by-products. Based on the results, lactate levels and citrate synthase activity were regulated by Mel independently of its receptor in SKOV-3 cells, but in the CAISMOV-24 cells they were dependent on Mel receptors.

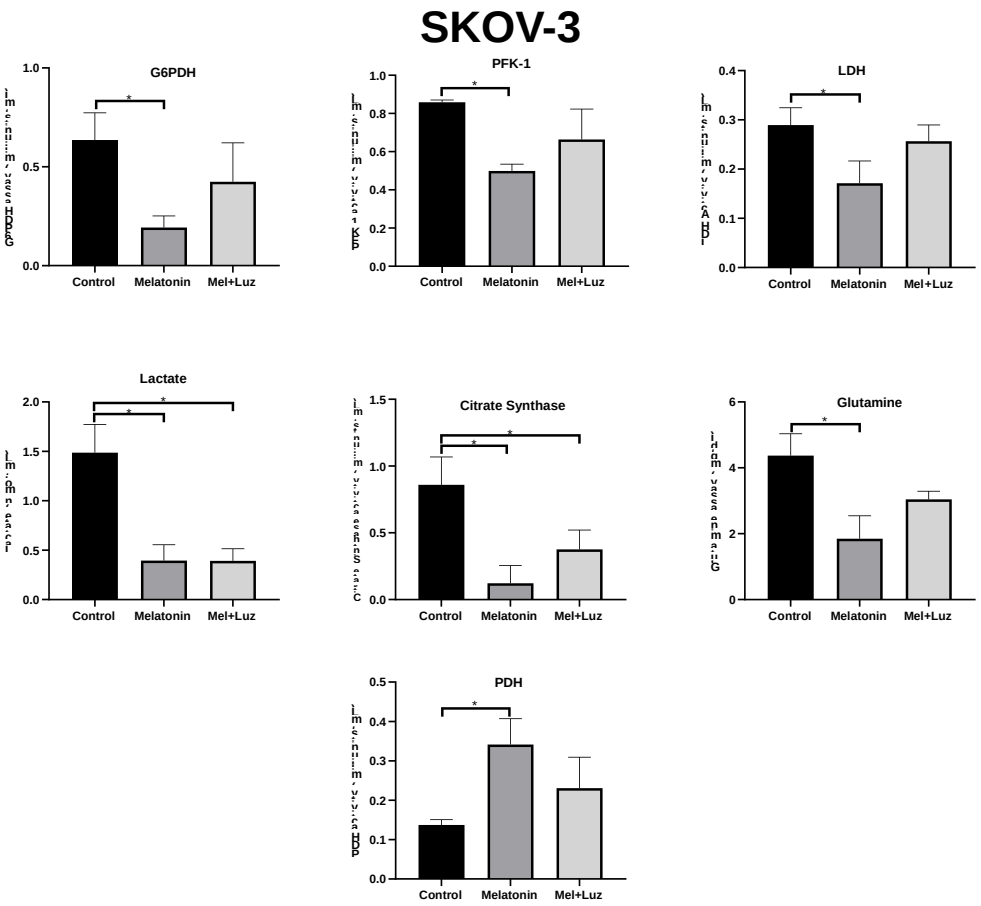

Figure 5. Enzymatic activity of PFK-1, G6PDH, LDH, PDH, and citrate synthase, and the levels of lactate and glutamine in SKOV-3 cells in response to melatonin and luzindole after 24h treatment exposure. Data are expressed as the mean ± SD. *P<0.05. All samples were assayed in triplicate and in the same run. One-way ANOVA complemented by Tukey's test. G6PDH: glucose-6-phosphate dehydrogenase; LDH: lactate dehydrogenase; PDH: pyruvate dehydrogenase complex; PFK-1: phosphofructokinase 1.

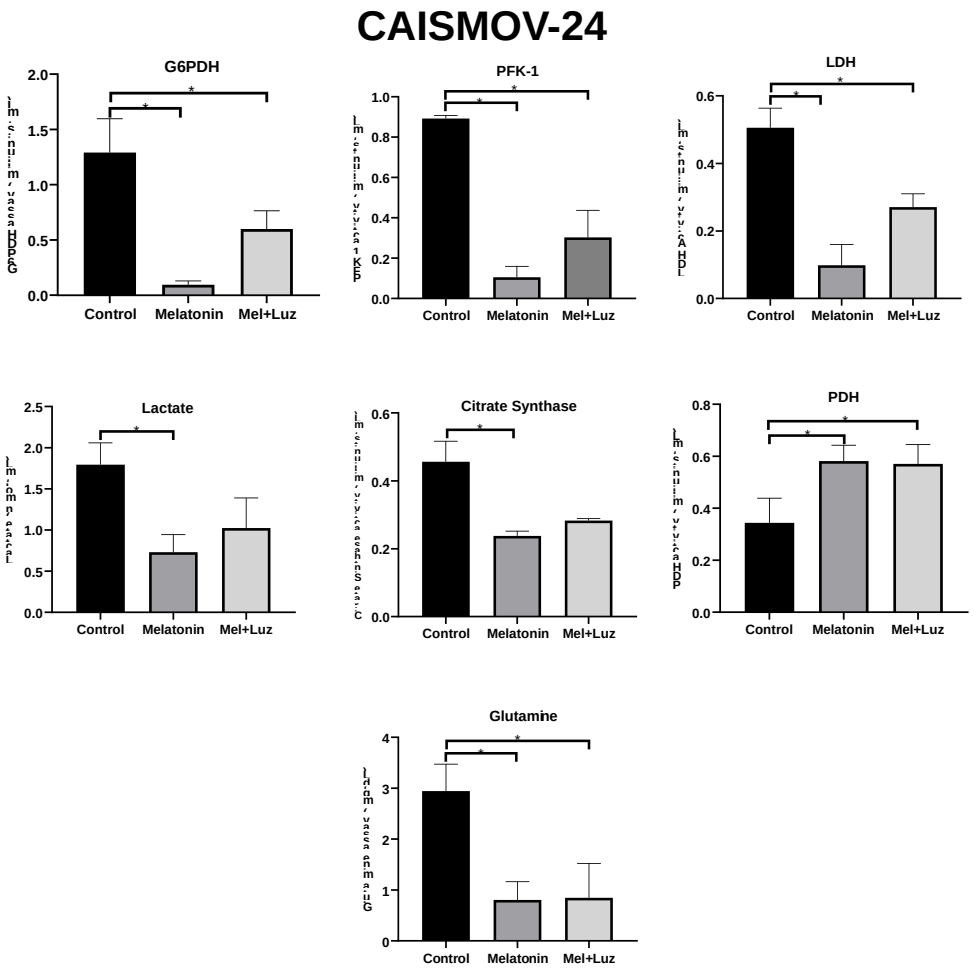

Figure 6. Enzymatic activity of PFK-1, G6PDH, LDH, PDH, and citrate synthase, and the levels of lactate and glutamine in CAISMOV-24 cells in response to melatonin and luzindole after 24h treatment exposure. Data are expressed as the mean ± SD. *P<0.05. All samples were assayed in triplicate and in the same run. One-way ANOVA complemented by Tukey's test. G6PDH: glucose-6-phosphate dehydrogenase; LDH: lactate dehydrogenase; PDH: pyruvate dehydrogenase complex; PFK-1: phosphofructokinase 1.

3.4. Melatonin reduces CREB, JNK, NF-kB, and AKT levels in SKOV-3 and CAISMOV-24 cells regardless of its membrane receptor activation.

Using a multiplex assay to assess the total amount of protein per cell volume, it was possible to observe whether treatments with Mel and Mel with luzindole influenced the concentrations of CREB, JNK, NF-kB, and AKT in the two cell lines (Figures 7 and 8). CREB levels were significantly reduced by Mel in SKOV-3 and CAISMOV-24 cells ($p<0.001$) and caused a greater reduction in the presence of luzindole ($p<0.001$). JNK levels were also significantly reduced by Mel and Mel combined with luzindole in both OC cells ($p<0.001$). AKT levels showed the same reduction response to Mel treatments in SKOV-3 and CAISMOV24 cells ($p<0.001$). The same occurred with the group combined with luzindole in

both cell lines ($p<0.05$). Finally, either Mel alone or Mel with luzindole induced a significant drop in the concentration of NF-kB in SKOV-3 cells ($p<0.001$). In CAISMOV-24 cells, Mel combined with luzindole also showed a decrease in NF-kB levels compared to the Mel group ($p<0.001$). These findings demonstrate the Mel's multiple actions in OC cells which may contribute to the ability of Mel to inhibit this cancer type. Except for NF-kB levels in CAISMOV-24 cells, these biological responses occurred independently of Mel receptors since the groups Mel alone or combined with luzindole showed a similar effect.

3.5. Concentrations of p38, P70S6K, ERK1/2, STAT3 and STAT5 are reduced in OC cells after melatonin treatment.

We also investigated the actions of Mel combined or not with luzindole on signaling factors involved with OC progression (Figures 7 and 8). The p38 levels were only reduced with Mel and luzindole in the two OC strains ($p<0.001$). P70S6K levels were significantly decreased in both OC cells. While in SKOV-3 cells this effect occurred with Mel ($p<0.001$) and Mel plus luzindole ($p<0.001$), in the CAISMOV-24 cells, this reduction appeared in the combination group ($p<0.001$). Similar results were observed for ERK1/2 and STAT3. The levels of ERK1/2 were reduced in the SKOV-3 cells after treatments ($p<0.001$) and in the CAISMOV-24 cells only with the combination of Mel with luzindole ($p<0.05$). STAT3 levels were reduced in SKOV-3 cells after both treatments ($p<0.001$) but only after combination treatment in CAISMOV-24 cells ($p<0.001$). STAT5 was significantly reduced only in SKOV-3 cells considering both treatments ($p<0.05$). By attenuating the concentration of these factors, Mel likely impacts OC growth, expansion, and neovasculogenesis. Most of the Mel's effects were independent of its membrane receptors.

