UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de São José do Rio Preto

Janesly Prates

Interação funcional e molecular da proteína anexina A1 e do quimioterápico cisplatina no carcinoma de colo de útero

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Financiadoras: CNPq Proc.140883/2014-2
CAPES Proc.99999.000615/2016-01

Orientadora: Profª. Drª. Sonia Maria Oliani

Coorientadora: Profª. Drª. Flávia C. Rodrigues Lisoni

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Prates, Janesly.
Interação funcional e molecular da proteína anexina A1 e do quimioterápico cisplatina no carcinoma de colo de útero / Janesly Prates. -- São José do Rio Preto, 2018
121f. : il.

Orientador: Sonia Maria Oliani
Coorientador: Flávia C. Rodrigues Lisoni
Tese (doutorado) – Universidade Estadual Paulista “Júlio de Mesquita Filho”, Instituto de Biociências, Letras e Ciências Exatas

1. Apoptose. 2. Colo uterino – Câncer. 3. Anexina A1. I. Universidade Estadual Paulista "Júlio de Mesquita Filho". Instituto de Biociências, Letras e Ciências Exatas. II. Título.

CDU – 616-006.6

Ficha catalográfica elaborada pela Biblioteca do IBILCE
UNESP - Câmpus de São José do Rio Preto

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05 de junho de 2018

DEDICATÒRIA

Dedico este trabalho aos pilares da minha vida, meus pais, Joacyr e Alaide, meus verdadeiros mestres que dignamente me apresentaram à importância da família, ao caminho da honestidade e são colaboradores das minhas conquistas. Meus mais profundos agradecimentos pelas sábias lições, apoio e amor sincero.

À Profa. Dra. Sonia Maria Oliani, meu agradecimento pela oportunidade, orientação e confiança à mim outorgada, permitindo meu crescimento profissional e pessoal.

À minha coorientadora, Profa. Dra. Flávia C. Rodrigues Lisoni, pela amizade, carinho, humildade e pelas sugestões e interação com este trabalho que me trouxe mais experiência e amadurecimento.

AGRADECIMENTOS

Primeiramente e acima de tudo agradeço a DEUS pela fé que me mantem viva e fiel à vida honesta de trabalho e estudo, por me iluminar e me dar forças nos momentos difíceis. Agradeço por mais essa vitória!

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pelo financiamento da bolsa de Doutorado (processo 140883/2014-2).

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela bolsa de Doutorado Sanduíche (CAPES/MINCyT, projeto n°259/14, processo 99999.000615/2016-01), permitindo minha estadia em Córdoba/Argentina, a qual tem sido de grande importância.

Ao Programa e professores da Pós-graduação em Biociências do Instituto de Biociências, Letras e Ciências Exatas (IBILCE/UNESP), por possibilitar a realização desse trabalho.

Ao Departamento de Biologia do Instituto de Biociências, Letras e Ciências Exatas de São José do Rio Preto, e aos funcionários da Seção de Pós-graduação pela atenção e colaboração.

À Profa. Dra. Solange Correa Garcia Pires D'Avila, do Departamento de Análises Anatomopatológicas do Hospital de Base de São José do Rio Preto, por ter cedido e avaliados as amostras teciduais de colo de útero.

À Profa. Dra. Sandra Helena Poliselli Farsky e a aluna Silvana Sandri (USP-SP), pela disponibilidade e auxilio na técnica de ciclo celular.

À Profa. Dra. Silvia Graciela Correa, do *Centro de Investigaciones en Bioquímica Clínica e Inmunología* (CIBICI-CONICET-UNC), *Universidad Nacional de Córdoba*, Córdoba, Argentina, pela oportunidade de desenvolver o doutorado-sanduíche, pelas suas ideias, acolhimento e amizade.

Às Profas. Dra. Ana Paula Girol e Dra. Cristiane Damas Gil pelas contribuições, dedicação e amizade.

Aos queridos companheiros do Laboratório de Imunomorfologia do IBILCE, Mab Corrêa, Rafaela Molás, Caroline de Freitas Zanon, Lucas Ribeiro de Azevedo, Thaís Bravo, Juliana P. Gusson e Julliene Guaraldi, uma equipe a qual é exemplo de trabalho com alegria e entusiasmo. Em especial à Jusciéle Brogin Morelli e ao Alexandre Dantas Gimenes pelas preciosas dicas, apoio, discussões, técnicas e aprendizado.

Aos amigos da *Universidad Nacional de Córdoba*, em especial à Lisa Macció Maretto, Bibiana Barrios e Coni Savid Frontera pelas informações, estadia e acolhimento.

Aos meus familiares e demais amigos, pelo apoio e incentivo, que de uma ou de outra maneira deram crédito ao meu trabalho.

A todos, o meu sincero agradecimento.

“O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis.”

José de Alencar

RESUMO

O câncer de colo de útero apresenta altas taxas de incidência e mortalidade, e sua progressão depende de características genéticas e interações das células tumorais, epiteliais e inflamatórias. A maioria dos protocolos de tratamentos, neste tipo de câncer, utiliza a Cisplatina (Cis). No entanto, este quimioterápico induz efeitos colaterais, como a nefrotoxicidade, por isso alternativas terapêuticas tem sido estudadas, como a proteína anexina A1 (ANXA1), que apresenta ações anti-inflamatória, antiproliferativas e também modula a expressão do gene Inibidor de Diferenciação ao DNA 1 (*ID1*) no processo tumoral. Com essas considerações, o objetivo do trabalho foi investigar a interação molecular dos mediadores ANXA1 e ID1 com a Cis em linhagem celular, e relacionar com tecidos neoplásicos intraepiteliais no processo tumorigênico cervical. As células SiHa (carcinoma epidermóide de cérvice), tratadas com o peptídeo da anexina A1 (AC_{2-26}), Cis e AC_{2-26}+Cis, foram avaliadas nos ensaios de proliferação, citotoxicidade, migração celular, apoptose, imunolocalização e expressões dos genes *ANXA1* e *ID1,* e das proteínas ANXA1, ID1 e ERK1/2 (Quinase Regulada Extracelularmente 1/2). As amostras de tecidos cervicais foram analisadas histopatologicamente e as expressões proteicas de ANXA1 e ID1, por imuno-histoquímica. Os tratamentos, em todos os grupos, diminuíram a proliferação e migração celular, sem efeitos citotóxicos. Enquanto, Cis e AC_{2-26}+Cis induziram as células aos processos de apoptose tardia, diminuição da expressão do ID1 e aumento da ANXA1, indicando a atividade antineoplásica da cisplatina. A administração Cis e AC_{2-26}+Cis inativou ERK1/2 fosforilada e induziu a translocação nuclear da ANXA1. As expressões das proteínas ID1 e ANXA1 foram observadas nas amostras teciduais de Neoplasias Intraepiteliais Cervicais (NIC), com intensa imunomarcação de ID1 em NIC III. Em conjunto, os dados obtidos nesse trabalho fornecem entendimento adicional do mecanismo de ação da cisplatina na modulação dos mediadores *ID1* e *ANXA1* no processo tumorigênico. Esses

achados podem delinear novas estratégias para a intervenção terapêutica no câncer de colo de útero.

Palavras chave: linhagem SiHa, câncer de cérvice uterina, apoptose, ID1, translocação nuclear

ABSTRACT

Cervical cancer presents high rates of incidence and mortality, and its progression depends on genetic characteristics and tumor interactions, epithelial and inflammatory cells. Most treatment protocols, in this type of cancer, use Cisplatin (Cis). However, this chemotherapeutic induces side effects such as nephrotoxicity, so alternative therapies have been studied, such as the Annexin A1 protein (ANXA1), which exhibits anti-inflammatory, antiproliferative actions and also modulates expression of the Inhibitor of Differentiation to DNA 1 *(ID1) gene in the tumor process. With these considerations, the objective of this work was to investigate the molecular interaction of the ANXA1 and ID1 mediators with Cis in cell line, and to relate with intraepithelial neoplastic tissues in the cervical tumorigenic process. SiHa (cervical epidermoid carcinoma) cells treated with the annexin A1 (AC_{2-26}) peptide, Cis and AC_{2-26}+ Cis, were evaluated in the proliferation, cytotoxicity, cell migration, apoptosis, immunolocalization and gene expression assays ANXA1 and ID1, and of the ANXA1, ID1 and ERK1/2 (Extracellular signal-regulated kinase) proteins. Cervical tissue samples were analyzed histopathologically and the protein expression of ANXA1 and ID1 by immunohistochemistry. Treatments, in all groups, decreased cell proliferation and migration, without cytotoxic effects. While, Cis and AC_{2-26}+ Cis induced the cells to processes of late apoptosis, decreased expression of ID1 and increased ANXA1, indicating the antineoplastic activity of cisplatin. Cis and AC_{2-26}+ Cis administration inactivated ERK1/2 phosphorylated and induced nuclear translocation of ANXA1. Expressions of the ID1 and ANXA1 proteins were observed in tissue samples from Cervical Intraepithelial Neoplasia (CIN), with intense ID1 immunolabeling in CIN III. Together, the data obtained in this work provide additional understanding of the mechanism of action of cisplatin in the modulation of mediators ID1 and ANXA1 in the tumorigenic process. These*

findings may outline new strategies for therapeutic intervention in cervical cancer.

Key words: SiHa line, cancer of the uterine cervix, apoptosis, ID1, nuclear translocation

LISTA DE FIGURAS

Manuscrito

LISTA DE ABREVIATURAS E SIGLAS

%: porcentagem

>: maior

<: menor

≤: menor ou igual

°C: graus célsius

cm2: centímetros quadrados

mg: miligrama

µg: microgramas

mM: milimolar

µM: micromolar

min: minutos

mL: mililitro

µL: microlitros

ng: nanograma

nm: nanômetro

rpm: rotação por minuto

ACTB: β-actina

ALXR/FPR2: Receptor peptídeo formil 2

ANXA1: Anexina A1; Proteína Anexina A1

AC_{2-26} ou Ac2-26 : peptídio da porção N-terminal da proteína ANXA1

BSA: Albumina Sérica Bovina

Br: Brasil

bFGF: Fator Básico de Crescimento Fibroblasto

Ca^{2+}: íon cálcio

Cis: Cisplatina

cDNA: DNA complementar

CO_2: dióxido de carbono

Ct: Cycle threshold

DNA: Ácido desoxirribonucleico

DEPC: água ultrapura, tratada com Dietil Pirocarbonato

EDTA: ácido etilenodiamino tetra-acético

ERα: Receptor de Estrógeno Alfa

ERK1/2: Quinase Regulada Extracelularmente 1/2 (Extracellular signal-regulated kinase)

(p) ERK1/2: Quinase Regulada Extracelularmente 1/2 fosforilada

GAPDH: Gliceraldeído-3-fosfato desidrogenase

HLH: Domínio Hélice-Alça-Hélice

HPV: Papilomavirus Humano

IBILCE: Instituto de Biociências, Letras e Ciências Exatas

ID1: Ibinidor de ligante de DNA-1, proteína dominante negativo helix-loop-helix

IL-1β:Interleucina 1 Beta

INCA: Instituto Nacional do Câncer

JEC: Junção Escamo Colunar

kDa: kilodalton

LDHA: lactato desidrogenase A

LOTE: tampão de diluição Tris-EDTA

Log2: escala logarítmica de base 2

mA: miliampère

Meio MEM (Earle): mistura de sais enriquecida com aninoácidos, vitaminas e compostos essenciais para crescimento celular

MAPK: Proteíno-Quinases Ativadas por Mitógenos (Mitogen Activated Protein Kinases)

Na_3VO_4: Ortovanadato de sódio

NF-κB: Fator de crescimento nuclear kappa B

NIC: Neoplasia Intraepitelial Cervical

pb: Pares de base

PBS: Tampão Fosfato Salino

PBEA: PBS + Albumina

PCR: Reação em cadeia da polimerase

pH: Potencial hidrogeniônico

PLA2: Fosfolipase A2

p65: Fator Nuclear NF-kappa-B subunidade p65

RIPA: Tampão de lise

RNA: Ácido Ribonucleico

RNAi: RNA de interferência

RT: transcriptase reversa

SDS: Dodecil Sulfato de Sódio

SFB: Soro Fetal Bovino

SiHa: linhagem celular derivada de células de carcinoma epidermóide de cérvice

SiHa controle (sem tratamento)

TBS: Tris-buffered Saline

TBST: Tris-buffered Saline + Tween 20

TLRs: receptores toll-like

TUBA6: Tubulina Alfa 6

Tween 20: Poliexietileno Sorbitano Monolaurato

U: unidades

UK: Reino Unido

UNESP: Universidade Estadual Paulista

UV: luz ultravioleta

V: volts

VEGF: Fator de crescimento vascular endotelial

Δ: delta

Sumário

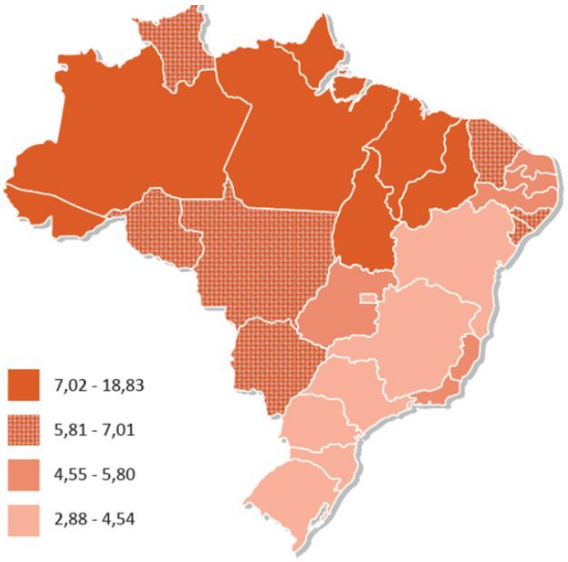
1. INTRODUÇÃO

O câncer de colo de útero ou cérvice é o quarto mais comum em mulheres e o sétimo mundialmente mais frequente (SMALL et al., 2017). Apresentando altas taxas de incidência e mortalidade, é um importante problema de saúde pública. A cada ano surgem cerca de 530 mil novos casos, sendo a quarta causa de morte em países em desenvolvimento, com aproximadamente 275 mil mortes por ano (FERLAY et al., 2015; TORRE et al., 2017).

No Brasil, o câncer de colo de útero ocupa a terceira posição, com a maior incidência na Região Norte (Figura 1). Dados do Instituto Nacional do Câncer (INCA) mostraram 16.370 novos casos, para o biênio 2018-2019, com um risco estimado de 15,43 a cada 100 mil mulheres. Aproximadamente 35% das mulheres diagnosticadas com carcinoma de colo uterino têm a doença recorrente, com 90% desses achados nos três anos após o tratamento inicial (PECTASIDES et al., 2008). Assim, terapias alvo e estratégias de quimioterapias e radioterapias são essenciais para a redução da mortalidade por essa malignidade.

Figura 1 - Distribuição dos 10 tipos de câncer mais incidentes em mulheres estimados para 2018 e representação espacial das taxas ajustadas de incidência de câncer de colo de útero por 100 mil mulheres, no Brasil

	Localização Primária	Casos	%
Mulheres	Mama Feminina	59.700	29,5%
	Cólon e Reto	18.980	9,4%
	Colo do Útero	16.370	8,1%
	Traqueia, Brônquio e Pulmão	12.530	6,2%
	Glândula 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 2018 (modificado).

No câncer de cérvice o agente etiológico bem estabelecido é o Papiloma Vírus Humano (HPV) o qual pode produzir lesões papilares na ectocérvice. Os subtipos HPV16 e 18, que apresentam potencial oncogênico, são comumente identificados no câncer de colo de útero, e responsáveis mundialmente por 70-80% dos casos (LI et al., 2011; DAS et al.,2015). Estudo mostrou que após a conclusão de tratamentos com radio e quimioterapia, em câncer avançado, foi observada a persistência do HPV, correlacionando esse vírus com recidivas precoces (MAHANTSHETTY et al., 2018). Múltiplos parceiros sexuais contribuem para o risco de infecção pelo HPV, além do alto número de partos normais, contraceptivo oral e tabaco que são fatores de risco para o desenvolvimento desse tipo de câncer (SOTO-DE-LEON et al., 2011; CROSBIE et al., 2013; ROURA et al., 2016).

A detecção precoce desta malignidade, por meio de testes de triagem colpocitológica (Teste de Papanicolaou), monitoramento e tratamento de lesões precursoras auxiliaram para a redução de incidência e mortalidade em países desenvolvidos (GUIDO, 2017). O desenvolvimento e a introdução de vacinas em adolescentes também contribuíram efetivamente para prevenção de infecção pelo Papiloma Vírus Humano (HPV) (WALKER et al., 2017).

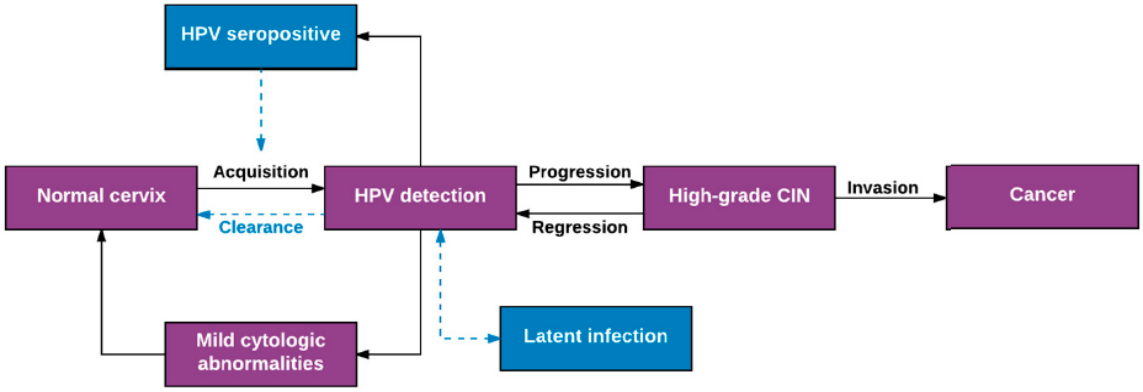
O HPV apresenta tipos de alto e baixo risco desses, 14 são considerados de alto risco (16, 18, 33, 45, 31, 58, 52, 35, 59, 56, 51, 68, 39, 66), e estudos verificaram que infecções pelo HPV16, 18 ou 45 são mais propensos a evoluírem para carcinoma escamoso celular de cérvice uterina do que os outros tipos (CLIFFORD et al., 2003; ROBADI; PHARAON; DUCATMAN, 2018).

A infecção pelo HPV pode sofrer regressão naturalmente, porém a persistência de HPV de alto risco pode levar ao desenvolvimento de Neoplasia Intraepitelial Cervical (NIC) e câncer de colo uterino (Figura 2) (GRAVITT; WINER, 2017).

O câncer cervical é precedido por alterações na zona de transformação e na Junção Escamo Colunar (JEC) denominadas Neoplasia Intraepitelial Cervical (NIC) com três graus de severidade e diretamente relacionadas à

proporção de células atípicas substituindo o epitélio cervical. NIC I é considerado uma displasia, com células atípicas restritas ao terço inferior do epitélio. NIC II apresenta displasia moderada, com células atípicas e inúmeras figuras de mitose ocupando a metade inferior do epitélio. A displasia grave e carcinoma in situ (NIC III) mostra a presença de células atípicas em todo o epitélio espessado, com um mínimo de diferenciação e maturação na superfície. Nesse estágio pode haver amplificação da alteração ao longo dos canais das criptas endocervicais e focos de microinvasões, além de elementos mitóticos e mitoses anormais presentes em todas as camadas. NIC I representa baixo risco de evolução para carcinoma, enquanto NIC II e NIC III estão associados com alto risco de desenvolvimento, sendo um importante precursor da malignidade invasiva (BASU et al., 2018).

Figura 2 - Modelo esquemático da história natural da infecção pelo HPV e câncer cervical.



