Universidade Estadual de Londrina

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MELATONINA MODULA A ATIVIDADE DE LACTATO DESIDROGENASE E REDUZ VIABILIDADE E POTENCIAL MIGRATÓRIO DE CÉLULAS TUMORAIS HUH7.5

Londrina
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Dissertação de Mestrado apresentada à Universidade Estadual de Londrina - UEL, como requisito parcial para a obtenção do título de Mestre em Patologia Experimental.

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Londrina, 28 de Julho de 2022.

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“Os que se encantam com a prática sem a ciência são como os timoneiros que entram no navio sem
timão nem bússola, nunca tendo certeza do seu destino”.
(Leonardo da Vinci)

"A ciência é a mãe do conhecimento, mas as opiniões geram ignorância"
(Hipócrates)

RESUMO

CRUZ, Ellen Mayara Souza. **Melatonina modula a atividade de lactato desidrogenase e reduz viabilidade e potencial migratório de células tumorais Huh7.5**. 2022. 49 p. Dissertação (Mestrado em Patologia Experimental) - Universidade Estadual de Londrina, Londrina, 2022.

O Carcinoma Hepatocelular (CHC), uma neoplasia primária derivada dos hepatócitos, é um tipo agressivo de câncer, apresentando apenas 18% de sobrevida em 5 anos, baixa responsividade e recorrente quimiorresistência. Diversos estudos têm buscado por compostos alternativos capazes de diminuir o potencial proliferativo e que sejam efetivos no tratamento do CHC. A melatonina é uma indolamina secretada principalmente pela glândula pineal, contudo outros órgãos são capazes de produzir melatonina para seu próprio uso e em menor concentração, dentre eles o fígado. Estudos já demonstraram efeitos potenciais da melatonina em inibir a proliferação celular, antiangiogênese, imunomodulador e antimetastático. O presente estudo teve por objetivo explorar a atividade antitumoral da melatonina contra a linhagem celular de carcinoma hepatocelular HuH7.5. Inicialmente, foi avaliada a viabilidade celular da linhagem tratada com a melatonina (0,50 – 4 mM) nos tempos de 24 e 48h por ensaio de 3-(4,5-Dimethylthiazol-2-yl) -2,5-Diphenyltetrazolium Bromide (MTT). Após este ensaio, foram escolhidas as concentrações de 2 e 4 mM para os demais experimentos. Foram avaliadas alterações morfológicas e capacidade de migração celular por microscopia eletrônica de varredura e ensaio de cicatrização de ferida. Também foram feitas imunomarcações para N-caderina e TGF. Para avaliar as alterações no metabolismo energético foram dosadas as concentrações de glicose e lactato, bem como a atividade da enzima lactato desidrogenase (LDH). Realizou-se também o ensaio de clonogenicidade, para análise de formação de colônias. As células tratadas com 2 e 4 mM apresentaram migração comprometida, além de alterações morfológicas como redução na microvilosidade, rompimentos nas junções aderentes e dano a membrana celular. Houve redução acentuada de N-caderina e TGF após os tratamentos. A melatonina reduziu os níveis de glicose, lactato e LDH intracelular, bem como o número de colônias formadas. Desse modo, foi possível verificar a ação citotóxica e antiproliferativa direta da melatonina sobre a linhagem tumoral, sugerindo a melatonina como uma candidata promissora para ser avaliada como coadjuvante no tratamento do CHC.

Palavras-chave: hepatocarcinoma; efeito *warburg*; melatonina; metabolismo.

ABSTRACT

CRUZ, Ellen Mayara Souza. **Melatonin alters energy metabolism and reduces cell migration on the tumor cell line HuH7.5**. 2022. 49 p. Dissertation (Master's in Experimental Pathology) - University State Londrina, Londrina, 2022.

Hepatocellular Carcinoma (HCC), a primary neoplasm derived from hepatocytes, is an aggressive type of cancer, presenting only 18% of 5-year survival, low responsiveness, and recurrent chemoresistance. Several studies have searched for alternative compounds capable of decreasing the proliferative potential and that are effective in the treatment of HCC. Melatonin is an indolamine secreted mainly by the pineal gland, but other organs, such as the liver, are capable of producing melatonin for their own use and in lower concentration. Studies have already demonstrated potential effects of melatonin in inhibiting cell proliferation, antiangiogenesis, immunomodulator and antimetastatic. The present study aimed to explore the antitumor activity of melatonin against the hepatocellular carcinoma cell line HuH7.5. Initially, the cell viability of the melatonin-treated cell line (0.50 - 4 mM) was evaluated at 24 and 48h by 3-(4,5-Dimethylthiazol-2-yl) -2,5-Diphenyltetrazolium Bromide (MTT) assay. After this assay, the concentrations of 2 and 4 mM were chosen for the remaining experiments. Morphological changes and cell migration capacity were evaluated by scanning electron microscopy and wound healing assay. Immunolabeling for N-cadherin and TGF was also performed. To assess changes in energy metabolism, glucose and lactate concentrations were measured, as well as lactate dehydrogenase (LDH) enzyme activity. The clonogenicity assay was also performed to analyze colony formation. Cells treated with 2 and 4 mM showed impaired migration, as well as morphological alterations such as reduction in microvillus, disruption of adherens junctions and damage to the cell membrane. There was a marked reduction in N-cadherin and TGF after the treatments. Melatonin reduced the levels of glucose, lactate and intracellular LDH, as well as the number of colonies formed. Thus, it was possible to verify the direct cytotoxic and antiproliferative action of melatonin on the tumor lineage, suggesting melatonin as a promising candidate to be evaluated as an adjuvant in the treatment of HCC.

Key-words: hepatocarcinoma; warburg effect; melatonin; energy metabolism.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

AKT	do inglês, *serine/threonine kinase*
ATP	Adenosina trifosfato
CHC	Carcinoma hepatocelular
DNA	do inglês *Deoxyribonucleic acid*
IARC	do inglês, *International Agency for Research on Cancer*
IL-1 β	Interleucina 1 beta
IL-17	Interleucina 17
IL-18	Interleucina 18
IL-6	Interleucina 6
IFN-γ	Interferon *gamma*
LDH	Lactato desidrogenase
MEC	Matriz extracelular
Mel	Melatonina
mM	Milimolar
MT1	Receptores específicos de membrana 1
MT2	Receptores específicos de membrana 2
NF-κB	do inglês, *Factor Nuclear Fator nuclear kappa B*
NK	do inglês, *Natural Killer*
OMS	Organização Mundial da Saúde
PDGF-R	do inglês, *Platelet-derived growth factor receptors*
PI3K	do inglês, *Phosphoinositide 3-kinases*
SCN	do inglês, *Suprachiasmatic Nucleus Supraquiasmático*

TCA:	do inglês, *Tricarboxylic acid*
TEM	Transição epitélio-mesenquimal
TGF-β	do inglês, *Transforming growth factor beta*
TLRs:	do inglês, *Toll-Like Receptors*
TNF	do inglês, *Tumor Necrosis Factor*
VEGF	do inglês, *Vascular Endothelial Growth Fator*
VEGFR	do inglês, *Vascular Endothelial Growth Factor Receptors*

SUMÁRIO

1. INTRODUÇÃO

1.1 ASPECTOS GERAIS DO CANCER

O câncer é uma das quatros doenças com a maior taxa de mortalidade, considerado um dos principais problemas de saude pública (SUNG et al., 2021). De acordo com dados da IARC (*International Agency for Research on Cancer*), em 2020 os canceres de maior incidência foram de mama (2,2 milhões) e pulmão (2,2 milhões), enquanto o câncer de fígado ocupou o 6º lugar, com mais de 905 mil casos (SUNG et al., 2021) (**Figura 1A**). Já em relação às taxas de mortalidade, o câncer de fígado levou a morte de 830 mil pessoas por ano, ficando em terceiro lugar, atrás somente do câncer colorretal e pulmão (**Figura 1B**) (BRAY et al., 2018)

O câncer é uma doença em que diversos fatores podem estar envolvidos e caracterizada pelo aumento descontrolado de células (DIORI KARIDIO; SANLIER, 2021). Dentre os principais eventos associados à iniciação e progressão da doenças citam-se os danos ao DNA e/ou falha nos processos de reparo; como consequência pode haver instabilidade genômica e mutações de genes específicos associados aos processos de diferenciação, replicação, sobrevivência celular, invasão, metástase, inflamação e geração de energia (SINGH; KUMAR; PANDEY, 2018)

A carcinogênese pode ser dividida em quatro etapas: iniciação, promoção, conversão maligna e progressão do tumor. Na iniciação, moléculas iniciadoras ou agentes carcinogênicos entram em contato com a célula, e promovem danos irreversíveis ao DNA, causando mutações. Agentes promotores são responsáveis por aumentar a taxa de proliferação, levando a um maior número de células mutadas, permitindo a evolução para um fenótipo metastático (etapa de promoção). A conversão maligna é a transformação de uma célula pré-neoplásica em um fenótipo maligno e, que alterações genéticas diversas e distintas se acumulam. A progressão tumoral compreende a expressão do fenótipo maligno e a tendência dessas células adquirirem características mais agressivas ao longo do tempo. Além disso, o processo metastático envolve a capacidade das células tumorais de secretarem proteases que permitam a disseminação pela corrente sanguínea e invasão de novos tecidos (MARRASSINI; ANESINI, 2017; PIRETTO et al., 2019).

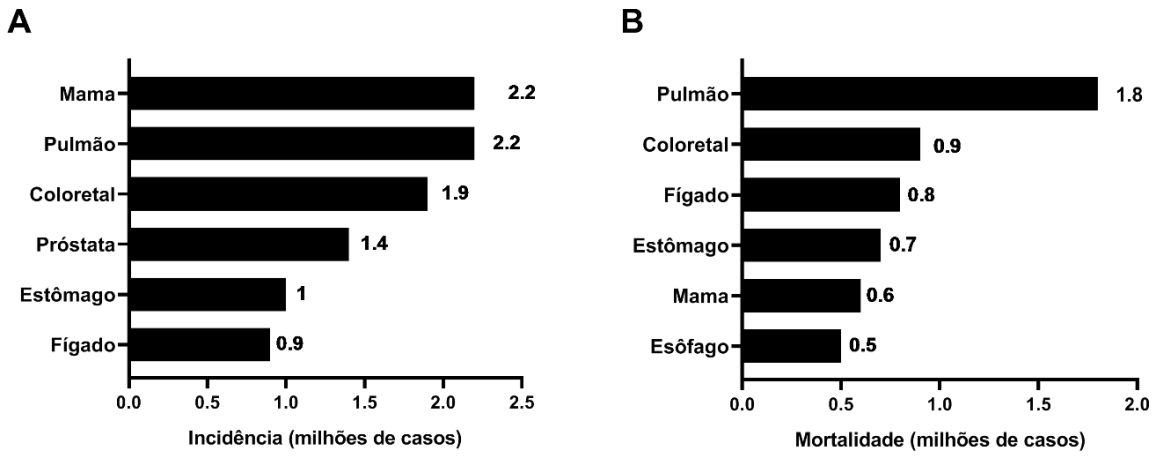


Figura 1 – Número estimado de novos casos e de mortalidade em todo o mundo, consideranto ambos os sexos e todas as faixas etárias. Adaptado de Globocan.

1.2 CARCINOMA HEPATOCELULAR

Existem dois tipos principais de cânceres primários de fígado - carcinoma hepatocelular (CHC) e colangiocarcinoma intra-hepático (CIH); subtipos menos frequentes são angiossarcoma, hemangiossarcoma e hepatoblastoma. Enquanto o CIH se origina dos ductos biliares, o CHC surge dos hepatócitos, as principais células parenquimatosas do fígado (KULIK; EL-SERAG, 2019; SINGH; KUMAR; PANDEY, 2018).

O CHC tem como principais fatores de riscos agentes virais (Hepatite B e C), consumo excessivo de etanol, consumo de aflatoxinas e a esteatose hepática não-alcóolica e, causas menos frequentes incluem hemocromatose hereditária, deficiência da Alfa1-Antitripsina, hepatite autoimune, algumas porfirias e a doença de Wilson (GOMES et al., 2013)**(Figura 2)**. Exceto em países onde a infecção com os vírus da hepatite ocorre precocemente, os casos de câncer de fígado são mais comuns em pessoas acima de 75 anos e acometem mais homens do que mulheres (YANG; ROBERTS, 2010). Pertinentemente cabe salientar que embora o número de pessoas infectadas com os vírus das hepatites B e C tenda a diminuir globalmente, estima-se que o número de pessoas com síndrome metabólica, diabetes e doença hepática gordurosa não alcóolica aumentem, assim elevando a incidência de CHC (YANG et al., 2020).

A incidência de CHC tem aumentado ao longo dos anos em diversos países. Nos EUA, nas últimas três décadas, esse número triplicou, alcançando em 2020 o número de 42.284 casos. Embora no Brasil os dados a respeito desse carcinoma sejam escassos, a incidência em 2020 foi de 12.674 casos, e número de mortes de 12.139. Ainda, no mundo todo, em 2020, foram diagnosticados 905.677 novos casos, com taxa de mortes estimada em 830.180 (IARC, 2022), revelando, portanto, a agressividade deste tipo de tumor; o câncer de fígado é o sexto tipo de neoplasia mais comum na população mundial e em relação a taxa de mortalidade ocupa o terceiro lugar em números absolutos (**Figura 3**).