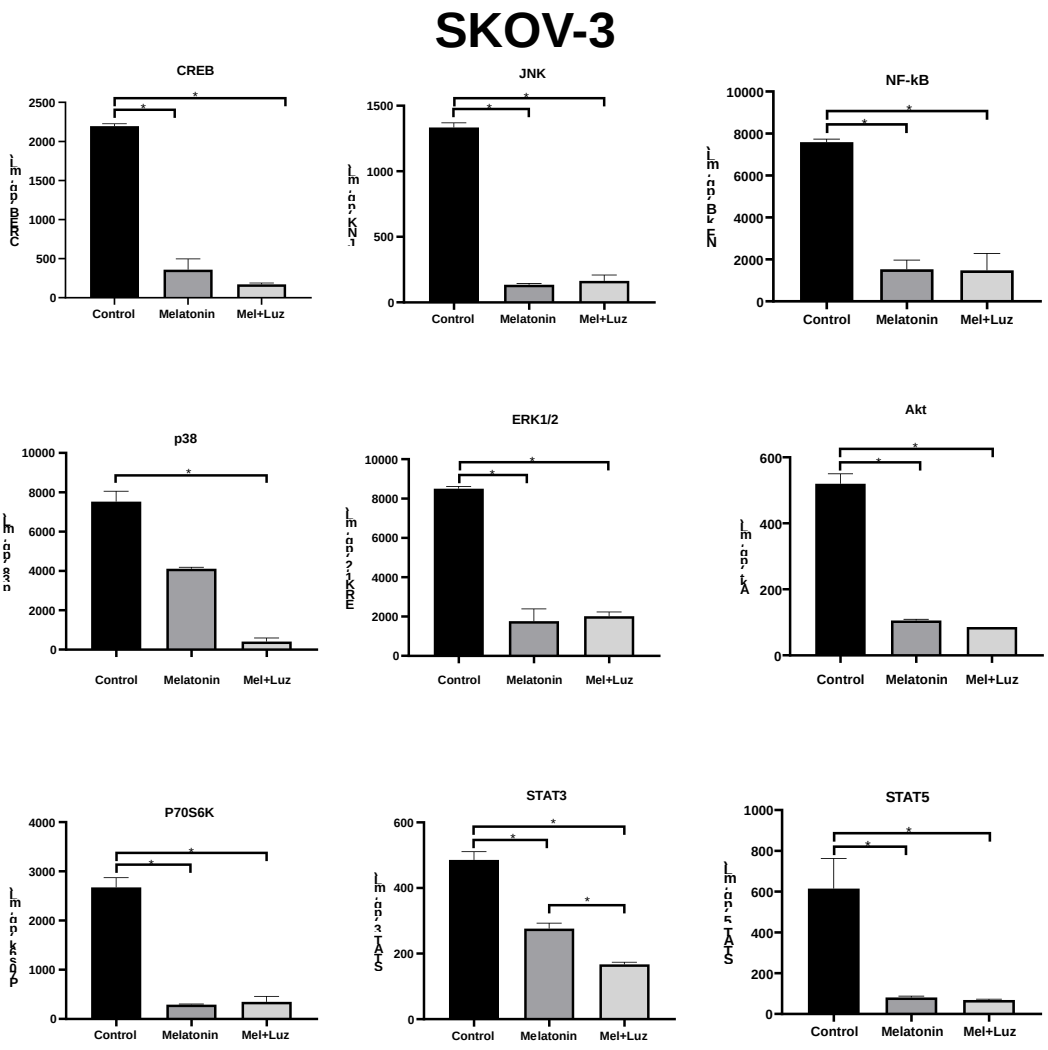

Figure 7. Multiplex analysis of cell signaling-related molecules in SKOV-3 cells in response to melatonin and luzindole. Concentrations of CREB, JNK, AKT, NF-kB, p38, ERK1/2, STAT3, STAT5, and P70S6K were evaluated in the OC cell extracts after 24 h of treatment. Data are expressed as the mean ± SD. *P< 0.05. The samples were assayed in triplicate and in the same run. One-way ANOVA complemented by Tukey's test. CREB: cAMP response element-binding protein; JNK: c-Jun N-terminal kinase; AKT: Protein kinase B; NF-kB: factor nuclear kappa B; p38: mitogen-activated protein kinase; ERK1/2: Ras-dependent extracellular signal-regulated kinase (ERK)1/2; Ribosomal protein S6 kinase (P70S6K); STAT3: Signal Transducer and Activator of Transcription 3; STAT5: Signal Transducer and Activator of Transcription 5

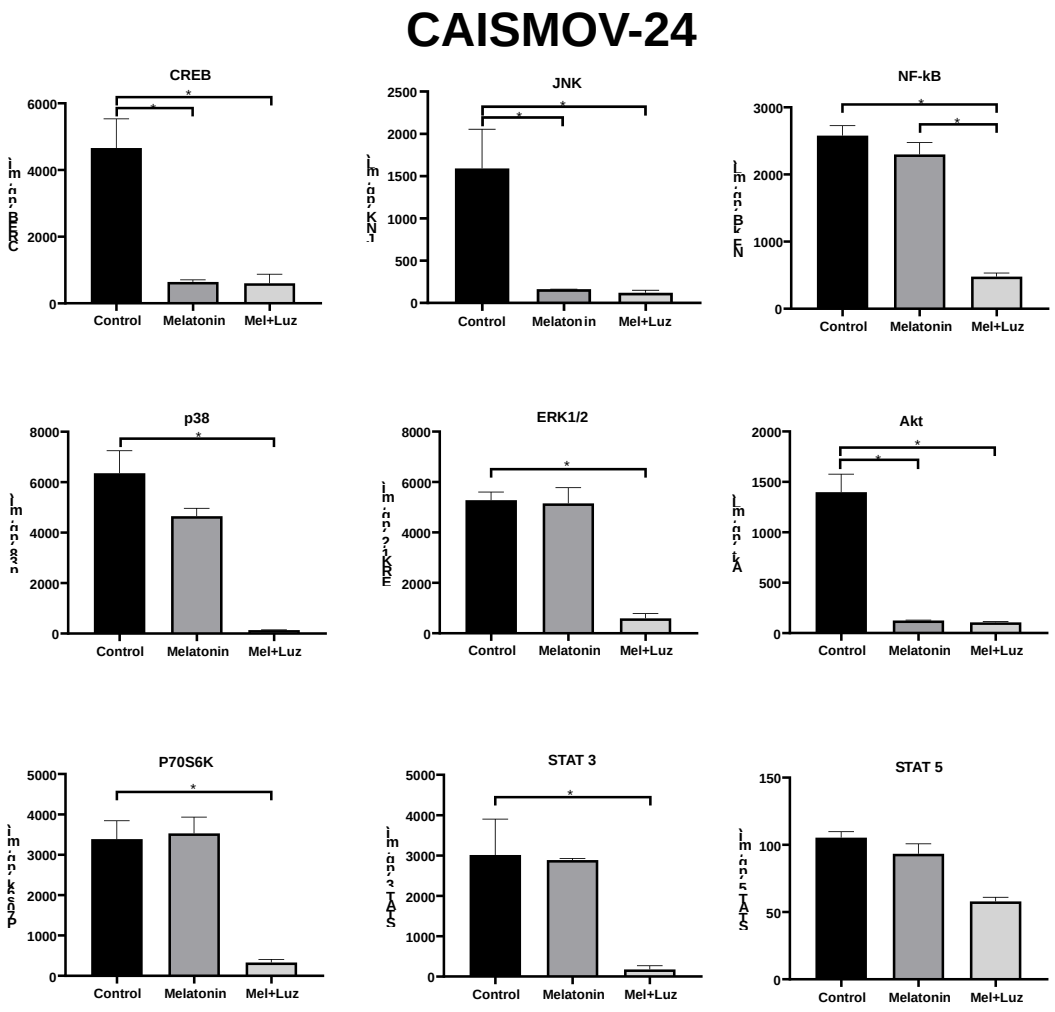

Figure 8. Multiplex analysis of cell signaling-related molecules in CAISMOV-24 cells in response to melatonin and luzindole. Concentrations of CREB, JNK, AKT, NF-kB, p38, ERK1/2, STAT3, STAT5, and P70S6K were evaluated in the OC cell extracts after 24 h of treatment. Data are expressed as the mean ± SD. *P< 0.05. The samples were assayed in triplicate and in the same run. One-way ANOVA complemented by Tukey's test. CREB: cAMP response element-binding protein; JNK: c-Jun N-terminal kinase; AKT: Protein kinase B; NF-kB: factor nuclear kappa B; p38: mitogen-activated protein kinase; ERK1/2: Ras-dependent extracellular signal-regulated kinase (ERK)1/2; Ribosomal protein S6 kinase (P70S6K); STAT3: Signal Transducer and Activator of Transcription 3; STAT5: Signal Transducer and Activator of Transcription 5.