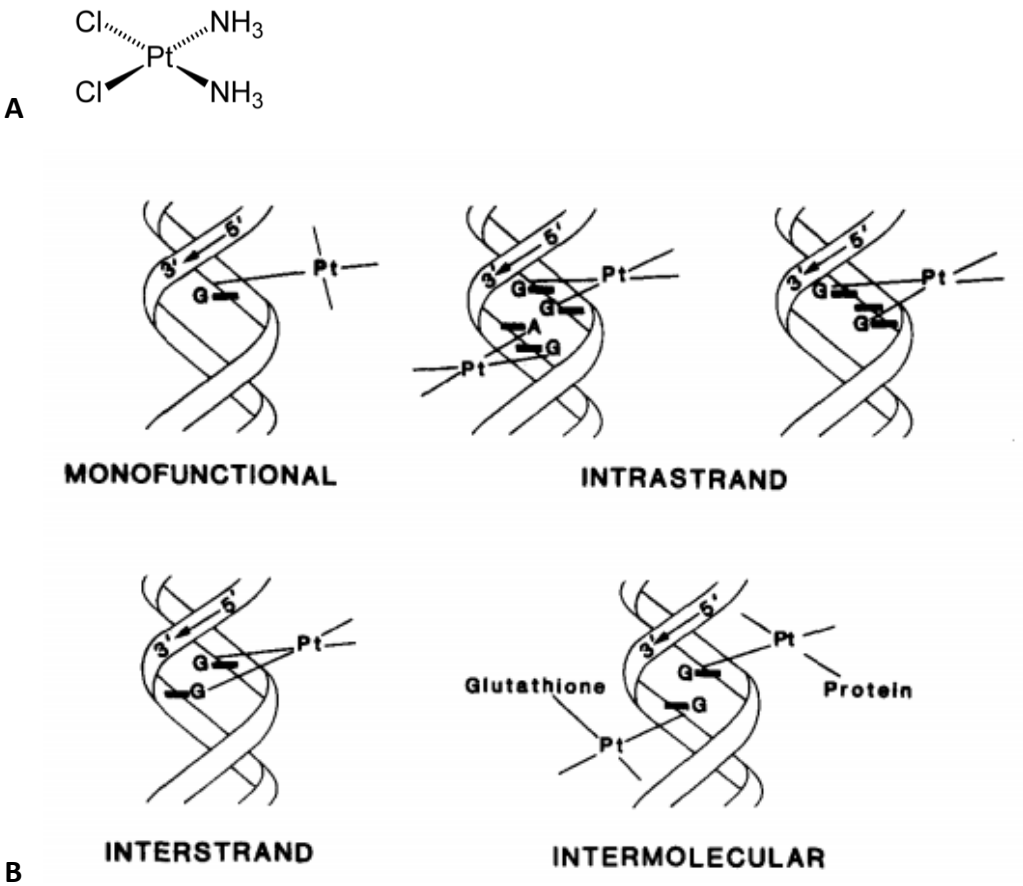
Fonte: GRAVITT; WINER, 2017. Em roxo, parâmetros que representam o modelo da história natural e em azul, as incertezas.

Atualmente, o tratamento para lesões pré-malignas inclui técnicas de crioterapia, termo coagulação, procedimentos cirúrgicos, como conização e histerectomia, e o câncer de colo uterino é tratado com cirurgia seguido de quimioterapia e radioterapia, ou ambos (BASU et al., 2018). O quimioterápico

Cisplatina (cis-diaminodicloroplatina II ou cis-DDP) foi aprovado pela FDA (American Food and Drug Administration) em 1979, e é amplamente utilizado como recurso terapêutico no câncer (SHERMAN; SUZANNE E.; LIPPARD, 1987).

Quimicamente, a cisplatina é composta por um átomo central de platina circundado por átomos de amônia e cloro em posição cis (Figura 3A), e se liga ao DNA na base purina na posição N7 para formar ligações inter e intra-fita, podendo formar ainda aduto monofuncional ou se ligar a uma proteína (Figura 3B) (SHERMAN; SUZANNE E.;LIPPARD, 1987; JAMIESON; LIPPARD, 1999).

Figura 3 - Estrura da cisplatina e adutos de DNA.



Fonte: EASTMAN, 1987. [A] Representação química da estrutura da cisplatina. [B] Estruturas de adutos no DNA formadas pela cisplatina.

A ligação mais prevalente intra-fita ocorre em GG (65%), GA (25-30%) e GNG (1-3%) (EASTMAN, 1987; CHANEY et al., 2005). A atividade antineoplásica da cisplatina tem como alvo principal o DNA celular, onde induz a formação de adutos que posteriormente interferem na transcrição e replicação desencadeando o processo de apoptose (SUO;LIPPARD;JOHNSON, 1999; LI et al., 2014; LI et al., 2015). No entanto, essa droga pode interagir com diferentes componentes celulares como fosfolipídeos e RNA (WANG; LU; LI, 1996; HOSTETTER; OSBORN; DEROSE, 2012). Os efeitos celulares que envolvem esse quimioterápico revela sua ação sobre diversas vias de sinalizações (p53 e folato) e processos epigenéticos (modificações de histonas) (SIDDIK, 2003; LICONA et al., 2017).

Estudos apontam resultados importantes no tratamento do carcinoma de cérvice como a associação da cisplatina a outras drogas, tais como a Gencitabina (HASHEMI et al., 2013), Paclitaxel (CHENG et al., 2014) ou agentes hormonais (SEGOVIA-MENDOZA et al., 2015). No estágio inicial do tumor de cérvice foi confirmada a eficácia da cisplatina em controlar ou retardar o crescimento tumoral (SUPRASERT et al., 2007). No entanto, esse quimioterápico pode induzir gastrotoxicidade, mielossupressão, neuropatia, reações alérgicas e, principalmente, nefrotoxicidade (OH et al., 2014; YANAGIMOTO et al., 2016).

Várias alterações genéticas e epigenéticas ocorrem durante o processo de tumorigênese do colo de útero (STEENBERGEN et al., 2014; LI et al., 2017b). Entre essas, destacamos a metilação da região promotora de genes supressores de tumor que favorecem o seu silenciamento e resultam na carcinogênese de cérvice (ZHANG et al., 2011, 2012). Além disso, os oncogenes E6 e E7 codificam proteínas multifuncionais que inativam os supressores tumorais, p53 e pRb, respectivamente (NARISAWA-SAITO; KIYONO, 2007). Essa instabilidade no genoma desencadeia o bloqueio do ciclo celular e desregulação dos checkpoints devido a perda dos supressores tumorais, ocasionando o processo tumorigênico (INCASSATI et al., 2006). Proteínas E7 do HPV de alto risco (16, 18 e 31) tem maior afinidade pela

proteína Rb, inativam CDK1 p21 e p27 e se ligam ativando as ciclinas E e A. Enquanto as proteínas E6 de HPV de alto risco tem afinidade maior pela proteína p53. Essa interação E6-p53 direciona os polimorfismos e os fatores de risco para o desenvolvimento de câncer de cólo de útero, uma vez que, o p53 é polimórfico na posição 72 e a variante Arg72 é bem mais susceptível à degradação pelo E6 (ROBBINS e COTRAN, 2010).

O colo uterino é altamente sensível ao hormônio estrógeno e, durante o ciclo menstrual, as células epiteliais proliferam e se diferenciam à medida que os níveis desse hormônio aumentam, resultando em epitélio hiperplásico sem mudanças patológicas. No entanto, uma contínua estimulação hormonal pode levar à neoplasia, pois há evidências de que o Receptor de Estrógeno Alfa (ERα) é necessário para o desenvolvimento e manutenção tumoral em associação à infecção pelo HPV de alto risco (CHUNG et al., 2013; RAMACHANDRAN, 2017).

A carcinogênese de colo de útero também está relacionada com processos de angiogênese e inflamatórios. A angiogênese ocorre em tumores que secretam mediadores tais como fator de crescimento endotelial vascular (VEGF-A) e fator básico de crescimento fibroblasto (bFGF) (SALGADO et al., 2004). A via do VEGF regula a angiogênese e, portanto, é alvo de terapias dirigidas, apresentando resultados satisfatórios com efeitos anti-angiogênicos (WANG et al., 2015). A presença de infiltrado inflamatório é de suma importância no processo de neovascularização. Uma vez que os tumores recrutam células hematopoiéticas, em especial os monócitos, que compõem a massa tumoral e parecem estar diretamente envolvidos na progressão e metástase (CONDEELIS; POLLARD, 2006).

O influxo de células inflamatórias é uma característica do microambiente tumoral, sendo esse aspecto muito discutido na progressão neoplásica (ZIJLMANS et al., 2009). Componentes celulares e humorais apresentam impacto na iniciação e progressão tumoral, evidenciado pela inflamação envolvida na tumorigênese e frequentes de áreas de inflamação crônica, como observado na associação da pancreatite crônica e colite ulcerativa e o surgimento de tumor no pâncreas e intestino, respectivamente (CLEVERS,

2004; FARROW et al., 2004; GALDIERO; MARONE; MANTOVANI, 2017). A infiltração de células do sistema imunológico e mediadores liberados também constituem as principais razões para mudanças pró-inflamatórias no microambiente do colo uterino (BERTI et al., 2017) e próstata (THAPA; GHOSH, 2015), contribuindo para o desenvolvimento do câncer.

No início do processo neoplásico, as células inflamatórias e os fatores produzidos regulam o crescimento, a migração e a diferenciação de todos os tipos celulares no microambiente tumoral, incluindo as células neoplásicas, endoteliais e fibroblastos. Posteriormente, as células neoplásicas utilizam alguns mecanismos inflamatórios para invasão de outros tecidos, como adesão celular ao endotélio vascular, migração e diapedese. Perretti (1997) já havia destacado a semelhança da metastatização com esses estágios do processo inflamatório.

As células inflamatórias secretam numerosas citocinas, fatores de crescimento e quimiocinas, que estimulam a proliferação, inibem a apoptose, induzem a morfogênese e geram espécies reativas de oxigênio capazes de lesar o DNA (COUSSENS; WERB, 2002). Em consequência, promovem um ambiente propício para o processo neoplásico, facilitando a instabilidade genômica (RONNOV-JESSEN; PETERSEN; BISSELL, 1996; TLSTY; HEIN, 2001; GASHI et al., 2018).

A prevenção de danos teciduais e manutenção da homeostasia é controlada por mediadores anti-inflamatórios, dentre estes destacamos a Anexina A1 (ANXA1), uma proteína de 37 kDa, que age como modulador da inflamação (CHOUTHURY; FLOWER, 1988; SILISTINO-SOUZA et al., 2007; COSTA et al., 2018). Dessa forma, o estudo do envolvimento dessa proteína no carcinoma de cérvice pode ser útil na caracterização do desenvolvimento tumoral.

A ANXA1 (Figura 4) foi o primeiro membro caracterizado de uma superfamília de proteínas que se ligam aos fosfolipídios de forma dependente de cálcio (RESCHER; GERKE, 2004). Essa proteína foi inicialmente denominada lipocortina pelo fato de mimetizar a ação anti-inflamatória dos

glicocorticóides, por meio da inibição da síntese de eicosanóides e fosfolipase A2 (PLA2), afetando dessa forma componentes da reação inflamatória e liberação do ácido araquidônico (FLOWER, 1988; PERRETTI; GAVINS, 2003).

Figura 4 - Estrutura da proteína Anexina A1 (ANXA1).

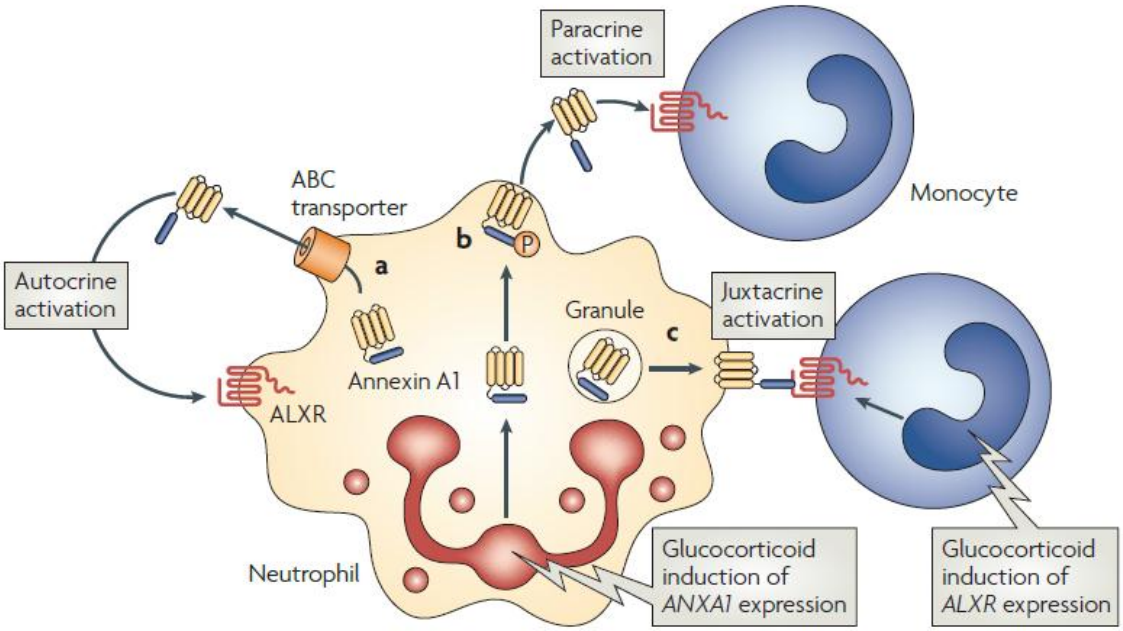
Fonte: RESCHER; GERKE, 2004. [A] Representação esquemática da estrutura primária, com destaque da região ativa N-terminal. [B] lustração do arranjo tridimensional dessa proteína.

A ANXA1 é capaz de se ligar a fosfolipídeos carregados negativamente de maneira dependente de cálcio (Ca2+) e apresenta uma estrutura conservada de cerca de 70 aminoácidos repetidos. Esta ligação é reversível e a remoção de Ca2+, por agentes alquilantes, conduz a liberação de anexinas a partir da matriz de fosfolipídeos (GERKE; MOSS, 2002). Estruturalmente, as anexinas compreendem dois domínios: uma pequena região N-terminal, variando em comprimento e composição, e um domínio central, região C-terminal, formado por quatro a oito dobras repetidas de uma sequência conservada de 70 a 80 aminoácidos (RESCHER; GERKE, 2004). O domínio N-terminal é específico para cada membro dessa família e interage com diferentes ligantes, enquanto a região C-terminal é responsável pela afinidade ao cálcio, e consequente ligação aos fosfolipídios.

ANXA1 apresenta ações anti e pró-inflamatórias e está envolvida em diversas vias de sinalização, sendo liberada endogenamente de maneira autócrina e parácrina (Figura 5) (PERRETI; D'AQUISTO, 2009). As propriedades dessa proteína foram avaliadas em modelos experimentais de

inflamação aguda, crônica e sistêmica (DAMAZO et al., 2006; GIL et al., 2006; GASTARDELO et al., 2009; GIROL et al., 2013; MOLÁS et al., 2017).

Figura 5 - Mobilização da ANXA1 em células ativadas e seu potencial modo de ação.



Fonte: PERRETI; D'AQUISTO, 2009. Após a ativação celular, através da adesão a monocamadas de células endoteliais, a anexina A1 intracelular é mobilizada para a membrana plasmática. Dependendo do tipo de célula, a anexina A1 é então externalizada e/ou secretada através de um dos três mecanismos: (a) através da ativação do transportador (ABC) de ligação ao ATP; (b) através da fosforilação da resíduos da serina amino-terminal 27 seguido pela localização da membrana em domínios lipídicos específicos antes de se deslocar para o folheto externo da membrana plasmática e ser secretada; (c) ou através da fusão de granulos contendo anexina A1 com a membrana plasmática, seguida da libertação de anexina A1. Na presença de $Ca^{2+} \geq 1$ mM, a anexina A1 extracelular sofre mudança conformacional que leva à exposição da região N-terminal e liga ao seu receptor ALXR (conhecido como FPR2). A anexina A1 pode funcionar de maneira autócrina, parácrina e justacrina (envolvendo o contato célula-célula) para ativar a sinalização ALXR. A via da anexina A1 – ALXR pode ser manipulada por glicocorticoides, que induzem a expressão do gene que codifica anexina A1 (ANXA1) e ALXR por células imunes inatas, aumentando os efeitos desse circuito antiinflamatório.

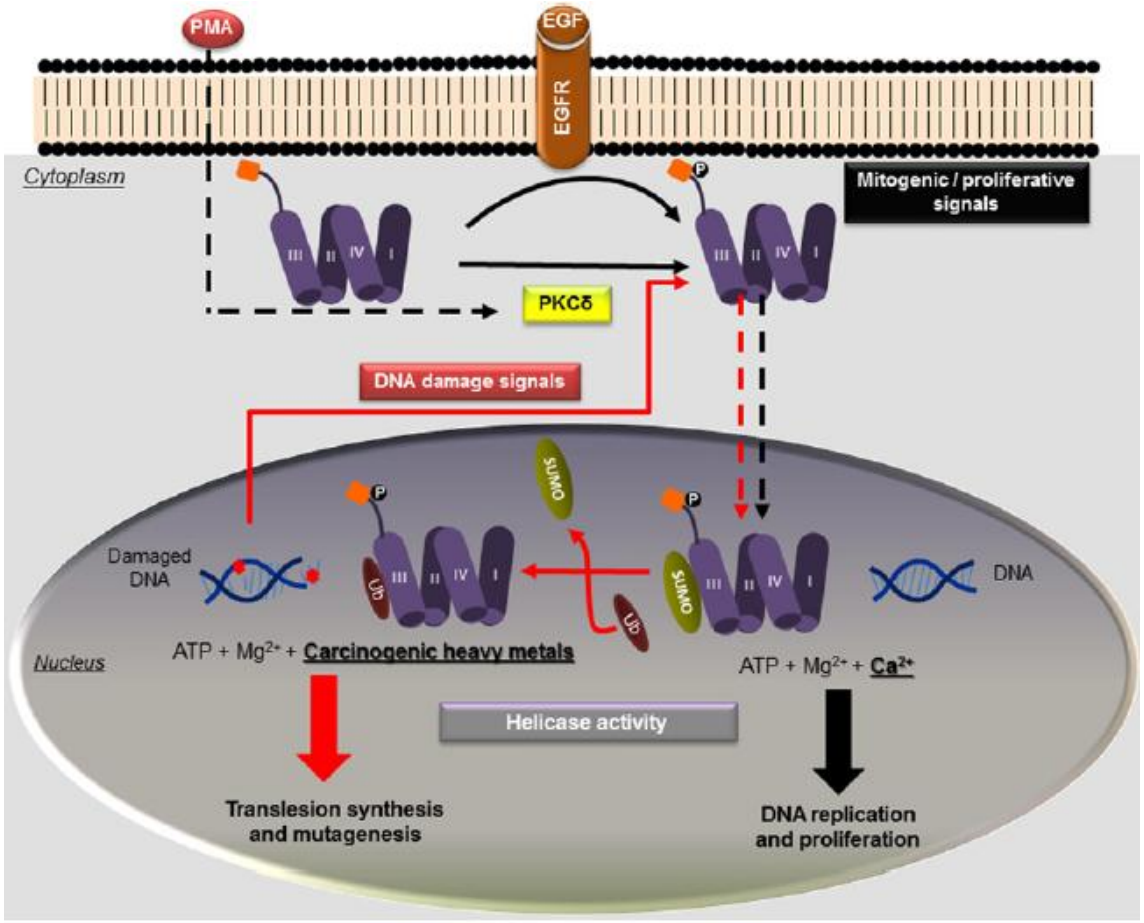
Na artrite inflamatória, estudo com microvesículas de neutrófilos mostrou que a ANXA1, direcionada aos condrócitos pelo receptor FPR2/ALX, eleva a deposição da matriz extracelular e diminui a degradação da cartilagem (HEADLAND et al., 2015). Por outro lado, na doença pulmonar obstrutiva crônica, a superexpressão da ANXA1 foi associada à obstrução do fluxo aéreo, por afetar a função dos fibroblastos pulmonares (LAI et al., 2018).

Além dos efeitos anti-inflamatórios, a ANXA1 tem papel na proliferação, diferenciação celular e apoptose (RESCHER; GERKE, 2004; LIM; PERVAIZ, 2007; CARDIN, 2017), indicando sua relação com a tumorigênese. No entanto, esse papel ainda não está totalmente esclarecido, pois sua expressão ocorre de maneira diferente, dependendo do tipo do tumor. A desregulação da ANXA1 está associada com a progressão e o desenvolvimento tumoral em vários tipos de câncer (GUO; LIU; SUN, 2013; JORGE et al., 2013), exercendo efeito sobre a proliferação e invasão de células tumorais (HAN et al., 2017).

Estudo em câncer de cabeça e pescoço mostra baixa expressão de ANXA1 em amostras teciduais de carcinoma de laringe, no entanto, a administração do peptídeo da AC_{2-26} que confere ação biológica através do receptor FPR2/ALX, inibiu o crescimento de células Hep-2 (GASTARDELO et al., 2014). Diferentemente, a superexpressão dessa proteína foi detectada em câncer hepático (LIN et al., 2014) e de mama (SOBRAL-LEITE et al., 2015; OKANO et al., 2015). Interessantemente, em carcinoma esofágico há perda da ANXA1 (PAWELETZ et al., 2000; YAZDIAN-ROBATI et al., 2017), enquanto o adenocarcinoma de esôfago apresenta expressão elevada dessa proteína e esse aumento correlaciona com estágios avançados do tumor e metástases (WANG et al., 2006).

A ANXA1 é encontrada na membrana plasmática, citoplasma, associada às organelas e no núcleo (RESCHER; ZOBIACK; GERKE, 2000; MUSSUNOOR; MURRAY, 2008). A presença dessa proteína no compartimento nuclear está relacionada com o desenvolvimento tumoral (Figura 6), e sua translocação do citoplasma para o núcleo ocorre por danos no DNA, estímulos proliferativos e associados à fosforilação (BOUDHRAA et al., 2016).

Figura 6 - Envolvimento da ANXA1 nuclear no câncer.