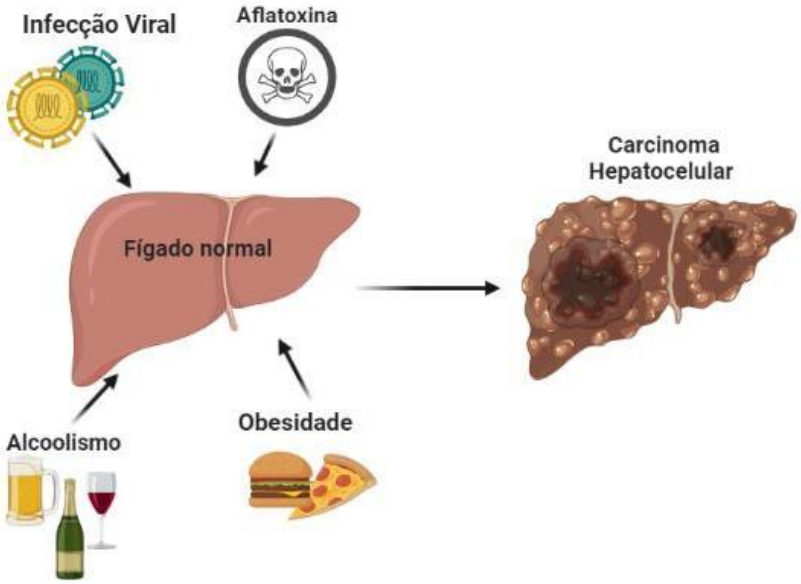


Figura 2 - Fatores etiológicos do CHC. Fonte: Adaptado de SARKAR, 2021

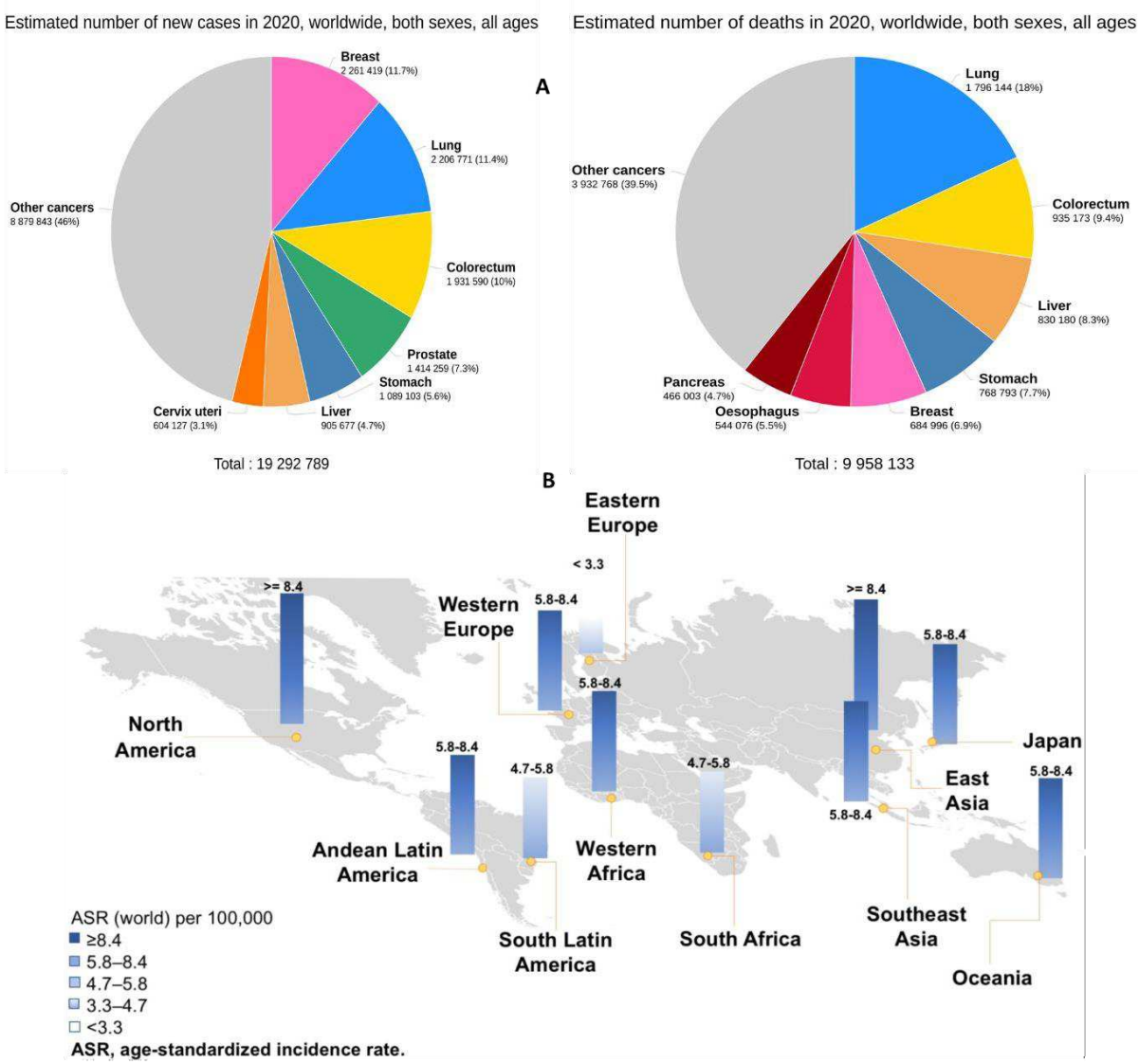


Figura 3. Número do Carcinoma Hepatocelular: (A) Incidência e taxa estimada de mortalidade estratificadas por tipos de cânceres, no ano de 2020 (Fonte: GLOBOCAN 2020; retirado de International Agency for Research on Cancer (IARC), 2022.) e (B) incidência de CHC, por área geográfica (retirado de Hou et al., 2022).

Existem várias linhas celulares para o estudo do HCC (Huh-7, Huh-1, HepG2, Hep3B, JHH-5, JHH-7, Bell-7402, SNU-449, entre outros). Huh-7 é uma linha celular imortal composta de células tumorigénicas semelhantes a epiteliais. A linha foi estabelecida por (NAKABAYASHI, 1982)a partir de uma linha de células cancerígenas derivadas de hepatócitos bem diferenciadas, originalmente retiradas de um tumor hepático num homem japonês de 57 anos. Huh7.5, uma sub-linha de Huh7, que para além dos estudos do cancro do fígado também foi estabelecida como uma linha celular altamente permissiva para replicar o RNA do vírus da hepatite C. In vitro, a homogeneidade da população celular assegura vários ciclos de replicação relativamente seguros, mas esta característica é também descrita como uma limitação dos estudos in vitro, porque os tumores in vivo são, em regra, bastante heterogéneos

em termos celulares (AO et al., 2017). As células do mesmo tipo de tumor, como o HCC, podem diferir nas suas respostas aos tratamentos e também nos mecanismos moleculares envolvidos na progressão da doença. Por exemplo, as estirpes de HepG2 e Huh7 diferem na tolerância e sensibilidade à doxorubicina, sorafenibe e melatonina, sugerindo uma sobrevivência celular distinta (DUBBELBOER et al., 2019).

1.3 HEPATOCARCINOGÊNESE

O desenvolvimento do CHC é um processo complexo que envolve dano inflamatório, necrose e regeneração de hepatócitos, associado à deposição de tecido fibroso (FORNER; REIG; BRUIX, 2018). A inflamação crônica do fígado (hepatite) pode levar à fibrose e cirrose hepática, elevando as chances de desenvolvimento do CHC(LOPES; BORGES-CANHA; PIMENTEL-NUNES, 2016). Dentre os mecanismos moleculares envolvidos, têm-se a incapacidade de inibição de crescimento, ativação de eventos neoangiogênicos, manutenção do ambiente inflamatório e morte celular, sendo essas características-chave da tumorigênese (URBAN-WOJCIUK et al., 2019).

O sistema imune inato é responsável pela primeira resposta do organismo a um desafio imunológico. Esse sistema é composto de barreiras físico-químicas e componentes humorais como sistema complemento, macrófagos, granulócitos e células dendríticas (BERNSMEIER; VAN DER MERWE; PÉRIANIN, 2020). Ao serem desafiadas por um antígeno, essas células respondem através de uma série de mecanismos que incluem fagocitose, geração de espécies reativas de oxigênio e citocinas pró-inflamatórias (LUPI et al., 2019). Dentre essas estão: fator de necrose tumoral (TNF), interleucina 1-β (IL-1-β), IL-6, IL-17, IL-18, interferon gama (IFN-γ) e agonistas de receptores do tipo Toll (do inglês, TLR, *toll-like receptor*) (BERNSMEIER; VAN DER MERWE; PÉRIANIN, 2020).

1.3.1 METABOLISMO ENERGÉTICO DA CÉLULA TUMORAL

Essencialmente tanto células sadias como células tumorais dependem das mesmas maquinarias enzimáticas para a manutenção da homeostase celular. No entanto, células cancerosas possuem características (hallmarks) que as diferem de células normais; dentre essas citam-se a proliferação sustentada e altas taxas de replicação, evasão do sistema imune, capacidade de invasão e metástase, imortalidade, produção de estímulos angiogênicos e também alteração do

metabolismo energético (SENGA; GROSE, 2021). Em células tumorais as mutações ocorridas e as interações alteradas estão intimamente ligadas a modulações heterogêneas do metabolismo energético celular, levando a uma complexa reprogramação de vias do metabolismo da glicose, lipídeos e aminoácidos. Essas mudanças metabólicas favorecem o desenvolvimento, sobrevivência, invasão e progressão tumoral.

Nos últimos anos diferentes grupos de pesquisa vêm se dedicando a estudar em detalhes as alterações que ocorrem em vias relacionadas ao metabolismo energético de células tumorais. NWOSU.(2018) relataram centenas de genes com expressão diferenciada em linhagens de CHC distintas. Alguns dos produtos desses genes são enzimas que controlam a taxa de fluxo de intermediários das vias de síntese e β-oxidação de ácidos graxos, do catabolismo e síntese de aminoácidos, enzimas mitocondriais que participam do ciclo do citrato e relacionadas à cadeia de transporte de elétrons, bem como enzimas importantes envolvidas com a utilização de glicose. Um já bem caracterizado fenótipo de células tumorais é o chamado efeito Warburg (VAUPEL; MULTHOFF, 2021). Esse fenômeno prediz que células de um tumor, mesmo em condições de aerobiose, utilizam preferencialmente a via glicolítica ao invés da fosforilação oxidativa a fim de se adaptar ao microambiente tumoral e atender suas demandas energéticas e de biossíntese. Em partes, essas alterações são dependentes da ativação da via de PI3K/AKT/mTOR e sinalização por MYC e HIF-1. Ainda que já bem estabelecido, o efeito Warburg ainda carece de elucidações a respeito das consequências de sua ocorrência. Nesse contexto, o estudo do metabolismo do lactato por exemplo, trará certamente novas contribuições para a área da bioquímica metabólica. O lactato, até pouco tempo atrás, era considerado apenas um subproduto do metabolismo da glicose, porém estudos em andamento do nosso grupo, bem como de outros autores, têm apontando para o papel modulador dessa molécula em células tumorais (CUCIELO et al., 2022a; DANHIER et al., 2017; SAN-MILLÁN; BROOKS, 2017; VANDER HEIDEN; DEBERARDINIS, 2017). Enzimas-chaves para utilização da glicose (GLUT, hexoquinase, PFK1, piruvato quinase, G6PDH, LDH, entre outras), além de apresentarem expressão alterada, podem também desempenhar funções não-canônicas que favorecem a sobrevivência de células tumorais; uma discussão detalhada sobre papéis de enzimas não-relacionados ao metabolismo energético pode ser consultada em (SNAEBJORNSSON; SCHULZE, 2018; YU; LI, 2016)

O metabolismo de aminoácidos também pode estar alterado no câncer (VAZQUEZ et al., 2016). São vários os aminoácidos que têm seu metabolismo modulado diferencialmente: a glutamina, o glutamato e a alanina, que participam de reações ana- e cataplerótica; a serina, envolvida em vias de biossíntese de purinas e pirimidinas; a metionina e mecanismos de metilação; arginina e via da poliamina, entre outros (VAZQUEZ et al., 2016). Por exemplo, a glutamina é convertida em glutamato por desaminação realizada pela enzima glutaminase-1 (GLS1); o metabolismo aumentado da glutamina permite a viabilidade e proliferação de células tumorais em condições de hipóxia. Já a glutamato desidrogenase (GDH), em células saudáveis, catalisa a conversão controlada de glutamato a α-cetoglutarato (α-KG), no entanto, em células tumorais GDH apresenta baixa atividade, e para compensar, a expressão de isoenzimas da aspartato transaminase mitocondrial 1 e 2 (GOT1; GOT2) estão aumentadas (SHANWARE et al., 2011).