4. Discussion

In this study, we report that Mel alleviates the harmful impact of Warburg-type metabolism and tumor signaling factors in OC cells by decreasing the expression and activities of important players involved with aerobic glycolysis, glutaminolysis, and tumor progression. More recently, Mel has been documented to have a beneficial effect in reversing the glycolytic metabolism of tumor cells [19,29–32]. The results of the present study also support these findings in OC cells. In addition to its action on energy metabolism, the enzyme

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) also acts on DNA replication and repair, nuclear tRNA export, apoptosis, endocytosis, exocytosis, and cytoskeletal organization [33–35]. Studies have indicated that GAPDH is differentially expressed in several types of cancer, including OC [33,36,37]. In OC, the overexpressed GAPDH is involved in tumor development and metastatic processes as it leads to an increase in glycolysis as well as in cell motility [38,39]. Notably, Mel significantly decreased the expression of GAPDH in both OC cells, thus demonstrating its effectiveness in OC metabolism and revealing a receptor-independent effect.

PFK-1 is responsible for the conversion of fructose-6-phosphate into fructose 1,6bisphosphate and acts as a rate-limiting enzyme for glycolysis [40,41]. An increase in PFK-1 expression could cause the inactivation of the PDH complex, leading to the suppression of the Krebs cycle and consequently depressing oxidative phosphorylation. This mechanism diverts ATP production to glycolysis, favoring the Warburg effect [42,43]. Mel reduced the activity of PFK-1 in OC cells, thereby preventing glycolysis typical of cancer cells. In addition to the molecules involved in cellular respiration, Mel also regulates other processes related to glycolysis, such as the pentose phosphate pathway (PPP). G6PDH is the first enzyme of PPP [44], and its overexpression is linked to the stage of cancer development, cell cycle regulation, DNA repair, tumor size, invasion, metastasis, survival rate, and chemoresistance, which creates a favorable environment for tumor progression [45–47]. Mel therapy not only reduced G6PDH expression but also its activity in the OC strains, thus revealing a key role against the production of ribose-5-phosphate and NADPH. PDH is a complex of interdependent enzymes, one of which is pyruvate dehydrogenase E1α. PDH is inhibited by another enzyme called PDK [48]. PDH, which converts pyruvate to acetyl-CoA, is often downregulated in tumors that utilize Warburg type metabolism. This prevents pyruvate transport into the mitochondria where it would be converted to acetyl CoA. Since acetyl CoA is a cofactor for the conversion of serotonin to acetyl serotonin, its loss limits AANAT activity, the rate-limiting enzyme that transfer acetyl group of acetyl CoA to N-acetyl-serotonin during mitochondrial synthesis of Mel [10]. Under these conditions, when pyruvate is retained in the cytosol it is converted to lactate by the enzyme lactate dehydrogenase [19,49].

Lactate is then released from the cell resulting in the acidification of the tumor microenvironment [19]; this aids the processes of invasion, progression, and metastasis of tumor cells [50]. HIF-1α is a transcription factor related to the Warburg effect in tumor cells. Under low oxygen concentrations, HIF-1α stimulates the transcription of genes responsible

for tumor aggressiveness [51]. HIF-1α specifically stimulates the expression of PDK, which acts by inactivating the PDH complex [52] and prevents the formation of acetyl CoA. As observed in our experiments, via reducing the expression of HIF-1α, Mel contributes to reversing the aerobic glycolysis impairing OC cell metabolism [31]. Herein we found that although the levels of PDH were reduced in the OC cells, its enzymatic activity was significantly higher after Mel treatment. This Mel -induced change would redirect glucose oxidation to the mitochondria, reversing Warburg-type metabolism in a receptor-dependent manner for SKOV-3 cells but independent for CAISMOV-24. Also supporting these findings, Mel reduced the activity of LDH and lactate levels in OC cells, thus preventing their harmful effects and corroborating the canonical route of the glycolytic pathway.

For tumor cells to proliferate, an energy supply from both glucose and glutamine sources is needed since they provide energy and intermediates for the synthesis of macromolecules [53]. When glucose is at low concentrations, tumor cells convert glutamine into lactate faster than normal cells, thus ensuring a glucose-independent metabolic pathway and favoring the Warburg effect [54]. We observed that Mel reduced glutamine levels in OC cells, thus likely lowering the production of acetyl CoA and explaining the previously observed reduced Mel concentration in cancer cell mitochondria [15,55]. CS catalyzes the first reaction of the TCA cycle and is thought to regulate energy generation in mitochondrial respiration playing a central role in carbohydrate metabolism [56]. Evidence has shown that CS activity is altered in different types of cancer [57]. An increase in CS expression is observed in malignant OC cell lines (SKOV3 and A2780) compared with benign OC and normal human ovarian surface [58]. Our results suggest that reduced CS activity caused by Mel may help to explain the ability of the indoleamine to disrupt energy production leading to impaired cellular metabolism; further investigation will shed light on this mechanism.

We further measured multiple cells signaling kinases associated with OC aggressiveness. There are studies proving the relationship of P70S6 kinase, a component of the PI3K/Akt pathway, with OC [59]; an increase in 70 is related to OC aggressive phenotypes [60]. Our findings showed a reduction in p70 levels after Mel treatment, which may attenuate cell-growth-linked processes. Studies have shown that 70% of OC cases have dysregulated PI3K/AKT signaling [61]. Changes in AKT activity can alter the invasion, migration and autophagic activity of tumor cells, making this pathway a promising target for therapeutic intervention in OC [61–64]. Treatments with Mel and cisplatin alone and in combination showed the inhibition of the PI3K/Akt signaling pathway as well as the increase of oxidative stress resulting in OC cell death [65]. Using in vivo and in vitro models of OC, we previously

documented the ability of Mel to reduce AKT levels [55,66]. Our current analyses further revealed that Mel reduced the MAPK-JNK-ERK1/2 pathway; these pathways regulate several cellular processes such as cell cycle progression, adhesion, migration, survival, differentiation, metabolism, and proliferation [67]. Studies have documented that the MAPK-JNK-ERK1/2mTOR pathway is involved in resistance associated with autophagy and cell survival in several cancer cell lines [68–70]. Mel attenuated the expression of JNK and ERK1/2 in both cell lines, thereby inhibiting the aggressive potential of OC. The p38 kinase regulates many cellular functions, including migration, proliferation, differentiation, stress response, apoptosis, and cell survival, since it has high affinity for different substrates [71,72]. There is paucity data that relate to the effects of p38 in OC; our results showed a reduction of p38 after Mel exposure. We also showed that Mel significantly reduced STAT3 and STAT5 levels in OC cells.

STAT3 and STAT5 are signal transducers and transcriptional activators belonging to a large family of transcription factors [73]. They are involved in proliferation, escape from apoptosis, angiogenesis, cell invasion and migration, and inactivation of antitumor immunity [74]. The STAT3 and STAT5 levels are increased in OC compared to normal tissue [75]. In this context, Mel inhibits transcription factors that activate key pathways in tumor development. Moreover, Mel promoted the reduction of CREB and nuclear factor kB (NF-kB). CREB is a transcription factor complex with many functions such as upregulating the methylation of histones H3 and H4, acting on the initiation of the transcriptional machinery [76,77]; CREB is overexpressed in tumors such as Hodgkin's lymphoma, melanoma, prostate, lung, gastric, esophageal, pancreatic, breast, and OC, and when it is negatively regulated, a reduction in the expression of genes that control the hallmarks of cancer occurs [78–81]. Although there is a lack of studies involving CREB in OC, our findings support the role of Mel in negatively regulating its expression in the two OC cells. The same occurred with NF-kB, which is involved in several processes participating in the transcription of genes mainly related to inflammatory actions and tumor processes [82,83]. Mel also effectively reduced this nuclear factor in SKOV3 cells, contributing to the inhibition of the inflammatory processes in the OC microenvironment.

The main limitations of this study include (I) the lack of a complete investigation of the metabolic enzymes involved in energy acquisition by the cells (II) when luzindole is combined with Mel an even more distinct biological response occurred at targets; a possible explanation to this fact is that the unbound Mel could enter the cells by alternate routes, thereby interfering with intracellular activation of signaling molecules.

5. Conclusions

Mel reversed the Warburg type metabolism of OC cells by regulating critical players altered in energy metabolism associated with tumor expansion and dissemination. There was also a reduction in lactate and glutamine levels, along with attenuation of oncogenic signaling molecules associated with OC aggressiveness, which directly or indirectly act in the promotion and activation processes in the tumor microenvironment. Since CAISMOV-24 resembles its primary malignant cells and survives well in ascitic fluid suspensions, it showed greater resistance to treatments, especially considering oncogenic signaling. The results of the current study document the significant therapeutic potential of Mel in arresting OC tumor progression; the effects of Mel were mostly generated independent of the classic membrane receptors, MT1 and MT2, especially in SKOV-3 cells. This work represents a milestone in the investigation of Mel's role in energy metabolism and provides an overview of how it acts mechanistically by curbing the energy machinery altered in OC cells, finally pointing to a new horizon for the treatment of OC (Figure 9).