Fonte: BOUDHRAA et al., 2016. A translocação do ANXA1 do citoplasma para o núcleo é iniciada por sinais mitogênicos/proliferativos (setas pretas) ou após danos no DNA (setas vermelhas). O tratamento com PMA (*phorbol 12-myristate 13-acetate*) induz a ativação de PKCδ (proteína quinase C), que permite a fosforilação de ANXA1 e sua relocalização ao núcleo. Da mesma forma, a ativação do EGFR (receptor do fator de crescimento epidérmico) pelo seu ligante cognato (EGF) induz a fosforilação (do resíduo Tyr21) e a translocação nuclear de ANXA1. Uma vez no núcleo, ANXA1 é saturada (dentro de sua terceira repetição em Lys257) e estimula a replicação do DNA através da sua atividade helicase (setas pretas). Após o dano ao DNA, a ANXA1 é translocada do citoplasma para o núcleo, onde é monoubiquitizada. Pela atividade de helicase e presença de metais pesados (As^{3+} e Cr^{6+}), a ANXA1 monoubiquitizada permite a síntese da translesão e a mutagênese (setas vermelhas).

Estudos apontam que a ANXA1 modula a expressão de algumas vias gênicas, como por exemplo, o gene Inibidor de diferenciação ou Ligação ao DNA 1 (ID1) (PRATES et al., 2015), sendo esse gene indicado como um

oncogene, pois atua na iniciação e progressão tumoral (LASORELLA; UO; IAVARONE, 2001; LI et al., 2017a).

ID1 não é um fator de transcrição, mas um regulador transcricional de genes alvos que controla negativamente o domínio hélice-alça-hélice (HLH) (ZHENG et al.,2004). Apresenta superexpressão em diversos tumores sólidos (RUZINOVA; BENEZRA, 2003), correlacionando resistência à tratamentos, mau prognóstico e metástase (SCHINDL et al., 2001, 2003). A família de proteínas ID é um grupo de pequenas moléculas de curto comprimento (13 a 20 KDa), que pertence a família de fatores de transcrição HLH pelo qual inibe os fatores de transcrição básica de ligação ao DNA. Em mamíferos, esse grupo apresenta quatro subtipos (ID1-4) que participam de vários processos celulares (BENEZRA et al., 1990; PERK; IAVARONE; BENEZRA, 2005).

As regiões N-terminal e HLH do ID1 são responsáveis por causar deformidades no centrossomo e centríolo, contribuindo para a instabilidade genômica e iniciação de tumores. Essa confirmação se deu ao analisar várias células tumorais, incluindo linhagens de carcinoma cervical (SiHa, HeLa, CaSki e A33A), com elevado nível de expressão endógena de ID1 (MANTHEY et al., 2010).

Investigações têm sugerido que os HPVs de alto risco contribuem para a progressão tumoral pela regulação de ID1, em câncer de cérvice (DARNEL et al., 2010), e que níveis de expressão desse gene estão relacionados com processos de angiogênese, diferenciação e metástase.

Nos tumores de pâncreas (LEE et al., 2004) e esofágico de células escamosas (LUO et al., 2012) foram observadas superexpressões do ID1 relacionada com o processo de angiogênese, com alta densidade de vasos intratumorais. Alto nível de ID1 foi detectado em câncer de próstata, sugerindo participação no desenvolvimento e progressão do tumor, assim como prognóstico ruim e baixa sobrevida (OUYANGE et al., 2002; PONZ-SARVISÉ et al., 2014).

Com essas considerações, estratégias para entender a expressão da ANXA1 e ID1 em amostras teciduais de cérvice e relacionar os efeitos da

administração da cisplatina em associação com o peptídeo da ANXA1 nas células tumorais, nossos estudos podem representar um grande avanço no entendimento funcional e molecular da regulação gênica no câncer de colo de útero.

2. OBJETIVOS

2.1. OBJETIVO GERAL

O objetivo deste trabalho foi investigar as atividades do quimioterápico cisplatina e interação molecular com as proteínas anexina A1 (ANXA1) e inibidora de ligação (ID1) envolvidos no processo tumorigênico do carcinoma epidermóide do colo uterino.

2.2. OBJETIVOS ESPECÍFICOS:

Nas células da linhagem SiHa (carcinoma epidermóide de cólo de útero) preservadas no meio de cultura controle e tratadas com peptídeo da anexina A1 (AC_{2-26}), cisplatina (Cis) e associação AC_{2-26}+Cis avaliamos:

i. Os efeitos dos tratamentos sobre o índice de proliferação e migração celular;

ii. A citotoxicidade dos tratamentos por meio do ensaio de viabilidade celular;

iii. A avaliação do ciclo celular e apoptose por citometria de fluxo;

iv. A expressão gênica de *ANXA1 e ID1* por PCR quantitativa;

v. A expressão das proteínas ANXA1, ID1 e ERK1/2 por *Western blot*;

vi. A localização da proteína ANXA1 pela reação de imunofluorescência.

Nas amostras teciduais de pacientes com neoplasia intraepitelial cervical (NIC) avaliamos:

i. A expressão das proteínas endógenas ANXA1 e ID1 por imuno-histoquímica.

3. MANUSCRITO

Effect of cisplatin modulates Annexin A1 and Inhibitor of Differentiation to DNA 1 expression in cervical cancer cells

Manuscrito submetido à revista "The FEBS Journal"
ISSN: 1742-464X

Effect of cisplatin modulates Annexin A1 and Inhibitor of Differentiation to DNA 1 expression in cervical cancer cells

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Key words: cisplatin, ANXA1, ID1, nuclear translocation, cervix,

Research Article: Cancer Therapy and Prevention

Novelty and impact: Our studies have shed new light on the mechanism of action of cisplatin (Cis) and have identified that this chemotherapeutic agent decreases inhibitor of differentiation to DNA 1 (ID1) and increased annexin A1 (ANXA1) by modulating the expression of these genes in tumor cells. The results suggest that these mediators may serve as an effective strategy to overcome the side effects of chemotherapy in the treatment of cervical cancer.

ABSTRACT

Cervical cancer presents high rates of incidence and mortality, and its progression depends on genetic characteristics and interactions of tumor, epithelial and inflammatory cells. The objective of this study was to investigate the molecular interaction of the annexin A1 (ANXA1) and inhibitor of differentiation to DNA 1 (ID1) mediators with the chemotherapeutic cisplatin (Cis) in cell line as well as relate to intraepithelial neoplastic tissues in the cervical tumorigenic process. SiHa cells (cervix squamous cell carcinoma) treated with the annexin A1 peptide (AC_{2-26}), Cis and AC_{2-26}+Cis, were evaluated in the cell proliferation and migration, cytotoxicity, apoptosis, immunolocalization, expression of *ANXA1* and *ID1* genes and ANXA1, ID1 and ERK1/2 proteins. Cervical tissue samples were analyzed histopathologically and the expression of ANXA1 and ID1 protein evaluated by immunohistochemistry. All treatments decreased cell proliferation and migration, without presenting cytotoxic effects. Cis and AC_{2-26}+Cis induced the cells to processes of late apoptosis, decreased ID1 and increased ANXA1 expression, indicating the antineoplastic activity of this chemotherapeutic. Cis and AC_{2-26}+Cis administration inactivated phosphorylated ERK1/2 expression and induced ANXA1 nuclear translocation. Expressions of the ID1 and ANXA1 proteins were observed in tissue samples from Cervical Intraepithelial Neoplasia (CIN), with intense ID1 immunolabeling in CIN III. Together, the data obtained in this work provide additional understanding of the action mechanism of cisplatin in the modulation of ID1 and ANXA1 mediators in tumorigenic process. These findings may outline new strategies for therapeutic intervention in cervical cancer.

3.1. INTRODUCTION

Cancer of the cervix is the fourth most common in women and the seventh most frequent in the world [1]. There are approximately 530,000 new cases each year, which becomes it the fourth leading cause of death in developing countries, with approximately 275,000 deaths per year [2]. The early detection of this neoplasm through colpocitological screening (Papanicolaou Test), monitoring and treatment of precursor lesions has helped to reduce incidence and mortality in developed countries [3]. The development and introduction of vaccines in adolescents has also contributed effectively to the prevention of human papillomavirus (HPV) infection, however the incidence remains high [4].

Currently, the treatment for cervical cancer includes surgical procedures, as well as chemotherapy and radiotherapy [5]. Cisplatin (cis-diaminodichloroplatin II or cis-DDP) has been approved by the American Food and Drug Administration (FDA) in 1979, and is widely used as a therapeutic remedy in cancer [6]. The antineoplastic activity of cisplatin has as its main target cellular DNA, where it induces the formation of adducts that subsequently interferes in the transcription and replication, triggering the apoptosis process [7].This drug may interact with different cellular components such as phospholipids and RNA[8]. However, this chemotherapeutic may induce gastrotoxicity, myelosuppression, neuropathy, allergic reactions and mainly nephrotoxicity [9].

At the beginning of the neoplastic process, the inflammatory cells and the factors produced regulate growth, migration and differentiation of all cell types in the tumor microenvironment, including neoplastic, endothelial and fibroblast cells. Subsequently, neoplastic cells use some inflammatory mechanisms for invasion of other tissues, such as cell adhesion to the vascular endothelium, migration and diapedesis. Perretti (1997) [10] had already highlighted the similarity of metastatization with these stages of the inflammatory process. Prevention of tissue damage and maintenance of homeostasis is controlled by anti-inflammatory mediators, including Annexin A1 (ANXA1), a 37 kDa protein, which acts as a modulator of inflammation [11]. ANXA1 was the first

characterized member of a superfamily of proteins that bind phospholipids in a calcium-dependent manner [12].

ANXA1 is capable of binding to negatively charged phospholipids in a calcium (Ca2+) dependent manner and has a conserved structure of about 70 repeated amino acids [13]. Structurally, annexins comprise two domains: a small N-terminal region, varying in length and composition, and a central domain, C-terminal region, formed by four to eight repeated folds of a conserved sequence of 70 to 80 amino acids [12]. The N-terminal domain is specific for each member of this family and interacts with different ligands of these proteins, while the C-terminal region is responsible for calcium affinity and consequent binding to the phospholipids.

In addition to anti-inflammatory effects, ANXA1 plays a role in cell proliferation, cell differentiation and apoptosis [14], indicating that it is related to tumorigenesis. However, this role is still not well understood, since its expression occurs differently depending on the type of tumor. Dysregulation of ANXA1 is associated with the progression and tumor development in several types of cancer, exerting an effect on the proliferation and invasion of tumor cells [15].

ANXA1 is found in the plasma membrane, cytoplasm, associated with organelles and in the nucleus [16]. The presence of this protein in the nuclear compartment is related to tumor development, and its translocation from the cytoplasm to the nucleus occurs due to DNA damage, proliferative stimuli and phosphorylation [17]. Studies indicate that ANXA1 modulates the expression of some gene pathways, such as, for example, the Inhibitor of DNA binding 1, HLH protein (ID1) gene [18], which is indicated as an oncogene, acting on tumor initiation and progression [19].

ID1 is a transcriptional regulator of target genes that negatively controls the helix-loop-helix (HLH) domain [20] has overexpression in several solid tumors [21], correlating resistance to treatments, poor prognosis and metastasis [22]. The ID family of proteins is a group of small molecules of short length (13 to 20 KDa) that belongs to the family of HLH transcription factors by which it inhibits the basic transcription factors of DNA binding. In mammals, this group

presents four subtypes (ID1-4) which participate in several cellular processes [23].

The N-terminal and HLH regions of ID1 are responsible for causing centromere and centriole deformities, contributing to genomic instability and tumor initiation [24]. Investigations have suggested that high-risk HPVs contribute to tumor progression by ID1 regulation in cervical cancer [25] and that levels of expression of this gene are related to processes of angiogenesis, differentiation and metastasis. High level of ID1 in prostate cancer is related to poor prognosis and low survival [26].

Given the importance of the ANXA1 and ID1 proteins in tumorigenic processes, the objective of this work was to investigate the activities of the chemotherapeutic cisplatin and its molecular interaction with the annexin A1 (ANXA1) and Inhibitor of DNA binding 1 (ID1) proteins involved in the cervical tumorigenic process.

3.2. RESULTS

3.2.1. AC_{2-26} and Cis decrease cell growth, migration and low cytotoxicity

The SiHa cell line showed a decrease in proliferation, after 48 hours of treatment with AC_{2-26} ($p<0.01$), cisplatin (Cis) and AC_{2-26}+Cisplatin (AC_{2-26}+Cis) ($p<0.001$). The cells maintained a reduction in proliferation after 72 hours of treatment ($p<0.001$) with no difference between the Cis and AC_{2-26} +Cis groups (Fig. 1A).

The treatments with Cis and AC_{2-26} +Cis reduced cell viability after 24 ($p<0.01$), 48 and 72 hours ($p<0.001$), and, although these treatments reduced viability, they were low cytotoxicity, because the cell death did not reach 50% (Fig. 1B).

The treatments AC_{2-26}, Cis and AC_{2-26} +Cis decreased the cell migration. In the wound healing assay, the control cells closed the area in 120 hours, the AC_{2-26} presented closure as well as to the control ($p>0.05$), differently from the treatments Cis and AC_{2-26} +Cis ($p<0.001$) (Fig. 1C, 1D). The occupation rates of area were expressed in relation to time 0 (zero) hour.

The Transwell migration assay confirmed the effect of chemotherapeutic action on chemotaxis in this cell line. The control group had a higher number of transmigrated cells in relation to treatments AC_{2-26} ($p<0.001$), Cis ($p<0.001$) and AC_{2-26} +Cis ($p<0.001$) (Fig. 1C, 1E).

3.2.2. Cis and AC_{2-26} +Cis direct cells to late apoptosis and necrotic process

Cell cycle analysis was performed to investigate whether inhibition of cell growth alters the progression of the cycle, in SiHa cell line. The G0/G1 population decreased from 77.4 ± 4.94% (control) to 44.4 ± 8.11% (Cis) ($p<0.0001$) and 48.4 ± 7.64% (AC_{2-26}+Cis) ($p<0.0007$) (Fig. 2A). These results obtained in the FITC/PI annexin-V apoptosis assay corroborate with those observed in the cell cycle, in which cells in late apoptosis and necrosis were observed with Cis and AC_{2-26} +Cis treatments ($p<0.001$) (Fig. 2B, 2C). In view of these results, we sought new mechanisms that could justify this well-established effect of cisplatin. Among the targets of interest, the endogenous expression of ANXA1, ID1 and ERK1/2 gene was highlighted.

3.2.3. ANXA1 and ID1 Gene and protein expression are modulated by Cis and AC_{2-26}+Cis

Analysis of mRNA expression by real-time PCR demonstrated increase in *ANXA1* and reduction of *ID1* gene expression in the Cis and AC_{2-26}+Cis treated groups (Fig. 3A). The analysis of protein expression by Western Blot indicated equivalence in the modulation observed in the quantitative PCR, characterized by the increase of ANXA1 and reduction of ID1 protein expression in the groups Cis (ID1: $p<0.001$) and AC_{2-26} +Cis (ANXA: $p<0.001$; ID1: $p<0.0001$) (Fig. 3B, 3C). These findings indicate that the chemotherapeutic treatment Cis exerts an effector action on the modulation of expression of the ANXA1 and ID1 genes and proteins.

3.2.4. Inhibition of phosphorylated ERK1/2 by Cis and AC_{2-26} +Cis

Cis and AC_{2-26} +Cis treatments inhibited ERK phosphorylation (p-ERK1/2), but this result was not observed after AC_{2-26} treatment (Fig. 4). Interestingly, total ERK1/2 was not detected in any group, suggesting that Cis decreases cell proliferation and induces apoptosis by MAPK/ERK1/2 signaling pathway in the SiHa cell line.

3.2.5. Nuclear translocation of ANXA1 is induced by Cis and AC_{2-26} + Cis in SiHa cells

Immunofluorescence analysis shows ANXA1 positive cells predominantly in the cytoplasmic control cells and AC_{2-26} treated (Fig. 5). This pattern was modified in cells treated with Cis and AC_{2-26} +Cis (Fig. 5), where ANXA1 protein expression was observed in the cytoplasm and nucleus.

3.2.6. Increased endogenous expression of ID1 protein in cervical biopsies

Cervical fragments obtained in evolutionary degrees of Cervical Intraepithelial Neoplasia (CIN I, CIN II and CIN III) show coilocytic cells with clear perinuclear halos and condensation of the surrounding cytoplasm (Fig. 6A, 6B) and presence of inflammatory cells (Fig. 6C).

Immunoreactivity was observed for the ANXA1 (Fig. 6D-6F) and ID1 (Fig. 6G-6I) proteins, predominantly in the basal portion of the epithelium in CIN I and II and intense in CIN III of ID1 (Fig. 6I).

Densitometric analyzes revealed increased expression of the ID1 proteins in the nucleus and cytoplasm of the epithelial cells during the progression of the neoplasic process. There was no differentiation in nuclear and cytoplasmic ANXA1 expression (Fig. 6K).

In ID1 expression, a significant difference in CIN III ($p<0.001$) was observed in relation to CIN I and CIN II. While in the cytoplasm, CIN III ($p<0.05$) was higher in relation to CIN I and CIN II (Fig. 6L).

3.3. DISCUSSION

A recent study by our research group, involving the tumorigenic process in the SiHa cervical carcinoma tumor line, shows that the mimetic peptide treatment of the ANXA1 protein (AC_{2-26}) modulates expression of inhibitor of differentiation/ DNA binding 1 (ID1) gene [18]. In the current project, we related the effect of cisplatin (Cis) and AC_{2-26} on SiHa cells. We have shown that administration of cisplatin induces the apoptosis process by decreasing ID1 and increasing ANXA1 gene and proteins expression, effects that may reveal a potential therapeutic approach for cervical cancer.

In the SiHa cells, our studies showed a decrease in cell proliferation and migration after treatment with AC_{2-26}, corroborating previous findings in our laboratory [18]. As expected, cisplatin-treated cells entered into apoptosis, with consequent reduction of cell proliferation and migration. In addition, the cytotoxic agent, which induces DNA replication, inhibits cell replication and transcription, and promotes cell cycle blockade [9,27].

Considering that the cisplatin classic mechanism of action is to induce cell death [28,29], we investigated probable modulations of genes and/or proteins that could complement this process. The AC_{2-26} treatment does not interfere in the expression of the ANXA1 and ID1 genes and proteins, while cisplatin, associated or not to the peptide, induces an increase in the expression of ANXA1 and a decrease in ID1 genes and proteins. Increased expression of ANXA1 in the SiHa cell line was also observed by Calmon and colleagues [30], showing that the protein is related to carcinogenesis human papillomavirus (HPV) mediated. This protein was also indicated as a regulator of cisplatin resistance in lung adenocarcinoma [31] and in colorectal carcinoma [32]. Differently, the decrease of ANXA1 was observed in nasopharyngeal carcinoma tumor cell lines, promoting cell growth and blockade of apoptosis [33], as well as in patients with cervical cancer who received neoadjuvant chemotherapy (paclitaxel and cisplatin) [34].

Whereas the ANXA1 is related to apoptosis by the ERK1/2 phosphorylation pathway [35], the next step was to investigate whether the treatments modulate this pathway. The control and AC_{2-26} groups showed ERK1/2 (p-ERK1/2) phosphorylation without increased apoptosis. In contrast,

cisplatin treatments inhibited p-ERK1/2 expression leading to cell death. Corroborating our studies, the administration of the peptide induced the phosphorylation of ERK1/2 in macrophages [36] and in skin allograft [37]. Similar action was observed by Dang and colleagues [38] in the ovarian cancer cell line after administration of metformin-associated cisplatin, showing a reduction in cell viability, inducing apoptosis by the inactivation of p-ERK1/2. On the other hand, rodent study shows that the high endogenous expression of ANXA1 or treatment with AC_{2-26} decreases the activation of ERK1/2 [39]. The results obtained in this work suggest that, in the SiHa tumor cell line, cisplatin promotes cell death through the classic MAPK signaling pathway, when inactivating p-ERK1/2.

The results showed that, in addition to increased expression of ANXA1, cisplatin induces translocation of ANXA1 from the cytoplasm to the nucleus. Our research group had already detected the expression of ANXA1 in the plasma membrane, cytoplasm and nucleus of neutrophils after induction of experimental peritonitis [40]. This change in intracellular localization may be a possible response to DNA damage induced by this chemotherapeutic agent [41]. Other studies have shown that nuclear translocation of ANXA1 induces apoptosis via p53-Bid-Caspase-3 in neurons [42], and regulates IL-1β expression by p65 in retinal cells[43], both in deprivation-reperfusion model. Our hypothesis, that cisplatin modulates the expression and intracellular localization of ANXA1, with a focus on apoptosis, corroborates data from the literature, however, the exact role of these translocations to the nucleus still needs further elucidation.

Other molecular targets such as ID1 were related to apoptosis in cancer cells [44]. Our analyzes show a reduction in the expression of ID1 after treatment with cisplatin, confirming this relationship. Studies indicate that ID1 silencing decreases cell invasion [44], cell proliferation and directs for apoptosis [45] in hepatocellular carcinoma. Suppression of ID1 also reduces cell proliferation and migration in prostate cancer [46]. Recently, Huang and colleagues [47] mechanistically demonstrated that miR-381 interfered in the activation of nuclear factor NF-κB by repressing ID1, with consequent suppression of cell growth in non-small cell lung cancer. These associated data

propose that the *ID1* gene may be a potential therapeutic target for the regulation of the apoptotic pathway in response to the chemotherapeutic cisplatin.