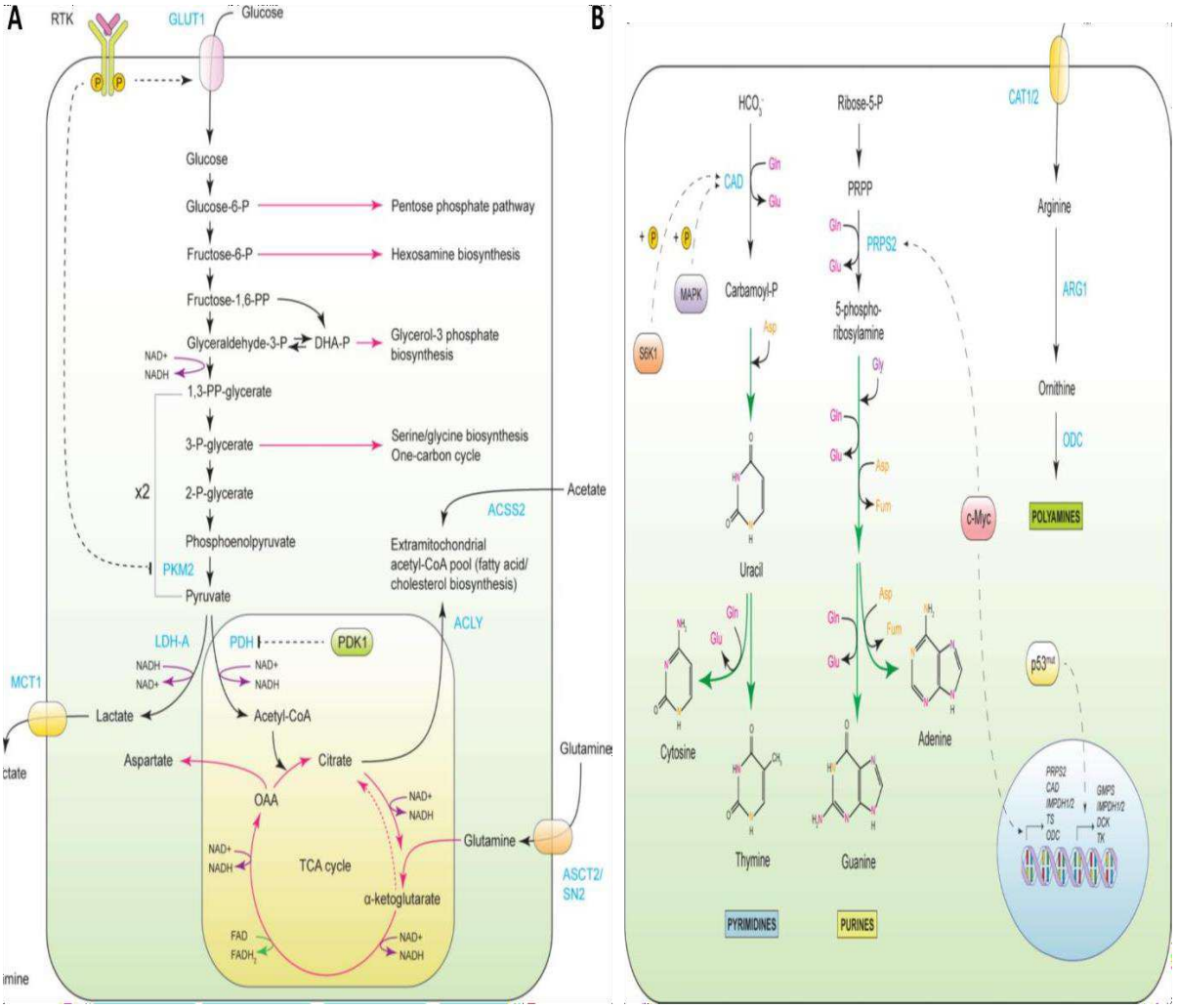
O metabolismo dos lipídeos pode ser reprogramado em células tumorais. Além de fornecer blocos para construção de membranas e serem combustíveis essenciais para o metabolismo celular, os lipídeos são fundamentais para sinalização intra- e extracelular e substratos importantes para as modificações pós-traducionais de proteínas. A relação entre lipídios e células tumorais é de fato bastante importante, inclusive já foi demonstrado que o conteúdo e o tipo de lipídio dietético, exerce a capacidade de modulação em alguns tipos de tumores (VARELA-LÓPEZ et al., 2021). A geração de energia pelas mitocôndrias está acoplada com a atividade do ciclo do citrato, mas também com a oxidação de ácidos graxos. As duas vias garantem o catabolismo de ácidos graxos que além de suprir a alta demanda por ATP, também gera equivalentes redutores importantes para sustentar a proliferação tumoral. Intermediários aumentados da β-oxidação, ciclo do citrato e de outras vias desreguladas no câncer são considerados oncometabólitos. O perfil metabólico amplo de 928 linhagens tumorais de mais de 20 tipos de cânceres pode ser consultado em (LI et al., 2019). A **Figura 4** resume algumas das vias que podem estar alteradas em células tumorais.

1.1.1 METÁSTASE

A transição epitélio-mesenquimal (TEM) é um processo que envolve um alto nível de plasticidade celular, frequentemente ativado durante a invasão de

células cancerosas. Sabe-se que o lactato induz “dormência” metabólica e está envolvido no processo de TEM, promovendo aumento de fator de crescimento endotelial (VEGF), o qual está envolvido na neoangiogênese, aumentando as metaloproteinases que degradam a matriz extracelular e membrana basal, facilitando a migração e invasão dessas células (KANG et al.,2019; LIN et al., 2020; PAYEN et al., 2016).

A TEM primeiramente foi descrita como parte integrante da morfogênese no desenvolvimento embrionário, e posteriormente observada em cicatrização de feridas e metástase do câncer (BAJ et al., 2020; YEUNG; YANG, 2017). Consiste um processo fisiológico, em que ocorre a transformação de células epiteliais imóveis em células mesenquimais móveis, as quais sofreram remodelação do citoesqueleto e aquisição da capacidade migratória devido à perda de contatos intercelulares e polaridade (**Figura 5**) (BAJ et al., 2020; LUU, 2021).



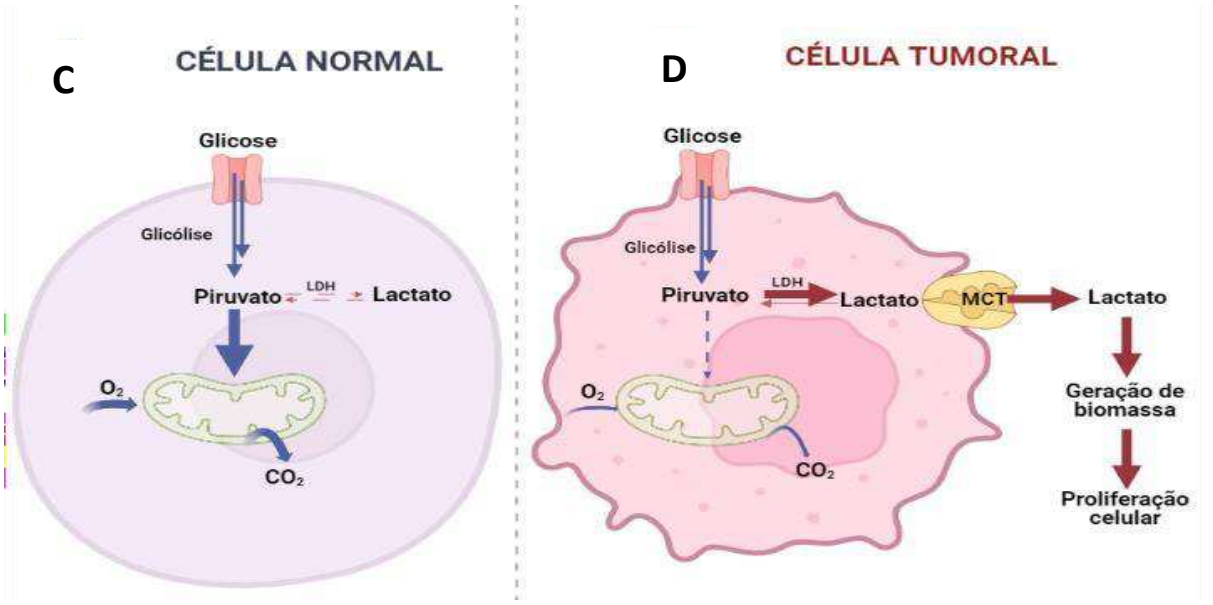


Figura 4. Metabolismo energético e células tumorais: (A) representação esquemática das vias relacionadas ao metabolismo da glicose e de ácidos graxos e (**B**) representação esquemática de vias relacionadas ao metabolismo do nitrogênio. (**C**) e (**D**) representações com ênfase no metabolismo do Piruvato. Figuras retiradas de Pavlova et al., 2016 e Kim & Baek, 2021.

Figura 5 - Invasão Celular

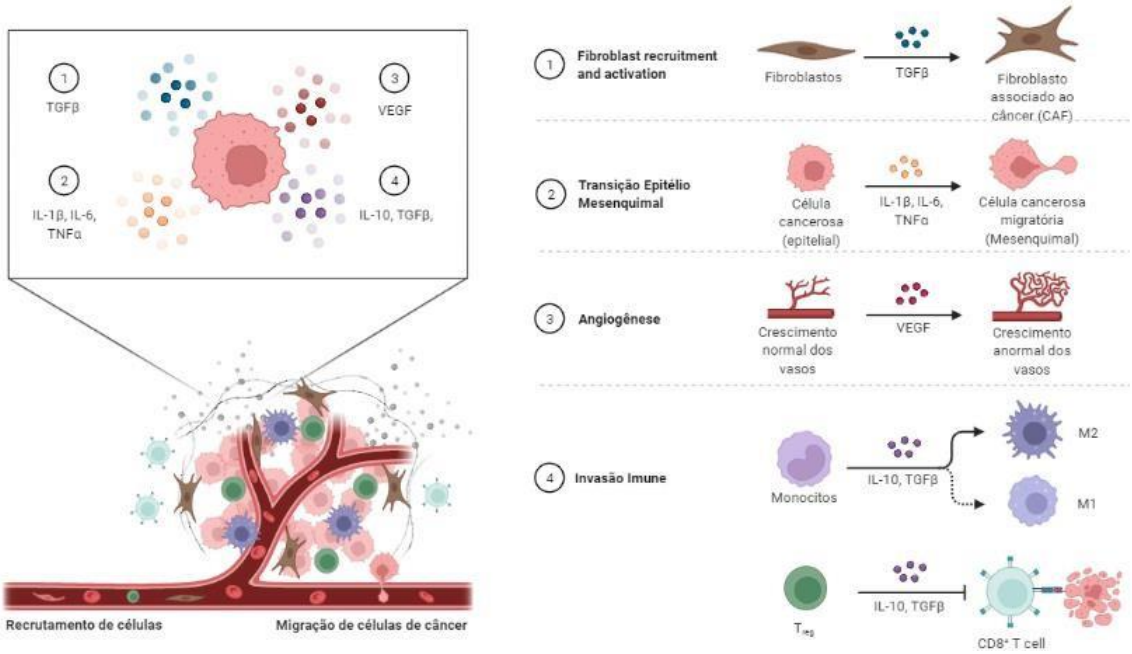


Figura 5 - O Tumor Microambiente e invasão: Visão geral das alterações associadas ao câncer.
Fonte: Adaptado de (KIM; BAEK, 2021).

Sabe-se que a disseminação tumoral para órgãos vitais é uma das principais causas de morte em pacientes com câncer e a TEM está envolvida nesse processo

(LUU, 2021). As caderinas, proteínas transmembranas, atuam como moduladores durante o crescimento do organismo, assim, células que sofrem TEM apresentam nível de expressão diminuído de proteínas epiteliais (E-caderina), e aumentado de genes mesenquimais (N-caderina e vimentina) (YU et al., 2019). A N-caderina (caderina tipo I clássica), é um membro da família de moléculas de adesão dependentes de cálcio, que medeiam diretamente a adesão célula-célula. No desenvolvimento fisiológico, a N-caderina desempenha papel importante nos processos morfogenéticos durante a formação de tecidos cardíacos e neurais. Esta proteína é regulada positivamente, enquanto a E-caderina é regulada negativamente durante a TEM em cânceres, e essa “transição de caderinas” (*cadherin switch*) está associada a características migratórias e invasivas (LOH et al., 2019; MROZIK et al., 2018).

O fator de transformação de crescimento beta (TGF-β, do inglês, *transforming growth factor-beta*) é uma citocina secretada que desempenha papéis cruciais em muitos processos celulares, incluindo inibição do crescimento, remodelação da matriz extracelular (MEC), TEM, migração celular, invasão e imunossupressão. Consequentemente, alterações na sinalização de membros da família TGF têm sido implicadas em muitas doenças, incluindo câncer (HAO; BAKER; DIJKE, 2019; XIE et al., 2018). Durante a fase inicial da tumorigênese, o TGF-β atua como supressor tumoral, induzindo essas células à apoptose. Em estágios posteriores, quando as células tumorais adquirem mutações oncogênicas, essa citocina perde sua função supressora e funciona como promotor de tumor, estimulando TEM, aumentando a expressão de marcadores mesenquimais, como N-caderina e vimentina, e reduzindo a expressão de marcadores epiteliais, como E-caderina (XIE et al., 2018; ZHAO et al., 2018). A mudança de isoforma de caderinas, com aumento da expressão de N-caderina, foi associada com menor taxa de sobrevida em pacientes com CIH e esse fenômeno foi atribuído ao TGFB-1 que foi capaz de ativar a migração e invasão celular em um modelo in vitro com células HuCCT (ARAKI et al., 2011).