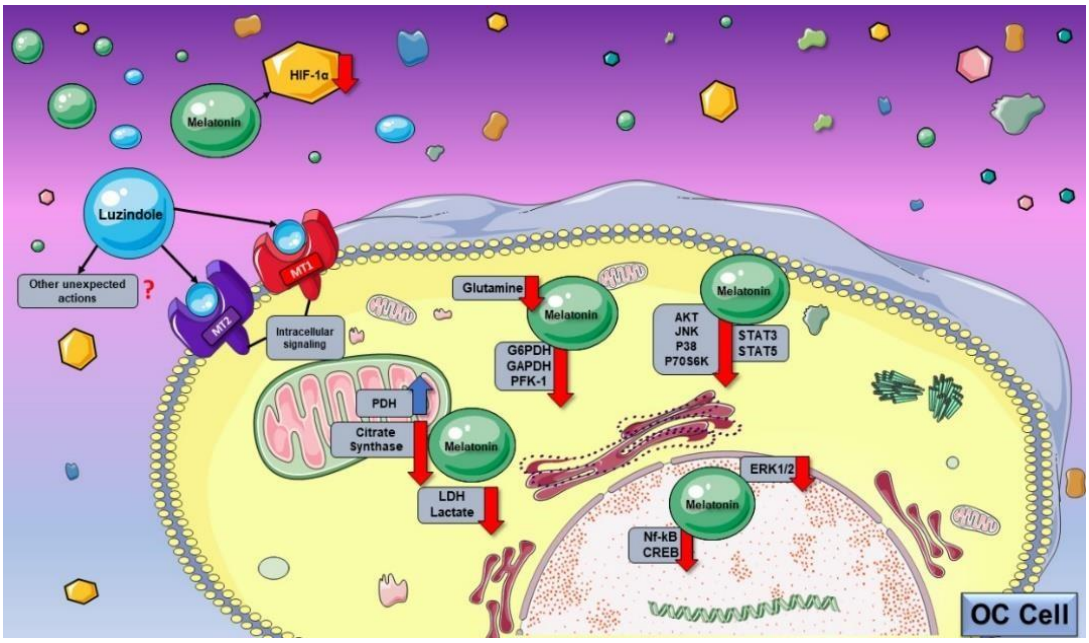

Figure 9. This image summarizes the main findings of the study. By acting on OC cell, melatonin reduces the levels of lactate and glutamine in addition to the enzymatic activities of LDH, PDH, G6PDH, GAPDH, PFK-1, and citrate synthase. Also, melatonin was capable of altering the concentrations of NF-kB, ERK1/2, CREB, AKT, JNK, p38, P70S6K, STAT3, and STAT5. Most of these melatonin-mediated cellular mechanisms may be occurring in different compartments of the OC cell and some of them were independent of its membrane receptor activation.

Author Contributions: Conceptualization, Henrique Silveira, Fábio Rodrigues Seiva and Luiz Gustavo Chuffa; Data curation, Henrique Silveira; Formal analysis, Roberta Cesário, Renan Vígaro, Leticia Gaiotte, Maira Cucielo, Fernando Guimarães, Fábio Rodrigues Seiva, Debora Pires de Campos Zuccari and Luiz Gustavo Chuffa; Investigation, Henrique Silveira, Fábio Rodrigues Seiva and Debora Pires de Campos Zuccari; Methodology, Henrique Silveira, Roberta Cesário, Renan Vígaro, Leticia

Gaiotte, Maira Cucielo, Fernando Guimarães and Debora Pires de Campos Zuccari; Visualization, Russel Reiter; Writing – original draft, Henrique Silveira and Luiz Gustavo Chuffa; Writing – review & editing, Russel Reiter. The authors read and approved the final version of this manuscript.

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CAPÍTULO 2

Artigo a ser submetido ao periódico científico "*Pathology Research and Practice"*

Melatonin reduces cell motility and antioxidant defenses in ovarian cancer cell lines.

[1]Department of Structural and Functional Biology, UNESP - São Paulo State University, Institute of Biosciences, Botucatu, 18618-689, São Paulo, Brazil.

[2] Hospital da Mulher "Professor Doutor José Aristodemo Pinotti" – CAISM, UNICAMP; Campinas; São Paulo; Brasil.

[3] Department,of Molecular Biology, Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, SP, 15090–000, Brazil.

[4] General Biology Department, Biological Sciences Center, State University of Londrina, Londrina (UEL), PR, Brazil.[5] North of Paraná State University (UENP), Biological Science Center, Bandeirantes, PR, Brazil.

[6] Department of Cellular and Structural Biology, UTHealth, San Antonio, TX 78229, USA.

*****Luiz Gustavo de Almeida Chuffa, Department of Structural and Functional Biology, Institute of Biosciences of Botucatu, UNESP - São Paulo State University, Botucatu, São Paulo, Brazil, Zip Code: 510; P.O Box: 18618–689, Rubião Júnior, s/n, Botucatu, SP – Brazil, Phone: +55 (14) 3880-0027. Email: luiz-gustavo.chuffa@unesp.br

Abstract

Ovarian cancer (OC), a highly recurrent and fatal tumor,of female reproductive system, poses diagnostic challenges due to its generic symptoms and resistance to established treatments. Melatonin (Mel) is an indoleamine produced by the pineal gland with well-known properties that act against tumor progression and exhibit a pro-oxidative effect on tumor cells. This study explores the impact of Mel on the antioxidant defenses of OC cells (SKOV-3 and CAISMOV-24), focusing on both its receptor-dependent and independent effects. Cell viability was assessed using the MTT method with IC_{50} values of Mel determined. Viability curves were estimated, and cytotoxicity was detected using a microplate reader. Cell invasion and migration assays were conducted using Geltrex®-coated transwell chambers for invasion and standard chambers for migration. Mel concentration was measured using a commercial ELISA kit, and the antioxidant system was analyzed by preparing supernatants to assess glutathione (GS), reduced glutathione (GSH), oxidized glutathione (GSSG), catalase (CAT), glutathione-S-transferase (GST), and superoxide dismutase (SOD). SOD and CAT activities were determined through enzyme assays, and GSH, GT, GSSG, and GST levels were quantified using specific protocols. Mel treatment stimulated its own intracellular synthesis, reducing cell viability in both cell lines. Notably, Mel, independent of its membrane receptors, inhibited migration and invasion, demonstrating its antitumoral potential. By investigating antioxidant systems, we observed pro-oxidative effects of Mel due to the reduced activity of superoxide dismutase (SOD), catalase, and glutathione enzymes. The modulation of these antioxidants by Mel demonstrates its multifaceted role in OC, emphasizing its therapeutic potential against this challenging malignancy. This study contributes with novel insights into Mel's receptor-independent actions, providing a foundation for further research in OC therapy.

Keywords: Ovarian Cancer, Melatonin, Oxidative stress, Antioxidant Defense

1. Introduction

Ovarian cancer (OC) is a prevalent subtype affecting women's reproductive systems, characterized by high recurrence and mortality rates [1]. This mortality rate is associated with

its late diagnosis due to multiple symptoms that are often generic and their aggressive recurrence, even with already established treatments and surgical techniques [2,3]. Tumors undergo metabolic modifications to fulfill energy, replication, and redox balance needs, [4] These changes are recognized as key aspects of cancer metabolism, marked by features such as tissue incursion and metastasis, elusion of growth inhibition in cells, rapid proliferation, sustained cell viability, resilience against cell demise, initiation of the angiogenesis pathway, evasion of the immune response, and modified cellular vitality [5,6].

Melatonin (Mel) is a lipophilic molecule converted from serotonin by the enzymes acetylserotonin O-methyltransferase (ASMT) and arylalkylamine N-acetyltransferase (AANAT) [7]. Mel has been characterized by actions that may either depend on or be independent of its membrane receptors (MT1 and MT2). While primarily produced in the pineal gland at night, other reports have shown its synthesis in different tissues and cells with local actions [8]. Mel has several functions in normal metabolic conditions including its antioxidant action by removing reactive oxygen and nitrogen species and stimulating DNA repair mechanisms [9]. In some cancer cells, Mel has oncostatic functions associated with prooxidative function whereas reducing the migration and invasiveness of cancer cells while inducing apoptosis. [10,11].

Free radicals (FR) are a generic term that encompasses unstable and reactive atoms or molecules. In biological systems, FR can be separated into two classes: reactive oxygen species (ROS) and reactive nitrogen species (RNE) [12]. Superoxide anion ($O2^{\bullet-}$), hydroxyl radical (OH•), alkoxy radical (RO•), hydrogen peroxide (H_2O_2), and hypochlorous acid are considered ROS and they are normally generated during cellular metabolism; nitrogen dioxide (NO_2), nitroxyl (HNO) and peroxynitrite (ONOO-) are examples of RNE produced from nitric oxide and by the NADPH oxidase (NOX) system [12]. In healthy cells, there is a delicate balance between the production and removal of FR, which ensures their physiological effects [13].

FR affects gene expression, cell growth and differentiation, modulate metabolic reactions, and regulate transcription factors activation, in addition to acting as intra and intercellular signaling molecules [14–17]. In OC cell lines, there is initially a significant increase in ROS production, thus favoring tumorigenesis. This elevated ROS results from NADPH oxidase activity and mitochondrial metabolism, as this increase is diminished by mitochondrial complex I inhibitors [18]. ROS increases the resistance to platinum-derived therapies leading to a poor prognosis and accelerating OC growth [19]. In addition, the increase in ROS is

associated with dysregulation of microRNAs (miRNAs) which are linked to tumor progression; in serous epithelial OC, there is overexpression of miR-483, but it does not occur in the non-serous OC [20–22].