Considering the importance of translational research, cervical biopsies of patients with different stages of neoplasia were used to evaluate the expressions of ANXA1 and ID1. We did not observe a difference in ANXA1 expression in biopsies among the degrees of neoplasia. Wang and colleagues [48] evaluated cervical fragments in the different stages of the neoplasia, observed decreased expression of ANXA1 in relation to tumor progression. However the increase of ANXA1 was observed in colorectal tumors [49], cholangiocarcinoma [50] and esophageal adenocarcinoma [51].

Increased expression of ID1 was also observed in the biopsies of patients with high degree of dysplasia (CIN III). In this aspect, similar results were obtained in investigations of cervix tumors [22], prostate [26] and breast [52]. In endometrial carcinogenesis, the elevated ID1 level is associated with the highest histological grade and invasiveness [53]. With the considerations related to ID1 and ANXA1 we suggest that the increase in the expression of these proteins is associated with the tumor progression of the uterine cervix cells.

With these considerations, we pointed out that the increase in ID1 expression is associated with the progression of the tumor stage. Although the expression of ANXA1 did not present a modification in the biopsies analyzed, in the in vitro assays, treatment with cisplatin increased the expression of this protein, triggering cell death. Thus, we suggest that either ID1 or ANXA1 could be used as molecular targets prognostics in cervical tumorigenesis.

Together, our results provide a better understanding of the role of the oncogene *ID1* and *ANXA1* in the tumorigenic process, and additional knowledge of the mechanism of action of cisplatin on the modulation of these genes in this type of cancer. These findings may outline new strategies for the therapeutic intervention of cervical cancer.

3.4. MATERIALS AND METHODS

3.4.1. Cell culture and treatment protocols

The SiHa cell line originally established from an epidermoid carcinoma of the cervix purchased from ATCC (ATCC, Rockville, Maryland, USA) were grown in MEM-Earle medium (Cultilab, Campinas, SP, Brazil), supplemented with 10% fetal bovine serum (FBS) (Cultilab), 1% non-essential amino acids and 1% antibiotic/antimycotic (Invitrogen Corporation, Carlsbad, CA, USA) and maintained at 37°C under 5% of carbon dioxide (CO_2) and 95% relative humidity.

To determine the optimal length of treatments, SiHa cell line were cultivated initially in: (a) Control, only with complete MEM medium; (b) $AC_{2\text{-}26}$ submitted to the addition of the $AC_{2\text{-}26}$ peptide of Annexin A1 (Ac-MVSEFLKQAWFIENEEQEYVQTVK - Invitrogem, USA) at 3µM concentration [18]; (c) Cisplatin (Cis) submitted to the addition of this chemotherapeutic (Libbs, Brazil) at a concentration of 5 µg/mL [54] and (d) $AC_{2\text{-}26}$ +cisplatin ($AC_{2\text{-}26}$ +Cis) at the referred concentrations. The experiments were performed at times of 4, 24, 48 and 72 hours.

3.4.2. Growth curve and viability

The SiHa cell line was seeded following the above treatments and proposed times. Morphology was observed daily and cells quantified in the Countess® Automated Cell Counter (Invitrogen, Carlsbad, USA, UK). To evaluate proliferation, counted cells were compared to an untreated growth curve (Control) and the results expressed as number of $x10^4$ cells. Measurement of cell viability was performed with CellTiter 96® AQueous One Solution Assay (MTS tetrazolium compound), according to the manufacturer's protocol (PROMEGA, USA). The reading was performed after 4 hours of reagent incubation (in the oven at 37°C, 5% CO2, humidified) in ELISA reader at the wavelength of 492nm (Thermo Plate, Tp-Reader Basic). The experiments were performed in triplicate.

3.4.3. Wound healing assay

A wound healing assay was used to determine cell migration. SiHa cell line were seeded in 12 well plates and after adherence to the culture plate and 90% confluency, a scratch with a tip was performed in the center of the well, forming a groove. Cells were washed with PBS and cultured at 37°C with 5% CO2, with the proposed treatments at the times of 4, 24, 48, 72, 96 and 120h. Only this experiment extended the time to observe the closure of the groove. The cell migration was observed with images obtained before and after treatments using a camera (Olympus DP73, Japan), coupled to the microscope (Olympus, Tokyo, Japan), using a 40x objective. The distance of cell migration was measured in four random fields. All samples were tested in triplicate. The quantitative analysis of the slot closure area was analyzed using Image J-Image Processing and Analysis in Java (NIH, Bethesda, MD, USA, software).

3.4.4 Transwell migration

Cells were grown in 25cm^2 flasks with different treatments and incubated for 72 hours (Statistically significant time chosen for the other assays). Afterwards, cells were trypsinized and loaded in the amount of 3x10^4 onto the Transwell-type inserts (Corning Costar, New York, USA) using 8µm pore filters in 24-well plates. In the upper portion of the chamber was added 150µL MEM medium without FBS and in the lower 500µL MEM 10% FBS. The plate was incubated for 24 hours in a CO_2 oven at 37°C. Fixation of the lower portion cells was performed with 4% paraformaldehyde in PBS for 30 minutes and stained with Crystal Violet solution for another 30 minutes. The inserts were then photographed in four random fields.

3.4.5. Cell cycle analysis

Cells were plated on culture for 24 hours for adhesion. Then, they were incubated with MEM without supplementation for 24 hours for synchronization. At the end of the period, cells were treated with proposed treatments. After 72h incubation, cells were harvested, whashed with cold PBS and fixed with ice cold 70% ethanol and incubated for 1 hour on ice, washed with PBS and centrifuged for 5 minutes, repeating the procedure twice. At the end, cells were

resuspended with PBS and incubated with RNAse (10mg/ml - Sigma) and 0.5 ul Propionate Iodide (1mg /ml-Sigma). The cell cycle was analyzed using FACSCanto flow cytometer (Becton Dickinson, San Diego, CA, USA). Data were analyzed through FlowJo software (Tree Star Inc., Ashland, OR, USA).

3.4.6. Annexin V-FITC/PI assay to analyze apoptosis

Evaluation of cell death was performed using the Annexin V Kit (FITC Annexin V Apoptosis detection kit I, Becton Dickinson, San Jose, CA, USA) according to the manufacturer´s protocol. Briefly, SiHa cells were grown in culture flasks as described for 72h, disaggregated, washed twice with cold PBS and resuspended in Annexin V Binding Buffer at a concentration of $1x10^6$ cells/mL. FITC Annexin (5µL) V and Propidium Iodide (5µL) were added to cells, incubated at room temperature in the dark for 15 minutes. After that time, 400µL of the 1X binding buffer was added and the cells analyzed by flow cytometer (GUAVA easyCyte ™, Millipore).

3.4.7. RNA Extraction and Reverse Transcription

Total RNA was extracted from the SiHa cell line after treatments using the RNeasy® Mini Kit (Qiagen, Germany), following the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using High Capacity cDNA-ReverseTranscription Kit (Applied Biosystems, Foster City, CA) using 1µg of total RNA (according to manufacturer's recommendations) and stored at -20°C.

3.4.8. Quantitative real-time PCR

The expression of *ANXA1* and *ID1* genes was determined by Real Time PCR, performed in triplicate, on a StepOnePlus System 2.2.3 (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacture’s recommendations. The endogenous *TUBA6*, *GAPDH* and *ACTB* genes were used as reaction normalizers (Table 1 - Supplement 1). The data obtained from the PCR were analyzed from the Ct (Cycle Threshold) values of each sample, the means of the triplicates being calculated and the mathematical model Pfaffl (2001).

3.4.9. Western Blot analysis

Expression of ANXA1, ID1, ERK1/2 and (p)ERK1/2 proteins were examined in SiHa cells by SDS-PAGE, then transferred to nitrocellulose paper (Immobilon 0.45 mm, Millipore) in a wet transfer apparatus (Bio-Rad). The blots were incubated with the respective secondary antibodies diluted in TBST-Tween 20 for one hour at room temperature, and membranes were developed using Clarity^{TM} Western ECL Substrate (Bio-Rad,USA) for the detection of reactive bands by chemiluminescence. For samples standardization, the same membranes were incubated with the anti-human β-actin antibody (Cell signaling Tecnology). The densitometric analysis was performed by the Image J program (NIH, Bethesda, USA).

3.4.10. Immunofluorescence

For ANXA1 detection, SiHa cells were grown in coverslips, fixed in paraformaldehyde 4% for 24h,washed in PBS, Tween 20 (0.4%), blocked with BSA 1% diluted in 3% normal goat serum and then incubated with polyclonal rabbit anti-ANXA1 antibody (Invitrogen, USA) (1:100 in goat normal serum 1.5%). After washing, the cells were incubated with fluorescent antibody anti-rabbit IgG (H+L) Alexia Fluor 488 (Molecular Probes, Eugene, Oregon, USA), (1:100) in goat normal serum 1.5% for 1h. The slides were mounted with FluoroshieldTM with DAPI (Sigma, Ca, USA). Goat normal serum was used in the reaction control. The cells were analyzed using a filter with wavelength of 488nm, in AXIOSKOP 2-Mot Plus Zeiss microscope (Carl Zeiss, Jena, Germany).

3.4.11. Tissue specimens of uterine cervix

Paraffin-embedded cervical tissue blocks (n=24) were obtained from the Anatomopathological Analysis Bank, collected between January 2013 and December 2015 and assigned by the Pathology Department of FAMERP (Faculty of Medicine of São José do Rio Preto - SP, Brazil). Samples (CIN I=8; CIN II=8; CIN III=8), included in paraffin, were obtained from patients with a mean age of 38.6 years, analyzed and characterized by a pathologist. The

study was approved by the Research Ethics Committee Plataforma Brasil with the opinion 1.412.751 (CAAE: 46392515.0.0000.5466).

3.4.12. Immunohistochemistry and expression of ANXA1 and ID1 proteins

Sections of paraffin-embedded cervical tissues (3µm) were submitted to the antigen exposure using citrate buffer (0.01 M, pH 6.0) and the endogenous peroxide activity was blocked. After, they were incubated with antibody primary: (a) Anti-annexin A1, 1:3000 (rabbit, polyclonal, Invitrogen, USA) diluted in 5% PBS-BSA, incubated overnight at 4°C and (b) Anti-Id1, 1:200 dilution (rabbit, monoclonal, Abcam/USA) diluted in TBS/BSA, incubated for 1 hour at 37°C. After washing, the sections were incubated with secondary antibody, staining streptoavidin (Histostain®-SP Broad Spectrum Kit - HRP, Invitrogen, Paisley, UK) e DAB substrate (3,3-diaminobenzidine, Liquid DAB Substrate Kit, Invitrogen, Paisley, UK). Sections were counterstained with hematoxylin. Densitometric analysis of the ANXA1 and ID1 proteins were performed 20 points in four fields, for each region studied, using a 40x objective, for a mean related to the intensity of the immunoreactivity. The analyses were performed in the image analyzer, using the software AXIOVISION (Zeiss). Subsequently, the data were calculated and demonstrated as mean ± S.D. of the mean optical density (DOM).

3.4.13. Statistical analysis

All data were analyzed using Prisma® GraphPad software version 5.00. Results were presented as mean ± standard error of the mean (SEM) and statistical analysis was performed by analysis of variance for multiple comparisons (ANOVA), followed by the Bonferroni adjustment. P values less than 0.05 were considered to indicate statistically significant results.

3.5. ACKNOWLEDGMENTS

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (Grants 308144/2014-7 to S.M.O. and 140883/2014-2 to J.P.) and Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP (Grant 2016/02012-4 to S.M.O.), Brazil.

3.6. AUTHOR'S CONTRIBUTIONS

J.P. conducted the experiments

J.B.M. assisted in the western Blot and participated in the discussion of the manuscript

A.D.G. assisted in the Immunofluorescence

J.M.B. assisted in the real-time PCR

S.C.G.P.A. provided and reviewed paraffin-embedded cervical tissue blocks

S.S. performed cell cycle

S.H.P.F. assisted in the cell cycle

F.C.R.L. assisted in elaborated the project and participated in the discussion of the manuscript

S.M.O. elaborated the project, conducted the experiments and wrote the manuscript

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3.8. FIGURE LEGENDS

Figure 1. Effects of treatments with AC_{2-26}, Cis and AC_{2-26}+Cis on cell proliferation, cytotoxicity and migration**. [A]** Quantification of SiHa cells at the time of 4, 24, 48 and 72 hours in the control (complete MEM medium only) and after administration of AC_{2-26} (3μM), Cis (5 μg/mL) and AC_{2-26}+Cis. **[B]** Colorimetric method of cellular cytotoxicity (MTS). **[C]** Phase contrast microscopy and area measurements show the space of the cell monolayer groove (0, 24, 72 and 120 hours) and Transwell cell migration (20X objective) after staining with crystal violet. **[D]** Statistical analyzes of occupied area/migration rates expressed in relation to 0 hour. **[E]** Quantification of cells that migrated. Values *P*1 symbol = p <0.05; 2 symbols = p <0.01; 3 symbols = p<0.001. Symbols: * vs control; # vs AC_{2-26}.

Figure 2. Cell cycle analysis and apoptosis in SiHa cells after 72h of treatments. [A] Analyses of cell cycle progression. Data show mean ± S.E.M. of three independent experiments performed in unicate. **[B]** Analyses of apoptosis. Dotplots with axis x = FITC- Annexin V and y = propidium iodide. In the double negative population (FITC -Annexin V-/PI-) are viable cells; in FITC-positive cell populations (FITC-Annexin V +/PI-) are the cells in initial apoptosis; cells positive for Propionate Iodide (FITC -Annexin V-/PI+) the necrotic cells; double positive cells (FITC - Anexin V+/PI+) in late apoptosis or necrosis process. Representative images of three independent experiments.

Figure 3. Expression of ANXA1 and ID1 by Real-Time PCR and Western Blot in SiHa cells. [A] The expressions of the *ANXA1* and *ID1* genes were analyzed after treatments AC_{2-26}, Cis and AC_{2-26}+Cis, relative to control. The red line (≥ 1.0 or ≤ -1.0) is equivalent to the difference of the significant expression, based on logarithm 2. **[B]** Expressions of the ANXA1, ID1, and β-Actin proteins used as endogenous control. **[C]** Data statistics demonstrated as arbitrary units of the relative expressions of ANXA1 37kDa, 33kDa (cleaved) and ID1.

Figure 4. Detection of the ANXA1 cell location by immunofluorescence. SiHa cells after treatments with the peptide AC_{2-26}, cisplatin (Cis) and AC_{2-26}+

Cis (72 hours). ANXA1 in green (Alexa fluor 488) and blue nuclei (DAPI). ANXA1 with dense cytoplasm (yellow arrows) in the control and AC_{2-26} groups. The expressive expression of cytoplasmic immunoreactivity and nuclear expression positivity (red arrows) after treatments Cis and AC_{2-26}+ Cis. Bar: 20μm.

Figure 5. Western Blot protein expression in SiHa cells. Expressions of ERK1/2 and pERK1/2 were analyzed after treatments with AC_{2-26}, Cis and AC_{2-26}+Cis in relation to the control. β-actin, used as endogenous control.

Figure 6. Endogenous expressions of the ANXA1 and ID1 proteins in Cervical Intraepithelial Neoplasia (CIN). [A-C] Representative histopathological analyzes of CIN I, CIN II and CIN III with presence of coilocytic cells and inflammatory process cells. Hematoxylin and Eosin. Immunoreactivity to ANXA1 **[D-E]** and ID1 **[G-H]** in CIN I and CIN II in the basal portion of the stratified squamous epithelium. **[I]** CIN III increased ID1 expression throughout epithelial extension. **[J]** Absence of immunoreactivity in the control of the reaction. Counterstain: Hematoxylin. Bars: 50μm. Densitometry of the immunoreactivity of ANXA1 **[K]** and ID1 **[L]** in the nucleus and cytoplasm. Values *P* 1 symbol = $p < 0.05$; 2 symbols =$p < 0.01$; 3 symbols = $p < 0.001$. Symbols: * vs CIN I; # vs CIN II.

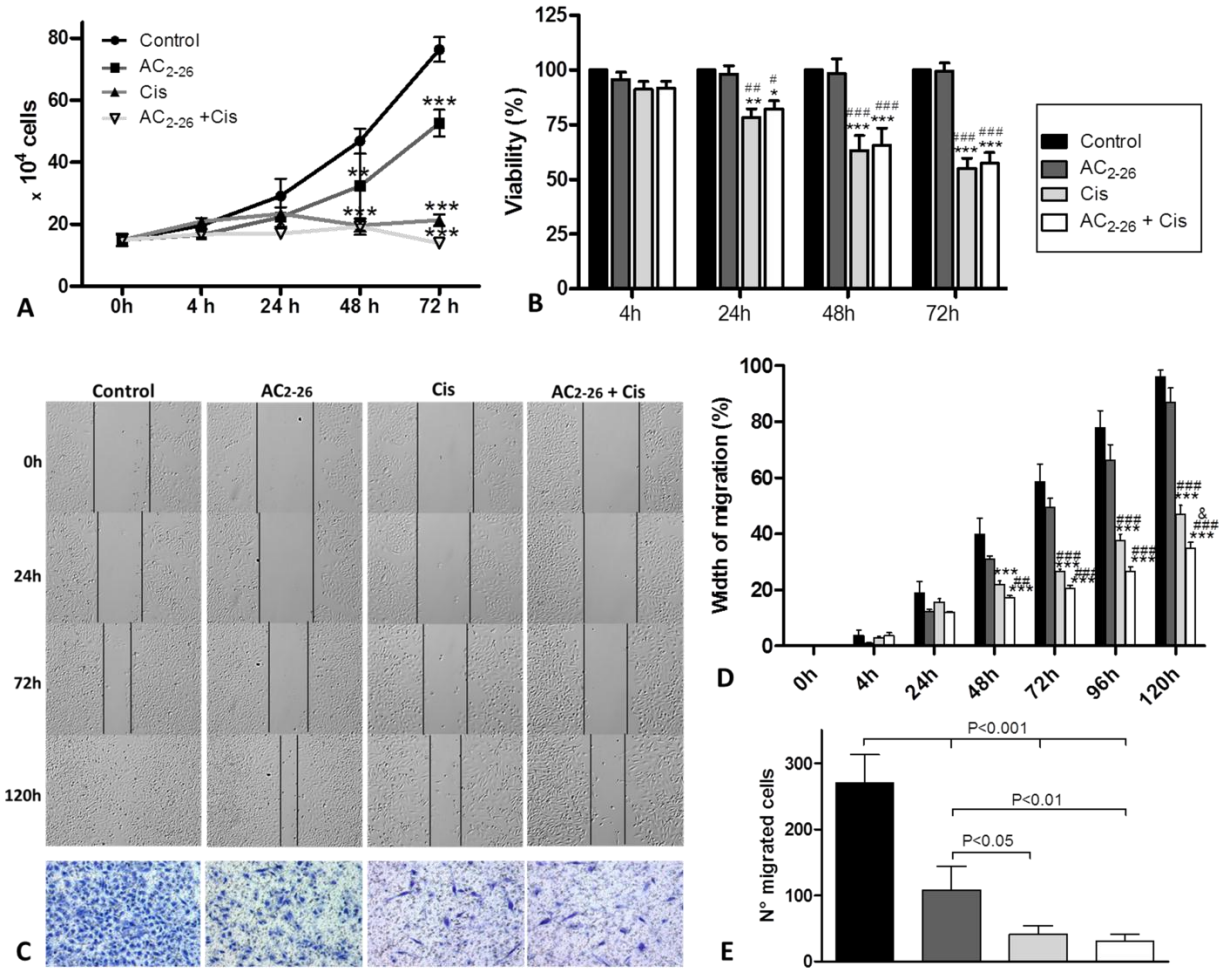

Figure 1

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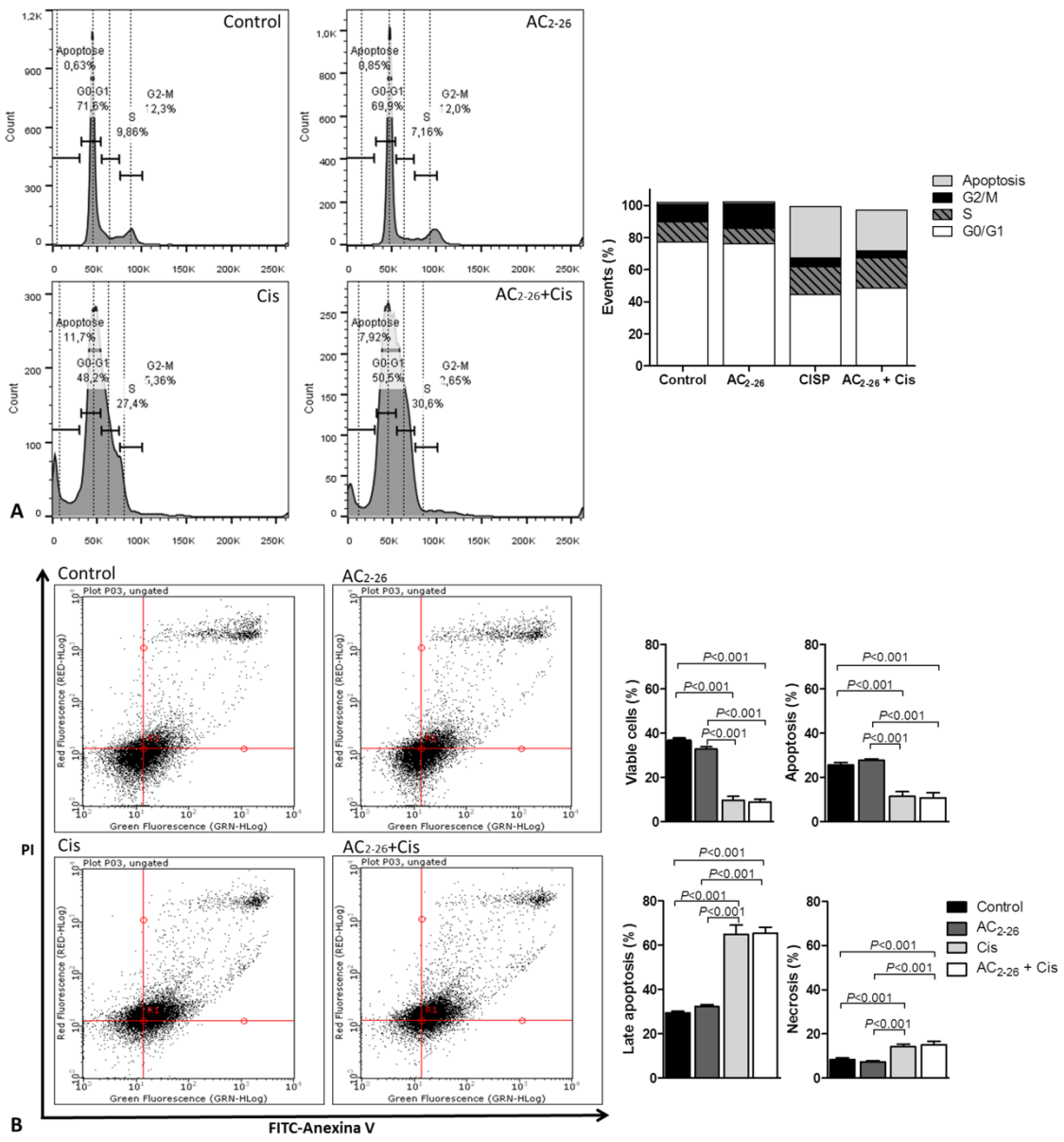