1.2 TRATAMENTO

O tratamento do CHC é particularmente complexo devido ao seu desenvolvimento silencioso e lento. As opções de tratamento para CHC podem consistir em terapias cirúrgicas (ressecção, ablação e transplante de fígado),

procedimentos que apenas os pacientes diagnosticados em estágios iniciais são submetidos. Enquanto terapias não cirúrgicas, que podem ser direcionadas a pacientes com funções hepáticas preservadas consistem em quimioembolização transarterial, através da injeção de um quimioterápico como doxorrubicina, cisplatina e mitomicina seguido por embolização arterial a partir da infusão de partículas como microesferas de amido, miçangas de polivinil ou bobinas metálicas e radioterapias (FORNER; REIG; BRUIX, 2018; REIG et al., 2021). Contudo, a maior parte dos pacientes acabam sendo diagnosticados em estágios avançados, apresentando pior prognóstico, alta recorrência e baixa resposta aos quimioterápicos e radioterapia (KULIK; EL-SERAG, 2019).

A opção de tratamento medicamentoso alvo-direcionada para o CHC é relativamente recente. A partir dos resultados de trials como o SHARP (Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol), o inibidor múltiplo de kinases, sorafenibe, foi aprovado para o tratamento sistêmico da doença, no ano de 2007, e desde então diversas novas drogas e regimes terapêuticos vêm sendo experimentados (RINALDI et al., 2021). O sorafenibe, um inibidor oral multiquinase tem sido considerado o tratamento padrão para pacientes com CHC avançado. Seu mecanismo de ação envolve a inibição da atividade de tirosinas quinases envolvidas na angiogênese e progressão tumoral, incluindo o receptor do fator de crescimento endotelial vascular (VEGFR, do inglês, *vascular endothelial growth factor receptor*), o receptor do fator de crescimento derivado de plaquetas (PDGF-R, do inglês, *platelet-derived growth factor receptors*), e tem como alvo as quinases Raf envolvidas na via MAPK/ERK. No entanto, o sorafenibe apresenta alto custo e está associado a eventos adversos, além de alguns pacientes tratados não apresentarem respostas ao medicamento (LLOVET et al., 2008; MARISI et al., 2018). Como terapia de segunda linha, três quimioterápicos são aprovados para o tratamento de CHC, são eles: regorafenibe, cabozantinibe e ramucirumabe. O regorafenibe consiste em um inibidor multiquinase direcionado ao VEGFR1-3; o cabozantinib é um inibidor multiquinase com atividade única contra VEGFR2 e ramucirumab é a única terapia guiada por biomarcadores para o CHC (LLOVET et al., 2008, 2021).Contudo, a eficácia dessas drogas é limitada devido aos efeitos colaterais (perda de cabelo, inflamações na boca, perda de apetite, náuseas e vômitos, diarreia, infecções recorrentes, hemorragias e fadiga) que acometem parcela significativa dos pacientes tratados (KEATING, 2017;

LENCIONI et al., 2014), mas sobretudo pela quimiorresistência causada pela interação de diversos mecanismos moleculares complexos (MARIN et al., 2018).

Neste contexto, tem sido cada vez mais estudada a utilização de compostos que possam atuar como agentes terapêuticos sobre o CHC, devido à alta letalidade e à todas as complicações encontradas no tratamento desta doença. Produtos naturais derivados de uma variedade de fontes podem estimular muitas vias fisiológicas que podem ser benéficas para doenças persistentes como o câncer. A melatonina tem sido intensamente estudada por seus efeitos antitumorais, pró-apoptóticos, anti-angiogênicos e quimiopreventivo no câncer e em diversas doenças (TALIB et al., 2021). Este hormônio é produzido de forma natural pela glândula pineal, mas pode ser encontrado também em plantas e fungos, fornecendo proteção contra diferentes tipos de estresses (ARNAO; HERNÁNDEZ-RUIZ, 2006; HARDELAND; POEGGELER, 2003)-RUIZ, 2006; HARDELAND; POEGGELER, 2003). Desta maneira, acredita-se que a melatonina possa exercer atividades antiproliferativa e quimiopreventiva para o câncer de fígado.

2. MELATONINA

A melatonina (Mel) ou N-acetil-5-metoxitriptamina (**Figura 6**) é uma indolamina lipofílica que possui efeitos sistêmicos e celulares já descritos há tempos (Revisado por (REITER et al., 2021). A síntese da Mel, a partir do triptofano, ocorre na ausência de luz majoritariamente pela glândula pineal, mas outros órgãos também possuem a maquinária para síntese da Mel, como o fígado por exemplo (VENEGAS et al., 2012). Na literatura a melatonina já demonstrou atuar como uma “espada de dois gumes” (double-edged sword), isto é, enquanto atua como potente antioxidante protetor em células sadias, em células tumorais a Mel pode aumentar a produção de RL, atuando, portanto, de maneira pró-oxidante (SAMEC et al., 2021). A dose, duração e o tipo celular são determinantes para os efeitos do tratamento com melatonina.

A melatonina possui potencial quimioterápico em muitas formas de câncer humano e pode aumentar a eficácia de drogas anticancerígenas (por exemplo, sorafenibe, doxorrubicina e cisplatina) regulando vias de sinalização diferentes (LIN et al., 2017). Os efeitos atribuídos à Mel em células tumorais são, resumidamente, antiproliferativo, anti-inflamatório, promotor de apoptose e antimetastático (**Figura**

7A). Os efeitos da Mel sobre o metabolismo energético mais recentemente vêm ganhando espaço na literatura especializada; Reiter et al. (2020a) propõem que as alterações em vias metabólicas importantes que ocorrem nas células tumorais podem se beneficiar com o tratamento com a melatonina (**Figura 7B**). Nosso grupo de pesquisa vem há anos elucidando os efeitos benéficos da Mel em tumores de ovário (CESÁRIO et al., 2022; CHUFFA et al., 2016; CUCIELO et al., 2022b; REITER et al., 2020) e mais recentemente começamos a estudar os efeitos da Mel no hepatocarcinoma, in vitro.

O papel da Mel na saúde do fígado e hepatócitos já foi demonstrado e revisado por (SATO et al., 2020). Também, em uma recente revisão sistemática foram elencados diversos estudos que avaliaram os efeitos benéficos do tratamento com Mel isolada ou em associação; esses estudos utilizaram linhagens celulares e modelos animais distintos. De 34 trabalhos in vitro, apenas 7 utilizaram a linhagem Huh7.5, sendo a linhagem HepG2 a mais estudada (FERNÁNDEZ-PALANCA et al., 2021). Além disso, de 21 estudos in vivo, nenhum deles avaliou a associação da Mel com Cisplatina. Entretanto, essa associação já foi experimentada em células HepG2, e foi demonstrado que o tratamento em associação da Mel com o quimioterápico estimulou a progressão da autofagia (BENNUKUL; NUMKLIANG; LEARDKAMOLKARN, 2014), inibiu a proliferação celular e incrementou os efeitos da cisplatina em células de CHC, atuando no nível da expressão gênica (MI; KUANG, 2020).

Figura 6: Estrutura química da melatonina.

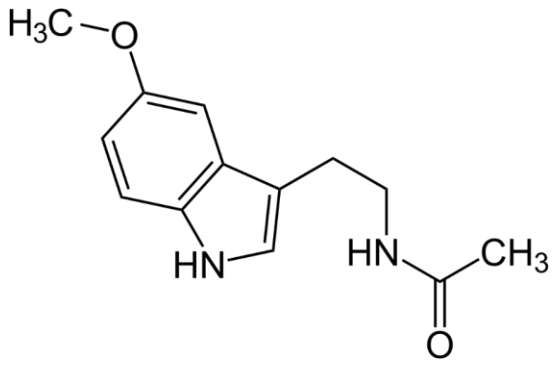


Figura 6 - Estrutura química da melatonina. Fonte: Pubchem**.**

Atualmente os tratamentos para o CHC são acompanhados por graves efeitos adversos e quimiorresistência. Por conseguinte, a proposição de novos medicamentos

adjuvantes com atividade seletiva contra as células tumorais e menor efeito citotóxico em células sadias permanece mandatória. Assim, este trabalho visou avaliar o efeito citotóxico da melatonina sobre a linhagem celular HuH7.5 de HCC e identificar possíveis mecanismos envolvidos.

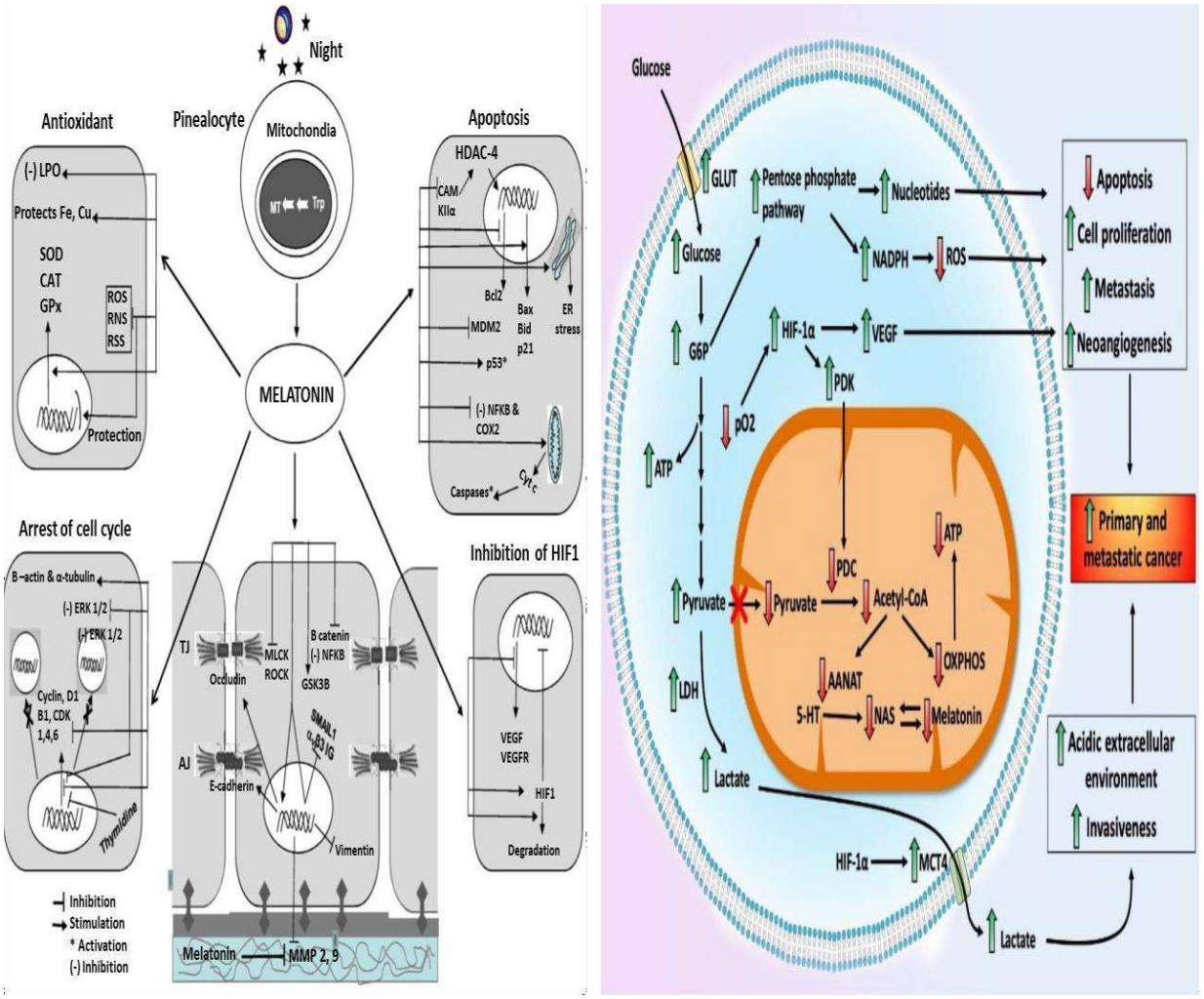


Figura 7. A melatonina e o metabolismo tumoral: (A) representação esquemática dos mecanismos de ação da melatonina em células tumorais. (retirado de Samanta et al., 2020) e (**B**) representação esquemática de alterações metabólicas que ocorrem em células tumorais (retirado de Reiter et al., 2020).

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3. OBJETIVOS

3.1 OBJETIVO GERAL

- Investigar o perfil antiproliferativo, a motilidade celular e o consumo de glicose de células tumorais humana de CHC, da linhagem HuH7.5. após o tratamento com melatonina.