Cells employ antioxidant systems, categorized as either enzymatic or non-enzymatic to counteract the damage caused by ROS. Key enzymes in this process include superoxide dismutase (SOD), catalase (CAT,), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd), among others. It is important to consider that an antioxidant, to be efficient, does not necessarily need to be ubiquitous, but rather be able to react quickly with FR, cross biological barriers, not have a high excretion rate, be available when needed and be in adequate concentrations in the cell. Although additional investigation is needed, Mel has been documented to promote an increase in ROS via different mechanisms [24,25]. In head and neck cancer, Mel induced ROS by reversing mitochondrial electron transport which generated the anti-growth effect of cancer cells [26]. Mel also induces apoptosis mediated by the stimulation of ROS synthesis in breast cancer as well as in mesangial cells, highlighting its potential to increase the harmful effects of ROS in both malignant or non-malignant cells [24,27,28]. To date, the role of Mel in the oxidative system of OC remains unexplored. Therefore, we examined the impact of Mel, acting via its membrane receptors or not, on the antioxidant defenses of OC cells, as well as its impact on cell migration and invasiveness.

2. Materials and Methods

2.1 Cell culture and reagents

The SKOV-3 cell line (ATCC® HTB-77) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), while the CAISMOV-24 cell line was obtained from the Women's Hospital Prof. Dr. José Aristodemo Pinotti Caism (UNICAMP, Campinas, SP, BRAZIL). SKOV-3 cells were cultivated using RPMI medium (Gibco, Paisley, UK), whereas CAISMOV-24 cells were nurtured in DMEN HAM'S F-12 (LGC, Cotia, BR). Both cell types received 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin - streptomycin solution (100 µg/ml) (Gibco). The cells were placed in a humidified environment at 37°C with 5% CO_2. Cellular expansion was carried out in distinct 75 and 25 cm^2 culture flasks (Costar, Cambridge, MA, USA). Upon achieving 80% of confluence, the cell supernatant was carefully extracted. Afterwards, the cells underwent two washes with 10% phosphate-buffered saline (PBS; Oxoid Limited, Hampshire, UK). Subsequently, trypsin/EDTA solution (Gibco) was employed to detach the cells from the flask surface.

2.2. Experimental design and treatments

To investigate the effects of Mel alone and in combination with luzindole (Luz) on OC cells, we first determined the safe concentrations according to the IC_{50} method (MTT assay). Mel concentrations of 3.4 µM for SKOV-3 and 7 µM for CAISMOV-24 cells were selected for a 24-hour treatment period. Luz, a Mel receptor antagonist),was used at a concentration of 1 µM for both cell lines. To understand the effect of Mel in combination with Luz on cancer cells, we divided each cancer cell line into three experimental groups: Control: cells exposed only to the culture medium containing 100 µL of DMSO solution as the vehicle; Mel: cells exposed to Mel at concentrations of 3.4 µM for SKOV-3 and 7 µM for CAISMOV-24, plus vehicle; and Mel + Luz: cells exposed to the combination of Mel and Luz, plus vehicle. In the combination group, Luz was administered first, followed by the preestablished concentration of Mel after 30 minutes. Mel and Luz were dissolved in DMSO (5%) to maintain the molarity values indicated by the manufacturers. All experimental assays were performed in biological and technical triplicates.

2.3. Cell viability (MTT assay)

Cell viability was analyzed using the MTT method based on the IC_{50} to determine appropriate Mel concentrations. After the confluence rate, SKOV-3 and CAISMOV-24 cells were trypsinized, seeded in 6-well plates at a density of $5x10^5$ cells per well, and cultured with appropriated medium supplemented with 10% FBS. After cell adherence, Mel and Luz were added to the culture medium following the experiment design with specific concentrations (2.0, 3.4, 5.0, and 7.0 µM). Viability curves were estimated after 24 hours of treatment using the MTT solution (5mg/mL). To detect cytotoxicity, the reactions were replicated in a 96-wells plate and read in a microplate reader (Epoch, Bio Tek Instruments, USA).

2.4. Cell invasion and migration assays

The evaluation of SKOV-3 and CAISMOV-24 cell invasiveness was performed using 24-well plates. A thin layer of Geltrex® was added to each well, covering the lower polyethylene terephthalate (PET) membrane. SKOV-3 and CAISMOV-24 ($1x10^5$ cells) were added to the top of the insert and received standard medium without FBS. The invasive potential was analyzed based on the cells' ability to cross the gel barrier and the PET membrane through the pores, being chemotactically attracted by the lower coverage of the culture medium containing 5% FBS. The plates were placed in a CO_2 atmosphere at 37ºC for

24 hours. After the incubation period, cells were fixed in methanol for 10 minutes, and the remaining cells were removed by scraping. Migrated cells were stained with a 0.1% toluidine blue solution and photographed with a 5X objective using an inverted microscope (ZeissAxiovert®). For the migration assay, a similar experimental procedure was used, except that Geltrex® was not added to the transwell chamber. All experiments were performed in triplicate based on four fields and submitted to automatic cell count using LUNA-II® (Logos Biosystems, South Korea)

2.5. Measurement of melatonin concentration

Mel levels were determined in both SKOV-3 and CAISMOV-24 cells using a human-specific commercial ELISA kit (EH3344, Fine Test), according to manufacturer's instructions. Absorbance was read at 450 nm on a microplate reader (Epoch, Bio Tek Instruments, USA). Results were interpolated from standard curves generated by plotting the concentration of the standards against their absorbance, and results are presented in pg/mL.

2.6. Preparation of the supernatant for analyzing the antioxidant system

After Mel treatment, the culture medium was discarded, and PBS was added to the cells. The cells were lysed through three cycles of freezing (-80 ºC) and thawing (37 ºC) for 30 min each. After this process, protein levels were quantified and used to normalize the results of total (GS), reduced (GSH), and oxidized glutathione (GSSG), catalase (CAT), glutathione S-transferase (GST), and superoxide dismutase (SOD).

2.7. Activity of SOD and CAT

After treatment with Mel and Luz, the cells (5 x 10^5 cells/mL) were washed with PBS (pH 7.4) and re-plated for all the analyzes. SOD enzyme activity assay was performed according to [29] with modifications. The reaction medium was composed of methionine (13 mM), NBT (75 µM), and riboflavin (4 mM) in phosphate buffer (50 mM, pH 7.8). 10 µL of the cell extract was added, and the samples were exposed to fluorescent light (13W) for 5 min. Control samples (no enzymes) and the blank (containing all reagents but not exposed to light) were used. Readings were performed using a MultiSkan-Skyhigh microplate reader at 560 nm and at 25°C. To assess CAT activity, cells were lysed, and a reaction medium was prepared as described by Aebi [30]. Spectrophotometric reading were conducted at 240 nm over a duration of 1 minute, with measurements taken at 15-second intervals.

2.9. Measurement of GSH, GT, GSSG, and GST.

To quantify the levels of reduced glutathione (GSH) and total glutathione (GT), the protocol proposed by Rahman, Kode and Biswas [31] was carried out with modifications, using 5,5'-ditiobis 20-nitrobenzoic acid (DTNB) in the homogenate, evidenced by yellow formation. To determine total glutathione (TG) levels, DTNB, nicotinamide-adenine dinucleotide phosphate (NADPH), and glutathione reductase were used in the homogenate. GT and GSH levels were measured at 412 nm (Multiskan GO, Thermo Scientific), and results were expressed as µmol/mg protein. GSSG levels were calculated [32] considering reaction stoichiometry. To evaluate glutathione S-transferase (GST) activity, 1-chloro-2,4dinitrobenzene (CDNB) and potassium phosphate buffer were used. Absorbance was measured at 340 nm for 160 seconds, with 5 measurements at 40-second intervals [33], and results were expressed as ymol/mg protein.

2.10. Statistical analysis

Data were analyzed using One-way ANOVA and presented as mean ± standard deviation (SD). Significant results were complemented by Tukey's test. Statistical significance was set at $P < 0.05$ for all analyses. Results were analyzed and generated using GraphPad Prism 9.0 scientific graphing software (GraphPad Software, San Diego, CA).

3. Results

3.1. Mel treatment stimulates intracellular Mel synthesis by SKOV-3 and CAISMOV-24 cells.

Based on MTT analysis, we identified the cytotoxic effect of Mel (IC_{50}) which was previously established at 3.4 µM for SKOV-3 cells and 7 µM for CAISMOV-24 cells. Luzindole, which functions as an antagonist of MT1/2 Mel receptors, was used to determine whether the Mel's effect is dependent or independent of their receptors. The intracellular concentrations of Mel were measured in the OC cells by the enzyme assay (ELISA). Mel treatment elevated the levels of intracellular Mel synthesis compared to the control group, especially in SKOV-3 cells. After blocking MT1/2 receptors, Mel promoted an increase in its own intracellular levels in both SKOV-3 and CAISMOV-24 cells (1.96-fold increase vs. Control and 2.08-fold increase vs. Control, respectively). More importantly, Mel alone in the presence of its receptors, increased the levels of its intracellular synthesis in SKOV-3 cells

(1.75-fold increase vs. Control), thus corroborating our expectation since Mel is quite reduced in these OC cells (Figure 1).