Figure 2

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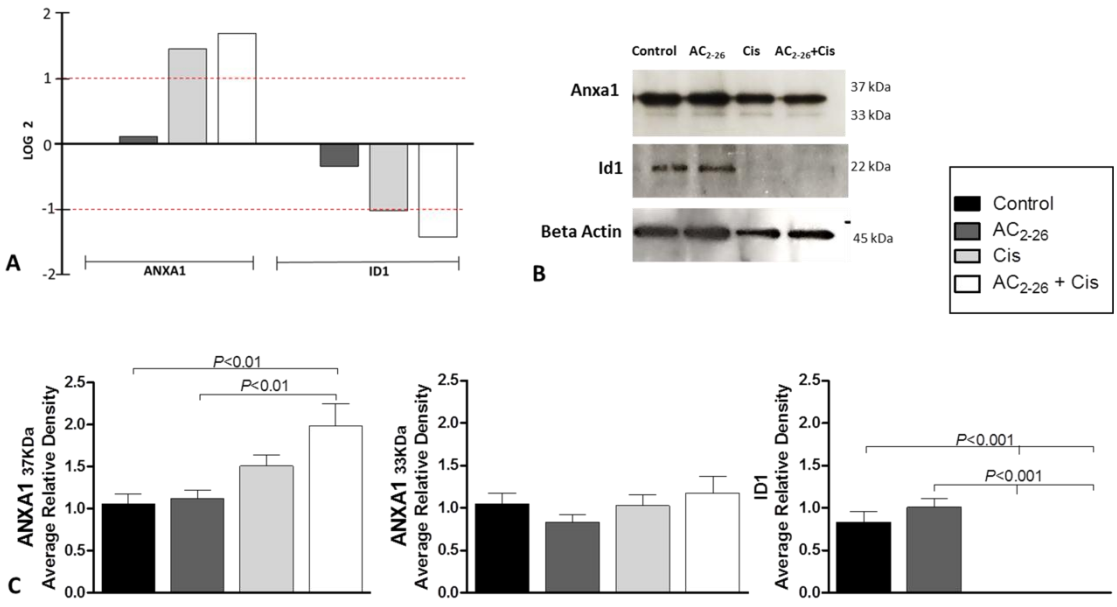

Figure 3

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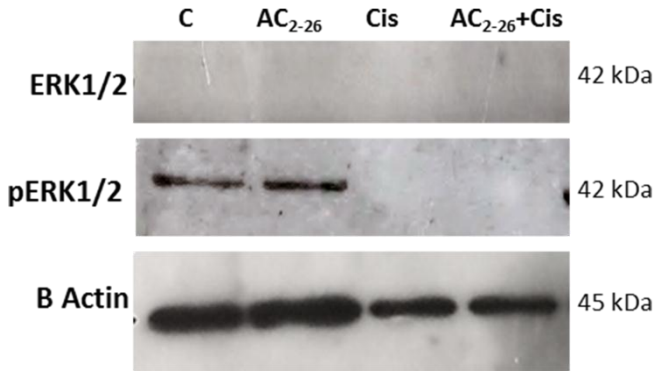

Figure 4

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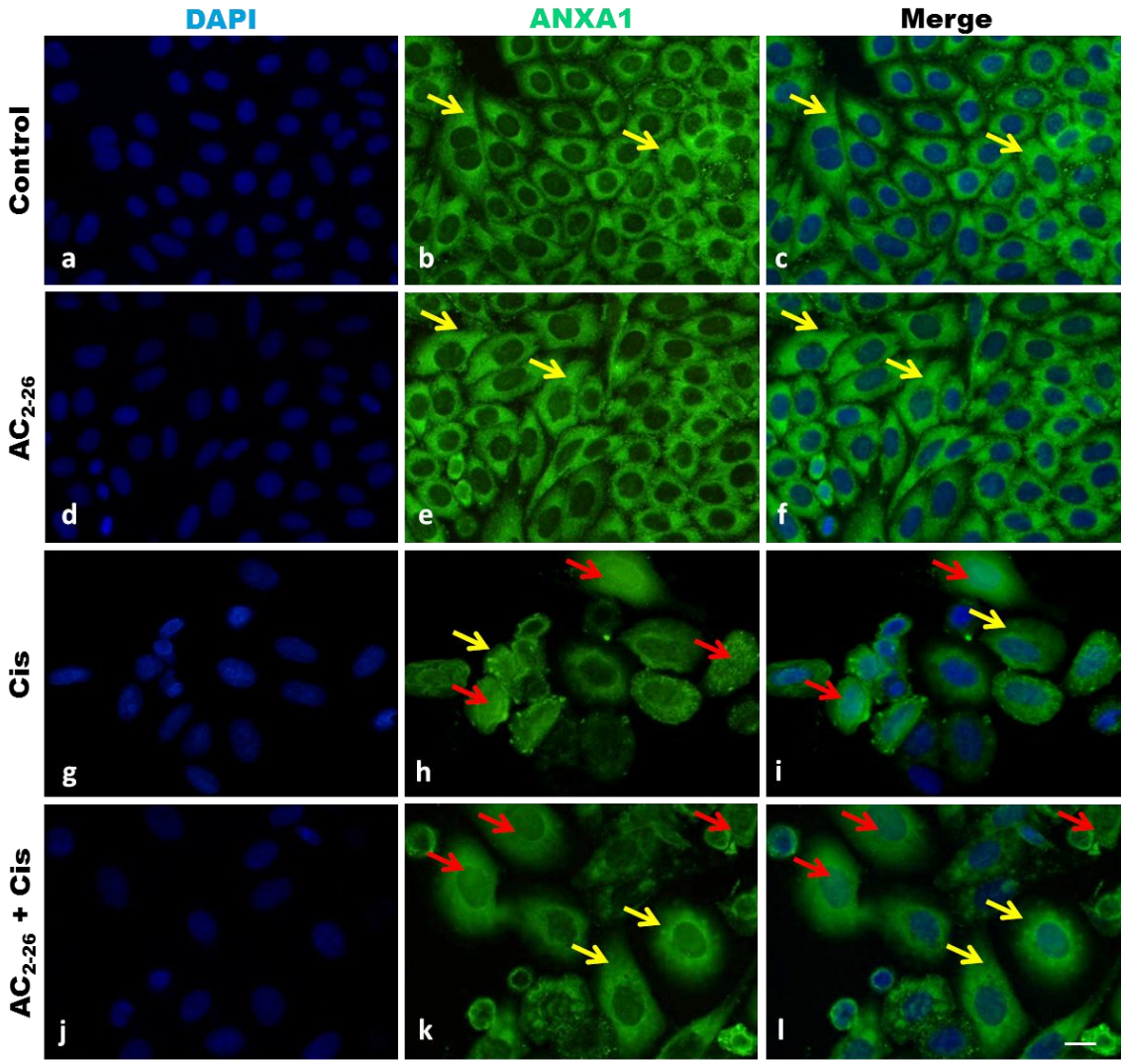

Figure 5

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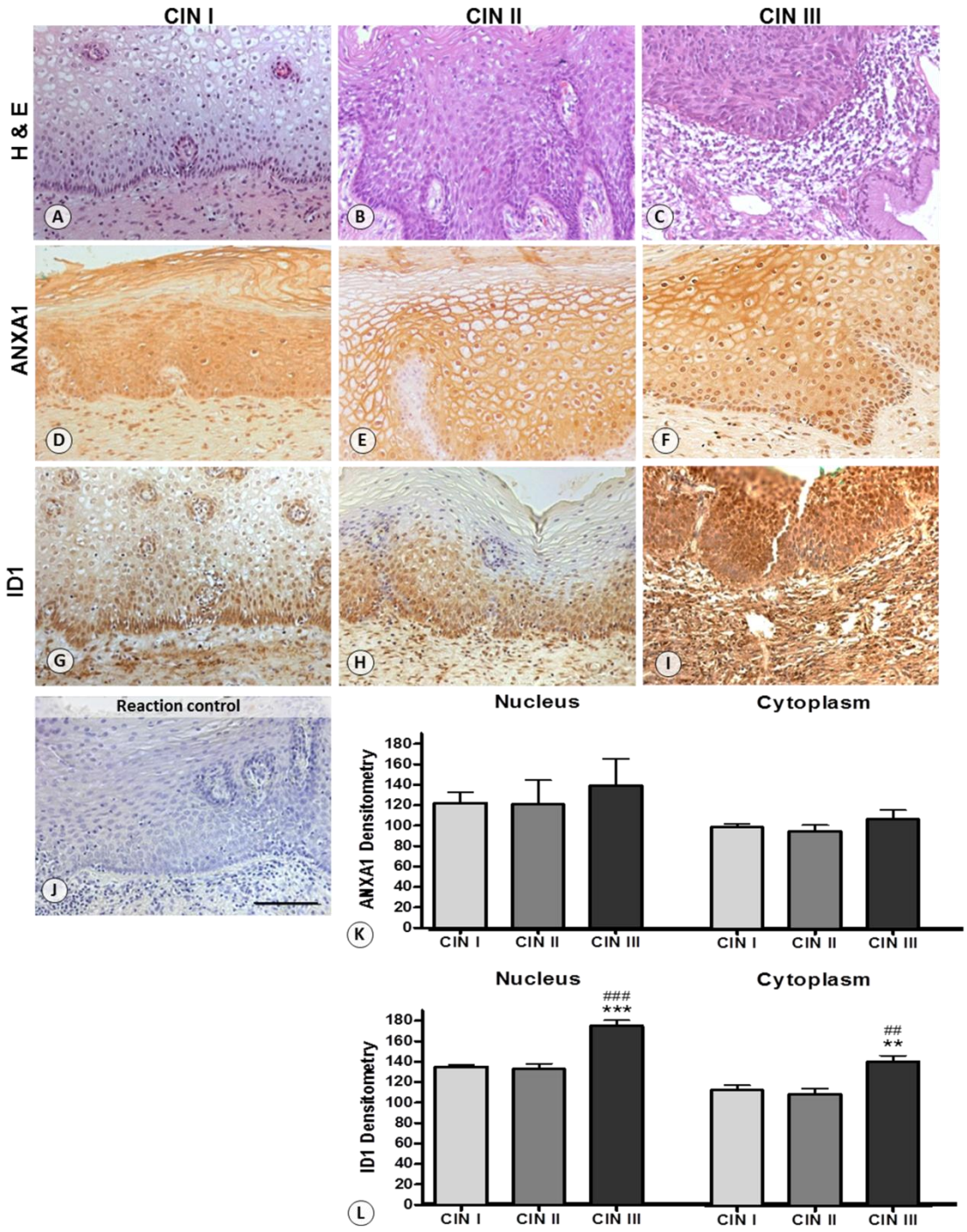

Figure 6

PRATES et al., 2018

4. DISCUSSÃO

O câncer de colo de útero é um problema de saúde pública (OGAWA et al., 2017; OGILVIE et al., 2017; INCA, 2018) e, entre as opções de tratamentos destacamos cirurgia, quimioterapia, com frequente uso da cisplatina (MONK; TEWARI; KOH, 2007) ou combinação de quimioterapia e radioterapia (MARCHETTI et al., 2017). Estudo recente do nosso grupo de pesquisa, envolvendo o processo tumorigênico em linhagem tumoral de carcinoma cervical SiHa, mostra que o tratamento com peptídeo mimético da proteína ANXA1 (AC_{2-26}) modula a expressão do gene Inibidor de diferenciação ou ligação ao DNA1 (ID1) (PRATES et al., 2015).Esses dados, que conduziram ao atual projeto, foram relacionados com o efeito da associação cisplatina e AC_{2-26} nas células SiHa. No presente estudo, mostramos que a administração da cisplatina induz o processo de apoptose pela modulação de ID1 e aumento da expressão da ANXA1, efeitos que podem revelar uma potencial abordagem terapêutica para o câncer de colo de útero.

Nas células SiHa, nossos estudos mostraram diminuição na proliferação e migração celular após tratamento com $AC_{2-26,}$ corroborando achados prévios realizados em nosso laboratório (PRATES et al., 2015). Resultados semelhantes foram obtidos nas células Hep-2, em câncer de laringe (SILISTINO-SOUZA et al., 2007) e nas células HaCat, HeLa e SiHa, com adição do meio condicionado, no estudo de microambiente tumoral em câncer de cérvice (CARDIN, 2017). Como esperado, as células tratadas com cisplatina também reduziram proliferação e migração. Esse quimioterápico, ao formar adutos no DNA, inibe a replicação celular e transcrição, além de promover o bloqueio do ciclo celular (YASUKAWA et al., 2016; OH et al., 2014; PÉREZ-ROJAS et al., 2016). Quando os tratamentos peptídeo e cisplatina foram associados, o efeito sobre a proliferação e migração celular não foi potencializado.

Considerando que a ação clássica da cisplatina é sinalizada para a morte celular, como observado nos carcinomas de cérvice (LI et al., 2014), mama (LI et al., 2015) e em linhagens de carcinoma gástrico (LI et al., 2012), nós investigamos as possíveis vias desse processo nas células SiHa . Com

esse objetivo, as expressões de mRNA e proteica da ANXA1 e ID1 foram avaliadas. O tratamento com AC_{2-26} não interfere na expressão dos genes estudados, enquanto a cisplatina, associada ou não ao peptídeo, induz aumento da expressão da ANXA1 e diminuição de ID1. A expressão aumentada de ANXA1, na linhagem SiHa, também foi observada por Calmon e colaboradores (2016), mostrando que a proteína aumenta o potencial carcinogênico mediada pela infecção do Papiloma Vírus Humano (HPV). Essa proteína foi apontada como regulador de resistência à cisplatina no adenocarcinoma de pulmão (WANG et al., 2014) e no carcinoma colorretal (ONOZAWA et al., 2017). Diferentemente, a diminuição da ANXA1 foi observada em linhagens tumorais de carcinoma nasofaríngeo, promovendo crescimento celular e bloqueio da apoptose (LIAO et al., 2018), assim como em pacientes com câncer de cérvice que receberam quimioterapia neoadjuvante (paclitaxel e cisplatina) (LIU et al., 2011).

Investigações têm mostrado que o aumento da ANXA1 exerce efeito antiproliferativo por meio da ativação da via de transdução de sinal ERK1/2 em diferentes linhagens celulares (ALLDRIDGE; BRYANT, 2003) e em células tumorais de mama (ANG et al., 2009). Com base nos dados da ação da ERK1/2, o passo subsequente deste trabalho foi investigar se os tratamentos propostos influenciam a apoptose pela fosforilação de ERK1/2 nas células SiHa. Nas análises proteicas realizadas, os grupos controle e AC_{2-26} apresentaram fosforilação de ERK1/2 (p-ERK1/2), sem aumento da apoptose. corroborando nossos resultados, a administração do peptídeo induziu a fosforilação de ERK1/2 em macrófagos (DUFTON et al., 2010), e aumentou significantemente sua expressão no aloenxerto de pele (TEIXEIRA et al.,2012). Por outro lado, estudo com roedores mostra que a expressão endógena elevada de ANXA1, ou tratamento com AC_{2-26}, diminui a ativação de ERK1/2 (VAGO et al., 2012). Enquanto, após os tratamentos Cis e AC_{2-26}+Cis, ocorre inibição da expressão de p-ERK1/2. Ação semelhante foi observada por Dang e colaboradores (2017) na linhagem celular de câncer ovariano após administração da cisplatina associada à metformina, mostrando redução na viabilidade celular e indução da apoptose pela inativação de p-ERK1/2. Os resultados obtidos neste trabalho sugerem que, na linhagem tumoral SiHa, a

cisplatina promove morte celular pela via clássica de sinalização MAPK, ao inativar ERK1/2.

Em função do entendimento relacionado com o aumento da expressão de ANXA1 nos grupos tratados com cisplatina, foi avaliada a localização intracelular dessa proteína nas células. Os resultados obtidos mostraram expressão citoplasmática da ANXA1 nos grupos controle e AC_{2-26}. Enquanto, após tratamentos Cis e AC_{2-26}+Cis, a expressão citoplasmática foi observada densa e positividade nuclear. Estes resultados apontam que a cisplatina, não somente aumenta a expressão da ANXA1 no citoplasma das células SiHa, como também no núcleo, como possível indução da translocação da ANXA1 em resposta aos danos de DNA induzidos por esse quimioterápico. Nosso grupo de pesquisa já havia detectado a expressão de ANXA1 na membrana plasmática, citoplasma e núcleo, por imunocitoquímica ultraestrutural, em neutrófilos após indução de peritonite experimental (OLIANI et al., 2001). Recentemente, Zhao e colaboradores (2018) demonstraram que o acúmulo de ANXA1 nuclear regula expressão de IL-1B por meio do *p65* e induz apoptose nas células de retina expostas a isquemia-reperfusão. Esses estudos relacionam essa proteína à apoptose (LI et al., 2016; ZHAO et al., 2018) e a capacidade de resposta aos danos no DNA (CHOI et al., 2012). Nossa hipótese, de que a cisplatina modula a expressão e localização intracelular da ANXA1, com direcionamento para morte celular, corrobora dados da literatura. No entanto, o exato papel fisiológico dessa translocação para o núcleo precisa de mais esclarecimentos.

Para complementar a investigação do efeito da cisplatina na resposta tumoral nas células SiHa, foi avaliada a expressão do oncogene *ID1*. Nossas análises mostram redução da expressão do *ID1* após tratamento com cisplatina, sugerindo envolvimento com o processo de apoptose. Estudos em carcinoma hepatocelular apontam que o silenciamento de *ID1* diminui a invasão de células (CHO et al., 2016), a proliferação celular e direciona para o processo apoptótico (YIN et al., 2017). Na literatura tem sido relatado que a supressão de *ID1* reduz a proliferação e migração das células no câncer de próstata (STRONG et al., 2013). Recentemente, Huang e colaboradores (2018)

demonstraram que o miR-381 interfere na ativação de NF-kB por meio da repressão de ID1, com consequente supressão do crescimento celular em câncer de pulmão de células não pequenas. Esses dados associados propõem que o gene *ID1* pode ser um potencial alvo terapêutico da regulação da via apoptótica em resposta ao quimioterápico cisplatina.

Os dados *in vitro* motivaram o estudo interdisciplinar que visa acelerar a troca bidirecional entre ciência básica e clínica para transferir resultados de pesquisas básicas para as aplicadas. Com este objetivo, biópsias de colo de útero de pacientes, com diferentes fases da neoplasia (Neoplasia Intraepitelial Cervical - NIC I a NIC III), foram coletadas para avaliar as expressões de ANXA1 e ID1.

As análises com a proteína ANXA1 não apresentaram expressão diferencial dentre os graus de neoplasia. Estudo do nosso laboratório mostrou aumento da ANXA1 em glândulas não diferenciadas de endométrio ectópico (PAULA et al., 2014), assim como outras investigações em tumores colo retal (YDY et al., 2016), colangiocarcinoma (HONGSRICHAN et al., 2013) e bexiga (LI et al., 2010). Ressaltando que no adenocarcinoma esofágico, a alta expressão apresentou relação com o avançado estágio tumoral, recorrência e pobre prognóstico, efeitos relacionados com a progressão tumoral (WANG et al., 2006). Enquanto, Wang e colaboradores (2008) avaliaram fragmentos de colo uterino nos diferentes estadiamentos da neoplasia, observando diminuição da expressão da ANXA1 em relação a progressão tumoral.