3.2 OBJETIVOS ESPECÍFICOS

- Determinar a viabilidade celular da linhagem tumoral HuH7.5 após o tratamento com melatonina em 24 e 48 h;
- Observar as alterações morfológicas das células tumorais após o tratamento com melatonina 24 e 48 h;
- Investigar o efeito da melatonina sobre a capacidade migratória e clonogênica da linhagem HuH7.5;
- Verificar o efeito da melatonina sobre o consumo de glicose e produção de lactato.

4. PRODUÇÃO CIENTIFICA

Artigo: Melatonin reduces proliferation, migratory potential and alters morphology through metabolic changes in the HuH7.5 tumor cell line.

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Melatonin modulates the Warburg effect and alters the morphology of hepatocellular carcinoma cell line resulting in reduced viability and migratory potential

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ABSTRACT

Aims: Hepatocellular Carcinoma (HCC) is a primary neoplasm derived from hepatocytes with low responsiveness and recurrent chemoresistance. Melatonin is an alternative agent that may be helpful in treating HCC. We aimed to study in HuH 7.5 cells whether melatonin treatment exerts antitumor effects and, if so, what cellular responses are induced and involved.

Main methods: We evaluated the effects of melatonin on cell cytotoxicity and proliferation, colony formation, morphological and immunohistochemical aspects, and on glucose consumption and lactate release.

Key findings: Melatonin reduced cell motility and caused lamellar breakdown, membrane damage, and reduction in microvillus. Immunofluorescence analysis revealed that melatonin reduced TGF and N-cadherin expression, which was further associated with inhibition of epithelial-mesenchymal transition process. In relation to the Warburg-type metabolism, melatonin reduced glucose uptake and lactate production by modulating intracellular lactate dehydrogenase activity.

Significance: Our results indicate that melatonin can act upon pyruvate/lactate metabolism, preventing the Warburg effect, which may reflect in the cell architecture. We demonstrated the direct cytotoxic and anti-proliferative effect of melatonin on the HuH 7.5 cell line, and suggest that melatonin is a promising candidate to be further tested as an adjuvant to antitumor drugs for HCC treatment.

1. Introduction

Chemotherapeutic agents are still the most widely used options, often with a good prognosis, for treating different types of cancers [1]; however, the need to reduce the occurrence of adverse effects and increase the effectiveness of drug therapies further drives the study of possible adjuvant compounds. Melatonin (*N*-acetyl-5-methoxytryptamine) is a small lipophile indoleamine produced by humans and other non-related species possessing potential physiological and pharmacological pleiotropic effects [2–4]. Although melatonin is essentially synthesized and secreted by the pineal gland, other organs can also synthesize melatonin independently of pineal regulation [5].

Liver cancer is the fourth most common cause of cancer-related death worldwide and hepatocellular carcinoma (HCC) accounts for

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about 90 % of the cases. Although the number of HCC associated with viral hepatitis infections has decreased, the escalating number of obese individuals may increase the incidence of HCC [6,7].

The classic role of melatonin in regulating the circadian cycle has been supplanted by several other functions of this endogenous hormone, such as a potential tool for treating some types of cancer [8,9]. Melatonin provokes several distinct responses in tumor development and progression, modulating various pathways related to angiogenesis, cell proliferation, migration and invasion [10]. Furthermore, melatonin can modulate tumor energy metabolism, thereby inhibiting the glycolytic pathway [2,11].

Some of the hallmarks of tumor cells are altered energy metabolism, sustained proliferation, high replication rate, invasion, and metastasis [12]. Briefly, tumor cells, to account for their altered phenotype, need advantages in energy production; this is achieved, for example, through the so-called Warburg effect, in which cells consume glucose more rapidly and divert its catabolism to the reduction of pyruvate into lactate, even under aerobic conditions and independently of mitochondrial dysfunction [13]. Furthermore, epithelial cell transformation to a mesenchymal phenotype is another key feature acquired by tumor cells to successfully initiate intrahepatic and extrahepatic metastasis [14]. In this context, the overexpression of proteins such as N-cadherin and *TGFB1* are involved in this epithelial-mesenchymal transition (EMT) process and is found in distinct HCC cell lines [14–17].

HuH 7.5, a HuH 7 subline, in addition to liver cancer studies, was also established as a highly permissive cell line to replicate the hepatitis C virus RNA [18]. Several studies have lent themselves to the evaluation of the effects of melatonin on HepG2 [19], but to date we have not been able to find any study that has evaluated the effects of melatonin specifically in HuH 7.5. The deeper the investigation in different cell lines, the more robust answers we may gather about HCC management. This is because solid tumors are often quite heterogeneous in cellular and molecular aspects [20]. Cells from the same tumor type differ in their responses to treatments and in the molecular mechanisms involved in disease progression. For example, HepG2 and HuH7 cells differ in tolerance and sensitivity to doxorubicin and melatonin, suggesting distinct cell survival responses [21–23]. In addition, an extensive number of animal and human studies have documented the safety of short-term use of melatonin, as well as its high tolerability and the capacity to attenuate the adverse effects of chemotherapy [24,25]. This, together with its relatively low cost, makes melatonin a suitable candidate to be tested as an adjuvant in treating HCC.

Currently, there are no curative options for most patients with HCC; for those for whom therapeutic regimens are available, discontinuation of therapy due to side effects is not uncommon, and the relapse rate is high. Therefore, there is an emergency for developing new adjuvant drugs with greater selective activity against tumor cells and less adverse effects on healthy cells [26]. We evaluated the cytotoxic, antimigratory, and antiproliferative effects of melatonin and determined its effects over glucose and lactate levels and the morphological alterations in the HCC cell line HuH 7.5.

2. Materials and methods

2.1. Cell viability and determination of the experimental groups

Melatonin (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 1 % dimethyl sulfoxide (DMSO) (GIBCO, Invitrogen, New York, USA). HuH 7.5 cells were initially seeded and cultured in RPMI (Life technologies, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS) (GIBCO, Invitrogen, New York, USA), 100 U/mL Penicillin and 100 μg/mL Streptomycin (Santa Cruz Biotechnologies, Dallas, TX, USA) and maintained at 37 °C in a humidified atmosphere of 5 % CO_2.

To assess the effects of melatonin on cell viability, HuH 7.5 cells (1×10^4) were seeded in 96-well microplates and treated with increasing concentrations of melatonin for 24 and 48 h. Subsequently, the culture medium was removed and 100 μL of MTT solution (0.05 mg/mL) was added to quantify the formation of formazan crystals using a microplate reader (Thermo Scientific Multiskan) at 540 nm. For the MTT assay, the following were also considered as a negative control group (cell and culture medium), a vehicle control group (cell, culture medium and DMSO), and a positive control group (cell, culture medium and hydrogen peroxide). From the results of the MTT assay, the 50 % cytotoxic concentration (CC_{50}) was calculated by logarithmic regression, and doses of 2.0 and 4.0 mM of melatonin were tested for 24 and 48 h in the experiments described below. To compare the cytotoxic potential of melatonin in healthy cells, Hacat (primary epidermal keratinocytes) cell line were also submitted to MTT assay.

2.2. Clonogenic assay

To investigate whether melatonin interferes with the ability of HuH 7.5 cells to form new colonies, the protocol described by Franken et al. [27] was used. Briefly, cells (1.5×10^5) were seeded in a 6-well plate and treated with melatonin. Then, cells were trypsinized, the supernatant was removed and 500 cells/well were seeded in a 24-well plate and maintained in untreated medium for 14 days, with the medium being renewed every 3 days. Subsequently, the colonies were incubated with resazurin (60 μM) for 2 h, and the fluorescence intensity was quantified in the Glomax® fluorescence reader (520 nm excitation and 580 nm emission). After that, colonies were fixed in methanol + acetic acid solution (3:1) for 5 min and stained with crystal violet (0.5 %) for 15 min.

2.3. Migration assay

HuH 7.5 cells (1×10^6) were seeded into 6-well microplates and incubated until around 90 % confluence. Subsequently, a wound (cell-free) area (T0 area considered as 100 %) was made in the monolayer by gently passing 10 μL pipette tips into the bottom of each well. The cells were treated with 2.0 mM and 4.0 mM melatonin. Photomicrographs (200× objective lens) were performed using the EVOS microscope (Life Technologies, CA, USA), at different times (0, 12, 24, 36, and 48 h). Cell migration was evaluated as free area (region without cells) using the Image Pro Plus Program Software, and the percentage decrease of the area characterized the cell migration index.

2.4. Immunofluorescence analysis

After melatonin treatments, cells were pretreated with 10 % Triton X and 10 % FBS solutions for 15 min each, and incubated overnight at 4 °C with mouse antihuman *TGFB1* and N-cadherin (1:1000 dilution monoclonal antibody, Santa Cruz Technologies). Next, they were rinsed 5 times in saline 0.9 % for 5 min, protected from light, and then incubated at 37 °C for 60 mi with a secondary antibody (1:2000 dilution, Texas Red goat anti-mouse IgG, Abcam). Subsequently, the sections were rinsed twice in saline 0.9 % for 5 min each and incubated with 4,6 diamidinofenilindol (DAPI, Sigma Aldrich), 5 mg/mL solution, for 30 min. The slides were mounted with glycerol and kept protected from light under a fluorescence microscope for later observation. The intensity and localization of the immunoreactivity were analyzed with the Motic BA 410E fluorescence microscope and MOTICAM ProS5 Plus. The merge of DAPI and Texas Red images was performed using the Image J software (NIH, USA).

2.5. Morphological analysis of HuH 7.5 cells by scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

SEM was performed to analyze morphological changes in cell surface topography. HuH 7.5 cells treated with 2 mM and 4 mM for 24 h and 48 h at 37 °C and 5 % CO_2 were fixed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer for 60 min. Then, cells were dehydrated with

increasing ethanol concentrations (30–100 %), submitted to critical point drying and metalized with gold for visualization on a high-resolution double beam electron microscope FEI SCIOS.

TEM was performed to evaluate the ultrastructural changes in treated HuH 7.5 cells. For this, cells were fixed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer for 60 min, post-fixed with 1 % OsO4, 0.8 % potassium ferrocyanide and 10.0 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer for 1 h. After, samples were dehydrated in increased concentration of acetone (30–100 %) and embedded in EPON™ epoxy resin. Ultrathin sections (60–70 nm) were obtained, mounted on copper grids and contrasted with uranyl acetate and lead citrate. Analysis was performed on JEOL JEM-1400.

2.6. Lactate dehydrogenase (LDH) activity

To evaluate the LDH activity, HuH 7.5 cell line (1×10^6) was seeded in 24-well microplates and submitted to melatonin treatment regimens. After trypsinization, the supernatant was collected to measurement