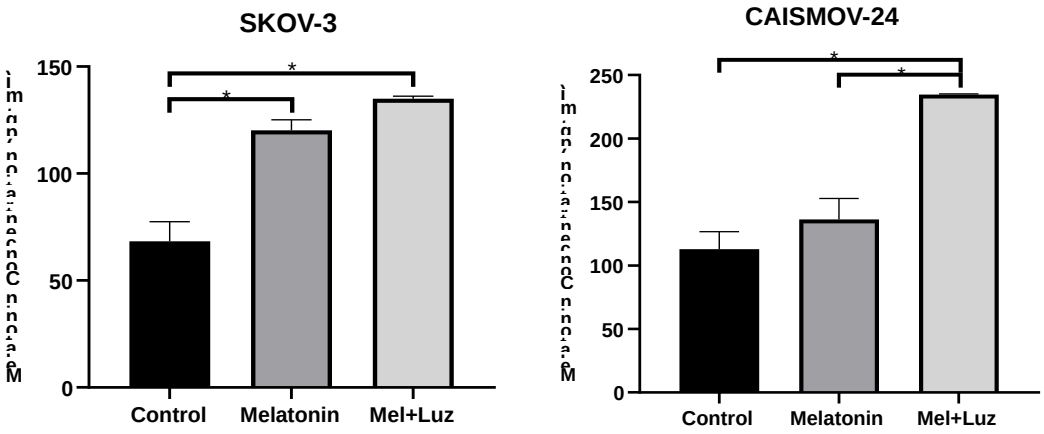

Figure 1. Intracellular Mel concentration after treatment with Mel and Luz in the SKOV-3 and CAISMOV-24 cell lines. Data were expressed as mean ± SEM of triplets. * P<0.05.

3.2. Mel reduces the migratory and invasive capacity of SKOV-3 and CAISMOV-24 cells regardless of the MT1/2 receptor activation.

To investigate the effects of Mel and luzindole on the migratory and invasive potential of SKOV-3 and CAISMOV-24 cells, we used transwell inserts in 24-well plates. The invasive potential of SKOV-3 and CAISMOV-24 cells was reduced by Mel alone (2.45- and 3.58-fold decrease vs Control groups, respectively), and after the combination of Mel and luzindole (2.17fold decrease vs Control group in SKOV-3 cells and 2.38-fold decrease vs Control group in CAISMOV-24 cells; Figure 2 and 3 A, B). The results also showed that Mel alone significantly reduced the migration of both SKOV-3 and CAISMOV-24 cells (2.03- and 2.29-fold decrease vs Control group, respectively; Figure 2 and 3 C, D). When Mel was combined with luzindole, a significant decrease in cell migration was also observed considering both OC cells (1.67-fold decrease vs. Control in SKOV-3 cells and 2.02-fold decrease vs. Control in CAISMOV-24).

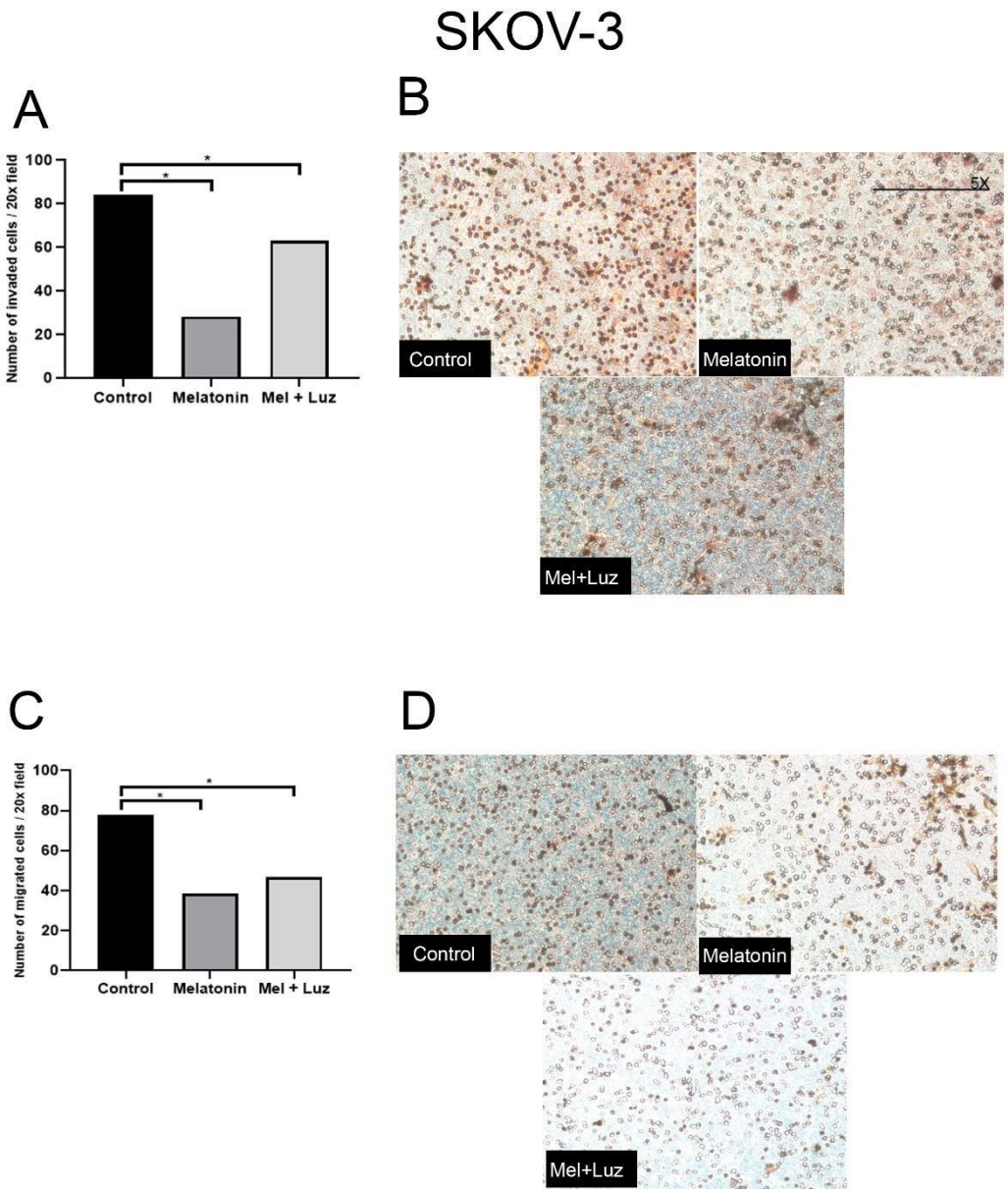

Figure 2. Effects of Mel and Luz on the invasion and migration of SKOV-3 cells. A) Effect of Mel and Luz on the invasive capacity of SKOV-3 cells. B) Images of invaded cells after treatments. C) Effect of Mel and Luz on the migratory potential of SKOV-3 cells. D) Images of migrated cells after treatments. Mel: melatonin; Luz: luzindole. Data were expressed as mean ± SEM of the triplets. *P<0.05.

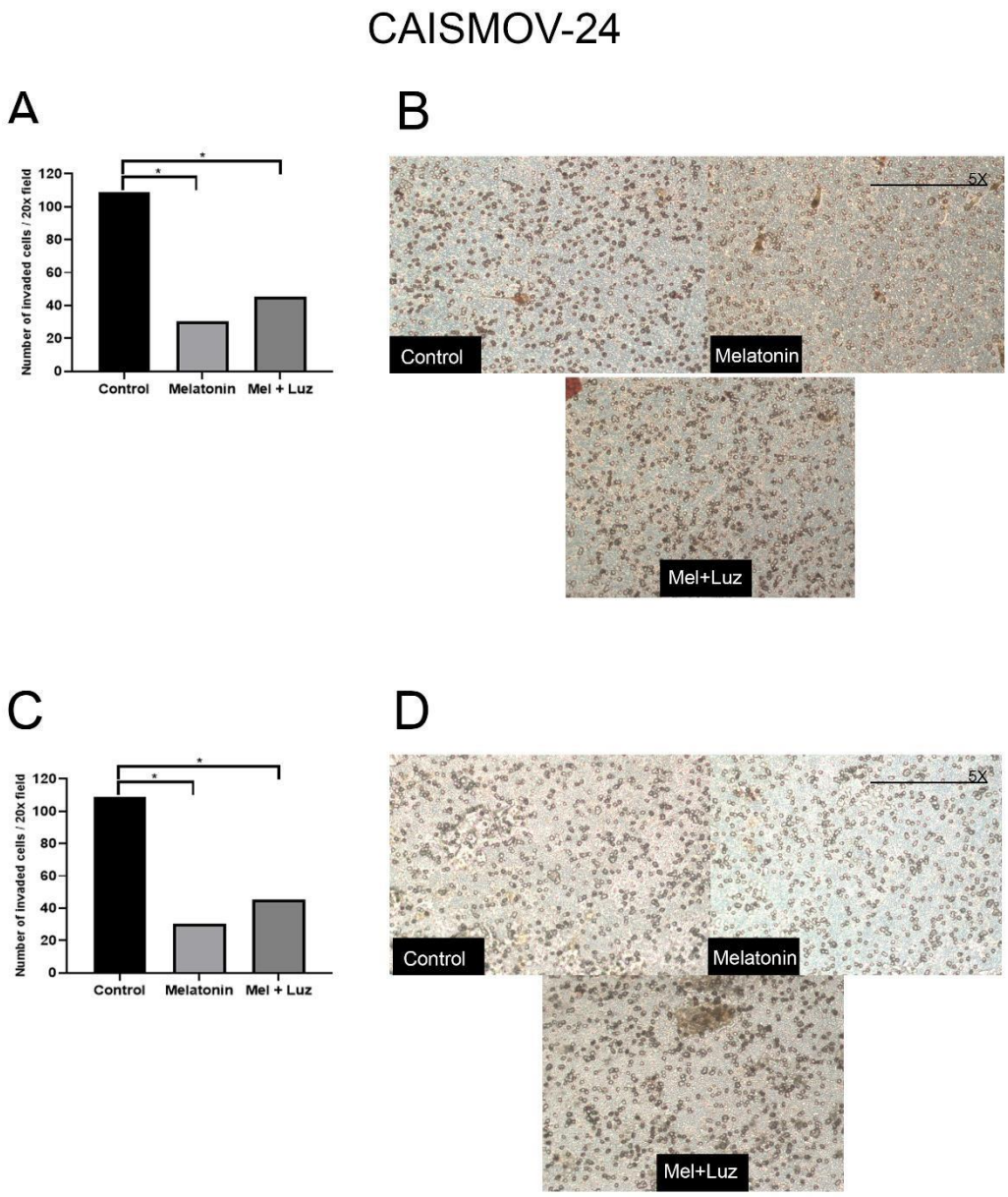

Figure 3. Effects of Mel and Luz on the invasion and migration of CAISMOV-24 cells. A) Effect of Mel and Luz on the invasive capacity of CAISMOV-24 cells. B) Images of invaded cells after treatments. C) Effect of Mel and Luz on the migratory potential of CAISMOV-24 cells. D) Images of migrated cells after treatments. Mel: melatonin; Luz: luzindole. Data were expressed as mean ± SEM of the triplets. *P<0.05. Iguais?!