A expressão aumentada de ID1 foi observada nas biópsias das pacientes com alto grau de displasia (NIC III). Corroborando nossos achados, Schindl e colaboradores (2001) observaram elevado nível de ID1 em amostras de câncer de cérvice. Na carcinogênese endometrial, o nível de ID1 elevado é associado com o maior grau histológico e de invasividade (TAKAI et al., 2001). Nesse aspecto, resultados semelhantes foram obtidos em investigações de tumores de esôfago (LI et al, 2009), cólon (O'BRIEN et al., 2012) e próstata (PONZ-SARVISÉ et al., 2014; RICHTER et al., 2014). Com as considerações relacionadas com ID1 sugerimos que o aumento na expressão está associado

com a progressão tumoral das células de colo uterino, apresentando potencial alvo terapêutico.

Concluindo, nossos resultados fornecem melhor entendimento sobre o papel do oncogene *ID1* e *ANXA1* no processo tumorigênico, e adicional conhecimento do mecanismo de ação da cisplatina sobre a modulação desses genes na linhagem de carcinoma cervical. Esses achados podem delinear novas estratégias na intervenção terapêutica do câncer de colo de útero.

5. CONCLUSÕES

Os resultados obtidos nas células SiHa controle e tratadas com peptídeo AC_{2-26}, cisplatina (Cis) e associação peptídeo e cisplatina (AC_{2-26}+Cis), permitem concluir:

1. A proliferação e migração celular são reduzidas após os tratamentos e não são citotóxicos.

2. Os tratamentos com Cis e AC_{2-26}+Cis induzem as células à apoptose e necrose celular.

3. As expressões dos genes ANXA1 e ID1 e respectivas proteínas são moduladas após tratamentos Cis e AC_{2-26}+Cis.

4. A fosforilação de ERK1/2 é inibida após os tratamentos Cis e AC_{2-26}+Cis.

5. A localização intracelular de ANXA1 é citoplasmática em todos os tratamentos, e com positividade nuclear após Cis e AC_{2-26}+Cis.

6. As amostras teciduais, obtidas de biópsias de pacientes nos diferentes graus evolutivos de Neoplasia Intraepitelial Cervical (NIC), apresentam elevada expressão de ID1 em NIC III.

Associadas, essas conclusões mostram que a ANXA1 e o ID1 são mediadores importantes no desenvolvimento tumoral e o tratamento com o quimioterápico Cisplatina atua modulando esses mediadores. Desse modo, nossos resultados podem direcionar terapias inovadoras no tratamento de câncer de colo uterino.

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Conditioned medium of endothelial cells and annexin A1 peptide modulate cervical tumourigenesis

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Running title. Effect of the peptide Ac2-26 on the cervical tumourigenesis

Keywords: cervix cancer; ANXA1; carcinogenesis; inflammation; peptide treatment.

Disclosures

The authors have declared that no competing interests exist.

ABSTRACT

Cervical cancer is one of the leading causes of cancer death in women worldwide, and microenvironment influences can interfere with the tumour process. In the different phases of the tumour the inflammatory process can be observed, and the anti-inflammatory protein annexin A1 (ANXA1) has been associated with cancer progression and metastasis, suggesting its role in regulating tumor cell proliferation. The effect of the N-terminal peptide Ac2-26 of ANXA1 on the uterus carcinoma cells were evaluated using a conditioned medium of HUVEC cells (endothelium) (HMC) to conduct the co-culture with HaCaT cell line (normal) and HeLa cell line (cervical cancer). We performed in vitro experiments to further investigate the cellular proliferation, wound healing, viability and apoptosis assay, besides the modulation of gene expression related to inflammatory pathways, by quantitative PCR. Our results showed that the cervical cancer cells had a decrease in proliferation, while changes in cellular morphology, motility, and viability were not observed. The HeLa cells showed an increase in apoptotic cells, and an upregulation expression of MMP2, while COX2, EP3 and EP4 presented a downregulation after the HMC stimulus and Ac2-26 treatment. In conclusion, Ac2-26 treatment may modulate cellular and molecular mechanisms in the cervical carcinogenesis, and indicate this peptide as a possible therapeutic alternative, and its use should be better explored.

INTRODUCTION

Cervical cancer is one of the main gynaecological cancers around the world, and has been associated with the human papillomavirus (HPV) and/or bacterial infection, such as Chlamydia trachomatis and Neisseria gonorrhoeae [1,2]. Cervical neoplasm is associated with persistent HPV in more than 95% of cases, particularly in people with impaired immune system functions [2,3]. In overview, cervical cancer is the seventh most frequent type of tumour and fourth most common in women, disregarding non-melanoma skin cancer [2,4]. Diagnosis may be ascertained using the papanicolaou test, which assists in the identification of persistent inflammation, caused by the HPV, and in cases of positive results, the treatment may be initiated [5].

Besides infections, the cancer microenvironment also influences the tumour development process because it is very different from the corresponding health tissue [6]. The microenvironment has been characterised by its unsettled extracellular matrix composition, increased microvessel density, and abundance of inflammatory cells and fibroblasts with an activated phenotype [6–8]. There is evidence that the cancer progression and development does not rely only on the genetic characteristics, but also on the interaction between the cancer and stromal cells, including endothelial cells, immune cells, fibroblasts, adipocytes and inflammatory cells [6]. The different tumour phases that are associated with inflammation are considered an imminent threat, directly connected to the development process [9]. Therefore, the anti-inflammatory effects of annexin A1 (ANXA1) represent a key role in the modulation of the inflammatory response.

ANXA1, which is also expressed by the tumour cells, acts as a modulator of the inflammatory process and has been linked to the tumourigenesis process [10]. The studies involving ANXA1 and cancer are controversial, nevertheless they indicate that this protein may be a target for new therapeutic interventions, and used as a potential biomarker [11]. Their functions are specific to each type of cancer and there is evidence to indicate that its regulation and subcellular localisation have been linked to the development, invasion, metastasis, progression, and treatment resistance of tumours [12–15].

Structurally, the protein have a C-terminal core, representing 80% of its composition, this portion is common to all members of the Annexin superfamily, and has four repeated homologous sequences and the ‘type 2’ domain to calcium linkage [11]. The variable N-terminal core is unique in length and sequence to each member of the family, and includes potential phosphorylation, glycosylation and peptidase actions sites [16,17].

The biological activity of ANXA1 can be reproduced by the first 26 amino acids of the N-terminal core [18] or by some smaller peptides [19]. Since these studies, it has become common practice to use these molecules in experimental models of acute [20–23], chronic [24] and systemic inflammation [25], and also in vitro studies using different cancer cell lines [26–29]. Recently, the use of the peptide was evaluated in skin allograft [30], and in inflammatory ocular disease [21,31].

There are evidences of a relationship between ANXA1 expression and cervical tumourigenesis. To ascertain upregulation of the phosphorylated protein, according to the disease progression, samples from dysplasia and cervical cancer stages I, II, and III was used [32]. Other work showed that ANXA1 was downregulated in all stages of the disease [33], and another one analysing healthy, stages I, II, and III, and invasive cancer samples demonstrated that the protein expression levels corresponded to the disease progression [34].

The ANXA1 contributions to the tumourigenesis are still not well known, and considering its role in inflammation, it is an important area of research. The available data also points to the controversies in the expression of this protein in cervical carcinogenesis, indicating a possible research field.

Considering the important role of ANXA1 in the inflammatory response and tumour, we analysed the activity of the synthetic peptide of the ANXA1 protein in a cervical carcinoma cell line, along with the conditioned medium of endothelial cells, to help elucidating the processes in the tumour microenvironment, and, moreover, we intend to expand the understanding of ANXA1 as a therapeutic alternative.

RESULTS

Ac2-26 peptide response

The HaCaT cell line showed an increase in proliferation, after 72 hours (Figure 1A), and motility, closing the wound in the experiment, after 24 hours (Figure 1B). In the HeLa cell line it was possible to observe that the proliferation decreased after 2, 24, 48 and 120 hours (Figure 1A), while increased the motility after 24 and 48 hours (Figure 1B). The cytotoxicity was not observed in either cell lines in all experimental times analyzed (Figure 1C). The late apoptosis was decreased in both cell lines after the treatment (Figure 2A). The gene expression showed an upregulation of all genes analyzed in the HaCaT cell line, and of EP4, MMP2 and MMP9 in the HeLa cell line (Figure 2B).

Conditioned medium of Endothelial cells (HMC) and Ac2-26 peptide response

In the HaCaT cell line, the secreted factors of endothelial cells (HUVEC) without the peptide treatment (HMCS) increased the proliferation after 24 hours (Figure 3A). Associating the secreted factors of endothelial cells with the Ac2-26 treatment, it was possible to observe an increase of the proliferation in 48 and 120 hours, but a decrease in 72 hours (Figure 3B). The motility decreased after 24 hours in HaCaT cells (Figures 3C, 3D) after the induction with the conditioned medium without and with the treatment (HCMS and HCMT). Moreover, both conditions showed cytotoxicity to these cells only in 48 hours (Figures 3E, 3F).

Already in the HeLa cell line, it was showed a decrease in the proliferation after 24 hours (Figure 4A) by the secreted factors of endothelial cells without treatment (HMCS), while induction with HMC and the peptide treatment decreased the proliferation in 72 hours, but increased in 24 and 48 hours (Figure 4B). The motility increased after 4 hours in HeLa cells, after the induction with the conditioned medium without and with the treatment (HCMS and HCMT), excepted in 24 hours that showed a decreased after HMCT induction, but only statistically significant in 120 hours (Figures 4C, 4D). As in

the HaCaT cell line, HeLa cells showed cytotoxicity only in 48 hours (Figures 4E, 4F).

The late apoptotic cells was increased in the HaCaT cell line (Figures 5A, 5B) after the induction with the conditioned medium without and with the treatment (HCMS and HCMT), while decreased in the HeLa cells (Figures 5C) after the induction with the conditioned medium without the treatment (HCMS). It was also possible to observe, in the HeLa cells after the induction with the conditioned medium with the treatment (HCMT) an increase in apoptotic cells, however still showing more viable cells (Figures 5C, 5D).

The secreted factors of endothelial cells without the peptide treatment (HMCS) upregulated EP3, EP4 and MMP2 genes expression in the HaCaT cell line, while downregulated COX2, EP3 and EP4 genes and upregulated MMP2 gene expression in the HeLa cell line (Figure 6A).

The peptide treatment upregulated the COX2, EP3, EP4 and MMP9 genes expression in the HaCat cell line and also upregulated the EP4 and MMP9 genes expression in the HeLa cell line. Already the association of the secreted factors of endothelial cells with the Ac2-26 treatment upregulated MMP2 gene in HaCat cells and downregulated COX2, EP3 and EP4 genes expression in HeLa cells, but upregulated the MMP2 gene expression (Figure 6B).

DISCUSSION

Cervical cancer is related to HPV infection, and microbial infection is associated with 20-25% of the cancer cases worldwide [35]. It has been observed that the start of the carcinogenesis is more associated with persistent inflammation, as a result of infections or autoimmunity [36]. Besides the infections, the tumour microenvironment also plays a role in the tumourigenesis, due to its complex structure, in which different factors may influence and modify the cancer [37–39], and our experiments demonstrate this heterogeneity.

Our results demonstrate that the cervical cancer cells exhibit a decrease in growth when treated with the Ac2-26 peptide, and stimulated with the conditioned medium of endothelial cells. This is an important issue in our

understanding of the cancer of this protein, and these conditions also result in an increase in motility after 120 hours, and the maintenance of viability, the stimulation been cytotoxic only in 48 hours. Regarding the apoptosis and gene expression, it was observed an increase in apoptotic cells and the downregulation of COX2, EP3 and EP4, and upregulation of MMP2.

The pro and anti-inflammatory effects of ANXA1, a protein known to be an endogenous glucocorticoid, depends on the type of the ligand, and have been described previously [12]. ANXA1 protein was demonstrated to initiate the p38 mitogen activated pathway and the Ac2-26 peptide promotes the initiation of the c-Jun N-terminal protein kinase pathway (JNK) [40]. The MAPK family pathway is one of the most important cascades responsible for reading the extracellular stimulus and unleashes a response in the cells, and one of these pathways is JNK, a signalling cascade known to be activated by stress [41]. The JNK cascade may act phosphorylating different molecules, among them is the p53 [42]. Through this action, we could suggest that the proliferation decrease, after the Ac2-26 treatment and HMC stimulation, may be activating the MAPK family and targeting the p53, resulting in the cell cycle arrest.

The proliferation decrease found in this study is corroborated by [26,27], which also observed this phenomenon in cancer cell lines after treatment with ANXA1. One of the authors indicates that ANXA1 may act as a tumour suppressor, possibly acting on the ERK1/2 pathway, also a signalling cascade from MAPK family [27]. This response may result in overexpression of this pathway, which modifies the expression of the molecules involved in the cell cycle, resulting in arrest in the G1/S phase [43]. Thereby, adding to the suggestion that the ANXA1 protein may have an anti-proliferative role in the cervical cancer cells through the MAPK family, as observed after the peptide treatment and stimulation with HMC.

The cytotoxicity assay demonstrated that the cells were as viable as the healthy control cells. Therefore, although the proliferation decreased after stimulation with the HMC and peptide treatment, the cells remained viable, making it possible to proceed with the cellular processes. There was an association between NF-κB protein and ANXA1, suggesting that the protein

activate this pathway promoting metastasis [44], which could be a result of the maintenance of the motility.

The apoptosis analysis showed that the majority of the cancer cells were viable, but some were in apoptosis and late apoptosis. The cervical cancer apoptosis may be altered because of HPV18 infection. This virus has E6 and E7 domains, which express oncoproteins, and they are involved with p53 and retinoblastoma (Rb) degradation [45–47]. The E6 protein, specifically, can form a complex, which causes p53 polyubiquitination and its degradation by the proteosome [45,48]. The p53 gene is also associated with the control of apoptosis by regulating the expression of two genes, BCL2 (anti-apoptotic) and BAX (pro-apoptotic) [49]. ANXA1 is related to apoptosis regulation, according to [11], some research groups suggest the involvement of this protein as a pro-apoptotic factor, whereas other groups indicate its role as an anti-apoptotic factor. The activation of the MAPK pathways could also have an important role in the apoptotic process, and a range of anti-cancer substances activates the MAPK cascade with the aim to promote the apoptosis in the cancer cells [41]. Although there were a greater number of viable cells, we could observe an increase in apoptotic cells after the peptide treatment and stimulation with HMC.

There are studies with ANXA1 showing its pro and anti-inflammatory responses, also associating its expression with metastasis [50]. During the invasion process, it is suggested that a cell population, called ‘leader’ cells, invade the adjacent matrix, opening space to the following cells [51]. It is necessary that the ‘leader’ cells secrete factors in order to initiate the process, which will provide a path for the other cells; one of these factors is MMP [52]. [53] have observed that ANXA1 shows expression patterns related to specific tumour types, and they have associated the protein cleavage with melanoma cell aggressiveness. The same group showed that in melanoma cells, the administration of Ac2-26 peptide activates formyl peptide receptors (FPRs) and MMP2 gene expression [54]. In our current work, the level of MMP9 gene expression equalled to the control, after HMC stimulation and peptide treatment, and the upregulation of MMP2 was also observed in the healthy HaCaT cells,

therefore this pattern could be due to the secreted paracrine factors of the endothelium, possible as an attempt of restoring a healthy environment.

Pro-carcinogenic functions have been attributed to the COX2 gene, supporting the strategy of gene suppression; some of the processes related to COX2 are apoptosis inhibition, proliferation increase, and induction of angiogenesis [55–57]. The available literature shows that this gene may be triggered by different factors, for instance, bacterial lipopolysaccharide, interleukin (IL)-1β, IL-2, tumour necrosis factor α, epidermal growth factor, and transforming growth factor β; and it may be blocked by anti-inflammatory factors, such as corticosteroids, IL-13, IL-10, and IL-4 [55,58,59]. In this work, it was possible to observe that the peptide treatment and HMC stimulation downregulated the COX2 gene expression in the Cervical cancer cells. Prostaglandin E2 (PGE2) is one of the most abundant COX2 products due to the fact that PGE2 exists in all cell types [55]. Besides stimulation of pain and inflammation, PGE2 participates in proliferation, apoptosis, and metastasis mechanisms [60,61]. PGE2 initiates its actions through specific G-protein-coupled membrane receptors, namely, EP1, EP2, EP3, and EP4, with each of them initiating a specific cellular pathway [62]. The EP2 and EP4 receptors stimulate adenylate cyclase to increase cyclic adenosine monophosphate (cAMP) production, a glycogen synthase kinase-3 (GSK3) activator involved in the cell cycle [55]. Cyclic AMP promotes β-catenin phosphorylation, and degradation by the proteasome 26S, stopping the cell cycle [55]. The EP3 receptor has the opposite action to that described above; its interaction with PGE2 leads to the downregulation of adenylate cyclase and cAMP decrease [60].

The culture with HMC stimulation, in the HeLa cells, showed that the treatment did not alter cell motility, but it did result in a decrease in cell proliferation, and in COX2, EP3, and EP4 gene expression. The remaining production of PGE2 could be acting through the EP4 receptor, culminating in a decrease of cellular growth after treatment, which could be interfering with apoptosis, since a lower number of cells were observed in this cellular process.

Our research studied for the first time the interaction of ANXA1 with the conditioned medium of endothelial cells, an attempt to simulating the tumour microenvironment. It has been possible to observe the likely existence of a complex interaction, and the diverse array of secreted factors may influence cellular and molecular mechanisms in different ways. The diverse types of tumours exhibit different biological roles and signalling pathways when ANXA1 is involved, this contradiction is explained by the differentiated expression patterns and biological behaviours of this protein [11]. And also the roles of ANXA1, its calcium binding to the C-terminal core, which binds to the phospholipids, may determine the expression specificity and promote its functional diversity [16].

We suggest that along with the tumour cells secreted paracrine factors, ANXA1 diminishes proliferation, and does not alter the cellular cytotoxicity. It was possible to observe an increase in apoptotic cells and a downregulation of COX2, EP3 and EP4. Altogether, these data provide further insight into the protective effect of Annexin A1 and its mimetic peptides to the cervical tumourigenesis, and more research needs to be carried out to confirm its use.

MATERIALS AND METHODS

Cellular culture

Three cell lines were used, human umbilical vein endothelial cell (HUVEC) line, human immortalized keratinocytes (HaCaT) and human cervical adenocarcinoma cells infected with HPV18 (HeLa). The HUVEC cell line were cultured in MEM-Earle medium (Cultilab, Br), pH 7.5, supplemented with 10% foetal bovine serum (BSA) (Cultilab, Br), 1% antibiotic/antimycotic (Invitrogen, Carlsbad, USA, UK), and 1% L-glutamine (200 µM) (Sigma Aldrich, USA). The HaCaT cell line were cultured in MEM-Earle medium (Cultilab, Br), pH 7.5, supplemented with 10% foetal bovine serum (BSA) (Cultilab, Br), 1% antibiotic/antimycotic (Invitrogen, Carlsbad, USA, UK) 1% L-glutamine (200 µM) (Sigma Aldrich, USA), 1% non-essential amino acids 10mM (Sigma Aldrich, USA), and 1% sodium pyruvate 100 mM (Sigma Aldrich, USA). The HeLa cell line were cultured in MEM-Earle medium (Cultilab, Br), pH 7.5, supplemented

with 10% foetal bovine serum (BSA) (Cultilab, Br), 1% antibiotic/antimycotic (Invitrogen, Carlsbad, USA, UK) 1% L-glutamine (200 µM) (Sigma Aldrich, USA), and 1% non-essential amino acids 10mM (Sigma Aldrich, USA). A total of 106 cells from each cell line were seeded in 75 cm2 culture flasks and kept at 37 °C in an atmosphere of 5% CO2.

Cellular co-culture

The co-culture was performed with the conditioned medium of the HUVEC (HMC) cell line, which was used in the co-cultures with the other two cell lines. The HMC was obtained after the HUVEC culture and when the cells had acquired 80% confluence. The medium (HCM) was collected after 24 hours of culture, from the HUVEC cell line medium without treatment (HMCS) and with treatment (HMCT), the treatment was performed with the N-terminal Ac2-26 peptide of ANXA1 (Ac-MVSEFLKQAWFIENEEQEYVQTVK) [63], at a concentration of 10 µg/mL [27]. The co-culture was performed at a 1:1 dilution, according to Rodrigues-Lisoni et al. (2010).