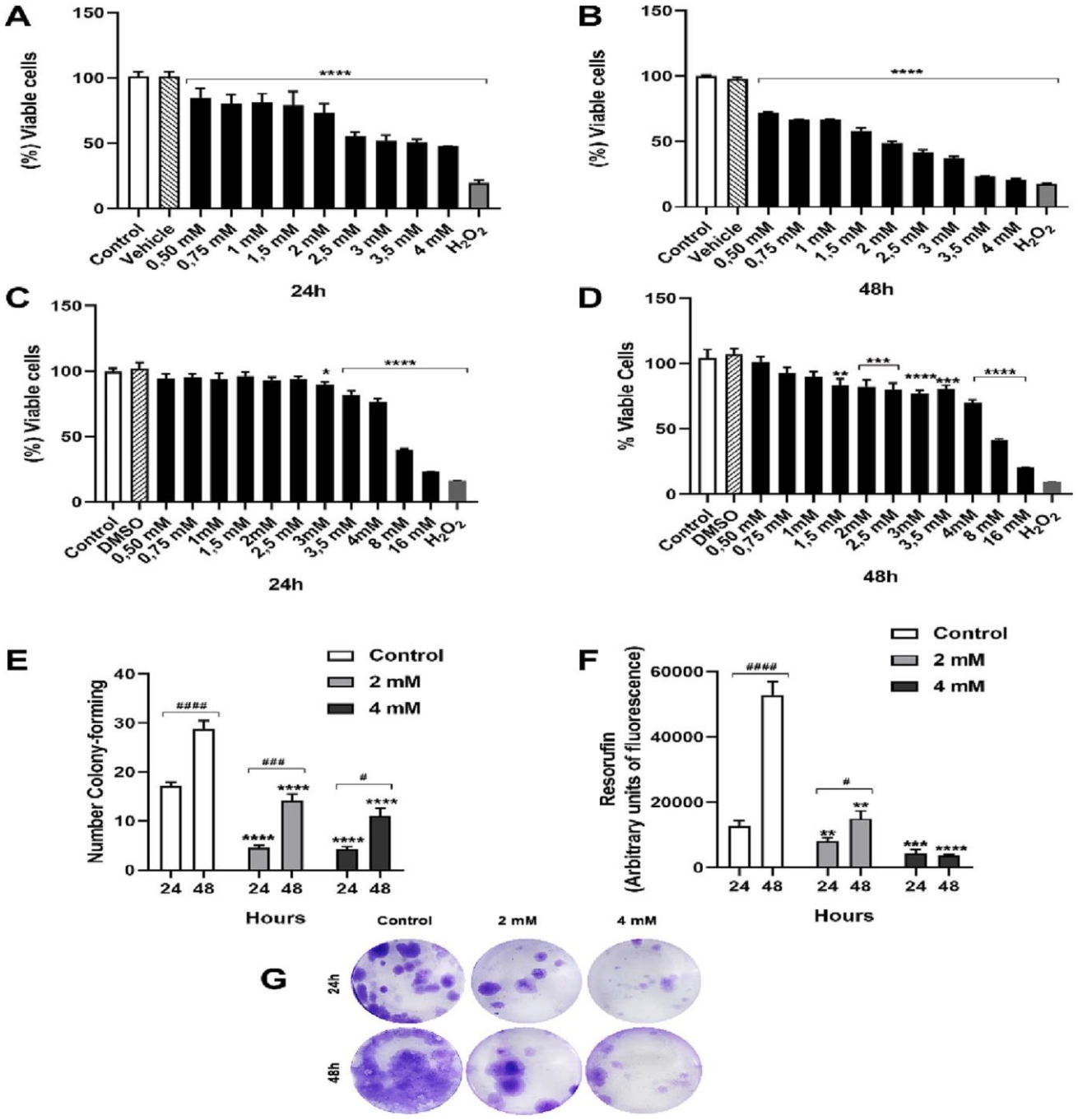

Fig. 1. Melatonin treatment reduced cell viability and clonogenicity of the HuH 7.5 line. HuH 7.5 and Hacat cells were treated with melatonin for 24 and 48 h. 24 h-MTT assay for HuH 7.5 (A), 48 h-MTT assay for HuH 7.5 (B), 24 h-MTT assay for Hacat (C) and, 48 h-MTT assay for Hacat (D). After determining the concentrations to be tested, the clonogenic assay was performed on the HuH 7.5 line. The cells were treated with melatonin at concentrations of 2.0 and 4.0 mM for 24 and 48 h. Then, the cell medium was renewed and cells were allowed to grow for 14 days in an untreated medium. Quantification of the number of colonies following melatonin treatment (E), and the resazurin assay (F). (G) Clonogenic assay photo-documented confirming the results. Data are expressed as the mean ± SEM of 3 independent experiments performed in triplicate. * ($p < 0.05$); *** ($p < 0.001$); **** ($p < 0.0001$) comparing treated groups with control. $^{\#}$ ($p < 0.05$); $^{\#\#\#}$ ($p < 0.001$); $^{\#\#\#\#}$ ($p < 0.0001$) comparing 24 and 48 h treated groups.

extracellular LDH. For the determination of intracellular LDH activity, the wells were first washed twice with PBS and water was added to perform 3 repetitions of freezing at —80 °C and thawing in a heated bath, in order to lysis the cells. The LDH assay was performed according to the kit instructions (Gold Analisa Diagno´stico, Minas Gerais, Brazil).

2.7. Glucose and lactate levels

To verify the consumption of glucose by HuH 7.5 cell line, 1×10^6 cells were seeded into 24-well microplates and treated with melatonin. After 24 and 48 h, 1 μL of the supernatant was aliquoted and transferred to a 96-well plate and 100 μL of Glucose-PP reagent (Gold Analisa Diagnostics, Minas Gerais, Brazil) was added and incubated for 10 min at 37 °C, following the kit instructions. Glucose uptake was compared with non-treated HuH 7.5 cells and was calculated as relative glucose uptake. For intracellular lactate measurement, cells were lysed and the procedures were described according to the kit datasheet (Katal Biotecnolo´gica, Belo Horizonte, MG, Brazil). Spectrophotometric reads were performed using a microplate reader (Thermo Scientific Multiskan).

2.8. Statistical analysis

All data were tested for normality distribution by the Shapiro-Wilk test. Statistical differences were obtained after analysis of variance (ANOVA), followed by the Tukey test for multiple comparisons using GraphPad Prism 8 (GraphPad Software, California, USA). Data were expressed as mean ± SEM and were considered significant when meet a p-value < 0.05.

3. Results

3.1. Melatonin reduces viability and alters HuH 7.5 colony formation capacity

We initially investigated whether melatonin had cytotoxic effects on the HuH 7.5 and Hacat cell lines. For HuH 7.5 cells, melatonin reduced tumor cell viability at both 24 and 48 h compared with the control group ($p < 0.0001$). Although all concentrations reduced cell viability, significant reductions were seen starting at 2.5 mM, during 24 h (reductions of 45 % or higher); after 48 h of treatment with melatonin, a drastic reduction started at 2.0 mM (52 %) (Fig. 1A, B). For Hacat cells, 24 h of exposure to melatonin drastically reduced the cell viability only at concentrations of 8 and 16 mM, with a reduction of 60 % and 77 %, respectively. At 48 h, there was a significant reduction in cell viability in different concentrations, but a pronounced reduction occurred from 8 mM on, with 59 % and 80 % respectively (Fig. 1C, D).

The clonogenic survival assay was performed to demonstrate viable cells that could still proliferate and form colonies after melatonin was removed from the culture medium. As expected, cells allowed to proliferate for a longer period had higher initial numbers of colonies. After melatonin treatment, we observed a reduction in the number of colonies compared with the respective control group (Fig. 1E). At 24 h, the control group contained an average of 17 colonies, and the treated groups (2.0 and 4.0 mM) 4.5 and 4.2 colonies, respectively. At 48 h, these values were 28.0, 14.0, and 11.0 colonies for the control, 2.0 and 4.0 mM groups, respectively. Corroborating these findings, both concentrations of melatonin reduced HCC cell proliferation after 24 and 48 h of treatment, as demonstrated by the assay with resazurin fluorescence (Fig. 1F). Representative images were taken after colonies staining, which confirmed the previous data (Fig. 1G).

3.2. Melatonin reduces the migratory capacity of HuH 7.5 cells and EMT biomarkers

To evaluate the influence of melatonin on cell migration, a wound-healing assay was performed. After treatment, images were captured at 0, 12, 24, 36, and 48 h after scraping (Fig. 2A). As shown in Fig. 2B, the wound gaps in the melatonin-treated groups were quantified to be significantly larger than in the control group. After 12 h, the wound closure was 43 % reduced in the control group and treatment with 2.0 and 4.0 mM of melatonin inhibited migration by 30 % and 9 %, respectively. After 24 h, wound closure was approximately 66 % reduced in the control group, and 54 % and 34 % reduced at melatonin concentrations of 2.0 and 4.0 mM, respectively. The same pattern of reduced migration was seen after 36 h. Finally, at 48 h, we observed a 79 % wound closure in the control group, and 48 % and 26 % in the group treated with 2.0 and 4.0 mM of melatonin, respectively. In addition, after 48 h of treatment with 4 mM of melatonin, most of the cells are also loose, which is an indication that they have lost their adhesion properties. To confirm these findings, we conducted immunofluorescence analysis for *TGFB1* and N-cadherin, and showed a drastic reduction in the expression of these EMT biomarkers, reinforcing the ability of melatonin to regulate proliferative and migratory events (Fig. 3A, B).

3.3. Melatonin drastically alters HuH 7.5 morphology

Scanning electron microscopy (SEM) was used to identify alterations in cell morphology after melatonin treatment. At both 24 and 48 h, the control groups exhibited firm adhesion to the substrate, an intact cell membrane, presence of lamellipodia, and microvilli on the cell surface (Fig. 4A–B, A'–B'). Conversely, tumor cells treated with 2 mM melatonin, for 24 and 48 h, showed a reduction in the microvilli and indications of membrane damage. It can be clearly seen a disruption of cell junctions at 24 and 48 h after melatonin treatment with 2.0 mM (Fig. 4E, E'). The concentration of 4.0 mM of melatonin caused reduction in microvilli, membrane damage, and cell membrane shrinkage in both periods of treatment (Fig. 4H, J, J'). The loss of microvilli and cell-to-cell adherence is clearly demonstrated in the transmission electron microscopy (TEM) images. The treatment with melatonin reduced significantly the microvilli projections of Huh 7.5 cells compared with the control group (Fig. 5).

3.4. Melatonin reverses Warburg-type metabolism in the HuH 7.5 cells

To analyze the initial steps of glucose metabolism, lactate dehydrogenase (LDH), glucose, and lactate assays were performed. Extracellular LDH activity was increased in melatonin-treated groups compared with the control group (Fig. 6A), evidencing that melatonin may disrupt the membrane integrity of HuH 7.5 cells. Otherwise, the intracellular LDH activity was reduced following both melatonin concentrations and exposure periods (Fig. 6B). The activity in the control group was 220 U/L, while in the groups treated with melatonin, after 24 h, averages were 180 and 150 U/L with 2.0 and 4.0 mM, respectively. At 48 h, the control group had an average of 194 U/L and the groups treated with 2.0 and 4.0 mM melatonin, 150 and 135 U/L, respectively.

In addition to a decreased LDH activity, glucose uptake and lactate production were reduced after melatonin treatment at 24 and 48 h. Glucose uptake was significantly dropped after treatment with 2.0 and 4.0 mM melatonin (Fig. 6C). The lactate concentration was reduced in both melatonin-treated groups, during 24 h, compared with the control group (Fig. 6D). The levels of lactate were 20 mg/dL in the control group, whereas lactate levels were 14 and 9 mg/dL in the groups treated with 2.0 and 4.0 mM melatonin, respectively. At 48 h, an even more significant reduction was observed after melatonin treatment.

4. Discussion

When diagnosed in the early stages, HCC may be treatable with surgical resection or transplantation, or locally through ablation, chemoembolization, and radioembolization; however, with the advance of

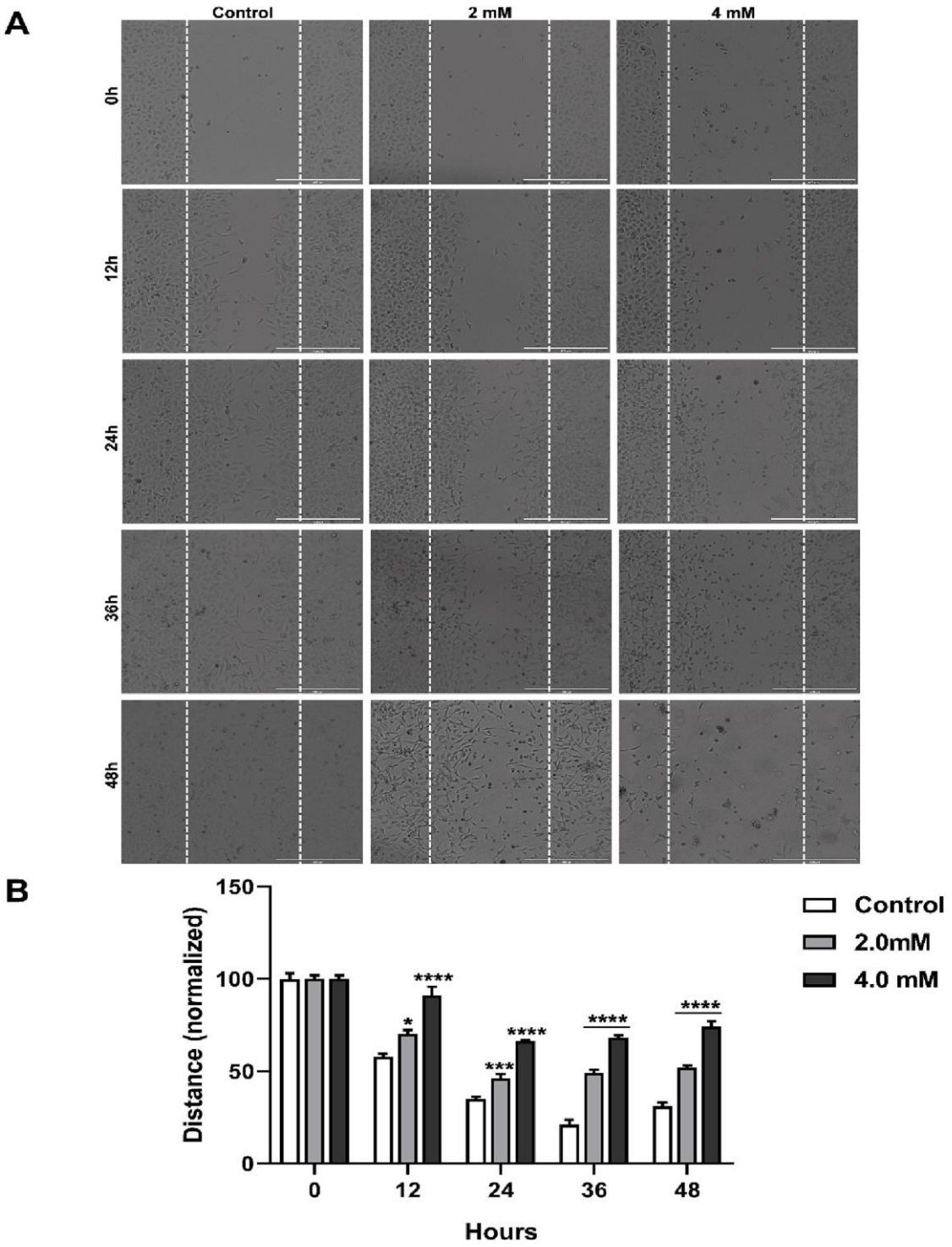

Fig. 2. Melatonin treatment reduced the healing capacity of HuH 7.5 cells. Wound-healing assay was performed at overtime (0, 12, 24, 36, and 48 h) (A) and the distance traveled from the free area of these treated cells was quantified using Image J software (B) Data are expressed as the mean ± SEM of 3 independent experiments performed in triplicate. * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$) *vs.* Control 24 and 48 h.