3.3. Mel differentially regulates the antioxidant enzymes in OC cells.

SOD levels were only reduced by Mel in combination with luzindole in the CAISMOV24 cells (p=0.027). Regarding Catalase levels, a significant reduction was observed after Mel treatment in both OC cells (p<0.05 for CAISMOV-24 and p = 0.012 for SKOV-3). The presence of luzindole restored catalase levels close to that of control groups, thus showing a receptor dependent response. The changes in glutathione activities following treatments with Mel and luzindole varied differently between the two OC cell lines (Figures 4 and 5). In SKOV-3 cells, Mel alone significantly decreased the levels of GT (p<0.001) and GSSG (p<0.001) compared with the control group. The combination of Mel and luzindole also showed a decrease in the levels of GT (p=0.003) and GSSG (p<0.001). Conversely, this combination increased the levels of GST compared with control group (p<0.001) and Mel alone (p=0.002). Notably, in CAISMOV-24 cells, Mel alone significantly reduced the activities of GT (p<0.05), GST (p<0.05), GSSG (p<0.05), and GSH (p<0.05) compared with the control group, whereas Mel combined with luzindole only reduced GST levels compared with the control group (p<0.001). These results demonstrate an opposite effect of Mel favoring pro-oxidative processes in the OC cell.

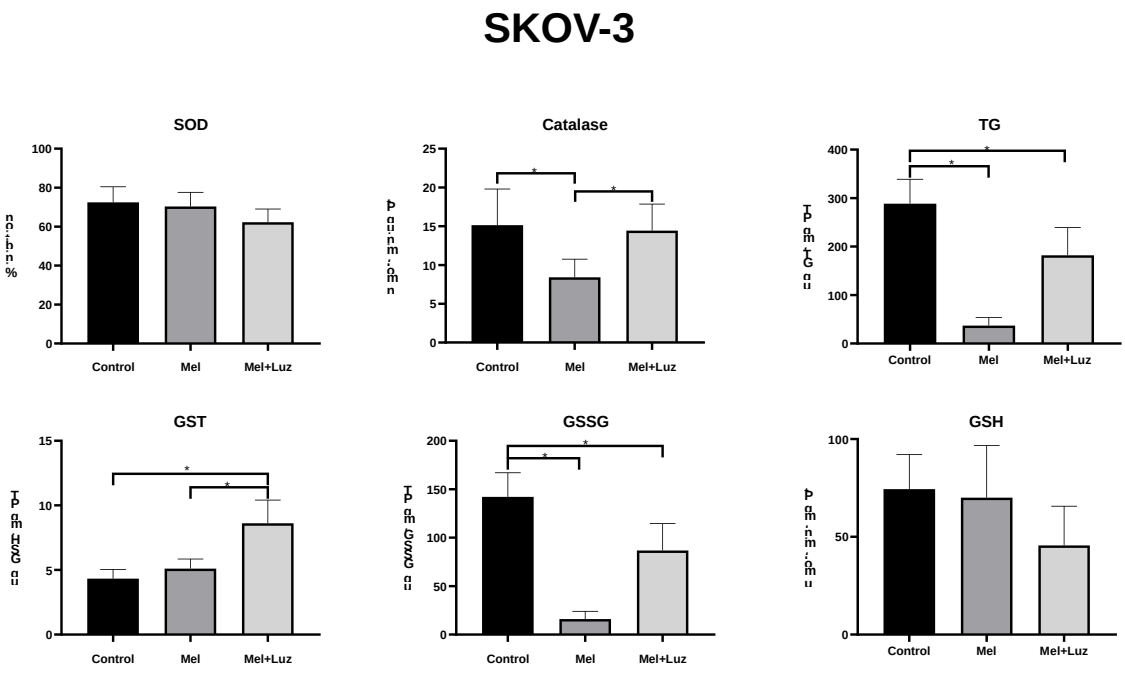

Figure 4. Enzymatic activities of SOD, Catalase, GT, GST, GSSG and GSH in SKOV-3 cells in response to Mel and luzindole after 24h of treatment exposure. Data are expressed as the mean ± SD. *P<0.05. All samples were assayed in triplicate and in the same run. One-way ANOVA complemented by Tukey's test. SOD: superoxide dismutase, GT: Total Glutathione, GST: Glutathione-S-transferase, GSSG: Oxidized glutathione, GSH: Reduced glutathione.

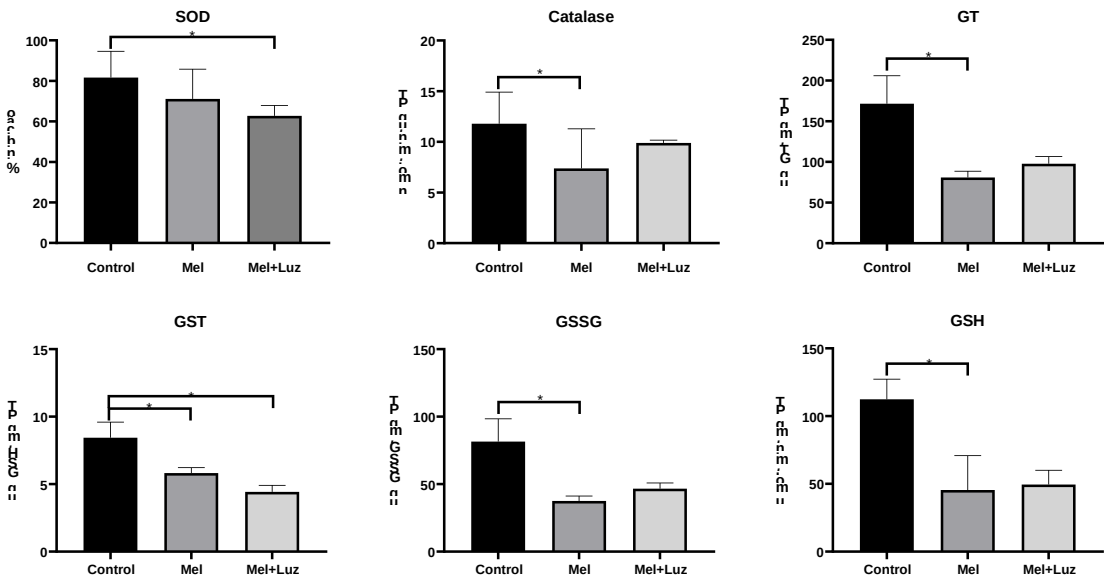

Figure 5. Enzymatic activities of SOD, Catalase, GT, GST, GSSG and GSH in CAISMOV-24 cells in response to Mel and luzindole after 24h of treatment exposure. Data are expressed as the mean ± SD. *P<0.05. All samples were assayed in triplicate and in the same run. One-way ANOVA complemented by Tukey's test. SOD: superoxide dismutase, GT: Total Glutathione, GST: Glutathione-S-transferase, GSSG: Oxidized glutathione, GSH: Reduced glutathione.

4. Discussion

Herein we report the effects of exogenous Mel being capable of increasing its own intracellular levels, leading to attenuation of the invasive and migratory capacity of OC cells in a receptor-independent manner. We also provide new information regarding the interference of Mel in the enzymatic antioxidant system of OC cells which may adversely impact their survival and growth.

Firstly, we observed an increase in Mel synthesis in both OC cell lines, mainly in SKOV3 cells following Mel treatment, in the presence of Luz or not, and in CAISMOV-24 cells only after the combination of Mel and Luz. We previously documented an increase in the intracellular concentrations of Mel synthesized in SKOV-3 cells after treatment with 3.2 mM of Mel [34]. Greater Mel synthesis may contribute to the antiangiogenic process, prooxidative, and pro-apoptotic pathways and may promote modifications in the metabolic profile of tumor cells [35]. The elevation in intracellular levels of Mel appear to be receptor-independent since the combination of Mel with luzindole also promoted an increase in the availability of the indolamine. Treatment with Mel can disinhibit the enzymatic activity of the pyruvate dehydrogenase complex (PDC), by first reducing hypoxia-inducible factor 1-alpha (HIF-1α), a factor highly expressed in hypoxic conditions of the tumor microenvironment, which

stimulates the pyruvate dehydrogenase kinase (PDK) enzyme. After PDC disinhibition, Mel can be synthesized in mitochondria, and oxidative phosphorylation is reestablished [34,36].