Pharmacological treatment

The cell lines were cultured in complete medium, as described above, and subsequently, they were submitted for co-culture with HMC and treated with the N-terminal Ac2-26 peptide of ANXA1 (Ac-MVSEFLKQAWFIENEEQEYVQTVK) [63], at a concentration of 10 µg/mL for six different time points (2, 4, 24, 48, 72 and 120 hours) to perform the proliferation, wound healing, and cytotoxicity assays. After the analysis of these experiments one time point was chosen to continue the other assays. The experimental groups are described below:

- HaCaT
- HaCaT + Ac2-26
- HaCaT + HMCS
- HaCaT + Ac2-26 + HMCT
- HeLa
- HeLa + Ac2-26
- HeLa + HMCS
- HeLa + Ac2-26 + HMCT

This study was performed to analyze the response of Ac2-26 peptide treatment in cancer cell line, in that way we develop the experiments

only with the cells and the peptide. After analyzing the results and with the attempt of mimicking the tumour microenvironment we added the conditioned medium of endothelial cells (HMCS and HMCT), along with the peptide treatment, and observed if in these conditions the peptide would exhibit different results.

Proliferation assay and cellular morphology analysis

To analyse the cell proliferation in the HaCaT and HeLa cells, a growth curve was performed. To count the number of cultured cells, they were seeded at a concentration of 3 x 104 in 1 mL of complete medium for 24 hours. After this period, the medium was replaced with a serum free medium, with the purpose of maintaining the same cellular phase. After a further 24 hours, this medium was replaced again with complete medium, with the addition of specific HMC and Ac2-26 peptide, according to the experimental groups. The cells were analysed and counted at six different time points (2, 4, 24, 48, 72 and 120 hours). The cellular morphology was evaluated with inverted microscopy, using an Olympys CKX41.

Wound healing assay

The HaCaT and HeLa cells were distributed in 12-well plates, after reaching adherence and confluence, a wound was made in the centre of the well. Subsequently, the cells were subdivided according to the experimental groups and analysed at 0, 2, 4, 24, 48, 72, and 120 hours. The cellular motility was monitored with images obtained using a photographic camera coupled to the microscope. The wound areas were determined in six different microscopic fields and quantified using the 'Image J – Image Processing and Analysis in Java' software.

Cytotoxicity and viability assay (MTS)

The cells were subdivide into the experimental groups and handled according to the manufacture's protocol from the CellTiter 96®AQUeous One Solution Cell Proliferation Assay (Promega), which uses the tetrazolium

compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS], and an electron coupling reagent [Phenazine ethosulfate (PES)]. MTS reagent was added and absorbance at 490 nm was recorded using ELISA plate reader. The analyses were made at the time of 2, 4, 24, 48, 72, and 120 hours, and were evaluated by comparing the cellular viability across the experimental groups, as well as the IC50 calculus (inhibitory concentration to 50% of the cells).

Flow cytometry

The cells were subdivided properly into the experimental groups and handled according to the manufacture's protocol for the FITC Annexin V Apoptosis Detection Kit I (BD PharmingenTM), to investigate viability, apoptosis, late apoptosis, and necrosis, and utilised the Guava Easy Cyte (MILLIPORE) equipment to perform the analysis.

Selection of the genes

The genes were previously selected from potential markers in the tumour microenvironment, evaluated in the studies of [64,65]. The metabolic pathways from these potential markers were evaluated and some related genes were also chosen. The gene selections took into consideration those that were potentially involved in the tumour invasion and inflammatory processes.

The genes selected were: prostaglandin-endoperoxide synthase 2 (PTGS2), also known as cyclooxygenase 2 (COX2); prostaglandin E receptor 3 (EP3 or PRGER3); prostaglandin E receptor 4 (EP4 or PTGER4); matrix metallopeptidase 2 (MMP2); matrix metallopeptidase 9 (MMP9); and TIMP metallopeptidase inhibitor 1 (TIMP1). The specific primers for each transcript were designed with Primer3 (http://frodo.wi.mit.edu/cgibin/primer3/primer3.cgi), as shown in table 1.

Quantitative real time PCR

The reactions were performed in the 7500 Fast Real-Time PCR system thermocycler (Applied Biosystems), all the reactions were carried out in a final

volume of 20 µL with 100 ng of cDNA, SYBR® Green PCR Master Mix, and 100 nM of each primer (F and R). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was the best endogenous control gene when tested alongside ACTB (Actin beta). The analyses were performed from the cycle threshold (Ct) of each sample accordingly [66].

Statistical analysis

All assays were performed with three independent experiments. Graph Pad version 6.0 was used to perform all statistics. The Kolmogorov–Smirnov normality test was used to analyze distribution. Variance analysis (ANOVA) was used for between group comparisons, followed by the appropriate parametric or non-parametric post-hoc test. Statistical significance was set at a probability value of less than 0.05.

ACKNOWLEDGMENT

This work was supported by São Paulo Research Foundation – FAPESP (2013/24083-2 to FCR-L and 2016/02012-4 to SMO); National Council for Scientific and Technological Development – CNPq (Grant 208144/2014-7 to SMO); Coordination for the Improvement of Higher Education Personnel – CAPES for the scholarships (Postgraduation in Brazil and PDSE 99999.004402/2014-06) to LTC. We thank the PhD Egle Solito, from Queen Mary University of London, for the constant support, the supervision during the Sandwich Doctoral degree, and the professional development of LTC. We thank the Dra. Jusciéle Brogin Moreli, from São Paulo Federal University (Unifesp), for all the technical support.

AUTHOR CONTRIBUTION

LTC designed and performed all experiments and wrote the manuscript. JP contributed to the analysis of the flux cytometry. BRC performed the experiments to the quantitative PCR. EHT contributed to write the manuscript. SMO supported the experiments development and contributed to write the

manuscript. FCR-L coordinated the project, contributed in the experiments and write of the manuscript.

ABBREVIATIONS

HPV, human papilloma; ANXA1, Annexin A1 protein; Ac2-26, peptide of ANXA1; HMC, Conditioned medium of HUVEC cells; HMCS, non-treated conditioned medium of HUVEC cells; HMCT, treated conditioned medium of HUVEC cells; MAPK, mitogen-activated protein kinase pathway; ERK, extracellular signal-related kinases; E6 and E7, oncoproteins in the genome of the HPV16 and HPV18 virus; p53, tumor protein p53; BCL2, apoptosis regulator gene; BAX, BCL2 associated X apoptosis regulator; FRP, formyl peptide receptor.

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TABLE

Table 1. Primers to the studied genes

Oligonucleotide	Sequencia
COX2 anti-sense	5´ AGAAGGCTTCCCAGCTTTTG 3´
COX2 sense	5´ ATTCCCTTCCTTCGAAATGC 3´
EP3 anti-sense	5’ TCTCCGTGTGTGTCTTGCAG 3’
EP3 sense	5’ AGCTTATGGGGATCATGTGC 3’
EP4 anti-sense	5’ CCAAACTTGGCTGATATAACTGG 3’
EP4 sense	5’ CGAGATCCAGATGGTCATCTTAC 3’
MMP2 anti-sense	5’ CCGTCAAAGGGGTATCCATC 3’
MMP2 sense	5’ AAGTCTGGAGCGATGTGACC 3’
MMP9 anti-sense	5’ ATTTCGACTCTCCACGCATC 3’
MMP9 sense	5’ TTGTGCTCTTCCCTGGAGAC 3’
TIMP1 anti-sense	5´ TTTTCAGAGCCTTGGAGGAG 3´
TIMP1 sense	5´ ACTGTTGGCTGTGAGGAATG 3´

FIGURE LEGENDS

Figure 1. Response of Ac2-26 peptide treatment in HaCaT and HeLa cell lines in the proliferation, motility and citotoxicity. The cells were cultured in complete MEM medium and treated with Ac2-26 [10 µg/mL]. A. Proliferation; B. Motility; C. Cytotoxicity. Graphs with x = time (hours) and A y = number of cells x 104; B y = occupied area (percentage); C y = viability (percentage). P values < 0.05 were significant, 1 symbol = $p < 0.05$; 2 symbols $p < 0.01$; 3 symbols = $p < 0.001$. Symbols: * vs. HaCaT; # vs HeLa. Assays were performed with three independent experiments.

Figure 2. Response of Ac2-26 peptide treatment in HaCaT and HeLa cell lines in the apoptosis assay and gene expression. The cells were cultured in complete MEM medium and treated with Ac2-26 [10 µg/mL]. A. Apoptosis; B. gene expression. Graphs with A x = cellular process and y = cell

percentage in 10.000 events; B x = genes and y = logarithm base 2. P values < 0.05 were significant, 1 symbol = p < 0.05; 2 symbols p < 0.01; 3 symbols = p < 0.001. Symbols: * vs. HaCaT; # vs HeLa. Assays were performed with three independent experiments.

Figure 3. Response of Conditioned Medium induction and Ac2-26 peptide treatment in HaCaT cell line. The cells were cultured in complete MEM medium and stimulated with conditioned HUVEC cell medium (HMC), untreated (HMCS) (A-C-E) or treated (HMCT) (B-D-F) with Ac2-26, at a ratio of 1:1, and treated with Ac2-26 [10 µg/mL] (B-D-F). A-B. HaCaT Proliferation; C-D. HaCaT Motility; E-F. HaCaT Cytotoxicity. Graphs with x = time (hours) and A-B y = number of cells x 104; C-D y = occupied area (percentage); E-F y = viability (percentage). P values < 0.05 were significant, 1 symbol = p < 0.05; 2 symbols p < 0.01; 3 symbols = p < 0.001. Symbols: A-C-E * vs. HaCaT; B-D-F * vs HaCaT + Ac2-26. Assays were performed with three independent experiments.

Figure 4. Response of Conditioned Medium induction and Ac2-26 peptide treatment in HeLa cell line. The cells were cultured in complete MEM medium and stimulated with conditioned HUVEC cell medium (HMC), untreated (HMCS) (A-C-E) or treated (HMCT) (B-D-F) with Ac2-26, at a ratio of 1:1, and treated with Ac2-26 [10 µg/mL] (B-D-F). A-B. HeLa Proliferation; C-D. HeLa Motility; E-F. HeLa Cytotoxicity. Graphs with x = time (hours) and A-B y = number of cells x 104; C-D y = occupied area (percentage); E-F y = viability (percentage). P values < 0.05 were significant, 1 symbol = p < 0.05; 2 symbols p < 0.01; 3 symbols = p < 0.001. Symbols: A-C-E * vs. HaCaT; B-D-F * vs HaCaT + Ac2-26. Assays were performed with three independent experiments.

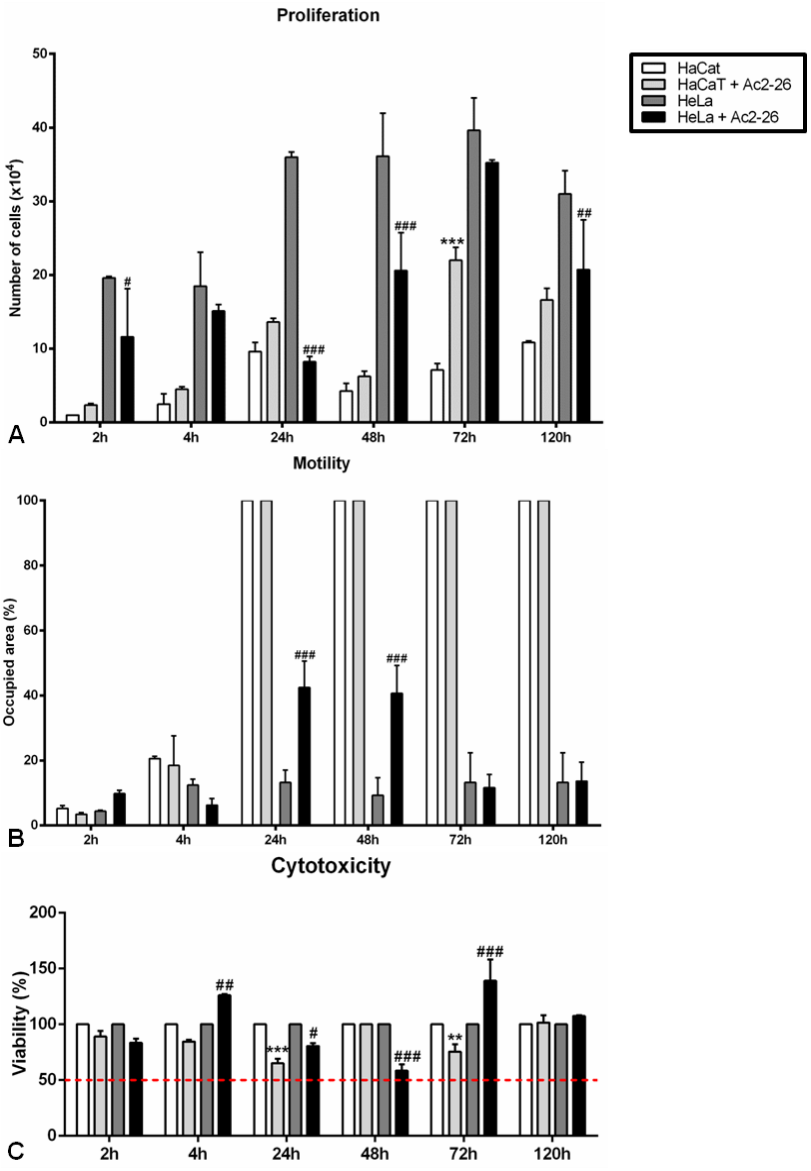
Figure 5. Response of Conditioned Medium induction and Ac2-26 peptide treatment in HaCaT and HeLa cell lines. The cells were cultured in complete MEM medium and stimulated with conditioned HUVEC cell medium (HMC), untreated (HMCS) (A-C) or treated (HMCT) (B-D) with Ac2-26, at a ratio of 1:1, and treated with Ac2-26 [10 µg/mL] (B-D). A-B. HaCat Apoptosis; C-D.

HeLa Apoptosis. Graphs with x = cellular process and y = cell percentage in 10.000 events. P values < 0.05 were significant, 1 symbol = p < 0.05; 2 symbols p < 0.01; 3 symbols = p < 0.001. Symbols: A-C * vs. Control of each group; B-D * vs Control + Ac2-26 of each group. Assays were performed with three independent experiments.

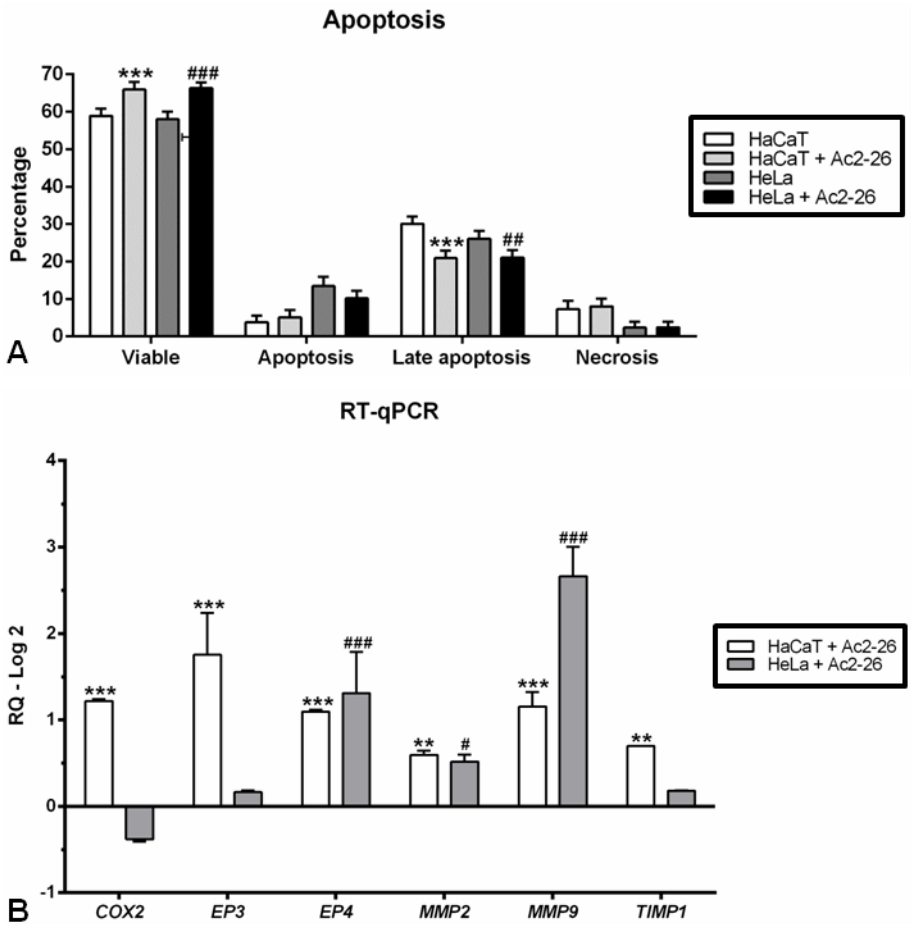
Figure 6. Response of Conditioned Medium induction and Ac2-26 peptide treatment in HaCaT and HeLa cell lines. The cells were cultured in complete MEM medium and stimulated with conditioned HUVEC cell medium (HMC), untreated (HMCS) (A) or treated (HMCT) (B) with Ac2-26, at a ratio of 1:1, and treated with Ac2-26 [10 µg/mL] (B). A. Gene expression after HMCS induction; B. Gene expression after Ac2-26 treatment and HMCT induction. Graphs with x = genes and y = logarithm base 2. P values < 0.05 were significant, 1 symbol = p < 0.05; 2 symbols p < 0.01; 3 symbols = p < 0.001. Symbols: A * vs. HaCaT; # vs HeLa; B * vs. HaCaT; $ vs HaCaT + Ac2-26; # vs HeLa; £ vs HeLa + Ac2-26. Assays were performed with three independent experiments.

FIGURES

1.


Proliferation
HaCat
HaCaT + Ac2-26
HeLa
HeLa + Ac2-26
Number of cells (x10⁴)
A
2h
4h
24h
48h
72h
120h
Motility
Occupied area (%)
B
Cytotoxicity
Viability (%)
C

2.