the disease, patients are unamenable to curative therapies [28]. The treatment of HCC, when resection or transplantation is not possible, relies on chemotherapy drugs often not curative due to tumor chemoresistance. Treatment with sorafenib alone or in combination has limited advantages for advanced cases of HCC; although sorafenib can prolong overall survival (OS) in HCC patients, its effectiveness is short-termed due to the development of resistant cell [29]. In oncology practice, it is currently well established that a combination of drugs has been shown to be a more successful option for treating different tumor types, including HCC [30]. Just to be in one example, the drugs atezolizumab/bevacizumab combined met the dual primary endpoint of significant improvement in OS and progression-free survival compared to sorafenib [1]; other drugs and combined regimens for HCC management are in clinical phases [26]. The therapeutic use of melatonin in aggressive tumors that have high mortality rates and limited curative options may shed light on this recurrent global challenge. Particularly, the benefic

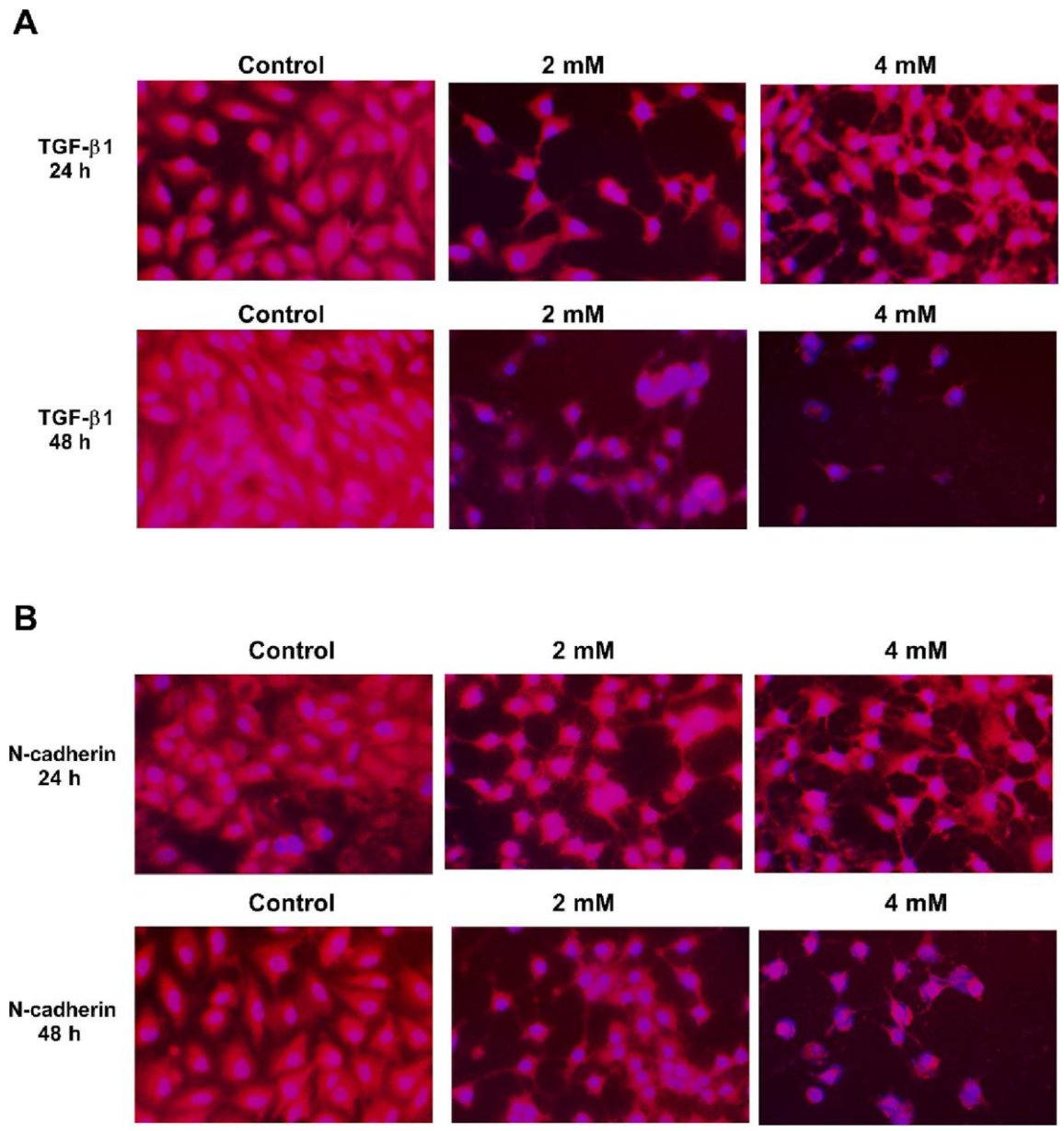

Fig. 3. Immunofluorescence analysis showed that melatonin treatment reduced the expression of *TGFB1* and N-cadherin in HuH 7.5 cells. HuH 7.5 cells were treated with the concentrations of 2.0 and 4.0 mM melatonin for 24 and 48 h.

adjuvant role of melatonin associated with approved chemotherapies has already been evidenced [31]. Melatonin can be used as a safe and well-known pharmacological molecule with promise results in helping treat HCC [32]. To achieve the desired therapeutic effects of this "*jack of all trades*," studies have shown that melatonin must generally be administered at higher concentrations compared to physiological ones; our group has recently demonstrated that melatonin levels are significantly reduced in ovarian cancer cells, emphasizing that their levels may differ across cellular compartments [2].

Abnormal cell proliferation is a prominent event in tumor growth and development. We examined the cytotoxic response of HuH 7.5 to melatonin at various concentrations and showed that melatonin exhibited an antiproliferative effect against HuH 7.5 cells. Using different concentrations of melatonin, similar results were obtained with HuH7 and HepG2 cell lines [32,33]; the concentrations of 2.0 and 4.0 mM are between the 1 and 5.4 mM concentrations tested in the previous studies. This finding corroborates the dose-dependent and cell-specific effects of melatonin treatment. Therefore, different concentrations may be adapted for melatonin to be effective.

The clonogenicity assay is a methodology for assessing sensitivity to radiotherapy, chemotherapy, as well as molecularly targeted therapy. Our study showed that melatonin was able to reduce the number of colonies, as well as their viability. In that same vein, the migratory capacity of HuH 7.5 cells was influenced by the treatment; we observed that melatonin inhibited complete wound closure. Interestingly, we suggested that at 48 h the melatonin-treated cells are already undergoing some type of cell death and further investigation may clarify this issue. Melatonin's capacity to inhibit or minimize cell migration is a critical point in the development of new antitumor drugs as invasion and adhesion are pivotal steps in the process of tumor malignance [34].

Metastasis is a process that involves the spread of cancer cells from the primary tumor to other organs, and this invasive characteristic contributes to tumor chemoresistance; the EMT is a key feature for the success of metastatic processes. Cell surface structures, such as tight, adherens, and gap junctions help maintain cellular architecture of the epithelium and mediate the adhesion of epithelial cells to their basement

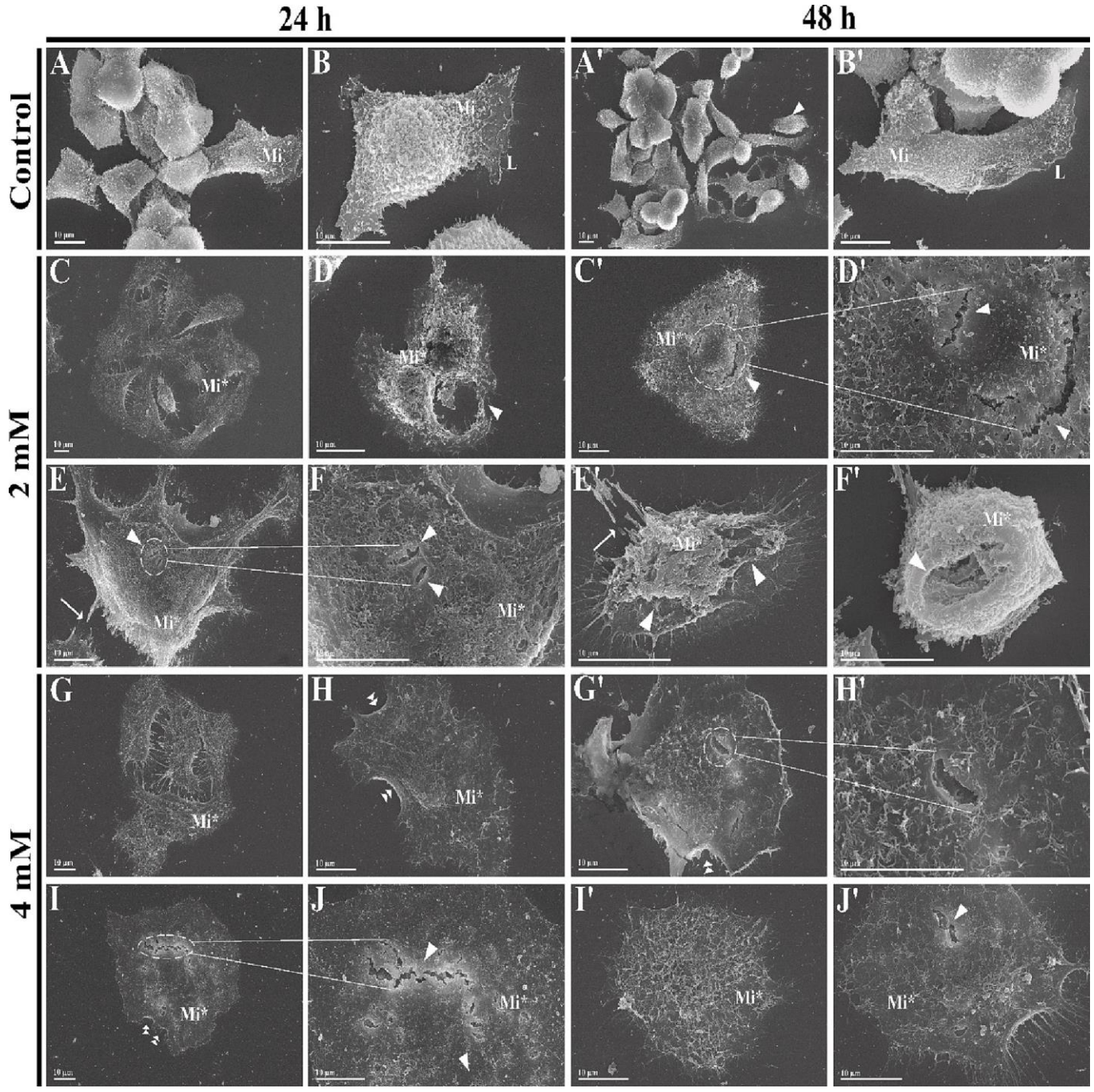

Fig. 4. Morphological changes in HuH 7.5 cells treated with 2.0 and 4.0 mM melatonin for 24 and 48 h. SEM images of (A–B) Untreated HuH 7.5 cells (24 h control); (A'–B') Untreated HuH 7.5 cells (48 h control); (C, D, E, F) HuH7. 5 treated with 2.0 mM melatonin for 24 h; (C', D', E', F') HuH 7.5 treated with 2.0 mM melatonin for 48 h; (G, H, I, J) HuH 7.5 treated with 4.0 mM melatonin for 24; (G', H', I', J') HuH 7.5 treated with 4.0 mM melatonin for 48 h. Lamellipodia (L); microvilli (Mi); reduced microvilli (Mi*); Cell membrane damage (white filled arrow); cell membrane shrinkage (two white filled arrows); cell junction disruptions (white thin arrow). Scale bars: 10 μm.

membrane by connecting the intermediate filaments of the cytoskeleton with the extracellular matrix. Lamellipodia and filopodia are projections of cytoskeletal actin proteins that play a key role in enhancing motility and metastasis [35,36]. Melatonin reduced lamellipodia and induced a disruption of cell junctions, confirming that the treatment can impair cell migration. Modulation of cell adhesion molecules associated with tight and adherens junctions has already been associated with melatonin [37]. Microvilli are extensions of the surface plasma membrane of cells, which enlarge the area available for the uptake of nutrients, cooperate with cell adhesion and are related to metastasis [38]. In pancreatic cancer tissues, increased basal microvilli are associated with invasiveness and proliferative potential of cells [39]. To the best of our knowledge, this is the first study to demonstrate the capacity of melatonin to alter the morphological architecture of HuH 7.5 cells; the reduction of the microvillus, confirmed through SEM and ETM, may be of relative importance to preventing cell migration.