The ability of Mel to decrease the invasive and migratory capacity of OC cells is well known [37–39]. In a recent study, our team observed a significant reduction in cell migration and invasion in SKOV-3 cells treated with 3.2- or 4-mM Mel after knocking down the MT1 receptor [39]; Similarly, using luzindole, a non-selective antagonist of MT1 and MT2 receptors, we observed comparable results in two OC cell lines via transwell inserts. Despite using Mel concentrations ranging from 3.4 µM for SKOV-3 and 7 µM for CAISMOV-24 cells, our results clearly demonstrated decrease in the invasive and migratory capacity of OC cells, regardless of the activation of Mel receptors. The precise mechanisms by which Mel regulates OC cell migration and invasion remain incompletely understood. Previous studies have shown that Mel exerts anti-migratory effects via the MAPK and PI3K pathways in OC cells [37,39]. In other cancer types like colon and breast cancer, Mel attenuated cell invasion, migration, and survival by targeting PI3K/AKT and NF-κB signaling pathway [40] or by downregulating the p38 MAPK pathway [41]. Besides its passive diffusion, Mel can enter tumor cells using other membrane transporters like PEPT1/2 and GLUT1 [39,42], suggesting its potential to act independently of MT1/2 receptors, exerting anti-tumoral effects on cell migration and invasion.

As we have seen, defects in mitochondrial metabolism can increase the production of ROS and RNS, resulting in OS. Cells are equipped with an antioxidant defense system that includes enzymes such as SOD, CAT, and glutathione derivatives to combat the excessive and harmful production of these radical and non-radical species [43]. An excessive and permanent generation of OS is related to genetic/epigenetic events that could facilitate the tumorigenic process; however, increasing the generation of ROS and RNS in tumor cells is paradoxically used as a strategy to induce their death [44,45]. A potential agent in this strategy is the use of Mel, known for its antiproliferative effects and for interfering with angiogenesis and metastasis events; Mel acts by modulating the oxidant/antioxidant state of cells [45–49]. Moreover, the effects of Mel on tumor cells seem to be opposite of those occurring in normal healthy cells, that is, Mel acts as a pro-oxidant agent in tumor cells, thus reducing their antioxidant defenses [50,51].

Our results reinforce Mel’s pro-oxidant properties of in OC cells, particularly evident in CAISMOV-24 cells. Our study is pioneering in demonstrating a decrease in SOD activity when Mel was combined with luzindole, i.e., a receptor-independent response, and a

significant reduction in CAT levels after Mel treatment in both OC cells, suggesting that Mel acts directly as a modulator of these two antioxidant enzymes. Previous studies have linked Mel treatments to decreased enzymatic activities of SOD and CAT, along with elevated ROS levels observed in human colorectal cancer cells and hepatocellular carcinoma cells [52,53], resulting in apoptosis due to excessive oxidative damage.

In healthy tissues, Mel generally upregulates antioxidant enzymes, including the glutathione system, which plays a central role combating OS [49,54]. Here, we observed a marked decrease in total glutathione (GT) levels in both OC cell lines following treatment with Mel alone. When Mel receptors were blocked by luzindole, only SKOV-3 cells showed a significant decrease in GT levels, while CAISMOV-24 cells showed only a trend. The reduced form of glutathione (GSH) is one of the most important machineries on the front line of the antioxidant defense system. GSH maintains a redox state in subcellular compartments such as mitochondria and cytosol, and its increased levels may be involved in the chemoresistance of cancer cells [55]. In this study, GSH content was only reduced with CAISMOV-24 cells treated with Mel alone. It is also reported that Mel can exert a depletion on GSH levels in HepG2 cells [45,56], and in human myeloid leukemia cell line (U937) [53,57]. Under OS conditions, GSH can be directly converted to oxidized glutathione (GSSG) in the presence of ROS [45]. We observed a notable reduction in GSSG levels in both OC cells. Particularly, in SKOV-3 cells, GSH levels resembled those of the control group, while GSSG was significantly reduced by both treatments. Based on this GSH:GSSG ratio that favors GSH, we can hypothesize a more pronounced resistance of SKOV-3 cells to OS, facilitating the maintenance of GSH homeostasis. This contrasts with the observations in CAISMOV-24 cells.

Cancer cells can also gain resistance through overexpression of enzymes that can increase detoxification capacity and avoid the cytotoxic action of antitumor drugs [58]. More specifically, overexpression of glutathione S-transferases (GST) and efflux pumps in tumor cells can reduce the reactivity of various anticancer drugs, such as cyclophosphamide [59], cisplatin [60], and others [58]. In this study, OC cell lines showed opposite results regarding GST. While in CAISMOV-24 cells both treatments decreased GST activities, in SKOV-3 cells the combination of Mel with luzindole increased its activity. As reviewed in detail by [58], the increase in GST levels occurs through transcriptional activation mediated by the nuclear factor erythroid 2 p45-related factor 2 (Nrf2). However, further studies are needed to precisely determine the role of Mel and its receptors in the molecular pathways that regulate the activity and expression of genes in the glutathione system. Overexpression of GST and high levels of GSH are linked to the development and expression of chemoresistance [61]. In this context,

our treatment with Mel alone at 7 µM successfully promotes a decrease in GST and GSH levels in CAISMOV-24, a recently established human low-grade serous ovarian carcinoma cell line [62]. SKOV-3 cells, a non-serous ovarian cancer cell line, in turn, remained unchanged for these parameters after treatment of 3.4 µM of Mel alone, thus suggesting further evaluations with higher concentrations. This sounds plausible since it has been shown that high levels of Mel are required to induce ROS production in tumor cells [63].

5. Conclusion

Collectively, Mel treatment attenuates the migratory and invasive capacity of OC cells in a receptor independent manner while stimulating its intracellular synthesis. Moreover, the antioxidant enzymatic defenses were dampened by Mel, especially in the CAISMOV-24 cells. The blockage of MT1/2 receptors by the antagonist luzindole followed by Mel administration showed a tendency to soften the results. Indeed, pre-incubation of SKOV-3 cells with 10 µM luzindole revealed an increase in cell viability [37]. Based on our results, we believe that the function of Mel in SKOV3 and CAISMOV-24 is partially mediated by MT1 and MT2 receptors. Our data provide valuable insights into the regulatory role of Mel in modulating antioxidant enzymes in OC cells, shedding light on potential therapeutic avenues for managing this devasting disease.

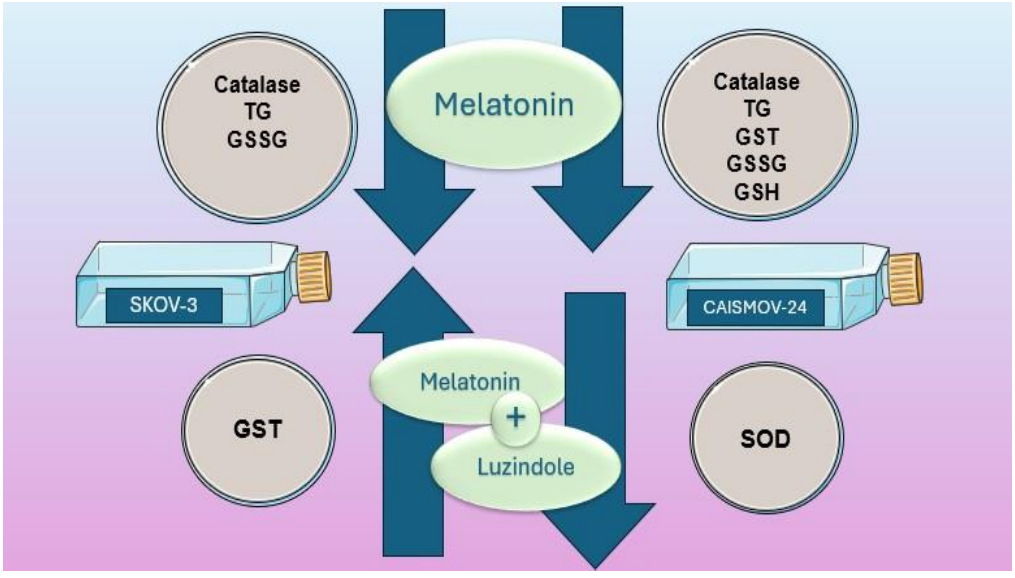

Figure 6: Schematic representation of Mel's impact on oxidative stress related factors in SKOV-3 and CAISMOV-24 OC. SOD: superoxide dismutase, GT: Total Glutathione, GST: Glutathione-S-transferase, GSSG: Oxidized glutathione, GSH: Reduced glutathione.

Author Contributions: Conceptualization, Henrique Silveira, Fábio Rodrigues Seiva and Luiz Gustavo Chuffa; Data curation, Henrique Silveira; Formal analysis: Roberta Cesário, Fernando Guimarães, Fábio Rodrigues Seiva, Vinicius Augusto Simão, Glaura Scantamburlo Alves Fernandes, Milena Cremer de Souza, Debora A. Pires de Campos Zuccari and Luiz Gustavo Chuffa; Investigation, Henrique Silveira, Fábio Rodrigues Seiva and Debora A. Pires de Campos

Zuccari; Methodology, Henrique Silveira, Roberta Cesário, Fernando Guimarães and Debora A. Pires de Campos Zuccari; Visualization, Russel Reiter; Writing – original draft, Henrique Silveira and Luiz Gustavo Chuffa; Writing – review & editing, Russel Reiter. The authors read and approved the final version of this manuscript.

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