Apoptosis
Percentage
70
60
50
40
30
20
10
0
***
###
***
##
HaCaT
HaCaT + Ac2-26
HeLa
HeLa + Ac2-26
Viable
Apoptosis
Late apoptosis
Necrosis
A
RT-qPCR
RQ - Log 2
4
3
2
1
0
-1
***
***
***
###
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#
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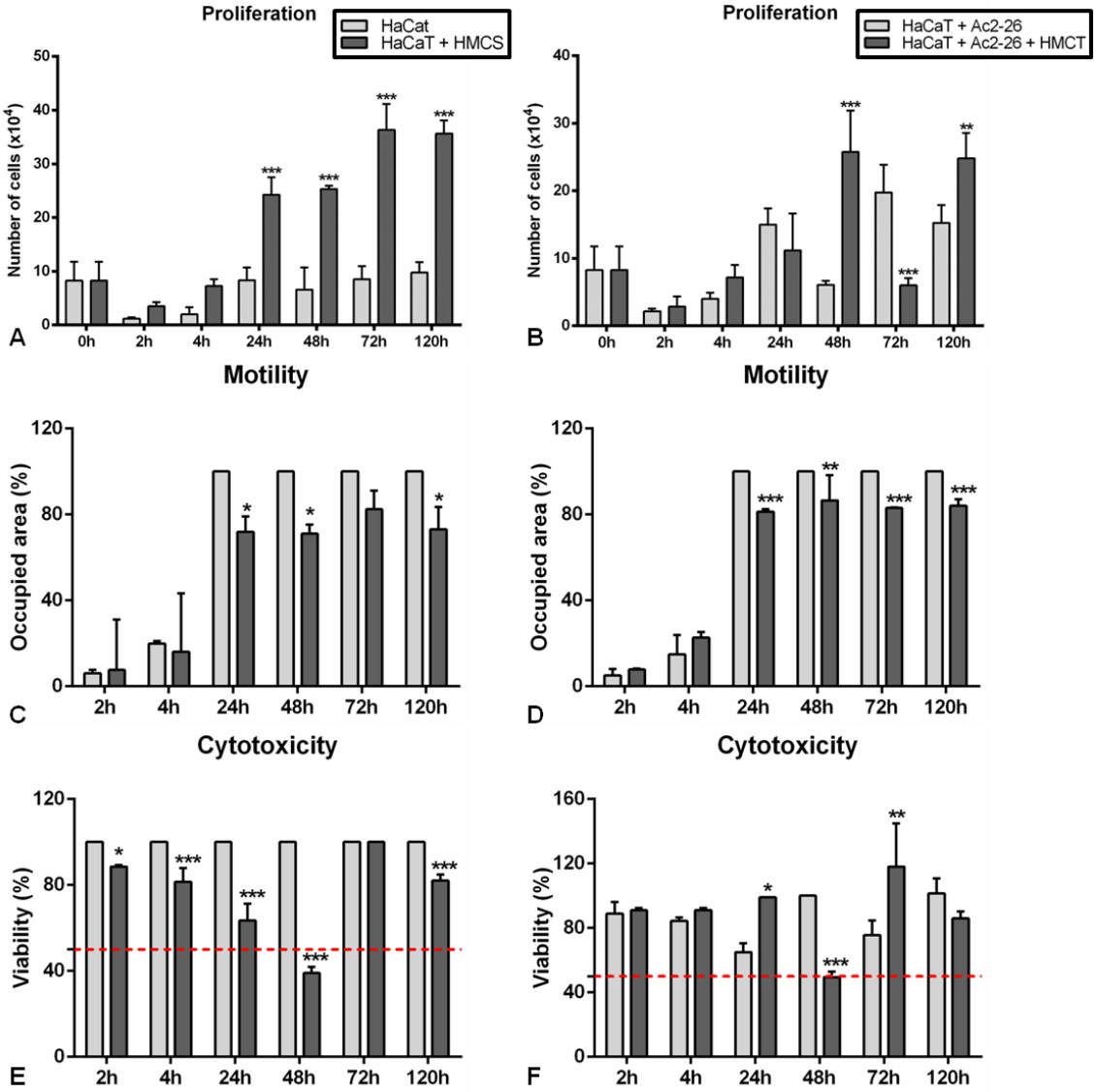
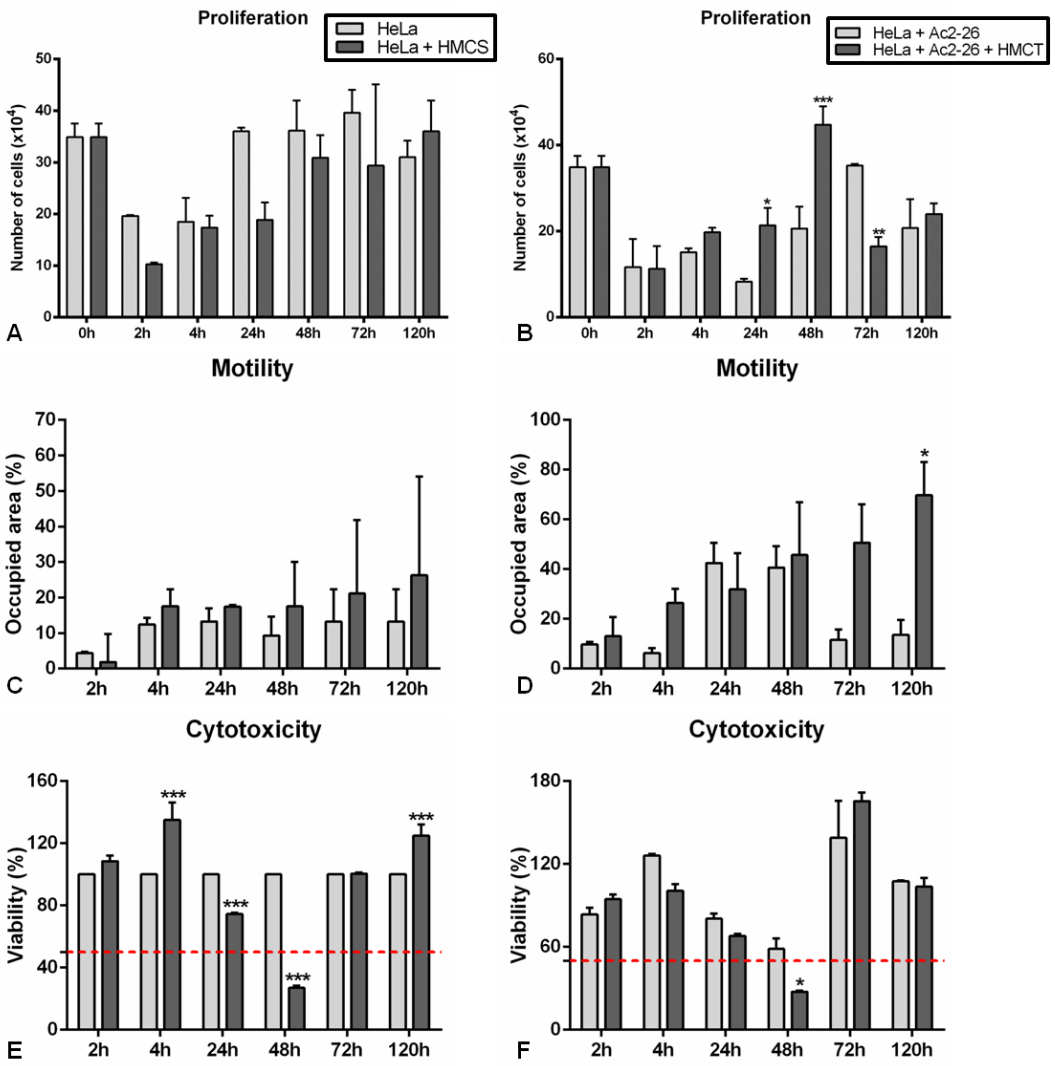
###
**
HaCaT + Ac2-26
HeLa + Ac2-26
COX2
EP3
EP4
MMP2
MMP9
TIMP1
B

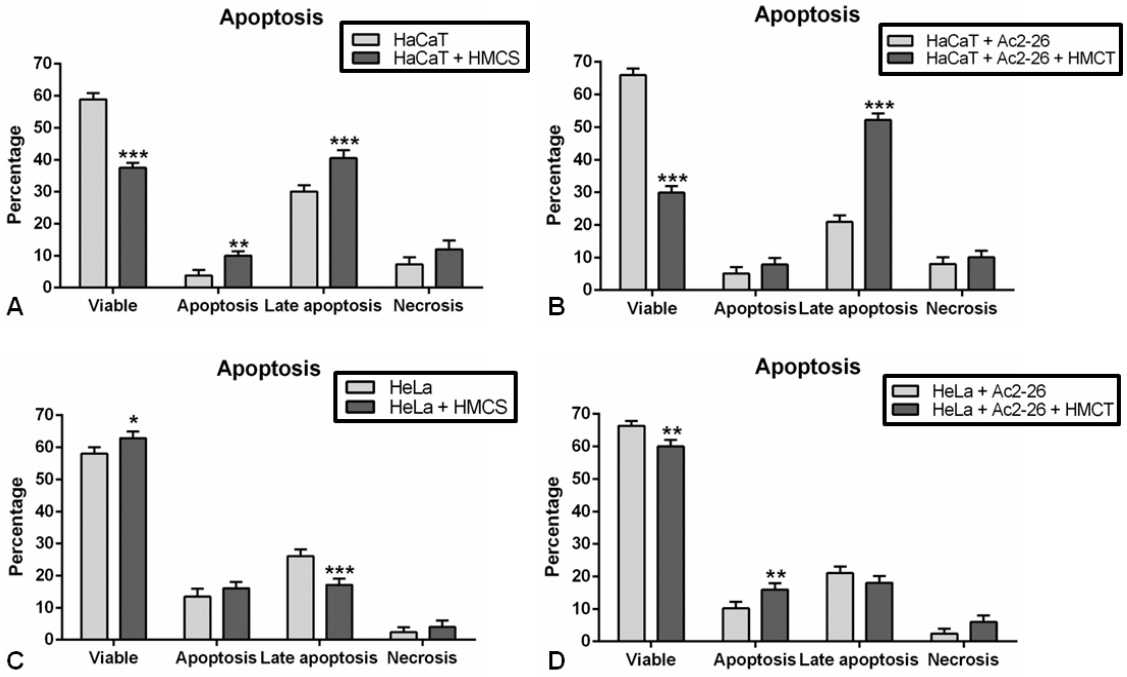
3.


Proliferation
HaCat
HaCaT + HMCS
Number of cells (x10⁴)
A
0h
2h
4h
24h
48h
72h
120h
Motility
Proliferation
HaCaT + Ac2-26
HaCaT + Ac2-26 + HMCT
Number of cells (x10⁴)
B
Motility
Occupied area (%)
C
Cytotoxicity
Occupied area (%)
D
Cytotoxicity
Viability (%)
E
Viability (%)
F

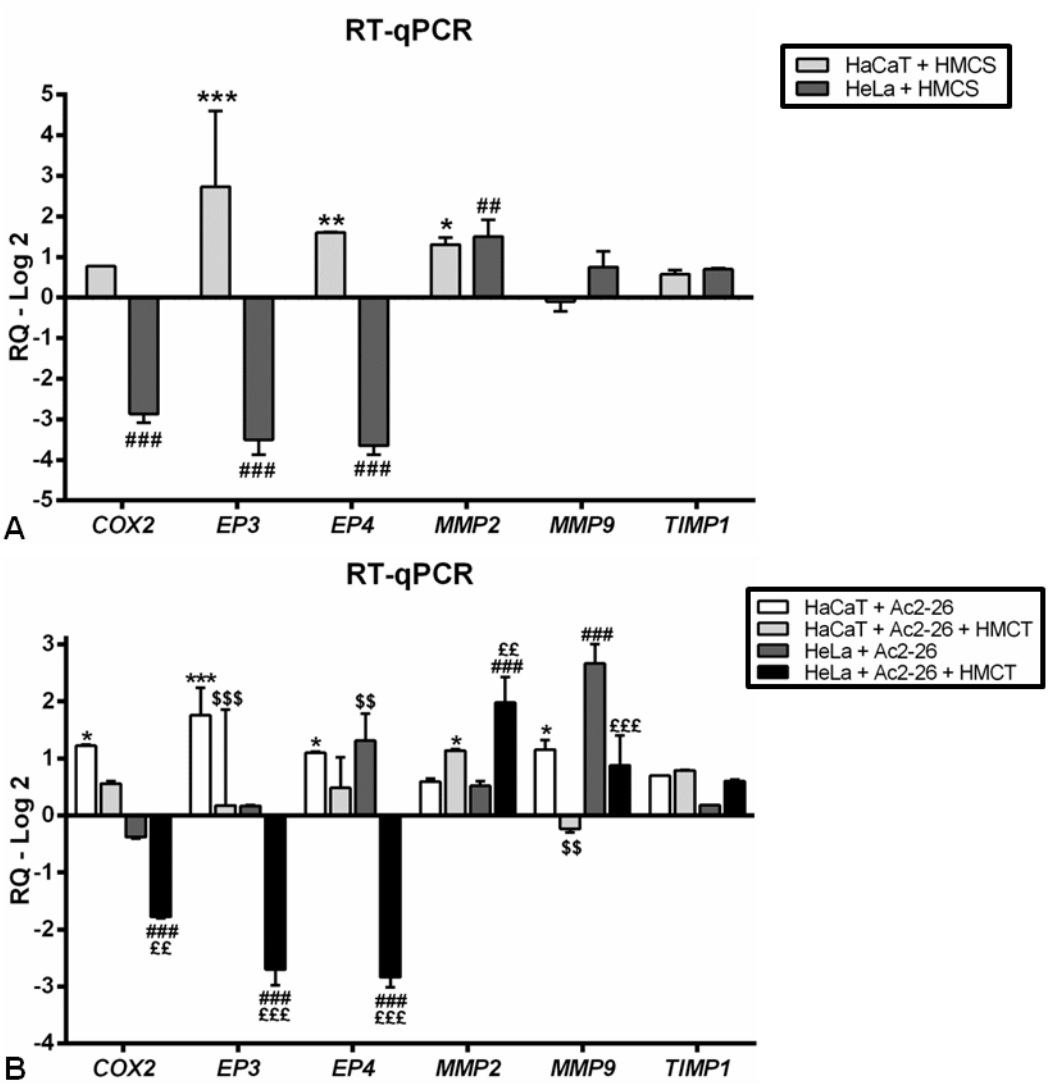
4.


Proliferation
HeLa
HeLa + HMCS
Number of cells (x10⁴)
A
Proliferation
HeLa + Ac2-26
HeLa + Ac2-26 + HMCT
Number of cells (x10⁴)
B
0h
2h
4h
24h
48h
72h
120h
Motility
Occupied area (%)
C
D
Cytotoxicity
Viability (%)
E
F

5.


Apoptosis
HaCaT
HaCaT + HMCS
Percentage
Viable
Apoptosis
Late apoptosis
Necrosis
A
Apoptosis
HaCaT + Ac2-26
HaCaT + Ac2-26 + HMCT
Percentage
Viable
Apoptosis
Late apoptosis
Necrosis
B
Apoptosis
HeLa
HeLa + HMCS
Percentage
Viable
Apoptosis
Late apoptosis
Necrosis
C
Apoptosis
HeLa + Ac2-26
HeLa + Ac2-26 + HMCT
Percentage
Viable
Apoptosis
Late apoptosis
Necrosis
D

6.


RT-qPCR
HaCaT + HMCS
HeLa + HMCS
RQ - Log 2
COX2
EP3
EP4
MMP2
MMP9
TIMP1
A
RT-qPCR
HaCaT + Ac2-26
HaCaT + Ac2-26 + HMCT
HeLa + Ac2-26
HeLa + Ac2-26 + HMCT
RQ - Log 2
COX2
EP3
EP4
MMP2
MMP9
TIMP1
B

ANEXO 2

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PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Interação funcional e molecular da proteína anexina A1 e do quimioterápico cisplatina no carcinoma de colo de útero

Pesquisador: Sonia Maria Oliani

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 3

CAAE: 46392515.0.0000.5466

Instituição Proponente: Instituto de Biociências Letras e Ciências Exatas/ Campus de São José do

Patrocinador Principal: MINISTERIO DA CIENCIA, TECNOLOGIA E INOVACAO

DADOS DO PARECER

Número do Parecer: 1.412.751

Apresentação do Projeto:

Aproximadamente 35% das mulheres diagnosticadas com carcinoma de colo uterino têm a doença recorrente, com 90% desses achados nos três anos após o tratamento inicial. Entre os fatores de risco para o câncer de colo uterino pode ser destacado a infecção pelo Papilomavírus Humano (HPV). Fatores de risco estão relacionados com características tanto do hospedeiro quanto do vírus, como exposição ao HPV, oncogenicidade viral, ineficiência da resposta imune e presença de cocarcinógenos Outros fatores também contribuem. A carcinogênese de colo de útero também está relacionada com processos de angiogênese e inflamatórios. Os tratamentos de câncer, em geral, utilizam a cisplatina como quimioterápico. A proteína anti-inflamatória anexina A1 (ANXA1) tem sido associada com a progressão em alguns tumores invasivos, sugerindo um papel na regulação da migração/invasão das células epiteliais. No entanto, os mecanismos moleculares pelos quais a ANXA1 modula essas respostas celulares não são completamente determinados. Os dados disponíveis sugerem que essa proteína pode ter um envolvimento significativo no câncer, por meio de cascatas de sinalização que incluem genes relacionados com o ciclo celular, diferenciação e apoptose. O projeto proposto envolverá as linhagens de células do colo do útero SiHa (carcinoma) e Ect1/E6E7 (normal) após tratamentos com a ANXA1 e do quimioterápico

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Continuação do Parecer: 1.412.751

cisplatina, com o objetivo de entender os mecanismos funcionais e moleculares de interação que envolvem a ANXA1 e o gene ID1. Poucos estudos têm investigado a proteína no câncer de colo de útero, assim como o efeito da administração concomitante da ANXA1 e quimioterápico. O entendimento dos processos que controlam a tumorigênese é essencial para o desenvolvimento de mediadores farmacológicos eficazes no tratamento dos processos neoplásicos. O projeto será desenvolvido no Laboratório de Imunomorfologia do IBILCE/UNESP e, em colaboração com a coordenadora do Laboratório de Biomarcadores Moleculares e Bioinformática Médica, da Faculdade de Medicina de Rio Preto (FAMERP) e profissional do campus de Ilha Solteira/UNESP.

Objetivo da Pesquisa:
Investigar, in vivo e in vitro, a interação da ação anti-inflamatória/antiproliferativa da ANXA1 e do quimioterápico cisplatina nas células carcinogênicas e interação desses mediadores no câncer de cérvix humano. Experimentos in vivo: Nas amostras de câncer de colo de útero avaliar: Expressão das proteínas endógenas ANXA1 e Id1 por meio da técnica de imuno-histoquímica (tecidos incluídos em parafina); Expressão dos genes ANXA1 e ID1, pela técnica de PCR quantitativa (amostras cirúrgicas); correlacionar a expressão protéica e gênica diferencial com o desenvolvimento do tumor. Experimentos in vitro: Nas células das linhagens SiHa e Ect1/E6E7 tratadas com peptídeo ANXA12-26, cisplatina e ANXA12-26 + cisplatina avaliar: Morfologia, índice de proliferação e migração celular por análise na microscopia de luz; Ciclo celular, analisando viabilidade, proliferação celular e apoptose, por citometria de fluxo; Expressão protéica diferencial pela tecnologia de proteômica; Expressão das proteínas endógenas ANXA1 e Id1 por Western blotting; Expressão dos genes ANXA1 e ID1, por PCR quantitativa; As vias de sinalização celular, das quais ANXA1 e ID1 participam, com auxílio dos bancos de dados já existentes.

Avaliação dos Riscos e Benefícios:
Apresentação e avaliação adequada de riscos e benefícios em projeto e faz referência a estes em TCLE de forma adequada. Riscos para as pacientes: as biópsias incluídas em parafina foram anteriormente coletadas por equipe médica e, após, armazenadas no arquivo do Serviço de Patologia. Enquanto, as amostras cirúrgicas serão coletadas em centro cirúrgico, com equipe médica especializada, seguindo os protocolos de padrões hospitalares e sem riscos previsíveis adicionais. Nesse projeto atual, apenas os fragmentos cirúrgicos obtidos serão utilizados para as análises experimentais e não serão incluídos em biobanco/biorepositório. Os benefícios dessa investigação estão relacionados ao entendimento do papel da ANXA1 no desenvolvimento do processo tumoral, com perspectiva do uso da proteína como marcador prognóstico ou alvo

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terapêutico

Comentários e Considerações sobre a Pesquisa:

Define equipe responsável pelo desenvolvimento do projeto, que será desenvolvido no Laboratório de Imunomorfologia do IBILCE/UNESP e, em colaboração com as Profas. Dra. Eloiza Helena Tajara da Silva, coordenadora do Laboratório de Biomarcadores Moleculares e Bioinformática Médica, da Faculdade de Medicina de Rio Preto (FAMERP) e Dra. Flavia Cristina Rodrigues Lisoni (UNESP, câmpus de Ilha Solteira). Deixa expresso em projeto e TCLE que a coleta da amostra em colo do útero é realizada de rotina para os casos de carcinoma do Colo do útero com amostra já coletadas anteriormente e também amostras a serem coletadas em centro cirúrgico do Hospital de Base, adicionalmente a esta, não interferindo na condução clínica posterior. Descreve o tamanho e obtenção de N amostral prévio e a realizar. Apresenta critérios de inclusão e exclusão claros. Apresenta forma de transporte e armazenamento adequadamente descritas ("...congeladas em nitrogênio líquido no interior de um tubo de criogenia e transportadas, em carro particular, do Hospital Padre Albino, Catanduva, SP para o Laboratório de Imunomorfologia (IBILCE-UNESP) de São José do Rio Preto. Após, serão armazenadas em freezer -80°C até o momento da realização dos experimentos."). Refere claramente que as amostras não comporão biobanco/biorepositório. Descreve o responsável pela coleta do material como responsável pelo esclarecimento e coleta do TCLE. Encaminha a declaração de ciência e autorização do Diretor Executivo da FUNFARME, Dr. Horácio Ramalho, e da FAMERP, Dr. Dulcimar D. Souza.

Considerações sobre os Termos de apresentação obrigatória:

Apresenta declaração de ciência/concordância/autorização do responsável pela Fundação Faculdade Regional de Medicina de São José do Rio Preto.

Apresenta declaração de ciência/concordância/autorização do responsável pela Faculdade de Medicina de São José do Rio Preto.

Recomendações:

Nenhuma.

Conclusões ou Pendências e Lista de Inadequações:

Nenhuma.

Considerações Finais a critério do CEP:

O Comitê de Ética em Pesquisa do IBILCE, em reunião de 15 de fevereiro de 2016, deliberou, por unanimidade, pela aprovação do presente projeto de pesquisa. Os relatórios parciais deverão ser

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encaminhados semestralmente, contando a partir desta data.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_516048.pdf	21/01/2016 11:02:02		Aceito
Projeto Detalhado / Brochura Investigador	ANEXOII_Projeto_JaneslyPrates.pdf	21/01/2016 11:01:07	Janesly Prates	Aceito
Declaração de Instituição e Infraestrutura	ANEXOI_assinatura_DrHoracio_HB.pdf	21/01/2016 11:00:03	Janesly Prates	Aceito
Outros	Oficio_Carta_resposta.pdf	21/01/2016 10:54:56	Janesly Prates	Aceito
Declaração de Instituição e Infraestrutura	Declaracao_DrAyder_versao2.pdf	21/09/2015 15:22:50	Janesly Prates	Aceito
Declaração de Instituição e Infraestrutura	Declaracao_HospPadreAlbino_Catanduva.PDF	21/09/2015 15:22:01	Janesly Prates	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	Declaracao_DrDulcimar_Diretor_FAMERP.pdf	21/09/2015 15:20:50	Janesly Prates	Aceito
Declaração de Instituição e Infraestrutura	Assinatura_DrHoracio_diretor_HB_FUNFARME.pdf	21/09/2015 15:19:47	Janesly Prates	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_Janesly_versao2.pdf	21/09/2015 15:15:18	Janesly Prates	Aceito
Folha de Rosto	folha_rosto_plat_Brasil.pdf	19/06/2015 09:58:30		Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	Declaração_Dra Solange.pdf	17/06/2015 09:47:29		Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

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Não

SAO JOSE DO RIO PRETO, 17 de Fevereiro de 2016

Assinado por:
Claudia Regina Bonini Domingos
(Coordenador)

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Manuscript ID
FJ-18-0334

Title
Effect of cisplatin modulates Annexin A1 and Inhibitor of Differentiation to DNA 1 expression in cervical cancer cells

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Date Submitted
24-Apr-2018

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