Cadherins, transmembrane proteins, act as modulators during organ growth, and are associated with tumorigenesis and tumor progression and immunology [40]. N-cadherin is positively regulated, while E-cadherin is negatively regulated during EMT in cancers, and this "cadherin

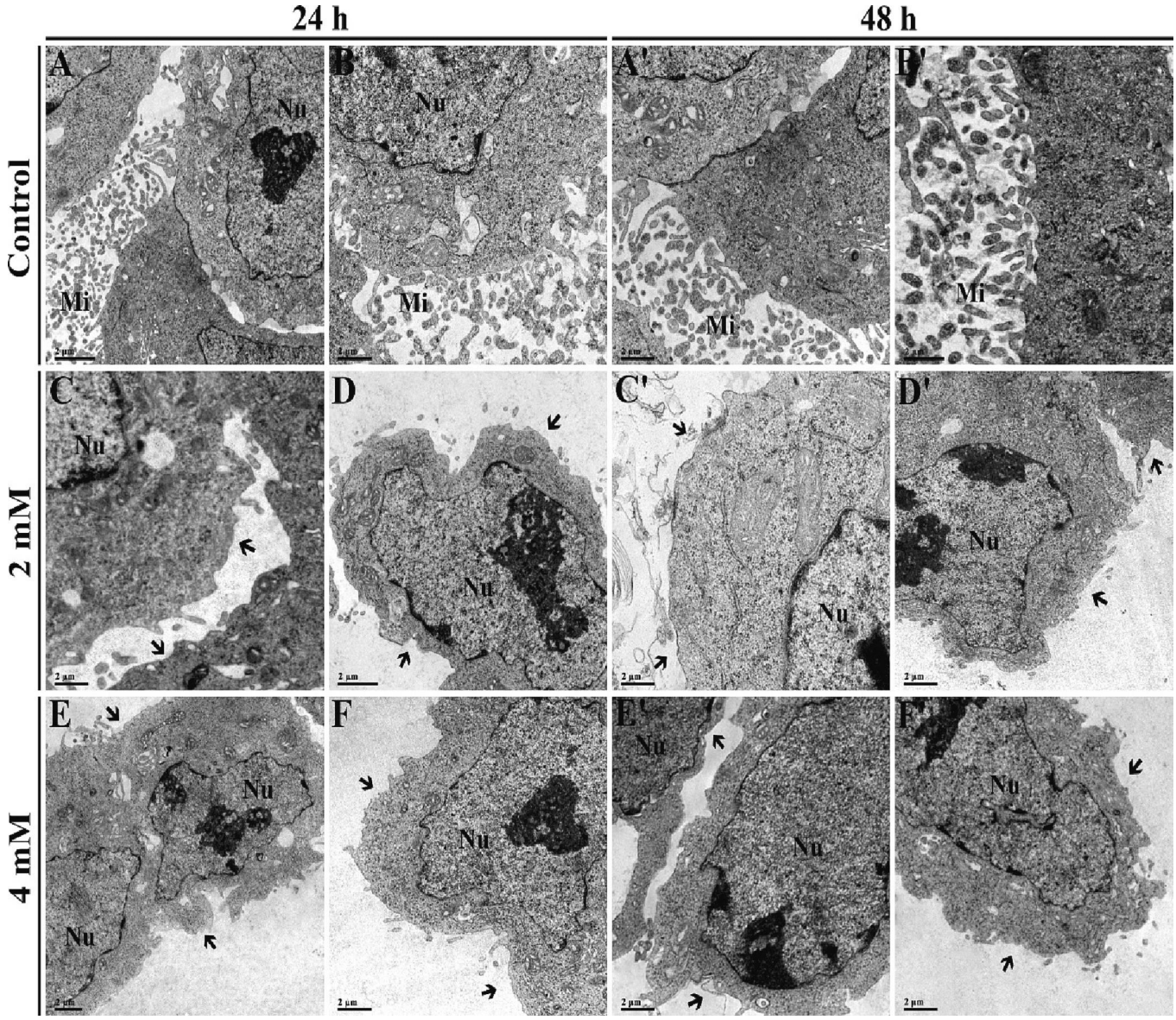

Fig. 5. Structural alterations in HuH 7.5 cells treated with 2.0 and 4.0 mM melatonin for 24 and 48 h. TEM images of (A–B) untreated HuH 7.5 cells (24 h control); (A'–B') untreated HuH 7.5 cells (48 h control); (C, D) HuH7. 5 treated with 2.0 mM melatonin for 24 h; (C', D') HuH 7.5 treated with 2.0 mM melatonin for 48 h; (E, F) HuH 7.5 treated with 4.0 mM melatonin for 24; (E', F') HuH 7.5 treated with 4.0 mM melatonin for 48 h. Microvilli (Mi); nucleus (Nu); microvilli reduction and/or destruction (black arrow). Scale bars: 2 µm.

switch" is associated with migratory and invasive characteristics [41,42]. Melatonin may regulate different pathways and act in a variety of downstream molecules and adaptors. By blocking the ERK signaling pathway in gallbladder cancer cells, melatonin reduced the expression levels of mesenchymal markers, such as N-cadherin [43]. Human bladder cancer cell lines, T24 and UM-UC-3, treated with melatonin, had reduce levels of EMT marker expression snail and vimentin [44]. Other mechanisms that may contribute to anti-migratory and anti-invasive effects of the indolamine include interruption of NF-kB cascade, over-expression of occluding through JNK/MAPK pathway modulation, and reduction of ROS-αvβ3 integrin-FAK/Pyk2 signaling pathways [37]. Detailed and deep review of the mechanistic actions of melatonin can be found in Gurunathan et al. [3] and Mehrzadi et al. [45].

Cadherin isoform switching, with increased expression of N-cadherin, was associated with lower survival rate in patients with extrahepatic cholangiocarcinoma and this phenomenon was attributed to *TGFB1*, which was able to activate cell migration and invasion in an *in vitro* model with HuCCT cells [46]. Transforming growth factor-beta (*TGFB*) is a secreted cytokine that plays crucial roles in many cellular processes, including growth inhibition, extracellular matrix (ECM) remodeling, EMT, cell migration, invasion, and immunosuppression. Consequently, alterations in TGF family member signaling have been implicated in many diseases, including cancer [47,48]. Melatonin was already found to reduce the expression of TGFB1 in an ovarian carcinoma *in vivo*-model [49]. The reduction we found in N-cadherin and *TGFB1* expressions helps explain the antimigratory effects of melatonin; in addition to the loss of junction showed by the electron microscopy, we confirm that the indolamine alters significantly HuH 7.5 structure, and this effect is of great therapeutic interest and deserves a deeper investigation in HCC patients.

The Warburg effect, or aerobic glycolysis, is the phenomenon in which tumor cells convert glucose to lactate even in the presence of oxygen, unlike normal body cells that perform the Krebs cycle and oxidative phosphorylation. In cancer cells, glucose uptake and lactate production are dramatically increased, even in the presence of oxygen and fully functioning mitochondria. The tumor cells use this pathway

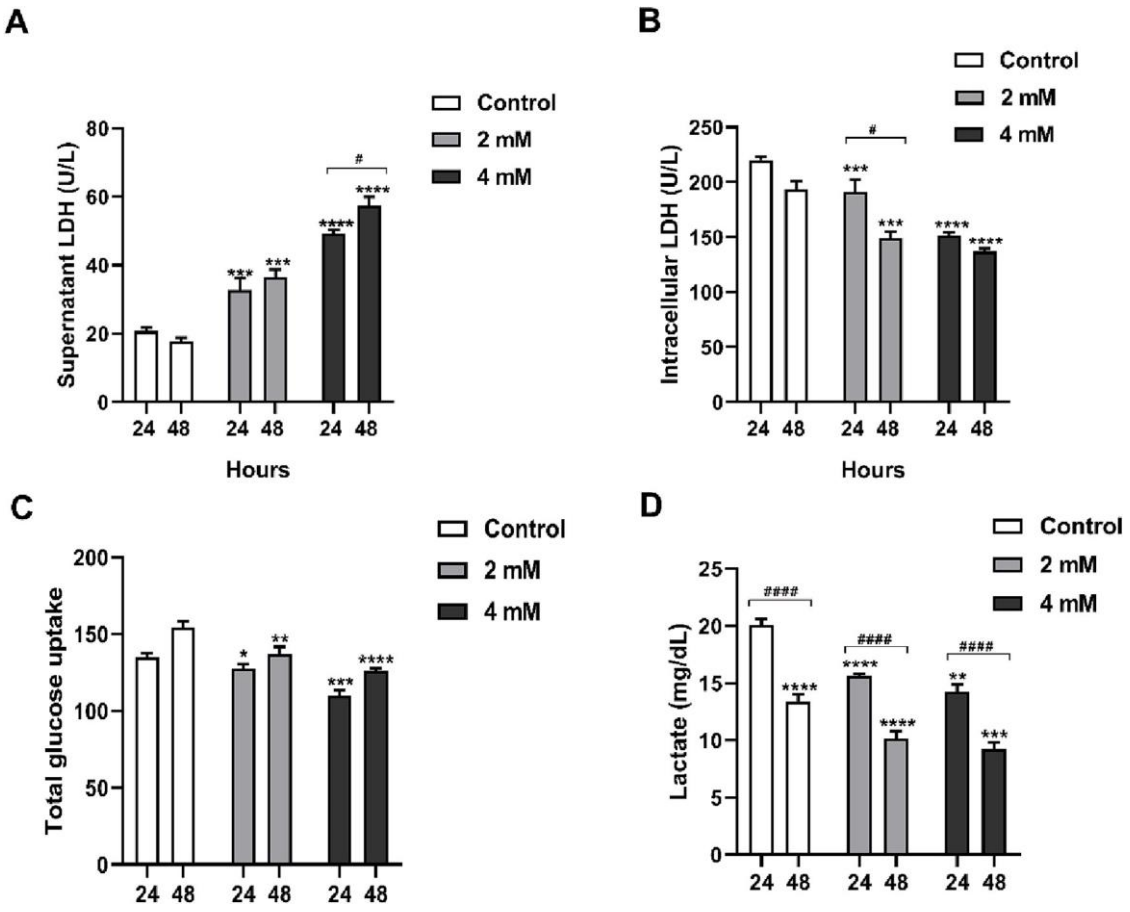

Fig. 6. Melatonin treatment altered the energy metabolism of the HuH 7.5. HuH 7.5 cells were treated with the concentrations of 2 and 4 mM melatonin for 24 and 48 h. The extracellular and intracellular activities of LDH enzyme (A) and (B), respectively was detected by spectrophotometer. In (C) an extracellular glucose assay was performed, and (D) intracellular dosage of mean lactate was performed Data are expressed as the mean ± SEM of 3 independent experiments performed in triplicate. * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$) *vs.* Control 24 and 48 h. $^{\#}$ ($p < 0.05$); $^{\#\#}$ ($p < 0.01$); $^{\#\#\#}$ ($p < 0.001$); $^{\#\#\#\#}$ ($p < 0.0001$) group comparison.

because energy generation occurs up to 100 times faster, sustaining the rapid tumor growth, and allowing metastatic progression and long-term survival [13]. After melatonin treatment, glucose uptake by HuH 7.5 cells was significantly reduced at both concentrations and periods. Human breast cancer cell lines have elevated growth rate when exposed to a high glucose level medium [50]; in this scenario, melatonin may be a promisor agent in the regulation of glucose metabolism in HCC. Novel candidate drugs to interfere in glucose metabolism are designed and currently tested for different types of tumors [51]. Whether the reduction in glucose uptake was caused by the reduction in microvilli projections or the lower capacity to capture glucose caused decreased microvilli projections needs to be further investigated.

Lactate is no longer considered just a waste product of aerobic glycolysis, but a signaling molecule and even an energy source for aerobic cancer cells through the "lactate shuttle" [52]. Lactate is produced by pyruvate reduction and can be oxidized back by cytoplasmatic lactate dehydrogenase (LDH). The higher extracellular LDH activity gives us an indication of cell injury caused by melatonin that may coincide with the cell membrane damage seen in scanning electron microscopy. The reduction in intracellular LDH activity caused by melatonin treatment confirms our hypothesis that melatonin modulates energy metabolism by decreasing the use of aerobic glycolysis, reverting to the oxidative phosphorylation pathway [2]. High levels of LDH help cancer cells proliferate by promoting the epithelial to mesenchymal transition, angiogenesis, increasing cell motility, invasion, and migration [53–55]. Whether the lower intracellular activity of LDH is a response to the lower glucose intake remains to be exploited. In any case, the ability of melatonin to modulate glucose and lactate metabolism, reverting the Warburg effect, is an important hallmark of the treatment with the indolamine in the HCC cell line. Furthermore, these are relevant findings as increased aerobic glycolysis favors tumor cell proliferation, chemoresistance, and metastasis.

5. Conclusion

In this study, we showed that *in vitro* treatment of HuH 7.5 cells with melatonin had an antiproliferative effect, decreased cell motility resulting in reduced cell viability and migration. These cytotoxic effects were accompanied by morphological changes, reduced biomarkers of EMT, and reversal of the Warburg-type metabolism.

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CRediT authorship contribution statement

E.M.S.C., V.M.C., J.M.B.M., M.C.S.: Methodology, Formal analysis, Investigation. T.F·S, F·S.R.I., E.S: Validation. D.L.B., R.R.B.M., M.S.M., C.P.: Methodology, Investigation, Resources. W.R.P., L.G.A.C.: Conceptualization, Resources, Writing - review & editing. F.R.F.S: Conceptualization, Writing - original draft, Supervision, Project administration.

All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

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